

## Article (refereed)

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**Klavsén, Signe Koch**; Madsen, Tom V.; **Maberly, Stephen C.**. 2011  
Crassulacean acid metabolism in the context of other carbon-  
concentrating mechanisms in freshwater plants: a review. *Photosynthesis  
Research*, 109 (1-3). 269-279. [10.1007/s11120-011-9630-8](https://doi.org/10.1007/s11120-011-9630-8)

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1 **Crassulacean Acid Metabolism (CAM) in the context of other carbon concentrating**  
2 **mechanisms in freshwater plants: a review**

3  
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14

1     **Abstract**

2     Inorganic carbon can be in short-supply in freshwater relative to that needed by freshwater  
3     plants for photosynthesis because of a large external transport limitation coupled with frequent  
4     depleted concentrations of CO<sub>2</sub> and elevated concentrations of O<sub>2</sub>. Freshwater plants have  
5     evolved a host of avoidance, exploitation and amelioration strategies to cope with the low and  
6     variable supply of inorganic carbon in water. Avoidance strategies rely on the spatial variation  
7     in CO<sub>2</sub> concentrations within and among lakes. Exploitation strategies involve anatomical and  
8     morphological features that take advantage of sources of CO<sub>2</sub> outside of the water column such  
9     as the atmosphere or sediment. Amelioration strategies involve carbon concentrating  
10    mechanisms (CCM) based on uptake of bicarbonate, which is widespread, C<sub>4</sub>-fixation which is  
11    infrequent and Crassulacean Acid Metabolism (CAM) which is of intermediate frequency.  
12    CAM enables aquatic plants to take up inorganic carbon in the night. Furthermore, daytime  
13    inorganic carbon uptake is generally not inhibited and therefore CAM is considered to be a  
14    carbon conserving mechanism. CAM in aquatic plants is a plastic mechanism regulated by  
15    environmental variables and is generally down-regulated when inorganic carbon does not limit  
16    photosynthesis. CAM is regulated in the long term (acclimation during growth), but is also  
17    affected by environmental conditions in the short term (response on a daily basis). In aquatic  
18    plants CAM appears to be an ecologically important mechanism for increasing inorganic carbon  
19    uptake, since the *in situ* contribution from CAM to the C-budget generally is high (18-55%).

20  
21    **Keywords:** CO<sub>2</sub>, elodeids, inorganic carbon, isoetids, macrophytes, regulation.  
22

## 1 **Inorganic carbon availability in freshwater habitats**

2 In terrestrial environments, autotrophic plants have evolved mechanisms and strategies that  
3 allow them to obtain the resources necessary for photosynthesis and growth such as water, light,  
4 nutrients and CO<sub>2</sub>. Of these, atmospheric CO<sub>2</sub> is most constant and so, coupled with the relatively  
5 high rate of diffusion of CO<sub>2</sub> in the gas phase, it seldom limits productivity in natural systems, or  
6 directly-affects ecological distribution. Nevertheless, some terrestrial plants have evolved carbon  
7 concentrating mechanisms (CCMs), such as C<sub>4</sub> carbon fixation and CAM, that may maximise  
8 carbon-uptake but also often solve problems caused by interaction with other environmental factors  
9 such as high temperature or shortage of water (e.g. Lüttge 2002; Keeley and Rundel 2003; Sage and  
10 Kubien 2003).

11 In contrast, in freshwaters, water is readily available but the concentration of CO<sub>2</sub> is highly  
12 variable and may range from close to 0 to more than 350 μmol L<sup>-1</sup> (Bowes and Salvucci 1989;  
13 Madsen and Sand-Jensen 1991). Because of a high transport limitation caused by low diffusion  
14 coefficients of CO<sub>2</sub> in water and substantial boundary layers, these concentrations are in the lower  
15 range of concentrations needed to saturate photosynthesis of freshwater macrophytes, where half-  
16 saturation concentrations often vary between 100 and 200 μmol L<sup>-1</sup> (Maberly and Spence 1983;  
17 Bowes and Salvucci 1989; Madsen and Sand-Jensen 1991; Maberly and Madsen 1998).

18 Furthermore, photosynthetic removal of CO<sub>2</sub>, which often generates very low CO<sub>2</sub> concentrations  
19 (e.g. Maberly 1996) also generates high concentrations of oxygen, producing conditions that  
20 favours photorespiration via the oxygenase reaction of Rubisco. *In situ* measurements have  
21 demonstrated that photosynthesis and growth of freshwater plants can indeed be limited by  
22 inorganic carbon (Madsen and Maberly 1991; Vadstrup and Madsen 1995).

23

## 24 **Responses to carbon-limitation in freshwaters**

1 Freshwater plants have evolved anatomical, morphological, biochemical, physiological and  
2 ecological strategies to counter this restriction (Bowes 1987; Bowes and Salvucci 1989; Madsen  
3 and Sand-Jensen 1991; Raven 1995; Maberly and Madsen 2002; Raven et al. 2008). These  
4 strategies can be broadly classified as: ‘avoidance’, ‘exploitation’ and ‘amelioration’.

#### 6 *Avoidance strategies*

7 This is perhaps the simplest strategy and relies on the ability of the plants to avoid low-CO<sub>2</sub>  
8 habitats or niches. In the aquatic habitat avoidance of low-CO<sub>2</sub> is possible due to the high within-  
9 and among-lake variation in concentration of CO<sub>2</sub>. For example, the freshwater moss *Fontinalis*  
10 *antipyretica*, which is restricted to the use of CO<sub>2</sub> (obligate CO<sub>2</sub>-user), could survive in a lake with  
11 substantial summer CO<sub>2</sub>-depletion by exploiting the niche just above the sediment surface with  
12 elevated CO<sub>2</sub> concentrations (Maberly 1985). Another example of plants avoiding low-CO<sub>2</sub> is  
13 macrophytes from streams, which benefit from the continuous replacement of CO<sub>2</sub>-depleted water.  
14 Finally, macrophytes from unproductive lakes do not experience the same severe CO<sub>2</sub>-depletion as  
15 plants from productive lakes and therefore macrophytes from these habitats are more likely to  
16 depend on CO<sub>2</sub> taken up from the water column than species from productive lakes (Maberly and  
17 Madsen 2002).

