

Distribution of Diurnal Acid Metabolism in Submerged Aquatic Plants Outside the Genus *Isoetes**

J. E. KEELEY and B. A. MORTON

Department of Biology, Occidental College, Los Angeles, CA 90041, U.S.A.

Abstract

Diurnal acid metabolism previously has been studied in detail in the submerged aquatic *Isoetes howellii* Engelmann where it is clearly indicative of Crassulacean Acid Metabolism. Other studies have shown it is widespread in the genus *Isoetes*. The present study shows that diurnal acid metabolism does occur in submerged aquatic species outside the genus *Isoetes*, and in distantly related families, but it is not common. A survey of thirty species distributed in seasonal pools and lakes, ranging from oligotrophic to eutrophic conditions, has revealed two with marked overnight acid accumulation: *Crassula aquatica* (L.) Schoenl. common in northern hemisphere seasonal pools and *Littorella uniflora* (L.) Aschers a common component of the littoral flora of oligotrophic lakes at high latitudes in Europe.

Crassulacean Acid Metabolism (CAM) recently has been demonstrated in the submerged aquatic *Isoetes howellii* Engelmann (*Isoetaceae*) (Keeley 1981a). There is substantial net CO₂-uptake in the dark which is fixed into malic acid. Phosphoenolpyruvate carboxylase activities are sufficient to account for net dark CO₂-uptake rates which in turn are sufficient to account for overnight rates of malic acid accumulation. Daytime deacidification results in a diurnal flux >100 μ-equivalents titratable acidity per g fresh matter. This carbon fixation pathway is shut off in the light and dark-fixed carbon moves from organic acids into 3-phosphoglycerate (PGA) and other C₃ pathway products. *Isoetes howellii* apparently lack functional stomata (as is the case in other aquatics, Sculthorpe 1967) thus they lack a diurnal pattern of changes in stomatal conductance typical of many terrestrial CAM plants. *Isoetes howellii* is capable of CO₂ uptake in the light which is fixed directly into PGA. There is strong evidence that diurnal fluxes in carbon availability in the seasonal pools *I. howellii* inhabits limits daytime carbon assimilation and has selected for nighttime CO₂-uptake.

It is very likely that all aquatic *Isoetes* species possess this pathway, although only 15 of the over 60 species in the genus have been surveyed (Keeley 1982). The species so far studied cover the range of variation in the genus; these include ones from North America, Central America, Europe, and Australia, from 10° to 53° latitude, 0 to 2850 m elevation, species permanently submerged, ones seasonally submerged and ones diurnally submerged. The leaves of all these aquatic species, while submerged, show marked diurnal changes in levels of titratable acidity and malic acid. Upon emergence, acid metabolism is lost. Thus, it is not surprising that the terrestrial *Isoetes nuttallii* A. Br. ex. Engelmann lacks acid metabolism even if artificially submerged.

Diurnal fluxes in titratable acidity or malic acid (malate) have recently been reported for several submerged aquatic flowering plants (Browse *et al.* 1980, Holaday and Bowes 1980, Beer and Wetzel 1981). The purpose of the present study was to survey submerged aquatic plants, covering a wide phylogenetical and ecological range, for evidence of diurnal acid fluctuations.

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METHODS

Species were sampled either in the field or in a few cases transplanted to aquaria. In most cases identifications were verified by outside sources (see acknowledgements). Vouchers have been deposited in LOC.

Habitats: In all but one case, the aquatic habitats were characterized as follows. Photon fluence rate was measured with a *LiCor LI-188B* integrating meter with *LI-190SB* quantum sensor at, and perpendicular to, the water surface. Specific conductance of the water was measured with a *YSI-33* conductivity meter at 25 °C. For pH determinations and titrations a *Photovolt 126-A* pH meter with combination electrode was used. Carbon dioxide and total alkalinity were determined titrimetrically on water samples kept on ice till assayed (usually within an hour). Titrations were done potentiometrically, at ambient temperatures, following procedures described in APHA (1976). CO₂-free 0.0227 N NaOH and 0.02 N H₂SO₄ were the titrants for CO₂ and alkalinity respectively. A pH 4.5 endpoint was used for total alkalinity on all samples except those from Siesta Lake and Woahink Lake where a pH 5.1 endpoint was used because of the very low alkalinity (as recommended by APHA 1976). Total CO₂ was calculated as described in APHA (1976).

