PHYSIOLOGICAL ECOLOGY OF MOUGEOTIA (ZYGNETACEAE)
FROM AN EXPERIMENTALLY ACIDIFIED LAKE

by

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PHYSIOLOGICAL ECOLOGY OF MOUGEOTIA (ZYGNETACEAE) FROM AN EXPERIMENTALLY ACIDIFIED LAKE.

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Filamentous green algae were collected in July, 1989, from metaphytic blooms that occurred in the acidified (pH 5.2) basin, but not an unacidified reference basin (pH 6.1) of Little Rock Lake, Vilas Co., WI. Isolates of a Mougeotia species, the dominant bloom former, and Spirogyra reflexa were cultured in SD11 medium at pH 5.5, with aeration. Measurements of O2 production in a factorial experiment revealed that optimal irradiance and temperature for photosynthesis in Mougeotia were 2500 μE·m⁻²·s⁻¹ and 25°C. Additional O2 evolution measurements showed that the optimal pH for Mougeotia photosynthesis was 8, but that net photosynthesis was positive from pH 8 to 3. Further photosynthesis studies indicated that Mougeotia was tolerant to concentrations of zinc and aluminum that were greater than levels observed in the acidified basin of the lake. Since inorganic carbon (Ci) is known to limit Mougeotia photosynthesis and growth in acidified lakes, the occurrence of carbonic anhydrase (CA) as a mechanism for uptake and concentration of Ci was investigated. No
CA activity was detected in *S. reflexa*. In contrast, both external and internal CA were measured in *Mougeotia* at pH 3.7 and at pH 8, by means of a potentiometric assay. By comparison to pH 8, at pH 3.7 external CA activity increased by a factor of about 2. An antibody to *Chlamydomonas* external CA was used to localize CA in the plasma membrane and cell wall of both *Chlamydomonas* and *Mougeotia*. A histochemical procedure that localizes CA in animal tissues gave positive results for pig liver, but did not work in any of the algae tested, possibly indication of differences in amount of enzyme activity between animal and algal CAs. Finally, when unaerated (DIC-limited) *Mougeotia* was grown in SD11 medium supplemented with 1% glucose, chlorophyll a levels were significantly higher than for cultures grown without sugar. Chloroplast morphology was also judged superior for sugar-supplemented cultures. The data suggest that *Mougeotia* possesses a DIC-concentrating system, and may also be able to import DOC (glucose).
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Chapter 1

Physiological ecology of the filamentous green alga *Mougeotia* (Zygnematales, Charophyceae) under acidic conditions.
Abstract

One of the earliest and most reliable indications of acid precipitation impacting freshwater systems is the development of littoral blooms of the filamentous green alga *Mougeotia* (Zygnematales, Charophyceae). Despite the frequency of this phenomenon, little is known about the ecology or physiology of *Mougeotia* that would explain this dramatic response. Field observations of depth distribution and seasonal abundance in Little Rock Lake, an experimentally acidified seepage lake in north-central Wisconsin, suggested that *Mougeotia* might prefer warmer temperatures and carry out net photosynthesis at fairly low light levels. To test these hypothesis measurements of net photosynthesis and respiration were made at 56 combinations of irradiance and temperature in a controlled environment facility. Optimum conditions were 25°C and 300-2300 μE-m⁻²-sec⁻¹. Over this range net photosynthesis averaged 40.4 mg O₂-g⁻¹-hr⁻¹. Respiration rates increased with both temperature and prior irradiance. Light-enhanced respiration rates were significantly greater than dark respiration rates following irradiance of 164 μE-m⁻²-sec⁻¹ or greater. *Mougeotia*’s photosynthetic response to temperature and irradiance explains aspects of its growth and distribution in acidified lakes but does not by itself explain the magnitude of this alga’s growth at low pH.

Key words: acidification, filamentous algae, *Mougeotia*, pH, photosynthesis, respiration, Zygnemataceae.
Introduction

Since the early 1970s acidification of freshwater systems by acid precipitation has been increasingly recognized as a widespread and significant environmental problem. One of the earliest and most reliable indications of acid precipitation impact on freshwaters is the development of littoral blooms or mats of filamentous green algae of the family Zygnemataceae. The genus *Mougeotia* is the most common bloom-forming alga in acid waters of Europe and North America. Despite the frequency with which *Mougeotia* is cited as an indicator of acidification, little is known about its ecology or physiology. There are no data to suggest why *Mougeotia*, rather than some other alga, should be so frequently associated with acidification. The most extensively studied feature of *Mougeotia* is phytochrome-mediated rotation of the laminate chloroplast (Haupt 1982, 1983; Wagner and Grolig 1992).

*Mougeotia* is a member of the family Zygnemataceae (class Charophyceae) which is characterized by unbranched filaments reproducing sexually by conjugation and production of resistant, diploid zygospores. Of some 784 described species of Zygnemataceae, there are 138 species of *Mougeotia* (Hoshaw and McCourt 1988). With the discovery of extensive mats of *Mougeotia* in streams on Signy Island, Antarctica (Hawes 1989), this genus has been recorded from every continent. Within the continental United States, McCourt et al. (1986) conducted an extensive survey of Zygnemataceae along a 31,000
km transect through 35 states. They found *Mougeotia* at 135 of 1000 sites examined. Only 6% of these 135 collections contained zygospores, essential for species identification. *Mougeotia* was not preferentially found in any specific habitat including flowing water, permanent ponds and lakes, or temporary pools. Recently, Sheath and Cole (1992) conducted on a survey of stream macroalgae across the entire North American continent, and found species of *Mougeotia* in streams in every biome from tundra through tropical rainforest except desert chapparal. In the three cases where species were identified, they were found in only one biome. Since *Mougeotia* rarely forms the zygospores which are necessary for species identification, surveys infrequently report species occurrence.

*Mougeotia* has also been reported from a variety of habitats in waters not apparently affected by acid precipitation. Hillebrand (1983) examined the development of floating mats of filamentous algae on several small ponds in nature reserves south of Amsterdam, the Netherlands. The ponds were all eutrophic and bicarbonate rich, with pH in the range of 7.5 to 9.9. *Mougeotia* was mainly observed in the algal mats from mid-June through July when water temperatures were around 20-25°C. *Mougeotia* was also observed in floating mats in coastal dune pools in the Netherlands (Simons 1987). At two ponds out of 25 studied in calcium carbonate-rich areas, *Mougeotia* reached a large biomass in spring. The pH in one of these pools was 7.1 to 7.8. But this alga was much more abundant at another site where the ponds were
calcium carbonate-poor with pH ranging from 5.5 to 8.2. Probably the most extreme environment from which \textit{Mougeotia} has been studied are streams on Signy Island in the maritime Antarctic (Hawes, 1989). These streams flow for 4-5 months from November/December to March/April, and algal biomass peaks about 2-3 months after stream flow begins. The pH of the streams ranges from less than 6 to greater than 7. Hawes (1989) found that \textit{Mougeotia} was generally found in the deeper streams. However, it was most abundant in a stream 15 cm deep with moderate current velocity, where it was attached to boulders and formed trailing strands 90 cm long. \textit{Mougeotia} has also been reported from the rocky littoral zones and streams around the Great Lakes. Parker (1979) found it in streams and nearshore areas along the north shore of Lake Superior, where it was most abundant when water temperatures were highest (11.5-18°C) in September. Sheath et al. (1988) also collected \textit{Mougeotia} from eight sites around Georgian Bay, the North channel, and their drainage basin in soft waters with low conductance and low phosphate levels. On the basis of these observations, species of \textit{Mougeotia} are able to grow in a wide range of environments from eutrophic to oligotrophic, across a range of pH levels and over a fair range of temperatures. Furthermore, it may occur as floating mats or attached strands in streams, but is rarely dominant under natural conditions. In contrast, freshwaters impacted by acid precipitation or experimental acidification are dominated by zygnematacean algae, particularly \textit{Mougeotia}. 
When the first reports of acid precipitation and its effects on lakes began to appear from Scandinavia (Almer et al. 1974, Grahn et al. 1974), the resulting blooms of Zygnemataceae and particularly *Mougeotia* were recognized (Hendry and Wright 1975, Hendry 1976). In North America, whole lake experimental acidification studies began in 1976 in the Experimental Lakes Area (ELA) of northwestern Ontario (Schindler et al. 1985). The pH of Lake 223 was lowered from an original value of 6.8 in 1976 down to 5.64 by 1979. At that point "filamentous algae of the genus *Mougeotia*, which had not been noticed previously, formed highly visible, thick mats in littoral areas" (Schindler et al. 1985). Once formed, the mats remained through the end of the study in 1984. Early in the process of acidifying Lake 223, Muller placed a series of tubular enclosures (2-2.5 m deep by 10 m diameter) into the same lake and acidified separate enclosures to pH 6, 5, and 4 while the lake and control enclosure were at pH 6.5. *Mougeotia* dominated all of the acidified enclosures. When the Plexiglas plates used as substrates were moved from the pH 4 enclosure, where they were covered with *Mougeotia*, to the control enclosure at pH 6.5, the algal filaments disappeared. However, the basis for their disappearance was not determined. The transplantation experiment does, however, clearly indicate that the proliferation of *Mougeotia* was linked to changes brought about by acidification.

Research on whole-lake experimental acidification has continued at ELA, and *Mougeotia* blooms continue to be an important correlate of
the acidification event. Turner et al. (1987) reported on a more extensive set of acidifications involving two additional small lakes, one acidified with sulfuric acid, the other with nitric acid. The results were compared to a lake previously acidified with sulfuric acid (Lake 223) and two neutral lakes as controls. The Zygnemataceae again dominated acidified lakes, with *Mougeotia* the dominant genus in lakes acidified with sulfuric acid. However, in lakes acidified with nitric acid, *Spirogyra, Zygogonium, Zygnema,* and *Mougeotia* were co-dominants. In their report on this experiment, Turner et al. (1987) were the first to suggest that the proliferation of filamentous green algae in acidified lakes might be related to the dynamics of dissolved inorganic carbon (DIC) in these lakes. Below pH 6.5, bicarbonate levels are very low, and at lower pH levels dissolved inorganic carbon levels may be reduced by exhaust to the atmosphere. Turner et al. (1987) suggested that if filamentous Zygnemataceae are better able than other algae to acquire carbon dioxide at low pH, they would gain a competitive advantage with acidification. Subsequently, Howell et al. (1990) measured DIC in two acidified lakes at 0.18-0.20 mg C·l⁻¹, and found even lower DIC levels (0.04-0.05 mg C·l⁻¹) inside aggregates of *Mougeotia* on a sunny day. These workers also investigated the possibility that photosynthesis was DIC limited by performing incubations of the algae at two DIC levels, 0.53 mg C·l⁻¹ and 0.91 mg C·l⁻¹; photosynthesis was 64% greater at the higher DIC level. These results suggest that there is some connection between the proliferation of certain filamentous green algae, pH and DIC.
Recent studies outside the ELA have tended to confirm the connection between low pH, DIC dynamics and *Mougeotia* growth, but have not quantified the interrelationships. In a recent survey of acidified streams in Scotland, Kiaross et al. (1993) found that algal species richness and diversity were highly correlated with pH. Fairchild and Sherman (1993) carried out a similar type of analysis on 12 softwater lakes in Pennsylvania which varied in pH from 4.4 to 8.8. Again, species composition was strongly related to pH. The proportion of green algae was highest in the more acidic lakes, and *Mougeotia* was almost solely responsible for the higher total biovolume and high proportion of green algae in those lakes. Fairchild and Sherman's (1993) analysis allowed them to determine a pH optimum for *Mougeotia* at 5.3 ± 0.8. In addition, Fairchild and Sherman placed nutrient enriched substrates in these lakes to observe species changes in response to nutrient additions. They observed that "the number of C [carbon]-enhanced species was greatest in the most acidic lakes". Thus, as lakes become more acidic, they become more dominated by filamentous green algae, especially *Mougeotia*, and are evidently carbon-limited.

Thus, several significant field studies have demonstrated the association between increased acidification and the proliferation of filamentous green algae, especially the Zygnemataceae, and specifically *Mougeotia*. Furthermore, several studies have indicated that lake acidification leads to a shift from bicarbonate to carbon dioxide as the
primary dissolved carbon species, with low total dissolved inorganic carbon (DIC) levels and carbon limitation of algal growth as a consequence (Turner et al. 1987, Howell et al. 1990, Turner et al. 1991, Fairchild and Sherman 1993). But why the Zygnemataceae and often Mougeotia? To answer this question we must examine the physiology of Mougeotia and specifically evaluate the importance of such factors as light, temperature, pH and carbon metabolism on photosynthesis (as suggested by Turner et al. 1987).

In 1984, the Wisconsin Department of Natural Resources and the Center for Limnology, University of Wisconsin-Madison began a joint long-term study involving experimental acidification of Little Rock Lake, located in north-central Wisconsin. As a seepage lake, it lacks inlets and outflows so its hydrologic budget is dominated by precipitation and evaporation (Watras and Frost 1989). The chemistry of the lake should therefore reflect that of rainwater. Little Rock Lake consists of two basins separated by a narrow isthmus, which facilitated its use in an experimental acidification study, since one basin could be acidified while the other served as a reference (control) basin. The treatment basin was 9.8 hectares in area, with an average depth of 3.9 m; the area of the reference basin was 8.1 hectares and the average depth 3.1 m. In 1984 the basins were separated by installation of a dacron fiber-polyvinyl curtain. Initially, the pH in both basins was 6.1, and over a period of years the pH in the treatment basin was gradually reduced by addition of H₂SO₄ to 4.6, the approximate pH of regional rainwater.
Algal mats of *Mougeotia* were observed in the treatment basin at pH 5.6 and reached their maximum at pH 5.2 in 1987 when they covered most of the littoral zone from 1-2 m, as well as much of the rest of the basin, extending to depths as great as 6 m (Webster et al. 1992). The mat of *Mougeotia* reached its largest area by mid to late summer. These observations suggest that this form of *Mougeotia* preferred warm temperatures and could carry out photosynthesis at fairly low light levels. In the present study, we examined this hypothesis by measuring photosynthetic oxygen production by *Mougeotia* cultured from Little Rock Lake, and exposed to a wide range of temperature and irradiance conditions in a factorial laboratory experiment. Separate papers will report on other aspects of the physiological ecology of Little Rock Lake *Mougeotia*, namely, its photosynthetic response to varying levels of pH and the heavy metals Zn and Al, and aspects of its carbon metabolism.

Materials and Methods

Pieces of the algal mat were collected in the summer of 1989 from the treatment basin near the curtain barrier which divides Little Rock Lake. The pH in the treatment basin at the time of collection was 4.6. Algal mat samples contained a mixture of filamentous algae including, in order of abundance, *Mougeotia*, *Spirogyra*, *Oedogonium*, and several species of cyanobacteria. Single filaments of *Mougeotia* were removed from the mat with forceps and dragged through 0.5% agar to remove epiphytes. Dragged filaments were then placed in small Erlenmeyer
flasks containing a defined medium designated SD11. SD11 is a modification of an algal growth medium named D11, whose composition has been previously described (Graham et al. 1982). Three changes were made to the D11 medium to make SD11 suitable for growth of *Mougeotia*. Glass distilled water was used, trace element solutions B7 and C13 (Gerloff and Fitzgerald 1976) were omitted, and the pH was adjusted to 5.5 with 1M sulfuric acid after aseptic addition of stock vitamins and bicarbonate to the autoclaved medium. Sulfuric acid was used to lower the pH of the medium because this was the acid used to reduce the pH of the Little Rock Lake treatment basin. Subsequent culture work revealed that growth of *Mougeotia* was enhanced by aeration. Stock cultures were therefore maintained with aeration in a culture room at an irradiance of 200-300 µE·m^{-2}·sec^{-1} (PAR) provided by Sylvania Cool White fluorescent lamps (F96T12CW) and 25 W incandescent bulbs, on a 12:12 light-dark cycle, at a temperature of about 20°C.

The isolates were confirmed to belong to the genus *Mougeotia* by using the techniques of Stabenau and Säftel (1989) for induction of gametangia. Putative *Mougeotia* was treated with low levels of nitrate (10^{-3} M) and low light levels (105 µE·m^{-2}·sec^{-1}) in aerated culture flasks at pH 5.5 for eight days. Subsequent production of gametangial branches demonstrated that the isolate was *Mougeotia*, rather than the similar genus *Tennagametum*, which may also occur in lakes as a response to acidification, but which divides off a small conjugating cells
from the larger vegetative cells prior to conjugation between filaments (Bourrelly 1966). However, since zygospores were not obtained, we were unable to determine the species identity of the Little Rock Lake isolate of *Mougeotia*.

Measurements of net photosynthesis and respiration in *Mougeotia* were conducted in a crossed gradients room of a controlled environment facility (the Biotron) located at the University of Wisconsin-Madison. The crossed gradients room is designed to allow simultaneous operation of four separate temperature corridors at right angles to an irradiance gradient. In addition to the crossed gradients room, an additional standard plant growth room was used to investigate the response of *Mougeotia* to temperatures (5°C) lower than could be maintained in the crossed gradients room. During the summer of 1991 all combinations of 5 temperature levels (5, 10, 15, 20, 25°C) and 8 irradiance levels (10, 20, 40, 82, 164, 334, 918, 2338 \(\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}\)) were investigated in a factorial experiment. During summer, 1992, all combinations of four temperature levels (20, 25, 30, 35°C) and the same 8 irradiance levels listed above were investigated. Thus, a total of 56 combinations of irradiance and temperature were studied with measurements at 20 and 25°C duplicated as a between-years control. Irradiances were measured outside and at the level of the algal cultures with a spherical quantum sensor (Biospherical Instruments). Temperatures were continuously monitored from the Biotron control room, and variation was held to ±1°C. The crossed gradients room and
standard plant growth room were programmed for a 16:8 light-dark cycle to replicate mid-summer daylength conditions.

