Diurnal Photosynthesis Cycle in Cam and Non-Cam Seasonal-Pool Aquatic Macrophytes

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DIURNAL PHOTOSYNTHESIS CYCLE IN CAM AND NON-CAM SEASONAL-POOL AQUATIC MACROPHYTES[1]

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Abstract. Seasonal pools undergo marked diurnal changes in pH, free carbon dioxide, and oxygen levels. Previous studies showed that *Isoetes howellii* utilized crassulacean acid metabolism (CAM) photosynthesis as a means of assimilating carbon at night when ambient carbon dioxide levels are high. However, much of the pool flora is not CAM. We hypothesized that coexistence under extreme carbon-limiting conditions would select for other photosynthetic characteristics in these non-CAM species. Quantitative carbon uptake measurements were made in the field on the CAM *Isoetes howellii* and the non-CAM *Eleocharis acicularis, Downingia bella,* and *Plagiobothrys undulatus.* Despite wide phylogenetic separation, these species are structurally convergent in that they all produce an aquatic stage with an isoetid growth form. They are functionally similar in that none appear to utilize bicarbonate, and all are carbon limited through much of the day, as indicated by a marked midday depression in carbon uptake that lasts through the afternoon.

All four species were capable of carbon fixation at night, although only in *Isoetes howellii* was this accompanied by an overnight accumulation of acid. It was estimated that dark fixation in *I. howellii* contributed up to 40% of its total carbon gain under submerged conditions, but was <1% under aerial conditions. The role of dark fixation in the other three, non-CAM, species is unknown. Short-term (1 s) steady-state ^{14}C tracer studies in the laboratory revealed that, under submerged conditions in the light, all species assimilated carbon into the C_3 photosynthetic product phosphoglycerate (PGA) and the C_4 photosynthetic products of malate, citrate, and aspartate. The proportion of label fixed into C_4 organic acids was greatest in *E. acicularis.* The ratio of RuBP carboxylase/PEP carboxylase was broadly similar in all species, ranging from 8.8 to 11.5.

When the pools dried down and the plants became aerial, photosynthetic rates increased and a midday depression such as occurred under submergence was not observed. Carbon fixation in the dark became negligible, and the RuBP carboxylase/PEP carboxylase ratio increased. Seasonal changes in biomass showed *I. howellii* and *E. acicularis* dominated the pools early in the season, and the biomass of the other two species increased later when the pools dried. The latter species, *D. bella* and *P. undulatus,* also had higher rates of photosynthesis under aerial conditions.

Key words: aquatic macrophytes; C_4; competition; crassulacean acid metabolism; photosynthesis.

INTRODUCTION

California vernal pools are temporary bodies of water that have attracted a great deal of scientific interest because of the number of endemic species that are considered threatened or endangered (see Zedler 1987 for review). This habitat is of interest too because many vernal pools are dominated by *Isoetes howellii* Engelmann (Isoetaceae), a primitive vascular plant with a well-developed CAM (crassulacean acid metabolism) pathway under submerged conditions (Keeley 1981, Keeley and Bowes 1982). It has been hypothesized that this pathway evolved in this aquatic macrophyte as a means of competing for carbon under daytime carbon limitation.

Carbon limitation results from several unique char-

acteristics of these environments. Vernal pools form in depressions underlain with a hardpan and fill during the winter and spring rainy season but are dry throughout the summer and fall drought typical of this mediterranean climate. Much of the flora is annual, and most species germinate underwater, some of which cue the timing of germination to submergence through a dependence upon anaerobic conditions for germination (Keeley 1988). These shallow pools are densely vegetated and during the warm days of early spring, photosynthetic demand is capable of greatly reducing the water concentration of free CO_2 early in the morning. The pool water is poorly buffered, thus as the free CO_2 is depleted the pH of the water rises, often changing from pH 6–7 at sunrise to pH 8–10 at midday. Additionally, the water becomes supersaturated with oxygen during the daytime. Consequently these pools are largely inhospitable for photosynthetic organisms during much of the day. Overnight, respiration by the pool flora and invertebrate fauna, as well as diffusion from the atmosphere (and the lower temperature), once

again replenishes the free CO_2 in the water and reduces the oxygen level.

Previous studies (Keeley and Busch 1984) showed that for the aquatic CAM plant *I. howellii*, CO_2 assimilation rates were highest just after sunrise when free CO_2 concentrations in the water were highest. As free CO_2 levels dropped, carbon assimilation rates likewise dropped and throughout much of the day when the inorganic carbon pool in the water was dominated by bicarbonate, carbon assimilation by *I. howellii* remained low. Nighttime carbon uptake was estimated to contribute one-third to one-half of the total daily gross carbon assimilation. In addition the CAM pathway of dark carbon fixation contributed to the carbon budget by refixation of respiratory CO_2. It was concluded that diurnal changes in carbon availability had selected for CAM photosynthesis in this species.

Isoetes howellii coexists with other aquatic macrophytes that do not exhibit CAM (Keeley and Morton 1982). We hypothesize that if CAM is of selective value as a means of competing for carbon in this carbon-limited environment, then coexistence of non-CAM species would be promoted by other photosynthetic characteristics that increase the ability of non-CAM plants to compete for carbon. One option would be the capacity to continue photosynthesis throughout the day by utilizing bicarbonate. Another option would be a CO_2-concentrating mechanism involving a (daytime) C_4-type fixation similar to that already described for the aquatic macrophyte *Hydrilla verticillata* (L.F.) Royal (Holaday and Bowes 1980, Bowes and Salvucci 1984).

For this study three non-CAM species, common to vernal pools in southern California, were selected; a perennial sedge *Eleocharis acicularis* (L.) R. & S. (Cyperaceae), and two dicotyledonous annuals, *Downingia bella* Hoover (Campanulaceae) and *Plagiobothrys undulatus* (Piper) Johnston (Boraginaceae). For comparison the perennial *Isoetes howellii* was included in all studies. The two dicot species are endemic to California vernal pools, whereas *E. acicularis* and *I. howellii* are distributed in other seasonally aquatic habitats in the western United States. All four species are structurally convergent in that they produce an "isoetid" growth form of cyclindrial leaves or culms often arranged in a rosette. For the two dicot species this isoetid growth form is eventually replaced by laminate-shaped leaves, which emerge from the pools and exist in an aerial environment.

Quantitative carbon fixation rates in the light and dark were determined during a 48-h period on four occasions throughout the spring growing season. During each sampling period total biomass of each species was measured. In the laboratory ^{14}C tracer studies of short-term carbon fixation products in the light and dark were done and assays of specific activity of RuBP (ribulose bisphosphate) carboxylase and PEP (phosphoenol pyruvate) carboxylase. Anatomical sections and slides were made of aquatic and aerial foliage.

METHODS AND MATERIALS

Study site

Field studies were conducted on a large ($\approx\frac{1}{2}$ ha) seasonal pool on Mesa de Colorado, Riverside County, California, USA (elevation 675 m). This pool was filled to a depth of 35 cm during the first sampling period on 19–20 March 1986, and there was no standing water during the fourth sampling period on 30–31 May 1986.

