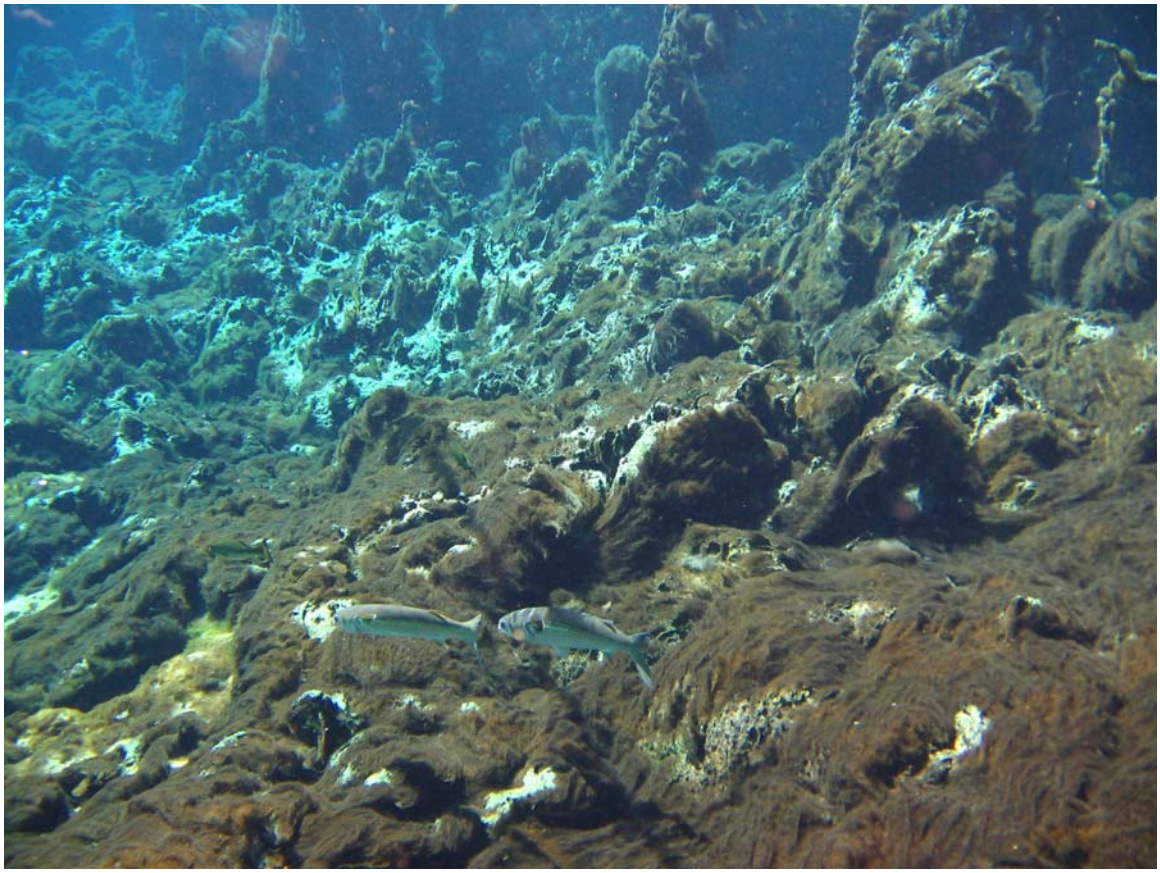
INTEGRATED INTERPRETATION OF SURVEY AND EXPERIMENTAL APPROACHES FOR DETERMINING NUTRIENT THRESHOLDS FOR MACROALGAE IN FLORIDA SPRINGS

LABORATORY EXPERIMENTS AND DISTURBANCE STUDY

DELIVERABLES FOR TASKS 2.1-2.8
DEP CONTRACT NUMBER S0291



SUBMITTED TO THE FLORIDA DEPARTMENT OF ENVIRONMENTAL PROTECTION
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Integrated interpretation of survey and experimental approaches for determining nutrient thresholds for macroalgae in Florida springs.

Description of deliverables for Task 2.1-2.8

Task 2.1-2.3

The deliverable for this task consists of 2 papers: one on Lyngbya wollei and one on Vaucheria sp. The papers describe the results of laboratory experiments on the light, conductivity and microelement requirements of the two taxa.

Effects of light, conductivity and microelements on the growth of *Lyngbya wollei.*

Effects of light, conductivity and microelements on the growth of *Vaucheria* sp.

Task 2.4

The deliverable consists of a short description of the results of field experiments on the recovery of mats of Lyngbya wollei and Vaucheria sp. after complete biomass removal.

Recovery of filamentous algal mats of Lyngbya wollei and Vaucheria sp. after disturbance.

Task 2.5-2.8

The deliverable for this task consists of 2 papers: one on Lyngbya wollei and one on Vaucheria sp. The papers describe the results of laboratory experiments on the effects of

nitrogen (in the form of nitrate and ammonia) and phosphorus on the growth of the two taxa.

Differential effect of PO_4, NO_3 and NH_4 on the growth of *Lyngbya wollei*

Differential effect of PO_4, NO_3 and NH_4 on the growth of *Vaucheria* sp.

In the Vaucheria sp. paper, not all of the results from the laboratory experiments conducted in small microcosms were included. Vaucheria sp. did not respond well to the handling procedure we originally used. In some of the experiments we observed a large variability within our data, and we did not observe any effects of the experimental treatments. When cutting off tips of Vaucheria sp., it is necessary to observe whether the protoplast was damaged in the process. It often can be seen under the dissecting scope when the tip is cut off. Our final solution to this concern was to cut tips off 2 days before the experiment and only use tips that were still healthy 48 hours later. The quality of the algal mat used for the experiments was crucial. If the Vaucheria sp. collected was healthy and vigorously growing in the field and sent to us within 24 hours from collection following the protocol that we developed, we were very successful with the experiments. All mats that I personally collected resulted in healthy growing tips for the experiments and good experimental results. Lyngbya wollei filaments have thick cell walls and can take more abuse than filaments of Vaucheria sp.

A protocol for collection of Vaucheria sp. for use in experiments

A kit to collect live Vaucheria sp. consists of a cooler, two ice packs and two 1 gallon Ziploc® bags. A mat that is intensively growing, has a bright green color, and is free of debris, sediments and invertebrates should be selected for collection. A fragment of a mat should be gently detached and rinsed in the site water by shaking while submersed. A 1 gallon bag should be filled with the site water and a well rinsed mat should be placed inside. The mat should have plenty of water and should not fill more than half of the bag when it returns to its original volume, leaving half of the bag filled just with the site water. The first bag should be placed inside the second bag, sealed and placed on an ice pack in a cooler and covered with an ice pack. If there is any empty space left, it should be filled with bubble wrap to make sure nothing is loose inside the cooler. The lid should be taped and sealed to the cooler using duct tape. The cooler should be shipped overnight and received within 24 hours from collection to assure that the algae will be alive and growing. Upon receipt of the shipment, the bag with algae should be taken out of the cooler and left on the counter for 2-3 hours until its temperature adjusts to room temperature. Then the algae can be examined, any debris should be removed, and the algae can be transferred to the growth media. The growth medium used to maintain fresh collections is a Z8Fl Fe200 medium modified to reflect the median chemistry of fresh water in Florida springs with reduced N and P concentration (Appendix 1).

Effects of light, conductivity and microelements on the growth of *Lyngbya wollei*.

Introduction

Most of the algae nutrition studies focus on the most important macronutrients: nitrogen and phosphorus. In the case of *Lyngbya wollei*, a cyanobacterium forming nuisance mats in lakes, ponds, rivers, streams and springs, studies have focused on the effects of nitrogen, phosphorus, salinity and light (Turner 1990, Speziale and Dyck 1992, Cowell and Botts 1994, Yin et al. 1997, Cowell and Dawes 2004). None of the studies looked at growth at very low light levels and at growth in different conductivity water (at the levels found in fresh water springs). There also are no studies on the effects of iron and micronutrients on the growth of *L. wollei.* The growth media used in other studies were BG11, Z8 and LM6E. Most of these media were developed to sustain algal growth in long-term batch cultures, however, not to optimize the short term growth. In this study we developed a new medium based on the Z8 medium that reflects the median chemistry of freshwater Florida springs. We also tested a new microcosm system for the growth of filamentous algae consisting of polyethylene microcentrifuge tubes.

The objectives of this study were: (i) to compare *L. wollei* growth rates in natural spring water to the ones in the growth medium and to test whether single or multiple filaments should be used while growing algae in microcentrifuge tubes, (ii) to investigate the growth of *L. wollei* at different levels of water conductivity, (iii) to investigate the growth of *L. wollei* under different light levels, (iv) to study the growth of *L. wollei* in media with different iron concentrations and (v) to compare the growth of *L. wollei* in the media with and without the addition of micronutrients.

Materials and methods

Five experiments were conducted using small algal microcosms (Table 1). The goal of any experiment investigating the effects of nutrients on algal growth is to achieve high replication, to test as many nutrient levels as possible, and to grow algae in a continuous culture with constant nutrient levels. To achieve high replication and a large number of treatments, we used microcentrifuge tubes as microcosms in which to grow algae. To achieve semi-continuous culture conditions, we kept the ratio between the algal biovolume and the volume of the growth medium very low, and we frequently changed growth media.

Experimental setup

Filaments of *Lyngbya wollei* were grown in 1.5 ml sterile microcentrifuge tubes filled with 1 ml of growth media. Growth media were changed every 48 hours. Racks made of clear polyethylene were placed on an orbital shaker (60 RPM). Microcentrifuge tubes with *L. wollei* were incubated under grow lights providing 25 μmol/m^2/s of light with the exception of the light gradient experiment. The unobstructed grow lights provided 250 μmol/m^2/s, thus, the desired light intensity was obtained by covering the racks with a neutral density film. The initial filament length was 1-2 mm. The filaments were photographed using a digital video camera system installed on a Leica MZ12.5 Stereomicroscope under 8x magnification at the beginning and at the end of each experiment. Filament length was measured on the digital images using Image J software (Rasband 2003). Incubation time was 11 or 12 days. Each treatment was replicated 10

times. Since some of the filaments were lost during media changes, the final number of replicates used for the data analyses was lower than set at the beginning of the experiment.

All experiments had a complete factorial ANOVA design with 1 or 2 factors. Experiment 1 had a 2 factor design with 3 media treatments (spring water from Alexander, spring water from Silver Glen and Z8Fl medium) and 2 algal biomass treatments (1 and 5 filaments of *L. wollei* in each microcentrifuge tube). Due to an error in preparation of the Z8Fl medium for this experiment, no nitrate was added to the medium. Experiment 2 had a 1 factor design with 5 conductivity treatments (100, 300, 500, 1000 and 3000 µS/cm). The desired water conductivity was obtained by a combination of salts reflecting the chemistry of spring water with different conductivity levels (Table 2). Experiment 3 had a 2 factor design with 15 treatments: a combination of 3 conductivity levels (100, 300, 1000 µS/cm) and 5 light intensities (1, 2.5, 5, 25, 250 µmol/m^2/s). Experiment 4 had 2 treatments: a growth medium with and without the addition of microelements. Experiment 5 had a 1 factor design, with 4 iron concentrations (0, 20, 200, 600 µg Fe/L). If not stated differently, all treatments had the same concentration of microelements, Fe, N, P, Ca, Mg, CO3 as in the standard Z8Fl medium (Appendix 1).

Growth media

A new growth medium was developed based on the Z8 medium (Kotai 1972). The Z8 medium was modified to simulate the median chemistry of macronutrients in Florida spring water (referred to as medium Z8Fl) (Appendix 1). All growth media were autoclaved or filter sterilized.

Water analyses

Ammonia nitrogen, Kjeldahl nitrogen, nitrite-nitrate nitrogen and total phosphorus concentrations were analyzed in the prepared growth media by FDEP (Table 3). A single sample of each growth medium was sent for the analyses. When the concentration of a compound was below the detection limit of the laboratory method, the method's detection limit is reported (Table 4). None of the water analyses results were designated as Q or Y. Total nitrogen was calculated as a sum of nitrite-nitrate nitrogen (nitrates) and Kjeldahl nitrogen. Water conductivity was measured using YSI 556 MSP.

Experimental conditions

All experiments were conducted in the walk-in growth room. The temperature in the growth room was set for 22.5ºC to reflect the average water temperature of the Florida springs. The minimum and maximum temperatures were recorded daily on a max-min thermometer.

Relative growth rates

Algal relative growth rate (RGR) in the small microcosm experiments was calculated using the following formula (Hunt 1990):

$$\text{RGR} = \frac{\ln(\text{Final Length}) - \ln(\text{Initial Length})}{\#\text{ of days}}$$

Data analyses

Data were analyzed using R statistical software (R Development Core Team 2006). Analysis of variance was conducted using a general linear model with car package. If

necessary, data were square root or 1/square root transformed to meet the assumptions of normality and variance homogeneity.

Results

Experiment 1 - growth of L. wollei *in spring water and in growth media*

Both growth media and the number of filaments in the microcosm affected the growth rate of *L. wollei* (ANOVA for medium effect $p<0.026$ and for number of filaments effect $p<0.022$) (Fig. 1). When 5 filaments were present in the microcosm, their growth rates were lower than in the microcosms with 1 filament. Overall, the growth of *L. wollei* was affected by the nitrate concentration in the water (Table 5). Due to an error in the preparation of the Z8Fl medium for this experiment, we were not able to truly test the medium in comparison to spring water, but in later experiments using the Z8Fl medium, we observed growth rates higher than the ones in spring water in this experiment.

Experiment 2 - growth of L. wollei *in water with different conductivity*

Lyngbya wollei grew well in water with conductivity from 100 to 1000 µS/cm. Its growth was reduced at 3000 µS/cm (ANOVA for conductivity effect $p<0.022$) (Fig. 2). Actual conductivities measured in the treatments were: 105, 331, 516, 963 and 2942 µS/cm. All treatments had the same nitrogen and phosphorus concentrations, indicating that the observed growth differences were the result of different conductivity levels in the growth media (Table 5).

Experiment 3 -growth of L. wollei *under different light levels and water conductivities*

Both light and conductivity affected the growth rates of *L. wollei* (ANOVA for light effect $p<0.001$; for conductivity effect $p<0.040$ and for interaction light*conductivity $p<0.004$). *Lyngbya wollei* grew even at very low light levels, but it had the highest growth rates at a light intensity of 25 µmol/m^2/s or higher (Fig. 3). Only at the lowest light level (1 µmol/m^2/s) water conductivity had an effect on the growth rates of *L. wollei* and at that light level filaments in 100 µS/cm conductivity water grew better than filaments in 1000 µS/cm conductivity water (TukeyHSD $p<0.010$).

Experiment 4 - growth of L. wollei *in the media with and without micronutrients added*

Surprisingly, addition of microelements to the growth media resulted in reduced growth rates (ANOVA for micronutrient effect $p<0.001$) (Fig. 4). The Gaffron's micronutrient mix used in the Z8 medium has relatively low micronutrient concentrations compared to other freshwater growth media. There was no difference in the nitrogen and phosphorus concentration between the different growth media (Table 5).

Experiment 5 - growth of L. wollei *in different iron concentrations*

Iron is an important micronutrient for the growth of *L. wollei*. (ANOVA for the iron effect $p<0.001$) (Fig. 5). When no iron was added to the growth media, very low growth rates of *L. wollei* were observed. Surprisingly, an iron concentration of 600 µg/L, found in the standard Z8 medium, had a negative effect on the growth of *L. wollei*. High growth rates were observed at 20 and 200 µg Fe/L. There were no differences in the nitrogen and phosphorus concentrations between the different growth media (Table 5).

Discussion

Lyngbya wollei is clearly a freshwater species, since it did not grow well in high conductivity water (Fig. 2). Speziale and Dyck (1992) and Cowell and Botts (1994) also observed reduced growth rates and loss of pigment when *L. wollei* was grow*n* in growth media to which salt or sea water had been added . *Lyngbya wollei* grew well at low light levels (Fig.3), as also described by Turner (1990) and Yin et al. (1997). It did not respond well to the addition of microelements and to a high concentration of iron in the growth media (600 µg Fe/L) (Fig. 5). This is opposite to *Lyngbya majuscula,* which has a very high iron requirement, 1400-6000 µg/L (Gross and Martin 1996). Microcentrifuge tubes used as microcosms for the growth of algal filaments worked very well, and using one 1-2 mm long filament worked better then using multiple filaments. Even though all chemicals used to prepare media were ACS certified, they have microelements in them as contaminants, and these are probably sufficient for *L. wollei* growth. Further study on the effects of particular microelements is needed to determine which micronutrient in Gaffron's mix is causing the reduction of growth rates. When conducting experimental work on *L. wollei,* growth media with reduced iron and micronutrient concentrations should be used as proposed in Appendix 1.

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Fig. 1

Box plot showing relative growth rates (RGR) or *L. wollei* in a factorial mesocosm experiment with 3 growth media (spring water from Alexander, spring water from Silver Glen and Z8Fl growth medium but with very low nitrogen and phosphorus content) and 2 biomass levels: 1 or 5 filaments (n=57).

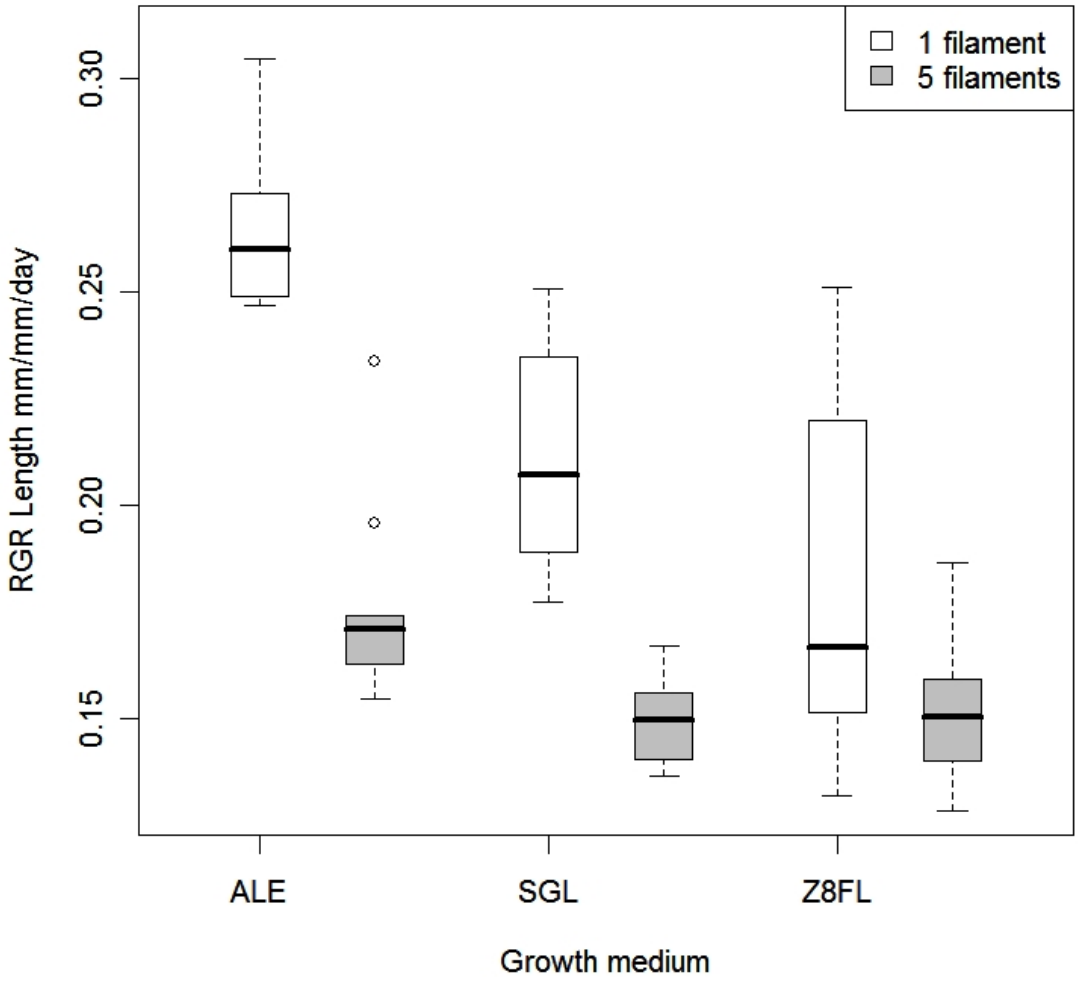

Fig. 2

Box plots showing relative growth rates (RGR) of *L. wollei* in different conductivity water (n=45). Boxes sharing the same letter do not differ (Tukey's test $p<0.05$).

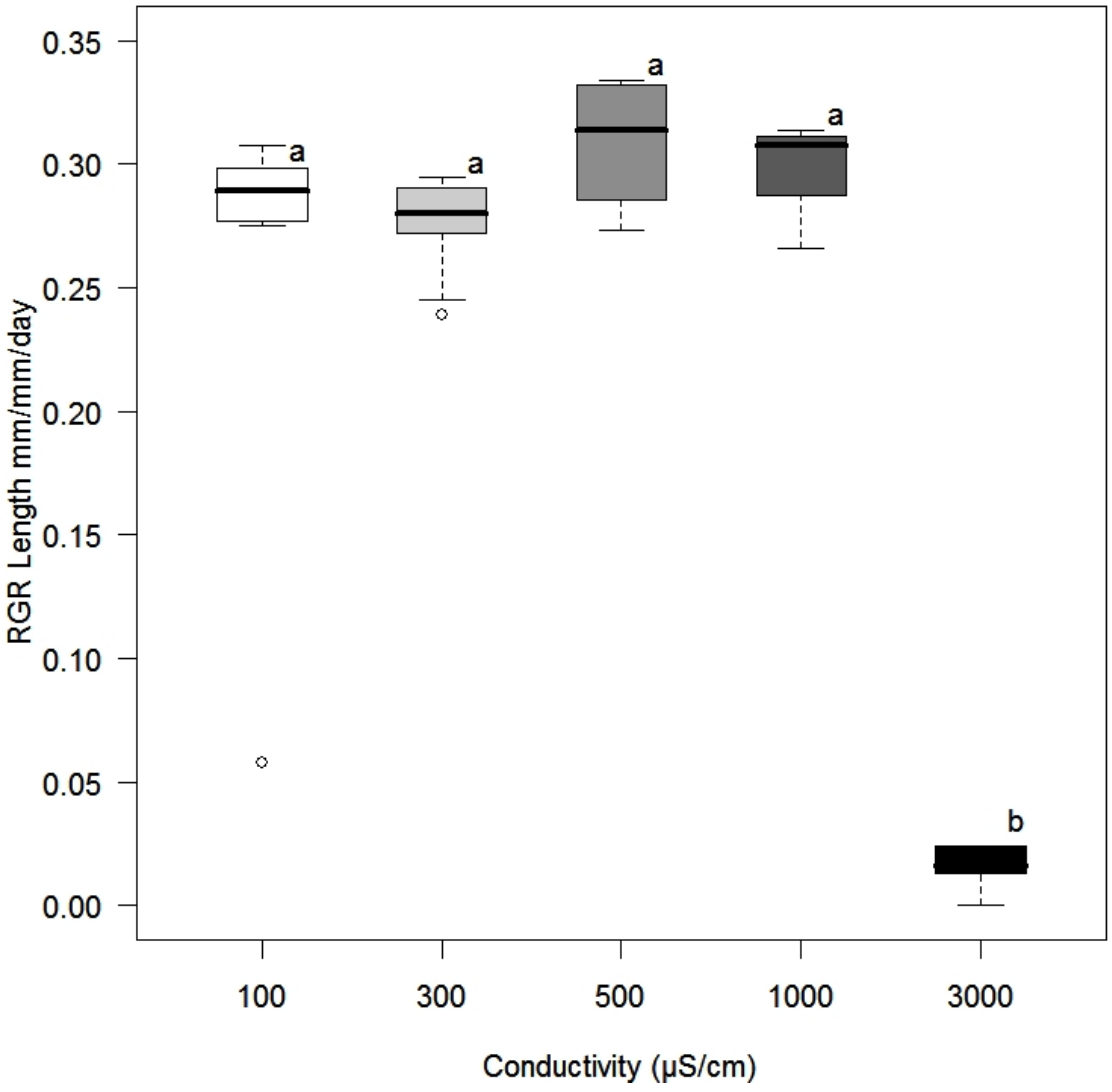

Fig. 3

Box plots showing relative growth rates (RGR) in a factorial mesocosm experiment with 5 light levels and 3 conductivity levels (n=121).

