Carbon Acquisition in Variable Environments: Aquatic Plants of the River Murray, Australia

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Declaration

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Abstract

This thesis considers the implications of changes in the supply of resources for photosynthesis, with regard for modes of carbon acquisition employed by aquatic plants of the River Murray. Carbon supplies are inherently more variable for aquatic plants than for those in terrestrial environments, and variations are intensified for plants in semi-arid regions, where water may be limiting. In changeable environments the most successful species are likely to be those with flexible carbon-uptake mechanisms, able to accommodate variations in the supply of resources.

Studies were made of plants associated with wetland habitats of the Murray, including *Crassula helmsii, Potamogeton tricarinatus, P. crispus* and *Vallisneria americana*. The aim was to elucidate the mechanisms of carbon uptake and assimilation employed, and to determine how flexibility in carbon uptake and/or assimilation physiology affect survival and distribution. Stable carbon isotopes were used to explore the dynamics of carbon uptake and assimilation, and fluorescence was used to identify pathways and photosynthetic capacity. The studies suggest that physiological flexibility is adaptive survival in changeable environments, but probably does not enhance the spread or dominance of these species.

V. americana is a known bicarbonate-user, and it is shown here that it uses the Crassulacean Acid Metabolism (CAM) photosynthetic pathway under specific conditions (high light intensity near the leaf tips) concurrently with HCO_3^- uptake, while leaves deeper in the water continue to use the C_3 pathway, with CO_2 as the main carbon source. However, *V. americana* does not use CAM when under stress, such as exposure to high light and temperature. The diversity of carbon uptake and assimilation mechanisms in this species may explain its competitive ability in habitats associated with the Murray. In this way it is able to maximise use of light throughout the water column. In shallow, warm water, where leaves are parallel to the surface, CAM ability is likely to be induced along the length of the leaf, allowing maximal use of carbon and light.

The amphibious *C. helmsii* is shown to use CAM on submergence, even where water levels fluctuate within 24 hours. This allows continued photosynthesis in habitats where level fluctuations prevent access to atmospheric CO_2. It appears that stable conditions are most favourable for growth and dispersal, and that the spread of *C. helmsii* is mainly by the aerial form.

Carbon uptake by *P. tricarinatus* under field conditions is compared with that of *P. crispus* to demonstrate differences in productivity associated with aqueous bicarbonate and atmospheric CO_2 use. *P. tricarinatus* uses HCO_3^- uptake to promote growth toward the surface, so that CO_2 can be accessed by floating leaves. Atmospheric contact provides access to light and removes the limitation of aqueous diffusive resistance to CO_2, thereby increasing photosynthetic capacity above that provided by submerged leaves.

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INTRODUCTION

1.1 Preamble

In the course of evolution, aquatic and terrestrial plants have confronted fundamentally different problems in obtaining the raw materials for photosynthesis (light, CO_2 and water). In terrestrial environments, water may be limiting, light is rarely so, and the atmospheric CO_2 supply is low but relatively stable. In aquatic environments, the supply of carbon is more variable and plants must avoid or cope with hypoxia. The problems of variable resources are compounded for semi-aquatic (amphibious) plants with tissues exposed to both air and water, and further intensified for plants in arid and semi-arid environments, where supply and demand follow a "boom and bust" trajectory (Walker *et al.* 1997). Although variable carbon supplies sometimes are disassociated from variable oxygen and water, plants often must adapt to simultaneous uncertainty in all three.

Freshwater angiosperms are secondarily aquatic. That is, they evolved from terrestrial angiosperms derived from aquatic ancestors (Raven 1995; Philbrick and Les 1996 ; Cook 1999). When plants invaded the land, problems of physical support and water loss were overcome through development of a vascular system, cuticle and stomata (Sculthorpe 1967; Hutchinson 1975). With the subsequent return to water, carbon availability was problematic, for two main reasons (Maberly and Madsen 2002). First, the CO_2 concentration in air (c. 360 ppm) remains constant over short periods of time, but the CO_2 concentration in water fluctuates diurnally and seasonally, depending on dissolved inorganic carbon and other chemical factors. Second, the resistance to gaseous diffusion in water is 10,000 times greater than in air.

Aquatic plants have developed specialised morphological and physiological mechanisms to aid carbon acquisition. Aerial and floating leaves and lacunae allow direct access to atmospheric CO_2, and bicarbonate provides an alternative when CO_2 is limiting. Carbon concentrating mechanisms, like the C_4 and Crassulacean Acid Metabolism (CAM) photosynthetic pathways, actively pump (or alter the uptake of) CO_2 to concentrate carbon around Rubisco. The ecological significance of these carbon-concentrating mechanisms has been little explored, especially in aquatic environments where they are likely to improve the growth, survival and competitive ability of plants.

Plants associated with the River Murray, south-eastern Australia, are governed by a changeable climate and flow regimes that are typical of dryland regions (Puckridge *et al.* 1998). The temporal and spatial scales of flow variability have been altered, however, by intensive regulation through diversions and construction of dams and weirs over the past century (Maheshwari *et al.* 1995). The effects have been to reduce discharge, limit exchanges between river and floodplain environments and promote greater seasonal and inter-annual flow stability. The plants best able to accommodate these changes are likely to have been those with flexible physiology, particularly in photosynthetic pathways.

This thesis explores the physiological nature of selected plants associated with the River Murray. It describes the different forms of carbon uptake employed by these plants, their dependence on C_3, C_4 and CAM photosynthetic pathways, and the implications of variability in the supply of the resources needed for photosynthesis.

1.2 The Murray-Darling Basin

1.2.1 Climate and hydrology

The 2570-km River Murray is part of the Murray-Darling Basin (MDB), extending over 1.073 million km^2 of south-eastern Australia, across latitudes 24-37°S and longitudes 138-151°E and through sub-tropical, sub-alpine, warm temperate and desert regions (Figure 1-1). The lowland regions of the basin are mainly arid or semi-arid, and the climate is typically Mediterranean. Annual rainfall varies from 1400 mm in the eastern highlands, where the Murray has its source, to less than 300 mm in inland areas (Mackay *et al.* 1988).

The 830-km 'Lower Murray' extends from the Murray-Darling junction to the river mouth in South Australia. Below the Darling junction, the Lower Murray flows within a 10-20 km floodplain with extensive wetlands and woodlands (Gill 1973). Near Morgan, where the river turns southward, the Murray enters a 30-m limestone gorge and the floodplain is constrained to 2-3 km, with 'channel margin swale' wetlands rather than oxbows (Pressey 1986).

The hydrologic behaviour of the Lower Murray is determined mainly by flows from the Murray rather than the Darling, which contributes only about 10 percent of the total. The annual discharge of the system is low but variable (0.617-49,271 GL in 1950-1980; (data 1950-80 Walker 1996), and was <4000 GL during this study. Natural flows were

seasonal, with peak flows in winter and early spring arriving in the lower reaches in late spring and early summer. Flow regulation, however, has caused major changes. These include seasonal stability in flow, reduced over-bank flooding and changed wetting and drying cycles in floodplain wetlands. Over 70% of wetlands along the Murray are permanently flooded by weir pools (Pressey 1986), although many would have dried and flooded seasonally prior to regulation. A series of 10 low-level weirs on the Lower Murray maintains close control over pool levels, and routine weir operations cause small daily fluctuations (c. 20 cm) in the tailwater downstream of each weir (Walker 1996). There have also been increases in turbidity and salinity, associated with regulation (Blanch *et al.* 1999).

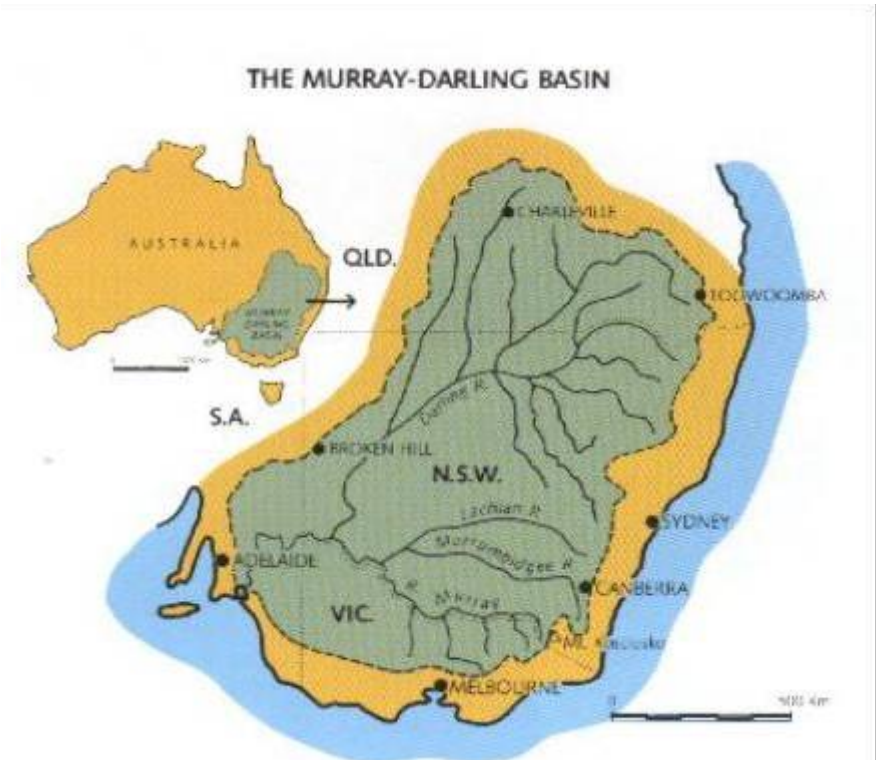


Figure 1-1 The Murray-Darling Basin, extending over 14% of mainland Australia.

The open channel is not a favourable habitat for aquatic macrophytes, due to variable flows, high turbidity and unstable sediments (Walker 1996). Wetlands and littoral habitats, however, provide sheltered environments, and the permanent pools between weirs also have encouraged the development of littoral plant communities (Lloyd *et al.* 2003). The regulated flow environment supports a low diversity of species compared to conditions that existed prior to the advent of regulation (Wilcox and Meeker 1991).

1.2.2 Vegetation

The riparian terrestrial vegetation is dominated by *Eucalyptus camaldulensis* (river red gum) and *E. largiflorens* (black box), *Acacia stenophylla* (river cooba), *Muehlenbeckia florulenta* (lignum) and introduced willows (*Salix* spp.). A variety of aquatic and amphibious plants occurs in wetlands and backwaters, and *Phragmites australis* (common reed) and *Typha* spp. (cumbungi) are conspicuous along the river margins.

Wetlands along the Lower Murray vary widely in size and shape, water chemistry and biota, and have a variety of flooding regimes (Boon and Brock 1994). Submerged aquatic species *Vallisneria americana* (water ribbon), *Potamogeton crispus* (curly pondweed) and *Myriophyllum* spp. (milfoils) are prevalent in wetlands and backwaters, especially in summer. In the weir pools, the distributions of aquatic and semi-aquatic littoral vegetation are influenced by water-level fluctuations (see Blanch *et al.* 2000).

The warm temperatures and high light conditions that prevail in most of the MDB, coupled with salinization and variable water levels, are favourable for C_4 and CAM plants (Still *et al.* 2003). These stressors are known to induce CAM in *Mesembryanthemum crystallinum* (Broetto *et al.* 2002), which occurs on the Murray floodplain, and may similarly affect other plants.

1.2.3 Photosynthetic diversity

The relative abundances of plants with different photosynthetic pathways are significant because they can affect nutrient cycling, water use and carbon cycling at an ecosystem level. The diversity of photosynthetic mechanisms reflects the availability of water. In the Murray-Darling Basin, it appears that most emergent plants are C_3, and many submerged macrophytes (e.g. *Vallisneria* spp., *Myriophyllum* spp., *Potamogeton* spp.) are known to be bicarbonate-users (Harris 1963; Keeley 1998).

Raven (1991) suggested that there may be interactions between the photosynthetic pathways of aquatic plants and their habitat. Keeley (1999) evaluated the photosynthetic diversity of dominant aquatic species in a community in the south-western United States and found that about half of the submerged species were clearly C_3, three were CAM, one was C_4 and three were likely C_3-C_4 intermediates. He suggested that stress is crucial in reducing the expansion of monocultures and maintaining open space for new species, and that seasonal aquatic habitats promote the coexistence of plants with diverse photosynthetic pathways. In the Lower Murray, inter- and intra-annual cycles in seasonal environments (albeit now less than under natural conditions) produce potential

dis-equilibrium conditions and these, according to Keeley's (1999) ideas, should promote a high diversity of photosynthetic and carbon-uptake mechanisms and species. Differential competitive success from year to year is likely to be emphasized by differences in carbon gain, related to differences in inundation and exposure tolerance of co-habiting species.

Carbon acquisition underwater poses a number of problems for aquatic plants with submerged leaves. First, free CO_2 is available to submerged leaves, because CO_2 is very soluble in water, but it diffuses much more slowly than in air and this can limit plant growth. To compensate, some submerged species obtain CO_2 from the sediment (Kimber *et al.* 1999). Second, inorganic carbon is present in water in forms other than CO_2, in quantities that are strongly influenced by pH. Free CO_2 is the principal form of carbon up to pH 7, while HCO_3^- and carbonate dominate at pH 7-10. Above pH 8.2, there is no free CO_2. The availability of these forms of carbon depends upon rates of hydration and dehydration, and consequently the concentration of total inorganic carbon. Long-term or sustained increases in pH will disadvantage those species which are obligate CO_2 users. The capacity of submerged plants in the MDB to use HCO_3^- has been established for *Ceratophyllum demersum*, *Potamogeton crispus* and *Vallisneria americana*, among others, and for the introduced *Elodea canadensis*. Bicarbonate transport is often assumed for *Chara* and *Nitella*, but evidence is lacking (Smith and Walker 1980; Walker *et al.* 1980).

1.3 Carbon supply and acquisition

1.3.1 Overview

Water levels in the streams and wetlands of the MDB vary spatially and temporally. Amphibious species need to endure submergence when levels are high and desiccation when levels are low. Submerged plants are not limited by water, of course, and gain support and buoyancy, but they must also cope with low carbon availability.

Carbon-concentrating mechanisms such as the C_4 and CAM photosynthetic pathways can improve photosynthetic efficiency when carbon availability is low, but factors such as light, salinity and oxygen concentration also affect assimilation. In air, carbon supply is controlled by stomata, which are sensitive to environmental factors such as light and humidity. In an aquatic environment, stomata are rarely present. Carbon gain in aquatic plants is enhanced by increasing the surface area in contact with water and increasing

the cell/environment transfer of inorganic carbon (Rascio 2002). This may be achieved, for example, by maintaining a large surface area: volume ratio of chloroplast-rich photosynthetic cells, actively altering pH in the surrounding medium and utilising C_4 and CAM mechanisms.

1.3.2 Atmospheric CO_2

The problems associated with accessing CO_2 in an aquatic environment can be overcome by the presence of floating or emergent leaves, or floral spikes, allowing access to atmospheric CO_2. Emergence of leaves from the water often, but not always, results in changed leaf morphology, known as *heterophylly,* where there are two distinct leaf forms adapted to photosynthesize in either air or water. One exception is elodeid species, which are not heterophyllous yet often become emergent during draw-down. Heterophyllous forms include species of *Marsilea, Ranunculus, Potamogeton* and *Myriophyllum*. In these species, heterophylly may be controlled by CO_2 concentrations surrounding the photosynthetic tissues (Bristow 1969).

Access to atmospheric CO_2 has obvious advantages for emergent plants, because of the lower diffusive resistance relative to water. Sand-Jensen (1999) showed that photosynthetic rates in four common amphibious species were 2-4 times higher in air than when submerged, even in water saturated with CO_2. The adaptive value of having both aerial and submerged leaves is unclear, although a greater photosynthetic capacity is obtained through access to atmospheric CO_2. Bowes (1987) suggested that a high photosynthetic capacity in aerial leaves does not necessarily mean that these have evolved in response to carbon stress at the whole plant level.

1.3.3 Alternative carbon sources

While terrestrial plants are limited to CO_2 as a carbon source, submerged species have access to bicarbonate (HCO_3^-), in concentrations determined by temperature and pH. Most aquatic plants favour CO_2, because it can be taken up from the surroundings without energetic cost, but bicarbonate concentrations are often many times higher than that of CO_2. Bicarbonate use is the most common carbon acquisition strategy in submerged angiosperms. High pH provides access to a larger pool of inorganic C, whereas low pH promotes conversion of HCO_3^- to CO_2.

Macrophyte use of bicarbonate is determined by species' affinities for HCO_3^-, HCO_3^- compensation points and the concentration (Falkowski and Raven 1997), all governed

in turn by the ambient pH (Allen and Spence 1981). The ratio of the different species of inorganic carbon in aquatic systems is related to the equilibration of dissolved CO_2 and the hydrated forms H_2CO_3 and HCO_3^- (Falkowski and Raven 1997):

$$CO_2 \Leftrightarrow CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO^{3-} \quad (1\text{-}1)$$

The concentration of HCO_3^- is often greater than that of dissolved CO_2. This may be why so few aquatics utilise C_4 and CAM photosynthetic mechanisms, which rely on dissolved CO_2 prior to conversion to HCO_3^- (Bowes *et al.* 2002). Macrophytes tend to have larger affinities for CO_2 than HCO_3^-, compared to plants such as micro-algae (Allen and Spence 1981). Bicarbonate use in macrophytes may be determined more by pH than by species preferences (Allen (1981).

Bicarbonate uptake has been observed in *Potamogeton crispus* (Allen and Spence 1981), *Littorella uniflora* (Robe and Griffiths 2000), *Vallisneria spiralis* (Webb *et al.* 1998), *Ranunculus aquatilis, Chara contraria* and *Gloeotrichia* sp. (Keeley 1999). Robe and Griffiths (2000) noted that, on emergence from the water, *Littorella uniflora* continues to take CO_2 from the sediment rather than from air; they suggested that the plants are 'poised for re-submersion'.

As with other concentrating mechanisms, the uptake of HCO_3^- increases the CO_2 concentration around Rubisco and reduces the inhibitory effects of photorespiration (Bowes *et al.* 2002). Species limited to using CO_2 tend to have a high affinity for CO_2, whereas species that use HCO_3^- often have a lower affinity for CO_2 (Maberly and Madsen 1998). In environments that are high in CO_2, plants typically do not have a mechanism for bicarbonate uptake, but where pH is high and HCO_3^- is abundant, plants are likely to be bicarbonate users (Maberly and Spence 1983).

Assimilation of carbon from HCO_3^- can continue until the pH of the water exceeds 9.0. At this point, photosynthesis can be restricted to only 10% of that observed at saturating CO_2 concentrations (Allen and Spence 1981). In contrast, macro-algae can photo-synthesise maximally at such pH values. Species that do not assimilate HCO_3^- can benefit from high levels due to the conversion of HCO_3^- to CO_2, especially across the boundary layer.

CO_2 and HCO_3^- levels in water can change rapidly, and acclimation by macrophytes also be fast, often within hours (Maberly and Madsen 2002), although days, sometimes months, are required for the response to be completed (Sand-Jensen 1987).

Potamogeton crispus responds quickly to changed CO_2 availability by altering its HCO_3^- affinity (hence its uptake capacity) within 7 days (Maberly and Madsen 2002).

High levels of inorganic carbon in sediment can be exploited directly by some aquatic rhizophytes (e.g. Littorella uniflora Robe and Griffiths 1998, and Vallisneria; Kimber *et al.* 1999). In these species, interstitial water and nutrients (including carbon) in the sediment can be accessed by diffusion to the roots (Barko *et al.* 1981). In *Littorella* and other isoetids, morphological and anatomical features such as a rosette growth form, high root to leaf biomass and a large air lacunal system provide for utilisation of sedimentary CO_2 (Madsen and Sand-Jensen 1991). Up to 99% of carbon fixed by *Isoetes dortmanna* is from the sediment (Raven *et al.* 1988). Use of sedimentary CO_2 requires a high root to shoot ratio and a high concentration of CO_2 in the sediment relative to that in the water (Madsen and Sand-Jensen 1991).

1.3.4 Temperature

Water temperature is buffered from diurnal terrestrial changes, but there may be a strong vertical temperature gradient, as much as 10ºC over a depth of 2 m. For most aquatic plants the optimal range for photosynthesis is 20-35°C, although some species of *Potamogeton* are able to photosynthesise below 2°C (Bowes 1985). Temperature affects CO_2 solubility and thereby has major implications for carbon uptake.

Solubility of CO_2 decreases with increasing temperature, but the diffusion coefficient of CO_2 in water at 25°C is twice that at 0°C (table 8.1 Raven 1984). Therefore, in aquatic plants, photosynthesis at low temperatures is not favoured to the extent suggested by the increased solubility of CO_2.

Temperature can also affect aquatic photosynthesis through its impact on photo-synthetic enzymes. At low temperatures the C_3 carboxylation enzyme Rubisco has decreased activity (Raven 1991), yet photorespiration also decreases. At low temperatures, provided diffusion is not hindered, the need for a carbon concentrating mechanism is decreased (Raven 1991).

1.3.5 pH

The effect of pH on photosynthesis in aquatic plants is mediated by its effect on the availability of inorganic carbon in water. The pH determines the balance between dissolved CO_2 and the bicarbonate concentration, per equation 1-1 above (Lawlor 2001). As the pH becomes more acidic, the conversion of HCO_3^- to CO_2 increases,

increasing the supply of CO_2 for photosynthesis (Brandrud 2002). CO_2 is unavailable at pH above 8.2 (Newman and Raven 1999); thus, submerged aquatic macrophytes growing in such waters often use HCO_3^- rather than CO_2 as a source of carbon. In water over 99% of CO_2 is in solution, but some is hydrated to carbonic acid, H_2CO_3, which dissociates to yield HCO_3^- and H^+.

1.3.6 Light

Light governs the depth distribution of aquatic plants (Raven 1984). Just below the water surface, Photon Flux Density (PFD) can be similar to that in terrestrial environments, depending on the time of day (Menendez and Sanchez 1998), but it is rapidly attenuated with depth. The rate of attenuation is dependent on factors such as turbidity and the presence of photosynthetic organisms. Submerged aquatics usually exhibit low photosynthetic rates at the same incident PFD relative to their terrestrial counterparts (Raven 1995), and often behave like 'shade' acclimated terrestrial plants (Mommer *et al.* 2005).

Submersed aquatic species are acclimated to low-light regimes under water, rather than to surface irradiance. This is observed through the light-response of photosynthesis as compared with irradiance, where submersed macrophytes demonstrate 'shade' characteristics, saturating at <500 μmol quanta m^{-2} s^{-1}, less than one-quarter of full sun-light (among others Salvucci and Bowes 1982). However, as suggested by Bowes and Salvucci (1989), shade characteristics may be a secondary result of the DIC diffusion resistance of water. High-capacity photosynthetic electron transport and CO_2 fixation systems may be unnecessary when a plant is already diffusion (CO_2) limited.

Although light is a primary limiting factor in aquatic environments (Maberly 1985), it may become damaging when combined with a stressor such as limiting water or carbon (Osmond *et al.* 1987).

Interactions between light and carbon uptake have been under-studied in aquatic plants. *Elodea canadensis*, *Potamogeton lucens* and other species exhibit a light-dependent polarity at the leaf surface, whereby OH- and H + ions are extruded from the adaxial and abaxial leaf surfaces (Prins *et al.* 1982), aiding the conversion of HCO_3^- to CO_2 and enabling photosynthetic carbon uptake even when pH is high (Bowes and Salvucci 1989). Enhanced CO_2 availability increases light-use efficiency and may allow plants to penetrate to greater depths (low light) where concentrations are higher due to re-mineralisation in the sediment (Maberly 1985).

1.3.7 Oxygen

Aquatic macrophytes have a lacunar system or continuous network of air spaces that provide surface area for exchange of CO_2 and O_2. Typically, rhizomes and roots grow in sediment with low oxygen levels, whereas aerial and floating leaves have contact with atmospheric oxygen for respiration. Photosynthesis during the day can cause development of a pressure differential between photosynthesising and non-photo-synthesising shoots and roots lower in the water column or sediment (Sorrell and Dromgoole 1996; Sorrell and Tanner 2000), resulting in bulk movement of gas along the shoots. This can occur either by diffusion down concentration gradients, or by pressure-driven convective flow. The pressure differential is created by temperature and humidity gradients between the atmosphere and internal air spaces (Schuette and Klug 1995; Sorrell and Dromgoole 1996). Oxygen build-up during photosynthesis is then countered by respiration. Pressure differentials have been recorded in floral spikes of the macrophytes *Myriophyllum* (Schuette and Klug 1995) and *Potamogeton* (Heilman and Carlton 2001).

1.3.8 Salinity

Solubility, hence the availability of carbon for photosynthesis, decreases with salinity (Falkowski and Raven 1997) and, according to Raven (1991), is likely to decrease the CO_2 diffusion coefficient such that saline water will contain less CO_2 than fresh water at the same temperature. For this reason, increased salinity may promote the occurrence of carbon-concentrating mechanisms. Sand-Jensen (1984) highlighted the relationship between the abundance of species with carbon-concentrating mechanisms and decreased CO_2 solubility in saline water.

1.3.9 Diffusive exchange

Concentrations of CO_2 in air and water under equilibrium conditions are similar (near 10 mmol m^{-3}) (Rascio 2002). The diffusive resistance in water, however, is 10,000 times higher than in air (Sage 2002) and there can be much greater changes, and larger gradients, in concentrations in aquatic systems due to the respiratory and photosynthetic activities of submerged plants. Diffusion across boundary layers in water influences total CO_2 conductance into photosynthetic tissues, especially under rate-limiting CO_2 conditions (Sand-Jensen and Frost-Christensen 1999). However, CO_2 diffusion into leaves also involves the cuticle, outer cell wall, mesophyll and carboxylation capacity of photosynthetic organs (Raven 1984). These factors vary with species and flow rate

(Sand-Jensen and Frost-Christensen 1999) and the leaf morphology of aquatic plants. Each species has evolved to maximise uptake of CO_2. This is achieved by having a high surface area to volume ratio, as in the highly-dissected leaves of *Myriophyllum* spp., or very thin leaves, as in *Vallisneria* spp. Similarly, many aquatic algae have thalli only a few cells thick (e.g. some Chlorophyta: (Maberly and Madsen 1998)). While a carbon-concentrating mechanism is another means of overcoming CO_2 limitation, if the plant has a carbon-concentrating mechanism or the ability to use HCO_3^-, high permeability would be a disadvantage by promoting leakage from the site of carboxylation.

The cuticle in many submerged aquatics is thin, but not necessarily highly permeable (Raven 1984). In the isoetids, use of carbon from the sediment through the roots may be possible because of the low cuticle permeability (see Sand-Jensen etal 1982 in Raven 1984). In addition, low cuticle permeability reduces leakage from carboxylation sites and aids carbon-concentrating mechanisms and HCO_3^- uptake.

1.3.10 Heterophylly

The problems associated with accessing CO_2 in an aquatic environment can be over-come by floating or emergent leaves, allowing access to atmospheric CO_2 and avoiding the problems of aquatic CO_2 diffusion. Emergence of leaves from the water often results in changed leaf morphology (*heterophylly:* Sculthorpe 1967; Nobel and Walker 1985), where there are distinct leaf forms for photosynthesis in either air or water.

Many factors play a role in the induction of aerial leaf morphology in heterophyllous species. Environmental conditions that typify summer, including high temperature (e.g. Deschamp and Cooke, 1984; Goliber and Feldman, 1990; Kane and Albert, 1982), long photoperiod (Bostrock and Millington, 1962; Cook, 1969; Webb, 1984) and high light intensity (e.g. Goliber, 1989), induce aerial leaf morphology in various species. Light quality also be significant.

Heterophylly occurs in many vascular plants, including dicots and monocots. Its widespread occurrence suggests that it has arisen several times by convergent evolution.

Regardless of the physiological advantages, the ecological significance of having aerial and submerged leaves is unclear. Heterophylly may increase the fitness of aquatic plants by decreasing leaf damage by mechanical forces or herbivores, by decreasing water loss or by enhancing photosynthesis. Cook and Johnson (1968) provided evidence that aquatic plants that routinely experience most variability in water levels

also exhibit the greatest degree of heterophylly. The paucity of heterophyllous species in populations from more stable environments suggests that there may be hidden energetic costs (DeWitt *et al.* 1998). Research on phenotypic plasticity is further summarized by Arber (1920), Sculthorpe (1967) and Hutchinson (1975).

1.4 Carbon assimilation

Some aquatic plants (e.g. *Littorella*) obtain carbon from the rooting medium, with direct CO_2 diffusion to Rubisco, but for most submerged aquatic plants carbon acquisition is diffusive, from the aqueous phase (Raven 1991). This yields a comparatively low rate of photosynthesis, due to the low diffusive capacity of CO_2 in water. As a result, many submerged aquatic plants rely on mechanisms that increase the concentration of carbon at the Rubisco active site. This effectively increases and stabilizes the supply rate to Rubisco, and increases photosynthetic efficiency and activity. Carbon-concentrating mechanisms increase the concentration of CO_2, not HCO_3^-. The inorganic form of carbon is important as CO_2 is the substrate for Rubisco. Raven (1995) suggested that carbon-concentrating mechanisms may have beneficial effects on the use of other resources such as nitrogen (Beardall *et al.* 1982), favouring species in harsh or stressful environments.

Carbon assimilation in plants occurs *via* three photosynthetic pathways. The most common and evolutionarily primitive is the C_3 pathway, where initial CO_2 fixation results in a 3-carbon compound. The other two pathways are carbon-concentrating mechanisms that increase CO_2 concentration around Rubisco. This is achieved by having two carboxylation steps, one in which CO_2 is initially fixed by the enzyme, PEP carboxylase, resulting in the production of a 4-carbon compound, and a second in which the carbon initially captured by PEP carboxylase is released as CO_2, then fixed by Rubisco in the C_3 pathway (More 1982; Ehleringer and Monson 1993; Apel 1994).

The C_3 photosynthetic pathway is inherently inefficient because of the oxygenase activity of the major carboxylating enzyme, Rubisco. Perhaps it is in response to this inefficiency that some plants have evolved carbon-concentrating mechanisms, C_4 and CAM, increasing the CO_2 concentration at the Rubisco active site.

C_4 photosynthesis converts CO_2 and PEP into a 4-carbon acid (oxaloacetate), using the enzyme PEP carboxylase within mesophyll cells. The C_4 acids are then transferred to the bundle sheath, where they are decarboxylated to produce CO_2. Rubisco and the PCR cycle then assimilate the CO_2. The two carboxylation steps occur simultaneously.

Crassulacean Acid Metabolism (CAM) uses similar mechanisms, but with temporal rather than spatial separation of the two carboxylation steps. Stomata are opened during at night to assimilate CO_2 and synthesize malate using PEP carboxylase; they are then closed during the day as the C_4 acids are decarboxylated and the resulting CO_2 fixed by Rubisco.

C_4 and CAM plants maintain high CO_2/O_2 ratios at the Rubisco active site and so reduce photorespiration to negligible levels.

CAM initially thought to be an adaptation to arid environments, as it significantly improves water-use efficiency. However, it was later identified in the freshwater plant *Isoetes howelii* (Keeley), and subsequently in other aquatic and littoral plants. The evolution of CAM in aquatic habitats is likely to have been a response to carbon rather than water limitations.

Molecular studies (Monson *et al.* 1986; Ehleringer and Monson 1993) indicate that only a few genes control the biochemical modifications in the C_4 pathway, and changes in pathway expression are relatively easy. Thus, there may be changes with age; there may be C3 and C_4 leaves in the same species, C_4 and CAM in the same plant and there may be switching from CAM to C_3 in the same leaf.

Light-use efficiency of the C_4 cycle is dependent on two factors, namely photo-respiration and ATP required for regeneration of intermediates. For every molecule of CO_2 fixed, two additional ATP molecules are required in the C_4 cycle, relative to the C_3 cycle, to regenerate PEP. Thus, the quantum yield of photosynthesis is lower in C_4 plants compared to C_3 plants. However, in C_3 plants photorespiration increases with temperature, and at temperatures above about 25°C there is a decrease in light-use efficiency (Ehleringer and Monson 1993). Photorespiration is very low in C_4 and CAM plants; thus, light-use efficiency remains constant as temperatures increase and the comparative efficiency at high temperatures is greater.

The distributions of terrestrial C_4 and CAM plants have been related to aridity and water use (for example Ehleringer and Monson 1993). Little is known of photosynthetic diversity in aquatic and littoral plants, even though they are significant producers. Productivity is highly correlated with photosynthetic output, and one might assume that photosynthetic efficiency would enhance productivity, especially when light and CO_2 are limiting. By altering either photosynthetic pathway, morphology or carbon source,

aquatic plants may be able to increase photosynthetic efficiency above that in the aquatic environment, achieving higher productivity.

Many aquatic and littoral plants are able to switch photosynthetic pathways. Carbon-concentrating mechanisms such as CAM and C_4 can be initiated by environmental factors. *Isoetes howelii*, *I. orcuttii*, *Crassula aquatica* (Keeley 1999) and *Littorella uniflora* (Robe and Griffiths 2000) utilise CAM and switch to C_3 on exposure to air. In contrast, re-emersion in water triggers a switch from C_3 to C_4 in *Eleocharis vivipara* (Ueno 1996). In terrestrial forms of *Mesembryanthemum crystallinum*, a non-reversible switch to CAM can be induced in response to stresses such as salinity (Luttge 1993), while other species can switch reversibly between CAM and C_3 in response to drought and salinity (e.g. Winter and Ziegler 1992).

In aquatics, a switch to CAM or C_4 is a response to CO_2 limitation (Keeley and Sandquist 1992), and a switch from CAM/C_4 to C_3 is related to the removal of CO_2 limitation (Robe and Griffiths 2000). In one unusual example, the centre of mats of *Hydrilla* undertake C_4 fixation due to midday depletion of CO_2, while on the outer edges the C_3 pathway is utilized (Keeley 1999; Bowes *et al.* 2002).

Crassulacean Acid Metabolism (CAM) is a mechanism where net CO_2 uptake occurs mostly at night though open stomata when evapotranspiration is low, and fixation by Rubisco occurs during the day, using light as an energy source. Carbon assimilation thus requires two carboxylating steps. In darkness, CO_2 enters through open stomata and is fixed by phosphoenolpyruvate carboxylase (PEPC) and assimilated into C_4 acids such as malate and aspartate. These accumulate in the vacuole and cause a diel change in pH, the hallmark of CAM plants. During the day, when stomata are closed, the C_4 acids are decarboxylated in the cytosol and Rubisco re-fixes the released CO_2 into the PCR cycle (the Calvin Cycle).

The two-part day/night process of carbon assimilation, where stomata remain closed during the day, suggests that CAM has evolved as a mechanism to reduce water loss (Winter and Smith 1996). In 1980, however, weak diel acid accumulation was reported in the aquatic *Hydrilla verticillata* (Holaday and Bowes), and Keeley (1981) reported CAM activity in the aquatic *Isoetes howellii*. These discoveries necessitated a rethink of the adaptive significance and evolution of CAM.

CAM has since been found in five families of aquatic plants. Representatives include succulents in the Crassulaceae, the genera *Vallisneria* and *Sagittaria* (Keeley 1998),

and isoetids such as *Isoetes* (Keeley 1981; Farmer and Spence 1985; Spence and Maberly 1985; Keeley 1998; Madsen *et al.* 2002) and *Littorella* (Keeley 1998; Robe and Griffiths 1998; Baattrup-Pedersen and Madsen 1999; Robe and Griffiths 2000; Maberly and Madsen 2002). To date, 69 aquatic species in 14 genera are known to have significant over-night acid accumulation (Keeley 1998).

CAM in aquatic species may provide an advantage in CO_2-limited environments, by enabling them to use higher night-time CO_2 levels, formed by dark respiration from non-CAM plants and sediment respiration. This confers a competitive advantage where there is dense submerged vegetation, hence competition for carbon during the day (Madsen *et al.* 2002). In the terrestrial environment, with access to atmospheric CO_2, an improved plant carbon balance influences competition (Wurth *et al.* 1998). Aquatic CAM species may also benefit from elevated night-time CO_2 concentration, and become better competitors. A higher internal CO_2 concentration suppresses photo-respiratory CO_2 loss and promotes carboxylation by Rubisco (Robe and Griffiths 1990).

Tradeoffs associated with CAM include substantial needs for carbon, nutrients and energy for the carboxylation and decarboxylation enzymes. However, as a high internal CO_2 concentration suppresses photorespiration, Rubisco is more efficient and this may possibly enhance nitrogen use by aquatic CAM plants (Baattrup-Pedersen (1999).

Rubisco is equally active in aquatic and terrestrial CAM plants, but PEPC has a significantly lower activity in aquatic plants (Dittrich et al. 1973 in Keeley 1998). PEPC is the enzyme responsible for C_4 acid accumulation, yet even with a lower activity, aquatic CAM plants still maintain night-time malate production comparable to that recorded for terrestrial CAM plants, with a lower investment in PEPC. Keeley (1999) suggested that higher ambient CO_2 levels at night, relative to air, reduce the need for large amounts of PEPC.

The ratio of Rubisco/PEPC in aquatic CAM plants generally midway between that observed for terrestrial C_3 and CAM plants (Keeley 1998). On exposure to the atmosphere the Rubisco/PEPC ratio increases and often causes a switch to C_3 metabolism. Greater CO_2 availability in the terrestrial (aerial) environment may cause the switch from CAM to C_3 in species of *Isoetes* and *Littorella* (Keeley 1998; Keeley 1999; Robe and Griffiths 2000)

Patterns of gas exchange in aquatic CAM plants are more complex than in terrestrial species, due to the fluctuating carbon source. Changes in carbon uptake mechanisms are

correlated with changes in ambient CO_2. Diel changes in carbon availability occur because of because of with respiratory CO_2 release from non-CAM species during the night and uptake during the day. Other effects on the gas-exchange characteristics of CAM plants are the extent to which the C_3 pathway is used and, in *Isoetes* and *Littorella,* the level of carbon uptake from sediment (Keeley 1998; Madsen *et al.* 2002).

Plasticity is a ubiquitous feature of aquatic CAM plants. CAM is intimately linked with environmental cues, and is affected by temperature, light level and water status (Dodd *et al.* 2002). In variable aquatic systems the necessity for photosynthetic plasticity is high. In terrestrial species, such as Crassulaceae, plasticity has been extensively explored (Teeri *et al.* 1981) and recent work on aquatic plants has revealed similar plasticity (Keeley 1998; Robe and Griffiths 2000; Cushman and Borland 2002; Griffiths *et al.* 2002; Holtum 2002; Maberly and Madsen 2002; Taybi *et al.* 2002).

Most flexibility probably is in C_3-CAM intermediates, which possess the capacity for induction depending on environmental conditions, especially water availability (Dodd *et al.* 2002). In terrestrial species, the induction of CAM is considered a stress response to maintain a positive carbon balance (Winter and Smith 1996); this is likely to be true for aquatic and amphibious plants.

Physiological and photosynthetic plasticity is observed in *Littorella uniflora* on exposure to air, where a switch from CAM to C_3 photosynthesis is a component of new leaf growth (Robe and Griffiths 1998). Adaptive characteristics of *L. uniflora* in a fluctuating environment include the accumulation of carbohydrate, and the lowering of water and osmotic potentials (Robe and Griffiths 2000).

In *L. uniflora* the rapid loss of CAM in newly emergent leaves and the lack of CAM in new terrestrial leaves demonstrate plasticity within a single plant (Robe and Griffiths 2000). In addition, permanently submersed leaves show gradual seasonal changes in CAM. Bicarbonate use from sediments is another way for *Littorella* to access and concentrate carbon when it is in limiting supply (Raven 1995).

The dominant view is that CAM is common in low-nutrient water bodies due to carbonate depletion, which favours a more efficient method of carbon assimilation (Keeley 1998). High densities of aquatic plants will deplete the CO_2 in water, competitively displacing those species lacking carbon-concentrating mechanisms. Thus, ephemeral pools are ideal environments for species able to concentrate carbon. Waters

high in nutrients are buffered against diel changes in pH. This may benefit species without.

1.5 Stable carbon isotopes

1.5.1 Composition and discrimination

Two isotopes of carbon are present in atmospheric CO_2: ^{12}C and ^{13}C, with natural abundances of 98.89% and 1.11%, respectively. During C_3 photosynthesis there is strong discrimination against the heavier isotope by the enzyme Rubisco, whilst in C_4 photosynthesis the enzyme PEPC discriminates less. Thus, terrestrial C_3, C_4 and CAM species have distinct carbon isotope signatures.

1.5.2 Sources of fractionation

Differences in stable carbon isotope composition ($\delta^{13}C$) between the tissues of C_4 and C_3 plants were first documented by Bender in 1968 (cited in O'Leary *et al.* 1992). Surveys show that $\delta^{13}C$ values, relative to the PDB standard (see below), in C_3 plants are from -30‰ to -20‰, while those for C_4 and CAM plants are -15‰ to -7‰ (Farquhar *et al.* 1989). This isotopic 'signature' has also been used as an indicator of the inorganic carbon source fot aquatic photosynthesis (e.g. Beardall *et al.* 1982; Farquhar *et al.* 1989; Keeley and Sandquist 1992; Hornibrook *et al.* 2000; Robe and Griffiths 2000; Adis and Victoria 2001; Cushman and Borland 2002; Wanek *et al.* 2002), and in submerged aquatics, to quantify diffusive resistance and the effect of boundary layers in gas exchange (O'Leary 1981; Raven *et al.* 1982; Raven *et al.* 1987; Farquhar *et al.* 1989; Adis and Victoria 2001).

1.5.3 $\delta^{13}C$ signals in aquatic systems

Most freshwater plants have the C_3 pathway, and isotopic signatures covering a broad range from -50 to -10‰; values, however, are more commonly from -30‰ to -12‰ (Boutton 1991). Reasons for this variability are the type of source carbon (HCO_3^- or CO_2), $\delta^{13}C$ of the source, the slower diffusion rate of CO_2 in water, membrane transport of HCO_3^- and the presence of an unstirred boundary layer (Osmond *et al.* 1981). The variation in $\delta^{13}C$ due to utilisation of different sources of carbon in one marine species (*Ruppia megacarpa*) is from -17.8‰ to -6.6‰ (Boyce *et al.* 2001). For these reasons, isotopic signatures alone cannot be used easily interpret fixation pathways. To employ

$\delta^{13}C$ values to better understand carbon acquisition and assimilation, data are needed on the chemistry of source carbon and environmental conditions during uptake.

The $\delta^{13}C$ signature of source carbon can vary considerably: HCO_3^- has a value of about +1‰, which is 7-11‰ less negative than CO_2 (Keeley and Sandquist 1992). The enzyme carbonic anhydrase enhances the establishment of chemical and isotopic equilibrium between CO_2 and HCO_3^-, resulting in a fractionation of -9.0‰ (O'Leary *et al.* 1992). For this reason, the $\delta^{13}C$ of the plant material will be affected by preferential uptake of HCO_3^-. *Vallisneria americana* and *Potamogeton crispus* both use bicarbonate and have $\delta^{13}C$ signatures of -18.2‰ and -16.9‰, respectively (LaZerte and Szalados 1982). Other carbon-concentrating mechanisms such as CAM may be present in these species and would alter the signature independently.

For aquatics that use HCO_3^-, the preference for either HCO_3^- or CO_2 is dependent on inter-species differences in the capacity for active transport of the former, and the relative concentrations of CO_2 and HCO_3^- across the boundary layer (Keeley and Sandquist 1992). The ratio of CO_2 to HCO_3^- is dependent on pH, total carbon levels, photosynthetic rate and level of turbulence (Keeley and Sandquist 1992). Fluctuating ratios of HCO_3^- and CO_2 diurnally and seasonally complicate interpretation of the isotopic signatures. Thus, drawing conclusions about photosynthetic pathway variation and control is difficult in species that utilise HCO_3^-.

Isotope ratios can reliably indicate photosynthetic pathways in terrestrial plants, but less so in aquatic plants. $\delta^{13}C$ values for terrestrial C_3 plants range from -20‰ to -35‰, while CAM or C_4 species have values between -17 and -7‰ (Ehleringer and Rundel 1989). Aquatic CAM species can be as high as -4‰ (Keeley and Sandquist 1992), similar to that for terrestrial CAM plants. C_3 aquatic plants, however, have a lower $\delta^{13}C$ than terrestrial species, falling into the range for both CAM and C_4 species, preventing easy identification of their photosynthetic pathway.

The basis of discrimination against ^{13}C in C_3 plants is that Rubisco discriminates against ^{13}C due to its lower reactivity (Farquhar *et al.* 1982). Isotopic discrimination can also vary with pH, temperature and metal ion concentrations (O'Leary 1981).

The isotopic signature is determined by mass spectrometry, where $\delta^{13}C$ is a measure of the deviation from the PDB standard, referring to belemnite from the Pee Dee Formation, South Carolina, with a $^{13}C/^{12}C$ of 0.01124 (O'Leary 1981):

$$\delta^{13}C(‰) = \left[\frac{{}^{13}C/{}^{12}C_{sample}}{{}^{13}C/{}^{12}C_{PDB}} - 1 \right] \times 1000 \quad (1\text{-}2)$$

However, variation within species, and between seasons and sites, complicates the interpretation of food webs (Boon and Bunn 1994). For example, *Vallisneria americana* varies from -27.6 ± 0.7‰ in spring to -17.1 ± 0.05‰ in summer (Boon and Bunn 1994). Similar ranges are reported for other amphibious species, especially submerged macrophytes.

V. americana has a typical $\delta^{13}C$ signature of about -18.2‰ (LaZerte and Szalados 1982). This can be altered, however, by any biophysical or biochemical carbon-concentrating mechanisms, changes in flow, temperature and pH and differences in boundary layers. Thus, Boon (1994) found low discrimination of $\delta^{13}C$ by *V. americana* during summer compared with spring; this could be due to any of the above factors, and perhaps a combination of all.

1.6 Fluorescence

There are three possible fates for the light energy absorbed by chlorophyll *a* in thylakoid membranes. First, it can be used to drive photosynthesis, secondly it can be lost as heat and thirdly it may be re-emitted at a longer wavelength as fluorescence (Schreiber 2004). Any change in the efficiency of one of these pathways will change the yield of the other two. Analysis of the chlorophyll fluorescence signal emitted by photosynthetic tissues gives information about changes in photochemistry and heat dissipation.

1.7 Pilot survey of $\delta^{13}C$ in River Murray species

In preparation for the research described in subsequent chapters, a pilot survey was undertaken to quantify the range of carbon uptake and acquisition mechanisms in aquatic and amphibious plants. Samples of leaf material were collected at sites on the River Murray between Lock 6 and Murray Bridge, South Australia, and from ponds on The University of Adelaide campus, in spring 2002. The youngest mature leaves were selected, to standardise for age. Leaves were dried for 48 hours at 40°C and ground to a fine powder, using liquid nitrogen to aid in grinding specimens with high cellulose content. Samples were then dried for 24 hours to remove condensed water. 20-25 μg of ground tissue was weighed into tin capsules and carbon signatures were determined

using a isotope ratio mass spectrometer and expressed in $\delta^{13}C$ notation relative to the PDB standard, according to section 2.5.

Table 1-1 shows that $\delta^{13}C$ values ranged from -10.7‰ to 30.9‰. Precision for each sample was >±0.1‰ and standard errors were <3.1‰. The submerged species (Group 1) had highest $\delta^{13}C$ values, with an average -22.1±1.48‰ (mean ± SE). Within functional groups, there were variations in $\delta^{13}C$ values, suggesting varying modes of carbon acquisition. Examples are *Sporobolus mitchellii* and *Cyperus exaltatus*, in Groups 3 and 4 respectively, both known to use the C_4 pathway (Takeda *et al.* 1985; Prendergast and Hattersley 1987). Variations in isotopic signature were small for the emergent macrophytes (Group 2), where the carbon source is more stable. Submerged plants were enriched relative to other functional groups, due in part to their ability to acquire carbon from HCO_3^-. In *V. americana* the ability to undertake low-level CAM could also explain the high carbon isotope signature. Submerged macrophytes exhibited $\delta^{13}C$ values between -30‰ and -12‰. Values of -50‰ to -10‰ are reported by others (Boutton 1991).