In preparation for Biotron experiments, *Mougeotia* was grown in large test tubes containing 50 ml SD11 medium at pH 5.5, and aerated via cotton plugged glass tubing inserted through rubber stoppers. Culture tubes were inoculated with *Mougeotia* and transferred to the Biotron within a single day. At least three replicate tubes were positioned at each of the 56 irradiance-temperature positions. Gray and black fiberglass screening were used to fine-tune irradiance levels. The algae were preconditioned to ambient conditions for at least one week prior to the start of photosynthesis measurements. Photosynthesis measurements were begun with algae in the 20 degree corridor and at the 164-334 μE·m⁻²·sec⁻¹ conditions because these were the closest to conditions under which stock algal cultures had previously been grown. This allowed algae placed in more extreme irradiance/temperature conditions to equilibrate for a greater length of time.

Net photosynthesis and respiration were measured in terms of oxygen evolution and consumption with an Orion Instruments oxygen electrode with a built-in stirrer. Algal filaments from a single replicate tube were transferred into a cylindrical Plexiglas chamber for photosynthesis measurements. These chambers were divided internally by a perforated Plexiglas plate, and had known volumes
ranging from 337 to 341 ml. Algal filaments were placed on one side of the perforated plate only. Chambers were then filled with fresh SD11 medium which had previously been equilibrated to the corridor (test) temperature, then sealed, without admitting air bubbles, with a Plexiglas plate lightly rimmed with silicone grease. The oxygen electrode was then inserted through a small hole into the chamber half opposite that containing the algal filaments, effectively isolating the unit from outside air. The perforated plate prevented algal filaments from becoming entangled with the electrode stirrer. The chamber and electrode were then placed on a magnetic stirrer covered with a square of Styrofoam to prevent heat transfer from stirrer to chamber. A screen-covered wooden frame was lowered over this assembly to achieve the appropriate level of irradiance. Irradiance was checked with the spherical quantum sensor at the level of the algal filaments. The oxygen electrode was connected to a pH meter calibrated to read directly in mg O₂·l⁻¹ with a precision of 0.01 mg O₂·l⁻¹. Oxygen readings were generally made at 5 min intervals for at least 30 min to obtain a regression line with slope in mg O₂·l⁻¹. At some positions, however, net photosynthetic rates were so high that readings were taken every 2.5 min for only 20 min. To measure respiration rates, the chamber and electrode were covered with a light-tight black plastic bag, and a black cloth slipped under the chamber to exclude light. After 10 to 15 min equilibration, readings of oxygen consumption were taken every 5 min for 30 min to determine the rate of oxygen use.
After each measurement of net photosynthesis and respiration was completed, the algal filaments were collected by filtration onto 14 μm pore size, 47 mm diameter Nuclepore filters (Nuclepore Corp.), then wrapped in labeled foil packets and plunged into liquid nitrogen. Algal filaments were freeze-dried, and the dry weight determined to 0.001g. Dry weights were in the range 0.010 g to 0.060 g. The slopes of the linear regressions for oxygen evolution and consumption were corrected for chamber volumes and dry weight to express net photosynthesis and respiration in \( \text{mg O}_2 \cdot \text{g}^{-1} \cdot \text{hr}^{-1} \). Gross photosynthesis is the sum of net photosynthesis and respiration.

Basic statistical calculations and analyses of variance (ANOVA) were performed on the net photosynthesis and respiration data using MINITAB release 8 for the IBM PC. The software sPlus (Statistical Sciences, Inc.) was used to fit polynomial equations to the data sets to generate response surface diagrams. A cubic polynomial model was fitted for net photosynthesis, and a quadratic polynomial for respiration using temperature and the natural logarithm of irradiance as predictive variables. Since the variance in both net photosynthesis and respiration changed as a function of irradiance and temperature, the polynomial models were fitted by weighting the responses proportional to the reciprocal of the predictive variables.
Results

Field observations indicate that blooms of *Mougeotia* tend to occur in summer months and at a range of depths in the water column. These observations suggest that *Mougeotia* prefers warmer temperatures and may have a relatively high temperature optimum for photosynthesis. They also suggest that the irradiance optimum for photosynthesis by this filamentous green alga may be relatively broad. Our factorial laboratory experiments provided a means of testing these hypotheses.

Laboratory experiments generated data on gross photosynthesis, net photosynthesis, and respiration in *Mougeotia* at 56 combinations of irradiance and temperature (Fig. 1 A-G). In Fig. 1 the bases of the bars rest on the 0.0 mg·O₂·g⁻¹·hr⁻¹ lines. Each bar represents the mean of three replicate measurements. Standard errors of the means were calculated for each position and appear in the figures as black vertical bars atop each bar for photosynthesis or respiration. Respiration rates are plotted as negative values to represent oxygen consumption.

At 5° C *Mougeotia* showed a positive net photosynthetic rate at all levels of irradiance except 10 µE·m⁻²·sec⁻¹, the lowest light level at which readings were made (Fig. 1A). The compensation point, where net photosynthesis is equal to zero, therefore lies between 20 and 10 µE·m⁻²·sec⁻¹ at 5° C. Net photosynthesis averaged 11.1 mg·O₂·g⁻¹·hr⁻¹
at the four highest irradiances. The optimum observed level of irradiance was 164 μE·m⁻²·sec⁻¹ (Table 1), although net photosynthesis was consistently high from 2300 down to 164 μE·m⁻²·sec⁻¹. Respiration rates increased with level of irradiance to which the algal filaments were exposed prior to being placed in the dark (Fig. 1A).

At 10°C, net photosynthesis was positive at all irradiances including 10 μE·m⁻²·sec⁻¹ (Fig. 1B). The compensation point must therefore lie below 10 μE·m⁻²·sec⁻¹. Average net photosynthesis at the four highest irradiances rose somewhat to 16.8 mg·O₂·g⁻¹·hr⁻¹, and the optimum level of irradiance shifted up to about 920 μE·m⁻²·sec⁻¹ (Table 1). Respiration rates again were largest when measured on algae exposed to the highest irradiances.

Net photosynthesis was again positive at all irradiance levels at 15°C, and therefore the compensation point lies below 10 μE·m⁻²·sec⁻¹ (Fig. 1C). Average net photosynthesis at the highest irradiances rose only slightly to 19.5 mg·O₂·g⁻¹·hr⁻¹ (Table 1). The observed optimum irradiance was 2300 μE·m⁻²·sec⁻¹; however, the individual measurements of net photosynthesis at 2300, 920, and 335 μE·m⁻²·sec⁻¹ overlap such that the mean values at each light level are not significantly different. It would be more accurate to state therefore that net photosynthesis plateaus above about 300 μE·m⁻²·sec⁻¹ at 15°C. The largest respiration rates were measured on filaments previously exposed to the highest light levels.
At 20° C net photosynthesis in Mougeotia at the four highest light levels rose to an average of 24.1 mg·O₂·g⁻¹·hr⁻¹ (Table 1). Rather than a distinct optimum irradiance, there is again a broad plateau of net photosynthetic rates above 200 μE·m⁻²·sec⁻¹ (Fig. 1D). Net photosynthesis is again positive at all light levels. Respiration rates remain low except at the higher irradiances.

Net photosynthesis is positive at all light levels at 25° C, but at 20 and 40 μE·m⁻²·sec⁻¹ it is just barely so (Fig. 1E). Above 300 μE·m⁻²·sec⁻¹ the net photosynthetic rates reach a maximum of 40.4 mg·O₂·g⁻¹·hr⁻¹ (Table 1) and maintain that rate through 2300 μE·m⁻²·sec⁻¹. The average values of net photosynthesis of three replicates at 2300, 920, and 335 μE·m⁻²·sec⁻¹ are 41.5, 39.8 and 40.0 mg·O₂·g⁻¹·hr⁻¹ respectively. Thus, the replicate measurements for maximum net photosynthesis at 25°C are highly consistent. The highest observed respiration rates also occur within this plateau region.

At 30° C net photosynthesis is no longer positive at all light levels. It is negative at 10 μE·m⁻²·sec⁻¹ and only weakly positive at 20 and 40 μE·m⁻²·sec⁻¹. As at 5° C, the compensation point lies between 10 and 20 μE·m⁻²·sec⁻¹ (Fig. 1F). The average net photosynthetic rate slides down to 36 mg·O₂·g⁻¹·hr⁻¹ at 30° C, and the optimum irradiance drops down to only 164 μE·m⁻²·sec⁻¹ (Table 1). What is particularly striking about 30°C, however, is the increase in the average respiration rate (Table 1). From 10 to 25° C, the average respiration rate increases only
slowly with temperature, but at 30°C average respiration almost doubles over the rate at 25°C. Respiration rates are highest for the filaments exposed to the highest light levels, but there are also some substantial respiration rates at intermediate light levels (Fig. 1F), where Mougeotia has clearly passed its optimal temperature.

At 35°C Mougeotia appears to be dying out. Net photosynthesis is negative at 900, 20, and 10 µE·m⁻²·sec⁻¹ (Fig. 1G). The small positive value for net photosynthesis at 2000 µE·m⁻²·sec⁻¹ is based on only one surviving replicate. Since the other two replicates died, it is likely that an average net photosynthetic rate for this irradiance level would be negative. Net photosynthesis is positive from 300 down to 40 µE·m⁻²·sec⁻¹ and averages 6.5 mg·O₂·g⁻¹·hr⁻¹ (Table 1). The optimum irradiance lies all the way down at 40 µE·m⁻²·sec⁻¹. Average respiration again rises sharply to a maximum of 12.8 mg·O₂·g⁻¹·hr⁻¹ (Table 1). Apparently, Mougeotia can only survive such an extreme temperature if light levels are fairly low.

The optimum temperature for this Little Rock Lake isolate of Mougeotia is 25°C. Higher than optimum temperatures result in a marked increase in respiration rates (Table 1). Optimum irradiance rises from 164 to 920 µE·m⁻²·sec⁻¹ when temperatures rise from 5 to 10°C. From 15 to 25°C, however, net photosynthesis essentially saturates by 300 µE·m⁻²·sec⁻¹ and then maintains a nearly constant plateau level on up to 2300 µE·m⁻²·sec⁻¹. The compensation point is
consistently low, generally below 10 μE·m⁻²·sec⁻¹ and only reaches as high as 10 mg·O₂·g⁻¹·hr⁻¹ between 20 and 40 μE·m⁻²·sec⁻¹ at 35°C. Thus, *Mougeotia* can generate positive net photosynthesis at very low light levels (10-20 μE·m⁻²·sec⁻¹) and saturate at moderate light levels (about 300 μE·m⁻²·sec⁻¹) or 14% of maximum irradiance tested. Both of these observations are consistent with the pattern of *Mougeotia* mat formation in nature, not on the water surface, but in the benthos of relatively clear, acidic northern lakes.

Analysis of variance (ANOVA) on the net photosynthesis data indicates that temperature, irradiance and their interaction are all significant (p<0.001) (Table 2). The original data matrix was missing three values and these were filled in by cell averages for the ANOVA averages. Overall, respiration rates, measured after net photosynthesis readings, increased with temperature from 10 to 35°C. The average respiration rate at 5°C was actually higher than that at 10 or 15°C (Table 1). Respiration actually increased very slowly from 10 to 25°C, but rose sharply at 30°C beyond optimum temperature for *Mougeotia*. Analysis of variance on respiration rates shows that temperature, irradiance, and their interaction are significant (p<0.001), but the F-ratio for temperature (55.7) is the largest (Table 3). The fact that light level has a significant effect on respiration means that the level of light to which the algal filaments are exposed for net photosynthesis has a significant effect on the respiration rate of these same filaments even after they are placed in the dark for 5-10 minutes before respiration rates are
measured. Respiration rates were also measured for algae after they had been in prolonged darkness (several hours). These are referred to as "dark respiration rates" to distinguish them from respiration measurements made shortly after photosynthesis measurements. Dark respiration rates were measured for three replicate samples of Mougeotia for each temperature level from 5 to 25°C. Since we are interested in the effects of light level and not temperature on respiration, these 15 values were pooled to form one set of dark respiration rates. We also pooled the respiration rates measured after net photosynthesis for each light level from 5 to 25°C. This process generated about 15 values for respiration at each light level to compare statistically to the 15 dark respiration measurements. The average respiration rates measured after net photosynthesis were significantly greater than the average dark respiration rate at 2300 µE·m⁻²·sec⁻¹ (p<0.001), 920 µE·m⁻²·sec⁻¹ (p<0.005), 335 µE·m⁻²·sec⁻¹ (p<0.025) and 164 µE·m⁻²·sec⁻¹ (p<0.025) (Table 4). At all other light levels the difference of means was not significant. This then is the basis of the effect of light level on respiration rates; the four highest light levels enhance the respiration rates significantly above the dark or basal respiration rate.

Photosynthesis and respiration data can be presented graphically as response surfaces in which the vertical axis is net photosynthesis or respiration and the horizontal axes are irradiance and temperature. In Figure 2 the observed values of net photosynthesis were used to
generate an interpolated response surface. In Figure 3, however, the response surface is a least-squares fit of a third-order polynomial to the same data. The two surfaces are clearly similar, but the polynomial surface is far smoother and of more use in predicting intermediate values. The polynomial model surface predicts that the physiological optimum for Mougeotia is 23°C and 1300 μE·m⁻²·sec⁻¹, while the observed optimum was at 25°C and 2300 μE·m⁻²·sec⁻¹. A response surface for the actual respiration data is shown in Figure 4. Even for the actual data the response surface is fairly smooth, at least at temperatures below 25°C. Figure 11 represents the response surface generated by a second-order polynomial fit by least-squares to the data set. The orientation of both figures is such that the greatest values of respiration are furthest from the viewer, back at 35°C and 2300 μE·m⁻²·sec⁻¹.

Discussion

Many algae have been examined for the effects of irradiance and temperature on growth. Among phytoplankton, growth effects of irradiance and temperature have been studied in Thalassiosira nordenskioldii (Durban 1974), Cryptomonas ovata (Cloern 1977), and Peridinium cinctum (Lindstrom 1984). However, phytoplankton are usually studied in terms of cell numbers rather than photosynthetic rates. It is therefore difficult to compare these types of studies to the present results with Mougeotia. Freshwater filamentous algae have not been examined as intensively as phytoplankton despite their abundance
in littoral areas where they may make a significant contribution to productivity.

One aspect of algal mat dynamics which we are presently unable to address is the potential role of grazers. Power et al. (1985) pointed out the importance of grazing minnows in the removal of *Spirogyra* in Oklahoma streams, but there are no studies that demonstrate a similar effect upon *Mougeotia*. Oakland (1980) stated that filamentous green algae began to increase when the pH was around 6.0, at the time when gastropods began to disappear from acid-impacted Norwegian lakes. What is needed is a field study in which substrates bearing luxuriant growths of *Mougeotia* from pH 5 systems are transplanted to pH 7 waters, such that potential grazers are exposed to part of the algal biomass and excluded from control portions. Exposed algal biomass could be monitored for numbers of mollusks or other invertebrates. An experiment of this sort might answer the question raised by Turner et al. (1991) when they asked if filamentous green algae might bloom under acidic conditions because they are no longer suppressed by grazers that would normally be present in neutral systems. An alternate hypothesis is that the apparently acidophilic zygnematacean green algae are competitively more successful in low DIC waters as compared to higher pH systems, where bicarbonate is more important than CO2 as an inorganic carbon source.
Collins and Boylen (1982) found optimal irradiance and temperature conditions for photosynthesis in the filamentous blue-green *Anabaena variabilis* Kutz. to be 35°C and 564 μE·m⁻²·sec⁻¹. The maximum observed rate of net photosynthesis was 2 g C·g⁻¹·day⁻¹ (Collins and Boylen 1982). For the purposes of comparison with photosynthetic rates expressed in terms of O₂ production, their net photosynthetic rate can be expressed in terms of O₂ production by assuming a photosynthetic quotient of one (1 mmole or 12 mg C fixed per 1 mole or 32 mg O₂ evolved) (Auer and Canale 1982). Such calculations reveal that *Anabaena variabilis* has a prodigious rate of net photosynthesis (220 mg O₂·g⁻¹·hr⁻¹), a fact which no doubt contributes to its success as a bloom-forming nuisance alga. It would be instructive to have comparable data for other major nuisance bloom forming cyanobacteria, to determine if they have similarly high rates of net photosynthesis.

Among eukaryotic green algae, the effects of irradiance and temperature on photosynthesis and respiration have been reported for *Cladophora glomerata* (Graham et al. 1982), *Ulothrix zonata* (Graham et al. 1985), *Pithophora oedogonia* (Spencer et al. 1985), and a *Spirogyra* species isolated from a nuisance bloom in a eutrophic lake (Graham et al. manuscript submitted). Optimal conditions for *Cladophora glomerata* were found to be 15°C and 300 μE·m⁻²·sec⁻¹, but even under optimal conditions maximum net photosynthesis was only 7.7 mg O₂·g⁻¹·hr⁻¹. *Pithophora oedogonia*, which is closely related to *Cladophora*
(both are members of the Cladophorales), exhibited a similarly low maximum rate of net photosynthesis (9.7 mg O$_2$·g$^{-1}$·hr$^{-1}$), but very different optimal temperature and irradiance conditions (26 °C. and 970 µE·m$^{-2}$·sec$^{-1}$). Ulothrix zonata (a member of the Ulvophyceae along with Cladophora and Pithophora), which has an optimum for growth at only 5° C, and 1100 µE·m$^{-2}$·sec$^{-1}$, fares only slightly better with a maximum net photosynthetic rate of 16.8 mg O$_2$·g$^{-1}$·hr$^{-1}$. These three filamentous green algae, which all form noticeable growths in eutrophic waters therefore have on average rather low rates of net photosynthesis, averaging only 11.4 mg O$_2$·g$^{-1}$·hr$^{-1}$ as a group.

