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Review of the cultivation program within the National Alliance for Advanced Biofuels and Bioproducts

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ABSTRACT

The cultivation efforts within the National Alliance for Advanced Biofuels and Bioproducts (NAABB) were developed to provide four major goals for the consortium, which included biomass production for downstream experimentation, development of new assessment tools for cultivation, development of new cultivation reactor technologies, and development of methods for robust cultivation. The NAABB consortium testbeds produced over 1500 kg of biomass for downstream processing. The biomass production included a number of model production strains, but also took into production some of the more promising strains found through the prospecting efforts of the consortium. Cultivation efforts at large scale are intensive and costly, therefore the consortium developed tools and models to assess the productivity of strains under various environmental conditions, at lab scale, and validated these against scaled outdoor production systems. Two new pond-based bioreactor designs were tested for their ability to minimize energy consumption while maintaining, and even exceeding, the productivity of algae cultivation compared to traditional systems. Also, molecular markers were developed for quality control and to facilitate detection of bacterial communities associated with cultivated algal species, including the *Chlorella* spp. pathogen, *Vampirovibrio chlorellavorus*, which was identified in at least two test site locations in Arizona and New Mexico. Finally, the consortium worked on understanding methods to utilize compromised municipal wastewater streams for cultivation. This review provides an overview of the cultivation methods and tools developed by the NAABB consortium to produce algae biomass, in robust low energy systems, for biofuel production.

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1. Introduction

1.1. Preface

Humans have marveled at the complexity and multiplicity of aquatic microorganism since drops of pond water were first examined under a microscope. Photosynthetic microorganisms were necessarily classified based on morphological differences in the descriptive era of biological science. Over time, chemical and biochemical characteristics began to take on more taxonomic significance as expounded by R.Y. Stanier [1] and more recently by others [2–3]. Even the notion of "species" as applied to microalgae has been reexamined recently with specific reference to the diatoms. The result was a more holistic approach to taxonomy, integrating biochemical, molecular and ecological characteristics that suggests the species concept has been applied far too broadly within the microalgae [4]. In sum, these studies provide a critically important backdrop to the current era. We know that primary production in aquatic systems is highly competitive and unstable in a biological sense [5]. Constant changes in nutrient levels, light intensity and temperature will trigger winners and losers in the competition for available light, inorganic carbon, nitrogen and phosphorus. Morphologically similar species with very different metabolisms will wax and wane with seasons and on more rapid time scales dictated with rainfall, wind, dust and diurnal temperature ranges. Large-scale microalgal cultivation systems will need to be designed to mitigate all of these ecological eventualities. Crop protection strategies against grazers [6], pathogens [7,8] and competitors [9,10] will be equally important.

Conceptually, the National Alliance for Advanced Biofuels and Bioproducts (NAABB) team was strongly influenced by the opportunities made available by the radical successes of reductionist biology: complete genome sequences, multiple "omic" analytical tools and detailed structure/function studies that provide both methods and metabolic insights needed for genetic manipulation of model microalgae. These tools provide tantalizing approaches to rapidly domesticate and improve wild microalgal species for renewable fuel production centered on neutral lipid synthesis [11] and secretion of pure hydrocarbons [12,13]. Well-funded start-up enterprises both within and outside the NAABB consortium set out to harvest this bounty of opportunity. Tightly coupled with this approach is the assumption that monocultures of elite algal strains can be effectively maintained in appropriately engineered cultivation systems. Modern agriculture provides a compelling model based on monocultures afforded by powerful methods of crop protection.

It is important to note that this approach has been questioned by two key studies: the final report of the DOE Aquatic Species Program (ASP) [14] and more recently in the report on Algal Biofuels from the National Research Council [15]. The ASP report cited repeated difficulties with maintaining desired strains in open raceway cultivation systems and went so far as to suggest better results might be obtained by cultivation of highly competitive local species with desired phenotypes over the use of elite strains of microalgae isolated elsewhere. The NRC study cites broader concerns regarding unsustainable requirements for energy, water and nutrients for elite genetically modified algae at scales required for production of 5% of the nations liquid fuel requirements. The NRC report provides important high-level guidance for future studies by identifying the significant barriers to sustainable algal biofuel production.

1.2. Approach

The NAABB cultivation studies outlined here reflect key design criteria with respect to large-scale cultivation. It was important to identify and select geographic locations that have high annual solar insolation and climatic conditions that can maintain pond water temperatures at elevated levels for most of the year. The NAABB cultivation teams attempted to identify those microalgae strains that exhibited high growth rates within the annual temperature range and water chemistries from the production sites. Also, there was a need to design relatively simple, cost-effective, and energy-efficient large-scale culture systems that could support high productivity, culture stability and help maintain elevated water temperatures during the cold season to sustain reasonable biomass productivities year round. Specific goals included the following:

- identify robust production strains that will perform reliably in specific geographic locations and seasons;
- develop methods and best practices for preventing large-scale culture crashes due to predators and competitors;
- develop methods for cultivation in low-cost media using agricultural grade nutrients, wastewater sources, and media recycling; and
- develop and demonstrate enhanced designs and operational methods that improve productivity of large-scale cultivation systems.

In response, the NAABB cultivation team executed the projects reviewed here. Highlights include publications that: i) demonstrated an effective raceway design (ARID) for temperature management in modified raceway systems [16–18]; ii) studied the energy efficiency of different cultivation systems [19–28]; iii) developed sensitive methods for detection of both closely and distantly related algal competitor strains and used these to monitor long-term cultivation [29,30]; iv) created detailed algal growth models for a *Scenedesmus* strain [31] as well as the most productive (*Chlorella sorokiniana* DOE1412) and stable (*Nannochloropsis salina*) organisms used by the NAABB consortium [32]; v) evaluated polyculture approaches to increasing pond productivity, stability and resilience [33]; and vi) identified a new approach to cultivation using extreme conditions of low pH and high temperature appropriate for evaporation control in photobioreactors [34,35].

The NAABB Consortium developed an R&D framework to begin addressing some of these major challenges and needs. The NAABB Cultivation team employed a variety of capabilities to execute the R&D framework. These include research tools (photobioreactors and specialized laboratory growth systems), small-pond/raceway testbeds and two large-pond testbeds for large-scale cultivation experiments and the production of algal biomass to support downstream processing R&D across NAABB [36].

As shown in Fig. 1, NAABB cultivation research was conducted across four major thrust areas:

- Cultivation Tools and Methods: Focused on developing new strain screening tools, systems, and models; sensors for cultivation; molecular diagnostics tools; and methods to control predators with environmental controls.
- Nutrient/Water Recycle/Wastewater Cultivation: Focused on nutrient studies, use of wastewater sources, and media recycle.
- Cultivation System Innovations: Focused on new raceway design to extend operation in cold season climates, airlift mixing systems, and computational fluid dynamic (CFD) models to improve raceway performance.
- Large-pond Cultivation/Biomass Production: Focused on scale-up of new strains, development of low-cost media, and production of algal biomass.
- Measured the productivity of different strains at testbeds using open raceway and photobioreactor systems

2. Technical accomplishments

2.1. Cultivation tools and methods

NAABB focused several efforts on developing new tools and methods for optimizing algal cultivation. These include new models and processes to select strains and cultivation conditions for maximum

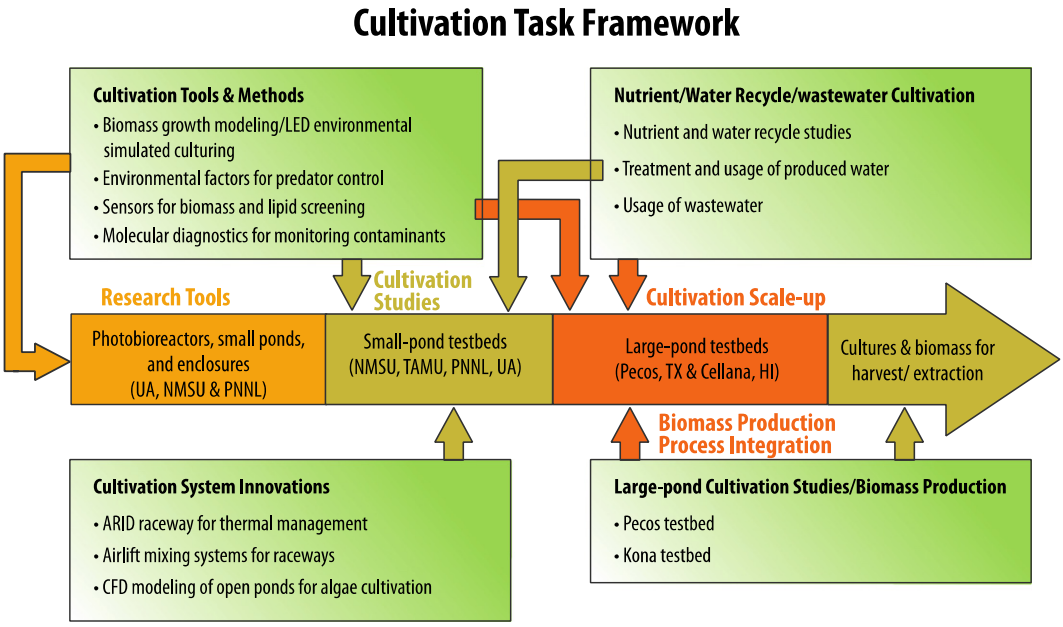

Fig. 1. The NAABB Cultivation task framework showing the various research tasks that fed into overall development of research tools, cultivation studies, and cultivation scale-up within the consortium.

productivity, methods to control predators using environmental factors and polycultures, and the development of a new molecular monitoring system and sensors.

2.1.1. Biomass growth modeling and climate-simulated culturing for strain screening

Light and temperature are the main abiotic determinants of biomass productivity of microalgae in photobioreactors and ponds operated under well-mixed and nutrient-replete conditions. For a pond at a given geographic location, the daily and seasonal fluctuations of sunlight intensity and water temperatures are determined by the prevailing climatic conditions at that site. The biomass productivity of a specific strain grown in this pond culture is then to a large degree determined by how the maximum specific growth rate is affected by sunlight intensity and water temperature. Thus, to optimize biomass productivities in outdoor ponds, it is necessary to identify not only geographic locations that have high annual solar insolation and climatic conditions that maintain pond water temperatures at elevated levels for most of the year, but also microalgae strains that exhibit high growth rates within the annual temperature range of the pond culture.

In order to accelerate the transition of promising microalgae from the laboratory into outdoor ponds, NAABB developed and tested a biomass growth model for determining strains with high biomass productivity potential [32]. For a given strain, the following four biological species-specific input parameters are measured and used as inputs into the biomass growth model:

- Maximum specific growth rate (μ) as a function of temperature;
- Maximum specific growth rate (μ) as a function of light;
- Rate of biomass loss in the dark (μ_{dark}) as a function of temperature during the dark period and average light intensity during the preceding light period; and
- Scatter-corrected biomass light absorption coefficient (k_{sca}).

An example of the data for a maximum specific growth rate matrix (7 light intensities × 7 temperatures) for *Chlorella sorokiniana* DOE1412 is shown in Fig. 2.

Using *Nannochloropsis salina* and *Chlorella sorokiniana* DOE1412 as the model strains, it was found that this *Chlorella* strain exhibits much higher maximum specific growth rates at the optimal temperature ($\mu = 5.9$ day^{-1} at 36 °C) and greater thermal tolerance (T_{max} ~ 45 °C) than *N. salina* ($\mu = 1.1$ day^{-1} at 26 °C, T_{max} ~ 35 °C) (Fig. 3).

Measurement of the maximum specific growth rates as a function of light intensity at different temperatures revealed that *Chlorella sorokiniana* DOE1412 is strongly photo-inhibited at lower temperatures (≤22 °C) and only slightly at higher temperatures. No photo-inhibition was found for *N. Salina* (data not shown).

Both strains lost significant amounts of biomass during the 10 h-long dark incubations, up to ca. 16% for *Chlorella sorokiniana* DOE1412 and 20% for *N. salina*. Biomass loss rates in the dark (μ_{dark}) increased with temperature and were positively correlated with the average light intensity the cells received during the preceding growth period (data not shown). The scatter-corrected biomass light absorption coefficient (k_{sca}) was determined for both strains from light attenuation profiles measured at different biomass concentrations in carboy cultures (data not shown).

Using these species-specific laboratory measurements together with sunlight intensity and pond water temperature data measured during an outdoor study in Arizona, the model-predicted and measured biomass concentrations compared reasonably well during the exponential and mid-linear batch growth phase, i.e., for the first 20 days following inoculation (Fig. 4). The sawtooth pattern of the model-predicted concentration curve reflects the periodic increase of biomass during the day, followed by biomass loss at night due to dark respiration.

Although the biomass growth model is useful for screening strains for the best candidates, it is important to validate the performance of the top strains in pilot-scale ponds simulating the light and water temperature conditions observed in outdoor pond cultures in selected geographic locations and seasons [37]. An indoor LED-lighted and temperature-controlled raceway pond that can be used to measure a strain's seasonal and annual biomass productivity under climate-simulated conditions was designed, built, and tested (Fig. 5).

