The influence of nutrients and light on the metabolic activity and buoyancy of *Microcystis aeruginosa* and *Anabaena circinalis*

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Summary

The cyanobacteria *Microcystis aeruginosa* and *Anabaena circinalis* are two nuisance, freshwater phytoplankton which produce toxins, taint the taste and odour of potable water and form surface blooms. To control these species it is necessary to understand their physiology and response to factors which favour their dominance. In this study a technique was developed to assess cell metabolic activity and to determine the influence of light and nutrients on gas vesicle synthesis and buoyancy regulation.

The fluorogenic probe fluorescein diacetate (FDA) was used to assess metabolic activity. FDA readily permeates cells and is hydrolysed by esterases which utilise the FDA as substrate releasing the fluorescent by-product fluorescein which is detected using a flow cytometer. FDA conversion to fluorescein occurred at a linear rate for the first ten minutes and was saturated at FDA concentrations exceeding 15 μM; assays used 40 μM FDA and 7 minute incubation time. FDA conversion was shown to be a reliable measure of metabolic activity and correlated with other measures of cell activity, photosynthesis and growth. The FDA conversion rate of nutrient or light limited cells decreased relative to replete cells. Upon replacement of the limiting resource the FDA conversion rate recovered in proportion to the degree of resource availability. Esterase activity was fuelled by ATP derived primarily from photosynthesis.

A nutrient bioassay based on the measurement of esterase activity following nutrient addition in nutrient limited algae was developed. This increased activity was detectable within 24 hours. The FDA nutrient bioassay was trialed on phytoplankton sampled from the lower River Murray, Australia. On several occasions nitrogen and phosphorus were shown to be limiting by both the FDA bioassay technique and by more conventional bioassays. However, this response was dependent upon the species present; N_2-fixing species were P-limited and other species N-limited. Bioassays revealed that between 8 and 78% of the total phosphorus in the non-chlorophyll fraction in the lower River Murray was bioavailable but the nitrogen in the same fraction was unavailable for growth.

The nutrient status of cyanobacteria affects the mechanisms which regulate their buoyancy. The gas vesicle volume of individual *Microcystis aeruginosa* cells and *Anabaena circinalis* filaments was measured indirectly by quantifying light side scatter with a flow cytometer. Nitrogen limitation reduced the gas vesicle pool and the rate of carbohydrate accumulation in cells of *Microcystis aeruginosa* relative to N-replete cells. During light exposure carbohydrate accumulated and more gas vesicles were required to maintain buoyancy. When nitrogen was limiting in the dark 69% of *Microcystis* cells were floating. After 24 hours of high light only 29% were buoyant. In contrast when nitrogen was not limiting 100% of cells

were buoyant after the dark treatment and 93% after light exposure. Phosphorus limitation did not affect the rate of carbohydrate accumulation or generate buoyancy loss as severely as nitrogen limitation.

Nitrogen limitation did not reduce the gas vesicle volume per cell in *Anabaena* cultures but phosphorus did. Sixty percent of *Anabaena* filaments grown at 100 µmol m^{-2} s^{-1} were buoyant after 16 hours dark, 44% were buoyant after 24 hours of continuous light at 150 µmol m^{-2} s^{-1}. Eighty percent of filaments were buoyant in all other nutrient and light treatments.

In contrast buoyancy loss was observed in a natural *Anabaena circinalis* population at Chaffey Dam, NSW, Australia. Filaments suspended at 70% of surface irradiance were all buoyant at dawn but 39% lost buoyancy through the day, all filaments experiencing less than 10% of surface irradiance maintained buoyancy. The addition of saturating concentrations of nitrate and phosphate reduced the incidence of buoyancy loss. With no added nutrients 96% of the filaments suspended at 70% of surface irradiance were buoyant at 08:00 but this was reduced to 38% by 17:00. In contrast when N and P were added 100% were buoyant initially and 88% buoyant at the end of the day. Although nutrient addition promoted buoyancy the irradiance cells were exposed to changed their filament size and carbohydrate concentration which affected the floating velocity of the filaments. Filaments suspended at 70% of surface irradiance had a mean floating velocity of 0.06 m h^{-1} initially but by the end of the day the velocity was 0.007 m h^{-1}. Filaments experiencing 1% of the surface irradiance floated at a rate of 0.215 m h^{-1} initially which increased to 2.01 m h^{-1} by 16:30.

Declaration

This Thesis contains no material which has been accepted for the award of any other degree or diploma in any other university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I consent to this thesis being made available for copying and loan, if accepted for the award of the degree.

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25 xi 1997

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Chapter 1

Introduction

1.1 Cyanobacteria

Cyanobacteria are an ancient group of photo-autotrophic prokaryotes and appear in the fossil record 2.5-3 billion years ago (Lau *et al.*, 1980). They have exploited many habitats and occur in terrestrial soil crusts and in symbiosis with lichens, sponges and free floating *Azolla*. Planktonic cyanobacteria have invaded the full spectrum of aquatic habitats with pelagic examples inhabiting fresh waters (eg *Anabaena* spp., *Microcystis* spp., *Aphanizomenon* spp., *Cylindrospermopsis* spp.), brackish/estuarine (eg *Nodularia* spp.) and marine systems (eg *Trichodesmium* spp.).

1.2 Factors contributing to cyanobacterial dominance

Shapiro (1990) summarised six hypotheses which have been postulated as features of cyanobacteria which allow them to out compete eukaryotic micro-algae and contribute to cyanobacterial dominance in eutrophic, relatively stable waterbodies. Blomqvist *et al.* (1994) added another two theories; the phosphorus storage hypothesis of Petterson *et al.* (1993) and the nitrogen species hypothesis. The eight explanations for cyanobacterial dominance are listed below (Shapiro, 1990; Petterson *et al.*, 1993; Blomqvist *et al.*, 1994).

- Water temperature hypothesis. Cyanobacteria have higher temperature optima and out compete other micro-algae in warm waters.
- Light hypothesis. Cyanobacteria have lower light requirements and out compete other microalgae when photon flux densities are low.
- TN/TP hypothesis. Low TN/TP ratios indicate low nitrogen availability which favours nitrogen-fixing and non N-fixing cyanobacteria.

- Buoyancy hypothesis. Some cyanobacteria possess gas vesicles which allow them to persist in stable water bodies. The regulation of buoyancy enables cyanobacteria to migrate vertically and optimise capture of the vertically separated resources, light and nutrients.

- Zooplankton grazing hypothesis. Zooplankton feeding on cyanobacteria is inefficient due to the large colony size, mucilage and potential toxicity. Other micro-algae are preferentially grazed.

- Carbon dioxide/pH hypothesis. When carbon dioxide availability is low (high pH) cyanobacteria out compete other micro-algae because they have low half-saturation constants for carbon dioxide and more readily utilise bicarbonate.

- Phosphorus storage hypothesis. Cyanobacteria are capable of storing phosphorus acquired from sediments reducing the requirement to compete for dissolved bioavailable phosphorus. Nitrogen fixing cyanobacteria are then capable of out competing other microalgae when nitrogen and/or phosphorus are in short supply.

- Nitrogen species hypothesis. Low inorganic-nitrogen concentrations favour nitrogen-fixing cyanobacteria but it has also been suggested that ammonium-nitrogen favours non-fixing cyanobacteria and nitrate-nitrogen favours eukaryotic microalgae. Cyanobacteria would out compete other micro-algae when the major nitrogen source is ammonium-nitrogen or when inorganic nitrogen is scarce.

Blomqvist *et al.* (1994) considered each hypothesis in the light of existing and new data and several of the theories failed to explain cyanobacterial dominance when reviewed critically. The buoyancy and buoyancy regulation of some cyanobacteria is unquestionably a factor contributing to cyanobacterial dominance. Buoyancy is not only advantageous when the water column displays persistent stability but also allows cells to remain suspended in water bodies which are periodically stable.

1.3 Buoyancy

1.3.1 Advantages of buoyancy and buoyancy regulation

Gas vesicles (Walsby, 1994) are proteinaceous structures within some cyanobacteria and provide buoyancy. Buoyancy and buoyancy regulation provide cyanobacteria with a significant ecological advantage in watercolumns which are persistently or periodically stratified when other phytoplankton tend to sediment. Buoyancy reduces sedimentation losses and increases photosynthesis by allowing colonies to float into more favourable light climates (Sherman and Webster, 1994; Walsby *et al.*, 1997). The regulation of buoyancy enables cells to:

- migrate between the optimal zones for nutrient and light capture when these resources are vertically separated (Ganf and Oliver, 1982)
- migrate and increase nutrient scavenging by increasing the volume of nutrient containing water the cell is exposed to (Kromkamp and Walsby, 1990)
- form deep water maxima at the preferred light intensity and avoid photoinhibitory irradiances (eg *Oscillatoria agardhii*, Utkilen *et al.*, 1985); chromatic adaptation increases the efficiency of the light harvesting antennae thus increasing light capture at the selected depth (Tandeau de Marsac and Houmard, 1988).

1.3.2 Mechanisms of buoyancy regulation

There are three mechanisms cyanobacteria employ to regulate buoyancy. Two of these involve changes in cellular constituents. Accumulated carbohydrate is dense and acts as ballast, decreasing cell buoyancy. Intermediates of photosynthesis may also increase turgor pressure to a point where weak gas vesicles are collapsed resulting in a net increase in density. Hydrostatic pressure can also be great enough, in deep water bodies, to collapse some gas vesicles. The third mechanism is the regulation of gas vesicle production (Walsby, 1987, 1994; Oliver, 1994).

1.3.3 Buoyancy regulation by turgor induced gas vesicle collapse

Turgor pressure is the force which the cell constituents exert on the inner cell wall (Raven and Johnson, 1986). Turgor increases with an increase of internal solutes as the cell becomes more hypotonic with respect to the external media. A rise in cell turgor can be generated by the

accumulation of photosynthates (Grant and Walsby, 1977) and by the uptake of (potassium) ions (Allison and Walsby, 1981).

Oliver and Walsby (1984) demonstrated that the loss of buoyancy can be caused by turgor induced gas vesicle collapse in cyanobacteria with weak gas vesicles. They conducted a series of experiments examining cell density, the density and concentration of ballast molecules, and gas vesicle volume from which they concluded that *Anabaena flos-aquae* regulates buoyancy primarily by turgor induced gas vesicle collapse and resynthesis of gas vesicles when turgor is reduced They found that the rise in ballast (particularly carbohydrate) would not by itself have been sufficient to negate the buoyancy originally provided by gas vesicles (Oliver and Walsby, 1984).. This was reconfirmed by Kinsman *et al.* (1991).

The turgor induced gas vesicle collapse buoyancy mechanism can only be employed in cyanobacteria with relatively weak gas vesicles. Current views are that regulation of buoyancy by changing the carbohydrate concentration in response to light is a much more rapid and widely observed buoyancy regulation mechanism (Kromkamp and Mur, 1984; van Rijn and Shilo, 1983, 1985; Walsby *et al.*, 1987, 1989, 1991; Kromkamp and Walsby, 1990; Ibelings *et al.*, 1991b). Oliver (1994) recognises this when he states, "the prominence previously given to the turgor pressure collapse mechanism has not been supported by recent evidence".

Kromkamp *et al.* (1986) concluded that ballast regulation was the principle regulatory mechanism in *Aphanizomenon flos-aquae*, a cyanophyte with weak gas vesicles. However, they performed experiments with cultures acclimatised to different light periods and discovered that these populations displayed different mechanisms of buoyancy regulation when transferred to high irradiance. Cells adapted to continuous light showed gas vesicle collapse (Kromkamp *et al.*, 1986). Cells adapted to decreasing photo-period by increasing the rate and efficiency which they store carbohydrate (Kromkamp *et al.*, 1986; Foy and Smith, 1980) enabling more polysaccharide to be stored. In continuous light cells cannot store carbohydrate as effectively and consequently an increase in carbohydrate results in an increase in the concentration of soluble intermediates of photosynthesis resulting in larger turgor pressure (Kromkamp *et al.*, 1986; Oliver, 1994).

1.3.4 Ballast change as a mechanism to regulate buoyancy

Gas vacuolated cyanobacteria with gas vesicles too strong to be collapsed by turgor pressure, such as *Microcystis* (Thomas and Walsby, 1985) and *Oscillatoria* (Utkilen, *et al.*, 1985a), regulate their buoyancy by changing the concentrations of the cellular constituents. Density differences between the colony and the surrounding media enable vertical migration. This mechanism is rapid as demonstrated by computer simulations of vertical migration (Kromkamp and Walsby, 1990). Ballast regulation is the most plausible mechanism for diel buoyancy changes (Kromkamp *et al.*, 1986).

1.3.5 Light, nutrients and ballast change

The polysaccharides, or carbohydrate, are the molecules which account for the greatest and most rapid change in cell density (Romans *et al.*, 1994). The process is driven by light but the ability to capture light and metabolise carbohydrate are also influenced by nutrient availability.

Photosynthesis and the rate of carbohydrate accumulation are dependent upon the irradiance the cell receives (Reynolds, 1984; van Rijn and Shilo, 1985; Kromkamp *et al.*, 1988; Ibelings *et al.*, 1991b; Kromkamp and Walsby, 1990). Carbohydrate concentration per cell is linearly related to the density change in *Oscillatoria agardhii* (Kromkamp and Walsby, 1990) and *Microcystis aeruginosa* (Brookes *et al.*, 1997). Consequently, turbidity, mixed depth and depth of a colony determine light exposure and the amplitude of vertical migration (Kromkamp and Walsby, 1990). Kromkamp *et al.* (1988) found that their *Microcystis* strain might always be buoyant under light limited conditions.

Nutrients have interesting but complex interactions with cyanobacterial growth and buoyancy regulation mechanisms. Oliver (1994) summarises the role of the major nutrients on buoyancy in the statement "buoyancy decreases when the major nutrients, nitrogen and phosphorus, are limiting" which is supported by studies by Klemer *et al.* (1982) and Konopka (1989).

Carbon fixed by photosynthesis is initially stored as soluble carbohydrate. The fate of this stored carbohydrate is dependent upon the nutrient status of the cell. When nitrogen and phosphorus are available the energy captured is utilised to synthesise metabolites and polymers for cell growth and carbohydrate does not accumulate (Turpin, 1991; Oliver, 1994).

Conversely when nitrogen and phosphorus are in short supply carbohydrate accumulates and buoyancy is reduced (Konopka *et al.* (1987a, b).

Konopka (1989) examining a population of *Aphanizomenon flos-aquae* found that phosphate affected the recovery of buoyancy by sinking filaments. Phosphate limited cells exposed to 100 μmol m^{-2} s^{-1} light lost buoyancy but cells in 2 μM phosphate required 200 μmol m^{-2} s^{-1} in order to lose buoyancy. This demonstrates how the buoyancy mechanism works in favour of the cell overcoming the vertical separation of light and nutrients. If the nutrient requirements of the cell are fulfilled then buoyancy is promoted in order that the cell's light requirements may also be met. If, however, the cell is under nutrient stress it sinks deeper where it may encounter more favourable nutrient conditions.

1.3.6 Light, nutrients and gas vesicle synthesis

Whether a colony or filament sinks or floats is determined by the density of the cellular constituents and the buoyancy provided by the gas vesicles. Change in cell buoyancy can also be achieved by altering the gas vesicle pool either by molecular control of gas vesicle synthesis or dilution due to growth (Hayes and Walsby, 1984; Konopka *et al.*, 1987 a, b; Oliver, 1994, Walsby, 1994).

Gas vesicle volume per cell is influenced by light (Konopka *et al.*, 1987b; Kromkamp *et al.*, 1988) and nutrients (Konopka *et al.*, 1987; Kromkamp *et al.*, 1989). Gas vesicles are composed of proteins but there is currently no studies which directly measure the influence of nitrogen on gas vesicle volume. Most studies examine the net response of nitrogen limitation on buoyancy without investigating all of the interacting factors giving rise to that response (eg Spencer and King, 1985, 1989). In this thesis a flow cytometer is used to estimate gas vesicle volume per cell by exploiting the light scattering properties of gas vesicles. This allowed rapid assessment of gas vesicle volume so numerous samples could be processed allowing multiple observations to be made through time of a series of treatments.

Konopka (1989) demonstrates the complexity of the effects of nutrients on buoyancy regulation and gas vesicle synthesis. The mechanisms contributing to buoyancy loss or gain can be

investigated individually but consideration of how the factors interact to determine whether a colony sinks or floats will be most beneficial to the advancement of our knowledge.

1.3.7 Buoyancy regulation studies

In this thesis the buoyancy strategies of two cyanobacteria are investigated, *Microcystis aeruginosa* Kütz. emend Elenkin and *Anabaena circinalis* Rabenhaerst. Hereafter these species are referred to as *Microcystis* and *Anabaena*. These species can coexist (Oliver and Ganf, 1988; Brookes *et al.*, 1994) although they display different morphology and physiology. *Microcystis* forms large colonies and *Anabaena circinalis* forms coiled, heterocyst containing filaments capable of fixing N_2. Although *Anabaena* and *Microcystis* can coexist one or the other may dominate under certain conditions. In Mt Bold Reservoir, South Australia, Oliver (1981) observed a shift in dominance from *Microcystis* to *Anabaena* as nitrogen became limiting.

Differences in buoyancy and growth responses to light, nitrogen and phosphorus will aid our understanding of why these species can coexist or occur individually. The interactions between ballast and gas vesicle volume are examined to determine whether a cell, colony or filament will float or sink in response to environmental stress or stimulus, migrate vertically or display persistent buoyancy.

1.4 Assessing cell metabolic activity and nutrient status

Seven of the eight hypotheses developed by Shapiro (1990) and Blomqvist (1994) to explain cyanobacterial dominance deal with physiological features which enable them to exploit their environment. It is often difficult to examine the effects of a particular stress or stimulus on micro-algal physiology in field situations because the phytoplankton community is composed of numerous populations which are not homogeneous or static, rather they are moving horizontally and vertically due to flow, wind-mixing and migratory mechanisms. Population increase is the best demonstration of organisms encountering favourable conditions. The rate of population increase offers an indication of how favourable conditions are, however, growth rates represent the mean response of the population to the environment over a significant time period. It would be advantageous to develop a rapid technique which measures the individual's

response to environmental stresses or stimuli in order that an estimate of population heterogeneity, proportion of active or stress tolerant cells can be made.

In this thesis a technique was developed which allowed measurements of metabolic activity to be made on individual cells. The technique used a high precision optical tool, a flow cytometer, to measure the fluorescent product of the fluorogenic probe fluorescein diacetate. FDA is hydrolysed by non specific esterases to fluorescein and acetate (Dorsey *et al.*, 1989). The hypothesis was that the more active the cell was the more FDA was hydrolysed and the greater was the fluorescein fluorescence.

1.4.1 Flow cytometry

The study of cellular activity, proliferation and differentiation has traditionally used microscopy in combination with staining technologies in order to obtain information. With the exception of direct cell counts most of the information is of "visual impressions" or qualitative data of some optical parameter and not exact numbers. Flow cytometers were developed to overcome this problem and to precisely measure high numbers of stained cells for medical and microbiological research. Flow cytometers are now used in a greater range of disciplines investigating a multitude of cellular phenomena.

Flow cytometers utilise lasers for excitation of single cells and have numerous detectors to make simultaneous, semi-quantitative measurements of multiple optical parameters. An application of flow cytometry is that it can be used to observe physiological phenomena which express themselves in the optical properties of cells (Balfoort *et al.*, 1992). These may include side or forward scatter, autofluorescence from pigments (eg chlorophyll or phycobiliproteins), immunochemical detection of biochemical compounds such as nucleic acids and proteins (Balfoort *et al.*, 1992; Vrielings, 1993; Costas and Lopez-Rodas, 1996) or fluorescent products of fluorogenic probes to assess cell viability and vitality (Widholm, 1972; Yentsch *et al.*, 1988; Selvin *et al.*, 1989; Thom *et al.*, 1993).

Viability and vitality stains are of particular interest when investigating population heterogeneity (Yentsch *et al.*, 1988), ecotoxicology and health assessment of individuals in different environmental conditions and stresses. Examples of these include survivorship assessment of

diatoms exposed to prolonged darkness (Selvin *et al.*, 1988), measurement of pollutant stresses on algal cells (Berglund and Eversman, 1988) and toxicity assessment using rotifers (Moffat and Snell, 1995).

1.4.2 Applications of flow cytometry to plankton research

In the past decade the use of flow cytometers in plankton research has increased tremendously. High-sensitivity flow cytometers have been constructed for picoplankton research (Frankel *et al.*, 1990) and portable phytoplankton specific cytometers developed for field research (Cunningham, 1990). The flow cytometer technology was adopted most enthusiastically by oceanographers where it has been used for identification and enumeration of algal cells and in conjunction with stains to assess metabolic activity and algal response to pollutants.

1.4.3 Identification and enumeration of algal samples

Considerable effort has been applied to estimating algal biomass in aquatic systems using in vivo fluorometric techniques, assays for chlorophyll and by direct counting of cell numbers. Direct microscopic determination of cell numbers is monotonous, time consuming and expensive particularly when the phytoplankton community is composed of numerous coexisting species. Flow cytometry has been used to enumerate "target" nuisance species using a quantitative injection system which records the number of events (cells) and the volume of sample measured (Borsheim *et al.*, 1989; Legner, 1990). An alternative method is to include beads of known concentration as an internal standard (Boucher *et al.*, 1991). Estimates of phytoplankton biomass in marine systems have also been determined using stains for DNA and chlorophyll autofluorescence which enables photosynthetic cells to be identified and biomass, which is linearly related to DNA, estimated (Boucher *et al.*, 1991).

In order to estimate specific cell number the optical features of the species of interest must first be identified so that it can be selected for analysis. Features of phytoplankton which can be utilised to identify them by cytometry include light scatter, deducing relative cell size and shape (Sharpless *et al.*, 1975; Cunningham and Buonnacorsi, 1992), side scatter which can be utilised to assess internal cellular complexity especially the presence of gas vesicles (Dubelaar *et al.*, 1987) and cell pigments. Chlorophyll fluorescence is a particularly useful parameter to measure (Neale *et al.*, 1989; Sosik *et al.*, 1989) as selection (gating) of chlorophyll bearing

particles effectively excludes non photosynthesising organisms and particulates. Accessory pigments such as the phycobiliproteins, phycocyanin and phycoerythrin, can be measured if the flow cytometer is fitted with a second laser (Cunningham, 1992) and are invaluable in distinguishing between green algae and the phycobilin containing cyanobacteria (Tandeau de Marsac, 1977; Tandeau de Marsac and Houmard, 1988). Further resolution in species discrimination can be achieved by obtaining pulse profiles from sensors which yields greater information on cell or colony size and morphology (Cunningham, 1992)

The measurement of multiple cell parameters culminates in a species, or group, specific "signature" or "finger print" which allows discrimination between phytoplankter based upon optical features (Yentsch and Yentsch, 1979; Yentsch *et al.*, 1983; Oldham *et al.*, 1985; Premazzi *et al.*, 1989; Balfoort *et al.*, 1992; Carr *et al.*, 1996).

Distinguishing between taxa and targeting certain species is achieved more readily by using immunochemical procedures to highlight the target species for detection by the flow cytometer. The procedure involves raising antisera directed against cell surface antigens of phytoplankton species in rabbits. Cell wall extracts may need to be used in some cases to avoid intoxication of the host animals (Vrieling *et al.*, 1993). Antibody activity was then analysed using a fluorescein isothiocyanate (FITC)-conjugated swine (or goat) anti-rabbit antibody (Shapiro *et al.*, 1989; Vrieling *et al.*, 1993). Cells are treated with the antisera then anti body and fluorescein labelled cells detected with a flow cytometer. Species specific antisera have been raised for a number of species of dinoflagellates (Shapiro *et al.*, 1989; Costas and Lopez-Rodas, 1996) and ultraplankton (Vrieling *et al.*, 1993) and most are specific to at least the genus level.

1.4.4 Vitality and Viability stains

The environment in which phytoplankton exist can change rapidly, for example, a rainfall event may increase stream turbidity and nutrient load or a water body may stratify rapidly in conditions of high solar input and low wind velocity (Imberger, 1985; Webster *et al.*, 1996). Sephton and Harris (1984) recognise this and state "primary productivity and community dynamics are influenced by the spatial and temporal interaction of nutrient availability and physical factors such as light, temperature and water column mixing. Natural phytoplankton

communities live in patchy, fluctuating environments ...". The cells in the water column respond rapidly to these environmental changes physiologically (Elrifi and Turpin, 1985; Davies and Sleep, 1989; Demers *et al.*, 1991; Wood and Oliver, 1995) but the evidence of these events take longer to manifest themselves in cell growth. Vitality and viability stains have been developed to allow rapid assessment of cell health and response to favourable environmental conditions or stress. Viability stains such as Rhodamine 123 have been used as tools for direct microscopic counting of viable cells in culture (Kaprelyants and Kell, 1992). This has proved to be a much quicker method than assessing viability by the number of microbial cells which can reproduce on nutrient agar following an incubation.

There are numerous commercially available stains but the vitality/viability stain used in these studies was the fluorogenic probe fluorescein diacetate (FDA). FDA is a non-polar, hydrophobic, lipophilic, colourless, non-fluorescent esterified compound of molecular weight 416 (Berglund and Eversman, 1988; Dorsey *et al.*, 1989). FDA readily penetrates the cell membrane (Breeuwer *et al.*, 1995) where non-specific esterases (Phosphodiesterase, Dorsey *et al.*, 1989; acetylesterase in the rotifer *Brachionus plicatilis*, Moffat and Snell, 1995) hydrolyse the FDA ester utilising the acetate as substrate leaving the fluorescent biproduct fluorescein (Dorsey *et al.*, 1989; Breeuwer *et al.*, 1995).

1.4.5 Metabolic activity studies

FDA and flow cytometry are explored as potential assays for metabolic activity of micro-algae and cyanobacteria. *Microcystis aeruginosa* was used to develop the staining protocol before a series of experiments were constructed to identify the metabolic response to light, phosphorus and nitrogen. The response to light was extremely rapid and it was postulated that the esterases converting FDA were fuelled by ATP derived from photosynthesis. The cellular response to nitrogen and phosphorus additions was slower because these elements had to be incorporated into cellular machinery. The response to nutrient additions allowed further development of the technique as a nutrient bioassay. Within 24 hours an assessment of cell nutrient status could be made which was a sufficiently short time period for the problems associated with long term incubations (Wood and Oliver, 1995) to be avoided. The FDA/flow cytometry nutrient

bioassay was then used to assess the nutrient status of phytoplankton in the lower River Murray.

1.5 River plankton and regulated rivers in Australia

The buoyancy of cyanobacteria enable them to persist in thermally stable water bodies where flow, water turbulence and wind mixing are low. The rivers within the Murray-Darling Basin typically display low summer flows providing physical conditions conducive to cyanobacterial growth. The dominant cyanobacteria in the Murray-Darling Basin include *Anabaena* spp., *Microcystis aeruginosa*, *Nodularia* and *Planktothrix*. Baker and Humpage (1994) conducted a survey of 130 sites within the Murray-Darling Basin during summer and autumn, 1990 to 1993. They found that *Anabaena* spp. were the dominant taxa at 61% of sites during the study period whereas *Microcystis* and *Nodularia* were dominant in only 13 and 12% of cases. *Anabaena* spp were the dominant taxa in 87% of the river sites and 66% of the swamp sites.

1.5.1 Flow and stratification

Flow in the River Murray is regulated by a series of weirs and consequently during low flow periods the river resembles a string of connected lakes (Walker and Thoms, 1993). Thermal stratification was disrupted by flow in Maude Weir on the Murrumbidgee River, NSW, (Webster *et al.*, 1996). When discharge through the weir decreased below 1000 ML day^{-1} stratification established and if this discharge was maintained for two weeks *Anabena circinalis* blooms developed.

A similar scenario was evident in the lower River Murray (Burch *et al.*, 1994). During periods of low flow diurnal thermal stratification established but was disrupted by late afternoon and nocturnal winds (pers. comm. Mike Burch, SA Water).

1.5.2 Flow and species dominance

Burch *et al.* (1994) and Webster *et al.* (1996) both observed increased cyanobacterial abundance during periods of low summer and autumn flow. Hötzel and Croome (1994) examining historical data collected from Burtundy on the Darling River, NSW, attribute changes in total cell density and community composition to flow and turbidity. They concluded

that seasonal temperature cycles and nutrient availability appeared less important than flow and turbidity in determining long-term variation in the algal population.

In contrast to the Darling River studied by Hötzel and Croome (1994) low nutrient concentrations are recorded in the River Murray (Mackay *et al.*, 1988). Therefore in addition to flow and turbulence, nutrients may also have a role in determining community composition and size of the algal crop in the lower River Murray. To examine nutrients in the lower River Murray nutrient analyses were performed and compared with results from growth and FDA nutrient bioassays. The nutrient status of the river phytoplankton was then assessed and the likely growth and buoyancy response predicted from laboratory studies. This information was then used to explain the dominance of *Anabaena* spp in the lower River Murray and the notable absence of *Microcystis aeruginosa*.

1.6 Cyanobacteria and water quality

Cyanobacteria are often considered nuisance organisms as they flourish in warm, stable, eutrophic conditions and produce cyanotoxins (Carmichael, 1992; Codd and Poon, 1988) and tastes and odours (Bowmer *et al.*, 1992; Hayes and Burch, 1989) which compromise water quality.

Awareness of cyanobacteria dates back to AD 77 when the Roman scholar Pliny the Elder described a cyanobacterial scum in the River Dnepr (Codd *et al.*, 1994). The first recording of cyanobacterial toxicity was documented by Francis (1878) detailing livestock deaths attributed to *Nodularia spumigena* in Lake Alexandrina, South Australia. Since then cyanobacterial "blooms" have been reported worldwide and toxic cyanobacterial blooms recorded in at least 25 countries (Codd and Poon, 1988).

Cyanobacterial toxins have been responsible for the deaths of a wide variety of animals including cattle, sheep, pigs and birds (Francis, 1878; Carmichael and Gorham, 1981; Codd and Poon, 1988). Dog fatalities have occurred following ingestion of dried cyanobacterial scums on the shoreline and invertebrate fauna and fish adversely affected within the water column (Codd, 1993; Demott *et al.*, 1991; Rabergh *et al.*, 1991). Cyanotoxins also pose a potential health hazard to humans. A significant link has been found between toxins and human

liver illness (Falconer, 1989) and some toxins may also promote tumour development (Falconer and Buckley, 1989). Perhaps the largest tragedy attributable to cyanotoxins was the death of numerous patients of a Brazilian renal hospital in which microcystin containing water was administered to patients resulting in terminal gastroenteritis and liver malfunction (Cyanotox discussion group- http//luff.latrobe.edu.au/~botbml/rocha.html).

There are two distinct groups of toxins produced by cyanobacteria, hepatotoxins and neurotoxins. Hepatotoxins specifically damage the liver cells of intoxicated organisms and neurotoxins act upon the nervous system. Anatoxin, produced by *Anabaena flos-aquae* acts as a post-synaptic neuromuscular blocking agent binding to acetylcholine receptors of torpedo electric tissue (Codd and Poon, 1988). Other neurotoxins show poisoning similar to marine paralytic shellfish poisons (Humpage *et al.*, 1994), saxitoxin and neosaxitoxin from marine dinoflagellates (Codd and Poon, 1988).

The hepatotoxins are the most common cyanotoxins with approximately 45 of 60 identified cyanotoxins being variants of the hepatotoxic cyclic pentapeptide microcystin (Carmichael *et al.*, 1988).

The volatile odorous compounds produced by cyanobacteria, such as geosmin and 2-methylisoborneol (MIB) are responsible for earthy-musty taste and odour taints in potable water (Hayes and Burch, 1989). These compounds are chemically stable (Hayes and Burch, 1989) and consequently persist in water bodies and continue to foul the water supply following the dispersal of the cyanobacterial population.

Water quality issues associated with cyanobacteria demand attention from scientists, managers and the community. Only with an understanding of the organisms and the physical and chemical climate of the water body can an effective management strategy be implemented to reduce the frequency and intensity of cyanobacterial "blooms".

Chapter 2

Protocol development of FDA staining technique

2.1 Introduction

Aquatic habitats experience variability in their physical and chemical characteristics in both temporal and spatial scales (Sephton and Harris, 1984). Phytoplankton in these habitats are differentially exposed to these features and respond to them as individuals, depending upon their specific physiological attributes. The individual response is manifested in the phytoplankton community composition which is then reflected in seasonal succession; eg. the dominance in a phytoplankton population shifting from cryptomonads to diatoms to green algae to cyanobacteria driven by decreasing water turbulence (Round, 1971).

Spatial microhabitat differences lead to heterogeneity within a population to the point where a proportion of individuals may be inactive and the active population may display a range of growth rates. A study of marine phytoplankton in the California and Maine Gulfs showed that only 15-30% of chlorophyll-fluorescing cells had measurable cell viability, detected using the fluorochrome fluorescein diacetate and fluorescence microscopy (Yentsch *et al., 1988*).

Cell counts in "open" aquatic systems give limited information about the growth rate of phytoplankton because the population sampled one day may have travelled downstream or dispersed laterally or vertically. Therefore, a useful tool in phytoplankton research would be an indicator of cell metabolic activity or photosynthetic productivity to account for intra- and interspecific heterogeneity and give insight into the primary productivity and growth potential of natural populations.

The metabolic activity of phytoplankton is often assessed using parameters associated with photosynthesis such as ATP formation, ^{14}C assimilation, oxygen evolution (Gilbert *et al.*, 1992) and more recently using fluorometry (Kolber and Falkowski, 1993; Schrieber *et al.*,

1994; Greene *et al.*, 1994). However, there exists a number of stains which are used routinely in microbiological fields to assess microbial viability and activity (Kaprelyants and Bell, 1992; Thom *et al.*, 1993) which may be suitable for use with phytoplankton (Klut *et al.*, 1988; Dorsey *et al.*, 1989; Klut *et al.*, 1989).

The aim of this research was to identify a staining technique which could rapidly assess the metabolic activity of phytoplankton in culture and therefore the stain needed to meet the following criteria: 1) the expression of the stain needed to be a function of enzyme or photosynthetic activity or related parameter; 2) readily permeate the cell; 3) be highly sensitive and easily measurable; 4) stain both eukaryote and prokaryote cells; and 5) emit a fluorescence not masked by the autofluorescence of the cell. Some stains were unsuitable for universally staining all phytoplankton taxa. An example is Rhodamine 123 which stains mitochondria, whose morphology may reflect a change in the cells metabolic activity (Klut *et al.*, 1989). However, mitochondria are not present in cyanobacteria and the outer membrane of gram negative bacteria are impermeable to Rhodamine 123 (Kaprelyants and Bell, 1992).

The stain chosen was the fluorogenic probe fluorescein diacetate (FDA) which is a nonfluorescent probe and readily permeates cells. Inside the cell non specific esterases cleave the acetate groups and the fluorescent product, fluorescein, remains (Dorsey *et al.*, 1989). The esterases responsible for FDA hydrolysis are involved with phospholipid turnover in the cell membrane which appears to be correlated with metabolic activity (Dorsey *et al.*, 1989).

The detection of fluorescent product was achieved by measuring fluorescence with a flow cytometer which also yielded data on light scatter and chlorophyll autofluorescence. Fluorescein and fluorescein derivatives are considered good fluorochromes for flow cytometry because they have high extinction coefficients and quantum yields and are readily loaded and measured in intact cells (Breeuwer *et al.*, 1995).

To validate the applicability of FDA and flow cytometry to phytoplankton research a rigorous protocol needed to be constructed, including standardisation of incubation time and FDA concentration. The aim was to develop a protocol for FDA staining and detection which could then be used to assess the metabolic activity of cyanobacteria under a range of nutrient and light

stresses. FDA conversion kinetics were also examined with emphasis on the factors which limit FDA conversion and cause fluorescence quenching. In particular answers to the following questions were sought.

2.1.1 What is hydrolysing FDA and where?

Bacteria can convert FDA (Chrzanowski *et al.*, 1984; Diaper and Edwards, 1994) and are often associated with the mucilage encompassing cyanobacterial cells. To ensure FDA conversion was due to intracellular esterases and not bacteria or esterases associated with the cell wall, FDA treated *Microcystis* cells were viewed with a confocal microscope to identify where fluorescent product was accumulating. Free bacterial forms were excluded from analysis by gating for chlorophyll positive cells with the flow cytometer.

2.1.2 What is the time dependence for FDA conversion to fluorescein?

There are a number of rigorous published techniques for staining with FDA (Moffat and Snell, 1995: Dorsey *et al.*, 1989: Breeuwer *et al.*, 1995). Time course and concentration dependent FDA conversion data are available for a number of cell types. Dorsey *et al.* (1989) have shown time dependent fluorescein increases (from FDA conversion) for four oceanic phytoplankton genera. These genera display a four fold difference in mean relative fluorescence (fluorescein) intensity per cell, which highlights the need to examine esterase kinetics for each species of focus. FDA concentration and time dependent FDA conversion was examined with *Microcystis aeruginosa* to ascertain the optimum FDA conversion and incubation time for future assays.

2.1.3 Is FDA conversion limited by uptake or esterase hydrolysis?

It is generally assumed that non-polar, esterified compounds, such as FDA, diffuse freely into intact cells (Breeuwer *et al.*, 1995). However, when using FDA as a probe for metabolic activity it is a critical assumption that the factor limiting FDA conversion to fluorescein is esterase activity, not cell permeability to FDA. Breeuwer *et al.* (1995) suggest that fluorescein formation is most likely limited by the hydrolysis rate, in contrast to 5-(and 6-) carboxyfluorescein formation from cFDA which is limited by the rate of influx. An aim of this work was to conclusively show that FDA conversion is esterase limited in phytoplankton. However, in a system where the measurable product (fluorescein) is a function of both influx

and enzyme activity it is difficult to isolate the two and investigate the uptake mechanism and hydrolysis kinetics separately. To overcome this an experiment was designed to compare the FDA conversion rates of cells with different FDA exposure times. The hypothesis was that if FDA readily permeates cells there will be sufficient intracellular FDA to saturate esterase activity (for some time period) irrespective of FDA loading time. This effectively isolated uptake from esterase conversion by varying uptake time but allowing the same time for esterase activity.

2.1.4 How does pH influence fluorescence quenching?

Oparka (1991) in a review of fluorescent probes identified efflux and pH dependence as two disadvantages of using fluorescein derivatives for research. Dorsey *et al.* (1989) state that fluorescein is hydrophilic and thus does not readily leak through healthy cell membranes. However studies by Prosperi (1990) and Breeuwer *et al.*, (1995) suggest there is significant fluorescein efflux. Martin and Lindquist (1975) found that fluorescein fluorescence was pH dependent which is important as ageing and dense phytoplankton cultures frequently have elevated pH levels. An experiment is reported here which examined FDA conversion at a range of pH values, and an optimum pH range was identified for all future assays.

2.2 Methods

2.2.1 Cyanobacterial cultures

Microcystis aeruginosa culture SHEP 100, supplied by Dr. Gary Jones, CSIRO Land and Water, was grown in unbuffered ASM-1 Media (Gorham, *et al.*, 1964) with supplementary carbon added as 150 µM $NaHCO_3$. Cultures were maintained in a culture room at 25 °C and grown under cool white fluorescent lights at an intensity of 100 µmol photons m^{-2} s^{-1} (hereafter units expressed as µmol m^{-2} s^{-1}), 15:9 light; dark cycle, in acid washed Erlenmeyer flasks prior to experimentation. Log phase cultures were used for the development of the protocol at a concentration which enabled at least 1000 events to be measured on the flow cytometer in one minute; usually $>2x10^4$ cells mL^{-1}. Cells used in analysis were selected based upon size, using forward scattering, and positive chlorophyll fluorescence.

2.2.2 Flow cytometer setup

The flow cytometers used in these studies were Becton Dickinson FACStar^{plus}, FACScan or FACStrak. The excitation light beam was supplied by an Argon laser (488nm; 50mW). The sample tubing was 200 μm and the nozzle 70 μm in diameter. The photomultiplier tube for green fluorescence (530 nm) was initially set such that untreated cyanobacterial cells appeared in the first decade of a log scale of relative (fluorescein) fluorescence. Fluorescein isothiocyanate (FITC)-labelled beads (Becton Dickinson cat #95-0002) were used to standardise the machine and allow day to day comparisons. Cells used in analysis were selected based upon size, using forward scattering, and positive chlorophyll fluorescence. The gas vesicles were collapsed prior to flow cytometric analysis to prevent light scattering interference.

2.2.3 Kinetics of Fluorescein diacetate uptake and conversion

*2.2.3.1 Concentration dependence of FDA hydrolysis**

Fluorescein diacetate (Sigma chemical Co.) was stored in acetone at -20°C at a stock concentration of 10 mg mL^{-1}. To ascertain the influence of external FDA concentration on FDA hydrolysis (a function of uptake and esterase activity) a range of FDA concentrations were prepared by adding varying amounts of the stock solution to 10 mL of deionised water. 300 μL of these solutions were then added to 400 μL of *Microcystis* culture which were incubated at room temperature for seven minutes prior to measurement of fluorescein using a FACStar^{plus} flow cytometer.

*2.2.3.2 Time course of FDA hydrolysis**

To examine FDA hydrolysis with time 40 μM (final concentration) FDA was added to *Microcystis* culture as above and the rate of accumulation of fluorescein measured at 2-3 min intervals using a flow cytometer.

2.2.3.3 The isolation of FDA uptake and hydrolysis to determine the rate limiting step

To determine whether FDA influx or esterase activity limits the rate of FDA hydrolysis an

* Experiments examining concentration and time dependence of FDA hydrolysis were conducted by Dr. Sean Geary based on the technique of Dorsey *et al.*, (1989). They are included here as a reference for future experiments.

experiment was designed to vary the uptake rate but maintain the incubation time of FDA in two treatments. For the first treatment *Microcystis* cells were incubated in 40 µM FDA and the hydrolysis of FDA, as indicated by fluorescein accumulation, was measured at one minute intervals using a flow cytometer.

The second treatment varied the time of exposure to a saturating concentration of FDA to assess whether sufficient FDA was loaded in one minute to satisfy substrate demand of esterases for some future period where external FDA concentrations are limiting. *Microcystis* cells were incubated in 40 µM FDA for one minute before they were diluted one in 25 into ASM-1 media, reducing the external FDA concentration to 1.6 µM. Fluorescein was again measured every minute using the flow cytometer.

2.2.3.4 pH dependent fluorescence quenching

pH may affect the expression of fluorescein fluorescence in two ways, either by altering the intracellular pH affecting enzyme function or by quenching the fluorescence (Martin and Lindquist, 1975). To determine over what range pH dependent fluorescence quenching occurs, log phase cultures of *Microcystis*, were transferred into pH adjusted ASM-1 media at a range of pH values (5.2, 6.1, 6.8, 7.3, 7.5, 8.2, 9.0, 9.2) immediately prior to incubation in 40 µM FDA for seven minutes at which time fluorescein was measured. The pH was adjusted using HCl and NaOH and the medium was not buffered. Some pH drift occurred between adjusting the pH and incubating the cells in this media, however, this was corrected for by measuring the pH immediately before experimentation.

2.3 Results

2.3.1 Accumulation of fluorescent product

The net accumulation and expression of fluorescein within cells is a function of FDA influx, fluorescein efflux and the pH dependent fluorescence quenching which all alter with time, FDA concentration and cell health. Confocal microscopy of FDA stained *Microcystis aeruginosa* cells revealed the presence of fluorescein throughout the cytoplasm indicating that the cleavage of the FDA molecule and accumulation of fluorescein occurred within the cell.

2.3.2 Time dependence of FDA hydrolysis

The accumulation of fluorescein within cells was linear over the initial 10 minutes in 40 µM FDA (figure 2.1). There was no further increase in fluorescence between 10 and 20 minutes. In order to use the rate of FDA conversion as an indicator of metabolic activity and allow comparisons between experiments a standard incubation time and FDA concentration were required. It was necessary to select a standard time for fluorescein measurement which was not confounded by the phenomena giving rise to the plateau in fluorescence after 10 minutes. A time of 7 minutes was chosen because the rate of conversion was still linear and sufficient fluorescein had accumulated to allow discrimination between treatments in future experiments. Fluorescein measurements henceforth are expressed as hydrolysis rates; fluorescein diacetate conversion rates (FDACR), with the units "relative fluorescence units min^{-1}".

2.3.3 Concentration dependence of FDA hydrolysis

The rate of FDA hydrolysis is dependent upon substrate availability (figure 2.2) and is saturated at FDA concentrations greater than 15 µM. However the shape of the curve approximates a Blackman curve (Salisbury and Ross, 1985), not the typical Michaelis-Menton enzyme-substrate curve, which suggests a diffusion restriction of FDA. The flattening of the FDA conversion rate (FDACR) at concentrations higher than 15 µM could be due to saturation of esterases or an indication that the maximal solubility of FDA in water has been reached. Breeuwer *et al.* (1995) suggest that FDA precipitates from solution at concentrations of approximately 10 µM. If the stock solution was pipetted in a fast stream into the water minimal precipitation occurred, this working solution was discarded after 45 minutes and fresh solution made.

2.3.4 The isolation of FDA uptake and hydrolysis to determine the rate limiting step

A simple experiment was designed to demonstrate that the rate of FDA conversion was determined by esterase activity not cell permeability. Cells were incubated either for seven minutes in a saturating concentration of FDA (40 µM) or in saturating concentrations for one minute prior to dilution into non-saturating concentrations (1.6 µM) for six minutes. Cells exposed for one minute displayed a similar rate of FDA hydrolysis as cells continuously

exposed to saturating levels of substrate (figure 2.3). This suggested that there was sufficient FDA uptake to satisfy esterase substrate demand and esterase activity, not influx, is the rate limiting step. It appears that some substrate limitation may be induced with time as cells loaded for one minute display no further accumulation of fluorescein after nine minutes, however, this is a function of experimental design not diffusion limitation.

2.3.5 pH dependent fluorescence quenching

Cells were placed in a series of pH adjusted media prior to incubation in FDA and fluorescein measurement to ascertain the impact of pH on fluorescein fluorescence quenching. The pH range examined was from 5.2 to 9.2. Fluorescein fluorescence was quenched at pH greater than 9 (figure 2.4). The maximal expression of fluorescein occurred at pH 6.8 with the more acidic media (pH = 5.2) showing only slightly less fluorescence.

2.4 Discussion

The hydrolysis of FDA follows first order kinetics in yeast (Breeuwer *et al.*, 1995) and in selected phytoplankton (Gilbert *et al.*, 1992). However, in *Microcystis* a few anomalies arise which may be due to properties of both FDA and *Microcystis*. If FDA hydrolysis was only a function of FDA availability and esterase activity the conversion of FDA to fluorescein (fig 2.1) should continue at a linear rate until substrate limitation occurred. However, there was a linear accumulation of fluorescein for nine minutes at which point no further net change occurred. There are a number of explanations for this, however it is more than likely due to a combination of several interacting factors. Substrate limitation is perhaps the most obvious possibility for a decrease in FDACR, however it was demonstrated that one minute of loading with FDA maximises FDACR (figure 2.3) so it is unlikely substrate limitation was induced in this experiment where external FDA concentrations were saturating. Oparka (1991) identified efflux and pH as potential problems of using FDA and these may partially explain the phenomena observed here. Fluorescein is negatively charged at physiological pH (Prosperi, 1990) and therefore the membrane potential would favour the efflux of fluorescein leading to a decrease in the intracellular fluorescein concentration. A further explanation for the apparent

decrease in FDACR could be the pH dependence of esterase activity and fluorescein fluorescence. The hydrolysis of the FDA molecule yields protons which could acidify the cytoplasm to a point where esterase function is impaired or fluorescein fluorescence is quenched.

The variation in fluorescent response of cells incubated in FDA at different values of pH highlights the need to ensure that all samples lie within the pH range 5-8, ageing cultures tend to have elevated pH.

The primary objective was to develop a protocol for the staining of phytoplankton to assess cellular metabolic activity and to investigate some kinetics and limitations of the technique. The hydrolysis of FDA was conclusively shown to be limited by esterase activity not membrane permeability.

In future experiments the FDA staining protocol was tested to discriminate between nutrient stressed and healthy populations. The primary assumption behind this work is that if a cell 's chemical and energy requirements are met then it will proliferate and the esterase activity, as indicated by FDACR, will be greater than an energy or nutrient limited population. However the development of this protocol has eluded to other factors which may be influencing fluorescein expression besides esterases alone. There is some evidence that acidification of the cytoplasm inhibits photosynthesis (Reid *et al.*, 1989) and may not only impair esterase function and reduce ATP supply but also quench fluorescein fluorescence. Additionally stressed cells have greater membrane permeability so efflux of fluorescein may be greater. Regardless of the mechanisms governing the expression of fluorescein the hypothesis remains the same; healthy cells will display greater fluorescein fluorescence following incubation with FDA than will less healthy cells.

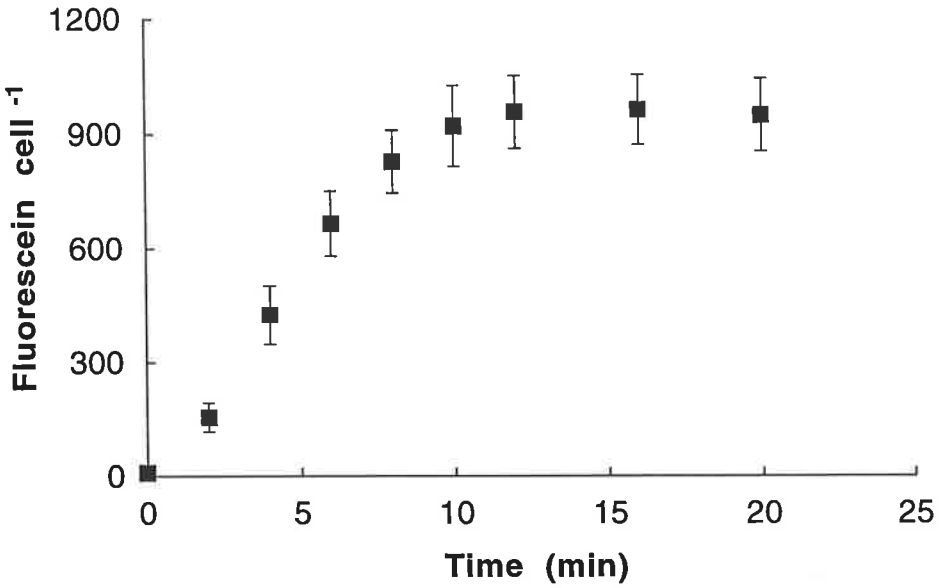


Figure 2.1 Time course for the net accumulation of fluorescein per cell by *Microcystis aeruginosa* . FDA was added at a concentration of 40 µM. Fluorescein is the product of FDA hydrolysis and was detected with a flow cytometer; excitation 488 nm, detection 530 nm.

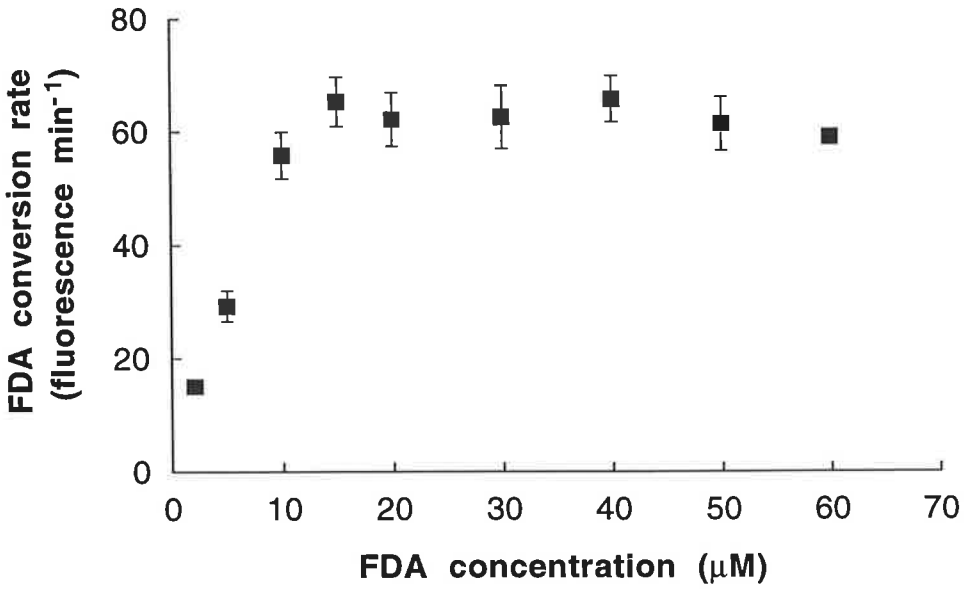


Figure 2.2 Relationship between the rate of FDA conversion to fluorescein (fluorescence $cell^{-1}$ min^{-1}) at various external FDA concentrations. Fluorescein fluorescence per cell was detected using a flow cytometer; excitation 488 nm, detection 530 nm.

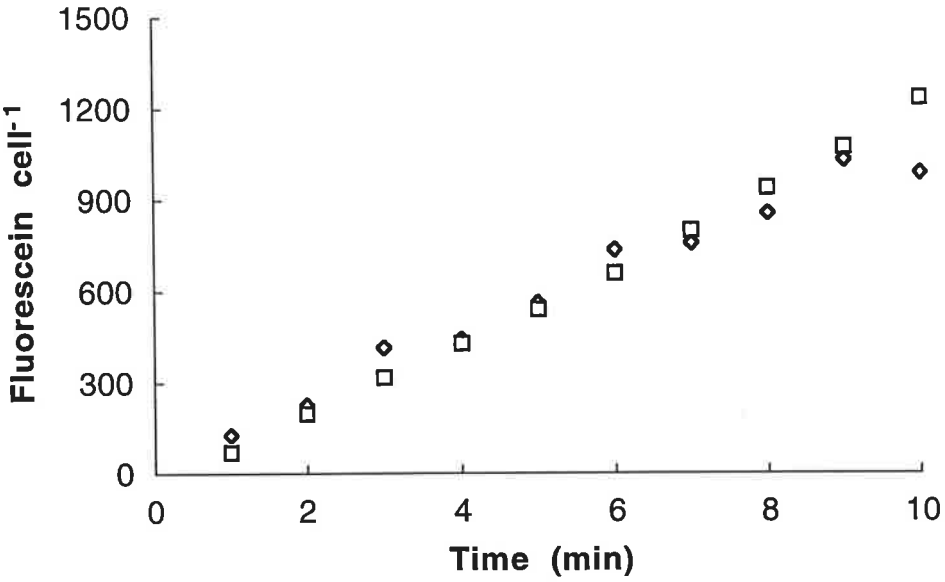


Figure 2.3 *Microcystis* cells were incubated in 40 µM FDA for either a) seven minutes (□) or b) one minute and then diluted to 1.6 µM for a further 6 minutes (◊). The rate of hydrolysis is independent of loading time indicating FDA conversion is limited by enzyme activity not uptake.

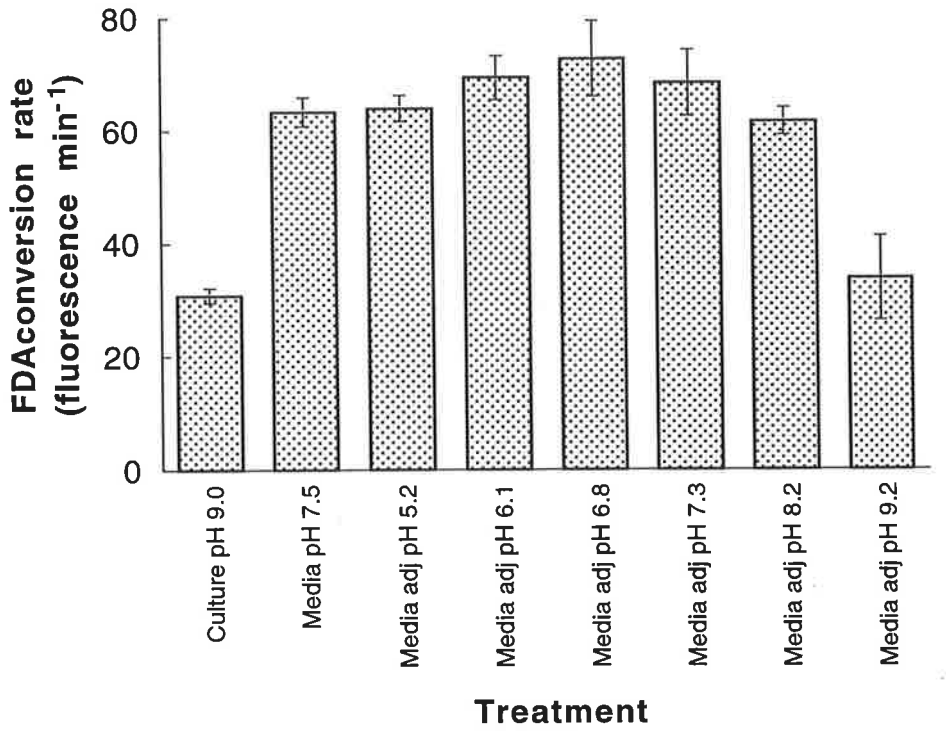


Figure 2.4 Relationship between rate of FDA conversion and external pH.

Chapter 3

Assessment of FDA and flow cytometry to determine cell health

3.1 Introduction

Aquatic environments have complex chemical and physical properties which display variation spatially and temporally. Temporal variation may be on a short time scale such as a rainfall event or turbulence (Reynolds, 1994) altering the nutrient load and optical properties of the water body, or on a long time scale with trends peculiar to seasons such as persistent stratification in mediterranean summers. Pelagic organisms are subjected to this environment and the phytoplankton respond according to their previous energy and nutrient history and their specific physiological attributes.

Light and nutrients are key elements for phytoplankton growth and the limitation of one or more of these factors limits the growth potential and ultimately the yield of a population (Hecky and Kilham, 1988). The nutrient and light history of an organism is manifested firstly in the physiology of individuals and secondly in the generation of new individuals. The cellular response to changes in the nutrient or light climate are rapid as evidenced by the rapid change in photosynthetic rate at different light intensities (Dubinsky *et al.*, 1987) or perturbations in the fluorescence from photosynthetic pigments in response to nutrient enrichment (Elrifi and Turpin, 1985; Wood and Oliver, 1995). At the population level it takes longer to observe growth in response to changes in the light or nutrient climate. The doubling of a population may take more than 24 hours (Reynolds, 1984) depending upon the species, the energy input and nutrient availability. Growth is difficult to measure directly in the field without isolating individuals from the environment which gave rise to the observed growth rate.

Light is attenuated as it penetrates a water body at a rate governed by the suspended particulates and dissolved substances. There is also a shift in the spectral composition of the light as

wavelengths are differentially attenuated (Kirk, 1981;1984). Turbulence and the mixed layer depth also influence exposure of phytoplankton to light as water movement carries suspended particles through a vertical light gradient. The interaction between light attenuation, water turbulence and the light dose phytoplankton receive is further complicated by passive and active migration mechanisms. Phytoplankton employ mechanisms which allow them to sink, float (Walsby, 1994), swim, migrate by self induced convection (Green and Diez, 1995) or attached vorticellids (Canter *et al.*, 1992) and even select a preferred light climate (Konopka, 1982; Ganf *et al.*, 1991).

Consequently a sample of phytoplankton taken from a particular depth need not necessarily represent the climate at that depth but an average of individuals migrating from different depths and micro-climates. Whittington *et al.* (1996) have used the maximum change in quantum yield of fluorescence from chlorophyll *a* to estimate the light history of phytoplankton and track their movement in Chaffey Dam, NSW. Chlorophyll fluorescence has also been used by Harris (1980, 1984) to estimate previous light history of phytoplankton and exposure to water turbulence.

FDA and flow cytometry have been used previously to investigate metabolic activity and the dark survival of microalgae (Dorsey *et al.*, 1989), however, no attempt has been made to examine the effect of light dose (light intensity*time) on cell metabolic activity. If FDA conversion rate (FDACR) increases with increasing light dose then this may provide a technique to determine the previous light history of phytoplankton in a water column and provide insight into the vertical migration of algae. FDACR was shown to be dependent on light dose (this chapter) and therefore all experiments which required day to day comparisons were sampled at the same time of day ensuring cells had received the same light dose and comparisons were valid.

Phototrophic organisms, such as cyanobacteria, utilise light as their primary energy source. Cyanobacteria are adapted for "shade" environments (Kirk, 1983), where light is often in short supply, and growth and photosynthesis are saturated at low light. The algal response to light is a rapid process as cells capture light, photosynthesise and distribute ATP for the maintenance of cell function. This rapid response is evident in measurements of photosynthesis such as

oxygen production or ^{14}C assimilation which occur almost instantaneously when cells are exposed to light.

A relationship exists between the photosynthetic activity and FDA hydrolysis in cells of different sizes incubated at 100 µmol m^{-2} s^{-1} (Dorsey *et al.*, 1989). In this chapter photosynthesis and FDA hydrolysis were measured in cells incubated at different irradiances to investigate the influence of photosynthesis on esterase activity. FDACR increased as light and photosynthetic activity increased which led to the hypothesis that ATP derived from photosynthesis fuelled esterases and FDA hydrolysis.

Light dependent FDA conversion could be due to light-sensitive enzymes or related to the photosynthetic process and esterases fuelled by photosynthetically derived ATP. The inhibitor 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea (DCMU) inhibits electron flow from photosystem II to I and thus inhibits oxygen production and reduction of $NADP^+$. If esterases were just light activated then DCMU should not affect their function and FDA conversion with and without DCMU should occur at the same rate. If the rate of FDA hydrolysis with and without DCMU is different it could be assumed that energy supply via photosynthesis limits and controls esterase activity, provided there is no direct effect of DCMU on esterases or FDA.

The physiological response of algae to light is evident on different time scales. The most rapid response to light is a change in photosynthesis. Growth as a result occurs over a much longer time scale. Experiments were designed to observe algal response to light over three time scales, 2 hours, 24 hours and 2-8 days. FDA conversion rate was compared with the traditional methods of measuring algal physiological response, namely photosynthesis and growth.

Nutrient concentrations in water bodies may display vertical gradients as nutrients are released from the sediments under reducing conditions associated with hypolimnetic oxygen depletion (Martinova, 1993) and stripped from the euphotic zone by sedimentation of algae (Hecky and Kilham, 1988). This difference in nutrient concentration ultimately affects the ability of water from different depths to support a phytoplankton population (Ganf and Oliver, 1982). The nutrients phytoplankton are exposed to, and can take up, is again governed by water movement,

vertical migration, the uptake kinetics of the phytoplankton, the bioavailability of the nutrients, and the flux rates of the nutrients to and from particulates and sediments.

Limnologists have developed numerous assays to estimate the nutrient status of water bodies and to determine phytoplankton response in terms of nutrient bioavailability and limitation. These assays are reviewed in Hecky and Kilham (1988) and Wood and Oliver (1995) and include chemical analysis of nutrient concentrations, algal growth bioassays, cellular ratios of carbon: nitrogen: phosphorus, pigment concentration and physiological assays for nutrient uptake rates. These techniques all have limitations (Hecky and Kilham, 1988; Wood and Oliver, 1995). These include time constraints, removing phytoplankton from the nutrient flux and species shift in bioassays. Consequently, alternative methods have been sought to measure phytoplankton nutrient status using technologies such as fluorometry to investigate perturbations in the chlorophyll *a* fluorescence in response to nutrient enrichment (Wood and Oliver, 1995).

The cellular response to an improved nutrient climate is rapid as nutrient limited cells have a high affinity for nutrients (chapter 6). In contrast to the very rapid increases in FDACR in response to light, changes in FDACR in response to nutrient addition takes longer to be manifested in metabolic activity. This is because the nutrients must first be incorporated into cell pigments, proteins, nucleotides and other cell components before esterase activity is increased.

FDA conversion is a measure of cell metabolic activity (Dorsey *et al.*, 1989; Breeuwer *et al.*, 1995; Moffat and Snell, 1995) and consequently offered potential as a bioassay to detect recovery of nutrient limited cells when the limiting nutrient was replaced. To avoid problems associated with long incubations detection of nutrient limitation needed to be rapid. Time course experiments were carried out to identify how long it would take for the cell metabolism to increase following enrichment with the limiting nutrients.

The value of FDA and flow cytometry in phytoplankton research depends upon its ability to discriminate between cells with different relative metabolic activities. It may also offer insight into the historical light and nutrient climate the organism has encountered. This chapter

evaluates the FDA protocol described in the previous chapter and tests whether it can be used to discriminate between metabolic activities of cells from different nutrient and light climates. The FDA conversion rate was compared with the more traditional assays; growth rate, nutrient bioassays and photosynthetic rate.

3.2 Methods and experimental design

3.2.1 Light and metabolic activity

The affect of light on the ability of *Microcystis* to hydrolyse fluorescein diacetate was examined over three time scales; 2 hours, 24 hours and 2-8 days. At the shortest time scale FDACR was related to photosynthetic capacity, and to growth rate at the longest time scale. An assessment of the effect of light intensity on FDA hydrolysis by *Microcystis* was conducted over a 24 hour period. All results are expressed as means of three replicates for each treatment and error bars indicate standard deviation.

3.2.1.1 Short term light induced changes in Microcystis *FDA hydrolysis*

An experiment was constructed to assess the potential of FDA in combination with flow cytometry to discriminate between populations grown at different light intensities. A log phase *Microcystis* culture (see Chapter 2 for culture and media description) was incubated in the dark for 9 hours prior to experimentation. Subsamples were then incubated at 6 light intensities (0, 15, 40, 80, 150, 250 μmol m^{-2} s^{-1}) for 2 hours after which the rate of FDA hydrolysis was measured. Fluorescein was measured per cell using a Becton Dickinson FACStrak flow cytometer following a 7 minute incubation in 40 μM FDA.

The photosynthetic rate was measured for each subsample at the light intensity it had previously been exposed to in order to compare FDACR with photosynthesis. Photosynthesis was measured as oxygen evolution using a Rank Brothers oxygen electrode with a water-cooled (25°C) perspex chamber mounted on top following the technique of Dubinsky *et al.* (1987). The algal suspension was continuously stirred within the chamber and illuminated by a projector fitted with neutral density filters to achieve the desired irradiance. The light intensity

was measured immediately in front and behind the chamber with a LiCor Li1000 quantum sensor and the average intensity estimated using the equation of Dubinsky *et al.* (1987).

3.2.1.2 Time series of FDA conversion by Microcystis *incubated at different light intensities*

The effect of light intensity and exposure time on the ability of *Microcystis* to hydrolyse FDA over a medium time period (24 hours) was examined in an experiment where cells were grown at three different light intensities (10, 50, 100 µmol m^{-2} s^{-1} on a 12:12 light:dark cycle at 25°C). After a 12 hour dark pretreatment these were incubated for eight hours at the light intensity used for growth followed by a 9 hour dark period and then another 7 hour light period to simulate a diel light:dark cycle. The FDA conversion rate was measured regularly throughout the course of the trial.

3.2.1.3 FDA hydrolysis by Microcystis *grown at different light intensities*

Microcystis cells were subcultured into 100 ml Erlenmeyer flasks and grown at 8 different light intensities (11, 23, 44, 63, 81, 100, 150 and 244 µmol m^{-2} s^{-1}) on a 12:12 light: dark cycle at 25°C. These experiments were sampled at the same time of day, every two days for eight days and metabolic activity estimated by the FDA hydrolysis technique. Growth rate was measured by microscopic determination of cell numbers on day zero and eight and using a Zeiss microscope and haemocytometer.

3.2.1.4 FDA measured activity in the presence and absence of DCMU

FDACR of *Microcystis* cells was measured in the presence or absence of an inhibitor of Photosystem II (DCMU) and with or without light exposure to determine whether esterases were light activated or directly related to the photosynthetic process. The photosynthetic rate and FDACR of log phase *Microcystis* cultures, which had been pretreated in the dark or 150 µmol m^{-2} s^{-1}, were measured with and without the addition of 10 µM DCMU immediately before FDA incubation. Treatments were replicated three times and FDACR expressed as the mean rate of fluorescein production per minute per cell.

3.2.2 The use of FDA to discriminate cells with different nutrient limited growth rates

The FDA staining protocol was tested for its ability to discriminate between phytoplankton grown in different nitrogen or phosphorus concentrations. Phosphorus limited cultures were established by collapsing the gas vesicles of a log phase *Microcystis* culture then centrifuging the cells, decanting the supernatant and resuspending the cells in P-free media; the centrifuging and resuspension step was repeated four times. Cultures were then grown in P-free media for seven days, to dilute the polyphosphate pool, prior to inoculation into ASM-1 media with eight different phosphate concentrations (0, 0.1, 0.2, 0.5, 1,2,5,10 μM) added as Na_2HPO_4. KCl was added to replace the potassium usually present as K_2HPO_4. The rate of FDA conversion by the P-limited cells and the growth rate were determined on days 0, 2, 4, 6, 8 after inoculation. Cultures were grown in triplicate in a temperature controlled (25°C) cabinet at 100 μmol m^{-2} s^{-1} (12:12 light:dark cycle). FDA conversion rate was measured after six hours light exposure on each sampling day to ensure that all samples had received the same irradiance so were comparable between days and observed differences were due to phosphate limitation not light.

A similar experiment was performed with nitrogen limited cultures, however these cells were only deprived of nitrogen for two days prior to inoculation into ASM-1 with nitrogen concentrations of 0, 2, 5, 10, 25, 50, 100, 200 μM. FDACR was measured and samples taken for cell counts on days 0, 3, 6. Culture conditions were the same as those described above for phosphate treatment. FDA conversion rate was measured following six hours of light exposure on each sampling day.

3.2.3 Evaluating the FDA staining protocol as a rapid assay to detect nutrient limitation

To evaluate the applicability of the FDA staining protocol as a rapid assessment of nutrient limitation it was necessary to establish that the technique could determine the individual influence of different nutrients. It was also necessary to determine the time period for the improved nutrient environment to be detected in the enzyme activity.

Nitrogen and phosphorus limited batch cultures were prepared using the method described above. Each culture was then divided into four 50 mL Erlenmeyer flasks and spiked with the following nutrient combinations (n=3). Nitrogen was added as nitrate and phosphorus as phosphate.

Nitrogen limited culture-spiked with 1) no nitrogen, 2) 100 µM N, 3)100 µM P, 4) 100 µM N and 100 µM P

Phosphorus limited culture-spiked with 1) no phosphorus, 2) 100 µM N, 3)100 µM P, 4) 100 µM N and 100 µM P

Nitrogen and Phosphorus limited culture-spiked with 1) no nitrogen and no phosphorus or 2) 100 µM N and 100 µM P

The ability of these cultures to hydrolyse FDA was estimated initially and then daily for three days using the FDA staining protocol described above.

Cultures were grown in triplicate in a temperature controlled (25°C) cabinet at 100 µmol m^{-2} s^{-1} (12:12 light:dark cycle). FDA conversion rate was measured following six hours of light on each sampling day ensuring all samples had received the same light exposure and so were comparable between days.

3.3 Results

3.3.1 Short term light induced changes in *Microcystis* FDA hydrolysis

Photosynthetic rate and FDA conversion rate increased with light intensity (figures 3.1, 3.2). Photosynthetic rate was greatest in cells at 250 µmol m^{-2} s^{-1} (400 nmoles 0_2 $(10^6$ cells$)^{-1}$ min^{-1}). The FDACR increased linearly (r^2=0.98) up to a light intensity of 80 µmol m^{-2} s^{-1} above which there was no further increase. The maximal FDACR corresponded with photosynthetic rates greater than 265 nmoles 0_2 $(10^6$ cells$)^{-1}$ min^{-1} (figure 3.3). An explanation could be that the non-specific esterases were receiving sufficient photosynthetically derived ATP at this photosynthetic rate to enable them to operate at their maximum level. Alternatively FDACR could be sustained by ATP generated by respiratory pathways utilising carbohydrate

stored during photosynthesis. The following experiment with DCMU clarifies these explanations.

3.3.2 FDA measured activity in the presence and absence of DCMU

Cells from dark and light pretreatments were treated with DCMU and the FDA conversion rate and photosynthetic rate measured to validate that the observed differences in FDACR at different light intensities were due to the photosynthetic response of the organisms. There was no net production of oxygen in the dark or in the light in the presence of DCMU (table 3.1) but a rate was measurable in the light in the absence of DCMU (780 ± 150 nmoles 0_2 $(10^6$ cells$)^{-1}$ min^{-1} at 150 µmol m^{-2} s^{-1}. The FDACR showed a similar pattern. The basal rate of FDA conversion in the dark was the same regardless of DCMU presence (27 relative fluorescein units min^{-1}). With a two hour light pretreatment the esterase activity increased to a level nearly four times greater than the dark measured rate (27 ± 2.79 in the dark compared with 102 ± 3.28 relative fluorescein units $cell^{-1}$ min^{-1} in the light). The FDACR measured in the dark and in the presence of DCMU may be fuelled by ATP derived from respiration.

	Dark		Light	
	-DCMU	**+DCMU**	**-DCMU**	**+DCMU**
FDACR	27 (2.79)	27 (0.66)	102 (3.28)	22 (0.5)
Photosynthetic rate	No net O_2 production	No net O_2 production	780 ±150	No net O_2 production

Table 3.1 To demonstrate that esterase activity was dependent on photosynthesis the FDA hydrolysis by *Microcystis* was measured in light, darkness and in the presence and absence of DCMU (an inhibitor of electron flow from Photosystem II). Units for FDACR are: relative fluorescein units $cell^{-1}$ min^{-1} and photosynthetic rate: nmoles 0_2 $(10^6$ cells$)^{-1}$ min^{-1} Values are the mean of three replicates and standard deviations are shown in parentheses.

3.3.3 Time series of FDA conversion by *Microcystis* incubated at different light intensities

The metabolic activity of *Microcystis* cells responded to light dose. Cells incubated at three different light intensities with alternating light/dark cycles displayed differential ability to

hydrolyse FDA dependent on light dose and previous light history (figure 3.4).. Cells grown in 10, 50 and 100 μmol m^{-2} s^{-1} had initial FDA conversion rates of 66, 168 and 264 relative fluorescein units min^{-1} respectively and upon exposure to light the FDACR $cell^{-1}$ increased to 100, 262 and 320 relative fluorescein units $cell^{-1}$ min^{-1} after eight hours. Following nine hours dark FDACR decreased but recovered in the second light period. This rapid response to light intensity and exposure time highlighted the importance of sampling at the same time on each day to enable day to day comparisons of results.

Whether the FDACR decreased to the minimum level instantaneously when the lights were switched off was not conclusively determined. Respiratory consumption of stored carbohydrate may have provided some ATP for esterase function and continued FDA conversion, however, the rapid decrease in FDACR when photosynthesis was inhibited with DCMU would suggest that FDACR would also decrease rapidly in the dark.

3.3.4 FDA hydrolysis by *Microcystis* grown at different light intensities

It was previously established that light intensity and exposure time impact upon *Microcystis* FDA hydrolysis in the short term. To determine the long term influence of light on FDACR *Microcystis* cultures were grown at eight light intensities for eight days and the growth and FDACR monitored. Growth, after eight days, was maximal and constant between 50 and 150 μmol m^{-2} s^{-1} (figure 3.5) at 0.4-0.45 ln units day^{-1}. At 244 μmol m^{-2} s^{-1} there appeared to be a slight decrease in growth rate relative to the maximum growth which may indicate some degree of photoinhibition.

A two way Anova was performed with FDACR as the response and light intensity and time as factors (the statistical package used was JMP (SAS, 1996); n=3, p=0.05) to examine whether there was any difference in the FDA conversion rate with time (figure 3.6). Results of this Anova (table 3.2) conclude that light intensity affected the FDA conversion rate, there was no effect of time, however, there was an interaction between time and irradiance. The interaction between light and time could indicate that the cells were adaptation to a particular light intensity. Alternatively this interaction maybe a negative response to a low light as energy became increasingly limited with the depletion of carbohydrate stores. It is emphasised again that measurements were taken at the same time each day so cells were exposed to light for the same

time prior to measurement ensuring that the effect of light intensity and exposure time were delineated.

Source	Nparm	DF	Sum of Squares	F ratio	Prob>F
Light intensity	7	7	8761271	80.84	<0.0001
Time	3	3	41594	0.8955	0.4485
Light intensity*time	21	21	1371425	4.2182	<0.0001

Table 3.2 Two way analysis of variance of data presented in figure 3.6 where light and time were assessed as factors giving rise to the observed FDA conversion rate response. Only light intensity has an effect on FDA conversion rate ($p<0.05$).

Figure 3.6 displays the FDA measured metabolic response of *Microcystis* grown at a range of light intensities. On day two the FDACR was maximal at 100 µmol m^{-2} s^{-1} and higher intensities and was approximately three fold greater than the FDACR at 10 µmol m^{-2} s^{-1}. The initial FDA conversion rate was 127 relative fluorescein units min^{-1} but by day two light dependent variability in FDA hydrolysis was evident (figure 3.6). The response of *Microcystis* incubated at different light intensities increased with increasing light intensity up to 100 µmol m^{-2} s^{-1} on day eight.

3.3.5 Assessment of the FDA staining protocol to recognise nutrient limited growth

Microcystis cultures were grown at different phosphorus and nitrogen limiting growth rates to assess the capacity of the FDA staining protocol to recognise nutrient limited growth

The growth rate of *Microcystis* increased with increasing initial phosphate concentration, approximating a Michaelis-Menten type curve saturating at about 2 µM (figure 3.7; k_m= 0.5 µM, μ_{max}=0.5 ln units day^{-1}). Metabolic activity at different phosphate concentrations displayed a similar pattern (figure 3.8). Initially the rate of FDA conversion was 32 relative fluorescence units $cell^{-1}$ min^{-1} but by day two differences were evident between phosphate treatments. With time the cells increased their metabolic activity at a rate dependent upon the

initial phosphate concentration until by day eight there was almost an order of magnitude difference between the phosphate limited and replete cultures.

Phosphate concentration and the time period following phosphorus exposure both had a significant effect on the FDACR determined using a two-way Anova (table 3.3, $p<0.05$) on data depicted in figure 3.8; phosphate concentration and time were considered as effects. The time dependence of FDACR for cells in different phosphate differed from the effect of light on FDACR which was independent of time.

Source	Nparm	DF	Sum of Squares	F ratio	Prob>F
Phosphate concentration	7	7	3835256	89.47	<0.0001
Time	3	3	111914	6.09	0.0011
P conc*time	21	21	2496006	19.41	<0.0001

Table 3.3 Analysis of variance table of a two way Anova of data presented in figure 3.8 where phosphate concentration and time were assessed as factors giving rise to the observed FDA conversion rate response. Phosphate concentration and time following inoculation into that P concentration both had a significant effect on FDA conversion rate ($p<0.05$).

The capability of the FDA assay to discriminate between cells from slightly different ambient phosphate concentrations prompted investigations into whether information obtained from metabolic studies could be used to predict future cell growth. The FDACR measured on day four was plotted against the growth rate determined from cell counts on days four and eight (figure 3.9). Although there was a linear relationship ($r^2=0.89$) between FDACR and growth rate (days 4-8) the slopes of plots of FDACR vs growth over different time periods differed. Consequently, the robustness of the FDA assay to predict future growth rate was diminished.

