UNIVERSITY OF CALIFORNIA SAN DIEGO

Culturing and co-culturing of the nitrogen-fixing cyanobacterium *Nodularia* in nitrogen-deplete media for biotechnological applications

A thesis submitted in partial satisfaction of the

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in

Biology

by

William Franklin Lambert IV

Committee in charge:

Professor James Golden, Chair
Professor Brian Palenik, Co-chair
Professor Jonathan Shurin

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The Thesis of William Franklin Lambert IV is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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Co-Chair

_____

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Dedication

For Mom and Dad

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ABSTRACT OF THE THESIS

Culturing and co-culturing of the nitrogen-fixing cyanobacterium *Nodularia*

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William Franklin Lambert IV

Master of Science in Biology

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Professor James Golden, Chair
Professor Brian Palenik, Co-chair

Algae are a promising feedstock for biofuels, but their growth on a large scale will require vast nutrient and energy inputs. Diazotrophic cyanobacteria, like the genera *Nodularia*, can fix their own nitrogen and reach very high densities in nitrogen-deplete growth media. *Nodularia* can also be co-cultured with diatoms, which can provide not only higher biomass yields than monocultures of either species, but also a wider range of lipid species including ω-3 polyunsaturated fatty acids (PUFAs) like α-linolenic acid, γ-linolenic acid, arachidonic acid, and EPA. Monocultures of *Nodularia* sp. WHNOD and

co-cultures with diatom strains were characterized under laboratory conditions and also at the UC San Diego Biology Field Station in both 100L photobioreactors and 800L ponds. Field station trials were performed over nearly 2 years, indicating that these strains are robust and can thrive in a wide range of conditions.

Introduction

Inexpensive, abundant liquid fuels are a cornerstone of modern society. However, the global rate of fossil fuel consumption far exceeds formation (Shafiee and Topal 2009), and adverse effects on the planet's climate are anticipated from further fossil fuel combustion (Vitousek 1994). Algae could serve as a renewable, nearly carbon dioxide-neutral feedstock for biofuel production, and can be grown on marginal land using seawater, brackish water or even wastewater (Chisti 2008). Algae's ability to grow on non-arable land is significant because it does not compete with food crops, a major issue with most other biofuel feedstocks (Ahmad et al, 2011). An algal culture can often double its biomass in a single day, which is significantly faster than terrestrial plants, like corn or switchgrass (Singh et al 2011). Some species of microalgae produce large quantities of lipids that can be converted to biodiesel and used without significant modifications in a diesel engine (Scragg et al 2003). The lipids can also be converted into "green crude" which is nearly chemically identical to fossil fuel-based crude oil (Kanda and Li 2011). This is not a coincidence, as it appears that prehistoric algae are one of the origins of the fossil fuels we use today (Charles 1916). Unlike other biofuels such as ethanol, algae-based fuels could act as a "drop in" solution that could utilize the current petroleum infrastructure around the world (Robertson et al 2011). One other significant advantage green crude has over ethanol is that it can be used to replace the other fractions of crude oil including petrochemicals (Singh and Gu 2010). These algae-based petrochemicals could be used to make a myriad of products we use each day – everything from plastics to adhesives.

Algae are also a promising source of other value added co-products like nutraceuticals. The cyanobacterium *Spirulina platensis* in particular has been grown on

an industrial scale for decades and its pigments and proteins marketed as health supplements (Colla et al 2007). In addition to pigments and proteins, algae also produce polyunsaturated fatty acids (PUFAs). A subset of PUFAs, the omega-3 fatty acids, are particularly valuable and have been documented to be beneficial to humans (Kris-Etherton et al 2003). Increased consumption of PUFAs has been shown to improve cardiovascular health in older adults (Lemaitre et al, 2003) and improve brain tissue integrity (Youdim et al 1999). Humans are missing the 12- and 15-desaturase enzymes necessary to synthesize α-Linolenic acid (ALA), so it must be obtained from plant-derived sources like oilseeds or algae (Holman 1998). ALA is integral to human health because it is used by the body as a precursor for other PUFAs like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and is considered an "essential fatty acid" (EFA) for this reason (Barceló-Coblijn and Murphy 2009). Oil from cold water fish, especially salmon and sardines, is currently the source of DHA and EPA in many fish oil dietary supplements (Rubio-Rodríguez et al 2010). While fish are able to synthesize DHA and EPA like humans, much of their DHA and EPA is originally biosynthesized by microalgae (Benemann 1992). It has been documented that microalgal PUFAs like EPA and arachidonic acid (AA) are bioconcentrated by zooplankton which are in turn further concentrated when eaten by fish that occupy an even higher trophic level (Kainz et al 2004). However, fish oil is not vegetarian and also further depletes already dwindling stocks of marine fish (Doughman et al 2007). In addition, there has been some debate on the safety of consuming fish oil due to its heavy metal content, especially methylmercury (Smith and Sahyoun 2005). For these reasons, microalgae are very attractive as a nutritional source of PUFAs.

Current biological research of algae-based biofuels and nutraceuticals has taken a number of approaches. Many labs are genetically engineering algae to possess more

desirable traits, while others are “bioprospecting” strains to attempt to identify a strain with a novel or desirable characteristic. Yet another focus is that of ecology. Research of land plants has shown that growing multiple species together can lead to higher biomass yields than growing single species on the same plot of land (Cardinale et al 2007). Similar studies with algae have been carried out and showed that in some cases this was true with algae as well (Shurin et al 2013). Polycultures of algae can also more efficiently remove nutrients from the growth medium than monocultures can as each species can thrive in its distinct niche (Hooper et al 2005). It has been demonstrated that growing multiple algal species together can yield more lipid per liter of culture as well as per cell (Stockenreiter et al 2012).

In addition to nutrient utilization and lipid accumulation, multiple species within a polyculture may provide one another with beneficial compounds. For example, nitrogen-fixing (diazotrophic) cyanobacteria are known to produce and excrete B vitamins (Bonnet et al 2010). Nitrogen-fixing cyanobacteria are also able to convert atmospheric nitrogen (N_2) to ammonia (NH_3) using the enzyme nitrogenase. Nitrogenase is irreversibly inhibited by oxygen, such as that produced by photosystem II, so the cellular machinery associated with nitrogen fixation and photosynthesis must be separated either temporally or spatially (Kumar et al 2010). Unicellular nitrogen-fixing cyanobacteria carry out photosynthesis during the day and fix nitrogen at night, avoiding this conflict (Johnson et al 1998). Filamentous nitrogen-fixing cyanobacteria contain two types of cells – photosynthetically active “vegetative” cells and specialized nitrogen-fixing cells called heterocysts. After differentiation from a vegetative cell, heterocysts lose photosystem II, meaning that oxygen is no longer evolved within the cell and they produce additional cell walls that protect the cell from atmospheric oxygen. It is thought that the heterocysts export glutamine or nitrogen-rich amino acids like arginine or

aspartate to the vegetative cells through a permeable membrane (Mariscal et al 2007). These amino acids are also stored within the cells in a non-protein branched polypeptide called cyanophycin (Meeks and Elhai 2002). It is not known if nitrogenous compounds are actively exported from the heterocysts into the surrounding growth medium, but it is possible that the septum between heterocysts and vegetative cells is not perfect or some form of passive diffusion may occur. In addition, the nitrogenous compounds fixed by diazotrophic cyanobacteria are likely introduced into the growth medium as the filaments die and lyse.

The idea behind co-culturing nitrogen-fixing cyanobacteria and diatoms is to take advantage of the biological phenomenon of nitrogen fixation and use the nitrogen-fixer to "feed" the diatoms nitrogenous compounds. It has been well documented that a marine system like this exists in some species of *Hemiaulus*, *Rhizosolenia* and *Chaetoceros* diatoms that contain endosymbiotic nitrogen-fixing cyanobacteria like *Richelia intracellularis* and *Calothrix rhizosoleniae* (Foster et al 2011). *Richelia* can fix 81–744% more nitrogen than is needed for their own growth and up to 97.3% of the fixed nitrogen is transferred to their diatom partners. There have been some studies detailing the transfer of fixed nitrogen from the marine filamentous cyanobacterium *Trichodesmium* to diatoms (Lee Chen et al 2011). However, it does not appear that there have been any studies using a co-culture of nitrogen-fixing cyanobacteria and diatoms to produce biofuels or nutraceutical compounds.

Materials and Methods

Monoculture isolation or strain collection details

The isolation locations of each strain can be found in Table 1.

16S / 18S rRNA sequencing

Cultures were centrifuged at 7000 RPM and DNA extraction of the resulting pellet was performed either using a DNeasy tissue kit (QIAGEN, Valencia, CA) with modifications (McCarren and Brahamsha 2005) or with a boiling preparation. For the boiling preparation the pellet was resuspended in 20 uL of molecular biology grade water with a small amount of Chelex 100 sodium form beads (CAS Number 11139-85-8) and heated at 100°C in a thermocycler for 20 min. Amplification of the 18S rRNA gene was performed using the Euk328F and EUK329R primers from Moon-van der Staay et al 2000. For cyanobacteria, the 16s rRNA gene and the internal transcribed spacer (ITS) between the 16s rRNA and 23s rRNA genes were amplified using cyanobacterial specific CYA106F (Nubel et al, 1997) and 340R (Janse et al 2003). 25 µL reactions contained 1 uL of DNA, 12.5 µL of GoTaq Green Master Mix (Promega, Madison, WI), 500 nM of each primer, and nuclease free water. Amplification for both 16S and 18S rRNA genes was performed using the touchdown method outlined in Janse et al. After an initial preincubation step at 94°C for 5 min, a total of 30 cycles were performed at 94°C for 1 min, annealing temperature for 1 min, and 72°C for 1 min. In the first 20 cycles, the annealing temperature decreased by 1°C after every second cycle, from 62°C in the first cycle to 52°C in the twentieth. After amplification, products were visualized on an agarose gel and a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) was used to obtain pure DNA. Sequencing was performed by Retrogen Inc (San Diego, CA) with internal primers used as needed. The primers used are shown in Table

2. Table 3 contains the 16S / 18S rRNA sequence of each strain which were determined as part of this thesis. Cultures are unialgal but have heterotrophic bacterial contaminants.

Establishment of co-cultures

Co-cultures were established by inoculating *Nodularia* sp. WHNOD cultures grown in nitrogen- and vitamin-deplete f/2 media (Guillard, 1975) with stock cultures of diatoms grown in nitrogen- and vitamin-replete f/2 media supplemented with silica. Before any experiments were carried out the co-cultures were transferred at least 5 times to ensure no residual nitrogen or vitamins remained from original stock cultures. Co-culture stocks of 50 mL were maintained in 125 mL flasks of nitrogen and vitamin-deplete f/2 media with no agitation at 25°C with constant 30 μE m^{-2} s^{-1} cool white fluorescent light. Some experiments were also carried out in 50 mL flat bottom culture tubes with 20 mL of medium or in 2800 mL Ferbach-style culture flasks with 1000 mL of growth medium. Growth did not appear to differ significantly using different types of glassware (Figure 30). All experiments, unless noted, were carried out in nitrogen- and vitamin-deplete f/2 growth medium. Co-cultures have been continuously maintained in this fashion for 1-2 years, depending on the culture.

Measurements of growth

Growth was tracked using chlorophyll a autofluorescence as a proxy of biomass. In situ chlorophyll a of some cultures was measured in a 10-AU Field and Laboratory Fluorometer (Turner Designs, Sunnyvale, CA) fitted with a red-sensitive photomultiplier and 450 nm excitation and ~670 nm emission filter set. Relative fluorescence units (RFU) of samples were quantified using the discrete sample averaging feature on the

fluorometer. Sample tubes were vortexed for at least 10 seconds prior to measurement to ensure homogeneity. Blank readings from sterile f/2 growth medium were subtracted from samples. In situ chlorophyll a autofluorescence of additional samples was measured using an Aquafluor portable fluorometer (Turner Designs, Sunnyvale, CA). Samples were dispensed into methacrylate cuvettes (Turner Designs cat. no. 7000-959) and mixed well by pipetting prior to measurement with a built-in averaging function. Blank readings of f/2 growth medium were taken and subtracted from samples. It should be noted that the relative fluorescence values obtained using the Aquafluor portable fluorometer are approximately 50 times higher than those obtained using the 10-AU Field and Laboratory Fluorometer. Optical density (OD) readings were taken on a Thermo Spectronic 20 at 750 nm (Thermo Fischer Scientific, Waltham, MA). Samples were vortexed prior to measurement and multiple readings were taken. Like fluorescence, blank values from f/2 media were subtracted from the sample values.

In addition to in vivo measurements, photosynthetic pigments were extracted and quantified using high performance liquid chromatography (HPLC) by Megan Roadman and Dr. Ralf Goericke (SIO). Pigments were extracted using a method adapted from Zapata et al. 2000. 25 mL of culture was filtered onto Whatman GF/F filters, stored at -80°C and extracted in 1.5 mL acetone. An internal standard (canthaxanthin) was added to the samples, which were analyzed on an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a Waters Symmetry C8 column (3.5 µm particle size, 4.6 × 150 mm, silica, reverse-phase; Waters, Milford, MA, USA). Pigments were eluted using a gradient method with two solvents: (A) a mixture of methanol, acetonitrile and an aqueous pyridine solution (0.25 M, pH = 5) (50:25:25 v:v:v); and (B) a mixture of methanol, acetonitrile, and acetone (20:60:20 v:v:v), according to the following times and proportions (time, %A, %B): (0, 100, 0), (12, 60, 40), (36, 0, 100), (38, 0, 100)

and (40, 100, 0). Fucoxanthin was used as the pigment marker for diatoms and an unknown pigment (retention time 32.2 min) was used for *Nodularia* sp. WHNOD. Both marker pigments were only present in one group and not the other.

Particulate organic carbon (POC) and particulate organic nitrogen (PON) measurements were performed by Shonna Dovel using a modified protocol she designed for the CCE-CalCOFI Methods Manual. An ECS 4010 CHNSO Analyzer (Costech Analytical Technologies, INC., Valencia, CA) was used for all measurements.

Outdoor cultivation

Outdoor cultivation was performed at UC San Diego's Biology Field Station. "Bag" photobioreactors were used for cultures grown inside the greenhouse (Figure 10 and Figure 11). These photobioreactors were made using 16" or 10" 10 mil low density polyethylene (LDPE) tubing (Landsberg, Pasadena, CA) hung from pallet racks. 3/8" HDPE tubing with 4 drilled holes at the bend was inserted into each bag and used to bubble air plumbed in from a blower fan. Bubbling rate was adjusted for each bag using a ball valve. Environmental controls in the greenhouse attempted to maintain a temperature of 22 ± 2 °C.

Pond cultures (Figure 12 and Figure 13) were grown in 320 gallon (1210 L) round stock tanks (part no. ARM-10138, Den Hartog Industries, Inc., Hospers, IA) inoculated with 800L of growth medium at a depth of approximately 40 cm. Ponds were mixed using an air stone and airlifters constructed with PVC pipe connected to a blower fan. A system was in place to bubble compressed CO_2 into the cultures, but was not used unless noted. All cultures were grown in nitrogen- and vitamin-deplete f/2 growth medium made using concentrated stocks of Proline f/2 Algae Feed (Pentair Aquatic Eco-

Systems, Inc., Apopka, FL). When CO_2 was used a 20 mM $NaHCO_3$ buffer was added to the growth medium. Sand-filtered seawater was brought from the SIO Pier and 0.2 µm filter-sterilized using a 30µm-15µm-10µm-5µm-0.2µm step-down filtration system. Cultures were harvested using a continuous flow centrifuge at 3000RPM and effluent was returned to ponds unless noted. Weather data was obtained from a Davis Vantage Pro2 weather station installed at the Biology Field Station (weatherunderground.com call sign KCALAJOL7).

Dry weight

47mm Whatman GF/F 0.7 uM glass fiber filters (cat. no. 1825-047) were placed inside folded foil pieces and dried inside glass petri dishes in an electric convection oven at 110°C for 2 hours. The masses of the dried filters and foil were then taken on a Mettler B5 single-pan analytical balance and noted. A Nalgene reusable filter tower (Thermo Scientific cat. no. 300-4100) was thoroughly rinsed with Milli-Q deionized water and a pre-dried GF/F filter was placed in the holder. A culture sample was then completely mixed and 12.5 mL was dispensed into the reservoir above the filter. A vacuum pump was attached to the filter tower and a suction of approximately 15 PSI was applied. As soon as the culture had run through the filter approximately 25 mL of 0.5 M ammonium formate (Sigma-Aldrich cat. no. 156264) was used to wash the filter to remove excess salts. After the wash was completed the filter was removed from the filter tower and placed back in the folded foil piece. Triplicates were always performed per sample. The filters were then dried at 110°C in the electric convection oven overnight. Once the filters were sufficiently dried the filters and foil were again weighed on the Metter B5 analytical balance. The dry weight of each culture was calculated using the following formula:

$$\frac{(M_f - M_i)}{V} = \frac{mg\ dry\ biomass}{liter\ of\ culture}$$

$$\frac{0.49845g - 0.48775g}{12.5\ mL} = 856\ mg/L$$

Fatty Acid Analysis

0.1-1 L of algal culture was centrifuged at 7500 RPM and stored at -80°C until analysis. Pellets were thawed and transesterification of lipid bound fatty acids was carried out by adding an excess of methanolic acid (1M HCl in methanol) to each pellet and incubating in a bead bath at 75 C for 1 hour. Fatty acid methyl esters (FAMEs) were extracted into approximately 2 mL of analytical grade hexane and transferred to 1.5 mL vials with septum. FAMEs were analyzed on an Agilent 7890A GC system connected to a 5975C VL MSD quadrupole MS (EI). Samples were separated on a 60m DB23 Agilent GCMS column using helium as carrier gas and a gradient of 110°C to 200°C at 15°C/min, followed by 15 minutes at 200°C. A final gradient of 200°C to 220°C at 5°C/min with a 1 minute hold at 220°C was applied to ensure all PUFAs had run through the column. Analysis of chromatograms was carried out using Agilent MSD Chemstation E.02.01.1177 software in conjunction with National Institute of Standards and Technology (NIST) library searches.

Hydrothermal liquefaction (HTT) of biomass

Samples of pond biomass grown at the UCSD Biology Field Station were provided to Sapphire Energy Inc and processed using their proprietary hydrothermal liquefaction process.

Toxicity analysis

Crude extracts were prepared using *Nodularia* sp. WHNOD grown in ponds at the field station and *Nodularia* sp. Las Olas grown in the laboratory. Approximately 1 L of very dense culture was centrifuged at 7500 RPM for 15 minutes and the supernatant decanted. The pellet was then transferred to a glass vial and extracted using a 2:1 mixture of dichloromethane (DCM) and methanol for 1 hour. The extract was partially purified with a C18 column and then run on a Thermo Finnigan Surveyor Autosampler-Plus/LC-Pump-Plus/PDA-Plus system coupled to a Thermo Finnigan LCQ Advantage Max mass spectrometer. A nodularin standard was run as well (Enzo Life Sciences cat. no. ALX-350-061-C050). Data were analyzed using the cyanobacterial molecular network developed in Dr. William Gerwick's laboratory (Yang et al, 2013).

