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EVALUATION OF ALGAE CONCENTRATION IN MANURE BASED MEDIA

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EVALUATION OF ALGAE CONCENTRATION IN MANURE BASED MEDIA

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Engineering
at the University of Kentucky

By
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Lexington, Kentucky

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ABSTRACT OF DISSERTATION

EVALUATION OF ALGAE CONCENTRATION IN MANURE BASED MEDIA

Algae can be used to treat wastewater and manure while producing a feedstock for renewable energy. Algae require nutrients to achieve their maximum growth and manure could provide those nutrients, thereby reducing the cost of algae production and the impact of manure treatment. Algae concentration during cultivation is a critical variable that is difficult to measure due to the high concentration of suspended solids present in manure. This dissertation addresses methods to measure algae concentration in the presence of manure solids.

Quantifying the algae concentration gravimetrically or by optical density was unreliable due to manure solids interfering with the measurement. Cell counting to determine algae concentration was accurate but time consuming, subjective, required dilution of concentrated samples and only small sample volumes could be measured. Chlorophyll extraction was a consistent method to determine algae concentration in manure based media, but the model had to be adjusted to account for solids interference. The proposed equation predicted chlorophyll concentration from *Chlorella vulgaris* in dairy manure better than the reference equation. Different algae strains (*Chlorella vulgaris*, *Cylindrocystis* sp, and *Scenedesmus* sp.) and manure sources (dairy, beef, swine, and sheep) were used to validate the proposed equation and all combinations had a linear relationship between actual and predicted chlorophyll concentration, but not all comparisons followed a 1:1 reference line. Even with chlorophyll extraction the manure solids interfered with the chlorophyll measurement and calibrations had to be developed based on manure type.

A method based on spectral deconvolution was used to quantify algae concentration in the presence of manure without chlorophyll extraction. Various manure-algae mixtures were scanned with a spectrophotometer. Algae concentration was accurately determined with the four manure sources. Measuring algae concentration required absorbance spectra from 600 to 700 nm and manure solids concentration between 280 and 350 nm. Spectral deconvolution was able to differentiate algae

concentration and manure solids concentration with a Pearson coefficient of 95.3% and 99.8% respectively. This method proved to be an accurate and efficient method for estimating algae and manure solids content in unprocessed samples. A critical factor was utilizing appropriate reference spectra.

KEYWORDS: Chlorophyll, spectral deconvolution, absorbance, nutrients.

Student's Signature

Date

EVALUATION OF ALGAE CONCENTRATION IN MANURE BASED MEDIA

By

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Director of Dissertation

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CHAPTER 1: INTRODUCTION

The unsustainability of using fossil fuels as a primary source of energy, compounded by its resulting environmental issues, demands a sustainable model of energy supply based on renewable resources such as sun, wind, water and crops. Numerous research projects have been conducted that focused on crops, which contain a high concentration of lipids or sugars that can be converted in to biofuel. The main cost of producing biofuels from crops is related to the raw material cost and competition with land used for cropland, pastureland, and forestland. Biofuels can also be produced from residues or waste products that may be acquired with zero or negative costs reducing the final product expense.

Utilizing residues is one method to reduce environmental impacts, recycle water and nutrients, and minimize the volume of residue being transported and treated. In terms of economics, it is very useful extracting valuable products from residues. This includes products, such as biogas, compost, ethanol, or even as a nutrient source for growing products like algae. Algae have a high oil concentration that could be converted into biodiesel. When compared to other oil crops, such as canola, palm, or soybeans, algae appear to be more productive, because their composition can achieve 80% oil on a dry weight basis; they grow very rapidly and require minimal resources (Sialve et al., 2009).

The oil from algae can be utilized after solvent or mechanical extraction and transesterified into biodiesel. Other routes to process algae include pyrolysis and hydrothermal liquefaction with further upgrading of the products. Residue from the conversion process (or unprocessed algae) can be digested biochemically using an anaerobic digester, producing energy to supply the process and carbon dioxide (CO_2) to saturate the algae growth media. Nutrient requirements for producing algae are a significant burden on the sustainability and cost of algae based biofuels. Manure could be used to supply nutrients to produce algae, which can be converted into biodiesel or other fuel sources (Wilkie and Mulbry, 2002).

Utilizing renewable energy is also motivated by a desire to reduce emissions of greenhouse gases that are believed to be responsible for climate change. The greenhouse

effect occurs because of the increase in greenhouse gases in the atmosphere absorbing thermal radiation (Houghton, 2005). Compounds that lead to the Greenhouse Gas phenomenon include water vapor, carbon dioxide, methane, nitrous oxide, ozone and chlorofluorocarbons (CFCs).The burning of fossil fuels alters the natural cycle of carbon, because fixed carbon is being burned and emitted into the atmosphere. According to Madigan et al. (2006), CO_2 levels during the past 40 years have increased by nearly 15% that has in large part triggered a period of steadily increasing global temperatures. Developing algae production systems would help reduce Greenhouse Gas emissions by capturing CO_2 and producing replacements for fossil fuels would be advantageous. If animal manure could be utilized for cultivation of algae, additional energy and environmental benefits would be possible.

Nitrogen, phosphorus, and potassium are major nutrients that are required by all plant life. Nitrogen is manufactured using the Haber-Bosch process to manufacture synthetic ammonia from natural gas. This process is energy intensive (52 MJ kg^{-1} N) and releases a large amount of global warming gases to the atmosphere in the form of carbon dioxide and nitrous oxide (Farrell et al., 2006). The CO_2 equivalent (CO_{2e}) Greenhouse Gas Emissions are 7.02 kg CO_2e kg^{-1} N, based on a 100 year global warming potential of 298 for N_2O (IPCC, 2006). Phosphorous and potassium also require a large quantity of energy and release greenhouse gasses during manufacture and use, although only about one sixth of the impact of nitrogen fertilizer (Farrell et al., 2006). Using waste products such as animal manure could improve the energy and environmental benefits of algae production (Mulbry et al., 2008)

1.1 **The Algae Group**

Algae are one of the major groups of microbial eukaryotes called Protists. Algae contain chloroplasts, which are organelles used by phototrophic organisms to conduct photosynthesis and obtain energy from light. Algae are also autotrophic organisms, which use water as an electron donor to reduce CO_2 into organic matter, fixing carbon in their biomass. Photoautotrophic organisms are the major organic matter producers in nature because they use energy from light and carbon from the atmosphere to produce biomass

and emit oxygen for aerobic organisms. Phototrophic organisms conduct photosynthesis during the day and respiration during the night (Chisti, 2007).

Algae require water, CO_2, light (primarily photosynthetically active radiation between the wavelengths of 400 to 700 nm), nitrogen, phosphorus, potassium, and relatively few additional minerals. Algae can be found in soil and aquatic habitats over a broad range of salinities, temperatures, and pH ranges. Phytoplankton species of algae live suspended freely in water, in contrast with benthic species of algae that live attached to surfaces within water (Madigan et al., 2006). Algae composition varies depending on the environment and species. However, an average composition was assumed by Neennan et al. (1986) to be 30% lipid, 20% carbohydrate and 10% metabolic intermediates, with an ash content of 8% and nitrogen content of 32%.

1.2 **Algae Production**

Algae grow naturally in a wide range of environments. Typical requirements for phototrophic algae include sunlight, CO_2, temperatures between 20 and 30°C, water and nutrients (primarily N, P, and K). Various algae species can be found growing in lakes, oceans, rocks and soil.

Algae have been grown on an industrial scale for different purposes such as treatment of organic residues, nutrient recovery for animal feed and fertilizer, human food, and production of biofuels. In industrial algae production, the ideal conditions may be provided, such as artificial light with the appropriate photoperiod and wavelength, consistent CO_2 supply, optimal temperature and essential nutrients like nitrogen (N) and phosphorous (P). Providing optimal conditions improves the algae growth rate and potentially improves the composition (oil, starch, protein) of the algae, although it increases the costs of the production.

Depending on the region's weather, algae can be produced in an open or closed system. Open systems usually are low-cost, but also lower productivity than closed systems. In open systems, there is free exchange to the environment, resulting in faster water evaporation and less efficient temperature, nutrient and pH control. Open systems

are cheaper to build and to maintain; they use natural light and temperature, and the media can be enriched with nutrients, although a portion of them may be lost to the atmosphere.

On the other hand with closed systems there is no free exchange between the media and the atmosphere. This allows for better environmental control including temperature, pH, and nutrient control. Closed systems are more expensive; require additional infrastructure and higher capital and operating costs to maintain. Inside a photo bioreactor, a portion of the CO_2 used to saturate the growth media does not become available for algae fixation. According to Doucha et al (2005), algae used about 38.7% of the CO_2 supplied and generated 1 kg of algae biomass per 1.74 kg of CO_2. Measuring the algae concentration and growth rate during cultivation are critical parameters for evaluating the feasibility of algae production. Algae require nutrients similar to land based crops that could be supplied by animal manure. Utilizing manure for algae production would reduce the environmental impact of land applied animal manure. However, organic solids from manure could interfere with the measurement of algae concentration. This dissertation addresses methods to improve the accuracy of measuring algae concentration in the presence of manure solids.

CHAPTER 2: PROJECT OBJECTIVES

The overall goal of this research was to develop a method to evaluate algae concentration in the presence of manure solids.

Specific project objectives were:

1. Evaluation of current algae concentration measurements with suspended solids.
2. Modification of equations for predicting chlorophyll concentration in the presence of manure solids.
3. Determination of the influence of algae species and manure types on the estimation of chlorophyll concentration.
4. Develop models to estimate algae concentration in samples containing raw manure using spectral deconvolution.

CHAPTER 3: LITERATURE REVIEW

3.1 Manure Characteristics

3.1.1 Ruminant Animals

Animal manure is the residue of animal digestion, containing various nutrients, organic residues, water, and numerous other compounds. Dairy, beef, and sheep are mammals and in addition are all classified as ruminant animals. Ruminants are herbivorous that possess a digestive organ called rumen in which cellulose and other polysaccharides are digested by microorganisms. Because the rumen is anoxic, anaerobic bacteria naturally dominate. The microbial fermentation of sugars released from these polysaccharides produce fatty acids that feed the ruminants. The microorganisms present in the rumen hydrolyze cellulose to free glucose, which is fermented into volatile fatty acids, CO_2 and CH_4. Ruminants are nutritionally superior to non-ruminants because this microbial protein is recovered and used by the animal (Madigan et al., 2006).

Dairy manure is one of the primary sources of manure that could be used as a nutrient source for algae production. Dairy cattle are frequently kept in confined operations that allow for easy manure collection and dairy facilities have a large quantity of wastewater from cleaning that needs to be disposed of. Sheep production is relatively small and a large percentage of beef cows are in a pasture based system that makes manure collection difficult.

According to Wen (2004), the composition of the raw dairy manure was 14.6% dry matter, in which 50.51% was carbon and 3.03% was nitrogen. These values were different compared to Hall et al. (1985), who found a dry matter content of approximately 26%. Manure composition values vary widely and depend on the bedding material, local weather conditions, feed rations, and the management of barns.

3.1.2 Dairy Manure Treatment Options

Management and treatment options for manure are needed in order to control odors and environmental pollution (Wilkie, 2005). The microbial decomposition of organic matter produces simpler compounds, recycles nutrients, and reduces the

pathogens present in the residue. A number of options are available to convert manure into energy, but a variety of factors limit the widespread conversion. Dairy cattle manure has a large amount of fat, from waste milk, which interferes negatively in anaerobic digestion. The presence of fat causes sludge flotation, formation of fat scum layers at the surface of the reactor, which do not digest and affect the anaerobic digestion process (Masse et al., 2001). Lignin will not degrade during anaerobic digestion. Since a substantial portion of the volatile solids in dairy waste are lignin, the percentage of volatile solids in cow manure that can be converted to gas is lower when compared to other manure and wastes (Burke, 2001).

The waste characteristics can be altered by simple dilution. Water will reduce the concentration of certain constituents such as nitrogen and sulfur that produce products (ammonia and hydrogen sulfide) that are inhibitory to the anaerobic digestion process. Dilution causes stratification within the digester. It is desirable to keep the separation or stratification in the digester to a minimum. Intense mixing, which requires electric power may reduce the stratification of dilute waste (Burke, 2001).

The biogas produced during anaerobic digestion is composed primarily of carbon dioxide and methane and is a renewable source of energy. It can be burned directly in heater and boilers, or used to generate electricity. If released to the atmosphere, both CO_2 and CH_4 are greenhouse gases. Anaerobic digestion has been proposed for converting algae to energy and could be coupled with livestock farms in the future (Vergara-Fernández et al., 2008; Nielsen and Heiske, 2011; Zamalloa et al., 2011).

An alternative manure treatment option is to cultivate algae to recover nutrients, produce protein, and vegetable oils. The use of algae for wastewater treatment is not recent, although there has been considerable recent interest (Oron et al., 1979; Brune et al., 2009; Sturm et al, 2012). Neennan et al., in 1986, pointed out the advantages of growing algae, which can grow even in saline water otherwise unsuitable for traditional agriculture. The maximum value of algae production found by Neennan (1987) was 60 g dry wt/m^2/day. The cell residue after lipid extraction can be anaerobically digested for the

production of CH_4 and CO_2. The CO_2 produced from anaerobic digestion would be used to saturate the material where algae are being grown, improving their productivity.

3.2 Algae Industrial Applications

3.2.1 Nutrient Recovery from Manure by Algae

Manure handling and treatment is a major expense and environmental burden associated with animal agriculture. Animal manure is an organic residue with high biochemical oxygen demand (BOD) and can contain pathogenic organisms, but it also contains water and nutrients. Manure cannot be disposed of in rivers and lakes because it contributes to the eutrophication process. Manure has residual protein and fiber fractions that could be used for feed, but the presence of pathogenic organisms limits that option. Using manure as a fertilizer is difficult because of the odor and high organic load. However, manure could be used as nutrient source to grow algae, associating two important benefits: manure treatment and the production of algae.

The manure used to grow algae could be fresh or the residual from anaerobic digestion. Wilkie et al. (2002) compared benthic algae grown on fresh and anaerobic digested residual dairy manure. They found a decrease in chemical oxygen demand (COD), nitrogen (N) and potassium (K) of 95%, 60%, and 93%, for algae grown on undigested manure, respectively. Wang et al. (2010) conducted studies using anaerobically digested manure as a media for cultivation of *Chlorella vulgaris*. They found an efficient removal of nutrients from dairy manure by algae as well as a high oil content in the algae produced.

The recovery of nutrients from organic residues is very important either on an environmental or economic basis. Animal manure usually contains N, P and K. Nitrogen can be lost to the atmosphere due to ammonia volatilization. K and P can be lost by soil percolation. Yet the same nutrients are bought to feed animals and fertilize crops, increasing the production and environmental costs. According to Wilkie et al. (2002), animal feed is commonly 50% or more of the cost with milk production.

Algae are being used to recover nutrients from manure and after processing as a feed ingredient to cattle. Algae grown on dairy manure can achieve a crude protein content of approximately 40% and could be used as a fraction of the dairy cattle's ration (Wilkie et el., 2002).

The estimated area required for treating the manure from 100 dairy cattle using algae raceways would be 1 hectare. The average production was 15 g of algae biomass m^{-2} day^{-1}, recovering around 60% of the original nitrogen and potassium (Mulbry et al., 2005). Without considering co-products from algae, treatment costs using algae have been estimated at $778/cow, which could be competitive to other treatment options in areas such as the Chesapeake Bay with restrictive regulations on cattle production (Mulbry et al, 2008).

3.2.2 Vegetable Oil Production

The other promising use for algae, besides manure treatment, feed and fertilizer is the conversion to renewable fuel. The high oil concentration in algae can be extracted from algae and converted to biodiesel. According to Chisti (2007) the only possible substitute for fossil diesel appears to be oil from microalgae. The two main reasons are the very fast growth of algae and the high oil content of these organisms. Microalgae can double their biomass in 24 hours and their oil content can exceed 80%. The main crops used currently to produce biodiesel compete with food and animal feed, such as corn and soybeans. Furthermore, algae have a higher productivity than the other oil crops, achieving more oil biomass per unit area. The oil yield from the primary crops is shown in Table 3-1. Algae would appear to be one of the most promising crops for vegetable oil production, out yielding soybeans by over 100 times.

Table 3-1 Oil Yield from Crops (Chisti, 2007).

Crop	Oil Yield (L/ha)
Corn	172
Soybean	446
Canola	1190
Jatropha	1892
Coconut	2689
Oil Palm	5950
Microalgae [a]	136,900
Microalgae [b]	58,700

[a] 70% oil (by wt) in biomass

[b] 30% oil (by wt) in biomass

3.2.3 Carbon Fixation

Algae, an autotrophic organism, require an inorganic carbon source to perform photosynthesis (Becker, 1994). Atmospheric air contains 0.03% of carbon dioxide, which can sustain algae growth, but below the maximum potential growth rate. Therefore, additional carbon dioxide can be supplied to increase the algae growth rate if sufficient light and nutrients are available.

Algae have been proposed as a method to fix carbon dioxide from the atmosphere. Vunjak-Novakovic et al. (2005) used a pilot-scale algae photo-bioreactor and found that CO_2 removal efficiency was 50.1% on cloudy days and 82.3% on sunny days from flue gas with a CO_2 concentration of 8%. Processes that produce CO_2 can use algal biomass to fix carbon and to avoid air pollution. The carbon fixation occurs by the accumulation of fatty acids and hydrocarbons in algae biomass, which can be converted to bio-oil or biogas.

Carbon dioxide is soluble in water and algae do not directly use CO_2, but instead bicarbonate and carbonate. The carbonic acid is a problem in algae cultures due to its potential change to the media pH. This means that a portion of the CO_2 used to saturate the growth media is not available for algae fixation. In the presence of water, the following reaction (Equation 3-1) may occur (Becker, 1994):

Equation 3-1

Chlorella vulgaris consumed 38.7% of an enriched CO_2 stream (6-8% by volume) and produced 1 kg of algae biomass from 1.74 kg of CO_2 (Doucha et al., 2005). The algae fixed 4.4 g CO_2 in 24 h with the enriched air stream compared to 3.0 g for atmospheric air.

Chlorella vulgaris is one example of an algae that can shift between an organic and inorganic carbon source according to the light availability (Becker, 1984). The presence of organic carbon is an alternative resource to the algae that may reduce the biomass loss during the dark period. Organic carbon could take the form of sugars that are supplied to algae during heterotrophic fermentation to increase the biomass and oil yield. Using animal manure as a nutrient source could also provide an organic carbon source to limit respiration losses during dark periods.

3.2.4 Heavy Metal Remediation

Algae are also being used to remove heavy metals from soil, water, and residues (Sekabira et al., 2011; Monteiro et al., 2012). The heavy metal concentration accumulated by algae depends on the algae species, the growth media and the management of systems such as the transportation and dryer system. Animal manure can contain heavy metals that may be removed by algae, which become toxic as feed if they are in excess of the maximum tolerable dietary levels (Li et al., 2005; Holzel et al., 2012). Algae production using animal manure could also aid in the removal of heavy metals from manure. However, the metals would likely accumulate in the algae and potentially create problems with downstream processing of the algae.

3.2.5 Soil Fertilizer

According to Becker (1994), nitrogen is the second most important element for algae growth and the form in which this nutrient is supplied has considerable influence on the biomass composition. Nitrate is reduced to ammonium, the preferable nitrogen source for algae (Equation 3-2). Nitrogen assimilation by algae is influenced by the pH of the media.

Equation 3-2

In media with a low nitrogen concentration, many algae reduce their respiration rate and increase their lipid reserve. However, in high nitrogen media, algae are able to increase biomass, primarily by increased protein and chlorophyll content. Table 3-2 summarizes the change in protein and total lipid content, percent dry biomass, for *Chlorella vulgaris,* with varying concentrations of ammonium (Becker, 1994). However, with lower N application rates the algae growth rate was lower.

Table 3-2 Protein and lipid concentration (% dry weight) of *Chlorella vulgaris* grown in media with varying nitrogen concentrations (Becker, 1994).

	N Concentration				
	0.0003%	0.001%	0.003%	0.01%	0.03%
Total Protein	7.79	11.1	19.9	28.9	31.2
Total Lipids	52.8	41.8	20.2	14.1	11.8

Other nutrients required for optimal algae growth are phosphorous, potassium and magnesium. Phosphorous is a critical nutrient for algae growth, because it is essential for many cellular processes such as biosynthesis of nucleic acids and energy transfer. Algae absorb phosphorus mainly as inorganic phosphate. Potassium is a nutrient needed by algae because of its role in photosynthesis, in addition it is important for protein synthesis and osmotic regulation. Magnesium is a central molecule for chlorophyll making it

essential to all algae growth (Becker, 1994). Animal manure has a blend of these essential minerals required by algae (Sutton et al., 1986).

Mulbry et al. (2005) demonstrated that algae grown on dairy manure can supply nitrogen and phosphorous equivalent to land applied fertilizer, and in addition, algal biomass does not have to be tilled into soil. Another advantage is that algae biomass works as a slow release fertilizer because only 3% of the nitrogen is available as mineral nitrogen at the time of application, avoiding ammonia volatilization and nitrogen lost by percolation. Besides that, algae are easier to transport and contain less pathogenic microorganisms than untreated manure.

3.3 Methods to Determine Algae Concentration

3.3.1 Cell Counting

Cell counting is a direct measurement procedure used to determine the concentration of many microorganisms, including unicellular green algae like *Chlorella vulgaris*. According to Madigan and Martinko (2006) cell counting has several limitations including: dead cells cannot be distinguished from live cells without staining methods, it is difficult to count small cells, it is difficult to achieve precision, cell suspensions with low density must be concentrated, motile cells must be immobilized and debris in the sample may be mistaken for microbial cells.

Some of those limitations are worse with algae samples in wastewater. Bertoldi et al. (2006) determined *Chlorella vulgaris* concentration in wastewater by counting the number of cells through light microscopy using a Neubauer hemocytometer. Mohan et al. (2009) also measured *Chlorella vulgaris* concentration by cell counting using a Neubauer hemocytometer. Algae were cultivated in a clear chemical media, in outdoor open ponds, where algae inoculum and water were added daily. Samples were taken every 5 days and they found 221 x 10^4 cells/ml on the 5^{th} day and 1224 x 10^4 cells/ml on the 25^{th} day.

Cell counting is very time consuming and a limited number of samples can be analyzed. Cell counting is a subjective test that is influenced by how individuals distinguish algae solids from non-algae solids. According to Becker (1994) cell counting

by microscopic methods should be used for qualitative estimations rather than quantitative estimations.

3.3.2 Dry Weight

Numerous methods have been developed to evaluate algae concentration using ovens. Aliquots of algae are placed in metal dishes and dried in a convection oven overnight (Liang et al., 2009). Ash-free dry weight is another direct measurement of algae biomass. The procedure involves filtering a known solution volume through a pre-combusted crucible with a glass fiber filter, drying the filter at 95^{o}C, and cooling in desiccators prior to weighing to determine the oven dry weight. The quantity of ash is determined by placing the filter in a furnace at 540°C for 4 hours (Zhu et al., 2007). The ash content of algae could be significant and varies depending on the media, mineral content of water, and algae species. Other issues with dry weight measurements are the potential interference of organic solids (i.e. manure particles or undigested feed) during filtering and oven drying. However, this method is accurate when measuring algae growth in standard chemical media, without the presence of organic solids. If ash-free dry weights are required, the glass fiber filters can become expensive and time consuming if a large quantity of samples are analyzed.

3.3.3 Fluorometer

A method used to estimate plankton density in nature is the fluorometer. This measurement is based on the capacity of chlorophyll molecules to fluoresce, where the chlorophyll absorbs light at one wavelength and emit light at a longer wavelength (Lorenzen, 1966). Based on the fluorescence magnitude, chlorophyll content is calculated using prediction equations. Thomas and Flight (1964) found that measurements of in vivo chlorophyll concentration by fluorescence were about 10 times less efficient than measuring extracted chlorophyll concentration.

3.3.4 Optical Density

Optical density is an indirect method in which the absorbance of light within a sample is measured. Wang et al. (2009) measured the algae growth rate (GR) as a

function of the optical density at 680 nm at time zero (OD0) and the optical density at 680 nm on day "t" (ODt). The growth rate could be calculated, according to the Equation 3-3:

Equation 3-3

Optical density is a rapid, low cost method to determine algae growth rate. However, suspended solids interfere with the optical density and accurately quantifying the algae growth rate using optical density could be difficult.

3.3.5 Chlorophyll Extraction

Algae are a large group of microorganisms that have chlorophyll *a* (chl a) as the primary photosynthetic pigment. The measurement of algae growth with a media containing suspended solids can be estimated by extracting chlorophyll from the cell and measuring the absorbance in a spectrophotometer. An aliquot of the algae solution is centrifuged, the supernatant is discarded, a solvent is added, and the algae resuspended to extract the chlorophyll. Equations have been developed for determining chlorophyll (chl a) concentration in a number of solvents. Chlorophyll is calculated as a concentration (mg l^{-1}) and the absorbance (A) measured at a wavelength specific to the type of solvent is measured using a spectrophotometer (Becker, 1994; Wellburn, 1994).

Chlorophyll extraction has been used to quantify the algae concentration from a number of species under a broad range of conditions (Sartory and Grobbelaar, 1984; Holm-Hansen and Riemann, 1978). Chlorophyll extraction has been used to quantify algae concentration during the treatment of urban wastewater (Martinez et al., 1999), treatment of wastewater from olive-oil extraction facilities (Hodaifa et al., 2007), and treatment of wastewater from pulp and paper plants (Tarlan et al., 2002).

3.4 UV Spectroscopy for Solids and Chemical Determination

3.4.1 Measurement of Suspended Solids

The ultra-violet spectrum can give relevant information about the constituents of a solution. UV absorbance has been used to estimate suspended solids from wastewater (Azema et al., 2001). Vaillant et al. (2002) developed a methodology for estimating suspended solids by spectral differences. In their study, total suspended solids were estimated by the difference between raw sample spectra and a settled sample:

$$ABS_{TSS}(\lambda) = ABS_{Raw}(\lambda) - ABS_{Settled}(\lambda)$$

According to Azema et al. (2002) the solids fraction in wastewater can be classified into four groups based on particle size: soluble (<0.001 μm), colloidal (0.001 – 1 μm), supracolloidal (1 - 100 μm), and settleable (>100 μm). Total suspended solids (TSS) were defined as the sum of supracolloidal and settleable fractions.

Thomas and Cerda (2007) developed a simple test to determine wastewater constituents by measuring the absorbance using UV spectroscopy. Nitrate concentration was determined at a wavelength of 210 nm, absorbance at 240 nm allowed for the discrimination between soluble organic matrix and suspended solids by subtracting the absorbance at 320 nm, which quantifies only suspended solids.

Wastewater is a heterogeneous material containing a large variety of organic and mineral matter. The spectral analysis of water and wastewater is disrupted by physical (e.g. diffuse absorption) and chemical (e.g. overlapping peaks due to competitive absorbance of compounds) processes (Thomas and Cerda, 2007). Due to those interferences, more robust methods have been developed to characterize heterogeneous materials.

There is a correlation between particle size and absorbance at a specific wavelength. Small particles are better detected at short wavelengths because the intensity of light scattered by a suspension decreases with higher incident radiation wavelength (Hulst, 1981). Besides particle size, the particle characteristics, such as form, color and composition, also influence light absorbance.

Particles with the same size can be “confused” by the measurements of total suspended solids. According to Figure 3-1 (Pouet et al., 2007), algae are included in total suspended solids based on their particle size (supracolloidal and seattleable fractions). Differentiating between algae solids and organic debris would require additional information besides particle size.

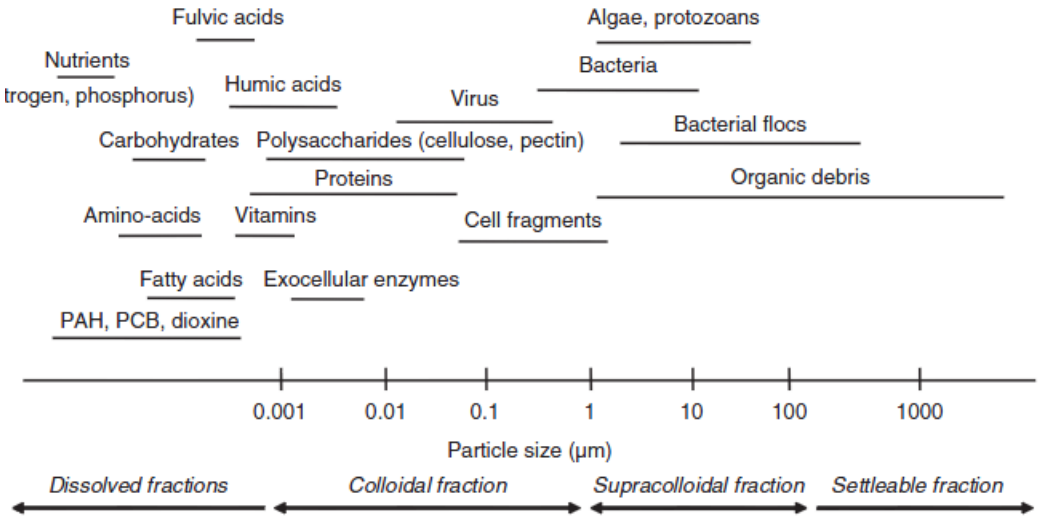

Figure 3-1 Particles size classification (Pouet et al., 2007).

3.4.2 Other Parameters Measured in Wastewater Using UV Spectroscopy

Ultra-violet absorbance has been used successfully to estimate total organic carbon in wastewater (Dobbs et al., 1992), living micro-organisms (Shibata et al., 1954), biochemical oxygen demand in slurry (Brookman, 1997), nitrates and surfactants (Roing et al., 1999), and urban water quality (Vaillant et al., 2002).

Light methods have been used to evaluate amino acids, sugars and carboxylic acids present in microalgae (Horton et al., 2011), macromolecular synthesis in microalgae under different nutrient conditions (Beardall et al., 2001; Stehfest et al., 2005) and lipid accumulation in microalgae under nitrogen limitation (Dean et al., 2010).

Roig et al. (1999) used UV-visible spectroscopy to determine the nitrogen and phosphorous content of wastewater. First, potassium peroxodisulfate was used to oxidize nitrogen and phosphorous into nitrate and orthophosphate ions, and then the ions were quantified by UV-visible spectroscopy.

3.4.3 Evaluation of Spectral Absorbance Data

Figure 3-2 summarizes the potential qualitative methods for analyzing spectral data. The first decision point is the number of spectra that will be handled. Quantifying algae concentration in samples will involve a set of spectra. This would require methods that use isosbestic points or hidden isosbestic points. Isosbestic points are specific wavelengths where the molar absorptivity of two chemical species are equal. Isosbestic points imply that the chemical species are linearly related (Thomas and Cerda, 2007).

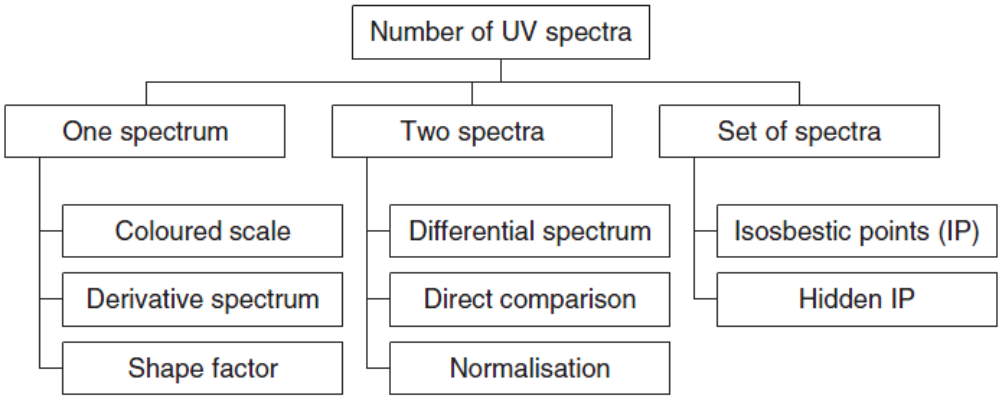

Figure 3-2. Qualitative methods for UV-visible spectra handling (Thomas and Cerda,2007).

Figure 3-3 shows some possible methods to quantitatively analyze UV-visible spectra absorbance data (Thomas and Cerda, 2007). Algae and manure solids are likely to show interference due to the size similarity of algae and organic debris. Statistical methods to resolve spectral data into components have been successfully applied to a number of heterogeneous mixtures (Thomas and Cerda, 2007).

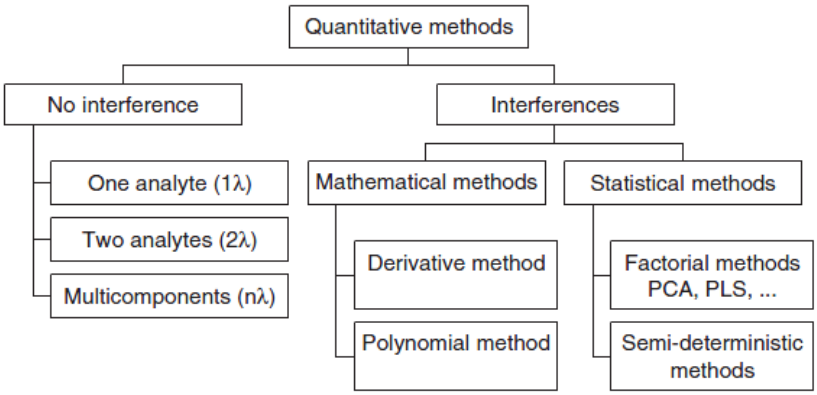

Figure 3-3. Quantitative methods for UV-visible spectra exploitation (Thomas and Cerda, 2007).

3.4.4 Statistical Methods

Multivariate analysis using Principal Component Analysis (PCA) or Partial Least Squares Regression (PLS) can be an alternative for rapid estimation of TSS by measuring a single wavelength. Lourenco et al. (2010) found a wavelength range could be selected for each PLS calibration model to minimize the influence of the particle size, shape, and composition in the light attenuated by the suspended solids. This allowed for the development of PLS models for the estimation of TSS under a wide range of conditions. The advantage of these models is the rapid estimation of TSS using either the absorbance at 550 nm or 860 nm (Lourenco et al., 2010).

Multiple-wavelength models can be more accurate than single-wavelength models, especially when the effluent to be analyzed was constantly varying (Sarraguca et al., 2009). Methods for TSS estimation based on multi-wavelength measurements constitute a potential alternative to turbidity measurements that could be applicable to a wider range of suspended solids characteristics (Sarraguca et al., 2009).

Sarraguca et al. (2009) used a PLS regression model to quantitatively monitor a sludge reactor using UV-visible spectroscopy. They measured the absorbance over a range from 250 to 380 nm to evaluate the change in total suspended solids content. The model was tested using 10 wavelengths and it was found that four variables (model

components) existed, in other words the variation in response could be explained by four wavelengths.

Very small particles can be distinguished and quantified in solutions and gasses using spectral absorbance. Azema et al. (2002) used optical methods to quantify the TSS, soluble matter, colloids, nitrates and surfactants concentration in wastewater. Gases, such as ammonia, emitted from manure, were studied by Galle et al. (2000) using FTIR (Fourier Transform Infrared) techniques. According to Galle et al. (2000), gases can be simultaneously determined by FTIR due their different spectral absorbance.

Stehfest et al. (2005) used spectroscopic techniques to determine nutrient stress and its effect on phytoplankton cellular composition, such as decrease in protein and increase in lipids under nitrogen limitation. FTIR spectral peaks can be assigned to distinct functional groups, like amides to detect proteins and esters to detect lipids and fatty acid concentration (Stehfest et al., 2005). A wide range of techniques and sample analyses have been performed using statistical techniques on spectral data.

3.4.5 Spectral Deconvolution

Depending on the procedure used to study UV absorbance of solids present in wastewater, a wide range of wavelengths could be used. The deconvolution method allowed the absorbance spectrum to be decomposed into a smaller number of characteristic spectra. These data can be further reduced to a wavelength range that is of interest to measure specific properties (Thomas et al., 1993). Vaillant et al. (2002) used absorbance data in the range of 205 to 330 nm to determine the total suspended solids using the deconvolution method. Spectral deconvolution has been shown to be effective with heterogeneous solutions by taking advantage of the different particle/light interactions that occur within the solution (Azema et al., 2002). Reference samples and spectra are required to perform the spectral deconvolution.

Escalas et al. (2003) used UV deconvolution to estimate dissolved organic carbon (DOC) present in municipal wastewater. The wavelength range chosen was 205 to 330 nm and four reference spectra for dissolved organic carbon were taken from the literature (Thomas et al., 1996). A Pearson coefficient of 95.4% was found for the regression

between predicted and measured values of DOC. Different sampling points were chosen from a wastewater treatment plant and the results accurately described the oscillations in DOC that occurred during treatment.

UV deconvolution was also used by Domeizel et al. (2004) to monitor the state of humification of composts. After extraction of humic substances, deconvolution of spectra samples was performed using three reference spectra for humic acid, fulvic acid and the non-humidified fraction. The ratios of deconvolution coefficients were used to evaluate evolution of humic fractions and accurately estimated the state of maturity of composts.

Deconvolution has been used to estimate many other substances and to monitor changes during the processing of various heterogeneous products. This method has been proven to be a quick, accurate method to determine the components of a solution either quantitatively or qualitatively. However, to use deconvolution methods, it is important that the reference spectra be chosen carefully so that it is representative of the samples analyzed.

Algae and suspended solids from manure can be of similar size, but their light absorbance characteristics can be used to distinguish them. Macromolecular components (e.g. lipids and proteins) and chlorophyll are two examples of substances that would differentiate algae from manure solids that could be determined using spectroscopic methods. The UV absorbance spectra of algae and manure could give relevant information and assist with the analysis of mixed sample.

CHAPTER 4: EVALUATION OF CURRENT TECHNIQUES FOR MEASURING ALGAE CONCENTRATION

There are numerous methods to measure the algae concentration that is required to calculate the algae growth rate and evaluate biomass production. To determine algae growth rates, the algae concentration within a sample needs to be precisely and repeatedly measured. Benthic algae produced with an algae turf scrubber are relatively easy to harvest and measure the concentration using oven methods. Accurately determining the algae concentration in a media with suspended solids is more difficult.

With unicellular suspended algae, i.e. *Chlorella* sp., algae concentration can be determined using a number of different methods that are evaluated in this chapter. These include direct measurement, such as counting the cells from a liquid sample. Sampling the algae and determining the dry weight of an aliquot using a convection oven would also provide the concentration. Indirect measurements of algae concentration include optical density at specified wavelengths that are correlated to chlorophyll or suspended solids concentration and therefore algae. Other indirect methods include chemically extracting chlorophyll that is correlated to algae concentration. Different reagents will be tested for extracting chlorophyll from algae in presence of manure. The objective of this chapter was to evaluate current techniques to measure the concentration of unicellular algae in the presence of suspended solids.

4.1 Materials and Methods

4.1.1 Materials

Dairy manure was collected from the lagoon at the University of Kentucky Dairy Research Farm in Lexington, KY and stored at 4°C in a dark refrigerator. Manure solids concentration was characterized by weighing a sample before and after oven drying at 105^{o}C for 24 hours (Standard Methods for Examination of Water and Wastewater, 1992). The pH was measured using a glass electrode pH meter.

The algae specie investigated was the unicellular green algae *Chlorella vulgaris* obtained from Carolina Biological Supply Company (Burlington, NC). Seed cultures of

Chlorella vulgaris were grown in a urea based media to provide seed inoculum for the experiments. Seed inoculum flasks were shaken and six flasks inoculated (3 flasks used a standard urea based medium and three flasks used a medium based on diluted dairy manure). Compressed atmospheric air from the building was mixed with anaerobic grade carbon dioxide to provide air with a CO_2 concentration of 5%. The flasks were incubated with a 16:8 hour light:dark photoperiod. Each flask was sampled by swirling the flask prior to pipetting a 22 ml sample. The algae concentration was determined using four methods: optical density (at a wavelength of 680 nm), oven dry weight, chlorophyll extraction, and cell counting using a Neubauer hemocytometer.

The urea medium was prepared following the proportions given in Table 4-1. Manure collected from the lagoon was relatively dilute due to management practices on the farm. As a result, the dairy manure was not further diluted prior to making the medium. The total solids concentration of the manure media was 5.2 mg/ml. Nutrient analysis of the manure and urea based media were performed by the University of Kentucky's College of Agriculture Regulatory Services using standard protocols for determining nutrient content of liquid animal manures and fertilizers. Macronutrients and micronutrients for the manure and urea based media are summarized in Table 4-2 and Table 4-3.

Table 4-1 Urea media composition.

Quantity	Units	Ingredient
1.1123	Grams	Urea
0.2400	Grams	Potassium Phosphate Monobasic
0.2195	Grams	Magnesium Sulfate Heptahydrate
0.1144	Grams	Calcium Chloride Dihydrate
0.0408	Grams	Ethylenediaminetetraacetate
2	Liters	Tap Water

Table 4-2 Macronutrients in urea and manure based media.

Medium	C %	N %	P %	K %	Ca %	Mg %
Urea	0.016	0.023	0.004	0.016	0.009	0.002
Manure	0.108	0.014	0.005	0.027	0.015	0.005

Table 4-3 Micronutrients in urea and dairy based media.

