Project M302

RAPID MEASUREMENT OF ALGAL BIOMASS, SPECIES COMPOSITION AND PHYSIOLOGICAL CONDITION

Project Leaders

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Natural Resources Management Strategy

Final Report Project M302

"Rapid measurement of algal biomass, species composition and physiological condition.

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December 1995.
**Project Team:**

This was a collaborative, two year project (NRMS Project M302) involving three research facilities and six research workers, Dr R.L. Oliver and M. Finke (Murray Darling Freshwater Research Centre), M. Burch (Australian Water Quality Centre), and Dr S Geary, J Brookes and Dr G Ganf (Botany Department, University of Adelaide).

**Introduction**

The basis of this project was the recognition that monitoring is an essential component of any management strategy. Although monitoring is considered by some as old fashioned, it incorporates the contemporary ideas of "continuous real time sampling and analysis", and so still plays a pivotal role in successful management. However, since detailed monitoring is time consuming it often leads to unacceptable time lags between identification of the problem and remedial action. Furthermore, time constraints and logistics often limit the number of samples that can be collected and processed. Since multiple samples are often required to evaluate the link between cause and effect, a lack of detail frequently means that limnologists are unable to provide succinct answers to what appear to be simple questions. This leads to poor management options.

Our solution to this problem was to embrace and develop technologies which have the potential to rapidly assess phytoplankton biomass, composition and metabolic state and to bring biological sampling and analysis into the same time frame as the monitoring strategies adopted in fluid mechanics.

The objectives of the project as stated in the original application were to evaluate the techniques of flow cytometry and active (dual flash) fluorometry for rapid determination of the biomass, composition and physiological condition of phytoplankton.

The background to the project relies on the hierarchy of relationships between light and nutrients which drive photosynthesis, and other metabolic activities and result in cell and population growth (Fig. 1). Active chlorophyll fluorescence and flow cytometry were used to evaluate these processes. Active chlorophyll fluorescence examines phytoplankton at the subcellular and cellular level whereas flow cytometry and the use of fluorescent probes are directed toward the cellular and population levels.

**Active fluorometry:**

Phytoplankton growth is dependent on photosynthesis to supply the cells with energy. Three key requirements in photosynthesis are; photon capture by the antenna of the photosystem, conversion of the light energy to chemical energy by a functional photosynthetic unit, and the capacity to process the chemical energy (Fig. 2). Environmental conditions that impact on these key processes play an important role in regulating phytoplankton primary production. The ability of different organisms to adjust their photosynthetic system to changing environmental conditions influences their competitive success and this affects the resultant community composition.

Photosynthesis can be investigated through an analysis of the changes in chlorophyll-a fluorescence that occur in response to alterations in photochemical processes. The basis of these relationships can be derived from a simple model (Fig. 3). In this model,
energy captured by chlorophyll-a molecules can be dissipated through two major pathways, it can either be used in photosynthesis, or if for some reason this cannot occur, then it can be re-emitted from the chlorophyll-a molecules in the form of light i.e. via fluorescence.

During the first step of photosynthesis energy captured in the antenna of a photosynthetic unit is used to transfer an electron to a molecule called $Q_A$ (Fig. 3 a), from where it is passed through an electron transport chain to generate energy that the cell can use. If the antenna captures a photon of light while $Q_A$ is still processing an electron from a previous photon hit, then the energy of the second photon cannot be transferred to $Q_A$ (the trap is shut) and instead it is dissipated as fluorescence (Fig. 3 b). In contrast, if electrons have passed from $Q_A$ into the electron transport chain when a photon is captured (the trap is open) then the energy enters the photosynthetic pathway via $Q_A$ and the fluorescence signal is minimal. As each cell contains many photosynthetic units, the intensity of the fluorescence signal will depend on the relative proportion of open and closed traps and as a result the chlorophyll-a fluorescence signal from a cell is variable.

