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Altering Growth Rates and Nutritional Qualities of Microalgal Feedstock with Symbiotic Bacteria

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Altering Growth Rates and Nutritional Qualities of Microalgal Feedstock with Symbiotic Bacteria

by

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Abstract

The cultivation of microalgae has many commercial purposes; it is integral in the farming of marine animals such as finfish, shrimp, and bivalves through its use as feedstock, and it has potential for use in renewable energy sources as a biofuel. Pink pigmented facultatively methylotrophic bacteria (PPFM) are known to live symbiotically on plants, feeding off of metabolic wastes and producing growth regulators and nutrients vital for plant development. These bacteria have also been isolated from algae and water samples. One strain of vitamin B12 over-producing PPFM has been previously isolated by our lab, and past research has indicated that co-culturing microalgae with PPFM can increase algal growth rates. Our research investigated the possibility of altering the growth rates and nutritional qualities of microalgae through the use of PPFM by conducting algae growth experiments and nutritional analysis. Microalgal species commonly used as feedstock for industrial bivalve aquaculture were supplemented with vitamin B12 over-producing PPFM. A significant difference in growth between PPFM supplemented and non-supplemented algal cultures was not seen consistently, while preliminary nutritional quality testing showed an increase in amino acid and lipid content for PPFM supplemented algal cultures over non-supplemented cultures.

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Chapter 1

Literature Review

1.1 Introduction

The cultivation of algae and microalgae is a billion dollar industry [1], and has formed the foundation of aquaculture and domestic farming of fish and shellfish [2]. Microalgae have found many uses as chemical extracts, health foods, and in pollution remediation [3] [4]. Importantly, microalgae represent a source of renewable oil-based biofuel that could potentially rival fossil fuels [5]. Bacteria have long been known to associate with microalgae in the wild, with mutual benefits for both parties [6]. A symbiotic relationship has been proposed in which bacteria feed off of microalgal exudate and excrete vitamin B12, an essential algal cofactor of strictly exogenous origin [7]. Preliminary research has shown other benefits for algae grown in the presence of bacteria, including enhanced growth and population stability [8].

Successful studies of the relationships between microalgae and bacteria could have profound implications and benefits for all fields of microalgal cultivation, and could help bring to fruition biotechnologies that have not yet reached economic feasibility [5]. Previous research in our lab has isolated strains of pink-pigmented facultatively

methylotrophic bacteria (PPFMs; genus *Methylobacterium*) which enhance the growth of plants through the excretion of vitamins, nutrients, and growth factors while feeding off of plant metabolic waste in a symbiotic relationship. Past research into PPFMs has led to the development of new technologies, and the bacteria have been isolated from algal samples. By studying the effects of PPFM supplementation on microalgal growth, our research hopes to explore potential new applications of biotechnology for the advancement of industrial microalgal cultivation.

1.2 Microalgae

Microalgae are an ancient group of organisms that include unicellular photosynthetic microbes, both prokaryotic (such as cyanobacteria) and eukaryotic. This group is distinct from macroalgae, which encompass multicellular species. Microalgal products range from food & food additives, nutritional supplements, chemical extracts, feedstock, and cosmetics. Microalgal culture has found widespread use in supporting the husbandry of marine animals such as finfish, shrimp, and mollusks [2]. They have also been studied extensively for their potential use as a source of renewable biofuels [9]. Basic research into the physiology of microalgae and novel cultivation methods has played a vital role in furthering the development of commerical uses for microalgae [3]. Many researchers recognize that the relationship between microalgae and bacteria cannot be ignored as an important component for consideration in microalgal cultivation, and represents a field with the potential to advance the development of new microalgal biotechnologies and improve the adoption of current ones [6] [10] [5] [11].

1.2.1 Aquaculture

The algal aquaculture industry is estimated to be worth USD $5-6 billion per year [12], with $1.25 billion of that being due to microalgae [1]. In the face of dwindling wild fisheries, many attempts have been made to raise aquatic animals domestically for harvesting through commercial aquaculture. Progress in this area has historically been impeded by difficulties in securing an adequate diet to support such animals [2]. Early efforts to raise animals such as shellfish, finfish, and bivalves made clear that domestic microalgal cultivation would be required. These economically important animals all require microalgal feed either directly in their diet at some point in their life cycle, or indirectly through prey items which require microalgae [3]. In particular, bivalves require large amounts of microalgal feedstock as larvae, with their appetite increasing exponentially with age [2]. In bivalve hatcheries, algal culture is estimated to account for 40% of costs [13], and is considered a bottleneck for many areas of aquaculture. Advancements in the feasibility and commercial success of raising aquatic animals for human consumption has been credited to research efforts focused on expanding basic understanding of algal physiology, growth conditions, and the combination of nutritional principles with the engineering of new culture systems [3].

One such advancement came with the development of a media enrichment formulation in which most marine microalgae can be grown successfully. Labeled as F/2 media, this quickly became a staple in aquaculture. Enrichment of filtered seawater with F/2 is still used today as the industry standard for the culture of feedstock species of microalgae [3]. Another innovation was found through testing combinations of algal species to find those which best supported the growth of commercially important aquatic animals. It was observed that wild algal populations did not necessarily provide animals with all required nutrients. Through analysis of compounds conserved through the food

chain, omega-3 fatty acids were identified as crucial for the growth and development of bivalves [3]. Among them, Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) were identified as necessary fatty acids for bivalve and oyster larval development, and microalgal species with high concentrations of these fatty acids were mixed together to form industry standard microalgal feedstocks [2] [3] [13]. Whereas wild strains of microalgae were susceptible to crashing unpredictably during cultivation, more easily grown strains high in lipids and omega-3 fatty acids were selected. Culture techniques were refined, with a preference for harvesting actively growing populations instead of those that had reached the stationary phase of growth and were nutrient limited [2] [13].

In addition to bivalve feedstock, cultivation of microalgae for other purposes has also benefited greatly from improvements in microalgal culture methods. Extracts of the antioxidant astaxanthin, derived from algal species of *Hematococcus*, have found wide use in farm raised salmonids. While not directly impacting the development of the fish, this compound naturally accumulates in the fish's flesh and imparts the distinctive color of salmon flesh [2] [3]. Similarly, *Spirulina* is often used in koi feed to impart the fish's vivid red and orange colors. The human market for microalgal food products has seen less growth however. Small markets have developed for high value health supplements such as *Spirulina* and carotenoid pigments from *Dunaliella*, but these have not reached the level of importance seen in aquaculture [2].

1.2.2 Biofuels & Biotechnology

More than 25,000 algal species have been recognized, but few species have found industrial usage outside of the field of aquaculture [1]. Microalgae can produce valuable products such as lipids, proteins, carbohydrates, and chemical extracts [14], but by far the most well known product is microalgal biofuel. Cultivation methods for microalgae

have not yet reached high enough efficiencies and low enough costs for microalgal oil based fuels to compete in modern markets dominated by inexpensive fossil fuels [5]. Still, microalgal biofuels offer several advantages over plant based biofuels. Microalgae grow faster and can contain more oil per dry biomass than oil crops, and do not compete for arable land that could otherwise be used for food production [5] [15]. Other advantages of microalgal biofuels include the ability to grow in brackish and salt water, and utilize nutrients contained in human and animal wastewater [14] [15].

Microalgal oils could be converted into biodiesel, jet fuel, and gasoline equivalents [5]. Due to the capture of carbon dioxide during algal growth, these biofuels would be essentially 'carbon neutral' [5], and could potentially use carbon emissions from power plants as a source of carbon dioxide [16]. Some species of algae have been reported to contain greater than 80% of dry biomass as lipid [14] [15]. Non-lipid byproducts of oil extraction can be used in animal feed and production of other compounds, or can be digested anaerobically to produce biogas for further energy generation [15].

The projected maximum yield of oil from algae greatly surpasses that of plant based oil sources, though these levels of production have not yet been reached [14]. Economic factors must be overcome before microalgal biofuels see widespread adoption [5] [14] [9] [15]. The feasibility of microalgal biofuel usage is directly tied to the price of petroleum based fossil fuels [5] [15]. More research into algal cultivation techniques is required in order to raise the efficiency of oil production and drive costs down [14]. The difficulty of harvesting oil from algal biomass remains one such challenge [14], but may be secondary in importance to the means of physical production of algae.

Currently, two main forms of cultivation of algae for biofuel and biotechnology have seen usage. Enclosed photobioreactors consisting of transparent tubes filled with algal growth media have proven to be highly efficient and maintain a stable, controlled

environment [5] [15] [4] [14] (Figure 1.1). However, costs of construction and labor needed to maintain and clean these systems is a major limitation. The primary competing approach for microalgal cultivation consists of shallow open ponds, with the parallel raceway pond being the most common [5] [15] [4]. These ponds have the advantage of being cheaper to construct and maintain, but suffer a major drawback in their disposition towards contamination [5] [14]. Since they are open to the environment, these ponds allow unwanted microbes and organisms to fall in. Bacteria, fungi, and fast-growing algae can quickly out compete a microalgal crop [5]. This problem is further exacerbated by the traditional usage of algal monocultures in cultivation, leaving room for invading organisms to thrive [5]. By recognizing the tendency of algae to associate with other microbes in the wild, a careful selection of beneficial co-inhabitants may increase the production efficiency of microalgae and help to protect them from contamination. A proposed "synthetic ecology" would occupy open niches in microalgal cultivation environments, and stabilize algal populations [5].

As cultivation techniques continue to be refined, the field of genetic engineering shows great promise for increasing yields of microalgal products [1] [15]. Varieties of genetically modified algae are under development for the direct production of biofuels and gasoline precursors [14], and cyanobacteria with mosquito larvicidal properties have already been produced [17]. Current research seeks to address challenges such as reduced growth rate commonly seen in transgenic algal strains. Other research goals include increasing the efficiency of oil production and mitigating the effects of photoinhibition [15]. The cultivation of transgenic algae is likely to be subject to regulatory action and environmental concerns [1], though companies such as Monsanto and Sapphire Energy have risen to meet these challenges and are actively pursuing genetically modified microalgal strains [18].

FIGURE 1.1: Microalgae growing in enclosed photobioreactors. Austin, TX.

1.2.3 Algae & Bacteria

The relationship between microalgae and microbes in the environment has been recognized as an important area for study and has shown great potential to benefit the fields of algal cultivation and aquaculture [6]. While some species of microalgae have been grown axenically in the past, researchers recognize that most microalgae do not naturally grow in axenic conditions [10]. Instead, they exist as part of larger ecosystems in conjunction with other organisms, the most numerous of which are likely to be bacteria [19] [5]. Bacteria have been shown to associate with algae in a host specific manner [6], and careful manipulation of these associations may benefit microalgal cultivation systems by stabilizing microalgal growth and preventing undesired contaminants from becoming established [5] [8]. Microalgal species grown in monoculture could greatly benefit from these bacterial associations, since these culture systems are very susceptible

to contamination [8] [4]. To date, the only algal monocultures that have proven to be commercially viable utilize microalgal species such as *Dunaliella* and *Spirulina* which are able to grow in extremes of pH or salinity [8].

Both bacteria and algae are numerous in aquatic ecosystems [19], and 16s rRNA gene sequencing has suggested that bacterial communities colonizing algal surfaces show a degree of host specificity [6]. The species of bacteria isolated from microalgae can sometimes differ greatly from those isolated from water samples [19], though it is often difficult to determine whether a given bacterium is physiologically and physically associated with host algal species or if they merely exist in the same aquatic habitat as the algal cells [6]. Research has shown that algae and bacteria are capable of maintaining these close associations, and some bacteria have even been found living inside of algal cells [19]. Algal cell surfaces represent a preferred habitat for growth by some bacterial species [19] [6], and the zone immediately surrounding the algal cell has been suggested to play a role analogous to the plant rhizosphere, the "phycosphere" [19] [20]. In this zone, algal cells excrete metabolites and photosynthate which can be used as nutrients by bacteria [6]. This algal exudate has been known to contain a wide variety of substances, including carbohydrates, lipids, amino acids, enzymes, and growth stimulators, along with many other organic molecules. These compounds have been shown to attract bacteria and influence their growth [20], and some isolated bacteria have shown the ability to specifically break down algal metabolites such as polysaccharides [6]. Studies have shown positive correlations between algal productivity and bacterial abundance [19]. Many examples of algal and bacterial symbiotic relationships have been demonstrated, wherein bacteria gain carbon based food sources from algae and excrete nutrients the algae require for growth, with growth of both species being stimulated [5] [4]. Not all

associations between bacteria and algae are mutually beneficial, with some algae showing the ability to produce antibiotic compounds [20], while certain bacterial species can have growth inhibiting effects on some algae [4] [8] [11].

Algae demonstrate an important relationship with bacteria through their need for vitamin B12 (cobalamin). This vitamin has been shown to function as a cofactor for vitamin B12-dependent methionine synthase. Early eukaryotes probably contained both B12-dependent and independent forms of methionine synthase, but Vitamin B12 auxotrophy has evolved independently several times with the loss of B12-independent forms of the methionine synthase enzyme [7]. The enzymes necessary for producing vitamin B12 have only been found in prokaryotes [7], and vitamin B12 synthesis by bacteria is likely to represent a major source of this vitamin in the water column [19]. About half of surveyed algae require vitamin B12, but none produce it themselves [5]. It has been strongly suggested that algal sources of vitamin B12 are almost exclusively bacterial in nature [7], though the extent to which this can be ascribed to algal-bacterial symbiosis has been questioned by some [21]. It seems likely that some algae and bacteria could exist in a symbiotic relationship, wherein they exchange fixed carbon for vitamin B12 and potentially other micronutrients [7].

It is clear that an understanding of the role of bacteria in the growth of microalgae can have important implications for the growth of microalgae in industrial pursuits [11], as demonstrated by past research. In one study, the growth of diatom species *Chaetoceros ceratosporum* and *Chaetoceros gracilis* were stabilized and stimulated with the addition of naturally derived bacteria [11]. Both microalgae and bacteria are used in the remediation of pollutants and waste water, and function better when used together. Bacterial growth and functionality in wastewater treatment facilities is limited by O_2 availability, which is linked to microalgal activity. Microalgae function to remove heavy

metals from water, but have difficulty growing in extremely polluted environments without the help of bacteria to first lower water toxicity [4]. The production of microalgal growth-supporting compounds by bacteria also suggests potential benefits to aquaculture [11]. The use of bacteria as a bivalve probiotic is a promising area of research, and could help protect bivalve larvae from diseases such as *Vibrio* by using microalgal consumption as a delivery vector for beneficial bacteria [10].

1.3 Pink Pigmented Facultative Methylotrophs

Pink pigmented facultatively methylotrophic bacteria (PPFM) are rod-shaped bacteria in the genus *Methylobacterium* [22]. They were first discovered on liverworts in 1969, where they were found to increase plant growth rate and size [23]. PPFM are ubiquitously distributed on plant surfaces, in soil containing organic matter, and in water. They have been isolated from the surface of hundreds of plant species [24], and can sometimes also be found intracellularly in herbaceous and woody plants [25]. Furthermore, PPFM can be spread through airborne soil particles, and plant leaves close to the ground have been found to contain greater numbers of PPFM colonies than those higher up on the plant [24].

The ability to metabolize methanol confers a unique advantage in the phyllosphere. This single-Carbon compound is given off by plants through metabolism and during active growth by the chemical breakdown of the cell wall for expansion. PPFM are able to exploit this unique source of metabolic fuel, which is unavailable for use by non-methylotrophic microbes. PPFM colonies are found in greater numbers on actively growing plant tissue where methanol excretion is highest [24]. The presence of PPFM also benefits the plant host, for these bacteria have been shown to produce growth regulators critical for plant development, such as auxins and cytokinins [26]. This symbiotic

relationship forms a proposed methanol cycle, wherein plant growth releases methanol which stimulates the growth of PPFM, who in return produce growth regulators which further stimulate plant growth [24]. The presence of PPFM has been shown to play an important role in plant seed germination, with seeds treated to remove PPFMs suffering a decreased germination rate, while similarly treated seeds subsequently re-inoculated with PPFM show a rescued germination rate that may even exceed that of untreated seeds [27]. Other research into PPFM has shown that they produce vitamins such as B12, which are required for plant development but not produced endogenously [24], and that they play a role in plant Nitrogen metabolism [28]. The degree to which these bacteria produce plant growth regulators has led some to hypothesize that they may be contributing the majority of growth factors involved in plant development [29]. Experiments in growing plant calli on vitamin and hormone deficient media has shown that PPFM bacterization is sufficient to induce the development of shoots and roots [30]. Benefits to crop plants can also be seen by spraying foliage with dilute methanol solution, subsequently resulting in improved plant growth, yield, and increases in the number of PPFM colonies [24].