Biomass sampling and specific leaf mass

At each sampling period aboveground biomass was sampled by collecting all plant biomass within 10 cm diameter plots ($n = 10$) randomly selected within the pool. In the laboratory these were separated by species and by reproductive vs. vegetative parts, oven-dried, and weighed. For the four species under study, fresh-to-dry mass ratios and specific leaf masses were determined on 10 samples as follows. On fresh foliage 30 2-cm segments were cut and top and bottom diameters measured. These were weighed, then oven-dried and reweighed. Total leaf area for a sample was calculated using the formula for surface area of a conical cylinder. Leaves were examined anatomically after being dehydrated through an alcohol series, embedded in paraffin, sectioned on a microtome, and stained. Photomicrographs were printed and cross-sectional area occupied by gas space was estimated by cutting out gas space from these prints and weighing before and after.

Carbon assimilation

In the field, rates of ^{14}C-carbon assimilation were determined on leaf/stem samples collected at 0600, 0900, 1200 and 1500 for measures of uptake in the light and at 0900 and 2400 or 2400 and 0300 for measures of uptake in the dark. Foliage was cut into 2-cm sections (0.25 g fresh mass) and incubated in 25-mL vials filled (and free of gas bubbles) with water collected from the pool at the time of sampling. After 10-min preincubation in a water bath at ambient light and temperature, experiments were initiated by injection of 185 kBq of $NaH^{14}CO_3$ (74 kBq/μmol) and then incubated for 30 min. Tests showed this level of $NaH^{14}CO_3$ altered the pH of the medium <0.1 unit. For measures of dark uptake, vials were covered with foil to eliminate any light penetration. Experiments were terminated by addition of boiling 80% methanol and killed samples were acidified and photobleached in sunlight.

Samples were returned to the laboratory, ground, and centrifuged for 10 min at 116.4 km/s^2 (11 870 g) and the supernatant, plus that from a methanol wash and a deionized H_2O wash of the pellet, was dried in an oven. The residue was resuspended in deionized H_2O, and the samples were counted in a 1:10 volume of Brays scintillation fluid on a LKB 1214 Rackbeta Liquid Scintillation Counter. Late in the season, emergent plants were sampled in air as follows: leaf sections

TABLE 1. Aboveground biomass of species collected from Mesa de Colorado vernal pool during the spring of 1986 ($n = 10$). On 20 March all foliage was submerged, on 30 April much foliage was aerial, and on 31 May all plants were terrestrial.

	Oven-dried biomass (g/m², $\bar{X} \pm$ SD)			
Species	20 March	11 April	30 April	31 May
Isoetes spp.*	164 ± 79	374 ± 243	560 ± 205	14 ± 31
Eleocharis acicularis	198 ± 205	260 ± 189	433 ± 237	56 ± 102
Downingia bella	5 ± 9	6 ± 10	4 ± 5	21 ± 41
Plagiobothrys undulatus	17 ± 34	74 ± 102	184 ± 127	52 ± 83
Total (includes six other species)	442 ± 217	897 ± 312	1058 ± 389	248 ± 244

* *Isoetes howellii* and *I. orcuttii*.

were placed on small screens in vials and 150 kBq of NaH^{14}CO$_3$ (74 kBq/μmol) was injected into 1 mL of 0.1 mol/L HCl beneath the screen. On all sampling occasions separate samples were returned on ice for chlorophyll determination. Foliage was ground on ice in 80% acetone and centrifuged for 20 min at 4°C and 116.4 km/s². Absorbance at 710 nm was subtracted from absorbances at other wavelengths prior to calculating chl *a* and *b*, as suggested by Sestak et al. (1971).

Acidity

Leaf samples (0.25 g fresh mass) were collected from the pools at 0600 and 1700, washed, blotted with tissue paper, and weighed. After grinding with 15.0 mL cold CO$_2$-free deionized H$_2$O, a 10.0-mL sample was immediately titrated with CO$_2$-free 0.01 mol/L NaOH to pH 6.4, and a 1-mL sample was deproteinized with an equal volume of 0.6 mol/L HClO$_4$ and returned to the laboratory for enzymic determination of malic acid (Gutmann and Wahlefeld 1974).

Environmental measurements

Photosynthetic photon flux density was measured with a LI-COR LI-188B integrating meter with a LI-190SB quantum sensor at the water surface. Temperature and oxygen in the water was determined with a YSI-5700 meter, and conductivity was measured with a YSI-33 meter at 25°. Water samples were assayed for alkalinity by titrating to pH 4.5 with 0.01 mol/L H$_2$SO$_4$ (American Public Health Association 1976). This endpoint was verified as the inflection point for alkalinity titrations of these waters. Free CO$_2$ was calculated from pH and alkalinity (Lind 1979). Chemistry of the interstitial water in the sediment was studied by burying dialysis bags (Spectrapor 1, Spectrum Medical Industries, Los Angeles, California, USA) filled with 200 mL of pool water after 7 d excavating them, measuring conductance and pH, and assaying for CO$_2$ and O$_2$.

^{14}C tracer studies

Plants were grown in artificial pools maintained on campus with substrate from the Mesa de Colorado site. The pools were kept filled with deionized water such that plants remained submerged. Emergent samples were maintained in an aerial environment for 2 wk prior to any assay. Foliage samples of 0.10–0.70 g were tied with a small thread into loose bundles. These were immersed in 25-mL stoppered serum vials filled with 60 mmol/L sodium potassium phosphate pH 6.0 buffer prepared fresh daily. Prior to injection of the isotope, samples were preincubated in the light for 15 min. Experiments were initiated by injection of 2800 kBq NaH^{14}CO$_3$ (330 kBq μmol). Vials were not stoppered, and after 1 s experiments were terminated by immersing leaf bundles in boiling 80% methanol on a heating plate and boiling was continued for several minutes. The sample was homogenized in a glass grinder and centrifuged at 116.4 km/s² for 20 min. The pellet was washed once in deionized H$_2$O and both supernatants combined. These were evaporated dry at 80° and then resuspended in 2 mL deionized H$_2$O. This was evaporated down to ≈500 μL and then centrifuged in capillary blood serum separator microcentrifuge tubes. An aliquot of this sample was taken for determination of total activity by liquid scintillation counting. Another aliquot was utilized for determination of labeled photosynthetic products.

Thin-layer chromatography (TLC) and electrophoresis were used to separate labeled products. Samples of 100 μL were spotted with capillary tubes on TLC cellulose (250 μm) covered glass plates (20 × 20 cm). Separation in the first dimension was with electrophoresis in pyridine : glacial acetic acid : H$_2$O (2:9:200) at pH 4.0 with solvent made fresh daily. The unit was an LKB Multiphor II with water circulating cooling plate (21 × 27 cm) maintained at 15°. Separation was run for 50 min at 900 V and 70–75 mA. Separation in the second dimension was done chromatographically in sec-butanol : acetic acid; H$_2$O (6:1:2) solvent made fresh daily. The solvent front was allowed to rise twice, the first time to 11 cm above the origin, after which the plates were blown dry under a cool airstream, and again to 15 cm above the origin. Autoradiographs were made by placing film sheets on the plates, wrapping tightly, and exposing for several days. Spots were identified by using the same separation techniques on authentic compounds and visualizing them by combinations of various stains. Spots detected by the autoradiographs were scraped from the plates, eluted in H$_2$O, and counted with liquid scintillation counting with an automatic quench curve for the cellulose.