18S rRNA deep sequencing analysis

Samples of two replicate *Nodularia* sp. WHNOD and *Nitzschia* sp. TJ-1 ponds were obtained on 27 June 2013 (Day 129 of Figure 19). 1 L of culture was centrifuged at 7500 RPM, the supernatant decanted and pellets frozen at -80C. DNA extraction was performed using a DNeasy tissue kit (QIAGEN, Valencia, CA) with modifications (McCarren and Brahamsha, 2005) and sent to Research and Testing Laboratory (Lubbock, Texas) for 18S rRNA sequencing using the Roche 454 FLX/FLX+ platform. Research and Testing Laboratory performed preliminary analysis of the data (denoising, clustering, trimming and chimera checking) and also carried out taxonomic identification of sequences using the NCBI BLAST database. Most sequences obtained were approximately 425-430 base pairs (bp). Select sequences were aligned in Geneious R6 using the built in Geneious alignment algorithm. Maximum likelihood (ML) trees of

alignments were performed with RAxML GUI 1.3 using the GTR+I+G algorithm and 100 bootstrap replicates.

Total nitrogen (TN) measurements

Nitrogen-deplete f/2 growth medium with normal concentrations of trace metals or f/20 concentrations of trace metals were used. One flask of each was inoculated with *Nodularia* sp. WHNOD, while one of each was not. All cultures were stirred with a magnetic stir bar. Samples of whole culture and filtrate were stored at 4°C in the dark and analyzed by Dr. Christopher Dupont on a Shimadzu TOC/TN analyzer.

Results

Initial Characterization of *Nodularia* sp.

During initial screening of candidates for biofuel feedstocks, the filamentous cyanobacterium *Nodularia* sp. WHNOD (Figure 1) stood out for its rapid growth rate, high maximal density, and ability to fix nitrogen. *Nodularia* has been proposed as an aquaculture organism before and was reported to have a similar crude protein content to *Spriulina* sp., but not as desirable of an amino acid profile (Pushparaj 1995). Additional lab experiments showed *Nodularia* sp. WHNOD to have fairly little change in growth rate across a wide salinity range (Figure 3). Another strain, *Nodularia* sp. Las Olas, was also used in some lab experiments. 16S rRNA analysis of *Nodularia* sp. WHNOD showed it to be most similar to *Nodularia harveyana* strain Bo53. *Nodularia* sp. Las Olas had the highest similarity to a number of *Nodularia spumigena* strains. A maximum likelihood 16S rRNA phylogenetic tree of these *Nodularia* isolates can be found in Figure 2.

Nodularia spumigena has been documented to produce a cyclic nonribosomal peptide called nodularin (Rinehart et al, 1988). Nodularin is similar to microcystin in that both are potent hepatotoxins that can severely damage liver cells and cause hemorrhaging in most animals (Pearson et al, 2010). *Nodularia spumigena* blooms along the North Sea Coast in Germany have been implicated in the death of dogs (Nehring, 1993) and nodularin has also been shown to stimulate liver tumor cell growth in rats (Ohta et al, 1994). Algicidal compounds have been identified in *Nodularia harveyana* (Volk, 2005). The main compound characterized, norharmane, was especially cytotoxic to other filamentous cyanobacteria like *Anabaena lutea* and *Arthrospira laxissima*, but had little to no effect on green algae. Acetone extracts of

Nodularia harveyana have also been documented to inhibit growth of some eubacteria, plant fungal pathogens and rotifers (Pushparaj et al, 1998).

In order to ensure that large scale culture would not pose potential health risks, a toxicity profile of both species was made. Cell extracts of both *Nodularia* sp. WHNOD and *Nodularia* sp. Las Olas grown in f/2 nitrogen- and vitamin-deplete medium had hits for four potential analogs (one of each) of tumonoic acids, malyngamide, hectochlorin and microcolin B. There were no hits for microcystin LR for either strain. Some analogs of malyngamide have been shown to be moderately ichthyotoxic (toxic to fish), slightly cytotoxic and possess antifeedant properties (Tan et al 2000). Hectochlorin is antiproliferative but not directly cytotoxic (Ramaswamy et al 2007). Microcolin B has been shown to be cytotoxic (Koehn et al, 1992) and also possess antifeedant properties (Nagle et al, 1998). Tumonoic acid does not appear to be known to possess any of these properties. Only *Nodularia* sp. Las Olas had hits for a structural analog for nodularin, but not for the standard.

Based on the results of the preliminary toxicity screening, *Nodularia* sp. WHNOD was selected for further trials and grown in “bag” photobioreactors at the UC San Diego Biology Field station. Unlike many of the other strains screened, *Nodularia* sp. WHNOD initially had little problem adapting to the conditions at the field station (growth data not shown).

Ponds of WHNOD were grown during the summer of 2012. One of the nitrogen- and vitamin-deplete f/2 ponds was grown for the duration of the summer and harvested twice, adding new growth medium each time (Figure 4A). The culture was able to recover after each harvest and again reach high densities. Growth rates for the first two

growths were 0.22 d^{-1} and 0.15 d^{-1}, respectively. Dry weight yields can be seen in Figure 4B.

Microscopic examinations of pond cultures revealed fairly low levels of contamination during the experiment. However, diatoms, especially *Cylindrotheca* sp., were almost constantly observed in the culture. This was surprising because the pond was nitrogen- and vitamin-limited.

Lab co-cultures

As a result of these observations, diatom isolates from Dr. Brian Palenik's culture collection were co-cultured with *Nodularia* sp. WHNOD in f/2 nitrogen- and vitamin-deplete medium. A control experiment was also performed by growing the diatom strains in nitrogen- and vitamin-deplete f/2 media with and without *Nodularia* sp. WHNOD (Figure 5). This experiment showed that both *P. tricornutum* CCMP2561 and *Nitzschia* sp. TJ-1 appear to reach higher densities when grown in the presence of *Nodularia* sp. WHNOD (t-test, $p < 0.01$, $p = 0.03$), while *Cylindrotheca* sp. GHCYL appears to reach higher densities when grown without *Nodularia* sp. WHNOD (t-test, $p = 0.02$). f/2 growth medium is seawater-based, so some growth of all diatoms could be expected as seawater contains up to 10 μM nitrate (Coale et al, 1996). However, the growth of *Cylindrotheca* sp. GHCYL is interesting in that *Nodularia sp.* WHNOD actually appears to inhibit its growth.

Cell count based growth data for single diatom co-cultures using WHNOD and *Phaeodactylum tricornutum* CCMP2561, *Cylindrotheca* sp. GHCOL or *Nitzschia* sp. TJ-1 can be seen in Figure 6A. Each species displays different growth kinetics. *P. tricornutum* stood out in lab co-cultures in that it featured a short lag phase, long growth

phase and reached a much higher final density than the other strains. *Cylindrotheca* sp. GHCYL remained in lag phase approximately 5 days longer than *P. tricornutum* and reached stationary phase at a much lower density. *Nitzschia* sp. TJ-1 appeared to have the slowest growth rate and longest lag phase of the three species. Its final density reached that of *Cylindrotheca* sp. GHCYL, but required an additional 25 days to do so. Figure 6B shows that similar growth kinetics were present when all three diatom species were co-cultured together with *Nodularia* sp. WHNOD. Co-cultures were also performed in the laboratory using *Nodularia* sp. Las Olas (Figure 6C). Fluorescence measurements of the cultures also showed the differences in yields among the cultures (Figure 7A/B) although fluorescence is usually a less accurate measure of yield.

In addition to these three species, another diatom, *Amphora* sp. SE4, was grown in co-culture. This strain grows almost exclusively in aggregates, which makes accurate counts in a hemocytometer nearly impossible. To get around this issue a pigment-based approach was used to quantify growth of the individual species within the co-cultures. *P. tricornutum* and *Nitzschia* sp. TJ-1 individual co-cultures were also analyzed in this fashion (Figure 8). These experiments were performed in f/2 nitrogen-deplete and vitamin-replete growth medium. *P. tricornutum* appeared to grow rapidly, reaching stationary phase after 10 days. *Amphora* sp. SE4 grew similarly to *Cylindrotheca* sp. in that its growth rate was rapid initially, but reached a fairly low final density. *Nitzschia* sp. TJ-1 exhibited a slower growth rate than the other strains, but grew to a higher final density.

The pigment data from day 17 of this experiment were used to back-calculate approximate POC (particulate organic carbon) and PON (particulate organic nitrogen) contributed by the diatoms in co-cultures. POC, PON and pigment data of monocultures

of each strain were used to establish relationships that were applied to co-cultures. An example calculation for diatom carbon in the *Nodularia* sp. WHNOD and *Nitzschia* sp. TJ-1 co-culture is shown below.

Nitzschia sp. TJ-1 monoculture POC to pigment marker relationship:

$$\frac{50618.49\ \mu g\ carbon/_{L}}{736.90\ \mu g\ fucoxanthin/_{L}} = 68.69\ \frac{\mu g\ carbon}{\mu g\ fucoxanthin}$$

Co-culture fucoxanthin measurement to carbon calculation:

$$8.56\ \mu g\ fucoxanthin/_{L} \times 68.69\ \frac{\mu g\ carbon}{\mu g\ fucoxanthin} = 588.27\frac{\mu g\ carbon}{L}$$

Nodularia sp. WHNOD monoculture POC to pigment marker relationship:

$$\frac{32687.27\ \mu g\ carbon/_{L}}{58.35\ \mu g\ cyano\ pigment/_{L}} = 560.16\frac{\mu g\ carbon}{\mu g\ cyano\ pigment}$$

Co-culture cyanobacterial pigment marker measurement to carbon calculation:

$$65.43\ \mu g\ cyano\ pigment/_{L} \times 560.16\ \frac{\mu g\ carbon}{\mu g\ cyano\ pigment} = 36652.39\frac{\mu g\ carbon}{L}$$

Calculating POC contribution by *Nitzschia* sp. TJ-1 in co-culture

$$\frac{588.27\frac{\mu g\ carbon}{L}}{36652.39\frac{\mu g\ carbon}{L}} \times 100\% = 1.58\%$$

Calculations for PON were carried out in a similar fashion. In addition to POC and PON, diatom chlorophyll a contribution was also calculated. An example calculation for the same the *Nodularia* sp. WHNOD and *Nitzschia* sp. TJ-1 co-culture timepoint is as follows:

Nitzschia sp. TJ-1 monoculture chlorophyll a to pigment marker relationship:

$$\frac{1566.21\ \mu g\ chlorophyll\ a/_{L}}{736.90\ \mu g\ fucoxanthin/_{L}} = 2.13\ \frac{\mu g\ chlorophyll\ a}{\mu g\ fucoxanthin}$$

Co-culture fucoxanthin measurement to chlorophyll calculation:

$$8.56\ \mu g\ fucoxanthin/_{L} \times 2.13\ \frac{\mu g\ chlorophyll\ a}{\mu g\ fucoxanthin} = 18.20\frac{\mu g\ chlorophyll\ a}{L}$$

Measured chlorophyll a for co-culture timepoint to calculating chlorophyll a contribution by *Nitzschia* sp. TJ-1:

$$\frac{18.20\frac{\mu g\ chlorophyll\ a}{L}}{1114.93\frac{\mu g\ chlorophyll\ a}{L}} \times 100\% = 1.63\%$$

Figure 9 shows a summary of these calculations for the 3 diatom strains. Each diatom species ranges from approximately 0.5%-2% of the total biomass in the co-cultures with *P. tricornutum* and *Amphora* sp. SE4 on the low end and *Nitzschia* sp. TJ-1 on the higher end.

Photobioreactor co-cultures

In addition to lab experiments, co-cultures were grown at the Biology Field Station on UC San Diego's campus. Initially trials in "bag" photobioreactors were carried out. Figure 10 and Figure 11 are photographs of co-culture bags growing in the greenhouse. Figure 16 compares the fluorescence of single diatom co-cultures of *P. tricornutum*, *Cylindrotheca* sp. GHCYL or *Nitzschia* sp. TJ-1 and a mixture of all three to a monoculture of *Nodularia* sp. WHNOD. This figure shows a much different picture than the lab trials. Both the *P. tricornutum* and *Cylindrotheca* sp. GHCYL single co-cultures contained very similar or slightly lower chlorophyll autofluorescence than the *Nodularia* sp. WHNOD monocultures while the *Nitzschia* sp. TJ-1 co-culture contained significantly more chlorophyll autofluorescence than the *Nodularia* sp. WHNOD monoculture. A similar trend is visible in the mixed diatom co-culture. Cell counts of the single diatom co-cultures (Figure 17A) and mixed diatom co-cultures (Figure 17B) also show that *Nitzschia* sp. TJ-1 reached the highest density of the diatoms grown in the greenhouse experiments. Initial growth rates calculated using fluorescence can be found in Table 4. Figure 18 contains the dry weight yields of these greenhouse co-cultures. The final yield of the *Nitzschia* sp. TJ-1 co-culture was higher than the *Nodularia* sp. WHNOD monoculture (t-test, $p < 0.01$). Co-cultures were also grown with supplemental CO_2 (Figure 14). Additional CO_2 did not significantly increase the growth rates (Figure 15) for either monocultures of *Nodularia* sp. WHNOD (t-test, $p = 0.12$) or co-cultures with *Nitzschia* sp. TJ-1 (t-test, $p = 0.84$).

Pond co-cultures

After trials in bags, two replicate *Nitzschia* sp. TJ-1 and *Nodularia* sp. WHNOD co-culture ponds were grown from mid February to early July 2013 (Figure 19). After

approximately 30 days the ponds were contaminated with a *Cylindrotheca* sp. that appeared very similar morphologically to the strain that was previously isolated from the field station and used in earlier lab and greenhouse trials. Other diatom species were visibly present in both of these cultures, but not in large enough numbers to allow manual counting.

Four more ponds, two *Nodularia* sp. WHNOD monoculture ponds and two *Nitzschia* sp. TJ-1 and *Nodularia* sp. WHNOD co-culture ponds, were started in early October 2013 (Figure 20). Figure 20E shows that the fluorescence values of the co-culture ponds were higher than those of the monoculture ponds, which could indicate the presence of diatoms in the co-cultures. Low numbers of small (2-4 μm) pennate diatoms were present in the WHNOD monoculture ponds. Similarly to previous greenhouse "bag" experiments, *Nitzschia* sp. TJ-1 growth tracked very closely to growth of *Nodularia* sp. WHNOD. On day 26 all four ponds were partially harvested and effluent added back, but only ponds 3 and 4 were able to fully recover afterwards. Dry weight measurements taken during the course of growth indicate that the overall biomass in each pond was relatively similar, as can be seen in Figure 21.

Analysis of 18S rRNA deep sequencing data

During the growth of the 2 replicate *Nodularia* sp. WHNOD and *Nitzschia* sp. TJ-1 co-culture ponds in winter and spring 2013, contaminating species, especially Cylindrotheca sp., had been observed after about one month of growth (Figure 19). During this period Pond 1 had consistently lower chlorophyll a autofluorescence and reached lower densities than Pond 2. On the sampling date (Day 129 of Figure 19) very few *Nitzschia* sp. TJ-1 had been microscopically observed in either pond, but *Cylindrotheca* sp. and numerous other diatoms were observed.

Figure 22A shows a breakdown of the 18s rRNA sequences obtained (3981 for Pond 1 and 4926 for Pond 2). Diatom sequences made up over 50% of the total sequences in Pond 1 and over 80% of the total sequences in Pond 2. Thraustochytrids made up the next largest group of sequences. Thraustochytrids are not very well studied, but are fairly common in the marine environment and appear to be involved with nutrient cycling and breakdown of detritus (Raghukumar 2002). Heterotrophic flagellates also had high representation. Most of these are likely bactivores or graze on small phytoplankton (Hall et al 1994). No ciliate sequences were found, but a small number of amoebal sequences from the genera *Vannella* and *Nolandella* were found. Amoeba are a major predator of algae and much work has been devoted lately to their grazing behavior (Simkovsky et al 2012). Few green algal sequences were recovered. The only representatives were *Chlorella* and *Nannochloris*. Of the diatom sequences (Figure 22B) all but one genus, *Chaetoceros*, were raphid pennate diatoms. A phylogenetic tree using the 18S rRNA sequences from these data and those of the lab strains can be seen in Figure 23. The *Cylindrotheca* sequences obtained appear to be very similar to that of *Cylindrotheca* sp. GHCOL as do the *Nitzschia* sequences for that of *Nitzschia* sp. TJ-1. However, the *Nitzschia* sequences differed from *Nitzschia* sp. TJ-1 by 4 bp, which could indicate that the *Nitzschia* diatoms in the pond after 129 days were not the same strain that was initially inoculated.

Fatty acid methyl ester (FAME) profiles

FAME profiles of lab monocultures (Figure 25A) were first obtained to establish baseline measurements without the worry of contamination. From these data, it was shown that *Nodularia* sp. WHNOD produced mainly C16:0, C16:1 and C18:2 fatty acids. In addition to these, WHNOD also produced C18:1, C18:2, C18:3, C18:4 and low levels

of C20:4 (arachidonic acid). Of particular note is C18:3 because both α-Linolenic acid and γ-Linolenic acid were produced. *Nodularia* sp. WHNOD did not produce any C14 derivatives, polyunsaturated C16 derivatives, or EPA. Like *Nodularia* sp. WHNOD, the fatty acid profile of *Nitzschia* sp. TJ-1 was comprised in large part of C16:0 and C16:1 but with the addition of C14:0 and higher levels of C20:4. *Nitzschia* sp. TJ-1 also produced polyunsaturated C16 derivatives and EPA. There was a noticeable shift between early and late stationary phase in the lipid profile of *Nitzschia* sp. TJ-1 from unsaturated fatty acids like C16:0 and C18:0 to polyunsaturated fatty acids like C20:4 (arachidonic acid) and C20:5 (EPA). Similar shifts were visible in the fatty acid profiles of both bags (Figure 25B) and ponds (Figure 25C). It is interesting to note that in co-cultures the lipids specific to *Nitzschia* sp. TJ-1 like C14:0, C16:2-4 and C20:5 (EPA) made up such a large amount of the total fatty acids even though the diatoms in the co-cultures did not make up a large portion of the total biomass.

Hydrothermal liquefaction (HTT) of biomass

HTT extracts more hydrocarbons from biomass and is becoming a more favored method of extraction over traditional methods (Biller and Ross, 2011). Figure 26 shows the hydrothermal liquefaction (HTT) yield percentage of biomass grown in ponds with the assistance of Dr. Shuyi Wang and Alice Tung. The yield for a *Nodularia* sp. WHNOD and *Nitzschia* sp. TJ-1 co-culture was higher than the yield for a *Nitzschia* sp. TJ-1 monoculture (t-test, $p = 0.01$), but not significantly higher than a *Nodularia* sp. WHNOD monoculture (t-test, $p = 0.06$). However, the *Nodularia* sp. WHNOD monoculture likely had some diatom contamination, masking the difference between it and the co-culture. In addition, the yield of the co-culture is higher than some eukaryotic algae like

Chaetoceros sp. SECHAET (t-test, $p > 0.01$), but still lower than that of the small green algae *Picocystis* SP. SENEW3 (t-test, $p > 0.01$).

Total nitrogen (TN) measurements

Figure 27 shows the TN production of *Nodularia* sp. WHNOD. The trace metal mixture in f/2 growth medium contains ethylenediaminetetraacetic acid (EDTA), a nitrogen containing compound, so cultures were grown with normal and 1/10 trace metal concentrations to lower the effects of EDTA on the measurements. None of the treatments tested appeared to have biological dissolved organic nitrogen (DON) production above baseline detection for the instrument (~2.3 μM TN). Thus it does not appear that WHNOD excretes nitrogen into the media at least in the absence of diatoms.

