Technical Memorandum 37

Isolation and culture of five species of freshwater algae from the Alligator Rivers Region, Northern Territory

A. Padovan

Supervising Scientist for
the Alligator Rivers Region

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TECHNICAL MEMORANDUM 37

ISOLATION AND CULTURE OF FIVE SPECIES OF FRESHWATER ALGAE FROM THE ALLIGATOR RIVERS REGION, NORTHERN TERRITORY

A. Padovan

Australian Government Publishing Service
Canberra, 1992

August 1992

ISSN 0810-9532
ISBN 0 644 25294 4

Supervising Scientist for the Alligator Rivers Region
P.O. Box 387, Bondi Junction
N.S.W. 2022, Australia

This Technical Memorandum was prepared by:

A. Padovan
Alligator Rivers Region Research Institute, Jabiru, N.T. 0886

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Printed for A.G.P.S. by Alken Press Pty. Ltd. – Smithfield

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Padovan, A. (1991). Isolation and culture of five species of freshwater algae from the Alligator Rivers Region, Northern Territory. Technical Memorandum 37, Supervising Scientist for the Alligator Rivers Region.

Of five freshwater algal species isolated from the Alligator Rivers Region, four were identified to specific level (one tentatively), and the other was identified to the genus. The taxa identified were: *Ankistrodesmus spiralis, Scenedesmus quadricauda, Scenedesmus dimorphus, Ankistrodesmus (falcatus?), Selenastrum* sp. All algal cultures were mono-specific but were not axenic.

Attempts were made to mass culture the algae so they could subsequently be used in food trials with the cladoceran, *Moinodaphnia macleayi*. *A. spiralis* was found to be unsuitable for mass culture because of its very slow growth rate and the formation of large aggregates in both non-aerated and aerated cultures. The other four species showed rapid exponential growth with no lag phase when cultured in a modified WC medium. *A. (falcatus?)* the smallest species measured on a dry weight basis, had the highest growth rate. The growth rates of the remaining three species were similar, with that of *S. dimorphus* being lowest.

1 INTRODUCTION

The use of Biological Toxicity Tests (BTT) for regular monitoring requires a constant and reliable supply of organisms. The Toxicology Section of the Alligator Rivers Region Research Institute (Office of the Supervising Scientist) at Jabiru, has used a variety of freshwater organisms to determine toxicity of uranium mine effluent waters that may be released into Magela Creek. Organisms tested include gudgeon (*Mogurnda mogurnda*) embryos, hydra (*Hydra viridissima* and *H.vulgaris*) and the cladoceran, *Moinodaphnia macleayi*.

In past years, the supply of *M. macleayi* from stock cultures kept in incubators has been unreliable, especially during the period leading into the Wet season. Problems encountered include death of animals, failure of mature females to produce consistent numbers of neonates, broods not being produced at their regular times, or the production of high proportions of males (also a sign of stress). BTT controls also experienced these problems on occasions, complicating the interpretation of results.

Varying water quality and inadequate nutrition may be contributing causes to the unreliability of cladocerans (water fleas) at these times, since all other environmental factors (e.g. photoperiod, light intensity, temperature) are kept constant. There could be several processes operating. Varying water quality may cause the cladocerans to be physiologically stressed (e.g. osmoregulatory stress). If a stressed animal was inadequately fed, it may not have the reserves (e.g. energy, metabolites) to survive that period of stress. Alternatively, varying water conditions could cause subtle alterations in the diet. This could, for instance, affect changes in the acquisition of nutrients directly, or indirectly, through affecting food quality. An indirect effect could occur due to changes in nutrient availability which would then be reflected in bacterial growth, thus affecting the cladocerans which graze on the bacteria.

On the basis of success reported in the literature it was decided to either substitute or supplement the diet of *M. macleayi* with live algae. The algal species investigated came from three genera—*Ankistrodesmus*, *Scenedesmus*, *Selenastrum*—all of which belong to the Chlorophyta (the green algae) and have previously been reported as being suitable for maintaining cultures of cladocerans. Due to regulations governing the introduction of foreign plants or animals into Kakadu National Park, algal species had to be isolated from waters within or associated directly with the park. The objectives of this project were to: isolate algae from each of the three genera mentioned above; describe a method for their mass culture and harvest; determine the growth characteristics of the isolated algal species to enable the yield and quality of cells harvested to be optimised; determine if optical density may be used as a reliable measure of population growth; investigate the measurement of cell dry weight as an alternative means of expressing algal biomass.

2 MATERIALS AND METHODS

2.1 Isolation of algal species

Field collections

Live cells were obtained by net tows (10 μm net mesh-size) in selected billabongs in the early 1991 Dry season (mid-June). Despite the target algal species being considered 'universal', field sites were chosen on their previous reported occurrence (Ling & Tyler 1986), and on the basis of site accessibility. The 3 sites sampled were Ja Ja, Mine Valley and Georgetown Billabongs. In addition, a surface sample was also collected from Nanambu

Creek (where it is intersected by the Arnhem Highway) which was supporting an obvious algal bloom. After collection, samples were stored in 150 mL plastic bottles and kept in the dark on ice.

On return to the laboratory, all samples were examined microscopically for the presence of at least one target species. Identification was based on descriptions in Ling & Tyler (1986). Samples were then transferred to sterile 500 mL Erlenmeyer flasks and placed in incubators.

Separation of algal species

All samples were filtered through a 200 μm plankton net to remove zooplankton and any large foreign particles. They were then, in turn, filtered through a series of 150, 100, and 20 μm plankton nets. Cells retained at each stage were backwashed with WC medium into 125 mL Erlenmeyer flasks and made up to approximately 30 mL. The surface sample from Nanambu Creek, dominated by *Microcystis aeruginosa*, was allowed to stand overnight in a 100 mL measuring cylinder, then buoyant cells were gently skimmed off the surface before passing the sample through the net series.

To ensure the survival of algae from each size fraction, 1 mL aliquots were transferred to sterile 250 mL flasks containing 50 mL of WC media at four concentrations, full (WC/1), half (WC/2), quarter (WC/4) and one fiftieth (WC/50) strength. In addition, these also served to increase algal biomass, with the possibility of target species increasing their relative abundance. Transfer to WC media also retarded the growth of diatoms, especially for samples from Ja Ja Billabong, as no silicate was added to the media.

All fractions were examined microscopically in a haemocytometer (see Appendix 1). Table 1 summarises observations made, noting fractions containing target species, the dominant species present, an indication of overall biomass, and the suitability of the fraction for streaking onto agar.

Fractions thought to have the greatest potential to yield target species were streaked out, in triplicate, across agar plates of media strengths WC/1, WC/2 and WC/4. The remaining fractions were streaked out only once on each media strength (WC/1, WC/2, WC/4, WC/50). Agar plates (inverted) were incubated as described above.

After approximately one week, green colonies were observed using the dissecting microscope. A glass Pasteur pipette tapered to a fine point (by heating and stretching) was used to transfer a single colony to a drop of sterile WC/2 liquid media placed on a glass microscope slide and a cover-slip positioned on top (both alcohol wiped). The colony was examined under the compound microscope to determine its type and the extent of contamination with other algae.

Slides of non-target species were discarded. Colonies of pure target species were transferred to sterile WC/2 media (50 mL in 250 mL sterile flasks) by using the cover-slip to scrape the colony from the slide. The entire cover-slip was dropped into the medium, and any liquid on the surface of the slide was washed into the flask using WC/2 medium.

Once algal cells attained a density high enough to colour the medium, they were streaked again on WC/2 agar to ensure purity both with respect to species and strain. After two weeks, colonies could be detected, and were subsequently transferred into sterile WC/2 media.

Liquid and agar culture media

Initially, algal samples were inoculated in a range of media strengths, WC/1, WC/2, WC/4 and WC/50 to determine their response. After initial screening of different strength media,

all algal species were cultured in half-strength WC medium (as modified by Guillard & Lorenzen 1972) - details are listed in Appendix 1.