The growth response of nitrogen limited *Microcystis* cells increased with increasing nitrate enrichment (figure 3.10) and after three days was maximal at 10 μM (0.75 ln units day^{-1}). Because the experiments were performed in batch culture the initial concentrations of nitrogen were depleted with time inducing nitrogen limitation. This was most evident in the low N

concentrations which displayed a shift in growth rate at concentrations 0, 2, 5, 10, 20 µM over the periods 0-3 days (k_m=11 µM; V_{max}=0.72 ln units day^{-1}) and 0-6 days(k_m=3 µM; V_{max}=0.61 ln units day^{-1}). Nitrogen limitation was also evident in the metabolic activity measurements (figure 3.11). Day three FDACR indicated that 0, 2, 5 µM were limiting and these concentrations displayed sub-maximal growth rates (figure 3.10). As the cultures grew and nitrogen was removed from the media there was evidence of induced limitation on day six at concentrations less than 50 µM which again relate to sub-maximal growth rates (figure 3.10).

The FDA staining technique successfully determined nutrient limitation as it was induced in the batch culture experiments. However, before this technique could be applied to field populations an enrichment protocol was required to show that the FDA staining technique could identify the limiting nutrient or nutrients. Nitrogen and phosphorus limited *Microcystis* cultures were "spiked" with media not containing the limiting nutrient, or enriched with nitrogen, phosphorus or nitrogen and phosphorus. The FDACR was monitored at daily intervals over three days to determine whether the FDA technique could identify the limiting nutrients.

All cultures displayed an increase in FDACR on day one relative to the initial FDACR (figures 3.12 and 3.13). This was probably due to subtle differences in media or light conditions. There were significant differences in the relative FDACR of cells from nutrient addition and control treatments. Nitrogen limited cultures without a nitrogen spike (-N and +P; figure 3.12) had FDA conversion rates of 50 and 43 fluorescence units $cell^{-1}$ min^{-1}, respectively. When nitrogen was replaced (+N and +N+P) the FDA conversion rate rose to 83 and 85 relative fluorescence units $cell^{-1}$ min^{-1} respectively and displayed daily increases in FDACR as cells responded to their improved nutrient climate.

Phosphorus limited *Microcystis* responded in a similar manner to nitrogen limited cultures upon replenishment of the limiting nutrient (figure 3.13). Relative to the control (-P) the +P and +N+P treatments had significantly higher FDA conversion rates by day one and a rate nearly three times as great by day two. This assay demonstrated the ability of the FDA technique to detect nutrient limitation and identify the limiting nutrient after only a 24 hour incubation. The response time necessary to detect nutrient limitation increased to 48 hours when the cells were limited of both nitrogen and phosphorus (One-way Anova, p=0.05, n=3: figure 3.14). The cell

activity was severely impaired because the cells were depleted of the essential nutrients, nitrogen and phosphorus, for seven days. The cells were probably moribund and consequently took longer to respond to the improved nutrient climate.

3.4 Discussion

3.4.1 Algal response to light

Phytoplankton migrating through the water column experience a vertical light gradient dependent upon the sub-surface light intensity and optical characteristics of the water column. Their response is the capture of solar energy, its conversion via photosynthesis to ATP, the distribution of that energy for cell maintenance and finally in the culmination of these factors, growth. In this chapter responses to light have been examined by the measurement of photosynthetic rate, metabolic activity and growth. The aim was to assess whether the FDA staining protocol could discriminate between subtle changes in the light and nutrient climates to which phytoplankton are exposed.

FDACR and photosynthesis displayed similar changes in response to changing light intensity, however, the esterase activity saturated at a lower irradiance. Photosynthesis was measured as changes in oxygen concentration and the biochemical pathways giving rise to this response are well established (Anderson and Beardall, 1991). The mechanism behind the response of the non-specific esterases to light are not as evident. It was suspected that esterases were fuelled by ATP derived from photosynthesis and respiration, although it was also possible that the esterases themselves were light sensitive giving rise to greater enzyme activity in higher light. Inhibition of FDACR in the presence of DCMU disputes the latter theory.

The fact that inhibition of electron flow from photosystem II by DCMU inhibited FDA conversion rate adds merit to the theory that the esterases converting FDA are fuelled by ATP derived from photosynthesis. Photosynthesis and ATP production are rapid responses to the ambient light intensity. Therefore, it is likely that the observed light dependent variation in the ability of cells to hydrolyse FDA is due to energy limitation induced by sub-optimal light conditions. The saturation of FDACR occurred at a lower light intensity (80 μmol m^{-2} s^{-1}) than

saturation of photosynthesis (approximately 150 μmol m^{-2} s^{-1}). This may be due to sufficient ATP being transferred to the esterases at this photosynthetic rate to allow maximal esterase activity corresponding to 265 nmoles 0_2 $(10^6$ cells$)^{-1}$ min^{-1} and 150 relative fluorescein units min^{-1} (figure 3.3).

Olesen and Ganf (1986) showed a similar cellular response to photosynthetic rate measuring the rate of protein synthesis and growth rate (Olesen and Ganf, 1986: figure 2d.); these functions became independent of photosynthetic rate at higher light intensities. In their work growth rate and protein synthesis were maximal and constant at photosynthetic rates greater than approximately 25 fmoles 0_2 $cell^{-1}$ min^{-1}. There are similarities between the FDA conversion rate and the specific rate of protein synthesis, as both are functions which give rise to growth and are correlated with growth (figure 3.9; Olesen and Ganf, 1986, figure 2d). It is possible that functions such as phospholipid turnover, FDACR and protein synthesis receive sufficient ATP to operate at maximal levels at sub-maximal photosynthetic rates and maximise growth.

The photosynthetic rate and FDACR were strongly correlated (r^2>0.98) at light intensities less than 80 μmol m^{-2} s^{-1}. Dorsey *et al.* (1989) also found a relationship between ^{14}C assimilation and FDA conversion rate for species of different sizes ranging from 3-12 μm diameter and grown at 100 μmol m^{-2} s^{-1}; larger cells had greater rates of both FDA hydrolysis and photosynthesis. It was evident from this study and Dorsey's *et al.* (1989) study that there was a link between the esterase activity and photosynthetic rate. It was postulated that this was due to esterases being fuelled by ATP derived from photosynthesis. It is interesting that FDA is also converted by non-photosynthesising organisms such as yeast (Breeuwer *et al.*, 1995), zooplankton (Moffat and Snell, 1995) and mammalian cells (Rotman and Papermaster, 1966) presumably fuelled and limited by the respiration rate. The dark rates of FDA conversion may be fuelled purely via respiration.

Light dependent FDACR monitored over eight days revealed similarities to the metabolic rate of cells exposed to different light intensities for only two hours (figure 3.6). Statistical analysis of data presented in figure 3.6 identified that light intensity had a significant effect on FDA conversion rate. There was an interaction between the light intensity and the day of sampling but day alone had no significant effect. Essentially what was measured was a short term

process and response to light but this interacted with time as cells acclimatised to the light intensity or suffered from prolonged exposure to sub-optimal light intensities.

Esterase activity in both the two hour light incubation (figure 3.2) and the eight day growth trial (figure 3.6) was maximal at light intensities exceeding 60-80 μmol m^{-2} s^{-1} which corresponded with the light intensity required for maximal growth (figure 3.5). Light limited FDA conversion rate at lower light intensities also corresponded to light limited growth. The explanation for the observed relationship between FDA conversion rate and growth is largely intuitive; cells exposed to limiting light intensities can not fix sufficient carbon or produce sufficient ATP to fuel the non-specific esterases involved in phospholipid turnover (and other cellular machinery, nitrogen metabolism etc.) and so cell division suffers. At the optimum light intensity these processes were satisfied both chemically and energetically and growth was maximal.

The slower increase in the metabolic activity of *Microcystis* following replenishment of limiting nutrients (figure 3.12 and 3.13) is in contrast to the rapid response to light intensity. The light limited cultures were grown in nutrient rich media and had the cellular machinery and nutrients available to efficiently capture and utilise light energy. The nutrient limited cultures were severely depleted in either nitrogen, which is a fundamental component for protein and pigments, or phosphate which is essential for energy transport and the nucleotides, DNA and RNA. Thus esterase response to a changing nutrient climate took longer than a response to a change in the light climate because the cellular machinery, including esterases, had to be resynthesised in order to maximise light capture, nutrient metabolism and metabolic activity of the cells.

The ability of FDA to detect variability in light intensity and exposure time prompts a cautionary note on sampling prior to measurement of FDA hydrolysis. In order for day to day comparisons to be made the cells must be subjected to a light history relevant to the sampling, either the same duration of light exposure or light intensity in the case of nutrient studies.

The FDA measured response to previous light history could be advantageous because it offers the potential for this technique to be applied to field populations to estimate their light history.

Previous light history data used in conjunction with data on thermal structure and current velocities will offer insight into plankton vertical movement.

Harris (1980) proposed that chlorophyll *a* fluorescence could be used to show if the water column is stable or actively mixed. By measuring the ratio of *in vivo* fluorescence and maximum fluorescence, following DCMU addition, of cells the light history and mixing conditions they have been exposed to can be approximated. A field example of this was demonstrated in Harris (1984) where there was a relationship between the fluorescence ratio and the mixing cycle as represented by Richardsons number (Harris, 1984; figure 4). An advantage of Chlorophyll *a* fluorescence is that there is a rapid response to light and rapid recovery in the dark (Vincent *et al.*, 1984) and consequently a clear relationship between downwelling surface irradiance and *in vivo* fluorescence per chlorophyll *a* (Keifer and Reynolds, 1992, figure 4). Light dependent chlorophyll fluorescence quenching has been used to track algal movement (Whittington *et al.*, 1996) using a PAM fluorometer (Schrieber *et al.*, 1994) incorporating surface irradiance, light attenuation and algal and water movement. The rapid response of chlorophyll fluorescence makes it possible to gain an estimate of water movement because vertical mixing and chlorophyll fluorescence quenching occur within the same time scale (Denman and Gargett, 1983; Harris, 1986).

It has been demonstrated here that FDA and flow cytometry are valuable in determining the moderate and long term light history of the cyanobacterium *Microcystis aeruginosa*. However, if they are going to be used to estimate short term changes in light climate it will be necessary to ensure that the esterase response time is rapid enough and the resolution fine enough to detect very short term light exposure to at least the time scale of vertical mixing (Denman and Gargett, 1983). It may be possible to combine the FDA technique and "pump and probe" chlorophyll fluorescence measured on a flow cytometer (Olson and Zettler, 1995) to gain more insight into long and short term algal response to light at the cellular level (Neale *et al.*, 1989) and subsequently algal movement.

3.4.2 Algal response to nutrients

Cellular response to an improved nutrient environment can be detected over a range of time scales. Nutrient enrichment is first observed in physiological functions; as perturbations in the

chlorophyll fluorescence signal (Wood and Oliver, 1995), nutrient uptake kinetics, ATP production and respiration (Healey, 1979). As the nutrients are incorporated into cellular machinery the response to nutrients becomes evident in enzyme activity, photosynthesis (Geider and La Roche, 1994; Greene *et al.*, 1994) and finally growth. Esterase activity is associated with the synthesis and turnover of phospholipid in membranes (Dorsey *et al.*, 1989) and so must be greatest during periods of high growth to maintain the rapid cell division. Nutrient limitation in this study was detected at the enzyme level by observing the ability of an enzyme group to metabolise a substrate.

Batch culture experiments with *Microcystis* grown in different concentrations of phosphate revealed that phosphate limitation was induced in concentrations less than 2 μM which was similar to the saturating concentration documented in Holm and Armstrong (1981). The esterase response to the improved phosphorus environment was clearly evident using FDA and flow cytometry, and displayed increases with increasing phosphate and time. There was good correlation between the measured FDA hydrolysis rate and the subsequent growth rate. However, attempts to model this and then predict future growth rate from the rate of FDA conversion were flawed by differences in the correlation constants (slope) between FDA and growth rates measured on different days. It was therefore concluded that FDA conversion rate is a valuable tool for estimating relative differences in cell health and relative growth rates but cannot, at this stage, be used to approximate absolute growth rates. It would be necessary to know the exact previous nutrient history in order to use this model to predict future growth.

Nitrate limitation in *Microcystis* batch cultures was detected by growth and FDA hydrolysis measurements (figures 3.10 and 3.11). Growth was maximal over the first three days at concentrations greater than 5 μM nitrate (approximately 0.075 mg L^{-1} nitrate as N). As cells proliferated the nitrate demand and the media nitrate concentrations became limiting to growth at initial nitrate concentrations less than 50 μM. It was apparent from figure 3.11 that the FDA technique, on day 6, was predicting even further nitrogen limitation at concentrations less than 50 μM.

The mean oxidised nitrogen for rivers in the Murray-Darling Basin range between 0.046 and 0.267 mg L^{-1} N as nitrate (Mackay *et al.*, 1988) which is sufficient to support a *Microcystis*

population . However, the measurements contributing to the mean range from 0.003 to 1.390 mg L^{-1} nitrate as N. Consequently there is sufficient nitrogen at certain times of the year but not others. Unpublished monitoring data of nutrients and cyanobacteria in the lower River Murray from SA Water suggest that saturating levels of nitrate (approximately 0.09 mg L^{-1} nitrate as N) occur typically in spring during peak flows. In summer when conditions favour some degree of thermal stratification and consequently cyanobacteria, the nitrate levels are very low, unsuitable for *Microcystis* growth, and the nitrogen fixing coiled *Anabaena* spp. dominate.

An assessment of nutrient status provides insight into how the phytoplankton are responding to the chemical environment and reflect the manifestation of nutrient fluxes, spatial variability, nutrient bioavailability, phytoplankton nutrient uptake kinetics and cellular physiology. To remove phytoplankton from their environment isolates them from some of the critical processes to which the algae are responding. The FDA assay to assess nutrient status required a 24 hour incubation to identify the limiting nutrients because the nutrients had to be incorporated into cell metabolism before they could affect cell function and increase FDA hydrolysis. Although this technique does require a short incubation, unlike the NIFT assay developed by Wood and Oliver (1995), the incubation time was less than one generation in a typical field population and it was considered safe to assume that the population sampled on day one was the population measured on day two.

The FDA assay correctly identified the limiting nutrient in each of the laboratory trials which highlights its value for rapid detection of nutrient limitation in field populations. The high resolution of the FDA assay and the strong relative relationship between FDA conversion rate and growth (3.9) indicate that with further refinement of the FDA nutrient assessment technique it may be possible to predict the degree of limitation.

Differences in metabolic activity have been detected between cells from natural populations using fluorochromes (Yentsch *et al.*, 1988). It is important to determine the factors giving rise to population variability. Investigations can then be undertaken to determine whether the observed effect is significant to growth and how different taxonomic groups respond to the environmental influence. With respect to cyanobacteria and other nuisance algae an understanding of their physiological response to environmental pressures is critical so that

management plans can be initiated, adopted and implemented to reduce the frequency and intensity of "cyanobacterial blooms" in water bodies. Flow cytometry offers the potential to separate the natural phytoplankton population into optically similar groups and thereby investigate the affect of improved light or nutrient conditions upon the metabolic activity of taxonomically distinct groups such as cyanobacteria versus eukaryotic algae.

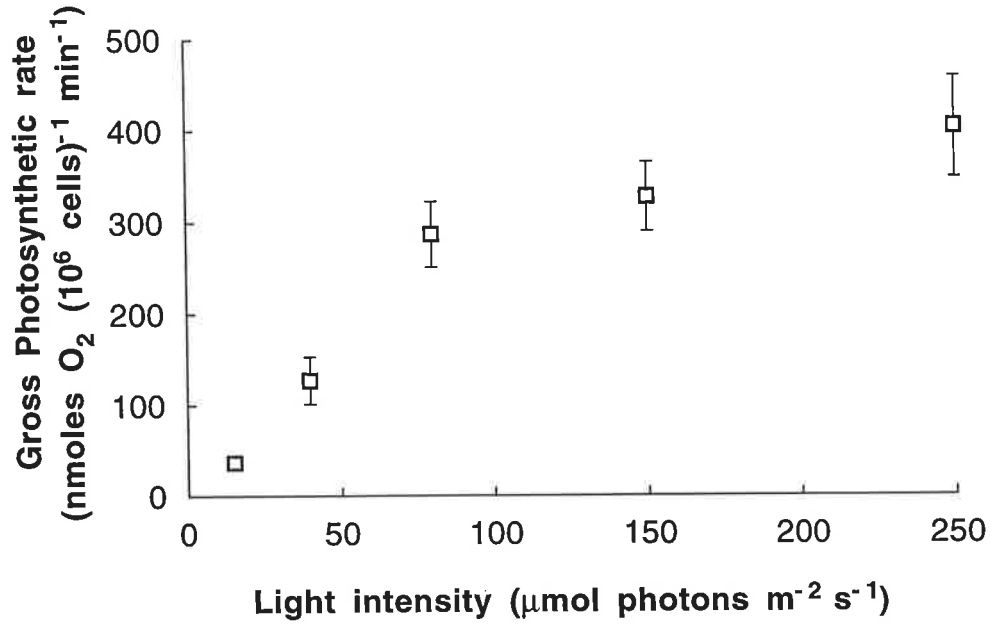


Figure 3.1 Photosynthesis-irradiance curve of *Microcystis aeruginosa* estimated using an oxygen electrode (error bars represent standard deviation)

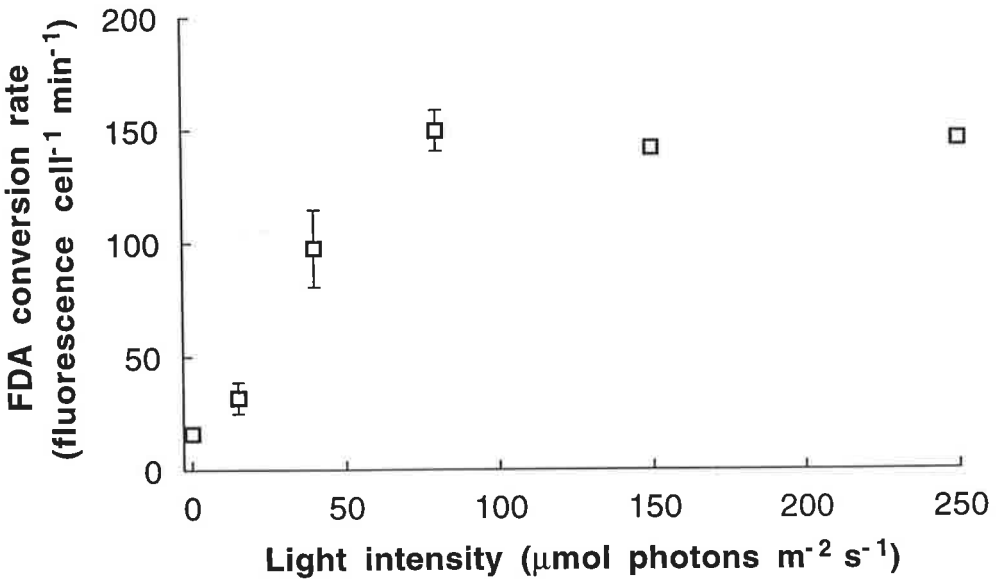


Figure 3.2 The FDA measured enzyme activity of *Microcystis* cells incubated at a range of light intensities for two hours was linear up to 80 μmol photons m^{-2} s^{-1}.

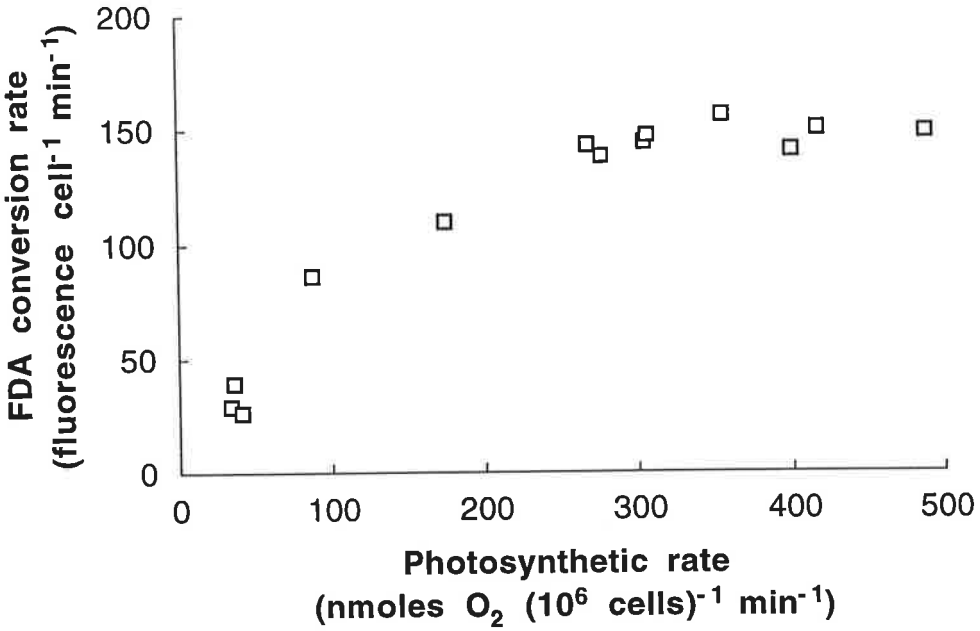


Figure 3.3 The relationship between photosynthetic capacity and FDACR suggests that hydrolysis of FDA by esterases is dependent upon energy derived from photosynthesis. At 265 nmoles O_2 (10^6 cells)$^{-1}$ min^{-1} sufficient ATP was produced to maximise esterase activity.

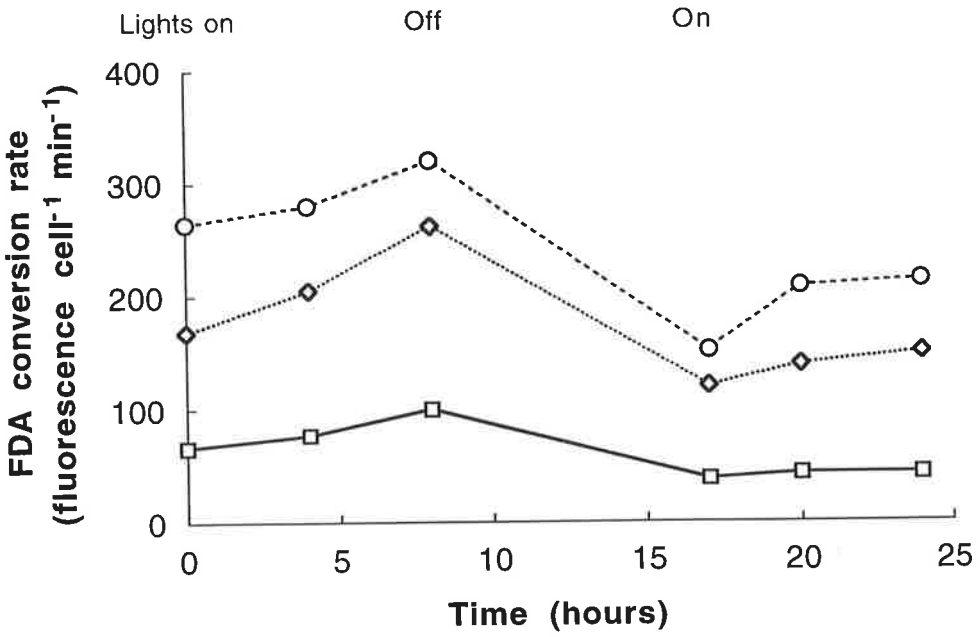


Figure 3.4 The FDA hydrolysis by *Microcystis* is dependent upon light intensity and exposure time; 10 (□), 50 (◊) and 100 (o) µmol photons m^{-2} s^{-1}. Following a dark period esterase activity is determined by the previous light history.

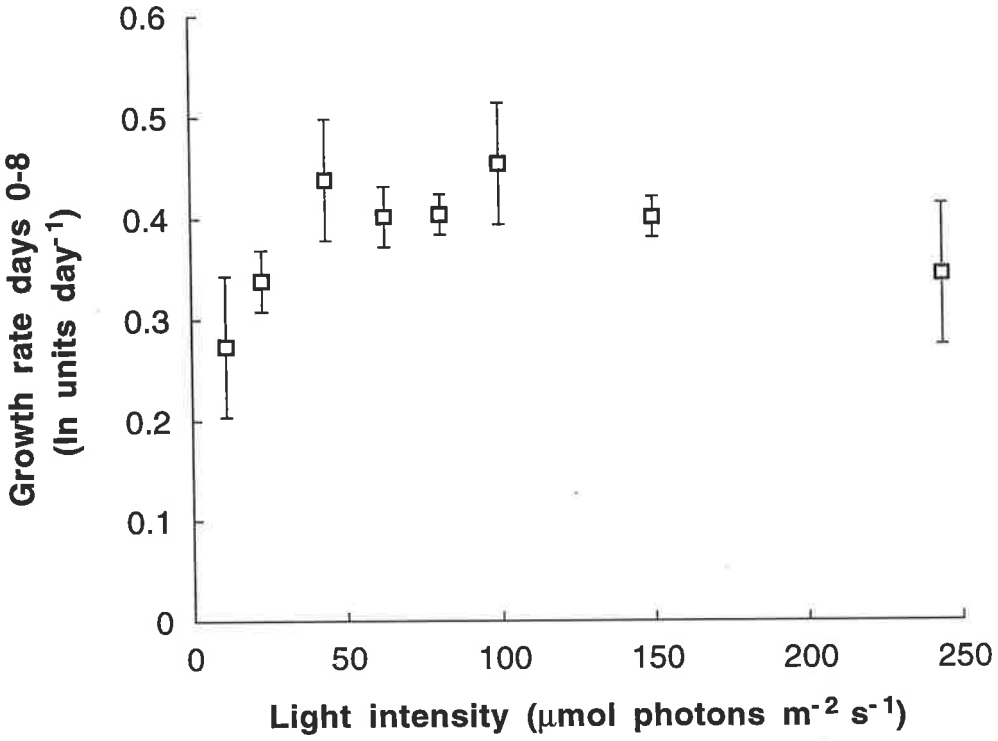


Figure 3.5 Light dependent growth rate of *Microcystis aeruginosa*

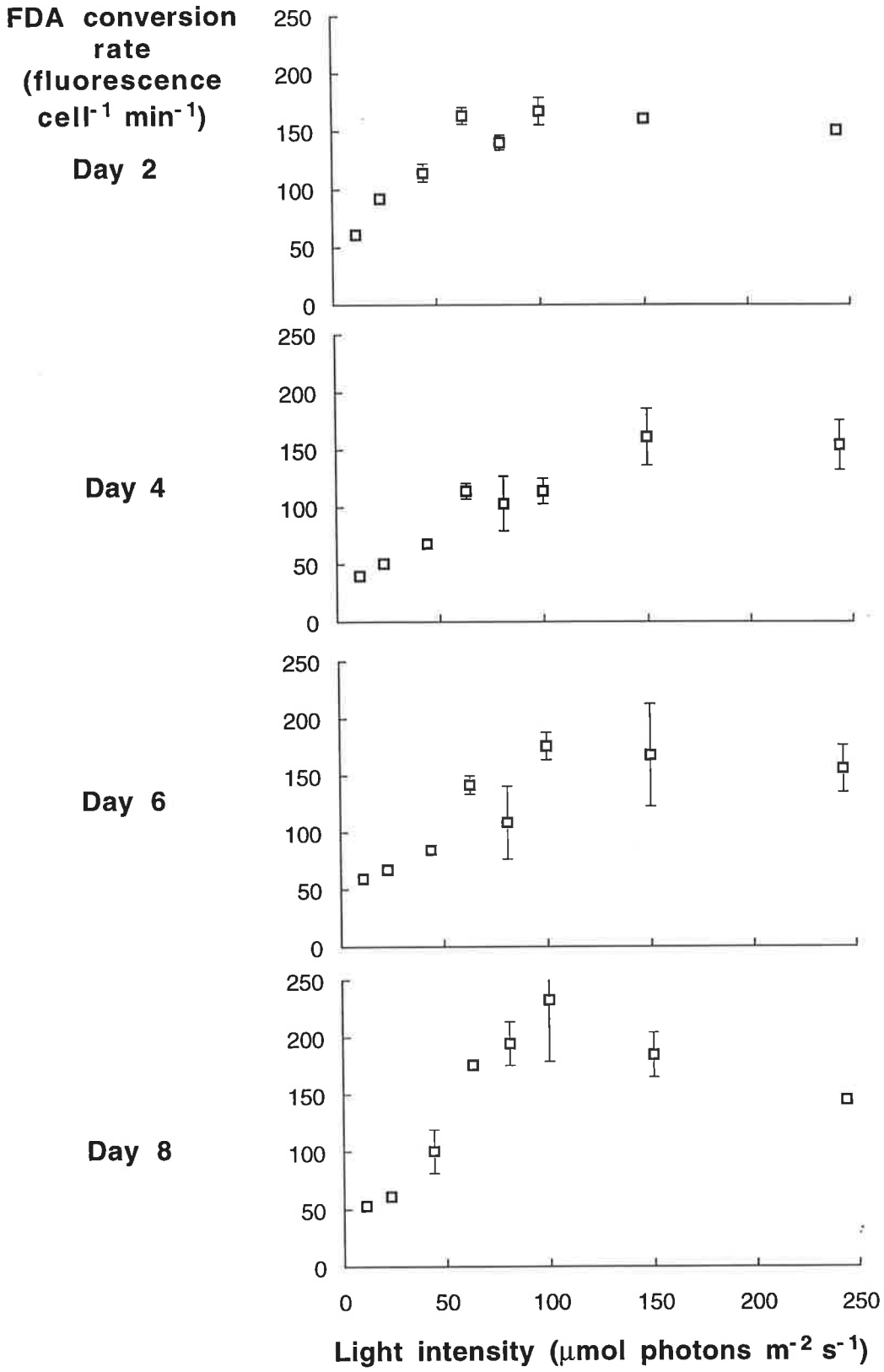


Figure 3.6 Esterase activity of *Microcystis* grown at different light intensities on a 12:12 light:dark cycle was determined by measuring the rate of FDA conversion to fluorescein. Cells were grown for eight days and FDA conversion rate estimated by measuring fluorescein on a flow cytometer following a 7 minute incubation in 40 μM FDA.

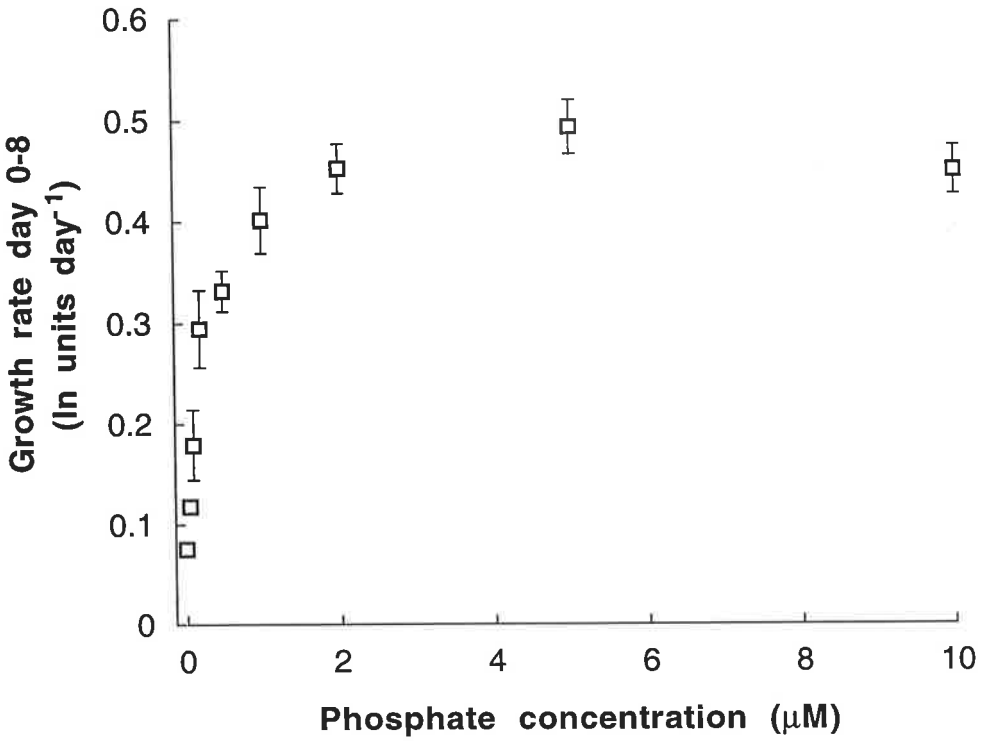


Figure 3.7 Phosphate dependent growth rate of *Microcystis aeruginosa* pretreated in phosphate-free media to reduce the polyphosphate pool and then inoculated into the different phosphate concentrations.

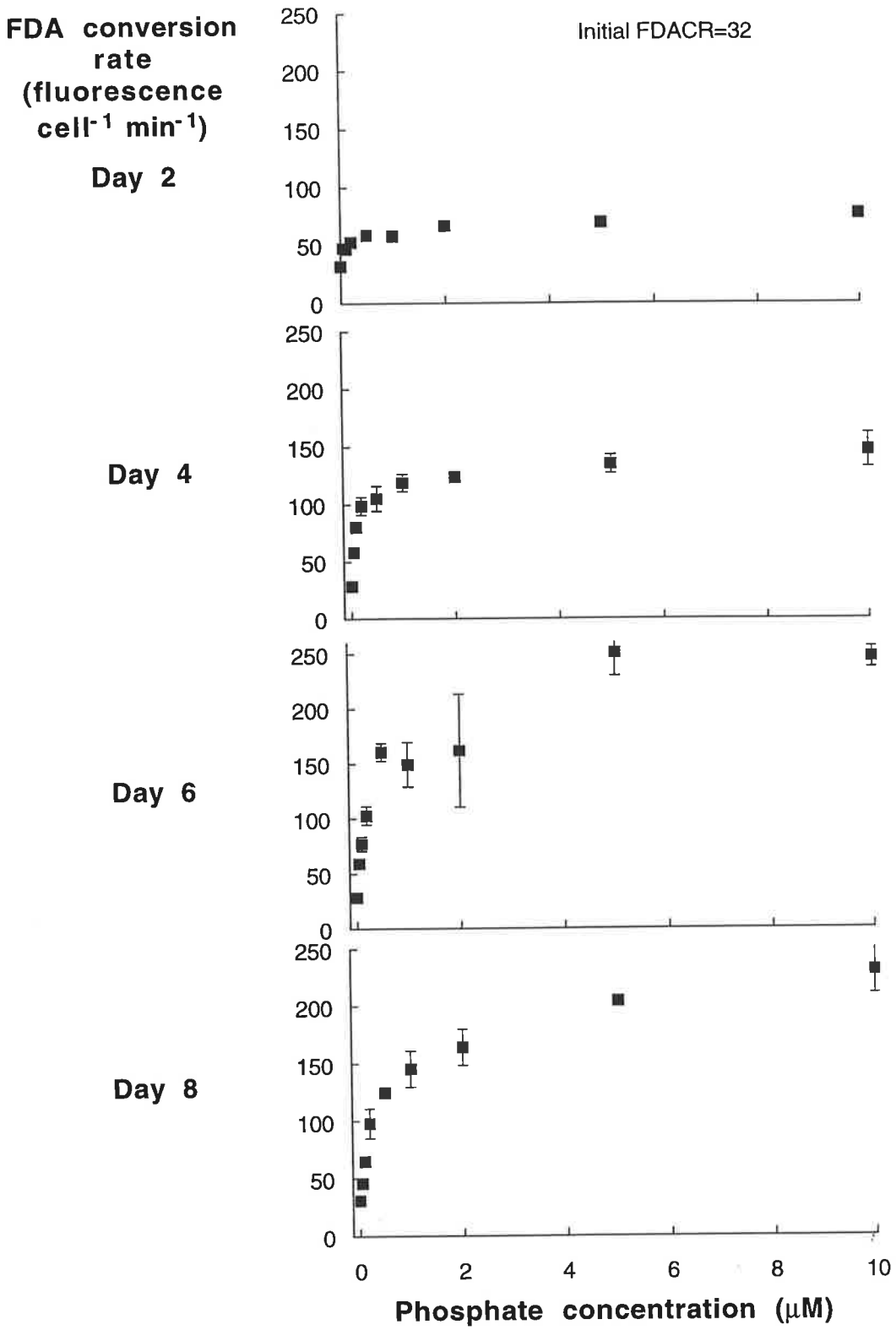


Figure 3.8 *Microcystis* cells inoculated into different phosphate concentrations increase their enzyme activity (estimated by FDA hydrolysis) in response to the improved phosphate climate.

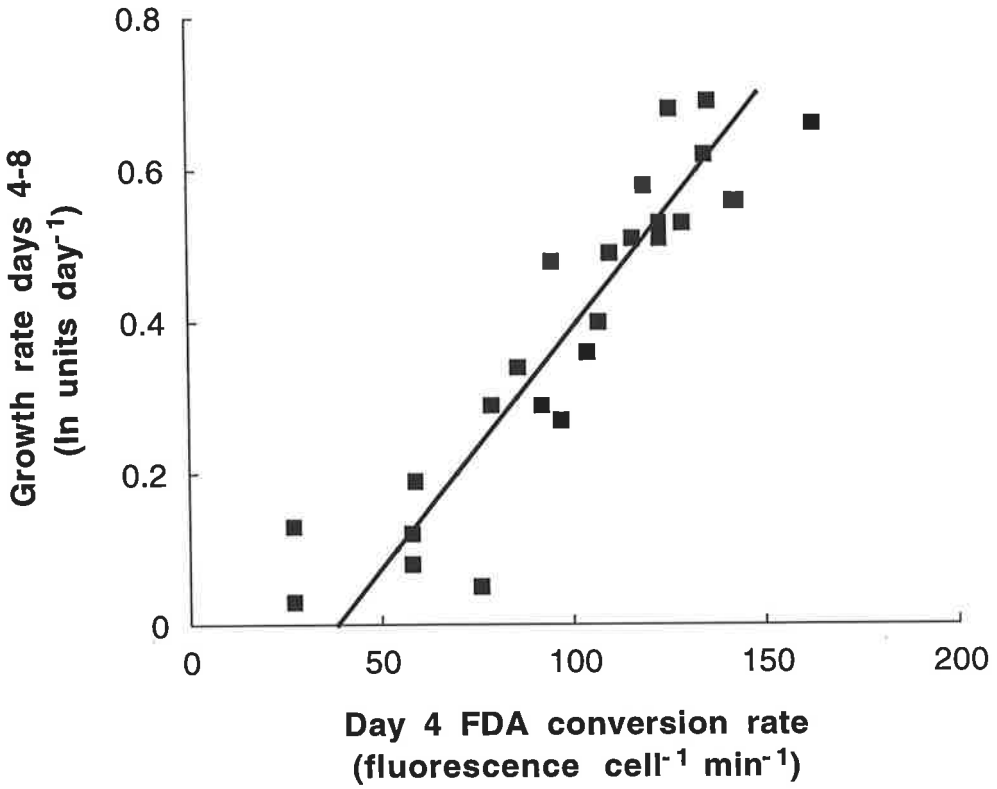


Figure 3.9 FDA conversion rate was plotted against future growth to investigate whether a model could be developed to predict future growth given a particular FDACR. FDACR is linearly related to future growth (r^2=0.89).

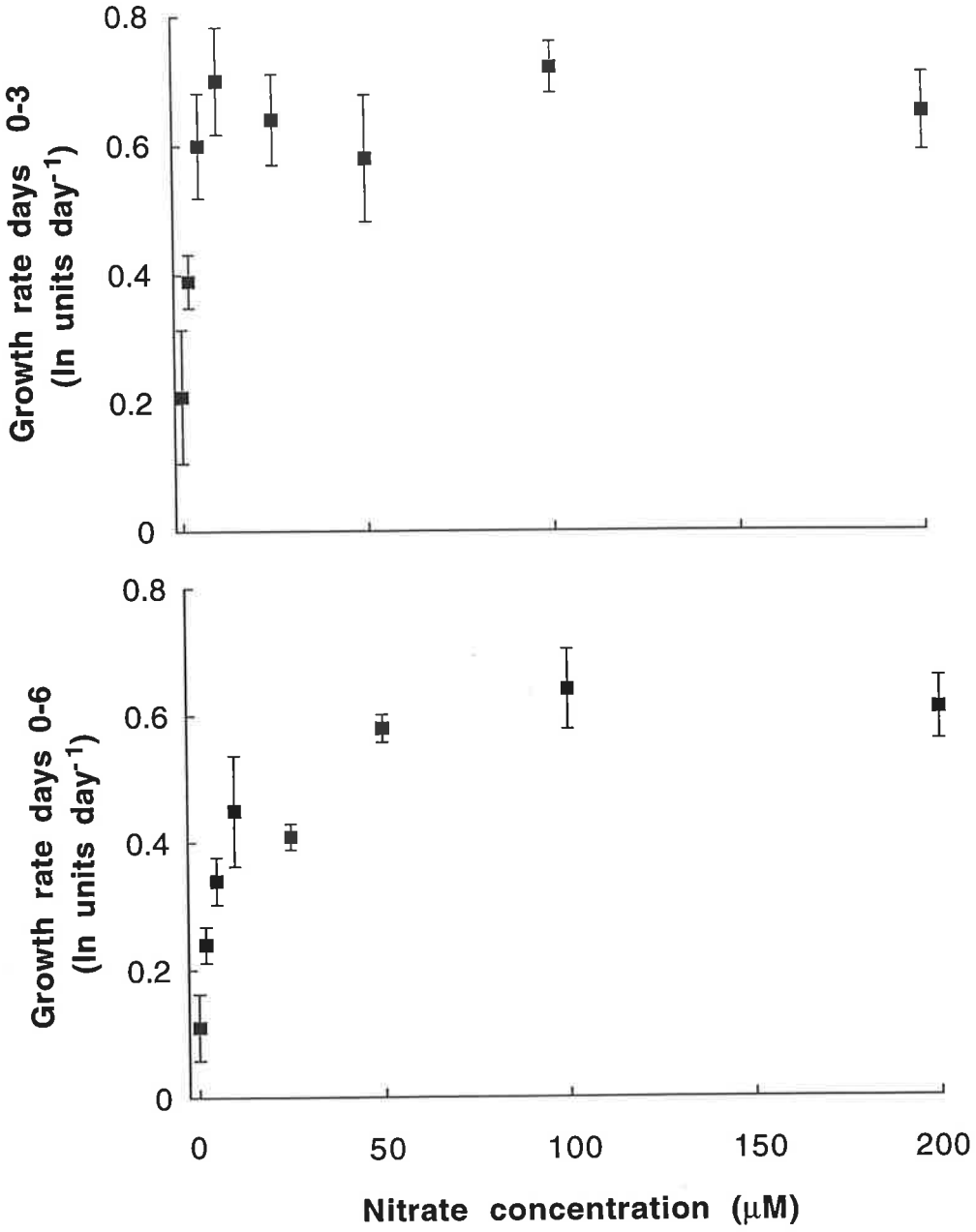


Figure 3.10 Nitrogen (nitrate) dependent growth of *Microcystis aeruginosa* over the first three days after inoculation (top) and over the six day experimental period (bottom graph). Growth was maximal at initial concentrations greater than 10 μM nitrate over the first three days but as N was incorporated into cells N limitation was induced, by day six, in treatments less than 50 μM initial concentration.

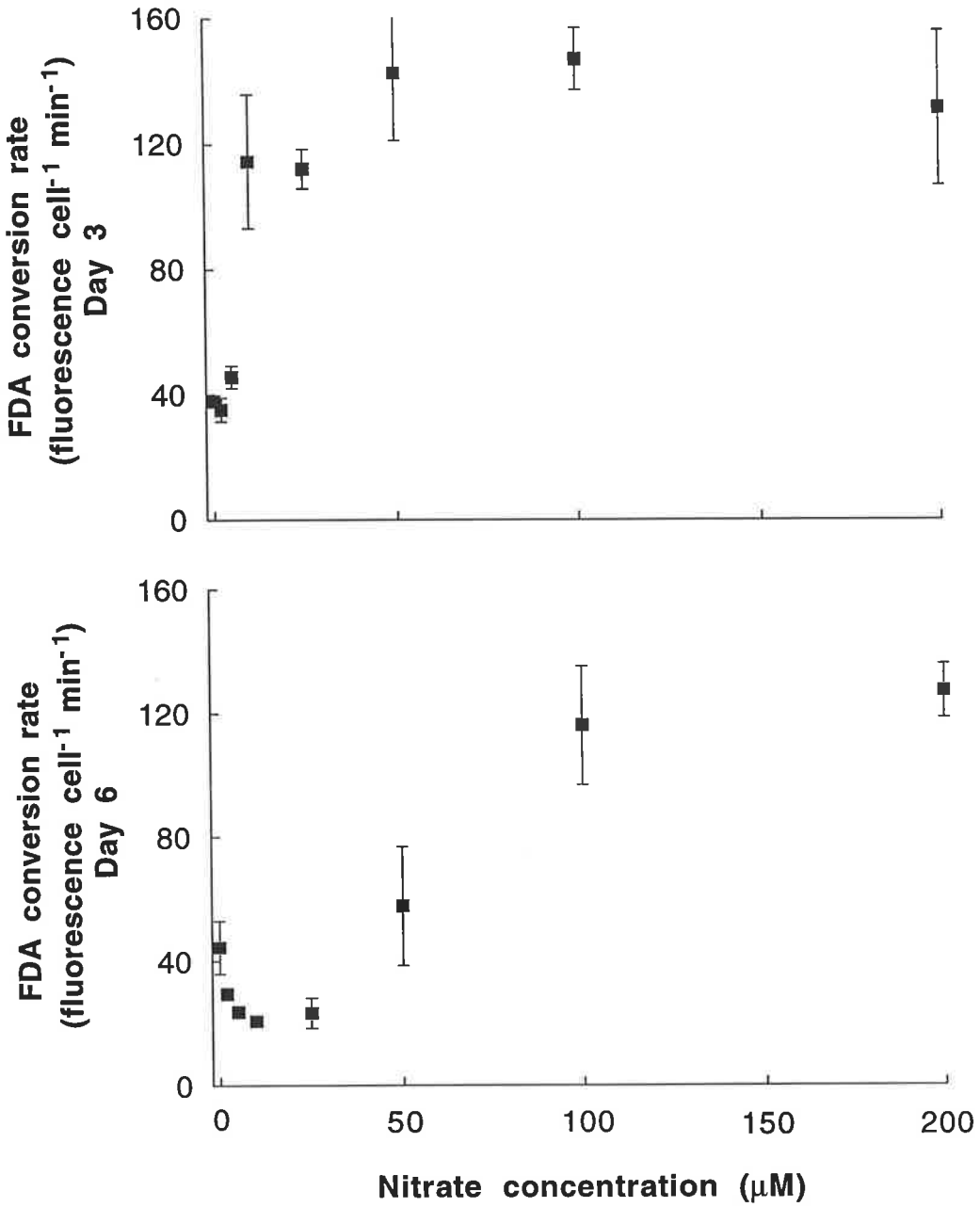


Figure 3.11 Enzyme activity of *Microcystis* grown in different nitrate concentrations and measured three (top) and six days (bottom) after inoculation.

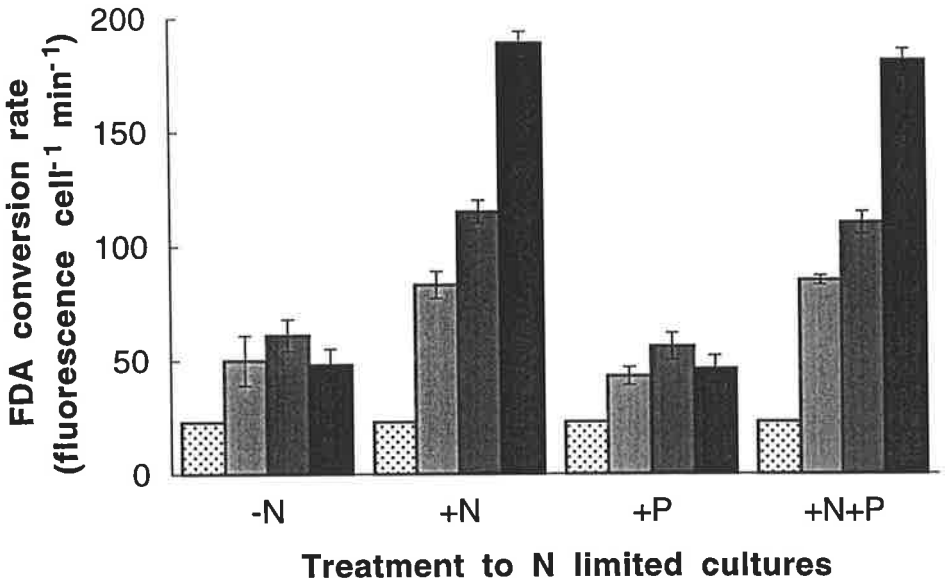


Figure 3.12 Nitrogen spikes to nitrogen deficient *Microcystis* induced a time dependent increase in metabolic activity; day 0 ⬚, 1 ▒, 2 ■, 3 ■ relative to the controls. Nutrient limitation was detected after one day.

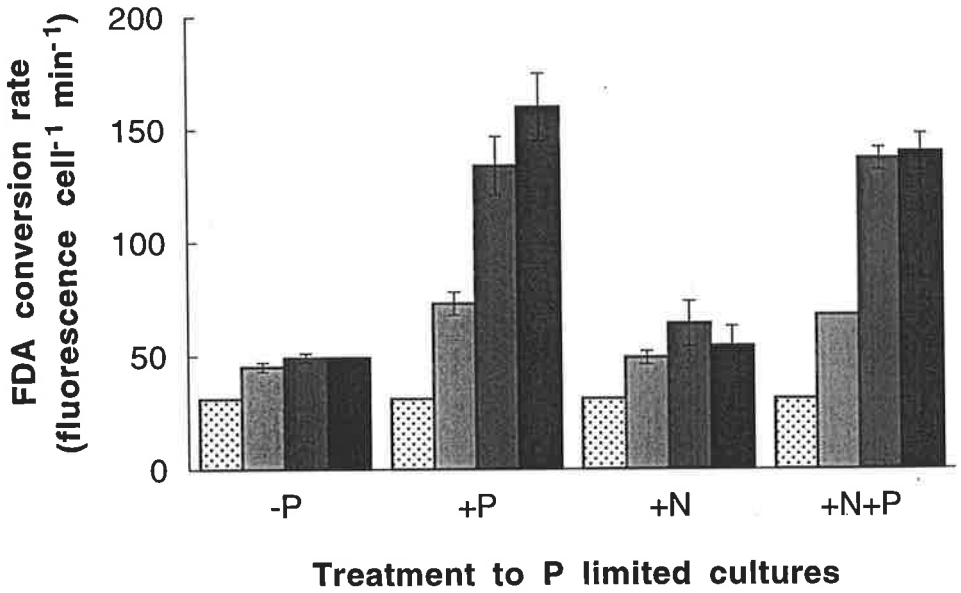


Figure 3.13 The replacement of phosphorus to phosphorus limited *Microcystis* generated an enzyme response which increased with time after spiking Day 0 ⬚, 1 ▒, 2 ■, 3 ■. Demonstrating the value of the FDA technique as a rapid assay to detect nutrient limitation and identify the limiting nutrients.

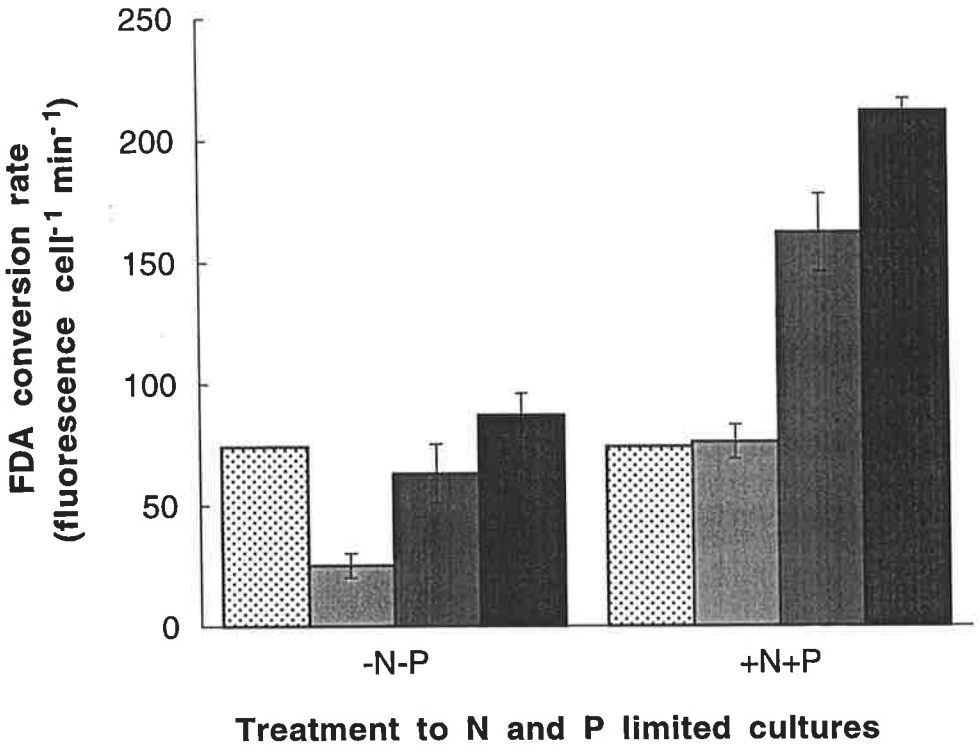


Figure 3.14 FDA conversion rate of nitrogen and phosphorus deficient *Microcystis* following N and P replacement. Initial ⊡, Day 1 following spike ■, 2 ■ 3 ■. The improved nutrient climate took longer to detect in N and P limited cultures because cell health was very low due to the severe limitation.

Chapter 4

Assessment of the FDA staining technique to detect nutrient limitation in field populations

4.1 Introduction

An assay which measures metabolic activity in laboratory cultures would be particularly useful if it could be adapted to study the eco-physiology of field phytoplankton populations. The FDA staining protocol was developed in chapter 2 and laboratory trials described in chapter 3. In this chapter the FDA staining protocol was trialed on field populations to detect nutrient limitation and estimate phytoplankton health in different aquatic habitats. In addition experiments were designed to utilise the technique to investigate some hypotheses of limnological importance. The technique was used to examine whether nutrients were limiting phytoplankton growth in the lower River Murray and whether backwaters were more favourable environments for phytoplankton growth than the main river channel.

The River Murray, 2500 km, in length, receives water from the Murray-Darling catchment particularly the Murray headwaters in the Snowy Mountains and the Darling River carrying water sourced from north east New South Wales and south east Queensland. The river is highly regulated and on the lower River Murray there are ten low-level weirs. The current management ensures that the water level at each lock is regulated to maintain the river at bank-full capacity (Walker and Thoms, 1993). The regulation of flow of the River Murray has reduced the seasonal variability of the hydrograph (Walker and Thoms, 1993) and in summer, during periods of low flow, the river resembles a string of connected lakes. Diurnal stratification occurs for short periods during low flows and when wind conditions permit (unpublished data, SA Water). As the river has meandered through the river valley it has cut new channels and formed floodplain billabongs, oxbows and backwaters.

Reynolds *et al.* (1991) and Reynolds (1994), investigating flow in river channels, discuss the theory of "dead-zones" which are local patches of slow- or non-flowing water. These retentive zones favour algal development and may act as seed populations within the river. Perhaps the most extreme example of retentive zones are the backwaters and oxbow lakes adjacent to large rivers such as the River Murray. The backwaters along the lower reaches of the River Murray are often connected to the main channel by narrow inlets and/or outlets and water may flow in or out depending on the hydraulic gradient and wind velocity. It has been hypothesised that these backwaters may act as a hydraulic store and support the growth of phytoplankton which then seed the main channel (pers. comm. Mike Burch and Peter Baker, SA Water). It has been demonstrated that the backwaters support higher cyanobacterial numbers than the main channel (pers. comm. Peter Baker, SA Water). Whether the longer retention time of the backwater or some other factors encourage greater algal growth in backwaters relative to the main channel is unknown. The backwaters are shallower than the main channel (<1m vs 5-12 m) and although often more turbid the mean irradiance phytoplankton encounter in mixed conditions is much greater in the backwaters. This suggests that phytoplankton from a backwater may display a greater metabolic activity than phytoplankton resident in the main river channel. This hypothesis was tested on a number of backwaters adjacent to the lower River Murray.

Monitoring of nutrient concentrations in the River Murray has been undertaken by various State water authorities and historical data of nutrient concentration between 1978 and 1986 are recorded in Mackay *et al.* (1988). The lower River Murray is typically higher in phosphorus than the Murray River waters east of the Darling confluence as the Darling consistently has a high nutrient load. Total phosphorus concentrations in the lower River Murray range from 0.005-0.713 mg L^{-1}; soluble reactive phosphorus, 0.002 mg L^{-1}; oxidised nitrogen, 0.01-0.72 mg L^{-1} and total Kjeldahl nitrogen, 0.13-2.77 mg L^{-1}. At the lowest soluble inorganic nitrogen and phosphorus concentrations these nutrients may limit phytoplankton growth. It is difficult to speculate on the bioavailability of the total nutrient pool as there are no data available on nutrient fluxes from lower River Murray sediments or suspended particulates. In this study methods are employed to examine the nutrient status of the resident phytoplankton community and estimate of the bioavailability of the nutrient pool.

The FDA nutrient-limitation detection assay was used to ascertain whether phytoplankton growth was nutrient limited and to identify the limiting nutrients. Two sampling regimes were used, one compared the nutrient status of phytoplankton in backwaters with those in the main channel; the other was a nutrient survey to identify if there were reaches with elevated nutrient concentrations giving rise to healthier phytoplankton populations.

Methods previously used to assess cell nutrient status include measurement of cell elemental ratios of carbon:nitrogen:phosphorus (Healey, 1979), measurement of photosynthetic pigments (Yentsch and Phinney, 1984; Herzig and Falkowski, 1989; Foy, 1993; Geider *et al.*, 1993) and carbohydrate to protein ratios (Ganf *et al.*, 1986). The major problem associated with using pigment changes to determine nutrient limitation is that light and nutrient limitation alter pigment composition and concentration (Tandeau de Marsac and Houmard, 1988; Falkowski and La Roche, 1991; Ganf *et al.*, 1991; Ohki and Fujita, 1992) and consequently it is difficult to isolate which factor is affecting the cell (Foy, 1993; Wood and Oliver, 1995). Assays which depend upon a response to a nitrogen or phosphorus addition to detect nutrient limitation, such as growth bioassays and the FDA conversion assay, often require an incubation in a defined light climate. In constant, saturating light relative differences in pigment content between treatments maybe assumed to be due to the treatment without confounding effects of light.

Chlorophyll *a* content per cell was compared with the metabolic activity measured as the rate of FDA conversion following the addition of nutrients to nutrient limited cultures. This allowed a reassessment of changes in chlorophyll content to determine nutrient limitation and a comparison between the two techniques. Measuring chlorophyll content per cell using a flow cytometer overcomes the problem of increasing Chl*a* concentration as a function of population growth. The relationship between chlorophyll per cell and the total chlorophyll , as an estimate of yield, was investigated.

Phytoplankton differ in response to nutrients in a manner which is determined by their physiology (Tilman *et al.*, 1986) and their physiological state. For example, cyanobacteria and diatoms have different nutrient requirements and nutrient uptake and growth characteristics (Holm and Armstrong, 1981). Different genera dominate in water with different nitrogen to phosphorus ratios, when at least one is in short supply, and will differentially display nutrient

limitation (Rhee and Gotham, 1980; Tilman *et al.*, 1986; Harris, 1994) ie nitrogen-fixing cyanobacteria dominate when the inorganic nitrogen levels are low.

Cell counts were undertaken to estimate relative abundance and identify which genera were responding to the different nutrient additions. The growth of different species may be limited by different nutrients depending upon the specific physiology of the phytoplankton (Suttle and Harrison, 1988). For example, nitrogen fixing cyanobacteria are more likely to display phosphorus limitation than nitrogen limitation. Chemical nutrient analysis, FDA bioassays and growth bioassays were used to assess the nutrient status of the water column, investigate how the phytoplankton were responding to their chemical environment and identify the limiting nutrients.

In chapter 3 a relationship between the metabolic activity (FDA conversion rate) and future growth was determined. It was concluded that there was not a universal relationship between FDACR and future growth, however, field trials present the opportunity to investigate this relationship further and determine the value of the FDA technique to model and predict relative future growth.

The macro nutrients, nitrogen and phosphorus, are often identified as the elements limiting phytoplankton growth (Hecky and Kilham, 1988; Elser *et al.*, 1990; Takamura *et al.*, 1992; Miyajima *et al.*, 1993; Wood and Oliver, 1995). However, some micro-nutrients may also limit cell function and growth. Silicon deficiency has been detected for diatom species (Healey, 1979; Reynolds, 1984; Miyajima *et al.*, 1993) in which silicate is incorporated, as an amorphous polymer, into the diatom's frustules (Miyajima *et al.*, 1993). Iron deficiency has also been observed in phytoplankton (Healey, 1979) particularly in marine systems (Geider and La Roche, 1994; Greene *et al.*, 1994). Iron is crucial for cell function, especially carbon and nitrogen reduction (Reuter and Peterson, 1987), as iron containing compounds serve roles in enzymes, pigment biosynthesis, the electron transport chain (Reuter and Ades, 1987; Reuter and Peterson, 1987) and in the repair of photosynthetic apparatus (Greene *et al.*, 1994).

Reuter and Peterson (1987) suggest that molybdenum may also be a potentially limiting micronutrient. Molybdenum is incorporated into the enzymes essential for nitrogen metabolism;

nitrogenase (in N_2 fixing species), and nitrate reductase (Hallenbeck *et al.*, 1982; Steeg *et al.*, 1986; Reuter and Peterson, 1987). It is possible that trace elements limit phytoplankton growth in the River Murray. To examine the potential importance of trace elements the river water was enriched with nitrogen, phosphorus, and the micronutrients, iron, molybdenum, manganese and silicon.

4.2 Methods

4.2.1 Nutrient survey

A sampling program was designed to assess whether there were areas along the lower reaches of the River Murray which have relatively higher nutrient concentrations which may be capable of supporting larger phytoplankton populations than low nutrient sites.

A single sampling was undertaken on March 6 and 7, 1996 and water sampled from a 217 km reach of the lower River Murray between Barmera and Nildottie, South Australia (Map 4.1). The flow at Lock 1 was 7438 ML day^{-1} on 06/03/96 and 7030 ML day^{-1} on 07/03/97. These flows create sufficient turbulence and water column instability to prevent the establishment of thermal stratification (pers. comm. Mike Burch).

Samples were collected in the middle of the main channel with a hose pipe integrating the entire depth of the water column. Three replicate samples were collected at each of the sites marked on map 4.1 and described below (table 4.1). Samples were kept in the dark and returned to the laboratory on the day of collection and bioassays performed. Chemical analysis was undertaken by Australian Centre for Water Quality, Bolivar, to determine conductivity, total dissolved solids, bicarbonate, alkalinity, chlorophyll, total Kjeldahl Nitrogen (TKN), ammonia, nitrate+nitrite, total phosphorus and filterable reactive phosphorus.

Site	Description	River kilometres from Mouth	Coordinates
1 Wachtels Lagoon	Grab sample (depth = approx 1m)	441	34° 15'S 140° 22'E
2 Lock 3	150 m upstream of Lock 3	433	34° 12'S 140° 21'E
3 Waikerie	500 m downstream of Ferry	382	34° 11'S 139° 59'E
4 Morgan	400 m upstream of Ferry	320	34° 02'S 139° 40'E
5 Lock 1	100 m upstream of lock 1	274	34° 21'S 139° 37'E
6 Devon Downs	Grab sample	225	34° 41'S 139° 39'E
7 Nildottie	Mid -channel at boat ramp	224	34° 41'S 139° 39'E

Table 4.1 Sites on the lower River Murray sampled in a nutrient survey

4.2.2 Bioassays and metabolic activity

Bioassays were prepared by pouring 40 mL of river water from each sample (n=3 at each site) into 100 mL Erlenmeyer flasks and enriched with either 100 µM $NaNO_3$ (final concentration), 100 µM K_2HPO_4 or 100 µM $NaNO_3$ and 100 µM K_2HPO_4; control flasks had no nutrients added. Flasks were incubated at 25°C, 120 µmol m^{-2} s^{-1} on a 12:12 light:dark cycle. Total DCMU-sensitive chlorophyll fluorescence was measured as a surrogate for phytoplankton biomass. Relative phytoplankton biomass was estimated initially and on day 5 using a Turner Model 111 fluorometer fitted with a blue excitation filter (CS5-60) and red emission filter (CS2-64) to detect chlorophyll fluorescence following the addition of 10 µM DCMU.

Phytoplankton metabolic activity was measured 24 hours after nutrient addition using the FDA technique described previously. The flow cytometer settings used for field populations were different than those used for *Microcystis aeruginosa* in the previous two chapters and therefore the results are on a different scale but are relative within experiments.

Statistics were performed using JMP statistical package (SAS, 1996). When variances between treatments were unequal the assumptions of analysis of variance were not satisfied and a Welch

anova used instead. Prior to statistical analysis homogeneity of variances was tested for using O'Brien, Brown-Forsythe, Levene and Bartlett's tests (p=0.05; SAS, 1996). If the variances were considered equal single factor anovas were executed. Dunnetts post-hoc tests were performed comparing each treatment with the control. The control was compared with the initial biomass to identify if there were sufficient nutrients initially in the system to maintain the population and sustain growth for five days. Nutrient spike treatments were compared with the control for biomass and FDA conversion rate. In the figures significance (p=0.05) is represented by letters;

a- indicates that the control is significantly different from the initial

b- the control is not significantly different from the initial

c- the treatment is significantly different from the control

d- the treatment is not significantly different from the control

4.2.3 Micronutrients and phytoplankton growth

The micronutrients iron, molybdenum, silicon and manganese were added in various combinations to river water to identify if they were limiting cell growth. Water was collected on March 19, 1996, at Swan Reach (34° 34' S 139° 36' E; 247 kilometres upstream from the Murray mouth) from the Swan Reach Ferry using a 2m long PVC pipe which collected an integrated sample over the euphotic zone. Three replicate sites were sampled spanning the width of the river; mid channel and 15 m from each bank. Iron was added as 10 µM $C_6H_5O_7Fe.H_2O$, molybdenum as 25 µM $Na_2MoO_4.2H_2O$, manganese as 0.9 µM $MnCl_2.4H_2O$ and silicon as 100 µM $Na_2SiO_3.5H_2O$. Citrate was included as a control for ferric citrate to test the affect of the chelator alone (Reuter and Peterson, 1987).

The flasks containing the river water and nutrient combinations were incubated at 100 µmol m^{-2} s^{-1} 12:12 light:dark cycle, 25°C and metabolic activity was measured, using the FDA technique, after 24 hours. Biomass was estimated after five days by measuring total, maximum chlorophyll fluorescence. Chlorophyll per cell was estimated on the flow cytometer following the addition of 10 µM DCMU to examine the effect of improved nutrient climate on photosynthetic pigments.

The variances were homogeneous for the FDACR data and a single factor ANOVA and Tukey-Kramer post hoc tests (p=0.05, n=3) were used to identify significant differences. The biomass data had heterogeneous variances so a Welch ANOVA was used with post-hoc tests as described above. Bars above the histogram in the results represent significantly similar groups at the p=0.05 level (Tukey-Kramer).

The relative effects of nutrients and light on chlorophyll content were examined in cultured *Microcystis aeruginosa* to examine the influence these variables have on photosynthetic pigments and reassess whether pigments can be used to detect cell nutrient status.

4.2.4 Light and *Microcystis* chlorophyll content

The effect of light on chlorophyll content in *Microcystis aeruginosa* was examined by growing three replicate cultures in ASM-1 media in eight light intensities (11, 23, 44, 63, 81, 100, 150, 244 µmol m^{-2} s^{-1}) on a 12:12 light:dark cycle (25°C) as described in chapter 3. Chlorophyll content per cell was estimated using a flow cytometer following the addition of 10 µM DCMU on days 2, 4, 6 and 8 following inoculation and incubation at the different irradiances.

4.2.5 Nitrogen and *Microcystis* chlorophyll content

Log phase *Microcystis* were deprived of nitrogen for two days prior to inoculation into 0, 2, 5, 10, 25, 50, 100, and 200 µM nitrate in otherwise normal ASM-1 to examine the impact of nitrogen deficiency on the chlorophyll content per cell. Culture conditions were 100 µmol m^{-2} s^{-1} on a 12:12 light:dark cycle and 25°C. Chlorophyll content per cell was estimated following the addition of 10 µM DCMU using a flow cytometer on days three and six following inoculation and incubation in the different nitrogen concentrations.

4.2.6 Backwater and main channel populations-who has it better?

The FDA staining technique and nutrient bioassays were employed to assess if there was any observable distinction in the metabolic activity and nutrient status of phytoplankton from the different environments.

A single survey was conducted on January 24, 1995, incorporating both main river channel and backwater sites described below. The connecting streams may act as inlets or outlets depending upon the wind velocity and hydraulic gradient between the main channel and the backwaters.

On the day of sampling the connecting channels at both Big Bend Lagoon and Devon Downs Lagoon were acting as inlets to the backwaters. It was therefore not possible to detect whether these backwaters were seeding the main channel. The sites sampled are marked on map 4.2 and described below:

Site	Location	Description
1 Nildottie	223 km from Murray Mouth	Net tow sampling in main channel
4 South Devon Downs lagoon	Adjacent to the main channel at 223 km	Grab sample, 0.6 metres deep
3 North Devon Downs Lagoon	Adjacent to the main channel at 225 km	Grab sample, very shallow, <0.4m
4 Devon Downs Lagoon inlet	At inlet 227 km from Murray Mouth	Grab sample
5 Downstream of Devon Downs inlet	225 km from Murray Mouth	Grab sample
6 Cliffs, upstream of Devon Downs outlet	228 km from Murray Mouth	Net tow.
7 Down stream of Big Bend Lagoon outlet	235.5 km from Murray Mouth	Net tow
8 Big Bend Lagoon South	Adjacent to main channel at 235.5 km	Grab sample
9 Big Bend Lagoon North	Adjacent to main channel at 237 km	Grab sample, taken near inlet so mainly river water
10 Big Bend Lagoon inlet	237 km from Murray Mouth	Grab sample, water flowing into lagoon at 1 ms^{-1}
11 Upstream of Big Bend Lagoon inlet	238 km from Murray Mouth	Net tow

Table 4.2 River and backwater sites sampled to assess the metabolic activity of phytoplankton from the two habitats

The ability of phytoplankton from sites 7,8,10 and 11 to hydrolyse FDA was used to estimate relative metabolic activity and to detect nitrogen or phosphorus limitation (as described above).

Another comparison was made between Wachtels Lagoon (site 1, Map 4.1)and the main river channel. Three sites were sampled; upstream of the lagoon outlet, in the centre of the lagoon and downstream of the lagoon outlet. Bioassays to detect nitrogen or phosphorus limitation

were performed using FDA and changes in biomass as indicators using methods described above.

The species composition of the field samples was determined by microscopic cell counts at the beginning of the experiment and following 5 days growth. Algal species were scored for presence or absence in twenty microscope fields of view (magnification 100x) as an estimate of relative abundance. Some species were not abundant enough to obtain resolution between sites so cell counts were performed using a Sedgewick Rafter chamber. Filaments were counted as biomass units.

4.3 Results

4.3.1 Nutrient survey

The survey conducted along the lower reaches of the River Murray yielded information on phytoplankton abundance and nutrient status. The initial biomass, as indicated chlorophyll a concentration was significantly higher at Devon Downs Lagoon than at the river sites (Table 4.4b; Single factor ANOVA, $p<0.001$, $n=3$; Tukey-Kramer all pairs comparison $p=0.05$).

Traditional nutrient bioassays and the FDA technique both detected nutrient limitation in the phytoplankton sampled from the lower River Murray (figure 4.1). The ability of phytoplankton from Wachtels Lagoon to hydrolyse FDA was similar for all nutrient treatments and not significantly different from the control after 24 hours (Single factor ANOVA, Dunnets compare with control post-hoc test, $p=0.05$, $n=3$). This indicates either nutrient sufficiency or that an element other nitrogen or phosphorus was limiting esterase activity. Alternatively 24 hours may not have been long enough for the inclusion of the limiting nutrient into the cell in order to increase FDACR. There was no growth in samples from Wachtels Lagoon upon the addition of nitrogen or phosphorus. This result suggests there was nutrient limitation. When nitrogen and phosphorus were both added to the Lagoon sample there was a significant increase in yield above the control and other treatments.

The FDA assay identified a combined nitrogen and phosphorus limitation at Lock 3 which translated to a higher yield in the growth bioassays. Nitrogen addition alone increased growth which may have been limited by phosphorus availability. The phosphorus treated sample did not show any growth because it was nitrogen limited. This trend was similar for Waikerie and Morgan sites where there was some growth with nitrogen limitation but significant growth with both nitrogen and phosphorus addition.

At the sites further downstream (Lock 1, Devon Downs Lagoon, and Nildottie) the FDA assay detected an increased metabolic activity in phytoplankton from Lock 1 spiked with nitrogen perhaps indicating a greater nitrogen than phosphorus limitation or less phosphorus limitation. The Nildottie sample only responded to N+P addition. Growth occurred in bioassays with nitrogen and nitrogen and phosphorus addition at all three downstream sites, indicating a nitrogen limitation. The percentage differences of final biomass and FDA conversion rate of nutrient enriched samples relative to the non enriched (table 4.3) identified an increasing growth response to the addition of nitrogen in the downstream sites. The nutrient gradient is also identifiable by comparing the FDA conversion rates. The apparent gradient in response to nitrogen addition may be due to a greater nitrogen limitation relative to phosphorus limitation or less phosphorus limitation at the downstream sites. The nutrient data (figure 4.4b) offer little insight into this as there is negligible difference in nutrient concentrations between river sites, although Devon Downs Lagoon does have higher concentrations than the main river channel.

In summary, nitrogen limitation was detected at one site using the FDA assay and at five sites using traditional growth bioassays. Phosphorus limitation was not detected with either assay. Combined phosphorus and nitrogen limitation was detected after 24 hours at four sites using the FDA conversion at every site after five days using growth bioassays.

	Nitrogen enriched		Phosphorus enriched		N and P enriched	
Site	**Biomass**	**FDACR**	**Biomass**	**FDACR**	**Biomass**	**FDACR**
Wachtels	150	85	105	110	389*	113
Lock 3	188*	117	114	123	756*	141*
Waikerie	168*	91	86	93	397*	121
Morgan	149	123	66	136	419*	168*
Lock 1	261*	125*	103	99	625*	158*
D.Downs	257*	130	109	93	604*	127
Nildottie	343*	115	117	108	755*	137*

Table 4.3 To standardise the growth and metabolic response of phytoplankton from different sites enriched with nutrients the percentage biomass or FDACR were calculated relative to the unenriched control. FDA and growth bioassays were measured 24 hours and five days after inoculation, respectively. (* denotes significantly different from control).

4.3.2 Chemical analysis -Nutrient survey

There was less soluble inorganic nitrogen (nitrate+nitrite) at the three downstream sites but all sites had soluble inorganic nitrogen concentrations below 0.1 mg L^{-1} which is considered to be the minimum concentration to maintain growth during a growing season (Reynolds, 1992). It is difficult to speculate how widespread, in a temporal sense, the apparent nutrient gradient is from a restricted sampling period. Reactive phosphorus concentrations were all at about the level of detection (0.004 mg L^{-1}, table 4.4b) and less than the 0.01 mg L^{-1} FRP concentration below which growth is considered to be P-limited (Sas, 1989). The water chemistry analysis showed a higher conductivity in Wachtels Lagoon and along with Devon Downs Lagoon the backwater sites had higher total dissolved solids, bicarbonate and calcium carbonate.

To estimate the fraction of the total phosphorus and total nitrogen incorporated in the chlorophyll containing particulate fraction the chlorophyll to phosphorus and chlorophyll to nitrogen ratios of Reynolds (1984, 1992) were used (Chl:P=1:1, Chl:N=7.7:1). The phosphorus and nitrogen in the non-chlorophyll bearing fraction were then calculated. Chlorophyll values, representing phytoplankton biomass, were highest in Devon Downs Lagoon (table 4.4b) and were twice that of the main river channel sites and correspond with higher total nitrogen and total phosphorus. Phosphorus and nitrogen incorporated into the

chlorophyll bearing plankton was estimated to be 19.01-46.8 μg L^{-1} and 129-360 μg L^{-1} respectively. Phosphorus and nitrogen not in the chlorophyll fraction were 41-84 μg P L^{-1} and 630-940 μg N L^{-1}.

The N:P ratio in the non-chlorophyll bearing particulate fraction ranged from 11.1-16.2 by weight. N:P ratios below 5 promote the dominance of cyanobacteria when nitrogen, phosphorus or both are limiting (Reynolds, 1984; Reynolds, 1992). Higher N:P ratios favour other phytoplankton. There is a significant nitrogen and phosphorus pool in the non-chlorophyll bearing fraction, however the flux rates of both nutrients from the particulates are not known.

Biomass data (table 4.3) illustrate that nitrogen enrichment increased the biomass but phosphorus had an insignificant effect on growth. For growth to occur when nitrogen is added phosphorus must be available. The soluble reactive phosphorus concentrations are below the level of detection, therefore, some fraction of the total phosphate pool, excluding phosphorus already incorporated into chlorophyll bearing particulates, must be bioavailable or internal stores used.

To estimate the bioavailable fraction of the total phosphorus the change in biomass was estimated using the difference in DCMU-sensitive chlorophyll fluorescence between the day 0 initial and day 5 +N treatment. From the equivalent day 0 chlorophyll data and DCMU sensitive chlorophyll fluorescence a ratio was calculated from which future chlorophyll concentrations could be approximated using chlorophyll fluorescence. DCMU-sensitive chlorophyll concentration was converted to a chlorophyll concentration using the calculated standard 1 μg L^{-1} chlorophyll = 5.7 DCMU-sensitive fluorescence units (from data in figure 4.1 and table 4.4b). The amount of phosphorus needed to support this increase in chlorophyll was calculated using the chlorophyll:phosphorus ratio of 1:1 (Reynolds, 1992) and expressed as a percentage of the total non-chlorophyll bearing phosphorus fraction.

Site	Increase in +N Chl fluorescence above initial (fluoro units)	Relative change in Chl (1µg L^{-1} = 5.7 Chl fluoro units	P incorporated in new growth (Chl:P=1:1)	TP in the non-Chl bearing Total P fraction (µg L^{-1})	% of total P which is bioavailable (%)
Lock 3	21	3.7	3.7	44	8
Waikerie	112	19.6	19.6	49	40
Morgan	92	16	16	46	35
Lock 1	133	23.3	23.3	48	49
Devon Downs	348	61	61	84	73
Nildottie	187	32	32	41	78

Table 4.5 Growth when nitrogen is added must be accompanied by the incorporation of phosphate into cells. When the soluble reactive phosphorus fraction is low this phosphate originates from the total phosphorus pool which is bound to particulates. The percentage of the phosphorus which is bioavailable can be estimated by how much P must be incorporated into cells to allow the observed increase in growth which is measured as increase in DCMU-sensitive chlorophyll fluorescence.