The low average maximum photosynthetic rate observed for freshwater members of the Ulvophyceae contrasts sharply with what thus far appears to be the case for members of the Zygnemataceae (Charophyceae). Spirogyra had a maximum net photosynthetic rate of 72.2 mg O$_2$·g$^{-1}$·hr$^{-1}$, which occurred at 1500 µE·m$^{-2}$·sec$^{-1}$ and 25°C (Graham et al. manuscript submitted). The present report demonstrates that the closely related Mougeotia has a maximum net photosynthetic rate of 40.5 mg O$_2$·g$^{-1}$·hr$^{-1}$ at 25°C and over the irradiance range 330-2330 µE·m$^{-2}$·sec$^{-1}$. Although this is lower than the rate observed for the Spirogyra isolate, it is considerably higher than the average for the ulvophytes described above. In view of these observations it would be useful to know if other zygnematacean algae exhibit such high photosynthetic rates. It is possible that this is one element of an explanation for their ability to form blooms.
Response surfaces are a convenient method for visualizing the interaction between irradiance and temperature on net photosynthesis and respiration, and comparing photosynthetic optima and productivity among taxa. The polynomial equations for such surfaces have been used in growth models along with models of nutrient uptake dynamics (Canale and Auer 1982). Most models, based on observed data, describe photosynthesis as a function of a single factor such as light (Steel 1978) or temperature (Shugart et al. 1974). In a few cases these models have included both irradiance and temperature (Lester et al 1974, Collins and Boylen 1982). Such combined empirical models can become mathematically cumbersome and often cannot generate negative values of net photosynthesis. When oxygen production is used as a measure of net photosynthesis, negative values can occur at extremes of temperature and/or irradiance. Polynomial equations can represent these negative values, but they are poor descriptors when values change rapidly or in a non continuous way across temperatures.

The polynomial equation that we have used for respiration in this and previous analyses included effects of both irradiance and temperature. Most empirical models assume that respiration varies only with temperature (Talling 1957, Steel 1978). Ganf (1974) and Gibson (1975) have shown that when algae are exposed to light before placing them in darkness, the light treatment stimulates dark oxygen uptake. Yallop (1982) found that respiration rates were elevated by 64-
113 % over basal dark respiration rates when algae were incubated in the light just prior to measuring respiration. When this investigator added the photosynthetic inhibitor dichlorophenyl-dimethylurea (DCMU) to culture media, the differences between basal dark respiration and respiration measured after light exposure disappeared (Yallop 1982). Light-enhanced respiration has been reported previously to occur in the green algae *Cladophora glomerata* (Graham et al. 1982), *Ulothrix zonata* (Graham et al. 1985), *Pithophora oedogonia* (Spencer et al. 1985), and *Spirogyra* (Graham et al. manuscript submitted). In contrast, light-enhanced respiration was not observed to occur in the filamentous red alga *Bangia atropurpurea* (Graham and Graham 1987). In *Mougeotia*, the average respiration rate was increased by 272% over the mean basal dark respiration rate when the alga was exposed to maximum irradiance (2330 μE m⁻² sec⁻¹) prior to measuring respiratory O₂ consumption (this report). The physiological basis for light enhancement of respiration rates is not known. However, since irradiance can have such a significant effect on respiration, it should be considered in any effort to model the growth of filamentous green algae.

Field observations (Webster et al. 1992) suggested that the *Mougeotia* isolate from Little Rock Lake might show a preference for warmer summer temperatures and also be able to carry out photosynthesis at rather low light levels. Our factorial laboratory measurements of photosynthetic rates support these hypotheses.
Mougeotia exhibited a temperature optimum at 25°C, consistent with observations of mat development in mid to late summer in Little Rock Lake. From 10-25°C, the light compensation point was less than 10 µE·m⁻²·sec⁻¹; thus, Mougeotia can produce at least some positive net photosynthesis even at very low light levels (less than one two-hundredth of full sunlight). It reaches maximum net photosynthesis at 200-300 µE·m⁻²·sec⁻¹ and maintains these rates up to and including 2300 µE·m⁻²·sec⁻¹ without evidence of photoinhibition. Such a net photosynthetic capacity is consistent with an alga which can cover most of a littoral zone from 0-2 meters depth and much of the rest of the basin down to 6 m. In the ELA studies, mats of filamentous green algae were confined to a shallow littoral band, perhaps because of a requirement for higher levels of irradiance or turbulent mixing as a source of DIC. In Little Rock Lake carbon levels may have been adequate to support mat development to a greater degree than observed in the ELA lakes.

This paper has addressed the role of temperature and light in the growth of Mougeotia, in order to increase our understanding of how these two physical parameters may affect mat development in acidified freshwater systems. We found that Mougeotia's temperature and irradiance optima explain aspects of the distribution of Mougeotia in acidified lakes. However, irradiance and temperature did not vary substantially between the treatment and reference basins of Little Rock Lake, whereas the massive Mougeotia mats occurred only in the
acidified basin. Thus, our light and temperature studies do not explain the magnitude of the *Mougeotia* growths that occur at low pH. Acidification of freshwater results in various changes in lakes that could potentially affect the magnitude of algal growth. These include alterations in the form and amount of DIC and DOC, release of various heavy metals, and changes in the biotic community composition. In a companion paper, we address the effects of pH and selected heavy metals on photosynthesis of *Mougeotia*, in an effort to understand how these factors might relate to mat development in acidified lakes. Additional papers focus upon aspects of *Mougeotia*’s carbon metabolism, including the potential for utilization of dissolved organic carbon for growth. Graham et al. (1994) demonstrated that glucose uptake and utilization can dramatically enhance growth of the charophyte *Coleochaete orbicularis* under DIC-limited conditions. This work, together with some semiquantitative measures suggesting sugar enhancement of growth of other charophycean algae (Mattox and Bold (1962), suggests that various charophytes (including members of the Zygnematales) may be able to import various forms of DOC to supplement limiting levels of DIC during growth. It is possible that DOC utilization could play an important role in explaining the occurrence of extensive growths of zygnematacean algae in acidified aquatic systems (where DIC is limiting).


TABLE 1. Average values of net photosynthesis at the three or four highest irradiances (5-30°C) and four intermediate irradiances (35°C), average respiration rates, and the optimum irradiance for Mougeotia at each temperature.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Average Net Photosynthesis (mg O₂·g⁻¹·hr⁻¹)</th>
<th>Average Respiration (mg O₂·g⁻¹·hr⁻¹)</th>
<th>Optimum Irradiance (μE·m⁻²·sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>11.1 (4)</td>
<td>4.5</td>
<td>164</td>
</tr>
<tr>
<td>10</td>
<td>16.8 (4)</td>
<td>3.3</td>
<td>920</td>
</tr>
<tr>
<td>15</td>
<td>19.5 (3)</td>
<td>4.0</td>
<td>335-2300</td>
</tr>
<tr>
<td>20</td>
<td>24.0 (4)</td>
<td>4.9</td>
<td>335-2300</td>
</tr>
<tr>
<td>25</td>
<td>40.5 (3)</td>
<td>4.9</td>
<td>335-2300</td>
</tr>
<tr>
<td>30</td>
<td>26.0 (4)</td>
<td>9.5</td>
<td>164</td>
</tr>
<tr>
<td>35</td>
<td>6.5 (4)</td>
<td>12.8</td>
<td>40</td>
</tr>
</tbody>
</table>
TABLE 2. ANOVA of net photosynthesis as a function of temperature and irradiance.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
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<td>7280.58</td>
<td>1213.43</td>
<td>48.86</td>
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</tr>
<tr>
<td>Light</td>
<td>7</td>
<td>10983.95</td>
<td>1568.99</td>
<td>63.18</td>
<td>0.000</td>
</tr>
<tr>
<td>Interaction</td>
<td>42</td>
<td>5080.22</td>
<td>120.96</td>
<td>4.87</td>
<td>0.000</td>
</tr>
<tr>
<td>Error</td>
<td>112</td>
<td>2781.37</td>
<td>24.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>167</td>
<td>26125.12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 3. ANOVA of respiration rates as a function of temperature and irradiance.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>6</td>
<td>1920.898</td>
<td>320.150</td>
<td>55.66</td>
<td>0.000</td>
</tr>
<tr>
<td>Light</td>
<td>7</td>
<td>744.243</td>
<td>106.320</td>
<td>18.48</td>
<td>0.000</td>
</tr>
<tr>
<td>Interaction</td>
<td>42</td>
<td>700.667</td>
<td>16.683</td>
<td>2.90</td>
<td>0.000</td>
</tr>
<tr>
<td>Error</td>
<td>112</td>
<td>644.268</td>
<td>5.752</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>167</td>
<td>4010.075</td>
<td></td>
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</tr>
</tbody>
</table>
TABLE 4. Statistical comparison of the mean dark respiration rate to the mean respiration rate at each light level with values pooled across the temperatures 5 to 25°C. *n.s.* denotes the difference of means is not significant at the 0.05 level (t-test).

<table>
<thead>
<tr>
<th>Irradiance Level (μE·m⁻²·sec⁻¹)</th>
<th>Mean Respiration Rate (mg O₂·g⁻¹·hr⁻¹)</th>
<th>Mean Dark Respiration Rate (mg O₂·g⁻¹·hr⁻¹)</th>
<th>Significance of difference in means</th>
</tr>
</thead>
<tbody>
<tr>
<td>2300</td>
<td>7.26 (n=15)</td>
<td>2.67 (n=14)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>920</td>
<td>6.29 (n=15)</td>
<td>2.67</td>
<td>p&lt;0.005</td>
</tr>
<tr>
<td>335</td>
<td>5.06 (n=15)</td>
<td>2.67</td>
<td>p&lt;0.025</td>
</tr>
<tr>
<td>164</td>
<td>4.07 (n=15)</td>
<td>2.67</td>
<td>p&lt;0.025</td>
</tr>
<tr>
<td>80</td>
<td>3.33 (n=14)</td>
<td>2.67</td>
<td>n.s.*</td>
</tr>
<tr>
<td>40</td>
<td>2.56 (n=15)</td>
<td>2.67</td>
<td>n.s.</td>
</tr>
<tr>
<td>20</td>
<td>2.05 (n=15)</td>
<td>2.67</td>
<td>n.s.</td>
</tr>
<tr>
<td>10</td>
<td>1.75 (n=15)</td>
<td>2.67</td>
<td>n.s.</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Gross photosynthesis, net photosynthesis and respiration in \textit{Mougeotia} as functions of temperature and irradiance. A. 5°C B. 10°C C. 15°C D. 20°C E. 25°C F. 30°C G. 35°C. Each bar is the mean of three replicates. Standard errors of the means appear as vertical lines on each bar.

Figure 2. Response surface for net photosynthesis in \textit{Mougeotia}. The grid of lines connects the actual observed values.

Figure 3. Response surface for net photosynthesis in \textit{Mougeotia}. The surface represents a least-squares fit of the third-order polynomial:

\[ 22.6889 - 13.6338 \times L + 3.6949 \times L^2 - 0.2945 \times L^3 -3.1729 \times T + 0.1696 \times T^2 -0.002926 \times T^3 + 0.5138 \times T \times L - 0.0108 \times T^2 \times L. \]

Figure 4. Response surface for respiration in \textit{Mougeotia}. The grid lines connect the actual observed data points.

Figure 5. Response surface for respiration in \textit{Mougeotia}. The surface represents a least-squares fit of the second-order polynomial: 

\[ -4.5344 + 0.3681 \times L - 0.1483 \times L^2 + 0.5554 \times T -0.02102 \times T^2. \]
Fig. 1

Light Intensity (µE • m⁻² • sec⁻¹)
Fig. 2

Net Photosynthesis Rate (mgO₂·gr⁻¹·hr⁻¹)

In(Light Intensity) (μE·m⁻²·s⁻¹)

Temperature (°C)
Net Photosynthesis Rate (mg CO₂·gr⁻¹·hr⁻¹)

In(Light Intensity) (µE·m⁻²·s⁻¹)

Temperature (°C)

Fig. 3
Fig. 5
Chapter 2

Effects of pH and selected metals on growth of the filamentous green alga *Mougeotia* (Zygnematales, Charophyceae) under acidic conditions.
Abstract

When acid precipitation begins to impact freshwater systems, littoral blooms of the filamentous green alga *Mougeotia* (Zygnematales, Charophyceae) frequently develop. Little is known about the physiological ecology of the alga to suggest why it responds so conspicuously to acidification. Field observations of its development in Little Rock Lake, an experimentally acidified seepage lake in north central Wisconsin, indicated that *Mougeotia* might have an optimum pH for growth around 5.0. Since a number of metals increase in concentration in waters undergoing acidification, it was also proposed that *Mougeotia* might be particularly tolerant of high concentrations of metals. To test these hypotheses we grew *Mougeotia* in culture at 8 pH levels, from pH 3 through 10, and measured net photosynthesis and respiration at each level. We also grew the alga over a range of zinc concentrations from 2 to 40 µg·l⁻¹ and aluminum from 10 to 300 µg·l⁻¹. *Mougeotia* was found to grow over a broad range of pH levels from 3 to 9 with an optimum, not near 5, but rather at 8, in contrast to expectation. It was, however, tolerant to levels of zinc and aluminum considerably higher than the elevated levels to which it had been exposed in the acidified basin of Little Rock Lake.

Key words: acidification, aluminum, filamentous algae, *Mougeotia*, pH, photosynthesis, zinc, Zygnemataceae.
Introduction

The acidification of freshwater aquatic systems by acid precipitation or acidic surface discharges has become a worldwide environmental problem. An early and conspicuous indication that acidification is impacting aquatic systems is the development of extensive growths of filamentous green algae of the family Zygnemataceae. The genus Mougeotia is one of the most commonly observed bloom-forming green algae in acid waters of Europe and North America, particularly in lakes subjected to experimental acidification (Schindler et al. 1985, Turner et al. 1987, Webster et al. 1992). Despite this fact, there is no data to suggest why Mougeotia, rather than some other filamentous alga, should so frequently form extensive blooms in acidified waters. In a previous paper (Arancibia et al. 1994), we examined the roles of irradiance and temperature in the physiological ecology of Mougeotia isolated from the acidified basin of an experimental seepage lake, Little Rock Lake, in north-central Wisconsin. We concluded that the photosynthetic response of Mougeotia to temperature and irradiance explained certain aspects of its seasonal growth and distribution in acidified lakes, but did not by itself explain the prolific growth of this alga at low pH. In the present paper we address the effects of pH and elevated levels of the trace metals aluminum and zinc on growth of Mougeotia.
We have used two approaches to examine pH and its effects on *Mougeotia*. Several researchers have surveyed the algal flora of streams, ponds or lakes and noted the pH of the water. We have gathered those in which *Mougeotia* was found, in order to obtain some idea of the range in pH conditions under which *Mougeotia* grows in natural and experimentally acidified freshwaters. Secondly, we have directly tested the effect of various pH levels on growth of *Mougeotia* by measuring net photosynthesis and respiration under constant light and temperature, but at a range of pH levels. On the basis of the extensive growths formed by *Mougeotia* in acidified freshwaters, we hypothesized that the pH at which net photosynthesis is optimal is around 5 to 6.