Plant assays: Leaf (and stem) samples were collected at 0600–0700 h and 1700–1800 h and kept on ice until extraction, usually within an hour. Leaf samples (0.2–0.5 g) were washed in deionized water, blotted dry and weighed on an *Ohaus 300* electronic balance. These were ground in a *Ten Broeck* with 15.0 cm³ cold CO₂-free deionized water and centrifuged. A 1.0 cm³ sample of supernatant was deproteinized with an equal volume of 0.6 N perchloric acid and later assayed for malic acid with the enzymatic procedure of Gutmann and Wahlefeld (1974). A 10.0 cm³ sample was immediately titrated with CO₂-free 0.01 N NaOH to pH 6.4 and pH 8.3. Any detectable diurnal change in acidity was \pm identical at the two pH endpoints; with one exception which is discussed below.

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RESULTS AND DISCUSSION

Submerged aquatic plants were sampled from two seasonal pools and five lakes. The localities and characteristics of these aquatic habitats are shown in Table 1. Seasonal pools typically show marked diurnal fluxes in pH and inorganic carbon levels (Keeley unpublished, Bamforth 1962) as seen for the ones in this study. The lakes involved in this survey represent the range from oligotrophic to eutrophic conditions.

Out of thirteen species surveyed from seasonal pools (Table 2) only one showed a marked diurnal change in titratable acidity and malic acid levels. This species, *Crassula aquatica*, is a submerged aquatic annual common in California "vernal pools" as well as seasonal pools throughout North America and Eurasia (Munz 1974).

Table 1

The seasonal pools and lakes from which submerged aquatic plants were sampled (or collected). Specific conductance and the diurnal range in pH and total carbon dioxide are presented for a single representative day, with $>1800 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR radiation at 1200 h, in spring (seasonal pools) or summer (lakes) 1981. Data was unavailable for Llynau Mumbur though conductivity, pH and CO_2 are likely very similar to Siesta Lake.

	Code	Location	Elevation [m]	Conductivity [$\mu\text{S cm}^{-1}$]	pH		Total CO_2 [g m^{-3}]	
					0600	1800	0600	1800
Seasonal pools:								
Mesa de Colorado	MdC	Riverside Co., CA	610	76	6.6	8.4	37	23
Mather	M	Tuolumne Co., CA	1375	73	6.6	7.6	75	45
Lakes:								
Siesta	S	Tuolumne Co., CA	2440	13	6.8	6.7	13	10
Llynau Mumbur	LM	Caernarvon, Wales	180	—	—	—	—	—
Woahink	W	Lane Co., OR	10	71	6.4	6.4	16	16
Birchlake	B	Tuolumne Co., CA	1375	99	7.0	7.4	66	65
Searsville	SRV	San Mateo Co., CA	110	750	7.5	7.8	225	203

Table 2

Overnight changes ($\bar{x} \pm S.D.$) in levels of titratable acidity (to pH 6.4) and malic acid for submerged leaves (and stems) of aquatic species from seasonal (vernal) pools; PM (1700–1800 h), AM (0600–0700 h), f.m. = fresh matter. See Table 1 for localities; * indicates plants collected from locality and sampled in cultivation.