The *Chlorella* outdoor pond culture experiment that was conducted in Arizona was repeated using the indoor LED-lighted and temperature-controlled raceways under climate-simulated conditions using

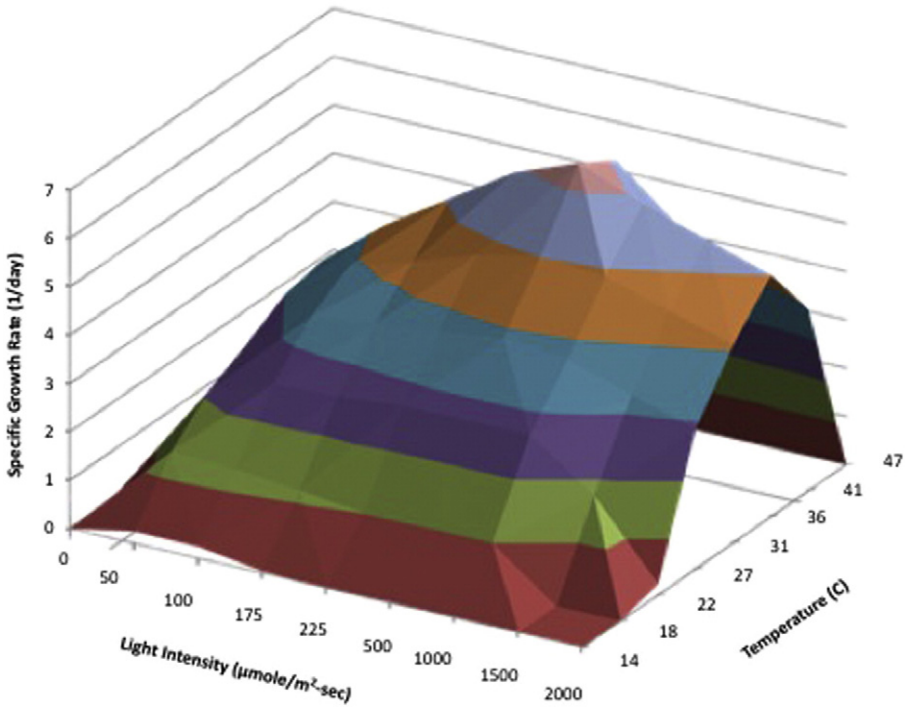

Fig. 2. Maximum specific growth rate matrix for *Chlorella sorokiniana* DOE1412 measured as a function of light intensity and temperature.

scripts of the sunlight intensity and water temperature fluctuations that were previously recorded during the outdoor study. Preliminary validation results (Fig. 6) indicate that the indoor LED-lighted and temperature controlled raceway is able to simulate the outdoor cultures. Testing in indoor ponds under climate-simulated conditions is a low-risk way to confirm a strain's superior performance before transitioning to cultivation in outdoor ponds. The integrated strategy, consisting of strain characterization, modeling of growth characteristics, climate-simulated testing, and outdoor pond testing and validation of the strain characteristics provided an efficient and cost-effective screening strains for their potential to exhibit high biomass productivities in outdoor ponds.

2.2. Environmental factors for predator control

Several limitations impede algal biofuel from attaining cost-effective commercial viability. These include the need for optimized production systems and stable, resilient algae cultures that are resistant to invading organisms. Within these systems, major contaminants have included unwanted bacteria, fungi, algae, and grazers [38–41]. NAABB examined environmental parameters that promote growth and lipid accumulation of *N. salina* while keeping invading organisms at a minimum. This included testing productivity and stability (resistance and resilience after disturbance by a grazer) of algae polycultures compared to monocultures.

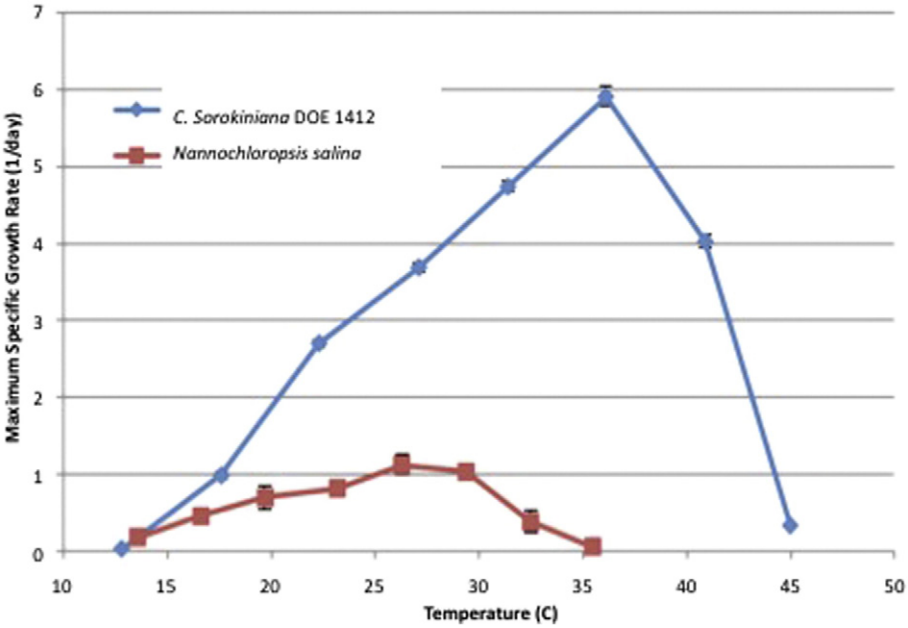

Fig. 3. Comparison of the maximum specific growth rate as a function of temperature for *Chlorella sorokiniana* DOE1412 and *Nannochloropsis salina*.

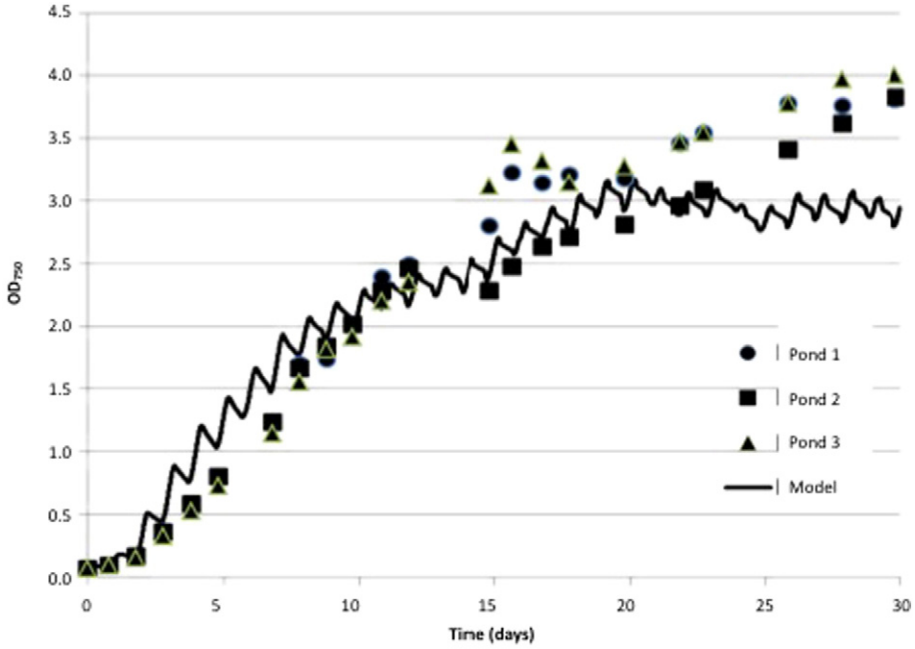

Fig. 4. Biomass growth model prediction compared to data from three separate outdoor pond cultures of *Chlorella sorokiniana* DOE1412.

In a series of experiments conducted in open aquaria in a greenhouse, we determined optimum salinity [42], pH, temperature and nitrogen source [43], to maximize *N. salina* production and minimize other algae competitors and predators (rotifers and ciliates). Table 1 shows the effects of salinity on invaders that appeared in *N. salina* cultures. We found that *N. salina* grows fastest at salt concentrations of 22–34 parts per thousand (ppt), pH 8–9 and a temperature around 20 °C. Highest biovolume was achieved using nitrate or a mixture of nitrogen sources (nitrate, urea, and ammonium) [44]. Lipid accumulation was enhanced when increasing salinity from 22 to 34 ppt upon reaching stationary phase [42]. Invaders were reduced at a salinity of 22 ppt (Table 1), pH above 8, and temperatures above 32 °C and using ammonium chloride as a nitrogen source. While *N. salina* still showed optimum growth at 22 ppt salinity and pH above 8, the higher temperatures and ammonium chloride had negative impacts on growth.

In a laboratory experiment, differences in algae monocultures versus polycultures were studied [33]. Polycultures consisted of 2, 4, and 6 species, where each of the 6 species was grown individually in the monocultures. Polycultures were assembled from equal numbers of big (potentially ungrazable) algae species and 3 small, fast-growing species.

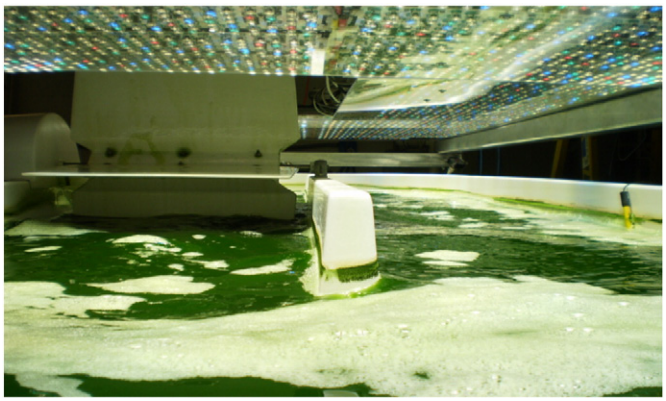

Fig. 5. NAABB partners built several LED based lighted and temperature controlled raceways, one shown here, to help develop and validate algae growth models along with the measurement and prediction of algae growth based on climate-simulated geographical location.

We demonstrated that growing several algae species together in polycultures (>4 species) may lead to a doubling of productivity and make algae cultures more resistant and resilient to disturbances by predators.

2.3. Sensors for biomass and lipid screening

Commercial-scale algae production for biofuels is comparable to crop production in traditional agriculture. Proper decisions concerning media addition, nutrient stressing, harvest, and invasion (by algae grazers, bacteria, or fungi) control should be made in a timely manner, in particular in open-pond conditions. Moreover, unlike traditional agriculture, where such decisions are typically made on a time scale of days, algae are very susceptible to environment change; therefore, management decisions should be made on a time scale of hours. Automated sensing and control systems can perform real-time monitoring of actionable data for production-scale ponds. NAABB developed and evaluated four prototype sensing systems: (1) an algae optical density (OD) sensor for biomass concentration measurement [45–48]; (2) Nile Red-staining-and-fluorescence-based (NRf-based) algal neutral-lipid quantification [49]; (3) near-infrared (NIR) and mid-infrared (MIR) analysis to characterize algal biomass composition [50]; and (4) algal thin-film infrared-attenuated total reflectance (IR-ATR) lipid sensor [51].

The OD sensor constantly pumped pond samples through the sensing chamber for wavelength-specific energy-transmission measurements [45–48]. The field test results of the OD sensor at the Pecos test facility (Fig. 7) demonstrated that the sensor could accurately measure the OD of the culture within the pond, trace algae growth, and pinpoint cultivation events such as media addition and culture transfer. LEDs at two central wavelengths were selected as the light sources for OD measurement. The first is in the red region, with a central wavelength of 670 nm, a spectral bandwidth of 25 nm, and a radiant flux of 2.5 mW (L6112-02, Hamamatsu Corp., NJ). The second LED is in the NIR region, having a central wavelength of 890 nm, a spectral bandwidth of 80 nm, and a radiant flux of 4.5 mW (L1915-02, Hamamatsu Corp., NJ). It should be noted that, for algae, a widely accepted wavelength for spectrometer-based OD measurement is at 750 nm (OD750). In this study, we calibrated the sensor output to OD750 to assess the sensor performance. The inclusion of two wavebands added robustness to the sensor,

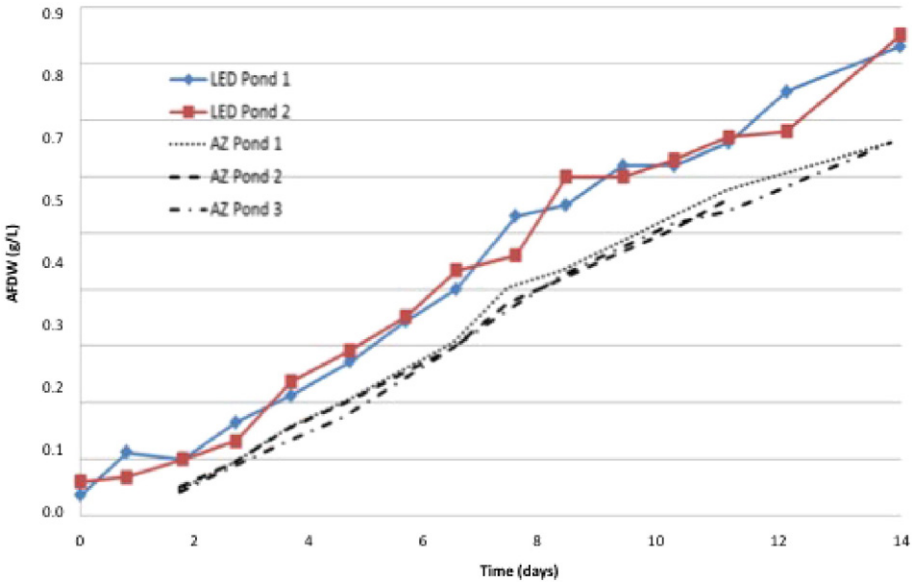

Fig. 6. Comparison of biomass growth in two duplicate LED climate-simulated raceways and three replicated Arizona outdoor pond cultures.

and may allow other algae parameters, such as chlorophyll content, to be estimated.