$FeCl_3.6H_2O$ was replaced by Fe-citrate/citric acid and silicate was omitted. In addition, half the nitrate was substituted with ammonium chloride on a mole for mole basis. $CaCl_2.6H_2O$ and $NaHCO_3$ were prepared then autoclaved separately, and upon cooling, were aseptically added to the rest of the media to avoid precipitation of $CaCO_3$. All stocks and media were made up using high purity Millipore Milli-Q water.

Agar medium was prepared by the addition of 1.5% agar powder to liquid media prior to autoclaving. To avoid condensation on inside surfaces of plates, agar was poured into pre-sterilised plastic Petri dishes while the agar was still liquid.

Growth conditions

Cultures were grown in sterile glass Erlenmeyer flasks ranging in size from 125 to 500 mL. Flasks were stoppered with non-absorbent cotton-wool covered with aluminium foil to prevent contamination with dust. Levels of media added never exceeded the widest diameter of the vessel, which ensured adequate gas exchange between media and air. Cultures were incubated in Labec temperature controlled incubators set at 27 ± 2°C. The incubators had an artificial lighting source with light intensity varying between 30 and 100 $\mu E/m^2/s$, photosynthetically active radiation (PHAR), depending on the distance from the light source (usually 30-60 $\mu E/m^2/s$ PHAR). Lighting was set to a 12:12 light/dark cycle.

2.2 Antibiotic treatment of cultures

The procedure outlined by Hoshaw & Rosowski (1979) was adopted, and is described below.

Antibiotic solution preparation

Immediately before use, 100 mg of penicillin G (Na salt) and 50 mg of streptomycin sulphate was dissolved in 10 mL Milli-Q water. Then 10 mg of chloramphenicol, dissolved in 1 mL 95% ethanol and mixed well, was added. Sterilisation was achieved by gently passing the solution through a 0.2 μm membrane filter and collecting filtrate in a sterile 100 mL Erlenmeyer flask.

Algal cultures

Exponentially growing cultures of *A. spiralis* and *Selenastrum* sp. grown in WC/2 media (100 mL in a 250 mL Erlenmeyer flask) were used in two separate trials. All cultures were grown in incubators at 27°C at a light intensity of approximately 30 $\mu E/m^2/s$ and a 12:12 light/dark cycle. Cultures were allowed to attain a density of about 2×10^5 cells/mL, ensuring a cell density of at least 100 cells/mL by the end of the treatment.

Treatment of cultures

Since dosages and contact times of antibiotics required to kill non-algal microbes were unknown, as well as each alga's sensitivity to the antibiotics, several antibiotic concentrations and different contact times were tested to determine the optimal treatment.

All glassware and solutions described were sterile; aseptic techniques were used throughout the study. To each of six 100 mL Erlenmeyer flasks containing 35 mL WC/2 media, was added 1 mL of algal culture, then one of the following volumes of antibiotic solution: 0.0875, 0.175, 0.35, 0.7, 1.4, and 2.1 mL (labelled 1 to 6). This gave a range of penicillin concentrations from approximately 20-500 mg/L and corresponding levels of the other two antibiotics. In a second trial, the antibiotic concentration was extended to include

1000 and 1667 mg/L by the addition of 4.2 and 7.0 mL of antibiotic solution. Each flask was well mixed and placed in the incubator. After 24 and 48 hours, a 1 mL sample was removed and added to 50 mL WC/2 media in a 100 mL Erlenmeyer flask and mixed well to stop the antibiotic treatment. All flasks were then replaced in the incubator.

Tests for bacterial contamination

Three bacterial media in both liquid and agar forms were used to test for the presence of bacteria in algal cultures treated with antibiotics. Agar plates for bacterial testing were used to test the possibility that there was no bacterial growth due to lack of oxygen in liquid media because liquid cultures could not be shaken. The 3 media used were i) 1% (w/v) Nutrient Broth, ii) SST media - glucose (1 g), tryptone (1 g) and yeast extract (0.5 g) in 100 mL Milli-Q water, and iii) Peptone-glucose media - 1 g of both glucose and peptone in 1 L of Milli-Q water. The agar equivalent of these media was made by adding 1.5% of powdered agar. All media were autoclaved at 121°C and 103 kPa for 15 minutes.

Liquid bacterial media (3 mL) were dispensed, in duplicate, to 15 x 2 cm sterile cotton-wool-stoppered glass tubes. Three drops of solution from selected antibiotic-treated cultures were used to inoculate these liquid media. Agar plates were divided into 4 sections. Solutions to be tested were aseptically streaked in duplicate (after re-heating loop between each streak) in a different sector. Autoclaved high purity (Milli-Q) water was used as a control. All plates and tubes were placed in the incubator under the same conditions as the algal cultures and were examined after 1, 2 and 6 days for evidence of algal or bacterial growth.

2.3 Growth of mass cell cultures

Populations of micro-algal species usually exhibit exponential growth due to increases in cell numbers as a result of binary fission. The following equation describes the growth of a population during the exponential phase of growth.

$$N_{t1} = N_o.e^k$$

where N_{t1} is the final cell density at time t, N_o is the initial cell density, and k is the growth rate as ln units per unit time.

Measurement of growth characteristics such as cell density at the end of exponential growth and the initial growth rate (k^1) enables:

i) maximizing the yield of cells obtained by harvesting at the greatest cell density before exponential growth ceases;
ii) determination when to initiate a culture to be harvested at a pre-arranged time; and
iii) avoidance of the need to constantly monitor the growth of the population each time it is initiated.

Mass cultures

Cultures of all five species were set up as described in Appendix 1, with an initial cell density of 10^4 cells/mL.

Measurement of biomass by optical density and cell counts

Approximately 20 mL sub-samples were taken each morning. After thorough mixing, 1 mL was set aside and preserved with 0.1 mL Lugol's Iodine for determination of cell counts using an improved Neubauer haemocytometer (see Appendix 1).

The remainder of the sample was used for measurement of OD at 438, 540, 678 and 750 nm (Sorokin 1979) in a Perkin-Elmer Lambda 2 UV/VIS spectrophotometer using a 1 cm path-length cuvette. To ensure cultures were not too dense and thus causing over-estimation of OD through scattering effects, samples were diluted 33% and 66% using non-sterile WC/2 media and re-measured.

Estimation of cell dry weights

A measured volume of cell concentrate of known cell density was filtered onto a pre-washed (100 mL Milli-Q water), dried (105°C, 24 hour) and pre-weighed (Cahn 29 Automatic Electrobalance) Whatman 2.7 cm GF/C filter. Filter and cells were re-weighed after drying. Four replicates were used for each species.

3 RESULTS

3.1 Isolation of algal species

Separation of algal species

Five algal species were isolated, three of which could be positively identified to species, one named tentatively, and one identified only to genus. They are: *Ankistrodesmus spiralis*, *Ankistrodesmus (falcatus?)*, *Scenedesmus quadricauda*, *Scenedesmus dimorphus and Selenastrum* sp. Due to the difficulty of positively identifying *A. falcatus*, it is designated throughout this paper as *Ankistrodesmus (falcatus?)*.

Fractions with the greatest potential to yield target species were N3, N4, M3, M4, G3 and G4. Between them, the genera *Scenedesmus*, *Ankistrodesmus* and *Selenastrum* were represented, with *Scenedesmus* being found in all 6 cases. N3 and M4 contained the greatest relative number of *Ankistrodesmus*. N3, G3 and G4 contained higher proportions of *Scenedesmus*; while M3 had the highest number of *Selenastrum* cells.

Liquid and agar culture media

It was apparent that varying the media strength of agar did not have dramatic effects on algal growth, as numerous algal colonies appeared in all concentrations.

Observations of algal growth in liquid culture media showed that no growth took place in WC/50 medium, and was minimal in full-strength (WC/1) medium. WC/2 medium, followed by WC/4 medium, produced the most growth. Consequently, WC/2 medium was used as the standard culture medium through-out this work (Appendix 1).

