The Effects of Water Flow, pH and Nutrition on the Growth of the Native Aquatic Plant, *Aponogeton elongatus*

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STATEMENT OF ORIGINALITY

"I hereby declare that the work presented in this thesis has not been previously submitted for a degree at any university. To the best of my knowledge and belief, this thesis contains material that is original and has not been previously published or written by another author except where due reference is made."

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Mark Norman Crossley

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LIST OF ABBREVIATIONS AND SYMBOLS

CH_3COOH	acetic acid
adapt	adaptor
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AC	alternating current
NH_4^+	ammonium ion
A	Amp
ANOVA	analysis of variance
et. al.	and others (Latin: et alabi)
AE	anion exchange mechanism
≈	approximate
aq.	aqueous
B	boron
BRI	bridge rectifier
BSP	British Standard Pipe
Ca	calcium
$CaCO_3$	calcium carbonate
CO_2	carbon dioxide
H_2CO_3	carbonic acid
CA	carbonic anhydrase
C	capacitor
Cu	copper
CAM	crassulacean acid metabolism
DH	degree of hardness (German)
^oC	degrees centigrade or Celsius
$\delta^{13}C$	delta 13 carbon
D	diode
DC or dc	direct current
DIC	dissolved inorganic carbon
dry wt.	dry weight or biomass

EC	electrical conductivity
e^-	electron
ETR	electron transport rates
⇆	equilibrium
Figs.	figures
eg.	for example (Latin: exempi gratia)
FAA	formalin/acetic acid/alcohol
C-4	four carbon eg oxaloacetic acid
g	gas or gram
GMA	glycol methacrylate
>	greater than
GND	ground
h	hours
H^+	hydrogen ion
ICP	inductively coupled plasma
I/O	input/output board
ID	internal diameter
Fe	iron
K	Potassium or kilo eg 1000 Ohm
LSD	least significant difference
LSM	least squares means
<	less than
LED	light emitting diode
r	linear correlation coefficient
r^2	linear correlation coefficient squared
L	litre
Log	logarithm
LS	longitudinal section
Mg	magnesium
MDH	malate dehydrogenase
Mn	manganese
m	meter
µF	microFarad

µM	microMolar
µmol	micromoles
µS cm^{-1}	microSiemens per centimetre
mL	millilitre
mg	milligram
mm	millimetres
nF	nano Farads
nm	nanometres
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced
N	nitrogen or Normality (chemistry: g equivalents L^{-1})
n	number of samples
O/C	open collector
O_2	oxygen
ppm	parts per million (mg/Kg)
%	percentage
PC	personal computer
PEP carboxylase	phosphoenolpyruvate carboxylase
P	phosphorus or probability
PAR	photosynthetically active radiation
±	plus or minus
pwr.	power
pH	power of hydrogen
PK	pyruvate kinase
QLD	Queensland
RLC	rapid light curves
Eh	redox potential
REG	regulator
R	relay
R or r	resistor
RuBP	ribulose 1, 5-bisphosphate
s	second
Na	sodium

cm^2	square centimetres
SD	standard deviation
SE	standard error
SAM	submerged aquatic macrophytes
S	sulphur
ie.	that is (Latin: id est)
C-3	three carbons eg phosphoglyceric acid
TDS	total dissolved salts
TS	Transverse section
Tr	treatment
USA	United States of America
V or v	volts
Zn	Zinc

ABSTRACT

The Australian native freshwater macrophyte, *Aponogeton elongatus*, is a decorative aquarium plant but little is known about the ecophysiology of its growth and environment. Formerly, plants were collected from the wild to supply the pet/aquarium retail industry in Australia and overseas. Conservation measures introduced by Australian State Governments now restrict this practice. To achieve an understanding of how environmental factors can be manipulated for optimum growth in cultivation, the long-term effects of stirred water, nutrition and water pH on *A. elongatus* growth were examined in two studies.

The plants were grown in sand filled pots in aquaria, under fluorescent lights (PAR; 100µmol m^{-2} s^{-1}) in temperatures from 22-24°C. Slow release fertiliser (Osmocote Plus^{R}) placed in the sand substrate supplied nutrition. A four-fold increase in plant growth in two months was found in stirred water with a pH level between 6.5 and 7.8 and fertile sediment. This macrophyte was also found to depend on its roots for nutrient uptake and it was the combined effect of nutrient availability and stirring that produced the strongest growth. The supply of dissolved inorganic carbon was the main factor in the plant response to stirred water, presumably due to the reduction in boundary layer resistance around the leaves. Dissolved free CO_2 was more readily assimilated than bicarbonate based on the strong growth response in water of pH 6.5 to 7.8 and strong correlation between the concentration of dissolved free CO_2 and plant growth.

It is apparent that the manipulation of nutrient level, dissolved free CO_2 and pH greatly influenced the growth of *A elongatus* in stirred water. These findings would benefit the commercial cultivation of this plant and give some insight as to the extent of *A. elongatus* adaptation to an aquatic environment.

PUBLICATIONS

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Background to Research

Aponogeton elongatus F. Muell. ex Benth, is an Australian native aquatic macrophyte, which belongs to the family Aponogetonaceae (van-Bruggen 1969; Aston 1973). The family consists of one genus with species occurring in Africa, Madagascar, India, South East Asia, New Guinea and Australia, all of which are perennial, herbaceous aquatics. *A. elongatus* is recognised overseas and in Australia as a decorative and hardy aquarium plant (Rataj and Horeman 1977; Elliot and Jones 1982). A number other species from Madagascar and tropical Asia are also popular aquarium plants including, *A. madagascariensis* (Lace Leaf Plant), A. *rididifolius*, A. *ulvaceus*, *A. crispus*, *A. undulatus*, *A. echinatus*, *A. boivinanus* and a range of hybrids (Stodola 1967; Rataj and Horeman 1977).

A. elongatus produces a crown of leaves from a tuber buried in the sediment (Plate1.1). Most of the leaves remain submerged but some floating leaves are produced during flowering (Sainty and Jacobs 1981; Sainty and Jacobs 1994). The submerged leaves are linear to narrow ellipsoid (2.5-55cm long x 0.5-5cm wide) with a prominent mid rib and longitudinal veins (Aston 1973). Leaf colour ranges from light green in low light to various shades of reddish to greenish brown in higher light intensities. The leaf margins are often undulate and minute fragrant yellow flowers are produced on an emergent spike.

This macrophyte grows predominantly submerged in still to flowing water in streams, creeks and rivers (Aston, 1973; Sainty and Jacobs 1994). Found only in permanent fresh water, rooted in mud or silt or in mixed sediment of silt, sand and gravel, it is often found growing in water shaded by overhanging trees as well as in full sun (Aston, 1973; Sainty and Jacobs 1981; Sainty and Jacobs 1994)(Plate1.2).

Plate 1.1. A medium sized ex-vitro specimen of *A. elongatus*, approx 6 months old, growing in a 100 mm (420ml) plastic pot.

Plate 1.2 An *A. elongatus* creek habitat with extensive riparian vegetation including species of *Melaleuca*, *Leptospermum* and *Syzygium*. In the foreground *A. elongatus* foliage intermingles with algae coated foliage of a *Myriophyllum* species. Other aquatic macrophytes in the same habitat include *Ottelia ovalifolia, Potamogeton* species and a narrow leaf form of *Vallisneria gigantea*.

The distribution *A. elongatus* was considered to range from the Kimberley region of Western Australia, the Northern Territory and on the East Coast of Australia, from northern New South Wales to the coastal regions of Cape York (Aston, 1973; Sainty and Jacobs 1994). However a recent study based on seed characteristics, has divided *A. elongatus* into five separate species; *A. kimberleyenis*, *A. lancesmithii*, *A. euryspermus*, *A. vanbruggenii* and *A. elongatus* (Hellquist and Jacobs 1998). *A. elongatus* has also been split in to two sub species; subsp. *elongatus* which rarely or never produces floating leaves and subsp. *fluitans* which commonly produces floating leaves. The distribution of *A. elongatus* subsp. *elongatus* includes north eastern New South Wales and the coastal regions of Queensland while *A. elongatus* subsp. *fluitans* occurs in the Petrie area of SE Queensland (Hellquist and Jacobs 1998).

From personal observation, the difference between these two sub species is questionable. Based on the location were the plants used in this study were collected, they are likely to be subsp. *elongatus*. In addition, from personal observation, in their natural habitat, these plants produce predominantly submerged leaves but in still water in holding tanks in high light intensity they produce mostly floating leaves with only a few submerged leaves. Hence, if the distribution of each subspecies given by Hellquist and Jacobs (1998) were ignored, they would also fit the description of subsp. *fluitans*. Perhaps the described differences between *A. elongatus* in the Petrie area compared to other areas are phenotypic expressions rather than genetic.

Apart from some morphological and taxonomic studies (van-Bruggen 1969; Aston 1973; Hellquist and Jacobs 1998) even less is known about the physiology of *A. elongatus*, particularly in relation to its growth habits, reproduction, responses to environment, and methods of propagation and culture.

The only information available comes from a limited range of aquarium hobbyist publications that are quite vague on this issue. Most recommend growing *A. elongatus* in water temperatures ranging from 22-25°C but giving the plant a period of rest during winter months in cooler water (15°C) (DeWit 1966; Stodola 1967; Rataj and Horeman 1977). Other than this *A. elongatus* is said to require water hardness

from 3°- 9° DH (24-72 mg L^{-1} $CaCO_3$), with a pH from 6.5 to 7.8 (DeWit 1966; Stodola 1967).

Most *Aponogeton* species produce a tuber and many, including some Australian species, go through an annual resting stage or dormancy period (Stodola, 1967; Aston, 1973; Sainty and Jacobs 1994). This appears to be an adaptation to climates with seasonal wet and dry periods (Stodola 1967; Sainty and Jacobs 1994). Under cultivation, in an aquarium, these plants often rot during the dormant period therefore aquarists commonly recommend removing the dormant plants from the aquarium and place them in moist media until growth recommences (Stodola 1967; Rataj and Horeman 1977; Elliot and Jones 1982).

As *A. elongatus* has a tuber, it has been assumed that an annual resting stage or dormancy period is a requirement. One Australian reference describes this as being a significant factor in the culture of this species (Elliot and Jones 1982). However, other references (DeWit 1966; van-Ramshorst 1991) claim that this species grows all year round in an aquarium without a rest period. In this study, propagating and growing *A. elongatus* in a range of experiments provided no evidence of dormancy or a set rest period. One large plant, although not used in any experiment described in this study, has been actively growing for three and a half years in a display tank under 24hr continuous light. The plants were also observed to have abundant healthy foliage when collected from a creek in the middle of the winter. It appears that provided conditions are suitable these plants are capable of growing continuously without any ill effects. Seasonal dormancy as described in some literature may be a form of plant quiescence in response to adverse environmental factors such as low nutrition or nutrient unbalance, low temperatures, and drought but this has not been tested.

Despite the recognition of *A. elongatus* as a decorative aquarium plant, in the past, the Australian aquarium plant industry has shown little interest in propagating this species because plants were obtained by exploiting natural sources. From personal observation, *A. elongatus* is not common throughout its natural range and may already be absent from some areas due to over exploitation. However, recent Queensland

State Government conservation laws offer some protection by restricting collection from the wild. This includes restrictions on the sale of native Aponogeton species in Queensland irrespective of the origin being intra or interstate (Queensland Department of Environment 1996; Queensland Department of Environment 1998). The sale of *A. elongatus* collected from the wild is prohibited however the sale of this aquatic macrophyte is permitted under certain conditions. One of these conditions is that the seller can prove that the plants have been propagated from cultivated mother stock.

Most Australian species of Aponogeton reproduce entirely by seed, however a tiny population of plants located in North Queensland are viviparous, that is they can reproduce vegetatively by producing plantlets on their flowering spikes (van-Bruggen 1969). van-Bruggen, (1969) was unable to fully classify this plant but in a more recent study by Hellquist and Jacobs (1998) it has been given the species name *A. proliferus.*

Most of the plants used in the experiments described in this study were propagate by tissue culture based on the techniques described by Polish researchers (Kukulezanka, et al. 1979; Kukulezanka, et al. 1980). One medium sized *A. elongatus* tuber can be cut up into about 50 tissue segments. Each explant (segment) is capable of producing 5-10 plantlets and with further subculturing, this number can be doubled. This means a potential increase from one tuber of 250 to 1000 plantlets. However, this multiplication rate has not been fully realised due to endogenous contamination (mostly bacterial and one or two fungi) that caused losses as high as 50 to 70% of explants. Repeated subculturing also proved to be of limited value as proliferation decreased with each successive subculture and contamination continued to reduce the number of cultures. The plantlets grow rapidly (three to four fold larger in two months) when planted in pots filled with sterile washed river sand, fertilised with Osmocote^{R} and placed in an aquarium.

Discussions with local aquatic plant nursery operators revealed they have had difficulty in growing this plant from seedlings, however one person observed that *A. elongatus* grows well in actively flowing creeks. Subsequent personal observations with both tissue cultured explants and seedlings suggest that a major improvement in

growth could be obtained by growing them in artificially stirred water compared to still or stagnant water.

Some initial questions arising from this observation lead to the formation of the objectives for this study. Was stirred water the main factor, or was some other factor contributing to this observed growth response. If stirred water was the main factor what were the properties of stirring the water that contributed to *A. elongatus* growth.

1.2 Significance of this Thesis

(1) *A. elongatus* is recognised overseas and in Australia as an attractive aquarium plant, which provides some opportunity for commercial production.

(2) It is an Australian native plant, which is considered endangered. Traditionally, all the material offered for sale in the Australian and overseas aquarium trade has been harvested from Australian rivers and creeks.

(3) This plant is not common throughout its natural range and may already be absent from some areas due to over exploitation.

(4) Since the introduction of the Queensland State Government Nature Conservation act, the Queensland commercial aquarium plant industry has shown considerable interest in obtaining information on the culture of this plant. . .

(5) The results of this study could lead to the development of effective commercial methods of culture that will reduce the danger of over exploitation of natural populations, illegal or otherwise.

(6) This is the first study into specific aspects of *A. elongatus* anatomy and physiology

1.3 Objectives and Thesis Structure

The development of effective methods of commercial cultivation depends on an understanding of how selected environmental factors ie temperature, light, water flow, nutrition, pH levels and dissolved inorganic carbon can be manipulated to obtain optimum growth. The overall objective of this study was to examine the effect of some of these factors on the growth of advanced ex vitro plantlets and seedlings of *A. elongatus*. This study also gives some insight as to the extent of *A. elongatus* adaptation to a range of limiting factors inherent to a natural aquatic environment.

Based on these objectives, Chapter 2 reviews the literature to identify the limiting factors of an aquatic environment and the range of aquatic plant adaptations. Chapter 3 examines the interaction between water stirring (water flow) and nutrition on the growth of *A. elongatus.* Chapter 4 examines the growth response of *A. elongatus* to varying levels of dissolved inorganic carbon (DIC) uptake as influenced by different pH and tests the hypothesis that the enhanced growth of *A. elongatus* in stirred water is due to increased DIC. Chapter 5 discusses the overall significance of the results and outlines future work.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This review is about the physiology and some morphology of aquatic plants in general. There has been almost nothing in the scientific literature on the physiology or anatomy of *Aponogeton*, particularly *A. elongatus*, but other aquatic plant (macrophyte) species have been more widely researched. It is generally believed that most aquatic macrophytes have terrestrial ancestors but they have become highly adapted to an aquatic environment (Sculthorpe 1967). Some are so well adapted that they can grow fully submerged at a rate equal to that of many terrestrial plants. This capacity for rapid luxuriant growth makes some of these plants serious aquatic weeds (Reiskind, et al. 1997). Although possibly originating from primitive terrestrial plants many aquatic macrophytes appear highly advanced as they have developed very specialised morphological, anatomical structures and physiological systems that enable them to flourish in an aquatic environment (Sculthorpe 1967).

Most aquatic macrophytes, including *A. elongatus*, produce flowers of similar form and structure to those of their terrestrial counterparts (Sculthorpe 1967). Often only the flowers have maintained their terrestrial form and they are commonly emergent or aerial. The majority of aquatic macrophytes still depend on either wind or insects for pollination. However, some highly specialised aquatic macrophytes have fully submerged flowers and are water pollinated (Sculthorpe 1967).

A range of environmental factors prevent terrestrial plants from surviving and growing under water. The submerged sediment (soil) is usually water logged, anoxic and often highly reduced (Gambrell and Patrick 1988). Saturation of foliage prevents transpiration, and 10^4 times greater diffusion distances restrict the availability of carbon dioxide and oxygen in water compared to air (Raven, et al. 1985; Keeley 1990)

2.2 Aquatic Sediment

One of the main reasons why water logged soils are devoid of oxygen is that the diffusion rate of oxygen through water logged soil is 10^{-4} the rate of gaseous diffusion (Gambrell and Patrick 1988). This means when soil becomes water logged, anoxic conditions develop rapidly as plant roots and soil microbes use up oxygen. Only a mere 3 or 4 mm of the sediment surface is oxygenated in permanently flooded sediments that are typically found at the bottom of ponds, lakes and swamps (Gambrell and Patrick 1988). Beyond this depth, the sediment is anoxic and often highly reduced. Bacterial facultative anaerobes can use up oxygen more rapidly than it can be replaced by diffusion from the sediment surface.

Waterlogged sediments are reduced because bacterial anaerobes and facultative anaerobes that exist in waterlogged sediment obtain energy from the reduction of oxides, causing an accumulation of soluble chemical reductants, some of which are highly phytotoxic (Armstrong 1978). Nitrate, an important plant nutrient, is reduced to nitrogen gas and rapidly lost to the atmosphere (denitrification), resulting in considerable losses of nitrogen from anaerobic sediments (Gambrell and Patrick 1988). Gambrell and Patrick Jr (1988) describe a simulated salt-water marsh losing the equivalent of 7.4kg nitrogen/ha/day due to denitrification. Apart from a lack of nitrates, sulphur is reduced to phytotoxic sulphide, and zinc is less available (Gambrell and Patrick 1988). Conversely, phosphorus, iron and manganese are more available in reduced sediments. Phytotoxic volatile fatty acids and hydrocarbons (methane) are also produced as a result of anaerobic respiration, particularly in sediments high in organic matter (Penhale and Wetzel 1983).

It is this accumulation of reductants, coupled with the accumulation of organic matter that can make some anoxic sediment strongly negatively aerated, providing a considerable oxygen sink (Armstrong 1978). Therefore plant roots growing under waterlogged conditions are subject to; a lack of oxygen, the presence of phytotoxins and a reduction in the availability of some plant nutrients (Armstrong 1978).

2.3 The Water Environment above the Sediment

The above ground (above sediment) environment for aquatic plants is also radically different from that experienced by terrestrial plants including aspects of light, temperature, dissolved oxygen, carbon dioxide, chemical composition, pH, conductivity, alkalinity and nutrient composition.

2.3.1 Light

Light is essential for both aquatic and terrestrial plants. Light can be broken into two main variables, quantity (irradiance or light intensity), and quality (spectral distribution)

2.3.2 Irradiance

The level of irradiance received by terrestrial plants from the sun is dependent on a range of factors including dust or smoke in the atmosphere, the amount of cloud, the time of year, the time of day, latitude, altitude, and the amount of shading by other plants. These factors also effect the level of irradiance received by submerged aquatic plants. However, when light passes through water the level of irradiance is further reduced. Some of this light is reflected at the water surface. Some is absorbed by various colloidal particles in the water or scattered (reflected) from particle to particle. This limits light penetration and causes it to follow a zigzag path in the water (Kirk 1994)**.** Therefore, not all light received by submerged aquatic plants comes from directly above; reflected light comes from all directions.

Wave action can also effect light particularly in shallow clear water. Very bright bands of light are formed by the convex part of a surface wave acting as a focusing lens. Conversely the concave part of the wave de-focuses or scatters the light causing zones of lower than normal light (Kirk 1994).

2.3.3 Spectral Distribution

The spectral distribution of light can change rapidly with water depth. In the sea, as sunlight penetrates to the depths, red light is the first to be absorbed followed by orange, yellow, green and finally blue. In relatively clear seawater at a depth of 15 meters most of the light is at the blue green end of the spectrum, which can be utilised by many forms of algae (Kirk 1994).

In fresh water, such as creeks, rivers and lakes, light behaves differently. Kirk (1994) described inland lakes as having no blue light in as little depth as one meter due to the presence of yellow materials such as clay colloids or organic material such as tannins or humus. These materials are not common in seawater. The spectral distribution of light at this depth consists of a broad band between red to green (750-550 nm) with a peak around yellow (580 nm).

Aquatic macrophytes may be more able to adapt to the absence of blue light in lake or river water at 1 m or more in depth than are algae. Chlorophyll *a* and *b* are the main photosynthetic pigments found in most macrophytes (higher plants) (eg *A. elongatus*). The absorption spectrum of chlorophyll *a* and *b* peaks around the red region (600-700nm) with a much larger peak around the blue region (350-500nm). Most algae have chlorophyll *a* and c_1 *or* c_2, which means they rely more heavily on getting their energy from the blue end of the spectrum (Kirk 1994).

2.3.4 Temperature

All plant growth and development is influenced by temperature and plants vary regarding the temperature range in which they can grow. For example the temperature range for growth of *A. elongatus* is 15 - 30° C, *A. crispus* 22 - 30°C, *A. undulatus* 22-28°C, *Egeria densa* 15 -25°C, *Elodea canadensis* 6.5-25°C (Sainty and Jacobs 1981) (Scheurmann 1993).

It is well known that diurnal temperature fluctuations in water can be much less than on dry land but this is dependent on the volume of water. Generally the larger the water volume the more even the temperature from night to day. Water tends to be slow in absorbing heat and releasing it. The larger the volume the smaller the surface area relative to the volume which reduces the rate of heat adsorption and release. Even seasonal differences in temperature can be much less in large bodies of water such as large lakes or oceans. In addition, the differences between maximum and minimum temperatures are much greater near the surface or in the shallows than in deeper water.

The temperature range for different aquatic macrophytes tends to be narrower than for terrestrial plants although many aquatic macrophytes can exist in shallow water of < 1m

where seasonal and diurnal temperature fluctuations are much greater (Sainty and Jacobs 1981; Bowes and Salvucci 1989).

2.4 Chemical Properties of Water

Some common parameters of water quality which effect water plants include; pH, alkalinity, water hardness (temporary, permanent), conductivity, dissolved gases (oxygen and carbon dioxide levels) and redox potential. Although these factors are treated separately in this review, they are somewhat interrelated, as the level of one factor can influence, or is linked to, the levels of the others.

2.4.1 pH

Natural sources of water vary greatly in pH. Aquatic macrophytes from different parts of the world tend to reflect this in their tolerance to different pH. According to one aquarium hobbyist publication, most cultivated aquatic macrophytes prefer pH in the range 5 – 7.5 (Scheurmann 1993). Aquatic macrophytes can tolerate a range of pH levels but they can vary in their tolerance of extremes.

A low pH effect on sediments for example can cause phytotoxic levels of some metals (manganese, aluminium,) and reduce the availability of dissolved CO_2 and other nutrients (calcium, magnesium) while a high pH can reduce the availability of phosphate, sulphate, iron, manganese (Gambrell and Patrick 1988; Jackson, et al. 1993; Handreck and Black 1994). The form of molecules present in solution can also be effected by pH. For example at a low pH, ammonia (NH_3) is ionised to ammonium (NH_4^+) due to a large number of hydrogen ions, while in solutions with pH above 7.2, ammonium ions (NH_4^+) are deionised back to ammonia (NH_3) (Frith, et al. 1993). With pH levels above 8.5, most of the dissolved carbon dioxide (CO_2) is converted to bicarbonate (HCO_3^-) or carbonate (CO_3^{-2}) (Sand-Jensen and Gordon 1984; Larkum, et al. 1989).

2.4.2 Alkalinity

Another water property is alkalinity. Alkalinity is a measure of the acid neutralising capacity of a solution (Faust and Aly 1981). The main contributors to alkalinity in natural

water supplies are bicarbonate (HCO_3^-) and to a lessor extent carbonate (CO_3^{-2}) and hydroxide (OH-) ions, but may also include, borates ($H_4BO_4^-$), phosphates ($H_2PO_4^{-2}$, PO_4^{-3}) and silicates ($H_3SiO_4^-$), usually in much lower proportions (Faust and Aly 1981). The relative proportions of bicarbonate and carbonate ions is determined by an equilibrium between atmospheric CO_2, dissolved CO_2, HCO_3^-, CO_3^{-2} and their interaction with pH and other pH dependant elements (Stumm and Morgan 1970; Faust and Aly 1981; Sand-Jensen and Gordon 1984).

Alkalinity measurement is of interest because it gives a measure of dissolved inorganic carbon (DIC) and pH buffering capacity (Stumm and Morgan 1970). Ions such as bicarbonate and carbonate increase the amount of acid that must be added to cause a unit decrease in pH (Faust and Aly 1981). They combine with the hydrogen ions supplied by the acid effectively removing them from solution, resulting in pH stability.

2.4.3 Water Hardness

Hardness is a commonly measured property of water. It is largely a measure of the quantity of calcium and magnesium ions in water but in some waters, iron and manganese can also contribute to hardness. Calcium ions are usually 3 to 10 times more common than magnesium ions (Frith, et al. 1993).

Hardness can be further divided into permanent and temporary hardness. Temporary hardness is due to the presence of calcium and magnesium bicarbonates in water. These can be easily precipitated out of solution as calcium and magnesium carbonate. Temporary hardness in water is formed when calcium or magnesium carbonate (limestone) is dissolved by carbonic acid in the water, forming soluble calcium bicarbonate. Carbonic acid is formed when carbon dioxide in the air dissolves in water. The following reactions illustrates this:

$$CO_2 + H_2O \leftrightarrows H_2CO_3$$

$$H_2CO_3 + CaCO_3 \leftrightarrows Ca(HCO_3)_2 \leftrightarrows 2HCO_3^- + Ca^{2+}$$

Permanent hardness is due to the calcium and magnesium ions remaining after temporary hardness has been removed. This has formed from soluble calcium and magnesium

compounds such as calcium chloride, calcium sulphate (relatively insoluble), magnesium chloride or magnesium sulphate (Frith, et al. 1993).

There is similarity between water hardness and alkalinity particularly with temporary hardness as the salts that contribute to hardness also contribute to alkalinity. The relationship however is complex (Frith, et al. 1993).

Hardness is expressed in a number of different scales. Australia, like the USA, and Great Britain, express hardness as mg L^{-1} of $CaCO_3$ (table 2.1) (Frith, et al. 1993):

Table 2.1
A comparison of two commonly used hardness scales

Australian Hardness Scale		**German DH scale**	
mg L^{-1} $CaCO_3$	Term	DH	Term
0 - 50	soft	0 to 5°	very soft
50 - 100	moderately soft	5 to 10°	soft
100- 200	slightly hard	10 to 20°	medium hard
200- 300	moderately hard	20 to 30°	hard
300- 450	hard	over 30°	very hard
over 450	very hard		

Another scale commonly used by aquarium hobbyists is the German Degree of Hardness (DH) (table 2.1). The Germans express water hardness in mg L^{-1} of calcium oxide (CaO). One German degree of hardness (1° DH) equals 17.9 mg L^{-1} CaO (Sterba 1967).

Aquatic plants are reported to vary in their response to water hardness. For example, *A. elongatus* prefers water hardness between 3 - 9° DH (24- 72 mg L^{-1} $CaCO_3$) (DeWit 1966); *A. crispus* 2 - 15° DH (16- 120 mg L^{-1} $CaCO_3$); *A. undulatus* 5 - 12°DH (40- 96 mg L^{-1} $CaCO_3$); *Egeria densa* 8 -18°DH (64- 144 mg L^{-1} $CaCO_3$) and *Cabomba aquatica* 2 - 8°DH (16- 64 mg L^{-1} $CaCO_3$); (Scheurmann 1993).

An increase in water hardness also contributes to salinity due to the increase in calcium and magnesium ions. However, unlike sodium, which is a major contributor to salinity, both calcium and magnesium are desirable plant nutrients.

2.4.4 Conductivity

Conductivity is the electronic measure of salinity. Salinity is the sum of all ions in solution. Salinity can be expressed in two ways; total dissolved salts (TDS) (mg L^{-1} or ppm), or electrical conductivity (EC). EC units used include deciSiemens/meter (dS m^{-1}), milliSiemens/cm (mS cm^{-1}) and microSiemens/cm (µS cm^{-1}). The term commonly used for measuring water is dS m^{-1}.

There is little information in the literature regarding the extent of aquatic macrophyte tolerance of salinity levels. However, aquatic macrophytes occur in a range of salinity levels from fresh water (0.1 – 0.8 dS m^{-1}), saline fresh water (0.9 to 2.4 dS m^{-1}) to brackish water (2.5 – 53 dS m^{-1}), or seawater (54 dS m^{-1})(Sainty and Jacobs 1994). Some occur only in fresh water (*A, elongatus*) while others appear to tolerate a wide range of salinity from freshwater to seawater (*Ruppia megacarpa*).

EC can be used to monitor the overall concentration of nutrients in solution. This is important when doing aquatic plant nutrition experiments as salinity levels can change over time. Evaporation of water in tanks increases salinity as does regular additions of water unless the water used is distilled water. EC also provides a quick but general indication of plant nutrient uptake.

2.4.5 Dissolved Gas levels (oxygen and carbon dioxide)

Monitoring oxygen levels is of prime importance in aquaculture as fish or invertebrates are users of oxygen. A range of factors determines the level of oxygen in water. These include; temperature, salinity and altitude (Frith, et al. 1993). In aquariums and tanks, high fish stocking rates and bacteria in the water commonly deplete the level of oxygen. Algae and higher plants can also deplete oxygen during periods of dark (night).

Oxidation-reduction potential (Redox potential; ORP) is commonly used to indirectly measure the amount of oxygen in water or submerged sediments. Theoretically, oxidation-reduction processes should be in equilibrium but in natural waters (rivers and lakes), many redox reactions are slow (Faust and Aly 1981). Also, there are differences in large deep bodies of water such as lakes. The surface waters in contact with atmospheric oxygen commonly have oxidation levels while deep in the lake, oxygen may be absent resulting in reducing levels (Faust and Aly 1981). Faust and Aly (1981) also suggest there may be intermediate levels in the lake where both oxidation and reduction reactions occur out of equilibrium. Aquatic plants as well as other organisms can create microenvironments, in which redox potentials differ from the surrounding macro environment (Wium-Anderson and Anderson 1972).

A range of electronic meters are available for measuring redox potential (Eh). They consist of a platinum electrode and a reference electrode from which the meter measures potential difference, this potential difference is then displayed on a digital readout as Eh values. Despite the fact that these meters are available to aquarium hobbyists as well as researchers, Faust and Aly (1981) suggest that proper interpretation of observed values can be very difficult. Reactions can occur on the platinum electrode that are non-reversible, which can influence the observed redox value leading to misinterpretation.

More recently, highly sensitive microelectrodes have become available that accurately measure oxygen and dissolved carbon dioxide levels and these are being used in aquatic plant research (Pedersen, et al. 1995).

Carbon dioxide, like oxygen, is also found dissolved in water. However, CO_2 reacts with water to form carbonic acid, which dissociates into bicarbonate ions and further into carbonate ions which inturn effects pH and alkalinity. This process is in equilibrium as follows (Saruhashi 1955; Kirk 1994):

$$H_2O + CO_2 \leftrightarrows H_2CO_3 \leftrightarrows H^+ + HCO_3^- \leftrightarrows 2H^+ + CO_3^{2-}$$

This equilibrium shifts to the right with increasing pH. At a pH between 6.7 to 8.8, approximately 80% of the inorganic carbon is in the form of bicarbonate ions (Larkum, et al. 1989; Kirk 1994). The distribution of CO_2, H_2CO_3, and CO_3^{2-} in pure water as a function of pH is illustrated in Fig 2.1.

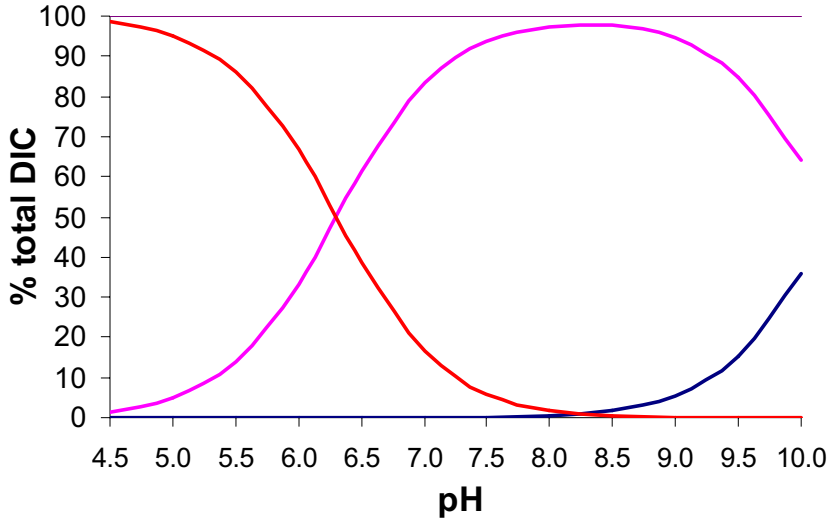

Fig 2.1 The effects of pH on percentage of (—) H_2CO_3*, (—) HCO_3^-, and (—) CO_3^- to (—) total DIC. Based on data calculated from formula and equilibrium constants provided by Stumm and Morgan (1970) for pure water at 25ºC open to the atmosphere. (H_2CO_3* = CO_2 aq + H_2CO_3)

2.4.6 Plant Nutrient Levels

Much the same plant nutrients are required for aquatic plants as for terrestrial plants. The main elements include nitrogen (in various forms ie ammonium, nitrate, nitrite ions) phosphate, potassium, magnesium, calcium, sulphate, and trace elements (iron, manganese, zinc etc). Aquatic macrophytes tend to take up more ammonium ions than nitrate. Work with a marine macrophyte *Ruppia maritima* showed that ammonia is taken up nine times faster than nitrate or nitrite (Thursby 1984).

2.5 Aquatic plant adaptations

2.5.1 Anoxia

Plants can be roughly grouped into the following categories according to their tolerance to root anoxia; intolerant terrestrial plants, flood tolerant terrestrial plants, marsh plants and submerged (and emergent) aquatic macrophytes. Plant tissues subject to a lack of oxygen

undergo anaerobic respiration. In aerobic respiration, pyruvate is broken down to Acetyl-Co-A plus CO_2. Acetyl-Co-A inturn is combined with oxyloacetate to form citrate within the Kreb Cycle. In anaerobic respiration, pyruvate from glycolysis is broken down to acetaldehyde, which in turn is broken down to ethanol. Intolerant terrestrial plants under flooded soil conditions typically show an acceleration of glycolysis coupled with increased production of alcohol dehydrogenase (Crawford 1978). The accumulation of ethanol causes membrane disintegration and eventually cell breakdown. In addition, the leakage of cell contents into the soil encourages the invasion of soil pathogens (Crawford 1978).

Flood tolerant terrestrial plants, marsh plants and submerged (and emergent) aquatic macrophytes have all overcome anoxia somewhat by adapting their metabolic mechanisms. This is achieved by one or more of the following; controlling the anaerobic metabolic rate, diversification of the end products of glycolysis or the coupling of metabolic pathways to avoid build up of toxic compounds (Crawford 1978) (Penhale and Wetzel 1983).

Flood tolerant macrophytes under anoxic conditions typically do not show acceleration of glycolysis or any change to alcohol dehydrogenase activity (Crawford 1978). Those plants capable of tolerating only brief periods of anoxia have the ability to retard anaerobic glycolysis and limit alcohol dehydrogenase activity. Adaptation to longer periods of anoxia requires diversification of the end products of glycolysis. Ethanol is replaced to a varying degree with less toxic products such as lactate found in some seeds, malate in marsh macrophytes, shikimic acid in some aquatic macrophytes and glycerol in *Alnus incana* (Crawford 1978).

Crawford (1978) suggests that in flood tolerant macrophytes the enzyme that converts malate to pyruvate is inhibited (Fig 2.2). He also mentions evidence that some of these plants produce oxalic acid, known to be a strong inhibitor of the malic enzyme. In this way, there is a reduction in ethanol in favour of malate.

Anoxia tolerant plants have been found to produce a range of metabolites under conditions of low oxygen (Crawford 1978). Crawford (1978) speculates that coupling of

metabolic pathways is beneficial in disposing of surplus protons and in increasing ATP yield. He cites the example of the production of shikimic acid, which is coupled with glycolysis and

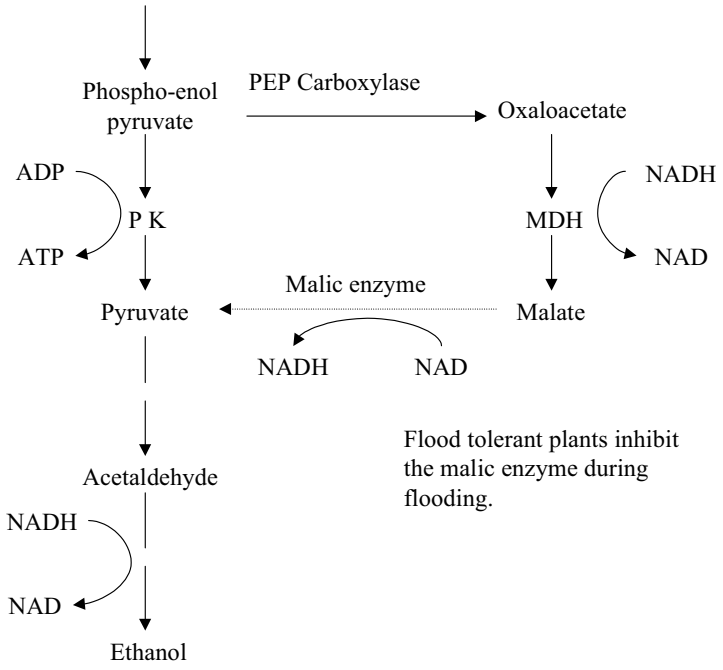

Fig 2.2 A possible metabolic separation between flood tolerant and intolerant macrophytes (Crawford 1978).

amino acid synthesis. Fig 2.3 (Crawford, 1978) illustrates the various methods of proton disposal and the range of end products produced by flood tolerant macrophytes resulting from prolonged periods of anoxia.

A good example of the coupling of metabolic pathways can be found in a fully submerged marine macrophyte, *Zostera marina* (eelgrass) (Pregnall, et al. 1984). This plant grows prolifically in dense beds while rooted in anoxic and highly reduced sediments. Under anoxic root conditions the amino acids alanine and γ-amino butyric acid rapidly accumulated and glutamate and glutamine declined. There was little increase in ethanol and malate remained static. Once the roots were placed under aerobic conditions both alanine and γ-amino butyric acid declined but glutamate and glutamine increased.

Pregnall et al (1984) proposed a metabolic model to account for these and other observed changes (fig 2.4). Pyruvate, instead of being converted to ethanol, is donated an amino group (transaminated) by glutamine to form alanine. At the same time glutamine losses that amino group to become glutamate which in turn is converted to γ-amino butyric acid by a decarboxylation reaction releasing CO_2 (Pregnall, et al. 1984).

It is interesting to note that the roots are normally adapted to periods of anoxia. If whole aquatic macrophytes are subject to anoxia there is usually a rapid accumulation of ethanol in the plant tissues (Crawford 1978) (Pregnall, et al. 1984). The foliage of most fully emersed aquatic macrophytes is normally unlikely to experience anoxia, as there is usually adequate supplies of dissolved oxygen in the surrounding water to sustain aerobic respiration.

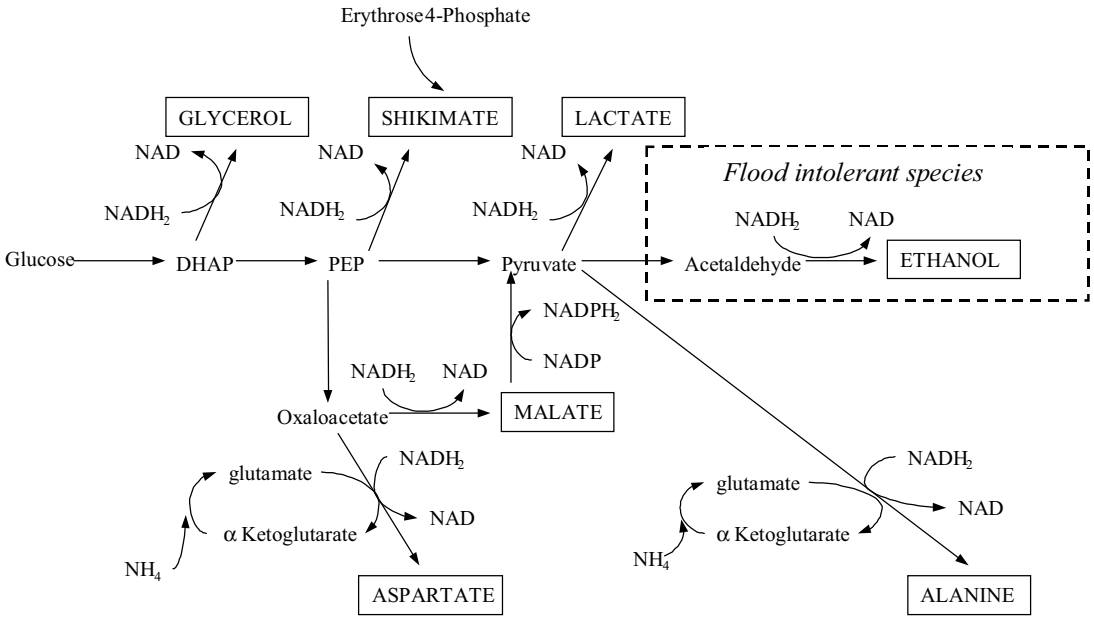

Fig 2.3 **'**The various means of proton disposal and range of end products in plants capable of enduring prolonged periods of anoxia' (Crawford 1978). .