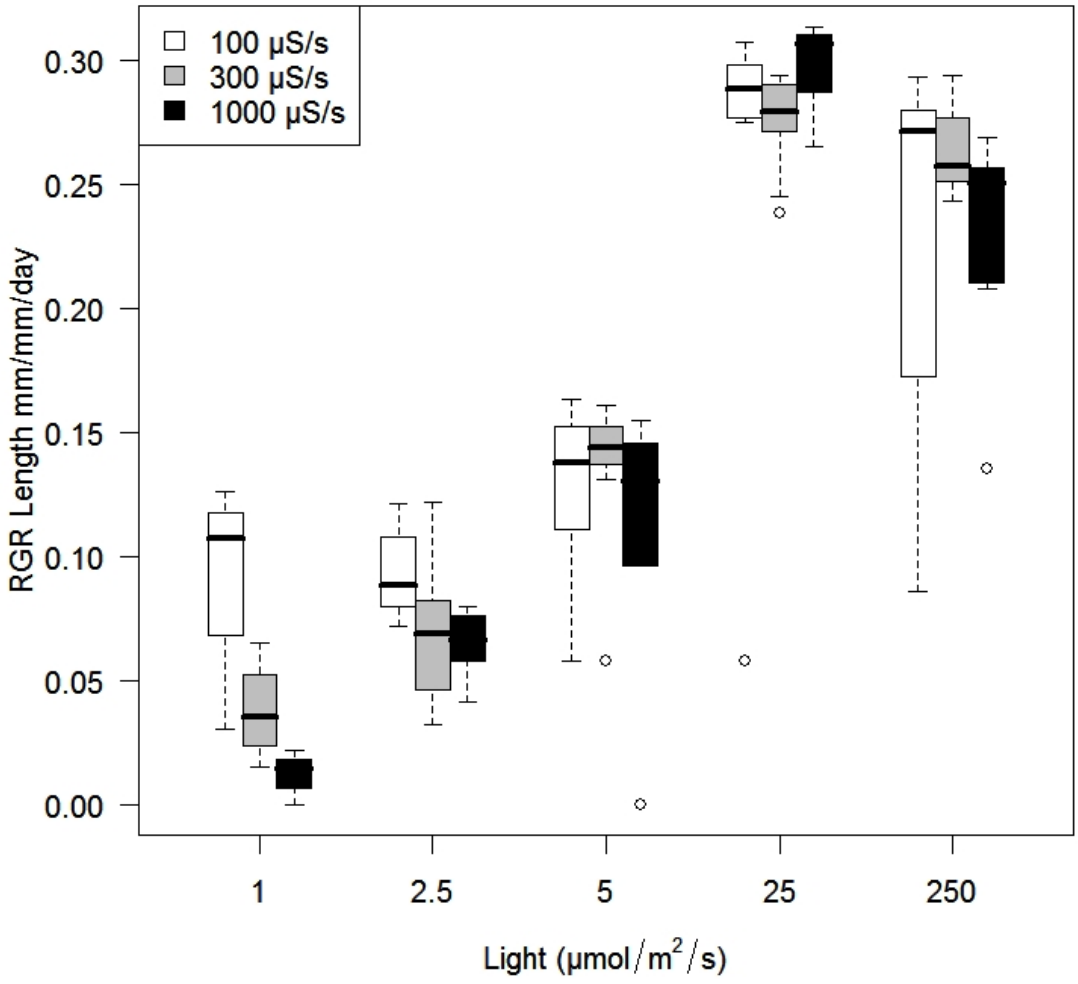

Fig. 4

Box plots showing relative growth rates (RGR) *L. wollei* in growth media with and without micronutrients added (n=17).

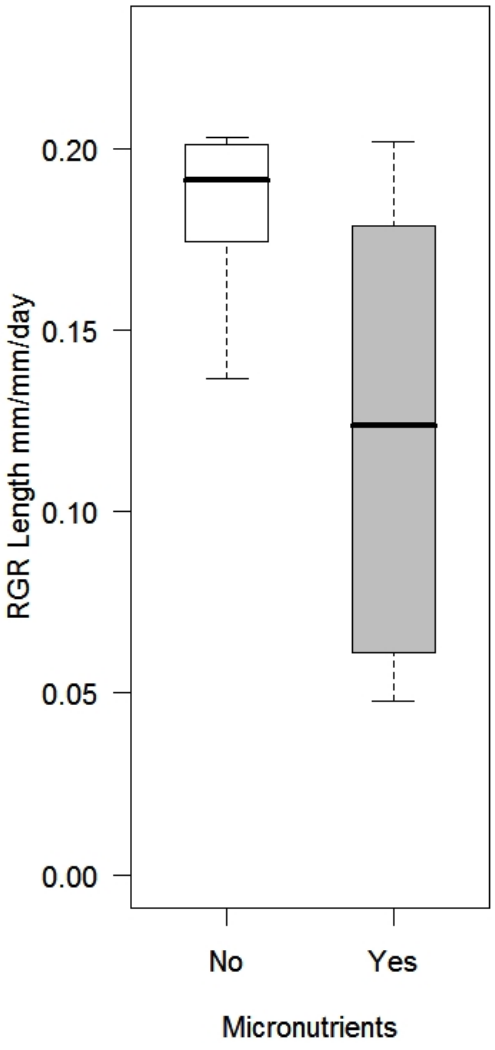

Fig. 5

Box plot showing relative growth rates (RGR) of *L. wollei* in 4 iron concentrations (n=37). Boxes sharing the same letter do not differ (Tukey's test $p<0.05$).

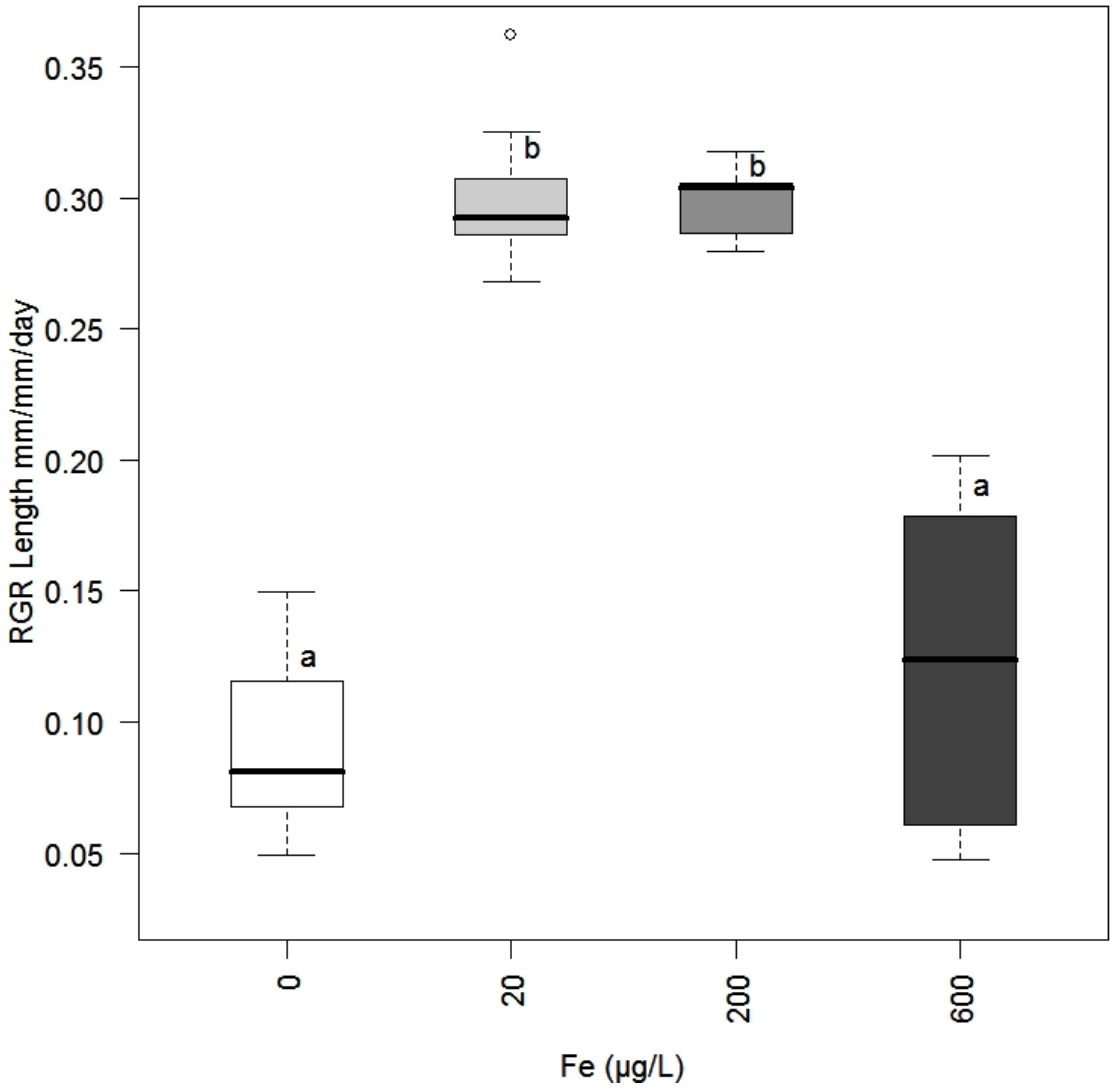

Table 1

Lyngbya wollei experiments. Experiment numbers in parenthesis refer to numbers used in the 2004 report (Stevenson et al. 2004).

Experiment (project experiment number)	Treatments	Algae collection location	Season	Growth medium	Mean min and max temperature (ºC)
1 (6)	Spring water, media and number of filaments in the tube	Ichetucknee	Summer	Z8Fl	20.6 & 24.9
2 (13)	Conductivity	Ichetucknee	Summer	Z8Fl	20.4 & 24.8
3 (13)	Light and conductivity	Ichetucknee	Summer	Z8Fl	20.4 & 24.8
4 (14)	Microelements	Ichetucknee	Summer	Z8Fl	20.6 & 24.8
5 (14)	Iron	Ichetucknee	Summer	Z8Fl	20.6 & 24.8

Table 2

Chemical composition of a component of growth media in experiments 2 and 3 responsible for desired conductivity in the medium.

Conductivity (µS/cm)	$CaCl_2 \cdot 2H_2O$ (mM)	$MgSO_4 \cdot 7H_2O$ (mM)	Na_2CO_3 (mM)	KCl (mM)	NaCl (mM)
100	0.116	0.101	0.198	0	0
300	0.496	0.203	0.792	0	0
500	0.747	0.284	1.293	0	0
1000	1.618	0.284	1.293	0.123	0.123
3000	2.739	0.284	1.293	0.875	0.875

Table 3

Methods used for analyses of water samples.

Parameter	Method	Description
Ammonia-N	EPA 350.1 (1983)	Ammonia in aquaeous matrices as mg N/L
NO2NO3-N	EPA 353.2 (1983)	Nitrite/Nitrate in aqueous matrices as mg N/L
N_KJEL_TOT	EPA 351.2 (1983)	Total Kjeldahl Nitrogen in aqueous matrices
Total-P	EPA 365.4	Total Phosphorus in aqueous matrices

Table 4

Detection limits of water chemical analyses. When the concentration of a compound was below the detection limit of the laboratory method, the method detection limit is reported.

Compound	Detection limit (mg/L)
Ammonia nitrogen	0.010
Total Kjeldahl nitrogen	0.060
Nitrates	0.004
Total phosphorus	0.004

Table 5

Measured concentration of nitrate, ammonia, total nitrogen and total phosphorus in the growth media used in the experiments.

Treatments	NH_4-N (µg/L)	NO_3-N (µg/L)	TN (µg/L)	TP (µg/L)
Experiment 1:				
Alexander spring water	22	53	220	41
Silver Glen spring water	13	19	180	24
Z8Fl	<10	6	270	42
Experiment 2 – conductivity:				
100 µS/cm	<10	1000	1360	94
300 µS/cm	18	1000	1350	74
500 µS/cm	<10	1000	1360	73
1000 µS/cm	<10	990	1320	77
3000 µS/cm	11	990	1370	69
Experiment 4 – microelements:				
not added	<10	1100	1370	110
added	13	1000	1200	100
Experiment 5 – Fe:				
0 µg/L	18	1000	1120	95
20 µg/L	16	1000	1190	94
200 µg/L	17	1000	1240	80
600 µg/L	16	1000	1220	110

Effects of light, conductivity and microelements on the growth of *Vaucheria* sp.

Introduction

Vaucheria sp. (Xanthophyce) is a common filamentous mat-forming species in streams, rivers and lakes (Entwisle 1990, McIntire et al. 1994, Necchi et al. 1994). There are many studies on the ecological and environmental requirements *of Vaucheria* sp. (Lakatos 1978, Gómez et al. 1994, Aboal et al. 1996). Many species of *Vaucheria* show high tolerance to different water salinities (Moreno et al. 2001), although they do prefer sites with high light availability (Ensminger et al. 2005) (Leukart and Hanelt 1995). *Vaucheria* sp. is also commonly used in studies on cell structure (Kataoka and Watanabe 1993, Tornbom and Oliveira 1993, Raven and Brownlee 2001, Takahashi et al. 2001). However, there are very few laboratory studies on the nutrition requirements of *Vaucheria* sp., and these studies focus on macronutrients such as nitrogen and phosphorus (Sudlow 1936, Ozimek et al. 1991).

The objectives of this study were: (i) to compare *Vaucheria* sp. growth rates in natural spring water to the ones in the growth medium and to test whether single or multiple filaments should be used while growing algae in microcentrifuge tubes, (ii) to investigate the growth of *Vaucheria* sp. at different levels of water conductivity, (iii) to investigate the growth of *Vaucheria* sp. under different light levels, (iv) to study the growth of *Vaucheria* sp. in media with different iron concentrations and (v) to compare the growth of *Vaucheria* sp. in media with and without the addition of micronutrients.

Materials and methods

Five experiments were conducted using small algal microcosms (Table 1). The goal of any experiment investigating the effects of nutrients on algal growth is to achieve high replication, to test as many nutrient levels as possible, and to grow algae in a continuous culture with constant nutrient levels. To achieve high replication and a large number of treatments, we used microcentrifuge tubes as microcosms in which to grow algae. To achieve semi-continuous culture conditions, we kept the ratio between the algal biovolume and the volume of the growth medium very low, and we frequently changed growth media.

Experimental setup

Filaments of *Vaucheria* sp. were grown in 1.5 ml sterile microcentrifuge tubes filled with 1 ml of growth media. Growth media were changed every 48 hours. Racks made of clear polyethylene were placed on an orbital shaker (60 RPM). Microcentrifuge tubes with *Vaucheria* sp. were incubated under grow lights providing 25 $\mu mol/m^2/s$ of light, with the exception of the light gradient experiment. The unobstructed grow lights provided 250 $\mu mol/m^2/s$, thus, the desired light intensity was obtained by covering the racks with a neutral density film. The initial filament length was 1-2mm. The filaments were photographed using a digital video camera system installed on a Leica MZ12.5 Stereomicroscope under 8x magnification at the beginning and at the end of each experiment. Filament length was measured on the digital images using Image J software (Rasband 2003). Incubation time was 11 or 12 days. Each treatment was replicated 10 times. Since some of the filaments were lost during media changes, the final number of

replicates used for the data analyses was lower then set at the beginning of the experiment.

All experiments had a complete factorial ANOVA design with 1 or 2 factors. Experiment 1 had a 2 factor design with 3 media treatments (spring water from Alexander, spring water from Silver Glen and Z8Fl medium) and 2 algal biomass treatments (1 and 5 filaments of *Vaucheria* sp. in each microcentrifuge tube). Due to an error in preparation of the Z8Fl medium for this experiment, no nitrate was added to the medium. Experiment 2 had a 1 factor design with 5 conductivity treatments (100, 300, 500, 1000 and 3000 µS/cm). The desired water conductivity was obtained by a combination of salts reflecting the chemistry of spring water with different conductivity levels (Table 2). Experiment 3 had a 2 factor design with 15 treatments: a combination of 3 conductivity levels (100, 300, 1000 µS/cm) and 5 light intensities (1, 2.5, 5, 25, 250 µmol/m^2/s). Experiment 4 had 2 treatments: a growth medium with and without the addition of microelements. Experiment 5 had a 1 factor design, with 4 iron concentrations (0, 20, 200, 600 µg Fe/L). If not stated differently, all treatments had the same concentration of microelements, Fe, N, P, Ca, Mg, CO_3 as in the standard Z8Fl medium (Appendix 1).

Growth media

A new growth medium was developed based on the Z8 medium (Kotai 1972). The Z8 medium was modified to simulate the median chemistry of macronutrients in Florida spring water (referred to as medium Z8Fl) (Appendix 1). All growth media were autoclaved or filter sterilized.

Water analyses

Ammonia nitrogen, Kjeldahl nitrogen, nitrite-nitrate nitrogen and total phosphorus concentrations were analyzed in the prepared growth media by FDEP (Table 3). A single sample of each growth medium was sent for the analyses. When the concentration of a compound was below the detection limit of the laboratory method, the method's detection limit is reported (Table 4). None of the water analyses results were designated as Q or Y. Total nitrogen was calculated as a sum of nitrite-nitrate nitrogen (nitrates) and Kjeldahl nitrogen. Water conductivity was measured using YSI 556 MSP.

Experimental conditions

All experiments were conducted in the walk-in grow room. The temperature in the grow room was set for 22.5ºC to reflect the average water temperature of the Florida springs. The minimum and maximum temperatures were recorded daily on a max-min thermometer.

Relative growth rates

Algal relative growth rate (RGR) in the small microcosm experiments was calculated using the following formula (Hunt 1990):

$$\text{RGR} = \frac{\ln(\text{Final Length}) - \ln(\text{Initial Length})}{\#\text{ of days}}$$

Identification of Vaucheria species

Since the presence of reproductive structures is necessary to identify the species of *Vaucheria,* we incubated a small fragment of the mat (Ott and Oldham-Ott 2003). The

mat fragment was placed onto a Petri dish filled with Z8Fl growth medium with 100 µg/L P and 1000 µg/L N and placed by the window. We were successful in observing reproductive structures in the samples collected from Manatee spring. The species was *Vaucheria geminata* (Prescott 1982).

Data analyses

Data were analyzed using R statistical software (R Development Core Team 2006). Analysis of variance was conducted using a general linear model with car package. If necessary, data were square root or 1/square root transformed to meet the assumptions of normality and variance homogeneity.

Results

Experiment 1 - growth of Vaucheria sp. *in spring water and in growth media*

Neither the growth media nor the number of filaments in the microcosm had an effect on the growth rate of *Vaucheria* sp. (ANOVA for medium effect $p<0.091$ and for number of filaments effect $p<0.622$) (Fig. 1). Due to an error in the preparation of the Z8Fl medium for this experiment, we were not able to truly test the medium in comparison to spring water. In later experiments using the Z8Fl medium, however, we observed growth rates higher than the ones in spring water in Experiment 1.

Experiment 2 - growth of Vaucheria sp. *in water with different conductivity*

Vaucheria sp. grew well in water with conductivity at 100, 300 and 1000 µS/cm, but its growth was reduced in water with conductivity at 500 and 3000 µS/cm (ANOVA for

conductivity effect p<0.016) (Fig. 2). However, when the means were compared using the Tukey HSD test, none of the differences among the means was significant at p=0.05. At the levels of conductivity tested, the effect of conductivity on growth of *Vaucheria* sp. was indefinite. Actual conductivities measured in the treatments were: 105, 331, 516, 963 and 2942 μS/cm. All treatments had the same nitrogen concentrations and the same phosphorus concentrations, indicating that the observed growth differences resulted from different conductivity levels in the growth media (Table 5).

Experiment 3 -growth of Vaucheria sp. *under different light levels and water conductivities*

Light affected the growth rates of *Vaucheria* sp. and conductivity did not (ANOVA for light effect p<0.001; for conductivity effect p<0.657 and for interaction light*conductivity p<0.046). *Vaucheria* sp. grew even at very low light levels, but the growth rates were very low (Fig. 3). The highest growth rates were observed at a light intensity of 25 μmol/m^2/s or higher.

Experiment 4 - growth of Vaucheria sp. *in the media with and without micronutrients added*

The addition of microelements to the growth media had no effect on the growth rates of *Vaucheria* sp. (ANOVA for micronutrient effect p<0.382) (Fig. 4). There was no difference in the nitrogen and phosphorus concentration in the growth media (Table 5).

Experiment 5 - growth of Vaucheria sp. *in different iron concentrations*

Iron is an important micronutrient for the growth of *Vaucheria* sp. (ANOVA for the iron effect p<0.001) (Fig. 5). When no iron was added to the growth media and when the iron

concentration in the media was 20 µg/L, very low growth rates of *Vaucheria* sp. were observed. Surprisingly, the iron concentration of 600 µg/L found in the standard Z8 medium had a negative effect on the growth of *Vaucheria* sp. High growth rates were observed at 200 µg Fe/L. There were no differences in the nitrogen and phosphorus concentrations measured in the growth media (Table 5).

Discussion

Vaucheria sp. had comparable growth rates in spring water and in the artificial growth medium, and there were no differences in the growth rate between 1 and 5 filaments incubated in 1 microcentrifuge tube (Fig. 1). We also did not observe a trend in the response of *Vaucheria* sp. to different conductivities of water (Fig. 2). Many species of *Vaucheria* are adapted to changes in water conductivity and are found from fresh water to marine environments (Moreno et al. 2001, Johanson 2005). *Vaucheria* sp. did not grow well at very low light levels, 1 and 2.5 µmol/m^2/s. The highest growth rates were found at a light intensity of 25 µmol/m^2/s. In the streams, *Vaucheria* sp. prefers areas well exposed to light and does not adapt to low light levels (Ensminger et al. 2005). We did not observe a difference in the growth rate of *Vaucheria* sp. incubated in the growth media with or without the addition of micronutrients. *Vaucheria* sp. is tolerant to high levels of metals in the water, much higher than the concentrations used in our experiments (Pirszel et al. 1995, Skowronski et al. 1998, Alessa and Oliveira 2001). We were, however, surprised that it did not prefer the media with added micronutrients. The optimum iron concentration for the growth of *Vaucheria* sp. was 200 µg/L. It did not grow well in the iron concentration of 600 µg/L found in the Z8 growth medium.

