Human & Environmental Health Risks Assessment of Algal Production Systems (9.6.1.5)

May 21, 2013
BETO Platform Review: *Algal Sustainability*

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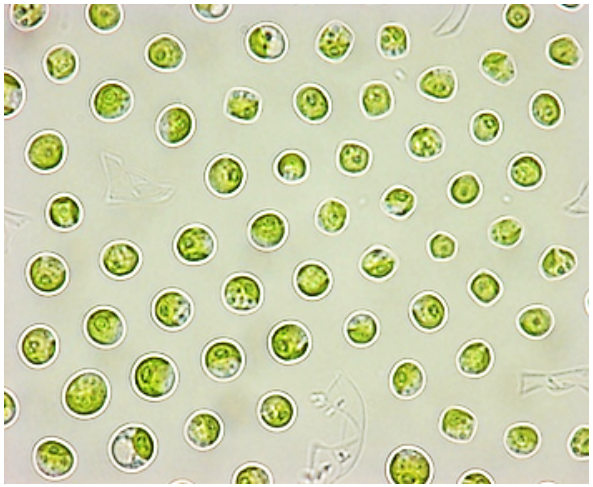
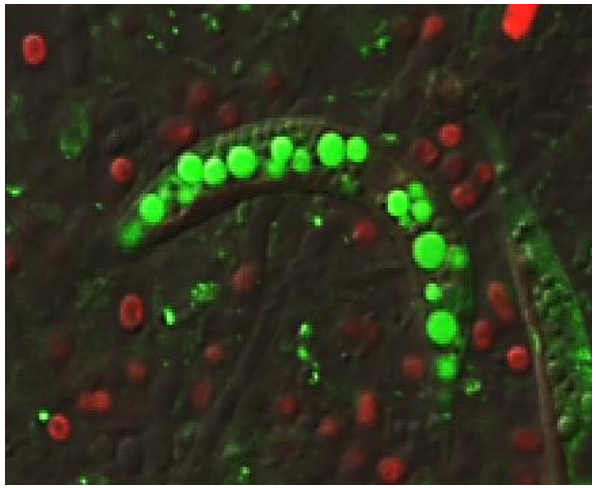
SRNL



Strains selection →



Production candidates →

Texas AgriLife (Pecos, TX)

Goals

- **Explore the potential for environmental or human health risks associated with large scale production of biofuel algae**
  - Potential reservoir for human pathogens or toxin producing microbes (SRNL)
  - Accumulation of metals or metalloids in algal biomass and water (LANL)
  - Emission of noxious, odorous, reactive volatile organic compound (VOCs) (TAMU-CC)
  - Production of cytotoxic metabolites (NOAA, SRNL)

- **Broader Relevance and Programmatic Fit**
  - Human health risks (cursory examination)
  - Social sustainability of algae biofuel production
  - Contribute to devise and operation of ‘smart’ systems

Quad Chart Overview

Timeline

9/01/10 - 9/30/12
NCTE thru April 2013

Project is complete

Barriers

- **Feedstock**
  - Safety
- **Sustainability**
  - Algae production
  - Social Acceptance
  - Resource re-use

Budget

Award	$1,340,319
FY11	$740,319
FY12	$600,000

LANL	$509,319 (37%)	
SRNL	$831,000 (62%)	
SRNL		$398,513 (48%)
NOAA		$221,295 (27%)
TAMU-CC		$211,192 (25%)

