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Anaerobic microbial communities can influence algal growth and nutrient removal from anaerobic digestate

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Graphical Abstract

Highlights

The digester microbial community doubled *Auxenochlorella protothecoides* growth
The microbial community increased nutrient removal by *A. protothecoides*
C. sorokiniana was not apparently affected by the microbial community
The microbial community had little impact on algal biomass composition

Abstract

The objective of this work was to test the impact of anaerobic digester microorganisms on algal growth, composition, and nutrient removal from digestate. Culture studies were carried out to determine the impacts of the microbial community on treatment of poultry litter anaerobic digestate by two strains of green algae: *Auxenochlorella protothecoides* and *Chlorella sorokiniana*. The results showed that the community doubled the growth of *A. protothecoides* but had no impact on *C. sorokiniana* growth. A similar result was observed for nutrient removal where the microbial community increased the capacity of *A. protothecoides* to remove ammonium and phosphate. The impact of the microbial community on biomass composition was minimal for both algae types.

Key words: Algae, anaerobic digestate, microbial community, Poultry litter, wastewater treatment

1. Introduction

The U.S. Department of Agriculture estimated in 2016 that the U.S. poultry industry, which includes broilers, layers, and turkeys, produces approximately 550 million tons of manure each year (Coker, 2017). Much of this manure is trapped in litter bedding

material which is typically applied to agricultural land as a source of nutrients for crops (Kelleher et al., 2002). Poultry litter is typically land-applied based on its nitrogen content, thus more phosphorus is applied than necessary for crop growth (Preusch et al., 2002). The result is over-accumulation of phosphorus in soils, resulting in nutrient runoff and eutrophication of natural waters (Wilkie, 2000).

Combining anaerobic digestion with algal cultivation has been explored as a means of recovering energy (Singh et al., 2010) and nutrients from poultry litter in an environmentally-sustainable manner (Singh et al., 2011). Anaerobic digestion converts complex organic molecules into biogas while mineralizing nitrogen and phosphorus nutrients, which are mostly retained in the digestate. Studies have shown that many algal strains can grow effectively on anaerobic digestate while sequestering nitrogen and phosphorus in their fixed, biological form (Cai et al., 2013a; Franchino et al., 2016; Woertz et al., 2009). The resulting algal biomass may also be suitable for biofuel production (Singh et al., 2011; Woertz et al., 2009) and feed ingredients (Ibekwe et al., 2017).

Other researches have demonstrated that algae can grow on wastes from swine (Bjornsson et al., 2013; Franchino et al., 2016), cattle (Kobayashi et al., 2013), poultry (Singh et al., 2011), winery (Higgins et al., 2017), and municipal wastes (Wang et al., 2019b; Woertz et al., 2009). Despite this apparent abundance of research on growing algae on wastewater, there is comparatively little knowledge regarding the impact that wastewater microbial populations have on algal growth, nutrient removal, and biomass composition. Understanding the impact of native microbial populations is important because engineers can potentially control the abundance of wastewater organisms entering the algal culture using clarification or filtration approaches. Research has shown that bacterial populations have significant potential to impact algal cultures in both positive and negative ways (de-Bashan et al., 2008;

Higgins & VanderGheynst, 2014; Higgins et al., 2016; Palacios et al., 2016). Previous work by Higgins et al. (2017), found that native microbial populations have significant impacts on algal treatment of winery wastewater but limited impact on algal growth rates. However, similar studies have not been carried out on manure-based anaerobic digestates. Moreover, the study by Higgins et al. did not investigate the impacts of the microbial community on biomass composition. The objective of this research was to grow algae on poultry slurry anaerobic digestate in order to test three hypotheses: 1) Growing algae in the presence of digestate microbes has minimal impact on biomass growth rates similar to the findings of Higgins et al. (2017), 2) Algae reduce nitrogen and phosphorus nutrient concentrations in the digestate proportional to algal growth, and 3) Microbes in the digestate help lower chemical oxygen demand (COD), including photosynthate secreted by algae, thereby improving water quality. These hypotheses were tested by culturing algae on anaerobic digestates of poultry litter slurry with and without the presence of the native digester microbial population. The latter was achieved using sterile filtration to remove microbes while minimally impacting digestate chemistry.

2. Methods

2.1 Litter collection and anaerobic digestion

Anaerobic digestate (AD) of poultry litter slurry was prepared in lab-scale digesters due to the lack of a regionally operating AD facility for poultry litter. The poultry litter was obtained from a local broiler farm in Eastern Alabama as previously described (Chaump et al., 2018), and served as the substrate for anaerobic digestion. Municipal sludge was used for digester inoculum and was collected from a local wastewater treatment plant's anaerobic digester. Both litter and sludge were analyzed for total and volatile solids as described previously (Chaump et al., 2018). Chaump et al. determined that an 80:20 volatile solids (VS) ratio of waste

poultry litter to municipal sludge inoculum was ideal to maximize removal of total solids (TS), VS, COD and total organic carbon (TOC) from the litter slurry on a volumetric basis. Digestion was carried out in three 2 L bottles with a VS loading of 10 g/L. One-way check valves were placed in the reactor lids to allow the escape of biogas into a water trap to ensure production of gas over time. The reactors were kept in a dark incubator at 37°C for 30 days; after which, the reactors were pooled and stored in a cold room (4°C) for use in the algae experiments. Two batches of digestate were prepared for experiments carried out in this study.

2.2 Experimental plan for algal treatment of digestate

Two batch culture experiments were conducted, each with a different strain of algae. The strains used for each experiment were *Auxenochlorella protothecoides* (UTEX 2341 (Higgins et al., 2015b)), and *Chlorella sorokiniana* (UTEX 2805). The following strains were selected for this study due to their ability to remove ammonium and phosphate from wastewater (Higgins et al., 2017). This strain of *A. protothecoides* is known to accumulate neutral lipids under nutrient stress and previous research revealed that the presence of *Escherichia coli* can stimulate nutrient uptake in this strain (Higgins et al., 2015a; Higgins & VanderGheynst, 2014). This strain of *C. sorokiniana* was chosen for its ability to grow on digestate (Wang et al., 2019b) and accumulate intracellular starch (Tanadul et al., 2014) which can be valuable for biofuel and feed production.

