Algal Research 51 (2020) 102041

Contents lists available at ScienceDirect

Algal Research

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

Growth, total lipid, and omega-3 fatty acid production by *Nannochloropsis* spp. cultivated with raw plant substrate

Jenna Y. Schambach[a], Anna M. Finck[a], Peter Kitin[c], Christopher G. Hunt[c], Erik R. Hanschen[a], Brian Vogler[a,d], Shawn R. Starkenburg[a], Amanda N. Barry[a,b,*]

[a] Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87544, United States of America
[b] Molecular and Microbiology Department, Sandia National Labs, Albuquerque, NM 87185, United States of America
[c] Forest Products Laboratory, Forest Biopolymer Science and Engineering, U.S. Forest Service, Madison, WI 53726, United States of America
[d] Currently at Conagen, Bedford, MA 01730, United States of America

A B S T R A C T

Improving productivity and lipid concentration in microalgae is important for the economic success of both biofuel and microalgae coproducts production. *Nannochloropsis* spp. are marine microalgae currently being grown at large-scale for the production of biofuel and lipid coproducts. Here we demonstrate improvements of growth and omega-3 production in *Nannochloropsis gaditana* CCMP526 and *Nannochloropsis oceanica* CCAP849/10 with plant substrate addition, a potentially economical option for increasing microalgal productivity. We examine growth in the presence of corn stover, switchgrass, sugarcane bagasse, and yard waste. By examining the microbial ecology of *N. gaditana* cultures with and without plant substrate and with and without antibiotics, we discovered a potential bacterial interaction in these cultures, but its presence is not necessary for algal growth improvements in the presence of plant substrate. Analysis of plant substrate morphology by scanning electron microscopy (SEM) after cultivation in media with and without *N. gaditana* shows a degradation of specific plant structural features and a colonization of the plant phloem by *N. gaditana*. An examination of *N. gaditana* in a Congo red plate assay indicates potential cellulolytic activity, but a preliminary examination of the potential cellulase transcripts does not reveal differential expression of a candidate in the presence of plant. This evidence demonstrates potential raw plant utilization by a marine microalga for increased productivity and provides a potentially economical option for increasing coproduct concentration at industrial scale without genetic engineering.

1. Introduction

Nannochloropsis is a genus of phototrophic microalgae with several species that exhibit high biomass and lipid accumulation rates, and robust growth in outdoor systems [1–6]. These characteristics make *Nannochloropsis* species a suitable feedstock for large-scale production of biodiesel and coproducts including eicosapentaenoic acid (EPA), an economically important nutraceutical [1]. *Nannochloropsis gaditana*, when grown with supplemental CO_2, exhibits high biomass with high lipid accumulation rates when grown to nutrient starvation [7,8]. Unlike other microalgae, such as *Chlamydomonas*, *N. gaditana* maintains lipid droplets, primarily composed of triacylglycerols, during linear growth in addition to the N deplete state [7,9]. The completion of a draft genome sequence for multiple strains of *N. gaditana* [10–12] and the development of robust genetic tools [8,13], make this strain a desirable candidate of study for optimized biofuel and biomass production.

A potentially economically feasible solution to optimize biofuel and biomass production includes growing microalgae mixotrophically using readily available plant carbon substrate. Under mixotrophic growth conditions, cells do not solely rely on light or organic carbon, and several have reported increased growth in *Nannochloropsis* sp. under this condition [14–19]. Recently, we characterized the first example of plant substrate utilization by the freshwater chlorophyte *Auxenochlorella protothecoides* UTEX 25 [20]. We observed an increase in overall growth and total lipid content when *A. protothecoides* was grown in the presence of switchgrass [20]. Genomic, transcriptomic, and proteomic analyses indicated that this alga carries a suite of cellulolytic enzymes and sugar transporters enabling it to directly degrade the plant material and use the liberated carbohydrates as an additional carbon source [20]. In contrast to *A. protothecoides*, *N. gaditana* is known to contain cellulose in its cell wall, and six potential glycosyl hydrolases involved in cellulose turnover have been identified [21]. However, it is unknown if these enzymes are used to degrade extracellular cellulose. Additionally, some studies have shown that *N. gaditana* is able to grow mixotrophically on glucose or glycerol [14,15,19], but the utilization of high molecular weight carbon sources by *N. gaditana* CCMP526 has not been demonstrated to our knowledge. In demonstrated mixotrophic growth of *Nannochloropsis* sp., studies show increases in biomass yield [14,16,17,19], overall lipid content [16,19,22], and/or EPA content

* Corresponding author at: Molecular and Microbiology Department, Sandia National Labs, Albuquerque, NM 87185, United States of America.
E-mail address: anbarry@sandia.gov (A.N. Barry).

https://doi.org/10.1016/j.algal.2020.102041
Received 29 April 2020; Received in revised form 4 August 2020; Accepted 4 August 2020
Available online 17 August 2020

[19,22].

In the present study, we explore the growth characteristics of *N. gaditana* CCMP526, and *N. oceanica* CCAP849/10 in the presence of several plant substrate types, as a potential strategy to increase biomass and lipid production at multiple scales. We characterize microalgal growth in flasks and mini raceway ponds, plant morphology before and after algal growth, and the bacterial community composition.

2. Materials and methods

2.1. Flask cultivation

N. gaditana CCMP526 (*N. gaditana* hereafter) was obtained from the Posewitz lab at Colorado School of Mines in Golden, CO. *N. gaditana* CCMP1894 was obtained from the National Center for Marine Algae and Microbiota at Bigelow Laboratory (NCMA, Maine, USA). *N. oceanica* CCAP849/10 (*N. oceanica* hereafter) was obtained from Scott Edmundson at Pacific Northwest National Laboratory (PNNL). Stock cultures were grown phototrophically in sterile f/2 media using artificial saltwater and maintained in exponential phase growth to avoid N limitation. The f/2 media composition used in all experiments unless otherwise stated was: 880 μM (75 mg L^{-1}) $NaNO_3$, 36 μM (5 mg L^{-1}) $NaH_2PO_4{\cdot}H_2O$, 11.7 μM (4.6 mg L^{-1}) $FeCl_3{\cdot}6H_2O$, 11.7 μM (4.4 mg L^{-1}) $Na_2EDTA\cdot 2H_2O$, 0.0393 μM (9.8 μg L^{-1}) $CuSO_2{\cdot}5H_2O$, 0.026 μM (6.3 μg L^{-1}) $Na_2MoO_4\cdot 2H_2O$, 0.0765 μM (22 μg L^{-1}) $ZnSO_4\cdot 7H_2O$, 0.042 μM (10 μg L^{-1}) $CoCl_2\cdot 6H_2O$, 0.91 μM (180 μg L^{-1}) $MnCl_2\cdot 4H_2O$, 0.0996 μM (135 μg L^{-1}) Vitamin B_{12}, 0.102 μM (25 μg L^{-1}) Biotin, 0.993 μM (335 μg L^{-1}) Thiamine HCl. Artificial seawater was prepared with 38 g L^{-1} of Instant Ocean Sea Salt in Milli-Q water. For each experiment, stock cultures were split into triplicate, 100 mL cultures in 250 mL Erlenmeyer flasks, with initial cell densities of approximately 1.0–2.0 × 10^6 cells mL^{-1}. We grew the cultures at 25 °C on an orbital shaker at 100 rpm, with a 12:12 h light:dark cycle at a light intensity of approximately 100 μmol m^{-2} s^{-1}. There was no CO_2 supplementation in these cultures. We monitored growth by performing daily cell counts using a hemacytometer, unless otherwise stated. Specific growth rates (μ) were calculated only for the log phase of growth using the equation:

$$\mu = \ln(N_2/N_1)/(t_2 - t_1) \quad (1)$$

where N_1 and N_2 are the cell counts (in cells mL^{-1}) at time 1 (t_1) and time 2 (t_2), respectively.

An initial screening of plant substrate utilization included the addition of 0.10% (*w/v*) milled corn stover (CS; *Zea mays*), sugarcane bagasse (SB; *Saccharum officinarum*), or switchgrass (SG; *Panicum virgatum*). The initial screening for *N. oceanica* also included 0.10% (*w/v*) yard waste, composed mostly of fresh grass clippings, from an untreated lawn in Los Alamos, NM, USA. Raw plant substrates were provided courtesy of Idaho National Laboratory (INL) Feedstock Library. Corn stover was collected by multi pass harvesting and ground to pass through a 2-in. sieve using a Vermeer BG480 grinder followed by a 1-in. sieve using a Bliss Hammermill (https://bioenergylibrary.inl.gov/Sample/BiomassInfo.aspx). Sugarcane bagasse was processed to pass through 6-in. screen (https://bioenergylibrary.inl.gov/Sample/BiomassInfo.aspx). Switchgrass was ground to pass through a 1-in. sieve using a Vermeer BG480 grinder (https://bioenergylibrary.inl.gov/Sample/BiomassInfo.aspx). All plant substrates for flask experiments were autoclaved for 20 min, at 121 °C and 15 psi. Once cultures reached early stationary phase (N deplete), the plant substrate was removed from the microalgae using a 40 μm Nylon cell strainer, algal cells were harvested by centrifugation, flash frozen in liquid N and stored at −80 °C, until FAME analysis. Nitrate (N) concentration was determined using Millipore colorimetric N test strips.

To examine the impact of having an additional surface in the flask for the microalgae to potentially adhere, we compared growth of *N. gaditana* in the presence of 2 mm wine cork (provided by Forest Concepts, LLC.), to growth of the microalgae alone. To explore the implications different tissues of the CS may have on growth, we grew *N. gaditana* in the presence of 0.10% (*w/v*) corn leaf stover cross-sections and diced stem (stalk, ~2 mm cubes) of Dairyland Seed variety DS-9804RA. Leaf and stem CS were uniformly cut to minimize any particle size effects. The corn leaf and stem were subsequently analyzed by SEM and fluorescence microscopy. In order to examine the effect of substrate concentration on growth, we grew *N. gaditana* in the presence of 0.10%, 0.25%, and 0.50% (*w/v*) CS. We grew cultures of *N. gaditana* in the dark with 0.10% (*w/v*) CS for 8 days to determine if there was heterotrophic activity by the algae.

Significant differences between growth rates were determined by analysis of variance and least-squares means for multiple comparisons with a Tukey *p*-value adjustment.

2.2. Pond cultivation

Plant utilization by *N. gaditana* and *N. oceanica* was examined at 50 L volume in six 100 L mini raceway ponds (MicroBio Engineering, San Luis Obispo, CA) situated in a greenhouse in Los Alamos, NM, USA. *N. oceanica* was grown in well water (provided by Qualitas Health, TX) supplemented with 1.76 mM (150 mg L^{-1}) $NaNO_3$, 0.230 mM (40 mg L^{-1}) K_2HPO_4, and 0.857 μM (0.295 mg L^{-1}) Fe EDTA during February 2019, and *N. gaditana* was grown in f/2 media during November 2019. 1200 mL cultures of inoculum were first grown in flasks for one week prior to the inoculation of one mini raceway pond at a 50 L volume. Once acclimated, the culture was drained from the pond and split among all six mini raceway ponds with a starting OD_{750} of approximately 0.20 at a 50 L volume. CS was added to every other pond ($N = 3$) to a final concentration of 0.25% (*w/v*) for *N. gaditana*, and 0.10% (*w/v*) for *N. oceanica*. Note the CS and media were not sterilized prior to inoculation. Each pond was equipped with cylindrical bubble diffusers and continuously aerated at a flow rate of approximately 400 mL min^{-1}, supplemented with CO_2 when the pH exceeded 8.1. The paddlewheel speed was 40 rpm and culture depth was maintained at 20 cm. For the *N. gaditana* experiment, the daily average temperature was 22 °C and the average incident irradiance was 850 μmol m^{-2} s^{-1}. For the *N. oceanica* experiment, the daily average temperature was 24 °C and the average incident irradiance was 835 μmol m^{-2} s^{-1}. Note the CO_2 was completely turned off during the night for the experiment with *N. gaditana*. Growth was monitored by performing daily cell counts using a hemacytometer. Significant differences between growth rates with CS and without for each species were determined by analysis of variance and least-squares means for multiple comparisons with a Tukey *p*-value adjustment.

