Effects of Growth-Promoting Bacteria in Suspended Green Algae Cultures for Bioenergy and Bioproduct Development

by

Haixin Peng

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Approved by

Brendan Higgins, Chair, Assistant professor, Department of Biosystems Engineering
Yifen Wang, Professor, Department of Biosystems Engineering
Yi Wang, Associate Professor, Department of Biosystems Engineering
Luz de-Bashan, Affiliate Associate Professor, Department of Entomology and Plant Pathology

Abstract

With the exhaustion of fossil-based fuels, microalgae have attracted great interest as a renewable energy feedstock. Microalgae are photosynthetic microorganisms with rapid growth and the potential for production of lipids, proteins, and carbohydrates. However, the capital costs of algae production have been prohibitive for commercial biofuel production. Efforts to further increase algal growth rates and lipid content have attracted significant attention over the past decades to improve biofuel cost-effectiveness. Nevertheless, a fledgling algal industry has emerged in the past decades, but it has primarily focused on protein, nutraceutical, and other high value products from algae. Efforts to improve algal growth rates, however, will benefit nearly all applications of algae. One promising approach is coculturing algae with bacteria to increase algae growth rates and production of biofuel precursors, achieving a win-win outcome. In the research described in this dissertation, efforts were made to improve our understanding of how bacteria alter growth and composition of suspended algae cultures, with a particular focus on plant-growth promoting bacteria (PGPB).

PGPB, such as *Azospirillum brasilense*, have the potential to significantly increase algal growth rates through a variety of mechanisms including the production auxin hormones such as indoel-3-acetic acid (IAA). In Chapter 3, a set of lab-scale photobioreactor experiments are described in which the effect of live *A. brasilense*, exogenous IAA, and spent medium from *A. brasilense* are studied on two green algae. *A. brasilense* and IAA were found to promote growth (11-90%) at the expense of energy storage product accumulation in suspended cultures of *Chlorella sorokiniana* and *Auxenochlorella protothecoides*. Co-cultures and exogenous IAA stimulated growth in both algae types, but the effect was stronger in *C. sorokiniana*. These same

treatments also suppressed neutral lipids (particularly triacylglycerol) and starch during exponential growth of *C. sorokiniana*. IAA and co-cultures suppressed starch in *A. protothecoides*. Spent medium from *A. brasilense* was also tested and found to promote growth slightly in *C. sorokiniana* but significant suppress growth in *A. protothecoides*. It also led to significantly different compositional changes compared to using live *A. brasilense*, indicating that bioactive constituents in *A. brasilense* secretions are transient or that physical cell attachment is important for ensuring adequate mass transfer of these constituents.

The finding that *A. brasilense* suppressed starch and neutral lipid content of algae raised questions about how *A. brasilense* mediates oxidative stress in algae. Many algae, including those in this study, are known to accumulate neutral lipid and starch under conditions that induce oxidative stress. Consequently, it was hypothesized that *A. brasilense* alleviates oxidative stress in algae, thereby promoting growth and suppressing energy storage products. Moreover, PGPB bacteria are known to alleviate the effects of stress conditions in several plants, but the stress-alleviating effects on the algae are not well understood. To evaluate the impacts of *A. brasilense* on oxidative stress in *C. sorokiniana* and the consequent changes in biomass composition, algae were co-cultured with *A. brasilense* under Cu and nitrogen stressors as described in Chapter 4. The results showed that both stressors induced oxidative stress and reduced chlorophyll content. Adding *A. brasilense*, and to a lesser extent, exogenous IAA, could partially rescue *C. sorokiniana* from the effects of oxidative stress. In fact, there was no significant difference in ROS levels between nitrogen-limited co-cultures and nitrogen-replete monocultures of *C. sorokiniana*. This indicates that *A. brasilense* could rescue the algae from the nitrogen limitation stress, which in turn explained why the presence of *A. brasilense* led to faster growth, higher chlorophyll content, and lower starch content, as we observed in this study.

The finding that the PGPB, *A. brasilense*, could promote green algae growth by 11-90%, depending on the algae strain, raised questions about how much more effective PGPB are compared to non-PGPB bacteria. Past research has shown that the non-PGPB, *E. coli*, can increase algal growth by similar margins. In Chapter 5, a side-by-side comparative study between a PGPB and non-PGPB organism is described. Efforts were made to understand the benefit of "universal" symbiosis mechanisms between algae and bacteria (e.g. cofactor exchange, dissolved O_2-CO_2 exchange) versus the benefits of PGPB-specific mechanisms (e.g. hormone exchange). The effect of the PGPB, *Azospirillum brasilense*, the non-PGPB, *Escherichia coli*, and a recently-isolated strain, *Bacillus megaterium,* were tested on three green algae: *C. sorokiniana* UTEX 2714, *A. protothecoides* UTEX 2341 and *C. sorokiniana* UTEX 2805. Results showed that, all three bacteria stimulated growth in *C. sorokiniana* UTEX 2714 and *A. protothecoides* UTEX 2341, but the effect was stronger in *C. sorokiniana*. They all led to significantly different compositional changes. Interestingly, the PGPB, *A. brasilense* slightly suppressed growth in *C. sorokiniana* UTEX 2805, although the effect was not statistically significant, whereas the other two bacteria significantly increased growth in this strain. This was surprising given that *A. brasilense* strongly promoted growth in *C. sorokiniana* UTEX 2714. Additionally, the algae biomass composition, nutrient uptake as well as algal photosynthate changes were measured. The latter indicated significant consumption and cycling of photosynthate, likely generating CO_2 for algae. Moreover, the riboflavin metabolite, lumichrome was also detected in co-cultures containing *A. brasilense* (0.4-0.6 ng/ml) and *E. coli* (5.5-13 ng/ml). A dose response study showed that lumichrome at 1 to 10 ng/ml led to small but statistically significant increases in growth of *C. sorokiniana* UTEX 2805 and *A. protothecoides*. Riboflavin metabolites and other vitamin cofactors from a wide range of bacteria likely confer

growth benefits to algae. Such mechanisms are present in interactions between algae and both PGPB and non-PGPB. In sum, understanding such coculture relationship details may provide guidance for the cost-effective algae bioenergy and bioproduct development.

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Chapter 1 Introduction

Natural resources, energy, and environmental degradation are three major challenges facing humanity in the 21st century. The National Academy of Sciences & Medicine has identified a number of Grand Challenges in the field of Environmental Engineering including sustainable supply of food, water and energy (Board, National Academies of Sciences, & Medicine, 2019). Such challenges also clearly fall under the purview of Biosystems Engineering. The increasing fossil fuel demand and the greenhouse gas emissions have led to serious environmental problems. Algae, as the "third generation" biofuel feedstock, has been of great interest for the various advantages such as adaptability to non-arable land, year-round cultivation, high productivity, and the production of high value molecules (Ahmad et al., 2011).

Microalgae are photosynthetic microorganisms with rapid growth and assimilate carbon dioxide into organic compounds including lipids, proteins, and carbohydrates. Compared to other plants, algae can grow on freshwater, saltwater, and wastewater. In addition, algae can produce a range of products including nutraceuticals, protein, and biofuel precursors (Laurens et al., 2017) and services such as wastewater treatment (Singh et al., 2016). Despite the great potential for microalgae, they are currently commercially viable only as a feedstock for high-value product synthesis or as aquaculture feed. For the algal biofuel market, increasing growth rates and lipid content were the most crucial barriers in reducing biofuel costs (Chisti, 2007). This is because

algae production requires significant capital investments and capital utilization is maximized by highly-productive organisms.

Coculturing algae with bacteria have been regarded as a great platform for increasing algae growth rates and production of biofuel precursors (Fuentes et al., 2016; Yao et al., 2019). Bacteria grow in the presence of algae in nature and are known to confer benefits to algae in many cases, including the exchange of metabolites (Munoz & Guieysse, 2006), exchange of oxygen and carbon dioxide (Bai, Lant, & Pratt, 2014), and through production of hormones (de-Bashan et al., 2008). Plant growth promoting bacteria (PGBP) have gained particular attention given their ability to promote growth in green algae via secretion of plant-growth promoting hormones (Bashan & Hartmann, 2009; Kim et al., 2014). As described in Chapter 3 of this dissertation, we first investigated whether the model PGPB, *Azospirillum brasilense*, and its secretion of the auxin hormone, indole-3-acetic acid (IAA), would simultaneously increase growth and accumulation of energy storage products in suspended green algae cultures. This turned out to be true for growth enhancement, but energy storage products were generally suppressed by *A. brasilense* and IAA.

Numerous studies confirmed that both chemical or physical stimulation stressors , such as temperature, salinity, nutrients, and chemicals stimulate accumulation of energy storage products such as neutral lipid and starch (Chokshi et al., 2017a; Markou & Nerantzis, 2013). These environmental stressors often lead to intracellular accumulation of reactive oxygen species, but

also suppress algal growth. Whether bacterial co-cultures can help algae simultaneously increase growth and accumulation of energy storage products has been suggested (Choix et al., 2012) but is still poorly understood. Given our findings in Chapter 3, we hypothesized that live *A. brasilense* and its secretion IAA could help alleviate oxidative stress in algae, thereby increasing algal growth but suppressing energy storage product accumulation. Experiments to test this hypothesis are presented in Chapter 4.

Then in Chapter 5, we raise the question how much more effective PGPB are compared to non-PGPB organisms during the algae coculture systems. The latter organisms do not make auxin hormones but have been reported to provide significant growth benefits through mechanisms like O_2-CO_2 exchange and cofactor provision.

Study Rationale and Research objectives

Objective 1: Quantify the impacts of *Azospirillum brasilense*, and its secretion of indole-3-acetic acid on growth and macro-composition in suspended green microalgae cultures (Chapter 3)

Objective 2: Determine the impacts of *Azospirillum brasilense* on oxidative stress in *C. sorokiniana* and the consequent changes in biomass composition (Chapter 4)

Objective 3: Determine the relative benefit of using a plant growth promoting bacteria *Azospirillum brasilense* over other non PGPB bacteria *Escherichia coli,* and *Bacillus megaterium* in terms of algae growth (Chapter 5).

This dissertation is mainly prepared from three manuscripts presented in Chapter 3-5. Chapter 3 has already been published in Algal Research:

1. Peng, H., de-Bashan, L. E., Bashan, Y., & Higgins, B. T. (2020). Indole-3-acetic acid from *Azosprillum brasilense* promotes growth in green algae at the expense of energy storage products. *Algal Research*, 47, 101845.

Chapter 2 Literature Review

2.1 Algae

To reduce the continued use of fossil fuels and global warming, the Renewable Fuel Standard (RFS) program mandates over 30 billion gallons per year of renewable biofuels by 2022 (Bracmort, 2018). International Energy Agency also released that the biofuels will target to grow 40% from until 2040 (Bioenergy, 2017). Compared with other biofuel feedstock, algae are commonly referred to third generation 'energy crops' and represent a key component to the sustainable biofuel industry in the long term (Behera et al., 2015). Algae capture more than 40% of the total carbon dioxide fixation across the world (Yun et al., 1997). Additionally, algae had the unique high oil productivity (50,000 to 140,000 L/ha/year), compared with corn at 172 L/ha/year and soybeans at 446 L/ha/year (Chisti, 2007).

The term algae encompass both prokaryotic (cyanobacteria) and eukaryotic organisms. The eukaryotes can be divided into macroalgae (multicellular) and microalgae(unicellular), with different range in size. Macro algae, also named seaweed, can achieve lengths of around 60 m (Barsanti & Gualtieri, 2014). Microalgae are the microscopic photosynthetic organism (smaller than 400 um), with less complex structure and typically more potential production of lipids. Compared with other higher plants photosynthetic efficiency (0.5-2.2%), algae can generally achieve more efficient conversation of light to fixed carbon (6-20%) due to the simple cellular

structure (Kumar et al., 2010). This is why algae vastly outperform plants in terms of aerial growth rates. Currently, 30,000-40,000 algae species has been reported, and around 10 species were commercially produced (Guiry, 2012). Different algae species live in a diverse range of environments, including ponds, rivers, lakes, seawater or even soil. Therefore, due to the diversity and high productivity, researches are trying to explore the algae in multiple applications, such as biofuel (Rawat et al., 2013), nutraceuticals (Yen et al., 2013) and pharmaceuticals (García & Galán, 2017) .

2.1.1 Algae biomass composition and cultivation

In the chloroplast, algae convert carbon dioxide into three major macromolecules (lipid, carbohydrates, protein) and other cellular compounds during photosynthesis. Depending on different species and environment, the algae biomass exhibits different variations. Generally, the lipid content is around 10-50% of total dry cell weight, with carbohydrates and proteins together accounting for about 60% of the mass (Spolaore et al., 2006).

Neutral lipid and starch are the main energy storage for the algae. For the lipid profile, it can be divided into polar lipids (known as structural lipids, such as phosphor glycerides, glycosyl glycerides), and nonpolar lipid (also known as storage lipid, such as acyl glycerol, free fatty acids). Sterile esters are also an important lipid component. The sterol and polar lipid (phospholipid) are used to construct the cell membrane to provide the selective transport and barrier for cells (Gurr et al., 2016). In terms of nonpolar lipid, the most primary part triacyl

glycerides TAG are the main energy storage for the algae, which help them survive in severe environment conditions. On the one hand, after extraction, the TAG could be trans esterified to C16-C18 fatty acid FAMEs, more commonly known as biodiesel. For algal biodiesel FAMEs made from palmitic acid, stearic acid, oleic acid, and linoleic acid are most valuable (Cha et al., 2011). Numerous studies tried to apply nutrient limitation (Peccia et al., 2013) and genetic engineering method (Rasala et al., 2013) to accumulate the lipid content in algae for biofuel production. Neutral lipid can be made into biodiesel. Algae with high lipid content are also more favorable for biocrude production through hydrothermal liquefaction. Nutrient stress is a widely-studied approach to increase algal lipid content (Peccia et al., 2013). With complete nutrient stress, the microalgae *Chlorella* obtained the highest 49% cellular neutral lipids (White, Anandraj, & Bux, 2011). Other stress sources may also be used to promote lipid accumulation in algae (Esakkimuthu et al., 2016). The fresh water microalgae *Chlorella vulgaris* and seawater *Tetraselmis chii* increased 20% lipid content by shear stress of a pulsed wave, after 8 days accumulation (Savchenko et al., 2017). Additionally, another approach is genetic manipulation. During fatty acid synthesis, the first rate-limiting synthesis step (from acetyl-CoA to malonyl-CoA), is catalyzed by the enzyme acetyl-CoA carboxylase ACCase (Roessler, 1990). Ruffing (2013) borrowed and cloned the ACC genes from the unicellular model green algae *Chlamydomonas reinhardtii* CC-503 and expressed them efficiently for FFA production in the cyanobacteria, *Scenedesmus elongatus* PCC7942, with 5.3-31.8 folds increase. In addition to

biofuel production, algal lipids have great potential in the nutraceutical industry (Yen et al., 2013). Certain algae are rich in polyunsaturated fatty acids PUFAs, such as docosahexaenoic acid DHA and eicosapentaenoic acid EPA. The microalgae *Nannochloropsis* could accumulate EPA about 22% for the total fatty acids at a salinities of 20 g/L (Mitra et al., 2015). Burja et al (2006) screened four marine algae *Thraustochytrids* from Atlantic Ocean and found DHA at around 5.18 to 83.63 mg/g biomass.

Algae carbohydrates are continuously storied in the cell wall and excreted by the cells. The polysaccharide contents vary greatly in different algal species (de Souza et al., 2019). The cellulosic inner cell wall of the marine microalgae *Nannochloropsis* was protected by the outer algaenan layer. They contained 68% glucose, and 4-8% ribose, xylose, fucose, rhamnose, galactose and mannose (Scholz et al., 2014). The freshwater microalgae *Scenedesmus* exuded the exopolysaccharides (mainly consisting of fucose and mannose), which bind and detoxify the copper in the environment (Lombardi et al., 2005). Also, algae starch could be the potential carbon sources for the ethanol production by fermentation or hydrolysis (Ho et al., 2013).

In the case of algae protein, RuBisCO is the major fraction in all photosynthetic organisms (Williams & Laurens, 2010). In industry, algae protein accounts for 18% of total protein market (Bleakley & Hayes, 2017). *Spirulina* contains 40-70% protein and various bioactive peptides, and is one of the most widely produced algae in industry (Ovando et al., 2018). Al-Dhabi and Valan Arasu (2016) collected 37 commercial *Spirulina* strains and found

the total amino acids were around 11.49-56.14 mg/100 g algae dry weight (the individual essential amino acid account for 17-39%). Moreover, as the algae biomass feedstock, the pigments and vitamins have to be considered since they are also of great value. All the pigments and vitamins vary greatly depending on the types of algae. The cyanobacteria pigment mainly consists of the chlorophyll, xanthophylls and carotenoids (Williams & Laurens, 2010). Within the carotenoids (i.e.β-carotene, lutein and astaxanthin), the astaxanthin was known for the strong antioxidant activity. Compared to shrimps and yeast, the algae *H. pluvialis* contained over 4% of total dry cell weight (Shah et al., 2016). The β-carotene from *D. salina* was also developed to grow in the seawater without being easily contamination (Tran et al., 2014).

2.1.2 Algae bioenergy and other commercial algae applications

As the non-food resources, algae could be one of the most important potential biofuel feedstocks (Ahmad et al., 2011). In the early 1950s, the use of algae for the methane gas production was first reported (Daniels, 1955). Then, using wastewater as a medium and nutrient source, algae biofuel production was actively studied with the energy crisis in 1970s (Benemann et al., 1978). In the 1980s, the focus of algae biofuel changed into the large-scale impacts for the fossil energy. From 1970 to the mid 1990s, the US government funded the Aquatic Species Program (ASP) program to develop the algal biofuel and co-production, with more than 3000 algae strains established (SHEEHAN, 1998). Later, DOE programs found the open raceway systems were more attractive because of the relative low cost, compared to

photobioreactors (Benemann, 2003). Over the last decades, synthetic biology and omics (genomics, proteomics transcriptomes and metabolomics) have been used to study algae (Guarnieri & Pienkos, 2015), in the hope that these tools could lead to breakthroughs in algal biotechnology. Most genetic modifications have been aimed at *Cyanobacteria* and a few eukaryotic microalgae such as *Chlamydomonas*, but the *Spirulina* was found to be extremely recalcitrant to transformation (García et al., 2017).

Although there is still no commercially-produced algal biofuel due to problems with cost-effectiveness, much research and development has been carried out in this eco-friendly area (Yen et al., 2013). A variety of pathways are available for processing algae into fuel and other products. A schematic for algae biomass conversion and bioprocessing is shown in **Figure 1** (Behera et al., 2015). In biofuel conversion processes, the algae cell wall was first broken down, then typically deconstructed through thermochemical, biochemical, transesterification, with high temperature (pyrolysis, gasification, hydrothermal liquefaction) or low temperature (with biological enzymes or chemicals fermentation) (Brennan & Owende, 2010). Specifically for algae biodiesel, through the transesterification of TAG with methanol, a mixture of fatty acid methyl esters FAME are obtained (Durrett et al., 2008). Typically, the short harvest cycle, strong adaptability and high productivity efficiency are the main advantages for algae biofuel (Hu et al., 2008). However, there are still some challenges for the commercial scale up production, from the upstream and downstream, such as: 1) algae biology, in particular the

desirable and stable algae strains; 2) algae cell cycle and TAG storage production; 3) sustainable large-scale culture systems; 4) scalable feedstock production 5) cost-effective harvest and biorefinery processing (Chisti, 2007; Shuba & Kifle, 2018).

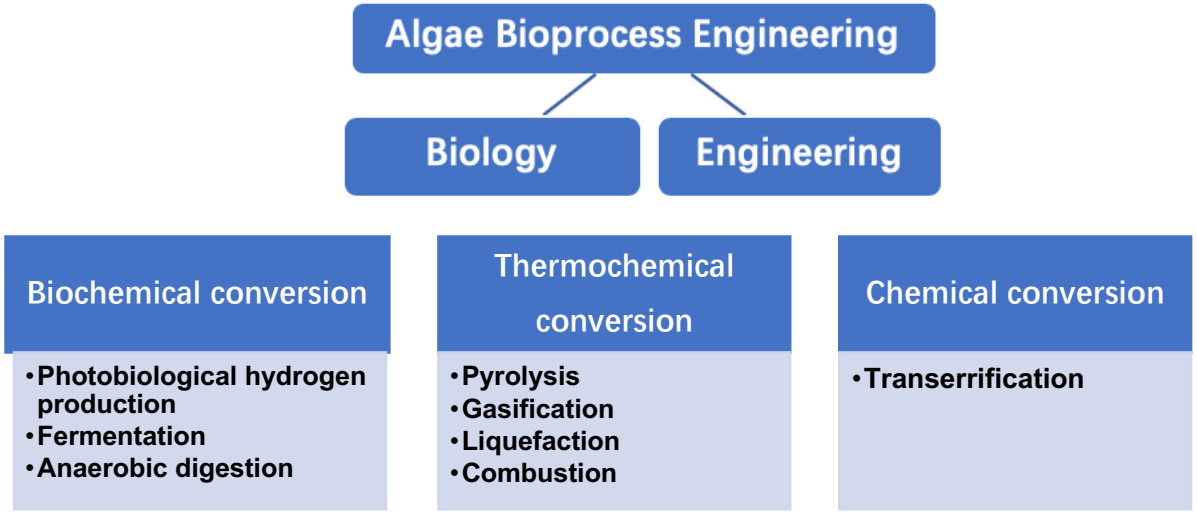

Figure 1 Algae biomass conversion and bioprocessing engineering as adapted from (Behera et al., 2015)

Other than the biofuel, some algae researches and industries have also been shifted into animal feeding, nutraceuticals, pharmaceuticals and cosmeceuticals (Yen et al., 2013). Generally, the global market for microalgae production was around $6.5 billion (Mobin & Alam, 2017). The commercial products were mainly from the *Chlorella*, *Spirulina*, and *D. salina*. There were over 70 *Chlorella* manufacturing companies all over the world. More than 50% *Spirulina* was used as the feed supplement and around 30% algae production was for animal feeding (Spolaore et al., 2006). So far, several major market players include the Organic *Chlorella* pills, *Spirulina* powder, EPA/ DHA dietary supplement, algae culinary oil, natural

algae astaxanthin, algae sustainable polymer, and some cosmetic production (algae hair coloring, facial mask, skin lotion) (Ariede et al., 2017; García et al., 2017).

2.2 Algae and bacteria co cultivation

In recent years, numerous studies have focused to investigate all kinds of growth-enhancing strategies, include mixotrophic growth (Higgins & VanderGheynst, 2014), genetic modification (Rasala et al., 2013), and cultivation conditions that maximize the photosynthetic efficiency (Granata, 2017). Specially, coculturing for algae and bacteria become a promising and hot topic platform nowadays. Maintaining axenic algae has proven to be costly due to ease of contamination during cultivation (Langer, 2008). Moreover, a significant body of research has shown that axenic algae often underperform those growing with bacteria (Yao et al., 2019). Artificial cocultivation could provide the solution for the bottlenecks in biomass production and high value coproduct synthesis (Padmaperuma et al., 2018).

In mixed cocultures, microorganisms (including microalgae) live in the communities with varying symbiotic relationships of competition, mutualism and commensalism relations. The effect of bacteria could be classified into beneficial, antagonistic and neutral on algae growth (Santos & Reis, 2014). Han et al. (2016) examined three algae *Tetraselmis chuii, Cylindrotheca. fusiformis* and *Nannochloropsis. gaditana*, with two bacteria. After 33 days of co-cultivation, the algae *T. chuii* and *C. fusiformis* had a remarkably higher cell density, however, the algae *N. gaditana* was clearly inhibited by the bacteria. Bacteria can inhibit algae by secretion of algicides

(Fu et al., 2012), nutrient competition (Helliwell et al., 2011) or cell wall degradation (Demuez & González, 2015). In the coculture of the algae *Emiliania huxleyi*, the bacteria *Roseobacter* initially promoted algal growth but ultimately led to algae death (Segev et al., 2016). Here, **Table 1** summaries the communication and application of algae-bacteria in recent decades (Fuentes et al., 2016).

2.2.1 Bacteria promote algae growth by hormone secretion

Plant growth promote bacteria PGPB are typically found in the rhizosphere of plants and secrete hormones which are known to promote certain green algae (Amavizca et al., 2017; Kim et al., 2014). The term Plant Growth Promoting Rhizobacteria (PGPR) was first used by Kloepper et al. (1989). Then, it was later extended to Plant Growth Promoting Bacteria (PGPB) by Bashan and Holguin (1998). They could directly or indirectly affect the plant growth, by mechanisms such as nitrogen fixation, hormone production, and other mechanisms of biocontrol (Beneduzi et al., 2012). The PGPB *A. brasilense* Cd and *B. pumilus* ES4, secreted volatile compounds, promoting the microalgae *C. sorokinianna* growth up to 6-fold increase (Amavizca et al., 2017). Lee et al. (2019) also showed that another PGPB bacteria *Achromobacter* sp. CBA4603 stimulated algae growth by the hormones and volatile compounds.

Table 1 Examples of algae-bacteria interaction, modified from (Fuentes et al., 2016)

Algae	Bacterium	Symbiosis factor	Results	Reference
L. rostrata	*M. loti*	Vitamin B_{12}	Bacterium delivered the vitamin and promoted the B12-independent algae growth; direct physical contact was not required.	(Kazami et al., 2012)
T. pseudonana	*R.pomeroyi*	Vitamin B_{12}	Bacterium recovered B_{12}-limited algae restoring growth rates comparable to those of axenic cultures supplemented with exogenous B_{12}. The biogeochemical metabolites DHPS in this coculture systems were crucial for marine carbon and sulfur cycles.	(Durham et al., 2015)
S. trochoidea	*Marinobacter*	Vibrioferrin	Bacteria promoted algal assimilation of iron by facilitating photochemical redox cycling of this nutrient	(Amin et al., 2009)
D. salina	*Halomonas*	Siderophore	Bacteria increased the algae cell densities around 6-fold under iron limited condition	(Baggesen, 2014)
Hemiaulus	*Richelia*	Nitrogen	Bacteria transferred 97.3% of the fixed nitrogen to the diatom partners.	(Foster et al., 2011)
C. sorokiniana	*A. brasilense*	Hormone IAA and volatile compounds	Volatile compound of PGPB *A. brasilense Cd* remotely increased the algae growth up to 6-fold.	(Amavizca et al., 2017)
Scenedesmus	*Pseudomonas*	Hormone IAA	Bacteria promoted the algae growth through secreting hormone IAA, and algae could selectively enhance IAA secretion in turn.	(Dao et al., 2018)
A. protothecoides	*E. coli*	Thiamine cofactor	Algae *A. protothecoides* cannot synthesis some vitamins de novo, so they use vitamins or precursors produced by bacteria during cocultivation.	(Higgins et al., 2016)
Chlorella	Bacteria in open culture	CO_2 exchange	For the mass transfer of carbon dioxide, the bacteria were helpful for inorganic carbon limitation.	(Bai et al., 2015)
C. sorokiniana	*Ralstonia*	CO_2 exchange	After coculturing, algae exhibited the highest growth rates and supported the fastest pollutant removal rate under photosynthetic oxygenation.	(Muñoz & Mattiasson, 2003)

Among all these plant growth promoters, the phytohormones are understood to be a key mechanism of interaction. The auxin phytohormone, indole-3-acetic acid (IAA), could serve as chemical messengers to coordinate cellular activities in plants (Steffen et al., 2009). It regulates the rate of cell elongation, division and expansion (Zhang & Van, 2014). The presence of IAA has been found both in unicellular and multicellular algae. Although the full aspects of algae hormone systems was limited, numerous studies showed the functions was similar with the higher plants (Lu & Xu, 2015; Tarakhovskaya, Maslov, & Shishova, 2007). In the study by Piotrowska-Niczyporuk and Bajguz (2014), the exogenous auxins acted in a concentration dependent manner on *Chlorella vulgaris* growth. Later, Amin et al. (2015) indicated that the bacteria *Sulfitobacter* could produce the IAA through the indole-3-acetonitrile (IAN), indole-3-acetamide (IAM) and tryptamine (TAM) pathways, therefore promote the algae *P. multiseries* cell division.