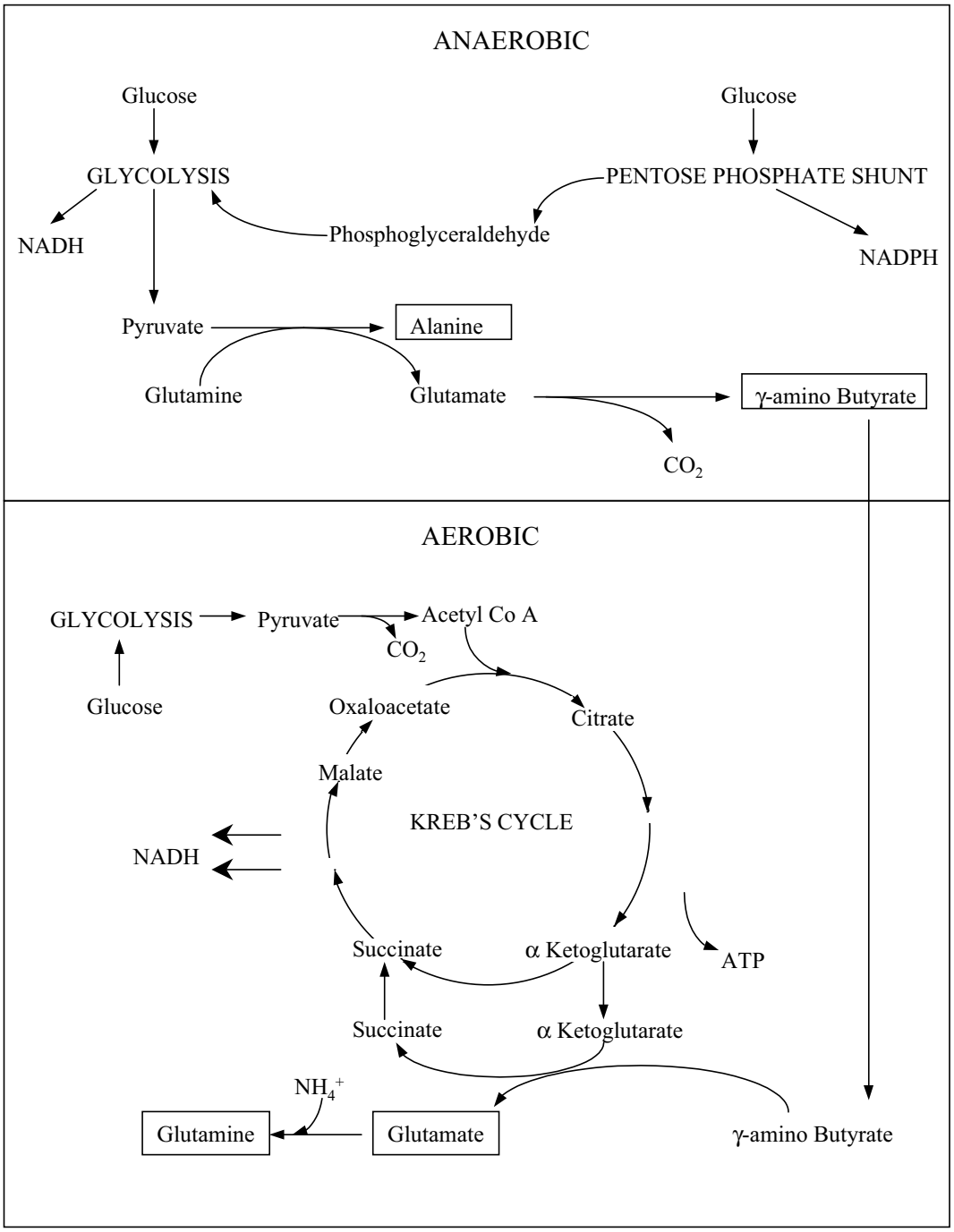

Fig 2.4 'A possible metabolic model that accounts for observed changes in metabolite levels in eelgrass roots during anaerobic and aerobic treatments' (Pregnall, et al. 1984)

2.5.2 Anatomical adaptations to anoxia

Almost all aquatic and marsh macrophytes have anatomical adaptations that enable them to grow in anoxic rooting substrate without dependence on metabolic processes. They have a continuous lacunar (ventilation) system throughout their leaves, stems, and roots, which generally provides more than adequate aeration of roots. Interestingly, gas spaces can also be found in some flood tolerant terrestrial macrophytes but are not usually as well developed as in marsh or true aquatic species (Sculthorpe 1967; Armstrong 1978; Crawford 1978).

Gas spaces (lacunae) commonly develop from spaces (chinks) between young cells, which enlarge by cell expansion, gaseous pressure and collapse of cells. They are often further enlarged by division and expansion of cells surrounding the air spaces (Sculthorpe 1967). In some species, there is no loss of cells and the lacunae are more numerous but not individually as large, forming a honey comb structure (Armstrong 1978). The site of lacunae development is commonly cortex tissue in stems and roots and in the mesophyll tissue of leaves (Sculthorpe 1967; Armstrong 1978). They can also develop in vascular tissue as xylem lacunae but are much smaller in cross section (diameter) than those that develop in cortex tissue (Sculthorpe 1967).

There are a number of advantages of root ventilation compared to metabolic methods of overcoming sediment anoxia. Anoxia is avoided by maintaining a supply of oxygen for root respiration, nutrient absorption and growth. In addition, some of the oxygen leaks out into the rhizosphere, oxidises reduced elements, which in turn reduces toxicity and improves nutrient availability (Armstrong 1978).

Work done with water lilies possibly illustrates how many marsh macrophytes and emergent aquatics ventilate their roots, tubers or rhizomes (Dacey 1991). Water lilies, like many other aquatic macrophytes, have a well-developed lacunar system. Dacey (1991) discovered that there was significant inflow of oxygen and other gases from young floating or emergent leaves, through these air spaces, to the under ground rhizomes and roots and out again into the atmosphere through older leaves. This could not be explained by simple diffusion. He found that heat from the sun caused evaporation of water within the leaves. The resulting water vapour pressure with young leaves was found to exceed

external atmospheric pressure. Consequently, there is a mass flow of gases, down lacunae to the roots. Apparently, the pores (stomata openings?) in young leaves are too small to allow mass flow to the outside atmosphere to equalise the pressure. The pores are large enough to allow diffusion of oxygen and carbon dioxide into the leaf. The oxygen concentration within the leaf is lowered compared to the atmosphere because of mass flow down the lacunae. This creates a diffusion gradient that allows more oxygen (as well as other atmospheric gases such as carbon dioxide) to rapidly diffuse into the leaf. Older leaves have much larger pores that allow mass flow out of leaves creating a 'flow through ventilation system' or 'biological steam engine' (Dacey 1991).

2.5.3 Photosynthetically driven oxidation

A considerable number of aquatic macrophytes grow effectively and often rampantly with predominantly submerged leaves. With submerged leaves, a flow through system for ventilating the root system is not possible. Yet, most of these plants have a fully functional lacunae system. Oxygen produced from photosynthesis is widely accepted as the means of oxygenating the root system in many fully submerged aquatic macrophytes (Wium-Anderson and Anderson 1972; Penhale and Wetzel 1983; Smith, et al. 1984; Thursby 1984; Barko, et al. 1986; Sorrell and Dromgoole 1987; Dennison 1988; Stevenson 1988; Larkum, et al. 1989; Sheppard and Evenden 1991; Pedersen, et al. 1995). Oxygen can also diffuse into foliage from the surrounding water during periods of darkness but this is considered only adequate to sustain plant organs that are above the substrate. It has been found that root oxygen loss is considerably greater during the day light period compared to the night period (Sand-Jensen, et al. 1982; Smith, et al. 1984; Thursby 1984; Sorrell and Dromgoole 1987). This implies that most of the oxygen transported to the roots is photosynthetic in origin. In fact, photosynthesis can produce considerably more oxygen than that needed for root respiration, which can result in extensive oxidation of the rhizosphere (Wium-Anderson and Anderson 1972; Pedersen, et al. 1995). This means that aerobic respiration and normal function of roots is highly dependent on the productivity of photosynthesis.

The mechanism of oxygen transport to the roots of fully submerged macrophytes is not clearly understood (Smith, et al. 1984; Sorrell and Dromgoole 1987; Larkum, et al. 1989). It has generally been considered to be simple gaseous diffusion but Smith et al (1984)

found for the marine macrophyte *Zostera marina*, that the measured O_2 transport rates, were 10^5 times faster than the known rates of gas phase diffusion. They based this on the limitations of lacunae diameters and distances of transport but it is not clear if they actually measured lacunae diameters or calculated likely diffusion rates based on theoretical parameters. Sorrell and Dromgoole (1987) found this was not the case for the freshwater macrophyte *Egeria densa*. They measured lacunar dimensions and calculated the diffusion gradient of oxygen that was required to drive their observed transport rate. They found that simple gas phase diffusion in the order of 5.49 X 10^{-2} cm^3 O_2 cm^{-3} m^{-1} was sufficient to account for observed transport rates. However Larkum (1989) speculates that as the lacunae are commonly pressurised to around 22kPa, mass flow may also be involved under certain conditions but this is yet to be substantiated.

The level of organic matter in sediment may effect the ability of aquatic macrophytes to oxygenate their rhizosphere (Barko and Smart 1983; Barko, et al. 1986; Barko and Smart 1986). Barko and Smart (1983) found that more than 5% (dry weight) organic matter in sediment can inhibit the growth of the following species; *Elodea canadensis*, *Hydrilla verticillata*, *Myriophyllum spicatum*, *M. aquaticum*, *Potamogeton nodosus*, and *Sagittaria latifolia*. However they also found that the partly emergent species; *M. aquaticum*, *P. nodosus*, and *S. latifolia* were less effected than the fully submerged species; *E. canadensis*, *H. verticillata*, *M. spicatum*. The explanation for the effects of sediment organic matter on plant growth is not clear. Barko and Smart (1983) suggest that growth inhibition results from the accumulation of phytotoxic organic carbon compounds during anaerobic decomposition. The reduced inhibition of emergent macrophytes compared to submerged macrophytes may be due to the greater rhizosphere oxidising abilities of the former. However in a later paper Barko and Smart (1986) found that sediment density effects growth to a greater extent than organic matter content. They hypothesised that the low density, high porosity associated with high organic matter content limits nutrient uptake by having long diffusion distances. Organic matter has effectively diluted the mineral nutrient concentration (Barko, et al. 1986).

It is worth remembering that, despite having photosynthetically driven oxidation of the root system and rhizosphere, submerged aquatic macrophytes (SAM) experience differing degrees of anoxia during the night when photosynthesis ceases (Pregnall, et al. 1984).

The severity and length of anoxia during the dark period depends on a range of factors. These include how highly reduced the rooting substrate is, the amount of oxygen present after photosynthesis ceases and to a limited degree, the amount of oxygen that can defuse down to the roots from the surrounding water above the substrate. Pregnall et al. (1984) found that a seagrass, *Zostera marina*, which grows in often highly reduced marine sediments, quickly switches to an anaerobic metabolic process upon nightfall. However Wium-Anderson (1972) found and Pedersen et al (1995) later confirmed that *Lobelia dortmanna* growing in sandy lake sediment was able to oxygenate the rhizosphere to a considerable depth (20cm). *Littorella uniflora* and *Isoetes lacustris* were also found to behave in a similar way to *Lobelia* (Wium-Anderson and Anderson 1972). There was sufficient oxygen produced in reserve for aerobic root respiration to occur over a considerable part of the night period before anoxia occurred (Pedersen, et al. 1995). The lake sediment where these plants were growing had low microbial oxygen consumption rates (Pedersen, et al. 1995) and was possibly less reduced compared to the marine sediments that *Zostera marina* commonly grows in. Penhale (1983) found that *Z. marina* has increased lacunae space when growing in more anaerobic conditions compared to the same species growing in less reduced sediment. They suggest that in more reduced sediment oxygen release rates from roots are greater than in less reduced sediments. Despite this, the effective depth of oxidation from the root surface may be less than in less reduced sediments. Both Pregnall et al (1984) and Thursby et al (1984) found limited penetration (0.75-1.25mm) of the oxidation zone around seagrass roots.

Other situations in which aquatic macrophytes may depend on anaerobic metabolic processes to avoid anoxia include periods of overcast weather or when water conditions are turbid, where light levels are insufficient for photosynthetically driven oxidation of roots (Pregnall, et al. 1984).

Interestingly, it is claimed that some aquatic macrophytes that ventilate their root system, are no more tolerant of anoxia than their terrestrial cousins should their ventilation system fail (Armstrong 1978; Pregnall, et al. 1984).

Most aquatic macrophytes depend on anaerobic metabolic processes at some stage of their life cycles to survive anoxic conditions. Anaerobic metabolic processes occur during the

germination of seeds such as *Rorippa nasturtium-aquaticum* (Sculthorpe 1967), *Typha latifolia* (Sculthorpe 1967); (Frankland, et. al. 1987) *Najas marina* (Frankland, Bartley et al. 1987) *Najas flexilis* (Wetzel and McGregor 1968). It is also possible that these processes also occur in quiescent tubers or rhizomes buried in anoxic sediment, in aquatic species that have a growing period followed by a leafless dormant period (*Aponogeton*, *Nymphaea* species). These plants are often dependent on anaerobic metabolism until leaves are produced and fully functional. Tubers and rhizomes of aquatic macrophytes are often high in carbohydrates such as starch. An adequate supply of carbohydrate is important for prolonged anaerobic respiration (Armstrong 1978), similar to aerobic respiration.

2.6 Photosynthesis

Aquatic macrophytes show many similarities to their terrestrial cousins in regard to photosynthesis. Nevertheless, there are also some distinct differences, particularly with emersed aquatics, which is a strong reflection of their successful adaptation to an aquatic environment.

Like some terrestrial plants that grow in deep shaded habitats, submerged aquatic macrophyte leaves, petioles and stems commonly have very thin cuticles and chloroplasts in the epidermis as well as in mesophyll cells (Sculthorpe 1967) (Larkum, et al. 1989). The mesophyll is usually homogeneous with no division into palisade and spongy mesophyll tissue. In some aquatic macrophytes ie, *Elodea canadensis* and *Najas graminea*, the leaves lack mesophyll cells, consisting entirely of two layers of photosynthetic epidermal cells (Sculthorpe 1967). Many SAM lack stomata or if they are present, they are non-functional (Sculthorpe 1967; Bowes 1985). Chloroplasts of most aquatic macrophytes, unlike the algae, contain chlorophyll a, b, and other pigments in similar proportion to terrestrial macrophytes growing in shady habitats. In fact, most SAM are considered to be shade plants (Bowes 1985).

Whether terrestrial or aquatic, the function of the photosynthesis is to capture light energy in the form of NADPH and ATP (light reaction). During the light reaction, light excited electrons from chloroplast pigments are transported to water molecules. The water molecules are split to produce oxygen molecules and hydrogen ions. Electrons from this

reaction are used along with the hydrogen ions to produce NADPH from $NADP^+$ while some hydrogen ions are used to produce ATP from ADP (Salisbury and Ross 1992). The oxygen produced is of course used by submerged aquatic macrophytes for root respiration and oxidation of the rhizosphere.

NADPH and ATP from the light reaction are used in the dark reaction for reduction and fixing of carbon dioxide (CO_2) to produce sugars and starch. The process or pathway in which CO_2 is fixed to produce carbohydrates is varied. Terrestrial macrophytes as well as most emergent aquatic and marsh macrophytes can be divided up into three main groups in depending on their photosynthetic carbon fixation pathways (Hartmann, Kofranek et al. 1988; Keeley 1990; Salisbury and Ross 1992). These are C-3, C-4 and CAM plants, each of which 'can be distinguished by certain specific physiological, morphological and biological characteristics' (Hartmann, Kofranek et al. 1988).

2.7 Dissolved Inorganic Carbon Uptake

As stated earlier, to overcome the environmental constraints of their habitat, SAM have developed a variety of different processes for collecting and fixing dissolved inorganic carbon (DIC), compared to their terrestrial or emergent cousins. Some of these adaptations involve modification of photosynthetic processes. Before discussing specific processes, it is relevant to briefly describe the availability of different forms of DIC in an aquatic habitat.

SAM occur in a variety of aquatic habits including rivers, streams, lakes, seasonal pools, and in the margins of oceans (Bowes 1985). As mentioned earlier (Section 2.4.5), DIC in water exists as dissolved CO_2, bicarbonate ions (HCO_3^-) and carbonate ions (CO_3^{2-}). The concentration of dissolved CO_2 in water, when in equilibrium with the atmosphere, is approximately 0.01mM which is comparable with the level of CO_2 that is available for terrestrial plants (Sand-Jensen and Gordon 1984; Bowes 1985). However, in fresh water dissolved CO_2 levels vary considerably not only between locations but also over time. Most freshwaters have pH levels that range from 7-8.5 with typical ratios of HCO_3^-: CO_2 ranging from 4 to140 (Sand-Jensen and Gordon 1984). In contrast, seawater is generally much less variable with higher and more stable HCO_3^- levels (2 mM) (Sand-Jensen and

Gordon 1984; Larkum, Roberts et al. 1989). Therefore, as bicarbonate occurs at much higher levels than dissolved CO_2, one could expect aquatic macrophytes to absorb bicarbonate ions as the main source of carbon for photosynthesis.

Many SAM are able to utilise bicarbonate except those that are adapted to very soft water (Raven, et al. 1985; Spence and Maberly 1985; Kirk 1994). Some aquatic mosses, for example, lack the ability to use bicarbonate (Spence and Maberly 1985; Stevenson 1988; Kirk 1994). Growth is more rapid if plants are able to utilise dissolved free CO_2 (Sand-Jensen and Gordon 1984; Kirk 1994). This includes well-known freshwater bicarbonate users such as *Elodea canadensis* and *Potamogeton crispus* (Stevenson 1988; Kirk 1994). However there are exceptions such as *Hydrilla verticillata* that have ten fold higher photosynthetic rates at pH 9 where DIC is almost entirely bicarbonate (Bowes 1985).

One of the possible reasons why facultative freshwater bicarbonate users are more common is that the uptake of bicarbonate requires expenditure of energy, which may not be advantageous in all situations (Keeley 1990). In marine habitats, due to the high and constant bicarbonate level, one would expect obligate bicarbonate users to be more common amongst sea grass species. Marine aquatic macrophytes are reported to have a greater affinity to bicarbonate than freshwater macrophytes (Stevenson 1988; Larkum, et al. 1989). However, Stevenson claims it is not clearly known whether bicarbonate uptake in marine aquatic macrophytes is influenced by the level of free CO_2 present. Although it is well recognised that many aquatic macrophytes can utilise bicarbonate, there is less certainty as to whether some of these plants fix carbonate directly or first convert bicarbonate to CO_2 (Miller 1985; Raven, et al. 1985).

Although there is a considerable variety of physical environments, they have some common factors. Submerged aquatic macrophytes are no different to their terrestrial or emergent aquatic cousins in that CO_2 has to be dissolved in water in order to enter photosynthetic cells. However, much longer diffusion pathways are experienced by SAM compared to terrestrial or emergent aquatic plants (Bowes 1985). At the same time, the diffusion rate of carbon dioxide in water (and oxygen) is 10^{-4} times than in air (Bowes 1985; Dennison 1988; Stevenson 1988). The distance that DIC or O_2 has to diffuse to reach the epidermis of a leaf is commonly named the boundary layer or the unstirred layer

(Smith and Walker 1980; Dennison 1988; Larkum, et al. 1989). The greater the boundary layer thickness the greater the diffusion resistance.

Not only is carbon essential for the photosynthetic process of producing carbohydrates but SAM, like terrestrial plants, require sufficient levels of carbon dioxide present in the cytosol to prevent photorespiration. High diffusion resistance can result in low uptake levels of CO_2 with corresponding high levels of photorespiration.

SAM have developed mechanisms for directly reducing the effect of photorespiration. Owttrim and Colman (1989) found that for *Myriophyllum spicatum*, net photosynthetic rate was not suppressed by high photorespiration. They suggest that *M. spicatum* is able to recycle carbon from photorespiration for refixation or that the plant has some mechanism for concentrating CO_2 at the site of rubisco. Sondergaard and Wetzel (1980) had earlier suggested recycling carbon from photorespiration and normal respiration was an effective way of reducing the reliance on less available external sources of DIC. They had looked at photorespiration and recycling of CO_2 in the submerged aquatic macrophyte *Scirpus subterminalis*. Hentschke (1997) found a significant level of photorespiration in *Potamogeton perfoliatus* L. He found that the magnitude of photo respiratory flux increases with the photosynthetic rate and is between 10 to 20% higher compared to terrestrial plants and is more variable. At the same time, its effect on net photosynthetic output decreases rather than increases. He suggests that SAM are able to reclaim both the carbon and energy lost in photorespiration. This means that the photorespiration pathway is linked with effective recycling and refixation of CO_2 (Hentschke 1997).

There are other ways the effects of photorespiration and diffusion resistance are reduced for aquatic macrophytes. These include water flow, waters with high DIC, substrate DIC and biophysical and biochemical DIC concentrating mechanisms.

2.7.1 Water Flow

SAM growing in flowing water have reduced diffusion resistance. Flowing water increases DIC uptake by reducing the boundary layer thickness (or diffusion resistance). Increasing water velocity from 0.03 to 0.46 cm s^{-1} reduces boundary layer thickness from 9.1 to 2.3 mm (Losse and Wetzel 1993). Even the thinnest layer is thicker than the

submerged leaf of < 1mm (Losse and Wetzel 1993). Increasing water velocity, and thereby reducing boundary layer thickness, gives a corresponding increase in net photosynthetic productivity (Chambers, et al. 1991; Madsen, et al. 1993). However, there is a limit to how much water velocity can increase and still give a net increase in photosynthetic productivity.

Generally in water velocities greater than 1 cm s^{-1} net photosynthesis is reduced rather than increased (Chambers, et al. 1991; Madsen, et al. 1993). Madsen et al (1993) reports that *Callitriche cophocarpa* growth rate increases up to a water velocity of 1.5 cm s^{-1}. Above this rate, growth starts to decline. However, work they did with *C. stagnalis* showed a net photosynthetic reduction of 25% when water velocity was increased from 1 to 4 cm s^{-1}. Madsen et al (1993) also found that in velocities above 1 cm s^{-1}, net photosynthesis drops and respiration increases. They found that 16-67% of the reduction in net photosynthesis was due to the increase in respiration. They conclude that this reduction in net photosynthesis is mainly due to mechanical stress from agitation and stretching of foliage.

Generally, once water velocities reach high levels ie >100 cm s^{-1}, increasing biomass loss is the main reason for a reduction in photosynthetic productivity (Chambers, et al. 1991). However this is species dependant as *Ranunculus aquatilis* can grow in average water velocities as high as 20 cm s^{-1}, some marine SAM grow in waters as fast as 30 cm s^{-1}, and some marine macro-algae > 100 cm s^{-1} without detrimental effects (Schwenke 1971; Boeger 1992).

Many SAM have foliage shapes, which help reduce boundary layer thickness in low water velocities (Raven, et al. 1985). For example, leaves with undulations are commonly found on a range of SAM including *A. elongatus*. Undulated margins of leaves are said to help create turbulence therefore reducing boundary layer thickness.

2.7.2 Waters with High DIC

Another way SAM overcome diffusion resistance is to grow in aquatic environments which support high natural respiration rates resulting in concentrations of DIC greater than that fixed by photosynthesis and lost to the atmosphere (Raven, et al. 1985).

Producing foliage close to the water surface is an alternative way SAM reduce this resistance (Raven, et al. 1985). This is a common growth habit of long stemmed SAM such as *Cabomba caroliniana*, *Ceratophyllum demersum*, *Egeria densa*, *Elodea canadensis*, *Lagarosiphon major*, *Hydrilla verticillata* and *Potamogeton* species. From personal observation, these plants often produce dense canopies just below the water surface.

2.7.3 Substrate DIC

Some SAM that grow in water deficient in DIC are able to supplement with DIC obtained via the roots from the rooting substrate. Sediments of water bodies such as lakes, ponds often contain accumulated remains of dead plant material. Raven et al (1985) claim that these sediments can have high CO_2 concentrations because of microbial activity. Pedersen et al (1995) describes how CO_2 is produced in otherwise anoxic substrate. They found that the submerged aquatic macrophyte, *Lobelia dortmanna* as a result of photosynthesis, released considerable quantities of O_2 from its roots into the surround root substrate during the day. During the night, there was enough accumulated O_2 to enabled aerobic microbial activity to produce CO_2. This is supplemented by CO_2 released from the roots because of respiration. During the day, this plant is able to reabsorb this accumulated CO_2 for utilisation in photosynthesis (Pedersen, et al. 1995). This plant commonly grows in sandy sediments in soft water of nutrient poor lakes. Other macrophytes also grow in this habitat and derive most of their CO_2 for photosynthesis from sediments. They are collectively called isoetids and include *Lobelia dortmanna*, *Stylites andicola*, *Littorella uniflora*, and *Eriocaulon decangulare* (Raven, et al. 1985; Pedersen, et al. 1995).

2.8 DIC Concentrating Mechanisms

Biophysical or biochemical DIC concentrating mechanisms also overcome diffusion resistance and minimise photorespiration in many SAM (Raven, et al. 1985). In some cases these DIC concentrating mechanisms are only active when the concentration of exogenous DIC levels are well below the threshold of that required for optimum photosynthesis (Raven, et al. 1985; Keeley 1990).

2.8.1 Biophysical

SAM are considered to have biophysical concentrating mechanisms when they are found to use only rubisco for DIC fixing, have a low CO_2 compensation point and have a high affinity for exogenous DIC (Raven, et al. 1985). Further evidence is the concentration of DIC within photosynthetic cells exceeding that which can be expected based on normal passive diffusion of exogenous DIC into cells. Raven et al (1985) suggests biophysical concentrating mechanisms are common to many SAM as well as to many species of fresh water and marine algae. It is not surprising that many SAM are able to utilise HCO_3^- as the bicarbonate concentration in water is generally much higher than carbon dioxide especially in high pH (8-9).

Evidence suggests various SAM use different kinds of DIC concentrating mechanisms. Three types of DIC concentrating mechanisms have been suggested for HCO_3^-. These are; HCO_3^- influx at the inner plastid envelope membrane, acid and alkaline zones, and/or active HCO_3^- transport at the plasmalemma (Raven, et al. 1985). Active HCO_3^- transport requires the presence of carbonic anhydrase within the cell to catalyse the conversion of bicarbonate to carbon dioxide for fixing by rubisco. This was confirmed by some researchers with the unicellular algae *Chlamydomonas reinharttii* but later research by others found that for SAM, carbonic anhydrase occurs mostly extracellularly (Raven, et al. 1985; Bowes and Salvucci 1989; Husic 1991; Rybova, et al. 1991; Newman and Raven 1993; Beer 1998).

Some SAM such as *Elodea nuttallii*, *Hydrilla verticillata* and *Potamogeton lucens*, produce acid and alkaline zones (pH polarity) on leaf surfaces (Prins and Helder 1985; Raven, et al. 1985; Bowes and Salvucci 1989; Eighmy, et al. 1991; Reiskind, et al. 1997). With *Hydrilla verticillata* the abaxial side of leaves has been found to have a pH of 4.0 and the adaxial side, pH 10.5 (Reiskind, et al. 1997). Raven et al (1985) suggests that HCO_3^- passively diffuses in the acid zone where it is transformed to CO_2. This in turn diffuses into the photosynthetic cells. This acid zone could be either in the boundary layer or within the cell wall (Larkum, et al. 1989). Prins and Helder (1985) found that for *Potamogeton lucens*, bicarbonate uptake is a 'chemiosmotically driven process'. They conclude that the conversion of bicarbonate to CO_2 is adjacent to the epidermis and is reliant on light dependent H+ extrusion causing an electrical potential difference across

the leaf. While not excluding the possibility of the alternate hypothesis of chemiosmotically driven H^+ and HCO_3^- co-transport across the plasmalemma to the interior of epidermal cells, they found no evidence to back this up. Instead, only CO_2 is thought to diffuse across the plasmalemma into the cytosol of leaf cells.

Whether this mechanism is theoretically able to supply enough CO_2 to account for measured uptake of HCO_3^- is considered contentious (Raven, et al. 1985). In fact, Eighmy et al (1991) showed direct uptake of bicarbonate across the plasmalemma in *Elodea nuttallii*, against the concentration gradient, in addition to leaf polarity. They describe a bicarbonate cation symport mechanism within the plasmalemma, which greatly surpassed the polarity mechanism in supplying DIC for photosynthesis. This means direct bicarbonate uptake into a cell but the mechanism of conversion of bicarbonate to CO_2 for RUBISCO was less clear. Reiskind et al (1997) showed a similar mechanism in *Hydrilla verticillata* and suggested a C-4 acid-based carbon concentrating mechanism in the chloroplasts

Other SAM such as *Myriophyllum* and *Vallisneria* species show no evidence of leaf polarity (Bowes and Salvucci 1989). Carbonic anhydrase (CA) located in the epidermal cell wall or the plasmalemma is considered to play an important role in catalysing the dehydration of bicarbonate to form CO_2 for many submerged aquatic macrophytes and unicellular algae (Bowes and Salvucci 1989; Husic 1991; Rybova, et al. 1991; Newman and Raven 1993; Reiskind, et al. 1997; Beer 1998; Axelsson, et al. 1999; Newman and Raven 1999; Rascio, et al. 1999). This appears to be an energy consuming process, as the uptake of bicarbonate into the plasmalemma requires H^+-ATPase (Rybova, et al. 1991).

However, even for plants using CA there are environmental conditions, such as high temperatures and irradiance and very low CO_2/HCO_3^- ratio, where direct uptake of bicarbonate is an advantage (Beer 1998). Axelsson et al, (1999) found in the macro-algae *Ulva lactuca* that direct bicarbonate uptake occurs across the cell plasmalemma via an anion exchange mechanism (AE), in coexistence with the CA mechanism. They proposed a simple model of the two mechanisms (Fig. 2.5).

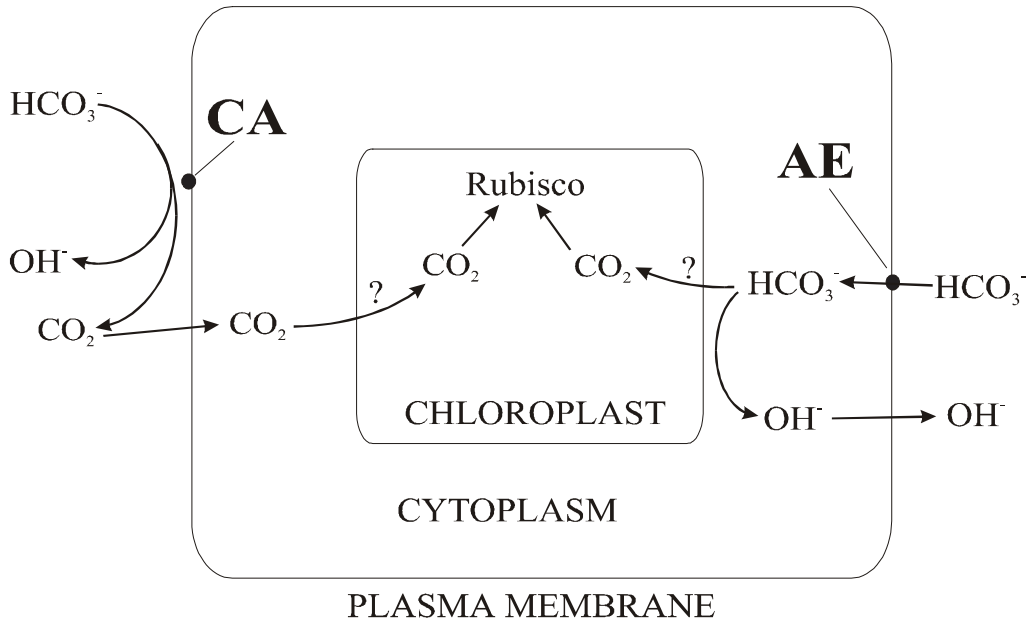

Fig.2.5 A diagram showing the two concurrent mechanisms of bicarbonate use by the macroalgae *Ulva lactuca* (Axelsson, et al. 1999).

Similar mechanisms have been found in the freshwater SAM *Ranunculus trichophyllus*. Rascio et al (1999) observed that when environmental dissolved CO_2 became low, there was an associated increase in apoplastic CA activity. A direct bicarbonate uptake mechanism was also proposed, based on evidence of photodependent H^+-ATPase activity in the transfer regions of the plasmalemma. However, in the large celled algae *Hydrodictyon reticulatum*, H^+-ATPase activity was found to be associated with CA activity in the plasmalemma (Rybova, et al. 1991).

At about the same time, Newman and Raven (1999) found that CO_2 was the main DIC species used by the chloroplasts of *Ranunculus penicillatus* ssp. *pseudofluitans*. They proposed that CO_2 was the only form of DIC crossing the plasmalemma. Apoplastic CA activity was confirmed but they did not test for H^+-ATPase activity in the plasmalemma.

2.8.2 Biochemical Concentrating Mechanisms

Two types of biochemical DIC concentrating mechanisms have been found in SAM. Like their terrestrial counterparts, some have been found to have either a type of C-4 carbon fixing or crassulacean acid metabolism (CAM).

2.8.2.1 C-4 photosynthesis

Some SAM are able to fix carbon in a similar manner to terrestrial C-4 plants. In most cases, it is a response to low ambient CO_2 as they revert to C-3 carbon fixing when CO2 is not limiting (Raven, Osborne et al. 1985; Keeley 1990). A well-studied plant that reflects this behaviour is *Hydrilla verticillata*. It reverts to C-4 acid synthesis in summer under long days, high temperatures and low ambient CO_2 levels (Bowes 1985; Raven, et al. 1985; Keeley 1990). Keeley (1990) points out that C-4 carbon fixing occurs simultaneously with C-3 carbon fixing within single cells for all SAM that have been identified as having C-4 acid synthesis. Besides *Hydrilla verticillata*, these include, *Vallisneria spirilis*, *Eleocharis acicularis, E. baldwinii* and *Orcuttia californica* (Keeley 1990; Uchino, et al. 1995).

Bowes (1985) and Keeley (1990) outline a model that describes C-4 fixation in SAM. Both suggest that the two carboxylase enzymes, PEP carboxylase and rubisco are separated into two locations within photosynthetic cells, rather than in separate cells as in terrestrial C-4 plants. PEP carboxylase is located in the cytosol and rubisco in the chloroplasts. CO_2 defusing into a cell is ether fixed by PEP carboxylase or by rubisco. Malate or other organic acids, products of C-4 synthesis, are transported from the cytosol to the chloroplasts where they are decarboxylated to produce CO_2 for refixing via rubisco into 3-PGA as part of the Calvin cycle. This results in a concentration of CO_2 at the site of rubisco that reduces photorespiration.

Kranz anatomy (the bundle sheath cells that surround the vascular system), typical of C-4 terrestrial plants has not been found in C-4 SAM (Raven, Osborne et al. 1985; Keeley 1990; Uchino, Samejima et al. 1995). Keeley (1990) describes an amphibious aquatic C-4 macrophyte *Orcuttia californica* that starts with submerged leaves followed by floating and emergent leaves. Both the floating and emergent leaves have typical Kranz anatomy. The juvenile submerged leaves lack this anatomy but were found to have similar turnover of C-4 fixation products as terrestrial C-4 plants (Keeley 1990).

2.8.2.2 Crassulacean Acid Metabolism

When a process resembling CAM was first discovered in SAM, it was with some surprise as CAM is usually associated with terrestrial succulents, adapted to an arid climate where water conservation is a requirement (Salisbury and Ross 1992). Interestingly it has been found in submerged plants of *Crassula aquatica*, but is also found in many Isoetids (Isoetaceae) such as *Littorella uniflora*, *Isoetes howelli* and *I. Lacustris* (Raven, Osborne et al. 1985). However not all Isoetids have CAM. Some aquatic species from other families such as Poaceae, Cyperaceae and Plantaginaceae also have CAM-like biochemical concentrating mechanisms (Keeley 1990; Uchino, et al. 1995).

In aquatic habitats, it is carbon rather than moisture limitation that has resulted in the development of CAM in some aquatic macrophytes. Keeley (1990) described most CAM aquatic macrophytes as occurring in habitats that are seasonally wet and then dry. During the wet season, the plants grow submerged in pools. These pools are subject to large diurnal fluctuations in DIC. During the day, dissolved CO_2 is rapidly taken up by dense aquatic vegetation so that CO_2 is almost completely depleted by mid morning (Keeley 1990). CAM macrophytes are unable to utilise bicarbonate (Keeley 1990). During the night, due to respiration from aquatic flora and fauna, dissolved CO_2 levels increase back to high levels. Therefore, in a similar manner to terrestrial CAM plants, aquatic CAM plants fix CO_2 during the night storing it as malate (Keeley 1990). Similarly, during the day malate is decarboxylated to release CO_2 within the cell where it is refixed via the C-3 Calvin Cycle (Keeley 1990). An adequate supply of CO_2 is therefore maintained to saturate photosynthesis and to prevent photorespiration.

Keeley (1990) points out that SAM either have no stomata or if stomata are present they are non-functional. The fixing of CO_2 between day and night is therefore dependent on the exogenous concentration of CO_2 rather than the opening and closing of stomata.

In conclusion SAM use a range of different mechanisms to overcome anoxic sediments and limited DIC, making them distinctively different from terrestrial macrophytes. Which or how many of these mechanisms any one individual species actually uses depends on their genetic make up or their particular habitat and possibly is a measure of the species adaptability to a range of aquatic environments.

A study of some of these mechanisms as used by A. elongatus grown in environmentally controlled aquaria is provided in the following chapters

CHAPTER 3

THE INTERACTION OF WATER FLOW AND NUTRIENTS ON AQUATIC PLANT GROWTH

3.1 Introduction

Aponogeton elongatus F. Muell. ex Benth, has been observed growing in slow to fast flowing waters (Sainty & Jacobs, 1994; Hellquist & Jacobs, 1998). For other submerged aquatic macrophytes (SAM), it is known that water flow from 1.5 -100 cm s^{-1}, depending on the species, increases net photosynthetic rate (Schwenke 1971; Carpenter et al., 1991; Chambers et al., 1991; Losse & Wetzel 1993). However, many of these studies have been based on short-term measures of photosynthetic rate or field measurements and observations. The significance of this study is that it measures long term growth rates of plants under controlled conditions in aquaria. Such long-term growth rates may reveal differences that are difficult to detect with short-term measures of photosynthetic rate (Titus et al., 1990).

While little is known about *A. elongatus,* for SAM in general, moderate water flow improves leaf uptake of nutrients as well as dissolved inorganic carbon (DIC), and oxygen (Smith & Walker, 1980; Larkum et al., 1989; Stevens & Hurd, 1997). It is also well recognised that flowing water reduces the thickness of the unstirred (boundary) layer that surrounds leaves of SAM (Schwenke, 1971; Carpenter et al., 1991; Chambers et al., 1991; Losse & Wetzel 1993; Madsen et al., 1993). The thinner the boundary layer, the lower the diffusion resistance to leaf uptake of nutrients and DIC (Smith & Walker 1980; Larkum et al., 1989; Stevens & Hurd 1997). Given its observed habitat, it is likely that growth of *A. elongatus* is greater in water of moderate flow compared to still water. This greater growth may be due to the improved foliar uptake of nutrients and/or DIC. The aims of this study were, to test the hypothesis that *A. elongatus* growth is increased by water flow (stirring), and to determine the relationship between water flow and nutrients.

3.2 Materials and Methods

3.2.1 Growth Conditions

This experiment was conducted from 13 July to 22 December 1998, in a controlled temperature room at the University of Queensland, Gatton. Air temperatures within the room were maintained at 22.4 ± 1.0°C (± SD). The mean daily water temperature was 23.7 ± 0.4°C (± SD). Light was provided by banks of 120cm long fluorescent tubes (40 watt Grow Lux Wide Spectrum® tubes) to give a mean photosynthetically active radiation (PAR) level of 101 ± 3 (± SE) μmol m^{-2} s^{-1} measured 5 cm above the water surface. Light was provided continuously throughout the experiment.

Twelve identical glass aquaria (tanks) were used, each 587 mm wide x 457 mm deep x 380 mm in height (100 L). Two tanks were placed under each light bank, partially surrounded by black polyethylene sheeting to exclude the extraneous light from other light banks. A 1:1 mixture of tap water and deionised water was used to reduce the concentration of nutrients, particularly phosphate, to minimise algal growth. Tanks were filled with this water to a height of 340 mm (90 L) and were topped up with deionised water weekly to replace evaporated water. The average electrical conductivity of the water was 237 ± 1μS cm^{-1} over all tanks.

Where required, water was stirred using submersible (4.6 watt) power water filters (Rio-PF400) installed in a back corner of each tank, providing a measured flow rate of 515 ± 5 L h^{-1} (± SE). The maximum water speed at pump outlets was approximately 182 cm s^{-1}. The swirling action created by each pump would have resulted in a wide range of reduced water speeds across a tank.

3.2.2 Experiment design

The experiment was a two-factor, split plot design with two main plot treatments (stirred and unstirred water) and three subplots (slow release fertiliser; 0 g, 0.5 g and 1 g). A pair of tanks under a single light bank were allocated the main plot treatments, one tank stirred, the other unstirred. Within each tank, individually potted plants received the sub plot fertiliser treatments; three plants to each sub-plot treatment (the experimental unit), giving nine plants per tank. Each of pairs of tanks under a single light bank represented one complete block and there were six blocks.

In an attempt to reduce experimental error, plants were ranked by total leaf length then allocated sequentially to the six blocks. Treatments were assigned randomly to the plots (tanks) and subplots (plants).

3.2.3 Preparation of potted plant material

One hundred and eight 100mm diameter plastic pots (420 ml) were lined internally with clear polyethylene bags, then 0.5g or 1g of controlled release fertiliser were placed in 72 of the pots before filling with sediment. The fertiliser was 8-9 month Osmocote Plus^{R} (16 N:3.5 P: 10 K:1.2 Mg plus trace elements). The approximate plant nutrient composition of the sediment at the start of the experiment is shown in Table 3.1. Due to the slow release nature of this fertiliser, these nutrients were not available to plant roots in the concentrations outlined in the table. The sediment was equal parts of washed fine white sand and coarse river sand (bulk density 1.43 g ml $^{-1}$) mixed with 1.7g of powdered iron (55.85% Fe) per pot.

Table 3.1.
The mineral composition of the sediment (µg g^{-1}) at the start of the experiment. This is based on one gram of the controlled release fertiliser Osmocote Plus^{R} per pot. (Calculated from data supplied by the fertiliser manufacturer).

Major elements	µg g^{-1}	Trace elements	µg g^{-1}
Nitrogen	265.7	Boron	0.3
Phosphorus	58.1	Copper	0.8
Potassium	166.0	Iron	6.6*
Sulphur	39.9	Manganese	1.4
Magnesium	19.9	Molybdenum	0.5
Calcium	0	Zinc	0.4

* Available iron levels may be much higher due to the addition of powered iron

A. elongatus plants, about 12 months old, were planted into the pots. The majority of plants were originally propagated by tissue culture from parent stock collected from a single site in southeast Queensland. However, 15 seedlings of similar size and maturity to the micropropagated plants, were also used to make up the required numbers.

The sediment was packed into each pot firmly around the plant, to within 15 to 20 mm of the rim of the pot. After this, granulated zeolite (2mm Horticulture Grade) was added to the top of the sediment to a depth of 10-15 mm. The plastic liner was raised above the rim

of the pot by about 20-30 mm to create a zone of unstirred water (boundary layer) above the potting mix. The placement of the fertiliser and inclusion of powdered iron in the medium, plus the plastic liner and zeolite, were all designed to minimise the diffusion of nutrients, particularly phosphate, from the potting mix into the surrounding water.

3.2.4 Plant measurements

Total leaf number, length (leaf blade base to leaf tip, ignoring the petiole) and blade width (at the widest portion of each leaf blade) of each plant was measured at the beginning of the experiment and in the 14th week. Leaf blades of non-senescent, fully developed leaves were measured.

For dry weight measurements, one plant was randomly selected from each treatment in the 22nd week. After removing the potting mix, each plant was divided into foliage, tuber and roots then fan forced oven dried at 60ºC for 5 days before being weighed.

3.2.5 Chlorophyll fluorescence

A portable submersible Diving-PAM (pulse amplitude modulated) Fluorometer (Walz, Effeltrich, Germany) was used to measure leaf chlorophyll fluorescence in the form of quantum (photochemical energy conversion) yield. These measurements were taken 11 weeks from the start of the experiment. For each measurement, electron transport rate (ETR) was determined over nine actinic light levels (4, 31, 54, 84, 155, 259, 372, 594, 1013µmole quanta s^{-1} m^{-2}) in the form of a rapid light curve (RLC). Each RLC was produced over 90 seconds within which there were 10-second periods of actinic irradiance and 1 second saturating pulses (Jones & Dennison, 1998). The youngest, non-shaded, mature leaf was measured on each of three plants from each treatment over four replicates. In addition, three floating leaves and three submerged leaves on outdoor plants of *A. elongatus* growing under 60 – 80% shade were measured. All leaf measurements were taken at one-quarter the length of the leaf blade from the leaf tip, avoiding the midrib and any other major leaf veins.

3.2.6 Nutrient levels in water

A 'Palintest® Photometer 7000'(Palintest Ltd, Tyne & Wear, England) and reagents from Palintest® kits were used to measure nitrate nitrogen, ammonium nitrogen, phosphate,

potassium, magnesium and iron in the water of each of the tanks at the end of the experiment following the instructions provided in the Palintest kits.