Samples for fatty acid methyl ester (FAME) analysis of the algal biomass were taken at the start of the experiment, when N concentrations reached 0 mg L^{-1} ($N = 0$), and when the ponds were harvested. Significant differences between FAME content with CS and without for each species were determined by Welch two sample *t*-test. Plant substrate was separated from algal biomass using a 40 μm Nylon cell strainer prior to FAME analysis. N concentrations were determined using Millipore colorimetric N test strips in conjunction with Hach low range N test kits (TNT 835) and a Hach D3900 spectrophotometer (Hach, Loveland, CO).

2.3. FAME analysis

Microalgal biomass was centrifuged at 5000 rcf for 10 min, then lyophilized prior to FAME analysis. Total lipids expressed as FAME (i.e. total lipids) were determined by gas chromatography coupled with flame ionization detection (GC/FID) according to National Renewable Energy Laboratory, Laboratory Analytical Procedure [23]. Briefly, we performed an acid-catalyzed transesterification reaction by adding 25 μL of 10 mg mL^{-1} methyl tridecanoate (C13:0ME, an internal standard), 200 μL of chloroform methanol (2:1, *v/v*), and 300 μL of 0.6 M HCl:methanol (2.1% *v/v*), to 5–10 mg of freeze-dried algal

biomass. The mixture was immediately incubated at 85 °C for 1 h. The released FAMEs were then extracted in 1 mL of hexane and analyzed using an Agilent 8890A Series GC/FID with a DB-WAX column (30 m length ·0.25 mm inner diameter ·0.25 μm film thickness (Agilent Technologies, Santa Clara, CA)). Calibration response factors from a GLC 461C 30-component FAME standard mix (Nu-Check Prep, Inc., Elysian, MN) were used to quantify each fatty acid after normalizing to the internal standard (C13:0ME). The summation of individual fatty acids gives the total lipid content as FAME and is represented as a percent of the dry weight for each sample. Significant differences between treatments were determined by analysis of variance and least-squares means for multiple comparisons with a Tukey *p*-value adjustment.

2.4. Bacterial sequencing

To assess the potential role bacteria may play in the degradation of CS, we cultivated *N. gaditana* alone, *N. gaditana* with gram positive antibiotics (100 μg mL^{-1} ampicillin, 30 μg mL^{-1} chloramphenicol, 100 mg mL^{-1} streptomycin, and 100 mg mL^{-1} tetracycline), *N. gaditana* with 0.10% (*w/v*) CS, *N. gaditana* with 0.10% (*w/v*) CS and gram positive antibiotics, and *N. gaditana* with 0.10% (*w/v*) CS and algal antibiotics (200 μg mL^{-1} hygromycin B and 600 μg mL^{-1} paromomycin). The CS used in this experiment was uniformly diced stem (described above). Microalgal and bacterial cell counts were performed using a BD Accuri C6 Plus flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ). The forward scatter threshold was set to 8000, and the fluidics were set to 66 μL min^{-1}. Mircoalgal populations were gated based on auto-fluorescence. Gating from an experiment where there was evident bacterial contamination in *N. gaditana* cultures was used to gate bacterial populations.

On the final day of this experiment, DNA was extracted from samples for 16S rRNA amplicon sequencing. DNA extraction was performed using the Zymo Research (Irvine, California) Quick-DNA Fungal/Bacterial Microprep Kit. Bead beating was performed for 40 s at 6 m/s using a MP Biomedical (Santa Ana, California) Fastprep-24 5G. Extracted DNA quality was assayed using a Qubit3 with a high sensitivity assay and an Invitrogen E-gel. 16S amplicon sequencing was performed using previously published primers [24,25], the V4 hypervariable region of the 16S rRNA gene was amplified from the DNA extracts during the first PCR step using the universal 515F forward primer (5' GTGCCAGCMGCCGCGGTAA 3') and uniquely barcoded 806R reverse primers (5' GGACTACHVGGGTWTCTAAT 3') for each sample. 16S amplicon sequencing was performed on an Illumina MiSeq machine (Illumina, Inc., San Diego,CA). The 16S rRNA compositional analysis was completed with QIIME (v1.9.1) [26], incorporated into the EDGE Bioinformatics platform (v2.3.0) [27]. A 94% nucleotide identity sequence clustering threshold (approximately genus level) was used to generate operational taxonomic units (OTUs). The representative sequence for each OTU was then queried against the GreenGenes database [28] for taxonomic classification assignment.

2.5. Cellulolytic activity analysis

To assess cellulolytic activity, Congo Red staining was performed as previously described [20]. In brief, *N. gaditana* was streaked onto f/2 agar plates enriched with 0.10% carboxymethyl cellulose (CMC). After 8 days the plates were stained for 15 min using Congo Red. The plates were rinsed with 1 M NaCl and clearing was observed.

2.6. Transcript analysis

N. gaditana was grown for 8 days with and without 0.10% (*w/v*) milled CS. The microalgae were separated from the CS, then 15 mL of each culture were pelleted at 3000 rcf for 15 min. The cells were resuspended in 1 mL DEPC water with RNase free glass beads and centrifuged at 3000 rcf for 15 min. Once supernatant was decanted, 1 mL of cold methanol was added. The pellets were vortexed for 10 min, then centrifuged at 20,000 rcf for 10 min. This step was repeated twice. Next, the pellets were resuspended in 200 μL cold DEPC water and 1 μL of RNaseOUT (added to the 200 μL DEPC immediately prior to resuspension). The samples were transferred to 5PRIME Phase Lock Gel Heavy tubes (QuantaBio, VWR) and 200 μL of phenol:-chloroform:isoamyl alcohol (25,24,1) was added. The samples were mixed, incubated on ice for 5 min, then centrifuged at 12,000 rcf for 12 min. Next, 200 μL of chloroform:isoamyl alcohol (24,1) was added, the samples were mixed and incubated on ice for 5 min. The phases were separated by centrifugation at 12,000 rcf for 12 min. The aqueous phase was transferred to a new 1.5 mL microcentrifuge tube and 20 μL 3 M RNase-free NaOAc and 500 μL 100% RNase-free ethanol were added. The samples were incubated at −80 °C for 1 h followed by centrifugation at 20,000 rcf for 15 min. The pellets were washed with 500 μL cold 75% RNase-free ethanol and dried in a Labconco 7,310,020 Refrigerated CentriVap Benchtop Concentrator (Labconco Corporation, Kansas City, MO) for 10 min. The pellets were resuspended in 20 μL DEPC water containing 1 μL of RNaseOUT.

Complementary DNA (cDNA) was synthesized from the RNA using the First Strand cDNA synthesis kit (New England Biolabs, Ipswich, MA), following the manufacturer's protocol. PCR primers were designed using the six putative glycosyl hydrolase family 9 sequences identified by [21] (Table 1). For each primer set, three 50 μL reactions were performed: a negative control (no cDNA), a reaction using cDNA from *N. gaditana* grown with 0.10% CS, and a reaction using cDNA from *N. gaditana* grown without CS. There were two positive controls using

Table 1
Forward (F) and reverse (R) PCR primer sequences for the six putative glycosyl hydrolase proteins identified by [12] and actin as a positive control.

Target gene	GenBank accession number	Primer sequence
actin (control)	NW005802401 F	TACCTTGATCGACCATGGCGGAAGAA
	NW005802401 R	TTGGAGATCCACATGCTCTGGAAGGT
cellulase	JU964548.1 F	ATGTCTTCTCCCTTCCAAGCCTACCAAAAA
	JU964548.1 R	TCAATACTCAGTGGGCAGCTTCTCCACGAG
b-endoglucanase	EKU20567 F	ATGATTAAAATGCGCACCGTGACCAGCGCG
	EKU20567 R	TTTAATCGCGGTGGTCACTTCGTTGCGGGT
cellulase	JU964585.1 F	ATGTTGTGCGTGCACACTCGCCAAAGTTTA
	JU964585.1 R	CTATCGCTTGTGGCCATTCCCATGACTTTA
cellulase	JU973032.1 F	ATGATGGACACCATCAAATGGGGTCTAGAC
	JU973032.1 R	CGGCTATGTCGGAGCCAGGATGCTTGGGGT
cellulase	JU981247.1 F	ATGCAGCATTCTGACATTGTCACGCCTCGA
	JU981247.1 R	TTAATACCGAATACCTCTCAGGCTCAAATG
endo-b-glucanase	AFJ68664 F	ATGTATATGATTCTGAAACCGCTGATTGCG
	AFJ68664 R	GGTAATAAAATCCGGGCATTTATCATGATG

actin gene primers along with cDNA from *N. gaditana* alone and *N. gaditana* cultivated with 0.1% (*w/v*) CS. PCR conditions were: Initial denaturation at 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 2 min, and final extension at 72 °C for 7 min. Products were run on a 1% agarose gel with a 1 kb DNA reference ladder (New England Biolabs, Ipswich, MA).

2.7. Light and electron microscopy

To determine if *N. gaditana* was indeed utilizing the plant substrate, the uniformly cut stem cubes were analyzed by light and scanning electron microscopy (SEM). Cubes were soaked in 1% sodium azide and stored in a 1:1:1 mixture of ethanol/glycerol/water at 4 C until use.

Light microscopy was performed on the surface of cubes, in glycerol, with UV (365 nm) excitation and 435 nm long-pass fluorescence emission. For SEM, cubes were repeatedly rinsed (50%, 25%, 0% ethanol), frozen in liquid N_2, freeze-dried, sputter-coated with gold, and observed at 10 kV. More than 30 vascular bundles of plant material with microalgae and 30 vascular bundles without microalgae were observed by both SEM and fluorescence microscopy. Fluorescence images (Fig. 6) were obtained with a Nikon Eclipse Ci fluorescence microscope with Dapi long pass filter and FLIR Grasshopper3 camera.

To provide supplementary evidence that cell aggregates seen in SEM were in fact microalgae cells, we performed conventional light and fluorescence microscopy on fragments of CS taken directly from *N. gaditana* pond cultures (Fig. S4B, C). Images were obtained with Zeiss Axioplan fluorescence microscope with Zeiss filter set 9 (BP 450–490, FT 510, LP 520) and a Nikon D700 camera.

2.8. Sugar analysis

NREL CS was recovered by cell strainer filtration after incubation with buffer (control) or *N. gaditana*, and the microalgae biomass separated by centrifugation and decanting. Sugar analysis was conducted on the corn, microalgae biomass, and liquid according to the method of [29]. Briefly, dry sample from each of the three biological replicates was acid hydrolyzed and the released sugars were separated by ion chromatography and detected by pulsed amperometric detection. Confidence intervals were calculated assuming normal distributions but unequal variances. The Welch-Satterthwaite approximation was used when combining averages.

3. Results and discussion

3.1. Growth of Nannochloropsis spp. with plant substrates

Raw plant substrates (CS, SB, and SG), roughly milled [INL Feedstock Library], were added to 100 mL cultures of *N. gaditana* to examine if they had any influence on overall growth. We observed improved biomass accumulation in all cultures containing plant substrate (Fig. 1A). Significant statistical differences between average cell densities of cultures with plant substrate versus those without are indicated by asterisks in Fig. 1A. The specific growth rate of *N. gaditana* increased by 13% when cultures contained CS (0.98 ± 0.06) relative to the microalgae alone (0.85 ± 0.05). This was the greatest improvement, although this was not statistically significant ($p = .287$). We saw a similar increase in overall growth and biomass accumulation when *N. gaditana* strain CCMP1894 was cultured with CS and SB (Fig. S1).

We also tested the addition of the above plant substrates, as well as yard waste, in flask cultures of *N. oceanica*, another commercially relevant alga (Fig. 1B). We saw improvement in specific growth rates in all cultures containing plant substrate compared to the microalgae alone, except for those containing yard waste. Similar to *N. gaditana*, *N. oceanica* cultures containing CS had the greatest biomass accumulation. Furthermore, we observed an increase in specific growth rate of 16% when cultures were grown with CS (0.58 ± 0.04) when compared to the microalgae alone (0.49 ± 0.04), but this was not statistically significant ($p = .596$). CS was selected as the plant carbon substrate to further investigate mixotrophic growth of *N. gaditana* and *N. oceanica*.