2.2.2 Other algae-heterotroph coculture mechanism

In addition to phytohormones, chemical and cofactor exchange are involved in algae growth promotion. Of particular importance are B vitamin cofactors (Croft et al., 2005) and amino acid (Palacios et al., 2016).

In terms of vitamins, more than 50% of microalgae are auxotrophic for vitamin B_{12} cobalamin, 20% require vitamin B_1 thiamine and 5% for B_7 biotin (Croft., 2006). Vitamin B_{12} is normally synthesized by bacteria with over 20 enzyme-catalyzed reactions, whereas algal autotrophically

had lost the enzyme cobalamin-independent methionine synthase (METE), and instead relied on the enzyme methionine synthase, METH, which needs B_{12} as a cofactor (Helliwell et al., 2011). The latter enzyme is more efficient and is preferentially used when B_{12} is available. Grant et al. (2014) found that bacteria *M. loti* could provide vitamin B_{12} for the algae *L. rostrata*, and obtain the photosynthate from the algae. Moreover, Higgins et al. (2016) suggested that the thiamine cofactor symbiosis could increase algae growth and double neutral lipid content, during the algae *Auxenochlorella protothecoides* and *scherichia coli* coculture system with mixotrophic condition.

Another well-known mechanism of symbiosis between algae and bacteria is the exchange of dissolve oxygen and carbon dioxide. Bacteria can obtain a supply of organic photosynthate and dissolved oxygen from algae and produce carbon dioxide in return. Bacteria can also mineralize exogenous organic carbon sources, such as those found in wastewater. During cultivation, carbon constitutes over 50% of the algae biomass, thus a sufficient supply of carbon dioxide is crucial to ensure rapid growth (Show et al., 2017). The solubility of CO_2 in water is also very low, and often the limiting nutrient, so mechanisms to generate dissolved CO_2 in situ are beneficial. When coculturing *C. vulgaris* with the yeast *Saccharomyces*, the microbial ecology in these systems was controlled by the CO_2 production, with 76% being used by algae (La & Taidi, 2019). Bai et al. (2015) investigated the mass transfer of carbon dioxide in a photobioreactor and found the carbon could limit the algal *Chlorella* growth. The bacteria were helpful in overcoming inorganic carbon limitation. The symbiotic relationship also benefits

bacteria. In the cocultivation of the algae *A. protothecoides* with *E. coli*, algal photosynthetic aeration increased the bacterial rate of organic degradation, with 18-66% faster COD removal when algae were present (Holmes et al., 2020).

For other mechanism, Angelis et al. (2012) reported that coculturing of the algae, *Agaricus blazei,* with the fungi *Vacromycetes* resulted in a 61% increase in the biomass productivity due to secretion of water soluble exopolysaccharides (EPS) production. Toi et al. (2014) stated that the N assimilation of algae *Dunalinlla tertiolecta* was improved after co-feeding with bacteria after 24 hours.

In case of application for algae-bacteria coculturing, the lipid content of algae *Characium* was increased 10% by unidentified extracellular compounds after co cultivation with heterotrophic bacteria *Pseudomonas* (Berthold et al., 2019). By co-culturing the *C. sorokiniana* L3 with other three dominant bacterial in the fermentation wastewater, 77.8% NH_4^+-N and 45.6% total PO_4^{3-}-P were removed compared to the axenic culture (Qi et al., 2018). The last decades have brought the exploration of the algae coculture systems, however, there are still some questions to be addressed. For example, constructing the potential coculture partner, revealing the unknow new signal, the tradeoff between optimal growth and lipid and starch accumulation, the sustainable reactor design (photo, stirred, fixed-bed, matrix immobilization, spent medium), the continuous or fed-batch cultivation (Fuentes et al., 2016).

2.3 Oxidative stress in algae

Under normal metabolic conditions, algae produce reactive oxygen species ROS, such as hydroxyl radicals(OH•), superoxide anions(O_2^-), and hydrogen peroxide(H_2O_2), which are more reactive than molecular oxygen (J. Hancock, R. Desikan, & S. Neill, 2001). The molecular O_2 accepts four electrons and two molecular H_2O are formed, but sometimes, they catch one electron at a time, yielding ROS. Among ROS, the hydroxyl radical is the most reactive in the oxygen species (Mallick & Mohn, 2000).

2.3.1 ROS production and metabolism in algae

For algae, there are several ways for the reactive oxygen species ROS production. In general, the reactive oxygen species are biologically produced as the natural byproduct during the various metabolic process such as photosynthesis and respiration, in mitochondria and chloroplasts (Mittler et al., 2011). Superoxide anion can be produced with various metabolic processes in the chloroplast or the antioxidation in the reduced mitochondrial electron-transport (Mallick & Mohn, 2000). In addition, the concentration of ROS increases when algae are subjected to certain stress conditions, including high light intensity (Lesser & Farrell, 2004), heat exposure (Ledford et al., 2004), metal exposure (Tripathi et al., 2006), and nutrient limitation (Zhang et al., 2013).

At higher levels, ROS overwhelms the cellular antioxidant defenses and becomes toxic. Lipid peroxidation was reported in higher plant cells after stress. ROS is easily targeted on the chain breakage for the polyunsaturated fatty acid (Sharma et al., 2012). Other than the lipid

peroxidation, excessive ROS levels can result in the irreversible oxidative damage for the protein and DNA, such as enzyme inactivation, peptide fragmentation, and DNA bases modification (Davies, 2000). After the ROS production exceeds the detoxification, all these damages occur.

To protect against ROS generation, the cell employs a number of enzymatic and non-enzymatic antioxidant defenses. There are enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and non-enzyme antioxidants, such as β-carotene and polyphenols (Apel & Hirt, 2004). Hence, the ROS level can be controlled to avoid the toxicity due to the algae oxidative stress in general. Intracellular SOD converts O_2^- to O_2 and H_2O_2, and is one of the first protectors against oxidative stress (Alscher, Erturk, & Heath, 2002).

For algal biotechnology, high levels of oxidative stress lead to significant biochemical changes in many algae including accumulation of neutral lipids (Sun et al., 2014), pigments (Udayan & Pandey, 2017), and starch (Markou & Nerantzis, 2013). Recent research has shown that environmental stressors such as nitrogen limitation are indirectly linked to lipid accumulation via oxidative stress pathways (Zhang et al., 2013). The neutral lipid can protect the cell from oxidative stress in some degree.

2.3.2 Algae biochemistry and ROS production under various stressors

Copper is an essential micronutrient in various physiological processes for low concentration. For high concentration, however, copper is known to reduce the efficiency of the photosystem due to the inhibition of electron acceptors on the reducing-side of photosystem II (Yruela et al.,

1991). Knauert and Knauer (2008) investigated the ROS in copper toxicity for two freshwater algae *Raphidocelis subcapitata* and *Chlorella vulgaris*, and found the species-specific sensitivities was caused by the difference in ROS defense systems. The copper toxicity affects the photosynthetic activity in short term and reduced the cell growth in long term. Algae cells were reported to accumulate two to four times more intracellular Cu^{2+} after 7 days exposure, less metal stress, but more than 30% higher superoxide dismutase SOD activity, compared to a short 6-hour copper exposure (Tripathi et al., 2006).

Various studies have found that nutrient depletion reduced biomass productivity but promoted the lipid content under stress condition. A stressor with nitrogen deficiency could maximize lipid accumulation by up to 90%, with high efficiency (Spolaore et al., 2006). When the nitrogen source is insufficient for the protein synthesis during algae growth, the excess carbon will be channeled into storage molecules such as neutral lipid and starch (Peccia et al., 2013). Oxidative stress can be the mediator for lipid accumulation under nitrogen deprivation. With 0.05 mM and 5 mM $NaNO_3$, the algae biomass were 495 mg/L and 1409 mg/L, but the lipid content increased by 35% at low nitrogen source (Yilancioglu et al., 2014b). Zhang et al. (2013) observed that during nitrogen starvation, photosynthesis efficiency decreased due to PSII damage. The 12-hour was the turning point from the early stress response to nitrogen-starvation. Furthermore, all other biotic and abiotic stress are also closely related to oxidative stress. Sharma et al. (2012) examined the excessive ROS under high salinity could disrupt the nucleic acids and

lipid peroxidation. Under excess light, compared to the wild-type algae *C. reinhardtii*, three genes level in the mutant (which lacked two photoprotective carotenoids and lutein) increased, involving the unexpectedly complex antioxidants and RNA level changes (Ledford et al., 2004).

Moreover, it is reported that the response of hormone auxin may respond to ROS induced signaling (Sharma et al., 2012). Joo et al. (2001) found that the auxin-induced gravity movement of the plant root was related to the ROS level. However, the interaction of ROS with the hormone signaling pathway is fragmental in algae cells. Piotrowska and Bajguz (2014) investigated that auxin hormones, including IAA, stimulated both enzymatic and non-enzymatic antioxidant systems in *Chlorella vulgaris*. Additionally, ROS was lower after two days of nitrogen starvation in algae *Scenedesmus dimorphus*, which could be associated with higher SOD and CAT level (Chokshi et al., 2017a).

Chapter 3 Indole-3-acetic acid from *Azosprillum brasilense* promotes growth in green algae at the expense of energy storage products

3.1 Background

As a renewable feedstock, microalgae have advantages over other food crops in terms of their rapid growth rate and ability to produce neutral lipids, starch, and protein (Brennan et al., 2010; Mata et al., 2010). Compared to other plants, microalgae can grow on freshwater, saltwater, and wastewater as well as use non-arable land. Consequently, algae have been considered as a source of biofuels (Salama et al., 2017), biofertilizers (Mulbry et al., 2005), and animal feed (Cole et al., 2016). Among microalgae, *Chlorella sorokiniana* (Bashan et al., 2016) and *Auxenochlorella protothecoides* (Higgins et al., 2015) have attracted attention for their high productivity, ability to grow on wastewater (de-Bashan, Trejo, Huss, Hernandez, & Bashan, 2008; B. Higgins et al., 2017), and their ability to accumulate energy storage products: namely starch and triacylglycerol (B. Higgins, A. Thornton-Dunwoody, J. M. Labavitch, & J. S. VanderGheynst, 2014; Higgins & VanderGheynst, 2014; Tanadul et al., 2014).

Despite the advantages of microalgae, they are currently commercially viable only as a feedstock for high-value product synthesis or as aquaculture feed. High capital investments in particular have hindered algal biofuel production and studies have shown that increasing growth rates and lipid content are the most crucial factors in reducing biofuel costs (Davis, Aden, &

Pienkos, 2011). Co-culturing algae with bacteria has been explored as a means of increasing algal growth rates (Antoun, et al., 2008; Higgins & VanderGheynst, 2014) and the production of biofuel precursors (B. Higgins, Labavitch, & VanderGheynst, 2015). Bacterial promotion of algal growth can occur via a range of mechanisms including synthesis of vitamin cofactors (Croft., 2006; Higgins et al., 2016; Kazamia et al., 2012), exchange of carbon dioxide and oxygen (Bai et al., 2014; Holmes et al., 2019), and through production of hormones (Amavizca et al., 2017b; de-Bashan et al., 2008).

As a well-studied example, *Azospirillum brasilense* is a plant growth-promoting bacteria (PGPB) that has also been found to enhance algal growth (Bashan & de-Bashan, 2010). *A. brasilense* produces the auxin hormone, indole-3-acetic acid (IAA), which has been found to stimulate growth in *C. sorokiniana* (Antoun, et al., 2008). Energy reserves such as neutral lipids and starch are of particular interest given their value as biofuel precursors (Griffiths & Harrison, 2009). Additionally, in the studies that have investigated lipids and starch production by algae in the presence of PGPB (Choix, et al., 2014; Choix, et al., 2012; Bashan, et al., 2002; Palacios, et al., 2016), nearly all of these studies have focused on cells that were immobilized in alginate beads. Moreover, analyses of lipids in the above studies have focused on total crude lipid as opposed to the more industrially-relevant neutral lipids. Past studies have suggested that *A. brasilense* and IAA increase crude lipid content, alter the fatty acid profile, and increase starch content under specific conditions (Choix et al., 2014; Choix et al., 2012; de-Bashan et al., 2002;

Palacios et al., 2016). While it is well-established that *A. brasilense* and IAA can promote the growth of certain algae strains, there has been a lack of systematic investigation of their impact on energy storage reserves in algae. The objective of this research was to determine the impact of *A. brasilense* and IAA on growth promotion and energy storage product accumulation in suspended cultures of *C. sorokiniana* and *A. protothecoides*. Based on research by others, we initially hypothesized that *A. brasilense* and IAA would simultaneously increase growth and accumulation of energy storage products. However, we show here that there is an apparent tradeoff whereby *A. brasilense* and IAA promote algal growth at the expense of storage products.

3.2 Materials and Methods

3.2.1 Experimental plan

C. sorokiniana (UTEX 2714) and *A. protothecoides* (UTEX 2341) were chosen as the model strains in this study for the aforementioned industrial relevance of these species. Moreover, interactions between *C. sorokiniana* and *A. brasilense* have already been studied by others (O. A. Palacios, Lopez, Bashan, & de-Bashan, 2019), providing a comparative foundation upon which we build. *A. protothecoides* has never been studied, to our knowledge, in association with *A. brasilense*. Within each algae strain, the experiment contained an axenic algae control group cultured on chemical N8 medium (*C. sorokiniana*) or N8-NH_4 medium (*A. protothecoides*). Experimental treatments consisted of co-cultures with live *A. brasilense* Cd (de-Bashan, Antoun, et al., 2008), axenic algae dosed with IAA, and axenic algae cultured on spent medium from *A.*

brasilense. To prepare the spent medium, *A. brasilense* was cultured for 96 hours in bottles filled with N8 (*C. sorokiniana* experiments) or N8-NH_4 medium (*A. protothecoides* experiments) supplemented with 1 g/L malate. The malate concentration was measured by HPLC using an Aminex 87H column using a previously-published method (Higgins & VanderGheynst, 2014) to verify that all malate was consumed by the end of the culture period. Spent medium was checked for its nitrogen content by ion chromatography and re-supplemented to restore it to the level found in the control medium. The pH was also re-adjusted to 7.2 with 3 M NaOH or HCl and then the spent medium was sterile filtered (0.2 μm). To add exogenous IAA to cultures, IAA was first dissolved in ethanol (100 mg/ml) and then added directly to the culture medium to achieve the desired IAA concentration. In separate experiments, we tested the impact of this small amount of ethanol and found it had no detectable effect on the growth of *C. sorokiniana*. Exogenous IAA was added to *C. sorokiniana* cultures at 50 mg/L because this concentration had previously been used successfully with this strain by Palacios et al. (Oskar et al., 2016). We confirmed that 50 mg/L IAA was appropriate for *C. sorokiniana* by running a dose-response experiment at 0, 25, 50, and 100 mg/L IAA. The result showed that 50 mg/L had slightly higher initial growth rates than the other levels (**Figure A1.1**). The effect of 50 mg/L IAA was also tested with *C. sorokiniana* at different inoculation densities (10^5-10^7 cells/ml) and we observed faster growth in the presence of IAA in all cases (**Figure A1.1**). In order to determine the appropriate exogenous IAA concentration for *A. protothecoides*, dose response experiments were

carried out with IAA concentrations ranging from 0 to 50 mg/L. The optimal IAA concentration of 3.1 mg/L was used in subsequent experiments (**Figure A1.1**). All experiments were conducted on batch cultures which were partially harvested at 72 hours and again at 120 hours which generally reflected late exponential and late log-phase of culture growth, respectively. All these experimental controls and treatments were carried out in biological triplicate.

3.2.2 Algae and bacteria cultivation

Freezer stock of *A. brasilense* were resuscitated and pre-cultured in liquid LB media at 30 °C for ~24 hours prior to co-inoculation. Cells were harvested by centrifugation (4,696 x *g*) for 5-10 minutes and the pellet was resuspended in sterile DI water. Optical density at 550 nm was used to quantify the cell density e.g. OD 0.05 = 10^7 cells/ml and appropriate volume was added to reactors to achieve a starting culture density of ~10^7 cells/ml.

Algae were initially plated and selected colonies were pre-cultured in 1 L bottles with N8 medium (Tanadul et al., 2014) for *C. sorokiniana* and N8-NH_4 medium for *A. protothecoides* (B. Higgins & VanderGheynst, 2014). Pre-cultures were checked for contamination by plating on rich ATCC No. 5 sporulation agar (ATCC, 2013). Pre-cultures were grown to a density of OD 0.2 at 550 nm (~10^7 cells/ml), then settled overnight to concentrate. The algae slurry was further concentrated through centrifugation at 4,696 x *g* for 5 minutes and the medium was decanted. The concentrated cells were used to inoculate 300 ml hybridization tubes filled to 200 ml with fresh medium. The target inoculation density for algae was 10^7 cells/ml (OD ~0.2). In co-

cultures, this yielded an inoculation ratio of roughly 1:1 with *A. brasilense*. Light microscopy images taken shortly after co-inoculation revealed that the true ratio of *A. brasilence* to algae may have exceeded the targeted 1:1 ratio (**Figure A1.2**). Illumination (170 μmol photons/m^2/s on a 14h:10h light-dark cycle) was provided by T5 growth lamps oriented horizontally and reactors were suspended in a 28 °C water bath. The pH was adjusted and maintained at 7.2 in all reactors with 3 M NaOH on a daily basis as needed. Sterile-filtered air (100 ml/min) was supplied to each reactor with supplementation of 2% CO_2 v/v. Reactors were stirred at 150 rpm. All cultures were handled in a biosafety cabinet using sterile technique.

The algae growth rate was determined through daily measurement of optical density at 550 nm. Thereafter, cells were removed from the sample by filtration (0.2 μm) and media samples were retained for analysis by subsequently-described liquid chromatography methods. At 72 hours and 120 hours ~80 ml of the cultures were harvested for detailed biomass composition analysis. Cells were washed and freeze dried as previously described (B. Higgins & VanderGheynst, 2014).

3.2.3 DNA extraction and quantitative PCR to determine *A. brasilense* abundance in co-cultures

Quantitative PCR was utilized to determine the abundance of *A. brasilense* in co-culture biomass. This approach has previously been used to quantify *E. coli* in co-culture biomass (B. Higgins & VanderGheynst, 2014). Briefly, known quantities of freeze-dried co-culture biomass were re-

suspended in dH_2O. Genomic DNA was extracted using the FastDNA Spin Kit (MP Biomedicals), using the bacterial lysis buffer as described in the manufacturer's instructions. The DNA concentration was determined by measuring the optical density at 254 nm. Known quantities of pure *A. brasilense* biomass were also used for DNA extraction and were serially diluted 10-fold to create a standard curve for qPCR. Primers were designed to amplify a specific segment of the 16S rRNA gene found in *A. brasilense*. In order to select primers that would be nearly universal across strains of *A. brasilense*, we aligned 16S rRNA gene sequences across five strains of *A. brasilense* using Geneious Software v. 11.0.3. We selected regions that were conserved within *A. brasilense* but that still lie on a hypervariable region of the 16S rRNA gene. The resulting primers were tested in BLAST to check for specificity to *A. brasilense*. The selected forward primer (5'-CTACCGCCAGTTGCCATCATT-3') and reverse primer (5'-CTTCGCATCCCACTGTCACC-3') amplify a 150-base portion of the *A. brasilense* 16S rRNA gene. They are located on hypervariable regions V7 and V8 of the 16S rRNA gene, respectively (Chakravorty, Helb, Burday, Connell, & Alland, 2007).

Quantitative PCR was carried out on a qTower3 instrument (Analytic Jena) using SYBR detection. The PerfeCTa SYBR Green FastMix (QuantaBio) and 0.5 µM of forward and reverse primers were used in a 20 µl reaction volume. Polymerase was activated for 10 minutes at 95 °C and thermocycling was carried out for 40 cycles as follows: 15 seconds at 95 °C, 15 seconds at 55 °C, and 30 seconds at 72 °C. A melt curve was obtained by reading fluorescence as the

temperature ramped from 72 °C to 95 °C. A standard curve was generated from dilutions of pure *A. brasilense* DNA extracts and used to quantify the amount of *A. brasilense* DNA in co-cultures. This amount of DNA was then correlated to total *A. brasilense* biomass quantity based on known quantities of pure cultures from which DNA was extracted as previously described (B. Higgins & VanderGheynst, 2014).

3.2.4 IAA analysis by liquid chromatography and mass spectrometry (LCMS)

IAA was analyzed using high pressure liquid chromatography-electrospray ionization-mass spectrometry HPLC-ESI-MS on an LCMS 2020 (Shimadzu). The HPLC system was equipped with a Thermo PolarAcclaim II C18 Column (3x150 mm, 3 μm particles) maintained in a 30 °C oven. Mobile phases A (95% 1 g/L ammonium acetate in Nanopure water, pH 5.0, 5% methanol) and B (100% methanol), were used in a gradient method with a flow rate of 0.5 ml/min. The following gradient was used: 0% phase B for 1 minute, ramp to 90% B from 1 to 7 minutes, hold B at 90% for 3 minutes, ramp back to 0% B from 10-11 minutes, and hold 0% B for 4 minutes. The mass spectrometer was initially run in positive ion ESI scan mode to confirm the dominant ions. These included m/z of 176 (H^+ adduct) and 214 (K^+ adduct) using a pure IAA standard in algal N8 medium. Subsequently, selective ion monitoring was used at m/z 214. The injection volume was 10 μl. Peak integration was carried out using LabSolutions software (Shimadzu).

3.2.5 Lipid analysis

Lipids were extracted from freeze-dried biomass using a modified Folch method as described previously (Higgins & VanderGheynst, 2014). The conventional gravimetric method (Folch, Lees, & Sloane Stanley, 1957) was conducted for total lipid content. The neutral lipid content was performed by adding Nile Red dye to lipid extracts followed by bleach to destroy the pigments according to Higgins et al. (2014) with the updates described in Wang et al. (Wang et al., 2019). Canola oil was used as a standard. Thin layer chromatography (TLC) was also used on select samples to qualitatively analyze the lipid profile with a particular focus on triacylglycerol content. The TLC conditions have been described previously (Higgins et al., 2014).

3.2.6 Chlorophyll analysis

Chloroform extracts from the modified Folch method were diluted 10-fold in acetone and absorbance was measured at wavelengths 645 and 663 nm. Chlorophyll a and b concentrations were then calculated using the following equations per Porra et al. (Porra, 2002):

$$\text{Chlorophyll a } (\mu g/ml) = 12.25(A663 - A750) - 2.55(A645 - A750) \quad \text{Eq. 1}$$

$$\text{Chlorophyll b } (\mu g/ml) = 20.31(A645 - A750) - 4.91(A663 - A750) \quad \text{Eq. 2}$$

3.2.7 Starch and cell wall analysis

After lipid extraction, the resulting cell pellet was washed 3 times with acetone and 3 times with water to remove residual soluble materials. The pellet was freeze dried and pulverized before undergoing enzymatic starch hydrolysis as described previously (Higgins & VanderGheynst,

2014). Briefly, an enzyme solution containing 100 mM acetate buffer, pH 5, 0.04% w/v sodium azide, 15 U/ml α-amylase and 6 U/ml α-amiloglucosidase was prepared. Starch was gelatinized and incubated in the enzyme cocktail overnight at 37 °C to convert starch into glucose. The digested samples were centrifuged at 12,000 x *g* for 10 minutes and the supernatant was recovered. A dinitrosalicylic acid (DNS) reducing sugar assay was used to measure the concentration of released glucose in the supernatant using glucose as a standard as previously described (Higgins & VanderGheynst, 2014). The enzyme blank was subtracted from the final results. The starch content was calculated by multiplying the glucose content by 0.9. The cell wall pellet (post starch digestion) was washed twice with dH_2O, freeze dried, and weighed.

3.2.8 Nitrogen and protein analysis

Nitrogen content and soluble protein content of the algal biomass were analyzed using a previously-described method (Higgins et al., 2015). Briefly, 1.5 mg freeze-dried algae were resuspended in 1.5 ml dH_2O and bead homogenized using a Beadruptor (OMNI). The HACH total nitrogen assay was performed on a portion of the resulting slurry per the manufacturer's instructions. Total nitrogen content was converted to crude protein content using a multiplication factor of 4.6 per Cole et al. (2016). In addition to a total nitrogen assay to measure crude protein, soluble protein was extracted and assayed using the bicinchonic acid (BCA) protein assay (Higgins et al., 2015) with bovine serum albumin as the standard. To accomplish this, an SDS buffer (150 mM sodium phosphate buffer adjusted to pH 7, 3 mM disodium EDTA, 0.3% SDS,

0.3% Triton X-100) was added to the homogenized slurry and underwent additional homogenization to extract soluble proteins. The BCA protein assay was carried out per the manufacturer's instructions and A562 was measured using a plate reader.

3.2.9 Ion chromatography for soluble nutrient analysis

Cation and anion chromatography were carried out using methods described in Chaump et al. (Chaump et al., 2018). Briefly, CS12 and AS22 columns (4x250 mm, Dionex) were used for cation and anion separation, respectively. ERS500 suppressors were used to reduce background noise and detection was carried out using a conductivity detector. Anion standards included chloride, nitrite, nitrate, phosphate, and sulfate. Cation standards included sodium, ammonium, potassium, magnesium, and calcium.

3.2.10 Statistical analysis

Statistical analysis was carried out in R (version 3.1.1) using the 'car' and 'agricolae' packages. ANOVA and Tukey's HSD tests were used for comparison across treatments. The Levene's test was used to check for violations of variance homogeneity before using ANOVA.

3.3 Results

3.3.1 Impacts of *A. brasilense* and indole-3-acetic acid on algal growth

Experiments were conducted with *C. sorokiniana* and *A. protothecoides* to determine the impact of live *A. brasilense* cells, exogenous IAA, and secretions of *A. brasilense* on algal growth. *A.*

brasilense secretions in spent media and exogenous IAA (50 mg/L) stimulated growth in *C. sorokiniana* but to a lesser extent than live *A. brasilense* (**Figure 2**). The co-culture group had 90% greater total growth, and exogenous IAA led to 30% greater growth in *C. sorokiniana* compared to control cultures. *A. protothecoides* was less responsive to live *A. brasilense* and exogenous IAA than *C. sorokiniana*. Co-culture growth was roughly 11% higher than the control after both 72 hours and 120 hours although only the former was statistically significant ($p = 0.01$). *A. protothecoides* was also much more sensitive to IAA than *C. sorokiniana* with an optimal exogenous dose of only 3.1 mg/L based on a dose response experiment (**Figure A1.1**). An early experiment in which 50 mg/L of IAA was used resulted in complete culture death (**Figure A1.3**). Spent medium led to significant growth suppression in *A. protothecoides* ($p < 0.001$) which subsided after 120 hours.