In our experiments, *Vaucheria* sp. showed a large variability of growth rates in all of the treatments. Also, in all of the experimental treatments, we found many filaments that did not grow at all (0 relative growth rate). This was due to the process of cutting short 1-2 mm tips of *Vaucheria* sp. for the experiments. Even though *Vaucheria* sp. is known for its ability to heal wounded areas, not all of the tips survived the process of being cut off. (Tornbom and Oliveira 1993) found that after excising a 3 mm long vegetative filament of *Vaucheria longicaulis,* the wound-healing process took several hours and new growth occurred 43 hours after wounding. Therefore, we changed our methodology in further experiments on *Vaucheria* sp. and incubated 1-2mm tips for 2 days in the Z8Fl growth medium (Appendix 1) before using them in the experiments.

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Fig. 1

Box plot showing relative growth rates (RGR) or *Vaucheria* sp. in a factorial mesocosm experiment with 3 growth media (spring water from Alexander, spring water from Silver Glen and Z8Fl growth medium but with very low nitrogen and phosphorus content) and 2 biomass levels: 1 or 5 filaments (n=50).

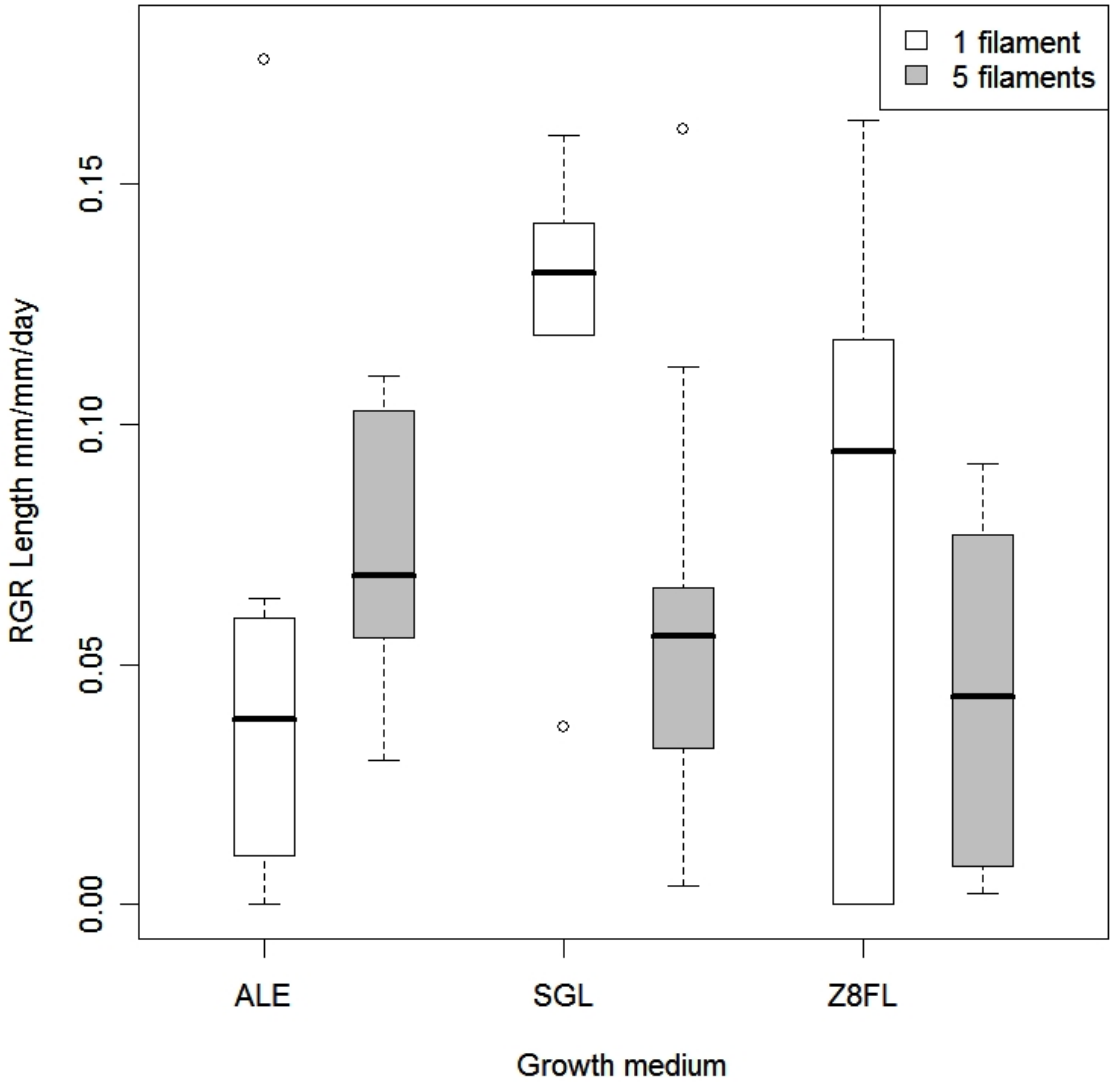


Fig. 2

Box plots showing relative growth rates (RGR) of *Vaucheria* sp. in different conductivity water (n=48).

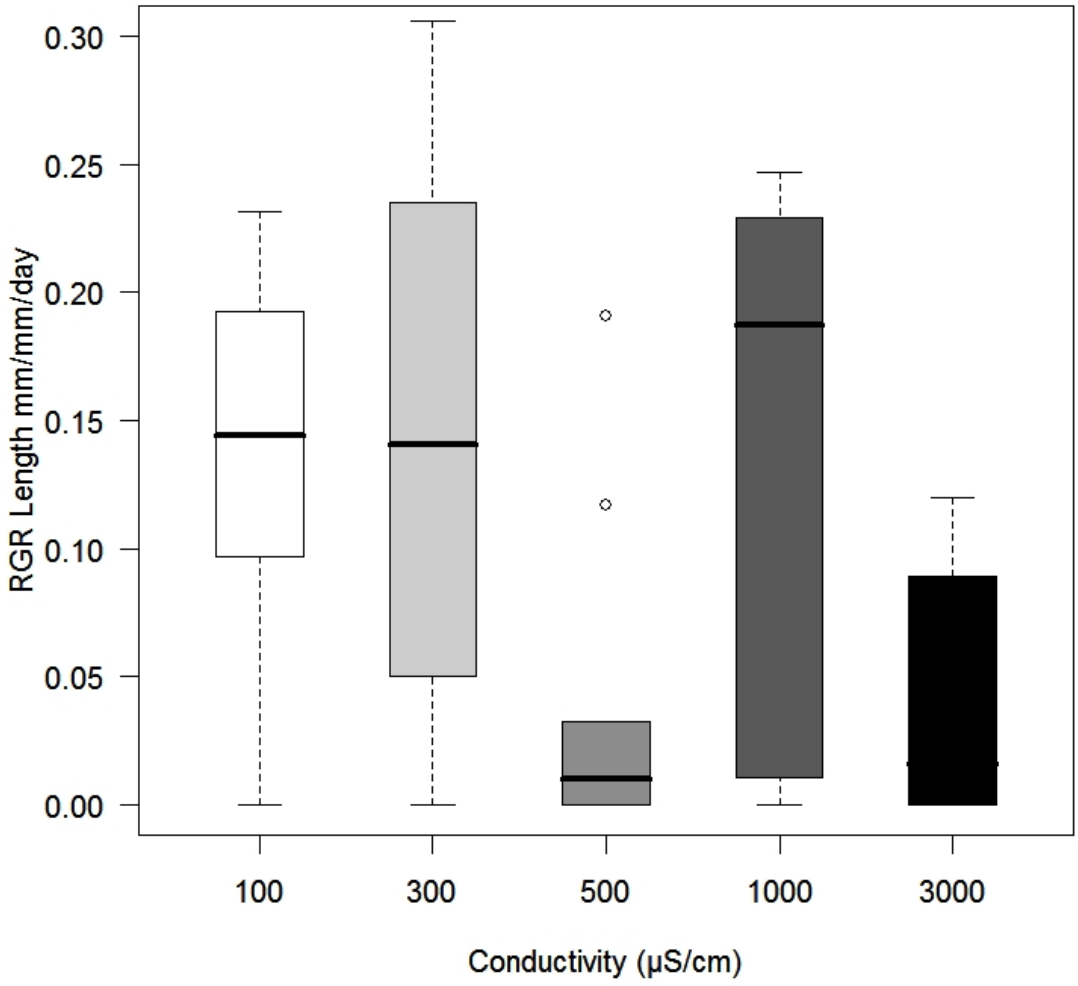


Fig. 3

Box plots showing relative growth rates (RGR) of *Vaucheria* sp. in a factorial mesocosm experiment with 5 light levels and 3 conductivity levels (n=121).

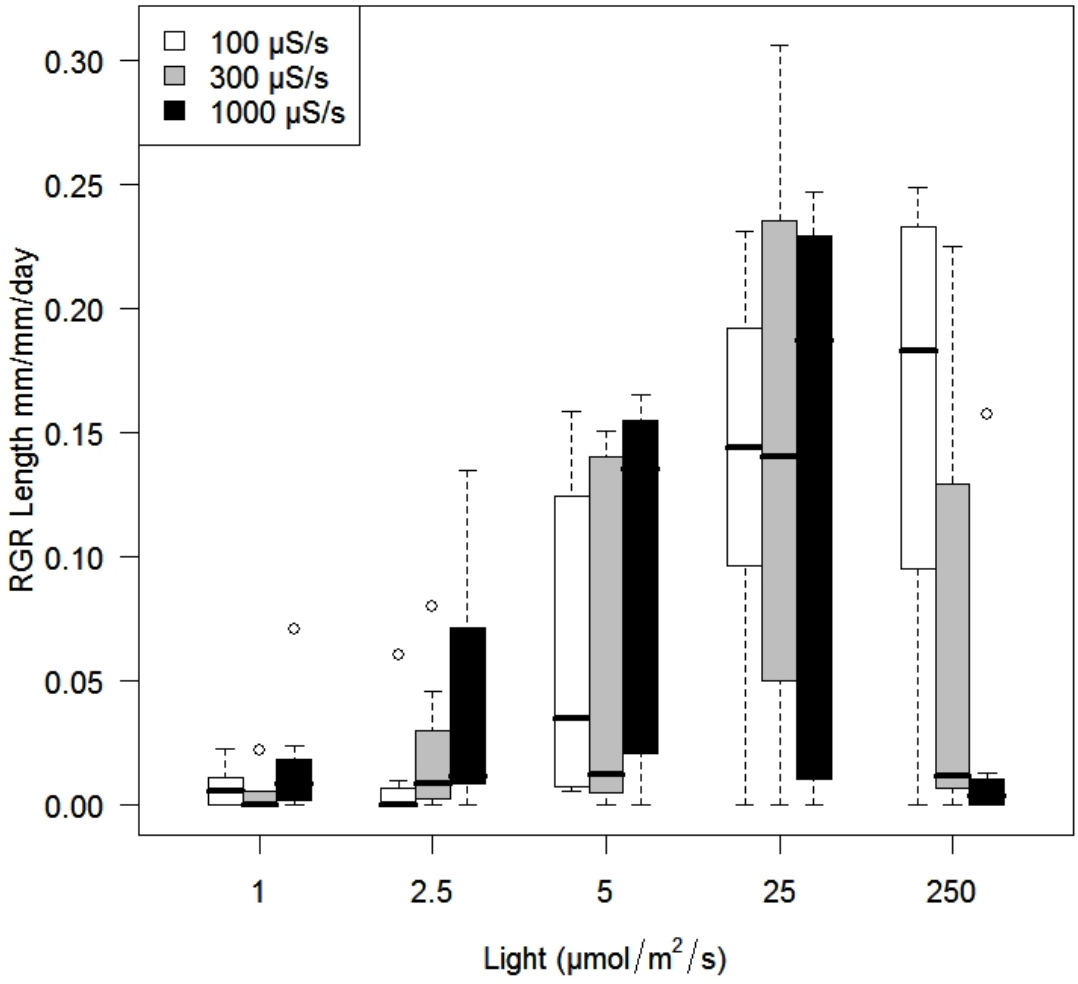


Fig. 4

Box plots showing relative growth rates (RGR) of *Vaucheria* sp. in growth media with and without micronutrients added (n=18).

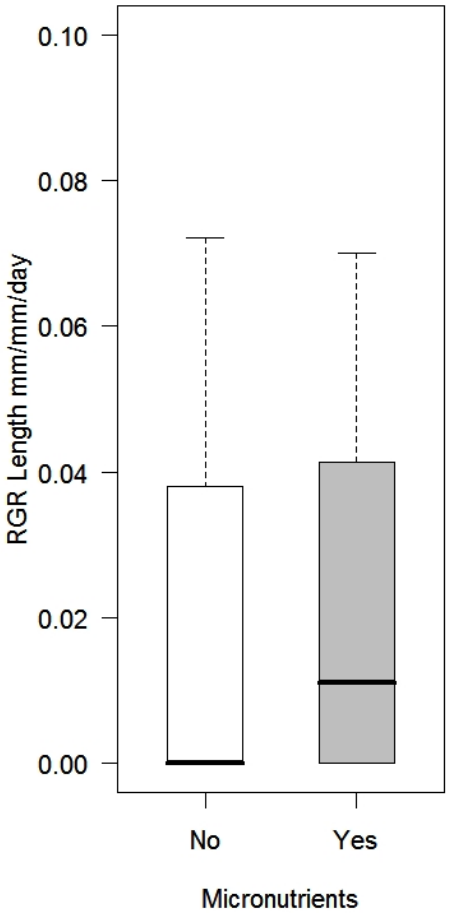


Fig. 5

Box plot showing relative growth rates (RGR) of *Vaucheria* sp. in 4 iron concentrations (n=39). Boxes sharing the same letter do not differ (Tukey's test $p<0.05$).

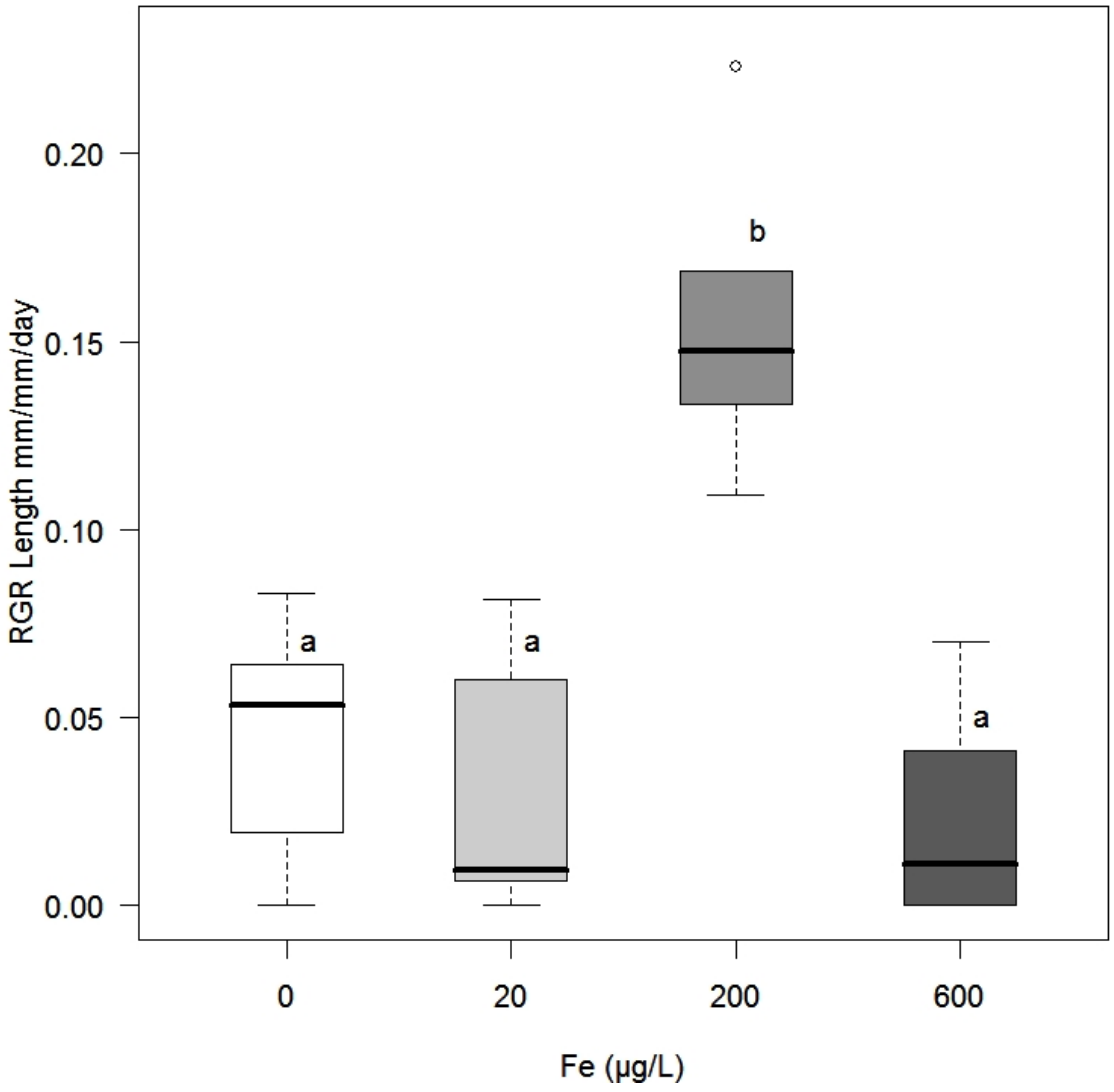


Table 1

Vaucheria sp. experiments. Experiment numbers in parenthesis refer to numbers used in the 2004 report (Stevenson et al. 2004).

Experiment (project experiment number)	Treatments	Algae collection location	Season	Growth medium	Mean min and max temperature (ºC)
1 (6)	Spring water, media and number of filaments in the tube	Manatee	Summer	Z8Fl	20.7 &25.0
2 (13)	Conductivity	Manatee	Summer	Z8Fl	20.4 & 24.7
3 (13)	Light and conductivity	Manatee	Summer	Z8Fl	20.4 & 24.7
4 (14)	Microelements	Manatee	Summer	Z8Fl	20.6 & 24.8
5 (14)	Iron	Manatee	Summer	Z8Fl	20.6 & 24.8

Table 2

Chemical composition of a component of growth media in experiments 2 and 3 responsible for desired conductivity in the medium.

Conductivity (µS/cm)	$CaCl_2 \cdot 2H_2O$ (mM)	$MgSO_4 \cdot 7H_2O$ (mM)	Na_2CO_3 (mM)	KCl (mM)	NaCl (mM)
100	0.116	0.101	0.198	0	0
300	0.496	0.203	0.792	0	0
500	0.747	0.284	1.293	0	0
1000	1.618	0.284	1.293	0.123	0.123
3000	2.739	0.284	1.293	0.875	0.875

Table 3

Methods used for analyses of water samples.

Parameter	Method	Description
Ammonia-N	EPA 350.1 (1983)	Ammonia in aquaeous matrices as mg N/L
NO2NO3-N	EPA 353.2 (1983)	Nitrite/Nitrate in aqueous matrices as mg N/L
N_KJEL_TOT	EPA 351.2 (1983)	Total Kjeldahl Nitrogen in aqueous matrices
Total-P	EPA 365.4	Total Phosphorus in aqueous matrices

Table 4

Detection limits of water chemical analyses. When the concentration of a compound was below the detection limit of the laboratory method, the method detection limit is reported.

Compound	Detection limit (mg/L)
Ammonia nitrogen	0.010
Total Kjeldahl nitrogen	0.060
Nitrates	0.004
Total phosphorus	0.004

Table 5

Measured concentration of nitrates, ammonia, total nitrogen and total phosphorus in the growth media used in the experiments.

Treatments	NH_4-N (µg/L)	NO_3-N (µg/L)	TN (µg/L)	TP (µg/L)
Experiment 1:				
Alexander spring water	22	53	220	41
Silver Glen spring water	13	19	180	24
Z8Fl	<10	6	270	42
Experiment 2 – conductivity:				
100 µS/cm	<10	1000	1360	94
300 µS/cm	18	1000	1350	74
500 µS/cm	<10	1000	1360	73
1000 µS/cm	<10	990	1320	77
3000 µS/cm	11	990	1370	69
Experiment 4 – microelements:				
not added	<10	1100	1370	110
added	13	1000	1200	100
Experiment 5 – Fe:				
0 µg/L	18	1000	1120	95
20 µg/L	16	1000	1190	94
200 µg/L	17	1000	1240	80
600 µg/L	16	1000	1220	110

Recovery of filamentous algal mats of *Lyngbya wollei* and *Vaucheria* sp. after disturbance.

Introduction

Algae in Florida springs are subject to physical disturbance, most commonly flooding and physical disturbance by large animals (large fish, manatees and alligators) or human activity (swimming, snorkeling, SCUBA diving). This results in sediment deposition which reduces light availability or direct biomass removal. The goal of this investigation was to simulate the effects of large animal herbivory and disturbance and the effects of sever flooding with turbid water that result in mass algal die off. In this study we monitored the regrowth of filamentous algal mats of *Lyngbya wollei* and *Vaucheria* sp. after direct biomass removal.

Materials and methods

This study was conducted at two locations: Manatee spring and Blue Hole in the Ichetucknee spring system. The dominant mat-forming filamentous alga at Manatee spring is *Vaucheria* sp (Stevenson et al. 2004). The Manatee spring waters are frequently turbid due to flooding from the Suwannee River, and as its name suggests, the spring is also frequently visited by manatees that graze on algae. The Blue Hole spring bottom is covered by macrophytes and mats of *Lyngbya wollei*. The Blue Hole spring stays clear even during severe flooding events. This study was conducted from January to May 2006. Three sites were selected within each spring. Each site consisted of 2

experimental plots (50cm x 50cm each) delineated by PVC poles driven into the sediments. All of the sites were within restricted areas that are not affected by direct human activity. At each site, the initial thickness of the mat was measured. There were no differences in the pretreatment mat thickness for the disturbed and control plots, indicating that there were no site-specific differences between the plots. After the initial measurement of mat thickness, one plot within each site was disturbed. Using a rake, all visible filamentous algae were removed down to the sediments. The recolonization of disturbed areas and algal growth on the control (undisturbed) plots were initially monitored every 2 weeks, and later less frequently depending on the recolonization rates. Mat thickness was measured with a ¼" diameter dowel at 13 evenly distributed points on each plot. On each observation date, the water depth, dissolved oxygen (DO), pH, conductivity and temperature were measured.

