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Carbon concentrating mechanisms maintain bloom biomass and CO_2 depletion in eutrophic lake ecosystems

Ana M. Morales-Williams[1,2,3], Alan D. Wanamaker[4], Jr., and John A. Downing[1,5]

[1]Department of Ecology, Evolution, and Organismal Biology, Iowa State University, 251 Bessey Hall, Ames, IA, 50011, USA

[2]Department of Ecology, Evolution, and Behavior, University of Minnesota-Twin Cities, 1475 Gortner Ave., Saint Paul, MN, 55108, USA

[3]Rubenstein School of Environment and Natural Resources, University of Vermont, 81 Carrigan Drive, Burlington, VT, 05405

[4]Department of Geological and Atmospheric Science, Iowa State University, 12 Science 1, Ames, IA, 50011, USA

[5]Minnesota Sea Grant, University of Minnesota-Duluth, 141 Chester Park, 31 West College St., Duluth, MN, 55812, USA

Correspondence: Ana M. Morales-Williams, mindymorales@gmail.com

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Abstract

Harmful phytoplankton blooms are increasing in frequency, intensity, and duration in aquatic ecosystems worldwide. In many eutrophic lakes, these high levels of primary productivity correspond to periods of CO_2 depletion in surface waters. Cyanobacteria and other groups of phytoplankton have the ability to actively transport bicarbonate (HCO_3^-) across their cell membrane when CO_2 concentrations are limiting, possibly giving them a competitive advantage over algae not using carbon concentrating mechanisms (CCMs). To investigate whether CCMs can maintain phytoplankton bloom biomass under CO_2 depletion, we measured $\delta^{13}C$ signatures of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) and phytoplankton particulate organic carbon ($\delta^{13}C_{phyto}$) in sixteen mesotrophic to hypereutrophic lakes during the ice-free season of 2012. We used mass balance relationships to determine the dominant inorganic carbon species used by phytoplankton under CO_2 stress. We found a significant positive relationship between phytoplankton biomass and phytoplankton $\delta^{13}C$ signatures, as well as a significant non-linear negative relationship between water column ρCO_2 and isotopic composition of phytoplankton, indicating a shift from diffusive uptake to active uptake by phytoplankton of CO_2 or HCO_3^- during blooms. Calculated photosynthetic fractionation factors indicated that this shift occurs specifically when surface water CO_2 drops below atmospheric equilibrium. Our results indicate active HCO_3^- uptake via CCMs may be an important mechanism maintaining phytoplankton blooms when CO_2 is depleted. Further increases in anthropogenic pressure, eutrophication, and harmful cyanobacteria blooms are therefore expected to contribute to increased bicarbonate uptake to sustain primary production.

Key words: Eutrophication, carbon cycling, Cyanobacteria, CCM, stable isotopes

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45 **1. Introduction**

46 Harmful cyanobacteria blooms (HCBs) resulting from anthropogenic eutrophication are
47 among the greatest current threats to inland water ecosystems, altering carbon cycling and
48 ecosystem function, impairing water quality, and endangering human health (Brooks et al., 2016;
49 Paerl et al., 2011; Visser et al., 2016). Forecasting models and macrosystem-scale analyses
50 suggest the occurrence of HCBs is driven by the interactive effects of land use, nutrient inputs
51 (nitrogen and phosphorus), climate, weather, and in-lake processes (Anneville et al., 2015;
52 Michalak et al., 2013; Persaud et al., 2015; Rigosi et al., 2014). Mechanisms determining
53 variability in timing and duration of these events in lakes, however, remain poorly understood
54 (Brooks et al., 2016), and it is unclear what the large scale feedbacks of sustained primary
55 production are on lake carbon cycling by phytoplankton. While temperate lakes have generally
56 been considered net sources of CO_2 to the atmosphere (Tranvik et al., 2009), eutrophic systems
57 can maintain both high levels of primary production and negligible concentrations of CO_2 in
58 surface water (Balmer and Downing, 2011; Gu et al., 2010; Laas et al., 2012), possibly
59 increasing the flow of dissolved inorganic C to organic C. Identifying drivers of the temporal
60 variability of bloom formation and maintenance will contribute to a better understanding of
61 carbon dynamics in lakes with high productivity.