Partners

Los Alamos NATIONAL LABORATORY EST. 1943

Jeri Sullivan
Babs Marrone
Taraka Dale

NOAA National Oceanic and Atmospheric Administration U.S. Department of Commerce

Peter Moeller

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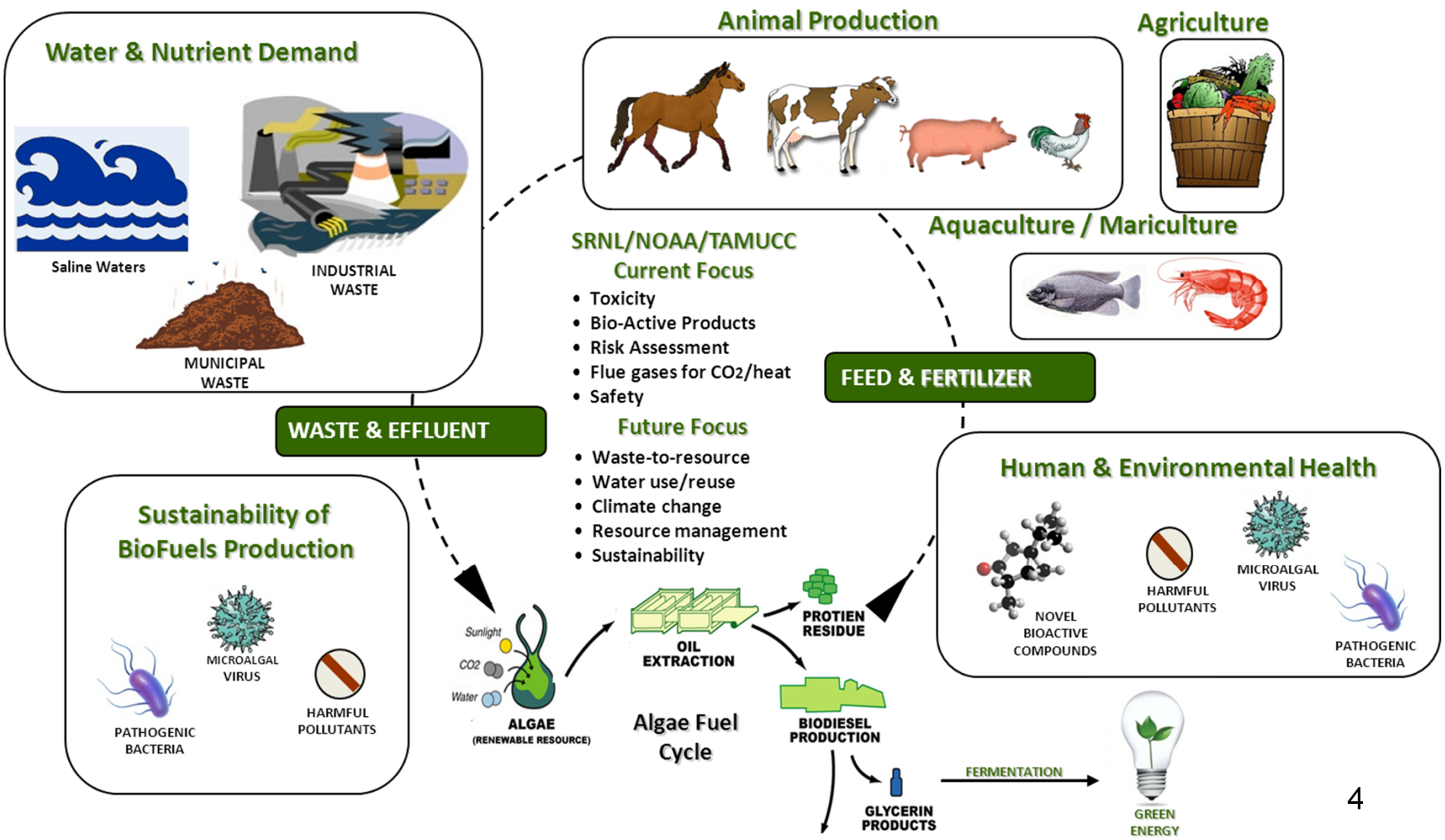
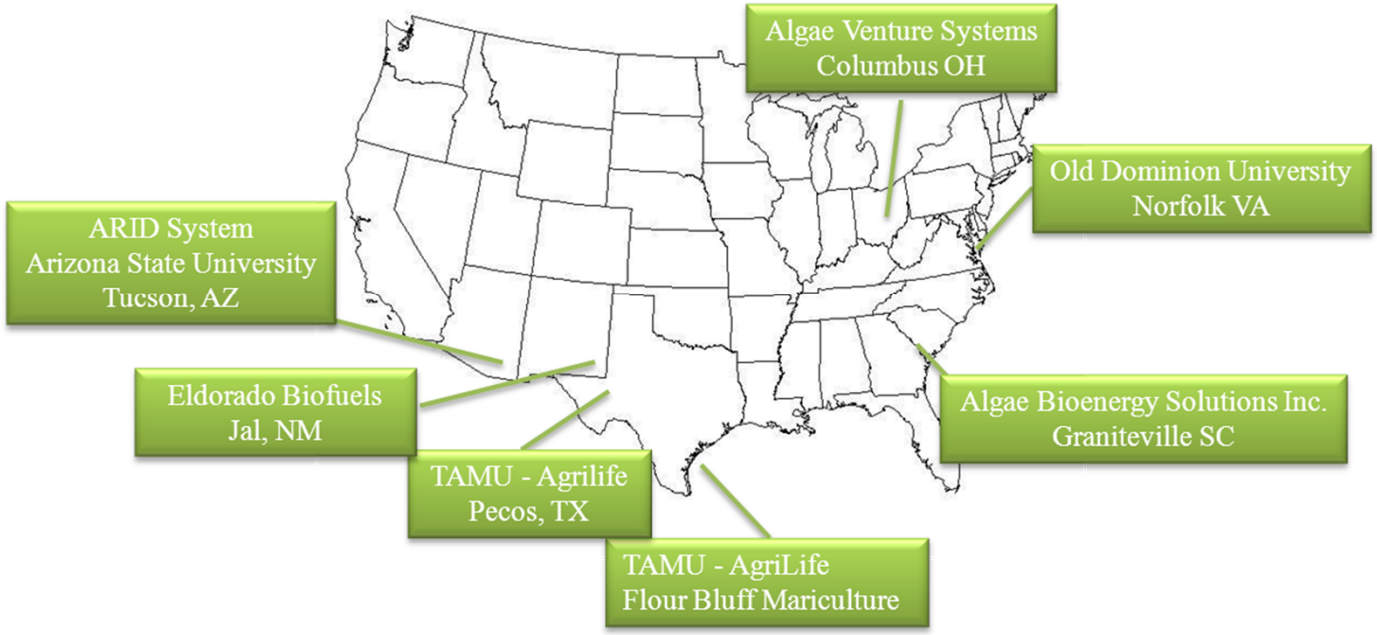
Expanding the Algal Fuel Cycle



