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Method Assessment for Microalgae Quantification in Wastewater Treatment and Biofuel Production

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Honors Thesis

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INTRODUCTION

Biofuel Production

As the world's population and energy demands increase, new systems and sources for waste treatment and fuel production must be researched and developed. Usage of fossil fuels is ever-increasing, but, by many estimations, the supply has peaked and will begin to fall rapidly. Even if the supply was sustainable into the future, the damaging effect of burning these fuels is not (Pittman et al. 2011). Anthropogenic greenhouse gas emissions are astronomical, and the entire planet is affected. Due to these effects, there is significant interest in "carbon-neutral" energy sources such as biofuels like ethanol or biodiesel, which are usually produced from sugar and corn or oil crops like soybean and sunflowers (Pittman et al. 2011). However these crop-based biofuels are not actually carbon-neutral or sustainable. They consume crops and cropland that could otherwise be used for food (Wurts 2010) and there is no feasible way for them to replace petroleum-based liquid fuels entirely because of the space and resources that would be required (Chisti 2007). So there is growing interest in a biofuel source that can produce a greater quantity of fuel with fewer inputs and on less land. Green microalgae have been proposed as an energy source due to their versatility, sustainable production, and high oil production rates. **Table 1**, a chart from *Conservation Biology*, shows a comparison between traditional biofuel crops and algae in terms of emissions, inputs, products, and land requirements.

Table 1

CROP	USED TO PRODUCE	GREENHOUSE GAS EMISSIONS* Kilograms of carbon dioxide created per mega joule of energy produced	USE OF RESOURCES DURING GROWING, HARVESTING AND REFINING OF FUEL				PERCENT OF EXISTING U.S. CROP LAND NEEDED TO PRODUCE ENOUGH FUEL TO MEET HALF OF U.S. DEMAND
			WATER	FERTILIZER	PESTICIDE	ENERGY	
Corn	Ethanol	81-85	high	high	high	high	157%-262%
Sugar cane	Ethanol	4-12	high	high	med	med	46-57
Switch grass	Ethanol	-24	med-low	low	low	low	60-108
Wood residue	Ethanol, biodiesel	N/A	med	low	low	low	150-250
Soybeans	Biodiesel	49	high	low-med	med	med-low	180-240
Rapeseed, canola	Biodiesel	37	high	med	med	med-low	30
Algae	Biodiesel	-183	med	low	low	high	1-2

Table 1 displays the relative inputs required, pollutant emissions released, ideal land use, and biofuel output of six common oil seed crops and algal biodiesel. Algal fuels are far more sustainable than oil crops due to their negative emissions and exceptionally low land requirement. Source: *Conservation Biology*

Wastewater Treatment

The treatment of wastewater from cities, sometimes including stormwater runoff, is complex and difficult. Municipal wastewater can contain toxic metals, nitrate and phosphate on a scale between 10 and 100 mg/L (Pittman et al. 2011), and high concentrations of organic carbon or biochemical oxygen demand (BOD). It is difficult to predict exactly what constituents will make up the wastewater from one cycle to the next, yet treatment plants are expected to meet water quality regulations regardless. A multistep system has been developed to clean most municipal waste, but it is labor, energy, and input intensive, and still additional steps are required to meet more stringent requirements for nutrient and metal concentrations.

Traditional Wastewater Treatment

Municipal wastewater treatment consists of two to three basic steps: primary, secondary, and sometimes tertiary (World Bank Group 2014). The waste stream is treated to remove suspended solids - physical particles that can settle out in the natural environment; biodegradable organics (BOD) - which can suddenly decrease the available oxygen in the receiving waters; pathogenic bacteria and other organisms; and mineral nutrients - including nitrates and phosphates (World Bank Group 2014).

First, the water is mechanically treated, with chemical additions only to speed up flocculation and removal. Suspended solids are screened or sedimented out. Primary treatment can reduce the BOD of the wastewater by 20-30% (World Bank Group 2014). Second, the water is biologically treated; secondary treatment can include various pond and wetland systems, trickling filters, and any other biological treatments to remove organic matter. Bacteria are added which will consume the dissolved organic matter and convert it into carbon dioxide and water (World Bank Group 2014). Secondary treatment also includes a second settling treatment to remove any residual waste as well as the treatment microbes. A well-run treatment plant with both primary and secondary systems can remove up to 85% of suspended solids and BOD from the waste stream (World Bank Group 2014).