Discussion

The experiments in Figure 5 show that *P. tricornutum* and *Nitzschia* sp. TJ-1 both reach higher cell densities when co-cultured with *Nodularia* sp. WHNOD, indicating that the cyanobacterium appears to be providing the diatoms with something they are limited for. However, such is not the case for *Cynlindrotheca* sp. GHCYL which could mean that it is inhibited by *Nodularia* sp. WHNOD or competing with it for nutrients. *Cynlindrotheca* sp. GHCYL was isolated as a contaminant at the field station, so its physiology or use of nutrients may be different than the other diatom species that are adapted for other environs. In addition, *Nitzschia* sp. TJ-1 has protease activity (Dr. Shuyi Wang, data not shown), which could allow it to break down detritus in the growth medium more effectively than *Cylindrotheca* sp. GHCYL. It may even be possible that *Nitzschia* sp. TJ-1 can hydrolyze surface associated proteins on *Nodularia* sp. WHNOD and use nitrogenous compounds from those to grow.

An important note to make is that none of the cultures used in these experiments were axenic. However, experiments performed at the field station were in a more or less open system and bacteria would likely be present in any algal biofuels growth system. Bacteria have been well-documented to play a key role in global nitrogen cycling. Some bacteria can fix nitrogen and others have the ability to oxidize ammonia to nitrite and finally to nitrate (Koops and Pommerening-Roser 2001). It is possible that ammonia produced by bacteria could be more readily accessible to the diatoms than what is produced by *Nodularia* sp. WHNOD. Regardless of the origin of the ammonia, nitrifying bacteria could have been providing the diatoms with nitrate in addition to what is naturally found in seawater. In addition, it has been documented that the Na_2-EDTA used as a chelator in f/2 trace metals will degrade by approximately 50% after 12 days of

exposure to sunlight and by 75% after 25 days (Metsärinne et al 2001). The EDTA in f/2 contains approximately 1.12 μM nitrogen, so it is possible that this nitrogen source may play a role as well. The TN measurements in Figure 27 show that there was some background dissolved organic nitrogen (DON) in the media blanks. However, the DON of the culture filtrate was not significantly higher than background, which makes the role of *Nodularia* sp. WHNOD or its associated bacteria unclear. This experiment was only performed one time without replicates, so it is possible that different results may have been obtained if it was repeated and measurement conditions optimized.

In some of the lab experiments a number of assumptions and simplifications were made. One of these has to do with the pigment and POC/PON data. While analyzing the pigment data it was assumed that pigment concentration per cell remains constant throughout growth and that the values are comparable between monocultures and co-cultures. It has been documented that during nitrogen stress chlorophyll fluorescence per cell increases in *Cyclotella nana* (Kiefer, 1973) while a four-fold reduction of chlorophyll a per cell occurs in *P. tricornutum* (Osborne and Geider 1986). This means that the cellular carbon to pigment or nitrogen to pigment ratios used to estimate diatom biomass may have severely over or underestimated the actual values.

One of the most noticeable disparities in the experiments performed is the difference in growth of the diatoms in the laboratory and the field station. This is likely due to a number of factors. While light and temperature in the laboratory were constant, these factors were quite variable at the field station. Irradiance at the field station can reach upwards of 1500 μE m^{-2} s^{-1} and may only be a fraction of that the next day due to cloud cover. In addition, cultures at the field station are only exposed to light for 8-12 hours a day compared to 24 hours a day in lab. Light limitation is also something to

consider. Cultures grown in the photobioreactors were well mixed, but reached OD values greater than 1, which would indicate that significant shading was occurring. The ponds tended to settle near the edges and middle, so manual mixing was required each day. This meant that some of the biomass was constantly light limited as only a small proportion of total irradiance would have penetrated 40 cm to the bottom of the pond.

Temperature was also likely a major factor. While the laboratory incubator maintains a constant temperature of 24°C, the field station was highly variable. Cultures were grown all times of year, with lows outside reaching 2°C during the winter and highs commonly reaching 30°C during the summer. Inside the greenhouse was slightly less variable due to the environmental controls, but temperatures routinely dropped well below or above the set values of 22 ± 2 °C. *Nitzschia* sp. TJ-1 did not fare well in ponds during cooler weather (Figure 19), indicating that it may be better suited for cultivation as a summer or fall strain. The deep sequencing data of the winter / spring ponds shown in Figure 22 indicated that other genera of diatoms, namely *Cymbella* or *Achnanthes,* may be better suited for growth during the colder times of year.

In addition, cultures in the lab were mostly grown in flat bottom 50 mL culture tubes or 125 mL flasks with no agitation, while cultures at the field station were grown with vigorous bubbling to ensure settling did not occur. This bubbling appeared to break the filaments of WHNOD into chains which were smaller than those normally observed in the laboratory. Laboratory cultures could have been grown with stirring, but that would introduce shear forces which are not present with bubbling. The particular bubbling dynamics of the field station would have been difficult to replicate in the lab as well.

Predation and competition are also things to consider at the field station. During the course of nearly two years of experiments, active grazing (by an amoeba) was only

observed on *Nodularia* sp. WHNOD on a single occasion. However, a number of bags of *Nodularia* sp. WHNOD crashed due to what appeared to be a bacterial infection. The crashes were associated with a dramatic increase of background bacterial levels. Healthy cultures treated with a 10% addition of infected culture had inhibited growth, while cultures treated with a 10% addition of a 0.2 μm filtrate of infected culture did not appear to be affected (Figure 29). *Nitzschia* sp. TJ-1 cultures were nearly cleared by contaminating amoeba on a number of occasions, both in bags and ponds. This seemed to occur more regularly in monocultures, but still occurred in co-cultures.

Growing a diazotrophic cyanobacterium like *Nodularia* sp. WHNOD on a large scale has a number of advantages over eukaryotic algae. Perhaps the main advantage is the ability to grow large quantities of biomass without adding additional nitrogen to the growth medium. This provides not only monetary savings, but energy savings as well, making the net energy output of the system much higher. Fixed nitrogen is currently produced on an industrial scale using the Haber-Bosch process which consumes 1-2% of the world's energy supply annually (Barry 2002). Nutrient use, especially nitrogen, is often the topic of algal biofuels life cycle analyses (LCAs) and it has been estimated that a 1 ha pond of eukaryotic *Dunaliella tertiolecta* would require 615 kg of N-P-K-S fertilizer and 815 kg of supplemental urea on an annual basis (Campbell et al 2009). In addition, further increases of industrial production of ammonia or urea would have detrimental effects on the environment by accelerating the global nitrogen cycle and introducing additional emissions of oxidized nitrogen (NO_x) into the atmosphere (Erisman et al 2010).

However, because diazotrophic cyanobacteria need to shunt carbon into synthesizing nitrogenase and the cellular machinery associated with nitrogen fixation

they do grow more slowly than non-nitrogen fixing microalgae. Although their growth rate is slower, growing diazotrophic cyanobacteria in nitrogen-deplete media allows them to easily outcompete eukaryotic algae. No qualitative data for prokaryotic abundance was obtained, but microscopic examinations of the cultures revealed that there was fairly little cyanobacterial contamination in the cultures, even from other diazotrophic genera, which could be due to algicidal compound production like those synthesized by *Nodularia harveyana.* In addition, even after four months of growth in a pond, few to no amoeba or ciliates were present in the cultures either, as evidenced by Figure 22. Lastly, *Nodularia* sp. WHNOD harvests exceptionally well using continuous centrifugation, even without the use of a flocculant. This is in contrast to smaller microalgae.

Each of these characteristics indicates that a *Nodularia* sp. WHNOD monoculture could be a good feedstock for either biofuels or nutraceuticals. However, growing a true *Nodularia* sp. WHNOD monoculture at the field station proved difficult. Various genera of diatoms were nearly always present in the cultures. This indicated that monocultures of *Nodularia* sp. WHNOD left a niche open that some diatoms were very readily able to fill. Rather than fighting against this, it would make more sense to fill this niche with a diatom strain that could produce desirable lipid species or other value added co-products. Co-culturing experiments in both the laboratory and at the field station showed that the chlorophyll a autofluorescence of co-cultures was often higher than that of *Nodularia* sp. WHNOD monocultures. This information, coupled with some of the dry weight data obtained, indicated that there was usually equal and sometimes higher biomass in co-cultures compared to monocultures. It appeared that only in the case of co-culturing *Nodularia* sp. WHNOD with *Cylindrotheca* sp. GHCOL that there can be lower total biomass with co-cultures, which means that the diatom strain used in co-

culture should be chosen carefully. In addition, co-cultures in the laboratory were attempted with the model centric diatom *Thalassiosira pseudonana*, but the diatoms went extinct in the culture after a few weeks. As both lab experiments and the deep sequencing data indicated, pennate diatoms appeared to be better suited for growth in co-culture with *Nodularia* sp. WHNOD.

In addition to biomass differences, co-cultures also had a markedly different lipid profile than monocultures. In the case of *Nodularia* sp. WHNOD, it did not produce EPA and only produced low levels of arachidonic acid, but did produce both α-linolenic acid (ALA) and γ-linolenic acid (GLA). The diatom *Nitzschia* sp. TJ-1 produced high levels of EPA and arachidonic acid, but not very significant levels of GLA or ALA. However, the combination of the two yielded a fairly comprehensive lipid profile, containing many desirable PUFAs and ω-3 fatty acids that are not often seen in a single organism (Lang et al 2011). While the diatoms did not make up a large proportion of the biomass in a co-culture, their lipid contribution was significant. Other methods of hydrocarbon extraction like hydrothermal liquefaction demonstrated that the yield of a *Nodularia* sp. WHNOD and *Nitzschia* sp. TJ-1 co-culture was higher than that of a *Nitzschia* sp. TJ-1 monoculture, but not significantly different than a *Nodularia* sp. WHNOD monoculture. This lack of significance could have been affected by diatom contamination in the monoculture pond. The HTT yield of the co-culture was also very comparable, and sometimes higher, than those of eukaryotic algae monocultures.

While not a perfect system, large scale co-culturing of diazotrophic cyanobacteria and diatoms provides a number of advantages over strictly eukaryotic monocultures. Nutrient, and therefore energy, inputs are lower, contamination can be minimized without

the addition of pesticides or fungicides, and a wide range of lipids and other co-products like pigments can be produced in a single culture.

Figures

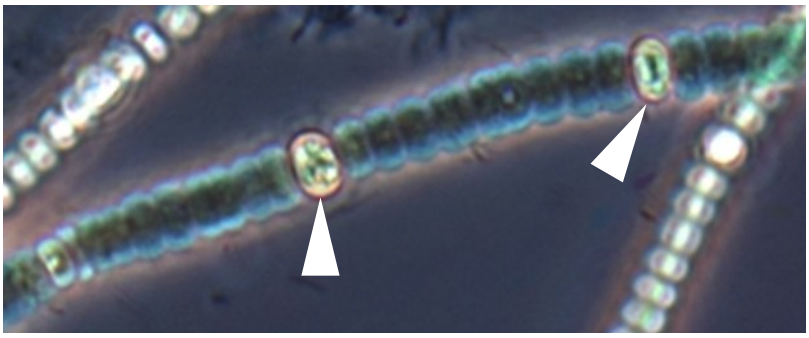


Figure 1 Brightfield image of *Nodularia* sp. WHNOD. Heterocysts denoted by white arrowheads.

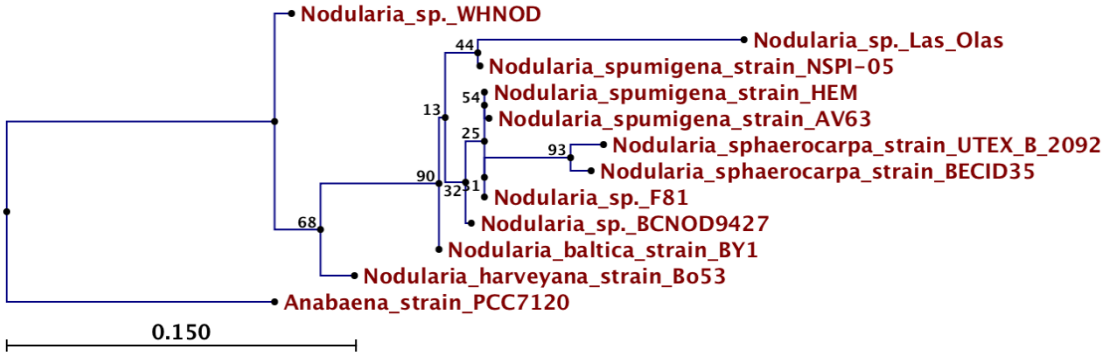


Figure 2 Maximum likelihood 16S rRNA phylogenetic tree of *Nodularia* isolates. Scale bar represents average number of amino acid replacements per nucleotide site.

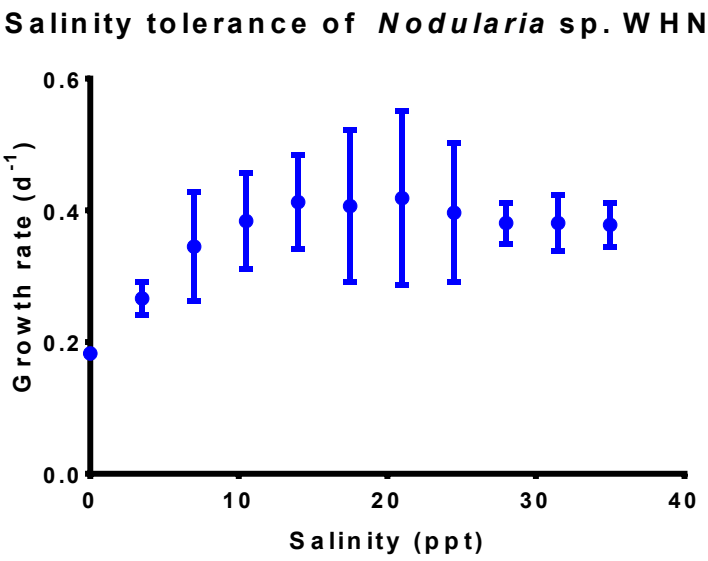


Figure 3 Salinity tolerance of *Nodularia* sp. WHNOD grown in the laboratory

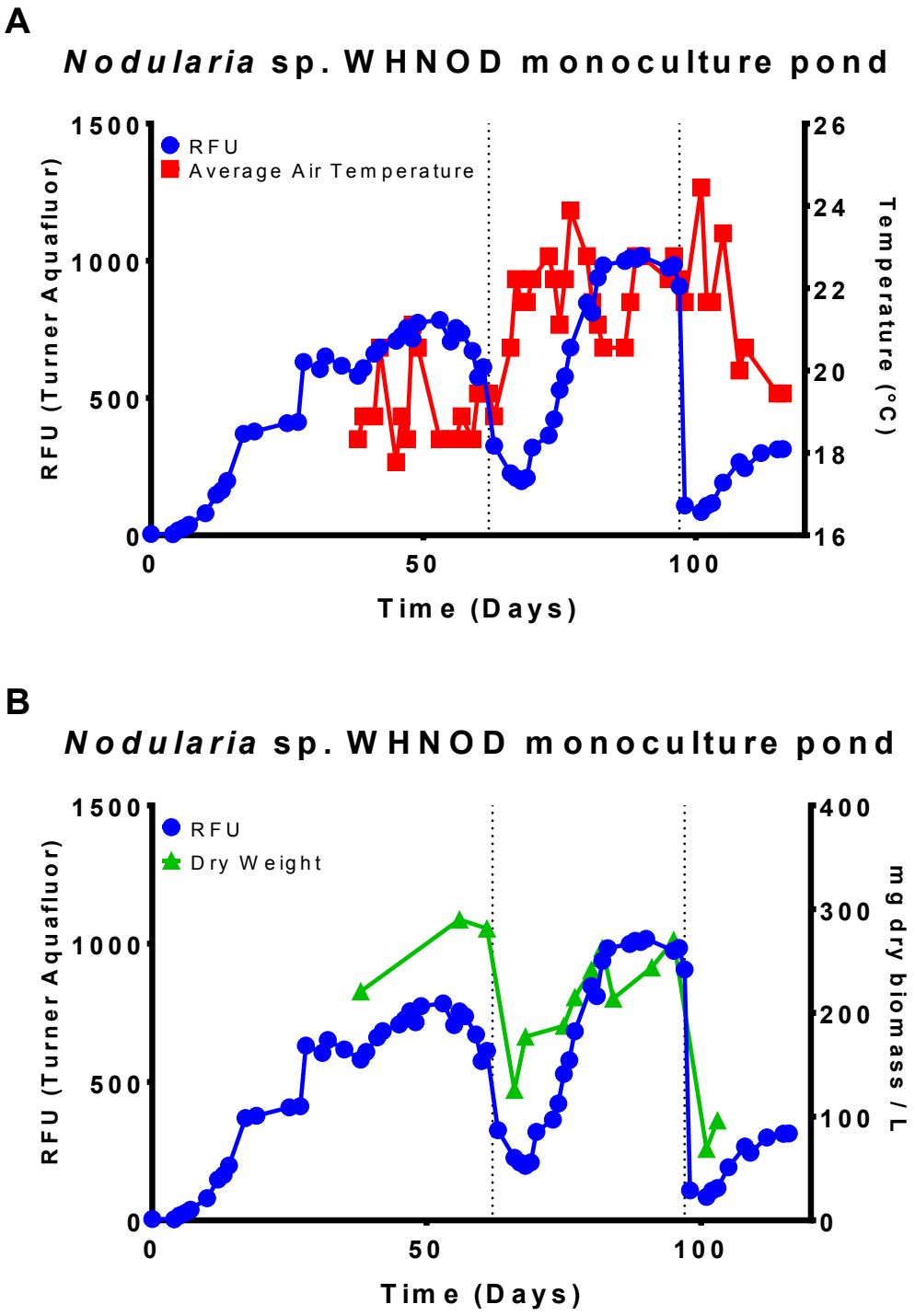


Figure 4 *Nodularia* sp. WHNOD monoculture pond grown at the Biology Field Station in summer 2012. Harvest points denoted by dotted lines.

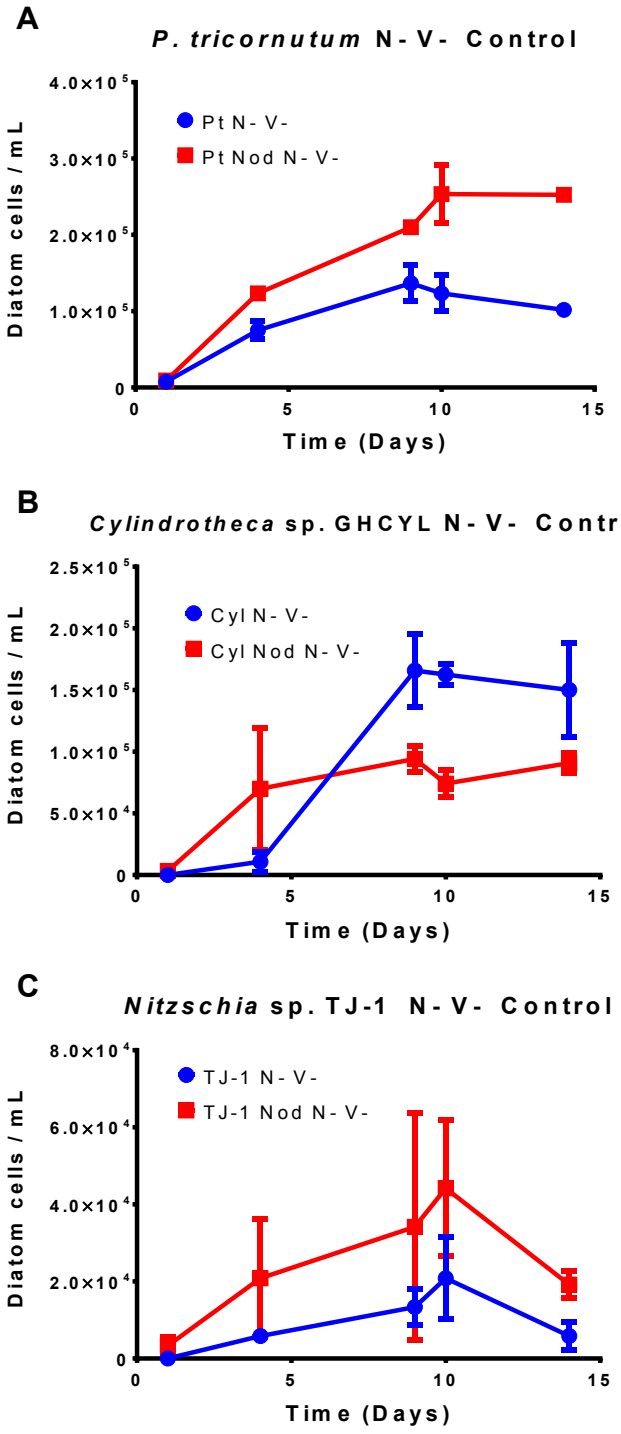


Figure 5 Control experiment of diatoms grown in nitrogen- and vitamin-deplete f/2 media with and without *Nodularia* sp. WHNOD in the lab.

