DOE Bioenergy Technologies Office (BETO) 2015 Project Peer Review

Realization of Algae Potential
Algae Biomass Yield Program

March 25, 2015
Technology Area Review

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New Mexico State University

AzCATI
Arizona Center for Algae Technology and Innovation

This presentation does not contain any proprietary, confidential, or otherwise restricted information

Goal Statement

- Develop an integrated process for producing 2,500 gallons of bio-fuel intermediate per acre per year through radical improvements in algal areal productivity and lipid content
- Successful demonstration will advance the DOE goal of producing 5,000 gallons per acre per year by 2022
- Benefits to the U.S./BETO include
  - Increasing the viability and deployment of renewable energy technologies
  - Spurring the creation of a viable domestic bio-industry
  - Evaluation of engineered productivity enhancements in elite strain versus an extremophile strain chosen for crop rotation purposes
  - Evaluation of Sequential HTL options for nutrient recycling

Quad Chart Overview

Timeline

- March 1, 2014
- Sept 30, 2016
- % Complete 40% (time)

Barriers

- Barriers addressed
  - Feedstock supply
  - Conversion R&D
  - Sustainability

Budget

	Total Costs FY14-15	Costs as of 12/31/14	FY 15 Costs	Total Planned Funding (FY 15-Project End Date (Non FFRDC)
DOE Funded		$543K		$4.265M
NMSU/ASU Pan Pacific NMC WSU		$99.9K $83.2K $24.7K $0		

*If there are multiple cost-share partners, separate rows should be used.

Partners

- Partners
  - New Mexico State, Washington State and Arizona State Universities
  - Pan Pacific, Algenol Biofuels
  - New Mexico Consortium
  - National Labs: Argonne, Los Alamos, Pacific Northwest
  - UOP-Honeywell (bio-crude feedstock analysis)

1 - Project Overview

- Genetic improvements in mixotrophic algae - LANL/NMC
  - 40% enhancement from chlorophyll antenna size optimization
  - 3 fold improvement in oil content from cellobiose utilization
- Horizontal photobioreactor cultivation - NMSU/ASU
  - EPA-exempt (40 CFR Part 725) outdoor testing of improved strains
  - Crop rotation for biological solution to temperature management
  - Calculate raceway productivity from climate simulation modeling
- Sequential Hydrothermal Liquefaction - WSU/NMSU/ASU
  - Enables C, N and P recycle to cultivation
  - Cleaner fuel (less N) with bio-char used for HTL heat integration
- Systems Modeling - Pan Pacific, Argonne, Algenol
  - LCA and TEA process models for iterative optimization of cultivation and pre-processing with mass and energy balance data
  - Modeling enables go/no-go decision for Phase 2

2 – Approach (Technical)

- *Key Challenges*
  - ✓ *Strain stability and thermo-tolerance via strain rotation*
  - ✓ *Cultivation mixing system, CO_2 delivery and energy consumption*
  - ✓ *Achieve harvest density of 2-3 g/L to lower harvesting costs*
  - ✓ *Heat integration and intermediate separations for SEQ-HTL*
  - ✓ *C, N and P recycle from HTL*

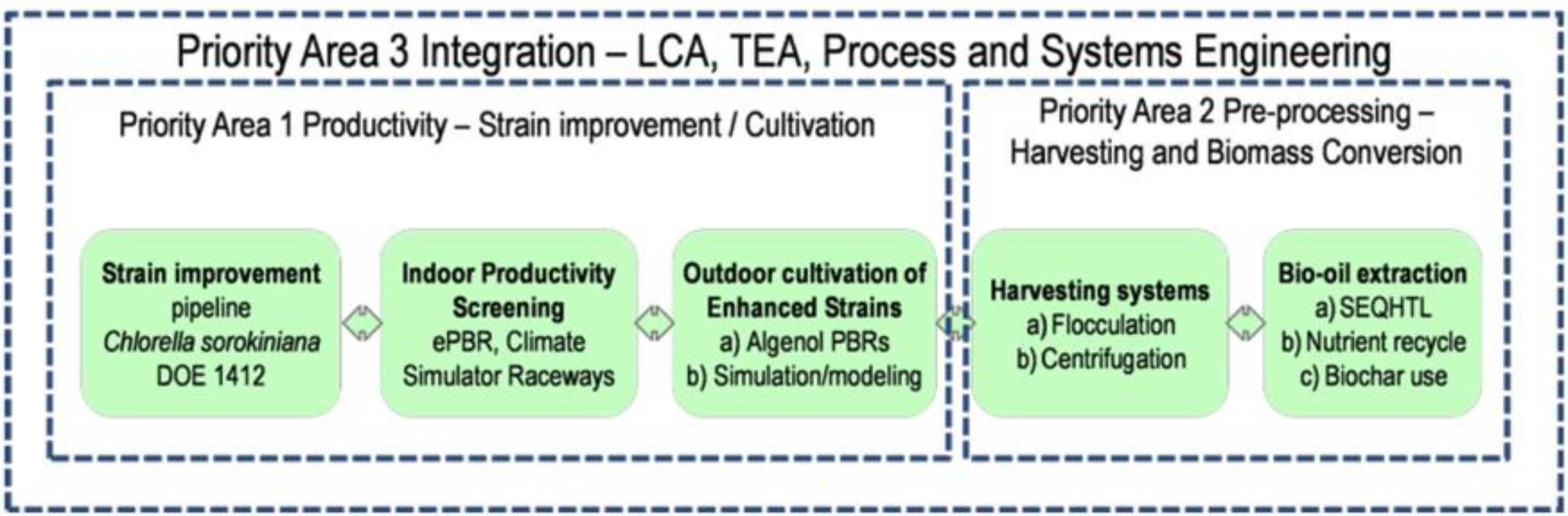

Figure 1. Schematic of the REAP research program. Integration activities define, track, and measure R&D in cultivation (Priority Area 1) and Pre-processing (Priority Area 2)

2 – Approach (Management)

- *Team includes all expertise required to execute the program*
  - ✓ *LANL/New Mexico Consortium – algal strain improvement*
  - ✓ *NMSU/ASU – outdoor cultivation and HTL testing*
  - ✓ *WSU – sequential hydrothermal liquefaction*
  - ✓ *PNNL – quantitative growth modeling*
  - ✓ *ANL – national leader in LCA*
  - ✓ *Pan Pacific – ASPEN modeling/TEA*
- *Two face-to-face meetings (Jan. ‘14 & ’15)*
- *Monthly team presentations via Adobe Connect*
- *PI Visits to WSU (July ‘14) & LANL/NMC (Feb ‘15)*

Aspen model of REAP unit operations

Aspen model of PBR cultivation for REAP process has successfully been constructed in five stages:

- Stage 1- Pre-Reactor Components
- Stage 2- Algal Growth
- Stage 3- Downstream Processing
- Stage 4- Model of SEQHTL unit operation
- Stage 5- Linkage of stages for complete process model

Aspen model of PBR cultivation

Outcomes:

- Integrated end-to-end Aspen system model for REAP process including CO_2 absorption, simulations of algal growth reactors for PBR, downstream processing and SEQHTL processing.

- Initial fully converged Mass and Energy balances for integrated REAP process.

- Initial costing of process economics using Aspen database.

3 – Technical Accomplishments/ Progress/Results

- *Strain improvement - Chlorella*
- *Cultivation trials, PBR mixing systems and CO_2 delivery*
- *Initial Sequential Hydrothermal Liquefaction Results*
- *TEA and LCA modeling*

Strain Selection

One elite strain, one extremophile

1. *C. sorokiniana* strain selection
   - UTEX 1230, 1228 and NAABB 1412
   - Genomes are only ~5% conserved
   - Initial genetic toolkit developed for 1230
   - Thermo-tolerance 1230 > 1412 > 1228
   - Productivity 1412 > 1230
   - Final selection for genetic enhancements is 1412
2. *G. sulphuraria* - summer season strain is a low-pH extremophile, tolerant to 56^{o}C, low lipid/high carbohydrate, versatile heterotroph
3. Strain rotation for temperature management

Objectives, Outcomes and Impacts by Priority Area

REAP pairs this cultivation approach with highly synergistic sequential hydrothermal extraction technology (SEQHTL). This is a two-stage process that: i) *extracts* non-lipid fractions for recycling to oil production; ii) *converts* the remaining lipid-enriched biomass directly to cleaner bio-fuel intermediate with lower nitrogen content; and iii) *eliminates* the need for unit operations involving drying or cellular disruption. Outdoor testing of enhanced strains in Algenol's inexpensive and scalable PBRs provides

Improved Algal Biomass Productivity – Priority Area 1		
Objectives	**Outcomes**	**Impacts**
1. Genetic enhancement of mixotrophic *Chlorella sorokiniana*	40% increase in biomass productivity and 300% increase in oil content	2500 gal/acre-yr by end of 2014 3500 gal/acre-yr by end of 2015
2. Enclosed PBR Cultivation Systems for EPA-exempt Outdoor Testing Enhanced Strains; Raceway Productivity Derived from Climate-Simulation Models	Outdoor PBR systems for >3 gAFDW per Liter autotrophic density and 6 gAFDW per Liter mixotrophic density >50% oil content by mass	Productivity of Greater than 25 g/m2-day at Lower CAPEX and OPEX vs. Harmonization Model (*1*)
Improved Pre-processing Technologies – Priority Area 2		
Objectives	**Outcomes**	**Impacts**
3. Innovative Harvesting and Dewatering Systems	Harvested algae resulting in 5% solids; dewatering resulting in 30% solids	Energy Expended Less than 10% of Energy Content direct input to SEQHTL
4. Sequential Hydrothermal Extraction Technologies to Produce Bio-Fuel Intermediate with C, N and P Recycle to Cultivation	Continuous SEQHTL process design balancing High Efficiency Bio-Oil Extraction and Bio-char Production for Heating Energy Requirement	Cleaner Bio-Fuel Intermediate qualified for UOP Fuel Upgrade Specifications
Technical Advances to Enable the Integration of the Algal Biomass Unit Operations – Priority Area 3		
Objectives	**Outcomes**	**Impacts**
5. Physical Integration and Systems Modeling	Iteratively Optimized Unit Operations for Algal Cultivation and Preprocessing	Optimum End-To-End Productivity with Mass and Energy Balance Data
6. Develop LCA, TEA and Process Model of Integrated Process	GREET, Techno-Economic and Aspen Models Enabling go/no-go Decision for Phase 2	Go Decision Triggers Engineering and Design for 1 Acre Integrated Pilot (FEL-2)

containment in line with standards administered by EPA TSCA (40 CFR Part 725) to allow outdoor cultivation of engineered strains. The PBR approach also allows higher harvest concentrations reducing energy demand. Biochar derived from liquefaction provides the heating source for SEQHTL, thus REAP

Progress towards antenna size optimization
Not quite there yet

Strain	Colonies screened	Transgenics (Chl a/b ratios)
Cs-1228 Chl a/b ratio (2.0)	300	2.0
Cs-1230 Chl a/b ratio (2.3)	350	3.0
Cs-1412 Chl a/b ratio (2.0)	400	3.4 (optimum is 5)

