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GROWTH OF BLUE-GREEN ALGAE IN THE MANUKAU (NEW ZEALAND) OXIDATION PONDS—II. EXPERIMENTAL STUDIES ON ALGAL INTERACTION

W. F. VINCENT* and W. B. SILVESTER†
Botany Department, University of Auckland, Auckland, New Zealand

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Abstract—An experimental approach was made to the interaction between the blue-green algae *Anabaena* and *Microcystis* and the normal oxidation pond dominant *Chlorella*. Methods employed were dialysis tubing, mixed culture with assay by *in vivo* absorbance, and by interaction tubes in which the algae were separated by a filter membrane. No dialysable inhibitor material in the oxidation ponds was detected with *in situ* experiments. In mixed culture *Chlorella* inhibits *Anabaena* only when starting concentration of *Chlorella* is high and that of *Anabaena* is low. On the other hand at all concentrations *Anabaena* quickly dominated *Chlorella*. These results were confirmed in interaction tubes where the algae were separated and the results extended to show *Microcystis* also inhibits *Chlorella* growth. Filtrate experiments indicate that both *Microcystis* and *Anabaena* produce a filterable substance capable of suppressing *Chlorella* growth but that *Chlorella* does not exert any influence on blue-green algae by way of soluble materials.

INTRODUCTION

In the previous study (Vincent & Silvester, 1979) it was shown that growth of blue-green algae in a well established sewage oxidation pond was strongly inhibited by high resident populations of green algae. Growth of blue-green algae was only possible during periods when green algal density was low and pond conditions of temperature and pH were sub-optimal for green algal growth. The simple interpretation of changing physical conditions of the ponds as the major factor in controlling algal dominance is strongly suggestive, but leaves open the whole problem of the complex interaction phenomena that exist in such a system.

A variety of approaches have been made to the study of algal interactions, both in the field and laboratory. In mixed culture experiments interactions between the components are difficult to analyse and different approaches have been made to resolving the interaction mechanisms. Vance (1965) separated the interacting species by a sintered glass filter welded into a glass tube. This approach was extended by Kroes (1971, 1972, 1973) who used a continuous flow system with in-line filters. These and other workers have emphasised the importance of extracellular products in algal interaction and competition.

It is well established that healthy algal cells liberate a significant proportion of total carbon fixed into their surrounding environment (Fogg, 1971). The diverse range of organic compounds released include carbohydrates, amino acids, organic-phosphates and vitamins, many of which produce effects of possible ecological importance such as growth inhibition, metabolic stimulation or induction of sexual development. For example, several strains of *Chlorella*, the genus dominating the Manukau ponds throughout most of the year, are known to produce inhibitory compounds termed 'chlorellins' (Scult, 1964). On the other hand Jacob (1961) and Vance (1965) have shown that blue-green algae are capable of suppressing a wide range of eucaryote algae.

Interactions between blue-green algae and *Chlorella* were investigated in the present study using mixed cultures, interaction tubes, and by filtrates to determine the possible competitive advantages of each group.

MATERIALS AND METHODS

Blue-green algal cultures *Anabaena* sp. (local isolate) and *Microcystis aeruginosa* (Cambridge culture collection) were grown and maintained on ASM inorganic nutrient medium (Gorham *et al.*, 1964). To assess the possible effect of low molecular weight chemicals on growth, polythene or dialysis bags containing algae and nutrient were suspended in the oxidation ponds and assayed after 4 days by cell count.

Algal interaction between *Anabaena* and *Chlorella* was investigated in mixed culture by growing each alga singly or in combination at various starting concentrations 5×10^3, 5×10^4 and 5×10^5 cells per ml. These were shaken under continuous fluorescent light (2500 lux) and constant temperature 28°C for 9 days and assayed by '*in vivo* absorbance' and by final cell count.

In vivo *absorbance*

The relative abundance of *Chlorella* and *Anabaena* in mixed culture was measured by making use of their different pigment composition. An opalescent glass screen

* Present address: Ecology Division, DSIR, P.O. Box 415, Taupo, New Zealand.
† Department of Biological Sciences, University of Waikato, Hamilton, New Zealand.

