Water Velocity and Light Intensity Effects on Carbon Stable Isotope Ratios ($\delta^{13}C$) of *Cladophora glomerata*

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UMBS REU 2007

Intraspecific variability in aquatic autotrophic carbon isotope ratios, $\delta^{13}C$, contests the precision and accuracy of current methods of producer-consumer association with stable isotope analyses. In attempt to understand the influence of physical environmental factors on $\delta^{13}C$ signatures of aquatic producers, the effects of water velocity and irradiance on $\delta^{13}C$ were examined in *Cladophora glomerata* with a controlled flume experiment. A highly significant inverse relationship was observed between water velocity and *C. glomerata* $\delta^{13}C$, likely due to velocity effects on boundary layer and carbon dioxide diffusion limitation. Light regime was not found be a significant independent affecter of $\delta^{13}C$, but did exert an interactive effect with velocity, accentuating degree/magnitude/difference of signature enrichment between high and low velocity regimes. It is probable that irradiance acts as an interactive affecter on $\delta^{13}C$ by 1) increase in photosynthetic rate, and thus carbon demand, accentuating overall carbon limitation and decreased isotopic discrimination at low water velocities; and 2) increased utilization of $\delta^{13}C$-enriched bicarbonate as a carbon source for photosynthesis with higher irradiance, contributing to overall signature enrichment. A field study examined *in situ* effects of water velocity and irradiance on $\delta^{13}C$ of *Cladophora spp.* and *Spirogyra spp.*; algal filaments growing in similar microhabitats varying in water velocity and canopy coverage were sampled and analyzed, but no correlations were found between water velocity or irradiance and $\delta^{13}C$. Lack of *in situ* support for trends observed in controlled experiments are likely attributable to very limited field sample sizes, myriad confounding and noisy factors of dynamic environmental systems, and lack of precision (to-species identification) in algal taxonomic identification.

Introduction

Carbon stable isotope ratios ($\delta^{13}C$) are widely used to deduce herbivore food sources and study food web dynamics of both terrestrial and aquatic ecosystems. Plants have been observed to exhibit characteristic ^{12}C and ^{13}C relative abundance signatures that vary by taxonomic group and are passed on predictably to consumers. As a minimal (0-1‰) isotopic enrichment occurs with trophic transfer, consumers retain carbon isotope ratios virtually identical to those of their food source(s). (Cornelisen et al. 2007; DeNiro and Epstein 1978; McCutchan et al. 2003). Comparison of isotopic signatures of consumers and local producers can be used to infer critical ecosystem energy bases and consumers' alimentary preferences. Accurate association of consumer and primary carbon sources, however, may be compromised by environmentally-induced variability in isotopic signature within aquatic producer groups.

Aquatic plant isotope variability (-3 to -35‰, as reported by Raven et al. 2002, as referenced in Cornelisen et al. 2007) has been found to greatly exceed the magnitude of predicted isotopic enrichment from food source to incorporation in consumer tissue (0-1‰). (Cornelisen et al. 2007; DeNiro and Epstein 1978; McCutchan et al. 2003), contesting the accuracy and precision of present consumer-producer linkage methods

using carbon isotope ratios. Terrestrial plants exhibit relatively narrow $\delta^{13}C$ ranges, closely related to their method of carbon fixation (C_3, C_4, or Crassulacean Acid Metabolism); the contrastingly broad, possibly continuous range of aquatic plant $\delta^{13}C$ signatures suggests an interplay between both physiological and physical/environmental factors in aquatic producer carbon isotope ratio determination. (Hecky and Hesslein 1995)

Though variability in autotrophic $\delta^{13}C$ exceeds that which can be accounted for by isotopic variability of inorganic carbon sources available to plants (atmospheric CO_2, biogenic CO_2, and weathered bicarbonate), (Finlay et al. 1999) it remains somewhat unclear how other environmental factors (*i.e.* light, temperature, water velocity, dissolved CO_2 concentration, equilibrium of CO_2 and H_2CO_3 interconversion reaction, pH) independently and concertedly affect aquatic plant $\delta^{13}C$ signatures. Autotroph interspecific signature overlap and degree of intraspecific $\delta^{13}C$ variability are also poorly understood. Studies suggest a significant effect of physical environmental factors on algal isotopic variability, even on small spatial scales (on the order of meters). (Raven et al. 1995; France and Holmquist 1997; Finlay et al. 1999; Cornelisen et al. 2007).