The facultative nature of methylotrophy exhibited in these bacteria also allows them to metabolize poly-Carbon compounds [22]. They derive their characteristic pink color from carotenoid pigments which act as a photoprotectant [22]. PPFM are especially hardy, and can withstand UV irradiation, desiccation, freezing, and heat. They secrete exopolysaccharides which aid in adhering to plant surfaces and promote colony formation and protection [24]. It is suspected that these compounds are responsible for clumping and adherence to glassware which is commonly seen in liquid cultures. While these bacteria can be isolated from a plethora of sources, they are extremely under-reported in microbiological surveys, most likely due to their slow growth and the

need for specialized media for their isolation. Previously, Dr. Mark Holland's laboratory has isolated many strains of PPFM from the environment, including strains that over-produce vitamin B12 and methionine. Bacteriophages that lyse the bacterium have also been isolated, and findings associated with the PPFM have generated patents and commercial interest. A start-up company, NewLeaf Symbiotics (`www.NewLeafSym.com`), is currently exploring methods to bring PPFM-containing products to market as plant probiotics. Of particular significance to this project are observations that PPFM are also associated with microalgae. Past research in our lab has shown that supplementation of microalgal cultures with PPFM increases the growth rate of *Neochloris* and *Chlamydomonas sp. in vitro* (Figure 1.3, Figure 1.4). In addition, supplementation with B12 overproducing PPFM strains increased growth rates of *Neochloris* compared to standard PPFM strains (Figure 1.3).

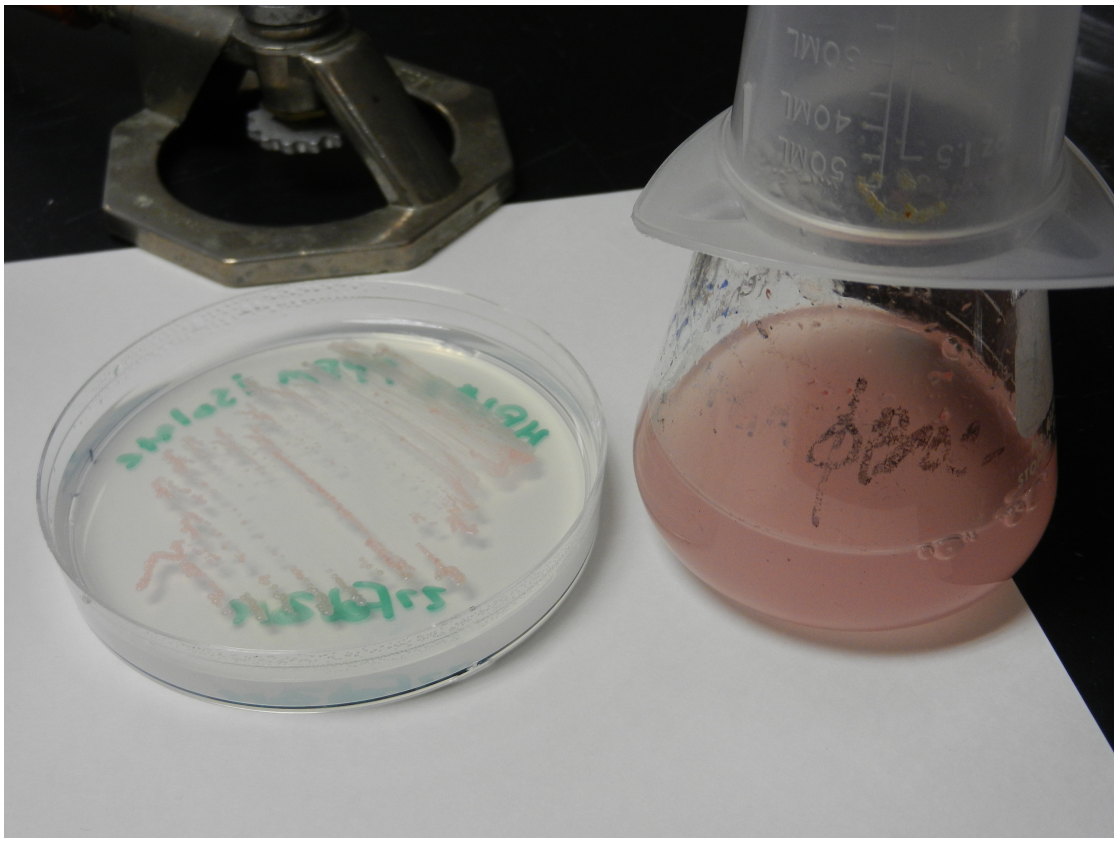

FIGURE 1.2: PPFM bacterial cultures in liquid and solid media.

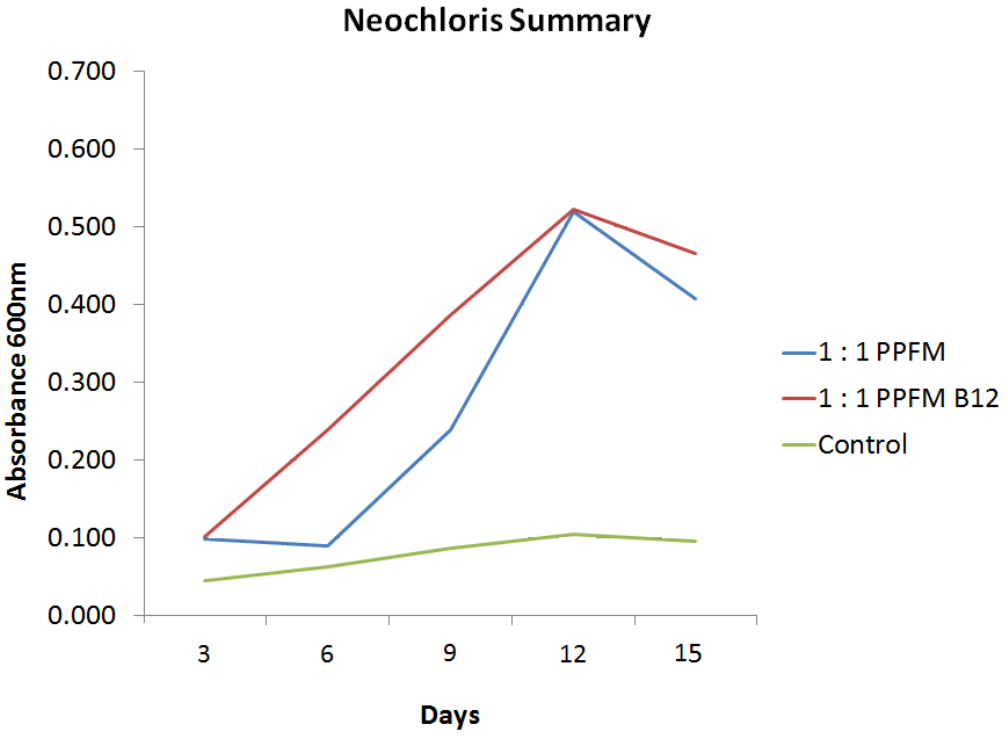

FIGURE 1.3: Optical density measurements showing the growth rates of *Neochloris sp.* grown in a 1:1 ratio of 'standard' PPFM bacteria to algal cells (blue), a 1:1 ratio of vitamin B12 over-producing PPFM to algal cells (red), and algal cells without bacterial treatment (green). Source: Davis and Holland 2010 (unpublished).

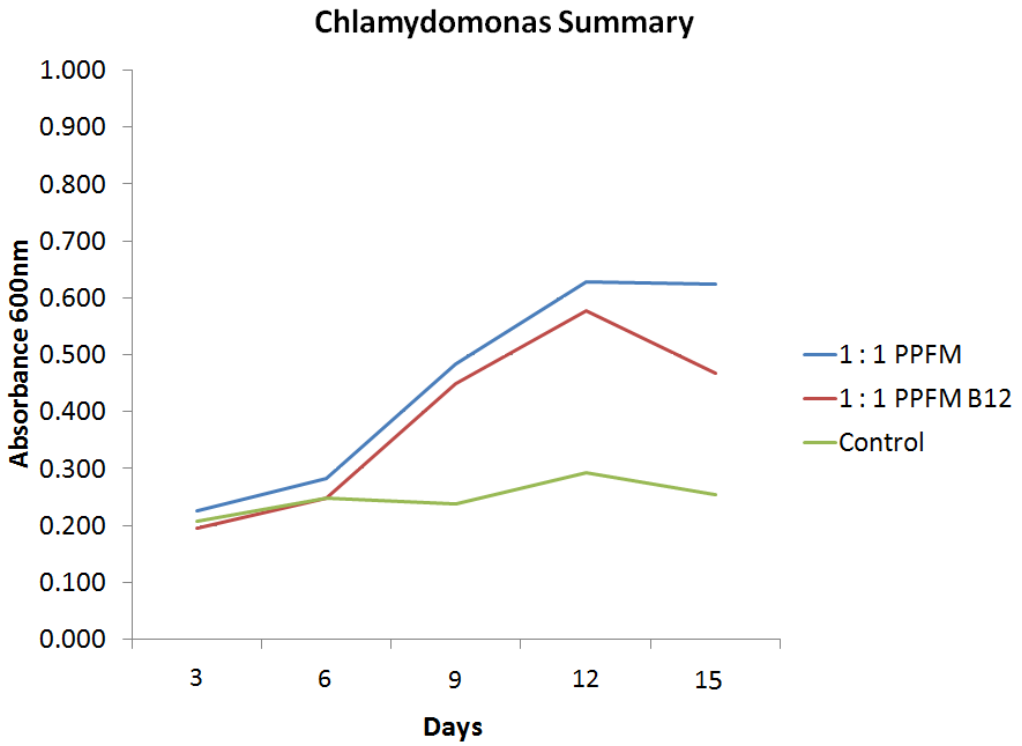

FIGURE 1.4: Optical density measurements showing the growth rates of *Chlamydomonas sp.* grown in a 1:1 ratio of 'standard' PPFM bacteria to algal cells (blue), a 1:1 ratio of vitamin B12 over-producing PPFM to algal cells (red), and algal cells without bacterial treatment (green). Source: Davis and Holland 2010 (unpublished).

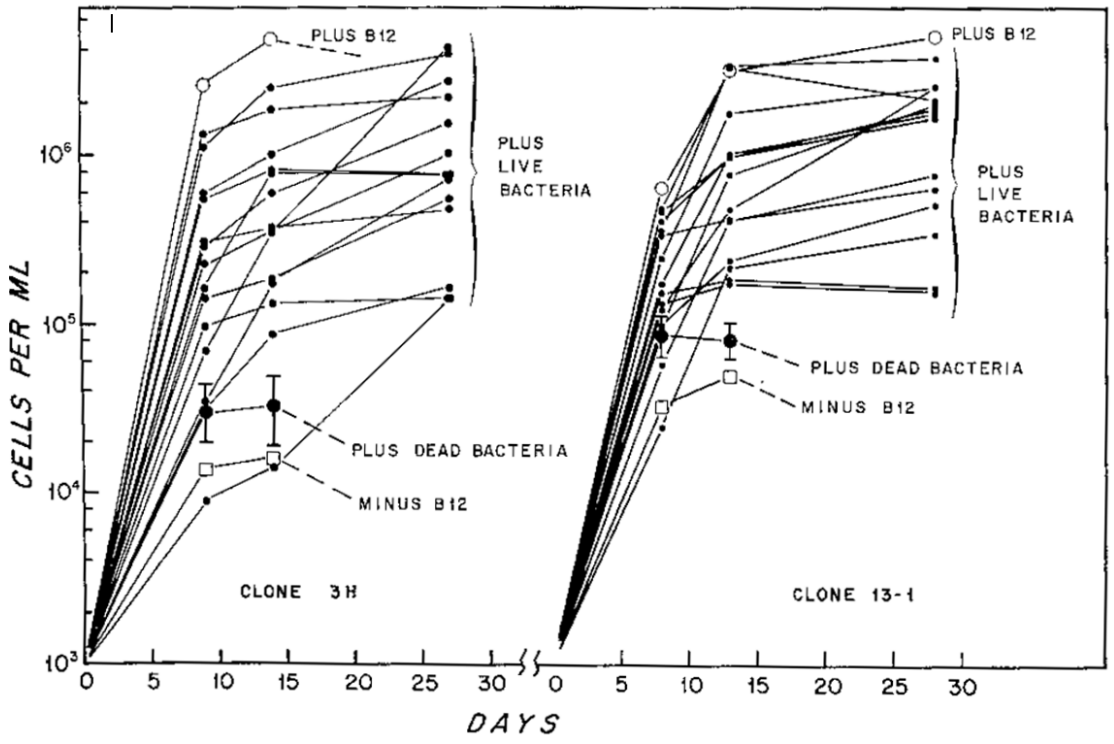

FIGURE 1.5: Growth of vitamin B12 requiring *Thalassiosira psuedonana* clones 3H (left) and 13-1 (right) with regular supplementation of vitamin B12, without added B12, with added B12 producing bacteria, and with dead B12 producing bacteria. Source: Haines and Guillard 1974 [31].

Chapter 2

Experimental Design

2.1 Goals

This project will test the applicability of using PPFM bacterization technology previously developed in our laboratory to improve the growth and nutritional quality of microalgae. Due to the cultural and geographic significance of the Chesapeake Bay and efforts to rejuvenate its native oyster population, emphasis will be placed on testing the use of PPFM bacterization with microalgal species vital to bivalve aquaculture. Species chosen will represent industry standard oyster feedstock (Table 2.1). Results will be evaluated in the context of their ability to benefit commercial microalgal production systems. Microalgal growth and experimental conditions will mimic as closely as possible real conditions seen in industrial aquaculture settings, including light intensities, day/night cycles, and standard F/2 media formulations. To simulate the commercial harvesting of microalgae at the peak of the exponential phase of growth, algal cell density on day seven of growth experiments will be used as a standardized threshold to evaluate for potential economic importance. Microalgal fatty acid composition is a critical factor in bivalve larvae development and will be a primary focus of algal nutritional analysis,

in addition to amino acid content. While our research focuses on aquaculture, this technology may also benefit other industries that require microalgal cultivation. The high lipid content of the microalgal species included in the study has implications for benefits to biofuel production, and increases in algal growth rate would benefit other commercial uses, such as the extraction of algal chemical products and pollution remediation.

2.2 Our Approach

Exploiting the symbiotic relationship between microalgae and naturally occurring bacteria could increase algal turnover rate and reduce the costs of algal growth. The potential for culture contamination may be reduced, and nutrients provided by beneficial bacteria may cut down on media supplementation required by algae cultivators. Healthier and more nutritious algae may provide a benefit to bivalve spat, particularly the oysters raised for use in Chesapeake Bay restoration projects. By utilizing this approach, insight would also be gained into the interactions and ecology of algae with bacteria in their environment. It should be recognized that PPFM are naturally occurring, and can be isolated from algal samples. As such, it can be expected that microalgae used in the study already have a natural microfauna including some amount of PPFM bacteria. Our approach seeks to increase the level of PPFM bacteria present through supplementation and inoculation with select strains. Algal samples included in the study will be tested for the presence of existing PPFM bacteria.

2.3 Competing Approaches

Competing approaches to the enhancement of algal aquaculture have focused on refinement of processing procedures, photobioreactor design, and even genetic modifications of the algae. Current methods of growing algae on a large scale involve the use of either

open systems such as parallel raceway ponds, or closed systems such as photobioreactors. While progress can be expected to continually improve the efficiency of these systems, their benefits are not mutually exclusive with the potential benefits offered by the co-culturing of microalgae with beneficial bacteria. In particular, the use of select PPFM strains isolated from wild populations will avoid regulatory difficulties involved in the use of genetically modified organisms [1].

2.4 Project Plans

1. Test algal cultures for the presence of PPFM. If present, isolate strains of PPFM from each algal culture.

2. Conduct microalgal growth experiments with and without PPFM supplementation. Emphasis will be placed upon previously isolated vitamin B12 overproducing PPFM strain *PPFM B12*. Determine if PPFM supplementation affects microalgal growth rates.

3. Assess the nutritional qualities of algae produced with and without PPFM supplementation.

Table 2.1: Algal species used in the study.

	Species	Attributes
1	*Tetraselmis chuii*	Chlorophyta - flagellate
2	*Chaetoceros mulleri*	Ochrophyta - diatom
3	*Isochrysis sp.*	Haptophyta - flagellate
4	*Thalassiosira pseudonana*	Ochrophyta - diatom

Chapter 3

Materials and Methods

3.1 Description of the work to be performed

1. Algal cultures will be plated on selective media for the isolation of PPFM bacteria.

2. Algal cell density of control and experimental PPFM treated cultures will be measured daily for 10 - 15 days with a fluorometer. Standard curves will be used to correlate chlorophyll autofluorescence with cell density.

3. A contract lab will analyze dry algal biomass in control and experimental PPFM treated samples to evaluate nutritional content.

3.2 Algal Species

Marine microalgae species used in the study are listed in Table 3.1. Strains of *Chaetoceros mulleri* and *Thalassiosira pseudonana* received from Horn Point Laboratory (HPL) [32] could not be isolated to remove contaminants, and were replaced with strains from The Culture Collection of Algae at the University of Texas at Austin (UTEX) [33]. Replacement UTEX strains were used in all algal growth experiments requiring these

respective species. PPFM isolation experiments were completed prior algal growth experiments, and instead used the HPL strains of these species.