62 Cyanobacteria have developed a suite of diverse strategies for obtaining and fixing carbon
63 and nutrients at growth-limiting concentrations. In addition to fixing atmospheric nitrogen , they
64 are able to maintain metabolic processes under severe CO_2 depletion by use of a carbon
65 concentrating mechanism (CCM; Badger and Price 2003; Raven et al. 2008). CCMs are present

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in many groups of aquatic photoautotrophs including green algae (Spalding, 2008) and diatoms (Hopkinson et al., 2016), as well as some higher plants. These mechanisms are thought to have evolved independently in eukaryotic algae and the cyanobacteria, corresponding to a large decrease in atmospheric CO_2 and doubling of O_2 approximately 400 million years BP (Badger and Price, 2003; Raven et al., 2008). Compared to less efficient eukaryotic CCMs, many cyanobacteria can concentrate dissolved inorganic carbon (DIC) to intracellular levels 1000 times greater than ambient concentrations (Raven et al., 2008).

The cyanobacterial CCM mechanism facilitates active transport of HCO_3^- across the plasma membrane, where it is accumulated in the cytosol, transferred to Rubisco-containing carboxysomes, and converted to CO_2 via carbonic anhydrases (Raven et al., 2008). In freshwaters, cyanobacteria use form 1B Rubisco, which facilitates acclimation to inorganic carbon depletion via high cellular affinity for CO_2 and HCO_3^- (Raven and Beardall, 2016; Raven et al., 2008; Shih et al., 2015). In addition to inorganic carbon availability, cyanobacterial CCMs are triggered by photosynthetically active radiation (PAR) and nitrogen availability. Because CCMs are energetically costly (Raven and Beardall, 2016), decreased PAR lowers cellular affinity for inorganic carbon (Giordano et al., 2005). Affinity increases with depletion of nitrate and iron, but decreases with depletion of NH_4^+, and does not have a consistent response to phosphorus limitation (Raven et al., 2008). CCM activation under carbon and nutrient stress thus may confer a competitive advantage to cyanobacteria via efficient carbon fixation when CO_2 is low (Badger and Price, 2003; Price et al., 2008).

Shifts to alternative carbon assimilation strategies result in measureable changes in isotopic fractionation. Stable isotopic signatures of phytoplankton are dependent both on the isotopic composition of their DIC source and the physiological mechanism used to acquire it.

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When phytoplankton use passive diffusion to take up ambient CO_2, photosynthetic fractionation resembles that of C3 terrestrial plants (Yoshioka, 1997), resulting in typical mean $\delta^{13}C$ signatures between -27‰ to -30‰ (Bade et al., 2004; Erez et al., 1998; O'Leary, 1988). In cyanobacteria and other phytoplankton, carbon fixation can be equally limited by carboxylation and active inorganic carbon transport into the cell. Cyanobacteria that are actively concentrating inorganic carbon via HCO_3^- uptake can have elevated $\delta^{13}C$ values as high as -8 to -11‰ (Sharkey and Berry, 1985; Vuorio et al., 2006). This is largely attributable to the isotopic signature of source material (Kaplan and Reinhold, 1999), as well as decreased carbon efflux when CCMs are active, resulting in reduced photosynthetic fractionation (-1‰ to -3‰; Sharkey and Berry 1985; Erez et al. 1998). Further, isotopic fractionation associated with active HCO_3^- uptake is negligible (Sharkey and Berry, 1985; Yoshioka, 1997). In other words, discrimination due to passive diffusion is reduced or negligible when active HCO_3^- uptake is occurring (Giordano et al., 2005). Thus, if CCMs are activated during cyanobacteria blooms in eutrophic lakes, we would expect the $\delta^{13}C$ signature of the phytoplankton to increase as ambient CO_2 is depleted, and photosynthetic fractionation factors to decrease as the community approaches a monoculture of phytoplankton using CCM.

