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1 **Crassulacean Acid Metabolism (CAM) in the context of other carbon concentrating**

2 **mechanisms in freshwater plants: a review**

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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_2 and elevated concentrations of O_2. 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_2 concentrations within and among lakes. Exploitation strategies involve anatomical and

8   morphological features that take advantage of sources of CO_2 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_4-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_2, elodeids, inorganic carbon, isoetids, macrophytes, regulation.

22

Inorganic carbon availability in freshwater habitats

1

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_2. Of these, atmospheric CO_2 is most constant and so, coupled with the relatively

5 high rate of diffusion of CO_2 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_4 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_2 is highly

12 variable and may range from close to 0 to more than 350 µmol L^{-1} (Bowes and Salvucci 1989;

13 Madsen and Sand-Jensen 1991). Because of a high transport limitation caused by low diffusion

14 coefficients of CO_2 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^{-1} (Maberly and Spence 1983;

17 Bowes and Salvucci 1989; Madsen and Sand-Jensen 1991; Maberly and Madsen 1998).

18 Furthermore, photosynthetic removal of CO_2, which often generates very low CO_2 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'.

5

6   *Avoidance strategies*

7   This is perhaps the simplest strategy and relies on the ability of the plants to avoid low-CO_2

8   habitats or niches. In the aquatic habitat avoidance of low-CO_2 is possible due to the high within-

9   and among-lake variation in concentration of CO_2. For example, the freshwater moss *Fontinalis*

10   *antipyretica*, which is restricted to the use of CO_2 (obligate CO_2-user), could survive in a lake with

11   substantial summer CO_2-depletion by exploiting the niche just above the sediment surface with

12   elevated CO_2 concentrations (Maberly 1985). Another example of plants avoiding low-CO_2 is

13   macrophytes from streams, which benefit from the continuous replacement of CO_2-depleted water.

14   Finally, macrophytes from unproductive lakes do not experience the same severe CO_2-depletion as

15   plants from productive lakes and therefore macrophytes from these habitats are more likely to

16   depend on CO_2 taken up from the water column than species from productive lakes (Maberly and

17   Madsen 2002).

18

19   *Exploitation strategies*

20   Since some of the anatomical and morphological adaptations allow exploitation of alternative

21   inorganic carbon sources besides CO_2 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_2; 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_2 from the interstitial water

4

1   in the sediment (sediment-CO_2). 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_2

5   is transported though 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_2 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_2, the lacunae facilitate transport of O_2,

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_2 equivalent is lost, when two O_2 equivalents are fixed by Rubisco.

8   Photorespiration is enhanced by a high $[O_2]:[CO_2]$ ratio near the active site of Rubisco and thus by

9   CO_2 depletion, high O_2 concentrations and high temperature (Bowes 1991). By increasing the

10   internal CO_2 supply and thereby increasing the $[CO_2]:[O_2]$ ratio internally, the operation of the

11   CCM can reduce photorespiration. Due to the higher internal CO_2 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_2 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_3-

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 (HCO_3^-) uptake 2/ C_4-fixation and 3/

7   Crassulacean Acid Metabolism (CAM).

8

9   *HCO_3^- uptake*

10   Uptake of bicarbonate from the bulk medium into the cell (HCO_3^- use) appears favourable in

11   most freshwaters since its concentration exceeds that of 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 CO_2 affinity and thus CO_2 is the preferred

14   inorganic carbon source when concentrations of HCO_3^- and 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 $HCO_3^-H^+$ symporter or indirectly via

19   acidification of the boundary layer, thereby shifting the chemical equilibrium towards 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_2 than species restricted to CO_2 alone (obligate CO_2-users) (Maberly and Madsen

2   1998; Madsen and Maberly 2003), which may impose an ecological cost at some sites.