Between 8 and 78 % of the non-chlorophyll bearing fraction was estimated to be bioavailable which is similar to estimates by Oliver *et al.* (1993) who measured desorbable and bioavailable phosphorus to be between 18 and 48 % of the total phosphorus. This may be an overestimate of bioavailability as chlorophyll per cell also increases with improved nitrogen conditions which would decrease the chlorophyll to phosphorus ratio. However, it is clear that a large proportion of phosphorus in the total pool was bioavailable which contrasts with nitrogen which was relatively unavailable as indicated by the low growth in the presence of phosphorus.

4.3.3 Micronutrients and phytoplankton growth

When added individually nitrogen and phosphorus were not limiting FDA conversion but when added together nitrogen and phosphorus limitation was detected at Swan Reach (figure 4.2). There was a significant increase (One-way ANOVA, Tukey-Kramer, p=0.05, n=3) in metabolic activity between the river sample (25 relative fluorescein units min^{-1}) and the nitrogen and phosphorus enriched sample (57 relative fluorescein units min^{-1}). Significance bars in figure 4.2 indicate how the treatments grouped in a Tukey Kramer test p=0.05; treatments covered by a bar were not significantly different to samples covered by the same bar but were significantly different to other treatments.

For trace element studies river water was enriched with nitrogen and phosphorus and then further enriched with trace elements. Phytoplankton in water with added iron, manganese and molybdenum did not alter the metabolic response relative to the phytoplankton enriched with just nitrogen and phosphorus, however, the presence of silicon significantly suppressed the metabolic activity of the river plankton relative to the N+P control. Two possible explanations for this are that the silicon compound or ion bound another crucial element reducing its bio-availability, or that silicon was toxic at that concentration. Citrate was added as a control for the chelator added with iron. The FDA measured metabolic activity following citrate enrichment was the highest recorded. The citrate may have been acting as a supplementary carbon source and enzyme substrate.

The relationship between FDACR and future growth, which was examined in chapter three (figure 3.9) was re-examined using data presented in figure 4.2. The biomass measured five days after the trace element enrichment revealed a similar trend to the FDA data and correlation analysis showed FDA conversion rate on day 1 was linearly proportional to the final biomass (r^2=0.66, n=3, $p<0.05$). FDA conversion rate measured five days after enrichment was more closely related to the final biomass (r^2=0.79, n=3, $p<0.05$) supporting evidence provided in figure 3.9 that FDACR is valuable in predicting relative growth.

4.3.4 Chlorophyll fluorescence as an estimate of nutrient limitation

Both light and nutrients influence the chlorophyll content per cell and consequently this has raised doubt as to whether photosynthetic pigments can be used to assess nutrient status of populations (Wood and Oliver, 1995). The chlorophyll content per cell is a cellular response to the ambient light and nutrient conditions the cell has encountered recently and potentially yields information on the light and nutrient history of the cell. The interacting effects of light and nutrients can be uncoupled by maintaining cells at a constant light intensity. Chlorophyll can be used as an indicator of nutrient status in experiments where there is a short incubation (24 hours) following the addition of various nutrients. Increased chlorophyll content in response to an improved nutrient climate could be used an indicator of nutrient limitation.

The chlorophyll fluorescence per cell was measured on day 5 on the flow cytometer after the addition of 10 μM DCMU. The chlorophyll concentration per cell was linearly related

(r^2=0.73) to the final biomass (figure 4.3a) which indicates that the estimated biomass (total maximum chlorophyll fluorescence) was a function of both increased cell numbers and increased chlorophyll per cell. The chlorophyll concentration per cell was also linearly proportional to the FDA conversion rate (figure 4.3b; r^2=0.84). This result raises the question,: can chlorophyll *a* fluorescence be used in place of FDA to detect nutrient limitation?

The chlorophyll content of *Microcystis* cells grown in different nitrate concentrations (figure 4.3c) showed that nitrogen limitation led to reduced chlorophyll content on day 3 at concentrations less than 5 μM. As nitrogen limitation occurred in cultures with higher initial nitrate (day 6, nitrate <100 μM) the chlorophyll content also decreased. In different light intensities (figure 4.3d) the chlorophyll content decreased as the irradiance increased.

4.3.5 Backwater and main channel populations

The metabolic activities of phytoplankton sampled from backwaters and the main river channel were compared to identify if there were any relative differences in growth potential (figure 4.4). Sites 2, 3 and 8 were all backwater sites and phytoplankton from these sites had the highest FDA conversion rates, and grouped together in a Tukey-Kramer post hoc test following a single factor ANOVA p=0.05, n=3, figure 4.4). Site 9 was also a backwater site but was sampled near the inlet and consequently the phytoplankton sampled may have originated from the river and not the backwater. Sites 2 and 8 also grouped with sites 1, 4, 6, 7, 10 and 11 indicating significant overlap between groups. Phytoplankton from site 5 had a lower metabolic activity, but grouped with the other river sites in a single factor analysis of variance. Whether the healthier phytoplankton were "seeding" the main channel could not be investigated because the connecting channels were inflowing to lagoons on the day of sampling.

Four sites from the above survey were chosen to assess the nutrient status of phytoplankton from Big Bend Lagoon (site 8), the inlet (site 10), and upstream (site 11) and downstream (site 7) from the inlet. Both nitrogen and phosphorus limitation was detected at site 7 using the FDA nutrient assay (figure 4.5). Nitrogen limited metabolic activity at site 8. Growth bioassays detected nitrogen limitation at sites 7, 8 and 11 and showed growth was stimulated by the addition of both nitrogen and phosphorus at all sites. Growth bioassays showed phosphorus limitation at site 8. The biomass yield, with nitrogen enrichment, was

approximately five times greater than the control at sites 7 and 11 but only about twice as much at sites 8 and 10. At sites 8 and 10 phosphorus limitation, which was detected with the FDA assay, may have reduced the final yield.

In summary N, P and N+P limitation was detected with FDACR at site 7 but only N and N+P limitation detected by growth bioassays. At site 8 FDACR detected N and N+P limitation but the growth bioassays detected limitation of N, P and N+P. The FDA technique failed to detect the N+P limitation at sites 10 and the N and N+P limitation at site 11. The inconsistencies between FDACR and growth bioassays could arise due to a limitation being induced in the flask after five days which was not present after one day and consequently would only show up in the growth bioassays not the FDA assays. Alternatively, a one day incubation following nutrient enrichment may not have been long enough to increase FDACR to a level greater than the control which was statistically significant.

Nitrogen limitation was again detected in April at a Lagoon further upstream, Wachtels lagoon (figure 4.6). Phosphorus limitation was also identified in the Lagoon using the FDA technique, however, this was not evident in the growth bioassays probably because of the extreme nitrogen limitation. The river populations revealed only nitrogen limitation. Nitrate and filterable reactive phosphorus concentrations were below detectable levels at this time (table 4.6).

Site	Total phosphorus as P (mg L^{-1})	Filtered reactive phosphorus as P (mg L^{-1})	TKN as N (mg L^{-1})	Nitrate+nitrite (mg L^{-1})
Upstream	0.078 (0.0057)	<0.005	0.68 (0.036)	<0.01
Lagoon	0.074 (0.0025)	<0.005	0.67 (0.055)	<0.01
Downstream	0.056 (0.0061)	<0.005	0.61 (0.078)	<0.01

Table 4.6 Water chemistry of samples collected at Wachtels Lagoon and in the adjacent river channel, upstream and downstream of the lagoon outlet.

In river populations few species achieve dominance (Reynolds, 1994), more usually a range of species coexist (Sommer *et al.*, 1993) and may become favoured under certain environmental conditions. To examine if there was a differential response of riverine phytoplankton to nutrient enrichment the relative abundance of the major species was estimated. Initial counts revealed differences in species composition between the river channel and the backwater. The cyanobacteria *Aphanothece* sp., coiled and straight *Anabaena* spp., and spiral cyanobacteria (tentatively identified as *Cylindrospermopsis* sp.) were more abundant in the backwater, along with *Ankistrodesmus* sp. (figures 4.7 and 4.8). *Aulacoseira* sp. on the other hand was more abundant in the main river channel.

The FDA and growth-nutrient assays had identified a nitrogen limitation in the main channel (figure 4.6) which was demonstrated again by the increase in biomass of the dominant river species upon nitrogen enrichment. In the Lagoon the N_2-fixing *Anabaena* spp. increased their relative biomass upon the addition of phosphorus. The non N_2-fixing cyanobacteria (*Aphanothece* sp.), green alga (*Scenedesmus* sp., *Actinastrum* sp., *Ankistrodesmus* sp., *Planktolynbia* sp.) and diatoms (*Aulacoseira* sp.) all displayed a relative nitrogen deficiency. The relative abundance of cyanobacteria in the backwater and absence in the main river channel may explain why the FDA and growth assays (figure 4.6) identified a phosphorus limitation in the backwater but not the river.

4.4 Discussion

An aim of this research was to demonstrate the applicability and value of the FDA and flow cytometry technique to identify nutrient status of natural phytoplankton populations.

4.4.1 FDA assay to assess the nutrient status of field populations

The FDA conversion assay detected significant nutrient limitation at various lower River Murray sites during the summer of 1996. The aim was to detect nutrient limitation or sufficiency with a minimum incubation time to overcome problems associated with longer incubations (Hecky and Kilham, 1988; Wood and Oliver, 1995) and ensure that the population sampled was the population measured. Consequently, a 24 hours incubation time was chosen since this was the

time nutrient limited culture took to respond to nutrient additions via FDA conversion rates (chapter 3; figures 3.12 and 3.13). On numerous occasions the mean FDA conversion rate following nutrient enrichment was greater than the control but was not statistically significant at the designated p=0.05 level. This may indicate that there was nutrient limitation which was not detected in 24 hours but may have been observed with a slightly longer incubation time or greater replication. Further refinement of the technique may increase its sensitivity by selecting for certain phytoplankton groups, based on optical characteristics, and measuring species specific FDACR response to nutrient enrichment.

4.4.2 Nutrient survey

The river is in continual motion and pelagic organisms are transported in the direction of net flow. It is not possible to determine from a single survey whether the higher biomass at Morgan was in response to favourable growth conditions upstream of Morgan or whether a local "dead-zone" (Reynolds, 1994) increased hydraulic retention. Phytoplankton responded to nitrogen enrichment at all sites but the greatest percentage increase occurred at the downstream sites. This may be because the phytoplankton were more nitrogen limited than phosphorus limited at these sites, or phosphorus less limiting which enabled a higher biomass yield. The apparent nitrogen limitation was observed further downstream but there was sufficient total nitrogen present to support a population. The relative nitrogen limitation may be caused by the loss of readily desorbed or available nitrogen to phytoplankton further upstream reducing the fraction of labile and bioavailable nitrogen at down stream sites.

Separation of the total phosphorus and total nitrogen into chlorophyll bearing and non-chlorophyll bearing particulate fractions enabled an estimate of nutrients potentially available to the phytoplankton. The bio-availability of nutrients depends on the rate and quantity of the flux from the particulates. N:P ratios of 11.1-16.2 would suggest that phosphorus would become limiting, if the flux rates were low and proportional to the above ratio, and the phytoplankton used nitrogen and phosphorus in amounts proportional to the Redfield ratio (100C:15N:1P, Redfield, 1958) which equates to N:P ratio of 7:1 by weight (Reynolds, 1984). This was not observed and nitrogen appeared to be limiting particularly in the growth bioassays. This maybe due to differential rates of flux and bioavailability of the two nutrients which could, in part, be

due to different desorption characteristics and microbial release. What would be more beneficial to our understanding of nutrient availability than N:P ratios is some estimate of the ratio of N:P flux rates and of different nitrogen and phosphorus species in the total nutrient pool. The nitrogen in the system was relatively unavailable for utilisation by algae but a large fraction of phosphorus was bioavailable.

4.4.3 Micronutrients and phytoplankton growth

No trace element limitation was observed in phytoplankton sampled from Swan Reach. There was a slight suppression of algal growth and depression of enzyme activity in the presence of silicon relative to the N+P control. Whether all species were negatively affected by the presence of silicon is unknown because species abundance was not estimated. If there was a differential response to silicon then when silicon concentrations in the water are high diatoms would be favoured. The nutrient requirements of diatoms would be satisfied but other species would suffer a toxic response leading to a reduction in resource competition.

Investigations into trace element limitation were all performed with excess nitrogen and phosphorus. It would be of interest to investigate the influence of trace element addition when nitrogen and phosphorus were in short supply. The phytoplankton's requirement for molybdenum and iron may be greater when nitrogen is limiting and a limitation more readily detected. Nitrogen fixing cyanobacteria demand up to ten times more iron than when they are growing at the same rate in nitrate (Reuter and Peterson, 1987), to optimise photosynthesis and supply reducing products to nitrogen fixation and nitrate reduction. Also when cyanobacteria are fixing nitrogen molybdenum is required in nitrogenase as well as nitrate reductase and consequently there is a greater Mo demand (Steeg *et al.*, 1986).

4.4.4 Species specific nutrient limitation

The Wachtels lagoon nutrient study identified phosphate limitation in the N_2-fixing cyanobacteria and nitrogen limitation in the other species. When other species dominate different nutrients may limit growth and biomass yield. Silicon limitation could control diatom abundance (Healey, 1979; Miyajima *et al.*, 1993) and iron and molybdenum limitation regulate nitrogen metabolism, photosynthesis and growth, particularly in the N_2-fixing cyanobacteria

(Hallenbeck *et al.*, 1982; Steeg *et al.*, 1986; Reuter and Ades, 1987; Reuter and Peterson, 1987; Geider and La Roche, 1994; Greene *et al.*, 1994).

A limitation of "bulk bioassays" using whole population growth is that the community, which is composed of numerous species, is treated as a single unit and response to nutrient enrichment recorded as a community average. The advantage of flow cytometry is that it can distinguish different phytoplankton taxonomic groups based on their optical characteristics (Yentsch and Yentsch, 1979; Oldham *et al.*, 1985; Carr *et al.*, 1996). Flow cytometry could then be used in conjunction with the FDA assay to observe the relative changes in FDA response for each genera and determine "what is limiting what?" (Harris, 1994). This was not tackled in the work presented here because the available flow cytometer had only a single laser and could therefore not detect phycocyanin and the optical characteristics of the riverine species were not properly characterised.

4.4.5 FDA conversion rate and final biomass yield

The nutrient assays described in this chapter were primarily to test the FDA technique which clearly identified the relative health and growth potential of phytoplankton in the various treatments. The correlation between FDACR and biomass increases confidence in the technique in predicting relative future growth. In chapter 3 FDACR predicted relative growth rate of phosphate-limited cultures but it was noted that absolute growth rate could not be predicted from FDACR. Evidence is provided here which demonstrates again the value of the FDA technique in assessing relative population differences in metabolic activity and growth potential. An estimate of relative limitation may be possible in the future involving the addition of various concentrations of nutrients and measuring the FDA conversion rate. Used in conjunction with chemical nutrient analysis and data on the phytoplankton species present in the water body the FDA nutrient bioassay will provide further insight into nutrient bioavailability.

4.4.6 Chlorophyll content per cell, FDA conversion rate and cell nutrient status

The relationships between FDA conversion rate, chlorophyll per cell and biomass suggest that chlorophyll per cell may be used as an indicator of nutrient status. The response of algal pigments to nutrients has been documented previously (Yentsch and Phinney, 1984; Foy,

1993). In assays where a short incubation is required the light regime is controlled and pigments offer further insight into cell nutrient status. In field studies the impact of light and nutrients cannot be delineated using chlorophyll concentration alone. By using techniques to assess the nutrient status of the phytoplankton (Wood and Oliver, 1995), and a knowledge of water and phytoplankton movement (Harris, 1980; Whittington *et al.*, 1996; chapter 3) further insight will be gained on which factors are controlling algal biomass.

4.4.7 Backwater and main channel populations

The comparison of phytoplankton from the backwaters and main channel highlights the problem of establishing what factors contribute to higher metabolic activity. Phytoplankton in the backwaters had higher metabolic activities, however it was impossible to determine whether this was due to a more favourable light or nutrient climate. The backwaters appear to provide a habitat more conducive to phytoplankton population development but not all species prefer them. *Aulacoseira* tends to persist in the main river channel where there is turbulence to maintain cells in suspension (Webster *et al.*, 1996). The Wachtels lagoon study identified that *Aulacoseira* was more abundant in the more turbulent main channel than in the backwater. Cyanobacteria, on the other hand, preferred the backwater perhaps because the light climate was better or the buoyancy mechanism was not over-ridden by flow induced turbulence. Increased retention time in the backwater may also allow the relatively slow growing cyanobacterial population to develop.

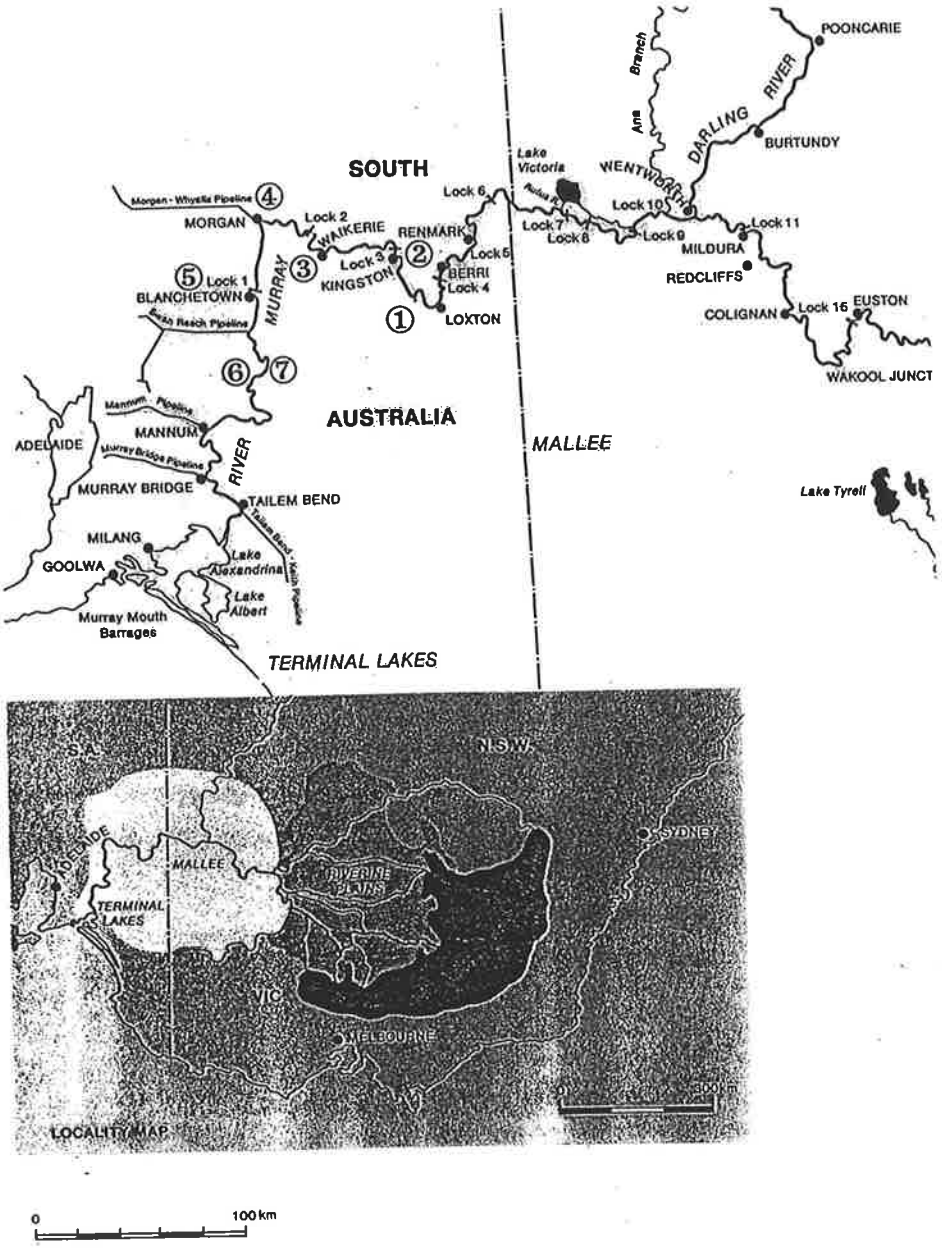
The species composition difference between Wachtels Lagoon and the main channel revealed a subtle difference in the relative nutrient deficiency between the two habitats. Cyanobacteria capable of fixing N_2 have a high demand and affinity for phosphorus and so phosphorus becomes limiting as well as nitrogen. In the main channel the nitrogen demand of *Aulacoseira* overshadows the phosphorus demand and consequently only a nitrogen limitation was detected. Light and nutrient interactions play a key role in determining species abundance and what nutrients become relatively limiting.

FDA and growth bioassays revealed a nitrogen limitation which was evident on several sampling occasions. Hecky and Kilham (1988) suggest that only the elements P, Fe, and Co are likely to become limiting in river water. Contrary to this phytoplankton sampled from the

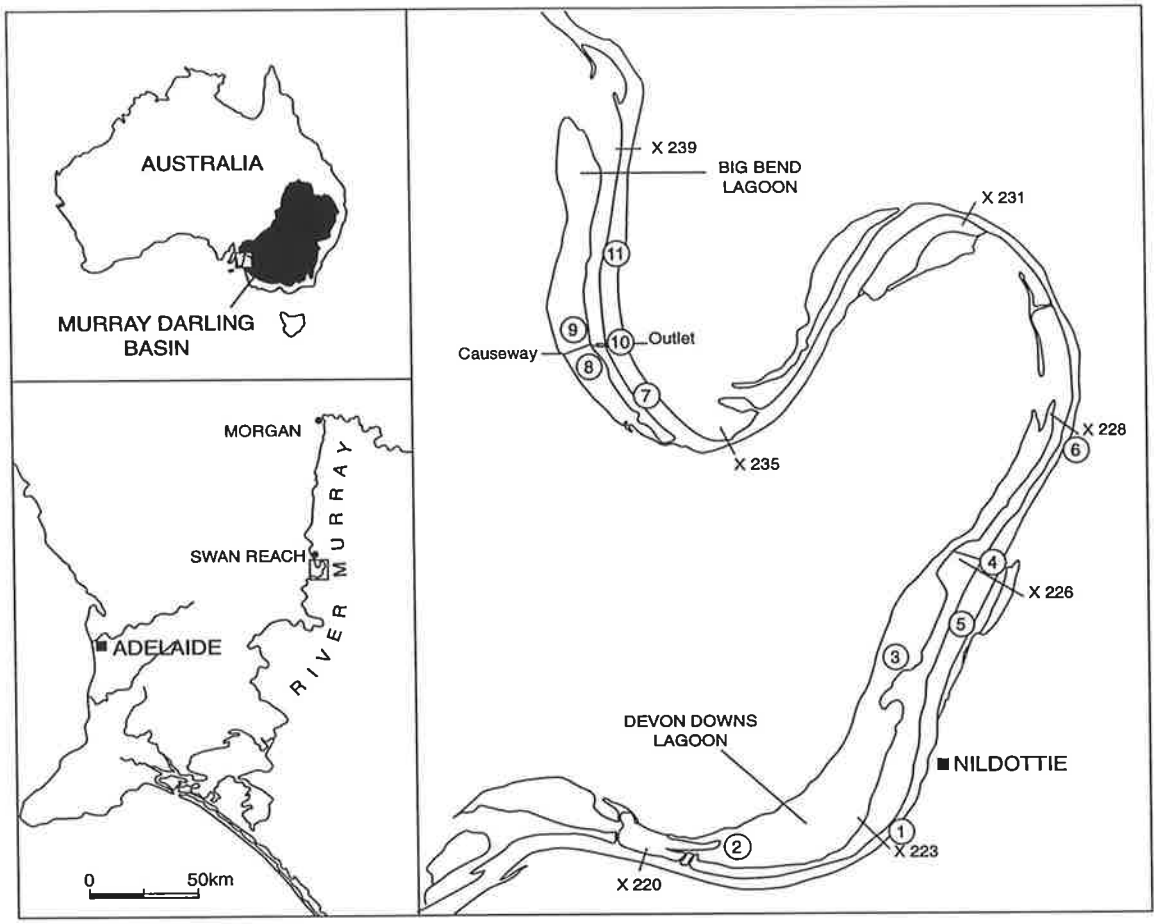
lower River Murray were conclusively shown to be nitrogen limited and it is possible that nitrogen limitation in Australian freshwaters is more important in determining species composition and yield than previously recognised. Elser *et al.* (1990) in a review of North American lake studies also found that nitrogen was limiting just as often as phosphorus. The paradigm that phosphorus limits algal growth in freshwaters (Hecky and Kilham, 1988) is questionable for although phosphorus may determine the maximum yield nitrogen also limits the growth of non N_2-fixing phytoplankton.

It has been demonstrated that the FDA technique can determine relative cell health and growth potential of laboratory and field phytoplankton and successfully identify limiting nutrients. There is scope to use the technique in other phytoplankton related studies including examining heavy metal toxicity and pollutant stresses (Berglund and Eversman, 1988; Gilbert *et al.*, 1992).

Nutrient limitation affects enzyme function, photosynthesis and growth which impact upon cell buoyancy by influencing gas vesicle production and carbon fixation and metabolism. The following chapters investigate the effect nitrogen and phosphorus limitation have on the buoyancy mechanisms and how these interact with light to effect the buoyancy status of the cell and ultimately the cells performance in a particular habitat.



Map 4.1 Sites along the Lower River Murray included in the nutrient survey on March 6 and 7, 1996



Map 4.2 Backwater and main channel sites on the Lower River Murray, South Australia. Samples were taken to compare the metabolic activity of phytoplankton inhabiting backwaters and the main channel and assess the nutrient status of the phytoplankton community.

Site	Conductivity ($\mu s\ cm^{-1}$)	Tot. dis. solids ($mg\ L^{-1}$)	Bicarbonate ($mg\ L^{-1}$)	Alkalinity as CaCO3 ($mg\ L^{-1}$)	Attenuation coefficient (m^{-1})
Wachtels Lagoon	832 (56)	443 (25)	76 (2)	62 (2)	2.62 (0.13)
Lock 3	519 (40)	283 (23)	63 (2)	52 (1)	2.75
Waikerie	531 (2)	290	63	52	2.53
Morgan	567 (3)	310	63	52	2.52 (0.06)
Lock 1	620 (9)	343 (6)	64 (1)	52	2.59
Devon Downs lagoon	540 (115)	460 (62)	73 (2.6)	60 (3)	6.12 (0.11)
Nildottie	643 (6)	353 (6)	66	54	2.24

Table 4.4a Water chemistry data for Lower River Murray sites sampled on March 6 and 7, 1996. The backwaters, Wachtels Lagoon and Devon Downs Lagoon exhibit higher concentrations of total dissolved solids, carbonate and bicarbonate than the main channel. Standard deviation in parentheses.

	TKN as N (mg L^{-1})	Ammonia as N (mgL^{-1})	Nitrate-nitrite as N (mg L^{-1})	Phosphorus total as P (mg L^{-1})	Filtered reactive P as P (mg L^{-1})	TN:TP ratio
Wachtels Lagoon	1.0 (0.035)	0.0077 (0.0006)	0.017 (0.0058)	0.059 (0.0052)	<0.004	16.9
Lock 3	0.86 (0.12)	0.010 (0.0015)	0.015 (0.0071)	0.063 (0.0045)	<0.004	13.6
Waikerie	0.85 (0.095)	0.017 (0.011)	0.02	0.066 (0.0017)	0.004	12.9
Morgan	0.88 (0.12)	0.008 (0.0017)	0.015 (0.0071)	0.066 (0.0021)	<0.004	13.3
Lock 1	0.89 (0.09)	0.0083 (0.004)	<0.01	0.07 0.0055)	0.004	12.7
Devon Downs Lagoon	1.3 (0.17)	0.0057 (0.0006)	<0.01	0.131 (0.011)	0.004	9.9
Nildottie	0.79 (0.091)	0.02 (0.02)	0.01	0.062 (0.0017)	0.0057 (0.0029)	12.7

	Chl a (µg L^{-1})	P in Chla fraction (µg L^{-1})	P not in Chla fraction (µg L^{-1})	N in Chla fraction (µg L^{-1})	N not in Chla fraction (µg L^{-1})	Non Chla N:P ratio
Lock 3	19.01 (1.95)	19.0	44	146.4	714	16.2
Waikerie	16.79 (0.32)	16.8	49	129.3	721	14.7
Morgan	19.14 (1.36)	19.1	47	147.4	733	15.5
Lock 1	21.16 (0.57)	21.2	49	162.9	727	14.8
Devon Downs Lagoon	46.80 (10.17)	46.8	84	360.4	940	11.1
Nildottie	20.79 (0.96)	20.8	41	160.1	630	15.4

Table 4.4b Water chemistry data for Lower River Murray sites. The amount of total nitrogen and phosphorus incorporated into chlorophyll bearing particles is estimated using the chlorophyll: nutrients ratios (Chl:N=1:7.7, Chl:P=1:1).

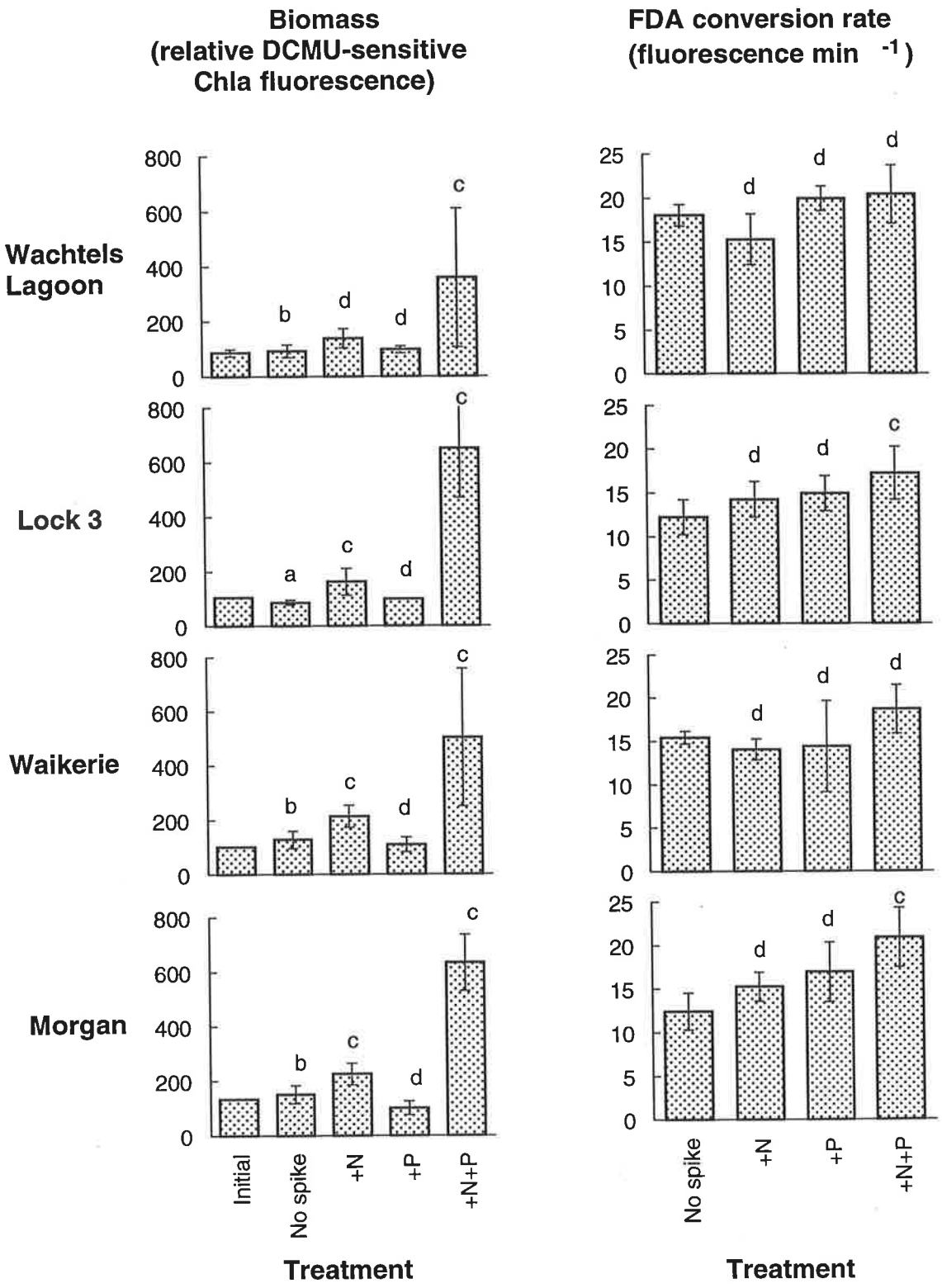


Figure 4.1 Growth and FDA bioassays of phytoplankton sampled from Lower River Murray sites and enriched with nitrogen, phosphorus or nitrogen and phosphorus. The control had no nutrient addition. Biomass was estimated as DCMU-sensitive chlorophyll fluorescence five days after enrichment and metabolic activity measured using the FDA technique described in chapter 3. (a- control is significantly different (sig. diff.) from the initial; b- control is not sig. diff. from the initial; c-nutrient spike treatment is sig. diff. from the control; d-nutrient treatment is not sig. diff. from the control).

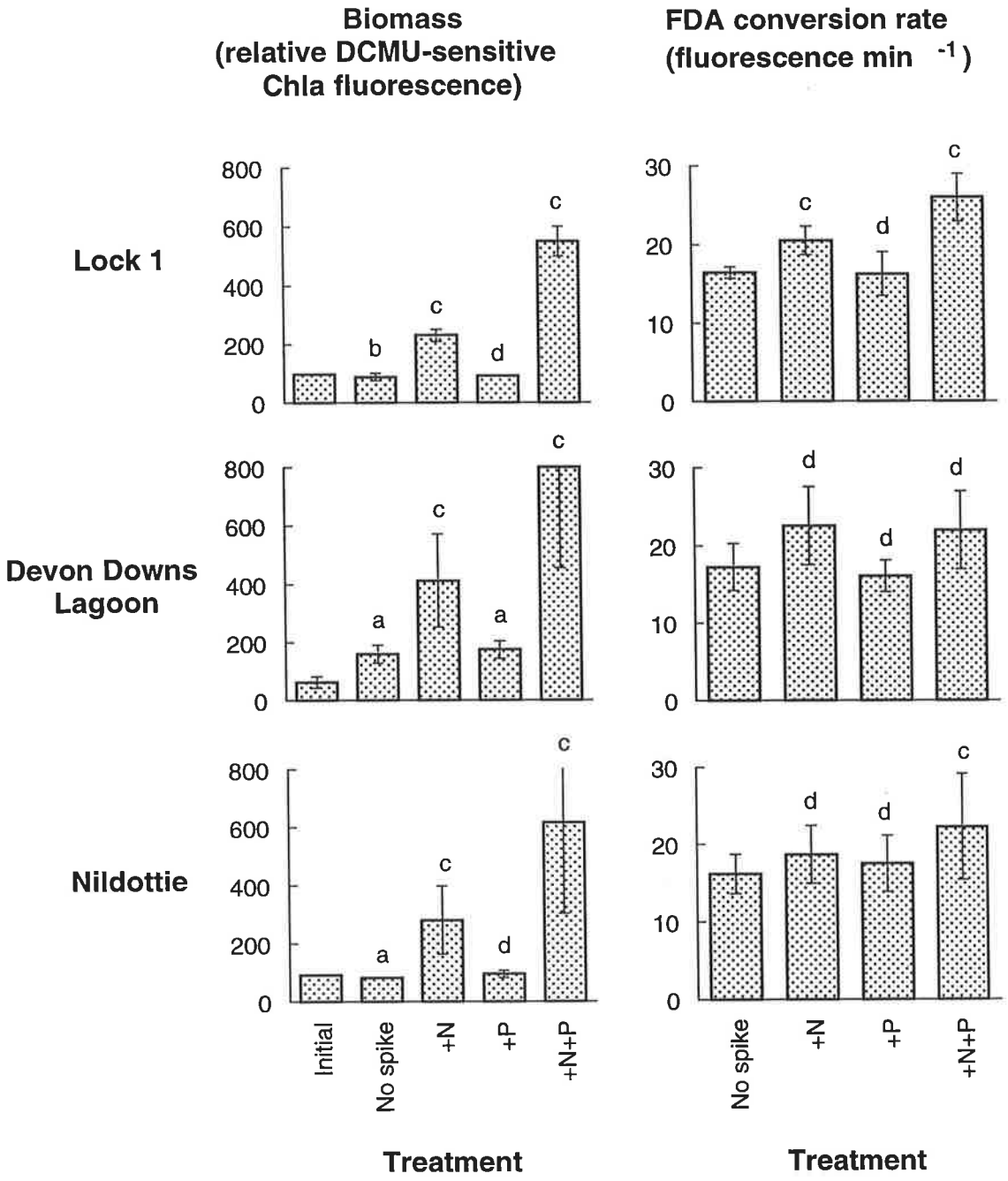


Figure 4.1 Continued.....

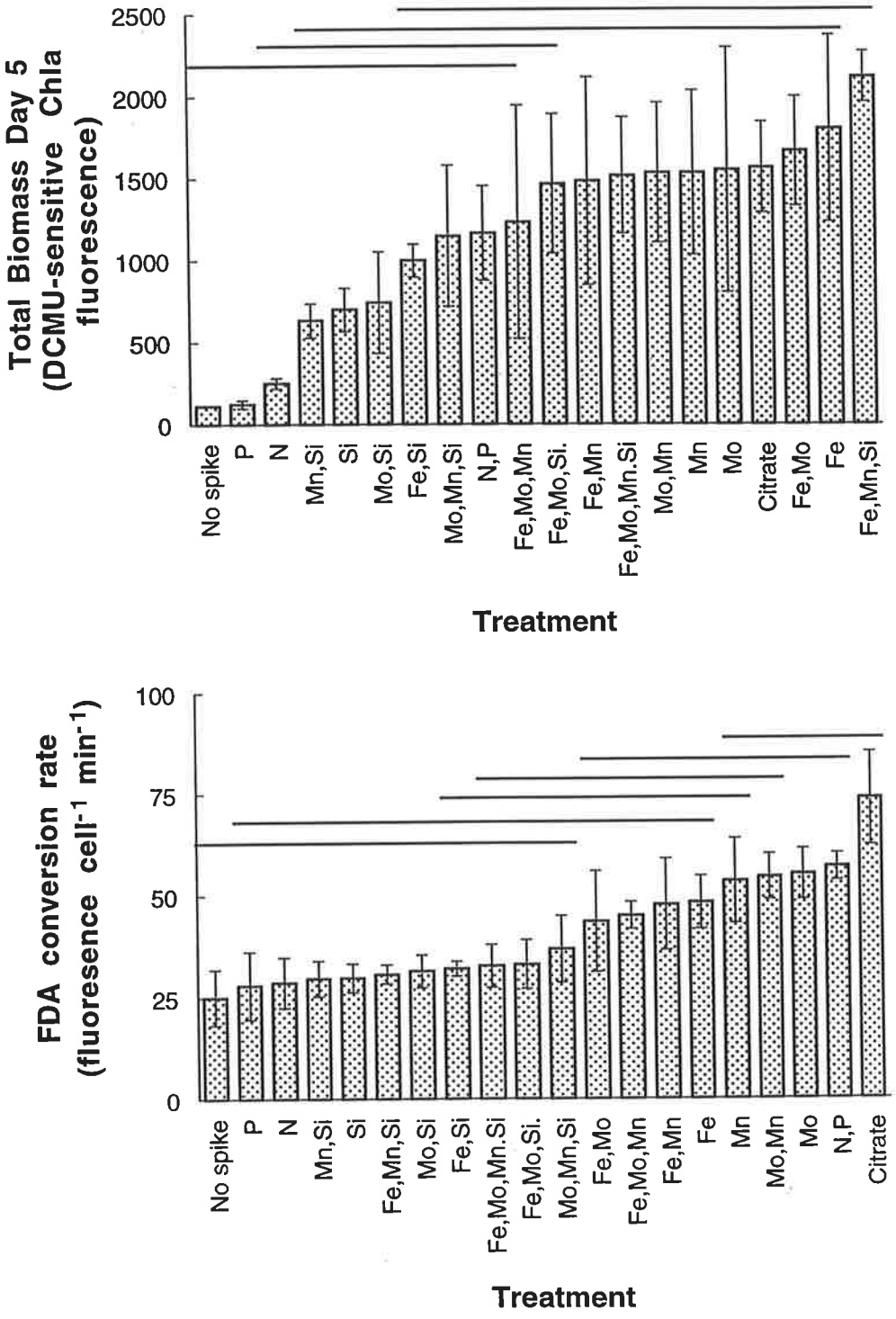


Figure 4.2 River water sampled from the River Murray at Swan Reach was enriched with nitrogen and phosphorus plus a range of trace elements. All trace element treatments had N and P added as these were known to be limiting. The control treatment was thus N+P. Silicon suppressed phytoplankton metabolic activity (bottom graph) but this did not necessarily transform to biomass (top graph) particularly in the presence of Fe+either Mn or Mo. Bars indicate how treatments grouped in an all pairs comparison post-hoc test (Tukey Kramer, p=0.05) following analysis of variance (One way ANOVA FDACR data; Welch ANOVA Biomass data)

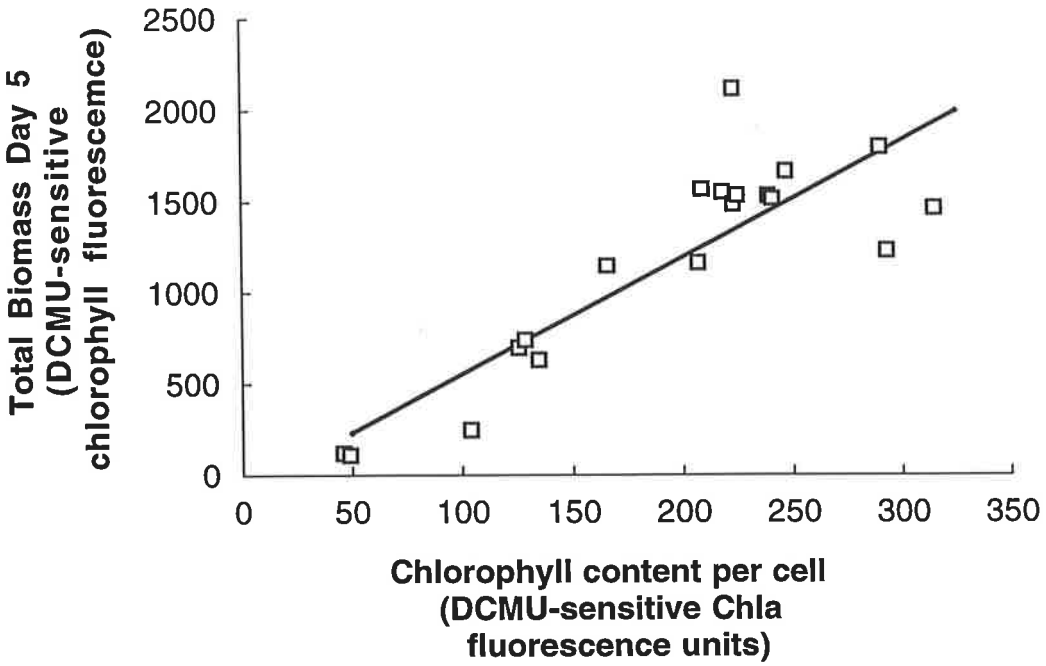


Figure 4.3a The chlorophyll content per cell, measured using a flow cytometer, was linearly correlated (r^2=0.73) with total biomass measured as DCMU-sensitive chlorophyll fluorescence on a flow cytometer

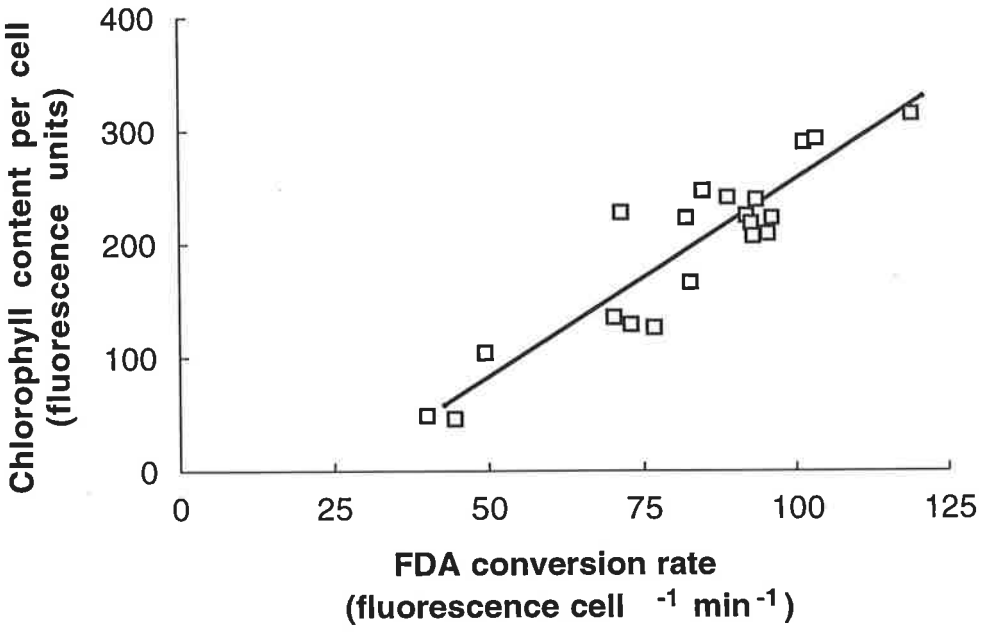


Figure 4.3b The relationship between chorophyll content per cell and enzyme activity (r^2=0.84) in cells grown in a range of trace element treatments.

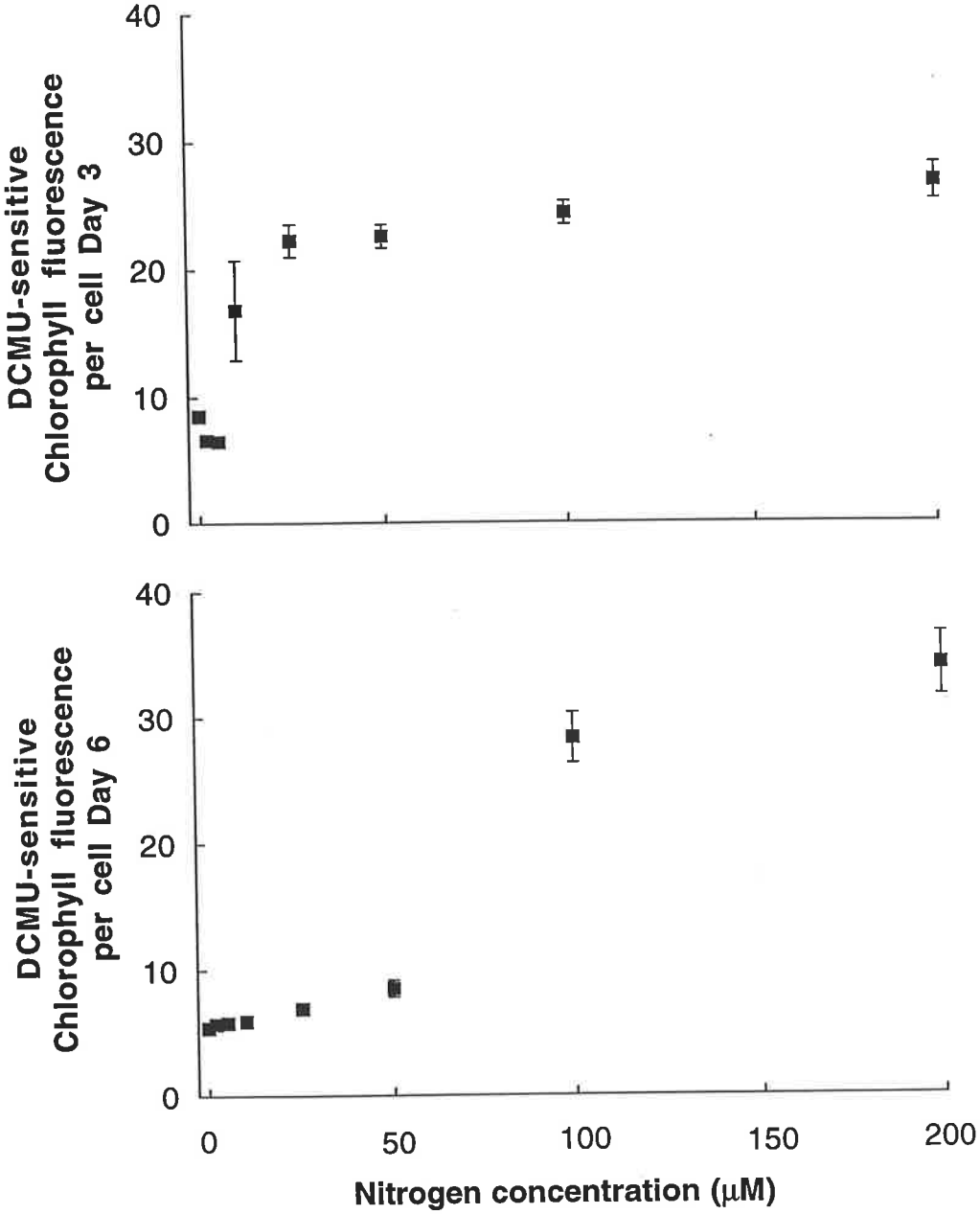


Figure 4.3c Nitrate dependent chlorophyll content of *Microcystis aeruginosa* cells three days after inoculation (top graph) and after six days (bottom graph).

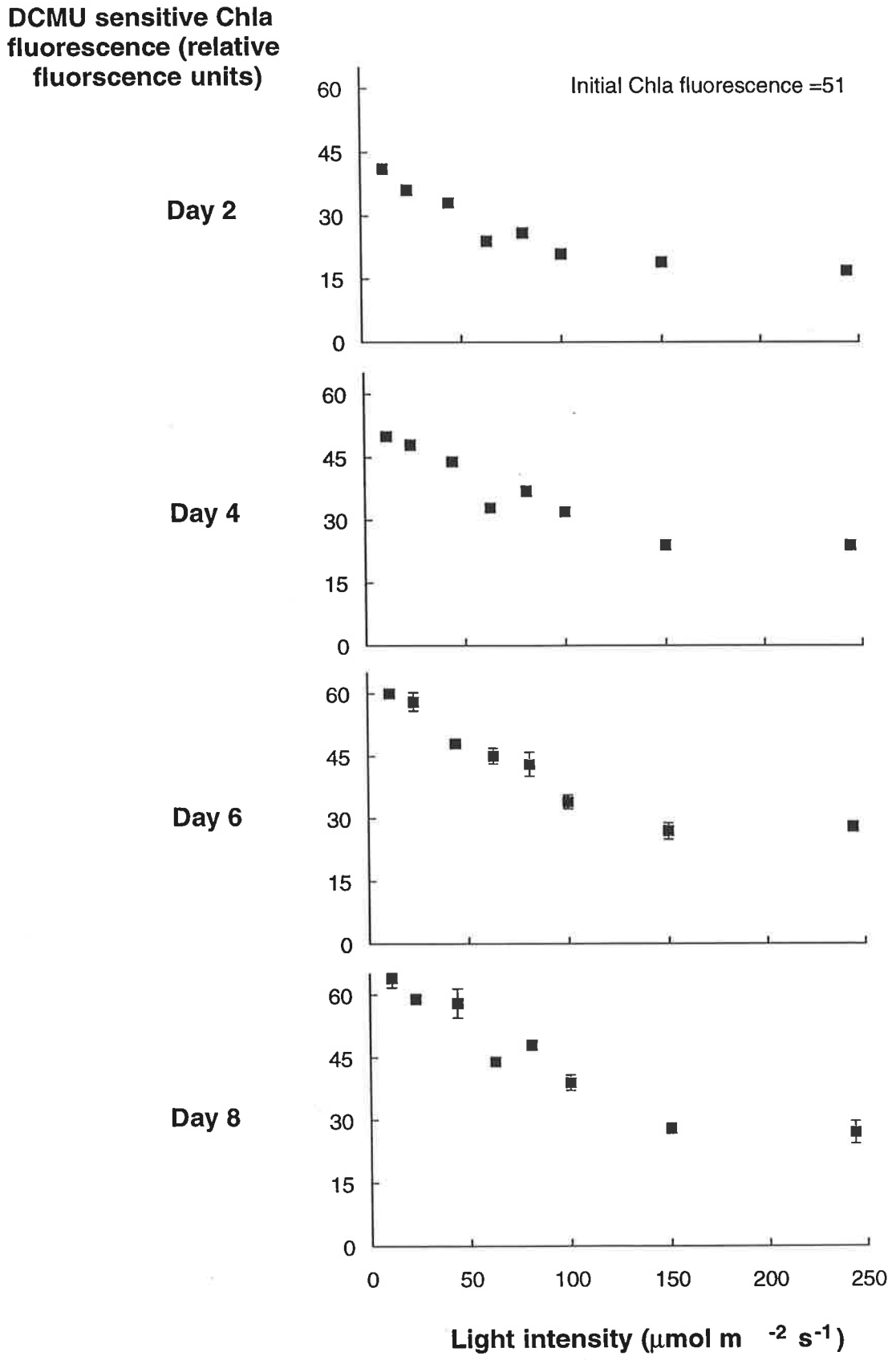


Figure 4.3d *Microcystis* cells change their chlorophyll content per cell in response to the light intensity they are exposed to. Relative chlorophyll content was estimated per cell using a flow cytometer following the addition of DCMU and was greatest in light limited cells.

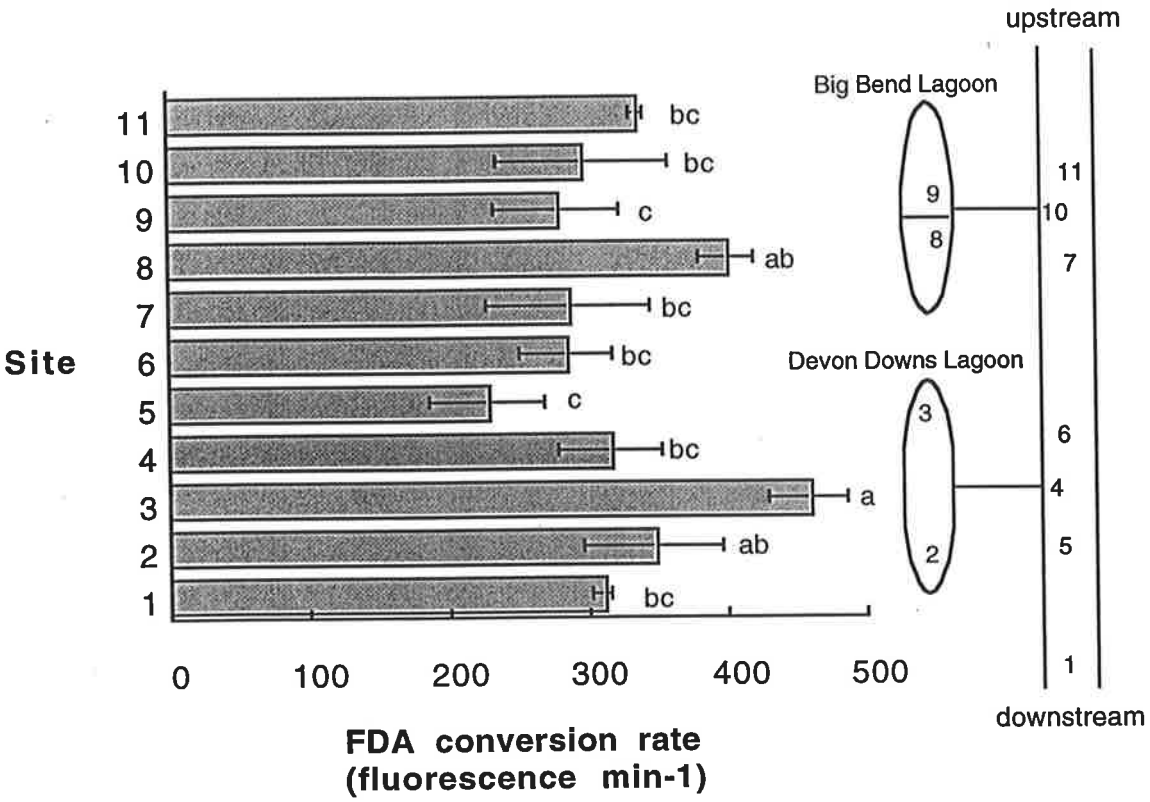


Figure 4.4 Phytoplankton sampled from backwater sites (2, 3, 8) grouped in a single factor ANOVA and Tukey-Kramer all pairs comparison test (p=0.05) and displayed slightly higher metabolic activities than phytoplankton of main channel origin. The metabolic activity was estimated following the hydrolysis of FDA by phytoplankton esterases. FDA conversion rate is proportional to relative growth rate indicating that the backwaters may be more conducive to phytoplankton growth than the main river channel. a, b and c represent statistically similar groups (p=0.05, n=3)

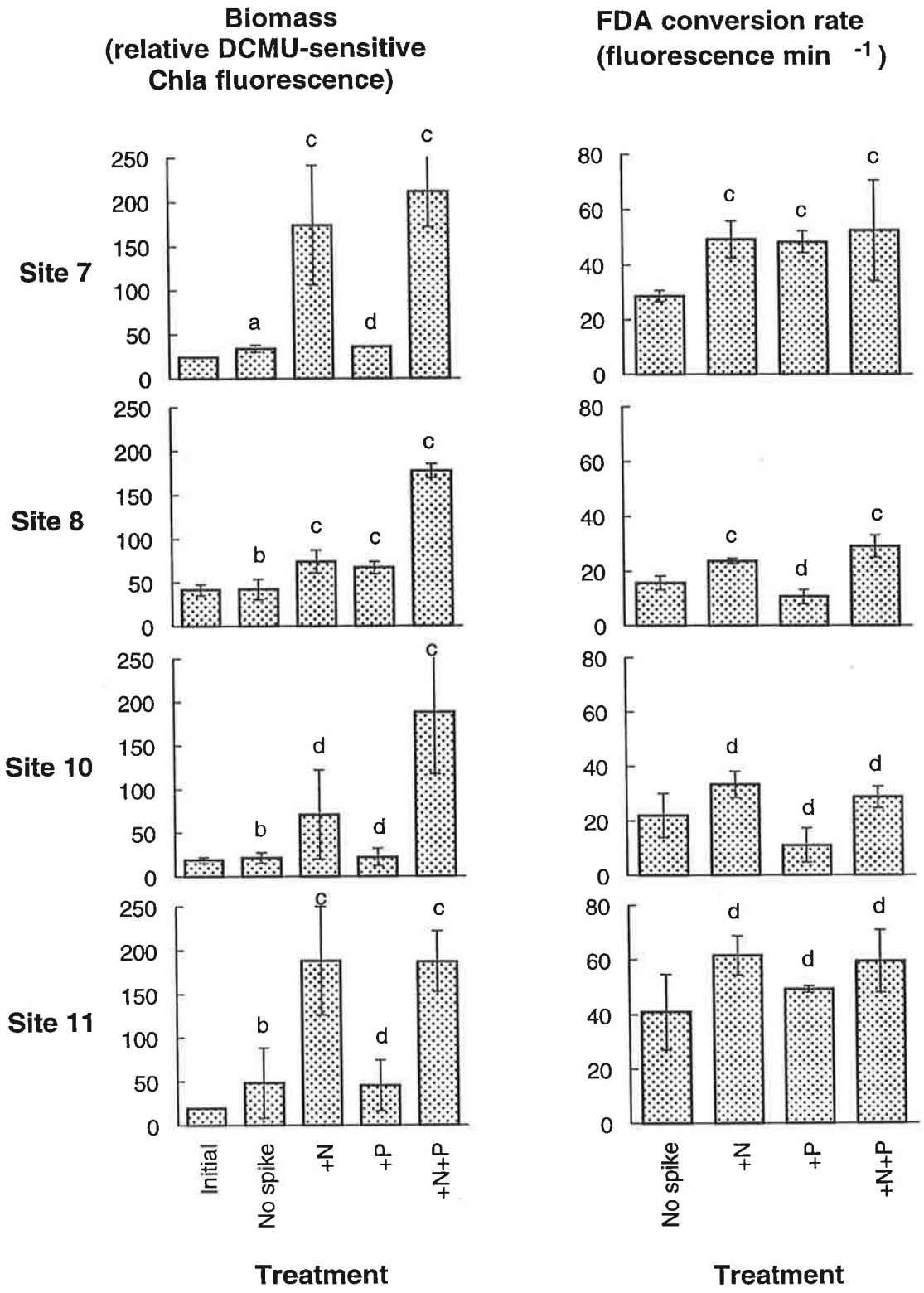


Figure 4.5 Growth bioassays and the FDA staining technique detected nitrogen limitation in the lower River Murray sites. In the main channel at site 7 the FDA technique revealed a P-limitation however this was not observed in the growth bioassays which may be due to a severe N-limitation ultimately determinig yield. (a- control is significantly different (sig. diff.) from the initial; b- control is not sig. diff. from the initial; c-nutrient spike treatment is sig. diff. from the control; d-nutrient treatment is not sig. diff. from the control).

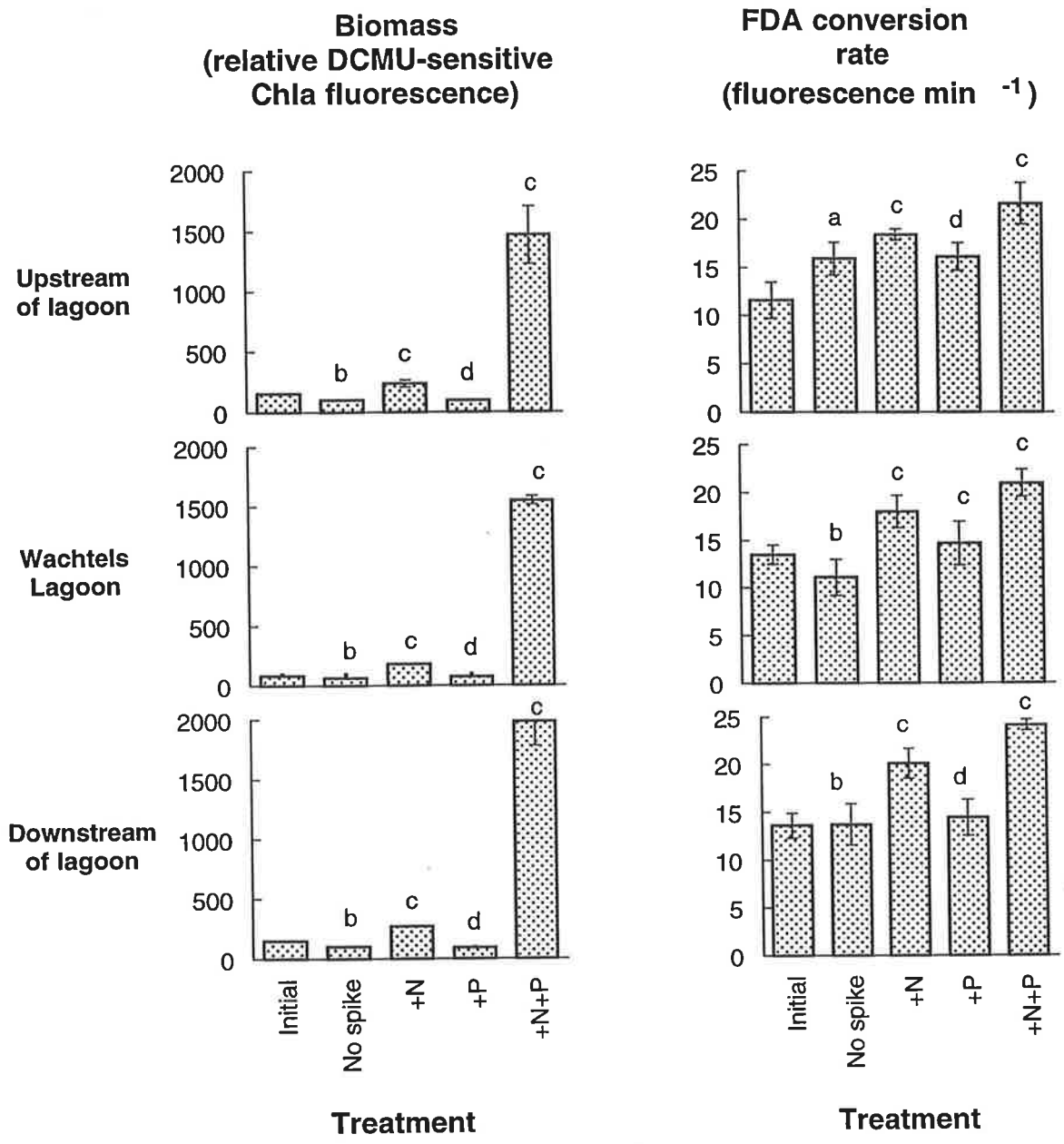


Figure 4.6 Nitrogen limitation was detected in Wachtels Lagoon and the adjacent main river channel (River Murray) using both growth and FDA bioassays. Phosphorus limitation was also detected in the lagoon which is due to an apparent P-limitation of nitrogen-fixing cyanobacteria (see text and figure 4.7). (a- control is significantly different (sig. diff.) from the initial; b- control is not sig. diff. from the initial; c-nutrient spike treatment is sig. diff. from the control; d-nutrient treatment is not sig. diff. from the control).

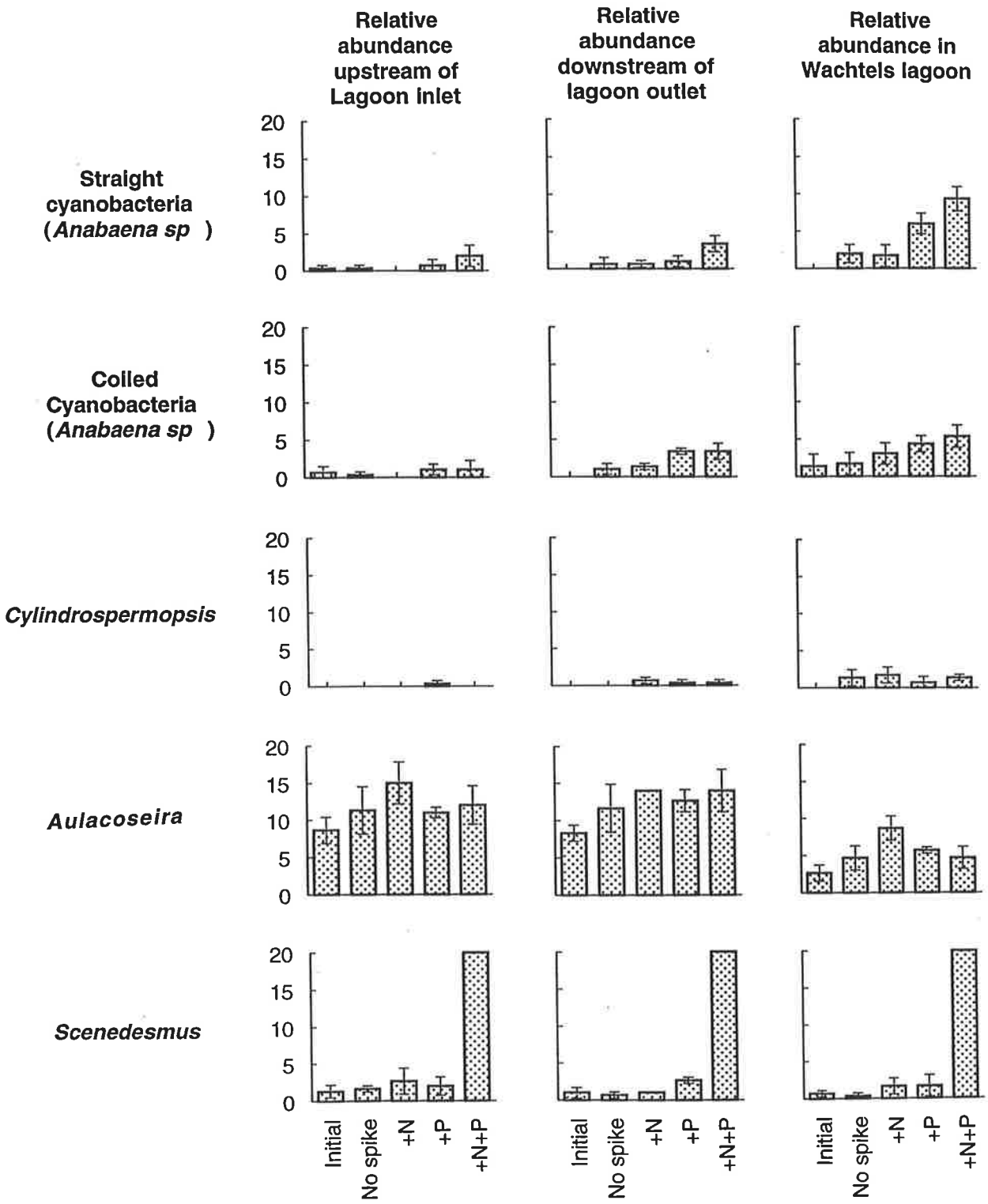


Figure 4.7 Species specific abundance and response to the addition of nitrogen , phosphorus, nitrogen and phosphorus and a no spike control following five day incubations. Phytoplankton were sampled from Wachtels lagoon and upstream and downstream of the Lagoon outlet. Abundance was estimated as presence in 20 microscope fields of view (100x).

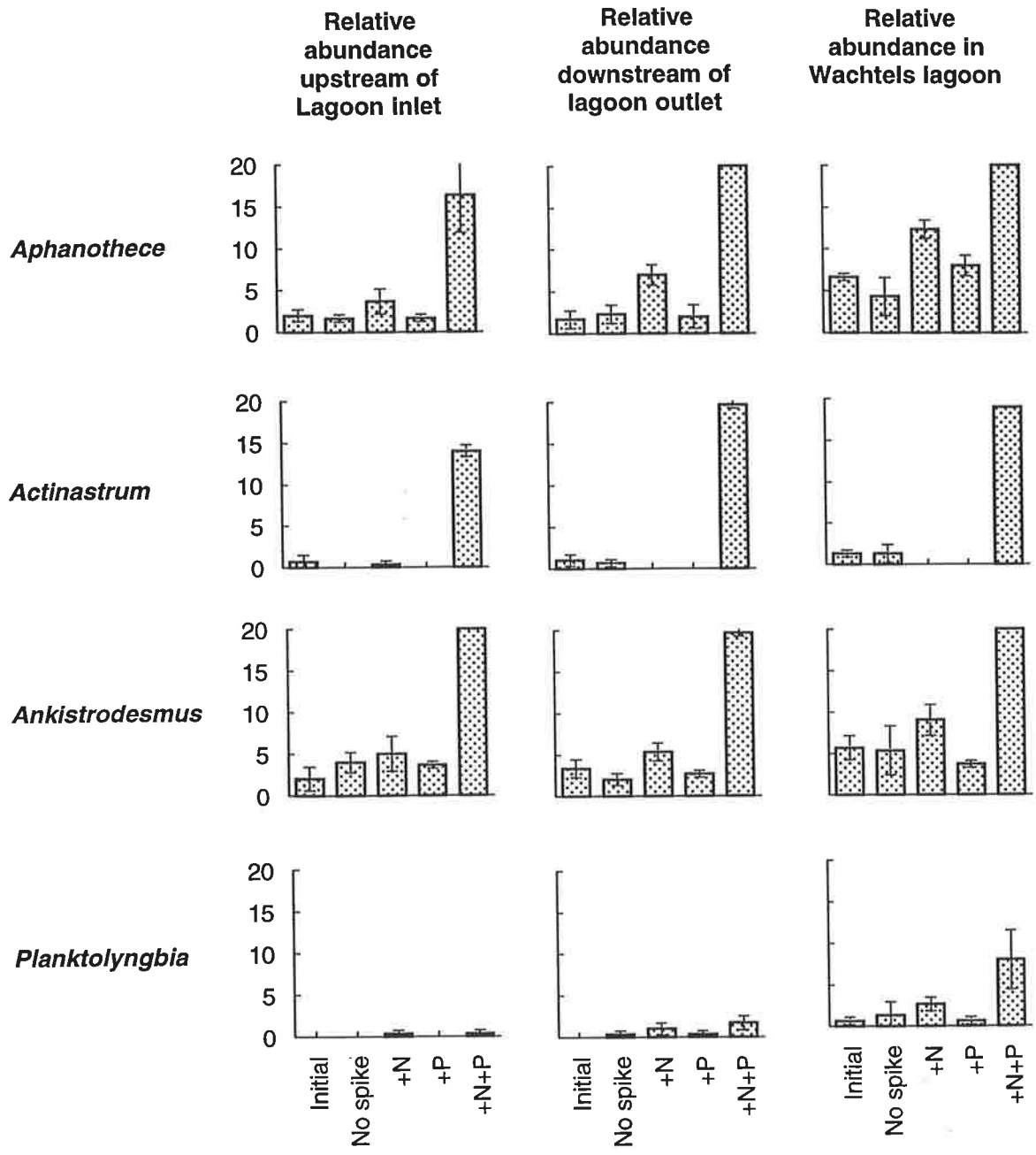


Figure 4.7 Species specific abundance and response to the addition of nitrogen , phosphorus, nitrogen and phosphorus and a no spike control following five day incubations. Phytoplankton were sampled from Wachtels lagoon and upstream and downstream of the Lagoon outlet. Abundance was estimated as presence in 20 microscope fields of view (100x).

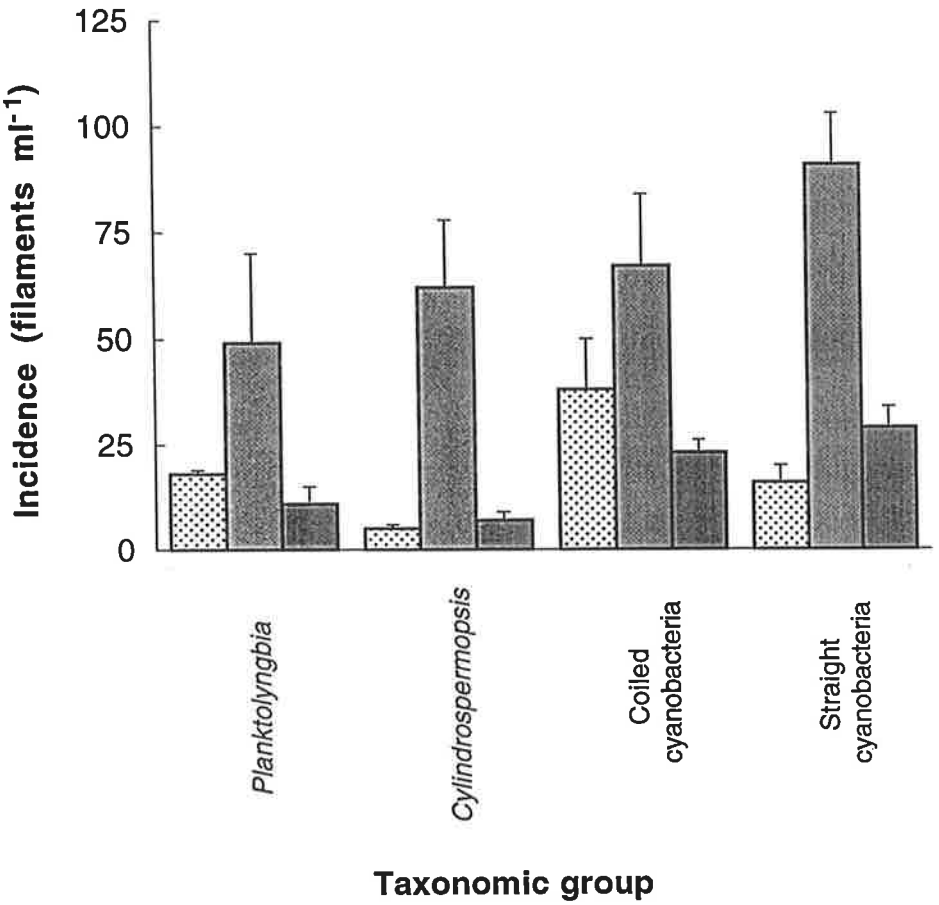


Figure 4.8 Incidence of filamentous cyanobacteria and *Planktolyngbia* in Wachtels Lagoon ( ), and in the adjacent main channel upstream ( ) and downstream ( ) of the lagoon outlet. The lagoon provides a habitat more conducive to cyanobacterial growth because there is greater hydraulic retention and greater mean average irradiance throughout the entire water column than the main channel. All species represented here were more abundant in the lagoon than the main river channel (One way Anova, Tukey-Kramer post-hoc test, p=0.05, n=3)

Chapter 5

Gas vesicle production in *Microcystis aeruginosa* and *Anabaena circinalis*

5.1 Introduction

5.1.1 Gas vesicles-definition and role

The success of certain cyanobacteria can be attributed to the presence of gas vesicles which provide cells with buoyancy, reducing losses by sedimentation and maintaining cells in a favourable light climate during periods of low turbulence (Reynolds and Walsby, 1975, Oliver, 1994, Walsby *et al.*, 1997).

The regulation of gas vesicle volume is one of three mechanisms by which cyanobacteria can regulate cell buoyancy and overcome the vertical separation of light and nutrients (Ganf and Oliver, 1982). Cell density is altered by carbohydrate accumulation during photosynthesis which provides ballast (Kromkamp and Mur, 1984; Thomas and Walsby, 1985; Utkilen *et al.*, 1985a; Kromkamp *et al.*, 1986; Kromkamp and Walsby, 1990). Collapse of gas vesicles caused by turgor pressure generated by osmotically active photosynthates (Dinsdale and Walsby, 1972; Grant and Walsby, 1977; Oliver and Walsby, 1984; Kinsman *et al.*, 1991) and potassium ions (Allison and Walsby, 1981) reduces cell buoyancy. The regulation of gas vesicle synthesis and dilution due to growth also changes the net cell density (Hayes and Walsby, 1984; Konopka *et al.*, 1987b; Oliver, 1994; Walsby, 1994). Therefore the gas vesicle pool present in a cell is a function of gas vesicle production, dilution due to growth and turgor induced gas vesicle collapse. Production of gas vesicles may be achieved by the synthesis of new gas vesicle protein and by the recycling of gas vesicle protein derived from collapsed gas vesicles (Hayes and Walsby, 1984).