Medium	Zn ppm	Cu ppm	Mn ppm	Fe ppm
Urea	0.686	0.909	0.488	8.158
Manure	1.619	2.543	4.307	90.98

4.1.2 Algae Cultivation Apparatus

The experiments were conducted inside a controlled environment chamber at 25^{o}C. Three shelving units were used, each with 3 shelves. Each shelf had two 1.2 m (four foot) long fluorescent light fixtures, each with 2 fluorescent bulbs, one cold (32 watts and 2850 lumens) and one warm (25 watts and 2400 lumens). The combination of a warm and cool light bulb provides light spectrally similar to sunlight (Dawson, 2010). Each pair of light fixtures per shelf was controlled using independent digital timers.

Air and CO_2 were supplied to a manifold constructed from 5 cm (2 in) PVC pipe, 0.76 m in length (30 in), sealed on both ends with pipe caps, and fitted with 27 hose barbs as exits for air distribution. Flexible plastic tubing of equal length with a 3 mm ID/6 mm OD (1/8 in ID, ¼ in OD) was used to distribute the gas to twenty-seven 500 ml Erlenmeyer flasks. An in-line nylon filter (13 mm diameter with 0.2 μm pore size) was connected to each flask to minimize contamination (Figure 4-1).

Figure 4-1 Shelving unit with lights and manifold.

4.1.3 Data Presentation

Algae concentration was measured using four methods over a 12 day growth period: cell counting, dry weight, optical density (absorbance at 680 nm), and chlorophyll extraction. After determining the algae concentration using the different methods, the concentration was plotted versus time.

4.1.4 Cell Counting

Algae cells were counted using pictures taken using a epifluorescence microscope (Zeiss AxioSkop Microscope; Carl Zeiss Jena GmbH, Zeiss Gruppe, Jena, Germany), with a resolution of 1300 x 1030 pixels and 12-bit digitization, and transmitted light illumination from 1.0x to 100x using a 35 W halogen lamp.

Two samples of 1 ml were placed on each side of the Neubauer hemocytometer. The average of both samples was used to calculate the concentration of algae cells per ml. The light microscope was used to take pictures with regular and fluorescent illumination. Because chlorophyll molecules fluoresce, the fluorescent picture allowed for the differentiation of algae cells from other suspended solids. Regular pictures were required

because the Neubauer hemocytometer squares were not visible in the fluorescent pictures. Pictures were superimposed to align the Neubauer hemocytometer squares to the fluorescent solids.

4.1.5 Dry Weight

Dry weight is a common method to evaluate the quantity of organic matter in a sample. Samples of 10 ml were taken from each flask and dried in an aluminum pan using a convection oven for 24 hours at 105^{o}C. Dry weight was calculated by subtracting the pan containing dried solid weight from the tare weight of the empty aluminum pan.

4.1.6 Optical Density

Optical density is an indirect method in which the absorbance of light within a sample is measured. Absorbance was read using a spectrophotometer (UV-Visible Evolution 60, Thermo Scientific, Waltham, MA), with a 1.0 nm spectral band width, Dual Silicon Photodiodes, Xenon Flash Lamp, and a wavelength range between 190 and 1100 nm. The specifications state the linear response range was up to 3.5 a.u. and the accuracy at 1.0 a.u. was ± 0.005 a.u. Plastic cuvettes of 1 cm^2 were used for spectrophotometer readings.

Wang et al. (2010) measured the algae growth rate (GR) as a function of the optical density at 680 nm at time zero (OD_0) and the optical density at 680 nm on day "t" (OD_t). The optical density range at 680 measured was from 0.2 to 5.5 during 22 days of algae growing in dairy manure. The growth rate was calculated, using the following equation:

Equation 4.1

4.1.7 Chlorophyll *a* Extraction

4.1.7.1 Chlorophyll *a* Extraction Procedure

Chlorophyll *a* extraction was performed using a 10 ml sample in a centrifuge tube. Each sample tube was centrifuged at 3000 rpm for 1 minute at 25°C according to

the method used by Becker (1994). The supernatant was discarded; 5 ml of reagent (either ethanol, methanol, acetone or dimethyl sulfoxide) was added to each sample tube and mixed using a vortex. Sample tubes were placed in a water bath at 40°C for 30 minutes to perform the chlorophyll extraction. A 1 ml sample was taken from each centrifuge tube and the absorbance measured using a spectrophotometer according to the appropriate wavelength recommended for each reagent (described below).

4.1.7.2 Reagent Comparison

Algae are a large group of microorganisms which have chlorophyll *a* as their primary photosynthetic pigment. The measurement of algae concentration can be estimated by extracting chlorophyll from the cells using various solvents. Chlorophyll concentration is determined by measuring the absorbance in a spectrophotometer (spectrophotometer described in Section 4.1.6). Equations have been developed for determining chlorophyll *a* concentration in a number of solvents, including acetone, methanol, ethanol, dimethyl sulfoxide (DMSO). Chlorophyll *a* is calculated as a concentration (mg/l) and the absorbance (A) measured using the spectrophotometer as a function of wavelength and solvent. Chlorophyll extraction is an option to evaluate algae concentration that could avoid the interference due to suspended solids.

The four reagents investigated to extract chlorophyll *a* from algae were ethanol, methanol, DMSO and acetone. Equations 4.2, 4.3, 4.4 and 4.5 were used to calculate chlorophyll *a* concentration (mg/l):

Equation 4.2[a]

Equation 4.3[a]

Equation 4.4[a]

Equation 4.5[b]

([a] Wellburn,A.R.,1994 ; [b] Becker,E.W.,1994)

Where the subscript corresponds to the absorbance at a specific wavelength.

The performance of the solvents was evaluated in an experiment with chlorophyll extraction from algae in urea medium, algae in dairy medium, and dairy manure with no algae addition. The same volume of algae grown in urea medium (5 ml) was used pure or mixed with 5 ml manure to perform tests. The chlorophyll extraction procedure was also performed for the manure sample with no algae.

4.2 Results and Discussion

4.2.1 Reagents Comparison

A number of preliminary experiments were conducted prior to using chlorophyll extraction to determine algae concentration. Acetone, methanol, ethanol, and DMSO have all been used previously to extract and quantity chlorophyll concentration. The performance of the various reagents and their influence on chlorophyll extraction from algae in urea and manure based media was determined.

Chlorophyll *a* extraction from algae in urea media, algae in manure media and raw manure media, in mg/l, for each reagent are presented in Table 4-4. Chlorophyll *a* extraction was calculated based on Equation 4.2, Equation 4.3, Equation 4.4 and Equation 4.5 for the respective reagents. The manure sample without algae inoculation was used as the control to evaluate the amount of chl *a* naturally present in manure.

Table 4-4 Chlorophyll *a* extraction using acetone, DMSO, ethanol, and methanol of *Chlorella vulgaris* in urea media, manure media, and untreated manure media at incubation times of 30 minutes and 24 h, and the respective standard deviation (Std Dev).

Reagent	Sample	30 min		24 hours	
		chl a (mg/l)	Std Dev	chl a (mg/l)	Std Dev
Acetone	Algae	7.50	0.29	6.97	0.12
	Manure+Algae	8.72	0.55	8.26	0.17
	Manure	2.23	0.01	1.97	0.12
DMSO	Algae	7.68	0.03	6.13	0.04
	Manure+Algae	15.08	0.49	8.07	0.88
	Manure	7.03	0.32	2.93	0.20
Ethanol	Algae	10.12	0.30	5.75	0.26
	Manure+Algae	10.93	0.73	9.53	0.31
	Manure	2.90	0.05	2.27	0.04
Methanol	Algae	8.15	0.08	5.01	0.04
	Manure+Algae	10.53	1.12	6.11	0.31
	Manure	2.33	0.05	1.51	0.01

Figure 4-2 shows the comparison between reagents for chlorophyll *a* extraction, in mg/l after a 30 minute extraction.

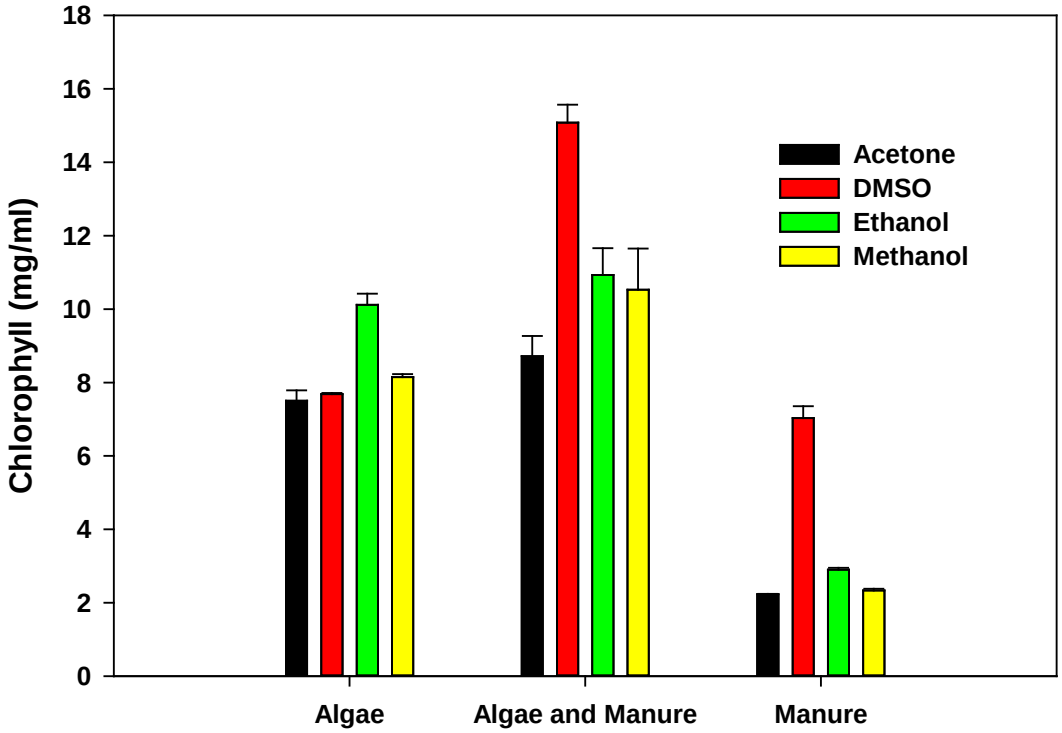

Figure 4-2 Chlorophyll a extraction from Chlorella vulgaris in urea and manure media and untreated manure after a 30 minute extraction in acetone, DMSO, ethanol, and methanol.

The interaction between reagent and extraction time was significant for the two conditions (algae in urea and algae in manure). It was not significant for the manure without algae. Untreated manure likely had algae and photosynthetic bacteria present because it was collected from a lagoon. The chlorophyll *a* concentration from manure samples was not different. Since the difference in absorbance readings after 24 hours in the presence of regents was not different, the absorbance read is more likely to be due solids interference.

The extraction time (30 minutes versus 24 hours) and solvent type (acetone, DMSO, ethanol, and methanol) resulted in significantly different concentrations of chlorophyll *a*. The results are presented in Table 4-5 and Table 4-6 respectively for algae in urea and algae in manure.

Table 4-5 ANOVA for time and reagent effect of chlorophyll extraction from algae in urea medium

Algae					
Source	DF	SS	MS	F	p
Reagent	3	0.85114	0.28371	9.68	0.0016
Time	1	34.4586	34.4586	1175.49	<.0001
Reagent * Time	3	12.9241	4.30804	146.96	<.0001

Table 4-6 ANOVA for time and reagent effect of chlorophyll extraction from algae in manure medium

Algae + Manure					
Source	DF	SS	MS	F	p
Reagent	3	8.4265	2.80883	5.45	0.0135
Time	1	66.1396	66.1396	128.22	<.0001
Reagent * Time	3	40.1229	13.3743	25.93	<.0001

The reduction in chlorophyll *a* concentration after 24 hours of extraction was expected. Reagents can cause chlorophyll molecules to breakdown, in addition enzymes are present that degrade chlorophyll (chlorophylase) that will reduce the chlorophyll concentration in a sample (Ritchie, 2006).

The difference between the average chlorophyll *a* concentration from each reagent and extraction time was tested using the Tukey test, at the 0.05 significance level and is shown in Table 4-7 (urea medium) and Table 4-8 (manure medium). Conditions that are significantly different at the 5% level are identified by different letters.

The chlorophyll *a* concentration of algae grown in urea using ethanol as the solvent was significantly higher than the other reagents, with a 30 minute extraction time. Methanol and DMSO extracted similar quantities of chl *a* after 30 minutes of extraction, while DMSO and acetone were also statistically the same after 30 min. After 24 hours, acetone presented the highest concentration of chlorophyll *a*. However, the chlorophyll concentration after 24 hours was significantly lower (due to degradation by chlorophylase and the solvent action) and a 30 minute extraction time would be recommended.

Table 4-7 Tukey's test for reagents and extraction time for chlorophyll *a* determination from algae in urea at the 0.05 significance level[1].

Algae					
30 min			**24 hours**		
Reagent	*Mean*	*Tukey*	*Reagent*	*Mean*	*Tukey*
Ethanol	10.1151	A	Acetone	6.968	C
Methanol	8.1532	B	DMSO	6.1263	D
DMSO	7.6752	B C	Ethanol	5.7513	D
Acetone	7.5006	C	Methanol	5.0127	E

[1]Significant differences at the 5% level are identified by different letters.

Like chlorophyll extraction from urea medium, chlorophyll extracted from algae in manure medium had higher concentrations after a 30 minute extraction versus 24 hours. DMSO provided the highest chlorophyll *a* concentration after a 30 min extraction.

Ethanol and methanol were statistically the same and methanol and acetone were statistically the same after a 30 min extraction. The lower concentrations with a 24 hour extraction time were expected due to chlorophyll degradation.

Table 4-8 Tukey's test for reagents and extraction time for chlorophyll *a* determination from algae in urea at the 0.05 significance level[1].

Algae + Manure					
30 min			24 hours		
Reagent	Mean	Tukey	Reagent	Mean	Tukey
DMSO	15.0809	A	Ethanol	9.5339	B
Ethanol	10.9251	B	Acetone	8.2601	C
Methanol	10.5345	B C	DMSO	8.0741	C
Acetone	8.715	C	Methanol	6.1069	D

[1]Significant differences at the 5% level are identified by different letters.

The quantity of chlorophyll *a* extracted from algae mixed in manure medium was different than algae grown in urea medium when chlorophyll was extracted using acetone, DMSO and methanol. The quantity of chlorophyll *a* extracted from algae mixed in manure medium was similar to algae in urea medium using ethanol as reagent. It was expected that the manure medium would have higher absorbance values of chlorophyll *a* than the urea medium due to solids interference.

Chlorophyll *a* concentration measured in manure samples using ethanol as the reagent resulted in 2.90 mg/l after 30 min compared to 10.93 mg/l in the algae plus manure sample. This confirmed that considerable background chlorophyll concentrations could be present in manure. In addition, it was visually noted that some suspended solids remained in the reagent after extraction of samples containing manure. It was possible

that suspended solids from the manure after chlorophyll extraction interfered with the spectrophotometer reading.

The extraction efficiency of the different solvents varied with the different media types. DMSO showed the highest concentration of chlorophyll *a* after 30 minutes and ethanol the highest value after 24 hours in manure media. The high chlorophyll *a* concentrations measured with DMSO were probably due to other factors. It was possible to see suspended solids, especially with the DMSO reagent, after the tube was vortexed, based on visual observation of the brown mixture color. Solids could have attached to the DMSO that caused the solids to stay in suspension longer than in ethanol, methanol and acetone. After 30 minutes it was obvious that the majority of the solids settled in ethanol, methanol and acetone, but a significant number of solids remained suspended in DMSO based on visual inspection. The high chlorophyll concentration measured with DMSO extraction was probably due to the interference of the suspended solids in the spectrophotometer reading, and not due to a more efficient extraction.

Based on the results, DMSO was eliminated as a potential solvent since solids likely interfered with the extraction. Acetone was also excluded because it required the use of glass tubing during the extraction procedure that could cause problems with transferring the sample, additional time, and the interference in the spectrophotometer plastic sample tubes. Acetone caused the plastic cuvettes to cloud in a short period of time potentially creating additional measurement variability.

Between ethanol and methanol, ethanol was chosen because it appeared to have the highest extraction of chlorophyll *a* from algae. There also appeared to be minor differences in its efficiency when extracting chlorophyll *a* from urea and manure based media, although not statistically different. In addition, ethanol is less toxic and cheaper than methanol.

In conclusion the four reagents investigated to extract chlorophyll *a* from algae were ethanol, methanol, DMSO and acetone. Acetone is very toxic and cannot be used in plastic sample tubes; DMSO held manure solids in suspension; and methanol is more toxic than ethanol. Ethanol is cheap, nontoxic, and easy to use, and because of that was

chosen as the reagent to be used for extracting chlorophyll from algae grown in manure and urea based media.

4.2.2 Initial measurements

At the beginning of cultivation, 10 ml of *Chlorella vulgaris* grown as a seed culture in urea medium was used to inoculate flasks containing 300 ml of media (urea or manure based). The initial chlorophyll *a* concentration (determined using ethanol as the solvent and equation 4.5), dry weight, pH and OD 680 were measured and are summarized in Table 4-9.

Table 4-9 Initial conditions of the inoculated flasks with urea and manure based media.

Media Type	Chlorophyll *a* (mg/l)	Dry Weight (mg/ml)	pH	OD680
Urea	0.217	1.085	6.67	0.012
Manure	1.694	3.125	7.39	1.066

The higher chlorophyll concentration in the manure based medium, compared to urea based medium, was due to chlorophyll present in the manure before inoculation with algae occurred. Dairy manure was collected from a lagoon, where it was highly probable that algae and other photosynthetic microorganisms were growing. Besides that, it was possible that solids within the medium contributed to a higher absorbance reading after chlorophyll extraction. Manure based medium had a higher initial dry weight and optical density (OD680) due to the quantity of suspended manure solids.

According to Becker (1994), the pH value of the medium is usually neutral or slightly acidic. The ideal pH for *Chlorella vulgaris* growth varies with temperature, metal ions and the presence of other microorganisms. Mayo (1997) determined the optimal pH

range to be between 6.4 and 6.8 for *Chlorella vulgaris* grown in cultures containing bacteria at 32^{o}C. The pH can also be adjusted between 5 and 6 in order to control oil yield (Liang et al, 2011). In general, the ideal pH for *Chlorella vulgaris* for optimal growth is approximately 7 (Liang et al. 2009; Hadjoudja et al. 2010). Wilkie and Mulbry (2002) found that a pH between 7 – 7.5 helped to minimize nitrogen losses due to ammonia volatilization. Although, the initial conditions of the urea and manure based medium were different, the nutrient profile, pH, and other variables should not affect the evaluation of the different measurement techniques.

4.2.3 Cell Counting

The first method investigated to determine algae concentration was direct counting of algae cells using a Neubauer hemocytometer. Two pictures were taken with fluorescent and conventional illumination and superimposed to aid in counting the cells.

Figure 4-3 shows a picture with regular illumination and Figure 4-4 is a picture with fluorescent illumination of an algae sample grown in urea medium for three days after inoculation. Pictures were imported into Photoshop (Adobe Photoshop Elements 10) to subtract the black background from the fluorescent picture (shown in Figure 4-5).

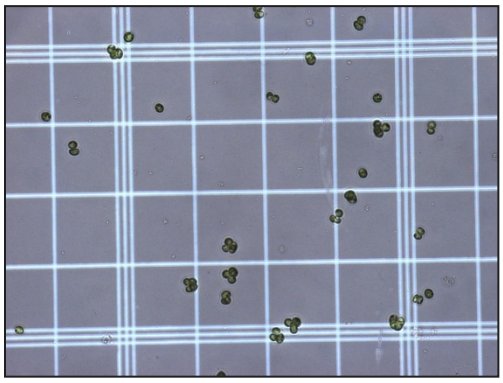

Figure 4-3. Picture of *Chlorella vulgaris* in urea medium with standard illumination.

Fluorescent pictures (Figure 4-4) appear black and are difficult to distinguish any features, but the algae cells are fluorescing in red. When the background is subtracted (Figure 4-5), it becomes possible to count the algae cells.

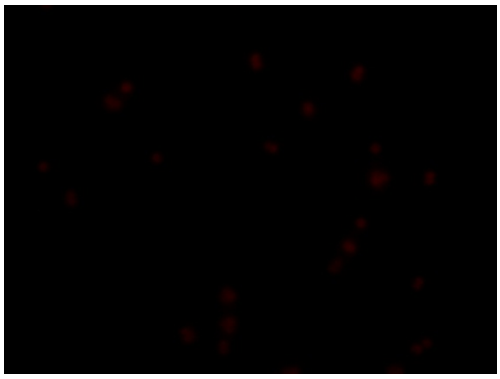

Figure 4-4 Fluorescent picture of *Chlorella vulgaris* in urea medium

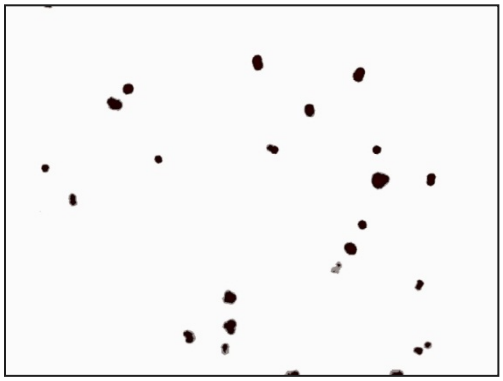

Figure 4-5 Fluorescent picture of *Chlorella vulgaris* in urea medium with the background removed.

However, it is not possible to see the squares of Neubauer Hemocytometer with fluorescent pictures or after removing the background. The picture taken with conventional illumination was superimposed on Figure 4-5 to permit the observation of the hemocytometer squares, solids and solids that fluoresce (Figure 4-6). The fluorescent solids were changed to green to aid the counting procedure.

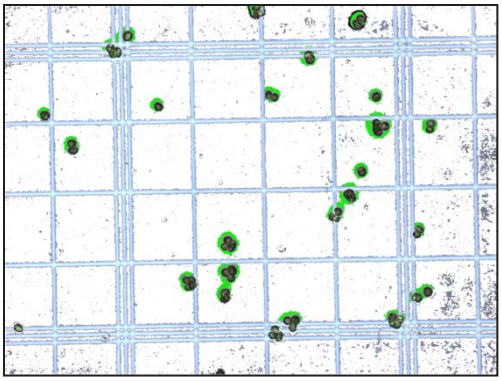

Figure 4-6 Fluorescent picture superimposed on the regular picture of *Chlorella vulgaris* in urea media

The same procedure described above was used for counting *Chlorella vulgaris* grown in dairy manure. It was possible to distinguish between algae and manure solids during the first week of growth due to differences in fluorescence. However, after 10 days it was not possible to count individual cells, because there were too many cells and they were too close to each other to distinguish individual cells (Figure 4-7).

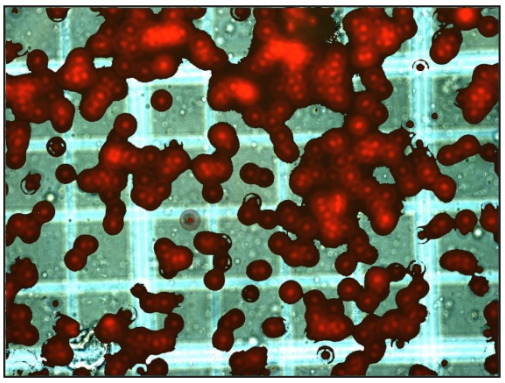

Figure 4-7 Fluorescent picture superimposed on a regular picture of *Chlorella vulgaris* in manure medium

Counting cells should be accurate with low algae concentrations and a clear medium such as urea. Errors are introduced in samples with a large quantity of suspended solids, either due to algae or other suspended solids. Counting cells under these conditions will lose accuracy as it becomes difficult to distinguish between individual solids. Figure 4-8 shows the algae growth curve in urea and manure media measured by

counting algae cells. Each point represents the average of 2 samples (each of 1µl) used with the Neubauer hemocytometer counted by 3 people. Error bars in all graphs represents the standard deviation.

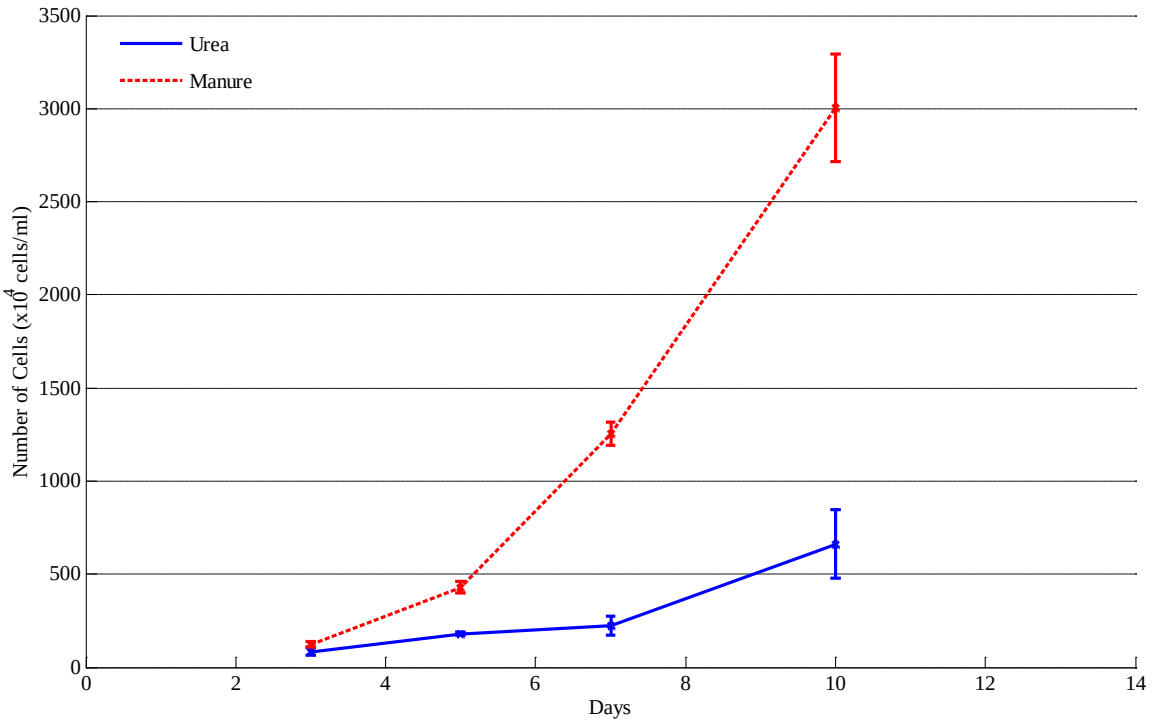

Figure 4-8 Concentration of *Chlorella vulgaris* grown in urea and dairy manure media determined by counting cells using a Neubauer hemocytometer.

The change in algae concentration, as measured by counting, was similar in urea and dairy manure until the third day. On the fifth day of growth there was a significant increase in the number of algae cells measured in the dairy manure medium. On the 5th day of cultivation, it appeared that the number of algae cells in the manure medium was higher than in urea medium. After 10 days however, the number of algae cells in the manure medium was more than three times greater than the number of cells in the urea medium.

The disadvantage with cell counting was the requirement for a person to count and make the determination if a suspended solid was an algae cell or manure solid. This could potentially result in a subjective test. In addition, the sample size (1 μl) used was very small and could potentially cause problems when sampling non-homogenous media and would likely lead to larger errors due to sampling effects. Counting was accurate in a relatively clear medium, but time consuming, variable in the presence of other solids, and a small sample size that could result in sampling error limits the applicability of cell counting to this project.

4.2.4 Dry Weight

Samples from the manure medium initially had high oven dry weights that remained steady during the first three days, probably due to changes in the characteristics of the manure solids (Figure 4-9). The increase in dry weight that began after the 5th day was probably due to a combination of solids decomposing, solids settling, and algae growth. At oven temperatures of 102°C, water is removed from the sample, but organic matter from the manure and algae cells remain. The oven dry weight does not distinguish between algae and manure solids. As a result, the oven dry weight would have organic solids from the manure that would not remain constant during the experiment, therefore using the oven dry weight to determine algae concentration was not feasible.

Other methods that could be used to improve the accuracy of the oven method would include filtration or centrifugation to remove manure solids, but not algae solids. Becker (1994) stated that separation processes such as centrifugation or filtration do not necessarily work for algae isolation. Some microorganisms pass through filters such as unicellular cyanobacteria and some unicellular algae. In addition, *Chlorella* has been shown to bind to solids (Bitton and Bianco-Peled, 2008; Johnson and Wen, 2010). Green algae, like *Chlorella vulgaris*, and some other microorganisms, produce an extracellular polysaccharide that attaches to solid particles (Zaadi et al., 2009). A number of filtration and centrifugation steps were investigated to improve the accuracy of the oven method in the presence of manure solids, but none were successful.

Figure 4-9 shows the change in algae concentration measured using dry weight.

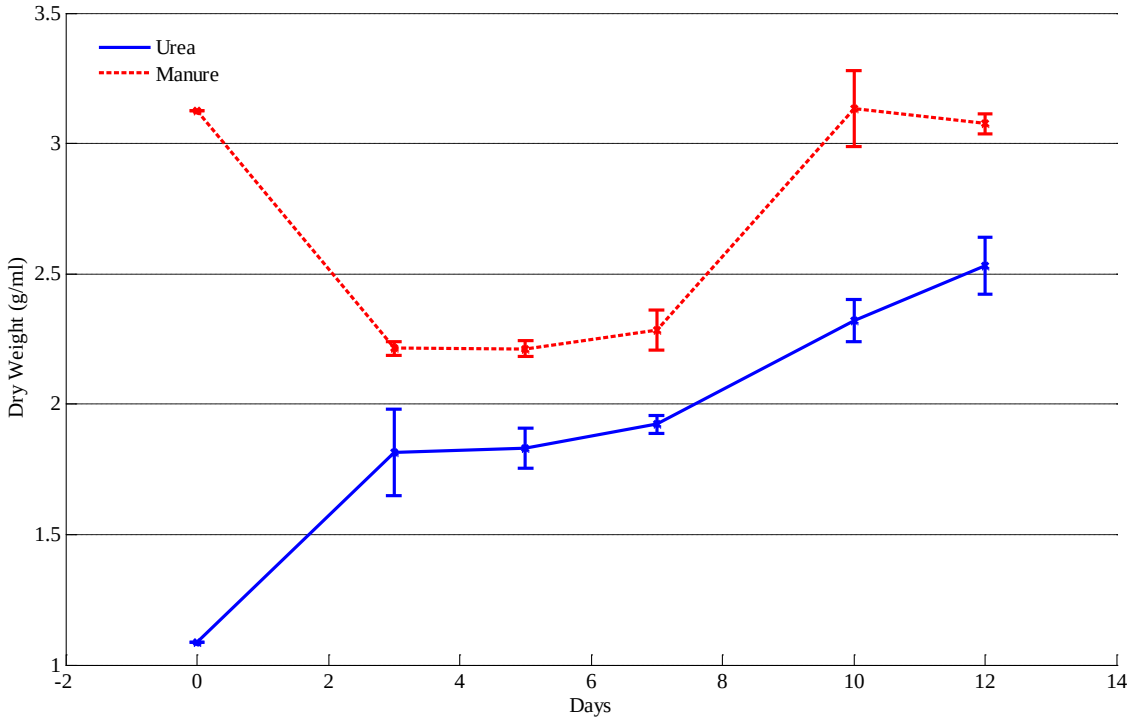

Figure 4-9 Concentration of Chlorella vulgaris in manure and urea measured by dry weight and initial dry weight corrected to zero.

Determining the algae concentration using dry weight was likely influenced by the presence of manure solids. However, this method is accurate and inexpensive when measuring algae concentration in standard chemical media with no organic solids other than algae. Separating manure from algae solids was not possible which limits this techniques applicability to this project.

4.2.5 Optical density

The optical density of algae grown in urea medium increased steadily until day 10 (Figure 4-10). The optical density in urea medium behaved as expected and increased

until day 10 when the algae began to die. When manure was used as the medium, the optical density of the medium showed an initial decrease, probably due to interference by suspended manure solids. This behavior was similar to the change in weight observed when the oven dry weight was measured. This was likely due to changes in the solids concentration (solids breaking down and settling) and not changes in the algae concentration. As the solids break down, nutrients are released and utilized by the algae which would partially explain the increase in OD observed starting on day 7. However, it was not possible to differentiate changes in solids concentration from algae concentration using OD.

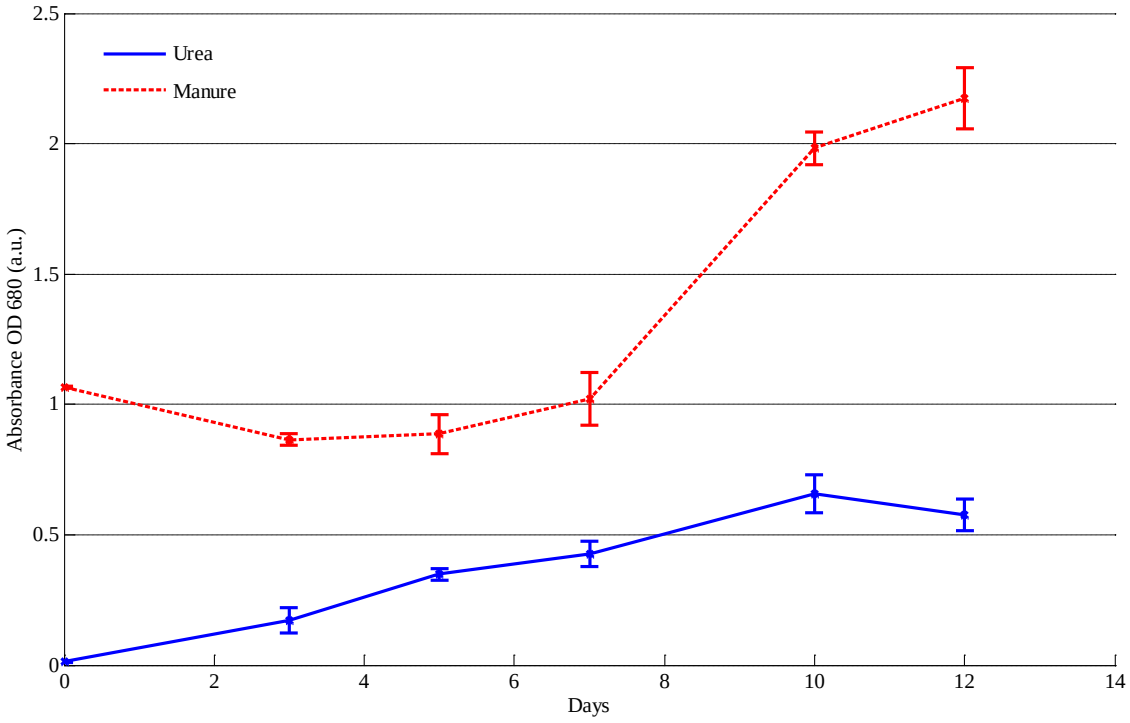

Figure 4-10 Concentration of *Chlorella vulgaris* grown in urea and dairy manure media determined by optical density.

The change in optical density does not solely represent algae growth, but represents the breakdown of manure solids, change in suspended solids concentration, and algae growth. Advantages of the method include low cost, minimal equipment and

supplies required to perform the measurements. However, the accuracy in the presence of suspended solids would make the method inappropriate for this study.

4.2.6 Chlorophyll *a* Extraction

Sample tubes were incubated for 30 minutes in a waterbath at 40^{o}C to perform the chlorophyll extraction. During this time the solids settled to the bottom of the centrifuge tube and chlorophyll was extracted into the ethanol. After incubation, a sample was taken to measure the absorbance and was assumed to be free of manure solids. Figure 4-11 shows the sample tubes after the extraction process in media with varying levels of algae and manure. Visually, the samples with 10 ml of algae and 2 ml of manure were greener (far left) than the sample with 2 ml of algae and 10 ml of manure that was brown (far right). As the concentration of manure increased additional suspended solids were observed in the reagent.

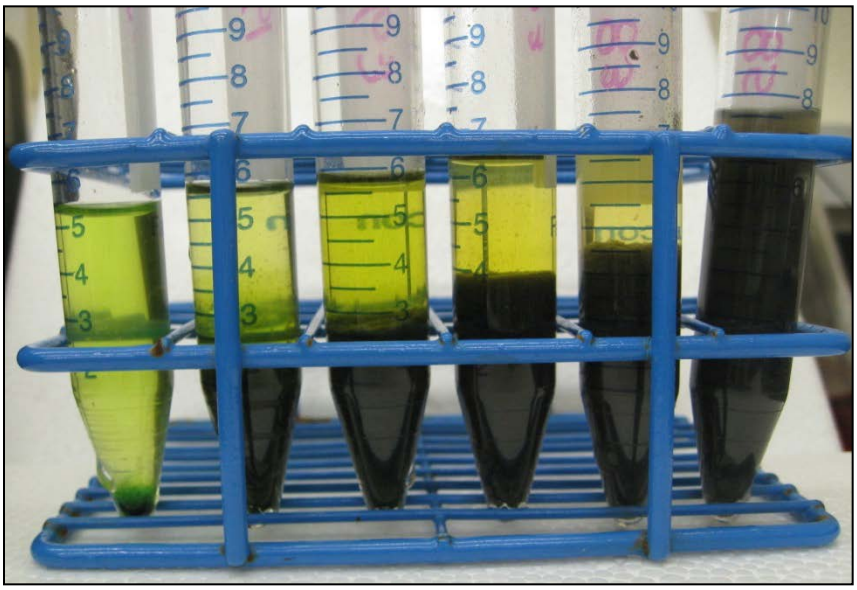

Figure 4-11 Sample tubes to perform chlorophyll extraction with varying manure volumes. From left to right the samples were no manure and 10 ml algae, 2 ml manure and 8 ml algae, 4 ml manure and 6 ml algae, 6 ml manure and 4 ml algae, 8 ml of manure and 2 ml algae, 10 ml manure.

The chlorophyll concentrations (Figure 4-12) were very similar in the manure and urea medium until day 7.

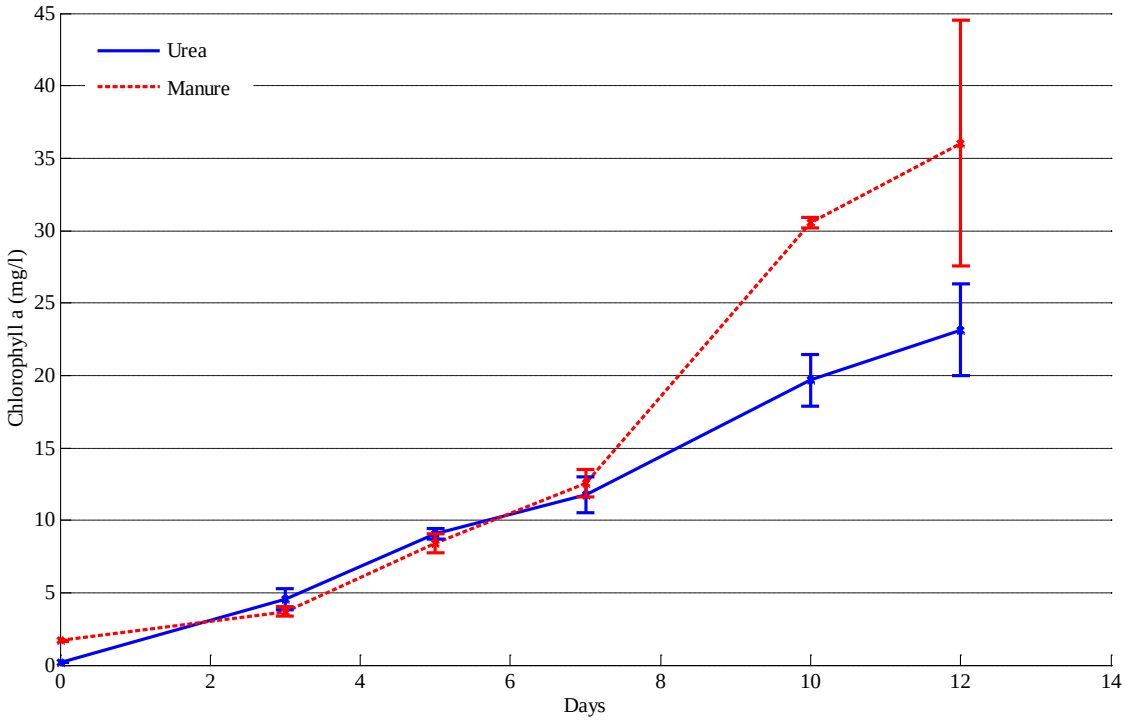

Figure 4-12 Growth of *Chlorella vulgaris* in manure and urea media measured by chlorophyll extraction in ethanol.

The chlorophyll concentrations presented a large standard deviation at day 12. Each point represents the average of a 10 ml sample from 3 different flasks. It is possible that algae grew differently in each flask. The air distributed from the manifold and lighting intensity could have varied between flasks. This could have resulted in the large variation in algae concentration measured on the 12th day.

