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Authors: Logan H. Hodgkiss, J. Nagy, Elliott P. Barnhart, Alfred B. Cunningham, & Matthew W. Fields

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# Cultivation of a native alga for biomass and biofuel accumulation in coal bed methane production water

L.H. Hodgskiss <sup>a,b</sup>, J. Nagy <sup>c</sup>, E.P. Barnhart <sup>d,b</sup>, A.B. Cunningham <sup>a,b,e</sup>, M.W. Fields <sup>b,c,e,\*</sup>

<sup>a</sup> Department of Civil Engineering, Montana State University, Bozeman, MT 59717, United States

<sup>b</sup> Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717, United States

<sup>c</sup> Department of Microbiology and Immunology, Montana State University, Bozeman, MT 59717, United States

<sup>d</sup> U.S. Geological Survey, Wyoming-Montana Water Science Center, Helena, MT, United States

<sup>e</sup> Energy Research Institute, Montana State University, Bozeman, MT 59717, United States

**Keywords:** Coal bed methane (CBM) production has resulted in thousands of ponds in the Powder River Basin of low-quality water in a water-challenged region. A green alga isolate, PW95, was isolated from a CBM production pond, and analysis of a partial ribosomal gene sequence indicated the isolate belongs to the Chlorococcaceae family. Different combinations of macro- and micronutrients were evaluated for PW95 growth in CBM water compared to a defined medium. A small level of growth was observed in unamended CBM water (0.15 g/l), and biomass increased (2-fold) in amended CBM water or defined growth medium. The highest growth rate was observed in CBM water amended with both N and P, and the unamended CBM water displayed the lowest growth rate. The highest lipid content (27%) was observed in CBM water with nitrate, and a significant level of lipid accumulation was not observed in the defined growth medium. Growth analysis indicated that nitrate deprivation coincided with lipid accumulation in CBM production water, and lipid accumulation did not increase with additional phosphorus limitation. The presented results show that CBM production wastewater can be minimally amended and used for the cultivation of a native, lipid-accumulating alga.

Different diatoms and algae can produce and accumulate a variety of value-added precursors (e.g., carbohydrates, fatty acids, and pigments) that can further contribute to reduced CO<sub>2</sub> emissions through utilization of atmospheric CO<sub>2</sub> [1]. Much of the reported algal biofuel research has focused upon exposing cultures to a range of environmental stresses prior to harvest ([1] and references therein), including nitrogen and phosphorus. In addition to carbon, nitrogen, and phosphorus, water is an inherent resource that has to be considered in the alternative energy sector. Water and nutrient availability are becoming an increasing concern in light of a rapidly increasing global population combined with decreasing reserves of fertilizer and the diminishing availability of clean water. The Committee on the Sustainable Development of Algal Biofuels (National Research Council) published a report in 2012 that indicated the large scale production of algal biofuels and bio-products would exert a significant strain on the already limited availability of nutrients and high-quality water resources. The report suggested that strategies were needed to reuse and recycle water and nutrients in future photo-synthetically-driven production facilities [2].

The volume of water used in the energy sector represented 15% of global freshwater withdrawals in 2010 [3], and coal-bed methane (CBM) production produces large amounts of water that can negatively impact the environment and the economy. According to the Wyoming Oil and Gas Conservation Commission [4], in 2014 the Powder River Basin produced 246,601,576 Mcf (Mcf = 1000 cubic feet) of gas and an associated 256,305,005 Bbls (8,073,617,107 gal) of water. Water produced by CBM extraction is typically stored in lined or unlined impoundments but can be used for agricultural purposes or discharged into streams with proper monitoring and treatment [5,6]. However, the production water can be problematic due to the high concentrations of Na<sup>+</sup> and total dissolved solids (TDS), and low concentrations of Mg<sup>2+</sup> and Ca<sup>2+</sup> observed in the majority of the produced water ([7]). Therefore, integrated approaches are crucial when developing future water, energy, and climate policy [8], particularly in regions with changing water demands.

Recently, the growth of microalgae in low-quality water sources for the purposes of remediation and/or biofuel production have been reported in the literature, and include dairy wastewater [9] and municipal wastewater [10]. A primary concern when cultivating microalgae is selecting a species that is a good fit for the selected conditions in

different parts of the country (e.g., water, light, nutrients). A recent study successfully grew a microalgal species in CBM production water in Australia in which a marine algae species was selected to accommodate the high salt content of the water [11]. However, the use of CBM production water from the Powder River Basin for this purpose has not been evaluated.