TABLE 3.1: Sources of algal species used in the study.

	Species	Sources
1	*Tetraselmis chuii*	Horn Point Lab
2	*Chaetoceros mulleri*	Horn Point Lab / UTEX
3	*Isochrysis sp.*	Horn Point Lab
4	*Thalassiosira pseudonana*	Horn Point Lab / UTEX

3.3 PPFM Strains

The following strains of PPFM were used for algal growth experiments.

- *PPFM 'Broccoli'*: a strain previously isolated from broccoli samples by the Holland lab, representing a 'standard' wild strain of PPFM
- *PPFM B12*: a vitamin B12 overproducing strain, previously isolated by the Holland lab and selected for producing higher levels of vitamin B12

It should be noted that vitamin B12 production is known in wild PPFM strains. Strain *PPFM B12* is notable for greater B12 production than 'wild' strains. The following strains of PPFM were isolated from algal strains provided by Horn Point Laboratory.

- *PPFM HPL1*: isolated from *Tetraselmis chuii*
- *PPFM HPL2*: isolated from *Chaetoceros mulleri*
- *PPFM HPL3*: isolated from *Isochrysis sp.*
- *PPFM HPL4*: isolated from *Thalassiosira pseudonana* Labs

3.4 Media Preparation

3.4.1 F/2 Algae Media

Guillards F/2 media was prepared according to FAO guidelines [34]. Media was adjusted to a salinity of 13ppm through the addition of 13g of Instant Ocean Aquarium Sea Salt Mixture [35] per final liter of media. Solid media formulations used an additional 15g of agar per liter. F/2 media was used for all experimental and stock liquid algal cultures, and for *Tetraselmis chuii*, *Chaetoceros mulleri*, *Isochrysis sp.*, and *Thalassiosira pseudonana* solid stock cultures in the form of plates and slants.

3.4.2 Vitamin B12 Deficient F/2 Media

Standard F/2 media preparations include supplementation with vitamin B12. Some experiments utilized F/2 media that did not include vitamin B12 supplementation. To ensure that trace levels of vitamin B12 were not present, all glassware used in the preparation of this B12 deficient media and in the completion of experiments was subjected to a 24 hour acid wash phase using concentrated HCl and tested for a final wash solution pH $\leq$1.5. When possible, plastic ware used for the preparation of media and for the completion of experiments came from fresh, factory-sterilized sources.

3.4.3 AMS Media for PPFM

Ammonium Mineral Salt (AMS) media (Table 3.2) was used for the growth, maintenance, and isolation of PPFM bacteria. Methanol (MeOH) was added to AMS media at 0.5% after autoclaving. Solid media preparations were formed by the addition of 15g of agar per liter and were poured into plates.

TABLE 3.2: Ammonium Mineral Salts (AMS) media preparation, pH adjusted to 6.8 (Media Formulation # 784 [36]). Methanol (MeOH) added at 0.5% after autoclaving. Agar added at 15g/L as needed.

	Compound	Molarity
1	K_2HPO_4	4.02mM
2	KH_2PO_4	3.97mM
3	$MgSO_4$	4.06mM
4	NH_4Cl	9.34mM
5	$CaCl_2$	1.36mM
6	$FeSO_4$	14.4μM
7	$ZnSO_4$	0.348μM
8	$MnCl_2$	0.152μM
9	H_3BO_3	4.84μM
10	$CoCl_2$	0.841μM
11	$CuCl_2$	58.6nM
12	$NiCl_2$	84.1nM
13	Na_2MoO_4	0.248μM

3.5 Stock Algae Culture Maintenance

Liquid algal cultures were maintained on an open bench top in liquid and solid F/2 media under fluorescent lamps set to a 12 hour on / 12 hours off cycle. Ambient lab lighting was measured as 1 PAR (Photosynthetically Active Radiation; μmol photons/m^2/sec) and considered insignificant for algal growth and maintenance. Light from other sources was attenuated. A raised wire shelf was constructed on the bench top to hold experimental algal cultures; stock algal cultures were stored beneath and adjacent to the shelf. Light levels beneath the shelf were measured at 23 PAR, while light levels adjacent to the shelf were measured at 42 PAR, as measured by the EMS 7 meter. Ambient temperatures in the lab were not controlled but measurements reported a temperature range of 19 - 26°C during the course of the study. Stock cultures were swirled by hand periodically, and subcultured regularly. Stock liquid cultures were kept in a 100-150mL sample volume, contained in 250mL flasks with loose caps. Smaller sized liquid cultures were maintained in 50mL Falcon Tubes [37] stored on a tilted rack, alongside stock algal cultures on agar slants. Background information on the principles of maintaining and growing algal

cultures was provided by the Culture Collection of Algae at the University of Texas at Austin [38].

3.6 Stock PPFM Culture Maintenance

PPFM bacterial cultures were grown in AMS media with 0.5% MeOH. Cultures were inoculated into a 50mL sample of sterile media, contained in 125mL flasks with loose fitting caps. Liquid cultures were maintained on a shaker table set to 200 rpm. Stock samples were kept on solid AMS media plates stored on the bench top or in a refrigerator at 4°C. Cryocultures were preserved in the deep freezer at -70°C either in a solution of 50% glycerol or 70μL DMSO per 1mL sample.

3.7 Cell Counting

Measurements of cell culture density were performed with a standard hemocytometer and a handheld manual counter. Samples were diluted as necessary. To aid in the counting of motile cells, SPOT [39] imaging software was used to take still photos of cell samples on the hemocytometer. Cell density measurements were converted into a final unit of cells/mL.

3.8 Light Measurements

A Burrage model EMS 7 Plant Canopy Analyser was used to measure light intensity, reported in PAR.

3.9 Algal Growth Experiments

Algal cultures were grown in 250mL or 300mL flasks with loose lids. Preliminary experiments used plain 250mL flasks for algal growth, while later experiments were conducted with custom 300mL sidearm flasks.Unless otherwise specified, algal growth experiments utilized six control samples of 100mL F/2 media inoculated with 1mL of stock algal culture, while experimental cultures utilized six samples of 100mL F/2 media inoculated with 1mL of stock algal culture along with a volume of stock PPFM culture adjusted to give a ratio of 5:1 PPFM to algal cells. PPFM inoculum volume ranged from 1μL to 137μL based on culture density. Inoculated flasks were arranged in a staggered fashion on a raised wire shelf on an open lab bench underneath a lamp containing 4 fluorescent light bulbs. Light intensity at this level was measured at 65 PAR. Lamps were controlled with a timer set to use a 12 hour on/off cycle, with the lamps turning on at 6:00 AM and shutting off at 6:00 PM. During algal growth experiments, measurements of cell density were taken daily, unless otherwise specified. A TD-700 model fluorometer [40] was used to measure chlorophyll *a* autofluorescence. A Spectronic 20 Genesys model spectrophotometer was used to record light absorbance.

A LabPro [41] meter with thermometer probe was used to record temperature during the course of the experiment. Temperature measurements were taken automatically at 15 minute intervals during experimental growth trials. Algal growth experiments lasted up to 15 days. Daily measurements of culture density were taken with fluorometry and spectrophotometry. Upon the conclusion of algal growth experiments, aliquots of each algal sample were saved in 50mL Falcon tubes for potential future nutritional analysis. Cryosamples were also preserved in a 1mL volume containing 50% glycerol. Samples were stored in freezer at -70°C.

3.9.1 Small Volume Algal Growth Protocol

Novel procedures were tested to reduce physical size and labor requirements of algal growth experiments. These procedures utilized 15mL Falcon Tubes on slanted racks as vessels for algal growth. This experiment utilized four categories of algal cultures in order to test changes in microalgal growth rate due to supplementation with *PPFM B12* in the presence of vitamin B12 and media that was deficient in vitamin B12. Twenty *Thalassiosira pseudonana* cultures were included, each with 10mL of media and a 500μL algal inoculum. The following parameters were used for culture setup:

- Control cultures with standard F/2 media containing B12 (n=5)
- Experimental cultures with standard F/2 media containing B12 and *PPFM B12* inoculation (n=5)
- Experimental cultures with B12 deficient F/2 media (n=5)
- Experimental cultures with B12 deficient F/2 media and *PPFM B12* inoculation (n=5)

Inoculum volume of *PPFM B12* was adjusted to provide a 5:1 ratio of PPFM to algal cells, based on the cell density of the algal inoculum. These criteria dictated a 1μL inoculum volume of *PPFM B12*. Cell density readings were performed with a Spectramax i3 plate reader [42] calibrated to detect chlorophyll *a* autofluorescence, which was correlated to cells/mL through the use of a standard curve. Excitation and emission wavelengths used were 436nm and 680nm, respectively. These wavelengths were selected in order to replicate measurement parameters of the TD-700 fluorometer used in other algal growth experiements. Fluorescence readings were taken on a multi-well slide with cover slip, with a 2.5μL sample volume in each well. These procedures ultimately reduced lab space requirements, but increased labor requirements.

3.9.2 Large Volume Algal Growth Protocol

Algal cultures were grown in 15L samples within 5-gallon glass carboys to obtain necessary biomass for nutritional profile analysis. Standard F/2 media formulation was used for both control and experimental samples (n=1). A mixed algal inoculum was used in both experimental and control replicates, including a 40mL sample of each of the following algal strains.

- *Tetraselmis chuii* (HPL)
- *Chaetoceros mulleri* (UTEX)
- *Isochrysis sp.* (HPL)
- *Thalassiosira pseudonana* (UTEX)

The experimental algal culture also included an inoculum of 15mL of *PPFM B12* stock, to give a final ratio of 5:1 PPFM cells per algal cell. Fluorescent lamps provided light while filtered air hoses were used for culture aeration and convection (Figure 3.4). Cultures were grown for nine days before harvesting. To obtain dry biomass, cultures were centrifuged at 20,000 rpm for 20 minutes. Media was decanted, and algal pellet was collected and dried for later nutritional analysis (Figure 3.5).

3.10 Optical Density Measurements of Algal Cell

3.10.1 Fluorometry

Measurements of chlorophyll *a* autofluorescence were recorded with the model TD-700 fluorometer [40]. Standard curves were used to correlate these values to cell density in cells/mL. The TD-700 emits light at a wavelength of 436 nm into a liquid sample contained in a 13mm cuvette. In response, chlorophyll *a* contained in the sample emits

light at a wavelength of 680nm. The emission is quantified and reported in relative fluorescence units (FSU) on a scale of 0 to 999. A fluorometer warm-up period of 10 minutes was used, and a secondary standard provided by Turner Design was used to calibrate the device. After inserting a sample, a stopwatch was used to take standardized readings after 15s to control for temperature changes and sample convection that could affect readings. For samples that reported an FSU greater than 999, a sub-sample was diluted and measured, and the resulting FSU was adjusted up based on the dilution factor. All samples were swirled to thoroughly mix algae before being measured. When experimental samples were grown in standard 250mL flasks, a 5mL sample was pippetted into a 13mm cuvette for measurement in the TD-700. In experiments that utilized 300mL sidearm flasks, it was not possible to close the lid on the TD-700 for normal measurement. To prevent external light from being read by the fluorometer and affecting measurements, all readings were taken in the dark. The TD-700 was tilted to one side to allow readings to be taken without spilling the algal culture in the sidearm flask.

3.10.2 Spectrophotometry

A spectrophotometer was used to measure light absorbance in algal culture samples. Light absorbance at a wavelength of 540 nm was measured and reported in relative absorbance units (ABS). Standard curves were used to correlate the ABS value to cell density in cells/mL. All experiments that utilized the spectrophotometer were carried out as described in the previous section (Figure 3.1). A sterile F/2 sample was used to blank the spectrophotometer prior to measuring.

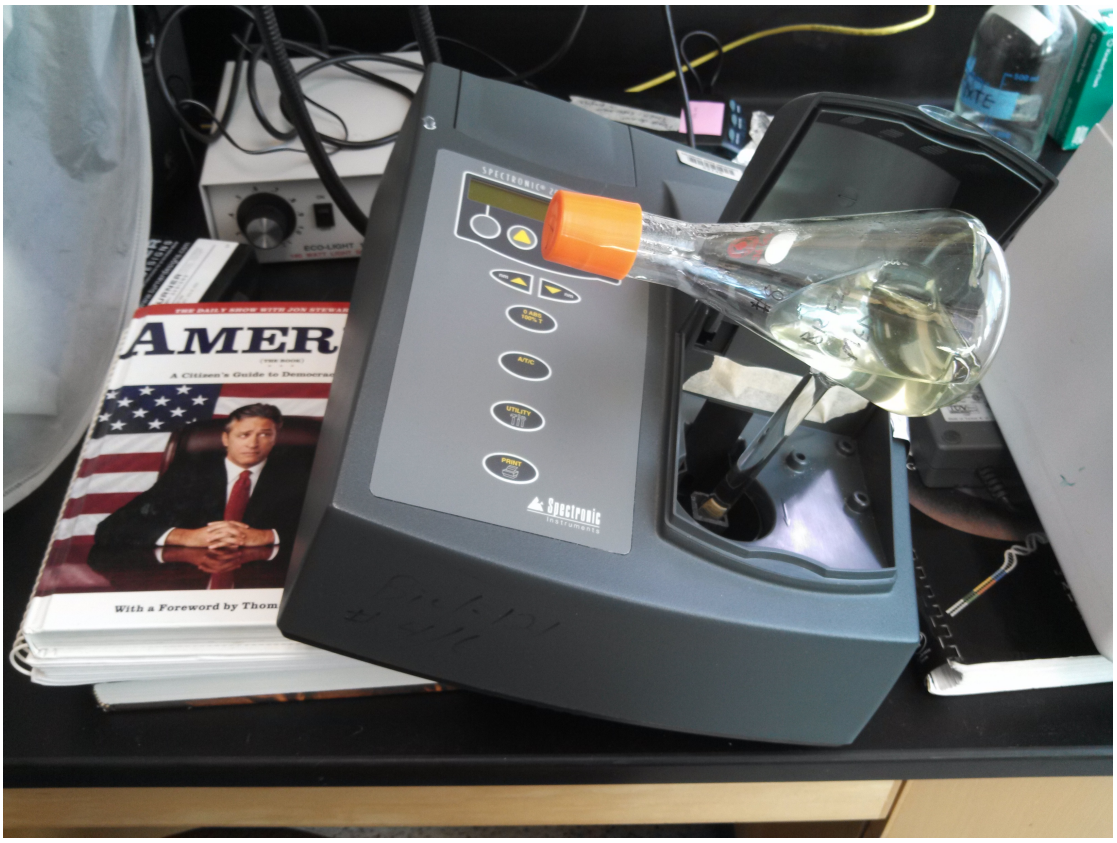

FIGURE 3.1: Flask containing algal sample inserted into spectrophotometer.

3.11 Temperature Recording

Temperature readings were recorded with a pair of simple thermometers one set on each side of the wire shelf. A Lab Pro [41] recording device with thermometer probe fastened to the center of the shelf was also used. The Lab Pro recorded temperature at 15 minute intervals for the course of algal growth experiments.

3.12 Microbial Isolation

3.12.1 PPFM Isolation

PPFM cultures were streaked on plates composed of solid AMS media containing 0.5% MeOH and 15g agar per liter. Single colonies were picked, and streaked on new plates. This process was repeated as necessary. Cycloheximide was added to media poured

FIGURE 3.2: A typical lab setup, with an algal growth experiment in progress. Sidearm flasks on the raised wire rack hold experimental samples. Algal stock samples were maintained on bench top below experimental samples to attenuate light.

into plates (12mg/L) to prevent the growth of eukaryotic contaminants while PPFM colonies developed. Naturally occurring PPFM strains were isolated from algal samples by plating algae on AMS + 0.5% MeOH. Resulting pink colonies were singled out, and streaked onto new plates. This process was repeated, and resulting PPFM strains were designated as stock cultures.

3.12.2 Algal Isolation

To purify algal stocks obtained from Horn Point Laboratory, samples were streaked onto solid F/2 media plates with 15g agar per liter. Single colonies were selected for *Tetraselmis chuii* and *Isochrysis sp.* and streaked onto fresh plates repeatedly until pure cultures could be obtained. Efforts to isolate *Thalassiosira pseudonana* and *Chaetoceros*

mulleri in this manner were not successful. Fresh cultures of these species were ordered from UTEX Algae Culture Collection instead.

3.13 PPFM DNA Isolation

DNA was extracted from PPFM samples using a protocol adapted from Simmon et. al. [43]. PPFM cells from stock cultures in liquid AMS media were washed twice with 1mL of sterile water. Cells were suspended in 50 μL of 10mM Tris, 1mM EDTA (pH 8.0) (TE) and sealed in sterile micro centrifuge tubes. Tubes were loaded into racks, with an empty rack atop and secured with tape to prevent the release of contents or contamination of samples during autoclaving. A jacketed, preheated autoclave set to 121°C was used to process the racks, with a a sterilization time of 1 min, 0 min dry time, 0 min purge time, and fast exhaust. The goal of this step was to disrupt bacterial cell walls through rapid depressurization to allow for extraction of DNA. The autoclaved material was cleared by micro-centrifugation for 5 min at 10,000 rpm. This cleared lysate was used directly in the PCR experiment [43].

