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Comparative adaptations of *Aphanizomenon* and *Anabaena* for nitrogen fixation under weak irradiance

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SUMMARY

1. *In situ* measurements of nitrogen fixation rates for *Aphanizomenon* in fertile Colorado lakes with low inorganic nitrogen concentrations demonstrated high efficiency of nitrogen fixation at low irradiance.
2. For study populations, rates of N_2 fixation in darkness and with alternating exposure to light and darkness were a higher percentage of light-saturated rates for *Aphanizomenon* than for *Anabaena*, suggesting storage of reduced metabolites at high irradiance that are used subsequently by *Aphanizomenon* when cells are forced by mixing into zones of low irradiance. Also, saturation of N_2 fixation occurred over a lower range of irradiance for *Aphanizomenon* than for *Anabaena*.
3. High efficiency of N_2 fixation in *Aphanizomenon* at low or fluctuating irradiance is complementary to its previously demonstrated high efficiency of photosynthesis at low irradiance. Nitrogen fixation rate was also strongly related to DIN concentration; fixation was highest at low DIN (maximum < 5 μg L^{-1}) but was also most vulnerable to photoinhibition under such conditions.
4. The fixation capabilities of *Aphanizomenon* under weak or varying irradiance could explain its commonly observed domination over *Anabaena* when transparency is low and available nitrogen is scarce.

Keywords: cyanobacteria, lake mixing, lake transparency, nitrogen fixation, photosynthesis

Introduction

Heterocystous cyanobacteria may become abundant in lakes and reservoirs when inorganic fixed nitrogen is depleted because their ability to fix N_2 offers a competitive advantage over non-fixing algal taxa (Schindler, 1977; Vitousek & Howarth, 1991; Smith & Bennett, 1999). *Aphanizomenon* and *Anabaena* are among the most common nitrogen-fixing genera in eutrophic lakes and reservoirs (Reynolds, 2006). Light availability may in part determine which of these two genera is dominant, as *Aphanizomenon* appears to be more tolerant of low transparency (Schreurs, 1992; De Nobel *et al*., 1998). The present study explores mechanisms that would explain the common predominance of *Aphanizomenon* in habitats where both light and inorganic nitrogen are scarce.

De Nobel *et al*. (1998) studied the photosynthetic and N_2-fixing capabilities of *Aphanizomenon* in comparison with those of *Anabaena*, its most common competitor in environments where N fixation is advantageous. Using one strain of *Aphanizomenon* and one of *Anabaena* derived from field populations in eutrophic Dutch lakes, they showed in culture that *Aphanizomenon* had a higher maximum efficiency of photosynthesis and a higher N fixation rate under weak irradiance, whereas *Anabaena* showed higher growth rates supported by N fixation under abundant irradiance. Thus, *Aphanizomenon* might be expected to predominate in environments with strong shading from dense algal populations, substantial vertical mixing, or both, whereas *Anabaena* would predominate where light is more abundant. Competition experiments with the two strains in culture, however, showed that

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Anabaena predominated under all conditions, including weak light, which implicates the importance of other factors yet to be identified.

The objective of the present study was to quantify light-response curves for nitrogen fixation in field populations of *Aphanizomenon*, for which such information has not been available, and to determine the effect of DIN concentrations on the light-response curve. A second objective was to quantify the comparative ability of *Anabaena* and *Aphanizomenon* to conduct nitrogen fixation efficiently in environments that show highly variable light availability during the daylight hours caused by circulation of cells within the mixed layer in lakes of very low transparency. Hypotheses associated with these objectives include (i) field populations of *Aphanizomenon* will show N fixation rates that are sensitive not only to irradiance but also to ambient DIN concentrations; (ii) the N fixation characteristics for field populations of *Aphanizomenon*, including the joint effects of irradiance and DIN availability, reflect adaptation for fixation under scarcity of irradiance; (iii) the response of field populations of *Aphanizomenon* and *Anabaena* to light exposure history, expressed experimentally as alternation of darkness and saturating irradiance, will differ substantially in ways that reflect adaptation to high irradiance (*Anabaena*) or low irradiance (*Aphanizomenon*).

