SAND2019-1992C

Chemical and Proteomic Profiling of Organic Compounds for Detecting Algal Interactions with Grazers and Commensal Bacteria

Kristen L. Reese[1,2]; Carolyn L. Fisher[3]; Christopher S. Ward[1], Matthew W. Moorman[4]; A. Daniel Jones[2]; Matthias Frank[1]; Miriam Windler[5], Alfred Spormann[5], Mary Lipton[6], Lindsey Anderson[6], Sam Purvine[6], Carrie D. Nicora[6], Patrik D'haeseleer[1], Xavier Mayali[1], and **Rhona Stuart[1]**, **Todd W. Lane** (twlane@sandia.gov)[3]

[1]Lawrence Livermore National Laboratory, Livermore, CA; [2]Michigan State University, East Lansing, MI; [3]Sandia National Laboratories, Livermore, CA; [4]Sandia National Laboratories, Albuquerque, NM; [5]Stanford University, Stanford, CA; [6]Pacific Northwest National Laboratory, Richland, WA

LLNL Biofuels SFA

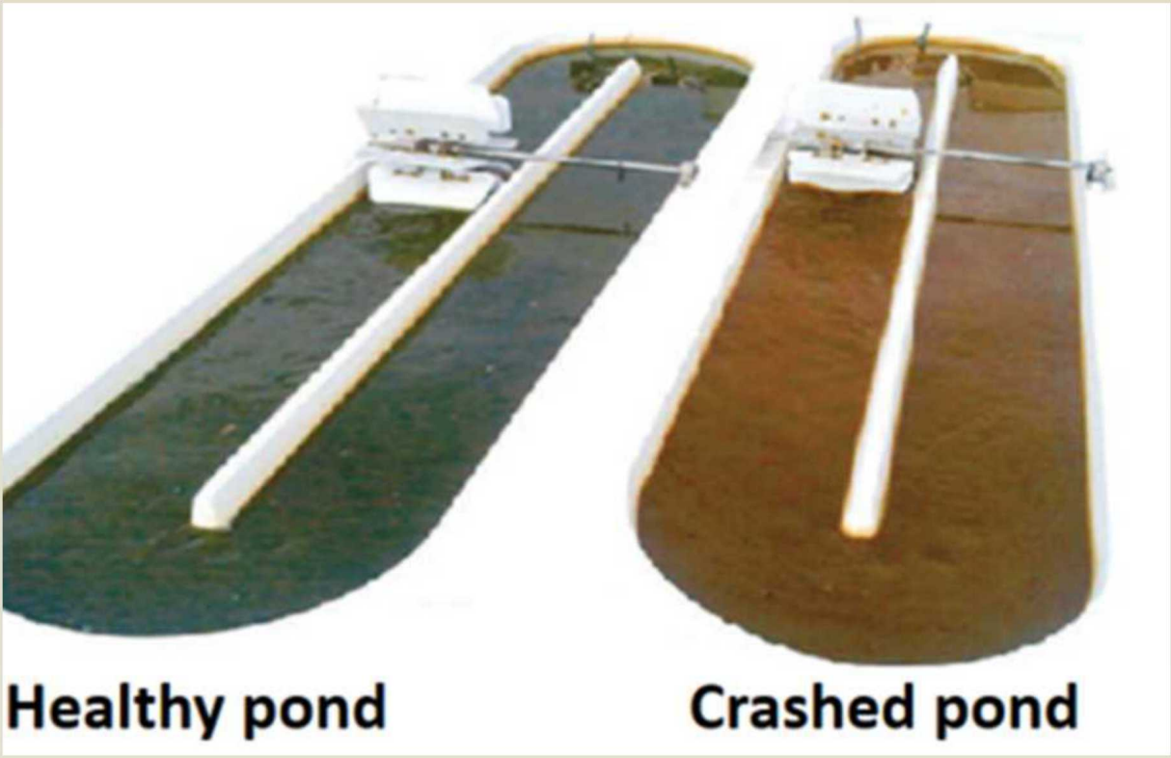
Systems Biology of Microbial Communities

https://bio-sfa.llnl.gov

Overview

Biotic interactions in algal ponds are both affected by and influence the chemical environment surrounding algal cells. As part of our SFA, we aim to disentangle various components of these chemical signals in order to better understand the mechanisms of how biotic interactions influence algal productivity and physiology. Here, we present two studies focused on different aspects—the first highlights the role of trophic interactions, identifying volatiles produced in algal-rotifer interactions, while the second examines algal-bacterial interactions at the molecular level in a model algal species.