3

4   *C_4-metabolism*

5   In addition to bicarbonate use, two inorganic carbon uptake mechanisms exist in freshwater

6   plants that are based on C_4-metabolism. They depend on carbon fixation via the enzyme

7   phosphoenol pyruvate carboxylase (PEPcase) either during the day (C_4) or during the night (CAM),

8   involving either a spatial (C_4) 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_4 photosynthetic pathway, but in contrast to terrestrial C_4 – which is normally

12   expressed constitutively – freshwater C_4 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_4 plants do not have Kranz-anatomy like most terrestrial C_4 plants. However, single-

15   cell C_4-metabolism has recently been observed in terrestrial plants and may be an overseen

16   phenomenon in freshwater plants (Edwards et al. 2004). C_4-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_2 to be taken up and fixed via night-time PEPcase activity and the

1   C_4 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-acidifcation and the released CO_2 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_2 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_3^-) is derived

8   from endogenous (respiratory CO_2) or exogenous sources (CO_2 from the bulk water or sediment-

9   CO_2). Use of HCO_3^- 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_2 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_2 concentration. Large daily CO_2 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_2 concentrations in the open water (Maberly 1996) and especially in weed beds (Van et al.

7   1976). In these lakes with large CO_2-fluctuations, plants with CAM are 1/ able to take up inorganic

8   carbon in the night, where the CO_2 concentration is higher and where competition for inorganic

9   carbon with non-CAM species is eliminated and 2/ less dependent on external CO_2 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_2 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.

4

5   *Long-term regulation of CAM*

6   Regulation of CAM is dependent on various environmental parameters e.g. light, CO_2,

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_2 interact in the regulation of CAM, and for the invasive elodeid, *C. helmsii,* low

11   light causes down-regulation, independent of the CO_2 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_2 availability

14   during growth, with low CO_2 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_2, CO_2

16   becomes saturating for photosynthesis which most likely triggers down-regulation of CAM. Down-

17   regulation of CAM at low light is ecophysiologically 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_2 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_2 (Table 1). However, the CO_2 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_2 concentrations causing down-regulation are likely to be related

3   to the CO_2 concentration needed to saturate photosynthesis, which for isoetids is relatively high

4   (half-saturation around 500-600 µmol L^{-1} CO_2) (Madsen et al. 2002). For *C. helmsii* the half-

5   saturation concentration of CO_2 is lower and was estimated to be ca. 100 µmol L^{-1} 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_2 *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_{10} of 0.6-0.7). In the summer, CAM was stimulated

16   by raised temperature and Q_{10} 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 δ13-C, since the δ13-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_2 from the air and

16   makes the plant less dependent on CO_2 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_2

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_2 and dark CO_2 uptake via CAM (Nielsen et al. 1996), the CO_2 concentration

23   experienced by the plant may not differ from the CO_2 experienced under water – and this may be

24   the reason for the lack of CAM down-regulation.

1

Short-term regulation of CAM

3   Light and CO_2 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_2 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_2, decarboxylation rates did not vary between CO_2 treatments,

16   but the decarboxylation period was longer and the minimum acidity level lower for low CO_2 grown

17   plants (Klavsen and Maberly 2010). In *L. uniflora* the rate of decarboxylation was generally high

18   under low external CO_2 concentration, but could be fully inhibited by high CO_2 (Madsen 1987c).

19   This indicates that CAM in *L. uniflora* operates under most natural CO_2 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

14

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_2 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_2:CO_2 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_2 derived from CAM could otherwise be lost. In *L.*

15   *uniflora* this was verified experimentally, since less than 2% of the CO_2 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_2 evolution relative to the external CO_2

22   uptake (and thus the O_2:CO_2 ratio) will be well above 1 during the decaboxylation phase. This was

23   found for *L. uniflora* and *I. lacustris* (Madsen 1987b), where the O_2:CO_2 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_2:CO_2 ratio above 1 during decarboxylation this may be because either 1/ external CO_2 is so

2   high that decarboxylation is inhibited or 2/ the high internal CO_2 obtained during decarboxylation