4.3 Comparison of Methods

Dairy manure is a heterogeneous mixture of numerous different compounds. Although dairy cattle are ruminants and able to digest cellulose, numerous other cellulose residues (bedding materials) might be present in the manure when it was collected. Starch and glycogen are common polysaccharides that are present and are digestible by many bacteria. Lactose is a disaccharide likely present in dairy manure from waste milk and milk house cleaning. Polysaccharides catabolized for microorganism growth are initially enzymatically hydrolyzed to monomeric or oligomeric units (Madigan et al., 2006). As the polysaccharides break down, nutrients are released, because the carbon and nitrogen cycles are closely interconnected. For example the rate of primary productivity (CO_2 fixation) is controlled by available nitrogen; high levels of ammonia stimulate primary production (Figure 4-13). Algae growth in manure and urea based media are expected to be different due the macro and micronutrients and the time lag associated with the carbon and nitrogen cycles.

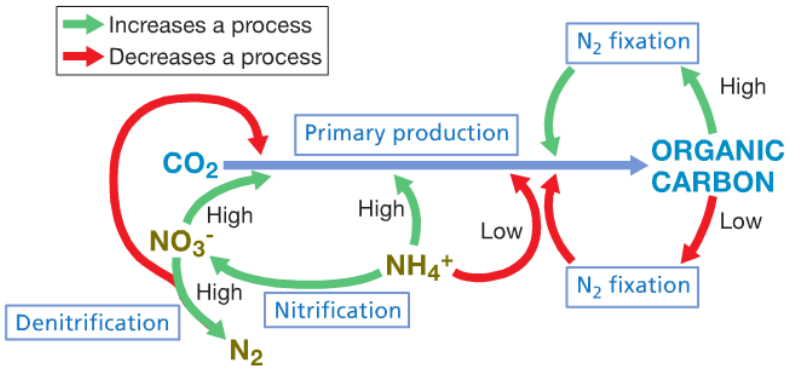

Figure 4-13 Carbon and Nitrogen cycles (Adapted from Madigan et al, 2006).

It was difficult to determine which method would perform the best for determining algae concentration in media with suspended solids. Optical density and dry weight were not accurate and were eliminated from further consideration. Cell counting should be accurate, but is time consuming, sensitive to operator judgment on differentiating algae from manure solids, and very small volumes are measured. It was believed that a method based on chlorophyll extraction would be most suitable for determining algae concentration in manure based media.

4.4 Conclusions

Manure based media complicated the measurement of algae biomass because of the interference of the manure solids in the media. Cell counting, dry weight, optical density, and chlorophyll extraction were investigated to determine algae concentration. *Chlorella vulgaris* concentration measured in dairy manure medium was not accurately quantified using optical density or dry weight. Cell counting should be accurate, but was time consuming, sensitive to operator judgment on differentiating algae from manure solids, and very small volumes were measured (1 μl). It was believed that a method based on chlorophyll extraction would be most suitable for determining algae concentration in manure based media. However, due to interference from residual manure solids a new calibration equation could be required. Four solvents are typically recommended for extracting chlorophyll from algae (ethanol, methanol, DMSO, and acetone). It was shown that extracting chlorophyll with ethanol was preferred. Acetone is very toxic and cannot be used in plastic sample tubes; DMSO held manure solids in suspension; and methanol is more toxic than ethanol. Ethanol is cheap, nontoxic, and easy to use, and because of that was chosen as the reagent to be used for extracting chlorophyll from algae grown in manure and urea based media.

CHAPTER 5: DEVELOPMENT OF A NEW EQUATION TO ESTIMATE CHLOROPHYLL CONCENTRATION OF A SAMPLE IN THE PRESENCE OF SOLIDS FROM MANURE

Procedures have been developed to extract chlorophyll *a* from algae and plant material using various regents and determining the chlorophyll *a* concentration based on the absorbance measured using a spectrophotometer. Equations have been developed to predict chlorophyll *a* concentrations in the presence of numerous solvents (Becker, 1994 ; Wellburn, 1994). Depending on the chlorophyll source (i.e. plant leaf, algae, bacteria) and species (corn versus oak leaf or *Chlorella vulgaris* versus other algae), a specific solvent can extract the chlorophyll. The proportions of the various chlorophyll types, such as "*a*", "*b*", "*c*", vary considerably between organisms. Besides that, other material present in the sample, such as suspended manure solids, could interfere with the extraction process or measurement of the absorbance. From preliminary experiments, the equations proposed by Becker (1994) to determine chlorophyll *a* concentration using ethanol as the reagent indicated a different result in the presence and absence of manure. The predicted chlorophyll *a* concentration was higher in the presence of solids that indicated the procedure should be adjusted.

The objective of the 5th chapter is to propose a new equation to predict chlorophyll *a* concentration extracted from algae in the presence of manure solids. The proposed model will correct the spectrophotometer absorbance data to estimate algae concentration in media with suspended solids.

5.1 Materials and Methods

5.1.1 Effect of Manure Solids on Spectral Absorbance

Algae, *Chlorella vulgaris,* were cultivated in Erlenmeyer flasks filled with 300 ml of urea media (Section 4.1.1). Different volumes of algae were added to sample tubes previously filled with 5 ml of manure and control tubes with no manure (Table 5-1, Figure 5-1) and were performed in triplicate. After the algae were added to manure, the tubes were shaken, and the chlorophyll *a* extraction was performed. Chlorophyll *a* was

extracted from the same volumes of algae in the control tubes with no manure added. Ethanol was used as the blank for absorbance readings in the spectrophotometer.

Table 5-1 Volume of *Chlorella vulgaris* grown in urea media added to dairy manure for determining the change in spectral absorbance due to the presence of manure solids.

Manure Samples		
Manure (ml)	Algae Added (ml)	Total Volume (ml)
5	0	5
5	2	7
5	4	9
5	6	11
5	8	13
5	10	15

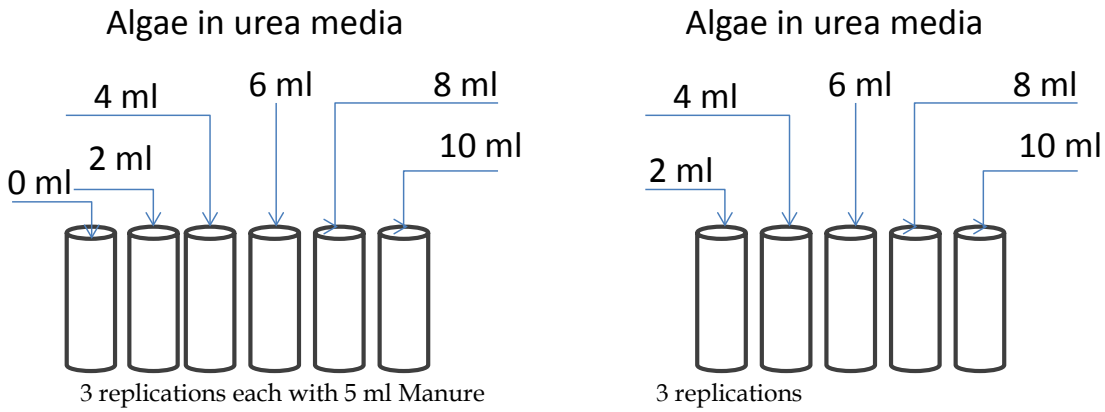

Figure 5-1 Schematic of algae grown in urea media added to manure and for controls to determine the change in spectral absorbance due to the presence of manure solids.

The absorbance of algae in urea media and algae mixed with manure was read in a spectrophotometer after chlorophyll *a* extraction, according to the procedure described in section 4.1.7.2. The absorbance was measured in the wavelength range from 200 nm to 700 nm in 5 nm increments. The most relevant wavelengths and absorbance peaks were chosen to formulate the new model. Absorbance at each wavelength corresponds to a specific type of chlorophyll or other component such as suspended solids.

5.1.2 Mathematical Formulation

The equation proposed by Becker (1994), was used as the reference method for chlorophyll *a* extracted from algae grown in urea medium (Equation 5.1). Becker's equation used the absorbance at two wavelengths (650 nm and 665 nm). The absorbance at a wavelength of 650 nm was related to chlorophyll *b* and absorbance at 665 nm was related to chlorophyll *a*, both wavelengths are in the red light range when ethanol was used as the solvent.

Becker's (1994) equation allowed for the determination of chlorophyll *a* and correct for the possible interference from chlorophyll *b*. This was done by determining the absorbance at 665 nm and subtracting the possible interference from chlorophyll *b* represented by the absorbance at 650 nm:

Equation 5.1

However, when chlorophyll is extracted from algae grown in manure based media, it is important to correct for the possible interference of suspended solids in the spectrophotometer readings. The correction for suspended solids could be accounted for by expanding Becker's model to a more general linear model:

Equation 5.2

Where "Y" is chlorophyll *a* concentration in mg/l, "β's" are fitted coefficients, "x" is the absorbance measured by the spectrophotometer at a specific wavelength "i", and "ε" is the error term.

The desired model for this study would be fit without an intercept. An intercept with this model would have little or no explanatory significance. According to Eisenhauer (2003), an intercept can be excluded if there are a priori reasons to believe that y=0 when x=0. For this equation it was desirable that chlorophyll *a* would be zero (y=0) when the absorbance read by the spectrophotometer was zero (x=0).

Hahn (1977) suggested that regressions should be performed with and without an intercept, and the standard errors compared to decide which model provided a superior fit. Eisenhauer (2003) suggested the models should be compared by calculating the square of the sample correlation between observed and predicted values, in order to choose the best model. Including or excluding the intercept term will be evaluated in this study.

5.1.3 Validation Samples

Following the chlorophyll extraction process (Section 4.1.7.1), the absorbance was measured between the wavelengths of 200 nm to 700 nm in 5 nm intervals. Absorbance curves were plotted for all tests for comparison and statistical modeling. Coefficients for chlorophyll *a* concentration extracted using ethanol from algae grown in urea medium was determined using the coefficients proposed by Becker (1994) (Equation 5.1). The samples grown in urea medium that followed the procedure proposed by Becker were used as the validation data for the models. The validation data set must represent the population (span the expected range of chlorophyll concentrations) in which predictions will be made.

5.1.4 Analysis and Evaluation

New coefficients for predicting chlorophyll *a* concentration extracted using ethanol from *Chlorella vulgaris* in the presence of dairy manure were established. The algae added to the dairy manure samples had a known chlorophyll *a* concentration (5.1.3). To develop the model, spectral absorbance data between 200 and 700 nm in 5 nm increments were collected from samples containing manure and algae grown in urea media (Section 5.1.1). The same procedure used to calibrate the model was repeated to

evaluate the impact of the intercept. Different volumes of algae were added to the same volume of manure to determine the possible interference from manure solids.

Actual: Chlorophyll *a* concentration calculated using Becker's equation with ethanol as the solvent from *Chlorella vulgaris* grown in urea media;

Predicted: Chlorophyll *a* concentration from *Chlorella vulgaris* grown in urea media and diluted with dairy manure calculated using the new equation with ethanol as the solvent.

5.2 Results and Discussion

5.2.1 Analysis of Absorbance

The absorbance curves from ethanol used to extract chlorophyll *a* from *Chlorella vulgaris* in urea media is shown in Figure 5-2. The graph shows the average of the three replications of the absorbance from 2, 4, 6, 8, and 10 ml of algae. It can be clearly seen that algae concentration had a considerable effect on the absorbance measured. Importantly the peaks at 665 nm increased proportional to the quantity of algae. For example, the absorbance from 2 ml of algae at 665 nm was approximately 0.4 a.u., while the absorbance from 10 ml of algae at 665 nm was 1.85 a.u. This indicated that the chlorophyll concentration increased nearly five-fold in line with the five-fold increase in algae volume. Interference due to chlorophyll *b*, as indicated by an absorbance peak at 650 nm, was not evident in *Chlorella vulgaris*.

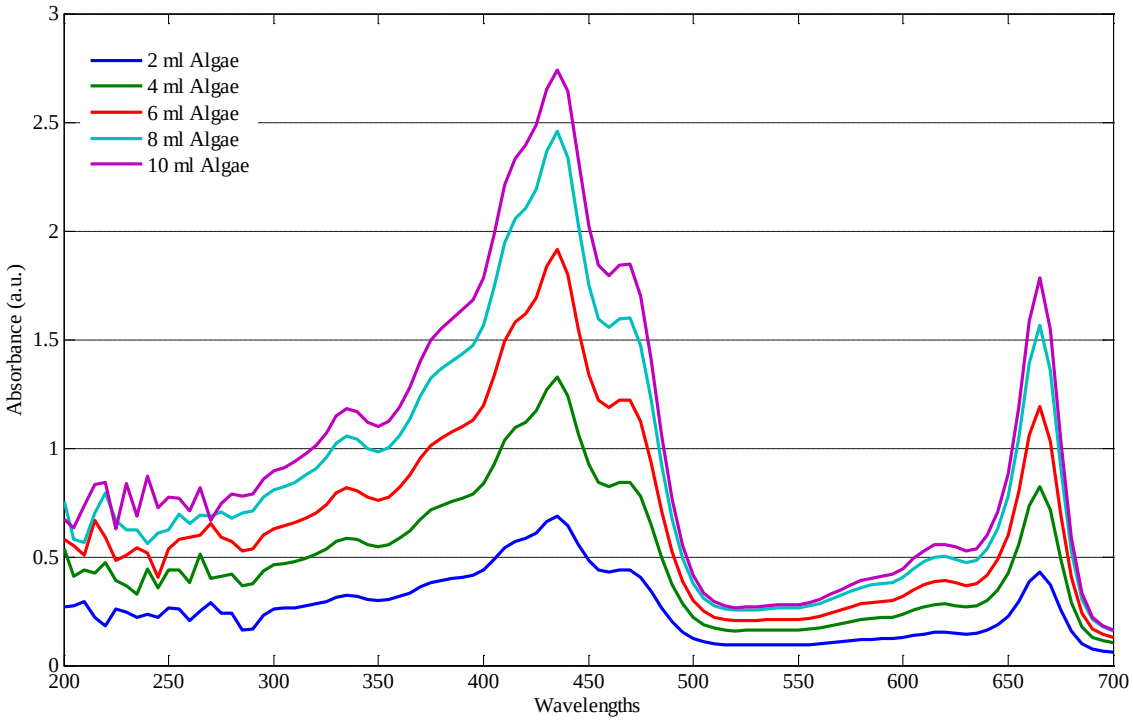

Figure 5-2 Absorbance of Chlorophyll a extracted using ethanol from Chlorella vulgaris (Three replicates are shown. The bottom set of lines corresponds to 2 ml of algae added followed by 4, 6, 8 and 10 ml).

The absorbance curves of chlorophyll *a* extracted from *Chlorella vulgaris* in dairy manure is shown in Figure 5-3. The graph shows the three replications of the absorbance when 0, 2, 4, 6, 8, and 10 ml of algae were added to 5 ml of dairy manure. It can be clearly seen that algae concentration had a considerable effect on the absorbance measured.

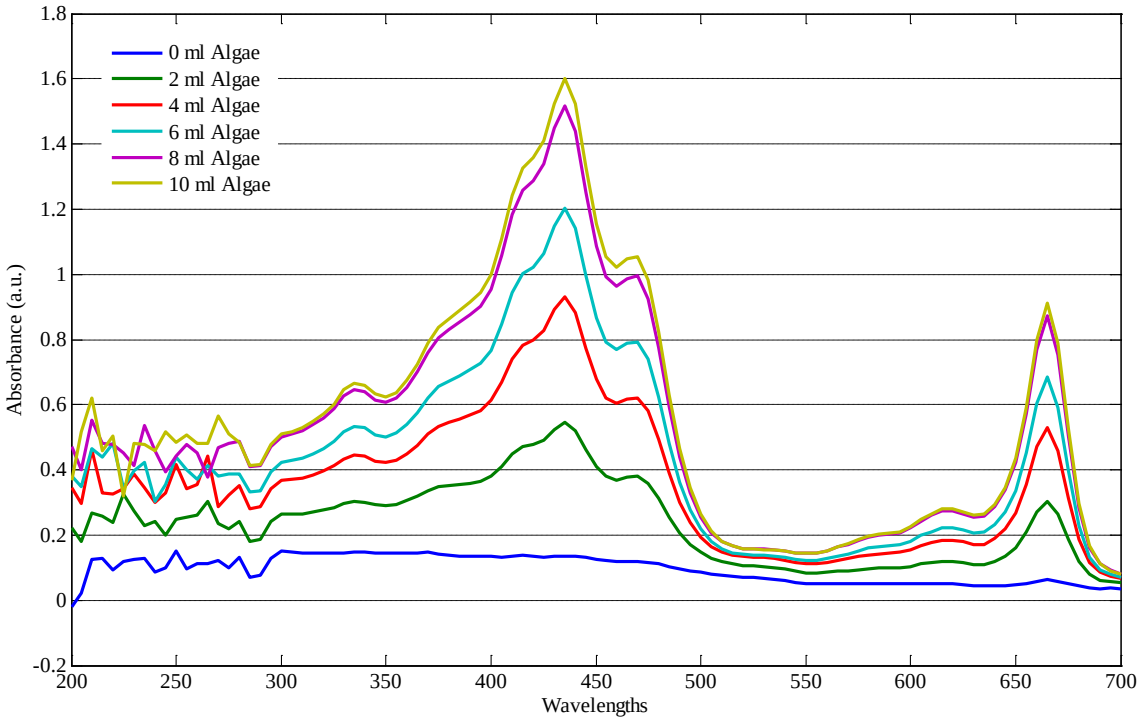

Figure 5-3 Absorbance of chlorophyll a extracted using ethanol from Chlorella vulgaris in dairy manure (three replicates are shown. The bottom set of lines corresponds to 0 ml of algae added to 5 ml of manure, (0:5) followed by (2:5), (4:5), (6:5), (8:5), and the upper set of lines corresponds to 10 ml of algae added to 5 ml of manure).

The peak observed near 430 nm was due to chlorophyll *a* and total carotenoids under blue light (Obertegger et al., 2011). According to Mosqueira et al. (1992), 430 nm is a desirable wavelength to use for the simultaneous detection of carotenoids and chlorophyll.

The peak at 665 nm was associated with chlorophyll *a* in the red light range (Obertegger et al., 2011; Becker, 1994; Rowan, 1989). It was possible to see clearly that higher absorbance readings were obtained due to the higher algae concentrations and therefore higher chlorophyll *a* concentration. There were no absorbance peaks evident at 430 nm or 665 nm in the absence of algae. There was not a peak at 650 nm of sufficient

magnitude to justify any correction for chlorophyll *b*. According to the preliminary experiments, chlorophyll *b* extracted from 10 ml of *Chlorella vulgaris* and calculated using Becker's equation (Equation 5.1) was an average of 5.65 mg/l while chlorophyll *a* averaged 14.50 mg/l.

There was a correlation between suspended solids, particle size and absorbance at specific wavelengths. Small particles absorb light at short wavelengths because the intensity of light scattered by a suspension decreases with higher incident radiation wavelength (Hulst, 1981). Other particle characteristics, in addition to size, that influence light absorbance are shape, color and composition. Higher absorbance readings were found at 430 nm due to manure solids.

Thomas and Cerda (2007) found a number of wavelengths in the UV spectrum that provide meaningful information of a wastewater sample. Absorbance at 210 nm corresponds to the presence of nitrate, 240 nm allowed for the discrimination between a soluble organic matrix and suspended solids and 320 nm was related to suspended solids only. However, wastewater is a heterogeneous material containing a large variety of organic and mineral material. Sources of interference during the spectral analysis of water and wastewater can occur due to physical (e.g. diffuse absorption) and chemical (e.g. overlapping peaks due to competitive absorbance of compounds) processes (Thomas and Cerda, 2007). Due to these interferences, more robust methods such as multiple wavelength regression have to be used to study a very heterogeneous material. Sarraguca et al. (2009) developed a method that utilized light in the range of 250 to 380 nm to estimate total suspended solids.

According to Vaillant et al. (2002), a range from 205 to 330 nm was used to evaluate total suspended solids by the deconvolution method and if the signal was saturated due to concentrated wastewater, sample dilution was needed. It was possible that the noise seen in Figure 5-3 at wavelengths below 300 nm were due to high concentrations of suspended solids, nitrates and the heterogeneity of the manure. Other small peaks observed in Figure 5-3 at 350 and 470 nm, were probably related to COD

and organic carbon (Matsche and Stumwohrer, 1996) and carotenoids (Lichtenthaler and Wellburn, 1983), respectively.

Absorbance at a wavelength of 665 nm has been commonly used to estimate chlorophyll *a* extracted in ethanol (Becker, 1994). Absorbance at 430 nm was related to both chlorophyll *a* and total carotenoids that would not be as beneficial to determine algal concentrations in this study. Therefore, the absorbance at 665 nm, related specifically to chlorophyll *a* will be used in the new model.

The impact of suspended solids was probably larger than the potential impact of the chlorophyll *b* concentration. In addition, peaks associated with chlorophyll *b* were not present in the samples (Figure 5-3). Model options were considered to correct for these factors using Equation 5.2.

5.2.2 Chlorophyll Concentration using Becker's Method

Chlorophyll *a* extraction from *Chlorella vulgaris* samples were used as the reference data. The reference data contained 18 measurements of chlorophyll concentration (Appendix B), with three repetitions of six algae volumes (0, 2, 4, 6, 8, and 10 ml), that had a chlorophyll concentration between 0 and 23 mg/l (Figure 5.4). These samples were used to perform the calibration to predict chlorophyll *a* concentration from algae mixed with dairy manure.

Chlorophyll *a* (mg/l) extracted from algae were plotted against the absorbance at 665 nm from the chlorophyll *a* extracted from algae (0, 2, 4, 6, 8, and 10 ml) diluted in 5 ml of dairy manure. A simple linear regression was performed to find the slope coefficients, with and without an intercept. It was believed that the primary wavelength associated with chlorophyll *a* concentration in manure would still be 665 nm based on the spectral absorbance data. The calibration data is shown on Figure 5-4.

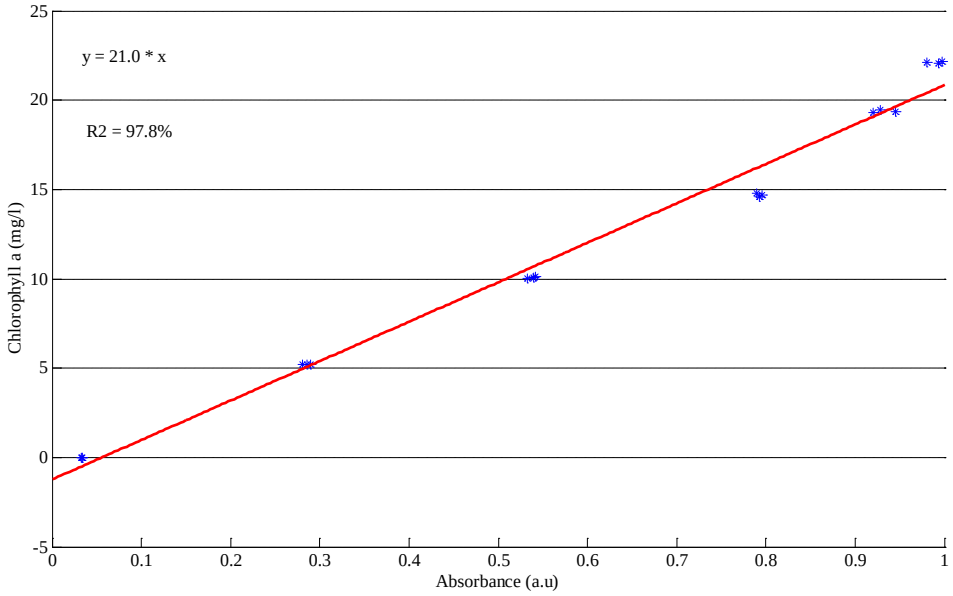

Figure 5-4 Calibration for chlorophyll a concentration from *Chlorella vulgaris* diluted with dairy manure.

The absorbance reading at the low range, where no algae were added to the manure media, were approximately 0.033 a.u. These absorbance readings are probably above the detection limit of the spectrophotometer which has a noise reading of 0.00025 at 0.0 a.u.

5.2.2.1 Calibration Equation with no Intercept

The equation proposed with no intercept using data from Figure 5-4 is provided in Equation 5.3.

Equation 5.3

Using linear regression with no intercept resulted in an R^2= 97.8 % and angular coefficient confidence interval between 19.8 and 22.2. An ANOVA table for the regression with no intercept is shown in Table 5-2.

Table 5-2 ANOVA for determining chlorophyll concentration from *Chlorella vulgaris* in dairy manure with no intercept term.

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	3584.07	3584.07	1406.55	0.00
Residual	17	43.32	2.55		
Total	18	3627.38			

The p-value determined by Analysis of Variance was smaller than 0.05, so we conclude that the correlation between absorbance at 665 nm from algae in manure media and Chlorophyll *a* was significantly different than zero. A residual plot of the regression with no intercept is shown in Figure 5-5. A small pattern in the residuals was evident at absorbance readings over 0.9 a.u. The residuals are very small (less than 0.1 mg/ml) with a chlorophyll concentration over 20 mg/ml. In terms of percentage error, the residual was only 0.5% of the total reading that was considered acceptable for this project. Another potential source of error at high chlorophyll concentrations could be saturation of the spectrophotometer with high absorbance readings. Although the specifications for the spectrophotometer indicated a linear range up to 3.5 a.u. and a noise level of 0.0008 at 2.0 a.u. However, the residual pattern was not considered important for this study.

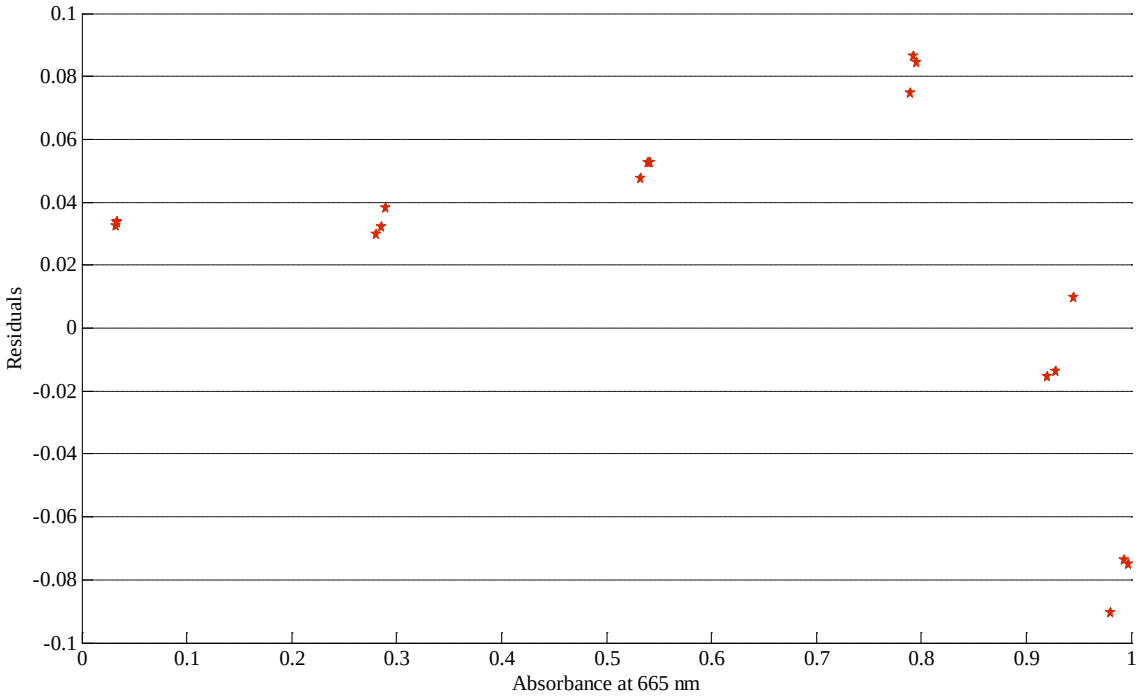

Figure 5-5 Residual plot of the regression for determining chlorophyll concentration with no intercept.

5.2.2.2 Calibration Equation with an Intercept

The proposed equation for chlorophyll *a* concentration in dairy manure when an intercept was included is:

Equation 5.4

The R^2 was 99.0 % with an angular coefficient confidence interval between 20.5 and 24.9. For the intercept the linear coefficient interval was between -2.8 and 0.2. An ANOVA for the regression that included an intercept is given in Table 5-3.

Table 5-3 ANOVA for determining chlorophyll concentration from *Chlorella vulgaris* in dairy manure with an intercept term.

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1038.65	1038.65	469.55	0.00
Residual	17	35.39	2.21		
Total	18	1074.04			

The Analysis of Variance resulted in a p-value smaller than 0.05, so we conclude that the correlation between absorbance at 665 nm from algae in manure medium and Chlorophyll *a* was significantly different than zero. The residual plot for the regression with an intercept is shown in Figure 5-6. Residuals for regression with the intercept had a small pattern, like the residual plot for the regression with no intercept. However, the residual pattern was not considered important from a practical standpoint as stated before.

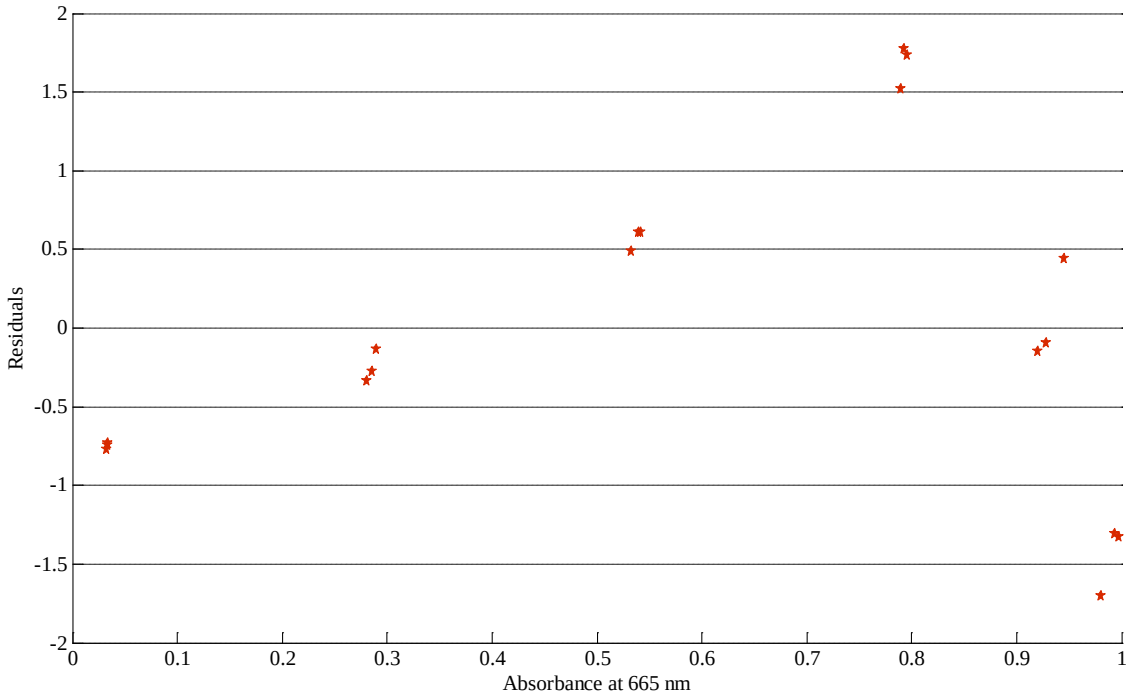

Figure 5-6 Residuals of the regression with an intercept term.

5.2.2.3 Comparison between Models With and Without an Intercept Term

Residual charts shown in Figure 5-5 and Figure 5-6 did indicate some trends and did not appear to be randomly distributed. Residuals are often analyzed from a graphical standpoint to detect abnormal behavior. If the model was correct and assumptions were satisfied, residuals should be random about zero. (Rawlings et al., 1998).

Both residual charts show a small pattern that remained very close to zero. The pattern evident at high chlorophyll concentrations was of acceptable accuracy (less than 0.5% of the actual reading), meaning there was no obvious inadequacy in the model with or without an intercept. Based on the ANOVA, both models were significant, which showed that the determination of chlorophyll *a* concentration in samples with manure was explained by the absorbance measurement at 665 nm using the spectrophotometer. Since there was no inadequacy for either model, we conclude that the intercept was not contributing significantly to the model and the intercept was neglected. From a practical

standpoint, if chlorophyll concentrations greater than 20 mg/ml were detected, samples would need dilution to work with the proposed model with no intercept term.

5.2.3 Analysis and Evaluation

In order to validate the model, an independent experiment was conducted using the same procedure described above, adding a known quantity of algae to 5 ml of dairy manure. The model developed in Equation 5.3 was used and the additional data set was used as validation. The validation of Equation 5.3 is summarized in Figure 5-7. The actual chlorophyll *a* reading was based on the algae sample from urea medium and Becker's equation. Predicted data was estimated using Equation 5.3 for the validation set. Based on Figure 5-7 the predicted value from Equation 5.3 followed the same trend as the actual chlorophyll *a* concentration. There was a non-zero n absorbance reading in the manure sample with no algae added due to the manure solids.

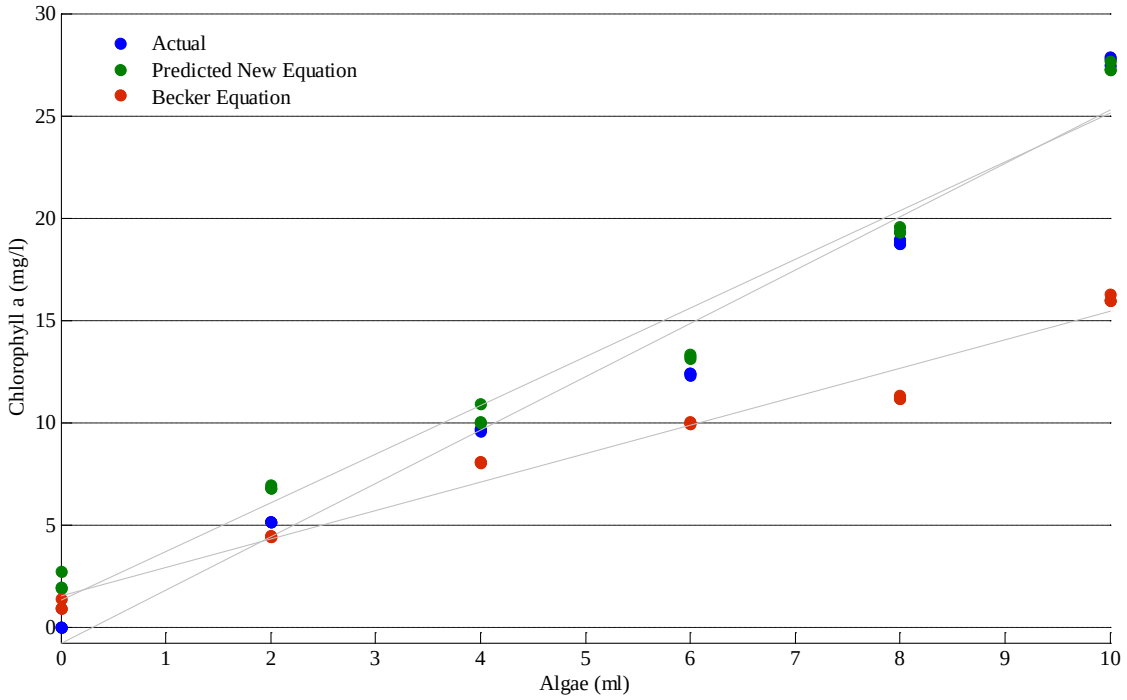

Figure 5-7 Validation data set 2 with 0, 2, 4, 6, 8, and 10 ml of *Chlorella vulgaris* added to 5 ml of dairy manure.

To evaluate the relation between predicted and actual values, chlorophyll *a* concentration from Becker's equation (Equation 4.5) was compared to the new model developed with no intercept (Equation 5.3) for the validation test (Figure 5-8). Slope was tested for the null hypothesis of: Ho: $\beta=1$; Ha: $\beta\neq1$. A t test was calculated using Equation 5.5:

Equation 5.5

Where "b" is the slope, "β" = 1 and "s_b" is the standard deviation of the slope.

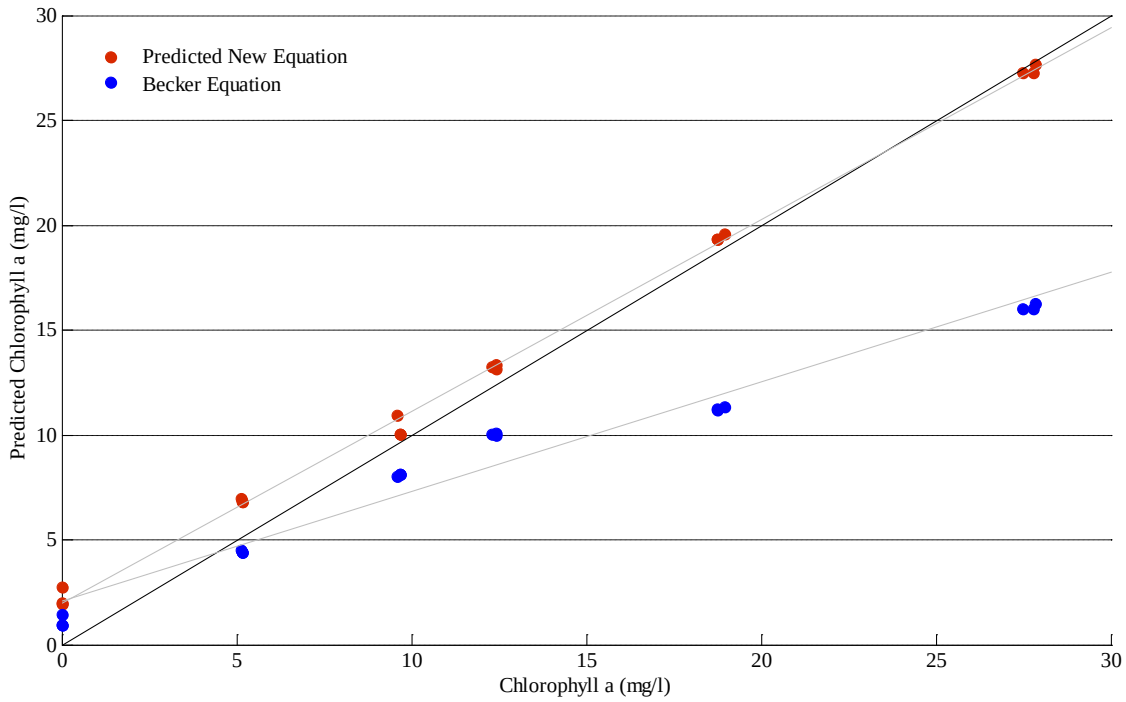

Figure 5-8 Validation data set of the predicted chlorophyll a concentration from *Chlorella vulgaris* in dairy manure using the new equation and Becker's equation.

Using the validation data set, the linear regression using the new equation had an R^2 of 99.1 %, a slope of 1.020 and a standard deviation 0.020. The resulting t statistic was calculated as:

Equation 5.6

For the same validation data set, linear regression was also run using Becker's equation for chlorophyll extracted from manure, in order to compare the new model to the reference. The reference model had an R^2 of 97.3 % with a slope of 0.632 and a standard deviation 0.024. And the resulting t statistic was determined to be:

Equation 5.7

For the validation data set, the reference *t*-statistic was $t_{0.05,18}$ = 2.88. The t-statistic calculated for the validation data set using the model proposed (Equation 5.3) was smaller the critical "t", therefore we fail to reject the null hypothesis (Ho:β=1) and conclude with 95% confidence that the slopes did not differ statistically from 1.

If the slope did not differ from 1, we conclude that the predicted values do not differ significantly from the actual values of Chlorophyll *a* concentration. In conclusion, the new model proposed for estimating Chlorophyll *a* from algae in manure medium presented an acceptable prediction with an independent validation set.

However, when using Becker's model for chlorophyll concentration extracted from algae in manure medium, the *t*-statistic was bigger than the critical *t*, and we reject the null hypothesis (Ho:β=1). We conclude with 95% confidence that the slope of actual versus predicted concentration differed statistically from 1. Since the slope differed from 1, we conclude that the predicted values differed significantly from the actual values of chlorophyll *a* concentration. Becker's model clearly underestimated the chlorophyll concentration when chlorophyll was extracted from algae in the presence of manure solids.

5.3 Conclusions

Chlorophyll concentration extracted from *Chlorella vulgaris* in dairy manure can be predicted. Chlorophyll concentration (mg/l) in ethanol was predicted using the following relationship:

spectrophotometer at high absorbance readings. Based on validation data, we can conclude that the new equation predicted chlorophyll concentrations better than the reference equation, which underestimated values.

Different species of algae are composed of different proportions of chlorophyll *a*, *b*, *c* and *d*. *Chlorella vulgaris* only had peaks evident due to chlorophyll *a*, so the relationship may not hold for other algae species and manure sources that are evaluated in Chapter 6.

CHAPTER 6: DETERMINATION OF THE INFLUENCE OF ALGAE SPECIES AND MANURE TYPES ON THE ESTIMATION OF CHLOROPHYLL CONCENTRATION

The goal of this chapter was to derive an equation/equations that would be used to determine chlorophyll concentration after extraction from mixed manure and algae samples. This would address concerns that manure type and/or the algae strain would influence the chlorophyll extraction procedure.

6.1 Materials and Methods

6.1.1 Algae and Manure Mixing Protocol

Algae inoculum was cultivated in 500 ml Erlenmeyer flasks filled with 300 ml of urea media (Section 4.1.1). Varying volumes of the algae inoculum was added to sample tubes previously filled with 5 ml of manure (Figure 6-1). The same volumes of algae were added to sample tubes without dilution as the control. No water was added to the algae only tubes because the tubes were centrifuged prior to chlorophyll extraction. Each sample tube was performed in triplicate.