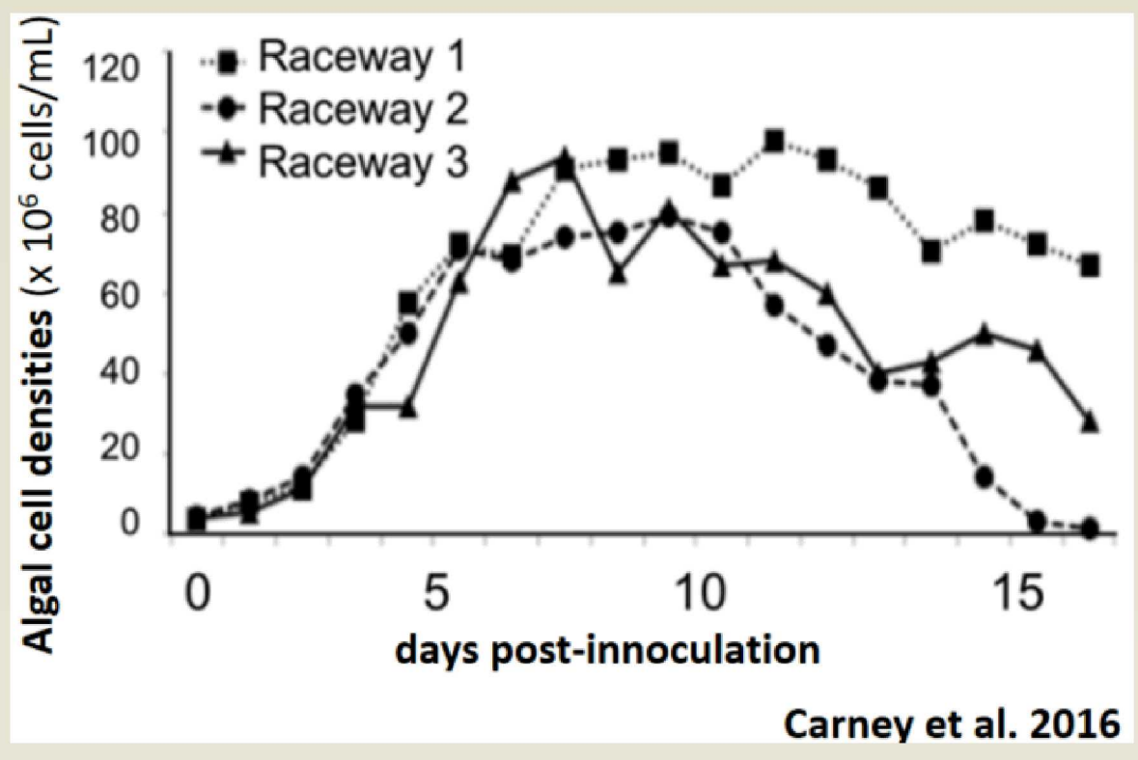
Approach & Rationale



Open algal ponds are likely to succumb to unpredictable, devastating crashes by one or several deleterious species. Developing methodology to mitigate or prevent pond crashes will increase algal biomass production, drive down costs for algae farmers, and reduce the risk involved with algae cultivation, making it more favorable for investment by entrepreneurs and biotechnology companies. Here, we show that specific algal-bacterial co-cultures grown with the biofuel green alga *Microchloropsis salina* prevented grazing by the marine rotifer, *Brachionus plicatilis*. With these data, we aim to define and develop an engineered-microbiome that can stabilize industrial *Microchloropsis salina* cultures by protecting against grazer-induced pond crashes. The establishment of protective algal-bacterial may be applicable to additional algal-grazer pairs and therefore may be a preferable, less expensive method than standard expensive interdiction techniques. Additionally, understanding the algal-bacterial interactions on an ecological level from a basic science approach will aid in the development of grazer-resistant algal production systems.

Algal population crashes cause **losses of up to 30% of annual crop production** from the typical open raceway system.

Richardson et al, 2014.



Carney et al. 2016

Pond crashes: *N. salina* growth in biological replicate raceways at Texas Agrilife. Raceways show moderate to severe **biomass loss** as a result of algal predation.

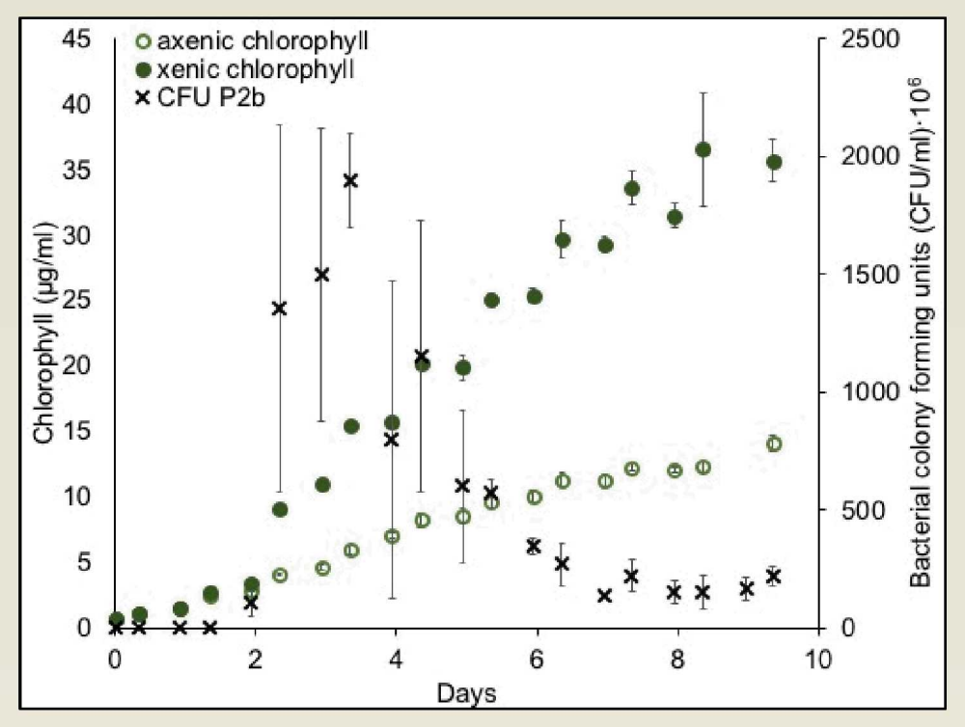
Investigating bacterial influence on algal biomass production—model co-culture

C. reinhardtii:

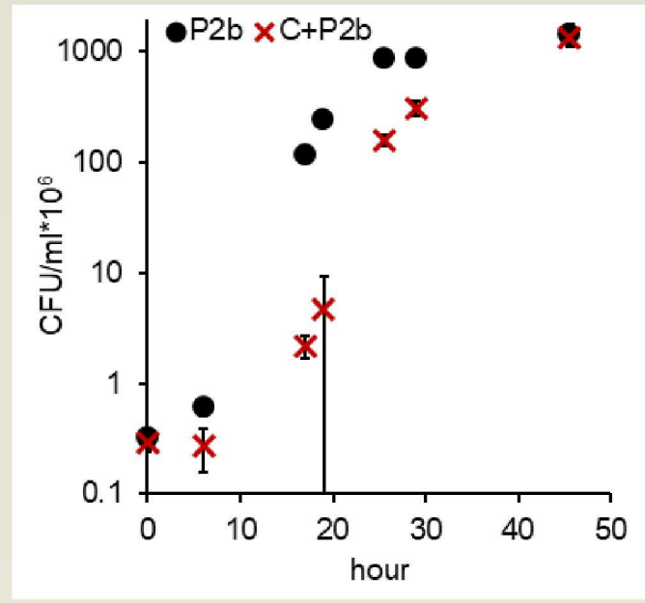
University of Minnesota

We combine growth and biomass assessments, microscopy and global proteomics profiling of a model system of one bacterial commensalist (*Arthrobacter* P2b) and one alga (*Chlamydomonas reinhardtii*) to ultimately understand the complex chemical and biotic interactions occurring in ponds at the molecular and gene level

Algal and bacterial growth:



