"Algae for Conversion of Manure Nutrients to Animal Feed: Evaluation of Advanced Nutritional Value, Toxicity, and Zoonotic Pathogens"

1) INTRODUCTION

Large concentrated/confined feeding operations (CAFOs) allow competitive animal production in a global market, but intensification of animal production requires intensification of manure recycling. Technologies and methods to recreate feed nutrients (crops) from waste nutrients, with minimal environmental impact, have not kept pace with CAFO development. The proposed work will begin to fill knowledge gaps that have slowed the advancement of a leading candidate technology for accelerated manure recycling — algae-based animal feeds. We will contribute to answering this question: Can manure nutrients be recycled into microalgae-based animal feeds both safely and affordably?

The long-term vision for algae animal feeds is based on the rapid growth rate of algae (>12 tons of crude protein per acre per year) versus conventional crops (1.2 tons /acre per cutting genetically engineered alfalfa), which allows more manure to be recycled per acre of land. This rapid assimilation of nitrogen into protein is also the basis for algae wastewater treatment. At dairies and swine farms, for example, algae production/wastewater treatment systems can be used to remove nitrogen (N), phosphorus (P), and dissolved organic matter from barn flush water, while producing high protein biomass. Such treatment is already commercial for municipal wastewater treatment in the US and for dairy in New Zealand, albeit with only a few installations and without algae feed production. Valuable nutritional oils are another component of microalgae that is produced commercially for the aquaculture industry and for human consumption (e.g., DSM-Martek, Solazyme). We are working towards CAFOs with land-efficient intensified manure-to-high-value-feed production via algae technology. The various technological parts are already commercial. What is needed is to integrate these technologies in a safe and affordable manner. The proposed project will also generate data helpful to the development of algae biofuels. While our main emphasis is production of proteins and oils with high nutritional value, the data and modeling of oil productivity and content will be directly applicable to algae biofuels.

Statement of Problem*:* The goal of this project is to cultivate microalgae on dairy farm wastewater to produce animal feed and to characterize the pathogen content of the feed. This information is an important element of the total risk analysis of algae feeds. The project brings together cutting-edge scientific resources in both algae production and pathogen characterization.

A strong livestock industry is essential to our nation's economy, a healthy food supply and viability of rural communities. As population pressure crowds agriculture, CAFOs have increased dramatically in number and size. Rather than animals grazing in pastures and rangeland, feed is brought to the animals confined in small areas. Dairies, cattle feed lots and poultry farms generate large amounts of waste. Dairy cows in California create twice as much fecal waste as the human population (dry weight basis; data NRCS 2008) and these wastes can contaminate ground waters and generate CO_2, methane and obnoxious odors. However, waste products from animal feeding operations can be a valuable resource supplying livestock feed supplements (LFS), energy and fertilizer for sustained production when utilized in an integrated biological system.

Nutrient contamination, primarily N and P, generated by CAFOs is leached from soils and can percolate into ground waters and/or be washed into freshwater reservoirs and the ocean through urban storm water systems. N enhances algal growth, resulting in uncontrolled algae blooms in freshwater and red tide blooms are frequently observed off the coast of southern California and are reported globally (Selner et al., 2003). Algal toxins and the hypoxia produced by decay of such blooms are associated with mortality of

marine fauna (Kudela and Cochlan, 2000). Moreover, many groundwater basins in the arid southwest, including the groundwater used by Cal Poly Pomona, have been historically under-used due to high nitrate concentrations. Consumption of drinking water with high nitrate levels has been associated with methemoglobinemia (blue-baby syndrome) and gastric cancer (Blowes et al., 1994). Excess P levels in waters are also associated with cyanobacterial blooms (Downing et al., 2001).

Feed costs are extremely variable and seriously impact the milk and meat industry (Bailey, 2007). In addition, current animal feed production competes for water and land use with human food sources. Microalgae have been frequently cited as having great potential as an animal feed (see detailed sections below). Their main advantage is that they are an extremely fast-growing crop, able to convert the waste nutrients in manure into raw material for animal feed. Thus, with microalgae, less land is required for waste management and feed production than with typical feed crops such as corn and alfalfa. Algae feed production has not become commercial previously due to many uncertainties, in particular potential pathogen and toxicity concerns, which are to be addressed in the proposed research.

Background*:* Microalgae have great potential both for sustainable bioremediation of wastewaters and as a feedstock for biofuels LFSs. Mass-culture of algae on manure N and P is an alternative to land spreading of manure effluents, particularly in the case of CAFOs where groundwater contamination is problematic and many CAFOs do not have affordable access to large tracts of land for manure application to soils. A highly productive crop is needed to remove manure N and P to prevent nutrient pollution in smaller land areas than are required by crops such as corn. Microalgae are photosynthetic organisms with high productivity (many strains double in less than a day) that remove eutrophying nutrients from water sources. They have been used for over 50 years in municipal wastewater treatment (see Oswald 1988 for review) and more recently for bioremediation of manure effluents (Woertz, Lundquist, et al., 2009, Mulbry et al., 2008). Some microalgae produce lipids, rather than carbohydrates, as their primary carbon storage molecule and thus, there has been considerable interest in coupling wastewater treatment to produce feedstock for renewable fuel production. Like higher plants, microalgae produce triacyglycerides that can be readily converted to fatty acid methyl esters, a substitute for fossil-derived diesel fuel. Microalgae also have high protein content and the ability to grow in saline or wastewaters not suitable for agricultural irrigation.

Current animal feed production competes for water and land use with human food sources. One of the major constraints to livestock production in this country as well as in many developing countries is shortage of protein (Preston and Leng 1987, Tareque and Saadullah 1988). In addition, lipid-rich feed supplements are used in the dairy industry to promote lactation (Papadopoulos et al., 2002). Because these supplements are relatively costly, Japanese and German workers explored the possibility of utilizing fast growing unicellular algae as a source of nutrients for humans and animals in the early 1950s (see Halama, 1990, for a review). Because of their ubiquity, *Chlorella* and *Scenedesmus* have been widely studied as a nutritional supplement. Protein content ranges from 50 to 60% of dry matter and except for methionine and cysteine, the essential amino acid profile is favorable for the nutrition of farm animals (Becker, 2007). Algae are also a rich source of carotene, vitamin C and K, and B vitamins (Halama 1990), highly unsaturated fatty acids (HUFAs), and bioactive compounds.

N is often the most limiting nutrient in both ecological and agricultural systems. Producing N-fertilizers is an energy intensive process consuming large amounts of fossil fuel to convert N_2 gas to ammonia. As fossil fuel prices escalate and atmospheric CO_2 rises, the global demand for technologies to drive sustainable agricultural practices and production of renewable fuels will increase. The work proposed here will couple waste management with biofuels and LFS production. Of note, on January 1, 2013, President Barack Obama signed a bill that enables algae-derived fuels to qualify as feedstock, achieving tax eligibility and parity ($1.01/gallon tax credit) with other biofuels (e.g., cellulosic feedstocks)(Biodiesel Magazine, 2013).

The proposed work will focus on several key processes that currently challenge the economically feasible use of integrated biological processes in agriculture waste management and feed and fuel production. Key among these is evaluation of the risks and benefits of algae animal feeds. The development and optimization of these processes and a detailed evaluation of cost-efficiency and environmental offset credits will provide a basis for implementation of the system and variations of the system for specific CAFOs.

***Algae Biomass*:** Microalgae are a phylogenetically diverse group of aquatic, photosynthetic, organisms that vary greatly in their metabolic capabilities, environmental adaptations and morphology. They lack the complex cellulose structures of land plants, can be highly productive (many strains can double in less than a day), and are rich in protein (See Becker, 2007 for review). Of interest for both algae biofuel and algae feed is the high amounts of oil (lipid) produced, primarily as triglycerides. Algal neutral storage lipids are similar in structure and molecular weight (carbon chains ranging from 12 to 22) to the oils extracted from terrestrial plants. Microalgae can have oil contents that vary from 15 to 77% of the dry weight (Banerjee et al., 2002; Chisti, 2007) compared with 4% in corn. The high oil content of some algal strains is also of interest to the dairy industry as lipids are a key component in feed supplements for lactating dairy cows.