Identifying beneficial uses for low-quality production water from underground coal seams will become increasingly important as domestic energy production from subsurface reservoirs increases to meet growing energy demands. An alternative use for CBM production water could be the growth of microalgae for biodiesel and biomass production. Due to photoautotrophic growth, algae utilize solar energy for growth and  $\text{CO}_2$  ( $\text{HCO}_3^-$ ) as the carbon source, but require a low-quality water source that does not deplete already stressed water supplies. In addition, CBM production water is inherently high in bicarbonate ( $\text{HCO}_3^-$ ) and pH, conditions that foster the utilization of dissolved inorganic carbon by photoautotrophs. Therefore, the low-quality water needs for algal biofuels and the wastewater streams from CBM production present an opportunity to couple water recycling, light-driven  $\text{CO}_2$  utilization, and production of value added products. We have isolated a native green alga from a CBM production pond in northeastern Wyoming and evaluated the potential for growth in CBM production water with the ability to accumulate lipids that can be converted into biodiesel. A native species provides the benefit of an organism that is already adapted to the water and climate conditions of the region, and we have identified the key nutrients required for the species to grow photoautotrophically in CBM production water and accumulate lipids during N-deprivation.

## 2. Methods

### 2.1. Materials

The alga, PW95, was isolated from a CBM production water pond in the Powder River Basin of northeastern Wyoming (44° 52.613'N 106° 54.700'W). All chemicals were of highest purity available.

### 2.2. Culture methods

#### 2.2.1. Culture isolation

Cultures were streaked on Bold's Basal Medium (BBM) agar plates in a 20 °C incubator with a 14:10 h light:dark cycle (7872 lx, cool white fluorescent). Subsequent colonies were streaked 3 times for the isolation of a single photoautotroph. DNA sequence analyses (18S and 16S) suggested a unialgal culture with no detected bacterial sequences, and heterotrophic plates (R2A) incubated at 20 °C in the dark did not display bacterial growth. Cultures were routinely checked for heterotrophic growth.

#### 2.2.2. Culture conditions

Liquid BBM was used routinely for culture maintenance and biomass production at 20 °C. Flasks were placed on a shaker rotating at 125 rpm to encourage mass transfer of  $\text{CO}_2$ . Flask experiments were inoculated from a stock culture of PW95 grown in liquid BBM. Each inoculum was centrifuged (1750  $\times g$  for 5 min) and washed twice with sterile CBM water or BBM depending on the intended use. CBM water was supplied from well FG-09 (45° 26' 5.8914"N 106° 23' 31.416"W) in the Powder River Basin, and this water was used for all experiments. CBM production water was filter sterilized using a bottle top 0.2  $\mu\text{m}$ , polyethersulfone (PES), Nalgene Rapid-Flow vacuum filter (Thermo Scientific) in order to better understand the direct responses of the algal isolate in CBM production water. Growth experiments at 20 °C were performed with biological duplicates in 500 mL Erlenmeyer flasks containing 250 mL of sterile CBM water or Bold's Basal Medium (BBM; 0.5 mM nitrate, 0.05 mM phosphate). Five different nutrient conditions were evaluated using CBM water: CBM water without additions; CBM water with

0.5 mM nitrate; CBM water with 0.5 mM nitrate and 0.05 mM phosphate; CBM water with 0.5 mM nitrate, 0.05 mM phosphate, and 0.3 mM magnesium sulfate; and CBM water with 0.5 mM nitrate, 0.05 mM phosphate, 0.3 mM magnesium sulfate, and micronutrients (i.e., trace vitamins and minerals) used in BBM [12]. All flasks were inoculated with 15 mL of inoculum from the same stock culture.

### 2.3. Analysis methods

#### 2.3.1. Determination of biomass dry weight

Nitrocellulose or acetate cellulose 0.2  $\mu\text{m}$  filters (25 mm diameter) were used to periodically determine the dry weight biomass of the cultures. Culture samples were vacuum filtered and rinsed with water to remove excess salts before air drying in an oven set at approximately 90 °C for 48 h. Filters were weighed before and after use to determine dry weight biomass.

#### 2.3.2. Determination of chlorophyll

Culture samples (1 mL) were centrifuged (16,162  $\times g$  for 15 min) in microcentrifuge tubes and 950  $\mu\text{L}$  of supernatant was frozen at  $-80^\circ\text{C}$  for water chemistry analysis. The cell pellet was submerged in 1 mL of 100% methanol, disrupted by sonication and vortexing, and stored at 4 °C for at least 24 h in a covered box. After 24 h, the samples were centrifuged (16,162  $\times g$  for 5 min). The supernatant was extracted and analyzed in disposable plastic cuvettes using a Shimadzu UV-1700 UV-VIS spectrophotometer at wavelengths of 665.2 nm, 652 nm, and 632 nm. Chlorophyll concentrations in  $\mu\text{g/mL}$  were estimated as previously described by Ritchie [13].