Phase I: Look and See

Phase I. **Conduct a cursory survey of different outdoor biofuel algae production systems for the occurrence of potential human pathogens and confirmed toxin producing microbes, evidence for metal/metalloid accumulation in biomass and water, the emission of noxious or hazardous VOCs and production of cytotoxic metabolites.**



Lab	**Responsibility**	**Method**	**Samples**
SRNL	Pathogens & Toxic Algae	QPCR	Total Biomass
LANL	Metals Analysis, Cytotox	ICP-MS, RT-PCR	Water & Biomass
NOAA	Cytotoxicity Assay	*In vivo,* LC-MS	Biomass
TAMU-CC	VOCs	SPME / GC-MS. NIST	Algae - Water

Composition of QPCR Array

PATHOGENS

Agent	**Target**	**Disease**
Francisella tularensis (biovars A, B)	*pdpD*¸ IS*Ftu2*	Tularemia
Bacillus anthracis	*sspE, cya, capB*	Anthrax
Yersinia pestis	*ypo*393, *caf1, pla*	Plague
Legionella pneuomphila	*16S rrn* (universal, serotypes)	Legionnaires disease
Giardia lamblia	*B-giardin, n=3*	Giardiasis
Cryptosporidium parvum	COWP	Cryptosporidiosis
Salmonella spp.	*invA*	Salmonella
Campylobacter jejuni	*mapA*	Gastroenteritis
V. cholera	*16Srrn, hlyA*	Cholera
V. anguillarum		Vibriosis
V. parahaemolyticus		Gastroenteritis

AUTHENTIC TOXIN PRODUCERS

CYANOBACTERIA

Agent	**Target**	**Toxin**
Nodularia	*ndaF*	Cyanotoxin, Nodularin R
Microcystis aeruginosa	*mcyB*	Microcystins
Cylindrospermopsis raciborkski	*cyn*	Cylindrospermopsin
Planktothrix	*stx*	Saxitoxin, Mycrocystin

DINOPHYCEAE

Agent	**Toxin**
Karenia brevis	Brevetoxin
Gambierdiscus toxicus	PSP toxin
Dinophysis ovum	DSP toxin
Alexandrium	PSP toxin
Lingulodinium polyedrum	Yessotoxin