#### 19 *Exploitation strategies*

20 Since some of the anatomical and morphological adaptations allow exploitation of alternative  
21 inorganic carbon sources besides CO<sub>2</sub> from the water, they are referred to as ‘exploitation  
22 strategies’. These include 1/ floating or aerial leaves, which enable freshwater plants to make use of  
23 atmospheric CO<sub>2</sub>; 2/ aerenchyma or lacunae within roots, stems and leaves, which allow gas  
24 transport by diffusion or mass flow and – linked to 2 – 3/ uptake of CO<sub>2</sub> from the interstitial water

1 in the sediment (sediment-CO<sub>2</sub>). Carbon uptake by floating or aerial leaves can make a major  
2 contribution to the carbon-balance of some freshwater plants (e.g. Prins and De Guia 1986, Nielsen  
3 and Borum 2008) and can also allow forced ventilation supplying oxygen and removing ethanol  
4 from the roots and hence promoting survival in anoxic sediments (Dacey 1980). The sediment-CO<sub>2</sub>  
5 is transported through the roots to the leaves in the lacunae system (Bowes 1987; Bowes and  
6 Salvucci 1989; Madsen and Sand-Jensen 1991; Madsen and Sand-Jensen 2006). Uptake of  
7 sediment-CO<sub>2</sub> is only significant in the functional group of isoetids because of their large root-  
8 allocation, well-developed lacunae and short stature (Raven et al. 1988; Madsen et al. 2002). In  
9 addition to enabling the exploitation of sediment-CO<sub>2</sub>, the lacunae facilitate transport of O<sub>2</sub>,  
10 produced in the leaves, to the roots.

11 Many submerged plants have evolved thin or dissected leaves – resulting in a large  
12 surface:volume ratio – and have chloroplasts positioned in the outermost cell layers of the leaf  
13 (Madsen and Sand-Jensen 1991) which may help to minimise transport limitation. Thin leaves may  
14 also match low areal-amounts of photosynthetic machinery to low areal-rates of inward carbon flux  
15 (Black et al. 1981). Although these anatomical and morphological adaptations may have evolved to  
16 reduce inorganic carbon limitation, their evolution could have been triggered by other  
17 environmental factors such as removal of water-shortage, response to shear-stress from water-flow  
18 and availability of nutrients or light.

19

## 20 *Amelioration strategies*

21 Physiological or biochemical adaptations, as opposed to the anatomical and morphological  
22 adaptations, most likely evolved to ameliorate inorganic carbon limitation. They are generally  
23 referred to as carbon concentrating mechanisms (CCMs) because they increase the concentration of

1 inorganic carbon around the active site of Rubisco (Bowes and Salvucci 1989; Madsen and Sand-  
2 Jensen 1991; Maberly and Madsen 2002; Raven et al. 2008).

3 CCMs are not ubiquitous in freshwater plants because their operation has both costs and benefits.  
4 The benefits may include increased carbon-uptake, reduced photorespiration, reduced  
5 photoinhibition and increased nutrient-use efficiency (Bowes and Salvucci 1989; Madsen and Sand-  
6 Jensen 1991; Raven et al. 2008). The photorespiration-cycle is an energy- and carbon-expensive  
7 mechanism, since one CO<sub>2</sub> equivalent is lost, when two O<sub>2</sub> equivalents are fixed by Rubisco.  
8 Photorespiration is enhanced by a high [O<sub>2</sub>]:[CO<sub>2</sub>] ratio near the active site of Rubisco and thus by  
9 CO<sub>2</sub> depletion, high O<sub>2</sub> concentrations and high temperature (Bowes 1991). By increasing the  
10 internal CO<sub>2</sub> supply and thereby increasing the [CO<sub>2</sub>]:[O<sub>2</sub>] ratio internally, the operation of the  
11 CCM can reduce photorespiration. Due to the higher internal CO<sub>2</sub> supply the CCM may also  
12 alleviate photoinhibition, since surplus energy may be dissipated via photosynthetic carbon  
13 assimilation (Osmond et al. 1993; White et al. 1996). Theoretically, the CCM, which increases the  
14 concentration of CO<sub>2</sub> around Rubisco, may increase the nutrient-use efficiency because of higher  
15 efficiency of the carboxylase activity of Rubisco (Ehleringer and Monson 1993). Higher  
16 carboxylase efficiency could reduce the Rubisco needed for a given amount of carbon fixation and  
17 thereby result in higher nitrogen-use efficiency (NUE). However, bicarbonate use is not increased  
18 under nutrient-deficient conditions, but rather depends on a sufficient nutrient-supply (Baatrup-  
19 Pedersen 1996). Similarly, for *Littorella uniflora* the relation between CAM and photosynthetic  
20 NUE could not be verified experimentally, although CAM was still present at low nitrogen  
21 concentrations (Baatrup-Pedersen and Madsen 1999).

22 On the flip side of the CCM-coin are the extra costs in terms of energy and nutrient demand  
23 needed to produce, maintain and run the CCM apparatus in addition to the basic costs of the C<sub>3</sub>-  
24 pathway into which it is an accessory (Madsen and Sand-Jensen 1991; Lüttge 2002; Madsen et al.