Initial experiments were carried out to determine the necessary rate of digestate dilution in order to achieve robust algal growth. Past research has shown that significant dilution is typically needed to support algal growth on anaerobic digestates (Cho et al., 2013; Franchino et al., 2016; Prandini et al., 2016). An initial screening study was carried out with *A. protothecoides* cultivated in biological duplicate on digestate diluted by factors of 2x, 4x, 8x, 16x, and 32x. All dilutions were carried out in sterile-filtered (0.2

μm) digestate diluted with dH_2O. The results indicated that 2x or 4x dilutions had the fastest growth and a follow-up study was conducted testing 1x, 2x, and 4x dilutions in triplicate to determine the final dilution rate to be used in subsequent experiments. The latter experiment confirmed that a 4x dilution was the optimal dilution rate for maximizing algal growth.

Once the optimal dilution rate was obtained, batch experiments were carried out for both algal strains to test the impact of digester microbes on algal growth. Each batch experiment contained triplicate control cultures grown on chemical medium: N8-NH_4 medium for *A. protothecoides* and N8 medium for *C. sorokiniana*. Because *A. protothecoides* is a thiamine auxotroph, the N8-NH_4 medium was supplemented with 500 μM thiamine (Higgins et al., 2016). Chemical media were sterilized by autoclave. Control cultures were included to provide baseline growth data and to verify that a healthy inoculum was used in experiments. Three experimental treatments were tested in triplicate on anaerobic digestate in order to study the impacts of algae and digester microorganisms on digestate treatment. These treatments included axenic algae cultures grown on sterile-filtered digestate, algae and digester microorganisms grown together, and digester microorganisms grown without algae. For treatments that contained digester microorganisms, digestate was clarified by centrifugation for 15 minutes at 5,000 rpm followed by vacuum filtration through a No. 2 Whatmann filter (8 μm) to remove large suspended particles. This filter is too course to remove individual bacteria. Axenic algae cultures were prepared through a step-filtration process. The step-filtration method started with the clarified digestate and removed bacteria by passing digestate through the following filters in sequence: 0.8 μm cellulose acetate, 0.45 μm cellulose acetate, twice through a 0.2 μm cellulose acetate filter, and finally through a sterile 0.2 μm filter apparatus (Thermo). The filter manufacturer has published literature showing that their membranes completely reject one of the smallest known bacteria, *Brevundimonas diminuta*.

Regardless, select sterile filtrates were checked for bacterial contamination by plating on ATCC No. 5 sporulating agar (ATCC, 2013) which is a rich medium that supports both algae and a range of heterotrophic organisms. Sterile filtration would never be used in a real wastewater process, but this experimental design allowed for quantitative testing of the specific contributions of algae and digester microorganisms to the treatment of litter digestate. Moreover, the experiment was designed to reveal synergistic or inhibitory interactions between algae and microorganisms native to the digestate.

2.3 Cultivation methods

Cultivation methods have been described in detail in Wang et al. (2019a). All cultures were handled in a biosafety cabinet using sterile technique. Each algae strain was initially plated on ATCC No. 5 sporulating agar. Single algae colonies were selected and inoculated into 1 L bottles filled with autoclaved chemical medium (either N8-NH_4 or N8 as previously noted) to produce inoculum for the experiments. Pre-cultures were grown under 140 mmol $m^{-2}s^{-1}$ PAR illumination on a 14:10 light/dark cycle. Aeration with 0.5 vvm humidified air supplemented with 2% CO_2 was introduced to the reactor through a sterile filter (0.2 μm). Cultures were mixed by stir bar at ~200 rpm and grown until optical density (OD) reached ~0.2 at 550 nm, similar to previously-described experiments (Wang et al., 2019a). All pre-cultures were re-plated on ATCC No. 5 agar in order to check for contamination. Pre-cultures were allowed to settle overnight and the supernatant was removed by vacuum under sterile conditions. The resulting algae slurry was used to inoculate the experimental bioreactors at a starting OD550 of ~0.15.

All experimental batch cultures were grown in 300 ml bioreactors filled with 200 mL of media or digestate as described in detail in Wang et al. (Wang et al., 2019a). For all experiments, anaerobic digestate was diluted 4-fold with deionized water in order to

support strong algal growth. All tubes used in the batch experiments and control media were sterilized by autoclave to avoid introduction of laboratory microbes into the system. The experimental cultures were grown in a room temperature water bath at 25 °C. Illumination, aeration, and mixing were the same as for the pre-cultures. Culture pH was adjusted with either 3M NaOH or 3M HCl, as needed throughout the experiments to maintain a pH of ~7.2. Samples were taken aseptically from the reactors every 24 hours for OD and water quality analyses. The OD was read for each sample at 550 nm and correlated to the final dry weight concentration at the end of the experiment. At the end of the 5-day experimental period, the culture biomass was harvested by centrifugation and freeze dried to determine the final biomass concentrations on a dry weight basis. Separate correlations between dry weight and OD550 were developed for each experimental treatment as previously described (Wang et al., 2019a). Correlations were used to estimate the biomass growth curve over the course of the batch experiment. Average productivity was calculated by subtracting the final dry weight biomass concentration from the initial concentration and dividing the result by the batch duration (120 hours).

2.4 Determination of soluble nutrients by ion chromatography

Ion chromatography was used to analyze a range of nutrient ions in digestate samples as described in a previously-published method (Chaump et al., 2018). The nutrient concentrations of particular interest were ammonium, nitrate, nitrite, phosphate, and sulfate. Each digestate sample was first filtered through a 0.2 μm filter before analysis. A Shimadzu Prominence High Pressure Liquid Chromatography instrument coupled to a conductivity detector was used for analysis. Cation analysis was carried out on a Dionex CS12 column coupled with a CERS500 suppressor at 59 mA. Anion analysis was carried out on a Dionex AS22 column coupled with an AERS500 suppressor at 26mA. A 20 μL injection volume was used with a column flow rate of 1 mL/min for cation

and anion analyses. A mobile phase of 20 mM methansulfonic acid in Nanopure water was used for cations, while 4.5 mM sodium carbonate and 1.5 mM sodium bicarbonate in Nanopure water was used to analyze anions. The resulting peaks were integrated using the Shimadzu LC Solutions software.

