

**Selection of High Performance Microalgae for Bioremediation of Nitrate-  
Contaminated Groundwater**

**Technical Report for Grant Number 01-HO-GR-0113**

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**Submitted by**

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## **Section 1.**

**Title:** Selection of High Performance Microalgae for Bioremediation of Nitrate-Contaminated Groundwater

## **Section 2.**

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## **Section 3.**

**Congressional District:** First Congressional District

## **Section 4.**

### **Description Information:**

#### ***A. Problem and Research Objectives:***

Clean and safe water is a precious and vulnerable resource. In Arizona, more than 40% of drinking water comes from groundwater. Over 1,000 wells across the State exceed the maximum contaminant level of  $10 \text{ mg L}^{-1}$  for nitrogen as nitrate in drinking water set by the US EPA. Major pollutant sources in Arizona include agricultural activities, wastes from industries, leaking underground storage tanks, septic tanks, landfills, mining and wastewater treatment plants. Many of the groundwater quality problems are located in the Phoenix and Tucson metropolitan areas, but groundwater quality problems are found in all of Arizona's 10 watersheds. Particularly, large portions of aquifers within the Salt River Valley, including areas in Glendale, Mesa, Chandler and Phoenix, contain groundwater with nitrate concentrations high enough to render the water unfit for potable use. In addition, high nitrate levels occur in Marana, St. David, Quartzsite, Bullhead City, Lake Havasu City and other areas. Septic tank discharges are common nitrate sources in rural areas of Arizona and have contaminated drinking water wells. Quartzsite, Bullhead City and Lake Havasu City are just a few locations with documented nitrate problems from septic tanks (*ADEQ's FY '02 Groundwater Assessment*).

High levels of nitrate in groundwater pose a serious health risk for some of Arizona's residents. It can be fatal to infants when nitrate is reduced to nitrite, and the latter combines with hemoglobin in the blood to form methemoglobinaemia and leads to a condition known as "blue baby syndrome" (Gangolli et al. 1994). Reduction of nitrate to nitrite can also be a risk to adults deficient in glucose-phosphate dehydrogenase. Moreover, nitrite can react with secondary amines or amides in water or food to form *N*-nitroso compounds that are potential animal carcinogens (Shank 1975; Pontius 1993). Long-term consumption of drinking water containing nitrate concentrations of  $\geq 18 \text{ mg L}^{-1}$  was reported to increase the risk of non-Hodgkin's lymphoma (Ward et al. 1996).

Nitrate removal from groundwater may be accomplished by microbial-based nitrification and denitrification, or chemically and physically-based technologies (such as ion exchange, reverse osmosis, electrodialysis and catalytic denitrification) (Kapoor and Viraraghavan 1997). However, these treatment processes are often difficult and expensive. They require input of external energy sources (e.g., electricity, organic carbon) and/or chemical additives, and generate concentrated waste-streams that then must be disposed. Shortage of surface water supplies coupled with a rapid increase in population places constant pressure on Arizona's cities and water supply utilities to treat and use available groundwater. Development of innovative, environmentally friendly and cost-effective sustainable technologies for treating nitrate-contaminated groundwater is becoming increasingly urgent.

Groundwater nitrate removal by engineered microalgal systems is an advanced concept. Microalgae require mostly simple mineral nutrients, such as nitrogen, phosphorous and inorganic carbon for growth and reproduction. By utilizing sunlight, microalgae convert, through photosynthesis, nitrate into organic compounds (such as proteins). Microalgae can exhibit growth rates that are an order of magnitude higher than other plants due to their extraordinarily efficient light and nutrient utilization. By taking advantage of various designs of engineered microalgal photobioreactors and high density algal culture techniques, large quantities of groundwater can be stripped of nitrate within a short period of time (Hu et al. 1996; 1998).

The long-term goal of the proposed research was to develop an advanced microalgal system for sustainable large-scale nitrate removal from nutrient-contaminated groundwater. The major objectives of this research grant proposal were to isolate high-performance algal species and to evaluate their nitrate uptake potential under various environmental conditions.

## ***B. Methodology***

Isolation and cultivation of microalgae: For isolation of high-performance algal species for maximum nutrient uptake potential, algal samples were collected from various water bodies throughout the metropolitan Phoenix area. Isolation of microalgae, including cyanobacteria, followed the procedure described in Allen (1973). Enrichment cultures for algal isolates were prepared using BG-11 growth medium (Rippka et al. 1979). Membrane filtered (0.45  $\mu\text{m}$  pore size) surface water and groundwater were used for nutrient uptake experiments.