The failure to achieve higher Chl a/b ratios in Cs-1228 and Cs-1412 transgenics may reflect DNA sequence dissimilarities with the Cs-1230 CAO RNAi gene (88% identity) or gene copy number (2X)

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Query  428   GATGTGCCGGTGGAGCACGGC  448
             |||||||| ||||||||||||
Sbjct  1222  GATGTGCCAGTGGAGCACGGC  1242
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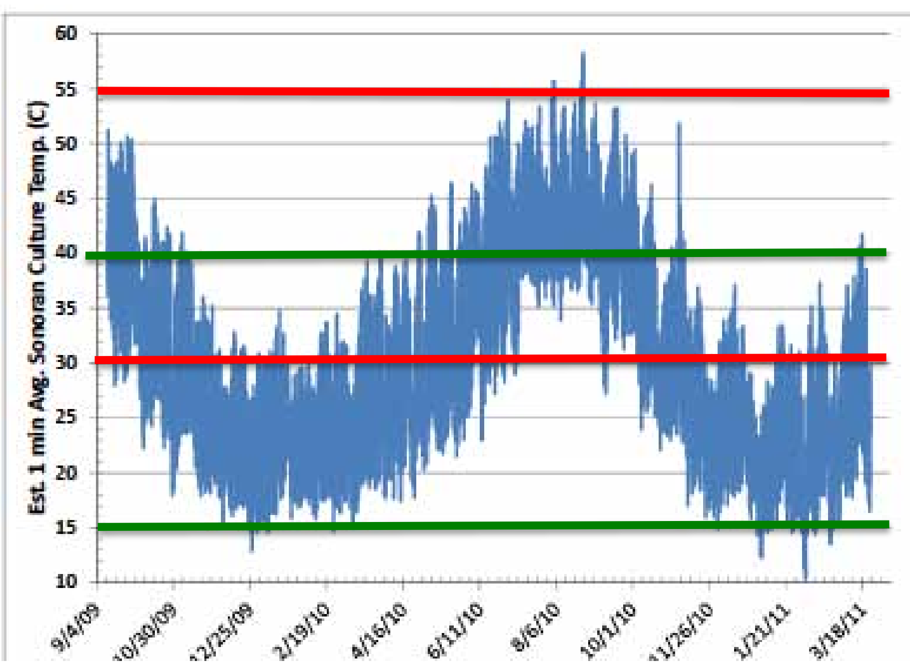
Thermo-tolerance Issues

Temperature data for 20 cm depth horizontal PBR.

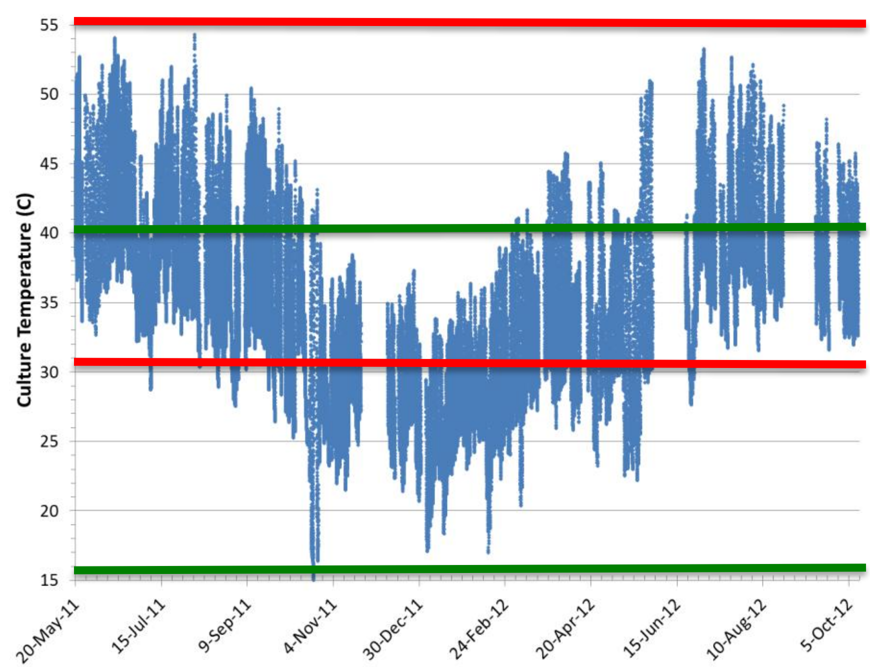
The area between the red lines indicates acceptable temperature range for *Galdieria*

The area between the green lines shows temperature range in which more temperate algae like *Chlorella* would grow.

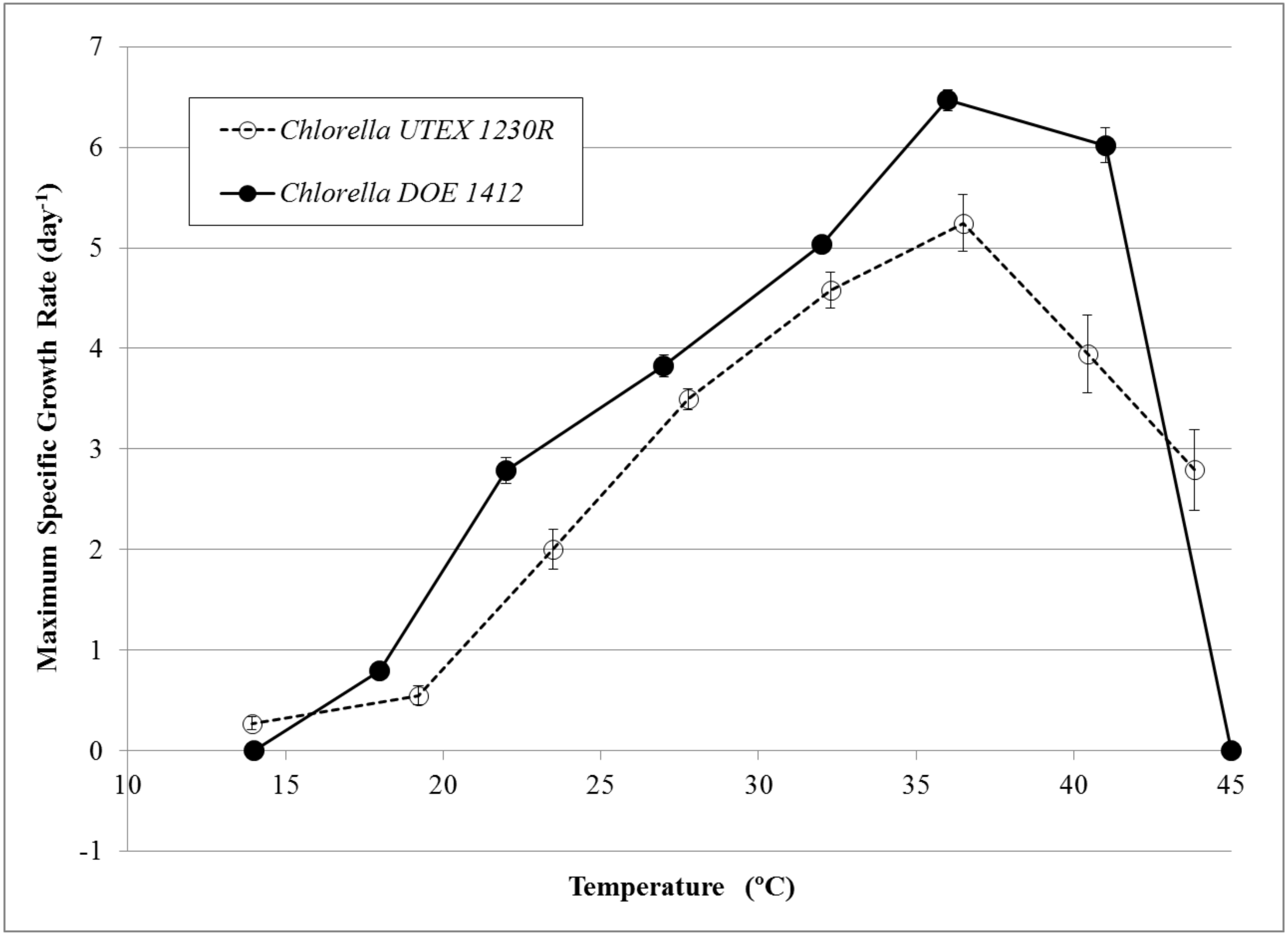
Sonora, Mexico



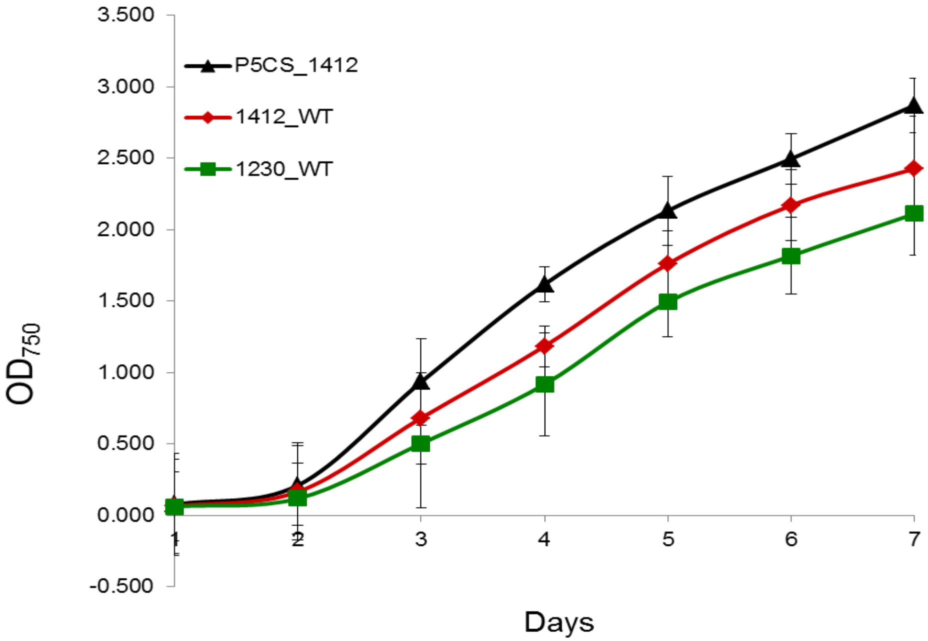
Fort Myers, FL

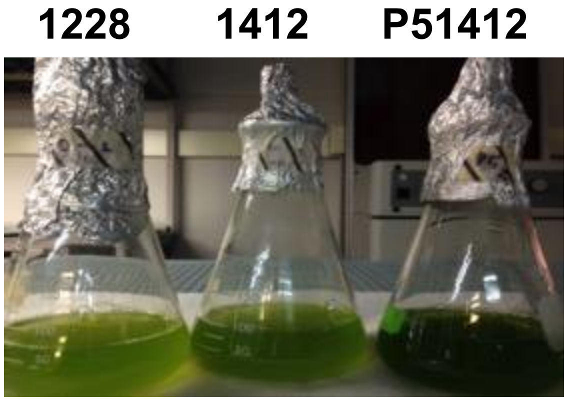


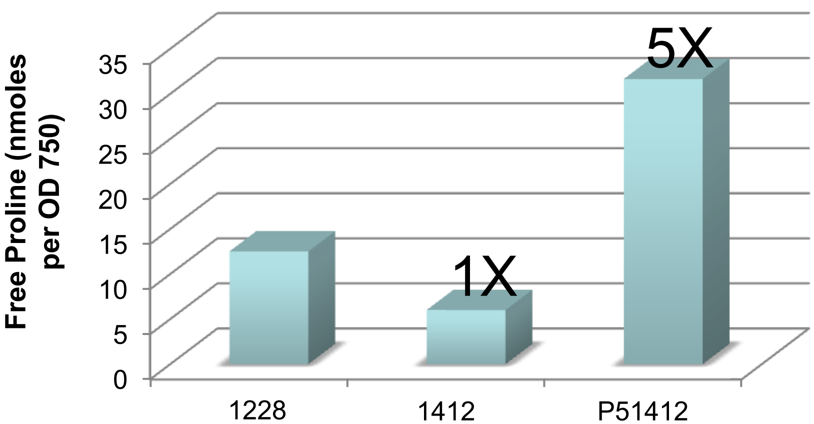
Maximum Specific Growth Rate as a Function of Temperature: *Chlorella* UTEX 1230R vs. DOE 1412



Enhanced heat tolerance in *Chlorella sorokiniana – 1412* mutants over-accumulating (5X) proline







Growth at 40 °C

- 1412 over-expressing 1-pyroline-5-carboxylate synthase gene (P5CS)
- P5CS transgenics have 20% greater productivity than the wild-type parent 1412.