Gas vesicles are hollow proteinaceous structures (Walsby, 1972) which are permeable to gas but impermeable to water (Walsby, 1969) and collapse irreversibly upon the application of

sufficient pressure (Hayes and Walsby, 1984). Gas vesicles have a very low density (120 kg m^{-3} in *Anabaena*; Oliver, 1994) and consequently decrease the net cell density. Gas vesicles may be present in sufficient numbers to provide buoyancy (Walsby *et al.*, 1991; Oliver, 1994) however this is dependent upon satisfying the energy and nutrient requirements for gas vesicle synthesis and the density of the cellular constituents which are also dependent upon light and nutrients (Klemer *et al.*, 1982; Spencer and King, 1985; Konopka *et al.*, 1987a, b; Kromkamp and Walsby, 1990; Klemer, 1991; Konopka *et al.*, 1993).

5.1.2 Measurement of gas vesicle volume

Gas vesicle volume can be measured directly using a capillary tube apparatus developed by Walsby (1982) and improved with the addition of a water jacket for thermal stability (Oliver and Walsby, 1984; Konopka *et al.*, 1987a; Oliver and Walsby, 1988) and replicate capillaries for triplicate measurements (Walsby *et al.*, 1992). The device comprises a 100 μm radius capillary with a reservoir bulb at one end. Algal suspension is poured into the reservoir and moves down the capillary. The end is stoppered, ensuring no air bubbles are present, and the entire tube inserted into a water jacket. Pressure is applied to the algal suspension which collapses the gas vesicles and causes a shift of the meniscus in the capillary, the pressure is then released and the meniscus retracts. The difference in the meniscus position before and after pressurisation is used to calculate the volume that the gas vesicles would have occupied (Oliver and Walsby, 1988; Walsby *et al.*, 1992).

Relative gas vesicle volume can also be estimated using a turbidimeter or nephelometer by exploiting the light scattering properties of gas vesicles (Walsby, 1973) which is due to differences in the refractive index of the gas in the vesicles and the surrounding cytoplasm. A number of turbidity ratios have been used to estimate relative gas vesicle volume such as that of Walsby (1973) where he describes relative gas vacuolation (RGV) with the following equation:

$$RGV = \frac{T_a - T_c}{T_c}$$

where T_a is the turbidity of the algal suspension prior to pressurisation and T_c the pressure insensitive turbidity due to the cells remaining following the collapse of all gas vesicles. Walsby (1973) states that this technique can not account for differences in pressure insensitive

turbidity due to pigment and sample heterogeneity, the presence of sediments or changes in carbohydrate content (Kromkamp and Mur, 1984) and consequently has limited application for comparing between different samples.

Kromkamp and Mur (1984) estimated the relative gas vesicle volume as the difference in light scatter before and after the collapse of all gas vesicles divided by the protein content of the suspension. This technique is limited by the fact that the protein content per cell is not conservative but changes with light/dark periodicity (Green, 1994) and the nutrient climate. Therefore, estimations of relative gas vesicle volume for cells grown in different nutrient concentrations and photon flux densities can not readily be compared using this technique.

A more accurate approach would be to calculate the relative gas vesicle volume as the change in light scatter before and after gas vesicle collapse on a per cell basis. To obtain the accuracy necessary to differentiate between treatments microscopic determination of cell numbers would need to be undertaken which is very time consuming for multiple samples. Flow cytometry offers the potential to examine light scattering by gas vesicles per cell and thus overcome this problem. Flow cytometry has been utilised previously to investigate light scatter by gas vesicles in *Microcystis aeruginosa* (Dubelaar *et al.*, 1987), however, to date there has been no attempt to employ this technology in ecophysiological studies of cyanobacterial gas vesicle accumulation in different light and nutrient climates.

The applicability of flow cytometry to estimate relative gas vesicle volume depended on its ability to detect light scattered by gas vesicles. Because a capillary apparatus to directly measure gas vesicle volume (Walsby *et al.*, 1992) was unavailable the flow cytometry technique was validated by comparisons of pressure collapse curves created using the flow cytometer and traditional nephelometry (Walsby, 1973). The volume of isolated gas vesicles was shown to be linearly proportional to turbidity by Ganf *et al.* (1989). Therefore, if side scatter measured using the flow cytometer following collapse of gas vesicles was similar to turbidity measured with a nephelometer then it could be assumed that side scatter on the flowcytometer is proportional to gas vesicle volume.

5.1.3 Gas vesicle volume and cell physiology

The two previous chapters identified physiological responses of phytoplankton to light and nutrients which were expressed as cell growth, photosynthesis and metabolic activity measured using fluorescein diacetate. Gas vesicles are proteinaceous and consequently the production and accumulation of gas vesicles within a cell will also be affected by light and nutrients. Whether a cell sinks or floats is a function of gas vesicle volume providing buoyancy and other dense cell constituents acting as ballast. The interaction of these will govern the cells density and ultimately the phytoplankton's future potential to scavenge for light and nutrients, maintain cell function and grow.

Microcystis aeruginosa and *Anabaena circinalis,* among other cyanobacteria, are notorious because they are often toxic (Carmichael and Gorham, 1981; Codd and Poon, 1988; Watanabe *et al.*, 1989, Carmichael, 1992; Brookes *et al.*, 1994) and form dense waterblooms (Reynolds and Walsby, 1975; Reynolds, 1987). *Microcystis aeruginosa* and *Anabaena circinalis* often coexist in reservoirs (Brookes *et al.*, 1994) but have different morphology and physiology which allow them to exploit different niches.

Anabaena circinalis is able to fix atmospheric nitrogen because of the presence of heterocysts which protect the N_2-fixing machinery from oxygen degradation, and consequently in a favourable light climate should not display nitrogen limitation when soluble inorganic nitrogen is low. Potentially nitrogen deficiency could be induced in this species through a light limitation which reduces the available reductant for nitrogen metabolism and assimilation. Ward and Wetzel (1980a) found growth of *Aphanizomenon flos-aquae* was not maintained in cells grown at low light when N_2 was the nitrogen supply. Iron limitation could also potentially be used to induce a nitrogen deficiency (Reuter and Peterson, 1987). An attempt was made to limit nitrogen in *Anabaena circinalis* by supplying synthetic air free of N_2 , however this was unsuccessful.

One advantage of buoyancy regulation and vertical migration is that it offers gas vacuolated cyanobacteria the opportunity to overcome the vertical separation of light and nutrients (Ganf and Oliver, 1982). If the surface waters are high in nutrients then the ability to vertically migrate does not offer added benefits to cells with respect to positioning them in the optimal

environments for nutrient and light capture. Buoyancy regulation may still function as a method to avoid damagingly high light intensity and photoinhibition but to a degree photo damage can be limited by the presence of carotenoids (Paerl *et al.*, 1983; Hirschberg and Chamovitz, 1995) which provide protection from UV wavelengths. Further more *Microcystis* has been shown to adapt to high surface irradiances within 48 hours (Ward and Wetzel, 1980b; Kohler, 1992).

Therefore a strategy cyanobacteria could adopt in nutrient rich surface waters is to increase production of gas vesicles relative to growth which would result in over buoyancy and the positioning of the cell in persistently high irradiances. Conversely, cells in nutrient deplete surface waters would be nutrient stressed which could lead to lower gas vesicle synthesis. This in turn would lead to a lower gas vesicle pool and facilitate conditions whereby buoyancy could be overcome by carbohydrate accumulation. Buoyancy regulation and migration deeper in the water column where nutrients are more abundant would be realised.

A similar argument can be developed to hypothesise what effect light has on gas vesicle volume. One would expect gas vesicle production to be greatest when light is in short supply to increase buoyancy and floating velocity and place cells in a more favourable environment for light capture. The aim of this study was to identify how the gas vesicle pool is affected by light and nutrient deficiency and discuss this in relation to cyanobacterial buoyancy.

Cell metabolic activity has been shown to be related to growth rate (chapters 3 and 4) and may also play a role in governing the rate of gas vesicle synthesis. Metabolic activity was measured using the FDA technique and related to the relative gas vesicle pool of *Microcystis* cells grown under different light conditions. Photosynthetic pigments change in response to the ambient light and nutrient conditions (chapter 4, Foy, 1993). Chlorophyll concentration has been shown to increase when light is low and decrease at high light intensities. Chlorophyll concentration is also relatively higher in cells which are nutrient sufficient than cells limited in nutrients (chapter 4). The above hypothesis predicts that gas vesicle synthesis would also be higher in low light and at high nutrient concentrations. Chlorophyll fluorescence was measured using the flow cytometer and compared with the relative gas vesicle volume to identify if a relationship existed between photosynthetic pigments and gas vesicle volume.

5.2 Methods

5.2.1 Algal cultures

The two cyanobacteria used in this study were *Microcystis aeruginosa* MASH01-A22 an axenic daughter of SHEP 100, isolated by Dr Gary Jones and obtained from CSIRO Marine Laboratories, Tasmania, and *Anabaena circinalis* Ana330E isolated by Peter Baker (SA Water) from The River Murray, Renmark, South Australia.

Microcystis aeruginosa was grown in ASM-1 media (Gorham *et al.*, 1964) with 150 µM supplementary bicarbonate at 25 °C and 100 µmol m^{-2} s^{-1} on a 12:12 light dark cycle unless specified and *Anabaena circinalis* was grown in WC media (Guillard and Lorenzen, 1972) in similar conditions. Three replicate samples were subjected to each treatment and a mean and standard deviation were calculated using replicate values.

5.2.2 Comparison of nephelometry with flow cytometry to estimate relative gas vacuolation

Cell side scatter was measured with a flow cytometer and nephelometer to validate the use of flow cytometry to measure relative gas vesicle volume. Gas vesicle collapse pressure curves (Walsby, 1973) were performed using the technique of Walsby (1973) and the flow cytometer following the sequential collapse of gas vesicles by the application of pressure. The light scattering properties of *Microcystis aeruginosa* suspended in either ASM-1 media or in 0.5 M sucrose were measured following increases in pressure using a Hach 2100 A turbidimeter (Ganf *et al.*, 1989; Brookes *et al.*, 1994) or with a Becton Dickinson FACStrak flow cytometer. The flow cytometer photomultiplier tube was set such that gas vacuolated cells appeared in the third or fourth decade of the side scatter plot to ensure they were on scale. The plots were standardised by setting the turbidity or side scatter of cells with maximum intact gas vesicles at atmospheric pressure as 100% (SSC_{atmos}), no gas vesicles remaining (SSC_{coll}) as 0% and intermediate values (SSC_{press}) calculated using the equation of Walsby (1971) where the percentage of gas vesicles remaining following the application of pressure is given as:

$$\frac{SSC_{press} - SSC_{coll}}{SSC_{atmos} - SSC_{coll}}$$

The establishment that flow cytometry was a valid tool with which to investigate relative gas vacuolation of phytoplankton enabled it to be used in future gas vesicle studies. The flow cytometer was utilised to estimate how gas vesicle volume was affected by energy and nutrient limitation in *Microcystis aeruginosa* and *Anabaena circinalis*.

5.2.3 Relative gas vesicle volume of *Microcystis*

5.2.3.1 Detection of cell parameters using a flow cytometer

FDA conversion rate was measured by the detection of fluorescein in cells following incubation in 40 µM Fluorescein diacetate for 7 minutes. Fluorescein was detected with a flow cytometer at 530 nm following excitation by an argon laser at 488 nm (see Chapter 3). Chlorophyll fluorescence per cell was also estimated upon the addition of 10 µM DCMU by detection with a flow cytometer at 680 nm.

Relative gas vesicle volume was estimated as the side scatter per cell, detected with a Becton Dickinson flow cytometer, of cells with intact gas vesicles minus the scatter of cells where all the gas vesicles had been collapsed. The flow cytometer outputs were standardised between days using FITC-labelled beads which accounted for machine variation and drift. The side scatter detectors were set such that the maximum relative side scatter was on scale in the third or fourth decade of the log plot. *Anabaena* side scatter per sample was higher than *Microcystis* and consequently different settings were used for the two species. Relative side scatter per cell was assumed to be linearly proportional to gas vesicle volume based on the evidence of Walsby (1971) who found a linear relationship between optical density and gas vesicle volume for isolated gas vesicles. This assumption still requires experimental validation.

5.2.3.2 Light and Microcystis *gas vesicle volume*

The effect of light on gas vesicle volume in *Microcystis aeruginosa* was examined by growing three replicate cultures in eight light intensities (11, 23, 44, 63, 81, 100, 150, 244 µmol $m^{-2}s^{-1}$) on a 12:12 light:dark cycle at 25°C as described in chapter 3. Growth rate was determined by microscopic enumeration of cell numbers using a Zeiss microscope and a haemocytometer.

FDA conversion rate, Chlorophyll content per cell and relative gas vesicle volume were all estimated on a per cell basis using a flow cytometer as described above on days 2,4,6 and 8 following inoculation and incubation at the different irradiances. Statistical analysis was performed using JMP statistical software (SAS, 1996).

5.2.3.3 *Nitrogen and* Microcystis *gas vesicle volume*

Log phase *Microcystis* were deprived of nitrogen for two days prior to inoculation into 0, 2, 5, 10, 25, 50, 100, and 200 μM nitrate in otherwise normal ASM-1 to examine the impact of nitrogen deficiency on the gas vesicle pool. FDA conversion rate, Chlorophyll content per cell and relative gas vesicle volume were all estimated on a per cell basis using a flow cytometer as described above on days three and six following inoculation and incubation at the different nitrate concentrations. Culture conditions were 100 μmol m^{-2} s^{-1} on a 12:12 light:dark cycle at 25°C. Growth rate was also estimated over the first three days and the entire six days by microscopic enumeration of cell numbers.

5.2.3.4 *Phosphorus and* Microcystis *gas vesicle volume*

Log phase *Microcystis* were deprived of phosphate for eight days to deplete the polyphosphate pool prior to inoculation into 0, 0.1, 0.2, 0.5, 1, 2, 5, and 10 μM phosphate in otherwise normal ASM-1 to examine the impact of phosphorus deficiency on the gas vesicle pool. Other culture conditions were as described above. FDA conversion rate, chlorophyll content per cell and relative gas vesicle volume were all estimated on a per cell basis using a flow cytometer as described above on days 2, 4, 6 and 8. Growth rates were calculated from cell counts performed on days 0, 4, and 8.

5.2.4 Gas vesicle volume of *Anabaena circinalis*

Gas vesicle studies of *Anabaena circinalis* using flow cytometry followed a similar protocol as the *Microcystis* gas vesicle studies, however, there were several notable differences in the detection of each species by the flowcytometer. *Microcystis* cells passed through the flow cytometer one cell at a time because the culture was unicellular and the small cell size (5 μm) allowed detection of individual cells. *Anabaena* on the other hand was filamentous and passed through the flow cytometer nozzle as small filaments which were measured as single units. The maximum length measurable was the width of the laser beam and each sampled unit was

approximately the same size based upon chlorophyll fluorescence per unit. Pressure within the sheath fluid of the flow cytometer caused by the fluidics of the machine were insufficient to cause any gas vesicle collapse.

5.2.4.1 Light and **Anabaena** *gas vesicle volume*

Anabaena circinalis cultures were grown in complete WC media at eight light intensities (10, 20, 40, 60, 80, 100, 150, 250 μmol m^{-2} s^{-1}). There were three replicate samples at each light treatment. Cell counts were performed using a Nikon inverted microscope and Sedgewick Rafter cell. *Anabaena* cells in the counting chamber had a biased distribution because they were grouped within filaments. To account for this filaments per volume were counted (>400 individual filaments) and multiplied by the average number of cells per filament averaged from 60 filaments. Growth rates were calculated from cell counts sampled on days 0, 4 and 8 of the experiment. Relative gas vesicle volume was measured on days 0, 2, 4, 6 and 8 with a Becton Dickinson flow cytometer and calculated as the relative difference in side scatter of cells with intact gas vesicles less the scatter of cells with collapsed gas vesicles.

5.2.4.2 Light dependent turgor pressure collapse of **Anabaena circinalis** *gas vesicles*

Turgor pressure in cyanobacteria increases as intermediates of photosynthesis (Oliver and Walsby, 1984) or potassium ions (Allison and Walsby, 1981) accumulate within cells. Oliver and Walsby (1984) and Kinsman, *et al.* (1991) both identified gas vesicle collapse by turgor pressure in species of *Anabaena*. To examine if there was gas vesicle collapse in *Anabaena circinalis* a log phase culture grown at 40 μmol m^{-2} s^{-1} at a 15:9 light: dark cycle at 25°C was incubated for 24 hours in 10, 20, 40, 60, 80, 100, 150 or 250 μmol m^{-2} s^{-1} (n=3). Relative gas vesicle volume was measured initially and following the 24 hour light exposure.

Turgor pressure was estimated, using the method of Walsby (1971; pressure collapse curves described above), as the difference in pressure required to collapse 50% of the gas vesicles in cells suspended in media and cells suspended in 0.5 M sucrose which reduces turgor to zero (Walsby, 1971; Walsby, 1972; Reynolds, 1972; Walsby, 1988).

The percentage of cells floating was estimated initially and following the 24 hour light exposure using a modification of the methods of Walsby and Booker (1980) and Oliver and Walsby (1988). One millilitre of cyanobacterial suspension was placed in a Sedgewick Rafter and let stand for 20 minutes before counting the number of filaments on the bottom of the chamber and the number of filaments on the top plane floating just under the cover slide using a Nikon inverted microscope. Carbohydrate content was determined using the phenol method of Herbert *et al.* (1971) and analytical glucose as a standard.

5.2.4.3 Nitrogen and **Anabaena** *gas vesicle volume*

Anabaena circinalis is heterocystic and able to fix atmospheric nitrogen reducing the likelihood of nitrogen limitation when soluble inorganic nitrogen levels are low. In this study nitrogen levels were controlled by growing cultures in varying levels of nitrate and controlling the atmospheric nitrogen by sparging a chamber in which the cultures grew with a synthetic air supply composed of 20% O_2, 0.3% CO_2 and the balance Argon carrier gas. The *Anabaena circinalis* used in this study was Ana330E which had been preconditioned to media containing no soluble nitrogen and fixed N_2 to satisfy its nitrogen requirements. Treatments were 0, 2, 5, 20, 50, 100 μM nitrate which were incubated in the presence of the argon gas, and two controls; 0 and 100 μM nitrate which were incubated in the presence of atmospheric nitrogen.

Cultures were grown at 100 μmol m^{-2} s^{-1} on a 15:9 light:dark cycle in a controlled temperature room (25°C). Three replicate samples were treated in each nitrogen concentration. Growth rate was calculated from cell counts on days 0, 4 and 8 and relative gas vesicle volume measured on a flow cytometer on days 2, 4, 6 and 8.

5.2.4.4 Phosphorus and **Anabaena** *gas vesicle volume*

Log phase *Anabaena circinalis* cultures were washed four times in P-free WC media containing KCl to replace the potassium which was normally present as K_2HPO_4. The *Anabaena* filaments were then incubated in phosphate free media to deplete the polyphosphate pool before inoculation into 0, 0.1, 0.2, 0.5, 1, 2, 5 and 10 μM K_2HPO_4 WC media. Other culture conditions were as described above. Relative gas vesicle volume was monitored every two days for the experiments eight day duration. Growth rate was also calculated over the eight day experimental period.

5.3 Results

5.3.1 Comparison of nephelometry with flow cytometry to estimate relative gas vacuolation

Comparative gas vesicle collapse-pressure curves, before and after the elimination of turgor pressure, were determined using nephelometry and flow cytometry to determine side scatter as an index of gas vesicle volume (figure 5.1). The curves were standardised to % gas vesicles remaining following the application of pressure. The apparent critical pressure and the critical pressure were calculated as the pressure required to collapse 50% of gas vesicles of cells suspended in normal media and in 0.5 M sucrose respectively. Turgor pressure was calculated as the difference between the apparent critical pressure and the critical pressure. The apparent critical pressure measured using nephelometry was measured as 280 kPa and by flow cytometry 240 kPa which represents a 15% difference between the two methods. There was only 5% difference between the critical pressure measured on the flow cytometer and the nephelometer; 600 and 568 kPa respectively (table 5.1). The turgor pressure measured on the nephelometer was 20 % lower than when measured using flow cytometry.

	P_a (kPa)	P_c (kPa)	P_t (kPa)
Flow cytometer	240	600	360
Nephelometer	280	568	288

Table 5.1 Apparent (P_a), critical (P_c) and turgor (P_t) pressures of *Microcystis aeruginosa* calculated from gas vesicle collapse pressure curves (figure 5.1). These were constructed from side scatter measurements made per cell using a flow cytometer or on a population using a nephelometer.

The good agreement between curves constructed using the nephelometer and the flow cytometer demonstrated that the flow cytometer could be used with confidence to estimate gas vesicle volume in cyanobacteria.

5.3.2 Light and *Microcystis* gas vesicle volume

Growth rate over the eight day experimental period was limited at photon flux densities less than 50 μmol m^{-2} s^{-1}, saturating between 50 and 150 μmol m^{-2} s^{-1} and slightly photoinhibited at 244 μmol m^{-2} s^{-1} (figure 5.2).

This experiment and the FDACR (figure 3.6) were reported in chapter three as part of *Microcystis*'s metabolic response to light experiment documented in chapter 3. In summary the FDA conversion rate increased with increasing light intensity up to a maximum rate at 100 µmol m^{-2} s^{-1}. At higher irradiances the FDACR was maximal and constant.

The chlorophyll content per cell was measured with a flow cytometer estimated as maximum fluorescence (F_m) following the addition of DCMU, which inhibits electron flow from Photosystem II. Cells incubated at different light intensities adjusted their chlorophyll pigments in response to the ambient irradiance (figure 5.3). By day two the chlorophyll response was evident and chlorophyll fluorescence decreased linearly relative to the irradiance up to 100 µmol m^{-2} s^{-1} and was minimal and constant at higher irradiances. The chlorophyll per cell on days 4,6nad 8, at each light intensity increased above the day two chlorophyll value. The general pattern was that the chlorophyll fluorescence decreased linearly with irradiance to 150 µmol m^{-2} s^{-1} and then was minimal and constant.

The initial relative gas vesicle volume per cell was 1191 relative side scatter units but by day 2 the gas vesicle volume per cell had increased above the initial at all light intensities (figure 5.4) and reached a maximum of 1603 relative side scatter units (SSC) at 44 µmol m^{-2} s^{-1}. Gas vesicle volume continued to increase with time at all light intensities but gas vesicles accumulated at a greater rate in higher light intensities as time increased: day 4 maximum, 1772 SSC at 44 µmol m^{-2} s^{-1}; day 6 maximum, 2130 SSC at 63 µmol m^{-2} s^{-1}; and day 8 maximum, 2417 SSC at 100 µmol m^{-2} s^{-1}. On day 8 the relative gas vesicle volume increased linearly with irradiance (r^2=0.93) up to 100 µmol m^{-2} s^{-1} but decreased at higher light intensities.

Day 8 relative gas vesicle volume was chosen for comparison with growth rate and metabolic activity (FDA conversion rate) because cells were in log phase growth, had acclimatised to that irradiance and the cellular response to the different light intensities was sufficiently large to differentiate between treatments.

The gas vesicle pool present within a cell is a function of production minus dilution by cell division and turgor induced gas vesicle collapse. In *Microcystis* gas vesicles are too strong to be collapsed by turgor pressure (Thomas and Walsby, 1985) so the measured relative gas

vesicle volume is determined only by production and dilution due to cell division. For a net increase in gas vesicle volume to occur the rate of gas vesicle production must exceed the growth rate. If there was reduced gas vesicle synthesis relative to growth rate the relative gas vesicle volume would decrease as a function of growth rate. If production and dilution of gas vesicles occurred at the same rate there would be no net change in gas vesicle volume per cell. The gas vesicle volume of *Microcystis* cultures exposed to a range of light intensities increased above the day 0 initial value of 1191 relative side scatter units at all irradiances (figure 5.4). The relative gas vesicle volume on day 8 was proportional to the rate of change of gas vesicle volume and was positively and linearly related to the growth rate (r^2=0.73; figure 5.5). This indicated that gas vesicle production in *Microcystis* exceeded, but was dependent upon, the growth rate.

In chapter three it was established that the cell metabolism, measured using FDA, was dependent on the ambient light intensity the cells were exposed to. The esterases involved in FDA conversion are fuelled by ATP derived from photosynthesis. Energy is also required for carbon and nitrogen metabolism which are essential for the maintenance of esterase function. A number of proteins are involved in gas vesicle production (Oliver, 1994; Walsby, 1994) which also demands energy, carbon and nitrogen. The linear relationship (r^2=0.75) between cell metabolic activity (FDA conversion rate) and the relative gas vesicle pool (figure 5.6) identifies that gas vesicle production is a function of light dependent cell metabolic activity.

5.3.3 Nitrogen and *Microcystis* gas vesicle volume

This experiment was conducted in conjunction with the *Microcystis* metabolic activity studies in response to nitrogen which was detailed in chapter 3 and consequently some of the data previously referred to is repeated here.

The growth rate of *Microcystis* grown in a range of nitrate concentrations was maximal and constant at concentrations exceeding 5 μM nitrate over the initial 3 days (figure 5.7) but as the cultures grew a nitrogen limitation was induced (days 0-6; see also chapter 3). FDA conversion rate on day three and day six detected the nitrogen limitation as sub-maximal FDA conversion rates corresponded to nitrogen-limited growth rates (figures 5.7 and 3.11).

The chlorophyll content per cell also changed in response to the nitrogen climate (figure 5.8) but displayed a different response to nitrate than was observed in the growth rate on day 3. At 5 μM initial nitrate concentration the chlorophyll content per cell was limited by nitrogen but this limitation had not yet been manifested in growth. On day six the chlorophyll content per cell was limited at nitrate concentrations less than 50 μM initial. In general the cell's chlorophyll content more closely resembled the FDA conversion response than the growth rate in nitrate (figures 5.8 and 3.11). In the light experiment chlorophyll content also more closely resembled FDA conversion rate than growth rate but in this case cellular chlorophyll content and FDA conversion rate were negatively correlated (r^2=0.93) except at the two highest light intensities where the relationship did not hold.

There was no universal relationship between chlorophyll content per cell and gas vesicle volume. Gas vesicle volume and chlorophyll content both increased with increasing nitrate but in increasing light relative gas vesicle volume increased while chlorophyll content decreased.

Gas vesicle protein may account for more than 10% of the total cell protein (Hayes and Walsby, 1984). Consequently, there was a significant nitrogen demand associated with gas vesicle production which was highlighted in figure 5.9. Gas vesicle volume increased with increasing initial nitrate concentration on day three up to 10 μM, at higher concentrations relative gas vesicle volume was maximal and constant. On day six relative gas vesicle volume per cell increased at 100 and 200 μM nitrate but at all lower initial concentrations there was a dilution in the gas vesicle pool per cell as nitrogen deficiency limited the rate of gas vesicle production relative to the growth rate. Figure 5.10 accentuates this point; on day three there was a linear relationship (r^2=0.81) between relative gas vesicle volume and the growth rate over the previous three days. By day six this relationship no longer held as the growth rate exceeded the rate of gas vesicle production except at the two highest nitrate treatments.

Relative gas vesicle volume on day six exhibited a relationship with cellular metabolic activity (FDA conversion rate) similar to that observed in the light treatments. The relationship was linear (r^2=0.91) and approximated by the equation: Relative gas vesicle volume on day 6=893.3+7.33*FDA conversion rate (day 6). There was no significant relationship between day three relative gas vesicle volume and day three FDA conversion rate.

5.3.4 Phosphorus and *Microcystis* gas vesicle volume

The growth response of *Microcystis* in a series of batch cultures containing media with different phosphate concentrations increased with increasing phosphate over the initial four days (figure 5.11), however, there was considerable variation around the mean of three replicates. The growth rate over the entire eight day experimental period was maximal and constant at about 0.38 ln units day^{-1} at phosphate concentrations exceeding 2 µM and could be approximated by a Michaelis-Menton curve (μ_{max}=2 ln units day^{-1}; k_m=0.5 µM).

Relative gas vesicle volume was 1560 relative side scatter units at the commencement of the experiment but decreased at all phosphate treatments by day two (figure 5.12). The greatest dilution of gas vesicles occured at concentrations greater than 2 µM phosphate which indicated that gas vesicle production was occurring at a rate relatively slower than the growth rate. On day four an increase in the gas vesicle volume occurred at all phosphate concentrations except the 0 µM treatment. The gas vesicle pool was diluted in cells grown in 0 and 0.1 µM phosphate on day 6 and further diluted by day 8. The relative gas vesicle volume was between 1380 and 1430 relative side scatter units for all other phosphate concentrations on day 8.

5.3.4.1 Heterogeneity of gas vesicle volume in different nutrients

Heterogeneity exists in natural populations, temporally and spatially, and is readily observed on large scales owing to differences in species composition, species abundance, toxin content (Carmichael and Gorham, 1981), metabolic activity (Yentsch *et al.*, 1988) and photosynthesis (Falkowski and Kolber, 1995) between sites. Within population heterogeneity is rarely assessed because most instrumentation and techniques usually do not provide resolution at the cellular scale. Flow cytometry offers the advantage of assessing optically expressed cell parameters on a per cell basis. Cells in culture, or indeed in wild populations, may not all exhibit the same metabolic or physiological state and consequently this will impact upon gas vesicle production and accumulation.

The relative gas vesicle volume of *Microcystis* cells deprived of phosphate (0 µM; day 8 sample from the above experiment) was compared with cells with an adequate phosphate supply of 10 µM (figure 5.13). The distribution of cells within the plots of side scatter versus chlorophyll fluorescence provide an indication of the heterogeneity exhibited in gas vesicle content between

individuals at each phosphate treatment. Cells grown in an initial phosphate concentration of 10 μM were more tightly clustered in their side scatter distribution (spanning 400 relative side scatter units) than cells deplete in phosphorus whose side scatter distribution spanned 800 relative side scatter units. The increased side scatter heterogeneity in low phosphate conditions indicated that these cells would exhibit different buoyancy characteristics due to different degrees of buoyancy provided by gas vesicles.

5.3.5 Light and *Anabaena* gas vesicle volume

Anabaena exposed to different irradiances on a 12:12 light:dark cycle were light limited at photon flux densities less than 100 μmol m^{-2} s^{-1} for the first four days (figure 5.14). The growth rate over the entire eight day experimental period increased approximately linearly with increased irradiance up to 150 μmol m^{-2} s^{-1} which supported the maximum growth rate of 0.39 ln units day^{-1}.

The flow cytometer instrument settings used to investigate optical features of *Anabaena* were different to the instrument settings used for *Microcystis* studies. Consequently the absolute differences in gas vacuolation of the two species could not be compared. However, the relative species response with respect to relative gas vesicle volume could still be compared for the two species limited by the environmental variables light, nitrogen and phosphorus.

Anabaena filaments grown for two days in different light environments showed no difference in their relative gas vesicle volume (figure 5.15). Some differences were evident on days four and six and a clear response pattern evident after eight days. On day eight gas vesicle production could not compensate for losses by cell division or possible turgor induced gas vesicle collapse as light intensity increased. There was approximately 25% less gas vesicles present at light intensities between 80 and 150 μmol m^{-2} s^{-1} and 40% less at 250 μmol m^{-2} s^{-1} relative to the four lowest light intensities. This trend is opposite to *Microcystis* where gas vesicle volume increased with increasing irradiance up to 150 μmol m^{-2} s^{-1} (figure 5.4).

The relative gas vesicle volume decreased with increasing growth rate (figure 5.16) further highlighting that production of gas vesicles could not match cell division or gas vesicle collapse.

5.3.6 Light dependent turgor pressure collapse of *Anabaena circinalis* gas vesicles

Relative gas vesicle volume of *Anabaena* cells incubated in different continuous light regimes was measured prior to and following 24 hours of light exposure (figure 5.17). There was no significant difference in the degree of gas vacuolation between the initial or light treatments (One way Anova, $p<0.01$; Tukey-Kramer Pair wise comparisons) except for the 250 µmol m^{-2} s^{-1} treatment which had a slightly depressed side scatter indicating less gas vesicles were present.

The turgor pressure of *Anabaena* cells at all treatments did not respond predictably to increasing irradiance (table 5.2). These results differ from studies by Kinsman *et al.* (1991) who found that turgor pressure increased by about 200 kPa in *Anabaena flos-aquae* incubated in 135 µmol m^{-2} s^{-1} for 24 hours and 100 kPa in cells at 5 µmol m^{-2} s^{-1}. There was no loss of buoyancy at any irradiance suggesting that there was sufficient gas vesicles present to counterbalance any increased carbohydrate accumulation due to photosynthesis. The lack of turgor and buoyancy change following 24 hours of light may indicate that the cells were moribund however the change in carbohydrate content in each treatment disputes this.

Light intensity (µmol m^{-2} s^{-1})	**Initial**	**10**	**20**	**40**	**60**	**80**	**100**	**150**	**250**
Turgor pressure (kPa)	415	422	422	422	371	407	368	400	400
% of filaments floating	91 (2.5)	92	92 (1.0)	93 (2.5)	92 (2.1)	88 (2.0)	89 (2.1)	89 (1.0)	88 (2.5)
Carbohydrate conc. ($g\ ml^{-1}$)	2.66 (0.9)	2.39	2.69 (0.5)	3.07 (0.3)	4.65	5.56 (0.8)	5.10 (0.6)	3.60 (1.6)	4.5 (0.7)

Table 5.2 Turgor pressure and the percentage of *Anabaena circinalis* cells floating following 24 hours exposure to various light intensities. Standard deviation is given in parentheses.

5.3.7 Nitrogen and *Anabaena* gas vesicle volume

To limit nitrogen in N_2-fixing *Anabaena circinalis* cultures flasks were incubated in a chamber which was sparged with O_2 and CO_2 in an argon carrier gas to limit atmospheric nitrogen in the system.

Over the first four days the growth rates were high and generally greater than 0.6 ln units day^{-1} (figure 5.18). Observation of the means alone suggested that there may have been a slight nitrogen deficiency relative to the high nitrate treatments and the nitrogen fixing control. The growth rate over the entire eight days was very high for all treatments and it appeared that a differential nitrogen limitation was not induced using the argon gas. It was possible that the nitrogen gradient between the chamber and the culture room was significant and allowed some atmospheric nitrogen into the chamber and therefore nitrogen limitation was not observed. The high light cultures experienced would have provided adequate energy to allow nitrogen fixation and consequently the nitrogen demands of the cells would have been met.

The relative gas vesicle volume of the *Anabaena* (figure 5.19) supports the conclusion that the cultures were not nitrogen limited as there was no apparent trend in the gas vesicle response to nitrogen after four days. On day two some interesting differences were observed with respect to gas vesicle volume. There was a slight increase in gas vesicle volume with increasing nitrate and which were also higher than the N_2-fixing control. A comparison of the two treatments exposed to atmospheric nitrogen revealed that the cultures with nitrate had a much greater gas vesicle volume than the treatment with no nitrate added. This phenomena may have been due to less energy being required to metabolise nitrate than atmospheric nitrogen (Ward and Wetzel, 1980a; Reuter and Peterson, 1987) leading to a more efficient reduction of nitrate to glutamate and incorporation of nitrogen into cell protein including gas vesicle protein.

5.3.8 Phosphorus and *Anabaena* gas vesicle volume

Anabaena cultures treated with 0.1 and 0.2 µM of phosphate recorded a loss of cells over the eight day experimental period (figure 5.20). However, the growth rate of cells plotted against the initial phosphate concentration still approximated a Michaelis-Menton substrate-growth curve with a maximum growth rate of 0.1 ln units day^{-1} for concentrations greater than 1 µM phosphate.

The gas vesicle volume on day two showed slight dilution of gas vesicle volume relative to the 0 µM phosphate control (figure 5.21). This dilution continued for cells in non-saturating concentrations of phosphate (<1 µM), except for the no phosphate treatment which maintained a higher relative gas vesicle volume. On day eight the relative phosphate dependent relative gas

vesicle volume approximated the shape of the growth curve. Linear least squares regression revealed that relative gas vesicle volume was linearly related to the phosphate limited growth rate (r^2=0.64; figure 5.22).

5.4 Discussion

Light and nutrients are the key "bottom-up" factors controlling phytoplankton in natural waters (DeMelo *et al.*, 1992). In the cyanobacteria these factors also affect buoyancy and buoyancy regulation which provide this group of organisms with a competitive advantage over other phytoplankton during extended periods of low turbulence. The effect nutrients and light have on the gas vesicle pool of cyanobacteria varies between species and it is essential to recognise this and identify the differences in conceptual models of buoyancy regulation. The comparison of gas vesicle accumulation in *Microcystis aeruginosa* and *Anabaena circinalis* revealed some interspecific differences which may help scientists and water managers to understand why some cyanobacteria dominate in certain habitats whilst others find different habitats or environmental conditions more favourable.

5.4.1 Flow cytometry to estimate relative gas vesicle volume

Side scatter, due to gas vesicles, measured using either the flow cytometer or nephelometer were comparable (figure 5.1). The major difference was the scale at which measurements were made; flow cytometry measured scatter per cell (*Microcystis*), or biomass unit (*Anabaena*), whereas the turbidimeter measured an algal suspension composed of many individuals. The use of flow cytometry to estimate relative gas vesicle volume provided a quick, highly accurate and repeatable technique which allowed population heterogeneity to be assessed.

Attempts to measure actual gas vesicle volume using a specially constructed capillary apparatus (Walsby *et al.*, 1992) were unsuccessful due to "leaky" seals which did not maintain a gas tight capillary. Therefore, it was not possible to provide estimates of absolute gas vesicle volumes and calibrate side scatter against gas volume. Absolute gas vesicle volume would permit calculation of the lift provided by gas vesicles and subsequently used to construct numerical

models of buoyancy and buoyancy regulation or include in existing models (Kromkamp and Walsby, 1990).

5.4.2 Gas vesicle accumulation in response to light

Microcystis at different light intensities showed a growth optimum at irradiances between 50 and 150 μmol m^{-2} s^{-1}. On the other hand *Anabaena* was light limited at irradiances less than 150 μmol m^{-2} s^{-1} although the maximum growth rates of both species were similar. This difference may be due to *Microcystis* displaying a greater adaptation to low light and utilising energy more efficiently. This is supported by data presented in Ganf (1980) where *Microcystis aeruginosa* was shown to have a lower K_s for photosynthesis (50 μmol m^{-2} s^{-1}) than *Anabaena circinalis* (105 μmol m^{-2} s^{-1}). K_s is an estimate of light capturing efficiency and was defined as the irradiance corresponding to 1/2 P_{max} where P_{max} was the maximum photosynthetic rate. The difference in growth rate between the species will also affect the rate of gas vesicle dilution due to growth at different irradiances and consequently buoyancy characteristics and the habitat they succeed in.

The relative gas vesicle volume of *Microcystis* increased above the initial volume at all light intensities and exceeded the growth rate resulting in greater buoyancy with time (figure 5.4). The relative gas vesicle volume in *Microcystis* was related to both growth (figure 5.5) and metabolic activity (figure 5.6). This was not surprising as both estimate cell health and are the product of carbon fixation, ATP production and nutrient metabolism which also govern gas vesicle protein production. This was inconsistent with the study by Reynolds and Walsby (1975) who documented an inverse correlation between growth rate and gas vesicle volume and observed the lowest gas vesicle volume in log phase cultures.

Observations made here that *Microcystis* increased gas vesicle volume in response to light does not support the work of Kromkamp *et al.* (1988). They found no light dependent regulation of gas vesicle content per unit protein and a decrease in gas vesicle volume per cell with increased growth in their strain of *Microcystis*. However, Kromkamp *et al.* (1989) found increasing gas vesicle volume with irradiance 40 hours after a phosphate pulse to phosphate limited cultures which was maximal at 50 μmol m^{-2} s^{-1} (Kromkamp *et al.*, 1989; fig. 4) This was similar to the light dependent relative gas vesicle volume recorded for *Microcystis* on day two (figure 5.4).

The shift with time from a maximum gas vesicle volume at 50 μmol m^{-2} s^{-1} on day two to a maximum at 100 μmol m^{-2} s^{-1} on day eight may represent adaptation to a higher light intensity. Alternatively, it may represent a slowing of growth which increases the rate of gas vesicle production relative to the growth rate resulting in increased gas vesicle accumulation per cell. Ward and Wetzel (1980b) and Kohler (1992) found that *Microcystis aeruginosa* took two days to adapt to a new light intensity. Why the 100 μmol m^{-2} s^{-1} cells displayed an apparent adaptation to light on day eight when adaptation takes only two days and cells at other light intensities did not respond remains unclear.

Conceptually one would expect greater gas vesicle accumulation in light-limited cells to provide greater lift and increase the flotation rate into more favourable light intensities. Why *Microcystis* doesn't follow this pattern is not immediately obvious but it may be related to the fact that it saturates growth at reasonably low light intensities. Ballast is accumulated slower or even lost at low light intensities and this coupled with the large colony size may move cells into the preferred light zone without relying on slow gas vesicle volume change.

Deacon and Walsby (1990) showed that their strain of *Microcystis aeruginosa* had the greatest gas vesicle volume at 30 μmol m^{-2} s^{-1} which decreased at higher irradiance. Cells incubated in the dark following light exposure formed more gas vesicles if their previous irradiance was high and they had adequate carbohydrate stores.

The response of *Anabaena*'s gas vesicle pool to light contrasts sharply with *Microcystis* and is similar to the response of *Oscillatoria redekei*, *Oscillatoria rubescens*, *Anabaena circinalis* and *Microcystis aeruginosa* reviewed by Reynolds and Walsby (1975), and in *Aphanizomenon flos-aquae* by Kromkamp *et al.* (1986) and Konopka *et al.* (1987b). In *Anabaena* the rate of gas vesicle production could not match the growth rate at higher light intensities and consequently the gas vesicle pool was diluted. Owing to the fact that the two species had similar maximum growth rates, and therefore dilution due to growth would be similar, it is feasible to conclude that gas vesicle production in *Anabaena* occurred at a slower rate than *Microcystis* under the experimental conditions used.

The different strategies employed by *Microcystis aeruginosa* and *Anabaena circinalis* are interesting because although *Microcystis* achieves a greater growth rate at lower light intensities it still invested energy into gas vesicle production more quickly than *Anabaena* to enable the rate of gas vesicle production to match and often exceed growth rate. *Anabaena* required a greater light intensity to maximise growth and retained gas vesicles at low irradiances to provide greater buoyancy and float upwards in the water column to maximise light exposure. These differences in response to light reflect the different strategies employed to regulate buoyancy.

The other factor which may reduce the gas vesicle volume of *Anabaena circinalis* is turgor pressure generated by osmotically active photosynthates (Oliver and Walsby, 1984; Kinsman *et al.*, 1991) or potassium accumulation (Allison and Walsby, 1981). This did not appear to be a major factor controlling buoyancy in *Anabaena circinalis* as the only reduction in relative gas vesicle volume evident following 24 hours of irradiance was at 250 μmol m^{-2} s^{-1} (figure 5.17).

Whether this was due to turgor induced gas vesicle collapse or dilution due to growth can not be determined using the flow cytometric technique alone. The total gas vesicle pool needed to be measured either by multiplying the relative gas vesicle volume by the cell number or measuring the absolute difference in gas vesicle volume with a capillary apparatus (Kinsman *et al.*, 1991) to determine if there was a net loss in gas vesicle space. Turgor pressure may have a more significant affect on a cell's buoyancy status when the cell is nutrient stressed, as the gas vesicle pool is reduced and intermediates of photosynthesis persist adding to cell turgor and density (Konopka *et al.*, 1987b).

Anabaena exposed to different light intensities for 24 hours did not lose buoyancy (table 5.1) suggesting there was sufficient gas vesicles present to maintain buoyancy and counterbalance the increase in density due to carbohydrate (Kromkamp and Mur, 1984; Thomas and Walsby, 1985; Utkilen *et al.*, 1985a; Kromkamp *et al.*, 1986; Kromkamp and Walsby, 1990). This persistent buoyancy has been observed previously (Walsby *et al.*, 1987; Walsby *et al.*, 1991) and will be discussed later in chapters six and seven.

5.4.3 Light-is it a case of timing?

In the range of experiments presented here cells were maintained at constant irradiances during the light exposure period (12:12 light:dark cycle). In natural water cells experience a fluctuating light climate. The photon flux density a cell experiences is a function of the diel solar input cycle, the optical properties of the water body and the position of the cell in the water column. The position in the water column will be governed by water movement and buoyancy regulation.

It has been demonstrated here that gas vesicle volume was determined by light dependent growth and gas vesicle production. Kromkamp *et al.* (1989) investigated phosphorus and light interactions on gas vesicle synthesis. They pulsed phosphate-limited cultures with phosphate and then incubated these cultures in the dark, or subjected them to five hour light periods at set intervals following the phosphate pulse. No gas vesicle formation occurred in the dark or when the five hour light period immediately followed the phosphate pulse. When the light pulse was given between five and fourteen hours after the phosphate pulse relative increases in gas vesicle volume were recorded; gas vesicle volume was measured 50 hours after the phosphate pulse.

The study by Kromkamp *et al.* (1989) revealed that gas vesicle synthesis is light dependent and is also dependent upon the periodicity of light:dark exposure following the nutrient pulse. When cells migrate vertically (Kromkamp and Walsby, 1990) they move through a light and nutrient gradient (Ganf and Oliver, 1982). The experimental design of Kromkamp *et al.* (1989) represented this. Different sized colonies or filaments migrate at different speeds and to different depths (Kromkamp and Walsby, 1990; Brookes *et al.*, 1997). Consequently they will experience light at different times following a nutrient uptake event which will affect gas vesicle synthesis and increase heterogeneity in the buoyancy characteristics of cells within a population.

Further evidence that light periodicity may affect gas vesicle protein is obtained by investigating the influence of light: dark cycles on cell protein. Green (1994) investigated the effect on cell protein of *Microcystis* exposed to one hour and 0.5 hour alternating light:dark periods. Cell protein remained constant with time in cells subjected to one hour light:dark fluctuations but increased linearly for 24 hours in cells at the 0.5 hour period light:dark treatment. The final cell protein was approximately four times greater in cells exposed to 0.5 hour periodicity light:dark

cycles than those at the one hour periodicity. Thomas and Walsby (1985) and Kromkamp *et al.* (1988) found that gas vesicle synthesis increased in proportion to total protein synthesis in light limited *Microcystis* cultures. If gas vesicle protein synthesis matched cell protein synthesis in the experiments of Green (1994) then cells experiencing half hourly transitions from light to dark would produce more gas vesicles and would be more buoyant than cells receiving hourly transitions. This may lead to over-buoyancy and may be a strategy to maximise light exposure in a rapidly fluctuating light environment.

Possible mechanisms giving rise to light-dependent gas vesicle synthesis are increased carbon fixation, ATP production via photosynthesis providing carbon for protein synthesis and ATP for cellular energy requirements. Photosynthesis also provides energy for nitrogen metabolism to glutamate for inclusion into amino acids and eventually proteins. There may also be photo-regulation of the gas vesicle genes controlling the rate of gas vesicle synthesis (Oliver, 1994).

5.4.4 Gas vesicle volume in response to nitrogen

Gas vesicles are proteinaceous and consequently there is a significant demand for nitrogen during gas vesicle synthesis. *Anabaena* is capable of fixing atmospheric nitrogen and so theoretically it may only be nitrogen limited when there is insufficient light driven photosynthesis to reduce N_2, nitrate and nitrite. Nitrogen may also be inefficiently incorporated into the cell when iron is limiting (Reuter and Peterson, 1987). In the *Anabaena*/nitrogen experiments detailed here nitrogen limitation was not induced which was most probably a result of technical difficulties in maintaining cultures free of atmospheric nitrogen. In future studies it will be necessary to ensure total N_2 removal.

Microcystis responded to increasing nitrate concentrations by increased growth rate, metabolic activity and rate of gas vesicle synthesis which is consistent with observations by Klemer *et al.* (1982) in *Oscillatoria rubescens*. This observation fits with conceptual reasoning of buoyancy regulation, a mechanism to overcome the vertical separation of light and nutrients. If the cyanobacteria's nutrient requirements are satisfied then more gas vesicles are produced, buoyancy is increased and the cell is capable of floating to a more favourable light climate in order to satisfy its energy needs.

A similar strategy was evident in *Anabaena* exposed to 24 hours of light which displayed constant buoyancy, although cell density would have increased as carbohydrate accumulated during photosynthesis (Thomas and Walsby, 1985; Kromkamp and Walsby, 1990). The *Anabaena* was grown in nutrient rich media and the cells nutrient requirements would have been met and sufficient gas vesicles synthesised to provide buoyancy in an attempt to maximise light exposure by floating upward in the water column. The interaction between nutrient limitation gas vesicle volume and ballast will be investigated in chapter seven.

It is recognised that nitrogen fixing cyanobacteria are favoured over other cyanobacteria in nitrogen deplete waters (Rhee, 1978; Rhee and Gotham, 1980; Presing *et al.*, 1996) and buoyancy may play a role in their ecological advantage. Non N_2-fixing species suffer a reduction in their relative gas vesicle volume when nitrogen stressed and experience an increased density, consequently cells would tend to sink out of the illuminated waters where they would experience light limitation. N_2-fixing species are more likely to retain buoyancy when ammonium and nitrate are low and remain in the illuminated surface waters until phosphorus becomes limiting which would act to decrease the gas vesicle pool and decrease buoyancy. This may explain why nitrogen fixing species are present in the River Murray where soluble nitrogen is low and the total nitrogen relatively unavailable to phytoplankton (Chapter 4). Population development of cyanobacteria such as *Microcystis aeruginosa,* which are suited to the physical conditions of diurnal stratification observed in the river (Ganf, 1974), would be inhibited by nitrogen limitation.

5.4.5 Gas vesicle volume in response to phosphorus

Oliver (1994) states that there is no phosphorus in gas vesicles and the effect of phosphate limitation on gas vesicle production is indirect either through ATP or nucleotides for RNA and DNA synthesis.

Konopka *et al.* (1987b) found no difference in the gas vesicle volume of *Aphanizomenon flos-aquae* grown at different dilution rates in phosphate limited continuous culture. A similar response was observed in *Microcystis* after eight days, except at the two lowest phosphate concentrations which had lower mean gas vesicle volumes. *Anabaena* on the other hand regulated its gas vesicle synthesis in a manner which was related to the initial phosphate

concentration and matched the growth rate. Why *Anabaena* has adopted a different response to phosphate limitation is unclear but the dilution of the gas vesicle volume under phosphorus stress will negatively affect buoyancy in this species. Nutrient limitation could cause *Anabaena* to lose buoyancy and sink deeper into higher concentrations (Ganf and Oliver, 1982) maintained by sediment fluxes (Martinova, 1993).

The gas vesicle volume and consequently the buoyancy of *Microcystis* was less affected by phosphate limitation than *Anabaena*. With a lower gas vesicle volume less carbohydrate is needed to overcome the buoyancy provided by the gas vesicles and consequently cells more rapidly migrate vertically in the water column and scavenge for nutrients (Brookes *et al.*, 1997).

5.4.6 Chlorophyll and relative gas vesicle volume

There was no universal relationship between the chlorophyll content and relative gas vesicle volume. Although chlorophyll and gas vesicle volume increased in improved nutrient climates, light increases resulted in higher gas vesicle pool but lower chlorophyll content. The gas vesicle volume of *Anabaena* responded in the opposite manner to *Microcystis* and decreased with increasing light and growth rate. This is the most commonly observed response (Kromkamp *et al.*, 1989; Deacon and Walsby, 1990). Therefore in *Anabaena* it is probable that chlorophyll content and the gas vesicle volume display similar responses to both light and nutrients. This relationship is probably coincidental because chlorophyll and gas vesicle protein production are completely separate processes. Chlorophyll degradation in the presence of high light occurs rapidly but the gas vesicle response is slower relying on slow production, high dilution by growth and high turgor to decrease gas vesicle volume.

5.4.7 Heterogeneity in gas vesicle volume and buoyancy

Figure 5.13 demonstrated the heterogeneity in gas vesicle volume displayed by *Microcystis* cells particularly when phosphate limited. When the gas vesicle volume is low less carbohydrate is required to lose buoyancy. Cells with low gas vesicle volume will display different buoyancy characteristics in response to light than cells with more gas vesicles. This will serve to increase heterogeneity in field populations as cells will experience light for different time periods, sink to varying depths and encounter a range of microhabitats and nutrient concentrations. Konopka *et al.* (1987b) also observed heterogeneity in the buoyancy

response of *Aphanizomenon flos-aquae* exposed to an increase in irradiance. They attribute their observed heterogeneity to differences in the rate of ballast accumulation or the amount of ballast required to overcome buoyancy, which is governed by the gas vesicle volume.

Population heterogeneity is an important aspect of phytoplankton communities with differences in buoyancy characteristics observed between species and within species. Heterogeneity needs to be considered when modelling buoyancy in cyanobacteria (Kromkamp and Walsby, 1990: Howard *et al.*, 1995) to recognise the bounds of the projected vertical migration and growth.

This chapter has examined gas vesicle volume as a mechanism influencing cell buoyancy and offering an advantage over other phytoplankton during periods of water column stability. Regulation of gas vesicle volume is one mechanism which affects cell buoyancy but cells also change their buoyancy by the photosynthetic accumulation and respiratory loss of carbohydrate, which acts as ballast. The following chapters examine how nutrients and light interact to govern ballast accumulation and loss and how this interacts with the gas vesicle pool to determine whether a cell sinks or floats and its pattern of vertical migration within a water column.

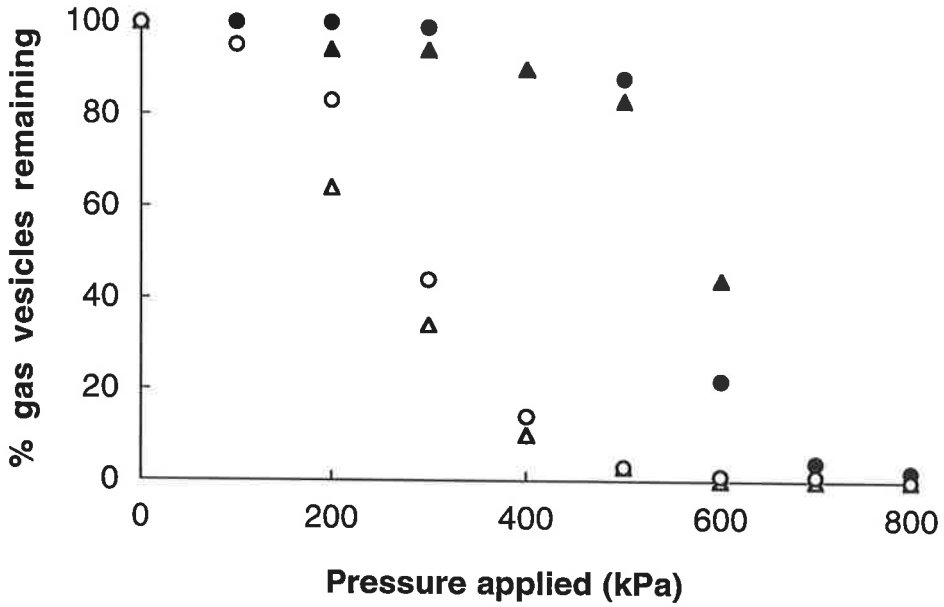


Figure 5.1 Gas vesicle collapse-pressure curves of *Microcystis aeruginosa* constructed by measuring light side scatter following the application of pressure on samples suspended in ASM-1 media (open symbls)or media + 0.5 μM sucrose (closed symbols) to eliminate turgor pressure. The agreement between samples measured using a nephelometer (Δ) and a flow cytometer (o) supports the use of flow cytometry in gas vesicle studies.

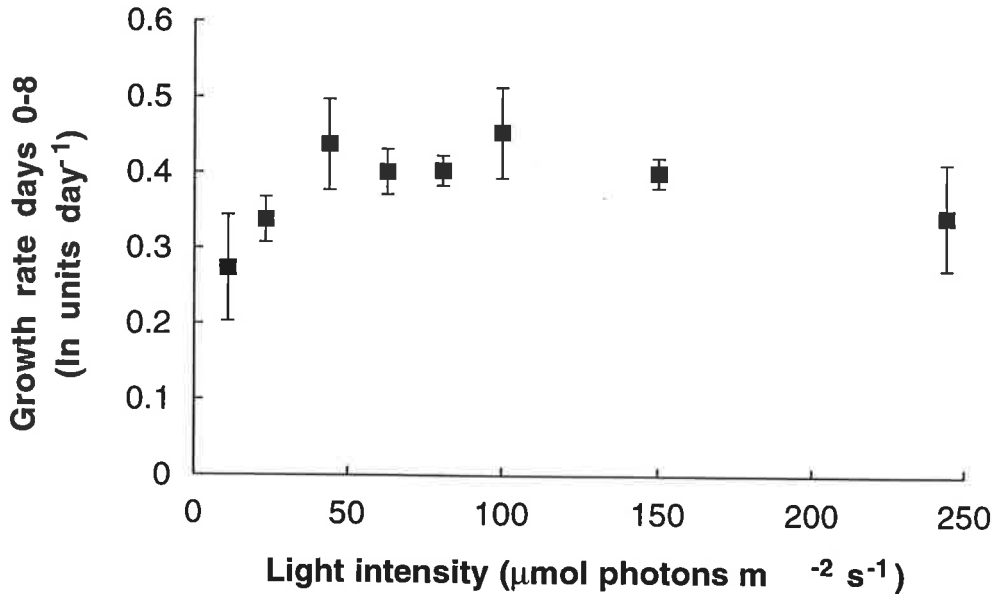


Figure 5.2 Light dependent growth rate of *Microcystis aeruginosa*

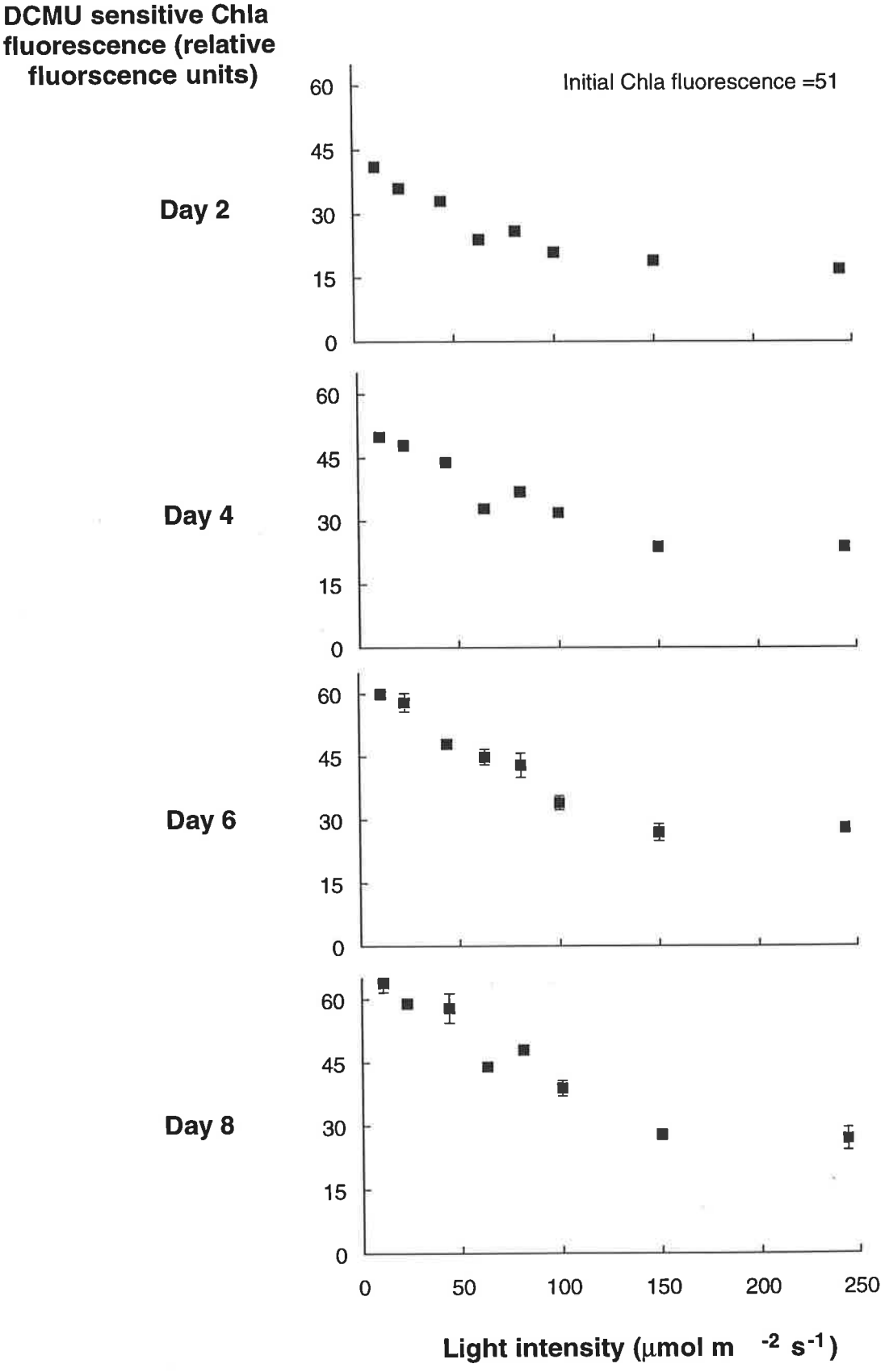


Figure 5.3 Relative chlorophyll content of *Microcystis* cells grown at different light intensities. Chlorophyll was measured per cell using a flow cytometer following the addition of DCMU .

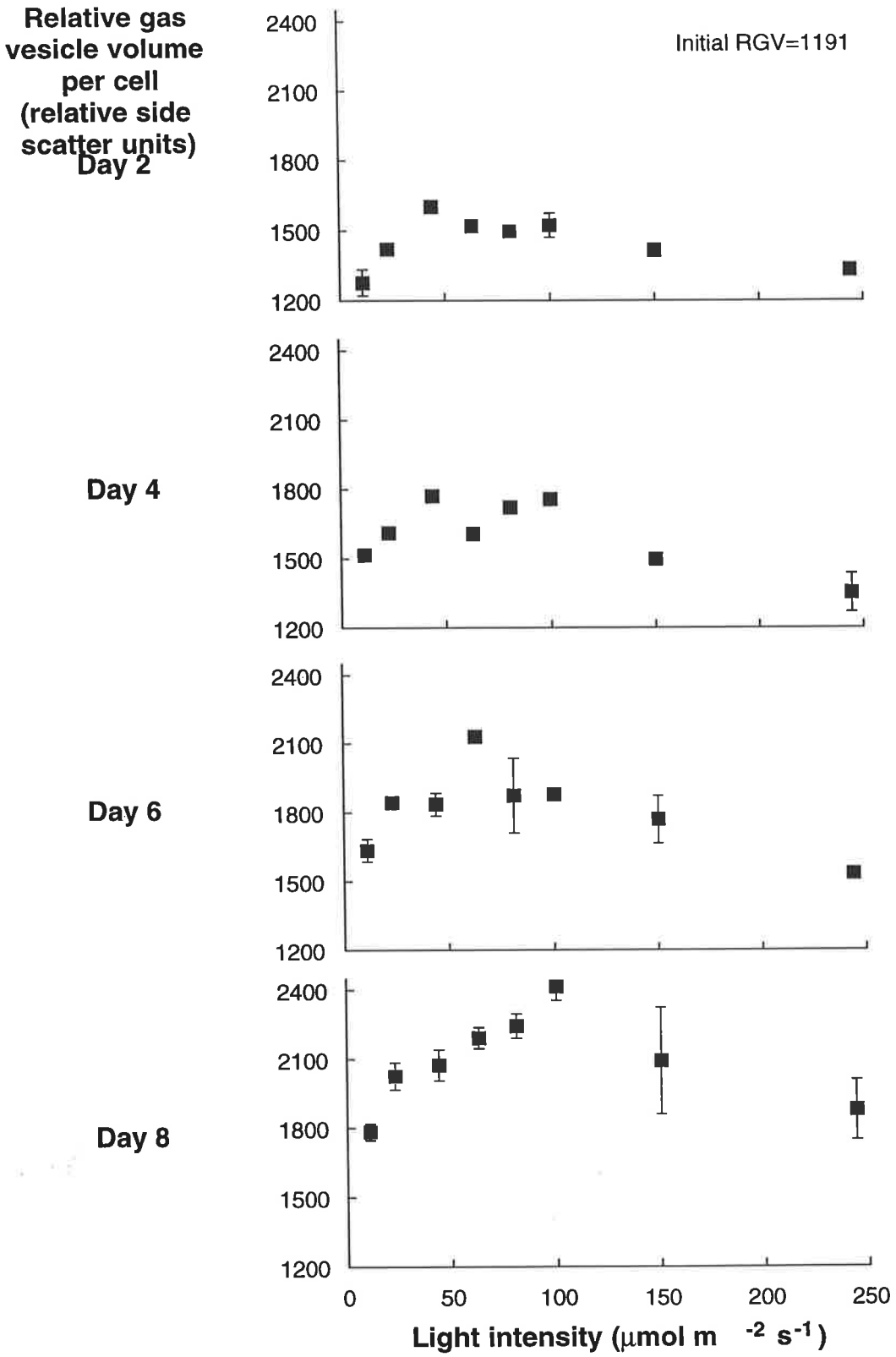


Figure 5.4 The relative gas vesicle volume of *Microcystis aeruginosa* cells, grown at different light intensities, was estimated by measuring side scatter per cell with a flow cytometer.

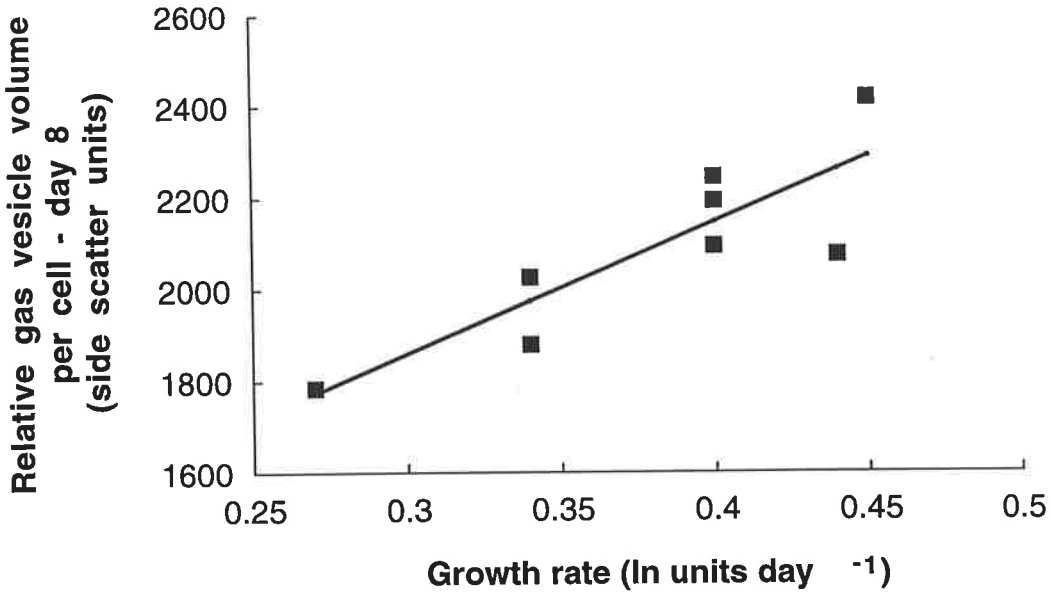


Figure 5.5 The gas vesicle volume , measured on day eight is positively correlated (r^2=0.73) to the light-limited growth rate.

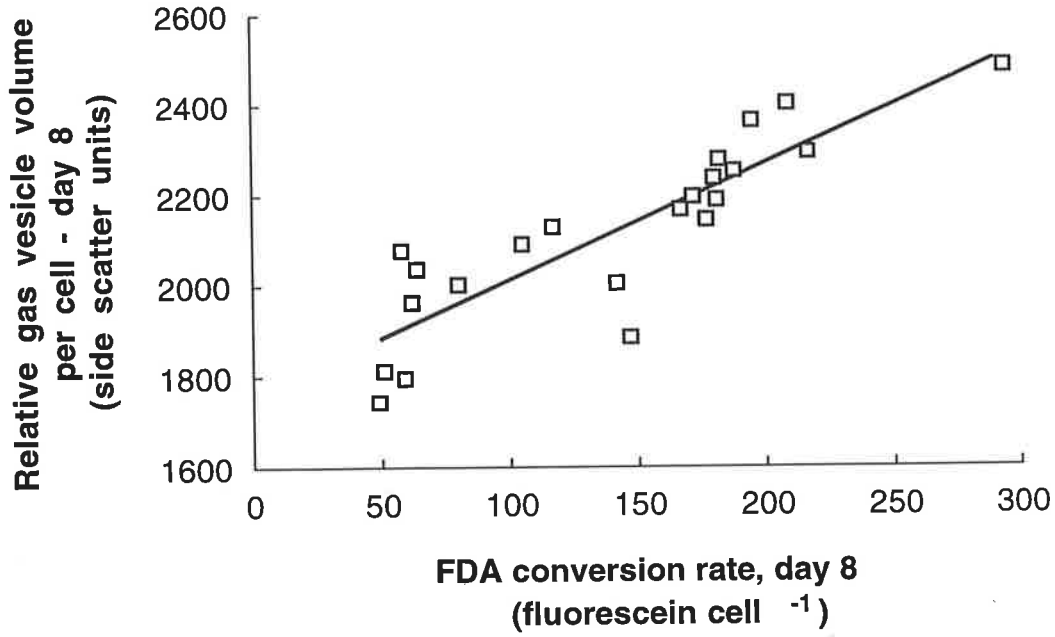


Figure 5.6 The relationship between relative gas vesicle volume (day 8) and cellular metabolic activity (r^2=0.75) measured using flow cytometry and the fluorogenic probe fluorescein diacetate of *Microcystis* grown at different light intensities.

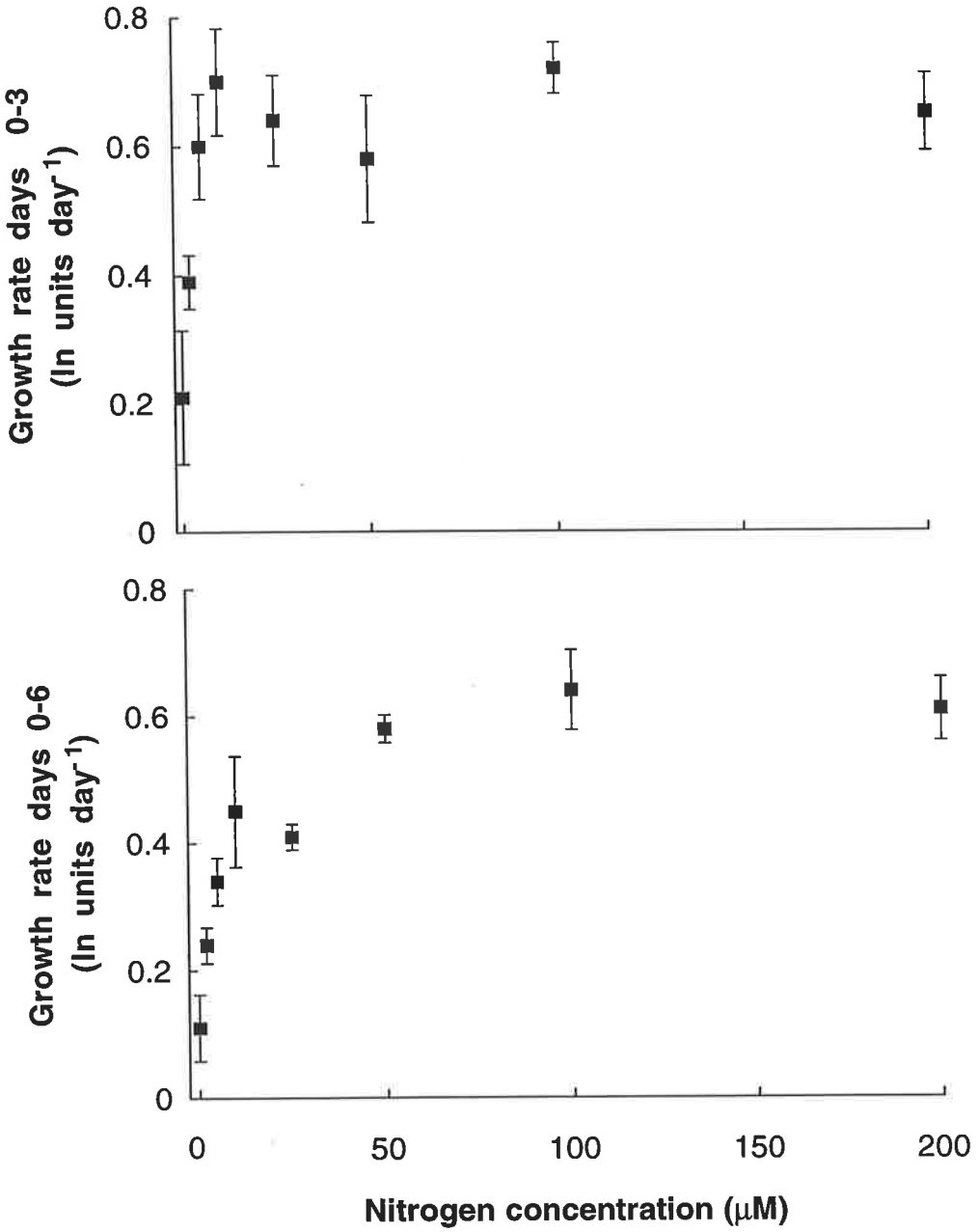


Figure 5.7 Nitrogen dependent growth of *Microcystis aeruginosa* over the first three days after inoculation (top) and over the six day experimental period (bottom graph).

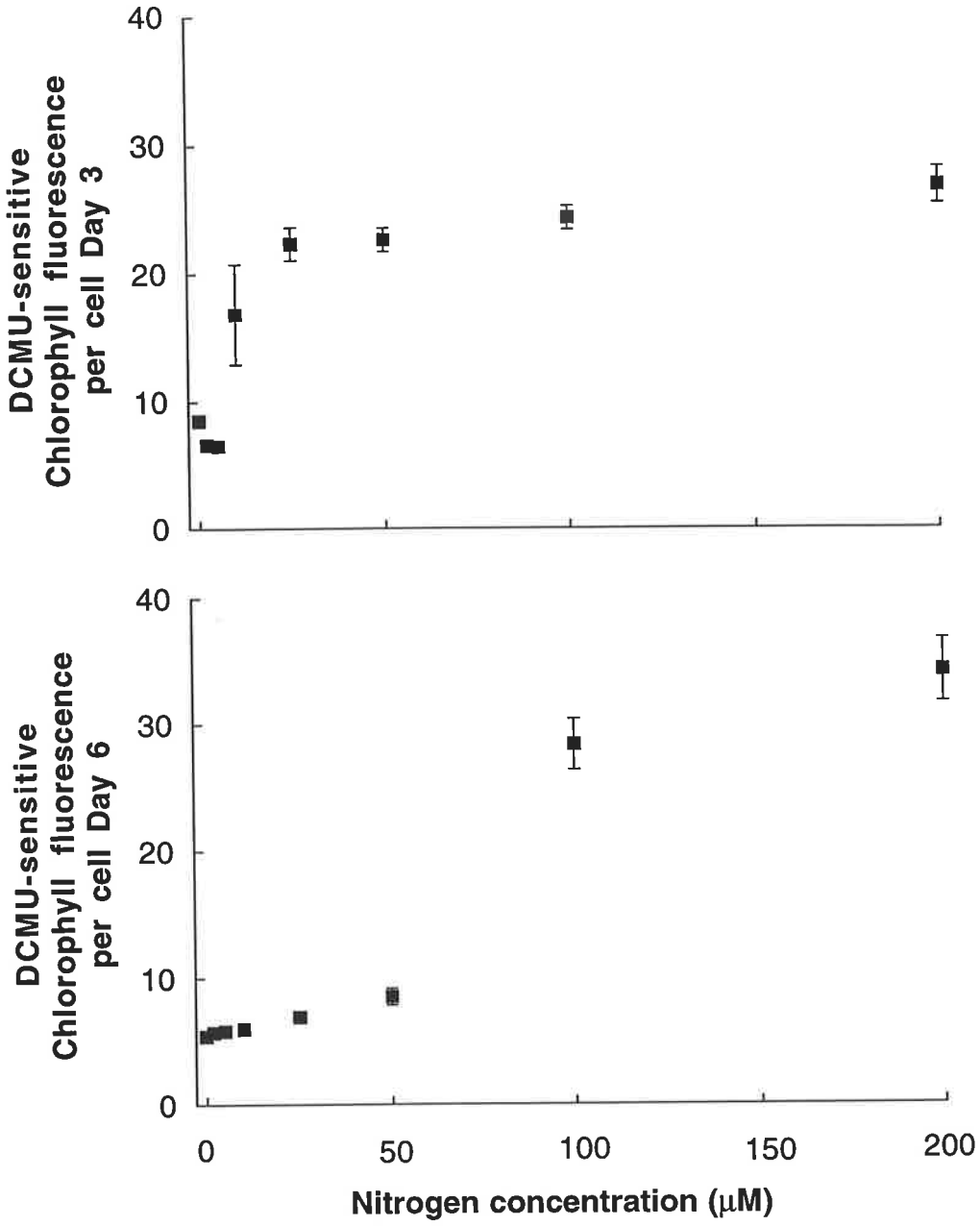


Figure 5.8 Nitrogen dependent chlorophyll content of *Microcystis aeruginosa* cells three days after inoculation (top graph) and after six days (bottom graph).