1.8 Thesis plan

This project evaluates the extent to which certain plants of the River Murray employ different forms of carbon acquisition, and considers the implications of variability in the supply of resources for photosynthesis. Methods common to most sections are outlined in **Chapter 2**. In **Chapter 3**, a simple system of Dissolved Inorganic Carbon (DIC) acquisition is explored in the amphibious *Crassula helmsii*, a C_3/CAM species, with regard for the effects of submersion on CO_2 uptake and assimilation. A more complex system of CO_2 and C in the submerged aquatic *Vallisneria americana,* another C_3/CAM species, is explored in **Chapter 4** (field studies) and **Chapter 5** (laboratory studies). **Chapter 6** compares the floating and submerged leaves of *Potamogeton tricarinatus* and the submerged leaves of *P. crispus*, both C_3 species. The hypotheses that underpin these investigations are explained in the respective introductory sections.

Table 1-1. Pilot study data. Stable carbon isotope signatures (mean (SE) ‰) of plants from the Lower Murray, sorted into functional groups after Brock (1997). § known C_4 species; * known CAM species.

Group 1-Submerged	
Ceratophyllum demersum	-25.1 (3.1)
Lepilaena australis	-23.4 (0.7)
Myriophyllum papillosum	-21.4 (0.3)
Vallisneria americana	-18.2 (0.6) *
Group 2-Permanent, stable hydrology	
Azolla	-30.9 (0.1)
Cotula coronopifolia	-30.4 (1.0)
Lemna minor	-29.0 (0.4)
Ludwigia peploides	-29.0 (0.3)
Bolboschoenus fluvialis	-27.7 (0.4)
Juncus ingens	-26.7 (0.3)
Juncus pauciflorus	-26.5 (1.0)
Juncus usitatus	-26.9 (1.0)
Marsilea drummondii	-27.1 (0.9)
Myriophyllum aquatorum	-30.8 (0.2)
Triglochin procerum	-25.5 (1.4)
Typha domingensis	-27.7 (0.3)
Group 3-Floodplain	
Arthrocnemum halocnemoides	-28.5 (0.8)
Bolboschoenus caldwelii	-29.7 (0.3)
Crinum flaccidum	-23.9 (1.1)
Eleocharis acuta	-26.1 (1.0)
Gnaphalium luteo-album	-28.7 (0.6)
Heliotropium supinum	-27.5 (0.5)
Muehlenbeckia florulenta	-30.9 (1.1)
Phyla canescens	-30.6 (0.3)
Sporobolus mitchellii	-14.8 (0.2) §
Stemodia florulenta	-28.6 (1.6)
Group 4-Widespread, tolerant of flooding and exposure	
Apalise australis	-29.1 (2.0)
Carex apressa	-27.5 (0.2)
Centipeda minima	-28.5 (1.9)
Cyperus exaltatus	-10.7 (0.3) §
Cyperus gymnocaulos	-29.7 (0.2)
Galena secunda	-29.7 (0.7)
Mimulus repens	-26.1 (0.4)
Nicotiana simulans	-26.8 (0.0)
Polygonum decipiens	-27.7(0.7)
Phragmites australis	-28.3 (0.7)
Sinapis alba	-30.2 (1.2)
Xanthium occidentale	-25.7 (0.2)
Sisimbrium orientale	-26.8 (1.5)

Chapter 2
MATERIALS AND METHODS

2.1 Titratable acidities

Leaf samples were collected in the hour before sunrise and the hour after sunset. Samples were rinsed in deionised water and a subsample of approximately 0.5 g was taken, immediately frozen and stored at -4°C. Leaf tissue for titration was weighed then boiled in 5 mL of Reverse Osmosis (RO) water for 20 min. The extract was cooled, filtered and titrated with 1 mM NaOH and 2-3 drops of phenolphthalein as indicator. As malate is dicarboxylic, half the quantity of NaOH used to neutralise sample is equivalent to the number moles of malate present in the sample hence overnight malate accumulated overnight. The data, however, are presented here as μmol H^+ g^{-1}fw.

2.2 Chlorophyll

Leaf tissue was collected (see respective chapters) and chlorophyll contents of shoot material measured according to Porra (1989). In each case, 0.1-0.3 g (fw) of tissue was weighed then ground in 2 mL 80% chilled acetone with a mortar and pestle kept on ice. A small amount of acid-washed sand was used to aid grinding. The extract was decanted into 30 mL centrifuge tubes and kept on ice in the dark. Washing twice with 1 mL 80% acetone brought the total volume to 4 mL. Extracts were centrifuged at 2500 rpm at 4°C for 10 min. Absorption of the supernatant was measured at 664 and 647 nm, corrected for absorption at 750 nm. Chlorophyll concentration of the supernatant (nmol mL^{-1}) was calculated as follows (Porra *et al.* 1989)

$$Chlorophyll\ a \quad (nmol/\mu L) = 13.71 * A^{664} - 2.85 * A^{647} \tag{2-1}$$

$$Chlorophyll\ b \quad (nmol/\mu L) = 22.39 * A^{647} - 5.42 * A^{664} \tag{2-2}$$

$$Chlorophyll\ total \quad (nmol/\mu L) = 19.54 * A^{647} + 8.29 * A^{664} \tag{2-3}$$

where A^{664} is the absorbance at 664 nm and A^{647} is the absorbance at 647 nm.

2.3 Oxygen evolution

Photosynthesis was measured as oxygen evolution in a Clark O_2 electrode. Chamber volume was 4 mL and a constant temperature of 25°C was used for all measurements.

Solutions of 4 mL were calibrated with N_2 to determine zero and bubbled air to set O_2 saturation before purging with N_2 to lower the dissolved O_2 to 15% or 1.5 mg L^{-1} and so reduce photoinhibition effects. For fixed pH measurements, 50 mM MES, MOPS and Bicine were used to hold solutions at pH at 5.5, 7 and 9, respectively. Approximately 0.05g of 2 mm sliced and washed leaf material was added, $NaHCO_3$ was added as appropriate to yield 0, 0.5 or 1 mM concentrations. The chamber was sealed with a pH probe, allowing continuous pH measurements, and stirred at a rate to just prevent oxygen bubbles forming on the cut surface. Lowering of free CO_2 due to bubbling with N_2 was overcome by adding HCO_3^- after the sealing of the chamber. This was possible for buffered controlled pH experiments, but not for pH drift experiments, due to the bicarbonate increasing the pH prior to the start of the experiment. A black cloth was subsequently placed over the system and respiration rate recorded. pH fluctuated no more than±0.02 units during buffered measurements.

2.4 Chlorophyll fluorescence

A Pulse-Amplitude Modulated fluorometer (mini-PAM, Walz GmbH, Effeltrich, Germany) was used for chlorophyll fluorescence measurements, with the leaf clip attached for dry measurements and a leaf distance clip used for submerged *in situ* measurements, retaining a constant distance and angle to the leaf surface. For aerial material, Photon Flux Density (PFD) was measured using the quantum sensor on the leaf clip and Electron Tranport Rate (ETR) was calculated by mini-PAM. For submerged material, however, the leaf clip could not be used and ETR was calculated manually from PFD values measured with an external quantum sensor.

2.4.1 Photosynthetic efficiency

Optimum quantum yield of PSII (Fv/Fm) can be determined with a dark-adapted sample (equation 2.4) because all reaction centres are open and heat dissipation is minimal in the dark. In contrast, effective quantum yield of PSII (ΦPSII) is measured in illuminated samples (equation 2.5).

In the dark: $$Optimum\ Quantum\ Yield = \frac{F_v}{F_m} \quad (2\text{-}4)$$

In the light: $$Effective\ Quantum\ Yield = \frac{F_m' - F}{F_m'} \quad (2\text{-}5)$$

F_v/F_m of shoot material was measured at dawn, so that samples had had many hours of prior darkness. Dark adaptation during the light period was for 20 minutes using black plastic bags (for field measurements) or lids (for mesocosms). This was considered adequate to eliminate acute photo-inhibition and to have a well-defined starting point for the Rapid Light Curves and to measure Non-Photochemical Qyuenching.

2.4.2 Rapid light curves

Rapid Light Curves (RLC) measure the effective quantum yield (ΦPSII) as a function of PFD, and indicate instantaneous photosynthetic activity (Ralph and Gademann 2005) by incorporating PFD changes (Schreiber 2004). RLC describe the relationship between ETR and PFD. The former was determined according to (Genty *et al.* 1989):

Electron Transport Rate $ETR = \Phi PSII \times PFD \times AF \times 0.5$ (2-6)

where ΦPSII is the effective quantum yield, the Absorption Factor (AF) is the percentage of incident light absorbed by the leaf and 0.5 compensates for ETR as a measurement of photosystem II only. Where AF = 0.84 is used, a 'relative' ETR is cited. This was assumed for *Crassula helmsii*, where an accurate measure of AF was not possible due to leaf structure.

RLC measurements were performed using nine incremental PFD steps from 0-2000 µmol m^{-2} s^{-1}, using the mini-PAM light source. Yield measurements were taken at each PFD as programmed in the PAM unit. A PFD calibration curve was made before and after each set of curves. Samples were shaded so that external light did not contribute to PFD. Each PFD was held for 10 seconds, long enough to allow relaxation of the fluorescence signal after the saturating pulse, but short enough not to influence fluorescence yield (Ralph and Gademann 2005).

2.4.3 Non-Photochemical Quenching

Measurements of Non-Photochemical Quenching (NPQ) require an initial dark adaptation measurement. Dark adaptation during the light period was maintained for 20 minutes using black plastic bags (field measurements) or lids (mesocosms). This was considered adequate to eliminate acute photo-inhibition and to provide a well-defined starting point for RLC and to measure NPQ (Rascher *et al.* 2000). Calculations of NPQ were then undertaken on data from the completion of the light response curves.

2.4.4 Absorbance

Due to differences in light transmittance across different media, absorbance factors were calculated for submerged and terrestrial leaves by two separate methods. Thick leaves and waxy cuticles on floating leaves meant that the submerged techniques were ineffective, while the extremely thin and cuticle-free submerged leaves resulted in large errors using the method for aerial leaves. A method developed by Carter & Knapp (2001) was used for floating leaves and one developed by Beer (1998) was used for submerged leaves. Light absorbed by submerged and floating leaves of *Potamogeton tricarinatus* was measured in the laboratory on leaf material collected from Ral Ral creek. Light absorption of *Vallisneria americana* was measured on leaves from mesocosms within one hour of excison from the plant. By measuring photon absorption of the different leaf types, an accurate comparison of photosynthetic light use efficiencies could be made (Frost-Christensen and Sand-Jensen 1995). An AF of 0.84 was used for *Crassula helmsii*.

Floating Leaves Absorbance of floating leaves was calculated by measuring reflectance from the top surface and transmittance through leaves with an integrating sphere. Light (PFD ~145 μmol $m^{-2}s^{-1}$) from a white light source (Hansatech Instruments) was passed through an integrating sphere and the resultant light measured with a sensor (LiCor LI190 quantum sensor coupled to a LI1000 data logger). Light reflectance was measured with the leaf at the 90° surface of the integrating sphere, where all possible incident light angles (0-90°) were measured. This gives a value of maximum reflectance; photographic paper was then used to obtain a 100% "reflectance" reading. Transmittance was measured with leaf material placed between the integrating sphere projector head and the light sensor, following Carter & Knapp (2001).

Submerged Leaves Submerged absorbance values were estimated using the leaf-layer technique (Beer *et al.* 1998; Beer and Björk 2000). They are calculated from the natural log of light transmittance through 0-4 leaf layers and plotted against the number of leaf layers (Figure 2-1). Leaves were layered on top of a LiCor LI-190 quantum sensor and the light reaching the sensor (Lr) was recorded. ln(Lr) was plotted against the number of leaf layers and a linear fit applied. The y-intercept (I) and slope (α) were determined for each leaf by regression, and the absorbance calculated as 1-exp(α). This holds for zero reflectance. Actual reflectance, which was 5.8±3.2%, was calculated as the difference between the fitted irradiance (I) and the PFD with no leaf layers.

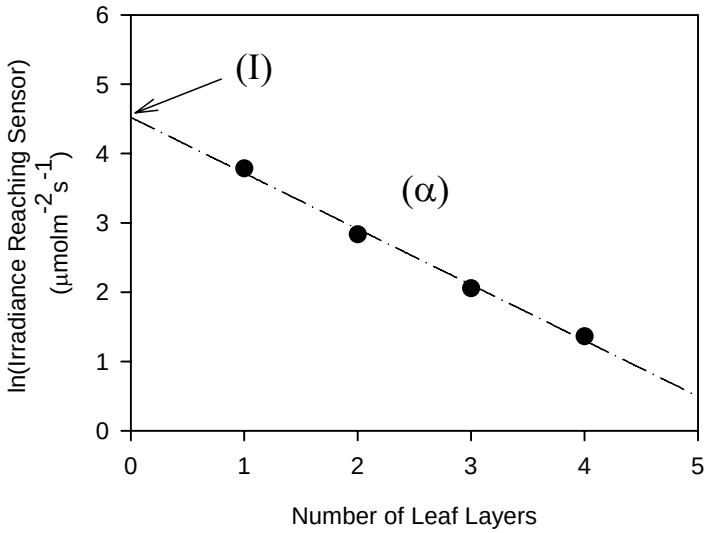


Figure 2-1. Calculation of absorbance factor using the leaf-layer technique. (I) is the y-intercept of the fitted least-squares regression, (α) is the slope of the line.

Submerged Leaves Submerged absorbance values were estimated using the leaf-layer technique (Beer *et al.* 1998; Beer and Björk 2000). Submerged leaf absorbance is calculated from the natural log of light transmittance through 0-4 leaf layers and plotted against the number of leaf layers (see Figure 2-1). Leaves were layered on top of a quantum sensor (LiCor LI-190) and the light reaching the sensor (Lr) was recorded. ln(Lr) was plotted against the number of leaf layers and a linear fit applied. The y-intercept (I) and slope (α) were determined for each leaf by regression, and the absorbance calculated as $1 - \exp(\alpha)$. This holds for zero reflectance. Actual reflectance, which was $5.8 \pm 3.2\%$, was calculated as the difference between the fitted irradiance (I) and the PFD with no leaf layers.

An absorption coefficient of 0.84 was used for calculations of relative ETR of *Crassula* as the conical leaf structure prevented the use of these techniques. As shown by (Beer *et al.* 2000) (see further Chapters 4-6), this factor is likely to be much lower for leaves adapted to submerged conditions. The leaves of *C. helmsii* are not heterophyllous, however, and the figure of 0.84 was deemed appropriate.

2.5 Stable carbon isotope fractionation

2.5.1 Plant material

Collection of leaf material is detailed in the relevant chapters. In general, leaves were dried at 40°C and finely ground with mortar and pestle. Liquid nitrogen aided the grinding of fibrous samples (samples then were re-dried for 24 hours at 40ºC). Approximately 1 mg dried ground leaf material was weighed into 6 × 4 mm tin cups (Elemental Microanalysis, Okehampton, Devon, UK). The cups were loaded into an auto sampler and combusted at 1020^{o}C to convert organic carbon into CO_2. The gases were swept by a helium carrier stream, purified using gas chromatography, chemically dried and directly injected into the source of an Isotope Ratio Mass Spectrometer (IRMS) (School of Earth and Environmental Sciences, Adelaide) or by Dr Charlie Schrimgeor (Scottish Crop Research Institute, Micromass Isoprime Stable Isotope Mass Spectrometer (Micromass, Manchester, UK)).

Isotope analysis was carried out using a Europa Scientific ANCA-NT 20-20 Stable Isotope Analyser with ANCA-NT Solid/Liquid Preparation Module (Europa Scientific, Crewe, UK), following Scrimgeour and Robinson (2003). It was operated in dual isotope mode using 1 mg samples of dried plant material (precision for $\delta^{13}C$: ~0.1 ‰). Working standards were 1 mg of a 1:4 leucine/citric acid mixture. $\delta^{13}C$ of the plant material was calculated using equation 2.7. $\delta^{13}C$ notation is expressed relative to the Pee Dee Belemnite limestone (PDB) standard, where the absolute concentration of ^{13}C (1.118‰) is arbitrarily set to 0 parts per thousand (‰).

$$\delta^{13}C\ ‰ = \left[\left(\frac{R_{sample}}{R_{standard}}\right) - 1\right] \times 1000, \qquad R = \frac{^{13}C}{^{12}C} \tag{2-7}$$

2.5.2 Extraction and measurement of carbohydrates

Soluble sugars were extracted from plant material using a procedure modified from Brugnoli (1988) and Borland (1994; 2002) and refined (Betson 2004) to give 1 mg for ^{13}C analysis. Aquatic tissue contains more water per gram of tissue than terrestrial plant material, so a greater fresh weight was required to extract sufficient carbon.

Leaf samples were collected as described in individual chapters for *Potamogeton tricarinatus, P. crispus*, *Vallisneria americana* and *Crassula helmsii*. Leaf material was ground in liquid nitrogen and 1 g material weighed into 2 mL Eppendorf tubes to which

0.5 mL 80% ethanol was added and mixed thoroughly. The solution was then incubated in a water bath at 80ºC for 30 min. Samples were spun at 13000 rpm (Sanyo Mini Centaur) for 15 min. Supernatant was collected and kept on ice. The pellet was washed 3 times with 0.5 mL 80% ethanol and each rinsing added to the supernatant. The washed pellet was retained for starch extraction.

The supernatant was then heated in a dry block at 80º C to evaporate most of the ethanol; the remaining100 μL of supernatant left was then diluted in 500 μL RO water, mixed and spun at 25000 rpm for 5 min to give a soluble carbohydrate fraction. The resulting pellet was washed three times in 250 μL RO water to give a total volume of 1.25 mL, and frozen. Removal of organic acids followed Borland (2002). Soluble fractions obtained as described above were purified passing sequentially through ion-exchange resins (cationic-Dowex 50x2-200, Acros, U.K., and anionic-Amberlite XAD-4, Acros, U.K.) to separate organic acids from the carbohydrate fraction.

Dowex (50x2-200, Acros, U.K.) cation exchange resin was washed with 1M HCl for 1 h and rinsed to neutrality with RO water. Amberlite (XAD-4, Acros, U.K.) anion exchange was washed in 1M NaOH for 1 h and rinsed to neutrality with RO water. Columns were constructed in a 5 mL syringe; 2 mL anion exchange resin acid then washed glass wool was layered in 2 mL cation exchange resin. A 23G needle controlled flow and residence time of the sample in the column, such that each pass of 2 mL took 2-3 min. Columns were rinsed five times with 3 mL RO water prior to loading 1 mL of sample onto the column. Washing with 3×10 mL of RO water gave a total sample volume of 30 mL. Samples were condensed manually using a rotary evaporator to approximately 1.5 mL and dried to 50 μL in a Speedivac (Uniscience, U.K) at 40Cº. The 50 μL was re-suspended to 80 μl of RO water and pipetted into tin cups. The solutions were then dried at 40ºC overnight and re-weighed prior to analysis.

Following initial separation from the soluble carbohydrate, as described above, the starch pellet was re-suspended in 1 mL 20% HCl on ice for 1 h. The supernatant was brought to 80% ethanol to precipitate the starch and cooled at 4ºC for 8 h. This was spun for 10 min and the starch pellet dried and re-suspended in 50 μL of water. This was loaded into tin cups, dried overnight at 40ºC for carbon isotope analysis as per bulk organic samples.

2.5.3 $\delta^{13}C$ and Total Inorganic Carbon determination

At each site, water was collected according to each chapter. Eight mL of filtered water was syringed into 12 mL evacuated Exetainers (Labco, High Wycombe, UK) at each site, stored on ice during transport and stored at -4ºC in the dark on return to the laboratory.

Each Exetainer was acidified with 0.1 mL 3 M HCl, shaken thoroughly and left overnight to release CO_2 into the 4 mL headspace. A 0.25 mL aliquot of the Exetainer headspace was removed and the CO_2 concentration measured against standards using an Infra-Red Gas Analyser (IRGA) (ADC 225MK3, Hoddesdon, England) fitted with a purpose-made CO_2 scrubbing inlet carrier tube (Figure 2-2). Standards of 1, 2, 3, 7, 10, 13, and 17 μL CO_2 were used to construct a standard curve. The peak height of the sample CO_2 given by the chart recorder attached to the IRGA was compared against standards to indicate the concentration of CO_2 for each sample.

Figure 2-2 Purpose-made CO_2 scrubbing inlet carrier tube attached to IRGA for analysis of CO_2 in headspace from Exetainers.

Remaining CO_2 in the Exetainer headspace was then purified cryogenically offline with a purification vacuum line (modified by Betson 2004 after Griffiths et al, 1990) by

cold-fingering with liquid nitrogen (-180°C) and acetone (-80°C). A needle was inserted into the Exetainer and the CO_2 passed through two cryogenic coils to remove water (-80°C) before CO_2 was trapped into a vial using liquid nitrogen (-180°C), as shown in Figure 2-3 below.

The $\delta^{13}C$ signature of the purified CO_2 from the dissolved inorganic carbon was run against an internal standard with a duel inlet mass spectrometer (PROVAC Services, Crewe, UK). Results are expressed in delta notation against Pee Dee Belemnite (PDB).

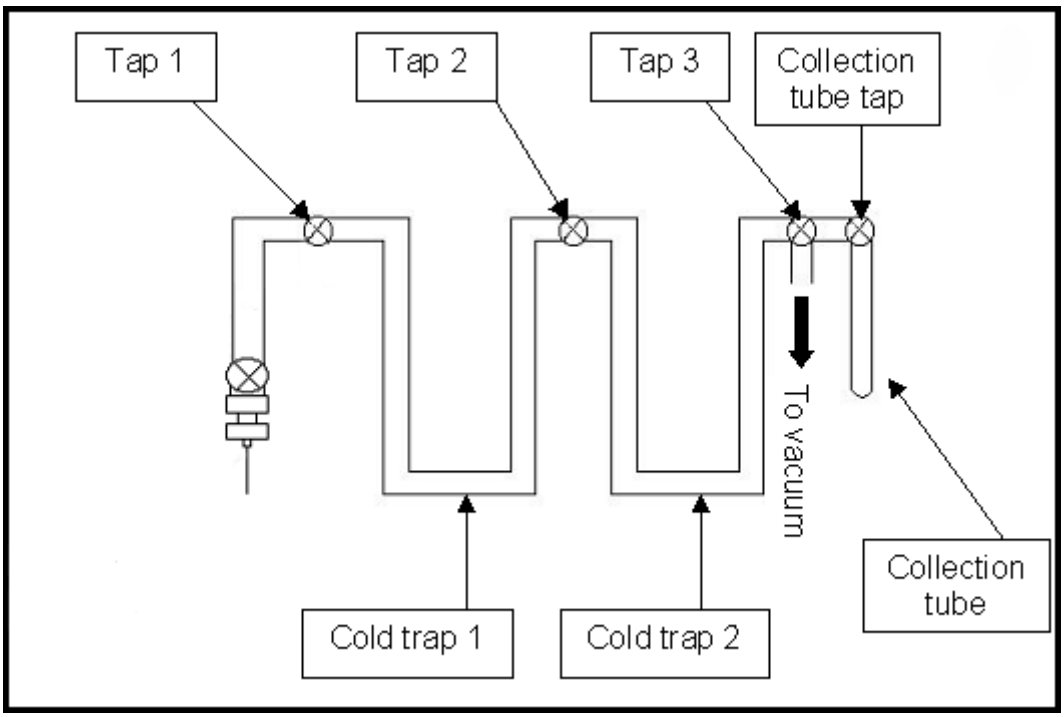


Figure 2-3. CO_2 purification line, modified with Betson (2004), used to purify CO_2 from Exetainers prior to mass spectrometry.

2.6 PEPc assays

The assay for phosphoenolpyruvate carboxylase (PEPc) activity followed Nimmo (1984). Shoot tissue, including stem and leaves, was ground in liquid nitrogen. About 0.5 g ground material was weighed and mixed with 1 mL extraction buffer (200 mM TRIS adjusted to pH 8.0 with 2 mM EDTA, 2% PEG20000, 10 mM L-malate, 1 mM benzamidine hydrochloride, 1 mM DTT, 350 mM $NaHCO_3$) at -4ºC. This was centrifuged at 13,000 rpm 4ºC for 90 sec (Sanyo Mini Centaur). The supernatant was de-salted to remove malate by passing through pre-packed NAP-5™ Sephadex columns

(Amersham Biosciences) and washed with 100 mM Tris base adjusted to pH 7.5 with 1 mM benzamidine hydrochloride, 1 mM DTT and 5% glycerol at -4ºC.

The eluate was then immediately used for enzyme analysis. The room temperature reaction mixture (1 mL) contained 650 µL 0.25 mM NADH, 50 µL 5 mM $MgCl_2$, 50 µL 10 mM $NaHCO_3$, 50 µL Malate dehydrogenase and 50 µL 2 mM PEP, to which 100 µl extract was added and mixed by inversion. Rate of NADH formation was determined with a spectrophotometer (Spectronic Unicam UV-300) against a standard NADH at 340 nm for 6 min. Soluble protein content of the extracts was determined after Bradford (1976). PEPc activity was calculated as µmol NADH s^{-1} mg^{-1} protein.

2.7 Water quality

2.7.1 Alkalinity

Site water samples were collected as described in each chapter. All samples were filtered through Whatman #1 filter paper to remove particulate matter. Alkalinity was assessed on 100 mL filtered water by the potentiometric method (Golterman *et al.* (1978). A titration curve of change in pH *versus* change in titrant volume was used to determine the inflection points at pH 8.3 and 4.5; these were used to determine equivalence points for calculations of alkalinity. Inflection points of maximum rate of change in pH per volume of titrant were identified by a sharp change in the linear rate of change in conductivity measured with a WP-84 meter (TPS, Springwood, Australia). Alkalinity and carbon species composition were then calculated:

$$Alkalinity\left(\frac{meq}{L}\right) = 1000\,(B)(C_a)/V_0 \tag{2-8}$$

$$Alkalinity\left(\frac{mg}{L}\,as\ CaCO_3\right) = 50044\,(B)(C_a)/V_0 \tag{2-9}$$

$$HCO_3^-\left(\frac{meq}{L}\right) = 1000\,(B-2A)(C_a)/V_0 \tag{2-10}$$

$$HCO_3^-\left(\frac{mg}{L}\,as\ HCO_3^-\right) = 61017\,(B-2A)(C_a)/V_0 \tag{2-11}$$

$$CO_3^{2-}\left(\frac{meq}{L}\right) = 2000\,(A)(C_a)/V_0 \tag{2-12}$$

$$HCO_3^- \left(\frac{mg}{L} as\ CO_3^{2-} \right) = 60009\,(A)(C_a)/V_0 \qquad (2\text{-}13)$$

where

A is the volume of titrant needed to reach the $CO_3^=$ equivalence point,

B is the volume of titrant needed to reach the HCO_3^- equivalence point,

Ca is the normality of acid titrant (eq/L) and

Vo is the initial volume of sample (mL).

2.8 Microscopy of leaf sections

Leaf material was collected according to each chapter and fixed within 24 h of collection. Tissues were fixed at room temperature in 3% glutaraldehyde in 0.025M phosphate buffer at pH 7.0 for 24 h. Progressive dehydration was undertaken involving 2 h in two changes each of methoxy ethanol (70%), ethanol (70%), propanol (70%) and butanol (70%). Infiltration and embedding were undertaken with a glycol methacrylate (GMA) solution added in equal volumes to the last change of butanol and allowed to infiltrate at 4ºC overnight. Subsequently, tissues were infiltrated with fresh GMA for two days, and the last change with butanol was repeated. Leaves were then embedded in gelatine capsules in fresh GMA before polymerising for 2 days at 55-60ºC. Prepared tissue was sectioned using a Reichert Jung microtome with glass knives and undertaken by the Microscopy Unit at The University of Adelaide. Sections were then stained with Periodic Acid-Schiffs and Toluidine blue O and mounted on slides.

2.9 Statistical analysis

Where necessary, one- or two-way analyses of variance (ANOVA) were applied using Minitab Statistical software (Release 14.1, ©Minitab Inc.). Normality and equal variances were confirmed using Kolmogorov-Smirnov and Bartlett's Test or Levene's Test, respectively (these assumptions were upheld unless otherwise stated). *Post hoc* testing employed the Tukey method. The Type I error rate (α) was set at 0.05.

Chapter 3
CARBON ACQUISITION IN CRASSULA HELMSII

3.1 Introduction

Crassula helmsii (Kirk) Cockayne is a small, succulent, semi-aquatic herb that occurs fully submerged, partially submerged or, in damp conditions, as a mat-like aerial form. The submerged form undertakes Crassulacean Acid Metabolism (CAM), and the aerial form uses the C_3 pathway (Newman and Raven 1995).

Most Crassulaceae occur in the deserts of South Africa and Central America. *Crassula* includes 300 species worldwide, including four obligate aquatic species. It is the only genus of the family native to Australia, where it is represented by the perennial, distinctively aquatic species *C. helmsii* (syn. *C. recurva*, *Tillaea recurva*), commonly known as Australian Swamp Stonecrop. In Australia, it is a common but not a dominant wetland plant, located mainly in the south-east (Figure 3-1). In the United Kingdom and other parts of Europe, however, it is a major aquatic weed. As a consequence, its ecology and growth requirements are well-documented (Dawson and Warman 1987; Dawson 1994; Dawson 1996; Leach and Dawson 1999; Leach and Dawson 2000).

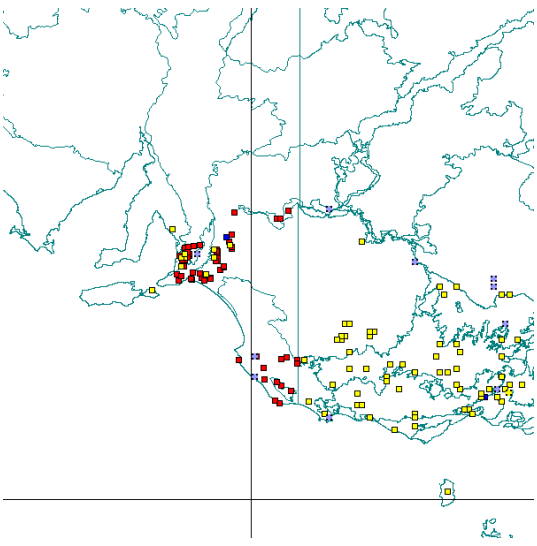


Figure 3-1. Distribution of *C. helmsii* in south-eastern Australia. Red squares are focused on the terminal lakes of the Lower Murray (and SE South Australia); yellow squares are sites in Victoria. Source: New South Wales Flora Online.

C. helmsii occurs in aquatic habitats, from deep dams to shallow river margins and along banks, but is most common in shallow lakes and lagoons. It tolerates seasonal temperatures from -6°C to 30°C (Leach and Newman 2001), grows in acid and alkaline water and persists even in salinities up to 6240 μS cm^{-1} (Hart *et al.* 1991). On banks and at wetland margins, where soil is damp, *C. helmsii* forms dense mats with succulent aerial leaves; in deep water (to 1 m) plants have an upright sparse growth form, with long inter-nodal lengths and thin, less succulent leaves. On emergence, there is a rapid switch from submerged leaves to emergent leaves and *vice versa*. As water recedes during summer in seasonal ponds, and along pond margins, the plants appear leafier and more compact than the shallow-water forms (pers. obs. MB). Branching is more frequent as inter-nodal distances decrease and the plants usually form a turf-like habit. *C. helmsii* can reproduce vegetatively from shoot fragments (5-10 mm) that contain a node. In autumn, short apical shoots with short internodes grow along the water surface.

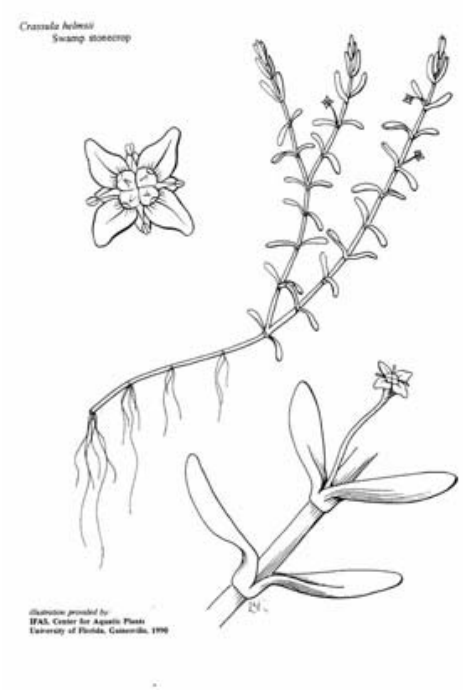


Figure 3-2. Aerial form of *Crassula helmsii*. Note the roots from nodes dependent on water availability. Source: Center for Aquatic Plants, University of Florida, Gainesville.

C. helmsii is a weed throughout England (Dawson and Warman 1987), where it became naturalised in the 1950s. It forms dense mats that reproduce vegetatively, out-compete native species and do not die back during winter. Isoenzyme data suggest that only one introduction occurred, probably from River Murray plants (Dawson 1994).

Crassulacean Acid Metabolism (CAM), the process whereby acid is accumulated at night, was named after the Crassulaceae. The submerged form of *C. helmsii* shows a marked diel fluctuation in malic acid, typical of CAM and first documented by Newman (1995). Use of pH drift methods (Newman and Raven 1995) showed that *C. helmsii* is unable to use HCO_3^- for photosynthesis. It has low photosynthetic rates even when CO_2 is not limiting. Cockburn (1998) recognised a number of sub-varieties of CAM. "Aquatic" CAM, in particular, appears to be highly flexible, but is concerned with efficient use of CO_2 rather than water stress. The diel separation of the activities of phosphoenolpyruvate carboxylase (PEPc) and Rubisco allow CO_2 acquisition at night, when there is no competition for CO_2 from non-CAM aquatic plants which are also releasing respiratory CO_2 (Keeley 1996). Aquatic CAM may serve to prolong the time available for uptake of CO_2 (Newman and Raven 1995). In aquatic CAM, loss of internally-released CO_2 by leakage to the environment is limited by the low rate of diffusion of CO_2 in the aqueous environment.

In this investigation, the central aim was to determine if physiological plasticity contributes to persistence of *C. helmsii* in environments where water level and water quality are prone to wide variations. It was postulated that *C. helmsii* remains competitive under changeable conditions by using CAM when submerged, and atmospheric CO_2 uptake by C_3-photosynthesis when emergent. Hypotheses were:

1. **Inducibility: CAM is initiated on submersion of aerial tissue.**

In Australia, *C. helmsii* usually occurs on damp ground but also occurs in still, shallow, fresh water (Frankston 2002). In the Murray-Darling Basin, *C. helmsii* generally grows alongside inland waters where fluctuations in water depth are small, frequent and rapid (within days), as in wind-driven seiches.

C. helmsii undertakes CAM when submerged (Newman and Raven 1995). Rapid induction of CAM on submersion may be essential to maintain a positive carbon balance in plants subject to changeable water levels. *C. helmsii* may switch pathways as a strategy to combat falling/rising water levels, hence changes in carbon availability, and would need to do so at a rate consistent with the changing hydrograph (Robe and Griffiths 2000).

2. **Flexibility: Submerged and aerial tissues have high background malate and PEPc, enabling a rapid switch to CAM on submersion.**

Strong CAM plants usually have low background acidity (e.g. Clusia Holtum and Winter 2005). High background PEPc and malate levels in C_3 aerial tissue may provide the mechanism for a

rapid (within days) switch to CAM after submergence. High background levels of PEPc and malate indicate C_3-CAM intermediates (Griffiths 1988; Holtum *et al.* 2005). While background PEPc levels have been explored in the C4 aquatic *Hydrilla verticillata*, where some increase in PEPc aids salt tolerance (Rout and Shaw 1998), PEPc levels of aquatic C_3-CAM switching plants have not been investigated.

3. **Photoinhibition: Photoinhibition in submerged CAM plants protects against absorption of excess light energy.**

Although diel fluctuations in CO_2 are the main driver for CAM (Keeley and Sandquist 1992), high light intensity often is a trigger in terrestrial and aquatic species, through lost efficiency due to photorespiration (Keeley and Rundel 2003). More efficient use of CO_2 with CAM may promote carbon assimilation under these conditions. On submergence, CO_2 availability is restricted due to diffusive resistance, but high light intensity may cause photo-inhibition. This means that the O_2:CO_2 ratio changes, O_2 out competes CO_2 for Rubisco and photorespiration is increased. It is proposed here that CAM is induced under 'high' or ‘excess' light conditions because there is a need to concentrate CO_2.

Previous work suggests that submerged leaves light less efficiently than aerial leaves, even though submerged plants tend to be similar to ‘shade’ plants (e.g. Mommer *et al.* 2005). This anomaly is explored. For leaf tissue to respond effectively to changes in water level and water quality, it needs to retain a capacity to absorb light and some protection from high light. Suppression of CAM in low light may be beneficial ecologically, as it releases energy for growth by reducing the energy needed for maintenance of CAM (Madsen 1987).

3.2 Methods

3.2.1 Carbon acquisition in a natural population

Site description

Riverglades is a permanent wetland on the River Murray immediately upstream of Murray Bridge, South Australia (35.07S, 139.1E). Since construction of weirs and barrages on the Lower Murray in 1922-1940, Riverglades has remained a mostly-permanent wetland, but in 2001-2003 artificial wetting and drying cycles were introduced by a community management group.

Field protocol

A small population of *C. helmsii* at Riverglades was used. Measurements were made in the austral summer, on 2 December 2003 and again on 2 January 2004. The plants occurred in small patches along the banks of the Southern Lagoon, where they were

partly submerged when wind caused a daily rise in the water level. Shoots usually consisted of stems with both aerial and submerged leaves. At each inspection, submerged and aerial leaves were assessed for a number of physiological parameters. Details are shown below (see also Chapter 2).

Before sunrise, a Pulse-Amplitude-Modulated fluorometer (mini PAM, Walz GmbH, Effeltrich, Germany) was used to measure dark-adapted Rapid Light-response Curves (RLC). Effective quantum yield and RLC were measured at 3-hour intervals throughout the day, following procedures described in Section 2.1. RLC were derived using nine incremental steps of PFD, from 0-800 μmol m^{-2} s^{-1}.

Submerged and aerial tissue was collected for titratable acidity at 3-hour intervals from dawn until dusk each day. Whole shoots (green photosynthetically-active stems) from five clumps per site were immediately frozen in liquid nitrogen and, on return to the laboratory, stored at -4ºC. Analysis then followed the procedure in Section 2.1.

Submerged and aerial shoots were collected at dawn and at midday for stable isotope analysis of soluble carbohydrates. Tissue was rinsed in deionised water, patted dry and frozen in liquid nitrogen before analysis according to Section 2.5. Shoots were collected at dawn and midday for bulk organic $\delta^{13}C$ analysis, stored in paper bags and, on return to the laboratory, dried at 40ºC for 48 hours. Analysis was according to Section 2.5. All other material was kept fresh in containers in Riverglades water for up to 24 hours. Chlorophyll content was assessed on 0.3 g material on the following day, according to Porra *et al.* (Porra *et al.* 1989), as in Section 2.2. Meteorological data included PFD recorded with a LiCor quantum sensor coupled to a Li1000 data logger, and air and water temperature recorded using a 1200 series multi-channel Squirrel Logger (Grant Instruments (Cambridge), UK) with thermocouples at 10 and 20 cm depth.

Water temperature and pH were measured at dawn and midday, and 8 mL samples were collected in 12 mL Exetainers. TIC and $\delta^{13}C$ were determined according to Section 2.5.

3.2.2 Impact of water regime on mesocosm plants

The impact of submergence on photosynthesis and carbon uptake of *C. helmsii* was assessed on mesocosm-grown plants, initially grown as emergent, atmospheric CO_2-using plants of the mat-like form. The "Control" remained emergent yet with waterlogged soil; the "Fully Submerged" treatment was top-flooded with no leaf exposed to the atmosphere; the "Partially Submerged" treatment was flooded half way

up stems and the "Emergent" treatment, like the control, accessed atmospheric CO_2 only but was allowed to drain fully.

Plant material

The mat-forming ecotype of *C. helmsii* was sampled from Paxton Pits Nature Reserve near St Neots, UK. Twenty-five 10-cm diameter pots of *C. helmsii* were grown for two months in a 50:50 mix of sand and loam plus 25 g slow-release fertiliser (Osmocote®, 6 month) per 10 L soil (equivalent annual loading: 100 g N m^{-2}). Pots were divided evenly between five small mesocosms with water depth midway between the soil and base of pot level, and maintained for two months to provide a waterlogged environment without submerging leaves or stems.

Pre-treatment

After two months' growth, physiological parameters were assessed on photosynthetically-active leaves and stems: titratable acidity (Section 2.1), chlorophyll (Section 2.2) and isotope discrimination of organics (Section 2.5) and carbohydrates (Section 2.5.2). *In situ* fluorescence measurements included dark-acclimated RLC and optimum quantum efficiency (Section 2.4), made on samples in the hour before dawn, and light-acclimated measurements of RLC and effective quantum efficiency undertaken every three hours until dusk (Section2.4). Morphological measurements on three shoots from each pot included fresh weight, total length, internodal length and leaf length. Sampled material was then dried for 48 hours at 40ºC and weighed.

Application of treatments

Pot positions were re-randomised following initial analyses to provide material of equal size in four pots per pond (Figure 3-3). Replicates were assigned to the same pond due to limited space and equipment; strictly, these are pseudoreplicates, and need to be considered cautiously in statistical analysis. Any replicated measurements per pot were averaged, so that each pot was considered a replicate. "Control" plants were kept at a consistent water level, such that water was midway between soil surface and base of pot. "Fully submerged" plants had all tissue below the surface. "Partially submerged" plants were grown with tips of the shoots emerging from the water surface. "Emergent" pots were fully drained (rainfall and occasional light watering provided for survival).

Figure 3-3. Five pots of *C. helmsii* per bin were grown and four evenly-grown plants were randomly assigned to three water regimes after two months' growth.

After initiation of the treatments, CAM activity was determined by titratable acidity was measured at dawn and dusk on days 1, 3, 12 and 30, according to Section 2.1. After 30 days, fluorescence measurements included 3-hourly optimum and effective quantum yield and RLC from dawn until dusk, according to Section 2.3. Leaf material was then harvested for stable isotope analysis, chlorophyll, PEPc activity and for titratable acidity (thus, dawn and dusk measurements). Approximately 5 g shoot material was collected for bulk organic analysis; this was rinsed in de-ionised water, dried at 40ºC for 48 hours and processed according to Section 2.5. A further 5 g shoot material was rinsed in de-ionised water and immediately frozen in liquid nitrogen; on thawing, extractions of carbohydrates and analysis of $\delta^{13}C$ followed the procedure in Section 2.5.2. Shoots for titratable acidities were frozen, stored at -4ºC and analysed according to Section 2.1. Chlorophyll was assessed immediately on 0.3 g material according to (Porra *et al.* 1989) (Section 2.2). PEPc assays followed Nimmo (1984) (Section 2.5.3).

Morphological measurements were made after 30 days' exposure to the three water regimes. Three shoots from each pot were collected and measured for fresh weight, total length, inter-nodal length and leaf length. Material was then dried for 48 hours at 40ºC and weighed. As noted, each pot was considered a replicate.

At the beginning and end of the experiment, water from the ponds was measured for pH and temperature at dawn and midday, and 8 mL water collected in 12 mL Exetainers at midday was assayed for pH, $\delta^{13}C$ and TIC, according to Section 2.5,.

3.2.3 Statistical analysis

Analyses of variance (ANOVA) were applied using Minitab software (Release 14.1, Minitab® Inc.). Assumptions of normality and equal variances were confirmed using Kolmogorov-Smirnov and Bartlett's Test or Levene's Test, respectively. Tukey's method was used for *post hoc* comparisons. The Type I error rate was set at $\alpha = 0.05$ or, in some cases (see text).

3.3 Results

3.3.1 *Impact of water regime on mesocosm plants*

Environmental factors

Maximum temperatures over the course of the experiment were 15-25ºC, with minimal overnight temperatures of c. 7ºC. Day length during the week of final assessment was about 10 h. Temperatures on 21 and 22 October 2004 were 7.7-15.4ºC and 10.6-17.2ºC, respectively. The pH of water in the mesocosms was 7.7-8.8, and total inorganic carbon was 25±5 mg L^{-1}.

Morphology

Shoot water content was highest in the submerged material

Figure 3-4). The large error associated with the emergent treatment was due to one outlier; once this was removed the difference between treatments became significant ($F_{4,4} = 7.37$, $P = 0.002$). All other comparisons were significant at either $\alpha = 0.05$ or 0.10 (fully submerged and partially submerged treatments).

The total length of stems in the control was similar to the initials. Other treatments showed significant increases after 30 days ($F_{4,4} = 12.57$, $P = 0.000$)

Figure 3-4b).

Inter-nodal lengths were not different between treatments ($F_{4,4} = 1.85$, $P = 0.172$). The growth of the partially-submerged treatment was horizontal, with new roots at nodes on the stem and leaves missing low on the stem. The emergent treatment appeared to form smaller, more compact and fleshy leaves, characteristic of a terrestrial CAM plant.

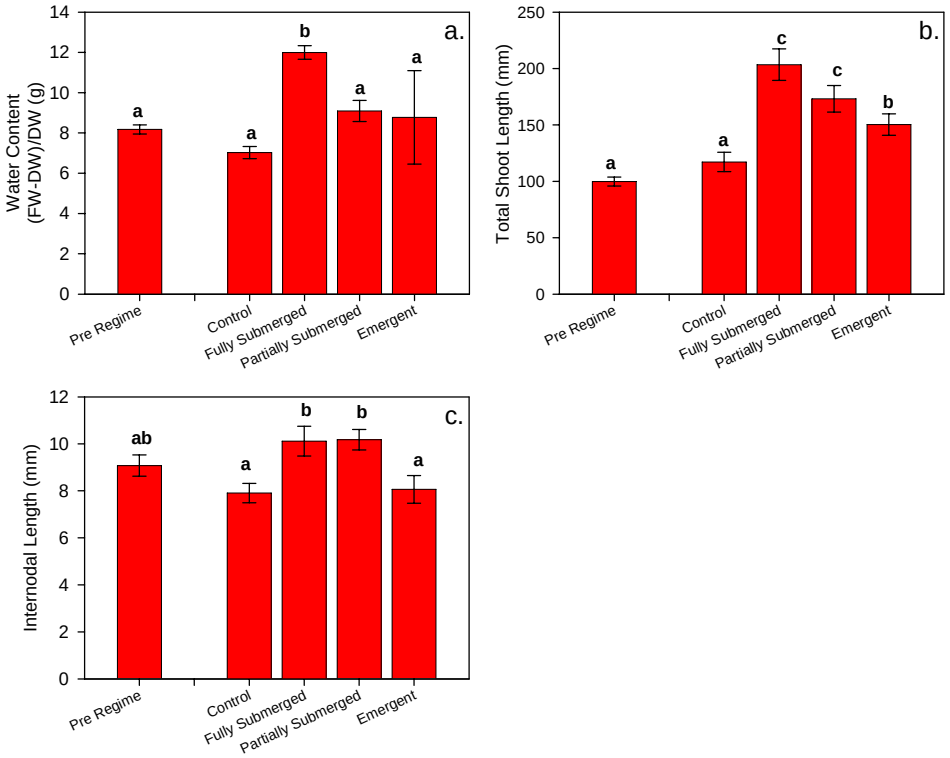


Figure 3-4. Water content (a), total length (b) and internodal length (c) after 30 days. Bars are standard errors (pre-regime, n = 16; final, n = 4). a and b signify differences at alpha = 0.05.