3.14 PPFM DNA Amplification and Extraction

Primers for PPFM 16s rDNA were obtained, with the following sequences:

F: `(5') AGAGTTTGATCCTGGCTCAG (3')`

R: `(5') ACGGCTACCTTGTTACGACTT (3')`

PCR reactions were prepared containing 0.2μM F primer, 0.2μM R primer, 1μL of PPFM DNA template where applicable, and raised to 25μL final volume with sterilized ePur water. One GE illustra PuReTaq Ready-To-Go PCR Bead [44] was used in each PCR reaction to provide necessary buffer and enzyme. The PCR cycler was set for a 95°C hot start for 5 min, followed by a cycle of 95°C for one min, 55°C for 1 min, and

72°C for 2 min repeated 35 times, and completed with a 72°C final extension phase before holding at 4°C. Samples were run on a 1% agarose Speed Buffer [45] gel with 1μL of 6x loading dye [46] and 5μL of PCR sample per gel lane. Gel was run at 80 Volts for 120 min in Speed Buffer [45] containing 250μg/L ethidium bromide stain. Negative control samples contained no DNA template, while positive control samples contained *PPFM 'Broccoli'* DNA template which has been proven to amplify with these primers in past experiments.

To extract DNA samples from the PCR gel for sequencing, a positive control sample was loaded at the far end of the gel to allow for approximation of the location of experimental samples. The PCR gel was placed over a UV illumination box and the positive control sample was allowed to fluoresce. Experimental samples were protected from fluorescence and potential UV degradation by a sheet of aluminum foil positioned under the gel (Figure 3.3). Experimental samples were cut from the gel and processed with a Wizard *Plus* SV Miniprep DNA Purification System kit to clean up the DNA for sequencing.

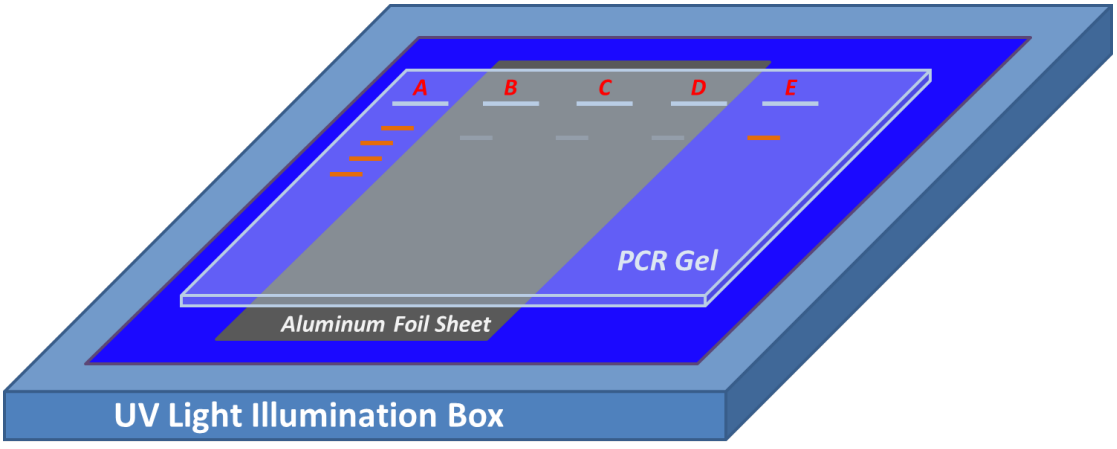

FIGURE 3.3: Diagram of DNA extraction method. PCR Gel includes base pair ladder (Lane A), experimental PCR samples (Lanes B, C, and D), and positive control PCR sample (Lane E). UV illumination box causes ethidium bromide treated DNA samples to fluoresce. Positive Control sample (Lane E) and base pair ladder (Lane A) allows for the approximation of experimental sample positions in the gel. Aluminum foil sheet protects the experimental samples from UV exposure and degradation. Experimental samples can be cut from gel and extracted.

3.15 PPFM DNA Sequencing

DNA samples were sent out for sequencing at the Clemson University Genomics Institute. Sequences were viewed with FinchTV and aligned with BLAST [47].

3.16 Nutritional Analysis

3.16.1 Vitamin B12 Assay

A Food Vitamin B12 ELISA Kit [48] was used to measure vitamin B12 levels in algal, bacterial, and media samples. Standard ELISA protocol was followed. Test sample results were measured with a Spectramax i3 plate reader [42].

3.16.2 Algal Nutritional Profile

Dry samples of experimental and control algae were analysed for nutritional profile by The University of Missouri Experiment Station Chemical Laboratories.

3.17 Data Analysis

Statistical analysis of data and generation of plots, graphs, and tables was performed in R [49] (version 3.2.0), along with RStudio [50] (version 0.98.1103). Nonparametric statistical testing methods were chosen due to low sample size among replicates during algal growth experiments.

FIGURE 3.4: Large volume (15L) mixed algal cultures grown for nutritional analysis.

FIGURE 3.5: Dry algal biomass for nutritional analysis. Approximately 2g shown, representing the entire dried algal content of one 15L mixed culture.

Chapter 4

Results

4.1 Algal Growth Experiments

It was hypothesized that supplementation of algal cultures with PPFM would cause a change in algal growth rates due to the release of nutrients and growth factors by the bacteria. Algal growth experiments were designed to test for this effect. Algal cultures were supplemented with PPFM bacteria at a ratio of 5:1 PPFM to algal cells, which was shown to be an optimal level based on previous laboratory experiments (Holland, unpublished). Experiments included control cultures that lacked PPFM supplementation, and all cultures were grown for 12 to 15 days. It should be noted that PPFM bacteria are ubiquitous in nature, and can be isolated from water and algal samples; experiments conducted in the course of the study succeeded in isolating naturally occurring PPFM from algal stocks used for growth experiments. Accordingly, algal growth studies tested for changes in algal growth from supplementation of PPFM above the level of naturally occurring bacteria using select lab PPFM strains.

In industrial settings, algae are grown only until the peak of the exponential phase of growth before harvesting [34]. For supplementation of algal cultures with PPFM

to benefit commericial microalgal cultivation, it was determined that this harvesting method must be reflected in algal growth experiments. Algal species included in the study require different lengths of time to reach the peak of the exponential growth phase. However, this point is reached by all included algal species within roughly six to eight days. To reflect this harvesting time frame consistently between algal species, we used measurements of algal culture density on the seventh day of growth as a benchmark to determine if changes in algal growth might benefit commercial cultivation methods. Algal strains included in the study represent economically important strains and consisted of industry standard species used as feedstock for oyster larvae.

4.1.1 Effect of *PPFM B12* Upon Algal Growth Rate

While many PPFM naturally produce vitamin B12, *PPFM B12* is a strain of vitamin B12 overproducing PPFM that has been previously isolated in the lab and produces B12 at a greater level than other naturally occurring strains. Many algal species are vitamin B12 auxotrophs, and require a source of exogenous vitamin B12 in order to grow [31]. It was hypothesized that supplementation with *PPFM B12* would cause an increase in algal growth rates due to increased vitamin B12 levels. Algal growth experiments were designed to test this hypothesis.

Algal growth curves conducted with *Tetraselmis chuii* grown with or without supplementation of *PPFM B12* at a 5:1 ratio showed no significant difference in cell density by day 7 ($p=0.937$; Figures 4.1 and 4.2).

Algal growth curves conducted with *Thalassiosira pseudonana* grown with or without supplementation of *PPFM B12* at a 5:1 ratio showed no significant difference in cell density by day 7 ($p=0.310$; Figures 4.3 and 4.4).

Algal growth curves conducted with *Chaetoceros mulleri* grown with or without

supplementation of *PPFM B12* at a 5:1 ratio showed no significant difference in cell density by day 7 (p=0.132; Figures 4.5 and 4.6).

Algal growth curves conducted with a combination of four aquaculture algal species (*Tetraselmis chuii*, *Isochrysis sp.*, *Thalassiosira pseudonana*, and *Chaetoceros mulleri*) grown with or without supplementation of *PPFM B12* at a 5:1 ratio showed a significant difference in cell density by day 7 (p=0.002; Figures 4.7 and 4.8).

A follow-up experiment using the same algal mixture grown in full F/2 media included supplementation of *PPFM B12* at a 50:1 ratio along with a 5:1 ratio. Control cultures included only algae. A significant difference in microalgal cell density between treatments and controls was not seen by day 7 (p=0.926; Figures 4.9 and 4.10).

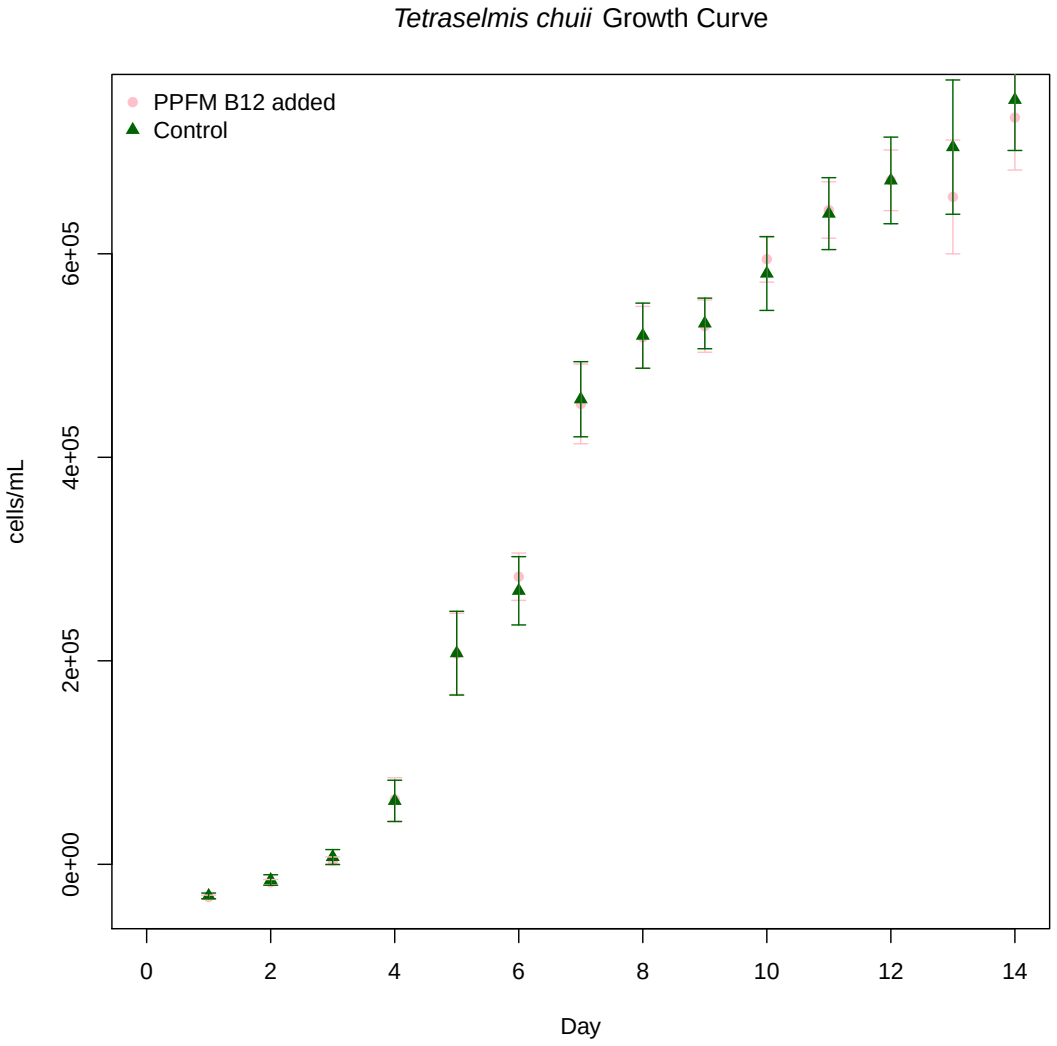

FIGURE 4.1: Growth curve for *Tetraselmis chuii* grown with *PPFM B12* at a 5:1 ratio of PPFM per algal cell. Points represent the average of six replicates while error bars represent standard deviation.

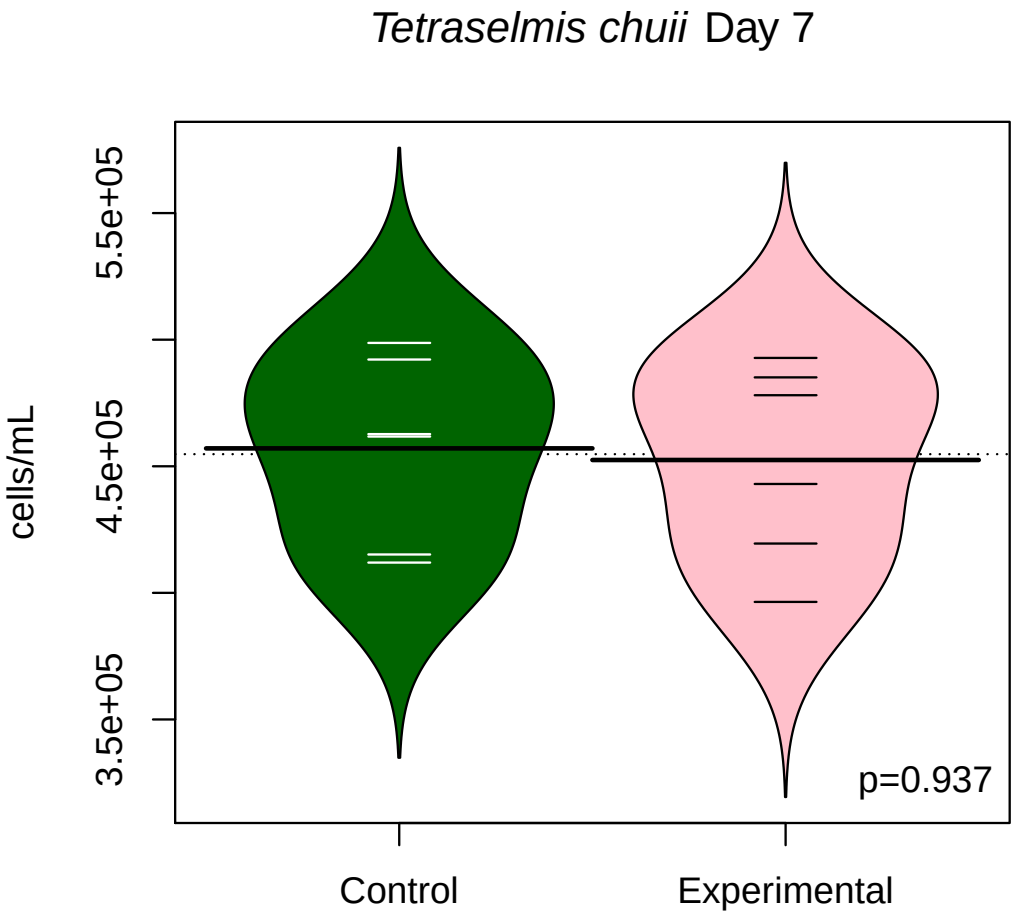

FIGURE 4.2: Beanplot for *Tetraselmis chuii* at the seventh day of growth. No significant difference was shown between Control and Experimental *PPFM B12* supplemented treatment groups (n=6, p=0.937 as measured by Mann-Whitney U test). Horizontal dashes represent individual data points, thick horizontal line represents group mean, dotted horizontal line represents overall mean, and 'bean' shape represents data distribution.