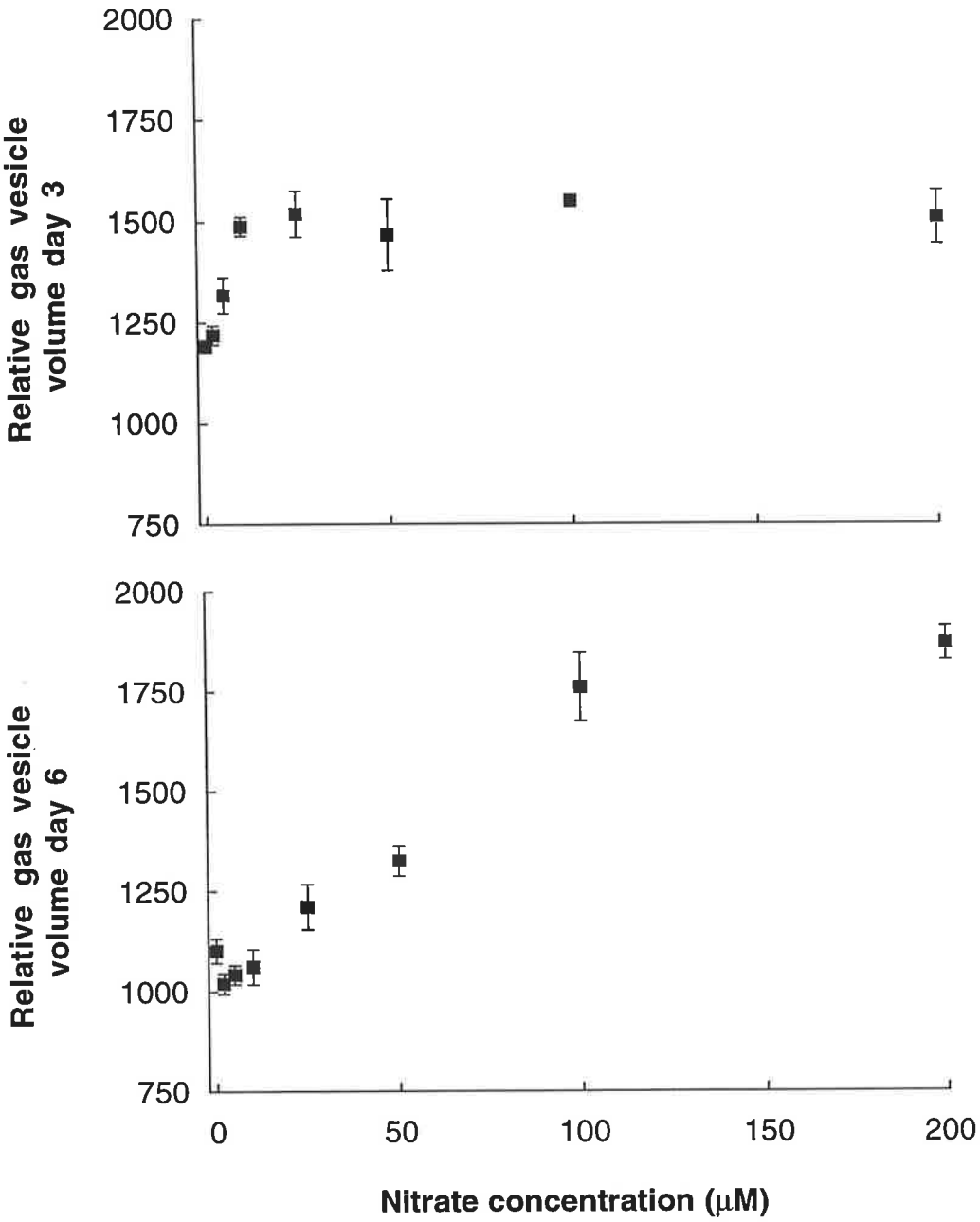


Figure 5.9 Gas vesicle volume of *Microcystis* grown in a range of nitrate concentrations and measured three (top) and six days (bottom) after inoculation. Relative gas vesicle volume was measured as side scatter per cell using a flow cytometer.

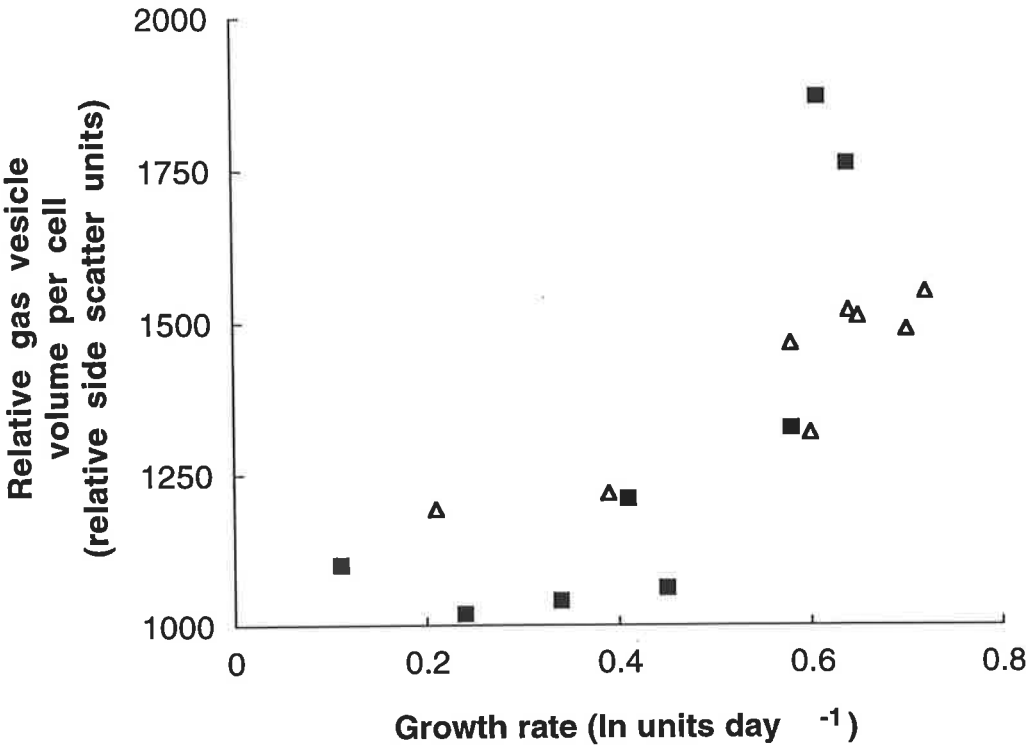


Figure 5.10 Relationship between relative gas vesicle volume and growth rate of *Microcystis* grown at different nitrate concentrations, measured on day three (Δ, r^2=0.81) and day 6 (■).

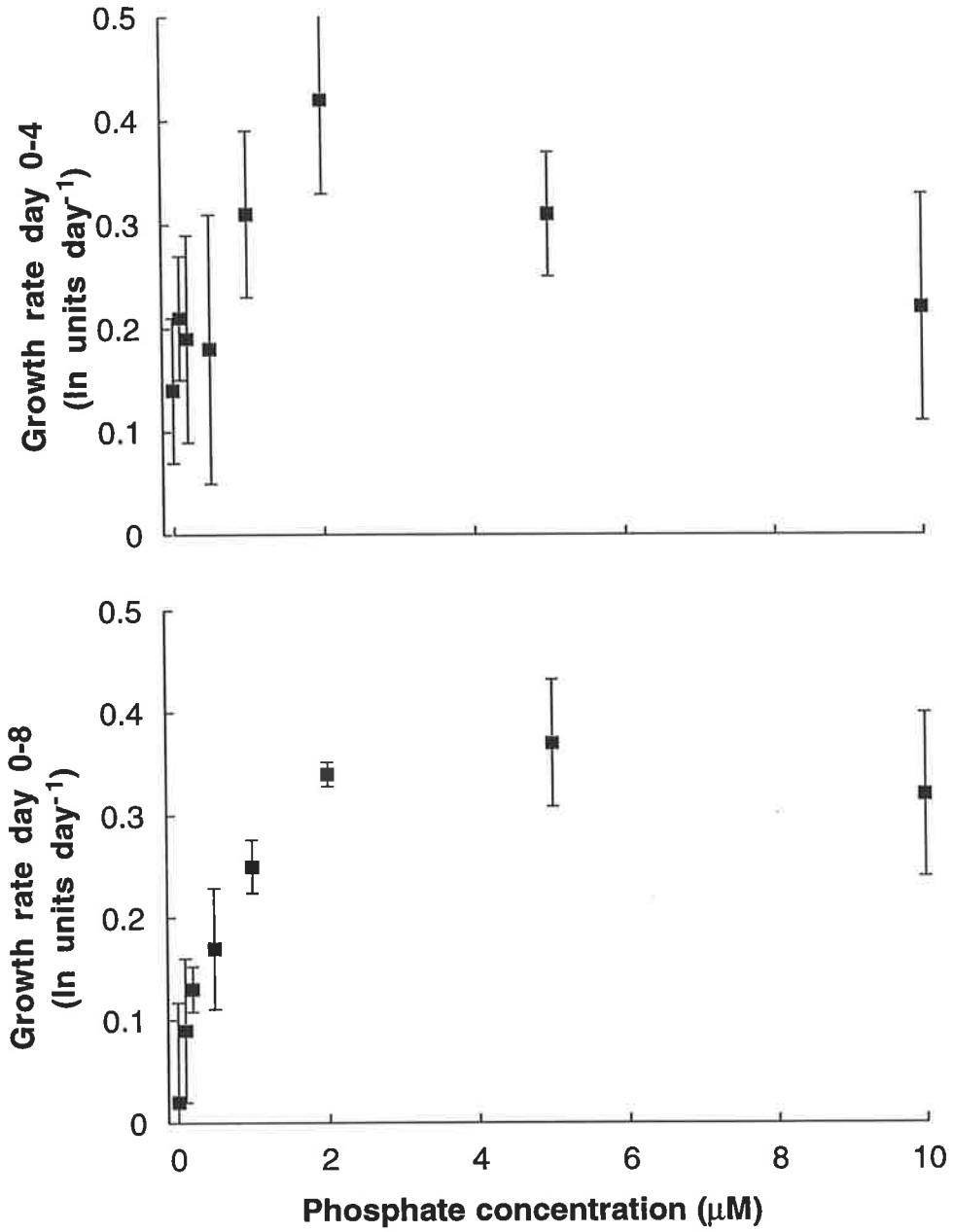


Figure 5.11 Phosphate dependent growth rate of *Microcystis aeruginosa* over the four days following inoculation (top graph) and over the entire eight day experimental period (bottom graph).

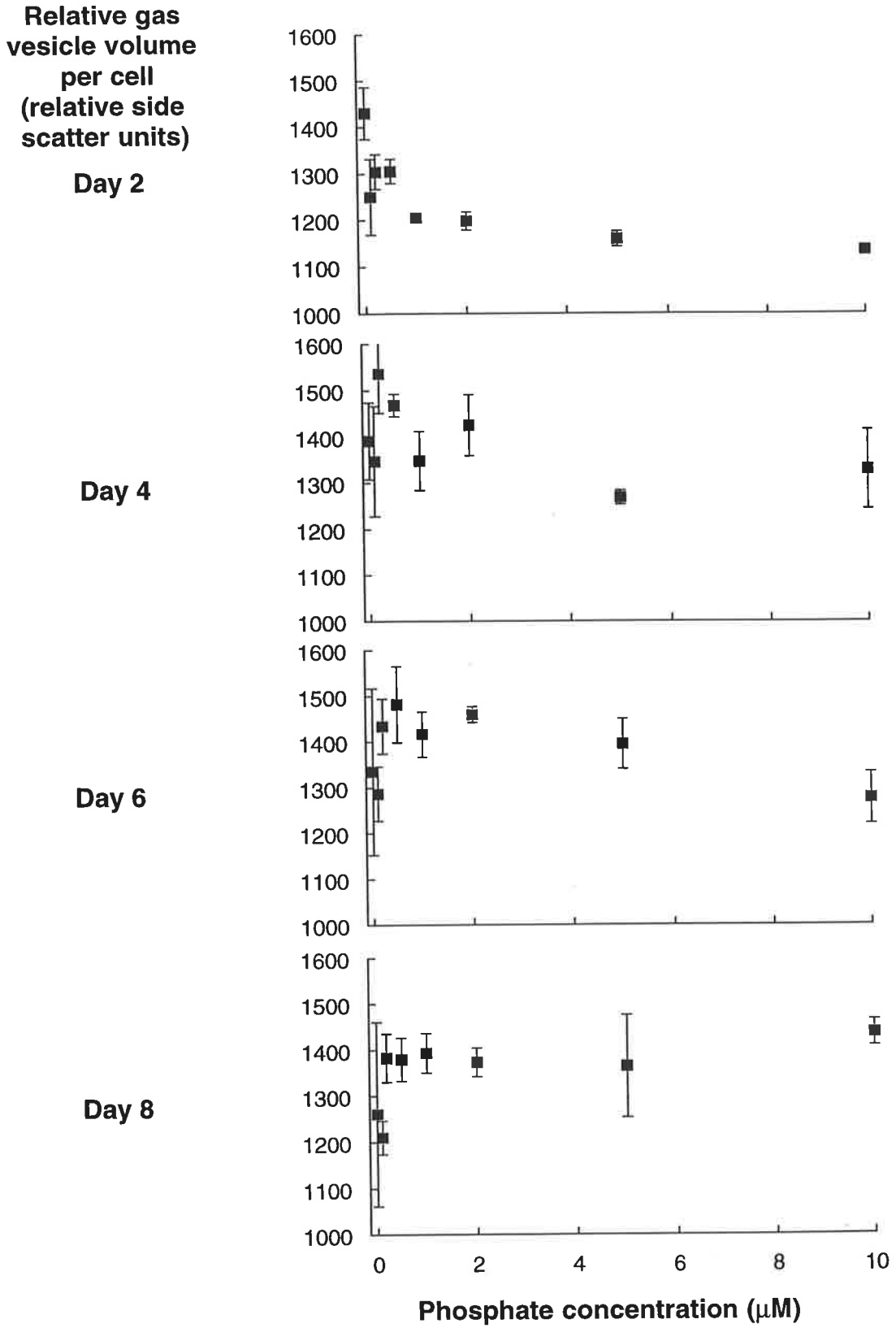


Figure 5.12 The gas vesicle volume of *Microcystis* cells grown at different phosphate concentrations measured as side scatter per cell using a flow cytometer.

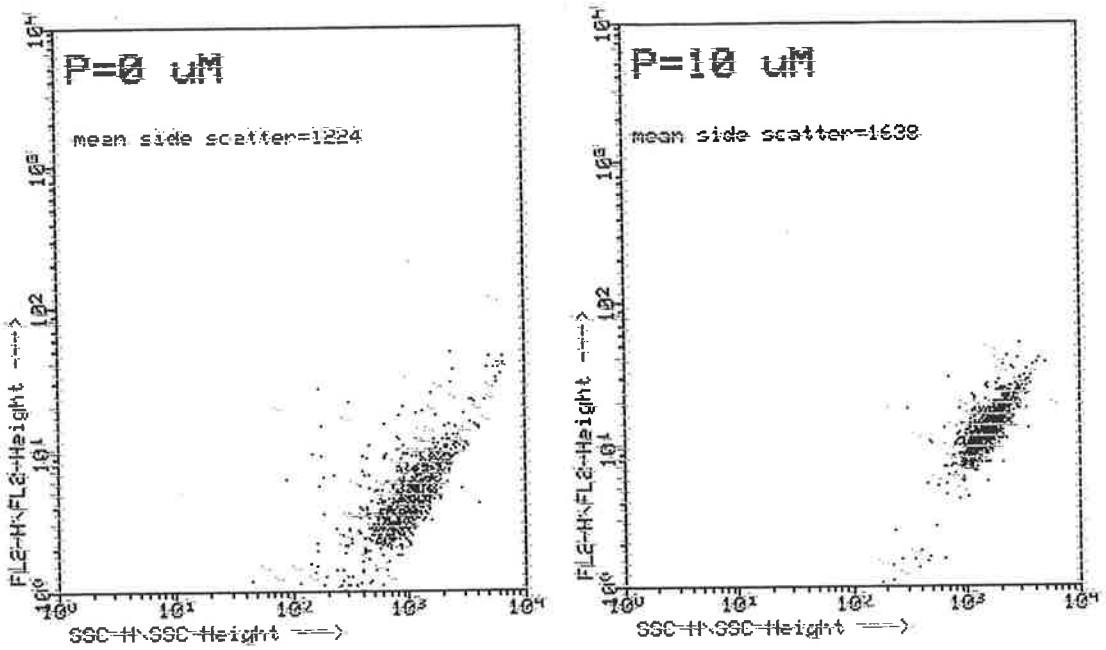


Figure 5.13 *Microcystis aeruginosa* was grown in 0 or 10 µM initial phosphate concentration in otherwise normal ASM-1 media. The relative gas vesicle volume per cell was measured using side scatter detectors on a flow cytometer eight days after inoculation. The plots show chlorophyll fluorescence (FL2) and side scatter (SSC). There is greater heterogeneity in gas vesicle volume (SSC) in cells deprived of phosphorus than cells grown in phosphorus sufficient media.

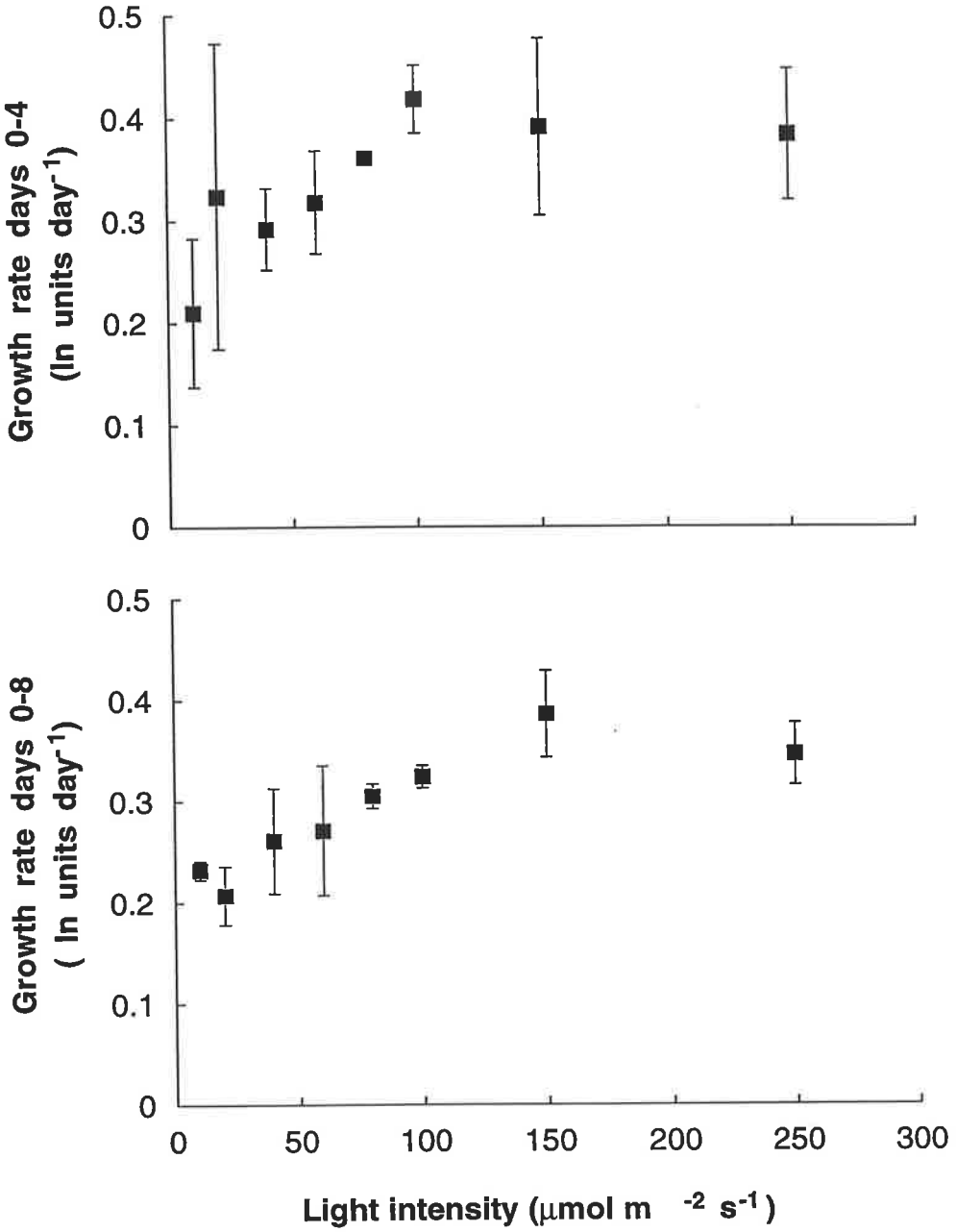


Figure 5.14 Light dependent growth rate of *Anabaena circinalis* over four days at different light intensities (top graph) and over the entire eight day experimental period (bottom graph).

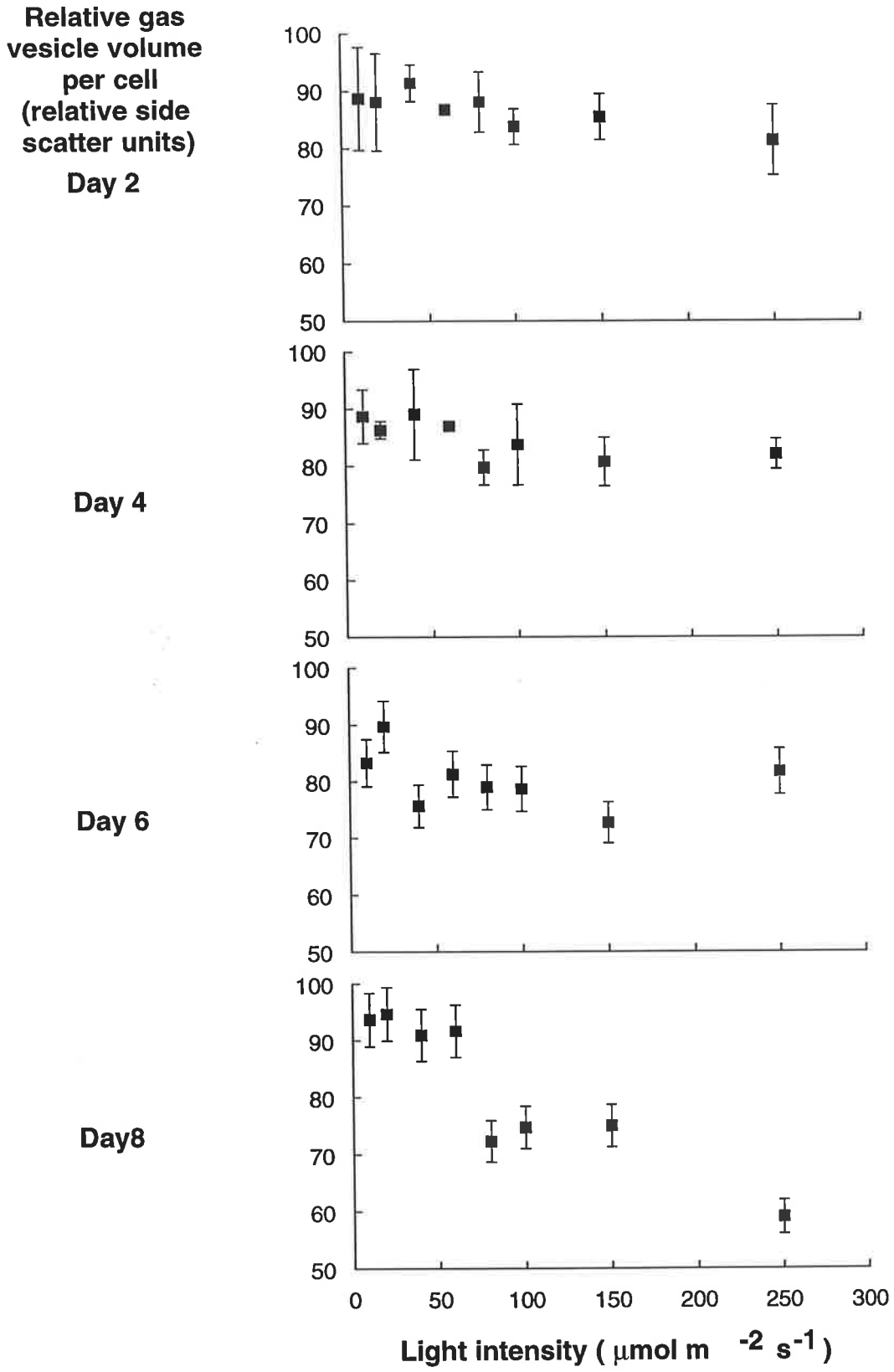


Figure 5.15 The relative gas vesicle volume of *Anabaena circinalis* grown at different light intensities was determined using a flow cytometer.

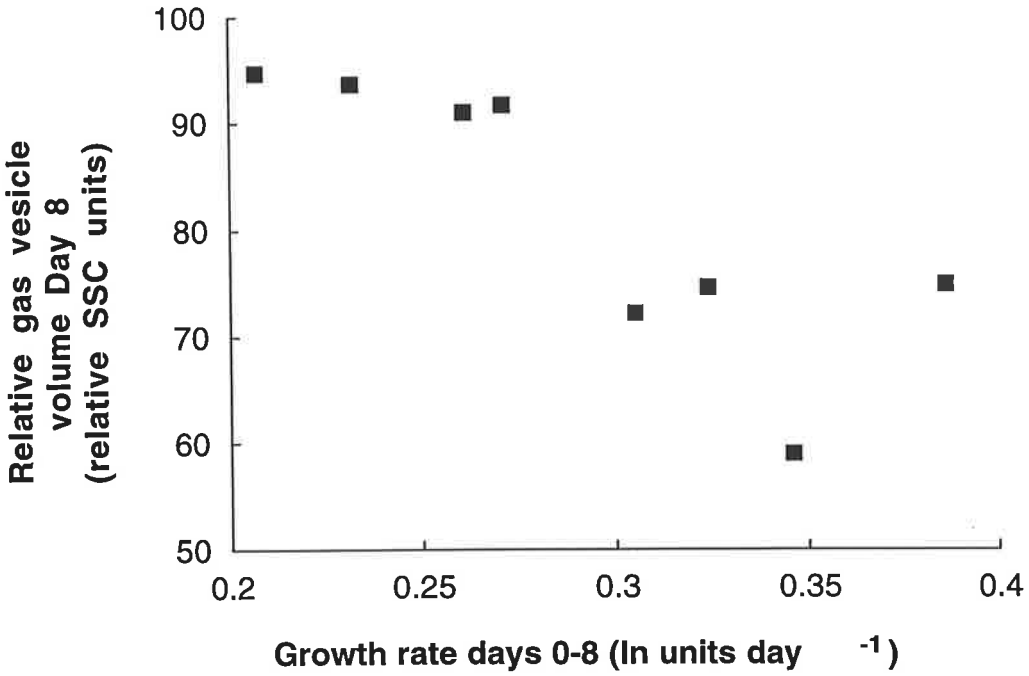


Figure 5.16 In *Anabaena circinalis* the relative gas vesicle volume per cell decreased with increasing growth rate in cultures incubated at different light intensities.

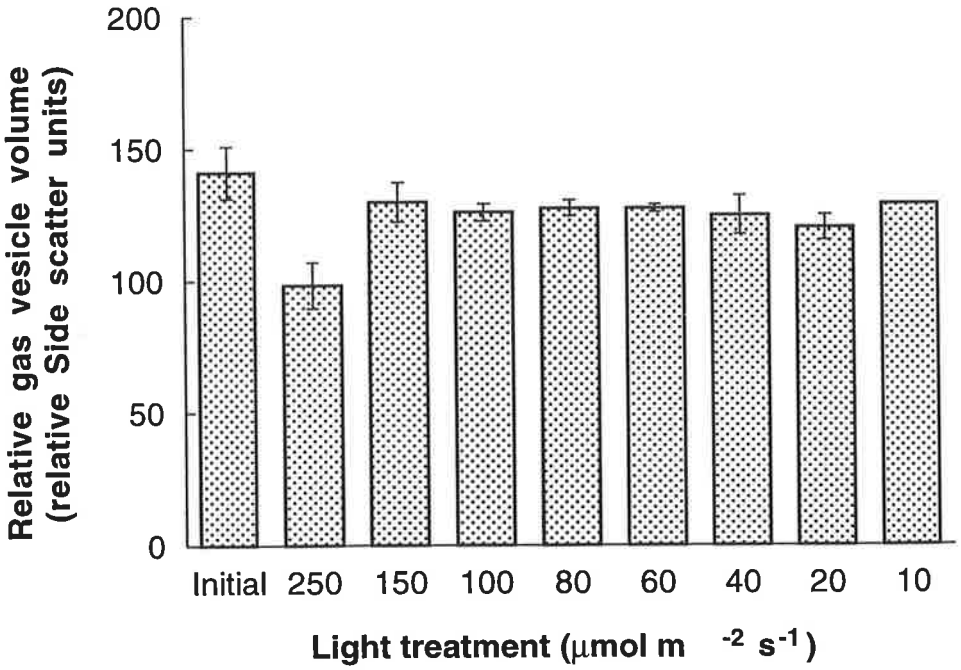


Figure 5.17 The relative gas vesicle volume of *Anabaena circinalis* was measured using a flow cytometer following 24 hours incubation at different light intensities to determine if there was a reduction in the gas vesicle volume due to turgor pressure collapse.

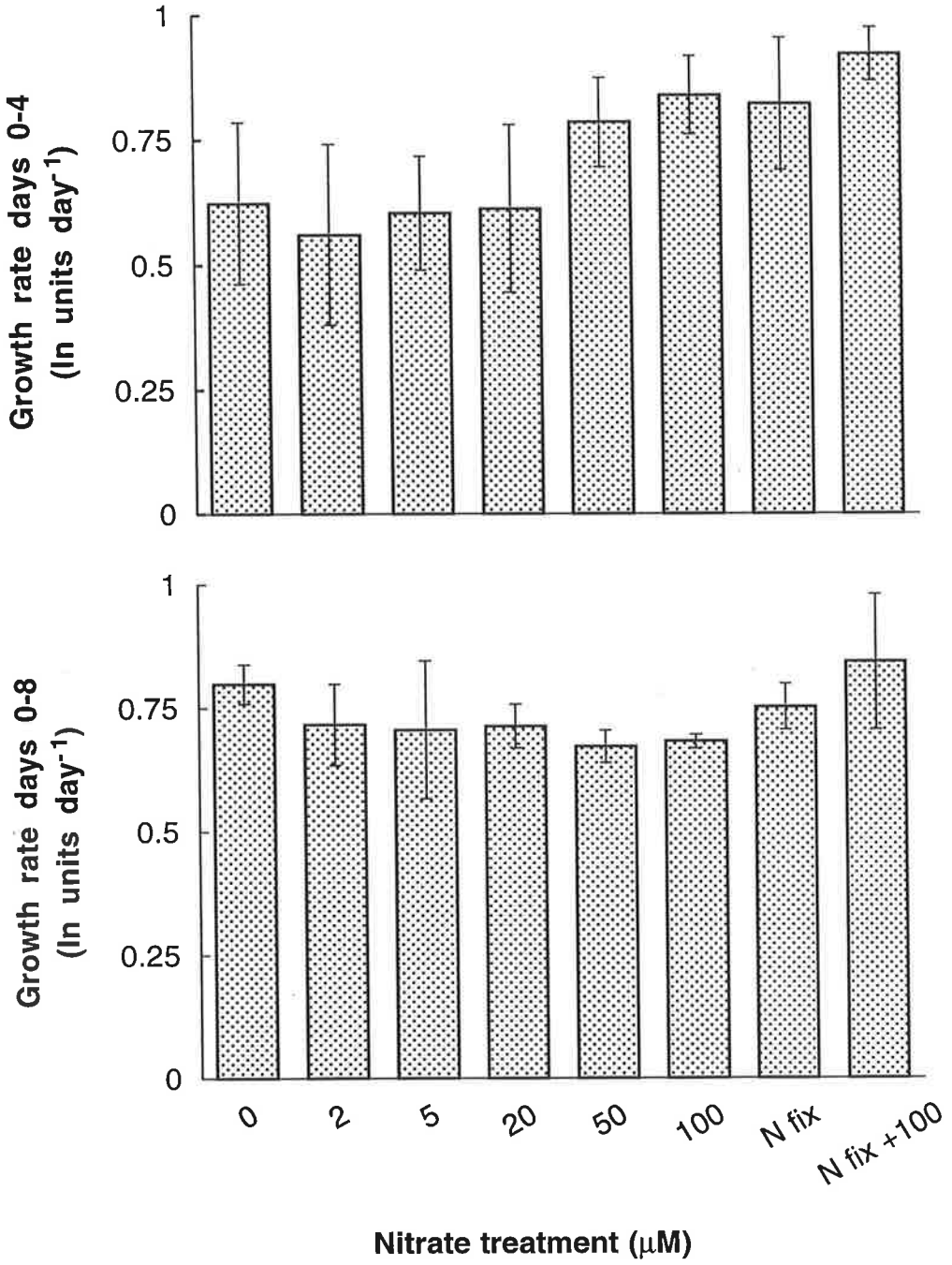


Figure 5.18 *Anabaena circinalis* was grown in different nitrate concentrations in a chamber flushed continuously with synthetic air (composed oxygen , carbon dioxide and argon replacing atmospheric nitrogen). The mean growth rates were measured over the first four days (top graph) and over eight days (top graph). The N fix treatments were exposed to atmospsheric nitrogen and had either no nitrate added (N fix) or 100μM nitrate (N fix+100).

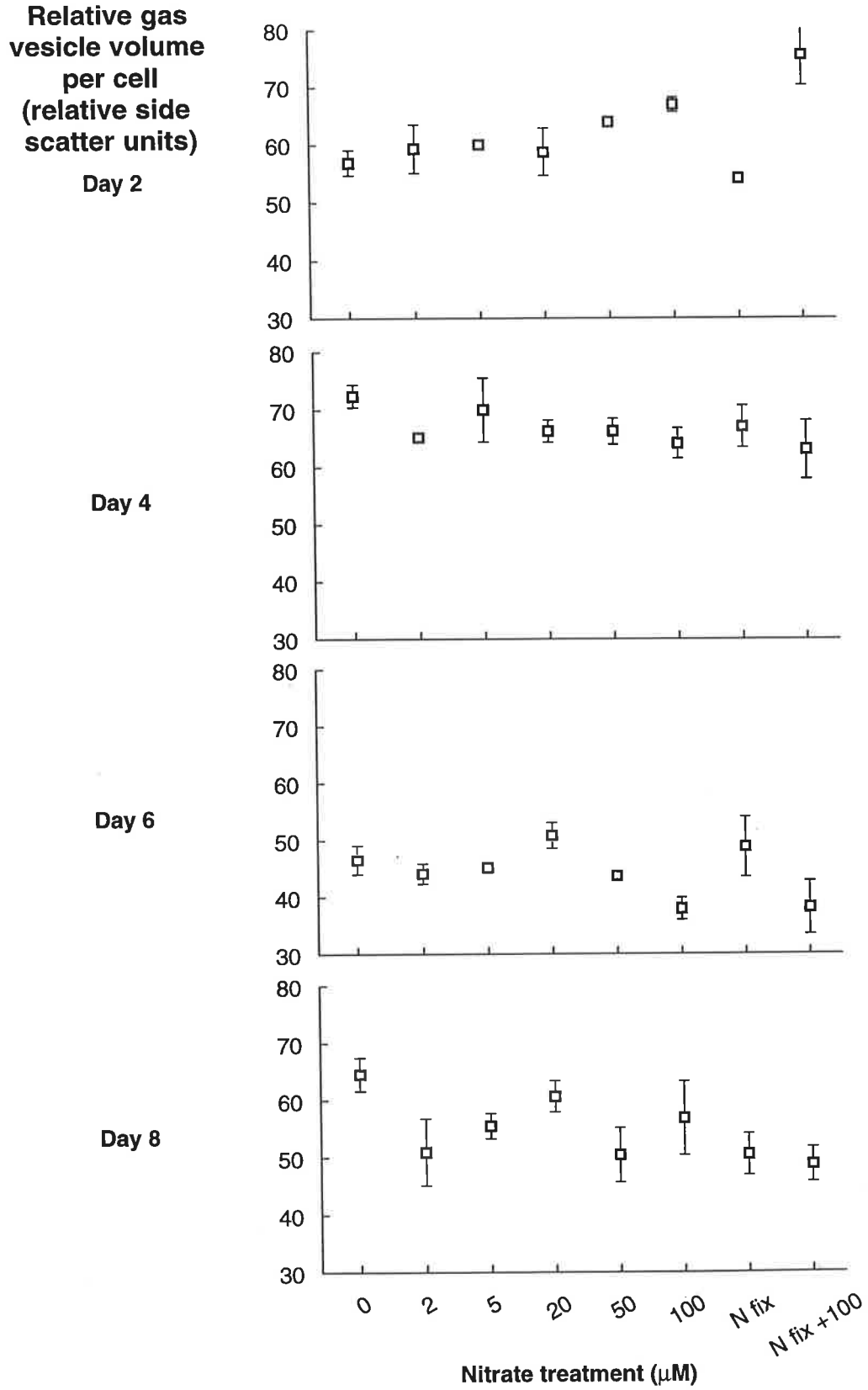


Figure 5.19 Relative gas vesicle volume of *Anabaena circinalis* cultures grown in different nitrate concentrations and flushed with N_2-free synthetic air. N fix treatments were incubated in normal air.

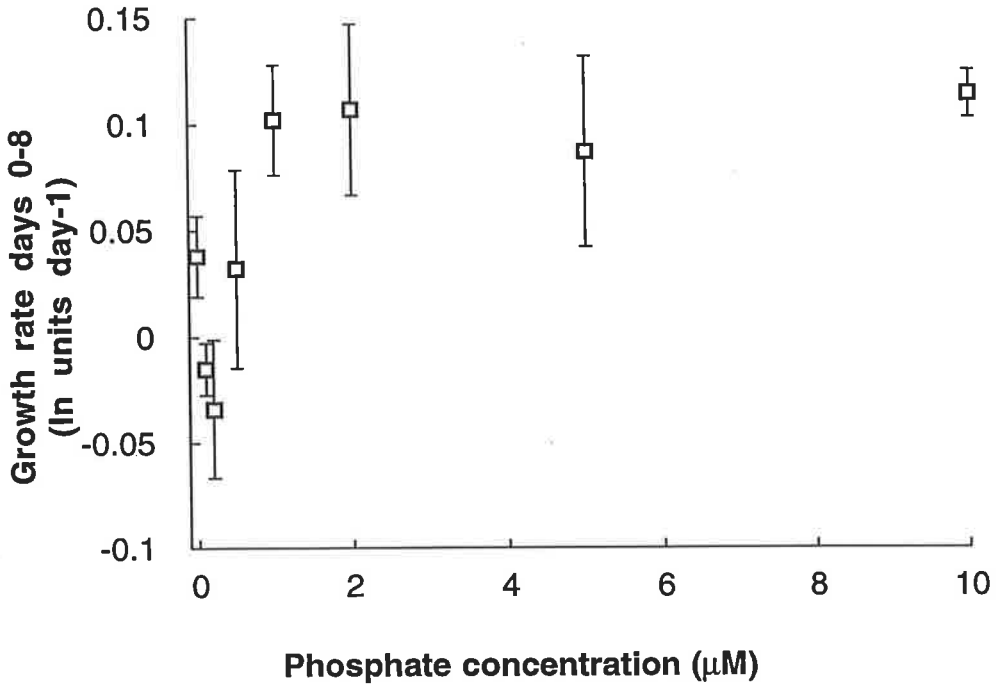


Figure 5.20 Phosphate dependent growth of *Anabaena circinalis*

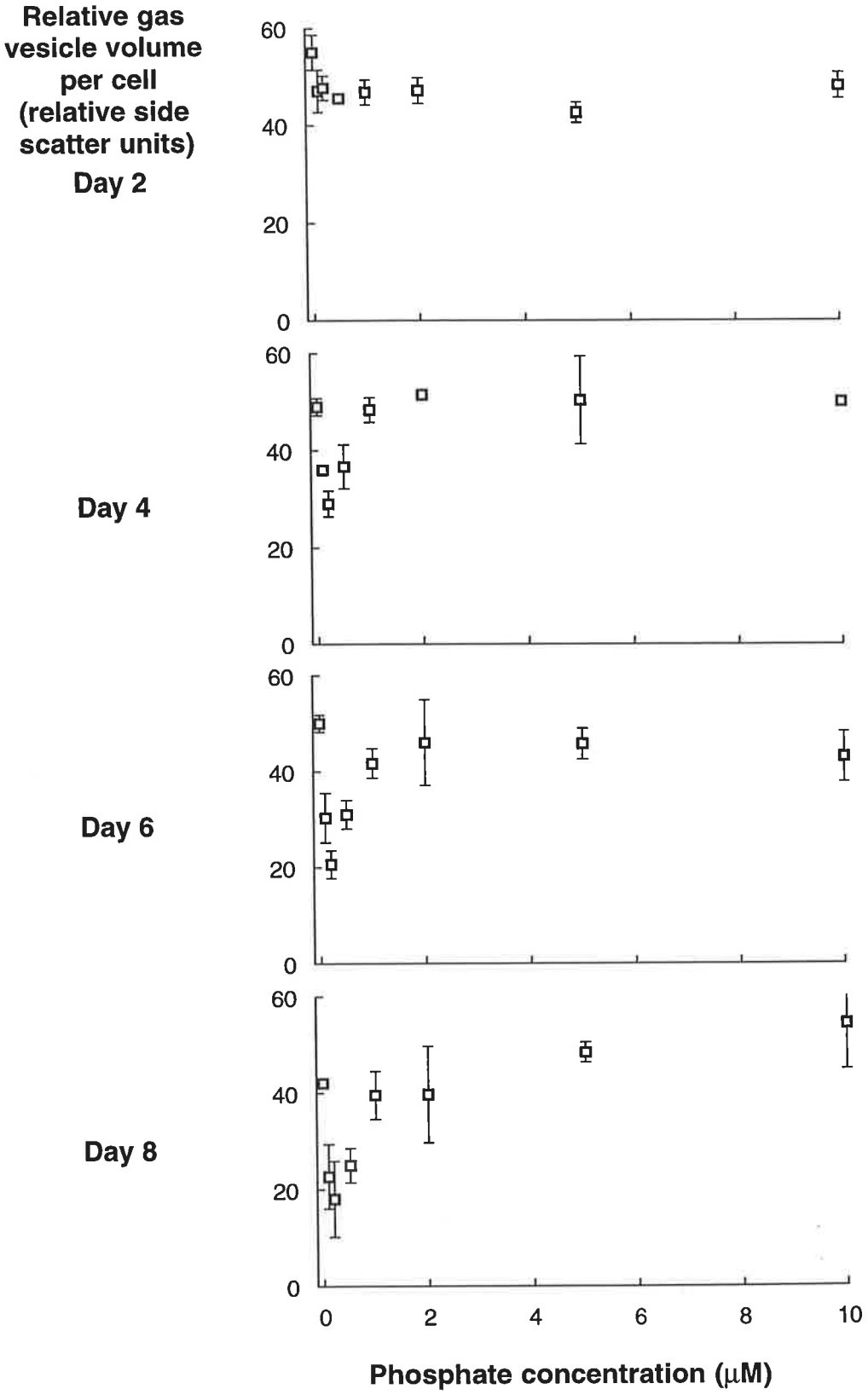


Figure 5.21 *Anabaena circinalis* grown at varying degrees of phosphorus limitation displayed some dilution of the gas vesicle pool per cell due to growth. As phosphate limitation was induced the gas vesicle pool was diluted at lower phosphate concentrations.

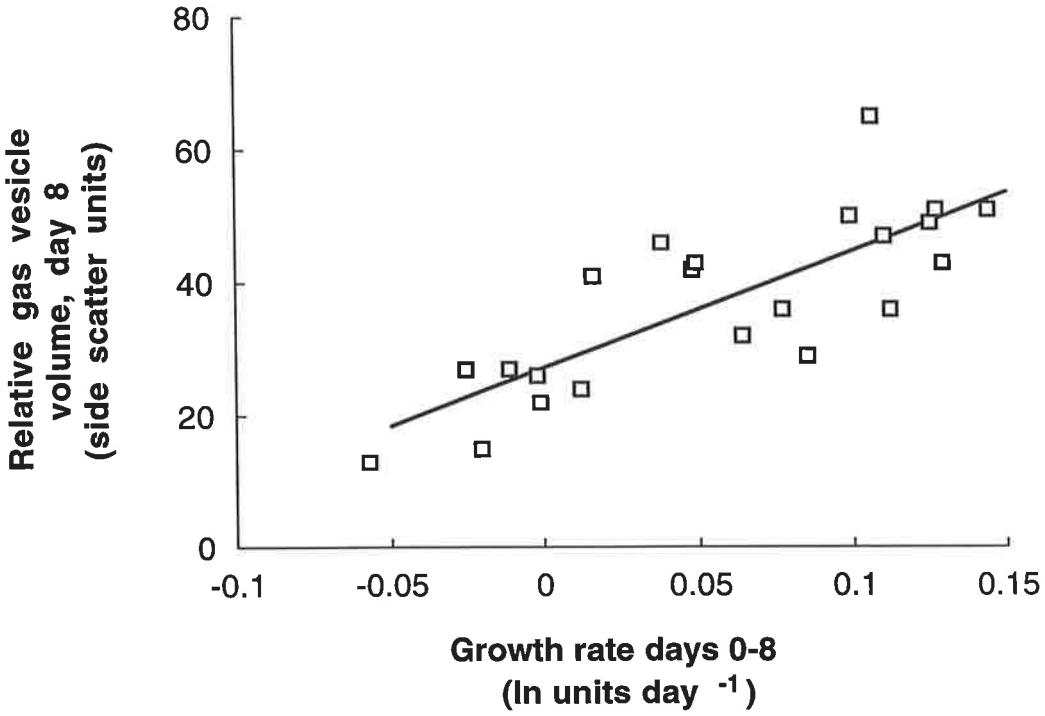


Figure 5.22 The gas vesicle volume of *Anabaena circinalis* (day 8) grown in different phosphate concentrations displayed a linear relationship (r^2=0.64) with the growth rate calculated over the eight day experimental period.

Chapter 6

The influence of nitrogen, phosphorus and light on ballast change and buoyancy regulation in *Microcystis aeruginosa* and *Anabaena circinalis*

6.1 Introduction

6.1.1 Buoyancy regulation

Reynolds and Walsby (1975), Humphries and Lyne (1988) and Shapiro (1990) amongst others have identified buoyancy regulation as an attribute of cyanobacteria which contributes to their dominance during high and late summer. The presence of gas vesicles enables cyanobacteria to persist in the water column when water turbulence is low and other phytoplankton, which rely on water movement to remain entrained in the illuminated surface waters, tend to sediment.

The regulation of buoyancy is a phenomena by which cyanobacteria adjust their net cell density and migrate vertically in the water column. The advantages of employing this strategy include i) overcoming the vertical separation of light, near the surface, and nutrients fluxing from the sediments (Ganf and Oliver, 1982),ii) increasing the volume of water which can be scavenged for nutrients (Reynolds, 1994; Brookes *et al.*, 1997) and iii) the avoidance of very high irradiances which can potentially damage pigments and photosynthetic machinery (Eloff *et al.*, 1976; Vincent *et al.*, 1984; Elser and Kimmel, 1985; Pierson *et al.*, 1994).

Buoyancy regulation can be achieved by three mechanisms. Alterations in the gas vesicle pool, due to either dilution by cell division or collapse by turgor pressure may reduce the gas vesicle pool to a level where buoyancy is overcome by the density of the cellular constituents (previous chapter; Dinsdale and Walsby, 1972; Grant and Walsby, 1977; Oliver and Walsby, 1984; Walsby, 1987; Kinsman *et al.*, 1991; Oliver, 1994; Walsby, 1994). Buoyancy can be regained by the respiratory loss of photosynthates, which reduces turgor, and by the synthesis of new gas vesicles. Turgor induced gas vesicle collapse and subsequent buoyancy regulation is only

evident in cyanobacteria with relatively weak gas vesicles (Oliver and Walsby, 1984; Kinsman *et al.*, 1991).

It is evident from recent literature that the regulation of cellular constituents is a more widely observed buoyancy regulation mechanism (van Rijn and Shilo, 1983, 1985; Kromkamp and Mur, 1984; Thomas and Walsby, 1985; Utkilen *et al.*, 1985a; Kromkamp *et al.*, 1986; Walsby *et al.*, 1987, 1989, 1991; Kromkamp and Walsby, 1990; Ibelings *et al.*, 1991a, b).

6.1.2 Density of cellular constituents

Cell buoyancy is overcome when the density of the cellular constituents exceeds the buoyancy provided by the gas vesicles. Some species of non gas vacuolated eukaryotic algae have densities which range between 1.02 and 1.18 kg m^{-3} (Oliver *et al.*, 1981) and cyanobacteria which have had their gas vesicles collapsed have densities in a similar range (Kromkamp and Mur, 1984; Oliver and Walsby, 1984; Thomas and Walsby, 1985; Ibelings *et al.*, 1991b). The cellular constituents which contribute to cell density are carbohydrate of density 1600 kg m^{-3} , protein (1300 kg m^{-3} ; Oliver and Walsby, 1984), cyanophycin granules (1394 kg m^{-3}), polyphosphate (2170 kg m^{-3}), and glycolipids which have a density of 1050 kg m^{-3} (Jacobson and Halmann, 1982; Thomas and Walsby, 1985; Oliver and Walsby, 1988; Oliver, 1994). Change in the concentration of these components will affect the net cell density. Carbohydrate content of cells changes in response to light and accumulates during nutrient stress. In low nitrogen conditions there may be a reduction in protein and cyanophycin and a similar reduction in polyphosphate when phosphorus is in short supply.

6.1.3 Change in cellular constituents

6.1.3.1 Polyphosphates

To overcome the problem of spasmodic or discontinuous phosphate supply the cyanobacteria have developed a mechanism whereby uptake can exceed immediate demand and phosphate is stored as polyphosphate in the volutin granules (Zaiss, 1978; Romans *et al.*, 1994). Polyphosphate contributes to cell density and displays a daily rhythm in concentration when phosphate is growth limiting. Polyphosphate is degraded during the day in order to enable photosynthetic activities (Zaiss, 1978). During dark periods intermediates of photosynthesis which contain phosphorus are metabolised and the phosphorus is stored as polyphosphate.

Cyanophycin granules are generally considered a nitrogen store and their concentration will be less under nitrogen stress as they are degraded to supply nitrogen to other cell functions.

The regulation of polyphosphate and cyanophycin may affect the net cell density and have implications for buoyancy regulation particularly when the ambient nutrient concentrations and fluxes are low. The relative importance of these compounds in rapidly regulating buoyancy is relatively less compared with the effect of carbohydrate change. For example, the average effects of polyphosphate on increased cell density is approximately 20% of that from carbohydrate accumulation in *Trichodesmium tenue* (Romans *et al.*, 1994).

6.1.3.2 Carbohydrate change and buoyancy regulation in response to light

There are numerous studies which document a decrease in buoyancy in gas vacuolate cyanobacteria exposed to high light (Walsby and Booker, 1980; Walsby *et al.*, 1983; Oliver and Walsby, 1984; Kromkamp and Mur, 1984; Kromkamp *et al.*, 1986; Konopka *et al.*, 1987a; Kromkamp *et al.*, 1988; Konopka, 1989; Spencer and King, 1989).

The prime mechanism of buoyancy regulation in cyanophytes, with gas vesicles too strong to be collapsed by turgor increases, is the accumulation of carbohydrate during photosynthesis and the respiratory consumption in subsequent dark periods (Kromkamp and Mur, 1984). When the density of cellular constituents exceeds the buoyancy provided by gas vesicles the cells become negatively buoyant (Oliver and Walsby, 1984) and sink. Buoyancy is restored when sufficient carbohydrate is consumed to lower the net cell density to a value less than the surrounding medium (ca 1000 kg m^{-3}).

Photosynthesis in cyanobacteria increases in response to light intensity and saturates at relatively low light relative to higher plants (Kirk, 1983; Edwards and Walker, 1983). Numerous models have been used to describe the photosynthesis-irradiance curve (Jassby and Platt, 1976; Henley, 1993; Zonneveld, 1997). The photosynthesis-irradiance curve is often described by a Michaelis-Menten equation (Kromkamp and Walsby, 1990; Davison, 1991; Zonneveld, 1997) and was used in this study to ensure parameters were compatible with other similar models. The rate of carbohydrate fuelled respiration is dependent on the cellular carbohydrate concentration (Olesen and Ganf, 1986) and in strictly controlled culture conditions

is described as linearly related to the previous light history (Kromkamp and Walsby, 1990; Brookes *et al.*, 1997). This is the most simplistic model of carbohydrate change in response to light photosynthesis and dark respiration and does not account for photoadaptation or photoinhibition (Henley, 1993).

Carbon is fixed from dissolved CO_2 and bicarbonate (Talling, 1976; Kirk, 1983) and incorporated into polysaccharide and stored as starch. The most rapid changes in the cellular carbohydrate content occur in the soluble fraction as carbon is fixed in response to light, utilised to assimilate nitrogen and respired to provide carbon skeletons for amino acids. It is changes in this fraction which give rise to the observed buoyancy regulation (Kromkamp and Walsby, 1990).

6.1.4 Effect of nutrients on photosynthesis and buoyancy regulation

It has been demonstrated that both nitrogen and phosphorus affect buoyancy in cyanobacteria (Klemer *et al.*, 1982; Konopka *et al.*, 1987a, b and others). This chapter deals primarily with changes in ballast in response to light and nutrients and so only papers relevant to that theme will be reviewed here. The previous chapter dealt with gas vesicle volume in response to light and nutrients and the following chapter will deal with the interaction between gas vacuolation, ballast change and buoyancy status in nutrient and light limited cultures.

Limitation of either nitrogen or phosphorus results in the accumulation of polysaccharide as energy captured exceeds the cell's requirements for growth and is stored, decreasing buoyancy (Konopka *et al.*, 1987a, b: Oliver, 1994). Nitrogen limitation affects both the cell's photophysiology (Fresnedo and Serra, 1992; Geider *et al.*, 1993) and the storage and utilisation of carbon (Turpin, 1991). Nitrogen limitation can affect both the apparent absorption cross section of Photosystem II and the efficiency of energy transfer from the light harvesting complex to the reaction centre II (Turpin, 1991).

Turpin (1991) in a review on the effects of inorganic nitrogen availability on algal photosynthesis and carbon metabolism cites numerous references which claim that nitrogen deficiency by cyanobacteria causes little change in the chlorophyll *a* content per cell but large reductions in phycobilin. This is contrary to reports in this thesis where nitrogen limitation

drastically reduced the chlorophyll content of *Microcystis* cells (previous chapter). A similar response to N-limitation was reported by Fresnedo and Serra (1992) working on *Phormidium laminosum*. Consequently one would predict both a decrease in the density of PS II in nitrogen limited cyanobacteria and a greater decrease in the antenna than would be predicted by a reduction in phycobilin alone. These factors act to decrease energy capture and utilisation by cyanobacteria and consequently impact on buoyancy regulation.

Nitrogen availability also affects carbohydrate metabolism and storage. When nutrients are in ready supply the endogenous carbohydrate stores decline and recent photosynthate is used in nitrogen assimilation and the provision of carbon skeletons for protein synthesis (Turpin, 1991). However, when cells are nitrogen limited they accumulate starch. Cells which are nutrient limited accumulate polysaccharide and tend to sink deeper in the water column where there is greater opportunity to scavenge for nutrients fluxing from sediments. Nitrogen limited cells readily take up NH_4^+ and can degrade the stored starch to provide reductant for nitrogen assimilation and inclusion into proteins, including gas vesicle protein, which would act to restore buoyancy. Cyanobacteria displayed greater buoyancy when nitrogen was available than when they were removed from the nitrogen source (van Rijn and Shilo, 1983, 1985).

6.1.5 Evidence of buoyancy regulation in the field

Buoyancy regulation allows vertical migration during periods of low turbulence, however the advantage of buoyancy regulation would often be over ridden by flow or wind induced turbulence in the mixed layer (Ganf, 1974; Imberger and Hamblin, 1982; Imberger, 1985; Spigel and Imberger, 1987; Humphries and Lyne, 1988; Webster, 1990; Kohler, 1992; MacIntyre, 1993; Hutchinson and Webster, 1994; Oliver, 1994; Webster and Hutchinson, 1994; Visser *et al.*, 1996). Many of the observations of buoyancy regulation in response to light have been in laboratory cultures.

Buoyancy regulation in a field population of *Microcystis aeruginosa* and *Microcystis flos-aquae* was observed by Ganf (1974) in Lake George, Uganda. He found that during periods of intense thermal stratification there was a significant reduction in the number of colonies at the surface but an increased concentration at depth. Smaller filamentous species such as *Lyngbya* spp and *Anabaenopsis* displayed a similar vertical redistribution which was not as marked as

for *Microcystis*. This could have been due a smaller greatest axial linear dimension (GALD, Reynolds, 1984) or a greater relative buoyancy giving rise to a slower sinking velocity. Ibelings *et al.* (1991a, b) identified buoyancy regulation in their strain of *Microcystis aeruginosa* as did van Rijn and Shilo (1985) who also demonstrated that *Oscillatoria* and *Spirulina* lost buoyancy when exposed to light. *Oscillatoria* has been shown to regulate its buoyancy in field populations and develop deep water maxima (Walsby *et al.*, 1983; Utkilen *et al.*, 1985b; Reynolds *et al.*, 1987).

Some populations of *Anabaena* species have displayed persistent buoyancy in New Zealand (Walsby *et al.*, 1987, 1989) and in the United Kingdom (Walsby *et al.*, 1991). In the New Zealand population of *Anabaena minutissima* there was little evidence of buoyancy regulation in natural conditions but when a sample was incubated at the surface there was loss of buoyancy (Walsby *et al.*, 1989). This may indicate that small scale turbulence was mixing the algal population and the average irradiance they received was not sufficient for carbohydrate accumulation to increase the cell density to a level where buoyancy was lost. On the other hand *Anabaena lemmermannii* was persistently buoyant even when maintained at high light intensity because the colonies were not able to accumulate sufficient carbohydrate in a day to overcome the excess buoyancy provided by the gas vesicles (Walsby *et al.*, 1991).

6.1.6 Factors affecting buoyancy regulation

6.1.6.1 Turbidity

The rate of buoyancy change and subsequent vertical migration is dependent upon the photon flux density the cell experiences. The efficiency of energy use depends on the cell photophysiology and metabolic state. The irradiance the cell receives is governed by the cells position in the water column and the optical properties of the water. Light is attenuated exponentially with depth as it is absorbed by particulates, gilvin, phytoplankton and water (Kirk, 1983; Ganf *et al.*, 1989).

As a cell receives light it photosynthesises at a rate determined by its specific physiology and the incident irradiance. The accumulation of polysaccharide during photosynthesis increases the net density of the cell and the buoyancy provided by the gas vesicles may be overcome. As a cell sinks deeper in the water the light it receives is reduced and consequently the rate of

photosynthesis and carbohydrate accumulation is reduced. Below the depth at which the light intensity corresponds to the compensation irradiance there is a net loss of carbohydrate. When carbohydrate levels are such that there is positive buoyancy the cell will rise again. The depth to which a cell migrates is dependent on the rate of carbohydrate accumulation and loss and thus turbidity and light penetration impact heavily on vertical migration (Kromkamp and Walsby, 1990).

Coupled with light attenuation is a shift in the spectral composition of the light (Kirk, 1983; Ganf *et al.*, 1989). Cyanobacteria have evolved secondary pigments, the phycobiliproteins, to maximise light absorption at particular wavelengths (Tandeau de Marsac, 1977; Tandeau de Marsac and Houmard, 1988; Falkowski and La Roche, 1991; Ganf *et al.*, 1991; Ohki and Fujita, 1992). There is generally a reduction in phycobilin concentrations under nutrient limitation so when nutrients are in short supply light capture and buoyancy regulation in turbid waters will be further impacted.

6.1.6.2 Factors governing sinking velocity- Stoke's law

The cell/colony/filament will sink or rise at a velocity governed by its density relative to the surrounding media and particle size. Velocity is approximated by Stoke's law modified to incorporate a coefficient of form resistance which accounts for non-spherical colony forms (Oliver *et al.*, 1981; Davey and Walsby, 1985, Reynolds, 1987; Oliver, 1994). At colony radii greater than 300 μm the colony experiences more turbulent drag and the observed velocities depart from those predicted by Stoke's equation (Reynolds, 1987). In this study Stoke's equation was used in modelling phytoplankton movement with no correction term for increased drag when particle size exceeds 300 μm. Discrepancies and assumptions are inevitable in ecosystem modelling but an awareness of where they exist aids in interpretation of the results. Stokes equation is described in the methods section under model parameters.

6.1.7 Modelling buoyancy regulation and vertical migration

In order to visualise what impact nutrients and light have on buoyancy regulation and vertical migration in a water column it is advantageous to model phytoplankton response to the physical environment. Kromkamp and Walsby (1990) created a computer model to simulate buoyancy regulation in phytoplankton. This simple model has been the basis for more complex models

including chaotic solutions, wind induced turbulence and growth (Sanderson *et al.*, 1992; Howard, 1993; Howard *et al.*, 1995, 1996). The Kromkamp and Walsby (1990) model was used in this study but the parameters for density change in response to light for *Microcystis* in different nutrient conditions and *Anabaena circinalis* are compatible for inclusion into the more complex models.

Physical parameters in the model include diel solar irradiance change and light attenuation by the water body to estimate irradiance at each depth and calculation of density change in response to that irradiance. The density of the cyanobacterial colony and a sinking or floating velocity is then recalculated. The periodicity of calculation was five minutes and simulation run time 24 hours. For each five minute interval the new position was estimated and irradiance, density change and sinking velocity calculated.

6.1.8 Experimental aims and approaches

The objective of this research was to investigate what effect nitrogen and phosphorus limitation had upon density change in *Microcystis* exposed to a range of light intensities. This information was used to model vertical migration and determine what size colonies were best able to overcome the vertical separation of light and nutrients in a water column during thermal stratification. It was found that nitrogen limitation significantly reduced the rate of density change whereas a reduction in the phosphorus concentration did not have such a severe impact. The buoyancy and density change of *Anabaena circinalis* was also examined to identify whether this species displayed a similar buoyancy mechanism to *Microcystis aeruginosa*. Although *Anabaena* changed its density in response to light it did not accumulate sufficient carbohydrate to overcome the excess buoyancy provided by the gas vesicles.

The nutrient uptake kinetics of cells differ depending on the cell's nutrient status with nutrient limited cells generally exhibiting higher affinity for the limiting nutrient (Morel, 1987; Kromkamp *et al.*, 1989). The phosphorus uptake kinetics of *Microcystis* were examined in order to place nutrient limited buoyancy regulation into context with cell physiology and the potential of a cell to scavenge for nutrients. Phosphate limited cultures had an uptake rate approximately ten times higher than cells in growth saturating concentrations.

6.1.9 Theory of optimal size for buoyancy regulation

Different phytoplankton species tend to favour certain habitats because they have functional attributes which enable them to exploit that habitat better than other species (Seip and Reynolds, 1995). Features of a habitat, such as turbidity, turbulence and nutrients, may vary seasonally favouring different phytoplankton groups and forcing a shift in the community composition, loosely termed seasonal succession. Reynolds (1984b) states that there are "sets of adaptive specialisations among the phytoplankton; each has evolved to suit particular characteristics of water bodies but none is ideal under all circumstances".

Within a species there may also be features of individuals or colonies which provide them with an advantage over other colonies under certain conditions. Colony size is a morphological feature of cyanobacteria which shows variability (Duarte *et al.*, 1990; Smith and Gilbert, 1995). Nutrient uptake, light capture, grazing pressures and velocity of vertical movement are affected by colony size. Small colonies are favoured when nutrients are sparse because of less diffusion limitation of nutrients into the colony centre and a lower nutrient subsistence quota (Shuter, 1978). Small colonies also limit "self-shading" of centre cells whereas larger colonies restrict light to inner colony cells, known as the "package effect" (Kirk, 1983). To some extent this is reduced by decreasing the chlorophyll content of outer cells and increasing the distance between neighbouring cells (Augusti and Philps, 1992). Large colonies are favoured over small colonies with respect to grazing losses as large colonies are excluded from the diet of herbivorous invertebrates (Holm *et al.*, 1983).

There may also be an optimal size for buoyancy regulation. Size is recognised in Stoke's law as a factor affecting the sinking or floating velocity of particles. Although buoyant microalgae flourish during summer months, when there is greater stability in the water column, thermal stratification is not absolute during this time and there may be wind or flow induced mixing events. Therefore in summer months the phytoplankton assemblage may encounter thermal stratification, intermittent or diurnal stratification or extended periods of mixing. An optimal size may exist for effective buoyancy regulation during persistent stratification and redistribution into the euphotic zone following a mixing event.

In this chapter the theoretical optimal size for effective buoyancy regulation by *Microcystis* is examined. The criteria for effective buoyancy regulation in this model were: i) the colony had to migrate a reasonable distance in the water column to maximise the available nutrient pool; ii) the average light intensity had to be greater than that necessary to saturate photosynthesis and growth; iii) it was assumed that there was a vertical separation of light and nutrients and that nutrient scavenging increased with time spent below the euphotic zone in higher nutrient concentrations. Numerous size classes were modelled in the Kromkamp and Walsby (1990) model to determine the theoretical optimal size in a water body with a light attenuation coefficient of 2 m^{-1}.

In intermittently mixed environments the survival and future production of individual colonies depends on their ability to position themselves in a favourable light climate when water stability reestablishes. Negatively buoyant species will tend to sink but small buoyant microalgae could also face losses if they do not reach the euphotic zone before their cellular stores became exhausted. In this chapter vertical redistribution of different sized colonies following a mixing event was simulated to examine what effect colony size had on floating velocity, vertical migration and the probability of reaching a favourable light climate before cell activity was impaired.

6.2 Methods

6.2.1 Cultures

The cultures used in this series of experiments were *Microcystis aeruginosa* SHEP 100 and *Anabaena circinalis* ANA330E grown in ASM-1 and WC media, respectively, as described in chapter 5.

6.2.2 Density change of nitrogen and phosphorus limited *Microcystis* cultures in response to light

Density change in response to light was examined for nitrogen and phosphorus limited *Microcystis* cultures and compared with cultures with adequate nutrients. Phosphate limited cultures were prepared by inoculating a log phase culture grown in complete ASM-1 media at

40 μmol m^{-2} s^{-1} 15:9 light:dark cycle, 25°C, into phosphate-free ASM-1. Cells remained in the phosphate-free environment for seven days to deplete the polyphosphate pool and induce a phosphate limitation. On day seven following inoculation the culture was placed in the dark for 16 hours to reduce the carbohydrate store before being subdivided into 15 flasks which were placed at five light intensities 152, 93, 60, 38 and 0 μmol m^{-2} s^{-1}, with three replicates at each intensity; temperature was maintained at a constant 25°C. Samples were taken for density measurements at hourly intervals during a five hour light incubation and subsequent four hour dark incubation.

Density was measured using Percoll density gradient centrifugation following collapse of gas vesicles using an adapted method of Oliver *et al.*, (1981). The density at which the algae banded was estimated by measuring the refractive index of the Percoll at that density and calibrating the Percoll gradient with beads of known density (Pharmacia Density marker beads; code no. 17-0459-01).

A similar method was employed to investigate density change in nitrogen-limited *Microcystis* cultures in response to light. Log-phase *Microcystis* cultures, grown in similar culture conditions to those described above, were inoculated into nitrogen free ASM-1 media and incubated for three days to induce a nitrogen limitation. Cells were then placed in darkness for 16 hours to reduce the stored carbohydrate then triplicate samples exposed to 250, 150, 80, 50, or 20 μmol m^{-2} s^{-1}. Density of the *Microcystis* cells was measured bihourly using the Percoll density gradient method (Oliver *et al.*, 1981) for a 10 hour light period at which point the lights were turned off and density decrease in the dark measured bihourly for six hours

Previous work has found a linear relationship between carbohydrate and density increase (Kromkamp and Walsby, 1990; Brookes *et al.*, 1997) in the light and subsequent decrease in the dark.

Density increase in light and decrease in the dark are linear and the rates were calculated using least squares linear regression. The rate of density change was then plotted against the irradiance or previous irradiance in the case of the dark treatments. From these plots rate constants were estimated for inclusion into a computer simulation of buoyancy regulation and

vertical migration developed by Kromkamp and Walsby (1990). The determination of these constants is discussed later in a description of model parameters.

The Kromkamp and Walsby (1990) model was extended to simulate vertical migration of colonies over 5 days. The parameters describing the physical conditions remained the same for each day but the starting depth and density of the colony at the beginning of each day was made equal to the final value from the previous day thus making the model continuous. Simulations were performed describing vertical migration of different sized *Microcystis* colonies (nutrient replete) following a mixing event which evenly distributed the colonies before the onset of thermal stability.

6.2.3 Buoyancy of *Anabaena circinalis*

To examine the buoyancy characteristics of *Anabaena circinalis*, a log phase culture grown at 40 μmol m^{-2} s^{-1} (15:9 light dark cycle, 25°C) was placed in darkness to reduce the carbohydrate pool and then triplicate flasks containing culture were placed at 300, 150, 80, 50 or 20 μmol m^{-2} s^{-1}. Samples were taken bihourly for a ten hour light exposure and a subsequent six hour dark period.

Samples were also taken to estimate the proportion of cells sinking or floating. This was done by placing 1 mL of culture suspension into a Sedgewick-Rafter chamber, allowing the cells to sink or float and measuring the number which had float to the plane just under the coverslip or sunk to the base of the chamber (Walsby and Booker, 1980). Results were expressed as percentage of cells floating.

6.2.4 Phosphate uptake by *Microcystis aeruginosa*

To examine the phosphorus uptake kinetics of *Microcystis aeruginosa* a time course and concentration dependence of uptake were undertaken followed by a comparison of phosphorus uptake by phosphate deplete and replete cultures.

6.2.4.1 Time course of Phosphorus uptake

A time course of phosphorus uptake was conducted by depriving *Microcystis* cells of phosphate for seven days, as described above, and using radioactive ^{32}P to measure phosphate influx. Following seven days in P free media *Microcystis* cells were pressurised to 1000 kPa

to collapse the gas vesicles (Brookes *et al.*, 1994) and resuspended in a simple buffered medium containing 2 mM EPPS, 0.75 mM CaOH, 0.1 mM KCl and pH adjusted to pH 8. 500 µL of culture solution was added to 4.5 mL of buffer solution containing 10 µM radioactively labelled phosphate which had a specific activity of 10000 counts min^{-1} $nmol^{-1}$. At set time intervals 0.5 mL of this solution was sampled and rinsed for 0.5 minutes with buffer solution on a 0.22 µm GS filter. Samples were then measured for phosphate influx using a scintillation counter. All uptake experiments were performed at 70 µmol m^{-2} s^{-1}, 22°C.

6.2.4.2 Concentration dependence of Phosphate influx

Concentration dependence was examined using a ten minute uptake time and the following radioactively labelled phosphate concentrations in buffer solution as described above; 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 µM P. Cells were rinsed with buffer solution for 0.5 minutes prior to counting on a scintillation counter.

6.2.4.3 Uptake by P-starved and P-sufficient Microcystis

The rate of uptake of phosphate by cells grown in growth saturating phosphate concentrations and cells deprived of phosphorus were compared to examine differential affinity for phosphorus. The nutrient sufficient population was grown in complete ASM-1 media and the P-limited culture in phosphate free ASM-1 as described above.

An estimation of internal inorganic phosphate was made on P-deplete and P-replete *Microcystis* by rinsing cells (centrifugation and resuspended in buffer solution) and boiling 400 µL of cells in buffer for 30 min. 900 µL of Bencini reagent (Bencini *et al.*, 1983) was then added before measuring the absorption at 350 nm and calculating inorganic phosphorus from a standard curve prepared using phosphate standards between 0 and 200 µM (Abs 350 nm = -0.0043+0.00206*Phosphate concentration; r^2=0.999)

6.2.5 Model Parameters

6.2.5.1 Equations describing density change with time in cyanobacteria

The rate of density increase with irradiance can be approximated by the equation.

$$dp/dt = C_1\left[\frac{I}{(K_I + I)}\right] - C_3$$

where dp/dt is the change in density with time (kg m-3 min-1), c_1 is the coefficient determining the increase in density with time (kg m-3 min-1), K_I is the irradiance at which the rate of density increase with time is half the maximal rate , I is the irradiance (µmol photon m-2s-1) and c_3 is the minimal rate of density decrease (corresponding to the basal rate of respiration).

The rate of density decrease following light incubation was a linear function dependent on the previous irradiance (I_a).

$$dp/dt = C_2 I_a - C_3$$

where c_2 is the light dependent rate coefficient describing the change in density with time [kg m-3 min-1 (µmol m-2s-1)-1] (Kromkamp and Walsby, 1990).

6.2.5.2 Equations describing model parameters

The light intensity cyanobacteria experience diurnally is determined by the surface irradiance, the attenuation coefficient of the water body and the depth of the colony.

The surface irradiance (I_o) can be described as a sine function of the maximum irradiance at noon (I_m):

$$I_o = I_m \sin\left(\frac{\pi}{D_L}\right)$$

equation 1

where D_L is the length of the light period from dawn to dusk.

As the surface irradiance penetrates the water column it is attenuated by gilvin, particulates and water. The irradiance a cyanobacterial colony experiences at depth (z) is calculated using the surface irradiance:

$$I_z = I_o e^{-kz}$$

equation 2

where k is the average downwelling attenuation coefficient (m^{-1}) and z is the depth (units are metres).

In the model the irradiance a cell experiences is calculated every 5 minutes. At depth z_1 the irradiance is I_{z1} at depth z_2 the irradiance is I_{z2}. To minimise the effect of step sizes the change in cell density is determined using the mean irradiance between two steps.

$$\overline{I_z} = \frac{(I_{z2} - I_{z1})}{(\ln I_{z2} - \ln I_{z1})}$$

equation 3

(Kromkamp and Walsby, 1990)

Colonies exposed to light photosynthesise and increase their density. The rate of density change of cyanobacterial cells is dependent upon the light intensity they experience.

The equation describing the light - dependent rate of density increase is:

$$dp/dt = C_1 \left| \frac{I}{(K_I + I)} \right| - C_3$$

equation 4

where dp/dt is the change in density, C_1, C_3 and K_I are rate constants .

Cell density is reduced by the consumption of accumulated carbohydrate. The equation describing the decrease in density with time dependent upon the previous light history of the colony is given by the equation.

$$dp/dt = C_2 I_a - C_3$$

equation 5

where I_a is the average irradiance and C_2 and C_3 are constants. A cyanobacterial colony at a certain depth (z) will increase density at a rate determined by its present irradiance and decrease density at a rate dependent on the irradiance it last experienced. A colony with an initial density p_1, exposed to an irradiance I_z at depth z for a time interval T will experience a change in density and the new density p_2 can be calculated by the equation.

$$p_2 = p_1 + f_1(p, I_z) - f_2(p, I_a) - C_3$$

equation 6

where p_1 is the previous density, $f1$ (p,I_z) describes the light-dependent increase in density with time given by equation 4. $f2$ (p,I_a) describes the decrease in density with time dependent on the previous averaged irradiance (I_a) using the linear rate constant derived in equation 5.

Therefore, the equation describing the new density is:

$$p_2 = p_1 + T\left\{C_1\left\lfloor\frac{\overline{I_z}}{(K_I + \overline{I_z})}\right\rfloor - C_2 I_a - C_3\right\}$$

equation 7

This initial value of irradiance is used in the calculation of the new density only as long as the colony remains at an irradiance greater than the compensation irradiance, I_c (ca. 0.5% of the mean surface irradiance over a day). If the colony sinks to a depth where the irradiance is less than I_c the I_a is replaced with a new value I_{a1} which is the integrated mean irradiance (I_z) which the colony has experienced since dawn. If the colony rises to a depth where $I_Z > I_C$ the previous irradiance is recalculated. The final value of I_a accumulated from dawn to dusk is used as the starting value for the following day.

To determine I_a the mean surface irradiance from dawn to dusk (I_o) is determined from the equation.

$$\overline{I_o} = I_m D_L/2 \int_0^{0.5D_l} \sin\left(\pi t/D_L\right)$$

equation 8

The mean irradiance I_d, at the bottom of the mixed depth z_d, is then calculated using the equation

$$I_d = \overline{I_o} e^{-kz}$$

(derived from equation 2)

Equation 3 is then used to calculate the average irradiance over depth and time (I_a); $\overline{I}_0$ is substituted for I_{z2} and I_d for I_{z1}.

The new density (p_2) calculated in equation 7 is the used to calculate the sinking velocity using Stoke's equation.

$$v = \frac{2gr^2(p_c - p')A}{9\phi n}$$

equation 9

where g is the gravitational acceleration, r the effective radius of the colony, p_c and p' the densities of the cyanobacteria and water, respectively. A is the proportion of cell volume relative to colony volume i.e. the packing efficiency, Ø the form resistance and n the viscosity of water. The form resistance is expressed as a dimensionless coefficient which describes the effect shape has on sinking rate. Spherical shapes have a form resistance of 1 and more elongate forms about 1.3 Plates and cylinder have much higher form resistances (2.3 - 5.5; Reynolds 1984). *Microcystis* is approximately spherical and has a form resistance of about 1.1

Water viscosity (n) is dependent upon water temperature. The temperatures at the surface, the bottom of the epilimnion and the bottom of the metalimnion can be fed into the model and the viscosity calculated using the equation.

$$n = \frac{\left\{10\exp\left[-1.65 + \frac{262}{(T+139)}\right]\right\}}{1000}$$

equation 10

However viscosity did not appear to significantly change the simulations and was excluded from the spreadsheet model (pers. comm. Jacco Kromkamp).

The new velocity from Stoke's law (equation 9) is used to calculate how far the colony sinks or floats during the time interval P.

$$z_2 = vP + z_1$$

equation 11

6.3 Results

6.3.1 Density change of nitrogen and phosphorus limited *Microcystis* cultures in response to light

Microcystis, which was either nutrient saturated, nitrogen limited or phosphorus limited, was exposed to different light intensities and the density change measured using Percoll density gradients. The rate of density change at each light intensity and in the subsequent dark period was calculated using least squares linear regression and plotted against the irradiance or previous irradiance (for dark measurements). The rate of density change for the phosphate-limited cultures was slightly faster relative to the nutrient saturated population (figure 6.1) for all light intensities. The maximum rate of density change for P-limited *Microcystis* was 11.18 kg m^{-3} h^{-1}, for the nutrient replete culture 10.02 and the N-limited culture 2.02 kg m^{-3} h^{-1}. The subsequent rate of density decrease in the dark was also greatest for the phosphate limited culture. Nitrogen limited *Microcystis* displayed the slowest rate of density change at all light intensities which indicated that the photosynthetic system was impaired and carbon fixation and carbohydrate accumulation reduced. The significance of these nutrient induced differences in the interpretation of field populations will be examined later via an extension of the Kromkamp and Walsby (1990) model.

6.3.2 Calculating the rate of density change in the dark and light

The rate constants describing the rate of density change in the light and dark were calculated directly from the graphs using equations 4 and 5 and are given in table 6.1.

Constant	*Microcystis* Nutrient sufficient	*Microcystis* N-limited	*Microcystis* P-limited	*Anabaena circinalis*
c_1	0.2081	0.04577	0.273	0.115
c_2	$2*10^{-4}$	$5.55*10^{-5}$	$8.133*10^{-4}$	$1.67*10^{-4}$
c_3	0.0415	0.0283	0.0405	0.0167
K_I	65	50	48	44

Table 6.1 Rate constants for inclusion into the Kromkamp and Walsby (1990) model of vertical migration for *Microcystis* cultures which are either nutrient replete, nitrogen or phosphorus limited. Constants are also included for the filamentous, nitrogen fixing cyanobacteria *Anabaena circinalis*.

6.3.3 Modelling vertical migration of N-limited, P-limited and nutrient replete *Microcystis aeruginosa*

To compare the vertical migration patterns of nutrient replete, nitrogen limited and phosphorus limited *Microcystis*, computer simulations of vertical migration were run using the constants in table 6.1. The model parameters describing colony size, light, starting density as well as the physical environment were: light attenuation coefficient k=2 m^{-1}; form resistance =1.1; maximum surface irradiance =1000 µmol $m^{-2}s^{-1}$; packing efficiency A=0.1; light period =13 hours; starting depth =0 metres; starting density = 980 kg m^{-3}; density range = 970 - 1100 kg m^{-3}. The colonies shown in figure 6.2 were radius 200 µm. In these computer simulations differences in gas vesicle volume were not accounted for. The interaction between gas vesicle volume and ballast will be investigated in chapter seven but results will be discussed in this chapter in context with the effect of gas vesicle volume.