Chlorophyll

Chlorophyll *a* concentrations were greatest, on a fresh weight basis, in fully-submerged and emergent treatments; this was highly significant compared with the control and significant when compared with the pre-regime and partially-submerged data ($F_{4,3}$ = 6.21, P = 0.01)

Figure 3-5a). The higher chlorophyll *a* in both the fully submerged and emergent treatments compared with other treatments is the cause of the higher total chlorophyll. Chlorophyll *b* was highest on a fresh weight basis in the fully-submerged treatment ($F_{4,3}$ = 2.96, P = 0.035). Higher chlorophyll on a fresh weight basis in the emergent treatment probably is an artefact of a lower fresh- to dry-weight ratio. The chlorophyll a/b ratio for all treatments was significantly higher than the control

Figure 3-5b) ($F_{4,3}$ = 3.72, P <0.014).

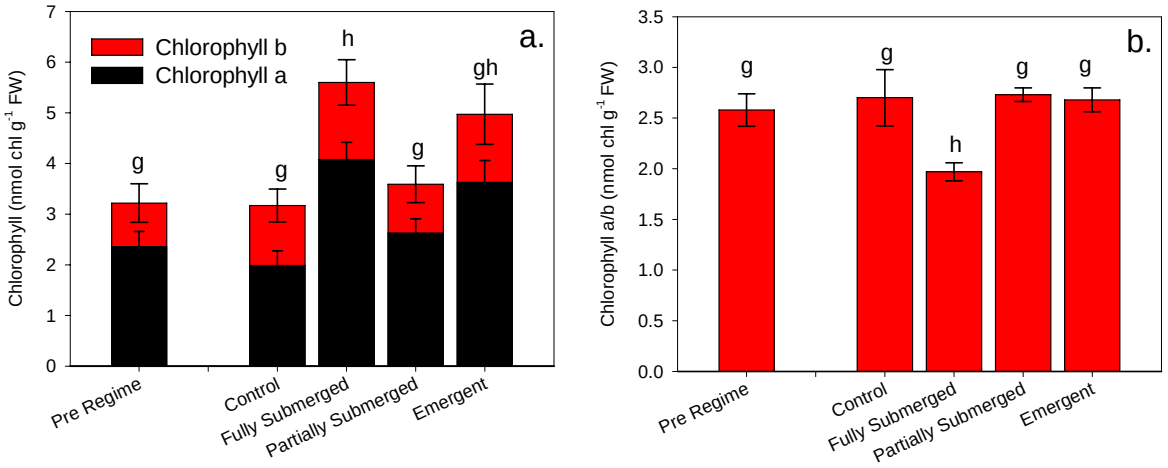


Figure 3-5. Chlorophyll after 30 days: (a) total chlorophyll, showing chlorophylls a and b and (b) chlorophyll *a*/*b* ratio. Pre-regime n = 16, treatments n = 4, where each replicate is the mean of 4 shoots. g and h signify differences at alpha = 0.05.

Titratable acidity

High background levels of acidity were evident in tissue prior to treatment and on days 1, 3 and 12 (Figure 3-6a), but not on day 30. The low background titratable acidity and low dawn acidity after day 30 may be a result of 5 days' low irradiance. Overnight accumulation in acidity, however, is in the same range as shown in Figure 3-6b.

The emergent pre-treatment tissue (day 0) accumulated 8 µmol H^+ g^{-1} fw overnight. On day 1, *fully submerged* tissue accumulated 24 µmol H^+ g^{-1} fw while *control, partially submerged* and *emergent tissue* accumulated only 12 µmol H^+ g^{-1} fw. Over the subsequent sampling days the fully submerged tissue accumulated about 10 µmol H^+ g^{-1} fw more than any other treatment. Due to the limited amount of tissue, it was not possible to pair measurements (hence standard errors are not shown); nevertheless, the means suggest a higher acid accumulation in fully submerged *C. helmsii* than in other treatments (Figure 3-6a).

After 30 days, the acidity in fully-submerged tissue showed a diurnal pattern of accumulation and loss. No such changes were recorded in other treatments (Figure 3-6). CAM is expressed in both leaf and stem tissue, and again is higher in fully-submerged tissue than in other treatments regardless of tissue type ($F_{1,11} = 14.61$, $P < 0.001$).

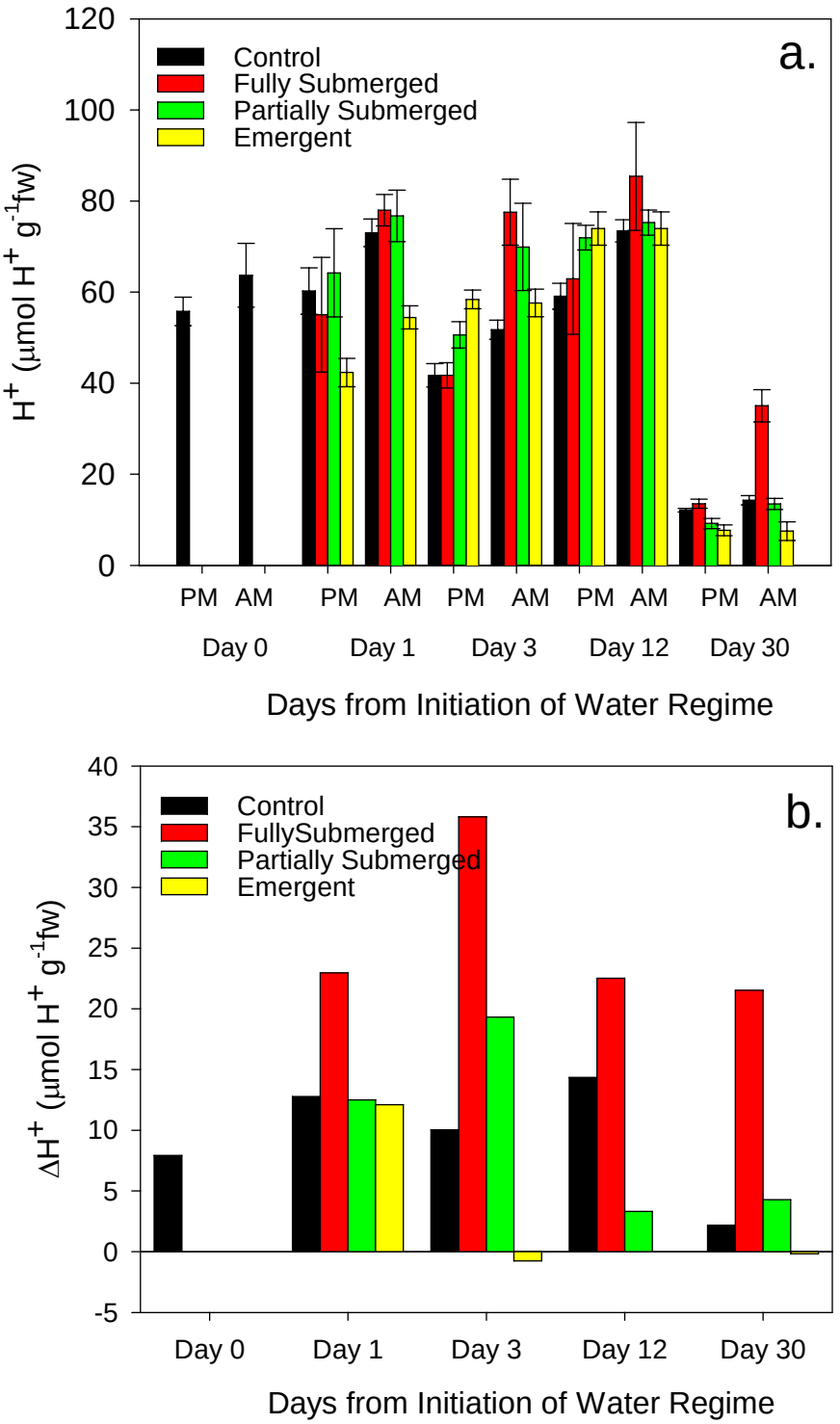


Figure 3-6. Dawn and dusk titratable acidities (a) and overnight accumulated acid (ΔH+) (b) for pre-treatment (day 0) and the control and three treatments (days 1-30).

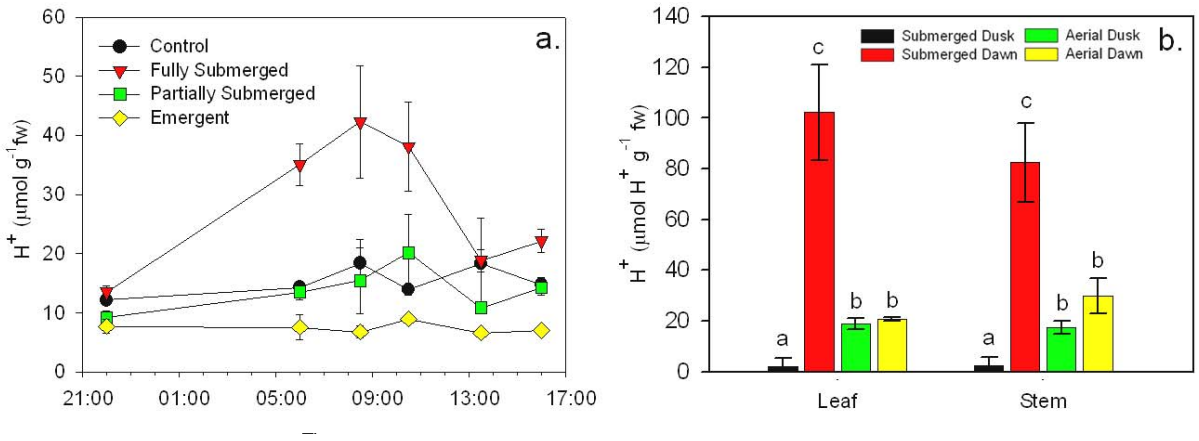


Figure 3-7. Diurnal change in acidity after day 30 for the *control* (■), *fully submerged* (●), *partially submerged* (▲) and *emergent* (◊) treatments (n = 4) (a) and titratable acidity in the leaf and stem at dawn on day 30. Bars are standard errors of the mean (n = 4) (b). a and b signify differences at alpha = 0.05.

PEPc activity

After 30 days, rates of PEPc activity in all treatments reached 300 µmol NADH s^{-1} (negligible compared with other CAM plants), with the partially submerged treatment being lower than all other treatments ($F_{3,3}$ = 8.05, P = 0.008) (Figure 3-8).

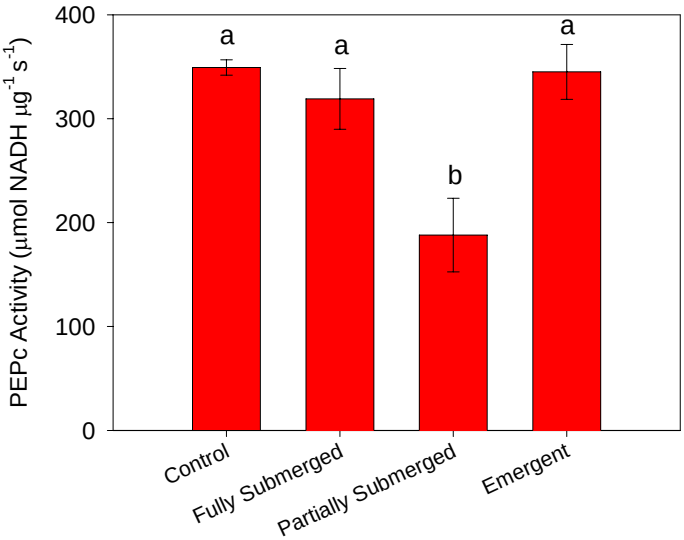


Figure 3-8. Dawn and dusk titratable acidities (a) and overnight accumulated acid (ΔH+) (b) pre-treatment (day 0) and for the control and three water regimes (day 1-30).

$\delta^{13}C$ organic

Fully-submerged *C. helmsii* were more enriched in $\delta^{13}C$ of bulk tissue (i.e. all tissue components) than in the pre-regime ($F_{2,3}$ = 13.25, P = 0.008), but did not differ from other treatments (Figure 3-9: n = 16 for pre-regime, n = 4 for treatments).

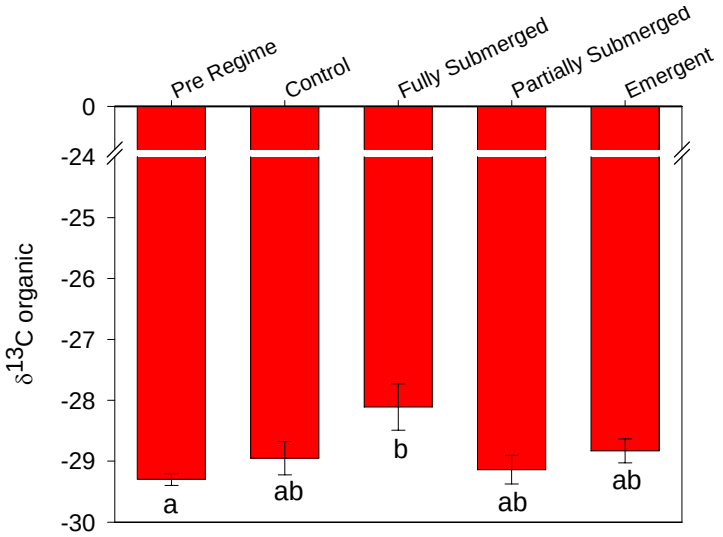


Figure 3-9. $\delta^{13}C$ for pre-treatment and control, fully-submerged, partially-submerged and emergent treatments after day 30. Bars are standard errors (n = 4). a and b signify differences at alpha = 0.05.

Light use

The optimum quantum efficiency (F_v/F_m) of the pre-regime and treatments showed a midday depression at maximum PFD (Figure 3-10a). This was most evident in the emergent treatment, which also had a strong recovery during the afternoon. The diurnal pattern of F_v/F_m in the fully-submerged treatment differed from other treatments. It started at 0.75±0.1, decreased during maximum PFD and remained low during the afternoon. In the control, partially-submerged and emergent treatments, F_v/F_m increased during the afternoon. The low value for the fully-submerged treatment at 07:00 h indicates that even after an 8-h dark period F_v/F_m did not recover from the previous day. Alternatively, it may never reach higher, irrespective of the duration of the dark period.

The depression in F_v/F_m during high PFD at midday probably is due to non-photochemical quenching (NPQ) as a result of photo-protective mechanisms. NPQ (a measure of thermal dissipation) increased between 09:00 h and midday in most treatments (except the control), during high PFD. The most striking feature is that fully-submerged tissue showed a further increase in NPQ over the afternoon, with a

maximum 4.5±0.5 at 16:00 h; in comparison, NPQ at 16:00 h in other treatments was negligible (Figure 3-10b).

Relative Electron Transport Rates (rETR) were low at 07:00 h because Rubisco inactivation) takes time to reactivate after prolonged dark periods. Rubisco activation increased for all treatments over the day. Fully-submerged plants showed a dramatic decline in rETR at 16:00 h compared to other treatments (Figure 3-11). This would be expected if they were using CAM and had exhausted their malate reserves.

Rapid Light response Curves (RLC) showed diel changes in efficiency of photosystem II). The initial slope of the curve (α) reflects the maximal photosynthetic quantum yield and was similar for all treatments. ETR_{max} (ETR calculated with the same absorbance for all leaf types) was similar for all treatments with emergent tissue. Highest ETR_{max} was at 12:00 h and 16:00 h, reaching 40-50 µmol $m^{-2}s^{-1}$. ETR_{max} for the fully submerged treatment, however, reached only 16.4±2.7 µmol m^{-2} s^{-1} at 12:00 h. Fully-submerged treatments were 20-50% of other treatments. As α was similar between treatments, calculated E_k (ETR_{max}/α) follows the same pattern as ETR_{max}. E_k of fully-submerged plants was low, between 40-70 µmol m^{-2} s^{-1}, while other treatments saturated at a PFD of 80-140 µmol m^{-2} s^{-1}. At 16:00 h, light saturation of fully submerged treatment occurred at extremely low PFD (<20 µmol m^{-2} s^{-1}), while other treatments were c. 150 µmol $m^{-2}s^{-1}$.

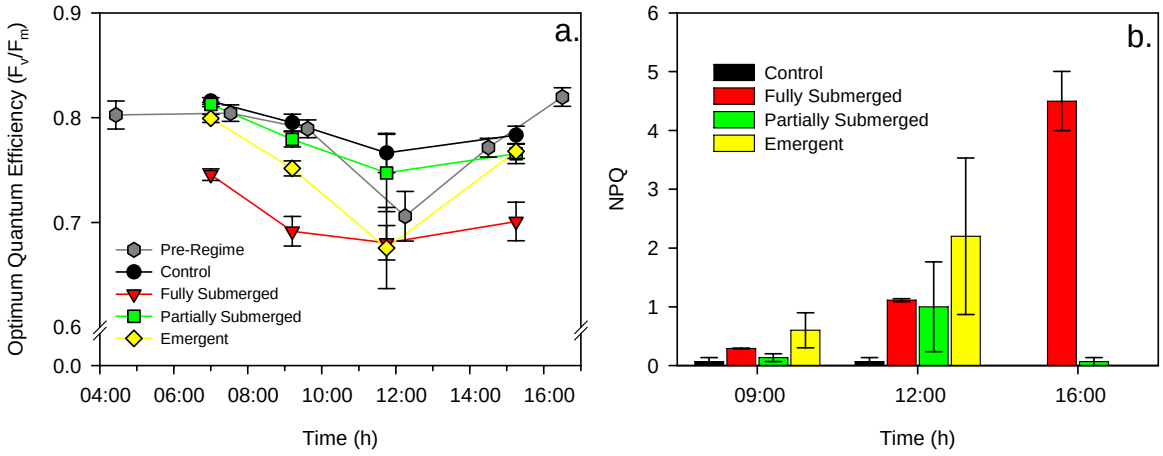


Figure 3-10. Diurnal optimum quantum efficiency (F_v/F_m) of each treatment after 30 days' treatment and the initial diurnal course pre-initiation of water regime (a) and non-photochemical quenching (NPQ) calculated from the ratio of a change in dark-measured fluorescence (F'_m) to measured fluorescence (F_m) at 9:00 h,

12:00 h and 16:00 h for each treatment (note that NPQ for the control and emergent treatment was zero) (b). Bars are standard errors (n = 3).

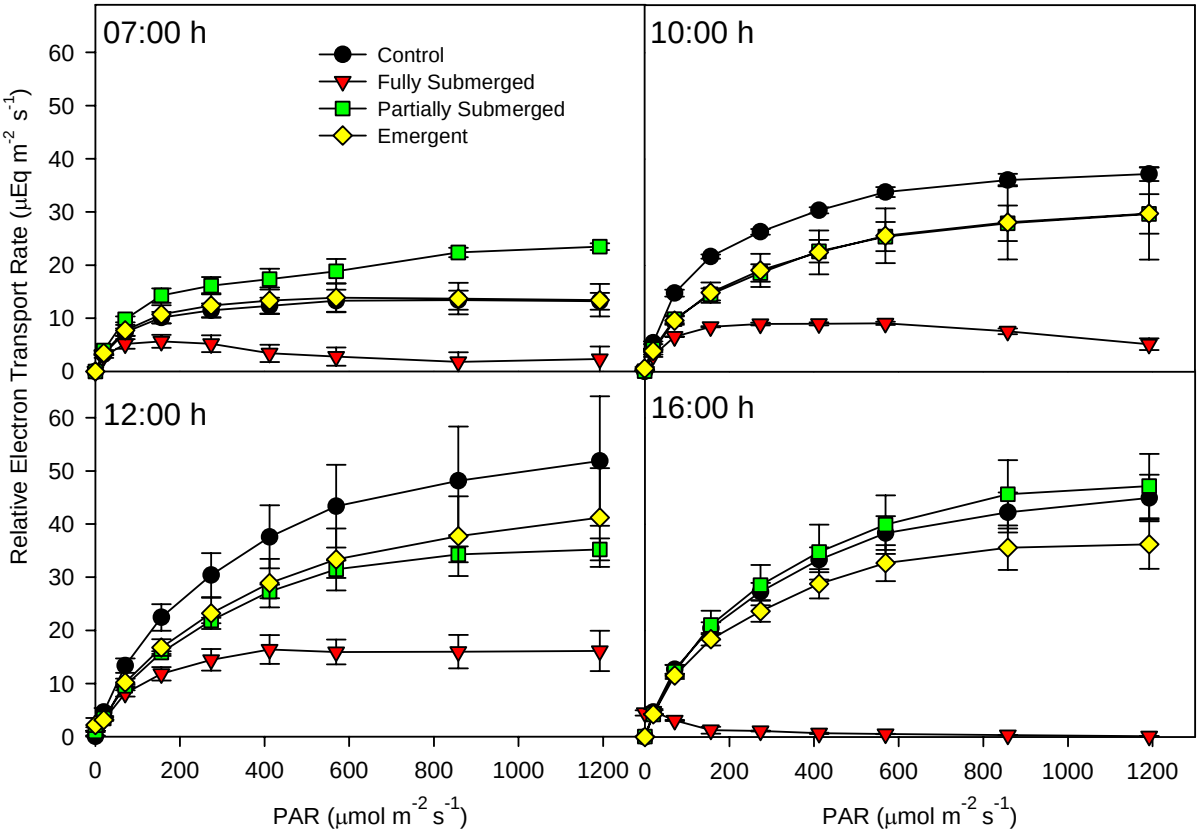


Figure 3-11. Relative ETR of each treatment after day 30 for four time points. *Control* (●), *fully submerged* (▼), *partially submerged* (■) and *emergent* (◆). Bars are standard errors (n = 3).

3.3.2 Carbon acquisition in a natural population

Climate and physical variables

A typical diurnal light regime (PFD 0-2000 µmol m^{-2} s^{-1}) occurred on both visits to Riverglades (Figure 3-12a). On 2 December 2003 there was patchy cloud cover, and on 2 January 2004 the sky was clear and bright. Water temperatures differed between two small (<2 m^2) populations, one sheltered by a stand of common reed, *Phragmites australis* (diurnal range 18-30ºC), and the other with an open aspect (18-36ºC) (Figure 3-12b). Minimal temperatures occurred at dawn.

Total Inorganic Carbon (TIC) of water (at midday) was high (15.1±1.7 mg C L^{-1}). Further analysis showed that at midday there was no free CO_2; rather, the available carbon was HCO_3^- and CO_3^- (Figure 3-13). The $\delta^{13}C$ was -14.43±0.02. pH measured at dawn was 7.13, and at midday was 10.40 (see Chapters 4-5).

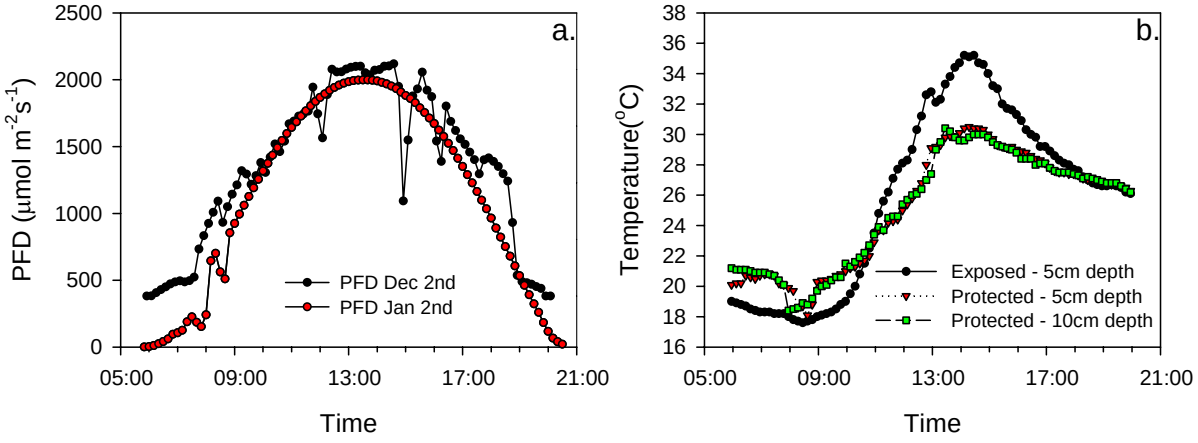


Figure 3-12. Diurnal incident PFD at Riverglades on each sampling occasion (a) and water temperature profiles of micro-sites on 2 January 2004 (b).

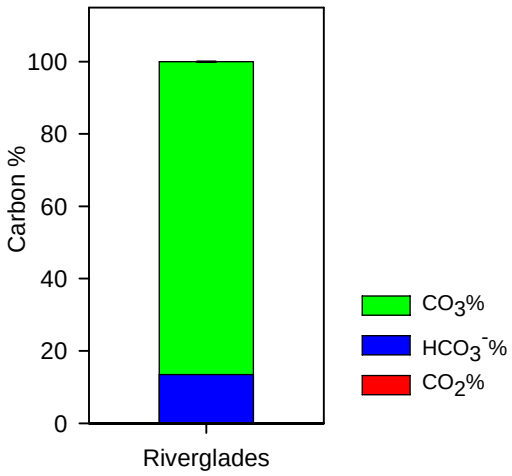


Figure 3-13. Analysis of carbon from water samples at Riverglades, midday, pH 10.

Titratable acidities

Background or dusk leaf titratable acidity levels were low (12-35 µmol g^{-1}fw) and although average amounts suggest that there was more accumulation in submerged leaves than in emergent leaves, this was not significant (background: $F_2 = 5.92$, $P = 0.072$ and accumulation: $F_2 = 0.17$, $P = 0.702$, respectively) (Figure 3-14).

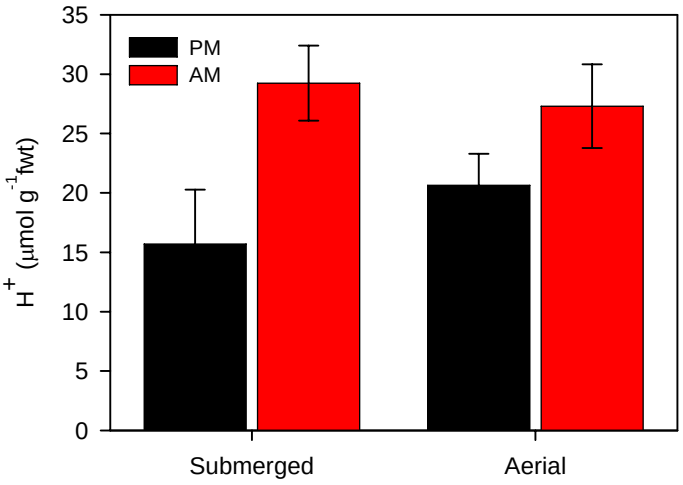


Figure 3-14. Titratable acidity in the hour after dusk (PM) and the hour before dawn (AM) of submerged and aerial leaves of different shoots of *C. helmsii* at Riverglades, January 2003. Bars are standard errors (n = 4).

$\delta^{13}C$ of bulk organic and purified soluble carbohydrates

The $\delta^{13}C$ of all submerged tissue was on average 2.5‰ more enriched than aerial tissue (Figure 3-15). There was little difference between soluble carbohydrate material in submerged and aerial tissue. Although there are no replicates as material was lost in preparation, there *appears* to be a difference between the dawn and dusk $\delta^{13}C$ of soluble carbohydrates, suggesting accumulation of more-enriched carbon during the night, in both aerial and submerged tissue. Dusk samples were similar to the bulk organic $\delta^{13}C$, whereas pre-dawn samples were more negative.

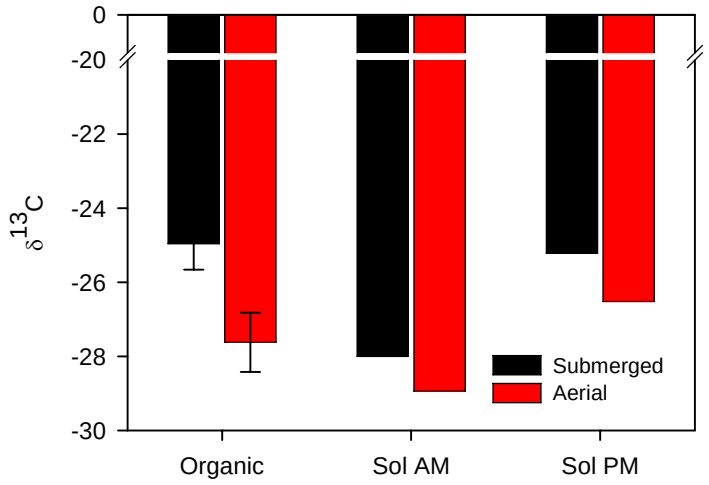


Figure 3-15. $\delta^{13}C$ of bulk organic carbon and purified soluble carbohydrates (dawn AM, dusk PM) for submerged and aerial leaves in January 2003 (a). Bars are standard errors (n = 3 for organic samples; no replication for carbohydrates).

Fluorescence

There was little difference between F_o/F_m in submerged and aerial tissue. The effective quantum yield in both tissue types decreased suddenly over the first few hours of daylight, remained at c. 0.3±0.1 during the middle of the day and recovered by dusk. Both aerial and submerged leaves of *C. helmsii* had typical dark-adapted (20 min) C_3 diurnal F_v/F_m profiles in January 2004 (Figure 3-16b), with a depression during the middle part of the day. Both aerial and submerged tissue reached a minimum at 13:30 h, with extremely low effective quantum yield (<0.4) indicating high stress (Maxwell 2002). Full recovery occurred over the afternoon.

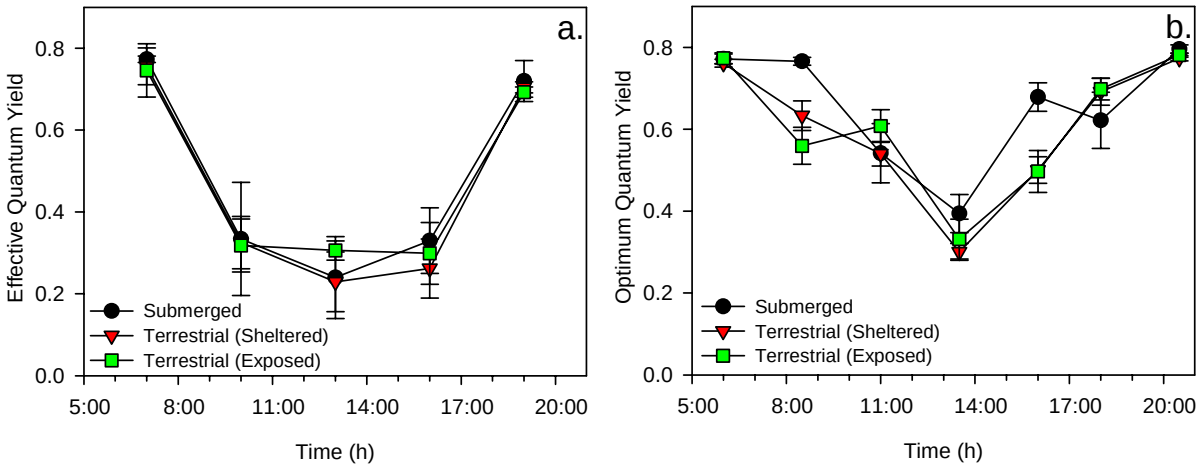
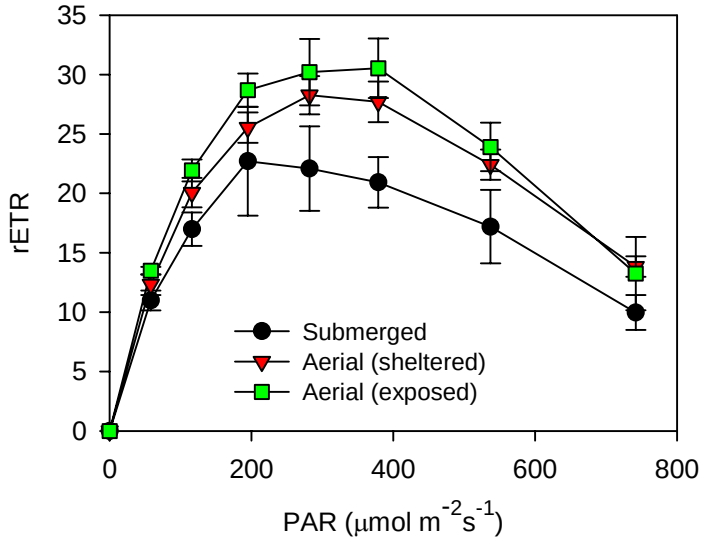


Figure 3-16. Diurnal Yield for submerged and aerial tissue (a) effective quantum yield (ΦPSII) and (b) dark-adapted optimum quantum yield (F_v/F_m) (b). Bars are standard errors (n = 10-20).



	α	ETR_{max} (µEq m^{-2} s^{-1})	E_k (µmol m^{-2} s^{-1})
Submerged	0.190	22.71	119.81
Aerial (sheltered)	0.212	28.26	133.03
Aerial (exposed)	0.232	30.52	131.30

Figure 3-17. Dark-acclimated relative Electron Transport Rate (rETR) for submerged and aerial tissue of *C. helmsii*. Bars are standard errors (n = 4).

The rETR decreased rapidly at PFD >400 µmol m^{-2} s^{-1} for submerged tissue, while the rETR of aerial tissue attained 400 µmol m^{-2} s^{-1} before decreasing. The rate of decline was sharpest for exposed aerial tissue and more gradual for submerged tissue. The

rETR was halved for each tissue type with PFD of 720 μmol $m^{-2}s^{-1}$ compared with respective $rETR_{max.}$ The dark-acclimated relative $rETR_{max}$ of submerged tissue was 6-8 μEq m^{-2} s^{-1} lower than aerial tissue. The onset of light saturation (E_k) also occurred earlier for submerged tissue at 119.81 μmol m^{-2} s^{-1} compared with >130 μmol m^{-2} s^{-1} for aerial tissue. No . difference was detected between exposed and sheltered sites for aerial tissue (Figure 3-17).

Measurements of rETR from RLC over the course of one day (Figure 3-18) indicate rapid and efficient use of low light and saturation at PFD over 500 μmol m^2 s^{-1}. ETR_{max} of aerial tissue was about 40 μEq m^{-2} s^{-1} at all times, regardless of site, until the last in the series at 18:00 h. At 18:00 h ETR_{max} of the sheltered site increased to 60 μEq m^{-2} s^{-1} compared with the exposed site, which remained at about 45 μEq m^{-2} s^{-1}. ETR_{max} was reached at progressively higher PFD until 15:00 h.

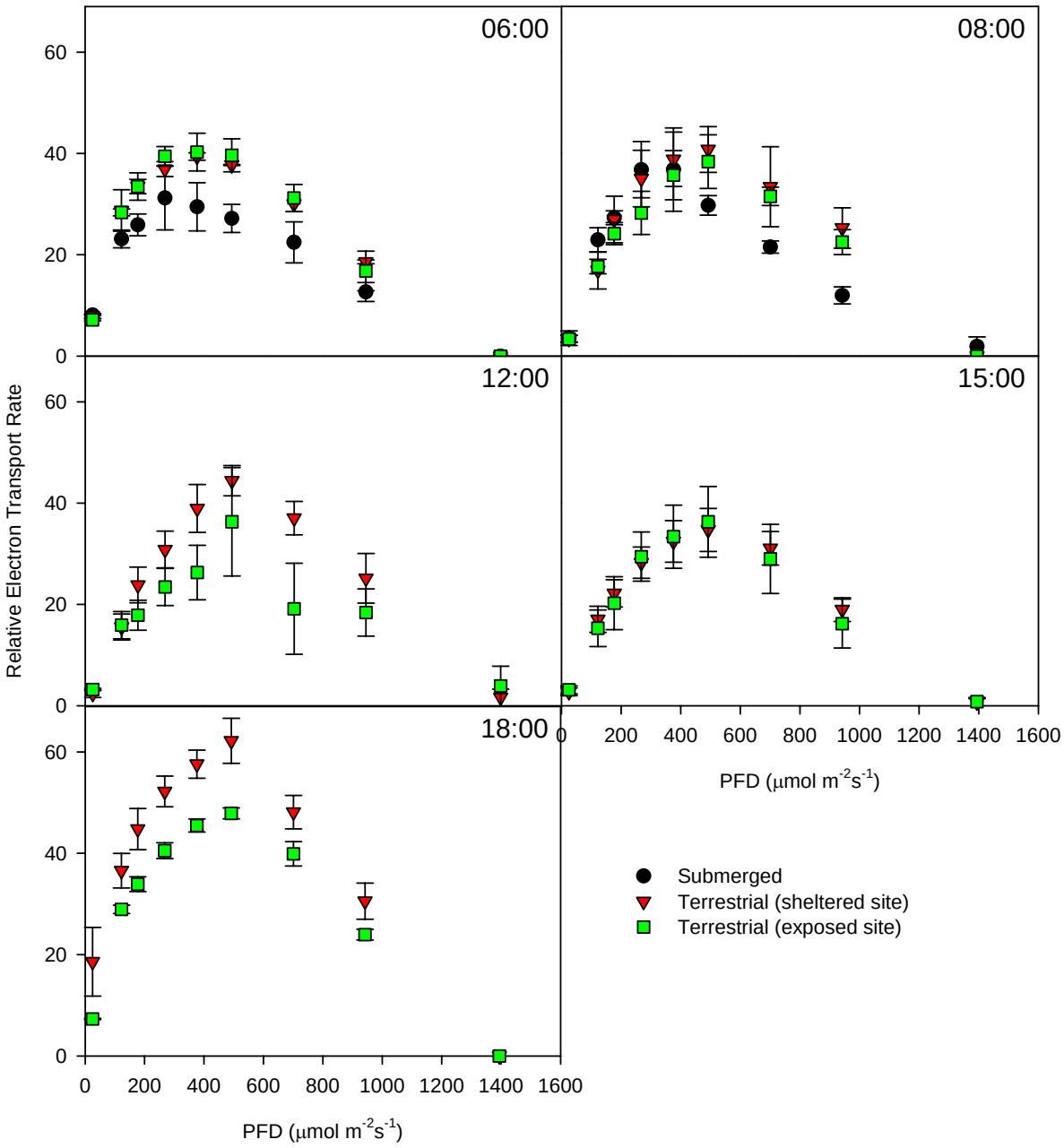


Figure 3-18. Diurnal rapid light response curves of *C. helmsii* at three locations over one day. Submerged (●), aerial sheltered site (▼), aerial exposed site (■). Bars are standard errors (n = 4-5). The water level decreased due to wind in the late morning, exposing material that was submerged. One point was removed from the midday reading of aerial exposed site as an outlier. Submerged parts became emergent with decreased water levels late in the day.

3.4 Discussion

3.4.1 *Impact of water regime change*

In controlled studies, CAM was initiated in *C. helmsii* within one day of submergence. This is consistent with reports that *C. helmsii* accumulates acid in submerged tissue (Newman and Raven 1995). Levels of accumulated acid were small and did not increase with continued submergence, yet *C. helmsii* retained photosynthetic ability in both aerial and submerged tissue. In partially-submerged shoots, contact with air prevented tissue becoming adapted for submerged photosynthesis, and may have suppressed CAM. The isotopic analysis shows a more-enriched $\delta^{13}C$ for submerged tissue and, recalling that *C. helmsii* is unable to use HCO_3^- (Newman and Raven 1995), this is consistent with CO_2 limitation and increased CAM activity (Raven *et al.* 1994).

Initiation of CAM within such a short time is likely to be due to increased diffusive resistance upon submersion, combined with the low proportion of $CO_{2(aq)}$ (<20%), causing a limitation in daytime CO_2, (Keeley and Rundel 2003). Levels of CAM did not increase over the 30 days, and indicate that there is no further acclimation to submergence. Levels of acidity of partially-submerged tissue were highly variable, perhaps because carbon was moved from submerged to aerial tissue.

Shoot water content was highest in submerged tissue, and reduced the chlorophyll content on a fresh weight basis. Yet, unlike obligate submerged leaves such as those of *Potamogeton* (Chapter 4), the submerged tissue had high total chlorophyll and a low chlorophyll *a*/*b* ratio, suggesting that submerged tissue was light-limited. This is probable as the mesocosms were based in England, and on this occasion there was less diffuse light compared with the high PFD and high diffuse light typical of field conditions in Australia.

There was higher background malate (20-40 μmol malate g^{-1} fw) in aerial and submerged tissue compared with the natural population (Section 3.4.2), probably associated with low fluctuations in day/night titratable acidity. On the other hand, PEPc activity was negligible. This may be due to inhibition by the relatively high malate, (post-translational regulation) (Winter 1980). Malate production might be stimulated in submerged tissue after it is utilised at the end of the light period.

Plants exhibiting strong CAM usually have low background acidity (e.g. *Clusia*: (e.g. Clusia Holtum and Winter 2005). Equally, relatively high background malate levels in *C. helmsii* appear to be associated with low fluctuations in day/night titratable acidity. Contrary to the significant malate levels, the activity of PEPc was negligible (<300 μmol NADH s^{-1} mg^{-1}) compared to other CAM plants (e.g. 13,000 μmol NADH $s^{-1}mg^{-1}$ for *Mesembryanthemum crystallinum)* (Davies 2005), and results from inhibition by high malate levels (Winter 1980). In addition to low CO_2, the diurnal decrease in malate over the day, coupled with decreased availability of CO_2 on submersion, may trigger PEPc activation. This is contrary to many terrestrial plants, where PEPc is activated by decreases in CO_2 concentrations (Dever *et al.* 1997). The assay measures the extractable PEPc, rather than activities *in vivo*, and so it is possible that the activation occurs under submerged conditions.

The combination of CAM on submergence and relatively high background malate suggests that *C. helmsii* switches between CAM and C_3 photosynthesis through submergence and emergence. Yet growth is often dominated by aerial tissue and the level of CAM observed in submerged tissue was low even in high light (max. 35 μmol g^{-1} fw, compared with other aquatics: 5-290 μmol g^{-1} fw) (Keeley 1999).

As a result of submergence, diffusion of gaseous CO_2 is restricted, light will be in excess for the balance of photosynthesis (assuming that tissue is not too deep in the water) and photorespiration is likely to increase. In these 'high' or 'excess' light conditions, CAM is adapted to maximise CO_2 uptake over the day-night cycle. In *Littorella*, the investment in the CAM is beneficial during growth at high light and low CO_2 conditions, but when growing at high CO_2 concentration or in low light, the benefits of CAM activity decrease (Madsen 1987).

Although the higher chlorophyll content in submerged leaves is consistent with a shade response, a number of indicators suggest light is in excess. Diel F_v/F_m of aerial tissue showed a midday decline associated with high PFD, attributed to ‘dynamic’ photo-inhibition (a short-term photo protection mechanism, Osmond 1994). Submerged leaves had a consistently lower F_v/F_m and higher NPQ compared with aerial leaves, supporting more substantial photo-damage (long-lasting photoinhibition, resulting in loss of functionality of PSII units). This was consistent with the RLC analysis, where ETR was consistently low and minimal in the late afternoon in submerged plants compared with aerial plants. If this latter time course represents the combination of CO_2 limitation and

chronic photoinhibition, the role of CAM in alleviating this response is evident during the late morning.

Most NPQ measurements were in the typical range for saturating PFD (0.5-3.5) (Maxwell and Johnson 2000). NPQ was usually the inverse of the maximum ETR, and because NPQ is linearly related to heat dissipation relative to the dark state, this implies that the tissues were stressed. The characteristic increase in NPQ in submerged leaves late in the afternoon is consistent with CO_2 limitation and excess light interception, typical of high-light leaves (Lovelock *et al.* 1998).

C. helmsii has low F_v/F_m and retains a capacity to dissipate excess light through NPQ, even under light-limited (submerged) conditions, because of CO_2 limitation.

3.4.2 Carbon acquisition in a natural population

Contrary to the mesocosm studies, neither submerged nor aerial leaves at Riverglades accumulated significant acid overnight. It is likely that wind action caused tissues to be alternately submerged and emergent, so that fluctuations of malate were not significant for carbon gain (net CO_2 uptake in air was possible). CAM activity in the amphibious macrophyte *Littorella uniflora* may depend on small environmental changes (Robe and Griffiths 1998), but the rapid water level changes at Riverglades do not initiate CAM.

C. helmsii retained photosynthetic ability in both aerial and submerged tissue, and all plants at Riverglades had a 'terrestrial' morphology even though lower parts were often flooded. Regular contact with air may have prevented tissue becoming modified for submerged photosynthesis, and may have suppressed CAM due to upper leaves retaining access to atmospheric carbon. There were large differences in $\delta^{13}C$, consistent with the effect of submersion on CO_2 limitation and/or CAM activity during tissue growth prior to the days when measurements were made (i.e. when water levels were less variable).

In both bulk material and soluble carbohydrates submerged tissue was 2.2‰ more enriched in ^{13}C than aerial tissue. As *C. helmsii* does not use bicarbonate (Newman and Raven 1995), the isotope signature is not enriched by source carbon and the difference probably is due to the comparative diffusive resistance in water *versus* air. The depleted carbohydrate signatures of submerged tissue at dawn and dusk are consistent with the overall bulk organic signals. However, the less negative carbohydrate $\delta^{13}C$ values at dusk are also consistent with reduced CO_2 supply, since at Riverglades there were

diurnal changes in pH during the day, and these would have reduced free CO_2 availability (see Chapter 4).

3.4.3 Hypotheses Revisited

1. Inducibility: CAM is initiated on submersion of aerial tissue.

The changeable environment at Riverglades may have prevented overnight acid accumulation, contrary to the mesocosm study and published reports that submerged tissue does accumulate acid (Newman and Raven 1995). Wind may have caused tissues to be alternately submerged and emergent, so that they did not exhibit CAM-like behaviour. In the experimental studies, CAM was initiated within one day of submergence. The carbon isotope composition of submerged leaves in both field and mesocosm were both consistent with CO_2 diffusion and/or CAM induction, and more experimentation would be needed to distinguish these contributions.

2. Flexibility: Submerged and aerial tissues have high background malate and PEPc, enabling a rapid switch to CAM on submersion.

The combination of CAM on submergence and relatively high background malate (15 μmol g^{-1} fw) suggest that *C. helmsii* switches between CAM and C_3 photosynthesis through submergence and emergence. Yet growth is often dominated by aerial tissue and the level of CAM observed in submerged tissue was extremely low, even in high light. According to Holtum *et al*. (2004; 2005), C_3-CAM plants with low overnight acid accumulation (<100 μmol gfw^{-1}) have an inclination to 'switch', and so are likely to have high background levels of malate, and conversely low background malate with high CAM-capacity (see also Kluge and Ting 1978). *C. helmsii* at Riverglades had low levels of background malate (<25 μmol gfw^{-1}). In some terrestrial C_3-CAM species (e.g. *Clusia*), CAM is indicated by a decrease in malate level at dusk, rather than merely by an increase at dawn (Zotz and Winter 1993). It appears that induction of CAM may not simply be a result of an increased capacity to accumulate acids, but exploitation of acids accumulated over a day.

In the mesocosm experiments there was more background malate (20-40 μmol malate g^{-1} fw) in aerial and submerged tissue compared to the natural population at Riverglades, where there was also low day/night titratable acidity. It appeared to be adequate to support the induction of CAM, and the high background levels of organic acids tend to support this hypothesis.

3. Photoinhibition: Photoinhibition in submerged CAM plants protects against absorption of excess light energy.

Light intensity is usually lower in water than in air, but submerged leaves showed consistently low F_v/F_m and ETR and, in particular, enhanced NPQ late in the day. It appears that the CAM activity was not sufficient to maintain photosynthetic electron transport throughout the day, and that photoinhibition occurred even though net photon flux was lower for the submerged leaves. These data therefore consistent with CO_2 uptake being limited by diffusion under water, with concomitant photoinhibition at lower light levels.

The amphibious *C. helmsii* is here shown to use CAM on submergence, even in environments where water levels fluctuate within 24 hours. This allows continued photosynthesis in habitats where water fluctuations prevent access to atmospheric CO_2. It is likely that stable conditions without photoinhibiting light are most favourable for growth and dispersal, and that the dominant spread of *C. helmsii* is by the aerial form. This is the likely reason for the invasion of *Crassula* in the UK. In Australia, the limited expansion of *C. helmsii* may be due to photoinhibition reducing photosynthetic output, while CAM triggered by high light and relatively high pH may allow it to persist in areas where it might otherwise die out. The physiological interactions underlying these differences are represented in Figure 3-19, below.