1 2002). Investment of nitrogen in various CCM enzymes or transport proteins may have a negative  
2 impact in a low-nutrient habitat. In low-light habitats or locations, the energetic cost of the CCM  
3 may be significant (Raven and Spicer 1996), since ATP and NADPH production limit  
4 photosynthesis at low light. However, in high-light habitats the energetic costs of the CCM are most  
5 likely irrelevant – or potentially affect plant performance positively by reducing photoinhibition.

6 The amelioration mechanisms include 1/ bicarbonate ( $\text{HCO}_3^-$ ) uptake 2/  $\text{C}_4$ -fixation and 3/  
7 Crassulacean Acid Metabolism (CAM).

8

### 9 *$\text{HCO}_3^-$ uptake*

10 Uptake of bicarbonate from the bulk medium into the cell ( $\text{HCO}_3^-$  use) appears favourable in  
11 most freshwaters since its concentration exceeds that of  $\text{CO}_2$  at pH values higher than ca. 6.4  
12 (Maberly and Spence 1983; Vestergaard and Sand-Jensen 2000; Madsen and Sand-Jensen 2006).  
13 However, the affinity for bicarbonate is lower than the  $\text{CO}_2$  affinity and thus  $\text{CO}_2$  is the preferred  
14 inorganic carbon source when concentrations of  $\text{HCO}_3^-$  and  $\text{CO}_2$  are similar (Bowes and Salvucci  
15 1989; Maberly and Spence 1989; Prins and Elzenga 1989). Bicarbonate use is by far the most  
16 frequently observed physiological mechanism for increasing inorganic carbon uptake and has been  
17 reported in about 50% of the investigated submerged angiosperms (Maberly and Madsen 2002).  
18 Transport of bicarbonate into the cell can occur directly via a  $\text{HCO}_3^- \text{H}^+$  symporter or indirectly via  
19 acidification of the boundary layer, thereby shifting the chemical equilibrium towards  $\text{CO}_2$ , which  
20 thereafter can diffuse into the cell (Prins and Elzenga 1989). Bicarbonate users have a competitive  
21 advantage and are generally most abundant in alkaline habitats, where pH and the absolute  
22 concentration of bicarbonate often are high (Maberly and Spence 1983; Vestergaard and Sand-  
23 Jensen 2000). In addition to energy costs, species that are able to use bicarbonate have a lower



1 affinity for CO<sub>2</sub> than species restricted to CO<sub>2</sub> alone (obligate CO<sub>2</sub>-users) (Maberly and Madsen  
2 1998; Madsen and Maberly 2003), which may impose an ecological cost at some sites.

3

#### 4 *C<sub>4</sub>-metabolism*

5 In addition to bicarbonate use, two inorganic carbon uptake mechanisms exist in freshwater  
6 plants that are based on C<sub>4</sub>-metabolism. They depend on carbon fixation via the enzyme  
7 phosphoenol pyruvate carboxylase (PEPcase) either during the day (C<sub>4</sub>) or during the night (CAM),  
8 involving either a spatial (C<sub>4</sub>) or temporal (CAM) separation of inorganic carbon fixation through  
9 PEPcase and Rubisco (Bowes and Salvucci 1989; Ehleringer and Monson 1993; Keeley and Rundel  
10 2003). The light-dependent PEPcase fixation of inorganic carbon in freshwater plants is analogous  
11 to the terrestrial C<sub>4</sub> photosynthetic pathway, but in contrast to terrestrial C<sub>4</sub> – which is normally  
12 expressed constitutively – freshwater C<sub>4</sub> is a plastic mechanism, induced under inorganic carbon  
13 limitation (Van et al. 1976; Salvucci and Bowes 1981; Reiskind et al. 1997). Furthermore,  
14 freshwater C<sub>4</sub> plants do not have Kranz-anatomy like most terrestrial C<sub>4</sub> plants. However, single-  
15 cell C<sub>4</sub>-metabolism has recently been observed in terrestrial plants and may be an overseen  
16 phenomenon in freshwater plants (Edwards et al. 2004). C<sub>4</sub>-metabolism appears to be relatively rare  
17 in freshwater plants, it has been observed in *Hydrilla verticillata*, *Egeria densa* (Bowes and  
18 Salvucci 1989; Madsen and Sand-Jensen 1991; Casati et al. 2000) and a number of freshwater  
19 grasses (Keeley 1998a, Ueno et al. 1988).

20

#### 21 *CAM*

22 CAM is primarily known from desert plants as an adaptation to enhance water conservation  
23 (Kluge and Ting 1978; Osmond 1978; Winter and Smith 1996; Cushman 2001; Dodd et al. 2002,  
24 Silvera et al. 2010). It enables CO<sub>2</sub> to be taken up and fixed via night-time PEPcase activity and the

1 C<sub>4</sub> product stored in the cell vacuole as malate, causing a decline in cell-sap acidity. During the day,  
2 malate is decarboxylated, resulting in de-acidification and the released CO<sub>2</sub> is fixed by Rubisco and  
3 enters the Calvin cycle (Fig. 1; Groenhof et al. 1988; Winter and Smith 1996; Nimmo 2000).

4 However, CAM is also present in some freshwater plants where it serves a different function.  
5 Unlike terrestrial CAM plants, where stomata are closed during the day, freshwater CAM plants  
6 have no stomata and CO<sub>2</sub> can potentially be taken up 24 hours a day (Osmond 1978; Keeley  
7 1998b). In freshwater plants, the inorganic carbon source for PEPcase fixation (HCO<sub>3</sub><sup>-</sup>) is derived  
8 from endogenous (respiratory CO<sub>2</sub>) or exogenous sources (CO<sub>2</sub> from the bulk water or sediment-  
9 CO<sub>2</sub>). Use of HCO<sub>3</sub><sup>-</sup> as the inorganic carbon specimen being transported into the cell has not been  
10 observed in aquatic CAM plants (Maberly and Madsen 2002). In addition to minimising or  
11 preventing respiratory carbon loss (potentially a positive carbon gain) in the night, freshwater CAM  
12 plants are able to concentrate CO<sub>2</sub> internally during the decarboxylation phase and thus CAM  
13 functions both as a carbon conserving mechanisms and a CCM (Keeley 1998b; Madsen et al. 2002).  
14 Freshwater CAM has been observed in five freshwater genera, *Isoetes*, *Littorella*, *Crassula*,  
15 *Sagittaria* and *Vallisneria* (Keeley 1998b) and is thus present in isoetids and elodeids.