2.5 Assessment of biomass composition

Biomass composition was carried out on freeze-dried biomass generated during the experiments using a previously-described analysis pipeline (Higgins & VanderGheynst, 2014; Wang et al., 2019a). Biomass composition analyses could not be completed for cultures that contained only AD bacteria due to insufficient biomass. The following analyses were carried out: crude lipid extraction using a modified Folch method, neutral lipid assay (Higgins et al., 2014), starch content using an enzymatic assay (Higgins & VanderGheynst, 2014), cell wall content, and total nitrogen content (Higgins et al., 2015a). Total nitrogen content was converted to crude protein content using a multiplication factor of 4.6 for algae per Cole et al. (2016).

2.6 COD determination

A HACH COD assay kit was used to analyze soluble COD remaining in the digestate at the beginning and end of the algae experiments. Digestate samples were filtered through a 0.2 μm filter before analysis to ensure that only soluble COD was measured. Digestates were diluted as necessary to ensure that samples were not above the upper limit of the assay range (1500 mg/L). The assay was conducted per the manufacturer's instructions and a HACH DR900 spectrometer was used to read absorbance.

2.7 Extraction of bacterial DNA and qPCR

DNA extraction and qPCR were carried out to quantify the relative fraction of bacteria within the mixed algal-microbe cultures. This approach has been previously used to measure total bacteria within complex algal-microbial communities (Higgins et al., 2017). Once the bacterial fraction of biomass was determined, it was converted into a biomass concentration and then into a rate of average growth over the 5-day culture period. A FastDNA Spin kit (MP Biomedicals, Solon OH) was used to extract bacteria DNA from the freeze-dried biomass per the manufacturer's instructions. The previously-described primers, 16Sv5F799mod3 and 16Sv5R926, were used to amplify a segment of the 16S rRNA gene (Higgins et al., 2017). These primers bind to conserved regions of bacterial 16S rRNA but mostly exclude chloroplast 16S rRNA (Hanshew et al., 2013). All qPCR reactions were prepared in a biosafety cabinet to ensure that the DNA extracts and any other qPCR materials were not contaminated by laboratory bacteria. The PCR reaction conditions have all been described previously (Higgins et al., 2017). Briefly, 20 µL reaction volumes consisting of PerfeCTa SYBR Green FastMix (QuantaBio) and 0.5 µM of forward and reverse primers were used. Template DNA was diluted 10-fold and 2 µl was added to each reaction tube. Dilutions of DNA extracted from known quantities of digester microbe biomass (obtained from the bioreactors without algae) were used as the standard to correlate gene abundance to digester microbe biomass. An analytic qTower3G and its software were used to run qPCR. Polymerase was activated for 10 minutes at 95 °C and thermocycling was carried out for 45 cycles with the following settings: 15 seconds at 95 °C, 15 seconds at 47 °C, and 30 seconds at 72 °C. After thermocycling, a melt curve was obtained by reading the fluorescence as the program ramped from 72 °C to 95 °C.

2.8 Statistical analysis

Averages and standard deviations were calculated in Microsoft Excel. The following statistical tests were carried out in R using the 'agricolae' package. Data from the full-factorial experimental design were analyzed using ANOVA and Tukey's HSD multiple comparison test. Data homogeneity of variance was tested with Levene's test and Brown and Forsythe's test. In all cases a p value of < 0.05 was considered significant.

3. Results and Discussion

3.1 Poultry waste and digestate characteristics

Poultry litter was anaerobically digested for roughly one month in order to create digestates suitable for testing algae cultures. The collected poultry litter contained 85% solids; while the municipal digester sludge contained 4% solids. 84% of the poultry litter solids were volatile, while 56% of the municipal sludge solids were volatile. The resulting anaerobic digestates were diluted four-fold and a range of chemical analyses were carried out to quantify soluble nutrients. The reason soluble nutrients were targeted was because these are generally retained in the liquid effluent after digestate solid-liquid separation and would therefore be available for algal growth. As noted in previous studies on cultivating algae on anaerobic digestate, solid-liquid separation is important for controlling digestate turbidity, thereby facilitating photosynthetic algal growth (Wang et al., 2019b). Two batches of anaerobic digestate were freshly prepared: the first batch was used for experiments with *A. protothecoides* and the second batch was used for experiments with *C. sorokiniana*. The four-fold diluted digestates were used for algae cultivation experiments. As shown in Table 1, some variation occurred between the two digestate batches, with particular differences in phosphate and sulfate levels. Differences in

sulfate could be explained by variable activity of sulfate reducing bacteria (Mizuno et al., 1998) whereas phosphate is susceptible to varying rates of precipitation with heavy metals such as iron (Möller & Müller, 2012).

3.2 Algal growth on different digestate concentrations

Past research has shown that full-strength ADs are generally inhibitory to algal growth. This has historically been attributed to light-limitation via turbidity and ammonium-inhibition (Cai et al., 2013b; Cho et al., 2013; Park et al., 2010). Inhibition is typically overcome through dilution (Cho et al., 2013; Franchino et al., 2016; Prandini et al., 2016), although recent developments have shown that biological treatment can also be used to overcome inhibition (Wang et al., 2019b). The initial ammonium concentration for the full strength digestate was high (878.3 ± 63.7 mg/L) so the impact of dilution on growth was tested in *A. protothecoides*. The results showed that a four-fold dilution yielded an average growth rate of 292 mg/L/d compared to 244 mg/L/d for a 2-fold dilution of digestate. A 4-fold dilution rate was therefore used in all subsequent experiments.

3.3 Impact of digestate microbes on biomass growth

It was initially hypothesized that growing algae in the presence of digestate microbes would have minimal impact on biomass growth rates given the results of a previous study on winery digestate (Higgins et al., 2017). This hypothesis was tested by comparing growth rates on sterile-filtered digestate (axenic algae) versus biomass growth on clarified digestate (digester microbes and algae present). Algal control cultures were grown on chemical medium to confirm that algal inoculum was robust in all experiments and provided a growth baseline for comparison with previously-published studies (Higgins et al., 2016; Tanadul et al., 2014). The results confirmed that the presence of digester microbes had minimal impact on biomass growth rates in *C. sorokiniana* cultures (Figure 1).

However, the presence of digestate microbes led to roughly double the biomass growth in *A. protothecoides* cultures compared to its axenic counterpart (Figure 1A). The mixed-culture biomass growth is the combination of growth from algae plus digester microorganisms. The growth of digester microbes in the absence of algae showed no detectable growth over five days, which is consistent with the results from a previous study on winery digestate (Higgins et al., 2017). These results suggest that, for *A. protothecoides*, either microorganisms promote algal growth, algae promote microorganism growth, or there is a combination of these two possibilities.