Tertiary treatment includes any additional processes to remove inorganic nutrients or disinfect the water. Disinfection may be achieved by adding chlorine or exposing the stream to UV light. Traditionally, nutrient removal includes chemical precipitation into an activated sludge, which is buried in a landfill or further treated into fertilizer (Pittman et al. 2011). Tertiary treatment can remove up to 99% of all impurities from sewage water,

but it is expensive and often requires technical experience and complicated equipment (World Bank Group 2014).

Algal Wastewater Treatment

This tertiary step is where the most intensive research on algal efficacy has been conducted (Pittman et al. 2011). Algae can be introduced at nearly any point in the treatment process, including before or after primary settling, after or instead of the addition of activated sludge, or as a tertiary step (Wang et al. 2009; Mallick 2002). Algae can be used to break down organic matter, to consume nitrogen and phosphorous nutrients, and to sequester heavy metals such as aluminum, calcium, iron, magnesium, and manganese (Wang et al. 2009). The microalgae of the *Chlorella* and *Scenedesmus* genus have shown to be particularly tolerant to the growing conditions found in wastewater effluent, as well as being adept at sequestering inorganic nutrients and metals (Pittman et al. 2011). Some species have been found to provide 80-100% removal of ammonia, nitrate, and total phosphorus from primary treated wastewater. This removal represents a huge cost savings and increase in sustainability, since the fertilizers that otherwise would have to be mined and shipped are considered pollution in wastewater streams.

Low-cost, environmentally friendly algal wastewater treatment has been studied since the 1950's (Pittman et al. 2011). The primary attraction of algal wastewater treatment lies in the relatively simple and inexpensive technology, as well as in energy savings (Mallick 2002). Algal wastewater treatment plants may not need to expend as much energy aerating their water as their traditional counterparts, for example, because of the photosynthetic oxygen production of the algae (Mallick 2002). In addition,

cultivating algae that can be used for other applications in wastewater treatment facilities could present huge savings on algae production.

Commercial Algae Production

Mass production of algae is achieved through cultivation in two main ways: photobioreactors and open ponds or "raceways" (Wurts 2010). Photobioreactors, more commonly used for small-scale research or demonstrations, are closed systems that pump water and algae continuously through a series of clear tubes, spheres, or plastic bags so that the algae is exposed to the maximum amount of light possible (Wurts 2010). **Figure 1** shows a basic photobioreactor schematic.

Figure 1

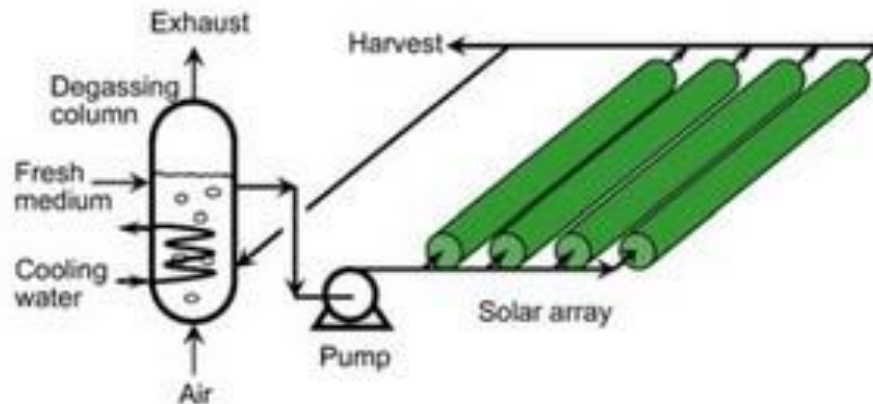


Figure 1 shows the fresh algae medium that will be pumped through the solar array to feed the growing algae. Gases are exchanged and fertilizers are added for the algae, and CO₂ is injected to encourage photosynthesis. Source: wiki.uiowa.edu

Open pond systems produce much larger quantities of algae, but at a slower rate (Lardon et al 2009). The ponds are each one to several hectares in size and farms require a large area of flat land - around 100 hectares - in a suitable climate for cultivation, where the temperature and sun are ideal for the highly eutrophic ponds (Lardon et al. 2009; Wurts 2010). A paddlewheel maintains ~25 cm/s movement in the ponds and pipes along the sides inject carbon dioxide into the water for the algae to use in photosynthesis (Lardon et al. 2009). Some open-pond production farms have found sources of wasted carbon dioxide to feed into these systems, such as that released from the flues of coal-fired power plants (Wurts 2010). Researchers at the University of Kentucky have estimated that over 2000 hectares of algae ponds would be needed to consume the carbon dioxide wasted by a single 500 MW power plant (Wurts 2010). Such sources of free recycled nutrients and other inputs may be crucial to making commercial algal production economically sustainable as it breaks into the market.