In the technique of “active fluorometry”, a weak flash of light is used to probe the status of the photosystem while a bright flash of light is used to pre-condition the system (Fig. 4 b). If the cells have been dark adapted so that all traps are open, then the energy of the probe-flash stimulates a minimal fluorescence signal ($F_o$) with most energy going to photosynthesis (Fig. 4a). The proportion of closed traps is increased by pre-conditioning the cells with a flash of light before measuring the fluorescence response to the weak probe light. By increasing the intensity of the conditioning flash (Fig. 4b) the number of closed traps can be increased, the fluorescence signal reaching a maximum ($F_m$) when all traps are closed. The relationship between trap closure and the intensity of the conditioning flash is then used to analyse the physiological status of the photosystem and to identify the impact of environmental conditions on primary production. If these measurements are made while cells are incubated at a particular light intensity, then information is obtained on the capacity of the cells to utilise the incident light.

Fluorometers capable of making these types of measurements on natural phytoplankton populations have not been commercially available until quite recently. In the original grant application it was anticipated that a Dual Flash Fluorometer would be constructed for the MDFRC by CSIRO. Construction is continuing on this instrument, however due to the numerous problems encountered in its manufacture a decision was made to use external funds to purchase a commercial instrument to ensure the continuation of this project. Additional funds were provided by CSIRO and MDFRC for this purpose. During the last year the Pulse Amplitude Modulated fluorometer (PAM fluorometer, WALZ Pty. Ltd.), which was originally designed for work with higher plants, had its range extended with the addition of a new, high-sensitivity cuvette holder, enabling it to be used on samples with moderate concentrations of phytoplankton cells. This instrument can measure changes in variable chlorophyll-a fluorescence under a wide range of conditions, even under continuous background irradiance.
Active fluorometry can be used to measure at least three major components of the photosynthetic process (Fig. 2 & 4); the effective absorption cross-section (ε) of photosystem II which is the size of the antennae that the photosystem uses to capture light; the maximum change in quantum yield (ΔΦ_MAX) which is a measure of the relative number of functional reaction centres; and photochemical quenching (q_P) which is a measure of the relative number of RC II sites that are open and indicates the capacity of cells to carry our photosynthesis under the experimental conditions. Individually each of these measurements provides information on the effectiveness of major steps in the light reactions of photosynthesis and can be used to assess the impact of changing environmental conditions on each of these processes.

**Flow cytometry**

Microscopy is traditionally used to identify and count freshwater phytoplankton but is time consuming. Flow cytometry can rapidly quantify the size, density (or granularity) and pigment composition of individual cells and is traditionally used in medical research. Flow cytometers (Fig 5) use an array of lasers to determine the scattering and fluorescent properties of algal cells suspended in a carrier fluid passing the array of lasers. Whether it can be adapted successfully for use in phytoplankton work depends upon its ability to recognise and distinguish phytoplankton species on the basis of size, density and pigment composition. Since these characteristics vary in phytoplankton and these variations have taxonomic affinities it is possible that flow cytometry could be used to rapidly identify and count algal cells.

To establish the validity of the technique unialgal cultures were used to define a combination of characteristics (signature tunes) which could identify the test species. The species chosen were representative of the major taxonomic groups found in freshwater. To test whether these signature tunes were faithful and unique the species were mixed together and the established signature tunes used to separate, count and identify individual species.

Since pigment composition as well as the density and size of phytoplankton vary according to environmental conditions it was also necessary to determine how conservative the characteristics chosen to identify the individual species were across environmental gradients.

**Fluorescent probes**

One of the objects of the program was to investigate the physiological condition of phytoplankton. It was anticipated that this would be done using active fluorometry, however because of the difficulties encountered with construction of the fluorometric instrument alternative techniques were sort. One promising method appeared to be the use of fluorescent probes. These probes are used to estimate metabolic activity and are usually colourless and of low molecular weight to facilitate passage across the cell membrane. Inside the cell enzyme activity cleaves the molecule and produces a fluorescent compound which can be detected with a flow cytometer. To test whether the production of the compound is related to a detectable change in metabolic activity cells were exposed to nutrient and light conditions and the conversion rate of the probe was measured.