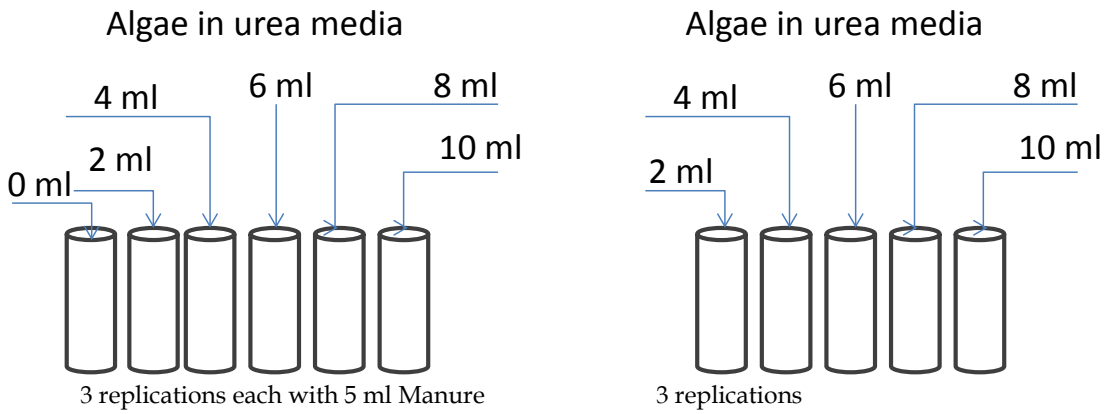

Figure 6-1 Dilution scheme of algae addition to manure prior and only to measuring chlorophyll *a* concentration.

The absorbance of algae and algae diluted in manure was read in a spectrophotometer (Section 4.1.6) after chlorophyll *a* extraction (Section 4.1.7). The

absorbance was measured between the wavelengths of 200 nm to 700 nm. The samples with no manure were used as the reference samples for chlorophyll *a* concentration using ethanol as the solvent and calculated using the equation proposed by Becker (1994) (Equation 5.1):

Equation 5.1

Predicted concentration values for chlorophyll *a* extracted from algae in manure were determined using the equation proposed in Chapter 5 (Equation 5.3):

Equation 5.3

6.1.2 Algae Species

Different algae species and animal manures were used to evaluate the influence of manure solids on chlorophyll extraction and the predicted chlorophyll *a* concentration. Algae species chosen were *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. All three algae are unicellular green algae, containing chlorophyll *a* as their main photosynthetic pigment. The prediction equation developed in chapter 5 (Equation 5.2) was developed for use with algae species that have Chlorophyll *a* as their primary photosynthetic pigment. All algae were grown in urea media prior to diluting with manure. Algae solids concentration of the three species are shown in Table 6-1 and were determined using the dry weight method described in Section 4.1.5.

Table 6-1 Algae solids content of *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. determined using dry weight.

Algae Solids (mg/ml)	***Chlorella vulgaris***	***Cylindrocystis* sp.**	***Scenedesmus* sp.**
	1.2	4.1	1.2

6.1.3 Manure type

Animal manures chosen were dairy, beef, swine and sheep that were collected from the University of Kentucky Research Farms near Lexington, KY. Three of the manure samples were from ruminant animals (dairy, beef, and sheep) and one from a monogastric (swine). In addition to different animal digestive systems, the manure varied in color, consistency and viscosity. Swine manure was a dark liquid without an obvious fiber fraction. The beef and sheep manure were solid with obvious fiber fractions from undigested feed and were diluted with water prior to mixing with algae. Dairy manure was collected from a lagoon and was already diluted and no additional dilution was needed. The total solids content was determined by dry weight (Section 4.1.5) for each manure source and is shown in Table 6-2. Dairy manure 1 and dairy manure 2 were taken from different farms and different seasons, although they had the same solids content.

Table 6-2 Total solids content determined using dry weight of manure samples after dilution with tap water.

	Dairy 1	**Dairy 2**	**Beef**	**Sheep**	**Swine**
TS Manure (mg/ml)	2.50	2.40	11.0	5.00	22.1

6.1.3.1 Dairy Manure

Dairy manure was collected from lagoons and two different farms and was already diluted and no additional dilution was needed (Figure 6-2). The sample was brown/red color and some large solids were present. However, after mixing the sample was relatively homogenous.

Figure 6-2 Sample of dairy manure.

6.1.3.2 Beef Manure

Beef manure was collected from pens at the Beef Research Center. The manure was mostly solid with a combination of bedding, manure solids, and urine. The materials was diluted with tap water and mixed using a vortex (Figure 6-3). In the undiluted sample, it was a dark brown color and a heterogeneous mix of solid fractions. Approximately, 10 g of manure was removed and diluted with 1 liter of tap water.

Figure 6-3 Sample of beef manure in the undiluted (left and top right) and diluted (bottom right) phase.

6.1.3.3 Sheep Manure

Sheep manure was collected from pens at the Sheep Research Facility. The material was solid with a mixture of manure solids, bedding, and urine, diluted inside the laboratory with tap water and mixed using a vortex (Figure 6-4). It presented a light brown color and was a heterogeneous mix of solids. Approximately, 5 g of manure was removed and diluted with 1 liter of tap water.

Figure 6-4 Sample of sheep manure undiluted (left and top right) and diluted (bottom right) phase.

6.1.3.4 Swine Manure

Swine manure was collected as a liquid and no additional dilution was performed. It was black in color and the liquid was very viscous with some small particles (Figure 6-5). Compared to the dairy manure sample that appeared similar to water, swine manure was much more viscous and much darker in color.

Figure 6-5 Sample of swine manure.

6.1.4 Data Analysis

In order to study the model proposed in Chapter 5 different algae species and manure sources were used. Equation 5.3 was used to determine the chlorophyll concentration of the algae samples mixed with manure and was plotted against the actual chlorophyll concentration. The actual chlorophyll concentration was based on Becker's equation from the undiluted algae samples. The prediction was evaluated by testing the intercept and slope of the actual and predicted values. Were the actual and predicted values are defined as:

- Actual: Chlorophyll *a* concentration calculated using Becker's equation with ethanol as the solvent from algae grown in urea media;

- Predicted: Chlorophyll *a* concentration from algae grown in urea media and mixed with 5 ml of manure (swine, dairy, sheep, or beef) calculated using the new equation developed in chapter 5 using ethanol as the solvent.

Analysis of variance was run for each regression to evaluate the linear relation between actual and predicted values. All slopes were compared using the "Multiple Comparison of Slopes" tool in Matlab.

6.2 Results and Discussion

6.2.1 Absorbance of Chlorophyll *a* Extracted From Manure

Chlorophyll was extracted from manure samples following the procedure described in Section 4.1.7 from 3 replications of 10 ml samples from each manure source. Absorbance spectra of the average of the 3 replications from each manure source are presented in Figure 6-6.

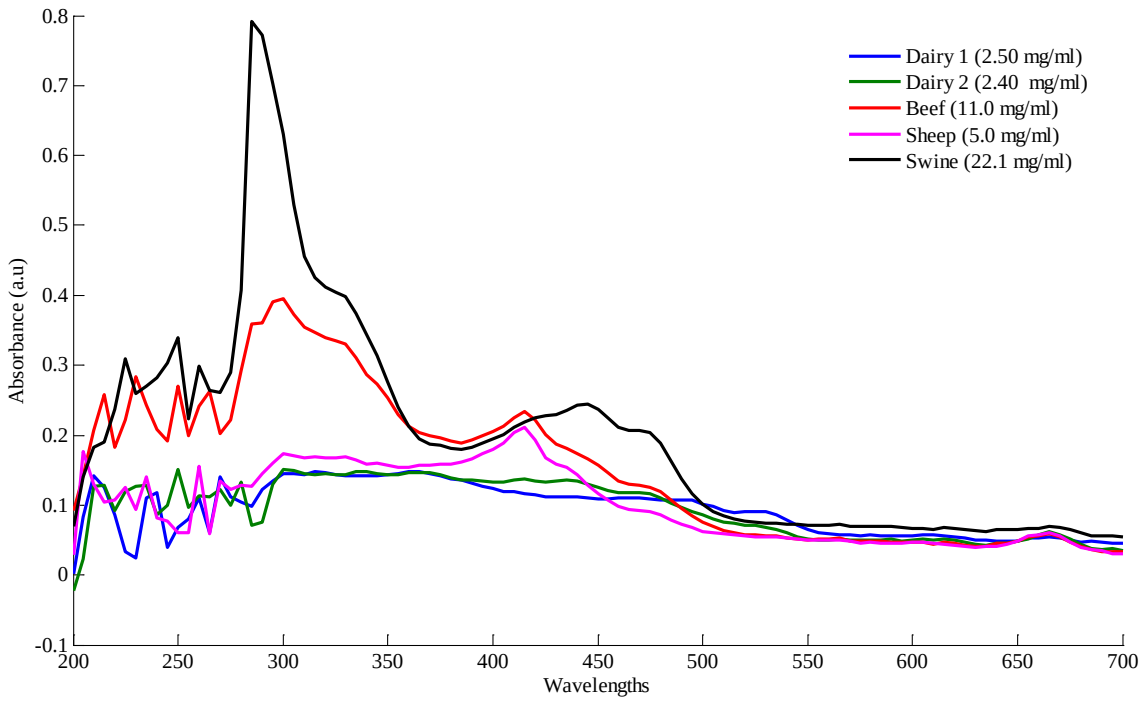

Figure 6-6 Absorbance of chlorophyll a extracted from a 10 ml sample dairy, beef, sheep and swine manure.

The four manure sources presented considerable noise below wavelengths of 280 nm due to the high concentration of suspended solids (Hulst, 1981; Azema et al.,2002; Vaillant et al. 1999). To reduce noise in the short wavelengths, the manure would need additional dilution. Above wavelengths of 300 nm the absorbance was smoother, with peaks at 430 nm, related to carotenoids, observed in swine, beef and sheep (Becker, 1994; Wellburn, 1994). Sheep manure presented a small peak at 665 nm, however with a very low absorbance (less than 0.1 a.u.). Similar trends were observed in all manure sources where the absorbance below 500 nm, curves were smoother with a relatively low absorbance. Based on the spectral absorbance data around 665 nm that corresponds to chlorophyll, it can be concluded that the initial chlorophyll concentration from the raw manure samples was negligible.

6.2.2 Absorbance of Chlorophyll *a* Extracted from Algae

Chlorophyll was extracted from the raw algae samples grown in urea media following the procedure described in Section 4.1.7 from 3 replications of 10 ml samples from each algae species. Absorbance spectra from each algae species were averaged and plotted in in Figure 6-7.

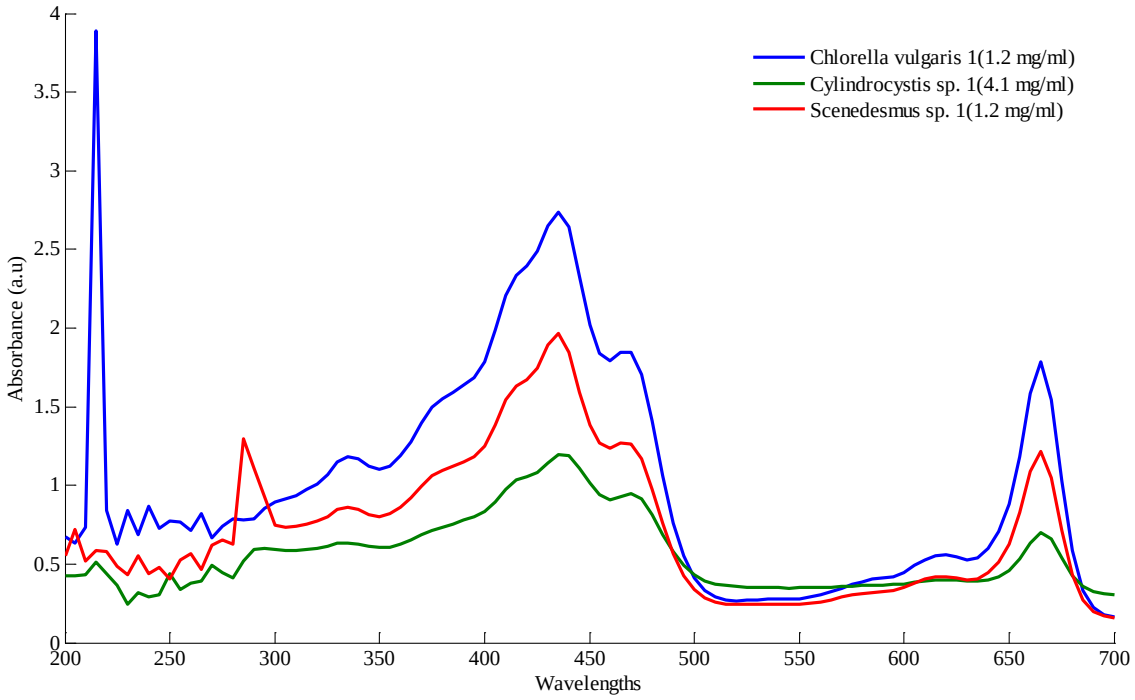

Figure 6-7 Absorbance of chlorophyll a extracted from a 10 ml sample of *Chlorella vulgaris, Cylindrocystis* sp. and *Scenedesmus* sp.

All algae samples had considerable noise in the absorbance values in the UV spectrum (primarily at wavelengths less than 300 nm). This was likely due to interference from suspended solids that could be reduced by additional dilution of the sample (Hulst, 1981; Azema et al., 2002; Vaillant et al. 1999). Additional dilution to reduce noise in the UV spectrum would reduce the sensitivity of the measurement in the 665 nm range where chlorophyll *a* absorbs.

Absorbance of chlorophyll *a* extracted from the three algae presented two peaks, one at 430 nm and one at 665 nm (Figure 6-7). There was also a small peak at 460 nm. The peak at 430 is related to carotenoids and peak at 665 nm is related to chlorophyll *a* (Becker, 1994; Wellburn, 1994).

6.2.3 Manure Source and Algae Species Mixtures

The four manure types and three algae species were numbered according to Table 6-3. The equation developed to predict chlorophyll concentration from each algae strain and manure source was based on the dairy 1 sample with *Chlorella vulgaris*. Dairy 1 and dairy 2 were different dairy manure samples, taken from different farms and different seasons, although they had very similar solids content.

Table 6-3 Numbering scheme for manure types and algae species mixtures.

	Dairy 1	Dairy 2	Beef	Sheep	Swine
Chlorella vulgaris	1	2	7	10	13
Cylindrocystis sp.	3	4	8	11	14
Scenedesmus sp.	5	6	9	12	15

6.2.3.1 *Chlorella vulgaris* in Dairy Manure

The dairy manure used for validation was a different sample then the one used for calibration in chapter 5. Samples were prepared following the procedure shown in Figure 6-1. The reference values (x-axis of Figure 6-8) were taken from *Chlorella vulgaris* grown in urea media, with chlorophyll extracted using ethanol, and the chlorophyll *a* concentration determined using Becker's equation (Equation 5.1). Figure 6-8 shows the predicted value of Chlorophyll *a* concentration from *Chlorella vulgaris* in dairy manure, calculated using the new proposed equation (Equation 5.3). Becker's equation was also used to evaluate the chlorophyll concentration from *Chlorella* in dairy manure. *Chlorella vulgaris* solids concentration used for this test was 1.2 mg/ml as determined using dry weight.

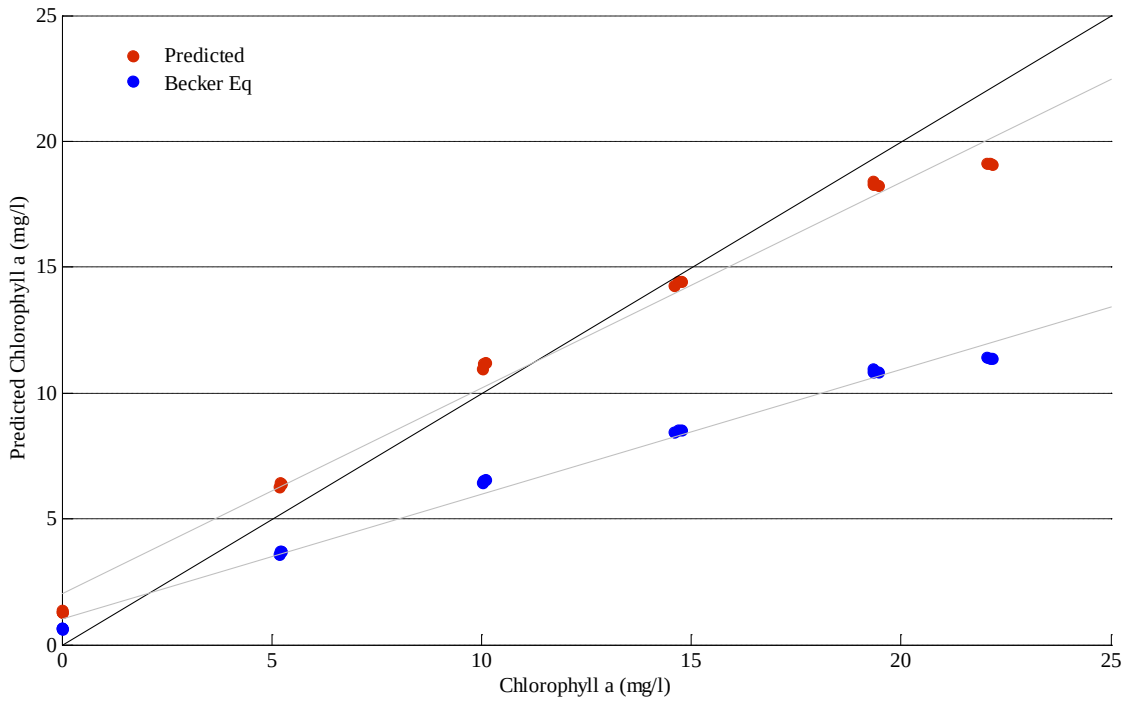

Figure 6-8 Predicted Chlorophyll a concentration after extraction of Chlorella vulgaris from dairy manure 2 showing a 1:1 reference line, predicted value from the proposed equation and Becker's equation.

The predicted values from the proposed equation (Equation 5.3) were close to the 1:1 reference line. Becker's equation (Equation 5.1) had a much smaller slope than the proposed equation and always underestimated the chlorophyll *a* concentration above 5 mg/ml. The poor performance of Becker's equation when used to evaluate chlorophyll concentration in mixed manure and algae samples was likely due to interference from suspended manure solids. It was believed that manure solids were resuspended into the ethanol when the sample tubes were vortexed for the chlorophyll extraction.

Table 6-4 shows the Analysis of Variance of the linear regression between predicted values predicted using the new equation and the actual chlorophyll *a* concentration.

Table 6-4 Analysis of Variance of predicted Chlorophyll *a* concentration from *Chlorella vulgaris* in dairy manure calculated using the proposed equation.

	df	*SS*	*MS*	*F*	*p value*
Regression	1	720.06	720.06	1493.58	0.00
Residual	16	7.71	0.48		
Total	17	727.77			

Since the p-value was smaller than 0.05, the regression was significant and there was a linear relationship between predicted (using the proposed equation) and actual concentration of Chlorophyll *a* extracted from *Chlorella vulgaris* in dairy manure.

6.2.3.2 Predicted Chlorophyll Concentration from Mixtures of Algae and Manure

All combinations of algae strains (*Chlorella vulgaris*, *Cylindrocystis* sp, and *Scenedesmus* sp.) and manure sources (dairy, beef, swine, and sheep) had a linear relation between actual and predicted chlorophyll concentration. This implied that a large part of the variation was being explained by a linear model. However, not all of the comparisons had a linear relationship that followed the 1:1 reference line. The new model proposed in Equation 5.2 would underestimate or overestimate the chlorophyll *a* concentration for some groups of data. Each combination of algae strain and manure source with the proposed equation and Becker's equation are summarized in the Appendix C with a corresponding ANOVA table.

6.2.4 Slope Comparison between Actual and Predicted Chlorophyll Concentration

In order to compare the slope between the actual and predicted chlorophyll concentration, all slopes were analyzed together. The actual concentration was determined using the algae grown in urea with no addition of manure (Figure 6-1) and chlorophyll concentration determined using Becker's equation. The predicted values for algae diluted into manure were determined using the proposed equation (Equation 5.3), developed using *Chlorella vulgaris* in dairy manure 1. The objective of this analysis was

to determine the applicability of Equation 5.3 with other manure types and algae strains. A multiple comparison of slopes test (aoctool ; multcompare) was performed using Matlab (version R2010b, Natick, MA) and the average slope and confidence interval is shown in Figure 6-9. Numbers 1 to 15 are the combinations of algae species and manure source (Table 6-3) described in this section. Number 16 is the 1:1 relation between actual and predicted values.

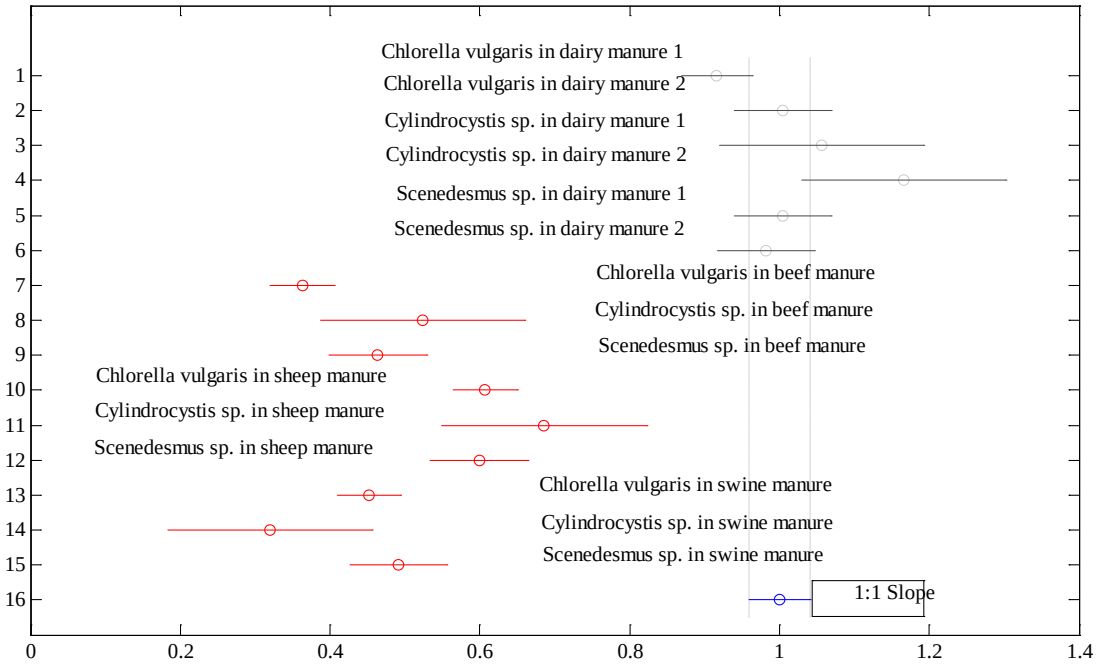

Figure 6-9 Multiple comparison of average slope and 95% confidence interval between predicted and actual chlorophyll concentration from varying manure and algae sources.

According to the analysis shown in Figure 6-9, nine combinations had a slope different than 1 ($\beta \neq 1$) and five combinations had a slope equal to 1 ($\beta = 1$). The five combinations with a slope equal to 1 were all samples with dairy manure. Large standard deviations in the slope were observed with samples containing *Cylindrocystis* sp. in all types of manure, likely due to the high algae solids content of the sample. The slope

comparison indicated that the prediction equation would be a function of manure type. Although, developing an equation for a specific manure source could be possible as seen by the performance using dairy manure. It was believed that the differences were probably due to how the manure particles were resuspended into the ethanol after vortex mixing. The particles suspended were likely interfering with the absorbance and varied by manure type.

6.2.4.1 Slope Comparison by Manure Source

6.2.4.1.1 Dairy

One equation was sufficient to estimate chlorophyll concentration from the three algae species in both dairy manure samples. This can be seen in Figure 6-9 with a slope of one that was within the 95% confidence interval. The comparison of the proposed equation and Becker's equation for each algae species and both dairy samples are given in the Appendix C (Figure C-1 and Table C-1 for *Chlorella vulgaris* in dairy manure 2, Figure C-2 and Table C-2 for *Cylindrocystis* sp. in dairy manure 1, Figure C-3 and Table C-3 for *Cylindrocystis* sp. in dairy manure 2, Figure C-4 and Table C-4 for *Scenedesmus* sp. in dairy manure 1, and Figure C-5 and Table C-5 for *Scenedesmus* sp. in dairy manure 2). The chlorophyll *a* extraction and concentration predicted using the new equation did not differ from the actual chlorophyll *a* concentration present in the dairy manure samples. The new equation proposed to predict chlorophyll *a* concentration in the presence of manure was calibrated using dairy manure and the good prediction was expected when tested with dairy manure. One equation fit the data from three algae species. However, *Cylindrocystis* sp. did have higher variability likely due to the much higher algae solids content of the samples.

6.2.4.1.2 Beef

The three tests that used beef manure and the three algae species had a slope significantly different from one (Figure 6-9). The Chlorophyll *a* concentration predicted using the new equation under predicted the chlorophyll concentration of *Chlorella vulgaris* in beef manure (Figure C-6 and Table C-6 in Appendix C). Different algae

strains in beef manure also had a slope significantly different from one for *Cylindrocystis* (Figure C-7 and Table C-7) and *Scenedesmus* (Figure C-8 and Table C-8). In all cases, Becker's equation when applied to samples with beef manure under predicted the chlorophyll concentration to a greater extent than the proposed new equation. In addition, beef manure with *Chlorella vulgaris* had a slope different from the samples with *Cylindrocystis* sp. and *Scenedesmus* sp. This indicated that algae strain could influence the prediction equation in the presence of beef manure. In all cases the predicted versus the actual was a linear relationship and Becker's equation had a slope less than the slope of the proposed equation.

A number of factors could have contributed to the under prediction with the new equation. Beef manure was diluted inside the lab, with tap water, and mixed with a vortex. It was possible that replicates were not representative due to the heterogeneity of the material. In addition, the solids concentration of the diluted beef manure was 11 mg/ml compared to a solid concentration of 2.5 mg/ml in the dairy manure used to develop the model. Changing the solids concentration of the beef manure could have changed the prediction performance of the new equation.

6.2.4.1.3 Sheep

Algae diluted into sheep manure had slopes that were statistically the same between algae species, but they were statistically different from one. Predicted chlorophyll *a* concentration using the new equation under predicted the actual chlorophyll concentration for *Chlorella vulgaris* (Figure C-9 and Table C-9), *Cylindrocystis* (Figure C-10 and Table C-10) and *Scenedesmus* (Figure C-11 and Table C-11). Becker's equation in the presence of sheep manure under predicted the chlorophyll concentration to a greater extent than the new equation.

Like beef manure, sheep manure was diluted inside the lab, with tap water, and mixed with a vortex. It was possible that the heterogeneity of the material interfered with preparing the replicates and the resulting absorbance readings. Solids concentration of sheep manure (5.0 mg/ml) was higher than dairy manure (2.5 mg/ml), which was another possible factor in the different slopes.

6.2.4.1.4 Swine

In swine manure, the three algae species had slopes significantly different from one. Chlorophyll *a* concentration predicted using the new equation under predicted the actual chlorophyll *a* concentration of each sample, with Becker's equation under predicting to a greater extent (Figure C-12, Table C-12, Figure C-13, Table C-13, Figure C-14, Table C-14). The sample with *Cylindrocystis* sp. had a slope different then the slope from the samples with *Chlorella vulgaris* and *Scenedesmus* sp.

Swine manure had a high solids concentration (22 mg/ml) and was a dark black color, which could have interfered with the absorbance readings. It was possible that the manure needed additional dilution to avoid interference due to the dark color.

6.2.4.2 Observations on Manure and Algae Interactions

In all manure samples, the variation in the slope of *Cylindrocystis* sp. was larger than *Chlorella vulgaris* and *Scenedesmus* sp. Based on data from Figure 6-7, *Cylindrocystis* sp. had a lower absorbance peak at 665 nm than *Chlorella vulgaris* and *Scenedesmus* sp. This would imply that *Cylindrocystis* sp. was lower in chlorophyll *a* than the other strains, especially considering it had the highest algae solids concentration.

Similar slopes were observed for each manure type that indicated a model could be developed based on manure type. Manure type appeared to contribute more variability to the proposed prediction equation than algae type. The proposed equation adequately predicted chlorophyll concentration with dairy manure, but under predicted the actual chlorophyll concentration for the sheep, swine, and beef manure. In all cases, Becker's equation under predicted the data to a greater extent than the proposed equation.

It was believed that differences in suspended manure solids were the reason that one model did not fit all of the data. During chlorophyll extraction, samples are centrifuged to remove the water before ethanol was added. After ethanol was added, the sample was vortexed and incubated for the chlorophyll extraction. The vortex mixer resuspended manure solids and the variations between manure type and concentration likely influenced the absorbance reading.

6.2.5 Performance of Becker's Equation with Manure Samples

According to the slope analysis shown in Figure 6-10, all the 16 combinations of manure sources and algae species had a slope different than 1 ($\beta \neq 1$) when using Becker's equation (the reference equation given in Equation 5.1) to predict chlorophyll concentration in the presence of manure. Large standard deviations in the slope were observed with samples containing *Cylindrocystis* sp. in all types of manure. This data indicated that Becker's equation would not work with algae samples in the presence of manure.

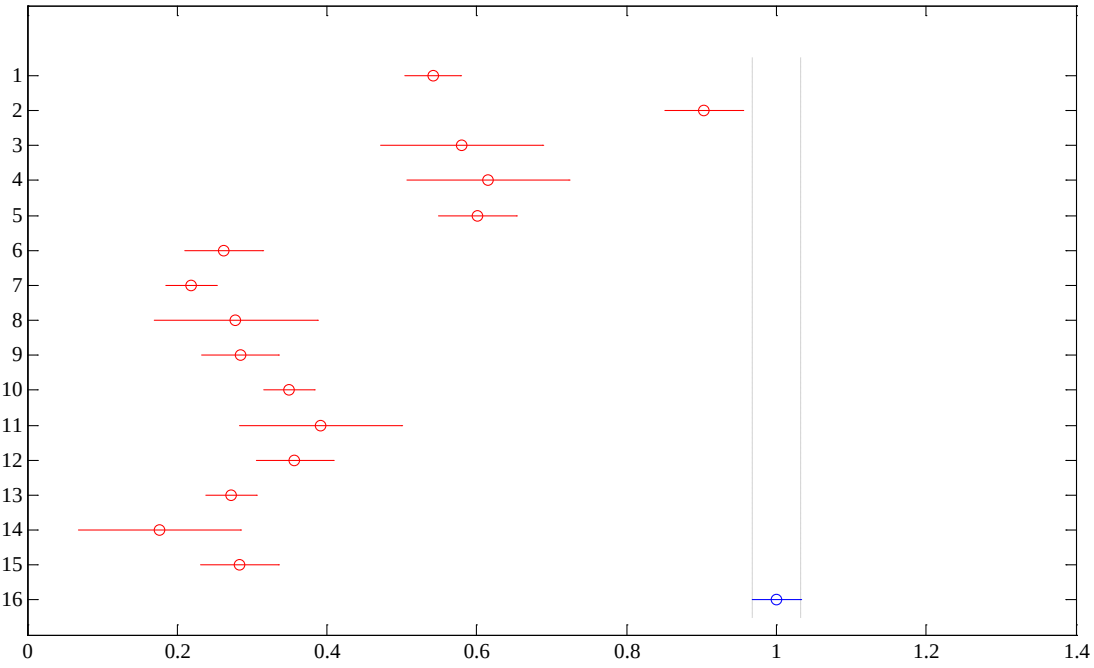

Figure 6-10 Multiple comparisons of average slope and confidence intervals between predicted and actual chlorophyll concentration from varying manure and algae sources calculated using Becker equation.

6.3 New Calibration Equations Based on Manure Type

The equation proposed to predict chlorophyll *a* concentration with manure solids accurately predicted the chlorophyll *a* concentration only with dairy manure samples. It

was expected that the proposed equation would perform well with dairy manure and *Chlorella vulgaris* since the equation was developed with this data set.

However, a linear relationship was observed between the absorption at 665 nm and the chlorophyll *a* concentration in the presence of manure solids. However, the linear relation was not the same for all combinations of algae species and manure source. For this reason, a calibration was developed for each combination of manure source and algae species used in this research. Absorbance readings at 665 nm were plotted against the actual chlorophyll *a* concentration for each combination and a linear regression performed. Due to the linear relationship observed in the data, only absorbance at 665 nm was considered.

6.3.1 Dairy Manure

The calibration equations for *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. in the two dairy manure samples based on the absorbance reading at 665 nm are presented graphically in Figure 6-11. All prediction equations were developed without an intercept term.

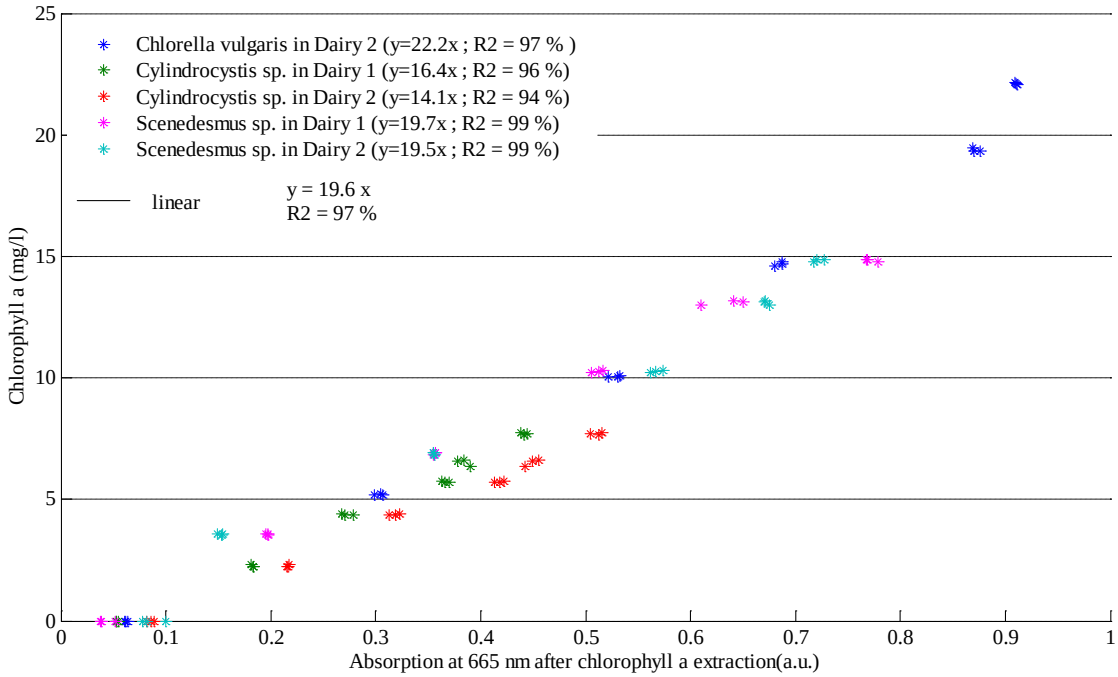

Figure 6-11 Model to predict chlorophyll concentration *Chlorella vulgaris, Cylindrocystis* sp. and Scenedesmus sp. in two dairy manures. (Slope and coefficient of determination is shown in parenthesis for each condition).

The calibration equation for *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. in dairy manure samples using the absorbance reading at 665 nm resulted in a slope of 19.6 and a Pearson coefficient of 97.0 %. The linear relationship between absorbance reading at 665 nm and chlorophyll concentration (mg/l) explains 97% of the variance in chlorophyll concentration (mg/l), using the three algae species in two types of dairy manure.

The non-zero absorbance reading when no algae were added to the sample was due to manure solids. When 2 ml of algae were added to manure, absorbance at 665 nm was approximately 0.2 a.u. A lower absorbance limit of 0.2 a.u. would probably be the recommended lower limit to predict chlorophyll concentration in the presence of dairy manure. The higher limit would be approximately 0.8 a.u. based on the potential outliers at absorbance's greater than 0.8 a.u. However, this could be partially due to the method

where 10 ml of algae were added to 5 ml manure in a 15 ml sample tube. The full sample tubes were centrifuged and supernatant removed. This could have interfered with the centrifugation process and the chlorophyll extraction was not consistent with the other samples..

6.3.2 Beef Manure

The calibration equations for *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. in beef manure samples using the absorbance reading at 665 nm are presented graphically in Figure 6-12. All prediction equations were developed without an intercept term.

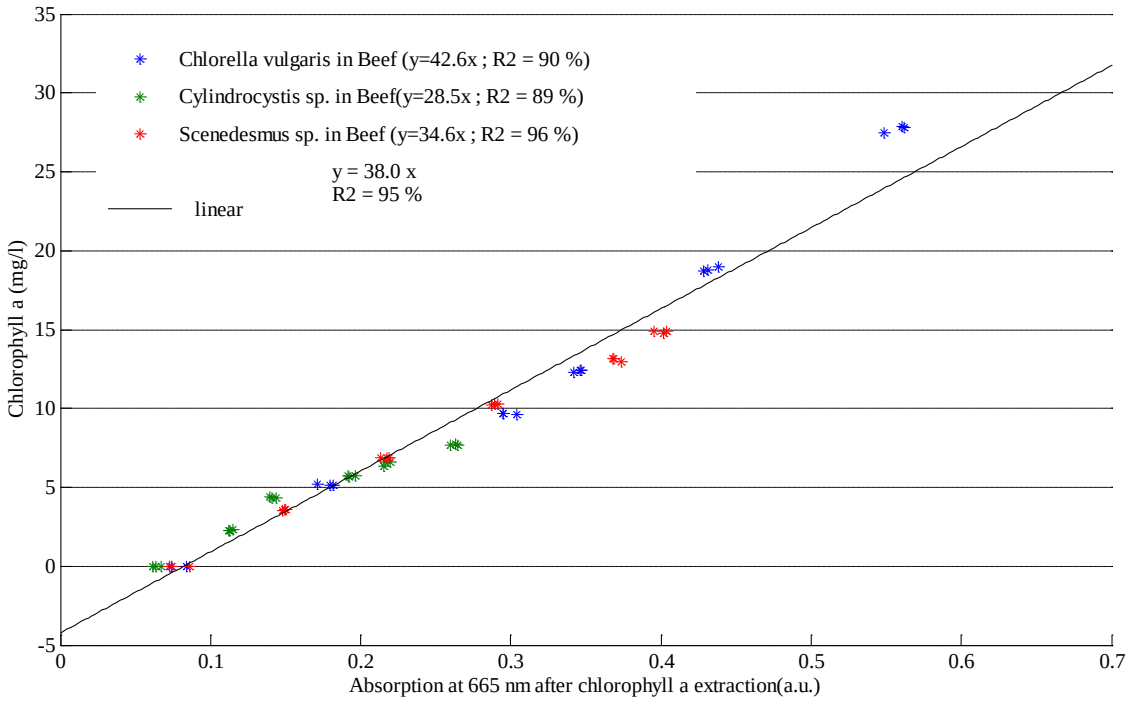

Figure 6-12 Model to predict chlorophyll concentration *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. in beef manure. (Slope and coefficient of determination is shown in parenthesis for each condition.

The calibration equation for *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. in beef manure samples using the absorbance reading at 665 nm resulted in a slope of 38.0 and a Pearson coefficient of 95.0%. The linear relationship between absorbance reading at 665 nm and chlorophyll concentration (mg/l) explains 95% of the variance in chlorophyll concentration (mg/l), using the three algae species in beef manure.

The absorbance reading measured when no algae were added was due to manure solids. When 2 ml of algae were added to manure, absorbance at 665 nm was approximately 0.2 a.u. Readings between 0.2 and 0.6 a.u. are probably most appropriate for measuring chlorophyll concentration in the presence of beef manure.

6.3.3 Sheep Manure

The calibration equations for *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. in sheep manure samples using the absorbance reading at 665 nm are presented graphically in Figure 6-13. All prediction equations were developed without an intercept term.