Influences of physical environmental factors on aquatic plant $\delta^{13}C$, as they are presently incompletely understood, may confound current methods of producer-consumer association, reliant on the assumption of insignificant spatial and temporal intraspecific variability in autotrophic $\delta^{13}C$. Improved characterization of the magnitude and mechanistic bases of environmentally-derived producer $\delta^{13}C$ variability may present the opportunity for higher-resolution analyses of food web dynamics, including spatial and temporal characterization of feeding habits and range.

The typical $\delta^{13}C$ values of common inorganic carbon sources--carbon dioxide (-7‰) and bicarbonate (+1‰)--fail to account for highly ^{13}C-depleted signatures of some plants with either C_3 or C_4 photosynthesis (reported by Throughton et al. 1974 as –25 to –30.5‰, and –11.9 to –15.2‰, respectively). (Throughton et al. 1974, as referenced in Wefer and Killingley 1986). Rubisco, the carboxylating enzyme in the C_3 carbon fixation pathway, kinetically discriminates against the heavier ^{13}C isotope, leading to production of simple sugars about –28 to –30‰ compared with source carbon dioxide (Farquhar et al. 1989 as referenced in Hecky and Hesslein 1995) when CO_2 is in excess. Discrimination against $^{13}CO_2$, though, declines with decreasing CO_2 availability. (Calder and Parker 1973; Pardue et al. 1976 as referenced in France et al. 1999) Physical factors, *i.e.* light and water velocity, likely affect CO_2 availability and thereby plant $\delta^{13}C$ signatures.

This study sought to investigate the effects of water velocity and light intensity, and the interactive effects of these two factors, on carbon isotope ratios of the filamentous green macroalga *Cladophora glomerata* (Chlorophyta). Occurring in both salt- and freshwater environments, it is most often attached to substrate by rhizoidal cells, and grows into dichotomously or trichotomously branching, cylindrical filaments (75-100μm diameter) up to 30 cm in length, often dangling off of the substrate surface. (Bold and Wynne 1978; Prescott 1968; Collins 1909) There is substantial morphological variability within the species. The robust nature and virtually ubiquitous occurrence of *Cladophora* species within a wide range of habitats makes them important producers and contributors to food webs of a variety of ecosystems, and they are often used in isotopic

analyses of ecosystem trophic structure. (Doucet et al. 1996; Rooker et al. 2006; Arroyo 2007; Turner and Rooker 2006)

A few studies have reported significant algal $\delta^{13}C$ enrichment with velocity in individual algal species, communities, and algae-feeding macroinvertebrates. (Moore, 2002; Trudeau and Rasmussen, 2003; Hecky and Hesslein, 1995; Finlay et al. 1999) Water velocity inversely affects boundary layer thickness; under low-velocity, stagnant conditions, boundary layer around plant cell walls is relatively large and carbon dioxide must diffuse a greater distance to reach an algal filament and be taken up for photosynthesis. As the diffusion coefficient of carbon dioxide and bicarbonate is about 10^4 times greater through water than air (Osmond et al. 1981), effects of boundary layer on aquatic plants are much more extreme compared with terrestrial plants. Increased difficulty of diffusion with thicker boundary layer decreases carbon dioxide supply to plants, and decreased isotopic discrimination with decreased supply likely accounts for enrichment of $\delta^{13}C$ signatures at lower water velocities.