Nitrogen limited *Microcystis* cells had the slowest rate of density change (figure 6.1) and were unable to accumulate sufficient carbohydrate in a day to over come the buoyancy provided by the gas vesicles and the colony remained at the surface (figure 6.2). The phosphate limited colony at the surface received light and was able to photosynthesise and accumulate sufficient carbohydrate to reduce the net cell density to a density less than water and the cell sank. As the colony moved vertically downwards the cell experienced lower light intensities and the rate of carbohydrate accumulation was less. At the depth corresponding to the compensation irradiance respiration exceeded photosynthesis and there was a net loss of density until buoyancy was restored and the colony migrated to the surface. *Microcystis* which had a growth-saturating nutrient supply followed a similar migratory pattern but stayed at the surface longer and migrated slightly deeper.

6.3.4 The effect of colony size on vertical migration and optimising light and nutrient capture

Buoyancy regulation and vertical migration are recognised as features of cyanobacteria which allow them to overcome the vertical separation of light and nutrients (Ganf and Oliver, 1982). Therefore, it could be postulated that features of an effective vertical migration must include maximising opportunities to capture light and nutrients which will in turn determine population

maintenance and growth. Colony size is one factor which affects sinking velocity and vertical migration. For the purposes of identifying what colony size is optimal to satisfy light and nutrient capture theoretical guidelines must be set to quantify these factors.

In this model the optimum light intensity was set at the intensity at which photosynthesis is saturated. This is approximately 150 μmol $m^{-2}s^{-1}$ (figure 3.1). The light climate was therefore determined to be optimal if the average irradiance a colony experienced over a 13 hour day was about 150 μmol $m^{-2}s^{-1}$. No account was taken of photoinhibition, photoadaptation, differential light capture of different wavelengths or the "package effect" where cells in the centre of a colony receive less light because light is absorbed by the outer cells..

The nutrient climate was a more difficult parameter to quantify in this type of model as it was not written in to the program. To optimise the nutrient climate a colony must encounter nutrient rich water and have a high affinity for the nutrient. A colony can potentially optimise nutrient capture by moving through a large vertical migration. This increases exposure to "new" water and increases the nutrient pool which can be scavenged while also minimising localised nutrient depletion. Nutrient scavenging potential can also be increased by migrating into waters which have higher nutrient concentrations. Soluble nutrient concentrations within the euphotic zone are often low as nutrients are taken up by phytoplankton and then lost by sedimentation. In this model time spent below the euphotic zone was considered a factor which increased nutrient scavenging potential of the cyanobacterial colony.

Simulations of vertical migration were calculated for N-limited, P-limited and nutrient saturated *Microcystis* colonies of different sizes. All physical parameters were the same as those described above. The size classes modelled were 5, 25, 50, 75, 100, 200, 400, 800, 1200, 1600, 2000 μm. The average light intensity the colony experienced, time spent below the euphotic zone and vertical distance travelled were calculated as estimates of the colonies potential to maximise light and nutrient capture and displayed in table 6.2.

Nitrogen-limited *Microcystis* colonies of all sizes remained at the surface, and experienced an average irradiance of 633 μmol photon $m^{-2}s^{-1}$, which was a function of the start density and a slow rate of carbohydrate accumulation. Nitrogen limited cells displayed heterogeneity in

buoyancy following a dark pretreatment (chapter 7) with 63% being buoyant. This heterogeneity in buoyancy can be attributed to different gas vesicle volumes (chapter 5) and ballast of cells. As the starting density of buoyant cells approached the density of water (density >995 kgm^{-3}) the rate of carbohydrate accumulation could overcome buoyancy but at lower starting densities cells remained persistently buoyant.

	P-limited *Microcystis*			**Nutrient replete *Microcystis***			**Nutrient replete *Anabaena***		
Colony radius (µm)	Average light intensity (pfd)	Time below euphotic zone (h)	Vertical distance travelled (m)	Average light intensity (pfd)	Time below euphotic zone (h)	Vertical distance travelled (m)	Average light intensity (pfd)	Time below euphotic zone (h)	Vertical distance travelled (m)
5	629	-	0.03	630	-	0.04	632	-	0.02
25	560	-	0.45	584	-	0.32	614	-	0.20
50	421	-	1.73	479	-	1.31	565	-	0.54
75	310	6.2	3.36	380	-	2.34	504	-	1.51
100	238	9.8	4.99	307	11.5	3.72	446	-	2.60
200	130	6.1	7.42	180	9.6	8.64	305	9.9	7.92
400	305	2.6	7.17	151	4.9	8.57	219	5.4	8.57
800	493	1.3	7.04	234	3.3	13.54	338	2.5	8.21
1200	501	0.9	8.32	241	2.6	18.43	409	1.7	10.30
1600	529	0.5	7.74	239	2.1	23.11	415	1.2	10.94
2000	504	0.4	9.6	236	1.9	30.10	410	1.0	12.96

Table 6.2 Computer simulations of vertical migration for nutrient replete and phosphate limited *Microcystis aeruginosa* and *Anabaena circinalis* of various colony radii were created using the model of Kromkamp and Walsby (1990). To exploit an environment where light and nutrients are vertically separated a colony must migrate between the optimal zones for light and nutrient capture. The average light intensity per day (pfd=photon flux density; µmol photon $m^{-2}s^{-1}$) was used as an estimate of the light climate the cell experienced and the vertical distance travelled and time spent below the euphotic zone were considered measures of the nutrient scavenging potential in order to determine the optimal size classes for effective buoyancy regulation.

Phosphorus-limited *Microcystis* colonies smaller than 50 µm did not travel more than 1.73 m vertically in one day and did not leave the euphotic zone (table 6.2). Under these conditions the light requirements of the cell were met but the nutrient scavenging ability of the colony was small and potentially limiting to colony maintenance and growth. As the colony size increased

colonies performed more effective migrations spending longer below the euphotic zone and travelled further which increased the volume of water which could be gleaned for nutrients.

Microcystis colonies with an adequate nutrient supply needed to be larger than 100 μm to migrate below the euphotic zone. Nutrient replete colonies, greater than 200 μm radius, travelled much further than P-limited colonies of the same radius. However, although larger colonies travelled further time spent below the euphotic zone decreased with increasing radius above 100 μm.

The theoretical optimum size for P-limited *Microcystis* was considered to be colonies with radius between 100 and 200 μm and between 100 and 800 μm for nutrient replete *Microcystis*. The optimum size theory is based on assumptions such as a colony experiencing sufficient light to maximise photosynthesis without becoming photoinhibited and scavenging the largest available volume of nutrient with higher nutrient concentrations. There are numerous limitations to this theory particularly with respect to the photophysiology and restricted nutrient uptake by colonial cells, however, in its simplest form it provides an appreciation of vertical migration and the role colony size plays in this.

6.3.5 Redistribution of different sized *Microcystis* colonies following mixing

Phytoplankton in a water column are subjected to redistribution by mixing events which may occur on an intermittent or regular basis. Colonies float at different rates depending on their size and therefore colony size and depth in the water column will determine how much light a colony receives following mixing which will affect photosynthetic performance and growth.

A simulation of the redistribution of different sized *Microcystis* colonies (nutrient replete) and subsequent buoyancy regulation following a mixing event was performed. This simulation was run over a five day period and the run conditions were: light attenuation coefficient k=2 m^{-1}; form resistance =1.1; maximum surface irradiance =1000 μmol $m^{-2}s^{-1}$; packing efficiency A=0.1; light period =13 hours; starting depth -variable, 0-10 metres; colony radius -variable, 10, 50, 100, 200, 400 μm; starting density = 980 kg m^{-3}; density range = 980 - 1100 kg m^{-3}.

100 μm colonies starting at different depths moved at velocities determined by size, initial density and light exposure. Following a mixing event the colonies were evenly distributed

through the 10 metre mixed layer but then turbulence in the water column reduced and movement was then due to colony density not water movement (figure 6.3). Colonies initially within the euphotic zone (2.3m) experienced light and change their net density and vertical position accordingly. As colonies floated up from lower depths and reached the illuminated surfaces water they accumulated carbohydrate and regulated their vertical position. With increasing time a greater proportion of the population arrived at the surface and "blooms" lasted for approximately 3 hours after dawn.

Aspects of the redistribution of cyanobacterial colonies of interest to water managers are bloom formation and limiting the survival of individuals. Colonies within the euphotic zone will experience light, proliferate and increase the probability of surface blooms. Rather than display simulations of all the size classes from all depths the aspects of interest are summarised in table 6.3. Small colonies (10 μm) were too small to move effectively. Over the five day simulation period they did not display significant vertical redistribution relative to their initial starting depth and only 30% of colonies were within the euphotic zone after 48 hours.

As the colony size increased colonies migrated faster toward the surface and the proportion of colonies within the euphotic zone and surface bloom duration increased. 400 μm colonies formed nocturnal surface blooms with the entire population at the surface within 15 hours. 50 μm colonies did not form surface blooms but a deep water maxima involving 40% of the population was evident at approximately 1.5 m depth after 48 hours.

The theoretical optimum size for redistribution and cell maintenance following a mixing event is greater than 100 μm. More than 80% of colonies with radius greater than 100 μm arrived at the euphotic zone within 48 hours and therefore light limitation and cell extinction were minimised. Smaller colonies may have suffered light limitation and reduced activity depending on the initial depth but larger colonies were better equipped to cope with a deep mixing events.

Colony radius (μm)	Redistribution Y-yes N-No	Synchronise movement in 5 days	Time to synchronise movement (hours)	Surface bloom duration	% which reached Zeu in 48 hours
10	N	N	∞	No bloom	30%
50	Y	If Z_0>4m	90	Deep water maxima at 1.5m	40%
100	Y	no but all within Zeu	60	3 hours	80%
200	Y	Y	24	4 hours	100%
400	Y	Y	15	14 hours	100%

Table 6.3 Buoyancy regulation of nutrient replete *Microcystis* cells with different starting depth was modelled using the Kromkamp and Walsby (1990) model to simulate vertical mixing followed by five days of stable water. Five colony radii were modelled and features of their migratory pattern of interest to water managers summarised. An example of a simulation is provided in figure 6.3. Z_0=depth at time zero.

6.3.6 The buoyancy regulation of *Anabaena circinalis*

6.3.6.1 % filaments floating

Cells were exposed to five different light intensities for ten hours of light and six hours of darkness. During this incubation the percentage of filaments floating was always greater than 95% which was consistent with the finding in table 5.1 where persistent buoyancy was also observed. This indicated that sufficient carbohydrate did not accumulate over the experimental period to overcome the buoyancy provided by the gas vesicles.

6.3.6.2 Density change of Anabaena in response to light

However, there was still a light dependent change in density. Density increased at a linear rate at each light intensity. The rate of density change in the light and subsequent dark period was plotted against irradiance and previous irradiance, respectively (figure 6.4). Constants were calculated from figure 6.4 for inclusion in the Kromkamp and Walsby (1990) computer simulation of vertical migration (table 6.1). The maximum rate of density change of *Anabaena* in the light was 4.9 kg m^{-3} h^{-1} which was about half the rate of density change of *Microcystis* in similar conditions. The slower rate of density change may partially explain why buoyancy was not overcome in cells with an adequate nutrient supply. When nutrients are in short supply buoyancy can be overcome (see the following chapter) and buoyancy regulation was modelled on this premise.

Vertical migration of different size *Anabaena circinalis* colonies was simulated for colony radii of 25, 50, 100, 200, 400 μm. Other model parameters were the same as those used in modelling *Microcystis* vertical migration. More size classes were modelled, however, these were not shown diagrammatically but data from them was included in table 6.2 to determine the theoretical optimum size for effective migration.

Figure 6.5 demonstrates the predicted vertical migration patterns of *Anabaena* of five different sizes. Although form resistance changes with size (Davey and Walsby, 1985) it was assumed to be a sphere for all colony sizes. Colonies less than 100 μm did not migrate below the euphotic zone (2.3 m) and thus were not considered effective scavengers for nutrients according to the optimal size class theory proposed earlier. The average size of *Anabaena circinalis* filaments sampled from the lower River Murray was 50 μm radius (pers. comm. Peter Baker, SA Water) which falls within the poor migrator class. It may be that small filaments rely on water turbulence to disrupt the vertical separation of light and nutrients and buoyancy is then just a mechanism to remain entrained within the water column.

Larger filaments migrated further (figure 6.5; table 6.2) but filaments greater than 200 μm spent less time below the euphotic zone. A filament size of radius 200 μm was determined to be the optimal size because the average irradiance was saturating, and the filament migrated a reasonable distance and spent a large proportion of the day below the euphotic zone scavenging for nutrients (table 6.2).

6.3.7 Phosphate uptake by *Microcystis aeruginosa*

To further investigate nutrient scavenging potential of *Microcystis* and place vertical migration and nutrient limitation into context phosphate uptake kinetics were also examined.

6.3.7.1 Time course of Phosphorus uptake

At a concentration of 10 μM, uptake of phosphate in unicellular P-starved *Microcystis* was linear for at least 90 minutes however, there was a slight slowing after 2 hours (figure 6.6). To estimate phosphorus uptake rate of *Microcystis* suspended in different ambient concentrations of phosphorus 10 minutes was selected as the influx time as influx was linear over this period.

6.3.7.2 Concentration dependence of phosphate influx

The rate of phosphorus influx was dependent on the ambient phosphorus concentration (figure 6.7) and followed Michaelis-Menton Kinetics; V_{max} = 24 nmol $(10^6$ cells h$)^{-1}$, k_s = 1 µM). Holm and Armstrong (1981) measured a k_s value of 1.23 µM P for their strain of *Microcystis aeruginosa.*

6.3.7.3 Uptake of P-starved and P-sufficient Microcystis

The nutrient scavenging potential of a cell depends on its ability to migrate into water with available nutrients and the nutrient uptake kinetics. *Microcystis* cells which had been deprived of phosphate had a lower internal phosphate concentration but a significantly higher uptake of phosphorus than cells which were grown in adequate phosphorus. The rate of phosphorus uptake by P-starved cells was nearly seven times greater than influx into P-sufficient cells (table 6.4)

Phosphorus status of *Microcystis*	Internal phosphate (nmol $(10^6$ cells$)^{-1}$	P influx (nmol $(10^6$ cells h$)^{-1}$
P-starved	6.6 (0.9)	20.0 (0.6)
	(n=3)	(n=5)
P-sufficient	11.6 (0.06)	2.95 (0.54)
	(n=3)	(n=5)

Table 6.4 Phosphorus deficient *Microcystis* cells had a lower internal phosphate but a greater phosphorus uptake rate. Influx solution = simple buffer solution + 10 µM Phosphate. Rinse t=30 s. (standard deviation shown in brackets)

6.4 Discussion

6.4.1 Buoyancy regulation of *Microcystis aeruginosa*

Nitrogen limitation altered the rate of density change of *Microcystis* cells in the light and dark relative to the nutrient replete cultures. Phosphorus limitation did not reduce the rate of density change relative to the control which was unexpected. It is known that phosphorus limitation moderately reduces the efficiency of Photosystem II (Geider *et al.*, 1993) however, in this study it is possible that photosynthesis was not P-limited. Cells had been incubated in P-free media for seven days which decreases the growth rate (chapter 3) but there may have been

sufficient mobilisation of stored polyphosphate to maintain photosynthesis. The internal phosphate content of *Microcystis* cells starved of phosphate for seven days was 57% of phosphate sufficient cells (table 6.4) which may have been adequate to maintain phosphate supply for ATP and nucleotide production. With extended low ambient phosphate concentrations one would anticipate a decrease in the internal phosphate and photosynthetic activity.

Nitrogen limitation decreased photosynthesis and the rate of carbohydrate accumulation and consequently the rate of density change at saturating irradiances decreased to 20% of the maximum measured values for nutrient sufficient cells. This resulted in an impaired buoyancy regulation mechanism and the gas vesicle pool would need to be very low and the initial density of the cells approaching the density of water for vertical migration to occur. Nitrogen limitation decreases the gas vesicle pool however if sufficient gas vesicles remain in each cell then severe N-limitation could disrupt the buoyancy regulation mechanism which could give rise to persistent buoyancy. Alternatively N-limited cells do not efficiently metabolise photosynthate (Turpin, 1991), have a reduced gas vesicle pool (chapter 5, Klemer, 1978) and may have a density greater than water. In the following chapter 37% of N-limited *Microcystis* cells were non-buoyant following a dark pretreatment and the balance accumulated sufficient carbohydrate in 24 hours to overcome buoyancy.

The loss and recovery of buoyancy allows colonies in thermally stratified water columns to migrate vertically and scavenge for nutrients. Simulations of vertical migration of colonies with different nutrient status revealed that nutrient sufficient and P-limited cells migrated effectively when they were large enough. An optimal size hypothesis was proposed for colonies in persistently stratified water columns and in nocturnally mixed water columns. In order to exploit a thermally and chemically stratified water body it is necessary for the colony to overcome the vertical separation between light and nutrients. The criteria for optimal size included sufficient light exposure and the colony had to travel vertically and spend time below the euphotic zone to maximise nutrient scavenging. Mierle (1985) suggested that there may be diffusion limitation of phosphate uptake at low concentrations. Vertical migration would, to some extent, overcome this.

The theoretical optimal size for P-limited *Microcystis* was 100-200 μm and for nutrient replete *Microcystis* 100-800 μm. The nutrient scavenging potential of P-limited *Microcystis* would be further enhanced by the increased affinity and rate of phosphate uptake relative to the P-sufficient cells (table 6.4).

Recovery from a mixing event and migration into the illuminated zone was also more rapid for larger colonies. This would increase cell activity and photosynthesis (Kohler, 1992; Walsby *et al.*, 1997) and decrease losses from extended dark exposure (Selvin *et al.*, 1988). From a water managers perspective small colonies are preferable because they may experience greater cell loss from dark exposure and do not appear to form persistent water blooms for as long as large colonies. The floating velocity and dark survivorship would also be useful in determining periodicity of mixing in lakes where intermittent destratification is considered a management option (Steinberg and Zimmermann, 1988). In nocturnally mixed-diurnally stratified water bodies, such as Lake George, Uganda (Ganf, 1974) large algae such as *Microcystis* may be favoured because they migrate quick enough to reach the euphotic zone during the day and capture light. Small algae would have a smaller probability of receiving sufficient light for cell maintenance depending on the euphotic and mixed depth.

The euphotic depth, initial start depth and the rate at which colonies travel following mixing determines the light dose the colony receives. Growth is dependent upon the previous light history. In chapter 7 cells were grown at different light intensities prior to a 16 hour dark treatment and 24 hours at 150 μmol m^{-2} s^{-1}. Cells initially grown at 100, 50 and 10 μmol. m^{-2} s^{-1} had a growth rate of 0.75, 0.43, 0.21 ln units day^{-1} respectively. Therefore, assuming there is sufficient nutrients, cells receiving a high (yet not inhibitory) light dose will display a greater growth. Size heterogeneity in a population will lead to heterogeneity in vertical migration, photosynthesis and growth.

Although large colonies may migrate more effectively than small colonies this may compromise the optimal size for other cell functions. For example, the growth rate of unicellular *Microcystis aeruginosa* culture was 1.11 ln units day^{-1} (Reynolds, 1984) whereas a colonial *Microcystis* culture displayed a growth rate of 0.48 ln units day^{-1} (Reynolds *et al.*, 1981). Small colonies have more efficient nutrient uptake, however, when nutrients are in short supply

colonies may induce a localised nutrient deplete zone around them and nutrient uptake would be limited by the rate of diffusion (Mierle, 1985). By migrating vertically colonies would increase the nutrient pool available and reduce localised nutrient limitation.

Light limitation of cells within the centre of a colony is also a risk involved in large colony size. This can be overcome by decreasing the amount of pigment in outer cells and increasing the distance between cells (Augusti and Philps, 1992). Increasing the amount of mucilage (Reynolds, 1984) encompassing the colony effectively increases colony size without compromising light harvesting capabilities. A remarkable strategy employed by *Microcystis aeruginosa* to maximise size and light capture was observed by Walsby and McAllister (1987) in Lake Okaro, New Zealand. These colonies were spherical and cells were concentrated on the periphery with the colony centre virtually hollow.

6.4.2 Buoyancy regulation of *Anabaena circinalis*

Anabaena circinalis filaments displayed persistent buoyancy at all light intensities throughout the experiment. Persistent buoyancy is a feature not uncommon in *Anabaena* species (Walsby *et al.*, 1987, 1989; chapter 5) and is explored further in chapter 7. In the modelling of *Anabaena circinalis* buoyancy regulation it was assumed that gas vesicle volume did not change in response to irradiance and was sufficiently low to allow cell buoyancy to be overcome by carbohydrate increase. Gas vesicle collapse by increased turgor pressure at high light intensities could also be a factor changing cell density and would act to change cell density more rapidly than by carbohydrate change alone. This would increase sinking velocity and the depth to which filaments migrate before buoyancy is restored.

The maximum rate of density change observed in *Anabaena* was approximately half the rate of density change in *Microcystis* allowing *Microcystis* to overcome buoyancy quicker. Growth of *Microcystis* was saturated at lower light intensities than *Anabaena* (chapter 5) and therefore it can potentially spend more time scavenging for nutrients particularly nitrogen, phosphorus can be stored and remobilised. *Anabaena* in waters low in inorganic nitrogen can fix N_2 and requires more light to do this. The difference in nutrient and light requirements of the two cyanobacteria therefore may determine the degree of buoyancy regulation and vertical migration displayed.

6.4.3 Limitations and assumptions of model

Inevitably assumptions are made in modelling biological and physical processes. A constraint of this computer model is that the biological data is collected from cells receiving a constant block irradiance which is unnatural (Ferris and Christian, 1991; Grobbelaar *et al.*, 1992). Photoadaptation, photoinhibition and temperature affects on photosynthesis are not accounted for. In a real system cells would move through a light gradient at different rates depending on their cell density, size and water movement. The irradiance they experience and the periodicity of light exposure would affect their photosynthesis (Grobbelaar *et al.*, 1992; Kromkamp *et al.*, 1992; Flameling and Kromkamp, 1997) and carbohydrate accumulation (Green, 1994). Further studies examining photosynthesis of cyanobacteria exposed to fluctuating light regimes will provide greater insight into how this affects buoyancy regulation. This could be done using apparatus such as the venetian blind setup of Kroon *et al.* (1992) or by tracking movement of algae in the water column and measuring fluorescence of photosynthetic pigments to estimate photosynthesis (Krause and Weis, 1991; Keifer and Reynolds, 1992; Kolber and Falkowski, 1993).

As cells migrate vertically they also experience a change in the spectral distribution of light (Kirk, 1983; Ganf *et al.*, 1989) which the model does not account for. Cells can adapt to different wavelengths by adjusting the concentration of phycobilin pigments (Falkowski and LaRoche, 1991, Ohki and Fujita, 1992) and increase their light harvesting potential when light is low and wavelength distribution skewed. Nutrient limited cells have reduced phycobilin concentrations and therefore may display inhibited production in turbid water bodies leading to impaired buoyancy regulation.

More field studies on the size distribution of *Microcystis in situ* photosynthesis and tracking of colony movement in water bodies with different mixing-stratification patterns will test the optimum size hypothesis and validate the model and provide better insights into cyanobacterial ecophysiology.

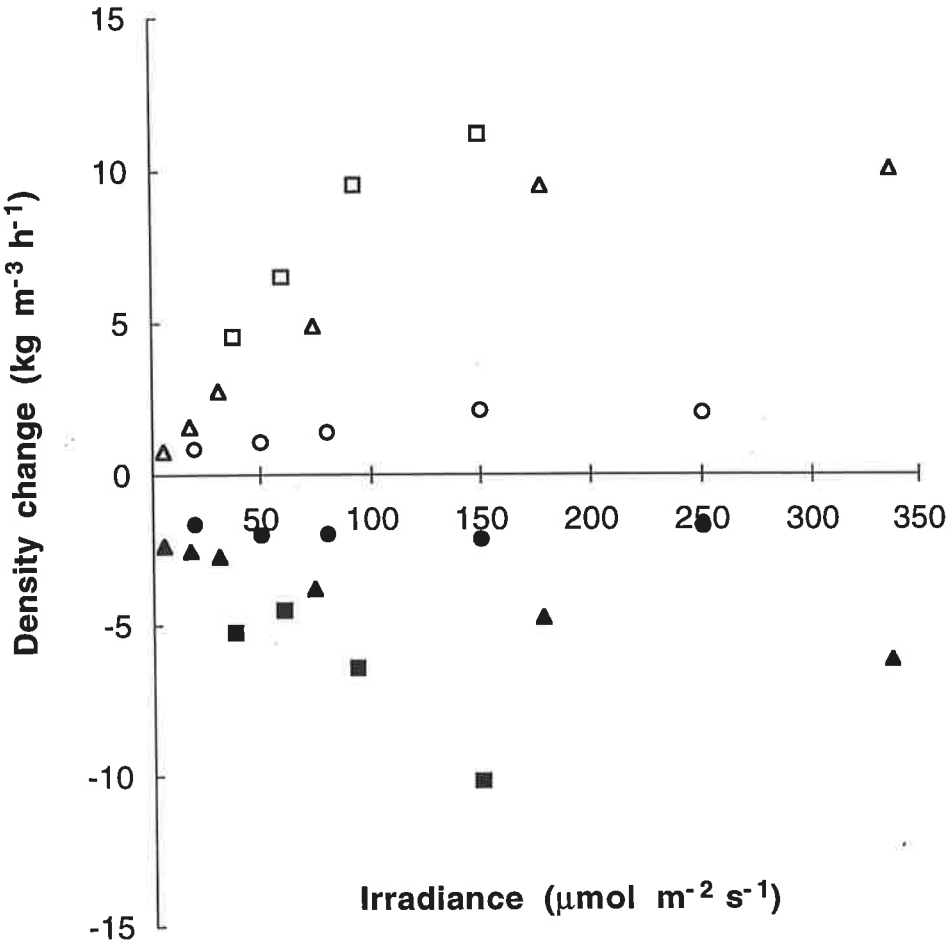


Figure 6.1 Light-dependent rate of density change. Phosphorus limited (□), nitrogen limited (○) or nutrient sufficient (△) *Microcystis aeruginosa* was transferred to different irradiances (hollow symbols) during which time the density change was measured using Percoll gradients. Cells were then transferred to the dark and density loss measured (filled symbols). The rate of density change was plotted against irradiance the cells were incubated at or the previous irradiance.

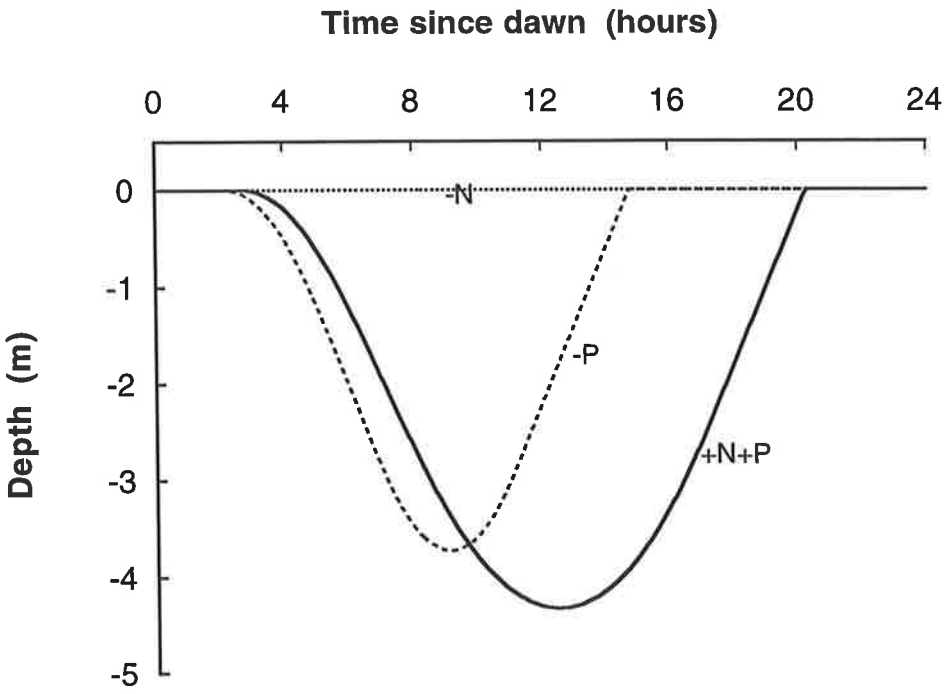


Figure 6.2 Computer simulations of vertical migration of P-limited, N-limited and nutrient replete *Microcystis aeruginosa*. Run conditions were form resistance Ø=1.1; packing efficiency A=0.1; start density=980 kgm^{-3}; density range 970-1100 kgm^{-3}; colony radius=200 µm; attenuation coefficient=2 m^{-1}; start depth=0 m; daylength=13 hours; maximum irradiance=1000 µmol $m^{-2}s^{-1}$.

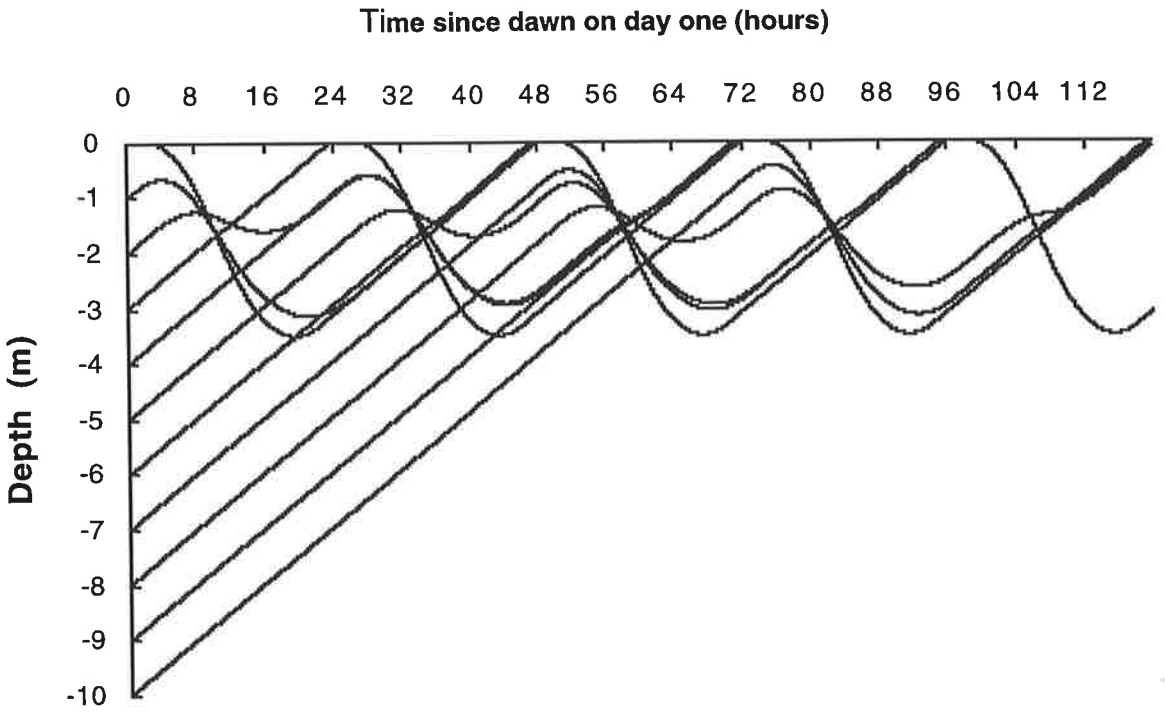


Figure 6.3 Simulation of vertical migration of 100 µm *Microcystis* colonies distributed throughout the watercolumn following turbulence. The Kromkamp and Walsby (1990) model was modified to run over 5 days. Run conditions were as for figure 6.2

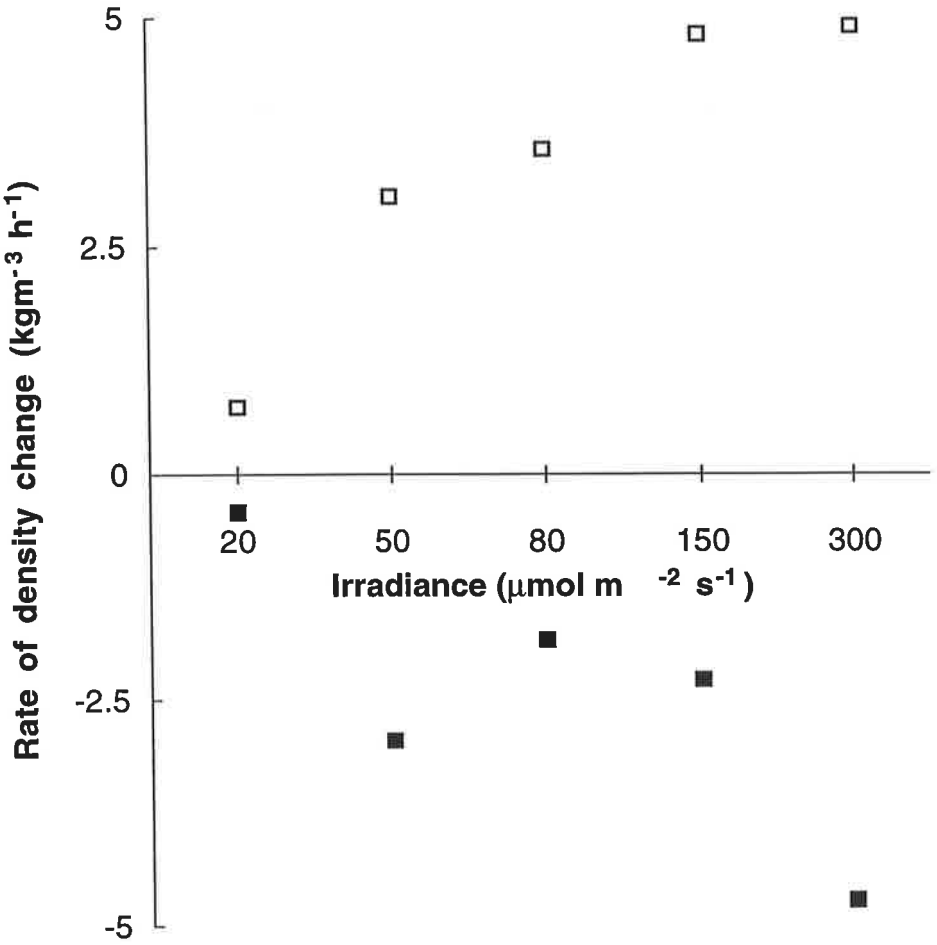


Figure 6.4 Light dependent rate of density change. An *Anabaena circinalis* culture was transferred to different irradiances for 10 hours (hollow symbols) before transfer to the dark for six hours (closed symbol). The rate of density change was calculated from bihourly measurements of density using Percoll gradients.

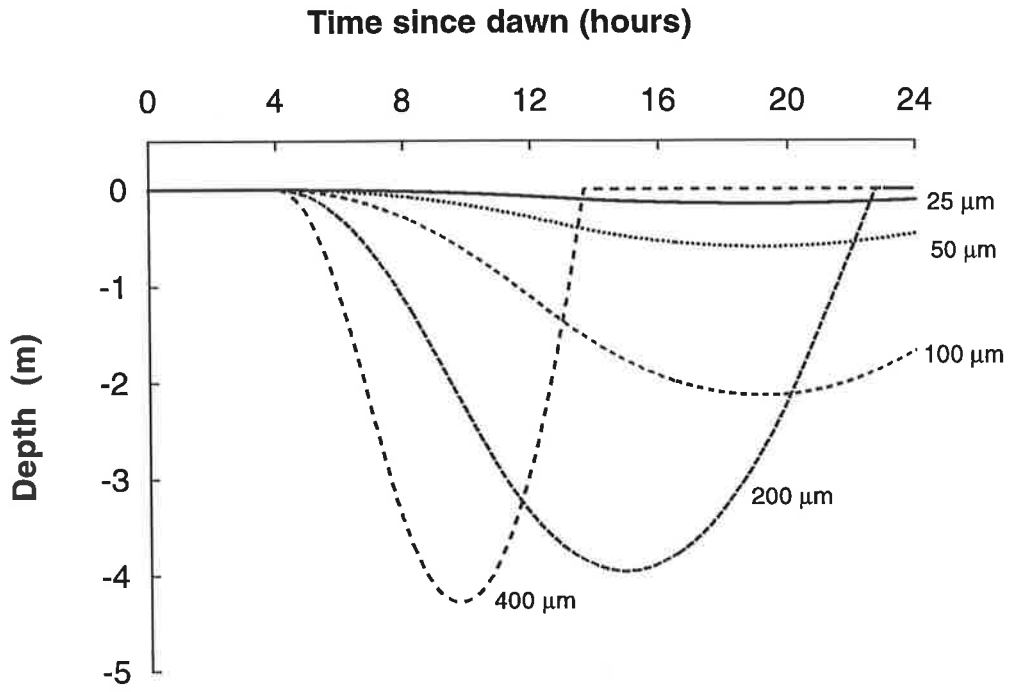


Figure 6.5 Vertical migration simulations of different sized *Anabaena circinalis* filaments. Run conditions were form resistance Ø=1.1; packing efficiency A=0.1; start density=980 kgm^{-3}; density range 970-1100 kgm^{-3}; colony radius=200 µm; attenuation coefficient=2 m^{-1}; start depth=0 m; daylength=13 hours; maximum irradiance=1000 µmol $m^{-2}s^{-1}$.

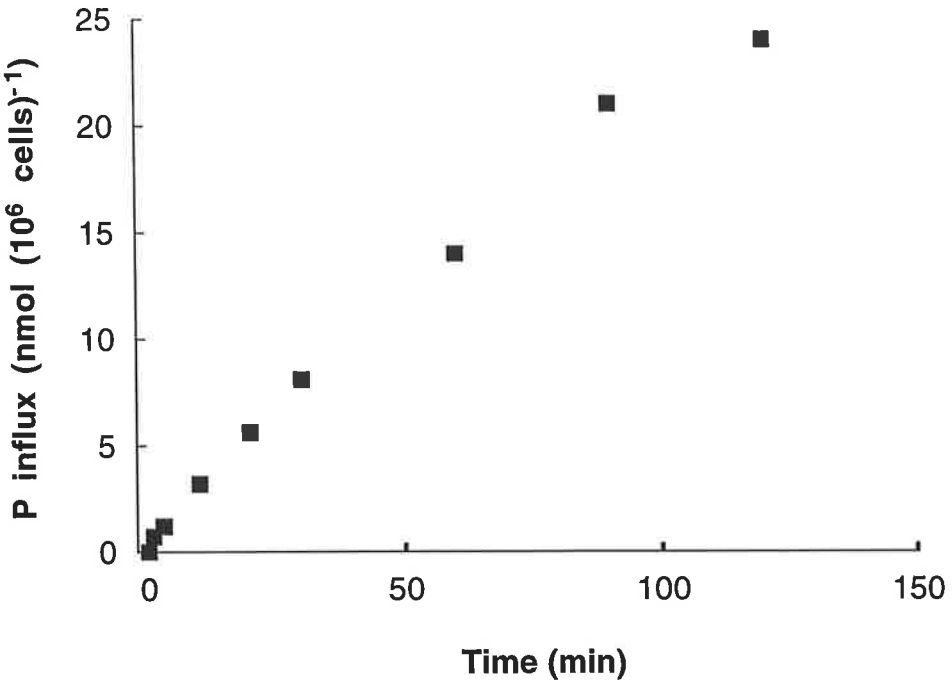


Figure 6.6 Time course of phosphate influx by P-starved *Microcystis aeruginosa*

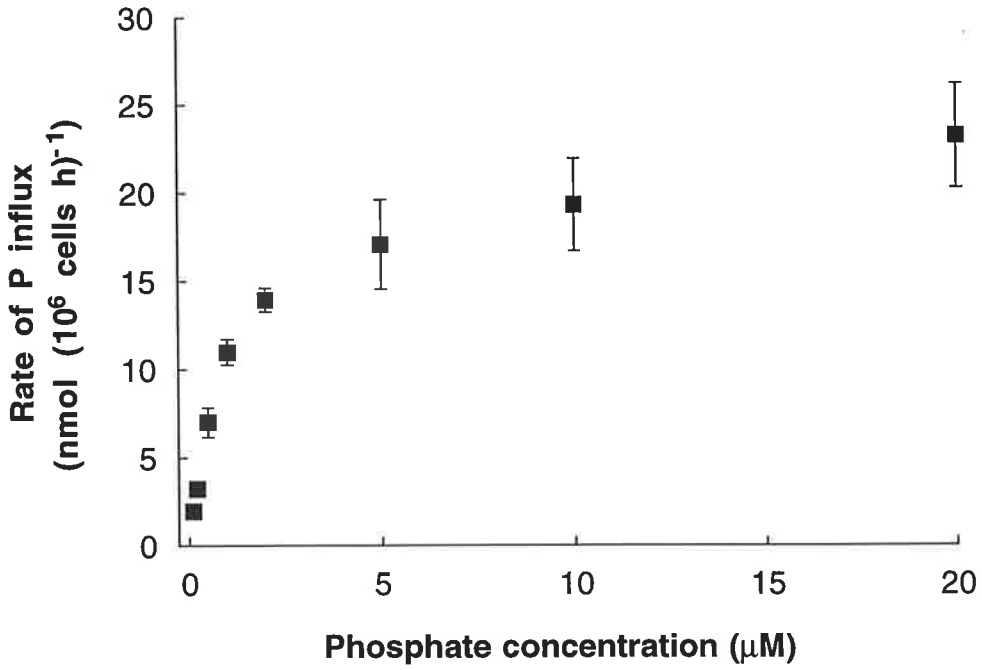


Figure 6.7 Concentration dependence of phosphate influx by P-starved *Microcystis aeruginosa*.

Chapter 7

The interaction of gas vesicles and ballast; factors determining buoyancy in nutrient and light limited *Microcystis aeruginosa* and *Anabaena circinalis*.

7.1 Introduction

The buoyancy status of a cell is determined by the density and concentration of the cellular constituents and the volume of gas vesicles present. Both are functions of the cell's nutrient and light history (Chapter 5 and 6). This chapter deals with the interaction between light, nitrogen, phosphorus and buoyancy regulation of *Microcystis aeruginosa* and *Anabaena circinalis*.

Oliver and Walsby (1984) used "ballast balance sheets" to examine the relationship between cell constituents, gas vesicle volume and buoyancy. This approach involved measurements of gas vesicle space and the mass of carbohydrate, protein, lipids and polyphosphate. The contribution of each component to cell density was calculated and the sum of all the components used to estimate the net cell density (Oliver and Walsby, 1984; Utkilen *et al.*, 1985a; Oliver *et al.*, 1985; Oliver and Walsby, 1988; Kinsman *et al.*, 1991; Oliver, 1994). In this study the cellular features of interest were the gas vesicle volume and carbohydrate content. Instead of creating balance sheets, measurements were made of the gas vesicle volume necessary to maintain buoyancy in cyanobacterial cells which had were exposed to saturating, or limiting, light and nutrients.

Previous chapters have shown that gas vesicle volume (chapter 5) and carbohydrate (chapter 6) change as a function of light and nutrients. The effect of nutrients and light as factors determining cell buoyancy are summarised here in order to detail the motivation for this work and set the scene for hypothesis development.

Nitrogen limitation generates a reduction in gas vesicle volume (chapter 5; Klemer, 1978; Klemer *et al.*, 1982) which coupled with less efficient carbohydrate metabolism (Herzig and Falkowski, 1989, Turpin, 1991) leads to a reduction in cell buoyancy (Klemer *et al*, 1982; van Rijn and Shilo, 1983; Spencer and King, 1985, 1989). Upon the addition of ammonium to nitrogen limited cultures cells rapidly recover buoyancy (van Rijn and Shilo, 1983; Konopka *et al.*, 1993).

In phosphate limited populations growth, which is normally a sink for photosynthate, is reduced and carbohydrate tends to accumulate rather than being metabolised (Konopka *et al.*, 1987b). Although this may be offset, to some extent, by a reduction in PS II efficiency relative to P-replete cells (Geider *et al.*, 1993) a reduction in gas vesicle volume as seen in chapter 5 would further act to decrease cell buoyancy.

Light limitation has the opposite affect to nutrient limitation. When light is in short supply gas vesicles accumulate in *Anabaena* cells (chapter 5) and other gas vacuolated species (Reynolds and Walsby, 1975; Utkilen *et al.*, 1985; Kromkamp *et al.*, 1986; Konopka *et al.*, 1987; Deacon and Walsby, 1990). Carbohydrate content is also low (if nutrients are not limiting) due to low carbon fixation, respiration and carbon metabolism and therefore buoyancy is high. Upon transfer to saturating light cells photosynthesise and accumulate carbon as polyglucose compounds. The net density of the cell increases and depending upon the gas vesicle volume may induce sinking (Thomas and Walsby, 1985; Utkilen *et al.*, 1985; Kromkamp *et al.*, 1986 Konopka *et al.*, 1987a; Kromkamp *et al.*, 1988).

From this summary of literature and combining the data presented in chapters 5 and 6 it is possible to predict the buoyancy response of gas vacuolated cyanobacteria with different nutrient and light histories to high or low irradiance.

If buoyancy regulation is a mechanism to overcome the vertical separation of light and nutrients there may be circumstances when buoyancy regulation offers no added benefit to the cyanobacteria. For example, if the surface waters are eutrophic there is no separation of light and nutrients. Similarly, if the water body is shallow, regularly mixed and sediments resuspended such as those described by Hamilton and Mitchell (1997) and Harding (1997)

there may be no requirement for buoyancy regulation but it may be advantageous to be persistently buoyant. Persistent buoyancy was observed in *Anabaena minutissima* (Walsby *et al.*, 1987; Walsby *et al.*, 1989), *Anabaena lemmermannii* (Walsby *et al.*, 1991) and cultured populations of *Anabaena circinalis* (chapter 5 and 6).

Persistent buoyancy is caused by either i) the maximum carbohydrate concentration is insufficient to offset the lift provided by gas vesicles, ii) cells never experience sufficient light to lose buoyancy ie in turbid mixed conditions; however, if the cells were maintained at high light they would lose buoyancy. iii) cells which accumulate at the surface may become photoinhibited and less active thereby displaying apparent persistent buoyancy or iv) small, buoyant colonies may rise to the surface and become trapped in the viscous surface layer (Webster and Hutchinson, 1994).

Filaments which are persistently buoyant may be selected for if the time scale of vertical mixing is short and the cells are able to exploit low light conditions (Walsby *et al.*, 1989). The nutrient and light conditions which would give rise to persistent buoyancy would be high nutrient availability and relatively low irradiance. Cells could display either persistent buoyancy or buoyancy regulation dependent upon their previous nutrient and light history, the number of gas vesicles present and the cells ability to metabolise photosynthates.

To demonstrate how the switch from persistent buoyancy to buoyancy regulation may occur in natural populations a hypothetical model was created. In a well mixed, turbid, eutrophic reservoir nutrients are entrained into the surface waters and cyanobacteria would display persistent buoyancy as gas vesicle production would be high relative to light-limited growth. Under these conditions the gas vesicle volume may be too great to be overcome by carbohydrate accumulation. With the onset of thermal stratification, water stability increases and the buoyant cyanobacteria float to the surface. However, with prolonged stratification non-buoyant phytoplankton would sediment stripping the surface waters of nutrients and generating a vertical nutrient gradient. Buoyant cells remaining at the surface would then experience a localised nutrient-limitation and growth and gas vesicle production would reduce accordingly. The cells may then reach a critical gas vesicle volume allowing buoyancy to be overcome by carbohydrate accumulation. Cells would then display vertical migrations as described in chapter 6.

To test this hypothesis *Anabaena* and *Microcystis* cultures were grown in different concentrations of nitrogen, phosphorus and a range of light intensities (limiting-saturating) which gave rise to cells with different gas vesicle volumes and ability to fix and metabolise carbohydrate. The buoyancy of cells prior to and following high light was recorded to determine if persistent buoyancy was a real phenomena in these species. Buoyancy was also measured following sequential collapse of gas vesicles to determine what gas vesicle volume was necessary to maintain buoyancy under each of the conditions. The species *Anabaena circinalis* and *Microcystis aeruginosa* were chosen for this study as they are common nuisance taxa in Australian water ways and display different morphological and physiological features. *Anabaena circinalis* is able to fix atmospheric nitrogen and therefore is able to avoid nitrogen limitation more readily and may be more prone to persistent buoyancy than *Microcystis aeruginosa.*

Buoyancy regulation of *Microcystis* has been demonstrated in field populations of *Microcystis* (Ganf, 1974; van Rijn and Shilo, 1985; Ibelings *et al*, 1991, Visser *et al.*, 1996) as has persistent buoyancy (Reynolds, 1973; Robarts, 1984). Laboratory studies identified persistent buoyancy in *Anabaena circinalis,* however, Reynolds (1975) showed *A.circinalis* did lose buoyancy in response to irradiance and therefore a field based trial was required to test laboratory observations. Chaffey Dam, NSW, Australia was chosen as the field site because there was a substantial population of *Anabaena circinalis* and therefore no shortage of experimental material. Chaffey Dam was also the site of an intensive CRC for Freshwater Ecology study on destratification and therefore high resolution data were available detailing physical and nutrient parameters. The natural population was sampled and experiments set up to examine buoyancy regulation and the effect of nutrients on buoyancy.

The success of buoyant cyanobacteria in nocturnally mixed water bodies is dependent on the ability of the filament or colony to position itself in a favourable light climate to satisfy its energy demands when stratification reestablishes. The rate of flotation is therefore an important aspect governing cyanobacterial survivorship. The flotation velocity can be approximated by Stoke's equation (Reynolds, 1984; Oliver, 1994; chapter 6) and is a function of the density, size and form of the filament or colony.

Numerous methods have been employed to measure the floating or sinking velocity of cyanobacteria and other microalgae. Ganf (1974) used a 2 m cylinder and measured the time it took for colonies to rise or fall through a 0.25 m zone. Titman (1975) used a similar approach and measured the continuous loss of algal biomass from a small window in a fluorometer cuvette and Nakamura *et al.* (1993) recorded movement with a microscopic video camera. Hutchinson and Webster (1994) developed a more elaborate system involving grid plates oscillating vertically in a flask containing algal suspension. Colonies which reached the surface could not be dislodged and the loss of biomass from the water column with time was measured. A new technique was designed in this study to estimate the flotation velocity of *Anabaena circinalis* which had been suspended at different depths to investigate what effect carbohydrate load and filament size had on floating rate.

7.2 Methods and experimental design

7.2.1 Cultures

Microcystis aeruginosa cultures SHEP 100 was grown in unbuffered ASM-1 media (Gorham *et al.*, 1964) with bicarbonate added as 150 μM $NaHCO_3$. *Anabaena circinalis* ANA 330E was grown in WC media (Guillard and Lorenzen, 1972). Cultures were grown prior to inoculation into the various treatments at 40 μmol m^{-2} s^{-1} on a 15:9 light: dark cycle at 25°C.

7.2.2 Relative gas vesicle volume and % cells floating

7.2.2.1 Nitrogen-limited Microcystis *and buoyancy status before and after high irradiance*

Log phase *Microcystis aeruginosa* cultures were deprived of nitrogen for two days prior to inoculation into 0, 10, 100 μM nitrate in otherwise normal ASM-1, n=3. Cultures were grown at 100 μmol m^{-2} s^{-1} on a 12: 12 light: dark cycle at 25°C for six days to allow relative nitrogen limitation prior to experimentation. To estimate the relative gas vesicle volume required to maintain buoyancy when ballast was either low or high the relative gas vesicle volume and percentage of cells floating was measured at the start and end of a 24 hour light period.

Cultures were pretreated in a dark environment to deplete the carbohydrate pool and then exposed to 150 μmol m^{-2} s^{-1} for 24 hours, which is a saturating irradiance for carbohydrate accumulation in *Microcystis* (Brookes *et al.*, 1997). Initially and following the 24 hour light treatment cells were subjected to pressures which progressively collapsed increasing numbers of gas vesicles 0, 100, 200, 250, 300, 350, 400, 500, 600, 700 kPa. Following each pressure increase relative gas vacuolation was measured using a FACStrak flow cytometer (chapter 5) and the percentage of floating and sinking cells estimated using a haemocytometer and Zeiss microscope (Walsby and Booker, 1980). This protocol was repeated for the experiments examining the influence of nitrogen, light and phosphorus on the buoyancy of *Microcystis* and *Anabaena* before and after exposure to saturating light.

Carbohydrate content was measured before and after light exposure using the phenol method of Herbert *et al.* (1971) following disruption of cells by sonication. Cell counts were obtained using a haemocytometer and Zeiss microscope.

7.2.2.2 Phosphorus-limited Microcystis *and buoyancy status before and after high irradiance*

Log phase *Microcystis* cultures were deprived of phosphate for eight days prior to inoculation into 0, 0.5 or 10 μM phosphate (n=3) in otherwise normal ASM-1 media. Cultures were grown at 100 μmol m^{-2} s^{-1} on a 12: 12 light: dark cycle at 25°C for six days to promote relative phosphate limitation prior to experimentation. The protocol described above was used to determine the relative gas vacuolation and percentage cells floating following incremental increases in pressure.

7.2.2.3 Light-limited Microcystis *and buoyancy status before and after high irradiance*

Log phase *Microcystis* cultures growing at 40 μmol m^{-2} s^{-1} light on a 15:9 light: dark cycle were inoculated in triplicate into complete ASM-1 media and incubated at three irradiances 10, 50, 100 μmol m^{-2} s^{-1} on a 12: 12 light: dark cycle, 25 °C. The protocol described above was used to determine the relative gas vacuolation and percentage cells floating following incremental increases in pressure.

7.2.2.4 Nitrogen-limited **Anabaena** *and buoyancy status before and after high irradiance*

To induce nitrogen limitation in *Anabaena* it was necessary to limit the concentration of atmospheric nitrogen and vary the nitrate concentration in the media. A daughter culture of *Anabaena circinalis* ANA330E preconditioned to media containing no soluble nitrogen was used in this study. *Anabaena* grown in WC media deplete in soluble inorganic nitrogen was inoculated into WC media containing 0, 20 or 100 μM nitrate. Atmospheric nitrogen was reduced by sparging the cultures with N_2 free air (chapter 5). Cultures were grown for six days at 100 μmol m^{-2} s^{-1} on a 12: 12 light: dark cycle at 25°C prior to experimentation. The protocol described above was used to determine the relative gas vacuolation and percentage cells floating following incremental increases in pressure.

7.2.2.5 Phosphorus-limited **Anabaena** *and buoyancy status before and after high irradiance*

Log phase *Anabaena circinalis* cultures were washed four times in P-free WC media containing KCl to replace the potassium which was normally present as K_2HPO_4. The washing procedure involved collapsing the gas vesicles of the cells by the application of 800 kPa pressure, centrifuging the sample at 25 g, removing the supernatant and resuspending in P-free WC. The centrifuging and resuspension steps were repeated four times. *Anabaena* filaments were grown in phosphate free media for seven days before inoculation into triplicate flasks containing 0, 0.5 and 10 μM phosphate in otherwise normal WC media. The cultures were maintained at 100 μmol m^{-2} s^{-1} on a 12: 12 light: dark cycle at 25°C for six days prior to experimentation. The protocol described above was used to determine the relative gas vacuolation and percentage cells floating following incremental increases in pressure.

7.2.2.6 Light-limited **Anabaena** *and buoyancy status before and after high irradiance*

Anabaena circinalis cultures were grown at 10, 50 or 100 μmol m^{-2} s^{-1} on a 12: 12 light: dark cycle at 25°C for six days prior to experimentation. The protocol described above was used to determine the relative gas vacuolation and percentage cells floating following incremental increases in pressure.

7.2.3 Field study-Chaffey Dam

7.2.3.1 Site description

Chaffey Dam is primarily an irrigation storage reservoir and is situated on the Peel River, upstream of Tamworth, NSW, Australia (31° 20' S, 151° 7.5'E). Land use in the catchment includes grazing on grassland and improved pasture in the valley and western slopes . Native grasslands and plantation forests make up the balance of land use on the steep eastern catchment.

The reservoir is classed mesotrophic-eutrophic and is persistently stratified for most of the year (pers. comm. Brad Sherman, CSIRO Land and Water, Canberra). Turnover can occur in late autumn or early winter (May, June), however, the water column does not remain mixed but restratifies. Differential cooling in winter has the effect of maintaining stratification even though the water temperature of the entire water body decreases (pers. comm. Brad Sherman). A compressed air bubble plume aeration system has been used in Chaffey Dam to attempt to destratify the water column and decrease the frequency and occurrence of nuisance "algal blooms" but was largely unsuccessful at deepening the surface layer.

7.2.3.2 Is there evidence of buoyancy regulation in a field population of **Anabaena circinalis?**

Two field trials were designed to examine the buoyancy status of cells following different light treatments and investigate whether *Anabaena circinalis* exhibited buoyancy regulation and lost buoyancy in field situations. One involved sampling the natural population throughout the day and scoring the number of filaments floating and in the second trial a sample of a natural population of *Anabaena circinalis* was bottled and suspended at different depths and buoyancy status assessed.

Sampling was carried out at the meteorological station at Chaffey Dam on April 15 1997. Water samples were collected at times 08:00, 13:00 and 16:00 hr with a 14.5 litre plankton trap and the sample concentrated through 30 µm mesh to approximately 40 mL. Samples were integrated over the depth of the plankton trap (400 mm and taken from depths; 0-0.4 m, 0.4-0.8, 0.8-1.2, 1.2-1.6, 2.0-2.4, 4.0-4.4, 6.0-6.4 and 8.0-8.4m). Samples were taken immediately back to the lakeside laboratory where the number of filaments floating or sinking

was estimated using a Sedgewick Rafter counting cell and an adapted method of Walsby and Booker (1980).

Bottle experiments were set up by collecting integrated water samples from between 20 and 60 cm depth from a single site, pooling these in a bucket, mixing well and transferring the water to clean, 1.25 litre PET bottles ensuring the removal of air bubbles when the bottles were capped. The initial cell concentration was 3.5 x 10^4 cells mL^{-1}. At dusk on the eve prior to experimentation (09/04/97) the samples were suspended at 0.3, 0.9, 1.9, 3.5 and 4.5 metres depth which corresponded to 67, 30, 10, 3, and 1% of surface irradiance respectively. A treatment which experienced an alternating light dark cycle was included. Bottles in this treatment started at 0.3 m at 08:00 h but moved from 0.3 m to 4.5 m after one hour and continued to alternate between the depths at hourly intervals throughout the day. A dark treatment was also included and consisted of bottles wrapped in black tape and suspended at 4.5 metres. Three replicate bottles were suspended at each depth. The percentage of filaments floating was measured using a Sedgewick-Rafter chamber at 08:00 h, 12:00, 17:00 and 08:00 h 24 hours after the commencement of the experiment.

7.2.3.3 What is the effect of nutrients on buoyancy status of **Anabaena circinalis?**

When the above experiment was performed (09/04/97) a nutrient treatment was included at each depth (n=3). These treatments had phosphorus and nitrogen added as 100 µg L^{-1} P as phosphate and 1 mgL^{-1} N as nitrate and were incubated for approximately 38 hours before the commencement of sampling. Comparisons were made between the no nutrient treatment and the treatments with added nutrients on 11/04/97 from samples collected at 0730 h before significant light exposure and at 17:00 h following a full day of sunlight. The percentage of filaments floating and sinking was measured using a Sedgewick Rafter chamber. A minimum of 100 filaments were counted.

7.2.3.4 Estimation of floating velocity of **Anabaena circinalis**

Floating was estimated using two techniques. When the mean filament size was small the method of Titman (1975) was used. This method estimated sinking or floating velocity by

measuring the loss of phytoplankton from an illuminated window tube using a Turner III fluorometer.

When the mean filament size was relatively large the method of Titman (1975) failed as the mean chlorophyll per filament was too great and the fluorescence measurement was off scale. To an extent this was accounted for by using neutral density filters but a more suitable technique was developed to measure sinking and floating velocity with a large water volume and migration distance so edge effects of the container were minimised.

To measure floating velocity using this technique a sequence of 10 to 15 replicate 50 mL Falcon centrifuge tubes were filled with a well mixed water sample containing the cyanobacteria. The tubes were gently mixed by inversion and left in dim light in a climate controlled (23°C), draft free room. At regular time intervals (approximately 1, 2, 3, 5, 10, 15, 20, 25, 30, 40, 50, 60 minutes) the top 5 mL and bottom 5 mL of one tube was carefully siphoned and the chlorophyll fluorescence (Fo) measured as a surrogate for biomass. The rate of accumulation of biomass at the surface or bottom of the tube was used to calculate a velocity of sinking or floating.

The fluorescence in each fraction was measured using a Pulse Amplitude Modulated (PAM-101, Walz, Effeltrich, Germany) fluorometer. Excitation was achieved with a low intensity LED at 655nm and detection of fluorescence was enhanced with a high sensitivity cuvette holder (ED-101US/D)(Schrieber *et al.*, 1994) (pers. comm. John Whittington, CRC for Freshwater Ecology, Albury). Biomass was estimated using the fluorescence parameter Fo, the fluorescence intensity in dark adapted cells with all PS II reaction centres open. The fluorescence in each fraction was measured after the samples were left in dim light (<10 µmol m^{-2} s^{-1}) for at least two hours to allow for relaxation of quenching. The background fluorescence was high and was estimated by measuring the fluorescence of water passed through a GF/C filter to remove chlorophyll-bearing particles. Background fluorescence was an essential parameter to measure so that the endpoint of the experiment when all cells were at the surface could be estimated.

An experiment was then designed to investigate the effect of light on the carbohydrate content of *Anabaena circinalis* and how this affected the rising or sinking velocity of filaments. A

phytoplankton sample was collected from a single site (DLWC buoy 2) by concentrating the plankton by net tows integrated from 10 m to the surface. The plankton net had a mesh size of 50 μm. A 30 litre water sample was also collected from the same site for resuspending the concentrated algal sample.

The concentrated algal sample was allowed to stand in a one litre measuring cylinder to allow particulates and non-gas vacuolated phytoplankton to sediment and then the top 950 mL containing the concentrated buoyant cyanobacteria was decanted off. The 30 litre water sample was filtered through a GF/C filter and the filtrate retained to resuspend the algal concentrate.

The algal suspension consisted mainly of *Anabaena circinalis* but there was some *Microcystis aeruginosa* present. This sample was put into 1.25 litre PET bottles and nutrients added as 100 μg L^{-1} P as phosphate and 1 mgL^{-1} N as nitrate. The bottles were suspended at 10:30 on 14/4/97 at depths 0.3m, 0.9m and 4.5m which corresponded to 70, 30 and 1% of I_o respectively. One of three replicate bottles was harvested at 08:30, 13:30 and 16:30 on 15/04/97 and the floating/sinking velocity measured using the method described above. Carbohydrate was measured using the anthrone method of Herbert *et al.* (1971) and cell number by microscopic enumeration using a Sedgewick-Rafter chamber.

7.2.3.5 Physical and chemical data

Vertical profiles of water temperature were measured continuously by a thermistor chain fixed to a raft in the centre of the dam. A metereological station was also installed on the raft which measured wind speed and direction, solar radiation, air temperature and humidity. This equipment was maintained by CSIRO Land and Water, Canberra and environmental monitoring data was provided by them. Nutrient analysis was performed by Tamworth Environmental Laboratories. Underwater light intensities were measured using LiCor cosine corrected underwater Quantum Sensor (LI-192B).

7.3 Results

7.3.1 Relative gas vacuolation and % cells floating, *Microcystis*

7.3.1.1 Nitrogen-limited Microcystis *and buoyancy status before and after high irradiance*

At atmospheric pressure following the dark pretreatment the relative gas vesicle volume of *Microcystis* was lowest in cells grown in 0 μM nitrate (1375 relative side scatter units per cell, SSC) and highest in 10 and 100 μM nitrate, 1735 and 1734 SSC (figure 7.1). With sequential increases in pressure, up to 700 kPa, there was a decrease in the relative side scatter per cell to a minimum of 209 SSC for cells grown in 0 and 10 μM and 256 SSC in the 100 μM treatment. Gas vesicles collapsed irreversibly (Hayes and Walsby, 1984) and the minimum side scatter represents scatter due to the cell alone. This is consistent with other gas vesicle collapse-pressure curves (Walsby, 1973; Ganf *et al.*, 1989).

Following 24 hours exposure to an irradiance of 150 μmol m^{-2} s^{-1} the *Microcystis* cells displayed a change in gas vesicle volume (0 kPa) which was dependent upon the nitrogen concentration they initially received (figure 7.1). The relative gas vesicle volume of the 0 μM treatment decreased from 1375 SSC before light exposure to 1311 SSC after light incubation. The relative gas vesicle volume of *Microcystis* cells in 10 and 100 μM nitrate increased their gas vesicle content from 1735 and 1734 SSC to 1839 and 2036 SSC, respectively. The minimum mean side scatter values increased for each treatment: 0 μM, 209 SSC before light to 273 SSC after light; 10 μM, 209 SSC to 247 SSC; 100 μM, 256 SSC to 453 SSC. These increases in side scatter following light exposure are consistent with cell size increases (Li, 1994) due to accumulation of cellular carbohydrate (table 7.1).

The percentage of buoyant cells prior to and following light exposure was dependent upon the presence of sufficient gas vesicles to decrease the net cell density to a density less than water (998 kg m^{-3}). At the commencement of the experiment only 63% of the 0 μM treatment *Microcystis* cells were buoyant as opposed to 99% and 100% for the 10 and 100 μM treatments (figure 7.2). Upon the application of pressure the relative gas vesicle volume per cell decreased (figure 7.1) generating a concomitant loss of buoyancy (figure 7.2). After the application of

400 kPa 63% of *Microcystis* cells grown in 100 μM nitrate maintained buoyancy whereas the 0 μM and 10 μM treatments had only 0% and 8% of cells floating.

Light exposure caused the accumulation of carbohydrate in *Microcystis* grown in all nitrate concentrations (table 7.1). The carbohydrate content of cells increased from 9 to 24 pg cell^{-1} in the 0 μM treatment which decreased the proportion of buoyant cells from 63% to 29% (figure 7.2). Similarly in the 10 μM treatment carbohydrate increased from 4.96 to 27.3 pg cell^{-1} and caused 80% of cells to sink (99% to 19% floating). *Microcystis* cells grown in 100 μM had more gas vesicle than the other two treatments and although the carbohydrate content increased from 6.88 to 19.6 pg cell^{-1} only 7% of cells lost buoyancy.

	Carbohydrate content (pg cell^{-1})	
Nitrate concentration (μM)	**Prior to light exposure**	**+ 24 hours at 150 μmol m^{-2} s^{-1}**
0	9.00	24
	(0.3)	(7.3)
10	4.96	27.3
	(0.46)	(5.6)
100	6.88	19.6
	(0.54)	(5.4)

Table 7.1 Soluble carbohydrate content of *Microcystis* cells grown in 0, 10 or 100 μM initial nitrate concentration. Cells were pretreated in darkness for 14 hours prior to light exposure for 24 hours.

The volume of gas vesicles required to maintain buoyancy increased as carbohydrate accumulated which increased the cell density. To estimate the relative gas vesicle volume required to maintain buoyancy the percentage of cells floating was plotted against relative gas vesicle volume (figure 7.3). The critical gas vesicle volume was determined as the relative gas vesicle volume required to maintain buoyancy in 50% of cells. Prior to light exposure a mean relative gas vesicle volume of 1261 SSC supported buoyancy in 50% of cells but following high light treatment the critical gas vesicle volume became 1952 SSC.

7.3.1.2 Phosphorus-limited Microcystis *and buoyancy status before and after high irradiance*

The initial mean gas vesicle volume was greatest in cells grown in media with no added phosphate (1710 SSC) and lowest in the 10 μM treatment (1503 SSC; figure 7.4). Cells grown in the 0.5 μM treatment had a mean relative gas vesicle volume of 1695 SSC which remained the same after 24 hours of light exposure. The relative gas vesicle volume increased in the 10 μM sample from 1503 to 2075 SSC after the light incubation. Increasing pressure decreased gas vesicle volume and consequently the percentage of cells floating (figure 7.5).

Although the gas vesicle pool per cell was less in the 10 μM samples, following dark pretreatment, than the 0 and 0.5 μM samples the pressure required to render 50% of cells non buoyant was 375 kPa in 10 μM but only 117 kPa and 283 kPa in 0 μM and 0.5 μM, respectively (figure 7.5). This was because the carbohydrate in the phosphate limited cultures was 26.4 pg cell^{-1} in 0 μM and 20.7 pg cell^{-1} 0.5 μM compared with the 7.3 pg cell^{-1} in the 10 μM treatment (table 7.2). Following 24 hours of light exposure the carbohydrate content per cell increased to 37.5, 27.3 and 22.7 pg cell^{-1} in the 0, 0.5 and 10 μM treatments, respectively. The increased carbohydrate content did not change the percentage of cells floating in the 0 and 0.5 μM treatments but resulted in a loss of buoyancy of 21% in the 10 μM sample (figure 7.5).

The critical relative gas vesicle volume required to maintain buoyancy in 50% of cells was calculated to be 1551 SSC for the 0 and 0.5 μM P cells after the dark pretreatment and 1575 SSC after the light treatment (figure 7.6). The small difference between the critical gas vesicle volume before and after high light reflects the small change in gas vesicle volume and carbohydrate (figure 7.6; table 7.2). When phosphate saturated growth (10 μM P) the critical gas vesicle volume required to maintain 50% buoyancy increased from 1194 SSC after the dark to 2018 SSC after the light treatment. Following high light these cells had three times their initial carbohydrate concentration hence the increase in the gas vesicle volume required to maintain buoyancy.

Phosphate concentration (μM)	Carbohydrate content (pg cell^{-1}) Prior to light exposure	+ 24 hours at 150 μmol photons m^{-2} s^{-1}
0	26.4	37.5
	(2.1)	(1.6)
0.5	20.7	27.3
	(3.4)	(2.2)
10	7.3	22.7
	(3.1)	(4.3)

Table 7.2 Soluble carbohydrate content of *Microcystis* cells grown in 0, 0.5 or 10 μM initial phosphate concentration. Cells were pretreated in darkness for 14 hours prior to light exposure for 24 hours.

7.3.1.3 Light-limited Microcystis *and buoyancy status before and after high irradiance*

The initial gas vesicle volume of *Microcystis* grown at 10, 50 and 100 μmol m^{-2} s^{-1} were similar (Anova, $p<0.05$, n=3). After 24 hours light the gas vesicle volume in the 100 μmol m^{-2} s^{-1} treatment was 1426, whereas, the 50 and 10 μmol m^{-2} s^{-1} treatments were 1582 and 1671 SSC, respectively (figure 7.7). This conflicts with the experiment detailed in chapter 5 where relative gas vesicle volume of *Microcystis* increased with increasing light up to 100 μmol m^{-2} s^{-1}. In this example the gas vesicle volume-light response of *Microcystis* more closely resembled the response of *Anabaena circinalis* (chapter 5) and literature trends (Reynolds and Walsby, 1975; Kromkamp *et al.*, 1988).

The data show there was no carbohydrate accumulation per cell during the light period (table 7.3). Although there was a net accumulation of carbohydrate in each culture this carbohydrate was used to fuel growth which was dependent upon the light intensity the cells had previously been grown at: cells grown at 100 μmol m^{-2} s^{-1} had a growth rate of 0.75±0.1 ln units day^{-1}; for the 50 and 10 μmol m^{-2} s^{-1} treatments the growth rates were 0.43±0.03 and 0.21±0.13 ln units day^{-1}, respectively, over the 24 hour light period.

Light intensity (μmol m^{-2} s^{-1})	Carbohydrate content (pg $cell^{-1}$) Prior to light exposure	+ 24 hours at 150 μmol m^{-2} s^{-1}
100	4.5	4.5
	(0.45)	(0.81)
50	7.4	6.6
	(0.84)	(0.34)
10	14.8	15.5
	(0.17)	(0.17)

Table 7.3 Soluble carbohydrate content of *Microcystis* cells grown at 10, 50 or 100 μmol m^{-2} s^{-1} on a 12:12 light:dark cycle prior to experimentation. Cells were then pretreated in darkness for 14 hours prior to light exposure for 24 hours.

75% of cells initially grown at 10 and 50 μmol m^{-2} s^{-1} lost buoyancy during exposure to 150 μmol m^{-2} s^{-1} for 24 hours (figure 7.8). Only 48% of cells lost buoyancy in the 100 μmol m^{-2} s^{-1} treatment. The small changes in carbohydrate did not forecast the large decrease in buoyancy that was observed.

Prior to light exposure a relative gas vesicle volume of 700 SSC was sufficient to maintain buoyancy in 50% of cells initially grown at 10 μmol m^{-2} s^{-1} but a greater gas vesicle pool was required to maintain the same proportion floating in the 50 μmol m^{-2} s^{-1} and 100 μmol m^{-2} s^{-1} treatments (910 and 1020 SSC respectively). This suggests that the cell density in cells without gas vesicles was greatest in the 100 μmol m^{-2} s^{-1} treatment. Cells in this treatment had the lowest carbohydrate content which suggests that some other cellular constituent was more abundant in these cells and was contributing significantly to the cell density.

7.3.2 Relative gas vacuolation and % cells floating, *Anabaena*

7.3.2.1 Nitrogen-limited Anabaena *and buoyancy status before and after high irradiance*

Anabaena circinalis used in this experiment were grown in conditions which attempted to preclude atmospheric nitrogen. The treatments included here were also used in an experiment detailed in chapter 5 investigating gas vesicle accumulation and growth in different nitrate concentrations with no free N_2 in the ambient environment. It was concluded in that experiment that N_2 was not satisfactorily removed and nitrogen limitation was not induced. However, this

provided useful insight into the buoyancy characteristics of *Anabaena circinalis* in field populations which would potentially only be nitrogen limited and reduce the gas vesicle pool when light is in short supply and nitrogen fixation limited. The settings on the flow cytometer to measure side scatter were different for *Anabaena* and *Microcystis* and therefore the relative units are at a different order of magnitude. These units are relative and do not represent absolute differences between the species. Units for relative gas vesicle volume are relative side scatter units and are abbreviated to ASSC for *Anabaena*.

The initial gas vesicle volume was greatest in the 0 μM nitrate (70 ASSC) treatment and lowest in the cells grown at 100 μM (46 ASSC) (figure 7.10). With increasing pressure the relative gas vesicle pool decreased in a manner typical of gas vesicle pressure-collapse curves. The apparent critical pressure required to collapse 50% of gas vesicles was lower in *Anabaena* following a dark period (approx. 100-250 kPa) than *Microcystis* (300-400 kPa) which is consistent with other reports (Brookes *et al.*, 1994). At the beginning of the light period there was no significant difference in the percentage of cells floating in each treatment (Anova, $p=0.05$, $n=3$); 98% of filaments were floating in the 0 and 20 μM treatments and 81% in the 100 μM treatment (figure 7.11). After the application of 600 kPa no gas vesicles remained intact and no filaments were buoyant.

24 hours of light exposure had little impact on the proportion of floating filaments relative to the initial at atmospheric pressure (figure 7.11). However, due to the accumulation of carbohydrate there was a shift in the critical number of gas vesicles required to maintain buoyancy in 50% of filaments; from 43 ASSC to 49 and 55 ASSC in the 0 and 20 μM treatments and from 40 to 43 SSC in the 100 μM treatment (figure 7.12).

The carbohydrate data for the *Anabaena* in this series of buoyancy studies was spurious and is therefore not included in support of the other data. The error in the carbohydrate data could have been due to the large relative error involved in counting cells in coiled filaments. Alternatively the results could have been confounded by the fact that the carbohydrate content in the samples was approaching the limit of detection of the assay. Rapid buoyancy change has been shown previously to be due to carbohydrate change in *Oscillatoria* (Kromkamp and Walsby, 1990) and *Microcystis* (Brookes *et al.*, 1997; this chapter). Carbohydrate content of

Anabaena circinalis cells was successfully measured on samples taken from Chaffey Dam (data following) and it was demonstrated that carbohydrate per cell increased in response to light. Therefore the assumption was made that change in the carbohydrate content per cell was giving rise to the buoyancy changes documented here.

7.3.2.2 Phosphorus-limited **Anabaena** *and buoyancy status before and after high irradiance*

At the commencement of the light period the relative gas vesicle volume of *Anabaena* grown in 0, 0.5 and 10 µM phosphate was 56, 57 and 50 ASSC and following light exposure was 48, 54 and 62 ASSC, respectively (figure 7.13). At atmospheric pressure gas vesicles supported buoyancy in 98% of filaments in the 0 and 0.5 µM treatments before high light and 88% after the high light period (figure 7.14). 92% of filaments in the 10 µM treatment were buoyant preceding light exposure but during the light period buoyancy was lost in a further 7% of filaments.

The sequential collapse of gas vesicles and measurement of the proportion of the population floating allowed estimates of the minimum relative gas vesicle volume required to provide buoyancy to 50% of filaments. The critical gas vesicle volumes were calculated directly from figure 7.15. The critical gas vesicle volume of cells in the 0 µM P treatment remained constant before and after light exposure at 45 ASSC. For filaments grown in 0.5 µM initial phosphate concentration the critical gas vesicle volume shifted from 41 to 50 ASSC as cells accumulated more carbohydrate and consequently a greater gas vesicle volume was required to maintain buoyancy in these filaments. In the 10 µM phosphate the gas vesicle volume required by filaments to maintain 50% buoyancy increased from 35 to 58 ASSC.

7.3.2.3 Light-limited **Anabaena** *and buoyancy status before and after high irradiance*

The side scatter attributable to gas vesicles was greatest in *Anabaena* cells grown at 10 µmol m^{-2} s^{-1} (21 ASSC versus 15 and 16 ASSC for *Anabaena* grown at 50 and 100 µmol m^{-2} s^{-1}) and was calculated as the side scatter with intact gas vesicles (0 kPa) less the scatter when all the gas vesicles had been collapsed (600 kPa) (figure 7.16). There was no change in the gas vesicle volume before or after light exposure (figure 7.16). 600 kPa was required to collapse all the

gas vesicles in the light experiment whereas in the nutrient experiments only 400 kPa was applied. This suggests that cells in the light experiment had significantly less turgor pressure which may be caused by reduced photosynthesis.

Buoyancy did not change in samples grown in 10 and 50 μmol m^{-2} s^{-1} and then incubated at 150 μmol m^{-2} s^{-1} for 24 hours (figure 7.17). However, a decrease in the percentage of filaments floating was observed in cultures grown at 100 μmol m^{-2} s^{-1} following 24 hours at 150 μmol m^{-2} s^{-1} (44% cf. 60% floating before light exposure).