The purpose of this study was to evaluate the importance of CCMs in maintaining high phytoplankton biomass during CO_2 depletion in eutrophic and hypereutrophic lakes. We hypothesized that photosynthetic fractionation would be tightly coupled with inorganic carbon limitation, resulting in decreased fractionation with shifts from atmospheric CO_2 to mineral HCO_3^- in the water column. We further hypothesized that phytoplankton isotopic composition and photosynthetic fractionation would correspond to CO_2 depletion in the water column, reflecting CCM activation during blooms that are intense enough to lower water column CO_2.

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2. Methods

16 lakes were chosen based on Iowa State Limnology Laboratory long-term survey data (total phosphorus and phytoplankton community composition, 2000-2010, data publically available at: http://limnology.eeob.iastate.edu/lakereport/) including lakes with flashy watersheds (Fraterrigo and Downing, 2008) and those with high and low interannual variability in Cyanobacteria dominance. Long term survey data were used only for site selection. Duplicate stable isotope samples for particulate organic and dissolved inorganic analyses were collected once following ice off in 2012, weekly May-July, bi-weekly in August, and monthly September-November (*n*=196). Standard physical, chemical, and biological parameters were measured at each sampling event using US-EPA certified methods, including total nitrogen (TN), total phosphorus (TP), chlorophyll a (Chl a), alkalinity, vertical depth profiles of temperature, pH, conductivity, and dissolved oxygen (DO), as well as meteorological data (air temperature, wind speed, barometric pressure). Aqueous carbon dioxide concentration was measured at 1 m using a Vaisala GMT2220 probe modified for water measurements (Johnson et al., 2009). Partial pressure of carbon dioxide (pCO_2) was determined using temperature, depth, and pressure corrections described in Johnson et al.(2009). Specifically, because pressure and temperature respectively increase and decrease sensor output relative to their calibration, measurements were reduced by 0.15% per unit increase hPa relative to calibration (1013 hPa), and increased 0.15% per unit hPa decrease. An additional correction for depth was added to the barometric pressure correction, because pressure is increased 9.81 hPa per 10 cm depth. Meausurements were taken at 1 m, equivalent to a 98.1 hPa increase. Similarly, measurements were increased by 0.3% per degree Celsius increase in water temperature above instrument calibration (25°C).

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All water chemistry was performed in the Iowa State Limnology Laboratory using United States Environmental Protection Agency (US EPA) certified methods. Total nitrogen was determined using the second derivative method described in (Crumpton et al., 1989). Total phosphorus was determined colorimetrically using the molybdate blue method (APHA, 2012). Samples for Chl a analysis were filtered onto GF/C filters which were frozen then extracted and sonicated in cold acetone under red light. Samples were then analyzed fluorometrically (Arar and Collins, 1997; Jeffrey et al., 1997). Alkalinity was determined by acid titration and reported as mg $CaCO_3$ L^{-1} (APHA, 2012). Field measurements of temperature, DO, pH, and conductivity were taken with a YSI multi-parameter probe.

Samples were analyzed by standard isotope ratio mass spectrometry methods (IRMS), and reported relative to the Vienna Pee Dee Belemnite in ‰ (Equation 1).

$$\delta^{13}C_{Sample} = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{VPDB} - 1] \times 1000 \quad \text{Eq. 1}$$

Samples collected for isotopic analysis of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) were filtered to 0.2 µm in the field using a syringe filter and cartridge containing a combusted GF/F prefilter (Whatman) and 0.2 µm polycarbonate membrane filter (Millipore). Samples were then injected into helium gas-flushed septa-capped vials with H_3PO_4 to cease biological activity and to sparge CO_2 (Beirne et al., 2012; Raymond and Bauer, 2001). $\delta^{13}C_{DIC}$ samples were measured via a Finnigan MAT Delta Plus XL mass spectrometer in continuous flow mode connected to a Gas Bench with a CombiPAL autosampler. Reference standards (NBS-19, NBS-18, and LSVEC) were used for isotopic corrections, and to assign the data to the appropriate isotopic scale. Average analytical uncertainty (analytical uncertainty and average correction factor) was ±0.06 ‰.