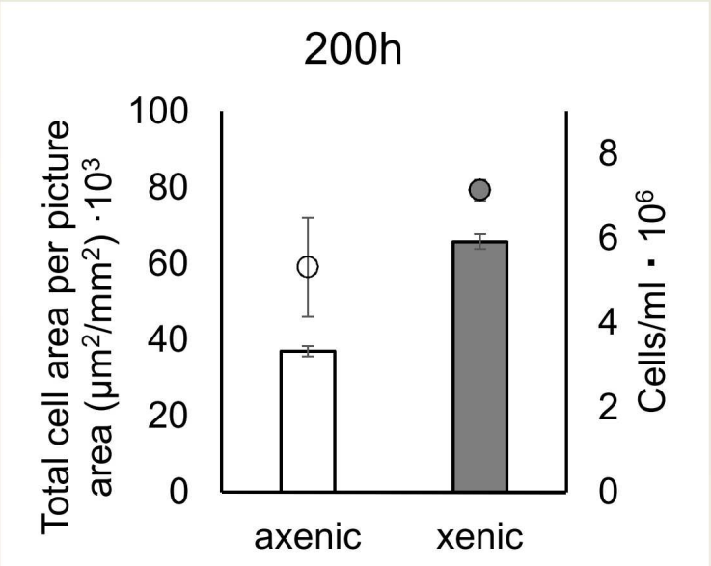
Chlorophyll measurements and bacterial colony forming units (CFU) over time in co-cultures and axenic cultures



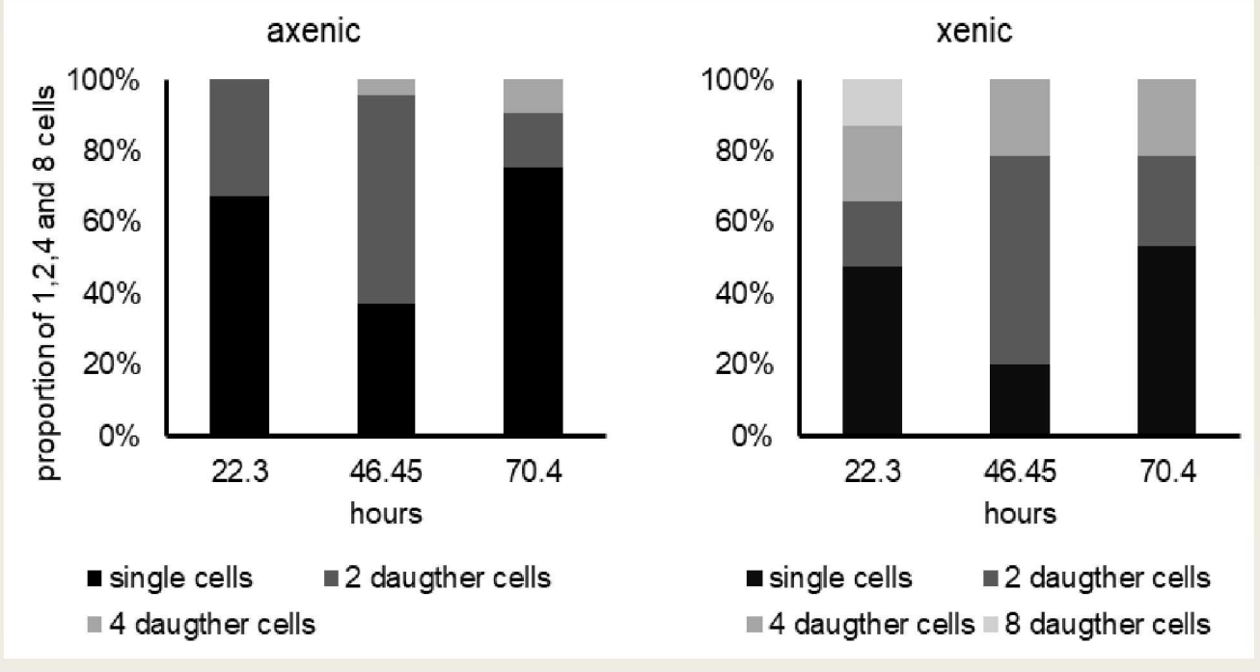
Bacterial counts (colony forming units) over time in co-cultures and pure cultures

- Co-culture with P2b increases *C. reinhardtii* chlorophyll up to 3x over axenic growth
- P2b grows similarly in co-culture as in isolate culturing

Algal cell morphology



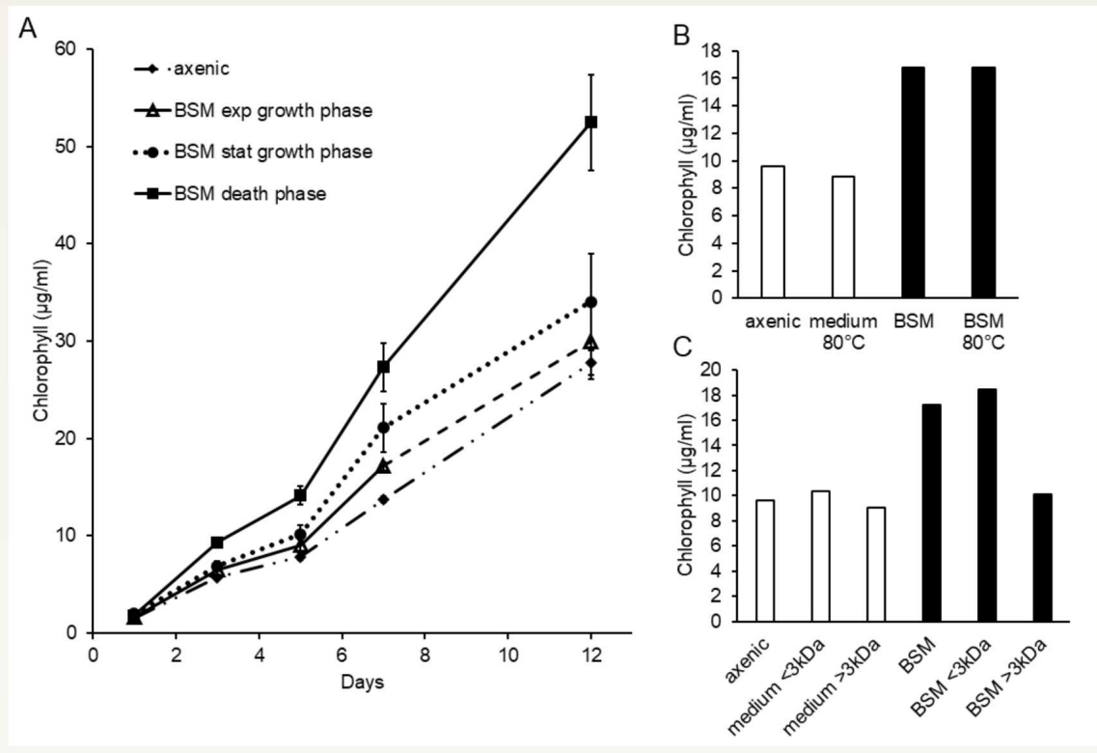
Microscopy-based analysis comparing algal cell area in axenic and co-cultures after 200 h of growth (using DAIME--Daims *et al* 2006. Environ. Microbiol. 8: 200-213)