Previous studies have reported up to 20‰ variability of $\delta^{13}C$ with light regime in the algal species *Desmarestia antarctica* (Wiencke and Fiscer 1990, as cited in Cornelisen et al. 2007), as well as significant enrichment of $\delta^{13}C$ signatures with higher irradiance. (Cornelisen et al. 2007; Kübler and Raven 1995; MacLeod and Barton, 1998; Wefer and Killingley, 1986) Light intensity directly affects photosynthetic rate, increasing plant carbon demand. This essentially decreases CO_2 supply relative to demand whenever CO_2 is not in excess, and the relative decrease in supply may account for decreased plant isotopic discrimination and observed $\delta^{13}C$ enrichment at higher irradiances. Compensation for higher carbon demands may manifest as higher overall CO_2 fixation and cellular retention, with decreased efflux of ^{13}C, producing $\delta^{13}C$ enrichment. (Cornelisen et al. 2007)

Carbon isotopic signature enrichment at higher irradiance may, in some species, be additionally be attributable to utilization of alternate dissolved inorganic carbon, particularly bicarbonate, with higher naturally occurring ^{13}C:^{12}C compared with carbon dioxide ($\delta^{13}C$ of HCO_3^- is ~+1‰, compared to ~-7‰ for CO_2). (Wefer and Killingley 1986) Under low levels of irradiance, carbon requirements for relatively slowed photosynthesis may be met principally by CO_2 uptake, but at higher irradiances some plants may rely more heavily on HCO_3^- uptake to meet higher photosynthetic carbon demands. (Cornelisen et al. 2007)

Cladophora glomerata seems capable of direct and indirect utilization of HCO_3^- as a carbon source for photosynthesis, in addition to dissolved carbon dioxide (Gessner 1959; Dahm 1926; Steeman-Nielsen 1947, 1949); Lindahl 1963 as referenced in Raven et al. 1982), by means of three distinct mechanisms: 1) extracellular/periplasmic carbonic anhydrase-catalyzed dehydration of HCO_3^- to CO_2, followed by CO_2 diffusive uptake; (Choo et al. 2002; Smith and Bidwell 1989, Haglund et al. 1992, Larsson and Axelsson 1999; Moroney et al. all as referenced in Choo et al. 2002); 2) direct HCO_3^- uptake by an ion exchange protein; (Choo et al. 2002; Drechsler and Beer 1991; Drechsler et al. 1993; Larson et al. 1997 all as referenced in Choo et al. 2002); 3) vandate-sensitive proton pumps (P-type H+-ATPase) (Kaplan et al. 1982; Thielmann et al. 1990 as referenced in Choo et al. 2002). Kübler and Raven (1995) suggest a resource allocation-based tradeoff between HCO_3^- use and light-harvesting efficiency, noting that *Palmaria palmata* exhibits compromised bicarbonate uptake under low light levels. Decreased HCO_3^-

uptake under light limitation likely contributes to $\delta^{13}C$ signature enrichment in organisms capable of direct or indirect use of alternate dissolved inorganic carbon sources for photosynthesis.

A 2007 study by Cornelisen et al. examining combined effects of irradiance and water velocity on $\delta^{13}C$ of the marine alga *Ulva pertusa* reports $\delta^{13}C$ enrichment under higher levels of irradiance, but counteraction of these effects at increased water velocities, suggesting a significant contribution of the interplay of light and stream flow factors to the environmental determination of algal $\delta^{13}C$. (Cornelisen et al. 2007)

It was hypothesized that differing carbon isotope ratios ($\delta^{13}C$) would be observed in *Cladophora glomerata* grown in different stream flow and light regimes, with an expected inverse relationship between water velocity and $\delta^{13}C$ and direct relationship between irradiance and $\delta^{13}C$. Additionally, light and water velocity variables were predicted to have an interactive effect on $\delta^{13}C$, producing greatest signature enrichment under conditions of high light and low velocity, and greatest depletion under low light and high flow.