We have used the same two approaches to examine the effects of aluminum and zinc on *Mougeotia*. There are a small number of studies in which researchers describe both the algal flora of a freshwater aquatic system and measurements of aluminum and/or zinc levels (Say and Whitton 1980, King et al. 1992, Sheath et al. 1982, Havens and Heath 1990, Kinross et al. 1993, Whitton 1970, Trollope and Evans 1976, Deniseger 1986). We have selected those studies in which *Mougeotia* was noted together with measured levels of aluminum and/or zinc to obtain a sample of the ranges of these metals under which *Mougeotia* can grow in various aquatic systems. In a very few cases, *Mougeotia* was observed in ponds or streams under certain conditions of pH or metals, but was absent from lower reaches of the same stream or nearby ponds with different pH and/or metal contents (Sheath et al. 1982,
Deniseger 1986). In these few instances it is possible to say that Mougeotia was absent because of the change in conditions because Mougeotia was definitely known to be present in the freshwater systems or in close proximity. The second method we used was to test growth of Mougeotia over a range of concentrations of aluminum and zinc in defined medium under constant conditions of temperature and light and the same initial pH. Nonacidified lakes, defined as those with pH from 6.0 to 7.8, generally have levels of aluminum from 10 to 65 μg·l⁻¹, and of zinc from 1 to 30 μg·l⁻¹ (Stokes 1983). Acidified lakes, defined as those having pH values in the range 4.1 to 5.3, have been reported to have substantially higher levels of both metals, and a wider range of levels than in non-acidified lakes. Aluminum levels had been reported to vary from 50 to 600 μg·l⁻¹, and zinc from as little as 3 to 122 μg·l⁻¹ in acidified lakes (Stokes 1983). Our isolate of Mougeotia came from the acidified basin of Little Rock Lake, a small seepage lake in north-central Wisconsin. In the acidified basin at pH 4.7, measured concentrations of zinc were only about 6 μg·l⁻¹, and of aluminum about 35 μg·l⁻¹ (King et al. 1992). These metal levels are low in comparison to those of other acidified lakes (Wright et al. 1980). We would expect that our Mougeotia isolate should be tolerant to these levels, since it formed extensive blooms under these conditions. We therefore tested levels ranging up to eight times greater than present in the basin of origin of our isolate in order to determine if it was tolerant of conditions more severe than it had yet encountered.
Materials and Methods

In the summer of 1989, pieces of algal mat were collected from the treatment basin of Little Rock Lake near the curtain barrier. At the time of collection, the pH in the treatment basin had been lowered to 4.6. The algal mat samples contained a mixture of filamentous algae including, in order of abundance, *Mougeotia*, *Spirogyra*, *Oedogonium*, and several species of cyanobacteria. Single filaments of *Mougeotia* were removed from the mat and dragged through 0.5% agar to remove epiphytes. Dragged filaments were placed in small Erlenmeyer flasks containing the defined medium SD11. This medium is a modification of an algal medium designated D11, whose composition has been described previously (Graham et al. 1982). Three changes were made to D11 to make an SD11 suitable for *Mougeotia* growth. Glass distilled water was used, trace element solutions B7 and C13 (Gerloff and Fitzgerald 1976) were omitted, and the pH was adjusted to 5.5 with 1M sulfuric acid after aseptic addition of stock vitamins and bicarbonate to autoclaved medium. Sulfuric acid was used to reduce medium pH because it was also the acid used to reduce the pH of the treatment basin of Little Rock Lake. Subsequently, it was determined that growth of *Mougeotia* in culture was enhanced by aeration. Stock cultures were therefore maintained with aeration in a culture room at an irradiance of 200-300 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{sec}^{-1} \) (PAR) provided by 25 W incandescent bulbs and Sylvania Cool White fluorescent lamps (F96T12CW) on a 12:12 light-dark cycle at a temperature of about 20°C.
Isolates were confirmed to belong to the genus *Mougeotia* by using the techniques of Stabenau and Säftel (1989) for induction of gametangia. Putative *Mougeotia* was treated with low levels of nitrate \((10^{-3} \text{ M})\) and low light levels \((105 \, \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1})\) in aerated culture flasks at pH 5.5 for eight days. Subsequent production of gametangial branches demonstrated that the isolate was *Mougeotia*, rather than the similar genus *Temenogametum*, which may also occur in lakes in response to acidification. *Temenogametum* does not produce elongate gametangial branches continuous with the vegetative cells, but instead divides off a small conjugating cell from vegetative cells. These conjugating cells then fuse without forming gametangial branches (Bourrelly 1966). Unfortunately, we were unable to obtain zygospores of *Mougeotia*, and therefore could not determine the species of the Little Rock Lake isolate.

To determine the growth response of *Mougeotia* as a function of pH, we measured net photosynthesis and respiration in *Mougeotia* from pH 3 to pH 10 under conditions of constant irradiance and temperature in a culture room. SD11 medium was prepared and 30 ml aliquots dispersed into large straight-walled test tubes. Three replicate tubes were set up for each pH level: 3, 4, 5, 6, 7, 8, 9, and 10 for a total of 24 tubes. The pH levels were achieved by addition of 0.5 M \(\text{H}_2\text{SO}_4\) or 1 M \(\text{NaOH}\). Small clumps of *Mougeotia* filaments were inoculated into the tubes, which were then stoppered and allowed to acclimate for three days without aeration at 20°C and 350 \(\mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}\). Irradiance levels
were checked with a spherical quantum sensor (Biospherical Instruments). At the end of the acclimation period, net photosynthesis and respiration were measured in terms of oxygen evolution and consumption with an Orion Instruments oxygen electrode equipped with a built-in stirrer. Algal filaments from a single pH replicate tube were transferred into a cylindrical Plexiglas chamber for photosynthesis measurements. These chambers were divided internally by a perforated Plexiglas plate and had known volumes ranging from 337 to 341 ml. Algal filaments were placed on one side of the perforated plate only. Chambers were then filled with fresh SD11 medium which had previously been equilibrated to the culture room temperature and pH adjusted to match that of the tube. The pH was checked and adjusted just prior to O₂ readings and rechecked and recorded immediately after readings. Chambers were sealed, without admitting air bubbles, with a thin Plexiglas plate lightly rimmed with silicone grease. The oxygen electrode was then inserted through a small hole into the chamber half opposite that containing the algal filaments, effectively isolating the unit from outside air. The internal perforated plate prevented algal filaments from becoming entangled with the built-in electrode stirrer. The chamber and electrode were then placed on a magnetic stirrer covered with a square of Styrofoam to prevent heat transfer from stirrer to chamber. Irradiance was checked at the level of the algal filaments with the spherical quantum sensor. The oxygen electrode was connected to a pH meter calibrated to read directly in mg O₂·l⁻¹ with a precision of 0.01 mg O₂·l⁻¹. Oxygen readings were made at 5 min
intervals for at least 30 min to obtain a regression line with slope in mg O$_2$·l$^{-1}$·min$^{-1}$. To measure respiration rates, the chamber and electrode were covered with a light-tight black plastic bag, and a black cloth slipped under the chamber to exclude light. After 10 to 15 min equilibration, readings of oxygen consumption were taken every 5 min for 30 min to determine the rate of oxygen use.

After each measurement of net photosynthesis and respiration was completed, the *Mougeotia* filaments were collected by filtration onto 14 μm pore size, 47 mm diameter Nuclepore filters (Nuclepore Corp.), then wrapped in labeled foil packets and plunged into liquid nitrogen. Algal filaments were freeze-dried and the dry weights determined to 0.001 g. Dry weights were in the range 0.012 g to 0.066 g. The slopes of the linear regressions for oxygen evolution and consumption were corrected for chamber volumes and dry weights to express net photosynthesis and respiration in mg O$_2$·g$^{-1}$·h$^{-1}$. Gross photosynthesis is the sum of net photosynthesis and respiration.

The growth response of *Mougeotia* to varying levels of the metals aluminum and zinc was examined by growing algal filaments in 20 ml volumes of SD11 in 50 ml aerated Erlenmeyer flasks under constant conditions of light (250 μE·m$^{-2}$·sec$^{-1}$) and temperature (20°C). Initial pH was adjusted to pH 4.7. Three replicate flasks were set up for each zinc concentration tested. Zinc levels examined were 2, 4, 6, 8, 10, 20, 30, and 40 μgl$^{-1}$, prepared from a 0.01M stock of ZnSO$_4$·7H$_2$O.
Aluminum levels were 10, 20, 30, 40, 50, 60, 100, 200, and 300 μg l⁻¹ prepared from a stock solution of 0.01M Al₂(SO₄)₃·18H₂O, with three replicates for each concentration. There were five control cultures. After three weeks of growth in the culture room, the algal filaments were collected onto 25 mm, 0.5 μm Nucleopore filters (Nuclepore Corp.), extracted in 2 ml of cold 100% methanol, and 1 ml spun down in a microfuge to remove cellular debris. The resulting extracted chlorophyll was transferred to a 1 cm cuvette for reading of chlorophyll a on a Beckman DU-600 spectrophotometer. Absorbance was read at wavelengths of 665 μm, 645 μm, and 630 μm, and readings were converted to chlorophyll a with the formula: chl a = 11.6 D₆₆₅ - 1.31 D₆₄₅ - 0.14 D₆₃₀ from Hansmann (1973).

Results and Discussion

Field data from Little Rock Lake indicated that although Mougeotia has been present in small amounts in this seepage lake before acidification and in the reference basin at pH 6.1 during experimental manipulation, it only began to form massive blooms at pH 5.6 and reached its greatest coverage at pH 5.2. On the basis of these observations we might expect Mougeotia to have an optimum pH for net photosynthesis in the range of 5.6 to 5.2. Although both zinc and aluminum were elevated in the acidified basin above concentrations in the reference basin, these levels were still quite low by comparison to other reported acidified lakes. There is no particular reason therefore,
to expect our isolate from Little Rock Lake to be especially tolerant of ten-fold higher levels of these metals. Our experiments on pH and metal concentrations provide a test of these hypotheses.

Our survey of published algal flora studies turned up a number of examples where Mougeotia has been noted to occur in both natural waters and acidified waters (Table 1). Both benthic and planktonic filaments of Mougeotia were observed in three tundra ponds at pH 8.1 to 8.2 in Canada (Sheath et al. 1982). At this pH, bicarbonate would be the most prevalent inorganic carbon source, and the ponds had 24-27 mg·l⁻¹ of inorganic carbon plus 21-37 mg·l⁻¹ organic carbon. In ponds in the Netherlands, Mougeotia occurred as floating mats associated with other filamentous green algae such as Spirogyra and Oedogonium (Hillebrand 1983, Simons 1987). In the remaining three examples of natural waters, Mougeotia was attached to substrates in waters of relatively low nutrients. In the group of 12 lakes in northeastern Pennsylvania, Mougeotia was much reduced in abundance at pH 6.4, and absent from five lakes of pH 6.8 to 8.8. The observed optimum pH for Mougeotia was 5.3 ± 0.8 (Fairchild and Sherman 1993).

In acidified waters, the abundance of Mougeotia increased dramatically around pH 5.6 in Experimental Lake 223 (Schindler et al. 1985) and in Little Rock Lake (Webster et al. 1992). Turner et al. (1987), however, noted that attached filamentous green algae began to proliferate in the upper littoral zone at around pH 6. Mougeotia was
common at every stream site in the Trossachs region of Scotland, an area heavily impacted by acid precipitation (Kinross et al. 1993). Maximum biomass of *Mougeotia* occurred at a pH of 5.2 in Little Rock Lake (Webster et al. 1992), a value very close to the optimum of 5.3 ± 0.8 derived from a study of natural softwater lakes by Fairchild and Sherman (1993). Together, these observations indicate that *Mougeotia* can occur under neutral to alkaline pH conditions but is rarely dominant. Only under acid conditions, with the critical pH around 5.6, does *Mougeotia* become conspicuously abundant. In acidified waters it apparently reaches its maximum abundance around pH 5.2-5.3.

The results of our experiments with net photosynthesis and respiration in *Mougeotia* as a function of pH of the culture medium are shown in Figure 1. Each bar is the mean of three replicates with the standard error shown at the top (or bottom) of the bars where large enough to be visible. Respiration is shown as negative oxygen production, and the base of all bars is the 0.0 mg O$_2$g$^{-1}$·h$^{-1}$ line. It was not possible to measure net photosynthesis at higher or lower pH levels than those shown because the filaments would die off rapidly. *Mougeotia* barely survives at pH 10, but grew well at pH 9. Photosynthesis was reduced at pH 3, and the alga died at lower pH levels. The optimum (or at least the maximum) was not pH 5 or 6, as might be expected from field observations in acidified waters, but was unexpectedly high at pH 8. Net photosynthesis was lower, but relatively constant from pH 4 to 7. *Mougeotia* was tolerant of a broad
pH range from 4 through 9. Thus, the results of the culture observations did not confirm our expectation based on field observations made under acidified conditions. They did, however, confirm the breadth of observations in Table 1, and explained why Mougeotia occurs at neutral to alkaline pH levels as well as acidic conditions.

The results of our survey of the literature for references on Mougeotia and the metals aluminum and zinc are summarized in Tables 2 and 3. The levels of these two metals reported from Little Rock Lake (King et al. 1992) are the lowest for both aluminum and zinc, of any reports cited in the Tables. The highest reported levels of both metals come from mining regions of western England. Apparently, the species or population of Mougeotia that occurs in Gillgill Burn, a stream system in Cumbria, England, can tolerate zinc levels to 25,600 μg·l⁻¹ and aluminum levels to 1410 μg·l⁻¹ (Say and Whitton 1980). Even at alkaline pH levels, some populations of Mougeotia can grow with aluminum levels of 600 μg·l⁻¹ (Sheath et al. 1982), and zinc concentrations of 500-3000 μg·l⁻¹ (Whitton 1970). On the basis of these observations we might not expect our Mougeotia isolate from Little Rock Lake to be particularly tolerant of elevated concentration of either zinc or aluminum. However, our isolate of Mougeotia proved to be relatively insensitive to elevated concentrations of both zinc and aluminum (Fig. 2). It did not demonstrate any significant depression of growth at levels of Zn more than 6 times what it had encountered in
the acidified basin of Little Rock Lake or at aluminum concentrations more than 8 times levels in the acidified basin. Despite its growth in an acidified basin with low metals concentrations, it was about as tolerant of zinc as *Mougeotia* from Myra Creek on Vancouver Island, British Columbia (Deniseger 1986) or to Al in the streams of the Trossachs in Scotland (Kinross et al. 1993). The levels of zinc and aluminum in Little Rock Lake are therefore neither inhibitory nor growth promoting, even if they were considerably higher than they are at present, to *Mougeotia* in the lake.

**Conclusions**

The population of *Mougeotia* present in Little Rock Lake was tolerant of low levels of ionic Zn and Al found in the acidified basin (King et al. 1992). Laboratory culture experiments revealed that this *Mougeotia* was also tolerant to much higher levels of these two metals than it had been exposed to in Little Rock Lake. High levels of Zn and Al were neither inhibitory nor enhancing of growth. As pH decreases, the concentration of free metal ions increases, and free metal ions are the form most likely to have an impact upon cell surfaces (Campbell and Stokes 1985). This effect appears to be countered by the increasing concentration of hydrogen ions at low pH which compete with the free metal ions for binding sites on cell surfaces (Peterson et al 1984). Thus, hydrogen ions act to protect cell surfaces from the same metal ions they have caused to increase.
There is relatively little information on toxic effects of Al and pH. Campbell and Stokes (1985) were unable to determine any consistent trend with decreasing pH for Al. Our results indicate that Mougeotia from Little Rock Lake was unaffected by Al at a level of 300 µg·l⁻¹. As indicated in Table 2, this level is as high or higher than indicated by most reports of Mougeotia and Al concentrations from various freshwater systems, except for the highly polluted streams of Gillgill Burn in Cumbria, England, where Mougeotia was found at some sites having Al levels of over 1400 µg·l⁻¹. Bringmann and Kuhn (1959) found Scenedesmus to be tolerant to 1500 µg·l⁻¹ of Al, but the highest tolerance reported is that of Foy and Gerloff (1972) who were able to select a strain of Chlorella pyrenoidosa tolerant to 48,000 µg·l⁻¹ Al at pH 4.2. Stokes (1983) has recommended that "much more attention needs to be paid to the lower trophic levels" with regard to aluminum tolerance.

Zinc is a widespread metal contaminant whose concentration in freshwater systems increases as pH decreases (Campbell and Stokes 1985). The tolerance to Zn shown by filamentous green algae varies widely depending on the concentration of Zn in the aquatic systems from which they were isolated (Whitton 1984). In the Rivers Hayle and Gannel, Foster (1982) found that Spirogyra and Mougeotia were typical of low metal pollution conditions. Gale et al. (1973), however, found both genera in Missouri streams polluted by mill and mine waste, and Whitton (1980) listed both genera as tolerant of high levels of Zn. As
shown in Table 3, *Mougeotia* has been found growing in waters with Zn concentrations up to 34,000 μg·l⁻¹ where mine wastes have polluted aquatic systems. Compared to these environmental levels, the concentrations in the acidified basin of Little Rock Lake are insignificant. In culture studies, our isolate of *Mougeotia* was tolerant of 40 μg·l⁻¹ of Zn, but this range places it in the same class as the *Mougeotia* in the unpolluted headwaters of Myra Creek on Vancouver Island. At the downstream site polluted by mining waste, the Myra Creek *Mougeotia* was greatly reduced in summer from 42.5 cells·mm⁻² at the upstream site to only 0.05 cells mm⁻² at the contaminated site (Deniseger 1986). Since pH was about 7 at both sites, *Mougeotia* may have been affected by increased levels of Zn from 16 μg·l⁻¹ upstream to 166 μg·l⁻¹ downstream. The Myra Creek *Mougeotia* population appears to be sensitive to Zn. The Little Rock Lake *Mougeotia* may also be a sensitive population, at least by comparison to the *Mougeotia* found in English streams and ponds polluted by mining waste.

Our culture experiments with net photosynthesis and respiration in *Mougeotia* as a function of pH revealed that this alga declines in productivity at pH 3 and dies off totally below pH 3. No one has experimentally acidified a lake to a pH below about 4.6 (Webster et al. 1992), and *Mougeotia* was still abundant in the acidified streams of the Trossachs in Scotland at pH 4.37 (Kinross et al. 1993). One field observation is however, congruent with our culture experiments. Sheath et al. (1982) surveyed the algal flora of a series of tundra ponds
Mougeotia was present in three ponds with pH values of 8.1 to 8.2. Nearby were four ponds acidified by burning lignite in an area known as the Smoking Hills. The lignite in these hills has been burning for at least 1000 years. Although present in nearby ponds, there is no Mougeotia in the four acidified ponds with pH values of 1.8, 2.0, 2.8, and 3.6. The first three are far too acid while the pond at pH 3.6 has around 18,000 µg·l⁻¹ of Al, as well as high levels of other metals.