3.2 Antibiotic treatment of cultures

Two initial attempts to produce axenic cultures of *A. spiralis* and *Selenastrum* sp. failed due to the sudden appearance of a fungal contaminant. Because of insufficient time during the course of this project to try different treatments (including fungicides) to determine a successful method, all subsequent work on these algal species was done on non-axenic cultures.

Table 1. Algae identified in various fractions of field samples from 4 sites.

Net Mesh Size (Fraction No.)	Ja Ja Billabong (J)	Nanambu Creek (N)	Mine Valley Billabong (M)	Georgetown Billabong (G)
> 200 μm	-zooplankton -*Spirogyra* -green colonies -detritus -dominated by diatoms -only flagellates are those caught in diatom/detritus -DISCARD	-*Microcystis*, low no. -mobile unicells -*Scenedesmus*, only 8 cells -few small pennate diatoms -DISCARD	-*Volvox* -*Spirogyra* -*Trachelomonas* sp. -zooplankton -*Oocystis* -*Microcystis* -DISCARD	-filamentous alga -zooplankton -detritus -few diatoms -few motile unicells -very low biomass -DISCARD
150-200 μm (1)	-very low biomass -diatoms -large flagellates	-*Scenedesmus dimorphus* -*Selenastrum* sp. -few pennate diatoms -very low biomass	-few diatoms -motile unicells -very low biomass -DISCARD	-1 large diatom -virtually no cells -DISCARD
100-150 μm (2)	-bulk are diatoms -*Trachelomonas* sp. -*Staurastrum* sp. -*Scenedesmus* sp. -*Peridinium?* -*Dictyosphaerium* sp. high no. -*Ankistrodesmus* sp.	-*Microcystis* -few motile unicells -pennate diatoms -*Scenedesmus* sp.	-motile unicells -diatoms -*Trachelomonas* sp. -*Staurastrum* sp. -*Kirchneriella* sp. -low biomass	-very low biomass -*Dictyosphaerium* - *Trachelomonas* -DISCARD
20-100 μm (3)	-*Kirchneriella* sp. -some diatoms -*Dictyosphaerium* sp. -unicells with flagellum (oval shaped) -*Staurastrum* sp. -*Ankistrodesmus* sp. -*Trachelomonas* sp.	-*Microcystis* -*Staurastrum* sp. -many pennate diatoms -*Scenedesmus* sp. high no. -*Selenastrum* sp. -*Dictyosphaerium* sp. -*Ankistrodesmus* sp. high no.	-high biomass -*Dictyosphaerium* sp. -diatoms -*Staurastrum* spp. -*Ankistrodesmus* sp. -*Trachelomonas* -motile unicells -*Scenedesmus* sp. -*Selenastrum* sp.	-*Kirchneriella* -*Dictyosphaerium* -*Oocystis* -motile unicells -diatoms -*Scenedesmus* -*Staurastrum* sp.
< 20 μm (4)	-motile unicells -*Trachelomonas* sp. -very few diatoms -low no. of cells	-very few algae -small motile diatom -*Selenastrum* sp. -pennate diatom -*Scenedesmus* sp. -*Staurastrum* sp.	-*Ankistrodesmus* sp. -motile unicells -diatoms -*Dictyosphaerium* sp. -*Trachelomonas* sp. -*Scenedesmus* sp.	-pennate diatom -*Trachelomonas* -motile unicells -*Scenedesmus* sp. -*Staurastrum* sp.

3.2 Antibiotic treatments of cultures

After 2 days, flasks containing the two lowest antibiotic concentrations for both algal species were infected with a fungus (hyphae could be seen under high power of a compound microscope). After 6 days, all treatments on *Selenastrum* sp. (including the 24 and 48 hour sub-samples) had fungal infection. For *A. spiralis*, all flasks except for the second and third highest antibiotic concentrations had a fungal infection. Eventually however, fungal contamination was evident in all treatments. In the second trial, the opposite happened with fungal growth occurring at the higher concentrations first, then appearing at the lower concentrations.

Finally, no algal growth was observed in all antibiotic treatments up to three weeks after commencement. Examination of some treatments showed algal cells to be either definitely dead or looking very yellow and so were considered dead.

Bacterial tests

Only the following cultures from the first trial were selected for bacterial testing; *A. spiralis* concentrations 4, 5, and 6, and *Selenastrum* sp. concentrations 1 and 6. All tests, including the three media types in both liquid and agar form, were positive after 24 hours. All controls (autoclaved Milli-Q water) were negative up until day 6.

3.3 Growth of mass cell cultures

Table 2 summarises cell counts and OD at various times for four of the five species isolated. *A. spiralis* was not included due to problems encountered with its growth (see Discussion below). In addition, *S. dimorphus* was found to be contaminated with a pennate diatom representing 4% of the total cell numbers on the harvest date.

Table 2. Algal cell counts and light absorbance of cell suspensions for each sampling day.

			Optical Density (nm)			
Species	Day	Cells/mL	750	678	540	438
Ankistrodesmus (falcatus?)	0.00	20,166	-	-	-	-
	2.25	1,117,875	0.063	0.090	0.087	0.115
	3.08	2,917,750	0.080	0.125	0.125	0.185
	3.98	3,388,000	0.114	0.174	0.171	0.243
	5.07	3,680,944	0.162	0.246	0.237	0.335
	6.02	7,167,188	0.186	0.292	0.291	0.430
Scenedesmus dimorphus	0.00	11,611	0.001	0.002	0.001	0.001
	1.00	37,583	0.009	0.012	0.012	0.017
	2.00	120,328	0.025	0.032	0.031	0.043
	3.00	203,194	0.057	0.076	0.068	0.090
	4.00	235,583	0.077	0.096	0.087	0.106
	5.00	285,083	0.091	0.109	0.097	0.121
Scenedesmus quadricauda	0.00	13,750	0.004	0.002	0.002	0.002
	1.00	41,556	0.009	0.009	0.011	0.015
	2.00	165,000	0.027	0.032	0.032	0.042
	3.02	544,500	0.081	0.093	0.088	0.108
	4.00	715,000	0.110	0.130	0.121	0.147
	5.00	1,054,625	0.142	0.169	0.160	0.197
Selenastrum sp.	0.00	15,278	-	-	-	-
	2.25	259,875	0.042	0.054	0.051	0.063
	3.08	984,500	0.070	0.097	0.092	0.123
	3.98	952,875	0.098	0.130	0.124	0.162
	5.07	853,875	0.139	0.175	0.166	0.199

Figure 1 shows increases in cell numbers over time expressed on a natural log basis to allow the length of the exponential phase of growth to be clearly identified. As expected, cell numbers initially increased at an exponential rate, and then began to plateau as nutrients and light intensity became limiting. In all cases, exponential growth commenced immediately after inoculation, with no lag phase evident in any of the plots.

Differences between the various algae can be seen in both the initial rate of growth (k^1, which is the initial slope of the curves, and calculated using equation 1) and also at the cell concentration at which exponential growth rate ceases. Table 3 summarises these values.