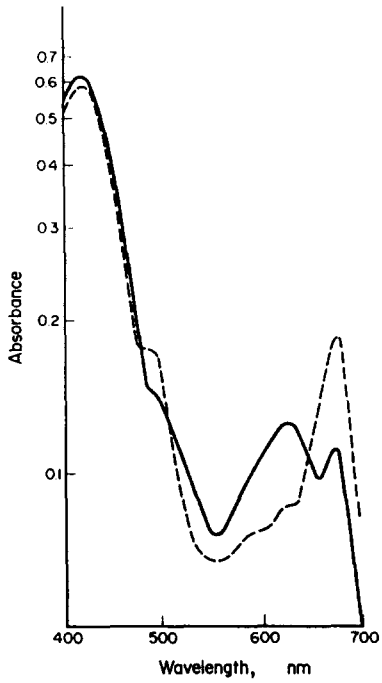

Fig. 1. Absorption spectra of suspended whole cells of *Anabaena* (continuous line) and *Chorella* (broken line). Spectra were run in a Beckman DB spectrophotometer using a pair of etched glass screens between cell suspension and phototube.

between cell suspension and phototube served to reduce the light scattering effect of the cell suspension (Shibata, 1958) and made it possible to separate the two absorption spectra as shown in Fig. 1. Based on the major differences in spectra the two components in mixed culture could be separated by an equation derived as follows:

Absorbance values at 675 nm and 615 nm can be summarized as follows:

Culture	Absorbance	
	615 nm	675 nm
Chlorella	A'_{615}	A'_{675}
Anabaena	A''_{615}	A''_{675}
Combined culture (*Chlorella* and *Anabaena*)	A_{615}	A_{675}

$$A_{615} = A'_{615} + A''_{615} \quad (1)$$
$$A_{675} = A'_{675} + A''_{675}. \quad (2)$$

From equations (1) and (2)

$$\frac{A_{615}}{A_{675}} = \frac{A'_{615}}{A'_{675} + A''_{675}} + \frac{A''_{615}}{A'_{675} + A''_{675}}.$$

Dividing through by $\frac{A'_{675}}{A'_{675}}$ or $\frac{A''_{675}}{A''_{675}}$,

$$\frac{A_{615}}{A_{675}} = \frac{\frac{A'_{615}}{A'_{675}}}{\frac{A'_{675}}{A'_{675}} + \frac{A''_{675}}{A'_{675}}} + \frac{\frac{A''_{615}}{A''_{675}}}{\frac{A'_{675}}{A''_{675}} + \frac{A''_{675}}{A''_{675}}}. \quad (3)$$

Defining $\frac{A_{615}}{A_{675}} = z$ the ratio of absorbance at 615 nm to 675 nm, for mixed culture,

$\frac{A'_{615}}{A'_{675}} = x$ the ratio of absorbance at 615 nm to 675 nm for *Chlorella*,

$\frac{A''_{615}}{A''_{675}} = y$ the ratio of absorbance at 615 nm to 675 nm for *Anabaena*,

and $\frac{A''_{675}}{A'_{675}} = r$ the ratio of absorbance at 675 nm for the blue-green alga to the absorbance at 675 nm for the green alga in mixed culture.

Equation (3) can now be reduced to:

$$z = \frac{x}{1 + r} + \frac{y}{1/r + 1};$$

multiply through by r/r;

$$z = \frac{x}{1 + r} + \frac{yr}{1 + r},$$

$$\therefore \quad z + zr = x + yr,$$

$$\therefore \quad r = \frac{x - z}{z - y}. \quad (4)$$

Therefore in a mixed culture the absorbance at 675 nm due to the blue-green alga is given by:

$$A''_{675} = \frac{r}{1 + r} \cdot A_{675} \quad (5)$$

and the absorbance due to the green algal component by:

$$A'_{675} = \frac{1}{r} \cdot A''_{675}. \quad (6)$$

The value z may be obtained from *in vivo* absorbance data for the mixed culture, and x and y can be obtained from test unialgal cultures grown under the same conditions of light, temperature and nutrients. From these three values, equations (4), (5) and (6) were used to calculate the absorbance values for the green algal and blue-green algal components of the mixed culture. The values obtained are only useful when pigment ratios remain constant during an experiment. This point is treated in detail in the discussion.