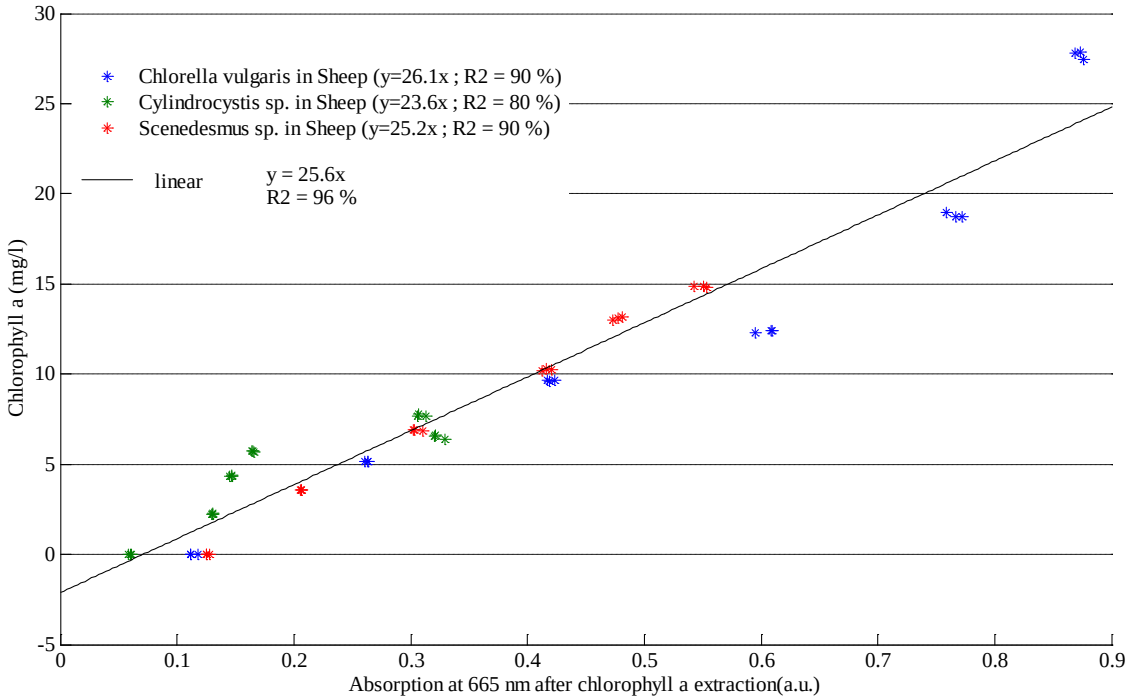

Figure 6-13 Model to predict chlorophyll concentration *Chlorella vulgaris, Cylindrocystis* sp. and *Scenedesmus* sp. in sheep manure. (Slope and coefficient of determination is shown in parenthesis for each condition).

The calibration equation for *Chlorella vulgaris, Cylindrocystis* sp. and *Scenedesmus* sp. in sheep manure samples using the absorbance reading at 665 nm resulted in a slope of 25.6 and a Pearson coefficient of 96.0%. The linear relationship between absorbance reading at 665 nm and chlorophyll concentration (mg/l) explains 96% of the variance in chlorophyll concentration (mg/l), using the three algae species in sheep manure.Based on Figure 6-13, the acceptable limits from measurement of chlorophyll in sheep manure is probably between 0.2 and 0.8 a.u.

6.3.4 Swine Manure

The calibration equations for *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. in swine manure samples using the absorbance reading at 665 nm are presented graphically in Figure 6-14. All prediction equations were adjusted without an intercept term.

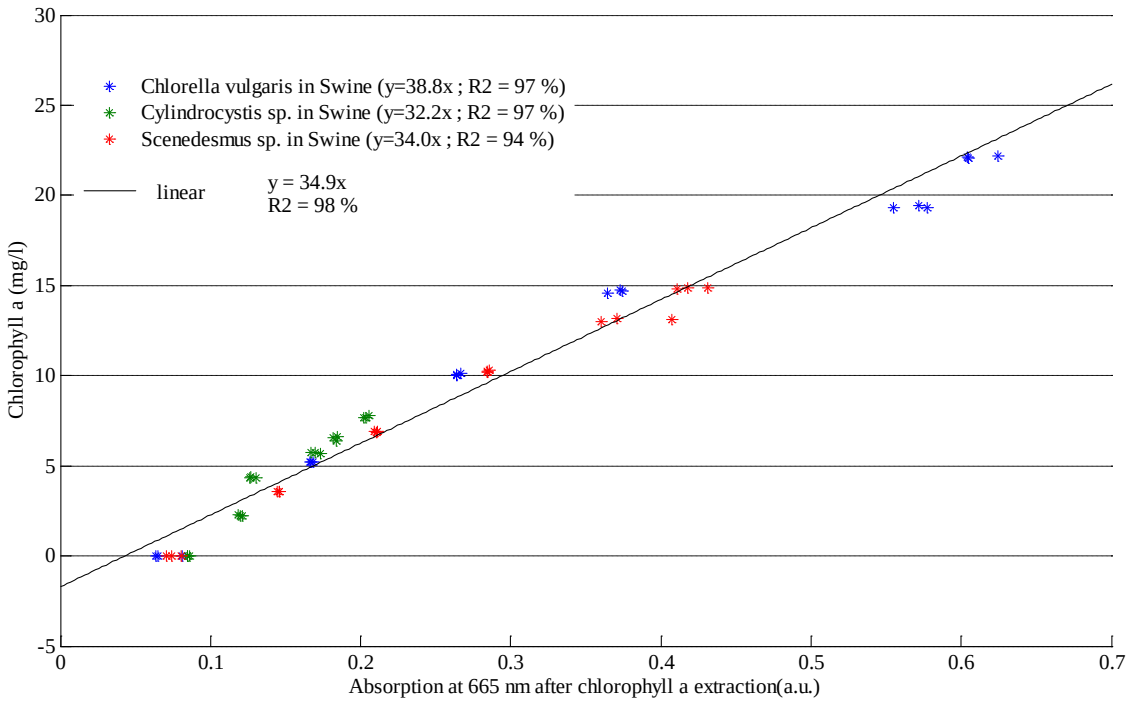

Figure 6-14 Calibration of chlorophyll extraction from *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. in swine manure.

The calibration equation for *Chlorella vulgaris*, *Cylindrocystis* sp. and *Scenedesmus* sp. in swine manure samples using the absorbance reading at 665 nm resulted in a slope of 34.9 and a Pearson coefficient of 98.0%. The linear relationship between absorbance reading at 665 nm and chlorophyll concentration (mg/l) explains 98% of the variance in chlorophyll concentration (mg/l), using the three algae species in swine manure. Based on Figure 6-14, the acceptable measurement range was probably

between 0.2 and 0.8 a.u. that corresponded to a chlorophyll concentration between 5 and 25 mg/ml.

6.3.5 Comparison of Dairy, Beef, Sheep and Swine Manure

The calibrations for each manure type with all three algae's are shown in Figure 6-15.

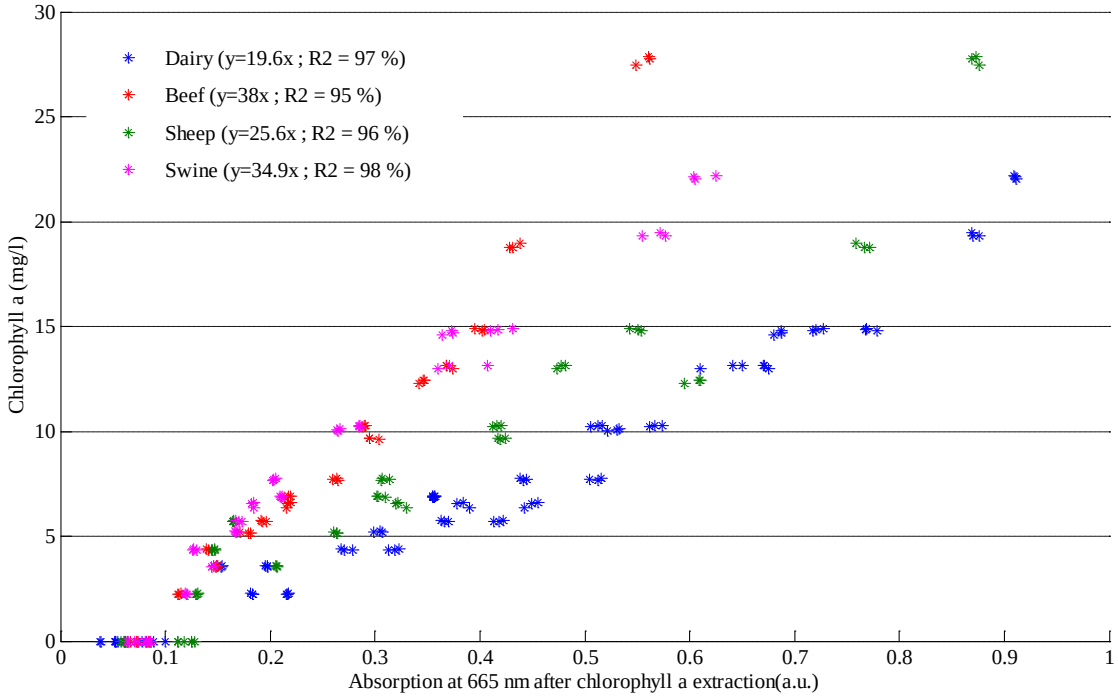

Figure 6-15 Calibration of chlorophyll extraction from dairy, beef, sheep and swine manure.

There was an obvious influence on the chlorophyll extraction due to the type of manure. However, the variations between algae strain were not as significant and one model for a specific manure type could be developed. Only the absorbance at 665 nm was required to develop a calibration model that would explain over 95% of the variation for each manure type. Separate models by manure type would be acceptable since farms will have only one type of manure at a facility.

The variation in the slope due to manure type was probably due to a number of factors. The two dairy manures were from different farms, coincidently with a similar solids content, that had similar calibration models. Dairy manure had the lowest solids content and smallest slope of the manures tested. However, the slope of the calibration was influenced by more than initial manure solids content. Swine manure had the highest solids content (22.0 mg/ml) and a smaller slope (34.9) than beef manure that had a solids content of 11.0 mg/ml and a slope of 38.0.

Other errors were probably introduced since beef and sheep manure were solid samples that were mixed in the laboratory prior to testing. Obtaining representative samples from the solid manure to produce a liquid substance could have introduced sampling errors. Swine manure had a very dark color relative to the other samples that could have interfered with the absorbance readings.

6.4 Conclusions

Mixtures of three algae strains (*Chlorella vulgaris*, *Scenedesmus* sp., and *Cylindrocystis* sp.) were mixed with four manure types (two samples of dairy, beef, sheep, and swine) to evaluate the performance of the proposed equation developed in Chapter 5 (Equation 5.3). It was determined that the proposed equation was only valid for dairy manure. Although, the equation fit all three algae strains with the two types of dairy manure.

The predicted versus actual chlorophyll concentration was linearly related, but the slope was not equal to one in beef, sheep, and swine manure. This implied that the calibration model developed for dairy manure with *Chlorella vulgaris* would not predict the chlorophyll concentration with other manure types. Becker's equation significantly under predicted the chlorophyll concentration for all four manure types and all three algae strains.

Calibration equations were developed for each manure type that successfully predicted the chlorophyll concentration from all three algae strains using the absorbance at one wavelength (665 nm). The chlorophyll concentration had a Pearson coefficient of

97 %, 95 %, 96 %, and 98 % for both types of dairy, beef, sheep, and swine manure, respectively. The slope of the calibration equation was different for all four manure types.

Other methods would need to be applied to handle the potential interference of manure solids on the estimation of algae concentration. Dairy, beef, sheep, and swine manure likely had suspended solids present after chlorophyll extraction that interfered with the absorbance readings. The primary goal is to determine the algae solids content, where chlorophyll concentration is correlated to algae solids. Chapter 7 outlines an alternative method to determine algae solids concentration in the presence of suspended manure solids.

CHAPTER 7: MODELING OF SUSPENDED SOLIDS AND CHLOROPHYLL USING ULTRA-VIOLET SPECTROSCOPY TO CORRECT FOR SOLIDS INTERFERENCE

Animal manure is a heterogeneous material containing a large variety of organic and mineral components. Chlorophyll *a* could be extracted from a mixed sample of manure and algae to determine the algae concentration. However, this resulted in a series of equations specific to the algae and manure source, which limits the usefulness of this technique. It would be preferable to avoid the chlorophyll extraction procedure and measure algae solids directly in the presence of manure solids with other methods.

UV spectroscopy can be used for differentiating solids in a heterogeneous material, but there are numerous difficulties. The analysis of water and wastewater using ultra-violet spectroscopy had difficulties due to interference by physical (e.g. diffuse absorption) and chemical (e.g. overlapping peaks due to competitive absorbance of compounds) processes (Thomas and Cerda, 2007). Unicellular green algae like *Chlorella vulgaris* can have a similar size to some suspended manure solids. Light absorbance in the 290 nm range would not be able to distinguish between algae and manure solids. Hypothetically, other wavelengths could be used to distinguish algae that have chlorophyll *a* and would have different light absorbance characteristics than manure solids. Macromolecular components (e.g. lipids and proteins) and chlorophyll are examples of substances that can be differentiated using UV spectroscopy methods (Azema et al., 2001.; Vaillant et al., 2002; Thomas and Cerda, 2007). The objective of this chapter was to develop an alternative method to determine algae solids in the presence of raw manure samples without extracting chlorophyll.

7.1 Materials and Methods

7.1.1 Materials

Algae inoculum was cultivated in 500 ml Erlenmeyer flasks filled with 300 ml of urea media (Section 4.1.1). Four algae species were used: *Chlorella vulgaris*, *Cylindrocystis* sp., *Scenedesmus* sp., and *Neospongiococcum* sp. All four algae are

unicellular green algae, containing chlorophyll *a* as their main photosynthetic pigment. The algae used to develop the measurement technique were from experiments on two different dates (September 2011 and April 2012) that had different media compositions. The compositions of the media are summarized in Table 7-1 for the September 2011 experiments (data set 1) and the April 2012 experiments (data set 2). The nutrient composition of data set 1 was four times greater than the composition of data set 2.

Table 7-1 Composition of media used for the two data sets.

Ingredient	Data set 1	Data set 2	Units
Urea	1.1123	0.2781	grams
Potassium Phosphate Monobasic	0.2400	0.0600	grams
Magnesium Sulfate Heptahydrate	0.2195	0.0549	grams
Calcium Chloride Dihydrate	0.1144	0.0286	grams
Ethylenediaminetetraacetate	0.0408	0.0102	grams
Tap Water	2	2	liters

Animal manure from dairy, beef, swine and sheep were collected from the University of Kentucky Research Farms near Lexington, KY, and are described in Section 6.1.3. UV absorbance was measured between the wavelengths 200 nm to 700 nm in 5 nm increments using the spectrophotometer described in Section 4.1.6.

7.1.1.1 Sample Preparation

Two data sets were used to develop the procedure. The first data set, “Data Set 1”, was collected in September, 2011. The mixtures of algae and manure were prepared using the same volume of manure (5 ml), varying volumes of algae from 0 to 10 ml and tap water was used to complete a constant 15 ml final volume (Table 7-2). There were a total of 100 samples with dairy, swine, beef, and sheep manure mixed with *Chlorella vulgaris,* and *Scenedesmus* sp. A total of 80 samples were used with dairy, swine, beef, and sheep manure mixed with *Cylindrocystis* sp. and *Neospongiococcum* sp.

Table 7-2 Volume of manure (dairy, beef, swine, or sheep), volume of algae grown in urea medium 1 (*Chlorella vulgaris*, *Scenedesmus* sp., *Cylindrocystis* sp. or *Neospongiococcum* sp.) and tap water added to each mixture for Data Set 1.

Manure (ml)	Algae Added (ml)	Water (ml)	Total Volume (ml)
5	0	10	15
5	2	8	15
5	4	6	15
5	6	4	15
5	8	2	15
5	10	0	15

The second data set, "Data Set 2", was collected during April, 2012. Both manure and algae volumes were varied to create the mixture (Table 7-3). This mixture should represent the conditions expected during algae cultivation where the proportion of manure solids would decrease as the algae solids increase. There were a total of 42 samples with dairy, swine, beef, and sheep manure mixed with *Chlorella vulgaris* and *Scenedesmus* sp. A total of 20 samples with dairy, swine, beef, and sheep manure were mixed with *Cylindrocystis* sp.

Table 7-3 Volume of manure (two dairy samples, swine, beef, or sheep) and volume of algae grown in urea medium 2 (*Chlorella vulgaris, Cylindrocystis* sp. and *Scenedesmus* sp.) used to develop the samples for Data Set 2.

Sample	Manure ml	Algae ml	Water ml	Total ml
Algae	0	10	0	10
Manure	10	0	0	10
Mixture	8	2	0	10
Mixture	6	4	0	10
Mixture	4	6	0	10
Mixture	2	8	0	10

7.1.2 Methods

7.1.2.1 UV Spectral Analysis

Spectral manipulation is a fast method that can be used for qualitative and quantitative analysis of samples (Gallot and Thomas, 1993). When light impinges on a cuvette containing the sample in the spectrophotometer, numerous optical processes occur such as absorption, transmission, reflection, refraction and scattering of light (Burgess 2007). The absorbance measured using a spectrophotometer is based on the Beer-Lambert Law for absorbance of light, and is calculated by:

Equation 7-1

Where "A" is the absorbance of light in absorbance units (a.u.), "I_0" is the intensity of a parallel beam of radiation of wavelength λ incident on a cuvette containing a sample, and "I" the intensity of the emerging beam, attenuated by the absorption process (Burgess 2007). However, the losses due to scattering and reflection are not considered with typical spectrophotometers available in laboratories. The presence of suspended solids and colloids of a heterogeneous material like wastewater cause

scattering effects and interferes with the absorbance readings (Vaillant et al., 2002). Approaches have been developed that would allow for a semi-deterministic deconvolution method that quantifies interferences as well as additional qualitative information included in the spectra shape (Vaillant et al., 2002; Thomas et al., 1996). These studies successfully quantified the concentration of wastewater components, such as organic carbon, chemical oxygen demand, total suspended solids, and nitrates.

The spectra of a mixture can be decomposed as a linear combination of reference spectra in a mathematical process referred to as deconvolution (Gallot and Thomas, 1993; Azema et al, 2002, Thomas and Cerda, 2002; Vaillant et al. 2002; Escalas et al., 2003; Domeizel et al. 2004). The deconvolution of the absorbance was proposed by Thomas et al. (1993) based on the relationship established for each wavelength:

Equation 7-2

Where the absorbance of a sample (A^s) at a specific wavelength (λ_j) can be represented by the sum of the absorbance's of the reference spectra at that wavelength (λ_j) multiplied by a linear coefficient (β_ι) plus an error term (ε_j). The reference spectra would be composed of "p" samples.

According to Gallot and Thomas (1993), the reference spectra can either be a pure component or a mixture of components. The reference spectra for this study were the undiluted manure and algae samples. This would allow for the determination of total solids concentration, in other words algae plus manure solids. Considering an algae sample grown in urea media as reference spectra 1 and a manure sample as reference spectra 2, Equation 7-2 can be written as:

Equation 7-3

This set of equation can be used in matrix form as proposed by Escalas *et al*. (2003) (Equation 7-4).

Equation 7-4

Where “S” is a matrix containing sample spectrums in columns and the corresponding absorbance at specific wavelengths is in “j” rows. The reference spectrum “R” for this study has 2 columns for the two reference spectra for algae and manure. The coefficients (β) were calculated using multiple regression in Matlab for each sample. The samples are the different algae and manure mixtures summarized in Table 7-2 and Table 7-3. Since two reference spectra were used, two coefficients were found for each sample, β_1 and β_2, that are associated to the reference algae and manure samples using Equation 7-5.

Equation 7-5

The spectra of the mixture can be restituted to check the performance of the deconvolution method (Escala et al, 2003). The spectra can be restituted ($\hat{S}$) using Equation 7-6:

Equation 7-6

7.1.2.2 Parameter Estimation

Coefficients estimated by the spectral deconvolution of each sample are used to calculate the desired parameters (total solids concentration). The parameters can be computed with the same linear combination of sample and reference spectra (Equation 7-7).

Equation 7-7

Where “P” is the parameter to be estimated based on the algae and manure references. The parameter “P” to be estimated in this study is the total solids (TS). Total solids of a sample can be calculated as the sum of the total solids of the reference spectra multiplied by the respective coefficient, and Equation 7-7 becomes Equation 7-8:

Equation 7-8

From the reference spectra, algae and manure solids were measured using the dry weight procedure described in Section 4.1.5. For each sample, solids from algae and solids from manure can be calculated by the multiplication of respective coefficients β to find the total solids concentration.

7.2 Results and Discussion

7.2.1 Typical Algae Absorbance Spectra

Absorbance of between 200 and 700 nm for the four algae species from data sets 1 and 2 were plotted (Figure 7-1) to compare the absorbance curves from each species. Algae concentrations were measured using the dry weight method described in Section 4.1.5.

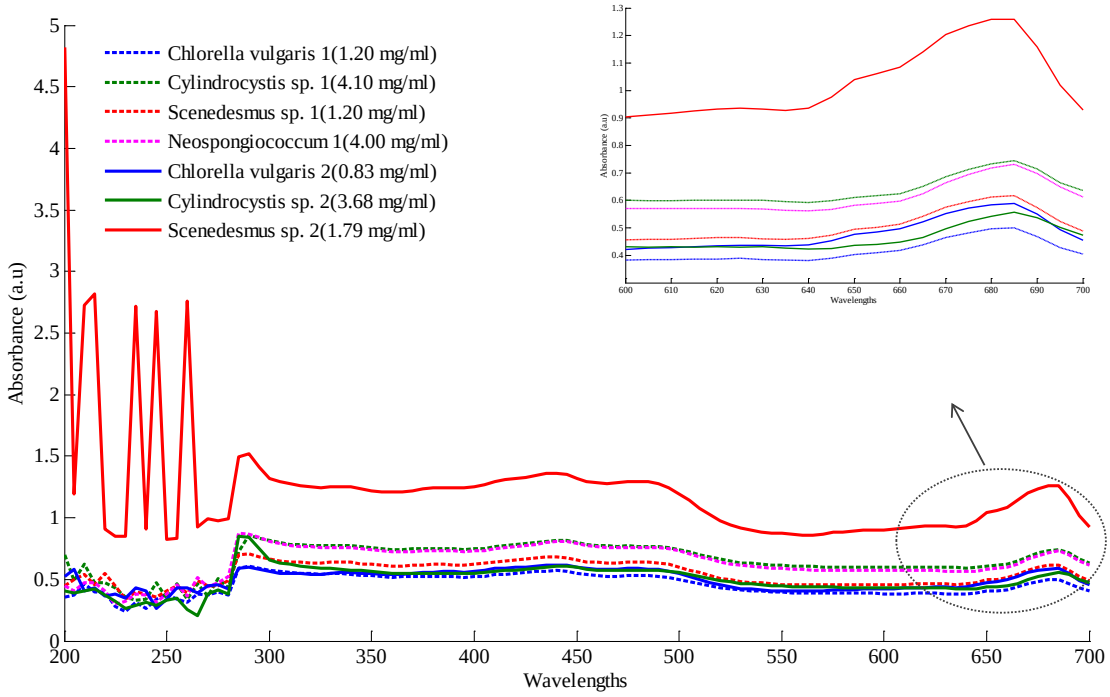

Figure 7-1 Absorbance of raw samples of algae (Chlorella vulgaris, Scenedesmus sp, Cylindrocystis sp and Neospongiococcum sp), from data set 1 and 2 between 200 and 700 nm.

The four algae species studied in this research presented similar absorbance curves, although the magnitude of the absorbance varied. All algae presented absorbance peaks at 290 nm and 680 nm, which are related to suspended solids and chlorophyll, respectively (Hulst, 1981).

The deconvolution method relates the absorbance curve from reference samples to the parameter to be estimated, which for this study were total solids. In order to compare the relation between the absorbance peak and algae solids between algae species, the absorbance at a wavelength of 680 nm were plotted against measured algae solids content (Figure 7-2).

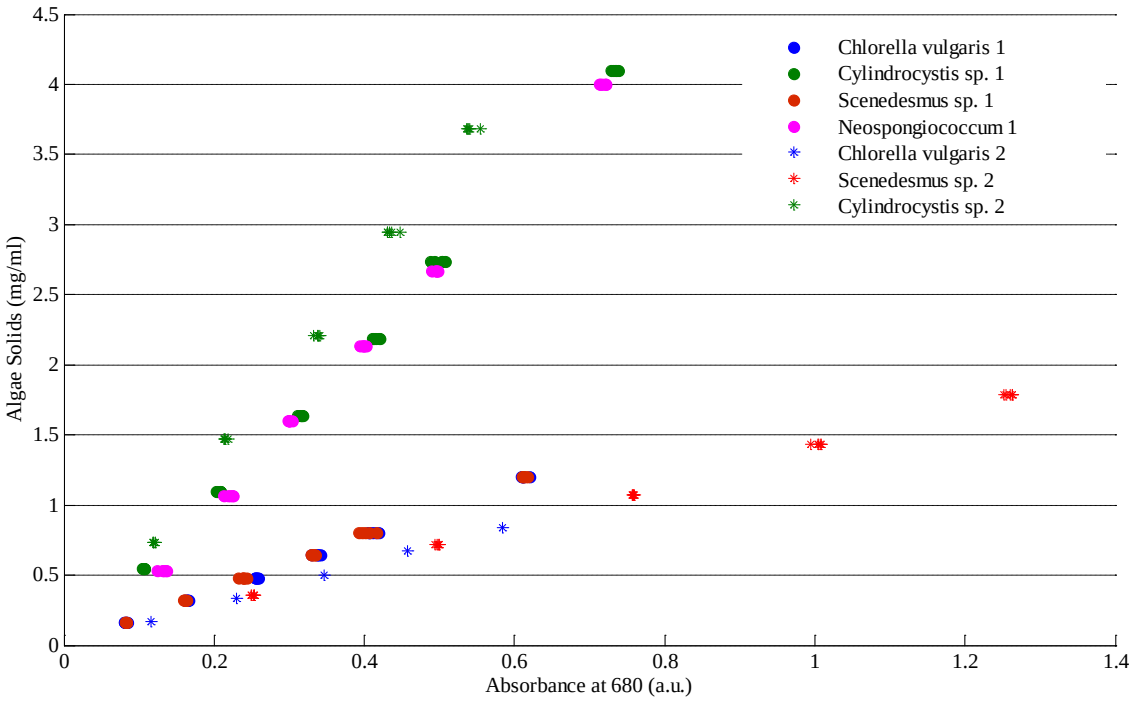

Figure 7-2 Absorbance at 680 nm and algae solids (mg/ml) of *Chlorella vulgaris*, *Cylindrocystis* sp., *Scenedesmus* sp., and *Neospongiococcum* sp. from data set 1 and 2.

The data appeared to group along two trends. *Chlorella vulgaris* and *Scenedesmus* sp. grouped along one line and *Neospongiococcum* sp. and *Cylindrocystis* sp. grouped

along another line. Absorbance at 680 nm is related to the chlorophyll *a* concentration of the algae. The difference in the behavior between the algae could be due to differences in chlorophyll concentration between the species and to the level of algae solids. *Cylindrocystis* sp. and *Neospongiococcum* sp. had higher algae solids concentration (approximately 4 mg/ml) compared to *Chlorella vulgaris* and *Scenedesmus* sp. that had a lower algae solids concentration (approximately 1 mg/ml). The smallest absorbance measured from the raw algae samples was greater than the spectrophotometer lower detection limit.

7.2.2 Typical Manure Absorbance Spectra

Manure sources used in this study were dairy, beef, sheep and swine. The absorbance spectra of each manure source over the wavelength range from 200 to 700 nm are presented in Figure 7-3.

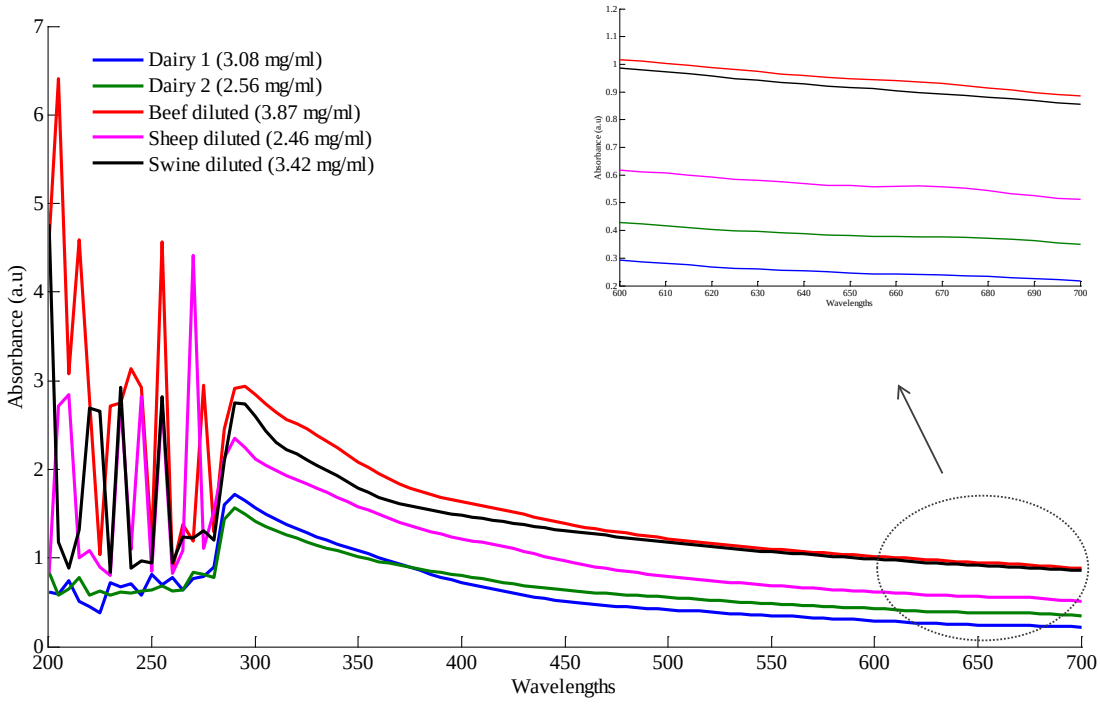

Figure 7-3 Absorbance of dairy, beef, sheep and swine manure, from 200 to 700 nm.

It was noted that the four manure sources studied in this research presented similar absorbance curves, albeit with different absorbance magnitudes. All manure samples presented peaks at 290 nm related to suspended solids and absorbance decreased steadily until 700 nm. The higher absorbance for swine and beef manure can be attributed to the higher solids concentration.

Some signal saturation with the spectrophotometer probably occurred at wavelengths below 280 nm. This wavelength range is used to estimate small particles and nutrients such as nitrates, dissolved organic carbon, BOD and COD (Thomas et al., 1993). Those parameters were not estimated in this study, although if the samples were diluted further they could potentially be determined.

The deconvolution method relates the absorbance curve to the parameter to be estimated, which for this study were total solids. In order to compare the relation between absorbance peaks and manure solids among manure sources, the absorbance at 680 nm was plotted against measured manure solids (Figure 7-4).

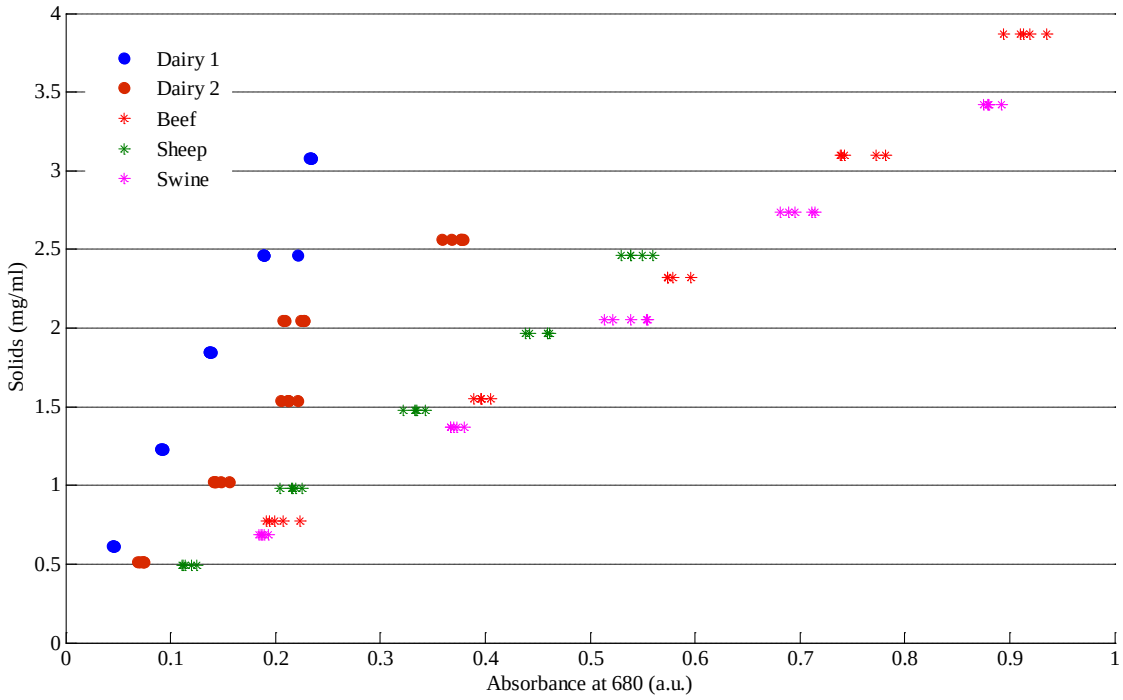

Figure 7-4 Absorbance at 680 nm of manure solids of dairy (two types), beef, sheep and swine manure.

There was a difference between dairy and the other manure sources when comparing the absorbance at 680 nm to the manure solids concentration. The two dairy sources absorbed less than other manures at 680 nm, even with a higher solids concentration. The three other manure sources, beef, sheep and swine, presented similar relation between solids concentration and absorbance at 680 nm. The lowest absorbance reading was greater than the lower detection limit of the spectrophotometer.

Manure solids from each source were also plotted against absorbance at 290 nm and results are presented in Figure 7-5. The absorbance at 290 nm was related to suspended solids and was used to estimate the concentration of manure solids.

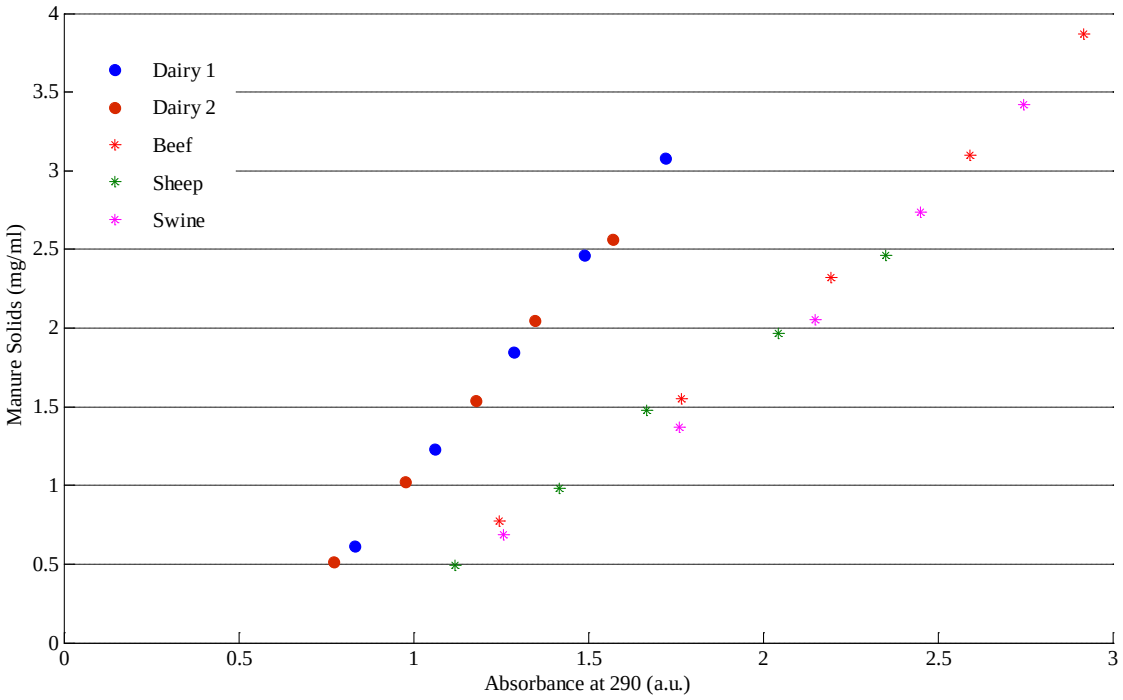

Figure 7-5 Absorbance at 290 nm of manure solids of dairy, beef, sheep and swine manure.

There was a difference between dairy and the other manure sources when comparing the absorbance at 290 nm and the manure solids concentration. The two dairy sources absorbed less than other manure at 290 nm even in higher solids concentration. The three other manure sources, beef, sheep and swine, presented a similar relation between solids concentration and absorbance at 290 nm. The spectrophotometer had a linear detection range up to 3.5 a.u. that was within the measured absorbances shown in Figure 7-5.

7.2.3 Reference Absorbance Spectra

The spectra used as the reference for manure was swine manure containing 3.42 mg/ml total solids from data set 2. The behavior of the absorbance curve was very similar among manure sources. However, beef and sheep manure were diluted inside the laboratory and were less homogeneous than dairy and swine manure, which were already

liquid samples and were not initially used as reference samples. Swine manure was chosen as the reference relative to dairy manure, because the swine manure had a higher absorbance and solids concentration, therefore it should represent a larger range of samples.

The spectra used as a reference for algae solids concentration was *Scenedesmus* sp. grown in urea media containing 1.79 mg/ml total solids from data set 2. This algae was chosen because it had a higher solids concentration and had a similar absorbance behavior to *Chlorella vulgaris*.

Since the main objective was to estimate algae concentration, wavelengths in the range from 600 to 700 nm were chosen as the reference. The highest peak around 680 nm was related to algae solids and was not found in manure spectra. The difference between the spectra of the reference samples (Figure 7-6) at this range helps the deconvolution process when predicting the concentration of the mixed sample.

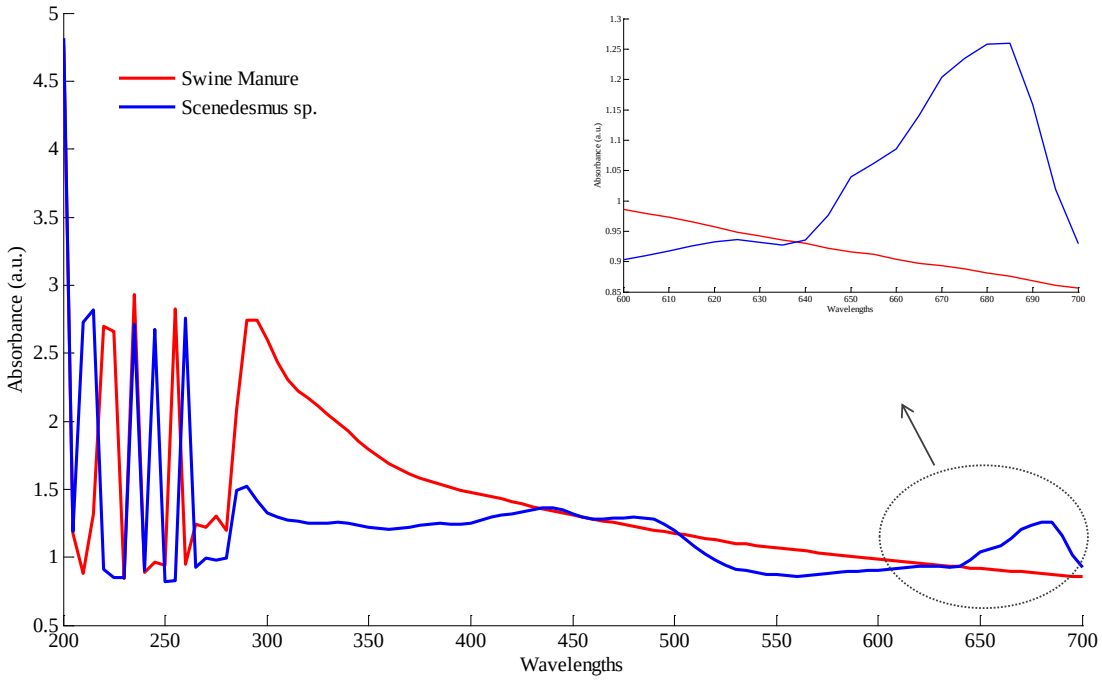

Figure 7-6 Reference spectra of swine manure and algae *Scenedesmus* sp. from data set 2 (April 2012).

7.2.4 Predicted Algae Solids Concentration

7.2.4.1 Predicted Algae Concentration from Data Set 1

A set of 100 samples were used to predict algae solids concentration. The 100 samples included different mixtures (Table 7-2) of *Chlorella vulgaris* in dairy, beef, sheep and swine manure; and *Scenedesmus* sp. in dairy, beef, sheep and swine manure. The reference spectra used were presented in Figure 7-6, for swine manure and *Scenedesmus* sp. from data set 2, over the wavelengths from 600 to 700 nm. Predicted algae solids are presented in Figure 7-7.