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156 To determine the isotopic composition of phytoplankton organic carbon ($\delta^{13}C_{phyto}$),
157 samples were filtered onto pre-combusted GF/C filters. Zooplankton and detritus were removed
158 manually from filtered samples using a dissecting microscope. Samples were gently fumed in a
159 desiccator for 24 h with 1N HCl to remove inorganic carbon, dried in a low temperature oven,
160 then pulverized using a mortar and pestle and analyzed with standard methods (above IRMS
161 connected to a Costech Elemental Analyzer). For organic isotope samples, three reference
162 standards (Caffeine [IAEA-600], Cellulose [IAEA-CH-3], and Acetanilide [laboratory standard])
163 were used for isotopic corrections, and to assign the data to the appropriate isotopic scale. The
164 average combined uncertainty for $\delta^{13}C$ was ± 0.17‰ (1 sigma, VPDB). For all isotopic
165 measurements, at least one reference standard was used for every six samples.
166 Photosynthetic fractionation factors were calculated using published temperature
167 dependent fractionation factors between carbon species following methods described in
168 Trimborn et al. 2009 (Mook, 1986; Trimborn et al., 2009); inorganic carbon fractions and total
169 DIC concentration were calculated using discrete CO_2, alkalinity, and pH measurements:
170

171 $$\delta^{13}C_{HCO_{3-}} = \frac{\delta^{13}C_{DIC}\,[DIC] - (\varepsilon_a[CO_2] + \varepsilon_b[CO_3^{2-}])}{(1+\varepsilon_a * 10^{-3})[CO_2] + [HCO_{3-}] + (1+\varepsilon_b * 10^{-3})[CO_3^{2-}]} \quad \text{Eq. 2}$$

172 $$\delta^{13}C_{CO2} = \delta^{13}C_{HCO3-}(1 + \varepsilon_a \times 10^{-3}) + \varepsilon_a \quad \text{Eq. 3}$$

173 $$\varepsilon_p = (\delta^{13}C_{CO2} - \delta^{13}C_{phyto}) / (1 + (\delta^{13}C_{phyto} / 1000)) \quad \text{Eq. 4}$$

174 In order to test the hypothesized relationships between phytoplankton isotopic
175 composition, photosynthetic fractionation, and ambient pCO_2 (n=196), we used a nonlinear
176 dynamic regression and ran 199 model iterations (SigmaPlot 12, Systat Software) resulting in
177 100% model convergence. The same approach was used to test the relationship between
178 photosynthetic fractionation (ε_p) and the isotopic composition of the DIC pool. The relationship

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between phytoplankton biomass as chlorophyll *a* (Chl *a*) and phytoplankton isotopic composition was analyzed using linear regression. Prior to analyses, data were tested for normality using a Shapiro Wilk test.

3. Results

Phytoplankton $\delta^{13}C$ signatures in this study ranged from -29.86 ‰ to -13.48 ‰ with an average -25.26 ± 2.8 ‰. The highest values were measured when algal biomass peaked (i.e., during blooms). We found a significant positive linear relationship between phytoplankton $\delta^{13}C$ and phytoplankton biomass (µg Chl *a* L^{-1}, $R^2 = 0.35$, $P < 0.001$, Figure 1), suggesting a shift from diffusive to active uptake of inorganic carbon during blooms. Over the course of this study, bloom conditions, defined as > 40 µg Chl *a* L^{-1} (Table 1; Bachmann et al. 2003), were observed in 46% of our observations with varying degrees of intensity.

We found a significant, positive, non-linear relationship between the stable isotopic composition of the DIC pool and photosynthetic fractionation (ε_p, R^2=0.72, P<0.001, Figure 2). Specifically, the lowest ε_p was observed when the $\delta^{13}C_{DIC}$ values were less than -8 ‰, or atmospheric levels. Below this level, ε_p decreased exponentially toward zero.