Microscopy-based analysis counting incidence of cells divided in two or multiple daughter cells

- Co-culture with P2b increases *C. reinhardtii* cell volume and total culture biomass
- Co-cultures also display higher proportions of multiple cell divisions, indicating cell cycle effects.

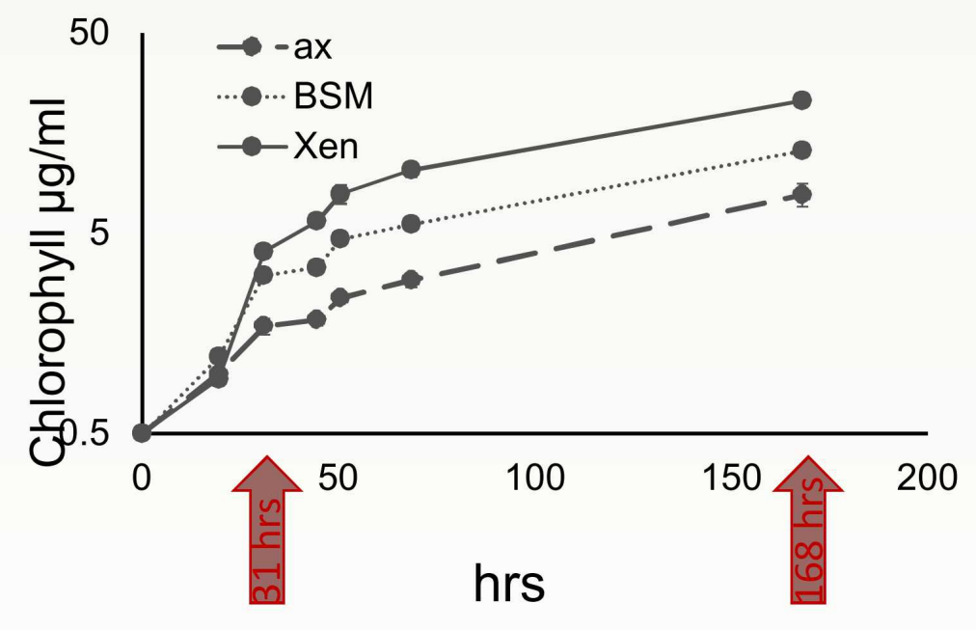
Bacterial secreted compounds



(A) Chlorophyll measurements of algal growth with bacterial spent media (BSM) from different bacterial growth phases.
(B) Comparing algal biomass with BSM and 80˚C-denatured BSM.
(C) Size-fractionated BSM. Controls included addition of similarly treated bacterial medium

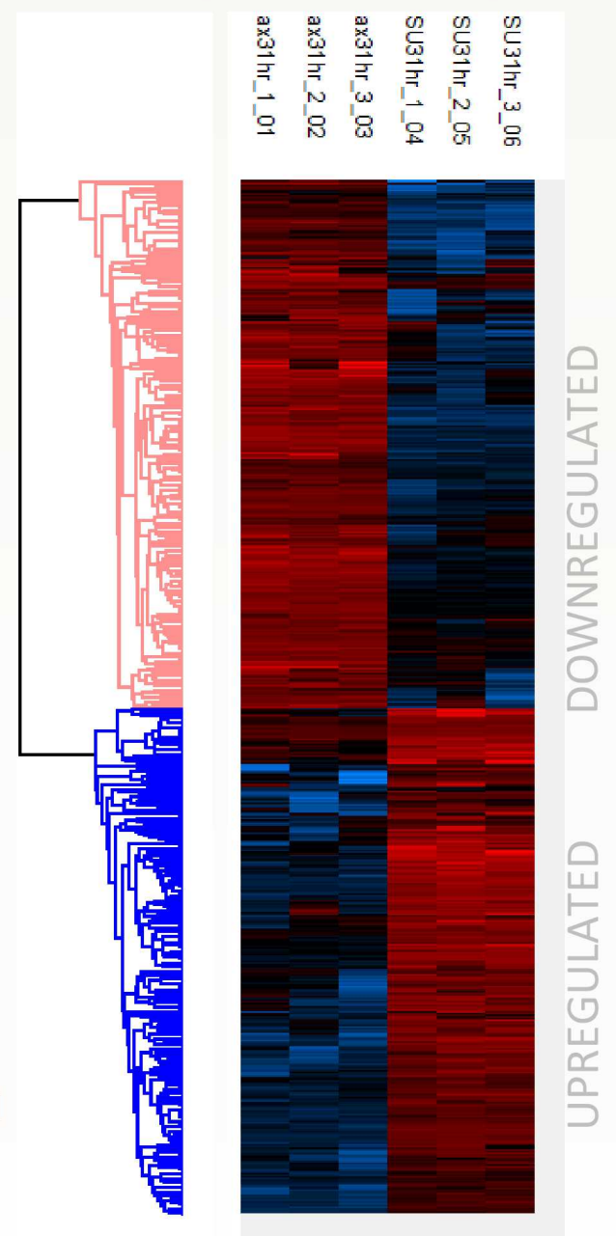
- Growth with P2b cell-free spent medium (BSM) exhibits a similar effect, indicating a potential role for secreted compound(s)
- Denaturing BSM did not alter the effect, indicating non-protein effector molecule
- Size fractionation of BSM indicates a size range of <30 kDA for the effector molecule