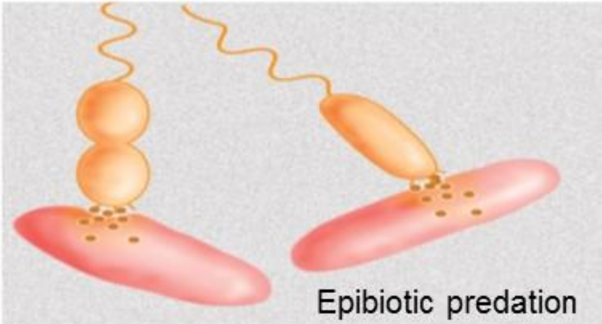
Angela Pedroso Tonon,
Los Alamos National Laboratory

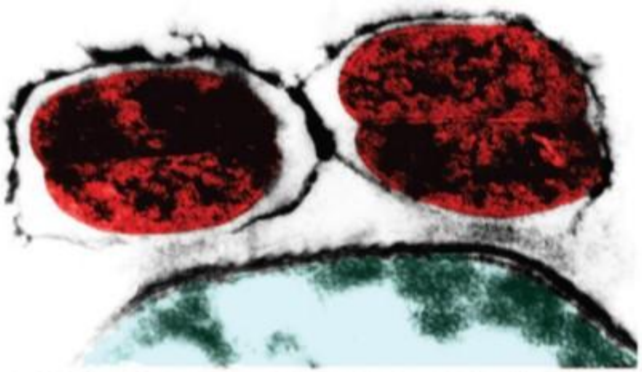
Vampirovibrio chlorellavorous causing *C. sorokiniana* crashes?

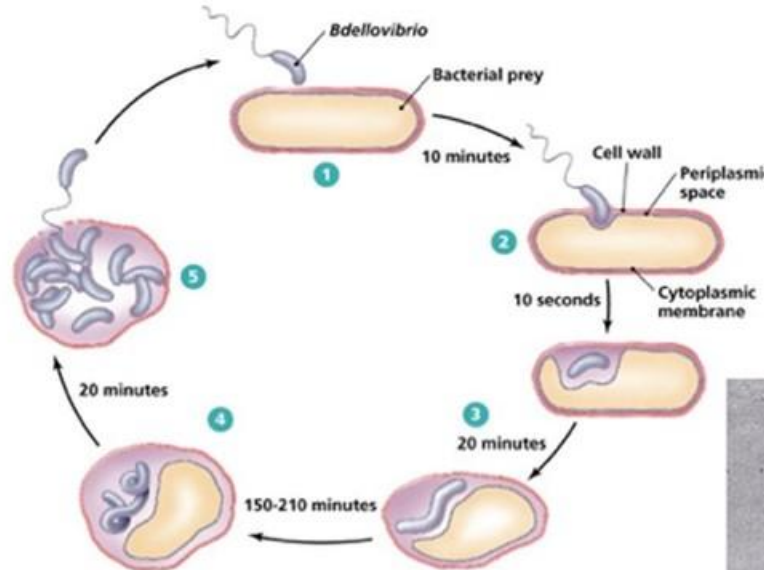
Results of PCR tests by Judy Brown @ Univ. Arizona

Case# - Sample#	Algal Strain	Sample Identification and specification (provided by sender)	Condition of Sample	Detection of *V.chlorellavorous* (positive/negative)	Restriction Digestion EcoRI, *V. chlorellavorous*-specific
14.06-1a	DOE1228 & Coelestrella	Sample Set 1, tube 1: Culture is 1228+Coelestrella grown in our hoop-house	Frozen Liquid Culture	Positive	Pooled and validated
14.06-1b	DOE1228 & Coelestrella	Sample Set 1, tube 2: Culture is 1228+Coelestrella grown in our hoop-house	Frozen Liquid Cultu	Positive	Pooled and validated
14.06-1c	DOE1228 & Coelestrella	Sample Set 1, tube 3: Culture is 1228+Coelestrella grown in our hoop-house	Frozen Liquid Cultu	Positive	Validated
14.06-2a	DOE 1412	Sample Set 2, tube 1: Culture is 1412 grown in outdoor open raceways from 5/27/14-	Frozen Liquid Cultu	Positive	Pooled and validated
14.06-2b	DOE 1412	Sample Set 2, tube 2: Culture is 1412 grown in outdoor open raceways from 5/27/14-	Frozen Liquid Cultu	Negative	N/A
14.06-2c	DOE 1412	Sample Set 2, tube 3: Culture is 1412 grown in outdoor open raceways from 5/27/14-	Frozen Liquid Cultu	Positive	Pooled and validated
14.06-3	DOE 1228	Sample Set 3: Culture 1228 in hybrid system floating water basin 8/14/14-8/29/14 (one	Frozen Liquid Cultu	Positive	Pooled and validated
14.06-4	DOE 1228	Sample Set 4: Culture 1228 grown in hybrid system floating water basin 9/4/14-9/24/14	Frozen Liquid Cultu	Positive	Pooled and validated
14.06-5a	DOE 1412	Sample Set 5, tube 1: Culture is 1412.1grown in outdoor open raceways	Frozen Liquid Cultu	Positive	Pooled and validated
14.06-5b	DOE 1412	Sample Set 5, tube 2: Culture is 1412.1grown in outdoor open raceways	Frozen Liquid Cultu	Positive	Pooled and validated

Predatory Bacteria: *Bdellovibrionaceae*

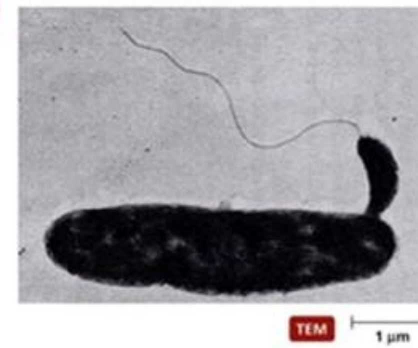






Epibiotic and periplasmic predation

Bdellovibrio

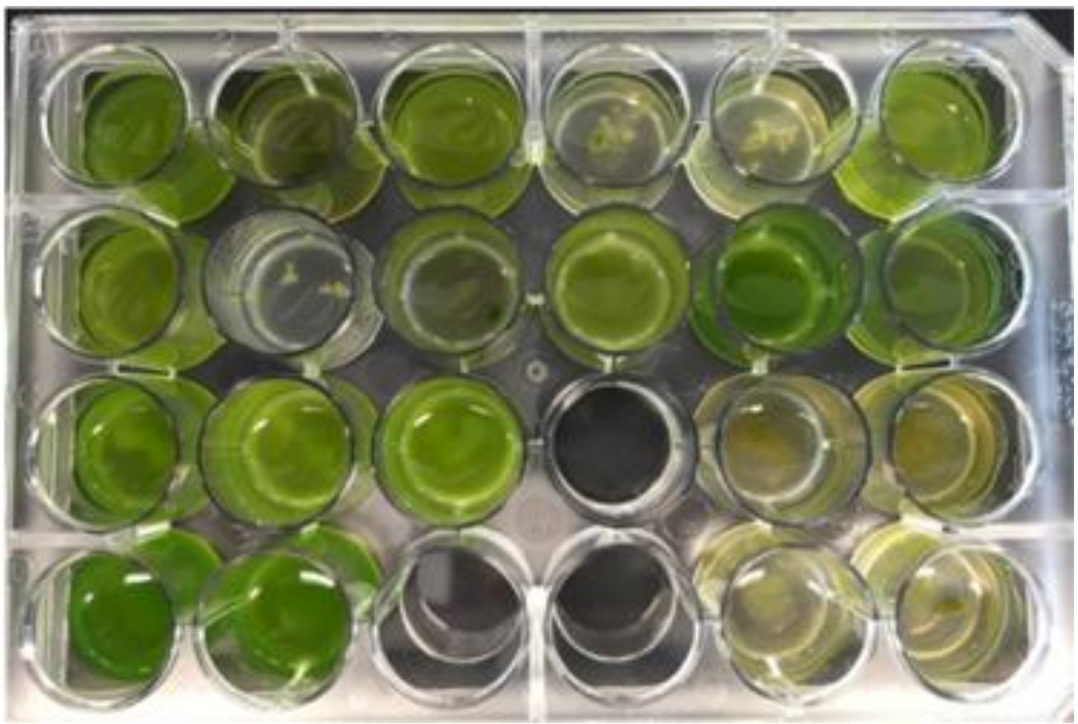


Vampirovibrio chlorellavorus:

- Obligate biotroph on Chlorella species
- Gram-negative bacteria
- 0.3 µm diameter-larger coccoid forms (0.6 µm) described (Coder and Star 1978)
- Single, polar non-sheathed flagellum for motility

Jurkevitch 2007; Guerrero et al. 1986;