16

### 17 **Habitats with CAM plants**

18 For CAM (and other CCMs) to be of ecological benefit, the plants with CAM must be growing  
19 in a habitat with limited inorganic carbon. One such low-carbon habitat is soft-water lakes, which  
20 are characterised by relatively low pH, very low total inorganic carbon concentration and  
21 bicarbonate concentrations that are too low to support bicarbonate-use. Here, plants with CAM are  
22 likely to have an ecological advantage, since inorganic carbon can be taken up throughout the day  
23 increasing carbon gain and thus enhancing the chance of survival. In agreement with this, several  
24 CAM species – including the isoetids *Isoetes spp.* and *Littorella uniflora* – belong to the plant

1 community typical of oligotrophic, soft-water lakes (Sand-Jensen and Søndergaard 1997; Keeley  
2 1996; Madsen et al. 2002).

3 CAM may not only raise the competitive ability of the plants in soft-water lakes, but also in  
4 habitats with large fluctuations in the CO<sub>2</sub> concentration. Large daily CO<sub>2</sub> variations occur in low-  
5 and high alkaline lakes with a high productivity, thereby giving rise to low daytime and high night-  
6 time CO<sub>2</sub> concentrations in the open water (Maberly 1996) and especially in weed beds (Van et al.  
7 1976). In these lakes with large CO<sub>2</sub>-fluctuations, plants with CAM are 1/ able to take up inorganic  
8 carbon in the night, where the CO<sub>2</sub> concentration is higher and where competition for inorganic  
9 carbon with non-CAM species is eliminated and 2/ less dependent on external CO<sub>2</sub> in the daytime –  
10 and thus CAM confers a competitive advantage upon these species relative to non-CAM species in  
11 these habitats. In accordance with this, isoetid-CAM species are often found in ‘seasonal-pools’,  
12 while CAM species such as the invasive *Crassula helmsii* can be found in high-alkaline, more  
13 eutrophic lakes (Keeley 1996, 1999; Dawson and Warman 1987). Thus, even in high-alkaline  
14 habitats with a relatively high inorganic carbon concentration during the daytime, CO<sub>2</sub> may be  
15 limiting and thus make the possession of CAM favourable. However, the reason why high-alkaline  
16 lakes are not a typical CAM-plant habitat is likely to be caused by the direct competition with  
17 bicarbonate-users, which can take advantage of the high bicarbonate concentration and tend to be  
18 larger, faster-growing species.

19

## 20 **CAM plasticity**

21 CAM is a plastic mechanism in freshwater plants which is consistent with its function as a  
22 carbon conserving and carbon concentrating mechanism: the regulation ensures that resource-  
23 allocation to energy- and nutrient-demanding uptake mechanisms is avoided when inorganic carbon  
24 does not limit photosynthesis (Bowes and Salvucci 1989; Maberly and Madsen 2002; Madsen et al.

1 2002). The regulation can involve long-term acclimation over weeks or months or short-term  
2 responses (during the 24 hour cycle) to external conditions and has been documented in isoetids and  
3 elodeids.

#### 5 *Long-term regulation of CAM*

6 Regulation of CAM is dependent on various environmental parameters e.g. light, CO<sub>2</sub>,  
7 temperature, nutrients and water level (Aulio 1985; Madsen 1987a; Robe and Griffiths 1990;  
8 Hostrup and Wiegleb 1991; Klavsen and Maberly 2009; 2010, Klavsen unpubl. data). However, the  
9 outcome of regulation of CAM is dependent on the interaction between these parameters (Table 1).

10 Light and CO<sub>2</sub> interact in the regulation of CAM, and for the invasive elodeid, *C. helmsii*, low  
11 light causes down-regulation, independent of the CO<sub>2</sub> concentration (Klavsen and Maberly 2010)  
12 (Table 1). For the isoetid *L. uniflora*, down-regulation of the CAM apparatus has also been  
13 observed at low light, although in this species down-regulation depends on the CO<sub>2</sub> availability  
14 during growth, with low CO<sub>2</sub> grown plants not reducing CAM activity (Madsen 1987a; Klavsen  
15 unpubl. data) (Table 1). In a low light regime, and particularly at moderate or high CO<sub>2</sub>, CO<sub>2</sub>  
16 becomes saturating for photosynthesis which most likely triggers down-regulation of CAM. Down-  
17 regulation of CAM at low light is ecophysiologicaly favourable because it removes the energy cost  
18 associated with maintaining and running the CAM cycle (Raven and Spicer 1996). Maintenance of  
19 the CAM apparatus in a low light regime may also be too costly in terms of nutrients. When CAM  
20 is not needed to enhance inorganic carbon uptake, nutrients associated with CAM can be allocated  
21 to acquisition of more limiting resources such as investments in light harvesting.