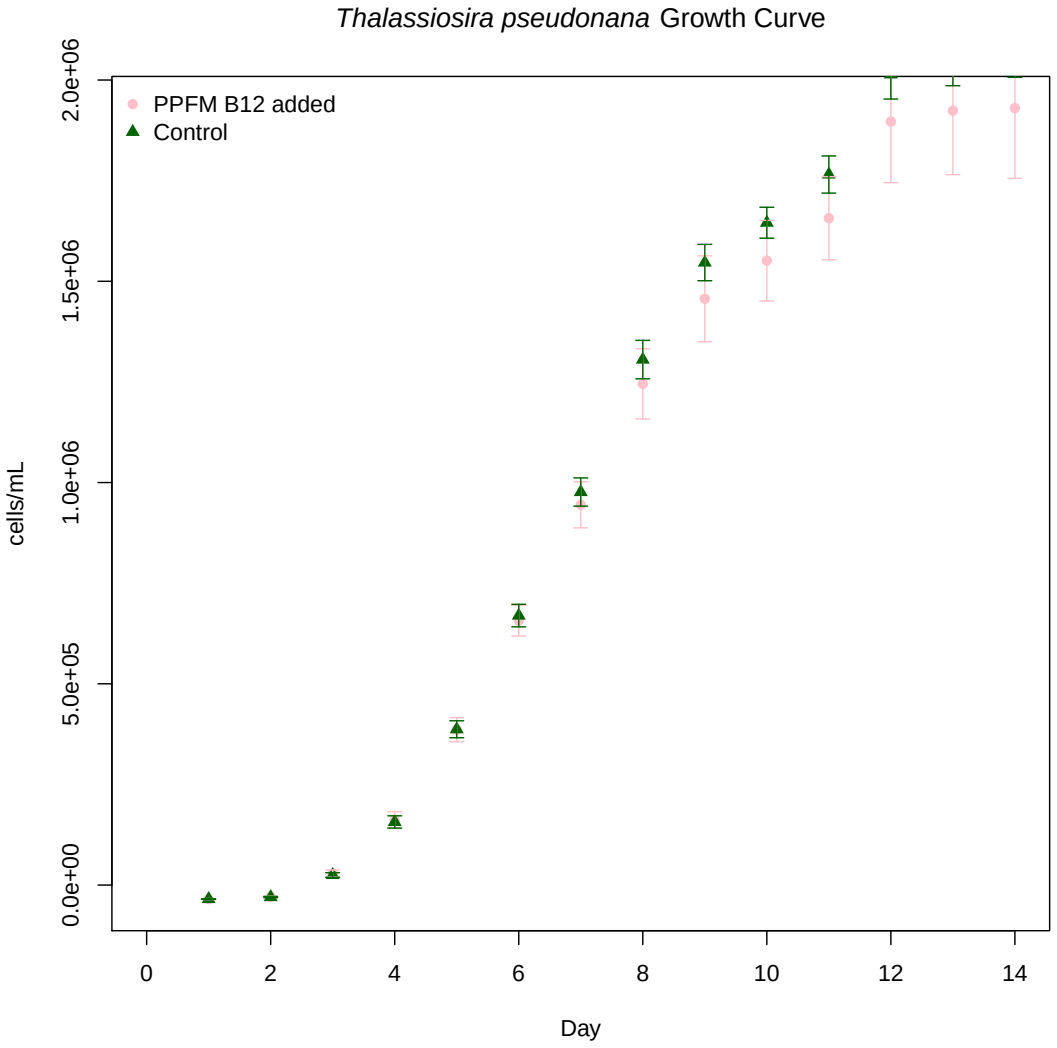

FIGURE 4.3: Growth curve for *Thalassiosira pseudonana* grown with *PPFM B12* at a 5:1 ratio of PPFM per algal cell. Points represent the average of six replicates while error bars represent standard deviation.

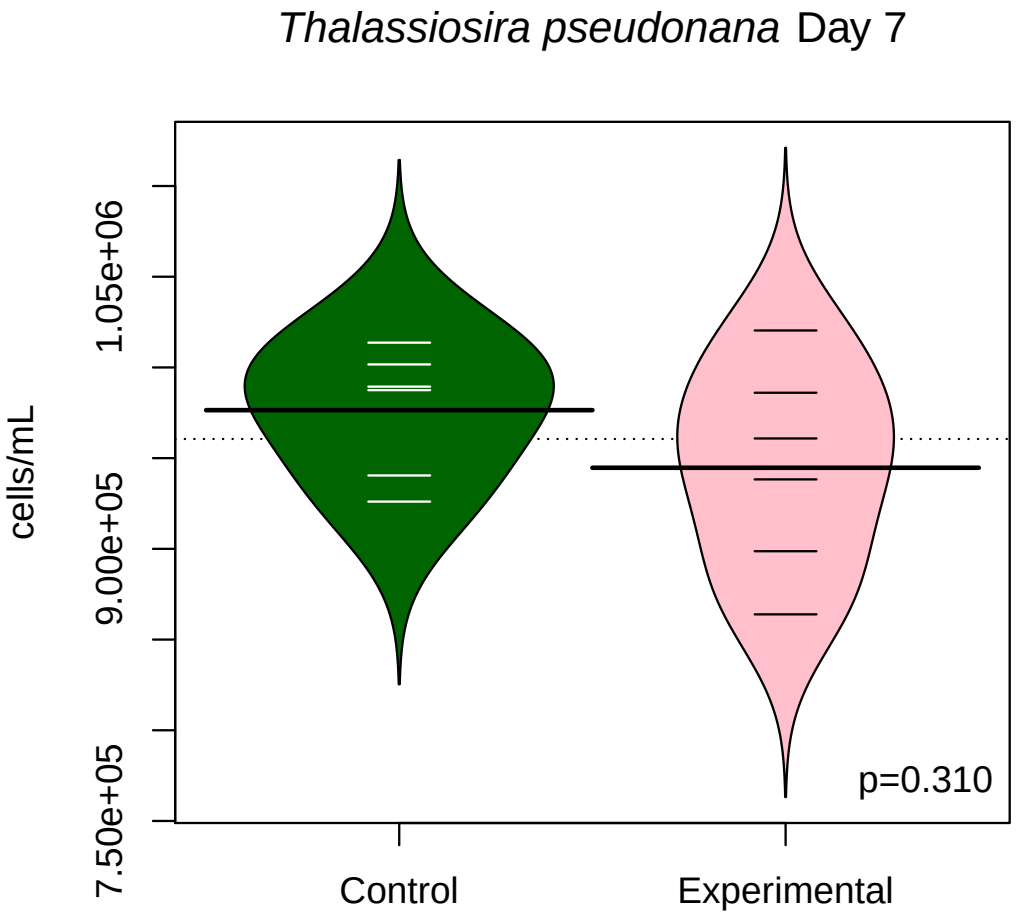

FIGURE 4.4: Beanplot for *Thalassiosira pseudonana* at the seventh day of growth. No significant difference was shown between Control and Experimental *PPFM B12* supplemented treatment groups (n=6, p=0.310 as measured by Mann-Whitney U test). Horizontal dashes represent individual data points, thick horizontal line represents group mean, dotted horizontal line represents overall mean, and 'bean' shape represents data distribution.

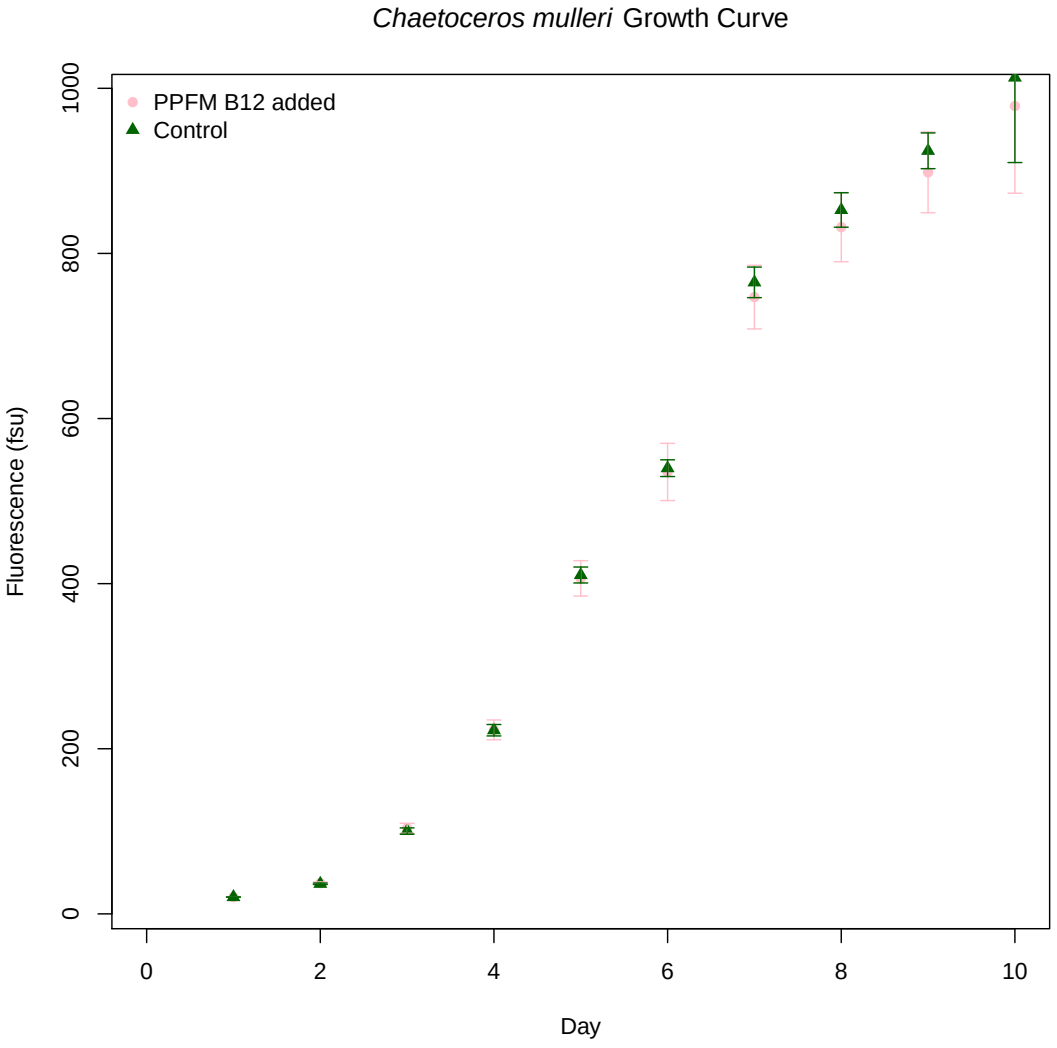

FIGURE 4.5: Growth curve for *Chaetoceros mulleri* grown with *PPFM B12* at a 5:1 ratio of PPFM per algal cell. Points represent the average of six replicates while error bars represent standard deviation.

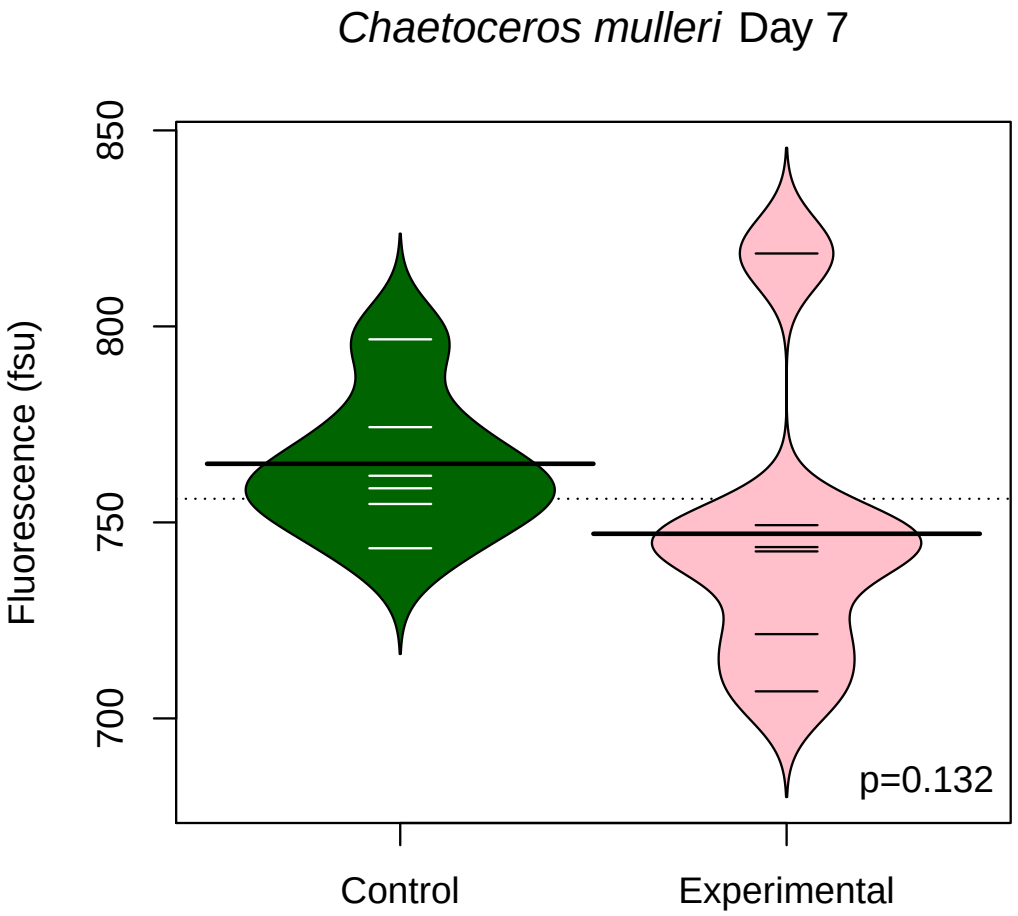

FIGURE 4.6: Beanplot for *Chaetoceros mulleri* at the seventh day of growth. No significant difference was shown between Control and Experimental *PPFM B12* supplemented treatment groups (n=6, p=0.132 as measured by Mann-Whitney U test). Horizontal dashes represent individual data points, thick horizontal line represents group mean, dotted horizontal line represents overall mean, and 'bean' shape represents data distribution.

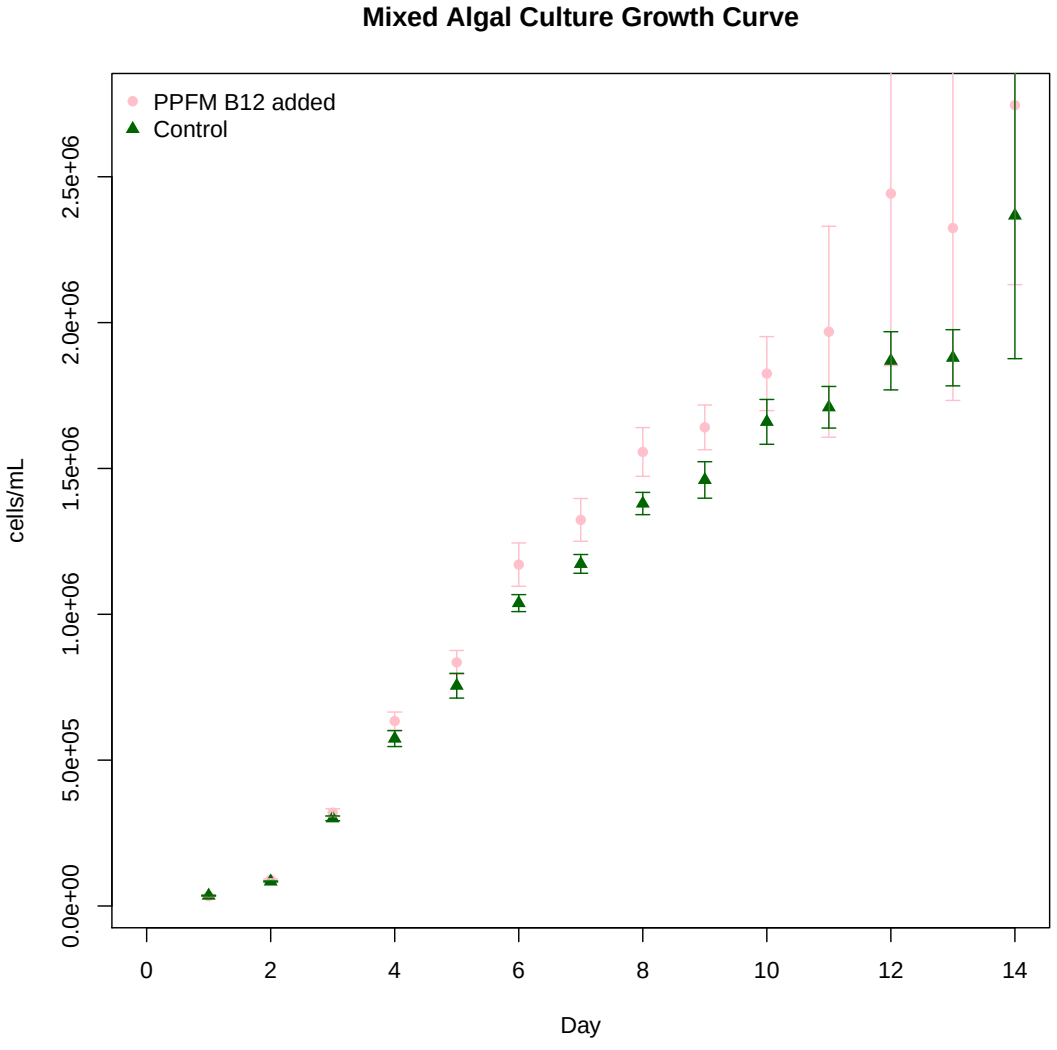

FIGURE 4.7: Growth curve for Mixed Algal Culture containing *Chaetoceros mulleri*, *Thalassiosira pseudonana*, *Tetraselmis chuii*, and *Isochrysis sp.* grown with *PPFM B12* at a 5:1 ratio of PPFM per algal cell. Points represent the average of six replicates while error bars represent standard deviation.