Algal growth measurement: Algal growth was measured using four different methods, depending on the nature of individual species, e.g., unicellular versus filamentous species: optical density, cell count, chlorophyll concentration, and dry weight analysis.

Optical density of the culture was measured with a UV-Vis spectrophotometer at a wavelength of 750 nm.

Cell numbers were determined by placing an aliquot of well-mixed culture suspension on a hemocytometer. Two fields ( $0.1 \text{ mm}^3$ ) were counted per each of two

hemocytometers. Average of four (4) counts were used to calculate cell concentrations. A linear regression equation between optical density and cell counts was established for individual algal species.

For dry weight measurement, a 20-ml aliquot of culture was filtered through pre-weighed Whatman GF/C filter paper. The filter paper was dried overnight in an oven at 100 °C. The difference between the final weight and the weight before filtration was the dry weight of the sample.

A 5-ml culture sample was harvested by centrifugation (14,000 rpm, 5 min), the resulting pellets was extracted with methanol at 4°C overnight. Absorbance of the supernatant at 665 nm was measured with a spectrophotometer.

Nutrient analysis: NO<sub>3</sub><sup>-</sup> and PO<sub>4</sub><sup>-3</sup> measurements were performed on a Bran-Luebbe TrAAcs 800 Autoanalyzer, a continuous flow wet chemistry autoanalyzer using the cadmium reduction method (APHA, #4-89). The instrument was operated according to the standard operating procedure provided by the manufacturer. The standards, QC, and reagents were prepared fresh the day of analysis. For nitrate nitrogen analysis, the standards were made from a 100 ppm concentration of sodium nitrate ranging from 0.01, 0.02, 0.05, 0.2, 0.8, 2.0, and 5.0 ppm. Every six samples the blank, QC, and drift were measured.

Nitrate uptake rate: Cellular nitrate uptake rate of individual algal species was calculated using the following equation:

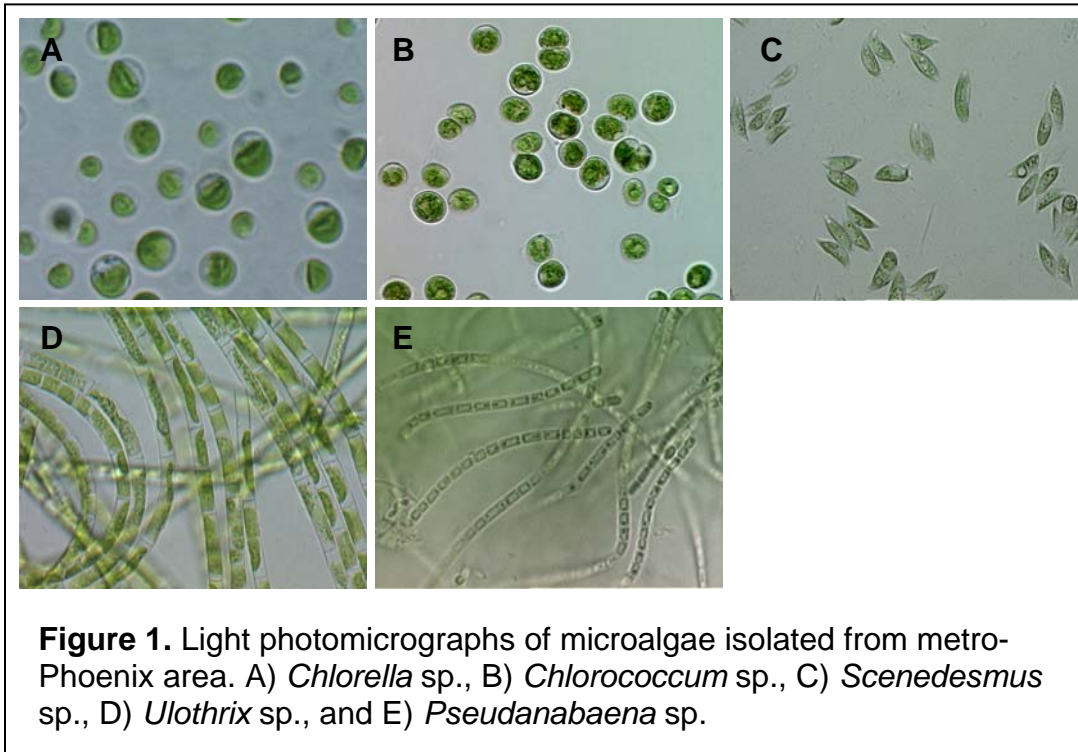
$$\text{Nitrate uptake rate (mg N L}^{-1} \text{ h}^{-1}) = (\text{Ln}N_2 - \text{Ln}N_1)/(\text{t}_2 - \text{t}_1);$$

Where  $t_1$  and  $t_2$  represent different time points, and  $N_1$  and  $N_2$  represent nitrate concentration in the growth medium at time  $t_1$  and time  $t_2$ , respectively.