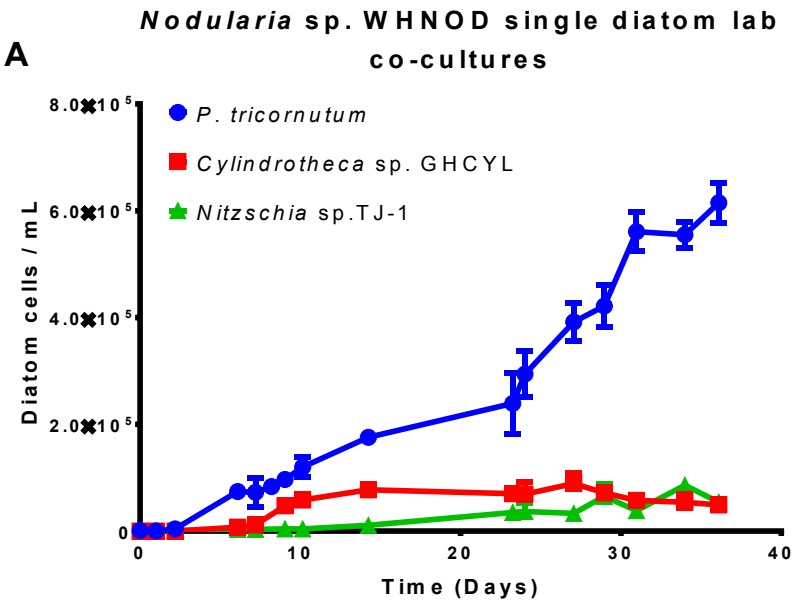


Figure 6A Cell counts of single diatom species co-cultured with *Nodularia* sp. WHNOD in nitrogen- and vitamin-deplete f/2 medium in the lab.

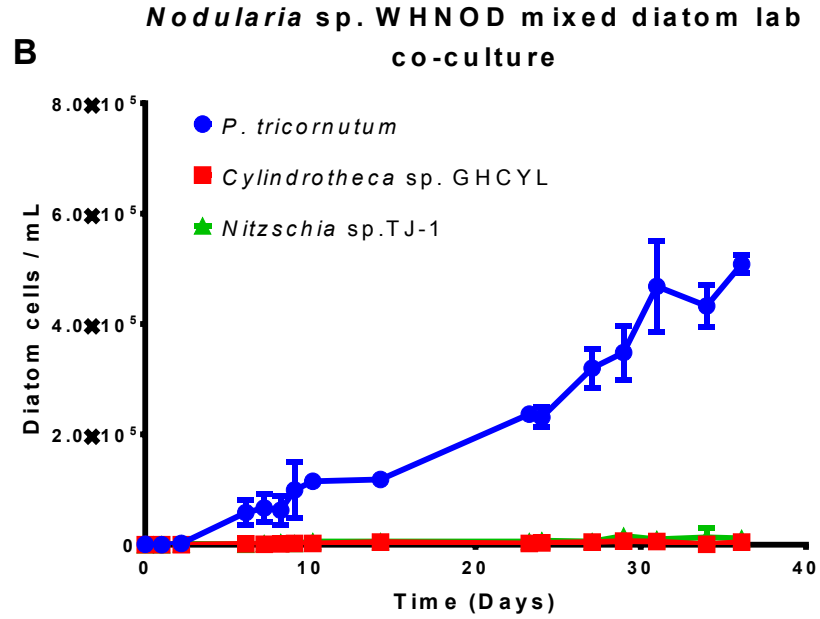


Figure 6B Cell counts of three diatom species co-cultured with *Nodularia* sp. WHNOD in nitrogen- and vitamin-deplete f/2 medium in the lab.

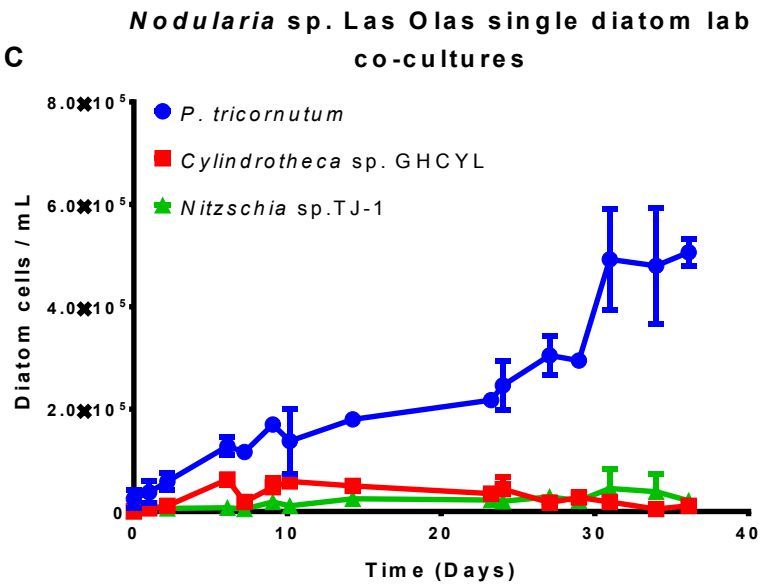


Figure 6C Cell counts of single diatom species co-cultured with *Nodularia* sp. Las Olas in nitrogen- and vitamin-deplete f/2 medium in the lab.

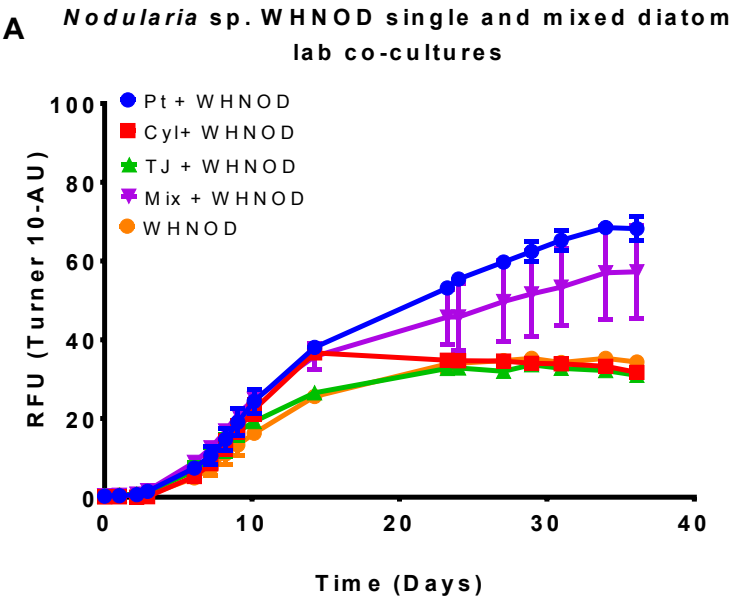


Figure 7A Fluorescence measurements of co-cultures of *Nodularia* sp. WHNOD with single and mixed diatoms in the lab. *Nodularia* sp. WHNOD monoculture shown for reference.

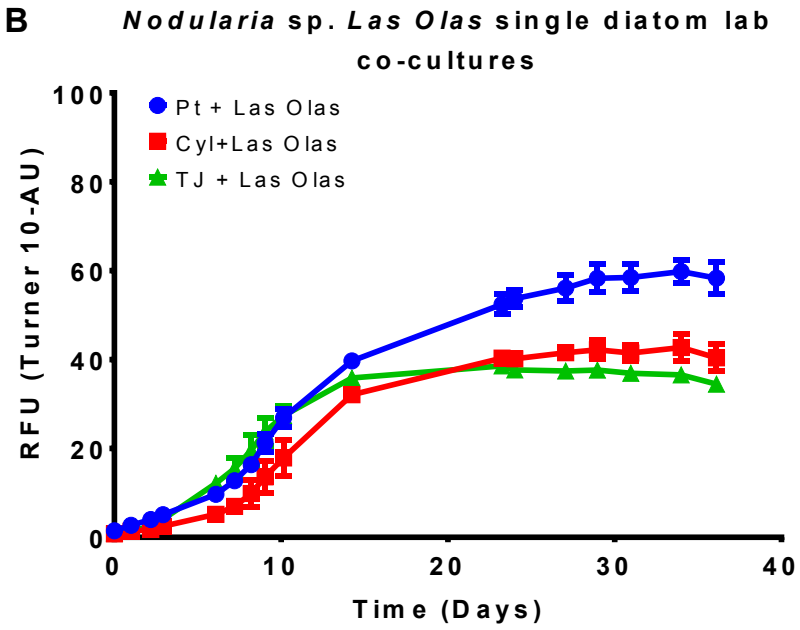


Figure 7B Fluorescence measurements of *Nodularia* sp. Las Olas co-cultured with single diatoms in the lab.

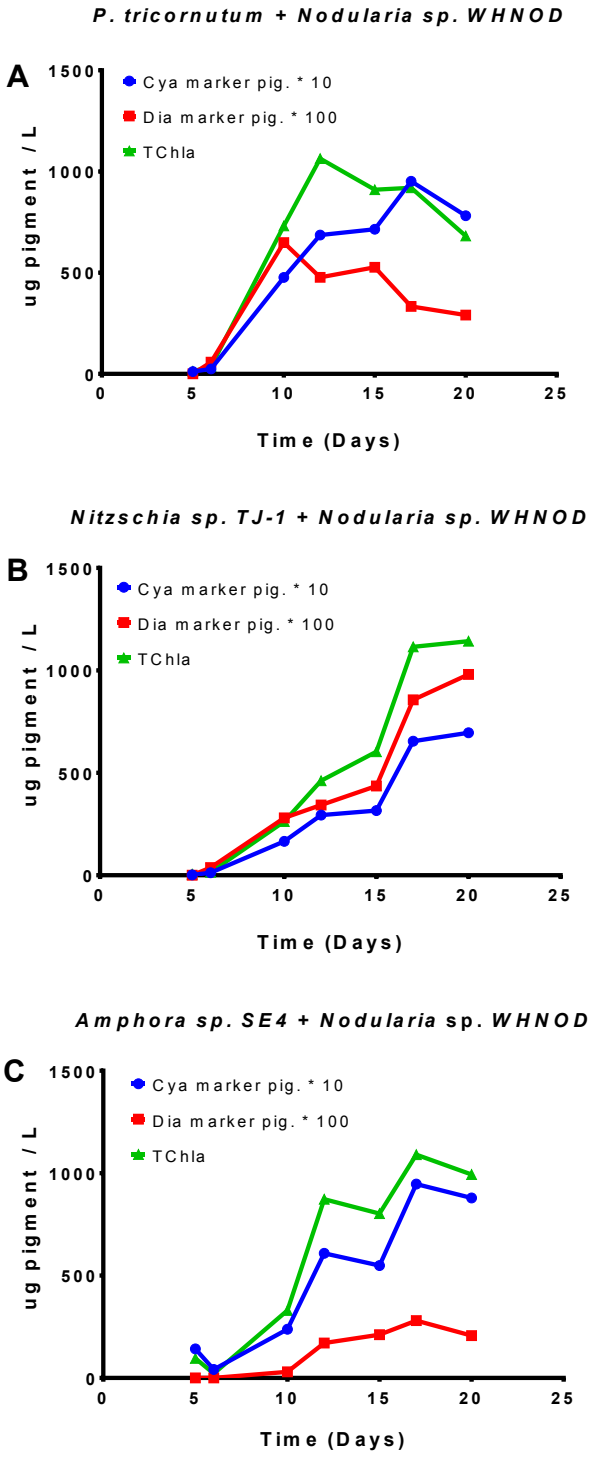


Figure 8 Use of pigment markers to track growth of individual species within co-cultures in the lab.

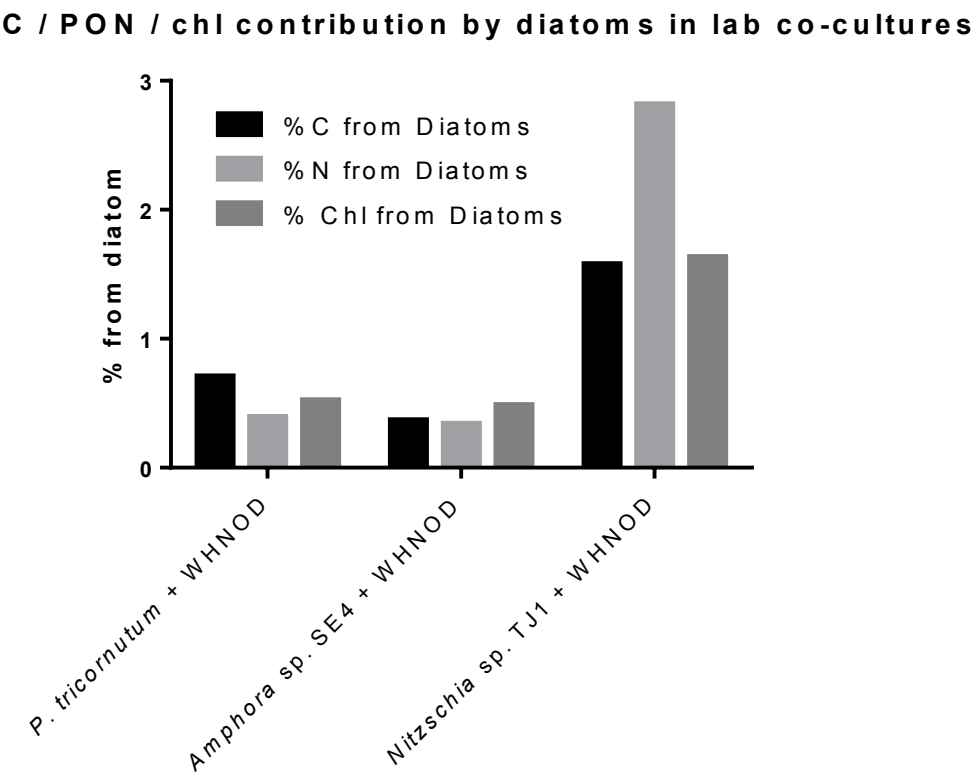


Figure 9 Use of POC / PON and pigment data to quantify diatoms in co-culture

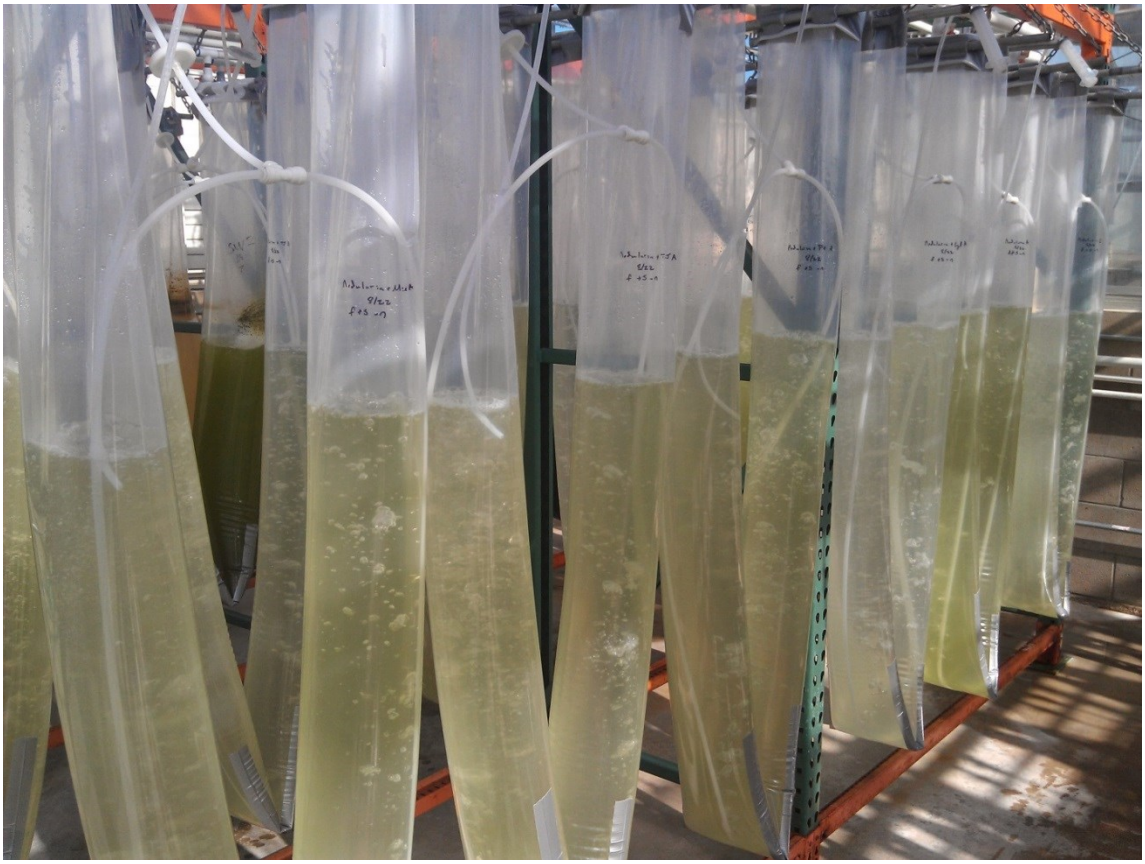


Figure 10 Photograph of *Nodularia* sp. WHNOD and diatom co-cultures in “bag” photobioreactors 5 days after inoculation. *Nodularia* sp. WHNOD monoculture is second from the right.