#### 2.3.3. Determination of lipid content

Lipid accumulation was tracked using a Nile Red staining method [14]. Culture samples (1 mL) were stained with Nile Red stock solution (4  $\mu\text{L}$  of 0.25 mg Nile Red/mL of acetone). Samples were stored in the dark for 5 min. The set time of 5 min was determined by performing a time assay with PW95 to determine the optimum time to measure Nile Red fluorescence as described by Chen et al. [15]. After 5 min, 200  $\mu\text{L}$  duplicates were pipetted into a 96 well plate and fluorescence was read using a Synergy H1 hybrid fluorometer/spectrophotometer reader. Gen5 microplate reader software was used to evaluate the fluorescence at an excitation of 480 nm and emission of 580 nm. Samples were diluted 2, 4, or 10 times as necessary to attempt to keep Nile Red measurements within a linear range [15].

#### 2.3.4. Determination of anion concentrations

Nitrate and phosphate concentrations were determined with a DIONEX ICS-1100 and Chromeleon Chromatography Management System with an ASRS 4 mm suppressor. A 4.5 mM sodium carbonate and 1.4 mM sodium bicarbonate eluent mix was used. Values for pH were measured using a standard bench top Oakton pH 11 Series meter.

#### 2.3.5. Determination of FAME content via GC-MS

Cultures were harvested once a Nile Red peak was observed and processed as described previously [16]. Flasks were transferred to 50 mL sterile Falcon tubes and centrifuged (4816  $\times g$  for 10 min). The supernatant was decanted and the biomass was flash frozen at  $-80^\circ\text{C}$  until the sample was lyophilized. After lyophilization, biomass was weighed in glass tubes, and submerged in 1 mL of toluene and 2 mL of sodium methoxide. The samples were vortexed and placed in a 90 °C oven for 30 min (samples were vortexed every 10 min). After 30 min, 2 mL of 14% boron trifluoride in methanol were added to each sample and vortexed. Samples were again incubated for 30 min at 90 °C (samples were vortexed every 10 min). When finished, 0.8 mL of hexane and 0.8 mL of 15% NaCl were added to each sample. Samples were incubated for 10 min and centrifuged (2500  $\times g$  for 2 min). The top layer in the tube (containing the lipids) was extracted and analyzed on an Agilent

Technologies 6890 N Network GC system to determine lipid concentration and composition.

2.3.6. Determination of SSU rRNA sequence

Cells for DNA extraction were taken through three freeze-thaw cycles with liquid nitrogen, and cells were lysed with sterile sand in a mortar and pestle (3 times). A FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA) was used to extract the algal DNA. Quality of the extracted DNA was checked by agarose gel electrophoresis and DNA yield was quantified using a Nanodrop UV-Vis spectrophotometer and a Qubit (1.0) Fluorometer. The 18S rRNA gene of the isolate was amplified using PCR and analyzed with Sanger sequencing through Functional Biosciences (Madison, WI). The 18S rRNA gene was PCR amplified (NS1, 519R, 576F, and 1148R) using the following parameters: initial 2-steps of 80 °C for 1.5 min, 94 °C for 2.0 min, and then 40 cycles of 94 °C for 0.5 min, 52 °C for 1.0 min, and 72 °C for 1.25 min with a final extension of 72 °C for 7.0 min. The nucleotide sequences were compared to the National Center for Biotechnology Information (NCBI) BLASTn database to determine the phylogenetic relationship of the isolate to previously observed sequences.

3. Results and discussion

3.1. Water chemistry of CBM production water

Comparison of geochemical results from the selected CBM production water and BBM indicate some nutrients were similar but the concentrations varied (Table 1). The tested CBM water was typically pH 8.1 to 8.5, and was bicarbonate dominant with a typical alkalinity of 23 meq/kg. The tested CBM water had a substantial amount of manganese and iron ( $3.6 \times 10^{-4}$  and  $3.58 \times 10^{-4}$  mM, respectively) when compared to standardized BBM. Typically, iron availability can be a point of concern for algal growth and supplementation of iron will increase biomass productivity [17]. Lack of iron does not appear to be a growth-limiting factor when using CBM water as a growth medium. However, magnesium and sulfate, two key nutrients in algal physiology [18,19], were 5-fold and 4-fold lower, respectively, when compared to standard BBM. From the water chemistry, a SAR (sodium adsorption ratio) value of 64.3 was calculated; therefore, the tested CBM water likely represents production water that could be harmful to soil health if used for irrigation purposes [20]. The following were unique to the tested CBM production water and not included for BBM:  $\text{Ba}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Si}$ ,  $\text{F}^-$ , and  $\text{Br}^-$ . In addition, the absence of nitrate and phosphate in CBM water was a major difference.

3.2. Isolate PW95 identity

Based upon the small subunit (SSU) rRNA sequence (600 nt), PW95 is predicted to be a member of the *Chlorococcaceae* or *Chlamydomonadaceae* family, and has 100% sequence identity with *Chlamydomonadaceae* KMMCC206 and 100% sequence identity with *Neosporangiococcus* H6904 [21]. *Chlamydomonadaceae* KMMCC 206 was isolated from fresh water in Korea and is part of the Korean Marine

Microalgae Culture Center, and *Neosporangiococcus* H6904 (UTEX 2284) was previously classified as *Chlorochytrium* [21]. The results indicate that isolate PW95 is a green alga that can be classified as a *Chlorophyceae*. However, it should be noted that despite the high degree of sequence homology between the SSU rRNA gene sequences for these isolates, the extent of phenotypic and/or genotypic similarity is not known.