The critical gas vesicle volume required to maintain buoyancy in 50% of filaments was calculated to be 13, 22 and 33 ASSC initially and 12, 22 and 34 ASSC following light exposure for the 50, 10 and 100 μmol m^{-2} s^{-1} treatments respectively. The small change in critical side scatter following 24 hours of light exposure suggests the cells were inactive and did not respond in a predictable manner. The minimum critical gas vesicle volume in this experiment was 12 ASSC whereas in the phosphate experiment it was 35 ASSC and in the nitrate experiment, 45 ASSC. From these data and the low apparent turgor pressure it was concluded that the cells were inactive. Prior to the experiment cultures were growing in the predicted manner and what caused all the cultures to be inactive is unclear. The trends observed in the other experiments were not observed in this experiment and consequently less emphasis is placed on these results as their applicability and value is questionable.

7.3.3 Field study-Chaffey Dam

7.3.3.1 Is there evidence of buoyancy regulation in a field population of Anabaena circinalis?

The majority of filaments in laboratory cultures of *Anabaena circinalis* Ana330E were persistently buoyant (chapter 5, this chapter). However, culture conditions do not truly simulate the natural environment and it was possible that buoyancy regulation was occurring in field populations. A sampling strategy was designed to determine if there was loss of buoyancy in *Anabaena circinalis* filaments throughout the day as thermal stratification became established and cells in the surface waters were exposed to relatively high irradiances.

Sunrise was at 06:30, incident surface light intensity reached a maximum of 1614 μmol m^{-2} s^{-1} at 12:40, sunset was at 17:30 (figure 7.19). The attenuation coefficient was 1.02 ln units m^{-1} giving rise to a euphotic depth of 4.5 metres. As light penetrated the water column the surface water absorbed solar energy and depth-temperature differences became apparent at approximately 09:00. Thermal stratification increased until a maximum temperature difference (2.9°C) between the surface and the euphotic depth (4.5 metres) was measured at 13:30 (figure 7.20). Thermal stratification in the surface waters broke down in the afternoon and there was less than 1°C difference between 0.1 and 14 m by 21:00.

The diurnal stratification enabled buoyant cyanobacteria to float toward the surface and encounter light. At 08:00 light exposure was minimal and greater than 97% of filaments sampled were buoyant (table 7.4). Vertical profiles of the water column at 12:00 revealed that 48 % of filaments in the surface had lost buoyancy and non-buoyant filaments were observed at all depths.

	% filaments floating at time:		
Depth (m)	**08:00**	**12:00**	**16:00**
0-0.4	97	52	81
0.4-0.8	99	68	91
0.8-1.2	100	71	86
1.2-1.6	99	75	86
2.0-2.4	99	81	86
4.0-4.4	97	89	68
6.0-6.4	99	92	78
8.0-8.4	99	74	75

Table 7.4 Vertical profiles of buoyancy status of *Anabaena circinalis* filaments sampled from Chaffey Dam with a plankton trap on 15/04/97. The percentage of filaments floating was measured using a Sedgewick Rafter counting cell and counting the number of filaments on the top plane (floating) and on the base of the counting chamber (sinking).

The detection of vertical migration is often difficult in field population as cells migrate up or down depending upon their ballast, gas vesicle volume and specific physiology. Thus cells at a particular depth may have migrated from the water above or below the depth at which they were sampled. Below the euphotic zone (4.5m) light intensity was minimal and non-buoyant filaments below 4.5 m must have migrated there from higher depths or moved there via water

currents. Non buoyant cells at 8 metres depth have migrated or travelled on water currents at least 7 metres in 4 hours. The proportion of filaments floating at the surface increased to 81% at 16:00 which represents a loss of non-buoyant filaments and a gain of buoyant filaments.

Buoyancy regulation and vertical migration is often considered a mechanism to overcome the vertical separation of light and nutrients (Ganf and Oliver, 1982). To place the above results in context with nutrient scavenging potential the vertical distribution of nutrient concentrations was analysed (table 7.4a). A nutrient gradient was evident for all measured parameters; nutrient concentrations increased with depth below the mixed layer. The cells which were not buoyant at 8m depth must have originated from the top one metre (see below) and would have travelled deeper than their current depth as they were negatively buoyant. This would have exposed them to higher nutrient concentrations than they would have been exposed to at the surface.

Depth (m)	NH_3 (mg L^{-1})	TKN (mg L^{-1})	NO_3 (mg L^{-1})	NO_2 (mg L^{-1})	TP (mg L^{-1})	SRP (mg L^{-1})
0.25	0.285	1.23	0.23	0	0.086	0.034
2	0.325	1.01	0.21	0	0.074	0.048
4	0.33	1.01	0.18	0	0.068	0.049
6	0.33	1.01	0.17	0	0.084	0.053
8	0.33	1.34	0.25	0	0.098	0.059
10	0.35	1.01	0.21	0	0.092	0.056
12	0.42	1.34	0.22	0	0.118	0.065
14	0.5	1.46	1.0	0	0.163	0.089
16	0.62	1.57	1.3	0.48	0.207	0.109
18	0.7	1.68	1.7	0	0.247	0.180

Table 7.4a Nutrient profile taken from Chaffey Dam, 16/04/97. Nutrient analysis was performed by Tamworth Environmental Laboratories.

In the natural population non-buoyant filaments were recorded as deep as 8 metres (table 7.4a). To establish at what depths the light intensity and subsequent photosynthesis was sufficient for cells to accumulate enough carbohydrate to overcome buoyancy another experiment was undertaken. *Anabaena circinalis* was bottled and suspended at different depths within the reservoir. The buoyancy of cells was recorded initially and twice during the light period. The light dose the cells received was estimated using the attenuation coefficient and data collected at the meteorological station.

Initially (08:00) all filaments were buoyant but 15 % of filaments lost buoyancy in the near-surface bottle (67% surface irradiance) by 12:00 and by 17:00 only 61% of filaments remained floating (table 7.5). At 0.9 metres buoyancy was lost in 9% of filaments by 17:00. At all other depths insufficient carbohydrate was accumulated to negate the lift provided by gas vesicles. This suggests that the non buoyant filaments observed at all depths in the above sampling table 7.4) must have originated from within one metre of the surface (the maximum irradiance was similar for both days 10/04/97 and 15/04/97; 1600 µmol m^{-2} s^{-1}). By 08:00 24 hours after the commencement of the experiment and following approximately 13 hours of darkness buoyancy was restored in filaments at all depths.

Anabaena filaments in the bottle which was moved alternately between 0.3 and 4.5 metres did not lose buoyancy even though the light dose they received over the day (13.23 µmol m^{-2}) exceeded the light dose of the 0.3 m sample at 12:00 (12.75 µmol m^{-2}) which generated buoyancy loss in 15% of filaments.

		08:00	12:00		17:00		08:00 +24 hours
Depth (m)	Percent surface irradiance	Percent filaments floating	Light dose µmolm^{-2}	Percent filaments floating	Light dose µmolm^{-2}	Percent filaments floating	Percent filaments floating
0.3	67	100	12.75	85 (6)	25.87	61 (2)	96
0.9	30	100	5.71	99 (1)	11.58	91 (1)	100
1.9	10	100	1.90	100	3.86	100	100
3.5	3	100	0.57	100	1.16	100	100
4.5	1	100	0.19	100	0.38	100	100
Dark	0	100	0	97 (3)	0	100	100
Moving (0.3-4.5)	1-67	100	5.40	100	13.23	99	100

Table 7.5 *Anabaena circinalis* collected from Chaffey Dam was bottled at suspended in the reservoir. The percentage of filaments floating was estimated throughout the day. The light dose was estimated using the light attenuation coefficient and surface irradiance data which was logged continuously at the meteorological station. (standard deviation in parentheses)

A computer model of vertical migration (Kromkamp and Walsby, 1990; chapter 6) was modified to simulate the vertical migration of the *Anabaena* population at Chaffey Dam on April 15, 1997. The model used physical data collected at the meteorological station moored in Chaffey Dam and laboratory data measuring the rate of density change of *Anabaena* in response to light (chapter 6). The surface irradiance throughout the day was approximated by a sine function of the maximum irradiance (1614 μmol m^{-2} s^{-1}, figure 7.19). The attenuation coefficient was 1.02 ln units m^{-1}.

Figure 7.20 illustrates vertical temperature profiles which provide an indication of water column stability. Throughout the night the surface waters mixed but as light penetrated the water column post dawn thermal stratification established. Kromkamp and Walsby (1990) simulated the vertical migration of plankton in a water column which was nocturnally mixed and stratified through the day. The effect of nocturnal mixing was modelled by overlaying simulations of single colonies starting at different depths. At dawn water stability increased and phytoplankton movement was assumed to be driven by buoyancy regulation not water movement.

The model parameters describing vertical migration in Chaffey Dam on 15/04/97 were: light attenuation coefficient k=1.02 m^{-1}; form resistance = 1.5; maximum surface irradiance =1614 μmol m^{-2} s^{-1}; packing efficiency A=1; light period =13 hours; starting density = 980 kg m^{-3}; density range = 980 - 1060 kg m^{-3}; average filament radius was approximated from the natural population, r=90 μm and were tangled helical trichomes (see Booker and Walsby, 1979, figure 3, plate b); the start depths 0, 2, 4, 6, 8m were used simulating a well mixed surface layer. The constants describing the rate of density change of *Anabaena circinalis* in response to light are given in table 6.1. The simulation started at dawn which was set as 07:00. Therefore 5 and 11 hours after dawn in the simulation (figure 7.21) correspond to 12:00 and 16:00, respectively, in table 7.4.

In the simulation of buoyancy regulation of *Anabaena* in Chaffey Dam (figure 7.21) the filaments which started at the surface did not accumulate sufficient carbohydrate to overcome buoyancy until 4 hours after dawn. They then migrated downward to a maximum depth of 7.3 metres approximately three hours after sunset. Filaments starting at two metres depth reached the surface three hours after dawn and persisted there for one hour before carbohydrate

accumulation overcame buoyancy. Filaments starting deeper than four metres did not reach the surface during the 13 hour daylight period. Although the filaments migrated to the depth that negatively buoyant filaments were found in the field sampling they did not travel as rapidly to that depth and the computer simulation did not match the field observations.

7.3.3.2 What is the effect of nutrients on buoyancy status of **Anabaena circinalis?**

Manipulating the ambient nitrate and phosphate concentrations did not alter the gas vesicle pool to a critical level where carbohydrate accumulation could overcome buoyancy in the *Anabaena circinalis* culture used in laboratory experiments (ANA330E). Buoyancy regulation and loss of buoyancy was observed in a natural population of *Anabaena circinalis* in Chaffey Dam (above). This presented an opportunity to investigate the influence nutrients had on the buoyant status of *Anabaena circinalis* suspended at different depths and receiving different irradiances.

Reservoir water containing a natural population of *Anabaena circinalis* was sampled from Chaffey Dam, bottled and divided into two treatments. The first treatment had no additional nutrients added and the second had nitrate and phosphate added in concentrations saturating for growth. Bottles were suspended in the reservoir at dusk approximately 38 hours prior to the commencement of sampling to allow cells to respond to the nutrient conditions. At 07:30 (initial) 96% of filaments with no added nutrients suspended at 0.3 metres were floating. 100% of filaments in all other treatments were floating (table 7.6)

Following a day of sunlight (maximum surface irradiance 1762 μmol m^{-2} s^{-1}) 38% of *Anabaena* filaments with no added nutrients suspended at 0.3m remained floating while 88% retained buoyancy in the added nutrient treatment. At 0.9 metres 31% of filaments with no added nutrients lost buoyancy but when nutrients were available all filaments were buoyant. All filaments suspended at 4.5 metres or moving hourly between 0.3 and 4.5 metres were buoyant.

Depth (m)	Control-no added nutrients 07:30	17:00	Nutrients added as nitrate and phosphate 07:30	17:00	Ave. light intensity µmol m^{-2} s^{-1}
0.3	96	38 (9)	100	88 (1)	436
0.9	100	69 (12)	100	100	195
4.5	100	99 (1)	100	100	7
Moving (0.3-4.5)	100	100	100	100	233

Table 7.6 The percentage of filaments floating in bottles suspended at three depths, and bottles moved hourly between 0.3 and 4.5 m. No nutrients were added to the controls but nitrate and phosphate were added to simulate nutrient enrichment. The average light intensity experienced at the various depths was calculated from continuous records of solar irradiance.

7.3.3.3 Estimation of floating velocity of Anabaena circinalis

An estimate of floating velocity of *Anabaena* was first obtained using the fluorometric method of Titman (1975). All filaments in this trial were floating, the average filament radius was 45 µm and the measured floating velocity was 2.88 m day^{-1}. A filament of similar size (45 µm) with a form resistance of 1.5 and density of 990 kg m^{-3} had a velocity of 2.03 m day^{-1} according to Stokes equation (chapter 6). The floating velocity measured using the method of Titman (1975) therefore agrees with predicted floating velocities calculated using Stoke's equation. Slight discrepancies may be due to error in the estimation of form resistance or cell density.

The *Anabaena circinalis* filaments at Chaffey Dam included numerous examples which were large and tangled. It was difficult to measure the floating velocity of these filaments using the method of Titman (1975) because the sample volume was small (approximately 4 mL), the chlorophyll fluorescence of each filament large and one measurement took approximately 2 hours to record. This resulted in individual filaments having a large impact on the biomass losses. Also numerous measurements could not be taken simultaneously as the fluorometer only held one sample cuvette. An alternative technique was developed which used a larger sample volume and allowed numerous treatments to be simultaneously measured.

7.3.3.4 An alternative technique to estimate floating velocity of cyanobacteria

To validate that Fo could be used as a surrogate for biomass a series of 50 mL tubes were set up with replicate algal samples in each. The top 5% of a single tube was sampled at each time and cell number and Fo measured. This gave rise to a time series of cell number and chlorophyll accumulation at the surface of the tubes.

The initial sample had 90,230 cells mL^{-1} of *Anabaena circinalis* and 45,977 cells mL^{-1} of *Microcystis aeruginosa*. The cell diameter of *Anabaena circinalis* was 4.67 µm (SD=0.71, n=72) and approximated a spherical shape of volume 53.32 μm^3. *Microcystis* cells had a mean diameter of 3.12 µm (SD=0.4, n=35) and an approximate cell volume of 15.9 μm^3. Cell number was converted to biovolume and plotted against Fo measured using the PAM (figure 7.22). There was a good correlation between Fo and biovolume of *Anabaena* (r^2=0.96). In the initial sample *Anabaena circinalis* accounted for 67% of cell number and 87% of cell volume. *Microcystis aeruginosa* made up the balance and accounted for 33% of cell number and 13% of cell volume. The biovolume of *Microcystis* was also correlated with Fo (r^2=0.75).

Reynolds (1984, table 7) identified that *Anabaena circinalis* had 50% more chlorophyll per volume than *Microcystis* and given that *Anabaena* cells have a volume approximately three times greater than *Microcystis* the chlorophyll content per cell of *Anabaena circinalis* is about five times that of *Microcystis*. Therefore *Anabaena* in the water sample would account for approximately 10 times more chlorophyll than *Microcystis*. The relationship between Fo and *Microcystis* biovolume is therefore probably fortuitous because *Anabaena* dominates in cell number (66%), biovolume (87%) and has more chlorophyll per volume than *Microcystis*. The good correlation between Fo and *Anabaena* biovolume validated this technique to estimate biomass and its use in future floating rate experiments.

The measurement of flotation rate was made by measuring the rate of accumulation of biomass at the surface (top 10%) of a series of tubes. The biomass (Fo) in the top 5 mL represented 10% of the total biomass and was set as 100% of original Fo. Increases in Fo above 100% represent flotation of filaments into the surface 5 mL and were expressed as a percentage of original Fo. If filaments were all the same size and floated at a constant rate then the increase in Fo at the surface should be linear with time. However, the accumulation was not linear and it

was evident that *Anabaena* filaments exhibited more than one rate (figure 7.23). The data could generally be described by the sum of two straight lines, a slow floating rate and a fast rate. All *Anabaena* in the fast class reached the top of the sample tube during the experiment. The slow rate was determined by using linear regression to calculate the slope of Fo increase after the fast class had reached the surface. Fluorescence attributable to the slow class was back calculated and subtracted from the initial Fo (100%) which gave the Fo of the fast class. It was then possible to calculate the proportion of the total Fo (10*initial Fo at surface) which was in each class (figure 7.23, B= proportion in fast class; C= proportion in slow class). The flotation rate was calculated using a formula adapted from Titman (1975).

$$\textbf{Flotation rate} = d\ (t_{0.5})^{-1}$$

where d was the mean distance travelled (400 mm) and $t_{0.5}$ was the time taken for 50% of the total biomass to accumulate at the surface.

Bottles containing 87% *Anabaena circinalis* and 13% *Microcystis aeruginosa*, by biovolume, were suspended in Chaffey Dam at depths corresponding to 70%, 30% and 1% of surface irradiance (0.3, 0.9, 4.5m) at 10:30 on 14/04/97. The mean filament diameter was 90.3 µm initially (table 7.7). The size of filaments was measured at 08:00, 13:30 and 16:30 the following day (table 7.7) and ranked into size classes which were compared with the initial size classes to evaluate if there were any significant differences in size distribution (Kolmogorov-Smirnov, p=0.05, significant differences at $p<0.05$ were indicated with * and $p<0.01$ with **, table 7.7).

Filaments suspended at 70% I_o had a similar size distribution at 08:00 and 13:30 but were significantly larger at 16:30, and at 08:00 in the 30% I_o treatment. Although the mean diameters of filaments suspended at 30% I_o at 13:30 (121.6 µm) and 16:30 (124.3 µm) were greater than the initial (90.3 µm) the size distribution was not significantly different. Filaments held at 1% of surface irradiance showed a significant increase in size relative to the initial. At 13:30 and 16:30 (table 7.7) the mean greatest axial linear dimension (GALD; Reynolds, 1984) had doubled from 90 to greater than 180 µm.

Light % surface	Time	Flotation rate (metres hour^{-1}) fast class	slow class	total pop	% fast class	Mean GALD (μm)	CHO pg cell^{-1}
Initial		0.744	0.026	0.389	32	90.3	
70	08:00	0.732	0.04	0.060	11	101.8	14.39
						ns	(2.7)
	13:30	0.387	0.034	0.046	8	101.9	29.22
						ns	(0.65)
	16:30	0.124		0.007	4	108.6	23.35
						*	(1.38)
30	08:00	0.598	0.03	0.144	26	127.5	14.55
						**	(4.44)
	13:30	0.837	0.02	0.036	16	121.6	28.56
						ns	(2.35)
	16:30	0.853	0.031	0.061	17	124.3	24.19
						ns	(0.4)
1	08:00	0.919	0.022	0.215	33	133.3	15.89
						*	(2.5)
	13:30	1.351	0.044	0.835	33	184.5	11.35
						**	(4.67)
	16:30	3.172	0.116	2.010	42	202.9	12.47
						**	(0.66)

Table 7.7 Algal suspension containing 87% *Anabaena circinalis* and 13% *Microcystis aeruginosa* was suspended at 0.3, 0.9 and 4.5 metres corresponding to 70%, 30% and 1% of surface irradiance. The mean diameter, size class distribution and carbohydrate content of cells were measured at three times during the day. Floating velocities were measured by calculating the rate of accumulation of biomass at the surface (5 mL) of a 50 mL tube. Biomass was estimated as Fo with a PAM fluorometer. (*,$p<0.05$; **,$p<0.01$; ns-not significant in comparison of size class distribution of each treatment with initial, Kolmogorov-Smirnov).

The surface irradiance (I_o) on 15/04/97 followed a similar sine pattern as illustrated in figure 7.19 with a maximum irradiance of 1614 μmol m^{-2} s^{-1} at 12:40. The floating velocity of the cyanobacteria when the experiment was deployed was 0.744 metres hour^{-1} in the fast fraction and 0.026 m h^{-1} in the slow fraction. This was similar to the floating velocity of filaments in the 70% light treatment at 08:00 the following day, 0.732 and 0.04 m h^{-1} in the two fractions (table 7.7). However, the proportion of filaments in the fast class at 70% I_o, at 08:00 (11%) was less than the initial (32%) which resulted in a slower flotation rate for the entire population

(0.060 vs 0.389 m h^{-1} initially). As the surface irradiance increased cells suspended at 70% I_o photosynthesised and accumulated carbohydrate which increased the net cell density and decreased the floating velocity of the fast fraction to 0.387 m h^{-1} by 13:30 and 0.124 m h^{-1} at 16:30. The proportion of the population in the fast class also decreased from 11% at 08:00 to 4% at 16:30 and the mean velocity of whole population decreased from 0.060 to 0.007 m h^{-1} over the same time period.

Filaments experiencing 30% of surface irradiance displayed an increased fast rate at the second and third sampling times relative to the 08:00 sample however the proportion of the population in that fraction decreased from 26% to 16% and the mean velocity of the entire population decreased from 0.144 to 0.036 m h^{-1} in the 5.5 hours between the first two sampling times. Between 08:00 and 16:30 the carbohydrate content of cells at the 30% treatment increased from 14.55 to 24.19 pg $cell^{-1}$ which was similar to the change in carbohydrate in the 70% I_o treatment (14.39-23.35 pg $cell^{-1}$) but the mean flotation velocities were greater in the 30% treatment. This could be due to the 30% I_o filaments (biomass units) being slightly larger. Alternatively, the 70% I_o filaments may have had relatively less gas vesicles than the 30% I_o filaments due to growth dilution, gas vesicle collapse by turgor or increased gas vesicle production relative to growth in the 30% treatment.

There was little change in the carbohydrate of filaments experiencing 1% of surface irradiance, 15.89 ± 2.5 pg $cell^{-1}$ at 08:00 and 12.47 ± 0.66 pg $cell^{-1}$ at 16:30. However, the mean filament (biomass unit) diameter and the velocities of both the slow and fast classes increased as did the proportion of filaments in the fast class. This resulted in greater mean velocities (0.215 m h^{-1} at 08:00, 0.835 m h^{-1} at 13:00 and 2.010 m h^{-1} by 16:30).

7.4 Discussion

7.4.1 Buoyancy of *Microcystis aeruginosa*

Microcystis aeruginosa fits some aspects of the hypothesis of persistent buoyancy proposed in the introduction but differs in others. The limitation of nitrogen resulted in a decrease in the gas vesicle volume to the extent that it was unable to maintain cell buoyancy upon the accumulation

of carbohydrate. When nitrogen was at a saturating concentration (100 μM) persistent buoyancy was observed in 93% of cells. However, when the phosphate concentration was manipulated (figure 7.5) the greatest buoyancy loss was observed in the highest P treatment (68% remaining floating). The high P and N treatments has were grown in similar media but there was heterogeneity in the buoyancy response to light. This could partially be explained by differences in photosynthetic activity or storage compounds which altered net cell density. Regardless of heterogeneity a significant proportion of the population was positively buoyant even after 24 hours of exposure to high light.

Nitrogen-limited *Microcystis* had a greater carbohydrate content per cell than nitrogen replete cells. Following high light exposure for 24 hours the carbohydrate content per cell was similar for all treatments. However, the rate of carbohydrate accumulation would have varied depending on photosynthetic efficiency (chapter 6, Geider *et al.*, 1993) and the partitioning of fixed carbon to carbohydrates and lipids (Herzig and Falkowski, 1989). It is possible that some N-limited cells would display persistent buoyancy because carbon fixation could not overcome gas vesicle buoyancy as seen in figure 6.2.

Although the cells grown in limiting phosphate concentrations had less gas vesicles than the high phosphate treatment, following the light treatment (figure 7.4), they displayed greater buoyancy than the high P treatment (figure 7.5). Phosphate limited cells metabolise stored polyphosphate to satisfy the cellular phosphate demand which results in a decrease in the polyphosphate concentration (Jacobson and Halmann, 1982). Polyphosphate has a density of 2170 kg m^{-3} (Jacobson and Halmann, 1982) and therefore a reduction in the polyphosphate pool would reduce the net cell ballast and less gas vesicles would be required to achieve positive buoyancy. The metabolism of carbohydrate is dependent upon nutrient availability (Konopka *et al.*, 1987b, Turpin, 1991) and phosphate limited cultures were less efficient at metabolising carbohydrate, however, only a small percentage of the population lost buoyancy. The reduction in polyphosphate in the phosphate limited treatments offset the carbohydrate accumulation and the net ballast could not overcome buoyancy.

Light limitation had a similar affect to nitrogen limitation on cell buoyancy which was not predicted from the hypothesis in the introduction. 99% of cells grown at 10 and 50 μmol m^{-2} s^{-}

^{1} were buoyant following a dark pretreatment but after an extended light exposure only 25% remained buoyant although these cells had more gas vesicles than the 100 μmol m^{-2} s^{-1} treatment which retained 52% buoyancy. A relative gas vesicle volume of 1420 SSC maintained buoyancy 50% of cells grown at 100 μmol m^{-2} s^{-1} but a much greater gas vesicle volume was needed by cells grown at 10 and 50 μmol m^{-2} s^{-1} to maintain buoyancy because these cells accumulated more carbohydrate (table 7.3). Carbon fixed in cells originally grown at 100 μmol m^{-2} s^{-1} was used in growth which was much greater than in the other two light treatments. Ward and Wetzel (1980b) state that laboratory cultures take 48 hours to adapt to a new light regime which is evident here as although cells received the same light treatment for 24 hours their growth rate was dependent on their previous light history. Cells circulating through a natural light gradient show greater plasticity to changing light regimes than was observed in cultures maintained at a single light intensity (Kromkamp *et al.*, 1992). Cells grown at different light intensities had the lowest carbohydrate concentration per cell of all the treatments, however, it is unclear what, besides carbohydrate, was contributing to the excess ballast giving rise to the significant loss of buoyancy.

Field based examinations of *Microcystis aeruginosa* buoyancy have shown both persistent buoyancy (Reynolds, 1973, Robarts, 1984) and buoyancy regulation (Ganf, 1974; Ibelings *et al.*, 1991a, b). In the study of Ibelings *et al.* (1991a, b) although buoyancy loss was observed in *Microcystis* a significant proportion of the population (>50%) retained buoyancy. Visser *et al.* (1996) found positive buoyancy in *Microcystis* sampled near a bubble plume destratifier but buoyancy was lost in colonies outside of the turbulent zone. The heterogeneity in buoyancy response has been attributed to different rates of carbohydrate accumulation and different gas vesicle volume (Konopka *et al.*, 1987a). Therefore, in a single population of *Microcystis* there may be individual colonies which display persistent buoyancy and form surface accumulations with the onset of thermal stratification and other individuals which display diel changes in buoyancy status and migrate vertically. Irradiance and nutrient availability may then alter the cell's buoyancy mechanisms and cause a switch to or from persistent buoyancy.

7.4.2 Buoyancy of *Anabaena circinalis*

Loss of buoyancy was minimal in *Anabaena* cultures grown at different nitrogen and phosphorus concentrations, however, there was some loss of buoyancy of cells grown at 100 μmol m^{-2} s^{-1}. Filaments used in the light experiment appeared to be inactive as they had less turgor pressure than the nutrient treatment filaments and there was no shift in the curves relating % of filaments floating to the gas vesicle volume (figure 7.18). It is unclear what caused this inactivity because at the commencement of the experiment cells displayed light dependent growth indicating activity.

From the laboratory studies it was apparent that *Anabaena circinalis* fitted the model of persistent buoyancy as although there was some buoyancy loss the majority of the population maintained persistent buoyancy. To extend this laboratory result to a natural population a field experiment was designed. The principal aim was to identify if buoyancy loss was occurring and the influence nutrients and light had on this process.

7.4.3 *Anabaena circinalis* at Chaffey Dam

Heterogeneity in the rate of vertical movement of individuals, vertical position and light exposure results in a population which at any one depth consists of sinking and floating biomass units travelling at different speeds. Consequently, it is difficult to track algal movement and therefore loss of buoyancy was considered to be indicative of buoyancy regulation and ability to migrate vertically.

Anabaena circinalis filaments sampled from the natural population did display loss of buoyancy contrary to the laboratory results but supported by Reynolds (1972) who measured negative flotation on freshly harvested filaments. The addition of nutrients resulted in an increased resistance to buoyancy loss which further supports the persistent buoyancy hypothesis. The increased resistance to sinking could have been due to greater gas vesicle production and more efficient carbohydrate metabolism. Walsby *et al.* (1989) reported persistent buoyancy in 88.9% of filaments after 9 hours of surface light intensities but at the end of the following day just 44.9% were buoyant. This represents the switch from persistent buoyancy to the ability to regulate and lose buoyancy in response to high irradiance and perhaps induced nutrient limitation within the bottles.

7.4.4 The implications of persistent buoyancy

Persistent buoyancy must be distinguished from bloom formation which is a different phenomena. Persistent buoyancy is a physiological state of a cell, colony or filament and refers to a cell maintaining buoyancy throughout the day whether the water column is mixed or stratified. Bloom formation is the accumulation of cells at the surface and may consist of persistently buoyant cells and cells which have reached the surface during their diel vertical migration. The computer simulation of vertical migration (Kromkamp and Walsby, 1990) illustrates that a surface accumulation need not be static but cells can join and leave the "scum" continuously.

Persistent buoyancy may be a phenomena peculiar to regularly mixed or eutrophic systems but it is an important feature from the cyanobacteria's point of view. Persistent buoyancy would not be advantageous when the surface water is not wind mixed and the population persists at the surface for extended periods. This leads to photo-oxidation of photosynthetic pigments (Eloff *et al.*, 1976) and cell senescence (Reynolds, 1973). The formation of "scums" or surface accumulations caused by cell buoyancy and light wind conditions (Hutchinson and Webster, 1994) lead to self-shading with perhaps the most extreme example being the "hyperscums at Hartbeespoort Dam, South Africa (Robarts, 1984; Robarts and Zohary, 1984; Zohary and Breen, 1989; Zohary and Madeira, 1990) which also reduced free gas exchange (Zohary and Madeira, 1990).

Persistent buoyancy can also lead to photoinhibition (Hader *et al.*, 1997) although it has been reported that photoinhibition did not occur in cultured *Microcystis* (Stone and Ganf, 1981). The light source used by Stone and Ganf (1981) may have lacked the longer UV wavelengths (280-340 nm) which are considered photoinhibitory (Falkowski, 1984). Robarts and Zohary (1992) found strong surface photoinhibition and Ibelings and Mur (1991) suspected some photoinhibition with their microelectrode work on *Microcystis* scums with small colonies displaying greatest photoinhibition.

Studies comparing the photosynthetic performance of bottled cells maintained at a single depth and cells migrating vertically show, more often than not, that it is advantageous to change vertical position and light regime to avoid photoinhibition (Falkowski, 1984).

Populations which typically display persistent buoyancy may require another forcing factor to induce sinking. Temperature may be such a factor. A decrease in buoyancy and sedimentation of cyanobacteria may occur in autumn (Reynolds, 1973) as the water temperature decreases and respiration of accumulated carbohydrate is less efficient (Ganf, 1974; Reynolds *et al.*, 1981; Thomas and Walsby, 1986; Robarts and Zohary, 1987; Visser *et al.*, 1996).

7.4.5 Vertical movement in Chaffey Dam

Sampling of the natural *Anabaena circinalis* population at Chaffey dam revealed that this species did lose buoyancy in response to light as Reynolds (1972) found. Bottle experiments suspended at different depths revealed that buoyancy loss only occurred at depths less than 0.9m by midday. Therefore non buoyant filaments sampled as deep as 8m which were non-buoyant must have originated from within the top one metre of the water column. This represents a downward velocity of approximately 2 metres hour^{-1}. Previous estimates of *Anabaena* floating and sinking velocity have been in the order of 0.004-0.21 metres hour^{-1} (Reynolds and Walsby, 1975; Seip and Reynolds, 1995). The *Anabaena* filaments were large intertangled coils and were large enough to be macroscopic. Their fast velocity reflects this as they displayed a velocity more often observed in large colonies of *Microcystis* (Ganf, 1974). A maximum flotation velocity of 3.172 metres hour^{-1} was measured directly for *Anabaena circinalis* (table 7.7) which shows that such velocities are possible.

As the filaments moved downwards they experienced a nutrient gradient and therefore the vertical migration increased the nutrient pool available to the filament. Some filaments were not buoyant at eight metres depth and would have migrated even deeper in the water column where nutrient concentrations were greater, particularly soluble phosphate.

A computer simulation (Kromkamp and Walsby, 1990) was used to model the vertical migration of *Anabaena* at Chaffey Dam using laboratory data on density change in response to irradiance. On the day of sampling the water column was nocturnally mixed and it was assumed that the algae were evenly distributed through the mixed layer. To simulate this numerous simulations were overlaid with colonies starting at different depths (Kromkamp and Walsby, 1990). Although the simulation showed non-buoyant colonies did migrate to about 7.5 metres they did not reach this depth until approximately 16 hours after dawn. In reality

colonies migrated to 8 metres in 4 hours. The discrepancy between the observed and modelled responses may be explained by a number of physical and biological factors.

Gas vesicle collapse by turgor pressure was observed in *Anabaena flos-aquae* (Oliver and Walsby, 1984; Kinsman *et al.*, 1991). The gas vesicles of *Anabaena circinalis* are weak enough to be collapsed by turgor pressure (Brookes *et al.*, 1994) although limited gas vesicle collapse has been observed in this species (chapter 5). It is possible that gas vesicle collapse was contributing to the loss of buoyancy observed in the field but was not accounted for in the computer simulation which models density change based solely on changes in the density of cellular constituents. Another factor which could have lead to differences between observed and modelled vertical migration is heterogeneity in filament size. The modelled population was of a single size representing the population average. In reality their was a large range of different sizes, larger filaments would migrate more rapidly although the maximum depth would be similar to smaller filaments (Kromkamp and Walsby, 1990). Coils could also migrate at different rates depending on their orientation (Davey and Walsby, 1985).

Alternatively the laboratory and field populations may have displayed differences in their physiology. The laboratory data were collected by measuring density change of *Anabaena* at fixed irradiances whereas the photophysiology of the field population needed to be flexible to efficiently capture light of different wavelength and intensity. Differences in the fixation of carbon and respiration of photosynthates could also explain some of the observed differences. Small water currents may override the buoyancy regulation of cyanobacteria and force cells from the surface to depth.

To gain better insight into algal vertical movement in field populations techniques which "track" algal movement will be valuable. These include FDA and flow cytometry (chapter 3) and using fluorescence parameters (Whittington *et al.*, 1996) to determine the previous light history of the organism to allow estimates of where it has migrated.

7.4.6 Floating velocity of *Anabaena circinalis*

The technique developed to measure floating velocity of *Anabaena circinalis* proved to be consistent and allowed multiple measurements to be made simultaneously. The good

relationship between Fo and biovolume increased confidence in fluorometry to measure biomass changes in this short experiment. Furthermore, it could be assumed that the measurements were of *Anabaena*, although *Microcystis* contaminated the sample, because *Anabaena* made up the bulk of the biovolume and has greater chlorophyll content per cell than *Microcystis*.

The flotation rate of the total population suspended at 70% of the surface irradiance was smaller at 08:00 than the initial flotation rate of the *Anabaena* when the experiment was set up approximately 22 hours earlier (table 7.7). This could be due to a carbohydrate accumulation or reduction in gas vesicle volume due to either growth dilution (chapter 5, Hayes and Walsby, 1984; Konopka *et al.*, 1987) or turgor induced collapse (Oliver and Walsby, 1984). Walsby *et al.* (1989) also observed a decrease in buoyancy of *Anabaena minutissima* on the second day of a field trial with bottles suspended at different depths. It is difficult to conclude what gave rise to the loss of buoyancy on the second day in their experiment because ballast and gas vesicle volume were similar on day one and two (Walsby *et al.*, 1989). In the Chaffey Dam experiment the fast and slow rate are about the same for the two days and it is the proportion of filaments in each class which gives rise to the different total population rates.

The previous experiment investigating buoyancy in *Anabaena* which had nitrogen and phosphorus added found buoyancy was retained in 88% of filaments following high irradiance. Although buoyancy was not lost by these filaments the accumulated carbohydrate still acts as ballast and had an affect on the floating velocity by changing the net filament density relative to the density of water. Change in carbohydrate did not account for all the differences in floating rate observed between *Anabaena* at different depths. Filaments suspended at 70% surface irradiance had a flotation rate of 0.007 metres hour^{-1} and filaments at 30% irradiance had a flotation rate of 0.061 metres hour^{-1} but the carbohydrate content per cell and filament size were not significantly different. The difference in flotation velocity was probably attributable to a reduction in gas vesicles in the 70% filaments from turgor pressure collapse of gas vesicles, dilution with growth or an increased gas vesicle pool in cells experiencing lower light intensities (chapter 5).

A decrease was observed in the number of filaments in the fast class and the total population velocity throughout the day in the 70% and 30% treatments which is due to a rise in cellular carbohydrate. Filaments at 1% increased their flotation velocity through the day in both the fast and slow class although there was no change in carbohydrate content per cell. The increased velocity could be attributed to an increase in the filament size and perhaps some increase in gas vesicle volume (chapter 5). Smith and Gilbert (1995) found seasonal variability in filament length but the rapid increase in filament size observed in this study was too rapid to be due to growth alone and appeared to be due to tangling of *Anabaena* trichomes as a strategy to increase size, floating velocity and light capture. Booker and Walsby (1979) postulated that tangling of helical forms may be a strategy small filaments could employ to increase size and floating velocity. This was shown to be the case for *Anabaena circinalis* (Reynolds, 1972) and *Oscillatoria* by Walsby *et al.* (1983). They found filaments aggregated to form larger colonies which moved more rapidly than single filaments. This appears to be an excellent strategy to increase floating velocity and move into a more favourable light climate.

The floating velocity of filaments has implications for cell survival. If the velocity is slow then the filament or colony may encounter localised nutrient limitation but if larger it can travel more quickly and scavenge a greater volume of water for nutrients (Brookes *et al.*, 1997). Travelling to the surface also enhances the light climate the filament is exposed to (Humphries and Lyne, 1988; Walsby *et al.*, 1997). However large colonies may display the "package effect" (Kirk, 1983) where there is self shading of inner cells, although this can be reduced by reducing the internal pigment concentration with increasing size (Augusti and Philps, 1992). The strategy observed here where colonies aggregate and disperse when necessary appears to be a very effective strategy to maximise light capture. Small filaments in nocturnally mixed water bodies may risk extended dark exposure and possible extinction (Selvin *et al.*, 1989) because they have low floating speeds, however, if they aggregate and float faster they increase light exposure and survival rate.

Faster colonies will reach the water surface and receive maximum irradiance but this offers a greater chance of photoinhibition than small colonies would encounter. However, in the study of Ibelings and Mur (1991) small colonies (<55 μm) showed the greatest inhibition but they

postulated that in natural populations the large colonies may shield small colonies from inhibitory light intensities if there are significant surface accumulations. Floating quickly is particularly advantageous following a deep mixing event. Walsby *et al.* (1997) calculated that net photosynthesis of the *Aphanizomenon flos-aquae* population increased by three times by floating upwards into higher irradiance following deep mixing.

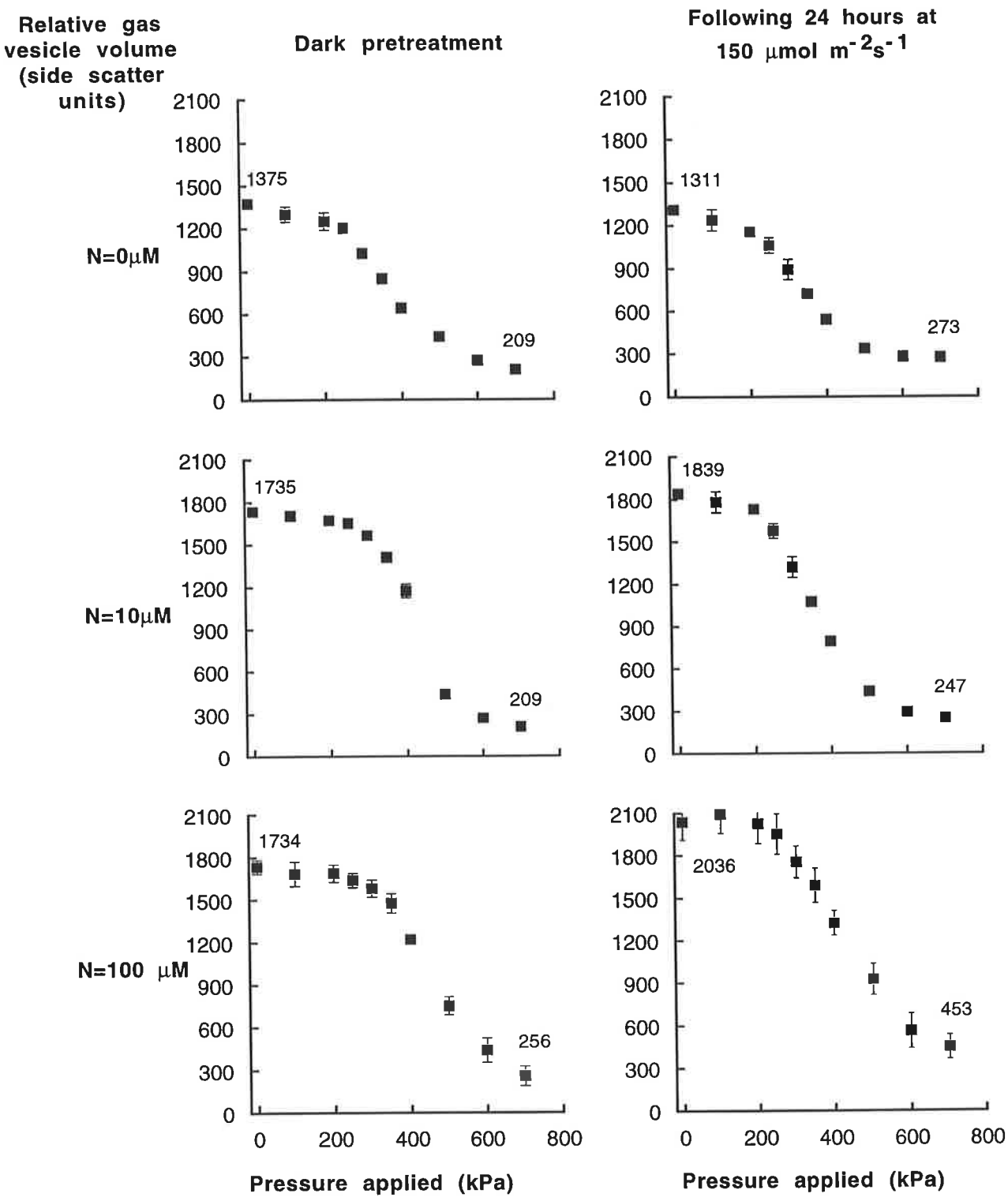


Figure 7.1 *Microcystis aeruginosa* was grown in one of three nitrate concentrations in otherwise normal ASM-1 for six days. Relative gas vesicle volume (RGV) was measured per cell using side scatter detectors on a flow cytometer following incremental increases in pressure. RGV was first measured after a dark preatment (left graphs) and following 24 hours of high light (right graphs). Maximum and minimum RGV values are shown on the graphs.

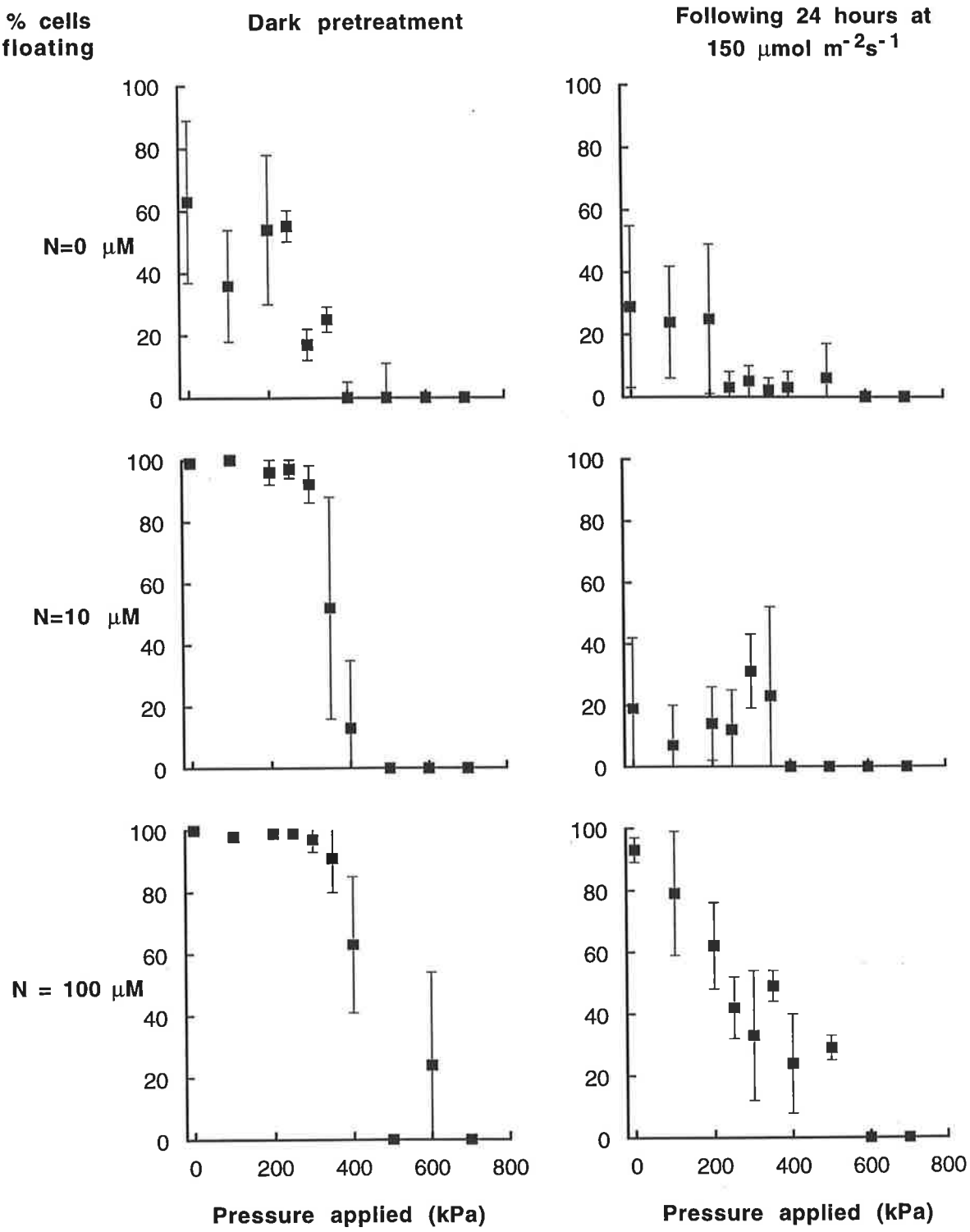


Figure 7.2 *Microcystis aeruginosa* was grown in one of three nitrate concentrations (n=3) in otherwise normal ASM-1 media for six days. Cells were then pretreated in darkness and the percentage of buoyant cells determined microscopically afetr incremental increases in pressure. Cells were then subjected to 24 hours of high light and the percentage of cells floating remeasured.

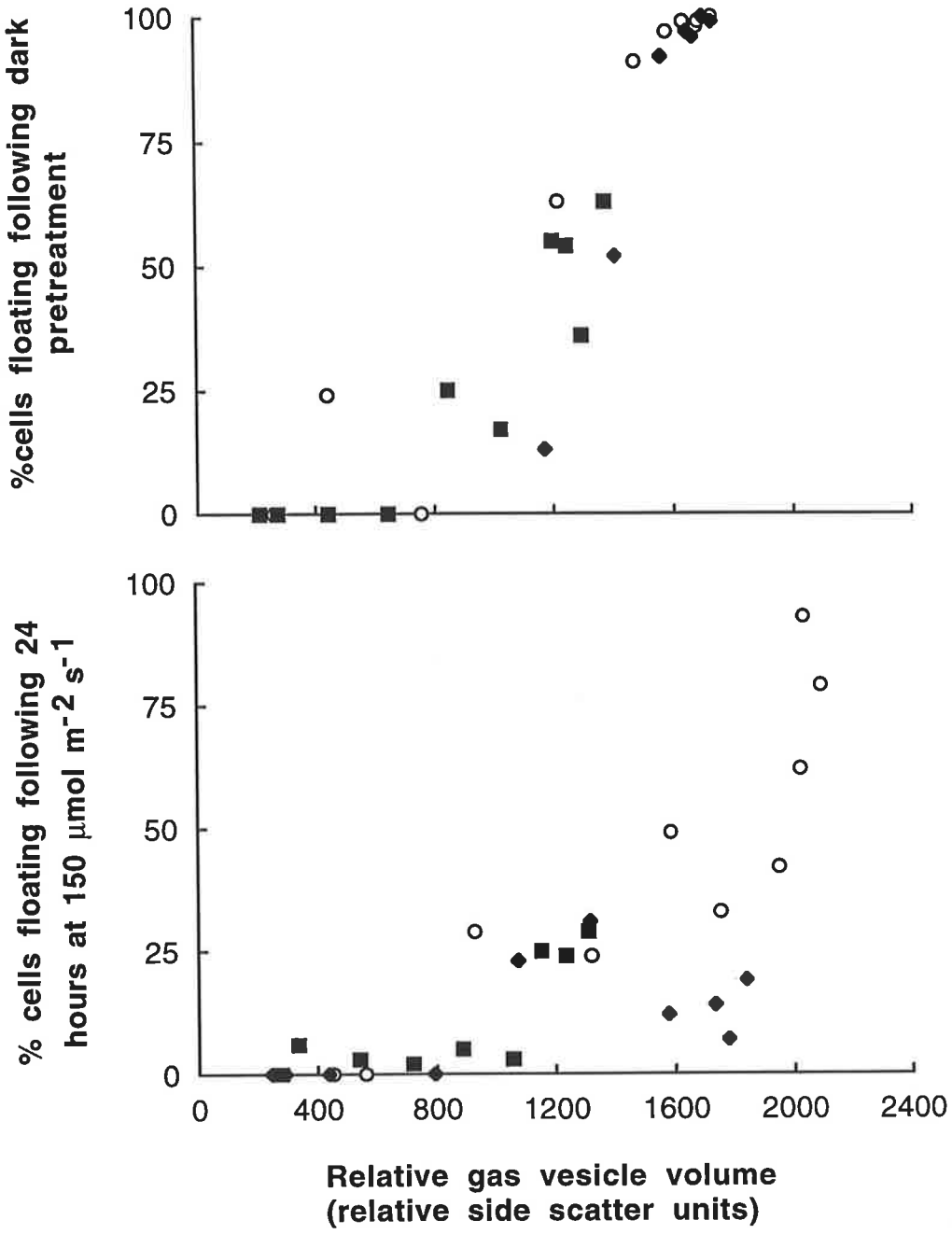


Figure 7.3 *Microcystis aeruginosa* was grown in either nitrate free conditions (■), 10 µM (◆) or 100 µM (o) initial nitrate concentrations. Following incremental increases in pressure the relative gas vesicle volume was measured with a flow cytometer and the percentage of buoyant cells determined microscopically. Measurements were taken before (top) and after (bottom) 24 hours of high light exposure. A greater relative gas vesicle volume was required to maintain 50% of cells buoyant after high light (1952 SSC) than following the dark pretreatment (1261 SSC) because carbohydrate accumulated increasing cell density.

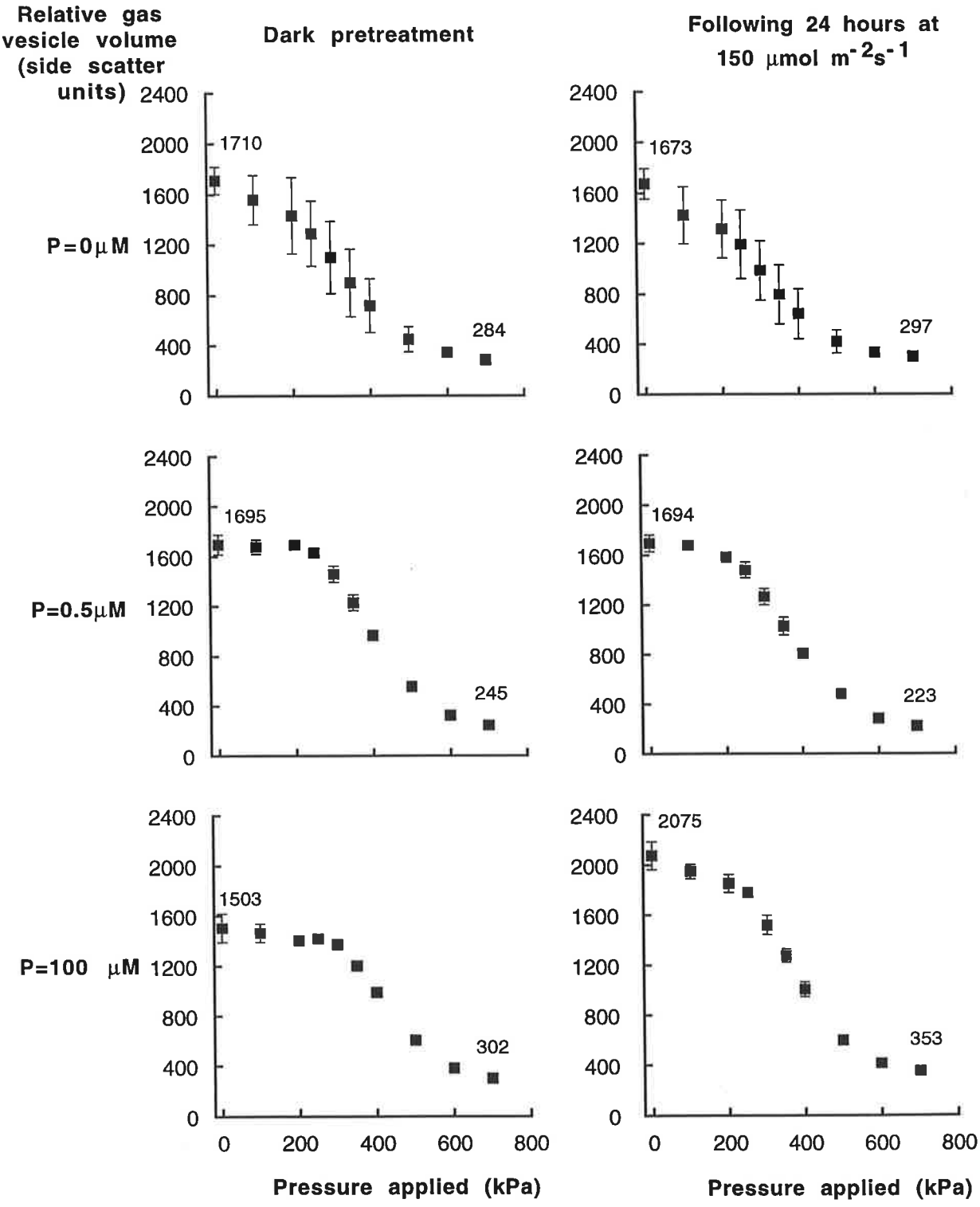


Figure 7.4 *Microcystis aeruginosa* was grown in three phosphate concentrations (n=3) in otherwise normal ASM-1. Relative gas vesicle volume (RGV) was measured per cell using a flow cytometer (side scatter) following incremental increases in pressure. RGV was first measured after a dark pretreatment (left) and then following 24 hours of high light (right graphs). Maximum and minimum RGV values are shown on the graphs.

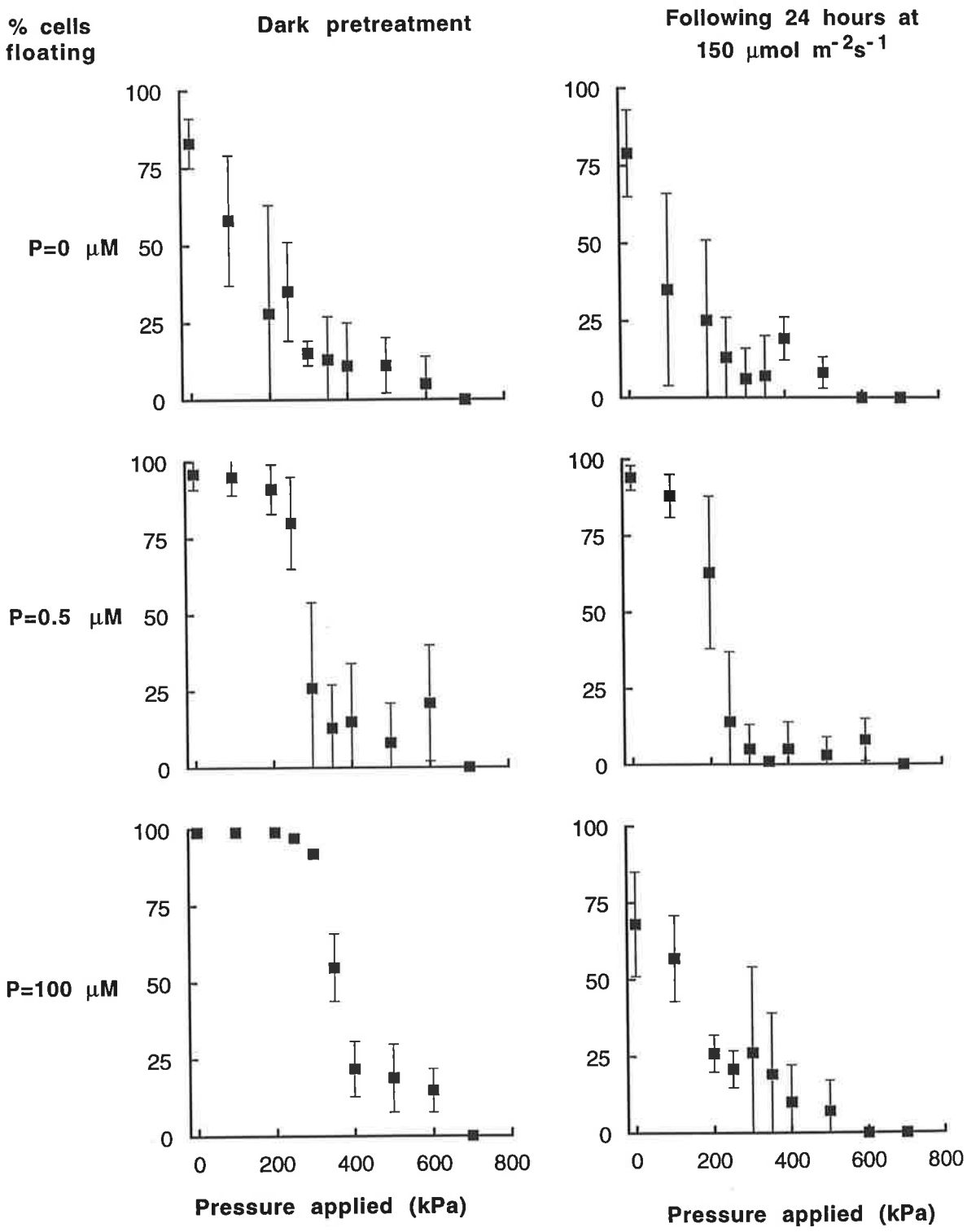


Figure 7.5 *Microcystis aeruginosa* was grown in one of three phosphate concentrations (n=3) in otherwise normal ASM-1. Cells were pretreated in darkness and the percentage of buoyant cells was measured following increases in pressure. Cells were then subjected to 24 hours of high light and the measurements repeated.

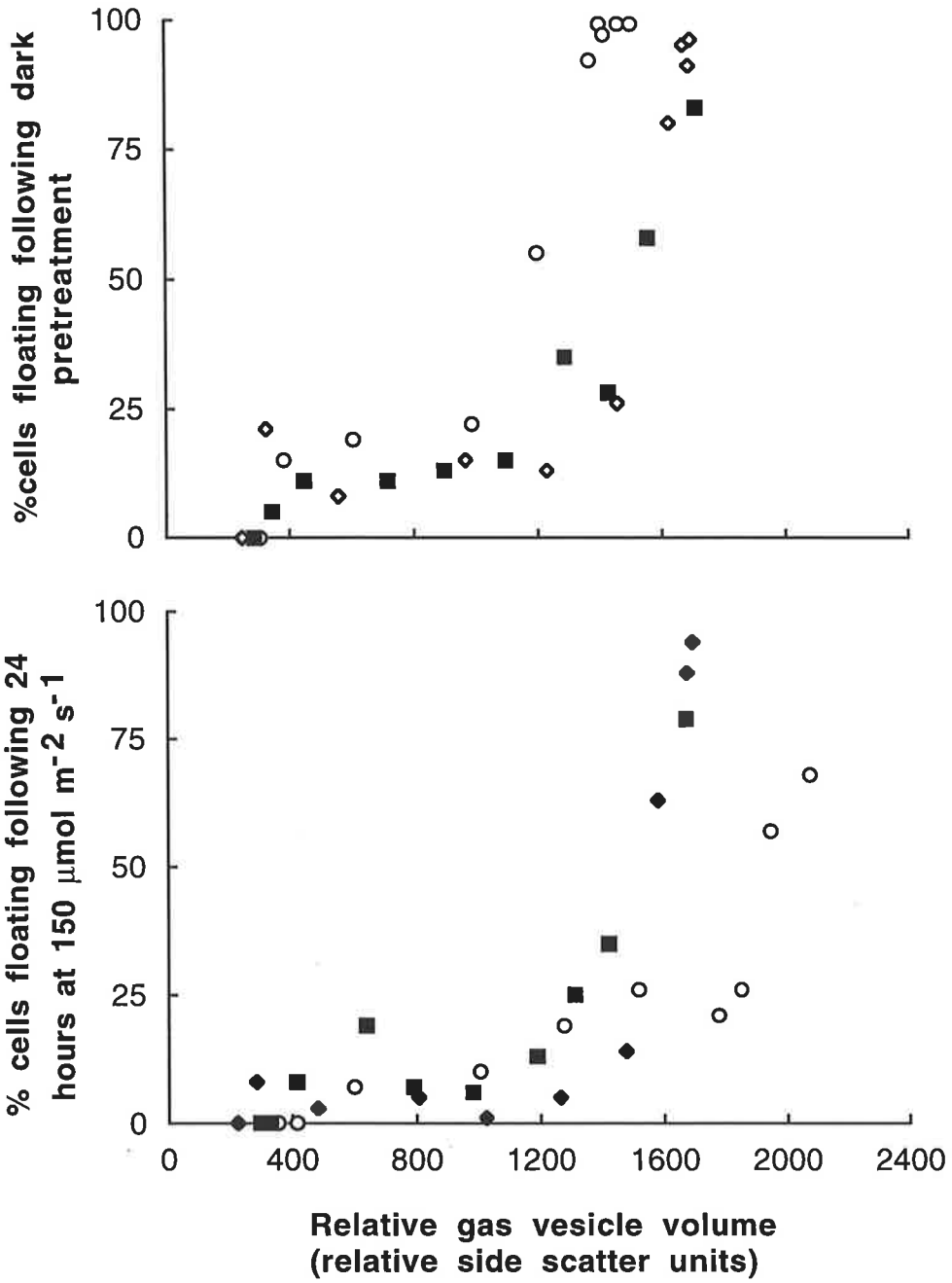


Figure 7.6 *Microcystis aeruginosa* was grown in either phosphate free(■), 0.5 µM (◆) or 100 µM (o) initial phosphate concentrations. Following increases in pressure the relative gas vesicle volume was measured with a flow cytometer and the percentage of buoyant cells determined microscopically. Measurements were taken before (top) and after (bottom) high light exposure. For cells grown in 0 and 0.5 µM P the RGV required to maintain buoyancy in 50% of cells increased from 1194 SSC before light to 1575 after high light. Cells in 100 µM initially required an RGV of 1194 SSC to maintain 50% of cells buoyant but this increased to 2018 SSC as cells accumulated carbohydrate in the light.

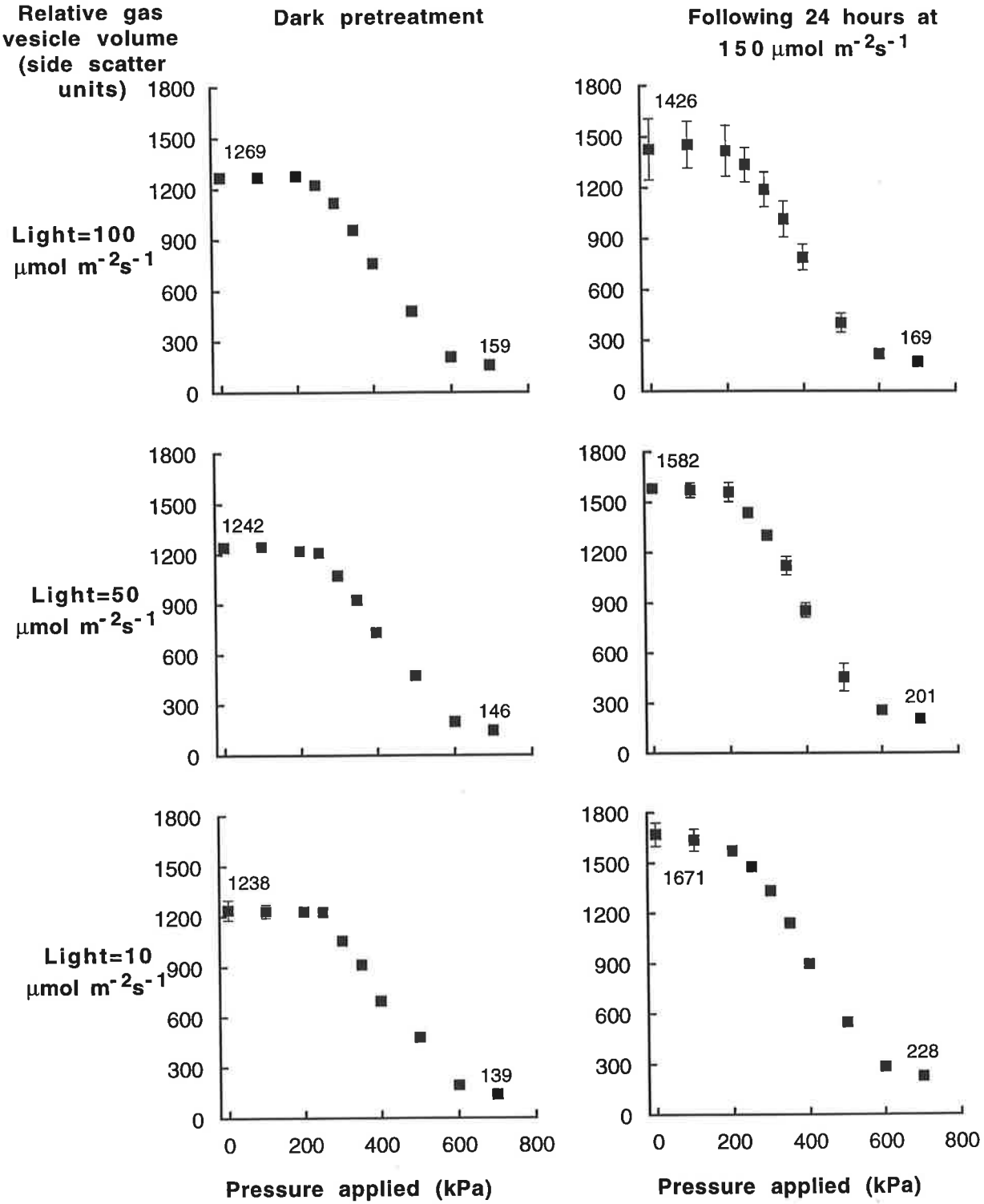


Figure 7.7 *Microcystis aeruginosa* was grown in complete ASM-1 media at three different light intensities. Relative gas vesicle volume (RGV) was measured as side scatter using a flow cytometer following incremental increases in pressure. RGV was measured after a dark pretreatment (left graphs) and again after high light exposure (right graphs). Maximum and minimum RGV values are shown on the graphs.

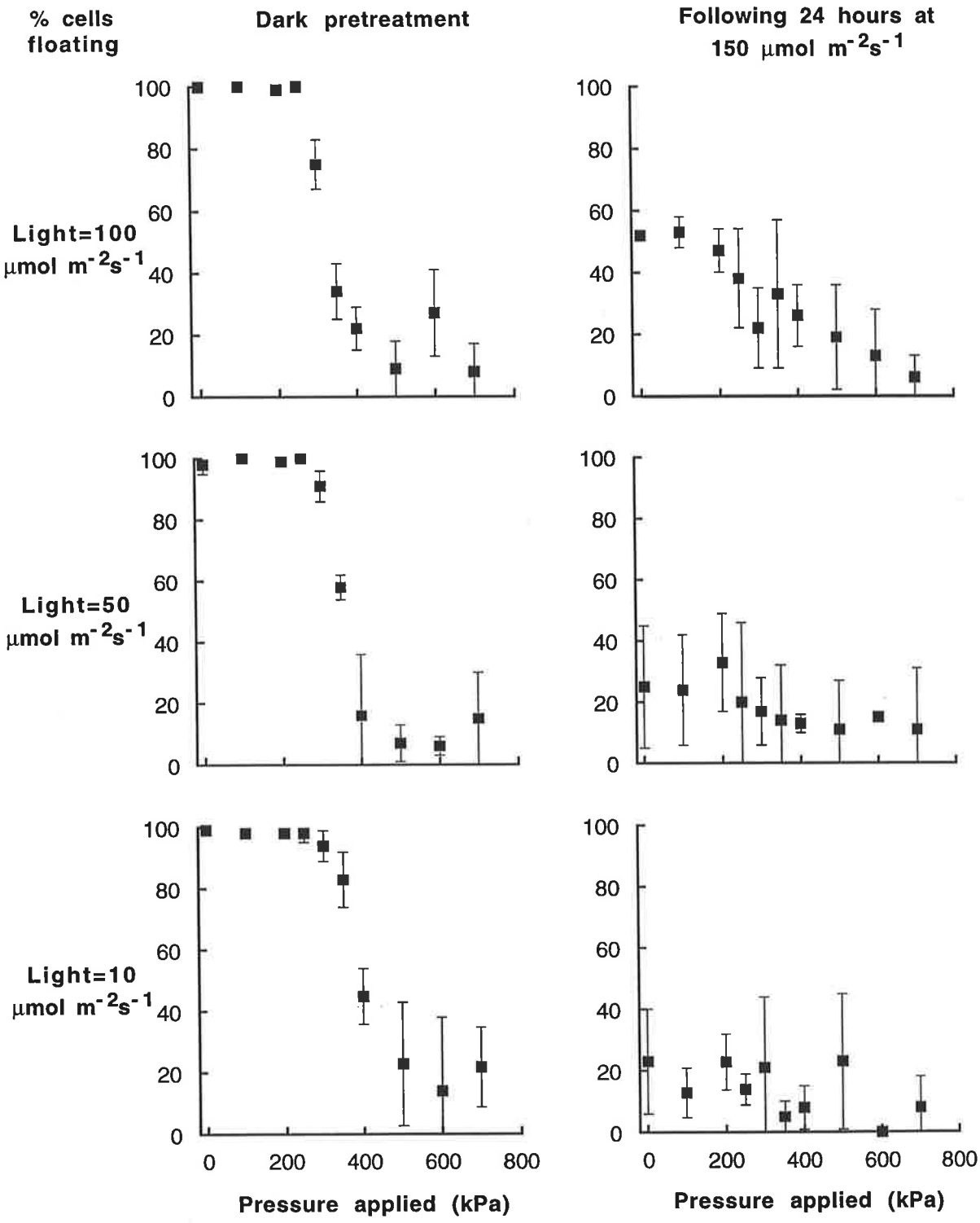


Figure 7.8 *Microcystis aeruginosa* was grown at three light intensities (n=3) in complete media. Cells were pretreated in darkness prior to being subjected to incremental increases in pressure and the percentage of floating cells measured. Cells were then exposed to high irradiance and the measurements repeated.

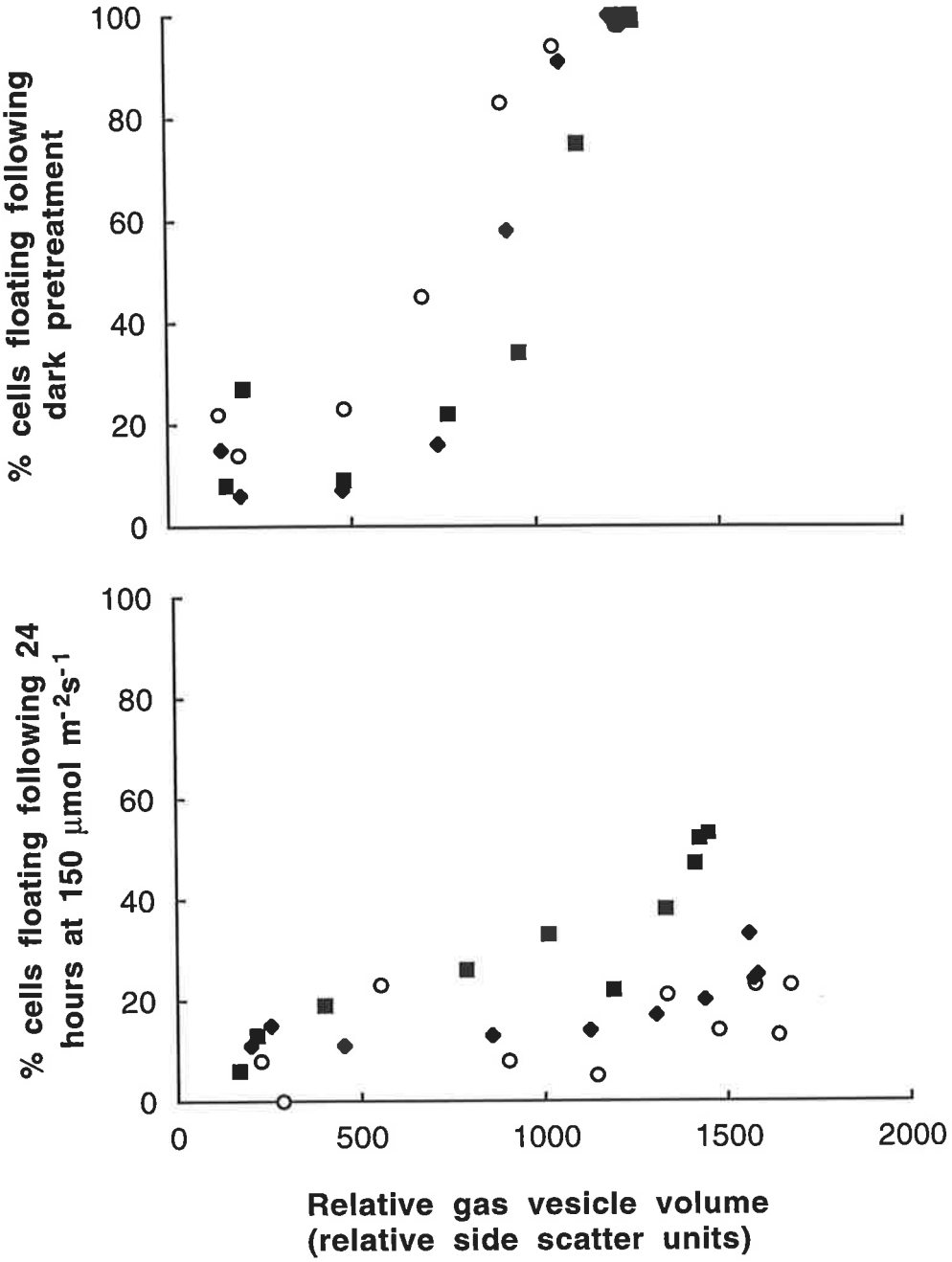


Figure 7.9 *Microcystis aeruginosa* was grown in three light intensities; 100 (■), 50 (◆) or 10 (o) µmol m^{-2} s^{-1} before treatment. Following incremental increases in pressure the relative gas vesicle volume (RGV) was measured with a flow cytometer and the percentage of buoyant cells estimated microscopically. Measurements were taken before (top) and after (bottom) 24 hours of high light exposure. The RGV required to maintain buoyancy in 50% of cells shifted from 1020 SSC before high light exposure to 1420 SSC after high light in the 100 µmol m^{-2} s^{-1} treatment.

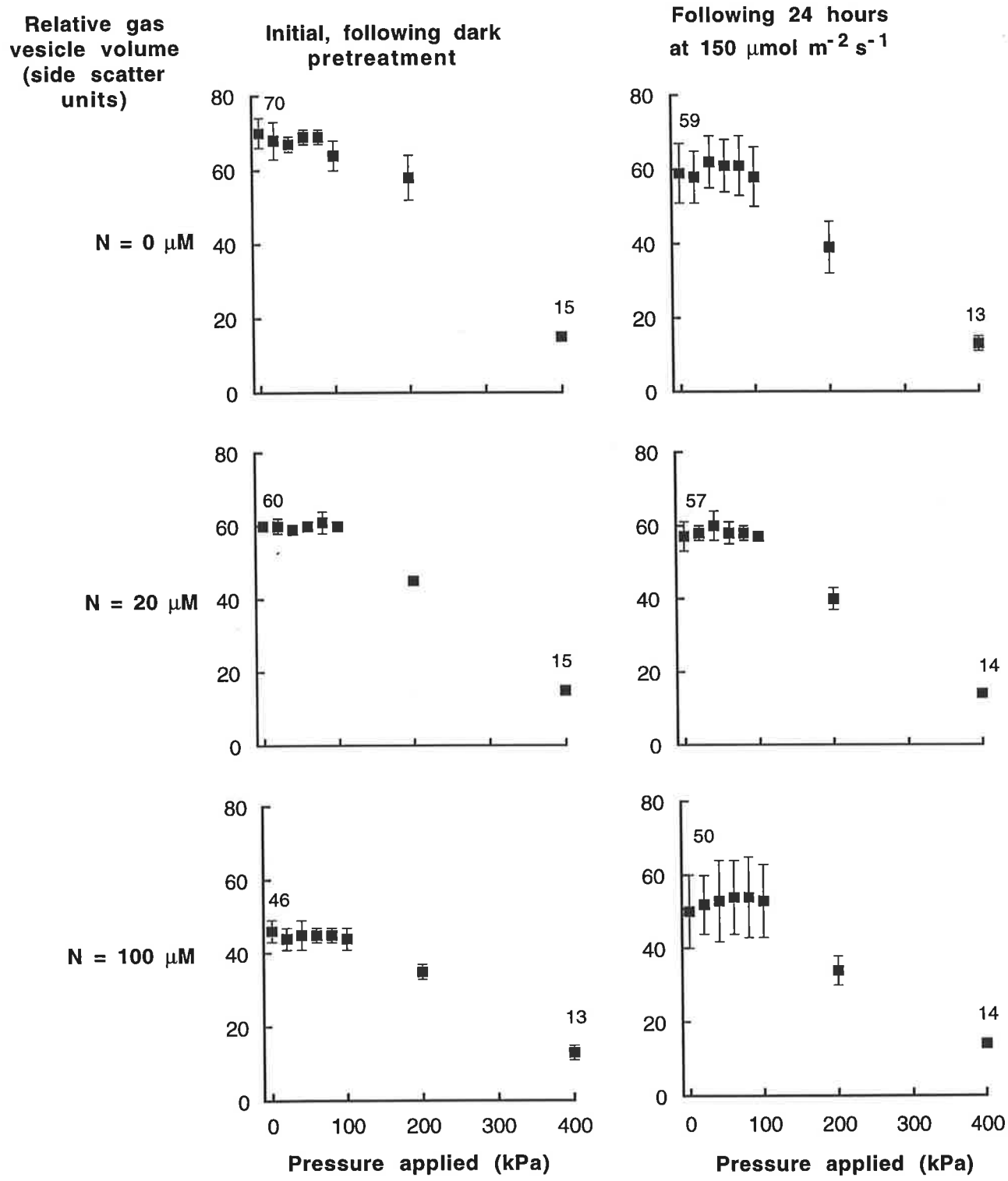


Figure 7.10 *Anabaena circinalis* was grown in one of three nitrate concentrations in otherwise normal WC media for six days. Relative gas vesicle volume (RGV) was measured per cell using side scatter detectors on a flow cytometer following incremental increases in pressure. RGV was first measured after a dark preatment (left graphs) and following 24 hours of high light (right graphs). Maximum and minimum RGV values are shown on the graphs.