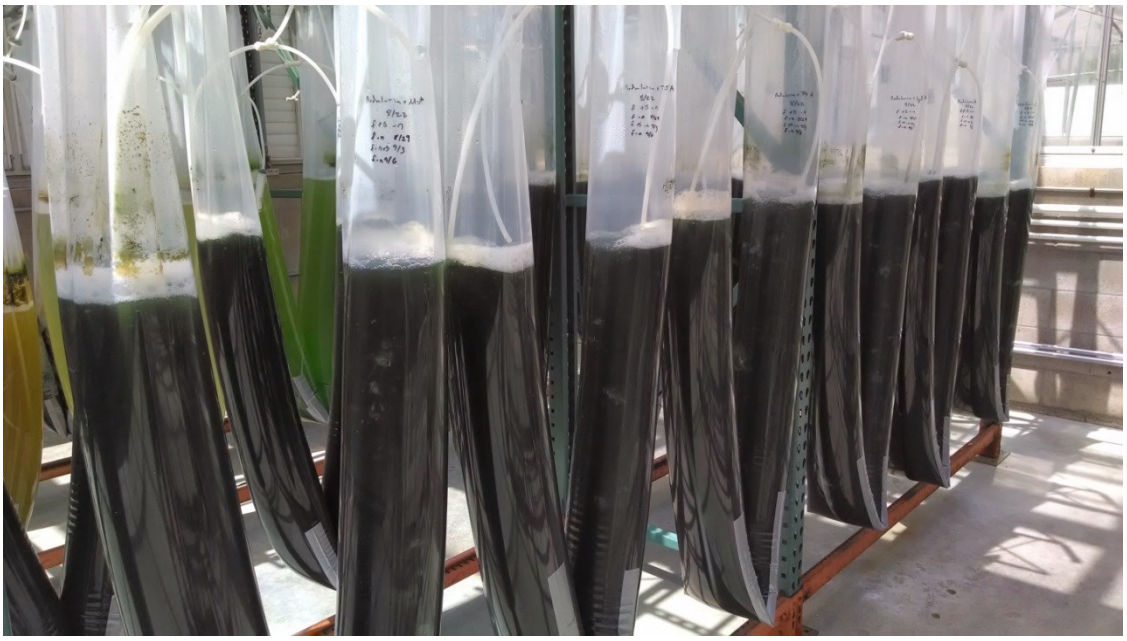


Figure 11 Photograph of *Nodularia* sp. WHNOD and diatom co-cultures cultures in “bag” photobioreactors 15 days after inoculation. *Nodularia* sp. WHNOD monoculture is second from the right.

Figure 12 Photograph of mini ponds of *Nodularia* sp. WHNOD and diatoms.

Figure 13 Photograph of mini ponds of *Nodularia* sp. WHNOD and diatoms.

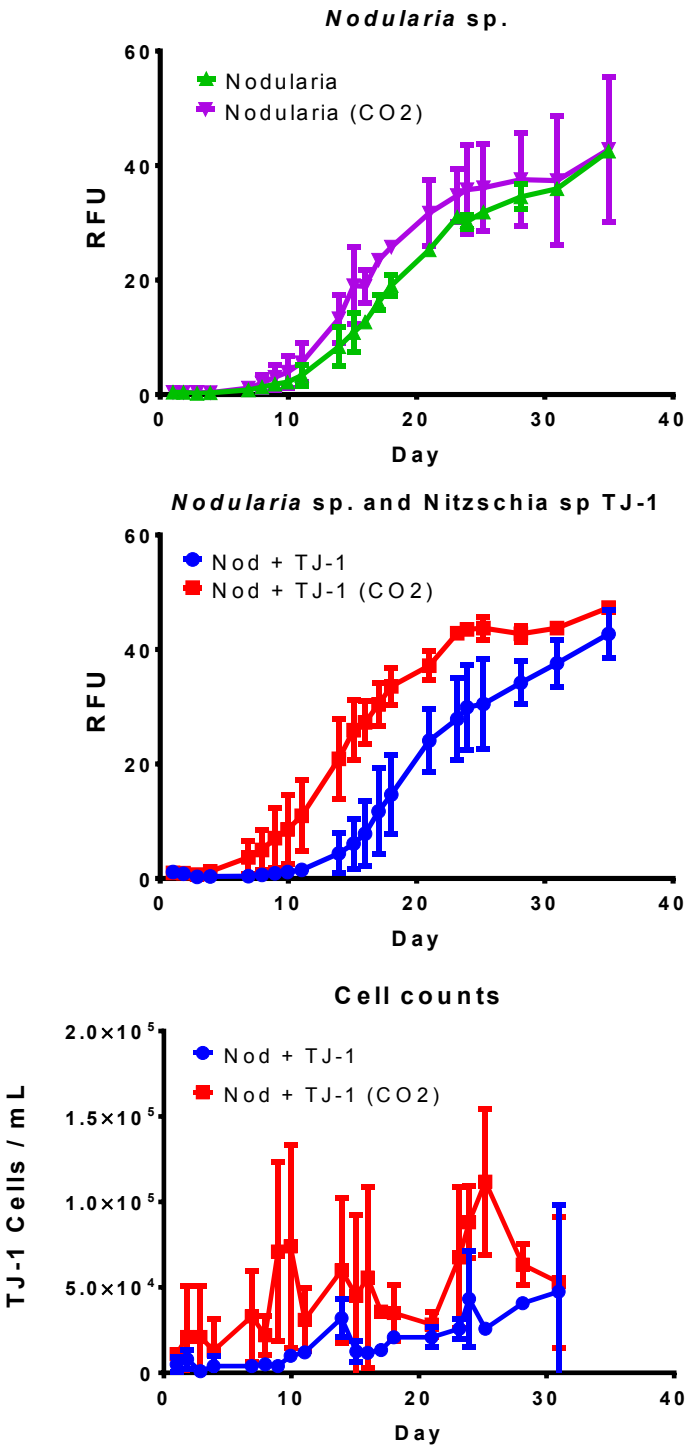


Figure 14 *Nodularia* sp. WHNOD and *Nitzschia* sp. TJ-1 co-cultures grown in the greenhouse with and without supplemental CO_2

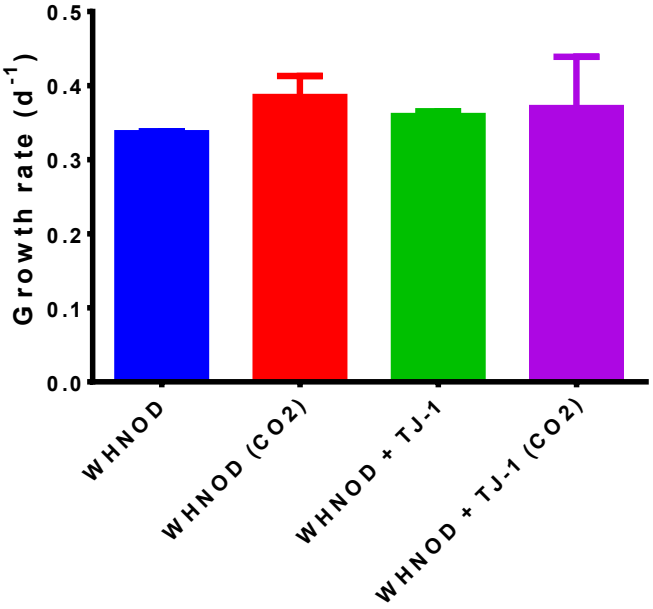


Figure 15 Growth rates of *Nodularia* sp. WHNOD and *Nitzschia* sp. TJ-1 co-cultures grown in the greenhouse with and without supplemental CO_2

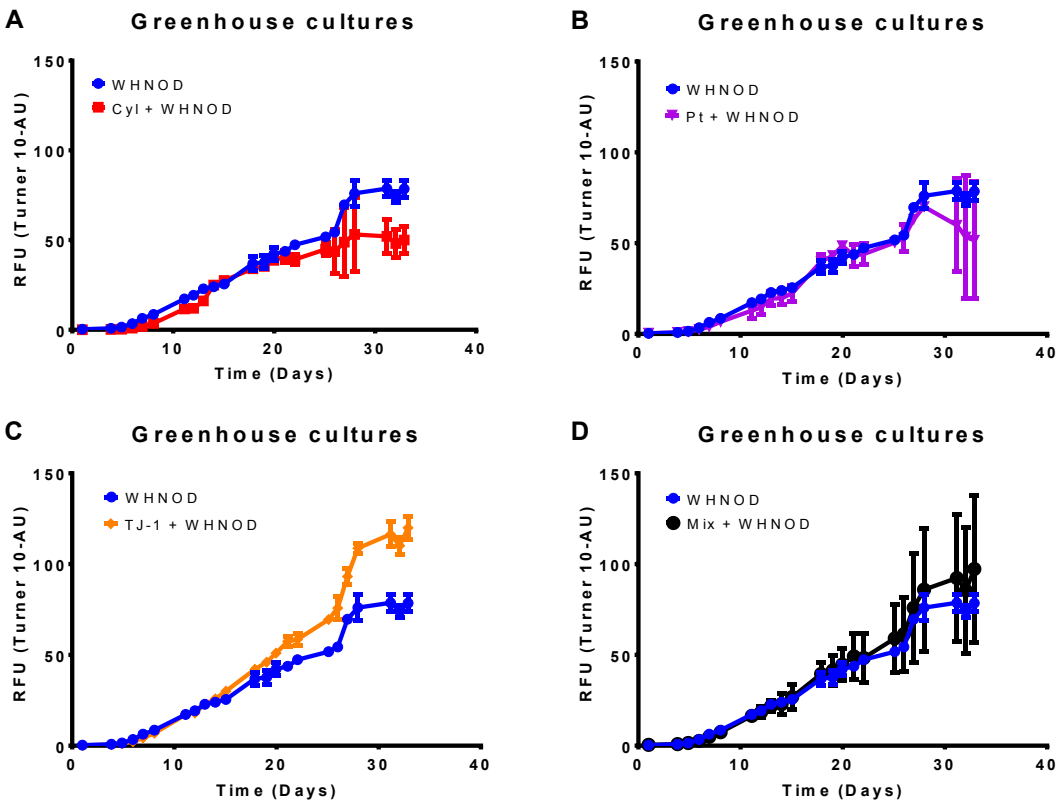
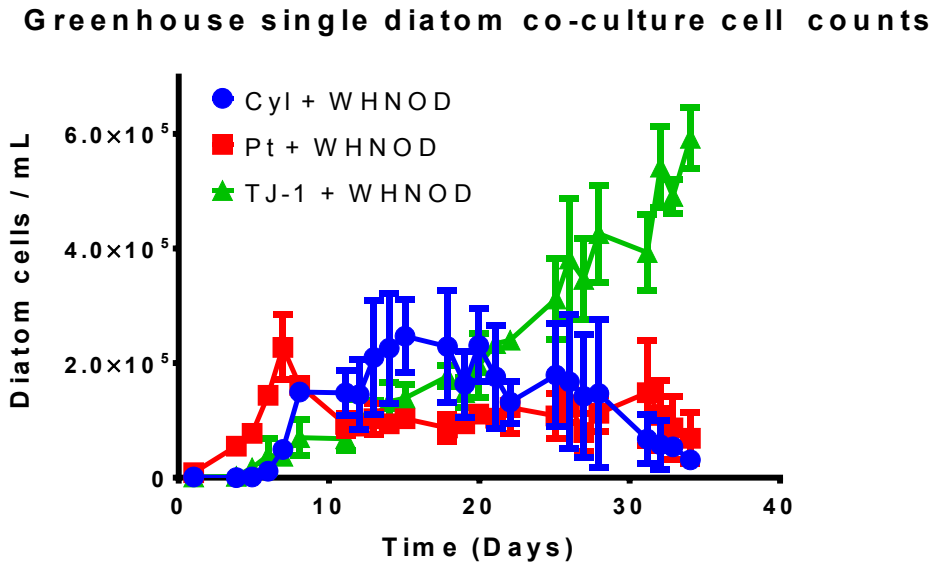


Figure 16 Fluorescence measurements of co-cultures grown in bags at greenhouse

A



B

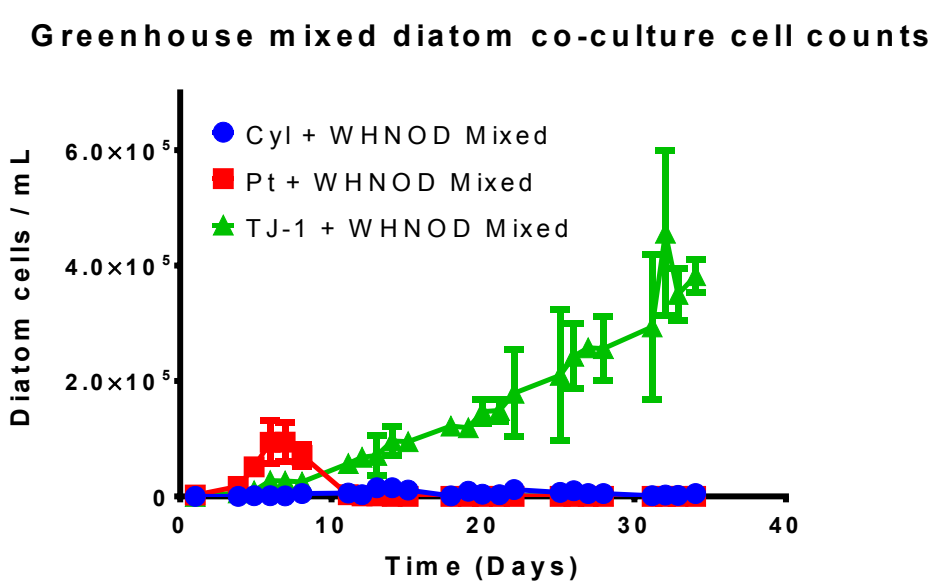


Figure 17 Cell counts of diatoms in greenhouse co-cultures

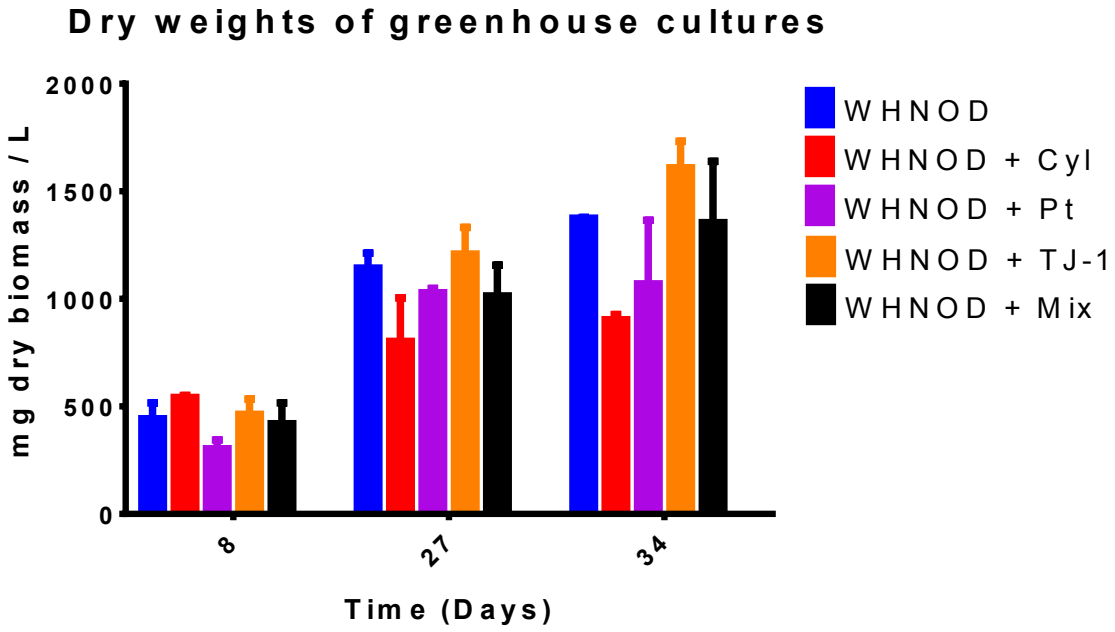


Figure 18 Dry weight yields of greenhouse cultures

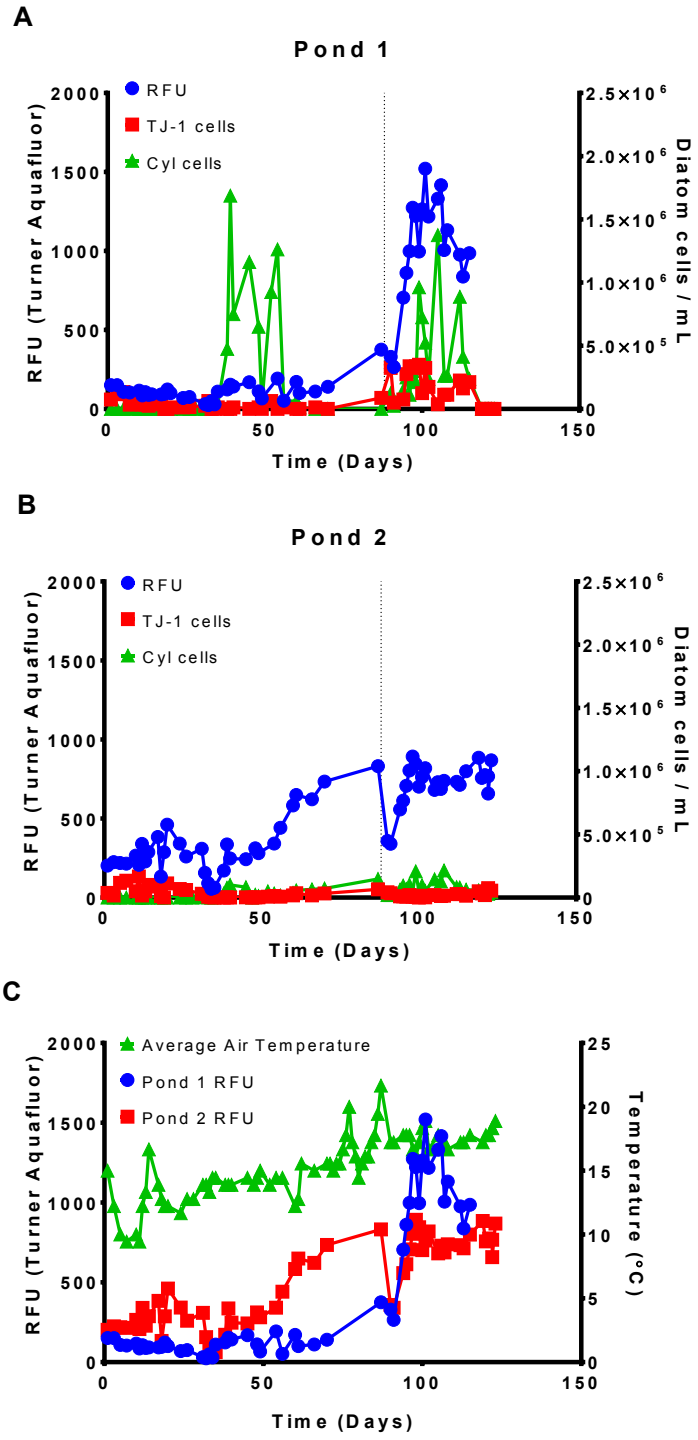


Figure 19 Replicate ponds of *Nitzschia* sp. TJ-1 and *Nodularia* sp. *WHNOD* grown in winter and spring 2013. Dotted line denotes harvest point.