**Aims**

The aims and significance of this project were:

- To assess active chlorophyll fluorescence, flow cytometry and fluorescent probes as techniques for the rapid determination of the biomass, composition and physiological condition of algae in relation to light and nutrients.
• To evaluate the use of these techniques for the rapid monitoring of algal populations.

**Methods and Results:**

**Active fluorometry:** Measurements on algal cultures:

*Maximum quantum yield of fluorescence (ΔΦ<sub>MAX</sub>):*

The maximum change in quantum yield of fluorescence is calculated as:

\[
ΔΦ_{MAX} = \left( f_M - f_0 \right) / f_M
\]

Under ideal growth conditions, ΔΦ<sub>MAX</sub> was found to have a maximum value of ca. 0.65 for *Anabaena circinalis*, *Microcystis aeruginosa*, *Melosira granulata* and *Selenastrum capricornutum*. In continuous cultures of the cyanobacterium *Microcystis aeruginosa* grown under phosphorus-limited conditions the maximum change in quantum yield of fluorescence (ΔΦ<sub>MAX</sub>) decreased in response to phosphorus limitation as a function of relative growth rate. This response is similar to that observed in several species of marine phytoplankton under nitrogen limited conditions (Kolber et al. 1988). Although a wider range of freshwater phytoplankton needs to be studied to confirm the generality of these findings, it appears that decreases in the maximum quantum yield of fluorescence can be indicative of nutrient limitation and may be used to predict the relative growth rates of phytoplankton.

*Effective absorption cross-section (<i>σ</i>):*

The effective absorption cross-section was determined from the rate of increase in variable chlorophyll-a fluorescence with an increase in intensity of the conditioning light flash (Fig. 4 and 6) as described by Falkowski et al. (1986).

The effective absorption cross-section of the light harvesting antenna changes in response to the light intensity encountered by the cells, generally increasing in size as cells acclimatise to lower light intensities and decreasing in size as they adjust to higher light intensities. Our results suggest that the range of adjustment varies between different phytoplankton species as does the average cross-section size. The common bloom forming cyanobacteria *Anabaena circinalis* and *Microcystis aeruginosa* generally have smaller cross-sections than common eukaryotic phytoplankton such as *Melosira granulata* (Table 1).

Table 1. Absorption cross-sections measured in cells grown under a range of light conditions in laboratory cultures

<table>
<thead>
<tr>
<th>Organism</th>
<th>Absorption cross-section (&lt;i&gt;A&lt;/i&gt;&lt;sup&gt;2&lt;/sup&gt;/ quanta)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anabaena circinalis</em></td>
<td>29 to 54</td>
</tr>
<tr>
<td><em>Microcystis aeruginosa</em></td>
<td>31 to 63</td>
</tr>
<tr>
<td><em>Melosira granulata</em></td>
<td>86 to 178</td>
</tr>
<tr>
<td><em>Selenastrum capricornutum</em></td>
<td>101 to 137</td>
</tr>
</tbody>
</table>
If the size range of the effective absorption cross-section is indicative of the light conditions to which a cell is best suited, then these results indicate that the bloom forming cyanobacteria are better adapted to higher light intensities than the eukaryotic algae.

**Photochemical quenching (qp):**

Photochemical quenching is a measure of the proportion of open reaction centres, i.e. the proportion of reaction centres able to process a photon and carry out photosynthesis. It is calculated as:

\[ q_p = \frac{f_0 - f_i}{f_M - f_0} \]

where \( f_i \) is the variable fluorescence of a sample exposed to a continuous background light prior to conditioning with a light flash (Fig. 7).

Experiments with the green alga *Selenastrum capricornutum* indicated that photochemical quenching responds to alterations in light intensity (Fig 8). Cells of *Selenastrum* acclimated to higher light intensities were better able to process electrons through the electron transport chain and so had higher levels of photochemical quenching, and a greater capacity for photosynthesis at higher light intensities. In contrast, cells acclimated to low light intensities showed a rapid decline in photochemical quenching with increasing irradiance indicating a reduced capacity for photosynthesis at higher light intensities.