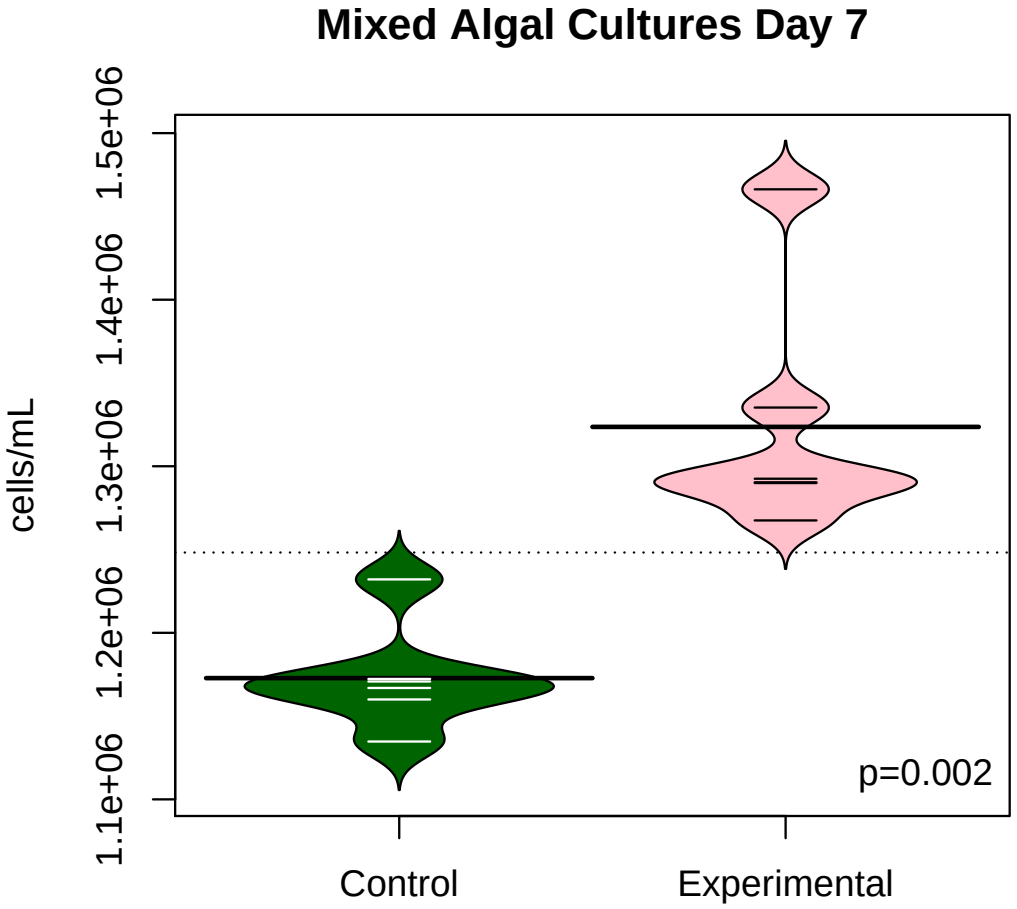

FIGURE 4.8: Beanplot for Mixed Algal Culture at the seventh day of growth. A significant difference was shown between Control and Experimental *PPFM B12* supplemented treatment groups (n=6, p=0.002 as measured by Mann-Whitney U test). Horizontal dashes represent individual data points, thick horizontal line represents group mean, dotted horizontal line represents overall mean, and 'bean' shape represents data distribution.

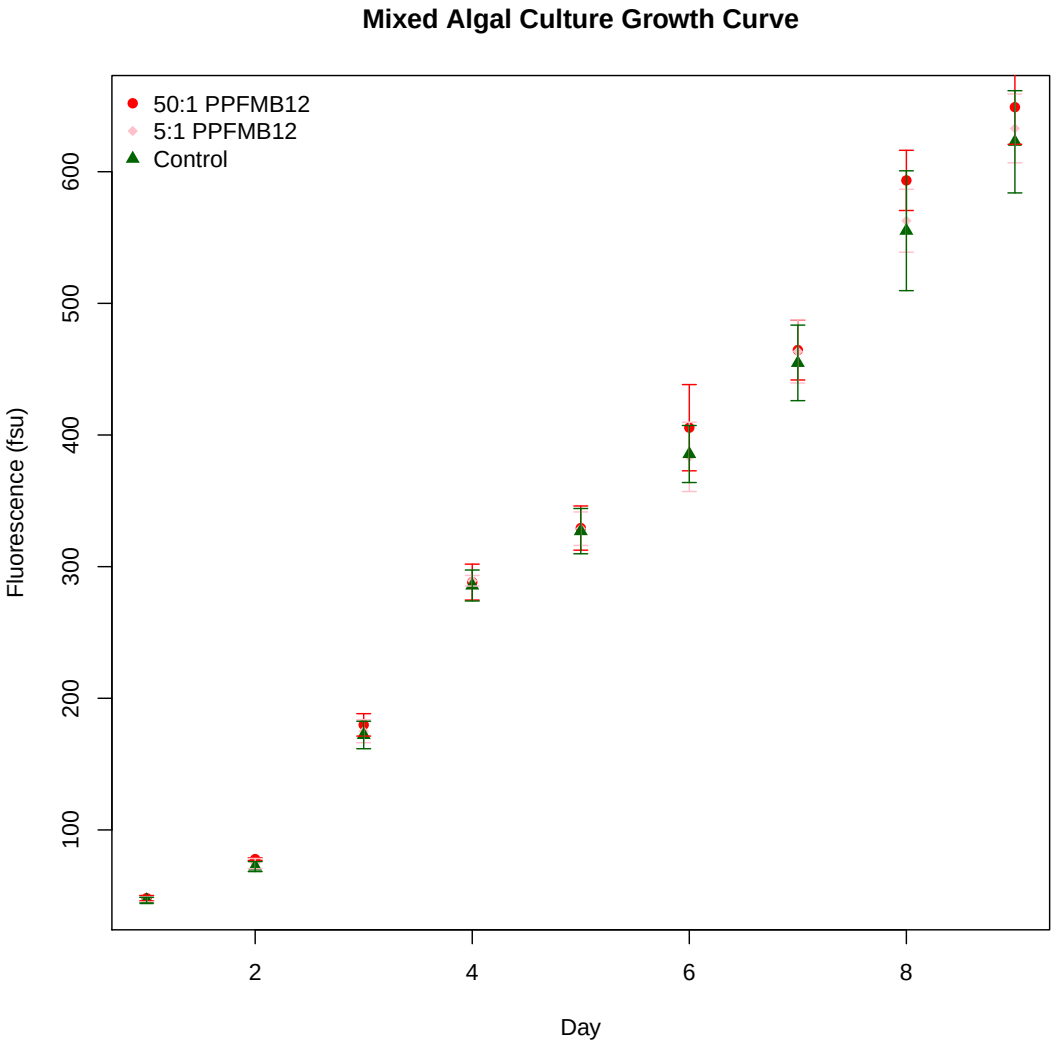

FIGURE 4.9: Growth curve for Mixed Algal Culture containing *Chaetoceros mulleri*, *Thalassiosira pseudonana*, *Tetraselmis chuii*, and *Isochrysis sp.* grown with *PPFM B12* at 50:1 and 5:1 ratios of PPFM per algal cell in full F/2 media. Control cultures included algae without PPFM supplementation. Points represent the average of four replicates while error bars represent standard deviation.

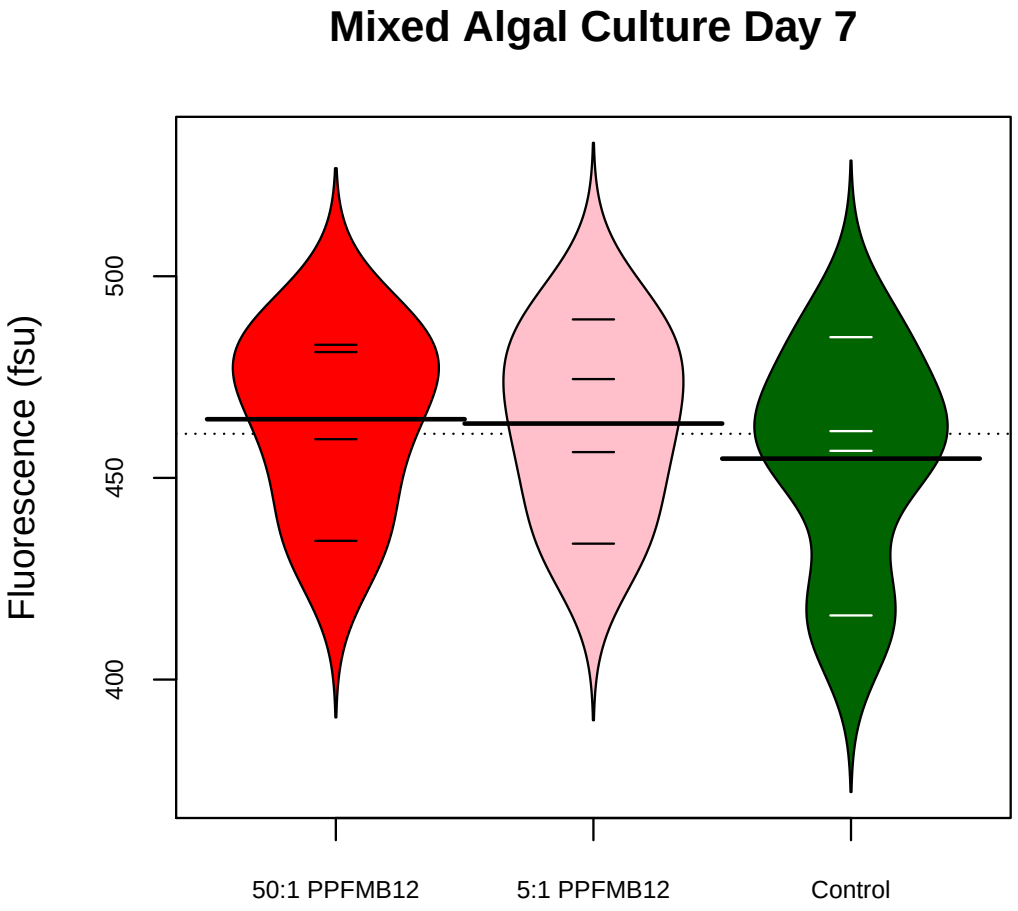

FIGURE 4.10: Beanplot for Mixed Algal Culture at the seventh day of growth. No significant difference was shown between Control and Experimental *PPFM B12* supplemented treatment groups (n=4, p=0.926 measured with Kruskal-Wallis test). Horizontal dashes represent individual data points, thick horizontal line represents group mean, dotted horizontal line represents overall mean, and 'bean' shape represents data distribution.

4.1.2 Effect of Deficient Media and PPFM Upon Algal Growth Rate

It was hypothesized that the vitamin B12 requirements of microalgae used in growth experiments may be met completely by the nutrients present in F/2 media, and that extra vitamin B12 produced by supplemental PPFM may not provide a benefit under these conditions. To test for the effects of PPFM-produced B12 independent of vitamin included in standard F/2 media formulations, growth experiments were designed to include algal cultures grown in F/2 media prepared without vitamin B12 (B12 deficient media) along with cultures grown in F/2 media that included B12 (full media).

To determine algal growth potential in vitamin deficient media, an algal growth experiment was conducted, using the following treatment groups (Figure 4.11):

- *Isochrysis sp.* supplemented with *PPFM 'Broccoli'* at a 5:1 ratio of PPFM to algal cells, in full media (n=3)
- *Isochrysis sp.* supplemented with *PPFM B12* at a 5:1 ratio of PPFM to algal cells, in full media (n=3)
- *Isochrysis sp.* in full media (n=3)
- *Isochrysis sp.* in deficient media (n=3)

Due to the reduced growth rate seen in the deficient media group, a follow up growth experiment was conducted, which included the following treatment groups (Figure 4.12):

- *Isochrysis sp.* supplemented with *PPFM B12* at a 5:1 ratio of PPFM to algal cells, in full media (n=3)
- *Isochrysis sp.* in full media (n=3)

- *Isochrysis sp.* supplemented with *PPFM B12* at a 5:1 ratio of PPFM to algal cells, in deficient media (n=3)

- *Isochrysis sp.* in deficient media (n=2)

A significant difference in algal growth was seen by day 7 (p=0.030, Figure 4.13). In both cases, the treatment groups lacking vitamin B12 in the algal media showed a reduced rate of growth. This reduced growth rate was partially rescued in treatment groups that lacked vitamin B12 in the media but included supplementation with *PPFM B12* (Figure 4.12). Differences in growth rate were not apparent for cultures grown in regular F/2 algal media, which contains vitamin B12 supplementation, for either experiment (Figures 4.11 and 4.12).

A follow up experiment tested for a similar difference in growth rate among these treatment levels using using the diatom *Thalassiosira pseudonana*, which is a known B12 auxotroph [31]. A new growth protocol was also tested, in which the algal cultures were grown in 10mL samples contained in 15mL Falcon tubes, with fluorescence measurements taken from 2.5μL sample measured in a Spectra Max i3 Plate Reader (Figure 4.14). A significant difference in growth between the four treatment groups was not seen by day 7 of growth (Figure 4.15). However, a difference in growth between the four treatment groups was seen by day 9 of growth (Figure 4.16). These results did not suggest that *PPFM B12* supplementation increased algal growth.

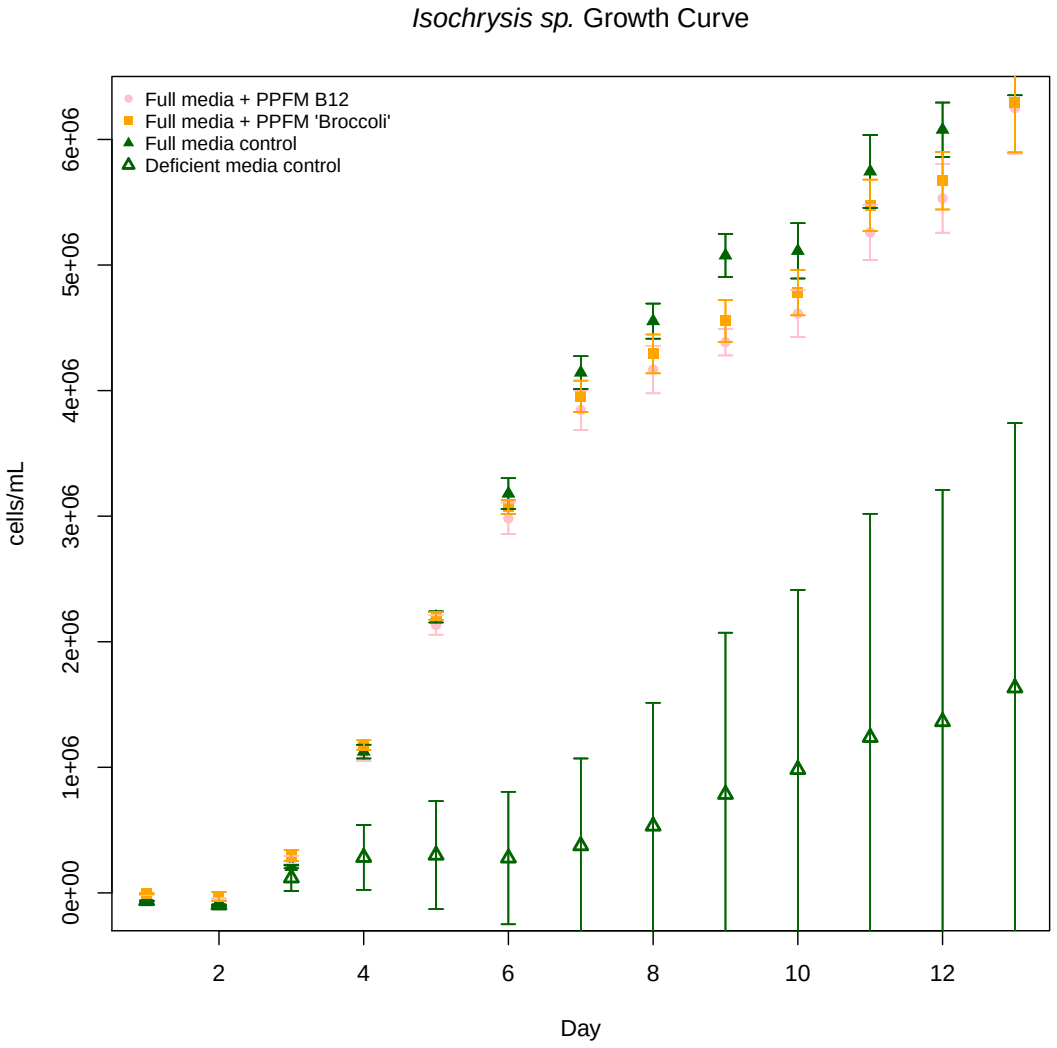

FIGURE 4.11: Growth curve for *Isochrysis sp.* grown with or without *PPFM B12* or *PPFM 'Broccoli'* at a 5:1 ratio of PPFM per algal cell, along with *Isochrysis sp.* grown in a media formulation deficient in vitamin B12 (n=3). Points represent the average of three replicates while error bars represent standard deviation.