Methods

Sample collection and acetylene reduction

Nitrogen fixation was measured *in situ* by the acetylene reduction method (Flett, Hamilton & Campbell, 1976) in five reservoirs of the Colorado Front Range and plains (Table 1). At each site, an integrated surface sample (0–1 m) was collected for nitrogen fixation incubations, phytoplankton counts and nutrient analyses. Subsamples were drawn into field rinsed, 50-mL gas-tight syringes (30 mL headspace). Acetylene was generated from the hydrolysis of calcium carbide in deionised water and stored in a 1-L bladder. Each syringe was inoculated with 5 mL of acetylene and agitated for 15 s. Gas from the headspace in two control syringes was immediately transferred to a 5-mL vacutainer to account for background ethylene in the water sample and acetylene source. The remaining syringes were incubated for 2 h, after which gas from the headspace of each syringe was transferred to a 5-mL vacutainer for ethylene analysis.

The ethylene concentrations were measured with a Shimadzu (Kyoto, Japan) 14-A gas chromatograph with a flame ionisation detector (330 °C) and a Porapak (Waters Associates, Framingham, MA, U.S.A.) N column (100–120 mesh) at an oven temperature of 80 °C. For each analysis, 3 mL of gas was withdrawn from a 5-mL vacutainer (Becton, Dickinson & Company, Franklin Lakes, NJ, U.S.A.) serum vial and injected into the instrument. The syringe carried a valve that was closed prior to withdrawal of the syringe from the vacutainer. Ethylene concentration was quantified by use of a standard curve prepared by analyses of standard concentrations of ethylene withdrawn from vacutainers in a manner identical to the procedure used for field samples. After accounting for variables affecting ethylene recovery (temperature and relative volume of headspace; Capone, 1993), ethylene production was converted to nitrogen fixation with a 4 : 1 ethylene/dinitrogen conversion ratio (Jensen & Cox, 1983).

Incubation designs

Two incubation designs were used in this study: 'light response' and 'light history'. For light-response incubations, syringes were exposed to a gradient of light intensities within the euphotic zone (>1% surface irradiance). On each date, three syringes were incubated at each of four depths at ambient irradiance (all within the mixed layer and not within or below the thermocline); in addition, two syringes were incubated in darkness.

The light-history incubations tested the effects on nitrogen fixation of intermittent irradiance, as would be characteristic of cells moved by vertical mixing between the euphotic and aphotic zones. Bottles were exposed to light (L) or darkness (D) >3 h prior to incubation, and to light (*L*) or darkness (*D*) during incubation (2 h); all four

Table 1 Locations and physical characteristics of the study sites

Study site	Latitude, °	Longitude, °	Altitude, masl	Surface area, ha	Maximum depth, m	Mean depth, m
Seeley Reservoir	40.467	−104.736	1434	52	6	3
Pelican Pond	40.173	−104.984	1471	15	4	2
Bear Creek Reservoir	39.652	−105.144	1690	47	10	6
Banner Lake	40.066	−104.553	1523	0.7	<1	<1
Jackson Lake	40.392	−104.074	1353	242	2	1

combinations of exposure were used: L*L*, L*D*, D*L* and D*D*. Water was collected before dawn and held in 1-L dark bottles (D, dark pre-treatment) or light bottles (L, light pre-treatment) until the beginning of the incubations at 11:00 hour. The L pre-treatment bottles were exposed to natural irradiance (217–656 μmol photons m^{-2} s^{-1}) for 3 h prior to the time of incubation, and the D pre-treatment bottles were held in darkness. Following each pre-treatment, nine syringes were incubated at the surface of the water column for the *L* treatment (exposed to light), and nine syringes were incubated for the *D* treatment (not exposed to light).