Methods

Water from the East Branch of the Maple River was pumped continuously into forty flumes/artificial streams, constructed at the University of Michigan Biological Station Stream Lab. *Cladophora glomerata* samples were collected from the downstream culvert of the East Branch of the Maple River below the Lake Kathleen dam in Emmett County, Michigan. Filaments with minimal visible epiphytic diatom flora were selected and cut on both ends with a scalpel to 3 cm lengths. Three filaments were doubled over and tethered with fishing line 1 cm above the downstream edge of ceramic tiles attached to the bottom of each flume. (APPENDIX A, Figure 4C) Algae were cultured for twelve days at variable water velocity (initial velocity settings of 5, 25, 40, and 70 cm/s with ten replicates). Half of the trials for each velocity category were shaded throughout the experimental period, while the other half were left exposed to full sun. (APPENDIX A, Figures 4A, 4B)

Bi-daily, measurements of discharge-per-unit-time were taken for each flume, and used to determine the average flume velocity over the experimental period. Bi-daily adjustments of water discharge and depth were made for individual flumes in order to attempt to achieve target categorical velocities.

After twelve days of culture, filaments were removed from tiles, rinsed with tap water, and cleaned of diatoms and detritus with forceps and a scalpel. Algal samples were dried at 60°C overnight, coarsely ground and disrupted with a metal spatula, treated with 0.2ml 0.1M HCl approximately 30 minutes to remove exogenous inorganic carbon from algal samples, and dried again overnight at 60°C. Samples were then weighed and analyzed by mass spectroscopy to determine by-weight carbon isotope signatures.

A field study was conducted to examine the *in situ* effects of water velocity on *Cladophora spp.* and *Spirogyra spp*. Four sample sites in Emmett County, Michigan in the East Branch of the Maple River were selected for presence of *Cladophora* or *Spirogyra* algal growth in multiple microhabitats of varying water velocity within a small spatial range (~50m?) within each site. (APPENDIX A, Figure 5) Water velocity was measured with an electromagnetic Marsh-McBirney flowmeter approximately 1 cm above the trailing end of *Cladophora* filaments. A denseometer held one meter above

algal growth was used to determine canopy cover. Water temperature, microsite water depth, and algal depth were also recorded. Ends of algal filament clusters were sampled (~2-4 cm in length), dried, acid-treated, and analyzed by the same procedure employed for flume experiment sample analysis.

Results

C. glomerata from flume experiments exhibited a range of $\delta^{13}C$ values of 4.84‰, from –33.66‰ to –28.82‰ (lowest-velocity full-sun treatment). There was a significant linear decline in *Cladophora glomerata* carbon isotope ratio ($\delta^{13}C$) with increasing water velocity ($y=-0.032x-30.185$; $R^2=0.5472$; Figure 1). This inverse relationship was also observed when full sun-exposed and shaded treatment groups were examined separately ($y=-0.0426x-29.654$; $R^2=0.6777$; Figure 2 and $y=-0.0226x-30.589$; $R^2=0.4432$; Figure 3, respectively).

Two-way analysis of variance was conducted twice, with velocity variable re-coding into to examine $\delta^{13}C$ variation attributable to water velocity and light variables; velocity data was re-coded into two and four categories (5.07-31.08 cm/s, 39.39-80.21cm/s; and 5.07-8.15; 18.95-31.08; 39.39-51.51; 59.28-80.21 cm/s) and compared in conjunction with dark (shaded) and light (unshaded) irradiance categories. Both tests showed a highly significant independent effect of water velocity, but not light regime ($p=0.758$; $p=0.716$) on $\delta^{13}C$ *Cladophora glomerata*. Both ANOVA tests suggest that light regime did, however, did exert a significant ($p=0.025$; $p=0.047$ for Light*Water Velocity parameters) interactive effect with velocity on $\delta^{13}C$, influencing the magnitude of velocity influence over algal carbon isotope signature. A greater difference in mean $\delta^{13}C$ of low and high velocity groups was found under high irradiance than for shaded tests. (Figure 4)

Cladophora and *Spirogyra* samples were collected from microsites of a wide range of water velocities--1 to 114 cm/s for *Cladophora* and 1 to 74 cm/s for *Spirogyra*. Field samples ranged in carbon isotope ratio from –35.44 to –25.65‰ for *Cladophora* and 35.14 to –23.2‰ for *Spirogyra*. Regression analyses of (limited) field data showed no significant correlation between either water velocity or canopy coverage and carbon isotope ratios of *Cladophora* or *Spirogyra* samples, either within sites or overall. Within sites, a maximum range/variation of *Cladophora* $\delta^{13}C$ signatures of 3.13‰ was observed, with a range of 9.79‰ overall for all *Cladophora* field samples. Maximum intra- and inter-site $\delta^{13}C$ variability of 11.95‰ was found for *Spirogyra* samples.