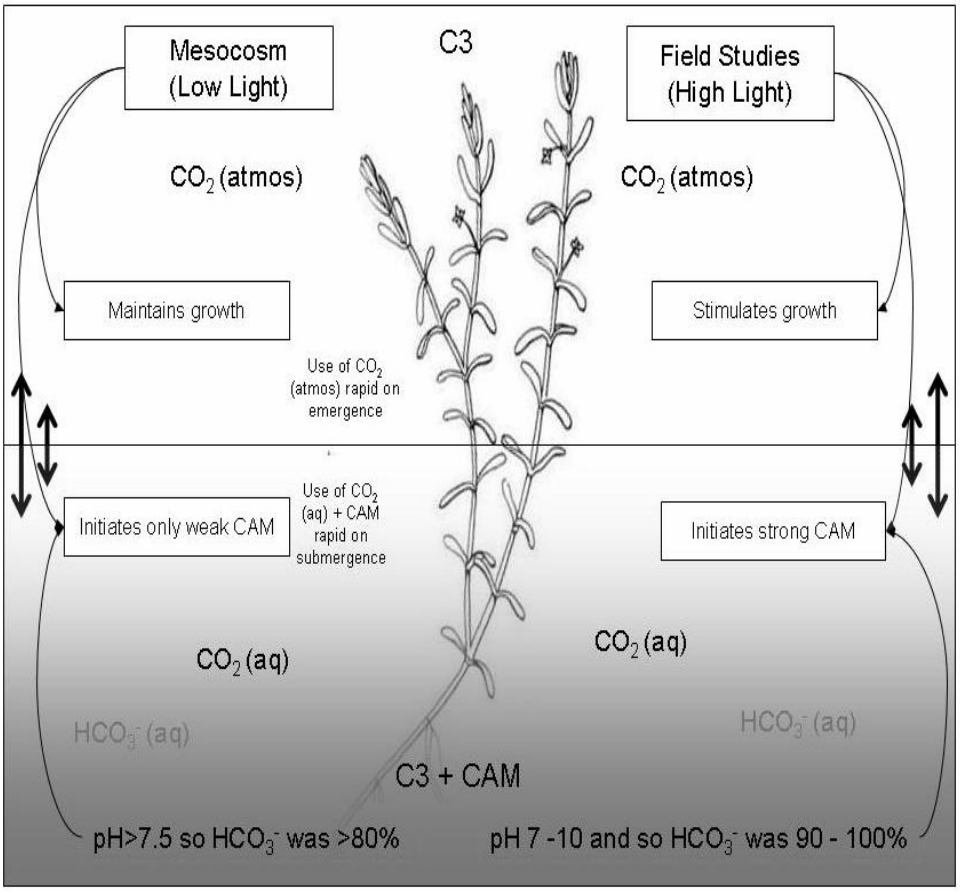


Figure 3-19. Conceptual model of the stimuli for C_3 and CAM pathways in natural and mesocosm-grown *C. helmsii*.

Chapter 4
FIELD STUDIES OF VALLISNERIA AMERICANA

4.1 Introduction

Vallisneria americana Michx. (ribbon weed) is a submerged aquatic macrophyte widespread throughout the world, including the Murray-Darling Basin (MDB). It is a dominant species in lentic and lotic habitats associated with the River Murray, South Australia and of interest because some members of the genus, notably *V. spiralis* Graeb., exhibit Crassulacean Acid Metabolism (CAM) (Keeley 1998; Webb *et al.* 1998). In these species, CAM is low-level, and its occurrence is seasonal and spatially patchy (Helder and van Harmelen 1982; Keeley 1998). The capacity for CAM enables the plants to offset carbon limitations that would be incurred by reliance on diffusive CO_2 (cf. Raven 1984). *V. americana* uses both CO_2 and HCO_3^- (Titus and Stone 1982; Keeley 1998; Webb *et al.* 1998), and is thereby able to maintain carbon uptake under changeable conditions (Bowes 1987). Although localised pH environments at the leaf surface allow use of HCO_3^- through conversion to CO_2 before uptake in some species such as Characeae (Prins *et al.* 1982), this has not been confirmed for *Vallisneria*.

Barko (1981) suggested that in *V. americana* irradiance and temperature are more important to seasonal growth, and particularly shoot biomass, than bicarbonate use in deep water. This was supported by Blanch (1998), who reported that changes in canopy morphology are associated with changes in turbidity, and that variations in relative growth rate are explained by irradiance. If the average midday irradiance between the water surface and sediment surface is greater than c. 30 μmol m^{-2} s^{-1}, then the plant will be able to maintain a positive growth rate. Blanch (1998) suggested that a high level of Rubisco activity may explain the shade tolerance of *V. americana* (Morris *et al.* 2004). Plants may survive for up to 3 months under irradiance deemed too low for growth (Blanch *et al.* 1998; Morris *et al.* 2004). *V. americana* can also tolerate extended periods with little photosynthesis (Madsen *et al.* 1991). Although the implications for low light due to turbidity have often been investigated for aquatic plants (Goldsborough and Kemp 1988; Hootsmans *et al.* 1996; Cenzato and Ganf 2001; Bunn *et al.* 2003; Mommer *et al.* 2005), it may be the variable and occasional high intensity of light in Murray wetlands, combined with variable water chemistry, that drive photosynthetic path diversity in *V. americana*.

Under optimal conditions, the carbon used by *V. americana* in basal and apical leaf sections is mainly CO_2. The capacity to switch to HCO_3^- depends on a number of factors, including water flow and stand density.

In this chapter, observations of fluorescence, carbon isotope fractionation in bulk material and assimilated carbon and sugars are used to explore mechanisms for carbon acquisition in *V. americana*. The objective was to determine the mechanisms governing CAM and bicarbonate use under field conditions like those prevailing in the Lower Murray. Further exploration of carbon acquisition and assimilation strategies in *V. americana* under a controlled environment is described in Chapter 5.

These investigations were guided by three hypotheses:

1. **The CAM pathway is used by *Vallisneria americana* in seasonally-isolated pools (typically shallow with high temperatures, variable light intensity and depleted in free CO_2 due to high pH). In contrast, there is low or negligible CAM in lakes and wetlands that retain connectivity with the parent river, and are physically and chemically more stable.**

Since the discovery of 'CAM-like' CO_2 uptake in *Vallisneria* (Willmer and Dittrich), *Vallisneria* spp. variously have been credited with a C_3, C_4, CAM and C_3-C_4 intermediate physiology (Helder and van Harmelen 1982; Keeley 1998; Webb *et al.* 1998; Sage *et al.* 1999). In *V. americana*, low-level, spatially heterogeneous CAM has been linked to 'vernal pools' (Keeley 1998) and seasonal fluctuations (Keeley 1998; Webb *et al.* 1998), as in *Isoetes howellii* (Isoeteaceae) and other aquatic CAM species (Keeley 1998). Seasonally-isolated pools develop where wetlands are flooded and subsequently isolated; they are prone to high light and wide diel fluctuations in pH, oxygen and temperature (for example, Suter *et al.* 1993)

In *V. spiralis,* diel changes in acidity have been confirmed (Webb *et al.* 1998), and ^{14}C labelling has shown that dark malate fixation is seasonal, albeit variable between plants. Helder (1982) concluded that *V. spiralis* was a C_3 plant, but remarked that the high level of C_4 acid production and malate accumulation were suggestive of CAM plants.

CAM in terrestrial species is often considered a stress response to maintain a positive carbon balance (Borland *et al.* 1996; Martin 1996). Diurnal fluctuations in seasonally-isolated wetlands may induce CAM in *V. americana* to help maintain carbon balance. Wetlands which have lost connectivity with the channel are typically shallow, warm,

high light environments with changeable water chemistry (Keeley 1998). In such wetlands it is proposed that CAM will be comparatively high, as for *Isoetes* (Keeley 1999). This is in contrast to wetlands that remain connected to the river and have a greater level of mixing, hence moderate fluctuations in water chemistry, light and temperature. Keeley (1998) noted that alkaline pools generally lack CAM species due to high pH and small diel changes in pH and CO_2 availability, and also observed that soft water lakes have a higher biomass of CAM species such as *Isoetes* and *Littorella*, due to greater diel changes in CO_2 and O_2.

2. In seasonally-isolated pools, where temperature, light and pH are often high, bicarbonate (rather than free CO_2) will be the principal carbon source.

Inorganic carbon in water exists as dissolved CO_2, bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}), in proportions determined, *inter alia,* by pH, temperature, the rate and extent of biological activity and degree of turbulence (e.g. Keeley and Sandquist 1992). Inorganic carbon supplies rarely are limiting where there is a low PFD (Johnston *et al.* 1992), but submerged plants in Murray wetlands are often exposed to variable and occasionally very high PFD (Thompson 1986).

Many macrophytes and fresh water algae can assimilate carbon from HCO_3^- until the pH of the water exceeds 9.0. At this point, the photosynthetic rate is restricted to 10% of that at saturating CO_2 concentrations (Allen and Spence 1981). Fluctuating ratios of HCO_3^- and CO_2 diurnally and seasonally complicate studies on aquatic plants. CO_2 and HCO_3^- in water bodies can change rapidly and aquatic macrophyte acclimation is fast; initiated in days or hours (Maberly and Madsen 2002), sometimes several months (Sand-Jensen 1987). In wetlands associated with the Lower River Murray, pH is rarely <6.1 but frequently reaches 9.4 (Tibby *et al.* 2003), temperature can reach 40ºC in shallow pools and salinity is high and variable, depending on source water inflows. Most wetlands in the MDB are alkaline (pH 7.3-9.7) due to the presence of bicarbonate (Scholz *et al.* 1999; Scholz *et al.* 2002). In water of pH 8.8, over 80% of the inorganic carbon is present as bicarbonate (Kirk 1983).

3. Bicarbonate use is independent of the photosynthetic pathway

Phosophoenyle Pyruvic Carboxylase (PEPc), the primary carboxylating enzyme during CAM, uses bicarbonate. Despite this, aquatic CAM species are suggested to lack the capacity for bicarbonate uptake (Keeley 1998), including *Isoetes lacustris* (Sand-Jensen 1987), *I. macrospora* and *Littorella uniflora* (Maberly and

Spence 1983; Maberly and Spence 1989), *Crassula helmsii* (Newman and Raven 1995) and *C. aquatica* (Keeley 1998). Bicarbonate uptake is usually seen as an alternative to CAM, yet both CAM and HCO_3^- carbon-concentrating mechanisms are used by *V. americana* (see (1) and (2) above).

For *V. americana* to make best use of carbon and light resources, a range of mechanisms is used for acquiring and assimilating carbon. Gradients of CO_2 from the leaf base to the tip and reverse gradient of O_2 combined with high CO_2 in leaf lacunae are suggestive of carbon drawn from the sediments (LaZerte and Szalados 1982; Kimber *et al.* 1999), although this has been disputed (Locy *et al.* 1983). Various combinations of photosynthetic pathways are apparent (Keeley 1998; Webb *et al.* 1998; Sage *et al.* 1999), while bicarbonate uptake is well-documented (eg. Titus and Stone 1982; Keeley 1998; Webb *et al.* 1998). It is likely that these carbon-uptake mechanisms do not 'switch', but represent a continuum depending on water chemistry and light.

4.2 Methods

4.2.1 Species description

V. americana Michx. (variously ribbon weed, strap weed, eelgrass, tape grass, water celery) is a member of the monocot family Hydrocharitaceae, and one of the most common aquatic plants in the southern MDB. In the Murray it forms dense, mono-specific stands with a near-continuous canopy in still and flowing (Blanch *et al.* 1998), turbid and clear water (MB, pers. obs.). Abundance, however, is strongly favoured by hydrological stability (Roberts and Marston 2000). Its range and abundance probably have increased with the construction of weirs and the intensification of flow regulation in the 20th century (Walker 2006). It is a perennial, submerged aquatic plant with ribbon-like leaves arising from a rosette on a short vertical stem. The leaves have rounded tips and raised veins; their length depends on growing conditions (MB, pers. obs.). In shallow wetlands they may be <10 cm, but in deep water they may attain 5 m. Net growth is limited to turbidities below 80 Nephelometric Turbidity Units (NTU) (Blanch *et al.* 1998). *V. americana* typically occurs at 1-2 m depth in the Murray, but flows from the tributary Darling River may produce turbidities of 100±600 NTU and growth is then limited to <1 m (Walker *et al.* 1994).

Growth is seasonal, and the canopy develops from spring through to late summer before dying back in early winter (Briggs and Maher 1985). The plants grow for several years before flowering (Jacobs and Frank 1997), and then flower through summer. They are dioecious (Aston 1973), with single white female flowers growing to the water surface on spiral stalks, and male plants that produce a head of numerous flowers on a short basal stalk. Dispersal to new habitats is *via* seed or plant fragments, but expansion within existing habitats is mostly vegetative (Roberts and Marston 2000).

There is some confusion regarding the regional status of the species, as the names *V. gigantea* Graeb. and *V. spiralis* L. were used until a decade ago (Black 1980; Lowden 1982; Jacobs and Frank 1997), and the latter still has some currency for resource managers. The Murray species probably all *V. americana* (Sainty and Jacobs 1994; Jacobs and Frank 1997; Romanowski 1998), while *V. nana* R. Br. occurs north of the MDB (Jacobs and Frank 1997).

4.2.2 Field sites

Three wetlands in the Lower River Murray were used to explore the carbon acquisition mechanisms exhibited by *V. americana*. A large population of *V. americana* at Brenda Park Lagoon was used to investigate the extent of CAM during summer 2003-2004, during a controlled draw-down of water level. In 2004-2005, Riverglades and Banrock Station wetlands both had extensive *V. americana* populations, and both retained water during measurements. Banrock and Riverglades wetlands were selected due to their differing water chemistry, to isolate and understand bicarbonate use and CAM. Riverglades had a high pH (>8.3) compared with Banrock (<8.3).

Brenda Park Lagoon

Brenda Park Lagoon is a temporary wetland above Lock 1 and 10 km downstream of Morgan, South Australia (34º04’56”S, 139º40’47”E). It is part of the Brenda Park Scott Creek wetland complex (870 ha), extending for approximately 10 river kilometres along the western bank of the Murray (Figure 4-2). It has a high regional and state-wide conservation rating. The lagoon has a managed wetting and drying cycle. *Phragmites australis* and *Muehlenbeckia florulenta* fringe the lagoon, and the submerged vegetation consists of *V. americana*, *Myriophyllum* spp. (water milfoil) and *Potamogeton crispus* (curly pondweed). During summer 2003-2004, *V. americana* was the dominant species.

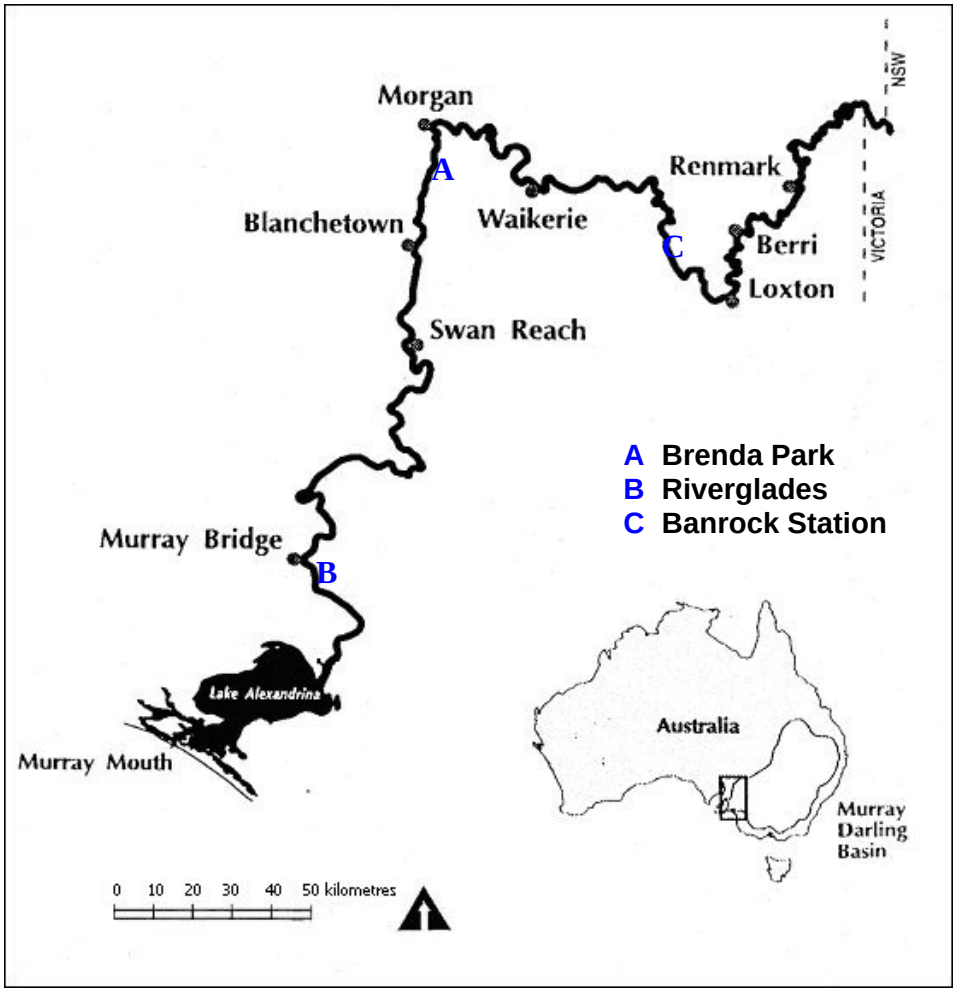


Figure 4-1. Field sites "Brenda Park" near Morgan, "Banrock Station" near Overland Corner and "Riverglades" between Mannum and Wellington. Source: map base from SA Department of Environment & Heritage

Brenda Park was filled to weir-pool level in November of 2002, and remained wet through 2003. A managed draw-down of 80 cm occurred from November 2003 until the lagoon was dry in February 2004. Dense populations of female *V. americana* (1-4 m^2) occurred to a maximum depth of 80 cm. Plants flowered in December 2003. Although pH was not measured, it is predicted that the dense bicarbonate-using plant material would have been pushing the pH beyond 10. The high pH, combined with high temperatures and little mixing, would have ensured that bicarbonate was the main form of carbon available.

Five submerged populations of *V. americana* (each 2-4 m^2) within Brenda Park Lagoon were assessed during December 2003. Four of these were re-assessed in early January, but the fifth had become exposed by falling water level. By late January, the water level

had dropped c. 1 m and had exposed all previous sites, so three additional populations that had not been exposed were assessed. Physiological measurements were undertaken on leaf tissue at 5-10 cm depth (total length 10-20 cm). This depth corresponds to the tip sections at the sites described below.

Figure 4-2. Brenda Park Lagoon, adjacent to the River Murray. Populations of *V. americana* were surveyed along a 100-m stretch of the lagoon. Source: Image ©2005Digitalglobe

Riverglades

Riverglades is situated upstream of Murray Bridge (35º05'52"S 139º17'50"). Naturally it would have been a temporary wetland with seasonal wetting and drying (1 flood in 2-3 years: (Jensen *et al.* 1999). Wind action during sampling altered the water level by up to 0.6 m (MB pers. obs.). Submerged vegetation consisted of a seasonally-variable

combination of *V. americana*, *Myriophyllum* spp. and *Potamogeton crispus* and *P. pectinatus*. *P. crispus* was dominant in late 2004, but was damaged by ducks early in 2005 and was virtually absent in February 2005. Male and female *V. americana* occurred grom 0.4-1.5 m depth, singly and in large populations throughout the wetland. A pH measurement >10 was observed during 2004, and attributed to the high biomass of bicarbonate-using plants. Riverglades therefore provided a system to investigate the bicarbonate use of *V. americana*. Five populations along the axis of the wetland, each c. 100 m apart, were assessed in December 2004 and January 2005.

Figure 4-3. Riverglades wetland, upstream of Murray Bridge. Note that the wetland has only one connection with the River Murray. Source: Image ©2005Digitalglobe

Banrock Station

The Banrock Station Wetland Complex is on the Murray floodplain downstream of Kingston-on-Murray (34º11'38"S 140º20'15"E). It is a *Wetland of International Importance* under the Ramsar Convention. In 1925, Lock 3 was installed in the river

adjacent to the complex. The weir increased the river water level upstream by 3 m and radically changed the hydrological regime of the wetland, permanently inundating areas that previously were seasonally-flooded. In 1992, semi-natural and intermittent wetting and drying cycles were reinstated. The wetland is surrounded by *Muehlenbeckia florulenta, Phragmites australis* and *Typha domingensis*. Open-water plants include sparse *Vallisneria americana* and *Potamogeton* spp and *Myriophyllum* spp..

A near-neutral, stable pH due to sparse vegetation and wind mixing provided a system to investigate bicarbonate- and CO_2-use in *Vallisneria* during summer 2004-2005. Five sites c. 2 m^2 and 3 m apart were assessed in December 2004 and January 2005. These were on either side of a boardwalk at the north-western end of the complex.

Figure 4-4. Banrock Station wetland complex, showing *V. americana* sites. Source: Image ©2005Digitalglobe

4.2.3 General methods

At Riverglades and Banrock Station, five leaves from five small populations of *V. americana* were divided into three sections for analysis, labelled "tip" (10 cm from the

tip or, where the leaf was prostrate on the surface, 10 cm from where it reached the surface), "mid" (middle section of leaf) and "base" (10 cm from the base of the plant). Leaf samples and fluorescence measurements were obtained with difficulty: (1) material was hard to access, samples were obtained using chest-high waders at Brenda Park and Banrock but a canoe was needed at Riverglades, (2) there was difficulty in finding the same leaf at different times (thus, leaves were not paired) and (3) changing leaf orientations caused problems with fluorescence measurements.

Water chemistry and light

Photon Flux Density (PFD) was logged at 10-min intervals between dawn and dusk, using a Li1000 data logger and sensor. For most sampling periods, PFD was measured in air at the surface and, where possible, 10 cm below the surface, using a calibrated underwater LiCor light sensor. On clear days, PFD was also measured at varying depths to determine light attenuation for the given day. Temperature was measured at various depths in plant stands and in open water, using thermocouples, and logged with a 1200 series, multi-channel Squirrel Logger (Grant Instruments (Cambridge), UK). Conductivity was measured with a WP-84 meter (TPS, Springwood, Australia) calibrated daily. Dissolved oxygen at Brenda Park was measured using a WP-82Y meter and YSI sensor (TPS, Springwood, Australia).

Fluorescence

In situ fluorescence measurements were made (see Chapter 2) every 3 hours between dawn and dusk. Light-acclimated Rapid Light Curves (RLC) and photochemical efficiency (ΦPSII) were determined at all sites, and dark-acclimated optimal photochemical efficiency (F_v/F_m) also was determined at Brenda Park. Three sections of each leaf were analysed (tip, middle, base). At Brenda Park, after each light-acclimated set of measurements, populations were dark-acclimated using black plastic bags for 20 min and 5-10 optimum photochemical yield (F_v/F_m) values were recorded. The fibre optic of the mini-PAM, however, determined the depth of *in situ* fluorescence measurements. Where depth was >1 m (Riverglades and Banrock), only tip and mid sections of the leaves were assessed by fluorometry.

Titratable acidities

Analysis of titratable acidities was undertaken on each of tip, middle and base of 5 replicate leaves per population, collected 30 min prior to sunrise and 30 min after sunset. Leaves were blotted dry, wrapped in aluminium foil and immediately frozen in

liquid nitrogen. Samples were transported in liquid nitrogen and stored at -4°C. Analyses were according to procedures in Chapter 2.

Chlorophyll

Chlorophyll analysis was undertaken on five replicates of each section per population collected at midday and stored in site water in the dark until return to the laboratory. Analysis was undertaken on the day following collection according to Porra (1989), following the procedure in Chapter 2.

$\delta^{13}C$ of plant tissue

Leaf tissue for organic $\delta^{13}C$ material included tip, middle and base of five replicate leaves per population collected at midday, rinsed in RO water, blotted dry and stored in paper bags during transport. Leaf sections were dried for 48 h at 40ºC and processed according in Chapter 2. Similar samples were collected for carbohydrate $\delta^{13}C$ analysis 30 min prior to dawn and 30 min after dusk. Leaf sections were rinsed in RO water, blotted dry, wrapped in foil and frozen in liquid nitrogen. Samples were transported in liquid nitrogen and stored at -20°C pending analysis according to Chapter 2.

4.2.4 pH drift under field conditions

On sampling days at Banrock, *in situ* incubations were undertaken with *V. americana* sections in small tubes. At 09:00 h, 2 L of water was collected and filtered with Whatman #1 paper. Filtered water was analysed for pH, temperature, conductivity and alkalinity according to Section 2.7.1, and five 8-mL replicates of filtered water syringed into 12-mL Exetainers for analysis of DIC and $\delta^{13}C$.

Approximately 0.5 g of tip, middle and basal leaf tissue was collected from five plants at 09:00 h and rinsed in deionised water. The 0.5-g sections were placed in 15-mL plastic tubes with 10 mL filtered water. Tubes were floated on the surface for 6 h, half under black cloth and half under natural sunlight. The pH was measured and 8 mL of remaining water collected and stored in Exetainers for analyses of DIC and $\delta^{13}C$ (Section 2.5.3). Leaf sections were blotted and stored for titratable acidity analysis according to Section 2.1.

4.2.5 Statistics

Analyses of variance (ANOVA) employed Minitab Statistical software (Release 14.1, ©Minitab Inc). Normality and equal variances were confirmed using Kolmogorov-

Smirnov and Bartlett's Test or Levene's Test, respectively. *Post hoc* comparisons were by the Tukey method. Alpha (Type I error rate) was set to 0.05 unless otherwise stated.

4.3 Results

The Results and Discussion are in three parts: (1) characterisation of CAM in *V. americana* (Brenda Park 2003-2004); (2) CAM and bicarbonate-use (Riverglades and Banrock 2004-2005); (3) CAM and bicarbonate-use in incubations (Banrock Station 2005).

4.3.1 Characterisation of CAM

Water characteristics

Brenda Park Lagoon had shallow, still water and high daily temperatures (summer water temperatures reached 38ºC at 16:00h).

Titratable acidity

Overnight acid accumulation was extremely low and patchy at Brenda Park in 2003 (Figure 4-6). Background acidity was negligible (<5 μmol H^+ g^{-1} fw). Average overnight H^+ accumulation was 19.7, 21.7 and 0 μmol H^+ g^{-1} fw on 5, 18 December 2003 and 2 January 2004, respectively. The pattern was inconsistent, however, as plants in four populations had significant overnight accumulation on both December dates (average 61, 77.8 μmol H^+ g^{-1} fw), and a patch within 20 m of other populations had no overnight accumulation at any time. All plants were male, and subject to a similar light regime. The January sampling used different populations of *V. americana*, as those sampled previously were exposed and desiccated. No overnight acid accumulation was recorded in the new populations. Leaves had browned and the water depth was <10 cm.

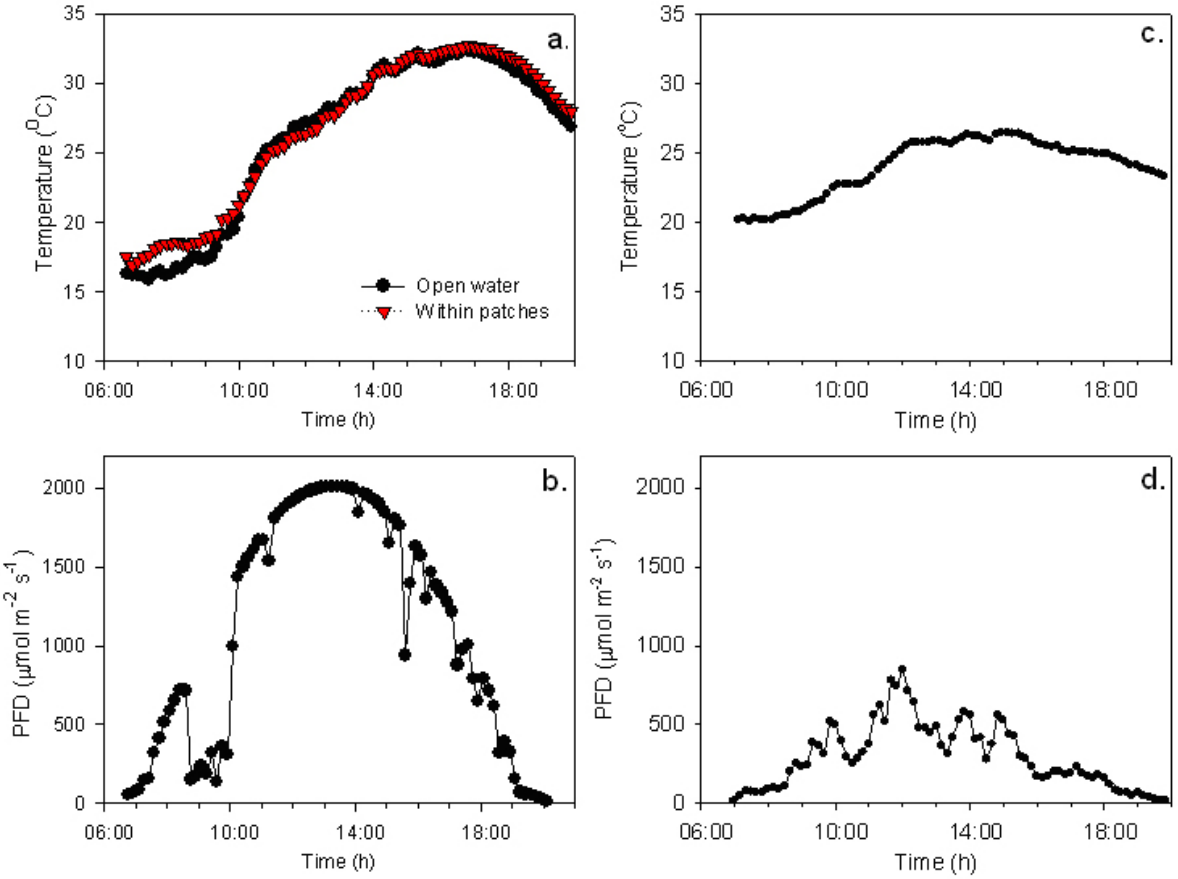


Figure 4-5. Water characteristics in *V. americana* populations and in open water and PFD at Brenda Park on 6 December 2003 and 6 January 2004. (a, c) diel temperature, (b, d) PFD at the water surface.

Drying Down

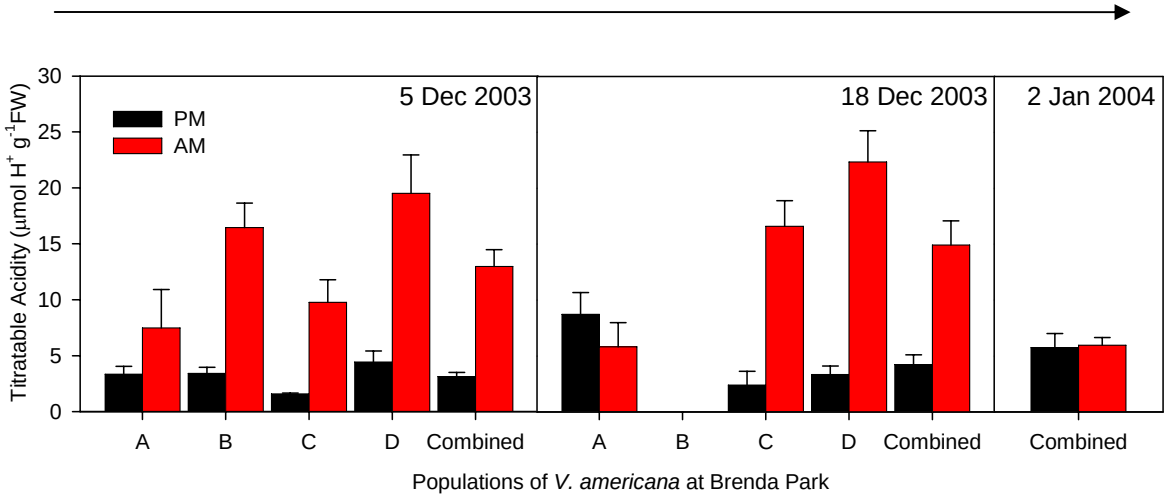


Figure 4-6. Titratable acidity at dawn and dusk in *V. americana* leaves at Brenda Park over three periods. Populations (A-D) are shown to indicate the patchiness of sites within sampling periods.

Fluorescence

Both dates in December indicated high PFD, with similar PFD on days preceding measurements, and temperatures increased from 15ºC to 34ºC (Figure 4-5). The dark-adapted photochemical efficiency (F_v/F_m) assessed in the hour before dawn was low, between 0.4-0.6 for most populations (non-stressed F_v/F_m usually is c. 0.8). Further assessment of ΦPSII every 3 hours indicated a sharp decrease after sunrise. This extreme minimum (0.03-0.08) was followed by a gradual recovery in ΦPSII from 11:00 h to exceed the pre-dawn figure (c. 0.6) by 19:30 h—this occurred on both dates in December

Figure 4-7). Population A had a dark-adapted F_v/F_m of only 0.25, significantly lower than other sites ($F_{4,4} = 7.65$, $P < 0.05$; $F_{4,4} = 4.42$; $P = 0.010$ for 6, 18 December, respectively). The pattern of recovery, however, was the same.

Measurements on 18 January were on different *V. americana* populations under low PFD and lower temperatures due to cloud cover. F_v/F_m was higher, indicative of lower light from the previous day, and the decrease in ΦPSII was less dramatic. There was a gradual recovery over the afternoon, similar to previous measurements.

The gradual recovery in ΦPSII was investigated by undertaking subsequent dark acclimated F_v/F_m during the day. F_v/F_m showed dramatic recovery at each site at 11:00 h after 20-min dark acclimation (Figure 4-8).

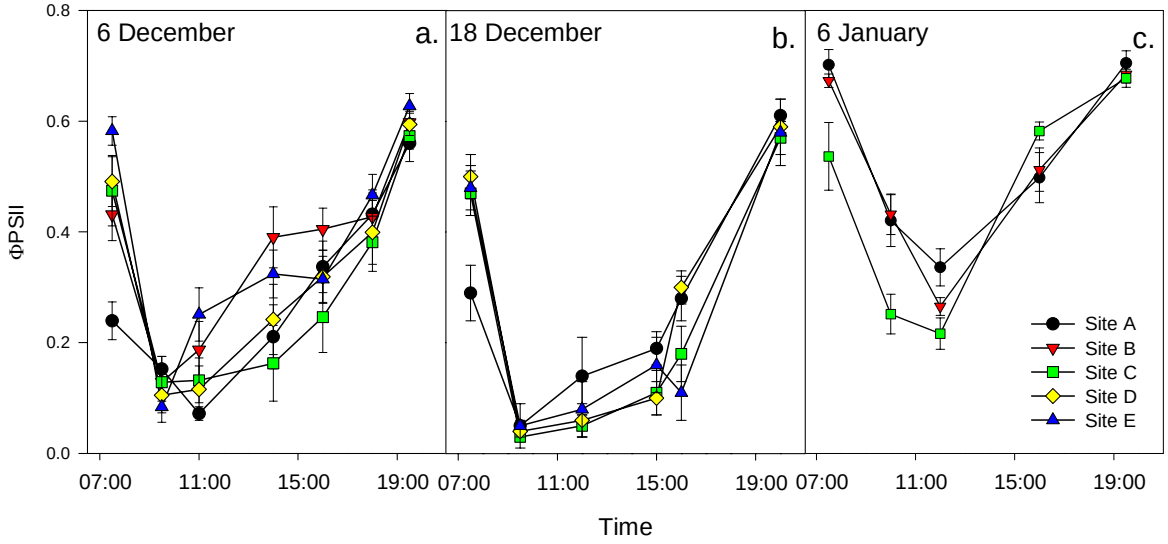


Figure 4-7. Diel effective quantum yields at Brenda Park on (a) 6 December 2003, (b)18 December 2003 and (c) 6 January 2004. Sites in 6 January 2003 are not the same as those in December.

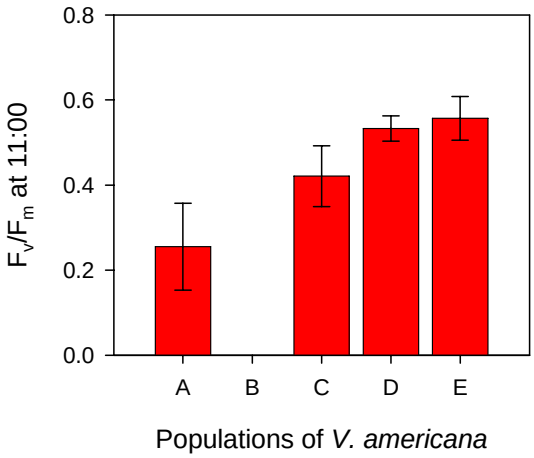


Figure 4-8. Dark-acclimated (20 min) F_v/F_m at 11:00 h for populations of *V. americana*.

4.3.2 CAM and bicarbonate use at two sites

Populations of *V. americana* at Banrock and Riverglades grew at water depths of 1-2 m. Both sites were connected to the main channel, and thus maintained a constant water level, but the Riverglades inlet was downstream and water turnover was minimal compared to Banrock. Both sites maintained stable water temperatures (Banrock 23-26ºC, Riverglades 24-25ºC). The most striking difference between sites was the pH, which was low and constant at Banrock (7.5; min 7.13, max 7.68), but was high and diurnally variable at Riverglades (min at dawn 9.1, max at 14:00 h 10.2).

Cloud cover was high on the first day of analysis at Banrock, but the day prior to measurements had a reasonably clear morning (Table 4-1). The day of 11 February was clear with little cloud cover, and 11 December and 8 February were overcast.

Table 4-1 Climatic data including rainfall, air temperature and cloud cover for day of analysis (day prior to analysis in brackets).

Site	Date	Rainfall (mm)	Temperature (ºC)		Cloud Cover (oktas)	
			Max	Min	09:00 h	15:00 h
Banrock	4 Jan 2005	4.6 (0.0)	26.4 (34.5)	15.4 (19.4)	6/8 (0/8)	6/8 (5/8)
	11 Feb 2005	0.0 (4.0)	27.1 (26.7)	12.5 (13.3)	1/8 (0/8)	1/8 (2/8)
Riverglades	11 Dec 2004	52.5 (1.2)	15.8 (17.4)	24.5 (27.6)	7/8 (3/8)	8/8 (8/8)
	8 Feb 2005	0.6 (0.0)	15.0 (16.7)	24.6 (31.4)	8/8 (4/8)	5/8 (4/8)

Titratable acidity

Due to inconsistent CAM at Brenda Park and the longer leaves at Riverglades and Banrock compared with Brenda Park, each leaf was divided into three parts: tip, middle and base. The background acidity was up to three times higher at Riverglades and Banrock compared with Brenda Park. Background acidity was the same for all populations at Riverglades ($F_{4,3} = 1.59$, $P = 0.22$) and there was no difference between the tip, mid and base of leaves ($F_{2,3} = 2.14$, $P = 0.14$) (Figure 4-9a). Conversely, Banrock had higher background acidity; there was a slight increase towards the tips during January, but this was not consistent (Figure 4-9a).

Acid accumulation (ΔH^+) data in Figure 4-9 are presented in two ways: (i) variation between base, middle and tip across all populations (a-c), and (ii) population variation within each site (d-f). As the background acidity was the same for all leaf positions (see above), absolute values at dawn were used for statistical analysis rather than ΔH^+.

At Riverglades, position on the leaf sampled affected the amount of overnight acid accumulation ($F_{2,35} = 4.96$, $P = 0.013$) (Figure 4-9a). Accumulation in tips was 58.3 μmol H^+g^{-1} fw, and the bases of leaves accumulated 24.9 μmol H^+ g^{-1} fw. Further, when each population is considered separately, dawn acidity at Riverglades varied between populations ($F_{3,34} = 12.02$, $P = 0.000$) (Figure 4-9d), but the plants still had progressively more accumulation towards the leaf tips ($F_{2,34} = 12.82$, $P = 0.000$) (Figure 4-9b). A significant interaction, however, indicates that the pattern of accumulation is different for each site ($F_{2,34} = 4.22$, $P = 0.005$).

At Banrock, there was a consistent, highly significant increase in acid accumulation from the base to the tip of the leaf on both dates (4 January: $F_{2,42} = 46.45$, $P = 0.000$; 11 January: $F_{2,42} = 17.73$, $P = 0.000$) (Figure 4-9b-c). Accumulation at the base of the leaves was consistently low (but for one data point) at 29±5 μmol H^+ g^{-1} fw across both sites and at all time periods, accumulation in the tips of *V. americana* leaves was high (114±8 μmol H^+ g^{-1} fw). Similarly, there was consistent accumulation in base and tip positions among Banrock populations, and mid positions sampled fluctuated between the two extremes. Population A, however, did not show any difference in accumulation along the leaf. Although the background amount of acid is higher at Banrock compared with Riverglades, the extent of accumulation was comparable.

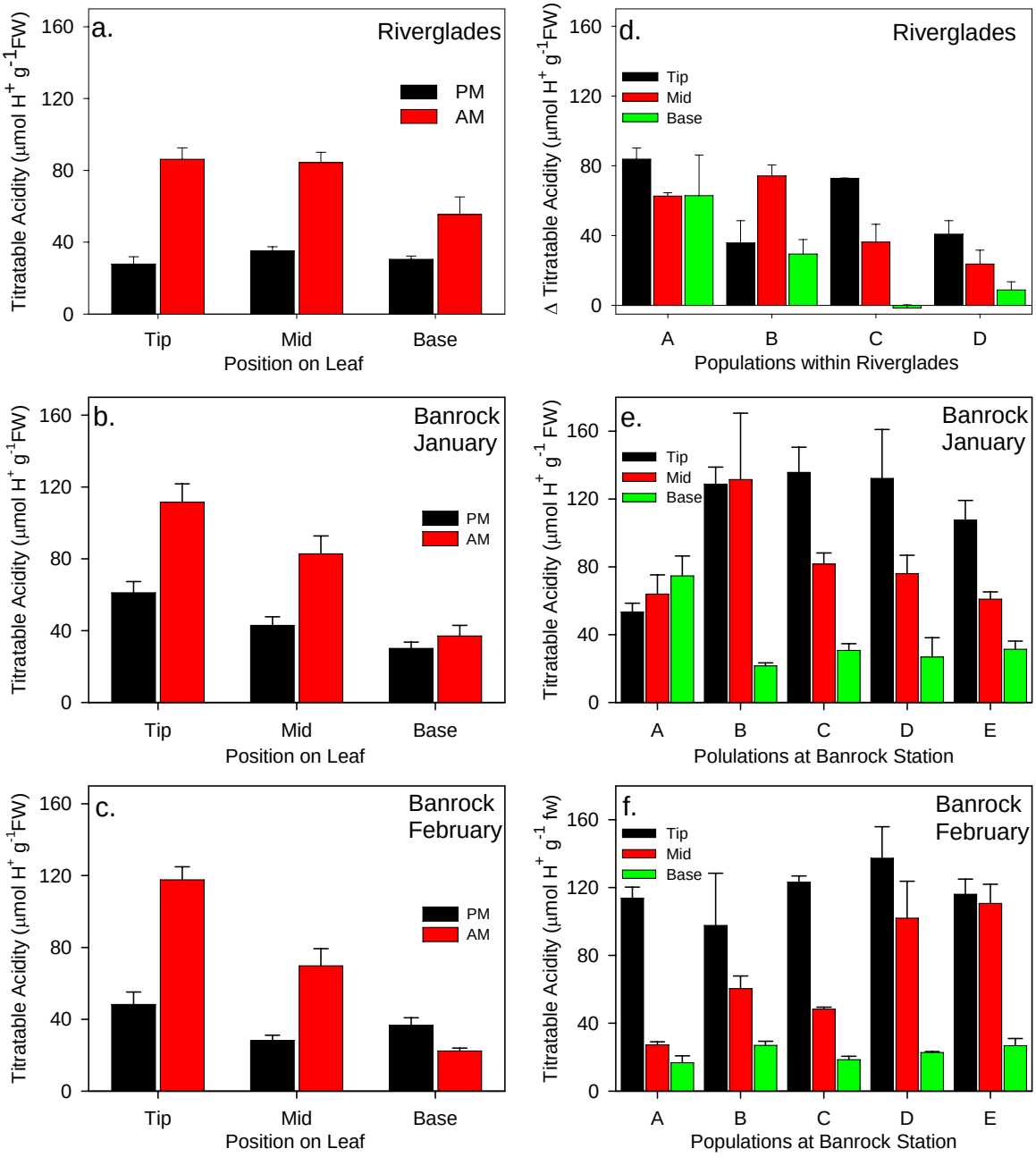


Figure 4-9. Titratable acidities for Riverglades and Banrock showing (a-c) pooled acidity differences between tip, middle and base of leaves for each site and (d-f) differences in dawn titratable acidity between populations. A-D represent separate populations of *V. americana* within sites.

Light response

There was higher $rETR_{max}$ at Banrock (20-30 µeq m^{-2} s^{-1}) compared with Riverglades (12.5-13.5 µeq m^{-2} s^{-1}) (Figure 4-10). Further differences were exhibited between positions on the leaves for both sites. At Riverglades there was no difference between maximum rETR of tip and mid sections of leaves, but both are double the rates of basal

sections. Total water depth was c. 40 cm, so tip measurements were at 10 cm depth, mid at 20 cm depth and base at 30 cm depth.

At Banrock, tip sections had a higher $rETR_{max}$ compared with mid sections. Total water depth was c. 100 cm, thus tip measurements were at 10 cm, mid at 40 cm (basal sections were not measured due to the limited length of the mini-Pam fibre optic). Pre-dawn measurements were significantly lower than midday measurements, highlighting the induction effects of light.

At both Riverglades and Banrock there was a steep initial rise in rETR. All Riverglades measurements were saturated by 50 µmol m^{-2} s^{-1}. At Banrock, however, tip sections saturated at 400 µmol m^{-2} s^{-1}, while mid sections gradually increased in response to increasing PFD, and the fitted curve suggests saturation after 1000 µmol m^{-2} s^{-1}. A decrease in rETR with PFD (photoinhibition) was not apparent in tip and mid sections at Riverglades, but occurred in basal sections after 400 µmol m^{-2} s^{-1}. There was no indication of photoinhibition in any sections at Banrock up to 1000 µmol m^{-2} s^{-1}.

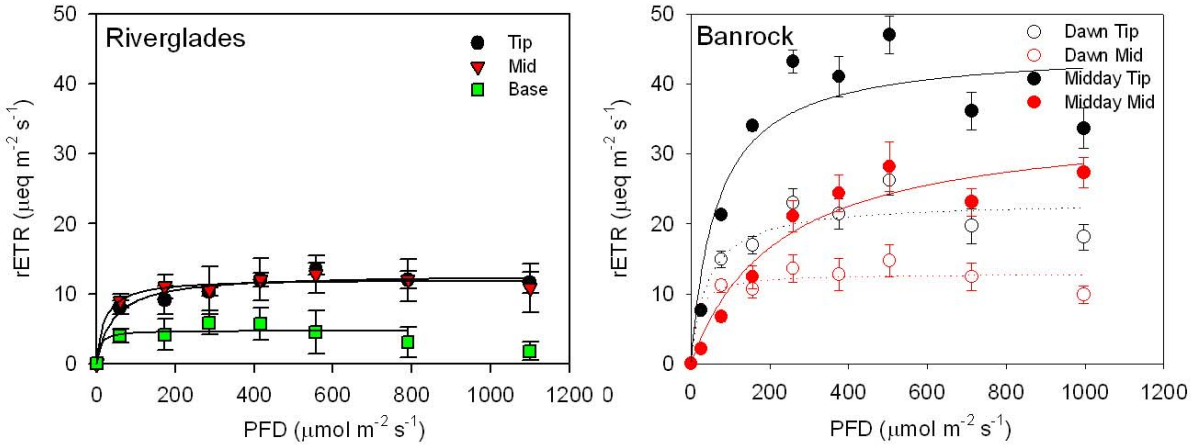


Figure 4-10 Rapid light response curves at (a) Riverglades at dawn and (b) Banrock at dawn and midday.