QPCR Array: Pathogens & Toxin Producers

Technique

QPCR offers rapid, paralleled molecular diagnostics
Amplification products short, QA/QC important, + controls essential

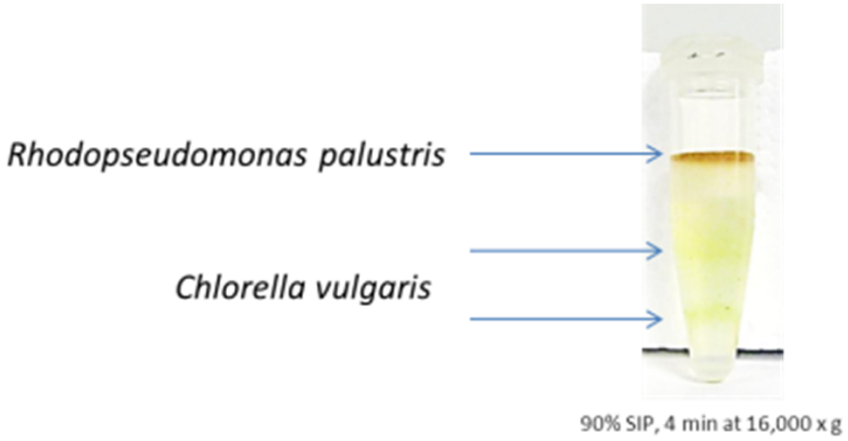
Results

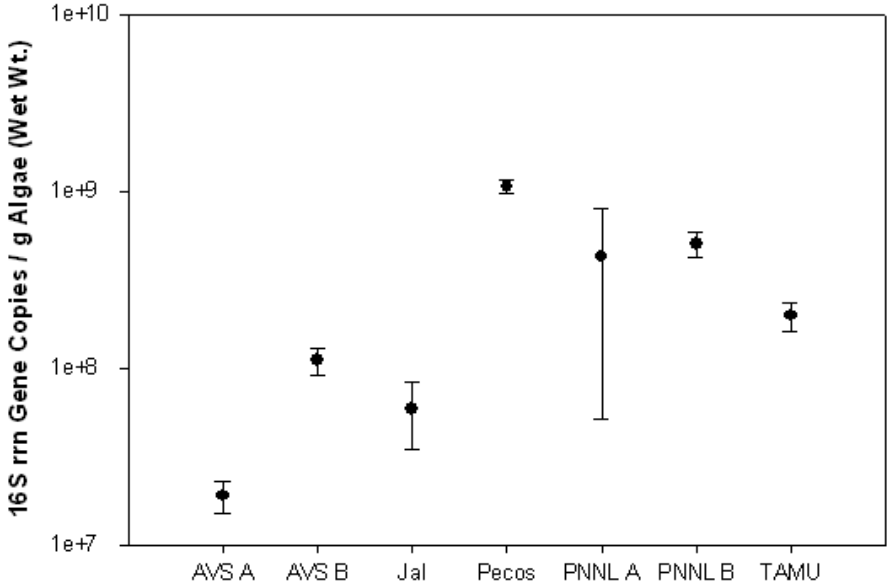
- >200 samples analyzed by direct lysis procedures
- Putative 'hits' at or below background
  - *Legionella spp.* is everywhere
  - Signatures for *Yersinia pestis* and *Microcystis sp.* detected but not confirmed
- Algae samples consistently positive for hemolytic activity
  - Indicator for *Streptococcus, Staphylococcus* but not confirmed
- Low target biomass

No Go

- Too many variables; time prohibitive, costly
  - QPCR is ideal for a defined set of targets
- Contaminant threats will differ site and system specific; not universal
  - *Prymnesium parvum*
- More appropriate, HT – unbiased methodologies
  - Microfluidic forensics – RapTOR (T. Lane, Sandia)
  - High throughput sequencing - bioinformatics

Outdoor Ponds – Raceways: Bacterial Loads





- Methodology developed to physically separate prokaryotes from unicellular algae
- Bacterial biomass high
  - Common despite different locations, conditions, operational procedures
  - Competition for nutrients and resources; need for IPM
- Beneficial bacteria associated with algae; not all ‘contaminants’ (*In review*)