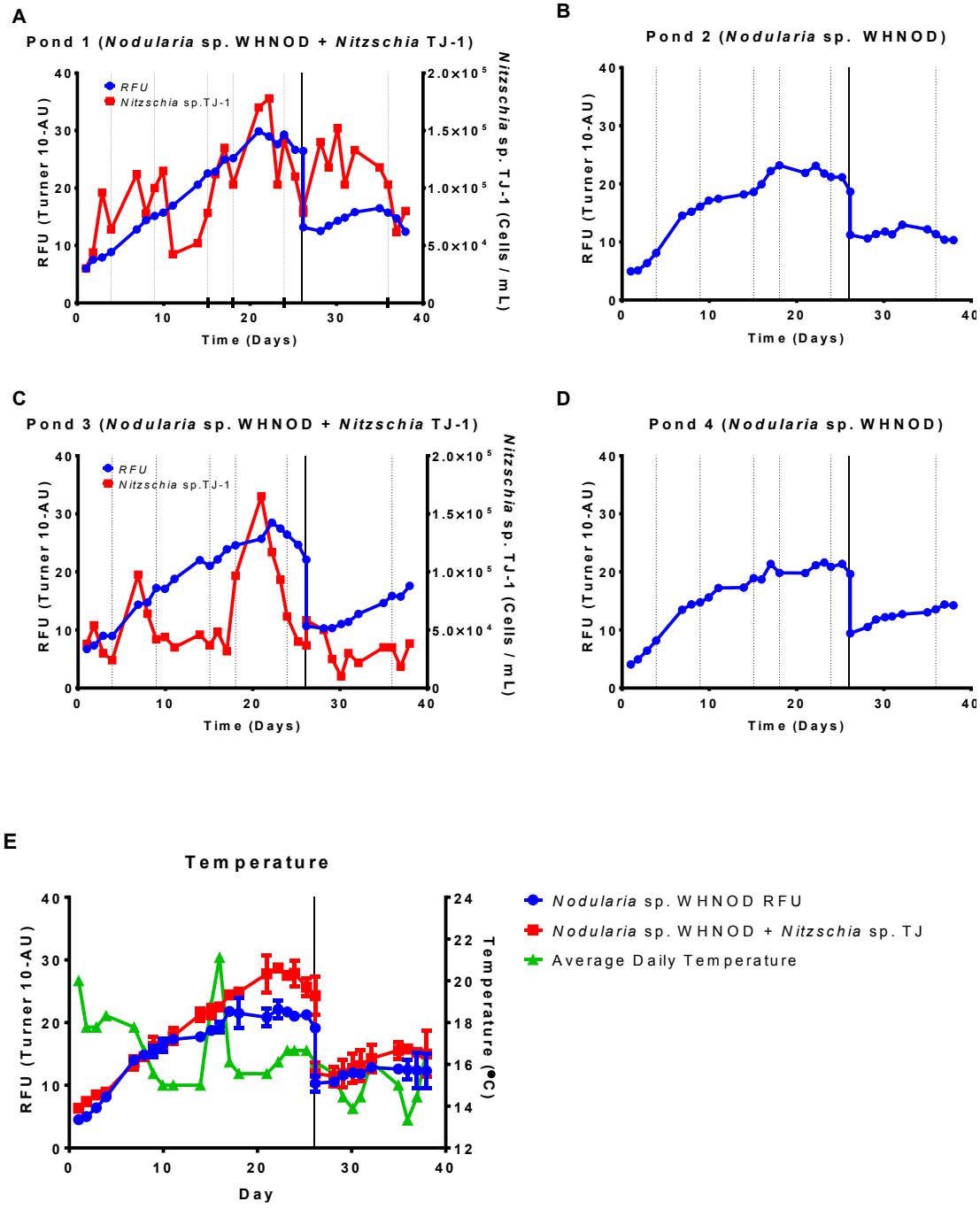


Figure 20 Replicate *Nodularia* sp. WHNOD monoculture ponds and *Nitzschia* sp. TJ-1 and *Nodularia* sp. WHNOD co-culture ponds grown in fall 2013. Solid line denotes harvest point and dotted lines denote nutrient additions.

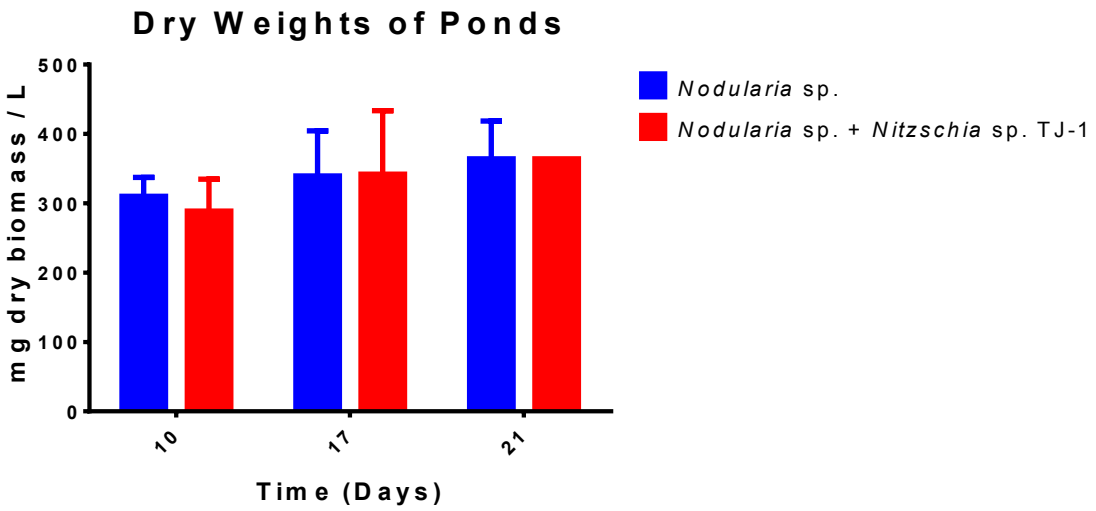


Figure 21 Dry weights of ponds shown in Figure 20.

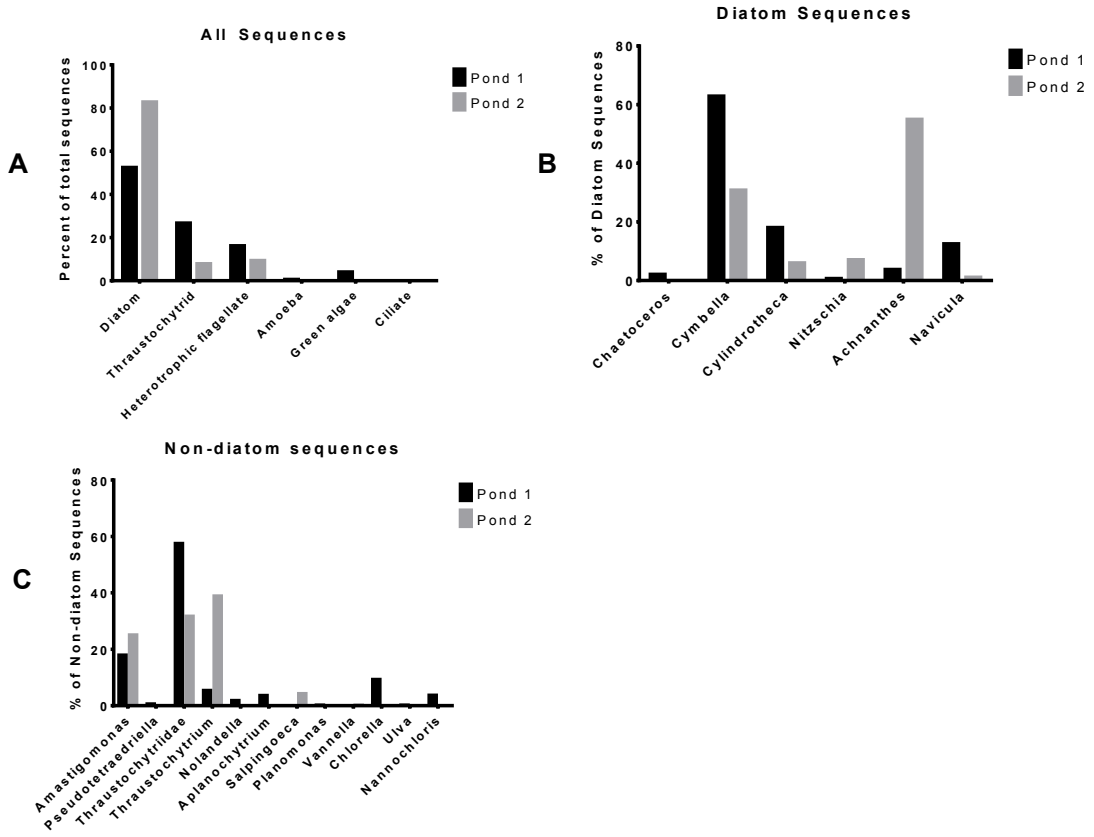


Figure 22 Breakdown of sequences obtained from winter / spring 2013 ponds

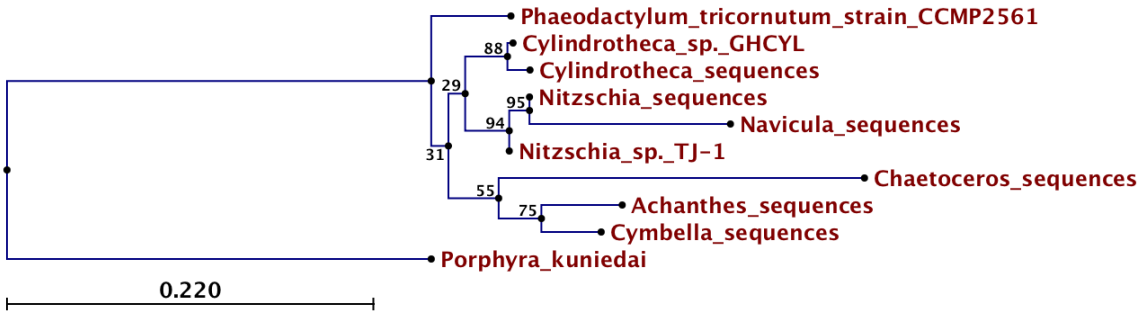


Figure 23 Maximum likelihood tree using diatom 18S rRNA sequences from Figure 22. Scale bar represents average number of amino acid replacements per nucleotide site.

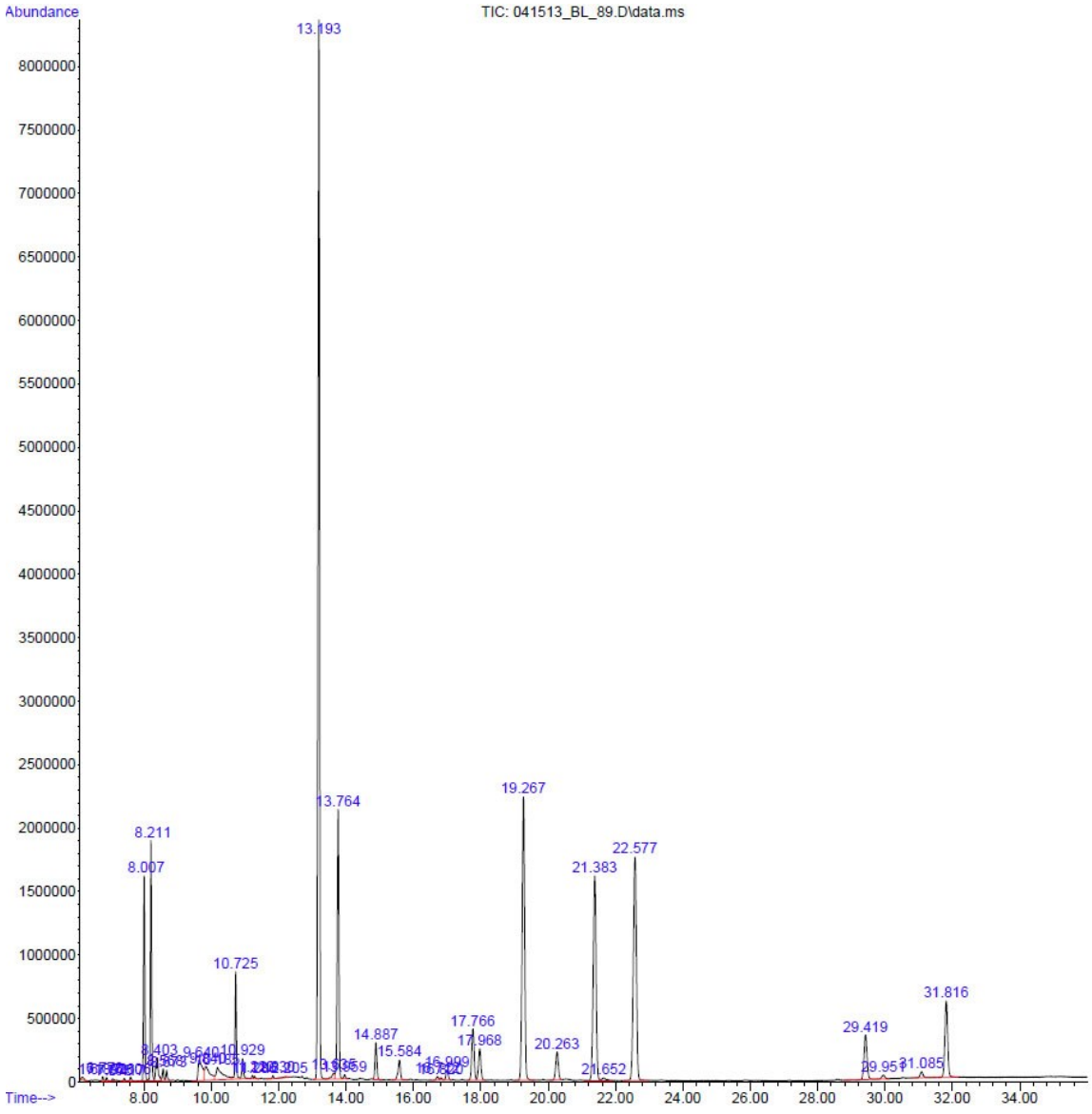


Figure 24 Example GC/MS chromatogram of co-culture FAME lipid profile

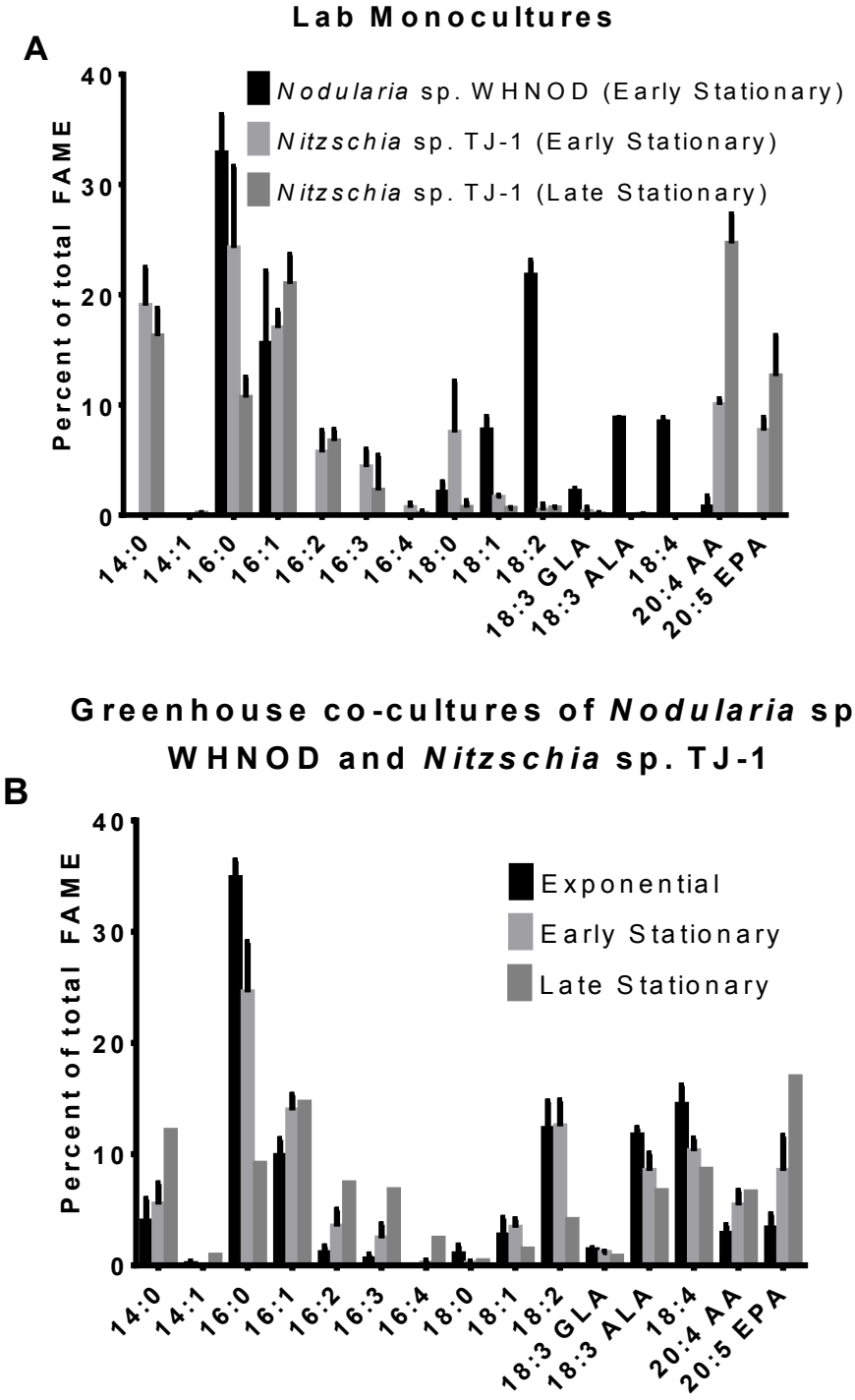


Figure 25 Lipid profiles of lab monocultures (A), greenhouse co-cultures (B) and Figure 20 pond co-cultures (C)

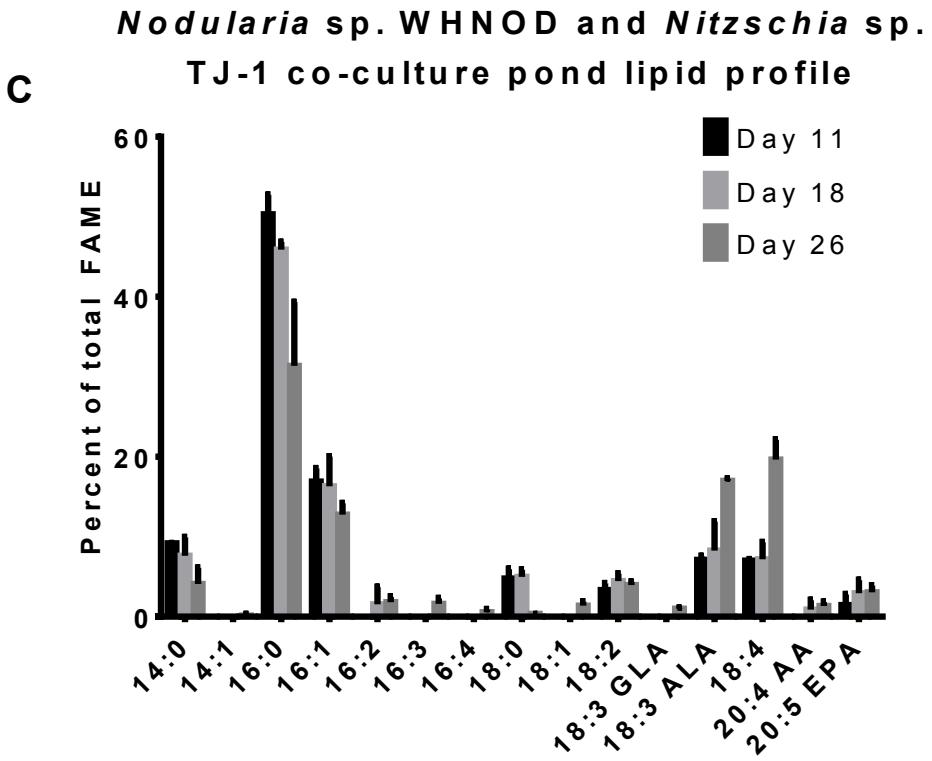


Figure 25 Lipid profiles of lab monocultures (A), greenhouse co-cultures (B) and Figure 20 pond co-cultures (C), continued