***Algae as Animal Feed*:** Microalgae are an essential food source in nature and in aquaculture operations in the rearing of molluscs, crustaceans and small fish (FAO, 1996). A large body of literature has been developed to explore the chemical composition of algae species for aquaculture (see Lavens and Sorgeloos, 1996, for review). The proximate composition of algae however, is species-specific (Brown, 1991) and strongly influenced by environmental parameters including light, temperature and nutrient levels (Herrero et al.,1991; Gatenby et al., 2003). Thus biomass production operations must be concerned with species composition, proximate composition, and the environmental influences on both. In general, depletion of specific nutrients results in slowed algal growth, protein levels decline while lipids and carbohydrates increase. Aquaculture operations seek algal strains rich in HUFAs, in particular eicosapentaenoic acid (20:5n-3, EPA), arachidonic acid (20:4n-6, ARA), and docosahexaenoic acid (22:6n-3, DHA), HUFAs of importance for marine organisms and essential fatty acids for humans.

The use of microalgae as animal feed is relatively recent and predominantly aimed at poultry because the high carotenoid content of many species improves the color of the skin and egg yolks. Multiple nutritional and toxicological evaluations demonstrated that algae biomass is a valuable feed supplement or substitute for conventional protein sources (Becker, 2007). In addition, a variety of potentially useful agricultural and pharmaceutical secondary metabolites, including anti-viral (Lee et al., 1998), anti-tumor and anti-microbial (Ordog et al., 2004) and immune-stimulatory (Pulz and Gross, 2004) agents are produced by microalgae, improving the product for consumption.

The nutritional quality of proteins is based on the amino acid composition. In the case of ruminants, all the essential amino acids can be synthesized by the rumen microorganisms, which should make ruminants independent of a dietary source of these. However, maximum rates of growth or milk production cannot be achieved without dietary amino acid supplements and non-ruminant animals cannot synthesize the essential amino acids fast enough to meet the animal's needs. Therefore those essential amino acids must be provided in the ration (Mac Donald, 2002). Most algae species have high protein content and favorable amino acid composition relative to WHO/FAO standards and have a relatively low content of potentially troublesome non-protein nitrogen (Becker, 2007; Lavens and Sorgeloos, 1996).

Carbohydrate utilization by animals depends on their digestion system. The cellulose content of algal cell walls (~10% of dry weight) will affect the digestibility by non-ruminant animals. In land plants, cellulose may

account for 20% to 50% (w/w) of the biomass (Becker, 2007). Microbial fermentation, however, enables ruminants to utilize cellulose efficiently and algal feed supplements have been used successfully to increase growth rates of calves (Chowdhury et al., 1995) and improve milk composition in dairy ewes (Papadopoulos et al., 2002). Algal cell walls vary among taxa (Domozych, 2011). In the eukaryotic algae currently used for large-scale production (i.e., the *Chlorophyceae*, such as *Chlorella and Scenedesmus spp.,* cell walls are composed of β-1,4-glucan and cellulose, Popper and Tuohy 2010, Domozych, 2011).

Algae feeding tests performed so far indicate that their overall digestibility is high (Becker, 2004, Abril et al., 2003). Algal lipids (DHA, and other Omega 3s) have a positive impact as an animal feed on healthy fat marbling in cattle (Stamey et al., 2012). The addition of microalga-derived astaxanthin to feed formulations enhances the color of the muscles of salmonids and poultry. This has a high biotechnological potential and culture techniques for the algae *Haematococcus pluvialis* are well developed for this purpose (Pulz and Gross, 2004).

Wastewater Treatment by Algae*:* A major cost factor for algal biomass production is the provision of water and nutrients (Davis and Aden, 2011), as well as generating revenue from the treatment service (Benemann and Oswald, 1996; Lundquist et al., 2010). Thus, a resource and economic synergy are possible.

Ponds are probably the most common technology used for treatment of municipal, agricultural, and aquacultural wastewaters in the US, with over 7,000 publicly-owned treatment pond and lagoon systems (USEPA, 2008). However, nutrient removal is an increasingly common regulatory requirement, and conventional ponds are not well suited for nitrogen and phosphorus removal. Newer pond technologies (e.g., paddle wheel-mixed raceway ponds, Figure 1, and newer variants of aerated lagoons) have advanced the reliability, effectiveness, and geographical range of pond treatment.

Bioflocculation of microalgae in raceway ponds has been demonstrated as a chemical-free means for removing excess algal suspended solids from pond effluent (Lundquist et al., 2011; Craggs et al. 2011; Figure 2), which has been the major drawback of ponds previously. Bioflocculation is also the preferred means to harvest algae for animal feed and biofuels due to its simplicity, low energy input, and lack of chemical residues (USDOE, 2010; 2012; Lundquist et al., 2010). The first large scale-up of algae biofuel technology is underway by Sapphire Energy, Inc. (San Diego, CA), with private and DOE funding. Considerable progress has been made over the past six years to develop and commercialize missing

Figure 1. Raceway pond with paddle wheels for wastewater treatment. Lundquist participated in the design of this full-scale facility for secondary treatment only, which was commissioned in 1998 and still operates.

Figure 2. Wastewater treatment and effective bioflocculation of algae at the SLO pilot ponds, June 2012. (Left) Primary clarifier effluents feeds the ponds. (Center) Bioflocculated algae grow and perform treatment. (Right) Settled pond effluent after 30 minutes in a continuous-flow settling tanks.

elements in the algae biofuels production chain. Enclosed bioreactors mitigate some of the problems of maintaining monocultures and predation issues, but capital and labor costs (Weismann et al., 1988) limit

their use to production of high-value products.

Making cost-efficient algae-based feed supplements, a relatively low value commodity, will require major advancements in several technologies. Other major hurdles to economically feasible algae-based biomass production include the use of strains adapted to the local environment, development of resource specific production and management systems and, in the short-term, coupling algaculture with mitigation of environmental problems and co-production of high value compounds.

Microalgae ponding systems were developed in the 1950s for municipal sewage treatment and this approach continues to serve as a starting point for the development of cost-efficient algae- biomass for feed and fuel production. Using the lessons learned from the past 50 years of research, this proposal describes a scenario where microalgae could be grown on wastewater (dairy effluent) to produce multiple benefits including a high quality effluent (i.e., stripped of nutrients), biofuels, and a high protein livestock feed supplement. The economic potential of the various byproducts of this process could offset the cost of production to produce a commercially viable biofuel.

The fundamental engineering design parameters were developed and the basic biological processes in bioremediation were described in high rate-ponds (Oswald, 1988). Although algae-based tertiary treatment is economically feasible, few municipal algae ponds attempt to control species composition or even harvest the algal biomass (Benemann and Oswald, 1996). At present, it is unlikely that the economics of liquid fuel production from algal biomass can be uncoupled from wastewater, feed production and CO_2 abatement credits. Significant improvements in several key technologies, such as strain selection, species composition, best cultivation practices and harvesting, are needed to advance the economics of algae-based waste treatment and biomass production.

Minimal nutritional requirements for algal growth can be estimated from the approximate molecular formula of algal biomass: $C_{(0.48)}$, $H_{(1.83)}$, $N_{(0.11)}$, $P_{(0.01)}$ (Grobbelaar, 2004). Municipal wastewaters typically have less N than P relative to algal biomass (Fulton, 2009). Although CO_2 limits algal growth in high rate oxidation ponds (Benemann and Oswald 1996), when CO_2 is supplemented, N then typically limits algal growth on municipal wastewaters (Benemann et al., 1977). N limitation has long been known to be a trigger for lipid synthesis in some algal species (Spoehr and Milner 1949, Collyer and Fogg, 1955) and is also associated with cyanobacterial blooms in wastewater ponds (Benemann et al., 1977). The standard process flow diagram for algae wastewater treatment-biofuels/feed production is shown in Figure 3. Maintenance of species composition, especially in outdoor ponds and when using wastewaters, is problematic (Benemann et al., 2008). Cultivation of *Spirulina* in open outdoor ponds has been a success story in commercial algaculture. This strain, described by Bernal Diaz (Diaz, 1632) in his chronicle of Cortez's conquest of Mexico, grows in nearly pure culture in the alkaline, high salt waters of Lago de Texcoco. While protein and lipid profiles are characteristic of some organisms, microalgae show great inter- and intra-specific variation in amino and fatty acid profiles and these profiles can be affected by environmental parameters (Roessler, 1990; Flynn et al., 1993; Shifrin and Chisholm, 1981).