	Family	Locality	N	Titratable acidity [$\mu\text{eq g}^{-1}$ (f.m.)]		Malic acid [$\mu\text{mol g}^{-1}$ (f.m.)]	
				PM	AM	PM	AM
CHLOROPHYTA							
		<i>Chara contraria</i> Braun ex. Kutzing	2	0 \pm 0	0 \pm 0	1 \pm 1	1 \pm 1
ANTHOPHYTA							
Monocotyledones:							
		<i>Eleocharis acicularis</i> (L.) R. & S.	6	3 \pm 2	9 \pm 4	5 \pm 3	6 \pm 2
		<i>Lilaea scilloides</i> (Poir.) Haum.	2	1 \pm 1	1 \pm 1	1 \pm 1	2 \pm 1
Dicotyledones:							
		<i>Barbarea orthoceras</i> Ledeb.	2	3 \pm 1	6 \pm 1	10 \pm 6	12 \pm 1
		<i>Callitriche longipedunculata</i> Morong.	2	0 \pm 0	1 \pm 1	2 \pm 1	2 \pm 1
		<i>Crassula aquatica</i> (L.) Schoenl.	4	9 \pm 10	112 \pm 9	17 \pm 5	53 \pm 9
		<i>Downingea cuspidata</i> (Greene) Greene	2	1 \pm 1	0 \pm 0	8 \pm 4	4 \pm 1
		<i>Elatine chilensis</i> Gay.	2	1 \pm 1	2 \pm 1	4 \pm 1	10 \pm 2
		<i>Eryngium aristulatum</i> Jeps.	2	1 \pm 1	2 \pm 0	12 \pm 2	9 \pm 1
		<i>Mentha arvensis</i> L.	2	0 \pm 0	0 \pm 0	2 \pm 1	3 \pm 2
		<i>Plagiobothrys undulatus</i> (Piper) Jtn.	4	0 \pm 0	0 \pm 0	8 \pm 3	10 \pm 2
		<i>Ranunculus aquatilis</i> L.	2	0 \pm 0	3 \pm 4	4 \pm 1	10 \pm 3
		<i>Ranunculus flammula</i> L.	2	0 \pm 0	0 \pm 0	9 \pm 1	8 \pm 1

Evidence of CAM in this member of the *Crassulaceae* family is perhaps not surprising. If the aquatic habit was the derived condition in the family, CAM in *Crassula aquatica* could be a conservative trait which presently is of little adaptive value. Several observations argue against this interpretation. As in all *Isoetes* species thus far examined (Keeley 1981b), overnight acid accumulation dampens out as *Crassula aquatica* becomes emergent (Table 3). This is consistent with the hypothesis that CAM has been selected-for in aquatic plants as a means of enhancing carbon gain in certain carbon-limited aquatic environments (Keeley 1981a, b). Additionally, apparently CAM is absent in the terrestrial taxon *Crassula erecta* (Table 3) which is very closely

Table 3

Overnight changes ($\bar{x} \pm$ S.D.) in levels of titratable acidity and malic acid in leaves and stems of the aquatic *Crassula aquatica* and the closely related terrestrial *Crassula erecta* (H. & A.) Berger (*Crassulaceae*); f.m. = fresh matter. Seeds were collected from dried pools (*C.a.*) or rock outcrops (*C.e.*) on Mesa de Colorado, Riverside Co., CA, and plants were sampled in cultivation.

Species	N	Titratable acidity [$\mu\text{eq g}^{-1}$ (f.m.)]		Malic acid [$\mu\text{mol g}^{-1}$ (f.m.)]	
		PM	AM	PM	AM
<i>Crassula aquatica</i>					
Submerged since germination	4	9 \pm 10	112 \pm 9	17 \pm 5	53 \pm 9
Emergent 2 weeks	2	2 \pm 1	30 \pm 0	26 \pm 1	35 \pm 5
<i>Crassula erecta</i>					
Drained soil (dry)	2	9 \pm 0	13 \pm 1	14 \pm 3	11 \pm 4
Submerged 1 week	2	2 \pm 1	3 \pm 0	9 \pm 1	14 \pm 3
Submerged 1 month	2	10 \pm 1	9 \pm 1	14 \pm 3	8 \pm 2

related to *C. aquatica* (the major taxonomic distinction is in number of flowers/axil and seeds/carpel; in the vegetative state, under similar conditions, they are difficult to distinguish from one another, Keeley pers. obs.). *Crassula erecta* does not exhibit CAM under natural drained conditions or artificial submerged conditions which it survives quite well (Table 3). *Crassula erecta* may be facultative CAM (Kluge and Ting 1978) though it has been tested under submerged conditions (in which *Crassula aquatica* exhibits CAM) and in its natural state under the driest conditions it is likely to be exposed to. Possibly the absence of CAM in the terrestrial *C. erecta* is related to the fact that this ephemeral annual is a drought avoider which completes its life cycle by mid-spring. This would be supported by the observation that apparently the only other known instance of a non-CAM member of the *Crassulaceae* is an annual species of *Sedum* (J. Teeri, unpublished).