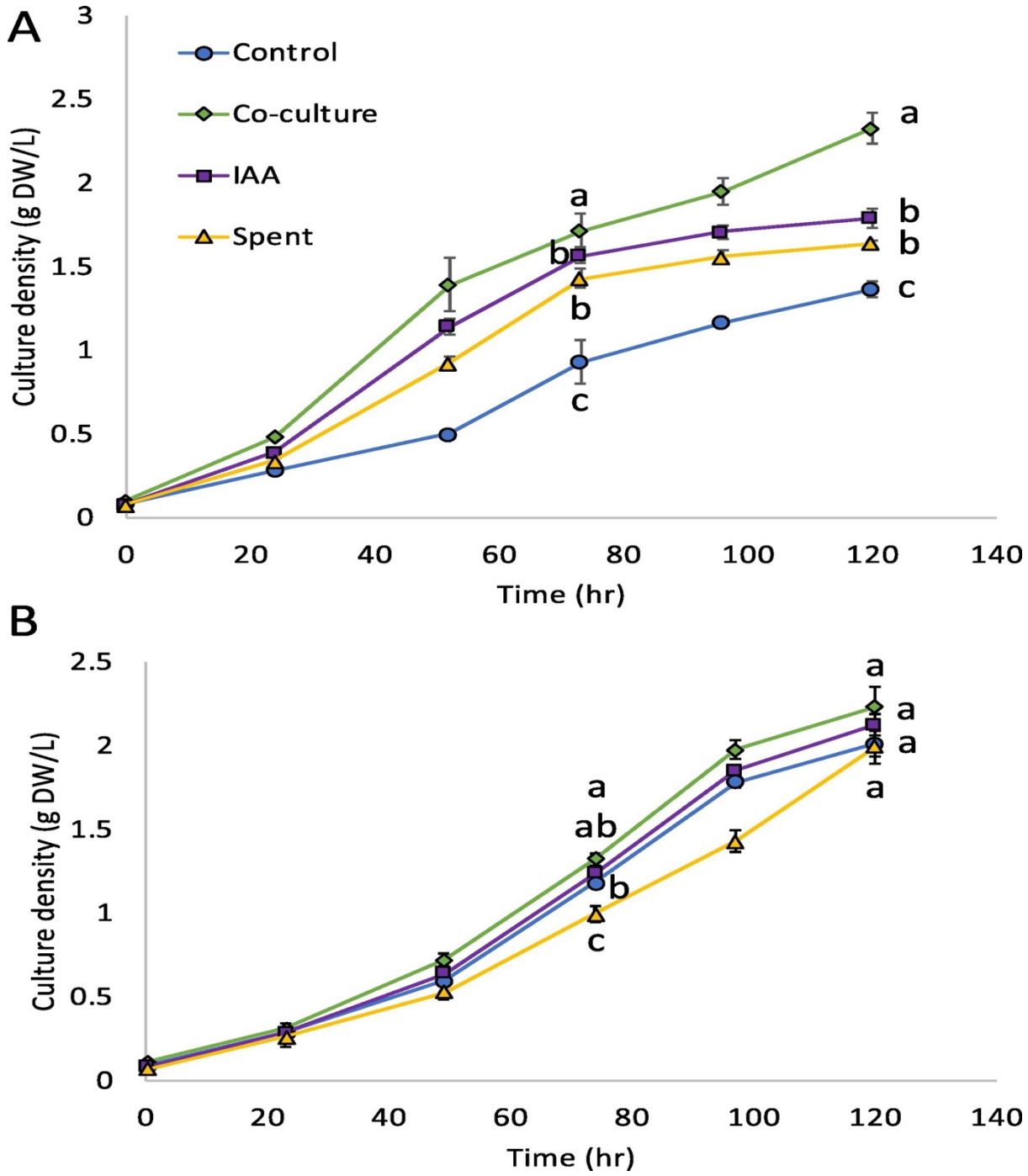

Figure 2 Growth curves for cultures of *C. sorokiniana* (A) and *A. protothecoides* (B). Culture density is determined on a dry weight (DW) basis. Error bars are standard deviations based on three biological replicates. Within a time point, data points with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

3.3.2 *A. brasilense* represented a small and decreasing fraction of biomass in co-cultures

It is not clear from the growth curve alone whether the additional biomass growth in co-cultures is an additive contribution from *A. brasilense* or if the bacteria promote algal growth. Thus, quantification of *A. brasilense* within the co-culture was carried out using qPCR to better understand its contribution to total biomass. After 72 hours, 5.7% of co-culture biomass was *A. brasilense* in *C. sorokiniana* cultures and this level declined to 1.3% of biomass after 120 hours. In *A. protothecoides* co-cultures, 0.6% of biomass was *A. brasilense* at 72 hours and this level declined to only 0.02% of co-culture biomass after 120 hours. The decline in relative abundance of *A. brasilense* is partly due to the dilution effect associated with algal growth but also suggests that the *A. brasilense* population stagnated or declined later in the culture period. The majority of co-culture biomass was algae (>90%) in all cases and the algal fraction of the co-culture growth rate was significantly higher than control cultures in all cases (**Table 2**). This indicates that the presence of live *A. brasilense* promoted algal growth. Although the exogenous IAA treatment significantly promoted growth in *C. sorokiniana* cultures, it was less than the total growth promotion effect of live *A. brasilense* in co-culture (**Table 2**). This indicates that other symbiotic mechanisms besides IAA impact algal growth.

Table 2 Algal and bacterial productivity (mg/L/d) over 72 hours and 120 hours periods

		Control	Co-culture[1]	IAA	Spent[2]
72-hour productivity	*C. sorokiniana*	195 (6)[3] d[4]	406 (42) a	270 (17) c	306 (20) b
	A. brasilense	-	22.0 (11)	-	-
	A. protothecoides	364 (1) b	401 (11) a	385 (6) ab	309 (15) c
	A. brasilense	-	2.4 (1.3)	-	-
120-hour productivity	*C. sorokiniana*	256 (11) c	442 (19) a	314 (11) b	292 (4) b
	A. brasilense	-	2.6 (1.5)	-	-
	A. protothecoides	384 (16) a	424 (25) a	409 (13) a	385 (19) a
	A. brasilense	-	0.08 (0.05)	-	-

[1]Algae in co-culture with *A. brasilense*

[2]Spent medium from *A. brasilense* cultures

[3]Values in parentheses are standard deviations based on 3 biological replicates

[4]Within a row, values with the same letter are not statistically different at the 0.05 level

3.3.3 Indole-3-acetic acid concentration

Given that IAA has been identified as one of the main mechanisms by which *A. brasilense* promotes plant and algal growth (de-Bashan et al., 2008), we quantified IAA levels in the medium of each experimental treatment (**Table 3**). With the exception of exogenous IAA additions, IAA levels were less than 150 μg/L in all cases and relatively high variability was observed among experimental replicates. In co-cultures of *C. sorokiniana*, we observed IAA levels that were up to 4 times higher than those observed in co-cultures with *A. protothecoides,* reflecting the higher relative abundance of *A. brasilense* in the former. In co-cultures with both algae strains, IAA levels first increased and then decreased with time, a pattern that reflects the decline in relative abundance of *A. brasilense* toward the end of the batch culture. It is apparent from the cultures with exogenous IAA that the hormone is consumed and/or degrades over time. Spent medium used for *C. sorokiniana* cultures also exhibited a decline in IAA over time given

the lack of organisms to replenish it. Spent medium used in *A. protothecoides* cultures contained little detectable IAA, indicating a batch effect that further underscores the high variability in IAA production by *A. brasilense*.

Table 3 IAA concentrations (μg/L) in the medium

	Time	Control	Co-culture[1]	IAA	Spent[2]
C. sorokiniana	0 hr	ND	10.5 (0.8)[3]	50,000	55 (15)
	48 hr	ND	120 (12)	12,083 (3108)	17 (10)
	96 hr	ND	98.2 (14.2)	3,777 (1077)	21 (12)
A. protothecoides	0 hr	ND	8.9 (4.9)	3,100	<LOQ[4]
	48 hr	ND	28.7 (35.1)	272 (128)	ND
	96 hr	ND	ND	<LOQ	<LOQ

[1]Algae in co-culture with *A. brasilense*
[2]Spent medium from *A. brasilense* cultures
[3]Values in parentheses are standard deviations based on 3 biological replicates
[4]LOD was 2.4 μg/L and LOQ was 7.2 μg/L
ND indicates that the peak area was below the LOD

3.3.4 *A. brasilense* promotes chlorophyll production

Chlorophylls *a* and *b* are key pigments for carrying out photosynthesis and it is hypothesized that IAA remodels chloroplasts and pigment levels to protect against photooxidative inhibition (Tognetti, Muhlenbock, & Van Breusegem, 2012). In this study, live *A. brasilense* and exogenous IAA stimulated production of chlorophylls *a* and *b* (**Figure 3**). Compared with the control group, exponentially growing (72 hours) co-cultures with *C. sorokiniana* led to more than tripling of the chlorophyll *a* level and a more than 5-fold increase in the chlorophyll *b* level. After 120 hours, the chlorophyll enhancement by co-cultures and IAA attenuated as cultures moved into the stationary growth phase. This result mirrors our observation that *A. brasilense*

levels in co-cultures declined over time, suggesting that growth-promoting effects and chlorophyll stimulation are transient. Spent medium also had less effect on chlorophyll levels than live *A. brasilense* or exogenous IAA during exponential growth. Similar trends were observed with *A. protothecoides* but the effects of live *A. brasilense* and IAA were much smaller and, in some cases, were not statistically significant. None of the treatments stimulated an increase in chlorophyll of more than 30% in *A. protothecoides* compared to the control cultures.

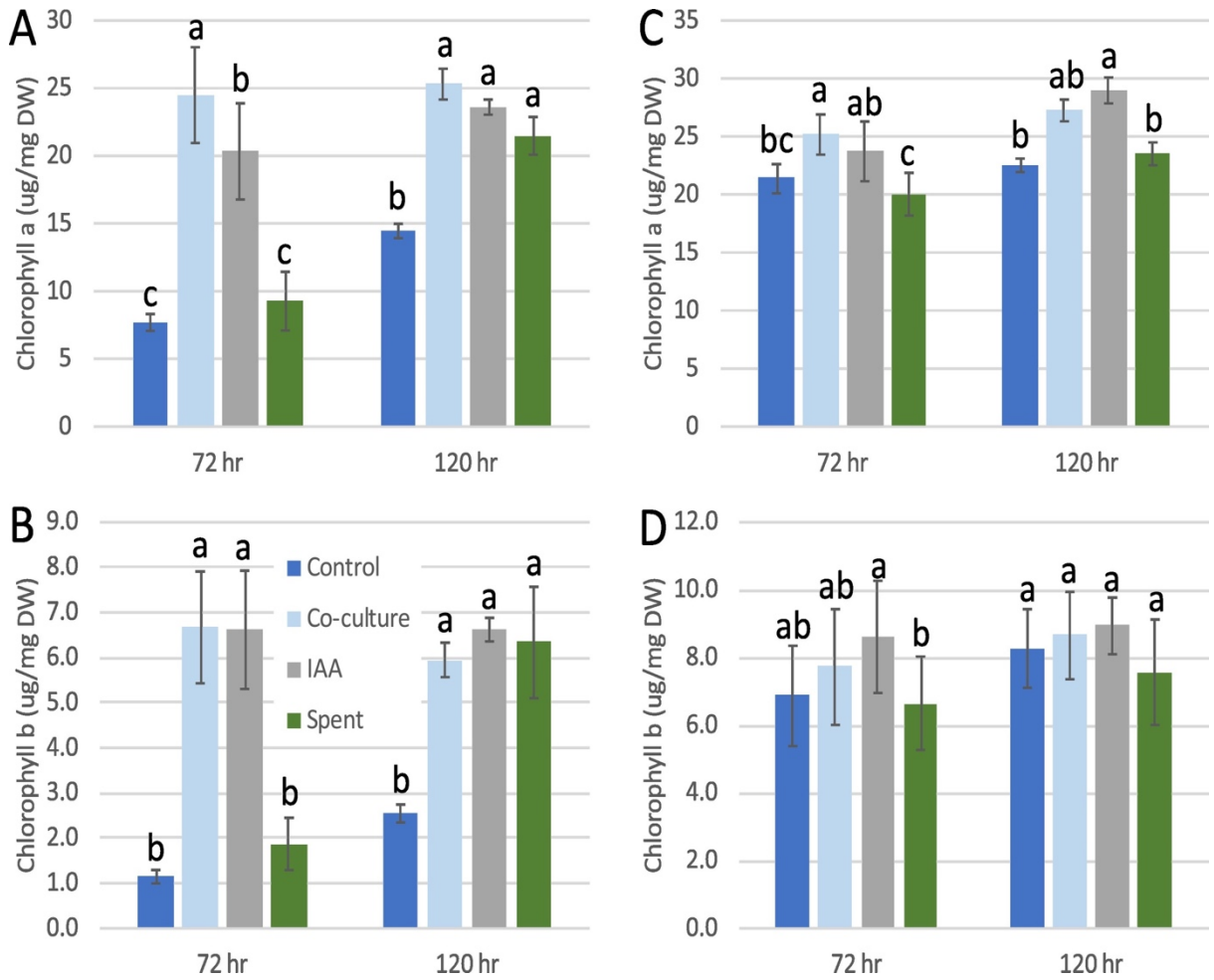

Figure 3 Chlorophyll a and b in *C. sorokiniana* (A and B) and *A. protothecoides* (C and D). Within a time point, data points with the same letter are not significantly different at the 0.05 level based on Tukey's multiple comparison test.

3.3.5 *A. brasilense* and IAA can suppress energy storage products in green algae

One of the objectives of this study was to better understand the impact of *A. brasilense* and IAA on algal biomass composition and particularly on energy storage products. We analyzed total crude lipid, neutral lipid, and starch content. Total crude lipid includes pigments, sterols, neutral lipids and polar lipids extracted in chloroform. Co-cultures, IAA, and spent medium groups all had higher total lipid content than controls for both algae types (**Figure 4**). This can be partially explained by the higher chlorophyll levels in these treatments.

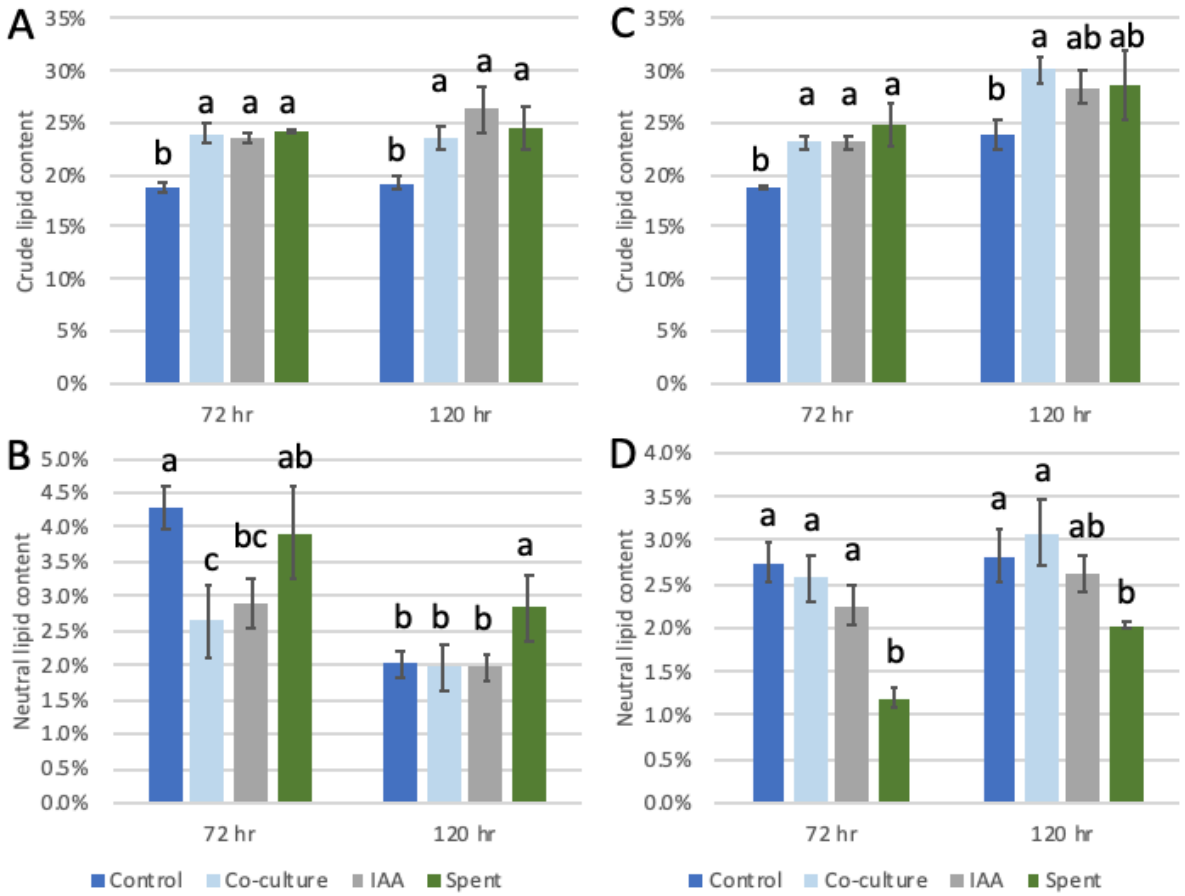

Figure 4 Crude lipid and neutral lipid content in *C. sorokiniana* (A and B) and in *A. protothecoides* (C and D, respectively). Content is measured on a dry weight basis.

Triacylglycerol (TAG) is one of the main neutral lipids and is a key energy storage molecule in eukaryotic algae (Breuer, Lamers, Martens, Draaisma, & Wijffels, 2013). TAG is also useful for biodiesel production. Data from the neutral lipid assay showed that neutral lipid only accounted for roughly 10-20% of total crude lipid depending on the growth condition and strain. Interestingly, live *A. brasilense* and exogenous IAA suppressed neutral lipid production in *C. sorokiniana* by 32-38% during exponential growth, which contrasts with results for total lipid content. This effect subsided during logarithmic growth (120 hours). In all treatments, neutral lipid content declined by late log growth (120 hours) but this decline was less severe for cultures grown in spent medium. It was interesting that live *A. brasilense* and exogenous IAA suppressed neutral lipid but spent medium from *A. brasilense* apparently did not. To determine if live *A. brasilense* and exogenous IAA were specifically suppressing TAG production, we performed TLC on the crude lipid extract on select samples. The results confirmed that TAG was indeed suppressed compared to the control culture (**Figure A1.4**). A somewhat different neutral lipid response was observed in *A. protothecoides* compared to *C. sorokiniana*. Live *A. brasilense* and exogenous IAA generally did not have a significant effect on neutral lipid content in *A. protothecoides*. However, spent medium significantly suppressed neutral lipid production ($p < 0.027$) and this persisted through late log growth.

Starch is another key energy storage polymer in *C. sorokiniana*. Similar to neutral lipid content, starch content was suppressed in exponentially-growing co-cultures and IAA culture

groups (**Figure 5**). At 72 hours, starch content of the co-culture and IAA groups had roughly one fourth of the starch content as the control and spent medium groups. The effect subsided during logarithmic growth as starch content decreased in control and spent medium cultures while increasing in co-cultures and those treated with exogenous IAA. *A. protothecoides* is a model lipid producer (under stress conditions) and consequently its starch content was much lower than that of *C. sorokiniana*. As with neutral lipid content, the lowest starch content in *A. protothecoides* was observed in spent medium. However, both co-cultures and exogenous IAA also had significantly lower starch content compared to control cultures although the percent differences were smaller in *A. protothecoides* than in *C. sorokiniana*.

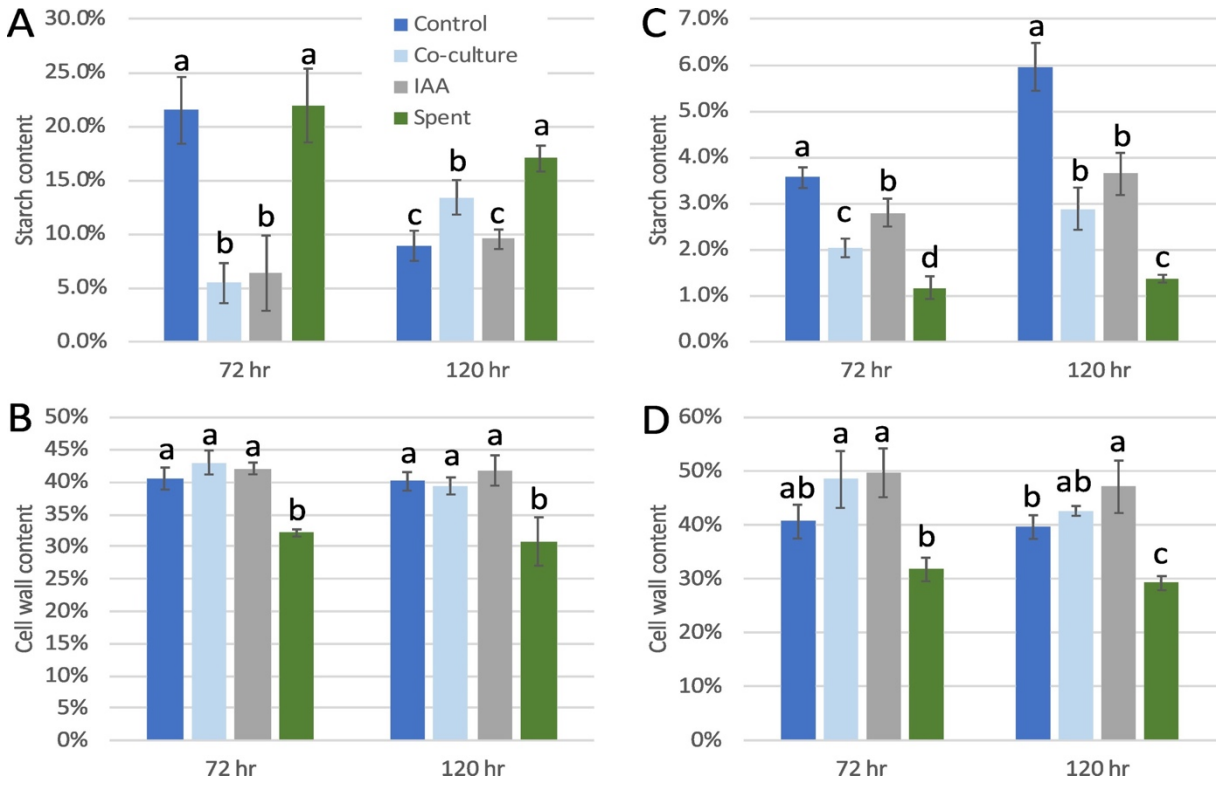

Figure 5 Starch content and cell wall content in *C. sorokiniana* (A and B) and in *A. protothecoides* (C and D, respectively). Content is measured on a dry weight basis.

Given IAA's strong suppression of starch and neutral lipid production in *C. sorokiniana*, we followed up with a dose-response study to further explore this strain's sensitivity to IAA. This experiment confirmed IAA's suppression of both starch and neutral lipid although there was a saturation effect whereby IAA levels above 10 μg/L did not result in additional suppression (**Figure A1.5**). The IAA level was at or below those observed in co-cultures with *C. sorokiniana*.

3.3.6 Spent medium reduced cell wall content in both algae

The cell wall serves a structural purpose and is made of polysaccharides in both of these algae species (Cheng, et al., 2011). IAA is known to impact the elasticity of the cell wall in plants, signifying potential structural modifications, and so cell wall content was analyzed here (Majda & Robert, 2018). Generally, neither co-cultures nor IAA had a significant impact on cell wall content in these algae strains (**Figure 5**). Interestingly, the use of spent medium from *A. brasilense* reduced the cell wall content by 20-26% in both algae types and this reduction was persistent through the log growth stage.

3.3.7 *A. brasilense* and indole-3-acetic acid increase protein content

Crude protein and soluble protein were determined by a nitrogen assay and the BCA assay, respectively. In general, soluble protein exhibited the opposite behavior of neutral lipid and starch. At the 72-hour time point, *C. sorokiniana* co-cultures and IAA cultures had roughly 2.5-

fold and 2-fold higher soluble protein, respectively, than control cultures (**Figure 6**). By 120 hours, these large differences disappeared although spent medium cultures had 37% lower soluble protein content than control cultures. This latter trend was the opposite of what was observed for starch and neutral lipids. In *A. protothecoides*, all of the treatments increased crude protein levels by 25-33% and soluble protein levels by 40-60% compared to control cultures at 72 hours. This was the opposite trend to what was observed with starch. In *A. protothecoides*, the higher protein levels observed in the three culture treatments generally persisted into the late logarithmic growth stage.

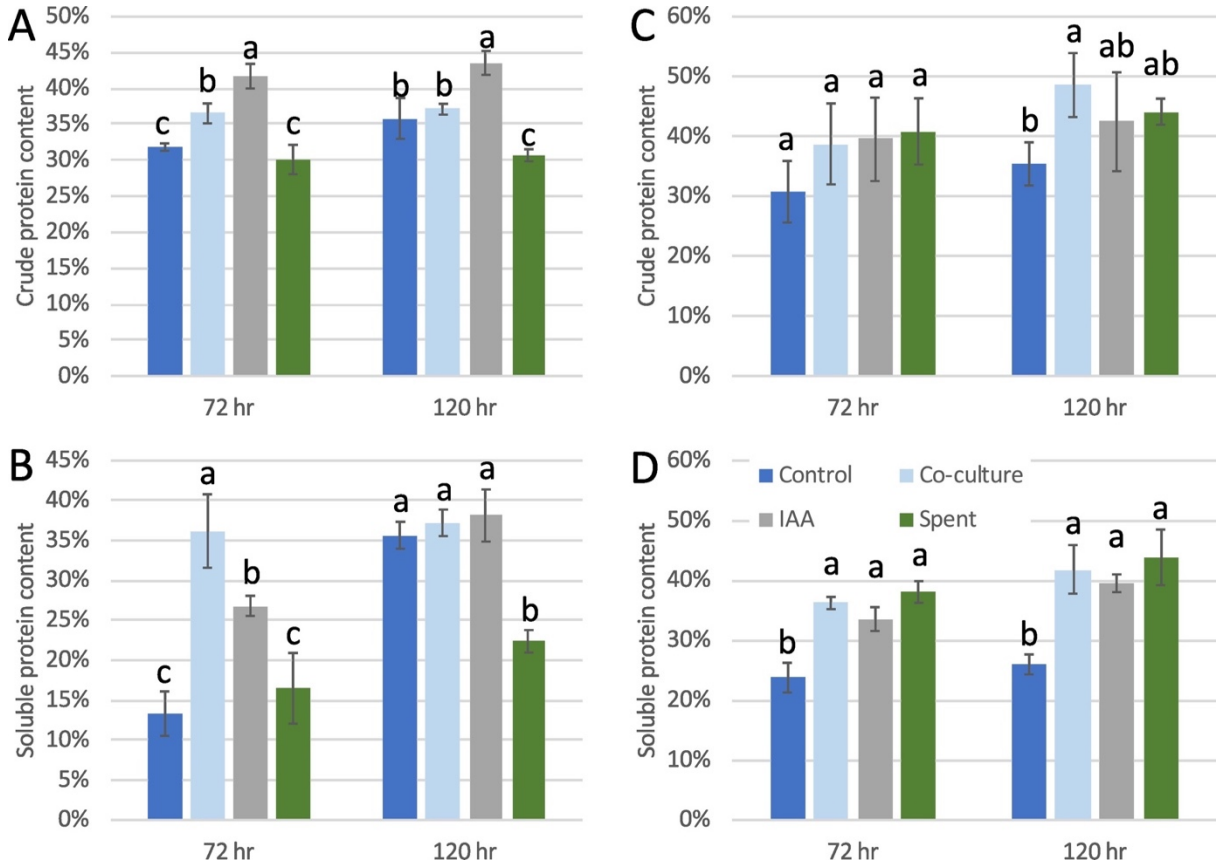

Figure 6 Crude protein and soluble protein content in *C. sorokiniana* (A and B, respectively) and in *A. protothecoides* (C and D, respectively).