A laboratory protocol was developed toward potentially automating NRf-based measurements with a high degree of sensitivity to neutral-lipid content [49]. A spectrofluorometer was used to identify NRf emission maxima and investigate the temperature effect; and a single-band fluorometer was used to investigate the effect of staining time and species. For all algae types, the NRf emission maximum was at 590 nm. Temperature had a large impact, with NRf intensity increasing almost proportionally with temperature. NRf signal increased from minute 0 to 4 after staining. Finally, NRf intensity was linearly correlated with neutral lipid content in algae culture. The particular strain of algae significantly affected NRf signal intensity, but within each strain NRf signal was highly correlated with lipid content ($R^2 = 0.96$ for *Nannochloropsis* sp. and $R^2 = 0.88$ for *Botryococcus braunii*).

NIR combined with MIR spectroscopy of dried algae samples was used in an effort to quantify several biochemical components in dry algae biomass [50]. Crude protein and heating value were estimated satisfactorily ($R^2 > 0.85$), followed by ash content ($R^2 > 0.57$), and neutral lipids ($R^2 > 0.45$). The absorption band at around 2920 cm^{-1} was strongly related to total neutral lipid content ($R^2 = 0.90$).

Finally, a simple, fast, low-cost, IR-ATR sensor was developed [51]. The sensor included an integrated convective heater to dry algae-mixture droplets onto an internal-reflection element (IRE). The main components of the sensor are an infrared light-emitting diode (LED) that emits energy at 3.4 μm (≈2940 cm^{-1}), a right-angle zinc-selenide IRE, and a photodiode to measure energy reflected through the IRE. A generalized model relating sensor measurements of two species to lipid content had a moderate root mean square error of 59 mg/g, but it showed a promising linear trend between sensor measurements and lipid content across multiple species. The time required for the sensor to take a measurement of one microalgae sample was 120 min, far less than that required with conventional laboratory methods.

Table 1
Effects of salinity on invaders appearing in *N. salina* cultures.

Salinity (ppt)	Phytoplankton		Zooplankton	
	Diatoms (organisms μL^{-1})	Cyanobacteria (organisms mL^{-1})	Ciliates (organisms mL^{-1})	Rotifers (organisms mL^{-1})
10	410 ± 78	1400 ± 950	2200 ± 900	8.3 ± 6.8
22	450 ± 140	6.7 ± 2.2	630 ± 430	0
34	890 ± 380	0	2500 ± 300	0
46	560 ± 110	0	2000 ± 1000	0
58	810 ± 380	0	970 ± 460	0

2.4. Molecular diagnostics for monitoring contaminants in algal cultivation

Given the depth of efforts related to strain discovery and improvement a key issue in cultivation was to determine the extent to which monocultures of elite strains could be maintained (see for example Reference [30]). Sensitive methods were developed for enumeration of elite algal varieties relative to "weedy" invader strains that are ubiquitous in the environment, and for cultivation management. The ideal monitoring strategy should be inexpensive and identify weedy algae long before they become prominent in cultures of elite varieties. NAABB developed and evaluated polymerase-chain-reaction–based (PCR-based) tools for monitoring contaminants [29]. In this work, primers were designed to amplify an approximately 1500-nucleotide region of the 18S rRNA gene from three major classes of algae: Bacillariophyceae, Eustigmatophyceae, and Chlorophyceae. These amplicons can be sequenced for definitive identification of strains, or they can be digested with a restriction enzyme to generate allele-specific fragmentation patterns for rapid, inexpensive characterization of strains and cultures. This work provides molecular tools to detect and monitor algal population dynamics and clarifies the utility, strength, and limitations of these assays. These include tools to identify unknown strains, to routinely monitor dominant constituents in cultures, and to detect contaminant organisms constituting as little as 0.000001% of cells in a culture. One of the technologies examined was shown to be 10,000× more sensitive for detecting contaminants than flow cytometry [29].

Another NAABB effort developed molecular monitoring tools using 16S ribosomal RNA (rRNA) gene [52] and polymerase chain reaction (PCR) amplification [53] for identifying and tracking bacterial communities associated with the different cultivated microalgal species. These assays monitored by using the 18S rRNA gene as a marker by PCR amplification [54] to assess the health of cultivated algal species, and anticipate, detect and mitigate pond crashes. The percent algal-associated bacterial community composition based on the 16S rDNA marker over an 8-week growth cycle of *Chlorella sorokiniana* DOE1412 in the Aquaculture Raceway Integrated Design (ARID) outdoor cultivation pond in Tucson, AZ are shown in Fig. 8. The percentages were obtained by cloning each 16S rDNA PCR product, followed by DNA

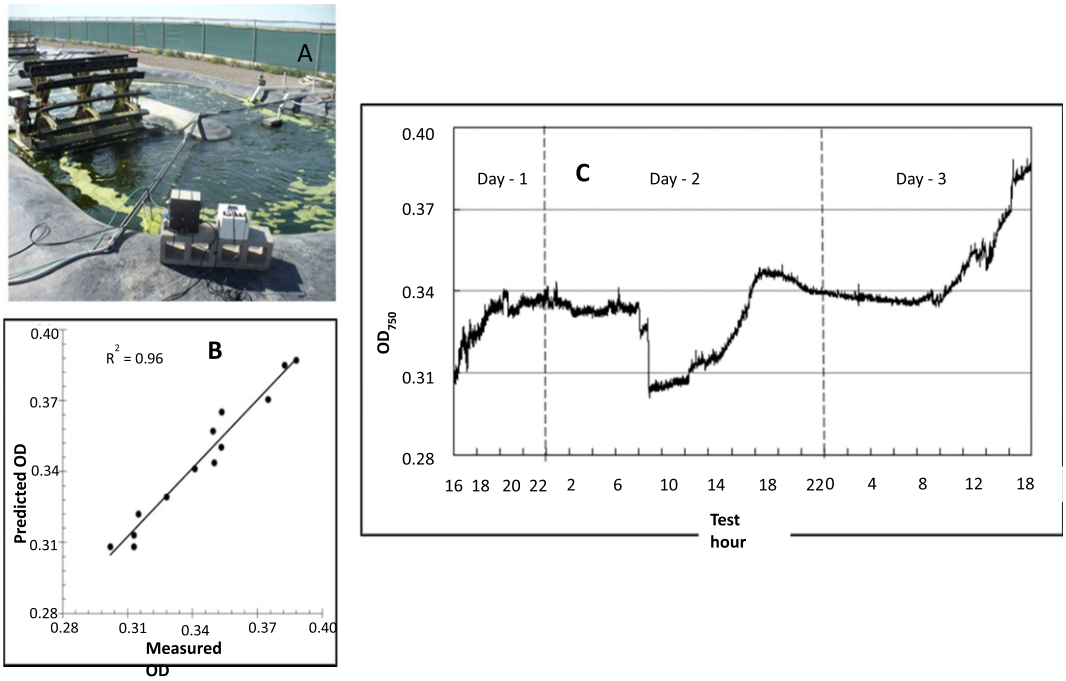

Fig. 7. A: Testing of the algae optical density (OD) sensor in and an open pond raceway. Shown are the different sensor modules including (left) the sensor housing with optical and electronic module inside, to which the miniature pump is mounted, and (right) the data acquisition module. B: Spectrometer measured optical density (OD) versus sensor measured OD in the open pond raceway test. C: The variation of optical density (OD) in the open pond raceway, as a function of cultivation time, measured by the OD sensor. The abrupt change in OD that occurred on Day 2 was due to growth media addition by a facility operator.

sequencing of thirty colonies per amplicon. Because algae contain chloroplasts with 16S rRNA genes, using the 16S rDNA as a marker allows for identification of algal species, and bacterial communities with a single molecular marker assay. Thus, in cultures for which *Chlorella* spp. (or other algal species) is the predominant community member (by PCR amplification; 30 clones), experience has shown this to be a positive indicator of health. In contrast, when invaders predominate the percentage of algal clones among PCR amplicons is fewer, and bacterial clones increase in number; thus, serving as an indicator of stress. In the growth cycle shown, the predominant bacterium is detectable as an observed

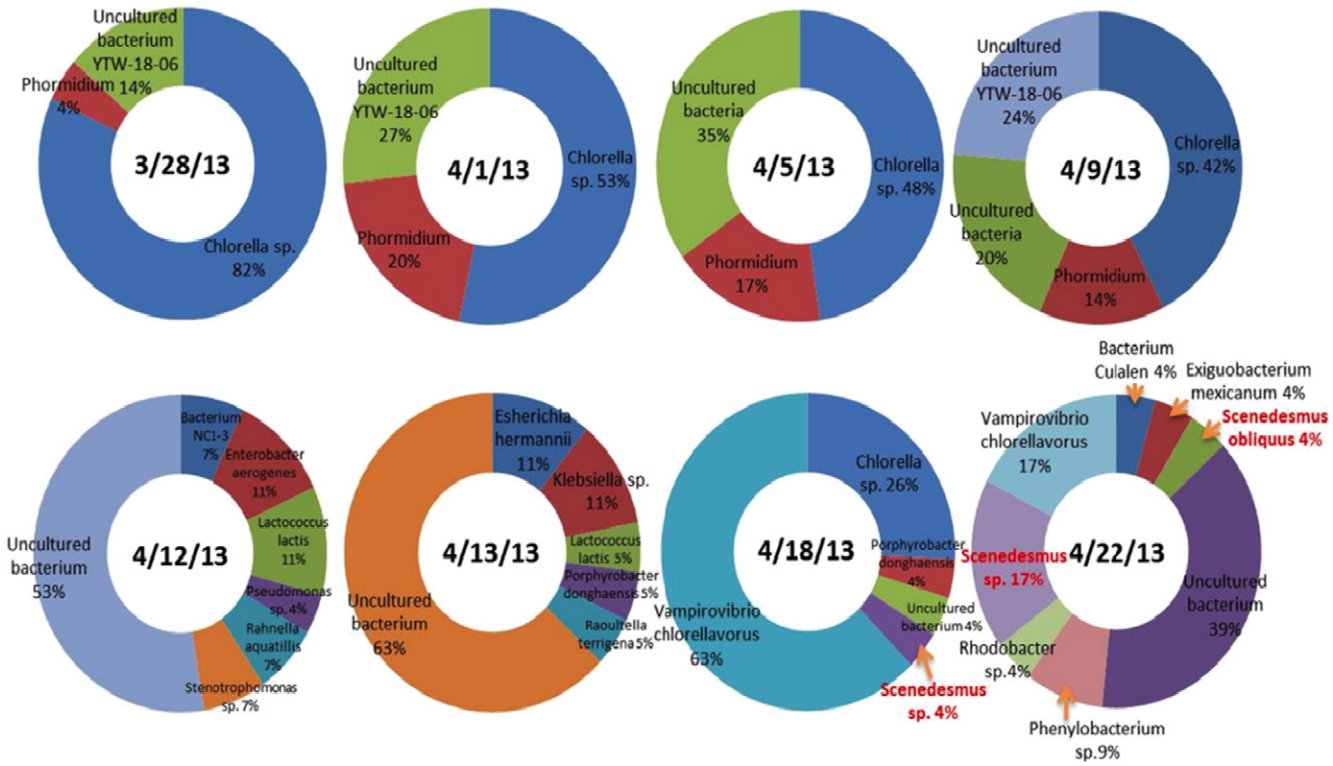

Fig. 8. Time course community analysis for *C. sorokiniana* culture in an ARID cultivation pond using 16S rDNA analysis by PCR. In the growth cycle shown, the predominant bacterium is detectable as an observed shift in bacterial community members predicted the eventual crash of the pond caused by the predator *Vampirobrio chlorellavorus*.