As of 2009, it was estimated that the cost of algal oil was 20 to 30 times higher than that of vegetable oil (Wurts 2010). University of Kentucky researchers projected the price of algal biodiesel to be as much as \$30/gallon. These exceptionally high pricetags are due to the high costs of resources and infrastructure required for large-scale algae production.

Specifically, critics cite the use of fossil fuels in the production process, the construction of the facilities, and in the harvesting and processing of the algae into biofuels (Pittman et al. 2011). Harvesting and processing the biofuel-bound algae do present some difficult engineering tasks. The algae that are most commonly studied for biofuel production are microalgae; they are difficult to harvest from open ponds due to their

small diameter and dispersal throughout the water (Lardon et al. 2009). The biomass can be harvested by centrifuging huge quantities of algae-water, but this is an expensive and demanding task that most plants cannot manage sustainably. Another option is to alter the pH of the water, causing the algae to flocculate naturally: up to 90% of the algal biomass can be collected by bringing the pH of the water up to 11 (Lardon et al. 2009). However, this requires chemical additives that can negatively affect the quality of the fuel produced (Wurts 2010). An efficient, low-cost harvesting method is sorely lacking, and multiple studies are underway to design one. Algae production has become an important research point as scientists recognize algae's versatility and potential, as well as the shortcomings of current methods and materials.

Traditional Biofuel Sources

Biofuel includes a few variations of renewable fuel such as ethanol, biodiesel, and combustible biomass. Biodiesel is often produced using oily or fibrous crops such as soybean, sunflower, rapeseed, corn, or switchgrass (Pimentel and Patzek 2005). However, in their 2005 comparison study, David Pimentel and Tad Patzek of Cornell and Berkeley Universities, respectively, found that every one of these fuel sources produce significantly less energy in biofuel than their production consumes in fossil fuels (Pimentel and Patzek 2005). At the low end, corn and soybean bioethanol only produce about 30% less energy than they consume to produce. At the high end, production using sunflower requires 118% more energy in fossil fuels than it can yield in ethanol (Pimentel and Patzek 2005). In addition to this excessive fossil fuel use, these sources all take up valuable agricultural land and impact the available food supply.

The widespread use of traditional biofuel sources - including transportational fuels like ethanol - is maintained by subsidies from the government and established infrastructure. Algae, or another source, may produce energy more efficiently, but do not benefit from the previously established distribution system that carries other biofuels (Lardon et al. 2009). Additionally, car engines and energy plants are already built to burn ethanol, biodiesel, and other traditional biofuels, and switching over to algal biofuel may require costly adaptations of those systems (Lardon et al. 2009).

Algae as Biofuel Source

If the fuel systems and algal biofuel can be adapted to work together, algae offers several advantages over oil crops for biofuel production (Wurts 2010). Algae grow and reproduce much faster than terrestrial plants. It is common for algae to double their biomass within 24 hours; some, during the maximum growth period, have shown consistent doubling times as low as 3.5 hours (Chisti 2007). Microalgae can establish a flourishing population in a matter of days, where the time frame for terrestrial plant growth is on the scale of months or even years (Wurts 2010). Also, algal production can be done in areas that are not conducive to crop growth, thereby preserving arable land for growing human and livestock food. Some researchers have even found that photobioreactors can be built near the coasts and use saline water, fortified with nutrients, for production (Chisti 2007). If the algae are produced and harvested in a low-input, high- efficiency system, the feasibility and sustainability of the algal biofuel easily surpasses that of the terrestrial crops.

Algal biofuel is most commonly produced through transesterification of lipids that are produced within the algal cells. The neutral lipids are isolated from the algae and converted into biodiesel (Pittman et al. 2011) that can be used for transportation fuel and industrial machines.