To evaluate the predicted shift in algal carbon assimilation strategies below atmospheric equilibrium, we used a nonlinear dynamic model to analyze the relationships between ambient pCO_2 and $\delta^{13}C_{phyto}$ across lakes and sampling events. We found that while no relationship existed between these variables above atmospheric equilibrium, there was a rapid, significant increase in $\delta^{13}C_{phyto}$ (Figure 3; R^2=0.58, P<0.001) and decrease in fractionation (Figure 4, R^2=0.66, P<0.001) as CO_2 was depleted below atmospheric equilibrium (393 ppm, NOAA Earth System Research Laboratory, http://www.esrl.noaa.gov/).

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202 **4.Discussion**

203 Our results indicate that alternative carbon assimilation strategies may be an important
204 mechanism sustaining HCBs in anthropogenically eutrophic and hypereutrophic lakes. While
205 previous studies found no predictive relationship between ambient pCO_2 and photosynthetic
206 fractionation (Bade et al., 2006), others have shown long term relationships between pCO_2 and
207 the isotopic composition of phytoplankton (Smyntek et al., 2012). Here we demonstrate that the
208 relationship between pCO_2 and photosynthetic fractionation exists only when pCO_2 drops below
209 atmospheric equilibrium during blooms. We found a similar clear breakpoint below atmospheric
210 equilibrium between pCO_2 and phytoplankton isotopic composition, together suggesting that
211 CCM mechanisms are switched on in phytoplankton communities when ambient water column
212 CO_2 is depleted below atmospheric levels.

213 The range of values for both $\delta^{13}C_{phyto}$ and ε_p associated with these trends is consistent with
214 previous laboratory and marine field studies demonstrating shifts from diffusive to active
215 inorganic carbon assimilation via CCM activation (Boller et al., 2011; Cassar, 2004; Erez et al.,
216 1998; Trimborn et al., 2009). Calculated photosynthetic fractionation was lowest during blooms,
217 consistent with phytoplankton CCM utilization. While other freshwater studies have
218 demonstrated similar variability in phytoplankton isotopic composition (Vuorio et al., 2006),
219 ours is the first to demonstrate the co-occurrence of decreased fractionation with CO_2 depletion
220 during blooms in eutrophic and hypereutrophic lakes. This mechanism likely provides a
221 competitive advantage to bloom-forming taxa when high productivity depletes ambient CO_2.

222 $\delta^{13}C_{DIC}$ values presented in Bade et al. (2006) were more negative than those measured in
223 our study, attributable to heterotrophic degradation of terrestrial organic matter in northern

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224 temperate oligotrophic to mesotrophic lakes (Bade et al., 2007). In contrast, $\delta^{13}C_{DIC}$ in our study
225 was relatively enriched in ^{13}C across all lakes and sampling events, with values ranging from -
226 12.5 to + 5.8 ‰, within the range of previously measured values for eutrophic lakes in the same
227 region (de Kluijver et al., 2014). Values in this range can be attributable to mineral dissolution
228 and geochemical fractionation of HCO_3^- at high pH values (Mook 1986; Boutton 1991; Bade et
229 al. 2004), and to biogenic methane production via acetate fermentation (Drimmie et al., 1991;
230 Simpkins and Parkin, 1993; Stiller and Magaritz, 1974). In oligotrophic/ mesotrophic lakes, these
231 differences correspond to higher average photosynthetic fractionation. In eutrophic/
232 hypereutrophic lakes, however, fractionation decreased with active uptake of mineral bicarbonate
233 (Sharkey and Berry, 1985).

234 We found a significant positive relationship between photosynthetic fractionation and
235 $\delta^{13}C_{DIC}$, which is opposite of what is generally expected in lakes. In other words, fractionation is
236 expected to increase with decreasing $\delta^{13}C_{DIC}$ values. Across trophic gradients (e.g., $\delta^{13}C_{DIC}$
237 values between -30 ~ + 5 ‰, Bade et al. 2004; de Kluijver et al. 2014, this study), these
238 relationships would be driven by decreased $\delta^{13}C_{DIC}$ with increasing biomass (i.e., blooms), and
239 decreased fractionation as CCMs are induced (Sharkey and Berry, 1985). In eutrophic and
240 hypereutrophic lakes, however, the range of $\delta^{13}C_{DIC}$ values are enriched overall. Our results
241 suggest that CCMs are functioning and fractionation is lowest when the DIC pool is sourced
242 from mineral dissolution and HCO_3^- is the predominant species (~ -15 to 0 ‰, Boutton 1991).
243 Fractionation increased in these lakes as $\delta^{13}C_{DIC}$ became more positive, possibly indicating a
244 groundwater –sourced CO_2 generated from organic acid decomposition prior to microbial
245 methanogenisis (Simpkins and Parkin, 1993).