Others have investigated the influence of organic carbon addition on overall growth with different strains of *N. gaditana* and *N. oceanica*. Menegol et al. reported a higher maximum specific growth rate and biomass accumulation of *N. gaditana* B-3 with 5 g L^{-1} glucose or 1 g L^{-1} glycerol as an additional carbon source but not acetate [19]. Ren et al. also saw an increase in growth rate with the addition of 10 mM glucose to *N. gaditana* CCMP527 cultures [14]. Li et al. reported enhanced growth and high acetate assimilation in *N. oceanica* with both sodium acetate and sodium bicarbonate in the growth medium [30]. Conversely, others did not observe increases in overall growth of *N. oceanica* at any of the tested concentrations of glucose [22,31]. Differences in culture conditions across the different studies likely play a large role in the variability of results.

In previous studies, structurally simple, soluble organic substrates like glucose or glycerol were used, and any increase in algal growth was attributed to organic carbon utilization [30]. While using CS as a carbon source has its advantages (e.g. low cost, carbon is not bioavailable for many organisms), it is insoluble and structurally complex making it more difficult to directly measure uptake, and definitively attribute the increase in *Nannochloropsis* growth to organic carbon utilization. Using cork, we were able to test if having insoluble particles present that provided an additional surface for the microalgae to potentially adhere to, ultimately promoted growth of *N. gaditana*. Suberin, the main chemical constituent of cork, is a complex biopolymer and the carbon therein is not directly bioavailable to the microalgae [32]. Since we did not see a difference in specific growth rate when the cork was present ($p = .445$), this provided further evidence that the CS may be a microalgae-accessible nutrient source (Fig. 1C).

To address the complex structure of CS and attempt to eliminate error due to surface area effects, we obtained precisely cut stem (stalk) and leaf stover cross-sections for growth studies.

We found greater biomass accumulation when *N. gaditana* was grown in the presence of stem compared to leaf ($p = .014$) (Fig. 1D). The corn stem or stalk portion has a greater concentration of cellulose and hemicellulose than the corn leaf (https://www.celignis.com/feedstock.php?value=27). Our previous studies with *A. protothecoides* indicate that some microalgae utilize hemicellulose [20]. We attempted to grow *N. gaditana* in the presence of the hemicellulose, xylan, extracted from SG, but bacteria overtook these cultures. We are, therefore, unable to conclude that *N. gaditana* can utilize the xylan fraction (see Fig. 4C).

We also investigated the ability of *Nannochloropsis* to grow heterotrophically by cultivating *N. gaditana* with and without CS in the dark (Fig. S2). We found that cultures containing CS maintained a higher average cell density for the majority of the experiment, although growth was still minimal. Some microalgae can overcome light limitation experienced in dense cultures through heterotrophic growth, but there are conflicting reports about the ability of *Nannochloropsis* spp. to grow heterotrophically [1,16,18,33,34]. *N. oculata* was unable to grow heterotrophically using glucose or acetate [34], whereas an unspecified *Nannochloropsis* sp. was capable of heterotrophic growth using glucose as the sole carbon source, although growth was slower compared to that under photoautotrophic conditions [16].

The regulatory mechanisms of mixotrophy are not well understood, and are unlikely to be universal across microorganisms [35]. It is thought that there may be a synergistic effect between photosynthesis and aerobic respiration, rather than simply switching from one metabolic mode to the other under corresponding conditions [35]. A genome-scale metabolic model for *N. gaditana* provides evidence that this alga is metabolically capable of mixotrophy, as flux balance analysis revealed a co-existence of electron transport from the light reactions and from the dark cycle with acetate or glucose as the simulated

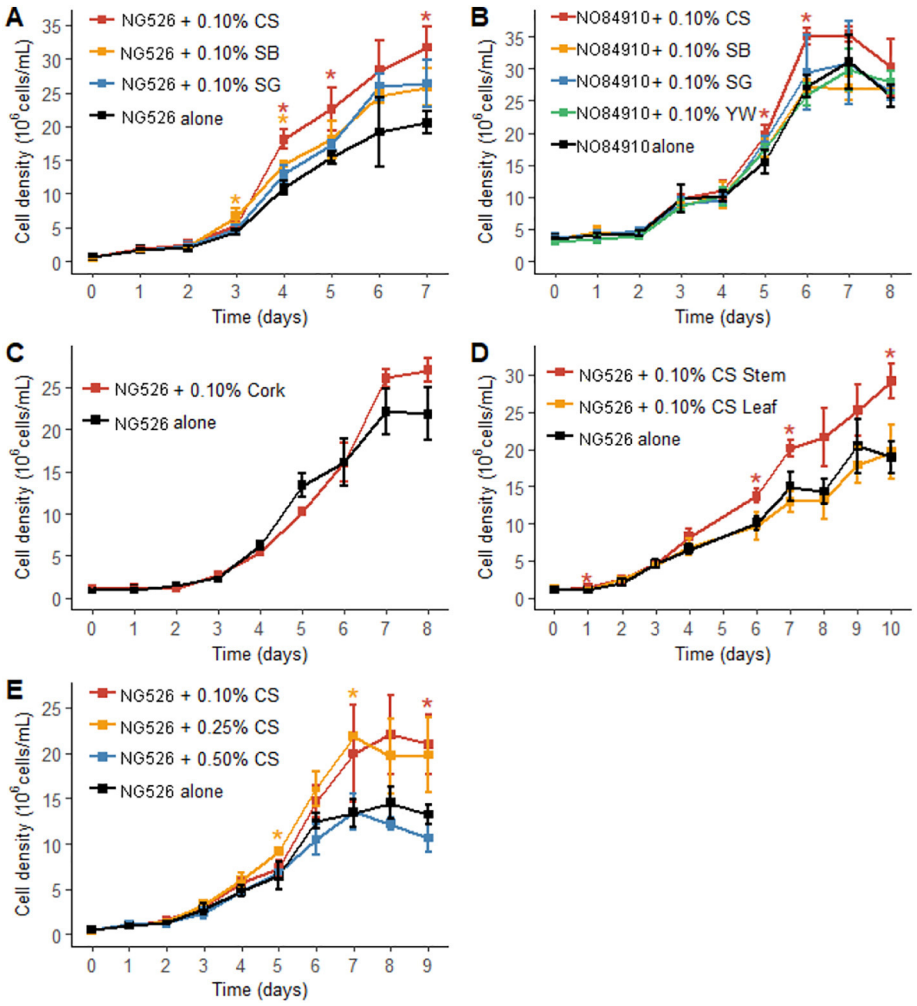

Fig. 1. Growth of *Nannochloropsis* spp. with and without plant substrates. **(A)** *N. gaditana* CCMP526 (NG526) with and without corn stover (CS), sugarcane bagasse (SB), switchgrass (SG); **(B)** *N. oceanica* CCAP849/10 (NO84910) with and without CS, SB, SG, and yard waste (YW); **(C)** Growth of *N. gaditana* CCMP526 with and without wine cork; **(D)** Growth of *N. gaditana* CCMP526 with and without precisely cut CS stem and leaf cross-sections; **(E)** Growth of *N. gaditana* CCMP526 with and without 0.10%, 0.25%, 0.50% (*w/v*) CS. Error bars indicate standard deviation of the mean for three biological replicates. Colored asterisks indicate the corresponding microalgae and plant substrate is significantly different from the microalgae alone ($p < .05$).

organic carbon source [36]. While we do not see appreciable growth when cultivating *N. gaditana* in the dark in the presence of CS, we cannot discount mixotrophy as we are likely witnessing this synergistic effect when in the light.

One of the main factors identified as a trigger for mixotrophic growth is the presence of a carbon source at an optimal concentration [35]. To determine the optimal concentration of plant biomass in the culture, we compared the growth of *N. gaditana* with three different concentrations of CS: 0.10%, 0.25%, and 0.50% (*w/v*). We saw improved biomass accumulation, and a 20–25% increase in specific growth rate in cultures containing 0.10% and 0.25% (*w/v*) CS compared to the control (Fig. 1E). In cultures containing 0.50% (*w/v*) CS growth was comparable to cultures with the microalgae alone, presumably due to increased light attenuation when more substrate is present. To test this, we measured the light intensity under flasks containing the three concentrations of CS and media alone. We found the average light intensity was much less with 0.50% (*w/v*) CS, at 48 μmol m^{-2} s^{-1}, whereas with 0.10% and 0.25% (*w/v*) CS it was 87 and 81 μmol m^{-2} s^{-1}, respectively.

We investigated CS addition to cultures of *N. gaditana* and *N. oceanica* at the 50 L scale in raceway ponds (Figs. 2A-E; 3A-E). For both strains, there was an overall increase in biomass accumulation in the ponds, with and without plant, when compared to the original flask experiments (Fig. 1A and B), likely due to CO_2 supplementation and increased irradiance experienced in the greenhouse. The average specific growth rate for *N. gaditana* cultures with CS (0.39 ± 0.02) was 30% greater than those grown without (0.27 ± 0.02, Fig. 2A). This was statistically significant ($p = .0021$). The average specific growth rate for *N. oceanica* cultures with CS (0.48 ± 0.04) was only 8% greater than those grown without (0.44 ± 0.04, Fig. 3A). Both of these strains have been reported to grow successfully in large-scale, outdoor, flat-panel photobioreactors, and/or raceway ponds, with the addition of pure CO_2 or industrial flue gases, under a variety of environmental conditions [4–6,19,37]. However, only one study was found that examined the effects of organic carbon addition to *Nannochloropsis* cultures at large-scale [19]. They also reported an overall increase in biomass concentration and biomass productivity of *N. gaditana*, although they used a continuous culturing technique, with the addition of glucose at 400 L in photobioreactors [19].

Pond cultures grown with CS were N-deplete one and two days after those grown without CS for *N. gaditana* and *N. oceanica*, respectively (Figs. 2B; 3B). This led to delayed entry into stationary phase for cultures with CS. Further investigation is needed to determine if these patterns are due to differences in N uptake and utilization when *Nannochloropsis* is grown mixotrophically [17,33] or if the plant substrate itself contributes to the total N pool, ultimately influencing N uptake kinetics. The difference in N availability in cultures grown with CS may contribute to the increase in final biomass density for both strains. Additionally, while not directly measured, both strains remained vibrantly green through the last day of growth with CS present, whereas the cultures of microalgae alone became visibly chlorotic during stationary phase (Figs. 2C-E; 3C-E).