Photochemical efficiency

At Riverglades, tip sections exhibited photoinhibition, with a decline in ΦPSII between dawn and midday and a corresponding increase in NPQ (Figure 4-11a,b). At Banrock light attenuation had a dramatic effect on midday ΦPSII differences between tip and mid sections of leaves (Figure 4-11c), hence NPQ (Figure 4-12d). Tip sections dropped to a ΦPSII of only 0.16 compared to mid sections, which remained at dark-adapted

levels of c. 0.77, assessed before dawn. ΦPSII assessed at midday was significantly lower in tip sections c. 10 cm from the surface. NPQ showed a corresponding increase in tip sections up to 2.8, while mid sections showed no increase in NPQ at midday. At Banrock in February, depressions in ΦPSII were patchy at each site and ΦPSII ranged from 0.22-0.50, with corresponding high NPQ (Figure 4-11). NPQ was considerably higher in February than January for the same reductions in ΦPSII.

Carbon use at sites with different water chemistry

The total inorganic carbon (TIC) content of the source water was variable between dates and sites, but all measurements are within a normal band for fresh water (1 ± 0.2 mmol L^{-1}) (Figure 4-12a). There were dramatic differences, however, in the source water $\delta^{13}C$ (Figure 4-12b). Water was considerably more enriched in ^{13}C in February than in January.

Further analysis of the inorganic carbon composition shows little difference in the carbon species at Banrock between dates, but a slightly high pH resulted in a move towards the carbonate scale and a decrease in CO_2. There was a striking difference in inorganic carbon species composition between the two sites (Figure 4-12c). Riverglades had much higher pH of 9.1-10.2, hence no CO_2, relatively low HCO_3^- and proportionally more CO_3^{2-}. Banrock on the other hand, maintained a neutral pH (7.5) and HCO_3^- was dominant with c. 7.5% CO_3^{2-} and c. 5% CO_2. This difference affords a comparison in carbon use of *V. americana* between the two sites.

The bulk organic $\delta^{13}C$ signature of the leaves was 2.5-3.5‰ more depleted than the source water, leaf $\delta^{13}C$ values ranged between -16.5‰ and -18‰. The $\delta^{13}C$ of the base of the leaves are slightly more enriched (c. 0.5‰), but there were no statistical differences ($F_{4,2} = 2.14$, $P = 0.214$) for either site. This value is lower than previous measurements (LaZerte and Szalados 1982), and 7‰ more enriched than a comprehensive study at Broken Creek in 2001-2002, which found a $\delta^{13}C$ of -24.9±0.30 for *Vallisneria* (pers com Gigney 2005). All starch and soluble carbohydrate data for each site were lost during sample purification, due to equipment failure.

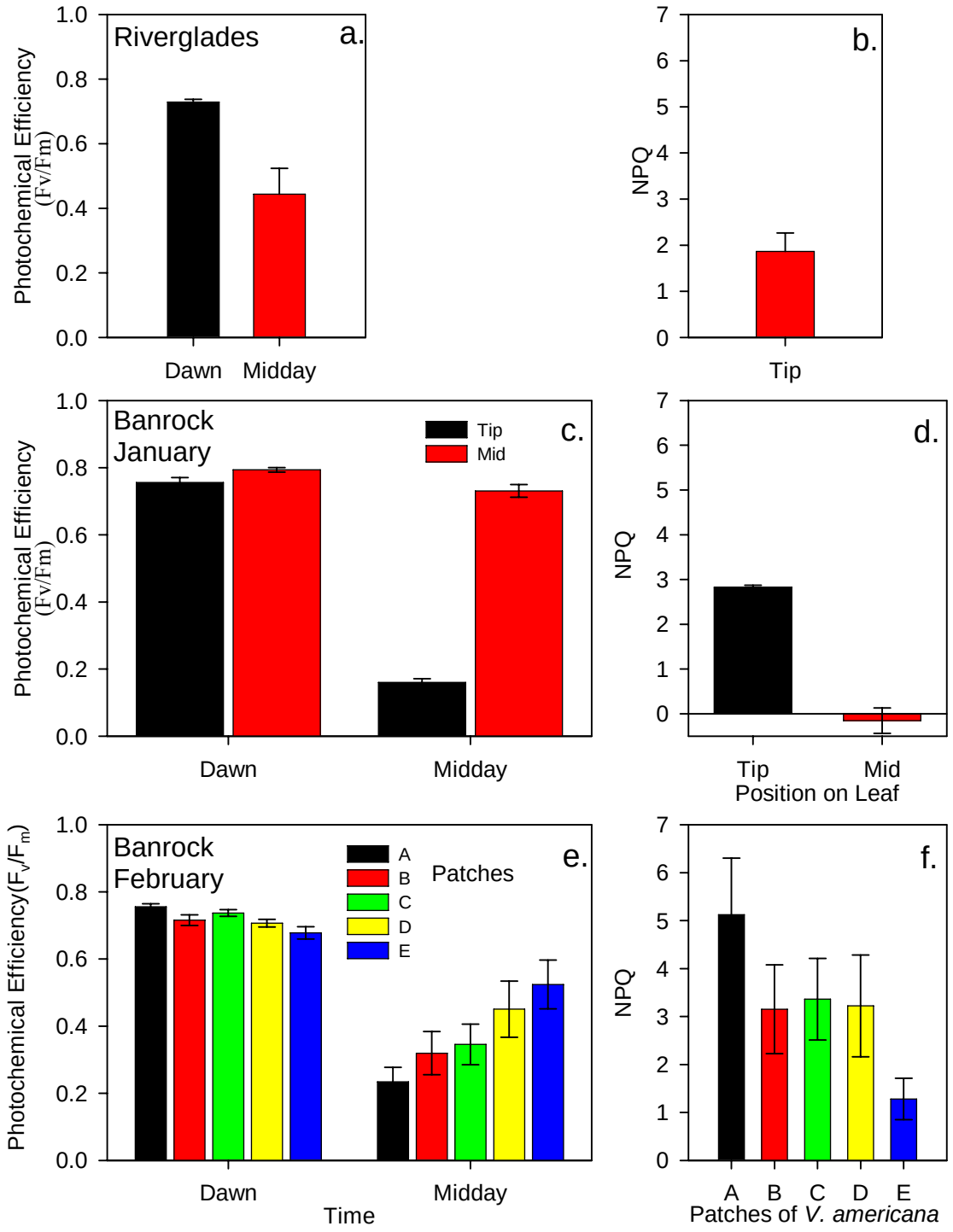


Figure 4-11. Photochemical efficiency (F_v/F_m) for leaf tips at (a) dawn and midday at Riverglades, (c) at Banrock during January, showing F_v/F_m (black) and Φ PSII (red), and (e) in February, documenting the range within sites at the tip (10 cm depth) of leaves. Non-photochemical quenching (NPQ) at midday (b) at Riverglades and (e) tip and middle of leaves at Banrock in January and (d) variability between sites in February.

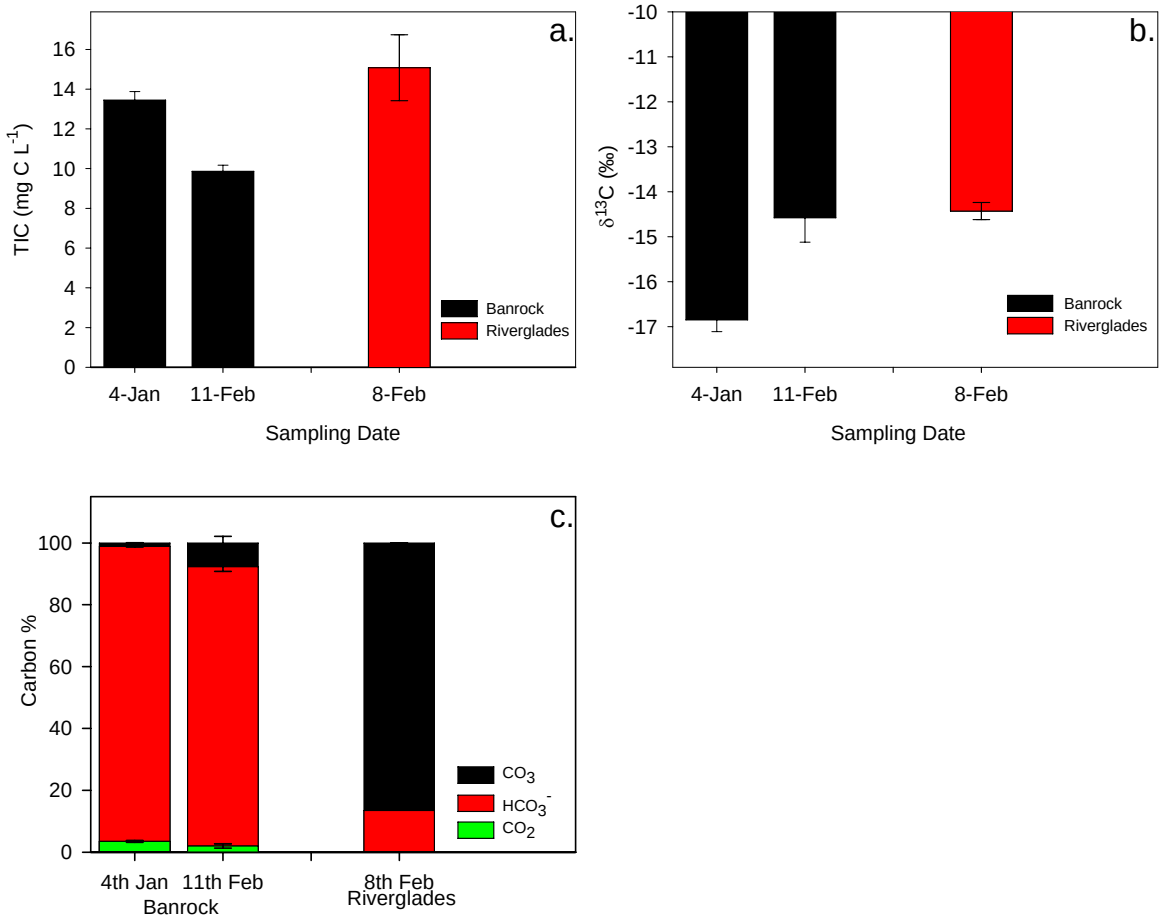


Figure 4-12. Dissolved organic carbon in acidified water samples from Banrock Station and Riverglades in 2005. (a) $\delta^{13}C$ of total inorganic carbon (b) TIC and (c) percentage of carbon species calculated using pH, temperature and salinity (Johnston 2004).

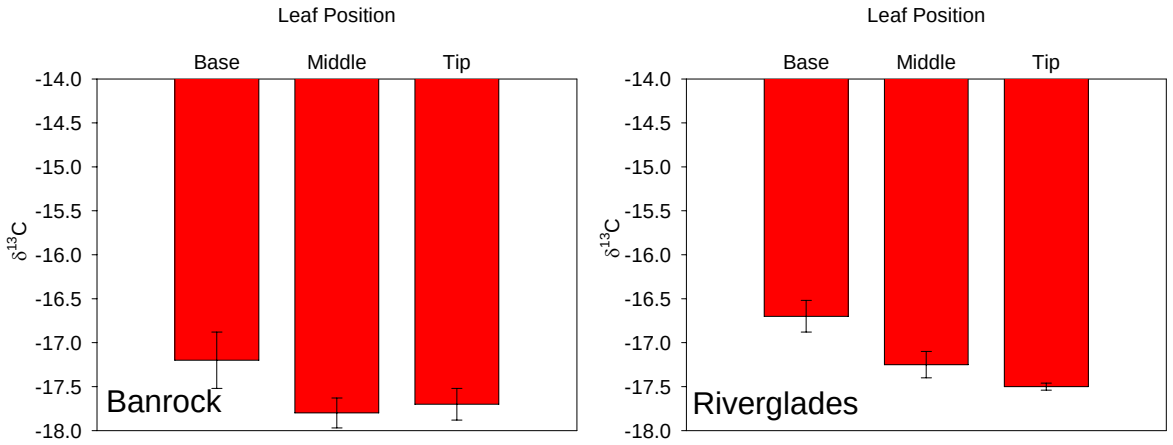


Figure 4-13. $\delta^{13}C$ isotope values along the leaf at (a) Riverglades and (b) Banrock Station.

4.3.3 Bicarbonate use in field incubations

Incubations of leaf material in closed containers under field conditions produced a dramatic increase in the pH of the water of tubes with leaf tips exposed to light (Figure 4-14a). This did not occur for other incubations exposed to PFD similar to that experienced by the leaf tips.

Titratable acidity was higher in the tip and mid sections of leaves compared with basal tissue for both dark (acid accumulation) and light (background) treatments. Neither dark accumulation ($F_{1,4}$ = 2.41, P = 0.147) or position along the leaf yielded a statistically significant difference after 6-h incubation ($F_{2,4}$ = 2.82, P = 0.099) (Figure 4-14b).

The total inorganic carbon left in tubes was variable, but more carbon generally remained in tubes with basal tissue than those with tip tissue, indicating that the use of carbon by tip tissue was predominantly in the light (Figure 4-14c).

Carbon speciation ($CO_3^{=}$, HCO_3^{-}, CO_2) was calculated from final pH, conductivity and temperature. There was a distinct shift of speciation in tip tissue in the light compared with other leaf sections, such that the carbon remaining in solution comprised 75% $CO_3^{=}$ and 25% HCO_3^{-}, with no free CO_2 present (Figure 4-14d). Other incubations did not show this pH increase, and did not show significant deviations in carbon species composition from the initial solution.

The $\delta^{13}C$ of the source dissolved inorganic carbon prior to the incubations was c. -18‰. There was no difference in $\delta^{13}C$ after 6-h incubation with basal leaf tubes in the dark or the light (Figure 4-14e). However, incubations that contained tip sections in the dark showed a dramatic enrichment of ^{13}C in water, and less-dramatic enrichment was evident for mid sections in the dark.

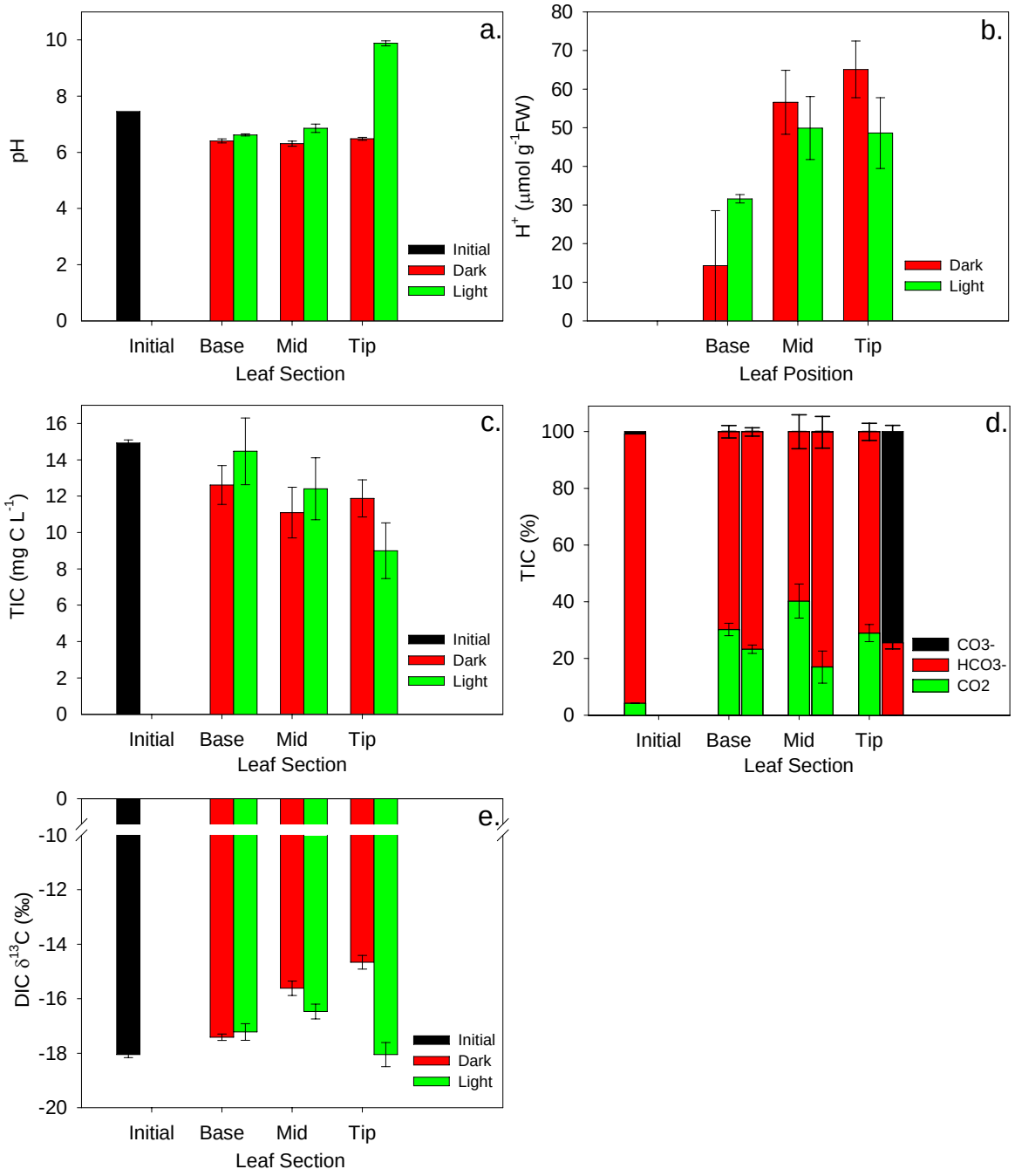


Figure 4-14. Final measurements from the controlled drift experiment on *Vallisneria* at Banrock in February 2005 under natural light or artificial dark, (a) final pH of solutions (b) titratable acidity of leaf tissue at conclusion of experiment, (c) final concentration of inorganic carbon of solution (d) proportions of each carbon species remaining in solution after the incubation and (e) $\delta^{13}C$ of dissolved inorganic carbon in solution.

4.4 Discussion

4.4.1 General Vallisneria system (Brenda Park)

There were low and variable amounts of CAM in Brenda Park populations, confirming CAM for *V. americana*, as for *V. spiralis* (Keeley 1998). The shallow, warm and productive lagoon at Brenda Park was expected to favour high CAM activity, and thereby confirm reports of CAM in *Vallisneria* in seasonal pools (Keeley and Sandquist 1991). However, CAM activity proved to be considerably lower than measured in other locations (for example, the mesocosms described in Chapter 7). Indeed, no CAM was observed late in the season when water was shallow and prone to fluctuations in light, oxygen content, pH (thus carbon speciation) and temperature. Plants produced numerous short leaves, presumably to maximise light interception (e.g. Blanch *et al.* 1998). High light and high temperature were associated with the ability to undertake CAM, and suggest that there is a light-intensity to enable CAM induction.

Background CAM activity was low but consistent (c. 4-8 μmol m^{-2} s^{-1}), typical of species with weak CAM ability (Holtum and Winter 1999). Four of five populations showed significant CAM on two sampling periods, and one showed no overnight accumulation of acid. This recalls previous studies where the occurrence of CAM has been disputed and attributed to seasonal pools (Keeley and Sandquist 1991).

The light responses of *V. americana* did not show a clear C_3 type pathway (Maxwell 2002) early in the season. This indicates multiple photosynthetic pathways. ΦPSII dropped suddenly at dawn then gradually increased over the remainder of the day. Predawn F_v/F_m values were lower than the proceeding evening ΦPSII and suggested a 'memory' effect of the previous high-light day. There was a dramatic decrease in effective quantum yield (ΦPSII) at dawn, implying quick saturation with low PFD, potentially due to CAM, and severely photo-inhibition as indicated by extremely low ΦPSII. During the progressive draw-down of water, photoinhibition was expected to progressively increase due to shallow water and higher light. The sharp decline in ΦPSII was surprising, and may be due to non-photochemical quenching; on further inspection with 20-min relaxation in darkness, however, F_v/F_m had noticeably improved with the same relative recovery for each population.

Consistent recovery in ΦPSII highlights a switch to C_3 photosynthesis after an initial use of overnight stored carbohydrate. So, in accord with the previous day's overcast

conditions during the last measuring period (compared with other sampling dates), the lack of a sudden decrease in ΦPSII may indicate a completely C_3 photosynthetic pathway for certain climatic conditions. This is supported by the lack of CAM in the latter sampling period.

4.4.2 CAM and bicarbonate use at Riverglades and Banrock Station

Riverglades and Banrock Station had distinctive carbon water chemistry, driven by pH (increasing due to biological activity at Riverglades and stabilising at Banrock by mixing due to connection with the river). The pH at Banrock remained under 7.7, and thus 95% of the carbon was present as HCO_3^- and 5% as CO_2. The pH at Riverglades fluctuated diurnally but free CO_2 was never detected.

CAM was again confirmed in *V. americana* at Riverglades and Banrock. Background acidity was relatively high and consistent along the leaf at Riverglades (25-35 μmol g^{-1}fw), yet was lower towards the bases of leaves at Banrock. High background acidity is often associated with low level CAM (Holtum and Winter 1999), and while CAM activity is higher than at Brenda Park the range of CAM is still low, with maximal accumulation of 60 μmol g^{-1}fw overnight. This is low compared to most CAM aquatics, where overnight accumulation of acids ranges from 5-290 μmol H^+ g^{-1} fw (70 species: (Keeley 1998). Relatively high levels of CAM were exhibited by leaf tips at Banrock and Riverglades, but at Banrock—where water was deeper and light was lower—there was a sharp decline in CAM towards the bases.

High PFD is likely to enhance CAM induction in *V. americana*, as in many terrestrial C_3-CAM intermediates (e.g. *Mesembryanthemum crystallinum*: (Cockburn *et al.* 1996; Miszalski *et al.* 2001). Knowledge of the effects of light on CAM in aquatic plants is sparse, but low photorespiration has been attributed to high light in the 'C4-like' *Hydrilla* (Salvucci and Bowes 1983). It appears that high light may help induce CAM under carbon-limited situations (Cushman and Borland 2002; Grams and Thiel 2002). The slightly deeper water at Banrock causes less light to reach the basal tissue (10 cm from the sediment), and may thereby prevent CAM. At Riverglades, CAM was evident throughout the leaf, from the tip to 10 cm from the base, but there was more CAM towards the tips of the leaves (again, this is attributed to the higher light).

Unlike the initial study at Brenda Park, CAM was consistent across all populations at Riverglades and Banrock. This is attributed to the high, but less severe, light and temperature conditions compared with Brenda Park.

Photosynthetic capacity was higher at Banrock than Riverglades, indicated by the high ETR in overnight dark-acclimated leaves. ETR_{max} at Banrock was over twice as high for each position on the leaves compared with Riverglades, and in the leaf tips ETR_{max} reached 40 μmol μeq s^{-1}. Other fluorescence parameters show few differences, indicating similar photosynthetic efficiencies; any differences may be attributed to the light history and differences between the light climates at each site.

A commonality between sites is the extreme photoinhibition observed at midday for the tips of leaves compared with lower down the leaf, showing the high-light effect on photochemical efficiency. A high NPQ was observed with the low ΦPSII, but NPQ was above 3.5 (typical maximum at saturating intensity, Maxwell and Johnson 2000) for only one population of *V. americana* at Banrock. This was not critical photo-damage, as a full recovery was made overnight.

Even though low ETR was experienced by basal tissue, due to the low light, *V. americana* can survive up to 3 months with no light (Blanch *et al.* 1998; Morris *et al.* 2004), and is highly tolerant to severe light attenuation.

Carbon dynamics

Although dissolved inorganic carbon concentrations at sites were similar on any one sampling date, carbon species composition at Riverglades and Banrock differed substantially. Riverglades had much higher pH and (with source-carbon determined using dissolution and equilibration equations: (Johnston 2004) no CO_2, relatively low HCO_3^- and proportionally more $CO_3^=$ was available other than through dissolution.

Generally, leaf material $\delta^{13}C$ was similar to the source carbon (1-2‰ more depleted) and suggest slow diffusion of carbon in water, carbon supply becomes limiting and the usual isotope fractionation is not expressed. The organic $\delta^{13}C$, which reflects an integration of all carbon over the life of the tissue, was similar between sites, although slightly more enriched lower down the leaf than in the mid and the tip sections. Basal leaf sections had a $\delta^{13}C$ that was <1‰ more depleted than source water, while middle and tip sections consistently c. 2‰ more depleted. Possible causes may be temperature, photosynthetic capacity or bicarbonate uptake:

Temperature: CAM plants vary in $\delta^{13}C$ values with temperature as a result of changes in the balance between dark and light fixation. The water was not thermally-stratified, due to mixing, but there may have been stratification over the course of total carbon assimilation. Temperature alters the balance between carbon species due to changes of carbon solubility (Johnston and Kennedy 1998). An increase in temperature causes a more-depleted $\delta^{13}C$ value for terrestrial CAM plants (Osmond *et al.* 1976), and it is assumed here that it may have a similar effect on aquatic CAM. In this case, a higher temperature would be expected in the top of the water column and the tip and mid sections would exhibit a more-depleted $\delta^{13}C$ than the water column.

Photosynthetic capacity: lower rates of photosynthesis (e.g. basal leaf sections) reduce the amount of carbon being utilised. This may affect $\delta^{13}C$ values.

Bicarbonate uptake: Bicarbonate in the water has a much more enriched $\delta^{13}C$ signature (-5.84‰ at Riverglades on 8 February; -8.13 at Banrock on 4 January) compared with the overall signature of DIC (-16.5‰ in January; -14‰ in February). Use of bicarbonate then would cause a more-enriched signature, as observed in the basal tissue. However, higher light drives bicarbonate use, and this would counter the difference between the basal tissue and the mid and tip tissue.

4.4.3 Field incubations

A modified pH drift technique was used to investigate bicarbonate uptake under field conditions. There was a dramatic pH increase from 6.3 to 10 under natural light conditions for tip sections, demonstrating HCO_3^- use. No other sections demonstrated HCO_3^- uptake, even under the same light conditions.

The enrichment in $\delta^{13}C$ of inorganic carbon of solutions for tip and mid sections was light-induced, and no change was observed for any dark incubation. This is due to reduced fractionation in diffusion-limited (aquatic systems), and the high degree of enrichment implies that carbon was not limiting. Although the high pH meant that there was no free CO_2, submerged leaves may be continuing to access CO_2 though the continual dissociation of HCO_3^-. It is unknown how much carbonic anhydrase is affecting the uptake of HCO_3^- and CO_2 in this respect. As *V. americana* is a known bicarbonate-user, it is surprising that there was no pH increase in other treatments. The tips appear to be adapted to bicarbonate use with high light, while lower down the leaf

there is no bicarbonate uptake due to lower light (these sections do not have the capacity to switch to bicarbonate use). However, as shown by Raven (1994), the diffusive CO_2 entry and the active influx of CO_2 or HCO_3^- both give rise to alterations in $\delta^{13}C$, regardless of CAM effects on $\delta^{13}C$ through changes in fractionation by Rubisco or PEPc.

During the incubations there was no translocation of carbon down the leaf. Organic $\delta^{13}C$ in mid and tip sections from plants measured *in situ* (CAM and bicarbonate use at page 84) implies HCO_3^- use. However, the analysis suggests that mid and tip sections do not use HCO_3^-, at least not in the high light environment usually experienced by tips of *V. americana*. As *V. americana* is a monocot, developmental stages of leaf growth may play a role in the adaptation of carbon acquisition features and thus the $\delta^{13}C$, where leaf tissue is adapted to high light. Differences in other stable isotopes, such as ^{18}O, along monocot leaves are related to developmental stages (Helliker and Ehleringer 2002). A high-light adaptation may result in the ability to use HCO_3^- or to progressively undertake more CAM, but was not apparent in sections lower down the leaf.

4.4.4 Hypotheses Revisited

V. americana has great flexibility as a C_3-CAM intermediate, possessing the capacity for induction with certain environmental conditions. CAM in aquatic plants also allows a longer period of time for CO_2 uptake which will be of benefit when competition for carbon is high and light is high. CAM in *V. americana* appears to be less to do with carbon limitation but rather reflects the need to cope with and process excess light.

These investigations were guided by three hypotheses, discussed below:

1. **The CAM pathway is used by *Vallisneria americana* in seasonally-isolated pools (typically shallow with high temperatures, variable light intensity and depleted in free CO_2 due to high pH). In contrast, there is low or negligible CAM in lakes and wetlands that retain connectivity with the parent river, and are physically and chemically more stable.**

V. americana shows extraordinary plasticity in the expression of CAM in response to changes in environmental factors such as light, temperature and carbon. CAM was low and patchy at Brenda Park, where conditions were the most extreme. CAM at Riverglades was low, and focused in the tips, and relatively high at Banrock (which was connected to the river). These results are contrary to the hypothesis above. CAM was

limited to the tips of leaves and could not be induced in lower sections of leaves under similar high-light conditions. Although CAM in leaf tips may give *V. americana* an ecological advantage, promoting better performance under highly variable radiation, it seems more of a stress response to high light.

2. In seasonally-isolated pools, where temperature, light and pH are often high, bicarbonate (rather than free CO_2) will be the principal carbon source.

At both Brenda Park and Riverglades, pH rose considerably in the water suggesting that HCO_3^- using plants were effectively removing carbon from the water. Conversely pH remained near-neutral at Banrock: this probably demonstrates mixing of the water rather than the lack of HCO_3^- use. From incubations it is clear that tip sections use bicarbonate, but mid and base sections did not increase the pH and changes in $\delta^{13}C$ demonstrate the use of CO_2 rather than HCO_3^-. So while bicarbonate is not the principal carbon acquired throughout the leaf, the draw down of carbon, pH increase and the altered $\delta^{13}C$ probably means that HCO_3^- is the dominant source of carbon for tips. Where $HCO_3^-$is the primary source of carbon available during the day tips are likely to be the primary location for carbon uptake.

3. Bicarbonate use is independent of the photosynthetic pathway.

V. americana uses bicarbonate and also undertakes CAM, thus suggestions that aquatic CAM species lack the capacity for bicarbonate uptake (Keeley 1998) is unfounded. In addition, there is evidence (in tips only) that when bicarbonate is the predominant form of carbon available, CAM is highest and show that these can be used concurrently. The lack of bicarbonate use in deeper leaf sections also coincided with reduced ETR and reduced CAM and is probably a function of reduced light although due to significant CAM in mid sections of leaves when there was no indication of HCO_3^- uptake proves CAM also occurs with CO_2 use.

Higher rates of photosynthesis (high CAM and high ETR) at Banrock also coincided with low pH and more available CO_2 and suggest the increased capacity for photosynthesis with available free CO_2. High NPQ in tip indicates that light is in excess and is likely that carbon is limiting photosynthesis. Carbon limits to photosynthetic rate is possibly the reason for CAM in *V. americana*. Fluorescence data from Brenda Park indicated that there was possibly a switch from using predominantly stored organic acids during the morning to more C_3-like use of carbon during the day.

V. americana is a known bicarbonate user, and it is shown here that it uses the CAM photosynthetic pathway under specific conditions (high light intensity near the tips of the leaves) concurrently with HCO_3^- uptake, while leaves deeper in the water continue to use the C_3 pathway and prefer to use CO_2 as the main form of carbon. However, *V. americana* does not use CAM when under stress, such as extremely high levels of light and temperature. The diversity of carbon uptake and assimilation mechanisms in *V. americana* may explain its competitive ability in lentic and marginal lotic habitats associated with the Murray through its ability to maximise use of light throughout the water column. It appears that in shallow relatively warm water bodies, where leaves run parallel to the surface, that the CAM ability is likely to be induced along the length of the leaf and allow maximal use of available carbon and high light.

Chapter 5
LABORATORY STUDIES OF VALLISNERIA AMERICANA

5.1 Introduction

Vallisneria americana is known to use bicarbonate (Titus and Stone 1982), and may undertake Crassulacean Acid Metabolism (CAM) (Keeley 1998). Spence and Maberly (1985) suggested that aquatic CAM plants are not capable of bicarbonate uptake, but this has been questioned (Keeley 1998). Prins (1989) suggested that bicarbonate is used facultatively by *Vallisneria* spp., but there is no evidence of polarity in HCO_3^- uptake in *Vallisneria* (2002), the uptake is by diffusion of HCO_3^- rather than acidification of the external medium. As with other concentrating mechanisms, the uptake of HCO_3^- increases the CO_2 concentration around Rubisco and reduces the inhibitory effects of photorespiration (Bowes *et al.* 2002), yet high levels of photo- and dark-respiration are reported for *Vallisneria* (Helder *et al.* 1974). In *Vallisneria* spp., CAM may apply to small seasonal pools with greater activity in spring and autumn (Keeley 1998), but ^{14}C labelling has shown dark malate fixation was seasonal and inconsistent between plants. Helder and Van Harmelen (1982) considered the high level of C_4 acid production and malate accumulation in *V. spiralis* reminiscent of acid metabolism in terrestrial CAM plants, but concluded that it is a C_3 plant.

Isotopic analysis has shown that carbon can be accessed from sediment pore water (*V. spiralis* Kimber *et al.* 1999), but this is not so for *V. americana* (LaZerte and Szalados 1982). Studies of stable carbon isotopes have shown enriched $\delta^{13}C$ for *V. americana* (-18.2‰: LaZerte and Szalados 1982), consistent with HCO_3^- uptake and high diffusive resistance of CO_2 in water. These studies make no mention of the likelihood of alterations due to alternative pathway assimilation.

The aim in this chapter was to determine the conditions shaping photosynthetic control of CAM in *V. americana.* Hypotheses tested were:

1. ***Vallisneria americana* acquires CO_2 and HCO_3^- in proportions dependent on those in the water.**

   It is proposed that the environment (pH, temperature and buffering capacity) determines the preference for HCO_3^- or CO_2 uptake rather than a preference for HCO_3^-. This is supported by Allen (1981).

2. ***Vallisneria* is a C3-CAM intermediate, whereby the relative contribution of C_3 and CAM to carbon fixation varies with environmental conditions (light, temperature, and pH).**

   CAM in terrestrial plants is often induced by high light. *V. americana* is prevalent in shallow water and deeper turbid water, and so is exposed to high and low light. Optimising carbon uptake through switching between forms of carbon assimilation could help to optimise photosynthesis in this situation.

3. **The extent of CAM in *V. americana* is independent of carbon species: both HCO_3^- and CO_2 are used during dark uptake.**

   Following Hypotheses 1-2, the potential uptake and assimilation of carbon are independent of one another. Bicarbonate use is maximised under high light and so, when carbon is limiting, the ability to utilise HCO_3^- should provide additional day-time carbon. CAM should reduce photorespiration (Helder *et al.* 1974) and increase carbon during the dark.

5.2 Methods

5.2.1 Growth conditions

Mature plants were purchased from Tropica©, Denmark, and grown at Cambridge in summer 2003 in 50-cm deep ponds filled with bore water. Individual plants were grown in a 50:50 mix of sand and loam with 25 g added slow-release fertiliser (Osmocote® 6 month) per 10L soil (equivalent annual loading 100 g N m^2), capped by 2 cm clay to limit nutrient loss. The pond water stratified on warm, sunny days, attaining a maximal top-bottom differential of 5ºC. After two months, each plant had 6-12 leaves that were used over summer to test and refine techniques to assess carbon uptake and photosynthesis. In winter, the ponds were moved to a glasshouse to avoid frost and prepare for spring. In 2004, studies were made of carbon uptake and assimilation differences along leaves, and in 2005 the effects of light intensity was investigated on the same plants.

5.2.2 Experimental procedures

Three experiments were undertaken: (1) characterisation of carbon acquisition, (2) carbon acquisition along the leaf and (3) carbon acquisition in response to three light regimes. Procedures used are described in Chapter 2.

Characterisation of carbon acquisition

Physiological parameters were assessed *in situ* and in small-volume incubations. These included a) light response curves and light absorbance, b) level of malate, c) level of HCO_3^- use, d) oxygen evolution and e) integrated parameters:

a) Diurnal fluorescence and light response curves were obtained according to Section 2.3. Evaluation of ETR included an accurate calculation of light absorption by leaf tissue. Two methods were used for (1) submerged tissue and (2) emergent tissue, as described in Section 2.4.

b) Level of CAM was assessed by determination of malate levels on *in situ* material and that used in incubations. Leaf material was collected in the hour before sunrise and the hour after sunset, and titratable acidity was measured according to Section 2.1.

c) Bicarbonate utilisation was assessed by the pH drift method, using closed 12-mL incubations. Incubating solutions were tap water at 0.5 mM or 10 mM $NaHCO_3$, with pH brought to 6.5 by addition of deionised water and left overnight. 12 mL of solution was added to 15-mL plastic tubes and solutions then purged with N_2 prior to adding 0.5g of fresh leaf material, as described below. The 3-mL headspace of the tube was air. Tubes were then gently shaken horizontally for 6 h under 300 $\mu mol\ m^{-2}s^{-1}$. On completion, pH and temperature were measured and chlorophyll concentration of the leaves determined according to Section 2.2. Solutions subsequently were analysed for DIC and $\delta^{13}C$, as in Section 2.5.3.

d) Oxygen evolution was measured in small 4-mL incubations instantaneously and concurrently with pH, according to Section 2.3. Experiments assessed oxygen evolution at constant pH (using buffers described below) and also when pH was allowed to drift. For constant pH measurements, 50 mM buffers were used at each pH depending on their working pH range (pH 5.5: 2-[N-Morpholino]ethanesulfonic acid (MES), pH 7: 3-[N-Morpholino]propanesulfonic acid (MOPS) and pH 9: N,N-bis[2-hydrooxyethyl]glycine (BICINE)). Bicarbonate was added after the buffers to bring solutions to either 0.5 mm or 1.0 mM.

f) An integrated approach was undertaken to assess the relative amounts of CAM as might be compared with HCO_3^- use, chlorophyll and isotopic data from purified soluble carbohydrates and organic material. One leaf per plant was sectioned for a combination

of titratable acidities, HCO_3^- use by pH drift; influence of controlled pH on uptake and organic δ13C and soluble carbohydrates.

Carbon acquisition along the leaf

The pH drift and constant incubations were prepared with solutions as described above.

a) Temperature, alkalinity, pH, DIC and $\delta^{13}C$ were first measured in the initial solution as described in Section 2.5.3. Each tube then had 0.5 g fw of tissue added from three different sections: 5 cm above the sediment (hereafter the "base"); the exact midpoint between the sediment and the water surface ("mid") and 5 cm from water surface ("tip"). Tubes were incubated in a growth cabinet under constant temperature of 20°C and PAR of 300 μmol m^{-2} s^{-2}. pH was measured every 3 h, and after 8 h half of the leaf material was collected and solutions and material stored for analysis of titratable acidity, dissolved inorganic carbon, $\delta^{13}DIC$ and chlorophyll was undertaken immediately. The remaining material was then placed in the dark for a further 8 h before being collected for similar analyses. Titratable acidities were analysed according to Section 2.1; DIC and $\delta^{13}C$ were analysed according to Section 2.5.3.

Carbon acquisition and PAR

A pH-drift experiment was undertaken from a pH start point of 6.67. Before excising the leaves, a dark-adapted F_v/F_m was measured (as in Section 2.4) and the exact point marked with a permanent pen. Sections of 0.5 g fw tissue from the base, middle and tip were then incubated in 12 mL water in 15-mL tubes under PFD of 80 μmol m^{-2} s^{-1}, 230 μmol m^{-2} s^{-1} and 680 μmol m^{-2} s^{-2}, or low, medium and high light. The pH and temperature were measured before addition of tissue and then every 3 h. After 10 h, half the tubes from each light regime were collected and ΦPSII measured at the same position as the initial F_v/F_m.

Using a N_2 flushed needle, 8 mL of solution was syringed from tubes into evacuated Exetainers (Labco®). Leaf material was blotted dry and divided in two halves, one half was analysed for chlorophyll *a* and *b* using the method of Porra (1989) (Section 2.2), while the other half was stored at -4°C for analysis of titratable acidity (Section 2.1). Exetainers were stored horizontally at -4°C until analysed for TIC and $\delta^{13}C$.

The remaining tubes were placed in darkness at the same temperature for a further 10 h, and pH and temperature measured every 3 h. On completion of the dark period, material was exposed to the same analysis as the light incubation material.

Leaf sections

Sections were excised from material collected from Murray-Darling Basin wetlands, using a protocol adapted from O'Brien (1981) and detailed in Section 2.8.

5.3 Results

5.3.1 Characterisation of carbon acquisition

Light response

Rapid light response curves indicate that the tip of leaves had double the capacity of electron transport compared with middle and base (Figure 5-1a). ETR_{max} of the tip was c. 12 Eq m^{-2} s^{-1}, and middle and base sections reached 6 and 4 µEq m^{-2} s^{-1}, respectively. There was no photoinhibition in tip tissue and only slight photoinhibition in the middle and base sections at the highest PFD point (325 µmol m^{-2} s^{-1}). There was a high (40 µEq m^{-2} s^{-1}) ETR_{max} in the tips during the middle of the day (11:00, 13:30 h) (b), double that at 17:00 (20 µEq m^{-2} s^{-1}), while ETR_{max} at 09:00 and 19:00 h reached only 15 µEq m^{-2} s^{-1}.

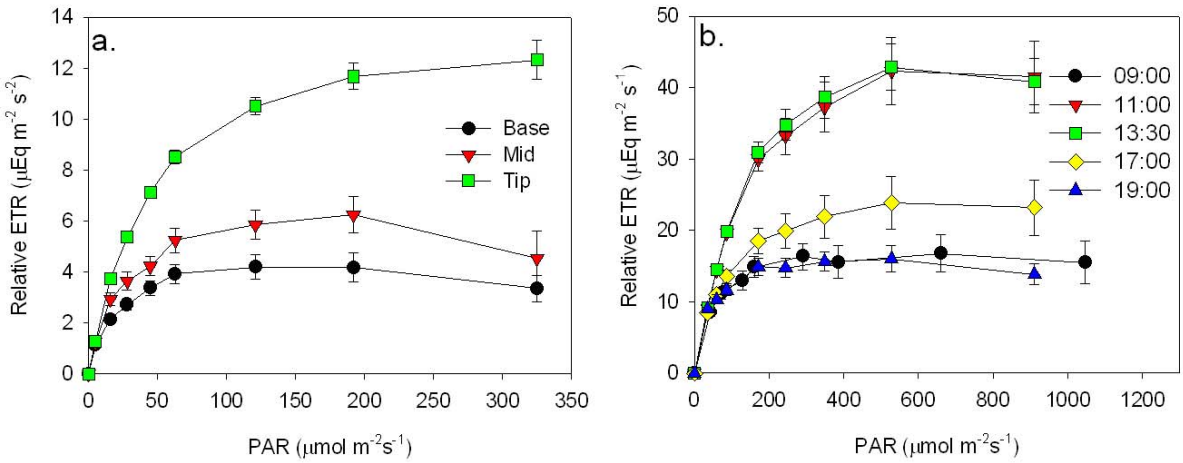


Figure 5-1. *In situ* rapid light response curves from base, middle and tip (leaf tissue) before dawn (a) and diurnal course of relative ETR (b). Error bars are standard errors.

Light absorption

V. americana has an absorbance factor (AF) of 0.70 for the tip, while the middle and base are 0.65 and 0.40, respectively (Figure 5-2a). There was no difference in reflectance along the leaf. Chlorophyll *a* increased towards the tip while chlorophyll *b* was relatively constant along the leaf (Figure 5-2b) such that *a/b* ratios decreased

towards the tip. Leaves were 90% thicker at the base of the leaf compared with the tip, yet had half the chlorophyll (Figure 5-2c).

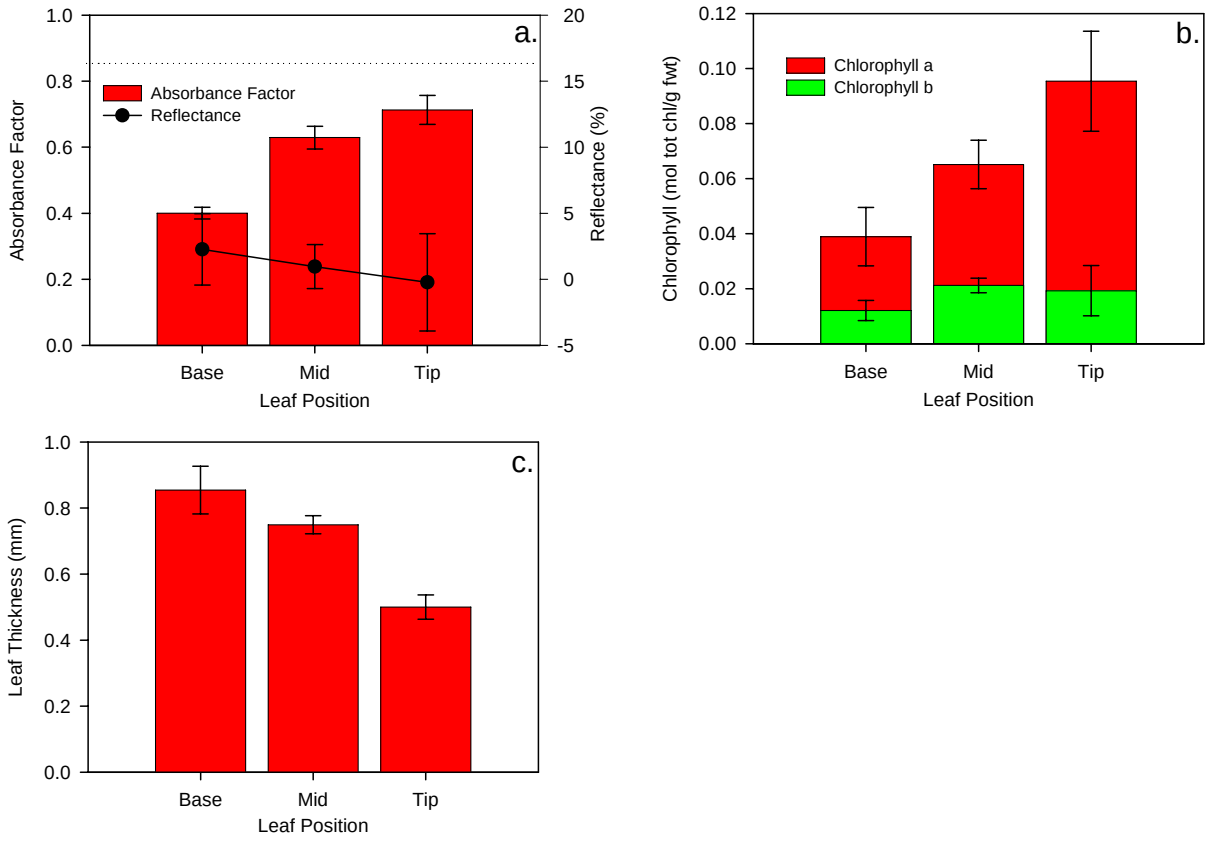


Figure 5-2. Absorbance factor and reflectance calculated from submerged measurements of each leaf section (a), Chlorophyll *a* and *b* content (b) and leaf thickness (c). Bars are standard errors ($n = 9$).

Crassulacean Acid Metabolism

Initial analysis of CAM indicated significant but variable malate accumulation over-night; the ΔH^+ was approximately 40 µmol H^+ g^{-1} fw. Dusk malate levels averaged 75 (10) µmol H^+ g^{-1} fw and increased overnight to 114 (40) µmol H^+g^{-1} fw at dawn.

Bicarbonate use

On warm, sunny days, the water in the 65-L ponds increased in pH from c. 6.5 to 10.4. Incubations of mid-section tissue in 12 mL of 0.5 mM and 1.0 mM bicarbonate solutions showed a similar pH increase from 6.5 to 10.7 (±0.54), regardless of the HCO_3^- concentration. This was likely due to the maximum pH for both concentrations being reached within the 6 h incubation time. In micro-assays of only 4 mL, the drift in

pH was less pronounced and rose from 6.5 to 9 over the course of 4 h, reflecting the shorter time incubation.