Fig. 9. Array of outdoor raceways at the Corpus Christi, Texas facility used for media optimization and polyculture experiments.

shift in bacterial community members predicted the eventual crash of the pond caused by the predator *Vampirobrio chlorellavorus* (ex Gromov and Mamkayeva 1972) Gromov and Mamkayeva 1980, a bacterium known to infect certain species in the genus, *Chlorella* (particularly *C. sorokiniana* and *C. vulgaris*) [55]. Among the other bacterial residents identified using the 16S rRNA as a marker, none but *V. chlorellavorus* has been reported to be an algal pathogen. It should be noted that some of the bacterial community members cannot be identified to genus and/or to species using the bacterial sequence references available in the GenBank database or other available databases that specialize in bacterial isolates, in part because taxonomic activities have not kept abreast of molecular marker approaches. Environmental factors conducive to algal growth contribute importantly to phycosphere health, since extreme conditions (i.e., cold, heat, sunlight) and nutrient shortages can cause 'abiotic' stress and at times make the algal culture more susceptible to predators, viral and other pathogens, and scavengers. Although not well defined, some bacteria associated with algae have been shown to positively or negatively influence growth, survival, and stability, greatly affecting feedstock yields [56]. Molecular monitoring tools have been shown to be effective in gauging the health of the phycosphere and show great promise for monitoring algal composition and algal-associated bacterial communities to serve as a forecasting system of environmental fluxes that may be detrimental to algal growth. In addition, molecular diagnostics have facilitated the early detection of at least one bacterial pathogen (e.g., *V. chlorellavorus*), and in advance of pond crashes. Management strategies are being tested to abate *Chlorella* spp. attack by this predator.

3. Nutrient/water recycle

A variety of bench scale and 1000 L scale cultivation studies were performed to determine strain-specific growth parameters. The scope included investigations of poly versus mono cultures, water recycle strategies and use of impaired waters, including produced water and wastewater. The majority of the work discussed in this section was done with the NAABB production strain *Nannochloropsis salina*. The suggested medium for cultivation of *N. salina* is denoted f/2, a well-defined growth medium for marine microalgae; hence the control case for all data presented is growth on f/2 medium.

3.1. Impaired water and nutrient studies

3.1.1. Small-scale nutrient and water-recycle studies

Initial experiments were done in 12 outdoor 3 m^2 raceways (shown in Fig. 9) located at one of the small test-bed locations in Corpus Christi, Texas, to investigate the effects of nitrogen source on productivity for *N. salina* in batch and semicontinuous cultures. Batch culture treatments continued to produce more biomass over the course of the study than the continuous cultures, with peak growth and time between harvests remaining consistent. Productivity averaged 12.8 g/m^2/d for batch cultures, compared to 10.9 g/m^2/d for the continuous cultures. In the nutrient regime study, nitrogen in the f/2 medium was provided as either ammonium or nitrate on an equimolar basis to determine the optimal growth response with respect to nitrogen source. No significant differences in production (AFDW [g/L]) were found between treatments. The ability to get the same production from the modified mix with ammonia and nitrate compared with the more expensive f/2 media greatly enhances the ability to produce biomass at reduced costs with this strain. Additional experiments were performed to compare the productivity of a monoculture of *N. salina* to mixed cultures of *Phaeodactylum tricornutum* and *N. salina*. Results suggest that the mixed culture grew better than or the same as the N. salina monoculture. In colder temperatures, the mixed culture did better suggesting that crop rotation strategies require more investigation.

Another important part of cultivating and characterizing algae is to recycle water. After algal cultures reach stationary phase, the water or

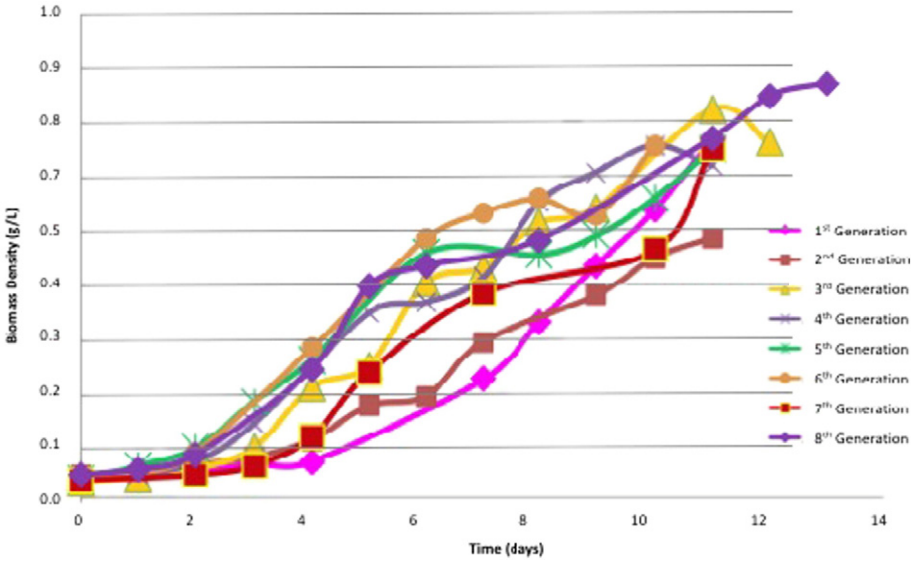

Fig. 10. Algal growth for *N. salina* as a function of generation cultured by recycling 90% of the spent media using fresh inoculum in batch shake flasks. Sufficient nitrogen and phosphorous were added to the recycled water so that the initial concentration was always the same.

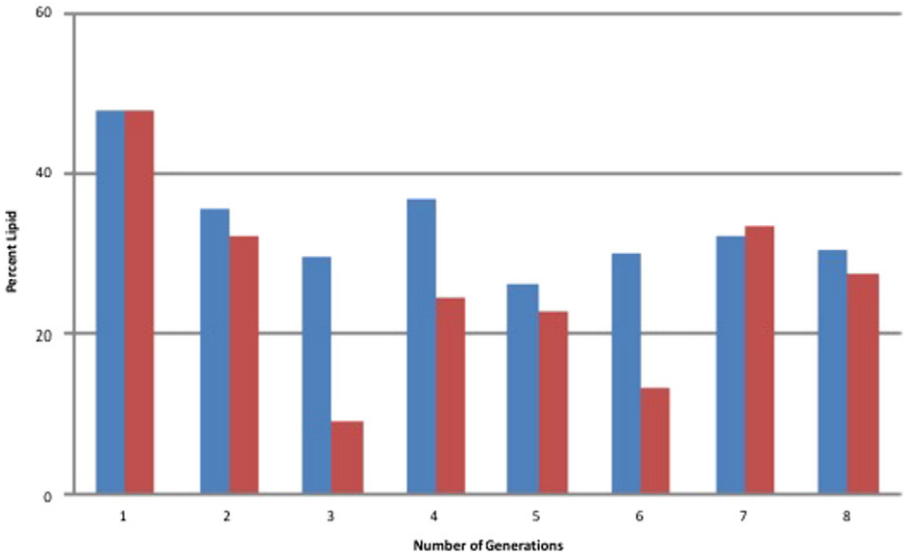

Fig. 11. Variation in lipid content through succeeding generations. Blue bars = media filtered with 0.2 μm filter. Red bars = unfiltered media. (n = 1 for each set).

spent media is separated from the algae and ideally recycled to the raceway. Recycling of spent media should occur until algal growth is inhibited severely. Fig. 10 shows growth of *N. salina* as a function of generation. A generation is defined as a batch culture started with fresh inoculum but the water (spent media) is recycled. Hence for this set of experiments 90% of the water was reused 8 times. Each time, sufficient nitrogen and phosphorous were added to the recycled water so that the initial concentration was always the same. When fresh inoculum and additional media are added, the algae grow well with very little change in biomass productivity as a function of generation. As shown in Fig. 11, the lipid percentage varied more than when fresh inoculum was used; however, overall, these results demonstrate that water can be recycled multiple times with some losses in overall lipid productivity to be expected.

In the field, some systems operate in fed-batch or semicontinuous mode as opposed to batch mode. In

this configuration, a percentage of the culture is removed and dewatered, the water is recycled to the reactor and additional nutrients are added but not additional inoculum. In these cases, the lipid percentage varied more than when fresh inoculum was used; however, overall, these results demonstrate that water can be recycled multiple times with some losses in overall lipid productivity to be expected.

3.1.2. Algae cultivation on wastewater

Use of municipal wastewater for algal cultivation is an area of great interest as it provides an inexpensive source of nutrients. As part of NAABB, four studies were done. The first study specifically targeted primary wastewater treatment in arid climates where unsustainable evaporative water losses preclude the use of open ponds for algae-based methods. The second study investigated whether or not the metals found in wastewater would be toxic to mesophilic algae. The two other studies investigated different types of wastewater (centrate and secondary) obtained from Southwestern wastewater treatment plants.

A complete treatment system operating on primary (settled) municipal wastewater was initiated with NAABB funding and evaluated at New Mexico State University [34,35]. It is specifically designed for use only in hot arid climates. The algae-based treatment occurs in inexpensive horizontal photobioreactors designed to minimize evaporative water losses by minimizing gas flow rates (Fig. 12). This system retains metabolically generated O_2 and CO_2 to maximize mixotrophic metabolism and boost biomass productivity. Measured O_2 in headspace at midday in summer did not exceeded 30%, while the enhanced CO_2 levels in head space dramatically reduced the probability of photorespiration, as in C4 plants. The entire system operates somewhat like green seed metabolism, whereby CO_2 released by pyruvate dehydrogenase for fatty acid synthesis is recaptured by photosynthetic cells [57,58]. In this case, the oxidation of reduced carbon via respiration by algae or heterotrophic microbes in the PBR can be recaptured by algae in the light zone (see previous section on Growth modeling and Reference [32]). The PBR system experiences passive solar heat gain in hot arid environments such that moderately thermophilic algae are required. *Galdieria sulphuraria* was chosen as the ideal algal component. In addition to thermotolerance to 56 °C, it possesses the most versatile heterotrophic capabilities known among the phototrophs [59]. *Galdieria* grows at low pH, which rapidly destablizes mesophylic neutral pH organisms in primary wastewater. The outdoor PBR system was shown to support the growth of *Galdieria* at productivities from 2 to 16 g/m^2/day and to remove N and P to discharge limits in batch mode within 4 days [34,35].

Acidophile-based wastewater treatment systems must also overcome challenges associated with the cost of acidification and potential effects of acid anions on downstream processes. *G. sulphuraria* naturally acidifies its growth medium [60] likely because of proton pumping after uptake of NH_4^+ and assimilation of NH_3. Data from Oesterhelt et al. [60] demonstrate that adjusting the wastewater to pH 6.0 might be sufficient. Additional experiments will be required to directly test this hypothesis. To assess the potential impact of sulfate ions remaining from

Fig. 12. Photobioreactor system for arid conditions that minimizes evaporative water losses shown in a culture for wastewater treatment with *Galdieria sulphuraria* at pH 2.5 and 1–2% CO_2 enrichment in the head space.

Table 2
Comparison of continuous flow metals toxicity data to metals levels in concentrated wastewater effluent from the Ina Road Water Pollution Control Facility in Tucson, Arizona.

Metal	EC_{50} from scientific literature (μg/L)	Actual conc. in VSEP treated Ina Road wastewater (μg/L)
Copper	50.2[a]	87.70
Zinc	240[b]	124.00
Cobalt	520[b]	n/a
Lead	680[b]	7.66
Nickel	410[b]	65.54

[a] J. L. Stauber, T. M. Florence, Mechanism of toxicity of ionic copper and copper complexes to algae. *Marine Biology* **94** (1987), 511.
[b] K. -C. Lin, Y. –L. Lee, C. –Y. Chen. Metal toxicity to *Chlorella pyrenoidosa* assessed by a short-term continuous test. *Journal of Hazrdous Metals* **142** (2007), 236.

acidification on downstream hydrothermal liquefaction (HTL) processing, *Galdieria sulphuraria* biomass was grown in outdoor PBRs like that shown in Fig. 12. H_2SO_4 was used for acidification and the resulting biomass concentrated to a 10% solid feed and converted to biocrude oil. The lipid content of the biomass was ~5% as total fatty acid methyl esters and the HTL biocrude yield was 19% by weight [61].

3.1.3. Other wastewater cultivation experiments

Two other studies investigated different types of wastewater (centrate and secondary) obtained from Southwestern wastewater treatment plants [62]. In these, the metals investigated as potential toxicants included those present at the highest concentrations in regional municipal wastewaters. Compounds and their respective half maximal effective concentration (EC_{50}) values (obtained using a particularly sensitive algal species) were as illustrated (Table 2). Initial experiments involved *N. salina* in batch cultures that were simultaneously exposed to various multiples of the EC_{50} concentrations. Subsequent work in this area was designed to determine which metal species were the predominant source of observed toxicity.