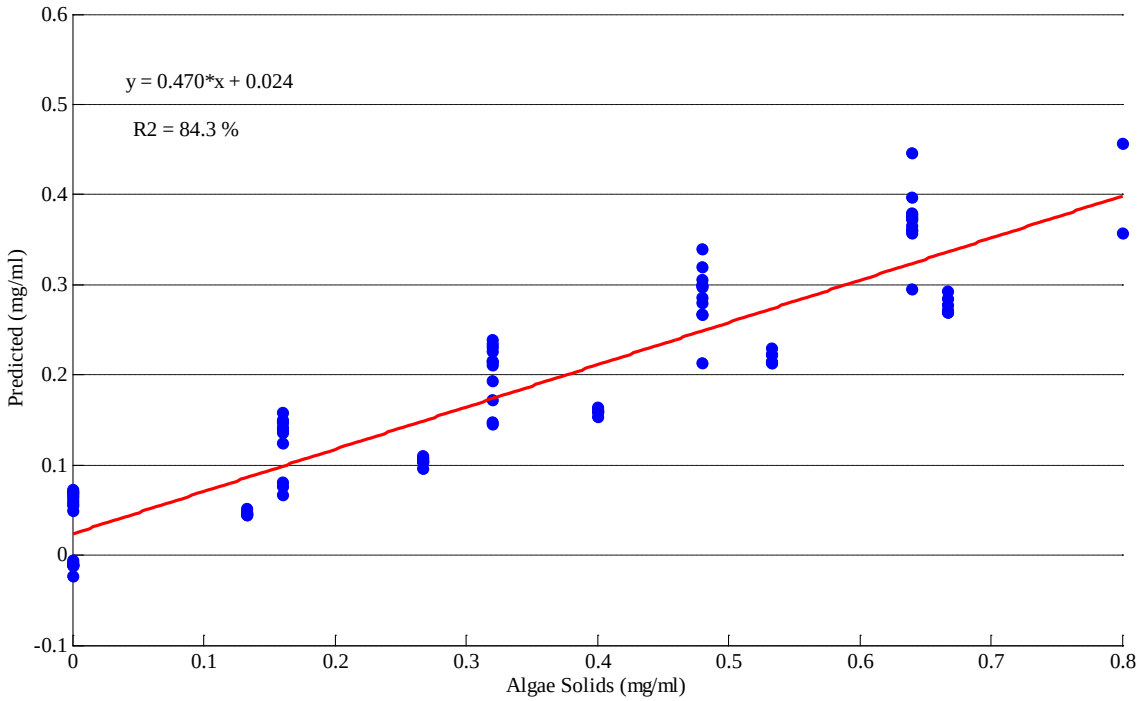

Figure 7-7 Predicted algae solids concentration of 100 samples from data set 1 using swine manure and *Scenedesmus* sp. as reference spectra from data set 2.

The predicted algae solids concentration underestimated the actual algae solids. This difference was probably due to the differences between algae concentration and absorbance of algae used as reference (data set 2) and the algae used in data set 1 (Figure

7-8). The algae from data set 2 had a higher absorbance for the same quantity of algae solids.

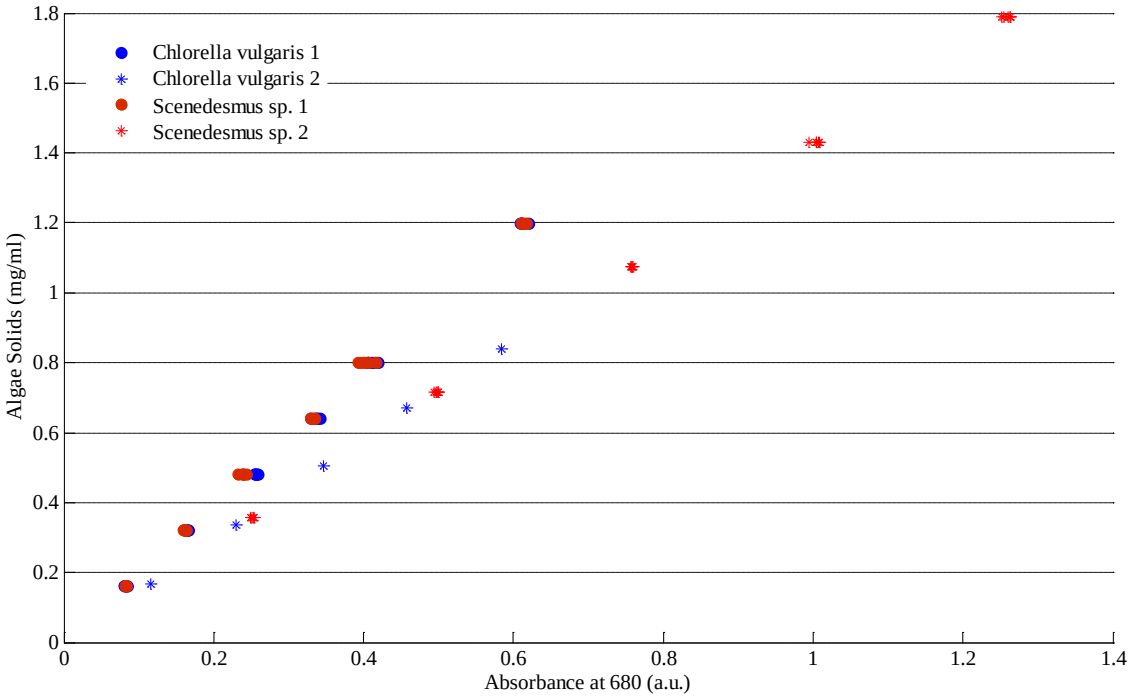

Figure 7-8 Relation between algae solids and absorbance for *Chlorella vulgaris* and *Scenedesmus* sp. from data set 1 and 2.

The reference manure and algae were from data set 2 collected on April, 2012, and the 100 samples tested were from data set 1, collected on September 2011. Algae have been continually cultivated since September 2011 and some genetic evolution could have occurred during that time period that would result in changed spectral absorbance characteristics. Urea media used in the laboratory for algae cultivation had different proportions of nutrients between data set 1 and data set 2, as shown in Table 7-1. Allen and Smith (1969) found evidence of nitrogen chlorosis in blue-green algae that changed the concentration of the phycocyanin that would also change the absorption characteristics around 680 nm. Nitrogen chlorosis is yellowing of plants due to insufficient nitrogen and the plant does not produce enough chlorophyll. Since the media

background was not used as a reference sample, changes in the media could have also influenced the spectral absorbance.

From Figure 7-8, it was possible to observe that the relation between algae solids concentration and the absorbance peak at 680 nm was very similar for *Chlorella vulgaris* and *Scenedesmus* sp. from the same data set. For this reason spectra of algae from the different data sets probably represent different reference sample spectra. Therefore, to predict algae solids concentration in samples from data set 1, a reference algae sample from data set 1 should be used.

7.2.4.2 Predicted Algae Concentration from Data Set 1 Using Reference from Data Set 1

Reference spectra for algae were selected from data set 1 and replaced the reference data initially used from data set 2. Reference spectra from data set 1 used swine manure and *Scenedesmus* sp., due to their higher absorbance and solids concentration compared to the other manure sources and algae species from data set 1 and consistency with data set 2. The absorbance of the reference sample spectra from data set 1 is presented in Figure 7-9, for swine manure and *Scenedesmus* sp. between the wavelengths from 600 to 700 nm.

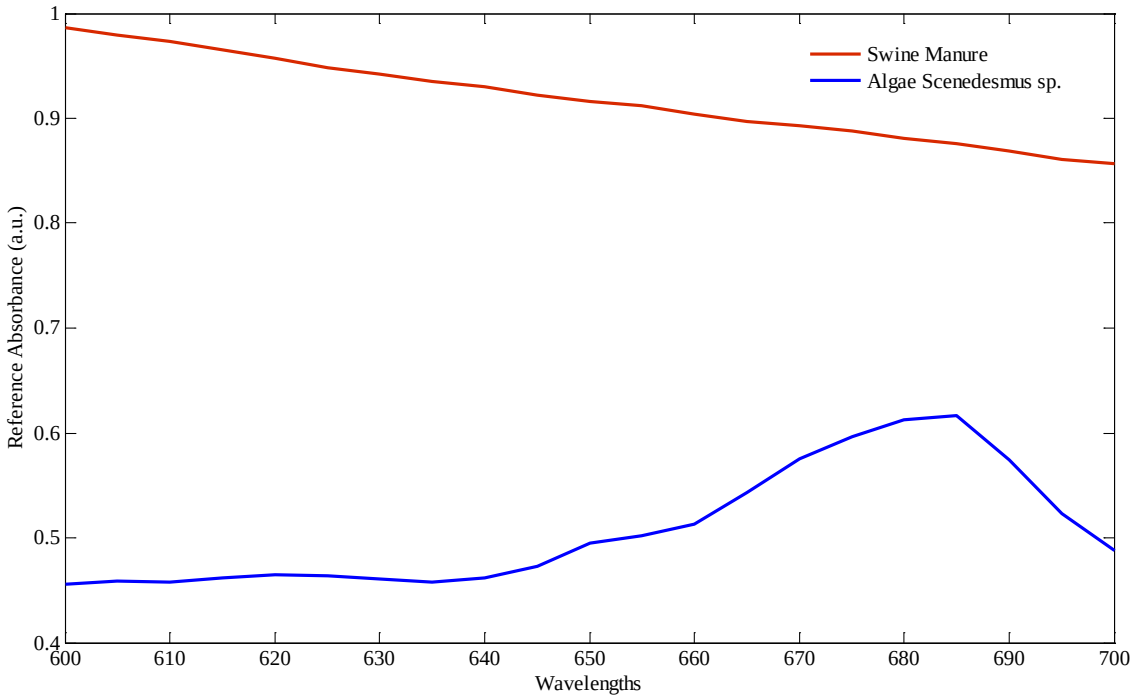

Figure 7-9 Reference absorbance spectrum from data set 1, with swine manure and *Scenedesmus* sp.

The spectra from the 100 manure and algae samples comprising data set 1 were used in the deconvolution method, using the new reference spectra presented in Figure 7-9. The predicted algae solids concentration for data set 1 is shown in Figure 7-10.

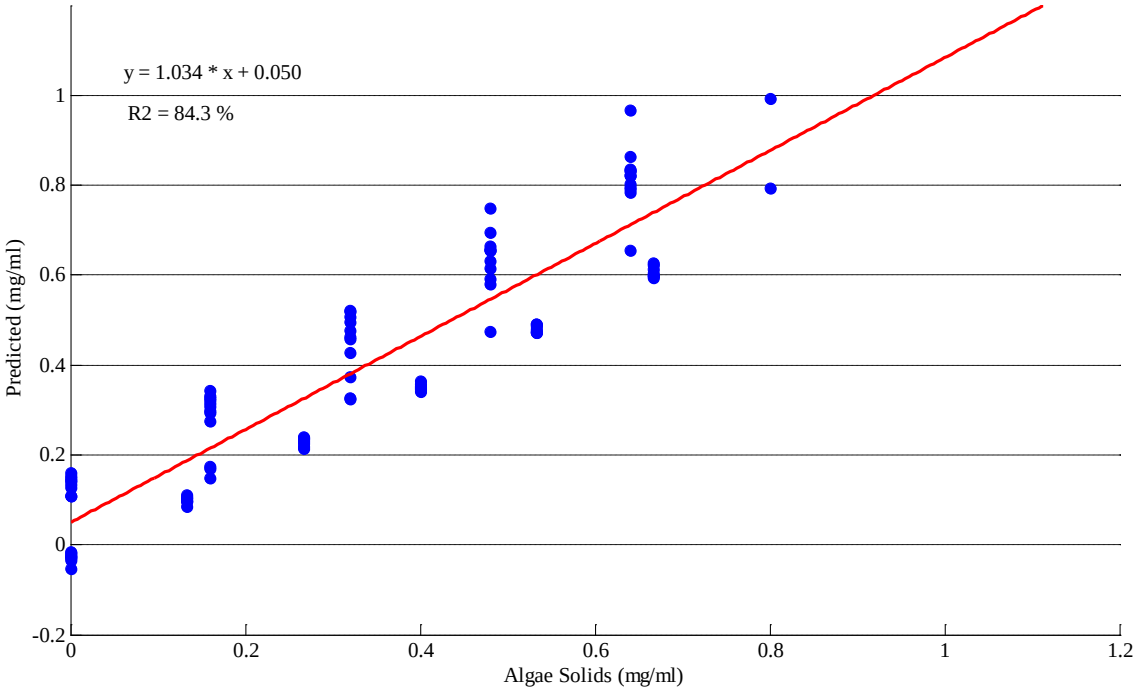

Figure 7-10 Predicted algae solids of 100 samples from data set 1, using reference spectra from data set 1.

A linear regression of the estimated versus actual algae solids concentration was performed using *Chlorella vulgaris* and *Scenedesmus* sp. in dairy, beef, swine, and sheep manure. Predicted algae solids were correlated to the actual values. The slope between the predicted and actual algae solids concentration approached 1 (1.034) and the intercept was near zero (0.0495 mg/ml). The Pearson coefficient was 84.3%. Parameter estimates, standard error, and t-statistic are presented in Table 7-4.

Table 7-4 Regression summary for the predicted versus actual algae solids using samples and reference spectra from data set 1, with *Chlorella vulgaris* and *Scenedesmus* sp. in dairy, beef, sheep and swine manure.

	Coefficients	*Standard Deviation*	*t Stat*	*P-value*
Intercept	0.050	0.019	2.632	0.009
Slope	1.034	0.045	22.978	0

Slope was tested for the null hypothesis of: Ho: $\beta=1$; Ha: $\beta\neq1$. A t test was calculated using Equation 5.5.

Equation 7.9

Where "b" is the slope, "β" = 1 and s_b is the standard deviation of the slope.

Equation 7.10

Reference "t" statistic was $t_{0.05,99}$ =1.98. The "t" statistic calculated for the regression was smaller than the critical "t", therefore we fail to reject the null hypothesis (Ho:$\beta=1$) and conclude with 95% confidence that the slope did not differ statistically from 1. The slope represents the estimate change in the predicted value when actual value increased by one unit. If the slope did not differ from 1, we conclude that the predicted values did not differ significantly from the actual values of algae solids.

The intercept was tested for the null hypothesis of: Ho: $\alpha=0$; Ha: $\alpha\neq0$. A t test was performed and the t statistic was calculated using Equation 7.11:

Equation 7.11

Where "a" is the intercept, "α" = 0 and "s_a" is the standard deviation of the intercept.

Testing the intercept resulted in a t stat of 2.656, while the reference t statistic was $t_{0.05,99}$ =1.98 (Table 7-4). The "t" statistic calculated for the regression was greater than the critical "t", therefore we reject the null hypothesis (Ho: α=0) and conclude with 95% confidence that the intercept did differ statistically from 0. An intercept different from zero represented a constant predicted value when the actual value was zero.

Algae concentrations desired within photobioreactors are around 1 mg/ml (need a reference). This would likely be the upper limit desired for prediction. A realistic detectable lower limit for the algae concentration would be 0.2 mg/ml that would correspond to the logarithmic growth phase (not sure if this is true, but add a reference if it is).

The change in reference spectra for an algae spectra from the same data set resulted in an accurate prediction of algae solids for all combinations of manure sources (dairy, beef, sheep and swine) and algae species (*Chlorella vulgaris* and *Scenedesmus* sp.).

7.2.4.3 Predicted Algae Concentration from Data Set 2 using Reference from Data Set 2

Data set 2 comprised the spectra of 42 samples of algae and manure samples to determine the algae solids concentration. The reference spectra used were presented in Figure 7-6, using swine manure and *Scenedesmus* sp. with the absorbance between 600 and 700 nm. The set of samples included different mixtures (Table 7-3) of *Chlorella vulgaris* in beef, sheep, swine, and two samples of dairy manure; and *Scenedesmus* sp. in two samples of dairy manure. The prediction of algae solids concentration is illustrated in Figure 7-11.

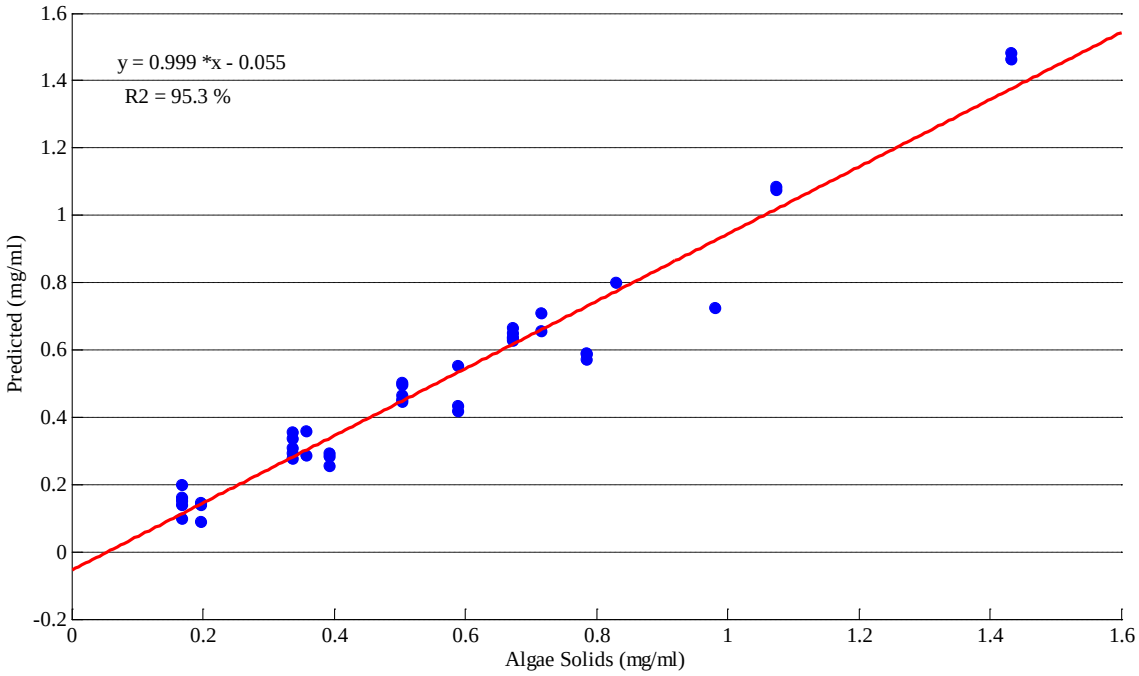

Figure 7-11 Predicted algae solids of from *Chlorella vulgaris* in dairy (2 samples), beef, sheep and swine manure and *Scenedesmus* sp. in dairy (2 samples) manure, using swine and *Scenedesmus* sp. as algae the reference.

A linear regression was performed on the estimated versus actual algae solids concentration of the data shown Figure 7-11. Predicted algae solids were very close to the actual values. The slope approached 1 (0.999) and the intercept was near zero (-0.055 mg/ml). The Pearson coefficient was 95.3 % and the parameter estimates are summarized in Table 7-5. The data that appear to be below the linear regression line (red line) represents 10 samples of *Scenedesmus* sp. which were diluted with tap water before mixing to manure.

Table 7-5 Regression summary for the predicted versus actual algae solids using samples and reference spectra from data set 2, with *Chlorella vulgaris* and *Scenedesmus* sp. in dairy, beef, sheep and swine manure.

	Coefficients	*Standard Deviation*	*t Stat*	*P-value*
Intercept	-0.055	0.022	-2.500	0.018
Slope	0.999	0.035	28.543	0.000

The slope parameter was tested for the null hypothesis of: Ho: $\beta=1$; Ha: $\beta\neq1$. The t test was calculated using Equation 5.5:

Equation 7.12

The reference "t" statistic was $t_{0.05,41}$ =2.02. The "t" statistic calculated for the regression was smaller the critical "t", therefore we fail to reject the null hypothesis (Ho:$\beta=1$) and conclude with 95% confidence that the slope did not differ statistically from 1. Since the slope did not differ from 1, we conclude that the predicted value of the algae solids concentration did not differ significantly from the actual value.

The estimate and t statistic for the intercept is given in Table 7-5. The "t" statistic calculated for the regression was slightly bigger than the critical "t", therefore we reject the null hypothesis (Ho:$\alpha=0$) and conclude with 95% confidence that the intercept did differ statistically from 0. If the confidence level was reduced to 90%, the reference "t" statistic became 1.68, and the intercept would be statistically zero.

The absorbance relationship at 680 nm with algae solids concentration was very similar between *Chlorella vulgaris* and *Scenedesmus* sp. as illustrated in Figure 7-12a. This indicated that using *Scenedesmus* sp. as a reference for both *Chlorella vulgaris* and *Scenedesmus* sp. was appropriate. The reference manure used, swine, had a similar absorbance curve as the other manure sources used (Figure 7-12b), although the magnitude of the absorbance value varied with manure solids concentration. This

similarity allowed for only swine manure to be used as a reference sample to represent all four manure types used.

Data set 1 had a smaller Pearson coefficient relative to data set 2. This was probably due to two reasons: difference in solids content of the urea medium between experiments and data set 1 had a wider range of samples. Suspended fertilizer and minerals in urea medium 1 would have been measured using the dry weight. This would have resulted in the algae solids concentration being over predicted by a constant quantity. Higher concentrations of minerals could have also changed the absorbance characteristics of the medium and/or the composition of the algae in a non-linear fashion. The quantity and diversity of samples from data set 1 was much broader. Data set 1 contained 100 samples from four algae species and four manure types. Data set 2 only contained 42 samples and *Scenedesmus* was only added to dairy samples.

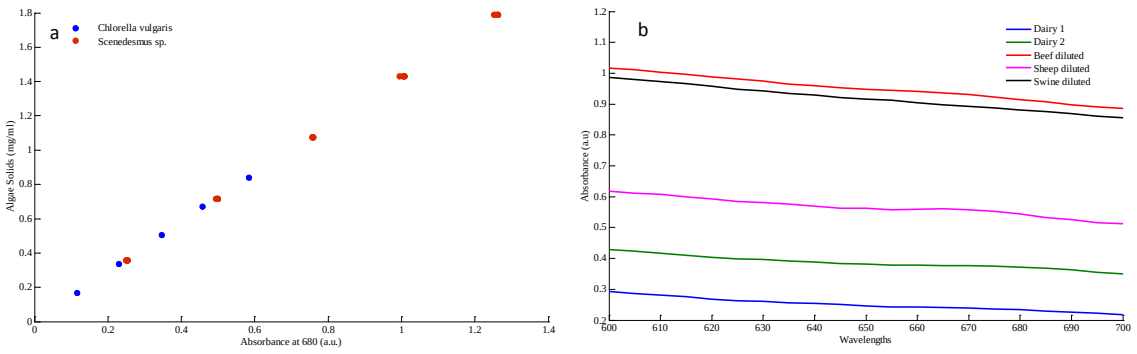

Figure 7-12 Relation between absorbance at 680 nm and algae solids of Chlorella vulgaris ans Scenedesmus sp (a); Manure sources (dairy 1, dairy 2, beef, sheep, swine) spectra from 600 to 700 nm (b).

7.2.4.4 Prediction of *Cylindrocystis* sp. and *Neospongiococcum* sp.

Algae species *Cylindrocystis* sp. and *Neospongiococcum* sp. presented a similar relationship between algae solids concentration with an absorbance peak at 680 nm (Figure 7-2). The reference spectra chosen for predicting algae solids concentration was *Cylindrocystis* sp., only this reference was used to predict the algae solids concentration

for samples with *Cylindrocystis* sp. and *Neospongiococcum* sp. The reference algae (*Cylindrocystis* sp.) from data set 1 were used because this sample had the highest solids concentration and absorbance when compared to *Cylindrocystis* sp. from data set 2 and *Neospongiococcum* sp. from data set 1. It was believed that this sample would be more useful as a reference due to its higher solids concentration. The reference spectrum for manure was the same swine manure from data set 2 that was also used for the *Chlorella* and *Scenedesmus* sp. mixtures. Figure 7-13 illustrates the reference spectra for *Cylindrocystis* sp. from data set 1 and swine manure from data set 2.

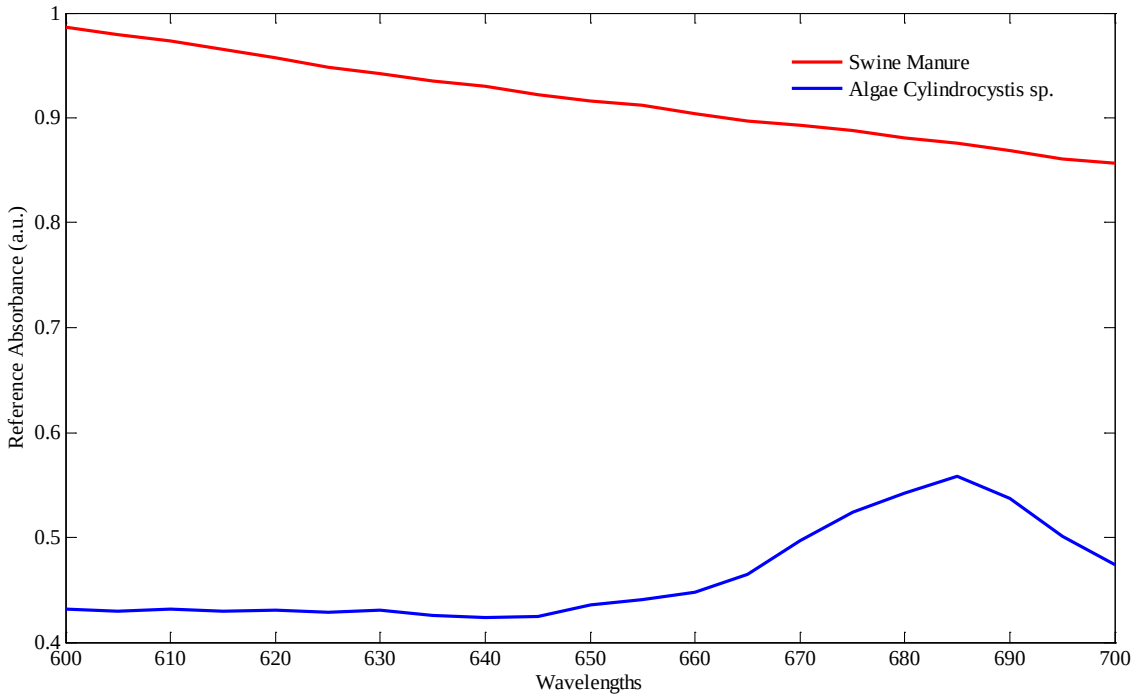

Figure 7-13 Reference spectra of swine manure from data set 2 and algae Cylindrocystis sp from data set 1.

The spectra from the 100 samples from data set 1 were used to estimate algae solids from *Cylindrocystis* sp. and *Neospongiococcum*. The 100 samples included different mixtures (Table 7-2) of *Cylindrocystis* sp. in dairy beef, sheep and swine

manure; and *Neospongiococcum* in dairy, beef, sheep and swine manure, from both data sets. Predicted algae solids are presented in Figure 7-14.

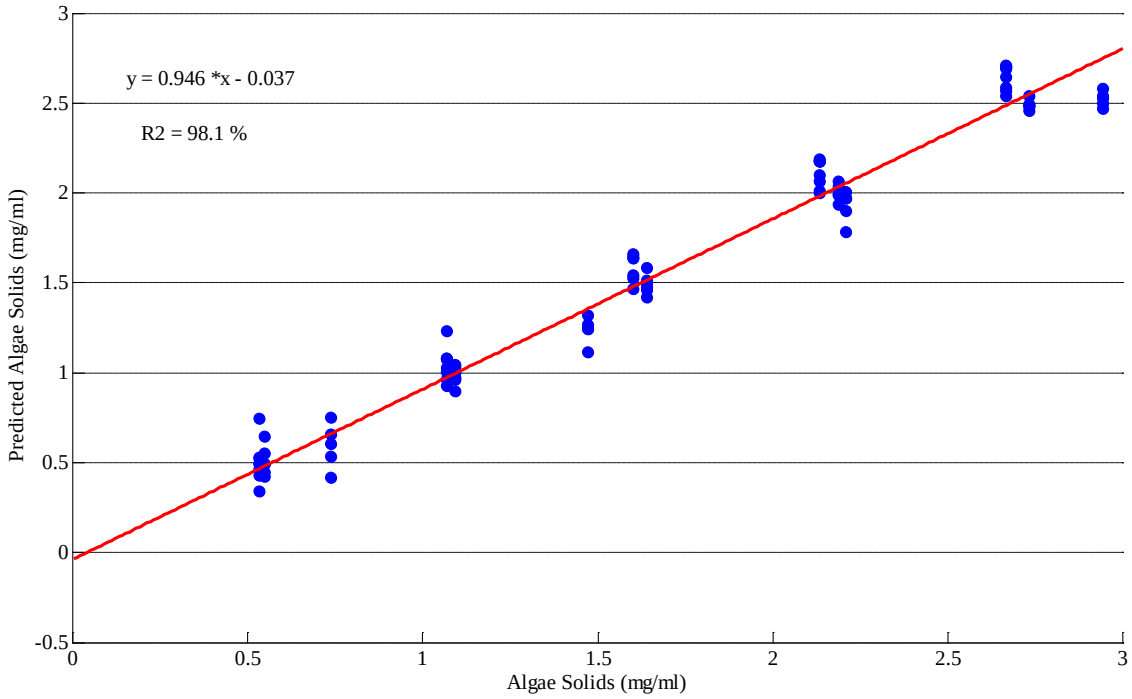

Figure 7-14 Predicted algae solids of *Cylindrocystis* sp. and *Neospongiococcum* sp. in dairy, beef, sheep and swine manure from both data set.

A regression of the estimated versus actual algal solids concentration was performed for all samples from data set 1 and 2. Predicted algae solids were very close to the actual values. The slope approached 1 (0.946) and the intercept approached zero (-0.037 mg/ml) with a Pearson coefficient of 98.1%. The results for the parameter estimation are presented in Table 7-6.

Table 7-6 Regression summary for the predicted versus actual algae solids using samples from data sets 1 and 2 with *Cylindrocystis* sp. and *Neospongiococcum* sp. in dairy, beef, sheep and swine manure[1].

	Coefficients	*Standard Deviation*	*t Stat*	*P-value*
Intercept	-0.037	0.023	-1.609	0.111
Slope	0.946	0.013	71.769	0.000

[1]Reference samples were swine manure (data set 2) and *Cylindrocystis* (data set 1).

Slope was tested for the null hypothesis of: Ho: $\beta=1$; Ha: $\beta\neq1$. A t test was calculated using Equation 5.5.

Equation 7.13

The "t" statistic was $t_{0.05,99}$ =1.98. The "t" statistic calculated for the regression was larger than the critical "t", therefore we reject the null hypothesis (Ho:$\beta=1$) and conclude with 95% confidence that the slopes do differ statistically from 1. Since the slopes differ from 1, we conclude that the predicted values differ significantly from the actual algae solids value.

Deconvolution was performed on the samples grouped by data set. Swine manure from data set 2 was the reference manure for both data sets, but the algae reference was *Cylindrocystis* from each respective data set. Actual and predicted algae solids concentration was plotted in Figure 7-15. Samples were grouped by data set and the differences between slopes were tested using Tukey's test at the 95% confidence interval. The slope comparison between predicted and actual algae concentration are presented in Table 7-7.

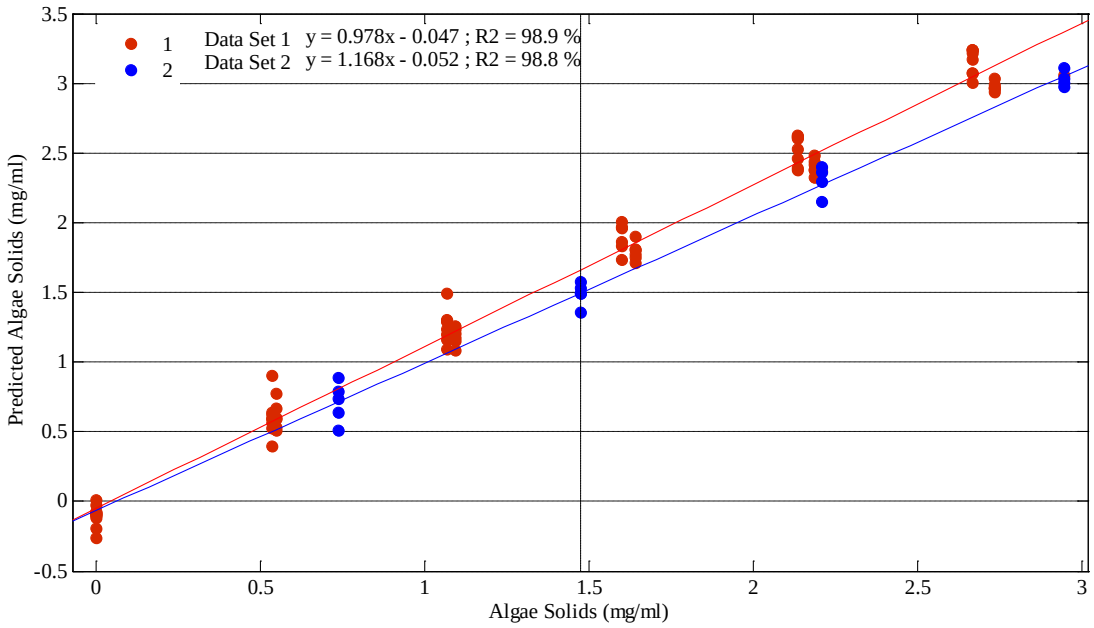

Figure 7-15 Slope comparison between predicted values of algae solids (*Cylindrocystis* sp. and *Neospongiococcum* sp.) from data set 1 and 2, using *Cylindrocystis* sp. from the from the respective data set and *Neospongiococcum* sp. and dairy, beef, sheep and swine manures.

The slopes from the two data sets were different. This matched the behavior seen with *Chlorella vulgaris* and *Scenedesmus* sp. that the algae reference should be from the current data set. Using an algae culture from data set 2 (April, 2012) as the reference for data set 1 (September, 2011) would not provide accurate estimates

A regression of the estimated versus actual algae solids concentration was run for 80 samples with *Cylindrocystis* sp. and *Neospongioccocum* sp. from data set 1. Predicted algae solids were very close to the actual values. The slope approached 1 (0.978) and the intercept approached zero (-0.047 mg/ml), with a Pearson coefficient of 98.9%. The results for the analysis of variance are presented in Table 7-7.

Table 7-7 Regression summary for the predicted versus actual algae solids using samples and reference spectra from data set 1, with *Cylindrocystis* sp. and *Neospongiococcum* in dairy, beef, sheep and swine manure from data set 1.

	Coefficients	*Standard Deviation*	*t Stat*	*P-value*
Intercept	-0.047	0.019	-2.474	0.019
Slope	0.978	0.012	81.500	0.000

Slope was tested for the null hypothesis of: Ho: $\beta=1$; Ha: $\beta\neq1$. A t test was calculated using Equation 5.5 and was found to be:

Equation 7.14

The reference "t" statistic was $t_{0.05,80}$ =1.99. The "t" statistic calculated for the validation data set was smaller than the critical "t", therefore we fail to reject the null hypothesis (Ho:$\beta=1$) and conclude with 95% confidence that the slope did not differ statistically from 1. Since the slope did not differ from 1, we conclude that the predicted values are statistically the same as the actual value of algae solids.

The intercept for the regression is given in Table 7-7 as "t Stat=-2.405" with a reference "t" statistic of 1.99 ($t_{0.05,80}$). The "t" statistic calculated for the regression was bigger than the critical "t", therefore we reject the null hypothesis (Ho:$\alpha=0$) and conclude with 95% confidence that the intercept did differ statistically from 0. Although, from a practical standpoint 0.047 mg/ml of algae solids was a relatively small fraction considering the samples were raw manure and algae.

Algae concentration desired inside a reactor is 1 mg/ml. Cylindrocystis sp and Neospongiococccum achieve much higher concentration during growth in enrlenmeyer flasks. The predictions presented in samples if no algae are due manure solids. A lower limit of 0.5 mg/ml of algae is recommended.

7.2.5 Manure Solids Estimation

7.2.5.1 Effect of Reference Spectra on Manure Solids Estimation

Manure solids were estimated for the 42 samples described in Section 7.2.5 (mixtures of *Chlorella vulgaris* in beef, sheep, swine, and two samples of dairy manure; and *Scenedesmus* sp. in two samples of dairy manure). The reference spectra for the swine manure and *Scenedesmus* sp. was presented in Figure 7-6 between the wavelengths from 600 to 700 nm. Deconvolution was performed and the relevant coefficients determined to predict manure solids in Figure 7-16.

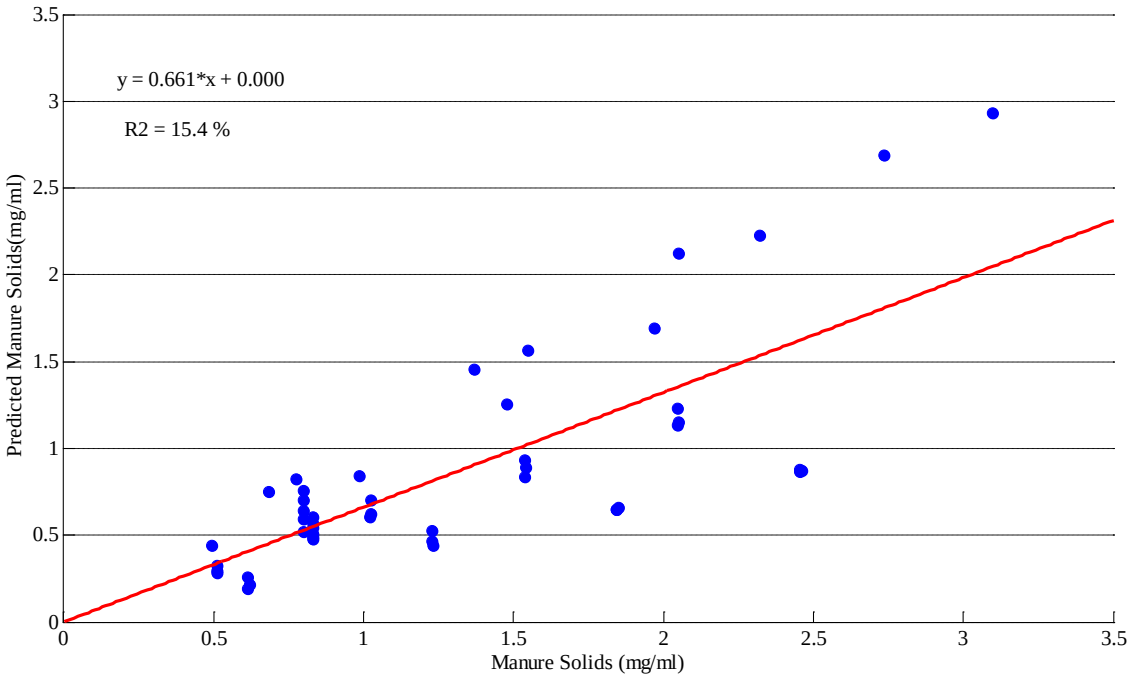

Figure 7-16 Predicted manure solids for dairy 1, dairy 2, beef, sheep and swine manure, from 600 to 700 nm.

A regression of estimated versus actual values of manure solids was performed for the 42 samples. Predicted values were scattered and the Pearson coefficient was very low (15.4%). It was possible that the wavelength range chosen was not responsive to manure solids. The range from 600 to 700 nm was used due to the peak around 680 nm

related to chlorophyll predict algae concentration. In order to predict manure solids, a different wavelength range needs to be chosen.

7.2.5.2 Manure Solids Estimation Using Different Wavelength Ranges

In order to estimate manure solids, the absorbance at wavelengths between 280 and 350 nm were investigated. According to (Azema, 2002; Sarraguca, 2009), absorbance at 290 nm was related to suspended solids. To evaluate the deconvolution method, the absorbance between 280 and 350 nm was used to capture the peak at 290 nm and avoid the noise below 280 nm. The absorbance below 280 nm was very noisy (Figure 7-1) and these wavelengths did not provide information related to manure or algae solids concentration. The objective was to pick the peak that represents manure solids concentration. The reference spectra representing manure solids (swine) and algae solids (*Scenedesmus* sp.) are shown in Figure 7-17.

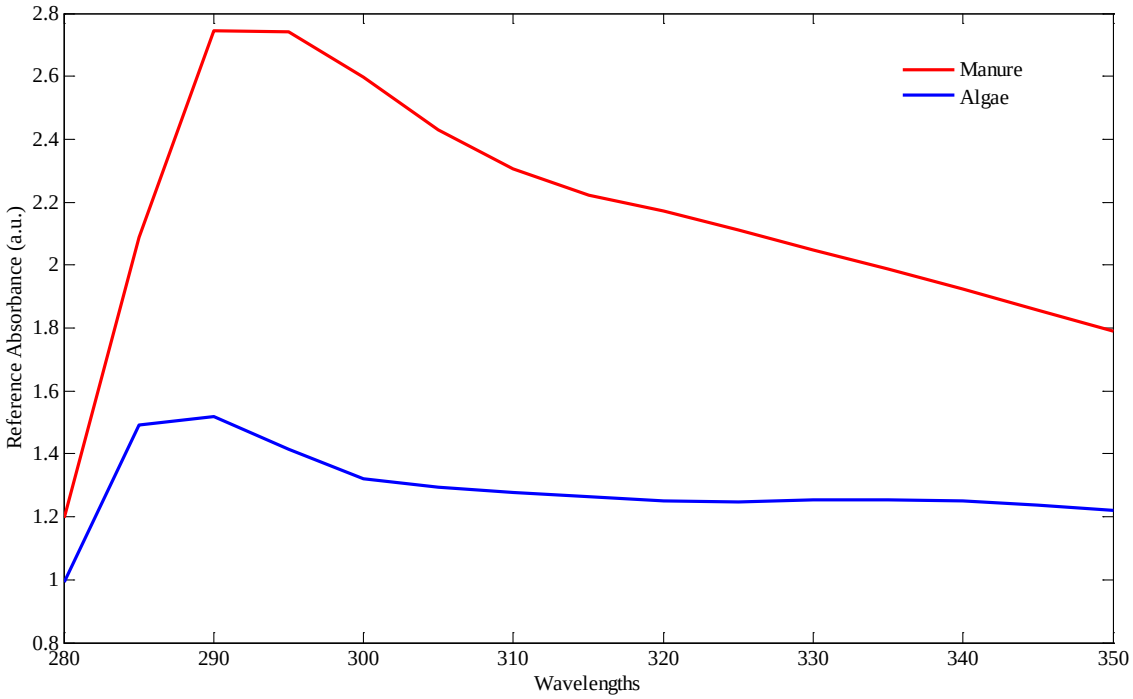

Figure 7-17 Reference absorbance spectra for determining manure solids (swine as reference) and algae solids (Scenedesmus sp. as reference) from 280 to 350 nm.