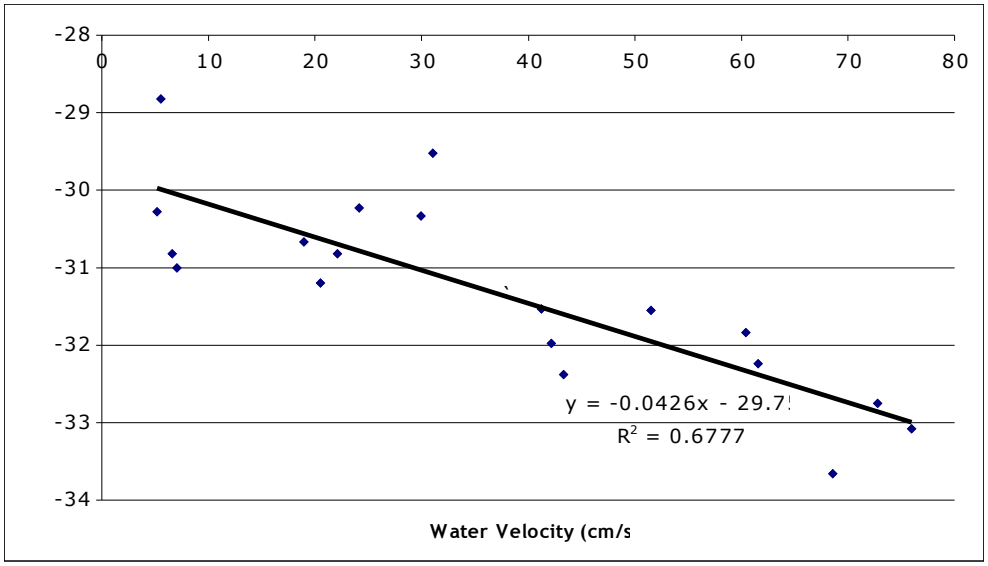

Figure 1. The effect of water velocity on $\delta^{13}C$ of *Cladophora glomerata* grown under high irradiance (no shading).

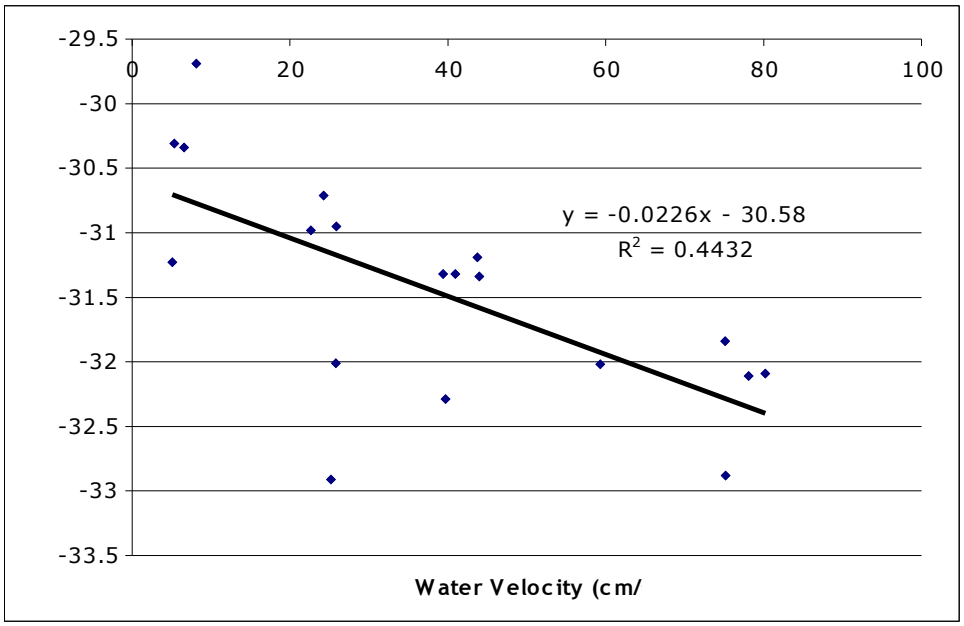

Figure 2. The effect of water velocity on $\delta^{13}C$ signature of *Cladophora glomerata* grown in shaded flumes.