Estimates of the amount of N and other nutrients that are removed from fields by CS harvest are variable across and within fields [38,39]. The bioavailability, concentration, and ultimate effect of nutrients originating from the CS and leaching into microalgae cultures is

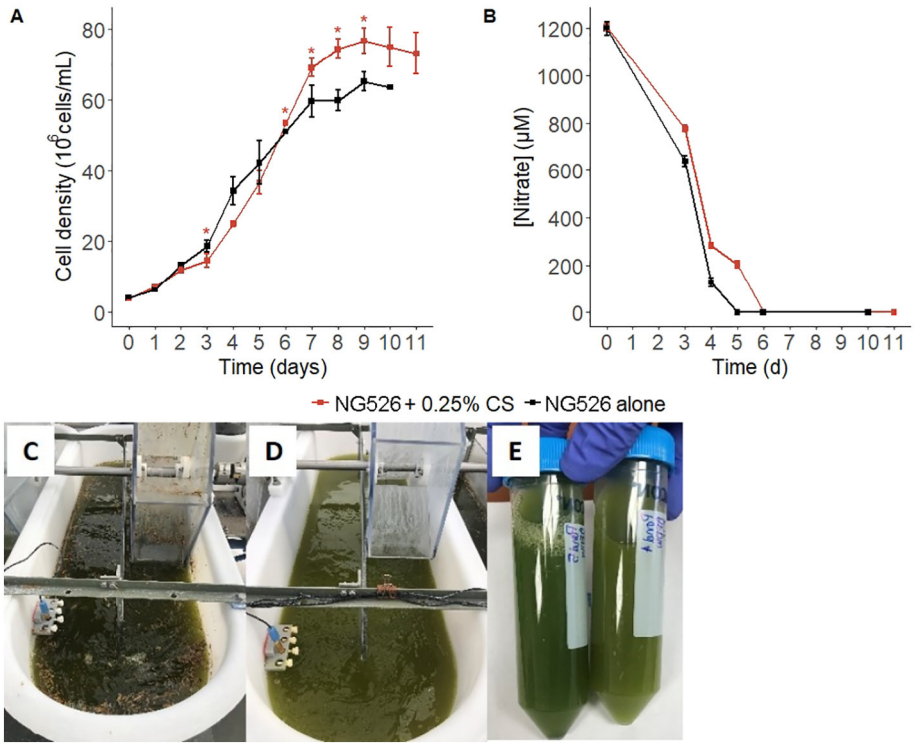

Fig. 2. Growth of *N. gaditana* CCMP526 (NG526) with and without 0.25% (*w/v*) corn stover (CS) at 50 L volume in raceway ponds. **(A)** Microalgae growth; **(B)** Nitrate (N) concentrations; **(C)** Photo of pond with microalgae and CS at the final time point; **(D)** Photo of pond with microalgae alone at the final time point; **(E)** Photo of concentrated microalgae samples from pond with CS (left), without CS (right). Error bars indicate standard deviation of the mean for three biological replicates. Images were taken on the final day of the experiment. Asterisks indicate microalgae and CS is significantly different from the microalgae alone ($p < .05$).

currently unknown. Plant substrates, such as CS, could prove to be a source for macro- and micronutrients, thus potentially improving the overall economics of algal biomass production.

3.2. Bacterial microbiome analysis

To determine if bacteria play a significant role, we grew *N. gaditana* with and without CS, with and without gram-positive antibiotics (Fig. 4). In cultures with CS present but without antibiotics, bacterial counts increased considerably in the first three days of culturing, declining thereafter (Fig. S3). We also saw a small increase in bacteria around the same time in cultures with the microalgae alone. 16S sequencing confirmed a greater relative abundance of bacteria in cultures with CS, likely explaining the beneficial effect on algal growth in the

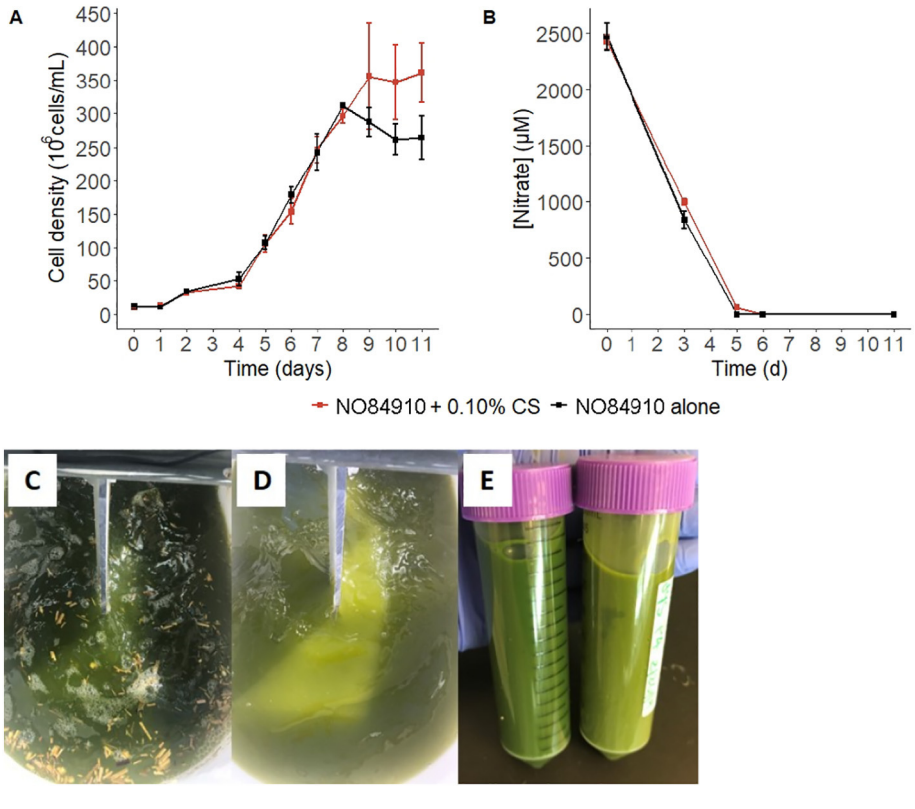

Fig. 3. Growth of *N. oceanica* CCAP849/10 (NO84910) with and without 0.10% (*w/v*) corn stover (CS) at 50 L volume in raceway ponds. **(A)** Microalgae growth; **(B)** Nitrate (N) concentrations; **(C)** Photo of pond with microalgae and CS at the final time point; **(D)** Photo of pond with microalgae alone at the final time point; **(E)** Photo of concentrated microalgae samples from pond with CS (left), without CS (right). Error bars indicate standard deviation of the mean for three biological replicates. Microalgae and CS were not statistical different the microalgae alone ($p > .05$).

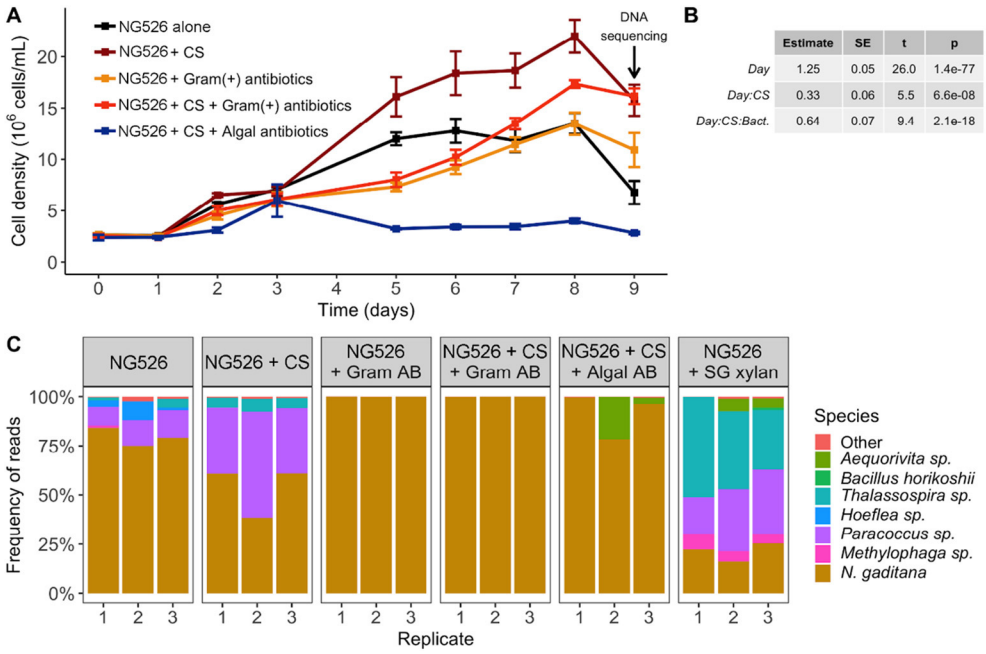

Fig. 4. (A) Growth of *N. gaditana* CCMP526 (NG526) with and without 0.10% (*w/v*) corn stover (CS), with and without gram positive (Gram(+)) antibiotics, or algal antibiotics; **(B)** Results from multiple linear regression using cell density as the response variable, Time (days) as an explanatory variable, and presence/absence of CS and Gram(+) antibiotics as categorical explanatory variables. The treatment including algal antibiotics was removed from this analysis due to lack of algal growth. Significance of Day indicates growth across all algal treatments. The Day:CS interaction demonstrates the significant effect of CS addition on algal growth. The Day:CS:Bacteria interaction demonstrates the significant effect of bacteria in the presence of CS on algal growth. **(C)** Relative abundance of *N. gaditana* CCMP526 chloroplast and bacterial 16S sequences from DNA. Gram AB and Algal AB refers to Gram (+) antibiotics, and algal antibiotics, respectively. SG xylan refers to xylan extracted from switchgrass.

presence of CS (Fig. 4B). *Paracoccus* sp. was identified by 16S sequencing as a major bacterial species in both cultures (Fig. 4C).

In all cultures with antibiotics, bacteria were effectively eliminated as confirmed by 16S sequencing and cell counts (Fig. 4C). Algal growth was dampened overall when the bacteria were eliminated, regardless of the addition of CS (Fig. 4B). This could indicate that the bacteria identified in cultures without antibiotics may be commonly associated with *N. gaditana* and promote algal growth. For example, *Paracoccus* sp. has been reported in species-specific association with *Chlamydomonas reinhardtii*, and are likely growth promoting [40]. Further investigation is necessary to determine if these bacteria employ cellulolytic enzymes and play an active role in the breakdown of the plant substrate. However, when comparing growth in the presence of antibiotics with and without CS, microalgae productivity was enhanced in CS amended cultures compared to the control, suggesting this effect is independent of the bacterial community composition (Fig. 4B). Finally, addition of CS to microalgae significantly improved algal growth (in the absence of bacteria) compared to a control culture of microalgae without bacteria (Day 7, one sided *t*-test $p = 1.2\times10^{-4}$; Day 8, one sided t-test $p = 1.8\times10$–6; Benjamini-Hochberg corrected; Fig. 4A). This demonstrates that corn stover has a significant effect on the growth of *N. gaditana* when alone.

We eliminated the microalgae population using Hygromycin B to investigate bacterial proliferation in the presence of the CS (Fig. 4C, "Algae + CS + Algal AB"). We did not find a substantial increase in bacterial counts even in the absence of algal growth, suggesting a potential symbiotic relationship between the residential bacterial community and *N. gaditana*. It is known that most microalgae live in association with a consortia of microorganisms that influence algal evolution, ecology, and physiology in many ways [41–43]. The microalgae also release dissolved organic matter, or signaling molecules, that nurture specific bacterial communities [41]. However, our knowledge is limited on these complex relationships and how they may change over time and under varying conditions.

3.3. Changes in plant morphology after N. gaditana growth

In order to determine the effect of *N. gaditana* growth on plant morphology, we examined the CS after incubation with the algal culture by scanning electron microscopy (SEM). As a control, CS was added to f/2 media without *N. gaditana*, under identical conditions over the growth period. Initial SEM suggested structural changes in samples with microalgae present. Corn stem cubes with and without *N. gaditana* growth showed intact epidermis, hypodermis, and ground parenchyma. Conversely, all of the 30+ vascular bundles with microalgae, observed by SEM or epi-fluorescence, suffered from phloem cell-wall degradation and collapse (Figs. 5A and C; 6). No missing or collapsed phloem cells were found in the cubes without microalgae (Fig. 5B and D). A tendency for porous cell walls after incubation, as opposed to compact and cleanly cut walls in control, also suggests cell wall dissolution in the presence of microalgae (Fig. 5C).

Physical associations between *Nannochloropsis* spp. and other fibrous biopolymers have been recently reported [42,43]. Our SEM image of presumed *N. gaditana* cells aggregated in the plant phloem (Fig. S4A) are strikingly similar to the images of *N. oceanica* clustered on fungal hyphae [42,43], indicating similar cell behavior in the presence of a structured carbon source. Additionally, red autofluorescence from microalgal chlorophyll can be seen around and within a CS stem fragment taken from a pond culture of *N. gaditana* (Fig. S4B and C). This further indicates that these cell aggregates are indeed microalgae and not bacteria.