S. dimorphus ceased exponential growth at the lowest cell density (approximately 120,000/mL).

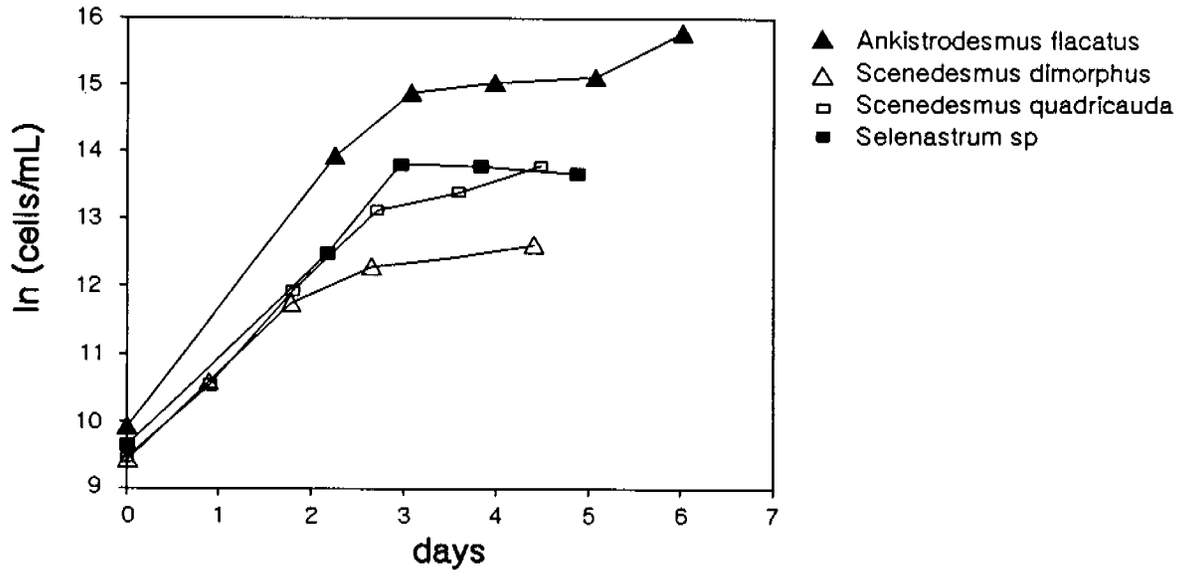

Fig 1. Growth of 4 algal taxa in half-strength WC medium after an initial cell density of 10^4 cells/mL; *Ankistrodesmus (falcatus?)* ▲ , *Scenedesmus dimorphus* △ *Scenedesmus quadricauda* □ , *Selenastrum* sp. ■

It required 3 days under the stated culture conditions for population growth to begin levelling off for each algal species, except for *S. dimorphus* which required 2 days (Table 3).

A. (falcatus?) had the highest growth rate. The rates of growth of the remaining 3 species were similar, with that of *S. dimorphus* being the lowest.

Cell weights (Table 3) varied up to 194 pg/cell for *S. dimorphus*. Values of dry weights inversely follow the same trend as k^1. The values of cell weights and k^1 agree with values obtained from other workers using the same or similar sized algae (Reynolds 1984).

Table 3. Growth parameters and cell weights of isolated species

Species	Density cells/mL End Expt.	k^1 Units/day	SE	n	Time (days) at End of Exponential Growth	Dry Cell Weight pg/cell	SE
A. (falcatus?)	2,500,000	1.649	0.135	3	3	6.4	0.02
S. dimorphus	120,000	1.169	0.003	3	2	194.0	0.67
S. quadricauda	550,000	1.234	0.035	4	3	44.3	0.44
Selenastrum sp.	980,000	1.332	0.072	3	3	41.7	0.32

Figures 2-5 shows the relationship obtained between cell counts and OD at each of the 4 wavelengths - see Table 2 for complete data. At any one cell density for any species, the highest OD measurements were read at 438 nm, followed by OD readings at 678, 540 and then 750 nm. The only exception was for readings at 678 and 540 nm for *A. (falcatus?)* which were very similar. However, all wavelengths used reflected similar trends in algal growth.

A very poor relationship exists between cells/mL and OD for *A. (falcatus?)* and *Selenastrum* sp. (Figs 2 and 5 respectively). In the case of *Selenastrum* sp. the relationship in the initial stages of growth between the two measures cannot be ascertained due to insufficient readings.

A much better relationship between OD and cells/mL can be seen for *S. dimorphus* and *S. quadricauda* (Figs 3 , 4). Linear regressions of the plots in Figs 6 and 7 are summarised in Table 4 (for 438 nm only). Slopes vary greatly between the two species, being greater for the larger *S. dimorphus*. A reasonable fit was obtained for both species with R-squared values being 0.992 or better.

Table 4. Regression of algal density (cells/mL) vs light absorbance OD_{438}

Species	Slope ($\times 10^{-7}$)	SE ($\times 10^{-7}$)	Constant ($\times 10^{-3}$)	SE ($\times 10^{-3}$)	R-sqrd	n
S. dimorphus	4.470	0.203	-3.558	5.014	0.992	6
S. quadricauda	1.860	0.068	6.600	6.363	0.995	6

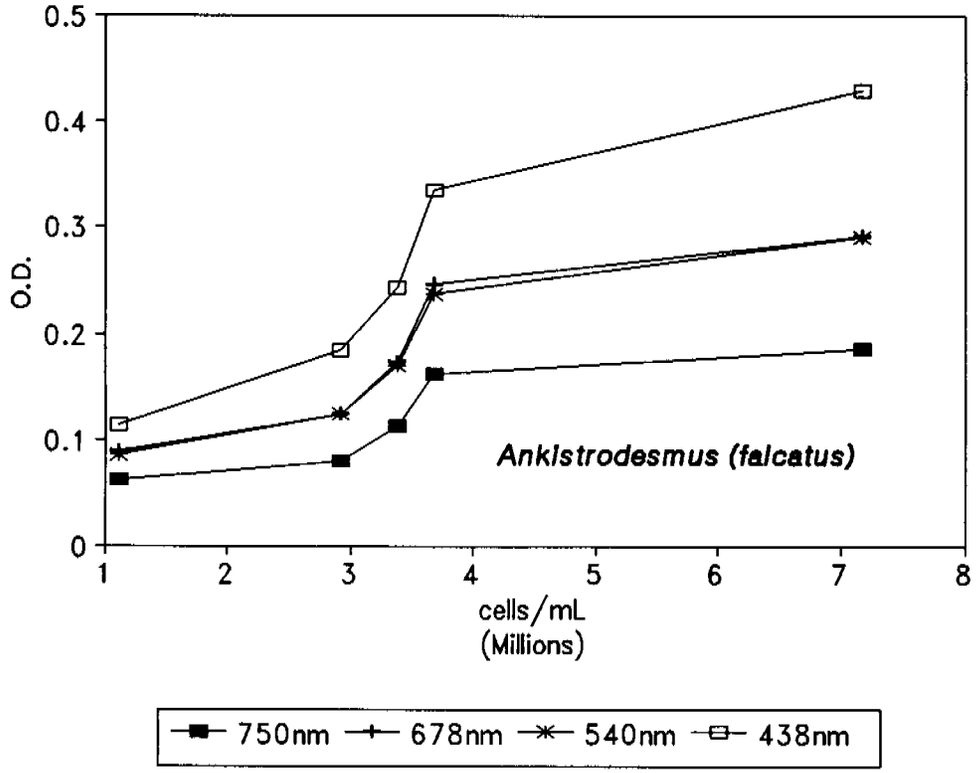

Fig 2. Relationship between cell density and optical density (OD) at various wavelengths for *Ankistrodesmus (falcatus?)* (see Table 2)