### ***C. Principal Findings and Significance***

#### Isolation of high-performance microalgae

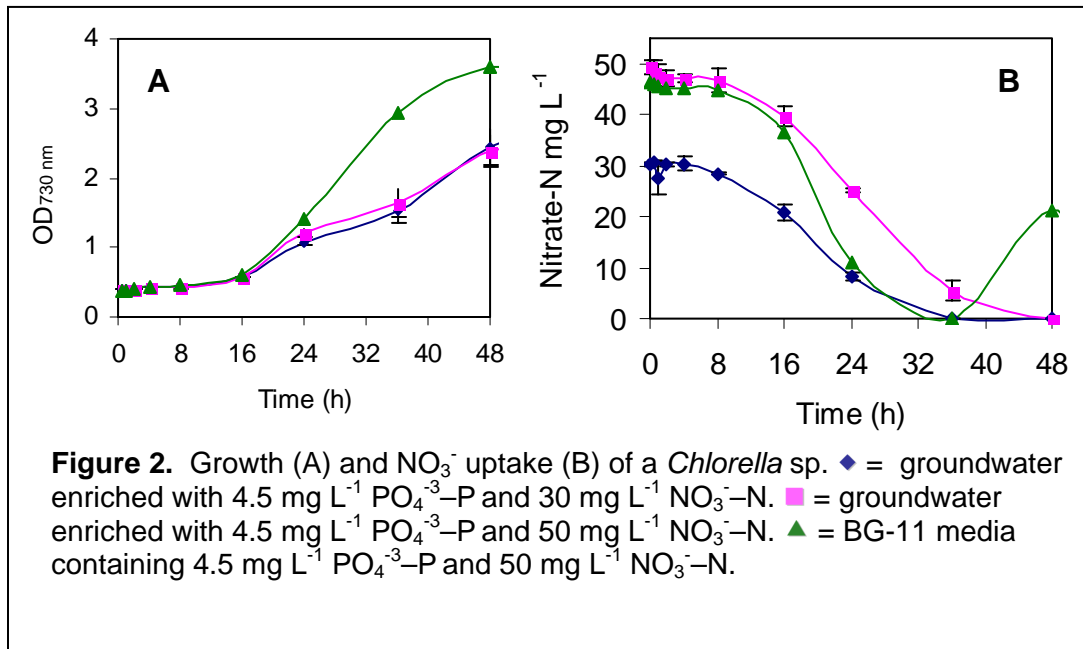
Frequent field sampling trips were made throughout the year to collect algal samples from diverse water environments including groundwater wells, surface canals, urban lakes, irrigation ditches, and wastewater lagoons, as well as private swimming pools. Three unicellular green microalgae, *Chlorella* sp., *Chlorococcum* sp., and *Scenedesmus* sp., one filamentous green alga, *Ulothrix* sp., and one filamentous cyanobacterium, *Pseudanabaena* sp., have been isolated and maintained in the laboratory. The photomicrographs of these algal isolates are shown in **Figure 1**.



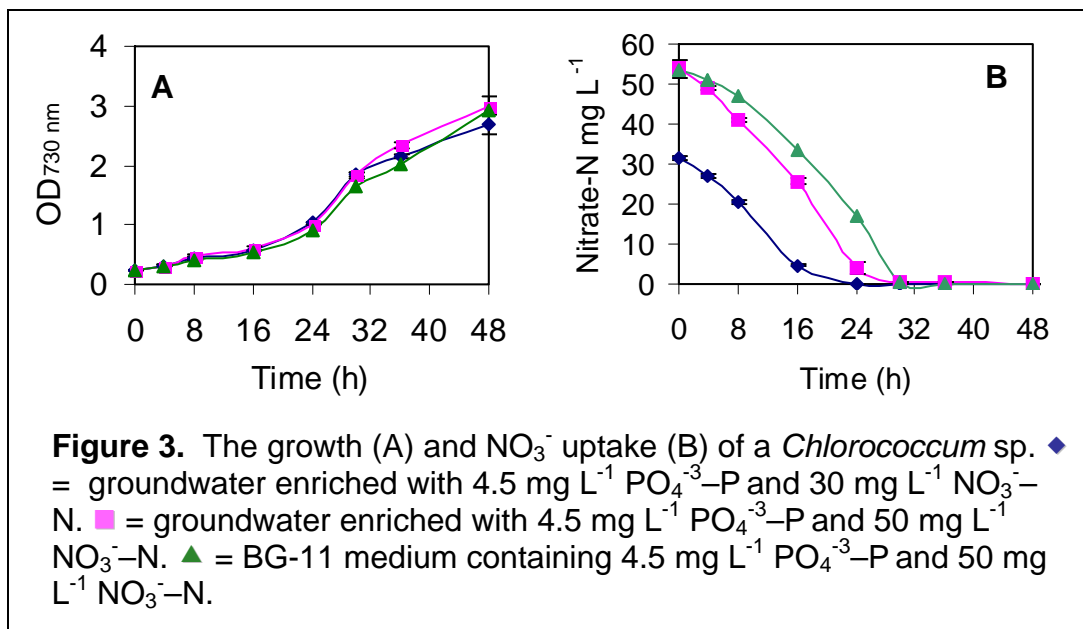
#### Comparative growth and nitrate uptake kinetics