Fifty percent inhibition of the *N. salina* growth rate was observed in the culture amended with the Table 2 metals at 11× their respective EC_{50} values (Fig. 13). That is, zinc and copper were present at concentrations near mg/L levels—exceptionally high relative to their typical concentrations in regional municipal wastewater effluent (Table 2).

After determining that *Nannochloropsis* can grow in water that contains >10× the amount of heavy metals typically found in wastewater effluent, centrate was investigated. The basic experimental strategy was to substitute either wastewater effluent or a nutrient-rich sidestream developed during the dewatering of biosolids for the source of macronutrients in the f/2 medium. Effluent or the sidestream flow comprised fractions of the total liquid volume ranging from 5 to 100%. Salts were added to maintain a near uniform ionic strength. Relative growth rates and lower than normal terminal optical densities were taken as indications of inhibition (Fig. 14; also, see Reference [62]).

Results indicate that the addition of centrate derived from biosolids dewatering increased both the rate and extent of growth of *N. salina* at ratios ranging from 5 to 25% v/v. Higher fractional additions inhibited growth (results not shown). Minor changes were apparent in the lipid compositions of the cells grown in centrate (Fig. 15). It is apparent that those cells produced a larger percentage of fatty acids that were not recognizable based on the authentic standards utilized here. Furthermore, centrate addition virtually eliminated production of fatty acid C18:1n9 at every level of centrate addition.

Additional tests were carried out using *Nannochloropsis salina* growing on secondary-treatment wastewater collected at the outflow before discharge or just before the chlorination stage from the Jacob A. Hands municipal wastewater treatment plant in Las Cruces, New Mexico. This plant uses an activated sludge protocol combined with a biological filter pretreatment (trickling filter) and a final chlorination and SO_2-dechlorination. This process produces an advanced secondary treated wastewater effluent. On bench-top shakers, without CO_2 addition, algal growth was significantly slower in the wastewater effluent as compared to the standard f/2 substrate (Fig. 16). The effect of wastewater bacteria on algal productivity and contaminant risk parameters was evaluated by comparing sterile wastewater to either raw treated wastewater or nutrient-amended treated wastewater. As expected, the algae grew to higher cell densities in the sterilized wastewater. For this water source, adding nutrients or fertilizer did not significantly increase the final cell density. We conclude that wastewaters, even partially treated to remove nutrients, are a viable source of nutrients allowing productivity levels similar to the ones obtained on standard growth media.

3.1.4. Future outlook for wastewater usage

The research program described has shown that the economic and environmental sustainability of a meaningful algal biofuels industry requires use of CO_2 and fertilizer nutrients that are not derived from fossil fuels [63–65] and that do not reduce the availability of fertilizer for agriculture. Recycling water or using otherwise impaired water can

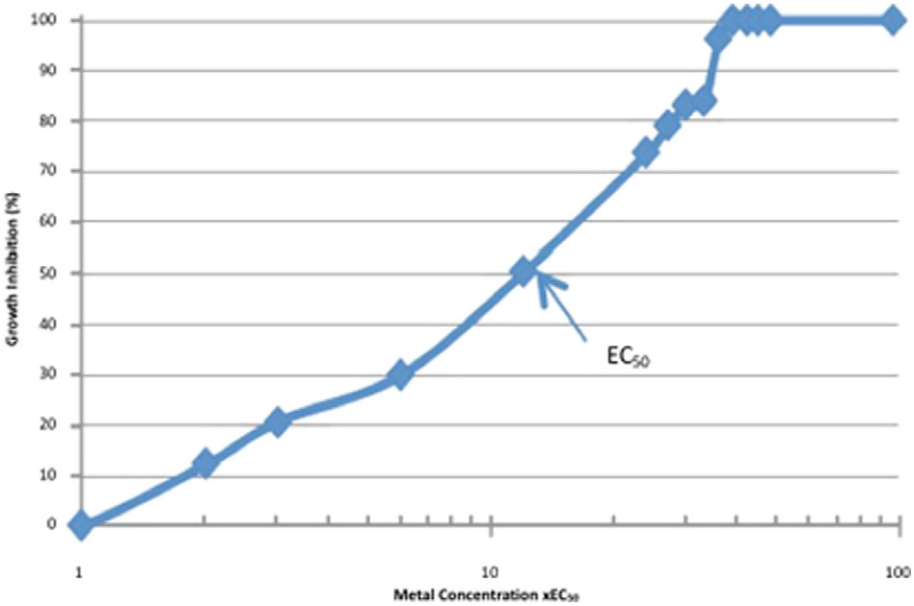

Fig. 13. Growth of *N. salina* in standard medium supplemented with mixtures of the Table 2 metals. (Metals concentrations were multiples of the EC_{50} values given in Table 2) [62].

further increase the sustainability of biodiesel production from algae [64–66]. One kilogram biodiesel requires approximately 3726 kg water, 0.33 kg nitrogen, and 0.71 kg phosphate if freshwater is utilized [66]. Therefore, the use of wastewater as the source of water and nutrients is requisite to the development of algal biofuel technology in Arizona and other parts of the semiarid Southwest. Straightforward calculations indicate that without nutrient recovery and reuse, the supply of municipal wastewater cannot satisfy large scale biofuel nutrient requirements. In their recent report titled Sustainable Development of Algal Biofuels in the United States (2012), the National Research Council of the National Academies concluded: "…with current technologies, scaling up production of algal biofuels to meet even 5% of U.S. transportation fuel needs could create unsustainable demands for energy, water, and nutrient resources…"[15]. Identification of alternative water and nutrient sources is necessary to make algal biofuels a sustainable energy resource. Municipal wastewater is among the most promising sources of water and nutrients (nitrogen, phosphorus, and trace elements) for algal growth. However, annual production of 39 billion L of algal biofuel, which is equivalent to 5% of annual U.S. demand for transportation fuels, requires at least 123 billion L of water, 6 million MT of nitrogen (N) and 1 million MT of phosphorus (P). Without recycling, it would take over 1×, 4×, and 5× the entire U.S. population, respectively, to generate sufficient wastewater to provide that much water, N, and P. Therefore, nutrient and water recycling/reuse are fundamentally critical for microalgae to be a sustainable energy source.

4. Cultivation system innovations

NAABB had several efforts focused on developing new innovative approaches for the design and operation of the algal cultivation systems to improve algal biomass productivity and the associated capital and operating costs. These include new pond designs, mixing systems, CFD models for improved raceway design and the use of photobioreactor (PBR) systems to produce high-yield inocula for large scale ponds.

4.1. ARID raceway system

One of the causes of decreased algae production in open ponds is diurnal and seasonal temperature variation. The ARID system (shown in Fig. 17) maintains temperature in the optimal range by changing the water surface area between day and night by draining the culture to a sump [17].

A finite-difference temperature model of the ARID raceway was developed in Visual Basic for Applications [16]. The model accurately simulated the temperature changes in the ARID raceway during winter cultivation experiments where the algal growth rate of *N. salina* in ARID and conventional raceways was compared. The ARID raceway remained 7–10 °C warmer than conventional raceways throughout the experiments.

NAABB efforts continued to make design improvements and energy evaluations of the ARID system [18,37,67]. Although the original ARID system was an effective method to maintain temperature in the optimal growing range, the pumping-energy input was excessive and the flow mixing was poor. Thus, an improved high-velocity raceway design was developed to reduce energy-input requirements (Fig. 18). This was accomplished by improving pumping efficiency, optimizing the operational hydraulic parameters, and using a serpentine flow pattern in which the water flows through channels instead of over barriers. A second prototype ARID system was installed in Tucson, Arizona, and the constructability, reliability of components, drainage of channels, and flow and energy requirements were evaluated. Each of the energy inputs to the raceway (air sparger, air bag blower, canal lift pump, and channel recirculation pump) were quantified, some by direct measurement and others by simulation. An algae growth model was used to determine the optimal flow depths as a function of time of year. Then the energy requirement of the most effective flow depth was calculated.

The Biomass Growth Model was added to the ARID raceway model. The accurate estimates of light transmission and temperature enable an accurate prediction of algae growth for various raceway configurations, depths, and operational schemes. The model was used to compare ARID raceway algae growth with conventional raceway algae growth at different flow depths and then to simulate daily and monthly production values for different scenarios. The model was run for Tucson, Arizona, and showed that the ARID raceway had much higher production than a conventional raceway in winter, significantly higher production in spring, the same production in the last month before the monsoon (June), and similar production during the monsoon months (July and August) (Fig. 19).

4.2. Airlift mixing systems for raceways

A new airlift-driven raceway reactor configuration was developed for energy-efficient algal cultivation and high CO_2 utilization efficiency (Fig. 20). Advantages of this configuration were demonstrated in a

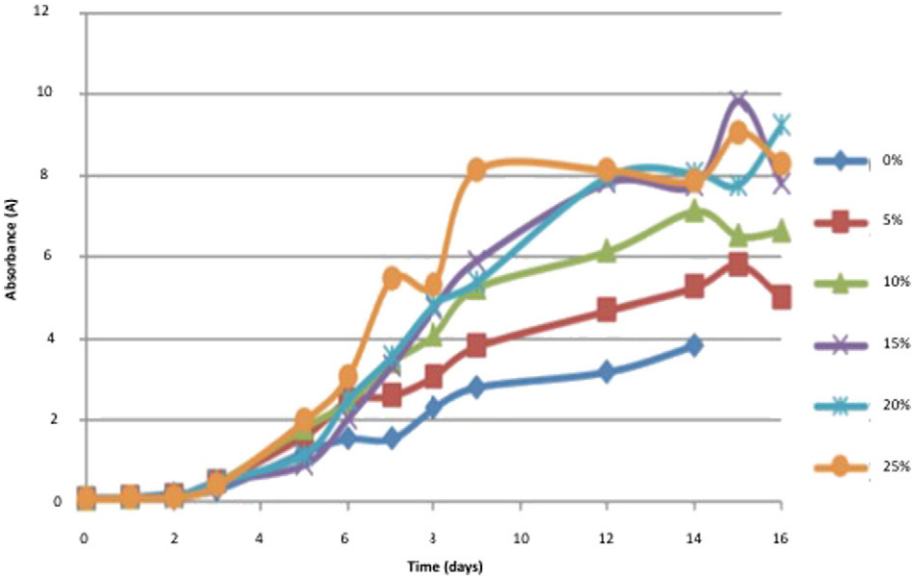

Fig. 14. Results for *N. salina* growing in normal growth medium with different percentage of centrate derived from the dewatering of digested sludge.

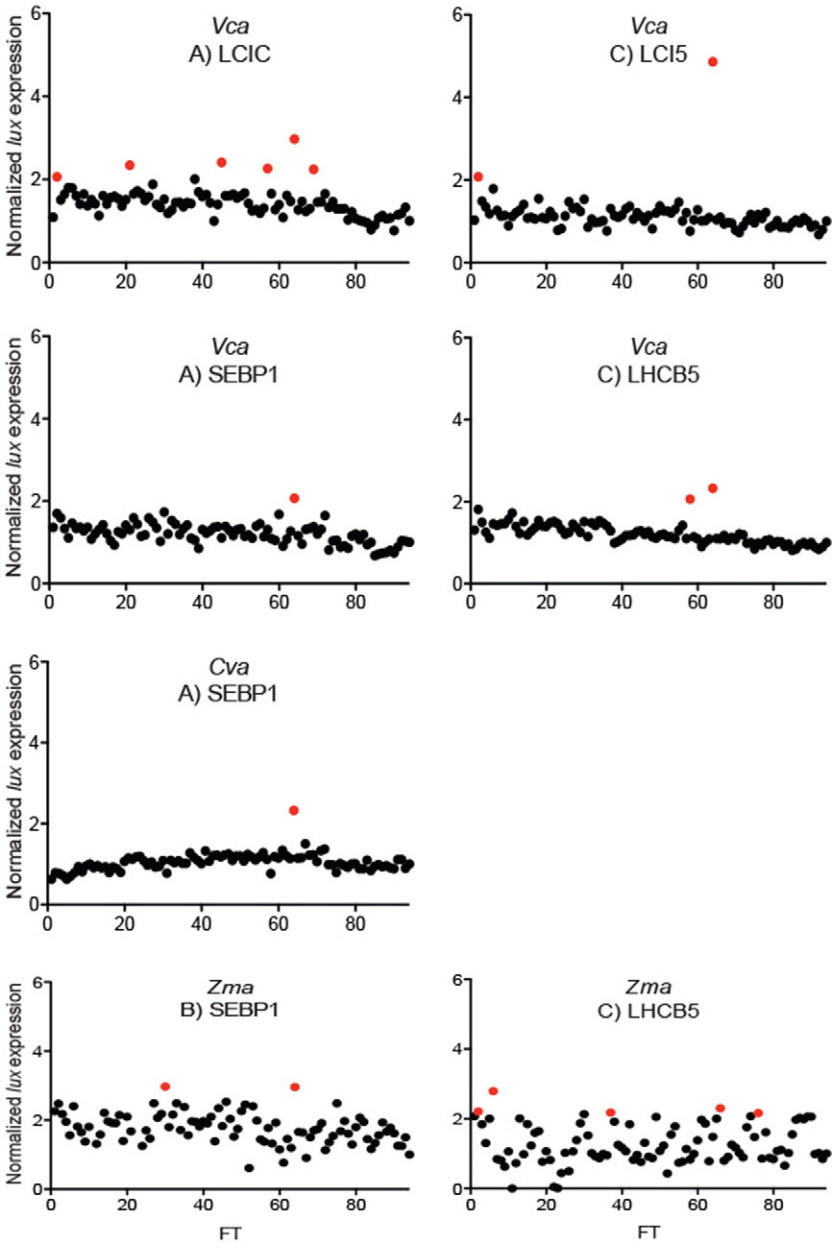

Fig. 14 (*continued*).