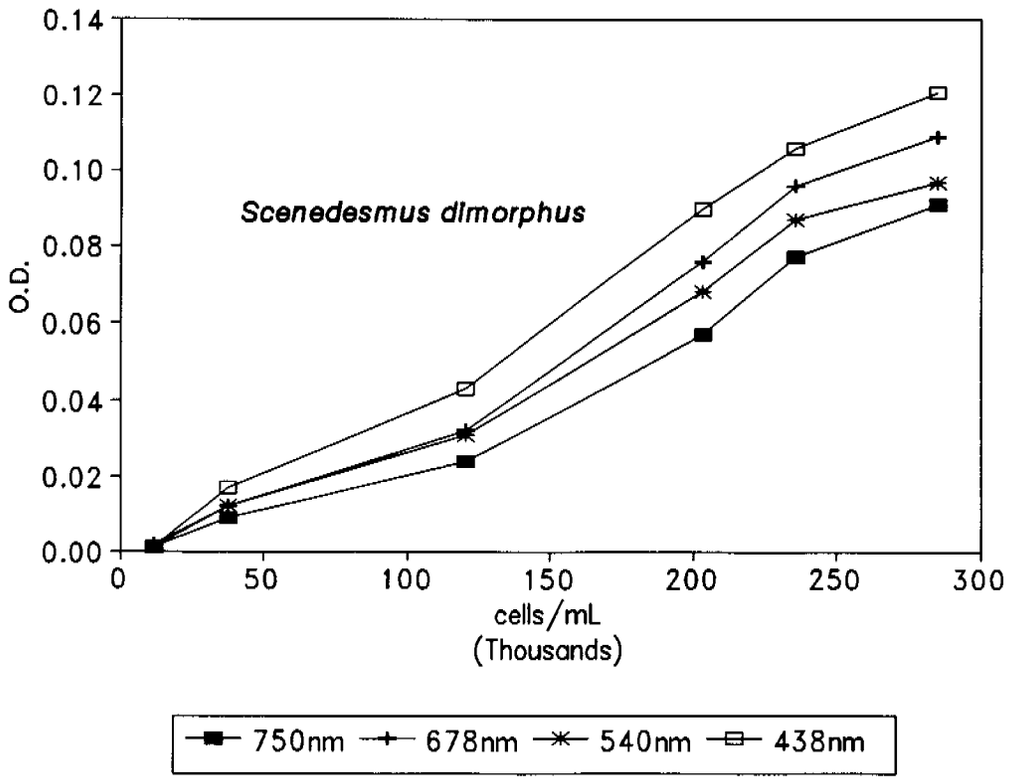

Fig 3. Relationship between cell density and optical density (OD) at various wavelengths for *Scenedesmus dimorphus* (see Table 2)

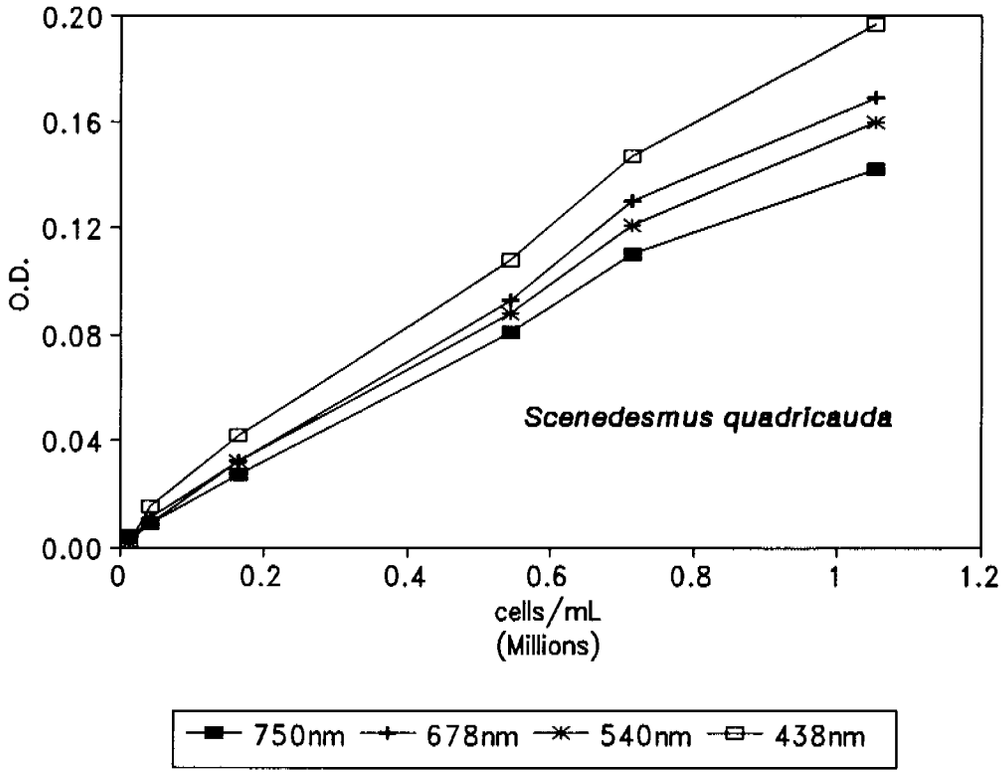

Fig 4. Relationship between cell density and optical density (OD) at various wavelengths for *Scenedesmus quadricauda* (see Table 2)

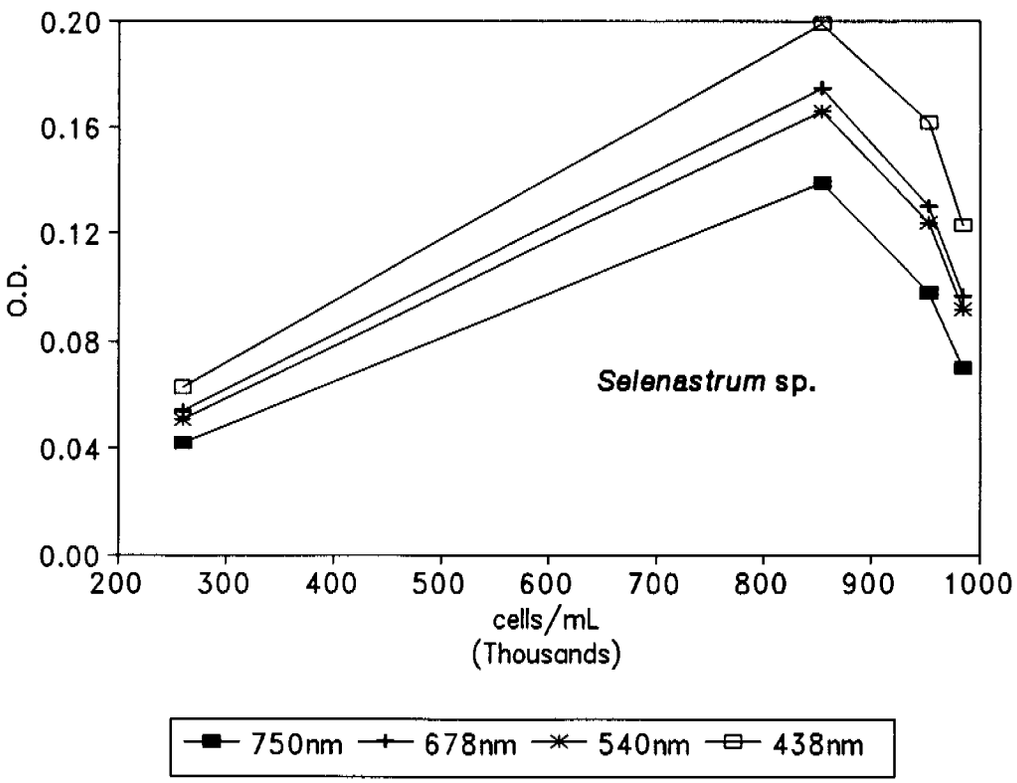

Fig 5. Relationship between cell density and optical density (OD) at various wavelengths for *Selenastrum* sp. (see Table 2)

4 DISCUSSION

4.1 Isolation of algal species

Separation of algae

The coarse separation of algae carried out prior to streaking ensured that i) species that were several times larger than the target species (including a large dominant diatom from Ja Ja Billabong) were eliminated, ii) target species were concentrated into a particular size fraction, and iii) attention could be paid, through the microscopic examination of each fraction and the location of target species, to only those agar plates streaked with desired fractions. All samples in the > 200 μm fraction, and fractions M1, G1 and G2 were discarded since either the target species was absent or biomass was too low for streaking onto agar.

Liquid and agar culture media

Screening with different strength liquid culture media was undertaken because there was a large difference in electric conductivity between natural waters (20-30 $\mu S.cm^{-1}$) and full-strength medium (184 $\mu S.cm^{-1}$), suggesting that algal cells transferred directly from the field into full-strength culture medium may not grow, or die due to osmotic shock.

All species were isolated from Nanambu Creek samples, despite being identified in fractions from Mine Valley, Georgetown and Ja Ja Billabongs (Table 1). The early and dominant occurrence of *Dictyosphaerium* colonies on all Ja Ja and Mine Valley Billabongs Plates resulted in these plates being discarded due to the reduced likelihood of locating target species. Consequently, attention was focused on agar plates streaked with samples from Georgetown Billabong, and Nanambu Creek, especially plates N3 and G3, in which few *Dictyosphaerium* were detected (Table 1).