3.3. Growth of isolate PW95 in sterile CBM water and BBM

The growth of PW95 was compared between unamended CBM water, amended CBM water (N, N + P, N + P +  $\text{MgSO}_4$ , N + P +  $\text{MgSO}_4$  + micronutrients) and a defined growth medium (BBM). The lowest biomass was in unamended CBM water, although some growth was observed (most likely due to carry-over nutrient and/or stored polymer) with a subsequent decline in biomass (Fig. 1). The fastest initial growth rate was observed with the N + P amendment that was approximately 2-fold the observed growth rate in unamended CBM water. The other conditions displayed initial growth rates that ranged from 0.03/h to 0.04/h, including the defined BBM, and these results indicated that the isolate could grow faster in amended CBM (i.e., N + P) water than BBM (Fig. 1). The biomass maxima for the tested conditions were 1.7-fold to 2-fold higher than unamended CBM water, and the addition of P increased the growth rate but not biomass maxima. Interestingly, the CBM water amended with all additions did not show higher biomass compared to the other amendments that had similar maximum biomass (approximately 0.3 g/l) at the tested N concentrations of 0.5 mM (Fig. 1). Even though phosphate was not added to the amendment with only CBM water and nitrate, it is possible that the microalgae in the inoculum had stores of internal phosphate that could be utilized once transferred to a medium lacking the nutrient [22].

All culture conditions displayed an increase in pH that ranged from 9.5 to 10.75, and the highest pH was observed with the three most amended culture conditions (Fig. 2). The nitrate-only amendment and BBM culture reached similar pH levels (10.3) before declining photosynthesis and subsequent pH decline.

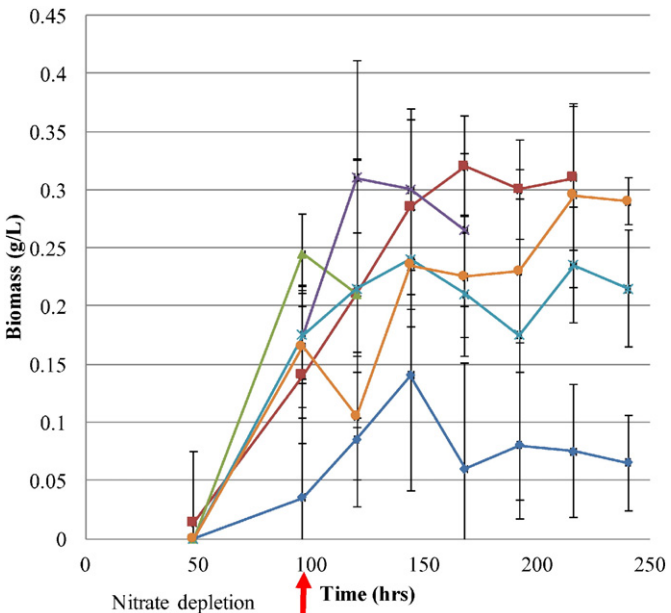


Fig. 1. Dry weight biomass of isolate PW95 grown in CBM water (♦), CBM water with 0.5 mM  $\text{NO}_3^-$  (■), CBM water with 0.5 mM  $\text{NO}_3^-$  and 0.05 mM  $\text{PO}_4^{3-}$  (▲), CBM water with 0.5 mM  $\text{NO}_3^-$ , 0.05 mM  $\text{PO}_4^{3-}$ , and 0.3 mM  $\text{MgSO}_4$  (●), CBM water with 0.5 mM  $\text{NO}_3^-$ , 0.05 mM  $\text{PO}_4^{3-}$ , 0.3 mM  $\text{MgSO}_4$ , and micronutrients (×), and BBM with 0.5 mM  $\text{NO}_3^-$  and 0.05 mM  $\text{PO}_4^{3-}$  (○). Arrows mark when nitrate was depleted. Error bars represent one standard deviation of the combined biological and technical replicates.