Given the apparent growth-promoting effects of the mixed culture in *A. protothecoides*, the fraction of mixed-culture biomass that was bacteria vs. algae was tested using qPCR. This analysis showed that only 0.64% (Table 2) of biomass in the mixed culture was bacteria with the remainder being primarily algae. Archaea typically make up less than one third of digester microbes compared to bacteria (Pampillon-Gonzalez et al., 2017) and likely declined in number under the fully aerobic conditions used in this study. Therefore, archaea likely account for a negligible fraction of the non-bacterial biomass at the end of batch cultivation. This result suggests that algal growth was promoted by the presence of live microorganisms in the mixed culture. In the case of *C. sorokiniana*, the bacterial fraction of biomass was also low (2.24%), indicating that the majority of the culture was algae (Table 2). Both experiments showed that placing digester microbes in aerated reactors by themselves led to a net loss of organisms over time (negative growth rate) whereas the presence of algae supported slow but positive bacterial growth. Significant growth of anaerobic digester microbes in the aerobic reactors was generally not expected. Limited COD, high oxygen, and low temperatures (25 °C in the aerobic

reactors versus 37 °C in the digester) all likely inhibited growth of anaerobic digester microbes. The temperature and volumetric aeration rate used in the bioreactors align with an activated sludge process operated in a temperate climate (Holmes et al., 2019).

These results contrast somewhat with past research on winery wastewater which showed that the presence of live microbes had no effect on *A. protothecoides* and mild suppression of *C. sorokiniana* growth compared to axenic cultures grown on sterile-filtered wastewater (Higgins et al., 2017). *A. protothecoides* is a known auxotroph of the thiamine metabolite, 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) and its derivatives (Higgins et al., 2016). The winery study concluded that *A. protothecoides* did receive benefits from microbial secretions of thiamine metabolites, thereby boosting algal growth. However, thiamine metabolites are notoriously unstable in the environment (Jenkins et al., 2007), potentially limiting their availability to algae in certain wastewaters. On the other hand, thiamine is synthesized by a wide range of bacteria and many can re-salvage degraded thiamine (Jenkins et al., 2007; Melnick et al., 2004). Thus, in the present study, the sterilized digestate may have contained deficient thiamine metabolites to support *A. protothecoides* growth but the presence of the microbial community could help overcome this deficiency. This result aligns with a range of literature on algal microbial ecology suggesting that cofactor auxotrophs, such as *A. protothecoides*, typically benefit from co-cultivation with other cofactor-producing microorganisms (Croft et al., 2006). Given that *C. sorokiniana* is a fully autotrophic organism, there was no such requirement for the presence of symbiotic bacteria. This result is consistent with the findings of Shetty et al. (2019) when they cultured an autotrophic strain of *Chlorella vulgaris* in the presence and absence of a microbial community.

Other possible explanations for *A. protothecoides* growth-promotion also exist including mutual exchange of oxygen and carbon dioxide (Bai et al., 2014) and availability of micronutrients. One possibility is that the filtration used to sterilize the digestate in

axenic cultures also removed precipitated trace metals. Magnesium, in particular, has been identified as a limiting metal in many digestates (Tan et al., 2016) and it is essential for chlorophyll production and nitrogen assimilation into microalgae (Bjornsson et al., 2013). Consequently, both the filtered and unfiltered digestates were analyzed for potentially limiting metal nutrients. The results indicate that neither nutrient was limiting in *A. protothecoides* cultures (Table 4). Consequently, inadvertent removal of magnesium via filtration is unlikely to explain differences in *A. protothecoides* growth in the presence and absence of digester microbes. Interestingly, *C. sorokiniana* cultures consumed all soluble magnesium, suggesting potential limitation, yet this apparently occurred in both axenic and mixed cultures.

Symbiosis through the exchange of oxygen and carbon dioxide is also unlikely to explain growth promotion of *A. protothecoides* because this effect would have also led to growth promotion of *C. sorokiniana* in mixed cultures. Moreover, heterotrophic bacteria, which consume oxygen and produce CO_2, also should have consumed soluble organics in the digestate. However, only negligible consumption of COD was observed in cultures containing digester microbes (see Section 3.6 for details). Moreover, there was actually a slight increase in COD in mixed cultures containing both microbes and algae. Such a result is not consistent with robust cycling of oxygen and CO_2 between algae and bacterial heterotrophs.

3.4 Biomass composition

It was initially hypothesized that the neutral lipid content in the mixed algal and bacteria cultures would be larger than the axenic algal cultures, due to increased competition for nitrogen resources between algae and digestate microorganisms. Such competition could lead to nitrogen limitation which is known to lead to neutral lipid accumulation in many oleaginous algae (Griffiths

& Harrison, 2009) including *C. sorokiniana* and *A. protothecoides* (Higgins et al., 2015). Moreover, microbial promotion of *A. protothecoides* growth has previously been observed to lead to nitrogen depletion followed by neutral lipid accumulation (Higgins et al., 2015). However, neutral lipid contents were less than 4% in all cases (Table 3) suggesting that neither of these strains accumulated neutral lipids to an appreciable extent. Typically stress conditions, particularly nitrogen limitation, are required to induce significant neutral lipid accumulation in these algae strains (Higgins et al., 2015a; Higgins et al., 2014). Sufficient nitrogen was available to all cultures as confirmed during subsequent analysis of nutrient levels in the media. In addition to neutral lipid content, total crude lipid content was measured which includes neutral and polar acyl lipids, sterols, and pigments. Overall, there was no significant difference in crude lipid contents between any of the treatments ($p > 0.0668$) (Table 3).

Other biomass composition markers including starch, cell wall, and crude protein were also measured, but here too, little difference was observed between cultures with and without digester microbes. Technically, the presence of digester microbes increased starch content in *A. protothecoides* ($p < 0.005$), but the difference between 0.6% and 1.6% starch content is unlikely to be of practical significance. These results indicate that the presence of digester microbes may not significantly impact the potential uses for algal biomass grown on digestate. Interestingly, there were dramatic differences in biochemical composition between cultures grown on control chemical medium and digestate. Specifically, *C. sorokiniana* produced significantly more starch and less protein and cell wall when grown on digestates ($p < 0.0039$) versus chemical medium. Given that the control media and digestate are chemically different, this result is not surprising, but underscores the plasticity of algal composition in response to chemical constituents. It was not the original intent to compare chemical medium to digestate; the former was simply included as a positive control. Regardless,

these vast differences in starch accumulation raise questions about specific chemical constituents in digestate that cause significant remodeling of algal cellular components.