3.2.7 Dissolved CO_2 levels

Total dissolved inorganic carbon (DIC) and dissolved free CO_2 (dissolved CO_2 and H_2CO_3) were measured after 12 weeks using titration techniques described by Frith et al (1993). One of the limitations with the dissolved free CO_2 technique is that it could not measure CO_2 levels below 4.5 μM. One sample was tested for each of 12 tanks.

3.2.8 Monitoring

A waterproof, hand-held 'TPS WP-80' (TPS Pty Ltd, Brisbane, Australia) pH meter was used to measure pH weekly and a waterproof, hand-held (TPS WP-84) electrical conductivity meter was used to measure tank water conductivity every 2 weeks throughout the experiment. Water temperature (in 4 tanks) and air temperature (2 locations) were measured hourly using thermocouples connected to a data logger ('Datataker 500', Pacific Data Systems Ltd, Brisbane, Australia)

3.2.9 Statistical Analysis

ANOVA were applied to the leaf area, dry weight and chlorophyll fluorescence data (SAS release 6.12) and LSDs computed (MS Excel version 97). Leaf area data were transformed to their square root before ANOVA. This was because the leaf area was estimated by multiplying the length and width of a leaf, which tended to inflate the error mean square in the ANOVA reducing the degree of precision in which the treatments were compared. The inflated error term was reduced by square root transformation of the leaf area data. The Dry weight data were transformed to their log to account for one outlier in each of the data sets of leaf, tuber and root. F tests and t tests were applied to DIC, pH, conductivity and temperature data using MS Excel. A significance level of $P< 0.05$ was adopted unless otherwise indicated.

3.3 Results

3.3.1 General observations

By the end of the experiment, the fertilised plants appeared much larger than the unfertilised plants (Fig 3.1). Plants fertilised with 1.0 g Osmocote were larger than those with 0.5 g Osmocote, particularly in the stirred tanks. Plants in the unstirred tanks lost more leaves and appeared smaller than those in the stirred tanks. The leaves of plants in stirred tanks were dark red to purple green while those in unstirred tanks were a paler green with little or no red or purple.

3.3.2 Leaf Morphometrics

The average leaf length and width, and hence leaf area, were greater on plants growing in stirred water than in unstirred water (Table 2.1). Plants without fertiliser had fewer and smaller leaves than those of the fertilised plants ($P < 0.001$). Leaf number and length were not significantly different between the two levels of fertiliser (0.5g and 1g) but the leaves were wider, and hence leaf area greater, on plants with 1g of fertiliser. There was no significant interaction between fertiliser level and ± stirring for these parameters.

Table 3.2
Effects of stirred or unstirred water and three levels of fertiliser on *A. elongatus* mean leaf number, length, width and area, after a 22 week growth period with a mean temperature of 24°C and PAR 101.6 µmol m^{-2} s^{-1}. Leaf area data were transformed to the square root before ANOVA and LSD comparisons (All means in table are from original untransformed data). All data are an average of six blocks. Means within a column having a common letter are not significantly different at 5% level. There are no significant two-factor interactions.

Treatments		Leaf Number per plant	Leaf Length (cm) per plant	Leaf Width (cm) per plant	Leaf Area (cm^2) per plant
Osmocote®	0g	22.6 a	221.4 a	35.2 a	400 a
	0.5g	31.8 b	452.6 b	63.0 b	973 b
	1.0g	33.9 b	533.6 b	75.7 c	1297 c
Stirred		31.5 a	484.5 b	67.4 b	1113 b
Unstirred		25.3 a	320.3 a	48.6 a	667 a
Interactions (A × B)					
Stirred	0g	22.9	255.1	40.8	497
	0.5g	33.3	543.6	72.5	1220
	1.0g	38.3	654.9	89.0	1623
Unstirred	0g	20.4	187.7	29.7	304
	0.5g	28.2	361.0	53.5	726
	1.0g	27.4	412.3	62.5	971

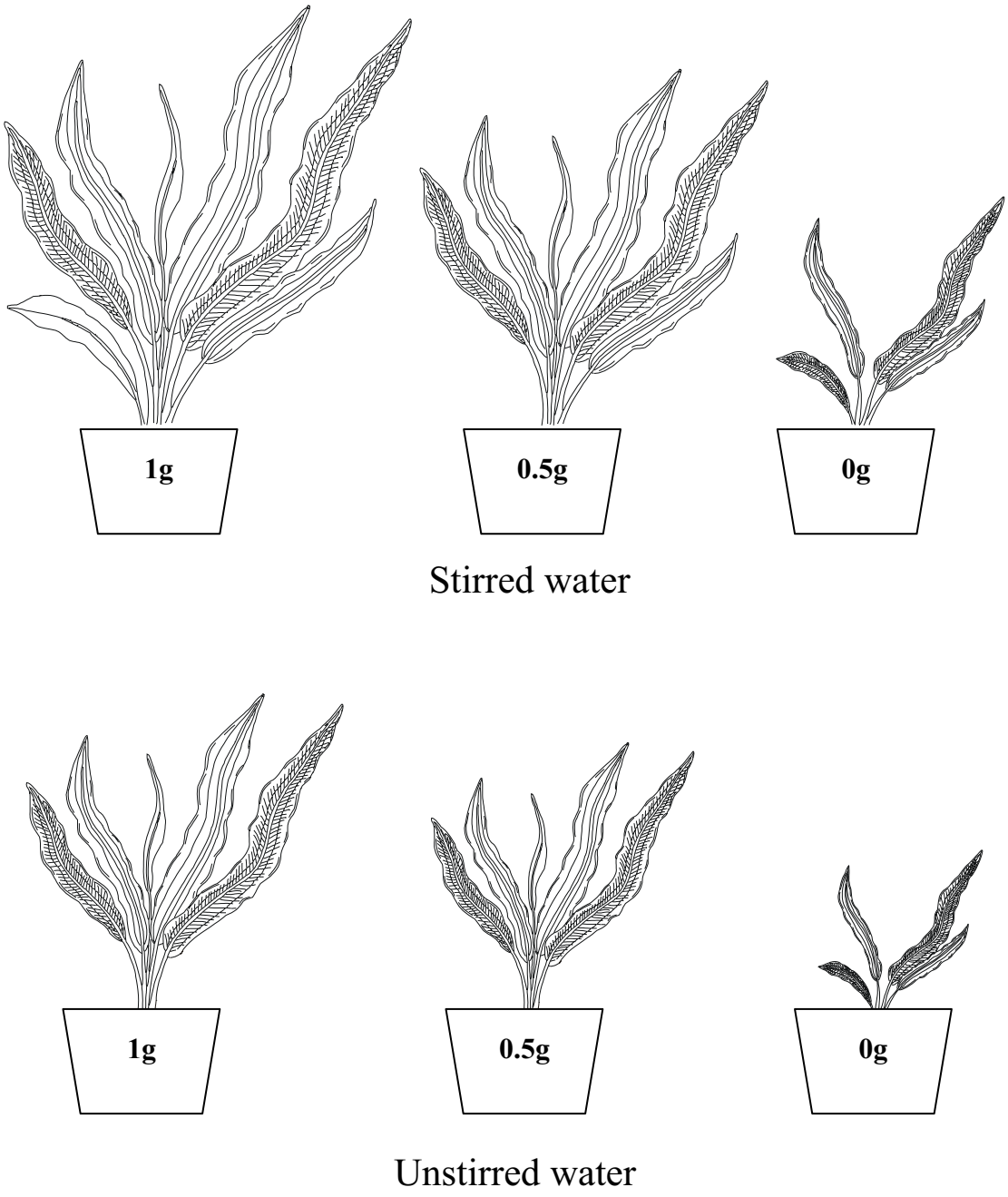

Fig. 3.1. The response of potted *A. elongatus* plants to three fertiliser levels (1, 0.5, 0g pot^{-1}) and stirred verses unstirred water. The drawings represent the actual leaf numbers (Table 2), and leaf areas are scaled in proportion to the leaf area means ± 2%.

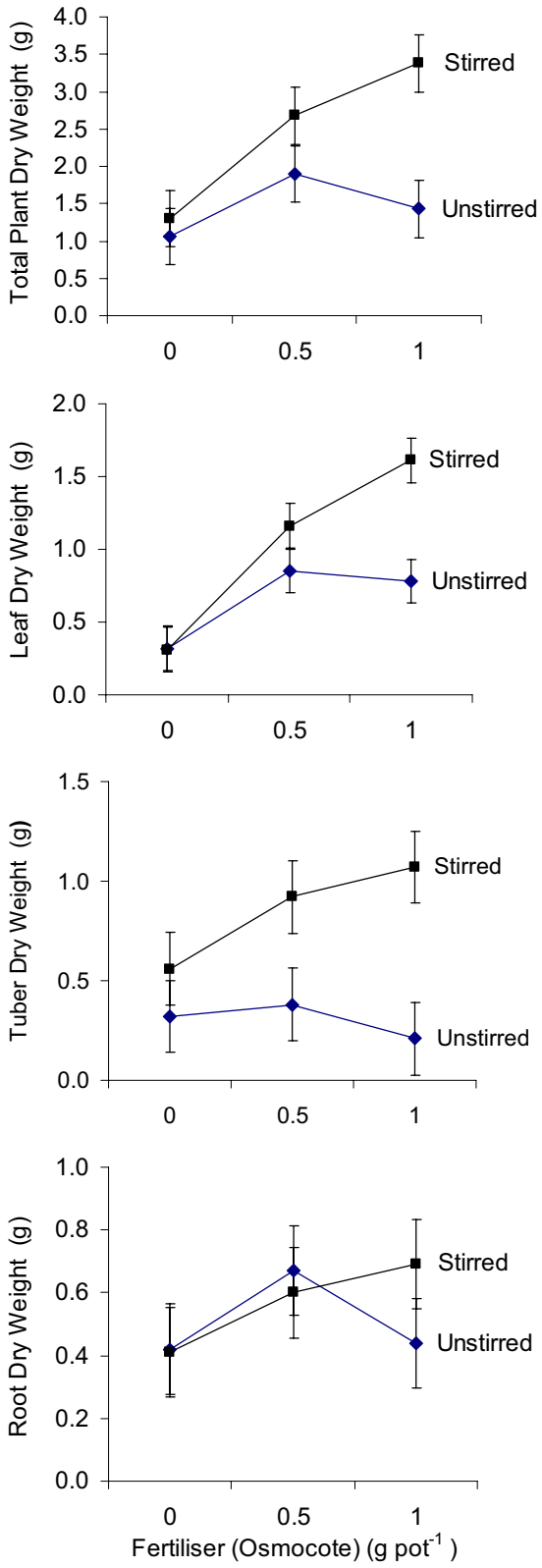

Fig. 3.2 Growth of *A. elongatus* after 22 weeks at a mean temperature of 24°C and PAR of 102 µmol m^{-2} s^{-1}. The only significant interaction occurred between leaf dry weights. The differences between the treatment means for roots are not significant. (Means ± 1 SE).

3.3.3 Dry Weight

Plants with the largest total dry weight were those growing in stirred water and fertilised with either 0.5g or 1g of Osmocote (Fig 3.2). The difference between 0.5g or 1g of Osmocote was not significant. Stirred water produced greater leaf dry weight than unstirred water at the highest rate of fertiliser (1g) but there was no difference with 0 or 0.5g.

Fertiliser application had no significant effect on tuber dry weight but tubers in stirred water had significantly greater dry weight than those in unstirred water ($P < 0.01$)(Fig 3.2). In contrast, there were no differences in root dry weight between plants growing in either stirred or unstirred water, or between different fertiliser levels (Fig 3.2).

3.3.4 Chlorophyll fluorescence

There was no significant difference between any treatments in maximum quantum yield or maximum electron transfer rate (ETR). The maximum ETR value (5.9 ± 3.3 (±SE) µmol e^- m^{-2} s^{-1}) of leaves growing in the tanks is similar to that for the rapid light curve measurements taken from submerged leaves of plants growing outdoors under 60-80% shade (9.0 ± 0.5 µmol e^- m^{-2} s^{-1}) (Fig 3). They fall well short of the maximum ETR of floating leaves (53 ± 11 µmol e^- m^{-2} s^{-1}) on the same outdoor plants (Fig 3.3).

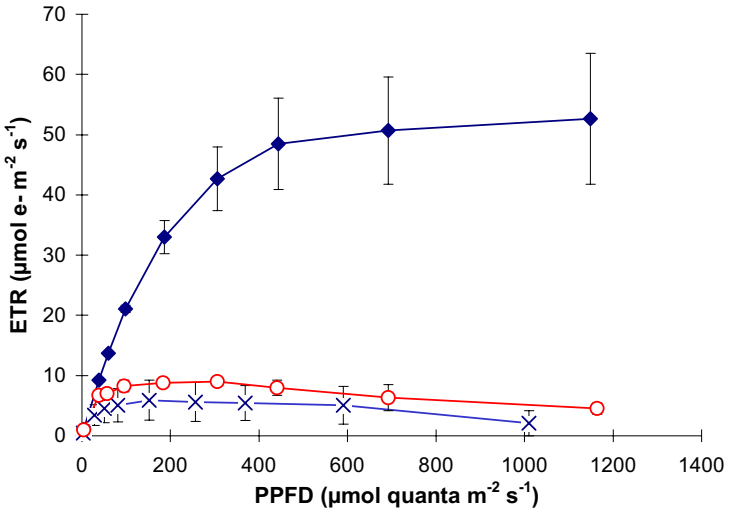

Fig. 3.3 Rapid light curves derived from electron transport rate (ETR) (µmol electron m^{-2} s^{-1}) verses photosynthetic photon flux density (PPFD) (µmol quanta m^{-2} s^{-1}). Floating leaves (♦), submerged leaves (○) of *Aponogeton elongatus* plants (mean of three) and mean RLC of three submerged leaves from three indoor tank grown plants from the experiment (**x**). Data presented as the mean ± 1 SE.

3.3.5 Water nutrient levels

There was considerable variation in nutrient levels between tanks (Table 3.3) but most nutrients except potassium (in some tanks) and magnesium were very low. The total nitrogen in the water was no more than 1%, phosphorus 3% and potassium 36% of that originally supplied as Osmocote. In contrast, magnesium was 45% higher than in Osmocote because of the high level in the tap water used (Table 3.3).

A comparison of the means using a paired t test revealed no differences in any nutrients between stirred and unstirred water tanks. However, nutrient levels (nitrogen, phosphorus and potassium) were more variable in unstirred tanks than stirred tanks using Two-Sample F-Test ($P < 0.008$). The exception was magnesium which was more variable in stirred tanks ($P = 0.008$).

3.3.6 Dissolved CO_2 levels

The dissolved inorganic carbon level (DIC) ranged from 941 – 1120 μM between tanks while dissolved free CO_2 ranged from 0 - 41 μM. For total DIC the variance in stirred tanks was no different from the variance in unstirred tanks, while dissolved free CO_2 in unstirred tanks was much more variable according to a two-sample F-test (Table 3.3). However, when the means were compared using a paired t test, Total DIC appeared higher ($P=0.054$) in stirred tanks than in unstirred tanks while there was no difference in dissolved free CO_2.

3.3.7 pH, Electrical Conductivity and Temperature

There was a steady rise in mean pH from 8.1 in the first week to pH 8.58 in unstirred tanks and pH 8.49 in stirred tanks after four weeks (Fig 3.4A). In week 10, the mean pH peaked at 8.74 in unstirred tanks and 8.54 in stirred tanks. The pH remained higher in the unstirred tanks than the stirred tanks through to the last measurement in week 20. The maximum pH recorded was 9.14 in an unstirred tank compared to 8.59 in a stirred tank. As indicated by the larger standard errors, the pH level varied much more between unstirred tanks than between stirred tanks (Fig 3.4A).

Electrical conductivity increased slowly from a mean of 238 to 294μS cm^{-1} at the end of the experiment. The differences between tanks became more pronounced towards the end of the experiment as indicated by the error bars (Fig 3.4B).

Table 3.3
Water nutrient concentrations, conductivity and temperature, 5 months from the start of the experiment. Total dissolved inorganic carbon (DIC) (µmole CO_2), dissolved free CO_2 and pH, measured after 3 months. The 'original water' data are estimates of minerals in the tank water at the start of the experiment (tap water plus deionised water 1:1), based on measurements from the tap water. 'Fertiliser per tank' is an estimate of the amount of nutrients supplied in the Osmocote (calculated from data supplied by the manufacturer).

Treatment	**N – NO_3**	**N – NH_4**	**Total N**	**P**	**K**	**Mg**	**Fe**	**Dissolved Inorganic Carbon**	**Dissolved Free CO_2**	**pH**	**Conductivity**	**Temperature**
	µM	µM	µM	µM	µM	µM	µM	µM	µM		µS cm^{-1}	°C
Mean Stirred	**0.36**	**0.71**	**1.07**	**1.16**	**3.30**	**411**	**0.18**	**1048**	**22.7**	**8.41**	**303**	**23.7**
Std Error	0.36	0.29	0.36	0.23	2.05	24	0.05	20	1.6	0.02	5	0.29
Mean Unstirred	**0.71**	**3.57**	**4.14**	**0.84**	**46.3**	**420**	**0.18**	**986**	**18.2**	**8.58**	**285**	**23.2**
Std Error	0.36	2.14	0.03	0.06	23.5	7	0.05	40	6.6	0.04	7	0.24
Original water	**3.57**	**0**	**3.57**	**1.29**	**46.0**	**267**	**0.18**					
Fertiliser per tank	**194**	**171**	**366**	**36.1**	**128**	**283**	**N. A.**					

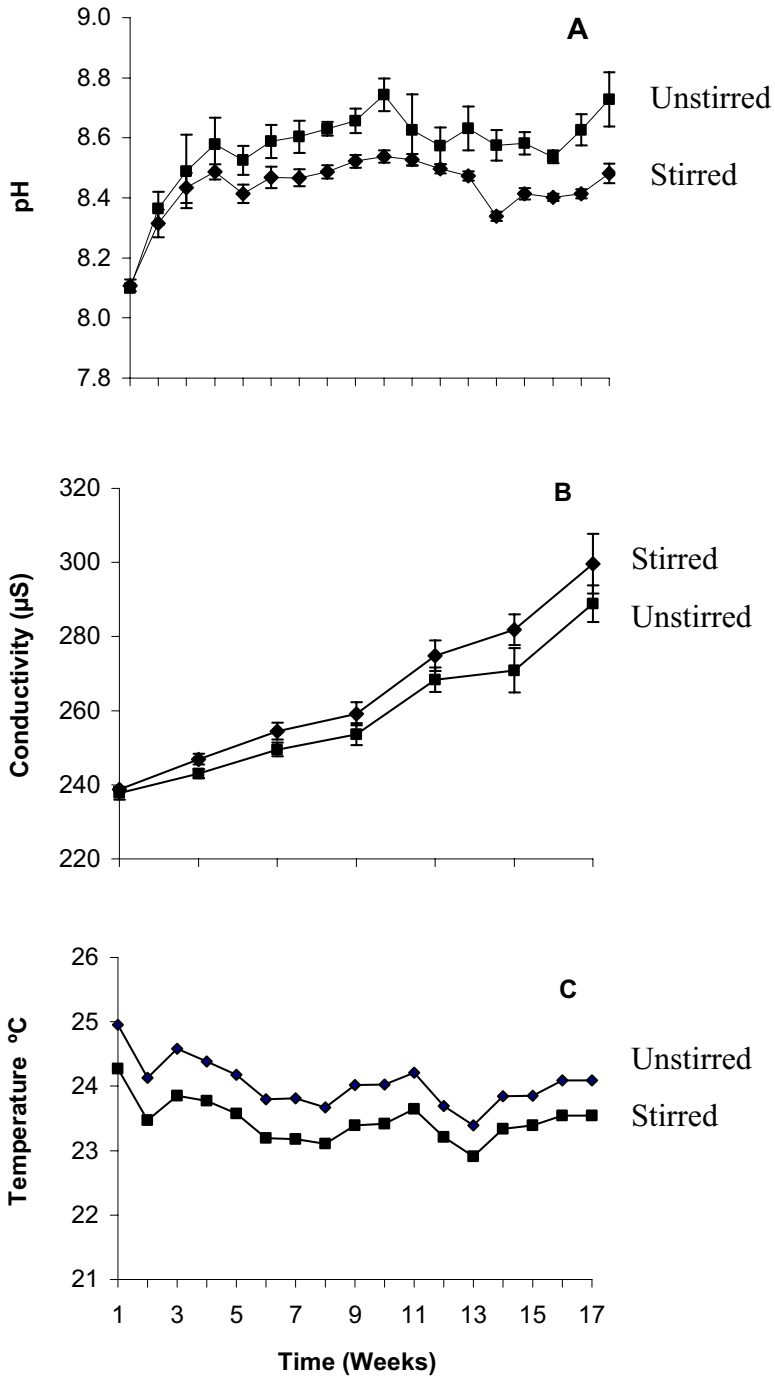

Fig. 3.4 Comparison of stirred and unstirred water over time: (A) pH (based on 18 weekly measurements; mean ± 1 SE, n = 6); (B) conductivity (based on seven measurement times; mean ± 1 SE, n = 6) and (C) mean weekly water temperatures.

However, there was no difference either in the variances or between the means of stirred and unstirred tanks.

Mean daily water temperatures in stirred tanks were 0.4-0.7°C warmer than in unstirred tanks ($P < 0.01$) (Fig 3.4 C). It is possible this temperature difference contributed to the difference in plant growth between unstirred and stirred water, but since the temperature difference is small, the effect is likely to be minimal.

3.4 Discussion

This study indicates that *A. elongatus* grows larger in flowing (stirred) water. Larger leaf area and leaf dry weight per plant were the main responses to increasing nutrient level and stirred water. Tuber weight was also greater in stirred water but was not significantly affected by fertiliser level, which suggests that some factor other than mineral nutrition limited growth in unstirred water.

A. elongatus and other species of *Aponogeton* in creeks and rivers, have longer leaves in rapidly flowing water (Islam, 1996; Hellquist and Jacobs, 1998). In this study, the leaves were both longer and wider but proportionally narrower in stirred water compared to unstirred water (based on length breadth ratio). However, in many SAM, light intensity and/or mineral nutrition may have a much greater influence on leaf shape compared to water velocity (Kirk, 1994; Spence, 1995). *A. elongatus* has been observed to have much thinner and broader leaves in dense shade caused by overhanging trees (Hellquist & Jacobs, 1998). This is similar to the leaves of land plants adapted to dense shade (Salisbury & Ross, 1992). In this study, the leaves are likely to be adapted to shade, as the plants were grown with an irradiance level equal to approximately 5% sunlight. Based on an approximate leaf thickness of 0.1mm, the plants had a leaf surface area to leaf volume ratio of approximately 20:1. Leaf surface area to leaf volume ratios equal to or greater than this are common in SAM (Madsen et al., 1993). Apart from more efficient light interception, thin leaves may be more efficient in nutrient or DIC uptake (Sculthorpe, 1967).

Roots of many aquatic macrophytes may have a primary role in nutrient uptake, particularly nitrogen and phosphate (Bristow & Whitcombe, 1971; Barko et al., 1988;

Rattray et al., 1991; Robach et al., 1995; Best, et al., 1996; Wigand & Stevenson, 1997). However, it has been shown that aquatic plant foliage can also take-up nutrients from the water (Bristow & Whitcombe, 1971; Rattray et al., 1991; Robach et al., 1995; Best et al., 1996). Whether nutrients such as phosphate are taken up by foliage or roots is dependent on the nutrient concentration gradient between interstitial water and the water above the sediment (Rattray et al., 1991; Best et al., 1996).

The present study indicates that *A. elongatus* obtained most of its nutrients from the substrate via its roots. Those plants without the added fertiliser grew poorly and were significantly smaller in leaf number, length and width, and leaf dry weight, even though the unfertilised plants and fertilised plants were growing in the same tank of water. Water flow (stirred water) is known to assist nutrient uptake via the foliage (Stevens & Hurd, 1997). Adding more nutrients directly to the water may improve nutrient uptake by the plants, without fertiliser in the sediment, but a more likely consequence is an algae bloom.

A substantial quantity of iron was added to each pot in the form of fine metallic iron particles. The aim was to restrict algal growth by reducing the solubility of phosphate. The root substrate is likely to be largely anoxic and iron in the substrate is likely to be present in reduced form (Gambrell and Patrick 1988). A narrow zone (≈2 mm) of aerobic substrate occurs near and extending to the substrate surface (Armstrong 1978; Gambrell and Patrick 1988; Caffrey and Kemp 1990). Any reduced soluble iron and phosphorus ions that passes into this aerobic zone as a result of diffusion would oxidise, precipitating out of solution as ferric phosphate (Gambrell and Patrick 1988).

High levels of iron are known to be toxic to aquatic plants (Barko et al. 1986). High iron restricts the availability of phosphorus and disrupts sulphur metabolism (Barko et al. 1986). Therefore, it is possible that in the oxidated root rhizosphere (Wium Anderson and Anderson 1972; Penhale and Wetzel 1983; Larkum et al. 1989; Pedersen et al. 1995; Smith et al. 1984;) a high level of soluble iron could severely reduce phosphate uptake. Iron powder was applied evenly to all treatments and plants grew well in the fertilised treatments, particularly in stirred water, with no visual evidence of phosphate deficiency. The unfertilised plants may have had phosphate deficiency among other nutrient deficiencies but this can not be attributed to iron toxicity. For particles of metallic iron (Fe) to get into solution, they first have to be oxidised (Fe^{2+} and Fe^{3+}). A short time after

mixing the iron in the sand substrate the potted media was placed under water. Microbial action would eventually render the substrate anoxic (Gambrell and Patrick 1988). Any oxidised iron (Fe^{3+}) would be reduced to a more soluble form (Fe^{2+}) (Gambrell and Patrick 1988) but it is possible only a tiny amount of the metallic iron particles was oxidised before the media became anoxic. Therefore, the impact of soluble iron on phosphate uptake could be minimal. It is also possible that phosphate uptake by the leaves offset the affects of high iron on phosphate uptake by the roots. A more detailed study is needed to fully test whether the iron level in the experiment reduced phosphate uptake sufficiently to limit growth.

There was a leaf dry weight interaction between stirred water and 0.5g, and 1g of Osmocote^{R} but not unstirred water. This suggests that fertiliser is not the limiting factor in unstirred water. It is more likely DIC uptake is the primary reason for the greater growth in stirred versus unstirred water. Perhaps the strongest indication that DIC is the limiting factor is tuber weight. Tubers produced by these plants are primarily storage organs rich in carbohydrates such as starch (based on starch iodine test), therefore tuber weight must be strongly linked to photosynthetic productivity. It is likely that tubers in stirred water are larger because the reduced boundary layer around leaves in stirred water allowed greater DIC assimilation than in unstirred water (Smith & Walker, 1980; Larkum, et al., 1989; Stevens & Hurd, 1997).

An interesting question is why pH was high overall. One unstirred water tank reached a pH of 9.14. Some tanks, particularly the unstirred tanks, had a small amount of algal growth, both benthic and pelagic. The uptake of dissolved free CO_2 by both the algae and *A. elongatus* could have contributed to the rise in pH (Kirk 1994). The uptake of bicarbonate could also contribute to the high pH. Other aquatics increase the pH by releasing OH^{-} ions in exchange for bicarbonate ions, for example *Elodea nuttallii* and blue green algae (Miller & Colman, 1980; Allen & Spence, 1981; Jahnke et al., 1991).

The slightly lower pH (difference 0.16) in stirred water could be due to the stirring action of the water increasing the rate of CO_2 replenishment from the surrounding air (Sand-Jensen, 1983). Conversely, in unstirred tanks there may be a rise in pH because of plant removal of CO_2 coupled with slower replenishment from the air.

The dissolved free CO_2 levels represents nearly double the air equilibrium value of 10μM (Stumm & Morgan, 1970; Bowes & Salvucci, 1989). This level of dissolved free CO_2 may still result in a deficit in stirred tanks, as boundary layer resistances although reduced, are still high, (Bowes & Salvucci, 1989). This is likely to be more evident under higher irradiance levels than were used in this study. The ability to use bicarbonate would be an advantage as there was approximately 46 times as much bicarbonate present as dissolved free CO_2.

It is plausible that stirring the water increased DIC uptake irrespective of whether the form used by the plant is bicarbonate or dissolved free carbon. Most fresh water macrophytes have a higher affinity for dissolved free CO_2 than bicarbonate (Allen & Spence, 1981; Sand-Jensen, 1983). *A. elongatus* may be able to use bicarbonate but have a preference for dissolved free CO_2. Clearly, further investigation is required to confirm whether this plant is able to use bicarbonate.

From personal observation, *A. elongatus* normally has predominantly submerged leaves but under conditions of high irradiance in still (stagnant) water, it can switch to predominantly floating leaves thus overcoming boundary layer limitations. Although this macrophyte is found only in permanent water, its habitat is subject to variable flow rates from stagnant water during droughts to rapid flow during high rainfall events. In our laboratory, floating leaves were not a feature in the unstirred treatments possibly because irradiance levels were low. Photosynthetic capacity (ETR) of outdoor floating leaves of *A. elongatus* was approximately 6 times greater than submerged leaves on the same plants and 9 times greater than either floating or submerged leaves in this study. The ETR of the outdoor floating leaves are comparable with that recorded on seagrasses in clear marine waters (Ralph et al., 1998). However, floating leaves are not as persistent as submerged leaves, usually undergoing senescence within 7 to 12 days of reaching full maturity compared to several months for submerged leaves. This continual replacement of floating leaves may offset any advantage of accessing carbon directly from the atmosphere. Further work is needed to determine why floating leaves are short lived and whether the production of floating leaves in higher light intensities can reduce dependence on flowing water for DIC uptake.

In conclusion, *A. elongatus* growth was limited by nutrient levels equal to or less than 0.5 g of fertiliser per pot. Above this nutrient level, the availability of DIC appears to be the main factor limiting growth for *Aponogeton* normally dependent on submerged leaves (Fig 3.5).

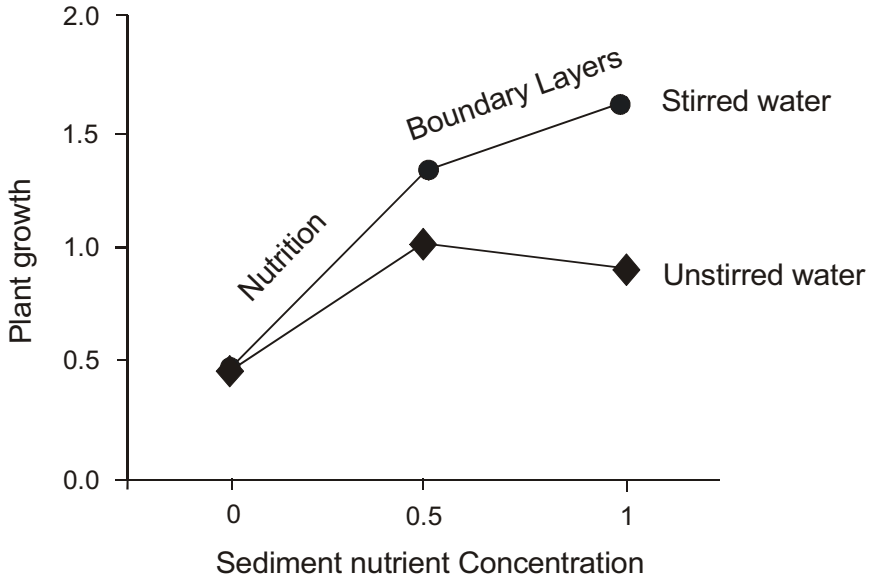

Fig. 3.5 The interaction of ± stirring and fertiliser level on leaf dry weight per plant in *A. elongatus*; (●) stirred, (♦) unstirred water. Below 0.5g of Osmocote, growth was limited by 'nutrient' supply. Above 0.5g plant growth in unstirred water was limited by the 'boundary layer'.

Presumably, stirred water improves DIC uptake due to the decrease in diffusion boundary layer resistance around the leaves (Larkum et al., 1989; Stevens & Hurd, 1997). The resulting increase in photosynthetic productivity enabled greater growth of both tuber and leaves. This provides some explanation for the observations of others that these macrophytes flourish in creeks or river systems with permanently flowing water (Sainty & Jacobs, 1994; Hellquist & Jacobs, 1998). Nutrient availability in different sediments is known to have a marked effect on SAM growth (Barko & Smart, 1986; Stewart, 1991; Best et al., 1996). This can be due to variation in nutrient concentration and/or sediment density (Barko et al., 1988; Boeger, 1992). The availability of nutrients in different sediments may limit growth and possibly the distribution of *A. elongatus* in rivers and creeks. However, growth will be limited even in sediments containing abundant plant nutrients without the effect of water flow on unstirred boundary layers.

Chapter 4

Dissolved Inorganic Carbon Limitation of Aquatic Macrophyte Growth under Controlled pH

4.1 Introduction

The aquatic macrophyte *Aponogeton elongatus* F. Muell. ex Benth. was found to grow best in stirred water, with an adequate supply of nutrients in the sediment (Chapter 3). Improved dissolved inorganic carbon (DIC) uptake was hypothesised as the main reason for the superior growth in stirred water.

Atmospheric carbon dioxide is absorbed by water until equilibrium is reached between the concentration of atmospheric CO_2 and dissolved CO_2 in the water (Stumm and Morgan 1970). In turn, this dissolved CO_2 reacts with water to form carbonic acid, which dissociates into bicarbonate ions and further into carbonate ions. This process is in equilibrium as follows CO_2 (g) $\leftrightarrows$ CO_2 (aq) + H_2O $\leftrightarrows$ H_2CO_3 $\leftrightarrows$ HCO_3^- + H^+ $\leftrightarrows$ CO_3^{-2} + $2H^+$ (Saruhashi 1955). These equilibrium reactions shift to the right with increasing pH (Kirk, 1994). Stumm and Morgan (1970) proposed a simple model to show characteristics of the carbonate system in pure water in equilibrium with the atmosphere at a set temperature of 25ºC. They proposed that increasing the pH of water increases total DIC but the molar concentration of dissolved free CO_2 remains unaffected.

In natural waters, the concentration of dissolved free CO_2 may vary considerably due to the interaction of constantly changing factors such as pH, temperature, total DIC, alkalinity, ionic strength, respiration and plant uptake for photosynthesis (Saruhashi, 1955; Stumm and Morgan 1970; Frith, et al. 1993). Natural levels of bicarbonate range from 0.02 to > 2000 µM and dissolved free CO_2 can range from 0 to 350 µM (Bowes and Salvucci 1989). Natural water bodies often have levels of DIC well above equilibrium levels (Titus, et al. 1990). A state of equilibrium between the concentration of atmospheric CO_2 and dissolved free CO_2 may be rare in biologically active natural waters (Kirk 1994).

Table 4.1 The effects of different pH or the concentration of carbon species on the rate of photosynthesis has been well demonstrated with the following aquatic macrophytes.
Note: Aquatic macrophytes with an asterisk (*) are seagrasses.

Aquatic macrophytes	**Researchers**
Batrachium aquatile (L.)	(Nielsen & Sand-Jensen 1989)
Berula erecta (Hudson) Coville	(Nielsen & Sand-Jensen 1989)
Callitriche cophocarpa Sendtner	(Nielsen & Sand-Jensen 1989)
Callitriche stagnalis Scop.	(Sand-Jensen 1983)
Ceratophyllum demersum L.	(Van, Haller et al. 1976)
Egeria densa Planch.	(Browse 1979)
Elodea canadensis L. C. Rich.	(Allen & Spence 1981; Elzenga & Prins 1988; Nielsen & Sand-Jensen 1989)
Elodea nutalli (Planch.)St.John	(Elzenga & Prins 1988; Jahnke et al. 1991)
Eurhynchium rusciforme Milde	(Allen & Spence 1981)
Fontinalis antipyretica L.	(Allen & Spence 1981)
Halodule uninervis (Forssk.) Aschers *	(Beer et al. 1977)
Halophila stipulacea (Forssk.) Aschers *	(Beer et al. 1977)
Hippuris vulgaris L.	(Allen & Spence 1981)
Hydrilla verticillata (L Fil.)Presl.	(Van et al. 1976)
Littorella uniflora (L.) Ascherson	(Nielsen & Sand-Jensen 1989)
Lobelia dortmanna L.	(Nielsen & Sand-Jensen 1989)
Marine Macroalgae	(Sand-Jensen & Gordon 1984)
Myosotis palustris L.	(Nielsen & Sand-Jensen 1989)
Myriophyllum alterniflorum Macoun	(Allen & Spence 1981)
Myriophyllum spicatum L.	(Allen & Spence 1981; Titus & Stone 1982; Nielsen & Sand-Jensen 1989)
Nitella flexilis (L.) Ag.	(Allen and Spence 1981)
Nuphar luteum (L.) Smith	(Smits et al. 1988)
Nymphaea alba L.	(Smits et al. 1988)
Nymphoides peltata (Gmelin) O. Kuntze	(Smits et al. 1988)
Potamogeton crispus L.	(Allen & Spence 1981; Sand-Jensen 1983; Nielsen & Sand-Jensen 1989)
Potamogeton densus L.	(Nielsen & Sand-Jensen 1989)
Potamogeton lucens L.	(Elzenga & Prins 1988)
Potamogeton natans L.	(Bodner 1994)
Potamogeton panormitanus Biv.	(Nielsen & Sand-Jensen 1989)
Potamogeton pectinalis L.	(Sand-Jensen 1983; Nielsen & Sand-Jensen 1989)
Potamogeton polygonifolius Pourr.	(Allen & Spence 1981)
Ranunculus aquatilis L.	(Allen & Spence 1981)
Ranunculus fluitans Lam.	(Bodner 1994)
Ruppia maritima L. *	(Sand-Jensen & Gordon 1984)
Scirpus subterminalis Tor.	(Beer & Wetzel 1981)
Sparganium emersum Rehman	(Nielsen & Sand-Jensen 1989)
Sparganium erectum L.	(Nielsen & Sand-Jensen 1989)
Sparganium simplex Huds.	(Sand-Jensen 1983)
Syringodium isoetifolium (Aschers.)Dandy *	(Beer et al. 1977)
Thalassodendron ciliatum (Forssk.) den Hartog *	(Beer et al. 1977)
Vallisneria americana A. Michaux	(Titus & Stone 1982; Titus et al. 1990)
Zostera marina L.*	(Sand-Jensen & Gordon 1984)

The effects of pH or different concentrations of carbon species on the rate of photosynthesis have been well demonstrated with other aquatic macrophytes (Table 4.1). This experiment differs from most of these studies in that it concentrates on long term growth rates in an open system as distinct from short term measures of photosynthetic rate in closed systems. Such long-term growth rates may reveal differences that are difficult to detect with short-term measures of photosynthetic rate (Titus, et al. 1990). A long-term growth experiment may not show typical equilibrium CO_2 levels as described by Stumm and Morgan (1970). It is inherently closer to a natural water environment in that it is not as completely controlled as a typical closed system experiment.

This experiment is possibly the first on the effects of different pH on *A. elongatus*. Little is known about the optimum conditions to grow *A. elongatus*. Aquarium hobbyist publications offer only vague comments on this topic. Elliot and Jones (1982) suggest that *A. elongatus* requires hard, slightly alkaline water. Others recommend slightly acid to slightly alkaline water with a pH between 6.5 - 7.8 (DeWit, 1966; Stodola, 1967).

The main aim of this experiment is to test the hypothesis that the enhanced growth of *A. elongatus* in stirred water is due to increased DIC uptake. A second aim is to examine the growth response of *A. elongatus* to varying levels of dissolved free CO_2 as influenced by different pH.

4.2 Materials and Methods

4.2.1 Growth Conditions

This experiment included three replications (runs) all implemented in the same controlled temperature room at the University of Queensland, Gatton as in Chapter 3. The first run commenced on 19 November 1999 and was completed in 89 days ending 16 February 2000. The second run commenced on the 3 March and finished on 25 June 2000 (114 days). The third and final run commenced on the 1 September 2000 and the last measurements were taken on 9 November 2000 (69 days). During run two, there was a premature severe loss of leaves in one treatment. Consequently, run two was extend well beyond the duration of run

one to allow time for the affected plants to recover. Run three was shorter than run one to avoid a recurrence of what happened in run two (See results section for further details).

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Within the room the mean air temperature was 22.9 ± 0.5 °C (± SD) and the water temperature 23.7 ± 0.8 °C (± SD) over all three runs (Table 4.2). The mean difference in temperature within blocks (pairs of tanks) was minimal for each of the three runs (Table 4.2).

Table 4.2
Environmental conditions of tanks for each of the three runs.
(Means ± standard deviation)

Runs	Air Temperature °C	Water Temperature °C	Water temp. difference (within blocks) °C	PAR. µmol m^{-2} s^{-1}
1	22.4 ± 0.4	23.5 ± 0.5	0.06 ± 0.04	91.4 ± 2.0
2	23.0 ± 1.1	24.6 ± 0.7	0.16 ± 0.13	114.6 ± 3.5
3	23.3 ± 1.2	23.1 ± 1.3	0.20 ± 0.09	93.6 ± 2.1

Light was provided by banks of 120cm-long fluorescent tubes of the same type and position as described in chapter 3. Table 6 shows the mean photosynthetically active radiation (PAR) level for each of the three runs, based on measurements taken 5 cm above the water surface. PAR was measured using a quantum light meter and sensor (Licor, inc, Lincoln, Nebraska, USA). Light was provided on a diel pattern of 14 h light followed by 10 h dark for all three runs.

Nine identical glass aquaria (tanks) were used, as described in chapter 3. Two tanks were placed under each light bank. Extraneous light from other light banks was excluded (chapter 3). All the tanks were filled with tap and deionised water to a height of 340mm, to give a conductivity of about 260 µs cm^{-1}over all tanks.

Submersible 4.6-watt power filters (Rio-PF400) stirred the water in all tanks. The tanks were topped up to the 340mm mark with deionised water weekly to replace evaporated water. To reduce excess algae growth the tanks sides were regularly scraped and the power filters cleaned. Occasionally (every two weeks in the run 3) the tanks were partially emptied

(approx. one third) and refilled with fresh water (1:1 tap and deionised water). This helped reduce plant nutrients in all tanks, as well as, sodium and algae in pH 9 tanks, and general microbial growth in pH 6.5 and 7.8 tanks.

4.2.2 Experiment design

The experiment was based on a balanced incomplete block design. The treatments were:

1. Ambient pH
2. Controlled pH 6.5
3. Controlled pH 7.8
4. Controlled pH 9.0.

The limited number of tank systems available prevented the experiment from being completed in a single run; therefore, the experimental design was balanced over three runs. All treatments were replicated twice in each run (fig.4.1).

Run 1

Block	I	II	III	IV
Tank 1	Tr2	Tr1	Tr1	Tr2
Tank 2	Tr3	Tr3	Tr4	Tr4

Run 2

Block	I	II	III	IV
Tank 1	Tr2	Tr1	Tr3	Tr1
Tank 2	Tr3	Tr2	Tr4	Tr4

Run 3

Block	I	II	III	IV
Tank 1	Tr1	Tr3	Tr2	Tr1
Tank 2	Tr3	Tr4	Tr4	Tr2

Fig. 4.1. Diagram of experiment layout showing treatments (tanks) (Tr) in pairs (blocks I to IV) where each treatment occurs with each other treatment twice over the three runs

Each treatment occurred twice with each other treatment over three runs. Each run contained all the above treatments and their replicates in four blocks of two aquaria (tanks) under a light box. Treatment pairs were randomly assigned to each of the four blocks. In addition, there were two reference tanks in run 1, and one reference tank in run 2 and 3 that contained ambient pH and stirred water without plants. These were not strictly part of the experiment design

All developing floating leaves were removed on a weekly basis to reduce the direct uptake of atmospheric carbon dioxide.

4.2.3 pH Control

A computer controlled digital /analog system was used to regulate pH of the water in each of six tanks. The controller was set to maintain the pH at 6.5, 7.8 and 9.0 (two tanks for each pH level). Refer to Appendix 2 for a description of this control system.