3.3.8 Medium nutrient content

Because nutrient limitation can have a large impact on lipid, starch, and protein content in these microalgae, we analyzed the media nutrient content over time in each culture. The results showed that only *A. protothecoides* cultures depleted nitrogen, and only at the 120-hour time point (**Figure 7**). Nitrogen was not depleted in any of the *C. sorokiniana* cultures. The speed of nitrogen removal roughly reflected the differences in culture growth among treatments with co-cultures typically consuming nutrients most rapidly. However, differences in nutrient levels between control cultures and co-cultures were not statistically significant. Spent medium cultures consumed nitrogen at a significantly lower rate than control cultures. In the case of *A. protothecoides*, this mirrored the slower growth rate in spent medium. *C. sorokiniana*, in contrast, did not have slower growth on spent medium but did have lower final protein and nitrogen content compared to control cultures. Phosphorus nutrients were not depleted in any cultures and removal rates were much lower than they were for nitrogen. This was expected given the higher molar requirement of nitrogen compared to phosphorus. Other key nutrients (sulfate, magnesium, calcium) were also not depleted (data not shown).

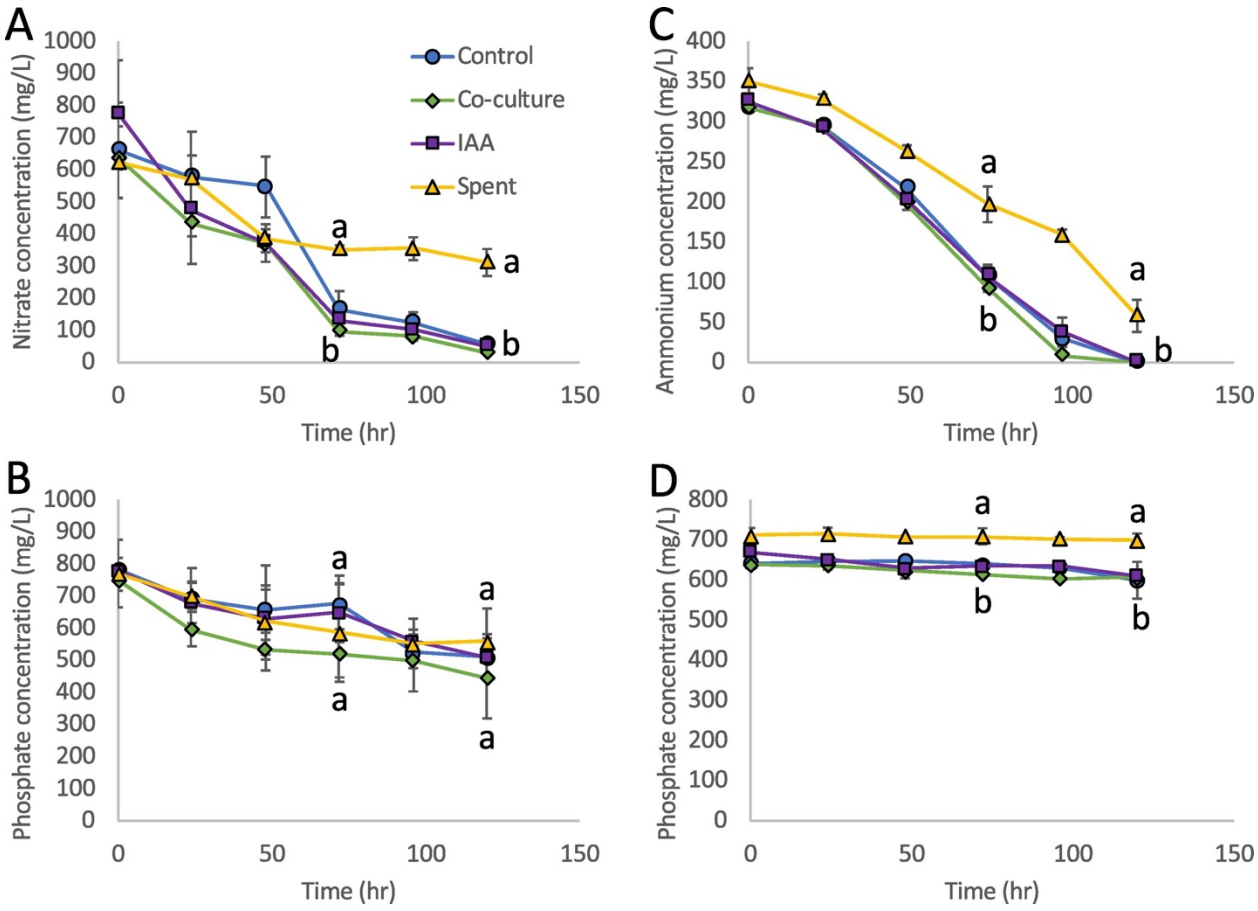

Figure 7 Nitrogen and phosphorus content in medium from *C. sorokiniana* (A and B, respectively) and in *A. protothecoides* (C and D, respectively) as a function of time.

3.4 Discussion

Our finding that *A. brasilense* and IAA stimulate growth in suspended cultures of *C. sorokiniana* was expected. Extensive research by others, almost exclusively focused on co-cultures immobilized in alginate beads, found that *A. brasilense* and IAA promote *C. sorokiniana* growth (de-Bashan et al., 2008; de-Bashan et al., 2002; Palacios et al., 2019; Palacios et al., 2016). In immobilized systems, microscope images reveal significant physical attachment of *A. brasilense*

to algal cells (Palacios et al., 2019). Based on our results, however, immobilization of cells is apparently not required to achieve growth promotion. Moreover, Amavizca et al. (Amavizca et al., 2017b) found that volatiles secreted by *A. brasilense* and other bacteria led to growth promotion in suspended algae cultures, underscoring the point that immobilizing algae and bacterial cells in a solid matrix is not required to achieve interactions. However, relatives of *C. sorokiniana* such as *A. protothecoides* did not exhibit the same extent of growth promotion when exposed to IAA. In fact, high levels of IAA suppressed growth, a finding also observed by Chung et al. (Chung et al., 2018) in the green algae *Desmodesmus*. These findings show that green algae have evolved significant differences in their response to the auxin hormone IAA.

The bulk concentrations of IAA observed in co-cultures in this study ranged from roughly one tenth to one third of the peak IAA concentrations in co-cultures studied by Palacios et al. (Palacios et al., 2016). We suspect that a significant factor was our utilization of LCMS to measure IAA rather than HPLC. LCMS is much more selective and excludes many molecules that co-elute and share spectral absorbance with IAA. A consequence of this result is that our exogenous IAA additions (based in part on work by Palacios et al.) were generally much higher than the bulk IAA concentration produced by *A. brasilense*. However, *A. brasilense* is known to attach to *Chlorella* cells (Palacios et al., 2019) and bulk concentrations are therefore not representative of what the algae cell likely experiences, given its close proximity to *A. brasilense,* even in a suspended culture. Light microscopy images taken a few hours after

inoculation revealed that partial agglomeration of *C. sorokiniana* and *A. brasilense* had already taken place in the suspended cultures (**Figure A1.2**). This could partially explain why the spent medium, with low bulk densities of IAA, generally led to lower algal growth than co-culture and exogenous IAA treatments. In addition to providing hormones, live *A. brasilense* may also serve as a source of CO_2 that stimulates algal growth. It is well-established that oxygen and CO_2 exchange between algae and bacteria has the potential for mutual benefits (Bai et al., 2014; Holmes et al., 2019; Oswald et al., 1953). In autotrophic cultures, such symbiosis relies on robust bacterial cycling of degradable carbon found in algal photosynthate which was not measured in the present study.

The present research also shows that exogenous IAA and co-cultures promoted *A. protothecoides,* but the effect size was smaller than with *C. sorokiniana*. Interestingly, spent medium suppressed *A. protothecoides* growth suggesting the initial presence of inhibitory molecules secreted by *A. brasilense*. There was no evidence that these spent medium cultures were nutrient-deprived and we may have overcompensated slightly when restoring ammonium levels to spent medium (**Figure 7**). Given that secreted inhibitory molecules are also likely present in co-cultures, we hypothesize that live *A. brasilense* simultaneously stimulates and suppresses *A. protothecoides* growth, leading to a muted growth promoting effect in co-cultures.

A. brasilense and IAA are also known to increase the abundance of chlorophyll and crude lipid content in *C. sorokiniana* (de-Bashan et al., 2002) and our results confirm this finding. Our

results also indicate that the impact of exogenous IAA and *A. brasilense* co-cultures on *C. sorokiniana* are most acute during the exponential growth phase compared to the logarithmic growth phase. This has also been observed by others (Palacios et al., 2019) and suggests that the signaling pathway induced by IAA is most relevant during active cell growth. In addition to timing effects, it is also clear from our data that IAA's impacts are highly strain dependent. *C. sorokiniana* apparently supported more robust *A. brasilense* populations in co-cultures and received greater growth promoting benefits than did *A. protothecoides*.

IAA, as one of the auxin hormones, is known to regulate the rate of cell elongation, division, and expansion in plants (Lau et al., 2009). Research in plants shows that the auxin binding protein, ABP1, is a critical regulator of cell division (Perrot-Rechenmann, 2010). Unfortunately, very little is known about the mechanisms of auxin signaling in green algae. Similar to plants, IAA and other auxin hormones have elongation effects on cells in brown algae (Bail et al., 2010) which are genetically distant from green algae and plants. Investigation of the genome of the brown alga, *Ectocarpus siliculosus*, indicates that several of the auxin-responsive genes found in *Arabidopsis* are not found in this algae (Bail et al., 2010) including ABP1. Thus the proteins involved in auxin signaling in algae exhibit high genetic variability even though the physiological outcomes: *e.g.* cell elongation and/or growth stimulation are similar. In green algae, growth stimulation but not cell elongation has been observed in response to *A. brasilense* (Palacios et al., 2019).

Azospirillum is known to help plants overcome stress conditions (Khalid et al., 2017) and auxin hormones specifically help plants adapt to stressful conditions (Tognetti et al., 2012). This stress-mitigating effect also extends to algae for which *A. brasilense* helps certain algae overcome pH, light, and salinity stresses (Bashan & de-Bashan, 2010) but the specific mechanisms remain unclear. Under a variety of stress factors, algae are known to accumulate energy storage products including starch (Markou & Nerantzis, 2013) and neutral lipids (Sun et al., 2014). This is particularly true of salinity stress (Chokshi et al., 2017b) as well as stress due to nutrient limitation (Chen et al., 2017; Higgins et al., 2014). However, our results showed that none of the cultures were nutrient-limited except for potentially A. *protothecoides* at 120 hours. This lack of stress was further reflected in the fact that none of the cultures had a neutral lipid content in excess of 5% of total dry weight whereas stressed *A. protothecoides* typically have neutral lipid content in excess of 20% (Higgins et al., 2015). Moreover, cultures with the most rapid nutrient removal rates, namely co-cultures, also had the lowest concentration of energy storage products.

The results here show that both IAA and *A. brasilense* suppressed energy storage products while promoting growth, protein, and pigment production, suggestive of a low stress/ high growth metabolic state. Although exogenous stress factors were not specifically applied to algae in the present experiments, it is possible that IAA and *A. brasilense* stimulate a chemical cascade leading to mobilization of energy resources for rapid growth. In plants, it is established

that certain auxin receptors are involved in tolerance to oxidative stresses and that there is cross-talk between auxins and signaling pathways associated with reactive oxygen species (ROS) (Tognetti et al., 2012). Specifically, auxin pathways help regulate hydrogen peroxide, antioxidant levels, and chlorophyll content in plants (Tognetti et al., 2012). We did not specifically test ROS or antioxidant levels in the algae used in this study, however.

Our finding that *A. brasilense* suppressed starch production in exponentially-growing *C. sorokiniana* was surprising because it contrasted with the findings of past researchers. Choix et al. showed that *A. brasilense* promoted starch production in both autotrophic (Choix et al., 2012) and mixotrophic (Choix et al., 2014) *C. sorokiniana*. Palacios et al. also showed that *A. brasilense* promoted starch production in autotrophic cultures (Palacios et al., 2016). In all of the above cases, *A. brasilense* and *C. sorokiniana* were cultured in alginate beads and starch was measured using a chemical assay (perchloric acid) which may hydrolyze residual material from the alginate beads, biasing the results. Underscoring this point is that total starch and total carbohydrate levels in one of these studies were nearly identical (Choix et al., 2012). In the present study, we used enzymes specific to starch and thereby avoided potential confounding factors from incidental degradation of other polysaccharides. It is also possible that growth in the beads alters the growth and metabolism of the algal cells compared to the suspended cultures investigated in the present study.

Interestingly, spent medium from *A. brasilense* led to significantly different compositional changes compared to co-cultures. Specifically, spent medium did not lead to suppression of starch and neutral lipids in *C. sorokiniana* but did lead to a reduction in cell wall content. Moreover, by 120 hours, spent medium led to a significant reduction in *C. sorokiniana*'s nitrogen and protein content which corresponded to its slower nitrogen uptake rate. This result indicates that chemical constituents produced by *A. brasilense* are transient in nature (e.g. IAA) and/or require close cellular proximity in order to achieve effective mass transfer. It is also apparent that the more stable secretions from *A. brasliense* can even be harmful to some algae species as demonstrated by the inhibition of *A. protothecoides* growth in spent medium. The spent medium used for *A. protothecoides* cultures had IAA levels that were detectable but below the limit of quantification.

The finding that *A. brasilense* and IAA can suppress rather than enhance energy storage neutral lipids and starch have implications for industrial deployment of this system. This plant growth-promoting bacteria and its auxin hormones may be most appropriate in applications demanding rapid algal growth with high protein content. However, in biofuel applications where lipids and starch are desired, this system may be deployed as part of a two-stage process. In the first stage, algae are grown with *A. brasilense* to maximize growth before transferring to a second stage where nutrient starvation stimulates lipid and/or starch production similar to processes described by others (Cheng et al., 2017; Su et al., 2011; Sun et al., 2014). This should

be possible in batch or fed-batch processes because our data show that *A. brasilense* and its effects die out over the course of batch culture.

3.5 Conclusions

The outcomes of this study suggest that *A. brasilense* and IAA may promote the growth of green algae but down-regulate energy reserves such as neutral lipids and starch in the process. Moreover, the effects of *A. brasilense* and IAA are highly strain dependent with stronger growth promotion in *C. sorokiniana* than was observed in *A. protothecoides*. Finally, secretions of *A. brasilense* found in spent medium resulted in very different compositional changes compared to using live *A. brasilense* and exogenous IAA. This indicates that bioactive constituents in *A. brasilense* secretions are transient or that physical cell attachment is important for ensuring adequate mass transfer of these constituents.

Chapter 4 *Azospirillum brasilense* reduces oxidative stress in the green microalgae *Chlorella sorokiniana* under different stressors

4.1 Background

Algae are defined as a third-generation feedstock and are used in biotechnological applications ranging from nutraceutical production (Udayan et al., 2017) and aquaculture (Spolaore et al., 2006) to wastewater treatment (Fernández et al., 2018). Algae are the fastest-growing photosynthetic organisms (Dismukes et al., 2008) yet they suffer from high production costs due mainly to large capital investments (Davis et al., 2011). Consequently, great research effort has been expended with the aim of further increasing algal growth rates in order to better utilize capital infrastructure. Many strategies have been explored for increasing algal growth including mixotrophic growth (Liang et al., 2009), genetic manipulation (Tredici, 2010), and utilization of synergistic relationships between algae and bacteria (Croft et al., 2006; de-Bashan et al., 2008; Higgins et al., 2016)

Product accumulation, including neutral lipids, pigments, or other desired molecules, has also been an intense area of research over the past decades (Fakhry & El Maghraby, 2015; Huntley & Redalje, 2007). Application of environmental stressors has been the most commonly utilized method of manipulating algal biomass composition. Both chemical and physical stressors, such as temperature (Markou & Nerantzis, 2013), salinity (Chokshi et al., 2017b), nutrient deprivation (Chen et al., 2017; Higgins et al., 2014), and direct application of reactive

oxygen species (Burch & Franz, 2016) have been employed to manipulate algal composition. These environmental stressors often lead to intracellular accumulation of reactive oxygen species including hydroxyl radicals, peroxyl radicals, superoxide anions, and hydrogen peroxide, which are more reactive than molecular oxygen (Hancock et al., 2001). More importantly for algal biotechnology, high levels of oxidative stress lead to significant biochemical changes in many algae including accumulation of neutral lipids (Sun et al., 2014), pigments (Udayan et al., 2017), and starch (Markou & Nerantzis, 2013). Recent research has shown that environmental stressors such as nitrogen limitation are linked to lipid accumulation via oxidative stress pathways (Yilancioglu et al., 2014a). However, the current paradigm is that stress-induced lipid and pigment accumulation leads to a trade-off in algal growth and photosynthetic efficiency (Dillschneider et al., 2013). In our past work, we have shown that co-culturing algae with bacteria can, in special cases, simultaneously improve growth and energy storage product accumulation (Higgins et al., 2015). However, understanding of how bacteria mediate stress responses, and thereby growth and product accumulation in algae is still an area of great uncertainty.

Plant growth-promoting bacteria are known to alleviate the effects of stress conditions in several species of plants (Khalid et al., 2017; Tognetti et al., 2012). To our knowledge, such stress-alleviating effects have not been studied in algae. *Azospirillum brasilense* is a well-studied plant growth promoting bacteria, normally found in the rhizosphere of plants. It secretes the

auxin hormone indole-3-acetic acid (IAA) (de-Bashan et al., 2008) as well as bioactive volatile organics including acetoin and 2,3 butanediol (Amavizca et al., 2017). Work by us and others has shown that this species has profound impacts on growth and biomass composition in multiple species of green algae (Choix et al., 2012; de-Bashan et al., 2002; Peng et al., 2020). In our previous study, we found that live *A. brasilense* and its secretion of the plant hormone indole-3-acetic acid (IAA) promoted growth in the green algae, *Chlorella sorokiniana*, while suppressing intracellular energy storage products in actively growing cells (Peng et al., 2020). We hypothesized that live *A. brasilense* and its IAA secretion could reduce oxidative stress in *C. sorokiniana*, thereby suppressing lipid and starch production. The aim of the present study was to test the capacity of *A. brasilense* to mitigate different stresses in *C. sorokiniana* that typically lead to the generation of reactive oxygen species. Thus, we investigated *C. sorokiniana* subjected to copper stress and nitrogen starvation stress conditions in the presence and absence of *A. brasilense*. Algal growth, intracellular ROS levels, pigment, neutral lipid content, and starch content were measured as response variables.

4.2 Materials and Methods

4.2.1 Experimental designs

The algae *Chlorella sorokiniana* (UTEX 2714) was cultivated under three different experimental setups. In the first experiment, we tested the effect of live *Azospirillum brasilense*, exogenous

IAA addition, and the use of spent medium from *A. brasilense* on growth and ROS formation by *C. sorokiniana*. In this experiment, There were no external stressorsand was described in detail in our previous study (Peng et al., 2020). Briefly, axenic *C. sorokiniana* cultures grown on N8-NH_4 medium (Higgins & VanderGheynst, 2014) served as a control. In the co-culture treatment, *A. brasilense* was co-inoculated with *C. sorokiniana* on a 1:1 cell basis with roughly 10^7 cells/ml of each. The exogenous IAA treatment involved dosing at 50 mg/L. The spent medium treatment involved culturing *A. brasilense* for 96 hours in N8-NH_4 medium supplemented with 1 g/L malic acid as a carbon source. This medium was readjusted to pH 7.2 with 3M HCl, re-supplemented with NH_4Cl based on the amount consumed by *A. brasilense*, and sterile filtered (0.2 μm) before *C. sorokiniana* inoculation. All experimental treatments and controls were conducted in biological triplicate for all experiments.

In the second experiment, copper stress was applied. Before conducting this experiment, the appropriate copper dose was first determined by carrying out a dose-response study with concentrations ranging between 5 μM and 100 mM was used (**Figure A2.1**). The 25-uM copper concentration, which slightly suppressed algal growth but did not kill the algae, was selected for subsequent stress experiment. The stress experiment was carried out in N8-NH4 medium. Control cultures received no copper treatment. The first treatment received 25 μM copper as copper sulfate, the second treatment received 25 μM copper along with 50 mg/L IAA, and the third treatment received 25 μM copper but was co-inoculated (1:1 cell basis) with *A. brasilense*.

In the third experiment, nitrogen limitation stress was applied. Control group were grown in N8-NH_4 medium and the first treatment involved growth on this same medium but with the addition of live *A. brasilense* (co-inoculated 1:1 cell basis). In the second treatment, axenic *C. sorokiniana* was inoculated into nitrogen-free N8-NH_4 medium (no ammonium) and in the third treatment, *A. brasilense* and *C. sorokiniana* were co-inoculated into the nitrogen-free N8-NH_4 medium.

4.2.2 Algae cultivation methods

For all experiments, stock algae cultures were grown in 1L bottles using N8-NH_4 medium for ~5 days, or to achieve an optical density (550 nm) around 0.2 as described previously (Wang et al., 2019). This corresponds to ~10^7 cells/ml. Stock cultures were inoculated from colonies of *C. sorokiniana* isolated from Bold 3N plates and handled in a biosafety cabinet using sterile technique. The reactors were outfitted with sterilized air filters (0.2 μm) to maintain axenic cultures during growth. The stock cultures were harvested by overnight settling and then further concentrated by centrifugation at 4500 g for 5 min. Then 6 mL algae concentration were used to inoculate the experimental bioreactor system which consisted of twelve tubular bubble column reactors (200 ml working volume) as previously described (Wang, et al., 2019).

For the cocultures, *A. brasilense* stock cultures were grown on LB medium at 30 °C for ~24 hours, as previously described (Peng et al., 2020). Optical density (550 nm) was measured and used to estimate cell number based on a previously identified correlation (OD 0.05 = 10^7

CFU/ml). After growth, the bacteria were centrifuged at 4500 g for 5 min and the medium was completely decanted. Cells were resuspended in 6 mL sterile N8-NH_4 medium and aliquoted into the experimental bubble column reactors to achieve a roughly 1:1 ratio with *C. sorokiniana*.

The experimental bubble column reactors were maintained in a 28 °C water bath, illuminated from the side by T5 growth lamps with 170 μmol photons/m^2/s on a 14h:10h light-dark cycle. All bioreactors were mixed by stir bar and received sterile-filtered air (100 ml/min) supplemented with 2% CO_2 v/v. Experimental cultures were carried out for 120 hours. Each day, all culture were adjusted the pH 7.2 with 3 M NaOH as needed, and a 2 ml sample was collected for optical density (550 nm) analysis as previously described (Wang et al., 2019). Half of each culture was harvested at 72 hours, which captures the exponential growth stage, and the remainder of the culture was harvested at 120 hours, which was selected to capture the logarithmic growth phase. The algae biomass was determined through gravimetric analysis and a unique correlation to OD 550 was established for each experimental treatment.

4.2.3 Reactive oxygen species ROS analysis determination

Reactive oxygen species ROS was detected with the cell-permeant dye, fluorescent probe carboxy-2'7'-dichlorofluorescein, carboxy-H2DFFDA (Invitrogen, USA). Carboxy-H2DFFDA is one of the most widely used fluorometric probe for detecting oxidative stress. The fluorinated derivative, H2DFFDA, exhibited improved photostability compared to chlorinated fluorescein derivatives (Invitrogen, 2006). The method was adapted from Knauert and Knauer (2008). First,

1.5 ml algae cells were transferred in 2 ml tubes, centrifuged 5 min at 12000 g to pellet. After washing with 0.5 M Tris-buffer, 5 μM carboxy-H2DFFDA dye reagent was used to resuspend the cells. The cells were incubated for 10 min and then transferred to a 96-well plate (200 μl/well). The fluorescence density was measured using the microplate reader (SpectraMax M2), with 488 nm excitation and 520 nm emission. The plate was incubated in the light for 1 hour and fluorescence was read every 20 min. The rate of fluorescence increase was determined and is considered to be proportional to the rate of intracellular ROS production. The rate of fluorescence increase was normalized by dividing by the algal culture density (dry weight concentration from the growth curve). Three biological replicates were performed with three technical replicates. The algae-free dye mixture was used for the negative controls.

4.2.4 Chlorophyll determination

Chlorophyll a and b were determined through extraction and absorption measurements as previously described (Peng et al., 2020). Briefly, the algae cells were extracted using a modified Folch method with bead disruption (B. Higgins & VanderGheynst, 2014). The chloroform phase was diluted 10-fold in acetone and optical density was measured at 663 nm, 645 nm, and 750 nm. Chlorophyll a and b contents were estimated using the following equations (Porra, 2002):

$$\text{Chlorophyll a } (\mu g/ml) = 12.25(A663 - A750) - 2.55(A645 - A750) \quad \text{Eq. 1}$$

$$\text{Chlorophyll b } (\mu g/ml) = 20.31(A645 - A750) - 4.91(A663 - A750) \quad \text{Eq. 2}$$

4.2.5 Energy storage product Neutral lipid and Starch determination

Extracts from the Folch method were also used to measure neutral lipid content using a Nile red assay procedure with canola oil as the standard as previously described (Higgins et al., 2014; Wang et al., 2019). Briefly, in a 96-well microplate, algae lipid extracts were dried and stained with a solution containing 1 μg/mL Nile red (Sigma, USA) solution. A bleach solution was then added to destroy the pigments, which quench the Nile red fluorescence signal. Finally, fluorescence was measured at 530 nm excitation and 575 nm emission.

For starch analysis, the DNS assay was used, as described previously in Chapter 3.

4.2.6 Statistical analysis

All experiments contained biological triplicates for treatments and controls. Means and standard deviations on the biological replicates were determined in Microsoft Excel. Statistical analyses were carried out using R, with the ‘car’ and ‘agricolae’ packages (Team, 2013). Analysis of variance (ANOVA) and Tukey's HSD test were used to separate the mean for different treatment groups. A p-value of less than 0.05 was considered significant.