At the other end of the pH range, the culture studies reported in this paper showed that Mougeotia could just barely survive at pH 10, grew well at pH 9, and had a maximum at pH 8 for net photosynthesis. In this respect, Mougeotia is similar to an isolate of Spirogyra studied by Simpson and Eaton (1986). Their Spirogyra peaked in photosynthetic oxygen production when the pH was 7 to 8; there was a sharp drop off at pH 9, and a more gradual decline from pH 7 down to pH 5. But if Mougeotia from an acidified lake really grows best at pH 8, why doesn't it bloom as a metaphytic mat in alkaline waters? Other factors besides light, temperature, pH and metals must be involved. Two possibilities are grazing pressure and competition with other algae (Turner et al. 1991). Since at alkaline pH levels bicarbonate is the principle source of inorganic carbon, Mougeotia might be at a competitive disadvantage at higher pH when competing for bicarbonate against other filamentous algae such as cyanobacteria. On Signy Island, Antarctica, Mougeotia does form extensive growths of attached filaments in cold water
streams at neutral pH; there are no invertebrate grazers in these streams (Hawes 1989). No one has done the critical experiment in which substrates covered with attached *Mougeotia* at pH 5.2-5.5 are moved to an environment at pH 7 or 8, with some substrates enclosed to restrict grazers, then the fate of the algal growths is monitored. In 1980, however, Muller came close when he moved substrates covered with *Mougeotia* from an enclosure at pH 4 to one at pH 6.5. The filaments disappeared, but he was not able to say why. If they had been outcompeted by some other attached alga, these would have been apparent upon recovery and examination of substrates. Algal competition does not therefore seem as likely an explanation in this instance as some form of grazing.

If grazers are important in controlling the growth of *Mougeotia* at higher pH levels, we still need to understand why the alga blooms so prolifically at lower pH. It is known that filamentous green algae begin to increase around pH 6 as gastropods disappear in Norwegian lakes (Okland 1980). The removal of grazing pressure is likely to be one important factor in the success of *Mougeotia* at low pH, but not the most important one. We still need to understand how it can grow so well under acidic conditions. *Mougeotia* can clearly tolerate elevated metal concentrations and acidic conditions which other filamentous algae cannot. We propose that the real "secret" of its success is its ability to effectively harvest CO$_2$ as a source of inorganic carbon, and to augment this by uptake of organic carbon at low pH. Turner et al. (1987)
were among the first researchers to point out the potential significance of low levels of dissolved inorganic carbon (DIC) as CO₂ in acidified waters. In subsequent papers they showed that DIC was very low in acidified lakes, even lower within mats of Zygnemataceae, and that additions of DIC to enclosures with filamentous algae (by bubbling CO₂ in water) resulted in a marked increase in productivity (Howell et al. 1990, Turner et al. 1991). Under acid conditions, mats of Mougeotia are carbon-limited. Fairchild and Sherman (1993) also observed that Mougeotia was DIC limited in natural softwater lakes in northeastern Pennsylvania. In summary, Mougeotia is tolerant of elevated metal concentrations and low pH, but these are not optimal conditions for this alga. Grazing pressure is probably reduced or eliminated at low pH, and this release from grazing may be another factor in the occurrence of Mougeotia blooms. But what really allows Mougeotia to grow abundantly in acid waters is its effectiveness in carbon uptake. We will explore carbon metabolism in two additional reports which focus on carbonic anhydrase as a mechanism for inorganic carbon (Cᵢ) accumulation, and uptake of dissolved organic forms of carbon by Mougeotia.


Table 1. Occurrence of *Mougeotia* and the pH of natural and acidified freshwater systems reported in the literature.

<table>
<thead>
<tr>
<th>Growth Form</th>
<th>Environment</th>
<th>pH</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benthic and planktonic</td>
<td>Tundra ponds, Cape Bathurst, Canada</td>
<td>8.1 - 8.2</td>
<td>Sheath et al., 1982a</td>
</tr>
<tr>
<td>Floatiing mats</td>
<td>Eutrophic ponds in nature reserves, the Netherlands</td>
<td>7.5 - 9.9</td>
<td>Hillebrand, 1983</td>
</tr>
<tr>
<td>Floatiing mats</td>
<td>Coastal dune ponds, the Netherlands</td>
<td>5.5 - 8.2</td>
<td>Simons, 1987</td>
</tr>
<tr>
<td>Attached filaments</td>
<td>Georgian Bay and North channel</td>
<td>6.6 - 7.8</td>
<td>Sheath et al., 1988</td>
</tr>
<tr>
<td>Attached to cobbles</td>
<td>Streams on Signy Island, Antarctica</td>
<td>6 - 7</td>
<td>Hawes, 1989b</td>
</tr>
<tr>
<td>Aggregates attached to substrates</td>
<td>Softwater lakes in northeastern Penn.</td>
<td>4.4 - 6.2</td>
<td>Fairchild &amp; Sherman, 1993c</td>
</tr>
</tbody>
</table>

(table continued on next page)
Table 1 continued

<table>
<thead>
<tr>
<th>Growth Form</th>
<th>Environment</th>
<th>pH</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metaphytic mats</td>
<td>Lake 223,</td>
<td>5.09 - 5.64</td>
<td>Schindler et al., 1985</td>
</tr>
<tr>
<td></td>
<td>Experimental</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lakes Area (ELA), Ontario</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metaphytic mats</td>
<td>Lake 223, ELA</td>
<td>5.0 - 5.2</td>
<td>Turner et al., 1987</td>
</tr>
<tr>
<td></td>
<td>Lake 302S, ELA</td>
<td>5.5 - 6.0</td>
<td></td>
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<tr>
<td>Algal mats</td>
<td>Seepage lake, north-central</td>
<td>4.7 - 5.6</td>
<td>Webster et al., 1992d</td>
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<td></td>
<td>Wisconsin</td>
<td></td>
<td></td>
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<tr>
<td>Attached to</td>
<td>Acidified streams, the Trossachs,</td>
<td>4.37 - 6.67</td>
<td>Kinross et al., 1993</td>
</tr>
<tr>
<td>substrates</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Mougeotia was absent from three nearby tundra ponds with pHs of 3.6, 2.0 and 1.8.

b There were no grazers in these cold streams.

c Authors reported an observed optimum pH of 5.3 ± 0.8 for Mougeotia.

d Maximum biomass occurred at pH 5.2.
TABLE 2. Occurrence of *Mougeotia* and concentrations of aluminum in various freshwater systems reported in the literature.

<table>
<thead>
<tr>
<th>Freshwater system</th>
<th>Location</th>
<th>pH</th>
<th>[Al] μg·l⁻¹</th>
<th>Source</th>
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<tr>
<td>30 stream sites on Gillgill Burn</td>
<td>Cumbria, England</td>
<td>6.2 - 7.3</td>
<td>50-1410</td>
<td>Say &amp; Whitton, 1980</td>
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<tr>
<td>Tundra ponds</td>
<td>Smoking Hills, Cape</td>
<td>8.2</td>
<td>&lt;600</td>
<td>Sheath et al., 1982</td>
</tr>
<tr>
<td>P4</td>
<td>Cape</td>
<td>8.1</td>
<td>&lt;300</td>
<td></td>
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<tr>
<td>P5</td>
<td>Bathurst, N.W.T., Canada</td>
<td>8.1</td>
<td>&lt;300</td>
<td></td>
</tr>
<tr>
<td>P6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>East Twin Lake</td>
<td>Portage Co., Ohio</td>
<td>4.5</td>
<td>200</td>
<td>Havens &amp; Heath, 1990</td>
</tr>
<tr>
<td>Little Rock Lake</td>
<td>North-central Wisconsin</td>
<td>4.7 - 5.1</td>
<td>26 - 35</td>
<td>King et al., 1992</td>
</tr>
<tr>
<td>15 stream sites</td>
<td>The Trossachs, Scotland</td>
<td>4.37 - 6.67</td>
<td>41 - 221</td>
<td>Kinross et al., 1993</td>
</tr>
</tbody>
</table>
TABLE 3. Occurrence of *Mougeotia* and concentrations of zinc in various freshwater systems reported in the literature.

<table>
<thead>
<tr>
<th>Freshwater system</th>
<th>Location</th>
<th>pH</th>
<th>[Zn] μg·l⁻¹</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>River Wear</td>
<td>Co. Durham, England</td>
<td>8.0</td>
<td>500 - 3000</td>
<td>Whitton, 1970</td>
</tr>
<tr>
<td>Pond #1</td>
<td>Swansea, Wales</td>
<td>?</td>
<td>34,000</td>
<td>Trollope &amp; Evans, 1976</td>
</tr>
<tr>
<td>30 stream sites</td>
<td>Cumbria, England</td>
<td>6.2 - 7.3</td>
<td>24 - 25,600</td>
<td>Say &amp; Whitton, 1980</td>
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<td>on Gillgill Burn</td>
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<tr>
<td>Myra Creek</td>
<td>Central, Vancouver Island, Canada</td>
<td>4.8 - 7.4</td>
<td>4 - 40</td>
<td>Deniseger, 1986</td>
</tr>
<tr>
<td>Little Rock Lake</td>
<td>North-central, Wisconsin</td>
<td>4.7 - 5.6</td>
<td>6</td>
<td>King et al., 1992</td>
</tr>
</tbody>
</table>
Figure Legends

Fig. 1. Photosynthesis and respiration in Mougeotia as a function of culture medium pH under constant light (250 μE·m⁻²·sec⁻¹) and temperature (20°C).

Fig. 2. Chlorophyll a production in Mougeotia as a function of Zn concentration (upper graph) and of Al concentration (lower graph).
Fig. 2
Chapter 3

Occurrence of carbonic anhydrase in filamentous Zygnemataceae, and the ecological implications.
Abstract

Filamentous green algae collected from metaphytic blooms appearing in an experimentally acidified lake (Little Rock Lake, Vilas Co. WI) were isolated into unialgal culture. Because acidified lakes are characterized by reduced levels of dissolved inorganic carbon (DIC), the dominant bloom former, a species of Mougeotia was examined for possible occurrence of an enzymatic mechanism to import and concentrate inorganic carbon (Ci) intracellularly. A potentiometric method was used to measure activity of carbonic anhydrase (CA) in Mougeotia at pH 3.7 and pH 8. External activity in Mougeotia was significantly smaller ($p < 0.005$) than internal activity at both pH levels. By comparison to the low pH (pH 3.7), internal activities were halved ($p < 0.025$) at the higher pH (pH 8) whereas external activities were doubled ($p < 0.0025$) at pH 8. An isolate of Spirogyra reflexa that co-occurred with Mougeotia but which did not bloom, was devoid of carbonic anhydrase activity. Ultrastructural immunogold labeling using a polyclonal antibody to Chlamydomonas external CA showed that Mougeotia has external CA associated with the inflated end walls. A histochemical procedure that localizes CA in animal tissues was positive for pig liver, but did not work for any of the algae tested, possibly because of differences in cellular enzyme activity levels between animals and algae.
Introduction

One of the earliest and most repeatable events marking lake acidification is the development of littoral blooms or mats of green filamentous algae belonging to the family Zygnemataceae, order Zygnematales, and class Charophyceae. Such nuisance growths occur both in lakes affected by acidic precipitation and those subjected to experimental acidification (Schindler et al. 1985, Turner et al. 1987). Extensive growths composed of the zygnematacean algae Mougeotia and Spirogyra also appeared during the course of an experimental acidification project conducted jointly by the Wisconsin Department of Natural Resources and The University of Wisconsin Center for Limnology in Little Rock Lake, Vilas Co., Wisconsin (Webster et al. 1992). In 1984, this hourglass-shaped, seepage lake was separated into treatment and reference basins by installation of a dacron fiber-polyvinyl curtain across the narrow isthmus region. Over a period of years the pH of the treatment basin was reduced from an initial 6.1 to 4.6 (the approximate pH of local rainwater), by addition of sulfuric acid (Watras and Frost 1989). During the acidification process, extensive algal mats were first observed when the pH was dropped to 5.6. The zygnematacean growths reached a maximum at pH 5.2, when they covered most of the littoral zone from 1-2 m, as well as much of the rest of the basin, extending to depths of 6 m (Webster et al. 1992). Although zygnematacean algae also occurred in the untreated reference basin of Little Rock Lake, where the pH remained unchanged at 6.1, they did not
produce macroscopically visible growths. The basis for the difference in magnitude of algal growth in the two basins of Little Rock Lake, and the regular appearance of Zygnemataceae in acidic lakes has not been elucidated. Turner et al. (1987) suggested that under low pH conditions, zygnematacean algae might be better competitors for dissolved inorganic carbon (DIC) than other algae. Certainly, DIC levels are reduced in natural waters below pH 6.5, because bicarbonate levels are very low.

Howell et al. (1990) demonstrated that low DIC levels limited photosynthesis by *Mougeotia* in two acidified lakes. These workers determined DIC to be 0.18-0.20 mg C·l⁻¹, with even lower levels (0.04-0.05 mg C·l⁻¹) measured inside algal aggregates on a sunny day. When algal samples were incubated at two DIC levels (0.53 and 0.91 mg C·l⁻¹), photosynthesis was 64% greater at the higher DIC level (Howell et al., 1990). In a nutrient enrichment study conducted in 12 Pennsylvania softwater lakes which varied in pH from 4.4 to 8.8, Fairchild and Sherman (1993) found that the number of Carbon-enhanced species was greatest in the most acidic lakes, and that these acidic lakes were dominated by filamentous green algae, especially *Mougeotia*. These two field studies strongly suggest that zygnematacean algae are probably carbon limited at low pH. Even so, the magnitude of their biomass suggests that they may possess mechanisms for efficient acquisition of carbon.
One common mechanism by which DIC limited algae are able to accumulate carbon for photosynthesis is by use of one or more forms of the enzyme carbonic anhydrase (CA) (Aizawa and Michayi 1986, Miyachi et al. 1985). Carbonic anhydrases are zinc metalloenzymes that catalyze the hydration of carbon dioxide and dehydration of carbonic acid. In alkaline waters, where the dominant species of DIC is bicarbonate, certain eukaryotic algae are known to utilize a wall-associated CA to convert bicarbonate to CO$_2$, which then diffuses into the cells (Coleman et al. 1984). In contrast, cyanobacteria are thought to lack a wall-based CA, and instead appear to use a bicarbonate pump to bring DIC into cells under alkaline conditions (Badger and Andrews 1982). However, in cyanobacteria an internal CA appears to function in the conversion of intracellular bicarbonate to CO$_2$ at the site of its fixation, aggregates of Rubisco (Ribulose bisphosphate carboxylase) known as carboxysomes (Badger and Price 1989). *Chlamydomonas* is known to produce both internal and external (wall-based) CA (Husic et al. 1989, Husic 1991). Since zygnematacean algae are able to achieve significant biomasses in acidic, DIC-limited waters, we investigated the possibility that isolates of *Mougeotia* and *Spirogyra* from the Little Rock Lake blooms possess a carbon accumulation system involving one or more forms of CA. We have taken three approaches: measurements of CA activity according to the potentiometric method of Giordano and Maberly (1989), chemical localization of CA at the light microscopic level using the Ridderstråle (1991) method, and ultrastructural
immunolocalization of CA as described for *Chlamydomonas* by Coleman et al. (1991).

**Materials and Methods**

**Sources of algae and culture conditions**

Isolates of *Mougeotia* sp. and *Spirogyra reflexa* were obtained from metaphytic algal growths occurring in the acidified basin of Little Rock Lake (Vilas Co., WI). The one-time collection was made from a depth of 2 m, during the summer of 1989, when the pH was 4.7. Algal filaments were isolated by dragging through soft agar to remove epiphytes, then maintained in SD11 medium Graham et al. (1982), at pH 5.5, in aerated flasks, at 20°C.

*Spirogyra reflexa* was identified on the basis of the size of zygotes that appeared spontaneously in old cultures. However, no zygotes have been observed in *Mougeotia*. The Little Rock Lake isolate was distinguished from *Temenogametum*, which is vegetatively very similar, by induction of characteristic conjugation bridges, according to the methods of Stabenau and Säftel (1989). Algae in aerated SD11 medium with low (10^{-4} M) nitrate were grown in low irradiance conditions (105 \mu E\cdot m^{-2}\cdot sec^{-1}). Cultures of *Chlamydomonas reinhardtii* were obtained from the University of Texas Culture Collection, and maintained in Bold's Basal Medium (BBM) (Nichols 1979).
**Carbonic anhydrase activity assays.**

Assays of external and total CA were conducted according to the procedures of Giordano and Maberly (1989), which are modified from the potentiometric method of Wilbur and Anderson (1948). The assay measures proton production as a result of CO$_2$ hydration, and is expressed in terms of Wilbur-Anderson units calculated from the equation $10([t_c/t_s]-1)$. Here $t_c$ is the time taken for the specified pH drop to occur in the absence of sample, and $t_s$ is the pH drop time in the presence of the sample. One Wilbur-Anderson (W-A) unit will cause the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per minute at $0^\circ$C. Internal CA activity is calculated as the difference between total and external activity.

The activity of the enzyme, either internal or external, is inducible to some extent by variations in DIC level (Sultemeyer 1993). Therefore, *Mougeotia* and *Spirogyra* were assayed at two different pH levels with three replicates. A week before assays were done, *Mougeotia* was transferred to SD11 medium at pH 3.7 and pH 8.0; *Spirogyra* was transferred to SD11 at pH 7 and pH 10; and *Chlamydomonas* was grown in BBM at pH 10. The pH was adjusted with 0.5 M sulfuric acid or 1 M sodium hydroxide. *Chlamydomonas reinhardtii* served as a known source of external carbonic anhydrase activity, and fresh spinach leaves were a known source of internal CA activity. Purified bovine erythrocyte carbonic anhydrase (EC 4.2.1.1) Sigma) was used as a positive control. 133 ng of enzyme was dissolved
in 10% glycerol to a final concentration of 8.06 μg·l⁻¹. Negative controls consisted of reaction mixture without added algal or plant samples.