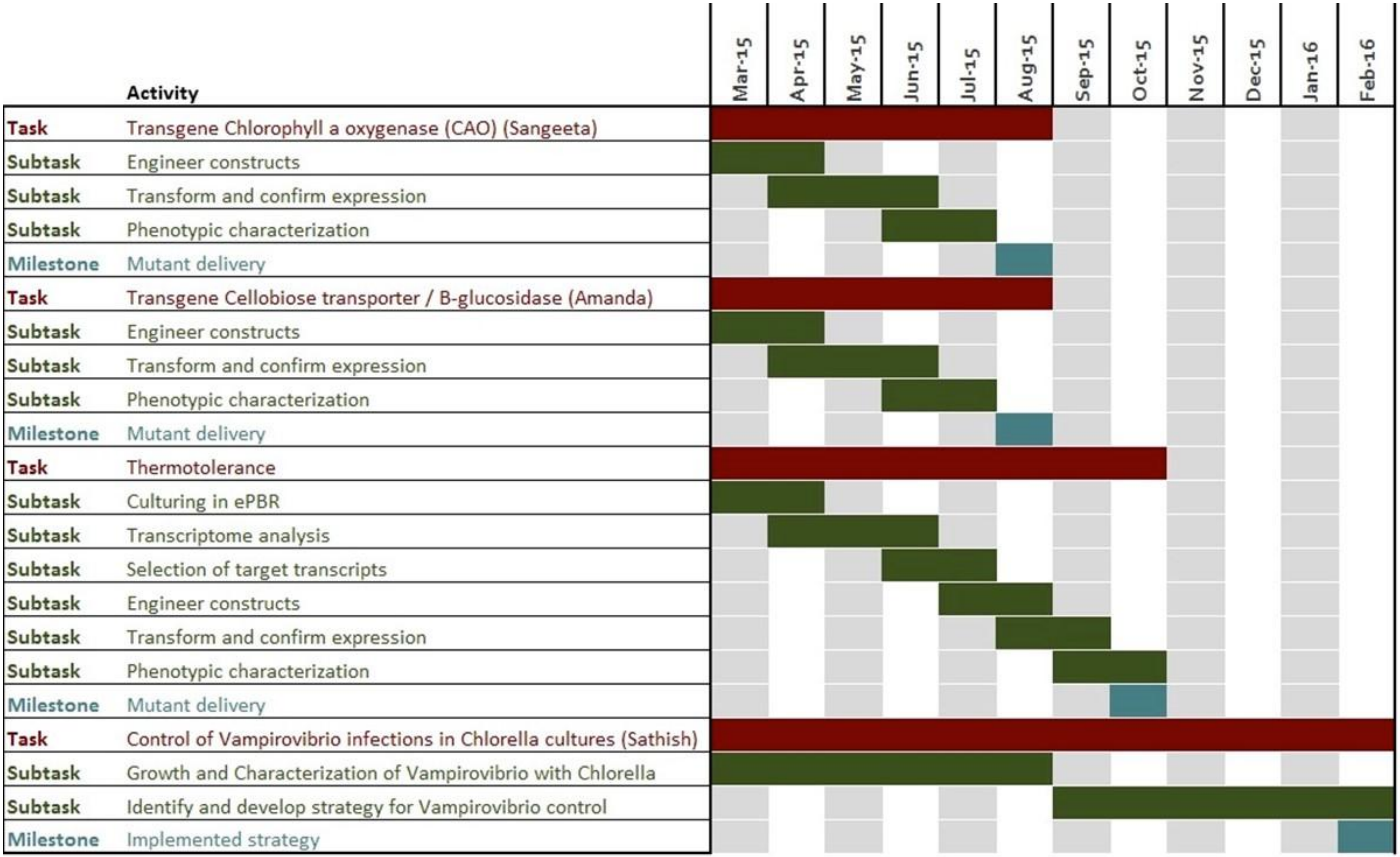
Anti-Microbial Peptides tested for inhibition of *Vampirovibrio* and



Dr. Satish Rajamani

A1- CecropinA
A2- CecropinB
A3- Maginin2
A4- W16-CA
A5- LactoferricinB
A6- MsrA
B1- Dermaseptin
B2- Mellitin
B3- TemporinA
B4- TemporinF
B5-TemporinL
B6- TemporinG
C1- Piscidin-1
C2- Piscidin-2
C3- Piscidin-3
C4- Empty well
C5- Kanamycin (50ug/mL)
C6- Carbenicillin (125mg/mL)
D1- Uninfected Cs1412
D2- Uninfected Cs1412
D3- Empty well
D4- Empty well
D5- Cs1412 Infected with Vampiro
D6- Cs1412 Infected with Vampiro

Genetic Enhancements Schedule



Cultivation System Overview

- Closed Plastic Photobioreactors
  - Mixing by paddlewheel
  - Mixing by hydraulic driven waterfoil (Algenol)
  - Hybrid tubular plastic with waterfoil

Horizontal Photobioreactors

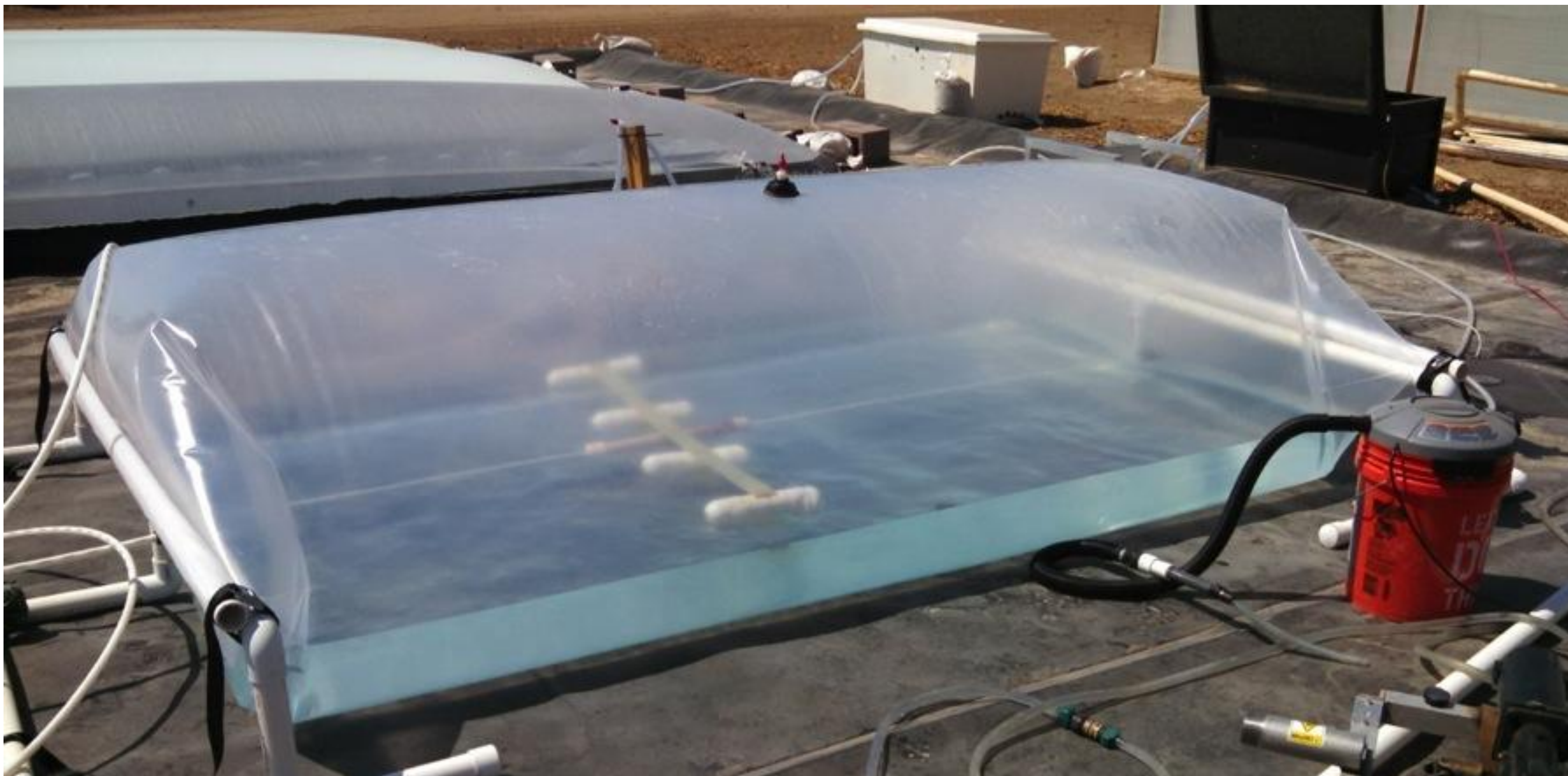
Enclosed raceway design, passive solar heating, **paddlewheel mixing**, expandable from 4 to >75 linear feet

100 L/m^2 at 10 cm depth

200 L/m^2 at 20 cm depth

Hybrid System

- Tubular Plastic Bag with hydraulic waterfoil mixing system
- Easier to set up, avoids weakness at bulkhead fitting at ends of the Algenol bags (leak prevention)
- Floated on a water basin to provide temperature control for summer growth of *C. sorokiniana*



Rapid Molecular Diagnostics for Contaminant Detection

List of PCR primers:

			Chlorophyta			Rhodophyta			
			Chlorellaceae			Cyanidiaceae		Galdieriaceae	
		~Product	*Coelastrella*	*Chlorella sorokiniana*		*C. caldarium/C. merolae*		*G. sulphuraria*	
Marker	Primer set	size (bp)	NMSU1	1412	1230	5510	MS1-YNP	5572	5587.1
Rubisco Large subunit	RbcL F/R	550	●	●	●	●	●	●	●
Euk. 18S rDNA	Cdm F/R	750	●	●	●	●	●	●	●
Algal 18S rDNA ITS1	ITS 1F/2R	350-400	●	●	●	●	●	●	●
Chlorella specific 18S rDNA	ChspeR/TresR	195	●	●	●	▬	▬	▬	▬
18S rDNA-ITS1	CdmF/ITS2R	1300	●	●	●	▬	▬	▬	▬
Chlorophyte plastid genes	UCP5 F/R	1300	●	●	●	●	●	▬	▬
Rhodophyte plastid genes	URP1 F/R	440-500	●	▬	▬	●	●	●	●
C. Merolae URA5.3	URA5F/6R	725	▬	▬	▬	●	●	●	●
Cox2-Cox3 intergenic spacer	Cox2f/3R	300-500	●	●	●	●	●	●	●
Simple Sequence Repeats	SSR9 F/R	573	▬	▬	▬	▬	●	▬	▬