4.3 Results

4.3.1 *A. brasilense* and its IAA secretions reduced algal ROS levels in the absence of an exogenous stressor

In our previous study (Peng et al., 2020), we found coculturing with the plant growth promoting bacteria *A. brasilense* and exogenous phytohormone IAA both stimulated growth in *C. sorokiniana* UTEX 2714, while suppressing energy storage products in growing cells. However, we never tested the level of intracellular reactive oxygen species in those studies. We therefore began this study by repeating our previous experiment while measuring algal growth and intracellular ROS. The growth patterns were similar to our previous study with growth in co-cultures > IAA treatment > spent *A. brasilense* medium treatment > axenic controls (**Figure 8A**). No exogenous stress source was applied in this experiment and yet significant differences in intracellular ROS were observed among treatments (**Figure 8B**). None of the culture treatments differed significantly from the control cultures immediately after inoculation. However, from 24-48 hours, which coincided with rapid algal growth, the presence of *A. brasilense*, IAA, and spent *A. brasilense* medium all significant decreases ROS levels compared to controls. The *A. brasilense* treatments decreased ROS levels by 65% to 69% compared to controls after 24 hours ($p < 0.01$). After 72 hours, the decreases in ROS induced by *A. brasilense* and spent medium were no longer statistically significant.

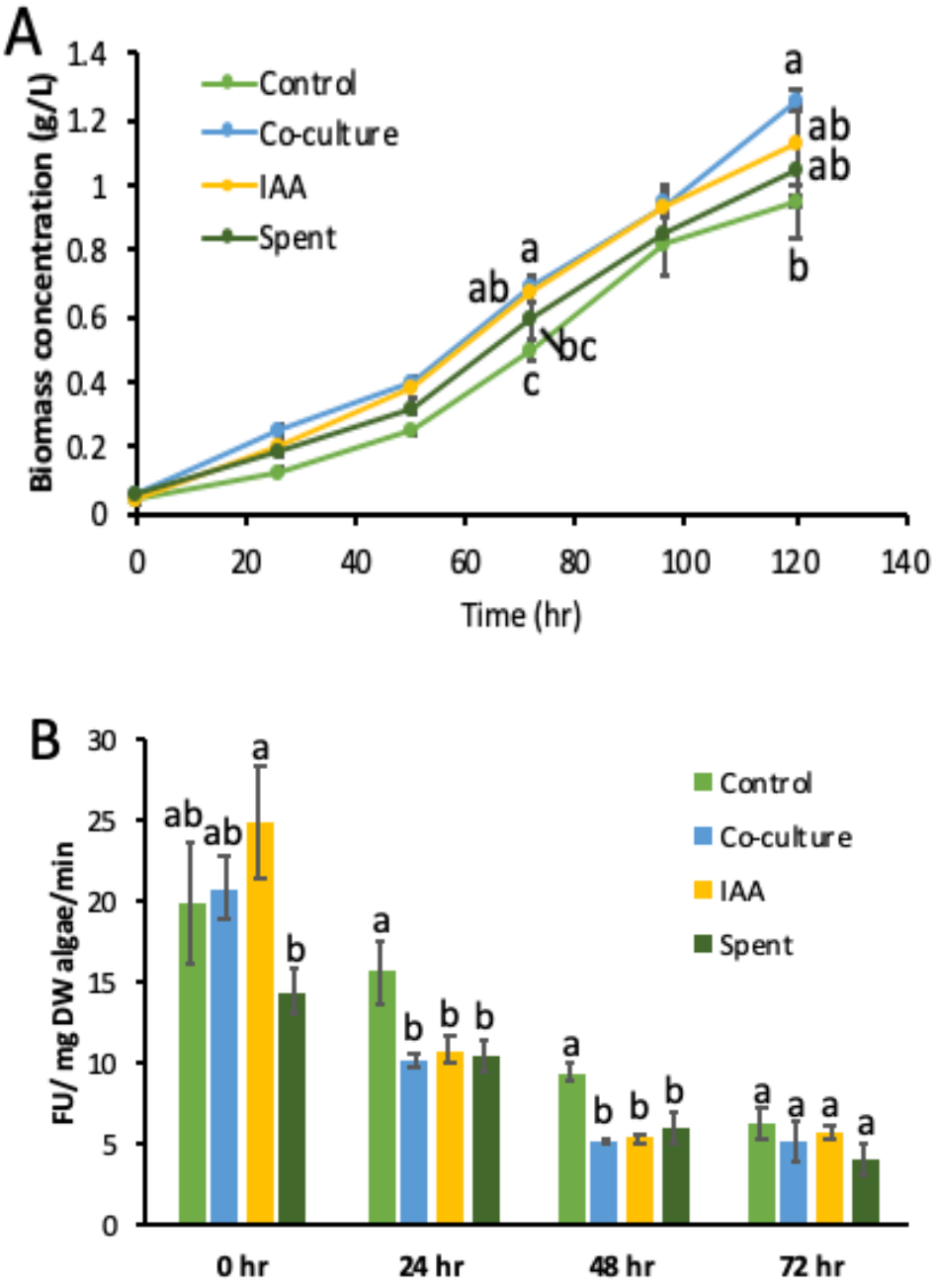

Figure 8 Growth curves for cultures of *C. sorokiniana* (A) and rate of intracellular ROS accumulation (B), in the absence of an exogenous stressor
(The fluorescence increase rate is determined and is considered to be proportional to the rate of intracellular ROS production. The rate of fluorescence increase is normalized by dividing by the algal culture density dry weight).

4.3.2 *A. brasilense* and IAA decrease algal ROS levels under a copper stressor

We next tested if *A. brasilense* and exogenous IAA could mitigate *C. sorokiniana* from an exogenous stressor in the form of copper sulfate. Results showed that the copper treatment suppressed algal growth by 20-32% compared to the control ($p < 0.001$) but that *A. brasilense* and exogenous IAA had a small but significant ($p < 0.01$) restorative effect (**Figure 9A**). After 120 hours, addition of *A. brasilense* or IAA promoted culture growth by 3.5% and 8%, respectively relative to the copper-only treatment.

Addition of the 25 μM copper stressor led to a sharp and significant increase in the ROS level for all time points except for 96 hours (**Figure 9B**). The addition of exogenous IAA and *A. brasilense* decreased the ROS levels by 31% and 35% ($p < 0.014$), respectively, compared to copper-only cultures at the zero-hour time point. However, the ROS levels in these groups were still nominally higher than the control group. After 72 hours, the ROS benefits associated with *A. brasilense* cocultures waned and ROS levels were no longer significantly different from the copper-only treatment. However, the positive effects of IAA persisted through 72 hours. After 96 hours, there was no significant difference in ROS level among any treatments.

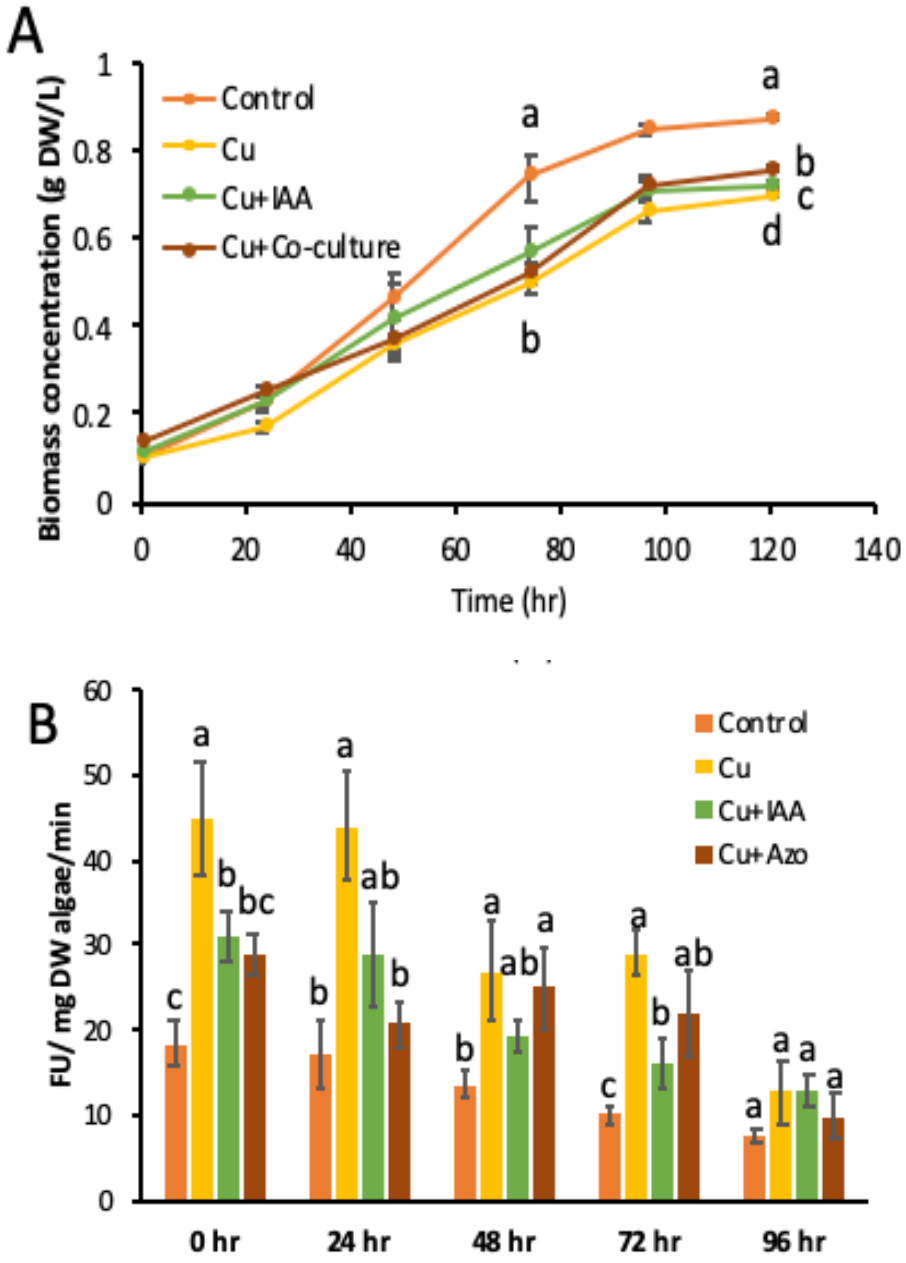

Figure 9 Growth curves for cultures of *C. sorokiniana* (A) and rate of intracellular ROS accumulation (B), under a copper stressor

(The fluorescence increase rate is determined and is considered to be proportional to the rate of intracellular ROS production. The rate of fluorescence increase is normalized by dividing by the algal culture density dry weight).

4.3.3 Co-culturing algae with *A. brasilense* alleviated stress associated with nitrogen limitation

The effects of nitrogen limitation were investigated in the presence and absence of live *A. brasilense*. In the absence of *A. brasilense*, nitrogen limitation suppressed growth compared to control cultures (**Figure 10A**). After 120 hours, the culture density in the control cultures was roughly 3 times higher than the N-limited cultures ($p < 0.001$). Co-culturing *C. sorokiniana* with *A. brasilense* led to growth stimulation regardless of whether N was limited or not. In N-replete cultures, *A. brasilense* led to 36% higher culture density by 72 hours ($p = 0.001$) but this difference waned after 120 hours compared to the *C. sorokiniana* monoculture. In N-limited cultures, A. brasilense led to a 30% higher culture density after 72 hours ($p = 0.07$) and a 44% higher culture density after 120 hours ($p < 0.001$) compared to monocultures.

Under nitrogen limitation stress, the intracellular ROS level increased significantly ($p < 0.012$) compared to control cultures across all time points (**Figure 10B**). Coculturing with *A. brasilense* decreased the ROS level in both the N-replete and N-limited medium groups. It is noteworthy that when adding *A. brasilense* to the N-limited group, there was no significant difference in ROS level compared to the control group at 24 and 48 hours. This suggests that *A. brasilense* could fully alleviate oxidative stress due to N limitation in *C. sorokiniana,* at least temporarily. By 72 hours, the control group had lower ROS levels than the co-culture group subjected to nitrogen limitation.

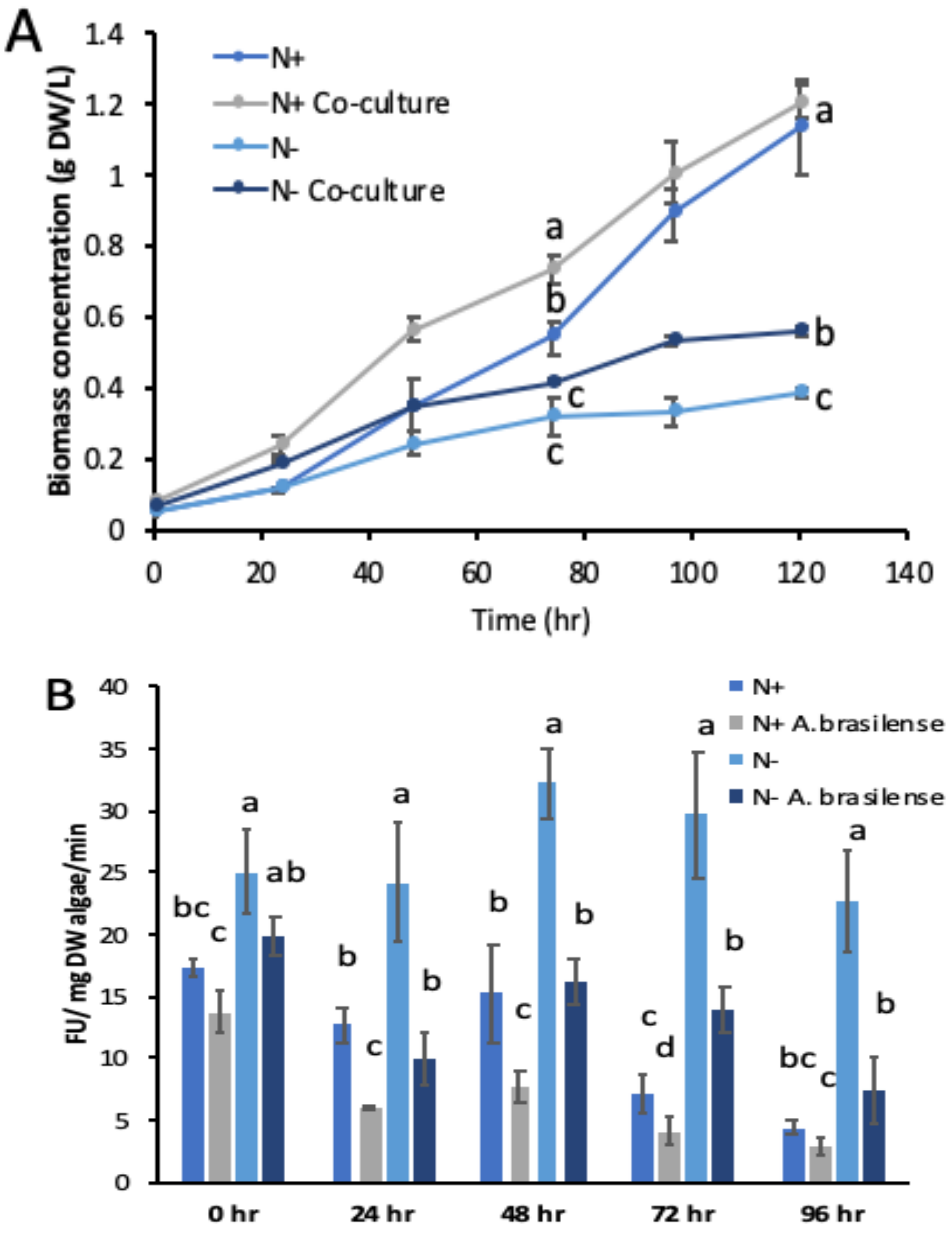

Figure 10 Growth curves for cultures of *C. sorokiniana* (A) and rate of intracellular ROS accumulation (B), under nitrogen limitation

(The fluorescence increase rate is determined and is considered to be proportional to the rate of intracellular ROS production. The rate of fluorescence increase is normalized by dividing by the algal culture density dry weight).

4.3.4 Changes in pigment chlorophyll content in response to stressors

Chlorophyll levels are known to decline in conjunction with increases in oxidative stress, including stress induced by copper (Qian et al., 2009) and nitrogen limitation (Zhang et al., 2013). Here, we measured chlorophyll a and b in response to copper (**Table 4**) and nitrogen stressors (**Table 5**).

Under copper stress, the chlorophyll a level, measured at 72 hours and 120 hours, was significantly lower than control cultures ($p < 0.04$). Interestingly, the addition of IAA suppressed chlorophyll content further although it was not significantly lower than the copper-only treatment. Coculturing *C. sorokiniana* with live *A. brasilense* in the presence of copper led to 12% higher chlorophyll a compared to the copper-only treatment but this differences not statistically significant ($p = 0.66$). The copper treatments had no significant effect on chlorophyll b content.

Additionally, nitrogen limitation stress led to significantly lower chlorophyll a and b content compared to nitrogen replete cultures. Compared with the control group, the nitrogen-limited group had only roughly 20-30% of the chlorophyll a content of the control cultures ($p < 0.001$). Coculturing with *A. brasilense* increased the chlorophyll level significantly in the N-replete cultures ($p = 0.043$). *A. brasilense* also increased chlorophyll levels by 21% in N-limited cultures at 72 hours but the difference was not statistically significant ($p = 0.4$).

Table 4 Effect of Copper stressor on algal biomass composition

		Control	Cu[1]	Cu + IAA[2]	Cu + Co-culture[3]
72-hour	Chlorophyll a (μg/mg DW)	21.0 (2.1) a	15.4 (2.1) b	12.8 (1.0) b	17.3 (2.6) ab
	Chlorophyll b (μg/mg DW)	5.1 (0.7) a	4.8 (1.3) a	4.2 (0.3) a	6.0 (1.4) a
	Neutral lipid (%)	2.2 (0.13) ab	2.6 (0.22) a	2.0 (0.12) b	2.1 (0.16) b
	Starch content (%)	17.2 (0.7) a	18.2 (1.4) a	16.5 (1.7) a	14.3 (2.0) a
120-hour	Chlorophyll a (μg/mg DW)	22.8 (1.7) a	19.6 (3.5) b	17.4 (1.1) b	19.4 (3.0) b
	Chlorophyll b (μg/mg DW)	6.0 (1.6) a	6.8 (0.9) a	5.3 (0.3) a	5.6 (0.2) a
	Neutral lipid (%)	2.7 (0.30) a	1.7 (0.23) b	1.6 (0.29) b	1.7 (0.14) b
	Starch content (%)	21.4 (1.9) a	10.6 (1.3) b	11.3 (1.9) b	11.1 (2.5) b

[1]Algae were grow under 25 μM copper stress

[2]The IAA treatment included exogenous IAA at 50 mg/L to *C. sorokiniana* cultures, under 25 μM copper stress

[3]Algae were in coculture with *A. brasilense*, with 1:1 cell ratio, under 25 μM copper stress.

Table 5 Effect of Nitrogen limitation stressor on algal biomass composition

		N+ (control)	N+ Co-culture[1]	N- [2]	N- Co-culture[3]
72-hour	Chlorophyll a (μg/mg DW)	18.9 (3.0)[4] b[5]	23.3 (1.1) a	5.6 (0.3) c	6.8 (0.2) c
	Chlorophyll b (μg/mg DW)	4.1 (1.7) a	5.7 (0.7) a	1.5 (0.1) b	1.5 (0.2) b
	Neutral lipid (%)	2.1(0.11) ab	1.8 (0.22) b	2.8 (0.36) a	2.5 (0.35) a
	Starch content (%)	12.0 (2.2) b	10.0 (1.0) b	46.6 (5.9) a	42.1 (2.9) a
120-hour	Chlorophyll a (μg/mg DW)	20.7 (2.2) a	24.2 (2.4) a	4.2 (0.2) b	4.9 (0.7) b
	Chlorophyll b (μg/mg DW)	4.6 (0.8) a	5.0 (0.8) a	1.2 (0.1) b	1.7 (0.3) b
	Neutral lipid (%)	1.8 (0.33) b	1.6 (0.20) b	3.8 (0.65) a	2.9 (0.32) ab
	Starch content (%)	12.5 (5.2) c	15.9 (4.5) bc	47.3 (7.3) a	28.6 (2.7) b

[1]Algae were in coculture with *A. brasilense*, with nitrogen

[2]Algae were under nitrogen limitation

[3]Algae were in coculture with *A. brasilense*, with 1:1 cell ratio, under nitrogen limitation stress

[4]Values in parentheses are standard deviations based on 3 biological replicates

[5]Within a role, values with the same letter were not statistically different at the 0.05 level.

4.3.5 Changes in neutral lipid and starch content in response to stressors

Neutral lipid and starch are the two main energy storages in *C. sorokiniana*. Stress conditions lead to accumulation of neutral lipid and starch in many algae (Higgins et al., 2014; Markou & Nerantzis, 2013; Sun et al., 2014). Cu stress led to a 15% increase in neutral lipid content 72 hours compared to control cultures, but the difference was not statistically significant (**Table 4**). However, adding IAA or *A. brasilense* led to a significant reduction in neutral lipid content compared to the copper-only cultures ($p < 0.029$). After 120 hours, neutral lipid was roughly two thirds that of the control in all copper-containing cultures. Starch levels followed the same pattern as neutral lipid, but no significant differences were found at 72 hours among any treatments. Copper had a suppressive effect on starch by 120 hours but IAA and *A. brasilense* had no effect.

Nitrogen limitation stimulated neutral lipid and starch production (**Table 5**). Compared with nitrogen-replete medium, the nitrogen-limited group experienced a 2-fold increase in neutral lipid content after 120 hours ($p = 0.001$). It took some time for the strength of this effect to materialize as the difference in neutral lipid content between N-replete and N-limited cultures were not statistically significant at 72 hours. Adding *A. brasilense* to the cultures slightly decreased the neutral lipid, but the effect was not statistically significant. Similar trends were found for starch content except that the strength of the effect from N limitation was stronger

(nearly 4-fold increases) and more immediate compared to that observed with neutral lipids. Adding *A. brasilense* led to little difference in starch content in the nitrogen-replete cultures but led to a 40% decline in starch content in nitrogen-limited cultures at 120 hours ($p < 0.01$).

4.4 Discussion

In this study, we confirmed that co-culturing *C. sorokiniana* with live *A. brasilense* or growing it with the auxin hormone IAA could reduce the oxidative stress. It is already well established that copper and nitrogen limitation induce oxidative stress and reductions in chlorophyll content (Knauert & Knauer, 2008; Qian et al., 2009) and was confirmed for this algae strain. Oxidative stress regulates the algal biochemical composition, promoting lipid and starch accumulation in this strain, similar to findings by others (Burch & Franz, 2016; Yilancioglu et al., 2014a). That said, while both copper and nitrogen limitation clearly induced oxidative stress and suppressed growth and chlorophyll content, the copper treatments had a much smaller impact on neutral lipid and starch content compared to nitrogen limitation.

Copper is known to decrease the efficiency of the photosystem (Knauert & Knauer, 2008) due to inhibition of electron acceptors on the reducing-side of photosystem II (Yruela et al., 1991). Photosystem inhibition is caused by copper-induced ROS accumulation and this effect can be reversed through antioxidant application (Knauert & Knauer, 2008; Yruela, et al., 1996). Other researchers have found that long-term copper exposure induces the activation of algal

antioxidant defense systems and increases lipid peroxidation (Tripathi et al., 2006). Less information is available on copper's effect on lipid and starch accumulation in microalgae. Yang et al. (2015) found that copper significantly induced lipid in *Auxenochlorella protothecoides* UTEX 2341 (formerly classified as *C. minutissima* (Higgins et al., 2015)). In our study, we saw an insignificant increase in neutral lipid and starch content in *C. sorokiniana* in response to copper treatment despite a large increase in ROS. This suggests that, although ROS is linked to neutral lipid accumulation in *Chlorella*, it is not the sole controlling factor. Nitrogen limitation also induced oxidative stress but also led to a significant increase in neutral lipid accumulation and a large increase in starch production in *C. sorokiniana*.

Our results confirm the hypothesis that coculturing *C. sorokiniana* with *A. brasilense* decreased ROS levels under some stress conditions. These include conditions without exogenous stress, under copper stress, and under nitrogen limitation. Interestingly, our data showed that there was no significant difference in ROS levels between nitrogen-limited co-cultures and nitrogen-replete monocultures of *C. sorokiniana*. This indicates that *A. brasilense* could mitigate the algae from the stress it would normally experience under N limitation. This in turn can explain why the presence of *A. brasilense* leads to faster growth and higher chlorophyll content as we observed in this study as well as our previous study (Peng et al., 2020).

This reduction in oxidative stress is linked in part to the secretion of the auxin hormone, IAA, by *A. brasilense*. The exogenous dose of IAA in both the unstressed and copper-stress

cultures showed a significant reduction in intracellular ROS at several time points. Piotrowska-Niczyporuk and Bajguz (2014) also showed that auxin hormones, including IAA, stimulate both enzymatic and non-enzymatic antioxidant systems in *Chlorella vulgaris*. Antioxidants scavenge ROS (generated in the presence and absence of exogenous stressors), leading to higher growth and chlorophyll content as we observed in this study. ROS is already known to be a key governing factor in the accumulation of neutral lipids in algae (Burch & Franz, 2016; Yilancioglu et al., 2014a). Thus, suppression of ROS by *A. brasilense*-produced auxins was expected to suppress neutral lipid and starch accumulation in *C. sorokiniana*. The data generally support this hypothesis but with some ambiguity. In nitrogen-limited cultures, the presence of *A. brasilense* suppressed starch production but the suppression of neutral lipid production was not statistically significant. In copper-stressed cultures, *A. brasilense* led to a small but statistically significant decrease in neutral lipid content but the decline in starch content was not statistically significant. In other words, the ability of *A. brasilense* and IAA to reduce oxidative stress is clear. The ability of *A. brasilense* to suppress energy storage product accumulation via reductions in oxidative stress, led to relatively small effect sizes. *A. brasilense* is also known to fix nitrogen (Souza et al., 2014) and this could have been a confounding factor in the nitrogen-limitation study. However, the fact that *A. brasilense* also significantly suppressed ROS in nitrogen-replete and copper stressed cultures indicate that the aforementioned antioxidant stimulation mechanism is likely dominant.

The biotechnological implications of auxin-producing bacteria, such as *A. brasilense*, on algae cultures are well-established (Amavizca et al., 2017). They induce faster algal growth and the ability to withstand stressful environmental conditions. Our results may also help to explain why past researchers have found that *A. brasilense* helps certain algae overcome pH, light, and salinity stresses (de-Bashan & Bashan, 2010). However, our current and recent findings indicate that these positive attributes come at the cost of energy storage products which are often valued for biofuel applications (Peng et al., 2020). This study shows that this tradeoff is mediated in part through the auxin-induced antioxidant mechanisms that suppress ROS and thereby suppress accumulation of neutral lipids and starch.