To further investigate *Anabaena/Chlorella* interactions the two algae were grown in 'interaction tubes' in which the algal species were physically separated by a membrane filter but remained in chemical contact. The tubes were modifications of the design by Vance (1965) and consisted of two L-shaped glass tubes 25 mm diameter with ground faces clamped together holding a membrane filter (0.45 μm) between the two compartments. To each arm was added 30 ml of ASM medium plugged with cotton wool and autoclaved. Following inoculation with the test algal species the tubes were placed on a shaker (70 rev/min) at constant temperature and continuous light. Growth was followed by removal of samples every one or two days for cell counting or absorbance measurements. The constant gentle movement of the tubes prevented clogging of the membranes by algae. Tests with dyes verified that dissolved substances rapidly exchanged between compartments and there was no cross contamination of cultures at any time.

Further tests for extracellular products were made on filtrates of batch cultured *Anabaena*, *Chlorella* and *Microcystis*. Cultures were grown in shaken flasks, centrifuged, filtered and the filtrate added to test algae in nutrient solution.

Table 1. Initial and final cell count data for growth of *Anabaena* and *Microcystis* grown for 4 days in polythene or dialysis tubes suspended in the Mangere circulation channel in mid-January 1974

	Cell No. 10^5 ml^{-1}
Anabaena	
Initial	1.0
Final polythene	6.6 ± 1.2
Final dialysis	8.4 ± 0.2
Microcystis	
Initial	1.0
Final polythene	2.9 ± 0.4
Final dialysis	3.0 ± 0.3

Each figure represents the mean of 3 replicates ± 1 S.E.M.

RESULTS

Growth of blue-green algae in ponds

Earlier experiments showed (Vincent & Silvester, 1979) that blue-green algae were totally inhibited by pond conditions during summer. To test whether this effect was due to low molecular weight compounds, *Anabaena* and *Microcystis* were inoculated into polythene and dialysis bags and suspended in the circulation channel in mid-January. After four days both species showed significant growth with little difference between final concentration in polythene or dialysis bags (Table 1). These results show that the dominant pond algae *Chlorella* and *Euglena* do not exert their dominance by a dialysable chemical substance.

Mixed culture experiments

The effects of interaction were tested in the laboratory by mixed batch culture experiments which were assayed by the '*in vivo* absorbance' method. Using three starting concentrations of *Anabaena* 0.05, 0.5 and 5.0×10^5 cells per ml *Chlorella* was added to 0, 0.05, 0.5 and 5.0×10^5 cells per ml. The final results of a nine day growth period show that only at the lowest initial concentration of *Anabaena* did *Chlorella* inhibit (Table 2). This is confirmed in the *in vivo* absorbance assay which shows the growth assayed over the whole nine days (Fig. 2). These results show in more detail that the high *Chlorella* concentration had an initial effect on *Anabaena* growth, but only at the lowest *Anabaena* concentration was this inhibition maintained (Fig. 2c). Thus the *in vivo* absorbance