To characterize high-performance algal species, five algal isolates were compared in terms of growth potential and nitrate uptake rate. All cultures were grown in 300-ml glass column reactors at 25 °C and 185  $\text{mol m}^{-2} \text{s}^{-1}$  light. Aeration was provided by compressed air enriched with 1~2%  $\text{CO}_2$  to affect culture mixing. Algae grew in either groundwater or surface water containing 30 to 50  $\text{mg L}^{-1}$  nitrate-N. As the control, BG-11 growth medium was used to support maximum algal growth under the given conditions.

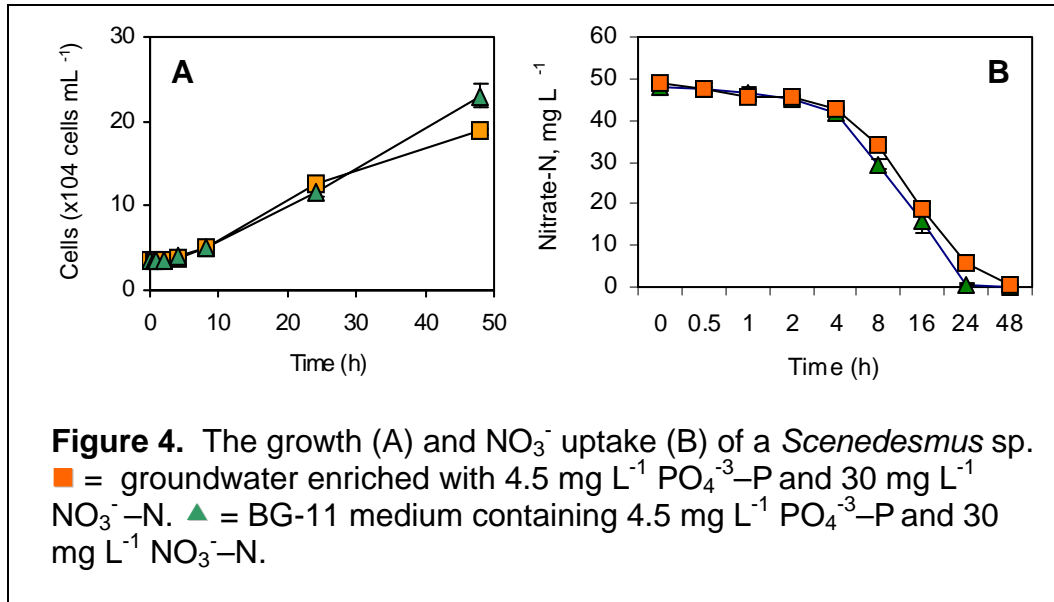
*Chlorella* sp. – This organism grew well in the groundwater and assimilated nitrate. However, the cells grew more rapidly and removed nitrate faster in the BG-11 artificial growth medium than in the natural groundwater (**Figure 2**). However, 50  $\text{mg L}^{-1}$  nitrate-N was reduced to levels below 10  $\text{mg L}^{-1}$  nitrate-N from the BG-11 growth medium by *Chlorella* cells within first 24 h, whereas 36 h was required to reach the same reduction level using the groundwater as culture medium.



*Chlorococcum* sp. – Cells exhibited similar growth potential both in groundwater and in the BG-11 growth medium, suggesting higher tolerance of *Chlorococcum* cells to groundwater than observed for *Chlorella* cells. As shown in **Figure 3**, complete removal of 50 mg L<sup>-1</sup> NO<sub>3</sub><sup>-</sup>-N occurred in both cultures within 32 h.