3.4. Sugar analyses of CS after microalgae growth and initial examination of known potential glycosyl hydrolases of N. gaditana

To examine plant carbon utilization by *N. gaditana*, we examined total sugars in CS with and without microalgae growth (Table 2) and in the supernatant (without microalgae), which includes dissolved sugars (Table 3). CS comprises the stalks, leaves, leaf sheaths, tassels, cobs, and cob stems, each with its own chemical composition and all subject to variability [44,45]. The major components of CS include glucan, xylan, soluble fractions, and lignin [45]. There were no significant differences in carbohydrate content of the retentate.

However, when we analyzed the sugar content of filtrates after incubation, arabinan, galactan, and mannan levels in *N. gaditana* incubated with CS were almost exactly the sum of *N. gaditana* cells and CS incubated separately, indicating no interaction (Table 3). Glucan and xylan were less abundant in *N. gaditana* incubated with CS than the CS filtrate alone, suggesting that *N. gaditana* metabolized glucan and xylan from the solution, however only glucan had a *p*-value clearly suggesting significance. This indication that only certain sugars appear to be used by the microalgae is similar to what we saw in our previous work with

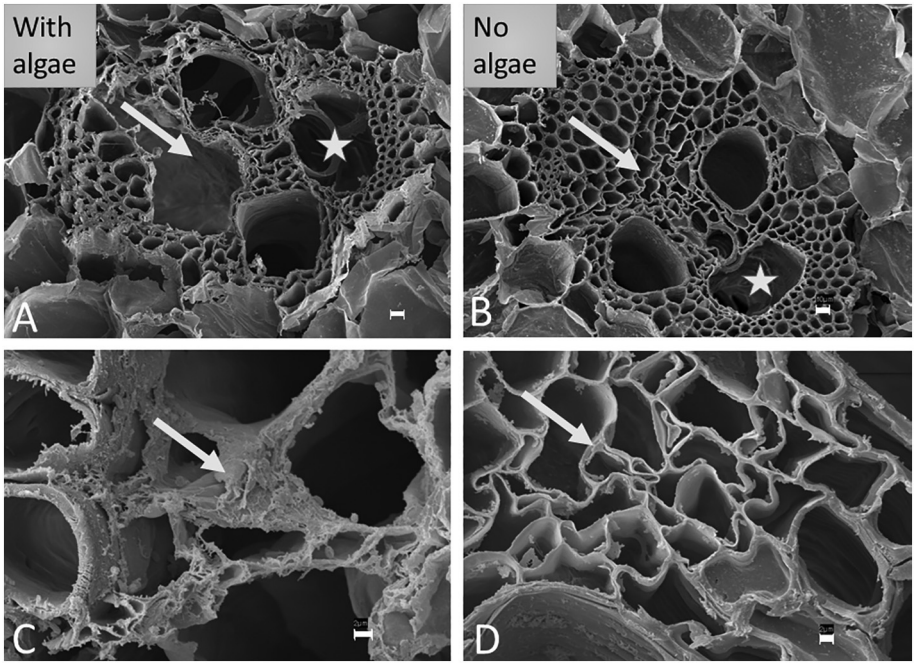

Fig. 5. (A, C) Scanning electron microscopy (SEM) images of corn stem cubes in microalgae cultures; **(B, D)** SEM images of corn stem cubes in media alone control. Arrows point to phloem cells. Stars indicate the lacunas of the vascular bundles. Missing or collapsed phloem cells can be observed in the plant material in microalgal cultures. Most of the phloem is missing in **(A)**. Degraded phloem cell walls in **(C)**. Scale bars = 10 μm (A and B); 2 μm (C and D).

A. protothecoides [20]. Interestingly, glucan is the primary storage carbohydrate in *N. gaditana* [46], which suggests that this microalga has the necessary molecular machinery for glucan metabolism.

In addition, we grew *N. gaditana* on agar plates containing carboxymethylcellulose (CMC) under continuous light or in the dark and stained with Congo Red to determine if *N. gaditana* exhibits cellulolytic activity that can act on external cellulose. We observed evident clearing of CMC on both plates, with substantially more clearing of CMC on plates grown in continuous light, likely due to the increased algal growth (Fig. S5A).

Previous work has identified six putative cellulases, glycosyl hydrolase family 9 proteins, in *N. gaditana* [21]. This family of proteins is capable of hydrolyzing the glycosidic linkages in cellulose and hemicellulose [47]. After isolating *N. gaditana* total mRNA grown with and without CS, we attempted to amplify the six transcripts by RT-PCR. Of the six, only two (JU964585 and JU973032) had single amplicons (Fig. S5B). We did not detect transcripts from JU981247 and AFJ68664. Multiple RT-PCR products were seen when we attempted to amplify JU964548 and EKU20567, and therefore, we cannot make inferences about mRNA expression. These preliminary results indicate no difference in presence or absence of mRNA of these six putative cellulases when *N. gaditana* is grown with and without CS. It is hypothesized that these proteins are used for cell wall cycling, as the cell wall of *N. gaditana* is primarily composed of cellulose [21]. Future transcriptomic analysis of *N. gaditana* grown with and without plant substrate aims to clarify whether these proteins also play a role in hydrolyzing plant substrate.

3.5. Lipid content of Nannochloropsis spp. cultivated with plant substrate

We performed FAME analysis on the harvested algal biomass from the initial growth experiment to determine if the addition of plant substrate had an impact on total lipids as FAME (total lipids hereafter) and fatty acid composition of *N. gaditana* and *N. oceanica*. Total lipids, on average, increased from 30% (algae alone) to 44%, 46%, and 34% of the total biomass in *N. gaditana* when CS, SB, and SG were present in the culture, respectively ($p = .079$) (Fig. 7A). On the contrary, the total lipids for *N. oceanica* cultures with plant substrate were, on average, slightly less than (SB, SG) or equal to (CS) that of the algae alone, with the exception of cultures containing yard waste (Fig. 7C). It is unclear if this increase in total lipids in cultures containing yard waste is due to carbon utilization, or a stress response related to the presence of this substrate.

Increased lipid accumulation under mixotrophic growth has been reported for several microalgal genera [18,48,49]. Growth simulations of the genome-scale metabolic model for *N. gaditana* also showed an increased in the flux of fatty acid biosynthesis in the presence of organic carbon [36]. Excess carbon under mixotrophic growth may be

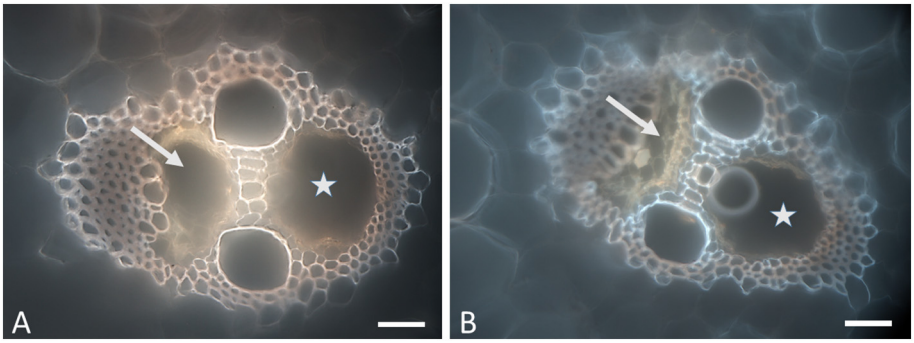

Fig. 6. Fluorescence microscopy showing corn stem cubes in microalgae cultures. **(A)** Missing material of phloem in the vascular bundle; **(B)** Collapsed cells of phloem. Arrows point to the position of phloem in the vascular bundles. Stars indicate the lacunas of the vascular bundles. Scale bars = 50 μm.

Table 2
Compositional analysis of corn stover (CS) (% of total mass) incubated with and without *N. gaditana* CCMP526 (NG526). Values in percent of sample mass. Half width of 95% confidence interval in parentheses. Yield refers to the sum of ash, lignin, and total carbohydrate.

	Ash	Klason Lignin	Arabinan	Galactan	Glucan	Xylan	Mannan	Tot Carb	Yield
CS	0.9 (0.9)	13.4 (1.8)	3.2 (0.8)	0.6 (1.2)	33.1 (3.4)	18.6 (1.9)	0	55.4 (4.4)	69.8(7.0)
NG526 + CS	0.6 (1.1)	14.1 (1.1)	3.5 (1.0)	0	33.6 (2.1)	18.5 (1.9)	0	55.7(4.7)	70.4(5.5)

Table 3
Average (Avg) micrograms of sugar in supernatant of *N. gaditana* CCMP526 (NG526) alone, corn stover (CS) alone, the sum of NG526 alone + CS alone, NG526 and CS, and 95% confidence intervals (95% C.I.). *P* values represent the difference between NG526 alone + CS alone vs. NG526 and CS incubated together.

	Arabinan		Galactan		Glucan		Xylan		Mannan	
	Avg	95% C.I.	Avg	95% C.I.	Avg	95% C.I.	Avg	95% C.I.	Avg	95% C.I.
NG526 alone	0.0	N/A	54.6	18.6	49.1	37.3	9.0	8.6	16.0	11.4
CS alone	30.9	19.9	55.3	43.8	109.5	30.1	59.2	30.1	0.0	N/A
Sum	31.0	19.9	110.0	37.5	159.0	31.4	68.0	27.4	16.0	11.4
NG526 + CS	29.5	16.9	100.9	64.7	78.5	11.5	44.0	18.3	20.2	43.8
p value	0.797		0.656		0.002		0.051		0.716	