The predicted manure solids concentration using deconvolution and the wavelengths between 280 and 350 nm are presented in Figure 7-18.

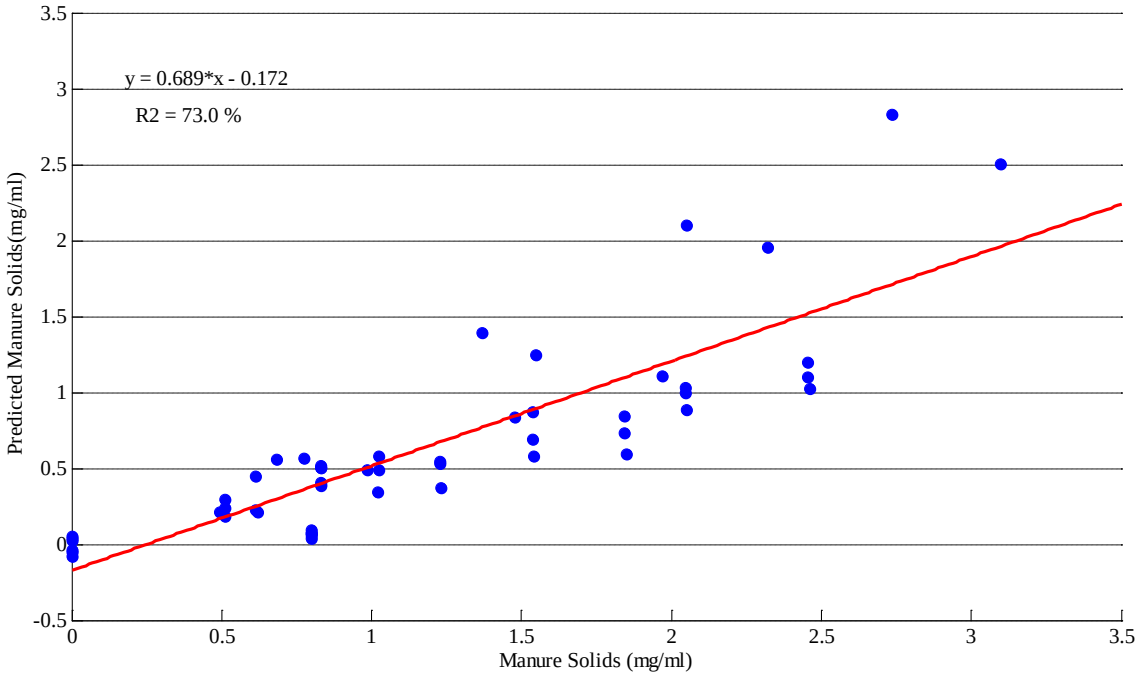

Figure 7-18 Predicted manure solids concentration for dairy 1, dairy 2, beef, sheep and swine manure when absorbance data between 280 and 350 nm was used.

A regression of the estimated versus actual manure solids concentration for the 42 samples using absorbance data between 280 to 350 nm was performed. Predicted values were less scattered when absorbance data between 280 and 350 nm was used relative to the prediction with absorbance data between 600 to 700 nm (Figure 7-16). This was evident by the increase in the Pearson coefficient that increased from 15.4% to 73.0% when the wavelength range was adjusted.

7.2.5.3 Manure Solids Estimation for Each Manure Type

Manure solids were estimated using deconvolution and developing a model specific to each manure type. Based on the manure type, a corresponding reference manure was picked that had no algae added to the sample. Predicted manure solids were

analyzed by manure type with varying levels of algae added. For dairy manure, a set of 24 samples were picked from data set 2, which included two dairy manure samples (dairy 1 and dairy 2). The reference spectra used were from dairy manure 1 and *Scenedesmus* sp. The predicted dairy manure solids concentrations are presented in Figure 7-19.

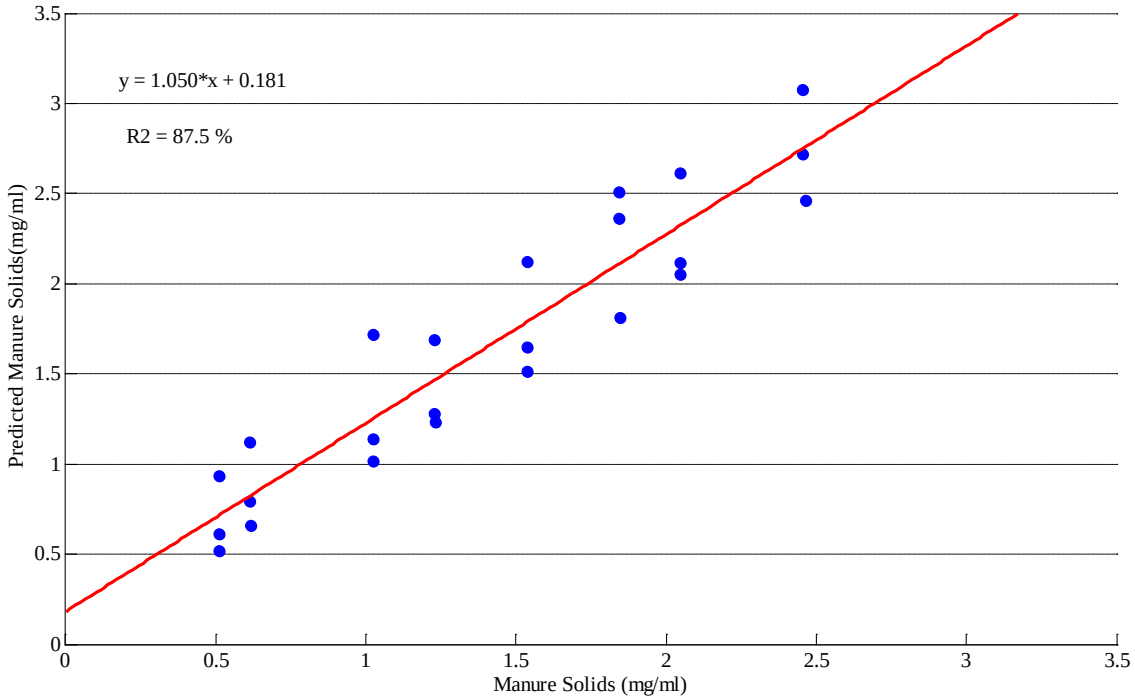

Figure 7-19 Predicted manure solids of dairy using spectra from 280 to 350 nm.

The same procedure was repeated for the other three manure sources and the solids were predicted using spectral deconvolution and the corresponding pure manure spectra as the reference. Algae reference was the same *Scenedesmus* from data set 2. Predicted solids from beef, sheep and swine manure are presented in Figure 7-20.

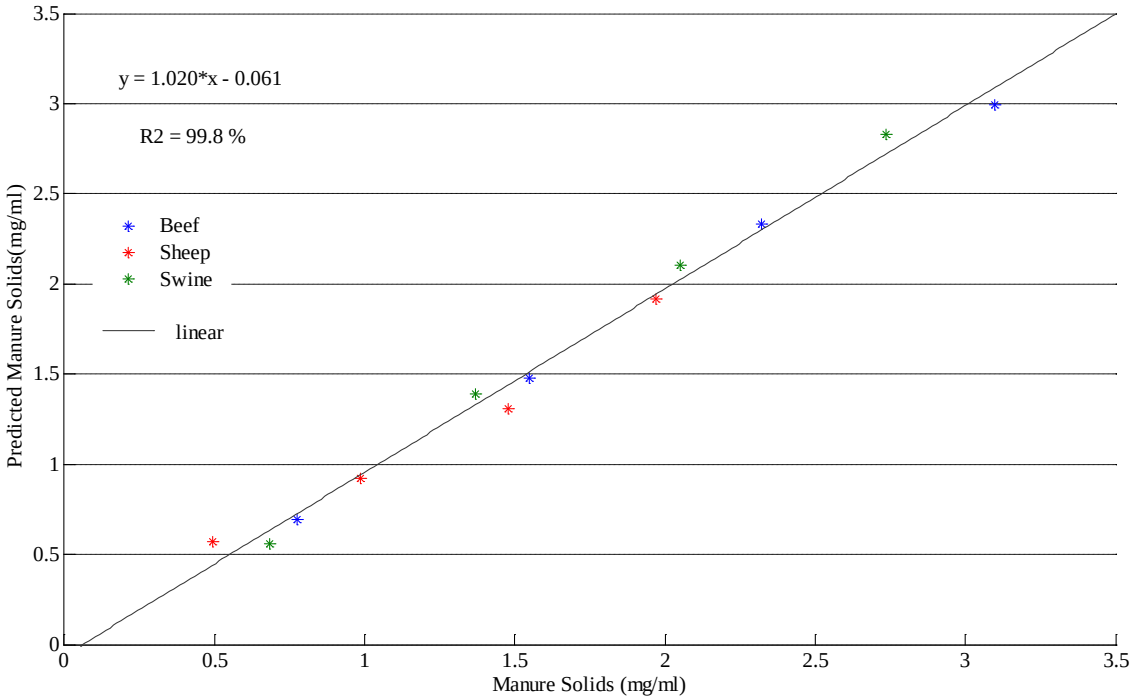

Figure 7-20 Predicted manure solids of dairy, beef and sheep, using wavelengths from 280 to 350 nm.

Very accurate estimates of manure solids concentration could be developed for specific manure types. The slope was statistically equal to one; however there was a non-zero intercept. To predict manure solids concentration, the absorbance needs to be measured between 280 and 350 nm.

7.3 Conclusions

Spectral deconvolution was successfully used to determine the algae and manure solids concentration in mixed, unprocessed samples. Two data sets from September, 2011 and April, 2012 were used to demonstrate the application of spectral deconvolution. Variations in algae and/or media require consistent selection of algae reference spectra. Algae from September, 2011 and April, 2012 had different spectral characteristics that could have been due to evolution or media changes.

Spectral characteristics were similar between *Chlorella vulgaris* and *Scenedesmus* sp. and using *Scenedesmus* sp. as the reference was sufficient to determine the algae solids concentration from each strain. However, the spectral behavior of *Cylindrocystis* sp. and *Neospongiococcum* sp. was different than *Scenedesmus* sp. Using *Cylindrocystis* as the references was sufficient to predict the algae solids concentration of both *Cylindrocystis* sp. and *Neospongiococcum* sp.

If the appropriate algae sample was chosen with absorbance data between 600 and 700 nm, the following results were found:

1. Algae solids concentration from *Chlorella vulgaris* and *Scenedesmus* sp. were accurately determined (Pearson coefficient between 84.3% and 95.3%) in samples with dairy, beef, sheep, and swine manure.
2. Algae solids from *Cylindrocystis* sp. and *Neospongiococcum* sp. were measured (Pearson coefficient of 98.1%) in samples with dairy, beef, sheep, and swine manure.

Manure solids concentration was not accurately predicted if the absorbance data between 600 and 700 nm was used. However, if absorbance data between 280 and 350 nm was used the accuracy of the prediction improved with a Pearson coefficient of 73.0%. If additional accuracy was desired, the data had to be segregated by manure type and the appropriate reference sample used, resulting in:

1. Dairy manure solids concentration from two sources could be predicted with a Pearson coefficient of 87.5%.
2. Models specific to beef, sheep, and swine to determine manure solids concentration were developed with a Pearson coefficient of 99.8%.

The deconvolution method proved to be an accurate and efficient method for estimating algae and manure solids concentration in unprocessed samples. A critical factor was utilizing appropriate reference spectra to determine the algae and manure solids concentration. Although, only two reference samples were required to provide accurate estimates.

The techniques developed should accurately predict algae concentration within the range of 0.2 to 1.0 mg/ml. This would be an acceptable range for the majority of systems that would be cultivating algae.

CHAPTER 8: FUTURE WORK

There are several areas in which the studies presented in this work can be extended. Besides the estimation of algae and manure solids concentration in raw samples, the spectral deconvolution could be also used to correct for solids interference in chlorophyll extraction readings. It was concluded from chapter 6 that the manure solids interfere in chlorophyll readings after extraction. However, it was also observed that there is a linear relation between each manure source and the actual chlorophyll concentration. Spectral deconvolution could correct the solids interference using appropriate reference spectra.

Another area that can be further explored is the estimation of constituent parameters in the manure-algae mixture such as nitrates, organic carbon, surfactants, BOD, COD and other living organisms (Dobbs et al., 1992; Shibata et al., 1954, Brookman, 1996; Roing et al., 1999; Vaillant et al., 2002.). An extra sample should be taken to be diluted in order to study the spectra of small dissolved and colloidal fractions. From the same diluted samples, macromolecular (amino-acids and lipids) synthesis in microalgae can be study using light methods (Beardall et al., 2001; Stehfest et al., 2005). Changes in media nutrients concentration and algae macromolecular synthesis could be studied together.

In addition to estimation of solids from mixtures, spectral deconvolution seems to be an option to estimate algae concentration in chemical media such as urea. Although chemical media can have some unabsorbing components, which contributes to solids weight but does not contribute to changes in spectral shape, it can be possible to find a representative reference spectra set.

In conclusion, the study of mixture components concentration using spectral deconvolution is a large area to be explored. The advantages of this method are that many samples can be quickly scanned by spectrophotometer and low cost since samples can be studied unprocessed (raw).

REFERENCES

Allen, M. M. and Smith, A. J. 1969. Nitrogen chlorosis in blue-green algae. Archives of Microbiology. 69(2): 114-120.

APHA, AWA, WPCF. 1992. In Standard Methods for the Examination of Water and Wastewater, 18th ed.; American Public Health Association: Washington, D.C.

Azema, N., Pouet, M. F., Berho, C. and Thomas, O. 2002. Wastewater suspended solids study by optical methods Colloids and Surfaces A. Physicochemical and Engineering Aspects. 204: 131-140.

Beardall, J., T. Berman, P. Heraud, M.O. Kadiri, B. R. Light, G. Patterson, S. Roberts, B. Sulzberger, E. Sahan, U. Uehlinger and B. Wood. 2001. A comparison of methods for detection of phosphate limitation in microalgae. Aquat. Sci. 63: 107–121.

Becker, E.W. 1994. Microalgae Biotechnology and Microbiology. Cambridge University Press, New York.

Bertoldi, F. C., E. Sant'Anna, M.V.C Braga, and J. L. B. Oliveira. 2006. Lipids, fatty acids composition and carotenoids of *Chlorella vulgaris* cultivated in hydroponic wastewater. Grasas y Aceites 57(3): 270-274.

Bitton, R., H. Bianco-Peled. 2008. Novel Biomimetic Adhesives Based on Algae Glue. Macromol. Biosci. 8: 393–400.

Brookman, S. K. E. 1997. Estimation of biochemical oxygen demand in slurry and effluent using ultra-violet spectrophotometry. Water res. 31: 372: 374.

Burgess, C. 2007. The Basics of Spectrophotometric Measurement. In: UV-Visible Spectrophotometry of Water and Wastewater. Techniques and Instrumentation in analytical chemistry. 27: 21-45.

Burke, D. A. 2001. Dairy Waste Anaerobic Digestion Handbook. Environmental Energy Company. www.makingenergy.com

Brune, D. E.; Lundquist, T. J.; Benemann, J. R. 2009. Microalgal Biomass for Greenhouse Gas Reductions: Potential for Replacement of Fossil Fuels and Animal Feeds. Journal of Environmental Engineering-Asce. 135(11): 1136-1144.

Chisti, Y. 2007. Biodiesel from microalgae. Biotechnol. Adv. 25: 294–306.

Dawson, J. T. 2010. Personal communication. Pittsburg State University, Pittsburg, KS.

Dean, A. P., Sigee, D. C., Estrada, B., Pittman, J. K. 2010. Using FTIR spectroscopy for rapid determination of lipid accumulation in esponse to nitrogen limitation in freshwater microalgae. Bioresource Technology. 101: 4499-4507.

Dobbs, R. A., R. H. Wise, R. B. Dean. 1992. The use of UV absorption measurement for monitoring the total organic carbon content of water and wastewater. Water Res. 6: 1173-1180

Domeizel, M., A. Khalil, P. Prudent. 2004. UV spectroscopy: a tool for monitoring humification and for proposing an index of the maturity of compost. Bioresource Technology. 94(2): 177–184.

Doucha, J., Straka, F., Livansky, K. 2005. Utilization of flue gas for cultivation of microalgae (*Chlorella* sp) in an outdoor open thin-layer photobioreactor. J. Appl. Phycol. 17: 403–412.

Eisenhauer, J. G. 2003. Regression through the Origin. Teaching Statistics. 25(3).

Escalas, A., M. Droguet, J. M.Guadayol, J. Caixach. 2003. Estimating DOC regime in a wastewater treatment plant by UV deconvolution. Water research. 37: 2627-2635.

Farrell, A. E.; Plevin, R. J.; Turner, B. T.; Jones, A. D.; O'Hare, M.; Kammen, D. M. 2006. Ethanol can contribute to energy and environmental goals. Science. 311(5760): 506-508.

Galle, B., Kelmedtsson, L., Bergqvist, B., Ferm, M., Tornqvist, K., Griffith, D. W. T., Jensen, N. O., Hansen, F. 2000 Measurements of ammonia emission from spreading of manure using gradient FTIR techniques. Atmospheric Environment. 34: 4907-4915.

Gallot, S., and Thomas, O. 1993. Fast and easy interpretation of a set of absorption spectra: theory and qualitative applications for UV examination of waters and wastewaters. Fresenius J Anal Chem. 346: 976-983.

Hadjoudja, S., V. Deluchat, M. Baudu. 2010. Cell surface characterization of Microcystis aeruginosa and *Chlorella vulgaris*. Journal of Colloid and Interface Science. 342: 293–299.

Hahn, G. J. 1977. Fitting regression models with no intercept term. Journal of Quality Technology. 9(2): 56–61.

Hall, S. J., Hawkes, D. L., Hawkes, F. R., Thomas A. 1985. Mesophilic anaerobic digestion of high-solids cattle waste in a packed bed digester. J Agric Eng Res. 32(2): 153–62.

Hodaifa, G.; Martínez, M. E.; Sánchez, S., 2008. Use of industrial wastewater from olive-oil extraction for biomass production of *Scenedesmus* obliquus. Bioresource Technology. 99(5): 1111-1117.

Holm-Hansen, O.; Riemann, B. 1978. Chlorophyll *a* Determination: Improvements in Methodology. Oikos. 30(3): 438-447.

Holzel, C. S.; Muller, C.; Harms, K. S.; Mikolajewski, S.; Schafer, S.; Schwaiger, K.; Bauer, J. 2012. Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. Environmental Research. 13: 21-27.

Horton, R. B., Duranty, E., McConico, M., Vogt, F. 2011. Fourier Transform Infrared (FT-IR) Spectroscopy and Improved Principal Component Regression (PCR) for Quantification of Solid Analytes in Microalgae and Bacteria. Applied Spectroscopy. 65(4): 442-453.

Houghton, R.A., 2005. Aboveground forest biomass and the global carbon balance. Global Change Biol. 11, 945–958.

Hulst, H. C. van de. 1981. Light scattering by small particles.Dover publication INC New York. 470.

Johnson, M. B., and Wen, Z. 2010. Development of an attached microalgae growth system for biofuel production . Appl Microbiol Biotechnol. 85: 525–534.

Li, Y.; McCrory, D. F.; Powell, J. M.; Saam, H.; Jackson-Smith, D. 2005. A survey of selected heavy metal concentrations in Wisconsin dairy feeds. Journal of Dairy Science. 88(8): 2911-2922.

Liang, G., Mo, Y., Tang, J., Zhou, Q. 2011. Improve lipid production by pH shifted-strategy in batch culture of *Chlorella* protothecoides. African Journal of Microbiology Research 5(28): 5030-5038

Liang, Y.; Sarkany, N.; Cui, Y. 2009. Biomass and lipid productivities of *Chlorella vulgaris*; under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnology Letters. 31(7): 1043-1049.

Lichtenthaler, H. K., and A. R. Wellburn. 1983. Determinations of total carotenoids and Chlorophylls *a* and *b* of leaf extracts in different Solvents. Biochemical Society Transactions. 11: 591–592.

Lourenco, N. D., Paixao, F., Pinheiro, H. M., Sousa, A. 2010. Use of Spectra in the Visible and Near-Mid-Ultraviolet Range with Principal Component Analysis and Partial Least Squares Processing for Monitoring of Suspended Solids in Municipal Wastewater Treatment Plants. Applied Spectroscopy. 64(9): 1062-1067.

Lorenzen C.J. 1966. A method for the continuous measurement of in vivo chlorophyll concentration. Deep-Sea Res.,13:223-227.

Madigan, M. T., Martinko, J. M. 2006. Brock Microbiology of Microorganisms. Southern Illinois University Carbondale, Pearson Prentice Hall. 12.

Martinez, F. and Orus, M. I. 1991. Interactions between Glucose and Inorganic Carbon Metabolism in *Chlorella vulgaris* Strain UAM 101. Plant Physiol. 95: 1150: 1155.

Masse, L. Kennedy, K. J. Chou, S. 2001. Testing of alkaline and enzymatic pretreatment for fat particles in slaughterhouses wastewater, Bioresour. Technol. 77: 145–155.

Matsche, N., and Stumwohrer, K. 1996. UV absorption as control parameter for biological treatment plants. Water science and technology. 33(12): 211-218.

Mayo, A. W. 1997. Effectc of Temperature and pH on the Kinectic Growth of Unialga *Chlorella vulgaris* Cultures containing bacteria. Environment Research. 69(1): 64-72.

Miao, X., Wu, Q., Yang, C., 2004. Fast pyrolysis of microalgae to produce renewable fuels. J. Anal. Appl. Pyrol. 71: 855–863.

Mohan, N., Hanumantha, P. R., Ranjith R. K., Sivasankaran, S. and Sivasubramanian, V. 2009. Studies on mass cultivation of *Chlorella vulgaris* and effective harvesting of biomass by low cost methods. J. Algal Biomass Utln. 1(1): 29–39.

Monteiro, C. M.; Castro, P. M. L.; Malcata, F. X. 2012. Metal uptake by microalgae: Underlying mechanisms and practical applications. Biotechnology Progress. 28(2): 299-311.

Mosquera, M. I. M., B. G. Rojas, and M. L. G. Guerrero. 1992. Rapid Method of Quantification of Chlorophyll s and Carotenoids in Virgin Olive Oil by High-Performance Liquid Chromatography. J. Agric. Food Chem. lQQ2. 40: 60-63.

Mulbry, W., E. Westhead, C. Pizarro, and L. Sikora. 2005. Recycling of manure nutrients: use of algal biomass from dairy manure treatment as a slow release fertilizer. Bioresource Technology. 96: 451–458.

Mulbry, W.; Kondrad, S.; Pizarro, C.; Kebede-Westhead, E. 2008. Treatment of dairy manure effluent using freshwater algae: Algal productivity and recovery of manure nutrients using pilot-scale algal turf scrubbers. Bioresource Technology. 99(17): 8137-8142.

Neenan, B., D. Feinburg, A. Hill, R. Mcintosh, and K. Terry. 1986. Fuels from Microalgae: Status, Potential, and Research Requirements. Solar Energy Research Institute, Golden, CO SERI/SP. 231-2550.

Nielson, H. B., Heiske, S. 2011. Anaerobic digestion of macroalgae: methane potentials, pre-treatment, inhibition and co-digestion. Water Science and Technology. 64(8): 1723-1729.

Obertegger, U., H.A. Smith, G. Flaim & R.L. Wallace. 2011. Using the guild ratio to characterize pelagic rotifer communities. Hydrobiologia, 662:157-162.

Oron, G.; Shelef, G.; Levi, A.; Meydan, A.; Azov, Y. 1979. Algae-Bacteria ratio high-rate ponds used for water treatment. Applied and Environmental Microbiology.38(4): 570-576.

Pouet, M. F., N. Azema, E. Touraud, O. Thomas. 2007. Physical and Aggregate Properties. In: UV-Visible Spectrophotometry of Water and Wastewater. Techniques and Instrumentation in analytical chemistry. 27: 145-161.

Rawlings, J. O., S. G. Pantula, and D. A. Dickey. 1998. Applied regression analysis: a research tool. Springer-Verlag, New York, New York, USA. 2.

Ritchie, R. J. 2006. Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynth Res. 89: 27–41.

Roig, B., C. Gonzalez, O. Thomas. 1999. Simple UV: UV-visible method for nitrogen and phosphorus measurement in wastewater. Talanta. 50: 751–758.

Roig, B., Thomas, O. 2003. UV spectrophotometry: a powerful tool for environmental measurement. Management of Environmental Quality: An International Journal. 14(3): 398-404.

Rowan, K. S. Photosynthetic pigments of algae. 1989. Cambridge University Press, Cambridge.

Sarraguca, M. C., Paulo, A., Alves, M. M., Dias, A. M. A., Lopes, J. A., Ferreira. 2009. Quantitative monitoring of an activated sludge reactor using on-line UV-visible and near-infrared spectroscopy. Anal Bioanal Chem. 395: 1159-1166.

Sartory, D. P.; Grobbelaar, J. U. 1984. Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia. 14(3): 177-187.

Sekabira, K.; Origa, H. O.; Basamba, T. A.; Mutumba, G.; Kakudidi, E. 2011. Application of algae in biomonitoring and phytoextraction of heavy metals contamination in urban stream water. International Journal of Environmental Science and Technology. 8(1): 115-128.

Sialve, B., Bernet, N., Bernard, O. (2009) Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnology Advances. 27, 409:416.

Shibata, K., A. A. Benson, and M. Calvin. 1954. The absorption spectra of suspensions of living microorganisms. Biochim. Biophys. Acta. 15:461-470.

Stehfest, K.; Toepel, J.; Wilhelm, C. 2005. The application of micro-FTIR spectroscopy to analyze nutrient stress-related changes in biomass composition of phytoplankton algae. Plant Physiol. Biochem. 43: 717-726.

Sturm, B. S. M.; Peltier, E.; Smith, V.; DeNoyelles, F. 2012. Controls of microalgal biomass and lipid production in municipal wastewater-fed bioreactors. Environmental Progress & Sustainable Energy. 31(1): 10-16.

Sutton, A. L.; Nelson, D. W.; Kelly, D. T.; Hill, D. L.1986. Comparison of solid vs. liquid dairy manure applications on corn yield and soil composition. Journal of Environmental Quality. 15(4): 370-375.

Tarlan, E.; Dilek, F. B.; Yetis, U. 2002. Effectiveness of algae in the treatment of a wood-based pulp and paper industry wastewater. Bioresource Technology. 84(1): 1-5.

Thomas J. B., and W. F. G. Flight. 1964. Fluorescence responses of chlorophyll in vivo to treatment with acetone. Biochim. Biophs. Acta. 79: 500-510.

Thomas, O., Theraulaz, F., Domeizel, M., Massiani, C. 1993. UV Spectral Deconvolution: A valuable tool for waste water quality determination. Environmntal technology. 14: 1187-1192.

Thomas, O., V. Cerda. 2007. From Spectra to Qualitative and Quantitative Results. In: UV-Visible Spectrophotometry of Water and Wastewater. Techniques and Instrumentation in analytical chemistry. 27: 21-45.

Vaillant, S., Pouet, M. F., Thomas, O. 1999. Methodology for the characterization of heterogeneous fractions in wastewater. Talanta. 50: 729-736.

Vaillant, S., Pouet, M. F., Thomas, O. 2002. Basic handling of UV spectra for urban water quality monitoring. Urban Water. 4: 273–281.

Vergara-Fernández, A. G. Vargas, N. Alarcón, A. Velasco. 2008. Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. Biomass and Bioenergy. 32(4): 338–344.

Vunjak-Novakovic, G., Y. Kim, X. Wu, I. Berzin, J. C. Merchuk. 2005. Air-Lift Bioreactors for Algal Growth on Flue Gas: Mathematical Modeling and Pilot-Plant Studies. Ind. Eng. Chem. Res. 44: 6154–6163.

Wang, L., M. Min, Y. Li, P. Chen, Y. Chen, Y. Lui, Y. Wang, and R. Ruan. 2009. Cultivation of Green Algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant. Appl Biochem Biotechnology. 162(4): 1174-1186.

Wang, L., Y. Li, P. Chen, M. Min, Y. Chen, J. Zhu, and R. Ruan. 2010. Anaerobic digested manure as a nutrient supplement for cultivation of oil-rich green microalgae Chlorella sp. Bioresource technology. 101: 2623-2628.

Wellburn, A. R., 1994. The spectral determination of chlorophyll a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J. Plant Physiol. 144: 307–313.

Wen Z, Liao W., Chen S. 2004. Hydrolysis of animal manure lignocellulosics for reducing sugar production. Biores Technol. 91(1): 31–9.

Wilkie, A. C. 2005. Anaerobic Digestion of Dairy Manure: Design and Process Considerations. In: Dairy Manure Management Conference. NRAES. 176: 301-312.

Wilkie, A. C., and W. W. Mulbry. 2002. Recovery of dairy manure nutrients by benthic freshwater algae. Bioresource Technology. 84: 81–91.

Zaadi, E.; M. Segoli, Eldridge, D. J.; Groffman, P. M.; Boeken, B.; Shachak, M. 2009. Relationship among soil surface properties, hydrology and nitrogen cycling along a climatological gradient in drylands. Geophysical Research Abstracts. 11.

Zamalloa, C., E. Vulstek, J. Albrecht, W. Verstraete. 2011. The techno-economic potential of renewable energy through the anaerobicdigestion of microalgae. Bioresource Technology. 102(2): 1149–1158.

Zhu, C. J., Y. K. Lee. 1997. Determination of biomass dry weight of marine microalgae. Journal of Applied Phycology. 9: 189–194.

Appendix A

Table A- 1 Concentration of *Chlorella vulgaris* in urea and manure media measured using chlorophyll extraction (mg/ml).

Chlorophyll *a* Extraction (mg/l)						
	12-Nov	15-Nov	17-Nov	19-Nov	22-Nov	24-Nov
	0	3	5	7	10	12
Urea	0.22	4.54	9.09	11.78	19.67	23.13
Manure	1.69	3.70	8.41	12.55	30.53	41.00

Table A- 2 Concentration of *Chlorella vulgaris* in urea and manure media measured using dry weight

Dry Weight (mg/ml)						
	12-Nov	15-Nov	17-Nov	19-Nov	22-Nov	24-Nov
	0	3	5	7	10	12
Urea	1.1	1.8	1.8	1.9	2.3	2.5
Manure	3.1	2.2	2.2	2.3	3.1	3.1

Table A- 3 Concentration of *Chlorella vulgaris* in urea and manure media measured using optical density

Optical Density (a.u.)						
	12-Nov	15-Nov	17-Nov	19-Nov	22-Nov	24-Nov
	0	3	5	7	10	12
Urea	0.012	0.170	0.349	0.427	0.658	0.578
Manure	1.066	0.865	0.886	1.023	1.982	2.173

Table A- 4 Concentration of *Chlorella vulgaris* in urea and manure media measured using cell counting.

Cells Counting (x 10^4 algae cells/ml)					
	12-Nov	15-Nov	17-Nov	19-Nov	22-Nov
	0	3	5	7	10
Urea	5	84.2	178.8	222.5	662.5
Manure	10	123.3	430.0	1252.5	3005.0

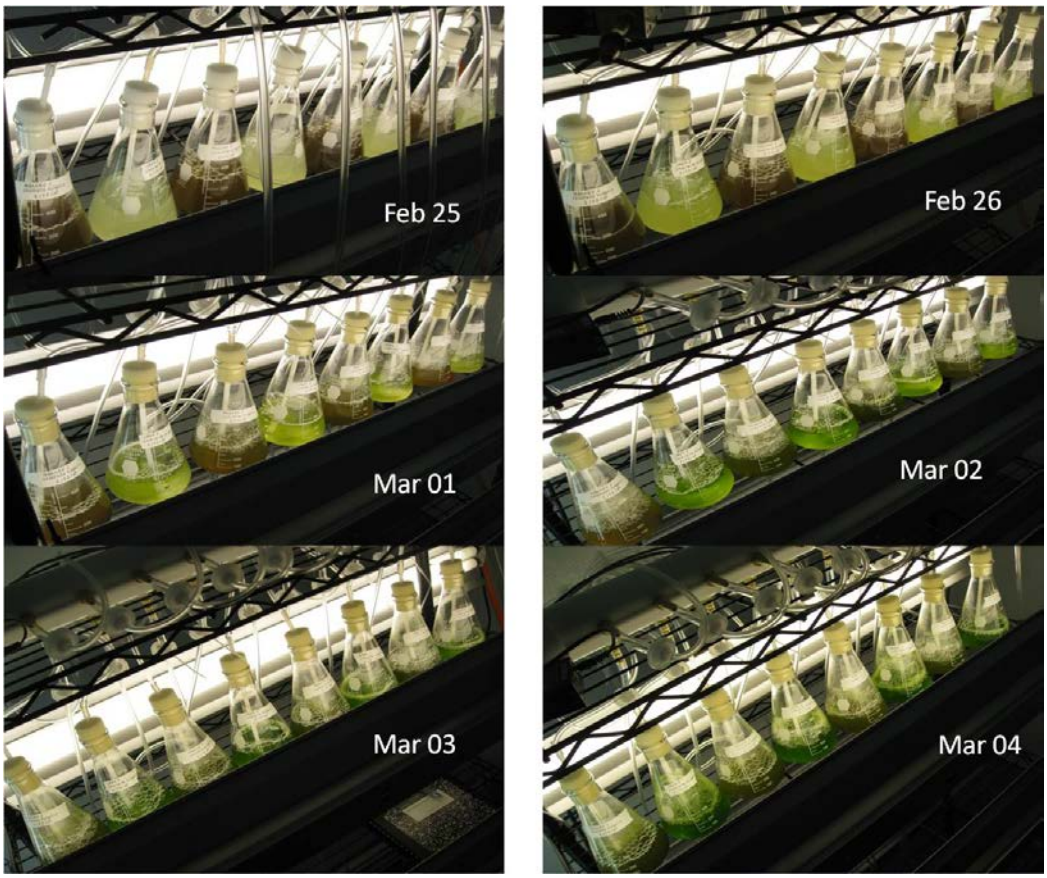

Figure A- 1 *Chlorella vulgaris* growing in manure and urea media.

Appendix B

Table B- 1 Calibration Data for chloropyll *a* concentration extracted from *Chlorella vulgaris* diluted with dairy manure.

Actual Chlorophyll *a*	A 665
mg/l	a.u.
0	0.0325
0	0.0334
0	0.034
5.18938	0.2809
5.22737	0.2852
5.19704	0.2898
10.03569	0.5331
10.1007	0.5412
10.05868	0.5394
14.60571	0.7931
14.77788	0.7896
14.69109	0.7953
19.32961	0.9198
19.4628	0.928
19.34279	0.9456
22.13033	0.9202
22.05924	0.9071
22.17484	0.9324

Table B- 2 Validation Data set 2 for chlorophyll extraction from samples with 0,2,4,6,8 and 10 ml of *Chlorella vulgaris* added to 5 ml of dairy manure.

Algae	Chlorophyll *a*(mg/l)		
ml	Actual	Predicted	Becker Eq.
0	0	2.7573	1.41115
0	0	1.9761	0.9509
0	0	1.9299	0.94033
2	5.15528	6.8225	4.41902
2	5.14534	6.9443	4.50891
2	5.17917	6.8204	4.41986
4	9.605	10.9167	8.02706
4	9.67734	10.007	8.09303
4	9.68265	10.0406	8.10449
6	12.42379	13.1486	9.95387
6	12.30531	13.2284	10.00246
6	12.4168	13.3376	10.06502
8	18.74464	19.3137	11.17694
8	18.75991	19.3305	11.20923
8	18.97463	19.5678	11.34505
10	27.47745	27.2706	15.98127
10	27.77606	27.2622	15.99625
10	27.85041	27.6759	16.27897

Appendix C

Chlorella vulgaris in Dairy Manure

The dairy manure used for this validation is a different sample then the one used for calibration in chapter 5. Figure C-1 shows the predicted values of Chlorophyll *a* extraction from *Chlorella vulgaris* in dairy manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Chlorella vulgaris* in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* *a* extracted from *Chlorella vulgaris* in dairy manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Chlorella vulgaris* solids concentration was 1.2 mg/ml.

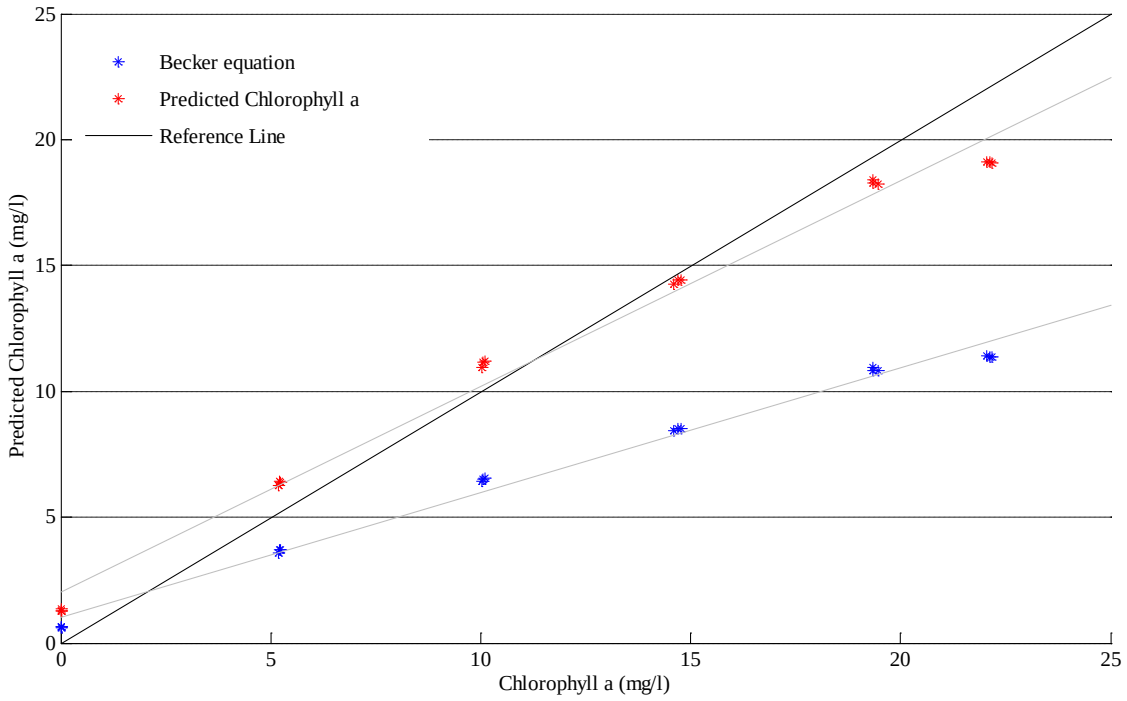

Figure C-1 Chlorophyll *a* extraction from *Chlorella vulgaris* in dairy manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) are close to the reference line (1:1), although the slope seems to be slightly smaller. The reference equation (Equation 5.1) presented a smaller slope, underestimating the results.

Table C-1 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-1 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Chlorella vulgaris* in dairy manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	720.06	720.06	1493.58	0.00
Residual	16	7.71	0.48		
Total	17	727.77			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Chlorella vulgaris* in dairy manure.

Cylindrocystis sp. in Dairy Manure

a. Dairy manure sample 1

Figure C-2 shows the predicted values of Chlorophyll *a* extraction from *Cylindrocystis* sp. in dairy manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Cylindrocystis* sp. in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Cylindrocystis* sp. in dairy manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Cylindrocystis* sp. solids concentration was 4.1 mg/l.

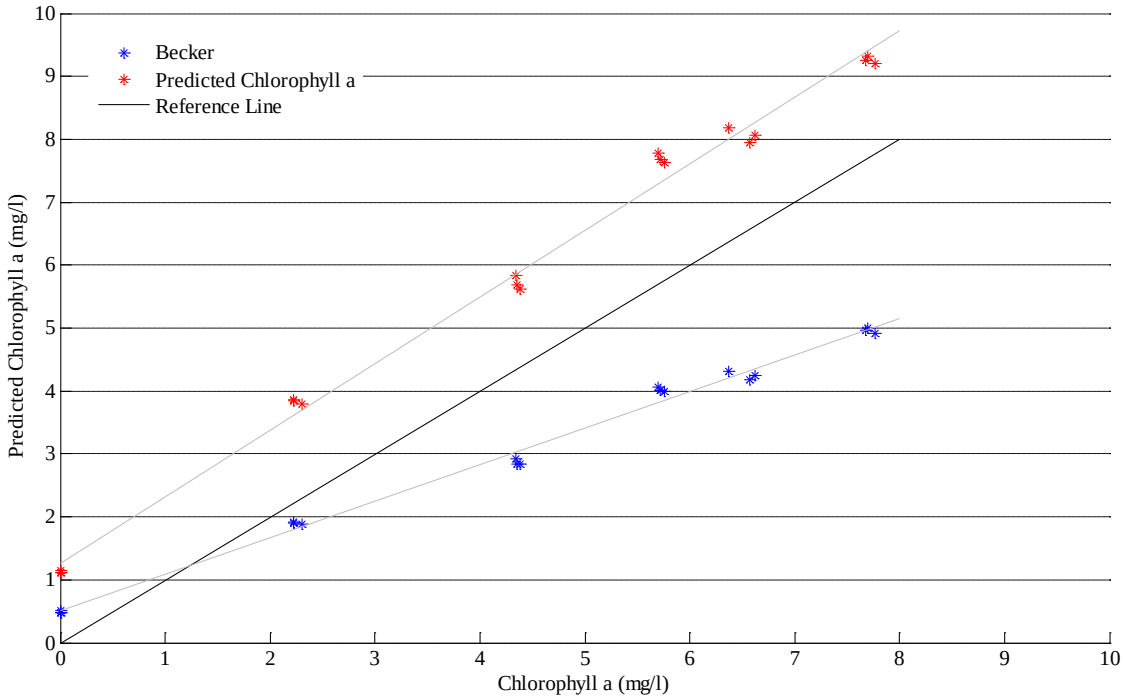

Figure C-2 Chlorophyll *a* extraction from *Cylindrocystis* sp. in dairy manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference line showed similar slope tendency, but a difference in results. Values predicted by the reference equation are smaller than the ones predicted by the new equation and the reference line.