The US Department of Energy Aquatic Species Program has done extensive research on the oil production capabilities of microalgae, and have found them to be significantly greater than oilseed crops (Pittman et al. 2011). Of course, the algal species that is cultivated and the conditions under which it is grown are both major factors in potential lipid and biodiesel production. The microalgal genus that is commonly studied for this application, *Chlorella*, have been shown to produce much higher levels of lipids when exposed to nitrogen starvation, but this condition necessarily decreases the total biomass production (Lardon et al. 2009). Normal fertilization levels cause the alga to produce a lower ratio of lipids to biomass, which leads to a greater total lipid production through greater biomass production and more versatility in the final product because there is more algae to convert into fuels (Lardon et al. 2009). This study highlights the need to find a sustainable and inexpensive source of fertilizer for biofuel algae, since the nitrogen and phosphorus inputs required are expensive.

Combining Wastewater Treatment and Fuel Production

The ideal algal species for wastewater treatment would grow well under a variety of physical and chemical conditions because many treatment systems operate outdoors and treat a range of waste inputs, so they are subject to seasonal and compositional variation. Treatment systems are also subject to contamination by bacteria, zooplankton, and other algal species (Pittman et al. 2011), so the algae must be able to maintain

production under these conditions. The algal species must also grow relatively well regardless of variations in temperature, light level, nutrient concentration, pH, and water quality (Wurts 2010). The ideal species for biofuel production would produce a high level of neutral lipids, such as triacylglycerol (Pittman et al. 2011), compared to the quantity of biomass. Algae also produce proteins and carbohydrates, which can be used for fuel production; however, lipids have been shown to have over twice the caloric content of either proteins or carbohydrates (Lardon et al. 2009), so they are the most likely to be used as a viable energy source. Other important factors for both of these applications include maximum biomass production in a short time span, good performance in monoculture with a resistance to contamination, and ease of harvest (Wurts 2010).

One of the most commonly studied strains for meeting nearly all of these criteria is *Chlorella sp.* *Chlorella* is a unicellular, green microalgae which is easily cultured in wastewater conditions, produces a high lipid-to-biomass ratio, and has been shown to sequester high amounts of nutrients and metals from wastewater (Pittman et al. 2011). *C. vulgaris* has been shown to remove up to 90% of nitrogen and 80% of total phosphorus from primary settled wastewater (Pittman et al. 2011). It is also tolerant of a variety of wastewater conditions, resistant to contamination by undesirable organisms, and well-adapted to growing in monoculture. *Chlorella* is difficult to harvest or quantify, however, due to its small size and dispersal in the water.

OBJECTIVE

This study seeks to compare existing methods for quantifying growth and lipid production in terms of data accuracy and reliability, required sample volume, required volume of hazardous chemicals, and processing time; with the ultimate goal of producing a reliable method that is cleaner and less labor intensive.

MATERIALS AND METHODS

This objective was to be completed by comparing the three traditional quantification methods (microscope cell count, lipid separation, and chlorophyll extraction) to two modern methods (the Coulter Counter and the microplate reader) using a microalga that could be adapted to either algae application. *C. vulgaris* was chosen as the 'ideal' species based on its potential as a wastewater treatment or biofuel production cornerstone.

Algal Cultures for Comparison

C. vulgaris on agar slants was purchased from UTEX algae center at the University of Texas at Austin (Austin, Texas) and maintained in Bristol media (NaNO₃ (25 mg/L), CaCl₂·H₂O (2.5 mg/L), MgSO₄·7H₂O (7.5 mg/L), K₂HPO₄ (7.5 mg/L), KH₂PO₄ (17.5 mg/L), NaCl (2.5 mg/L), and peptone (1 g/L)) at 26°C for inoculation. Liquid cultures of *C. vulgaris* were periodically isolated on agar plates prepared in the same media to ensure the purity of the strain. For this method comparison, the algae were cultivated in jars plugged with autoclaved cotton balls, covered with autoclaved

aluminum foil, and placed on a stir plate in a 26-27 °C warm room lit continuously by fluorescent ceiling lights and three T5 high-output aquarium lights. The algae were grown for roughly ten days to a sufficient density for analysis.

Samples of the algal solution were diluted according to the amounts in **Table 2** and duplicates of each sample were made for comparison among the three traditional and two modern quantification methods.