● PCR product amplified

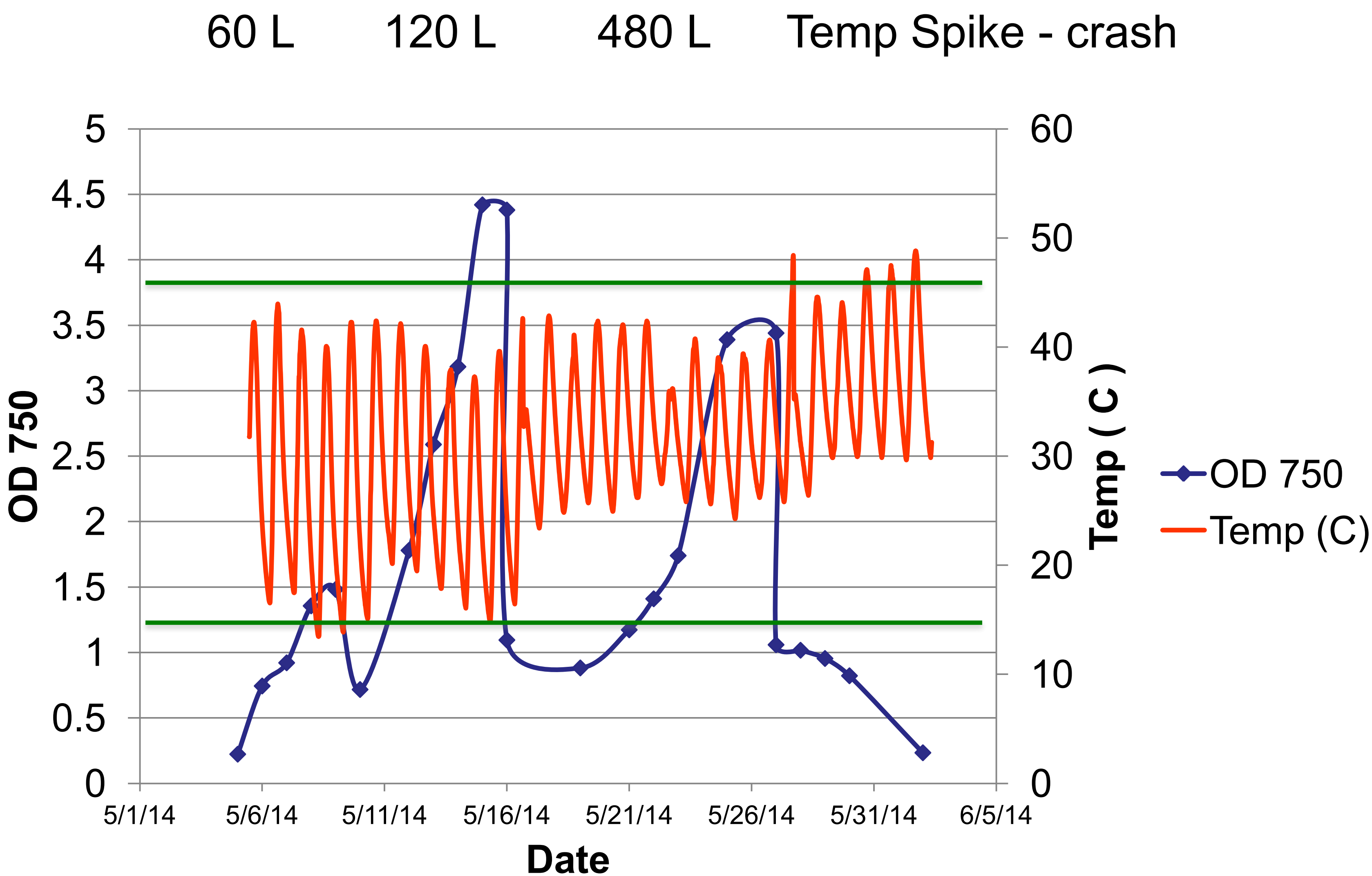
▬ No PCR product amplified

Black box: universal (euk/algae) primer set; green box: green algae specific primer set; and red box: red algae specific primer set.

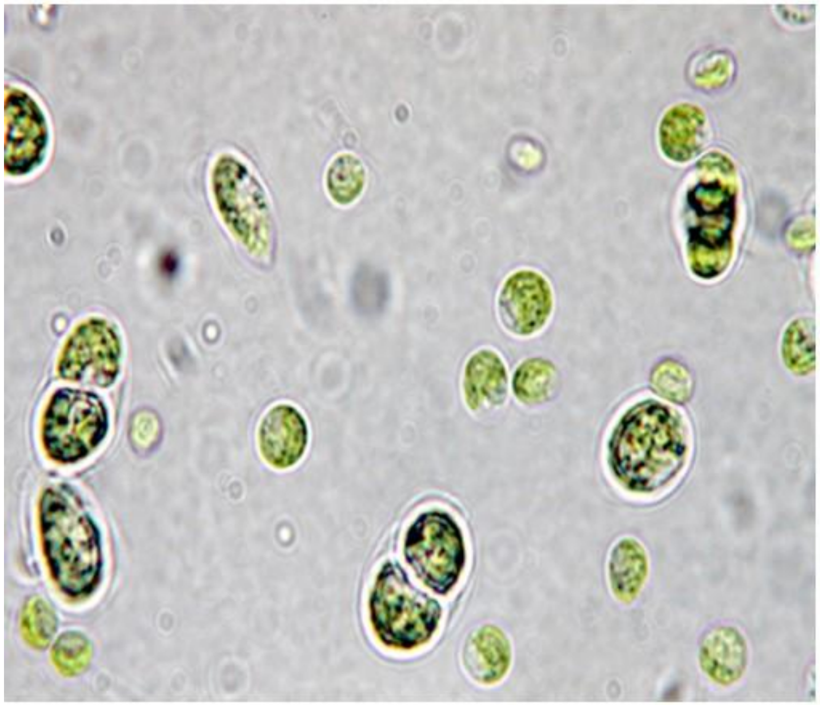
Chlorella sorokiniana NAABB 1412.1

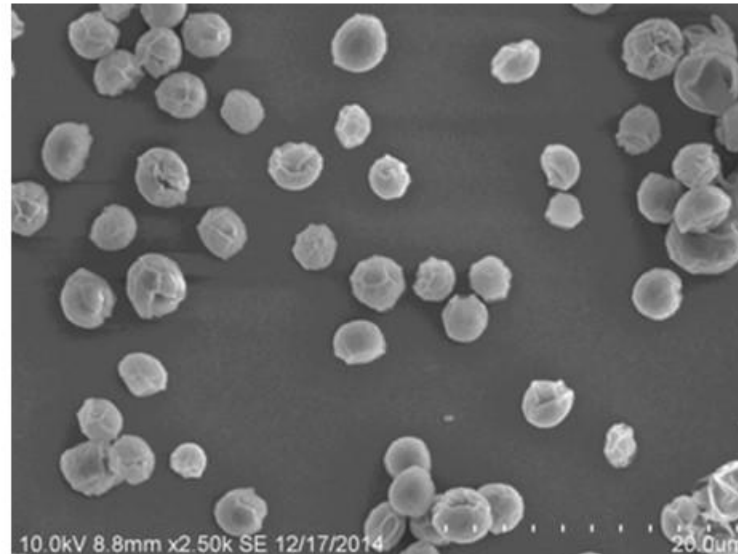
Peak Growth in enclosed horizontal PBR at 30 g/m2/day

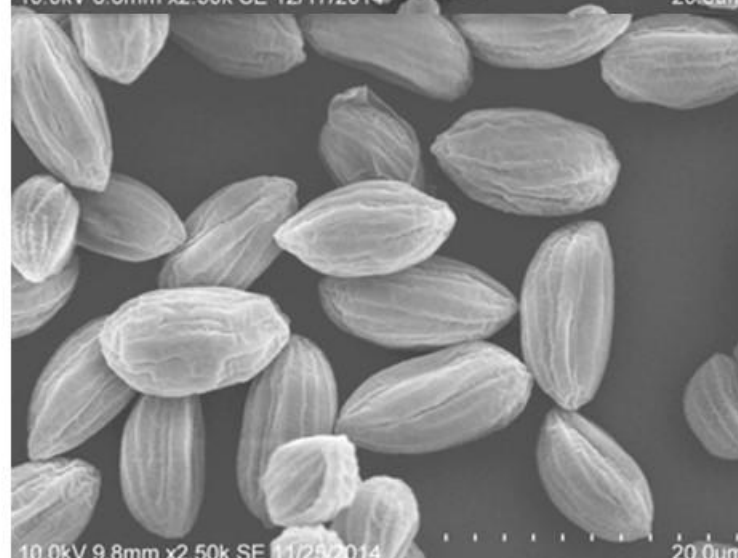
May 2014 in Las Cruces, NM



Biological Risk #2
Competitor Algae - Coelastrella



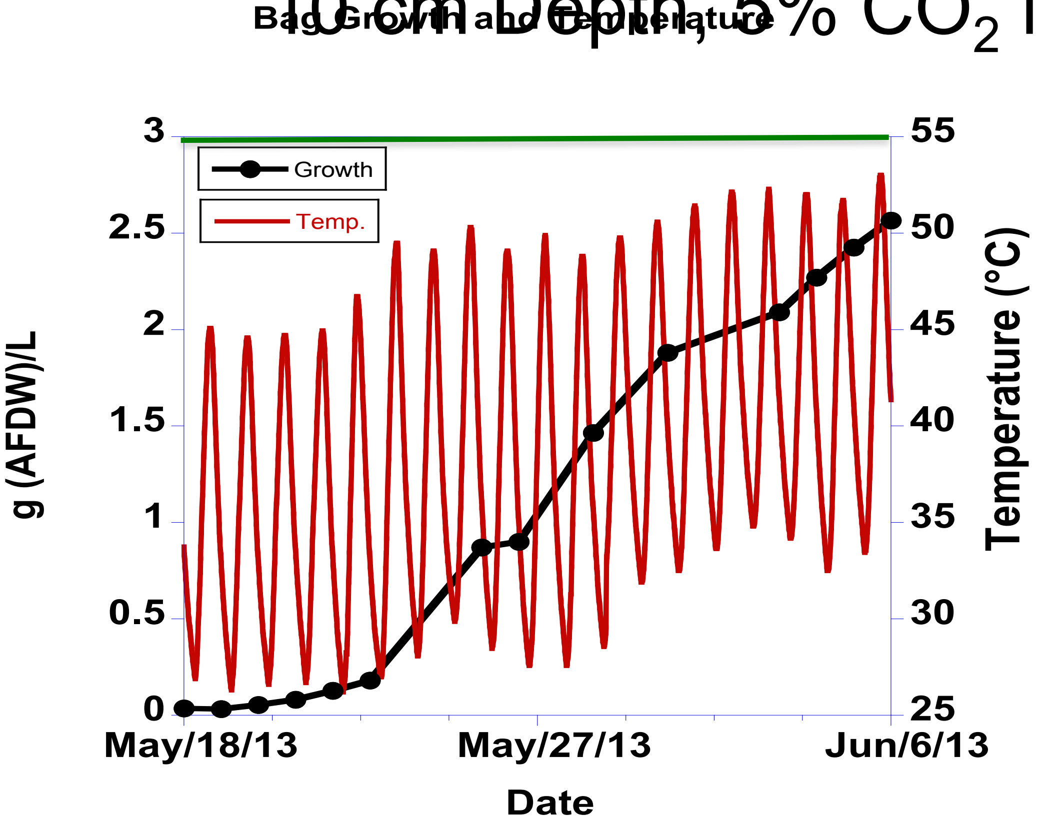




Electron micrograms - Peter Cooke

Strain isolation and characterization – Mark Seger

2013 Outdoor Growth of *Galdieria sulphuraria CCMEE 5587.1*** in an Enclosed Horizonal PBR 10 cm Depth, 5% CO_2 in Air, @ 2 L/min