The survey of aquatic plants from various lakes (Table 4) showed that out of 17 species assayed only *Littorella uniflora* exhibited strong diurnal changes in titratable acidity and malic acid. *Littorella uniflora*, an aquatic member of the *Plantaginaceae* family is a common component of the littoral flora of soft-water (low carbon) oligotrophic lakes at high latitudes in North America and Europe. The restriction of *L. uniflora* to such habitats is consistent with the hypothesis that CAM has been selected (in aquatic plants) as a mean of enhancing carbon gain under carbon limiting conditions. However this species is known to take up CO₂ from the interstitial water in the sediment and transport it from the roots to the leaves (Sondergaard 1979), thus the extent

Table 4

Overnight changes ($\bar{x} \pm S.D.$) in levels of titratable acidity (to pH 6.4) and malic acid for submerged leaves (and stems) of aquatic species from lakes; PM (1700–1800 h), AM (0600–0700 h), f.m. = fresh matter. See Table 1 for localities; *indicates plants collected from locality and sampled in cultivation.

	Family	Locality	N	Titratable acidity [$\mu\text{eq g}^{-1}$ (f.m.)]		Malic acid [$\mu\text{mol g}^{-1}$ (f.m.)]	
				PM	AM	PM	AM
CHLOROPHYTA							
<i>Chara contraria</i> Braun ex. Kutzing	(Characeae)	SRV	2	0 \pm 0	0 \pm 0	7 \pm 2	8 \pm 4
<i>Spirogyra</i> sp.	(Zygnemataceae)	SRV	2	0 \pm 0	0 \pm 0	10 \pm 1	7 \pm 3
BRYOPHYTA							
<i>Amblystegium riparium</i> (Hedw.) BSG	(Hypnaceae)	BL	2	0 \pm 0	0 \pm 0	11 \pm 0	14 \pm 0
<i>Fontinalis antipyretica</i> Hedw.	(Fontinalaceae)	S	2	13 \pm 3	13 \pm 1	8 \pm 3	12 \pm 5
ANTHOPHYTA							
Monocotyledones:							
<i>Echinodorus berteroi</i> (Spreng.) Fassett	(Alismataceae)	SRV	2	0 \pm 0	0 \pm 0	9 \pm 3	3 \pm 1
<i>Elodea canadensis</i> Mich X.	(Hydrocharitaceae)	BL	2	6 \pm 3	5 \pm 6	9 \pm 1	16 \pm 3
<i>Potamogeton crispus</i> L.	(Potamogetonaceae)	SRV	2	0 \pm 0	0 \pm 0	10 \pm 2	5 \pm 1
<i>Potamogeton illinoensis</i> Morong.	(Potamogetonaceae)	SRV	2	0 \pm 0	0 \pm 0	5 \pm 3	2 \pm 1
<i>Potamogeton pectinatus</i> L.	(Potamogetonaceae)	SRV	2	0 \pm 0	0 \pm 0	3 \pm 4	9 \pm 1
<i>Sagittaria cuneata</i> Sheldon	(Alismataceae)	S	2	3 \pm 1	10 \pm 1	13 \pm 4	18 \pm 3
<i>Scirpus setaceus</i> L.	(Cyperaceae)	W	4	0 \pm 0	7 \pm 1	10 \pm 3	20 \pm 3
<i>Sparganium angustifolium</i>	(Sparganiaceae)	S	2	7 \pm 1	8 \pm 1	17 \pm 4	16 \pm 2
Dicotyledones:							
<i>Ceratophyllum demersum</i> L.	(Ceratophyllaceae)	SRV	2	2 \pm 2	2 \pm 3	15 \pm 1	8 \pm 3
<i>Limosella acaulis</i> L.	(Scrophulariaceae)	SRV	2	3 \pm 1	0 \pm 0	6 \pm 1	6 \pm 3
<i>Littorella uniflora</i> (L.) Aschers	(Plantaginaceae)	LM*	4	19 \pm 22	112 \pm 14	21 \pm 14	66 \pm 8
<i>Myriophyllum brasiliense</i> Camb.	(Haloragaceae)	SRV	2	0 \pm 0	0 \pm 0	10 \pm 2	6 \pm 1
<i>Nuphar polysepalum</i> Engelm.	(Nymphaeaceae)	S	2	14 \pm 1	10 \pm 3	5 \pm 2	6 \pm 2