Although five lakes were included in the study, the distribution of experiments across these lakes was dictated by the unpredictable occurrence of substantial populations of either *Anabaena* or *Aphanizomenon*; no experiments were conducted on mixed populations of these two genera. For the study of light response, which focussed on *Aphanizomenon*, experiments were conducted on two of the lakes (Pelican Ponds seven dates; Bear Creek Reservoir, one date). In addition, one light-response experiment was performed for *Anabaena* on one date at Seeley Reservoir, primarily to confirm that the nitrogen fixation curve parameter I_k, which has been measured in a number of *Anabaena* field populations, is similar to published values for this genus. Light-history experiments, which have not been conducted previously for either genus, were performed twice for *Anabaena* (Banner Lake) and twice for *Aphanizomenon* (Pelican Pond, Jackson Lake).

Water sampling

Water samples for nutrient analyses were filtered with Whatman (Maidstone, Kent, U.K.) glass fibre filters (GF/C; effective pore size *c.* 1 μm). Nitrate concentrations were analysed by ion chromatography, and ammonium was measured colorimetrically (Koroleff, 1976). Soluble reactive phosphorus (SRP) was determined by an ascorbic acid-molybdate method (Murphy & Riley, 1962), as was dissolved phosphorus (TDP), following persulphate digestion (Valderrama, 1981; Lagler & Hendrix, 1982).

Preserved phytoplankton samples were examined in an Utermöhl sedimentation chamber with an inverted microscope under phase contrast illumination at 400× magnification. Heterocyst density was estimated for each sample from counts over transects of known area in the sedimentation chamber (Lewis & Levine, 1984).

Surface irradiance was measured with a LiCor (Lincoln, NE, U.S.A.) Li-190 quantum sensor. Irradiance within the water column was measured with a LiCor Li-192 underwater quantum sensor. The PAR (400–700) attenuation coefficients (K, m^{-1}) were derived from irradiance profiles. Irradiance at all incubation depths was estimated from the attenuation coefficient and surface irradiance measured during the incubation.

Light-response curves

Light-response curves for N_2 fixation were constructed according to the method of Lewis & Levine (1984), as adapted from the photosynthesis equation of Platt & Jassby (1976). Although a rectangular hyperbola model can be used for this purpose (Staal *et al.*, 2002), the equation from Lewis & Levine (1984) incorporates physiological parameters other than I_k (light saturation threshold), including light inhibition, and allows direct comparison with previous work by Lewis & Levine (1984). The equation is as follows:

$$F = F_s\,[1 - e^{-a}]\,e^{-b} + F_{dark} \quad (1)$$

where $a = \alpha I F_s^{-1}$, and $b = \beta I F_s^{-1}$, F is the heterocyst-specific rate of nitrogen fixation, F_s is the maximum achievable rate of nitrogen fixation in the absence of inhibition, and F_{dark} is the rate of nitrogen fixation in the absence of light [all with units nmol N $(10^6$ heterocyst$)^{-1}$ h^{-1}]. Alpha (α) is the slope of the initial ascending limb of the light-response curve and β is the slope of the descending limb of the light-response curve ($\beta > 0$ indicates light inhibition of nitrogen fixation). Both α and β are expressed as nmol N $(10^6$ heterocyst$)^{-1}$ h^{-1} (μmol photon m^{-2} $s^{-1})^{-1}$. The independent variable, I, is irradiance (μmol photon m^{-2} s^{-1}). The parameters F_s, α and β were estimated by nonlinear regression with Marquardt's algorithm (Conway, Glass & Wilcox, 1970).

Two derived values also can be calculated. F_m is the maximum gross fixation rate with the same units as F_s and F_{dark}:

$$F_m = F_s[\alpha/(\alpha+\beta)][\beta/(\alpha+\beta)]^{\beta/\alpha} + F_{dark} \quad (2)$$

Because F_s is the rate of fixation in the absence of inhibition, F_s and F_m are equal on dates with no inhibition. The second derived value indicates the onset of full light saturation (I_k).

$$I_k = F_m/\alpha \quad (3)$$

Results

The concentration of nitrate N was low (<30 μg L^{-1}) on all sampling dates and below detection (<5 μg L^{-1}) on most dates (Table 2). The concentration of ammonium-N was