Of the target algal genera that this project aimed to isolate, possibly all had been successfully removed from the field and cultured in the laboratory. This includes two species of *Ankistrodesmus*, two species of *Scenedesmus* and one species of *Selenastrum*. Two other algal taxa, resembling *Chlamydomonas*, were isolated and cultured; however, final identification was not possible and so the results were not included. Although all the algal isolates were mono-specific, they were not axenic.

4.2 Antibiotic Treatment of Cultures

An initial attempt was made at treating isolated algal cultures with antibiotics with the aim of killing all foreign organisms and rendering the cultures axenic. The advantages of axenic cultures are i) data obtained from experiments using axenic cultures truly reflect the characteristics of the alga, and are not marred by artifacts due to the presence (or products) of bacteria or fungi, ii) stock cultures on agar slopes and in liquid media can be kept with minimal maintenance for very long periods. This latter point is not only true because of a saving in time and other resources, but also because of the decreased risk in the loss of the culture. Loss of the culture could occur due to large scale contamination by bacteria or fungi leading to competition with algae for resources, and/or death of algae because of the production of algicides by the bacteria or fungi.

The similar response amongst all bacterial tests indicates that a single test can be selected for future use. Nutrient broth in the liquid form would be the best choice as its preparation requires the least amount of time. The conflicting responses in fungal infection between the two trials for both algal species makes any interpretation difficult. However, there are three factors which were apparent: 1) treatment with these antibiotics leads to a massive increase in fungal contamination, 2) bacteria were not completely eliminated by the antibiotics, and 3) algal cells were not surviving the treatments even at the lower concentrations.

The contrasting results may be explained by bacteria producing a compound(s) which suppresses fungal growth and/or out-competing fungi for available nutrients. The addition of antibiotics reduces the number of live bacteria allowing fungi to dominate the cultures. Throughout this project, fungal contamination has only occurred in these treatments and never in other algal cultures. Consequently the presence of fungi requires algal cultures to undergo additional treatments to make them axenic. Because of the time limitations on this project, no other treatments were attempted, and non-axenic cultures were used in all subsequent work. Although axenic cultures were not established, the non-axenic cultures could still be considered successful as the five species were cultured in large quantities. Axenic cultures have considerable benefits, but the most important aspect of the work was to be able to provide enough algal culture to use as a food supplement or substitute in tests with cladocerans; the results indicate that one of these algal species from a non-axenic culture is a suitable supplement.

Finally, the death of algal cells suggests that either lower concentrations of antibiotics or a completely different class of compounds, should be used. However the former suggestion cannot be applied since increasing the dosage did not kill all bacteria. One possibility is to 'clean up' algal cultures of bacteria as much as possible before commencing the antibiotic treatment. Some suggestions are given in the following section.

4.3 Alternative treatment methods

Apart from the maintenance of sterile techniques, a number of other measures can be taken to reduce the incidence of bacterial or fungal contamination.

Eliminating or minimising selected nutrients in culture solutions reduces non-algal growth. Nutrients may be medium components or substances released by culture cells; the quantity of organic compounds released is a function of algal density and 'health' of the cells. This may require algal cultures to be continuously renewed by transferring small aliquots of exponentially growing cultures into fresh media; cell density should be kept at a minimum. Aeration can be used to increase algal growth by keeping cells in suspension and in constant contact with nutrients and in the light field.

Reducing or minimising the foreign cells in cultures may not necessarily lead to axenic cultures, which can be achieved through either chemical or physical means, or a combination of both.

Physical methods involve separating fungi, bacteria and their spores from algal cells through either repeated washings or continuous streaking across agar plates. Centrifuged algae that are re-suspended may be briefly treated in an ultrasonic bath prior to further centrifuging to dislodge any bacterial cells or spores attached to their cell walls (Hoshaw & Rosowski 1979).

Chemical methods involve the use of bactericides and fungicides to kill foreign organisms, or static compounds which inhibit their growth but which are not lethal. These compounds can be either organic molecules or inorganic ions. For example, potassium tellurite is noted as being a bacteriostatic agent (Hoshaw & Rosowski 1979), and copper is used as a fungicide, as well as a common algicide (Garraway & Evans 1984).

4.4 Growth of mass cell cultures

The mass culture and harvest of micro-algae requires knowledge of the growth characteristics of the organism to optimise the quality and the quantity of cells obtained. Cells are usually harvested during the exponential growth phase when cell growth and division are at a maximum, not during senescence when cell activity is greatly diminished due to lack of nutrients or light. The last point is of particular importance when cultures are grown on a regular basis. However, these parameters are only valid for a particular set of growth conditions (temperature, light intensity, aeration and nutrients).

Nevertheless, this does not completely remove the need to occasionally check on the population's progress during the course of growth. When large numbers of cultures are running simultaneously, estimating cell density by measuring the optical density (OD) of the culture is a much quicker method than cell counts. However its accuracy depends on various factors, including the presence of bacteria during algal growth which will boost OD, and the form of the organism such as aggregation of cells and cell size. The OD measured for each species during growth therefore needs to be calibrated against cell density.

Finally, the size of isolated algae varies greatly between species, from the small *Chlamydomonas*-like species to the relatively larger types such as *Selenastrum* and *Scenedesmus*. Therefore, measurements such as cell density do not accurately reflect the true quantity of food grazing animals are receiving. This is of particular importance when comparing the effect of different algal types on animal growth and reproduction. A better alternative measure of algal biomass can be expressed as cell dry weight. This allows the absolute combined mass of algal protein, sugars, lipids, etc. fed to animals to be calculated, and is therefore independent of cell size.

Four of the five algal species were successfully mass cultured with all four species showing an initial exponential growth rate with no lag phase.

A. spiralis was not mass cultured due to various problems that were encountered. *A. spiralis* was found to have a very slow observed growth rate in both non-aerated and aerated cultures. Increasing the light intensity from about 30 to 90 $\mu E/m^2.s^{-1}$ did not increase algal growth. The large cells of this species formed aggregates visible to the naked eye. These factors reduced the likelihood of it being a suitable alga to grow on a routine basis. More importantly, it might not be an acceptable food source for *M. macleayi* because of the formation of aggregates.

Although in the wild and in non-aerated media *Selenastrum* sp. and *S. quadricauda* also tend to form large aggregates or colonies, the aeration of cultures had the effect of dispersing cells of *Selenastrum* sp. and reducing the size of *S. quadricauda* colonies from 4 cells down to 2 cells. Aeration of cultures of *S. dimorphus* also produced a greater proportion of colonies composed of 4 cells instead of 8 (approximately 25% of total colonies).

Success with using optical density as a measure of algal biomass was restricted to the species listed in Table 4. The slopes and constants derived from the data can be used to estimate cell density in future cultures but these values would not be as precise as those derived from direct cell counts. However, optical density in the case of *S. dimorphus* and *S. quadricauda* is still useful. Optical density could be used for rapidly assessing the stage of growth of these cultures by comparing the calculated cell density to Figs. 2 and 3.

The absence of any lag phase in Figure 1 was due to cells immediately beginning rapid growth after they were transferred into 2 L flasks. This is probably a result of using an inoculum that was exponentially growing at the time of use. The subsequent calculation of growth rates showed that the highest rates of growth (k^1) were observed for the smaller species, as measured as cell dry weights (Table 3).

The smallest species *A. (falcatus?)*) requires less time after cell division to grow to a size where cell division can occur again. This may be due to smaller cells having a shorter distance to transport nutrients from uptake sites to where they are finally assembled, or the relatively larger surface area to volume ratio of smaller cells, leading to potentially higher rates of nutrient uptake (Reynolds 1984). The exponential relationship between the surface area and volume (which is also a feature of cell size and shape) and growth rate is also reflected by a similar exponential relationship in Table 3 between cell weights (as a measure of cell size) and growth rates.