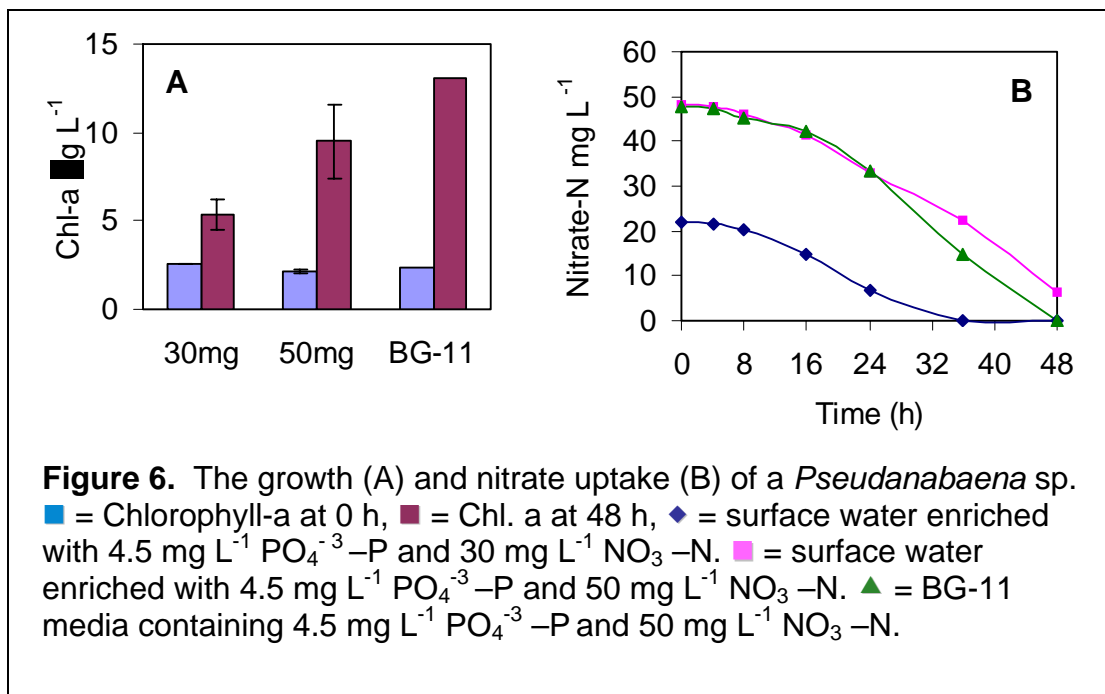
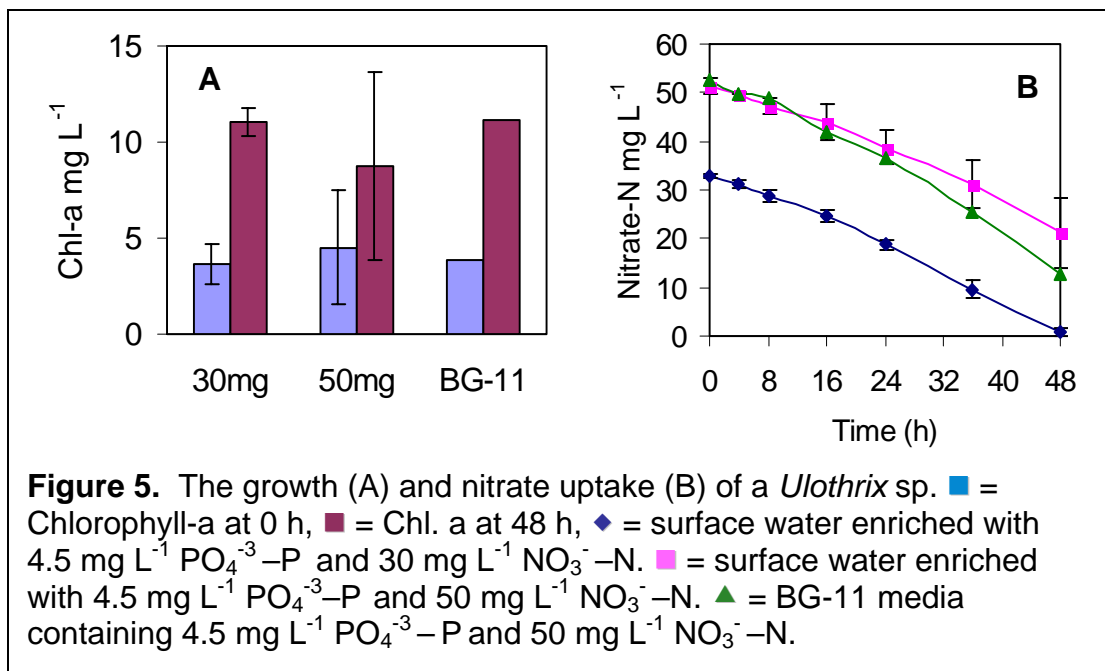