4.5 Conclusion

In the present study, we investigated oxidative stress in the green algae, *C. sorokiniana* UTEX 2714, in co-culture with the plant growth promoting bacteria, *Azospirillum brasilense*. This relationship was studied in the absence of an exogenous stressor, under copper stress, and under nitrogen limitation. We confirmed that copper and nitrogen limitation induced algae oxidative stress and reductions in chlorophyll content. In all cases, the presence of *A. brasilense* lowered the concentration of intracellular reactive oxygen species (ROS) while promoting chlorophyll content. This effect was driven, at least in part, by *A. brasilense's* secretion of the auxin hormone, indole-3-acetic acid, which is already known to mitigate stress in plants. The findings of the

present study show that stress mitigation by *A. brasilense* resulted in suppressed accumulation of starch and neutral lipid that otherwise occur under conditions that induce oxidative stress. In fact, *A. brasilense* could almost completely rescue *C. sorokiniana* from the effects of nitrogen limitation stress with no significant difference in ROS level from the axenic control cultures. The biotechnological implication of these findings is that co-culture strategies with *A. brasilense* (and similar PGPB) are most effective for high growth applications. A second growth stage may be needed to induce accumulation of desired products.

Chapter 5 Comparison of algae growth in the presence of PGPB and non-PGPB organism

5.1 Background

Algae have attracted attention as a feedstock for biofuel production, wastewater treatment, and synthesis of nutraceuticals. Algae have very high growth rates (15 to 50 MT/ha/year) and, for certain oleaginous algae, their oil yields can reach 20,000 to 60,500 L/ha/year (Weyer et al., 2010). The higher end of these ranges remains aspirational; however, despite their fast growth, algae suffer from high production costs. Much of these costs are driven by high capital investment and studies have shown that increasing algal growth rates further is one of the most important ways to reduce feedstock costs (Davis et al., 2011). Research on improving algal growth rates has therefore been a subject of intense research interest.

Growth-enhancing strategies include mixotrophic growth (Higgins & VanderGheynst, 2014; Liang et al., 2009), genetic modification (Negi et al., 2020), and cultivation conditions that maximize photosynthetic efficiency (Granata, 2017). Another approach that has been investigated is the co-cultivation of algae with bacteria (Amavizca et al., 2017; Higgins & VanderGheynst, 2014). This latter approach is particularly attractive because bacteria naturally grow in the presence of algae in nature and are known to confer benefits to algae in many cases (Bai et al., 2014; Croft et al., 2006). Moreover, purposely growing algae with bacteria can help

alleviate the challenge of maintaining pure algal monocultures which are notoriously unstable and susceptible to culture crashes (Godwin et al., 2018).

Mechanisms of symbiosis between algae and bacteria include exchange of metabolites such as vitamin cofactors (Croft et al., 2006), amino acids (Palacios et al., 2016), and phytohormones (de-Bashanet al., 2008). Exchange of oxygen and carbon dioxide can also be significant and beneficial (Bai et al., 2014), particularly under conditions with degradable organic carbon present (Holmes et al., 2019). Plant growth promoting bacteria (PGBP) have gained particular attention given their ability to promote growth in green algae via secretion of plant hormones (Amavizca et al., 2017; Kim et al., 2014; Peng et al., 2020). In nature, PGPB are typically found in the rhizosphere of plants, but research by us and others has shown that these bacteria can grow in suspended algae cultures as well as in alginate bead systems with algae (Choix et al., 2012; Peng et al., 2020). Two PGPB, *A. brasilense* Cd and *B. pumilus* ES4, promoted a 6-fold increase in growth of *C. sorokiniana* due in part to secretion of volatile organic compounds including acetoin and 2,3 butanediol (Amavizca et al., 2017b) which are known to stimulate growth in plants (Ryu et al., 2003). Lee et al. (2019) also showed that another PGPB, *Achromobacter* sp. CBA4603, can stimulate algal growth via provision of hormones and volatile compounds. Given these findings, the benefits of PGPB toward algal growth are now widely recognized.

In our previous study, we found that the PGPB, *A. brasilense*, significantly increased the growth of *C. sorokiniana* (UTEX 2714), driven in part by the secretion of the auxin hormone indole-3-acetic acid (IAA) (Peng et al., 2020). However, algal growth in the presence of live *A. brasilense* was significantly higher than growth in the presence of exogenous IAA or spent medium from *A. brasilense*. This indicates that other symbiotic mechanisms associated with live bacteria are important (Peng et al., 2020). Our past results also raised questions about how much more effective PGPB are compared to non-PGPB. The latter organisms generally do not make plant hormones but often provide significant growth benefits to algae (Higgins & VanderGheynst, 2014). Indeed, Amavizca et al. (2017) showed that the non-PGPB *E.coli* can provide growth benefits to green microalgae, but their study only investigated the impact of volatile metabolites exchanged between bacteria and a single strain of green microalgae. In their study, it was assumed that *E. coli* provision of CO_2 was the main mechanism of symbiosis. To date, there has not be a comprehensive evaluation of PGPB versus non-PGPB impacts on algal growth in suspended cultures.

The objective of this study was to test the benefits of a model PGPB versus non-PGPB in a side-by-side study, evaluating improvement in algal growth rates and impacts on biomass composition. In this study, we tested the impact of the model PGPB, *Azosprillum brasilense,* versus the non-PGPB *Escherichia coli.* We also included a strain of *Bacillus megaterium* which was originally isolated from one of our contaminated algae cultures and had exhibited growth-

promoting properties (Higgins et al., 2018). These bacteria were tested with three green algae strains, *Chlorella sorokiniana* (UTEX 2714 and UTEX 2805) and *Auxenochlorella protothecoides* (UTEX 2341). Biomass growth, algal-bacterial biomass ratio, biomass composition, and nutrient uptake were measured as responses. We found that there was little benefit in growth promotion by *A. brasilense* versus *E. coli* and this prompted further investigation of a newly suggested growth-promotion mechanism that is expected to be nearly universal among bacteria: riboflavin metabolites. Research by Heo et al. (2019) showed that riboflavin metabolites from bacteria are likely to be an important mechanism of growth promotion in *Chlorella*. The riboflavin derivative, lumichrome, is a known plant-growth promoting hormone which stimulates shoot growth and starch accumulation in plants (Gouws et al., 2012; Phillips et al., 1999) but its impacts on algae have hardly been studied (Lopez et al., 2019). Because it is derived from riboflavin, lumichrome synthesis is expected in a wide range of PGPB and non-PGPB. The results of this study will provide insight into the effects of PGPB-specific growth promoting mechanisms (e.g. auxin and volatile organic compounds) versus other growth-altering mechanisms that are ubiquitous in bacteria.

5.2 Materials and Methods

5.2.1 Microalgae and bacteria growth conditions

Three microalgae strains were tested in the presence of three bacterial species. The microalgae tested were *C. sorokiniana* (UTEX 2714), *C. sorokiniana* (UTEX 2805), and *A. protothecoides* (UTEX 2341, formerly classified as *Chlorella minutissima* (Higgins et al., 2015)). *C. sorokiniana* UTEX 2714 a strain of unicellular green microalgae that was isolated from secondary effluent of a wastewater treatment pond (Bashan et al., 2016). *C. sorokiniana* UTEX 2805 was also isolated from wastewater stabilization ponds and is extremely tolerant to high temperature and light intensity (de-Bashan et al., 2008). Given their origins, both of these strains have promise for wastewater treatment (Wang et al., 2019) and also production of starch (Peng et al., 2020; Tanadul et al., 2014). *A. protothecoides* UTEX 2341 is a eukaryotic alga that grows rapidly and accumulates very large amounts of neutral lipid (up to 57%) under nitrogen-limited conditions (Illman et al., 2000). This strain has also proven effective in wastewater treatment (Higgins et al., 2017).

Both *C. sorokiniana* strains UTEX 2714 and UTEX 2805 were pre-cultured in sterilized N8 medium (Tanadul et al., 2014), and *A. protothecoides* UTEX 2341 was pre-cultured in sterilized N8-NH_4 medium (Higgins & VanderGheynst, 2014). These algae stock cultures were established from colonies scraped from plates to ensure axenic cultures. All cultures were handled in a biosafety cabinet using sterile technique.

The bacteria, *A. brasilense* Cd*, E. coli* ATCC 25922*, and B. megaterium* (isolate) were reactivated in liquid LB medium from axenic freezer stocks. Bacterial cultures were grown at 30 °C for 12 to 24 hours at 120 rpm on an orbital shaker until an optical density (OD) > 0.2 at 550 nm.

5.2.2 Experimental Design

Each microalgae strain was cultivated in 5-day batch cultures in monoculture (control) and with each of the three bacteria species: *A. brasilense*, *E. coli*, and *B. megaterium*.

The stock cultures of bacteria and algae were centrifuged at 4500g for 5 minutes to concentrate them for inoculation into experimental photobioreactors. Bacterial cell pellets were completely decanted to remove LB medium prior to resuspension in sterile algal medium. Microalgae and bacteria were inoculated at a ~1:1 ratio into the 12 experimental photobioreactors. Cell counts were estimated based on previously-established correlations between OD 550 nm and viable colony counts. All the batch experiments were carried out in 300 ml bubble column photobioreactors with a working volume of 200 ml as previously described in detail (Wang, et al., 2019). Briefly, the reactors were suspended in 28 °C water bath with horizontal illumination of 170 μmol photons/m^2/s, operated on 14h:10h light and dark cycle. Compressed air supplemented with 2% CO_2 was filtered (0.22 μm) and bubbled into the reactors at 100 ml/min. Reactors were stirred at ~150 rpm by stir bar.

Biomass productivity was measured according to Wang et al. (2019). Briefly, 2 ml samples were collected using a sterile syringe every 24 hours and adjustments to pH 7.2 were made as necessary with 3M NaOH. Optical density (OD) at 550 nm was measured every 24 hours and unique correlations to the algal dry weight concentration for each treatment after final culture harvest. For the harvest processing, each sample was collected after 72 h and 120 hours, washed with dH_2O to remove salts, and freeze-dried. The 72-hour time point captured the exponentially growing cells and the 120-hour time point captured the log growth stage. All sampling was performed in the biosafety cabinet using sterile technique.

5.2.3 DNA extraction and qPCR amplification

Genomic DNA was extracted from freeze-dried co-culture biomass using the FastDNA Spin Kit following the manufacturer's instructions. DNA was also extracted from known masses of pure cultures of each of the three bacteria. Quantitative PCR (qPCR) was conducted to determine the mass fraction of bacteria in the co-cultures, an approach that has already been described in detail elsewhere (Higgins & VanderGheynst, 2014; Peng et al., 2020). Briefly, primers specific to *A. brasilense* (Peng et al., 2020), *E. coli* (Higgins & VanderGheynst, 2014), and *B. megaterium* were used to amplify a segment of the 16S rRNA gene unique to these bacteria. In this work, we developed a set of primers unique to *Bacillus* species. This was accomplished by aligning 16S rRNA gene sequences from four species of *Bacillus* and identifying regions that were conserved

in *Bacillus* but also different from its near relatives including *Thermobacillus* and *Staphylococcus*. Gene alignments were performed using Geneious software and selected primers were checked for melting temperature and dimer complexes using IDT's Oligo Analyzer tool. The primers used are listed in **Table 6**. Dilutions of DNA obtained from the known quantities of pure bacteria were used to generate standard curves for the qPCR analysis. The PerfeCta SYBR Green Fast Mix (Quanta Bio) and 0.5 µM of forward and reverse primers were used in a 20 µl reaction volume. PCR was carried out on a qTower 3 (Analytic Jena) using the following thermal cycling conditions: 95 °C for 15 sec, 50-55 °C for 15 sec, and 72 °C for 30 sec. The amplification efficiency was in the range of 96%-102% for all primer sets.

Table 6 Primers used for qPCR amplification of bacteria in the three coculture systems

		Sequence (5'-3')	Annealing Temperature (°C)	Amplicon Size (bp)
A. brasilense	Forward	**CTACCGCCAGTTGCCATCATT**	55	148
	Reverse	**CTTCGCATCCCACTGTCACC**		
E. coli	Forward	**CAAGACCAAAGAGGGGGACC**	55	110
	Reverse	**TCAGACCAGCTAGGGATCGT**		
B. megaterium	Forward	**AATCTTCCGCAATGGACG**	51	96
	Reverse	**CACTTGTTCCCTAACAAC**		

5.2.4 Determination of Indole-3-acetic acid levels

Culture samples were centrifuged at 12,000 g for 5 min, then filtered through 0.2 □m syringe filters to remove cells. Quantitation of IAA was determined using high pressure liquid chromatography-electrospray ionization-mass spectrometry HPLC-ESI-MS with an LCMS 2020 (Shimadzu), as previously described (Peng et al., 2020). A Thermo PolarAcclaim II C18 Column

(3x150 mm, 3 um) was used for separation. Mobile A was 95% ammonium acetate (1 g/L) in Nanopure water, pH 5.0 and 5% methanol; Mobile B was 100% methanol. Flow rate was 0.5 ml/min with a gradient method, and the injection volume was 10 µl. The mass spectrometer was run in scan mode with positive ion ESI to confirm the dominant ions first. Then, analyzed with sim mode at m/z of 176 (H^+ adduct) and 214 (K^+ adduct). Dilutions of pure IAA were used as a standard. Peak integration was analyzed in LabSolutions software (Shimadzu).

5.2.5 Determination of pigment chlorophyll

The content of chlorophyll a and chlorophyll b were analyzed after 72 hour and 120 hours, as described previously (Peng et al., 2020). The algae freeze dryer powder was homogenized by Beadruptor (OMNI) and break the cell wall. Then, the chlorophyll a and b were extracted in modified Folch solvent 90% methanol and concentration was spectrophotometrically determined by measuring optical density at wavelengths 645, 663 and 750 nm. The chlorophyll a and b content were determined as follows (Porra, 2002):

Chlorophyll a (µg/ml) = 12.25(A663 – A750) – 2.55(A645 – A750) Eq. 1

Chlorophyll b (µg/ml) = 20.31(A645 – A750) – 4.91(A663 – A750) Eq. 2

5.2.6 Determination of neutral lipid

Total lipids were extracted using a Folch method as previously described (Higgins & VanderGheynst, 2014). The chloroform extracts were used to measure neutral lipids using a previously-described microplate assay (Higgins et al., 2014) with the modifications described in Wang et al., (2019). Neutral lipids are one of the most important energy storage products in algae and are a biodiesel precursor. Canola oil was used for the standard.

5.2.7 Determination of nutrient uptake condition and COD assay

The soluble nutrient status of cultures was detected using ion chromatography as described by Chaump et al. (2018). For anion chromatography, the chloride, nitrite, nitrate, phosphate and sulfate were analyzed. An AS22 column (Dionex) and basic eluent (4.5 mM sodium carbonate and 1.5 mM sodium bicarbonate, 1 ml/min) were used. Cation chromatography was used to detect sodium, ammonium, potassium, magnesium, and calcium. A CS12 column (Dionex) and acidic eluent (20 mM methane sulfonic acid, 1 ml/min) was used.

The HACH COD kit was used to quantify the chemical oxygen demand for the coculture systems. Samples were diluted 2-fold to ensure the results were between 200-1500 mg/L. Each sample was read three times on a HACH DR900 spectrophotometer and the average was taken.

5.2.8 Measurement of lumichrome by liquid chromatography and time of flight mass spectrometry (LC-MS-TOF)

Analysis was carried out on a Waters UPLC and QTOF Premier mass spectrometer at the Auburn University Mass Spectrometry Center. The same column and mobile phases were used as for the IAA analysis. The flow rate was slowed to 0.3 ml/min and the following gradient was used: 0-1.66 min at 5% B, 1.66-11.66 min ramp linearly to 90% B. Hold 90% B until 15 min, then ramp to 5% B from 15-16 min and hold until 21 min. Electrospray ionization was used in negative scan mode with collision cell energy at 5 eV. The ions used for quantification of lumichrome were m/z 241.077 (M-H) and 198.072.

5.2.9 Data analysis

Results were statistically analyzed using R software. Analysis of variance (ANOVA) was used to determine if significant differences exist among treatments; and Tukey's HSD was used to separate the mean with a significance level ($p<0.05$). Before the ANOVA, the violations of variance homogeneity were checked using Levene's test.

5.3 Results

5.3.1 Impacts of bacteria on algal growth

Three algae strains were grown in the presence of three different bacteria. Partial culture harvests were completed at 72 hours and 120 hours to capture transient effects on biomass composition. In general, all three bacteria were found to promote algal growth but marked differences in the

effect size were observed among individual pairs (**Figure 11**). Greater differentiation was also observed at 72 hours which marked the end of the exponential growth stage compared to 120 hours which represented logarithmic growth.

All three bacteria promoted growth in *C. sorokiniana* UTEX 2714, yielding a 31-39% increase in biomass yield at 120 hours versus the control. At 72 hours, the order of growth enhancement was *A. brasilense* > *E. coli* > *B. megaterium* > control. Total biomass is a combination of both algal and bacterial biomass and thus some of the growth enhancement could be due to bacterial growth. None of the cultures were provided with organic carbon sources, however, so any bacterial growth promotion was driven by secretion of algal photosynthate. Thus, all cellular growth was ultimately driven by photosynthesis. Despite being a member of the same species, *C. sorokiniana* UTEX 2805 exhibited remarkably different behavior in response to bacterial stimulation compared to UTEX 2714. The PGPB, *A. brasilense*, suppressed growth by 1-3% compared to the control but the difference was not statistically significant. In contrast, *E. coli* and *B. megaterium* had growth stimulating effects, resulting in 16% and 13% higher biomass yields, respectively, by 120 hours. In *A. protothecoides*, all three bacteria had a strong growth-promoting effect but the effects of *B. megaterium* at 72 hours were significantly ($p < 0.001$) weaker than the other two bacteria. At 72 hours, *A. brasilense* and *E. coli* led to 69% and 64% higher biomass yield compared to the control, but no significant difference was observed between these two bacteria.

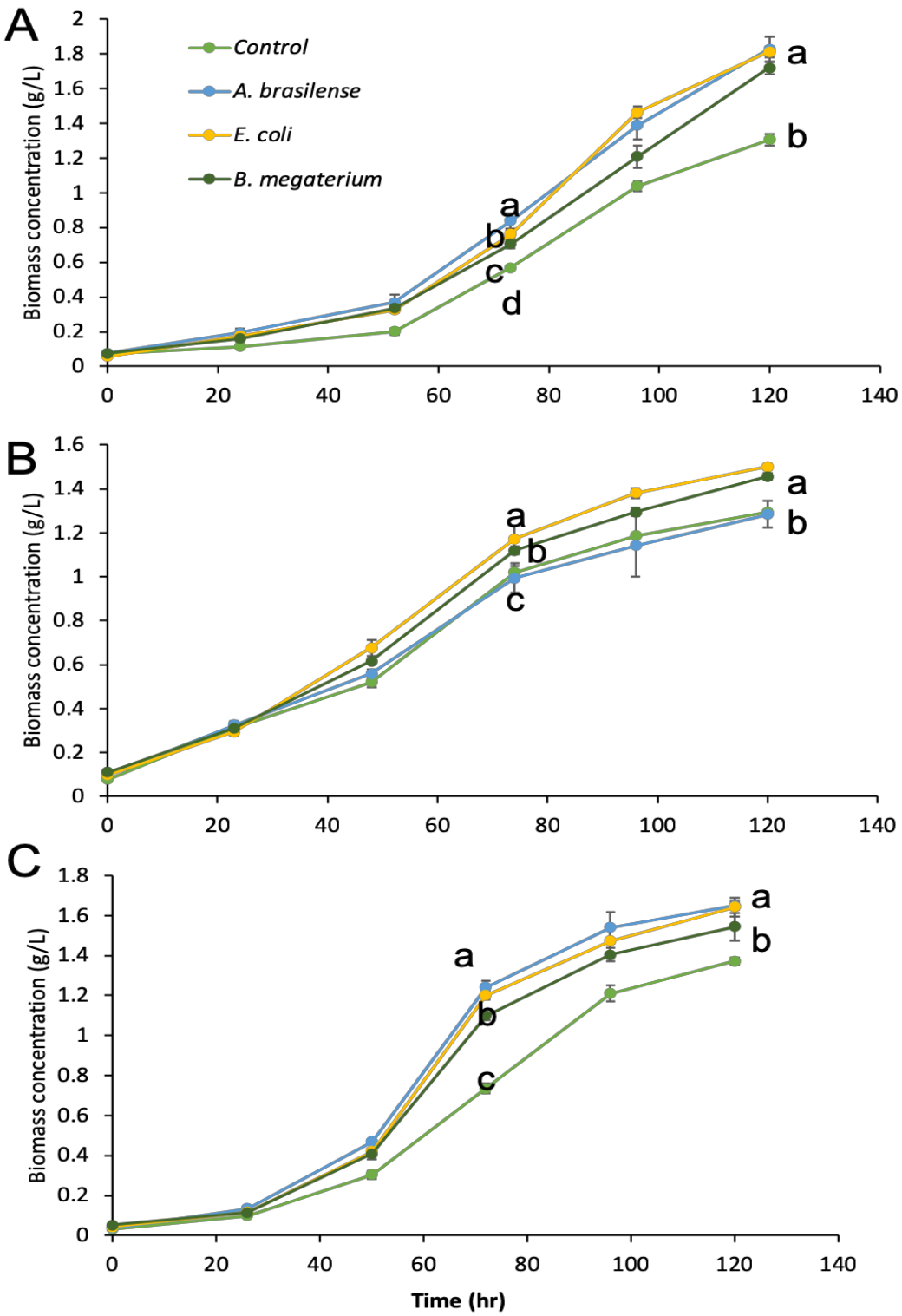

Figure 11 Growth curves for cultures of *C. sorokiniana* UTEX 2714 (A), *C. sorokiniana* UTEX 2805 (B) and *A. protothecoides* UTEX 2341 (C).

Remarkably, the PGPB, *A. brasilense*, only outperformed the non-PGPB, *E. coli*, in the promotion of growth in one algae strain. It also significantly underperformed *E. coli* in one of the algae strains (UTEX 2805). Given that IAA is known to be toxic to certain algae at high doses (Peng et al., 2020) and that this phytohormone is secreted by *A. brasilense* (de-Bashanet al., 2008), we carried out an IAA dose response experiment with UTEX 2805. The result showed that IAA concentrations ranging from 3.1 mg/L to 25 mg/L of IAA promoted this algae's growth whereas a concentration of 50 mg/L had a slight suppressive effect (**Figure A3.1**).

5.3.2 Microalgal and bacterial quantification

Total biomass production is a function of both microalgal and bacterial biomass. To better understand changes in the size of the algal versus bacterial population in these cultures, qPCR was used. All mixed algal-bacterial cultures were inoculated at a target rate of 1:1 on a cell basis which corresponds to roughly 9-16% bacterial biomass on a mass basis. *A. brasilense* populations roughly doubled (both *C. sorokiniana*) or remained stable (*A. protothcoides*) within the first 72 hours. Thereafter, populations declined dramatically. *E. coli* populations roughly doubled over the first 72 hours when in the presence of all three algae followed by a substantial decline as the algal culture growth slowed. *B. megaterium* populations were stable over the first 72 hours in the presence of all three algae followed by a large decline by 120 hours. In all cases, the vast majority of biomass (>95%) was algae by 72 hours of cultivation and over 99% algae by

120 hours. **Table 7** shows that the growth promoting effects of co-cultivation predominantly benefitted algae despite the transient growth of bacterial in the first 72 hours for certain co-culture pairs.

Table 7 Algal and bacterial concentrations (mg/L) over the batch culture

Species	Time	Strain	**Control**	***A. brasilense***	***E. coli***	***B. megaterium***
C. sorokiniana	0 hours	UTEX 2714 Bacteria[1]	61	61 8.9	61 13.1	61 13.3
	72 hours	UTEX 2714 bacteria	495 (<1)[2] d[3]	737 (14) a 16.6 (1.9)	684 (5) b 23.4 (4.8)	609 (7) c 13.4 (4.3)
	120 hours	UTEX 2714 bacteria	1305 (34) b	1827 (73) a 0.8 (0.4)	1812 (31) a 1.3 (0.4)	1717 (34) a 2.0 (0.7)
C. sorokiniana	0 hours	UTEX 2805 bacteria	76	76 9.6	76 15.2	76 16.9
	72 hours	UTEX 2805 bacteria	726 (6) c	689 (21) c 25.3 (9.6)	885 (18) a 30.9 (11.1)	761 (3) b 16.0 (1.4)
	120 hours	UTEX 2805 bacteria	1294 (11) b	1275 (59) b 8.9 (4.2)	1498 (14) a 2.2 (0.8)	1455 (12) a 1.8 (0.7)
A. protothecoides	0 hours	UTEX 2341 bacteria	52	52 10.3	52 12.3	52 13.6
	72 hours	UTEX 2341 bacteria	579 (19) c	924 (23) a 8.8 (2.2)	910 (14) a 25.4 (5.7)	794 (10) b 10.5 (2.7)
	120 hours	UTEX 2341 bacteria	1372 (17) b	1648 (22) a 1.7 (0.3)	1642 (46) a 0.4 (0.1)	1543 (68) a 0.1 (<0.1)

[1]Algae in co-culture with *A. brasilense; E. coli; B. megaterium*

[2]Values in parentheses are standard deviations based on 3 biological replicates. For inoculum (0 hour), only one biological replicate was measured.

[3]Within a row, values with the same letter are not statistically different at the 0.05 level based on Tukey's HSD test.

5.3.3 Indole-3-acetic acid analysis

Indole-3-acetic acid IAA is a plant growth promoting hormone synthesized by *A. brasilense* which can also promote algal growth (de-Bashanet al., 2008). IAA was detected in all co-cultures containing *A. brasilense*. In co-cultures containing *E. coli* and *B. megaterium*, no IAA was detected (**Table 8**). When *A. brasilense* was cultivated with *C. sorokiniana* UTEX 2714, IAA concentrations over the course of the batch culture were: 11.8 μg/L at 0 hour, 84.0 μg/L at 24 hours and 60.3 μg/L at 96 hours. These IAA levels were 3-5 times higher than those in co-cultures of *A. brasilense* with *C. sorokiniana* UTEX 2805 (16.5-22.6 μg/L) and *A. protothecoides* UTEX 2341 (12.5-16.2 μg/L). These results confirmed that the PGPB *A. brasilense* was unique among this set of bacteria in its ability to secrete IAA.

Table 8 IAA concentrations (μg/L) in the medium

	Time	Control	*A. brasilense*[1]	*E. coli*	*B.megaterium*
C. sorokiniana UTEX 2714	0 hour	ND	11.8 (0.6)	ND	ND
	48 hours	ND	84.0 (3.1)	ND	ND
	96 hours	ND	60.3 (2.7)	ND	ND
C. sorokiniana UTEX 2805	0 hour	ND	<LOQ[4]	ND	ND
	48 hours	ND	16.5 (2.8)	ND	ND
	96 hours	ND	22.6 (1.2)	ND	ND
A. protothecoides UTEX 2341	0 hour	ND	<LOQ	ND	ND
	48 hours	ND	16.2 (1.4)	ND	ND
	96 hours	ND	12.5 (0.9)	ND	ND

[1]Algae in co-culture with *A. Brasilense*

[2]Values in parentheses are standard deviations based on 3 biological replicates

[4]LOD was 2.4 μg/L and LOQ was 7.2 μg/L

ND indicates that the peak area was below the LOD

5.3.4 Pigment Analysis

A range of phytohormones produced by PGPB, including IAA, are known to increase algal chlorophyll content (Tate et al., 2013). It is hypothesized that auxin phytohormones such as IAA remodel chloroplasts and pigment levels (Tognetti et al., 2012), thereby increasing growth rates.