3   inhibits external CO_2 uptake. This implies that the CCM is working less efficiently and external

4   CO_2 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 C4 activity, the plant may benefit from

8   CAM, since decarboxylation occurs around midday, where the inorganic carbon demand is likely to

9   be greatest (Klavsen and Maberly 2010). Furthermore, CAM may help conserve carbon, since

10   respiratory CO_2 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; Klavsen 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_2:CO_2 ratios (Fig. 4)). Due to the plasticity of CAM, the influence of night-time CO_2

22   uptake on daily CO_2 uptake in photosynthesis can vary significantly depending on the

23   environmental conditions. Thus, the contribution from CO_2 derived from CAM to daily

24   photosynthesis varies from 0 to 95%. The latter estimate of the contribution from CAM was found

for *L. uniflora* and *I. lacustris* at an external CO_2 concentration of 30 µmol L^{-1}. At higher external

CO_2 concentrations the night-time CO_2 uptake via CAM in relation to daily photosynthetic carbon

uptake was reduced to 34-38% (Madsen 1987b) due to higher uptake of external CO_2 and

potentially partial inhibition of decarboxylation. In *L. uniflora* grown at low light, the contribution

from CAM-derived CO_2 to photosynthesis was high (62%), but lower than in plants grown at high

light (81%) (Robe and Griffiths 1990). Also in *I. howellii* ca. 30-50% of daily CO_2 uptake in

photosynthesis was estimated to derive from night-time uptake through CAM (Keeley and Busch

1984). Another estimate of the contribution from CAM to the carbon budget was made on *L.*

uniflora, in which 40-55% of the annual carbon gain derived from CAM (Boston and Adams 1985,

1986).

For the elodeid *C. helmsii*, however, no oxygen peak is observed during decaboxylation (Fig. 4))

and thus the benefit from CAM is in principle lost. However, CAM may still be favourable to the

C-gain of the plant, if the external CO_2 concentration is low. In *C. helmsii* the *in situ* contribution

from CAM to daily photosynthesis varied from 18 to 42%, depending on depth of growth and time

of year (Klavsen and Maberly 2009). Most likely these estimates are valid as contributions from

CAM to the daily carbon balance, since almost all respiratory CO_2 in the night was refixed via

CAM and since roots make up a very small part of the total plant biomass in this species. Thus, in

natural populations of freshwater CAM species, CAM appears to be of high ecophysiological

significance for the carbon balance. These estimates are in agreement with estimates for terrestrial

facultative CAM plants, in which 10 to nearly 100% of the carbon fixation in daily photosynthesis

derive from CAM (Winter and Holtum 2002; Lüttge 2004).

Night time CO_2 uptake

CAM potentially enables the plants to take up inorganic carbon 24 hours a day, although this is probably not realised in all species (Keeley 1998b; Madsen et al. 2002; Klavsen and Maberly 2010). Even though external CO_2 is not taken up at night, CAM can still be considered a carbon conserving mechanism, since re-capture of respiratory endogenous produced CO_2 through the operation of CAM can reduce or eliminate C-loss in the night and thereby influence C-gain positively (Keeley and Busch 1984; Madsen 1987c; Robe and Griffiths 1990; Madsen et al. 2002). The contribution of re-captured respiratory CO_2, otherwise lost to the surroundings, to the total CO_2 uptake via CAM is dependent on the external CO_2 concentration, but often makes up a substantial part of the night-time inorganic carbon fixation. For *L. uniflora* between 30 and 99% of night-time CO_2 uptake via CAM derives from CO_2 produced in respiration (Richardson et al. 1984; Smith et al. 1985; Madsen 1987b,c; Boston et al. 1987; Robe and Griffiths 1990) and for *I. howellii* values of 50-66% have been found (Keeley and Busch 1984). Since respiratory CO_2 under natural conditions rarely makes up the total night-time CO_2 uptake, this implies that CO_2 uptake though CAM is at least partly dependent on the external CO_2 availability, which potentially can lead to inorganic carbon limitation at night (Klavsen and Maberly 2010). However, the length of the night period – although not realised under field conditions – can compensate for low external CO_2 availability (Keeley and Bowes 1982; Madsen et al. 2002). Thus, plants relying on CO_2 primarily derived from endogenous sources can reach the same maximum CAM activity as plants incubated in a high CO_2 medium. Respiratory CO_2 can potentially make up the entire night-time carbon uptake through CAM under low external CO_2 in both *C. helmsii* and *L. uniflora*, since the rate of respiration can exceed the rate of CO_2 uptake through CAM (assuming a constant CO_2 uptake in CAM, a constant respiratory rate and a respiratory quotient of 1) (Boston et al. 1987; Klavsen and Maberly 2010).