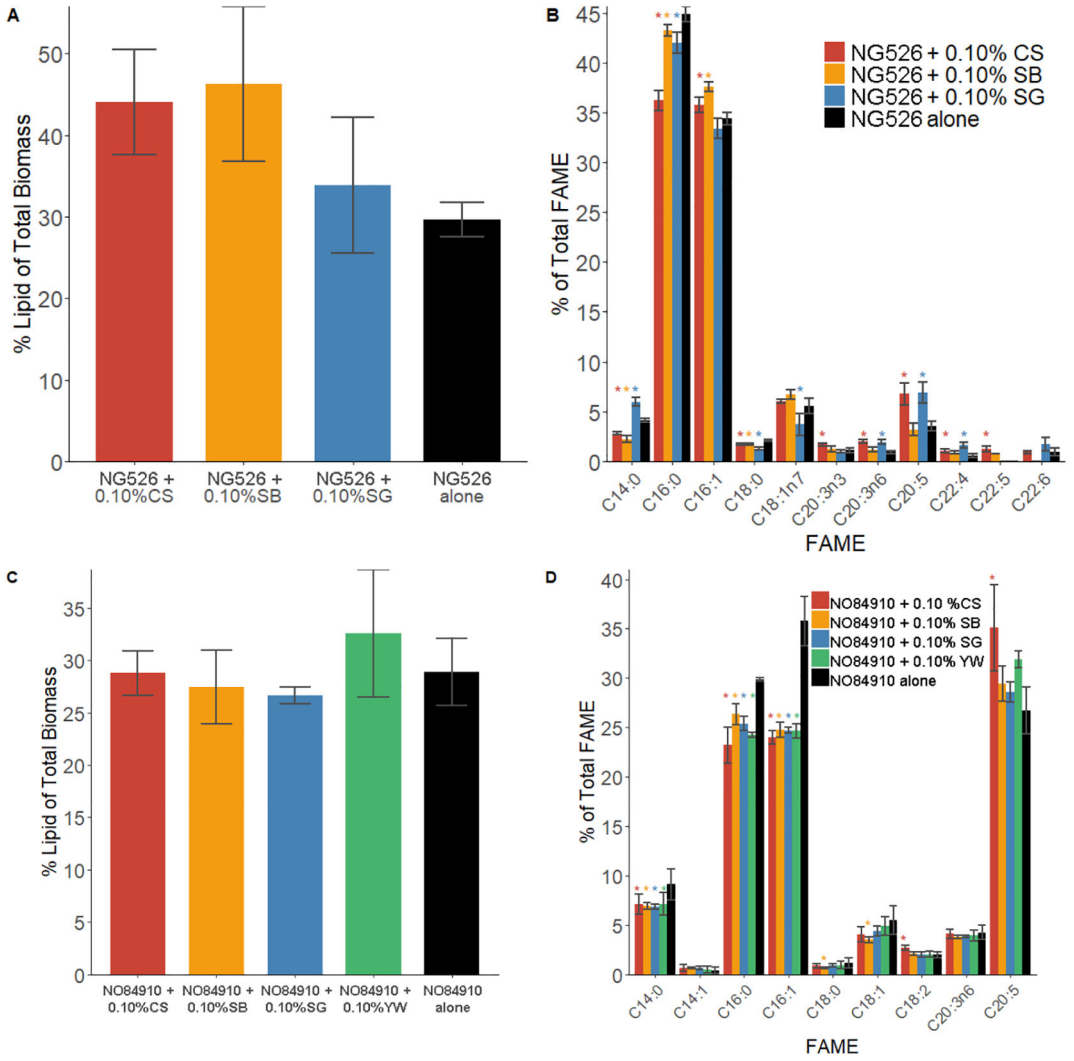

Fig. 7. Total lipids as fatty acid methyl esters (FAME) and FAME profiles of *Nannochloropsis* spp., with and without plant substrates, on the final day of cultivation in flasks. **(A)**Total lipids as FAME for *N. gaditana* CCMP526 (NG526) with and without CS, SB, SG; **(B)** FAME profile for *N. gaditana* CCMP526 with and without corn stover (CS), sugarcane bagasse (SB), switchgrass (SG); **(C)** Total lipids as for *N. oceanica* CCAP849/10 (NO84910) with and without CS, SB, SG, and yard waste (YW); **(D)** FAME profile for *N. oceanica* CCAP849/10 with and without CS, SB, SG, YW. Error bars indicate standard deviation of the mean for three biological replicates. Total lipids as FAME are represented as a percentage of the total biomass. Only individual FAMEs with values of 1% or greater of the total FAME content for any one treatment were included. Colored asterisks indicate the corresponding microalgae and plant substrate is significantly different from the microalgae alone ($p < .05$).

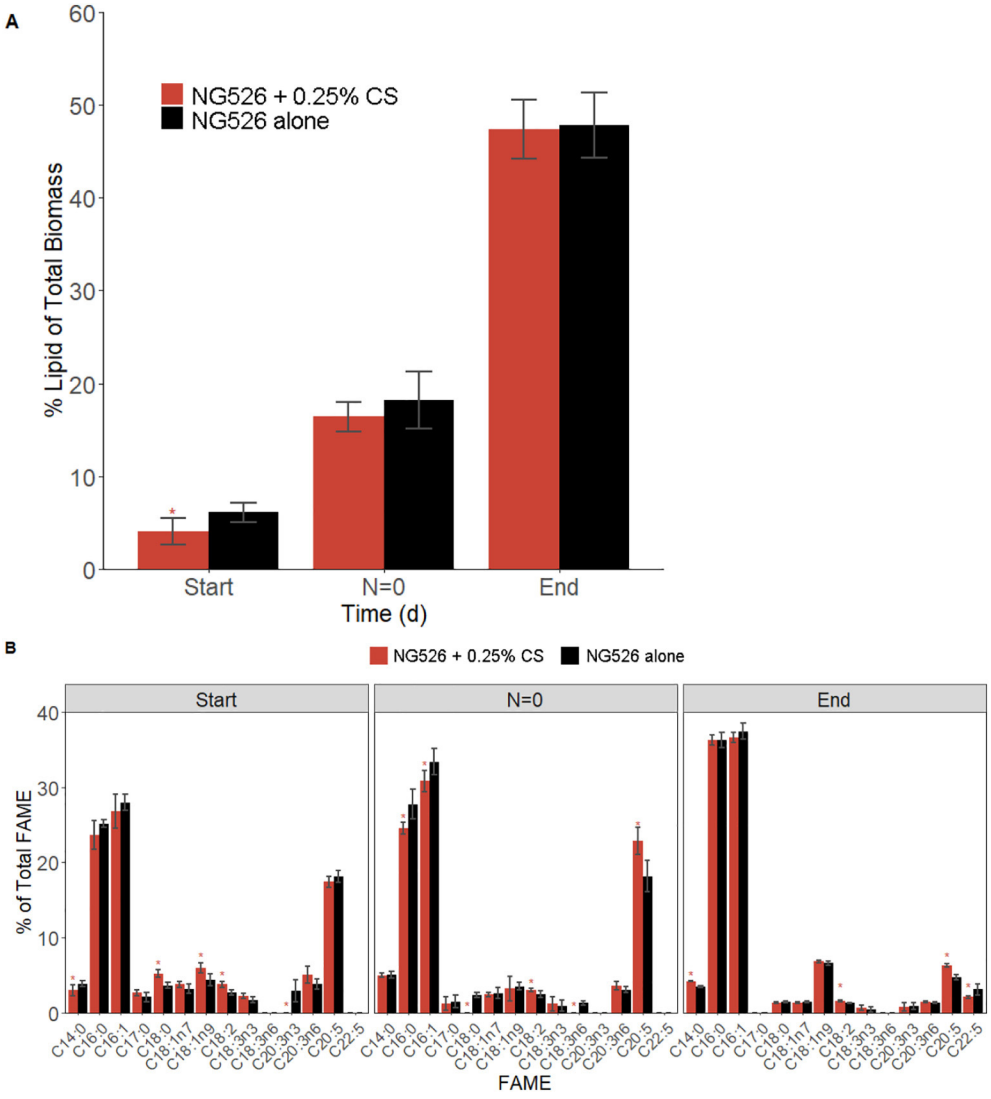

Fig. 8. Total lipids as fatty acid methyl esters (FAME) and FAME profile of *N. gaditana* CCMP526 (NG526), with and without 0.25% (*w/v*) corn stover (CS), at 50 L volume in raceway ponds, at the start ($T = 0$), when nitrate (N) concentration reached 0 mg/L (N = 0; $T = 5$ for algae alone, $T = 6$ for algae and CS), and at the end ($T = 10$ for algae alone, $T = 11$ for algae and CS). **(A)** Total lipids as FAME; **(B)** FAME profile. Error bars indicate standard deviation of the mean for three biological replicates. Only individual FAMEs with values of 1% or greater of the total FAME content for any one treatment were included. Asterisks indicate algae and CS is significantly different from the algae alone ($p < .05$).

channeled into lipid biosynthesis resulting in the observed increase in lipid content in the presence of plant substrate; however, this remains to be investigated with plant carbon substrate. Furthermore, the increased light attenuation experienced in cultures with plant substrate may also trigger lipid biosynthesis as a stress response [18]. However, saturation irradiance (I_k) has been shown to be lower in mixotrophic versus photoautotrophic cultures of *Nannochloropsis* sp., indicating that cells are less light-dependent [16].

We expected an increase in total lipid content, similar to that seen in *N. gaditana*, when plant substrate was present in *N. oceanica* cultures, although this was not the case. This could suggest that increased lipid accumulation in response to the plant substrate is not common across different *Nannochloropsis* spp. Other mixotrophic growth studies with *N. oceanica* are contradictory in regard to the resulting lipid content. No significant difference was found in total lipid content when *N. oceanica* KMMCC-359 was cultured with 2% glucose in the medium [31]. Another study found that as the glucose concentration in the media increased, the lipid content also increased, reaching a peak of 43% of the dry cell weight with 25 g L^{-1} glucose supplementation [22]. However, it could be that under the conditions tested here, and at the point in which we harvested the cells from the culture, excess carbon is more readily stored as carbohydrates in *N. oceanica* [50]. Future investigations of the *Nannochloropsis* transcriptome hope to provide insights on potential differences in carbon partitioning in the presence of plant substrate.

Similar to a previous study of *N. gaditana*, we observed a high abundance of palmitic (16:0) and palmitoleic acid (16:1), as well as minor amounts of myristic (14:0) and oleic acid (18:1) [10] (Fig. 7B). *N. oceanica* had a similar fatty acid composition to *N. gaditana*, in addition to minor amounts of linoleic (18:2) and dihomo-gamma-linolenic acid (C20:3n6) (Fig. 7D). This is in agreement with a previous study of *N. oceanica* [22]. Notably, when CS was present in the culture with either strain, the EPA (C20:5) content significantly increased compared to the cultures of microalgae alone ($p = .00043$, $p = .023$ for *N. gaditana,* and *N. oceanica,* respectively). The increased biomass yield together with the greater EPA content in the presence of plant substrate resulted in a greater overall yield of EPA, a valuable product that could aid in improving the economics surrounding biofuel production from *Nannochloropsis* sp.

When scaled up to 50 L in the raceway ponds, the average total lipids for *N. gaditana* cultures at harvest was 47% of the total biomass, irrespective of plant substrate presence (Fig. 8A). When compared to the total lipids in the flask experiment (Fig. 7A), there was only a slight increase in the total lipid content of *N. gaditana* when CS was present, but a 1.6-fold increase in total lipids for the microalgae alone. This could suggest that the CO_2 supplementation or increased irradiance in the ponds could have more of a substantial effect on lipid accumulation than the presence of plant substrate. Others have reported an increase in lipid content when cultures were grown in excess CO_2 [51]. We also saw no difference in the end point average total lipids for *N. oceanica,*

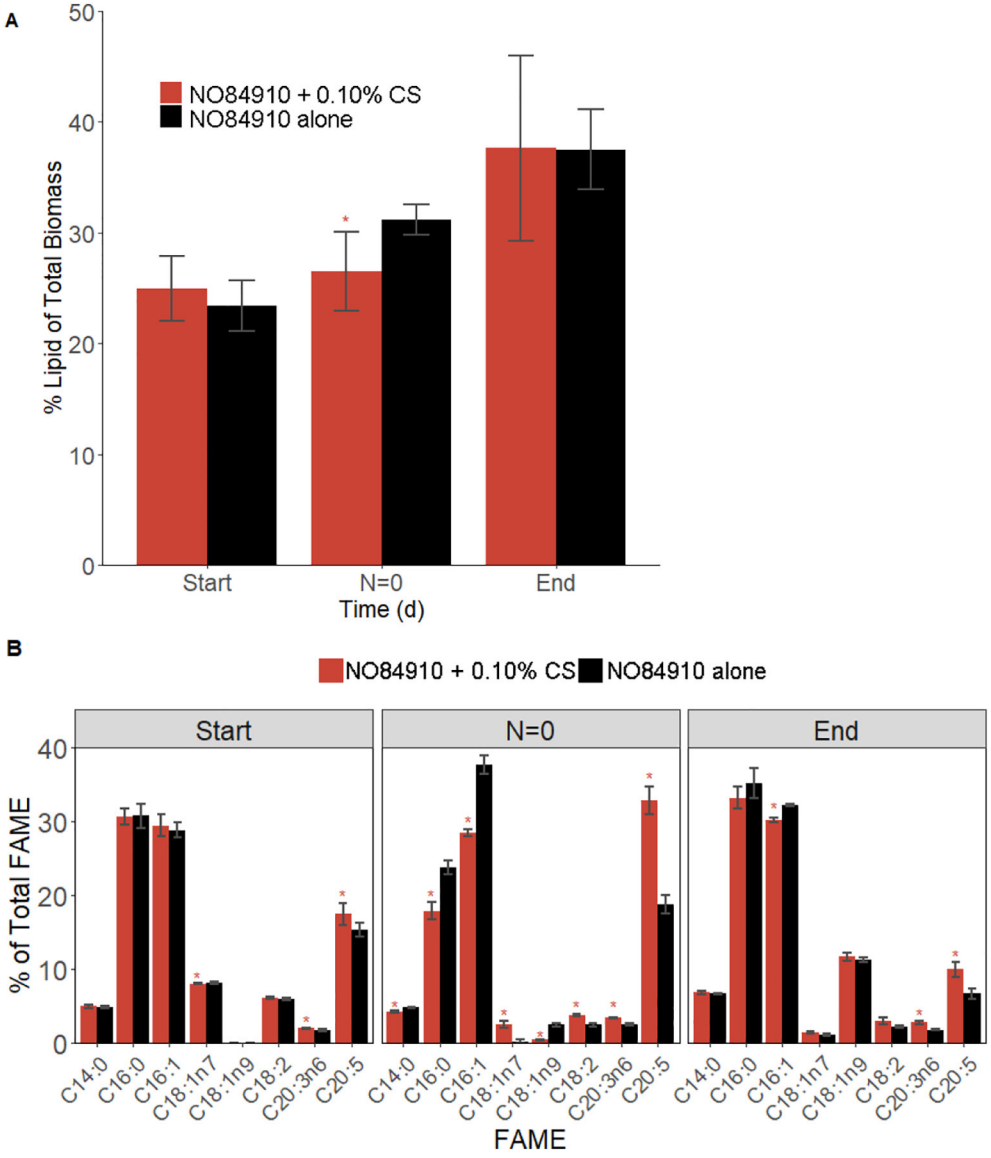

Fig. 9. Total lipids as fatty acid methyl esters (FAME) and FAME profile of *N. oceanica* CCAP849/10 (NO84910), with and without 0.10% (*w/v*) corn stover (CS), at 50 L volume in raceway ponds, at the start (T = 0), when nitrate (N) concentration reached 0 mg/L (N = 0; T = 5 for algae alone, *T* = 7 for algae and CS), and at the end (T = 11). **(A)** Total lipids as FAME; **(B)** FAME profile. Error bars indicate standard deviation of the mean for three biological replicates. Only individual FAMEs with values of 1% or greater of the total FAME content for any one treatment were included. Asterisks indicate algae and CS is significantly different from the algae alone ($p < .05$).

with and without CS, at 37% of the total biomass (Fig. 9A).