As in agriculture, each specific algae production site, considering for example, location, season and water quality available, will require different strains adapted to local conditions (Sheehan et al., 1998). The diversity of algal species, interspecies variations in metabolism as well as the effect of cultivation practices on proximate composition, have led to high interest in the field for rapid and effective techniques for screening algal physiological processes. Open ponds present a number of obstacles to monoculture production including rapid variations in temperature, light, and invasion of competing algae species and grazers. These problems will require rapid means to monitor ponds for species composition, quantify growth rate, identify algae stress, and assess time of harvest to improve productivity and avoid pond crashes. The

current proposal seeks funding to adapt technologies developed by molecular microbiologists and aquatic ecologists to monitor algal populations, growth and physiological processes under controlled conditions in the lab at Cal Poly Pomona (CPP) and in demonstration-level wastewater treatment ponds at Cal Poly San Luis Obispo (SLO).

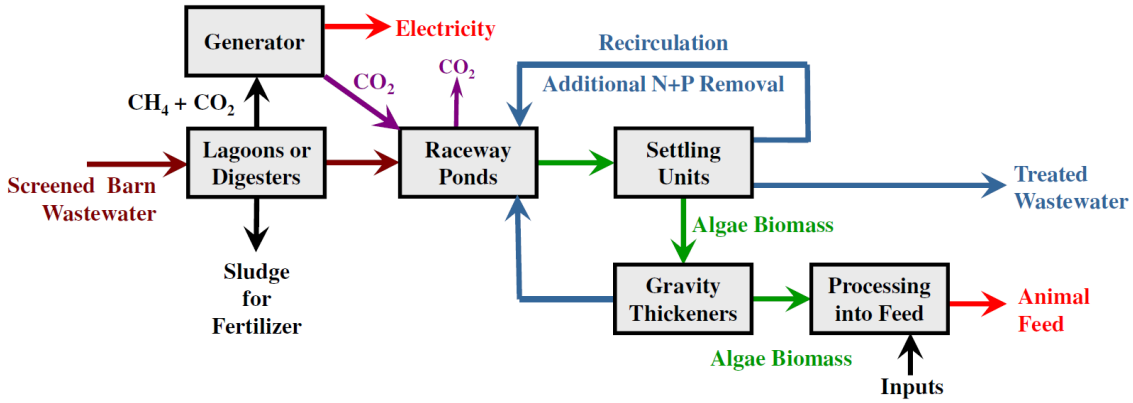

Figure 3. Envisioned process flow diagram for algae wastewater treatment and animal feed production. The proposed research will develop information on nutritional algae production in Raceway Ponds, with supplemental CO_2 provision. The algae also serve to remove nutrients and organic matter from the wastewater. Bioflocculation of the algae biomass allows harvesting in the Settling Units. This dilute material is thickened and then processed into feed through dewatering, drying, and pelletizing. Pasteurization parameters to achieve safe feed will be determined.

Microbial Community Dynamics: Ponding operations may benefit from investigation of population and community dynamics using recent advances in next generation sequencing techniques (e.g., 454-pyrosequencing and Illumina) and microarray protocols (e.g., PhyloChip). High-throughput sequencing is a promising method, as it provides enough sequencing depth to cover the complex microbial communities (Shendure and Ji, 2008). For these reasons, we believe that the elucidation of bacterial and algal taxa based on deep sequencing may provide some insights into bacterial and algal communities and their interactions in algal ponds.

GeoChip, another high-throughput metagenomic technology, has provided powerful tools for examination of microbial interactions at the community level in greater depth. The GeoChip is a gene array designed to study the functional structure and metabolic diversity of microbial communities (He et al., 2007; He et al., 2010) and has been used widely to assess the functional communities of various ecosystems (Hazen et al., 2010; Liang et al., 2011; Lu et al., 2012; Zhou et al., 2012, Wang et al., 2009). The newest version, GeoChip 4.0, contains more than 82,000 probes representing genes from over 400 functional categories (Zhou et al., 2012; Wang et al., 2009).

In synergy with feed and biofuel production, algae-based wastewater treatment is a low cost, simple process compared to conventional wastewater treatment. Furthermore, algae systems have ~50% lower energy consumption compared to conventional mechanical treatment technologies (Downing et al, 2002; Lundquist et al., 2010; Craggs et al., 2012). The combinations of these two aspects of algae biotechnology are topic of the present proposal and are described below. Key, however, to synergistic feed and fuel production using wastewaters (dairy effluent) is an understanding of pathogens and potential toxins (i.e., bacterial and cyanobacterial toxins) introduced to the system either from manure or during growth and processing of the algae biomass.

Manure pathogens: Manure-borne pathogens include*: Salmonella, Listeria, Clostridium, Campylobacter* spp., pathogenic *E. coli* (such as Shiga toxin-producing *E. coli*), *Mycobacterium paratuberculosis,*

Cryptosporidium parvum and many viruses (Anonymous, 2012; Sobsey et al., Available Online). Zoonotic pathogens associated with cattle include *Yersinia* spp., *Leptospira* spp., *Coxiella burnetii*, and the parasitic protozoa *Giardia lamblia* (Klein et al, 2010b). CAFOs produce large amounts of waste in California (Cole et al., 1999). A mid-size dairy of 1,000 cows will produce more than 12,000,000 kg of manure per year (McGarvey et al., 2004) and the manure is usually stored/processed on-site which increases the risk of transporting bacterial pathogens by a number of mechanisms, e.g., via water. Enteric bacterial pathogens that cause much of foodborne illnesses, have been reported to survive for long periods in manure (Pillai et al., 2002; Kudva et al., 1998). Survival of potentially pathogenic organisms in manure depends on many factors; e.g., type of slurry or manure pH, dry matter content, temperature, numbers and type of pathogen present, and presence of competing organisms (Kirk, Available Online).

Cyanobacteria and their Toxicity: Cyanobacteria (also called cyanophytes), are small, unicellular or colonial prokaryotic organisms that have great diversity of form and habit of growth. These characters were used as the basis for genus and species identification until recently when genetic techniques have come to the fore (Stewart and Falconer, 2008). A number of cyanophyte species produce potent hepatotoxins or neurotoxins that can be transferred through the food web where they may kill other life forms such as zooplankton, shellfish, fish, birds, marine mammals and humans that feed, either directly or indirectly, on them (Warrington, 2001). Freshwater cyanobacteria include: *Anabaena circinalis, Anabaena flos-aquae, Aphanizomenon flos-aquae, Aphanizomenon ovalisporum, Cylindrospermopsis raciborskii, Gloeotrichia echinata, Lyngbya, Microcystis aeruginosa, Nodularia spumigena, Nostoc, Oscillatoria, Schizothrix* and *Scytonema* (Warrington, 2001). *Nodularia spumigena*, was the first cyanobacterium to be identified in the scientific literature as the cause of livestock poisoning in 1878 (Francis, 1878). Toxins are known to bioaccumulate in some aquatic invertebrates, zooplankton and mussels, and in marine mammals (Warrington, 2001), shellfish, prawns and fish (Mulvenna et al., 2012).