Filamentous algae and the spinach leaf samples were assayed on a fresh weight basis, whereas *Chlamydomonas* was assayed on a haemacytometer cell count basis. Fresh weight of intact algal or plant material was measured immediately prior to the assay. Filamentous algal biomass was collected on 14 μm pore size polycarbonate filters, then dried between layers of filter paper prior to weighing. Fresh weights ranged from 0.1 to 0.7 g. Small, whole spinach leaves were blotted dry with filter paper prior to determining fresh weight.

**External CA activity measurement.**

CA assays were conducted at 7°C, at an irradiance of 20 μE·m⁻²·s⁻¹, in flasks containing 15 ml phosphate buffer (25 mM, pH 8.36) which was continuously stirred. In order to prevent heating of the reaction flask by the magnetic stirrer, a piece of Styrofoam was inserted between flask and stirrer. Reactions were carried out at the nonphysiological temperature of 7°C because the reaction time would be too fast to be measured at a higher temperature. The fastest mixing time of a mechanical stirrer is about one second, and that sets a lower limit to the time resolution of the technique (Forster II 1991). The usual times recorded for the control were in the range of 80 to 120 seconds and for the samples tested about 10 to 30 seconds. Total activity minus external activity represented internal activity. Precision in
measurement of sample and liquid volumes, as well as constant
temperature are required because the assay is highly sensitive to
variation in these parameters. A combination pH electrode (Orion) was
inserted into the reaction flask and allowed to equilibrate before
initiation of the reaction. To measure external activity intact algae or
plant leaves were added to assay flasks, and the reaction initiated by
addition of 1.5 ml of double distilled water saturated with CO₂.
Saturation was accomplished by introduction of CO₂ from a compressed
gas cylinder to cooled (5°C) water for 20 minutes. CO₂ saturated water
maintained in ice at 0°C had a pH of 4.5.

Total CA activity measurements.

Total activity was measured in extracts of 1 ml of fresh
Chlamydomonas culture, and the same filamentous algal or spinach
samples used in the assay of internal activity (see above). Algal
filaments were refiltered onto 14 μm polycarbonate membranes then
algae or spinach leaves were ground in a mortar containing 3-10 ml tris-
borate buffer at pH 8.36 and liquid nitrogen. Graham and Smillie's
(1976) tris-borate buffer formulation included 300 mM tris adjusted to
pH 8.36 with a saturated solution of boric acid, 25 mM dithiothreitol
(DTT), 5 mM EDTA, 0.5% w/v bovine serum albumin, 0.10% v/v
Triton X-100, and 2% w/v Polyclar AT. Although DTT can inhibit CA
even in very small amounts (0.1 mM) (Sultemeyer 1993) it was not a
cause of concern in our assay. DTT was used in concentrations of 25
mM. However, we found that Polyclar AT contained in the buffer
borate is a chemical that inhibited purified bovine erythrocyte enzyme by 2.8%. Inclusion of Polyclar AT in the formulation is thought to prevent inhibition of the CA activity by phenolic compounds that may be present in plant tissue extracts. Although phenolics are known to occur in Mougeotia (Wagner and Grolig 1992), no problems were encountered when we eliminated Polyclar AT from the buffer mixture.

Although Giordano and Maberly (1989) recommended longer centrifugation times at low speed when extracting the enzyme, there were no problems when shorter (4 minutes) centrifugation was applied at higher rpm (4000). Extracts were assayed immediately after extraction. However, Giordano and Maberly (1989) found conservation of activity in samples kept in ice in the dark for 99 hours. After addition of 0.1 ml of supernatant to assay buffer in reaction flasks, the pH was adjusted to 8.36 with 0.1 M HCl. Then the reaction was initiated with 1.5 ml CO2-saturated, double distilled water at 5°C. The time required for the pH to reach 7.8 was recorded and converted to W-A units, as in the assay of total CA activity described above.

Histochemical localization.

Algal, plant and animal tissue samples were treated according to the Ridderstråle method (Ridderstråle 1991). In this procedure (Sexton 1978) tissue sections are placed in a medium which contains NaHCO3 as the substrate, together with KH2PO4, CoSO4 and H2SO4. Carbonic anhydrase dehydrates the bicarbonate ions to give CO2 and OH as
products. The tissue is floated on the surface of the incubation medium, so that the CO\textsubscript{2} released can immediately escape into the air. This results in local tissue alkalization due to an accumulation of OH\textsuperscript{-}. A divalent trapping ion, cobalt, forms a salt at the site of tissue alkalization. The precise nature of this salt is unknown and it may be a carbonate, phosphate or carboxylate, or perhaps a mixture of these. The cobalt salt is visualized for light microscopy by conversion to cobalt sulfide.

A week prior to the localization procedure, Mougeotia filaments were transferred to SD11 medium, at pH 4.7 and pH 8.0; Spirogyra was transferred to SD11 at pH 6.2 and pH 8.0; and Chlamydomonas was transferred to Bold's basal medium at pH 10. The pH levels were adjusted with 0.5 M H\textsubscript{2}SO\textsubscript{4} and 1 M NaOH. Fresh pig liver served as a positive control since it is known to have carbonic anhydrase (Ridderstråle 1991).

Algal and animal samples were fixed by immersing small pieces in Karnovsky's fixative for 4 hours at room temperature. *Chlamydomonas reinhardtii* cells were gently centrifuged before fixation to concentrate cells in a pellet, then resuspended in the fixative. The centrifugation was repeated for each solution change. Karnovsky's fixative was prepared with 0.5% glutaraldehyde, 4% paraformaldehyde in buffer phosphate (100 mM, pH 7.2). The low glutaraldehyde concentration is necessary to prevent loss of enzyme activity. After
fixation, samples were washed 3 to 4 times in buffer phosphate, then dehydrated in increasing concentrations of ethanol (15%, 30%, 50%, 70%, 95%, 100%, 100%) in 15 minute steps. Cells and tissues were infiltrated in LR White resin (Polysciences) according to the following schedule: 1 part resin : 2 parts 100% ethanol for one hour, 2 parts resin : 1 part 100% ethanol for one hour, and two changes to pure resin for two hours each time. Curing of the resin was done in eppendorf tubes at 50\(^\circ\) C for 24 hours. Sections approximately 0.25 microns thick were made with a glass knife using a Sorvall MT-2B ultramicrotome. Sections were transfer directly from the glass knife to the surface of water droplets in a depression slide for storage until transferred to the incubation medium. The incubation medium consisted of solution I (10 ml 0.2 M CoSO\(_4\) + 60 ml 0.5 M H\(_2\)SO\(_4\) + 100 ml 0.06 M KH\(_2\)PO\(_4\)) and solution II (3.75 g NaHCO\(_3\) in 200 ml double distilled water). These solutions should be prepared just prior to incubation. Sections were transferred into the incubation medium for 8 minutes, then allowed to float for 1 minute on a rinsing solution (0.006 M phosphate buffer pH 5.9), prior to transfer to a blackening solution for 3 minutes. Blackening solution was prepared in a hood by filtering 0.5% [NH\(_4\)\(_2\)]S through a filter paper on which CoS crystals have been deposited. The CoS is made by mixing 2 ml of CoSO\(_4\) and 2 ml [NH\(_4\)\(_2\)]S in water, then pouring it onto the filter paper. After blackening, sections were rinsed twice for 1 minute in distilled water, then mounted on a slide. Sections were examined and photographed with a Zeiss Axioplan microscope.
Ultrastructural Immunolocalization.

A week prior to experiments, *Mougeotia* was transferred to aerated 250 ml vessels with SD11 at pH 9.1 and pH 4.3. *Chlamydomonas reinhardtii* was cultured for a week in aerated flasks in Bold's basal media (Stein 1979) at pH 10.6 and pH 6.2. In each case the pH was adjusted with 0.5 M H2SO4 and 1M NaOH.

*Mougeotia* filaments (and pelleted *Chlamydomonas reinhardtii* cells that were resuspended in each change of solution) were fixed by successive transfer through a series of Karnovsky's fixative at different strengths in phosphate buffer (Pi) at pH 7.2 (Karnovsky : buffer Pi, 1:2; 1:1; 2:1; 100% Karnovsky). Fixative contained 0.5% glutaraldehyde and 4% paraformaldehyde in phosphate buffer (100 mM, pH 7.2). An alternative formulation contained 2.5% glutaraldehyde and 2.5% paraformaldehyde in phosphate buffer (100 mM, pH 7.2). Algal samples remained for at least 6 hours in each fixative step, at room temperature. After fixation the material was washed 3 to 4 times in phosphate buffer, then samples were dehydrated in increasing concentrations of ethanol (15%, 30%, 50%, 70%, 95%, 100%, 100%) in 15 minute steps. Cells and tissues were infiltrated in LR White resin according to the following schedule: 1 part resin : 2 parts 100% ethanol for one hour, 2 parts resin : 1 part 100% ethanol for one hour, and two changes to pure resin for two hours each time. For embedding, algae were transferred to fresh resin in weighing dishes, then another weighing dish floated on top of the resin in order to exclude air. Resin was polymerized at 50°C for 24 hours.
Silver or gold sections were made with a diamond knife, and a Sorvall MT-2B ultramicrotome, and collected on nickel mesh grids.

Immunolocalization procedures followed Coleman et al. (1991), with a few modifications. The polyclonal antibody for external carbonic anhydrase of *C. reinhardtii* was prepared and donated by Dr. J. Coleman (Coleman and Grossman 1984). Sections were first blocked by incubation with 5% dry milk and 5% goat serum with 0.1% BSA (Bovine Serum Albumin, 0.1% w/v in TBST), 15 minutes in each blocking solution. The sections were then incubated in a 1:500 dilution of the primary antibody in PBS at room temperature for 1 hour. Incubations were followed by three washings in TBST (Tris-buffered saline, 100 mM Tris, 500 mM NaCl, 0.3% Tween 20). Sections were again blocked for 15 minutes in 5% goat serum. Sections were then incubated in a 1:25 dilution of gold conjugated goat anti-rabbit IgG (secondary antibody) for one hour, washed for 10 minutes in TBST, and finally washed in Milli-Q water prior to staining in uranyl acetate (2% aqueous) for 20 minutes in the dark. Control experiments were performed by incubation of one of the grids in non-immune serum and other grid by omission of primary antibody. Grids were dried with filter paper and examined and photographed with a JEOL JEM 2000-EX transmission electron microscope.
Results

*Mougeotia* (Fig. 1) was the dominant alga in the metaphytic blooms that were sampled. *Spirogyra reflexa* was second in dominance, followed by *Oedogonium* sp., several species of cyanobacteria, and diatoms. *Mougeotia* was found to have both external and internal carbonic anhydrase, detected by activity measurements (Table I). External CA was also detected by ultrastructural immunolocalization (Fig. 2). Light microscopic examination revealed that the Little Rock Lake isolate of *Mougeotia* typically exhibit inflated endwalls (Fig. 1). At the ultrastructural level these inflated endwalls show a moderate degree of gold labeling (Fig. 2). Gold labeling of these wall structures is noticeably heavier than adjacent cytoplasmic regions. The ultrastructural appearance of the inflated endwalls was similar to the appearance of these structures in unfixed specimens viewed by light microscopy.

Although internal CA activity was identified by the potentiometric assay, it was not detected with immunolocalization. A very small amount of gold labeling occurred in cytoplasmic and plastid compartments, but cell preservation was not good enough to achieve definitive results (Figs. 3-6). Carbonic anhydrase activity was not detected in *S. reflexa* (Table I). Although histochemical localization (Ridderstråle method) was positive for animal tissue (pig liver) (Fig. 7 A, B), it was negative for *Mougeotia, Spirogyra* and *Chlamydomonas.*
By comparison with the internal CA activity, external activity represented 4% to 16% of the total activity in *Mougeotia* at pH 3.7 and pH 8 respectively (Table I). Mean internal activity at pH 3.7 was 1.61 times greater than at pH 8 (p < 0.025), mean external activity at pH 8 was 2.59 times greater than at pH 3.7 (p < 0.0025) (Table I). Spinach (which served as the positive control for internal CA) showed a mean internal activity of 2303.5 W-A units gr⁻¹ (Table I), about half of that obtained for *Mougeotia* at pH 8. *Chlamydomonas reinhardtii* was the positive control for both internal and external carbonic anhydrase. In *Chlamydomonas* 63% of the total activity of the enzyme was attributable to the internal enzyme, at the pH tested (pH 10) (Table II).

**Discussion**

In view of the important role played by carbonic anhydrase (CA) in algal carbon acquisition, and the occurrence of more than one form of CA located within separate compartments in algal cells, it is important to survey the occurrence of CA in the cells of other organisms. Such an exercise sheds light on the relationships among these various forms of CA, and helps to explain the results obtained in the present study.

Carbonic anhydrase has been reported to occur in procaryotes; protists, including marine and freshwater algae (Tsuzuki and Miyachi 1989); animals; and land plants. Viruses may also exhibit a CA-like
protein (Niles et al. 1986). Carbonic anhydrase is one of the most abundant proteins in C3 plant leaves (Atkins et al. 1972). The known C3 plant CAs have a high degree (76%) of sequence identity to each other, and the cytosolic CA that occurs in mesophyll cells of the C4 plant maize is about 60% identical to other plant CAs (Fukuzawa et al. 1992). However, CA is virtually absent from root tissue (Chegwidden 1991), suggesting tissue-specific patterns of expression. In mammals, seven isozymes have been identified, with tissue specific patterns of occurrence. Of those, CAI, CAII, CAIII, and CAIV have been fully characterized. Related enzymes occur in birds, reptiles, amphibians and fish. Invertebrates such as corals, oysters, and sea urchin embryos, utilize CA in the formation of CaCO₃ shells or exoskeletons (Silverton 1991).

The function of CA in animal tissues has been extensively reviewed by Dodgson (1991), and offers some interesting parallels to CA function in algal and plant physiology. For example, in pulmonary capillaries, erythrocyte CAII converts stored bicarbonate to CO₂ which then can diffuse to lung alveoli (Swenson and Maren 1978). Alternatively, liver mitochondrial CA converts CO₂ to bicarbonate, the substrate for carbamyl phosphate synthetase (Silverman 1991), which is essential for urea formation (Dodgson 1991). Carbonic anhydrase also facilitates diffusion of CO₂, for example, from the lens in the process of reducing CO₂ and bicarbonate concentration gradients between the lens and aqueous humor (Friedland and Maren 1981). Analogous functions
in plants include plastid-based generation of CO$_2$ as a substrate for plant Rubisco (Spalding 1983), production of bicarbonate as a substrate for phosphoenolpyruvate carboxylase in C$_4$ plants (Sultemeyer et al. 1993), and in microalgae, enhancement of DIC diffusion from outside cells toward sites of the carboxylation reaction (Aizawa and Miyachi 1986, Smith and Bidwell 1989). Because the Ridderstråle technique assays CA activity, we expected to detect CA in algae as well as animal tissues. The fact that there was a negative result with all the algae tested might be explained by differences in amounts of enzyme in algae as compared to animal tissues. Alternatively, our results may be based upon substantial differences between animal and plant forms of CA.

Plant CAs are much less sensitive to inhibitors such as sulfonamides (ethoxyzolamide and acetazolamide) than are animal CAs, and plant CAs are richer in the sulfur-containing amino acids methionine and cysteine (Graham et al. 1984). Comparative analysis of amino acid sequences of mammalian, prokaryotic, algal, and plant CAs led Fukuzawa et al. (1992) to propose that animal and certain algal CAs were of separate origin from CAs of prokaryotes and plants. Their study has important implications for interpretation of the CA assay and immunolocalization results described in the present report, and therefore will be considered in more detail.

The *Synechococcus* (cyanobacterial) gene *icfA*, known to be involved in carbon fixation, encodes a 272 amino acid polypeptide
located 20 kb downstream from \textit{rbcLS}. The protein product is believed to be a CA because it is 22\% similar to the pea and spinach chloroplast CAs (Fukuzawa et al. 1992). Sequence similarity between cyanobacterial CA and plastid CA of plants is to be expected, since it is well established that plant chloroplasts share ancestry with cyanobacteria (Gray, 1992). Interestingly, the \textit{Synechococcus icfA} gene product amino acid sequence is 31\% similar to the \textit{E. coli cynT} gene product (Fukuzawa et al. 1992). This protein was earlier regarded as a cyanate permease, but is now thought to be a carbonic anhydrase that functions in the bicarbonate-dependent metabolism of cyanate (Guilloton et al. 1992). A 5 amino acid Zn-binding region is conserved in \textit{icfA}, \textit{cynT}, and plant plastid CAs (Fukuzawa et al. 1992). Thus, the ancestry of the plant chloroplast CA appears to be well documented and extremely ancient.

Plant chloroplast CA is encoded in the nucleus, synthesized on cytoplasmic ribosomes, and targeted to the chloroplast by means of transit peptides (Tsuzuki and Miyachi 1989). In contrast, as expected, targeting sequences do not characterize the \textit{Synechococcus icfA} or the \textit{E. coli cynT} products (Fukuzawa et al. 1992). Sometime during evolution of plant plastids, the CA and \textit{rbcS} genes moved to a nuclear location (possibly together?), in the same way that \textit{tufA} location has changed from plastid to nucleus during the evolution of the charophyte-embryophyte clade (Baldauf and Palmer 1990, Baldauf et al. 1990). The first phylogenetic occurrence of nuclear location for the plant chloroplast CA gene is, however, unknown, and it is not known
whether green algal CAs are nuclear or plastid encoded. Interestingly, the carboxyl-terminal region of *icfA* is not conserved in higher plant plastid CAs. Because cyanobacterial CA is believed to be associated with carboxysomes, and higher plant plastids lack these Rubisco aggregations, Fukuzawa et al. (1992) suggested that the carboxyl-terminal end of the *Synechococcus* gene product might target the protein to carboxysomes. However, this hypothesis has not been tested by analysis of mutants in which the putative targeting sequence is altered.