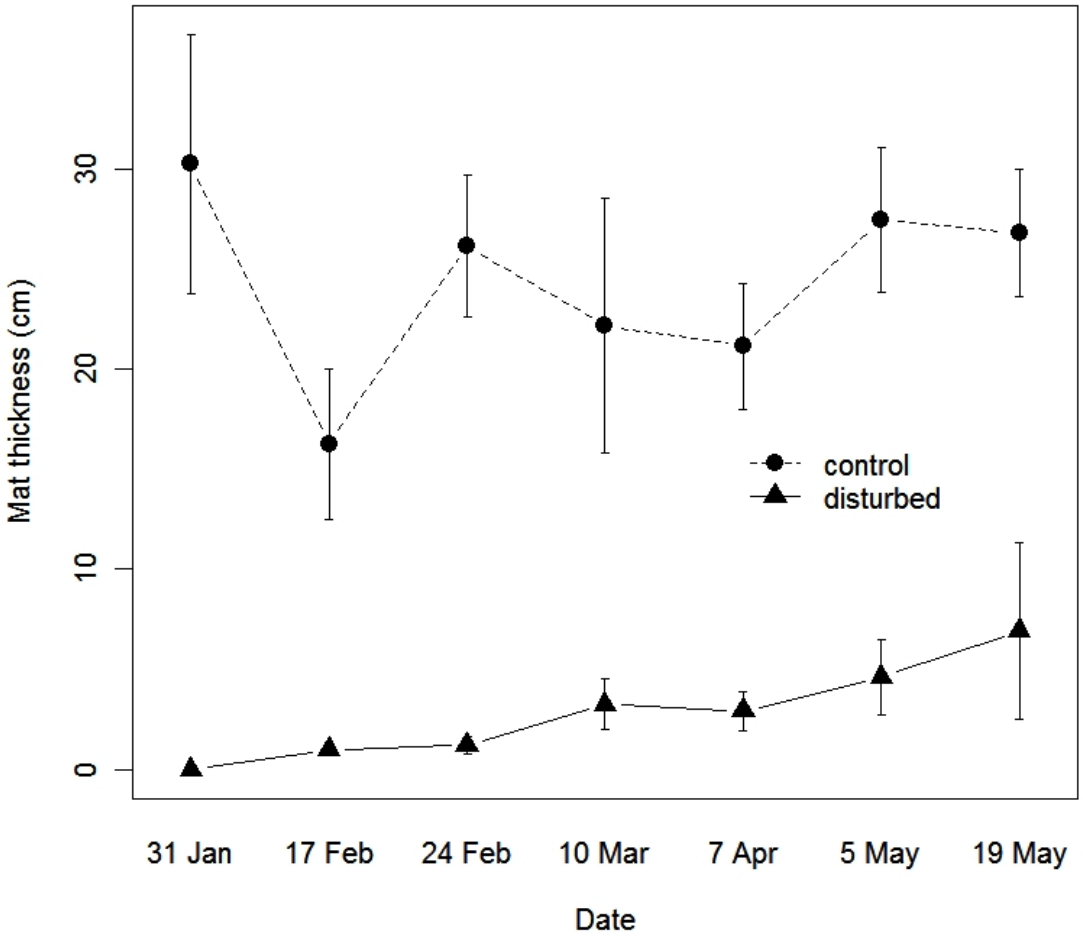
Data were analyzed using SAS statistical software (SAS 2000) (repeated measures ANOVA with LS means test) and R statistical software (R Development Core Team 2006). Data were square root transformed to reduce deviations from normality and homoscedasticity.

Results

The two populations studied had very different recovery rates after disturbance. The control mats of *Lyngbya wollei* did not change in thickness during the course of this study. Disturbed plots of *Lyngbya wollei* slowly grew back but did not recover from thedisturbance even after 108 days (repeated measures ANOVA: time effect $p<0.0013$, disturbance effect $p< 0.0021$) (Fig. 1).

Fig. 1

Average thickness of the mats of *Lyngbya wollei* at Blue Hole in Ichetucknee springs. Disturbed sites were completely scraped on the 31 of January and control sites were undisturbed.

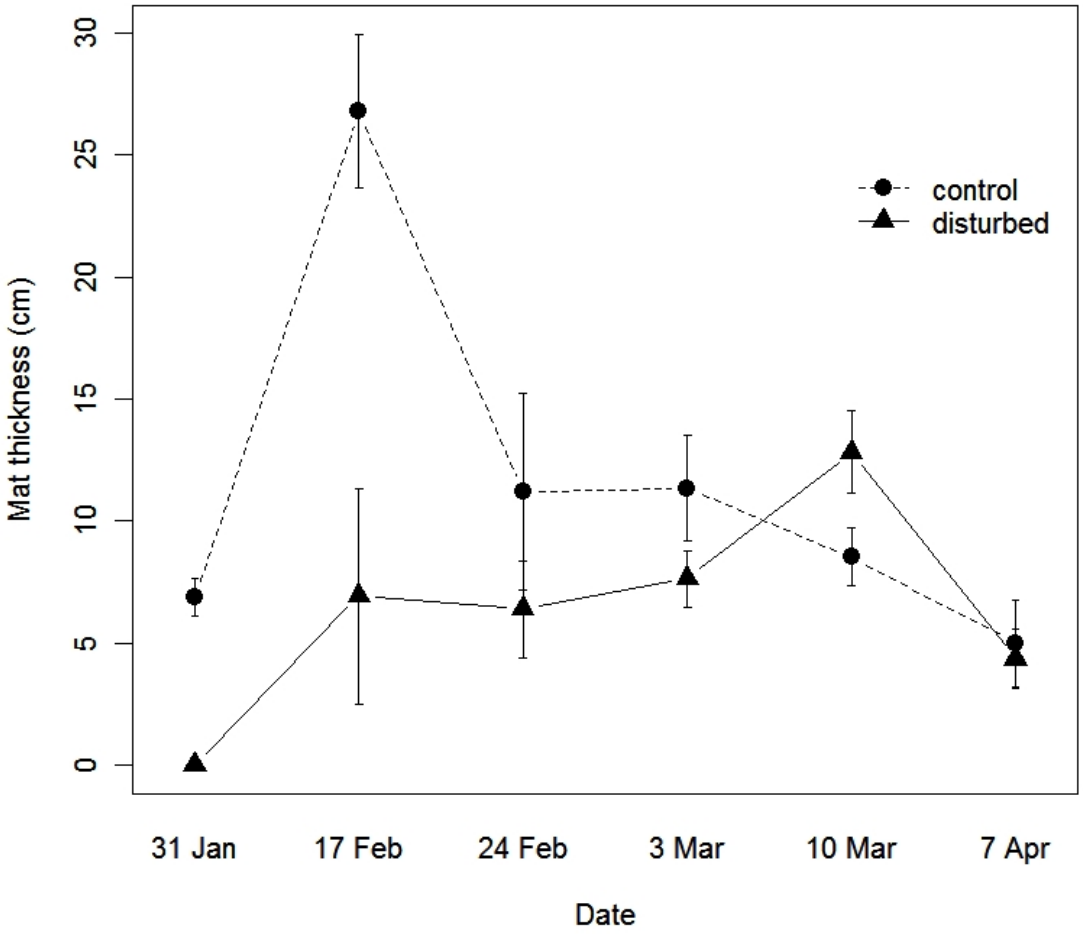


Vaucheria sp. in the control plots had the thickest mats on February 17. Later, mat thickness decreased and in April control plots were senescing and decomposing. Disturbed *Vaucheria* sp. recovered very quickly, and after 24 days there was no

difference in the thickness of the mat at the disturbed and control sites (LS means for 24 Feb p=0.2067, for 3 Mar p=0.3108, for 10 Mar p=0.2600, 7 Apr p=0.8269). By the end of our study, the control mats had begun to senesce and disintegrate (Fig. 2).

Fig. 2

Average thickness of the mats of *Vaucheria* sp. at Manatee spring. Disturbed sites were completely scraped on January 31 and control sites were undisturbed.



The two sites studied had comparable water parameters, however, Manatee springs is characterized by a higher amplitude of changes in conductivity and dissolved oxygen (Table 1).

Table 1

Water parameters at the two studied sites.

Spring	Conductivity (µS/cm)	Dissolved oxygen (mg/L)	pH	Temperature (ºC)
Blue Hole	279-312	2.3-2.6	6.8-7.7	21.35-21.43
Manatee	285-502	1.0-2.4	6.7-7.8	21.35-22.1

Discussion

Even though in our lab experiments the relative growth rates of *Vaucheria* sp. and *Lyngbya wollei* were similar, *Vaucheria* sp. recovered very quickly after disturbance and *Lyngbya wollei* did not recover even after 108 days. Both sites studied are characterized by high nutrient levels (Stevenson et al. 2004). The difference between the two taxa in the speed of recovery after disturbance can be explained by their different means of reproduction and the differences in their growth forms. *Vaucheria* sp. can reproduce asexually by fragmentation of the mat and through production of zoospores, akinets and aplanospores (Starmach 1972, Johanson 2005). It can also reproduce sexually by formation of antheridia and oogonia. The mat is formed from multinucleate filaments

and attached by colorless rhizoids to the substrate. *Lyngbya wollei* reproduces only by fragmentation of trychomes (Komárek and Anagnostidis 2005). The fact that *Vaucheria* sp. can reproduce much faster than *L. wollei* probably explains why *Vaucheria* sp. is the dominant taxon in springs along the Suwannee River. Manatee, Fanning and Guaranto springs are dominated by mats of *Vaucheria* sp. These springs also frequently experience the intrusion of turbid waters from the Suwannee River during floods.

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Differential effect of PO_4, NO_3 and NH_4 on the growth of *Lyngbya wollei*

Introduction

Lyngbya wollei (Farlow ex Gomont), family Oscillatoriace, a filamentous blue green alga with unusually thick, often over 60 μm wide, trichomes, forms nuisance growths in many Florida springs. In the algae surveys of 68 Florida spring sites conducted in 2003 and 2006 (Stevenson et al. 2004), and unpublished data) it was found that large mats were most commonly formed by *L. wollei.* This filamentous alga is found in water bodies throughout the southeastern United States (Speziale and Dyck 1992, Cowell and Botts 1994, Cowell and Dawes 2004), Texas (Owens et al. 2005), Canada (Macbeth 2004) and other locations. *Lyngbya wollei* can fix atmospheric nitrogen (Phlips et al. 1992) but large mats are often found at sites with high nitrate concentrations (Cowell and Botts 1994, Stevenson et al. 2004).

In the natural environment the growth of filamentous algal mats is highly affected by many biotic and abiotic factors. Even though *L. wollei* mats are not readily consumed by herbivores (Camacho and Thacker 2006) in Florida springs the mats are disturbed by large vertebrates and people visiting the springs. To successfully study nutrient effects on the growth of algae, the mats have to be moved to controlled laboratory conditions. Several studies investigated the effects of nitrogen and phosphorus on the growth of *L. wollei* (Turner 1990, Cowell and Botts 1994, Yin et al. 1997, Cowell and Dawes 2004). These studies used only a few concentrations of nutrients in their experiments and none of them investigated the threshold levels of nutrients for the growth of *L. wollei.* .

This study focused only on *L. wollei* from Florida springs. The objectives of this study were: (i) to investigate the effects of a wide range of concentrations of nitrogen (in the form of nitrate and ammonia) and phosphorus on the growth of *L. wollei*, (ii) to determine whether nitrate or ammonia is a preferred source of nitrogen for *L. wollei*, (iii) to study the importance of the size of algal mats when *L. wollei* was grown under different nutrient levels, and (iv) to investigate whether *L. wollei* populations show seasonal differences in their growth rates and whether populations from different Florida springs differ in their growth rates as well.

Materials and methods

Two experimental systems were used to study the growth of filamentous algae: small microcosms and large microcosms. The small microcosm system allowed a large number of replicates but restricted the experimental unit to a short algal filament. The large microcosm system could accommodate small filamentous algal mats of varying sizes but restricted the number of replicates. We conducted 9 small microcosm experiments and 3 large microcosm experiments (Table 1). The nutrient levels used in the experiments reflected phosphate and nitrate concentrations observed in Florida springs water. Ammonia concentrations used in the experiments were higher than those observed in Florida springs water but in the range of those observed inside the *L. wollei* mats (Task 3 of the report – the study conducted by Albertin and Sickman).

Small microcosm experiments

Filaments of *L. wollei* were grown in 1.5 ml microcentrifuge tubes filled with 1 ml of growth media. Growth media were changed every 48 hours. Racks made of clear polyethylene were placed on an orbital shaker (60 RPM) and incubated under grow lights providing 25 μmol/m^2/s of light. To achieve this light intensity, the racks were covered with neutral density film. Light intensity of 25 μmol/m^2/s was found to be the optimal intensity for the growth of individual filaments in microcentrifuge tubes. The initial filament length was 1-2mm. The filament was photographed using a digital video camera system installed on a Leica MZ12.5 Stereomicroscope under 8x magnification at the beginning and at the end of each experiment. Filament length was measured on the digital images using Image J software (Rasband 2003). Incubation time was 11 or 12 days. Each treatment was replicated 10 times, or 5 times if *L. wollei* from two locations (2 different springs) was used. Since some of the filaments were lost during the media changes, the final number of replicates used for the data analyses was lower than set at the beginning of the experiment. All experiments had a complete factorial ANOVA design with 1 or 2 factors. Experiments 1, 2 and 3 had a 2 factor design with 10 nutrient treatments each (PO_4-P concentrations: 1, 5, 10, 20, 30, 40, 60, 80, 100 and 250 μg P/L) and 2 population location treatments. The NO_3-N concentration in experiment 1, 2 and 3 was 1000 μg/L. Experiment 5 had a one factor design with 11 nutrient treatments (NO_3-N concentrations: 10, 30, 60, 125, 250, 500, 750, 1000, 1500, 2500 and 5000 μg N/L) and experiments 6, 7 and 8 had a 2 factor design with 10 nutrient treatments (NO_3-N concentrations: 10, 30, 60, 125, 250, 500, 750, 1000, 2000 and 5000 μg N/L) and 2 population location treatments. Experiment 10 had a 1 factor design with 11 nutrient

treatments (NH_4-N concentrations: 10, 30, 60, 125, 250, 500, 750, 1000, 1500, 2500 and 5000 μg N/L). Experiment 12 had a 2 factor design with 16 nutrient treatments: a combination of 4 ammonia concentrations (20, 100, 500 and 1000 μg N/L) and 4 nitrate concentrations (20, 100, 500 and 1000 μg N/L). The PO_4-P concentration in experiments 5, 6, 7, 8, 10 and 12 was 100 μg/L.

Large microcosm experiments

The large microcosm system consisted of a flat platform with Plexiglas cylindrical raceways (Manoylov 2005). The platform was placed on an orbital shaker table (60RMP) in a walk-in growth room. The platform was illuminated with fluorescent grow lights providing 150 μmol/m^2/s of light. Each microcosm was filled with 100 ml of growth medium. The media were changed daily. At the beginning and at the end of the experiment, small fragments of *L. wollei* mats were held between a Nitex screen and gently patted dry with a paper towel to remove excess water, then weighed (fresh mass). At the end of the experiment, all samples were processed for dry mass (DM). At the beginning of each experiment, 10 algal samples from each initial mass treatment were patted dry in between a Nitex screen, weighed to measure the initial fresh mass (FM), dried in the oven, and weighed again (DM). This allowed the calculation of the initial dry mass of the experimental samples based on their initial fresh mass. All samples for DM were dried in the oven at 105ºC for 24 hours (Eaton et al. 1995). All experiments had a complete factorial ANOVA design with 2 experimental factors: nutrient concentration and initial biomass. Experiment 4 had a total of 15 treatments that were a combination of 5 phosphate concentrations (PO_4-P concentrations: 2, 10, 30, 60, 150 μg

P/L) and 3 initial fresh mass levels (0.01g, 0.05g, 0.2g). The NO_3-N concentration in experiment 4 was 1000 µg/L. Experiment 9 had a total of 15 treatments that were a combination of 5 nitrate concentrations (NO_3-N concentrations: 20, 125, 500, 1000, 2500 µg N/L) and 3 initial fresh mass levels (0.01g, 0.05g, 0.2g). Experiment 11 had a total of 15 treatments that were a combination of 5 ammonia concentrations (NH_4-N concentrations: 20, 125, 500, 1000, 2500 µg N/L) and 3 initial fresh mass levels (0.01g, 0.05g, 0.2g). The PO_4-P concentration in experiment 9 and 11 was 100 µg/L.

Relative growth rates

Algal relative growth rate (RGR) in the large microcosm experiments was calculated using the following formula (Hunt 1990):

$$\text{RGR} = \frac{\ln(\text{Final Mass}) - \ln(\text{Initial Mass})}{\#\text{ of days}}$$

In the small microcosm experiments, algal initial and final filament length was used instead of algal mass.

Growth media

A new growth medium was developed based on the Z8 medium (Kotai 1972). The Z8 medium was modified to simulate the median chemistry of macronutrients in Florida spring water (referred to as medium Z8Fl) (Appendix 1). We originally used a Z8Fl

Fe600 medium that had the original concentration of iron (600 µg/L) found in the Z8 medium. Later we used Z8Fl Fe200 with the iron concentration reduced to 200µg/L, because we found an Fe concentration of 600µg/L to have a suppressing effect on the growth of *L. wollei* sp. We used the Gaffron micronutrients mix as used by Kotai (1972). To 1L of the final growth medium we also added 10ml of $CaCl_2 \cdot 2H_2O$ solution (stock solution 18.3g $CaCl_2 \cdot 2H_2O$ in 1L of deionized water), 10ml of $MgSO_4 \cdot 7H_2O$ solution (stock solution 7g $MgSO_4 \cdot 7H_2O$ in 1L of deionized water), and 10ml of Na_2CO_3 solution (stock solution 13.7g Na_2CO_3 in 1L deionized water). We used $NaNO_3$ and NH_4Cl as sources of nitrogen and K_2HPO_4 as a source of phosphorus. When $NaNO_3$ gradient was investigated, NaCl was also added to keep the Na concentration constant in all the treatments. In the experiments with K_2HPO_4, gradient KCl was added to keep the K concentration constant in all of the treatments, following the experimental design of (Ahlgren 1977).

Experimental conditions

All experiments were conducted in the walk-in growth room. The temperature in the growth room was set for 22.5ºC to reflect the average water temperature in Florida springs. The minimum and maximum temperature was recorded daily on a max-min thermometer.

Water analyses

Water samples of prepared growth media and from experimental macrocosms were sent to FDEP for analyses. On last day of experiment a sample of water in which algae were growing for 24 hours was collected for analyses.

Data analyses

Data were analyzed using SAS statistical software (SAS 2000) (repeated measures ANOVA) and R statistical software (R Development Core Team 2006). Logistic regression analysis was conducted using drc package for R (Ritz and Streibig 2007). All final and initial filament length and small mat biomass data were first tested using repeated measures ANOVA to test the significance of nutrient effect and time effect (indicating algal growth). Next, factorial analysis of variance was used to test the effect of nutrient concentration, season and *L. wollei* population location on relative growth rates (RGR). A lowess smooth line was fitted through the data for general assessment of the trends in the data. If a significant nutrient effect was found in the ANOVA analysis, a logistic curve was fitted through the RGR data. If the logistic curve did not provide a good fit and data were distributed along a straight line, a linear regression was calculated. Various logistic models were fitted through the data (Ritz and Streibig 2005, Ritz and Streibig 2006) and the best fitting models are presented in the results. The best fitting models for our data were: four parameter logistic model (l4()), four parameter logistic model with variance calculated as a power function of the mean to account for heterogeneity of the data (l4() varPower), Monod model (calculated using code for

Michaelis-Menten model from the drc package) and five parameter Brain-Cousens hormesis model (Borchardt 1996, Schabenberger et al. 1999, Ritz and Streibig 2007). When seasonal effects were compared using factorial ANOVA, only data from experiments conducted using exactly the same growth media and for populations collected from the same locations during different seasons were used. When population effects were compared, only experiments conducted in the same media and season were used. All data sets were checked for normality and heterogeneity of variance, and, where necessary, data were transformed for the ANOVA analyses. All analyses using the drc package were conducted on untransformed RGR data.

Results

Lyngbya wollei grew in all experiments under all nutrient concentrations. Relative growth rates (RGR) varied from 0 to 0.40. Low nutrient levels resulted in low growth rates and high nutrient levels resulted in high growth rates, with the exception of ammonia treatments. In these, the highest NH_4 concentrations in the small microcosm experiments resulted in reduced growth rates.

Effects of PO_4

In the small microcosm experiments, an increase in phosphate phosphorus had a significant effect on the growth of *L. wollei* (Fig 1). The relative growth rate data were well described by a logistic dose-response curve (Fig. 1, Table 2). The threshold concentration of phosphate phosphorus for the growth of *L. wollei* was 17.5 µg/L - the Effective Dose of 50% growth saturation (ED_{50}). The mean growth rate in the

experiments conducted on the *L. wollei* population from the Ichetucknee in the spring of the year (0.29±0.08 mm/mm/day) was higher than in the fall (0.20±0.10 mm/mm/day) (ANOVA for the season effect, P<0.001). The three populations of *L. wollei* studied did not differ in their RGRs (ANOVA for the location effect, P<0.543).

In the large microcosm experiments, *L. wollei* mats with smaller initial biomass grew faster than larger mats, especially in high phosphate concentrations (ANOVA for biomass effect, P<0.001) (Fig.2). A dose response curve was successfully fitted only through the lowest initial biomass data. The threshold phosphate phosphorus concentration was 49 µg/L (Fig.2, Table 3).

Effect of NO_3

In the small microcosm experiments, an increase in nitrate nitrogen had a positive effect on the growth of *L. wollei* (Fig. 3). The RGR data were well described by a logistic dose-response curve (Fig. 3, Table 2). The threshold nitrate nitrogen concentration for the growth of *L. wollei* was 88.1 µg/L (ED_{50}). The mean growth rate in the experiments conducted on the *L. wollei* population from the Ichetucknee in the spring of the year (0.27±0.08 mm/mm/day) was higher then in the fall (0.19±0.08 mm/mm/day) (ANOVA for the season effect, P<0.001). Among the three studied populations of *L. wollei*, the Ichetucknee population (0.22±0.10 mm/mm/day) had lower relative growth rates than the Silver Glen and Alexander populations (0.24±0.09 mm/mm/day and 0.24±0.08 mm/mm/day) (ANOVA for the location effect, P<0.001).

In the large microcosm experiments, *L. wollei* mats with smaller initial biomass grew faster than larger mats (ANOVA for biomass effect, P<0.001) (Fig.4). Dose response curves were fitted through the data. The threshold nitrate nitrogen concentration was 519

µg/L for the 0.01g initial biomass and 546 µg/L for the 0.05g initial biomass (Fig.4, Table 3).

Effect of NH_4

In the small microcosm experiments, an increase in ammonia nitrogen resulted in increased growth rates up to a 796.3 µg/L NH_4-N concentration, where it reached the highest growth rate of 0.27 mm/mm/day (Fig. 5). The RGR data were well described by the Brain-Cousens hormesis curve (Fig. 5, Table 2).

In the large microcosm experiments, *L. wollei* mats with smaller initial biomass grew faster than larger mats in high ammonia concentrations (ANOVA for biomass effect, $P<0.001$) (Fig.6). Dose response curves were fitted through the 0.01g and 0.05g initial biomass data. The threshold ammonia nitrogen concentration was 248 µg/L for the 0.01g initial biomass and 227 µg/L for the 0.05g initial biomass (Fig.6, Table 3).

Combined effect of NO_3 and NH_4

In the small microcosm experiments, nitrogen was added in the form of ammonia, nitrate or a combination of those two forms. Both nitrate and ammonia nitrogen had a positive effect on the growth of *L. wollei* (ANOVA for NO_3 effect, $P<0.001$, for NH_4 effect $P<0.001$) (Fig.7). We also found that the form of nitrogen used did not matter and only an absolute concentration of nitrogen had an effect on the growth rates of *L. wollei*. The RGR data were well described by the Monod model (Fig. 8, Table 2). The half saturation constant K_μ was reached at a 104µg/L combined nitrogen concentration.