The basic approaches used to measure toxic cyanobacteria include cell counts using microscopy, *in vivo* systems to analyze toxicity and advanced analytical chemistries (Rasmussen et al., 2007). Genetic methods have recently been used for identification of cyanobacteria. Molecular diagnostics target identification of informative DNA, RNA or unique proteins, such as toxins they produce (Baker et al., 2001, Rasmussen et al., 2007, Schembri et al., 2001). Real-time PCR (qPCR) relies on knowledge of the DNA sequence of the genes that encode cyanobacterial toxins, necessitating DNA sequencing so that specific PCR assays for those genes can be designed and tested (Kurmayer and Kutzenberger 2003; Vaitomaa et al., 2003; Neilan et al., 1995). A variety of biological methods, many of which search for specific bioactivity of known toxins use bioassays with bacteria, invertebrates, vertebrates, mice, fish and *in vitro* cell cultures as the test organisms (Blaha and Marsalek, 2000). This study, strongly justifies investigations of presence of cyanobacteria and their toxins in algal biomass since manure is rich in nutrients that can stimulate cyanobacterial growth in feed destined for livestock.

Preliminary Data on Wastewater Treatment: The main remaining challenges in algae-based wastewater treatment are: **(a)** maintaining cultures that consistently bioflocculate for sedimentation harvesting (mechanical or chemical harvesting is expensive or may be problematic for feed production) and **(b)** nutrient removal during winter. CPSLO has been operating a large pilot plant at a municipal wastewater treatment plant for over a year, making appreciable progress on these topics (described below). In addition, CPSLO has access to full-scale 6-acre raceway treatment facilities for future studies.

Nitrogen Removal: The total ammonia nitrogen (TAN) removal results from the new CPSLO 30-m^2 ponds has been quite encouraging for all three hydraulic residence times (HRTs) tested (Figure 4). For single ponds, removals were 84-94%, depending on residence time. Ponds-in-series were also tested, with the

initial pond HRT being 3 days and the subsequent pond HRT being 4 days. The TAN residual in the second pond was below the detection limit of 0.5 mg/L N.

Bioflocculation and Settling Results: Bioflocculation of algal-bacterial total suspended solids (TSS) allows the material to be inexpensively separated from the water without addition of chemical coagulants. The effluent of the pilot settling tanks at the Cal Poly pilot plant have generally contained 10-40 mg/L TSS (Figure 5). These settling results are a vast improvement over previous raceway pond results. For example, a raceway system treating municipal wastewater in California had average effluent TSS values of 111 mg/L after poor settling (Green et al. 1995). The wastewater raceway facility has also been used to study the biofuel potential of wastewater algae. CPSLO has achieved the equivalent of 600-700 gal/acre-year of algae oil production (Kelly, 2012) without any attempt to optimize for oil or protein production through nutrient stress. In the lab, CPSLO has achieved up to 50% total lipid content in nutrient stressed cultures (Kelly, 2012). GC/MS analysis of the oils showed a predominance of C-16 and C-18 carbon chains, which are also valuable for animal nutrition.

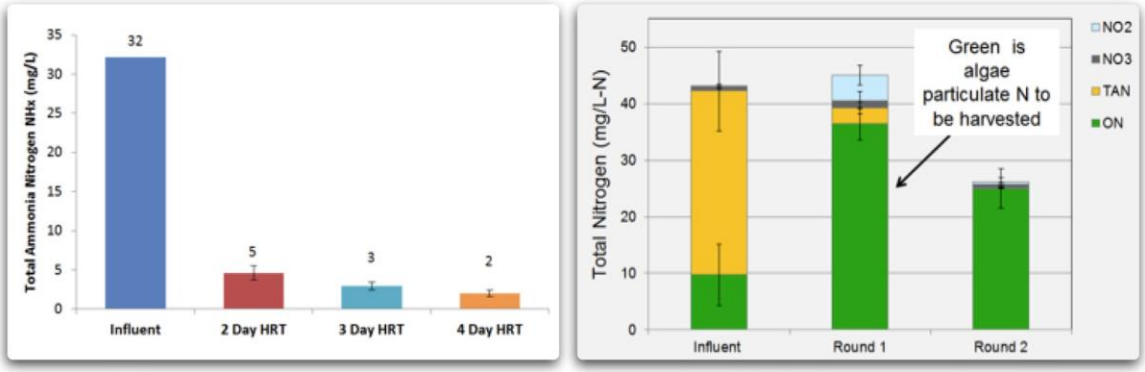

Figure 4. (Left) Total ammonia nitrogen removal in single ponds with hydraulic residence times (HRTs) of 2, 3, and 4 days during February-March 2012. (Right) Ammonium conversion into algal biomass containing ~50% crude protein. Round 1 and 2 are ponds-in-series with intermediate harvesting of algae (3-day HRT followed by 4-d HRT with CO_2 addition during April-May). A small amount of ammonium was converted to nitrate (NO3).

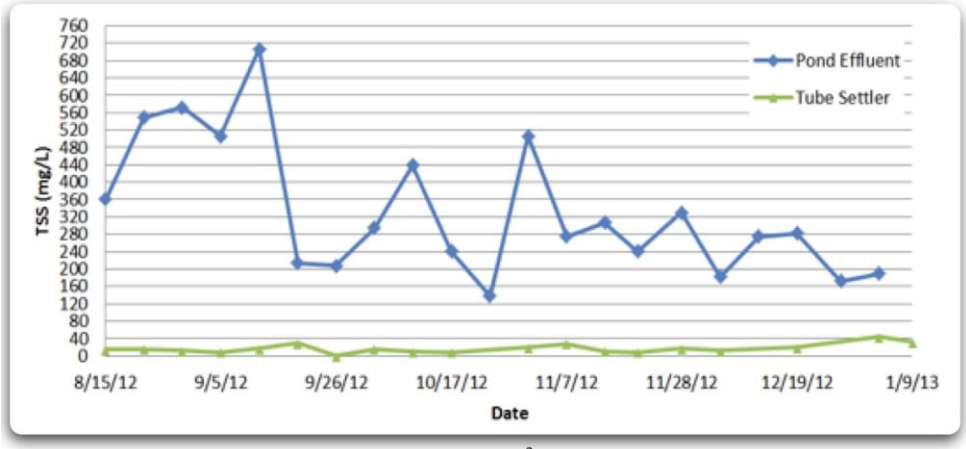

Figure 5. Suspended solids concentrations in a 30-m^2 raceway pond and remaining after sedimentation harvesting by a pilot tube settling tank, at the CPSLO Algae Field Station. The excellent biomass recovery was caused by bioflocculation of the culture. Developing such bioflocculated cultures in dairy wastewater is a major goal of the proposed research.

Preliminary data on Algae Physiology and Composition (CPP): A culture collection of over 200 oil-rich strains has been established at Cal Poly Pomona by seasonally sampling agricultural fields, estuaries and

marine sites with significant coliform levels. The group has identified ~30 strains by 18S rRNA and Internal transcribed spacer (ITS) sequence analysis (Personal Communication: Co-PI Murry). Strains were characterized for the kinetics of growth, lipid, protein and nucleic acid biosynthesis and photosynthetic efficiency in batch culture under nutrient-deficient and -replete culture conditions (Huang, 2012).

2) Rationale and Significance

Rationale: The need to control manure-derived nutrient pollution is straining the confined animal production industry. California is the top milk producing state in the US and has some of the strictest nutrient pollution regulations. But in the San Joaquin Valley, many dairies do not have affordable access to more land for manure application/disposal. A highly productive crop is needed that will convert manure N into feed but in smaller land areas than crops such as corn. Algae are a candidate feed with annual yields typically 7-13 times greater than soy or corn. Beyond 40-50% protein content, algae contain fatty acids, a favorable amino acid content, pigments, and vitamins that are valuable in animal feeds, especially for adding value to milk. Advanced molecular biology tools will allow us to gather needed information on the risks and benefits of algae animal feeds.

Relationship of the project's objectives to one of the Program Area Priorities: The objectives of the current research proposal address the goals of Program Priority Area - A1401 pertaining to Soil, Air, and Water Processes in Agroecosystems. Starting with dairy feed supplements, the project will lead the way towards an algae-based feed industry that employs advanced nutritional features to enhance agricultural products (e.g., milk protein, and poultry pigment) while assisting farmers to meet manure management challenges and environmental concerns. Furthermore, our research approach will address topics rarely covered in the algae field, i.e., interrogating potential toxicity of algae and their association with zoonotic pathogens from manure. Our approach is unique in that it integrates and addresses food/feed safety issues along with algae production techniques and waste management exploiting the diverse expertise of the Co-PIs.