Past research on *C. sorokiniana* by Tanadul et al. showed starch contents in the range of 20-25% when grown on 2% CO_2 and the same N8 medium used in this study (Tanadul et al., 2014). Notably the starch levels observed by Tanadul et al. were higher than those observed in control cultures from this study, but they were roughly half those observed in poultry litter digestate (42-43%). Research by Kobayashi et al. also showed that starch content roughly doubled from 10% to 20% when *C. sorokiniana* was grown on diluted cattle manure digestate compared to Bold's Basal chemical medium (Kobayashi et al., 2013). Other strains of *C. sorokiniana* have been observed to accumulate starch (up to 60%) under conditions of high light intensity (1000 μmol m^{-2} s^{-1}), high CO_2 (10%), and low nitrogen (Cheng et al., 2017). Light intensity was only 140 μmol m^{-2} s^{-1} in the present study and nitrogen was still abundant (>175 mg/L NH_4), making these explanations unlikely. Alkalinity in digestate is typically high (Franchino et al., 2013) and therefore could have contributed to additional CO_2 supplementation above the nominal 2% level used in the air stream. Future work is required to untangle the chemical underpinnings of starch accumulation in *C. sorokiniana* when grown on digestate.

3.5 Nutrient removal from digestate

It was hypothesized that nitrogen and phosphorus nutrients would be removed proportionally to the observed algal growth. Monitoring the nutrient concentration throughout the 5-day batch experiments allowed for determination of the nutrient removal rate. Nitrite was not detectable in any of the reactors throughout the study which is typical of anaerobic digestates except in those with

significant denitrification activity (Percheron et al., 1999). While nitrate and sulfate were present, their concentrations remained the same over the 5-day experiment in all treatments for each of the algal strains.

Ammonium removal in the mixed culture of *A. protothecoides* was double that of the axenic algae culture (Figure 2A). The fact that nitrite and nitrate levels did not change in these cultures indicates that this additional ammonium removal likely resulted from cellular uptake or volatilization rather than nitrification carried out by bacteria. *C. sorokiniana* had lower overall ammonium removal rates than *A. protothecoides,* and digester microbes did not significantly impact its ammonium removal rate ($p > 0.57$). Ammonium was not fully removed by either strain with *A. protothecoides* removing 33% and 52% for axenic and mixed cultures, respectively. *C. sorokiniana* removed 23% and 19% of ammonium in axenic and mixed cultures, respectively. Differences in removal rates between the strains can be partially explained by the fact that they were grown on different digestate batches with different chemical composition.

Because ammonium removal can occur through either cellular uptake or through ammonia volatilization (pH in the cultures was maintained at 7.2) (Cai et al., 2013a), the nitrogen content of the biomass was measured to estimate removal by cellular uptake (Figure 2B). Cellular uptake refers to the ability of algae and microbes to assimilate extracellular nitrogen sources, e.g. ammonium, into intracellular nitrogen which can be utilized for amino acid synthesis and other biochemical processes (Cai et al., 2013a). These results indicated that 48% and 52% of the ammonium removed in *A. protothecoides* cultures was due to cellular uptake in axenic and mixed cultures, respectively. Of the ammonium removal in *C. sorokiniana* cultures, 52% and 73% of removal was by cellular uptake

in axenic and mixed cultures, respectively. Given the lack of nitrite or nitrate production in any of the reactors, the remainder of the ammonium was assumed to be lost to volatilization during reactor aeration, a result also observed by others (Riano et al., 2011).

Similar to ammonium, *A. protothecoides* mixed cultures removed phosphate nearly 30% faster than their axenic counterparts (Figure 2C). Axenic *C. sorokiniana* had similar phosphate removal to axenic *A. protothecoides*. However, digester microbes had no significant impact ($p > 1$) on *C. sorokiniana*'s ability to remove phosphate from the digestate, mirroring the culture growth data. The results indicated that both nitrogen and phosphorus removal were roughly proportional to biomass growth rates: faster growth led to more rapid nutrient removal as expected. Thus, strategies to increase algal growth can improve nutrient uptake. This was especially apparent when growing *A. protothecoides* with the digester community compared to growing it in monoculture. Enhancing phosphorus uptake is particularly important when devising new technologies to treat poultry litter, given concerns about its high phosphorus content (Preusch et al., 2002). In addition to growth rates, cellular uptake of nutrients is governed by changes in biomass composition. Algae with higher protein content will utilize more nitrogen than those rich in polysaccharides and lipids, all else held constant, given the high nitrogen content of amino acids (Cai et al., 2013a). *C. sorokiniana* had a very high starch content and low nitrogen content (2.1-2.3%) relative to cultures grown on chemical medium (Table 3). Thus, despite rapid growth, *C. sorokiniana* did not remove ammonium as quickly as *A. protothecoides*.

3.6 COD removal through biological treatment

It was initially hypothesized that digester microbes would further consume COD in the digestate despite the switch to an aerobic environment. This should be possible if populations of facultative anaerobes are present. However, none of the biological

treatments led to large changes (>20%) in COD levels although there were trends in the data. The presence of axenic algae led to statistically significant increases in COD over the 5-day culture period (Figure 3), suggesting release of soluble photosynthate into the digestate similar to findings on winery wastewater (Higgins et al., 2017). It is well established that algae secrete a range of organic molecules into the surrounding environment including organic acids (Tolbert & Zill, 1956), lipids (Kind et al., 2012), and extracellular polymeric substances (Xiao & Zheng, 2016). Mixed cultures of *A. protothecoides* and *C. sorokiniana* also exhibited modest increases in COD but to a lesser extent than their axenic cultures. Due to the small effect size, the mixed cultures had COD levels that were neither significantly different from the initial digestate nor different from the axenic treatments. This suggests that the increase in COD, presumably triggered by release of algal photosynthate, was muted by the presence of wastewater microbes. COD generation by algae may also partially explain how the presence of algae reversed the decline in bacterial populations. Acting alone, cultures with digester microbes generally yielded insignificant decreases in COD. These results were somewhat surprising because the changes in COD were much smaller than those observed in a similar study carried out on winery wastewater, however, the general trends were similar. In that study, the presence of microbes led to 38% reductions in COD including consumption of algal photosynthate (Higgins et al., 2017). In that system, an oxidation tank was utilized between the digester and algal system which is an important distinction from the present study. Aerobic microorganisms from the oxidation tank may have been better suited to COD removal in the aerobic environment of the algal reactors, thus explaining some of the observed differences between the studies.