Global proteomic profiling



Three treatments for proteomic profiling: (1) Axenic (*C. reinhardtii* alone); (2) + P2b (Co-culture, xen) and (3) +bacterial spent media from P2b culture (BSM)

1. LCMS/MS (Orbitrap, PNNL)
2. Analysis pipeline developed using MaxQuant and Perseus (Cox and Mann 2008. Nat Biotechnol. 26:1367-72; Tyanova et al., 2016. Nature Methods 13:371-740)

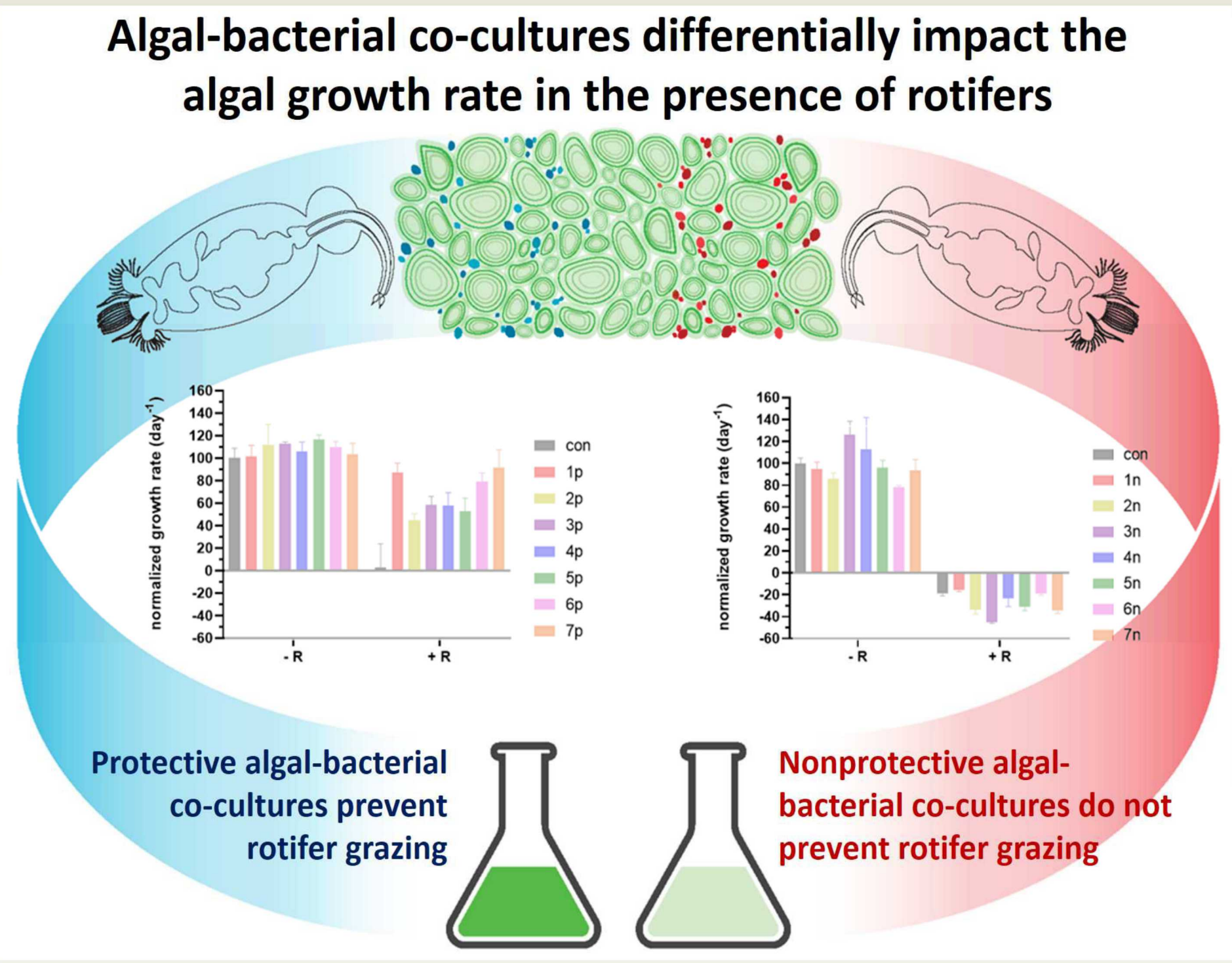


Significant differentially expressed algal proteins in the co-culture vs axenic culture at 31 h time point

Differentially expressed algal proteins in co-culture compared to axenic:
- Downregulation of flagellar proteins
- Upregulation of cell-cycle related processes (e.g. cyclins, cyclin dependend kinases, DNA replication etc.)