Table 2

Dilution Factor	Algae/Bristol Stock (mL)	Pure Bristol (mL)
0	330	0
1	165	165
5	66	264
10	33	297
50	6.6	323.4
100	3.3	326.74

Table 2 displays the six dilutions made of the algae stock solution. The data shown in the results section are a result of these six dilutions.

Biomass or Cell Count

Traditional Microscope Method

The Standard Method for microscopy cell counts (USEPA 160.2, SM 2540-D) was used as the traditional biomass quantification method. For the three densest algal sample solutions, one milliliter of solution was vacuum filtered, and for the three less dense samples, two milliliters were filtered. The filters were mounted on glass slides and five pictures were taken at random points on the slide at 100x magnification. The cells in each picture were counted by hand and the total number of cells per mL was calculated for each sample.

Modern Coulter Counter Method

The solution chosen for our objective was to replace microscopy with cell counts by the Coulter counter machine. The Coulter counter works on the Coulter principal, by measuring the number and duration of electrical impulses formed when particles are pulled through an aperture alongside an electric beam. For this process only 100 μL of the algal sample was required, and it was mixed with 9.9 mL isotone. The Counter drew the sample through a 100 μm aperture and tracked the amount and length of electrical disturbances which are counted as particles or cells. Based on the average size of *C. vulgaris* cells, particles from 2.2 - 7 μm were considered to be algae cells.

Chlorophyll Production

Traditional Spectrometry Method

The traditional method chosen for chlorophyll a quantification was standard method (SM 10200-H ESS Method 150.1 Spectrophotometric). Chlorophyll is an important constituent that can be used for algae quantification and that would be vital to algal wastewater treatment. For this method, 50 mL of the algae sample was vacuum filtered through a 0.45 μm membrane filter, along with magnesium carbonate as a preservative. The filters were frozen overnight, then dissolved in 90% acetone solution; the chlorophyll was mechanically separated from the cells by sonication, then the samples were kept in the dark overnight at 4°C. The chlorophyll was then brought back into solution by vortexing and analyzed via spectrophotometer. The absorbance at wavelengths of 750, 663, 645, and 630 μm was then used to calculate the amount of algae per mL of sample.

ModernMicroplateMethod

The microplate reader for chlorophyll analysis (Synergy H1 Multi-Mode Microplate Reader, Biotek Instruments, Inc., Winooski, VT) method requires only 100 μm of sample in a clear-bottomed microtiter plate (Corning 3603, Corning, Tewksbury, MA). The samples were analyzed using excitation at 440 nm and emission at 685 nm, and chlorophyll concentration was calculated from fluorescence (Held 2011).

Lipid Production

TraditionalBligh/MutjabaMethod

The traditional method chosen for the quantification of lipids was a modified Bligh/Dyer method (1959) proposed by Mutjaba (2012) which included extracting the lipids, separating them from the cell mass, and then drying and weighing the lipids. This method required 50 mL of sample to be centrifuged and separated by pipette. A chloroform/methanol solution was used to extract the remaining lipids, and the sample was centrifuged again. The lipids were separated by pipette, and the extraction solution was added a second time, followed by another round of centrifugation to achieve separation, and the lipids were collected on tins to be dried in an oven overnight and then weighed.

ModernMicroplateMethod

The method chosen to replace the lipid separation method was a microplate fluorescence analysis which utilized only 100 μL of sample and 100 μL of 2x Nile Red

dye. The absorbance of each sample was measured at 600 nm and 680 nm, then the Nile Red was added and allowed to absorb for ten minutes. Then fluorescence was measured at 530 nm excitation and 570 nm emission wavelengths, and these data could be compared to a standard curve to calculate total lipid concentration in the sample.

RESULTS

It was the objective of this research to identify and explore a method of quantifying microalgae that is less time-consuming, less labor-intensive, more intuitive, more accurate, more repeatable, and more sustainable than traditional quantification methods and compare it to traditional methods using a representative green microalga.

Table 3

Parameters	Method/Instrument	Volume Req. (mL)	Time Req.	Chemicals Req.
Chlorophyll A	SM 10200-H, ESS 150.1 / Spectrophotometer	50	2 days	13 mL 90% Acetone
Chlorophyll	Held / Microplate	0.1*	1 minute	None
Cell Count	Coulter Counter	0.1	5 min/sample	9.9 mL Isotone
Cell Count	Microscope Method	1 to 2	3 hours	None
Lipids	Modified Bligh-Mutjaba	50	2 days	2.5 mL Chloroform, 3 mL Methanol
Lipids	Held/Microplate	0.1*	12 minutes	100 µL Nile Red Dye
* These variables are measured using the same 0.1 mL of sample.				