Discussion

In our experiments *Lyngbya wollei* grew in all experimental conditions (Table 4). A maximum doubling rate of 0.4 was lower or similar to the growth rates of other species of filamentous algae (Borchardt 1996, Hall and Payne 1997). On the other hand. the doubling rates of *L. wollei* in our study were higher or similar to the growth rates of *L. wollei* observed by others (Cowell and Botts 1994, Yin et al. 1997, Cowell and Dawes 2004). Cowell and Dawes (2004) investigated the effect of nitrate on the growth of small mats of *L. wollei* in a continuous culture and found that the growth rate increased when the nitrate concentration was equal to or above 600 µg N/L. When we examined the growth of *L. wollei* mats in different nitrate concentrations, we found a threshold concentration of 518-546 µg N/L, which is in agreement with the results of Cowell and Dawes (2004). Turner (1990) and Yin et al. (1997) experimented with very high nutrient concentrations. These concentrations were above the highest concentration used in this study. Some of the low growth rates observed by Turner (1990) and Yin et al. (1997) were possibly the result of nutrient toxicity. In our study we did not find *L. wollei* to show a preference for ammonia or nitrate as a source of nitrogen, which agrees with the results of Turner (1990). In this study growth rates of single filaments were saturated at lower nutrient levels than growth rates of algal mats. Also, different size mats of *L. wollei* were used, and withlarger mat sizes, the saturating nutrient concentration was increased as well. Overall, for individual filaments, the nitrate concentration in almost all Florida springs (Stevenson et al. 2004) were above threshold concentrations for the growth of *L. wollei*, and in many of the springs the nitrate concentrations were above the threshold concentration for the growth and establishment of mats. Once mats develop,

they can recycle nutrients within the mat, thus, their growth is less dependent on the nutrients available from the water column. The water phosphate concentration in many Florida springs is above the threshold concentration for the growth of individual filaments. However, it was below 50 µg/L, the threshold concentration for the growth of mats, in 76% of the springs surveyed (Stevenson et al. 2004).

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Fig 1

Relative growth rates (RGR) of *Lyngbya wollei* in different phosphate phosphorus concentrations (n=271). A four parameter logistic curve, with variance as a power function of the mean, was fitted through the combined data from small microcosm experiments (experiments 1, 2 and 3). Curve parameters are in Table 2.

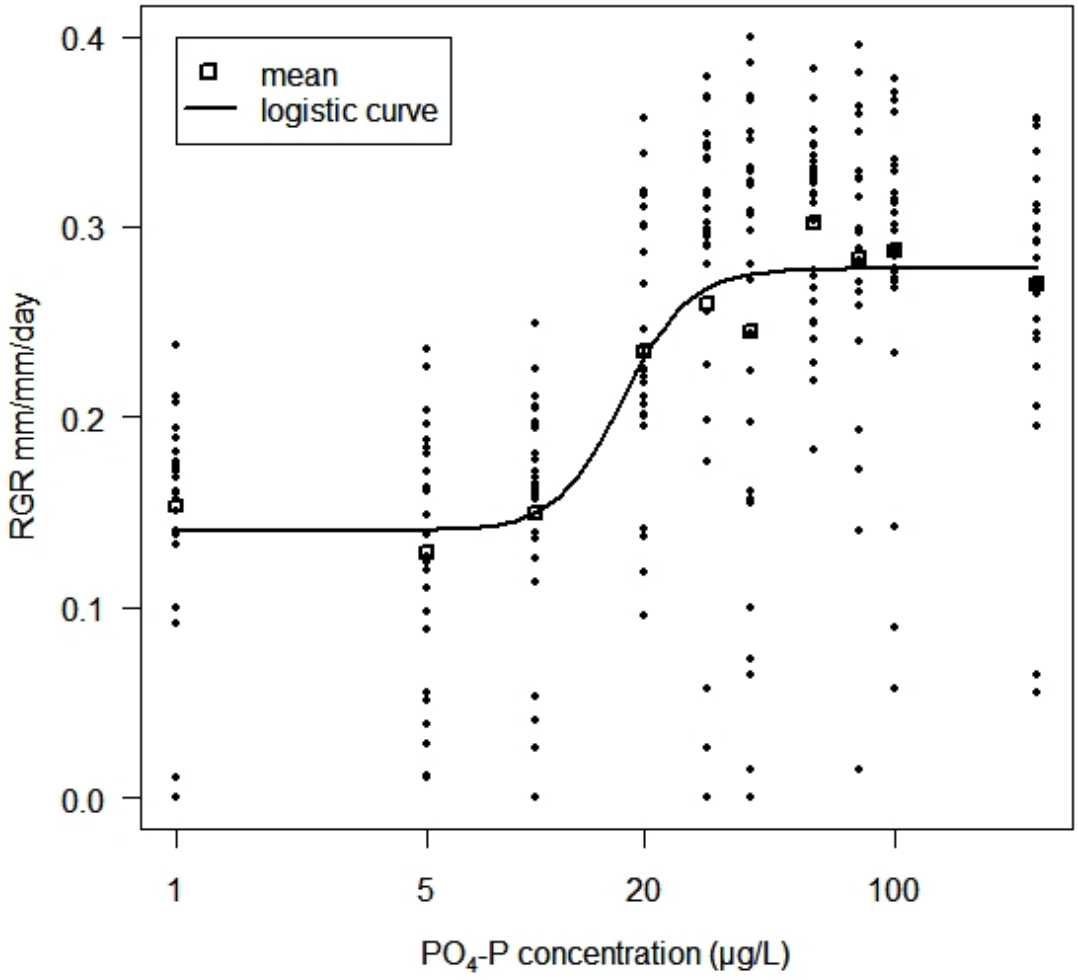

Fig. 2

Relative growth rates (RGR) of *Lyngbya wollei* in a factorial experiment with 3 initial biomass levels (0.01g, 0.05g, 0.2g) and 5 phosphate phosphorus concentrations (n=45). A four parameter logistic curve and 2 regression lines (initial biomass 0.05g R^2=0.05, P<0.41; initial biomass 0.2g R^2=0.21, P<0.086) were fitted through the data from a large microcosm experiment (experiment 4). Curve parameters are in Table 3.

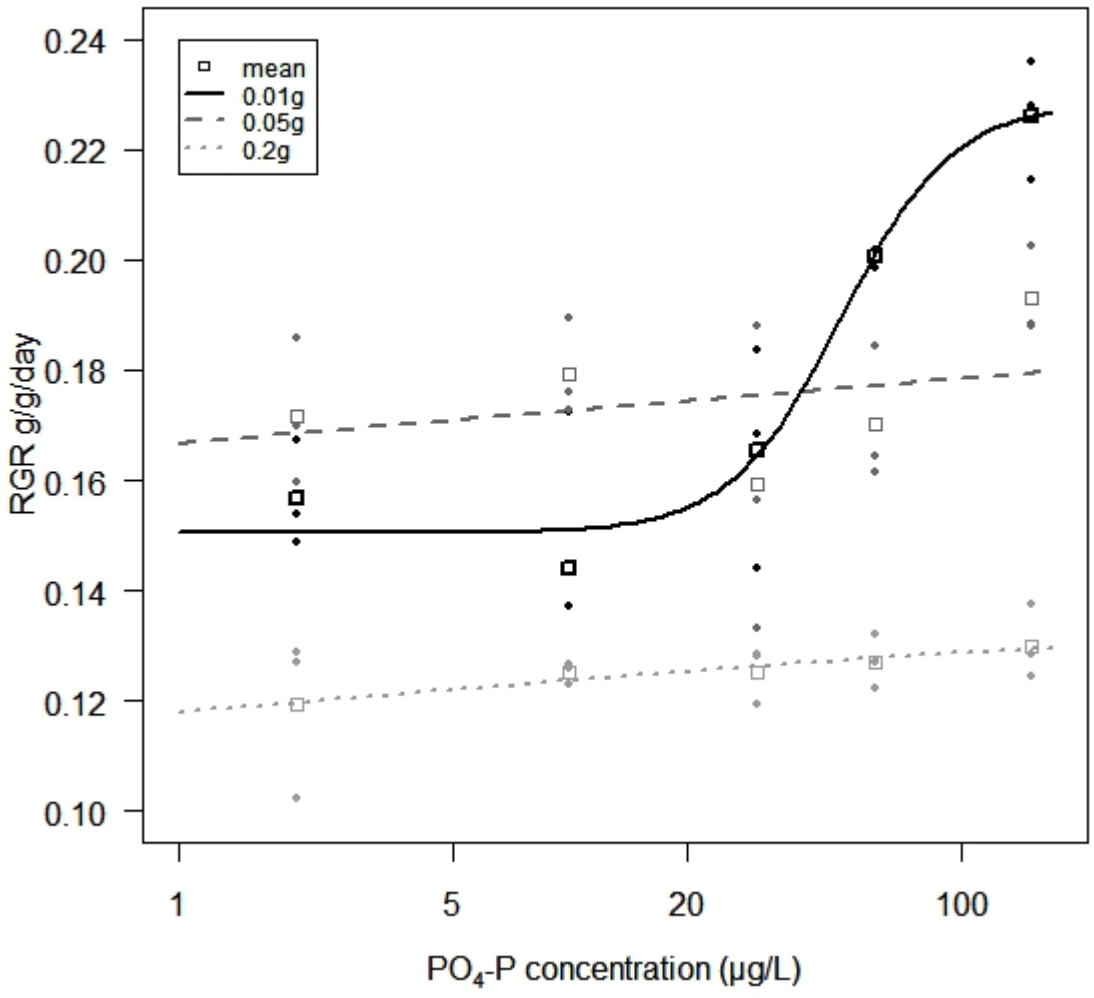

Fig. 3

Relative growth rates (RGR) of *Lyngbya wollei* in different nitrate nitrogen concentrations (n=380). A four parameter logistic curve, with variance as a power function of the mean, was fitted through the combined data from small microcosm experiments (experiments 5, 6, 7 and 8). Curve parameters are in Table 2.

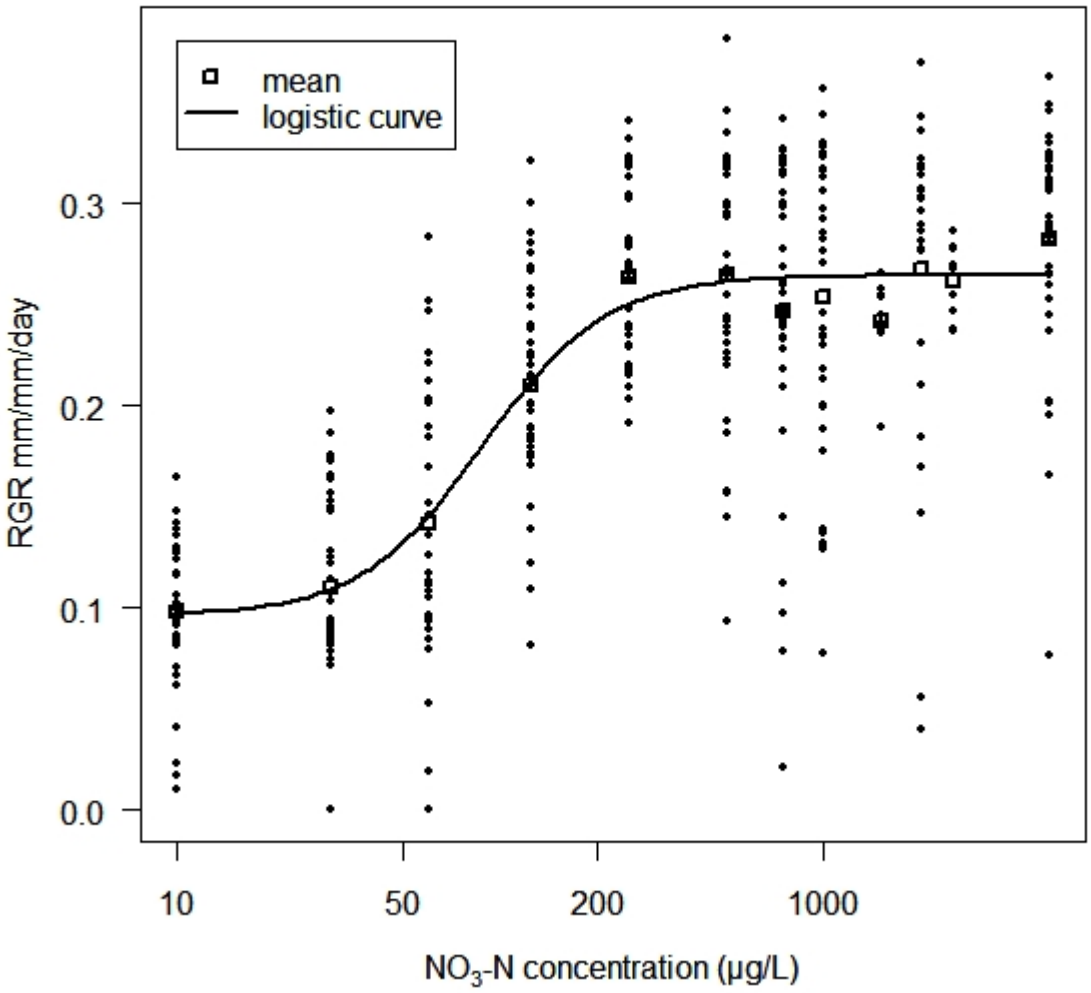

Fig. 4

Relative growth rates (RGR) of *Lyngbya wollei* in a factorial experiment with 3 initial biomass levels (0.01g, 0.05g, 0.2g) and 5 nitrate nitrogen concentrations (n=45). Four parameter logistic curves were fitted through the data from a large microcosm experiment (experiment 9). Curve parameters are in Table 3.

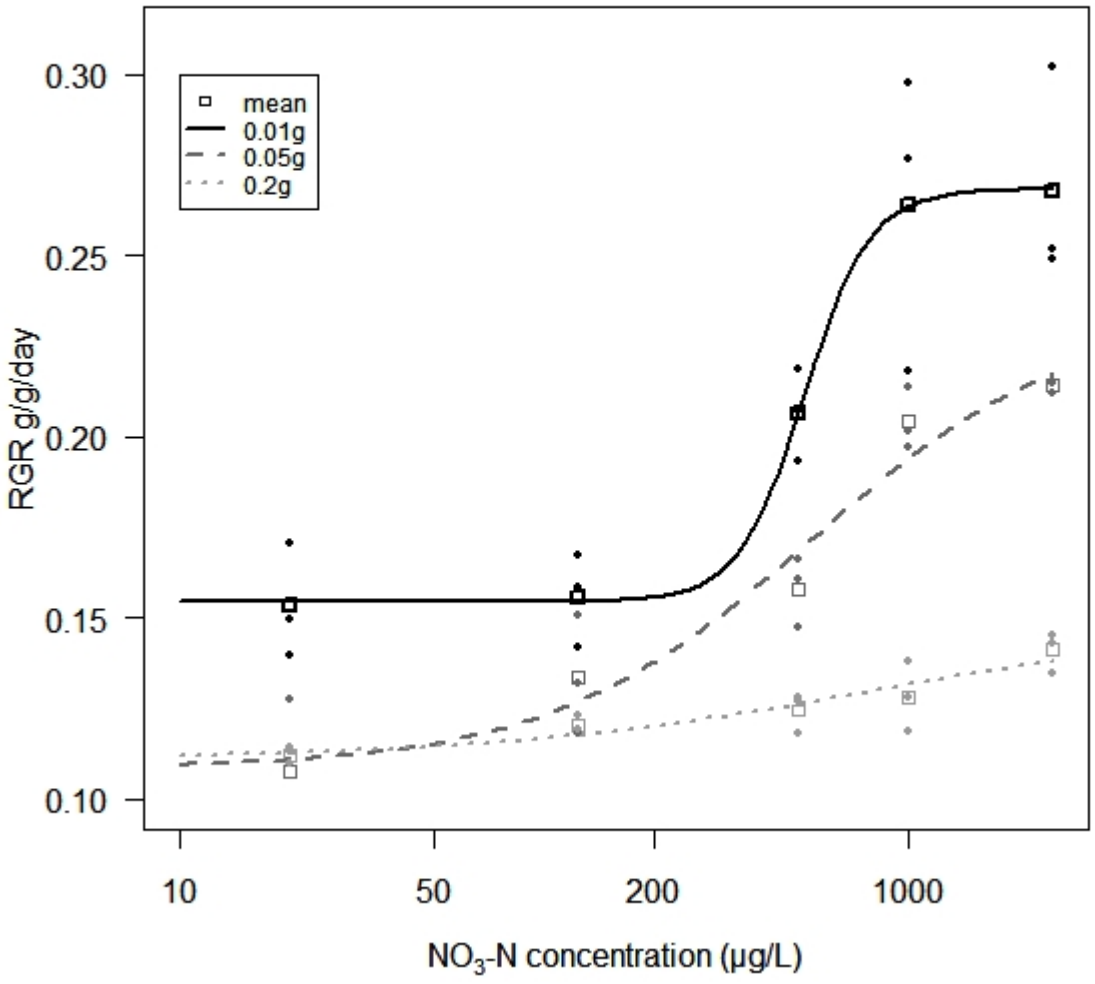

Fig. 5

Relative growth rates (RGR) of *Lyngbya wollei* in different ammonia nitrogen concentrations (n=101). A five parameter Brain-Cousens hormesis logistic model was fitted through the data from a small microcosm experiment (experiment 10). Curve parameters are in Table 2.

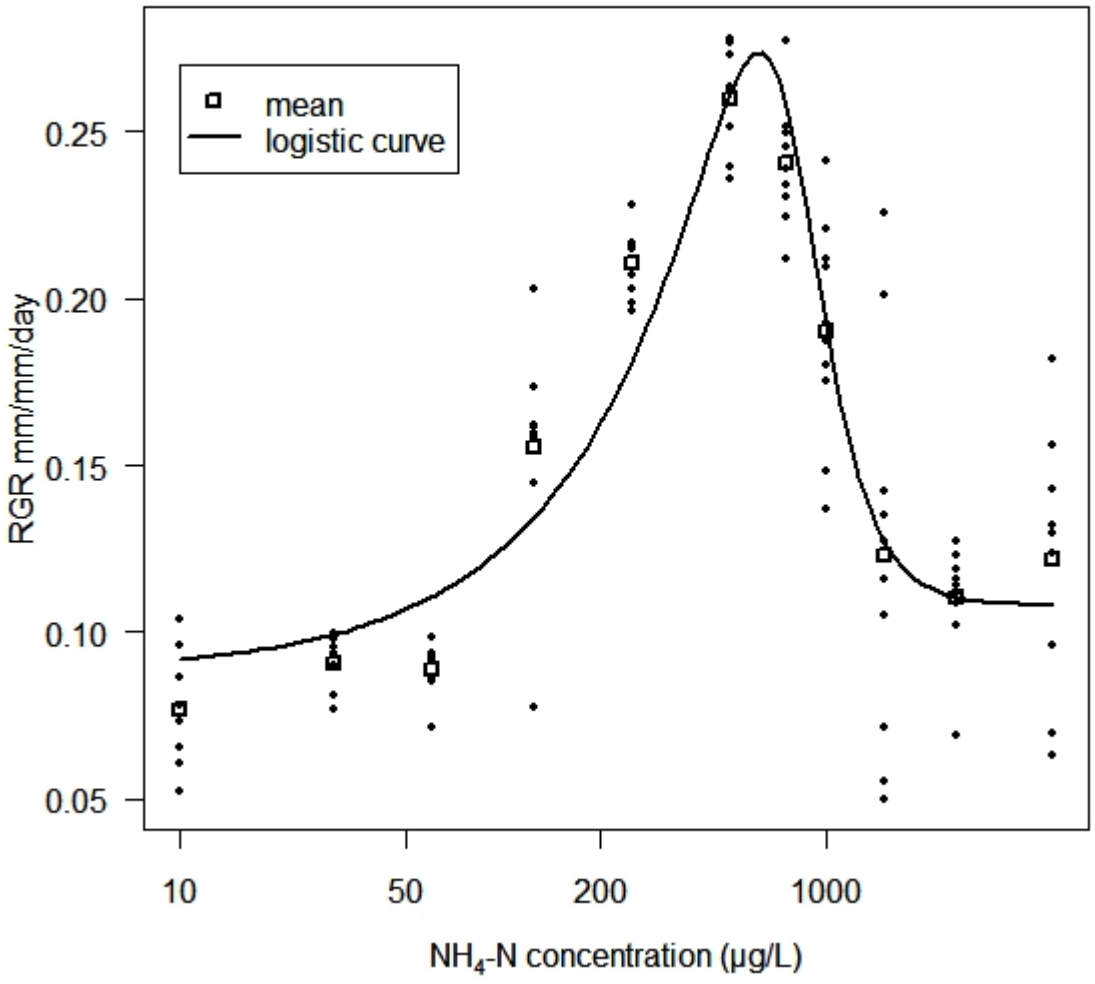

Fig. 6

Relative growth rates (RGR) of *Lyngbya wollei* in a factorial experiment with 3 initial biomass levels (0.01g, 0.05g, 0.2g) and 5 ammonia nitrogen concentrations (n=45). Two four parameter logistic curves and a regression line (initial biomass 0.2g R^2=0.45, P<0.006) were fitted through the data from a large microcosm experiment (experiment 11). Curve parameters are in Table 3.

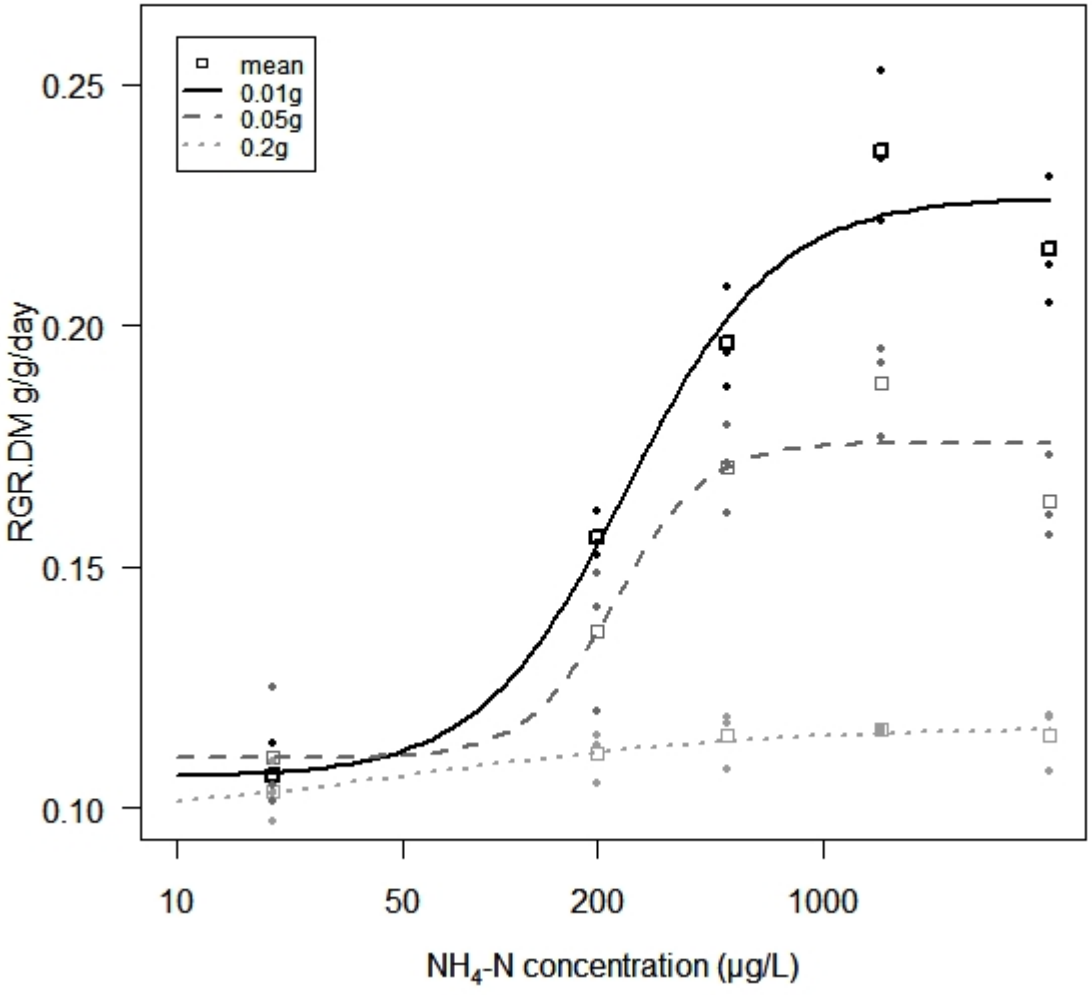

Fig. 7

Boxplot of relative growth rates (RGR) of *Lyngbya wollei* in a combination of different ammonia and nitrate nitrogen concentrations (experiment 12).