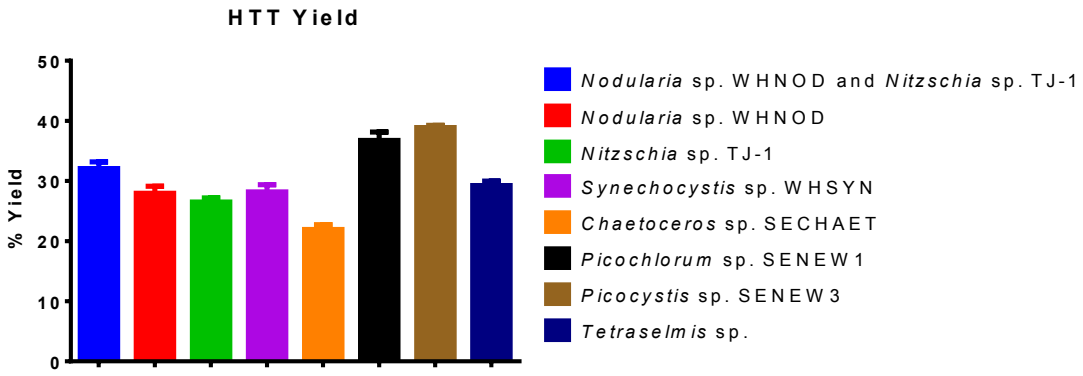


Figure 26 Hydrothermal liquefaction (HTT) of biomass from ponds

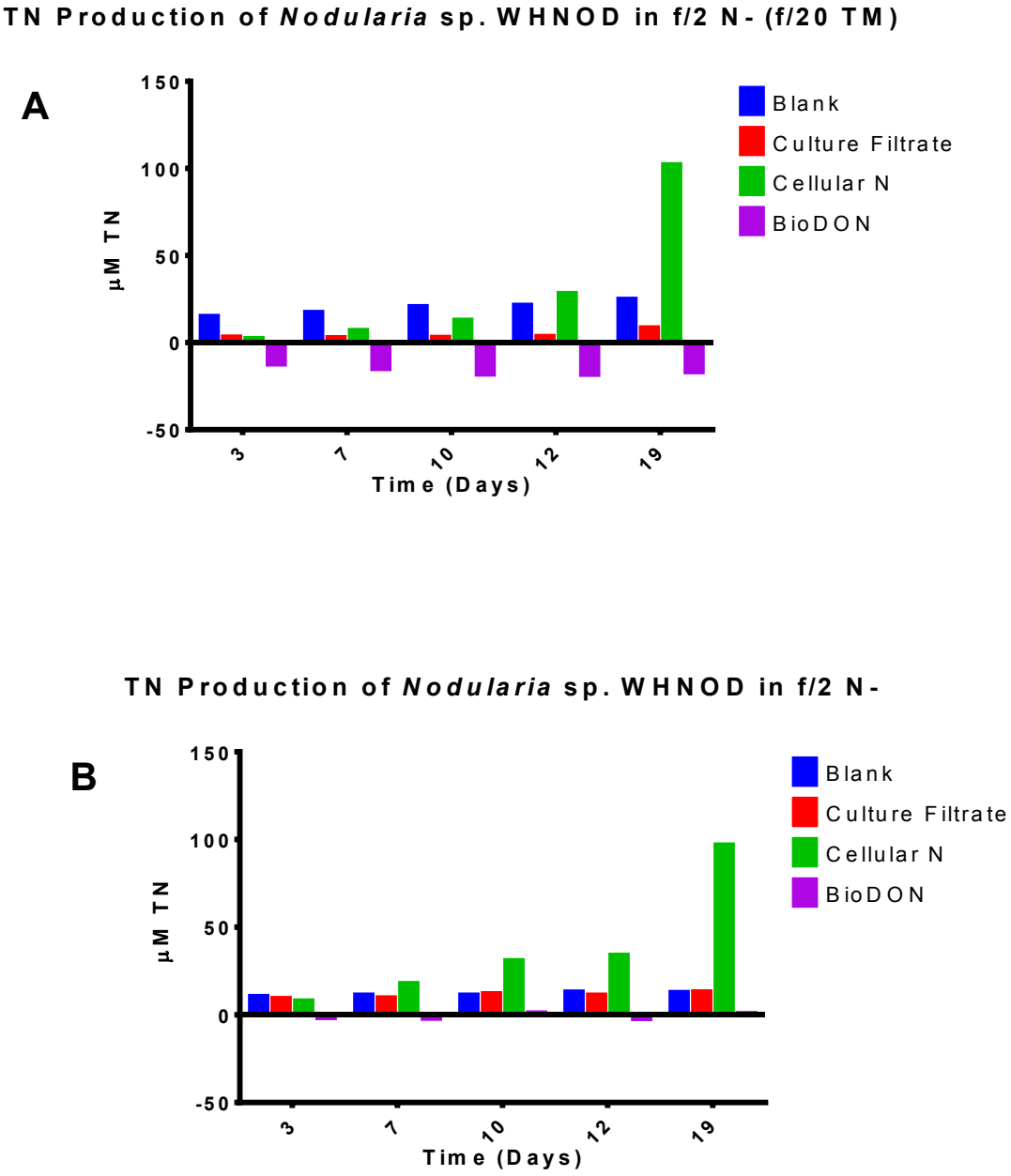


Figure 27 Total nitrogen (TN) production of *Nodularia* sp. WHNOD in nitrogen deplete f/2 growth media with f/2 and f/20 levels of trace metals (TM)

Tables

Table 1 Isolation locations of strains used

Strain Name	Isolation Location
Nodularia sp. WHNOD	Estuarine sand sample from Woods Hole, MA
Nodularia sp. Las Olas	Near Las Olas Mexican restaurant in Cardiff, CA
Nitzschia sp. TJ-1	Tijuana River Estuary, Imperial Beach, CA
Cylindrotheca sp. GHCYL	Pond at UCSD Biology Field Station, La Jolla, CA
Amphora sp. SE4	San Elijo Lagoon, Encinitas, CA
Phaeodactylum tricornutum	Strain CCMP2561

Table 2 Primers used for PCR amplification of 16S / 18S rRNA genes

Name	Target	Primer Sequence (5'-3')	Published Origin
EUK328F	18S	ACCTGGTTGATCCTGCCAG	Moon-van der Staay et al, 2000
EUK329R	18S	TGATCCTTCYGCAGGTTCAC	Moon-van der Staay et al, 2000
EUK502F	18S	GGAGGGCAAGTCTGGT	Worden, 2006
EUK1174R	18S	CCCGTGTTGAGTCAAA	Worden, 2006
CYA106F	16S	CGGACGGGTGAGTAACGCGTGA	Nubel et al, 1997
CYA359F	16S	GGGGAATYTTCCGCAATGGG	Nubel et al, 1997
CYA781R(a)	16S	GACTACTGGGGTATCTAATCCCATT	Nubel et al, 1997
CYA781R(b)	16S	GACTACAGGGGTATCTAATCCCTTT	Nubel et al, 1997
1492R	16S	ACCTTGTTACGACTT	Lane, 1991
CSIF	16S	G(T/C)CACGCCCGAAGTC(G/A)TTAC	Janse et al, 2003
373R	ITS	CTAACCACCTGAGCTAAT	Wilmotte et al, 1993
ULR	23S	CCTCTGTGTGCCTAGGTA TC	Iteman et al, 2000

Table 3 16S / 18S rRNA sequences of strains used

Strain Name	**16S rRNA sequence**
Nodularia sp. *WHNOD*	CGCGTGAGAATCTAGCTTCAGGTCGGGGACAACCACGG GAAACTGTGGCTAATACCGGATATGCCGAGAGGTGAAAG GCTAGCTGCCTGAAGATGAGCTCGCGTCTGATTAGCTAG TTGGTGTGGTAAGAGCGCACCAAGGCGACGATCAGTAGC TGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACA CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTT CCGCAATGGGCGAAAGCCTGACGGAGCAATACCGCGTG AGGGAGGAAGGCTCTTGGGTTGTAAACCTCTTTTCTCAG GGAAGAAAAAAATGACGGTACCTGAGGAATAAGCATCGG CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGC AAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAG GTGGCCATGTAAGTCTGCTGTTAAAGAATGAGGCTCAAC CTCATCAAAGCAGTGGAAACTACACGGCTAGAGTGCGTT CGGGGTAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGT AGATATCAGGAAGAACACCAGTGGCGAAGGCGCTCTACT AGGCCGCAACTGACACTGAGGGACGAAAGCTAGGGGAG CGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAAC GATGGATACTAGGCGTGGCTTGTATCGACCCGAGCCGTG CCGGAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACG CACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCG CACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGC GAAGAACCTTACCAAGACTTGACATGTCGCGGATCTTTCT GAAAGGAAAGAGTGCCTTCGGGAGCGCGAACACAGGTG GTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG TTAAGTCCCGCAACGAGCGCAACCCTCGTTTTTAGTTGCC AGCATTAAGTTGGGCACTCTAGAGAGACTGCCGGTGACA AACCGGAGGAAGGTGGGGATGACGTCAAGTCAGCATGC CCCTTACGTCTTGGGCTACACACGTACTACAATGCTACGG ACAAAGGGCAGCTACACAGCAATGTGATGCAAATCTCAG AAACCGTAGCTCAGTTCAGATCGCAGGCTGCAACTCGCC TGCGTGAAGTAGGAATCGCTAGTAATTGCAGGTCAGCAT ACTGCAGTGAATTCGTTCCCGGGCCTTGTACACACCGCC CGTCACACCATGGAAGCTGATCACGCCCGAAGTCGTTAC CCCAACTTTTCGGAGAGGGGGATGCCGAAGGCAGGATTG GTGACTGGGGTGAAGTCGTAACAAGGTAGCCGTACCGGA AGGTGTGGCTGGATCACCTCCTTTTAGGGAGACCTAATC CACTCAGACATCGAAAGCAAATTGCCAATAGATAATGAGA TGGTCATCTCTAGGTCGGTCGTGAATATT

Table 3 16S / 18S rRNA sequences of strains used, continued

Strain Name	**16S rRNA sequence**
Nodularia sp. Las Olas	TGCAGTCGANCGGTCTCTTCGGAGATAGTGGCGGACGG GTGAGTAACGCGTGAGAATCTAGCTTTAGGTCGGGGACA ACCACGGGAAACTGTGGCTAATACCGGATATGCCGAGAG GTGAAAGGCTTGCTGCCTAAAGATGAGCTCGCGTCTGAT TAGCTAGTAGGTGTGGTAAGAGCGCACCTAGGCGACGAT CAGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGGAC TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGG GGAATTTTCCGCAATGGGCGAAAGCCTGACGGAGCAATA CCGCGTGAGGGAGGAAGGCTCTTGGGTTGTAAACCTCTT TTCTCAAGGAAGAAAAAAATGACGGTACTTGAGGAATAAG CATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA GGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGT CCGCAGGTGGCTGTGTAAGTCTGCTGTTAAAGAATCTAG CTTAACTAGATAAAAGCAGTGGAAACTACATAGCTAGAGT GCGTTCGGGGTAGAGGGAATTCCTGGTGTAGCGGTGAAA TGCGTAGATATCAGGAAGAACACCAGTGGCGAAGGCGCT CTACTAGGCCGCAACTGACACTGAGGGACGAAAGCTAGG GGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGT AAACGATGGATACTAGGCGTGGCTTGTATCGACCCGAGC CGTGCCGGAGCTAACGCGTTAAGTATCCCGCCTGGGGA GTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGG GCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGC AACGCGAAGAACCTTACCAAGACTTGACATGTCGCGAAC CCCTTTGAAAGGAGGGAGTGCCTTCGGGAGCGCGAACA CAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGAT GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTTTT AGTTGCCAGCATTAAGATGGGCACTCTAGAGAGACTGCC GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTC AGCATGCCCCTTACGTCTTGGGCTACACACGTACTACAAT GCTACGGACAAAGGGCAGCTACACAGCGATGTGATGCAA ATCTCACAAAACCGTAGCTCAGTTCAGATCGCAGGCTGC AACTCGCCTGCGTGAAGTAGGAATCGCTAGTAATTGCAG GTCAGCATACTGCAGTGAATTCGTTCCCGGGCCTTGTAC ACACCGCCCGTCACACCATGGAAGCTGGTCACGCCCGAA GTCGTTACCCCAACTGTTCGCAGAGGGGGATGCCGAAGG CAGGTCTGGTGACTGGGGTGAAGTCGTAACAAGGTAGCC GTACCGGAAGGTGTGGCTGGATCACCTCCTTTTAGGGAG ACCTAACCCAAGTCAAAAGTCAAAAGTCAAAAGTCAAAAG TCAAAAACAGACTGAAGACAAGAAATAGACGTAAACTTGA GGTCTAACCTAGGTCGTTCGAGAATTGGTGAAAGCTTTCA AAGTATTTTTGGTTCAGTTCATTCATGGACATCTCAACTTG ATTTGGAATAAAGATTCAGCAATTGATTGTTTTGAACAACC GAACTGCTGGGTAATTATCCAGCCAGAACCTTGAAAACTG CATAGTAACGCGAAAAAAAGCAGGCAGTGAAAAAAGTCA AAAGTCAAAAGTCAAAAGTTAAAAGTCAAAAACTGGAAGC TGAGACAAAAGACAAAAAACTGCATGAAAAAGCCAATGTA TTGTGGTCAAGCTAATAAGGGCTGACGGTGGATACCTAG GCNCACA

Table 3 16S / 18S rRNA sequences of strains used, continued

Strain Name	**18S rRNA sequence**
Amphora sp. SE4	TGCAAGTCTAAGTATAAATCCTTTACTTTGAAACTGCGAAT GGCTCATTATATCAGTTATAGTTTATTTGATAGTCCCTCAC TACTTGGATAACCGTAGTAATTCTAGAGCTAATACATGCG TCAATACCCTTCTGGGGTAGTATTTATTAGATGGAAACCA ACCCCTTTGGGGTGATGTGGTGAATCATAATAAGCTTGCG GATCGCCGGTGGCGATGGATCATTCAAGTTTCTGCCCTA TCAGCTTTGGATGGTAAGGTATTGGCTTACCATGGCTTTA ACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGG AGCCTGAGAGATGGCTACCACATCCAAGGAAGGCAGCAG GCGCGTAAATTACCCAATCTTGACACAAGGAGGTAGTGA CAATAAATAACAATGCCGGGCCTTATAGGTCTGGCAATTG GAATGAGAACAATTTAAACCCCTTATCGAGGATCAATTGG AGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCT CCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCG TAGTTGGATTTGTGGTGGTGCCTGGGGTCCAATTTTGGTA CTTTCGGGGACTGCCATCCTTGGGTGGATCCTGTGTGGC ATTAGGTTGTCGTGCAGGGGATGCCCATCGTTTACTGTG AAAAAATTAGAGTGTTCAAAGCAGGCTTATGCCGTTGAAT ATATTAGCATGGAATAATGAGATAGGACCTTGGTACTATT TTGTTGGTTTGCGCACCGAGGTAATGATTAATAGGGACAG TTGTGGGTATTCGTATTCCATTGTCAGAGGTGAAATTCTT GGATTTCTGGAAGACGAACTAATGCGAAAGCATTTACCAA GGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGA AGATGATTAGATACCATCGTAGTCTTAACCATAAACTATG CCGACAAGGGATTGGCGGGGTCTCGTTACGTCTCCGTCA GCACCTTATGAGAAATCACAAGTTTTTGGGTTCCGGGGG GAGTATGGTCGCAAGGCTGAAACTTAAAGAAATTGACGG AAGGGCACCACCAGGAGTGGAGCCTGCGTCTTAATTTGA CTCAACACGGGAAAACTTACCAGGTCCAGACAAAGTGAG GATTGACAGATTGAGAGCTCTTTCTTGATTCTTTGGTTGG TGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTG GTTAATTCCGTTAACGAACGAGACCCCTGCCTGCTAAATA GTCCTTTGAGTGATTTTCACTGAATTGGGCTTCTTAGAGG GACGTGCATTCTATTAGATGCAGGAAGATAGGGGCAATA ACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGC GCGCTACACTGATGCATTCAACGAGTTTCCTTGGCCGAG AGGTCTGGGCAATCTTTTGAACGTGCATCGTGATAGGGA TAGATTATTGCAATTATTAATCTTGAACGAGGAATTCCTAG TAAACGCAGATCATCAATCTGCATTGATTACGTCCCTGCC CTTTGTACACACCGCCCGTCGCACCTACCGATTGGATGG TCCGGTGAAGCCTCGGGATTGTGACTGGTGCCTTTACTG GTGTTGGTTGCGAGAACTTGTCTAA

Table 3 16S / 18S rRNA sequences of strains used, continued

Strain Name	**18S rRNA sequence**
Nitzschia sp. TJ-1	TGCATGTCTAAGTATAAATCTTTTACTTTGAAACTGCGAAC GGCTCATTATATCAGTTATAGTTTATTTGATAGTCCCTTAC TACTTGGATAACCGTAGTAATTCTAGAGCTAATACATGCG TCAATACCCTTCTGGGGTAGTATTTATTAGATTGAAACCAA CCCCTTCGGGGTGATGTGGTGATTCATAATAAGCTTGCG GATCGCATGGCTTTGCCGGCGATGGATCATTCAAGTTTCT GCCCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCAT GGCTTTAACGGGTAACGGGAAATTAGGGTTTGATTCCGG AGAGGGAGCCTGAGAGACGGCTACCACATCCAAGGAAG GCAGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAG GTAGTGACAATAAATAACAATGCCGGGCCTTTGTAGGTCT GGCAATTGGAATGAGAACAATTTAAACCCCTTATCGAGTA TCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAA TTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAA AAGCTCGTAGTTGGATTTGTGGCAGTCGTGTGCATCCCG GCATTCGTGCCGGTGTTTGCTAGCGTCGCCATCCTTGGG TGGAACCTGTGTGGCATTAGGTTGTCGTGCAGGGGATGC CCATCGTTTACTGTGAAAAAATTAGAGTGTTCAAAGCAGG CTTATGCCGTTGAATATATTAGCATGGAATAATAAGATAG GACCTTGGTACTATTTTGTTGGTTTGCGCACCAAGGTAAT GATTAATAGGGACAGTTGGGGGTATTCGTATTCCATTGTC AGAGGTGAAATTCTTGGATTTTTGGAAGACGAACTACTGC GAAAGCATTTACCAAGGATGTTTTCATTAATCAAGAACGA AAGTTAGGGGATCGAAGATGATTAGATACCATCGTAGTCT TAACCATAAACTATGCCGACAAGGGATTGGCGGAGTCTC GTTTTGTCTCCGTCAGCACCTTATGAGAAATCACAAGTCT TTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT AAAGAAATTGACGGAAGGGCACCACCAGGAGTGGAGCCT GCGGCTTAATTTGACTCAACACGGGAAAACTTACCAGGTC CAGACATAGTGAGGATTGACAGATTGAGAGCTCTTTCTTG ATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTG GATTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCC CTGCCTGCTAAATAGCTTGTCGAGTGAATTTTCACTGGAT GAAGCTTCTTAGAGGGACGTGCATTCTATTAGATGCAGGA AGATAGGGGCAATAACAGGTCTGTGATGCCCTTAGATGTT CTGGGCCGCACGCGCGCTACACTGATGTATTCAACGAGT TTTTCCTTGGCCGAGAGGCCTGGGCAATCTTTTGAACGT GCATCGTGATAGGGATAGATTATTGCAATTATTAATCTTGA ACGAGGAATTCCTAGTAAACGCAGATCATCAATCTGCATT GATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCAC CTACCGATTGAATGGTCCGGTGAAGCCTCGGGATTGTGG CGAGTTTCCTTCATTGGGAATTTGTCGCGAGAACTTGTCT AA