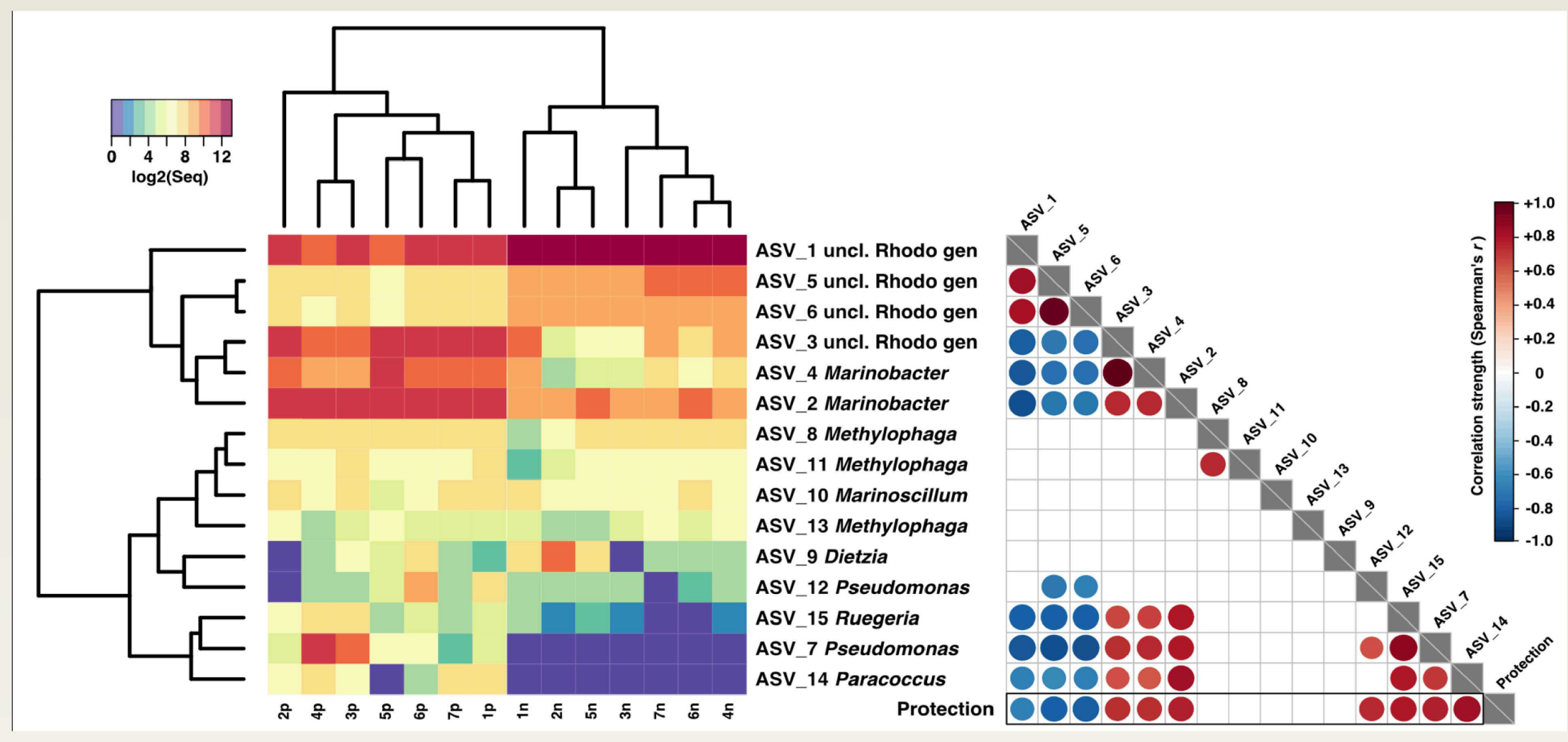
Proteomics results support growth results suggesting P2b influences *C. reinhardtii* cell cycle processes

Outcome & Implications



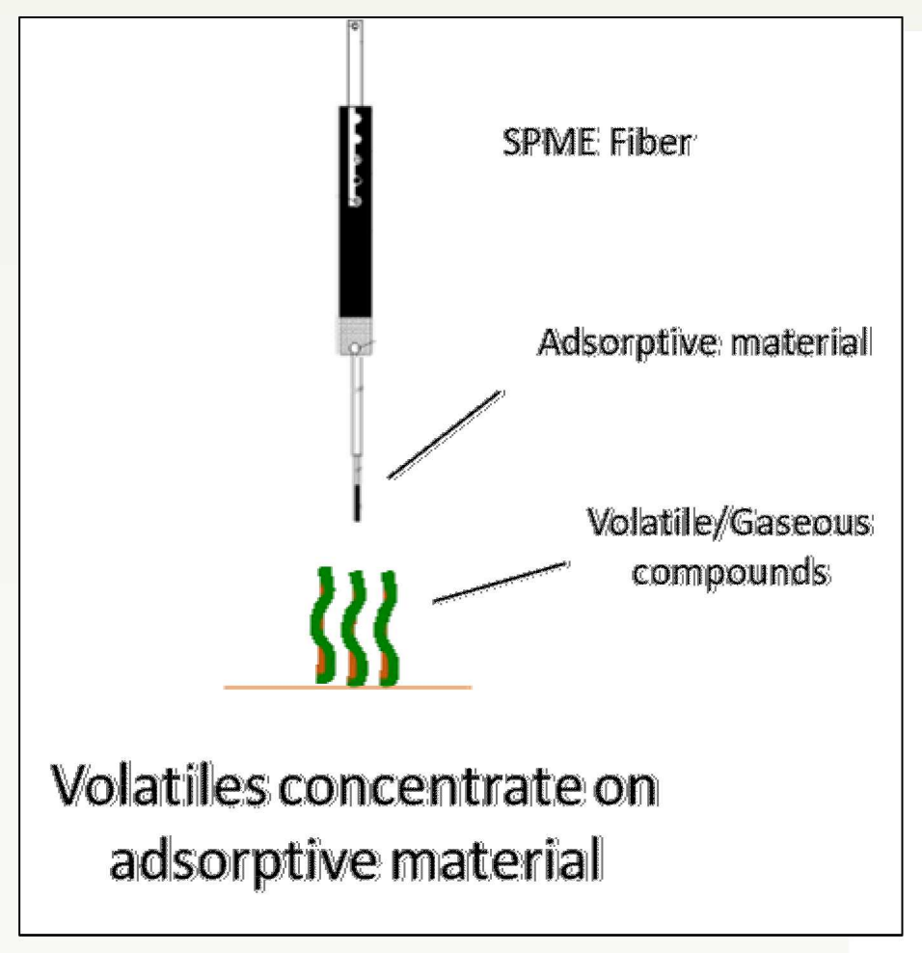
Protective vs, nonprotective algal-bacterial co-culture summary showing decreased algal productivity in the presence of rotifers for axenic control but increased productivity for protective algal-bacterial co-cultures.

We obtained seven algal-bacterial co-cultures from crashed rotifer cultures, maintained them in co-culture with *Microchloropsis salina*, and used a microalgal survival assay to determine that algae present in each co-culture were protected from rotifer grazing and culture crash. After months of routinely diluting and maintaining these seven algal-bacterial co-cultures, we repeated the assay and found the opposite result: none of the seven bacterial communities protected the microalgae from rotifer grazing. We performed 16S rRNA gene amplicon sequencing on the protective and nonprotective co-culture samples and identified substantial differences in the makeup of the bacterial communities. Protective bacterial commuities consisted primarily of Alphaproteobacteria (Rhodobacteraceae) and Gammaproteobacteria (*Marinobacter*, *Pseudomonas*, *Methylophaga*) while nonprotective bacterial communities were less diverse and missing many putatively crucial members. We compared the seven protective communities with the seven nonprotective communities and we correlated specific bacterial amplicon sequence variants with algal protection. Our data support inoculating production ponds with a diverse, protective microbiome may be a novel, low-cost method to reduce the frequency of pond crashes. We describe the establishment of algal-bacterial co-cultures and demonstrate their effectiveness in reducing microalgal loss to rotifer grazing. Engineering protective algal-bacterial co-cultures to save algal ponds from crash will reduce production costs for algal industry and drive down the costs for algal biofuel and biodiesel production.