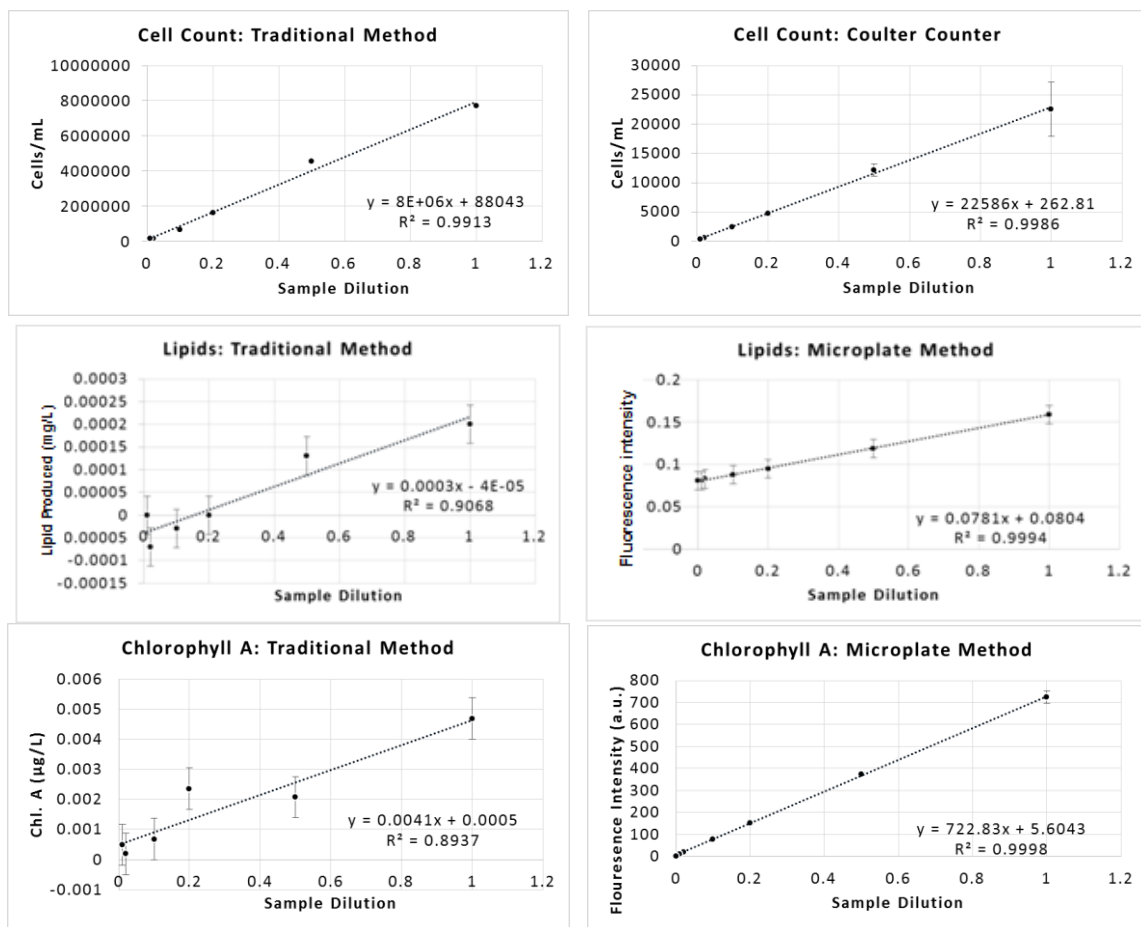
Table 3 shows the time and inputs required for each of the six methods compared.

The relative time, chemical input, and sample volume required for each of the six methods are shown in **Table 3**. The modern microplate and Coulter Counter methods require less time and effort; they require a smaller sample volume; and the microplate treatments require far less harmful chemicals than traditional methods.

Clearly the modern methods are more researcher- and environmentally- friendly than the traditional methods. They may also be more accurate.