Table 1  
Nutrients observed in CBM water and BBM.

| Medium                 | Nutrient (mM)    |                  |                 |                               |      |
|------------------------|------------------|------------------|-----------------|-------------------------------|------|
|                        | Ca <sup>2+</sup> | Mg <sup>2+</sup> | Na <sup>+</sup> | K <sup>+</sup>                | B    |
| CBM water              | 0.10             | 0.06             | 25.65           | 0.12                          | 0.03 |
| BBM                    | 0.17             | 0.30             | 1.10            | 0.06                          | 0.18 |
| Supplemented CBM water | 0.17             | 0.36             | 26.75           | 0.19                          | 0.18 |
|                        |                  |                  |                 |                               |      |
| CBM water              | Mn <sup>2+</sup> | Fe               | Cl <sup>-</sup> | SO <sub>4</sub> <sup>2-</sup> |      |
|                        | 3.6E-04          | 3.58E-04         | 1.79            | 0.09                          |      |
| BBM                    | 7.28E-06         | 1.79E-05         | 0.94            | 0.38                          |      |
| Supplemented CBM water | 3.72E-04         | 3.76E-04         | 2.13            | 0.41                          |      |

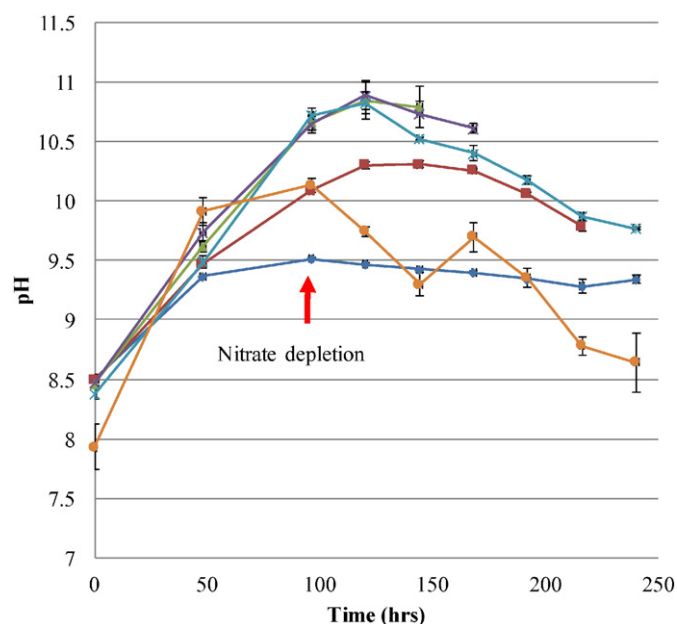


**Fig. 2.** pH of CBMW when grown in CBM water (◆), CBM water with 0.5 mM NO<sub>3</sub><sup>-</sup> (■), CBM water with 0.5 mM NO<sub>3</sub><sup>-</sup> and 0.05 mM PO<sub>4</sub><sup>3-</sup> (▲), CBM water with 0.5 mM NO<sub>3</sub><sup>-</sup>, 0.05 mM PO<sub>4</sub><sup>3-</sup>, and 0.3 mM MgSO<sub>4</sub> (●), CBM water with 0.5 mM NO<sub>3</sub><sup>-</sup>, 0.05 mM PO<sub>4</sub><sup>3-</sup>, 0.3 mM MgSO<sub>4</sub>, and micronutrients (○), and BBM with 0.5 mM NO<sub>3</sub><sup>-</sup> and 0.05 mM PO<sub>4</sub><sup>3-</sup> (●). Arrows mark when nitrate was depleted. Error bars represent one standard deviation of the combined biological and technical replicates.

Lipid accumulation was not observed in cultures grown in BBM, un-amended CBM water, nor CBM water amended with all additions, whereas CBM water with added N, added N + P, and added N + P + MgSO<sub>4</sub> did display increased lipid accumulation based upon Nile Red fluorescence (Fig. 3). Of these conditions, each that had phosphate reached a lipid accumulation peak more quickly than the condition without added phosphate. The rate of lipid accumulation was slightly lower for the N-only amendment (1.5-fold), and the increase