Enzyme assays

Leaves were ground on ice in buffer (50 mmol/L Tris-HCl, 10 mmol/L MgCl$_2$, 0.1 mmol EDTA, 5 mmol/L isoascorbate, 10 g/L PVP-40), one sample at pH 8.0 and one at pH 8.5. Aliquots were taken for chlorophyll and protein assays; these were maintained on ice in the dark until the enzyme assay was completed. The enzyme extract was centrifuged at 116.4 km/s^2 for 5 min at 4°. This supernatant was assayed immediately. Both enzymes were assayed at 25°, using $^{14}CO_2$ fixation techniques and liquid scintillation counting of acid stable products. RuBP carboxylase was assayed in the active form as described by Lorimer et al. (1977). Experiments were initiated by addition of RuBP substrate and terminated after 1 min by the addition of 6 mol/L HCl. PEP carboxylase was assayed according to the procedure of Van et al. (1976) with a 2-min incubation. Experiments were initiated by addition of PEP substrate and terminated by addition of 6 mol/L HCL saturated with 2,4-dinitrophenylhydrazine to stabilize oxalacetic acid (OAA). Use of this compound required a separate quench curve. Soluble protein was determined with the Lowry method as modified by Bergmeyer (1974:172–174).

RESULTS

Biomass and morphology

During the first sampling period, aboveground biomass was sampled from both the center of the pool (an area of ≈ 500 m^2) as well as the periphery. Total biomass per unit area was nearly double in the center of the pool. *Isoetes howellii* and *Eleocharis acicularis* dominated the center of the pool, constituting over 80% of the biomass, whereas in the periphery these two species made up only 27% of the total biomass. In the periphery of the pool, species diversity was greatest and other important species were *Plagiobothrys undulatus* and *Eryngium aristulatum* Jepson. Very few terrestrial species from the adjacent grassland occurred within the boundary of the pool. By the second sampling period the periphery of the pool was largely dried down, and sampling was restricted to the center of the pool.

Throughout the period in which the pools were filled, the center was dominated by *Isoetes* species (*I. howellii* and *I. orcuttii* A. Eaton) and *Eleocharis acicularis* (Table 1). In the early part of the season (mid-March), water depth was ≈ 35 cm, and plants were entirely submerged. By late April the water depth was half this, and plant growth was partially aerial. When visible water disappeared completely, *I. howellii* and *E. acicularis* withered rather quickly, whereas *Downingia bella* and *Plagiobothrys undulatus* were more resistant to the terrestrial conditions (Table 1).

Individual plant size varied markedly among these species. From the second to the third sampling periods, dry mass per plant ($\bar{X} \pm$ SD, $n = 10$) increased from 0.73 ± 0.46 to 1.46 ± 0.74 g in *I. howellii,* from 0.15 ± 0.11 to 0.24 ± 0.21 g in *I. orcuttii,* 0.12 ± 0.09 to 0.35 ± 0.15 g in *D. bella,* and 0.02 ± 0.01 to 0.07 ± 0.05 g in *P. undulatus. Eleocharis acicularis* spreads by underground rhizomes so distinguishing an individual plant is difficult; individual rosettes remained at 0.03 ± 0.02 g during this period. By the second sampling period in early April nearly 100% of the two *Isoetes* species were reproductive, but none of the other species were, and by the third sampling period in late April only 3% of the *P. undulatus* were reproductive. None of the *E. acicularis* or *D. bella* were reproductive until the pools dried down in May; at the last sampling period, 100% of the *D. bella* and *P. undulatus,* but only 40% of the *E. acicularis,* were reproductive.

The four species under study are somewhat convergent in having an isoetid growth form of cylindrical leaves or stems with extensive lacunae (>50% cross-sectional area is gas space). *Isoetes howellii* produced a rosette of leaves (1.8–2.0 mm diameter) each with four large lacunae that occupied 81% of the cross-sectional space (Table 2). Stomata were present, but their pores were occluded by wax. *Isoetes howellii* leaves grew to a length of 15–25 cm and as the pool dried down, previously submerged leaves became aerial leaves. The only obvious changes in the foliage were that the leaves initiated under aerial conditions were approximately half the diameter of submerged leaves and the proportion of lacunal gas space was 39%. Aerial leaves had a lower fresh/dry mass ratio (10.7 ± 0.4, $n = 10$, cf. Table 2), a thicker cuticle, and stomatal pores were functional.

Eleocharis acicularis produced cylindrical leaves, which did not exceed 10 cm length. They were distributed in rosette-like clusters at nodes along a lateral underground rhizome. Anatomically they were similar to *I. howellii* except they had five lacunal chambers and the leaves were very narrow (0.3–0.4 mm diameter), with a low fresh/dry mass ratio (Table 2) and less biomass per unit surface area. Foliage changes upon emergence were similar to those observed for *I. howellii.*

The two dicot species were distinct in that there were marked differences between submerged and aerial foliage (e.g., Fig. 1). *Plagiobothrys undulatus* germinated under water and produced a rosette of semicylindrical leaves (1.5–2.0 mm diameter). The submerged foliage of *D. bella* was a wide (1.8–2.0 mm diameter) cylindrical stem with a very high fresh/dry mass ratio and high surface area per unit biomass (Table 2), but the sparsely distributed leaves were very thin and narrow and represented a minor part of the photosynthetic surface area of the submerged foliage. *Downingia bella* and *P. undulatus* lacked stomata in the submerged foliage but produced laminate aerial leaves with substantially less gas space (not quantified) and abundant stomata (e.g., *P. undulatus* shown in Fig. 1).

Epidermal cells in all species lacked chloroplasts, but

TABLE 2.   Submerged foliage characteristics of species collected from Mesa de Colorado vernal pool ($n = 10$).

	Fresh/dry mass ratio	Specific leaf mass* (g/m², $\bar{X} \pm$ SD)	Airspace (% of cross-sectional area)
Isoetes howellii	15.7 ± 4.0	13.6 ± 1.6	81
Eleocharis acicularis	7.0 ± 1.0	9.8 ± 2.4	66
Downingia bella	30.9 ± 6.6	8.3 ± 1.0	67
Plagiobothrys undulatus	15.6 ± 6.3	10.9 ± 3.3	54

* Dry mass per unit leaf surface area.

they were abundant in the tissues surrounding the large lacunal gas spaces, both around the periphery of the foliage as well as in the center. None of the species had Kranz anatomy.