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_2 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_2

10   in the water is low.

11

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6

7

8

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_2 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_2 (ca. atmospheric equilibrium) and high light (thereby

6 increasing decarboxylation) and in the night plants were incubated in a medium with high CO_2

7 (>500 mmol m^{-3}) (thereby increasing night-time CO_2 uptake via CAM).

Species	Free CO_2 ($\mu mol\ L^{-1}$)	Light ($\mu mol\ m^{-2}\ s^{-2}$)	Temp. (°C)	Actual CAM ($\mu eq\ g^{-1}$ FW)	Potential CAM ($\mu eq\ g^{-1}$ FW)	Ref.
L. uniflora	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[a]	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[a]	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[b]	50	19-20	35	-	5
	50/1000[b]	300	19-20	112	-	5
	80/600[c]	350	18-19	110	-	6
	20/1000[c]	50	18-19	60	-	6
C. helmsii	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

Ref 1: Madsen (1987), Ref 2: Baatrup-Pedersen and Madsen 1999, Ref 3: Boston and Adams 1987, Ref 4: Klavsen unpubl. Data, Ref 5: Robe and Griffith 1990, Ref 6: Robe and Griffith 1994, Ref 7: Klavsen and Maberly 2009, Ref 8: Klavsen and Maberly 2010.

[a]estimate based on an irradiance of 10-16 mol photons m^{-2} day^{-1}

[b]plants were grown in natural sediments. The free CO_2 concentrations of the water and interstitial water were 50 and 1000 µmol L^{-1} respectively.

[c]plants were grown in natural sediments. The free CO_2 concentrations of the water and interstitial water were either 20 or 80 and 600 and 1000 µmol L^{-1} respectively.

Figure captions

Fig. 1. The Crassulacean Acid Metabolism (CAM) cycle. Dark CO_2 fixation occurs through the

enzyme, PEPcase, and the sources of inorganic carbon are either of endogenous origin (respiration)

or of exogenous origin (water or sediment-CO_2). The grey area represents reactions occurring in the

dark, while the white area contain daytime reactions. The round circle symbolises the cell vacuole.

Modified from Winter and Smith (1996).

Fig. 2. *In situ* CAM activity measured in the isoetids *Isoetes lacustris, I. bolanderi, I. kirkii* and

Littorella uniflora and in the elodeid *Crassula helmsii*. Data are modified from Keeley *et al*. (1983),

Boston and Adams (1985), Rattray *et al*. 1992 and Klavsen and Maberly (2009).

Fig. 3. Rates of inorganic carbon uptake and oxygen evolution in the isoetids *Littorella uniflora*

(left panel) and *Isoetes lacustris* (middle panel) and oxygen evolution in the elodeid *Crassula*

helmsii (right panel). *Crassula helmsii* was grown and photosynthesis measured at low CO_2 (22

mmol m^{-3}), but decarboxylation did not start until after 2 hours after light onset. High CAM activity

results in high O_2:CO_2 ratios (*L. uniflora* and *I. lacustris*), if external inorganic carbon uptake is not

inhibited by decarboxylation. Data modified from Madsen (1987a) and Klavsen

and Maberly (2010).