Glacial acetic acid (50%) and sodium hydroxide (1N) were used to adjust pH. There was one acid and one base-dosing unit per tank. Each dosing unit was comprised of a 1 litre schott bottle with a (2.5mm ID) spigot (glass tube outlet) at the base, flexible smoothbore teflon plastic tubing (8 and 6 mm ID), two 1/8" BSP stainless steel barbed hose fittings, and one solenoid (fig.4.2). Acid or base was gravity fed via the plastic tubing from the schott bottles to the solenoids. When the solenoids were activated a small volume of acid or base was released via tubing to a tank

The twelve 1/8" BSP (model 151112) (Goyen Distributors Ltd) valox plastic solenoids were fitted with weatherproof 24volt DC coils. The solenoids were the smallest available with a 1.4mm orifice. These functioned well throughout the three runs but some leaked (externally) after a period when used with sodium hydroxide. More chemically resistant solenoids were available but were much larger and two to three times more costly.

4.2.4 Preparation of potted plant material

For each run, forty eight 100mm diameter plastic pots (420 mL) were lined with clear polyethylene bags and 1g of 8-9 month controlled release Osmocote Plus^{R} fertiliser (16 N: 3.5 P: 10 K: 1.2 Mg plus trace elements) was placed in the pots before filling with sediment.

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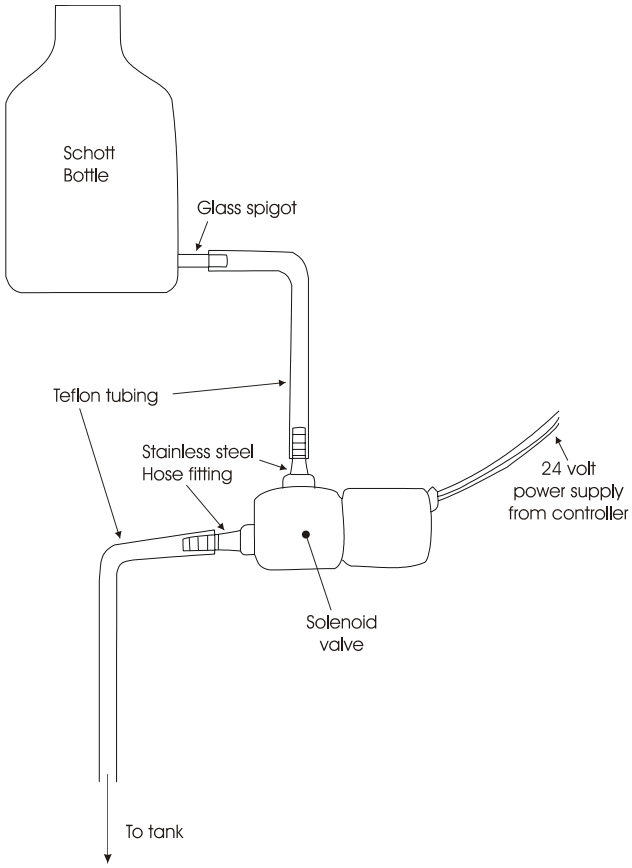

Fig 4.2 Acid/base dosing equipment comprised of a 1 litre schott bottle with a (2.5mm ID) glass tube outlet (spigot) at the base, flexible smoothbore teflon plastic tubing (8 and 6 mm ID), two 1/8" BSP stainless steel barbed hose fittings, and one solenoid

The approximate nutrient composition of the sediment at the start of each run is shown in Table 2 in Chapter 3

Due to the slow release nature of this fertiliser, these nutrients are not immediately available to plant roots in the concentrations outlined in the table. Independent measurements of the release rate of similar 8-9 month Osmocote^{R} have shown that 49 to 52 % of the nutrients in the fertiliser still remained after approx. 4½ months (133 days) of shaking the fertiliser in water at temperatures between 21 to 25°C (Lamont, et al. 1987; Handreck and Black 1994). The sediment that was used was similar to chapter 3. It was assumed that the natural plant nutrient level in washed sand would be negligible compared to the amount of fertiliser applied.

Approximately 6 month old seedlings of *Aponogeton elongatus* were used in the first run. The second and third runs used 8 and 12 month old tissue cultured stock of *Aponogeton elongatus*. The plants were potted in a similar procedure as that outlined in Chapter 3 except the powered iron (55.85 % Fe) was mixed with granulated zeolite (2 mm Horticulture Grade) and the mixture added to the top of the sediment to a depth of 10-15 mm at a rate of 0.2 g of powdered iron per pot (266 mg L^{-1} of sediment). This was followed by a layer of fine gravel (1mm to 8 mm in size).

Before potting, the plants were labelled, shaken to remove excess water and their weight recorded. The plant material, as in the first experiment, was more variable than desired. In an attempt to reduce variability in each run, all the available plants were ranked according to their weight. After ranking and after discarding any outliers, 48 plants were selected for each of the three runs and divided into 6 groups of 8 plants according to plant weight. Each group of 8 plants was then randomly distributed to the 8 tanks until all the tanks contained six plants (each tank was subsequently referred to as an experimental unit).

4.2.5 Plant measurements

For each plant, total leaf number, length and width, and plant dry weight were measured as with Chapter 3. Dry weight was determined for all 48 plants from each run.

4.2.6 Chlorophyll fluorescence

As with Chapter 3, a portable submersible Diving-PAM Fluorometer, was used to measure leaf chlorophyll fluorescence. Single quantum yield ([Fm'-F]/Fm') measurements and rapid light curves (RLC) of light adapted leaves and maximal yield (Fv/Fm) from dark-adapted (10 minutes) leaves were taken at the end of each run. For RLC measurements, electron transport rate (ETR) was determined over nine actinic light levels (ie; 1, 7, 19, 28, 40, 70, 110, 180, 267 μmole quanta s^{-1} m^{-2}). For single yield measurements of light adapted leaves, one leaf was measured on each of six plants from each treatment over two replicates. For single maximal yield measurements of dark adapted leaves and RLCs of light adapted leaves, one leaf was measured on each of three plants from each treatment over two replicates. All leaf measurements were taken at similar leaf positions as in Chapter 3.

4.2.7 Carbon Isotopes

An analysis of percentage carbon, delta carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) was carried out on the dried plant material from run one. Dried tuber material from run two was also analysed because of a shortage of material from run one in some treatments after inductively coupled plasma (ICP) analyses. A steel ball grinder finely ground the plant material after which the plant material was analysed by Analytical Services, Botany Department UQ using a Europa Mass Spectrometer.

4.2.8 Nutrient Levels in Plants and Water

ICP measurements of major nutrients and selected micronutrients were carried out on the plants and water in each tank in run 1. Water samples were taken at the beginning and at the end of the first run. Plant samples were taken at the end of run one to measure dry weights and for ICP analysis. The dried leaves tubers and roots from each experiment unit were first ground to a coarse dust using an electric grinder (Culatti grinder, type 14-580S, Glen Creston Ltd, Stanmore, England). Weighed samples (0.1 gram) were digested with nitric acid/perchloric acid. The samples were then analysed by Analytical Services, School of Agriculture and Horticulture, UQ, Gatton using standard ICP procedures with a flame spectrophotometer. The following elements; P, K, Mg, Ca, Na, S, Fe, B, Mn, Cu, and Zn were measured. Water from each of the pH treatment tanks and reference tanks in runs 1 and 2 were also analysed.

4.2.9 Dissolved CO_2 Levels

Total dissolved inorganic carbon (DIC) and dissolved free CO_2 (dissolved CO_2 gas and H_2CO_3) were measured using a titration technique described by Frith et al (1993). Three or more samples were tested for each of nine tanks for each run. One of the limitations with this technique is that it cannot measure free dissolved CO_2 levels below 4.5μM. The technique is also based on the assumption there is no free dissolved CO_2 above pH 8.3 (Frith, et al. 1993).

4.2.10 Monitoring

A waterproof hand held pH meter 'TPS WP-80' (TPS Pty Ltd, Brisbane, Australia) and a waterproof hand held electrical conductivity meter (TPS WP-84) were used to measure tank

water pH and conductivity once a week throughout the experiment. The pH meter was also used to check the calibration of the pH controller.

Water temperature (in all tanks) and air temperature (1 location) were measured hourly using thermocouples connected to a data logger ('Datataker 500', Pacific Data Systems Ltd, Brisbane, Australia).

4.2.11 Statistical Analysis

ANOVA and least squares means (LSM) were applied to the leaf morphology, plant biomass, chlorophyll fluorescence and DIC data (SAS release 6.12). Leaf area data was square root transformed before ANOVA. Dry weight data, plant turnover time, and DIC data were log transformed. This was to account for heterogenous variances where variances tended to be proportional to the mean for each treatment in each of the data sets of leaf, tuber and root (Gomez and Gomez 1984). Where data has been log transformed, equivalent (back transformed)(antilog) means have been used in tables and graphs in preference to the original estimated means. The original estimated means were invalid as they were based on data that did not comply with one of the ANOVA assumptions (see above). Because the balanced incomplete block design used in this experiment is also by definition a partially unbalanced design the means have to be estimated to take into account the effects of blocks and runs (Ott, 1993). A significance level of $P< 0.05$ was adopted unless otherwise indicated.

4.3 Results

4.3.1 General Observations

Generally, the pH 6.5 to 7.8 treatments, in all three runs, produced the largest plants with a relatively dense mass of foliage that filled the tanks. Towards the end of each run, some mutual shading and reduced water flow was observed. The later is likely to have reduced overall photosynthetic rate and subsequent growth. This stage was accompanied by an excessive loss of leaves (Section 4. 3. 2).

The water became increasingly cloudy towards the end of each run in pH treatments 6.5 and 7.8, due to the growth of single-celled organisms. Microscopic examination revealed large numbers of bacteria and protozoans with some algae. The micro-organisms also covered the

sediment, pots and leaves of the plants making them slimy to handle. The sliminess increased with run duration and coincided with leaf loss, thus run three had the least build up of micro-organisms and the least leaf loss. Micro-organism growth did not occur in the higher pH treatments (ambient pH and pH 9.0) in all the runs.

In the ambient pH and pH 9.0 treatments plants grew much more slowly and did not reach a stage of mutual shading. The plants in the ambient pH were under similar conditions as the plants in the first experiment (stirred water with 1g of slow release fertiliser), however, they produced only half the expected growth. Algae control was more difficult at these higher pH treatment levels.

4.3.2 Comparison of runs 1, 2 and 3

A foliage disease resulted in substantial leaf loss in all three runs. A description of this disease is in the Appendix 4. The first major incidence of this disease was in run one towards the end of the plant establishment period but before pH control was established. Almost all the plants rapidly developed foliage symptoms resulting in almost complete defoliation. All plants recovered, replacing lost foliage to varying degrees once pH control commenced but recovery was much slower in the ambient and pH 9.00 treatments. There was no major foliage loss before commencement of pH control in runs 2 and 3.

In all three runs, there was a loss of leaves towards the end in pH 6.5 and to some extent in pH 7.8. The symptoms towards the end of the run were different from those described in the Appendix 4, in that the leaves first yellowed along the margins followed by dark brown necrosis spreading inwards towards the leaf mid rib. Young, partially or fully developed leaves were the first to succumb.

The worst affected plants were in pH 6.5, in run two, as the plants became almost fully defoliated by 49 days after commencement of pH control and after a period of relatively strong growth. Plants in the pH 7.8 maintained most of their foliage. By the end of the run, the plants in pH 6.5 treatments had produced new leaves to replace those lost, but many of the leaves were small and distorted. This is evident in the low average leaf area and dry leaf biomass of plants in treatment 6.5 in run 2 compared to plants in treatment 7.8 (Fig. 4.3 & 4.4). The duration of run 2 was extended to give pH 6.5 treatment plants a chance to recover some foliage however they failed to catch up with plants in treatment 7.8 despite an extra 66 days.

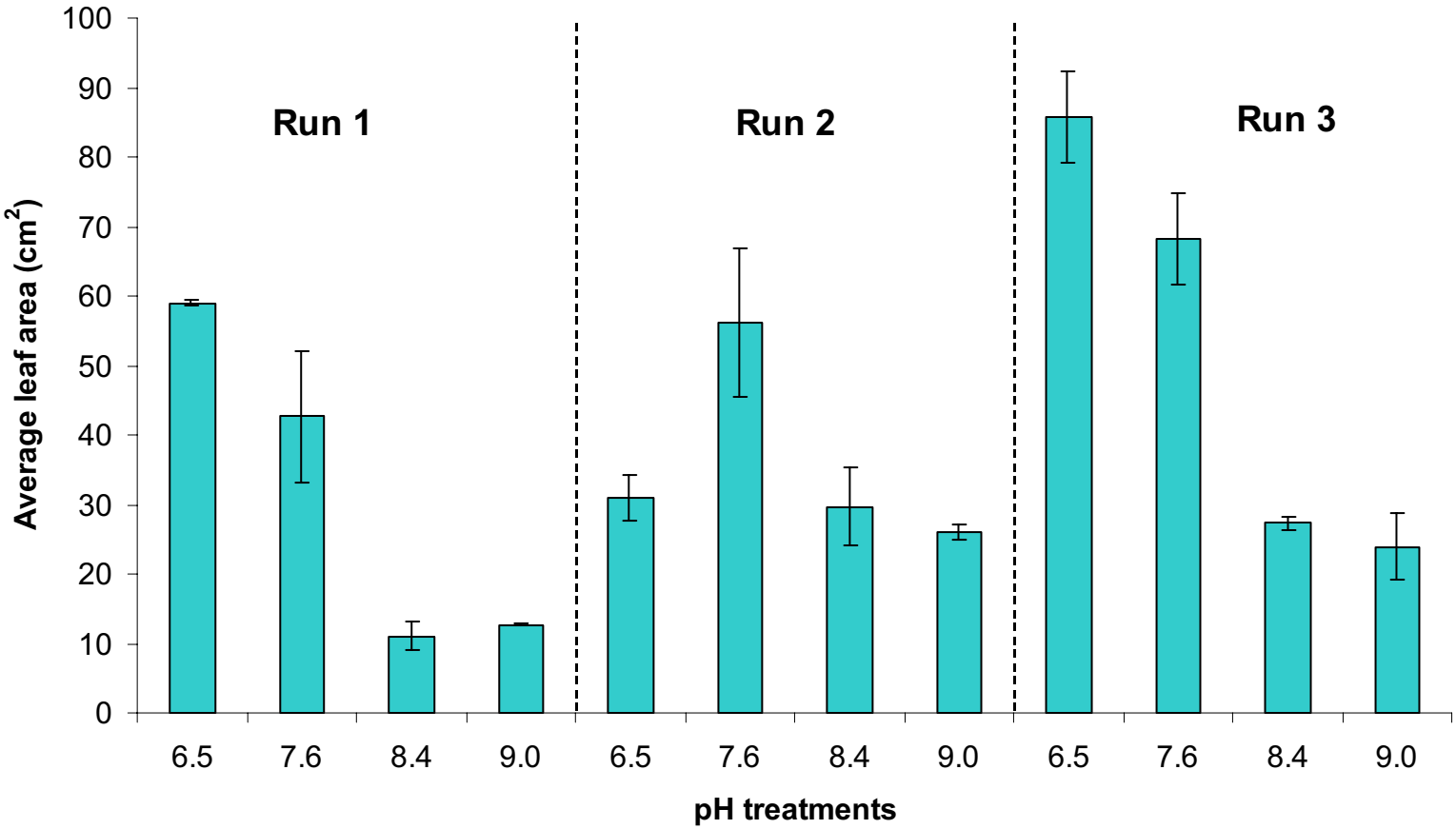

Fig. 4.3. The effect of four pH treatments on average area of an *A. elongatus* leaf for each of the three runs (means ± SE). The duration of run one was 89 days, run two 114 days and run three 69 days.

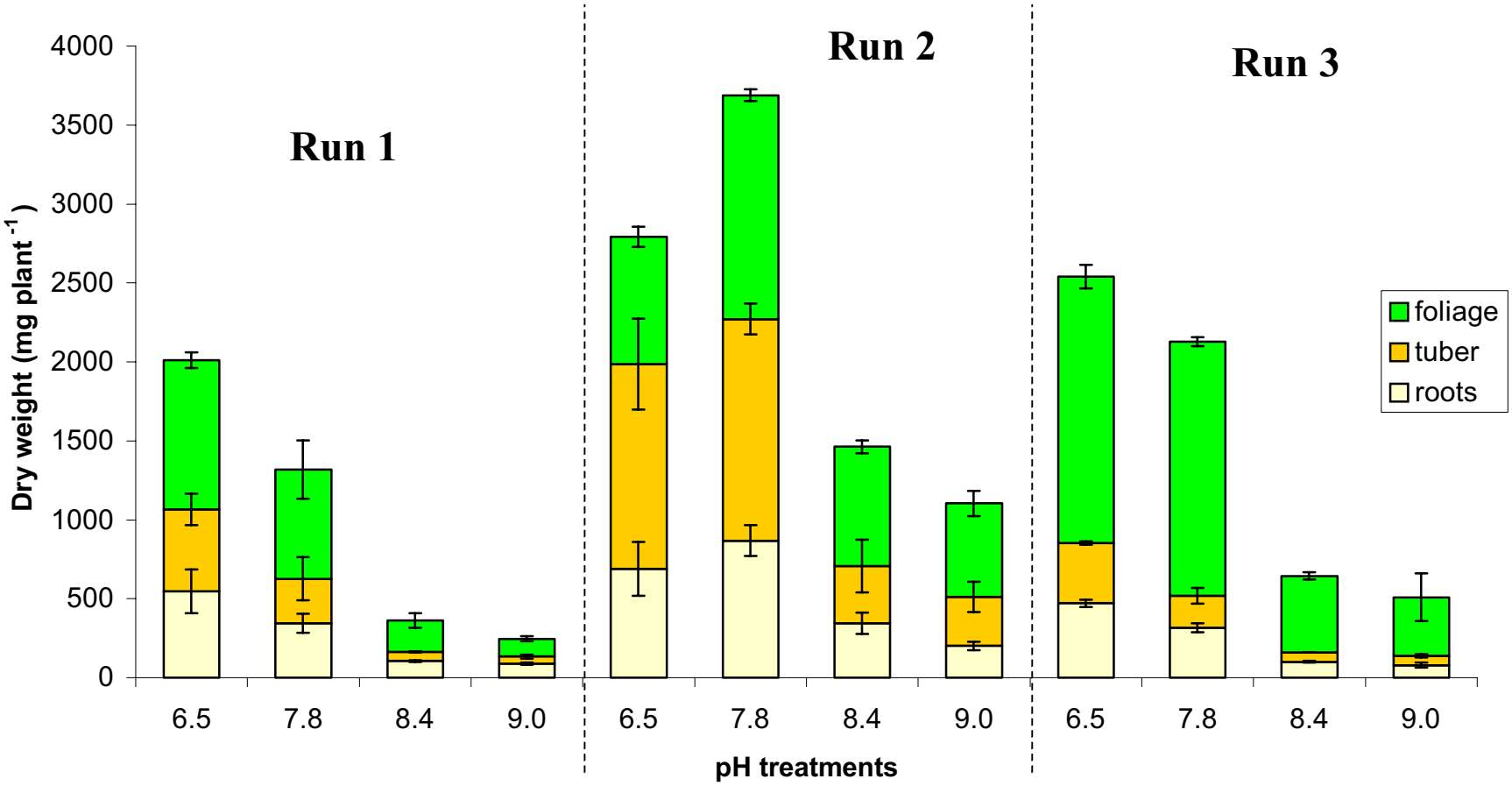

Fig. 4.4. The effect of four pH treatments on *A. elongatus* dry weight for each of the three runs (means ± SE). The duration of run one was 89 days, run two 114 days and run three 69 days.

The .dry weight data for each of the three runs clearly illustrates the effects of the pH treatments (fig 4.4). In runs 1 and 3, the tuber biomass of plants in pH treatment 6.5 was greater than for plants in pH 7.8. In run 2 there was no difference in tuber biomass between pH 6.5 and 7.8, which is likely due to a reduction in photosynthetic output because of leaf loss in pH 6.5. The differences in overall biomass between runs 2 and 3 are most likely due to the different periods of growth (fig 4.4). The plants in run one would likely have had greater overall biomass compared to run 3 if it was not for the complete defoliation that occurred near the beginning of run one.

The loss in biomass from the plants in pH 6.5 compared to pH 7.8 starting midway through run 2 markedly reduced the mean biomass across the three runs and therefore skewed the results. Leaf loss is apparently treatment related in that it occurred to varying degree in all three runs in treatment pH 6.5. The severity of leaf loss in run 2 suggests that *A. elongatus* may be more susceptible to this disease at lower pH but it is assumed that this severe defoliation effect in pH 6.5 in run 2 is a secondary, uncontrolled factor unrelated to plant response to CO_2. Therefore, the pH 6.5 treatment in run 2 was treated as missing data in all ANOVA and mean comparisons of leaf morphometrics and biomass data.

4.3.3 Leaf Morphometrics

Plants growing in pH 6.5 and 7.8 produced greater foliage area and average leaf area compared to plants in pH 8.4 (ambient) and pH 9.0 (Fig. 4.5). In the two lower pH levels the leaves were not only longer but also proportionally narrower (ie greater leaf length: width ratio) than plants in pH 8.4 and pH 9.0 (Fig. 4.6 & 4.7). The average leaf length was greater on plants in pH 6.5 compared to pH 7.8 but average leaf width was similar. All leaf dimensions were less in pH 8.4 and 9.0 with no significant difference between these two treatments. Leaf number was not significantly different between all treatments (Fig. 4.5).

4.3.4 Biomass

The *A. elongatus* plants in pH treatments, 6.5 and 7.8 produced the most growth in wet biomass (wet weight at end of each run less initial wet weight) (Fig 4.8) and higher total, foliage, average leaf, tuber and root dry biomass ($P<0.001$) (Fig. 4.9 & 4.10). Plants growing in pH 6.5 had significantly larger tuber dry biomass per plant than in pH 7.8 (Fig 4.9). Average leaf and root biomass showed a similar pattern of response to pH (not significant).

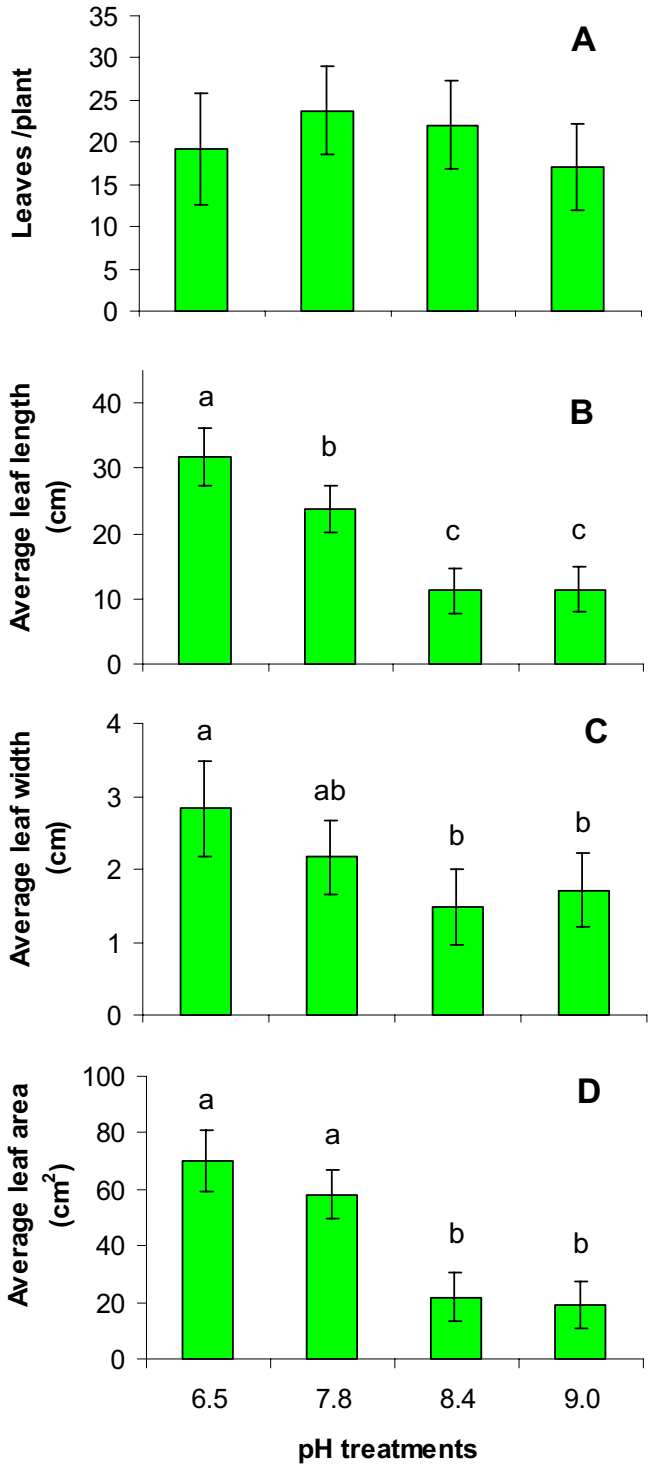

Fig 4.5 The effects of pH on *A. elongatus* (A) plant leaf number and (B) average leaf length, (C) width and (D) area. Treatments with a common letter or no letters (alphabet notation above bars) are not significantly different at 5% level. Error bars are 95% confidence intervals. The leaf area data was square root transformed before ANOVA and LSM comparisons and the square of square root transformed data is presented. The pH level 8.4 ± 0.2 (mean ± SD) indicates the ambient (control) pH treatment level and is based on data collected weekly with a pH meter.

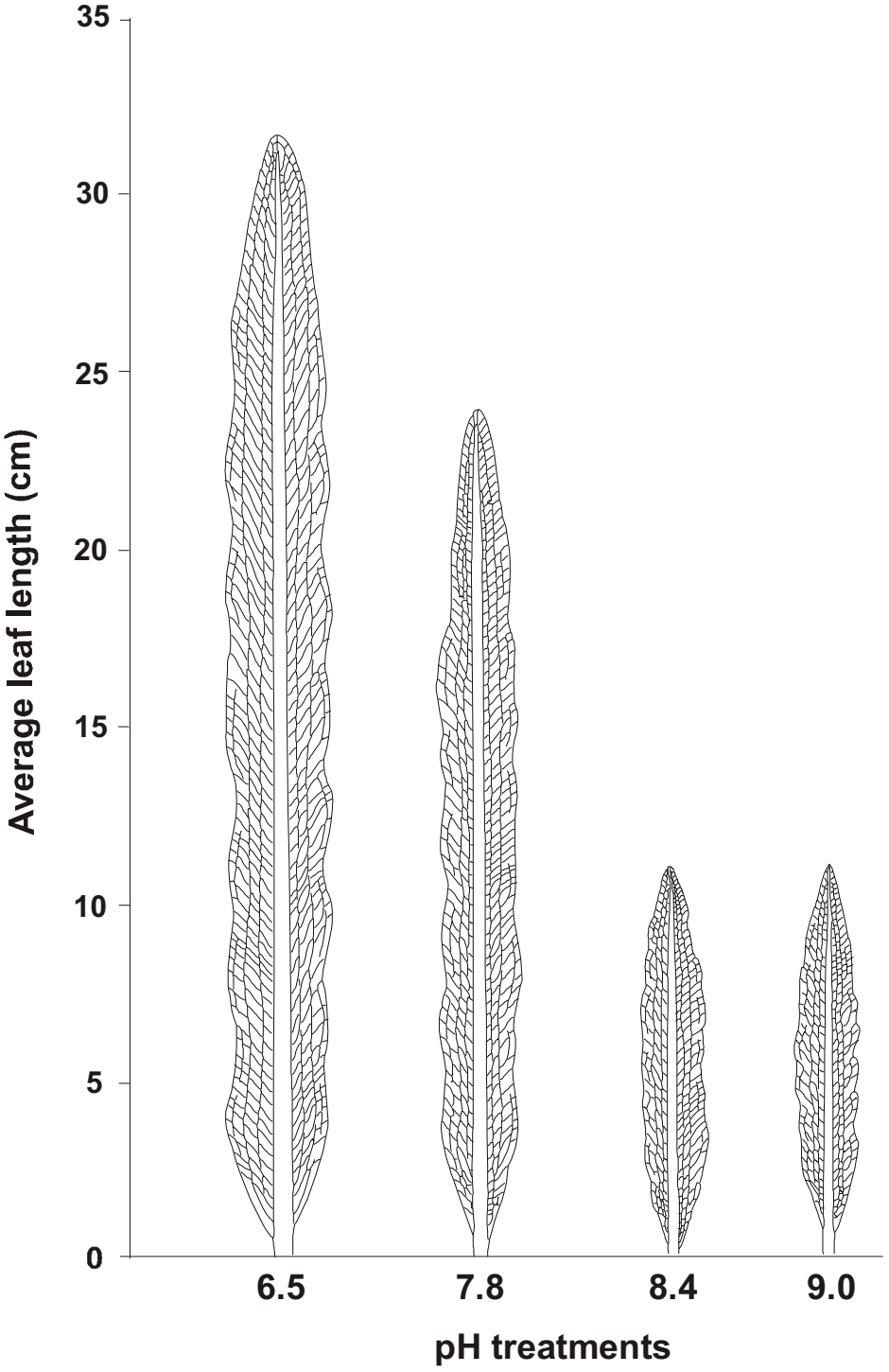

Fig. 4.6 The effect of pH on *A. elongatus* average leaf size and shape. Leaf drawings are scaled to average leaf length and width (means ± 6 %) (fig 4.5). The 8.4 indicates the mean ambient pH

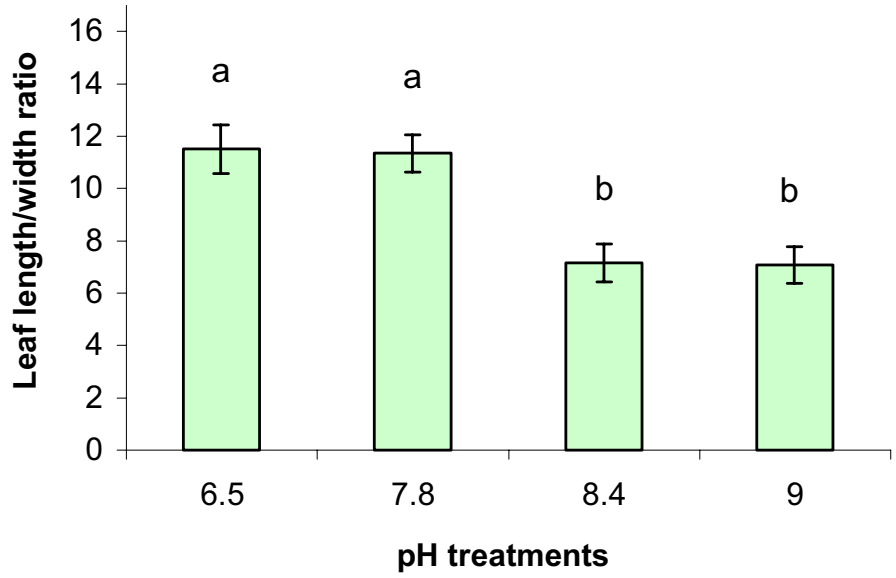

Fig. 4.7 The effects of pH on leaf length to width ratio. Treatments with a common letter (alphabet notation above bars) are not significantly different at 5% level. Error bars are 95% confidence intervals. 8.4 ± 0.2 (± SD) is the mean ambient pH

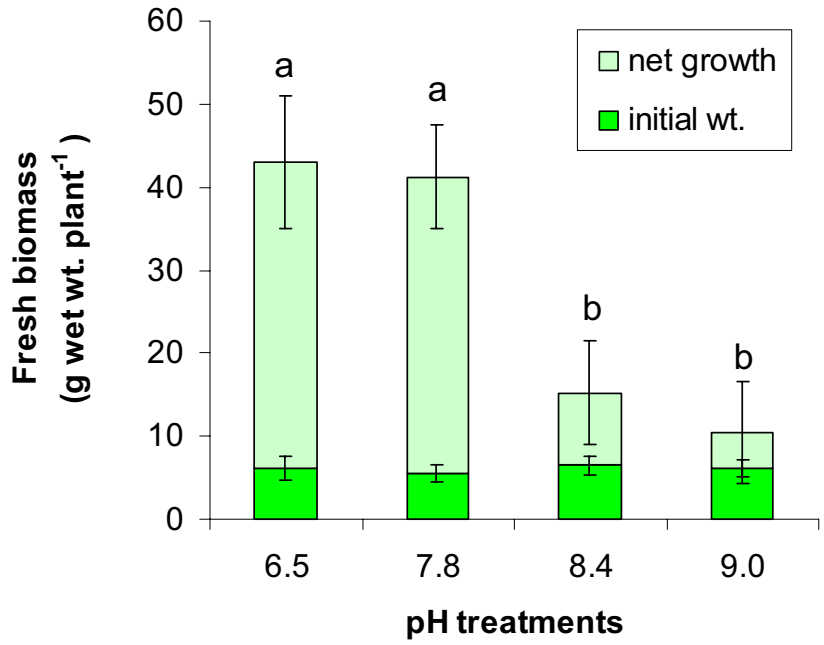

Fig. 4.8 The effect of four levels of pH over three runs on initial plant fresh biomass (wet weight) at the start of a run and on the net growth in fresh biomass by the end of a run. Treatments with a common letter (above columns) are not significantly different at 5% level. Error bars are 95% confidence intervals. The 8.4 is the mean ambient pH

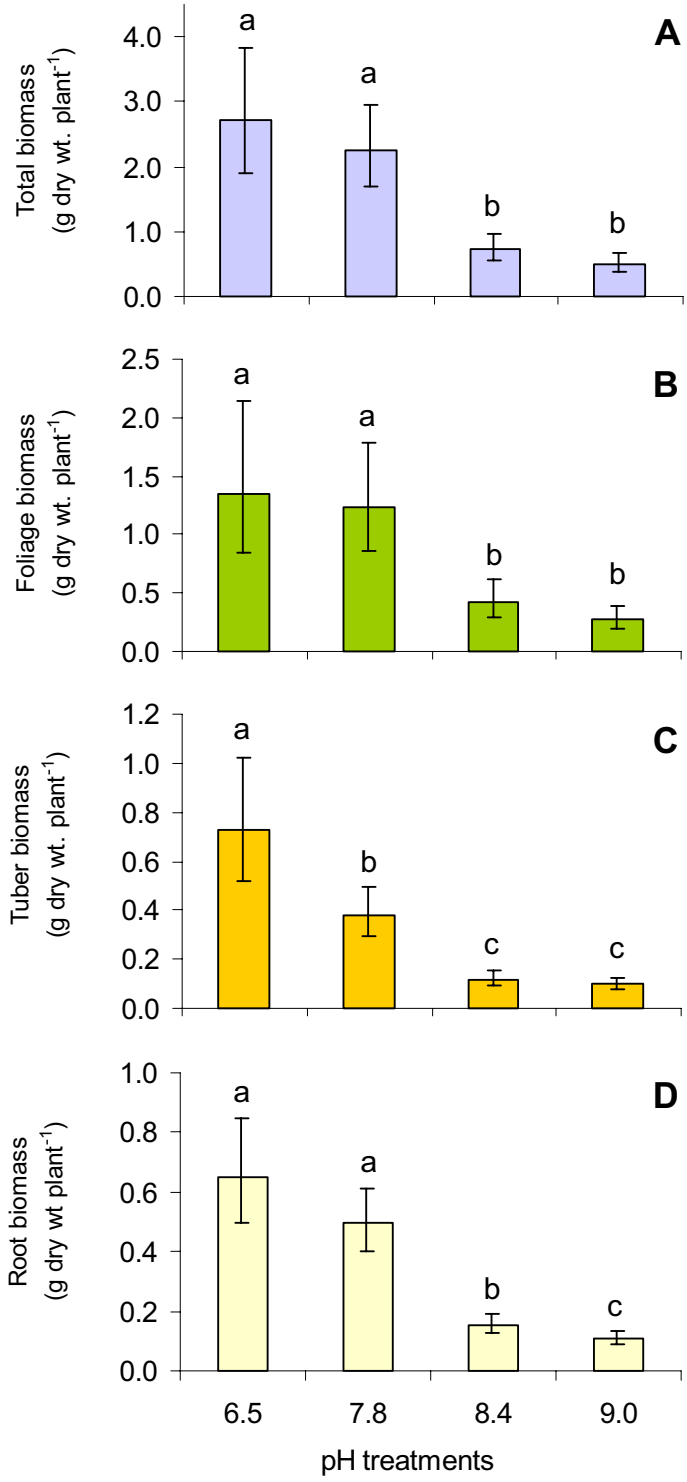

Fig. 4.9 The effects of pH on (A) mean total biomass (grams dry weight per plant) and biomass of (B) foliage, (C) tuber and (D) roots. Treatments with a common letter (alphabet notation) are not significantly different at 5% level. Error bars are 95% confidence intervals. The original data was transformed before ANOVA and LSM comparisons. These bar charts are based on the equivalent (back transformed, antilog) means of log10 data. The 8.4 is the mean ambient pH.

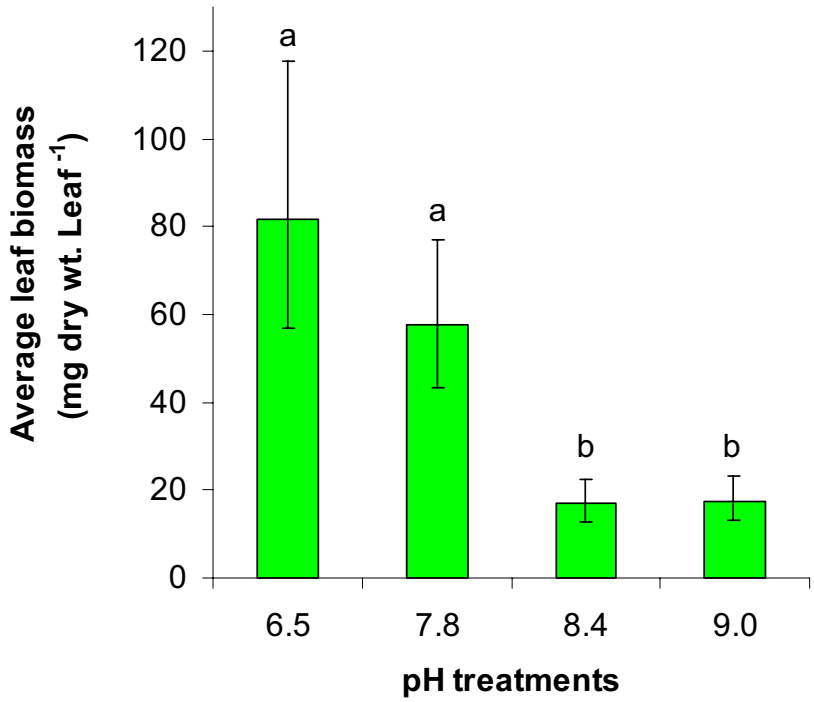

Fig. 4.10 The effect of pH on the biomass (dry weight) of an average leaf. The original data was transformed before ANOVA and LSM comparisons. This bar chart is based on the antilog of log10 data. Treatments with a common letter (above columns) or no letter are not significantly different at 5% level. Error bars are 95% confidence intervals. The 8.4 is the mean ambient pH.

Plants growing in pH 8.4 had significantly larger root dry weights compared to pH 9.0 (Fig 4.9). There was no significant difference in wet biomass, total dry biomass and foliage dry biomass, between pH 6.5 and 7.8 or between pH 8.4 and 9.0.

The relative proportions of foliage, tuber and roots of an average plant were similar for each of the pH treatments (Fig 4.11). This suggests that overall plant size was the main difference between the pH treatments

Plants in pH treatments 6.5 and 7.8 had the greatest turnover, taking significantly fewer days to double initial wet biomass than plants in treatments 8.4 and 9.0 (Fig 4.12). There was no significant difference in turnover between pH 6.5 and 7.8 or between pH 8.4 and 9.0.

Specific leaf weight (leaf dry weight per one square centimetre of leaf area) tended to be higher at pH 6.5 or 7.8 but the differences were not significant because of considerable variation within each treatment (Fig 4.13).

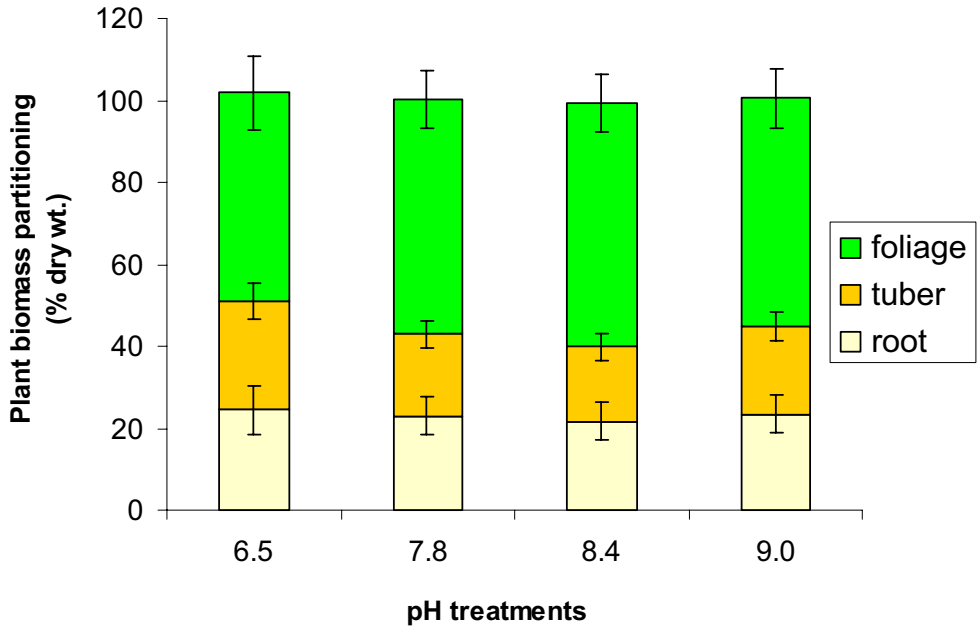

Fig. 4.11 The effects of pH on biomass (% dry weight) partitioning into foliage, tuber and root of an average plant. Treatments were not significantly different at 5% level. Error bars are 95% confidence intervals. The 8.4 is the mean ambient pH.

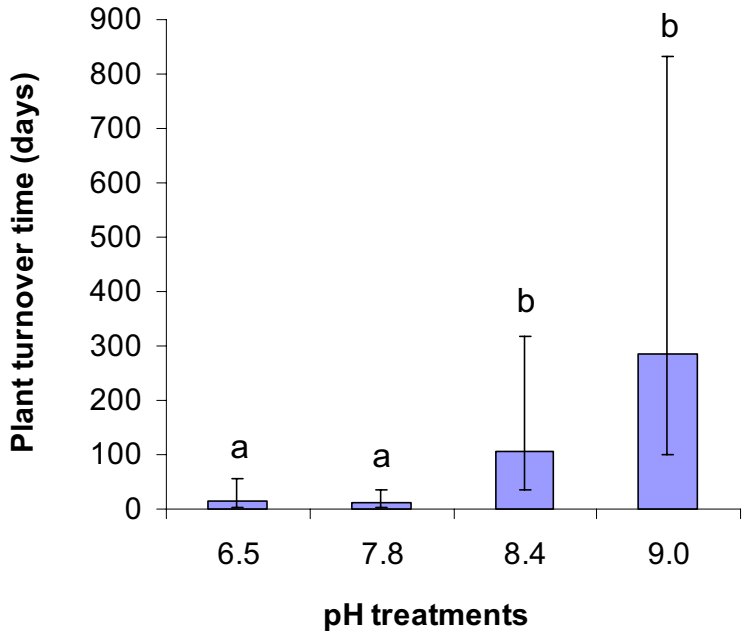

Fig. 4.12 The effect of pH over three runs on plant turnover time (defined as the number of days to double the initial fresh biomass). The original data was transformed before ANOVA and LSM. This bar chart is based on the antilog of log10 data. Treatments with a common letter (above columns) are not significantly different at 5% level. Error bars are 95% confidence intervals. The 8.4 is the mean ambient pH.