*Scenedesmus* sp. – Like *Chlorococcum* sp., *Scenedesmus* exhibited similar growth and nitrate uptake rates in the groundwater and the BG-11 growth medium. The nitrate concentration decreased from 50 mg L<sup>-1</sup> NO<sub>3</sub><sup>-</sup>-N to below the detection level within 24 h (**Figure 4**).



*Ulothrix* sp. – This alga exhibited similar growth and nitrate removal potential in surface water and BG-11 growth medium (**Figure 5**). When compared to the unicellular algal species described above, this filamentous alga performed poorly in terms of growth and nitrate removal. For instance, the *Ulothrix* culture resulted in a three-fold increase in biomass over a period of 48 h. In contrast, the *Scenedesmus* culture resulted in nearly nine-fold increase in algal biomass over the same period of time. As a result, by the end of 48 h cultivation period, only about 50% of the 50 mg L<sup>-1</sup> NO<sub>3</sub><sup>-</sup>-N was removed in the *Ulothrix* culture.

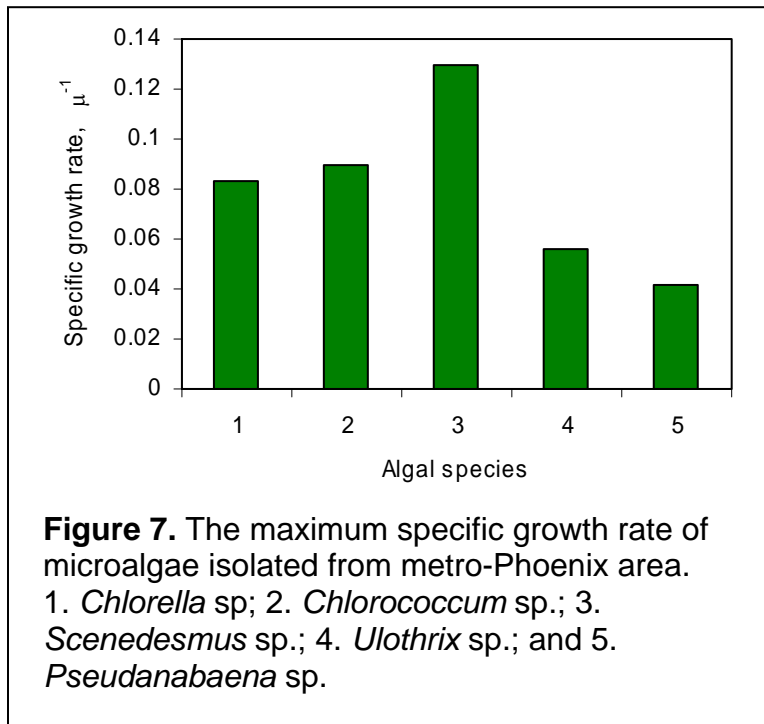
*Pseudanabaena* sp. – This filamentous cyanobacterial species growth and nitrate uptake rate was similar in the surface water and in BG-11 growth medium (**Figure 6**). The overall performance of the *Pseudanabaena* culture was better than that of *Ulothrix* species in terms of nitrate uptake rate. On the other hand, this species did not perform as well as the unicellular species.



Specific growth rate of isolated algal species

**Figure 7** shows the maximum specific growth rates of all five isolated algal species in a batch model under our culture conditions. It demonstrated that the unicellular algal species exhibit higher specific growth rates than the filamentous ones, paralleling the higher nitrate uptake rates. Among the three isolated unicellular green

algae, *Scenedesmus* sp. appears to be the more desirable candidate for high performance nitrate removal.