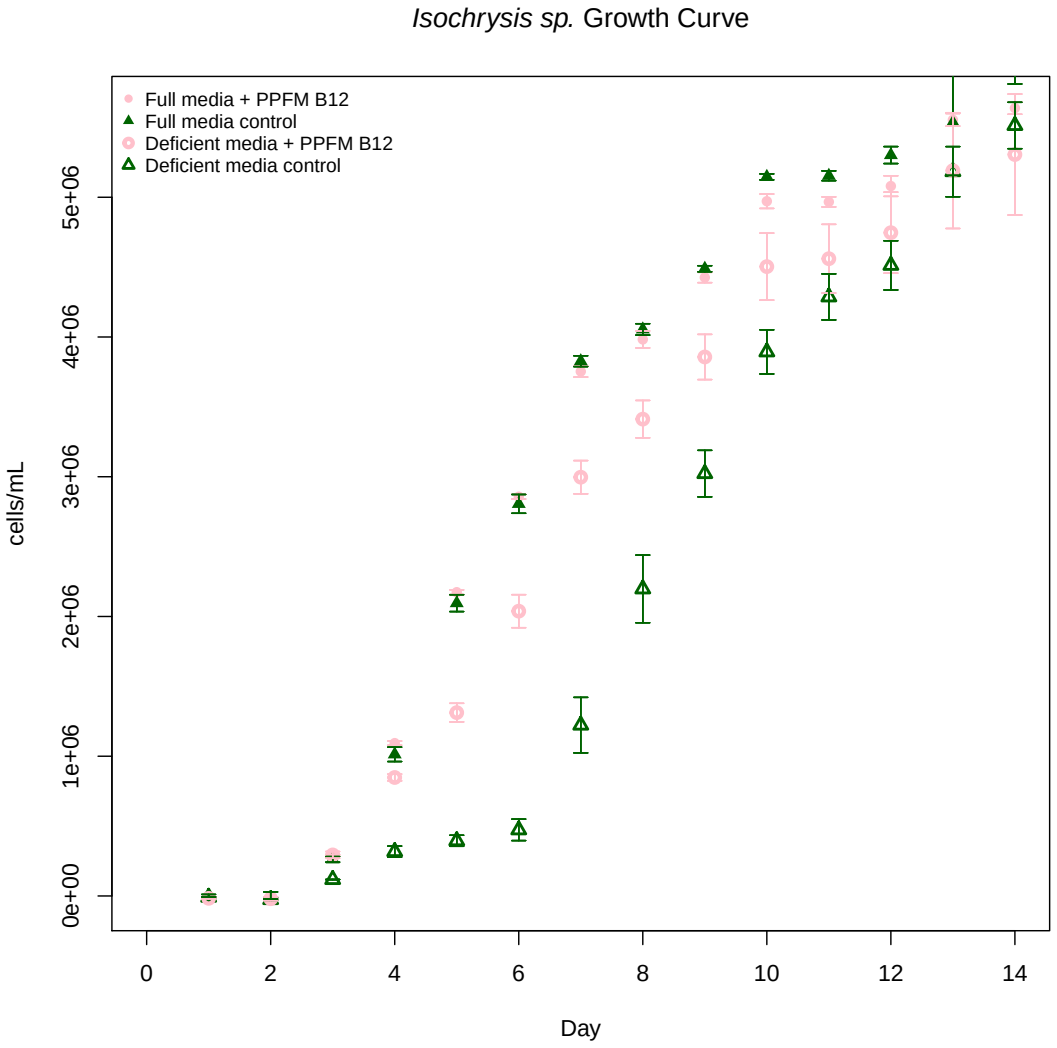

FIGURE 4.12: Growth curve for *Isochrysis sp.* grown in full media with or without *PPFM B12* (n=3), and in vitamin B12 deficient media with *PPFM B12* (n=3) or without PPFM supplementation (n=2; one culture removed due to contamination).

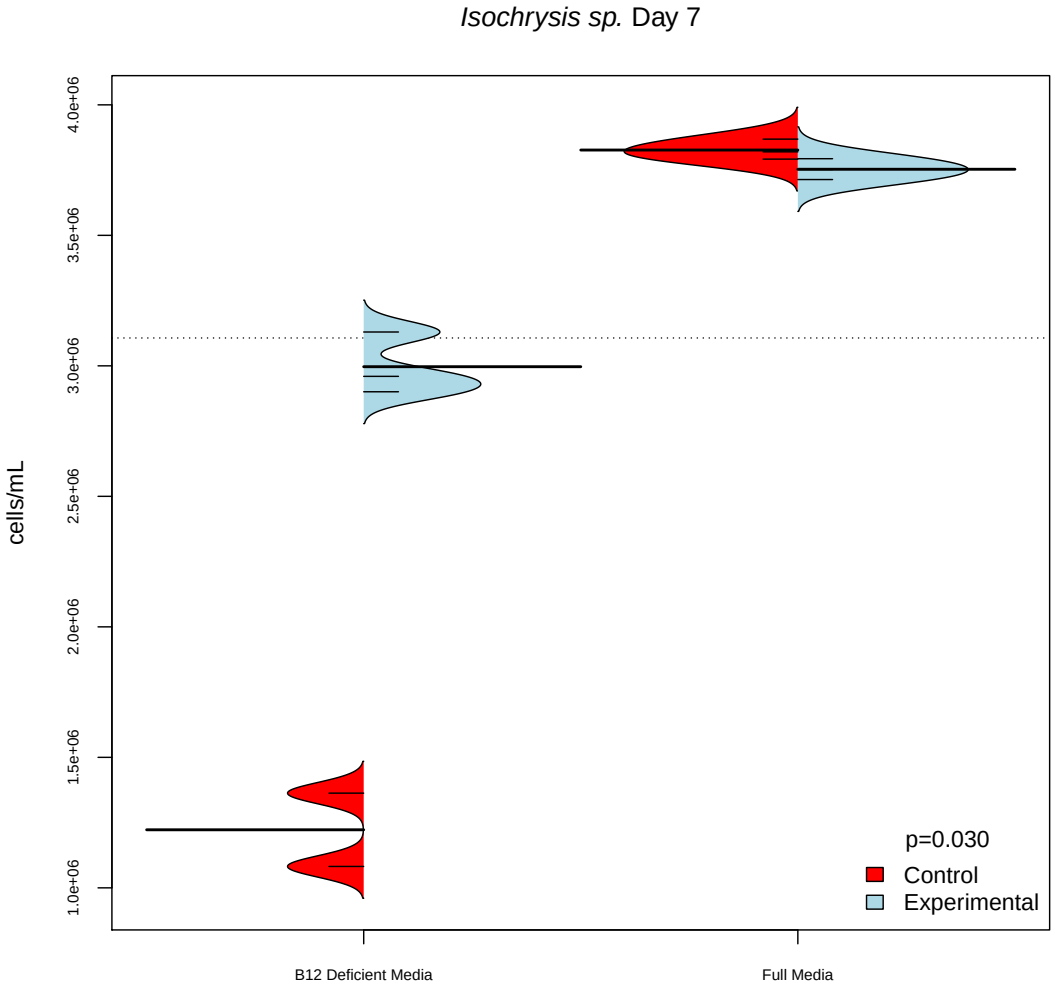

FIGURE 4.13: Beanplot for *Isochrysis sp.*, showing a difference in growth on day seven between four treatment groups (p=0.030, calculated with Kruskal-Wallis test). Control samples (red) included *Isochrysis sp.* grown in either full or deficient media. Experimental samples (blue) included *Isochrysis sp.* grown with *PPFM B12* supplementation at a 5:1 ratio of PPFM to algal cells in either media preparation. Horizontal dashes represent individual data points, thick horizontal line represents group mean, dotted horizontal line represents overall mean, and 'bean' shape represents data distribution.

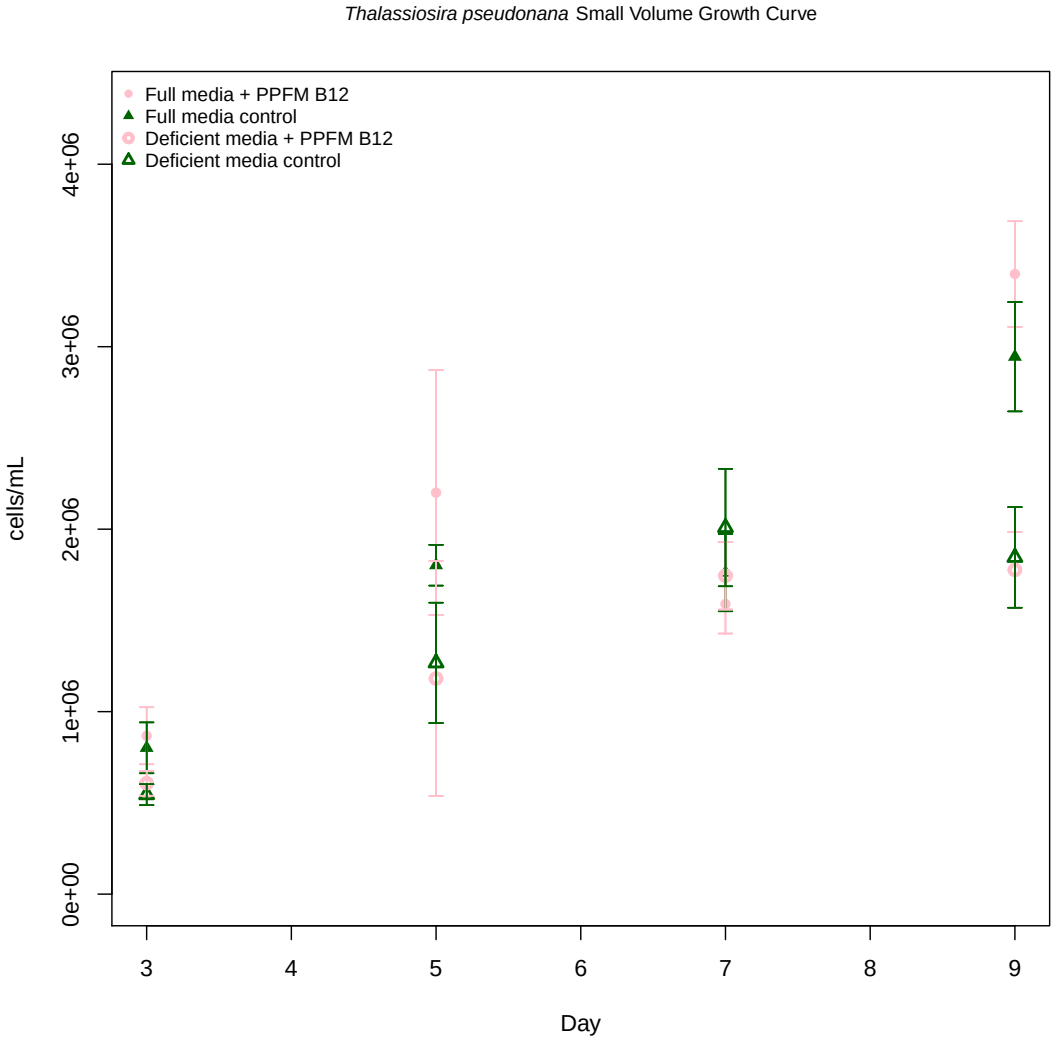

FIGURE 4.14: Growth curve for *Thalassiosira pseudonana* grown in 10mL cultures, with full media with or without *PPFM B12* and in vitamin B12 deficient media with or without *PPFM B12* at a 5:1 ratio of PPFM per algal cell (n=5). Points represent the average of three replicates while error bars represent standard deviation.

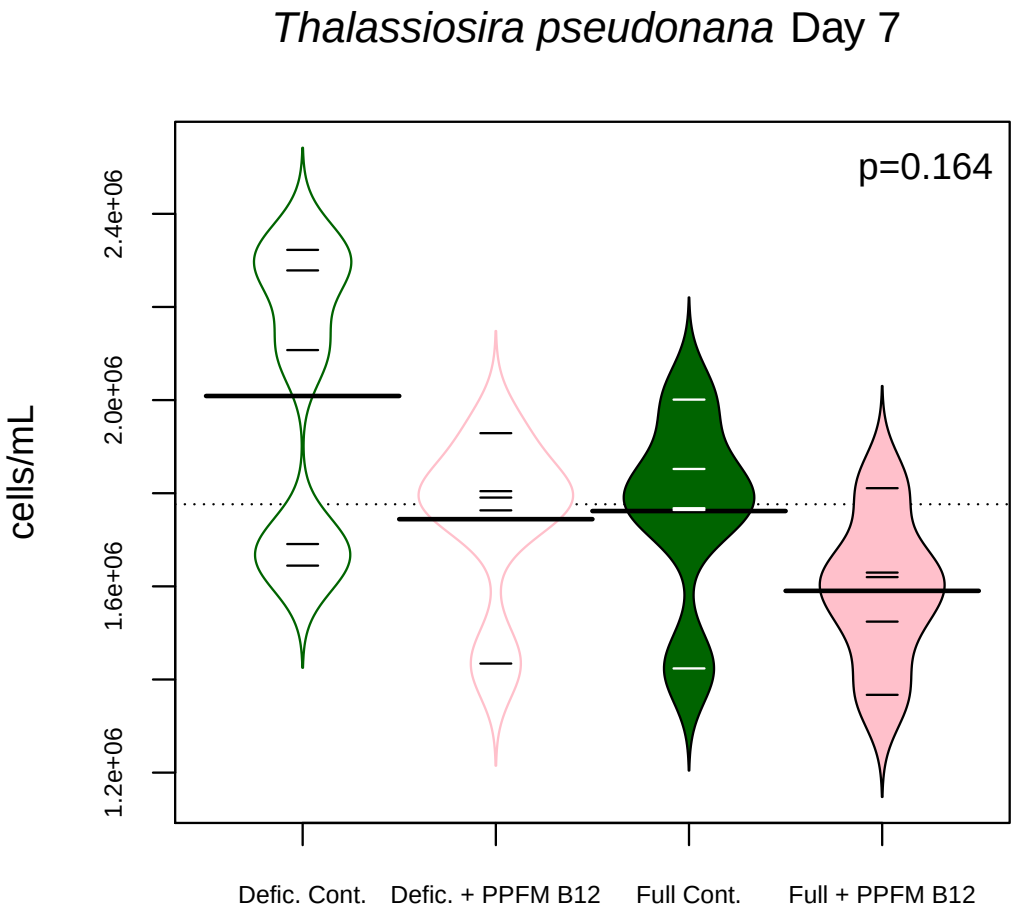

FIGURE 4.15: Beanplot for *Thalassiosira pseudonana*, day seven, showing no significant difference between media and *PPFM B12* treatment groups (n=5, p=0.164 measure with Kruskal-Wallis test). Horizontal dashes represent individual data points, thick horizontal line represents group mean, dotted horizontal line represents overall mean, and 'bean' shape represents data distribution.

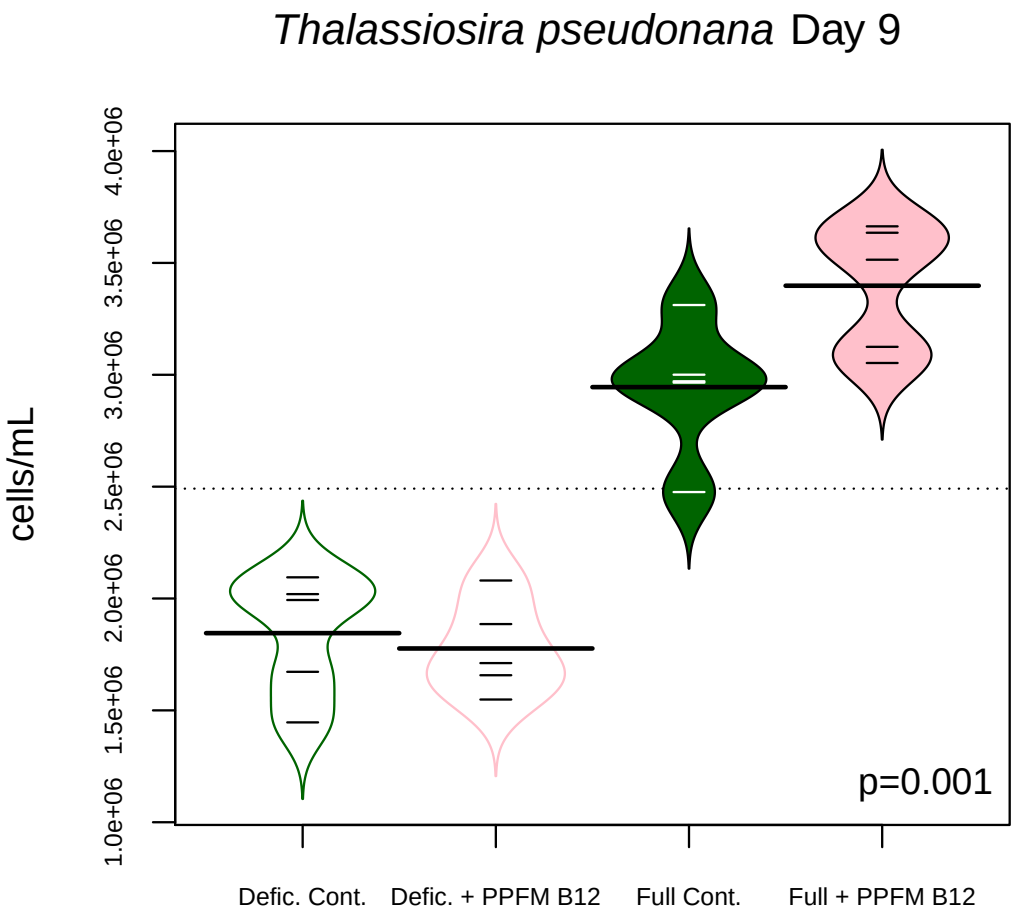

FIGURE 4.16: Beanplot for *Thalassiosira pseudonana*, day 9, showing a significant difference between treatment groups (n=5, p=0.001 measured with Kruskal-Wallis test). Horizontal dashes represent individual data points, thick horizontal line represents group mean, dotted horizontal line represents overall mean, and 'bean' shape represents data distribution.