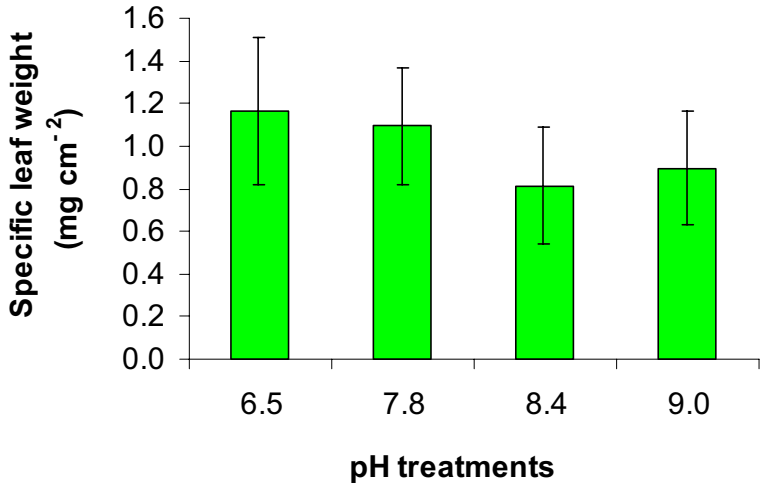

Fig. 4.13 Specific leaf weight (mg of biomass per cm^2 of leaf). Treatments with a common letter (above columns) or no letter are not significantly different at 5% level. Error bars are 95% confidence intervals. The 8.4 is the mean ambient pH.

4.3.5 Chlorophyll fluorescence

Despite significant differences in leaf morphometrics, biomass, and turnover, the pH treatments had no apparent effect on photosynthetic efficiency measured as leaf chlorophyll fluorescence. There were no significant differences between pH treatments in dark adapted and light adapted quantum yield and electron transport rates (ETR) (table 4.3).

Table 4.3

Effects of pH on *A. elongatus* leaf fluorescence as quantum yield and electron transport rate (ETR).

This table contains yield and ETR data from single measurements of dark adapted leaves (10 minutes) and light adapted leaves and maximum yield and ETR data from rapid light curves (RLC) from light adapted leaves. For the single yield measurements ETR was calculated using the formula ETR = quantum yield x PAR x ETR factor (Leaf absorbtance) x 0.5 (Ralph, et al. 1998). Mean ambient PAR near leaf surface was 20 µmol quanta m^{-2} s^{-1} and average leaf absorbtance 0.73. All data is an average of three runs. All Means in each column are not significantly different at 5% level. Means ± SE.

pH Treatments	6.50	7.80	Ambient	9.00
Quantum Yield (µmol quanta m^{-2} s^{-1})				
Dark adapted (10 min) (Fv/Fm)	0.733 ± 0.006	0.742 ± 0.006	0.740 ± 0.006	0.728 ± 0.006
Light adapted (ΔF/Fm')	0.712 ± 0.011	0.688 ± 0.011	0.703 ± 0.011	0.669 ± 0.011
Maximum RLC (ΔF/Fm')	0.721 ± 0.011	0.723 ± 0.011	0.724 ± 0.011	0.708 ± 0.011
ETR (µmol quanta e^- m^{-2} s^{-1})				
Dark adapted (10 min)	17.4 ± 0.1	17.6 ± 0.1	17.6 ± 0.1	17.3 ± 0.1
Light adapted	16.6 ± 0.6	15.7 ± 0.6	16.5 ± 0.6	16.3 ± 0.6
Maximum RLC	14.7 ± 1.0	13.8 ± 1.0	12.2 ± 1.0	11.6 ± 1.0

4.3.6 Percentage Carbon and Carbon Isotope Ratios in Plant Tissues

There was no significant difference in percentage carbon in *A. elongatus* plants between treatments (Fig.4.14). Interestingly this data is consistent with there being large amounts of polysaccharide in the tubers. Iodine solution applied to a tuber showed the presence of starch but microscope examination of tuber cell contents showed the presence of another polysaccharide, inulin in numerous plastids (Appendix 1). Inulin (mostly fructose), like starch (mostly glucose), is about 40% carbon and the tubers contain on average 40% approx carbon. The lower percentage carbon in the roots is possibly due to the relatively high mineral levels (Table 4.4).

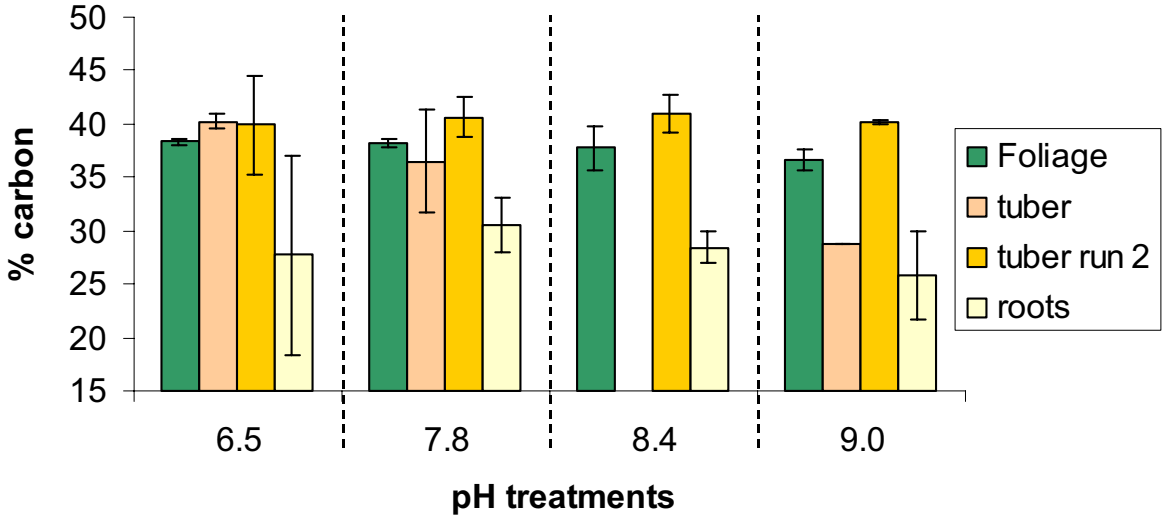

Fig. 4.14 The effect of four levels of pH on percentage carbon content in foliage, tubers and roots of *A. elongatus* in run 1 (Due to insufficient tuber material in treatments pH 8.4 and 9.0, tubers in run two were also analysed). Based on means ± SD (n = 2). The 8.4 treatment indicates the mean ambient pH (control)(Fig 4.2).

The delta carbon data shows a trend of decreasing discrimination against carbon13 with increasing pH (Fig. 4.15). Plotting the combined delta carbon data from roots, tuber and foliage against pH revealed a strong positive correlation ($r = 0.8$, $r^2 = 0.61$, $P < 0.01$, $n = 15$). Regression analysis on the same data revealed a highly significant linear increase in plant delta carbon 13 content with pH in this experiment (Fig 4.16).

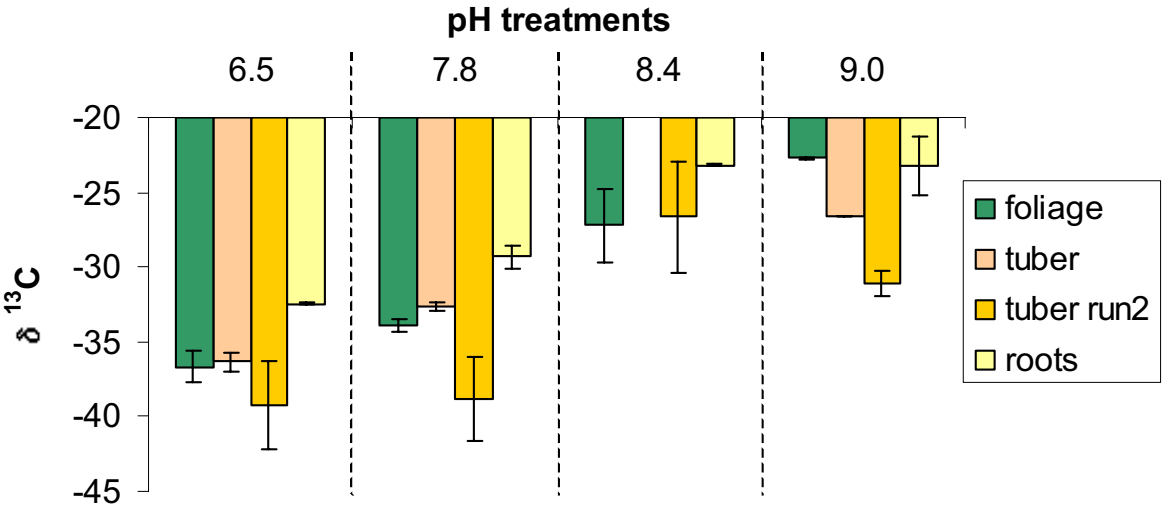

Fig. 4.15 The effect of pH on delta carbon ($\delta^{13}C$) content in foliage, tubers and roots of *A. elongatus* in run 1 (Due to insufficient tuber material in treatment pH 8.4 and 9.0, tubers in run two were also analysed). The 8.4 is the mean ambient pH (control). $\delta^{13}C$ data are means ± SD (n = 2)

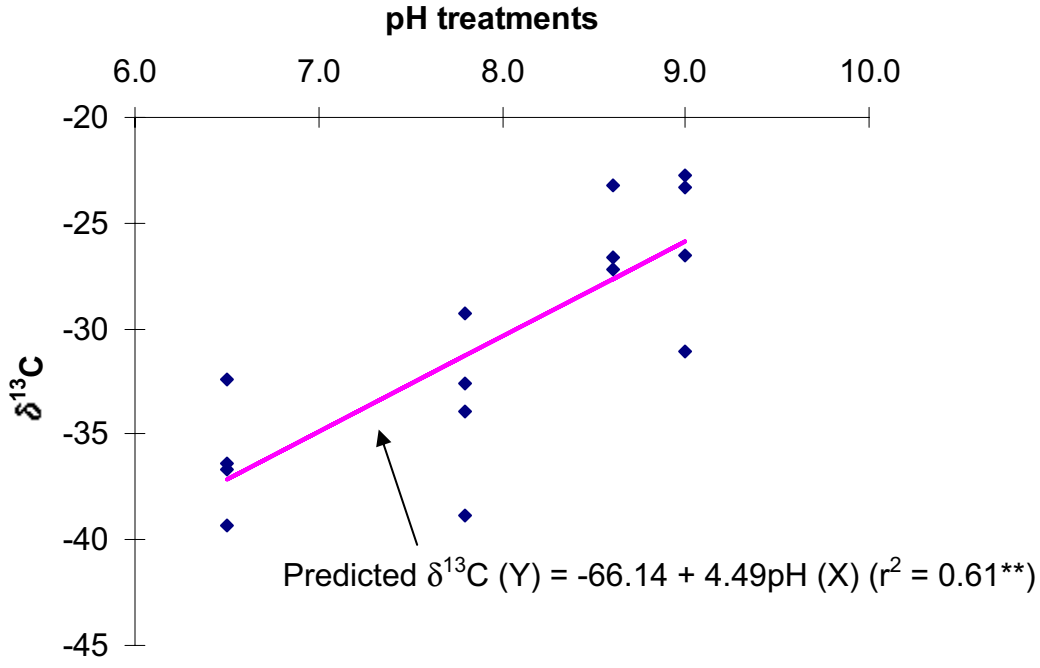

Fig. 4.16 The relationship of (◆) delta carbon ($\delta^{13}C$) content of *A. elongatus* (foliage, tubers and roots) to pH in run 1 (Includes tuber $\delta^{13}C$ data from run 2). A line fit plot based on linear regression of 15 means (n = 2).

4.3.7 Dissolved CO_2 Levels

Total dissolved carbon (DIC) and dissolved free carbon were found significantly different between treatments (Fig. 4.17). Because the variance was heterogenous, the data was log transformed before ANOVA.

The highest total DIC occurred in the pH 9.0 treatment (Fig 4.17). The second highest DIC level was in pH 7.8 treatment, but this level was not significantly different from the ambient pH (8.4) treatment. The lowest DIC occurred in pH 6.5, which was significantly lower than pH 7.8 but was not significantly different from pH 8.4. A reference tank without plants (mean pH 8.05 ± 0.17)(not included in the ANOVA) had similar DIC levels to the pH 6.5 and 8.4 pH treatments.

Both free dissolved CO_2 and free CO_2 as a percentage of total DIC decreased with increasing pH (Fig 4.17 graphs B & C,). There was a significant negative linear relationship ($R^2 = 0.95$) between pH and free CO_2 (Fig 4.18). There was also significant linear relationship between free dissolved CO_2 (μM) available in each pH treatment and root, tuber and average leaf biomass. The strongest linear relationship was between the free dissolved CO_2 and tuber dry biomass (grams) ($r^2 = 0.9993$, $P < 0.01$, $n = 4$) (Fig 4.19B). There was also a significant positive linear relationship between free dissolved CO_2 levels and average leaf dry biomass ($r^2 = 0.95$, $n = 4$) and root dry biomass ($r^2 = 0.93$, $n = 4$) (Fig 4.19A & C). The intercept values from regression analysis for average leaf and root were not significantly different ($P > 0.05$) from zero grams dry weight.

There was no significant linear relationship between free dissolved CO_2 levels and total plant dry biomass. Total foliage biomass per plant was not significantly different in treatment pH 6.5 compared to pH 7.8 despite the average leaf on plants in pH 6.5 having greater biomass and length. This suggests that plants in pH 6.5 had fewer leaves. Indeed, mean leaf number per plant is less in pH 6.5 compared to plants in pH 7.8 (not significant). This may be a reflection of the disease effect, despite the elimination of the worst effected pH 6.5 treatment from run two data. There is also no linear relationship between total DIC and plant biomass.

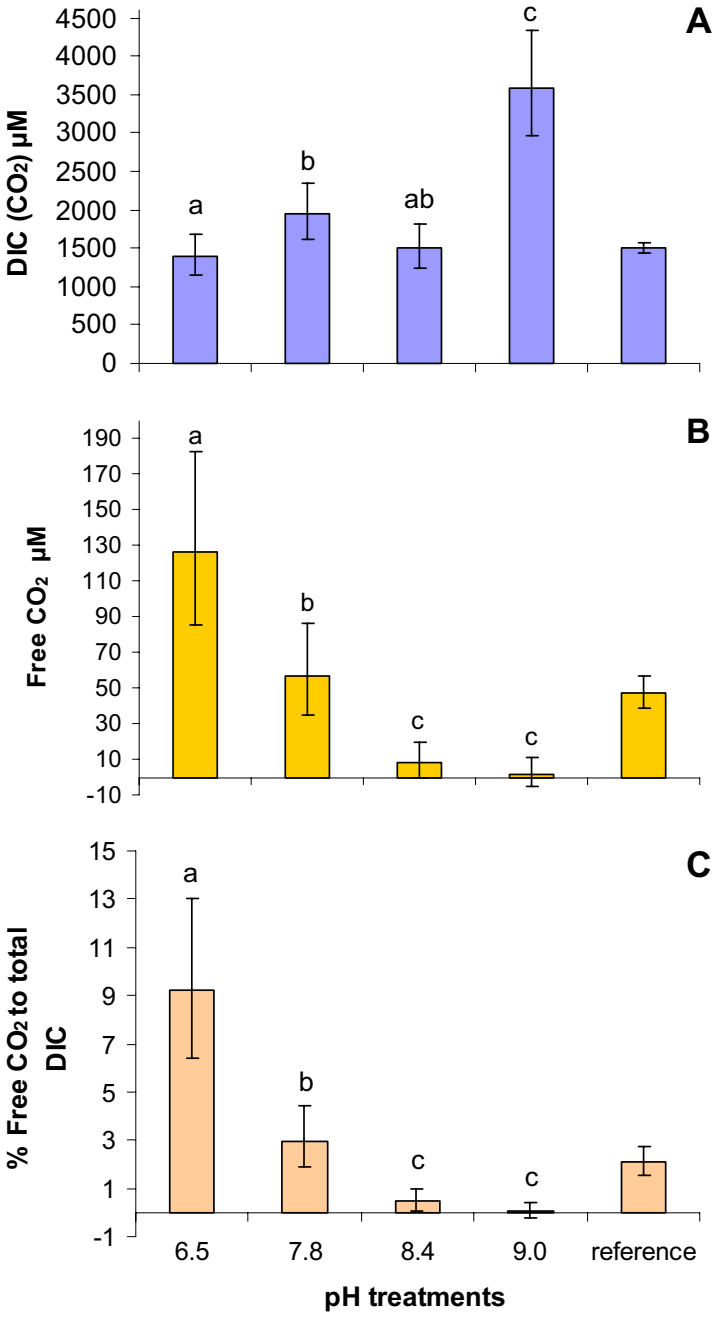

Fig 4.17 The effects of pH on (A) total dissolved inorganic carbon (DIC) as µM of CO_2, (B) free CO2 as µM and (C) percentage of free CO2 to total DIC. Treatments with a common letter (alphabet notation above bars) are not significantly different at 5% level. Except for the reference, error bars are 95% confidence intervals. The original data was transformed before ANOVA and LSM comparisons. These bar charts are based on the antilog of log10 data. The 8.4 is the mean ambient pH. A reference tank without plants although not part of the original analysis was included in each run (mean and STD Error (n = 6)).

Note that the dissolved free CO_2 levels in the pH 9.0 treatments were below measurable levels and treated as zero data. The dissolved free CO_2 levels in the pH 9.0 treatment given in fig 4.17 were estimated means of this unknown data produced by SAS ANOVA according to the Incomplete Block Design.

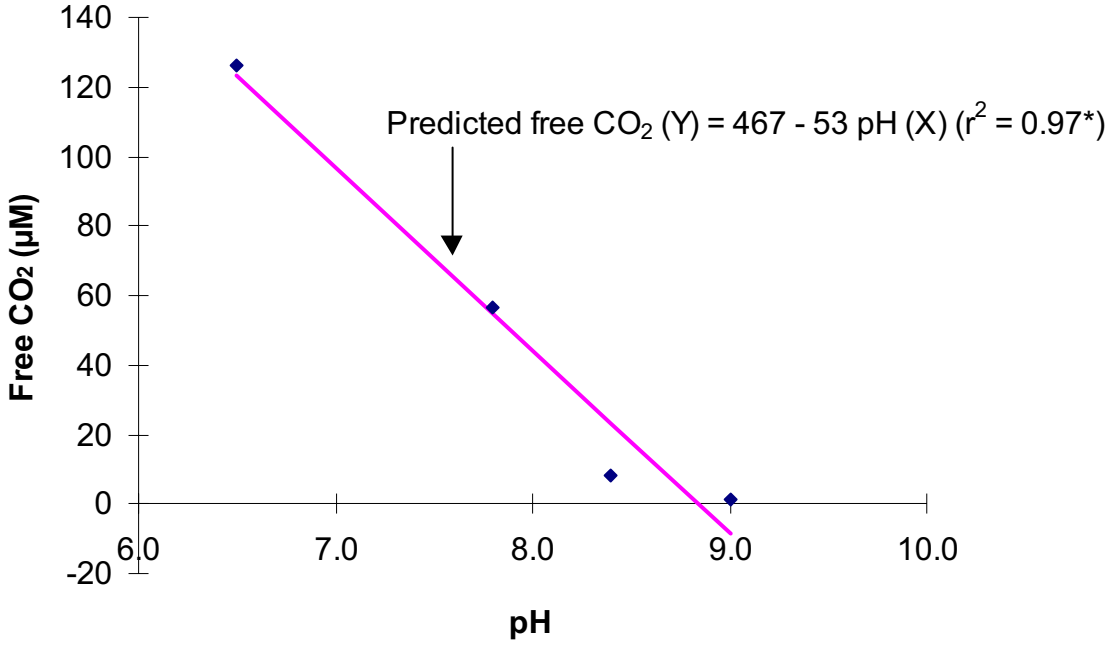

Fig. 4.18 The relationship of free CO_2 (◆) to pH and predicted free CO_2 (—) to pH for the three runs. The fitted line is based on linear regression.

4.3.8 Plant and Water Nutrient Levels

Only plants in run one were measured for plant nutrients, which limits any statistical analysis of the data. The nutrient content of the water above the sediment was based on two runs, which allowed statistical analysis (Tables 4.4 and 4.5, Appendix 3, Figs A3.1 to A3.7).

There were some differences in plant and water nutrient levels between pH treatments. For leaves the highest percentage nitrogen, potassium, calcium, boron, copper and zinc occur in the pH 8.4/9.0 treatments and the lowest in pH 6.5/7.8 treatments (Tables 4.4 and 4.5, Appendix 3 Figs A3.1 to A3.7). Potassium in the foliage was much lower in pH 6.5 compared to the other treatments (Table 4.4, Appendix 3 Fig A3.2). Potassium and boron were also much higher in the leaves compared to tubers and roots in pH 8.4/9.0.

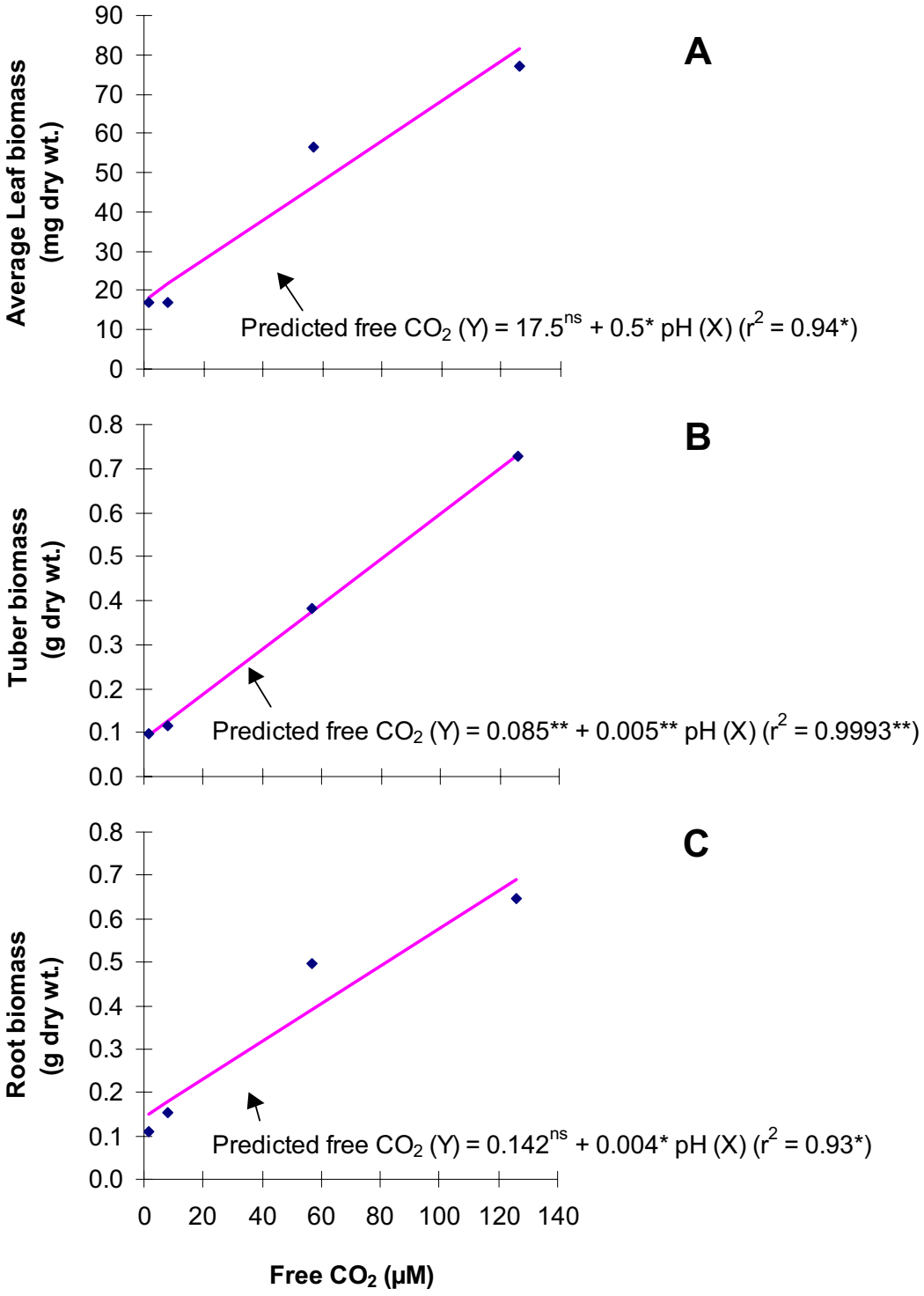

Fig. 4.19 The relationship of (A) average leaf, (B) tuber and (C) root biomass (mg or g dry weight) to free CO_2 (µM) for three runs. The symbols (◆) indicate the biomass points and the line (—) the predicted biomass. The fitted line is based on linear regression. The intercept and slope for tuber are highly significant (**)($P < 0.01$). The slope for leaf and root are significant (*)($P < 0.05$). The intercepts for average leaf and root are not significant (ns)($P > 0.05$).

Sodium was an exception to the above trends in that the percentage in foliage is much higher in pH 6.5/7.8 treatments than in pH 8.4/9.0 treatments (Table 4.4, Appendix 3 Fig A3.4). It was also much higher in foliage compared to roots and tubers particularly in the pH 6.5/7.8 treatments. Like boron, these sodium levels appear to have no clear relationship to the levels in the water in the different pH treatments. Magnesium, iron and manganese were also marginally higher in leaves in the 6.5 treatment compared to the other treatments (Table 4.4, Appendix 3 Figs A3.3, A3.5 and A3.6). This may reflect a pH effect on nutrient availability, as these nutrients are known to be more available at lower pH. With the possible exception of magnesium, this was not the case in tubers and roots where levels were similar between treatments. Iron levels in the water were also much higher in the pH 6.5 treatment. Conversely magnesium levels in pH 6.5 were no different to the levels in pH 8.4 while manganese levels were similar across all treatments ($P > 0.05$).

For tubers, pH 8.4/9.0 treatments generally had a higher level of all the nutrients tested compared to the pH 6.5/7.8 treatments. Nutrient levels in roots were more varied between treatments. Nitrogen, sulphur, sodium, manganese, iron, copper and boron levels were similar in all treatments. Calcium and zinc in roots tended to increase with pH with the highest levels in pH 9.0 (Table 4.4 and 4.5, Appendix 3, Figs A3.3 and A3.7). In comparison, percentage phosphorus and potassium in roots have tended to increase with decreasing pH with the highest levels in pH 6.5 (Appendix 3 Fig A3.2). Similarly, magnesium was higher in pH 6.5/7.8 than in pH 8.4/9.0 (Appendix 3 Fig A3.3). Iron in the roots was substantially higher than in the foliage and tubers over all treatments (Appendix 3 Fig A3.5).

The nutrients were variable in the water surrounding the plants. However, phosphorus, boron, manganese, copper and zinc were not significantly different between treatments ($P > 0.05$). The concentrations of these nutrients were higher in all four treatments compared to control tanks without plants and to tanks with pots but without plants. The pots without plants were filled with the same sediment as in the pH treatments but fertiliser was not added.

There was a general reduction in the level of potassium with decreasing pH, but there was not a significant difference between pH treatments 6.5 and 7.8 pH (Appendix 3 Fig A3.2). The levels of potassium in the water in the pH 6.5 and 7.8 treatments were lower than the levels in

the control tanks with no plants. This suggests the water in the lower pH treatments was depleted of potassium due to foliar or root uptake. There is a weak linear correlation between foliar potassium content and water content ($r = -0.92$, $r^2 = 0.84$, $n = 4$). The lack of strong correlation was most likely due to potassium uptake via the roots from the fertiliser supplied in the sediment. It is possible that foliar uptake was supplementary to root uptake. There was possibly a negative linear correlation between potassium levels in the water and foliage dry weight ($r = -0.95$, $r^2 = 0.90$, $P = 0.051$, $n = 4$)(based on logged data). In comparison, the boron levels in the leaves appear to have no relationship with the levels in the water.

Calcium levels in water were highest at pH 6.5 and 8.4 treatments and lowest at pH 9.0. There was a similar pattern with magnesium although there is no significant linear relationship between them. There was also no relationship between calcium, magnesium in the water and plant levels of these nutrients. The level of calcium at pH 9.0 was lower than in the reference tanks without plants and the reference tanks containing pots with sediment but no plants. Like potassium, this suggests depletion of calcium. There was a significant negative linear correlation between calcium levels in water and sodium levels ($r = -0.97$, $r^2 = 0.95$, $n = 4$) or total dissolved inorganic carbon (DIC) ($r = -0.98$, $r^2 = 0.96$, $n = 4$).

Levels of sulphur in the water are significantly lower in the pH 6.5 and 7.8 treatments compared to the pH 8.4 and 9.0 treatments. This is not strongly reflected in plant sulphur levels except for tubers, which appear significantly lower in pH 6.5 and 7.8 treatments. Foliage levels may also be marginally lower in the pH 6.5 and 7.8 treatments. The sulphur levels in water appeared depleted in the pH 6.5 and 7.8 treatments, as sulphur was higher in the control tanks without plants or control tanks containing pots with sediment without plants. This may be due to plant uptake as there was a near significant negative linear correlation between the levels of sulphur in the water and foliage (and total plant) dry weight ($r = -0.94$, $r^2 = 0.88$, $P = 0.056$, $n = 4$).

Sodium levels in the water were significantly lower in the pH 6.5 and 8.4 treatments compared to the pH 7.8 and 9.0 treatments. This pattern was similar to boron, DIC and EC levels. There was a significant linear relationship between sodium and EC ($r = 0.955$, $r^2 = 0.91$, $n = 4$) (Fig 4.20). The pH controller tended to oscillate between additions of sodium hydroxide and acetic acid to maintain the set pH in the water of pH 7.8 treatment tanks. Additions to the pH 9.0 treatment tanks were almost entirely sodium hydroxide and to pH 6.5 treatment tanks were almost entirely acetic acid. This means that pH 7.8 and 9.0 treatments

Table 4.4

Effects of pH on the primary and secondary nutrients contained in the dry biomass of *A. elongatus* in run one and in water in runs 1 and 2.
Plant nutrient data is mean ± standard deviation (SD) (n = 2). Water data is an average of two runs from ANOVA and LSD comparisons. Means in columns with a common letter are not significantly different at 5% level. Additional data is provided from the start of run one before pH treatments were started (run 1) (n =1), and from two reference tanks. One reference tank contained pots filled with sediment but with no plants (run 1)(n =1) and means ± SD and other reference tanks contained water without pots or plants (run 1 and 2) (n = 2). All additional data is from water with ambient pH. Nitrogen was not measured in water.

	Treatment	**N**	**P**	**K**	**Ca**	**Mg**	**S**	**Na**
	pH	**g/100g**	**g/100g**	**g/100g**	**g/100g**	**g/100g**	**g/100g**	**g/100g**
Foliage	6.5	2.91 ± 0.02	0.33 ± 0.01	2.38 ± 0.15	0.73 ± 0.01	0.34 ± 0.02	0.30 ± 0.02	2.00 ± 0.15
	7.8	3.42 ± 0.07	0.35 ± 0.05	4.15 ± 0.58	0.82 ± 0.03	0.27 ± 0.01	0.32 ± 0.04	2.44 ± 0.89
	8.4 (Ambient)	4.41 ± 0.57	0.41 ± 0.04	4.99 ± 0.16	0.97 ± 0.05	0.27 ± 0.01	0.37 ± 0.04	0.87 ± 0.05
	9.0	4.33 ± 0.04	0.43 ± 0.06	4.69 ± 0.01	1.05 ± 0.05	0.28 ± 0.00	0.39 ± 0.00	1.29 ± 0.03
Tuber	6.5	0.36 ± 0.16	0.14 ± 0.01	0.59 ± 0.06	0.18 ± 0.09	0.07 ± 0.01	0.12 ± 0.00	0.10 ± 0.03
	7.8	0.34 ± 0.01	0.19 ± 0.03	0.94 ± 0.27	0.39 ± 0.20	0.12 ± 0.04	0.19 ± 0.05	0.17 ± 0.10
	8.4 (Ambient)	2.24 ± 1.86	0.52 ± 0.10	1.48 ± 0.04	1.07 ± 0.13	0.18 ± 0.00	0.37 ± 0.02	0.38 ± 0.07
	9.0	2.94 ± 0.08	0.46 ± 0.17	1.09 ± 0.16	0.77 ± 0.16	0.16 ± 0.01	0.39 ± 0.05	0.39 ± 0.02
Roots	6.5	2.34 ± 0.89	0.20 ± 0.02	3.67 ± 0.50	0.70 ± 0.02	0.21 ± 0.01	0.51 ± 0.19	0.43 ± 0.10
	7.8	2.57 ± 0.15	0.20 ± 0.01	3.50 ± 0.73	0.91 ± 0.11	0.22 ± 0.04	0.43 ± 0.02	0.51 ± 0.02
	8.4 (Ambient)	2.79 ± 0.01	0.16 ± 0.01	1.29 ± 0.17	1.40 ± 0.04	0.16 ± 0.02	0.38 ± 0.01	0.45 ± 0.05
	9.0	2.57 ± 0.39	0.17 ± 0.01	0.91 ± 0.20	1.69 ± 0.03	0.13 ± 0.00	2.37 ± 0.01	0.43 ± 0.01

		N	**P**	**K**	**Ca**	**Mg**	**S**	**Na**
		mg/L	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**	**mg/L**
Water	Start of run 1	-	0.010	2.63	16.5	6.4	4.3	17.4
	6.5	-	0.016 a	0.44 a	22.0 a	8.0 b	1.9 a	21.0 a
	7.8	-	0.018 a	0.60 a	16.5 b	6.7 a	2.0 a	66.8 b
	8.4 (Ambient)	-	0.015 a	1.47 b	20.2 ab	8.8 b	3.8 b	37.8 a
	9.0	-	0.014 a	3.27 c	9.9 c	6.8 ab	3.3 b	90.4 b
	Pots only	-	0.005	2.30	16.3	6.0	2.7	23.9
	no pots	-	0.005 ± 0.007	4.40 ± 0.57	22.9 ± 4.7	9.1 ± 1.8	5.0 ± 0.9	29.4 ± 5.9

Table 4.5

Effects of pH on the micronutrients contained in *A. elongatus* dry biomass in run one and in water in runs 1 and 2.

Plant micronutrient data is mean ± standard deviation (n = 2). Water micronutrient data is an average of two runs from ANOVA and LSD comparisons. There was no significantly different between treatments at 5% level. Additional data is provided from the start of run one before pH treatments were started (run 1) (n =1), and from two reference tanks. One reference tank contained pots filled with sediment but with no plants (run 1)(n =1) and other reference tanks contained water without pots or plants (run 1 and 2) (n = 2) (means ± SD). All additional data is from water with ambient pH.

	Treatment pH	Fe mg/kg	B mg/Kg	Mn mg/Kg	Cu mg/Kg	Zn mg/Kg
Foliage	6.5	3100 ± 100	15.5 ± 21.9	174.8 ± 46.1	43.9 ± 1.7	240.9 ± 89.2
	7.8	1500 ± 800	37.7 ± 49.8	103.0 ± 2.4	63.2 ± 26.0	308.6 ± 90.7
	Ambient (8.4)	1500 ± 800	115.4 ± 33.9	81.0 ± 21.4	94.6 ± 5.3	677.0 ± 187.8
	9.0	1900 ± 200	156.2 ± 9.4	90.5 ± 6.6	86.9 ± 12.3	907.1 ± 0.0
Tuber	6.5	1000 ± 200	0.0 ± 0.0	114.8 ± 11.2	18.6 ± 1.7	220.9 ± 137.1
	7.8	3100 ± 2600	0.3 ± 0.4	270.0 ± 204.2	37.0 ± 1.6	425.3 ± 215.0
	Ambient (8.4)	4300 ± 0	31.7 ± 43.7	482.1 ± 146.0	113.7 ± 22.4	1460.6 ± 225.3
	9.0	5500 ± 1400	35.5 ± 17.5	592.4 ± 164.4	88.4 ± 13.0	957.4 ± 466.5
Roots	6.5	14600 ± 3500	8.4 ± 9.7	347.0 ± 15.3	130.0 ± 10.1	326.3 ± 108.5
	7.8	12200 ± 4600	18.9 ± 25.2	344.0 ± 53.7	129.0 ± 38.1	626.3 ± 240.0
	Ambient (8.4)	11100 ± 900	34.2 ± 30.2	342.6 ± 25.2	183.3 ± 6.0	1520.7 ± 234.4
	9.0	22500 ± 2300	38.6 ± 24.7	366.2 ± 52.0.	127.2 ± 37.8	2167.2 ± 135.9

		Fe mg/L	B mg/L	Mn mg/L	Cu mg/L	Zn mg/L
Water	Start	0.002	0.022	0.004	0.013	0.001
	6.5	0.242 ± 0.264	0.053 ± 0.014	0.083 ± 0.025	0.014 ± 0.002	0.017 ± 0.003
	7.8	0.002 ± 0.000	0.079 ± 0.014	0.001 ± 0.025	0.017 ± 0.002	0.017 ± 0.003
	Ambient (8.4)	0.002 ± 0.000	0.045 ± 0.014	0.004 ± 0.025	0.018 ± 0.002	0.016 ± 0.003
	9.0	0.002 ± 0.000	0.093 ± 0.014	0.017 ± 0.025	0.020 ± 0.002	0.019 ± 0.003
	Pots only	0.002	0.030	0.003	0.015	0.001
	No pots	0.002 ± 0.000	0.043 ± 0.010	0.0031 ± 0.0001	0.018 ± 0.004	0.002 ± 0.000

tended to accumulate sodium ions. There was also a significant linear relationship between sodium water levels and total DIC ($r = 0.91$, $r^2 = 0.83$, $n = 5$) and boron water levels ($r = 0.93$, $r^2 = 0.87$, $n = 5$).

Iron levels in the water were generally very low in runs one and two, except in pH 6.5 treatment. In run one, only one of the two pH 6.5 treatment tanks had high iron levels. In run two, both pH 6.5 treatment tanks had high iron levels in the water and the mean iron level was 34 times higher than the mean iron content in run one.

pH

The pH controller consistently maintained the set pH levels, 6.5, 7.8 and 9.0 throughout all three runs, though some initial set up adjustments at the beginning of each run and the occasional calibration adjustment was required (See Appendix 2, Fig A2.3). The ambient pH, not regulated by the controller, varied more between the three runs. Before plants were placed in the tanks for each run, the mean pH of the water was 7.8 ± 0.3 (± SD). After plants were placed in the tanks, the pH in the ambient tanks steadily rose, peaking as high as pH 8.9 in the third run.

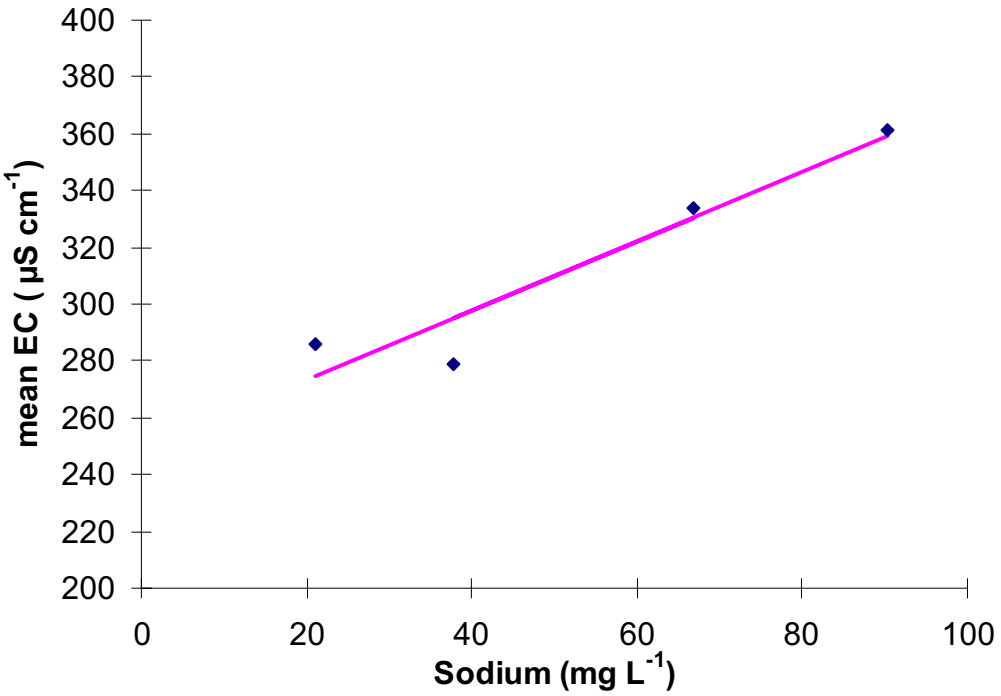

Fig. 4.20 The relationship of average EC (micro Siemens per centimetre) to sodium (milligrams per litre) for three runs. The symbols (◆) indicate the mean EC points and the line (—) the predicted EC. A line fit plot based on linear regression.

4.3.9 Electrical Conductivity

A combination of evaporation and the regular topping up of water resulted in a small increase in electrical conductivity (EC) for the pH 6.5 and ambient treatments (control), and a reference tank (without plants in each run) from a mean starting level of 262.3 ± 2.7µS cm^{-1} (Fig. 4.21).

The maximum EC in all treatments depended largely on the duration of each run. The pH 7.8 and 9.0 treatments gained much higher EC largely due to the regular additions of sodium hydroxide by the pH controller. Approximately 6 times as much sodium hydroxide was applied to the pH 9.0 tanks compared to the pH 7.8 tanks. Maximum levels of EC in run three were more consistent between treatments due partly to the shorter duration of the run and to more frequent partial water changes. The average EC levels of all three runs show a similar trend to the maximum levels in each run.

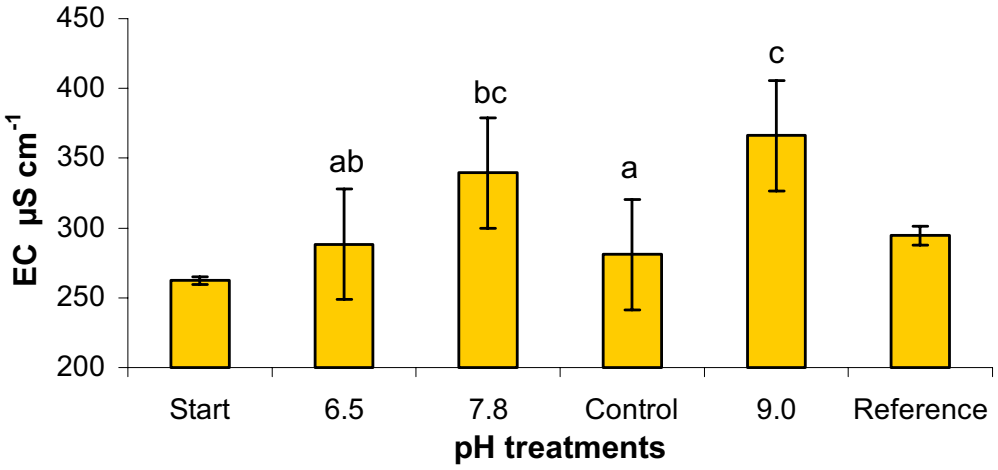

Fig. 4.21 The effects of pH on salinity (average electrical conductivity) (EC µS cm^{-1}) over three runs. Treatments with a common letter (alphabet notation above bars) are not significantly different at 5% level. Error bars above the four treatments are 95% confidence intervals. A reference tank without plants in each run and the average EC at the start of each run, although not part of the original analysis, has been included (means ± Std error)(start; n = 24, reference; n = 39).