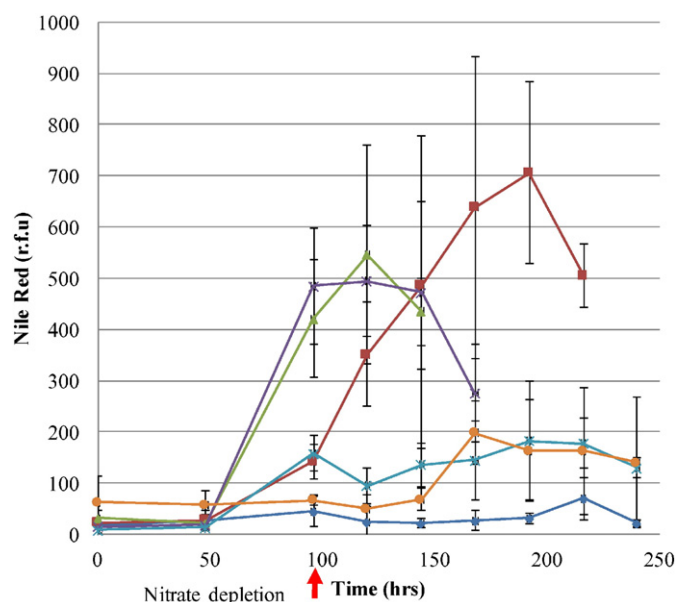


**Fig. 3.** Lipid production of CBMW when grown in CBM water (◆), CBM water with 0.5 mM NO<sub>3</sub><sup>-</sup> (■), CBM water with 0.5 mM NO<sub>3</sub><sup>-</sup> and 0.05 mM PO<sub>4</sub><sup>3-</sup> (▲), CBM water with 0.5 mM NO<sub>3</sub><sup>-</sup>, 0.05 mM PO<sub>4</sub><sup>3-</sup>, and 0.3 mM MgSO<sub>4</sub> (●), CBM water with 0.5 mM NO<sub>3</sub><sup>-</sup>, 0.05 mM PO<sub>4</sub><sup>3-</sup>, 0.3 mM MgSO<sub>4</sub>, and micronutrients (○), and BBM with 0.5 mM NO<sub>3</sub><sup>-</sup> and 0.05 mM PO<sub>4</sub><sup>3-</sup> (●). Arrows mark when nitrate was depleted. Error bars represent one standard deviation of the combined biological and technical replicates.

was delayed but sustained for a longer period of time compared to the other two conditions (N + P or N + P + MgSO<sub>4</sub>). Altered lipid accumulation (rate, duration, and peak) has been reported for both green algae and diatoms in response to varying combinations of N and P limitation [23–25]. The results indicated that algal biomass and lipid could be accumulated in nitrate-amended CBM production water.

In each condition that accumulated lipids, a strong correlation was observed between nutrient depletion and the onset of lipid accumulation (Fig. 4). This correlation has been previously documented for green algae and diatoms [26,27]. Theoretically, a lack of nitrogen causes an inability to produce new biomass, therefore, halting cell division and growth. This is supported by transcriptomic studies that have shown a depletion of nitrogen causes an overexpression of lipid biosynthesis and pentose phosphate pathway genes along with reduced expression of fatty acid degradation genes [27,28].

A higher pH was observed for each condition that accumulated a substantial amount of lipids (Fig. 2). pH stress has been observed to coincide with lipid accumulation in other microalgae [24,29]; however, pH may also be a consequence of increased photosynthesis (and thereby increased photosynthate). It can be difficult to determine whether the lipid accumulation was a result of high pH, nitrate depletion, or a combination of the two conditions. In this study, nitrate depletion was possibly the cause of lipid accumulation because the N-deprived state was observed before the spike in Nile Red fluorescence, whereas the highest pH values were observed in tandem with the Nile Red peaks.

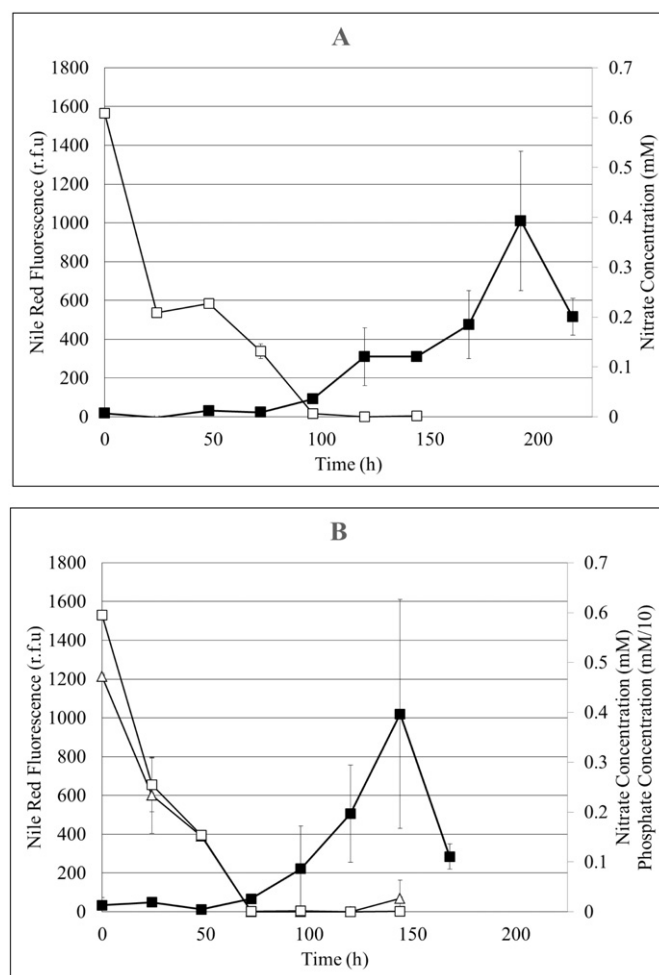


**Fig. 4.** (A) Lipid accumulation (■) and nitrate depletion (□) in CBM water with 0.5 mM NO<sub>3</sub><sup>-</sup> and (B) lipid accumulation (■), nitrate depletion (□), and phosphate depletion (Δ) in CBM water with 0.5 mM NO<sub>3</sub><sup>-</sup> and 0.05 mM PO<sub>4</sub><sup>3-</sup>. Phosphate concentration is multiplied by 10 for scaling purposes. Error bars represent minimum and maximum of technical duplicates.