On a fresh mass basis, *E. acicularis* had the highest chlorophyll levels and the other species were half this (Table 3). These other three species all showed a decrease in chlorphyll between the second and third sampling period but an increase upon emergence (Table 3). Chlorophyll *a* constituted 73–75% of the total and, upon emergence, all species showed a few percent increase in the percentage of chlorophyll *a*.

Quantitative carbon assimilation and acidity

Leaf acidity measurements (data not shown) followed patterns reported previously (Keeley and Morton 1982). *Downingia bella* and *Plagiobothrys undulatus* had no overnight increase in leaf acidity or malic acid. *Eleocharis acicularis* showed no consistent overnight increase in acidity, although occasionally there was observed a slightly higher (e.g., 1–5 µmol/g fresh mass) malic acid level in the morning. Overnight acidity [H⁺] increased substantially in *Isoetes howellii* under

submerged conditions, from a high during the second sampling period (10–11 April) of 192 µmol/g fresh mass to a low of 115 during the third sampling period (29–30 April). On the final sampling period (30–31 May) when the plants were growing terrestrially, no detectable change in acidity was noted for any species.

The diurnal changes in water chemistry were similar through the season (Figs. 2–4). Typically, free CO_2 levels were highest at sunrise and close to zero through the afternoon, while O_2 concentrations followed an opposite pattern. On the first sampling date (19–20 March) early morning water temperatures were below 10°C, and the morning depletion of CO_2 was relatively slow (Fig. 2). Three weeks later water temperatures were substantially warmer, and CO_2 was depleted from the water very rapidly between 0600 and 0900 (Fig. 3). During both April sampling periods (Figs. 3 and 4) the water pH rose to above pH 10 in the afternoon, suggesting that some component of the aquatic flora was consuming bicarbonate in addition to free CO_2. During this period there was an extensive bloom of the blue-green alga *Oscillatoria limosa*.

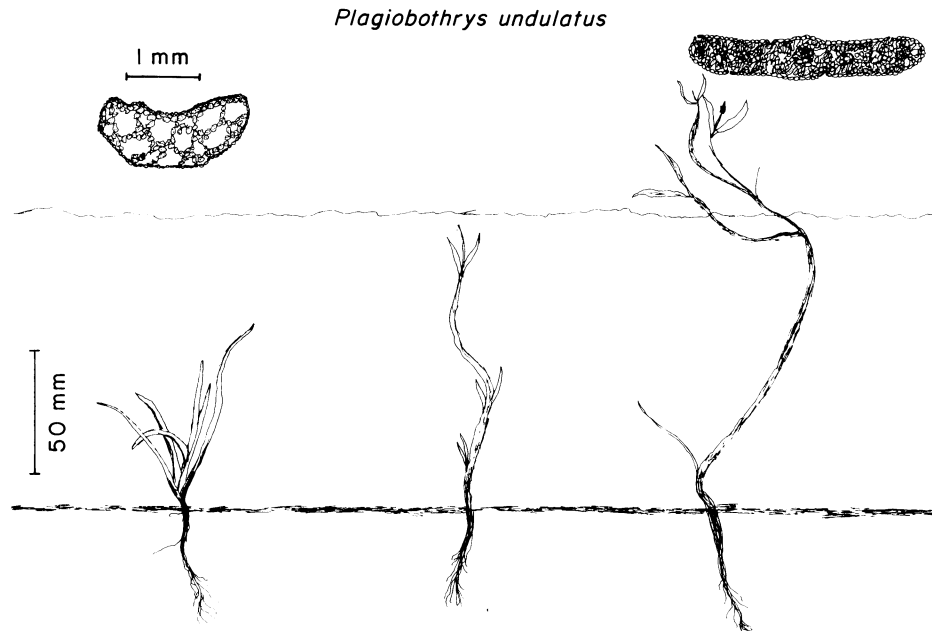Chemistry of the interstitial water in the sediment

FIG. 1.   Schematic sketch of *Plagiobothrys undulatus* morphological and anatomical changes in foliage from submerged to aerial conditions.

TABLE 3. Chlorophyll levels in species collected from Mesa de Colorado vernal pool (n = 3).

	Total chlorophyll (μg/g fresh mass, $\bar{X} \pm$ SD)			
	20 March	11 April	30 April	31 May
Isoetes howellii	560 ± 77	597 ± 41	393 ± 64	734 ± 9
Eleocharis acicularis	1536 ± 158	1380 ± 22	1721 ± 200	1559 ± 11
Downingia bella	433 ± 69	351 ± 20	344 ± 24	409 ± 2
Plagiobothrys undulatus	594 ± 77	869 ± 31	446 ± 33	512 ± 9

was studied by burying, during the second sampling period, dialysis bags filled with pool water and then excavating during the third sampling period and assaying. Two of these were sampled in the morning and two in the afternoon, but no difference was noted so these results are pooled (n = 4); specific conductance = 329 S/cm, pH = 6.5 ± 0.2, free CO_2 = 4.2 ± 0.6 mol/m³, and O_2 = 0.054 ± 0.034 mol/m³.

Throughout the season the daytime pattern of carbon assimilation was similar between the CAM *Isoetes howellii* and the other three, non-CAM, species (Figs. 2–5). Early in the season (Fig. 2) maximum carbon uptake rates were observed at 0900, whereas later in the season (Figs. 3 and 4) photosynthetic rates were highest at 0600. This seasonal change is most likely tied to the colder water temperature and the slower rate of CO_2 depletion from the water early in the season compared to later in the season. On the three sampling dates when the pools were in existence (Figs. 2–4), carbon uptake rates by all species declined between 0900 and 1200 as the CO_2 concentration in the water declined.

Carbon uptake rates in the dark were closely linked to levels of CO_2 in the water. Between 2100 and 2400, CO_2 concentration in the water increased markedly, as did carbon uptake rates by *Isoetes howellii* (Figs. 2 and 3). Of particular interest is the observation that the other "non-CAM" species showed measurable rates of dark carbon fixation on all sampling dates (Figs. 2–4).

By the third sampling date (late April; Fig. 4), the pool had shrunk in size, and the water depth was only ≈10 cm. Furthermore, the specific conductivity was higher, alkalinity was substantially higher, early morning free CO_2 levels were low, daytime O_2 concentrations were high, and daytime carbon fixation rates by the plants were generally lower. This was particularly evident in *Eleocharis acicularis*, which had higher daytime carbon uptake rates than the other species early in the season, but by the third sampling period carbon fixation rates were substantially reduced. At this time most plants, in particular *Isoetes howellii*, had obvious infestations of epiphytic algae, and the rates of carbon fixation measured with the ¹⁴C technique include contributions from these parasites. Also at this time the submerged plant parts were not as vigorous in appearance as they were earlier in the season. The CAM pathway in *I. howellii* was also not operating as it had earlier in the season; the maximum rates of carbon

uptake in the dark were far lower at this time, more or less on the order of that observed for the other, non-CAM, species.