Table 3 16S / 18S rRNA sequences of strains used, continued

Strain Name	**18S rRNA sequence**
Cylindrotheca sp. GHCYL	GTTATAGTTTATTTGATAGTCCCTTACTACTTGGATAACCG TAGTAATTCTAGAGCTAATACATGCGTCAATACCCTTCTG GGGTAGTATTTATTAGATTGAAACCAACCCCTTCGGGGTG ATGTGGTGATTCATAATAAGCTTGCGGATCGCATGGCTTT GCCGGCGATGGATCATTCAAGTTTCTGCCCTATCAGCTTT GGATGGTAGGGTATTGGCCTACCATGGCTTTAACGGGTA ACGGGAAATTAGGGTTTGATTCCGGAGAGGGAGCCTGAG AGACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGTA AATTACCCAATCCTGACACAGGGAGGTAGTGACAATAAAT AACAATGCCGGGCCTTTGTAGGTCTGGCAATTGGAATGA GAACAATTTAAACCCCTTATCGAGTATCAATTGGAGGGCA AGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATA GCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTG GATTTGTGGTGTACTTCCGCGGCCCGTCACTATGTGATG GAGCTTGCTGAAGTCGCCATCCTTGGGTGGATCCTGTGT GGCATTAAGTTGTCGTGCAGGGGATGCCCATCGTTTACT GTGAAAAAATTAGAGTGTTCAAAGCAGGCTTATGCCGTTG AATATATTAACATGGAATAATAGGATAGGACCTTGGTACT ATTTTGTTGGTTTGCGCACCAAGGTAATGATTAATAGGGA CAGTTGGGGGTATTCGTATTTCATTGTCAGAGGTGAAATT CTTGGATTTTTGAAAGACGAACTACTGCGAAAGCATTTAC CAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGAT CGAAGATGATTAGATACCATCGTAGTCTTAACCATAAACT ATGCCGACAAGGGATTGGTGGAGTTTCGTTATGTCTCCAT CAGCACCTTATGAGAAATCACAAGTCTTTGGGTTCCGGG GGGAGTATGGNCGCAAGGCTGAAACTTAAAGAAATTGAC GGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTT GACTCNACACGGGAAAACTTACCANGNCCANACATANTG AGGATTGACAGATTGAGAGCTCTTTCTTGATTCTATGGGT GGTGGTGCATGG

Table 3 16S / 18S rRNA sequences of strains used, continued

Strain Name	**18S rRNA sequence**
Phaeodactylum tricornutum CCMP2561	CCTGCCAGTAGTCATACGCTCGTCTCAAAGATTAAGCCAT GCATGTCTAAGTATAAATCTTTTACTTTGAAACTGCGAATG GCTCATTATATCAGTTATAGTTTATTTGATAGTCCCTTCAC TATTTGGATAACCGTAGTAATTCTAGAGCTAATACATGCG TCAATACCCTTCTGGGGTAGTATTTATTAGATTGAAACCAA CCCCTTCGGGGTGATGTGGTGATTCATAATAAGCTTGCG GATCGCATGGCTTTTGCCGGCGATGGATCATTCAAGTTTC TGCCCTATCAGCTTTGGATGGTAGGGTATTGGCCTACCAT GGCTTTAACGGGTAACGGGAAATCAGGGTTTGATTCCGG AGAGGGAGCCTGAGAGACGGCTACCACATCCAAGGAAG GCAGCAGGCGCGTAAATTACCCAATCCTGACACAGGGAG GTAGTGACAATAAATAACAATGCCGGGCCTTTCTAGGTCT GGCTTTTGGAATGAGAACAATTTAAACCCCTTATCGAGGA TCCATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAA TTCCAGCTCCAATAGCGTATATTAATGTTGCTGCAGTTAA AAAGCTCGTAGTTGGATTTGTGGTGGGCCCGGTGGCTCG GCCTTAGTTGGCCGTTGCTGTTTTGGGTCCGCCATCCTT GGGTGGAATCAGTGTGGCATTAAGTTGTCGCGCTGGGGA TGCCCATCGTTTACTGTGAAAAAATTAGAGTGTTCAAAGC AGGCTTACGCCGTTGAATATATTAGCATGGAATAATGAGA TAGGACCTTGGTACTATTTTGTTGGTTTGCGCACCGAGGT AATGATTAATAGGGACAGTTGGGGGTATTCGTATTCCATT GTCAGAGGTGAAATTCTTGGATTTCTGGAAGACGAACTAC TGCGAAAGCATTTACCAAGGATGTTTTCATTAATCAAGAA CGAAAGTTAGGGGATCGAAGATGATTAGATACCATCGTA GTCTTAACCATAAACTATGCCGACAAGGGATTGGCGGGG TTTCGTTACGTCTCCGTCAGCACCTTATGAGAAATCACAA GTCTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAA ACTTAAAGAAATTGACGGAAGGGCACCACCAGGAGTGGA GCTTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCA GGTCCAGACATAGTGAGGATTGACAGATTGAGAGCTCTTT CTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTG GTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGA CCCCTGCCTGCTAAATAGCCCAGTGAGTGAATTTCACTGA CCAGGGCTTCTTAGAGGGACGTGCGTTCTATTAGACGCA GGAAGATAGGGGCAATAACAGGTCTGTGATGCCCTTAGA TGTTCTGGGCCGCACGCGCGCTACACTGATGCATTCAAC GAGTGTTTTTCCTTGGCCGAGAGGCCTGGGCAATCTTTT GAACGTGCATCGTGATAGGGATAGATTATTGCAATTATTA ATCTTGAACGAGGAATTCCTAGTAAACGCAGATCATCAAT CTGCATTGATTACGTCCCTGCCCTTTGTACACACCGCCCG TCGCACCTACCGATTGGATGGTCCGGTGAAGCCTCGGGA TTGTGACCAGTGCCTTTATTGGTGTTGGTTGCGAGAACTT GTCTAAACCTTATCATCTAGAGGAAGGTGAAGTCGTAACA AGGTTTCCGTAGGTGAACCT

Table 4 Initial growth rates of cultures shown in Figure 16

Culture	Growth rate (d^{-1})
Nodularia sp. WHNOD monoculture	0.7029
Nodularia sp. WHNOD and *Cylindrotheca* sp. GHCYL	0.6585
Nodularia sp. WHNOD and *P. tricornutum*	0.5060
Nodularia sp. WHNOD and *Nitzschia* sp. TJ-1	0.6496
Nodularia sp. WHNOD and mixed diatoms	0.5575

Table 5 FAME profiles of lab monocultures

Fatty acid species	Nodularia sp. (Early Stationary)	Nitzschia sp. TJ-1 (Early Stationary)	Nitzschia sp. TJ-1 (Late Stationary)
14:0	0.00%	19.00%	16.24%
14:1	0.00%	0.00%	0.16%
16:0	32.86%	24.24%	10.66%
16:1	15.56%	16.97%	20.97%
16:2	0.00%	5.69%	6.71%
16:3	0.00%	4.35%	2.23%
16:4	0.00%	0.67%	0.14%
18:0	2.06%	7.49%	0.67%
18:1	7.71%	1.59%	0.60%
18:2	21.75%	0.41%	0.64%
18:3 GLA	2.14%	0.28%	0.08%
18:3 ALA	8.78%	0.00%	0.04%
18:4	8.44%	0.00%	0.00%
20:4 AA	0.71%	9.99%	24.64%
20:5 EPA	0.00%	7.66%	12.61%

Table 6 FAME profiles of *Nodularia* sp. WHNOD and *Nitzschia* sp. TJ-1 co-cultures grown in the greenhouse

Fatty acid species	Exponential	Early Stationary	Late Stationary
14:0	3.94%	5.47%	12.09%
14:1	0.12%	0.00%	0.87%
16:0	34.81%	24.54%	9.14%
16:1	9.83%	13.93%	14.63%
16:2	1.10%	3.49%	7.38%
16:3	0.54%	2.43%	6.76%
16:4	0.00%	0.17%	2.40%
18:0	1.00%	0.16%	0.37%
18:1	2.68%	3.38%	1.42%
18:2	12.26%	12.50%	4.07%
18:3 GLA	1.36%	1.13%	0.78%
18:3 ALA	11.69%	8.50%	6.68%
18:4	14.43%	10.26%	8.60%
20:4 AA	2.83%	5.39%	6.56%
20:5 EPA	3.32%	8.49%	16.92%

Table 7 FAME profiles of *Nodularia* sp. WHNOD and *Nitzschia* sp. TJ-1 co-cultures grown in ponds (Figure 20)

Fatty acid species	Day 11	Day 18	Day 26
14:0	9.17%	7.62%	4.09%
14:1	0.00%	0.00%	0.15%
16:0	50.30%	45.88%	31.36%
16:1	16.88%	16.29%	12.76%
16:2	0.00%	1.55%	1.82%
16:3	0.00%	0.00%	1.60%
16:4	0.00%	0.00%	0.57%
18:0	4.77%	4.98%	0.35%
18:1	0.00%	0.00%	1.38%
18:2	3.34%	4.44%	3.95%
18:3 GLA	0.00%	0.00%	0.99%
18:3 ALA	7.09%	8.26%	16.98%
18:4	7.01%	7.17%	19.63%
20:4 AA	0.00%	0.88%	1.33%
20:5 EPA	1.45%	2.93%	3.05%

Supplementary Data

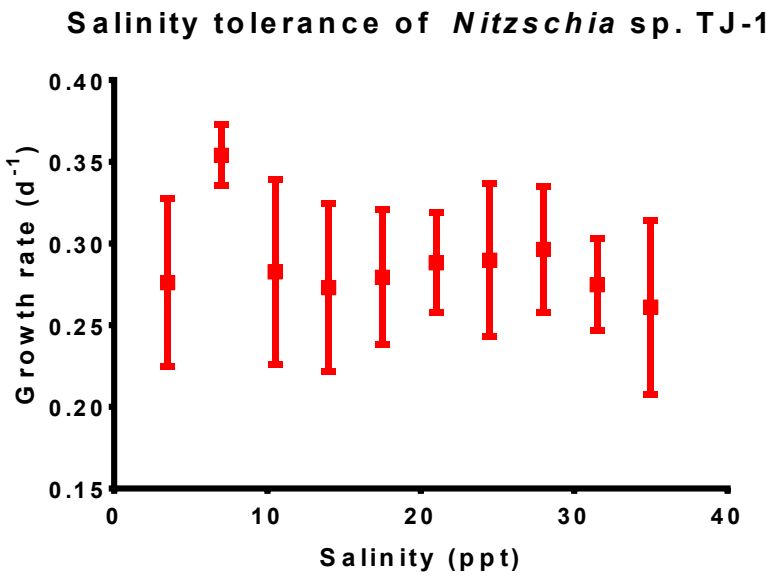


Figure 28: Salinity tolerance of *Nitzschia* sp TJ-1

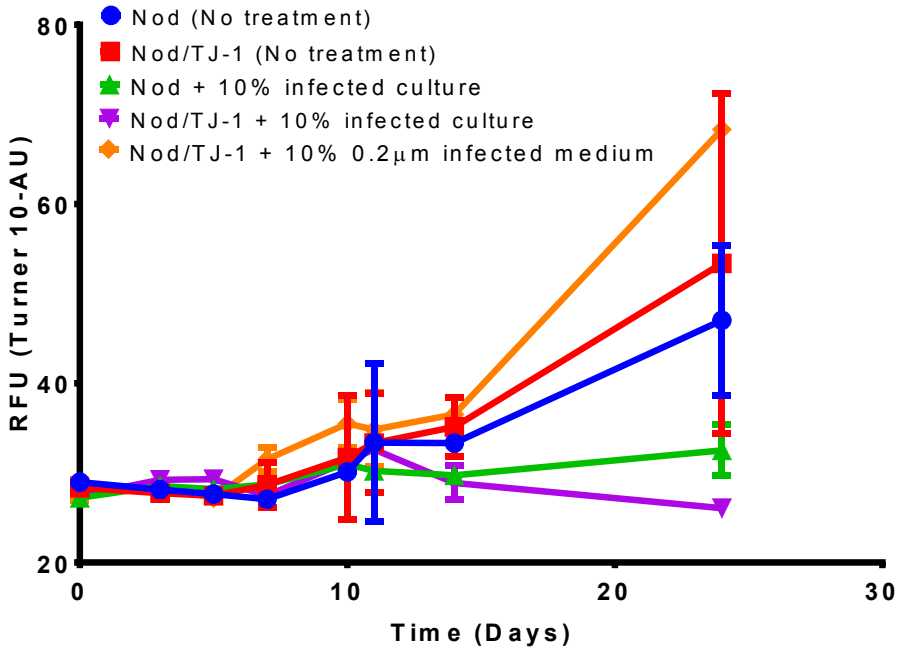


Figure 29 Growth of TJ-1 and WHNOD with additions from crashed cultures

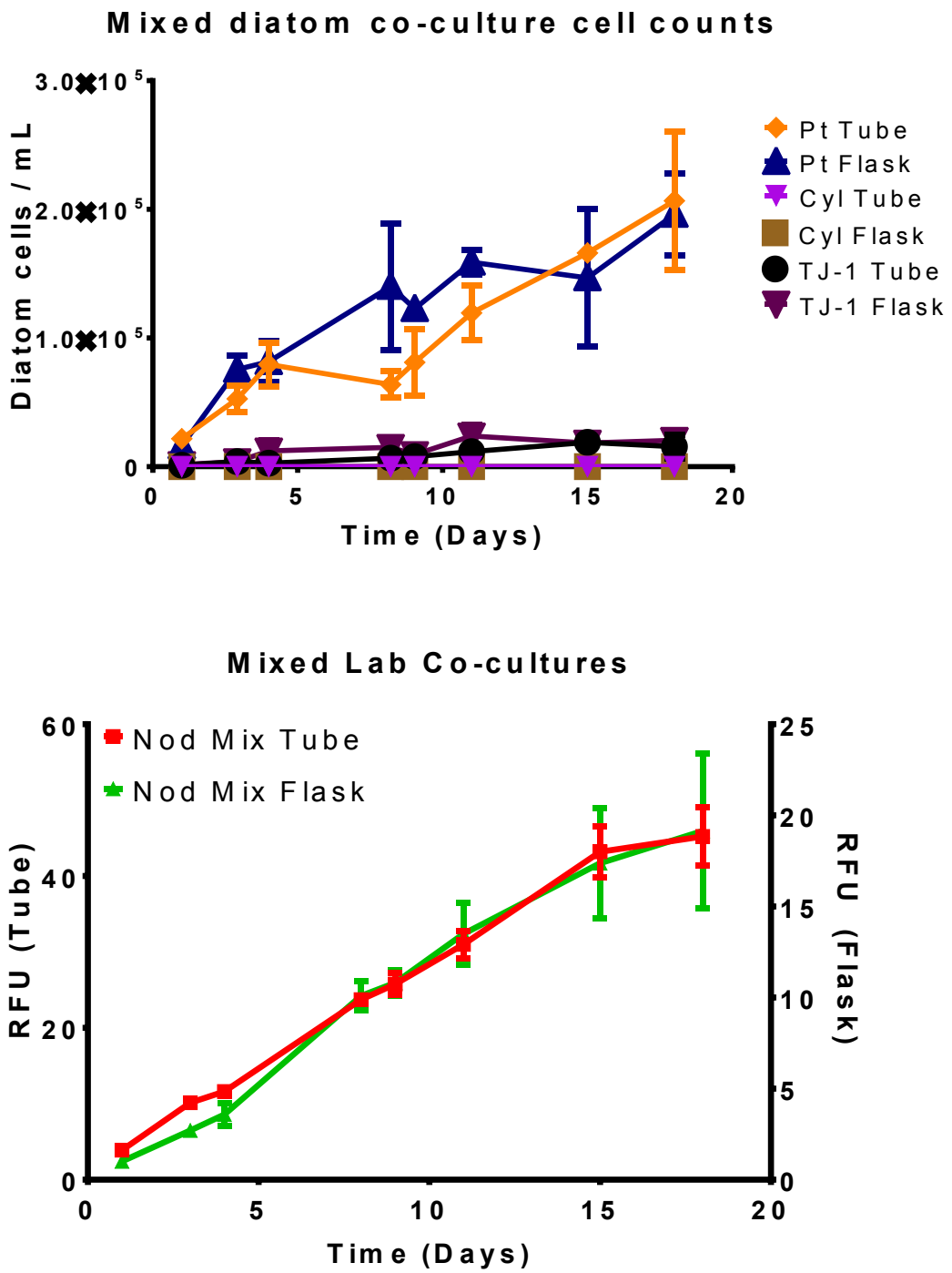


Figure 30 Growth of *Nodularia* sp. WHNOD and mixed diatoms in 50 mL tubes and 2800 mL flasks

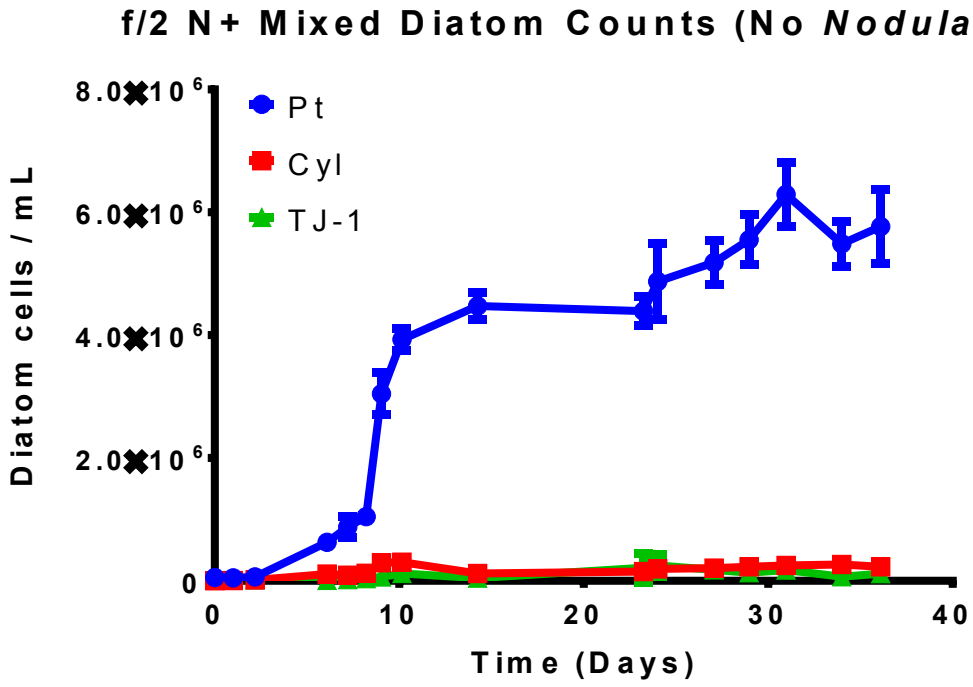


Figure 31 Cell counts of mixed diatoms grown in nitrogen-replete media in the absence of *Nodularia* sp. WHNOD

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