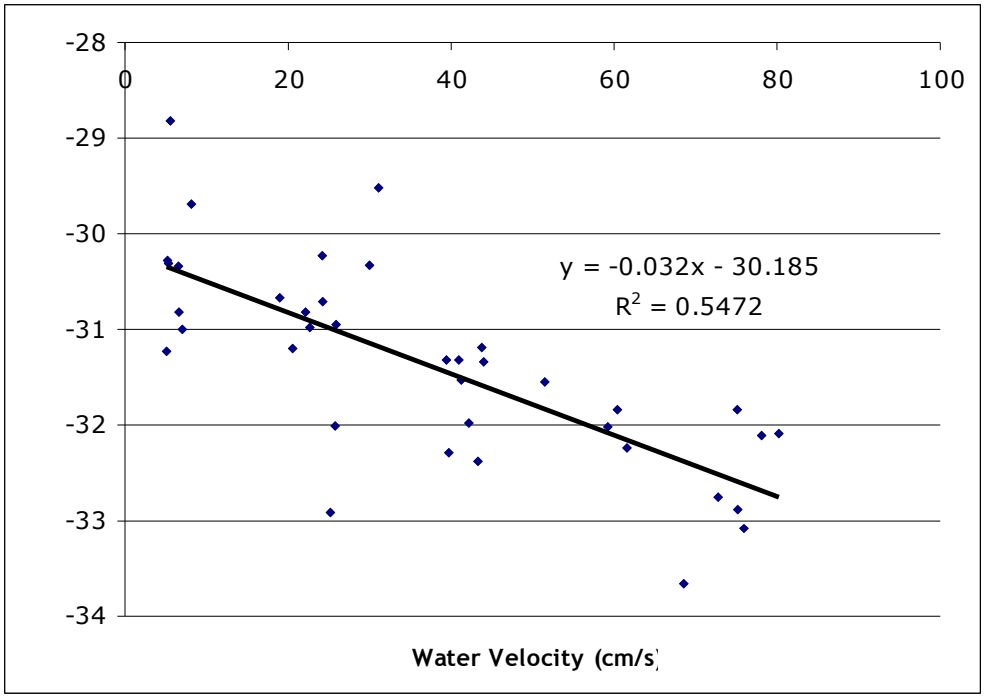

Figure 3. Overall effect of water velocity on $\delta^{13}C$ of *Cladophora glomerata*, including both shaded and full sun-exposed treatments.