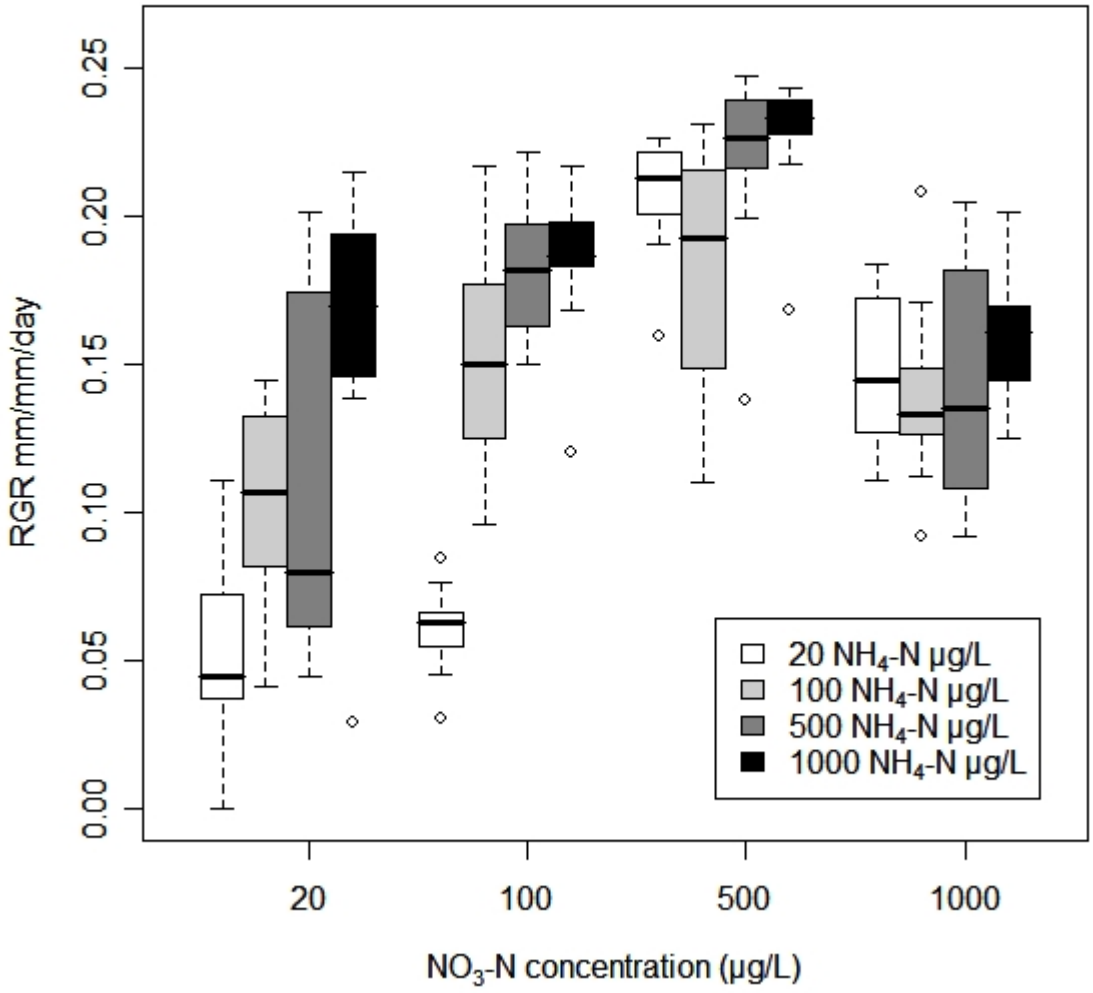

Fig. 8

Relative growth rates (RGR) of *Lyngbya wollei* in different nitrogen (sum of NO_3-N and NH_4-N) concentrations (n=153). A Monod logistic curve starting at y_0=0 was fitted through data from small microcosm experiment (experiment 12). The assumption that y_0=0 was used since there were no data in the low range of concentrations. Curve parameters are in Table 2.

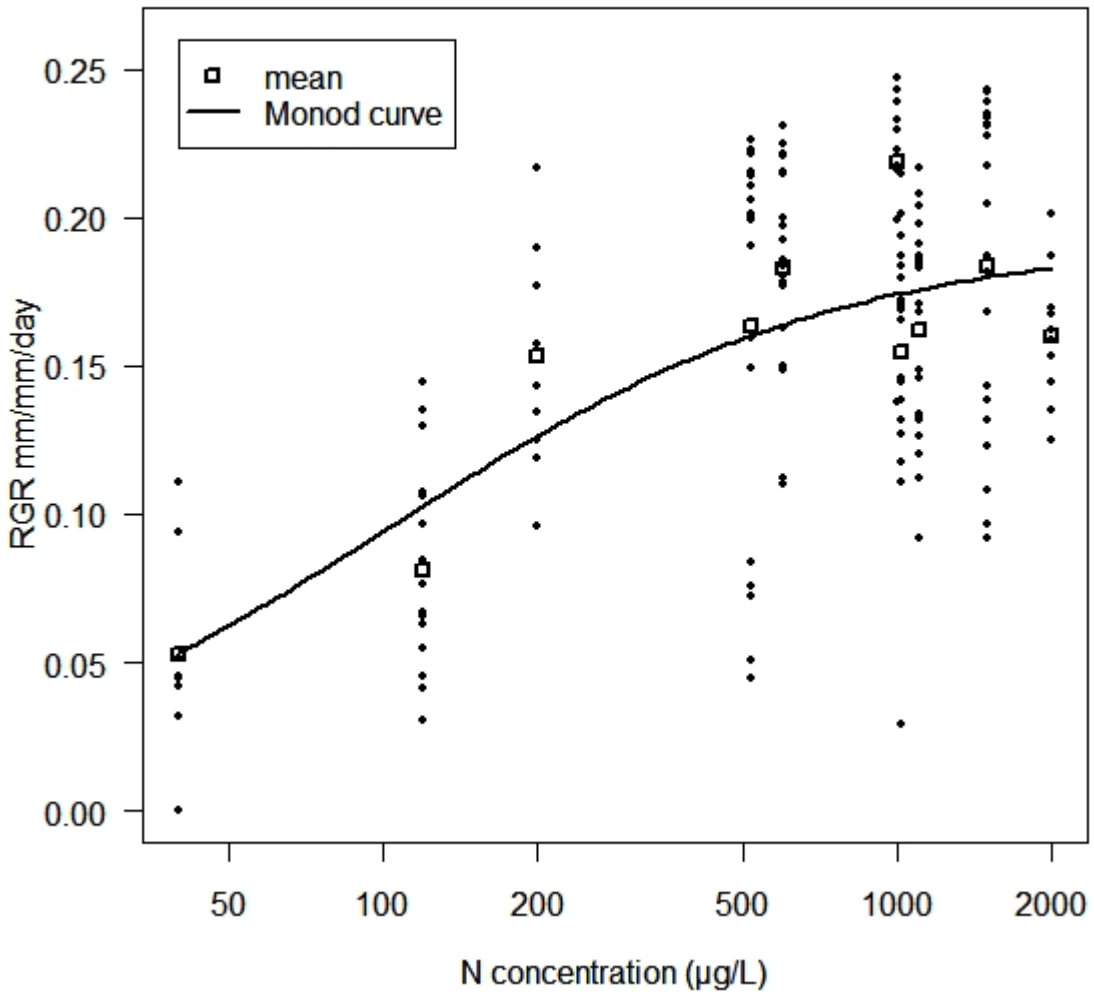

Table 1

Lyngbya wollei experiments. Experiment numbers in parenthesis refer to the numbers used in the 2006 report.

Experiment (project experiment number)	Micro cosm	Nutrient gradient	Algae collection location	Season	Growth medium	Mean min and max temperature (ºC)
1 (18)	Small	PO_4-P	Ichetucknee and Silver Glen	Fall	Z8Fl Fe200	20.8 &24.3
2 (19)	Small	PO_4-P	Ichetucknee and Silver Glen	Fall	Z8Fl Fe200	21.1 &25.3
3 (21)	Small	PO_4-P	Ichetucknee and Alexander	Spring	Z8Fl Fe200	21.5 &24.9
4 (8)	Large	PO_4-P	Silver Glen	Spring	Z8Fl Fe600	21.1 & 25.4
5 (3)	Small	NO_3-N	Silver Glen	Spring	Z8Fl Fe600	21.9 & 24.2
6 (16)	Small	NO_3-N	Ichetucknee and Silver Glen	Fall	Z8Fl Fe200	20.9 & 24.5
7 (17)	Small	NO_3-N	Ichetucknee and Silver Glen	Fall	Z8Fl Fe200	20.8 & 24.3
8 (20)	Small	NO_3-N	Ichetucknee and Alexander	Spring	Z8Fl Fe200	21.5 &24.9
9 (9)	Large	NO_3-N	Ichetucknee	Spring	Z8Fl Fe600	20.6 & 22.3
10 (4)	Small	NH_4-N	Silver Glen	Spring	Z8Fl Fe600	21.5 & 24.5
11 (12)	Large	NH_4-N	Silver Glen	Fall	Z8Fl Fe600	22.0 & 26.7
12 (2)	Small	N (NH_4 and NO_3)	Silver Glen	Spring	Z8Fl Fe600	21.0 & 24.3

Table 2

Parameters of the logistic dose-response model applied to the *Lyngbya wollei* RGR data from small microcosm experiments, where *d* and *c* denote the upper and lower limit of algae growth rates at infinitely low and high concentrations of nutrient. ED_{50} (also called *e*) denotes the concentration required to increase algal growth rate by half between the upper and lower limits and *b* is proportional to the slope (the rate of change) of the curve around ED_{50}. Significant parameters are in boldface. ED_{10} and ED_{90} denote the concentration required for 10% and 90% growth saturation. For the Brain-Cousens hormesis model, *c* and *d* are the upper and lower asymptote of the response. The *b* and *e* columns are related to the rate of change and point of inflection of the curve, respectively; and *f* indicates the initial rate of increase at low concentrations. For the Monod model, μ_{max} is the maximum specific growth rate and K_μ is the half saturation constant. Significant parameters are in boldface.

Nutrient	Model	*d* or μ_{max} (mm/mm/ day)	*c* (mm/mm/ day)	*b*	ED_{50} (µg/L) *e* or K_μ	ED_{90} (µg/L) or *f*	ED_{10} (µg/L)
PO_4	l4() varPower	**0.28±0.01**	**0.14±0.01**	**-4.65±1.39**	**17.50±1.39**	28.10±3.80	10.90±2.00
NO_3	l4() varPower	**0.26±0.00**	**0.10±0.01**	**-2.29±0.31**	**88.10±6.14**	230.05±32.06	33.74±5.28
NH_4	Brain-Cousens	**0.09±0.01**	**0.11±0.01**	**5.18±0.71**	**796.3±43.5**	0.00037±0.00004	-
N (NO_3 and NH_4)	Monod	**0.19±0.01**	-	-	**104.2±21.5**	-	-

Table 3

Parameters of the logistic dose-response model applied to the *Lyngbya wollei* growth response data from large microcosm experiments, where *d* and *c* denote the upper and lower limit of algae growth rates at infinitely low and high concentrations of nutrient. ED_{50} denotes the concentration required to increase the algal growth rate by half between the upper and lower limits and *b* is proportional to the slope of the curve around ED_{50}. Significant parameters are in boldface. ED_{10} and ED_{90} denote the concentration required for 10% and 90% growth saturation.

Nutrient and biomass	Model	*d* (g/g/day)	*c* (g/g/day)	*b*	ED_{50} (µg/L)	ED_{90} (µg/L)	ED_{10} (µg/L)
PO_4 x 0.01g	l4()	**0.23±0.01**	**0.15±0.01**	**-3.05±1.41**	**49.15±9.74**	100.9±42.8	23.9±8.3
NO_3 x0.01g	l4()	**0.27±0.01**	**0.15±0.01**	-4.78±4.00	**518.6±47.9**	820.6±353.7	327.7±116.1
NO_3 x0.05g	l4()	**0.24±0.03**	**0.11±0.02**	-1.19±0.87	**546.0±250.0**	3440.0±5490.0	86.66±106.1
NO_3 x0.2g	l4()	**0.15±0.03**	**0.11±0.02**	-0.83±1.19	808.2±2015	11283±61207	57.88±396.8
NH_4 x 0.01g	l4()	**0.23±0.01**	**0.11±0.01**	**-1.90±0.47**	**247.51±39.26**	788.5±246.4	77.70±26.74
NH_4 x 0.05g	l4()	**0.18±0.01**	**0.11±0.01**	-3.11±1.63	**226.95±41.86**	460.2±205.8	111.9±42.78

Table 4

Summary of *L. wollei* growth parameters.

Parameter	Single filaments			Mats		
	PO_4-P	NO_3-N	NH_4-N	PO_4-P	NO_3-N	NH_4-N
Lowest nutrient concentration tested	1 µg/L	10 µg/L	10 µg/L	2 µg/L	20 µg/L	20 µg/L
Average growth rate at lowest nutrient concentration	0.15	0.10	0.08	0.15	0.11	0.11
Highest nutrient concentration tested	250 µg/L	5 mg/L	5 mg/L	150 µg/L	2.5 mg/L	2.5 mg/L
Average growth rate at highest nutrient concentration	0.27	0.28	0.12	0.19	0.22	0.16
Average maximum growth rate	0.30	0.28	0.26	0.19	0.22	0.19
Lowest concentration at maximum growth rate	60 µg/L	5 mg/L	0.5 mg/L	150 µg/L	2.5 mg/L	1.5 mg/L
Minimum concentration at which growth was observed	1 µg/L	10 µg/L	10 µg/L	2 µg/L	20 µg/L	20 µg/L
Difference between maximum and minimum growth rate	0.15	0.18	0.18	0.04	0.11	0.09
Average threshold concentration	17.5 µg/L	88µg/L	796 µg/L	49 µg/L	532µg/L	238 µg/L

Differential effect of PO_4, NO_3 and NH_4 on the growth of *Vaucheria* sp.

Introduction

Vaucheria sp. (Xanthophyce) is a common filamentous mat-forming species in streams, rivers and lakes (Entwisle 1990, McIntire et al. 1994, Necchi et al. 1994, Aboal et al. 1996). It is often found at high nutrient sites, and its presence can be a symptom of system eutrophication (Lakatos 1978, Simons et al. 1994, Biggs 1996). *Vaucheria* sp. can also be found in the springs and areas of ground water input into streams (Davis and Gworek 1973, Moreno et al. 2001). In a survey of 60 sites within 28 spring systems in Northern and Central Florida, we found *Vaucheria* sp. mats at 33 sites (Stevenson et al. 2004). At Manatee, Fanning and Guaranto springs, more than 70% of the spring bottoms were covered by *Vaucheria* sp. Even though *Vaucheria* sp. is commonly found in the fresh, brackish and marine water systems around the world, there are few laboratory studies on its nutrient requirements (Sudlow 1936, Ozimek 1990).

This study was conducted on *Vaucheria* sp. in Florida springs. The objectives of the study were: (i) to investigate the effects of a wide range of concentrations of nitrogen (in the form of nitrate and ammonia) and phosphorus on the growth of *Vaucheria* sp., (ii) to determine whether nitrate or ammonia is a preferred source of nitrogen for *Vaucheria* sp., (iii) to study the importance of the size of algal mats when *Vaucheria* sp. was grown under different nutrient levels, and (iv) to investigate whether *Vaucheria* sp. populations from different Florida springs differ in their growth rates.

Materials and methods

Two experimental systems were used to study the growth of filamentous algae: small microcosms and large microcosms. The small microcosm system allowed a large number of replicates but restricted the experimental unit to short algal filaments. The large microcosm system could accommodate small filamentous algal mats of varying sizes but restricted the number of replicates. We conducted 4 small microcosm experiments and 3 large microcosm experiments (Table 1).

Small microcosm experiments

Filaments of *Vaucheria* sp. were grown in 1.5 ml microcentrifuge tubes filled with 1ml of growth media. Growth media were changed every 48 hours. Racks made of clear polyethylene were placed on an orbital shaker (60 RPM) and incubated under grow lights providing 25 μmol/m^2/s of light. To achieve this light intensity, racks were covered with neutral density film. A light intensity of 25 μmol/m^2/s was found to be the optimal intensity for the growth of individual filaments in microcentrifuge tubes. Healthy, growing tips of *Vaucheria* sp. 1-2mm in length were cut from the filaments. Originally these tips were used directly for the experiments. However, we found that many of the cut off tips of *Vaucheria* sp. were damaged in the process and did not grow. Therefore, we changed our methodology and incubated 1-2mm tips for 2 days in Z8Fl growth medium (Appendix 1) with 100μg/L P and 1000μg/L N before we used them in the experiments. For experiment 1 and 3, tips were pre-incubated for 48 hours, and for experiments 4 and 6, tips were used right after cutting. A tip of *Vaucheira* sp. was photographed using a digital video camera system installed on a Leica MZ12.5 Stereomicroscope under 8x magnification at the beginning and at the end of experiment.

Filament length was measured on the digital images using ImageJ software (Rasband 1997-2006). Incubation time was 11 or 12 days. Each treatment was replicated 5 times if *Vaucheria* from two locations (2 different springs) was used. If *Vaucheria* from one location was used, each treatment was replicated10 times. . Since some of the filaments were lost during the media changes, the final number of replicates used for the data analyses was lower then the number set at the beginning of the experiments. All experiments had a complete factorial ANOVA design with one or two factors. Experiment 1 had a two-factor design with 10 nutrient treatments (PO_4-P concentrations: 1, 5, 10, 20, 30, 40, 60, 80, 100 and 250 μg P/L) and 2 population location treatments. The NO_3-N concentration in experiment 1 was 1000 μg/L. Experiment 3 had a two-factor design with 10 nutrient treatments (NO_3-N concentrations: 10, 30, 60, 125, 250, 500, 750, 1000, 2000 and 5000 μg N/L) and 2 population location treatments. The PO_4-P concentration in experiment 3 was 100 μg/L. Experiment 5 had a one-factor design with 11 nutrient treatments (NH_4-N concentrations: 10, 30, 60, 125, 250, 500, 750, 1000, 1500, 2500 and 5000 μg N/L). The PO_4-P concentration in experiment 5 was 100 μg/L. Experiment 7 had a two-factor design with 16 nutrient treatments: a combination of 4 ammonia concentrations (20, 100, 500 and 1000 μg N/L) and 4 nitrate concentrations (20, 100, 500 and 1000 μg N/L).

Large microcosm experiments

The large microcosm system consisted of a flat platform with Plexiglas cylindrical raceways (Manoylov 2005). The platform was placed on an orbital shaker table (60RMP) in a walk-in growth room. The platform was illuminated with fluorescent growth lights providing 150 μmol/m^2/s of light. Each microcosm was filled with 100 ml of growth

medium. The media were changed daily. At the beginning and at the end of the experiment small fragments of *Vaucheria* sp. mats were gently patted dry with a paper towel, while held in between a Nitex screen, to remove excess water, and weighed (fresh mass). At the end of the experiment all samples were processed for dry mass (DM). At the beginning of each experiment 10 algal samples from each initial mass treatment were patted dry in between a Nitex screen, weighed to measure initial fresh mass (FM), dried in the oven, and weighed again (DM). This allowed for the calculation of the initial dry mass of the experimental samples based on their initial fresh mass. All samples for DM were dried in the oven at 105ºC for 24 hours (Eaton et al. 1995). All experiments had a complete factorial ANOVA design with 2 experimental factors: nutrient concentration and initial biomass. Experiment 2 had a total of 15 treatments that were a combination of 5 phosphate concentrations (PO_4-P concentrations: 2, 10, 30, 60, 150 µg P/L) and 3 initial fresh mass levels (0.01g, 0.05g, 0.2g). The NO_3-N concentration in experiment 2 was 1000 µg/L. Experiment 4 had a total of 15 treatments that were a combination of 5 nitrate concentrations (NO_3-N concentrations: 20, 125, 500, 1000, 2500 µg N/L) and 3 initial fresh mass levels (0.01g, 0.05g, 0.2g). The PO_4-P concentration in experiment 4 was 100 µg/L. Experiment 6 had a total of 15 treatments that were a combination of 5 ammonia concentrations (NH_4-N concentrations: 20, 125, 500, 1000, 2500 µg N/L) and 3 initial fresh mass levels (0.01g, 0.05g, 0.2g). The PO4-P concentration in experiment 6 was 100 µg/L.

Relative growth rates

Algal relative growth rate (RGR) in the large microcosm experiments was calculated using the following formula (Hunt 1990):

$$RGR = \frac{\ln(\text{Final Mass}) - \ln(\text{Initial Mass})}{\#\text{ of days}}$$

In the small microcosm experiments, the algal initial and final filament length was used instead of algal mass.

Growth media

A new growth medium was developed based on the Z8 medium (Kotai 1972). The Z8 medium was modified to simulate the median chemistry of macronutrients in Florida spring water (referred to as medium Z8Fl) (Appendix 1). We originally used Z8Fl Fe600 medium that had the original concentration of iron (600 µg/L) found in the Z8 medium. Later we used Z8Fl Fe200 with the iron concentration reduced to 200µg/L, because we found an Fe concentration of 600µg/L to have a suppressing effect on the growth of *Vaucheria* sp. We used the Gaffron micronutrients mix as used by Kotai (1972). To 1L of the final growth medium we also added 10ml of $CaCl_2 \cdot 2H_2O$ solution (stock solution 18.3g $CaCl_2 \cdot 2H_2O$ in 1L of deionized water), 10ml of $MgSO_4 \cdot 7H_2O$ solution (stock solution 7g $MgSO_4 \cdot 7H_2O$ in 1L of deionized water), and 10ml of Na_2CO_3 solution (stock solution 13.7g Na_2CO_3 in 1L of deionized water). We used $NaNO_3$ and NH_4Cl as sources of nitrogen and K_2HPO_4 as a source of phosphorus. When the $NaNO_3$ gradient was investigated, NaCl was also added to keep the Na concentration constant in all of the treatments. In the experiments with K_2HPO_4, gradient KCl was added to keep the K concentration constant in all of the treatments, following the experimental design of (Ahlgren 1977).