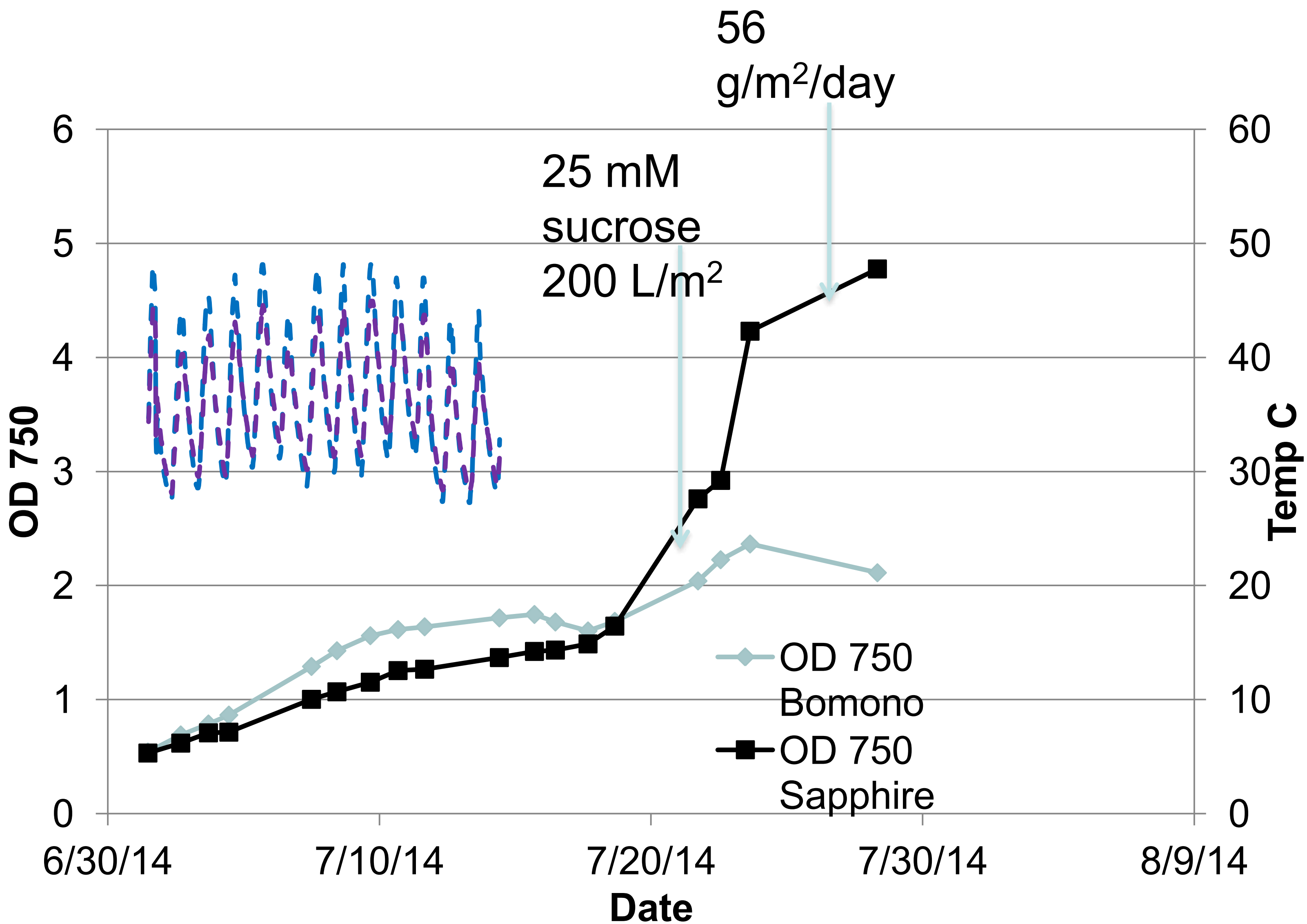


y = 0.165x + 0.159
R^2 = 0.978

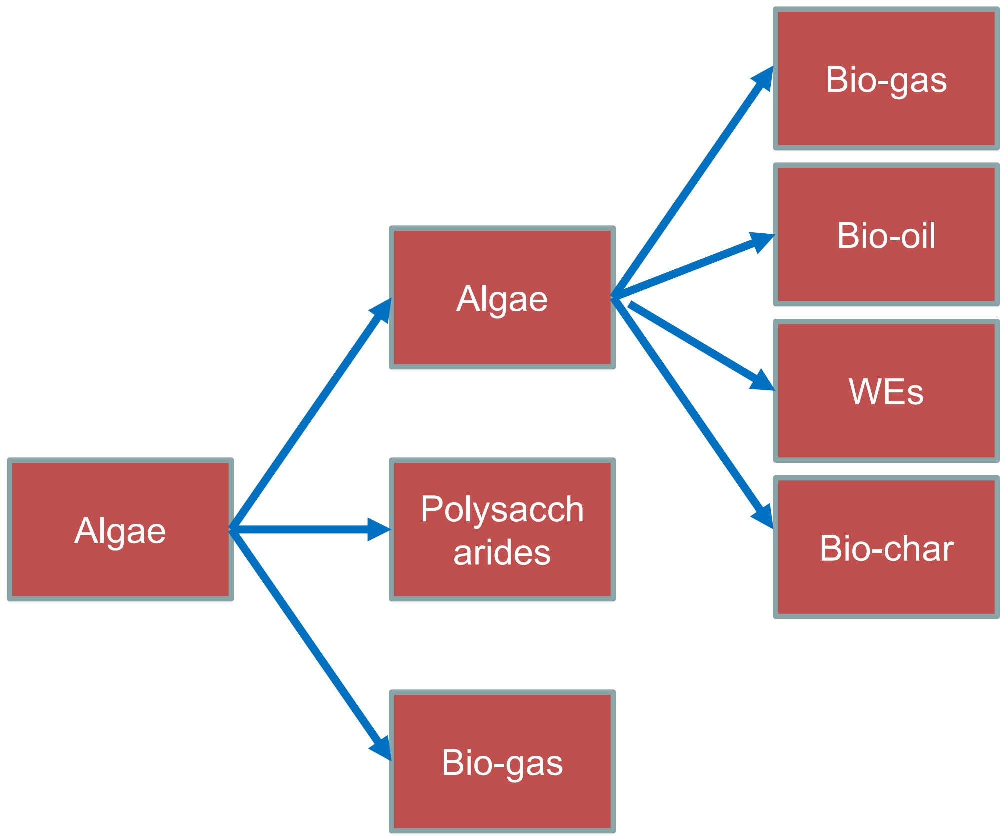
PEAK Growth
0.165 grams/L/day
16.5 grams/m²/day

** Retrospective DNA analysis documented this was actually a mixture of *G. sulphuraria* and *C. merolae*

Effect of Sucrose on Outdoor Growth Rate of *Galdieria sulphuraria*



SeqHTL-Reaction Network



Stage 1
2160C

Stage
240C

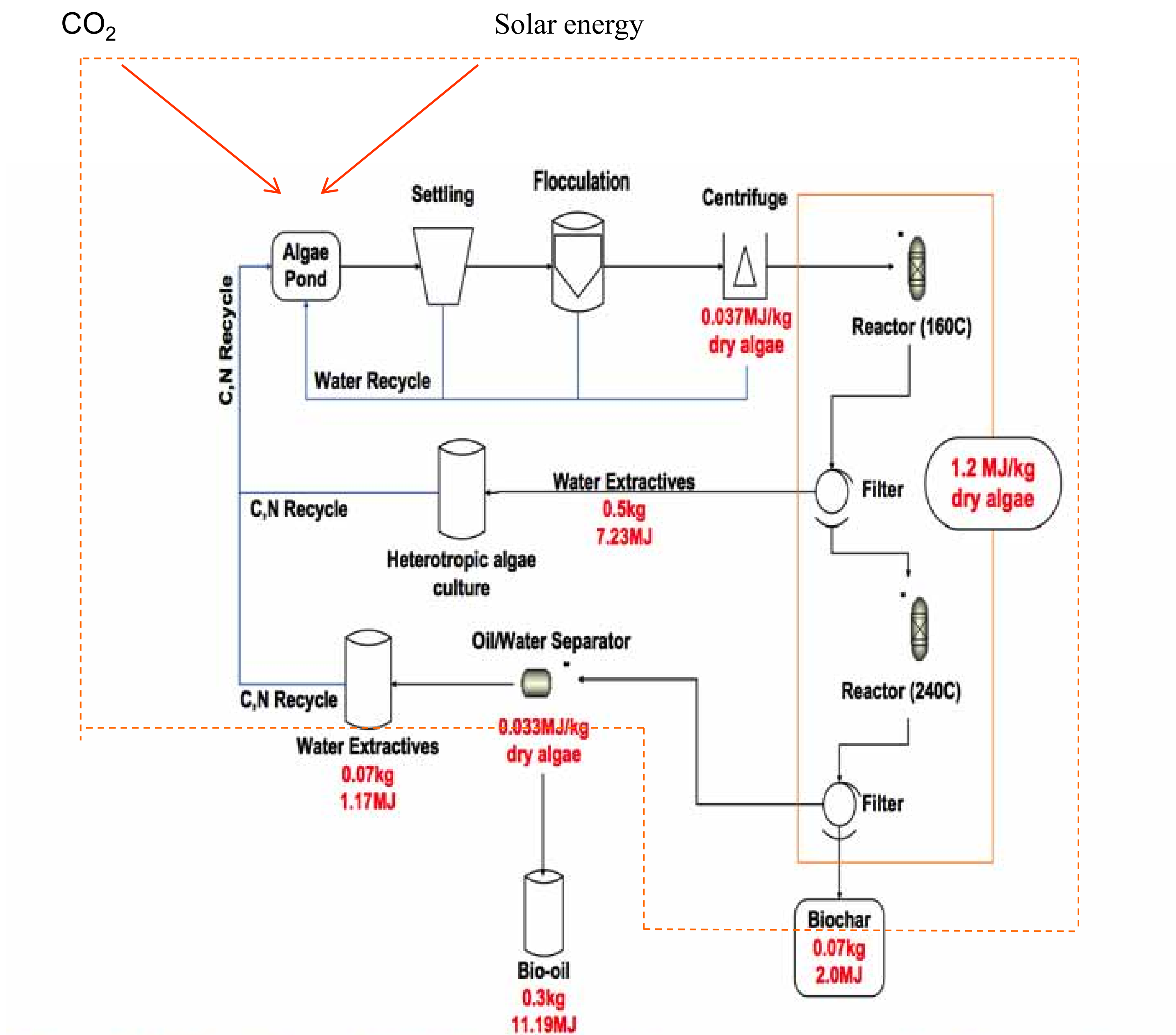
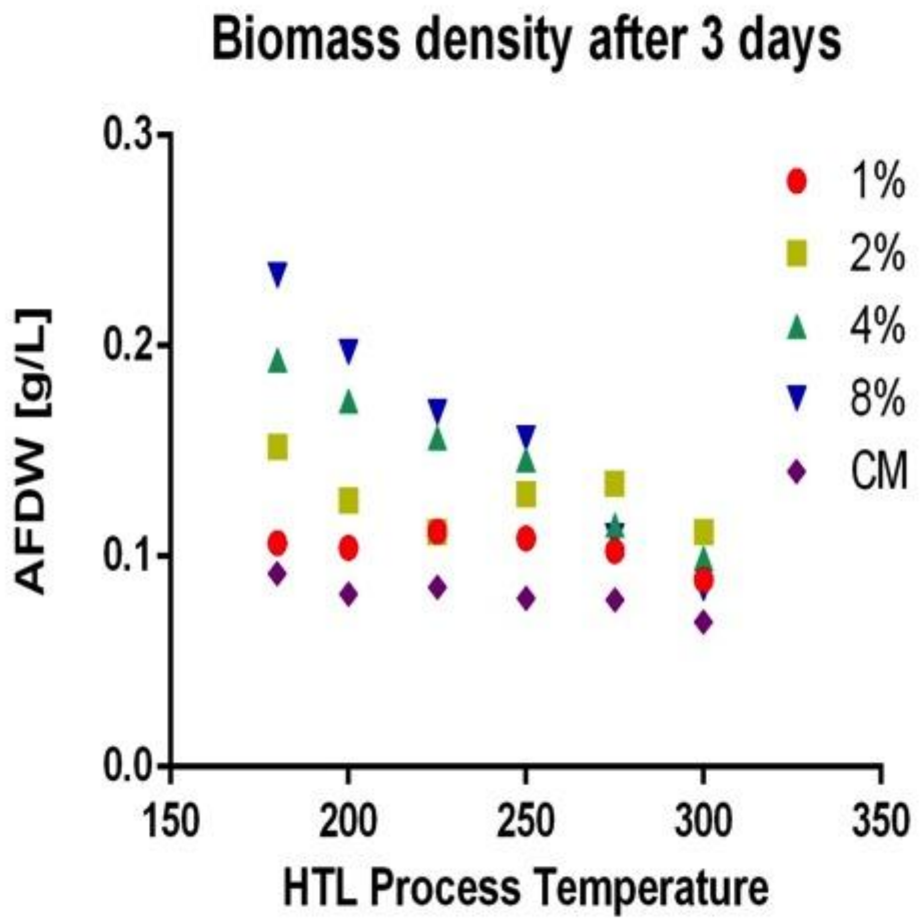
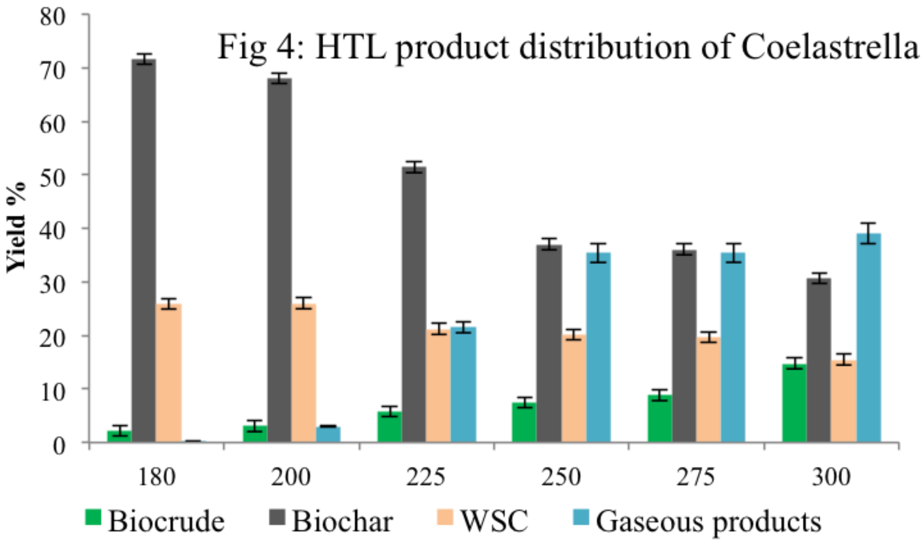