**Photosynthesis:**

Fluorescence measurements of individual photosynthetic processes provide insight to the adaptability of the cells and their responses to changing environmental conditions. However a critical test of these fluorescence measurements is that they can be combined together to determine the rate of photosynthesis. An equation to do this was proposed by Falkowski and Kolber (1993) but has not been extensively tested. Lack of time during the two year life of this project precluded the possibility of testing the model, however this has since been achieved under defined laboratory conditions with cultures of *Anabaena, Microcystis* and *Melosira*. In these experiments, rates of photosynthesis estimated from fluorescence measurements and expressed in terms of oxygen evolution were successfully matched with rates measured directly using an oxygen electrode (Fig. 9).

**Field Measurements:**

In the limited time available we were not able to apply these techniques to field samples. Since this initial work, successful field measurements have been made that provide exciting insights to phytoplankton primary production. A typical observation when taking primary production measurements over 24 hour periods, is a depression in the rate of photosynthesis during the afternoon. Although suggestions have been made about the likely cause of this decrease in photosynthesis, a clear demonstration of its cause has not been provided. Measurements made on a pond dominated by green algae including *Ankistrodesmus* indicated that the afternoon depression in
photosynthetic rate was due to a reduction in the maximum change in quantum yield (Fig. 10) with no change in the effective absorption cross-section or photochemical quenching.

The size of $\Delta \Phi_{\text{MAX}}$ decreases when cells are exposed to particularly high light intensities where photoinhibition of photosynthesis occurs. In these situations changes in $\Delta \Phi_{\text{MAX}}$ cannot be interpreted solely as responses to nutrients, but must be compared with other variables describing the behaviour of the photosynthetic process, particularly the effective absorption cross-section and the extent of photochemical quenching.

**Flow cytometry**

Three instruments were used in this study: Coulter Epics Profiler 2 & 3, Becton-Dickson FacScan 2 and a Becton-Dickson FacStarPlus. The FacStarPlus was fitted with a macrosort accessory which permitted cell sorting. Excitation at 488 and 620 nm are used to estimate the size, granularity and pigment composition of individual cells (Table 2). A signature tune for individual species was obtained by combining characteristics determined by the laser settings.

Table 2. Excitation and emission wavelengths used to determine cell characteristics using flow cytometry.

<table>
<thead>
<tr>
<th>Laser excitation</th>
<th>Emission</th>
<th>Cell Characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>488 nm</td>
<td>forward scatter</td>
<td>size</td>
</tr>
<tr>
<td>&quot;</td>
<td>side scatter</td>
<td>density /granularity</td>
</tr>
<tr>
<td>&quot;</td>
<td>575 nm</td>
<td>phycocerythrin</td>
</tr>
<tr>
<td>&quot;</td>
<td>680 nm</td>
<td>chlorophyll</td>
</tr>
<tr>
<td>620 nm</td>
<td>650 nm</td>
<td>phycocyanin</td>
</tr>
</tbody>
</table>

A major problem encountered was the size of the biomass units relative to the nozzle aperture. Particles >70 $\mu$m caused the nozzle to block. Various solutions to this problem were investigated with the aim of dispersing colonies and filaments without destroying the constituent cells. Treatment of the sample with sodium hydroxide (0.025 M) and or $H_2O_2$ (7.5%) for 10 to 120 min at room or 37°C was unsuccessful. Similarly, the use of pectinase (0.5%) and lysozyme or cellulase (10 – 50 units ml$^{-1}$ ) plus lysozyme for 2 hours did not disperse the biomass units.