23 L version of the configuration under artificial lighting and laboratory conditions and in an 800 L version under natural light and outdoor conditions [23,24].

Results from side-by-side growth studies conducted with *N. salina* in the two raceways, one with the modified air-lift system and the other an identical raceway with a standard paddlewheel (Fig. 21), are summarized in Fig. 22. The CO_2 input into paddlewheel and airlift systems was identical on a culture volume basis. The higher biomass and lipid production due to the efficient CO_2 supply resulted in higher net energy gain in the airlift-driven raceway than in the paddlewheel-driven raceway. The net energy output of the paddlewheel-driven raceway is estimated as 0.03 W/L whereas that in the airlift raceway is 0.15 W/L.

Based on the laboratory tests and the field tests conducted in this research, the proposed airlift-driven raceway can be seen to be more energy-efficient than the traditional paddlewheel-driven raceway. To quantify energy efficiency, biomass productivity per unit energy input is used rather than the traditional measure of volumetric biomass productivity. Based on this measure, performance of the airlift reactor configuration is shown to be comparable to or better than those reported in the literature for different PBR designs [24]. In light of the improved energy-efficiency and the higher CO_2 utilization efficiency demonstrated in this study under laboratory and outdoor conditions, the proposed airlift-driven raceway design holds promise for cost-effective algal cultivation. The mathematical model of the airlift-driven raceway developed as part of this study was validated using growth data on two different algal species under indoor and outdoor conditions. The predictive ability of this model was shown to be high.

4.3. Applications of PBR systems with high biomass yields

Discussions of microalgal cultivation systems have typically focused on either open raceway systems or closed PBRs but this is likely to be a

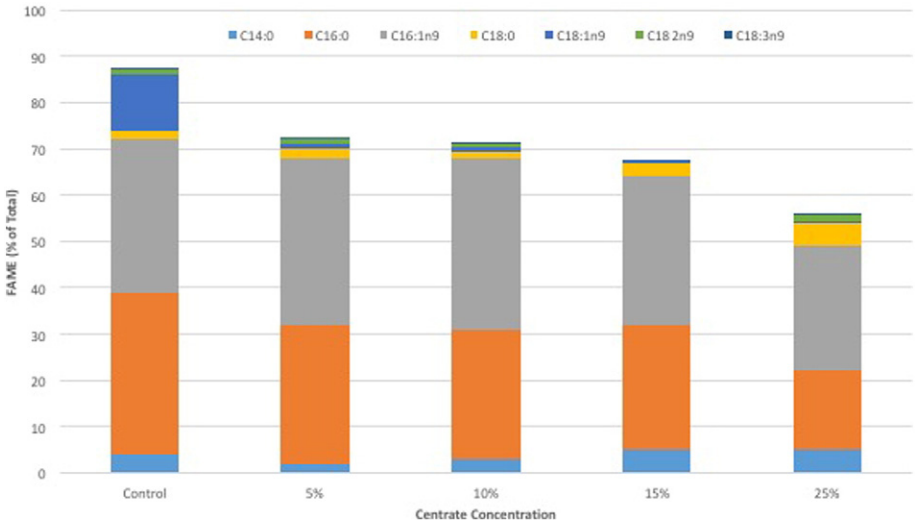

Fig. 15. FAME profiles (% of total) for *N. salina* grown in mixtures of f/2 medium and digester centrate.

false dichotomy as there are applications best achieved using one or the other or both options in concert [30,34,68,69]. For example, the use of highly efficient PBR systems for quality-controlled inoculum production at maximum rates for open raceway ponds could likely improve yields and cultivation system stability and reliability. PBRs integrated with large-scale open raceway production systems also represent a risk-mitigation system that can quickly repopulate a large-scale open raceway pond facility after a culture crash. PBR volumetric productivities can be 10-fold higher than open raceway ponds due to shorter light-paths and better control of culture parameters such as light, temperature, CO2, and mixing.

Biomass and lipid productivity data for *Nannochloropsis salina* were collected from operations of the Solix Algredients Inc. PBR system over several years conducted in a serial batch mode with a portion of each harvest used to start the next batch [30]. Harvest densities ranged between 2 g/L and 3 g/L. Some batches were harvested at lower densities due to low growth rates in low light periods during winter cultivation. A select number of batches were inoculated as low as 0.25 g/L and harvested at 6 g/L. Several sensitive tools were used in follow up studies to demonstrate that *N. salina* cells dominated these cultures in terms of cell numbers [29]. Together, these studies document multi-year, stable cultures of this strain. Nevertheless, even this sophisticated photobioreactor system did not support pure monocultures.

NAABB tested a newer version of the Solix Algredients Inc. PBR (AGS4000) with the same media and culture, *N. salina* CCMP1776 from the previously mentioned Solix studies. The lower range of

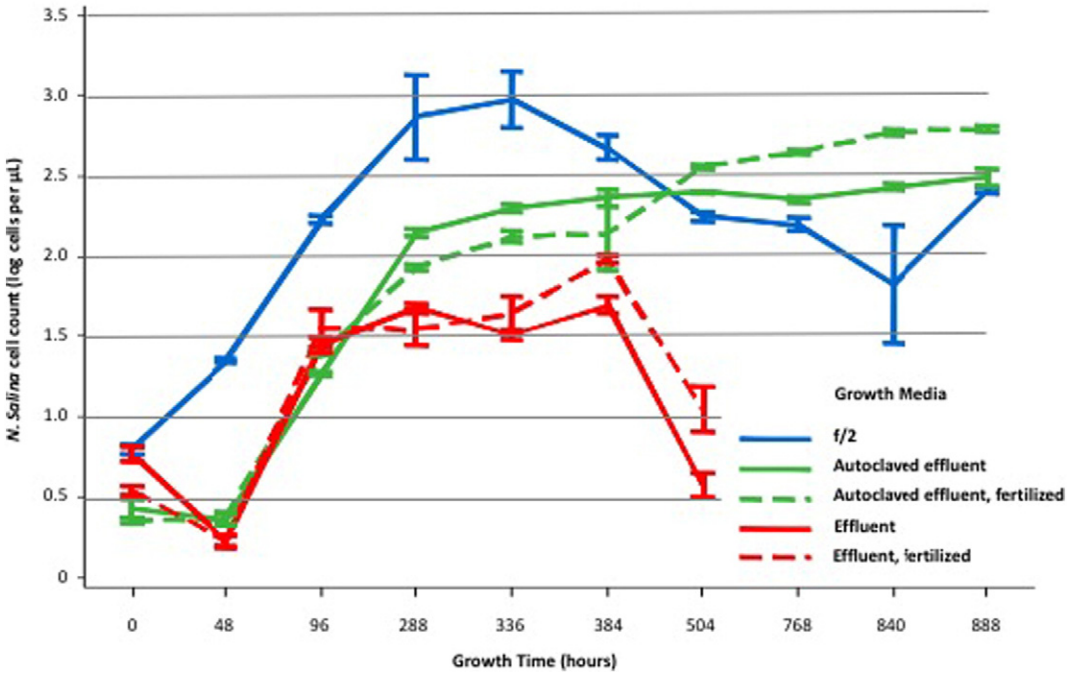

Fig. 16. Comparisons of growth of *N. salina* in cultures based on f/2 media or wastewater secondary effluent (autoclave sterilized and with fertilizer addition) from a municipal wastewater plant in New Mexico.

Fig. 17. ARID raceway in operation.

required inoculation densities and upper range of harvest densities was investigated with this system. Harvest densities as high as 6 g/L were observed in the same period. To achieve this growth range the system was fed a second batch of nutrients four days after inoculation. The ability of the system to operate at high linear growth rates over these density ranges supports the use of the system as an industrial scale cultivation technology for both stand-alone production and as an inocula source for large-scale integrated PBR/open-raceway pond systems.

Fig. 23 shows a data plot for a number of production runs in the Solix PBR over the past several years indicating the relationship between final culture density (x axis) and lipid content (y axis) as a percentage of dry weight. This data plot shows that yields exceeding 5 g/L with 50% lipid have been achieved using the Solix PBR system. Moreover, these results have been confirmed in both small- and large-scale PBR systems with efficient use of nutrients and CO_2. Actual operation of these PBR systems to produce inocula for open ponds would most likely focus on rapid biomass productivity under nutrient-sufficient conditions versus lipid accumulation under nutrient limitation, since this would significantly increase biomass productivity for providing seed for large-scale pond systems.

4.4. Large-pond cultivation/biomass production

An important aspect of the NAABB program was algal cultivation to provide biomass for downstream processing and analysis. Two sites were utilized: the Texas Agrilife facility at Pecos, Texas (Fig. 24A), and the Cellana facility in Kona, Hawaii (Fig. 24B). At Pecos, five algae strains, starting with *N. salina* as the baseline strain and four other selected strains, were cultivated. For each alga strain, two media were compared

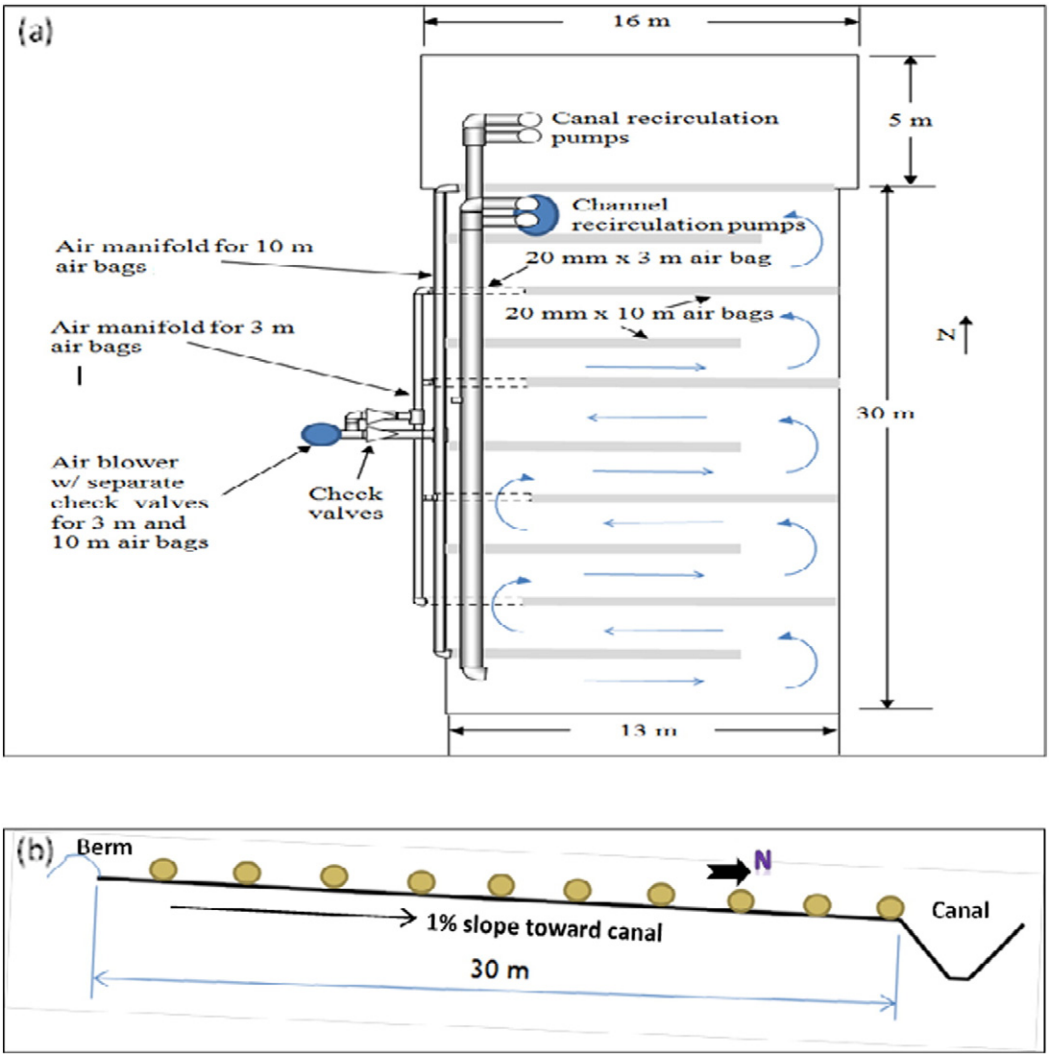

Fig. 18. Improved ARID pond system design, (a) ARID dimensions (general view), (b) profile of the channel's cross-section.