4.4 Discussion

An exceptional experimental system open to the atmosphere was used for this study. This long-term growth study, using a unique experimental system to control pH (See appendix 2), has identified major effects of pH on levels of DIC, and plant growth, morphology and composition. There was also a major shift in plant $\delta^{13}C$ composition and changes in plant nutrient balances.

4.4.1 DIC uptake

4.4.1.1 Effect of pH on CO_2 concentration

Most experiments on the effect of pH on aquatic plants and the supply of free dissolved CO_2, have been in systems closed to the atmosphere (Nielsen 1960; Van, et al. 1976; Browse 1979; Beer and Wetzel 1981; Titus and Stone 1982; Beer and Eshel 1983; Sand-Jensen 1983; Sand-Jensen and Gordon 1984; Miller 1985; Elzenga and Prins 1988; Smits, De Lyon et al. 1988; Nielsen and Sand-Jensen 1989; Jahnke, et al. 1991; Bodner 1994). Open system experiments have been avoided because it was believed that at air equilibrium, free dissolved CO_2 will remain the same irrespective of pH and total DIC (Stumm and Morgan 1970; Beer and Eshel 1983; Sand-Jensen 1983; Titus, et al. 1990). Given that the atmospheric CO_2 concentration is 0.035%, the corresponding aqueous phase free CO_2 at air equilibrium (25°C) is approx 10µM (Stumm and Morgan 1970; Bowes and Salvucci 1989). However, in this experiment continuous regulation of pH resulted in changes in free dissolved CO_2 in the water. Thus the dissolved free CO_2, values in this study do not reflect this theory of an equilibrium with the air. At pH 6.5 the dissolved free CO_2 was approximately 13 times the theoretical value while pH 7.8 was 6 times. The control treatment with the ambient pH of 8.4 was the closest with 0.82, while pH 9.0 was equal to or less than 0.16 times the theoretical value.

There was approximately 5 times as much dissolved CO_2 in the control treatment (pH 8.4) compared to pH 9.0, however, the difference was non-significant (based on ANOVA and LSD comparisons of means) due to high levels of variance. Since the technique used to measure dissolved free CO_2 was not sensitive enough to measure values below 4.5 µM, all the measurements of dissolved CO_2 from the pH 9.0 treatment that were less than 4.5 µM were given zero values. Zero values were also given to three out of five measurements of the ambient pH treatments. It was difficult therefore to show that there was a difference in dissolved free CO_2 levels between the control treatment (pH 8.4) and pH 9.0. In any case the

leaf morphometric and plant biomass results with the exception of roots, are consistent with there being no difference in dissolved free CO_2 levels. The roots had greater biomass in the control treatment compared to pH 9.0. Whether this can be associated with a difference in dissolved CO_2 or another pH effect is not clear.

Nevertheless, the plants in the control treatment may have had access to more dissolved free CO_2 than is possible in the fixed pH 9.0 tanks. The actual pH in the control treatments fluctuated by 0.2 (SD) over time around the mean of pH 8.4. This is based on the assumption that dissolved free CO_2 was essentially zero above pH 8.3 (Frith, et al. 1993) and that pH would be below 8.3 some of the time in the control tank. This is reflected in run 2 where the plants in the control had accumulated 23% more biomass than the plants in pH 9.0. The duration of this run was 22% longer than the other two runs, which may give enough time for the difference to become more evident.

It is worth noting that the reference tanks (with out plants) had a average pH of 8.05 and a dissolved free CO_2 concentration more than 3 times the theoretical air equilibrium value given by Bowes (1989). The CO_2 levels in the water appear to have more resemblance to natural water bodies than to Stumm's (1970) pure water equilibrium with atmospheric CO_2 model.

4.4.1.2 CO_2 rather than HCO_3^- is the major DIC source

In this study, plant growth closely paralleled the concentration of dissolved free CO_2. There is no correlation between plant growth and the level of total DIC but there is almost perfect correlation between tuber dry weight and dissolved free CO_2. The carbon content of the tubers is largely stored carbohydrate. However, as the data used was gross tuber dry biomass, the net growth in dry biomass (value unknown) (gross dry weight less dry weight at start) is likely to have a value of less than or equal to zero at the zero CO_2 level (intercept). This could mean that that A. *elongatus* is dependent on dissolved free CO_2. However, as there is less certainty about the dissolved free CO_2 concentration above approximately pH 8.2, A. *elongatus* may still be able to use bicarbonate when there is a deficiency of dissolved CO_2.

Eighty percent of aquatic macrophytes supplement free dissolved CO_2 with bicarbonate (Spence and Maberly 1985) however, they are more readily able to assimilate dissolved CO_2. Photosynthetic rates have been shown to be greatly increased by lowering pH or directly

increasing free dissolved CO_2 (Nielsen 1960; Sand-Jensen 1983; Nielsen and Sand-Jensen 1989). Browse (1979) showed that total resistance to bicarbonate uptake is 8 to 12 times' that of free dissolved CO_2 for *Egeria densa*. Similarly marine seagrass species that are known to use bicarbonate still have six times greater photosynthetic rates when free dissolved CO_2 levels in seawater are increased (Beer 1998). For the plants in this study, bicarbonate uptake in pH of 8.4 or greater may have partially compensated for the deficiency of free dissolved CO_2. However, the higher uptake resistance of bicarbonate may still have result in reduced plant growth compared to plants growing with abundant free dissolved CO_2.

Plants in pH 6.5 (run 3) in this study had approximately five times greater biomass and gained 18 times more carbon than plants in pH 9.0 where they had greater than 80 times more dissolved free CO_2. The decrease in dissolved free CO_2 with high pH did not give a proportional decrease in growth which suggests that the decline in free CO_2 from pH 6.2 to 9.0 was partly overcome by bicarbonate utilisation (Titus and Stone 1982). Conversely it could be equally argued that the reduction in growth in pH 9.0 is proportional to the reduction in availability of dissolved free CO_2 and that the increase in growth at low pH was not proportional to the increased availability of dissolved free CO_2. *A. elongatus* may be able to obtain enough dissolved free CO_2 at pH 9 to provide the growth found at this pH, without using bicarbonate, despite very low dissolved free CO_2 levels. Sand-Jensen (1987) suggests that any CO_2 assimilated by the plant is soon replenished by more CO_2 from bicarbonate due to the equilibrium between dissolved free CO_2 and bicarbonate ions in solution.

It is difficult to know how low the dissolved free CO_2 levels were in the pH 9 (or pH 8.4) because they were below measurable levels. Chloroplasts in *A. elongatus* leaf epidermis cells have direct contact (via diffusion boundary resistance layer) with the external DIC pool as well as to the internal recycled CO_2 pool in the lacunae (Appendix 1). Even in well-stirred conditions, boundary layer resistance to DIC uptake is very high (Bowes and Salvucci 1989; Larkum, et al. 1989). If the movement of CO_2 into a plant is by simple diffusion (Bowes and Salvucci 1989; Kirk 1994) then the photosynthetic epidermal cells will have to be strong sinks for the low dissolved free CO_2 found in pH of 9.0 to enter the cells (Raven, et al. 1985). A facilitated diffusion process eg carbonic anhydrase or an active DIC uptake process involving bicarbonate (Larkum, et al. 1989) seems more likely.

4.4.1.3 Threshold effect

Most of the leaf morphology, plant biomass, growth rate, turnover time, and specific leaf biomass data differed abruptly between pH 7.8 and 8.4, indicating a threshold effect in plant response (Fig 4.22). Dissolved CO_2 is the most likely environmental factor (other than pH) changing in concentration from 2.5 mg L^{-1} at pH 7.8 to < 0.2 mg L^{-1} above pH 8.4. However the presence of acetic acid in the two lower pH treatments(pH 6.5, 7.8) and the absence of acetic acid in the control and high pH treatments (pH 8.4, 9.0) may also be a factor. It is possible that microbial action broke down the acetic acid in the two lowest pH treatments 6.5 and 7.8, with respired CO_2 adding to the dissolved free CO_2 (Hite and Cheng 1996). As the application of acetic acid was a continual process, this could have maintained elevated free CO_2 levels. Approximately three times as much acetic acid was applied to the pH 6.5 treatments compared to the pH 7.8 treatments, which may explain the difference in CO_2 levels between these two treatments. The observed bloom of microorganisms in the pH 6.5 and 7.8 treatments towards the end of each run adds further weight to this possibility.

Acetic acid can also react with carbonate minerals to form bicarbonate ions, ie $CH_3COOH + Ca\ CO_3 = Ca^{+2} + HCO_3^- + CH_3COO^-$ (Hite and Cheng 1996). However, there was no clear indication of higher Ca levels in the water of pH 6.5 and 7.8 compared to the control treatments where no acetic acid was added. Organic acids such acetic acid can also react with ferric iron compounds to produce more soluble ferrous ions and bicarbonate in acidic anaerobic (reduced) conditions eg $CH_3COOH + Fe(OH)_3 + 14H^+ = 8Fe^{+2} + 2HCO3^- + 20H_2O$ (Hite and Cheng 1996). This may provide an explanation for the high iron levels in the water in the pH 6.5 treatments.

Acetic acid may have had a direct beneficial effect on growth, however, this seems less likely given there is no evidence to show that acetic acid has a regulatory effect on plant growth. Nevertheless, the possibility that acetic acid contributed to the level of dissolved CO_2 in the two lower pH treatments cannot be ignored.

4.4.2 Growth and Morphological effects by pH

Plants in the lower pH treatments not only were substantially larger in leaf, tuber and root biomass but the shape of the leaves were also different compared to the higher pH treatments. Increased nutrient supply is unlikely to be the causal factor since all treatments received the

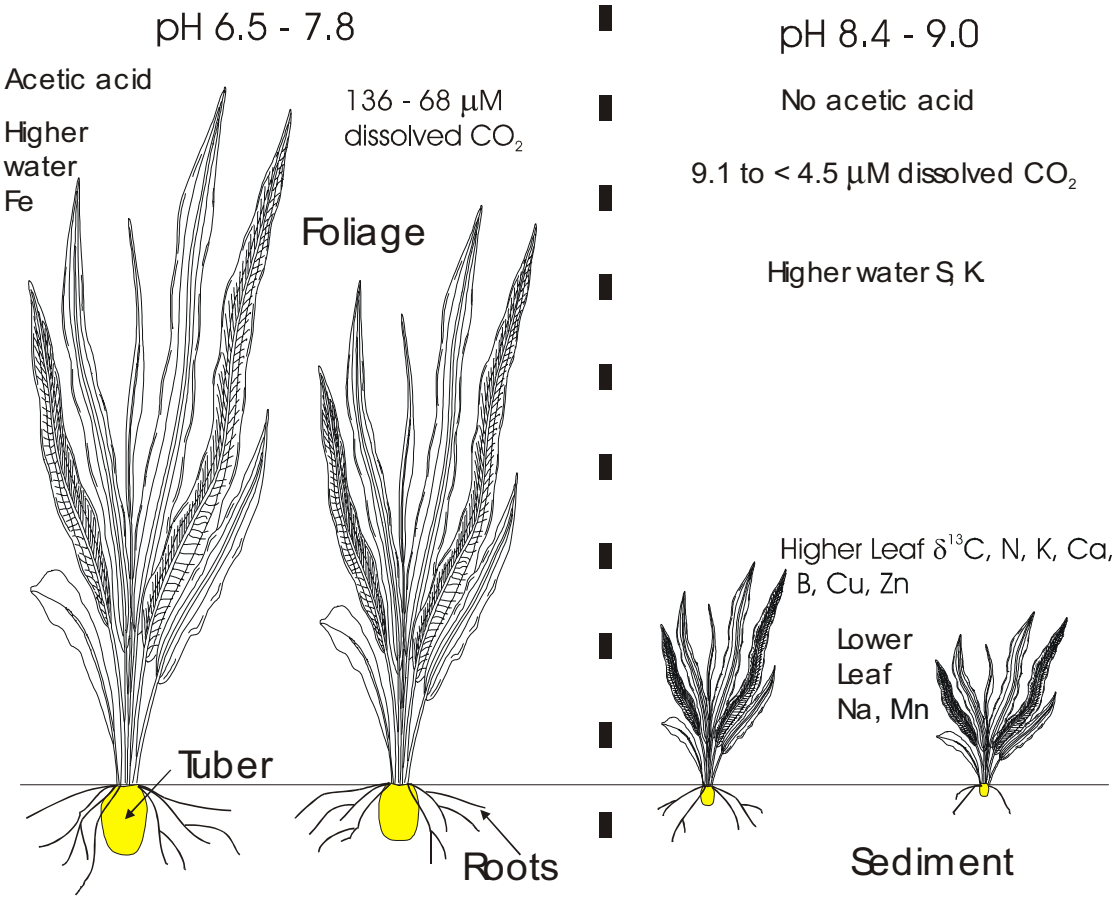

Fig. 4.22 Drawings of *A. elongatus* plants illustrating the threshold effect of pH 6.5 – 7.8 treatments verses pH 8.4 – 9.0 treatments. Drawing is scaled (± 5%) to tuber and root dry weight, leaf number and average leaf areas.

same level of fertiliser and there was no clear pattern of increased nutrient concentrations in the water. Similarly, the response to stirred water verses unstirred water, particularly tuber growth, was independent of the levels of minerals supplied (Chapter 3)

For *A. elongatus,* it appears that adequate nutrition is important to ensure maximum leaf number but ultimate leaf size and shape is also influenced by dissolved free CO_2 level. The irradiance level and nutrition are widely recognised as having a major influence on leaf shape eg (Kirk 1994; Spence 1995; Hellquist and Jacobs 1998). Rattray (1991) has demonstrated that DIC is more important than nutrients on growth rate, however, there is no recognition of the effect of DIC on leaf size and shape.

4.4.3 Chemical composition

4.4.3.1 Macronutrients and Micronutrients

Nutrient levels in plants and water were measured to see if there were any direct effects of pH on nutrient availability. It was expected that the availability of nitrogen, calcium, magnesium, iron, manganese, boron, copper and zinc would be greatly reduced at pH 9.0 based on the effect of pH on the nutrient availability in most terrestrial soils and growing media (Handreck and Black 1994). Aquatic sediments are similar in that low pH coupled with mild redox increases the availability of microelements ie, Al, Cu, Fe, Mn and Zn (Gambrell and Patrick 1988; Jackson, et al. 1993). However, a number of nutrients (N, P, K, Ca, Na, B, Cu, Zn) tend to be higher in plant tissues in the pH 8.4/9.0 treatments in comparison to the pH 6.5 and 7.8 treatments. The comparatively large amount of fertiliser present may have offset any pH effect on nutrient availability (Handreck and Black 1994). In addition, pH effects on the sediment would be minimal as sand generally has little cation exchange ability.

Since these are the only known data on the plant nutrients in *A. elongatus*, establishing whether the nutrient levels are typical for the species is difficult. Based on leaf nutrient analysis of *Aponogeton natans* and many cultivated terrestrial plants, most plant nutrient levels across all treatments in run one are well above typical deficiency levels (Dorofaeff 1974; Shardendu and Ambasht 1991; Reuter and Robinson 1997). This supports the suggestion from Chapter 3 that the fertiliser level of one gram per plant was more than adequate to maintain growth for the duration of each run.

It is likely that growth was limited in the high pH levels due to low levels of dissolved CO_2. Growth dilution may explain the lower nutrient levels in the lower pH treatments (Titus, et al. 1990). Some micronutrients could be approaching toxic levels although it is apparently normal for higher levels to occur in aquatic plants compared to terrestrial plants, as micronutrients are more available in anoxic sediments (Barko, et al. 1986). In general, plants are also capable of absorbing nutrients in excess of immediate requirements and holding these nutrients in various sites within the plant (Reuter and Robinson 1997). These nutrients are remobilised only when required.

Iron levels in the leaves in all treatments (0.146 to 0.314 g $100g^{-1}$) may not be exceptionally high compared to the range of leaf levels found in other aquatic macrophytes. Natural iron levels in the leaves of other Aponogeton species are not known but leaf levels ranging from 0.0265 to 0.320 g $100g^{-1}$ have been found in *Vallisneria americana*, *Hydrilla verticillata* and *Nymphoides peltata* (Titus, et al. 1990; Stewart 1991; Smolders, et al. 1994). Smolders (1994) describes iron deficiency symptoms with leaf levels of 0.0118 g $100g^{-1}$ in *Nymphoides peltata*. Iron levels may vary widely in freshwater aquatic macrophytes. The iron levels are much higher in the roots but this could be due to deposits of insoluble ferric iron (iron oxide sheath) on the outer surfaces of roots (rhizosphere) as a result of oxidation of soluble ferrous iron (Sheppard and Evenden 1991; Wigand and Stevenson 1997). The formation of this iron oxide sheath is said to restrict phosphorus uptake due to iron phosphate precipitation (Jones 1975; Barko, et al. 1986; Wigand and Stevenson 1997). However, there is no clear evidence of high phosphate levels on the roots that can be related to iron phosphate precipitation. Natural levels of phosphorus range from approx. 0.08 to 0.12 % in the leaves of *A. natans* (Shardendu and Ambasht 1991). The plant levels of phosphate appear more than adequate in this experiment, which confirms the speculation in chapter 3 that the fertiliser supplied a level of phosphorus sufficient to offset any restriction due to high iron levels.

Sodium and calcium cations may play a role in balancing the uptake of bicarbonate, as they are usually abundant in leaves of submerged aquatic macrophytes and (Prins and Helder 1985; Barko, et al. 1986). In this experiment, both calcium and sodium were much higher in leaves compared to the surrounding water in all treatments. The highest calcium (1.4 times the level in pH 6.0) occurs in leaves in the pH 9.0 treatment with the lowest calcium level in the water. This suggests that the plants in pH 9.0 were taking up calcium to facilitate the uptake of bicarbonate. However, pH 9.0 also had the second to lowest sodium in leaves (0.6 times the level in pH 6.0) and the highest sodium level in the water (4.3 times the level in pH 6.0). It is difficult to relate this directly to bicarbonate uptake but this plant may have an in-built mechanism that limits sodium uptake in more saline waters. It is also possible that sodium is excreted from leaves to facilitate the uptake of other more essential cations, ie potassium or calcium (Sculthorpe 1967). The depletion of calcium in the water at pH 9.0 may also be due to a reaction with carbonate resulting in precipitation and deposition of calcium carbonate (limestone) on the glass sides of the tanks (Beer, et al. 1977). These deposits are most noticeable on the sides of pH 9.0 tanks making cleaning difficult. Higher

levels of carbonate are normally present at pH 9 (Stumm and Morgan 1970; Beer, et al. 1977; Beer and Eshel 1983).

4.4.3.2 Delta carbon

There are two stable carbon isotopes ^{12}C and ^{13}C, of which ^{12}C is 100 times more common than ^{13}C (Raven, et al. 1987). In brief, photosynthesis discriminates against the heaver isotope and delta ^{13}C ($\delta^{13}C$) is a measure of this discrimination. The more positive the $\delta^{13}C$ value the less photosynthetic discrimination against ^{13}C. The $\delta^{13}C$ values in this experiment suggest bicarbonate use, as there is less plant discrimination against ^{13}C with increasing pH. Smith and Walker (1980) suggest the closer the plant $\delta^{13}C$ is to the $\delta^{13}C$ of bicarbonate in the water (eg 0 ‰) the more the plant is dependent on bicarbonate as a source of carbon for photosynthesis. Alternatively, the decreasing plant discrimination with increasing pH may be the result of the corresponding depletion in CO_2 and internal carbon recycling (Abel 1984).

4.4.4 Photosynthesis

Photosynthetic activity as measured by PAM fluorometer (quantum yield and ETR) was similar across all treatments. Quantum yield measurements of dark-adapted leaves give a measure of photosystem II photochemical efficiency (Ralph, et al. 1998) which appears to be the same across all treatments despite the differences in pH, dissolved CO_2 and plant growth. Quantum yield measurements may not discriminate between external dissolved carbon dioxide fixation in the low pH treatments and efficient recycling of internal carbon and /or external bicarbonate use in the higher pH treatments (Hentschke 1997). The use of a carbon concentrating mechanism that uses bicarbonate either directly or indirectly is considered a more energy consuming process compared to the uptake of dissolved CO_2 (Owttrim and Colman 1989; Eighmy, et al. 1991). Photosystem II photochemical energy may be partly consumed in alternate chemical pathways to the Calvin cycle, assisting in the utilisation of bicarbonate but resulting in less photosynthetic productivity over time compared to the lower treatments (pH 6.5 and 7.8) where dissolved CO_2 is more abundant.

It is not currently known whether *A. elongatus* has the ability to internally recycle CO_2 from respiration, including photorespiration, back into photosynthesis but it is well documented in other aquatic macrophytes. Sondergaard and Sand-Jensen (1979) suggest that *Littorella uniflora* (a plant with a similar growth habit to *A. elongatus*) does not have efficient recycling because the lacunae are only in the major leaf veins. *A. elongatus* is similar to *L. uniflora* in

that the majority of the leaf laminar is only three or four cells thick, however microscopic studies have revealed that unlike *L. uniflora* there are interconnecting gas filled lacunae between the upper and lower epidermal cells that are linked to extensive lacunae in the leaf veins (Appendix 1). It appears therefore that *A. elongatus* has the apparatus to efficiently recycle internal CO_2, which would partially compensate for the lack of sufficient external dissolved free CO_2 at pH 9.0.

Alternatively, as the duration of each quantum yield and ETR measurement is a few minutes, it is possible there were some small undetectable differences between treatments that only become evident after a much longer period (weeks) as accumulated plant biomass.

4.4.5 Conclusions

The results of this experiment are consistent with the hypothesis that growth of *A. elongatus* is limited by the supply of dissolved inorganic carbon (DIC) rather than nutrients in the water. Furthermore, these results support the earlier argument (Chapter 3) that the increase in growth in stirred water was due to enhanced supply of DIC rather than increased nutrient uptake.

The effect of pH on plant nutrient availability was minimal with no obvious detrimental effects on plants in pH 9.0 other than less dissolved free CO_2. Fertiliser levels were apparently more than adequate for growth in all treatments. Growth dilution was the main reason for lower plant levels of some nutrients in the lower pH treatments (pH 6.5-7.8). The high iron levels had only a minimal effect on plant phosphate levels.

A question that arose in the previous experiment (Chapter 3) was why the pH was so high overall. One still water tank had reached pH 9.14. Was this purely the result of passive plant uptake of dissolved carbon dioxide (CO_2 aq) or were the plants utilising a DIC concentrating mechanism which elevates pH and involves the use of bicarbonate (Chapter 2).

In this study (current chapter) the growth of plants in the pH 8.4 and 9.0 treatment in run 2, the major shift in delta ^{13}C, and calcium/sodium levels in the leaves do not preclude the likelihood that a DIC concentrating mechanism is involved. Alternate chemical pathways may explain why photochemical efficiency was the same across all treatments despite

differences found in growth. However, the evidence is not strong enough to exclude the possibility that these plants were only able to use dissolved free CO_2.

Nonetheless, there was a large response in plant growth and morphology to dissolved free CO_2 particularly in the lower pH treatments, with a clear threshold effect between the low pH (6.5-7.8) treatments and high pH (8.4-9.0) (Fig 4.22). This suggests *A. elongatus* is highly dependent on dissolved free CO_2 but may have some ability to concentrate DIC when dissolved free CO_2 is low. If such ability exists, it is likely to be an energy consuming process, which results in much reduced growth compared to plants growing in moderate to high dissolved free CO_2.

The likely dependence on free dissolved CO_2 possibly reflects the plants habitat and distribution ie creeks and river systems with permanently flowing water (Chapter 3)(Sainty and Jacobs 1994; Hellquist and Jacobs 1998). Flowing waters of wetter climatic zones with extensive riparian vegetation tend to be more acidic (pH 6.0 to 7.0) (Rataj and Horeman 1977) and have inherently less bicarbonate than more alkaline waters (Stumm and Morgan 1970).

Chapter 5

General Discussion and Conclusions

5.1 The effects of stirred water, nutrition and pH

Aponogeton elongatus is a predominantly submerged aquatic macrophyte. It is widely grown as an aquarium plant but little is known about the ecophysiology of its growth and development. This thesis investigates aspects of the interaction between *A. elongatus* and its aquatic environment, particularly the acquisition of mineral nutrients and dissolved inorganic carbon (DIC).

The growth of all plants is dependent on acquisition of nutrients and CO_2. The aquatic environment differs from the terrestrial environment in that, on the one hand, the surrounding water is potentially a ready source of soluble nutrients whilst on the other hand, the water limits the supply of gaseous CO_2. The experiments reported in chapters 3 and 4 sought to determine the primary sources of nutrients and DIC for *A. elongatus*, and how their supply is affected by two environmental parameters, water flow and pH.

The growth of *A. elongatus* was greater when the water in the aquaria was stirred rather than still (Chapter 3). This was not due to enhanced supply of nutrients since the primary source of nutrients was shown to be from the substrate via the roots. Plant growth was dependent on the level of minerals provided in the substrate, and there was a significant increase in plant growth where the supply of minerals to individual plants was increased even though they shared the same water environment (Section 3.4).

An alternative explanation for the response to stirring of the water is that it increased the availability of dissolved inorganic carbon (DIC) by reducing boundary layer resistance around the leaves (Section 3.4).

This DIC can be in the form of bicarbonate or dissolved free CO_2 the balance depending on the pH of the water. When the pH of the water was regulated at set

points between 6.5 and 9.4 plant growth was greatest between 6.5 and 7.8 (Chapter 4). Given that pivotal point in the growth response to pH coincides with the theoretical transition in the equilibrium between free CO_2 and bicarbonate as pH approaches 8.2, it clearly suggests that *A. elongatus* more readily assimilates dissolved free CO_2 than bicarbonate. This conclusion is supported by the strong relationship between the level of dissolved free CO_2 and plant growth (Chapter 4).

Under the range of conditions tested, the growth of *A. elongatus* was greatest when the plants were grown in stirred water at a pH of 6.5 or 7.7, with adequate minerals supplied in the substrate. These conditions gave an approximately four-fold increase in the growth of A. elongatus over a two month period compared to the control conditions.

The clear preference for dissolved free CO_2 does not preclude the possibility that these plants may be able to concentrate DIC when dissolved free CO_2 is low (Chapter 4). There is certainly some advantage in being able to directly use bicarbonate as a source of carbon, as the concentration is generally 10 fold higher than dissolved free CO_2. A carbon concentrating mechanism may be involved as indicated by some of the data in this study (chapter 4). This includes the shift in plant delta carbon and the shifts in potassium, calcium and sodium levels in leaves and in solution with increasing pH. *A. elongatus* also appears over time to become more efficient in obtaining DIC in the ambient and pH 9.0 treatments. The longer duration of run two resulted in much greater plant growth in the ambient pH and pH 9.0 treatments compared to the other two shorter runs (runs 1 & 3) extrapolated to a similar duration (chapter 4). Perhaps this plant requires time to adjust to low dissolved free CO_2 levels. This is certainly true of another aquatic macrophyte, *Hydrilla verticillium*. This aquatic macrophyte can be induced over a period of 10 days to change from C-3 to a separate C-4 photosynthetic state in conditions of low dissolved free CO_2 concentrations (Bowes and Salvucci 1989; Reiskind, et al. 1997). A prolonged period of conditioning (approx. two to three months) in low dissolved free CO_2 levels may be required before assessing *A. elongatus* for its ability to concentrate DIC.

5.2 Recommendations

Further work is needed to establish whether *A. elongatus* is able to concentrate DIC and to determine the mechanism. This could include determining CO_2 compensation and oxygen inhibition points of leaves from plants grown with low free CO_2 conditions compared to plants with high CO_2. A comparatively low photorespiratory state with low free CO_2 would add further weight to the existence of a DIC concentrating mechanism (Reiskind, et al. 1997). Analysing leaves for the presence or absence of external and internal levels of carbonic anhydrase and for the presence of pH polarity on leaf surfaces may also give some insight as to possible DIC concentrating mechanisms.

A. elongatus, from observation, produces more floating leaves in higher light intensities, which is likely to be a response to a diminished supply of dissolved free CO_2. Further work could be done to clarify that a restriction in dissolved free CO_2 is the factor that causes the plant to produce floating leaves and whether floating leaves are more productive as in terms of tuber dry weight gain than submerged leaves (Chapter 3).

For optimum growth and cultivation, the following factors need further investigation:

(1) The effects of different water flow rates on plant growth to determine optimum water speeds.

(2) The effect of applying CO_2 to the water at a rate that keeps the pH between 6.5 to 7.8 could also be investigated. The longer-term problem of excess microorganisms apparently associated with using acetic acid could be avoided using this method (Chapter 4).

(3) Inorganic acids such as 70% sulphuric acid with 30% nitric acid could also be used to keep the pH below pH 7.8 (Titus, et al. 1990). However, there may not be the same level of dissolved free CO_2 using inorganic acids as was found in this study (chapter 4) using acetic acid. It is generally accepted that the level of dissolved free CO_2 is not effected by changes in pH in water exposed to the air (Stumm and Morgan 1970; Beer and Eshel 1983; Sand-Jensen 1983; Titus, et al. 1990). To fully test this, perhaps the second experiment (Chapter 4) is worth

repeating using inorganic acids instead of acetic acid.

(4) A closer look at plant nutrition may also be advantageous for commercial production, especially nitrogen, phosphorus and potassium (N: P: K) and perhaps calcium and magnesium. Slow release fertilisers of different N, P and K ratios could be evaluated for more optimum nutrition.

(5) Isolation of the pathogen that caused plant disease in both experiments and the development of control measures would also be useful (Appendix 4)

(6) Finally, some aspects of propagation also need further work. Tissue culture was sufficiently successful to provide most of the plant material required for the experiments described in this thesis. However as mentioned in the introduction (chapter 1), endogenous contamination was a problem and proliferation declines with repeated subculturing. This creates a demand for fresh plant material for tissue culture. If the method of tissue culture used to produce the plants for experiments 1 and 2 (Chapters 3 and 4), were adopted by commercial aquatic plant producers, without eliminating the problems, the requirement to harvest plant material from the wild would be reduced but may not be eliminated.

(7) Further work is also needed to develop techniques that enable rapid establishment of seedlings in large numbers. Seedlings could be a useful means of commercial propagation as these plants produce large amounts of seed that germinates very easily. Yet, turning these freshly germinated seedlings into large numbers of fully established young plants is more difficult. This may not be necessary, however, as even small numbers of established seedlings could prove to be the means of maintaining sufficient stock for tissue culture. Once the seedlings are established, tuber growth will be rapid using stirred water, ample nutrition and a plentiful supply of dissolved free CO_2.

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Appendix 1

Some Aspects of *Aponogeton elongatus* Anatomy

1.1 Introduction

Almost nothing is known about the anatomy of *A. elongatus*. As an aquatic macrophyte with a majority of fully submerged leaves and a few floating leaves, it should contain anatomical modifications that would demonstrate successful adaptation to a submerged aquatic environment.

Modifications common to many submerged aquatic macrophytes but not all, include schizogenous development of an air filled lacunae system that extends from the leaves to the roots often with cellular diaphragms subdividing the lacunae (Sculthorpe 1967; Larkum, et al. 1989). Lacunae commonly occur in the cortex tissues of stems and roots and in the mesophyll of leaves (Sculthorpe 1967). The main function of lacunae in aquatic macrophytes is to allow the passage of gases such as oxygen and carbon dioxide between the leaves and the roots.

Lignified cell walls are uncommon. Stomata in submerged leaves, if present, are usually non-functional and the cuticle is thin or absent (Sculthorpe 1967; Bowes 1985). Leaves are often much thinner than those of terrestrial plants and may be as little as three cells thick (Sondergaard and Sand-Jensen 1979; Hentschke 1997). Chloroplasts often occur in the epidermal cells of leaves.

The main aim of this study was to determine the anatomical features of *A. elongatus* cells and tissues that show adaptation to an aquatic environment.

1. 2 Method

A whole plant that had the sediment washed from the roots was given to a Histologist in the Botany Department, UQ for sectioning and preparation of permanent mounts of root, tuber, petiole, and leaf tissues for light microscopy.

In brief the method was:
The selected small pieces of plant tissues were first fixed in 50% FAA (formalin/acetic acid/alcohol). This was followed by dehydration with a graded series of ethanol and tertiary butyl alcohol solutions of rising concentration. After this, with a series of standard procedures, the plant tissues were infiltrated with paraffin wax in a 60°C oven for approx 24 hrs. The wax/tissue blocks were then sectioned on an A. O. Spencer 810 Rotary Microtome at a thickness of 7µm. The sections were then mounted on glass slides and stained with 1% aqueous Safranin Orange and 1% ethanol/clove oil based green stains in separate procedures before mounting a cover slip. The staining colours nuclei and lignified cell walls red, cytoplasm, and non-lignified cell walls green.

At a later time, after receiving the results of the above, some fresh leaf TS sections were prepared and cut (approx thickness 200µm) by hand using a razor blade and mounted in water for microscopic examination.

Selected sections were photographed using a microscope-mounted camera containing slide film. The developed slides where then scanned into a computer.

1.3 Results and Discussion

Microscopic examination of the permanently mounted microtome sections proved disappointing due to extensive tissue collapse which occurred during preparation. The transverse sections of leaf were the worst affected, with almost all the cellular tissue having folded up into a crumpled mess (Plate A1.1). Thicker tissues faired better as areas of leaf such as the midrib and other major leaf veins suffered less damaged than the thinner parts of the leaf laminar (Plates A1.4 & A1.5). The tissue sections of petiole and roots suffered this damage, to a varying degree, mainly in the outer cortex tissue and epidermal cells (Plates A1.6, A1.7, A1.10, A1.11, A1.12 & A1.13.). Similar to leaves, the smaller the root sections the greater the damage. The fragile nature of the thin cellulose cell walls was the likely cause of the tissue damage based on discussion with the histologist who prepared the tissues. He suggested the delicate nature of the cell walls, coupled with the effects of alcohol and molten wax, resulted in irreversible tissue collapse.

Although the fresh samples of leaf tissues were difficult to cut very thinly, microscopic examination, of the tissue, revealed considerable detail about the cellular structure of the leaf laminar (Fig A1.1, Plates A1.2, & A1.3). The cells in the leaf laminar have very thin cell walls, which possibly explains why obtaining sufficiently thin sections of intact cells was difficult. Chloroplasts are present in both the mesophile and epidermal cells. The black zones in the middle of the leaf laminar are air bubbles, which are trapped within lacunae. The lacunae are between sparsely packed mesophile parenchyma cells in the middle of the leaf laminar sandwiched between the upper and lower epidermis cells and one possibly two layers of more tightly packed mesophile cells (Fig A1.1 and plate A1.3). A continuous air filled lacuna apparently exists due to the gaps within the layer of sparsely packed mesophile parenchyma cells, based on microscopic examination.

The number of layers of cells that make up a transverse section of an A. elongatus leaf depends on the position on a leaf from which the section was taken. From observation, the leaf laminar is thicker near the midrib and substantially thinner at the leaf margins. Microscopic examination of transverse sections of young immature leaf, in the tuber apex, has revealed only two cell layers, the upper and lower epidermis in the leaf margins but up to 4 or 5 layers closer to the midrib (Plate A1.9). No lacunae are present where there is only two cell layers. However, based on other aquatic macrophytes, it is possible that when the leaf matures and become photosynthetically active, the resulting O_2 gas pressure forces the epidermis layers apart resulting in the development of lacunae (Sculthorpe 1967). This occurs because oxygen diffuses slowly in water and tends to accumulate in intercellular spaces when photosynthetic oxygen output exceeds the rate of diffusion into the surrounding water (Sculthorpe 1967; Gambrell and Patrick 1988).

Transverse sections of leaf midrib show that a large percentage of the midrib cellular volume is occupied by lacunae (Plates A1.4 & A1.5). The remaining volume is taken up by at least three vascular bundles and large parenchyma cells, which surround the lacunae. The presence of lacunae in the midrib and laminar of a leaf perhaps explains why the leaves are buoyant in the water and float upwards when cut from the rest of the plant. Damaged, diseased or old leaves can sometimes develop a water soaked

appearance and loose their buoyancy possibly because lacunae in the leaf laminar have become deflated due to a reduction in photosynthetic output, perhaps allowing water to flood in.

The transverse and longitudinal sections of petiole also indicate extensive lacunae (Plates A1.6, A1.7, A1.10) which most likely extend from the leaf laminar and midrib to the tuber. It is worth noting that the cuticle if present on leaf and petiole epidermis, is very thin. No distinctive stomata structures were observed but stomata have been found on submerged leaves of another species *A. natans* from India (Paliwal 1976). These stomata were smaller and fewer in number than those on the upper surface of floating leaves of this species and were considered non-functional and a relic from a more terrestrial ancestor. A thin permeable cuticle therefore is an advantage in an aquatic environment to reduce resistance to the exchange of dissolved inorganic carbon, oxygen and nutrients between the water and the plant cells of a submerged leaf (Sculthorpe 1967).

The lucunae in the midrib and petiole form early in leaf development (Plates A1.8, A1.9). Even quiet small leaf primordia show the presence of lacunae amongst developing cortex cells and vascular bundles (Plate A1.9). The longitudinal sections of the petioles show the presence of diaphragms of small cells that subdivide the lucunae (Plate A1.10). Cellular diaphragms are thought to prevent extensive flooding of the lucunae from wounds and to provide lateral support (Sculthorpe 1967). It is known in other aquatic macrophytes that these diaphragms have pores that are thought to behave like stomata in terrestrial plants in regulating the flow of gases (Larkum, et al. 1989). Although these diaphragms could restrict the flow of oxygen or other gases, calculations on other aquatic macrophytes have shown that diaphragms contribute less than 2.5% of the total resistance to gaseous diffusion within lucunae (Sorrell and Dromgoole 1987; Larkum, et al. 1989).

The root transverse sections are badly effected by cellular collapse and distortion especially the epidermis, exodermis and outer cortex (Plates A1.11,A1.12,A1.13). The inner cortex, endodermis and the vascular cylinder were least effected in Plates A1.11 but even this is damaged in Plate 1.12. The root section depicted in Plate

A1.12 suffered more damage possibly because it was smaller. However, the arrangement of the damaged cortex tissue indicates the presence of lacunae.

The vascular cylinder of the root is dominated by two adjacent metaxylem with comparatively thick cell walls. There is some reddish stain in these cell walls, which indicates they are partially lignified (Plate A1.13). Adjacent to them is some much smaller protoxylem also with lignified cell walls and the thinner blue walled cells surrounding them are possibly phloem and associate parenchyma tissue. Around this inner core is a ring of thin walled cells which is possibly the pericycle followed by several incomplete rings of cells with moderately thick walls of which the inner most is the endodermis. The cells in the major break in the rings are likely to be passage cells (Plate A1.13).

The stem of *A. elongatus* is a swollen tuber mostly composed of parenchyma storage cells (Plates A1.14 & A1.15). Microscopic examination of transverse sections of tuber has revealed the storage cells contain numerous plastids. In each plastid, a dark red hilum like centre is visible but typical starch layering is not discernible. The contents of the plastids are very similar to sphaerocrystals of inulin found in dahlia tubers (Fahn 1969). Inulin is another polysaccharide similar to starch but composed mainly of fructose molecules (Abercrombie, et al. 1961; Tootill 1984).

If the thin cell walls had been less fragile, more information could have been obtained about the anatomy of *A. elongatus*, especially the shape and structure of leaf and root tissue. However, tissue collapse may be avoided by trying other techniques for preparing permanent mounts for light microscopy. The tissue damage may have occurred during either the dehydration and infiltration stages (O'Brian and McCully 1981). Assuming the cellular damage was due to exposure to paraffin wax, one technique worth trying is to substitute paraffin wax, a non-polar hydrophobic material, for a polar embedding agent such as glycol methacrylate (GMA)(O'Brian and McCully 1981). Another technique worth trying has been successfully used on the leaves of the submerged aquatic macrophyte *Ranunculus trichophyllus* (Rascio, et al. 1999). In brief, Rascio, et al.(1999) fixed sections of *R. trichophyllus* leaf in glutaraldehyde with sodium cacodylate buffer followed by osmium tetroxide in the

same buffer. The tissue was then dehydrated in solutions of ethanol and propylene oxide and embedded in araldite before sectioning with an ultramicrotome.

This study has confirmed the existence of thin cell walls that generally lack lignification, the extensive lucunae in leaves, petioles and roots, the presence of diaphragms and leaves reduced to a minimum of two cells thick at leaf margins, all of which indicate that *A. elongatus* is well adapted to an aquatic environment. Internal recycling of CO_2 seems more likely (Chapter 3 and 4) given the presence of extensive lacunae. The carbohydrate storage cells that occupy most of the tuber provide further evidence that an *A. elongatus* tuber can be used as a measure of photosynthetic performance and DIC acquisition (Chapter 3 and 4).

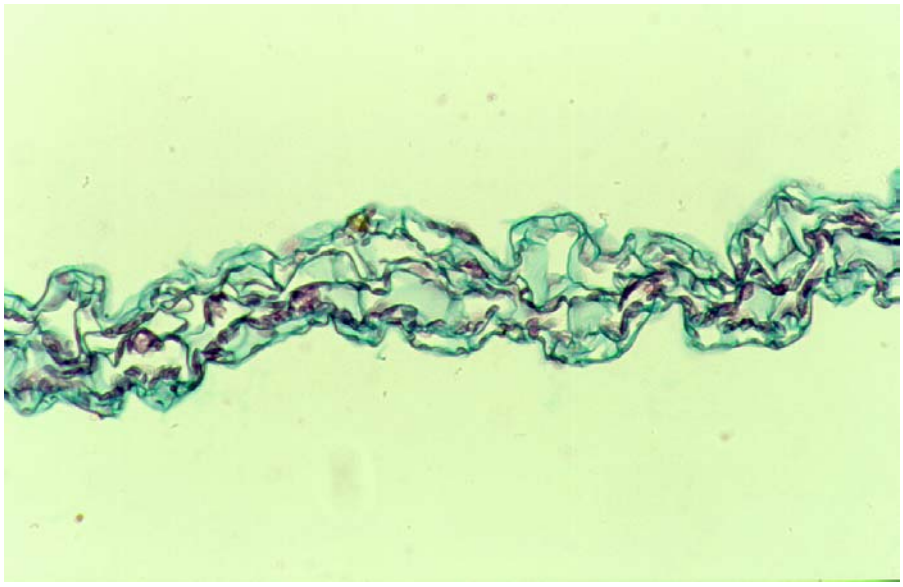

Plate A1.1
A transverse section of an *A. elongatus* leaf laminar (×270). Tissue collapse during the preparation of this specimen for permanent mounting makes identifying the cellular components and their arrangement unattainable.

Plate A1.2
A freshly cut transverse section of living *A. elongatus* leaf laminar. The black zones in the middle of the leaf section are gas filled bubbles occupying lacunae. (×360).