22 At light intensities saturating for photosynthesis and low CO<sub>2</sub> availability, CAM is generally up-  
23 regulated (Madsen 1987a; Robe and Griffiths 1990; Klavsen and Maberly 2010). At saturating  
24 light, CAM is generally decreased with raised CO<sub>2</sub> (Table 1). However, the CO<sub>2</sub> concentration, at

1 which down-regulation is triggered, is very different in *C. helmsii* and *L. uniflora*. The reason for  
2 the differences in the absolute CO<sub>2</sub> concentrations causing down-regulation are likely to be related  
3 to the CO<sub>2</sub> concentration needed to saturate photosynthesis, which for isoetids is relatively high  
4 (half-saturation around 500-600 μmol L<sup>-1</sup> CO<sub>2</sub>) (Madsen et al. 2002). For *C. helmsii* the half-  
5 saturation concentration of CO<sub>2</sub> is lower and was estimated to be ca. 100 μmol L<sup>-1</sup> from the data  
6 from Klavsen and Maberly (2010). Regarding *L. uniflora* contrary results on CAM regulation at  
7 high light have been found, since CAM down-regulation is not triggered by high CO<sub>2</sub> *per se*  
8 (Madsen 1987a, Baatrup-Pedersen and Madsen 1999), thereby emphasising the interactive effect of  
9 environmental parameters on CAM.

10 In agreement with light affecting the regulation of CAM, CAM varies with season and thus light  
11 intensity (Boston and Adams 1985; Klavsen and Maberly 2009). Indirectly, seasonal regulation  
12 indicates regulation of CAM by temperature in *L. uniflora* and *C. helmsii*. For *L. uniflora* regulation  
13 of CAM by temperature has been observed, since *L. uniflora* appears to optimize CAM at or close  
14 to ambient temperature (Klavsen unpubl. data). This implies that *L. uniflora* performed better at low  
15 than high temperature in the winter months (Q<sub>10</sub> of 0.6-0.7). In the summer, CAM was stimulated  
16 by raised temperature and Q<sub>10</sub> was 1.4-1.8 (Klavsen unpubl.). In contrast to terrestrial CAM plants,  
17 the seasonal variation in CAM cannot easily be determined by differences in δ<sup>13</sup>-C, since the δ<sup>13</sup>-C  
18 values in aquatic plants vary depending on factors such as inorganic carbon source and diffusion  
19 resistance (Keeley and Sandquist 1992).

20 The seasonal regulation of CAM by light and temperature is in agreement with CAM acting as a  
21 CCM to enhance inorganic carbon uptake under environmental conditions with inorganic carbon  
22 depletion. In the summer – where CAM is highest (Fig. 3, Boston and Adams 1985; Klavsen and  
23 Maberly 2009) – high temperature and irradiance as well as long daylength enhance the  
24 photosynthetic rate and the overall daily photosynthesis and thus increase the inorganic carbon

1 demand and the need for CAM. The need for an up-regulated CCM is further accentuated by a  
2 potentially higher photorespiration because of higher temperature in summer.

3 Regulation by nutrients appears to be of minor importance, although nutrient-depletion lowers  
4 CAM in *L. uniflora* grown at high light (Madsen 1987a; Robe and Griffiths 1994; Baatrup-Pedersen  
5 and Madsen 1999). This is consistent with the higher nutrient demand in the production and  
6 maintenance of the CAM apparatus, including CAM-related enzymes and tonoplast transporters.  
7 Theoretically, but not experimentally verified (Baatrup-Pedersen and Madsen 1999), a higher  
8 nitrogen use efficiency due to the operation of CAM may have balanced the extra nitrogen cost.

9 Freshwater CAM plants growing in the near-shore area of the littoral zone or in seasonal pools  
10 can be exposed to air. In the water-land transition, CAM is often fully or partially down-regulated  
11 (Keeley et al. 1983; Keeley and Busch 1984; Aulio 1985; Keeley 1999; Robe and Griffiths 2000).  
12 This is explained by higher inorganic carbon availability caused by the  $10^4$  times higher diffusion  
13 rate in air compared to water. Contemporary with CAM being down-regulated, *L. uniflora* also  
14 acclimates to the aerial life by traits such as low lacunal volume, high Rubisco activity and  
15 production of stomata, which enables the terrestrial life-form to make use of CO<sub>2</sub> from the air and  
16 makes the plant less dependent on CO<sub>2</sub> from the sediment and from CAM. However, contrary  
17 results on CAM regulation in the shift from water to land occur, since CAM is not always down-  
18 regulated in the land-form (Farmer and Spence 1985; Aulio 1986) and exposure to atmospheric CO<sub>2</sub>  
19 *per se* therefore does not trigger down-regulation. The factor triggering CAM regulation in the land-  
20 form may be water-vapour concentration, thereby down-regulating CAM, when the water-vapour  
21 concentration is low (Aulio 1986). However, since the land-form of *L. uniflora* can still rely on  
22 sediment CO<sub>2</sub> and dark CO<sub>2</sub> uptake via CAM (Nielsen et al. 1996), the CO<sub>2</sub> concentration  
23 experienced by the plant may not differ from the CO<sub>2</sub> experienced under water – and this may be  
24 the reason for the lack of CAM down-regulation.