Table 2 Study sites, nutrient concentrations, algal abundance and transparency

Site	Nitrate-N, μg L^{-1}	Ammonium-N, μg L^{-1}	SRP, μg L^{-1}	TDP, μg L^{-1}	DIN:TDP, mass	Seston N:P, molar	Chl *a*, μg L^{-1}	Heterocyst density, 10^6 heterocysts L^{-1}	Secchi depth, cm
Seeley Reservoir									
8 August	0	19	2	35	0.7	8.3	47	5.8	38
Pelican Pond									
16 August	0	5	149	236	0.0	7.7	205	8.3	20
17 August	12	4	151	223	0.1	6.0	281	5.7	17
31 August	0	11	141	221	0.0	14.9	234	7.5	15
6 September	0	19	109	195	0.1	8.6	269	6.8	13
7 September	0	64	112	175	0.5	8.0	224	7.5	13
12 September	0	260	169	231	1.4	3.7	165	4.4	15
14 September	0	26	111	205	0.2	6.9	311	5.1	14
21 September	11	14	114	172	0.2	16.3	159	4.6	14
Bear Creek Reservoir									
23 August	25	51	47	61	1.5	6.9	25	6.5	155
Banner Lake									
3 October	0	14	11	46	0.5	10.9	37	0.7	27
6 October	0	14	9	49	0.5	11.8	51	3.1	37
Jackson Lake									
12 October	0	31	5	64	1.0	28.5	1095	2.7	10

also low (<30 μg L^{-1}), except on two dates in Pelican Pond and one date in Bear Creek Reservoir. A high concentration of chlorophyll *a* (Table 2) suggests that the demand for inorganic nitrogen was high relative to supply on most incubation dates. Furthermore, a low ratio of nitrogen to phosphorus in all of the lakes suggests either actual or incipient N deficiency (i.e. a molar N:P ratio for seston <30 and a DIN:TDP mass ratio <6; Morris & Lewis, 1988; Downing, Watson & McCauley, 2001).

Light-response incubations

Nitrogen fixation by *Aphanizomenon* showed a sharp linear increase in the low range of irradiance (Fig. 1; Table 3); median for α was 1.2 nmol N $(10^6$ heterocyst$)^{-1}$ h^{-1} (μmol photon m^{-2} $s^{-1})^{-1}$. Saturation irradiance (I_k) was consistently low for *Aphanizomenon*, which indicates a maximum rate of nitrogen fixation even near the lower limit of the photic zone (median = 31 μmol m^{-2} s^{-1}; Table 3). Typically, high irradiance did not substantially inhibit nitrogen fixation of *Aphanizomenon*, even when surface PAR irradiance exceeded 1000 μmol photons m^{-2} s^{-1} (Fig. 1). On two dates, weak inhibition occurred, and nitrogen fixation was strongly inhibited on one date (16 August, Pelican Pond). In the absence of light, the mean rate of nitrogen fixation was 25% of the light-saturated rate (Table 3).

Estimated values for all four of the parameters of the light-response equation showed a reciprocal relationship with concentration of DIN (Fig. 2). The curves in Fig. 3 show the light-response equation of *Aphanizomenon* at six concentrations of DIN, as derived from the N fixation equation at specific DIN concentrations (Fig. 2).

The shapes of the contour lines shown in Fig. 3 are based on mean values for response to irradiance and DIN as shown in the experiments on light response of *Aphanizomenon*. The variance around these contour lines is of interest. The lower panel of Fig. 3 shows variance, expressed as standard error, around the mean response for the upper contour line. Standard error was derived from a simulation algorithm that produced 1000 independent estimates of the contour line by random sampling of the curve parameters based on their frequency distributions as shown in the last line of Table 3. Confidence intervals are widest for the uppermost curve and are progressively narrower for the lower curves (confidence limits not shown for lower curves).