4. Conclusions

The presence of AD microbes roughly doubled the growth of *A. protothecoides* but had no effect on *C. sorokiniana* growth. *A. protothecoides* growth promotion likely stems from provision of thiamine metabolites by live bacteria. Biomass composition in both strains were unaffected by the presence of microbes but digestate chemistry led to very large increases in starch content and declines in protein content relative to cultures grown on chemical medium. Removal of ammonium and phosphate was roughly proportional to culture growth, although ammonia volatilization was also an important nitrogen removal mechanism. Axenic algae increased COD slightly although the presence of AD microbes muted this effect.

E-supplementary data of this work can be found in the online version of the paper.

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References

1. ATCC. 2013. ATCC Medium 5: Sporulation Agar.

2. Bai, X., Lant, P., Pratt, S. 2014. The contribution of bacteria to algal growth by carbon cycling. *Biotechnology and Bioengineering*, **112**, 688-695.

3. Bjornsson, W.J., Nicol, R.W., Dickinson, K.E., McGinn, P.J. 2013. Anaerobic digestates are useful nutrient sources for microalgae cultivation: functional coupling of energy and biomass production. *Journal of Applied Phycology*, **25**(5), 1523-1528.

4. Cai, T., Park, S.Y., Li, Y. 2013a. Nutrient recovery from wastewater streams by microalgae: Status and prospects. *Renewable and Sustainable Energy Reviews*, **19**, 360-369.

5. Cai, T., Park, S.Y., Racharaks, R., Li, Y. 2013b. Cultivation of *Nannochloropsis salina* using anaerobic digestion effluent as a nutrient source for biofuel production. *Applied Energy*, **108**, 486-492.

6. Chaump, K., Preisser, M., Shanmugam, S.R., Prasad, R., Adhikari, S., Higgins, B.T. 2018. Leaching and anaerobic digestion of poultry litter for biogas production and nutrient transformation. *Waste Management*.

7. Cheng, D., Li, D., Yuan, Y., Zhou, L., Li, X., Wu, T., Wang, L., Zhao, Q., Wei, W., Sun, Y. 2017. Improving carbohydrate and starch accumulation in Chlorella sp. AE10 by a novel two-stage process with cell dilution. *Biotechnology for Biofuels*, **10**(1), 75.

8. Cho, S., Lee, N., Park, S., Yu, J., Luong, T.T., Oh, Y.-K., Lee, T. 2013. Microalgae cultivation for bioenergy production using wastewaters from a municipal WWTP as nutritional sources. *Bioresource Technology*, **131**, 515-520.

9. Coker, C. 2017. Poultry Litter Digestion. *BioCycle*, **58**(8), 50.

10. Cole, A.J., Neveux, N., Whelan, A., Morton, J., Vis, M., de Nys, R., Paul, N.A. 2016. Adding value to the treatment of municipal wastewater through the intensive production of freshwater macroalgae. *Algal Research*, **20**, 100-109.

11. Croft, M.T., Warren, M.J., Smith, A.G. 2006. Algae need their vitamins. *Eukaryot Cell*, **5**(8), 1175-83.

12. de-Bashan, L.E., Antoun, H., Bashan, Y. 2008. Involvement of the indole-3-acetic acid produced by the growth-promoting bacterium *Azospirillum spp.* in promoting growth in *Chlorella vulgar*is. *Journal of Phycology*, **44**, 938-947.

13. Franchino, M., Comino, E., Bona, F., Riggio, V.A. 2013. Growth of three microalgae strains and nutrient removal from an agro-zootechnical digestate. *Chemosphere*, **92**(6), 738-744.

14. Franchino, M., Tigini, V., Varese, G.C., Mussat Sartor, R., Bona, F. 2016. Microalgae treatment removes nutrients and reduces ecotoxicity of diluted piggery digestate. *Science of The Total Environment*, **569–570**, 40-45.

15. Griffiths, M., Harrison, S.L. 2009. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *Journal of Applied Phycology*, **21**(5), 493-507.

16. Hanshew, A.S., Mason, C.J., Raffa, K.F., Currie, C.R. 2013. Minimization of chloroplast contamination in 16S rRNA gene pyrosequencing of insect herbivore bacterial communities. *J Microbiol Methods*, **95**(2), 149-55.

17. Higgins, B., Gennity, I., Fitzgerald, P., Ceballos, S., Fiehn, O., VanderGheynst, J. 2017. Algal-bacterial synergy in treatment of winery wastewater. *Nature Clean Water*, **1**(6).

18. Higgins, B., Labavitch, J.M., VanderGheynst, J.S. 2015a. Co-culturing *Chlorella minutissima* with *Escherichia coli* can increase neutral lipid production and improve biodiesel quality. *Biotechnology and Bioengineering*, **112**(9), 1801-1809.

19. Higgins, B., Thornton-Dunwoody, A., Labavitch, J.M., VanderGheynst, J.S. 2014. Microplate assay for quantitation of neutral lipids in extracts from microalgae. *Analytical Biochemistry*, **465**, 81-89.

20. Higgins, B., VanderGheynst, J. 2014. Effects of *Escherichia coli* on mixotrophic growth of *Chlorella minutissima* and production of biofuel precursors. *PLoS One*, **9**(5), e96807.

21. Higgins, B.T., Gennity, I., Samra, S., Kind, T., Fiehn, O., VanderGheynst, J.S. 2016. Cofactor symbiosis for enhanced algal growth, biofuel production, and wastewater treatment. *Algal Research*, **17**, 308-315.

22. Higgins, B.T., Nobles, D., Ma, Y., Wikoff, W., Kind, T., Fiehn, O., Brand, J., VanderGheynst, J.S. 2015b. Informatics for improved algal taxonomic classification and research: A case study of UTEX 2341. *Algal Research*, **12**, 545-549.