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In eutrophic lakes, both phytoplankton isotopic composition and fractionation appear to be strongly related to pCO_2 availability below a critical equilibrium point. In less productive northern temperate lakes, however, CO_2 is a poor predictor of photosynthetic fractionation (Bade et al., 2006). Our lowest modeled fractionation values reflected active uptake of HCO_3^-, supported by elevated phytoplankton isotopic values. In contrast, northern temperate lakes had a narrower range of phytoplankton isotopic composition (more negative on average), and much higher ambient CO_2 concentrations, both attributable to heterotrophic degradation of terrestrial carbon. These results indicate inorganic carbon availability drives photosynthetic fractionation in eutrophic lakes, but that other processes likely control it (e.g., temperature) in low-nutrient ones.

Our results have important implications for how HCBs may be sustained in anthropogenically eutrophic systems. It is well established that high nutrient concentrations result in high phytoplankton biomass (Heisler et al., 2008). It is less clear, however, what mechanisms cause variability in timing and duration of blooms among eutrophic and hypereutrophic lakes. CCMs may provide a competitive advantage to Cyanobacteria when high primary productivity depletes ambient CO_2. This mechanism may allow blooms to be sustained for weeks to months at a time with negligible concentrations of CO_2 in the water column (Cotovicz et al., 2015). While nutrient reduction is ultimately critical in the prevention of blooms (Heisler et al., 2008; Rigosi et al., 2014), the mechanism presented here provides insight into causes of bloom duration and intensity at high nutrient concentrations.

Our results show that eutrophic lakes function substantially differently than less impacted surface waters. Temperate lakes are generally considered sources of CO_2 to the atmosphere (Tranvik et al., 2009). We demonstrate that phytoplankton CCM use allows dense phytoplankton to grow at low CO_2 and may facilitate extended periods of high primary production, CO_2

depletion, and atmospheric CO_2 uptake in surface waters. These processes may increase sediment C burial and the export of autochthonous organic C (Heathcote and Downing, 2011; Pacheco et al., 2014), and may have the potential to increase methane emissions from anoxic sediments (Hollander and Smith, 2001). Our work demonstrates fundamental differences in inorganic carbon utilization between northern temperate and agricultural, eutrophic lakes. Because the extent of impacted, high nutrient lakes is predicted to increase with the food demands of a growing human population (Foley et al., 2005), understanding mechanisms driving carbon cycling in these systems will be critical in evaluating the impact of HCBs on global carbon cycles.

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459 **Figure legends**

460 **Figure 1** Linear relationship between phytoplankton $\delta^{13}C$ and chlorophyll *a*, indicating isotopic
461 enrichment increased with phytoplankton biomass. Dashed line indicates phytoplankton bloom
462 conditions, defined here as >40 µg Chl *a* L^{-1} (Bachmann et al., 2003).

463 **Figure 2** Relationship between the stable isotopic signature of the ambient DIC pool and
464 photosynthetic carbon fractionation . Vertical line indicates an atmospheric DIC source (7.8 ‰,
465 VPDB).

466 **Figure 3** Relationship between the stable isotopic ambient pCO_2 concentration in surface water
467 and the stable carbon isotopic signature of the phytoplankton community. The vertical line
468 indicates atmospheric equilibrium when samples were collected (393 ppm).

469 **Figure 4** Relationship between photosynthetic fractionation (εp) and pCO_2. Vertical line
470 indicates atmospheric CO_2 equilibrium when study was conducted (393 ppm).

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Table 1. Summary data for lakes included in this study. Total phosphorus (TP), total nitrogen (TN), and chlorophyll *a* (Chl *a*) are reported as average values of all sampling events (ice free season, April to November 2012)± standard deviation.