Photosynthesis

Oxygen evolution was highest at pH 5 where CO_2 was the main form of inorganic carbon (Figure 5-3). This occurred on both fresh weight (not shown) and on a chlorophyll basis. Oxygen evolution was lowest at pH 9 where there was 50% each of HCO_3^- and CO_3^{2-} but no CO_2 available. The back reaction (HCO_3^- to CO_2) was fast and may include the observed carbon uptake.

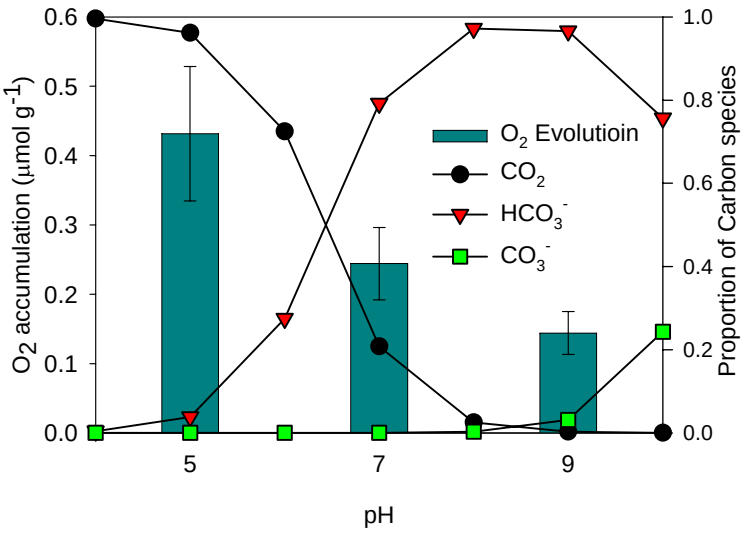


Figure 5-3 Photosynthesis represented by oxygen evolution at pH 5, 7 and 9. Overlaid is the proportion of each inorganic carbon species (Hutchinson 1975) over this pH range at 20ºC. Mean±standard error (n = 5).

Leaf sections

Monocot characteristics are evident in sections of *V. americana* (Figure 5-4). In all mid, tip and base sections there is a low density of chloroplasts and many large air spaces, characteristic of submerged leaves. There are two airspace layers in the basal sections, compared with only one in the middle and tip sections. This accounts for increased thickness often observed at the base of leaves. Chloroplasts (stained blue) do not have any consistent arrangement within cells or across the leaf.

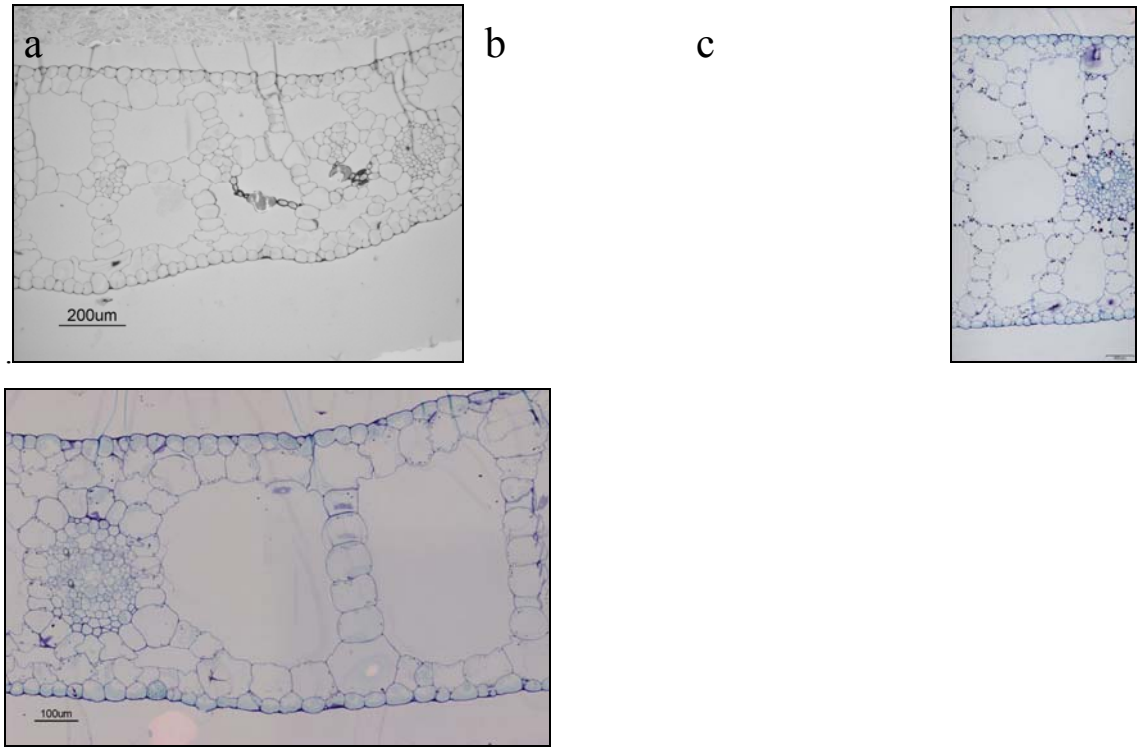


Figure 5-4. *V. americana* with chloroplasts stained in sections from (a) base (b) mid and (c) tip leaf sections at 10X magnification of *V. americana*.

5.3.2 Carbon acquisition along the leaf

Titratable acidities

There was small but significant CAM in the tip and middle sections with an average overnight accumulation of 25μmol H^+ g fw^{-1} ($F_{5,1} = 15.73$, $P = 0.000$), but no acid accumulation at the base of the leaves (Figure 5-5a).

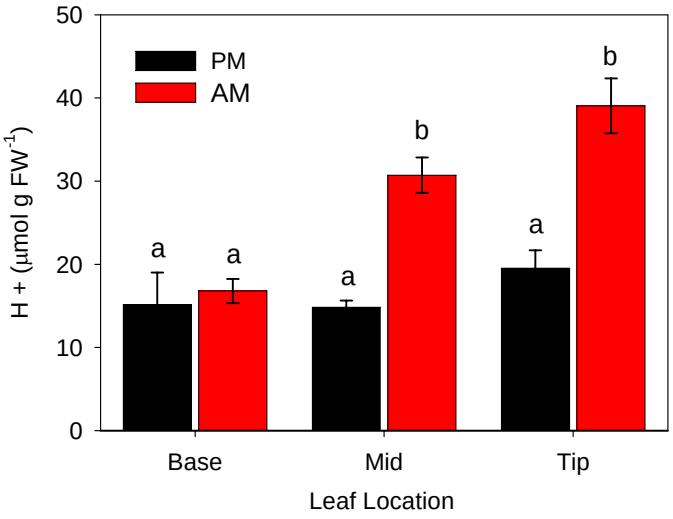


Figure 5-5. Titratable acidity (H^+) for each position on the leaf, measured from samples collected at dawn and dusk. Significant differences are shown by a, b. Bars are standard errors (n = 5).

pH drift incubations

The middle and the tip of the leaves caused an increase in pH from an initial 7.5 to 10.5. In the dark, a decrease in pH of approximately 0.3 was consistent for all parts of the leaf. There was negligible change in pH in control solutions in either the light or the dark. In the light, there was more bicarbonate used by the middle and tip sections than in base sections (Figure 5-6). A pH of 10.5 probably represents the maximum capacity for the solution, given the alkalinity (Talling 1976).

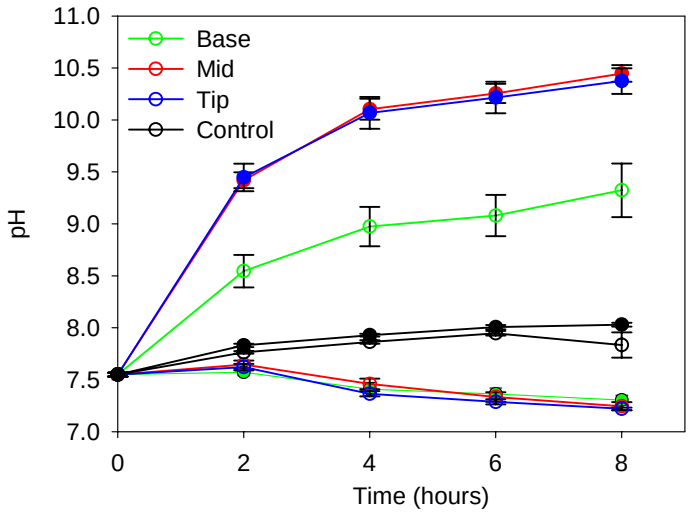


Figure 5-6. pH drift for each position on the leaf in light (○) and dark (●) (b). Bars are standard errors (n = 3).

DIC and $\delta^{13}C$

The initial total alkalinity of the solution was 98.12 mg L^{-1} and consisted of 87% carbonate alkalinity (85.36 mg L^{-1}). For tip and mid sections, water alkalinity decreased to 60.72 and 57.64 mg L^{-1} respectively over the 6 h. Alkalinity of the base section medium was double that of other sections: 113.08 mg L^{-1} (82.72 mg L^{-1} as carbonates).

Carbon uptake is shown by a decrease in DIC of approximately 50% in the tip solutions, regardless of collection after the dark or light phase, while the middle position on the leaf showed only decrease in DIC of solution collected after the light (Fig 5-7a). The DIC decrease in the tip is hypothesised to be due to C_3 photosynthesis during the light and CAM during the dark. The base tissue solution was equivalent to the control and shows that there was no influence of the leaf material during either the dark or light phase.

There was strong $\delta^{13}C$ enrichment in all final solutions, regardless of location on the leaf for light incubations (Figure 5-7). Solution enrichment, thus discrimination by Rubisco, was more pronounced for the base and middle sections than tip sections. Sections incubated in the dark showed less change in $\delta^{13}C$. Compared with the pH drift this appears to be regardless of carbon species availability.

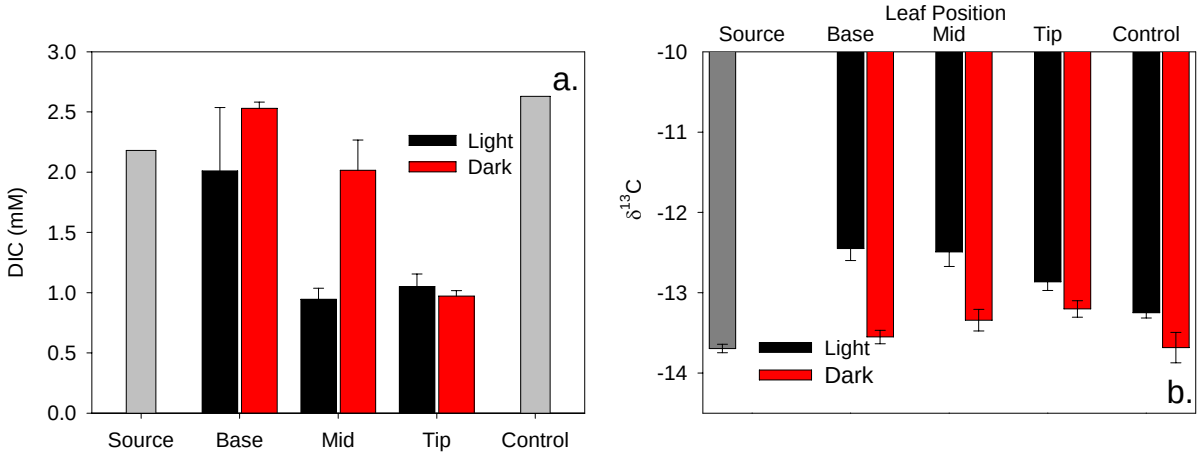


Figure 5-7. Total DIC (a) and δ13C of incubating medium at conclusion of experiment for leaf sections (b). Bars are standard errors (*n* = 3-5).

5.3.3 Carbon acquisition at three light levels

Chlorophyll

The structure of *V. americana* leaves used in the study meant that over their length they had acclimated to a gradation of light. *Tips* of leaves are defined here to be 5 cm from

the water surface; the *middle* of leaves was the exact middle between water surface to the base of the plant and the *base* was 5 cm above soil level. There is a reduction of total chlorophyll down the leaf while growing *in situ*. The normal daily maximum light exposure to each position on the leaves was approximately 600, 200 and 80 μmol $m^{-2}s^{-1}$, respectively. In addition, the middle and base of leaves would have been exposed to differing amounts of shading by upper portions of leaves (this was not taken into consideration when selecting sections to harvest).

Both chlorophylls *a* and *b* contributed to the total chlorophyll difference between the base and tip of leaves in material used in this experiment ($F_{8,2}$ = 21.12, P = 0.000). The chlorophyll *a*/*b* ratio was the same regardless of position on the leaf or light treatments. Light treatments were pooled in the statistical analysis of chlorophyll.

Titratable acidities

There was no consistent trend in overnight malate accumulation. Malate was accumulated in tip and middle of leaves incubated at 80 and 600 μmol $m^{-2}s^{-1}$, but the difference between accumulated malate in the light compared with the dark treatment was only significant at 100 μmol m^{-2} s^{-1} for the middle sections ($F_{2,2}$ = 12.93, P = 0.023) (Figure 5-8).

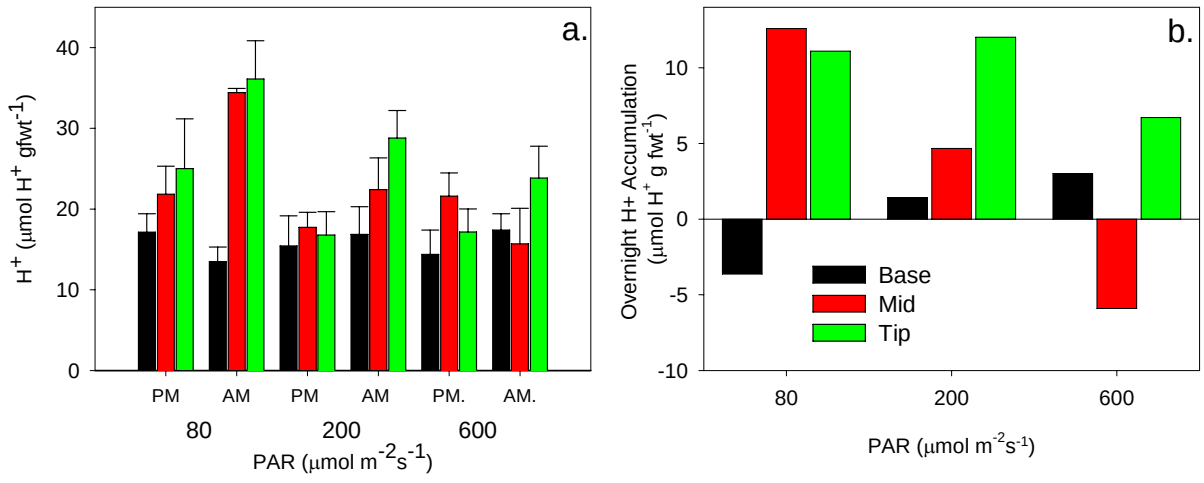


Figure 5-8. Titratable acidity representing malate for each of three light regimes for base, middle and tip of leaves for (a) and overnight accumulation (b). Bars are standard errors (n = 3).

pH drift

During the light phase, pH increased in all treatments for each leaf section (Figure 5-9). The tip and mid sections followed the same pattern of pH increase over 10 h of light, from pH 6.6 to 10, and the base and control samples increased by 1.5. There was a slight, unavoidable temperature increase of 2°C. The base did not differ from the control. Significant interaction between light and leaf sections ($F_{2,2}$ = 2.67, P = 0.04) indicates that the effect of light on pH varies between leaf positions. The first time point for pH was taken on still water having acclimated with atmospheric CO_2 overnight. In retrospect, this value was not the most appropriate to use in this instance as pouring water into tubes would have altered the pH due to mixing and the difficulty in measuring pH quickly in this poorly buffered water. Subsequent experiments suggest that a value closer to 7.5 may be more appropriate (indicated on the figure). The control tubes reflect the stabilised yet poured solutions of the initial set up and these should be used for comparison rather than the initial solutions.

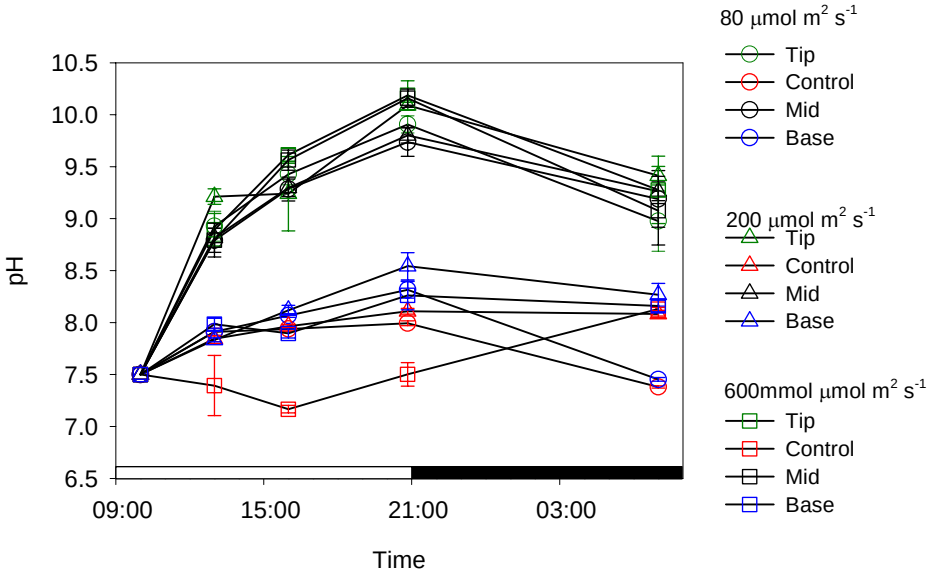


Figure 5-9. pH change over 20 h under three light treatments of 80 (○), 200 (Δ) and 600 (□) µmol m^{-2} s^{-2} for three positions along the length of the leaf (tip, middle and base). From 09:00 until 21:00 h leaves were exposed to light; from 21:00 until 09:00 h leaves were in darkness. Bars are standard errors (n = 3).

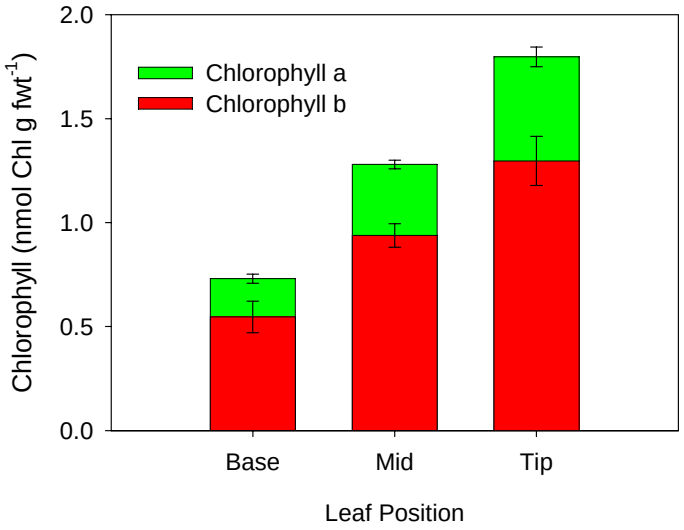


Figure 5-10. Titratable acidity representing malate for each of three light regimes for base, middle and tip of leaves for (a) and overnight accumulation (b).Bars represent standard errors (n = 3).

Fluorescence

After 10 h of incubation, F/F_m' decreased over most incubations ($F_{8,2}$ = 15.22, P = 0.000), and this was dramatic in the base of leaves under 200 and 600 $\mu molm^{-2}s^{-2}$ light which decreased by 70% (Figure 5-10). F/F_m' was significantly different ($F_{8,2}$ = 83.31, P = 0.000) between different positions on the leaves. After 10 h of dark, recovery in F/F_m' was noticeable, but F/F_m' was still lower than the dark pre-treatment measurements for both the light treatment ($F_{8,2}$ = 9.91, P = 0.001) and position on the leaf ($F_{2,1}$ = 16.73, P = 0.000).

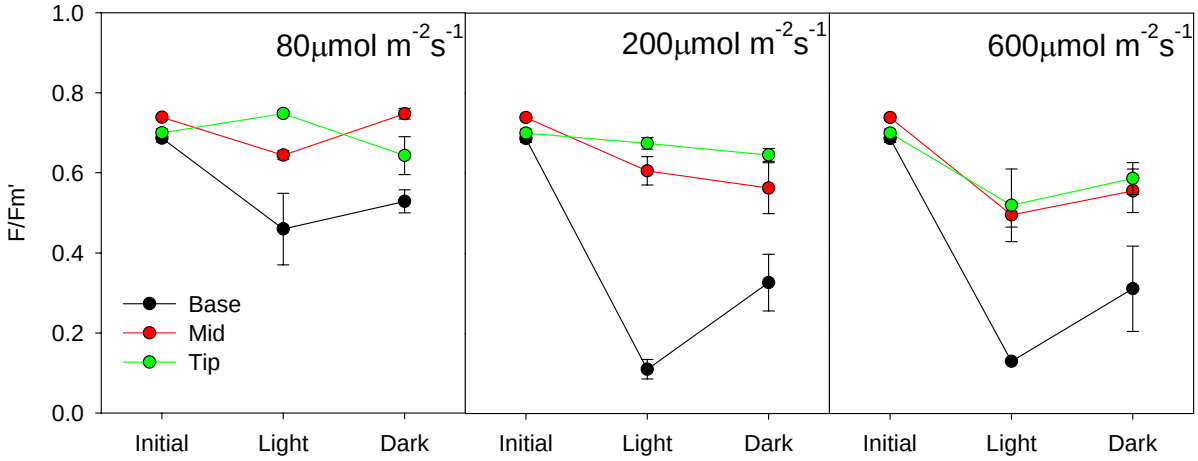


Figure 5-11. $F/F_{m'}$ measured before treatment, after 10 h light and after a subsequent 10 h dark period for light levels of 80 µmol $m^{-2}s^{-1}$, 200 µmol $m^{-2}s^{-1}$ and 600 µmol $m^{-2}s^{-1}$. Bars represent standard errors (n=9 for initial measurements, n=3 for treatment measurements).

After the light incubation, non-photochemical quenching (NPQ) was greater for medium and high light than for low light for each position on the leaves (Figure 5-12). This was pronounced for the base of the leaves, while other sections had negligible NPQ after the light period.

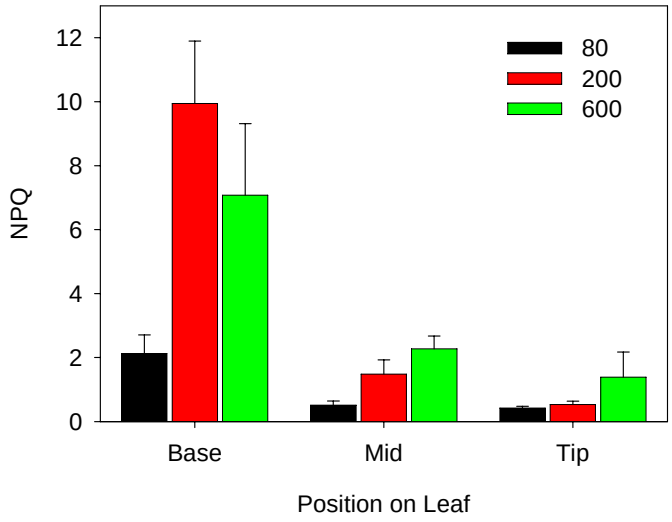


Figure 5-12. Chlorophyll *a* and *b* concentrations for each section of the leaves, treatments were pooled. Bars represent standard errors (n=9).

DIC and $\delta^{13}C$

For the light intensities used, the position of leaf sample significantly effected the final DIC concentration ($F_{2,1}$ = 52.06, P = 0.000) (Figure 5-13b). The tip and middle of leaves halved the initial DIC, while base samples did not alter the final DIC concentration; it is assumed that this is due to carbon uptake rather than precipitation of carbonates with pH increase along with temperature and the concentration of carbonate.

There was consistent ^{13}C enrichment of the medium in incubations run with the base of leaves, indicating that Rubisco had a discriminatory effect on carbon (Figure 5-13a). This is the opposite for the middle and tip of leaves, which show a more negative final medium for samples collected both before and after the dark period signifying that the leaf material became more enriched in ^{13}C. This is pronounced for the middle and tip from the high light treatment collected after the dark period with a $\delta^{13}C$ of -17.50‰ and -16.75‰ compared with the original medium $\delta^{13}C$ of -13.9‰, and is also significant for the middle and tip at all other light intensities.

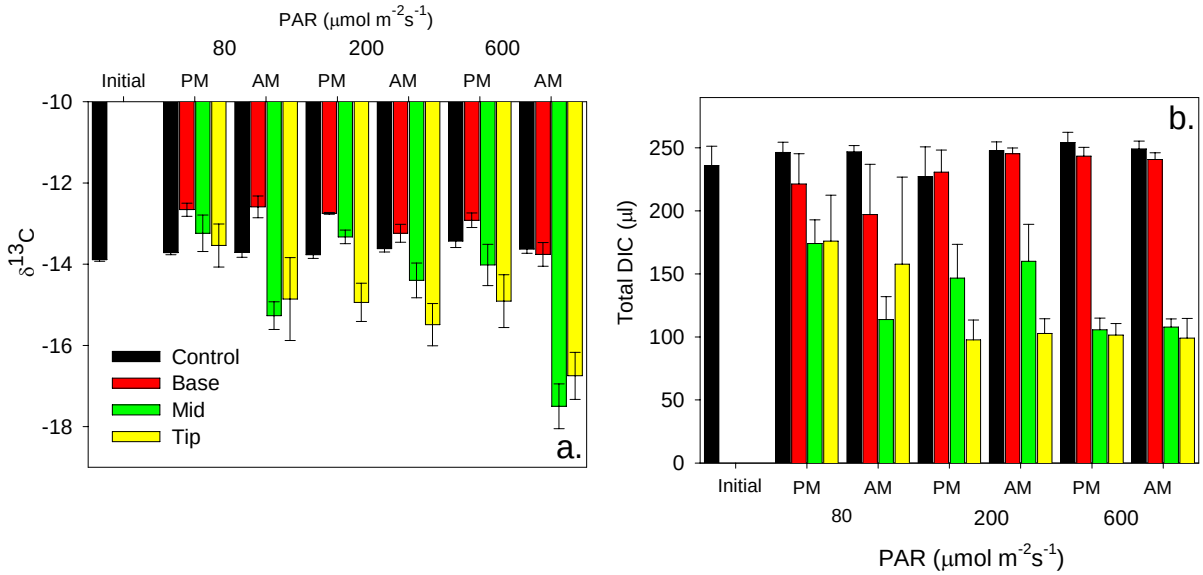


Figure 5-13. $F/F_{m'}$ measured before treatment, after 10 h light and after a subsequent 10 h dark period for light levels of 80 µmol $m^{-2}s^{-1}$, 200 µmol $m^{-2}s^{-1}$ and 600 µmol $m^{-2}s^{-1}$. Bars represent standard errors (n=9 for initial measurements, n=3 for treatment measurements).

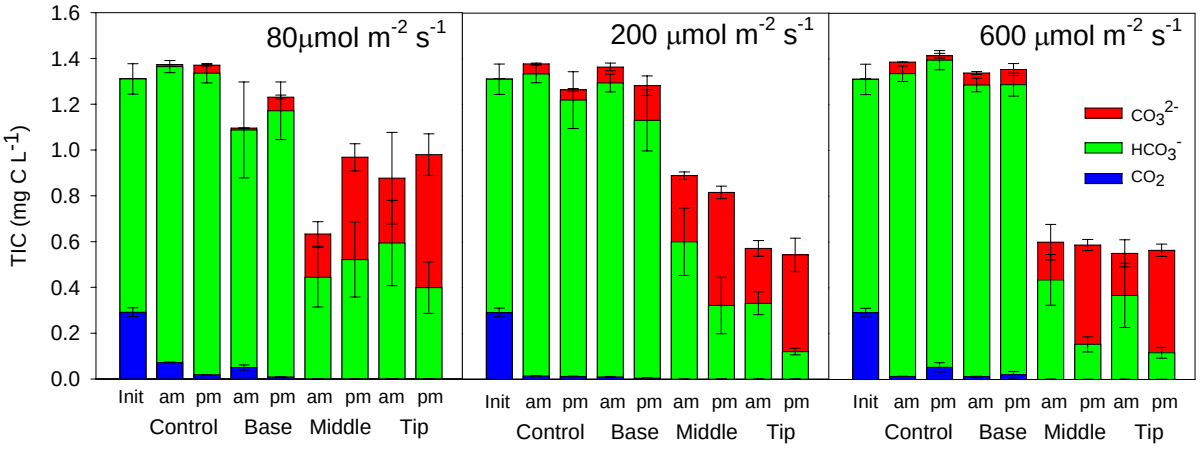


Figure 5-14. Calculated NPQ for each position on leaf for the three light treatments. Bars represent standard errors (n = 9).

DIC and $\delta^{13}C$

For the light intensities used, the position of leaf sample significantly effected the final DIC concentration ($F_{2,1}$ = 52.06, P = 0.000) (Figure 5-13b). The tip and middle of leaves halved the initial DIC, while base samples did not alter the final DIC concentration; it is assumed that this is due to carbon uptake rather than precipitation of carbonates with pH increase along with temperature and the concentration of carbonate.

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-16.75‰ compared with the original medium $\delta^{13}C$ of -13.9‰, and is also significant for the middle and tip at all other light intensities.

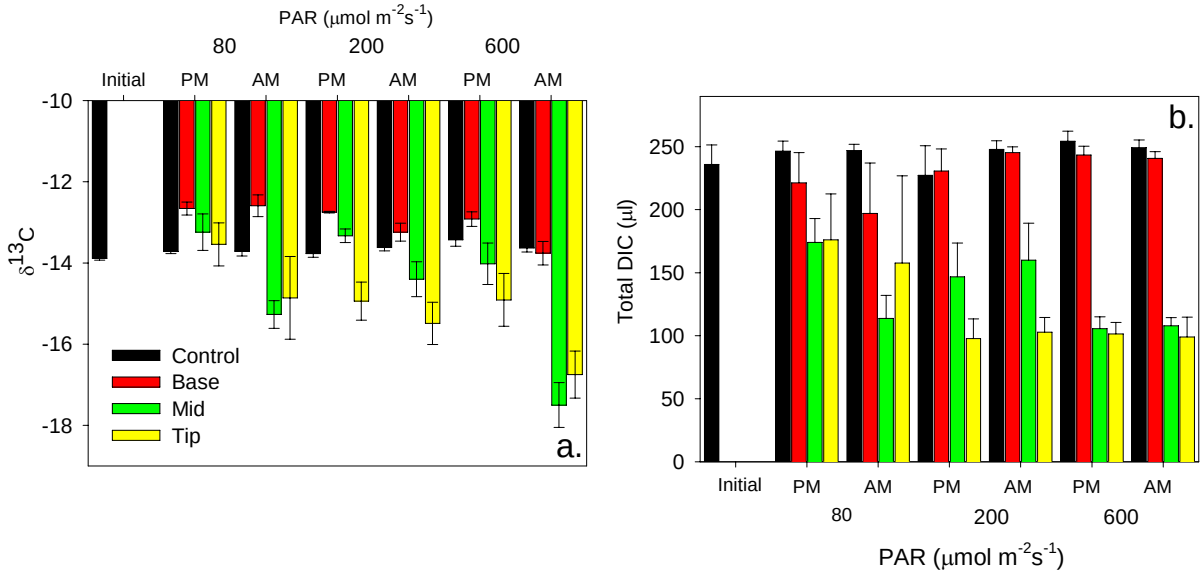

Figure 5-15. $\delta^{13}C$ of incubating medium at conclusion of experiment (a) and total DIC (b) for each light treatment and collection point. Bars are standard errors (n = 3).

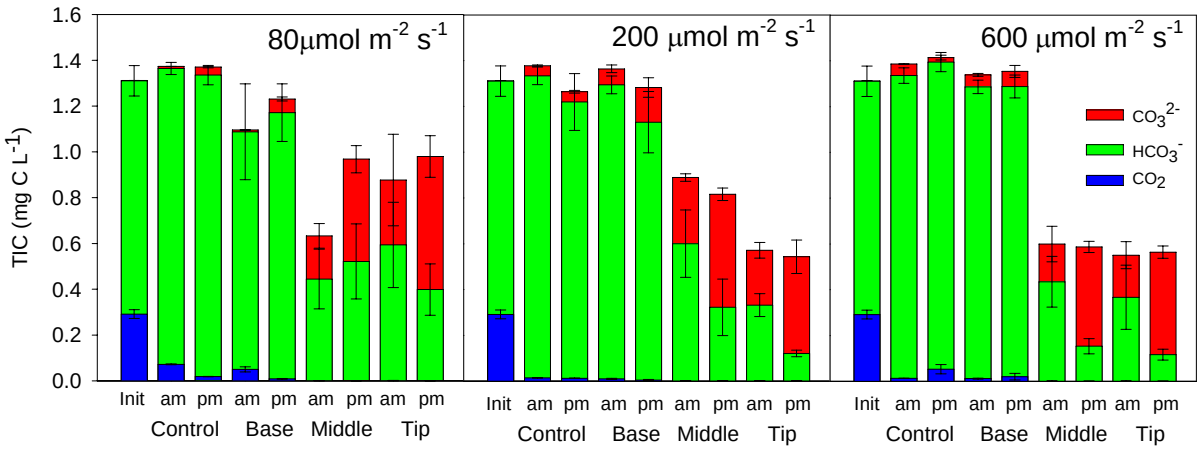

Figure 5-16. Inorganic carbon (CO_2, HCO_3^-, $CO_3^{=}$) as a function of pH, temperature, TIC and conductivity, determined by Johnston's equations (AM Johnston pers. comm.) at the completion of dark or light periods for low (80 µmol m^{-2} s^{-1}), medium (200 µmol m^{-2} s^{-1}) and high (600 µmolm^{-2} s^{-1}) light. Bars are standard errors (n = 3-5).

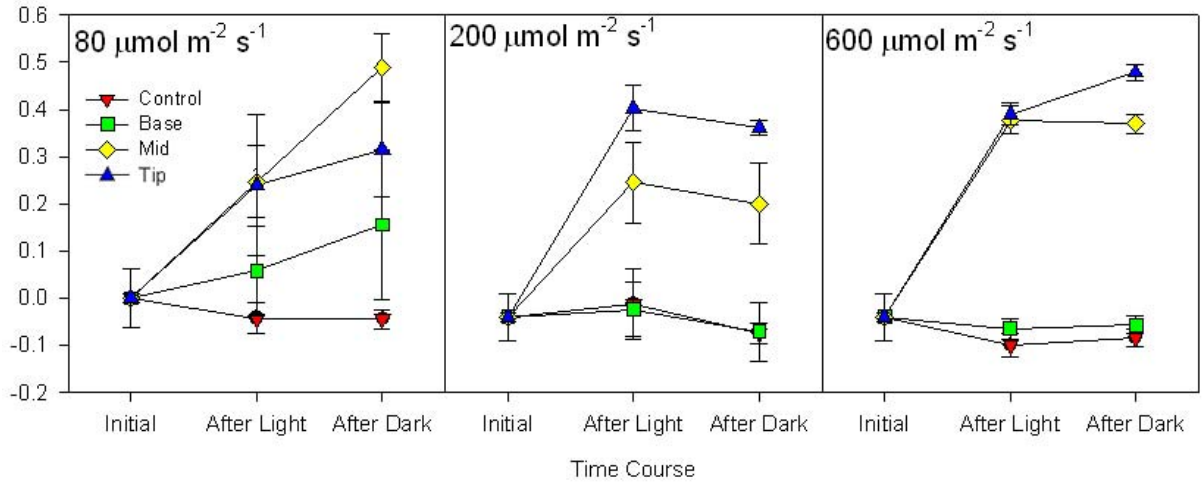

Figure 5-17. Assimilation calculated from the difference between initial and final DIC concentrations for low, medium and high light incubations, collected after the light period (red) and after the following dark period (green). Bars are standard errors (n = 3-5).

TIC and $\delta^{13}C$ data were explored further using equilibration and speciation factors (AM Johnston pers comm.) to determine the proportion of carbon species in the final solutions (Figure 5-14). Solutions used to incubate tip and middle section had no CO_2 and had proportionately more $CO_3^{=}$ and HCO_3^-, showing the preferential uptake of CO_2 by these leaf sections. The base incubations did not have any free CO_2 remaining, yet there was no drawdown of HCO_3^-, showing the inability of the base tissue to extract significant amounts of carbon from the water.

The lack of CO_2 and the increased CO_3^{2-} in the remaining tip and middle solutions as the TIC decreases shows preferential CO_2 and HCO_3^- use by the leaves. The base samples do not have free CO_2 remaining in solution, but there is still a shift towards reducing HCO_3^-. The same pattern is present for each light treatment, however, becomes more pronounced as light increases.

Carbon assimilation was calculated from the difference in initial medium and final DIC concentration and corrected for incubation time (Figure 5-15). Final assimilation measurements showed high levels for the middle and tip of leaves for all light levels, while no further change in carbon was observed during the dark, indicating that there was not a significant amount of carbon assimilated during the dark period.

However, the progressive $\delta^{13}C$ calculated for each of the light intensities and shows changes in the $\delta^{13}C$ and indicates the magnitude of CAM occurring after the period of

light (Figure 5-16). This suggests that basal tissue does result in discrimination probably due to the low amount of Rubisco and high light results in discrimination of both mid and tip tissue, probably due to the higher amounts of Rubisco being induced to undertake CAM with high light.

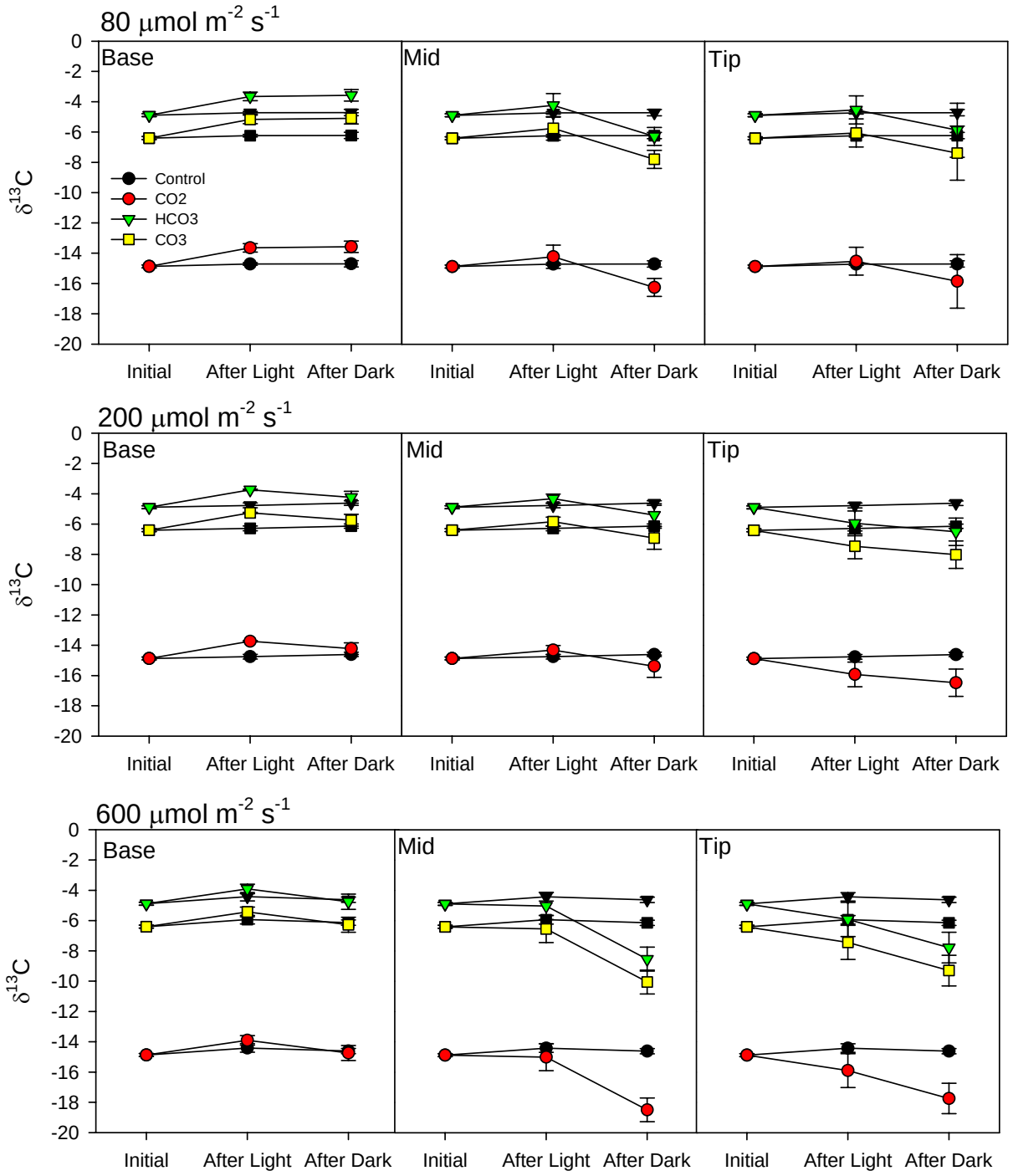

Figure 5-18. $\delta^{13}C$ for carbon species (CO_2, HCO_3^-, $CO_3^{2=}$) for each light level alongside controls. Bars are standard errors (n = 3-5).

5.4 Discussion

The results are discussed chronologically to show the progression of understanding of the carbon uptake and assimilation mechanisms in *V. americana*.

5.4.1 Characterisation of carbon acquisition

Differences in the relative ETR between the leaf sections reflect the light climate the leaves are usually exposed to 80 μmol m^{-2} s^{-1}, 200 μmol m^{-2} s^{-1} and 600 μmol m^{-2} s^{-1}. The diurnal course of rapid light response curves do not show a midday depression rather the maximal ETR is occurs during the middle of the day with lower ETR reached at dawn and during the late afternoon. This indicates that photoinhibition only kicks in during the later stages of the afternoon or possibly that ETR is dependent on the previous day light history

Electron transport rates are calculated using an absorbance factor to account for light absorbed by the chlorophyll rather than reflected or refracted by the leaf surface. For terrestrial plants this factor is approximately 0.84 and is used widely as a relative factor for comparison. *V. americana* had an AF considerably lower than 0.84; tissue within 5 cm of the tip was 0.7, the middle was 0.65 and the base was 0.40. The absorbance value was correlated with chlorophyll *a* content and inversely correlated to leaf thickness, which was probably a function of leaf water content. While submerged plants are often likened to shade plants, it would be expected that submerged leaf material would be optimised for light capture. Results show the opposite and indicate that light is not the primary limiting factor of photosynthesis; it is proposed that the limiting factor is likely to be carbon uptake, while at depth neither light nor carbon warrant the production of high amounts of chlorophyll.

The hypothesis that *V. americana* undertakes CAM as proposed by Keeley (1998) is upheld, however, the extent of overnight acid accumulation is extremely small and varies at different times of the year, in different plants, between leaves and even differs

within one leaf. Weak CAM has been attributed to only a 4 μequiv H^+ change in tropical ferns (Holtum and Winter 1999).

V. americana is a bicarbonate user and can efficiently extract both CO_2 and HCO_3^- from the water, this occurred with a pH increase of up to 4 units, although the upper limit is dependent on alkalinity (Talling 1976) a pH of 10.5 was often reached. Even though *V. americana* is proven to use bicarbonate effectively, maximum rates of oxygen evolution were observed at pH 5.5 where over 90% of carbon was present as CO_2 compared with just 10% HCO_3^-. Where HCO_3^- became increasingly more prevalent (between pH 7 and 9) there were reduced rates of oxygen evolution confirming that *V. americana* higher photosynthesis using CO_2 than HCO_3^-. These instantaneous responses to free CO_2 demonstrates that *V. americana* has the capability to use free CO_2 instantly, however, this may not extend to longer term studies such as Titus (1992) where low pH was found to significantly depress *V. americana* growth, yet Titus (1992) also found that enriched CO_2 levels result in greater biomass production at low pH. Low sensitivity to CO_2 enrichment at high pH also demonstrates the higher CO_2 uptake ability of *V. americana* (Barko *et al.* 1991).

The effectiveness of pH drift comparisons assumes constant alkalinity (discussed further in the following section). This assumption was not initially measured for these incubations and may invalidate the technique; however, regardless of alkalinity it does highlight the ability to instantaneously withdraw carbon from the environment regardless of carbon species available.

The results show that *V. americana*:

- Undertakes a small but significant amount of CAM.
- Uses a combination of bicarbonate and carbon dioxide in carbon acquisition that is dependent on availability of each carbon species, however, there is a preference for use of CO_2 above HCO_3^-.
- The absorbance of tissue within 5 cm of the tip was 70%, the middle was 65% and the base was 40%. This corresponds with studies by Beer and co-workers (1982; 1989; 1998; 1998; 2000; 2000) who found values of less than 84%. The absorbance was correlated with chlorophyll *a* content and inversely correlated to leaf thickness, which was probably a function of leaf water content.

5.4.2 Carbon acquisition along the leaf

Carbon acquisition experiments undertaken along leaves of *V. americana* provide a comparison of tissue acclimated to different irradiances. Differences in chlorophyll and structure between leaf sections show adaptations to various light regimes and may also imply different functions along a leaf. Regardless, the length of the leaf was used to assess a variety of carbon uptake mechanisms present in *V. americana*. It was hypothesised that higher light levels would induce CAM, this was found to be the case where tips of the leaves were shown to accumulate more acid than lower down the leaf. In small incubations significant but small amounts of overnight acid were accumulated on a fresh weight basis in both middle and tip of leaves and while none was accumulated in the base of leaves when the same light was given in the preceding light period. This may be due to the reduced amount of chlorophyll in the base of leaves.

The middle and tip sections also had greater uptake of bicarbonate compared with the base in the light. Slight decreases in pH occurred over the dark period, reflecting re-equilibration of carbon species in leaf water or a pH change with respiration and suggest that bicarbonate is not used during CAM. The mode of bicarbonate uptake in *V. americana* is not well established although it is known that ions are transported across the upper and lower leaf surface (Prins *et al.* 1980) whether different cells are involved in these processes is not known. Many species of the Hydrocharitaceae use either a polarized transport system across the leaves (e.g. *Potamogeton* & *Elodea*) or an anitport system where transport is at the same location (e.g. *Scenedesmus* and suggested for *Chara*). Suggestions of a light sensitive transport in some species (Higginbotham 1973) is supported here where carbon acquisition is not undertaken in the dark regardless of the CAM processes occurring, however, both light and dark induction of polarity have been documented in *Chara*.

During the course of pH drift experiments, the removal of DIC is assumed to occur at constant alkalinity (Stumm; Allen and Spence 1981; Maberly and Spence 1983; Prins and Elzenga 1989). The assumption of constant alkalinity is based on each carbon molecule removed from the medium being replaced stoichiometrically by an OH^- molecule and because there is no change in the ratio of bases and protons in the surrounding medium, the alkalinity remains constant. In these pH drift studies, the alkalinity did not remain constant with a slight rise in alkalinity from all samples.

Inorganic carbon was removed from the medium by tip sections after both light and dark periods while uptake was not show by the middle section of leaves after the dark period. This is consistent with the pH drift results; however, base sections do not extract carbon from solution.

Two different mechanisms of carbon uptake occur in the light and dark periods. Carbon uptake over night was detected in the tip at the same level as daytime carbon uptake. Combined with the lack of discrimination, this shows that CO_2 and not HCO_3^- is used during CAM. Respiratory carbon cycling in middle and lower sections may have occurred thus keeping a stable carbon level in the medium. If this was the case then it is likely there would have been a complementary decrease in pH with the output of CO_2 likely with respiration-this did not occur.

The base had higher discrimination in light incubations and shows greater activity of Rubisco and thus C3 tendency down a leaf, conversely this difference along the leaf may reflect the increasing influence of PEPc towards the tip. This is supported by the increasing acidity accumulation towards the tip.