23. Holmes, B., Paddock, M.B., VanderGheynst, J.S., Higgins, B.T. 2019. Algal photosynthetic aeration increases the capacity of bacteria to degrade organics in wastewater. *Biotechnology Bioengineering*.

24. Ibekwe, M.A., Murinda, S.E., Murry, M.A., Schwartz, G., Lundquist, T. 2017. Microbial community structures in high rate algae ponds for bioconversion of agricultural wastes from livestock industry for feed production. *Science of The Total Environment*, **580**, 1185-1196.

25. Jenkins, A.H., Schyns, G., Potot, S., Sun, G., Begley, T.P. 2007. A new thiamin salvage pathway. *Nat Chem Biol*, **3**(8), 492-7.

26. Kelleher, B.P., Leahy, J.J., Henihan, A.M., O'Dwyer, T.F., Sutton, D., Leahy, M.J. 2002. Advances in poultry litter disposal technology – a review. *Bioresource Technology*, **83**(1), 27-36.

27. Kind, T., Meissen, J.K., Yang, D., Nocito, F., Vaniya, A., Cheng, Y.-S., Vandergheynst, J., Fiehn, O. 2012. Qualitative analysis of algal secretions with multiple mass spectrometric platforms. *Journal of Chromatography A*, **1244**, 139-147.

28. Kobayashi, N., Noel, E.A., Barnes, A., Watson, A., Rosenberg, J.N., Erickson, G., Oyler, G.A. 2013. Characterization of three Chlorella sorokiniana strains in anaerobic digested effluent from cattle manure. *Bioresource Technology*, **150**, 377-386.

29. Melnick, J., Lis, E., Park, J.-H., Kinsland, C., Mori, H., Baba, T., Perkins, J., Schyns, G., Vassieva, O., Osterman, A., Begley, T.P. 2004. Identification of the Two Missing Bacterial Genes Involved in Thiamine Salvage: Thiamine Pyrophosphokinase and Thiamine Kinase. *Journal of Bacteriology*, **186**(11), 3660-3662.

30. Mizuno, O., Li, Y.Y., Noike, T. 1998. The behavior of sulfate-reducing bacteria in acidogenic phase of anaerobic digestion. *Water Research*, **32**(5), 1626-1634.

31. Möller, K., Müller, T. 2012. Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review. *Engineering in Life Sciences*, **12**(3), 242-257.

32. Palacios, O.A., Gomez-Anduro, G., Bashan, Y., de-Bashan, L.E. 2016. Tryptophan, thiamine and indole-3-acetic acid exchange between Chlorella sorokiniana and the plant growth-promoting bacterium Azospirillum brasilense. *FEMS Microbiol Ecol*, **92**(6), fiw077.

33. Pampillon-Gonzalez, L., Ortiz-Cornejo, N.L., Luna-Guido, M., Dendooven, L., Navarro-Noya, Y.E. 2017. Archaeal and Bacterial Community Structure in an Anaerobic Digestion Reactor (Lagoon Type) Used for Biogas Production at a Pig Farm. *J Mol Microbiol Biotechnol*, **27**(5), 306-317.

34. Park, J., Jin, H.-F., Lim, B.-R., Park, K.-Y., Lee, K. 2010. Ammonia removal from anaerobic digestion effluent of livestock waste using green alga *Scenedesmus* sp. *Bioresource Technology*, **101**(22), 8649-8657.

35. Percheron, G., Bernet, N., Moletta, R. 1999. Interactions between methanogenic and nitrate reducing bacteria during the anaerobic digestion of an industrial sulfate rich wastewater. *FEMS Microbiology Ecology*, **29**(4), 341-350.

36. Prandini, J.M., da Silva, M.L.B., Mezzari, M.P., Pirolli, M., Michelon, W., Soares, H.M. 2016. Enhancement of nutrient removal from swine wastewater digestate coupled to biogas purification by microalgae Scenedesmus spp. *Bioresource Technology*, **202**, 67-75.

37. Preusch, P.L., Adler, P.R., Sikora, L.J., Tworkoski, T.J. 2002. Nitrogen and phosphorus availability in composted and uncomposted poultry litter. *J Environ Qual*, **31**(6), 2051-7.

38. Riano, B., Molinuevo, B., Garcia-Gonzalez, M.C. 2011. Treatment of fish processing wastewater with microalgae-containing microbiota. *Bioresour Technol*, **102**(23), 10829-33.

39. Shetty, P., Boboescu, I.Z., Pap, B., Wirth, R., Kovács, K.L., Bíró, T., Futó, Z., White, R.A., Maróti, G. 2019. Exploitation of Algal-Bacterial Consortia in Combined Biohydrogen Generation and Wastewater Treatment. *Frontiers in Energy Research*, **7**(52).

40. Singh, K., Lee, K., Worley, J., M. Risse, L., C. Das, K. 2010. Anaerobic Digestion of Poultry Litter: A Review. *Applied Engineering in Agriculture*, **26**(4), 677.

41. Singh, M., Reynolds, D.L., Das, K.C. 2011. Microalgal system for treatment of effluent from poultry litter anaerobic digestion. *Bioresource Technology*, **102**(23), 10841-10848.

42. Tan, F., Wang, Z., Zhouyang, S., Li, H., Xie, Y., Wang, Y., Zheng, Y., Li, Q. 2016. Nitrogen and phosphorus removal coupled with carbohydrate production by five microalgae cultures cultivated in biogas slurry. *Bioresource Technology*, **221**, 385-393.

43. Tanadul, O.U., Vandergheynst, J.S., Beckles, D.M., Powell, A.L., Labavitch, J.M. 2014. The impact of elevated CO2 concentration on the quality of algal starch as a potential biofuel feedstock. *Biotechnology and Bioengineering*, **111**(7), 1323-1331.

44. Tolbert, N.E., Zill, L.P. 1956. EXCRETION OF GLYCOLIC ACID BY ALGAE DURING PHOTOSYNTHESIS. *Journal of Biological Chemistry*, **222**(2), 895-906.

45. Wang, Q., Peng, H., Higgins, B.T. 2019a. Cultivation of Green Microalgae in Bubble Column Photobioreactors and an Assay for Neutral Lipids. *JoVE*(143), e59106.

46. Wang, Q., Prasad, R., Higgins, B.T. 2019b. Aerobic bacterial pretreatment to overcome algal growth inhibition on high-strength anaerobic digestates. *Water Research*, **162**, 420-426.