However, further work is needed to discern the mechanism(s) of lipid accumulation in the described isolate in terms of photosynthetic rates at different light intensities associated with changes in nitrate levels and pH.

A lack of lipid accumulation in CBM water without added nutrients can likely be attributed to the poor growth from an absence of nitrate and phosphate. In the conditions with more replete nutrients (N, P,  $\text{MgSO}_4$ , and micronutrients; BBM) in which lipid accumulation was not observed, nitrate and phosphate were confirmed to be depleted at the same time as the other growth conditions that did accumulate lipids. A possible explanation could be the presence of micronutrients in these conditions that decouples nutrient deprivation and lipid accumulation. A recent study evaluated the proteome of *Chlamydomonas reinhardtii* and observed that vesicular proteins involved in lipolysis were negatively affected when Zn, Cu, Mn, and Fe were lacking [30]. Perhaps the presence of micronutrients allowed these enzymes that degrade lipids to stay functional and therefore prevent a lipid accumulation event. The presence of micronutrients could also override the signaling associated with short-term deprivation of macronutrients and/or interactions with potential heterotrophs under mixed culture conditions, but further work is needed to discern cellular responses in low-quality water amended with different nutrients, particularly for algal species that are indigenous to terrestrial environments.

For all cultures, except CBM with only nitrate, chlorophyll was observed to decrease when nitrate was depleted (Fig. 5). This response to nitrate depletion has been previously documented [31]. A possible explanation for the decline in chlorophyll could be the damaging effects of reactive oxygen species formed from oxidative stress during photosynthesis. A recent study showed an increase in antioxidant enzymes after nitrogen depletion indicating that the microalgae were experiencing oxidative stress [32]. Concurrently, microalgae have been observed to degrade proteins and RNA as well as down regulate protein synthesis to adapt to a lack of nitrogen in the environment [28]. The combination of these two responses may be a result of recycling nitrogen contained in chlorophyll that is balanced with the need for some photosystems to be maintained for the harvesting of light energy.

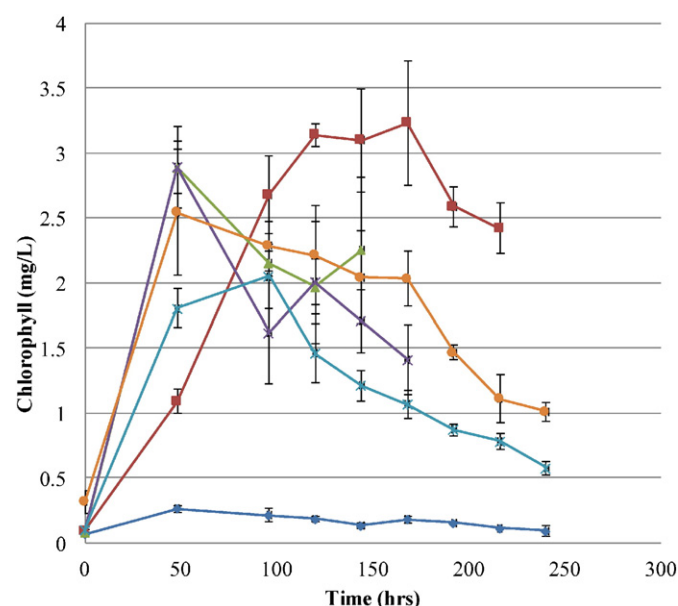


**Fig. 5.** Total chlorophyll (a, b, and c) of CBMW grown in CBM water ( $\blacklozenge$ ), CBM water with 0.5 mM  $\text{NO}_3^-$  ( $\blacksquare$ ), CBM water with 0.5 mM  $\text{NO}_3^-$  and 0.05 mM  $\text{PO}_4^{3-}$  ( $\blacktriangle$ ), CBM water with 0.5 mM  $\text{NO}_3^-$ , 0.05 mM  $\text{PO}_4^{3-}$ , and 0.3 mM  $\text{MgSO}_4$  ( $\blacklozenge$ ), CBM water with 0.5 mM  $\text{NO}_3^-$ , 0.05 mM  $\text{PO}_4^{3-}$ , 0.3 mM  $\text{MgSO}_4$ , and micronutrients ( $\bullet$ ), and BBM with 0.5 mM  $\text{NO}_3^-$  and 0.05 mM  $\text{PO}_4^{3-}$  ( $\circ$ ). Shaded area shows time frame when nitrate was depleted. Error bars represent one standard deviation of biological and technical duplicates.

It is interesting to note that under the condition of nitrate-only in CBM water, chlorophyll steadily increased and was maintained longer compared to the other conditions, and this coincided with biomass as well. These results suggested that a nitrate-only condition more analogous to environmental conditions for the isolate favored biomass and lipid accumulation.