Base tissue showed clear discrimination of ^{13}C against the DIC in the medium during the light, however, little change in removal of carbon from the medium thus strengthening the suggestion of cycling and possibly the rapid cycling proposed by (Cockburn 1985) where respiratory CO_2 may be used in photosynthesis.

This experiment illustrates that:

During the light, tips of *V. americana* leaves used HCO_3^- and CO_2, and tissue in the middle and base to a lesser extent. The tissue in the middle and base of leaves used proportionately more CO_2 than HCO_3^- compared with tip tissue

During the dark, tip tissue undertakes CAM using CO_2 not HCO_3^-, while tissue in the middle of the leaf undertakes considerably less CAM and basal tissue undertakes virtually no CAM.

5.4.3 Carbon acquisition at three light levels

The hypothesis that CAM would be triggered in the short term by high light and possibly as a stress response was tested in small incubation experiments under three light levels. Results highlighted the complexity of CAM in *V. americana* and ultimately

suggest a more complicated CAM cycling process rather than an ordinary C3-CAM intermediate.

From these experiments in is clear that the extent of bicarbonate use by the different portions of the leaves is not associated with the CAM ability of the leaves. Bicarbonate use was greater in upper portions of the leaves and negligible in the base of the leaves on a fresh weight basis, however, chlorophyll differences between the sections could affect the amount of bicarbonate able to be used by the leaf.

Submerged aquatic macrophytes are often compared with shade plants, therefore a lower chlorophyll *a*/*b* ratio was expected towards the base of leaves where there is a higher amount of shading which would allow a greater efficiency of light utilisation under light limited conditions (Anderson *et al.* 1988) which was expected towards the base of the leaves. This, however, was not the case, rather there was a decrease in total chlorophyll and the ratio was the same.

The decrease in F_v/F_m after each light period indicates photosystem II decreases in efficiency and in particular a decrease in the ability of reaction centres to operate. This is independent of the amount of chlorophyll and so (for the base of leaves in particular) this shows the inability of the photosystem to cope with the incident PAR. The recovery after 10 h of dark show some level of photo repair, however, a significant amount of this reduced yield is likely to be photo damage and small photo protection mechanisms at least in the base of leaves.

NPQ measurements of middle and tip sections all fell in the typical range at saturating intensities (0.5-3.5) (Maxwell and Johnson 2000), however, light above 200 μmol m^{-2} s^{-1} caused a dramatic increase in NPQ for the base of the leaf. The decrease in maximal fluorescence shown by the increase in NPQ was especially apparent in the base of leaves under high light. Strongly stimulated by stress, this is the best signal from the plant in the attempt to keep the electron flow constant and usually amounts to heat dissipation.

It was hypothesized that high light would stimulate more CAM. Over night acid accumulation was extremely small and inconsistent throughout all treatments and may have been due to the reduced light conditions the plants were exposed to prior to the experiment compared with the earlier incubation experiment. The highest ΔH^+ was

observed in the tip and middle sections exposed to low light rather than that expected from high light.

For most tissue sections the resulting $\delta^{13}C$ signature reflects the particular carbon species (CO_2 or HCO_3^-) being used by the leaf tissue. However, the high-light tip and middle sections had much less discrimination and in fact a preference for the heavier carbon isotope. Thus there is an additional effect causing the signature change that is not explained by diffusion of CO_2 or HCO_3^-. The strongly depleted medium left by the tip sections after the dark period hint at CAM, however, could be a result of preferential bicarbonate uptake or preference of the heavier isotope by carboxylation enzymes. As discussed in the previous section, HCO_3^- was not used during CAM, and given that there was virtually no overnight acid accumulation, it is likely there is a different explanation. An alternative mechanism such as CAM cycling may cause the enrichment in the medium with no net H^+ increase overnight.

Carbon uptake is dependent on both the position on the leaf and the light intensity absorbed. This is consistent at light levels above 200 μmol m^{-2} s^{-1}, however, at 80 μmol m^{-2} s^{-1} carbon uptake is inconsistent between positions. These data are not split into carbon species so may reflect a change in carbon species being used or simply a cycling of carbon over a light and dark period.

The draw down in CO_2 and HCO_3^- observed towards the tip was most pronounced for the high light treatment, and could be due to a number of factors:

1. Re-equilibration of carbon in solution
2. Physical processes, including temperature or pH changes
3. Physiological processes (Respiration, Carboxylases: PEPc and Rubisco or Acidification)

Significant assimilation was shown by the middle and leaf tip with a draw down in carbon species leaving solutions void of CO_2 and depleted in HCO_3^- and 4‰ more negative than the original solution and at least 2‰ more negative than after the light period. The dark period following the high light appears to have reversed the carbon proportions (with an increase of HCO_3^- / CO_3^{2-} in solution) yet the $\delta^{13}C$ becomes more depleted, total carbon levels, however, do not alter. The hypothesis is that this shift is due to carbon cycling or depletion of the medium due to PEPc activity, however, other factors need to be considered first such as re-equilibration, pH, temperature and

physiological processes such as respiration. A more negative $\delta^{13}C$ signature would suggest a relative proportional decrease of HCO_3^- compared to CO_2, however, results show the opposite and each carbon species signal becomes more negative which suggests the source carbon pool for the dark changes is simply more negative.

Abiotic factors

Temperature: A decrease in temperature will increase HCO_3^- and decrease CO_3^{2-}. However, the $\delta^{13}C$ did not change across all lights treatments even though the small decrease in temperature would have affected all treatments.

pH: The pH change from 10 to 9 during the dark will also increase HCO_3^- and decrease CO_3^{2-}. However, it is unclear what is driving the decrease in pH, and whether this magnitude of decrease can cause the change in carbon species composition.

A drop in pH of 1 unit will shift the carbon equilibrium such that at 25ºC, HCO_3^- will increase 5 fold while CO_3^{2-} will decrease similarly. Effectively results showed a 2-fold HCO_3^- increase while the CO_3^{2-} almost halved. So theoretically there should have even been a greater change in carbon species pools. But, fractionation is not altered by pH rather the speciation into HCO_3^- and CO_3^{2-} is a function of pH and the $\delta^{13}C$ of the solution changes according to the enrichment factors (Zhang *et al.* 1995):

$$CO_2 \rightarrow H_2O \rightarrow H_2CO_3 \qquad e = -1.2‰ \qquad (5\text{-}1)$$

$$H_2CO_3 \rightarrow H^+ + HCO^{3-} \qquad e = +9.1‰ \qquad (5\text{-}2)$$

$$HCO^{3-} \rightarrow H^+ + CO_3^{2-} \qquad e = -2.0‰ \qquad (5\text{-}3)$$

Therefore, proportionally more HCO_3^- will cause the solution to become less negative, which is the opposite of the $\delta^{13}C$ values observed.

Indirect effect of precipitation: Carbonates may have precipitated out of solution on to the leaves and effectively been removed from the assessment of DIC. $CaCO_3$ has a very low solubility (0.87×10^{-8} mol L^{-1} at 25ºC) and precipitation occurs at pH greater than c. 8.5 as the dissolved CO_2 is minimal and then even a small reduction in CO_2 will set the equilibrium to produce enough calcite to exceed the $CaCO_3$ precipitation

$$Ca(HCO_3)_2 \Leftrightarrow CaCO_3 \downarrow + H_2O + CO_2$$

Respiratory release of CO_2, however, would pull calcite out of precipitation rather than cause precipitation. So as shown, precipitation would only occur if CO_2 was being removed from the solution. It is more likely that dark respiration is taking place so that CO_2 is being added to the solution. This would do the cause the $CaCO_3$ that had precipitated to come into the solution and effectively increase the DIC.

Physiological-Respiration: Respiration may well occur over the dark period such that CO_2 is released from the leaf and cause an increase in carbon pools in the medium (which was not observed). Any release of CO_2 would cause a decrease in pH and is likely to be the cause of the shift of the equilibrium in the direction observed. However as shown previously this speciation shift does not cause an enrichment of the water, rather it will cause depletion.

Factors that may cause the depletion of water include the use of PEPc as the primary carboxylase which does not discriminate against the heavier isotope.

Other uptake mechanisms such as bicarbonate uptake would also cause this depletion. Bicarbonate uptake, however, does not occur in the dark. Previous experiment demonstrated this, however, the techniques do rely on pH increase over the light period to demonstrate HCO_3^- use by pH drift. Carbon cycling through respiratory carbon and combined with use of a non-discriminatory enzyme such as PEPc is perhaps possible, even with no change in H^+.

While the base of the leaves appear to undertake C3 photosynthesis with a consistent enrichment in ^{13}C of the medium in incubations, this opposite is true for the middle and tip of leaves where analysis of the final medium after the dark period suggest the leaf material became more enriched. This enrichment indicates the use of PEPc rather than Rubisco as the primary carboxylation enzyme. This is most pronounced for the middle and tip from the high light treatment collected after the dark period with a $\delta^{13}C$ of -17.50‰ and -16.75‰ compared with the original medium $\delta^{13}C$ of -13.9‰.

This experiment illustrates that:

- High light triggers HCO_3^- use in the tips of *V. americana* leaves but not in the middle or base of *V. americana* leaves.

- Very small amounts of CAM can have dramatic effects on the carbon species proportions (detectible in small volumes) in the dark. This is most pronounced when combined with high light during the previous light period.

5.4.4 Hypotheses Revisited

Vallisneria americana acquires CO2 and HCO3- in proportions dependent on those in the water.

Both light intensity and dark/light period change the proportion of HCO_3^- versus CO_2 use and so the above hypothesis does not hold. During the light, tips of *V. americana* leaves used both HCO_3^- and CO_2 while the tissue in the middle and base of leaves used proportionately more CO_2 than HCO_3^- compared with tip tissue. Bicarbonate use is stimulated in high light and it appears that in *V. americana* this is an acclimation and can not be triggered in tissue acclimated to low light and thus acclimated to CO_2 use.

***Vallisneria americana* is a C3-CAM intermediate, whereby the relative contribution of C3 and CAM to carbon fixation in *V. americana* varies with the environmental conditions (light, temperature, pH).**

The hypothesis that *V. americana* undertakes crassulacean acid metabolism as proposed by (Keeley 1998) is upheld by overnight acid accumulation, however, the extent is extremely small and varies at different times of the year, in different plants and leaves. This level of weak CAM has been documented in tropical ferns (Holtum and Winter 1999) where down to 4 μequiv H^+ has been observed.

V. americana is a C3-CAM intermediate with patchy CAM between plants, within plants and within leaves. CAM was highly dependent on light and was present at different times dependent on light levels, pond location, time and season. Higher CAM was shown in the tips of leaves this being related to the amount of light the plant is adapted to rather than the instantaneous light climate applied, such that short term changes in light climate (such as might be observed with a decrease in water level) will not allow plants to switch to CAM instantaneously. The extent of this, however, is unclear as the patchiness of CAM in *V. americana* is large.

Bicarbonate use of *V. americana* was well documented with reports of pH increases, however, along with CAM, *V. americana* uses CO_2 rather than HCO_3^- during CAM dark uptake.

In this study, the lack of a consistent trend in malate accumulation in *V. americana* highlights the variability in CAM. The short-term acclimation expected with 10 h of high light did not occur; it is possible that the carbohydrate reserves require a much longer acclimation to light. Yet, the significant and consistently negative signatures left behind in the incubating medium by tip and middle at high light, and the lack of malate accumulation measured could imply CAM cycling (only nocturnal respiratory CO_2 is fixed by PEPc (Cockburn 1985)) where the nocturnal flux through PEP carboxylase is used simply to reduce the loss of CO_2 at night via respiration.

This has been suggested in passing by (Cockburn 1998) where *Vallisneria spiralis* has show CAM patterns of $^{14}CO_2$ acquisition and isotope discrimination, however, with a lack of overnight acid accumulation. In this study overnight accumulation has been demonstrated in some (however inconsistent) instances. This may provide evidence towards rapid-cycling CAM as suggested by Cockburn (1998) where the CO_2 acquiring and CO_2 reducing phases of CAM alternate entirely within the photoperiod.

Thus the patchy nature of overnight malate accumulation appears to be a function of CAM cycling rather than fluctuating C3-CAM intermediate status of *V. americana*. CAM cycling possibilities in *V. americana* could well do with further investigation.

The extent of CAM in *V. americana* is independent of carbon species acquired such that CAM will use both HCO_3^-- and CO_2 forms of carbon during dark uptake.

Uptake of carbon during the dark by CAM appears to be dominated by CO_2 rather than HCO_3^-. The mechanisms surrounding this preference are unclear. This may be an environmental effect where respiration causes a change in the conditions immediately surrounding the leaf; however, results suggest that CO_2 is assimilated while HCO_3^- is left behind in solution overnight.

Chapter 6
PHOTOSYNTHESIS COMPARED IN TWO POTAMOGETON SPECIES

6.1 Introduction

Plasticity in carbon acquisition among aquatic macrophytes facilitates resource capture (Maberly and Madsen 2002), and is a common response to changes in growth conditions (Madsen *et al.* 1996). This chapter examines the interplay between the utilisation of light and carbon (both CO_2 and HCO_3^-) in two *Potamogeton* species, one of which is heterophyllous and was thus proposed to have an enhanced ability to 'hedge its bets', and so maintain CO_2 uptake in habitats with a variable light climate.

Potamogeton crispus L. (curly pondweed) and *P. tricarinatus* F. Muell. and A. Benn. Ex A. Benn. (1892) (floating pond weed) are rooted plants common in wetlands associated with the River Murray. *P. crispus* is a fully-submerged perennial monocot, with stems up to 1 m long and leaves 2-6 cm long, with narrow, crenate margins. *P. crispus* occurs in lentic and lotic habitats throughout the world, but is native to Eurasia and Australia. It can grow in clear to turbid, polluted waters and in alkaline or brackish waters (Stuckey 1979). Although seed is produced, dispersal is essentially through turions, and once established, the plants form colonies from rhizomes. In most of its range, *P. crispus* typically reaches peak biomass in late spring to early summer, forms turions, then declines and "survives" the warmer months in a dormant state (Woolf and Madsen 2003). In the River Murray, *P. tricarinatus* and *P. crispus* are associated with the middle and upper weir pools, where water levels may vary by an average of up to ±20 cm daily (Walker *et al.* 1994).

P. tricarinatus occurs in still or slow-flowing water up to 2.5 m depth, on muddy bottoms in virtually all kinds of freshwater environments (Aston 1973). It is an heterophyllic species with alternate leaves that may be all submerged, or some submerged and others floating. Heterophylly has a key role in how adaptive aquatic plants are to changing environmental conditions such as water level and light (Wells and Pigliucci 2000). Phenotypic plasticity is known to be advantageous in heterogeneous environments (Bradshaw 1965), like those encountered in wetlands. Differences may occur between species, among species, or even within a single plant or leaf (Winn and Evans 1991).

Heterophyllous leaves may reflect a developmental transition or a plastic response to environmental conditions (phenotypic plasticity) (Wells and Pigliucci 2000), or an interaction between the two (Winn and Evans 1991; Winn 1996). The phenotype, however, is dependent on both environmental and genetic factors (Cronin and Lodge 2003). Inducible changes in phenotype are viewed as an adaptive trait to deal with environmental variability (Grime *et al.* 1986). In addition to access resource to aid growth, heterophylly can be mediated by environmental cues and phenotypic change caused by resource limitation (Smith-Gill 1983).

Within-individual differences exist in morphological, anatomical and physiological characteristics of leaves along environmental gradients (e.g. light and carbon) (Winn and Evans 1991). Leaf morphology, however, can have a similar response to more than one environmental cue. For example, morphological changes of submerged leaves in response to low PFD are similar to those in response to low CO_2 availability (Mommer *et al.* 2005). Morphological responses to submergence, hence carbon limitation, are often similar to those for light limitation (Mommer *et al.* 2005), and similar between species (Wells and Pigliucci 2000). Comparisons are shown below in Table 6-1, modified from Wells and Pigliucci (2000).

Table 6-1 Characteristic responses to light PFD (sun vs. shade leaves) and submergence (submerged vs. emergent leaves) in terrestrial and heterophyllous aquatic species, respectively. [a]Depends upon generalized leaf form: monocot leaves typically become more linear (sometimes wavy at margins), and dicots more lobed/divided, underwater. [1]Lewis (1972) and references therein. [2]Givnish (1987) and references therein. [3]Sculthorpe (1967). Table modified from Wells and Pigliucci (2000).

Trait	**Response to decreased light in PFD in terrestrial species[1,2]**	**Response to submergence in heterophyllous aquatic species[3]**
Specific leaf area	larger	larger
Leaf thickness	thinner	thinner
Leaf margins	less lobed and/or toothed	variable[a]. more linear/lobed
Stomatal frequency	lower	lower
Mesophyll	reduced palisade layer	reduction to complete absence of (shorter cells and fewer layers) palisade layer
Venation	reduced vein density	reduced vein density
Cuticle	reduced	reduced or absent
Epidermal cells	larger, more undulate margins	Larger, long and narrow
Location of chloroplasts	more epidermal	found in epidermis

Two *Potamogeton* species (*P. tricarinatus*, *P. crispus*) co-occur in extremely turbid, deep water (>1 m) at Ral Ral Creek, a small anabranch stream entering the River

Murray near Remark, South Australia. *P. crispus* occurs as isolated individuals or in groups (area < 4 m^2) within patches of *P. tricarinatus.* The latter occurs as three more or less distinct morphs, described more fully later, and demonstrates considerable morphological plasticity.

P. crispus has submerged leaves only, and so relies solely on dissolved CO_2 and bicarbonate (HCO_3^-); photosynthesis is primarily limited by diffusive resistance to CO_2 (Madsen and Breinholt 1995) and light. *P. tricarinatus* has submerged leaves which acquire carbon like those of *P. crispus*, but because it can also have floating leaves it may access atmospheric CO_2.

The effect of submergence, in part, limits the carbon supply through the increased diffusive resistance of water to CO_2. Other physiological and morphological responses, however, are similar to those caused by low light (Vervurenm *et al.* 1999; Mommer *et al.* 2005), and it is difficult to isolate the causal factor. The effects of carbon limitation are well understood, but it is not known how these effects may interact with those of a strong vertical gradient in PFD (as in the highly turbid Lower Murray). However, increased carbon supply gained through access to HCO_3^- may increase light use efficiency and thus aid photosynthesis (Andersen and Pedersen 2002).

Increased diffusive resistance in water is usually the limiting factor controlling photosynthesis for submerged leaves (Maberly and Spence 1983) and aerial leaves function to enhance CO_2 acquisition (Madsen and Breinholt 1995). High turbidity reduces the penetration of light, such that carbon may no longer be the only factor limiting photosynthesis. The high diffusive resistance of carbon is predicted to remain limiting for all but the deepest of leaves.

In terrestrial plants, shade leaves are characterised by a broad, flat morphology and high chlorophyll concentration per leaf area to maximise light capture. Photosynthesis is typically light limited and photosynthesis usually saturates at PFDs below that of sunlit leaves. In the aquatic habitat, submerged leaves are analogous to shade leaves. Where the lower light saturation of photosynthesis compared with floating leaves and the large surface area to volume morphology enhances carbon acquisition (Mommer and Visser 2005).

Mommer *et al.* (2005) and Vervurenm *et al.* (1999) suggested that responses to carbon supply on submergence are analogous to responses to low PFD. Atmospheric CO_2 is

easier to acquire than aqueous CO_2 or HCO_3^- due to the high diffusive resistance of CO_2 in water, so that floating leaves serve to better access carbon.

What are the adaptive advantages of heterophylly? This chapter explores one central hypothesis:

Heterophylly in *P. tricarinatus* is an adaptive trait to accommodate a strong vertical light gradient and a rapid transition from DIC to atmospheric CO_2 as a carbon source for photosynthesis.

P. tricarinatus exhibits differences in leaf morphology, anatomy and physiology in response to heterogeneous environments, and should have a higher fitness relative to less plastic species (Wells and Pigliucci 2000) such as *P. crispus,* where the submerged form is dependent on a minimum light climate yet can tolerate variable inorganic carbon conditions. In *P. crispus* under light limited environments, the affinity for both CO_2 and HCO_3^- is reduced and photosynthetic capacity is suppressed (Maberly and Madsen 2002).

The morphology of emergent leaves aids carbon uptake in air with use of stomata while submerged leaves have a large surface area to volume ratio, where HCO_3^- uptake is further facilitated by high light in the upper water column. The trade-off between light capture and carbon uptake ability (Kübler and Raven (1995) may be exploited by *P. tricarinatus* to optimise carbon acquisition strategies at different light levels.

In floating leaves, high PFD coupled with high carbon availability enables high rates of carbon fixation. The effectiveness of carbon acquisition changes with light availability by adjusting the carbon uptake apparatus to the type and availability of carbon (Maberly and Madsen 2002). Light use efficiency is then coupled with carbon use efficiency (Andersen and Pedersen 2002).

When atmospheric CO_2 is assimilated through aerial leaves, a depleted $\delta^{13}C$ signature (~ -29‰) reflects the C_3 pathway of assimilation through the discrimination of Rubisco (O'Leary 1981). Submerged leaves show a less depleted $\delta^{13}C$, due to the decreased fractionation associated with high diffusive resistance coupled with the more enriched HCO_3^- source (Osmond *et al.* 1981; Raven *et al.* 1987). Measurements of $\delta^{13}C$ should then indicate the source of carbon.

6.2 Methods

6.2.1 Species description

The Potamogetonaceae is a small but morphologically diverse family, ubiquitous in still or flowing, fresh or slightly brackish water. *Potamogeton tricarinatus* and *P. crispus* are common representatives, alongside familiar freshwater and estuarine genera such as *Ruppia*, *Thalassodendron* and *Zostera*.

The *P. tricarinatus* complex is currently being divided into several species (pers comm. Wilkins 2005); it is likely that *P. tricarinatus* in this study will become "*P. sulcatus*" and *P. tricarinatus* will refer to a species in Northern Queensland. The name "*P. tricarinatus"* is used here to facilitate comparisons with earlier studies.

P. tricarinatus is an heterophyllic aquatic herb with alternate leaves which may be all submerged, or some may be submerged and others floating. The latter usually are ovate or elliptical, 7-10 cm long and 5-7 cm wide, with a waxy surface, while submerged leaves are 10-15 cm long, 2-10 cm broad and only a few cells thick (Aston 1973). *P. crispus* is a submerged perennial, with stems up to 1 m long and leaves 2-6 cm long with a narrow, minutely toothed and curly margin (Aston 1973).

P. tricarinatus individuals at Ral Ral Creek represented different stages of growth (Figure 6-1). Submerged leaves were assigned a typology, useful for assessment of light and carbon use, as follows:

'Type S-S' submerged leaves on plants that were fully submerged,

'Type S-F' submerged leaves on plants that also had floating leaves.

The most common type (S-F) consisted of 2-6 floating leaves and up to 10 submerged leaves; these were often found in sparse groups or on the edges of larger more dense populations. Fully submerged plants (S-S) had elongated rather than elliptical leaves. *P. crispus* occurred individually or in small populations of less than 4 m^2, among and between larger patches of *P. tricarinatus.*

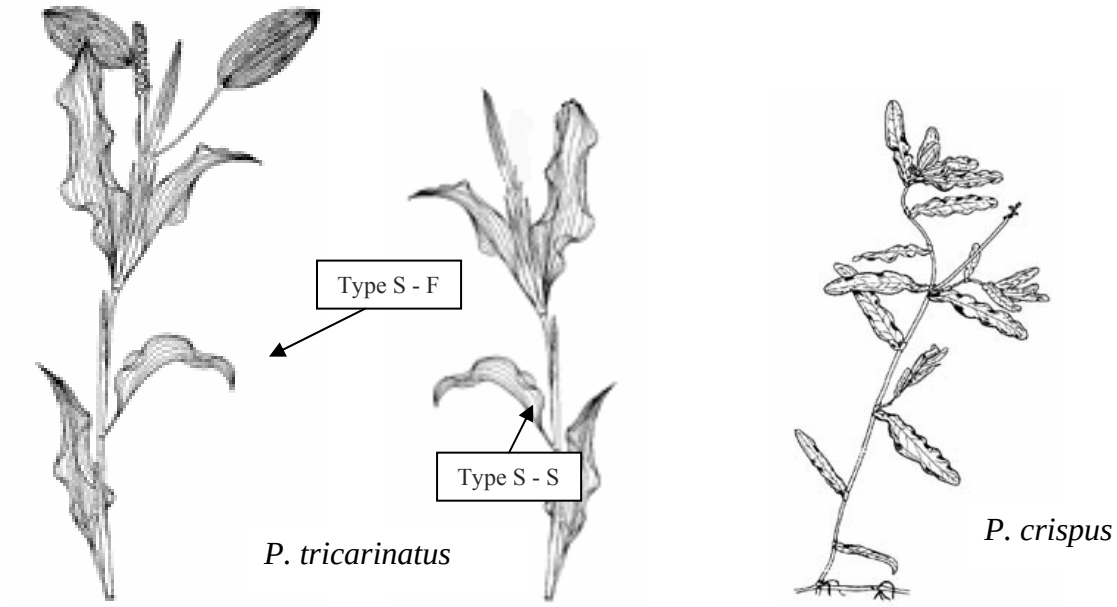

Figure 6-1. *Potamogeton tricarinatus* and *P. crispus*, showing different leaf types

6.2.2 Site Description

Ral Ral Creek is an anabranch of the River Murray, located in North Renmark (56º12'52.5"N 140º33'29.1"E). Figure 6-2). *P. tricarinatus* and *P. crispus* were sampled twice during 2005: during low light conditions on January 5 and in high light conditions on February 12. Ral Ral Creek is a permanent stream, with water level affected by a downstream weir (Lock 5) and floodplain wetlands (e.g. Lake Merreti). During January and February 2005 there were dense clumps of *P. tricarinatus* and sparse *P. crispus, Vallisneria americana* and *Myriophyllum* spp. Depth was 1-2 m.

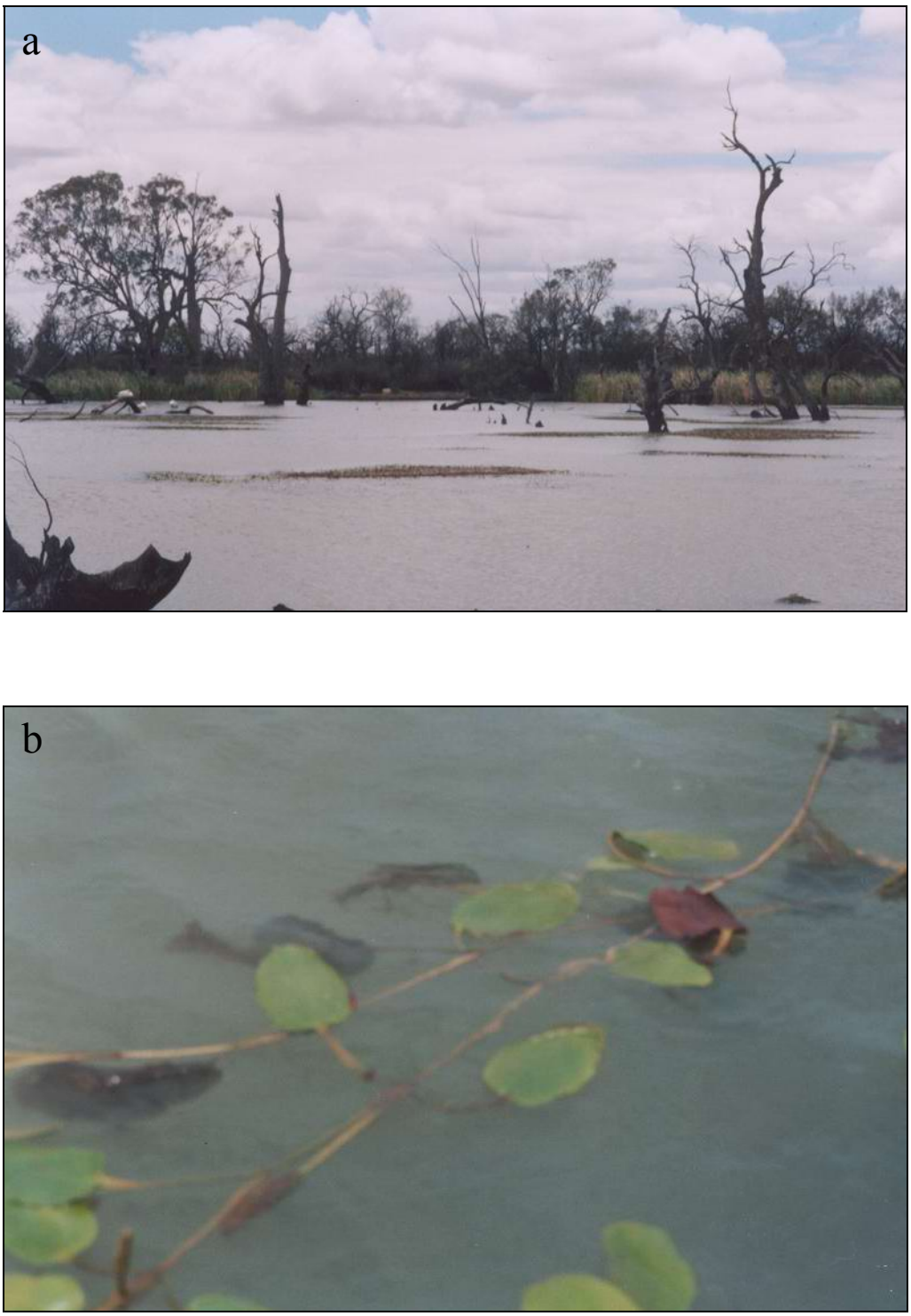

Figure 6-2. Ral Ral Creek with *P. tricarinatus* showing (a) floating leaves in small and large clumps and (b) floating leaves. Turbidity of the water is high and the submerged vegetation cannot be seen in this photograph.

6.2.3 General methods

On 12 January and 2 February 2005, between 11 and 3 pm, leaf and water samples were collected and *in situ* water characteristics analysed. Leaf samples were collected from a canoe, from four of ten large (>10 m^2) clumps of *P. tricarinatus* about 5-10 m apart along a 150 m stretch of Ral Ral Creek. At each time, measurements were undertaken on 4 leaves, and 10 leaves of each type were collected (floating leaves and submerged leaves from S-F plants and submerged leaves from Type S-S plants). No distinction was made between floating leaves on plants with or without submerged leaves. Collected material was stored for analysis as described below.

Water chemistry and light

Measurements of pH, conductivity and temperature were undertaken at midday from 4 locations within the 100 m stretch of water, using a hand held pH meter and thermometer. Measurements were in both open water and within the patches of *P. tricarinatus*. Water was collected from beneath the surface in 500 mL containers and filtered through Whatman #1 filter paper. Alkalinity was assessed on 100 mL of filtered water by the potentiometric method per Golterman *et al.* (1978) (Section 2.7.1). Total Inorganic Carbon (TIC) and $\delta^{13}C$ of TIC were measured from 8 mL of filtered water syringed into evacuated Exetainers (Labco, High Wycombe, UK) on site; these were stored at -4ºC in the dark until analysis. Stable carbon isotope signature of the inorganic carbon was assessed on return to the laboratory after acidification with hydrochloric acid (Section 2.5.3).

Photon flux density (PFD) was measured in the air before and after sampling and before and after fluorescence measurements, using a Li1000 data logger and sensor. In addition, light penetration was measured at 5 locations to 40 cm depth in 5 cm increments using a calibrated underwater LiCor light sensor.

Chlorophyll

Four replicates of each leaf type were collected for chlorophyll assessment and stored in water in the dark. Chlorophyll *a* and *b* were measured on the following day, according to Porra *et al.* (1989) (Section 2.2).

Fluorescence

A Pulse-Amplitude Modulated fluorometer (mini-PAM, Walz GmbH, Effeltrich, Germany) was used for all chlorophyll fluorescence measurements, using the leaf

distance clip. Standard settings and calculations were used, according to Section 2.3. Four populations were sampled between 11:00 and 14:00 within a 200 m section of Ral Ral creek; each leaf per population was considered a replicate. Effective quantum yield (ΦPSII) and rapid light curve measurements were undertaken on floating and submerged leaves *in situ.* Leaves were then removed, kept in water and dark-adapted for 20 minutes using black plastic bags. Optimum quantum yield (F_v/F_m) and dark-adapted rapid light response curves were determined in succession (Section 2.4).

Leaves for absorbance measurements were collected, stored in river water in the dark and measurements undertaken in laboratory conditions no more than two days after collection. Transmittance and reflectance of floating and submerged leaves were measured, and absorbance calculated according to Section 2.4.4.

Stable isotope fractionations

Leaves for isotope analysis were collected from floating and submerged plants at midday, rinsed in deionised water and patted dry. A modified procedure (Brugnoli *et al.* (1988)) was used to extract soluble sugars for $\delta^{13}C$ (Section 2.5.2). Leaves for bulk organic carbon isotope analysis were stored in paper bags and dried at 40ºC for 48 hours before analysis according to Section 2.5.3. Leaves for carbohydrate analysis were frozen immediately in liquid nitrogen and stored at -80ºC. However, during final stages of processing a drying oven over-heated, destroying most of these samples. $\delta^{13}C$ values from samples recovered from the oven are presented, but there are no replicates.

Microscopy of leaf sections

Leaf sections were made two days after collection from four replicates of each leaf type collected from Ral Ral Creek and stored in water in the dark using a protocol adapted from O'Brien (1981) (see Section 2.8).

6.2.4 Statistics

Results were analysed with Minitab 14.1® (Minitab Inc., State College, PA) software. Analyses of variance (ANOVA) were used to compare treatment means (α= 0.05).

6.3 Results

6.3.1 Morphology of floating and submerged leaves of P. tricarinatus

There were large differences in morphological characteristics of floating and submerged *P. tricarinatus* leaves (Figure 6-3). Type S-F submerged leaves were linear and narrow, and Type S-S submerged leaves were ovate or elliptical. The submerged leaves of *P. crispus* did not have variable morphology.

Floating leaves of *P. tricarinatus* had typical C_3 leaf structure, with stomata evident on the adaxial side (exposed to air), but not on the abaxial side (in contact with the water). There was a distinct adaxial palisade mesophyll approximately 200 μm thick and a clear epidermis, while large air spaces were apparent towards the abaxial side were approximately 300-400 μm thick (Figure 6-3a).

Submerged leaves had no palisade mesophyll and had only one cell layer between air spaces and water; this was also evident in the floating leaves on the adaxial surface in contact with the water (Figure 6-3b). Vascular tissue was similar in size, cell size and partitioning between the xylem and phloem.

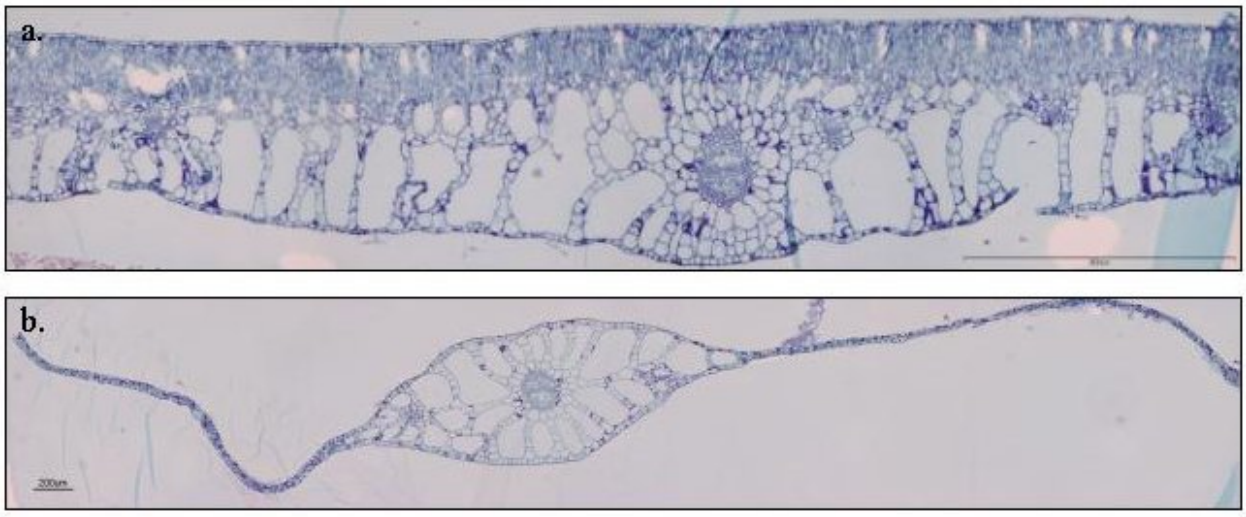

Figure 6-3. Cross-sections of the two leaf types of *Potamogeton tricarinatus*: (a) floating leaf, scale 1 mm and (b) submerged leaf, scale 200 μm.

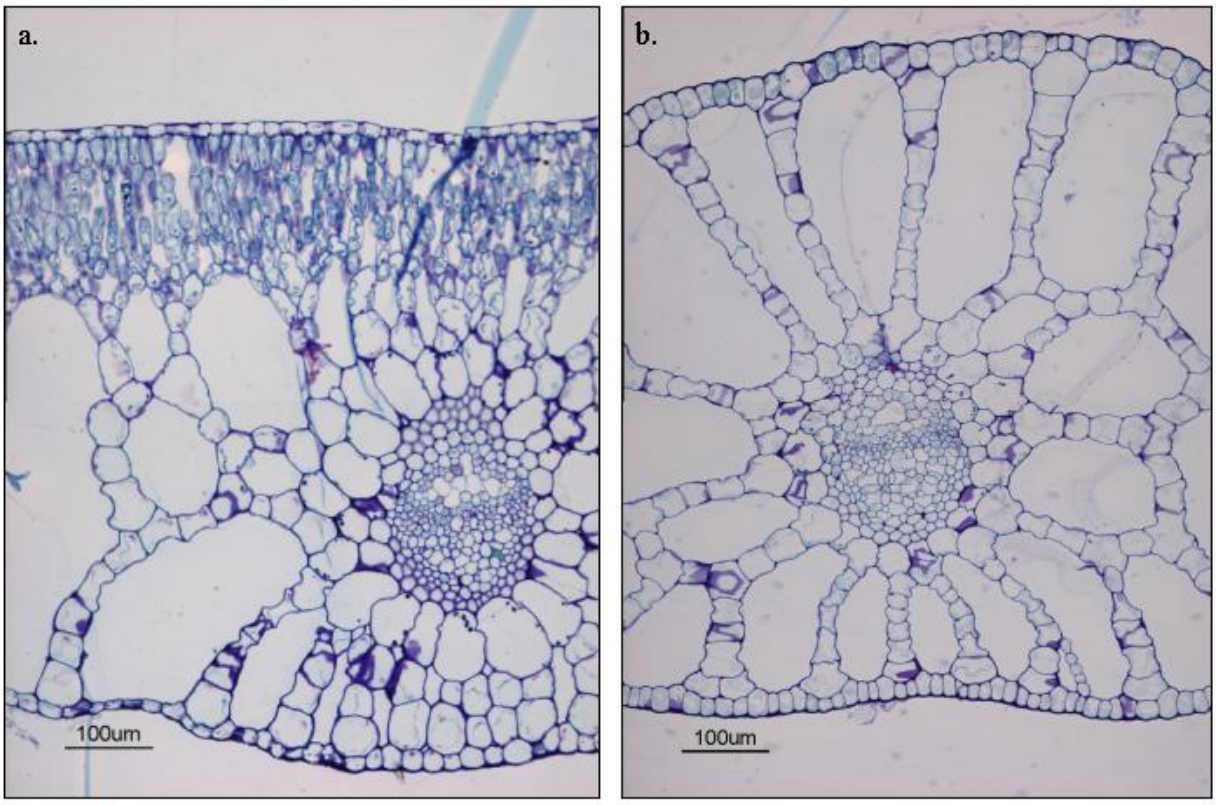

Figure 6-4. Magnified cross-sections of (a) floating leaved *Potamogeton tricarinatus* and (b) submerged *P. tricarinatus* leaves at 20x magnification. Scale 100 μm.

Chlorophyll content

In leaf sections, chlorophyll in the palisade of the floating leaves is stained with Toluidine Blue O; the location of chlorophyll is not clear in the submerged leaves (Figure 6-5). Floating leaves had a chlorophyll concentration of 0.91 nmol Chl g^{-1} fw while submerged leaves had a concentration of 0.51 nmol Chl g^{-1} fw. Total chlorophyll of floating leaves was almost twice that of submerged leaves ($F_{1,9} = 23.63$, $P = 0.001$), due to both higher chlorophyll *a* ($F_{1,9} = 39.54$, $P = 0.000$) and chlorophyll *b* ($F_{1,9} = 9.44$, $P = 0.015$). The chlorophyll *a*/*b* ratio of floating leaves was 1.6 which was significantly higher than submerged leaf chlorophyll *a*/*b* ratio of 1.1 ($F_{1,9} = 88.37$, $P < 0.005$) (Figure 6-5a).

Light absorbance

PFD absorbed by submerged leaves ($AF_{15} = 0.51 \pm 0.01$) was 60% lower than that assumed for most terrestrial leaves (84%) (Figure 6-5b), but was higher for floating leaves at 92%.

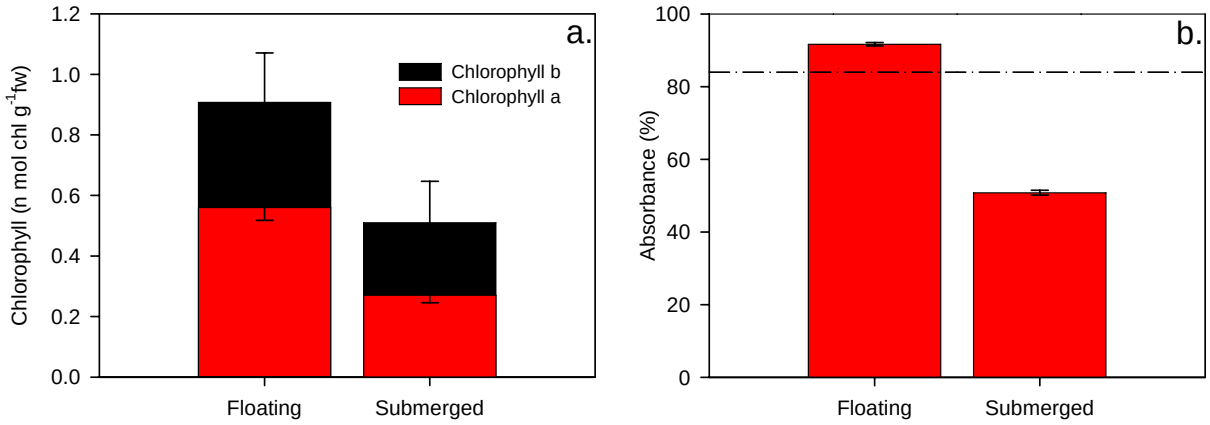

Figure 6-5. Chlorophyll *a* and *b* of floating and submerged leaves of *Potamogeton tricarinatus* (a). Bars are standard errors.

6.3.2 Carbon partitioning between submerged and floating leaves

Water chemistry

The pH of Ral Ral creek ranges between 6.2 and 9.5 (Anonymous 1997). During 2005, the pH was stable and averaged 7.2±0.2 on 5 January and 7.16±0.0 on 12 February; differences were negligible between water within the floating populations of *P. tricarinatus* and open water. Water temperature at 10 cm depth was 21.6ºC±0.1 on 5 January and a constant 22.7ºC on 12 February; conductivity was stable at 217 µS cm^{-2} for both dates.

Ral Ral Creek is generally not a well-buffered system: total inorganic carbon in January was 11.3±0.43 mg C L^{-1} and in February this decreased to 7.35±0.58 mg C L^{-1}. Total alkalinity or acid-neutralizing capacity (ANC) (OH^- + CO_3^{2-} + HCO_3^-) was 1.25 mmol L^{-1}, equivalent to calcium carbonate alkalinity of 62.55 mg L^{-1}. As the pH was below 8.3, the alkalinity was represented by carbonates (CO_3^{2-} + HCO_3^-). Total carbon, incorporating free CO_2 (CO_3^{2-} + HCO_3^- + CO_2), was 1.61 mmol L^{-1}.

Two sources of carbon were available, namely atmospheric CO_2 and aqueous carbon including $CO_{2\,(aq)}$, $HCO_{3\,(aq)}^-$ and $CO_{3\,(aq)}^{2-}$. Total dissolved inorganic carbon content in February was 0.1 mmol L^{-1} lower than January ($F_{1,14}$ = 30.99, P = 0.000) (Figure 6-6a). Due to the relatively neutral pH most carbon was calculated to be present as HCO_3^- (91.86-91.49%), CO_2 contributed less than 10% (7.7-8.1%) with negligible CO_3^{2-} (Figure 6-6a). The February δ^{13}DIC (-13.13±0.27) was significantly more enriched than water sampled during January (-15.17 ± 0.29) ($F_{1,14}$ = 24.28, P = 0.000) (Figure 6-6b). δ^{13}C of atmospheric CO_2 is close to -6‰ and following C_3 photosynthesis and fixation

by Rubisco it is assumed that plant material would typically be between -25‰ and -30‰. The proportion of carbon species remained consistent between January and February, while the individual $\delta^{13}C$ for each carbon species reflect the overall more enriched $\delta^{13}C$ of the water during February (Figure 6-6b).

Table 6-2. Carbon species proportions and $\delta^{13}C$ signature as calculated from pH, temperature, Conductivity and total inorganic carbon modelled using equations from equilibrium, solubility and dissociation constants (Johnston 2004).

	HCO_3^-		CO_2		CO_3	
	$\delta^{13}C$	%	$\delta^{13}C$	%	$\delta^{13}C$	%
January	-6.43	91.86	-16.15	7.71	-7.89	0.43
February	-4.26	91.49	-14.11	8.11	-5.75	0.40

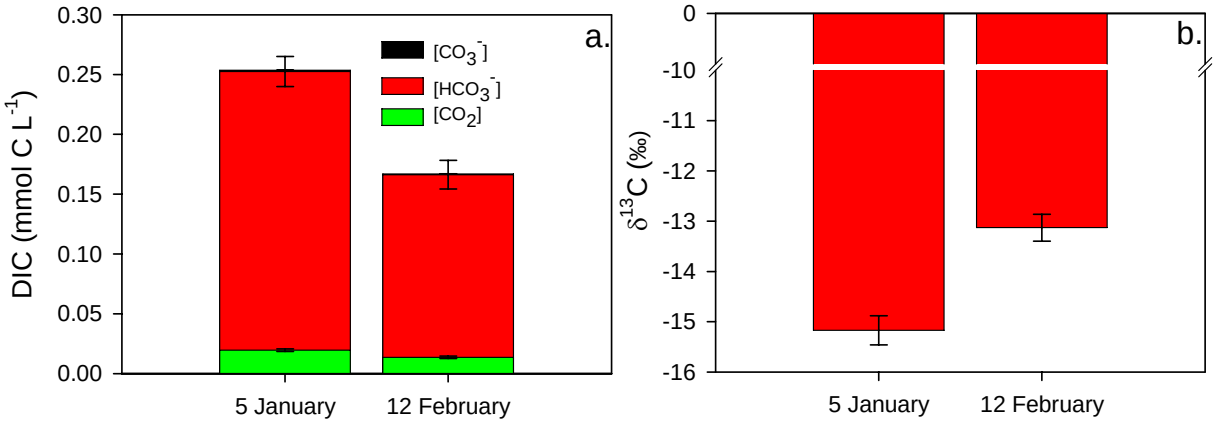

Figure 6-6 Total inorganic carbon (a) and $\delta^{13}C$ of dissolved inorganic carbon (b) from Ral Ral Creek on sampling dates. Bars are standard errors (n = 5).

Carbon partitioning between leaf types

One of the principal aims of this work was to trace the carbon source for submerged and floating leaves by investigation of acquired and assimilated carbon. Dissolved inorganic carbon (DIC) had a $\delta^{13}C$ signature of -15.17±0.29 in January and a $\delta^{13}C$ of -13.13±0.27 in February. Atmospheric CO_2 as a source can be traced through a combination of known fractionation factors. $\delta^{13}C$ of atmospheric CO_2 is close to -6‰ and following C_3 photosynthesis and fixation by Rubisco it is assumed that plant material would typically be between -25‰ and -30‰.