Long-range improvement in sustainability of US agriculture: We aim to develop methods to control the content and characteristics of the lipids, carbohydrates, and proteins in algae biomass to maximize its feed value. At the same time we will work to maximize the output of algae biomass, which will also improve the wastewater treatment performance of the process. The capability to control the biomass composition would allow the biomass produced to be optimized for various types of feed rations. In addition, algae culture conditions will affect pathogen die-off during cultivation in wastewater media. The cultures will be grown both in a pilot plant at the CPSLO dairy and in the lab.

Methods and feasibility*:* Methods to be used in carrying out the proposed project, including the feasibility of the methods: Algal biomass will be analyzed for P, N, protein, carbohydrates, and profiles of fatty and amino acids. Pathogen and algal communities extant in raw and feed-processed (i.e., pelletized, heat-treated) algal biomass will be analyzed using metagenomics. Potential toxicity of algal biomass will be studied using toxicity evaluation of cell–free extracts on cultured mammalian cells. The researchers have decades' experience in algae production, wastewater treatment, and food safety. The four aims of the project are hereby summarized, with details provided in the subsequent section.

Specific Aims List:

Aim 1: Generate experimental field data and calibrate optimization models. For treatment, expected removals are 85-95% biochemical oxygen demand and soluble N and 40-80% soluble P removal, depending on culturing technique and season.

Aim 2: Maximize the nutritional value of produced algae for animal feed. The cultures will be optimized to produce biomass at a high rate while also having the highest value composition for feed (in terms of lipids,

digestibility, essential fatty and amino acid profiles, including balanced protein and carbohydrate concentrations).

Aim 3: Optimize pathogen inactivation methods. Pathogens will die-off in the ponds and during disinfection processing of the harvested biomass. Inactivation rates for representative pathogen indicators will be determined under various algae cultivation conditions and during trials with several biomass disinfection techniques. The optimal combination of pond conditions (e.g., high pH) and biomass processing (e.g., pasteurization) will be determined to achieve needed log inactivation of pathogens, which is typically 1- >4 log10 reduction (Sobsey et al., Available Online).

Aim 4: Quantify and control any cyanobacterial toxins. qPCR assays described by Al-Tarineh et al. (2012 a and b) will be used and optimized to reliably determine the copy number of cyanotoxin biosynthesis genes, as well as an internal cyanobacteria 16S rDNA control, in a single reaction. The latter detects for presence of cyanobacteria. If toxins are detected, measures will be taken to control invasion of the ponds by cyanotoxin-producing cyanobacterial strains.

3) Approach, Methods, and Outcomes

Task 1: Pilot Plant Work: *Generate experimental pilot plant data and calibrate optimization models.* (Responsible Party: Lundquist, CPSLO).

1.1 Experimental Overview: Both laboratory and pilot plant algae cultivation will be used to develop cultivation and bioflocculation harvesting methods and to identify preferred algal strains for testing in the field at CPSLO. Examples of lab strategies that could be scaled-up are inoculation with high performance strains, timing of stress imposition, and CO_2 provision regimes. The rates of pathogen inactivation and cyanobacterial toxin production will be determined. The pilot plant work at CPSLO will be described first.

1.2 Apparatus and operations: Four identical algae production tanks will be installed adjacent to the waste lagoons at the 300-head CPSLO dairy, located on campus. The site is well developed and has been used extensively for various non-algal treatment pilot plant research, led by Co-PI Lundquist and sponsored by NIFA and USEPA. The algae tanks are paddle wheel-mixed raceways that simulate standard 30-cm deep algae production ponds. As shown below (Figure 6), these four 3-m^2 tank ponds were used previously for municipal wastewater treatment research (Ward, 2011). These ponds will be operated as two sets of duplicates to conduct controlled experiments to determine parameters to be used in optimization of wastewater treatment and algae feed production. Biomass settling tanks will be used for harvesting and re-inoculation experiments.

Figure 6. Raceway algae production ponds to be used in the proposed research.

1.3 Media: The influent to the algae ponds will be the flush water storage lagoons at the CPSLO dairy. Such storage lagoons are used at thousands of flush dairies in the western US and swine farms throughout the US. Lagoon water at the CPSLO dairy has been completely characterized in a series of recent theses and papers (Kane, Henemann, Fooks, Adler, Thompson; available on request). For example, soluble N concentrations are typically 500-1000 mg/L depending on dairy operations and season. N concentrations

can be decreased by dilution with well water or clarified pond effluent, and increased by addition of N fertilizer. Bottled CO_2 will be bubbled into the ponds for some experiments to eliminate any inorganic C limitation on algae growth. (Flue gas, from an electrical generator for example, would be substituted for CO_2 in full scale systems.)

1.4 Algal Strains: Our municipal wastewater ponds typically are dominated by five main genera—*Actinastrum, Micractinium, Scenedesmus, Cyclotella,* and *Chlorella*, which occur in polyculture with the ratio of genera changing seasonally. We have not discerned any relationship between treatment performance and dominant algae genus. In the proposed research, initially such indigenous "volunteer" strains will be used. However, any exceptional strains or cultivation methods developed in CPP lab studies will be implemented in the ponds. Particular algae genera can be promoted through frequent inoculation from lab cultures or by re-inoculating with algae harvested from the pilot ponds. In this circumstance, we would determine the relationship between level of dominance and level of inoculation effort (e.g., inoculation mass rate (mg/L-d) needed to maintain dominance).

1.5 Model Development: The experiments will be designed to estimate parameters in a biological-chemical model of algae cultivation. This model, implemented in JMP software (SAS Institute), will be used to optimize wastewater treatment performance and feed value/safety.

(a) For wastewater treatment, maximizing the mass removal of soluble organic matter, N, and P will be the main objectives (i.e., kg/m^2-d removed). Bioflocculation of algal biomass for sedimentation harvesting is another requirement for both treatment and feed production. **(b)** For feed production, the goal will be maximizing the value of the feed in terms of the lipid and amino acid profiles, protein, and carbohydrates (i.e., starch and simple sugars), or overall energy content. (**c)** A third objective is production of algae biomass with decreased pathogen concentrations, which will lower the pathogen (bacteria and cyanobacteria is our focus) load on the biomass disinfection equipment, possibly reducing cost or risk.

The major factors in the model are typical of algae production and biological wastewater treatment models (e.g., solar radiation; temperature; hydraulic and solids residence times; and concentrations of biomass, inoculum, inorganic and organic carbon, nutrients, and dissolved oxygen), with additional terms for ammonia volatilization, nitrification, denitrification, and pathogen inactivation. An algae composition sub-model will be attempted, with the most significant relationships expected to be: **(1)** biomass N content being proportional to biomass lipid content (Craggs et al., 2012) and **(2)** media soluble N concentration being proportional to biomass crude protein content. Many of the parameters are expected to be specific to the dominant algae genus being grown. Pathogen inactivation rate is anticipated to be mainly a function of pH, temperature, hydraulic residence time, and biomass concentration, as commonly modeled for treatment ponds (USEPA, 2011). Only bacterial and cyanobacterial pathogen indicators will be quantified in this project rather than viral and protozoan indicators in order to constrain the budget in this initial investigation. Literature results will be used to project the inactivation of viruses and protozoan cysts based on bacterial inactivation.

Duplicate ponds will almost always be used in our experimental designs to assess uncertainty. However, the project will be restricted to four pilot ponds, at least initially, due to the high cost to build and monitor these units. Thus, the field experiments will not have sufficient replication for simple statistical hypothesis testing. Instead the data collected during the experiments and across seasons will be used to calibrate the models described above. JMP will provide data on statistical significance for the fitted parameters. If time allows after calibration data are collected, additional data will be collected to test the predictive ability of the models.