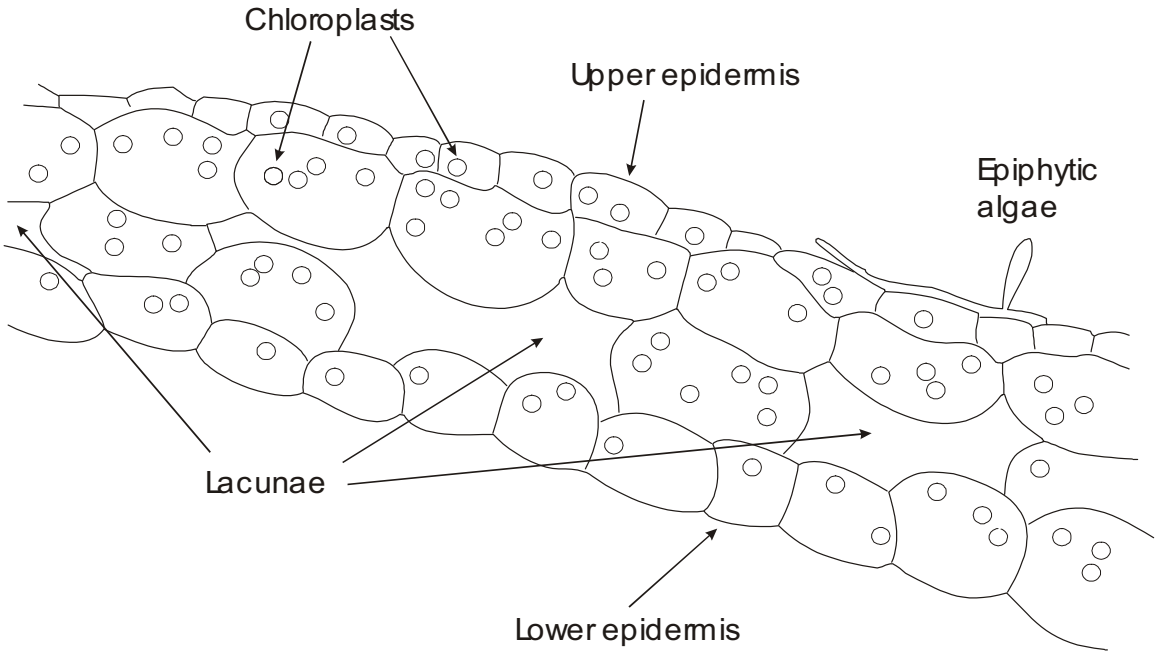

Fig. A1.1
An interpretive drawing of plate1.2 showing the cellular structure of an *A. elongatus* leaf transverse section. (Approx. actual size ×380).

Plate A1.3
A second freshly cut transverse section of a living *A. elongatus* leaf (×360). Chloroplasts are present in all the cells as well as black air filled bubbles occupying lacunae in the middle layer of the leaf.

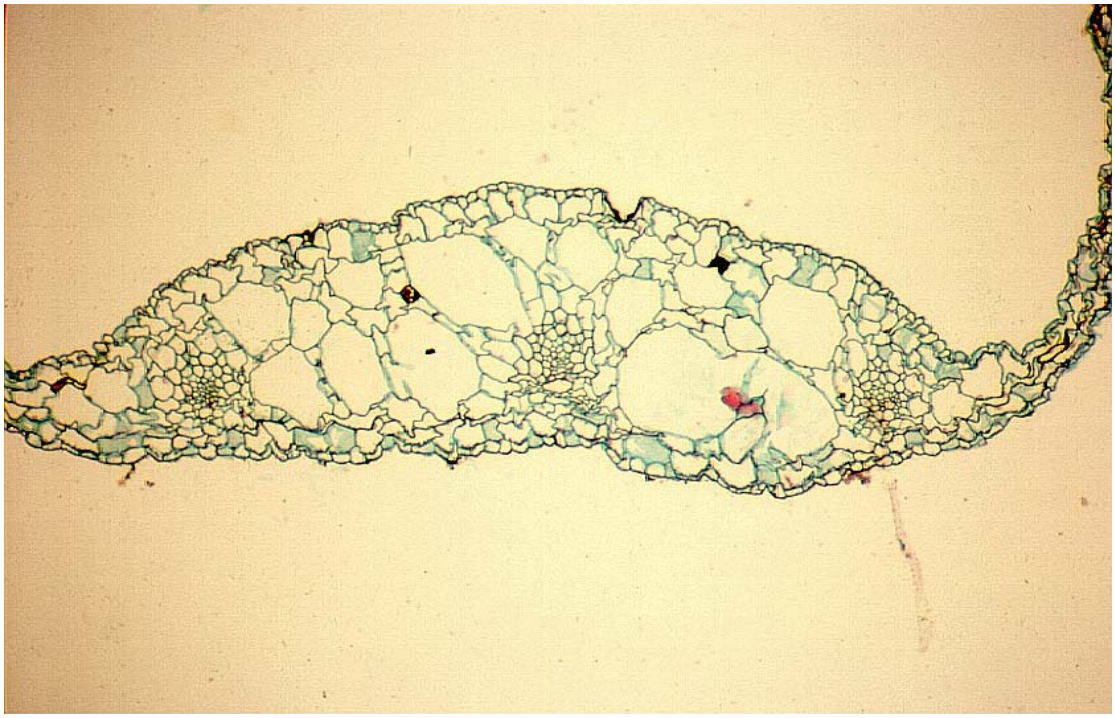

Plate A1.4
A transverse section of an *A. elongatus* leaf midrib (×50). The lacunae and three vascular bundles can be clearly seen.

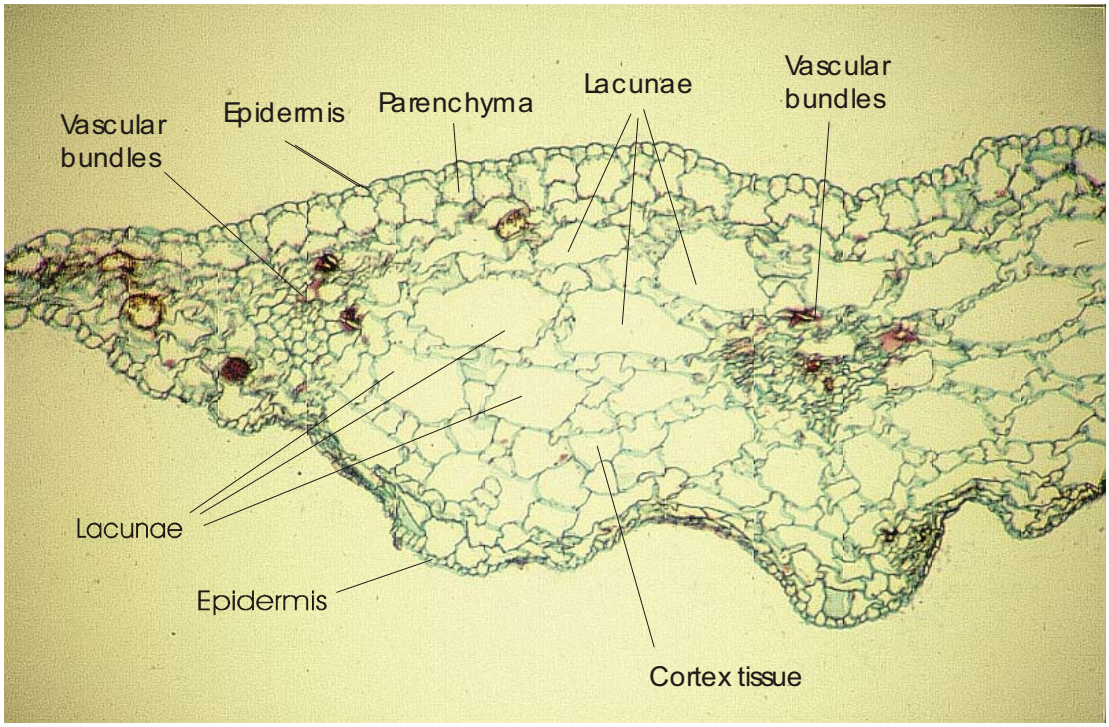

Plate A1.5
A second transverse section of an *A. elongatus* leaf midrib (×75). A closer view of the extensive lacunae and other cellular components.

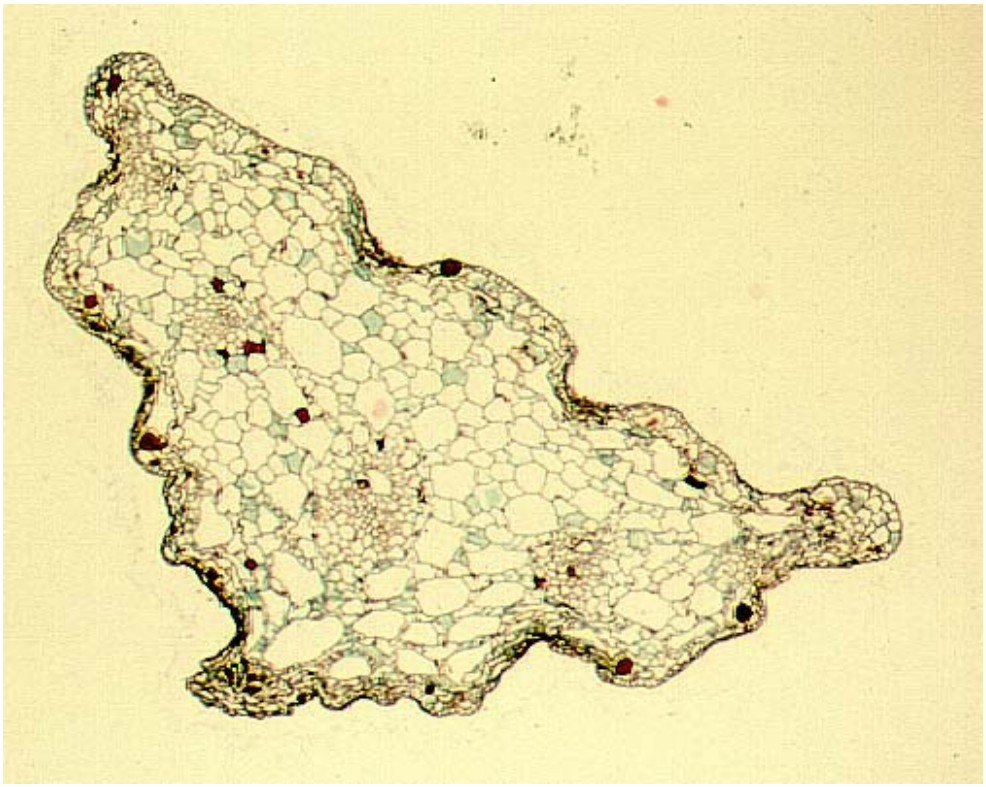

Plate A1.6
A transverse section of an *A. elongatus* leaf petiole (×80). Similar to the leaf midrib, the lacunae can be clearly seen.

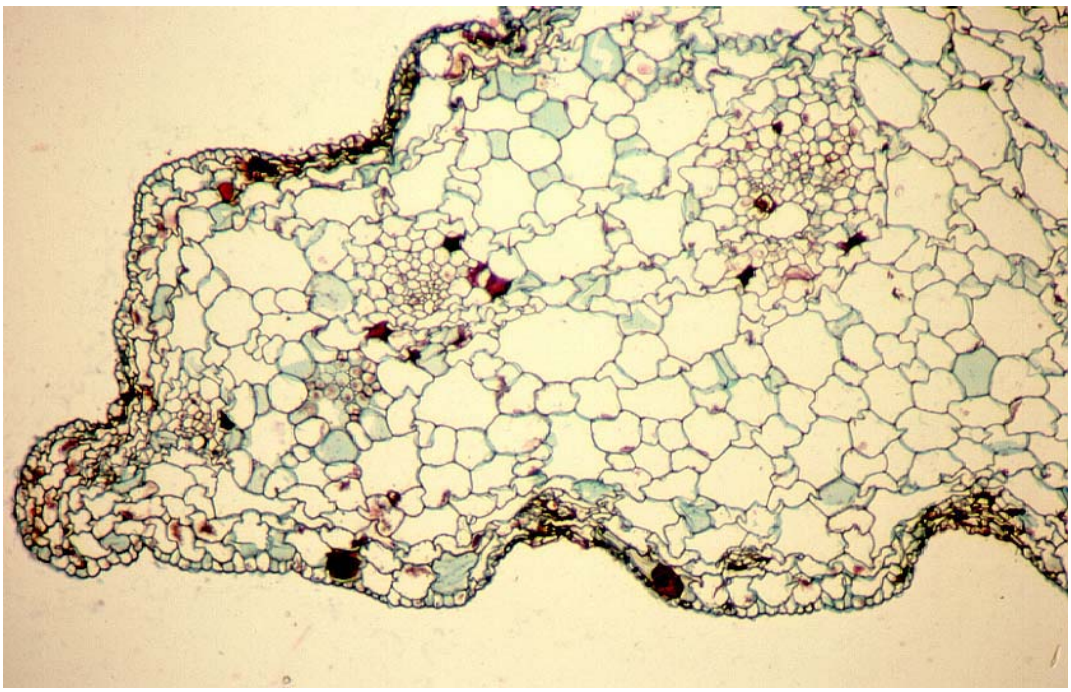

Plate A1.7
A higher magnification of a transverse section of an *A. elongatus* leaf petiole (×140). The vascular bundles, some epidermis cells, and large parenchyma cortex cells surrounding the lacunae can be seen despite considerable cellular distortion.

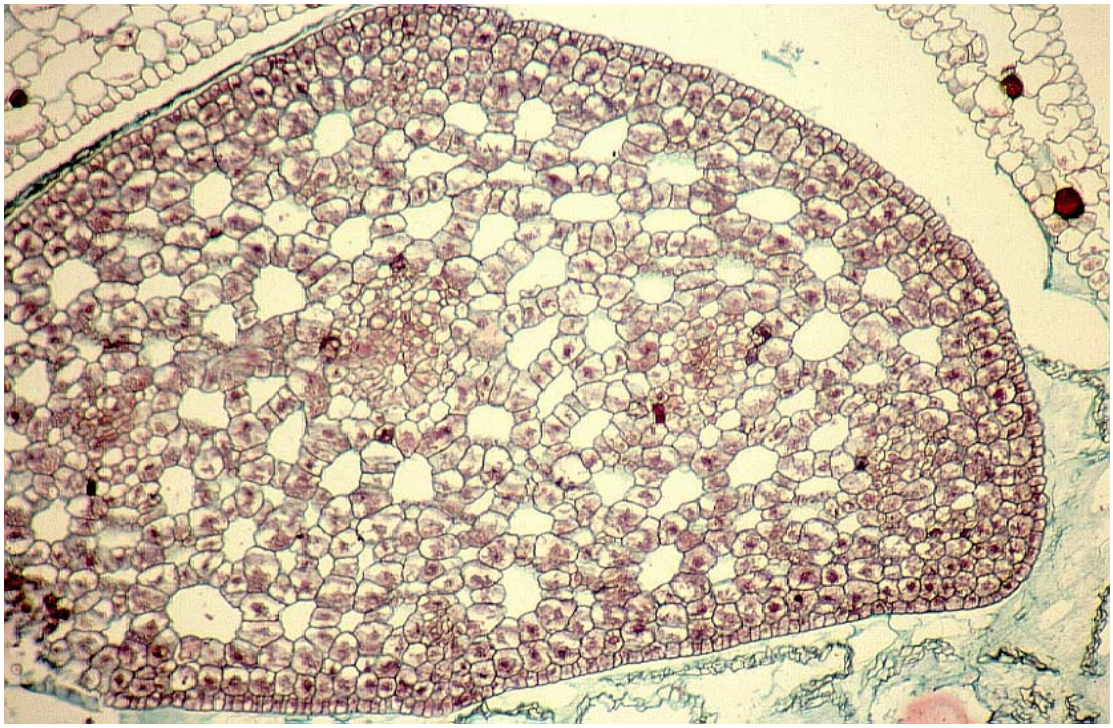

Plate A1.8
A transverse section of an *A. elongatus* leaf/petiole primordia at the apex of a tuber (×95). The lacunae appear well developed at this stage of development.

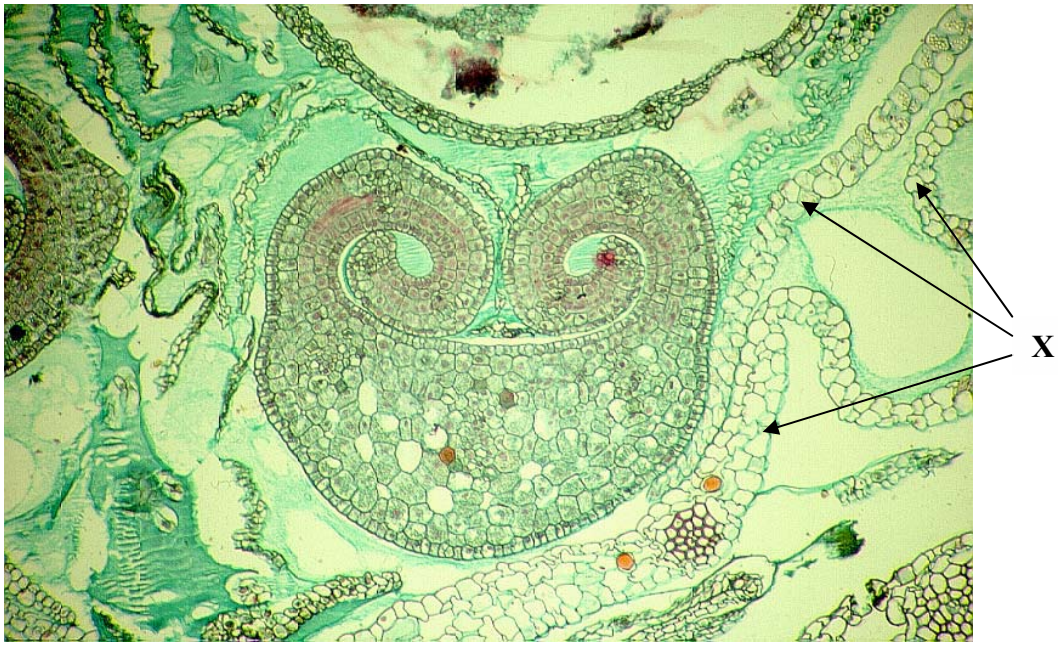

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Plate A1.9
A transverse section of a less developed *A. elongatus* leaf primordium in the apex of a tuber (×320). The lacunae are forming even at this early stage. The more advanced leaf segment (**X**) shows that a leaf is only two cells thick at leaf margin but increases to greater than four cells closer to the midrib (not pictured).

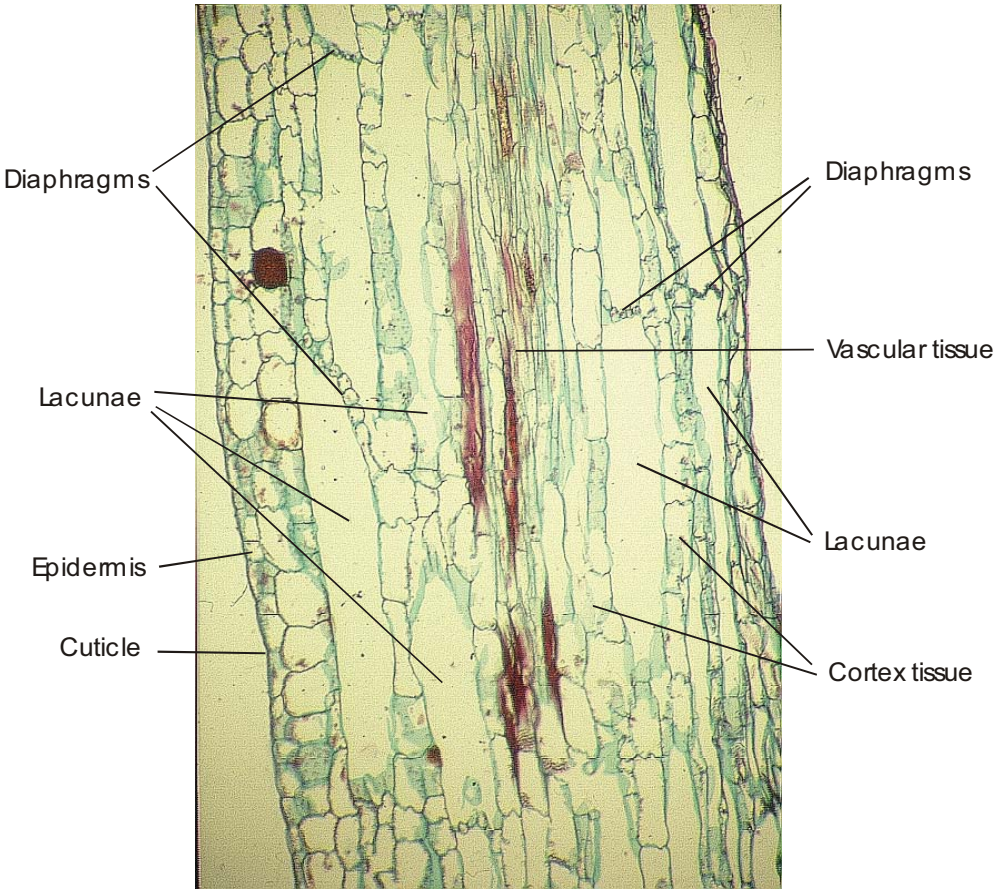

Plate A1.10
A longitudinal section of an *A. elongatus* leaf petiole showing epidermis, cortex and vascular system (×85). The cortex contains lacunae with diaphragm cells.

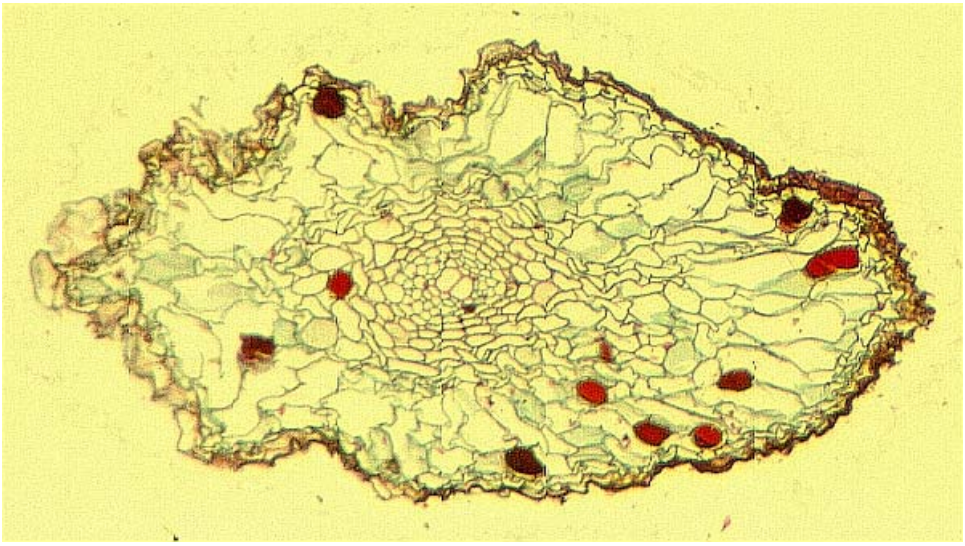

Plate A1.11
A transverse section of an *A. elongatus* root (×150). The vascular cylinder is distinctive but lacunae are difficult to distinguish due to extensive cellular collapse.

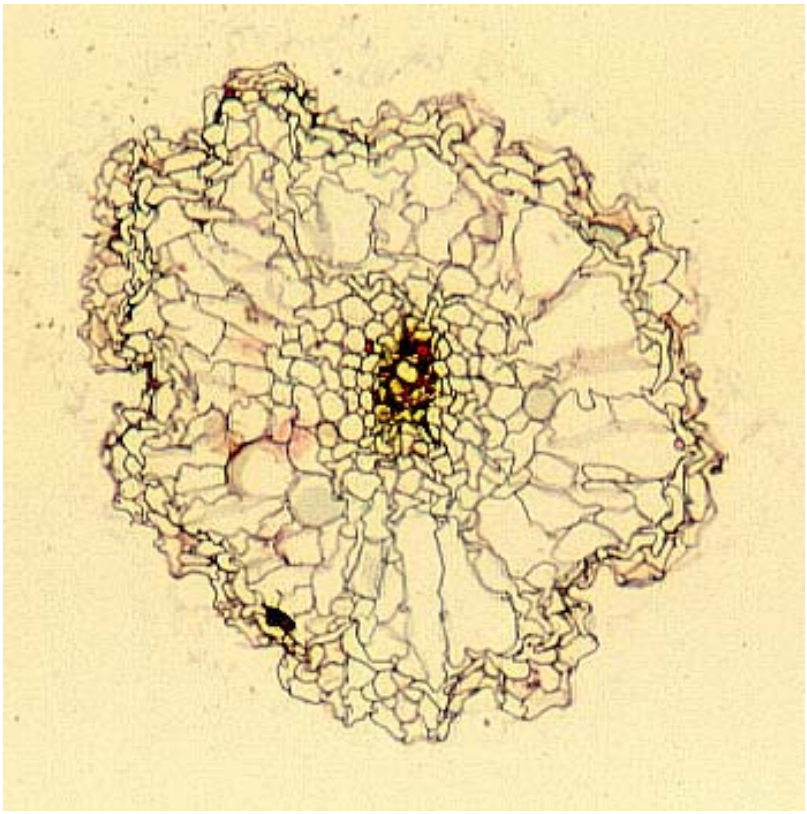

Plate A1.12
A transverse section of another *A. elongatus* root (×170). The vascular system is less distinctive but the lacunae although difficult to distinguish are likely to be in the cortex.

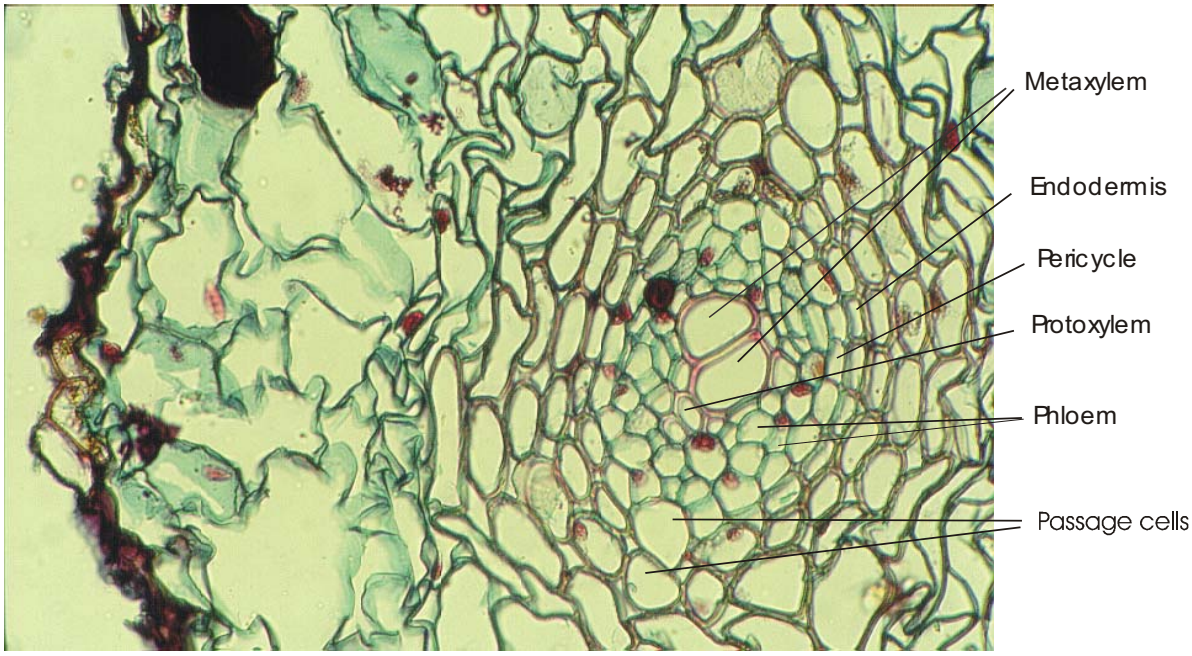

Plate A1.13
A closer look at part of an *A. elongatus* root transverse section (fig 12)(×250). The vascular cylinder with two large metaxylem is quite distinctive but the lacunae are difficult to distinguish from the collapsed cells in the cortex and epidermis.

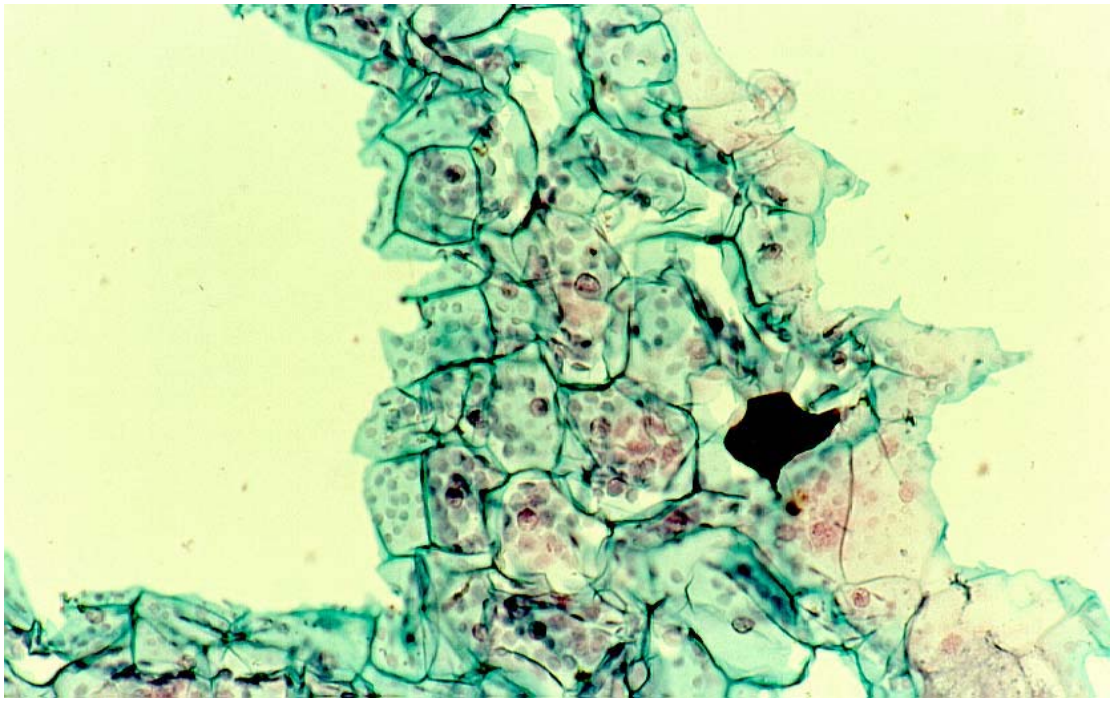

Plate A1.14
Storage cells from an *A. elongatus* tuber. Cell nuclei and smaller plastids are visible (×110).

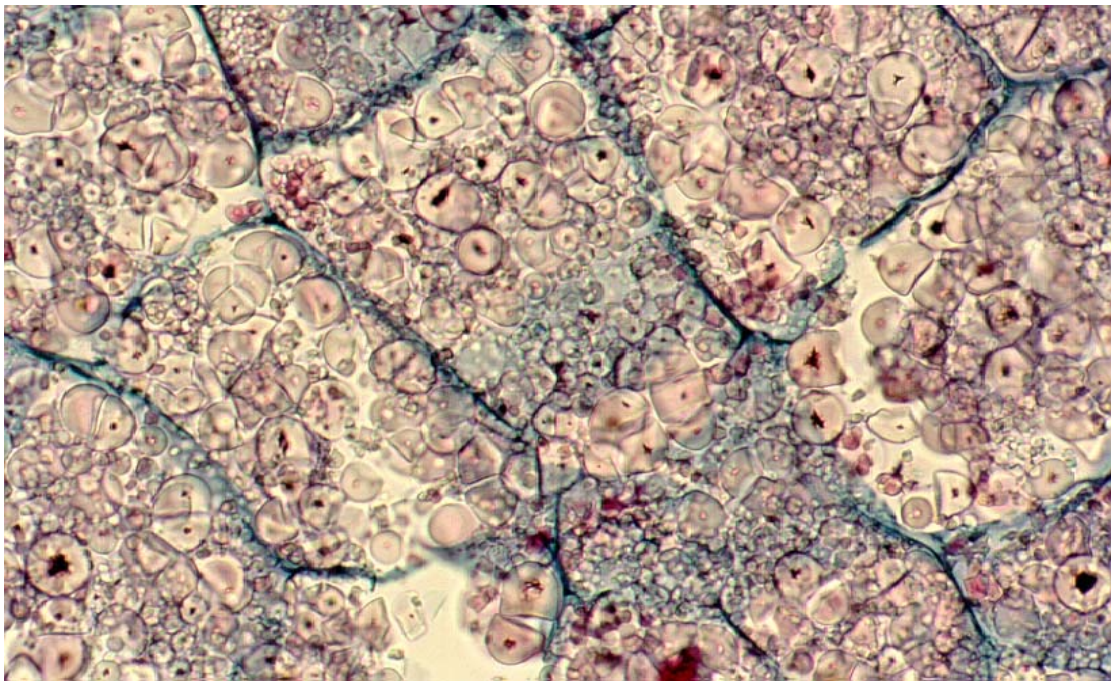

Plate A1.15
A closer look at *A. elongatus* tuber storage cells with plastids (×480). In each plastid, a dark red hilum like centre is visible but typical starch layering is not discernible. The plastids closely resemble sphaerocrystals of the polysaccharide inulin (Fahn, 1969).

Appendix 2

A low cost computer based electronic control system for regulating pH levels in six aquaria.

2.1 Introduction

The long term effects of different water pH levels on the growth of the submerged aquatic macrophyte Aponogeton elongatus are described in chapter 4. Over 15 papers have been written on the effects of pH on other aquatic macrophytes, mainly on photosynthetic rate rather than on plant growth (eg Sand-Jensen 1983; Nielsen and Sand-Jensen 1989). Some of these experiments have pH treatments consisting of small volumes of solution (1 to 5 ml) that are buffered to reduce pH change over relatively short periods (usually minutes)(Titus and Stone 1982; Owttrim and Colman 1989; Jahnke, et al. 1991). Long term growth experiments such as that described in chapter 4 use much larger volumes of water (100 litres plus) and may last several months (Titus, et al. 1990). The option of using buffered solutions to maintain a range of set pH levels may still be possible but would be more complex and difficult. A simpler method is to adjust pH around a set pH level with acid and base. This could be done manually but the need to make frequent adjustments to avoid large fluctuations around a set pH value and the need to make adjustments to a range of fixed pH values in different tanks makes some form of automation desirable.

An electronic controller can make adjustments more frequently and potentially more precisely than is possible with manual adjustment. There are a number of commercially available pH controllers but most of them can only deal with one station. This means multiple equipment is needed to enable monitoring and control of a number of different independent solutions all with different fixed pH levels. Multiple controllers of this type were used by Titus et al (1990) who successfully carried out a long term growth experiment on *Vallisneria americana*. Such pH control systems range from (in Australian dollars) $460 - $1700 each, excluding dosing equipment. More sophisticated multi-station controllers are also available but are considerably more expensive.

An alternative was to develop and build a relatively inexpensive computer based multi-station control system out of readily available electronic components, with the ability to control different pH levels in a range of tanks.

2.2 Materials and Methods

This controller was built in mid September 1999 at the University of Queensland, Gatton as part of an experiment looking at the effect of pH on the growth of the Australian native aquatic macrophyte *Aponogeton elongatus* (Chapter 4). The experiment was concluded by the 9th October 2000.

The pH control system was based on a HP 486SX personal computer (PC) connected to a 8 bit parallel port interface card that can convert analog signals from sensors to digital signal and vis versa. This enabled computer control of a set of solenoids or motors, using appropriate MS DOS based software, with feedback from relevant environment sensors.

The parallel port interface card (Digital /Analogue)(input/output board) was a Dick Smith Electronics Discovery Series kit (cat: K-2805). This kit was comprised of unattached electronic components that had to be soldered to a circuit board (card) before the I/O card was operational. The circuit board and instruction manual were provided in the kit and there was some limited interface software (QBASIC) programs printed in the manual, that can be expanded to suit specific applications. The card has a maximum of 10 analog inputs and 8 digital outputs (the operating characteristics are outlined in the manual). A 25-pin extension cable was used to connect this card to the computer parallel port. The card was housed in a plastic case (Dick Smith Electronics 'Zippy Box'; cat: H-2851).

The analogue input side of the card was wired, using ribbon cable, to a 12 way terminal block attached to the top of the zippy box lid. The terminal block was used to receive the wires from six pH and four temperature sensors. A resistor (100 K) was attached to the input side of the I/O board for each of the thermistors (temperature sensors) to convert electrical resistance to voltage (voltage divider).

The output side of the I/O board was wired with a 500mm length of 12 way ribbon cable to a series of double pole, double throw 12 volt relays housed in another Zippy box. A circuit diagram of the relays is shown in Fig. A2.1. Although not essential, light emitting diodes (LEDS) were attached to the input side of each relay to visually indicate when a specific acid or base solenoid was being activated by the controller. The output sides of the relays were wired to two further terminal blocks screwed to the top of the lid. These were wired to the solenoids using lightweight 'fig8' two core electrical cable.

A Dick Smith mains power adaptor supplied 24 volts AC (1.5 amps) which was converted to 24 volts and 12 volts DC by a bridge rectifier and two regulators (power supply) housed in the jiffy box containing the I/O board (Fig A2.2). This powered the I/O board and the relays.

The experiment in which the controller was used required six pH controlled aquaria (tanks). Each tank had 2 solenoids, one pH probe and ± one temperature probe. For each tank one solenoid was used for base and one solenoid used for acid.
In total, 6 pH probes, 4 temperature sensors and 12 solenoids. There were 3 set pH levels; 6.5, 7.7 and 9.0 in the experiment. Two tanks were allocated to each of these set pHs. A more complete description of the acid and base dosing equipment that includes the solenoids is outlined in Chapter 4.

The temperature sensors were made by soldering thermi-resisters (thermistors) on to lightweight two core electrical lead. To fully insulate and waterproof, each thermistor was plunged into a small glass test tube filled with epoxy resin. As controller inputs were limited only 4 temperature sensors were used. These were placed to take account of any temperature differences between the tanks.

The six pH sensors proved to be most expensive items of the controller system. The pH probes were Australian made, plastic bodied ionode^{R} pH electrodes, model PBFC. They were attached via BNC connecters to single core microphone cable to a terminal block on the zippy box. The connectors allowed easy detachment for maintenance but the trade off was a small increase in electronic noise.

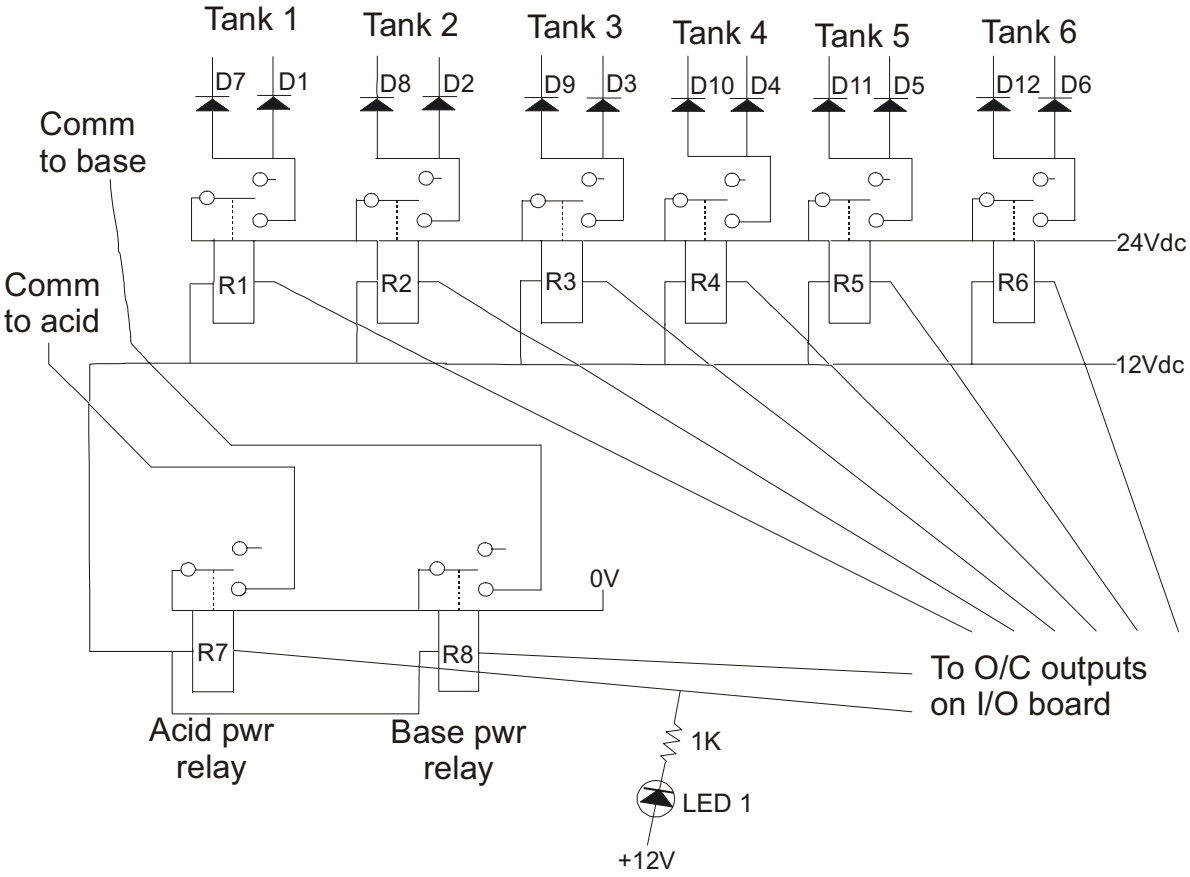

Fig A2.1. Circuit diagram of acid/base relay system. Consists of double pole/double throw 5 amp relays (R1-8), diodes (1N4007) (D1-12), eight resisters (1K) and light emitting diodes (LEDS 1-8). One LED and resistor was connected to each of the eight relay input wires from the open collector (O/C) outputs on the input output (I/O) board although only one LED and resistor is drawn on the diagram. The LEDS provided a visual indication of power supply to individual acid or base solenoids in each of the six tanks.

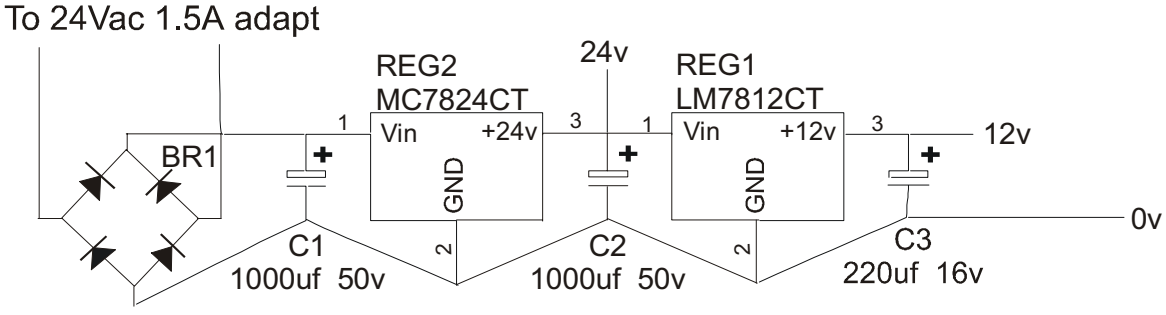

Fig A2.2 Circuit diagram of power supply that converts 24 volt AC from a Dick Smith mains power adaptor to 24 and 12 volt DC. Consist of a bridge rectifier (BR1), three capacitors (C1-C3) and two regulators (12 volt REG 1 and 24 volt REG 2)

Once the controller system was up and running there was a need to clean and check the calibration of the pH probes at least one every week against an accurate portable pH meter (TPS WP-80). The controller was periodically recalibrated for each of the pH probes.

2.2.1 Software

The MS Dos based control program was written in Turbo Pascal. It is able to monitor and maintain pH around the preset limits of a set pH for each of the six tanks sequentially and independently. For example if the pH of a tank is outside the preset limits the program modifies the pH by switching on the tank's acid or base solenoid to add preset amounts at preset intervals until pH reaches the preset limit. The program would activate either a base or acid solenoid if the preset upper and lower set limits of pH were exceeded (eg for pH 6.5, pH 6.40 – 6.60). The length of time a solenoid is activated determines the amount acid or base applied (adjustable) (eg 20 – 30 milliseconds to apply one ml of 1M NaOH). This time was related to the maximum flow rates of the acid or base dosing equipment (solenoids).