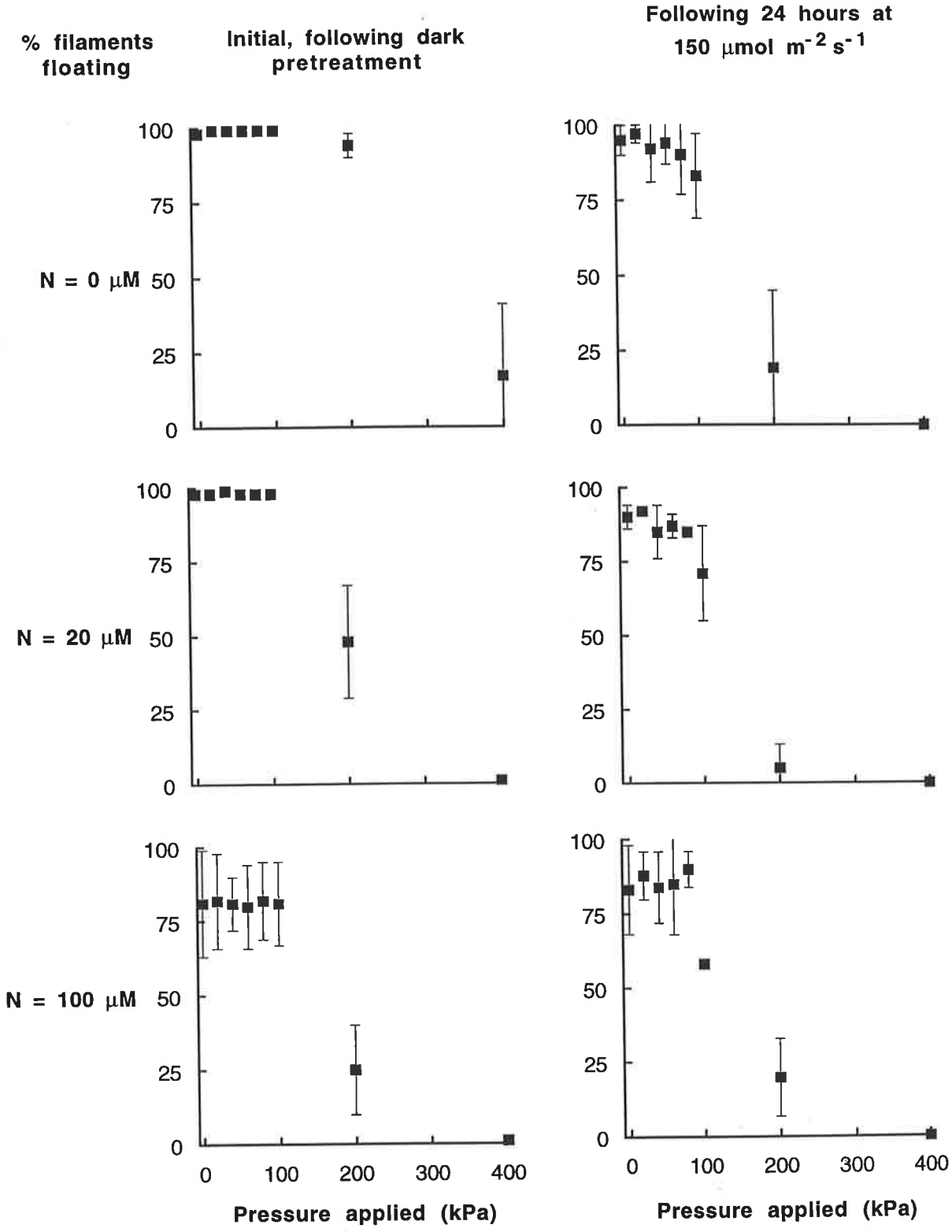


Figure 7.11 *Anabaena circinalis* was grown in one of three nitrate concentrations (n=3) in otherwise normal WC media for six days. Cells were then pretreated in darkness and the percentage of buoyant filaments determined microscopically after incremental increases in pressure. Filaments were then subjected to 24 hours of high light and the percentage of cells floating remeasured.

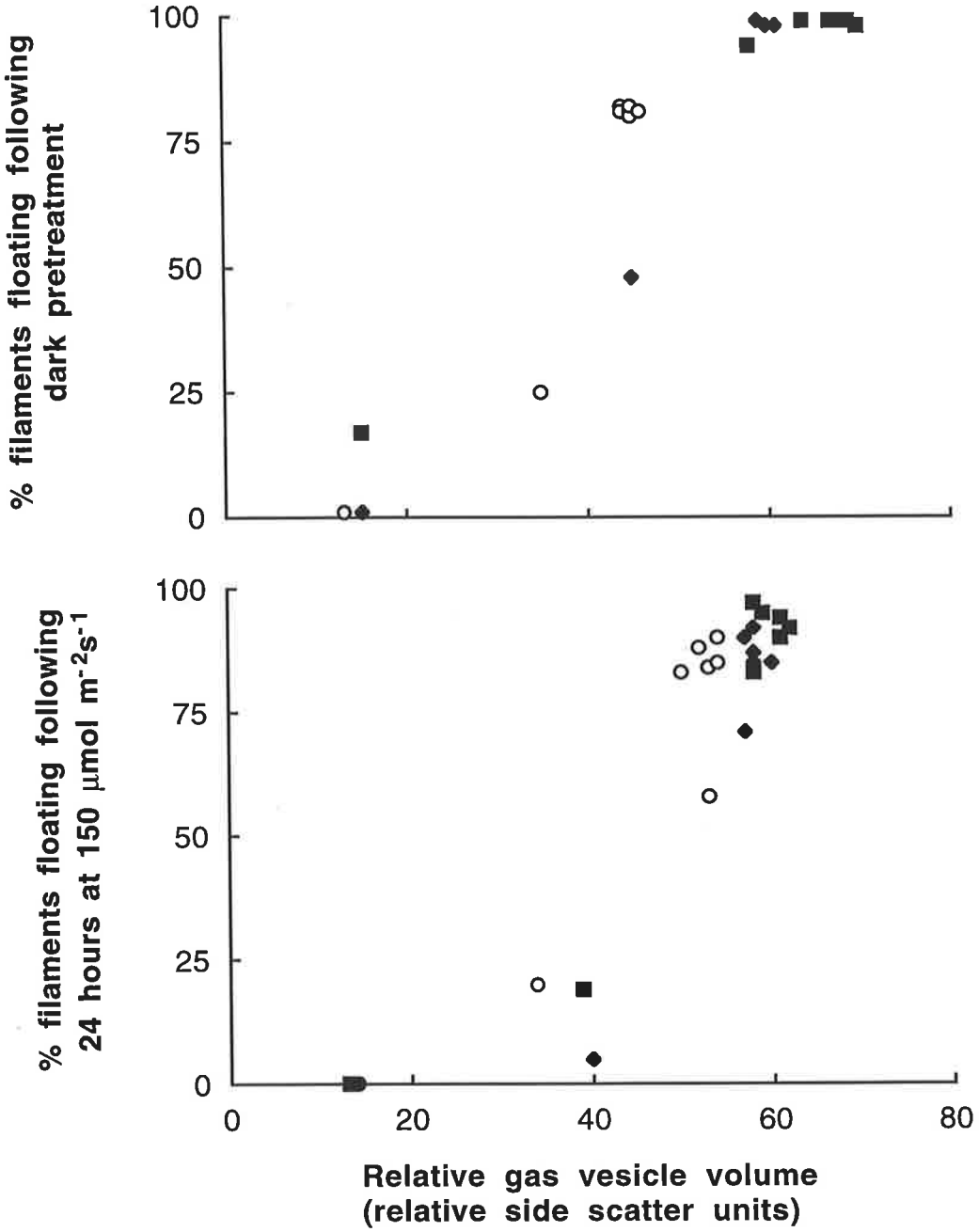


Figure 7.12 *Anabaeana circinalis* was grown in either nitrate free conditions (■), 20 µM (◆) or 100 µM (o) initial nitrate concentrations. Following incremental increases in pressure the relative gas vesicle volume was measured with a flow cytometer and the percentage of buoyant filaments determined microscopically. Measurements were taken before (top) and after (bottom) 24 hours of high light exposure. The critical number of gas vesicles required to maintain buoyancy in 50% of filaments increased from 43 ASSC to 49 and 55 ASSC in the 0 and 20 µM treatments and from 40 to 43 ASSC in the 100 µM treatment.

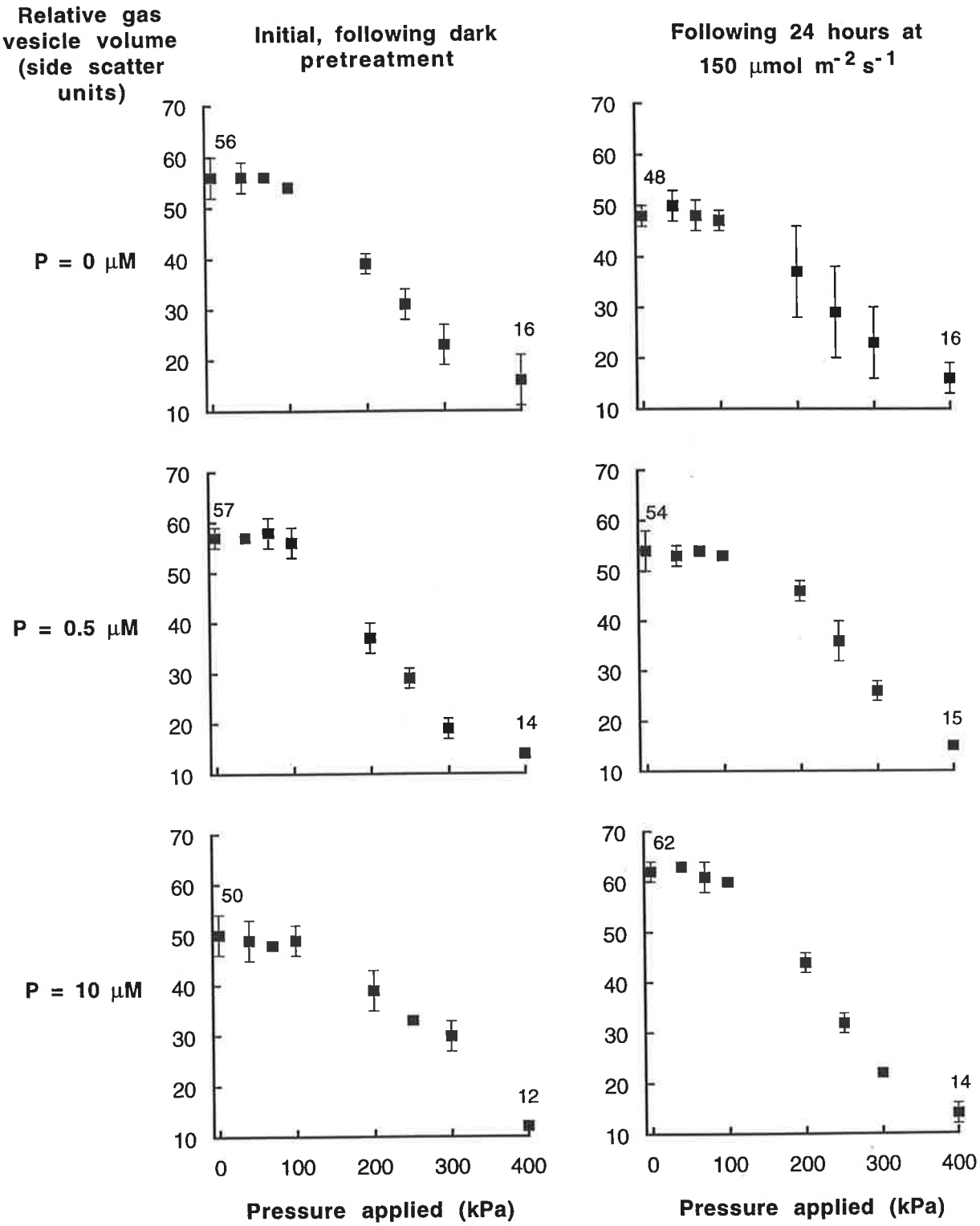


Figure 7.13 *Anabaena circinalis* was grown in three phosphate concentrations (n=3) in otherwise normal WC media. Relative gas vesicle volume (RGV) was measured per cell using a flow cytometer (side scatter) following incremental increases in pressure. RGV was first measured after a dark pretreatment (left) and then following 24 hours of high light (right graphs). Maximum and minimum RGV values are shown on the graphs.

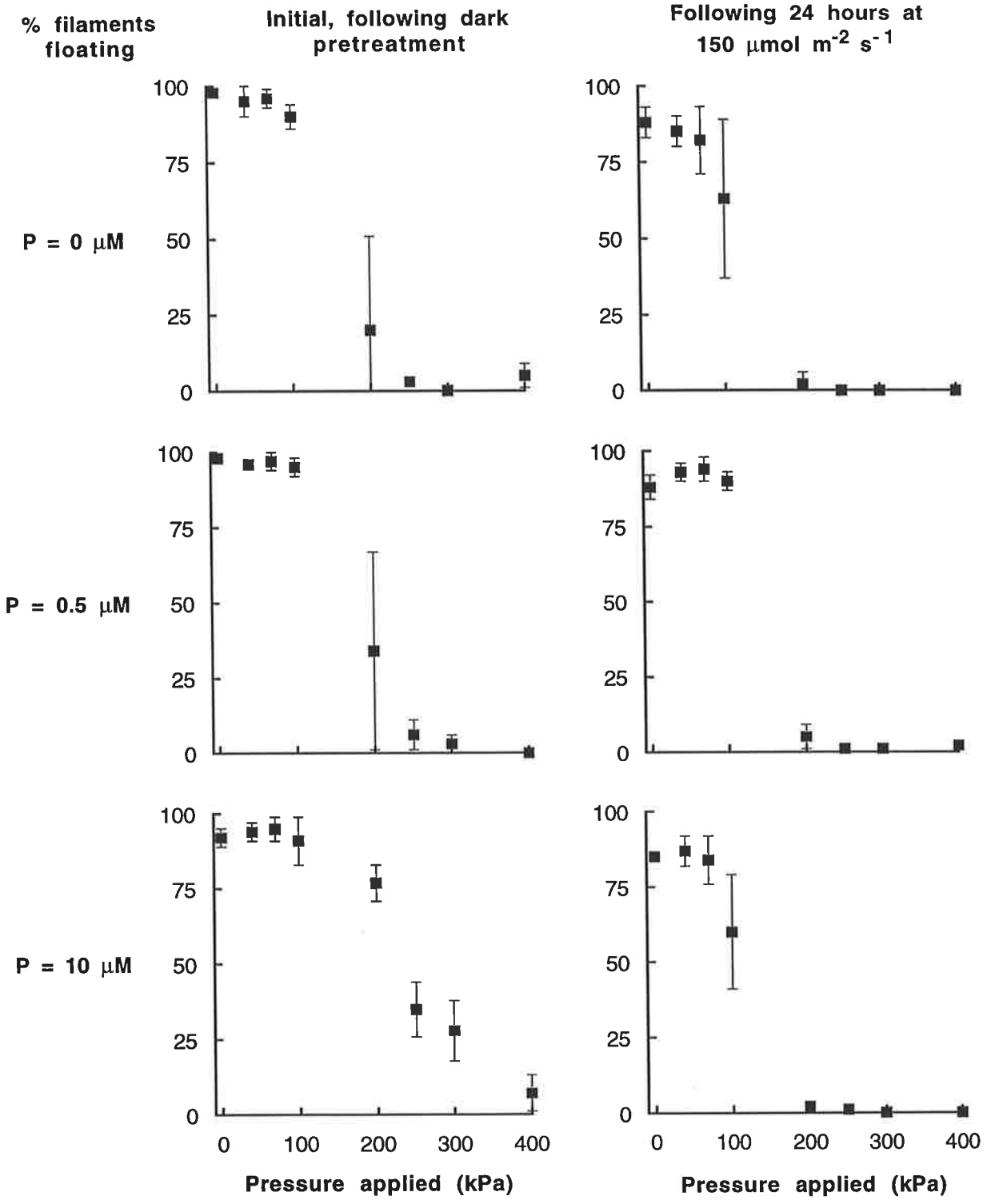


Figure 7.14 *Anabaena cirinalis* was grown in one of three phosphate concentrations (n=3) in otherwise normal WC media. Cells were pretreated in darkness and the percentage of buoyant filaments was measured following increases in pressure. Cells were then subjected to 24 hours of high light and the measurements repeated.

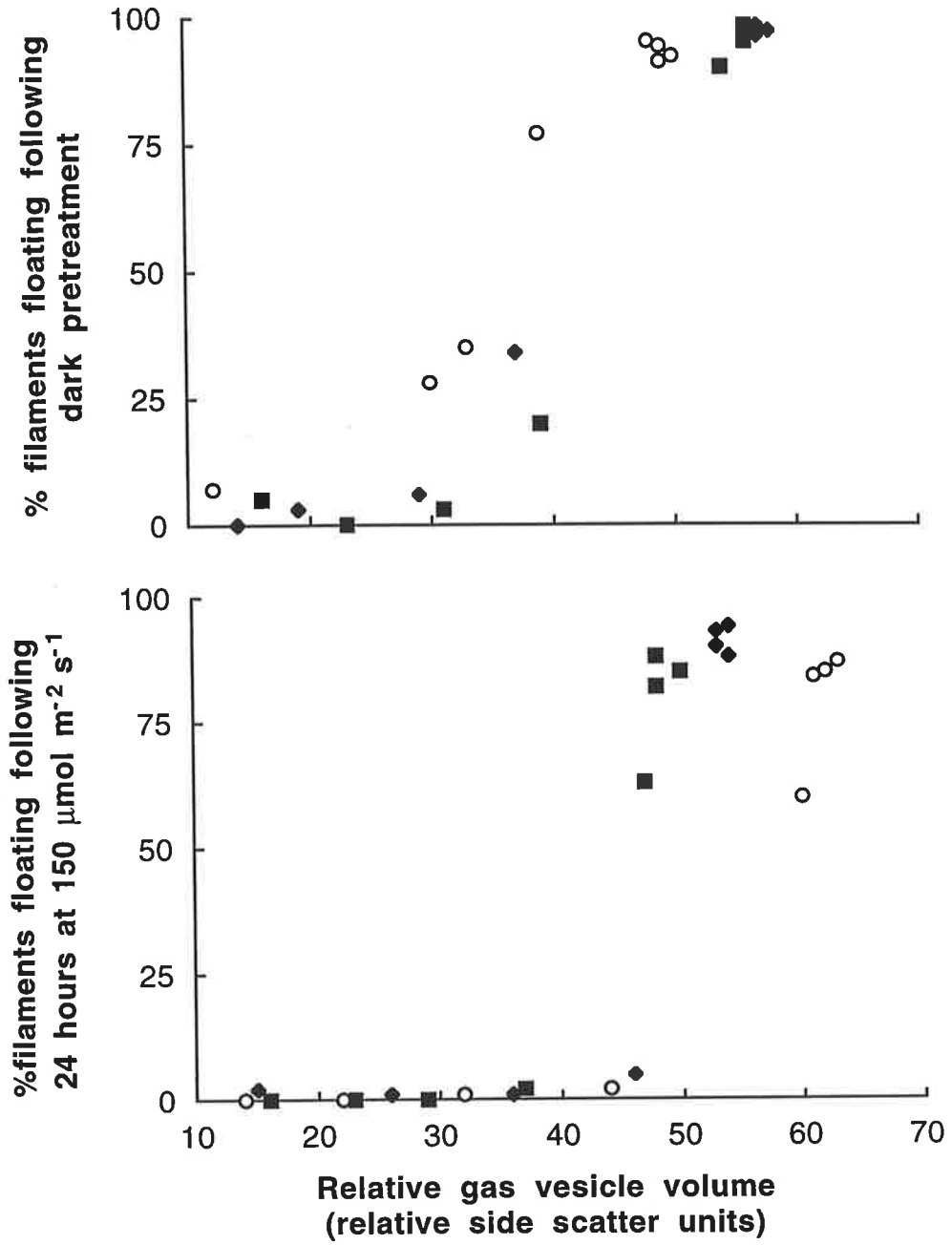


Figure 7.15 *Anabaena circinlalis* was grown in either phosphate free(■), 0.5 µM (◆) or 10 µM (o) initial phosphate concentrations. Following increases in pressure the relative gas vesicle volume was measured with a flow cytometer and the percentage of buoyant filaments determined microscopically. Measurements were taken before (top) and after (bottom) high light exposure. For cells grown in 0.5 µM and 10 P the RGV required to maintain buoyancy in 50% of cells increased from 41 to 50 and 35 to 58 ASSC respectively. For 0 µM P the critical gas vesicle volume did not change.

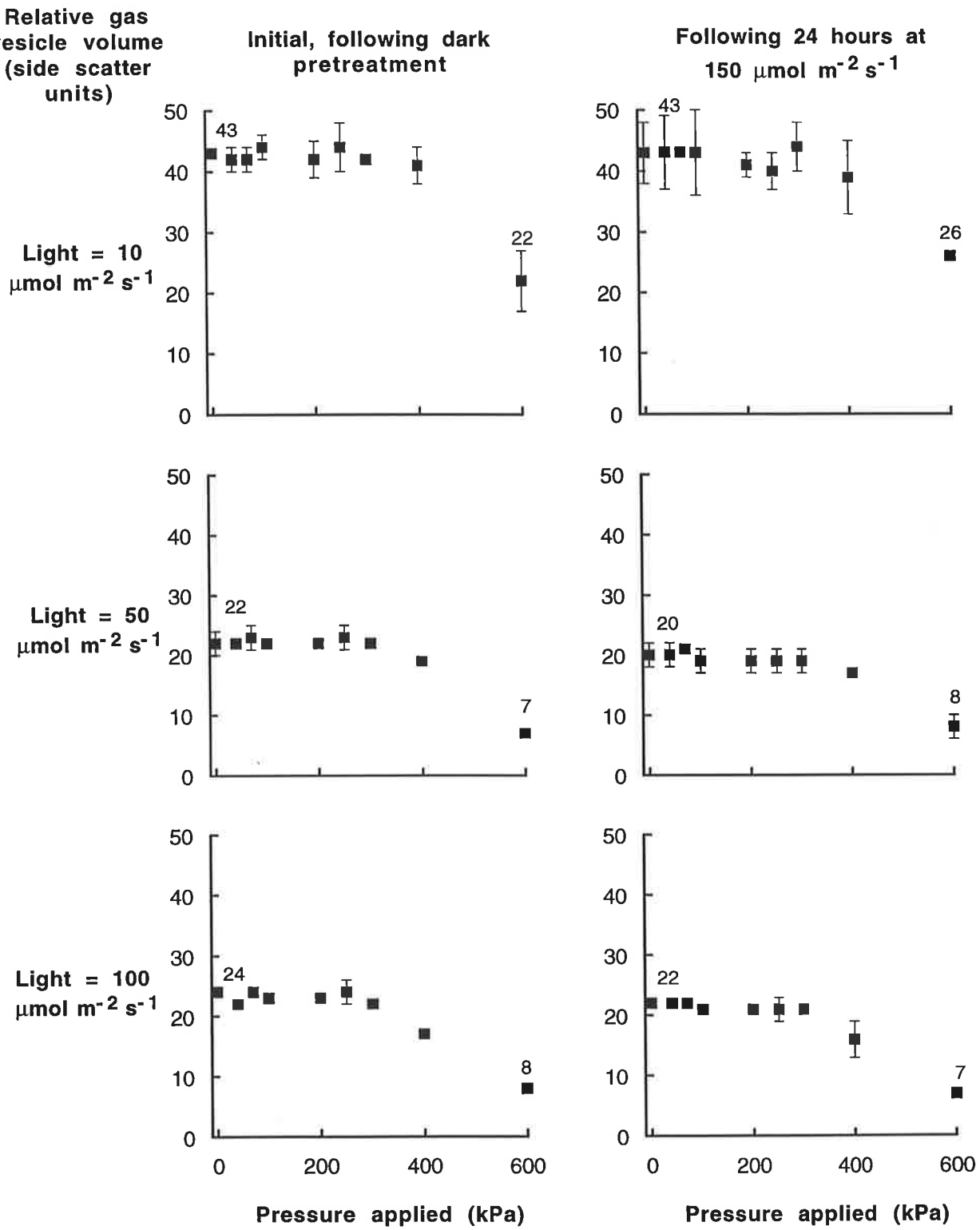


Figure 7.16 *Anabaena circinalis* was grown in complete WC media at three different light intensities. Relative gas vesicle volume (RGV) was measured as side scatter using a flow cytometer following incremental increases in pressure. RGV was measured after a dark pretreatment (left graphs) and again after high light exposure (right graphs). Maximum and minimum RGV values are shown on the graphs.

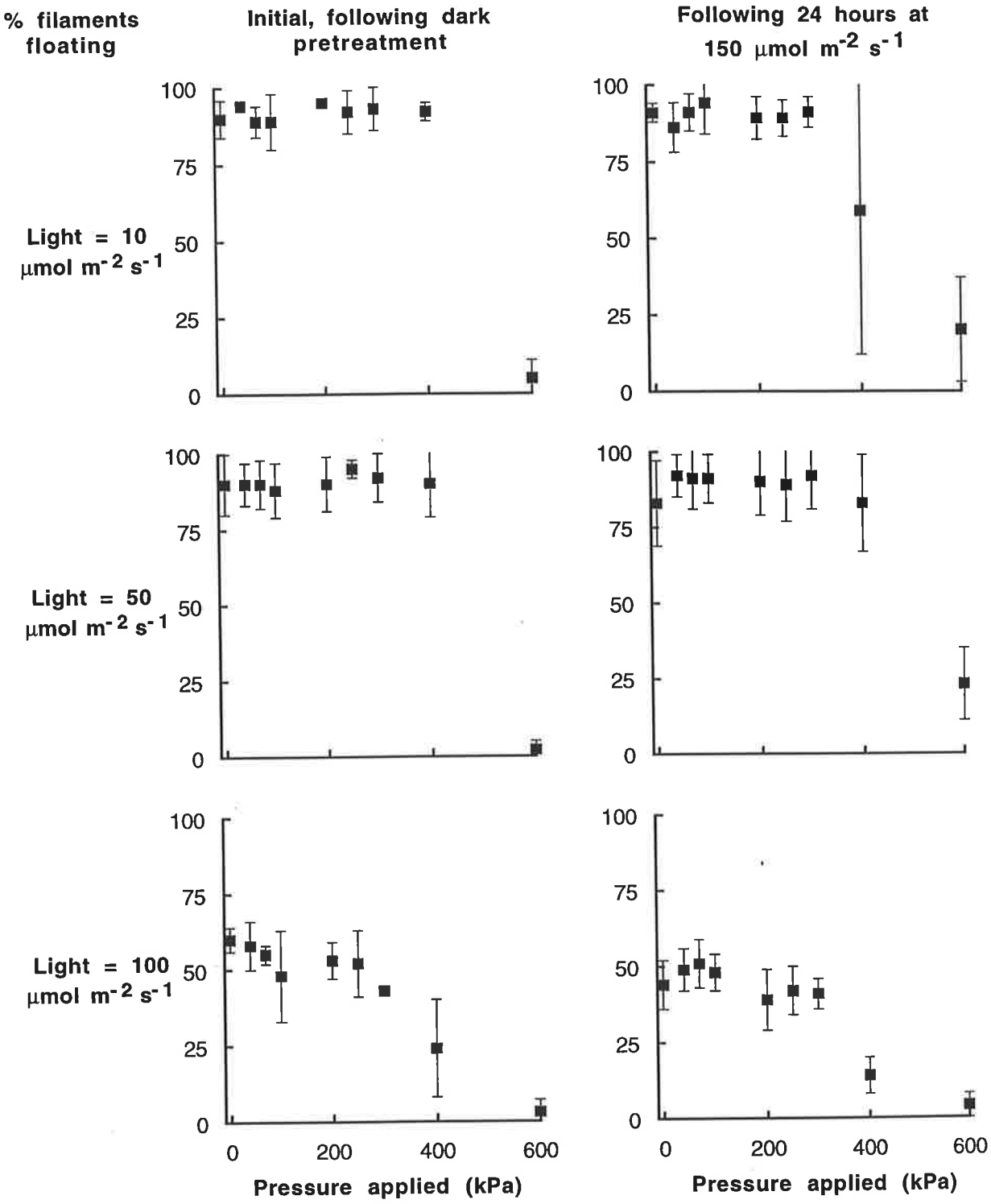


Figure 7.17 *Anabaena circinalis* was grown at three light intensities (n=3) in complete media. Cells were pretreated in darkness prior to being subjected to incremental increases in pressure and the percentage of floating filaments measured. Cells were then exposed to high irradiance and the measurements repeated.

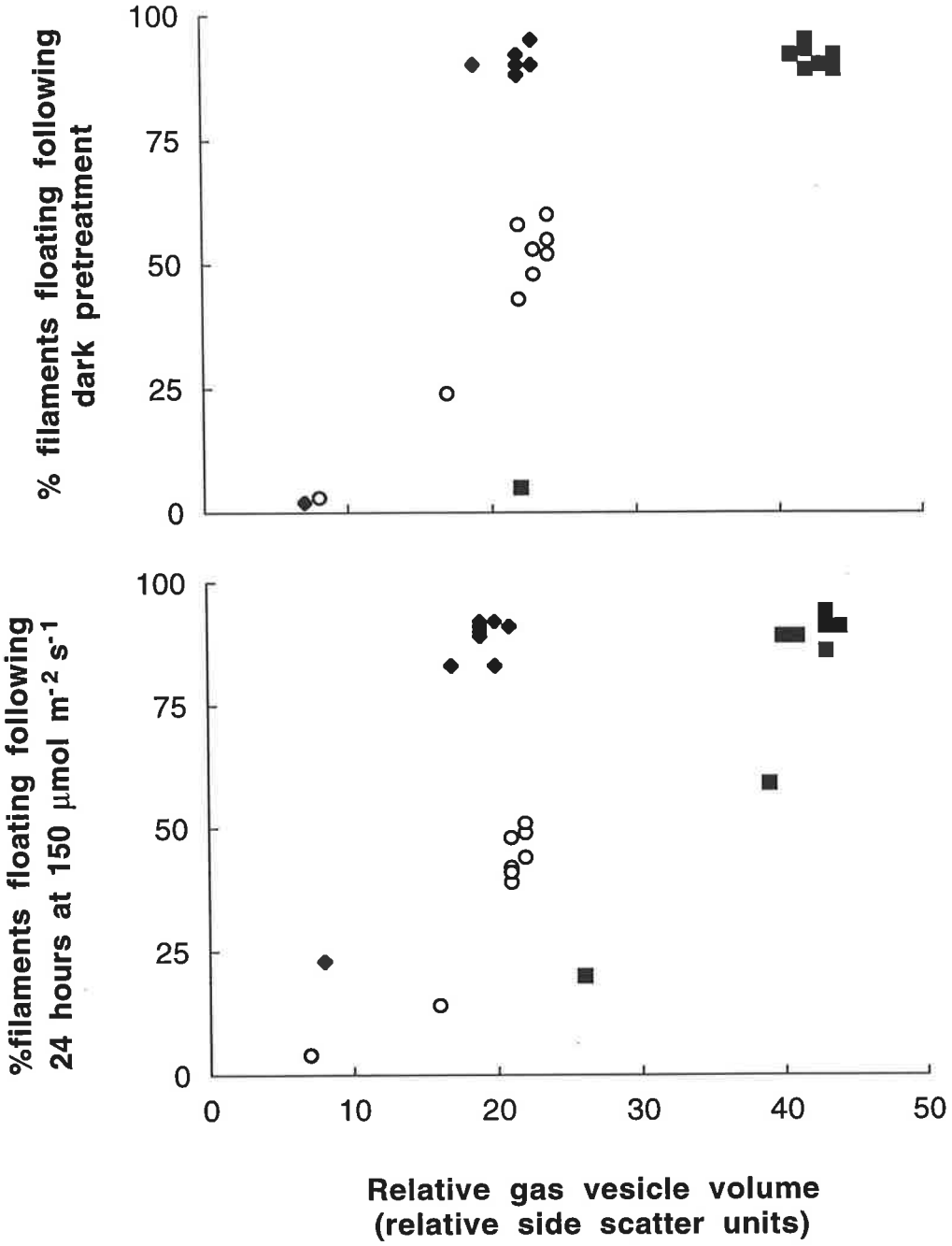


Figure 7.18 *Anabaena circinlis* was grown in three light intensities; 100 (■), 50 (◆) or 10 (o) µmol m^{-2} s^{-1} before treatment. Following incremental increases in pressure the relative gas vesicle volume (RGV) was measured with a flow cytometer and the percentage of buoyant filaments estimated microscopically. Measurements were taken before (top) and after (bottom) 24 hours of high light exposure. The RGV required to maintain buoyancy in 50% of cells did not shift significantly for any treatment following high light exposure.

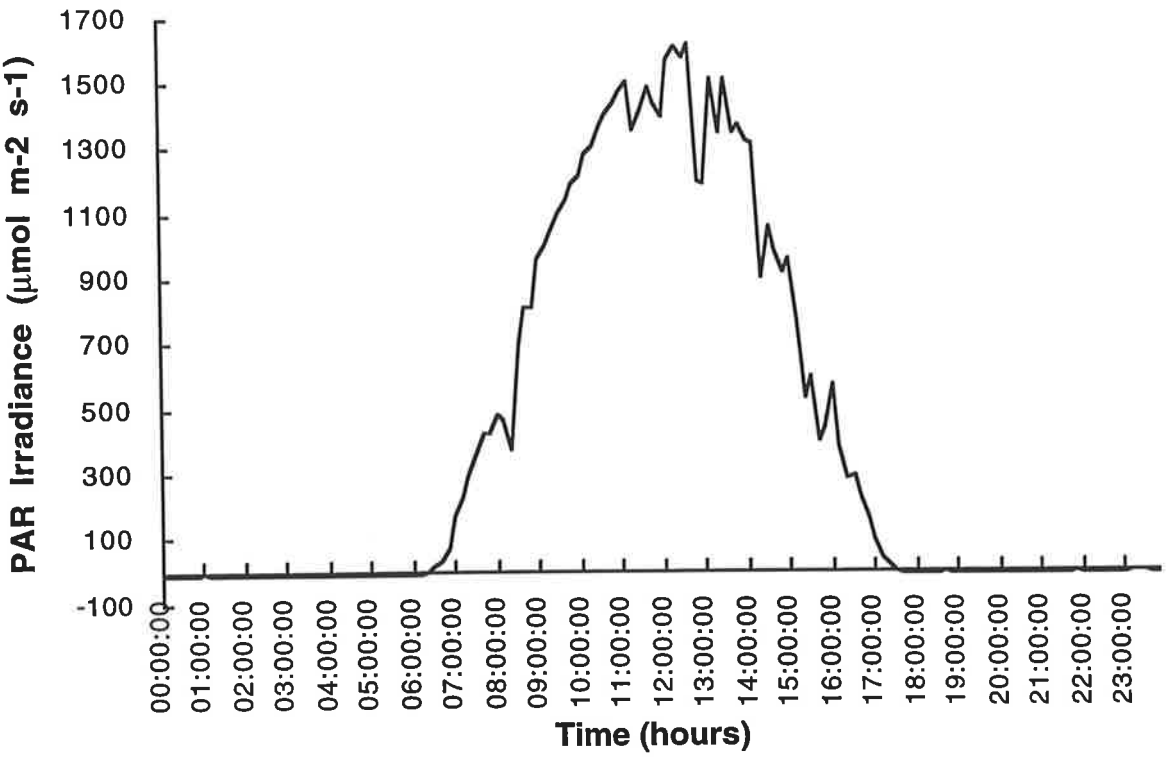


Figure 7.19 Solar irradiance (PAR; 400-700nm) measured on April 15, 1997 at Chaffey Dam, NSW.

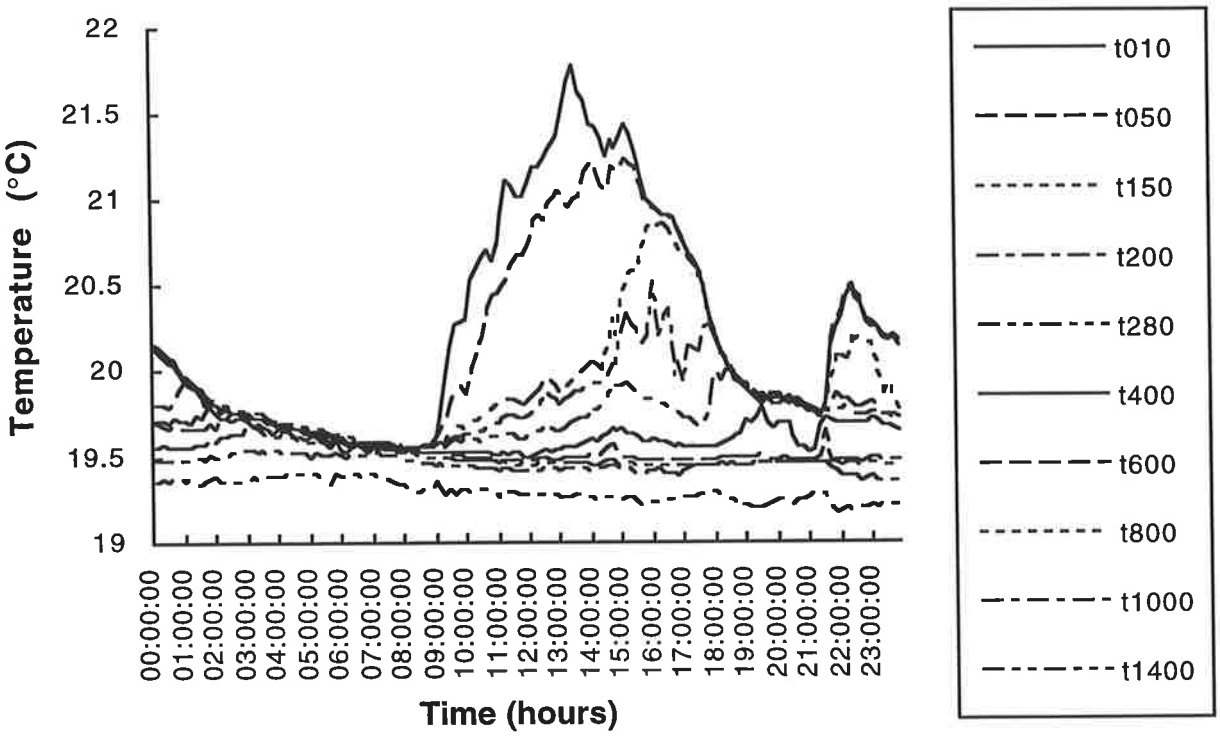


Figure 7.20 Vertical temperature structure measured at Thermistor chain 1, Chaffey Dam, on April 15, 1997. Readings were averaged over ten minutes. The legend details temperature data from thermistors deployed at different depths, measured in centimetres from the surface.

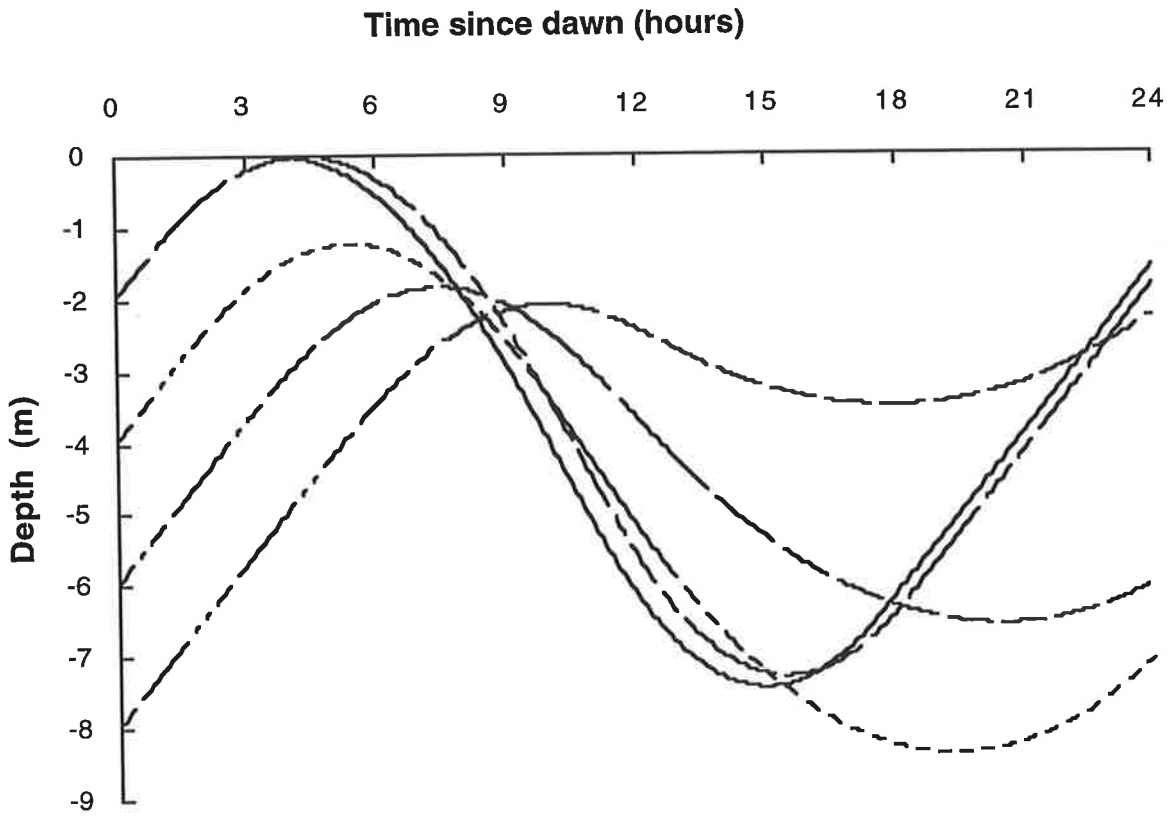


Figure 7.21 Simulation of *Anabaena circinalis* filament movement following nocturnal mixing which dispersed the population evenly throughout the eight metre epilimnion. The cyanobacteria encountered calm, stable conditions at the start of the simulation (dawn). Run conditions were selected which represented the physical conditions at Chaffey Dam on April 15, 1997. Light attenuation coefficient k=1.02 m^{-1}; maximum surface irradiance =1614 µmol m^{-2} s^{-1}; packing efficiency A=1; form resistance ϕ=1.5; starting density =980 kg m^{-3}; density range 980-1060 kg m^{-3}; filament radius =90 µm.

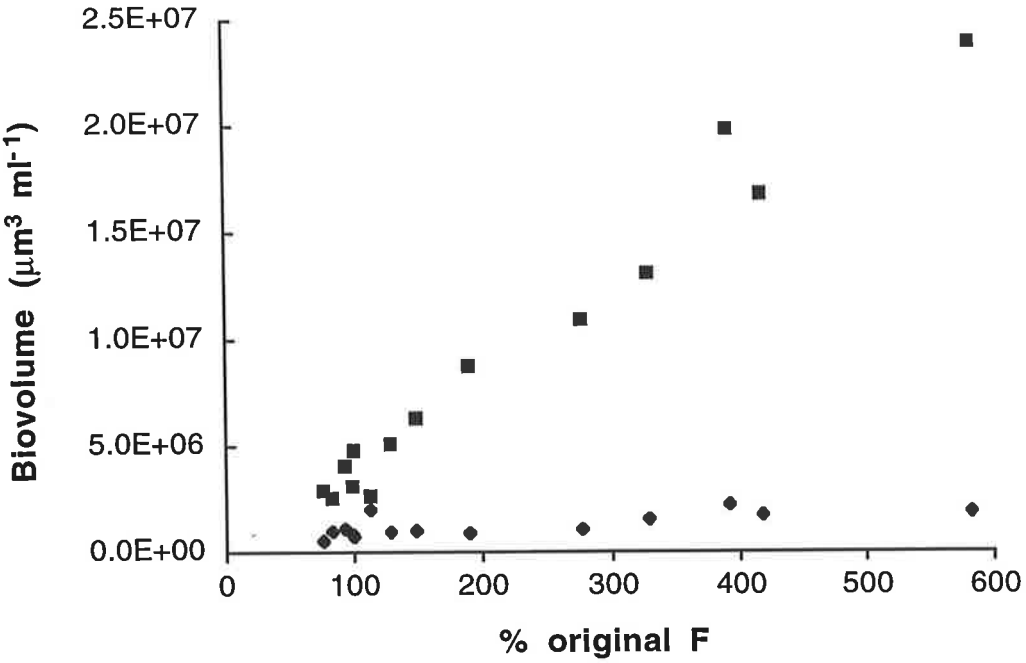


Figure 7.22 A series of tubes containing *Anabaena* (■) and *Microcystis* (◆) were set up and the top 5% was sampled at regular time intervals. As the colonies/filaments floated upwards the cell number in the top 5% increased which increased the biovolume and the chlorophyll fluorescence (Fo). To validate that Fo could be used as a surrogate for biomass cell counts were converted to biovolume and compared with Fo. The correlation between Fo and *Anabaena* biovolume is r^2=0.96 and *Microcystis* r^2=0.75.

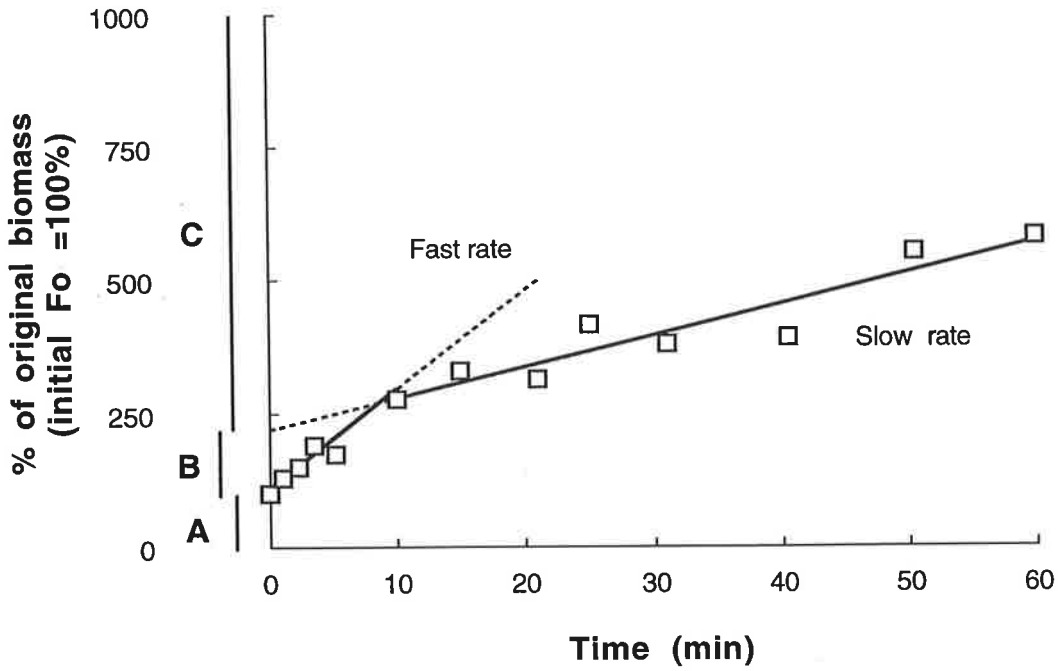


Figure 7.23 *Anabaena* suspension was placed in a series of tubes and the top 5% measured at regular time intervals in order to calculate flotation rate. Cells floating upwards increased the biomass (Fo) in the sample which could be divided into a slow and fast class. Rate of accumulation (floating rate) was calculated by linear regression. A= Fo at surface originally, B= proportion of population in fast class, C= proportion in slow class.

Chapter 8

Discussion

8.1 Flow cytometry and buoyancy

The major theme of this thesis was the influence of light and nutrients on cyanobacterial growth and buoyancy regulation. Two quite different approaches were used to understand this issue. The first developed a technique to examine the metabolic activity of phytoplankton in response to nutrient and light stresses using FDA and flow cytometry. This technique was further developed into a bioassay to determine nutrient availability or limitation in natural systems. The second approach was to examine the buoyancy response of cells given a particular chemical and light climate. The two become united as tools to examine cyanobacterial populations and manage water storages and rivers.

The rapid FDA nutrient bioassay can be used to determine the nutrient status of the cells and conceptual models of buoyancy regulation and growth used in broader models to determine the likelihood of surface accumulations and formulate the most effective strategy to control cyanobacterial biomass. For example, if the FDA assay detected no nutrient limitation in an *Anabaena circinalis* population then the buoyancy models would predict persistent buoyancy and a high likelihood of surface accumulations and therefore increased surface toxin concentrations. Solutions to control cyanobacteria would prioritise the disruption of surface blooms in the short term and a reduction of the nutrient load from the catchment in the long term. If the FDA nutrient bioassay detected nutrient limitation then managers would expect low growth and conceptual models of buoyancy response would predict functioning of buoyancy regulation, sedimentary losses of some cells and a decreased probability of prolonged surface blooms.

Several new techniques have been developed in this study including the use of FDA and flow cytometry to estimate cell nutrient status, flow cytometry to measure side scatter as a surrogate

for relative gas vesicle volume and a technique to measure floating velocity of phytoplankton. Each of these techniques has applicability to phytoplankton research beyond the uses addressed in this thesis, particularly the FDA-flow cytometry technique.

8.2 FDA assay and phytoplankton research

FDA and flow cytometry were used previously to assess phytoplankton metabolic activity, ecotoxicolgy and dark survivorship (Selvin *et al.*, 1989), but never extended to examine increased metabolic activity following relief from an environmental stress. The exploitation of this phenomena as a rapid nutrient bioassay may be extended with the advancement of flow cytometric and genetic technologies. It is possible to discriminate between different taxa based upon their specific optical characteristics (Yentsch *et al.*, 1983; Balfoort *et al.*, 1992) providing the flow cytometer has multiple lasers to excite different pigments (Premazzi *et al.*, 1989; Cunningham, 1992) and high resolution. With such a flow cytometer the natural phytoplankton community could be divided into different taxonomic groups and their response to FDA analysed individually. This would allow researchers to determine precisely what nutrients are limiting what phytoplankton rather than assessing whole community response which may mask subtle differences between taxa.

The FDA-flow cytometry assay also offers a technique to examine the relative bioavailability of phosphorus from different sediments. For example cells depleted of phosphorus could be exposed to different sediments in dialysis type containers (Kohler, 1997) and phytoplankton metabolic activity change used as an index of phosphorus flux and bioavailability. The value of this nutrient bioavailability assay would be appreciably enhanced when used in conjunction with established techniques examining phosphorus speciation, flux rates and bioavailability, such as those used in studies by Bradford and Peters (1987), Oliver *et al.* (1993) and Baldwin (1996).

Another application of the FDA-flow cytometry assay is in determining toxic metal stress which has been trailed by a number of research groups examining pollutant and algicide toxicity (Berglund and Eversman, 1988; Gilbert *et al.*, 1992; Arsenault *et al.*, 1993). Care must be taken in interpreting results from metal toxicity assays because lysed cells display different optical characteristics to intact cells and therefore the analysis would be biased toward intact cells which may display relative tolerance to the metal or pollutant stress.

8.3 Low nitrogen in the lower River Murray

The work detailed in this thesis was part of a larger collaborative project investigating the phytoplankton community of the lower River Murray, South Australia. In the lower River Murray minimum entitlement flows in summer allow thermal stratification to establish providing physical conditions conducive to cyanobacterial growth if sufficient nutrients are available. This project was initiated to examine advection and growth of cyanobacteria in the main river channel. The FDA technique was used to assess whether nutrient limitation was a factor controlling phytoplankton growth. As this work is unpublished a summary of techniques and major results are provided.

Eight sites were selected over a 54 km reach between Lock 1 and Nildottie (Map 4.1) and sampled weekly or twice weekly between December, 1994 and March, 1995. "Parcels" of water were tracked based on transport times and major phytoplankton taxa were recorded by microscopic enumeration of cell numbers allowing calculation of cell numbers.

Although thermal stratification occurred during the day by late afternoon wind disrupted thermal stratification and generally caused complete mixing; persistent diel stratification rarely occurred. Cell densities and growth rates of *Anabaena* species and the diatom *Aulacoseira* are summarised in Table 8.1. Although relatively high growth rates were measured for all three species these did not translate to high cell numbers as growth rates fluctuated between positive and negative values.

Species	# growth rates used to calculate means		Cell density (cells ml^{-1})		Growth rate (ln units day^{-1})	
	+	-	maximum	mean	maximum	mean
Anabaena circinalis	10	5	2800	500	0.428	0.037
Anabaena flos-aquae	21	6	5000	800	0.478	0.132
Aulacoseira granulata	15	12	1000	700	0.392	-0.012

Table 8.1 Cell densities and growth rates of major taxa in the lower River Murray, South Australia December-March, 1994-1995.

Chemical analysis revealed very low concentrations of filterable N and P which were often below minimum levels of detection. Growth bioassays indicated that 8-70% of TP (<0.1 mg L^{-1}) in the non-chlorophyll bearing fraction was bioavailable but the TN (<1 mgL^{-1}) in that fraction was essentially unavailable for growth. FDA-metabolic activity and growth bioassays were used to detect nutrient limitation. Nitrogen was often the limiting nutrient which conflicts with the paradigm that temperate freshwaters are phosphorus limited (Hecky and Kilham, 1988) but supports the hypothesis that semi-arid environments are N-limited. This study also challenges the public perception that the River Murray is a polluted and eutrophic waterway.

Microcystis was notably absent from the River Murray which may have been due to the very low inorganic nitrogen concentrations. The effects of low nitrogen availability on *Microcystis* are two fold, both growth and buoyancy are impaired. Gas vesicles are proteinaceous and account for 6-19% of cell protein (Oliver, 1994) and consequently there is a significant nitrogen demand involved in their production. Gas vesicle synthesis in *Microcystis* relies on soluble inorganic nitrogen whereas *Anabaena* spp. can use N_2 as a nitrogen source. Under nitrogen limitation in *Microcystis* the gas vesicle pool was reduced, carbohydrate was less efficiently metabolised and buoyancy was more readily overcome by increases in carbohydrate during photosynthesis. On the other hand in *Anabaena* without a source of inorganic nitrogen the gas vesicle pool remained intact. Only 63% of cells in a culture of nitrogen limited *Microcystis* were shown to be buoyant following a long dark period and therefore nitrogen limited *Microcystis* would not only suffer reduced growth but a proportion of the population would sediment during stable conditions reducing the population size.

8.3.1 Anabaena *and* Microcystis *in the River Murray*

Microcystis aeruginosa favours habitats which are persistently stratified (Kohler, 1992) and can tolerate disruption to stratification; eg diurnally stratified Lake George, Uganda (Ganf, 1974), Bautzen Reservoir, Germany (Kohler, 1992) and Lake Vinkeveen, The Netherlands (Ibelings *et al.*, 1991). Similar physical conditions were found in the lower River Murray, however, *Microcystis aeruginosa* did not invade the main river channel although a seed source was available in the backwaters adjacent to the river. Nitrogen could have been the factor limiting *Microcystis* population development instead the heterocystic nitrogen-fixing *Anabaena* spp.

were favoured. Although phosphorus may ultimately limit biomass, nitrogen and flow interact to control species selection.

During low summer flows thermal stratification can establish in the lower River Murray and favour cyanobacterial growth, however, nutrient limitation can alter this advantage. The majority of the flow during this experimental period was sourced from the Upper River Murray which historically has lower nutrient loads than the other major tributary, the Darling River (Shafron *et al.*, 1990). During periods where the Darling River contributes a higher proportion of flow greater nutrient concentrations may result giving rise to higher cyanobacterial biomass.

8.4 Buoyancy regulation in *Anabaena circinalis* and *Microcystis aeruginosa*

The buoyancy regulation mechanisms, including cellular carbohydrate change, gas vesicle synthesis, dilution of gas vesicle volume due to growth and turgor induced gas vesicle collapse, are all influenced by nutrients and light. The two cyanobacteria investigated in this thesis , *Microcystis aeruginosa* and *Anabaena circinalis*, displayed several differences in buoyancy response to light and nutrients particularly nitrogen. The basic buoyancy responses of each species in different growth conditions are summarised in Figure 8.1. Turgor induced collapse of gas vesicles was assumed to be less significant than the other two buoyancy mechanisms (Oliver, 1994) and was removed from the conceptual model to maintain simplicity. In general turgor pressure changes in response to carbohydrate accumulation and loss (Oliver and Walsby, 1984) and thus can be incorporated into the model as a factor further reducing buoyancy, in species with weak gas vesicles, as carbohydrate increases.

In this conceptual model carbon was also assumed to be available in saturating concentrations but for completeness the buoyancy response to carbon limitation is discussed in the text. If carbon was limiting an increase in buoyancy would be observed in the short term as cellular carbohydrate would be reduced but gas vesicle synthesis could be maintained on carbohydrate reserves (Spencer and King, 1989; Klemer, 1991). With sustained limitation of carbon, gas vesicle synthesis and consequently buoyancy would be reduced.

The model (figure 8.1) is specific for *Microcystis aeruginosa* and *Anabaena circinalis* and summarises work presented in this thesis. In general the physiological responses of

Microcystis and *Anabaena* to nutrient limitation lead to a reduction in buoyancy. The model presents the species *Microcystis* and *Anabaena* as the initial input representative of a seed source, either from vegetative cells or germination from a resting stage (eg. akinetes). The growth conditions are divided into limitation by either phosphorus, inorganic nitrogen or no nutrient limitation. Phosphorus limitation leads to a reduction in buoyancy predominantly from accumulation of carbohydrate (table 7.2) due to inefficient carbohydrate metabolism. A slight reduction in the gas vesicle pool may also result from phosphate limitation but this is dependent upon the degree of limitation (figures 5.12 and 5.21).

When nutrients are not limiting the growth of an organism then light may be limiting and was included as a growth condition. *Anabaena* and *Microcystis* displayed different responses to light and therefore buoyancy responses were modelled separately. When light was limiting the gas vesicle pool of *Anabaena* was greatest and decreased linearly with increasing irradiance (figure 5.15). Buoyancy in light limited *Anabaena* would be further enhanced by low photosynthesis and carbohydrate accumulation. The gas vesicle volume of *Microcystis* responded differently to *Anabaena* increasing with irradiance to a maximum at 100 µmol m^{-2} s^{-1} and then decreasing at higher light intensities (figure 5.4). Therefore, low light may or may not promote buoyancy via gas vesicle synthesis depending on the light intensity. Buoyancy would be promoted due to low carbohydrate accumulation.

Observed differences in the gas vesicle pool of *Microcystis* and *Anabaena*, in response to light, could be placed in context by examining the ecological implications of these phenomena. Growth rate of *Microcystis aeruginosa* saturated at 50 µmol m^{-2} s^{-1} (figure 3.5) whereas *Anabaena circinalis* growth rate saturated at 150 µmol m^{-2} s^{-1} (figure 5.14). The mean average light intensity needed by *Microcystis* to maximise growth was less than *Anabaena* and, therefore, *Microcystis* could afford more extensive buoyancy losses and deeper vertical migrations than *Anabaena*. When light was sufficiently high to saturate growth and photosynthesis both *Anabaena* and *Microcystis* experienced a dilution of the gas vesicle pool. Although carbohydrate increases in response to light, when nutrients are available carbohydrate is readily metabolised, does not accumulate in the long term and is thus included as a growth condition which promotes buoyancy.

When soluble inorganic nitrogen was low *Anabaena* was able to fix N_2, providing sufficient reductant, and overcome nitrogen limitation (figure 5.18). Under similar chemical conditions *Microcystis* was unable utilise N_2 as a nitrogen source and suffered nitrogen limitation, decreased growth (figure 5.7), decreased gas vesicle synthesis (figure 5.9) resulting in a reduced gas vesicle pool and decreased buoyancy (figure 7.2). Although nitrogen limitation leads to impaired carbohydrate metabolism (Turpin , 1991) carbon fixation was also diminished (figure 6.1) and carbohydrate did not accumulate to the extent of that observed in phosphorus limited cells (9 pg $cell^{-1}$ for *Microcystis* cells in 0 μM N following a dark pretreatment and 26.4 pg $cell^{-1}$ for cells grown in P-free conditions; tables 7.1 and 7.2).

The model (figure 8.1) summarises the previous growth conditions under which nutrients and light affect cellular carbohydrate and gas vesicle volume. The effect of these functions on cell buoyancy is indicated by (+) or (-). (+ indicates that the function acts to promote buoyancy; - indicates that the function reduces buoyancy). The net buoyancy response to the growth condition is shown prior to the forcing factor (+ the growth condition promotes buoyancy; - the growth condition reduces buoyancy). (-) does not necessarily imply that the cell is not buoyant but indicates reduced buoyancy relative to the maximum achievable buoyancy.

The forcing factor (high light) was included in the model to summarise the response of cells from different growth conditions to high light exposure. Cells photosynthesise and accumulate carbohydrate at different rates depending on their physiological state which is a function of their growth conditions. In general, exposure to high light results in carbohydrate accumulation leading to a reduction in buoyancy.

The buoyancy status of cells is dependent upon whether sufficient carbohydrate is accumulated to overcome lift provided by the gas vesicle pool. Cells may maintain buoyancy if they have a sufficiently large gas vesicle volume (+, persistently buoyant), or may lose buoyancy (-). (+/-) indicates that cells may or may not lose buoyancy, and represents heterogeneity in buoyancy response and gas vesicle volume between individuals. The heterogeneity in gas vesicle volume per cell may be a function of cell age. Cells which have recently undergone cell division have suffered a dilution of the gas vesicle pool and may be vulnerable to buoyancy losses following

light exposure. Older cells have had the opportunity to accumulate gas vesicles and may be more resistant to buoyancy loss with increases in dense polysaccharides.

The most obvious loss of buoyancy in response to light was observed in nitrogen-limited *Microcystis* cells. Persistent buoyancy was observed in *Anabaena* growing in high nutrient conditions (figures 7.11, 7.14 and table 7.6). Cells grown in other conditions may or may not lose buoyancy in response to light depending on the gas vesicle pool and carbohydrate accumulation rates of individuals within the populations.

The major difference in buoyancy between *Anabaena* and *Microcystis* occurs in response to low levels of soluble inorganic nitrogen. A proportion of the severely nitrogen limited *Microcystis* cells were unable to recover buoyancy even after an extended dark period (figure 7.2) whereas *Anabaena* displayed persistent buoyancy (figure 7.11). Nitrogen was implicated earlier as a factor determining what species dominate during low summer flows in the lower River Murray; low soluble inorganic nitrogen concentrations favouring heterocystic cyanobacteria.

In conditions conducive to cyanobacterial growth nitrogen availability and mixing may interact to select which species dominates. *Microcystis aeruginosa* and *Anabaena circinalis* are known to coexist (eg Mt. Bold Reservoir, Australia: Oliver and Ganf, 1988; Brookes *et al.*, 1994) but dominance by one species is also common eg. *Anabaena* dominance in the lower River Murray and the Murrumbidgee River, Australia (Webster *et al.*, 1996); *Microcystis* dominance in Lake George, Uganda (Ganf, 1974). *Anabaena* obviously has an advantage when soluble inorganic nitrogen is low, but when the nutrient requirements of both species are met then light may determine whether one species achieves dominance or whether they coexist.

Microcystis has a lower K_s for photosynthesis (50 µmol m^{-2} s^{-1}) than *Anabaena* (105 µmol m^{-2} s^{-1}) and also saturates growth at lower light intensities (50-150 µmol m^{-2} s^{-1} cf. *Anabaena* >150 µmol m^{-2} s^{-1}). It is therefore apparent that *Microcystis* could withstand a reduced light climate, resulting from greater mixing or turbidity, better than *Anabaena*. Further support for this hypothesis is provided by the different buoyancy responses of the two species. *Anabaena* displays more conservative buoyancy regulation than *Microcystis*. The rate of density change due to carbohydrate accumulation is approximately half the rate of *Microcystis* (5 kg m^{-3} h^{-1} vs

10 kg m^{-3} h^{-1} ; figures 6.1 and 6.4). Consequently *Microcystis* will dominate in habitats where it is advantageous to regulate buoyancy and migrate vertically, such as in waterbodies where nutrient and light resources are vertically separated. *Anabaena* would be favoured when mixing is low and *Microcystis* and *Anabaena* could coexist if nutrients were abundant. In stratified waters with low inorganic nitrogen *Anabaena* would dominate.

There is evidence to suggest that non-nitrogen fixing cyanobacteria have low competitiveness for nitrate nitrogen sources and high competitiveness for ammonium nitrogen sources (Blomqvist *et al.*, 1994). It appears that not only does thermal stratification or mixing determine species dominance but the species of nitrogen available is also important. A second conceptual model was developed to hypothesise which species or taxonomic groups would dominate under different physical and chemical conditions. The model is not exhaustive but rather provides a framework which can be modified to include other species, test specific case studies and develop hypotheses. The species presented are examples of taxonomic groups and do not necessarily represent specific successional patterns.

The model focuses on the degree of mixing and the species of the nitrogen which is available. The category boundaries are not absolute and it is anticipated that there is overlap between the categories in reality. Temperature and phosphorus were not considered although they may also affect the composition of the phytoplankton community (Watson *et al.*, 1997). Inspiration and concepts for this model were drawn from a multitude of sources particularly Blomqvist *et al.* (1994), Agbeti *et al.* (1997) and Rucker and Zippel (1997).

The model has the nitrogen species available along the Y-axis and the degree of mixing increasing along the X-axis (figure 8.2). During periods of water column stability indicated by persistent stratification the species which dominate must be able to resist sedimentation and remain suspended in the illuminated surface water for at least part of the day. Species able to do this include the microalgae with flagella and buoyant gas-vacuolated cyanobacteria. When soluble inorganic nitrogen is low the heterocystic nitrogen-fixing cyanobacteria dominate (eg *Anabaena*; figure 8.2). When the oxidised nitrogen species comprise the majority of the available nitrogen source then eukaryotic algae are favoured (Blomqvist *et al.*, 1994) and so dinoflagellates may dominate (eg *Ceratium*). Buoyant cyanobacteria may occur but would be

out competed by the eukaryotic species. When ammonium-nitrogen is the major nitrogen source then non-nitrogen fixing cyanobacteria may dominate. Cyanobacteria able to fix nitrogen may also occur in these conditions because it is more energetically favourable to utilise NH_4-nitrogen than N_2.

Intermittent mixing such as diurnal stratification favours buoyant species which are able to reduce sedimentation losses and similar species exist as in the persistently stratified system. Nitrate-nitrogen may be more readily available during intermittent mixing as ammonium fluxing from the sediments is entrained into surface water during mixing and oxidised. When ammonium-nitrogen is available increases in mixing may favour *Limnothrix* and even greater mixing favouring *Planktothrix*. When nitrate is the major nitrogen source the shift would be from Dinoflagellates to Bacillophyceae (eg *Rhizosolenia* and *Fragelaria*; Agbeti *et al.*, 1997) to Chlorophytes, Cryptophytes and Bacillariophyceae (eg *Aulacoseira*) with increasing mixing. With low soluble inorganic nitrogen and frequent mixing few species can persist (eg The lower River Murray) and these may represent the "ideal model" systems for formulating algal management strategies.

8.5 Floating velocity and its implications on reservoir management

The *Anabaena circinalis* population at Chaffey Dam displayed persistent buoyancy when nitrogen and phosphorus were added to bottled samples. Measurements of floating velocity of these filaments revealed that surface samples exposed to high irradiances floated slower than filaments suspended deeper in the water column. This outcome was expected from the principles of the ballast mechanism of buoyancy regulation (Oliver, 1994; Walsby, 1994) which predicted that carbohydrate accumulation would be greatest in filaments exposed to high irradiance resulting in a higher net density and slower floating velocity.

The result which was not predicted was the profile of increasing filament size with depth which further increased the floating velocity of filaments in low light. The increase in filament (biomass unit) size occurred too rapidly to be a function of growth and must be due to aggregation of filaments to form a larger unit. Presumably this phenomenon is due to cells exuding more mucilage when light it low and increasing their "stickiness". Mucilage is a complex polysaccharide (Reynolds, 1984) but whether the exudation of this compound

significantly reduced the internal carbohydrate pool, further enhancing cell buoyancy, is not known. Similar excreted mucilage has been observed in some marine diatoms and implicated in increasing flocculation and precipitating mass sedimentation of cells to the sea bed (Kiorboe and Hansen, 1993).

The floating velocity of cyanobacteria is an important feature of cyanobacterial ecology and has significant ramifications to cellular physiology and productivity. The floating velocity of a colony or filament determines how rapidly it can recover from a deep mixing event and regain position in a favourable light climate. Walsby *et al.* (1997) demonstrated that photosynthesis of *Aphanizomenon flos-aquae* increased nearly threefold by floating up after a deep mixing event. Filaments in the dark rely on reaching the light before cellular stores become exhausted. Small filaments with slow floating velocities may be selected against during periods of extended stratification and therefore by aggregating together in low light climates those filaments would significantly increase their survivorship and productivity.

8.6 Buoyancy regulation and algal movement

The movement of cyanobacteria in a water body is complicated and is affected by a suite of factors ranging from physical forces to chemical interactions and biological responses. These factors culminate in determining the vertical position of the phytoplankton, the photosynthetic productivity of the organisms and ultimately population growth. In natural systems it has been historically difficult to account for vertical movement and horizontal advection in measurements of growth and productivity. Models of buoyancy regulation and growth such as the Kromkamp and Walsby (1990) model of vertical migration and the expansion of this model to include wind mixing and growth (Howard *et al.*, 1996) provide insight into cyanobacterial dynamics but are limited by the heterogeneity of cyanobacterial response and site differences which complicates the calibration of these models.

Relatively new technologies such as the flow cytometry staining technique and fluorometry investigating algal photophysiology (Whittington *et al.*, 1996) show promise as techniques to estimate previous light history from which an estimate of previous vertical position and hence vertical migration can be made. Complemented by profiles of thermal structure, indicating the degree of mixing, it can be estimated what energy input and degree of mixing is necessary to

overcome buoyancy regulation and select for other phytoplankton taxa. From these data the efficiency of destratification systems could be increased as the mixing criteria required to control cyanobacterial biomass would be more accurately defined.

Further more, information on vertical migration coupled with estimates of productivity (Krause and Weis, 1991; Keifer and Reynolds, 1992), metabolic activity (FDA technology) and cell nutrient status (FDA technology; Wood and Oliver, 1995) will provide even greater insight into growth potential and estimates of population productivity in fluctuating light regimes. Fed back into models of reservoir water quality this information can then be used to improve reservoir management practices and water quality.

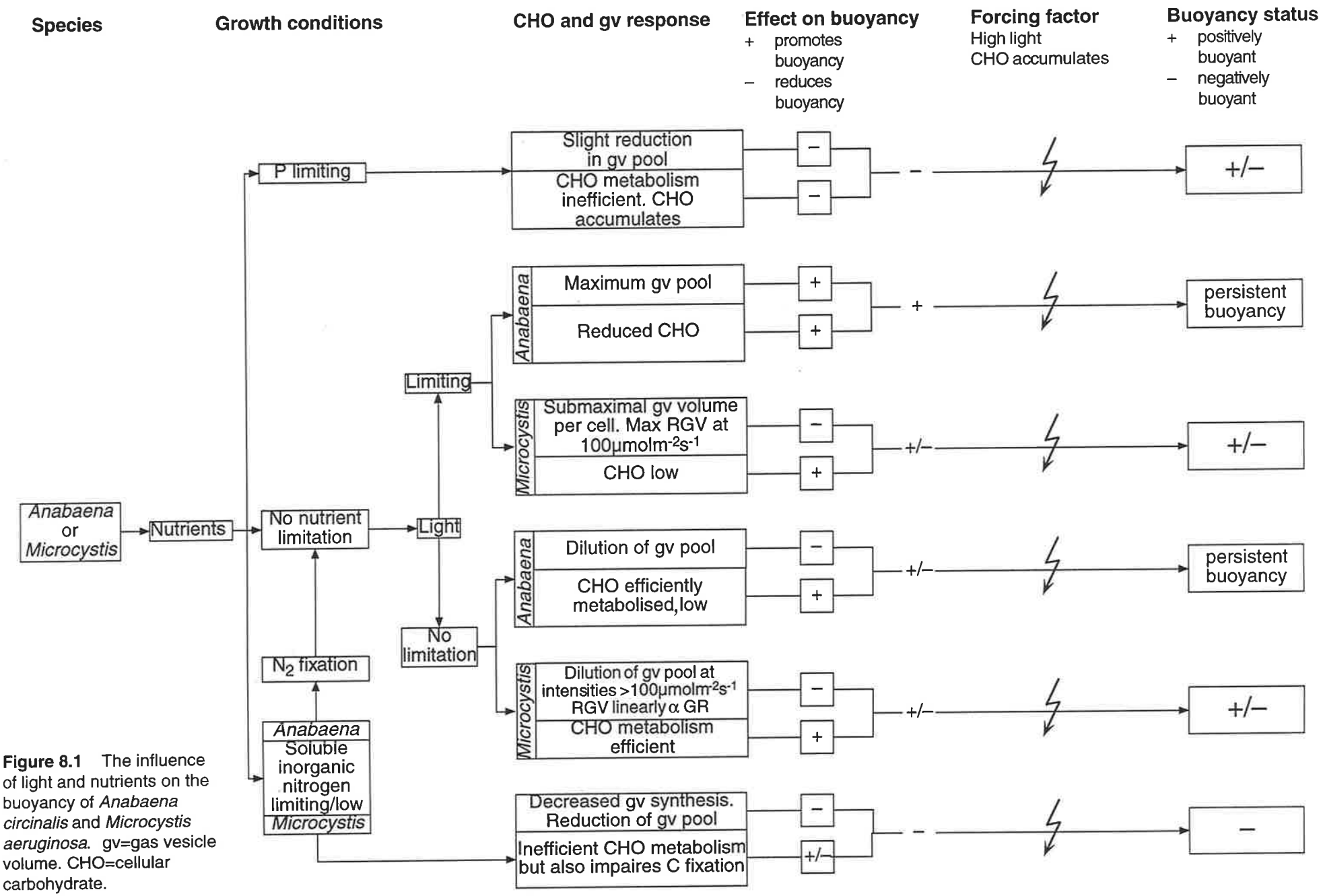


Figure 8.1 The influence of light and nutrients on the buoyancy of *Anabaena circinalis* and *Microcystis aeruginosa*. gv=gas vesicle volume. CHO=cellular carbohydrate.

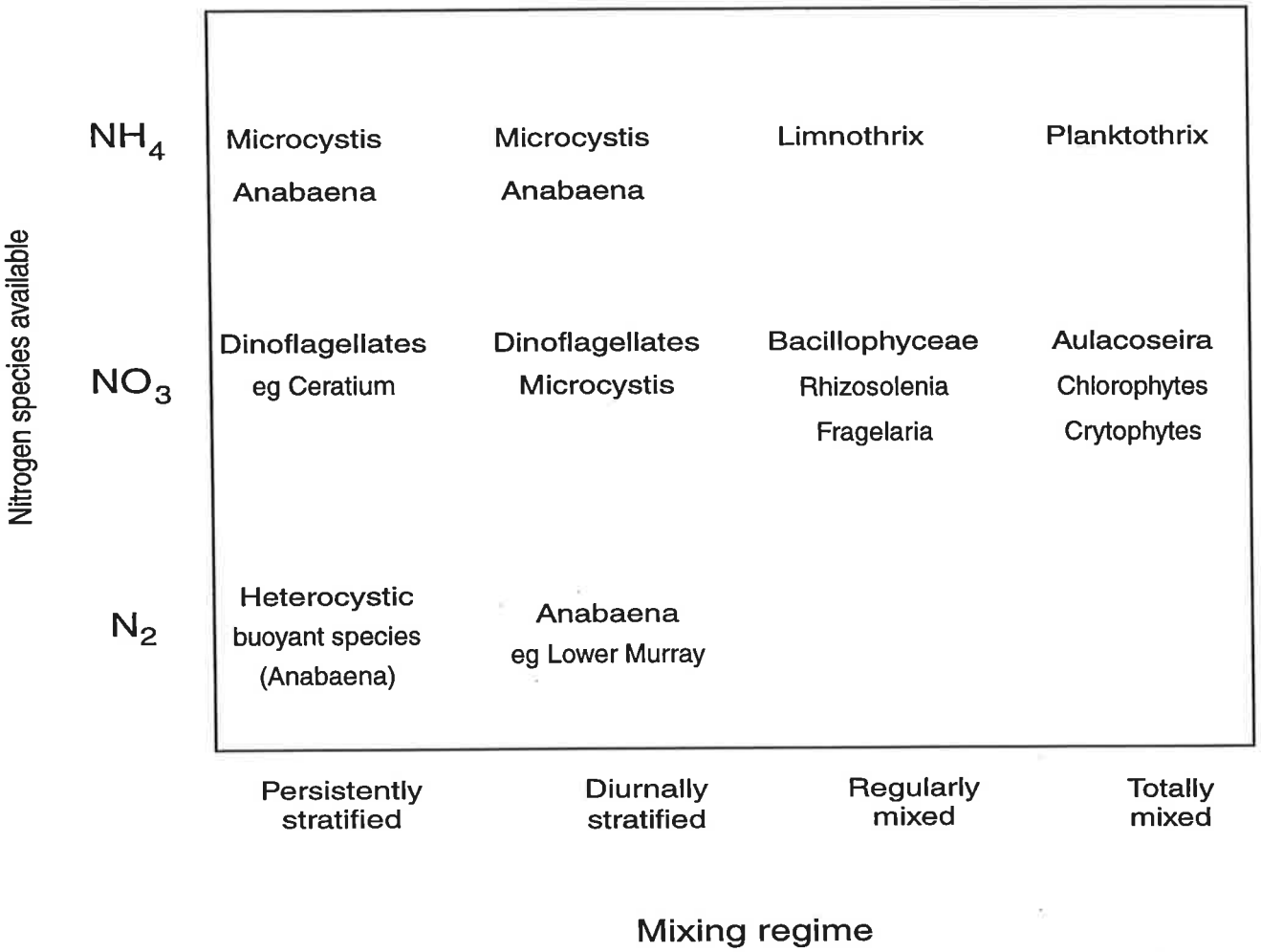


Figure 8.2 Prediction of phytoplankton species dominance under varying degrees of nitrogen availability and turbulence

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