4.2 PPFM DNA Isolation and Identification

Strains of native PPFM bacteria were isolated by streaking algal stock samples on plates of AMS media with 0.5% MeOH. Cultures were incubated at room temperature on the lab bench until single pink colonies could be sampled and isolated as described in Chapter 3.12. DNA was extracted from PPFM colony samples and amplified with PCR according to the methods detailed in Chapter 3.13 and 3.14. Strains *PPFM B12* and *PPFM 'Broccoli'* were included, and all samples underwent 16s rDNA sequencing. BLAST analysis returned no results for some samples. For samples that returned results, the following PPFM strains were identified:

- *PPFM HPL2*; Family *Methylobacteriaceae*
- *PPFM HPL4*; Family *Methylobacteriaceae*
- *PPFM B12*; Family *Methylobacteriaceae*

4.3 Vitamin B12 Assay

Levels of vitamin B12 were measured in algal, PPFM, and media samples using a competitive ELISA assay test kit as described in Chapter 3.16. Algal samples were sourced from aliquots of *Tetraselmis chuii* and Mixed Algal Cultures saved from algal growth experiments. Sub-samples were taken from each aliquot, with one sample undergoing homogenization followed by centrifugation, while the other underwent only centrifugation, as to test for differences in vitamin B12 concentration contained in algal cells versus only in the media. Vitamin B12 concentrations that returned negative values were adjusted to zero. No significance was seen in vitamin B12 concentrations among algal samples (Table 4.1).

Samples of *PPFM B12* and *PPFM Broccoli* were tested with the same method as algal samples using single sub-samples of each in order to verify the presence of vitamin B12 in PPFM cultures. A sample of F/2 media was also tested to verify the presence of vitamin B12 in the standard media concentration. All three types of samples returned positive values for vitamin B12 concentration (Figure 4.2). *PPFM Broccoli* samples measured higher concentrations of vitamin B12 than *PPFM B12*. These PPFM cultures were not normalized for age or cell density, which may explain the increased B12 concentration of *PPFM Broccoli* over *PPFM B12*. This test was designed only for the detection of vitamin B12 among PPFM and media samples, not to determine differences in B12 levels between bacterial cultures.

4.4 Algal Nutritional Analysis

Algal growth curve results and standard industrial practices suggested a mixed culture of four algal aquaculture species to be most suitable for preliminary nutritional analysis [34]. A mixed algal culture was used, with equal volumes of each algal species inoculum used in experimental and control samples. Dry biomass from mixed algal cultures grown with and without *PPFM B12* at a 5:1 ratio of PPFM per algal cell (n=1) were analyzed for protein, amino acid, and fatty acid content. Total Protein content was increased by 35% in the experimental algal culture, while Fatty Acid content increased by 115% (Table 4.3). Detailed results for amino acid content and fatty acid content are listed in Table 4.4 and Table 4.5.

TABLE 4.1: Vitamin B12 concentrations measured with competitive ELISA test (ng/mL, n=1). Negative concentration values adjusted to zero.

	SampleID	Material	Processing	Type	Species	Abs450nm	Concentration
1	SI21	Algae	Supernatant	Experimental	Mixed Culture	1.78	0.00
2	SI31	Algae	Supernatant	Experimental	Mixed Culture	1.35	2.75
3	SI61	Algae	Supernatant	Experimental	Mixed Culture	1.74	0.00
4	SH11	Algae	Supernatant	Experimental	Tetraselmis	1.75	0.00
5	SH21	Algae	Supernatant	Experimental	Tetraselmis	1.75	0.00
6	SH31	Algae	Supernatant	Experimental	Tetraselmis	1.70	0.00
7	SI71	Algae	Supernatant	Control	Mixed Culture	1.70	0.00
8	SI81	Algae	Supernatant	Control	Mixed Culture	1.84	0.00
9	SI91	Algae	Supernatant	Control	Mixed Culture	1.79	0.00
10	SH71	Algae	Supernatant	Control	Tetraselmis	1.70	0.00
11	SH91	Algae	Supernatant	Control	Tetraselmis	1.64	0.00
12	SH121	Algae	Supernatant	Control	Tetraselmis	1.75	0.00
13	HI21	Algae	Homogenate	Experimental	Mixed Culture	1.60	0.00
14	HI31	Algae	Homogenate	Experimental	Mixed Culture	1.66	0.00
15	HI61	Algae	Homogenate	Experimental	Mixed Culture	1.56	0.19
16	HH11	Algae	Homogenate	Experimental	Tetraselmis	1.57	0.02
17	HH21	Algae	Homogenate	Experimental	Tetraselmis	1.58	0.00
18	HH31	Algae	Homogenate	Experimental	Tetraselmis	1.50	0.83
19	HI91	Algae	Homogenate	Control	Mixed Culture	1.53	0.47
20	HI71	Algae	Homogenate	Control	Mixed Culture	1.54	0.41
21	HI81	Algae	Homogenate	Control	Mixed Culture	1.58	0.00
22	HH71	Algae	Homogenate	Control	Tetraselmis	1.56	0.21
23	HH91	Algae	Homogenate	Control	Tetraselmis	1.68	0.00
24	HH121	Algae	Homogenate	Control	Tetraselmis	1.56	0.20

TABLE 4.2: Vitamin B12 concentrations measured with competitive ELISA test (ng/mL, n=1).

	Material	Processing	Concentration
1	F/2 media	NA	1.79
2	PPFM B12	Supernatant	39.37
3	PPFM B12	Homogenate	41.19
4	PPFM Broccoli	Supernatant	44.93
5	PPFM Broccoli	Homogenate	45.87

TABLE 4.3: Summary of nutritional analysis results for mixed algal cultures. Increased nutritional qualities are in red. Values represent grams per 100g of sample.

	Compound	Algae Control	Algae + PPFM B12	% Change
1	Crude Fat	9.07	19.58	**115.88**
2	Crude Protein	21.80	29.46	**35.14**

TABLE 4.4: Amino Acid profile for mixed algae culture nutritional analysis. Increased nutritional qualities are in red. Values represent grams per 100g of sample.

	Compound	Algae Control	Algae + PPFM B12	% Change
1	Hydroxylysine	0.15	0.43	**186.67**
2	Histidine	0.33	0.60	**81.82**
3	Methionine	0.39	0.68	**74.36**
4	Lysine	0.79	1.26	**59.49**
5	Arginine	0.93	1.44	**54.84**
6	Tyrosine	0.59	0.89	**50.85**
7	Cysteine	0.20	0.30	**50.00**
8	Leucine	1.53	2.18	**42.48**
9	Proline	0.93	1.30	**39.78**
10	Glycine	1.09	1.52	**39.45**
11	Serine	0.80	1.11	**38.75**
12	Glutamic Acid	1.86	2.50	**34.41**
13	Phenylalanine	1.02	1.37	**34.31**
14	Aspartic Acid	1.79	2.40	**34.08**
15	Isoleucine	0.86	1.15	**33.72**
16	Valine	1.11	1.47	**32.43**
17	Threonine	1.00	1.28	**28.00**
18	Alanine	1.66	2.10	**26.51**
19	Hydroxyproline	0.17	0.21	**23.53**
20	Tryptophan	0.21	0.22	**4.76**

TABLE 4.5: Fatty Acid profile for mixed algae culture nutritional analysis. Increased nutritional qualities are in red. Values represent grams per 100g of sample.

	Compound	Algae Control	Algae + PPFM B12	% Change
1	DHA (22:6n3)	3.19	5.04	**57.99**
2	Linolenic (18:3n3)	5.14	7.61	**48.05**
3	Myristoleic (9c-14:1)	0.23	0.30	**30.43**
4	Myristic (14:0)	7.67	9.47	**23.47**
5	Linoleic (18:2n6)	3.37	4.14	**22.85**
6	Behenoic (22:0)	0.54	0.63	**16.67**
7	10c-17:1	1.53	1.60	**4.58**
8	Oleic (9c-18:1)	19.34	19.15	**-0.98**
9	Lignoceric (24:0)	0.16	0.14	**-12.50**
10	EPA (20:5n3)	4.58	3.93	**-14.19**
11	Stearic (18:0)	0.46	0.39	**-15.22**
12	C15:0	0.42	0.34	**-19.05**
13	C16:0 [Palmitic]	24.19	19.58	**-19.06**
14	Palmitoleic (9c-16:1)	15.25	11.95	**-21.64**
15	Margaric (17:0)	0.33	0.21	**-36.36**

Chapter 5

Discussion

5.1 Altering Algal Growth Rates with PPFM

It was hypothesized that supplementation of microalgal cultures with PPFM bacteria would affect growth rates of algae. Cell density at the seventh day of growth was used as a critical marker to mimic industrial algal harvesting during the peak of the exponential growth phase. Statistically significant results by day seven would suggest potential benefits to industrial aquaculture. Vitamin B12 over-producing PPFM strain *PPFM B12* was chosen for use in algal growth experiments due to its heightened production of vitamin B12, an essential nutrient for many algal species, and due to past success and intellectual property generated with this strain. A 5:1 ratio of PPFM cells per algal cell was used for initial inoculations based on past research that indicated benefits to algal growth at this level (unpublished). Algal species used for growth experiments were selected based upon industry standard aquaculture strains deemed optimal for use in oyster hatcheries. Some of the included species also have potential for use in microalgal biofuels due to their high lipid content. Algae was grown in F/2 media formulated based on industry standards.

The four algal aquaculture species failed to show significant differences in growth rate or cell density by the seventh day of growth when supplemented with *PPFM B12* as compared to control (Chapter 4.1.1). A mixed algal culture containing each of the four species showed a significant difference in growth rate by the seventh day of growth (Figure 4.8), but this effect was not seen in followup experiments, and was not affected by supplementation with greater levels of *PPFM B12* (Figure 4.10). No difference in algal growth was seen in *Isochrysis* cultures supplemented with a wild type strain *PPFM Broccoli* compared to the vitamin B12 over producing strain *PPFM B12* (Figures 4.11).

It was hypothesized that the presence of vitamin B12 in the standard formulation of F/2 algal media could mask the potential benefits of PPFM supplementation on algal growth and cell density. To test this, algal growth experiments were designed to include algal cultures grown in F/2 media prepared without vitamin B12 supplementation (vitamin B12 deficient media). Preliminary growth curves showed a decreased growth rate for *Isochrysis* grown in this deficient media (Figure 4.11). This effect was replicated in subsequent growth curves and partially rescued by supplementation with *PPFM B12*, with a significant difference in cell density by the seventh day of growth (Figures 4.12 and 4.13). However, this effect was not clearly replicated in *Thalassiosira*, a known vitamin B12 auxotroph (Figure 4.14). The difference seen in growth rates of *Isochrysis* cultures supplemented with *PPFM B12* in vitamin B12 deficient media suggests that algae grown in nutrient deficient media may benefit from supplementation with PPFM. This would include situations where algal growth is performed opportunistically; for example, algae grown near power plants for the remediation and recovery of carbon emissions [16]. The growth of algae for biofuel without the use of nutrient supplemented algal media could also benefit. Follow up studies could examine the potential benefit of PPFM supplementation in these cases by using larger sample sizes, different algal species, different

strains of PPFM, and different media formulations. A nutrient deficient media would not normally be chosen for use in commercial algae growing operations, making it unclear if PPFM supplementation would benefit industrial algal applications that make use of media with heavy nutrient supplementation.

5.2 Vitamin B12 Analysis

To determine if vitamin B12 levels in algal cultures were being affected by *PPFM B12* supplementation, and to confirm the production of vitamin B12 by this bacterial strain, concentrations of vitamin B12 (ng/mL) in PPFM and algal culture samples were measured with a competitive ELISA test kit. Samples of *Tetraselmis* and Mixed algal cultures saved from algal growth analysis experiments were tested. In order to determine if vitamin B12 was sequestered inside algal cells or present in media only, one set of sub-samples were homogenized to disrupt cells before centrifugation and supernatant testing, while other sub-samples were centrifuged without homogenization. Results suggested that a greater number of homogenized sub-samples contained detectable concentrations of B12 than non-homogenized samples (Table 4.1). It is important to note that the ELISA test returned negative values for many sub-samples, which were adjusted to a value of zero. Positive concentrations were not adjusted. These results draw into question the consistency of B12 measurements in algal samples.

Concentrations of vitamin B12 were also tested in PPFM and F/2 media samples in order to verify the presence of B12. Single sub-samples of *PPFM B12* and *PPFM Broccoli* were analyzed with and without homogenization. Contrary to the known vitamin B12 over-producing nature of strain *PPFM B12*, slightly greater concentrations of B12 were detected in *PPFM Broccoli* sub-samples. This test did not control for culture age or cell density, and used a single sub-sample per analysis, making it difficult to draw

conclusions about typical B12 concentrations in these strains. These results confirm known association of PPFM strains with vitamin B12 presence. A single sample of F/2 media returned a much lower, but discernible, concentration of vitamin B12 that was within an order of magnitude of the expected value based on media preparation (Figure 4.2).

Inconsistencies among algal growth curve and vitamin B12 analysis results lead to questioning of the activities and presence of supplemented PPFM bacteria post inoculation. While attempts to isolate natural PPFM colonies from algal stock cultures succeeded without issue, attempts to re-isolate supplemented PPFM strains from algal samples after growth analysis were inconsistent and complicated by loss of samples. Taken together, the fate of supplemented PPFM after initial inoculation in algal cultures is unclear and may be an important consideration for interpretation of study results. Importantly, PPFM were easily re-isolated from F/2 media 15 days after inoculation with PPFM alone in the absence of algae. This showed that PPFM could survive for the duration of the growth experiments in F/2 media. The possibility that supplemented *PPFM B12* were not surviving the duration of growth analysis experiments or were not establishing themselves sufficiently among the culture's microfauna when mixed with algal samples was not definitively ruled out.

5.3 Nutritional Analysis

Algal nutritional analysis experiments showed promising results, with an apparent difference in algal lipid and protein content (Table 4.3). Notably, levels of DHA were increased in *PPFM B12* supplemented algal culture compared to control (Table 4.5). This fatty acid is critical for oyster and bivalve growth and development [34], and suggest potential benefit to oyster and bivalve aquaculture. In contrast, levels of EPA, another fatty

acid essential for oyster larval development, decreased in algal culture supplemented with *PPFM B12*, but to a smaller degree than the increase in DHA. It is not clear if benefits from increased DHA levels would counteract potential negative effects from decreased EPA. Overall increases in lipid content of PPFM supplemented algal cultures also suggest more general benefits for microalgal aquaculture and biofuels, which depend on lipids as an energy source either for farmed aquatic organisms or for direct human usage. This study used a small sample size (n=1) due to physical constraints and requirements of processing large volume algal cultures. Follow up studies could determine if the measured changes in nutritional content of algal cultures are reproducible, and test the effects of different levels of PPFM supplementation or PPFM strains.

5.4 Conclusions

Algal growth rates were not consistently improved by supplementation with PPFM bacteria. This may have been influenced by the nutrient content of the algal media used in the study; algae grown in media deficient in vitamin B12 showed a reduced growth rate, which was partially rescued by PPFM supplementation (Figure 4.11, Figure 4.12). An increase in nutritional content of algae grown with PPFM supplementation was shown(Table 4.3). The effects of PPFM supplementation on algae grown in nutrient deficient media and on algal nutritional qualities would be strong candidates for follow up studies. While the purpose of the this study was to test for benefits of PPFM supplementation to microalgal cultivation under industry standard oyster aquaculture conditions, it is possible that algae grown in other conditions may benefit from PPFM supplementation.

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