Due to the likely delay in pH change after the addition of acid or base (time for mixing), there was a preset minimum time between each application (eg 1-5 minutes).

For pH monitoring, data from each of the pH probes was slope adjusted, based on the average of the four thermistors (temperature probes). Data was also automatically adjusted for asymmetry and slope variances for each pH probe. In addition, the program was designed for manual calibration of the pH and temperature probes.
The program was set up so the computer screen displayed tank number, date, time, set pH, actual pH and temperature conditions of each of the tanks, which was updated at regular intervals.

This data was recorded on the PC hard drive at regular time intervals and when pH adjustment occurred. The time intervals are adjustable from every 10 minutes to once every 24 hours. There was also provision for transferring this data from hard drive to floppy disk as a text (*.csv) file.

To allow for the occasional power interruptions the AUTOEXEC.BAT file was set up so that when the computer reboots the controller program automatically reloads. A copy of the controller program is available from our Internet site ie http:/www.aghort.uq.edu.au/staff/dpilon/misccode. You are welcome to further modify or refine it (provided it remains freeware).

2.3 Results and Discussion

2.3.1 Performance

There was excellent separation of the different pH treatments in all three runs as is shown for run 2 in Fig.A2.3. The controller also maintained consistent pH in all set pH treatments. Although the average recorded pH does not exactly match the set pH points they fit within a pH range allocated to each set point (table A2.1). It may be possible to set narrower limits around each pH set point with some refinements to the system. In particular a 10 to 16 bit Digital /Analogue card would have been better than the 8-bit card that was used in this system. A reduction in electronic noise may be possible with improved shielding between the pH probes and the controller. Although the solenoids proved adequate for the experiment, some improvement in pH control may have been possible if they had been more resistant to the effects of acid and base (acetic acid and sodium hydroxide) (Chapter 4). It is also important to ensure that the temperature sensors (thermistors) are electrically insulated from the water when submerged, as any leakage will effect the performance of the pH probes.

2.3.2 Cost

Table A2.2 shows the potential savings that were made in constructing a controller out of electronic components compared to purchasing the cheapest available 'off the shelf ' pH controller. The cost of the acid and base dosing equipment (solenoids) was not included, as this cost would occur irrespective of the type of controller that was used. However, the opportunity costs of the time involved in purchasing the different components, construction of the controller system and ironing out of bugs were not calculated, which may prevent a fair cost comparison with the cheapest available (Pinpoint^{R}) pH controller.

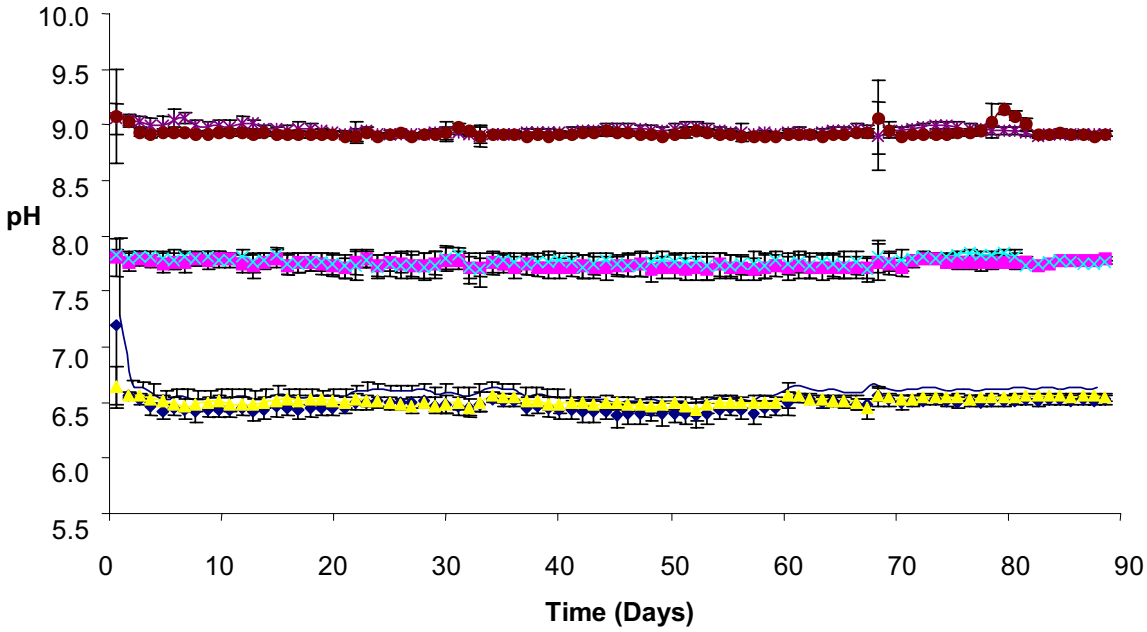

Fig A2.3 The effect of the controller on the pH of 6 tanks at the set pH levels of 6.5 (◆ ▲), 7.8 (■ ×) and 9.0 (✱ ●) (two independent tanks per pH level) in run 3 over time (days)(means ± SD)(n = 48). The plants were removed after the 69th day but the controller continued operating until switched off on the 89 day.

Table A2.1
A comparison of the set pH levels with the average pH achieved by the controller in each of six tanks over the time taken to complete each of three runs. These means ± STD are based on pH data measured and recorded by the controller every 30 minutes. (Run 1, n = 4319, run 2, n = 5685, run 3, n = 4000).

		Average pH (Means ± STD)		
Tank no	**Set pH**	**Run 1**	**Run 2**	**Run 3**
1	6.50	6.55 ± 0.09	6.59 ± 0.08	6.58 ± 0.08
2	6.50	6.59 ± 0.07	6.59 ± 0.08	6.62 ± 0.06
3	7.80	7.83 ± 0.11	7.88 ± 0.09	7.82 ± 0.09
4	7.80	7.84 ± 0.10	7.84 ± 0.08	7.84 ± 0.09
5	9.00	8.95 ± 0.11	9.04 ± 0.11	8.99 ± 0.05
6	9.00	8.87 ± 0.07	8.96 ± 0.05	8.97 ± 0.06

Table A2.2

A list of pH controller components and their cost (Total 1) compared to the cost of purchasing the cheapest available commercial "off the shelf" pH controllers (Total 2) at 4/11/99. The electronic components in the interface kit (I/O board) have not been listed in this table.

Item description	No	Cost ($)
486 SX 33 Personal Computer	1	0.00
pH electrodes plastic bodied ionode^{R}, model PBFC	6	594.00
Parallel port interface kit (analog/digital) (Cat. no. K 2805)	1	42.50
Power adaptor 240/24volt AC (Cat. no. M 9635)	1	39.95
Relays double pole/double throw, 12 volt, 5 amp	8	38.96
Plastic terminal blocks, 12 way, 10 amp	3	4.50
Resistors 1 K ohms	8	0.40
100 K	4	0.20
Thermistors 100 K	4	7.00
Capacitors 220 nF 16V	1	0.85
1000 uS 50V	2	1.70
Regulator 12 volts LM7812CT	1	3.00
Regulator 24 volts MC7824CT	1	4.00
LEDS	8	1.80
Diodes (1N4007)	12	1.20
Heat sink (clip on type)	1	2.00
Case mounted socket	1	7.00
Printer port extension cable (25 pin)	1	14.80
Ribbon cable (12 way)	1 m	2.01
Microphone cable (single core)	25 m	15.25
Electrical cable lightweight 2 core	20 m	20.00
"Zippy" box (Cat. no. H 2851)	2	11.30
Connectors (female) BNC	6	43.50
Solder		
Total 1		**855.60**
pH controllers (Pinpoint^{R}) Aquasonic Ltd	6	2784.00
Power adaptors 240/24 volt AC	12	479.40
Plus cost of Data logging equipment including sensors		
Total 2		**3264 +**

The Pinpoint^{R} pH controller has pH/temp probes included in the price (Table 2.2). This controller is only able to monitor and maintain one fixed pH. Apart from the cost of purchase, this Pinpoint^{R} controller comes with 240-volt AC outlets that require

conversion to a safer 24 volts DC using power packs (12 of them), an additional cost. This ‘off the shelf ‘ controller also has no data logging function and lacks flexibility. In contrast, the computer based pH control system that was used in this experiment is adaptable to a range of other uses that require simple multiple independent controls. This includes operating solenoids, electric motors and switches, based on physical environmental variables such as temperature, humidity, irradiance and oxygen/carbon dioxide level (Dick-Smith-Electronics). All that is needed is some reprogramming and different environmental sensors. Building this system is easy, provided some expertise in writing and editing software and basic knowledge of electronics is available.

Appendix 3

Graphs of mineral nutrients in plant tissues and water

The following graphs contain nutrient data of *A. elongatus* tissues (leaves, tubers and roots) and of the surrounding water in which these plants were grown. The data used to produce these graphs is also displayed in tables 8 and 9 in Chapter 4. The main aim for measuring plant and water nutrient levels was to assess the effects of pH on nutrient uptake. These effects are discussed in Chapter 4.

All of the nutrients were measured by ICP analysis as described in Chapter 4, except nitrogen, which can not be measured using this method. The concentration of nitrogen in plant tissue was obtained from the carbon and nitrogen isotope analysis (Chapter 4). The nitrogen concentration in the water was not measured (Fig A3.1). All the plant nutrient data was based on run one only (Chapter 4). The water nutrient data was based on runs 1 and 2, which allowed statistical analysis.

For the macronutrients, N, P, K, Ca, Mg, S, Na, the nutrient data is expressed in three forms, plant uptake (mg plant^{-1}), nutrient concentration (%) and nutrient efficiency (g plant dry weight mg^{-1} of nutrient). For microelements (Fe, B, Mn, Cu, and Zn), nutrient data was expressed as plant uptake (mg plant^{-1}) and nutrient concentration (ppm or mg kg^{-1}) but nutrient efficiency was excluded.

It is probable that this is the first nutrient analysis ever taken on *A. elongatus* tissues (chapter 4). The only other nutrient analysis of *Aponogeton* is some work done by Shardendu and Ambasht (1991). They analysed the tissues of *A. natans* (L.) Engle and Krause as well as other aquatic macrophytes growing in a natural lentic wet land in India. They measured N, P, K, Ca, Mg and Na of which N, P, K, and Na were between 10% to 60% lower than in *A. elongatus*. Apart from the fact that they are separate species and likely to have different nutrient requirements, *A. elongatus* was cultivated with sufficient fertiliser to insure mineral nutrition was not the limiting factor for growth (Chapter 4). This is less likely for any aquatic macrophyte growing in a more natural situation. This data should provide a useful point of reference for any further work that is done on the nutrition of *A. elongatus* either in the laboratory or in the field.

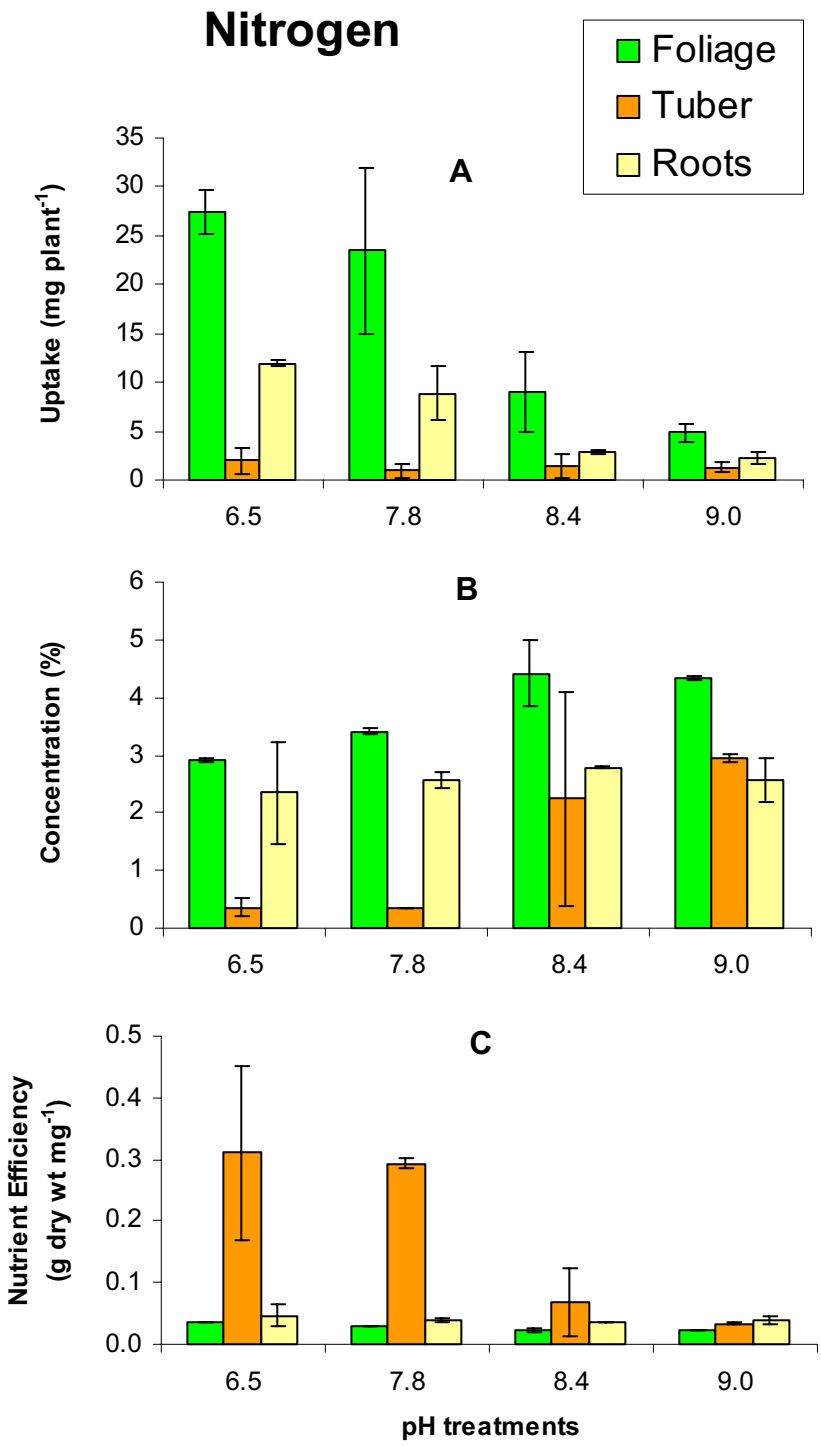

Fig A3.1 The effect of pH on nitrogen uptake (milligrams nutrient per average dry weight)(**A**) , the concentration of nitrogen (% of nutrient to dry weight)(**B**) and the nitrogen use efficiency (grams dry weight per milligram of nutrient)(**C**) of foliage, tubers and roots of an average *A. elongatus* plant in run 1. A mean pH level of 8.4 ± 0.2 (± SD) is the ambient pH (control). Based on means ± SD (n = 2)

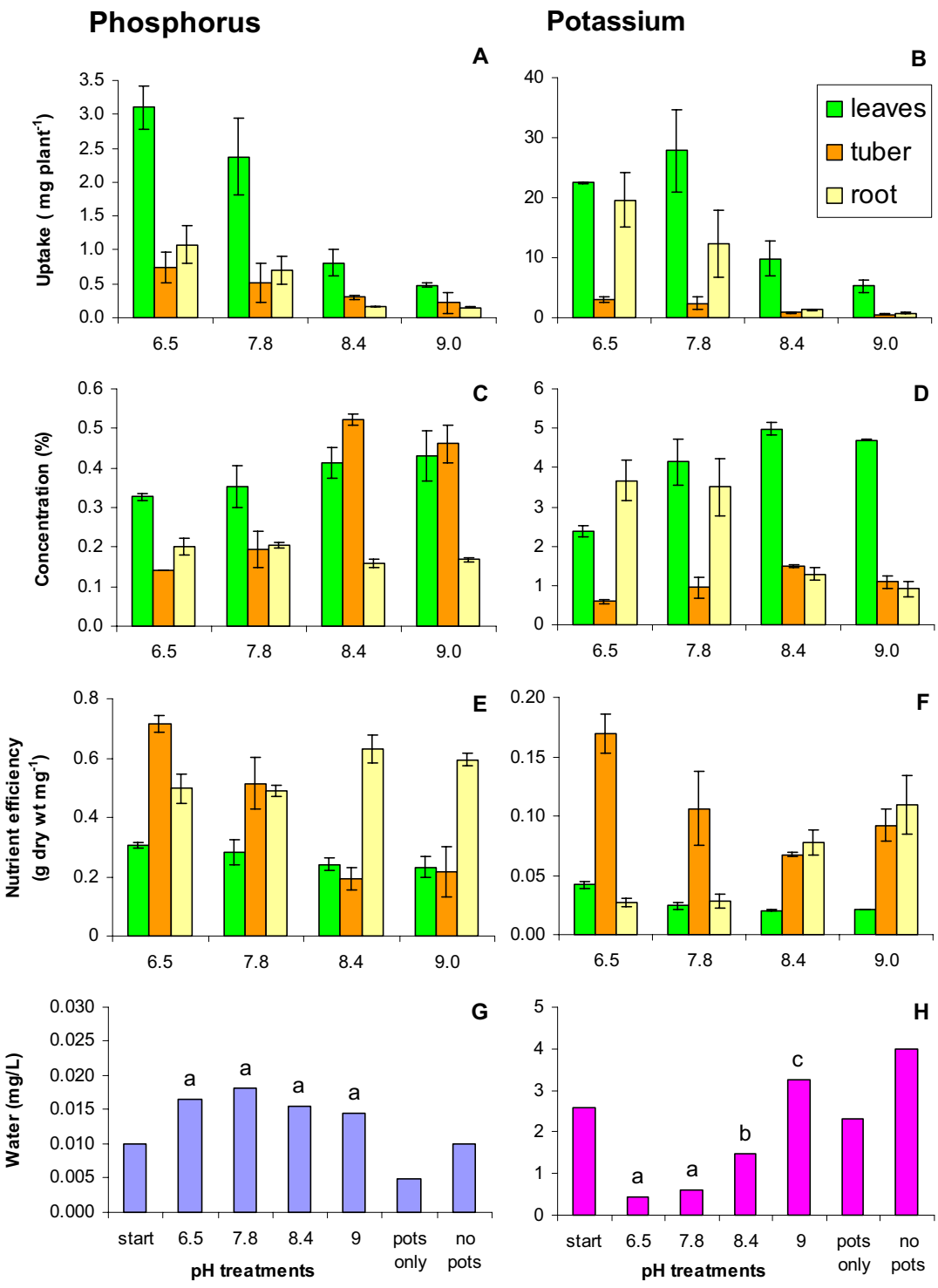

Fig A3.2 The effect of pH on, phosphorus and potassium uptake (milligrams nutrient per average dry weight)(**A & B**), nutrient concentration (% of nutrient to dry weight)(**C & D**) and nutrient use efficiency (grams dry weight per milligram of nutrient)(**E & F**) of foliage, tubers and roots of an average *A. elongatus* plant in run 1. Based on means ± SD (n = 2). Graphs (**G**) and (**H**) show the mean effect of pH on the nutrient content in the water (mg per litre) of two runs. Treatments with a common letter are not significantly different at 5% level. A mean pH level of 8.4 ± 0.2 (± SD) is the ambient pH (control).

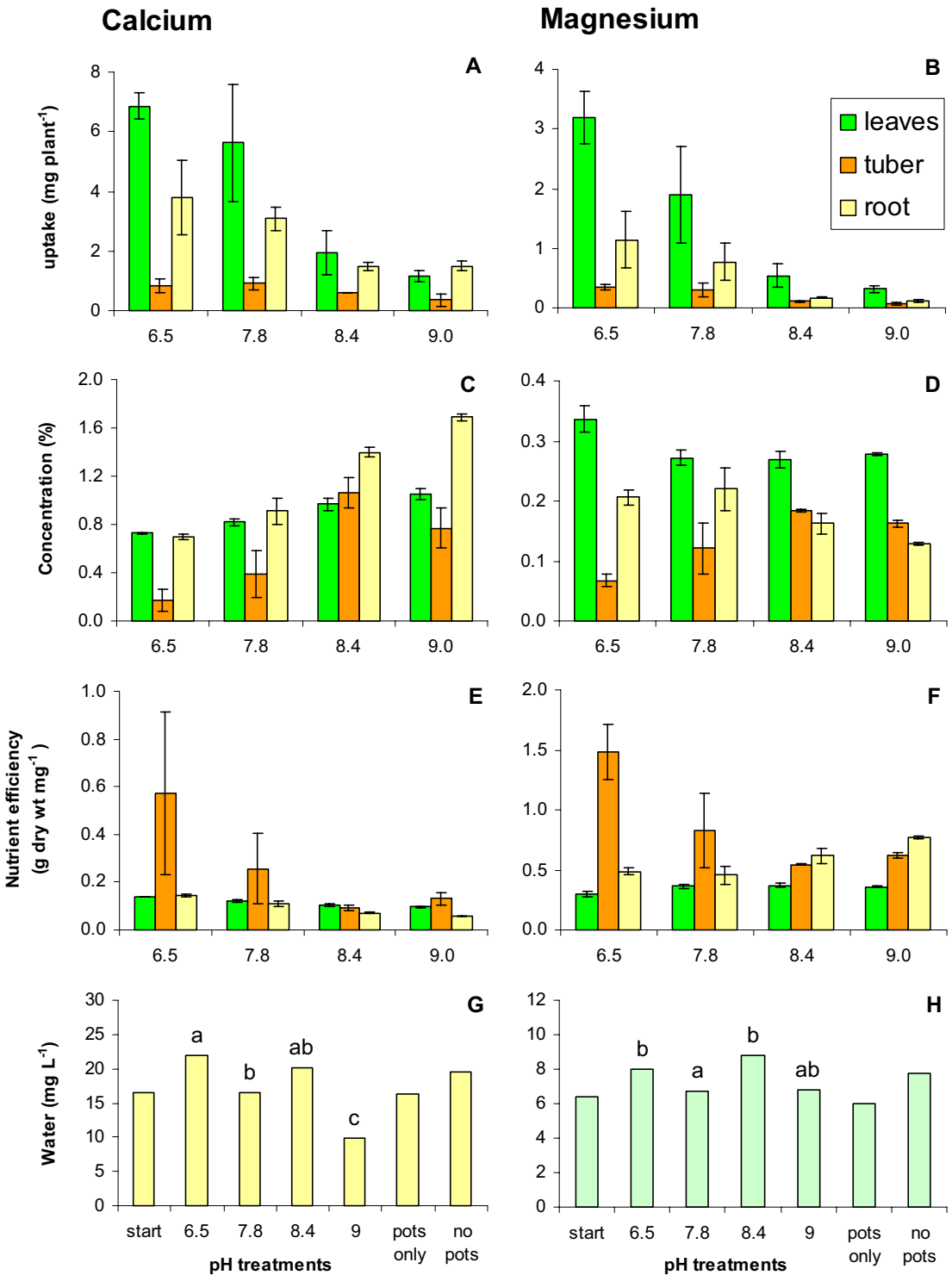

Fig A3.3 The effect of pH on, calcium and magnesium uptake (milligrams nutrient per average dry weight)(**A & B**), nutrient concentration (% of nutrient to dry weight)(**C & D**) and nutrient use efficiency (grams dry weight per milligram of nutrient)(**E & F**) of foliage, tubers and roots of an average *A. elongatus* plant in run 1. Based on means ± SD (n = 2). Graphs (**G**) and (**H**) show the mean effect of pH on the nutrient content in the water (mg per litre) of two runs. Treatments with a common letter are not significantly different at 5% level. A mean pH level of 8.4 ± 0.2 (± SD) is the ambient pH (control).

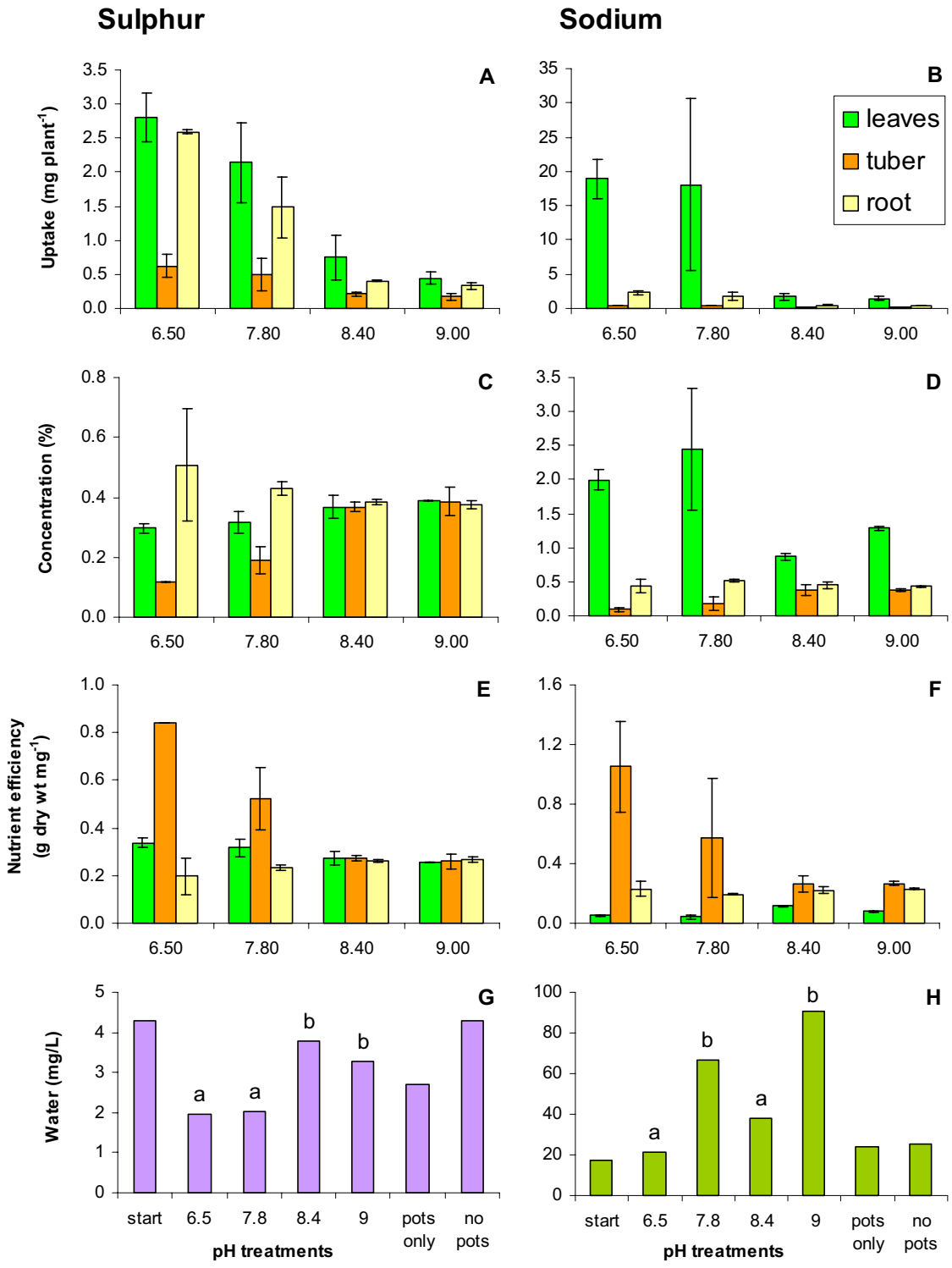

Fig A3.4 The effect of pH on, sulphur and sodium uptake (milligrams nutrient per average dry weight)(**A & B**), nutrient concentration (% of nutrient to dry weight)(**C & D**) and nutrient use efficiency (grams dry weight per milligram of nutrient)(**E & F**) of foliage, tubers and roots of an average *A. elongatus* plant in run 1. Based on means ± SD (n = 2). Graphs (**G**) and (**H**) show the mean effect of pH on the nutrient content in the water (mg per litre) of two runs. Treatments with a common letter are not significantly different at 5% level. A mean pH level of 8.4 ± 0.2 (± SD) is the ambient pH (control).

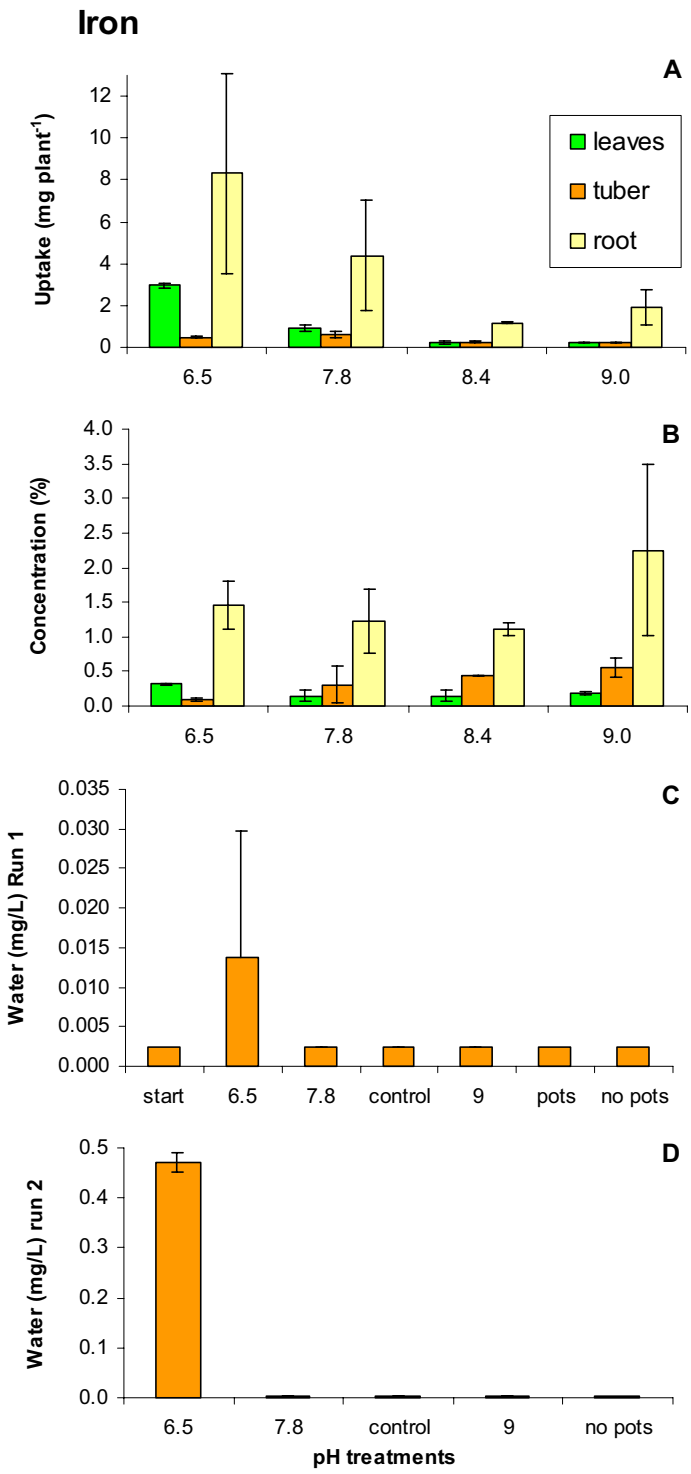

Fig A3.5 The effect of pH on (**A**) iron uptake (milligrams micronutrient per average dry weight) and (**B**) the iron concentration (% of micronutrient to dry weight) of foliage, tubers and roots of an average *A. elongatus* plant in run 1. Based on means ± SD (n = 2). Graphs (**C**) and (**D**) show the effect of pH on the iron content in the water (mg per litre) of run one (89 days) and run two (114 days). Based on means ± SD (n = 2). A mean pH level of 8.4 ± 0.2 (± SD) indicates the ambient pH treatment (control)

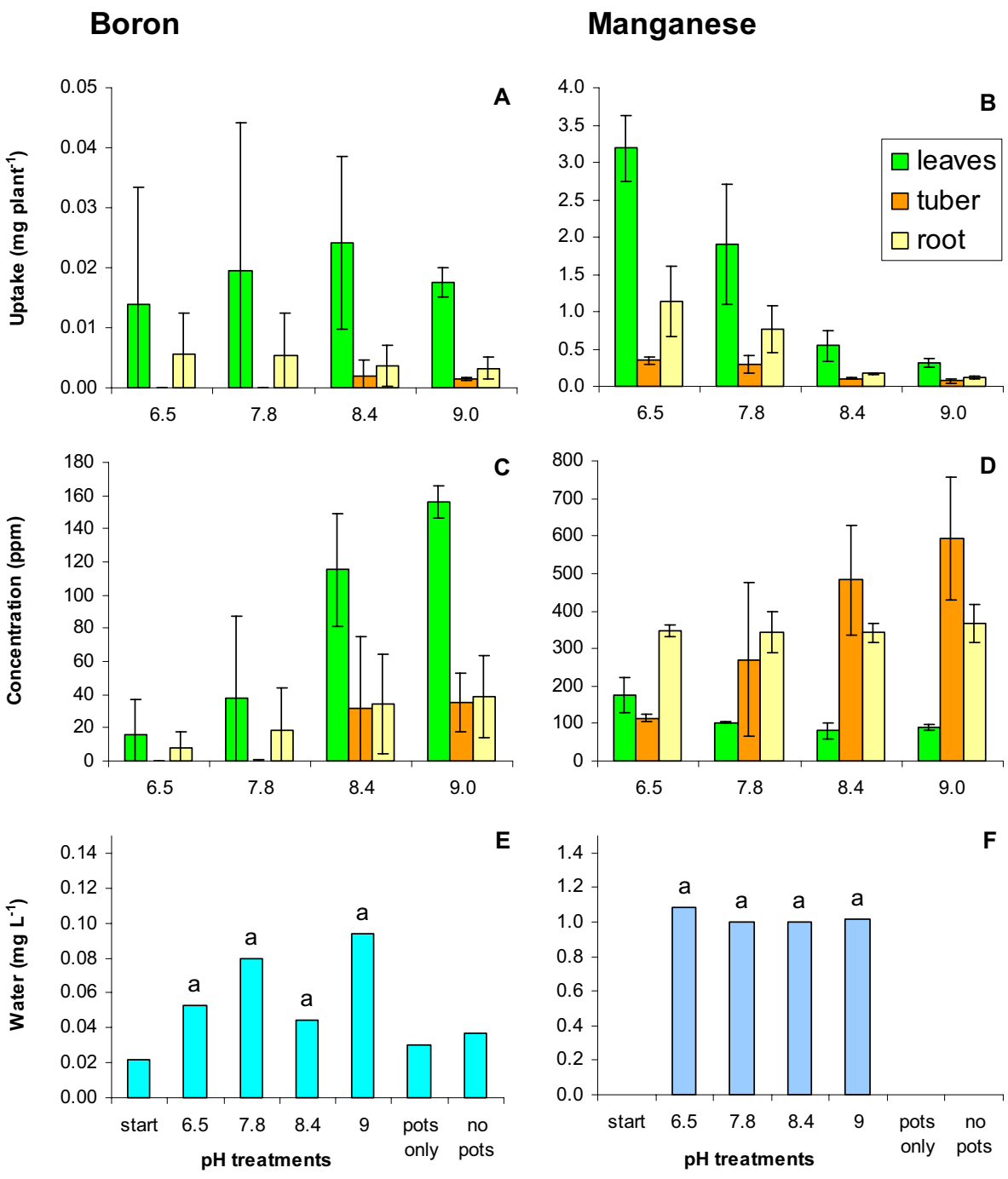

Fig A3.6 The effect of pH on, (**A**) boron and (**B**) manganese uptake (milligrams micronutrient per average dry weight) and (**C**) boron and (**D**) manganese concentration (ppm of micronutrient to dry weight) of foliage, tubers and roots of an average *A. elongatus* plant in run 1. Based on means ± SD (n = 2). Graphs (**E**) and (**F**) show the mean effect of pH on boron and manganese content in the water (mg micronutrient per litre) of two runs. Treatments with a common letter are not significantly different at 5% level. A mean pH level of 8.4 ± 0.2 (± SD) is the ambient pH (control).

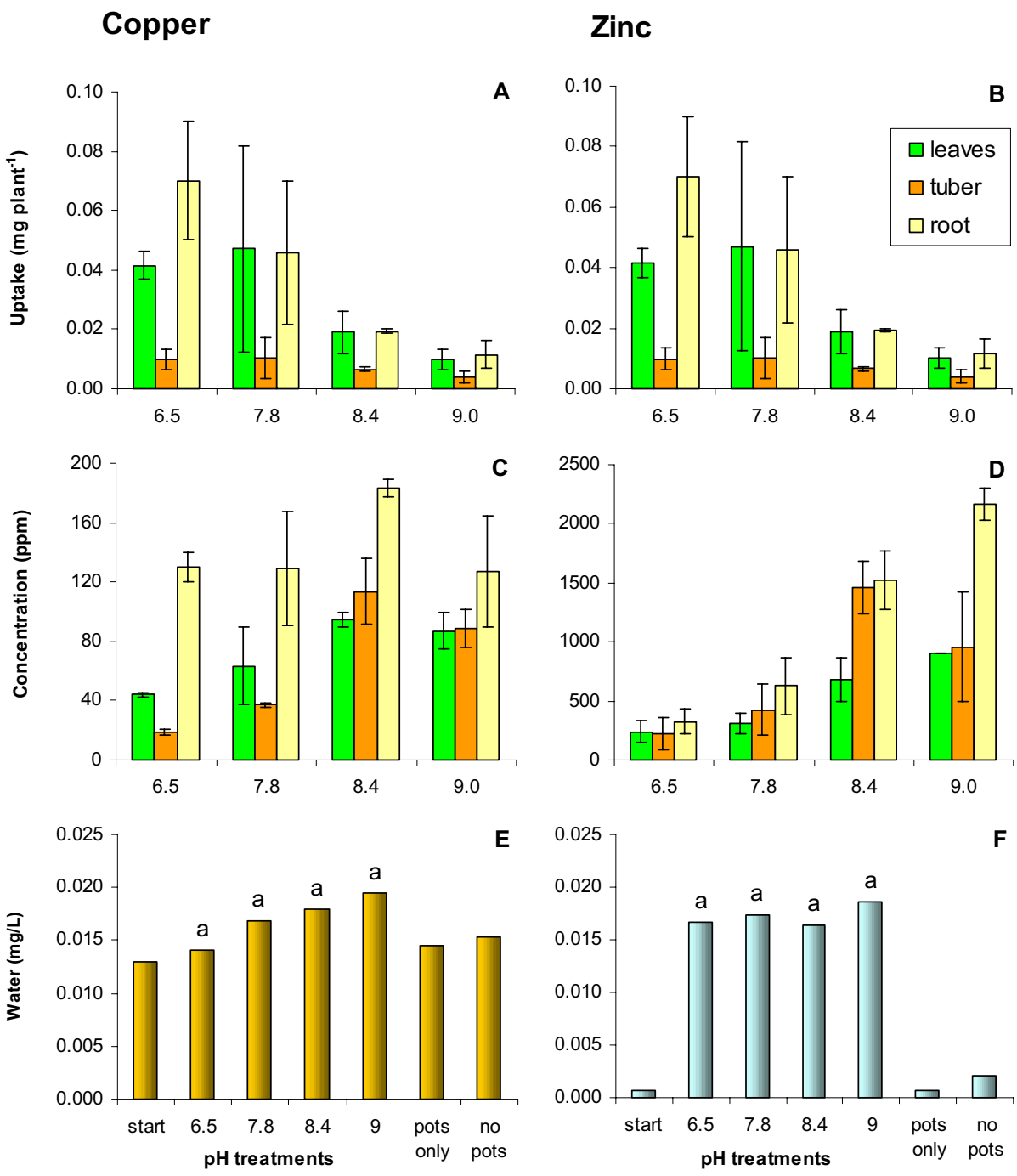

Fig A3.7 The effect of pH on, (**A**) copper and (**B**) zinc uptake (milligrams micronutrient per average dry weight) and (**C**) copper and (**D**) zinc concentration (ppm of micronutrient to dry weight) of foliage, tubers and roots of an average *A. elongatus* plant in run 1. Based on means ± SD (n = 2). Graphs (**E**) and (**F**) show the mean effect of pH on copper and zinc content in the water (mg per litre) of two runs. Treatments with a common letter are not significantly different at 5% level. A mean pH level of 8.4 ± 0.2 (± SD) is the ambient pH (control).

Appendix 4

Disease Observations

A foliage disease resulted in defoliation and stunting of effected plants in all three runs (chapter 4). This foliage disease was noticed in the first experiment (Chapter 3), but only a few plants developed severe symptoms. In Chapter 4 (experiment 2), this disease was more widespread and had a major effect on the results.

Milder symptoms consisted of black sunken lesions on young undeveloped leaves (leaf spikes) followed by leaf distortion during subsequent leaf development. More severe symptoms consisted of basal severing of leaf spikes as the lesions completely ringed them. Circular lesions also develop on older leaf blades, commonly on leaf margins (Plate A4.1) which eventually cause premature leaf senescence. This disease mainly affected foliage as tuber and roots were a normal healthy colour.

Plate A4.1 Photograph of foliage of *Aponogeton elongatus* showing circular leaf lesions on an older leaf. These lesions eventually coalesced resulting in leaf senescence.

Samples of infected foliage were plated out onto selective PAR (corn meal agar with antibiotics; Penicillin, Ampicillium and Rifampicin) growth media. A fungus found growing on this media was isolated and grown on PDA (potato dextrose agar) media A fast growing fungal species of *Pythium* (species unknown) was identified. Immature leaf parts of the Qld Umbrella Tree (*Schefflera actinophylla*), a known bait plant for *Pythium* and *Phytophthora* pathogens, were floated on top of the water in the infected tanks and uninfected tanks. Black lesions that developed on some of these leaf parts in infected tanks were also plated out and were found to contain the same species of *Pythium. S. actinophylla* leaves in uninfected tanks did not develop lesions. Although a number of species of this fungus are known plant pathogens, other species are solely saprophytic. Further study is needed to confirm whether this species of *Pythium* is able to cause disease on *Aponogeton elongatus* and to determine the degree of pathogenicity.

Acknowledgment

Many thanks to Greg O'Sullivan who did the work of isolating the fungus *Pythium* from *A. elongatus* leaf and stem lesions.

Appendix 5

Pest Observations

Leaf damage by water snails was another factor that contributed to experimental variability in experiments one and two. Although there was an attempt to avoid introducing snails to the experiment, a few tiny snails or snail's eggs were overlooked on some of the plants utilised in the experiment. Snail numbers had multiplied in some tanks by the end of the trial despite an attempt to remove snails during regular tank maintenance. This resulted in a low percentage of perforated leaves in some tanks. A number of different snail species appear to eat the leaves of *A. elongatus* although they may prefer selected types of alga (Plate A5.1 and A5.2). The snail species were not identified.

Plate A5.1 Snails with conical shells on an *A. elongatus* leaf.

Plate A5.2 Two types of snail on a leaf showing typical snail damage. The one at the top has a conical shell. The one near the base has a semi flat spiral shell.

Several insect pests have also been observed. An unidentified green aphid has been observed infesting the upper surfaces of floating leaves of mother plants grown in outdoor tanks. This apparently contributes to the premature senescence of the effected leaves.

Caterpillars of an unidentified moth species have also caused severe damage to floating leaves. When small these semi aquatic green-brown caterpillars fold over small sections of the leaf margins or, when larger, web together two overlapping floating leaves to protect themselves from predators.

Lastly the larvae of a Caddis Fly (?) species had totally defoliated a large *A. elongatus,* within a week. This plant had been flourishing in a display tank for three years. These larvae severed the leaves just above the tuber to provide short pieces (4 to 8 mm) of petiole to inhabit. None of these insects had a direct effect on the experiments outlined in this thesis.