Lake	*Latitude*	*Longitude*	*TP (μg L^{-1})*	*TN (mg L^{-1})*	*Chl a (μg L^{-1})*
Arrowhead	42.297218	-95.051228	25 ± 8	0.8 ± 0.1	10 ± 6
Badger	42.586161	-94.192562	58 ± 35	9.4 ± 5.7	33 ± 34
Beeds	42.770320	-93.236436	75 ± 48	7.4 ± 4.5	48 ± 40
Big Spirit	43.479377	-95.083424	46 ± 22	1.1 ± 0.3	22 ± 22
Black Hawk	42.296334	-95.029191	225 ± 118	2.4 ± 0.5	78 ± 35
Center	43.412607	-95.136293	104 ± 50	1.8 ± 0.2	41 ± 36
East Osceola	41.032548	-93.742649	195 ± 77	1.9 ± 0.4	80 ± 47
Five Island	43.145274	-94.658204	106 ± 50	2.1 ± 0.3	67 ± 37
George Wyth	42.534834	-92.400362	62 ± 22	1.0 ± 0.2	26 ± 7
Keomah	41.295123	-92.537482	106 ± 105	1.4 ± 0.6	44 ± 52
Orient	41.196669	-94.436084	397 ± 286	2.3 ± 1.2	144 ± 105
Lower Gar	43.352299	-95.120186	95 ± 35	1.6 ± 0.2	50 ± 23
Rock Creek	41.736936	-92.851859	115 ± 44	1.7 ± 0.4	52 ± 49
Silver (Dickinson)	43.439162	-95.336799	161 ± 85	2.1 ± 0.9	35 ± 58
Silver (Palo Alto)	43.030775	-94.883701	339 ± 206	2.5 ± 0.6	117 ± 60
Springbrook	41.775930	-94.466736	38 ± 25	1.8 ± 0.9	17 ± 14

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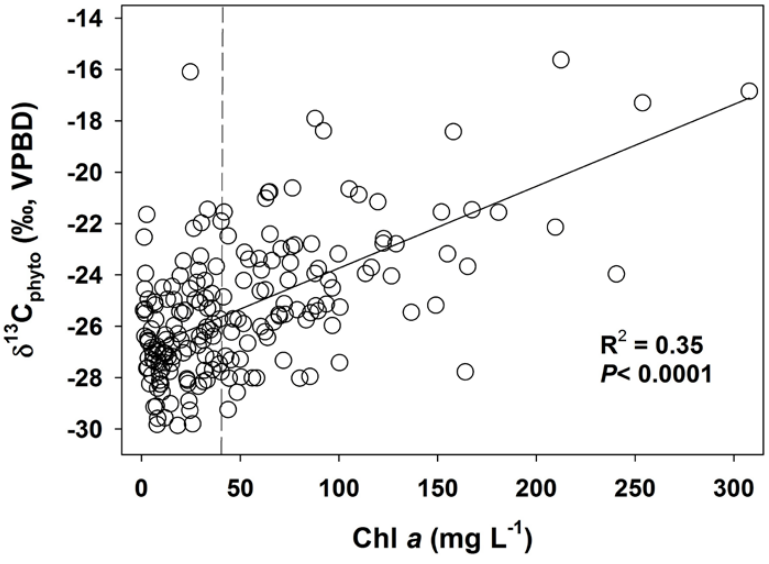
488 **Figure 1**