of carbon limitation is unknown. *Littorella uniflora* is commonly found in association with *Isoetes lacustris* L. which also exhibits diurnal acid metabolism (Keeley 1982). Both species have the "isoetid" growth form (den Hartog and Segal 1964) which suggests there may be a functional relationship between the "rosette of quill-like leaves" and diurnal acid metabolism.

Scirpus setaceus (Cyperaceae) may have a limited capacity for overnight acid accumulation (Table 4). Recently, a similar magnitude in diurnal fluctuation of malate was reported for *Scirpus subterminalis* Torr. (Beer and Wetzel 1981). Other submerged aquatic species reported in the literature to show diurnal acid fluctuations include *Hydrilla verticillata* Royal (Holaday and Bowes 1980) and *Egeria densa* Planch. (Browse *et al.* 1980).

In *Egeria* the accumulation of malate occurred during the day and levels decreased at night. A similar phenomenon has been observed in this study for several species sampled from the eutrophic Searsville Lake. *Echinodorus berteroi*, *Potamogeton crispus*, and *Ceratophyllum demersum* (Table 4) showed distinct daytime accumulation of malate/malic acid of approximately the same magnitude as observed for *Egeria*. For *E. berteroi* and *C. demersum* there was no diurnal change in titratable acidity at either the pH 6.4 or pH 8.3 endpoint. However *P. crispus* showed a marked increase, during the day, in titratable acidity (measured at pH 8.3, PM = 60 ± 21 vs. AM = 16 ± 3) and a marked diurnal change in extract pH (PM = pH 6.6–6.9 vs. AM = pH 8.1–8.2). Undoubtedly these patterns are unrelated to any sort of CAM pathway.

Scirpus subterminalis and *Hydrilla verticillata* thus represent the only reports, outside of *Isoetes* (Keeley 1982) and the species from the present study, of overnight acid accumulation in submerged aquatic plants. However, the level of acid accumulation in these two species is quite low relative to *Isoetes* species, *Crassula aquatica* or *Littorella uniflora* (Table 5).

Table 5

Overnight changes ($\bar{x} \pm$ S.D.) in levels of titratable acidity (to pH 8.3) and malic acid in submerged aquatic plants previously reported to show overnight accumulation of acids. *Isoetes howellii* and the two species from this study showing diurnal acid metabolism are included, though titratable acidity is given for pH 8.3 and malic acid is expressed on a chlorophyll basis for comparison with the other reports. PM (1500–1800 h), AM (0600–0700 h).

	Titratable acidity [$\mu\text{eq g}^{-1}$ (f.m.)]			Malic acid [$\mu\text{mol mg}^{-1}$ (chl)]	
	N	PM	AM	PM	AM
<i>Scirpus subterminalis</i> (Beer and Wetzel 1981)	1	—	—	3	28
<i>Hydrilla verticillata</i> (Holaday and Bowes 1980)	3	66 ± 1	67 ± 1	—	—
	3	62 ± 2	52 ± 3	—	—
	3	30 ± 6	51 ± 13	—	—
	3	40 ± 2	53 ± 4	—	—
<i>Isoetes howellii</i> (Keeley, unpublished)	2	28 ± 1	211 ± 37	63 ± 6	244 ± 42
<i>Crassula aquatica</i> (The present study)	4	51 ± 6	144 ± 16	46 ± 13	143 ± 24
<i>Littorella uniflora</i> (The present study)	4	44 ± 21	144 ± 9	57 ± 38	179 ± 22

CONCLUSIONS

Diurnal acid metabolism of the magnitude known in terrestrial CAM plants has previously been shown to be widespread in aquatic species of *Isoetes* (Keeley 1982). The present study has shown that diurnal acid metabolism does occur in aquatic species outside the genus *Isoetes*, and in distantly related families, but it is not common.