Table C-2 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-2 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Cylindrocystis* sp. in dairy manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	137.96	137.96	2243.40	0.00
Residual	16	0.98	0.06		
Total	17	138.94			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Cylindrocystis* sp. in dairy manure.

b. Dairy manure sample 2

Figure C-3 shows the predicted values of Chlorophyll *a* extraction from *Cylindrocystis* sp. in dairy manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Cylindrocystis* sp. in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Cylindrocystis* sp. in dairy manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Cylindrocystis* sp. solids concentration was 4.1 mg/ml.

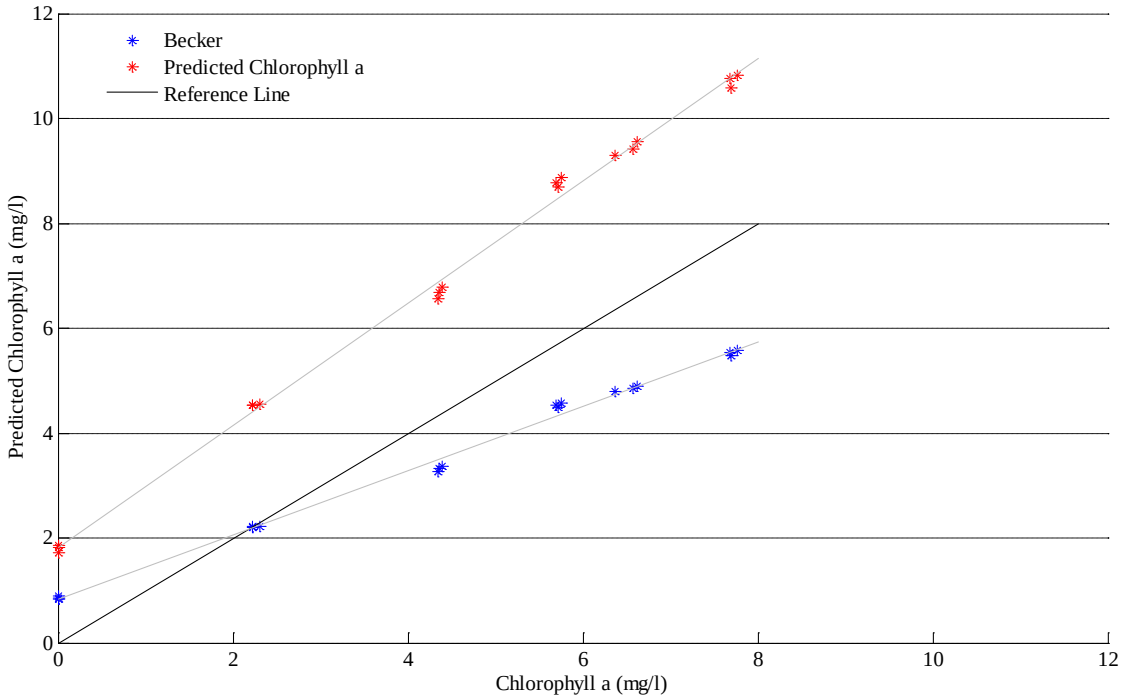

Figure C-3 Chlorophyll *a* extraction from *Cylindrocystis* sp. in dairy manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference line showed similar slope tendency, but different results. Values predicted by the reference equation are smaller than the ones predicted by the new equation.

Table C-3 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-3 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Cylindrocystis* sp. in dairy manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	168.07	168.07	5342.49	0.00
Residual	16	0.50	0.03		
Total	17	168.58			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Cylindrocystis* sp. in dairy manure.

Scenedesmus sp. in Dairy Manure

a. *Scenedesmus* sp. in dairy manure sample 1

Figure C-4 shows the predicted values of Chlorophyll *a* extraction from *Scenedesmus* sp. in dairy manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Scenedesmus* sp. in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Scenedesmus sp.* in dairy manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Scenedesmus* sp. concentration was 1.2 mg/ml.

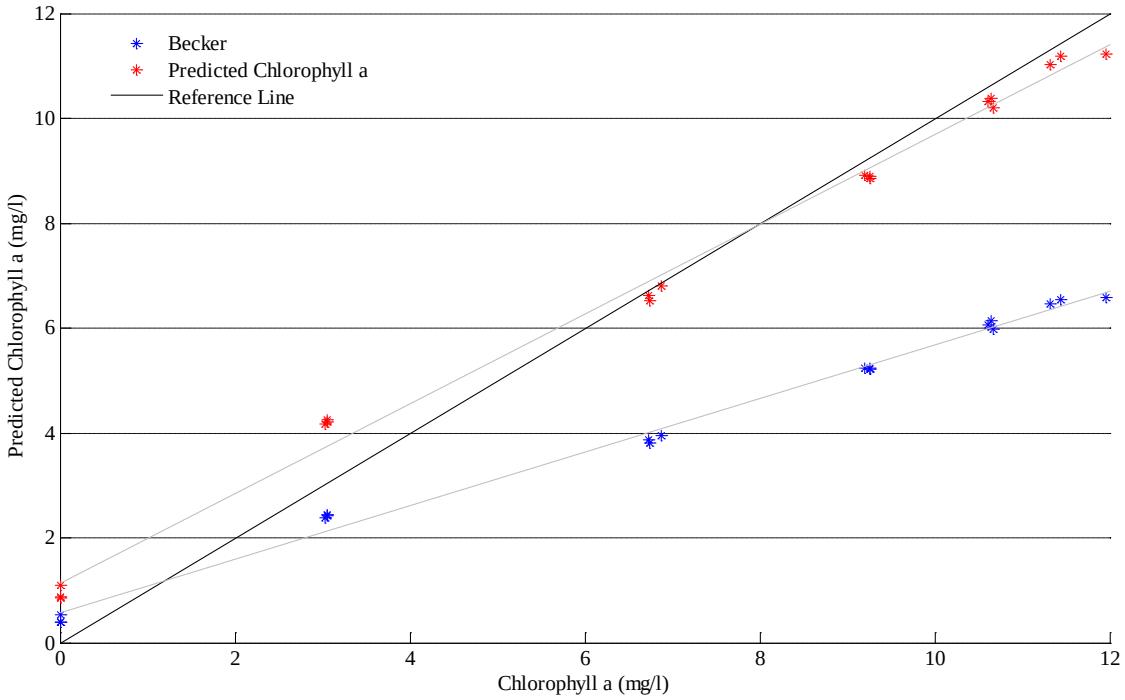

Figure C-4 Chlorophyll *a* extraction from *Scenedesmus* sp. in dairy manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference equation (Equation 5.1) showed are very close. Values predicted by the reference equation are smaller than the ones predicted by the new equation, and the difference becomes bigger when Chlorophyll *a* is more concentrated.

Table C-4 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-4 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Scenedesmus sp.* in dairy manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	492.54	492.54	2891.04	0.00
Residual	16	2.73	0.17		
Total	17	495.26			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Scenedesmus sp.* in dairy manure.

b. *Scenedesmus sp.* in dairy manure sample 2

Figure C-5 shows the predicted values of Chlorophyll *a* extraction from *Scenedesmus* sp. in dairy manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Scenedesmus* sp. in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Scenedesmus* sp. in dairy manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Scenedesmus* sp. solids concentration was 1.2 mg/ml.

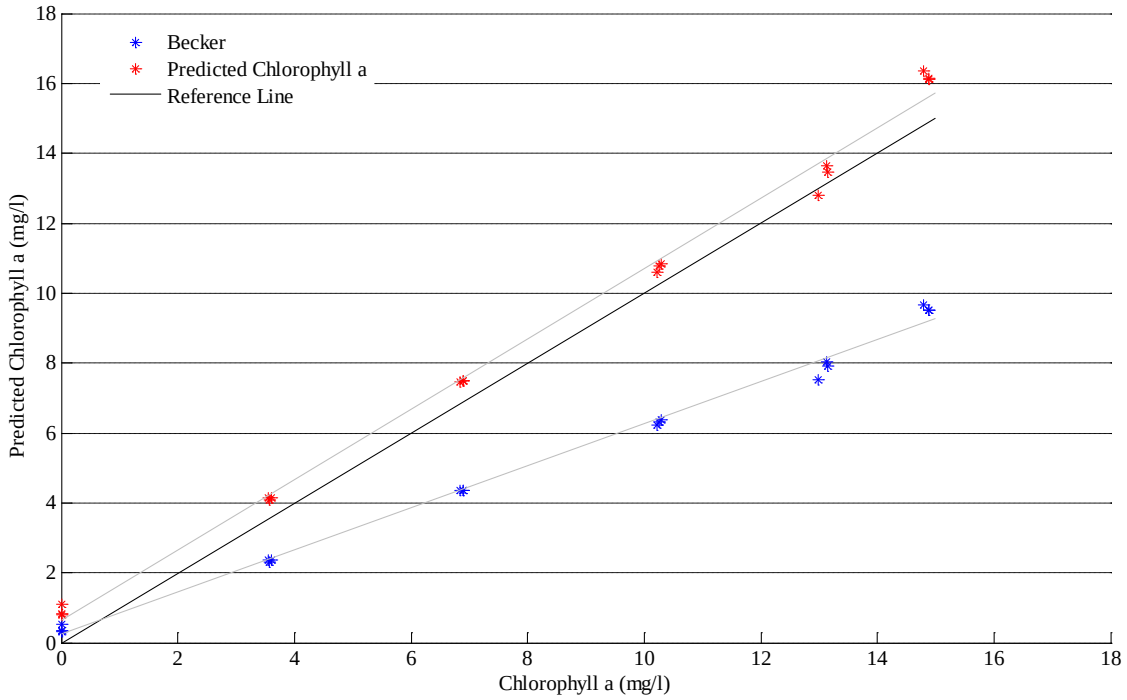

Figure C-5 Chlorophyll *a* extraction from *Scenedesmus* sp. in dairy manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference equation (Equation) showed are very close. Values predicted by the reference equation are smaller than the ones predicted by the new equation, and the difference becomes bigger when Chlorophyll *a* is more concentrated.

Table C-5 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-5 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Scenedesmus* sp. in dairy manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	470.49	470.49	717.11	0.00
Residual	16	10.50	0.66		
Total	17	480.98			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Scenedesmus sp.* in dairy manure.

Chlorella vulgaris in Beef Manure

Figure C-6 shows the predicted values of Chlorophyll *a* extraction from *Chlorella vulgaris* in beef manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Chlorella vulgaris* in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Chlorella vulgaris* in beef manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Chlorella vulgaris* concentration was 1.2 mg/ml.

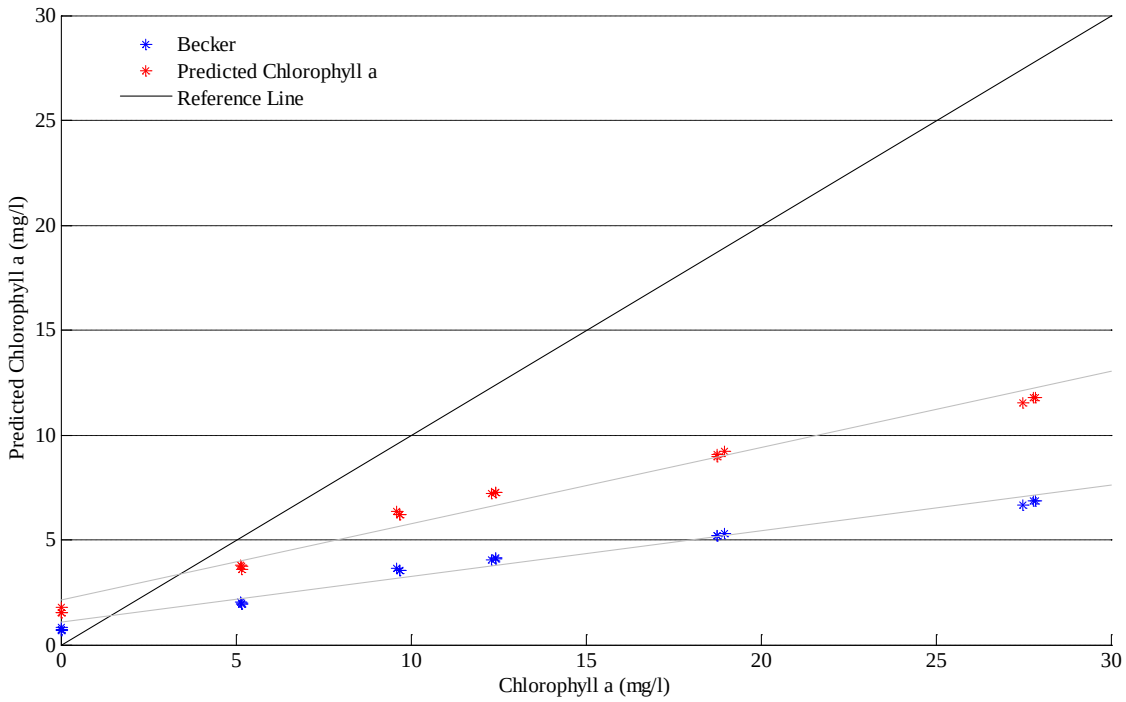

Figure C-6 Chlorophyll *a* extraction from *Chlorella vulgaris* in beef manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference equation (Equation) showed similar tendency. Values predicted by the reference equation are smaller than the ones predicted by the new equation, and the difference becomes bigger when Chlorophyll *a* is more concentrated.

Table C-6 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual chlorophyll *a*.

Table C-6 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Chlorella vulgaris* in beef manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	193.32	193.32	737.34	0.00
Residual	16	4.19	0.26		
Total	17	197.51			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Chlorella vulgaris* in beef manure.

Cylindrocystis sp. in Beef Manure

Figure C-7 shows the predicted values of Chlorophyll *a* extraction from *Cylindrocystis* sp. in beef manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Cylindrocystis* sp. in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Cylindrocystis* sp. in beef manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Cylindrocystis* sp. concentration was 4.1 mg/ml.

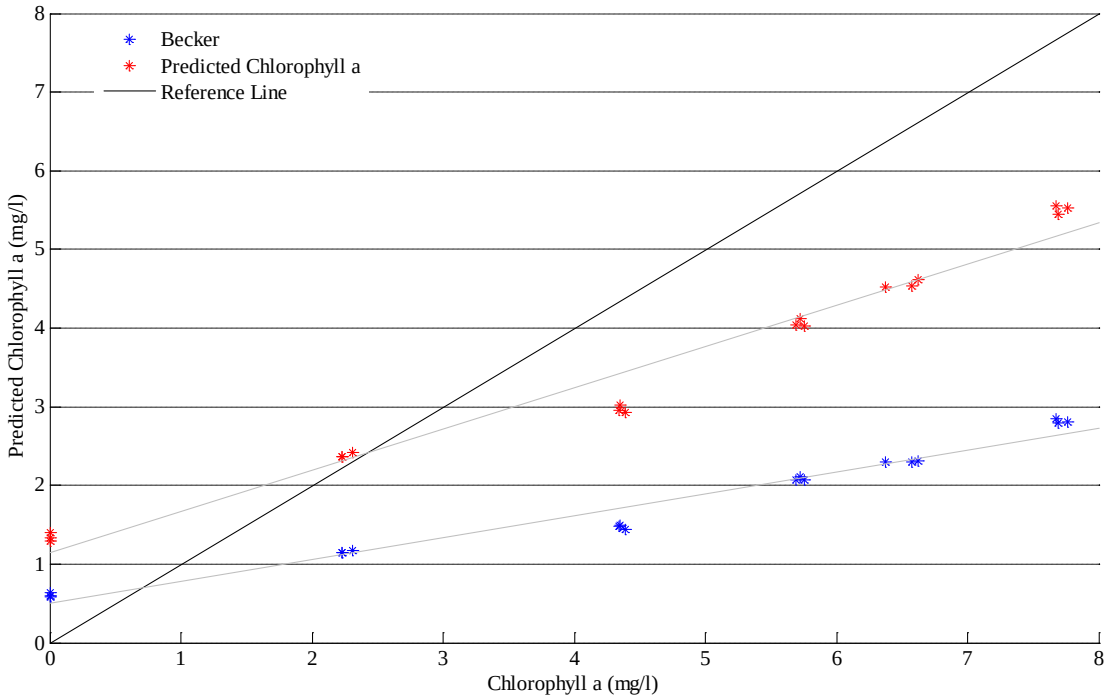

Figure C-7 Chlorophyll *a* extraction from *Cylindrocystis* sp. in beef manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference equation (Equation 5.1) showed results different than the actual Chlorophyll *a*.

Table C-7 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-7 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Cylindrocystis sp.* in beef manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	33.91	33.91	476.44	0.00
Residual	16	1.14	0.07		
Total	17	35.05			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Cylindrocystis* sp. in beef manure.

Scenedesmus sp. in Beef Manure

Figure C-8 shows the predicted values of Chlorophyll *a* extraction from *Scenedesmus* sp. in beef manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Scenedesmus* sp. in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Scenedesmus* sp. in beef manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Scenedesmus* sp. concentration was 1.2 mg/ml.

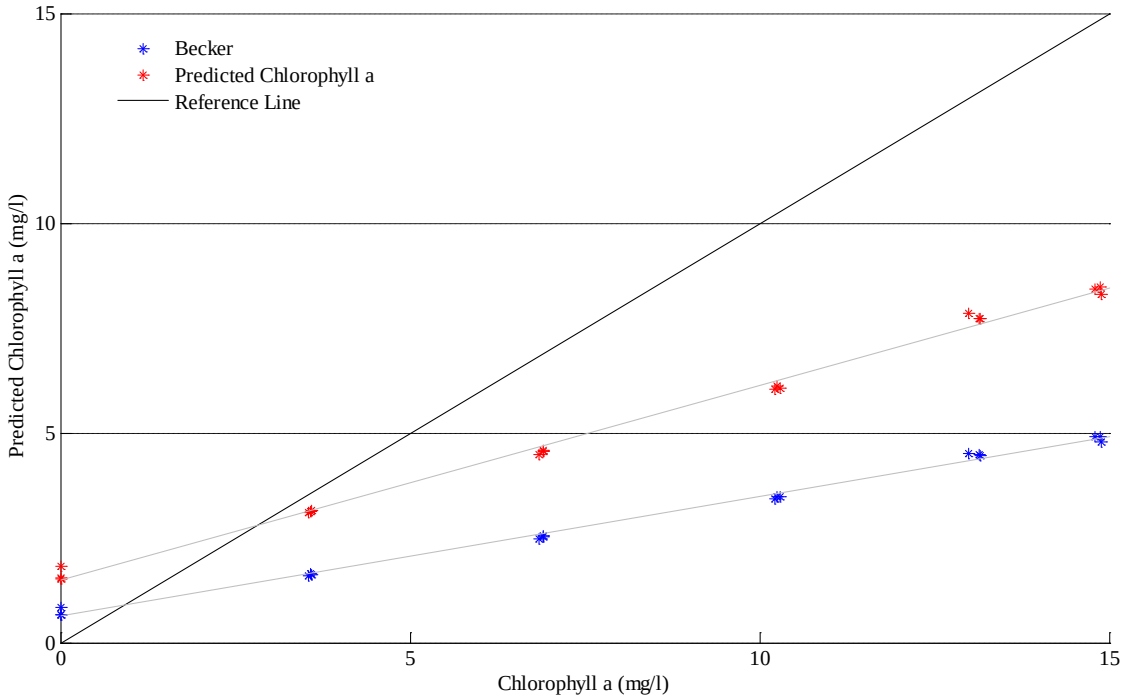

Figure C-8 Chlorophyll *a* extraction from *Scenedesmus sp.* in beef manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference equation (Equation 5.1) are different to the reference line (1:1).

Table C-8 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-8 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Scenedesmus sp.* in beef manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	104.93	104.93	3860.42	0.00
Residual	16	0.43	0.03		
Total	17	105.37			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Scenedesmus sp.* in beef manure.

Chlorella vulgaris in Sheep Manure

Figure C-9 shows the predicted values of Chlorophyll *a* extraction from *Chlorella vulgaris* in sheep manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Chlorella vulgaris* in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Chlorella vulgaris* in sheep manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Chlorella vulgaris* concentration was 1.2 mg/ml.

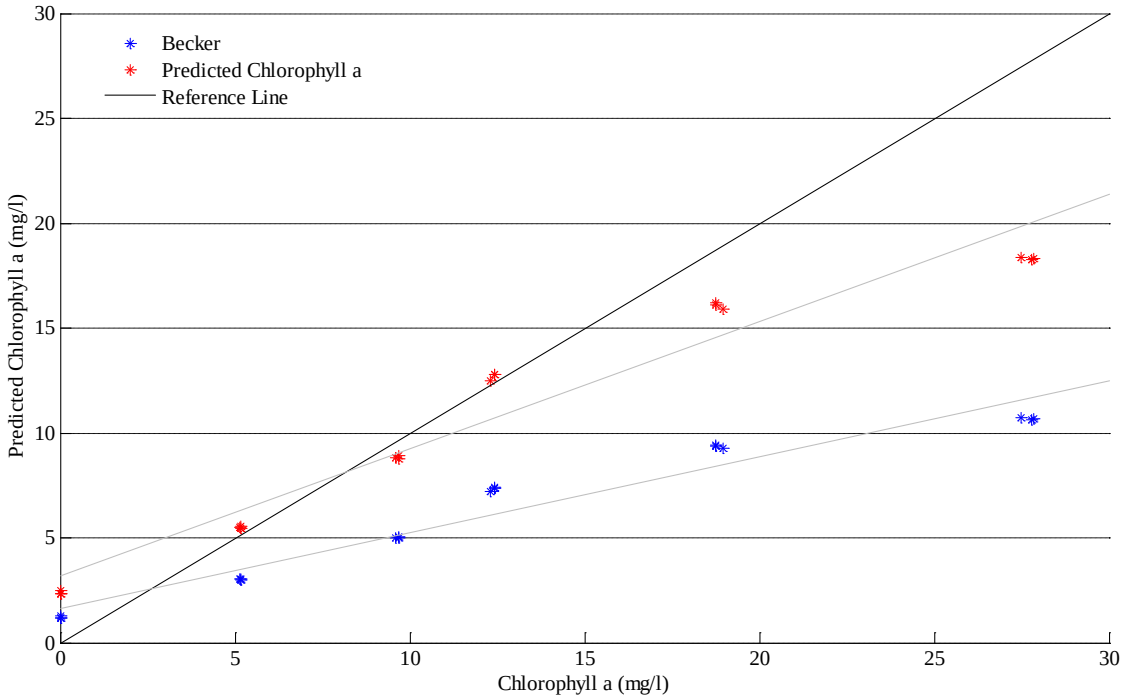

Figure C-9 Chlorophyll *a* extraction from *Chlorella vulgaris* in sheep manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference equation (Equation 5.1) are different. Both predicted values are very different from the reference line (1:1).

Table C-9 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-9 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Chlorella vulgaris* in sheep manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	540.63	540.63	279.08	0.00
Residual	16	30.99	1.94		
Total	17	571.62			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Chlorella vulgaris* in sheep manure.

Cylindrocystis sp. in Sheep Manure

Figure C-10 shows the predicted values of Chlorophyll *a* extraction from *Cylindrocystis* sp. in sheep manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Cylindrocystis* sp. in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Cylindrocystis* sp. in sheep manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Cylindrocystis* sp. solids concentration was 4.1 mg/ml.

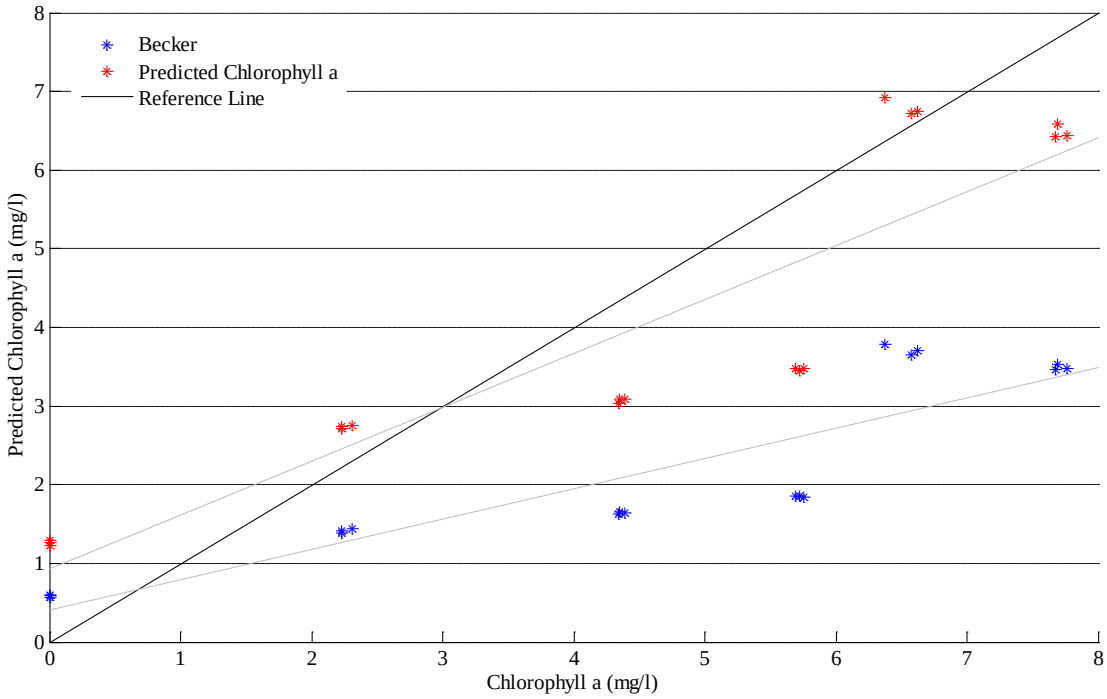

Figure C-10 Chlorophyll *a* extraction from *Cylindrocystis* sp. in sheep manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference equation (Equation 5.1) are very scattered.

Table C-10 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-10 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Cylindrocystis* sp. in sheep manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	58.16	58.16	63.60	0.00
Residual	16	14.63	0.91		
Total	17	72.79			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Cylindrocystis* sp. in sheep manure.

Scenedesmus sp. in Sheep Manure

Figure C-11 shows the predicted values of Chlorophyll *a* extraction from Scendemsus in sheep manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Scenedesmus* sp. in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Scenedesmus* sp. in sheep manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Scenedesmus* sp. solid concentration was 1.2 mg/ml.

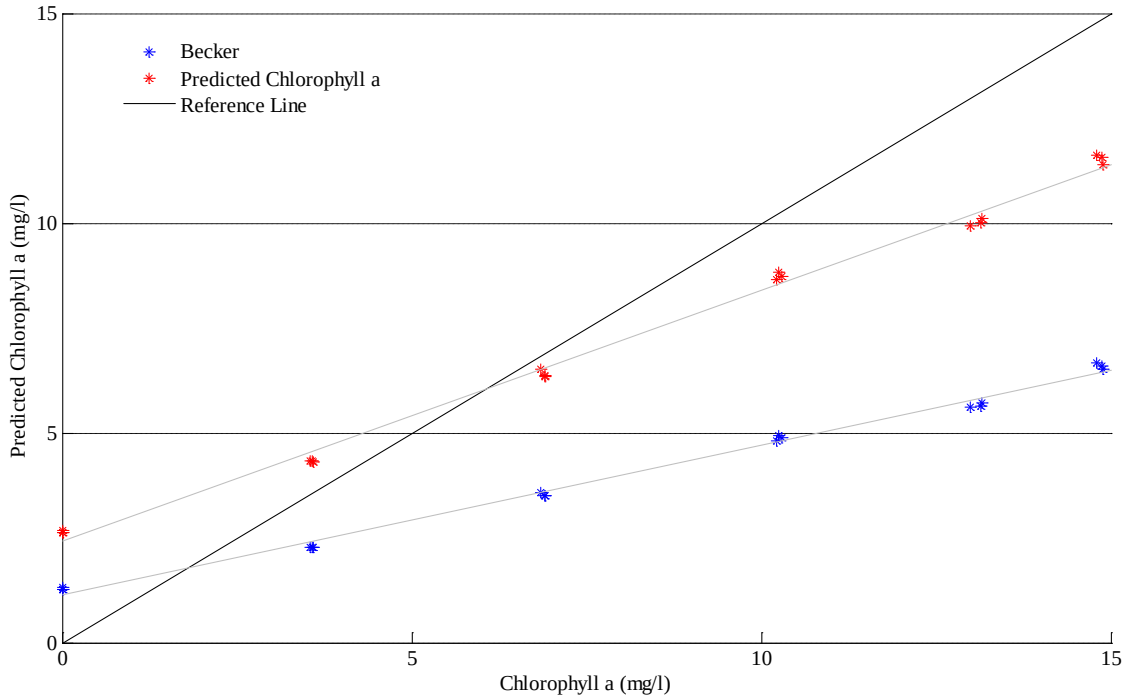

Figure C-11 Chlorophyll *a* extraction from *Scenedesmus* sp. in sheep manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) are closer to the reference line than results for the reference equation (Equation 5.1).

Table C-11 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-11 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Scenedesmus* sp. in sheep manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	175.20	175.20	3242.45	0.00
Residual	16	0.86	0.05		
Total	17	176.06			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Chlorella vulgaris* in dairy manure.

Chlorella vulgaris in Swine Manure

Figure C-12 shows the predicted values of Chlorophyll *a* extraction from *Chlorella vulgaris* in swine manure, calculated by the new equation proposed (Equation 5.3), and plotted against the Chlorophyll *a* extracted from *Chlorella vulgaris* in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Chlorella vulgaris* in swine manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Chlorella vulgaris* solid concentration was 1.2 mg/ml.

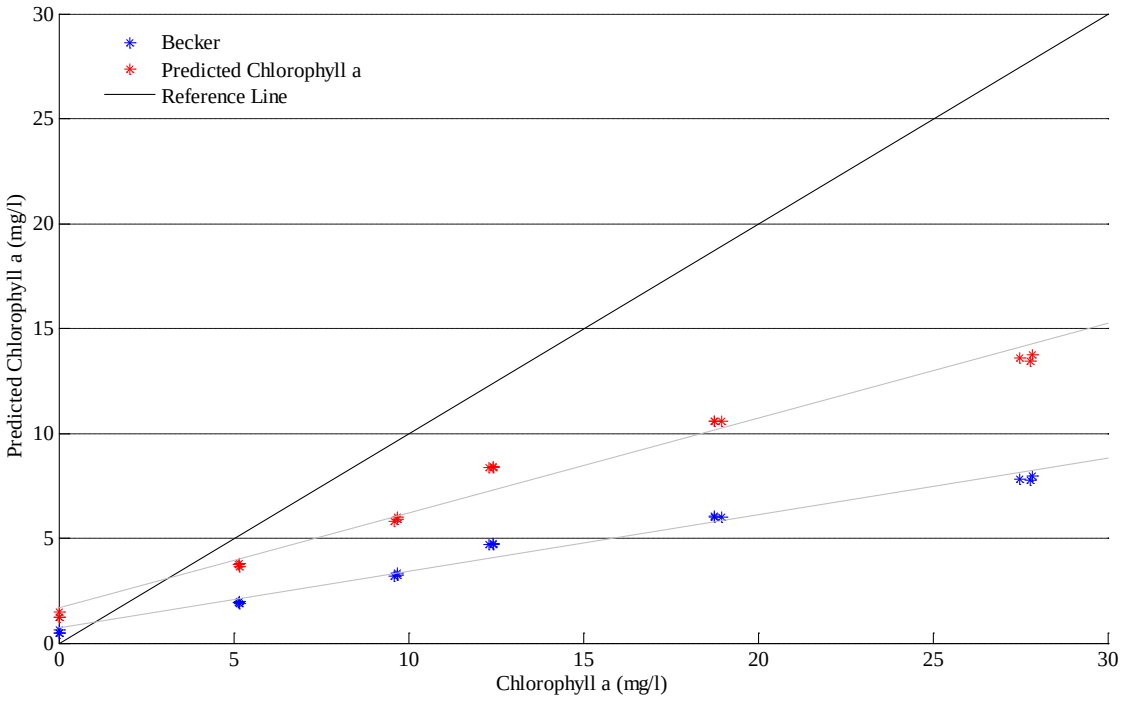

Figure C-12 Chlorophyll *a* extraction from *Chlorella vulgaris* in swine manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) are closer to the reference line than results for the reference equation (Equation 5.1).

Table C-12 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-12 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Chlorella vulgaris* in swine manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	299.47	299.47	776.35	0.00
Residual	16	6.17	0.39		
Total	17	305.64			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Chlorella vulgaris* in dairy manure.

Cylindrocystis sp. in Swine Manure

Figure C-13 shows the predicted values of Chlorophyll *a* extraction from *Cylindrocystis sp.* in swine manure, calculated by the new equation proposed (Equation 6.2), and plotted against the Chlorophyll *a* extracted from *Cylindrocystis* sp. in urea media, measured by the reference equation (Equation 6.1). Chlorophyll *a a* extracted from *Cylindrocystis sp.* in swine manure was also calculated by the reference equation (Equation 6.1) and plotted in the same chart in order to compare results to the new equation (Equation 6.2). *Cylindrocystis sp.* solids concentration was 4.1 mg/ml.

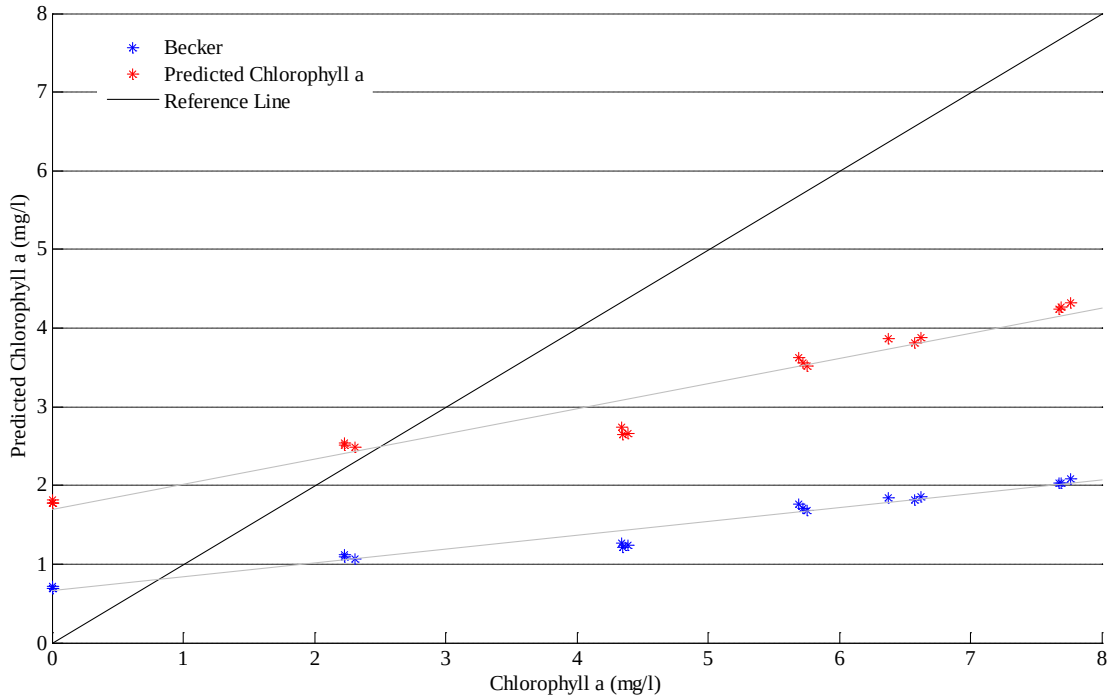

Figure C-13 Chlorophyll a extraction from *Cylindrocystis* sp. in swine manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference equation (Equation 5.1).

Table C-13 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-13 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Cylindrocystis* sp. in swine manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	12.62	12.62	315.07	0.00
Residual	16	0.64	0.04		
Total	17	13.26			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Chlorella vulgaris* in dairy manure.

Scenedesmus sp. in Swine Manure

Figure C-14 shows the predicted values of Chlorophyll *a* extraction from *Scenedesmus* sp. in swine manure, calculated by the new equation proposed (Equation 5.3), plotted against the Chlorophyll *a* extracted from *Scenedesmus* sp. in urea media, measured by the reference equation (Equation 5.1). Chlorophyll *a* extracted from *Scenedesmus* sp. in swine manure was also calculated by the reference equation (Equation 5.1) and plotted in the same chart in order to compare results to the new equation (Equation 5.3). *Scenedesmus* sp. solids concentration was 1.2 mg/ml.

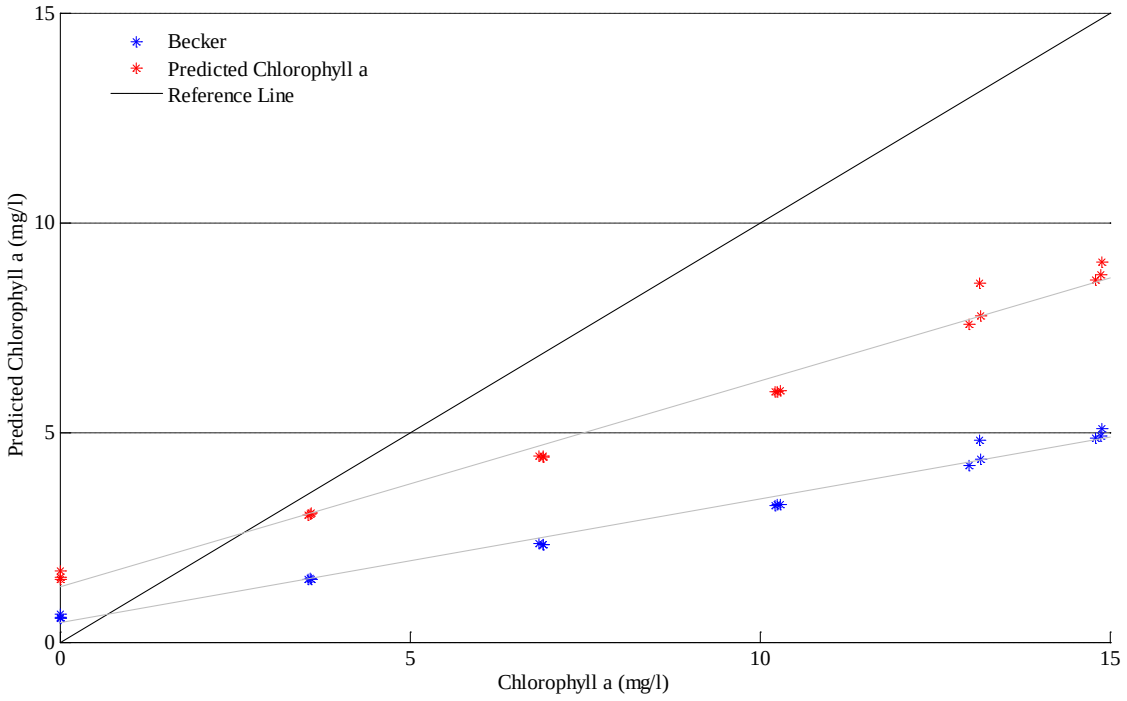

Figure C-14 Chlorophyll a extraction from *Scenedesmus* sp. in swine manure: actual vs predicted values.

Results for the new equation proposed (Equation 5.3) and reference equation (Equation 5.1).

Table C-14 shows the Analysis of Variance of the linear regression between values predicted by the new equation and the actual Chlorophyll *a*.

Table C-14 Analysis of Variance of Predicted Chlorophyll *a* extracted from *Scenedesmus* sp. in swine manure.

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1.00	117.80	117.80	1095.76	0.00
Residual	16.00	1.72	0.11		
Total	17.00	119.52			

Since p-value is smaller then 0.05, regression is significant and there is a linear relation between Predicted and Actual values of Chlorophyll *a* extracted from *Chlorella vulgaris* in dairy manure.

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REFEREED ARTICLES

Amaral, M. F. P., R. Gates, D.G. Overhults, I.F.F. Tinoco, H. Li, R. Burns, H. Xin, J. Earnest. 2008. ANALYSIS OF DIFFERENT METHODS TO COMPUTE AMMONIA CONCENTRATION AND EMISSION RATE In: VIII International Livestock Symposium, Foz do Iguacu, Brazil.

Amaral, M. F. P., R. Gates, E. Wilkerson, D.G. Overhults, I.F.F. TINOCO, R. Burns, H. Xin, J. Earnest. 2007. Comparison Between Two Systems for Ammonia Emission Monitoring in Broiler Houses In: ASABE International Meeting, Colorado, USA.