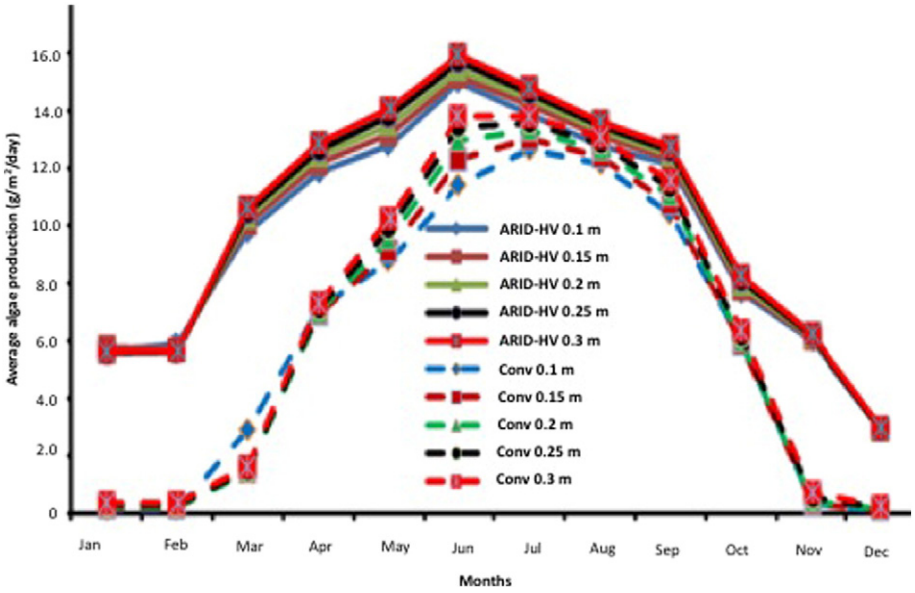

Fig. 19. Simulated comparisons of ARID and conventional raceways (at different depths) productivities throughout a one-year representation in the Tucson, Arizona, area using an algal growth model.

and productivities were determined (lipid percentage, ash percentage), and batches were grown in 23,000 L open ponds with paddlewheels. At Kona, Cellana's ALDUO™ large-scale cultivation "hybrid" system of PBRs and open ponds was utilized. Each production system consists of six 25,000 L PBRs and three 450 m^2 production ponds. All fluid transfers—including inoculations, nutrient additions, and harvest volumes—were operated and monitored by a remote process-control system.

4.4.1. Media and growth optimization

The first step performed prior to large-scale cultivation was to optimize the media to reduce the costs associated with growing algae at the 23,000 L scale. This was accomplished by replacing the nitrate with urea, the potassium phosphate with a mixture of monoammonium phosphate and potash (potassium source), and the iron citrate with iron chloride. Each component was evaluated separately and the lowest quantity of replacement chemical that did not result in a decreased growth rate was used. The cost and quantity information for a common freshwater *Chlorella* sp. cultivation medium, BG-11 was compared to a much less expensive media developed for use in the field. The new media recipe is 90% lower in cost than the standard BG-11 media.

Once the species had completed the media optimization testing at bench scale, intermediate scale tests were conducted in two medium (200 L) raceways located in a greenhouse (Table 3). Nine species were tested using this process at the Pecos site. Cultures of *Chlorella sorokiniana* DOE1412 were scaled-up from the bench to 800 L raceways on the BG-11 versus optimized media. The biomass productivity, lipid

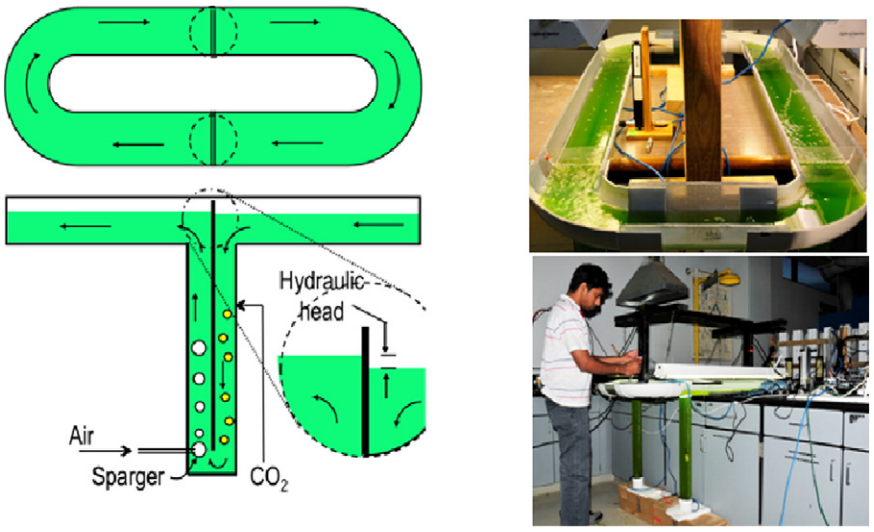

Fig. 20. The airlift section consists of a downcomer and a riser. CO_2 is sparged midway of the downcomer, while air is sparged from the bottom of the riser. Due to the lower density of the air-water mixture in the riser, the bulk fluid flows down the downcomer and up the riser, creating the circulation in the raceway. As the bulk fluid flows down the downcomer, the CO_2 bubble swarm is carried downwards by the drag force and to the riser section. While enabling mixing/circulation, this configuration provides longer detention time of the CO_2 bubbles compared to the traditional raceways where the CO2 is sparged from the bed of the raceway.

Fig. 21. Outdoor installation showing 800 L airlift-driven and paddlewheel-driven raceways side-by-side for comparison studies.

productivity, and FAME profile were monitored for both media formulations. Fig. 25 shows the cultivation data as a function of time. Essentially the media was added slowly, starting with 5 L of culture in 20 L of media, then 30 additional liters were added, followed by 30 L more. Subsequently, the cultures were transferred outdoors and the volume was set to 250 L and media added up to 800 L. The algae grew as well on the optimized, less expensive media as on the BG-11 media; however, the optimized media (PE-001A) is 10 times less expensive. The lipid content and lipid profile are shown in Fig. 26. This strain provides lipids with many unsaturated bonds and primarily consist of C18 compounds that are readily converted to fuels. The lipid profiles are similar for the two media regardless of reactor type.

Additionally, Cellana conducted strain screening and optimization experiments using its midscale cultivation system, which is a standalone system of 24 PBRs and pond simulators, each of 200 L capacity. Cellana focused on *N. oceanica*, strain KA19 (isolated on the Big Island of Hawaii), and optimized pH, salinity, total nitrogen, and cultivation time.

4.4.2. Production

At the large scale, five consortium strains were grown in 23,000 L open pond raceways in Pecos. Additionally, Cellana cultivated three species in their production facility: *N. oceanica* KA19, *Pavlova pinguis* C870 and *Tetraselmis* sp. (strain KA33). Table 4 provides the amount of biomass provided to the consortium for downstream processing studies. On average, a productivity of 10 g/m^2/day was obtained at both sites. More detail on the long-term and seasonal productivities from the Pecos testbed and other algal cultivation facilities is provided in the Sustainability review section. Full 100% media recycle (solid nutrients added to recycled water) showed no adverse effects on media composition, biomass, or lipid yield. However, changes in the concentration of divalent and transition metals over time in the cultivation system and algae remain unaccounted for. These changes appear to be nominally the inverse of total salinity variations. Over 15 cycles of growth using recycled media were accomplished without large increases in salinity. The use of the recycled media reduces the use of new well water and retains salts that would otherwise be purchased for addition, thereby drastically reducing the quantity of water withdrawn from the local shallow aquifer and potentially reducing costs.

4.4.3. Lessons learned from large-scale cultivation studies

There were several lessons learned at the large-scale related to scalability, media recycle, pond depth, ash content, contamination, and process integration. Overall, data collected at mid-scale matched up very well with that at large-scale in terms of the biomass productivity, pond cycle, and the biochemical composition of the biomass. This confirms that mid-scale production systems are useful research tools that simulate microalgae performance in large-scale ponds in a cost-effective manner.

An important aspect of cultivation is the use of media recycling. It is extremely important to reuse as much water for cultivation as possible to reduce input costs. Studies were performed in the lab using ion chromatography (IC) and analysis performed to determine nutrient uptake of each individual alga species as well as to determine the chemical balance of the media after the algae had been removed from suspension. During 2011 and 2012, media was recycled, showing no significant drop in productivity or lipid accumulation. It should also be noted that over time microelements present within the media increased in concentration within the recycled media the more it was reused for cultivation, suggesting a reuse limit. Also, recycled media had to be treated using specific amounts of bleach to remove any potential contaminants that were present over time. Alternative methods for sterilization of the recycled media are ongoing and included UV treatment similar to what is seen in the wastewater and aquaculture industries.

Pond depth, depending on the time of year, has an effect on the overall performance of the algae cultures. During the summer months,

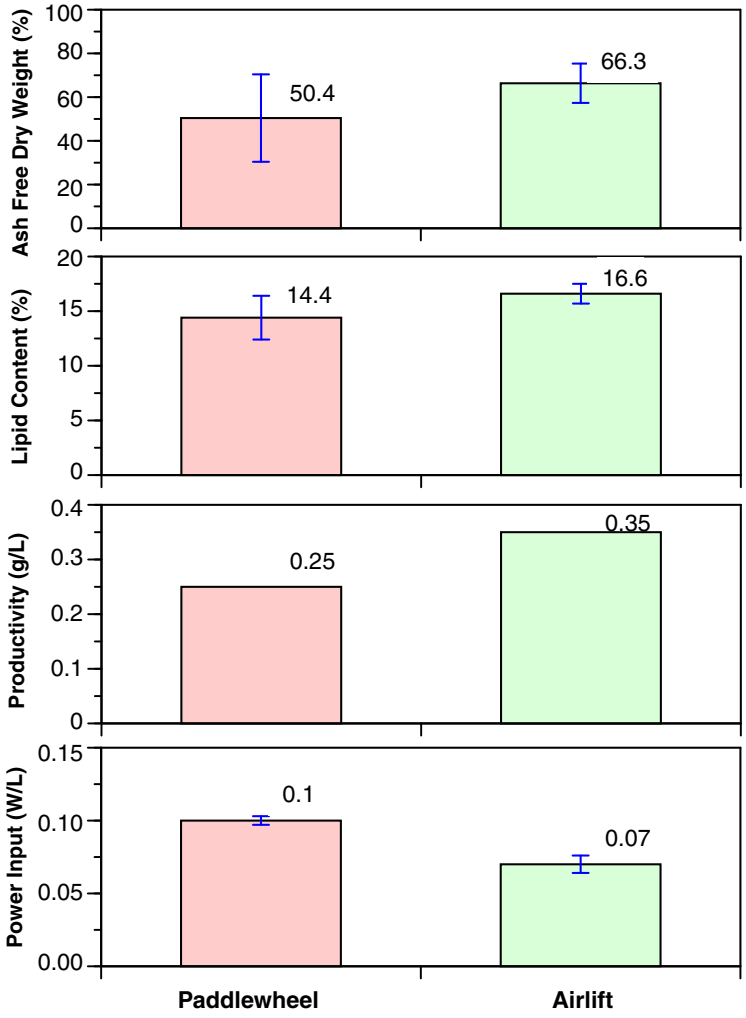

Fig. 22. Comparison of performance characteristics for the airlift-raceway versus paddlewheel-driven raceway. Species: *N. Salina*. Test duration: 12 days. Ash-free dry weight [%] with respect to dry weight indicates the organic content of the biomass.

increasing the speed of the paddlewheel and operating at a depth of 4–7 in. can reduce the overall temperature of the algae culture, keeping it more protected from overheating and thus maintaining high growth rates during the more extreme months of the year. However, this strategy will not work in arid regions with high evaporation rates and limited water resources. Slowing the paddlewheel down in the winter to help reduce evaporation and increasing the pond depth to provide more thermal protection helps prevent the culture from getting too cold, allowing the growth rates to stay competitive during the winter months. A reduction in growth rate was observed in the winter months due to temperature fluctuations and lower light levels, but through proper management and culture care, the Pecos facility has been able to stay operational year round.

One other lesson learned at the large scale is related to ash content management. In large open areas, especially in the Southwest, dust frequently blows into the ponds. The dust increases the ash content of the culture and is undesirable in the downstream processes. A strategy was developed utilizing partial harvests to minimize the amount of dirt in the cultures. However, efficient harvesting methods that minimize dust require further investigation.