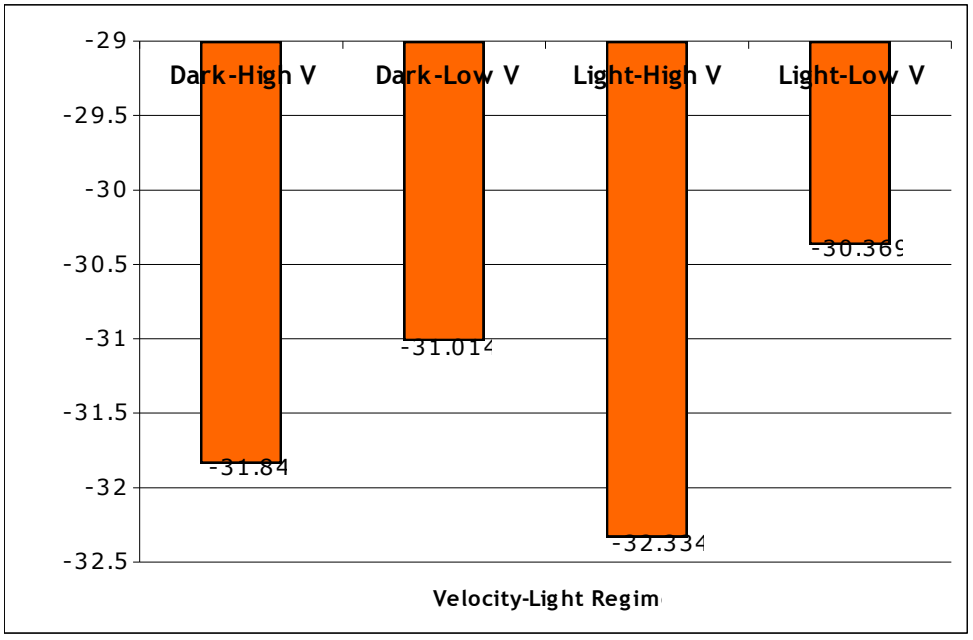

Figure 4. Interactive effects of water velocity and light on *Cladophora glomerata* $\delta^{13}C$: light-velocity categorical means from 2-way ANOVA with categorical re-coding of velocity variable (two categories) (Water velocity: $p<0.0005$; Light: $p=0.758$; Light*Velocity: $p=0.025$)

Discussion

Controlled flume experiment findings of an inverse relationship between $\delta^{13}C$ signature and water velocity provide support for the prediction that water velocity directly affects carbon isotopic discrimination in *Cladophora glomerata*. It is likely that the influence of water velocity on $\delta^{13}C$ is exerted principally through boundary layer effects on carbon dioxide diffusion. Increased boundary layer thickness and CO_2 diffusion limitation with higher velocity lowers isotopic discrimination by Rubisco. With CO_2 diffusion being more rate-limiting than the carboxylation reaction of Rubisco with CO_2 and ribulose biphosphate, CO_2 is consumed virtually immediately as it reaches Rubisco, allowing for little expression of enzymatic kinetic preference for the lighter ^{12}C isotope. (Geider and Osborne, 1992)

Lack of observation of a distinct, independent influence of light regime on $\delta^{13}C$ in flume study results suggests, perhaps, that increased carbon demands for photosynthesis at high irradiance are of minimal magnitude and importance compared with other factors influencing carbon limitation, such diffusion rate. Alternately, the two light regimes tested may have been insufficiently different to allow observation of significant light-driven effects on $\delta^{13}C$.

Light regime was, however, a significant affecter of $\delta^{13}C$ when considered interactively with water velocity. The ANOVA test revealed a greater difference in means between the high and low velocity categories under high light regime than between high and low velocity treatments grown under low light. Low velocity boundary effects decreasing carbon dioxide diffusion contribute more significantly to carbon limitation under high light conditions, under which carbon demand is higher. Increased carbon demands compound the effects of velocity-induced diffusion limitation and reduction in CO_2 availability on overall carbon dioxide limitation; water velocity boundary layer effects, then, contribute more strongly to overall carbon dioxide limitation, and thereby to decreases in isotopic selectivity, reflected by $\delta^{13}C$.

Additionally, irradiance may contribute to $\delta^{13}C$ enrichment by way of induction or increase of bicarbonate uptake and utilization in photosynthesis of *C. glomerata*. Fixation of bicarbonate-derived carbon, with an average source $\delta^{13}C$ value with 8‰ enrichment compared with carbon dioxide, may contribute substantially to intraspecific $\delta^{13}C$ variability. Resource diversion to light-harvesting efficiency mechanisms at lower light levels has been observed to compromise bicarbonate use in photosynthesis; higher light levels drive more rapid photosynthesis and provide the energy required to operate cellular machinery implicated in bicarbonate uptake and use (carbonic anhydrase, membrane transport systems). Greater contribution of bicarbonate to photosynthesis-assimilated carbon, relative to the contribution of more ^{13}C-depleted CO_2 would result in enrichment of plant $\delta^{13}C$ signatures. Both photosynthetic rate increase with irradiance, leading to exacerbation of water velocity-induced carbon limitation, and increased use of bicarbonate relative to carbon dioxide species likely contribute $\delta^{13}C$ enrichment with irradiance, and the observed interactive effect of velocity and irradiance on $\delta^{13}C$.