1.6 Experiments and Variables: The major pond operation variables to be evaluated include the hydraulic/organic loading rate, recycling of settled biomass as inoculum, algal strains, and recycle ratio of treated effluent for dilution. Past research and hydraulics scale-up constraints have fixed raceway pond depth at 30-cm (Weissman et al., 1988).

The initial methods of control to be tested are hydraulic residence time (2-14 days) and organic loading rate (10-50 g/m^2-day of biochemical oxygen demand), which will result in different N and organic carbon mass loadings to the ponds. This N provision rate is expected to be the main determinant of the lipid and protein content of the algae. When total soluble N concentrations of >10 mg/L are maintained, we expect the protein content and productivity to be maximized, and when N concentrations is <10 mg/L, the result should be higher lipid and carbohydrate content and productivity. Organic loading is expected to be the main factor in bioflocculation, along with harvested biomass recycle ratio. Organic loading can be studied independently of residence time by diluting the influent with well water or by recirculating clarified pond effluent.

During the first 18 months, we will grow a green algae polyculture (e.g., *Chlorella, Scenedesmus, Micractinium*) and focus on protein production. The high rate of nitrogen assimilation for protein production is expected to lead to a high rate of nitrogen mass removal, which is compatible with fulfilling the wastewater treatment objectives. During the final 18 months, we will grow diatom algae (e.g., *Cyclotella, Navicula, Nitzschia*) and focus on nutritional lipid production. Based on Co-PI Lundquist's experience with municipal wastewater treatment, *Cyclotella* can be made to dominate the culture through continuous harvesting and re-inoculation (E. Ripley, in preparation). During the experiments emphasizing nutrient removal and lipid production, if the inorganic carbon concentration of the wastewater is insufficient to allow for near-complete nutrient removal, bottled CO_2 will be bubbled into the ponds to allow nutrients to become limiting. CO_2 provision will be pH controlled.

For experiments on pathogen inactivation, some ponds will not receive CO_2 and be operated with sufficient residence time to allow the algae to increase pH. Carbon-limited algae cultures often raise pH >10, which is well-known to accelerate die-off of bacterial and viral pathogens, and possibly trigger increase of lipid content in algae. Maintaining the moderate pH of 8.0-8.5 with CO_2 addition will be beneficial to the biomass production rate, but will likely decrease the pathogen inactivation rate. Thus, pH is a variable which will need to be optimized using the proposed study's estimate of the pH sensitivity of pathogen die-off and lipid content. The CPP lab studies, to be described further below, will develop preliminary information on many of the above topics before they are tested at pilot scale.

Wastewater pilot studies must always contend with the uncontrollable variables of weather and influent characteristics. However, in past projects, we have used multivariate modeling to successfully account for these environmental variables and determine statistically significant growth or treatment parameters (e.g., Lundquist 2006; Kane, 2011). A similar approach will be used in this dairy effluent project.

1.7 Sampling and Analysis: The above experiments require analyses of samples of influent and effluent at least once per week for the following (done by CPSLO): Total and volatile suspended solids, total lipid content, total ammonia nitrogen, nitrate, nitrite, and alkalinity. Twice per month the following will be determined: lipid characterization, total biochemical oxygen demand, and soluble carbonaceous biochemical oxygen demand. Extensive analytical quality control procedures are followed for every batch of samples processed. Fresh samples will be sent to CPP for determination of soluble proteins, nucleic acids, carbohydrates and organic N (crude protein; sent to commercial lab). The algal and bacterial community structures will be determined in detail by CPP and the Ibekwe Lab. In addition to the chemical analytes, each sample will be evaluated under the microscope for algal genera, relative algal vs. bacterial

biomass in flocs, and prevalence of stalked ciliates, which are important players in bioflocculation. Micrographs will be taken to maintain an ongoing record. A dissecting scope will be used to enumerate and identify algae-grazing zooplankton, such as *Daphnia*, *Cyclops*, rotifers, and ostracods.

Task 2: Laboratory Culturing: Rapidly identify and test strains and cultivation methods (Murry).
Experimental Overview: Lab cultures will be optimized to produce algae at a high rate while also attempting to have the highest value algae composition for feed (in terms of lipids, carbohydrates, proteins, digestibility, valuable fatty and amino acid profiles, balanced protein and carbohydrate concentrations). Biomass and species composition, characterization of proximate composition, and photosynthetic function by PAM fluorometry will also be monitored routinely. Cultivation methods developed at CPP and SLO will be evaluated at pilot scale (Task 1). Examples are scale-up inoculation with high performance strains, timing of imposition of stresses, strain selection by recycling of harvested biomass, mixing and CO_2 provision regimes. Assessments on the engineering, costs, and lifecycle impacts of full-scale algae wastewater/biomass facilities will be updated based on results from the pilot pond facility using dairy wastes.

2.1 Strain Selection: The proposed study should lead to the development and characterization of native algal strains capable of both bioremediation of wastewater and biomass production for feed and fuel. Our goal is to produce algae with favorable nutritional characteristics, high digestibility, valuable fatty and amino acid profiles, balanced protein and carbohydrate concentration by developing modern analytical methods to study community structure (Task 3) and rapid fluorometric techniques for pond management. Our approach to strain selection is focused on existing cultivation candidates, unlike most current efforts that sample from the environment. It is well known from limnology studies (McCormick and Cairns, 1994) and municipal ponding operations (Benemann et al., 1977b; Lundquist et al., 2012) that certain algae genera dominate the population at different seasons and in response to nutrient and other environmental parameters. We will isolate several strains each season that we already know thrive in the production setting (at SLO, *Actinastrum, Micractinium, Scenedesmus, Cyclotella,* and *Chlorella*) by sampling from poly-cultures growing in algae production facilities at SLO. Dominant strains identified morphologically will be isolated directly using a single-cell micromanipulator as described earlier (Huang et al., 2013), and studied in pure culture in a high-throughput system and bioreactors (Huang, 2012) at Cal Poly Pomona.

2.2 Development of Rapid Diagnostic Tools for Pond Management: Culturing of axenic strains dominant in SLO ponds under controlled laboratory conditions at CPP will allow for development of rapid monitoring techniques to manage production sites including species control, proximate composition and various lipid and protein induction strategies, zooplankton control and optimization of cell harvest in wastewater treatment.

Axenic algal isolates will be screened for patterns of lipid, protein and nucleic acid biosynthesis as a function of growth phase under varying light and nutrient regimes**.** We propose to stain cells *in vivo* with fluorescent dyes to determine soluble protein, nucleic acids and lipid content in cell populations and correlate fluorescent measurements with traditional biochemical techniques (see Aim 2). In batch culture, protein, lipid and nucleic acid biosynthesis is not well synchronized and tremendous variations have been observed microscopically between individual cells in axenic culture (Liu and Lin, 2001; Roessler, 1990; Chelf, 1990; Nihei, et al., 1954).

Axenic algal isolates will be cultured in well-controlled bioreactors sparged with air/CO_2 mixes. Cells will be sampled daily, permeabilized with DMSO and stained with BoDipy (Invitrogen), fluorescein (FI) and propidium iodide (PI) and fluorescence emission measured as described earlier in our lab using a microplate reader (Huang, 2012). Nile red (NR) and BoDipy staining *in vivo* coupled with fluorometric

detection has been used to quantify total lipid content in our lab where a good linear correlation is found between gravimetrically determined total lipids and fluorescence (Huang, 2012). Correlation of fluorescent signals in intact cells with isolated lipid, protein and nucleic acid content determined by traditional means (Aim 2) will determine if fluorescent staining is quantitative for these properties. The goal is to later develop flow cytometry using fluorescent stains as a tool to rapidly quantify biochemical composition as well distinguish between algal strains and relative size of the microbial population in production ponds.