Figures 2 - 4 show the r^2 values and standard errors of each of the treatments; the results of traditional methods on the left, and the modern methods on the right. The values displayed by the traditional methods were surprisingly accurate, although their error margins were slightly larger. However, the r^2 value for each of the modern treatments is noticeably higher than for the traditional methods. This indicates more precise and repeatable results.

Figures 2 - 4



Figures 2 - 4 show a graphical view of preliminary results gained from each of the quantification methods at different sample dilutions. Note that these graphs are not for direct comparison of cell or constituent count, but for a comparison of the accuracy and error of each of the treatments.

DISCUSSION

This research achieved the objective of designing improved quantification methods for green microalgae for use in biofuel production and wastewater treatment. These modern methods using Coulter Counter for cell counts and microplate for chlorophyll and lipid measurement are more reliable and efficient than microscope cell counts and lipid extraction and chlorophyll absorbance measurements. It is hoped that research in the fields of biofuels and water quality will benefit by becoming more sustainable and streamlined.

Cell Count

The microscope cell count method requires relatively little volume of the algae sample, but it is cumbersome and time consuming, and prone to human error. *C. vulgaris* are mostly uniform spherical cells, but after the stress of filtration and slide-mounting, the cells break apart or clump together. In dense samples, the cells can be too thick to count. They can be deceptively spread or clumped on the slides, as well as misshapen or torn. The main benefit to the traditional microscope method is that it can be used to quantify a dilute sample of essentially any microbe. However, the procedure takes a few minutes to complete per sample and is labor-intensive; when available, a simpler procedure would be of benefit to researchers on large-scale projects.

The Coulter counter represents an improvement in sample size, accuracy, and time required over the traditional cell count method. Each sample takes only seconds to process, and discrepancies in cell sizes are mitigated by the Counter's method. Plus the Counter provides a quick way to check monoculture samples for contamination, because cells of other sizes appear as spikes on the cell count graph.

Chlorophyll Production

The traditional chlorophyll spectrometry method uses a considerable volume of sample and volatile chemical inputs. It also becomes a three-day process and requires several steps to move from samples to analysis. This process would be useful for nearly any green monoculture, but not for mixed species. A quicker alternative method, with fewer steps and chemical inputs, would make research on green algae more efficient and sustainable.

The microplate method measures chlorophyll content in a matter of seconds, even for several dozen samples. It requires no additional chemical inputs and no additional machinery or technology beyond the microplate analyzer. In addition, the sample that is analyzed this way is not compromised, so it can be used to measure other parameters as well, such as lipid production.

Lipid Production

The traditional lipid separation method was convoluted, frustrating, and difficult to reproduce with precision due to how error-prone it is. The results are inconclusive and inaccurate, and the process takes many hours in addition to an overnight drying period. Multiple inputs of volatile chemicals are required and several pieces of equipment are needed to complete the process.

The microplate reader is very fast and simple to use. It eliminates many of the chemical inputs required for traditional algae quantification methods, and requires a much smaller sample which can be used for multiple analyses. In addition to chlorophyll and lipid content, the microplate reader may be used for a variety of biomass, nutrient, and other quantifications or analyses.

These modern methods are more user-friendly, less time-consuming, and more precise than their traditional counterparts. Because they generally require fewer chemical inputs, they are also more environmentally friendly.

Taken separately, it would appear that algae could be used successfully for either wastewater treatment or biofuel production. However, vast amounts of fertilizer, fresh water, and fossil fuels could be saved by combining both of these applications. Algae grown in a wastewater treatment facility can sustainably clean nutrients, organic carbon, and toxic metals out of the water and then can be harvested and converted to biomass, biodiesel, or other renewable biofuels (Pittman et al. 2011). Additionally, replacing oilseed crops such as corn or soybean with algae for biodiesel production will lessen the negative impact that the use of these crops for biofuel production has on the food supply and agricultural land availability (Wurts 2010).

The methods explored by this research are optimal for a monoculture of unicellular microalgae, such as *Chlorella vulgaris*, because they are easily suspended and relatively uniform in shape. This species was chosen as the test subject for this research because it has been thoroughly researched in the past and found to be an optimal choice for both wastewater and biodiesel applications. Other species have been found to be prime candidates for these two applications as well. Some future research may include testing the modern Coulter Counter cell counting and microplate chlorophyll and lipid fluorescence methods on filamentous or dimorphous algae, as well as comparing to a new method for a combination of species.

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