Light-history incubations

The light-history incubations simulated the pattern of light exposure experienced by cells moved by mixing between the euphotic (*c.* upper 50 cm; Table 2) and aphotic zones (Fig. 4). Pre-incubation treatments generated samples with divergent light histories. Prior light exposure increased the rates of nitrogen fixation during subsequent incubations, demonstrating the connection between nitrogen fixation and the history of light exposure for populations dominated either by *Aphanizomenon*

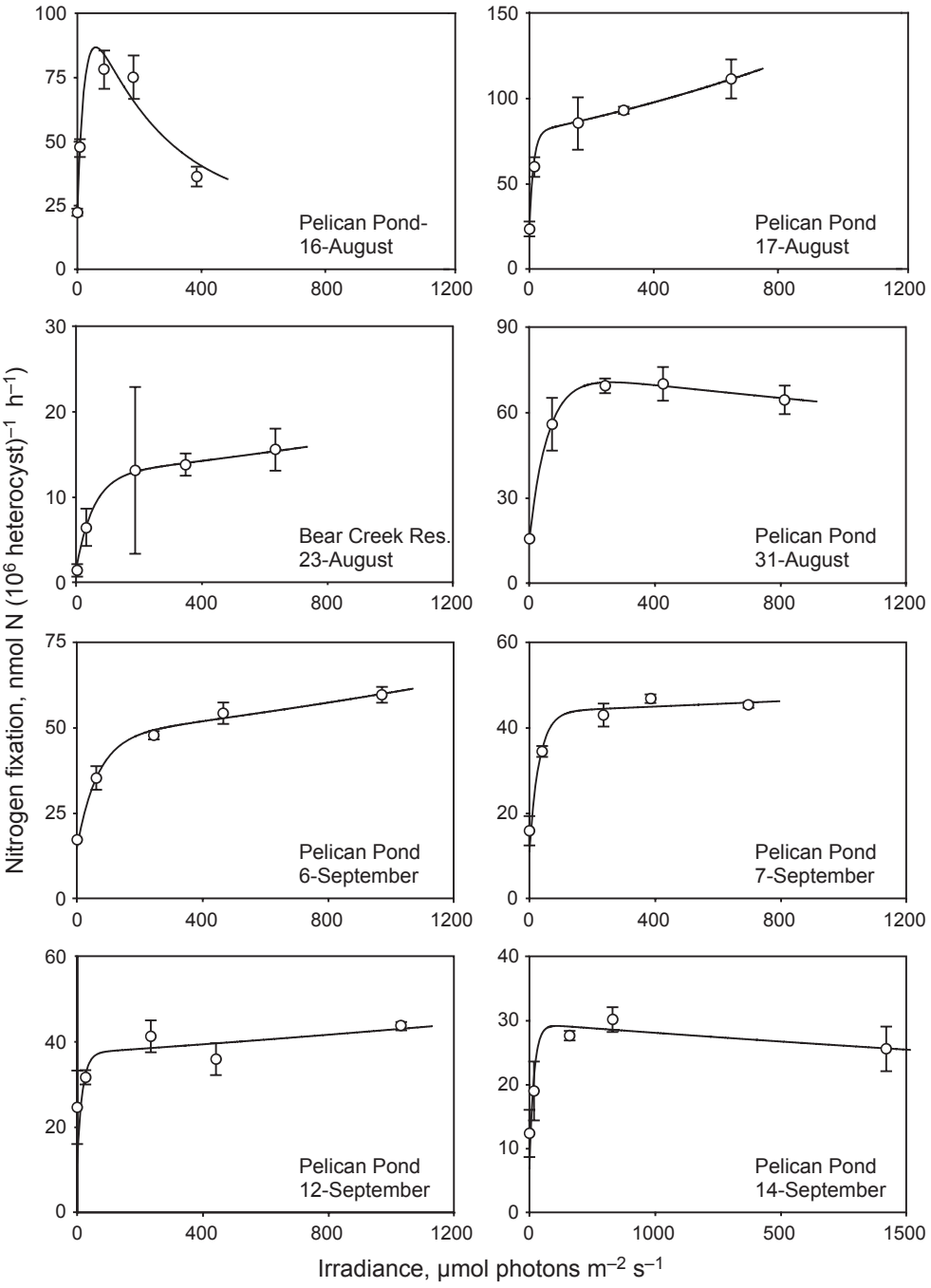

Fig. 1 Light-response curves for *Aphanizomenon*, fitted according to the method of Lewis & Levine (1984). Note that scales differ across panels.

or *Anabaena*. Among the four treatments (L*L*, L*D*, D*L*, D*D*), the highest rates of fixation were measured in the L*L* treatments. Fixation was lowest in the D*D* treatment (Fig. 1). Intermediate rates of fixation were measured in the L*D* treatment (>D*D*) and the D*L* treatment (>L*D*). In the D*D* and D*L* incubations, *Aphanizomenon* fixed N_2 at a higher percentage of its maximum rate (i.e. N_2 fixation in L*L* treatments) than did *Anabaena*.