1

## 2 *Short-term regulation of CAM*

3 Light and CO<sub>2</sub> does not only affect the diel CAM cycle in the long term (after an acclimation  
4 period), but also in the short term and thus on a daily basis and this effect has been observed in both  
5 laboratory and field (Keeley et al. 1983; Keeley and Busch 1984; Boston and Adams 1985; Madsen  
6 1987a; Hostrup and Wiegleb 1991; Robe and Griffiths 1990; Rattray et al. 1992; Klavsen and  
7 Maberly 2010; Klavsen unpubl.). Generally, malate decarboxylation appears to be dependent on the  
8 demand for inorganic carbon relative to its supply rate during the day. Thus, it has been found that  
9 high CO<sub>2</sub> availability and/or reduced light intensity, e.g. caused by an overcast sky, affect the  
10 amount of malate being decarboxylated, thereby resulting in lower decarboxylation rates – or  
11 complete inhibition of decarboxylation – and/or higher minimum acidity level at the end of the light  
12 period. Contrary, a high photosynthetic carbon-demand increases the decarboxylation rate and  
13 lowers the minimum acidity level obtained in the evening (Boston and Adams 1985; Madsen  
14 1987b; Robe and Griffiths 1990; Rattray et al. 1992; Klavsen and Maberly 2010). However, in *C.*  
15 *helmsii* grown under low and high CO<sub>2</sub>, decarboxylation rates did not vary between CO<sub>2</sub> treatments,  
16 but the decarboxylation period was longer and the minimum acidity level lower for low CO<sub>2</sub> grown  
17 plants (Klavsen and Maberly 2010). In *L. uniflora* the rate of decarboxylation was generally high  
18 under low external CO<sub>2</sub> concentration, but could be fully inhibited by high CO<sub>2</sub> (Madsen 1987c).  
19 This indicates that CAM in *L. uniflora* operates under most natural CO<sub>2</sub> conditions, although the  
20 long-term regulation of CAM, e.g. due to seasonal changes, will affect the actual CAM activity  
21 (Boston and Adams 1985; Klavsen and Maberly 2009)

22 Light not only affects decarboxylation, but also affects photosynthesis and eventually the pool of  
23 starch being synthesised during the day. In the night, starch is broken down in glycolysis and serves  
24 as the precursor for phosphoenol pyruvate (PEP) – the acceptor-molecule for night-time fixation of

1 inorganic carbon via PEPcase (see Fig. 1) (Kluge and Ting 1978; Osmond 1978; Winter and Smith  
2 1996). Thus, the light intensity the previous day can potentially have implications for malate (and  
3 thereby acidity) accumulation in the night. This indirect effect of light on CAM has been observed  
4 in *C. helmsii*, where high concentration of CO<sub>2</sub> only had significant effect on the acidity build up in  
5 the night after exposure to high daytime light intensity (Klavsen and Maberly 2010). It should be  
6 noted that in *I. bolanderi* the starch pool is not always sufficient to account for the malate build-up  
7 in the night (Keeley et al. 1983), indicating a role for another carbohydrate precursor-molecule or  
8 alternatively that starch production occurs from other carbohydrates simultaneously with starch  
9 breakdown.

10

#### 11 **Decarboxylation and O<sub>2</sub>:CO<sub>2</sub> ratios**

12 The regulatory pattern of CAM indicates that CAM functions as a CCM in freshwater  
13 macrophytes. However, for CAM to act as an effective CCM, the photosynthetic rate should at least  
14 balance the rate of decarboxylation, since CO<sub>2</sub> derived from CAM could otherwise be lost. In *L.*  
15 *uniflora* this was verified experimentally, since less than 2% of the CO<sub>2</sub> resulting from daytime  
16 decarboxylation was lost (Smith et al. 1985; Madsen 1987b) and since the photosynthetic rate  
17 exceeds the decarboxylation rate in both *L. uniflora* and *C. helmsii* (Klavsen and Madsen 2008;  
18 Klavsen and Maberly 2009). In agreement with this, photosynthesis and CAM have been shown to  
19 be positively coupled in *L. uniflora* (Klavsen and Madsen 2008).

20 For CAM to operate efficiently as a CCM, and thus for decarboxylation to influence the rate of  
21 photosynthesis positively, it would be anticipated that the O<sub>2</sub> evolution relative to the external CO<sub>2</sub>  
22 uptake (and thus the O<sub>2</sub>:CO<sub>2</sub> ratio) will be well above 1 during the decarboxylation phase. This was  
23 found for *L. uniflora* and *I. lacustris* (Madsen 1987b), where the O<sub>2</sub>:CO<sub>2</sub> ratio was up to 3.5 during  
24 decarboxylation (Fig. 4). If the oxygen evolution does not increase considerable and thus give rise



1 to O<sub>2</sub>:CO<sub>2</sub> ratio above 1 during decarboxylation this may be because either 1/ external CO<sub>2</sub> is so  
2 high that decarboxylation is inhibited or 2/ the high internal CO<sub>2</sub> obtained during decarboxylation  
3 inhibits external CO<sub>2</sub> uptake. This implies that the CCM is working less efficiently and external  
4 CO<sub>2</sub> will not be taken up 24 hours a day, thereby minimising C-gain. For *C. helmsii* no considerable  
5 change in oxygen evolution was observed during decarboxylation (Fig. 4). This may question the  
6 concept of CAM as a CCM in this species. However, since decarboxylation appears to be delayed in  
7 *C. helmsii*, maybe due to a circadian rhythm or daytime C<sub>4</sub> activity, the plant may benefit from  
8 CAM, since decarboxylation occurs around midday, where the inorganic carbon demand is likely to  
9 be greatest (Klavsén and Maberly 2010). Furthermore, CAM may help conserve carbon, since  
10 respiratory CO<sub>2</sub> can be re-captured in the night.