In late May there was no standing water, and in the aerial environment there were marked differences in carbon fixation rates among the four species (Fig. 5). The annual dicots *D. bella* and *P. undulatus* had carbon fixation rates substantially higher than under submerged conditions and higher than *I. howellii* and *E. acicularis*. Unlike the submerged situation, plants in the aerial environment showed increased photosynthetic rates between 0900 and 1500. At this time carbon uptake in the dark was negligible relative to light uptake.

Total gross carbon gain was estimated from curves in Figs. 2–5 (Table 4). During most of the period of submergence, carbon assimilation by *I. howellii* exceeded that of associated species. Late in the season when the pools were greatly contracted in size, total carbon gain was significantly reduced for *I. howellii* and *E. acicularis*. The proportion of carbon contributed by fixation in the dark was estimated at ≈40% at midseason for *I. howellii* and from 11 to 36% for other species. After the pools had dried, carbon assimilation was greatest for the dicot species *D. bella* and *P. undulatus*, and for all species, uptake in the dark was relatively insignificant.

Qualitative patterns of carbon assimilation

Laboratory studies of carbon fixation pathways showed that after 1 s steady-state ¹⁴C-labeling in the light, all species had label in phosphoglycerate (PGA) and in organic acids (Table 5). The proportion of label in organic acids ranged from one-third in *P. undulatus* to over half in *E. acicularis*. Thus, all four species in the light showed C_4 fixation. The organic acid products, however, varied; in *D. bella* citrate was the dominant organic acid labeled, in *I. howellii* it was malate, and in *E. acicularis* label was equally distributed between malate, citrate, and aspartate (data not shown). After 10 min steady-state ¹⁴C labeling in the dark, all species showed substantial activity in malate, but other products were also labeled (Table 6).

Under submerged conditions, *Isoetes howellii* had the lowest specific activities for both RuBP carboxylase (RuBPcase) and PEP carboxylase (PEPcase), although the ratio of RuBP/PEPcase was broadly similar in all species, ranging from 8.8 to 11.5 for submerged foliage
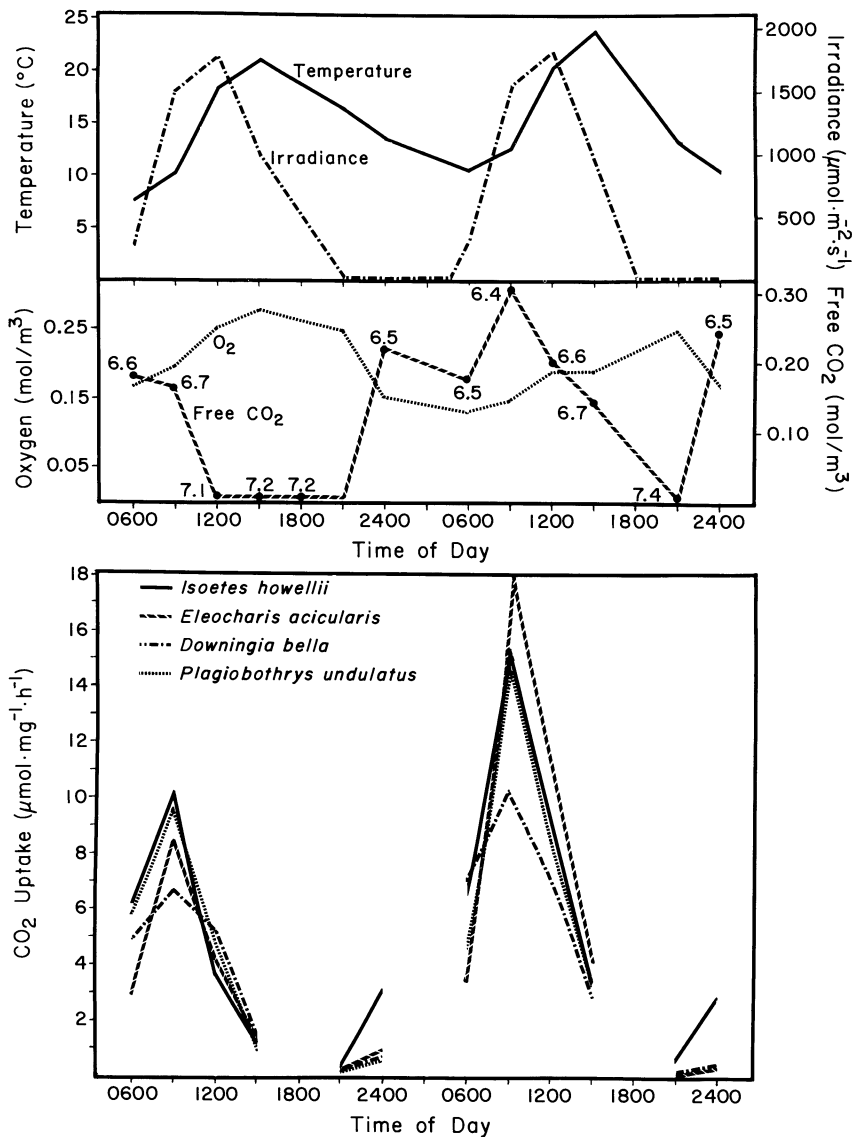
FIG. 2. Irradiance at the water surface, water temperature, oxygen concentration, and free CO_2 (pH adjacent to line) in water, and CO_2 uptake rates per unit mass of total chlorophyll in the light (measured at 0600, 0900, 1200, and 1500) and dark (measured at 2100 and 2400), by species in Mesa de Colorado pool, 18–19 March 1986. Total alkalinity (as Ca^{++}) ranged from 0.15 to 0.19 mol/m³. Specific conductance was 50 μS/cm.

(Table 7). Upon emergence, all species showed marked increases in soluble protein level and increases in the activity of RuBP carboxylase and the ratio of RuBPcase/PEPcase.

DISCUSSION

Under submerged conditions, all four aquatic macrophytes have daytime carbon uptake patterns that are broadly similar. The sharp drop in photosynthesis, which parallels the morning depletion of free CO_2 from the water, indicates that if bicarbonate is utilized, these species do so very poorly, although all species did continue carbon fixation during the afternoon when the

water was well above pH 8. This suggests perhaps some ability to utilize bicarbonate, however, it is likely that afternoon carbon uptake is more severely curtailed than indicated in Figs. 2–4 because, in situ, carbon depletion in the leaf boundary layer would be greater than in the vials due to the experimental manipulation, which generated some level of agitation of the water.