Discussion

The relationship between irradiance and N fixation in field studies supports the proposal of De Nobel *et al.* (1998) that *Aphanizomenon* is generally more efficient than *Anabaena* in using low irradiance for N fixation (Fig. 5). Marine populations may differ, as shown by data for *Aphanizomenon* in the Baltic Sea (Stal & Walsby, 2000).

Table 3 Parameters of eqn 1 from Marquardt's algorithm for one experiment on *Anabaena* and light experiments on *Aphanizomenon*

Site	F_s*	F_m*	$\alpha^\dagger$	$\beta^\dagger$	F_{dark}*	$I_k^\ddagger$	R^2
Anabaena							
Seeley Reservoir							
8 August	263	186.9	1.3	0.156	7	198	0.24
Aphanizomenon							
Pelican Pond							
16 August	90	86.7	3.7	0.355	22	24	0.72
17 August	56	80.0	4.4	−0.038	24	13	0.66
31 August	60	70.6	1.0	0.013	15	60	0.46
6 September	33	46.8	0.5	−0.011	15	64	0.84
7 September	33	43.5	1.0	−0.003	11	31	0.76
12 September	26	37.1	1.5	−0.005	12	17	0.48
14 September	23	29.1	1.2	0.003	7	18	0.52
Bear Creek Reservoir							
23 August	11	12.2	0.2	−0.004	1	53	0.40
Mean + SE (*Aphan.*)	41 + 9	51 + 9	1.7 + 0.5	0.039 + 0.045	13 + 3	45 + 10	–

*nmol N (10^6 heterocysts)$^{-1}$ h^{-1}.
†nmol N (10^6 heterocysts)$^{-1}$ h^{-1} (μmol photons m^{-2} s^{-1})$^{-1}$.
‡μmol photons m^{-2} s^{-1}.