Two techniques, an ultraturrax homogenizer operated at 60 cycles s$^{-1}$, and the repeated (5-10 times) drawing up and ejecting of a suspension through a 27 gauge needle were successful for a variety of species including: Chlamydomonas, Chlorella, Cyclotella, Cryptomonas, Microcystis, Melosira and Ochromonas. Unfortunately, these techniques were unsatisfactory for Anabaena and Nodularia where there was a significant and excessive cell breakage.

**Signature tunes of unialgal cultures**

The characteristics of ten algal species (Table 3 ) show that the intensity of the response differs significantly between species. For example, the three cyanobacteria have high levels of phycocyanin whereas in the other species phycocyanin is either absent or at a low level. It is through a combination of these characteristics that a specific signature tune for each species is derived.
Table 3. Characteristics of size, density, phycoerythrin (pe), phycocyanin (pc) and chlorophyll a (chl) of ten algal species obtained via flow cytometry.
Level of activity ( ) = not present, (+) = low, (++) = medium, (+++) = high.

<table>
<thead>
<tr>
<th>Species</th>
<th>FS (Size)</th>
<th>SC (Density)</th>
<th>PE (575 nm)</th>
<th>PC (650 nm)</th>
<th>Chl (680 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcystis</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Anabaena</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Nodularia</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Chlamydom.</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Scenedesmus</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Botryococcus</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Ochromonas</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Mallomonas</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Cyclotella</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Cryptomonas</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

These results are expressed graphically in Figure 11 and demonstrate that flow cytometry can rapidly identify a range of algal species.

Species composition and separation
Six species with pre-determined signature tunes were mixed together and analysed using the FacStar-Plus (Fig. 12). The two dimensional, dot-plot graphs identified six groupings based upon chlorophyll and phycocyanin (Fig. 12a). The R1 group were gated out and represent non-fluorescing particles. The remaining groups (R2 to R6; Fig. 12 c-f) were delineated via their side- and forward-scattering properties, however, R6 represented two groups (Fig. 12 g) which were further separated upon their pigment characteristics (Fig. 12 h & i). Figure 12 j shows the superimposed images for all six species based upon their scattering properties.

From these data it was concluded that, at least for culture populations grown under uniform light and nutrient conditions, flow cytometry was able to identify and separate species. The time for this analysis was ca. 30 min but does require prior knowledge of the organisms and machine calibration.

Enumeration of cell numbers
Cell numbers can be estimated by either recording the number of events (cells ?) in a fixed sample volume or by mixing a known concentration of marker beads with the algal suspension. For the Coulter Epics Profiler both fixed volume and marker bead techniques consistently under-estimated cell concentration by 40 - 50% compared with manual counts. Using the FacScan and a constant flow rate cell numbers were underestimated by 40 - 70%.

Comparison of manual and automatic counting using phycoerythrin (PE) labelled beads in conjunction with the FacStar-Plus gave satisfactory results (Table 4) for the three species tested.
Table 4. Comparison between manual and automatic cell enumeration techniques using the FacStar-Plus in conjunction with PE-labelled beads.

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>MANUAL</th>
<th>AUTO</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYCLOTELLA</td>
<td>2.4 \times 10^6</td>
<td>2.2 \times 10^6</td>
</tr>
<tr>
<td>CHLAMYDOMONAS</td>
<td>1.32 \times 10^5</td>
<td>1.37 \times 10^5</td>
</tr>
<tr>
<td>MICROCYSTIS</td>
<td>1.88 \times 10^3</td>
<td>2.06 \times 10^5</td>
</tr>
</tbody>
</table>

Although cell enumeration via flow cytometry and PE-labelled beads is rapid (once set up the counting procedure takes < 1 min) there are problems. Commercial flow cytometers used in medical science are not designed to analyse long and convoluted filaments as found in species such as Anabaena and Nodularia. Furthermore, and for understandable reasons, the operators of medical flow cytometers are reluctant to permit major changes to the flow cytometer settings. The pulse-processing technique is possible but the problem of the random orientation of filaments in relation to the laser beam must be overcome. In conclusion, laboratory tests using three commercial flow cytometers have demonstrated the practical advantages of flow cytometry, but further research is required to provide reliable techniques for the dispersion of cells in species which form colonies and or filaments. The European community is currently constructing a flow cytometer for specific use with algal samples (Europa flow cytometer, University of Plymouth, U.K.) and this should be investigated.