Carbon fixation pathways do show some degree of variation between species. *Plagiobothrys undulatus* has largely C_3-type carbon fixation during the day, whereas *Eleocharis acicularis* has largely C_4-type carbon fixation (without Kranz anatomy). This biochemical pathway could be of important selective value under the
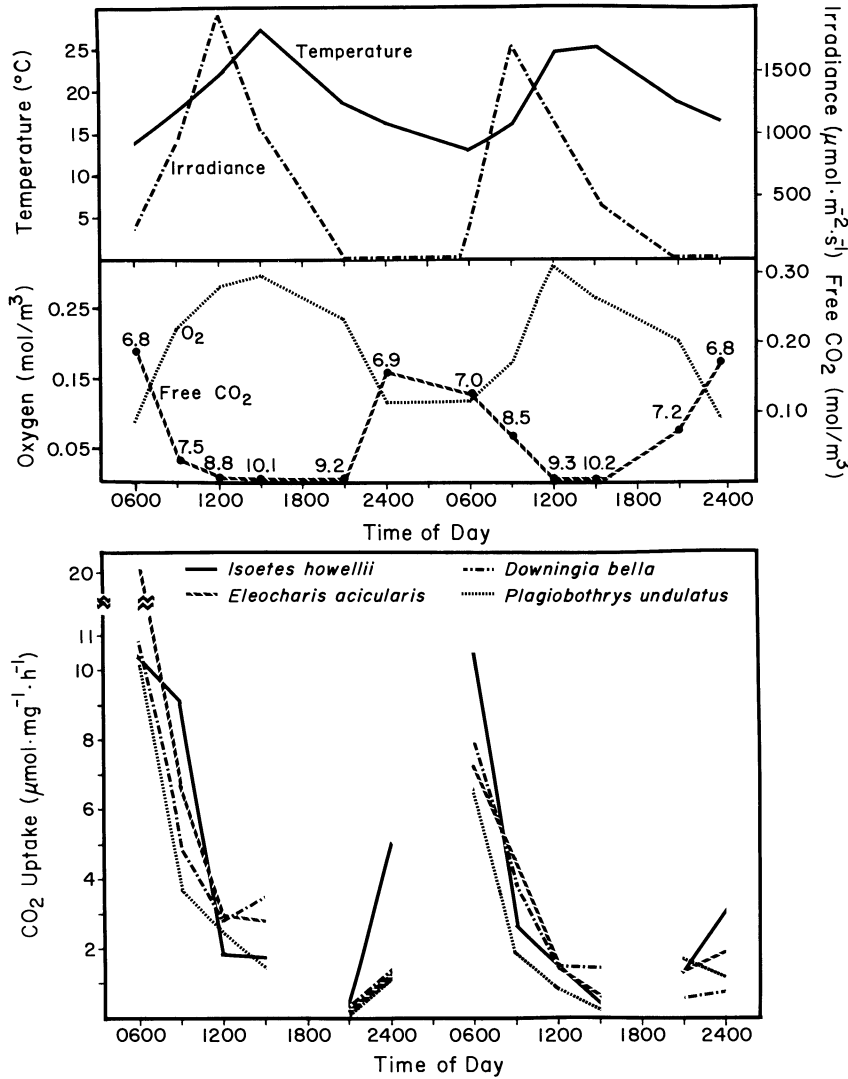
FIG. 3. Irradiance at the water surface, water temperature, oxygen concentration, and free CO_2 (pH adjacent to line) in water, and CO_2 uptake rates per unit mass of total chlorophyll in the light (measured at 0600, 0900, 1200, and 1500) and dark (measured at 2100 and 2400), by species in Mesa de Colorado pool, 10–11 April 1986. Total alkalinity (as Ca^{++}) ranged from 0.23 to 0.28 mol/m³. Specific conductance was 56 µS/cm.

low CO_2–high O_2 conditions experienced by these plants during the day. Further studies are required to determine the exact role β-carboxylation plays in the photosynthesis of these species.

Under submerged conditions the most obvious photosynthetic difference between these species lies in the well-developed CAM pathway evident in *Isoetes howellii*. Data presented here and in Keeley and Busch (1984) indicate that carbon fixation in the dark constitutes one-fourth to one-half of the total daily carbon assimilation, and, based on the level of overnight acid accumulation, a substantial role in conservation of carbon through refixation of respiratory CO_2.

For other species, at certain times during the season, dark carbon fixation may also be significant (Table 4).

These other species do not show the typical CAM pathway of acid accumulation overnight, and it would appear that although dark fixation may have an impact on the overall carbon gain it is not through the photosynthetic PCR cycle. The biochemical fate of carbon fixed in the dark by non-CAM species studied here remains to be elucidated. In some cases dark fixation may represent 10–20% of the total carbon fixation, however, the products are variable and do not appear to accumulate overnight as in CAM photosynthesis; rather they are turned over in the dark and are thus not involved in daytime photosynthesis. In many respects this is similar to what is observed in many species of marine macroalgae in the Phaeophyta (Kremer 1979).
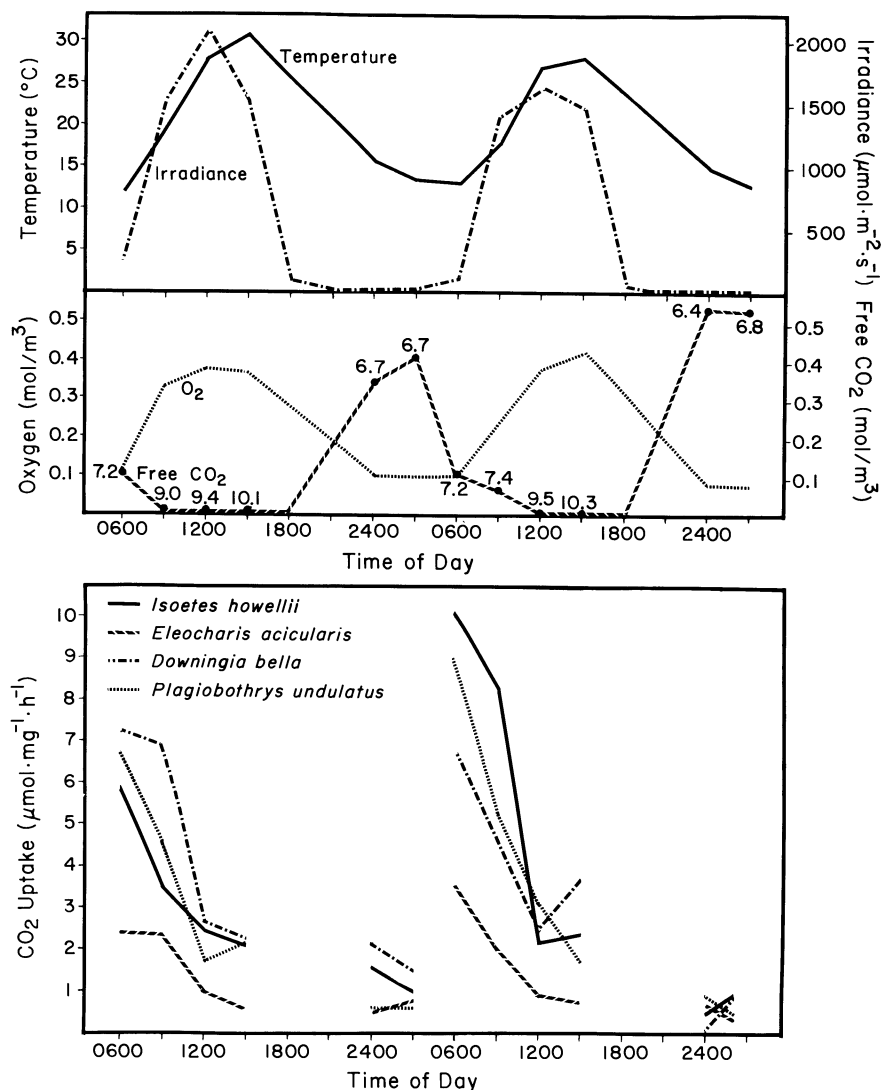
FIG. 4. Irradiance at the water surface, water temperature, oxygen concentration, and free CO_2 (pH adjacent to line) in water, and CO_2 uptake rates per unit mass of total chlorophyll in the light (measured at 0600, 0900, 1200, and 1500) and dark (measured at 2400 and 0300) by species in Mesa de Colorado pool, 29–30 April 1986. Total alkalinity (as Ca^{++}) ranged from 0.37 to 0.42 mol/m³. Specific conductance was 85 μS/cm.