Further work will involve identifying notable chemical signals (also known as "info chemicals") from liquid and headspace analysis to better investigate the chemical signaling present within protective and nonprotective algal-bacterial co-cultures. Previous communication with collaborators has determined that xenic algal cultures have more chemical signatures than axenic cultures.

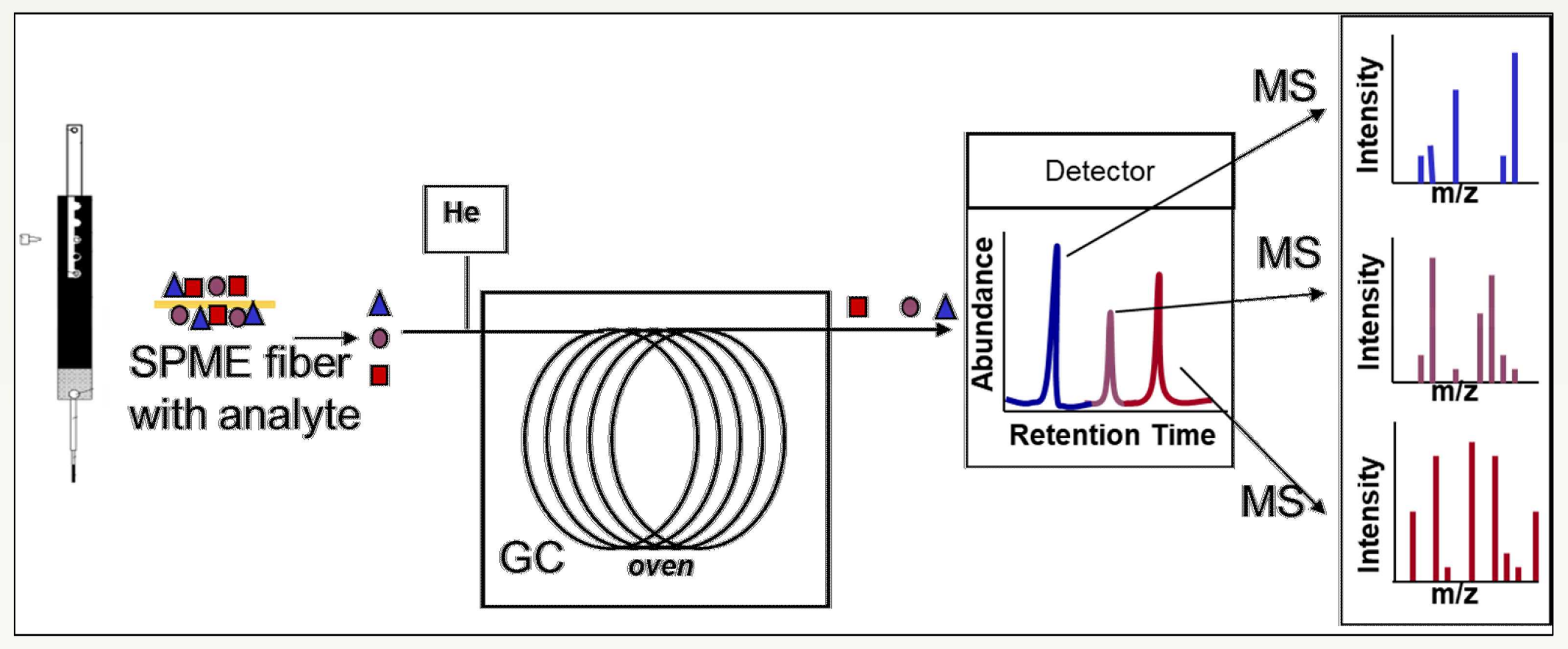
VOCs Sampled



SPMEs used to collect volatiles organic compounds (VOCs).

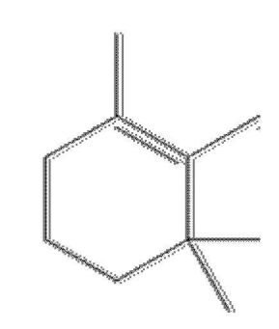
Experimental Setup: Growing *N. salina* and *B. plicatilis* cu
(1) with simulated sunlight sources (2) were sampled wit
SPME fibers (3); AVOCs characterized using GC-MS

AVOCs Data Acquisition



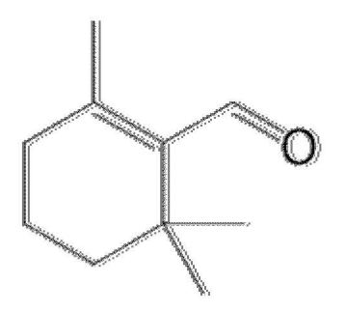
Microchloropsis salina was grown at 5M cells/mL in four 15-L cultures of chemically-defined, enriched artificial seawater with air and 1%-CO_2 sparged through bubblers. VOC profiles were sampled from all cultures after 24-48 hours of growth using 65 µm polydimethylsiloxane-divinylbenzene SPME fibers with 60-minute exposures. Algal density was monitored via chlorophyll fluorescence and cell counts. After 48-hours of growth, *Brachionus plicatilis* were added (88 rotifers/mL) to two algae cultures. VOC profiles were monitored for 2-4 additional timepoints after inoculation. An untargeted GC-MS approach characterized VOCs using 70 eV electron ionization on a quadrupole mass analyzer. Subsequent characterization, alignment, and preliminary identifications were made through analysis of deconvoluted experimental spectra with comparisons to the NIST14 spectrum database (Match>70%) and retention index matching. Notable differences were observed in VOC profiles emitted from infected and non-infected cultures, with select VOCs varying over time. Characterization of VOCs indicative of rotifer infections depended on reliable VOC fingerprints from each condition: 1) *M. salina*-only cultures, *B. plicatilis*-infected *M. salina* cultures, 3) enriched seawater controls, and 4) SPME fiber blanks. Volatiles were attributed to each condition if meeting the following conservative requirements: 1) presence in at least two out of three replicates at a timepoint, 2) greater than 10x abundance of corresponding abundance in media-only control. The addition of *B. plicatilis* to healthy cultures of *M. salina* produced an abundance of putatively identified carotenoid-derived products such as trans β-ionone and 4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2-butanone. Concurrent to VOC analysis, daily measurements of live algal density confirmed that rotifer-inoculated cultures displayed lower live algal counts relative to the uninoculated controls.