Productivity is dependent on photosynthesis which is influenced by a variety of environmental factors including nutrient, light, oxygen and heat stress and intrinsic light saturation characteristics of strains. Here we propose to use PAM fluorometry as a sensitive means to determine quantum efficiency ($F_V/F_{M)}$, non-photochemical quenching (NPQ) and rapid light curves of specific strains under experimental conditions in the lab and later in the field in polyculture. Samples of axenic cultures will be removed during batch growth (described above), for Y(II), ETR, FV/FM, Fv/Fo and NPQ measurements using a OS1p with Algae Cuvette (Opti-Sciences) as described (MacIntire et al., 1997; Schrieber et al., 1995; Huang, 2012). Rapid light curves will be generated using the OS1p as they allow measurements in variable light intensities common in mixed ponds. Light saturation curves will also be generated using Clark-type polarographic oxygen electrodes (Qubit Systems) in a water-jacketed cuvette using a variable tungsten light source as described earlier (Murry and Wolk, 1989). Irradiance will be measured with a Li-Cor quantum counter (Li-185) and sensor (Li-192) from inside the cuvette. Photosynthetic rates at increasing light levels will be calculated as gross photosynthesis (the sum of net photosynthesis and the dark respiration rate) to assess strain specific light saturation and identify photo-inhibition. The data will be correlated with PAM fluorometry measurements.

Quantum efficiency or yield (F_V/F_M), is widely used to evaluate the health of ecosystems and associated variables that directly or indirectly affect the photosynthetic apparatus in terrestrial and aquatic systems (see Schreiber, 2004 for review). The F_V/F_M dark adapted test is a measurement ratio that represents the maximum potential quantum efficiency of Photosystem II if all capable reaction centers were open. Healthy terrestrial leaves have a Fv/Fm of 0.832±0.004 (Kromcamp and Forester, 2003), and any loss of this ratio is often one of the earliest and most sensitive indicators of physiological stress in aquatic systems (Parkhill et al., 2001; Baker, 2008). F_V/F_M has both photochemical and non-photochemical components (Baker 2004). Rapid Light Curves provide information on the saturation characteristics of electron transport (Schreiber 2004). The light saturation rate as measured by Rapid Light Curves highly correlates with the concentration and maximum activity of Rubisco (Macintyre et al., 1996 and 1997). Non-photochemical quenching (NPQ) occurs in almost all plants and algae to protect against excessive light (Muller et al., 2001). Algae are susceptible to light saturation and productivity can become photo-inhibited at light levels well below maximum PAR radiation (Weissman and Benemann, 1978; Walker, 2009). Because protein and lipid biosynthesis is fed by PS carbon fixation, key questions to be addressed by this work are to determine light saturation levels, a species dependent variable, and how N-deprivation influences PS efficiency, lipid and protein biosynthesis, cell viability and growth. These parameters can also provide information on optimum culture parameters such as mixing rates to optimize light saturation, aeration rates, nutrient levels and pH.

Task 3: Maximize the compositional value of the produced algae for animal feed.

The biochemical composition of specific strains of algae and mixed populations grown in ponds is critical to the use of the biomass for feed and biofuels. The goal is to determine proximate composition relative to nutritional value and identify strain specific physiological responses to environmental variables in order to

identify strains favorable for feed and fuel, and/or to determine the cultivation conditions that lead to the highest value algae biomass. Because amino acid and fatty acid composition, nucleic acid and carbohydrate content along with digestibility are key to developing feed supplements for specific target animals, analysis of these parameters will be carried out using axenic strains grown under controlled lab conditions and in samples harvested from CPSLO ponds.

Cells will be harvested and disrupted using a bead-beater for determination of soluble protein (Bradford, 1976; Bio-Rad microplate method), carbohydrates (Leyva et al., 2008) and nucleic acids (using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE) at CPP. Isolated protein samples from select strains and mixed cultures grown at CPSLO will be sent to a lab (UC Davis) for amino acid profile analysis. Lipid profiles will be determined in log phase and late stationary phase under N-replete and N-limited conditions. Aliquots of each sample will be shipped to CPSLO for lipid analysis including total lipid content (Bigogno, 2002) as a percentage of algal dry weight and analysis of fatty acid methyl esters (FAMEs) using GC-MS analysis of trans-esterified lipids, to establish the lipid profile of select strains. Analysis of FAMEs will establish characteristic fatty acid profiles of each strain and determine whether this is a stable characteristic of the strain or subject to environmental influences. To allow comparisons of colorimetric analysis with traditional feed analysis, samples will also be sent to a commercial lab (SDK Laboratories) for analysis of moisture, dry matter, crude protein, acid detergent fiber, and associated components of importance.

The high costs associated with algal production, especially of monocultures, and temporal variations in proximate composition still pose problems for feed operations. Our approach will be to characterize the dominant strains individually and in poly-culture. As we learn more about the community structure, this may facilitate the development of suitable biomass for feed. Several algal species, each rich in specific nutrients that the others may lack would allow formulation of a balanced diet for the animal much in the same way that animal production facilities blend food sources to meet the specific nutritional requirements of the target animal.

Task 4: Optimize pathogen inactivation methods

4.1 Reductions of some pathogens by some animal waste treatment processes have been determined in laboratory and pilot scale field studies (reviewed by Sobsey et al., Available online). In general, thermophilic processes, such as pasteurization, thermophilic digestion and composting, are capable of producing extensive (>4 log10) pathogen inactivation, and therefore, resulting treated residuals are likely to contain only low pathogen concentrations. Desiccation or drying to very low moisture levels (<1%) has been shown to result in extensive (>4 log10) inactivation of pathogens in municipal biosolids and in soils (Sobsey et al., Available online). Several options for pelletization (pasteurization) and drying will be evaluated for their effectiveness in reducing pathogen loads.

4.2 Pond Pathogen Quantification and Characterization

Liquid samples will be collected from 30-cm deep algae raceway ponds at the 300-head Cal Poly campus (San Luis Obispo) dairy farm where extensive manure management research is ongoing. Survival of pathogens such as *E. coli* O157:H7, *Listeria monocytogenes*, *Campylobacter jejuni* and *Salmonella* spp. (Nam et al., 2005a; Nam et al., 2005b; Nguyen et al., 2004) will be studied using plate count, real-time PCR and reverse transcriptase real-time PCR (Ibekwe et al., 2002; Nam et al., 2005a; Nam et al., 2005b; Nguyen et al., 2004) Real-time PCR (qPCR) will also be used to quantify the total bacterial counts in the pond sample. Universal bacterial 16S rRNA primer pairs 534F 5'CCAGCAGCCGCGGTAAT 3' and 783R

and 5'ACCMGGGTATCTAATCCKG 3' (Sakai et al., 2004) will be used in 20 µl reactions with 10 µl of SsoFASTTM EvaGreen Supermix (Bio-Rad, Hercules, CA, USA), 2 µl of each primer (12.5µM stock), and 2 µl template DNA (Ibekwe et al., 2002). Bacterial cell counts will be estimated by comparisons of threshold cycle (Ct) values to an *E. coli* O157:H7 genomic DNA standard curve. Ct levels are inversely proportional to the amount of target nucleic acid, hence number of organisms, in the sample.

4.3 Biomass Disinfection Processing

A trend in municipal wastewater treatment is pasteurization of treated effluent using waste heat from natural gas electrical generator. Large dairies with digesters will have waste heat available for pasteurization and drying. High-protein algae will be pelletized with high carbohydrate feeds to create a balanced feed. The heat of pelletization also contributes to pasteurization. Cal Poly SLO has a research feed mill for producing such blended feeds in future research. In proposed work, autoclave and drying parameters will be evaluated to determine time-temperature relationships needed for disinfection.