The green flagellate *Chlamydomonas* appears to possess at least three distinct CAs, a 45 kD chloroplast form, a 37 kD external form, and a poorly characterized 110 kD enzyme that may be cytosolic (Husic et al. 1989). Coleman et al. (1991) used a gold-conjugated antibody to achieve ultrastructural localization of the external CA of *Chlamydomonas* to the cell wall. Prior to this work it was uncertain whether the external CA was located in the periplasmic space or the cell wall. These workers also compared the amino acid sequence of the *Chlamydomonas* wall CA with that of spinach chloroplast CA, and found no major regions of similarity. Similarly, Fukuzawa et al. (1992) considered the amino acid sequence of the *icfA* product to be unrelated to that of *Chlamydomonas* external CA. Furthermore, *Chlamydomonas* external CA is as sensitive as animal CAs to inhibitors such as acetazolamide (Graham et al. 1984). In contrast, *Chlamydomonas* internal CA is 3 orders of magnitude less sensitive than external CA to sulfonamide inhibitors (Husic et al. 1989). On aggregate, these findings suggest that the external and internal
(chloroplast) CAs of *Chlamydomonas* may be of independent phylogenetic origin, with the wall-bound enzyme more similar to animal CA, and the plastid enzyme more closely related to CA of cyanobacteria and plant plastids, as suggested by Fukuzawa et al. (1992). This concept is supported by a higher degree of immunological similarity between the chloroplast CAs of *Chlamydomonas* and higher plants than between the external CA of *Chlamydomonas* and plant chloroplast CAs in Western blots (Husic et al. 1989). An anti-spinach CA antibody reacts only very weakly with the *C. reinhardtii* external CA, whereas the same antibody exhibits significant cross-reactivity with *Chlamydomonas* chloroplast stromal extract (Husic et al. 1989).

On the other hand, Husic et al.'s (1989) Western blot work demonstrates that an antibody to *Chlamydomonas* wall CA clearly recognizes CA in *Chlamydomonas* chloroplast and cytosolic extracts. This could explain the small amount of localization that we observed in *Mougeotia*'s cytoplasm and chloroplast. However, our results could also be explained by inadequate blocking procedures.

It should be noted that plant and algal CAs may vary substantially in molecular weights, with (in decreasing MW order) dicotyledon and fern CAs at 180 kD, *Chlorella* and *Chlamydomonas* CAs at 165 and 150 kD respectively, *Chara coronata* CA at 90 kD, and grass CAs of 42 and 45 kD (Graham et al. 1984). There are probably multiple forms of CA in plants and algae. Until the numbers and types
of CA genes occurring in algae and plants have been determined, sequence comparisons and immunological results should be interpreted conservatively. However, we offer here a tentative explanation for the apparent discrepancy between the immunological results observed by Husic et al. (1989), and the difference between animal plus external *Chlamydomonas* CA and plant CAs in terms of protein size, amino acid sequence, and sensitivity to sulfonamide inhibitors noted by Fukuzawa et al. (1992).

Fukuzawa et al.'s (1992) work elegantly traces the phylogeny of plant chloroplast CAs back to eubacterial origins, and also provides evidence for CA gene transfer to the nuclear genome sometime during the evolutionary origin of plant chloroplasts. Once transferred to the nucleus, gene duplication and diversification may be postulated, along with development of a diversity of transit peptide sequences. Various CAs could then be targeted separately to chloroplasts, cytoplasmic compartments, and the endomembrane system for secretion to the cell wall. Postulated differences in length and sequences of transit peptides and differences in protein function (e.g. hydration of carbon dioxide or dehydration of carbonic acid) could explain the evolution of differences in protein size and gene sequences that have been observed. In an analogous system involving evolution of a large superfamily of related hexose transporters (Marger and Saier, 1993), dramatic sequence differences coexist with amazingly similar structure including 12 transmembrane alpha helices (Griffith et al. 1992). For example, a
comparison of eight, almost certainly homologous D-glucose transporters (including yeast, human, and the flowering plant *Arabidopsis*), revealed only 7% identical amino acids (Sauer and Tanner 1993). Such new data emerging from the field of protein evolution, suggests the occurrence within organisms of paralogous genes encoding families of homologous proteins that vary substantially in amino acid sequence and compartmentation, yet perform the same or similar functions. Conservation of critical regions necessary for common catalytic function might explain cases such as that of Husic et al. (1989), of immunological recognition of divergent, but functionally similar proteins by an antibody raised against only one of them. Further study of the expression and sequences of genes that confer carbonic anhydrase activity upon the cells of algae, plants, and other organisms may contribute substantially to our understanding of patterns in protein evolution. Such studies would also help elucidate ecological roles of CA in aquatic plants and algae that may be important in terms of global carbon cycling.

It is significant that *Mougeotia* responds to low pH (indirectly, limiting DIC levels) by increasing internal CA activity and decreasing external CA activity. This suggests an important role for internal CA in inorganic carbon (Ci) accumulation under low pH conditions. We propose five models that explain various ways in which internal and external CA might be involved in carbon uptake by *Mougeotia* (Figs. 8-12). Models shown in Figs 8 and 9 postulate the occurrence of a
bicarbonate pump protein in the plastid envelope. This is consistent with Graham et al. (1984), who speculate that the CO2 concentrating system in algae involves both a bicarbonate pump in the chloroplast envelope, and cytoplasmic and chloroplast stroma carbonic anhydrases. This concept is also consistent with evidence for hypothesized occurrence of a bicarbonate pump in blue-green algal cell membranes (Tzusuki and Miyachi 1989), since chloroplasts are derived from blue-green algae (Gray 1992). Fig 8 also postulates occurrence of a bicarbonate pump protein in the cell membrane of Mougeotia; a cell membrane bicarbonate pump has also been suggested to occur in another member of the charophycean lineage, Chara (Lucas 1985). In contrast Figs 10-12 illustrate models that do not rely upon occurrence of a plastid envelope bicarbonate pump. These models indicate that inorganic carbon moves across the plastid envelope as CO2. This is consistent with Tzusuki and Miyachi's (1989) model for function of carbonic anhydrase in eukaryotic algae. Fig. 10 does not postulate the existence of a cytoplasmic CA or a cell membrane bicarbonate pump. In contrast Fig. 11 and 12 illustrate models that involve cytoplasmic CA. The model shown in Fig. 12 also suggest the occurrence of a cell membrane bicarbonate pump. The occurrence of a cytoplasmic CA would be advantageous in facilitating diffusion of CO2 in the cells under DIC limiting conditions. Testing these models would require carefully done ultrastructural immunolocalization of CA. If it were possible to isolate chloroplasts, it might be possible to deduce the existence of envelope bicarbonate pumps by measuring bicarbonate uptake. Some of the methods
suggested by Aizawa and Miyachi (1986), such as uptake of radiolabeled bicarbonate, could be helpful in deciding whether or not a cell membrane bicarbonate pump is present.


Table I: Activity means (n= replicates) of the enzyme carbonic anhydrase in W-A units*g^{-1}(FW). Total activity is the sum of external and internal activity. Standard deviation is given in parenthesis. One Wilbur -Anderson (W-A) unit will cause the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per minute at 0°C. F. W. = freshweight, negative values are equivalent to zero activity.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>pH</th>
<th>Total Activity</th>
<th>External Activity</th>
<th>Internal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mougeotia</td>
<td>3.7</td>
<td>6339.8</td>
<td>282.3</td>
<td>6057.5</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td>(1035.4)</td>
<td>(42)</td>
<td>(1011.1)</td>
</tr>
<tr>
<td>Mougeotia</td>
<td>8.0</td>
<td>4474.5</td>
<td>732.8</td>
<td>3741.8</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td>(444.8)</td>
<td>(32.0)</td>
<td>(457.8)</td>
</tr>
<tr>
<td>Spirogyra</td>
<td>7.0</td>
<td>-0.7</td>
<td>-0.3</td>
<td>0</td>
</tr>
<tr>
<td>(n=3)</td>
<td></td>
<td>(4.2)</td>
<td>(0.5)</td>
<td></td>
</tr>
<tr>
<td>Spirogyra</td>
<td>10</td>
<td>-51.6</td>
<td>-0.5</td>
<td>0</td>
</tr>
<tr>
<td>(n=3)</td>
<td></td>
<td>(48.0)</td>
<td>(0.7)</td>
<td></td>
</tr>
<tr>
<td>Spinach</td>
<td>---</td>
<td>2303.5</td>
<td>-0.1</td>
<td>2303.5</td>
</tr>
<tr>
<td>(n=4)</td>
<td></td>
<td>(28.3)</td>
<td>(0.8)</td>
<td>(28.3)</td>
</tr>
</tbody>
</table>

Activity is calculated according to the formula $U=10 \left[ \frac{T_c}{T_s} - 1 \right]$, where $U=W-A$ unit, $T_c$ and $T_s$ are the time in minutes for the pH to drop from 8.2 to 7.8 for the control (no specimen) and the sample respectively.
Table II: Activity means (3 replicates) of the enzyme carbonic anhydrase in W-A units*cell⁻¹ for *Chlamydomonas reinhardtii* at pH 10. Total activity is the sum of external and internal activity. Standard deviation is given in parenthesis. One Wilbur-Anderson (W-A) unit will cause the pH of a 0.02 M Trizma buffer to drop from 8.3 to 6.3 per minute at 0°C.

<table>
<thead>
<tr>
<th>Total Activity</th>
<th>External Activity</th>
<th>Internal Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1.56 \times 10^{-3}$</td>
<td>$5.77 \times 10^{-4}$</td>
<td>$9.89 \times 10^{-4}$</td>
</tr>
<tr>
<td>$(2.70 \times 10^{-4})$</td>
<td>$(6.52 \times 10^{-5})$</td>
<td>$(3.28 \times 10^{-4})$</td>
</tr>
</tbody>
</table>

Activity is calculated according to the formula $U=10 \left[ \frac{t_c}{t_s} \right] - 1$, where $U=W-A$ unit, $t_c$ and $t_s$ are the time in minutes for the pH to drop from 8.2 to 7.8 for the control (no specimen) and the sample respectively.
Figure Legends

Fig. 1. Mougeotia sp. isolated from Little Rock Lake, grown in SD11 medium supplemented with 1% glucose, and aerated. Cell morphology resembles that of field-collected filaments. Chloroplast are flat plates that extend from one side to the other, and from one end to the other end of cells. Note that end walls are separated by an inflated space, the middle lamella. X13,700.

Fig. 2. Little Rock Lake isolate of Mougeotia grown in SD11 medium at pH 4.3, then processed for ultrastructural immunolocalization of carbonic anhydrase. The relative distribution of gold particles suggests localization of CA in the pectinaceous, inflated middle lamella, and perhaps also with the end cell wall per se. X14,500.

Fig. 3, 4. Gold labelling patterns suggest the possibility that CA is localized to lateral walls, the cell membrane, and peripheral cytoplasm, but cell preservation is not good enough to define the location of peripheral CA more definitely. X22,400.

Fig. 5, 6. Only very small amounts of gold label are associated with the chloroplast and pyrenoid. White areas are starch granules were once located
Fig. 7. Histochemical localization of carbonic anhydrase in (A) pig liver. Dark areas show salt precipitates where carbonic anhydrase is present. Section is 0.25 μm thick incubated for 8 minutes. (B) pig liver, untreated. Pictures were taken with a Zeiss Axioplan.

Fig. 8-12. Models proposed to explain inorganic carbon uptake by Mougeotia, involving external and internal carbonic anhydrase. The models explain what would be the function of the external enzyme at either low (pH < 6) or high pH (pH > 6). It is proposed the presence of bicarbonate pumps at cell membrane and/or chloroplast membrane (Figs 8, 9 and 12). A cytoplasmic carbonic anhydrase may have an accumulation role aiding in the CO₂ diffusion (Fig. 9 and 11). Internal CA may be accumulating CO₂ at the site of RuBisCo inside the pyrenoid (Figs. 8-11). Another alternative is that CA may be accumulating inorganic carbon in the form of bicarbonate to be converted back to CO₂ closer to the pyrenoid (Fig. 12).
Fig. 8
Fig. 9
Fig. 10.
Fig. 11
Fig. 12

Diagram showing the transport of CO₂ and HCO₃⁻ across the cell wall, through the cell membrane, and into the plastid membranes. The diagram illustrates the pH-dependent transport of CO₂ and HCO₃⁻, with CO₂ entering the cell through a pump protein in the periplasmic space, and HCO₃⁻ entering the cytoplasm. The pyrenoid contains Rubisco, which catalyzes the fixation of CO₂ into HCO₃⁻.
Chapter 4

Occurrence and ecological significance of glucose utilization by charophycean algae: Glucose enhancement of growth and morphology of Mougeotia.
Abstract

The filamentous charophyte *Mougeotia* forms nuisance level metaphytic growths in lakes that have been affected by acidic precipitation or experimental acidification. Although a number of hypotheses have been offered to explain the occurrence of these blooms, no single ecological correlate has been identified. In view of the fact that there is now evidence that various charophycean algae are able to import sugars such as glucose and use them to enhance growth, the possibility that *Mougeotia* might be able to use dissolved sugars under dissolved inorganic carbon (DIC) limiting, low pH conditions was explored. An isolate of *Mougeotia* was obtained from an acidified lake in Wisconsin, treated to reduce bacterial population to low levels, then grown without aeration in an inorganic medium (pH 4.3) that was supplemented with 1% glucose for a period of three weeks. Chlorophyll a levels were significantly higher ($0.005 < p < 0.01$, df=9) from those of identical cultures grown under the same conditions, but in a medium lacking supplemental sugar. In addition, glucose supplementation of inorganic culture medium appeared to enhance the morphological appearance of chloroplasts, whether cultures were DIC limited (nonaerated) or not (aerated). Although further studies are needed to substantiate these results, the findings suggest the possibility that *Mougeotia* might be able to supplement photosynthesis by importing and utilizing dissolved organic carbon in the form of glucose under low pH, DIC limiting conditions in nature. It is suggested that
dissolved organic carbon (DOC) utilization may be a factor in the formation of *Mougeotia* blooms in acidified lakes.

**Introduction**

The filamentous zygnematacean green alga *Mougeotia* (Zygnematales, Charophyceae) occurs in a wide variety of habitats, but is particularly well known for formation of conspicuous metaphytic blooms in softwater, low pH lakes (Fairchild and Sherman 1993), lakes affected by acidic precipitation (Dillon et al. 1987), and lakes subjected to experimental acidification (Schindler et al. 1985, Turner et al. 1987, Webster et al. 1992). However, despite attempts to correlate these blooms with various ecological factors, no satisfactory explanation for their occurrence has been validated by critical tests. Hypotheses that have been suggested include: 1. direct effects, such as acid-tolerance by zygnematalean algae, but not other, potentially competitive algae (Hendrey, 1976), 2. indirect effects of acidification such as reduction of herbivores that keep the algae under control at higher pH (Hendrey 1976), 3. the occurrence of unique responses to physical factors such as light and temperature that allow zygnematacean algae to bloom in acid lakes (Turner et al. 1987), 4. the existence in zygnematacean algae of highly efficient mechanisms for accumulation of dissolved inorganic carbon (DIC) under acidic conditions (Turner et al. 1987), and 5. resistance by the bloom-forming algae to heavy metals that may occur at higher concentrations in acidic waters than in neutral ones (Stokes
1981). Several of these concepts have been tested (Hendrey 1976), and one hypothesis, release from herbivory control, has been at least tentatively refuted (Hendrey 1976).

A significant body of recent evidence suggests that an additional explanation should also be considered—that Mougeotia and its close relatives are able to supplement DIC-limited photosynthesis with importation of exogenous DOC, with the effect that growth is enhanced. Graham et al. (1994) recently demonstrated that growth of the charophyte Coleochaete orbicularis under DIC limiting conditions was significantly enhanced by supplementing the inorganic culture medium with sugar. These workers found that nonaerated cultures grown for 7 weeks in an inorganic medium supplemented with 1% glucose produced as much as 13.6 times the biomass of clonal cultures grown for the same length of time in the same medium without glucose. Cultures not supplemented with glucose exhibited morphological abnormalities, whereas the morphology of glucose supplemented cultures was identical to that of natural collections. Furthermore, addition of 1% glucose to nonaerated cultures yielded chlorophyll a concentrations 20 times higher after 4 weeks growth than cultures grown under the same conditions without added glucose. These effects were observed only under conditions of DIC limitation, and were not observed when cultures (whether supplemented with glucose or not) were continuously aerated. Thus, glucose uptake is probably regulated by environmental levels of DIC. These results were
interpreted as evidence that *Coleochaete* is capable of loading glucose and using it to maintain normal growth and morphology under DIC-limiting conditions (Graham et al. 1994).

Additional, semi-quantitative data indicate that growth of other charophytes, including the desmid *Mesotaenium* (Taylor and Bonner, 1967) and strains of *Klebsormidium* and *Stichococcus* (*Mattox* and *Bold*, 1962), is also enhanced by glucose supplementation. Although additional surveys need to be done, the available evidence suggests that glucose uptake may be plesiomorphic for members of the charophyte clade, i.e., an ancient character inherited by the whole group from ancestral forms (Graham et al., 1994). Considerable recent molecular evidence supports this hypothesis.