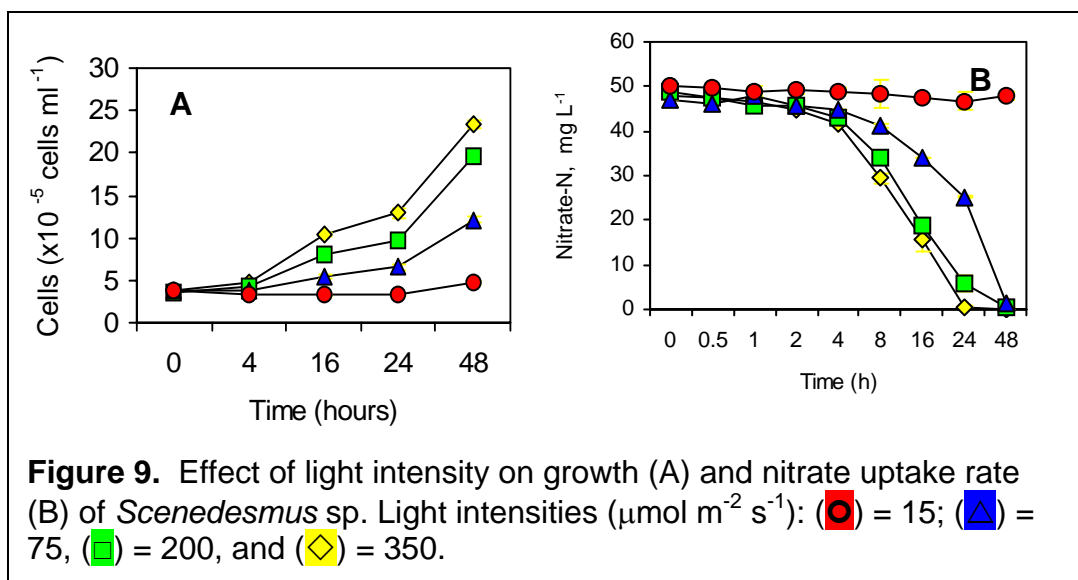
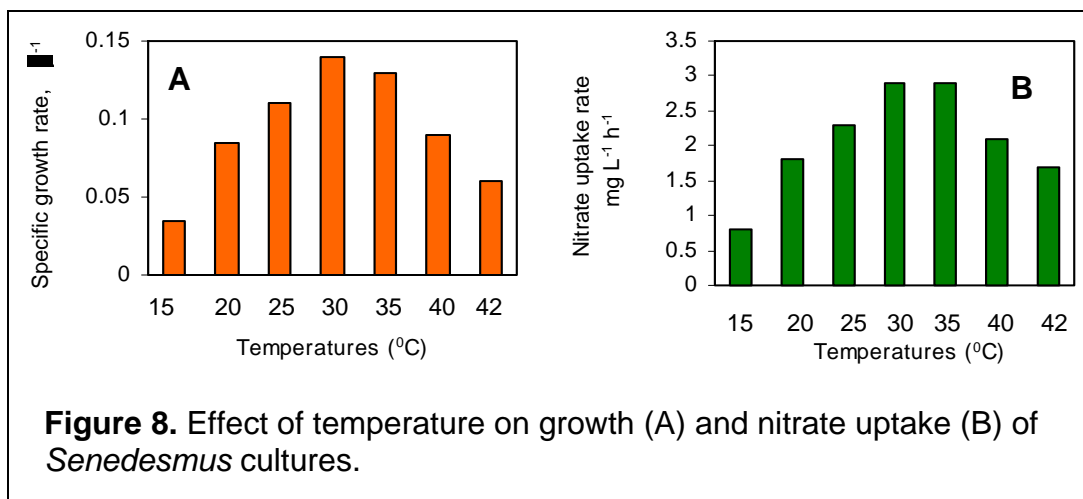


#### Effect of temperature on growth and nitrate uptake

*Scenedesmus* sp. was subjected to further investigation to determine the optimal growth temperature and temperature tolerance for nitrate removal. It appears that the alga can tolerate a broad temperature range for growth and nitrate uptake, with the optimal temperature being from 30 to 35 °C which also results in the maximum cellular nitrate uptake efficiency (**Figure 8**). The high temperature tolerance of *Scenedesmus* sp. makes this organism particularly useful for mass culture outdoors in the Phoenix area.

#### Effect of light intensity on growth and nitrate uptake

As expected there was a positive relationship between light intensity and algal growth and nitrate uptake in cultures of *Scenedesmus* sp. Little growth and nitrate uptake occurred in cultures exposed to 15  $\text{mol m}^{-2} \text{s}^{-1}$  light. As light intensity increased from 15- to 350  $\text{mol m}^{-2} \text{s}^{-1}$ , the maximum specific growth rate increased from 0.035 to 0.12  $\text{h}^{-1}$ , resulting in proportional increase in nitrate uptake (**Figure 9**). These results indicate that cellular nitrate uptake is a growth-dependent process: the higher the algal growth rate the higher the cellular nitrate uptake rate. Therefore, any efforts in improving algal growth rates will likely lead to enhancement in nitrate removal.