How conservative are signature tunes of individual species?
The intensity of any response is a function of the environmental conditions, for example cell concentrations of phycobiliproteins and chlorophyll may vary over an order of magnitude depending upon the intensity and spectral distribution of the light. Similarly, the intensity of side and forward scattering is a function of the size of the biomass unit and the degree of gas vacuolation. Our conclusion is that the signature tunes of individual species are not conservative properties but may vary over an order of magnitude.

Recognition of this variability lead to an investigation of whether or not it could be used to assess the physiological state of phytoplankton and address the following problems:
- Was it possible to distinguish between active and moribund cells within a population?
- Could flow cytometry be used to rapidly assess nutrient status of cells?
- Could flow cytometry be used to predict future growth rates from a knowledge of the current metabolic activity?

Physiological state
Traditional methods for assessing the physiological state of algal populations have often relied upon algal growth bioassay and or cellular composition or a mixture of both techniques. These have met with varying success and are often time consuming. New technologies which offer alternative and more rapid analysis include:
- Flow cytometry in conjunction with metabolic fluorescent probes
- Active chlorophyll fluorescence.

Fluorescent probes and flow cytometry
The probe we chose to investigate was Fluorescein Di-acetate (FDA) which has been used previously and has many of the characteristics we required. FDA is a colourless low-molecular weight compound which readily passes across the cell membrane. Within the cell it is
converted into fluorescein by non-specific esterases which are involved in the turn-over of phospholipid in cell membranes. We argued that esterase activity should reflect metabolic activity and since the synthesis of cell membranes is an essential precursor to cell division should also reflect cell growth.

Excitation of the sample at 488 nm and collection at 530 nm using a flow cytometer permitted the determination of the rate of FDA conversion to fluorescein. To test whether the rate of conversion of FDA was linear with time, and whether the rate was substrate dependent, experiments were carried out on two species (Chlamydomonas and Microcystis). The results (Fig. 13) showed conversion was linear over the first 15 minutes and a rate could be determined from the initial slope. In subsequent experiments the rate of conversion was calculated for the first seven minutes. In addition, (Fig. 14) the rate of conversion was substrate dependent with a saturating, final concentration of 40 μM.

**FDA conversion and growth rate**

To assess the relationship between FDA conversion rates and growth rates, cultures of Microcystis were exposed to light intensities of 10, 50 and 100 μmol m$^{-2}$ s$^{-1}$; the rate of FDA conversion and the growth rates were determined after three days. The results (Table 5) showed that in these nutrient saturated cultures growth rate was strongly correlated with light intensity ($r^2 = 0.98$). The slope of the line relating relative fluorescence per cell to time, ie the relative FDA conversion rate (RFC; $r^2 = 0.998$) increased as the light intensity increased, the slope being minimal at the lowest light intensity and highest at the maximum intensity. This correlation was also observed for the relation between growth rate and RFC.

Table 5. Growth rates and relative FDA conversion rates (RFC min$^{-1}$) of Microcystis aeruginosa after cultures were grown for three days at three light intensities.

<table>
<thead>
<tr>
<th>Intensity (μmol m$^{-2}$s$^{-1}$)</th>
<th>Growth rate (day$^{-1}$)</th>
<th>RFC min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.13 ± 0.01</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>50</td>
<td>0.37 ± 0.01</td>
<td>121 ± 4</td>
</tr>
<tr>
<td>100</td>
<td>0.48 ± 0.01</td>
<td>165 ± 4</td>
</tr>
</tbody>
</table>

These data demonstrate that under the culture conditions imposed, growth rate and RFC are positively correlated, which suggests that RFC maybe used to predict growth rates in the field providing a series of observations are taken over time.