Consequently, this shorter generation time is reflected in a higher rate of growth. Larger cells, as those belonging to *S. quadricauda* and *Selenastrum* sp. (approximately 10 times larger), and especially *S. dimorphus* (approximately 40 times larger) need to attain a larger size before the next division can re-occur. This leads to a longer generation time and therefore a slower observed rate of growth.

The great variations in cell weight are an important factor to consider when designing feeding experiments, as quantity of food supplied to animals measured simply as cell density is not a good measure. If the cell weights shown in Table 3 are adjusted so that *S. dimorphus* is 1.0 unit of mass for a given cell density, then the corresponding number of cells of the other species required to give the same mass of cells needs to be increased by a factor of 30.1, 4.4 and 4.7 for *A. (falcatus?), S. quadricauda* and *Selenastrum* sp.

Finally, one of the aims of these trials was to determine when cultures should be harvested to maximise both quality and quantity of cells. Table 3 summarises the cell densities at the end of exponential growth and the time taken to reach that stage. This information is important for the efficient routine production of these organisms. If the conditions of growth described, and in the protocol in the Appendix 1, are adhered to, cultures can be harvested with confidence after a certain period of growth. This removes the need to constantly monitor growth which is very time consuming.

ACKNOWLEDGEMENTS

I would like to thank Drs Ross Hyne and Greg Rippon for their comments and help in the preparation of this manuscript. Dr J R Merrick is acknowledged for editorial assistance.

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APPENDIX 1

Technique Details for Algal Culture and Harvesting

Medium solution and preparation (Guillard & Lorenzen 1972)

		Stock
Major salts (separately add 1 mL each per L)		g/500 mL
$CaCl_2.2H_2O$		18.35
$MgSO_4.7H_2O$		18.45
$NaHCO_3$		6.3
K_2HPO_4		4.35
$NaNO_3$	(add 0.5 mL/L)	42.5
NH_4Cl	(add 0.5 mL/L)	26.75
Iron solution (combine; add 0.1 mL/L)		g/100 mL
Citric acid ($C_6H_8O_7.H_2O$)		3.35
Ferric citrate ($FeC_6H_5O_3.5H_2O$)		3.35
Trace metals (combine; add 1 mL/L)		g/500 mL
Na_2EDTA		2.18
$CuSO_4.5H_2O$		0.005
$ZnSO_4.7H_2O$		0.011
$CoCl_2.6H_2O$		0.005
$MnCl_2.4H_2O$		0.09
$Na_2MoO_4.2H_2O$		0.003
H_3BO_3		0.5
Vitamins (combine; add 0.25 mL/L)		mg/250 mL
Thiamin HCl		100
Biotin		0.5
Vitamin B_{12}		0.5

Procedure

1) Prepare above stock solutions by adding the salts to high purity (Milli-Q) water and autoclave (except for K_2HPO_4, which can be sterilised by filtration) to retard any fungal or bacterial growth.

2) Add all stock solutions to the volume of Milli-Q water required, except for $CaCl_2$ and $NaHCO_3$ stock solutions.

3) Adjust pH to between 7.1 and 7.3 using 1M NaOH and 10% HCl.

4) Autoclave volumes less than 1.5 L for 30 minutes or for 40 minutes for greater volumes, at 121°C and 103 kPa.

5) On cooling, aseptically add $CaCl_2$ and $NaHCO_3$ stock solutions.

6) Allow medium to sit for at least 24 hours (or better 48 hours) to allow expelled gases from liquid to re-equilibrate with the air.

NB: To make up WC/2 medium, only add half the volumes of stocks indicated above.

Estimation of algal cell density

To measure the growth of algal cultures, a small sub-sample (1-5 mL) is aseptically removed for microscopic counting, and the time and date noted. Since the immediate quantification of cell density is usually not possible, a preservative such as Lugol's Iodine (see endnote) is added to the sample. This instantly stops algal growth by killing the cells, and prevents bacterial and fungal infection from destroying the sample. A secondary advantage of Lugol's solution is that iodine is absorbed by the cell walls, thereby increasing the cells density and reducing the time needed for them to settle out in the haemocytometer (see below).

Surface and side diagrams of a haemocytometer are shown in Fig. 6a. Each slide is composed of two chambers, each containing a ruled grid (Fig. 6b). Note that each grid is made up of nine squares of equal size (marked A). Each square is 1 mm by 1 mm, and the distance between the base of the grid and the bottom of the cover-slip is 0.1 mm. Consequently each grid has a volume of 0.1 mm^3.

1) Moisten raised polished surfaces (marked C, Fig. 6a) with finger, and centre cover-slip on top. Press down firmly with thumbs to secure cover-slip to slide (cover-slip should remain attached even if slide is vigorously shaken !).

2) Place slide on level surface, and transfer a drop of well mixed culture with a Pasteur pipette to the edge of the cover-slip. The sample is drawn into the chamber by capillary action. Remove pipette when chamber is full to avoid over-filling (there should be no liquid in the depressions surrounding the three sides of the chamber or protruding from where the chamber was loaded). Re-mix the sample and load other chamber.

3) Allow sufficient time for the cells to settle to the bottom of the chamber. Focus up and down to ensure there are no cells still in suspension. Larger cells such as *Scenedesmus* and *Selenastrum* settle out rapidly (< 1 minute) whereas smaller species such as *A. falcatus?*, and especially Unicells, may take as long as 3-4 minutes. Both chambers should be counted as soon as possible as the samples will dry up and eventually start contracting. This should be avoided, and the chamber rinsed and re-loaded in the event of it happening.

4) The choice of grid to use while counting is a function of cell density and the operator. At very low cell densities, the entire chamber can be counted. At higher densities, a smaller part of the chamber is counted e.g. the squares making up the outer 4 corners of the grid (see A, Fig. 6b). At very high densities, count the centre square or its divisions. If the density is too high, the sample should be diluted prior to loading the cell.

The general rules to bear in mind in selecting which squares to count are; i) if possible, at least 100 cells should be counted per chamber, and ii) count cells from as wide an area as possible. For example, if a corner square (A in Fig. 6b, composed of 16 smaller squares) is sufficient to give a count of 100 cells, it would be better to count 4 of the smaller squares in each of the 4 corners (see B Fig. 6b) which will still result in 16 smaller squares being counted).