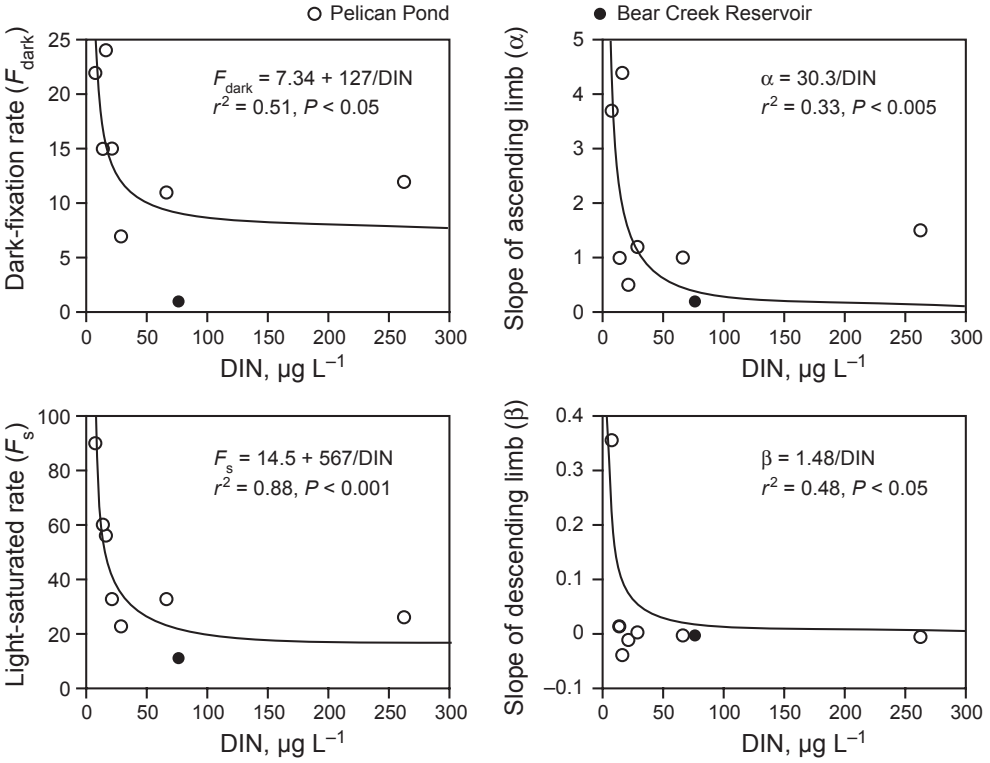

Fig. 2 Relationships between the parameter values of the light-response equation for *Aphanizomenon* and concentrations of dissolved inorganic N (DIN).

Thus, *Aphanizomenon* should predominate in low nitrogen environments with high light attenuation, high mixed layer thickness or both, whereas *Anabaena* should dominate under the opposite conditions. The outcome of laboratory competition in which *Anabaena* dominated consistently at all light intensities (De Nobel *et al*., 1998) remains to be explained. De Nobel *et al*. (1998) propose a role for allelopathy in field populations, but another

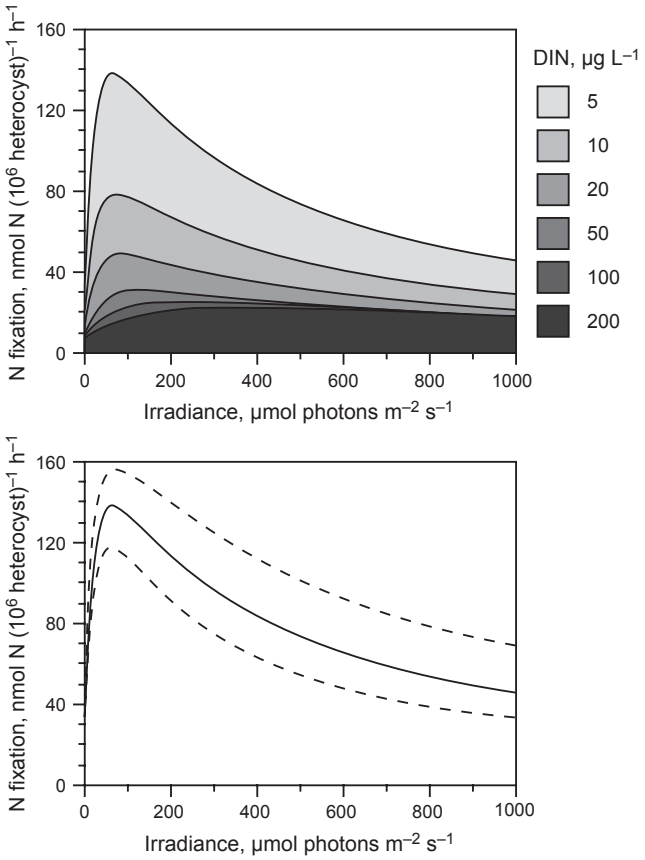

Fig. 3 Variation of the light response of nitrogen fixation in *Aphanizomenon* over a range of DIN concentrations (upper panel). Parameters of the light-response equation are derived from the relationships shown in Fig. 2. The lower panel shows the results of a simulation for the upper contour line of the upper panel as a means of estimating variance (SD) around the contour line.