11

## 12 **CAM in relation to C-gain**

13 For CAM to be of ecological significance as a carbon conserving mechanism, CAM must first of  
14 all be present in the field. Although the *in situ* CAM activity is dependent on long term (e.g. season)  
15 and short term regulation (e.g. day-to-day changes in, for example, irradiance), significant *in situ*  
16 CAM activities have been found in several aquatic CAM species (Fig. 3) (Keeley et al. 1983;  
17 Boston & Adams 1985; Rattray et al. 1992; Klavsén and Maberly 2009). In addition to CAM being  
18 present under natural conditions, CAM must contribute considerably to the carbon gain to act as a  
19 carbon conserving mechanism. For *L. uniflora*, CAM undoubtedly contributes in a net positive  
20 carbon gain, since decarboxylation does not inhibit the external inorganic carbon uptake (resulting  
21 in large O<sub>2</sub>:CO<sub>2</sub> ratios (Fig. 4)). Due to the plasticity of CAM, the influence of night-time CO<sub>2</sub>  
22 uptake on daily CO<sub>2</sub> uptake in photosynthesis can vary significantly depending on the  
23 environmental conditions. Thus, the contribution from CO<sub>2</sub> derived from CAM to daily  
24 photosynthesis varies from 0 to 95%. The latter estimate of the contribution from CAM was found

1 for *L. uniflora* and *I. lacustris* at an external CO<sub>2</sub> concentration of 30 μmol L<sup>-1</sup>. At higher external  
2 CO<sub>2</sub> concentrations the night-time CO<sub>2</sub> uptake via CAM in relation to daily photosynthetic carbon  
3 uptake was reduced to 34-38% (Madsen 1987b) due to higher uptake of external CO<sub>2</sub> and  
4 potentially partial inhibition of decarboxylation. In *L. uniflora* grown at low light, the contribution  
5 from CAM-derived CO<sub>2</sub> to photosynthesis was high (62%), but lower than in plants grown at high  
6 light (81%) (Robe and Griffiths 1990). Also in *I. howellii* ca. 30-50% of daily CO<sub>2</sub> uptake in  
7 photosynthesis was estimated to derive from night-time uptake through CAM (Keeley and Busch  
8 1984). Another estimate of the contribution from CAM to the carbon budget was made on *L.*  
9 *uniflora*, in which 40-55% of the annual carbon gain derived from CAM (Boston and Adams 1985,  
10 1986).

11 For the elodeid *C. helmsii*, however, no oxygen peak is observed during decarboxylation (Fig. 4))  
12 and thus the benefit from CAM is in principle lost. However, CAM may still be favourable to the  
13 C-gain of the plant, if the external CO<sub>2</sub> concentration is low. In *C. helmsii* the *in situ* contribution  
14 from CAM to daily photosynthesis varied from 18 to 42%, depending on depth of growth and time  
15 of year (Klavsen and Maberly 2009). Most likely these estimates are valid as contributions from  
16 CAM to the daily carbon balance, since almost all respiratory CO<sub>2</sub> in the night was refixed via  
17 CAM and since roots make up a very small part of the total plant biomass in this species. Thus, in  
18 natural populations of freshwater CAM species, CAM appears to be of high ecophysiological  
19 significance for the carbon balance. These estimates are in agreement with estimates for terrestrial  
20 facultative CAM plants, in which 10 to nearly 100% of the carbon fixation in daily photosynthesis  
21 derive from CAM (Winter and Holtum 2002; Lüttge 2004).

22

23 **Night time CO<sub>2</sub> uptake**

1 CAM potentially enables the plants to take up inorganic carbon 24 hours a day, although this is  
2 probably not realised in all species (Keeley 1998b; Madsen et al. 2002; Klavsen and Maberly 2010).  
3 Even though external CO<sub>2</sub> is not taken up at night, CAM can still be considered a carbon conserving  
4 mechanism, since re-capture of respiratory endogenous produced CO<sub>2</sub> through the operation of  
5 CAM can reduce or eliminate C-loss in the night and thereby influence C-gain positively (Keeley  
6 and Busch 1984; Madsen 1987c; Robe and Griffiths 1990; Madsen et al. 2002). The contribution of  
7 re-captured respiratory CO<sub>2</sub>, otherwise lost to the surroundings, to the total CO<sub>2</sub> uptake via CAM is  
8 dependent on the external CO<sub>2</sub> concentration, but often makes up a substantial part of the night-time  
9 inorganic carbon fixation. For *L. uniflora* between 30 and 99% of night-time CO<sub>2</sub> uptake via CAM  
10 derives from CO<sub>2</sub> produced in respiration (Richardson et al. 1984; Smith et al. 1985; Madsen  
11 1987b,c; Boston et al. 1987; Robe and Griffiths 1990) and for *I. howellii* values of 50-66% have  
12 been found (Keeley and Busch 1984). Since respiratory CO<sub>2</sub> under natural conditions rarely makes  
13 up the total night-time CO<sub>2</sub> uptake, this implies that CO<sub>2</sub> uptake through CAM is at least partly  
14 dependent on the external CO<sub>2</sub> availability, which potentially can lead to inorganic carbon  
15 limitation at night (Klavsen and Maberly 2010). However, the length of the night period – although  
16 not realised under field conditions – can compensate for low external CO<sub>2</sub> availability (Keeley and  
17 Bowes 1982; Madsen et al. 2002). Thus, plants relying on CO<sub>2</sub> primarily derived from endogenous  
18 sources can reach the same maximum CAM activity as plants incubated in a high CO<sub>2</sub> medium.  
19 Respiratory CO<sub>2</sub> can potentially make up the entire night-time carbon uptake through CAM under  
20 low external CO<sub>2</sub> in both *C. helmsii* and *L. uniflora*, since the rate of respiration can exceed the rate  
21 of CO<sub>2</sub> uptake through CAM (assuming a constant CO<sub>2</sub> uptake in CAM, a constant respiratory rate  
22 and a respiratory quotient of 1) (Boston et al. 1987; Klavsen and Maberly 2010).

23

## 24 **Conclusions**

1 CAM is found in aquatic plants belonging to both the functional group of isoetids and elodeids. In  
2 both types of CAM plants, CAM is regulated in relation to environmental cues – in agreement with  
3 CAM functioning as a CCM in aquatic plants. For both isoetid CAM-species (*Isoetes sp.* and *L.*  
4 *uniflora*) and the elodeid *C. helmsii*, CAM appears to be of high ecological importance, since  
5 inorganic carbon uptake via CAM contributes significantly to the carbon budget. For *C. helmsii* –  
6 but not the isoetid CAM-plants – external inorganic carbon uptake seems to be inhibited by  
7 decarboxylation, which will lower the significance of CAM. However, CAM may still help  
8 conserve carbon, since respiratory CO<sub>2</sub> loss can be eliminated by re-fixation through PEPcase in the  
9 night. Furthermore, for *C. helmsii*, CAM may be beneficial when the external concentration of CO<sub>2</sub>  
10 in the water is low.