In summary, four green algae (*Chlorella* sp., *Chlorococcum* sp.; *Scenedesmus* sp., and *Ulothrix* sp.) and one cyanobacterium (*Pseudanabaena* sp.) were isolated from various water environments in metro Phoenix area. Comparative growth and cellular nitrate uptake kinetics were studied among these algal isolates. The specific growth rate ranged from 0.035 to 0.14 h<sup>-1</sup> with *Scenedesmus* sp. exhibiting the highest growth rate and *Pseudanabaena* sp. the lowest. Compared to the filamentous isolates, the unicellular species exhibited higher specific growth rates. The nitrate uptake rate was species-specific, and hence the algal species that exhibited higher growth rates assimilated nitrate more rapidly. As the high-performance algal strain, *Scenedesmus* sp. was subjected to further investigation, aiming at identifying the optimal culture conditions for sustainable nitrate removal. The specific growth rate and nitrate uptake rate increased with increasing light intensity from 10- to 250 mol m<sup>-2</sup> s<sup>-1</sup>. *Scenedesmus* sp. also exhibited a broad temperature tolerance, from 15 to 42 °C, with 30 to 38 °C resulting in the highest nitrate uptake rate. The average nitrate uptake rate

of 2.6 mg N-NO<sub>3</sub><sup>-</sup> L<sup>-1</sup> h<sup>-1</sup> which occurred in cultures of *Scenedesmus* sp. is ca. 40% to 150% higher than those reported for nitrate removal by other microalgae and cyanobacteria.

The proposed project objectives have been successfully fulfilled, and the work represents a major milestone in the effort to demonstrate that microalgae have potential as an advanced engineered biological system for large-scale nitrate bioremediation. Continuation of this research is necessary in order to develop a highly efficient and cost-effective large-scale photobioreactor, and to reassess the growth physiology and nitrate uptake potential of *Scenedesmus* sp. under outdoor conditions.

## **Section 5. Publication Information**

### a. Articles in Refereed Scientific Journals

None yet

### b. Book Chapters

None

### c. Dissertations

None completed, but two in progress as follows:

Natalie Case

“Screening and characterization of high-performance microalgae for bioremediation of nitrate-contaminated waters”

M.S. Dissertation

School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501

Mike Bellefeuille

“Microalgae-based nitrate bioremediation: from laboratory to pilot scale”

B.S. Dissertation

School of Life Sciences, Arizona State University, Tempe, AZ 85287-4501

### d. Conference Proceedings:

1. Natalie Case, Milton Sommerfeld, and Qiang Hu (2004) A Search For High Performance Microalgae To Remediate Nitrate-Contaminated Groundwater: Concept And Preliminary Results.

*48<sup>th</sup> Annual Meeting of the Arizona-Nevada Academy of Sciences, April 10, 2004, Midwestern University, Glendale, Arizona (Poster).*

2. Mike Bellefeuille, Qiang Hu, and Milton Sommerfeld (2004) Removal Of Nitrate From Agriculture Runoff Using The Green Alga *Scenedesmus* sp. *48<sup>th</sup> Annual Meeting of the Arizona-Nevada Academy of Sciences, April 10, 2004, Midwestern University, Glendale, Arizona (Poster).*

## **Section 6. Student Support**

Two (2) students (a undergraduate, Mike Bellefeuille, and a graduate student, Natalie Case) have benefited from this grant. They have had the opportunity to participate in the novel concept of advanced microalgal bioremediation and get hands-on experiences in applying biological principles to a real world problem, groundwater contamination. Through this one-year project, the students have learned new techniques and how to independently carry out experiments ranging from field water and algal sampling, laboratory screening of microalgae for high-performance strains, growth medium preparation, and algal cultivation and physiological characterization, as well as nutrient (i.e., NO<sub>3</sub><sup>-</sup>, PO<sub>4</sub><sup>-3</sup>) analysis. They have also been exposed to a variety of scientific instruments and devices, including light microscopy, spectrophotometry-based pigment analysis, photobioreactor operation and maintenance, and nutrient analysis. The results obtained thus far have been so encouraging that they will continue working on this topic for their research thesis.

Section 104 Base Grant supported

Undergraduate 1

Masters 1

Ph.D. 0

Post-Doc. 0

Total: 2

## **Section 7. Notable Achievements and Awards**

None

## **Acknowledgements**

The authors gratefully acknowledge the technical assistance of Marisa Masles. This research was supported by grants from the USGS 104B Grant Program.

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