Two reviews of membrane transport proteins catalog the occurrence of a major superfamily of over 50 transporters, characterized by a common motif of 12 transmembrane-spanning alpha-helices, in bacteria and various groups of eukaryotes (Griffith, 1992; Marger and Saier, 1993). One group of these proteins, a cluster of homologous sugar transporters, links such disparate organisms as *E. coli* and *Synechocystis* among prokaryotes, to eukaryotes including *Saccharomyces cerevisiae*, *Homo sapiens*, and green algae and plants. The green alga *Chlorella kessleri* possesses a 57kD proton-hexose cotransporter (Hexose Uptake Protein 1--HUP1) that is 47% identical in deduced amino acid sequence to that of the 57 kD Sugar Transporter Protein 1 (STP1) of the
flowering plant *Arabidopsis* (Sauer and Tanner, 1993; Sauer, Friedlander, and Graml-Wicke, 1990). The *Chlorella* HUP1 protein is a proven plasma membrane hexose carrier; mutants in which this protein are affected lack an inducible sugar uptake system (Sauer, 1986). Recent physiological work also indicates the occurrence of proton-glucose cotransport proteins in the bryophyte *Polytrichum* (Renault, et al., 1992). Since a substantial body of molecular, biochemical, and cellular evidence links charophycean algae directly to the ancestry of bryophytes and vascular plants (summarized in Graham, 1993), ancient characters inherited by land plants from prokaryotes must have been filtered through the charophyte lineage. In fact, portions of sugar transporter genes homologous to those of *Chlorella* and *Arabidopsis* have been amplified or cloned and sequenced from several bryophytes and charophytes, including species of *Coleochaete*, *Klebsormidium*, and zygnematalean algae (Wilcox, Graham, and Fisher, 1994).

These findings strongly suggest that *Mougeotia* and other zygnematacean algae probably possess homologous, regulated H\(^+\)-hexose cotransport genes. Hence, under DIC-limiting conditions these charophytes may be capable of importing DOC in the form of sugars, if these are present at sufficiently high concentration. Since DIC levels in acidic freshwaters have been shown to limit algal photosynthesis (Howell, et al., 1990; Fairchild and Sherman, 1993), algal capacity to harvest DOC as a supplement to photosynthetically generated fixed carbon could provide a major competitive advantage. Therefore, we
investigated the possibility that growth and/or morphology of cultured *Mougeotia* might be enhanced by glucose supplementation.

**Materials and Methods**

An unidentified species of *Mougeotia* was isolated from an algal mat occurring in the treatment basin of Little Rock Lake, Vilas Co., WI, the site of an experimental acidification study conducted jointly by the Wisconsin Department of Natural Resources and the Center for Limnology, University of Wisconsin, Madison. At the time of collection, the pH of the water was 4.6, and samples were obtained from a depth of approximately 2 meters. Single zygnematacean filaments presumed to be *Mougeotia* were isolated from other algae with forceps, and dragged through 0.5% agar to remove epiphytes. Isolates were maintained in SD11 medium (Graham et al., 1994), a modification of D11 medium whose composition was described by Graham et al., 1982), in aerated flasks at 20 degrees C, at an irradiance of 200-300 uEm^2^s^-1 (PAR), provided by cool white fluorescent lamps and 25W incandescent bulbs, on a 12:12 light-dark cycle.

Isolates were determined to be *Mougeotia*, rather than the very similar genus *Temenogametum*, by applying Stabenau and Säftel's (1989) methods for inducing characteristic reproductive structures. Algae were grown in SD11 medium having low levels (10^-4^ M) nitrate, at low irradiance (105 uEm^-2^s^-1), under aeration. Although zygospores (which
allow species identification) were not obtained, the conjugation bridges characteristic of *Mougeotia* were observed.

Cultures containing only low numbers of bacterial cells, as determined by microscopic examination, were obtained by serial dilution of a suspension of *Mougeotia* filaments that had been cut into small pieces with a sterile scalpel blade. Replicate individual *Mougeotia* filaments were then transferred by sterile pasteur pipette to small flasks containing SD11 medium at pH 4.3, which was supplemented with 1% glucose in order to screen for gross bacterial or fungal contamination. Flasks were then aerated to generate the biomass necessary for subsequent experiments.

The effects of glucose supplementation on growth of *Mougeotia* was assessed by comparing chlorophyll a levels in 10 replicate cultures grown in SD11 supplemented with 1% glucose with 10 replicate cultures grown in SD11 without added sugar. Chlorophyll a determinations were by the methods of Hansmann (1973), with the use of a spectrophotometer. Special attention was paid to achieving a homogeneous inoculum that was equally partitioned to the experimental containers. A culture of *Mougeotia* having low bacterial numbers (generated by the process described in the previous paragraph) was cut into small pieces with sterile scalpel blades, then agitated to achieve uniform distribution of filaments in medium. 0.5 ml of filament suspension was distributed to each of 20 small culture tubes.
containing 10 ml of either SD11 medium plus glucose, or SD11 medium alone. Care was taken to avoid pipetting visible algal clumps, and the filament suspension used as an inoculum was agitated prior to withdrawal of each aliquot. Capped tubes were randomly distributed within each of two test tube racks (to avoid shading) and placed in a growth room at 23°C and an irradiance of 100 μEm⁻²s⁻¹, which are optimal conditions for Mougeotia photosynthesis (as ascertained in Chapter 1). Both fluorescent and incandescent light sources were used to provide full spectrum PAR radiation. Upon random occasions, tubes were randomly redistributed in order to avoid possible positional effects. Algae were allowed to grow without aeration for a period of 3 weeks, whereupon filaments were harvested in low light by filtration onto 5 um pore size Nuclepore filters (Nuclepore Corp.). Two ml of absolute methanol were poured into culture tubes to extract chlorophyll from algae that remained attached to the glass walls, then filters with algal filaments were added back to the appropriate tube. Tubes were agitated until algal filaments were devoid of color, as judged by visual monitoring, kept chilled in an ice bath. The contents of tubes were centrifuged at 4000 rpm for 6 minutes just prior to chlorophyll a determinations. Treatment and control means were compared with a t-test.

Samples of Mougeotia filaments grown under four aeration/medium combinations (aeration in absence of supplemental glucose, aeration with supplemental glucose, no aeration in absence of
supplemental glucose, and no aeration with supplemental glucose) were photographed for comparison of cell morphology, using a Zeiss Axioplan photomicroscope.

Results and Discussion

When Mougeotia cultures were grown for three weeks without aeration, the mean chlorophyll a concentration was $3.8 \pm 1.0 \text{ mg l}^{-1}$ for 10 glucose supplemented cultures, and $2.3 \pm 0.8 \text{ mg l}^{-1}$ for 10 cultures grown under the same conditions, except that glucose was not added to the medium. Application of the $t$-test indicated that this difference was significant at the 0.01 level ($0.005 < p < 0.01$). These results suggest that glucose-supplemented cultures were able to grow more rapidly because glucose was imported by the algae and used for growth when DIC in the medium reached limiting levels.

Previous experiments with the charophyte Coleochaete demonstrated that the means of various measures of growth (including chlorophyll a), in cultures that were supplemented with glucose or not, became more dramatically different as the length of the experiment increased. We believe that this effect can be explained as the result of increasing carbon limitation of photosynthesis and growth with time. In the present experiment, cultures were harvested after only three weeks of growth, and probably had not completely exhausted DIC resources. It is expected that if the experiment had been extended for a
week or two, that a larger differences in chlorophyll a concentration between glucose supplemented and non-supplemented cultures would have been observed. Further studies of longer duration are needed to better define the extent of the glucose effect on growth of *Mougeotia* as judged by chlorophyll a concentrations.

Commonly, chloroplasts of *Mougeotia* cells grown without addition of glucose, whether aerated or not, exhibited morphological abnormalities as compared to field collected specimens. *Mougeotia* chloroplasts are normally thin, flat, rectangular plates having regular edges (Bech-Hansen and Fowke 1972), but chloroplasts of culture isolates are often distorted, with irregular shapes. Although some normal appearing plastids occurred in cultures that were not glucose-supplemented, most cells contained plastids that were irregular in outline, and often shrunken (Fig. 1,2). In contrast, the majority of cells in cultures that were both aerated and which were supplemented with glucose, possessed normally shaped chloroplasts (Fig. 3). The chloroplasts of cultures that had been grown without aeration, but in glucose-supplemented medium were more normal in appearance than those grown without glucose, but were somewhat more irregular in outline (Fig. 4) than those of aerated, sugar-supplemented cultures. It is very difficult to devise an objective method of quantifying these differences in chloroplast morphology, and in the absence of appropriate measures few conclusions can be made regarding these observations. However, our observations suggest that the presence of
glucose in the culture medium may have a positive effect upon chloroplast morphology even when cells are not DIC limited. Further studies of this possible effect, perhaps at the ultrastructural level, are needed to determine whether glucose affects morphology of *Mougeotia* chloroplasts.

It may tentatively be concluded, however, that as in some other charophytes (Graham et al., 1994) glucose may enhance growth and morphology of *Mougeotia* under DIC-limiting conditions. This suggests the possibility that growth of this alga in acidified lakes may be influenced by the presence of dissolved organic carbon in the form of sugars.

In a classic and much cited paper, Wright and Hobbie (1966) suggested that in lakes algae are generally not able to compete with bacteria for assimilable forms of dissolved organic carbon such as glucose. This conclusion was based upon measurement of levels of glucose in a eutrophic lake (L. Erken, Sweden), and by comparing the 14C-glucose uptake kinetics of a bacterium isolated from the same lake, with those of a *Chlamydomonas* sp. isolated from a different lake. Wright and Hobbie found that the bacterium possessed a high-affinity glucose uptake system capable of harvesting even the small amounts of glucose present in the lake, whereas the comparatively inefficient *Chlamydomonas* glucose uptake system would not normally allow the alga to compete with bacteria for low levels of glucose. Yet many
freshwater eukaryotic algae are known to be capable of assimilating various forms of exogenous organic carbon, including glucose (Neilson and Lewin, 1974).

A recent study has documented significant enhancement of growth of the charophyte Coleochaete when inorganic culture medium is supplemented with glucose, under DIC-limiting conditions (Graham et al., 1994). These results, together with some other work (Mattox and Bold, 1962), and the data obtained in this study, indicate that charophytes may generally be capable of loading and using DOC in the form of glucose. This suggests an unresolved paradox. Why should freshwater algae retain capacity to utilize a resource that is not generally available to them? Together, some very recent studies documenting dissolved inorganic carbon (DIC) limitation of algal growth in acidic, softwater lakes (Fairchild and Sherman, 1993) and lakes affected by acidic precipitation or experimental acidification (Howell et al., 1990), coupled with observations that low pH may inhibit bacterial assimilation of glucose in lakes (James, 1991), suggest an answer.

In a study of 12 softwater lakes in Pennsylvania which varied in pH from 4.4 to 8.8, Fairchild and Sherman (1993) observed that species composition was strongly related to pH, with the proportion of green algae highest in the more acidic lakes. Mougeotia, with a high abundance at pH 5.3 ± 0.8, was almost exclusively responsible for the higher total biovolume of algae in the acidic lakes. As a result of
nutrient enrichment experiments, these workers also determined that the most acidic lakes harbored a greater number of "C-enhanced" algal species. These results strongly suggest that these algae, including *Mougeotia*, are DIC-limited in acidic waters.

Howell et al. (1990) observed that zygnematacean algae such as *Zygogonium*, *Mougeotia* (or *Tennogametum*), and *Spirogyra* formed large metaphytic mats or clouds in Plastic Lake, pH 5.6 and Lake 302, when experimentally acidified to pH 5.0. These workers measured DIC in these two lakes at 0.18-0.20 mg C l\(^{-1}\), and found even lower DIC levels (0.04-0.05 mg C l\(^{-1}\)) inside mats of *Mougeotia* on a sunny day. They also examined the effect on algal photosynthesis of increasing DIC levels. They achieved an increase in the DIC level of natural water samples without altering pH by the simple, but imaginative method of adding a small amount of distilled water that had been aerated with CO\(_2\) gas. When the DIC was 0.91 mg C l\(^{-1}\), photosynthesis was 64% greater than at a DIC level of 0.53 mg C l\(^{-1}\). These results indicate that photosynthesis by *Mougeotia* and other zygnematacean mat-forming algae was carbon limited in these acidic lakes.

James (1991) studied the relationships among DOC, pH, and other factors and bacterial densities in several Florida acid lakes. Bacterial densities either increased or decreased with DOC, depending upon the DOC level, and the proportion of organic acids in the lake. This finding suggests that bacterial densities may reflect lake-dependent conditions,
and thus may not represent a quantity that may be accurately applied in intra-lake comparisons. However, acid deposition explained much of the variability observed in the ability of bacteria to assimilate glucose, with acid deposition inversely related to bacterial use of glucose in clear lakes. James (1991) notes that the effect of acid precipitation upon bacteria would not have been detected by measures of bacterial densities alone, and that measurement of radiolabelled glucose uptake was necessary to identify the negative effect of acidity upon bacterial metabolism.

It is clear that further studies focused upon the relationships between DIC and DOC levels, pH, bacterial densities and metabolism of sugars, and growth of charophyte algae need to be done. However, it may be tentatively concluded that in lakes impacted by acid precipitation or experimental additions of acid, zygnematalean algae are able to grow to bloom proportions in the face of significant carbon limitation. Postulation of extremely efficient cellular mechanisms (such as carbonic anhydrase--see Chapter 3) for acquiring carbon do not entirely explain this phenomenon, for if zygnematacean cells were capable of acquiring sufficient DIC to account for bloom levels of growth, the algae would not respond to DIC enrichment experiments as they do (Howell et al., 1990; Fairchild and Sherman, 1993). The experiments of both Howell et al. (1990) and Fairchild and Sherman (1993) clearly indicate that within blooms that form in low pH waters, Mougeotia is carbon-limited. Where is Mougeotia getting the carbon
necessary to explain the formation of dramatic metaphytic growths? The experimental results described here, and in previous work (Graham et al., 1994) suggest that under conditions in which bacterial ability to metabolize dissolved sugars such as glucose is reduced (i.e. low pH, James (1991)), dissolved sugars may be available at levels high enough that algae may be able to load and metabolize them. This hypothesis will require testing by intensive study of the sugar uptake kinetics of zygnematacean algae such as Mougeotia that bloom in low pH waters, and by correlative determinations of sugar levels in acidified waters and the metabolic efficiencies of bacteria that inhabit acidic water, as compared to neutral or high pH systems.

If substantiated, however, a hypothesis for algal utilization of DOC at low pH could resolve the apparent conflict between Wright and Hobbie's (1966) results and the undoubted ability of various algae to import and metabolize sugars (Nielson and Lewin, 1974). Wright and Hobbie's (1966) work related to processes occurring in a eutrophic lake, in which pH (though undefined) was most likely higher than that of acidified lakes where Mougeotia and other zygnematacean algae bloom. In fact, most work focused upon the relationship between carbon (and other nutrients) and algal growth, has been done in neutral-higher pH systems, whereas, by comparison, low pH and oligotrophic systems are comparatively unstudied. It is possible that various groups of algae, including chrysophytes (Sandgren, 1988), may respond to the
comparatively low DIC levels in oligotrophic and acidic lakes by upregulating cellular sugar transport machinery.

Further, Hobbie and Wright's (1966) experiments on glucose uptake kinetics were conducted with *Chlamydomonas*, which is only distantly related to charophycean algae (Pickett-Heaps, 1975). Indeed, it has been proposed that charophytes may have been derived from endosymbiotic associations independent of those generating other green algae (Graham, 1993). In view of these phylogenetic considerations, unqualified extrapolation of the *Chlamydomonas* hexose uptake kinetic data generated by Wright and Hobbie (1966) to charophytes (or chrysophytes) is unwarranted.

Algal importation of organic carbon in DIC-limited aquatic systems is potentially important in global carbon cycling, because this process may interfere with rapid bacterial conversion of glucose to CO₂. Algal carbon that is buried in the sediments of acidified lakes (or in peat in acid *Sphagnum* bogs) may be regarded as a form of long-term C-storage, and may represent an important, although unquantified, component of CO₂ drawdown. In view of the potentially important role that algae, especially Zygnematales and other charophytes may play in these processes, considerably more attention needs to be directed toward elucidation of the mechanisms used by algae to obtain DOC. In particular, it will be important to determine how many sugar transporter proteins are involved in algal DOC import, the comparative
kinetics of bacterial and charophyte sugar transport at low pH, and the molecular basis of regulation of sugar transport gene regulation in bacteria and algae that occur in softwater, low pH waters.


Figure Legends

Fig. 1. *Mougeotia* filaments grown for two weeks in SD11 medium, without aeration and not supplemented with glucose. Chloroplast outline is often irregular and somewhat shrunken. X2,400.

Fig. 2. *Mougeotia* filaments grown for six weeks in SD11 medium, with aeration, but not supplemented with glucose. Chloroplasts look quite irregular and shrunken. X1700.

Fig. 3. *Mougeotia* filaments grown for several weeks in SD11 medium, with aeration and supplemented with glucose. Chloroplasts are normally shaped and have a regular outline. X2,700.

Fig. 4. *Mougeotia* filaments grown for two weeks in SD11 medium, without aeration, but supplemented with glucose. In that chloroplasts extend to the cell ends, they are normal in appearance, but exhibit a somewhat irregular outline. X2,000.