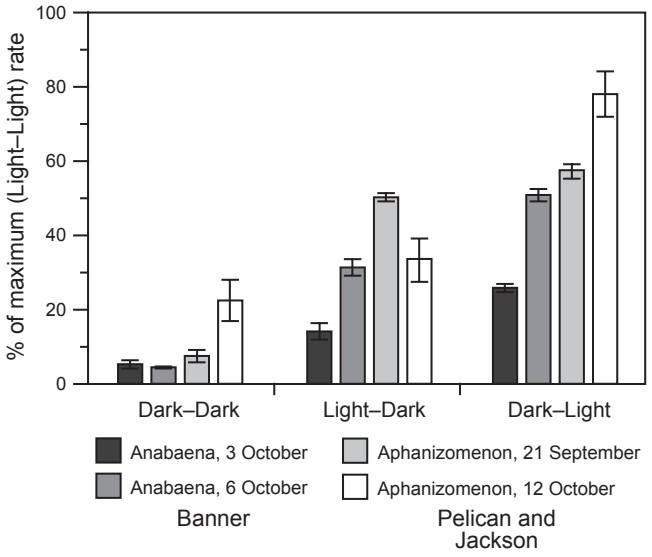

Fig. 4 Nitrogen fixation rates for *Aphanizomenon* and *Anabaena* from light-history experiments. Rates measured in the D*D*, L*D* and D*L* treatments are compared to the maximum rate (L*L* treatment) for each genus, with standard errors.

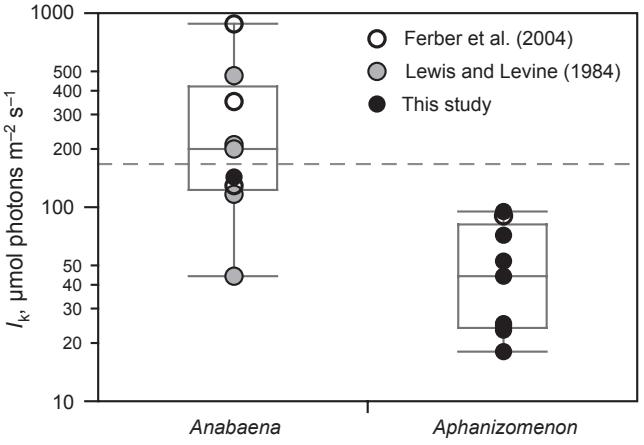

Fig. 5 Saturation irradiances (I_k) for *Anabaena* and *Aphanizomenon* in natural freshwater populations. Data are from Ferber *et al*. (2004; 0–1 m depth), Lewis & Levine (1984; 1 m depth), and this study (0–1 m depth).

possibility is related to the ability of *Aphanizomenon* to conduct significant N fixation over intervals of darkness, as shown in the present study.

The light-history experiments reported here show that *Aphanizomenon*, when responding to substantial irradiance following darkness, reaches a higher percentage of its maximum N fixation rate than *Anabaena*. In addition, *Aphanizomenon* shows substantial N fixation in complete darkness over extended intervals. Thus, it appears that *Aphanizomenon* stores reduced metabolites when light is abundant for use later for dark fixation of N. These observations may explain the lower efficiency of *Aphanizomenon* in fixing N when light is abundant because part of the N fixation is in effect deferred through storage of metabolites. Significant N fixation by *Aphanizomenon* in darkness combined with water column mixing through which low transparency produces alternating light and darkness for cells during the day could explain the dominance of *Aphanizomenon* over *Anabaena* in the field but not in culture, where the vertical mixing effect on irradiance is absent.

The strong effect of DIN concentration on the light response for N fixation reveals new aspects of the environmental control of N fixation for *Aphanizomenon*. When irradiance is below 100 µmol m^{-2} s^{-1}, N fixation rises rapidly if DIN concentrations are below 50 µg L^{-1}, but much less rapidly above 50 µg L^{-1}. This negative feedback of external DIN on N fixation rates is expected. Not expected is the strong decline in N fixation at

irradiances above 100 μmol m^{-2} s^{-1} when DIN is scarce. Figure 3 shows that maximum fixation, as well as the degree of significant photoinhibition, is highest at the lowest concentrations. A combination of these two effects causes the efficiency of N fixation to be highest at low irradiances accompanied by very low DIN concentrations (e.g. below 20 μg L^{-1}). *Aphanizomenon* is adapted not only for efficient N fixation under low irradiance, but also under strongly varying irradiance, as would be encountered by phytoplankton during bloom conditions in shallow lakes. If the characteristics of *Aphanizomenon* for N fixation reported here and by De Nobel *et al.* (1998) prove to be global attributes of *Aphanizomenon* in inland waters, the conditions for predominance of *Aphanizomenon* may be generally predictable from the light environment and concentration of inorganic N.

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