**RFC and nutrient status of cells**

The relationship between RFC and cell nutrient status was determined by growing populations of Microcystis in P-free media. After starvation RFC was between 4 and 5. On the addition of P over a range of concentrations between 0 and 10 μM (Table 1) there was no immediate increase in RFC, but by the second day RFC at the higher concentration had increased 5-fold, whereas the populations exposed to the lower concentrations showed no response. This trend continued so by day four RFC at all concentration above 0.1 had increased significantly above the control, and by the ninth day those at the highest P concentration had RFC values two orders of magnitude greater than the initial samples.
Table 6  Relative FDA conversion rates (RFC) after 2, 4 and 9 days of *Microcystis* populations initially starved of P and subsequently resuspended in media at P concentrations between 0 and 10 μM.

<table>
<thead>
<tr>
<th>[P] μM</th>
<th>DAY 2</th>
<th>DAY 4</th>
<th>DAY 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>8.5</td>
<td>9.9</td>
</tr>
<tr>
<td>0.1</td>
<td>4.4</td>
<td>15.3</td>
<td>41.8</td>
</tr>
<tr>
<td>0.2</td>
<td>4.6</td>
<td>26</td>
<td>67.2</td>
</tr>
<tr>
<td>0.5</td>
<td>6.1</td>
<td>41.6</td>
<td>110.2</td>
</tr>
<tr>
<td>2</td>
<td>26.8</td>
<td>221.9</td>
<td>147.7</td>
</tr>
<tr>
<td>5</td>
<td>24.5</td>
<td>172.4</td>
<td>634.2</td>
</tr>
<tr>
<td>10</td>
<td>23.4</td>
<td>212.6</td>
<td>735.4</td>
</tr>
</tbody>
</table>

These data demonstrate that FDA maybe used to determine the phosphorus status of cells. Similar results were obtained in experiments where the cells were starved of nitrogen.

The growth rates of the cells at different phosphate concentration provided a range of growth rates. These were plotted against the RFC on day four to assess how well this value would predict the subsequent growth rate over the interval 4 - 10 days (Fig. 15). The linear relationship suggests that the rate of FDA conversion is a powerful predictor of future growth.

**Conclusion**

The objectives of the project were achieved despite unforeseen technical difficulties with instrumentation. Both techniques were successfully trialed, but there was insufficient time to overcome all the problems encountered and to exploit the techniques to their full potential under field conditions. In retrospect, it was probably too short a period to fully develop the capacity of instrumentation which was either owned or to be built by third parties, and it was fortunate in this case that we were able to adopt alternative strategies. Much of the exciting progress in the project occurred towards the end of the two years after the period of detailed instrument assessment. It was a great shame that, despite this progress, funding could not be found to continue the project for a further year as this would have enabled the project team to fully capitalise on the expertise it had developed and would have maximised the return on the project.

The principal findings of the project were that the techniques could provide:

- Rapid estimates of algal photosynthesis as confirmed by oxygen measurements
- Quantification of key functional processes in photosynthesis
- Identification of adaptive ranges for specific taxa
- Data on functional processes related to nutrient/light conditions
- Rapid analysis of individual cell numbers in populations and communities
- Quantification of nutrient/light limited growth through the analysis of FDA uptake

These results provide a basis for:

- Understanding the occurrence of problematic species based on adaptive capabilities of their photosynthetic system and growth rates under various nutrient and light conditions
• Assessing responses of algal populations to management options related to alterations in the physical and chemical environment.

These findings are not only consistent with the original objectives in meeting the technical targets, but have contributed to the development of new concepts associated with algal growth and photochemistry. For example, the work with FDA demonstrated it was possible to determine whether the metabolic activity of a community or an individual population was light or nutrient limited. In addition, the PAM techniques showed that very rapid photochemical measurements could be used to assess the response of communities to alterations in the light climate. The results suggest that by combining the techniques an analysis of communities, populations and individual cells could be obtained in "real time" and used to assess the impact of management options.