Our results show that chlorophyll a enhancement occurred in the presence of all three bacteria tested but to differing degrees (**Figure 12**). In the case of *C. sorokiniana* UTEX 2714, co-cultivation with *A. brasilense*, *E. coli*, and *B. megaterium* resulted in a 2.25, 2.16, and 1.93-fold increase in chlorophyll a, respectively, compared to control cultures at 120 hours. At both 72 hours and 120 hours, however, no significant difference was observed between *A. brasilense* and *E. coli* in terms of chlorophyll a enhancement in this algae. For the other two algae, *A. brasilense* did not lead to a significant change in chlorophyll a content. In contrast, *E. coli* led to a 50% increase in chlorophyll a in *C. sorokiniana* UTEX 2805 (at 72 hours) and a 54% increase in *A. protothecoides* UTEX 2341 (at 120 hours). *B. megaterium* produced the smallest increases in chlorophyll a with the exception of *A. protothecoides* at 120 hours.

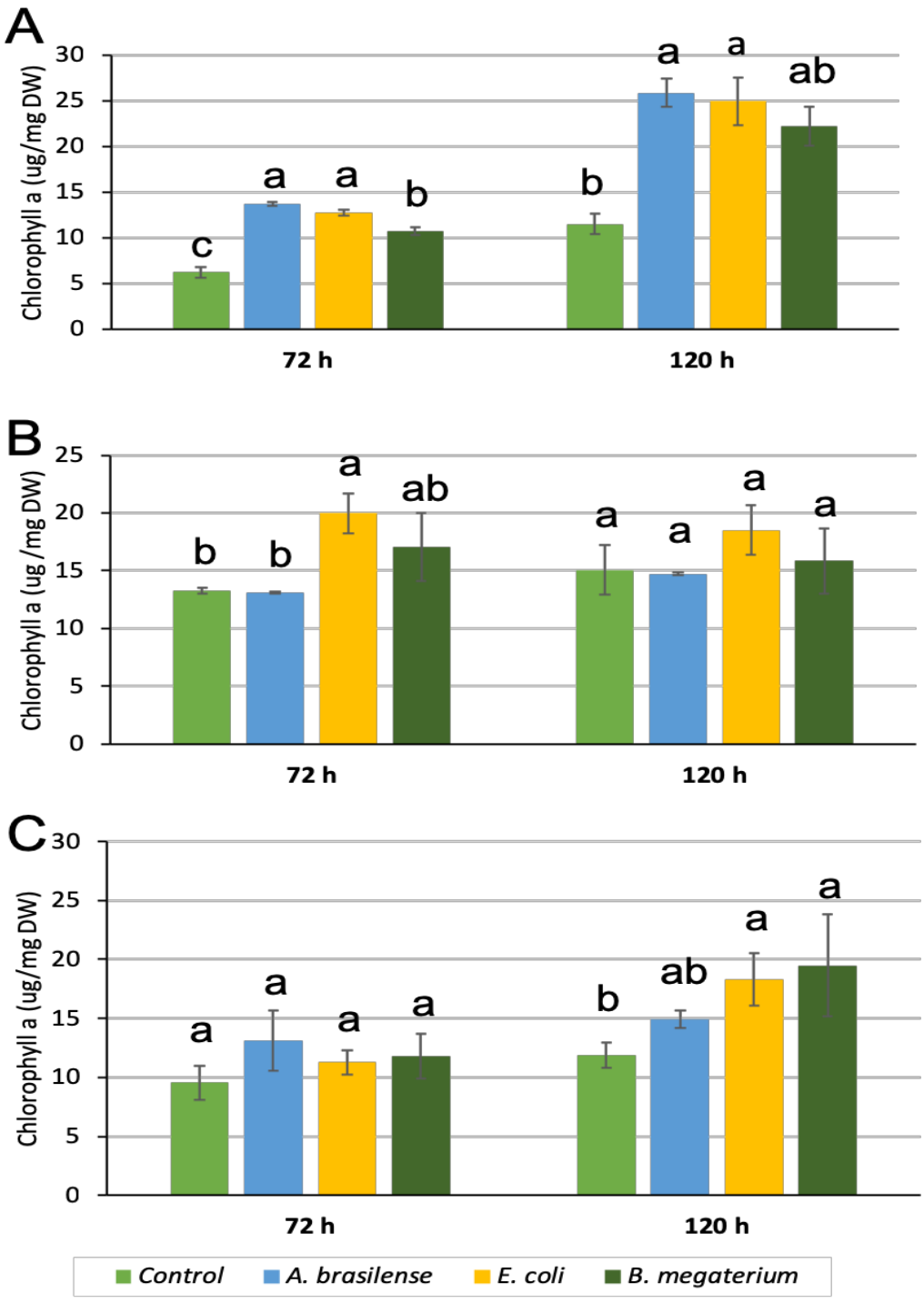

Figure 12 Pigment chlorophyll a content in *C. sorokiniana* UTEX 2714 (A), *C. sorokiniana* UTEX 2805 (B) and *A. protothecoides* UTEX 2341 (C).
Within a time point, data points with the same letter are not significantly different at the 0.05 level based on Tukey's multiple comparison test.

5.3.5 Neutral lipid analysis

Bacteria are known to have significant impacts on biomass composition in algae with acute effects on storage products such as neutral lipids (Higgins et al., 2015; Peng et al., 2020). **Figure 13** shows the impact of coculture treatment on algae energy storage product, particular the neutral lipid content change.

A. brasilense suppressed neutral lipid content by 37% in *C. sorokiniana* UTEX 2714 and in *A. protothecoides* UTEX 2341 by 16% compared to control cultures at exponential stage 72 hours. The effect subsided by stationary stage 120 hours. *E. coli* and *B. megaterium* had no significant effect on neutral lipid content in *C. sorokiniana* UTEX 2714. In fact, none of the bacteria had significant effects on neutral lipid content in either *C. sorokiniana* UTEX 2805 or in *A. protothecoides* UTEX 2341 with one exception. At 72 hours, *E. coli* increased neutral lipid content in *C. sorokiniana* UTEX 2805 by 42% ($p = 0.01$).

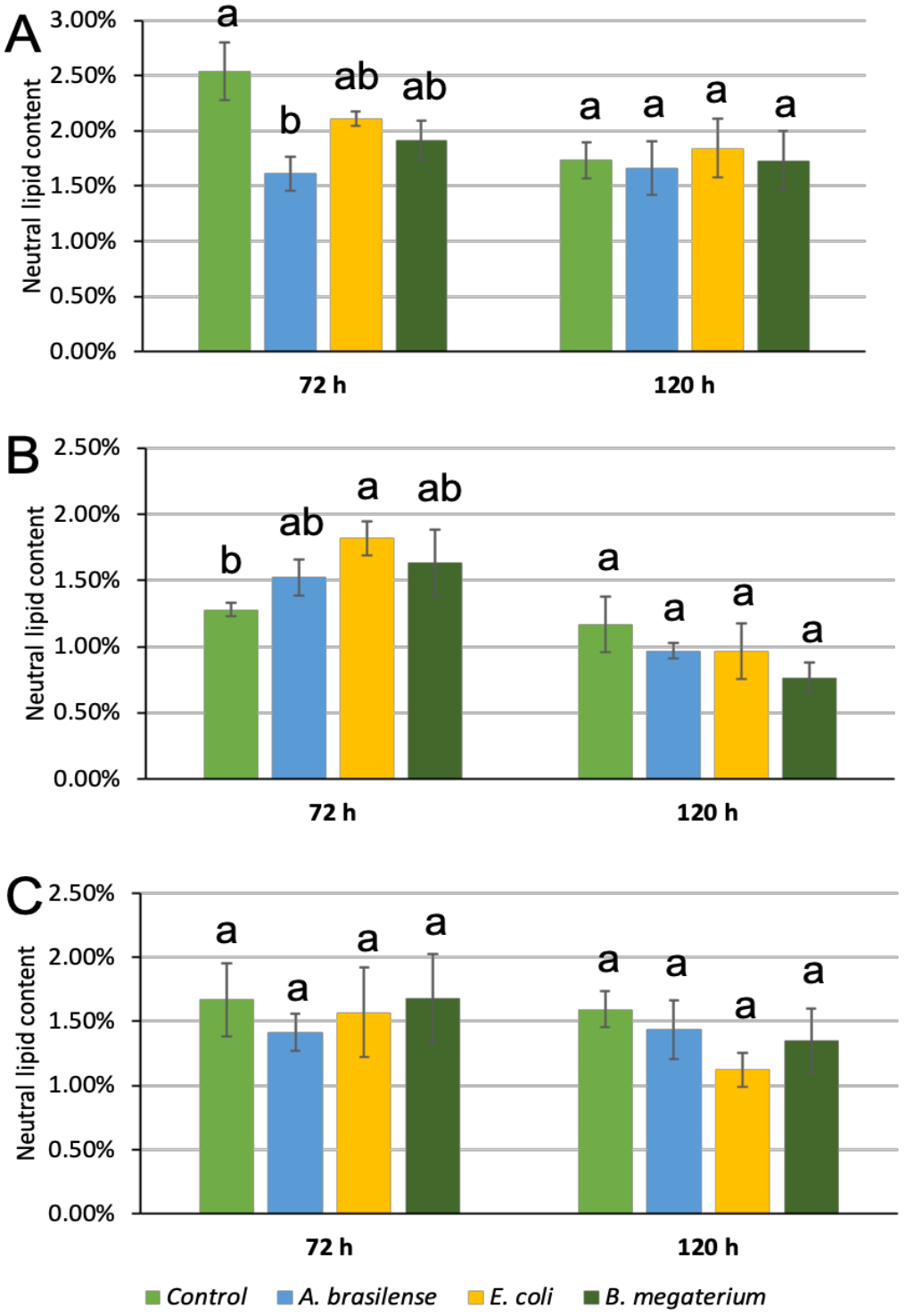

Figure 13 Neutral lipid content in *C. sorokiniana* UTEX 2714 (A), *C. sorokiniana* UTEX 2805 (B) and *A. protothecoides* UTEX 2341 (C).

5.3.5 Nutrient uptake analysis

Algal nutrient uptake rates are important for applications involving wastewater treatment. The nitrogen and phosphate source were the main nutrients taken up during culture growth. The nutrient uptake changes during all these coculture treatment are shown in **Figure 14**.

For *C. sorokiniana* UTEX 2714 and *A. protothecoides* UTEX 2341, the nitrate removal plots roughly mirrored the growth curves. After 72 hours, the *A. brasilense* and *E. coli* co-cultures with *C. sorokiniana* UTEX 2714 decreased the nitrate concentration to 251 mg/L and 267 mg/L, respectively, compared to the control group 369 mg/L ($p < 0.01$). After 120 hours, there was no significant difference. In the case of UTEX 2805, non PGPB bacteria *E. coli* declined into 130 mg/L compared to the control at 347 mg/L. However, the nitrate concentration for PGPB bacteria *Azo* was 322 mg/L, which was not significantly different with the control group. And *A. protothecoides* UTEX 2341 was provided with ammonium as the nitrogen source given its inability to consume nitrate (Higgins et al., 2017). After 96 hours, there was no ammonium in the PGPB *A. brasilense* co-culture group, below 5 mg/L for the non-PGPB *E. coli* and *B. megaterium*, and 60 mg/L ammonium in the control group.

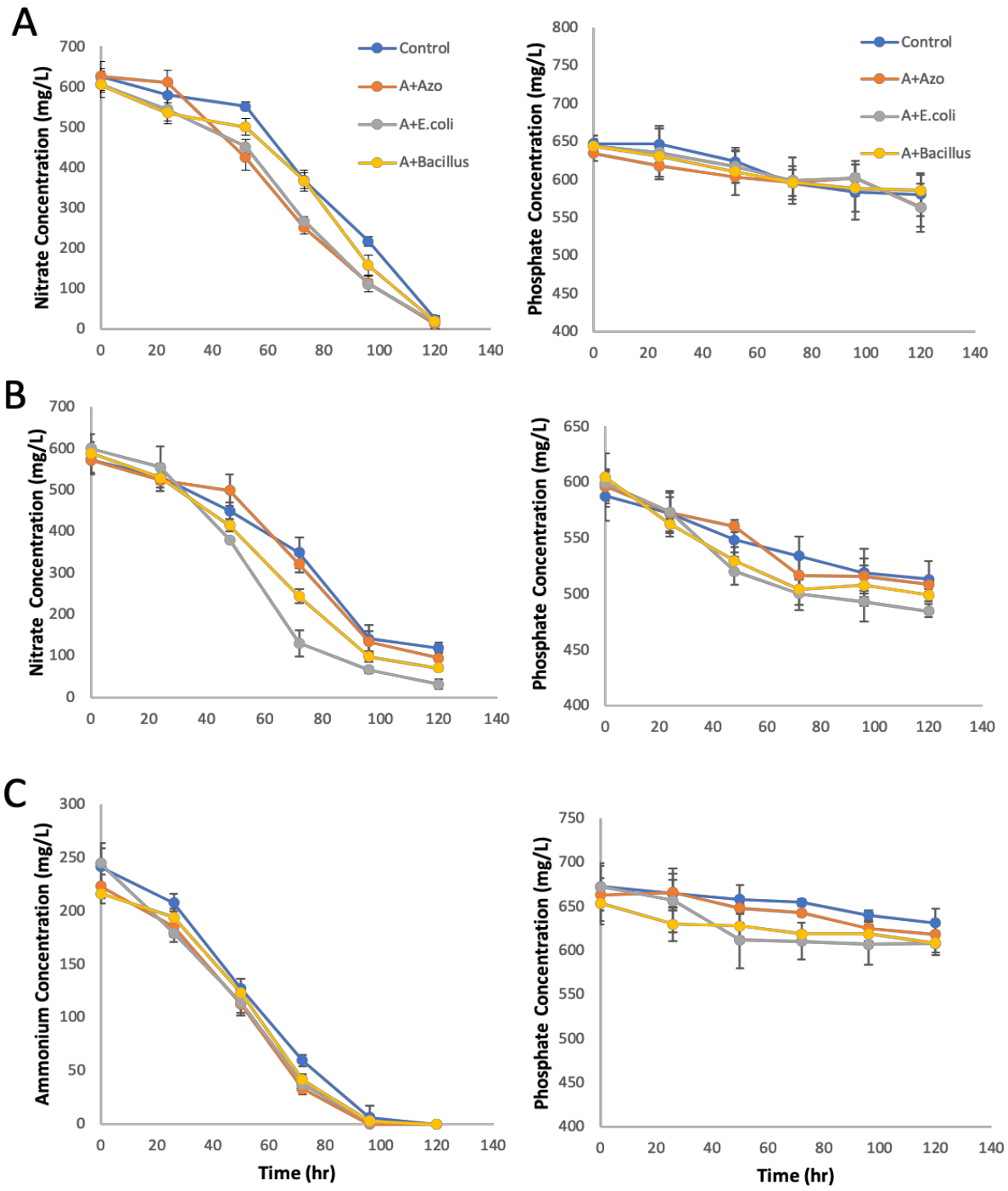

Figure 14 Nitrogen and phosphorus content in medium from *C. sorokiniana* UTEX 2714 (A), *C. sorokiniana* UTEX 2805 (B) and *A. protothecoides* UTEX 2341 (C).

5.3.6 Chemical oxygen demand COD as a measure of algal photosynthate

Algal secretion of organic photosynthate into the surrounding environment is a well-known phenomenon and a mechanism of symbiosis with bacteria (Higgins et al., 2017; Kazamia et al., 2012). At its simples level, bacterial consumption of photosynthate for respiration leads to CO_2 production which can further stimulate algal growth (Bai et al., 2014). This mechanism is expected to be fairly ubiquitous among heterotrophic bacteria provided they can consume algal photosynthate. We measured COD in the axenic algae cultures and in the co-cultures with bacteria after 72 hours (before significant die-off of the bacterial populations). COD was normalized against biomass concentrations to correct for significant differences in total cellular activity in each culture.

The presence of all three bacteria led to significant reductions ($p < 0.001$) in COD for both *C. sorokiniana* UTEX 2714 and *A. protothecoides* UTEX 2341 (**Figure 15**). In the case of *C. sorokiniana* UTEX 2714, roughly two thirds of the COD were consumed by bacteria, but no significant differences were observed among any of the bacterial strains. Roughly half of COD was removed by bacteria in co-cultures with *A. protothecoides* UTEX 2341. Only in the case of *C. sorokiniana* UTEX 2805 was there a difference in COD consumption among bacteria. *A. brasilense* cultures had COD levels that are not significantly different from the axenic controls whereas the other two bacterial led to significant declines in COD concentrations ($p < 0.001$).

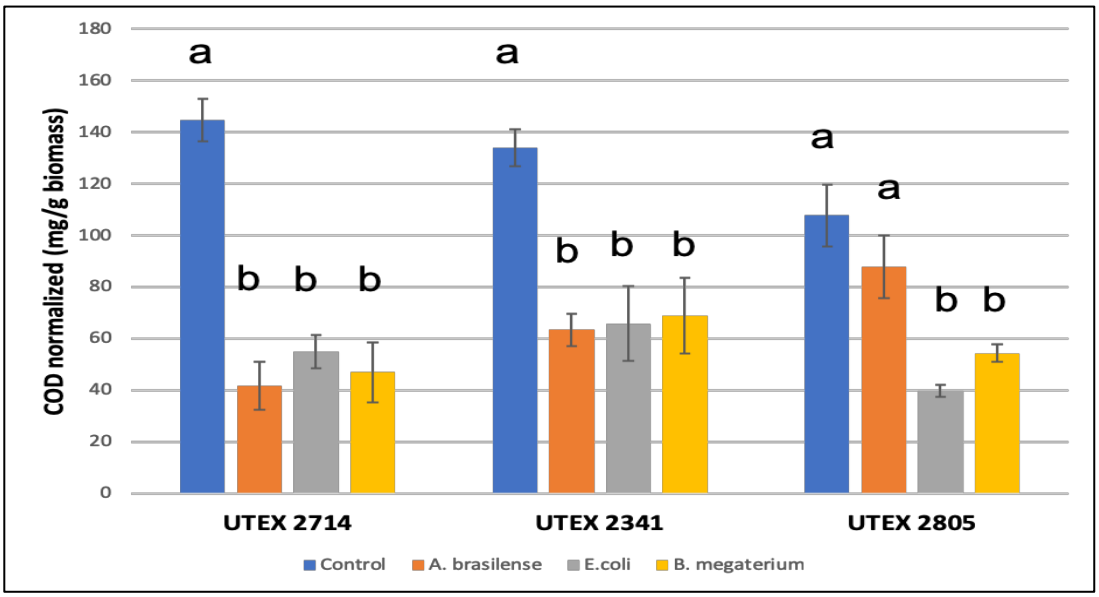

Figure 15 COD normalized in medium from *C. sorokiniana* UTEX 2714 (A), *C. sorokiniana* UTEX 2805 (B) and *A. protothecoides* UTEX 2341 (C).

5.3.7 Lumichrome production in co-cultures and growth impacts on algae

In addition to CO_2 provision from bacteria, symbiosis based on production of riboflavin metabolites such as lumichrome is expected to occur between algae and a wide range of bacteria. Riboflavin was not detected by LCMS-TOF in culture media during screening analysis of a subset of co-culture samples. However, lumichrome, which is formed by photooxidation of riboflavin, was detected at low levels in co-cultures containing *A. brasilense* (0.4-0.6 µg/L) and *E. coli* (5.5-13 µg/L) but not in co-cultures with *B. megaterium*. Given this finding, we ran dose-response studies with lumichrome at 0, 1, 10, and 100 µg/L to determine if this plant hormone has beneficial effects on the algae strains tested in this study (**Figure 16**). As *C. sorokiniana* UTEX 2714 was already tested for its response to lumichrome at these same concentrations by

Lopez et al. (2019), we only tested the two remaining strains. *A. protothecoides* exhibited a small (6%) but statistically significant increases in growth at 72 hours ($p < 0.038$) in response to 1 ng/ml. The higher lumichrome doses had no effect. *C. sorokiniana* UTEX 2805 experienced growth increases of 32% and 40% at 48 hours ($p < 0.045$) in response to 1 ng/ml and 10 ng/ml, respectively. Biomass concentrations at later time points were not significantly different.

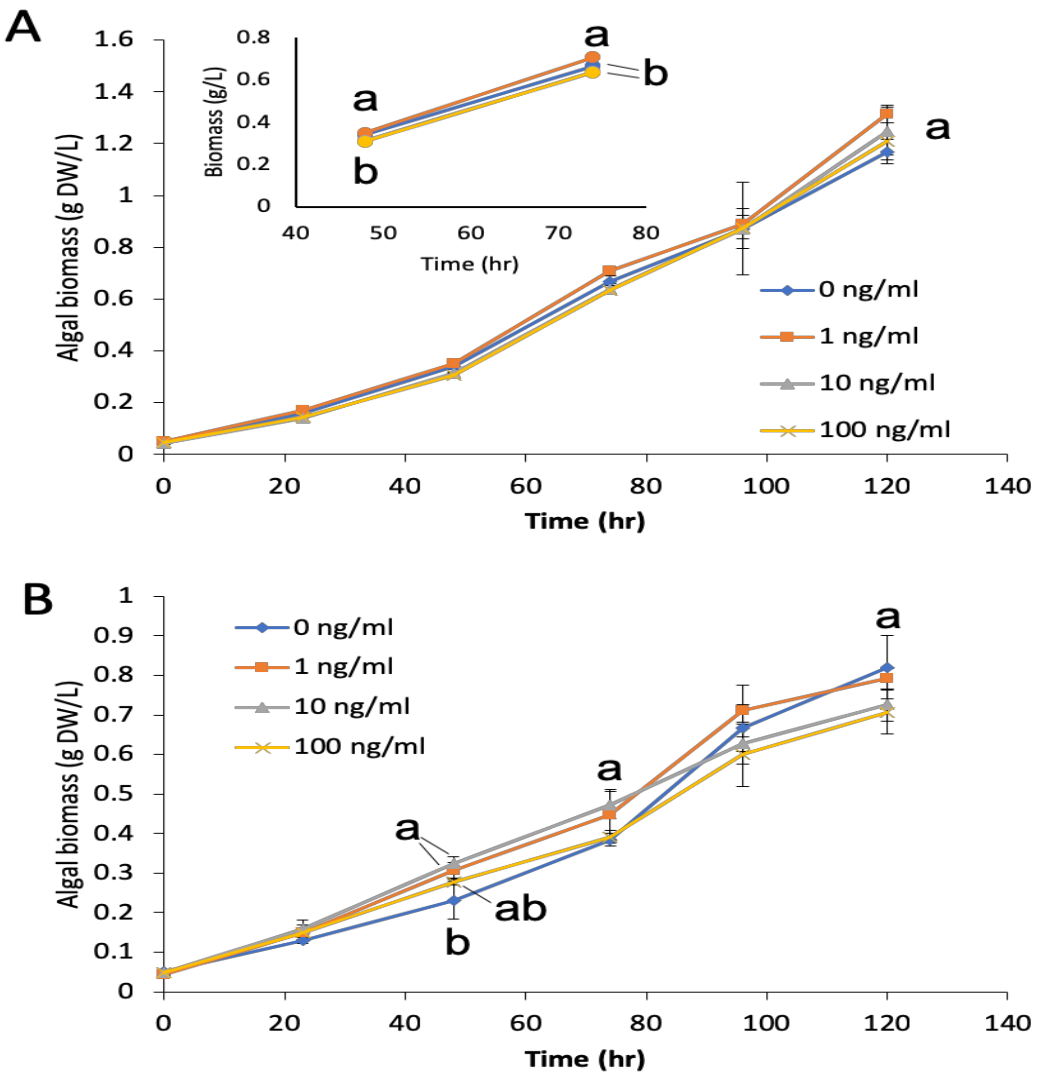

Figure 16 Lumichrome dose response study for *A. protothecoides* UTEX 2341 (A) and *C. sorokiniana* UTEX 2805 (B).

5.4 Discussion

Despite a strong research focus on algal interactions with plant growth promoting bacteria (Palacios et al., 2014), our results indicate few meaningful differences in algal response between the model PGPB *A. brasilense* and the non-PGPB *E. coli*. Both organisms enhanced growth to a similar extent in two of the tested green microalgae, and *E. coli* outperformed *A. brasilense* in *C. sorokiniana* UTEX 2805. Likewise, we observed similar impacts on chlorophyll between these two bacteria species despite research showing that auxin phytohormones specifically impact chlorophyll levels. While auxins (and IAA specifically) can in fact increase algal growth and chlorophyll content (Peng et al., 2020), our results suggest that other mechanisms of algal-bacterial interaction are equally, or possibly more important, than the effects of auxins and certain volatile hormones (acetoin, 2,3 butanediol) produced by PGPB (Amavizca et al., 2017). Moreover, it is clear that the effects of PGPB are not universally beneficial given the slight growth suppression observed in *C. sorokiniana* UTEX 2805, even though it is of the same species as *C. sorokiniana* UTEX 2714 which experienced clear growth benefits from *A. brasilense*. Our dose response study with IAA indicated that *C. sorokiniana* UTEX 2805 did not likely suffer from IAA toxicity and may have even received some benefit from this auxin hormone (with IAA levels <50 mg/L). Thus, some other mechanism is likely the source of growth inhibition. Such an outcome complicates use of PGPB at industrial scale where multiple algae and/or bacteria are often present in the culture. This point is underscored by suppression of

Nannochloropsis growth observed by Fulbright et al. (2016) in the presence of *Bacillus pumilus* which is already recognized as a PGPB (Amavizca et al., 2017).

Nevertheless, all three bacterial species in this study resulted in clear capacity for growth promotion across at least two algae strains despite their divergent lineages (two are members of the Proteobacteria and one is a Firmicute) and natural habitats. *E. coli* is native to the gastrointestinal tract of warm-blooded animals (Jang et al., 2017), *A. brasilense* is native to plant rhizomes (Cecagno et al., 2015), and *B. megaterium* is found in a diverse array of habitats including soil and water (Dobrzanski et al., 2018). This outcome is encouraging given the challenge of controlling which bacteria grow in large-scale algal cultures. This raises the question of which mechanisms of symbiosis are promoting algal growth given that only one of these three strains produce an auxin hormone.

It is now well-known that many algae are auxotroph for vitamin cofactors and that they typically rely on bacterial partner organisms to obtain these cofactors (Croft et al., 2006). In fact, *A. protothecoides* UTEX 2341 is an auxotroph for thiamine and the presence of bacteria confers significant growth advantages in the absence of an exogenous thiamine source (Higgins et al., 2016). In this particular study, exogenous thiamine was provided but thiamine is notoriously unstable (Jenkins et al., 2007) and bacteria-synthesized thiamine could replenish degraded thiamine. Research has shown that even in fully autotrophic algae such as *C. sorokiniana*, exogenous cofactors from bacteria can still confer advantage by allowing for use of more

favorable metabolic pathways (Croft et al.,2005; Higgins et al., 2016; Xie et al., 2013). This latter effect is particularly true for vitamin B12 which is synthesized by bacteria. In fact, *B. megaterium* is known as one of the most efficient producers of vitamin B_{12} (Eppinger et al., 2011) and this could explain some of the growth promotion observed in response to this strain.