Diurnal acid metabolism has been studied in great detail in *Isoetes howellii* where it is clearly indicative of crassulacean acid metabolism. My working hypothesis is that CAM was selected as a means of enhancing carbon gain in carbon limiting environments (Keeley 1981a, b). Evidence to date is consistent with this hypothesis. *Isoetes* species are largely restricted to either shallow seasonal pools or oligotrophic lakes (Keeley 1982), both of which are potentially carbon-limited environments (Table 1). In seasonal pools free-CO₂ is often depleted during the day and the consequent pH change means carbon is unavailable to those species unable to utilize HCO₃⁻ (Raven 1970). The extremely low inorganic carbon levels (for aquatic environments with a high diffusive resistance to CO₂) characteristic of oligotrophic lakes may likewise limit photosynthesis. Both conditions could place a selective advantage on nighttime CO₂ uptake.

The results of the present study are consistent with this hypothesis. *Crassula aquatica* and *Littorella uniflora* show marked diurnal acid metabolism; the former species is restricted to seasonal pools and the latter to oligotrophic lakes. Even the literature reports on *Scirpus subterminalis* and *Hydrilla verticillata* have implicated limiting carbon conditions as a possible explanation for the small overnight acid accumulation observed in those species.

REFERENCES

- APHA: Standard Methods for the Examination of Water and Wastewater. 14th Ed. — Amer. Publ. Health Ass., Washington 1976.
- Bamforth, S. S.: Diurnal changes in shallow aquatic habitats. — *Limnol. Oceanogr.* 7: 348—353, 1962.
- Beer, S. Wetzell, R. G.: Photosynthetic carbon metabolism in the submerged aquatic angiosperm *Scirpus subterminalis*. — *Plant Sci. Lett.* 21: 199—207, 1981.
- Browse, J. A., Brown, J. M. A., Dromgoole, F. I.: Malate synthesis and metabolism during photosynthesis in *Egeria densa* Planch. — *Aquat. Bot.* 8: 295—305, 1980.
- den Hartog, C., Segal, S.: A new classification of the water-plant communities. — *Acta bot. neerl.* 13: 367—393, 1964.
- Gutmann, I., Wahlefeld, A. W.: L-malate: Determination with malate dehydrogenase and NAD. — In: Bergmeyer, H. U. (ed.): *Methods of Enzymatic Analysis*. Vol. 4. Pp. 1585—1589. Academic Press, New York 1974.
- Holaday, A. S., Bowes, G.: C₄ acid metabolism and dark CO₂ fixation in a submerged aquatic macrophyte (*Hydrilla verticillata*). — *Plant Physiol.* 65: 331—335, 1980.
- Keeley, J. E.: *Isoetes howellii*: A submerged CAM plant? — *Amer. J. Bot.* 68: 420—424, 1981a.
- Keeley, J. E.: Diurnal acid metabolism in vernal pool *Isoetes*. — *Madrono* 28: 167—171, 1981b.
- Keeley, J. E.: Distribution of diurnal acid metabolism in the genus *Isoetes*. — *Amer. J. Bot.* 69: 254—257, 1982.
- Kluge, M., Ting, I. P.: *Crassulacean Acid Metabolism*. — Springer-Verlag, Berlin 1978.
- Munz, P. A.: *A Flora of Southern California*. — Univ. California Press, Berkeley 1974.
- Raven, J. A.: Exogenous inorganic carbon sources in plant photosynthesis. — *Biol. Rev. Cambridge phil. Soc.* 45: 167—221, 1970.
- Sculthorpe, C. D.: *The Biology of Aquatic Vascular Plants*. — E. Arnold Publ., London 1967.
- Sondergaard, M.: Carbon uptake by leaves and roots of *Littorella uniflora* (L.) Aschers. — *Aquat. Bot.* 6: 1—12, 1979.