11

## 12 **Acknowledgements**

13 This work was supported by a grant to SKK from the Danisch Research Council for independent  
14 research: Natural Sciences.

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6

7

8

1 **Table 1.** Regulation of CAM in aquatic CAM plants. Means of available data are presented. ‘-’  
2 indicates ‘not determined’. Plants have been growing and acclimated to conditions of CO<sub>2</sub> and light  
3 according to the ones given in the table. Actual CAM was measured as the diurnal change in acidity  
4 under growth conditions. Potential CAM was determined as the maximum diurnal acidity change:  
5 in the daytime plants were placed in low CO<sub>2</sub> (ca. atmospheric equilibrium) and high light (thereby  
6 increasing decarboxylation) and in the night plants were incubated in a medium with high CO<sub>2</sub>  
7 (>500 mmol m<sup>-3</sup>) (thereby increasing night-time CO<sub>2</sub> uptake via CAM).

<b>Species</b>	<b>Free CO<sub>2</sub></b> ( $\mu\text{mol L}^{-1}$ )	<b>Light</b> ( $\mu\text{mol m}^{-2} \text{s}^{-2}$ )	<b>Temp.</b> (°C)	<b>Actual CAM</b> ( $\mu\text{eq g}^{-1} \text{FW}$ )	<b>Potential CAM</b> ( $\mu\text{eq g}^{-1} \text{FW}$ )	<b>Ref.</b>
<i>L. uniflora</i>	60	40	15	35	65	4
	60	200	15	50	125	4
	100	450	15	66	87	1
	100	200	15	60	-	3
	100	1000	15	52	-	3
	130	300 <sup>a</sup>	18-28	50	55	2
	300	200	15	50	-	3
	300	1000	15	46	-	3
	500	40	15	15	60	4
	500	200	15	90	130	4
	900	300 <sup>a</sup>	18-28	140	180	2
	1000	1000	15	46	-	3
	1500	43	15	-4	30	1
	1500	450	15	70	79	1

	5500	450	15	-2	36	1
	50/1000 <sup>b</sup>	50	19-20	35	-	5
	50/1000 <sup>b</sup>	300	19-20	112	-	5
	80/600 <sup>c</sup>	350	18-19	110	-	6
	20/1000 <sup>c</sup>	50	18-19	60	-	6
<i>C. helmsii</i>	3	200	20	14	70	4
	20	40	15	-	83	4
	20	150	15	-	109	4
	22	30	20	15	23	7
	22	150	20	30	44	7
	22	23	20	-2	12	8
	22	230	20	35	60	8
	230	23	20	2	8	8
	230	230	20	18	30	8
	290	40	15	-	19	4
	290	150	15	-	12	4

1 Ref 1: Madsen (1987), Ref 2: Baatrup-Pedersen and Madsen 1999, Ref 3: Boston and Adams 1987,

2 Ref 4: Klavsen unpubl. Data, Ref 5: Robe and Griffith 1990, Ref 6: Robe and Griffith 1994, Ref 7:

3 Klavsen and Maberly 2009, Ref 8: Klavsen and Maberly 2010.

4 <sup>a</sup>estimate based on an irradiance of 10-16 mol photons m<sup>-2</sup> day<sup>-1</sup>

5 <sup>b</sup>plants were grown in natural sediments. The free CO<sub>2</sub> concentrations of the water and interstitial  
6 water were 50 and 1000 μmol L<sup>-1</sup> respectively.

7 <sup>c</sup>plants were grown in natural sediments. The free CO<sub>2</sub> concentrations of the water and interstitial  
8 water were either 20 or 80 and 600 and 1000 μmol L<sup>-1</sup> respectively.



1 **Figure captions**

2 **Fig. 1.** The Crassulacean Acid Metabolism (CAM) cycle. Dark CO<sub>2</sub> fixation occurs through the  
3 enzyme, PEPcase, and the sources of inorganic carbon are either of endogenous origin (respiration)  
4 or of exogenous origin (water or sediment-CO<sub>2</sub>). The grey area represents reactions occurring in the  
5 dark, while the white area contain daytime reactions. The round circle symbolises the cell vacuole.  
6 Modified from Winter and Smith (1996).

7

8 **Fig. 2.** *In situ* CAM activity measured in the isoetids *Isoetes lacustris*, *I. bolanderi*, *I. kirkii* and  
9 *Littorella uniflora* and in the elodeid *Crassula helmsii*. Data are modified from Keeley *et al.* (1983),  
10 Boston and Adams (1985), Rattray *et al.* 1992 and Klavsen and Maberly (2009).

11

12 **Fig. 3.** Rates of inorganic carbon uptake and oxygen evolution in the isoetids *Littorella uniflora*  
13 (left panel) and *Isoetes lacustris* (middle panel) and oxygen evolution in the elodeid *Crassula*  
14 *helmsii* (right panel). *Crassula helmsii* was grown and photosynthesis measured at low CO<sub>2</sub> (22  
15 mmol m<sup>-3</sup>), but decarboxylation did not start until after 2 hours after light onset. High CAM activity  
16 results in high O<sub>2</sub>:CO<sub>2</sub> ratios (*L. uniflora* and *I. lacustris*), if external inorganic carbon uptake is not  
17 inhibited by decarboxylation. Data modified from Madsen (1987a) and Klavsen  
18 and Maberly (2010).

19