Water analyses

Water samples of prepared growth media and from experimental macrocosms were sent to FDEP for analyses. On last day of experiment a sample of water in which algae were growing for 24 hours was collected for analyses.

Experimental conditions

All experiments were conducted in the walk-in growth room. The temperature in the growth room was set for 22.5ºC to reflect the average water temperature of the Florida springs. The minimum and maximum temperatures were recorded daily on a max-min thermometer.

Identification of Vaucheria species

Since the presence of reproductive structures is necessary to identify the species of *Vaucheria* from each batch of algae used in the experiments, we incubated a small fragment of the mat (Ott and Oldham-Ott 2003). The mat fragment was placed onto a Petri dish filled with Z8Fl growth medium with 100µg/L P and 1000µg/L N and placed by the window. We were successful in observing reproductive structures only in the samples collected from Manatee spring. The species was *Vaucheria geminata* (Prescott 1982)

Data analyses

Data were analyzed using SAS statistical software (SAS 2000) (repeated measures ANOVA) and R statistical software (R Development Core Team 2006). Logistic regression analysis was conducted using drc package for R (Ritz and Streibig 2007). All final and initial filament length and small mat biomass data were first tested using

repeated measures ANOVA to test the significance of nutrient effect and time effect (indicating algal growth). Next, factorial analysis of variance was used to test the effect of nutrient concentration and *Vaucheria* sp. population location on relative growth rates (RGR). A lowess smooth line was fitted through the data for general assessment of the trends in the data. If a significant nutrient effect was found in the ANOVA analysis, a logistic curve was fitted through the RGR data. If the logistic curve did not provide a good fit and data were distributed along a straight line, a linear regression was calculated. Various logistic models were fitted through the data (Ritz and Streibig 2005, Ritz and Streibig 2006) and the best fitting models are presented in the results. For our data, the best fitting models were: the four parameter logistic model (l4()), the four parameter logistic model with variance calculated as a power function of the mean to account for heterogeneity of the data (l4() varPower), the Monod model (calculated using code for the Michaelis-Menten model from the drc package) and the five parameter Brain-Cousens hormesis model (Borchardt 1996, Schabenberger et al. 1999, Ritz and Streibig 2007).

In allof the treatments in the small microcosm experiments we had filaments of *Vaucheria* sp. that did not grow at all. This is probably because *Vaucheria* sp. did not like to be handled. Precutting tips 2 days before the experiment partially resolved that problem, but we still had tips that did not grow. All initial data analysis was conducted on a complete dataset. With the 0 growth samples in our data set, we were not able to fit any of the logistic models. The results from the small microcosm experiments presented here are based on the dataset with the 0 growth samples removed (less then 10% of the

whole dataset). The 0 growth samples were randomly found in our datasets. There was no relationship between the experimental treatments and number of 0 growth samples. All data sets were checked for normality and heterogeneity of variance, and where necessary, data were transformed for the ANOVA analyses. All analyses using drs package were conducted on the untransformed RGR data.

Results

Vaucheria sp. grew in all experiments under all nutrient concentrations. Relative growth rates (RGR) varied from 0 to 0.35. In the small microcosm experiments, probably partially due to the cutting of filaments, we observed large variability in the growth rates for each treatment. In the large microcosm experiments, high nutrient levels resulted in high RGRs of *Vaucheria* sp.

Effects of PO_4

In the small microcosm experiments, an increase in phosphate phosphorus had a significant effect on the growth of *Vaucheria* sp. (Fig 1). The relative growth rate data were well described by a logistic dose-response curve (Fig. 1, Table 2). The threshold concentration of phosphate phosphorus for the growth of *Vaucheria* sp. was 11.6 µg/L - the Effective Dose of 50% growth saturation (ED_{50}). The two populations of *Vaucheria* sp. that were studied did not differ in their RGRs (ANOVA for the location effect, $P<0.418$).

In the large microcosm experiments, *Vaucheria* sp. mats with smaller initial biomass grew faster than larger mats in high phosphate concentrations (ANOVA for biomass

effect, P<0.001) (Fig.2). Dose response curves were fitted through the 0.01g and 0.05g initial biomass data. The threshold nitrogen concentration was 36.7 µg/L for the 0.01g initial biomass and 33.7 µg/L for the 0.05g initial biomass (Fig.2, Table 3).

Effect of NO_3

Inthe small microcosm experiments, an increase in nitrate nitrogen did not have an effect on the growth of *Vaucheria* sp. (Fig. 3). The two populations of *Vaucheria* sp. that were studied did not differ in their RGRs (ANOVA for the location effect, P<0.742).

In the large microcosm experiments, *Vaucheria* sp. mats with smaller initial biomass grew faster than larger mats (ANOVA for biomass effect, P<0.001) (Fig.4). Dose response curves were fitted through the data. The threshold nitrogen concentrations were 210 µg/L for the 0.01g initial biomass, 208 µg/L for the 0.05g initial biomass, and 499 µg/L for the 0.2g initial biomass (Fig.4, Table 3).

Effect of NH_4

In the small microcosm experiments, an increase in ammonia nitrogen resulted in increased growth rates up to approximately 500 µg/L NH_4-N concentration, where it reached the highest growth rate of 0.25 mm/mm/day (Fig. 5). The RGR data were well described by the Brain-Cousens hormesis curve (Fig. 5, Table 2).

In the large microcosm experiments, *Vaucheria* sp. mats with smaller initial biomass grew faster than larger mats in 200, 500 and 1500 µg/L ammonia concentrations (ANOVA for biomass effect, P<0.001) (Fig.6). Brain-Cousens hormesis curves were fitted through the data, and a good fit was achieved for the initial biomass of 0.01g and 0.05g (Fig.6, Table 3). A high ammonia concentration of 5000 µg/L resulted in reduced

growth rates and, in 4 cases, death of the mats and/or due to take over by coccoid cyanobacteria.

Combined effect of NO_3 and NH_4

In the small microcosm experiment, nitrogen was added as a combination of the two forms: ammonia and nitrate. Neither ammonia nor nitrate nitrogen had an effect on the growth of *Vaucheria* sp. (ANOVA for NO_3 effect, $P<0.797$, for NH_4 effect $P<0.584$) (Fig.7). Further, no effect was observed when both forms of nitrogen were summed and the growth of *Vaucheria* sp. was analyzed as a function of an absolute concentration of nitrogen (Fig. 8).

Discussion

Vaucheria sp. is usually found at locations with high nutrient concentrations. Leukart and Hanelt (1995) found *Vaucheria bursata* in a stream in Germany at phosphate concentrations above 200 µg/L and nitrate concentrations above 1700 µg/L Sabater et al. (2003), in his study of algal mats in the Llobregat River in Spain, found *Vaucheria bursata* at even higher levels of phosphate (400µg/L) and nitrate (3900 µg/L). In this laboratory study, we found a threshold concentration of phosphorus to be in the range from 11.6 to 36.7 µg P/L, and we also found that larger mats required higher phosphorus levels. The nitrate threshold concentrations were from 208 to 499 µg N/L. When ammonia was used as a source of nitrogen, we observed an increase in growth rates above 50 µgN/L. Above 1000 µgN/L, however, ammonia became toxic and growth rates were reduced (Fig. 5). *Vaucheria* sp. used ammonia and nitrate as a source of nitrogen

and we did not observe any preference for either. Ozimek (1990), in laboratory experiments, grew mat fragments of *Vaucheria dichotoma* and found higher growth rates in higher nutrient concentrations. The relative growth rates calculated based on Ozimek's (1990) data were in the lower range of those observed in this study ((0.01-0.05 mg/mg/day dry weight).

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Fig 1

Relative growth rate (RGR) of *Vaucheria* sp. in different phosphate phosphorus concentrations (n=65). A four parameter logistic curve was fitted through the data from the small microcosm experiments (experiment 1). Curve parameters are in Table 2.

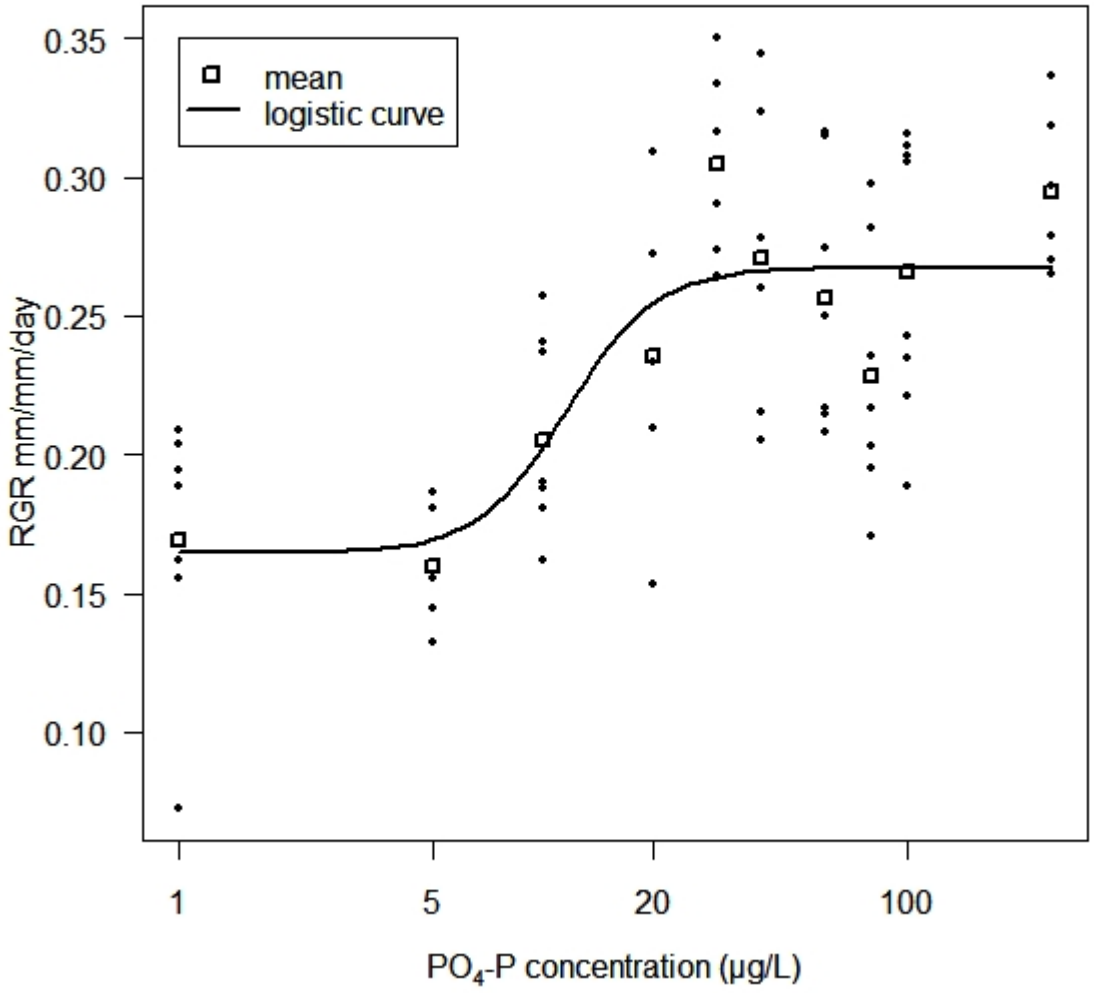

Fig. 2

Relative growth rate (RGR) of *Vaucheria* sp. in a factorial experiment with 3 initial biomass levels (0.01g, 0.05g, 0.2g) and 5 phosphorus concentrations (n=45). A four parameter logistic curve and a regression line (initial biomass 0.2g R^2=0.048, P<0.433) were fitted through the data from a large microcosm experiment (experiment 2). Curve parameters are in Table 3.

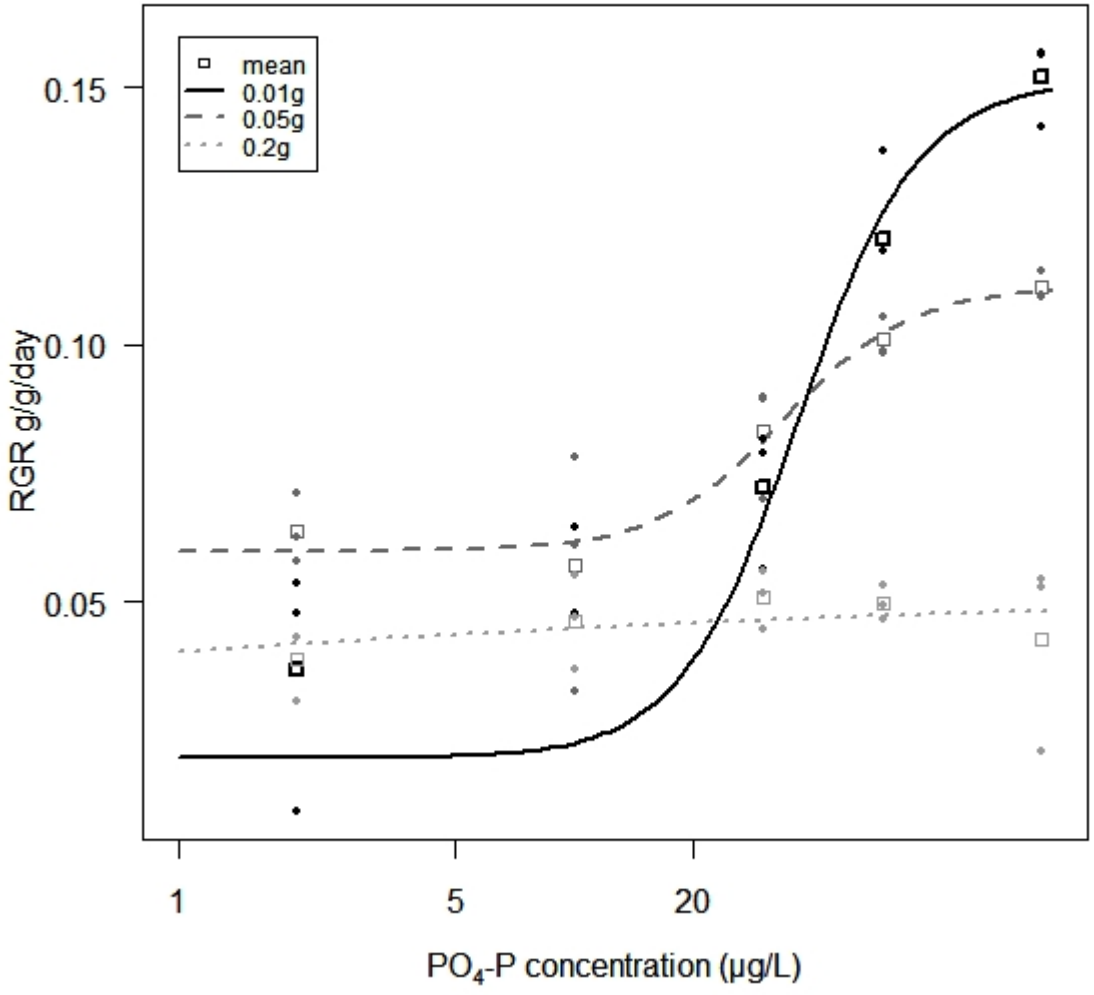

Fig. 3

Relative growth rate (RGR) of *Vaucheria* sp. in different nitrate nitrogen concentrations (n=68). Linear regression was fitted through the data from a small microcosm experiment (experiment 3) (R^2=0.01, P<0.414).

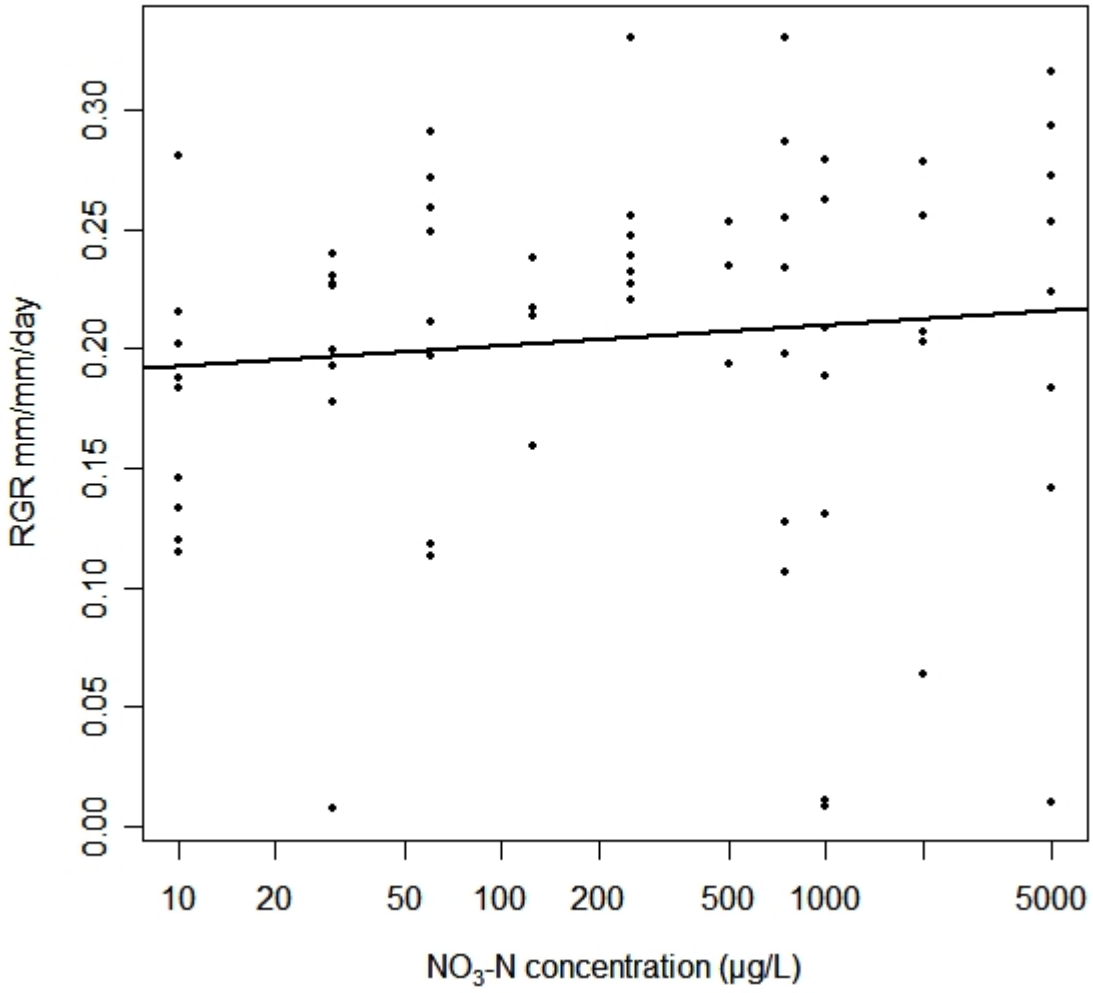

Fig. 4

Relative growth rate (RGR) of *Vaucheria* sp. in a factorial experiment with 3 initial biomass levels (0.01g, 0.05g, 0.2g) and 5 nitrogen concentrations (n=45). Four parameter logistic curves were fitted through the data from a large microcosm experiment (experiment 4). Curve parameters are in Table 3.

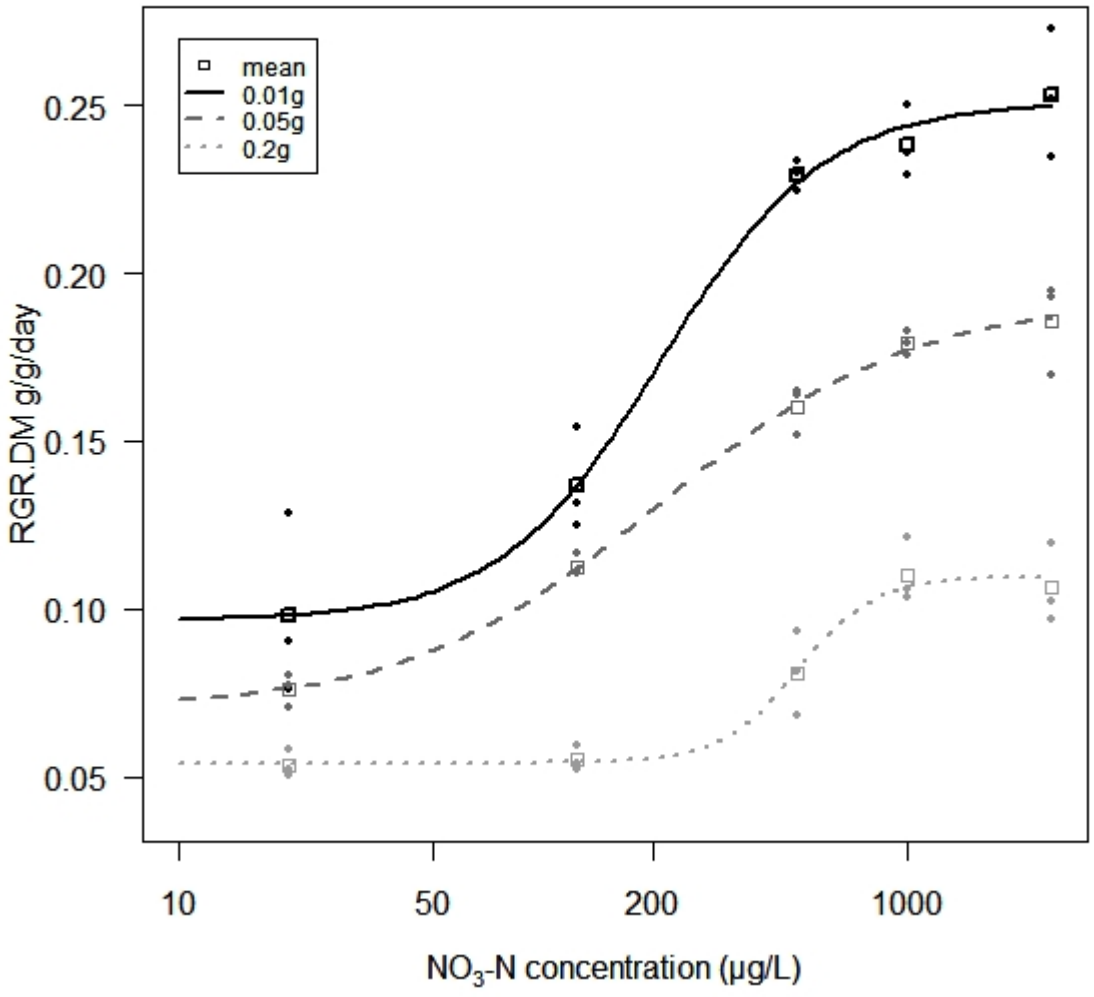

Fig. 5

Relative growth rate (RGR) of *Vaucheria* sp. in different ammonia nitrogen concentrations (n=58). Five parameter Brain-Cousens hormesis logistic model was fitted through the data from small microcosm experiment (experiment 5). Since missing data did not allow the calculation of the full model, point of inflection *e* was set at 500 µg/L after analysis of lowess smooth line. Curve parameters in Table 2.