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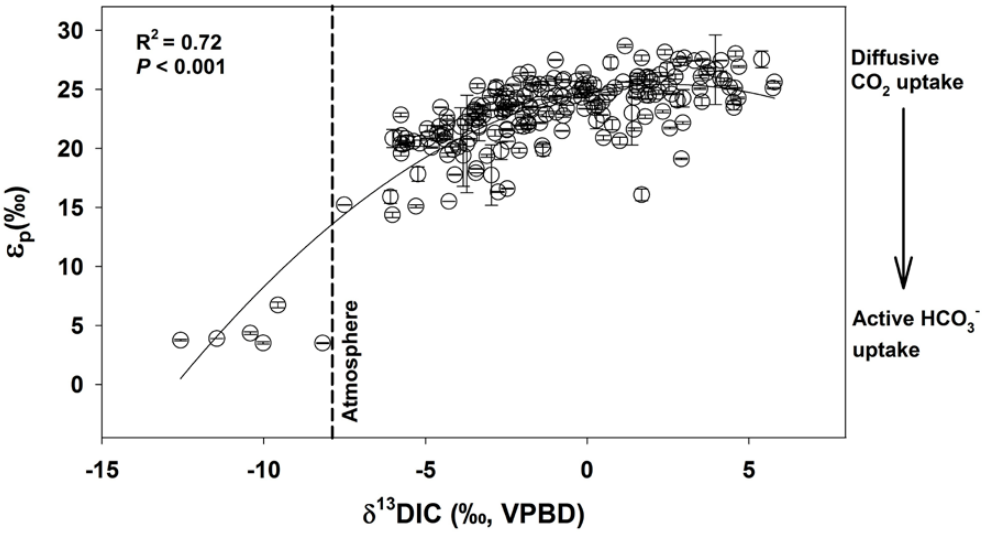
490 **Figure 2**



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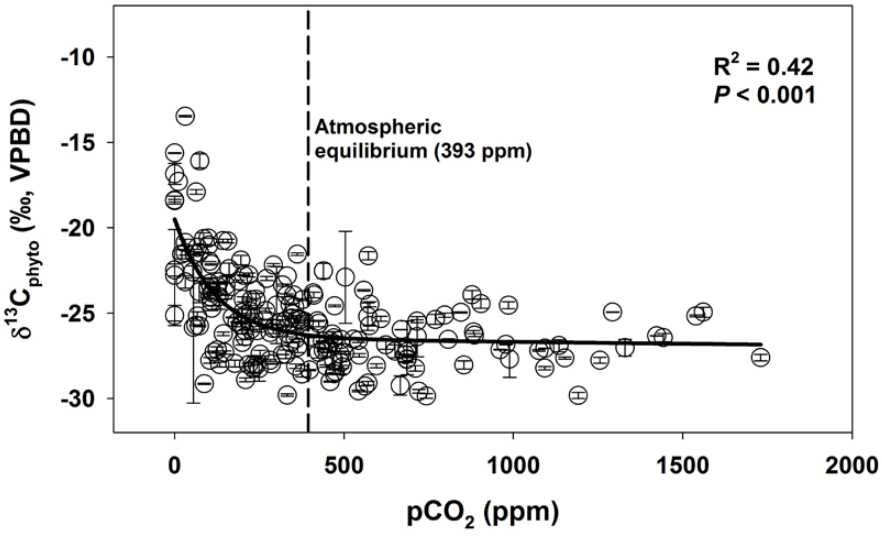
492 **Figure 3**



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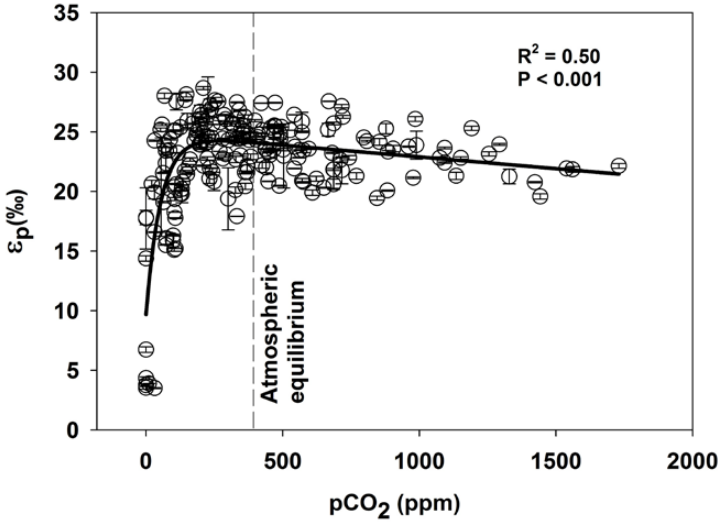
494 **Figure 4**



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