Outdoor Ponds – Raceways: Cytotoxicity

	Elutropic Solvent Series		
	DCM	MeOH	H_2O
Research Raceways: Saltwater			
Site A			
08-02-11		++	++
10-05-11		+	+
Site B			
07-27-11	+		
Commercial Ponds / Raceways: Freshwater			
Site A			
09-22-11	++	++	++
Site B			
10-04-11		+	+
Site C			
05-18-11	+++	+++	++

- Cytotoxicity assays conducted using mammalian cell line cultures
- Ion channel disruption; pituitary (Ca^{2+}), neuroblastoma (Na^{+}) cell line
- Cytotoxicity demonstrated for algal biomass
- Reactive metabolites unknown; LC-MS fingerprinting

Outdoor Ponds – Raceways: VOC Emissions

- Nuisance or noxious volatiles; Potential risk to workers
- Algal samples heated to 65°C, volatiles captured SPME – GC/MS; NIST
- Biochemical complexity. *Scenedesmus* >> *Nannochlorpsis*
- Numerous flavor – fragrance components (fruity, grass), odorous compounds
- Several dermal, inhalation hazards reliably identified. TOX thresholds

Isoprene & bromoform (ozone reactive), halogenated hydrocarbons, oximes (LD50, bacterial), FA, propanal (flammable)

- Origin of select compounds uncertain; biotic and/or abiotic

Dichloromethane, toluene, benzene

Phase II: Matrixed Grow-out

Phase II. **Execute an outdoor cultivation matrix to examine different combinations of biofuel algal strain, water composition and nutrition, operational parameters and harvesting frequency to identify statistical correlations between water chemistry, microbiological burden (i.e., contaminants and predators) and VOC emissions that might serve as reliable indicators of algal health and productivity**

▪ Strains

Nannochloropsis (OZ1), Chlamydomonas, Phaeodactylum, Cyclotella

▪ Scale & Growth Medium

- Replicated 1200L raceways
- f/2 and Pf4 (least cost formulation)
- Culture volume incrementally increased from 5 to 15 cm
- Redfield ratio maintained; 16:1 (N:P)

▪ Harvest

- 30% drawdown 2x per week
- Biomass harvested by centrifugation or biomesh bags
- Lipid production was determined

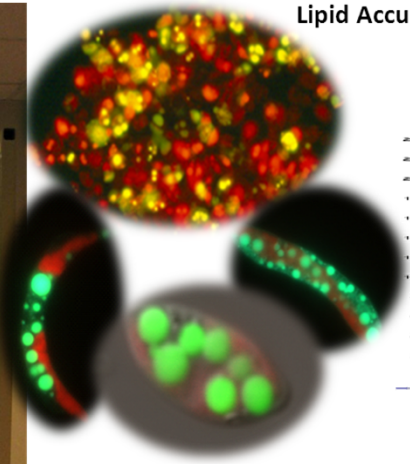
▪ Numerical counts recorded every 2 days
- >30% contamination (total counts) by 28 days; 2 cultures 48 days
- Rotifers, benthic diatoms, cyanobacteria, 2nd algae.
- No IPM

▪ VOCs were conditionally responsive and strain specific; *In review*
▪ Water chemistry analysis (LANL).

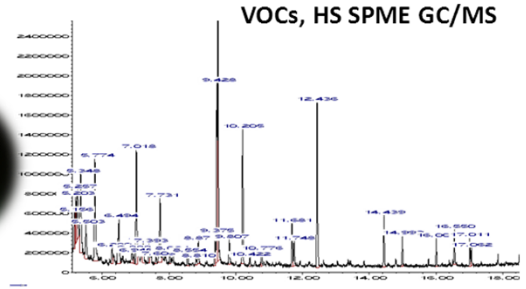
Cytotoxicity of Unicellular Chlorophytes

Biofuel strains are grown under nutrient replete and deplete Conditions implicated in triggering lipid accumulation.