Metabolites of riboflavin (Vitamin B2) also are known to alter growth and biomass composition in green algae (Lopez et al., 2019). Lopez et al. found that exogenous riboflavin in the range of 1-10 ng/ml could roughly double the growth rate of *C. sorokiniana*. Indirect production of the phytohormone lumichrome is another mechanism by which algae may benefit from a wide range of bacteria. Lumichrome is derived from riboflavin when it is exposed to light, such as occurs in algae cultures. Our results indicate that riboflavin may be short lived in algae cultures due to either rapid cellular uptake or photochemical conversion to lumichrome. Using LCMS-TOF analysis we observed low levels of lumichrome (0-10 ng/ml) in co-cultures with *E. coli* and *A. brasilense*. Research by others has shown that lumichrome increases the growth rate of many plants (Phillips et al., 1999). Heo et al. (2019) showed that *E. coli* mutants with upregulated riboflavin synthesis pathways were responsible for growth promotion in *Chlorella vulgaris*. Heo et al. suggested that riboflavin-derived metabolites such as lumichrome may be an important mechanism of algal growth promotion but studies on this topic are scarce. Only one study, to our knowledge has investigated the impact of lumichrome on green microalgae (Lopez et al., 2019) and they found that lumichrome significantly increases chlorophyll production but

did not promote the growth of *C. sorokiniana* UTEX 2714 (one of the strains also used in our study). In our study, we found that lumichrome did have a small but significant growth-promoting effect in *C. sorokiniana* UTEX 2805 and to a lesser extent, *A. protothecoides* UTEX 2341. It is clear that these small or, in the case of UTEX 2714, non-existent growth benefits from lumichrome cannot fully account for the growth benefits observed in co-cultures. Nevertheless, it may be one of several mechanisms that have additive or interactive effects. Symbiosis based on exchange of riboflavin metabolites is expected to occur between algae and a wide range of bacteria.

Exchange of carbon dioxide in exchange for dissolved oxygen is one of the oldest and most-studied symbiotic interactions between algae and bacteria (Oswald et al., 1953). This model relies on either an exogenous organic carbon source or robust cycling of organic photosynthate from algae to bacteria (Bai et al., 2014; Holmes et al., 2019; Yao et al., 2019). In our study, photosynthate was measured in aggregate through COD assays. Indeed, our results showed lower COD levels in cultures supplemented with bacteria compared to the algae-only controls. COD derived from algal photosynthate is well-established and past work has shown that this COD is a source of carbon for bacteria (Bankston & Higgins, 2020; Higgins et al., 2017). This result suggests that there was robust carbon dioxide cycling from bacteria to algae in these cultures. The benefit of this additional CO_2 was likely to be small, however, given exogenous CO_2 supplementation in the air supply stream to cultures.

Within each bacterium, it is apparent that different algae provided different levels of support for bacteria survival. For instance, the two strains of *C. sorokiniana* support higher populations of *A. brasilense* than does *A. protothecoides*, similar to previous findings (Peng et al., 2020). Interestingly, *A. brasilense* produced higher amounts of IAA in the presence of *C. sorokiniana* UTEX 2714 compared to UTEX 2805 even though similar *A. brasilense* population sizes were observed in both. This outcome could be driven by more favorable photosynthate from UTEX 2714 such as tryptophan secretions which have been previously documented (Palacios et al., 2016). Tryptophan is one of the precursors of IAA synthesis in *A. brasilense* (Zhao, 2012). All three bacterial species initially grew or held steady over the first 72 hours of the batch culture but then declined precipitously by 120 hours. The decline of bacterial populations in otherwise autotrophic algae reactors has been observed previously (Peng et al., 2020) and is likely a function of photosynthate availability. When algal growth slows, photosynthate production also likely declines, depriving bacteria of an organic carbon source and subsequently limiting CO_2 recycle to algae.

5.5 Conclusion

This study demonstrated that the PGPB, *Azospirillum brasilense*, the non-PGPB bacteria, *Escherichia coli*, and the recently-discovered bacteria *Bacillus megaterium*, promoted the growth of two algae strains, *C. sorokiniana* UTEX 2714 and *A. protothecoides* UTEX 2341. However,

the PGPB *A. brasilense* was no more effective at promoting growth in these two strains compared to *E. coli*, despite its secretion of the auxin hormone IAA. In a third algal strain, *C. sorokiniana* UTEX 2805, *A. brasilense* actually had a slight suppressive effect on growth whereas *E. coli* and *B. megaterium* resulted in significant growth benefits. Although IAA has been shown to result in some growth benefit to all three algae strains tested, it is apparent that other symbiotic mechanisms are of equal or greater importance. Exchange of O_2 and CO_2 between algae and bacteria was likely significant given that around 50-60% of the COD generated by algae was removed by the bacteria. Moreover, all three bacterial species initially grew or held steady over the first 72 hours of the batch culture (algal exponential growth) but then declined precipitously by 120 hours (algal log growth). The riboflavin metabolite, lumichrome, was also detected in low concentrations in co-cultures of the PGPB, *A. brasilense* (0.4-0.6 ng/ml), and the non-PGPB, E. coli (5.5-13 ng/ml). Lumichrome was found to have small but statistically significant growth benefits for *C. sorokiniana* UTEX 2805 and *A. protothecoides* UTEX 2341 in dose-response studies.

Chapter 6 Conclusion remarks and future work

With increasing concern about the world fossil fuel supply and environment pollution, algae can offer the sustainable, clean and environment friendly energy resources. The platform of coculturing green algae with bacteria is promising, but still have some challenge and concern.

In an effort to explore the more details and understanding of algae cocultivation systems, an attempt was made to develop the simplified green algae *C. sorokiniana* UTEX 2714, *C. sorokiniana* UTEX 2805, *A. protothecoides* UTEX 2341 with bacteria. Conclusions in Chapter 3 demonstrated that coculturing *C. sorokiniana* UTEX 2714 and the PGPB, *Azospirillum brasiliense,* promoted algae growth due in part to IAA secretion. This growth promotion occurred, however, at the expense of energy storage products in growing cells. The response to *A. brasilense* was stronger in *C. sorokiniana* than in *A. protothecoides*. Furthermore, in Chapter 4, the effect of *A. brasilense* on oxidative stress in *C. sorokiniana* UTEX 2714 and the consequent changes in biomass composition were investigated. Under 25 μM Copper stressor, the addition of exogenous IAA and *A. brasilense* decreased the ROS levels by 31% and 35%, respectively, compared to copper-only cultures at the zero-hour time point. Under the Nitrogen stressor, there was no significant difference in ROS levels between nitrogen-limited co-cultures and nitrogen-replete monocultures of *C. sorokinianam*, suggesting *A. brasilense* could rescue the algae under nitrogen limitation stressor. These results also partly explain why *A. brasilense* leads to suppression of energy storage products in *C. sorokiniana*.

It was clear from the results in Chapter 5, that co-culturing algae with the PGPB *A. brasilense* did not lead to any growth advantage compared to use of the non-PGPB, *E. coli*. The latter even outperformed *A. brasilense* in co-culture with one of the algae strains. Our results suggest that mechanisms that are not unique to PGPB (O_2-CO_2 exchange, vitamin metabolite exchange) may be more important than PGPB-specific mechanisms like auxin hormone production. All these results present a more comprehensive picture of the algae cocultivation systems for bioenergy and bioproduction development. Nevertheless, additional future research can be carried out to further improve understanding.

1) Given the results that the response to *A. brasilense* varied among green algae *C. sorokiniana* UTEX 2714 and UTEX 2805, there is strong interest in understanding more detailed mechanism between the organisms using omics method (genomics, proteomics transcriptomes and metabolomics).

2) The relative importance of different algae-bacteria mechanisms remains unclear. Future research could be conducted to test a variety of vitamin metabolites, separately and in combination. This would reveal if there are synergistic effects among metabolites. In addition, future research about algae-bacteria systems can be grown using pure air instead of air supplemented with 2% CO_2, which will provide more discoveries about the coculturing systems under inorganic carbon limitation. This is particularly helpful for quantifying the benefit of bacterial CO_2 generation.

3) For the algae reactive oxygen species ROS systems, there are also enzymatic and non-enzymatic scavengers. Under copper and nitrogen limitation stressors, the cellular crosstalk of ROS and scavenging antioxidant in the algae coculture systems is another interesting aspect for future research.

4) For the application, more algae coculture systems development from lab scale to the pilot scale such as growth in open tanks can be helpful for understanding how these bacteria persist and interact with other background microbial consortia. Also, more details about lipid profile and protein profile such as amino acid can be investigated. Additionally, the algae biomass conversion process and coproduct development such as fermentation, anaerobic digestion, hydrothermal liquefaction can be considered for biofuel applications.

5) Further investigation of techno-economic analysis and life cycle analysis can also advance the steps to where research may be most critical in improving algae cocultivation systems.

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Appendix Additional files

Appendix 1 (Additional files to Chapter 3)

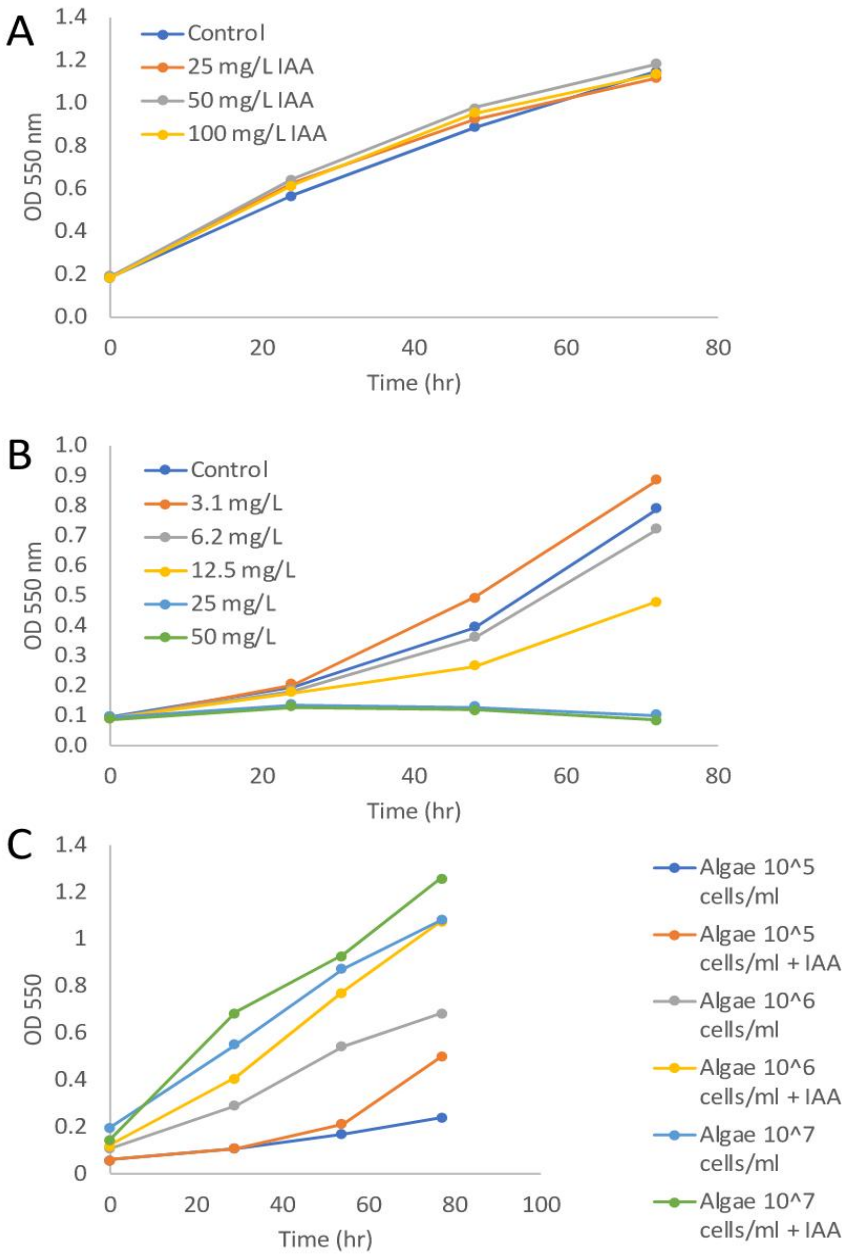

Figure A1.1: IAA dose response study for *C. sorokiniana* (A) and *A. protothecoides* (B); The effect of *C. sorokiniana* inoculum density on growth in the presence and absence of 50 mg/L IAA (C).

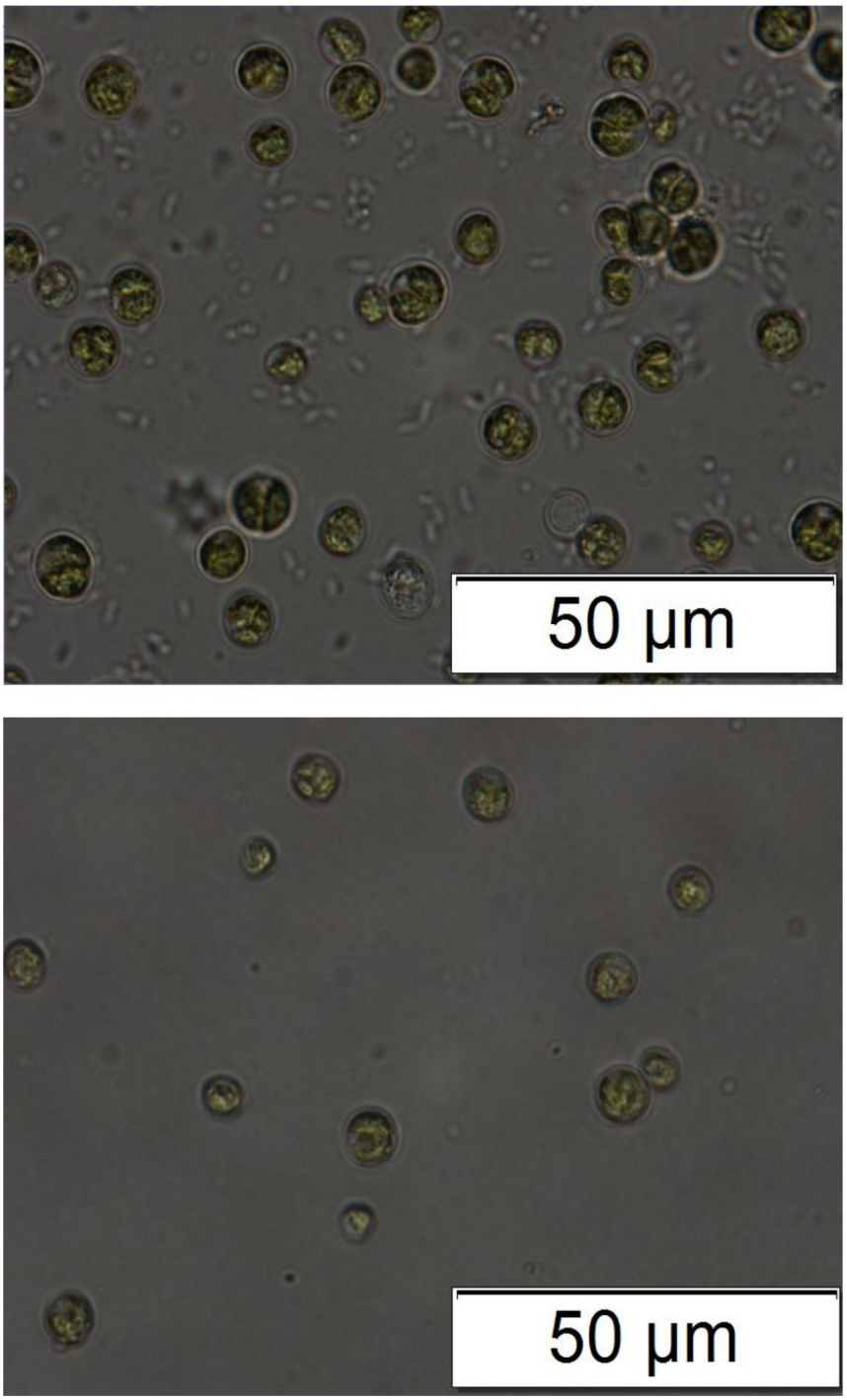

Figure A1.2: Light microscopy images of co-cultures of *C. sorokiniana* and *A. brasilense a* few hours after inoculation (A) and axenic *C. sorokiniana* in control culture (B).

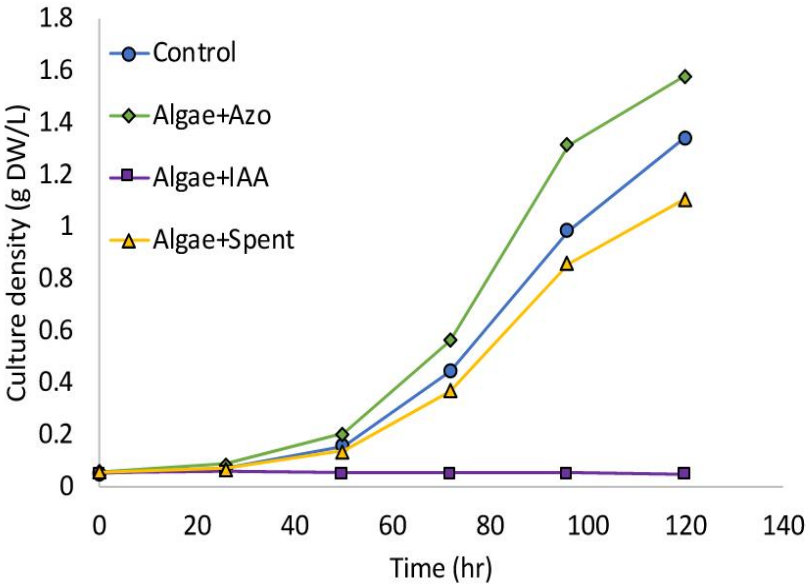

Figure A1.3: Cultivation of *A. protothecoides* under *A. brasilense a* few coculture and IAA treatment. The exogenous IAA level used was 50 mg/L, resulting in complete culture death.

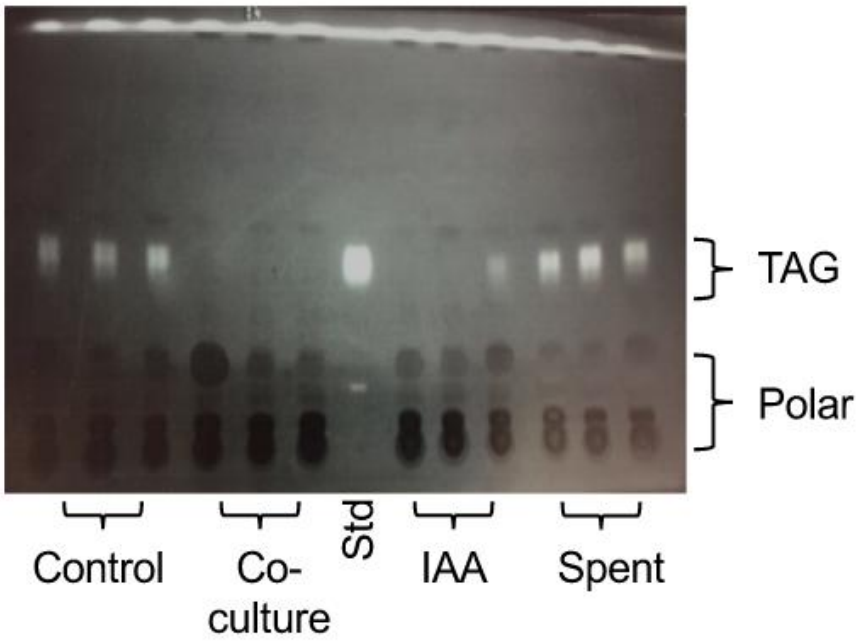

Figure A1.4: Thin layer chromatography of lipids from *C. sorokiniana* harvested after 72 hours. The exogenous IAA level used was 50 mg/L. Canola oil was used as the TAG standard.

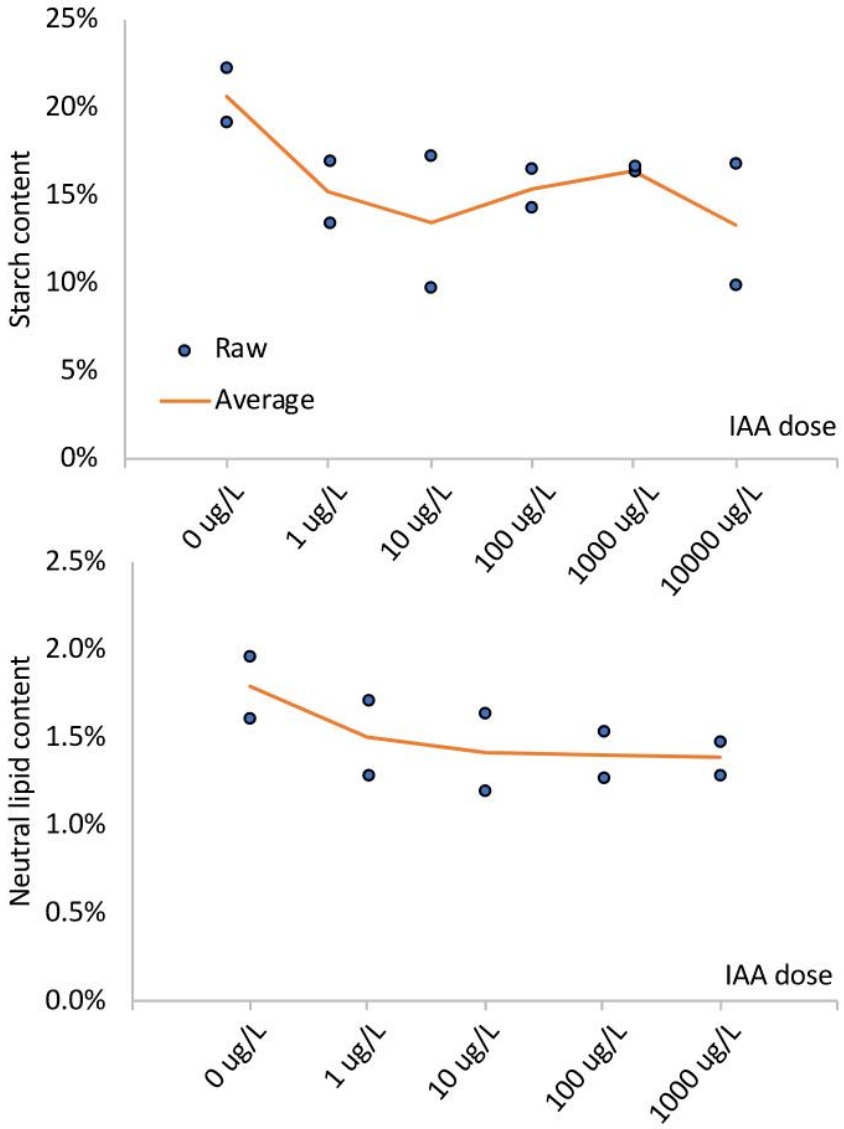

Figure A1.5: Dose response study with *C. sorokiniana* and IAA. Cultures were harvested at 96 hours, at the transition point between exponential and logarithmic growth (Lipid samples from cultures supplied with 10,000µg/L suffered from degradation during the freeze-drying process).

Appendix 2 (Additional files to Chapter 4)

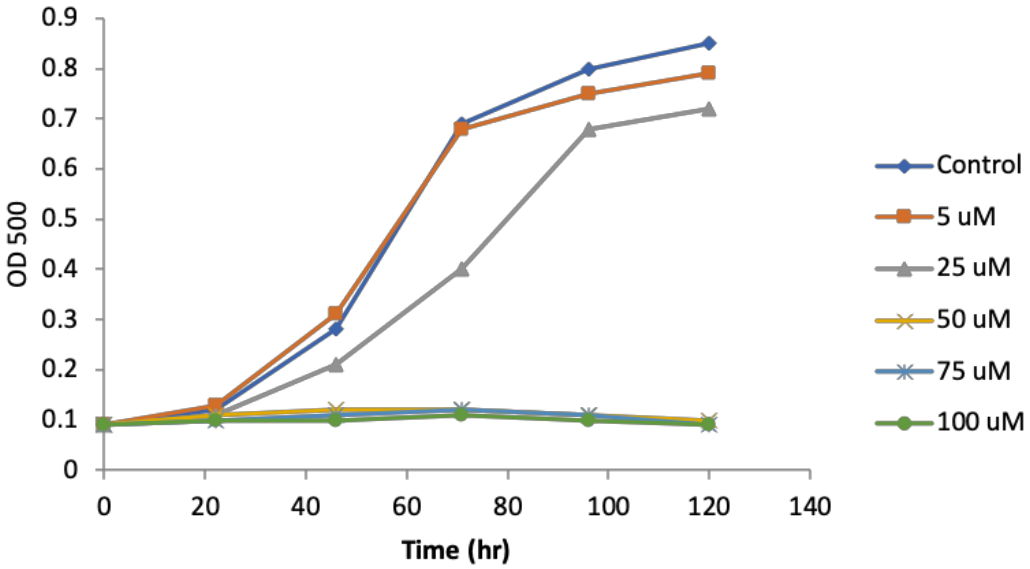

Figure A2.1: Copper dose response study with *C. sorokiniana* UTEX 2714, with copper concentrations ranging from 0-100 µM added to N8-NH4 medium. Each concentration was run in biological duplicate and points are the mean of the two replicates.

Appendix 3 (Additional files to Chapter 5)

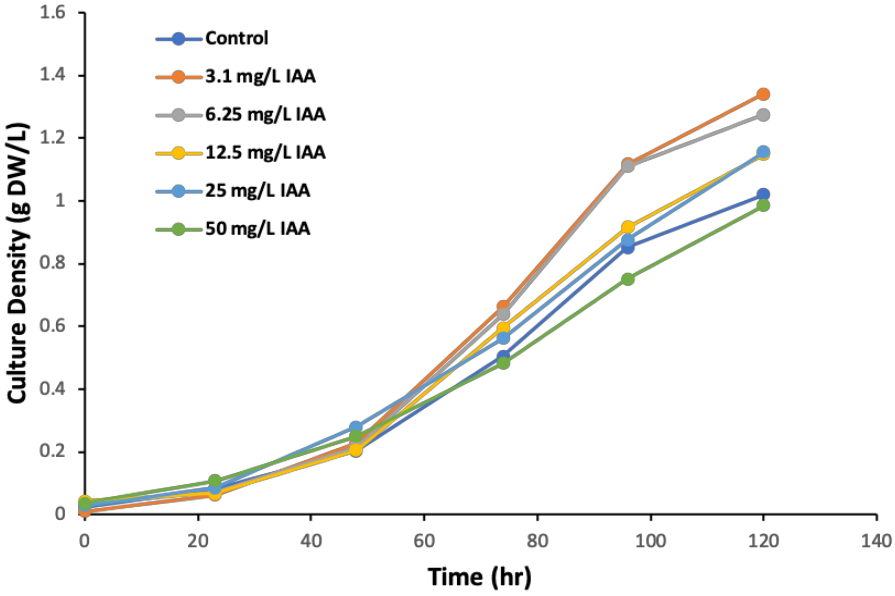

Figure A3.1: IAA dose response study with *C. sorokiniana* UTEX 2805.