Under conditions of significantly rapid water velocity, rate of carbon dioxide diffusion would be expected to exceed enzymatic rate of consumption by Rubisco (directly affected by irradiance). As carbon dioxide uptake and delivery cease to be rate-limiting, full expression of carboxylation reaction kinetics, including discrimination against ^{13}C, would become dominant in determination of plant $\delta^{13}C$. Past such a

threshold velocity, then, $\delta^{13}C$ would be continuingly determined by carboxylation kinetics and remain virtually constant even with increasing water velocity (ignoring effects of other factors on diffusion limitation and assuming no carboxylation rate changes, such as those induced by increased irradiance). Experimental observation of a non-asymptotic, fully linear relationship of $\delta^{13}C$ versus water velocity suggests that no such threshold velocity relative to tested irradiances was achieved, and carbon dioxide diffusive limitation played a key role in $\delta^{13}C$ determination under all tested conditions.

Field experiment results failed to provide support for trends observed in controlled experiments, and showed no clear correlations between water velocity or irradiance and algal $\delta^{13}C$. Lack of observation of velocity and light effects *in situ* is likely attributable to very limited sample sizes, lack of precision (to-species identification) in algal taxonomic identification of field samples, and myriad confounding and noisy factors of dynamic environmental systems.

Although similar flow rates were examined in both controlled and field studies, it is likely that much greater differences in nature of flow—laminar versus turbulent—were present in the field compared to flumes. Differences in turbulences among microsites may have significantly affected direction and speed of flow experienced immediately at the algal filament surface, contributing to $\delta^{13}C$ variability even among samples for which comparable larger-scale Marsh McBirney flow measurements were taken.

Canopy density may be a poor indicator of total or photosynthetically active irradiance reaching algal filaments, affected by many variables including water depth, turbidity, and turbulence. Furthermore, small-scale variation in nutrient availability or presence of nearby producers giving off $\delta^{13}C$-depleted carbon dioxide as respiration by-product may have confounded the observation of effects of water velocity and light as indicated by canopy coverage on algal $\delta^{13}C$ in dynamic natural environments. Further field experimentation is required to untangle confounding variables and support or negate water velocity and irradiance-related trends observed under controlled experimental settings.

APPENDIX A

Flume Experimental Set-Up at the University of Michigan Biological Station Stream Lab

Forty artificial streams [LENGTH] were constructed from plastic gutters, evenly distributed atop five different wooden tables (eight streams per table). Water from the East Branch of the Maple River was pumped into plastic drums with consistent discharge (pressure head??) elevated above and delivering water to flumes of each of the five tables. Discharge and gutter inclination were adjusted to create water velocities of approximately 5, 25, 40, and 70 cm/s. Water depth was manipulated with terra cotta tiles positioned at the discharge end of the flumes in attempt to achieve a depth of 1.5 cm in each. Replicates of each flow regime were randomly distributed across the five tables. Discharge and depth of flumes were measured bi-daily to calculate average actual velocity of each flume over the 12-day experimental period. Half of the artificial streams of each velocity regime were randomly selected for coverage with black shading cloth [DIMENSIONS] doubled over wire mesh structures and fitted over the portion of the flumes in which algal samples were grown.

Figure 5. (clockwise from left) **A**) one of five table with eight flumes with different water velocity; water was pumped from the East Branch of the Maple into bins with constant overflow and adjustable-volume nozzles delivering water to each flume. Half of all flumes were randomly covered with shading cloth (shown here on flumes 2, 4, 5, 6, and 7 from the left); **B**) five adjacent tables (total 40 flumes); bin overflow pipe shown in upper right; **C**) flume shown from above (water current shown running left-to-right in photograph) with glued-down tiles on bottom with fishing line tethering of algal filaments (*Cladophora glomerata*, left, and *Spirogyra* sp., right, not included in study due to culture limitations).

Figure 6. Map of field study sites: East Branch of the Maple River; Emmett County, MI

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