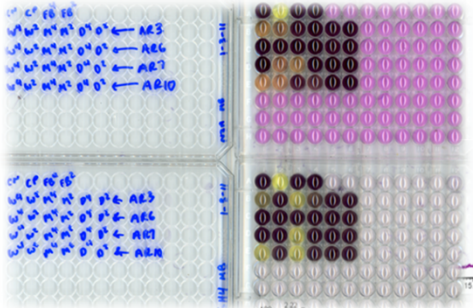
Lipid Accumulation



VOCs, HS SPME GC/MS



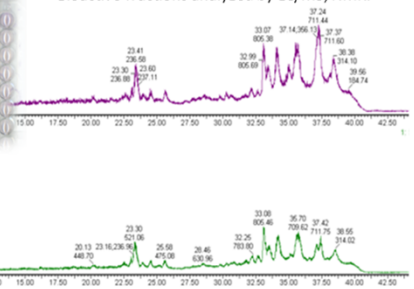
Ion Channel Disruptors



Extractions performed in water, methanol, dichloromethane.

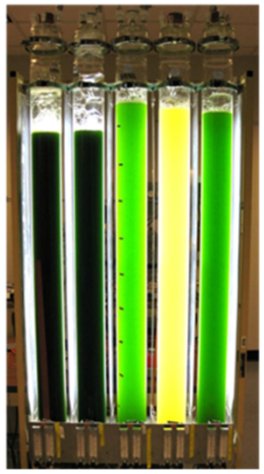
Fractions exposed to immortalized cell lines.
GH4C1 rat pituitary (Ca^{2+}),
N2A neural cell line (Na^{+}).

Bioactive fractions analyzed by LC/MS, NMR.



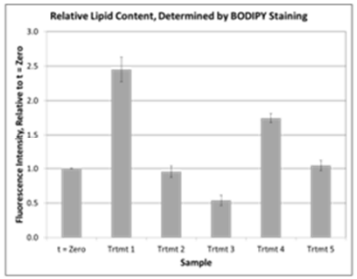
Explore the potential for cytotoxicity in freshwater biofuel algae. Examine unicellular green algae under nutrient replete and deplete conditions for TAG biosynthesis and production of bioactive metabolites.

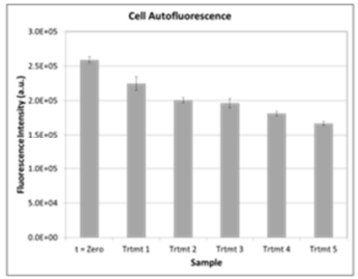
Production Scenarios Examined for Induction of Cytotoxicity, Growth Response, and Lipid Accumulation. *Chlorella* and *Scenedesmus* strains were grown in M9 or modified M8 medium (5 different conditions). Cytotoxicity induction examined in nutrient replete and nutrient deplete scenarios.



Preliminary characterization of bioactive metabolites from *C. vulgaris*

- ❑ 7 polar MeOH soluble, potentially novel non - FA 'toxins'
- ❑ Principal toxin is a small peptide or amino acid based structure
- ❑ High activity to mass ratio; implication for potency
- ❑ Toxins are active in the N2A cell bioassay
- ❑ Toxin masses are not produced in other growth conditions
- ❑ Non-polar toxic fractions appear to be fatty acid(s) derivatives

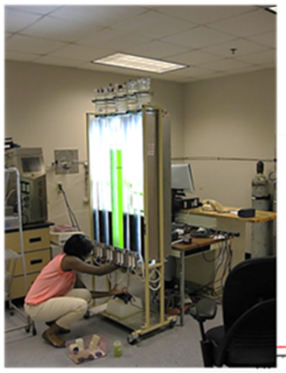


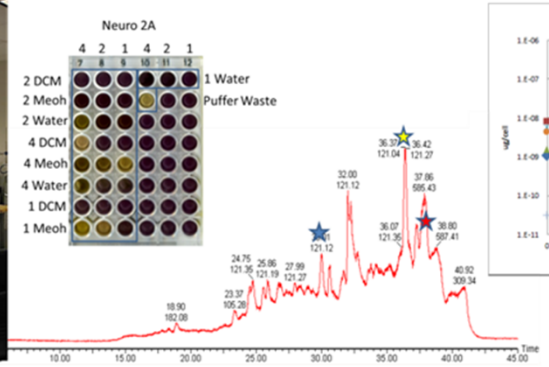


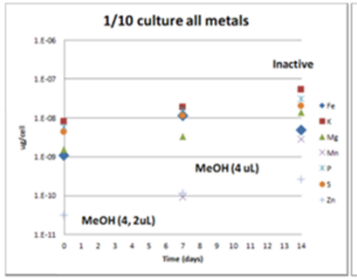
- ▪ **Freshwater microalgal candidates for biofuels are capable of synthesizing bioactive metabolites.**
- ▪ **We can reliably induce cytotoxicity in *Chlorella* and *Scendesmus* strains by restricting Fe availability.**