While we did not see the expected increase in the total lipid content in the presence of plant substrate in the ponds, EPA content in cultures with CS were 1.2 and 1.8-fold greater than controls at $N = 0$ for *N. gaditana* ($p = .0020$) and *N. oceanica* ($p = .0043$), respectively (Figs. 8B and 9B). Others have observed an increase in EPA content under N-replete conditions in *Nannochloropsis* spp. [52,53]. The presence of plant substrate and the subsequent delay of N starvation could actually promote EPA accumulation. The higher EPA content in cultures grown with CS could explain the vibrant green color, as there may be an association between Chlorophyll *a* and EPA production [54].

In a commercial setting, particularly one surrounding high-value products such as EPA, consistency of the algal biomass composition is a main concern. Advantages of semi-continuous and continuous culturing methods, as opposed to batch culturing seen here, are that the algal cells remain in exponential balanced growth and, in theory, give us more control over the physiological state of the microalgae [4,5,19,55]. This would lead to greater consistency in the biomass composition and potentially yield higher biomass production over time than in batch cultures [55]. Growing *N. gaditana* or *N. oceanica* mixotrophically with a plant substrate like CS, in a semi-continuous or continuous manner, could yield consistently high EPA content without sacrificing growth rate.

4. Conclusions

Here, we show improved biomass accumulation (Figs. 1–3) and EPA production (Figs. 7, 8) in *N. gaditana* and *N. oceanica* in the presence of plant substrate, particularly CS, at flask and mini raceway pond scale. These improvements, together with the physical association (Fig. S4), signs of CS degradations (Figs. 5, 6), and changes in sugar content (Table 3), when CS is in culture with the microalgae, suggest plant carbon utilization. While *Nannochloropsis* sp. have been used for large-scale production of products, biofuel production with this genus has stalled due to its phototrophic growth limitation. This study illustrates the growth potential for *Nannochloropsis* spp. when grown with raw plant substrates. The presence of this trait in both freshwater [20] and marine microalgae indicates that the utilization of hemicellulose or other plant nutrients may be more widespread in microalgae than originally thought, and may be a viable growth regime for increasing biomass and lipid-based products, including biofuels, in the future.

CRediT authorship contribution statement

J.Y.S. designed and contributed to all experiments, A.M.F. contributed to Congo Red staining, glycosyl hydrolase mRNA analysis, FAME analysis, and growth experiments of all microalgae with plant substrates, E.R.H. completed all bacterial and chloroplast sequencing and analysis with oversight by S.R.S., B.W.V. contributed to initial growth experiments of *N. gaditana* with plant substrates and primer design, C.H. and P.K. contributed SEM and sugar analyses of plant substrates with and without microalgae, A.N.B. managed the overall project, and conceived and designed experiments. All authors contributed to data analysis and to the writing of the article. All authors declare agreement to authorship and submission of the manuscript for peer review.

Declaration of competing interest

The authors declare no potential financial or other interests that could be perceived to influence the outcomes of the research. No conflicts, informed consent, human or animal rights applicable.

Acknowledgements

This work was supported under the Leveraging Algae Traits for Fuels (LEAF) project, awarded to A.N.B. and the Algal Translational Genomics project awarded to S.R.S. by the Department of Energy's Bioenergy Technologies Office, Office of Energy Efficiency and Renewable Energy, under agreement numbers NL0035866 (WBS 1.3.2.042) and NL0029949 (WBS 1.3.1.600), respectively. This work has been cleared for public release by Los Alamos National Laboratory (LA-UR-20-23298) and Sandia National Laboratories (SAND2020-7729 J).

Thank you to the INL Feedstock Library for milled plant substrates and to Mike Stanek at US Dairy Forage Research Farm, Prairie du Sac, WI, for the corn used to make uniformly cut sections. Thank you to Matthew Posewitz, Colorado School of Mines, Golden, CO, for *N. gaditana* and to Scott Edmundson at PNNL for *N. oceanica.* Thank you to Fred Matt and Steve Verril, Forest Products Laboratory, for the sugar analyses and statistics. Thank you to Qualitas Health for providing us with industrial-relevant water for the *N. oceanica* mini raceway pond experiments. Thank you to the Applied Genomics Team at Los Alamos National Laboratory for sequencing support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2020.102041.

References

[1] X.N. Ma, T.P. Chen, B. Yang, J. Liu, F. Chen, Lipid production from Nannochloropsis, Mar. Drugs. 14 (2016), https://doi.org/10.3390/md14040061.

[2] N. Moazami, A. Ashori, R. Ranjbar, M. Tangestani, R. Eghtesadi, A.S. Nejad, Large-scale biodiesel production using microalgae biomass of Nannochloropsis, Biomass Bioenergy 39 (2012) 449–453, https://doi.org/10.1016/j.biombioe.2012.01.046.

[3] J.C. Quinn, T. Yates, N. Douglas, K. Weyer, J. Butler, T.H. Bradley, P.J. Lammers, Nannochloropsis production metrics in a scalable outdoor photobioreactor for commercial applications, Bioresour. Technol. 117 (2012) 164–171, https://doi.org/10.1016/j.biortech.2012.04.073.

[4] A. San Pedro, C.V. González-López, F.G. Acién, E. Molina-Grima, Outdoor pilot production of nannochloropsis gaditana: influence of culture parameters and lipid production rates in raceway ponds, Algal Res. 8 (2015) 205–213, https://doi.org/10.1016/j.algal.2015.02.013.

[5] A. San Pedro, C.V. González-López, F.G. Acién, E. Molina-Grima, Outdoor pilot production of Nannochloropsis gaditana: influence of culture parameters and lipid production rates in flat-panel photobioreactors, Algal Res. 18 (2016) 156–165, https://doi.org/10.1016/j.algal.2016.06.011.

[6] A.R. López, S.B. Rodríguez, R.A. Vallejo, P.G. García, M.D. Macías-Sánchez, M.G. Díaz, R.G. Librán, F.J.F. Acero, Sustainable cultivation of Nannochloropsis gaditana microalgae in outdoor raceways using flue gases for a complete 2-year cycle: a circular economy challenge, J. Appl. Phycol. 31 (2019) 1515–1523, https://doi.org/10.1007/s10811-018-1710-0.

[7] R.E. Jinkerson, R. Radakovits, M.C. Posewitz, Genomic insights from the oleaginous model alga Nannochloropsis gaditana, Bioengineered 4 (2013), https://doi.org/10.4161/bioe.21880.

[8] I. Ajjawi, J. Verruto, M. Aqui, L.B. Soriaga, J. Coppersmith, K. Kwok, L. Peach, E. Orchard, R. Kalb, W. Xu, T.J. Carlson, K. Francis, K. Konigsfeld, J. Bartalis, A. Schultz, W. Lambert, A.S. Schwartz, R. Brown, E.R. Moellering, Lipid production in Nannochloropsis gaditana is doubled by decreasing expression of a single transcriptional regulator, Nat. Biotechnol. 35 (2017) 647–652, https://doi.org/10.1038/nbt.3865.

[9] Z.T. Wang, N. Ullrich, S. Joo, S. Waffenschmidt, U. Goodenough, Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless chlamydomonas reinhardtit, Eukaryot. Cell 8 (2009) 1856–1868, https://doi.org/10.1128/EC.00272-09.

[10] R. Radakovits, R.E. Jinkerson, S.I. Fuerstenberg, H. Tae, R.E. Settlage, J.L. Boore, M.C. Posewitz, Draft genome sequence and genetic transformation of the oleaginous alga Nannochloropis gaditana, Nat. Commun. 3 (2012) 610–686, https://doi.org/10.1038/ncomms1688.

[11] A.S. Schwartz, R. Brown, I. Ajjawi, J. McCarren, S. Atilla, N. Bauman, T.H. Richardson, Complete genome sequence of the model oleaginous alga nannochloropsis gaditana CCMP1894, Genome Announc. 6 (2018) 1–2, https://doi.org/10.1128/genomeA.01448-17.

[12] S.R. Starkenburg, K.J. Kwon, R.K. Jha, C. McKay, M. Jacobs, O. Chertkov, S. Twary, G. Rocap, R.A. Cattolico, A pangenomic analysis of the Nannochloropsis organellar genomes reveals novel genetic variations in key metabolic genes, BMC Genomics 15 (2014) 1–21, https://doi.org/10.1186/1471-2164-15-212.

[13] J. Verruto, K. Francis, Y. Wang, M.C. Low, J. Greiner, S. Tacke, F. Kuzminov, W. Lambert, J. McCarren, I. Ajjawi, N. Bauman, R. Kalb, G. Hannum, E.R. Moellering, Unrestrained markerless trait stacking in Nannochloropsis gaditana through combined genome editing and marker recycling technologies, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) E7015–E7022, https://doi.org/10.1073/pnas.1718193115.

[14] M. Ren, K. Ogden, B. Lian, Effect of culture conditions on the growth rate and lipid production of microalgae Nannochloropsis gaditana, J. Renew. Sustain. Energy. 5 (2013), https://doi.org/10.1063/1.4857375.

[15] Â.P. Matos, M.G. Cavanholi, E.H.S. Moecke, E.S. Sant'Anna, Effects of different photoperiod and trophic conditions on biomass, protein and lipid production by the marine alga Nannochloropsis gaditana at optimal concentration of desalination concentrate, Bioresour. Technol. 224 (2017) 490–497, https://doi.org/10.1016/j.biortech.2016.11.004.

[16] X. Fang, C. Wei, C. Zhao-Ling, O. Fan, Effects of organic carbon sources on cell growth and eicosapentaenoic acid content of Nannochloropsis sp, J. Appl. Phycol. 16 (2004) 499–503, https://doi.org/10.1007/s10811-004-5520-1.

[17] F. Pagnanelli, P. Altimari, F. Trabucco, L. Toro, Mixotrophic growth of Chlorella vulgaris and Nannochloropsis oculata: interaction between glucose and nitrate, J. Chem. Technol. Biotechnol. 89 (2014) 652–661, https://doi.org/10.1002/jctb.4179.

[18] B. Cheirsilp, S. Torpee, Enhanced growth and lipid production of microalgae under mixotrophic culture condition: effect of light intensity, glucose concentration and fed-batch cultivation, Bioresour. Technol. 110 (2012) 510–516, https://doi.org/10.1016/j.biortech.2012.01.125.

[19] T. Menegol, G.I. Romero-Villegas, M. López-Rodríguez, E. Navarro-López, L. López-Rosales, Y. Chisti, M.C. Cerón-García, E. Molina-Grima, Mixotrophic production of polyunsaturated fatty acids and carotenoids by the microalga Nannochloropsis gaditana, J. Appl. Phycol. 31 (2019) 2823–2832, https://doi.org/10.1007/s10811-019-01828-3.