The project outcomes clearly demonstrate the merit of these techniques and have confirmed our belief that these methods will play a key role in future research in this field. It is evident that further study is required, but this reflects the new and powerful avenues opened up by this investigatory project.

References:


Figure 1. Two techniques, active chlorophyll fluorescence and flow cytometry were used to evaluate the influence of light and nutrients on the activity at both the subcellular (photosynthesis, synthesis of metabolites and cell growth) and the cellular levels (metabolic activity, cell and population growth).

hv

ANTENNA → Chla → Photosynthesis

PHOTON CAPTURE

FUNCTIONAL PHOTOSYNTHETIC UNIT

ENERGY PROCESSING

Figure 2. Three major steps in photosynthesis.
Figure 3. A simple model describing changes in chlorophyll-a fluorescence as a function of the status of the photosystem (upper panel) open trap, (lower panel) closed trap.

\[ \Delta \phi_m = \frac{f_m - f_o}{f_m} \]

Fig. 4. Pictorial representation of the technique of active fluorometry to measure variable chlorophyll-a fluorescence: (upper panel) fluorescence response to (lower panel) light conditions. The formula for maximum change in quantum yield (\( \Delta \Phi_{\text{MAX}} \)) is shown.
Figure 5. Diagrammatic representation of a flow cytometer. Instruments such as the FacStar plus have the added benefit of sorting samples.

Measurements of Effective Cross Section

![Graph showing normalized variable fluorescence against flash intensity](image)

Figure 6. The relationship between increasing pre-conditioning light flash intensity and normalised variable fluorescence for three algal species. The effective absorption cross-sections ($\text{Å}^2$ quanta$^{-1}$) for each of the species is shown in the box and was calculated from the data following Kolber and Falkowski (1986).
Figure 7. Estimation of photochemical quenching from the fluorescence signal caused by a continuous background actinic light momentarily overlayed by a saturating flash. The formula for photochemical quenching is shown.

qp for Selenastrum Grown at Different Light Intensities

210 μmol m⁻² s⁻¹
Growth Irradiance

16 μmol m⁻² s⁻¹
Growth Irradiance

Fig. 8. The influence of light intensity on the photochemical quenching in cultures of Selenastrum capricornutum grown at two different light intensities.
Oxygen Electrode and Fluorescence Measurements of Anabaena Photosynthesis

- Oxygen Electrode
- PAM

Fig. 9. A comparison of photosynthesis-irradiance curves for *Anabaena* sp. estimated using an oxygen electrode and the PAM fluorometer.

$\Delta \phi_m$ and qP  Green Pond

Fig. 10. The effect of light intensity on the maximum change in quantum yield and photochemical quenching of algal samples taken at different times of the day from Green Pond. Sampling times are shown in the boxes.
Fig. 11. Upper panel: Fluorescence emissions at 680 nm (11 nm band width) of nine freshwater phytoplankton after excitation at 488 nm (50 mW) using a FacStarPlus flow cytometer. Lower panel: emissions at 650 nm (11 nm band width) after excitation at 620 nm (4 mW).
Fig. 12. A mixture of six freshwater phytoplankton (R2 - R7/8) were identified and sorted using a FacStarPlus. Each panel represents the progressive steps taken to separate the species using combinations of chlorophyll a (CHL), phycocyanin (PC) and forward scatter (FSC) and side scatter (SSC).
Fig. 13. Time course for the uptake and conversion of Fluorescein Di-acetate (FDA) to fluorescein measured as relative fluorescence in *Microcystis aeruginosa* (upper panel) and *Chlamydomonas reinhardtii* (lower panel). Error bars indicate standard deviations where these are larger than the symbols used.
Fig. 14. The relationship between FDA concentration (10 - 70 μM) and relative fluorescence in Chlamydomonas reinhardtii and Chlamydomonas reinhardtii.
Fig 15. Relationship between the rate of FDA conversion on day four (RFC min$^{-1}$) and the growth rate of a Microcystis aeruginosa culture from day four to ten.