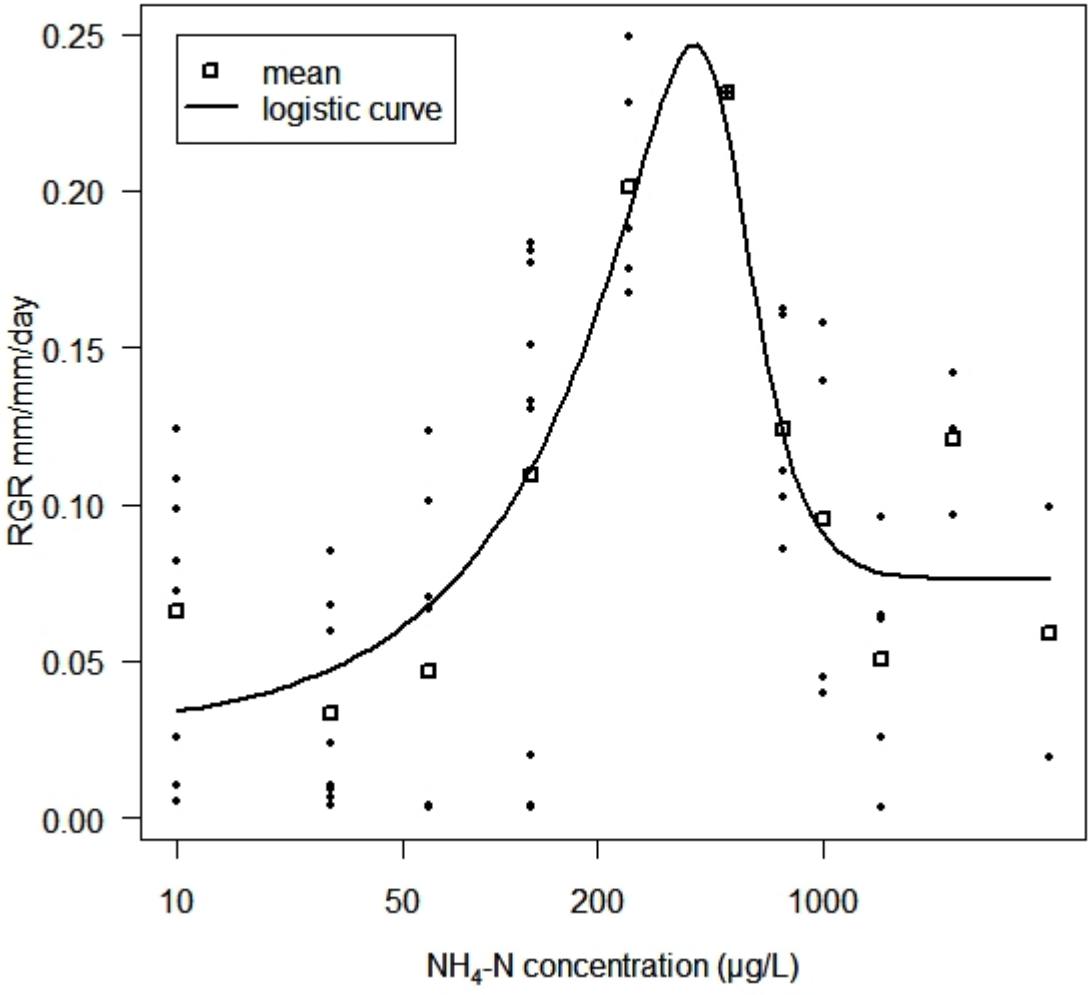

Fig. 6

Relative growth rate (RGR) of *Vaucheria* sp. in a factorial experiment with 3 initial biomass levels (0.01g, 0.05g, 0.2g) and 5 ammonia concentrations (n=41). Five parameter Brain-Cousens hormesis logistic model was fitted through the data from a large microcosm experiment (experiment 6). Since missing data from the 5000 µg/L treatment did not allow the calculation of the full model, point of inflection *e* was set at 800 µg/L after analysis of lowess smooth lines. Curve parameters are in Table 3.

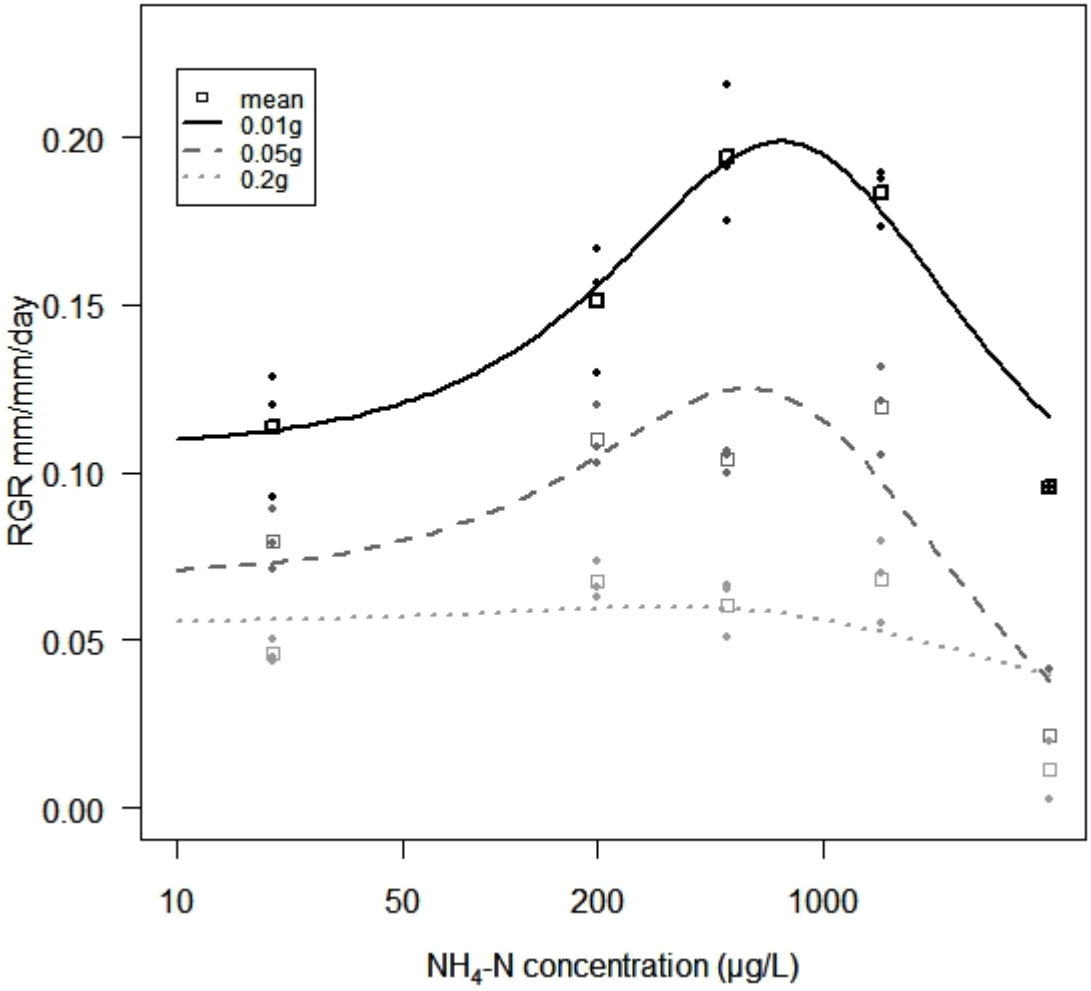

Fig. 7

Boxplot of relative growth rates (RGR) of *Vaucheria* sp. in a combination of different ammonia and nitrate nitrogen concentrations (experiment 7).

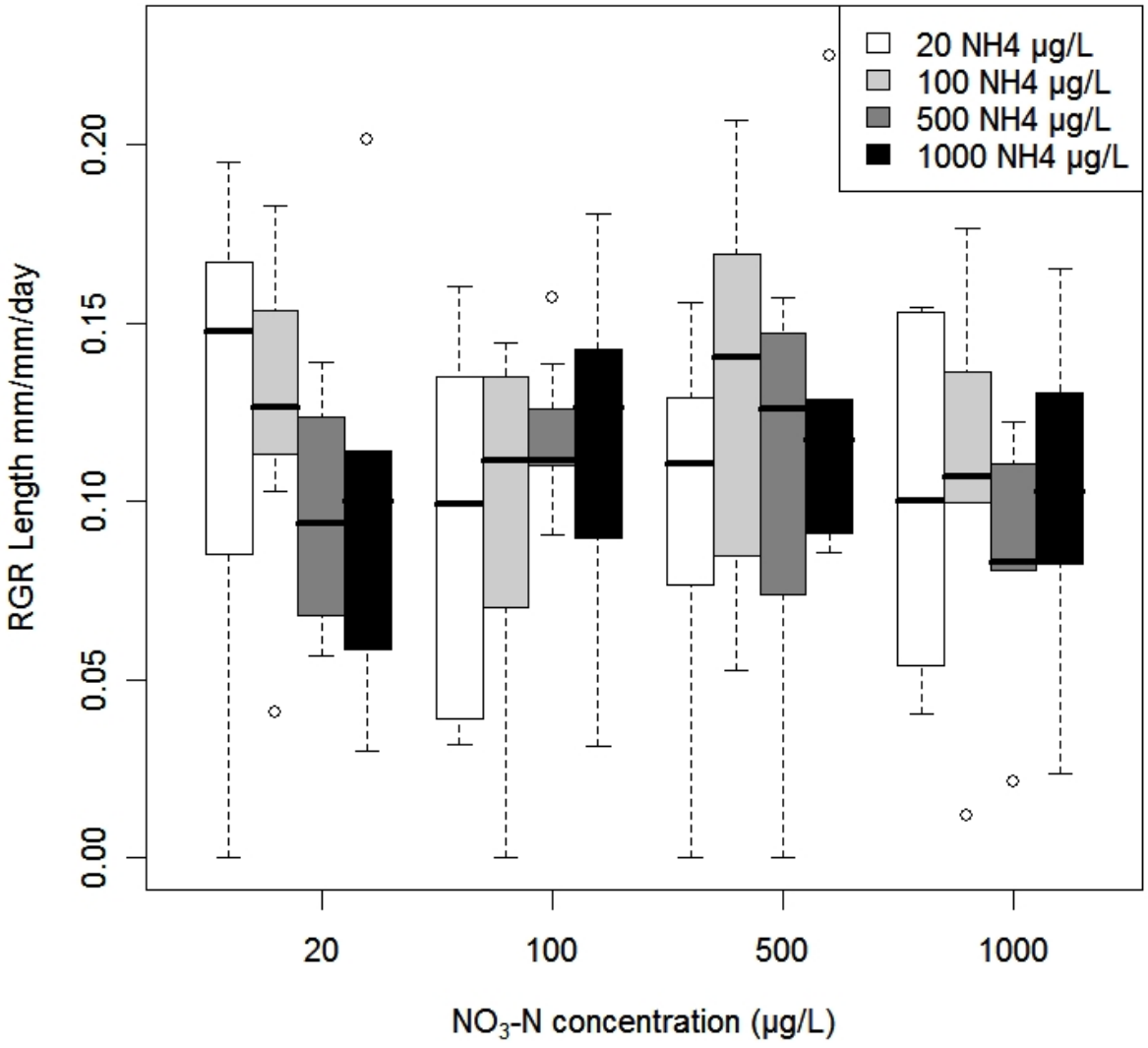

Fig. 8

Relative growth rate (RGR) of *Vaucheria* sp. in different nitrogen (sum of NO_3-N and NH_4-N) concentrations (n=99). Linear regression was fitted through the data from a small microcosm experiment (experiment 7) (R^2=0.03, P<0.097).

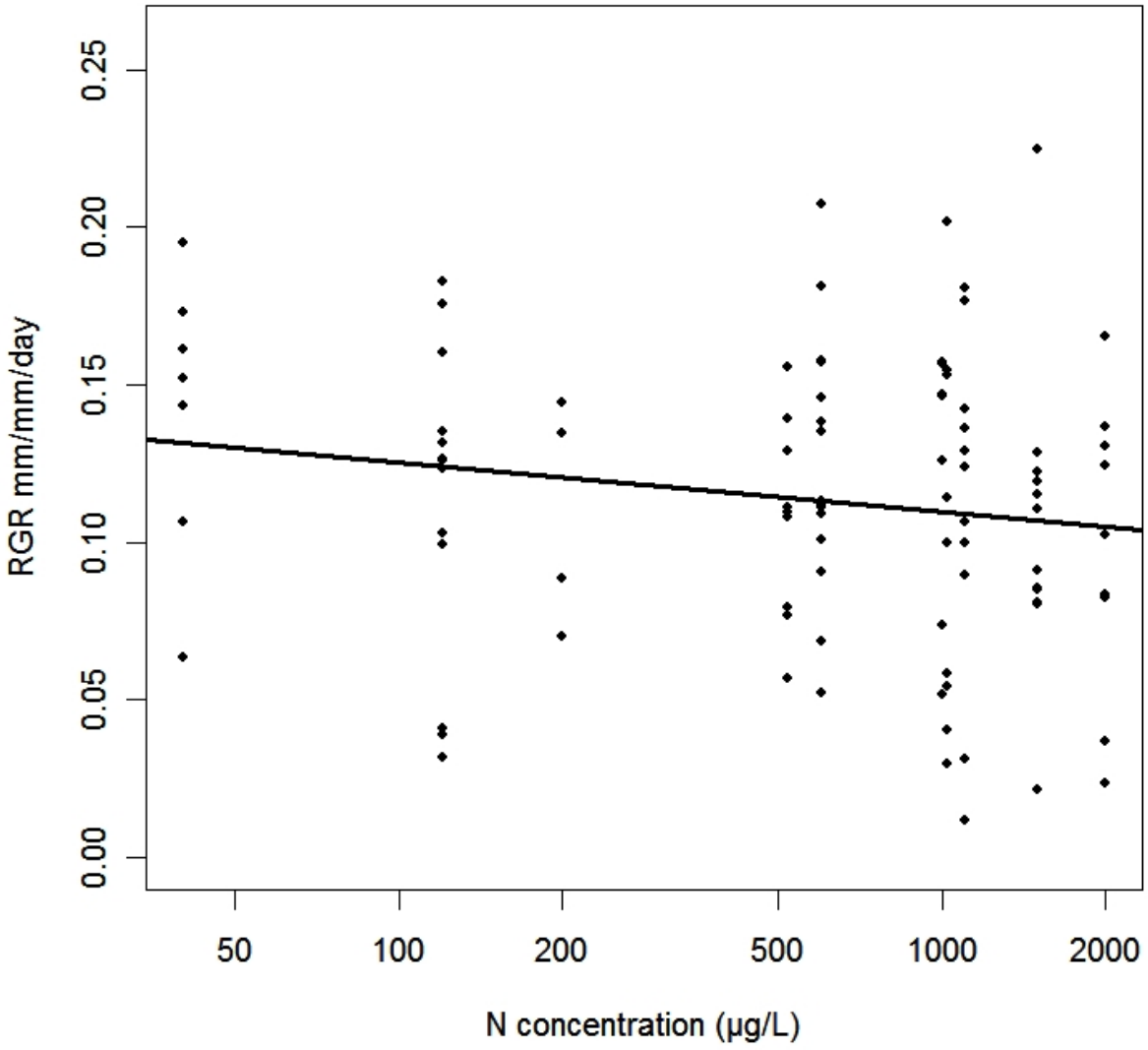

Table 1

Vaucheria sp. experiments. Experiment numbers in parenthesis refer to numbers used in the 2006 report.

Experiment (project experiment number)	Micro cosm	Nutrient gradient	Algae collection location	Season	Growth medium	Mean min and max temperature (ºC)
1 (21)	Small	PO_4-P	Ichetucknee and Alexander	Spring	Z8Fl Fe200	21.4 &24.9
2 (7)	Large	PO_4-P	Ponce de Leon	Winter	Z8Fl Fe200	20.2 & 24.0
3 (20)	Small	NO_3-N	Ichetucknee and Alexander	Spring	Z8Fl Fe200	21.3 &24.9
4 (9)	Large	NO_3-N	Alexander	Spring	Z8Fl Fe200	20.8 & 22.3
5 (4)	Small	NH_4-N	Manatee	Summer	Z8Fl Fe600	20.7 & 24.8
6 (11)	Large	NH_4-N	Ponce de Leon	Winter	Z8Fl Fe200	20.2 & 23.8
7 (2)	Small	N (NH_4 and NO_3)	Manatee	Fall	Z8Fl Fe600	20.8 & 24.4

Table 2

Parameters of the logistic dose-response model applied to the *Vaucheria* sp. RGR data from small microcosm experiments, where *d* and *c* denote the upper and lower limit of algae growth rates at zero and infinite concentrations of nutrient. ED_{50} (also called *e*) denotes the concentration required to increase the algal growth rate by half between the upper and lower limits and *b* is proportional to the slope (the rate of change) of the curve around ED_{50}. Significant parameters are in boldface. ED_{10} and ED_{90} denote the concentration required for 10% and 90% growth saturation. For the Brain-Cousens hormesis model, *c* and *d* are the upper and lower asymptote of the response. The columns designated *b* and *e* are related to the rate of change and the point of inflection of the curve respectively; and *f* indicates the initial rate of increase at low concentrations.

Nutrient	Model	*d* (mm/mm/day)	*c* (mm/mm/day)	*b*	ED_{50} (µg/L) *e*	ED_{90} (µg/L) or *f*	ED_{10} (µg/L)
PO_4	l4()	**0.27±0.01**	**0.16±0.01**	**-3.54±1.68**	**11.59±2.59**	21.53±8.19	6.24±2.22
NH_4	Brain-Cousens	**0.03±0.01**	**0.08±0.01**	**5.40±1.54**	set at 500	**0.0004±0.0001**	-

Table 3

Parameters of the logistic dose-response model applied to the *Vaucheria* sp. growth response data from large microcosm experiments, where *d* and *c* denote the upper and lower limit of algae growth rates at zero and infinite concentrations of nutrient. ED_{50} denotes the concentration required to increase the algal growth rate by half between the upper and lower limits and *b* is proportional to the slope of the curve around ED_{50}. Significant parameters are in boldface. ED_{10} and ED_{90} denote the concentration required for 10% and 90% growth saturation. For the Brain-Cousens hormesis model, *c* and *d* are the upper and lower asymptote of the response. The columns designated *b* and *e* are related to the rate of change and point of inflection of the curve respectively; and *f* indicates the initial rate of increase at low concentrations.

Nutrient and biomass	Model	*d* (g/g/day)	*c* (g/g/day)	*b*	ED_{50} (µg/L) *e*	ED_{90} (µg/L) of *f*	ED_{10} (µg/L)
PO_4 x 0.01g	l4()	**0.15±0.02**	0.02±0.01	**-2.89±1.34**	**36.68±8.95**	78.37±40.24	17.17±5.55
PO_4 x 0.05g	l4()	**0.11±0.02**	**0.06±0.01**	-2.71±3.60	33.67±20.61	75.78±107.9	14.96±15.31
NO_3 x0.01g	l4()	**0.25±0.01**	**0.10±0.01**	**-1.96±0.42**	**210.3±30.4**	644.0±202.2	68.67±16.62
NO_3 x0.05g	l4()	**0.19±0.01**	**0.07±0.01**	**-1.25±0.43**	**207.8±56.6**	1200±797.0	35.97±23.92
NO_3 x0.2g	l4()	**0.11±0.01**	**0.05±0.01**	-4.13±2.87	**499.2±68.9**	849.9.5±315.1	293.2±122.4
NH_4 x 0.01g	Brain-Cousens	**0.11±0.01**	0.06±0.00	**1.78±0.31**	set at 800	**0.00028±0.00007**	-
NH_4 x 0.05g	Brain-Cousens	**0.07±0.01**	-0.02±0.0	**1.67±0.59**	set at 800	0.00024±0.00001	-

Appendix 1

Z8Fl medium was derived from Z8 medium (Kotai 1972) for the culture of filamentous algae from Florida springs. It was adopted to reflect the Florida springs' water chemistry. The concentrations of nitrogen and phosphorus were reduced and the concentrations of calcium, magnesium and bicarbonate were increased. The original Z8 medium had an Fe concentration of 600µg/L. We found that this concentration reduced the growth rates of the algae, and we later used a concentration of 200µg/L. We used the original

concentration of microelements, but for future work this concentration probably should be reduced to one-half of the original concentration, because we found that the original concentration of microelements was limiting the growth of *Lyngbya wollei*. Solutions 1 and 2 were filter sterilized. Solution 5 was autoclaved.

To prepare 1 L of Z8Fl medium, fill a flask with 957ml of deionized water (DIH_2O), then add solutions 3, 4, 6 and 7 and autoclave. After autoclaving add solutions 1, 2, and 5. The prepared medium should be left for 24 hours for the gases to equalize. The target pH concentration should be around 8.5. The pH might have to be adjusted to that value.

1. Fe-EDTA 10ml (can be used at reduced level, then use 3ml)
10ml ●$FeCl_3$ solution (2.8 g $FeCl_3 \cdot 6H_2O$ dissolved in 100ml 0.1N HCl) and
9.5ml from an ●EDTA-solution (3.9g EDTA-Na_2 dissolved in 100 ml 0.1N NaOH)
mixed and filled up to 1000ml with distilled water.

2. Gaffron's trace element solution 1ml (can be used at reduced level, then use 0.5ml)

Stock solutions (kept separate)
Each in g in 100ml DIH_2O

●$ZnSO_4 \cdot 7H_2O$	0.287 g
●$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.088 g
●$Co(NO_3)_2 \cdot 6H_2O$	0.146 g
●$VOSO_4 \cdot 6H_2O$	0.035 g
●$Al_2(SO_4)_3K_2SO_4 \cdot 2H_2O$	0.474 g
●$NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$	0.198 g
●$Cd(NO_3)_2 \cdot 4H_2O$	0.155 g
●$Cr(NO_3)_3 \cdot 7H_2O$	0.037 g
●$Na_2WO_4 \cdot 2H_2O$	0.033 g
●KBr	0.119 g
●KI	0.083 g
●$Cu(SO_4) \cdot 5H_2O$	0.125 g

Take 10ml of each solution.

●H_3BO_3 3.10 g and $MnSO_4 \cdot H_2O$ 1.69 g in 1L DIH_2O. Take 100ml.

Final trace element stock solution: 12x10ml + 100ml + DIH_2O (780 ml) to 1L.

3. $NaNO_3$ solution 1ml
● 6.1g $NaNO_3$ in 1L DIH_2O

4. K_2HPO_4 solution 1ml
● 0.56g K_2HPO_4 in 1L DIH_2O

5. $CaCl_2 \cdot 2H_2O$ solution 10ml
● 18.3g $CaCl_2 \cdot 2H_2O$ in 1L DIH_2O

6. $MgSO_4 \cdot 7H_2O$ solution 10ml
● 7g $MgSO_4 \cdot 7H_2O$ in 1L DIH_2O

7. Na2CO3 solution 10ml
● 13.7 g Na_2CO_3 in 1L DIH_2O

References

Kotai, J. 1972. Instructions for preparation of modified nutrient solution Z8 for algae. Norwegian Institute for Water Research, publication B-11/69., Blindern, Oslo.