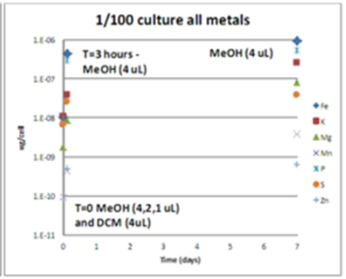
Is cytotoxicity and the biosynthesis of specific bioactive metabolites functionally linked to Fe starvation?

Dose Response of *Chlorella* Cytotoxicity to Iron Supplementation. *Chlorella* was grown in Fe – deplete M8 medium shown previously to induce cytotoxicity. The bioassay was used to confirm establishment of this phenotype. Different levels of iron were added back to the 'toxic' cultures and cytotoxicity response was measured over time independent of changes in cell densities.









- ▪ **Cytotoxicity (i.e., toxin potency or compound(s) abundance) responded to iron additions**
- ▪ **Cytotoxicity was more sensitive to incubation time than to the quantity of iron added to the culture.**

Chlorella Cytotoxicity

- **Fe is a required trace element for all living organisms**
  - Concentrations low due to uptake, utilization, competition
  - Intentional as a cost savings or to stimulate lipid biosynthesis
- **Fe linked toxicity in bacteria, fungi and eukaryotes – including microalgae**
  - Toxic Chlorophytes not expected; can produce bioactive metabolites
- **Cytotoxicity in *Chlorella* and *Scenedesmus* was consistent and reproducible**
- **1 *Chlorella* culture, 1 condition**
  - 10 – 12 MeOH soluble, LC – MS peaks were cytotoxic to N2A cell line
  - Several compounds are putative low MW peptides (MS-TOF)
  - Predominant 'toxin' likely a glycoside (NMR)
- **Cytotoxicity responded to Fe additions**
  - Metabolite mass or bio-reactivity alleviated
- **Evidence is mounting but a ways to go yet**
  - Partial ID, could enlighten pathway(s), environmental triggers unknown
  - Degree of conservation among *Chlorella* and *Scenedesmus* unknown
- Manuscript in preparation

Summary

- *Potential* for risk but actual risk was not quantified or assessed
- Results enlightening but proper risk assessment requires production system
- Opportunity to apply to standardized format; ATP^3 – Algae Testbed Facility

Future Work

Provide quantitative characterization of bioactive metabolites produced by biofuel algae to enable proper risk assessment to human health and the environment

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Publications and Presentations

Patent.

Priming Biofuel Algae for Efficient Photosynthetic Conversion of Carbon. This ID describes the application of a potent phytohormone to specifically and unanimously direct algal physiology into biosynthesis of the photosynthetic apparatus to maximize the capture and conversion of solar energy into biochemical energy.

Publications.

- Functional interactions explored between bacteria and chlorophytes. Bagwell et al., In Review.
- Evaluation of VOC compounds released by *Nannochloropsis* spp. Zimba et al., In Review.
- Experimental production of bioactive metabolites by *Chlorella* sp. Moeller and Bagwell. In Preparation.
- Recycling of metals in F/2 brackish water media during microalgae cultivation for algal biofuel production. Sullivan et al. In Review.

Proposals.

Applying ^{13}C – metabolic flux and functional genomics to resolve auxin response pathways in unicellular algae. Exploratory collaboration between DOE's Joint Genome Institute and the Environmental Molecular Sciences Laboratory.

Presentations.

Resolving complex microbial interactions for sustainable production of algae. The 34th Symposium on Biotechnology for Fuels and Chemicals (April 30 – May 3, 2012) in New Orleans, LA USA.

Cytotoxicity induction in algal strains used in biofuel production. The 2012 Algal Biomass Summit (Sept 24-24, 2012) in Denver, CO USA.