47. Wilkie, A.C. 2000. Fixed Film Anaerobic Digester, AgSTAR Press, US EPA. Washington DC.

48. Woertz, I., Feffer, A., Lundquist, T., Nelson, Y. 2009. Algae grown on dairy and municipal wastewater for simultaneous nutrient removal and lipid production for biofuel feedstock. *Journal of Environmental Engineering*, **135**(11), 1115-1122.

49. Xiao, R., Zheng, Y. 2016. Overview of microalgal extracellular polymeric substances (EPS) and their applications. *Biotechnol Adv*, **34**(7), 1225-1244.

Figure 1. Growth curves for algal and mixed cultures associated with *A. protothecoides* (A) and *C. sorokiniana* (B). Error bars are standard deviations based on n = 3 biological replicates. Values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test. N8-NH_4 medium was the chemical medium used to cultivate *A. protothecoides* and N8 was the chemical medium used to cultivate *C. sorokiniana*.

Figure 2. Average nutrient removal rates for algal and mixed cultures over 5-day batch. A) ammonium removal rate, B) algal nitrogen uptake rate, and C) phosphate removal rate are shown. Error bars are standard deviations based on n = 3 biological replicates. Values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

Figure 3. Chemical oxygen demand (COD) concentrations in digestate before and after treatment with algal and AD microbial cultures. Error bars are standard deviations based on n = 3 biological replicates. Values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

Table 1. Soluble components (mg/L) in poultry litter digestates diluted 4-fold

Component	Digestate 1	Digestate 2
COD	291 (9)*	249 (6.5)
Acetate	†	†
Ammonium	197 (8)	245 (21)
Nitrite	†	†
Nitrate	19.8 (0.2)	†
Phosphate	32.4 (1.7)	120 (2.9)
Sulfate	27.5 (0.2)	42.8 (0.2)
Chloride	40.0 (2.5)	44.3 (2.0)
Potassium	84.6 (3.2)	97.7 (9.2)

Sodium	13.1 (3.5)	31.5 (5.4)
Magnesium	2.8 (<0.1)	4.7 (0.7)
Calcium	18.5 (0.3)	17.7 (2.3)

*Reported values are averages of triplicate measurements; Standard deviations are in parentheses
†Not detectable.

Table 2. Bacterial and non-bacterial fraction of biomass growth over 5-day batch culture

Species Name	Treatment	Total Average Biomass Productivity (mg/L/d)	Bacteria Growth Productivity (mg/L/d)	Non-Bacterial Growth Productivity (mg/L/d)
A. protothecoides	Chemical medium (N8-NH4)	253 (21)* a #	-	253 (21) a
	Axenic algae	99 (11) c	-	99 (11) c
	Algae & AD Bacteria	169 (10) b	1.1 (0.3) a	168 (10) b
	AD Bacteria	-1.5 (0.9) d	-1.5 (0.9) b	-
C. sorokiniana	Chemical medium (N8)	218 (17) a	-	218 (17) a
	Axenic algae	207 (8) a	-	207 (8) a
	Algae & AD Bacteria	207 (14) a	4.6 (1.2) a	202 (14) a
	AD Bacteria	-5.0 (1.0) b	-5.0 (1.0) b	-

*Reported values are averages of biological triplicates; Standard deviations are in parentheses

[#]Within an algal strain and column, values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

Table 3. Biomass composition of algae and mixed cultures as a percent of dry weight

Species Name	Treatment	Neutral Lipid Content[*]	Crude Lipid Content	Starch Content	Cell Wall Content	Nitrogen Content	Crude Protein Content
A. protothecoides	Chemical medium (N8-NH_4)	- b [†]	26.8 (2.5) a	0.17 (0.01) b	51.0 (3.6) a	8.0 (0.1) a	36.8 (0.3) a
	Axenic algae	2.2 (0.8)[‡] a [#]	29.9 (3.6) a	0.62 (0.06) b	32.4 (1.2) b	5.01 (<0.01) b	23.0 (<0.1) b
	Algae & AD Bacteria	0.53 (0.43) b	22.2 (3.4) a	1.6 (0.6) a	34.5 (3.6) b	5.2 (<0.01) b	23.9 (<0.1) b
	AD Bacteria[¶]	-	-	-	-	-	-
C. sorokiniana	Chemical medium (N8)	0.20 (0.02) c	28.6 (2.5) a	4.7 (1.0) b	48.2 (3.2) a	7.2 (0.2) a	33.3 (1.1) a
	Axenic algae	2.1 (0.2) b	21.0 (0.9) b	43.1 (3.8) a	14.5 (1.3) b	2.1 (<0.01) b	9.6 (<0.1) b
	Algae & AD Bacteria	3.4 (0.6) a	23.1 (0.3) b	42.1 (2.2) a	23.7 (15.0) b	2.3 (<0.01) b	10.5 (<0.1) b
	AD Bacteria[¶]	-	-	-	-	-	-

[*]All values are percentages of harvested dry weight on a mass basis. Values do not add to 100% given overlap of some measured components across assays

†Not detectable.
‡Reported values are averages of three biological replicates; Standard deviations are in parentheses.
¶Biomass contents are not reported for AD bacteria due to insufficient biomass
#Within an algal strain and column, values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

Table 4. Magnesium and calcium concentrations in pre- and post-treated digestates

Species Name	Treatment	Initial Mg Concentration (mg/L)	Final Mg Concentration (mg/L)	Initial Ca Concentration (mg/L)	Final Ca Concentration (mg/L)
A. protothecoides	Sterile axenic algae	2.71 (0.06)	2.22 (0.26)	17.67 (0.23)	18.07 (1.10)
	Algae & AD Bacteria	2.82 (0.11)	1.46 (0.11)	18.54 (0.66)	19.29 (1.21)
	AD Bacteria	2.78 (0.03)	3.19 (0.29)	18.52 (0.27)	17.46 (2.54)
C. sorokiniana	Sterile axenic algae	4.17 (0.29)	-	15.12 (1.23)	7.93 (6.89)
	Algae & AD Bacteria	4.27 (0.66)	-	15.99 (1.59)	9.25 (8.01)
	AD Bacteria	4.69 (0.70)	6.42 (5.56)	17.74 (2.33)	15.25 (3.95)

*Reported values are averages of triplicate measurements for each treatment; Standard deviations are in parentheses.