Seasonal differences are evident in water chemistry and photosynthetic response. Comparison of the three sampling dates (Figs. 2–4) shows total CO_2 (in moles per cubic metre) varied over the 2-d period from 0.33– 0.64 in mid-March to 0.23–0.69 in early April to 0.32– 1.20 in late April. Over this time O_2 ranged from 0.25– 0.27 (Fig. 2) to 0.35–0.40 (Fig. 3) to 0.38–0.43 (Fig. 4), and minimum/maximum temperatures (°C) in-

TABLE 4. Gross 24-h carbon uptake per unit mass of total chlorophyll estimated from carbon uptake rates illustrated in Figs. 2–5.

	Gross CO_2 uptake* [μmol·mg⁻¹·(24 h)⁻¹]							
	19 Mar	20 Mar	10 Apr	11 Apr	29 Apr	30 Apr	30 May	31 May
Isoetes howellii	86	135	103	64	52	71	249	244
Eleocharis acicularis	59	119	95	54	23	24	105	85
Downingia bella	60	82	74	45	62	60	352	349
Plagiobothrys undulatus	67	103	42	36	48	56	339	332

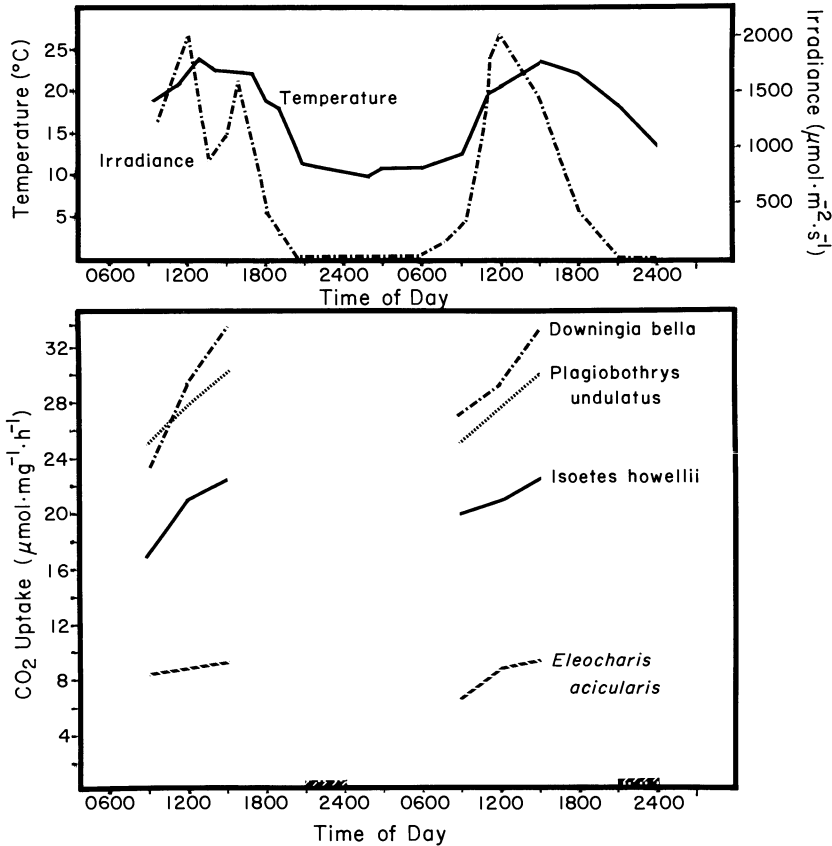* Estimated from 0600 to 0600 beginning on the date indicated.

FIG. 5. Irradiance and air temperature and CO_2 uptake rates per unit total chlorophyll mass in the light (measured at 0900, 1200, and 1500) and dark (measured at 2100 and 2400) by species growing terrestrially in dried-up pool on Mesa de Colorado, 30–31 May 1986.

creased from 7.8/24.3 to 13.3/27.5 to 12.4/30.5, respectively, and minimum/maximum pH ranged from 6.4/7.4 to 6.8/10.2 to 6.4/10.3, respectively. These patterns are typical as we have observed similar seasonal changes in other years (e.g., 1983 as described in Keeley and Busch 1984, and 1985, J. E. Keeley, *personal observation*), although the dates of pool filling and drying vary from year to year.

Early in the season (Fig. 2) free CO_2 depletion from the water is not as rapid as later in the season and midday carbon fixation is not reduced as much as later in the season. Throughout the season the daytime depletion of free CO_2 from the water plays a major role

in limiting daytime carbon gain. This is particularly evident when contrasted with the diurnal pattern of photosynthesis by aerial leaves (cf. Fig. 5). Late in the season (Fig. 4), when the pools are very shallow, daytime photosynthetic rates are generally lower, and this is particularly so for *E. acicularis*. At this time, dark carbon fixation by some of the species, e.g., *D. bella,* is equal to or even greater than for *I. howellii,* although not accompanied by overnight acid accumulation. Additionally, the overall vigor of the *I. howellii* is very greatly reduced. The cause of this decline in vigor is unknown, but as has been observed over years of cultivating this species it invariably loses vigor when maintained for extended periods of time under higher (e.g., >25°) temperatures. One hypothesis is that under higher nighttime temperatures respiration rates exceed fixation rates, reducing the overall net carbon gain.

Upon emergence, *I. howellii* and *E. acicularis* plants die relatively rapidly and *D. bella* and *P. undulatus* are a more dominant part of the flora. Under terrestrial conditions these two annual dicot species also have substantially higher photosynthetic rates (Fig. 5). As suggested by the increase in the RuBP carboxylase/PEP carboxylase ratio (Table 7), it would appear that the

TABLE 4. Continued.

Percentage contributed by CO_2 uptake in the dark						
19 Mar	20 Mar	10 Apr	11 Apr	29 Apr	30 Apr	30 May
30	19	39	43	23	10	3
14	7	11	34	25	17	2
11	3	15	14	25	11	1
10	8	23	36	12	10	<1

TABLE 5. Distribution of ^{14}C label into phosphoglycerate (PGA) and C_4 organic acids (O.A.) after 1 s steady-state labeling in the light (1000 μmol·m^{-2}·s^{-1}, at 25°C and air-equilibrium oxygen levels) for Mesa de Colorado pool species grown submerged in artificial pools. The difference between 100% and (O.A. + PGA) reflects label in other products.