Culture contamination is the most prevalent cultivation issue that was observed over the course of the NAABB cultivation projects. By using a batch cultivation system, contamination issues could be mostly contained, but from time to time either due to older cultures, rain events, or large dust storms, cultures would become contaminated during the production process. Microscope checks were performed on the batches at regular intervals to determine the rate in which each batch was becoming contaminated. An arbitrary threshold of 20% contamination was established to provide a decision point when cultures were deemed unusable. Contamination decreased when the ALDUO™ hybrid system, a combination of PBRs and open ponds, was used. Species-specific methods were also developed, such as the addition of salt to freshwater cultivation systems when the algae had some salt tolerance, pH shifts, and nutrient starvation.

The final aspect of large-scale cultivation is gaining an understanding of how changes in cultivation methodologies affect downstream processing. Ash content was one of the most significant issues for processing through harvesting and extraction equipment. Obviously, less is better; however, strategies to mitigate large quantities of ash are still required. Also, the addition of metals and high salt concentrations greatly affect the feed value and may require further cleaning of the bio-oil prior to conversion since these compounds affect catalyst life; hence, process integration is extremely important and crucial as the industry moves forward.

5. Conclusions and recommendations

Significant progress was made in all four major thrust areas shown in the Cultivation task framework (Fig. 1), thereby advancing toward the goal of cost-effective achievement of high annual biomass

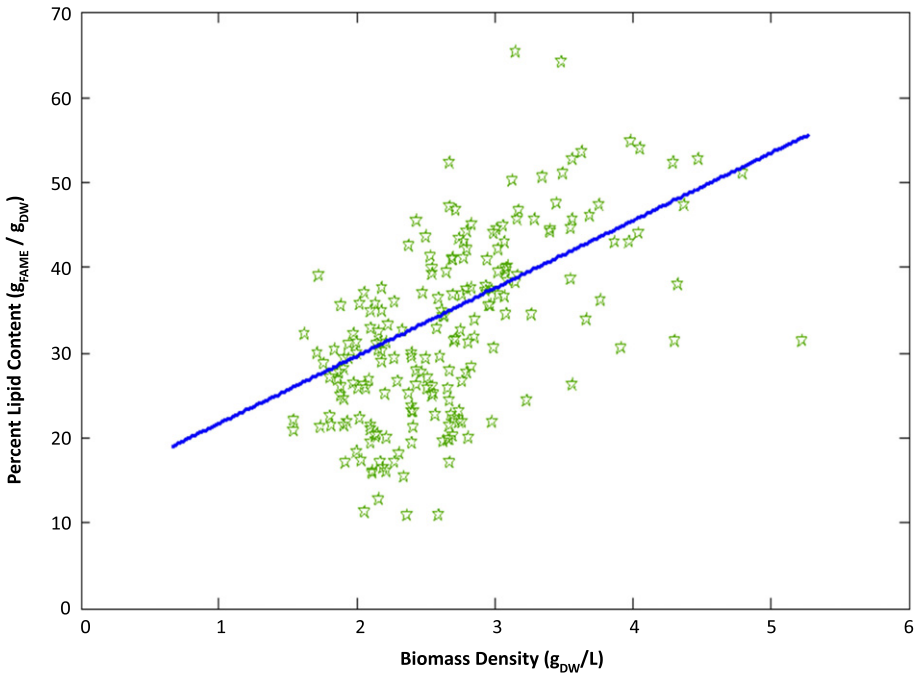

Fig. 23. Plot of *N. salina* biomass productivity for batches obtained over a period of three years with Solix PBR systems, shown as biomass density (x-axis) vs. percent lipid content (y-axis) for the batch. Blue line minimizing least square error shows, $R^2 = 0.37$.

productivities in robust outdoor pond and hybrid systems in an environmentally sustainable manner.

The key advance in our optimization and modeling was the development of a microalgae biomass growth model. This model utilizes experimentally determined species-specific parameters and was validated using outdoor pond cultivation data. The biomass growth model, in conjunction with the biomass assessment tool, enables the prediction of monthly and annual biomass productivities of a given strain in hypothetical outdoor pond cultures located across the United States. Furthermore, an indoor raceway pond with temperature control and LED lighting to simulate sunlight spectrum and intensity was designed and successfully operated under climate-simulated conditions. This system allows one to simulate the climate conditions of any geographical location and determine how algae will grow in a location of interest. This innovative modeling capability combined with the LED system can be used as a low-risk and cost-effective way of screening strains for their potential of exhibiting high biomass productivities in outdoor ponds, for finding the best match between a given strain and climate (i.e., geographic location), and for identifying the optimum pond operating conditions, thereby accelerating the large-scale cultivation of promising high-productivity strains while quickly eliminating suboptimal candidates.

Fig. 24. NAABB large scale algae production testbed facilities at A) Pecos, TX, and B) Cellana in Kona, HI.

Table 3
Biomass productivity for nine species tested at the NAABB testbed facility at Pecos, Texas

Strain name	Isolate ID	Suggested laboratory scale media	Maximum growth rate on suggested media ($g/m^2/d$)	Maximum growth rate on optimized media ($g/m^2/d$)
N. salina	CCMP1776	f/2 10×[a]	15.5	17.9
N. oculata	43-AM	f/2 10×[a]	16.7	18.6
Chlorella sorokiniana	DOE1412	BG-11	20.4	25.2
Desmodesmus sp.	DOE0043	BG-11	18.3	17.3
Chlorococcum sp.	DOE0202	BG-11	11.4	11
Chlamydomonadales sp.	DOE0101 (polleum)	BG-11	14.8	15.6
Scenedesmus obliquus	DOE0152	BG-11	16.9	14.8
Scenedesmus obliquus	EN-0004	BG-11	16.4	15.7
Chlorella sp.	DOE1095	BG-11	21.2	24

[a] f/2 10× refers to a 10-fold increase of $NaNO_3$ only relative to standard f/2 specifications.

It was demonstrated that microalgae can be successfully cultivated on municipal wastewater and produced water resulting from oil and gas exploration. Recycling water and media or the nutrients in waste biomass can further reduce the costs of inputs. A water management strategy that includes the use of low-cost impaired waters and recycle strategies for cultivation will be necessary for the anticipated large-scale production of microalgae biofuels.

With respect to the task of developing and operating innovative cultivation systems, the key advance was the modeling, testing, and design improvements of the ARID pond culturing system. This system provides improved temperature management, i.e., maintaining water temperatures within the optimum range for a given microalgae strain throughout the year. Modeling results and measurements demonstrated that water temperatures during the winter (in Arizona) remained 7–10 °C warmer than in conventional raceways. As a result of better temperature management, the ARID system was shown to have significantly higher annual biomass productivities compared to conventional raceways. In conjunction with engineered reductions in the energy use for pumping and mixing (i.e., use of a solar pumping system), cultivation in the ARID system was also shown to have significantly higher energy productivity (biomass produced per unit energy input) than conventional raceways. By extending the growing season and modulating temperatures, the impact of the ARID system could be profound by significantly increasing annual biomass productivities for any microalgae strain of choice. Collectively these improvements result in approximately an 18% reduction in cost of production of algal biomass in comparison to traditional open-pond systems with paddlewheels.

With respect to the task of large-pond cultivation and biomass production, a media cost reduction of 90% in chemicals was demonstrated over the use of typical (BG-11) laboratory media formulations. >1500 kg of biomass from eight different algal species was generated in the large-scale facilities at Pecos, Texas, and Kona, Hawaii for downstream processing and testing. By successfully demonstrating large-scale biomass production, significant progress was made toward the goal of commercial microalgae biofuels generation. A key development was the ability to move strains isolated from the prospecting effort from the laboratory to full production in outdoor pond systems, and subsequent downstream-processing of the strains to fuels and coproducts.

Future work should continue with the characterization of new strains using the LED climate-simulation system to optimize conditions for outdoor cultivation and to develop a crop-rotation strategy. Along with conducting long-term cultivation trials in established testbeds at different locations using NAABB strains and various pond designs (i.e., ARID system). The data from the above should be used to inform DOE harmonized models.

Scale up efforts should include cultivation in impaired waters with recycling, evaluating the water chemistry, along with quality, and impact on sustained productivity; and cultivation of GMO strains first in the LED climate-simulation system prior to outdoor trials. These efforts

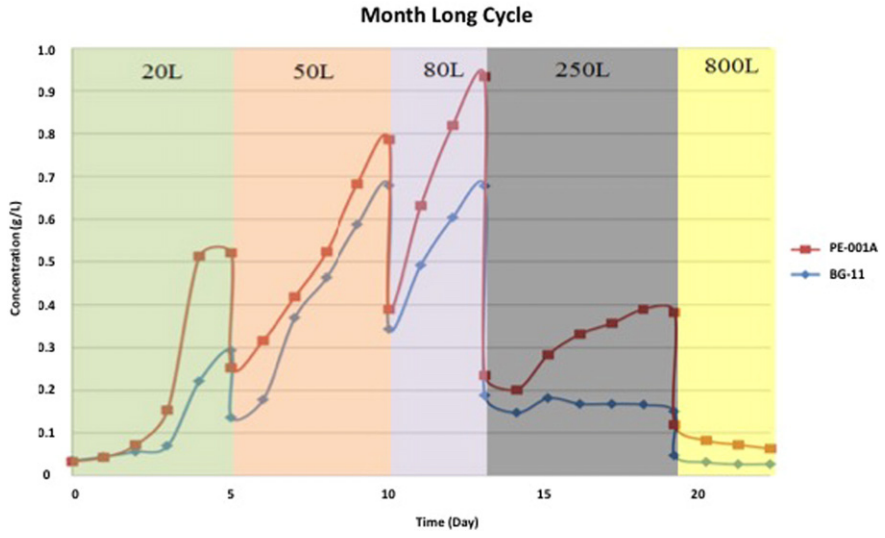

Fig. 25. Cultivation of *Chlorella sorokiniana* DOE1412 in traditional (blue) and optimized (red) media. The first 80 L of cultivation were done in PBRs, then the cultures were used as inoculum for an 800 L traditional raceway. Two media were compared. The cultures froze shortly after the volume was increased to 800 L.

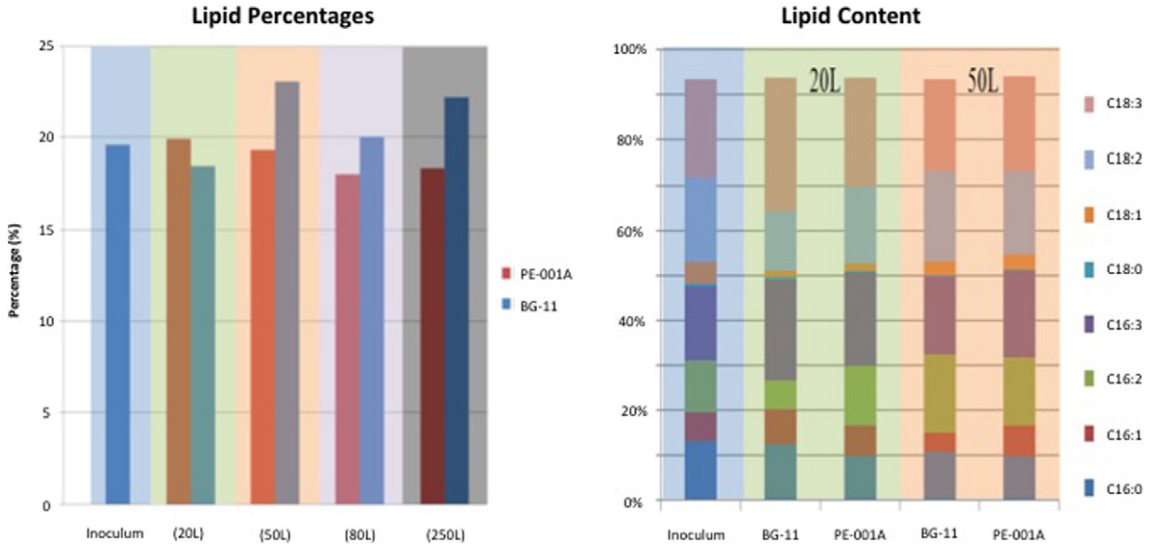

Fig. 26. Lipid profiles for *Chlorella sorokiniana* DOE1412 grown in PBRs and traditional raceways. Two different media were compared for cultivation.

Table 4
Summary of biomass produced for consortium on a dry weight basis.

Source	Strain	Harvested Biomass (kg)	Average Lipid (%)
2011 (Pecos)	*N. salina* (saltwater)	610.0	16.8
2012 (Pecos)	*Chlorella sorokiniana* DOE1412, *Chlorococcum* sp., *S. obliquus*, *Kirchenella* sp. (freshwater)	876.0	22.4
2013 (Cellana)	*N. oceanica* KA19 (saltwater)	50	35
	Pavlova pinguis C870(saltwater)	4	
	Tetraselmis sp. KA33 (saltwater)	50	< 15

should include mitigation strategies to minimize ash content and undesirable metals in algal biomass produced at large scale, and continue to demonstrate crop management strategies at scale. Finally, engineering optimization of pond design should continue in order to bring down capital and operating costs of large scale cultivation systems.

Acknowledgements

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