Task 5. Analysis of Microbial and Cyanobacterial Community Structure in Ponds

5.1 Bacterial will be harvested from pond water samples, and bacterial DNA will be isolated using the Power Water Extraction Kit (MO BIO Laboratories, CA) following the manufacturer's bead-beating protocol (Ibekwe et al., 2002; Ibekwe et al., 2003; Ibekwe et al., 2012; Ma et al., 2012; Ma et al., 2013). DNA molecular weight will be estimated on a 1.0% agarose gel using molecular weight standards. Fragments of the 16S rRNA-encoding region will be amplified from the purified DNA using the universal primer set 357F and 962R and will be subjected to 454 pyrosequencing analysis. Comparison of the bacterial communities among in different pond treatments will reveal the degree of specialization of different pond biota, and give us insights into the identity of the potential agents of negative and positive feedback on algal bloom. This will be done in parallel with GeoChip-based metagenomic studies with the fluorescent dye, Cy-3, using random primers (NimbleGen, Madison, WI). The hybridization will be performed on a Hybridization Station (MAUI, Roche, CA) at 42°C for 16 h with agitation. After washing the arrays will be scanned using an MS 200 Microarray Scanner (NimbleGen) at a laser power of 100% PMT (photomultiplier tube).
For the analysis of cyanobacterial and algal populations in ponds, we will use the large subunit (LSU) of the rRNA-encoding gene (Steven et al., 2012a and 2012b) from bacterial DNA described above. LSU rRNA phylogenies are generally in agreement with the more often employed ribosomal small subunit (SSU) rRNA phylogenies (Pei et al., 2009), encode more phylogenetic information because of greater length and higher sequence variance (Gutell et al., 1994; Pei et al., 2009), has greater representation in shotgun metagenomic datasets, approximately 2 : 1 compared with SSU rRNA genes, because of their larger size (Yilmaz et al., 2011), and finally may provide better predictions of phylogeny than classification of bulk reads (Segata and Huttenhower, 2011; Steven et al., 2012a and 2012b) using newly developed primers encoding plastid 23S rRNA genes from complex communities (Sherwood and Presting, 2007; Presting et al., 2008). Sequencing of 23S rRNA genes will be done using tag-encoded FLX–Titanium amplicon pyrosequencing (TETAP) which will be performed at the Research and Testing Laboratory (RTL, Lubbock, TX) as previously described (Dowd et al., 2008; Ma et al., 2013) using RTL protocols (www.researchandtesting.com). A DNA product of ~400 bp will be generated with the primers p23SrV_f1 and p23SrV_r1 as described by Sherwood and Presting (2007).

***5.2 Bioinformatics and statistics*:** Bacterial pyrosequencing population data will be analyzed by performing multiple sequence alignment techniques using the dist.seqs function in MOTHUR, version 1.9.1 (Schloss et al., 2009; Ma et al., 2013). All raw reads will be treated with the Pyrosequencing Pipeline Initial Process of the Ribosomal Database Project (RDP), as reported by Ma et al. 2013. These data will be used for α- and β- diversity analysis. For the GeoChip data, the signal intensity of each spot on the GeoChip will

first be normalized across samples by the mean of Cy-5 labeled universal standard signal intensities and then by the mean of Cy-3 labeled signal intensities of all hybridized spots within each sample. The normalized data will then be subjected to statistical analyses using the Vegan package in R 2.9.1 (The R Foundation for Statistical Computing, Vienna, Austria) and the pipeline developed at University of Oklahoma (http://ieg.ou.edu). Detrended correspondence analysis (DCA) will be applied to assess the overall functional compositions of pond communities.

Task 6: Quantify and control any cyanobacterial toxins

6.1 A quadriplex quantitative-PCR (qPCR) assay capable of detecting and quantifying toxin genes from the microcystin, nodularin, cylindrospermopsin and saxitoxin biosynthesis pathways described by Al-Tarineh et al. (2012a, 2012b) will be used. The primers and probes employed in this assay were designed from conserved regions within toxin biosynthesis genes of most of the representative cyanobacterial genera. The assay targets hepato- and neuro-toxigenic cyanobacteria of global significance. The toxin gene targets will be, the aminotransferase (AMT) domains located on the *mcyE/ndaF* modules of the microcystin and nodularin synthetases, respectively; the amidinotransferase (*cyrA*) gene involved in cylindrospermopsin synthesis; and the aspartate aminotransferase domain of *sxtA,* for saxitoxin synthesis. These genes encode key enzymes within their respective toxin biosynthesis pathways (Al-Tarineh et al. 2012a).

The amplified qPCR products will be initially visualized on 1.5% agarose gels stained with SYBR Safe (Invitrogen, Carlsbad, CA), to verify for amplification product formation and sizes of the products. Alternative primers and probes suggested by Rasmussen et al. (2007) will also be evaluated, if necessary.

6.2 Toxicity Assessment of Algal Biomass Using Cell Cytotoxicity Tests

Ideally, cytotoxicity assessment should be conducted before qPCR. The advantage of this approach is that it can pick up other toxicities not addressed by specific qPCRs that only target genes encoding for known toxins (Al-Tarineh et al., 2012a, Al-Tarineh et al., l 2012b). Potential toxicity of algal biomass (and stockfeed where there could be bioconcentration) will be studied using toxicity evaluation of cell–free extracts tested on cultured mammalian cells. The cytotoxicity assays will be conducted on well characterized targets, hepatocyte and neurocyte cell lines, acquired from the American Type Culture Collection (Manassas, VA). Cell culture and preparation for cytotoxicity tests will be conducted as described by Freshney (1994) and Murinda et al. (2003), following recommendations for use of the TC 20™ Automated Cell Counter (Bio-Rad). Known toxin-positive and -negative controls will be used to validate the assays. The TC 20 Cell Counter delivers reliable duplicate sample cell counts in 30 seconds using dual-chamber counting slides (Bio-Rad). The counting algorithm automatically identifies cells and excludes debris, thereby determining total cell count. For cell viability assessment – presence of trypan blue in the sample triggers cell viability analysis using images acquired from multiple focal planes during the focusing step. The TC 20's dynamic concentration range, of 5×10^4–1×10^7 cells/ml eliminates the need to dilute cells prior to counting, thus reducing errors associated with traditional sample dilutions. The counting algorithm successfully discriminates and counts individual cells within clusters of up to five cells, eliminating the need to extensively declump cells prior to loading into the counting slides. Cytotoxic positive samples will be tested for both presence and concentration of known cyanobacterial toxins using a quadriplex qPCR that targets the four major cyanobacterial toxins (Al-Tarineh et al., 2012a, Al-Tarineh et a., l 2012b), as previously described.

Project Timeline

Aim	Activities	Year 1	Year 2	Year 3
I	***Generate experimental field data and calibrate optimization models***			
	Task 1: Pilot Plant Work: Generate experimental pilot plant data and calibrate optimization models (**Lundquist Lab).**	x		
	Deliverables: Annual technical reports; Green algae models included in Year 2 report; Diatom models in Year 3 report		x	x
	Task 2: Laboratory Culturing: Rapidly identify and test algal strains and cultivation methods (**Murry Lab**).	x	x	
	Deliverables: Isolation of seasonally dominant algae and their characterization		x	x
2	***Maximize the compositional value of the produced algae for animal feed***			
	Task 3: Analysis of amino acid, fatty acid, nucleic acid, and carbohydrate content of dominant algae in raceway ponds (**Murry Lab: Ibekwe Lab** will assist on FAME* analysis)	x	x	
	Deliverables: Use of specific algal species, rich in specific nutrients to formulate food sources to meet the specific nutritional requirements of the target animal.		x	
3	***Optimize pathogen inactivation methods***			
	Task 4: Quantification of inactivation of pathogens in raceway ponds using plate count, real-time and reverse real-time PCR (**Murinda and Ibekwe Labs**).	x	x	
	Deliverables: provide reductions rates of some pathogens in raceway pond			
	Task 5: Analysis of microbial and cyanobacterial community structure and functions in ponds using pyrosequencing and GeoChip (**Ibekwe and Murinda labs**)	x	x	
	Deliverables: Reveal degree of specialization of different raceway pond biota, and identity of the potential agents of negative and positive feedback and their functions on pond algae			x
4	***Quantification and control of Cyanobacterial toxins***			
	Task 6: Detection and quantification of toxin genes from the microcystin, nodularin, cylindrospermopsin and saxitoxin biosynthesis pathways of cyanobacteria (**Murinda Labs**)	x	x	
	Deliverables: Data will be presented on the relationships between cynobacteria and their toxin genes in a way that will reveal the effects of biotic and abiotic factors on algae development to both scientists and nonscientists.		x	x

*FAME; fatty acid methyl ester analysis.