	Percentage of total label		
Species	Phospho-glycerate	Organic acids	n
Isoetes howellii	35 ± 10	46 ± 15	8
Eleocharis acicularis	35 ± 8	55 ± 7	4
Downingia bella	48 ± 12	40 ± 6	5
Plagiobothrys undulatus	56 ± 14	33 ± 9	10

C_3 pathway is the primary carboxylation pathway in all four species under terrestrial conditions. Dark carbon fixation represents an insignificant part of the carbon gain in the aerial environment.

One curious fact is the relatively low PEP carboxylase activity observed for species obviously involved in β-carboxylation. This is particularly evident in *I. howellii*, which has a well-developed CAM pathway. The magnitude of overnight malic acid accumulation is comparable to what is observed in terrestrial CAM species, yet the specific activity of PEP carboxylase is more than an order of magnitude lower than what has been reported for terrestrial species (cf. Dittrich et al. 1973). Our PEPcase values for *I. howellii* are comparable to those reported for other *Isoetes* species (Farmer et al. 1986). Although the PEPcase activities in aquatic *Isoetes* are far lower than for terrestrial CAM species with similar levels of overnight acid accumulation, it should be noted that PEPcase activity is sufficient to account for the total acid accumulation in *Isoetes* species. High PEPcase activities in terrestrial CAM species may be adaptive under arid conditions where there is a premium on high stomatal resistance, even at night, as a means of reducing water loss.

For all four aquatic species studied here, the ratio of RuBPcase/PEPcase suggests that activity of the C_3 pathway is far more important to carbon assimilation than is the C_4 pathway of carbon fixation. However, ^{14}C tracer studies indicate substantial C_4 acid fixation in some species. Once again it may be that the enzyme activities are not an accurate reflection of the activity of these pathways. For example, in all four species studied here, RuBP carboxylase activities are of the same order of magnitude as those reported for terrestrial C_3 species. However, carbon assimilation rates, as is typical of aquatic species in general, are more than an order of magnitude lower than the photosynthetic rates reported for terrestrial species. We hypothesize that the explanation may be tied to the dynamics of carbon fixation. Daytime C_3 fixation is largely restricted to a narrow window in time between sunrise and mid morning, after which conditions become unfavorable for RuBP carboxylase catalyzed fixation. Thus,

high specific activity of this enzyme may be a necessary requisite for taking advantage of the brief favorable period for carbon fixation.

CONCLUSIONS

This study focused on the degree to which coexistence of four vernal pool macrophytes depended upon divergent photosynthetic characteristics. All species exhibited a similar pattern of daytime carbon fixation, which closely tracked levels of free CO_2 in the water, suggesting a similar dependence upon this form of inorganic carbon for photosynthesis. Differences were demonstrated in biochemical route of carbon fixation with greater C_3-type fixation is some and greater C_4-type fixation in other species. Carbon assimilation in the dark was observed in all species. In *Isoetes howellii* this represents a substantial amount of carbon fixation and over a 24-h period this species is predicted to assimilate 25–40% more carbon than competing species. This species also dominates the pools under submerged conditions.

Eleocharis acicularis codominates these pools. On one sampling date carbon fixation rates were substantially greater than for the other species, but this was not consistent on other dates, and it is unknown what conditions induced the much greater rates of photosynthesis. One potential advantage this species has over *I. howellii* is the much greater photosynthetic surface area per unit biomass. Also the lower stature growth form of *E. acicularis* would put the photosynthetic tissues close to the sediment, which is richer in free CO_2 than the ambient water and thus in situ CO_2 levels may be greater in the boundary layer adjacent to the leaf surface. Another potential competitive advantage this species has under submerged conditions is a very active C_4-type fixation, which may act as a CO_2-concentrating mechanism under low carbon and high oxygen conditions typical through much of the day.

The two annual dicot species germinate under water and photosynthetically may be at a competitive disadvantage with respect to *I. howellii*. As the pools dry down these plants produce aerial foliage with photosynthetic rates higher than their submerged foliage and more than 40% higher than the aerial foliage of either

TABLE 6. Distribution of ^{14}C label after 10 min steady-state labeling in the dark (at 25°C and air-equilibrium oxygen levels) for Mesa de Colorado pool species grown submerged in artificial pools.

	Percentage of total label			
Species	Malate	Citrate	Aspar-tate	Other pro-ducts
Isoetes howellii	89	11	0	0
Eleocharis acicularis	65	1	34	0
Downingia bella	56	9	27	8
Plagiobothrys undulatus	44	18	28	10

TABLE 7. Specific activity of RuBP carboxylase and PEP carboxylase (CO_2 uptake rate per unit mass of total chlorophyll) at pH 8.0 and pH 8.5 ($n = 3$), and soluble protein ($n = 6$), for species from Mesa de Colorado pool. Data are means $\pm$ SD.

| | Carboxylase activity ($\mu mol \cdot mg^{-1} \cdot h^{-1}$) | | | | RuBPcase | Soluble protein (mg/g fresh mass) |
| | RuBPcase | | PEPcase | | PEPcase | |
	pH 8.0	pH 8.5	pH 8.0	pH 8.5		
Isoetes howellii						
Submerged	65 ± 28	70 ± 5	6 ± 3	8 ± 2	8.8	8.3 ± 3.1
Emergent	226 ± 85	181 ± 59	11 ± 6	8 ± 5	20.5	28.7 ± 3.4
Eleocharis acicularis						
Submerged	173 ± 39	136 ± 24	15 ± 2	12 ± 2	11.5	28.0 ± 9.9
Emergent	296 ± 86	170 ± 14	15 ± 2	13 ± 2	19.7	50.8 ± 3.2
Downingia bella						
Submerged	214 ± 58	175 ± 46	19 ± 9	22 ± 9	9.8	6.4 ± 3.7
Emergent	377 ± 50	257 ± 43	11 ± 7	14 ± 8	26.9	15.0 ± 3.3
Plagiobothrys undulatus						
Submerged	147 ± 84	70 ± 5	16 ± 5	15 ± 6	9.2	13.0 ± 4.4
Emergent	129 ± 5	83 ± 13	9 ± 1	7 ± 3	14.3	37.7 ± 4.5

I. howellii or *E. acicularis*. Thus, these plants compete with *Isoetes* and *Eleocharis* by escaping the aquatic milieu. These species were, on a biomass basis, relatively minor parts of the aquatic flora, however, upon emergence they represented a greater proportion of the flora.

Coexistence of the vernal pool flora is in all likelihood a function of factors other than just physiological adaptations in the competition for carbon. Since some species would seem to be at an advantage under submergence and others at an advantage in the aerial environment, then stochastic factors such as annual precipitation levels could over the long run play an important role. Thus, coexistence may be enhanced by a disequilibrium process that favors some species in very wet years when the pools remain for long periods of time and other species in dry years when the pools dry down relatively rapidly or even fail to ever fill.

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