[20] B.W. Vogler, S.R. Starkenburg, N. Sudasinghe, J.Y. Schambach, J.A. Rollin, S. Pattathil, A.N. Barry, Characterization of plant carbon substrate utilization by Auxenochlorella protothecoides, Algal Res. 34 (2018) 37–48, https://doi.org/10.1016/j.algal.2018.07.001.

[21] M.J. Scholz, T.L. Weiss, R.E. Jinkerson, J. Jing, R. Roth, U. Goodenough, M.C. Posewitz, H.G. Gerken, Ultrastructure and composition of the Nannochloropsis gaditana cell wall, Eukaryot. Cell 13 (2014) 1450–1464, https://doi.org/10.1128/EC.00183-14.

[22] M. Mitra, S. Mishra, Effect of glucose on growth and fatty acid composition of an euryhaline eustigmatophyte Nannochloropsis oceanica under mixotrophic culture condition, Bioresour. Technol. Reports. 3 (2018) 147–153, https://doi.org/10.1016/j.biteb.2018.07.013.

[23] S. Van Wychen, K. Ramirez, L.M.L. Laurens, S. Van Wychen, K. Ramirez, Determination of Total Lipids as Fatty Acid Methyl Esters (FAME) by In Situ Transesterification Determination of Total Lipids as Fatty Acid Methyl Esters (FAME by In Situ Transesterification Laboratory Analytical Procedure (LAP), (2015).

[24] J.G. Caporaso, C.L. Lauber, W.A. Walters, D. Berg-Lyons, J. Huntley, N. Fierer, S.M. Owens, J. Betley, L. Fraser, M. Bauer, N. Gormley, J.A. Gilbert, G. Smith, R. Knight, Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms, ISME J 6 (2012) 1621–1624, https://doi.org/10.1038/ismej.2012.8.

[25] J.G. Caporaso, C.L. Lauber, W.A. Walters, D. Berg-Lyons, C.A. Lozupone, P.J. Turnbaugh, N. Fierer, R. Knight, Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 4516–4522, https://doi.org/10.1073/pnas.1000080107.

[26] P.E. Li, C.C. Lo, J.J. Anderson, K.W. Davenport, K.A. Bishop-Lilly, Y. Xu, S. Ahmed, S. Feng, V.P. Mokashi, P.S.G. Chain, Enabling the democratization of the genomics revolution with a fully integrated web-based bioinformatics platform, Nucleic Acids Res. 45 (2017) 67–80, https://doi.org/10.1093/nar/gkw1027.

[27] J.G. Caporaso, J. Kuczynski, J. Stombaugh, K. Bittinger, F.D. Bushman, E.K. Costello, N. Fierer, A.G. Peña, J.K. Goodrich, J.I. Gordon, G.A. Huttley, S.T. Kelley, D. Knights, J.E. Koenig, R.E. Ley, C.A. Lozupone, D. Mcdonald, B.D. Muegge, M. Pirrung, J. Reeder, J.R. Sevinsky, P.J. Turnbaugh, W.A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, R. Knight, Correspondence QIIME allows analysis of high- throughput community sequencing data intensity normalization improves color calling in SOLiD sequencing, Nat. Publ. Gr 7 (2010) 335–336, https://doi.org/10.1038/nmeth0510-335.

[28] T.Z. DeSantis, P. Hugenholtz, N. Larsen, M. Rojas, E.L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, G.L. Andersen, Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB, Appl. Environ. Microbiol. 72 (2006) 5069–5072, https://doi.org/10.1128/AEM.03006-05.

[29] M.W. Davis, A rapid modified method for compositional carbohydrate analysis of lignocellulosics by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD), J. Wood Chem. Technol. 18 (1998) 235–252, https://doi.org/10.1080/02773819809349579.

[30] Y. Li, A. Huang, W. Gu, S. Wu, X. Xie, G. Wang, Effects of inorganic carbon and light on acetate assimilation by Nannochloropsis oceanica (Eustigmatophyceae) in mixotrophic cultivation, Eur. J. Phycol. 55 (2020) 64–75, https://doi.org/10.1080/09670262.2019.1660808.

[31] M.J. Jo, S.B. Hur, Growth and nutritional composition of eustigmatophyceae Monodus subterraneus and Nannochloropsis oceanica in autotrophic and mixotrophic culture, Ocean Polar Res 37 (2015) 61–71, https://doi.org/10.4217/OPR.2015.37.1.061.

[32] H.-W. Heldt, B. Piechulla, Phenylpropanoids comprise a multitude of plant secondary metabolites and cell wall components, in: H.-W. Heldt, B. Piechulla (Eds.), Plant Biochem, Fourth Ed., Fourth, Academic Press, 2011, pp. 431–449, , https://doi.org/10.1016/B978-0-12-384986-1.00018-1.

[33] N. Poddar, R. Sen, G.J.O. Martin, Glycerol and nitrate utilisation by marine microalgae Nannochloropsis salina and Chlorella sp. and associated bacteria during mixotrophic and heterotrophic growth, Algal Res. 33 (2018) 298–309, https://doi.org/10.1016/j.algal.2018.06.002.

[34] R. Vazhappilly, F. Chen, Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth, JAOCS, J. Am. Oil Chem. Soc. 75 (1998) 393–397, https://doi.org/10.1007/s11746-998-0057-0.

[35] B.N. Tripathi, D. Kumar, B. Nath, T. Dhananjay Kumar, Prospects and challenges in algal biotechnology (2017), https://doi.org/10.1007/978-981-10-1950-0.

[36] A.R. Shah, A. Ahmad, S. Srivastava, B.M. Jaffar Ali, Reconstruction and analysis of a genome-scale metabolic model of Nannochloropsis gaditana, Algal Res. 26 (2017) 354–364, https://doi.org/10.1016/j.algal.2017.08.014.

[37] B. Zhu, F. Sun, M. Yang, L. Lu, G. Yang, K. Pan, Large-scale biodiesel production using flue gas from coal-fired power plants with Nannochloropsis microalgal biomass in open raceway ponds, Bioresour. Technol. 174 (2014) 53–59, https://doi.org/10.1016/j.biortech.2014.09.116.

[38] J.E. Sawyer, A.P. Mallarino, Nutrient considerations with corn silage and Stover harvest, (2018), pp. 1–3, https://doi.org/10.31274/icm-180809-101.

[39] D.L. Karlen, J.L. Kovar, S.J. Birrell, Corn Stover nutrient removal estimates for Central Iowa, USA, Sustain 7 (2015) 8621–8634, https://doi.org/10.3390/su7078621.

[40] B.H. Kim, R. Ramanan, D.H. Cho, H.M. Oh, H.S. Kim, Role of rhizobium, a plant growth promoting bacterium, in enhancing algal biomass through mutualistic interaction, Biomass Bioenergy 69 (2014) 95–105, https://doi.org/10.1016/j.biombioe.2014.07.015.

[41] J. Lian, R.H. Wijffels, H. Smidt, D. Sipkema, The effect of the algal microbiome on industrial production of microalgae, Microb. Biotechnol. 11 (2018) 806–818, https://doi.org/10.1111/1751-7915.13296.

[42] Z.Y. Du, J. Alvaro, B. Hyden, K. Zienkiewicz, N. Benning, A. Zienkiewicz, G. Bonito, C. Benning, Enhancing oil production and harvest by combining the marine alga Nannochloropsis oceanica and the oleaginous fungus Mortierella elongata, Biotechnol. Biofuels. 11 (2018) 1–16, https://doi.org/10.1186/s13068-018-1172-2.

[43] Z.Y. Du, K. Zienkiewicz, N. Vande Pol, N.E. Ostrom, C. Benning, G.M. Bonito, Algal-fungal symbiosis leads to photosynthetic mycelium, Elife. 8 (2019) 1–22, https://doi.org/10.7554/eLife.47815.

[44] S.R. Decker, J. Sheehan, D.C. Dayton, J.J. Bozell, W.S. Adney, B. Hames, S.R. Thomas, R.L. Bain, S. Czernik, M. Zhang, M.E. Himmel, Biomass conversion, Handb. Ind. Chem. Biotechnol. Twelfth Ed. 2–2 (2012) 1249–1322, https://doi.org/10.1007/978-1-4614-4259-2_33.

[45] D.W. Templeton, A.D. Sluiter, T.K. Hayward, B.R. Hames, S.R. Thomas, Assessing corn Stover composition and sources of variability via NIRS, Cellulose 16 (2009) 621–639, https://doi.org/10.1007/s10570-009-9325-x.

[46] B.W. Vogler, J. Brannum, J.W. Chung, M. Seger, M.C. Posewitz, Characterization of the Nannochloropsis gaditana storage carbohydrate: a 1,3-beta glucan with limited 1,6-branching, Algal Res. 36 (2018) 152–158, https://doi.org/10.1016/j.algal.2018.10.011.

[47] S.M. Cragg, G.T. Beckham, N.C. Bruce, T.D.H. Bugg, D.L. Distel, P. Dupree, A.G. Etxabe, B.S. Goodell, J. Jellison, J.E. McGeehan, S.J. McQueen-Mason, K. Schnorr, P.H. Walton, J.E.M. Watts, M. Zimmer, Lignocellulose degradation mechanisms across the Tree of Life, Curr. Opin. Chem. Biol. 29 (2015) 108–119, https://doi.org/10.1016/j.cbpa.2015.10.018.

[48] M. Cecchin, S. Benfatto, F. Griggio, A. Mori, S. Cazzaniga, N. Vitulo, M. Delledonne, M. Ballottari, Molecular basis of autotrophic vs mixotrophic growth in Chlorella sorokiniana, Sci. Rep. 8 (2018), https://doi.org/10.1038/s41598-018-24979-8.

[49] C. Combres, G. Laliberté, J.S. Reyssac, J. de la Noüe, Effect of acetate on growth and ammonium uptake in the microalga Scenedesmus obliquus, Physiol. Plant. 91 (1994) 729–734, https://doi.org/10.1111/j.1399-3054.1994.tb03012.x.

[50] J. Jia, D. Han, H.G. Gerken, Y. Li, M. Sommerfeld, Q. Hu, J. Xu, Molecular mechanisms for photosynthetic carbon partitioning into storage neutral lipids in Nannochloropsis oceanica under nitrogen-depletion conditions, Algal Res. 7 (2015) 66–77, https://doi.org/10.1016/j.algal.2014.11.005.

[51] S.Y. Chiu, C.Y. Kao, M.T. Tsai, S.C. Ong, C.H. Chen, C.S. Lin, Lipid accumulation and CO2 utilization of Nannochloropsis oculata in response to CO2 aeration, Bioresour. Technol. 100 (2009) 833–838, https://doi.org/10.1016/j.biortech.2008.06.061.

[52] Y. Meng, J. Jiang, H. Wang, X. Cao, S. Xue, Q. Yang, W. Wang, The characteristics of TAG and EPA accumulation in Nannochloropsis oceanica IMET1 under different nitrogen supply regimes, Bioresour. Technol. 179 (2015) 483–489, https://doi.org/10.1016/j.biortech.2014.12.012.

[53] H. Hu, K. Gao, Response of growth and fatty acid compositions of Nannochloropsis sp. to environmental factors under elevated CO2 concentration, Biotechnol. Lett. 28 (2006) 987–992, https://doi.org/10.1007/s10529-006-9026-6.

[54] F. Guihéneuf, V. Mimouni, L. Ulmann, G. Tremblin, Combined effects of irradiance level and carbon source on fatty acid and lipid class composition in the microalga Pavlova lutheri commonly used in mariculture, J. Exp. Mar. Bio. Ecol. 369 (2009) 136–143, https://doi.org/10.1016/j.jembe.2008.11.009.

[55] W.J. Henley, The past, present and future of algal continuous cultures in basic research and commercial applications, Algal Res. 43 (2019) 101636, , https://doi.org/10.1016/j.algal.2019.101636.