(Trans) β



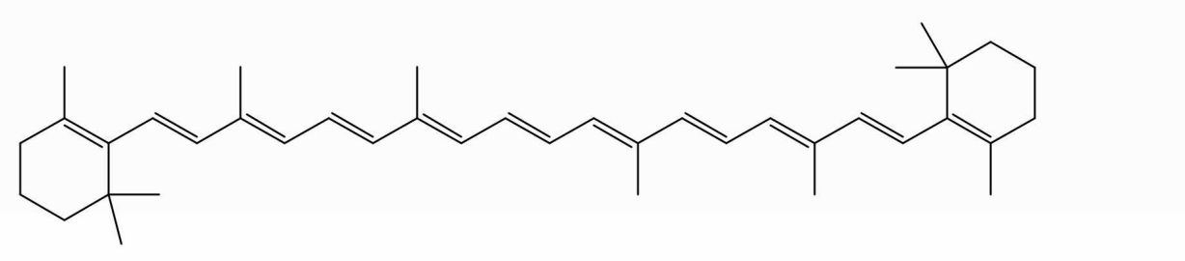
Chemical Formula: C
Exact Mass: 19

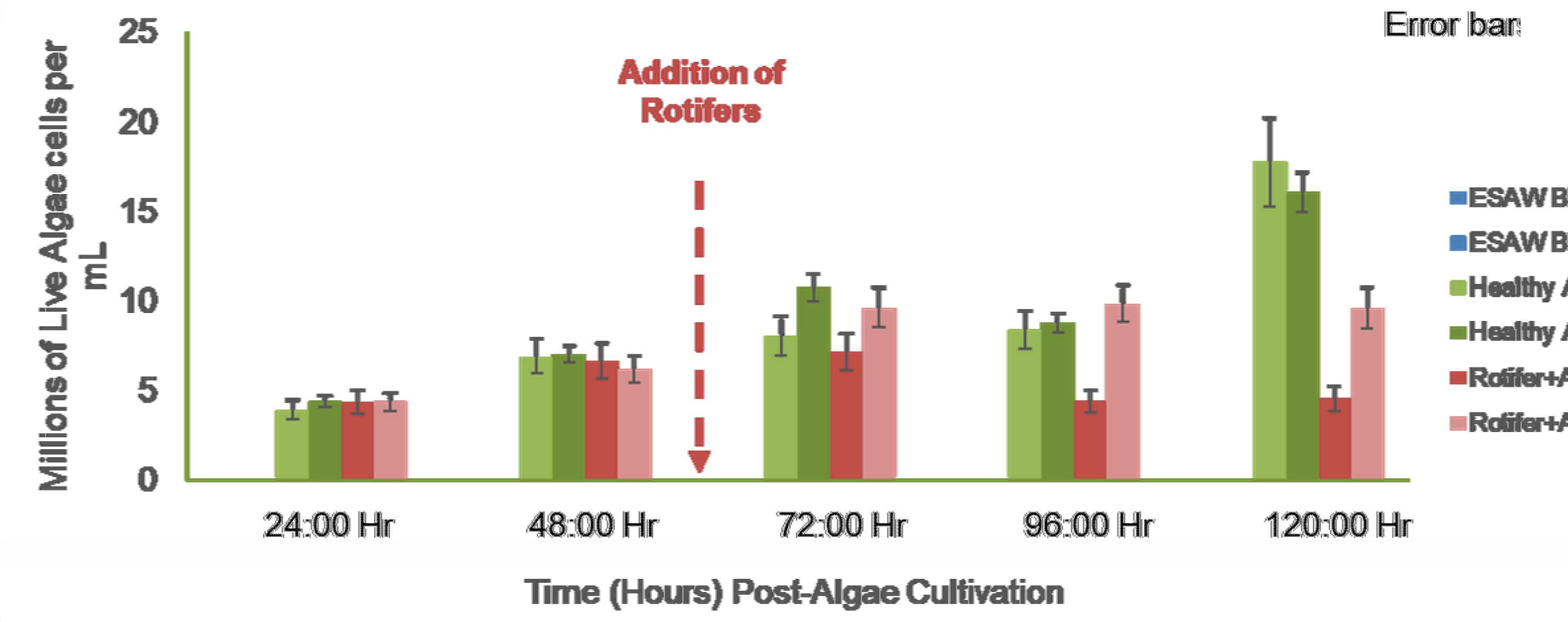
2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde



Chemical Formula: C10H16O
Exact Mass: 152.12

β-carotene





Conclusions

The peak areas of the carotenoid biomarkers were observed to increase when rotifers had consumed greater amounts of algae. Pertinent results will be reported detailing ongoing experiments in our lab, including 1) work with the closely-related algal species *Nannochloropsis gaditana* and *N. oceanica*, 2) additional rotifer and chytrid predators, and 3) evaluating characterized volatile targets in open-environment microalgal systems. Our work aims to increase the breadth and depth of reported algal and rotifer-specific VOCs, providing a tool to better define the chemical environment of microalgal ponds and facilitate greater algal biomass production.

- P2b increases *C. reinhardtii* chlorophyll, biomass and the proportion of multiple cell divisions, indicating pronounced physiological influence
- The effector molecule(s) are likely non-protein, small (<30 kDa) soluble compounds
- Proteomic profiling supports a global change of algal physiology influenced by the bacterium

References & Acknowledgments

[1] Croft, M. T., A. D. Lawrence, E. Raux-Deery, M. J. Warren, and A. G. Smith. 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438: 90–93.

Sandia National Laboratories is a multi-mission laboratory managed and operated by National Technology and Engineering Solutions of Sandia, LLC, a wholly owned subsidiary of Honeywell International, Inc., for the U.S. Department of Energy's National Nuclear Security Administration under contract DE-NA0003525. SAND2019-0896 A. The work was performed, in part, under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. LLNL-ABS-766797.

This research was supported by the LLNL Biofuels Scientific Focus Area, funded by the U.S. Department of Energy Office of Science, Office of Biological and Environmental Research Genomic Science program under FWP SCW1039. Work at LLNL was performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. LLNL-POST-XXXX