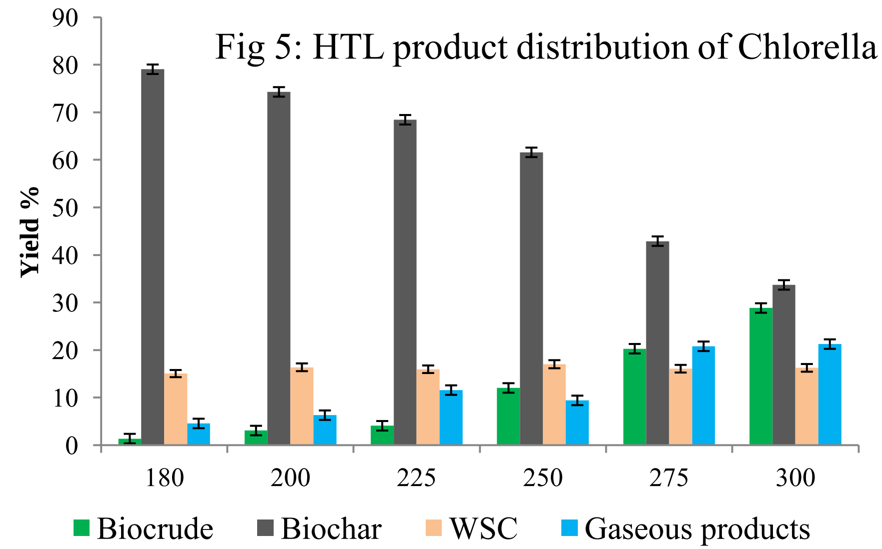
Figure 5. REAP wet Sequential Hydrothermal Liquefaction process process flow diagram with energ budget. Energy values are normalized to algal ash free dry weight.