Fully submerged (Type S-S) *P. tricarinatus* leaves had more enriched bulk organic material $\delta^{13}C$ (-17.79±0.93) compared with floating leaves (-24.76±0.73) ($F_{1,5}$=16.53, P =0.001); this was also the case for submerged leaves of *P. crispus* and *P. pectinatus* found at the site (Figure 6-7a). Submerged leaf $\delta^{13}C$ values are comparable to the

source carbon δ^{13}DIC of -15.17±0.29, representative of the time when structural carbon would have been assimilated, however, the extent of the δ^{13}C fluctuation in the time between January and February is unknown. The Type S-F submerged leaves had a δ^{13}C in the same range as floating leaves (similar to that expected of an atmospheric CO_2 using leaf (less than ca. -27‰).

Floating leaf soluble carbohydrate δ^{13}C had a more depleted δ^{13}C signature (-28.86‰±3.15) than either of the two submerged leaf groups (Figure 6-7b) and more depleted than the corresponding bulk organic δ^{13}C signature (by ca. 4‰). All carbohydrate pools were more depleted than source water δ^{13}C, and surprisingly more so compared with the enriched February DIC δ^{13}C. Soluble carbohydrate δ^{13}C of the type S-S submerged leaf was also more depleted than the corresponding bulk organic material δ^{13}C by ca. 6‰. In contrast, the Type S-F submerged leaves were more enriched. Although many samples were lost during processing, these pooled results do show trends in isotopic composition.

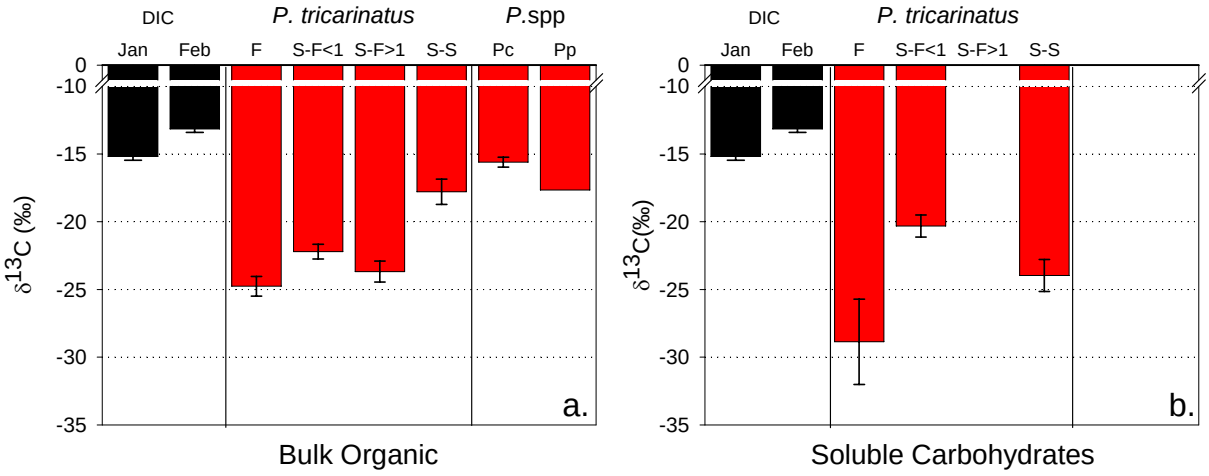

Figure 6-7. δ^{13}C of dissolved inorganic carbon and bulk organic leaf material from *P. tricarinatus*; *P. crispus* (Pc) and *P. pectinatus* (Pp) are also included as fully submerged leaves (a), and δ^{13}C of soluble carbohydrates for *P. tricarinatus* with similar symbols (b). Floating leaves (F), submerged leaves on plants that also had floating leaves (S-f), and submerged leaves on plants that are fully submerged (S-S). S-f leaves are also divided into leaves within 1 m of the surface (<1) and those at greater than 1 m depth (>1). Bars are standard errors (n = 4-5 for organics; n = 2-4 for soluble carbohydrates).

6.3.3 Light responses of floating and submerged leaves

Light climate

The two sampling days were assigned 'Low Light' (c. 700 µmol m^{-2} s^{-1}) and 'High Light' (ca. 1805±20 µmol m^{-2} s^{-1}) for 5 January and 12 February, respectively (Table

6-3). Maximum air temperatures varied less than 1ºC between sampling days while water temperature was constant at 22.7ºC. Historical records suggest that the conductivity of Ral Ral Creek seasonally fluctuates between 185 and 880 μS cm^{-2} (Anonymous 1997), and that salinity is lowest in spring, when water levels are highest. Conductivity was 180 μS cm^{-1} and 217 μS cm^{-1} during January and February 2005, respectively.

Table 6-3. Meteorological data from Renmark Research Centre (Rainfall and Temperature) and Loxton Research Centre (Cloud Cover) compared with PFD measured on the day. Cloud cover is measured in 8ths (oktas): 8 = overcast and 0 = clear.

		PFD_{max} (μmol m^{-2} s^{-1})	Air Temperature (ºC)		Cloud Cover (oktas)	
			Max	Min	9am	3pm
Low Light	4 /1/2005		26.4	15.4	6	6
	5/1/2005	700±100	26.2	15.4	5	7
High Light	11/2/2005		27.1	12.5	1	1
	12/2/2005	1805±20	27.0	14.5	3	0

Water in Ral Ral Creek is turbid. During February, reflectance accounted for a 49% drop in light at the surface, probably due to ripples from wind. Reflectance values, however, are more commonly between 11 and 22% in the River Murray at sites near the mouth of Ral Ral creek (Oliver 1990). Light attenuation ranges between 10 Nephelometric Turbidity Units (NTU) and 390 NTU. On 12 February 2005, PFD measured above the water surface was consistently high with little cloud cover (1800 μmol m^{-2} s^{-1}).

Fluorescence

Midday dark-acclimated F_v/F_m during January was the same for floating (0.74±0.04) and submerged leaves of *P. tricarinatus* (0.71±0.04) ($F_{1,7}$ = 4.18, P= 0.087). ΦPSII at midday was reduced in submerged (0.15±0.04) and floating leaves (0.41±0.12) ($F_{1,7}$ = 7.72, P= 0.032), relative to F_v/F_m.

In February a high Φ_{PSII} was maintained for floating (0.66±0.06) and submerged leaves (Type S-F; 0.69±0.04) (Figure 8b). *P. tricarinatus* type S-F leaves maintained high ΦPSII while the ΦPSII of *P. tricarinatus* type S-S leaves and *P. crispus* leaves remained depressed (0.49±0.07 and 0.52±0.07 respectively) ($F_{3,14}$ = 2.69, P = 0.098). Differences between the *P. tricarinatus* floating leaves and *P. crispus* were significant ($F_{3,15}$ = 4.43, P = 0.026).

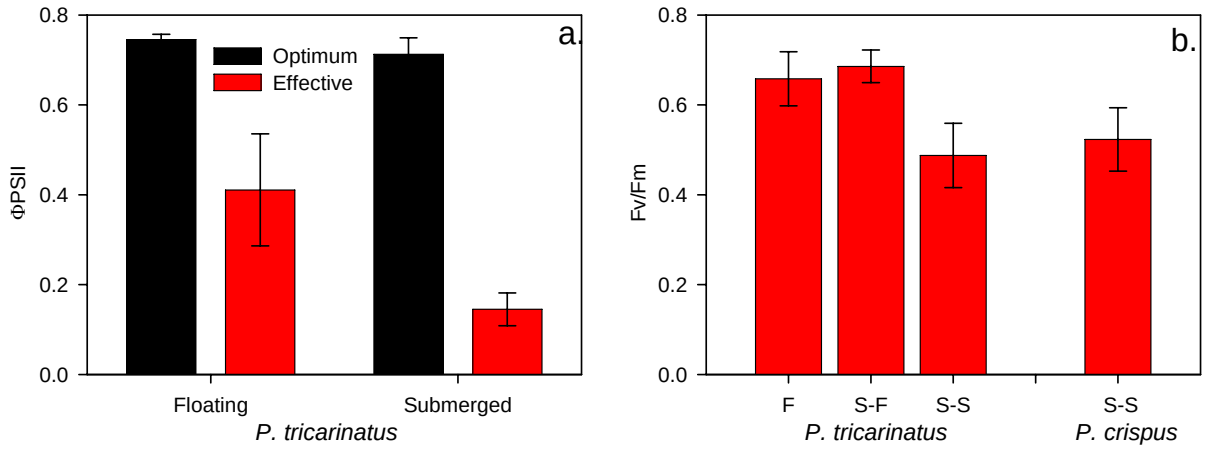

Figure 6-8. (a) Optimum (F_v/F_m) and effective (ΦPSII) quantum yield for floating and submerged leaves of *P. tricarinatus* on the 5 January 2005 and (b) F_v/F_m for three forms of *P. tricarinatus* (floating leaves, submerged leaves on stem with floating leaves and submerged leaves on a fully submerged stem) compared with submerged leaves of *P. crispus* measured on the 12 February 2005 measured at PFD of 1093±10.7. Bars are standard errors (n = 5).

Light Response Curves

Rapid Light Curves (RLC) demonstrate Electron Transport Rate (ETR) as a function of PFD (Figure 6-9), and essentially describe the photosynthetic capacity. Maximum Electron Transport (ETR_{max}) of floating leaves was consistently 2-3 times higher than submerged leaves. Floating leaves did not reach saturation, even at 1750 µmol m^{-2} s^{-1}. High ETR_{max} was reached for instantaneous and dark-acclimated RLC of floating leaves (260.9 and 174.8 µEq m^{-2} s^{-1}) during January measurements. Submerged ETR_{max} for dark acclimated leaves was 61.8 µEq $m^{-2}s^{-1}$ and instantaneous ETR_{max} was 36.3 µEq m^{-2} s^{-1}.

When ETR is calculated from absorbed rather than incident PFD for submerged leaves, the ETR_{max} is 40% lower. During high light during February under normal instantaneous light, ETR_{max} was considerably higher for floating leaves (341.1 µEq m^{-2} s^{-1}) yet lower for all submerged leaves (between 31-40 µEq m^{-2} s^{-1}), regardless of type and including *P. crispus*. Again, ETR calculated from absorbed rather than incident PFD results in an ETR_{max} 40% lower for submerged leaves.

Using incident PFD in calculations, the efficiency of light capture (α) is similar between floating and submerged leaves, however, as actual absorbed PFD is ca 60% lower than terrestrial leaves ($AF_{15} = 0.51 \pm 0.01$), α is calculated to be significantly

lower. Surprisingly, at high (supersaturating) PFD, there was no decline in ETR for floating or submerged leaves during any RLC.

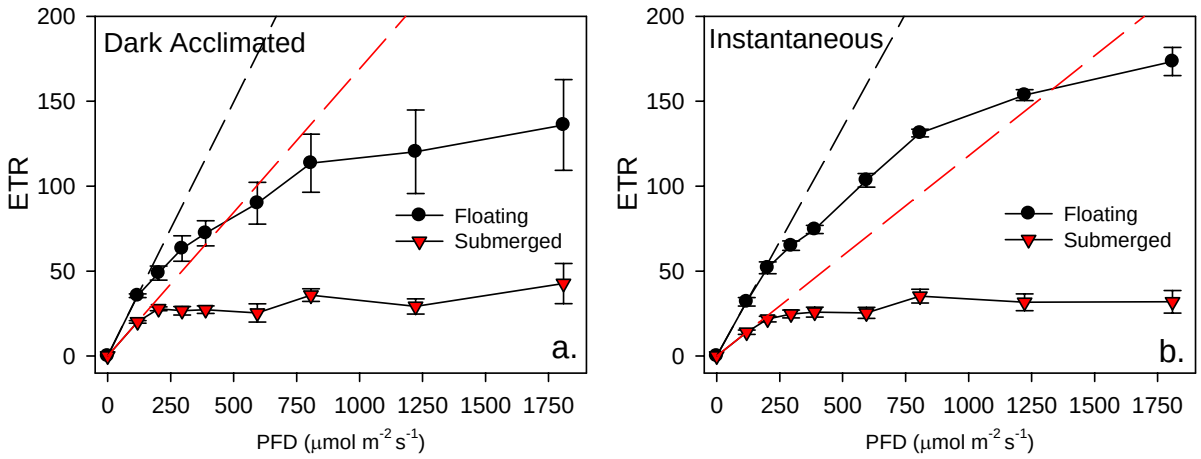

Figure 6-9. RLC of *P. tricarinatus* during January 2005; dark-adapted rETR calculated with absorbed light (a) dark-adapted ETR and (b) light-adapted rETR. Dashed lines represent the α for each leaf type.

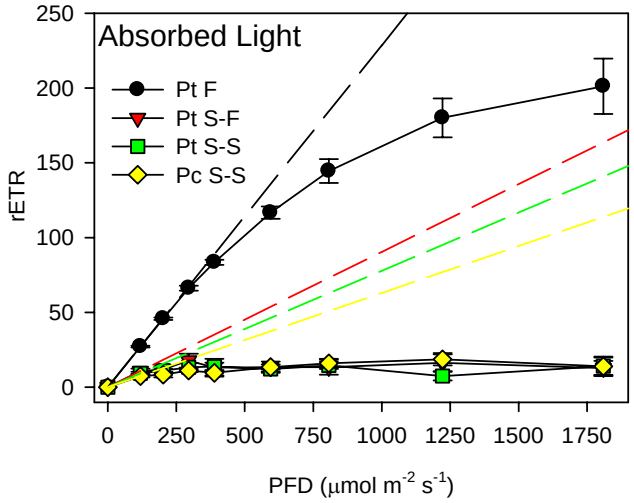

Figure 6-10. RLC of *P. tricarinatus* and *P. crispus* during February 2005; light-adapted rETR calculated with light absorbed by each leaf type. Bars are standard errors ($n = 4$).

6.4 Discussion

Results are discussed in three parts: (1) Morphology of floating versus submerged leaves, (2) Light responses of floating and submerged leaves, (3) Carbon partitioning between submerged and floating leaves. The main hypothesis is then discussed in light of the results.

6.4.1 Morphology of floating versus submerged leaves of P. tricarinatus

Plasticity in leaf morphology was indicated by changes in leaf size, shape, venation, chloroplast density and position, as well as cellular anatomy (Table 6-1,Figure 6-3, Figure 6-4). The large airspaces present in both submerged and floating leaves provide buoyancy for the leaf and continue into the stem and root to facilitate oxygen transport. In *P. tricarinatus,* some features help to balance carbon acquisition and light capture while others protect against sun and air damage associated with aerial living (Sculthorpe 1967). Carbon acquisition was maximised by use of stomata in floating leaves and in submerged leaves through a favourable surface (high) to volume (small) ratio including a thin (2-3 cells), flexible outer edge and lack of epidermis observed in leaf sections. In floating leaves, excess light is negated by a thick waxy cuticle to prevent radiation damage, while the well-developed palisade parenchyma containing many chloroplasts allowed ample sunlight capture. In submerged leaves, a palisade parenchyma layer was lacking due to the low and dispersed PFD of light underwater and even possibly as a mechanism against absorbing too much light.

Submerged leaves had less chlorophyll than floating *P. tricarinatus* and while amphibious plants have diverse and varying levels of chlorophyll (Nielsen 1993; Ueno 1996; Germ and Gaberscik 2003), these modifications exist to manage light capture in fluctuating light climates. Under high PFD levels and low availability of carbon, low chlorophyll prevents excess light absorption, whilst allowing net CO_2 fixation at reduced energy for production and maintenance of the photosynthetic apparatus. The combination of low chlorophyll and low photosynthetic capacity (low ETR_{max}) in the thin submerged leaves, compared with aerial leaves highlights that submerged leaves are not typical shade-adapted, slow-growing organisms (Figure 6-9 and Figure 6-10).

6.4.2 Light Responses of floating and submerged leaves

Light and carbon limitation

Relative ETR_{max} of submerged leaves was considerably lower than floating leaves which was expected (Woitke *et al.* 2004). Higher $rETR_{max}$ of floating leaves implies a larger demand for the total photosynthetic electron transport. However, contrary to terrestrial shade leaf adaptation (Salisbury and Ross 1995), chlorophyll concentration and light absorbed were both lower in submerged leaves compared with floating leaves. This suggests that the submerged leaves were acclimated to low light, and the lower $rETR_{max}$ in submerged leaves may relate to diffusion limitation rather than light limitation. Similarly, low carbon and low light each had a similar influence on

morphology of submerged leaves (Mommer *et al.* 2004; Mommer and Visser 2005). However, co-limitation of light and CO_2 probably occurs in many natural aquatic systems, and the physiological interactions between light and CO_2 have two important ecological implications for the natural growth of *P. tricarinatus*. While carbon in Ral Ral Creek is not likely to be limiting, dense populations may mean that carbon is available as HCO_3^- rather than CO_2 and thus requires additional energy for uptake. On the other hand, light is usually not limiting photosynthesis in leaves close to the surface or floating, and the present findings suggest that such plants should be able to exploit the limited CO_2 resource more efficiently as long as light is readily available. As suggested by Andersen and Pedersen (2002), increased carbon concentrations at depth or through use of HCO_3^- may alleviate light limitation at depth, or as in this case, with shading from upper canopy leaves.

Light absorbance of floating leaves was higher than submerged leaves, and comparable with most terrestrial leaves. Absorbance was greater than most terrestrial leaves (92%) and is consistent with other studies on *Potamogeton* species where absorbance has been recorded between 80 and 95% (Frost-Christensen and Sand-Jensen 1995).

Submerged leaves of *P. tricarinatus* were thin with sparse chlorophyll, and with 30-50% PFD transmitted through the leaf. Conversely, reflectance from the leaf surface was negligible due to the properties of light passage through water (amount of reflectance is decreased in water compared with in air Beer and Björk 2000). Light absorbance of submerged *P. tricarinatus* leaves by the leaf layer method (Beer and Björk 2000) was 51% compared with 91.6% for aerial or floating leaves. This 40% drop in absorbance is comparable with similar studies on submerged leaves, in particular, sea grasses; *Zostera marina:* 47-66% (Beer and Björk 2000) and *Cymodocea nodosa* 67-77% (Durako and Kunzelman 2002). The effects of lower light absorbance by submerged leaves has, however, been neglected in comparisons of submerged and floating leaves (Woitke *et al.* 2004). Producing a large number of relatively cheap leaves to intercept light allows maximum carbon uptake while distributing light capture.

Under water, light becomes more diffuse, this is similar to terrestrial canopies where diffuse light is more is more important than direct light (Jones 1992; Farquhar and Roderick 2003). The thin submerged leaves are economical to produce in terms of organic material and nutrients invested, and more efficient in terms of photon absorption capacity because of reduced internal self-shading (Frost-Christensen and Sand-Jensen 1992).

Balancing carbon and light resource capture

The production of new leaves allows acclimation to higher light environment closer to the surface. Any leaves that are produced early in the season or close to the surface that has not acclimated to high PFD are then chronically photo-inhibited. Such that there is a trade off with losing leaves to high light can be ameliorated by the production of new leaves acclimated to the light climate. Photoinhibition was higher in submerged leaves (low ΦPSII) than floating leaves (high ΦPSII). Full recovery after 20 minutes dark to a high F_v/F_m for both submerged and floating leaves shows no photo damage had occurred. Surprisingly, the ΦPSII of submerged leaves connected to stems with floating leaves was more comparable to floating leaves than to the submerged only stems. It is proposed that increased carbon supply from floating leaves allows a higher efficiency in submerged leaves connected to a stem with floating leaves, compared with carbon limitation in submerged only stems.

In turbid water, light extinction is rapid compared with many clear water studies where attenuation is assumed negligible (Blanch *et al.* 1998). Increased leaf surface area is a response to low light (Björkman 1981), and also a response to limiting carbon (Maberly and Spence 1983). Maximising carbon gain with broad, thin leaves then maximises light interception and submerged leaves became photo inhibited with high PFD.

Variation in the amount of chlorophyll directly influences light absorption and photosynthesis (Frost-Christensen and Sand-Jensen 1992). Floating leaves had chlorophyll in excess and are thick such that light transmittance is negligible. Amphibious plants, on the other hand, have diverse and varying levels of chlorophyll (Nielsen 1993; Ueno 1996; Germ and Gaberscik 2003). Submerged leaves had lower a/b ratios and lower total chlorophyll than floating *P. tricarinatus*. These differences in chlorophyll show that *P. tricarinatus* did not follow the sun and shade adaptations (Osmond *et al.* 1981), where shade plants have increased chlorophyll content and an a/b ratio of c. 2.1 (Anderson *et al.* 1988). Low chlorophyll combined with low ETR_{max} in the thin submerged leaves compared with aerial leaves illustrates that submerged leaves are not typical shade-adapted slow-growing structures. Chlorophyll content is the main predictor of photosynthesis of submerged leaves due to the light-harvesting capacity (Nielsen and Sand-Jensen 1989), here low chlorophyll concentration in submerged leaves acts to reduce light capture.

Basal leaves grow in low-light conditions and may be shade-adapted in addition to being carbon limited and remain so throughout the season due to light attenuation in the

turbid environment, coupled with shading by floating and higher submerged leaves. Submerged leaves growing higher in the water column get exposed to more light as they develop closer to the surface and so although they are adapted to high light, they still are carbon limited. These submerged leaves are carbon limited and exhibit photoinhibition characteristics. Submerged leaves on stems with floating leaves were contact with atmospheric carbon and had a greater operating efficiency than S-S leaves. It is proposed that carbon limitation was thus removed and so there was increased efficiency of light use. However, no difference was observed in the ETRmax of these leaves compared with the fully submerged stems; thus, although efficiency was much improved, no great photosynthesis was possible.

Many of the submerged leaves at the top of the stem on stems with floating leaves were degraded, with many of the leaf edge broken and reduced colour and completeness. This is attributed to expanding during higher light, and thus being adapted to this higher light, when overtopped by floating leaves (thus reducing the amount of light incident) these leaves can not with low light thus degrade/dieback. Although no fluorescence data was gathered for these degraded leaves, in the same situation, fully submerged stems in the same plane of water were not degraded and were not being shaded by floating material. Thus it is suggested that these fully submerged stems remained carbon limited and maintained sufficient light capture.

Although here it is assumed only one element was limiting, it may be possible that more than one element may limit photosynthesis. Access to HCO_3^- provides additional carbon which may in turn increase the light use efficiency as shown by Andersen and Pedersen (2002).

6.4.3 Carbon partitioning between submerged and floating leaves

Submerged leaves attached to a fully submerged stem had an enriched organic material $\delta^{13}C$, which is indicative of DIC use (Raven *et al.* 1987), while the depleted (<-25‰) organic $\delta^{13}C$ of floating leaves is indicative of atmospheric carbon use (-8‰) when Rubisco and other fractionation factors are considered. Crucially, the submerged leaves attached to stems with floating leaves had a depleted $\delta^{13}C$ similar to floating leaves and more suggestive of atmospheric carbon use assimilated during the growth. The lack of discrimination by submerged leaves is two-fold, it may imply either carbon limitation due to low diffusive rates or as HCO_3^- ($\delta^{13}C$ -6.43) was the main source of carbon available and will also give rise to an enriched $\delta^{13}C$ signature when assimilated.

It is not possible to confidently establish which of these factors is controlling the organic $\delta^{13}C$, however, as the submerged leaves were light stressed this indicates that diffusion limitation is more likely.

In *P. tricarinatus* ETR_{max} was almost 10 times higher for floating leaves and saturated at almost 20 times the E_k compared with submerged leaves. Fully submerged plants had healthy submerged leaves compared with leaves attached to stems with floating leaves; this provides evidence of ample carbon fixation and carbon contribution to growth with no aerial contract, even at these low ETR and PFD.

The problems associated with the interpretation of $\delta^{13}C$ in aquatic as compared to terrestrial plants have been discussed widely (Osmond *et al.* 1981; Beardall *et al.* 1982; LaZerte and Szalados 1982; Raven *et al.* 1982; Raven *et al.* 1987). The occurrence and use of HCO_3^-, increased diffusive resistances, variability associated with flow change and the variability of source in puts all serve to complicate the interpretation of DIC as a source. Further to this, the nature of inorganic carbon transport into cells is two fold; HCO_3^- can be actively transported while extra cellular HCO_3^- can also be converted to CO_2. This study, however, sought to qualitatively describe the carbon pools and translocation between them.

Carbon acquired from the water had an enriched $\delta^{13}C$ signature due to the combination of the enriched source DIC and the increased diffusional resistance. Submerged leaves of *P. tricarinatus*, *P. crispus* and other fully submerged macrophytes had $\delta^{13}C$ signatures of approximately -18±1‰, slightly more depleted than the $\delta^{13}C$ of DIC (approximately -15‰). Conversely, the $\delta^{13}C$ signatures of floating leaves were depleted in ^{13}C, corresponding to a $\delta^{13}C$ of approximately -27‰ indicating atmospheric CO_2 use.

Submerged leaves of type S-F, had $\delta^{13}C$ values (-22.6±2‰) that were no different from the floating leaves. This confirms that carbon was obtained from atmospheric CO_2 and that carbon allocation will switch where there is contact with atmosphere such that the bulk of carbon is acquired from atmospheric CO_2.

6.4.4 Hypotheses Revisited

This chapter examined the interplay between the utilisation of light and carbon in the heterophyllous *P. tricarinatus* and the submerged *P. crispus.* The primary aim was to determine what the adaptive advantages of heterophylly were for *P. tricarinatus*.

The advantages of heterophylly were explored in this chapter with the central hypotheses discussed below:

Heterophylly in *P. tricarinatus* is an adaptive trait to a strong vertical light gradient and a rapid transition from DIC to atmospheric CO_2 as a carbon source for photosynthesis.

Phenotypic plasticity is the property of a genotype to alter its development in response to changes in the environment (Schmal-hausen 1949). Alterations in physiological plasticity, such as the use of atmospheric and aqueous carbon (CO_2 and HCO_3^-) are necessary modifications pending emergence and submergence. However, similar to the work undertaken on *C. helmsii* (Chapter 3) morphological responses to carbon limitation are similar to those for light limitation (Mommer *et al.* 2005).

Submerged and floating leaves had equal optimal quantum yield (F_v/F_m), however, as shown with RLC parameters, submerged and floating leaves had very different photosynthetic capacities consistent with low light acclimation (Schreiber 2004). High light was exploited in floating leaves by maximising carbon uptake with large percentage of light absorbed even compared with terrestrial leaves. Submerged leaves just below the surface could not directly take full advantage of light available due to carbon diffusion limitation; however, as shown by the $\delta^{13}C$ data, atmospheric carbon was incorporated into these leaves

In heterogeneous environments changes to the availability of carbon and light may occur with changes in water level, turbidity or water chemistry, the latter often induced by the plants themselves through HCO_3^- uptake. Resource capture was optimised in floating leaves of *P. tricarinatus* by maximising surface area for carbon uptake. In submerged leaves, however, optimising available light was predicted to be more critical than carbon uptake in deep water while for leaves closer to the surface this trend would reverse. Most of the morphological features (such as increased surface to volume ratio) of submerged *P. tricarinatus* and *P. crispus* leaves serve to increase carbon uptake, yet at the same time light interception is improved, as is common with most aquatic plants (Mommer *et al.* 2005). However, the much lower chlorophyll in submerged leaves compared with floating leaves implies that carbon diffusion remains the key limiting factor.

Cook and Johnson (1968) provide evidence that populations of aquatic plants that experience the greatest heterogeneity in water levels, also exhibit the greatest degree of

heterophylly. The primary adaptation of heterophylly for *P. tricarinatus* is that deeper water submerged leaves maximise light capture, while nearer the surface, photosynthesis is optimised with a balance between carbon acquisition and light interception. Once the stem has reached the surface the floating leaves cover the surface and maximise both carbon and light acquisition. For this reason, heterophylly in *P. tricarinatus* appears to be a non-plastic developmental transition as suggested for other heterophyllous aquatics by Wells and Pigliucci (Wells and Pigliucci 2000). In this case the response to light is probably influenced by interactions between genotype and environment as occurs in some marine algae (Monro and Poore 2005) and while the adaptive phenology is not explored in this chapter it could be a significant mechanism in heterophylly. Many *P. tricarinatus* with several floating leaves were observed to have few submerged leaves; this can be attributed to two factors: 1) Energetic costs associated with heterophylly and thus a reduction in individual heterophylly as has been observed in populations from more stable environments (DeWitt *et al.* 1998) and 2) Shading of submerged leaves by the upper floating canopy.

Chapter 7 GENERAL DISCUSSION

For individual plants, particularly those with an amphibious habit, physiological adaptations are needed to accommodate to environmental changes. Rises and falls of water level, for example, may be countered by heterophylly, different forms of carbon uptake (e.g. reliance on CO_2 or bicarbonate) or different modes of carbon assimilation, in the form of C_3, CAM and C_4 photosynthetic pathways. This thesis explores these options through investigations of the carbon acquisition and assimilation characteristics of three diverse species. Each has more than one option to optimize carbon use, indicating the need for aquatic plants to combine plasticity in life form with flexibility in photosynthetic mechanisms. Insights into mechanisms, and switching between mechanisms, were gained by use of fluorescence and stable isotope techniques.

Chapter 3 describes a relatively simple system of DIC acquisition utilized by the amphibious *Crassula helmsii*, a C_3 and CAM species, with regard for the effects of submersion on CO_2 uptake and assimilation. **Chapters 4-5** consider a more sophisticated system of CO_2 and bicarbonate uptake in *Vallisneria americana*, a submergent C_3 and CAM species. **Chapter 4** presents field data; **Chapter 5** is laboratory-based. **Chapter 6** compares the options for CO_2 and bicarbonate uptake in two C_3 plants, *Potamogeton tricarinatus*, with floating and submerged leaves, and *P. crispus*, with submerged leaves.

These investigations were undertaken in the context of the River Murray, South Australia, in a semi-arid region where aquatic and terrestrial plants are often confronted by aseasonal changes that call upon flexible mechanisms for carbon uptake. CAM activity, for example, varies with seasonal conditions, leaf age, leaf position and local habitat conditions for individual plants. This is reflected in a changeable capacity for bicarbonate utilisation.

7.1 Study species

7.1.1 Crassula helmsii

Chapter 3 considers the responses of the amphibious *Crassula helmsii* to different water regimes, to determine whether CAM contributes to its persistence or spread in environments where water levels (and water chemistry) are prone to vary. It was postulated that *C. helmsii* would remain competitive under changeable conditions by

using CAM to assimilate CO_2 acquired from the water when submerged and atmospheric CO_2 uptake and C_3 photosynthesis when emergent.

C. helmsii does not use HCO_3^- but is reliant on CO_2 availability, however is shown to use CAM on submergence, even in environments where water levels vary within 24 hours. This allows it to maintain photosynthesis in habitats where submergence prevents access to atmospheric CO_2.

It is likely, however, that stable conditions are most favourable for growth, and that the spread of the species is predominantly by the aerial form. Under stable conditions the plant extends its stems so that the majority of leaves have access to atmospheric CO_2. Because rates of photosynthesis are elevated (compared with submerged leaves) when leaves have access to atmospheric CO_2, aerial leaves contribute the greatest fraction of the total carbon fixed.

The combination of CAM on submergence and relatively high background malate suggests that *C. helmsii* switches between CAM and C_3 photosynthesis to maintain carbon acquisition. Leaf mass, however, is often dominated by aerial tissue. While CAM may enable plants to re-fix part of the nocturnal respiratory CO_2, and perhaps facilitate the translocation of carbon, this is not enough to promote extensive growth in *C. helmsii*, rather survival until leave reach the surface. Rather than adapt to submergence by modifying its aerial (atmospheric CO_2 using) form, *C. helmsii* has a morph that use CAM under submergence. Although CAM capacity may have aided its invasion in the United Kingdom (Newman (1995)), this study suggests that the most invasive form in the UK is aerial mats on shallow ponds, where the plants would not be utilising their CAM ability. By extension, *C. helmsii* might be expected to be more competitive in the Murray, where seasonal water-level fluctuations are limited by flow regulation. At Riverglades, for example, wind-induced 5-10 cm changes of level in shallow water do not invoke CAM behaviour because the plants are not fully submerged.

In general, regimes where *C. helmsii* is dominant are exposed to daily changes in water level. CAM appears not to underwrite the invasive capability of *C. helmsii*; rather, it may predispose the plant for life on water-logged soils or in shallow ponds. In this species, CAM may be adaptive only for fully-submerged plants in deep, low-light water bodies.

7.1.2 Vallisneria americana

Chapters 4-5 highlight the role of plasticity in changeable conditions. *Vallisneria americana* lacks the option of heterophylly, but has physiological plasticity to enable survival in aquatic habitats where pH, temperature and the activities of other plants cause carbon pools to fluctuate in space and time. *V. americana* is a generalist rather than a specialist species, but readily adapts to constant conditions.

This study shows that *V. americana* uses the CAM pathway in seasonally-isolated pools, but there appears to be a threshold for CAM ability, possibly related to light intensity. The extent of CAM also varied with water chemistry, as expected, but also between small, localized populations. CAM in Riverglades which retained connectivity with the parent river, (so was physically and chemically more stable with lower pH and temperature) was lower than Banrock thus supporting the hypothesis. In these isolated water bodies extensive beds of *V. americana* influence the water chemistry thus restricting carbon use to those plants that can acquire HCO_3^-. However, where there is constant exchange with the river these plant induced changes are insufficient to overcome the influence of the incoming water thus *V. americana* does not have such a competitive advantage. Carbon signatures indicated both bicarbonate use and the differences in source water and water chemistry between sites. The water chemistry factors associated with isolated water bodies were driven by *V. americana* and provided evidence for bicarbonate use.

V. americana can acquire bicarbonate from the water and also undertake CAM, contrary to the suggestion by Keeley (1998) and reports by Sand-Jensen (1987), Maberly (1983; Maberly and Spence) and Newman (1995). This depends on the availability of each carbon species, although CO_2 generally is used in preference to bicarbonate. In the light, leaf tips used both CO_2 and HCO_3^-, and tissues from the middle and base of leaves used less of both, but proportionately more CO_2 than HCO_3^-. In the dark, leaf-tip tissue undertakes CAM, using CO_2 but not HCO_3^-; mid-leaf tissue undertakes less CAM and basal tissue performs virtually none.

The hypothesis that CAM is triggered in the short term by high light intensity, possibly as a stress response, was tested in incubator experiments under three light levels. The results highlighted the complexity of CAM in *V. americana* and suggested a more sophisticated CAM cycling process than is typical of intermediate C3-CAM species. It

appears that bicarbonate use by the different leaf portions is not associated with the CAM ability of the leaves.

High light triggers bicarbonate use in the leaf tips, but not in the middle or base of leaves. Low level CAM activity can have a dramatic effects on the mix of carbon species (detectible in small volumes) in the dark, however, the variability of overnight malate accumulation could be more a function of CAM cycling rather than the fluctuating C_3-CAM intermediate status of *V. americana*. This merits more study.

The plasticity of carbon acquisition in *V. americana* is reflected in its persistence in environments where other, less-flexible species are eliminated. In habitats with changing alkalinity, *V. americana* has a competitive advantage over species that do not use bicarbonate and species reliant on free CO_2. In addition the CAM mechanism extends the flexibility of *V. americana* providing another competitive advantage for carbon (see further Chapter 4).

7.1.3 Potamogeton tricarinatus

Chapter 6 is a comparative study of two *Potamogeton* species, confirming that heterophylly may confer a competitive advantage in some environments. In the deep, flowing Ral Ral Creek, heterophylly allows *P. tricarinatus* to grow rapidly and access high carbon and light resources at the water surface. In contrast, *P. crispus* has more robust submerged leaves able to cope with changeable water levels, and does not have specialized floating leaves. *P. crispus* is common in shallow waters where levels of pH, O_2, temperature and light are variable. It is also the more common of the two species along the margins of the Murray, where water levels vary erratically.

The submerged leaves of *P. tricarinatus* and *P. crispus* proved to be carbon- rather than light-limited, even in turbid environments. In submerged leaves photosynthesis is limited by increased diffusive resistance and, in turbid water, by increased light attenuation. Inorganic carbon use and light-harvesting efficiency are inversely related (Johnston *et al.* 1992; Kübler and Raven 1995), such that carbon uptake under low light is countered by more efficient light use (Kübler and Raven 1995).

Submerged *P. tricarinatus* leaves were severely carbon-limited, evident in the lack of discrimination against ^{13}C, and susceptible to photoinhibition even at low light levels. Carbon remains the primary factor limiting photosynthesis, even in turbid water, with submerged leaves able to utilize bicarbonate. Unlike most heterophyllous aquatics

(Spence and Maberly 1985), *P. tricarinatus* uses either bicarbonate or CO_2 as a carbon source. The bicarbonate used by submerged leaves should be ample, but the $\delta^{13}C$ data show that carbon is supplied from the atmosphere in quantities to overwhelm any aqueous carbon signal. These fully-submerged leaves are diffusion limited, with little discrimination against ^{13}C (Farquhar *et al.* 1989) and $\delta^{13}C$ signatures similar to those for Dissolved Inorganic Carbon (DIC). Thus, the floating leaves provide assimilated carbon from the atmosphere to the submerged leaves. In effect, heterophylly frees *P. tricarinatus* from reliance on dissolved carbon.

The stable isotope data show also that some carbon is transferred from submerged to floating leaves, as the latter had a slightly-enriched $\delta^{13}C$ signature (-25‰). This means that not all of the assimilated carbon is of atmospheric origin; rather, it is redistributed from submerged leaves or from the undersides of floating leaves.

7.2 Carbon Dynamics

7.2.1 Preamble

Each species investigated uses a variety of mechanisms to acquire carbon. Below is a brief discussion comparing the carbon acquisition methods (7.2.2) and the carbon assimilation methods (7.2.3).

7.2.2 Carbon acquisition

Crassula helmsii may adopt amphibious or fully-submerged habits. The submerged form has little capacity to use atmospheric CO_2, relies on physical support from the water and desiccates on exposure to the air. The amphibious form uses both atmospheric and aqueous CO_2, accessed by leaves of similar morphology, but atmospheric CO_2 evokes substantially more growth than aqueous CO_2 (cf. Mommer (2005)). Both aerial and submerged leaves have typical C_3 diurnal ΦPSII patterns and similar differences in ΦPSII or F_v/F_m between tissue types.

When subject to water levels that fluctuate daily, *C. helmsii* favours an aerial form suited to atmospheric CO_2 uptake. In still, deep water it develops a fully-submerged growth form, and in still, shallow water, it produces an amphibious, mat-like form that is reliant on atmospheric CO_2.

P. tricarinatus is flexible in its capacity to sustain photosynthesis under changeable pH and temperature conditions (which affect the relative amounts and availability of carbon species), and so can access a greater proportion of the aqueous carbon pool. In addition, it develops floating leaves able to utilize atmospheric CO_2, further extending the available carbon. *P. tricarinatus* thereby has access to carbon when competition is high, or where supplies are scarce, as in low-productivity environments.

The natural variability of pH in the wetlands studied allowed assessment of the effect of uptake of altered ratios of bicarbonate and aqueous CO_2. Wetlands disconnected from the river had considerable increases in pH due to HCO_3^- using submerged plants, in some wetlands pH reached 10.4. However, water mixing due to connectivity of the wetland to the river ameliorated the increase in pH such that it remained below 8.3.

Use of bicarbonate by *V. americana* is well documented causing the pH of the water to rise up to 10.4 through removal of HCO_3^- from the water and subsequent input of OH^-. This was shown to be highest in the tips of leaves exposed to high PFD but was virtually absent in the base of leaves. Due to attenuation of light, it is probable that light became a limiting factor in photosynthesis rather than carbon, thus there was no need for additional sources of carbon such as HCO_3^-. There was limited CO_2 uptake in the base of the leaves and demonstrates the functionality along the leaf length. Attenuation through water reduces the PFD reaching the base of the leaves such that they no longer function to actively acquire or assimilate carbon, rather support the leaves with a thick base. Through these studies undertaken at the field sites, it was found that bicarbonate use could be stimulated in tips of leaves when exposed to high PFD (>1000 μmol $m^{-2}s^{-1}$).

CO_2 and HCO_3^- in water bodies can change relatively rapidly and as a response aquatic macrophyte acclimation is fast, recently response has been measured as being initiated within hours (Maberly and Madsen 2002), yet sometimes days and sometimes up to several months (Sand-Jensen 1987). *Potamogeton crispus* shows a quick response time to changes in CO_2 availability by altering its HCO_3^- affinity (and thus its uptake capacity) and plateaus after 7 days (Maberly and Madsen 2002). Rather than switching source of carbon, *V. americana* appears to utilise both CO_2 and HCO_3^- uptake within the same leaf at the same time and thus does not require time to acclimate to different carbon sources.

Carbon uptake and assimilation varies along any one *V. americana* leaf due to light attenuation in deep water, this is similar to leaves sitting at varying depths of *P. tricarinatus*. Carbon uptake in leaves formed underwater such as in *V. americana* and *P. tricarinatus* differed substantially compared with leaves that become submerge such as when *C. helmsii* is top flooded. Acclimation through underwater development shows more efficient use of low light intensities than that observed when submerged are top flooded. In high light situations common in wetlands of the lower River Murray, carbon is the primary limiting factor due to diffusion limitation. In tips of Vallisneria, additional carbon acquisition through CAM enabled use of the high light towards the top of the water column where light still remained high and this may help to reduce light stress. Conversely the upper leaves of *P. tricarinatus* died due to the high level of light yet lower leaves remained active as light and carbon uptake were more balanced.

Plasticity in carbon acquisition as a response to growth conditions are a common phenomenon (Madsen *et al.* 1996) and is often observed in aquatic macrophyte where maximizing resource capture results in plastic allocation changes (Maberly and Madsen 2002).

Access to atmospheric CO_2 has its obvious advantages a lower resistance to diffusion. Rates of photosynthesis of four common amphibious species have been shown by Sand-Jensen and Frost-Christensen (1999) to be 2 to 4 times higher in air and growth significantly higher than when submerged, even in water 8 times supersaturated with CO_2.

7.2.3 Carbon assimilation

Submersion of aerially grown *C. helmsii* tissue induces CAM; this switch to CAM has been demonstrated to occur within 24 hours and is likely to be induced immediately. The benefit of CAM in *C. helmsii* is realised due to the increased submerged diffusive resistance, and is greater in plants that have developed under water, remain fully submerged and maintain an aquatic morphology. Low diffusivity in submerged tissue thus can benefit from additional carbon to match the PFD input and so to decrease the stress caused by excessive light.

In both natural populations and in controlled mesocosms *C. helmsii* undertook extremely low-level CAM (even in comparison with most C3-CAM plants Holtum and Winter 1999). At Riverglades it appears that short term fluctuations in water level (as

observed here due to wind) in the range of a few centimetres is enough to trigger CAM response, far more sensitive than first realised. In controlled studies, this instant response was repeated with the initiation of CAM within 1 day of submergence. Levels of accumulated acid, however, did not increase as was expected with continued submergence.

Comparatively large background levels of malate were shown in both aerial and submerged *C. helmsii*, these are associated with extremely low fluctuations in day/night titratable acidity is in keeping with (Holtum and Winter 2005) who found that high malate was maintained for the C_3-CAM *Clusia.* PEPc activity levels were low, probably due to inhibition by the relatively high Malate (Winter 1980) which might then be stimulated by submersion at the end of the day when malate is utilised. It is proposed that *C. helmsii* requires only small variations in PEPc to promote changes to uptake physiology. Small changes in acidity and malate are also consistent with small environmental changes, documented in a similar manner in the CAM amphibious macrophyte *Littorella uniflora* (Robe and Griffiths 1998).

Although the high chlorophyll implies that leaves can afford to capture more light, there are a number of indicators that suggest that light is in excess. Diel F_v/F_m of aerial tissue had a midday decline associated with high PFD attributed to 'dynamic' photoinhibition (Powles 1984; a short-term photo protection mechanism Osmond 1994). Submerged leaves had lower light use efficiency and substantial photo-damage (long lasting photoinhibition resulting in the loss of functionality of PSII units), with no recovery in F_v/F_m after 12 h where light was in excess of the normal ETR and non-photochemical quenching (NPQ) capacity (Krause 1988). Although (Flexas *et al.* 2001) suggest that often measurements of F_v/F_m give an impression of little photo inactivation of PSII when in reality many PSII units are dysfunctional, here photo inactivation is clearly demonstrated, further investigation into the separate parameters may provide more insight of timing and extent of photoinhibition.

In addition to CAM providing a mechanism to tolerate high light when either water levels drop or as tips grow towards the surface, low light C_3 photosynthesis provides a benefit at the other end of the spectrum where there is a competitive advantage in an ability to undertake photosynthesis in low light or under limiting DIC conditions, this perhaps explains the retention of C_3 photosynthesis in this species.

7.3 Conclusion

In the Murray-Darling Basin, water level fluctuations promote CAM in *V. americana* and *C. helmsii* and allow continued growth and possibly a competitive advantage. Small water level changes such as that experienced due to wind action and possibly small fluctuations due to lock pool height changes provide a system where CAM may be used to withstand changes to ensure survival rather than to promote growth and spread. In terrestrial species, it has often been noted that CAM operates more as a survival mechanism during stressful periods, helping to maintain carbon balance and prevent photoinhibition (Griffiths 1988; Borland and Griffiths 1990). From the studies reported in this thesis, it seems likely that an analogous situation holds for aquatic plants

Environmental changes such as water chemistry or the presence or absence of water for amphibious plants can be considered a stress and can help to explain plant species distribution (Osmond *et al.* 1987). Environmental changes stimulate changes in the routes used to acquire carbon such that under high pH, bicarbonate is more available and aquatic plants such as *V. americana* and *P. tricarinatus* have developed the ability to tolerate and even use this resource. While small confined water bodies were used for study purposes, *V. americana* is very common in flowing streams. It is possible that *V. americana* has developed its ability to use HCO_3^- as a means of competing in isolated water bodies, but retains its flexibility to allow continued survival during changes in environmental conditions rather than as a mechanism to actively use the resource.

Functional differences in photosynthetic ability and carbon uptake have important implications for local ecosystem dynamics allowing persistence of plants with flexibility in their exchanges of carbon, water, and energy. The distribution of photosynthetic mechanisms in aquatic plants is not based on the availability of water as with terrestrial CAM or C_4 species, rather it is based on the necessity to utilise a changing resource. As shown by the work undertaken in this thesis this is a mechanism to enable survival in changeable environments rather than to aid dominance and expansion. And as such 'switching' back to a more common carbon supply and photosynthetic pathway occurs when the resource becomes available.

Each of the species studied becomes dominant under specific conditions in particular areas. *V. americana* has extremely high biomass in relatively shallow warm waterbodies in the Lower River Murray where large amounts of leaf surface area are

near the surface, *P. tricarinatus* can dominate relatively deep channels while *C. helmsii* is a aquatic spreading invasive in many parts of the United Kingdom, although only becomes dominant in its endemic Lower River Murray habitat where there are only small change in water level.

While co-limitation of resources has been an accepted principle for at least twenty years (Andersen and Pedersen 2002), few aquatic studies have shown that the interacting effect of light and CO_2 may translate from photosynthesis into effects on growth (Maberly 1985). It has been shown that higher CO_2 near the bottom can increase the light use efficiency (Andersen and Pedersen 2002). This may be a considerable benefit for a species such as *V. americana* which has reduced chlorophyll content in lower parts of leaves.

Andersen and Pedersen (2002) suggest that additional CO_2 provides more carbon to the plant and therefore the plant can afford to invest less energy and resources in CO_2 uptake thus leaving more energy for optimising the light utilisation (i.e. more Chlorophyll can be produced without fatal consequences for the energy budget). The opposite may well be working efficiently with leaves of *V. americana* and *P. tricarinatus* close to the surface where high light allows resources to be put into accessing more carbon and thus the ability to concentrate carbon using CAM and or expending energy to use HCO_3^- and thus use carbon more efficiently.

Use of various mechanisms to acquire and assimilate carbon allows for survival with fluctuating water level and chemistry; however contrary to initial hypotheses, abundance and growth occur in areas of relatively stable hydrology.

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