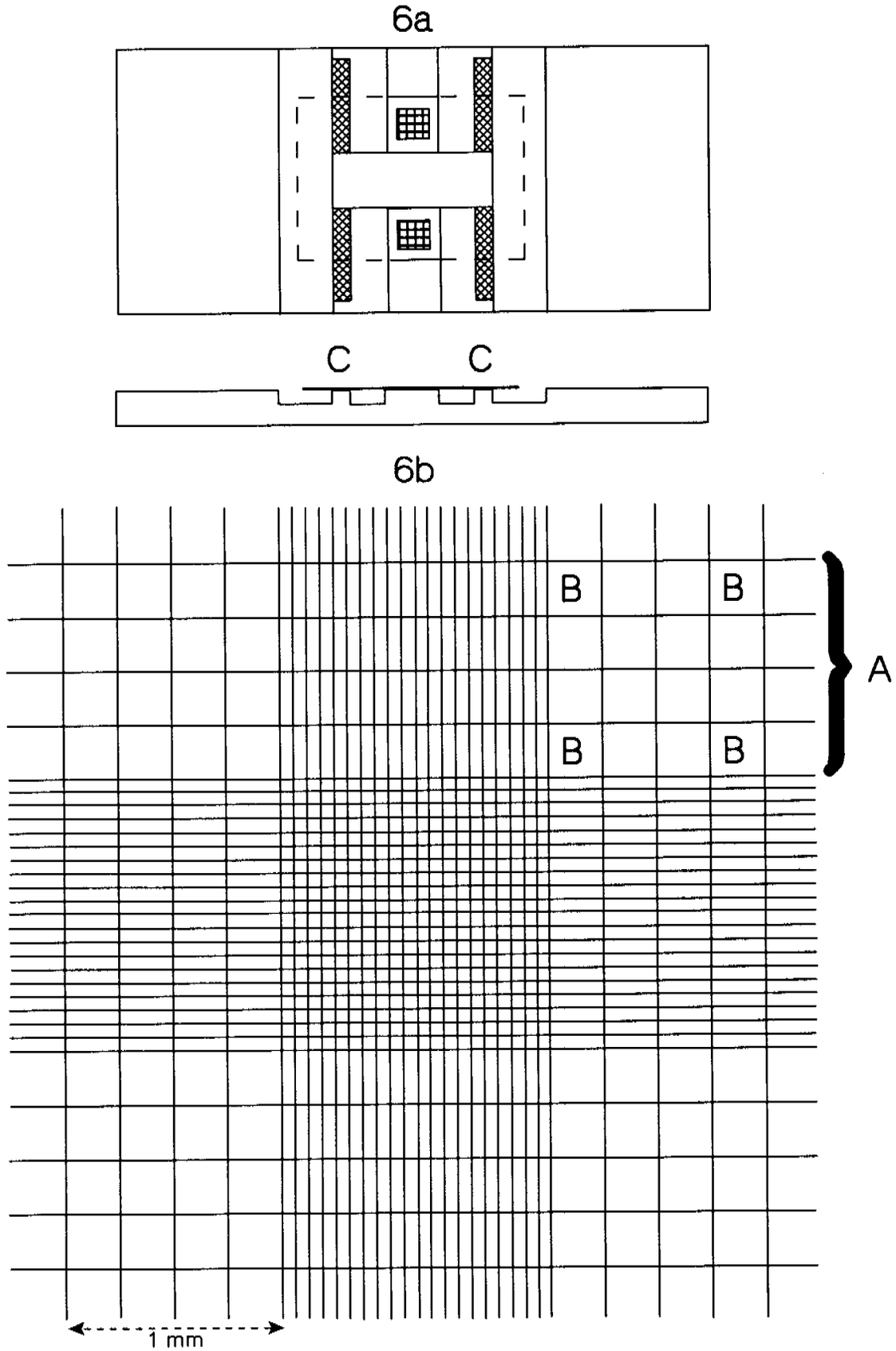

Fig 6. Surface and side illustration of (a) Hausser Haemocytometer; (b) the central ruled square mm areas with Neubauer pattern (approximately × 25)

Complications arise when cells lie on a line that borders the square being counted. Such cases need to be treated in a consistent manner by deciding which of the two vertical edges and which of the two horizontal edges are active i.e. will have any cells touching them included in the count. The other two non-active sides will not have any touching cells counted. The choice of active sides is an arbitrary one, but one which should be rigorously adhered to during any count. This rule applies whether the whole chamber or the smallest possible division is being counted.

5) Calculate cells/mL. As mentioned earlier, each grid can be divided into 9 smaller grids of equal size, where each one has an area of 1 mm^2 and a layer of solution above it 0.1 mm deep, giving a volume of 0.1 mm^3. Therefore the number of cells in any one of the 9 smaller grids, multiplied by 10^4 will give the number of cells per mL in the original solution (1 mL divided by 0.1 mm^3 equals 10^4). All other calculations are variations along the same theme. For example, if a total of 100 cells are counted in the 4 squares marked B in Fig. 6b, then the number of cells per mL is $100 \times 16/4 \times 10^4 = 4 \times 10^6$ cells/mL. If the entire chamber is counted (all 9 squares), then the calculation would be the number of counts divided by 9, and then multiplied by 10^4.

If appropriate, this value is then corrected for any dilution the culture may have underwent prior to loading the chambers, including dilution from the addition of Lugol's Iodine or any other cell preservative.

For a more detailed account the reader is referred to Guillard (1978), pp. 182-189.

Lugol's Iodine Solution: To 1 L water add 150 g KI, 50 g I_2 and 20 mL glacial acetic acid. Use 1-2 mL per 100 mL sample (Bellinger 1980).

Protocol for mass culture

All steps outlined below should be carried out aseptically in the Laminar Flow Cabinet using sterile media and glassware. Culture flasks are placed in the middle of the growth cabinets (40-50 $\mu E/m^2.s^{-1}$), set on a 12:12 light/dark cycle at 27°C and are continuously aerated.

1) Approximately 7 to 10 days before being required, transfer ≈ 2 mL of the required species from an older culture into a 250 mL Erlenmeyer flask to which 100 mL of WC/2 medium has been added. Cells should be exponentially growing by the time they are used.

2) Make up 2 L WC/2 medium in a 2 L Erlenmeyer flask, and adjust pH to between 7.1 to 7.3. Stopper with non-absorbant cotton wool wrapped around a 5 or 10 mL glass pipette which acts as the aerating tube. Pack some cotton wool into the top of the pipette to serve as an air filter. Autoclave for 40 minutes and complete medium on cooling. Allow medium to sit for at least 1 day before use.

3) On the day needed, aseptically sample inoculating culture for a cell count. Count both chambers on the haemocytometer, wash, re-load, then count both again (a total of 4 counts). Count at least 100 cells per grid. Average the cell counts and calculate the number of cells per mL.

4) Calculate the volume of inoculating culture needed in 2 L to get a cell density of 10^4 cells/mL. Add aseptically using either a sterile pipette or measuring cylinder. Place flask in the growth cabinet and attach air tube.

5) Table 4 derived from the growth characteristics determined for each species (Figure 1) indicates the time required for cultures to reach the end of exponential growth, and therefore time for harvesting.

Protocol for harvesting

1. Twenty four × 80 mL plastic centrifuge tubes are required to be placed in a refrigerated centrifuge fitted with a swing-out head. The culture flask is removed from the incubator and taken to the centrifuge room. Aseptic techniques are not possible or practical, but cleanliness of the work bench should be kept in mind. Each tube is filled to the 60 mL line, is pair-wise balanced and matched to the nearest 0.1 g, and placed on opposing sides in the swing-out rotor.

2. The controls are set to the maximum rotor speed (2800 rpm, 2000 × g), and the temperature set at approximately 15°C. A spinning time of 20 minutes is adequate. While one set of 12 tubes is spinning, another set of 12 can be prepared for the next run.

3. After each spin, the supernatant is removed through suction, using a glass Pasteur pipette directly attached to a venturi suction via flexible plastic tubing. A sufficient quantity of water (≈ 5 mL) is left behind to allow re-suspension of the pellet.

4. The re-suspended pellets are transferred and combined into the minimum number of tubes possible. Any remaining algal culture is transferred to the used tubes, balanced and re-spun. The empty tubes are rinsed out by adding 20-30 mL of 0.2 μm filtered control water (e.g. Magela Creek or Buffalo Billabong water), capped and vigorously shaken. To keep the volume of rinsing water used to a minimum, the same aliquot is transferred from one tube to another, then eventually to a final tube to be re-spun. This is repeated twice, or until the final aliquot appears clear.

5. The above procedure is repeated until all algal cells are concentrated into one tube. These cells are then washed twice with control water to remove any trace metals (from the culture media) by re-spinning, removing the supernatant, adding approximately 50 mL billabong water and shaking vigorously to re-suspend the pellet.

6. The pellet is finally re-suspended in approximately 50 mL of filtered billabong water. The exact volume of concentrate can be determined by weighing a dry marked tube before transferring pellets into it, and then re-weighing after the complete pellet is re-suspended.

7. Finally, the algal density of the concentrate needs to be measured to calculate what volume needs to be added to *Moinodaphnia* treatment vials to attain the pre-determined known cell density of 2×10^5 algae/mL. Transfer 1 mL of concentrate to a 100 mL volumetric flask and make up to the mark using filtered billabong water. Seal with Parafilm and mix well by inverting 5 times. Quickly break the seal and load the haemocytometer for a cell count. If algal density is too high, try a greater dilution (e.g. add 0.5 mL concentrate to a 250 mL measuring cylinder). Count at least 100 cells in each of 4 chambers (re-seal and invert 5 times before loading the haemocytometer for the second time), average the values and back-calculate to get the algal density of the concentrate.

8. The algal concentrate should be divided into 1 mL aliquots and stored in the dark at 4°C.

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