Pure Heterotrophic Growth of *G. sulphuraria*
Microtiter plate assay



DHTL- Coelastrella, Chlorella Nutrient Replete Cultures

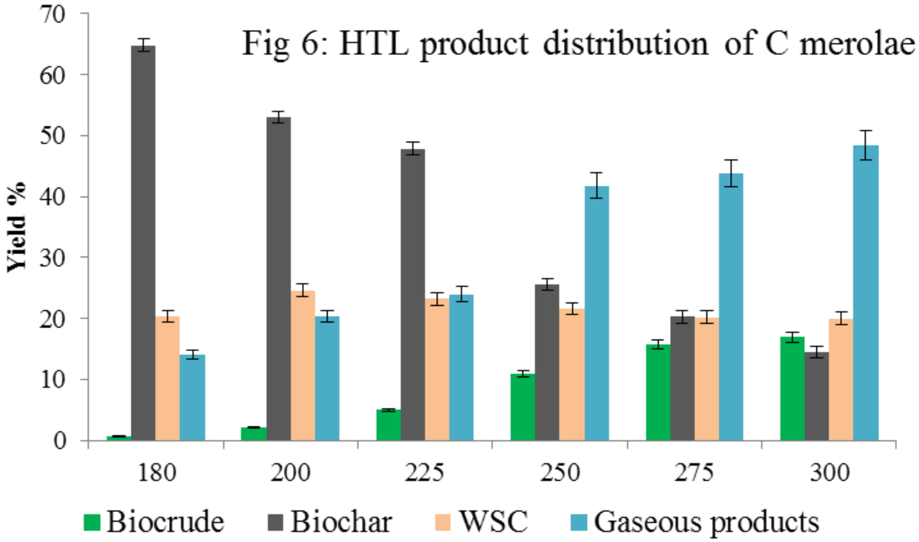


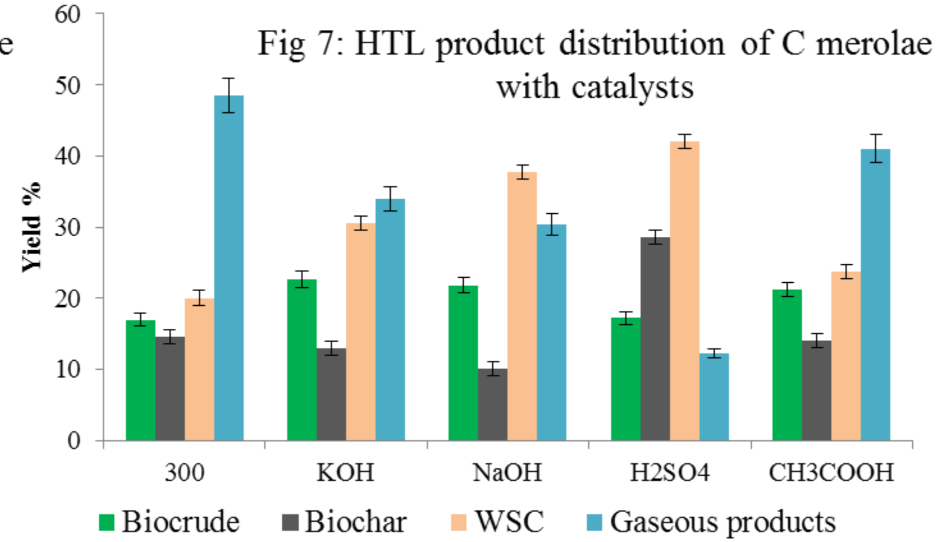


Max Bio-oil yield: 14.75%

Max Bio-oil yield: 33.6%

DHTL-*C. merolae/G. suphuraria* Nutrient Replete Cultures



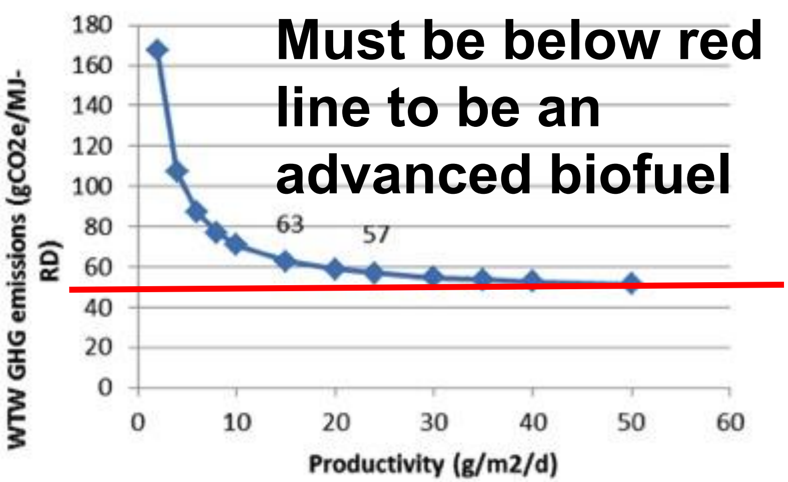


Max Bio-oil yield: 16.98%

Max Bio-oil yield: 21.22%

Results: Baseline Model

Emissions, gCO_2e/MJ-RD	REAP	Davis, 2014
Chlorella	57	32
Galdieria	63	37



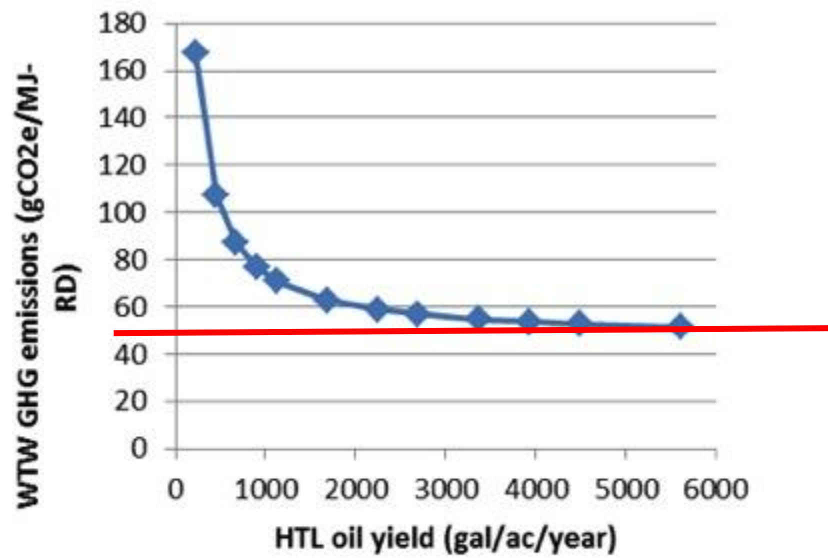
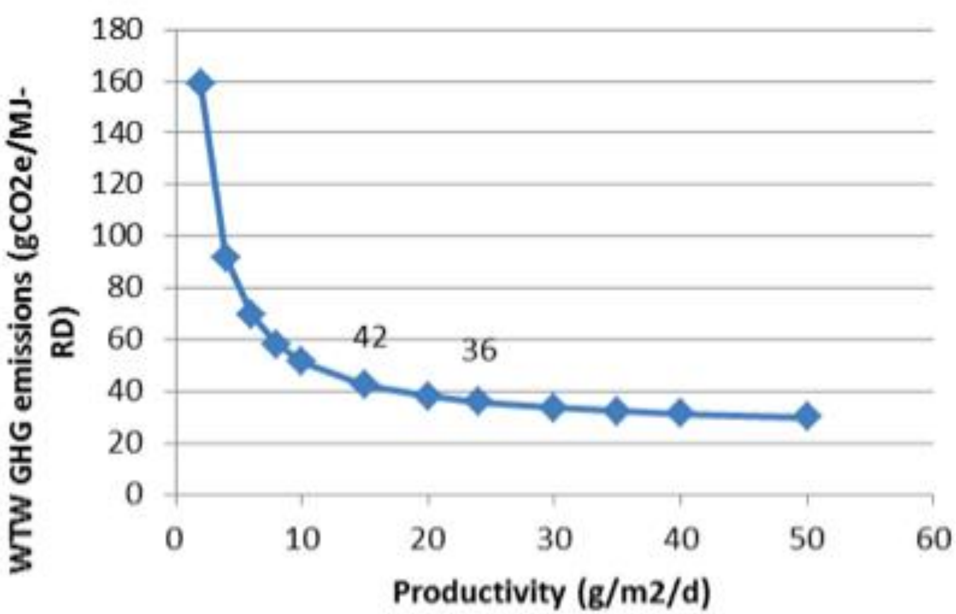


Figure 2: GHG emissions for hydrocyclone, centrifuge, and SEQHTL. The oil yield was computed by assuming 330 days/year, 3.1 kg algae/kg-oil, and 0.9 kg/L for the oil density

- REAP has high emissions with the baseline assumptions
- Cannot be overcome by increasing the productivity

Alternative Scenarios to reach 48 gCO2e/MJ

Scenario	*Chlorella* (24 g/m2/d)	*Galdieria* (15 g/m2/d)
Baseline	57 gCO_2e/MJ-RD	63 gCO_2e/MJ-RD
Belt filter press	50	57
18 wt% feed to HTL	44	51
Use biochar for heat	36	42



- Biochar in 80% efficient boiler, 29 MJ/kg (HHV)
  - Recovery energy neglected
- **Productivity must be kept above 12 g/m2/d to satisfy the emissions target**
  - An underestimate!

Constraints on Experiments

Systerm	Constraint
Cultivation	• Total energy < 48 KWh/ha/d • Likely need to be <u>lower</u> than this • If winter productivities are low, must reduce power further
HTL	• No solvents that require recycling? • Feed at 20 wt%? (under discussion) • Stage 2 yields measured with Stage 1 remnants from separation
Harvest / dewater	• Energy use less than that in current model • Cut costs

- Quick and dirty TEA says we must solve these while cutting costs
  - Solve problems by avoiding them
  - Simplify the system

5 – Future Work

- *Issues: <u>Staggered start</u> FFRDCs funded one year ahead of Universities and Sub-contractors who worked "at risk" through calendar 2014*
- *Genetic enhancements are lagging behind schedule*
  - *Strain issues, construct regulatory elements*
- *Low temperature water extractive reuse with* Galdieria *outdoors to enhance productivity to take advantage of high culture stability*
- *CO_2 utilization in Algenol vs paddlewheel horizontal PBRs*
- *Flocculant identification and dosing*
- *SEQ-HTL design (focus on separations & heat integration)*
- *Data collection with continuous flow HTL apparatus*
- *Supply numerous data needs for TEA and LCA*

Relevance/Key Objectives

- Addresses many of BETO's barriers

Aft-A	Biomass availability	Aft-B	Sustainable algae production	Aft-C	Biomass genetics
Aft-D	Sustainable harvesting	Aft-G	Feedstock properties	Aft-H	Integration and scale up
Aft-I	Feedstock processing	AFT-J	Nutrient / material recycle	Tt-B	Feed wet biomass

- Contributes to specific MYPP milestones
  - By 2016, review integrated R&D approaches for high-yielding algal biofuel intermediates
  - By 2018, demonstrate 2500 gal/yr of biofuel intermediate (non-integrated unit processes)

Summary of Important Results

- Good agreement between maximum predicted and maximum observed productivity of wild type *Chlorella sorokiniana* 1412 (30 $g/m^2/day$)
- Range of HTL bio-crude oil yields 17-33% in nutrient replete conditions, batch mode
- *Galdieria* cultivation is stable to carbohydrate addition for biomass productivity enhancement; wild-type readily uses cellobiose
- 14 external conference presentations, 2 papers, 0 patents

Questions?

2014 Productivity - *G. Sulphuraria* 5587.1

Depth	Productivity Values - g/m²/day					Mean Value (sd)
10 cm	**1.5**	**3.7**	**7.4**			**4.2 g/m²/day (3.0)**
Date Range	6/4/14-7/1/14	7/1/14-7/18/14	7/18/14-7/23/14			
Duration	28 days	18 days	5 days			
20 cm	**6.6**	**2.3**	**2.9**	**3.3**	**5.9**	**4.2 g/m²/day (1.9)**
Date Range	7/1/14-7/18/14	7/29/14-8/15/14	7/29/14-8/22/14	8/25/14-10/10/10	8/15/14-9/5/14	
Duration	18 days	18 days	25 days	46 days	21 days	

- Tested productivity and HTL yields on a single *G. sulphuraria* and a single *C. merolae* strain among the Acidophilic Red Algae (Cyanidiales).
- Lammers started a collaboration with Dr. Sherry Cady at PNNL who manages the Culture Collection of Microorganisms from Extreme Environments.
- Plan to screen more Cyanidiales for important phenotypes:
    - Cell wall thickness or lack of cell walls (transformability, Andreas Weber)

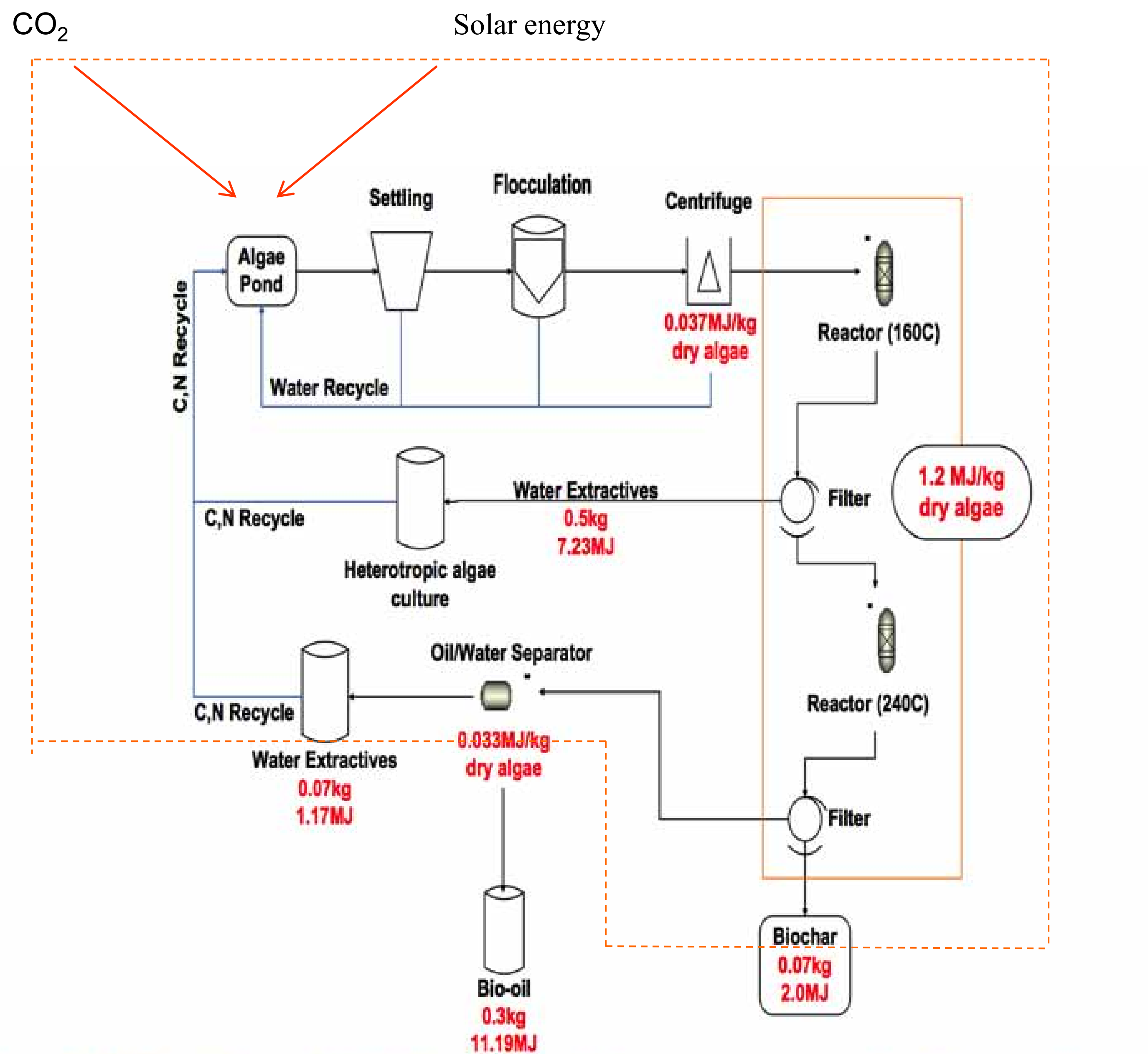


Figure 5. REAP wet Sequential Hydrothermal Liquefaction process process flow diagram with energ budget. Energy values are normalized to algal ash free dry weight.