### 3.4. Lipid analysis

Gas chromatography-mass spectrometry (GC-MS) analysis of the extracted lipids of trans-esterified samples revealed a total FAME content of 27%, 20%, and 25% weight of fatty acid/weight of biomass (w/w) for the conditions with added N, added N and P, and added N, P, and  $\text{MgSO}_4$ , respectively (Fig. 6). These values correspond to previously reported values in the literature for other algae and diatoms [33, 34]. All three lipid contents match very closely to values observed in *Dunaliella tertiolecta* (20% and 24% lipid content) when grown in CBM production water from Australia [11]. A primary difference between the two studies was the need to add a significant amount of salt to the coal seam gas water for optimum *D. tertiolecta* growth as the organism is a marine species. Our results highlight the advantage to using PW95 for growth in the tested CBM water, as the alga is indigenous to the region. Although primary nutrients such as nitrogen and phosphorus are needed, other amendments to the water are not needed for PW95 growth because low-quality CBM water is a natural habitat.

The predominant fatty acids in each case were palmitic acid (C16:0), oleic acid (C18:1 ( $\omega$ -9)), linoleic acid (C18:2 ( $\omega$ -9,12)), and  $\alpha$ -linolenic acid (C18:3 ( $\omega$ -9,12,15)). These fatty acids match those predominantly observed in other plant-based sources of biodiesel such as canola and palm oil [35], implying that they could be effectively utilized as a biodiesel source. When compared to other algal species, PW95 is lacking in palmitic acid accumulation and contains a higher amount of C:18 unsaturated fatty acids, especially  $\alpha$ -linolenic acid [34]. Interestingly, linolenic acids can be a value added product because of use as a dietary supplement as an omega-3 fatty acid. The decrease in lipid content observed in the conditions with phosphate could be a result of a non-optimal phosphate concentration. Varying the phosphorus concentration has been observed to have an effect on the amount of lipids produced by microalgae and diatoms [25,36].

However, more research needs to be done to characterize the species in terms of maximal biomass and lipid accumulation. The isolate PW95 can grow on ammonium as well as nitrate (Hodgskiss and Fields, unpublished data), and the ability to assimilate other nitrogen sources

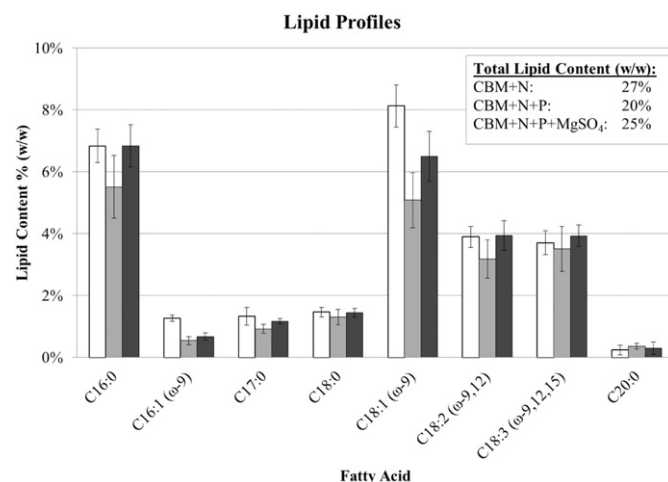


**Fig. 6.** Lipid composition of PW95 grown in CBM water with 0.5 mM  $\text{NO}_3^-$  (white), CBM water with 0.5 mM  $\text{NO}_3^-$  and 0.05 mM  $\text{PO}_4^{3-}$  (light grey) and CBM water with 0.5 mM  $\text{NO}_3^-$ , 0.05 mM  $\text{PO}_4^{3-}$ , and 0.3 mM  $\text{MgSO}_4$  (dark grey). Lipid content is % w/w of fatty acid to biomass. Error bars represent one standard deviation among technical and biological duplicates.

could prove beneficial when determining how to best supplement CBM water in the Powder River Basin, particularly low-cost nitrogenous waste products. Concurrently, moving from laboratory scale to pilot scale experiments with outdoor conditions (e.g., light and temperature) and non-sterile CBM water are planned for future work to assess the cellular responses and lipid accumulation of this novel environmental isolate in low-quality CBM production water.

#### 4. Conclusion

In summary, a green algae species, PW95, was isolated from a coal bed methane production pond in northeastern Wyoming. Preliminary identification based upon SSU rRNA gene sequences suggested isolate PW95 belongs to the *Chlorococcaceae* family. The native isolate accumulates lipid after nitrate depletion in CBM production water amended with nitrate. The highest lipid content achieved was 27% (w/w) when only nitrate was added to CBM water. The addition of other nutrients to CBM production water resulted in similar biomass maxima but lower lipid contents. The predominant fatty acids were C16:0, C18:1 ( $\omega$ -9), C18:2 ( $\omega$ -9,12), and 18:3 ( $\omega$ -9,12,15).

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