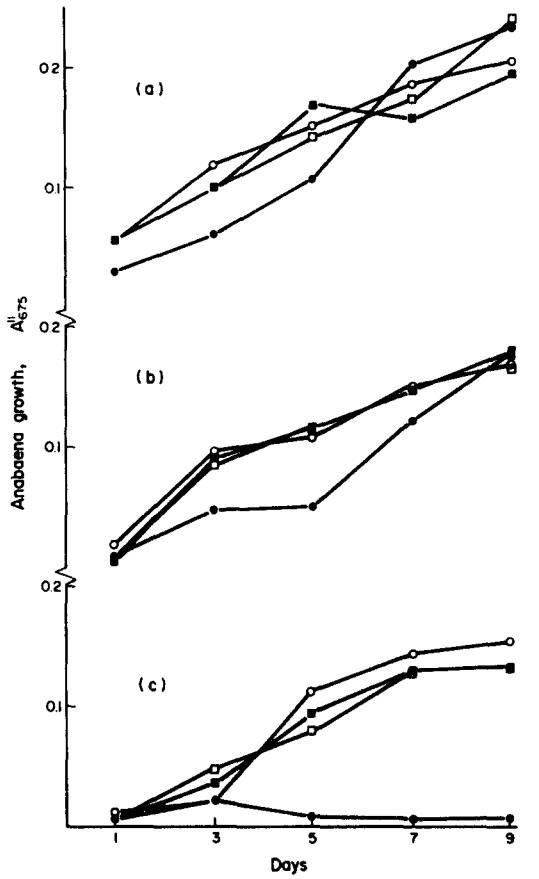

Fig. 2. Growth of *Anabaena* in mixed culture with *Chlorella* in ASM, shaken at 125 rev/min under 2500 lux continuous fluorescent light at 28°C. Growth assayed by relative absorbance at 675 nm (A'_{675}). Initial concentration of *Chlorella* (cells per ml): zero ○; 5.0×10^5 ●; 5.0×10^4 □; 5.0×10^3 ■. Initial concentration of *Anabaena* (cells per ml): 5.0×10^5 Fig. 2a; 5.0×10^4 Fig. 2b; 5.0×10^3 Fig. 2c.

method generally confirms the final growth data but gives an insight into the growth dynamics of the cultures. However the inhibition in terms of cell numbers (Table 2) is somewhat less than anticipated in the *in vivo* absorbance (Fig. 2c) indicating a possible pigment change.

In the reciprocal experiment in which final *Chlorella* numbers are plotted (Table 3) the result is conclusive in that *Chlorella* is inhibited at all levels of *Anabaena* concentration. This result is expanded by the *in vivo* absorbance results (Fig. 3) in which there is

Table 2. Final cell counts for *Anabaena* grown in mixed culture with *Chlorella* for 9 days in ASM under 2500 lux fluorescent light at 28°C

Initial conc. of *Chlorella* (10^5 ml^{-1})	Initial conc. of *Anabaena* (10^5 ml^{-1}) 0.05	0.5	5.0
0	66	96	120
0.05	69	95	104
0.5	71	78	120
5.0	39	94	103

Each figure represents the mean for 2 replicates.

Table 3. Final cell counts for *Chlorella* grown in mixed culture with *Anabaena* for 9 days in ASM under 2500 lux fluorescent light at 28°C

Initial conc. of *Anabaena* (10^5 ml^{-1})	Initial conc. of *Chlorella* (10^5 ml^{-1})		
	0.05	0.5	5.0
0	84	116	171
0.05	19	35	80
0.5	7	11	62
5.0	0.4	9	44

Each figure represents the mean for two replicates.

both quantitative and qualitative confirmation of the ability of *Anabaena* to suppress growth of *Chlorella*.

Interaction tube experiments

The interaction was further studied in interaction tubes whereby similar initial algal concentrations were separated by a membrane filter and their growth followed for eight days. The growth curve for *Anabaena* (Fig. 4a) confirms that *Chlorella* does not inhibit *Anabaena*; in fact the presence of *Chlorella* is shown to stimulate *Anabaena* growth and this is confirmed as a significant effect in the final cell counts (Table 4).

The inhibitory effect of *Anabaena* on *Chlorella* is also confirmed in interaction tubes (Fig. 4b) where growth is reduced to half that of control tubes (Table 4).

An exactly comparable set of results is obtained using *Microcystis* (Fig. 5) in which *Chlorella* is strongly inhibited by *Microcystis* but stimulates the blue–green algal growth; the effect however with *Microcystis* is not observed until late in the growth phase.

Filtrate experiments

Cultures of *Anabaena*, *Chlorella* and *Microcystis* were raised to 10^7, 10^8 and 5×10^6 respectively and the supernatant was filtered (0.45 μm) and 30 ml of filtrate added to 70 ml cultures of the three algae in ASM. The results for *Anabaena* (Fig. 6a) show no significant effect of added filtrates while for *Microcystis* (Fig. 6b) the *Chlorella* filtrate gave a significant enhancement of growth.

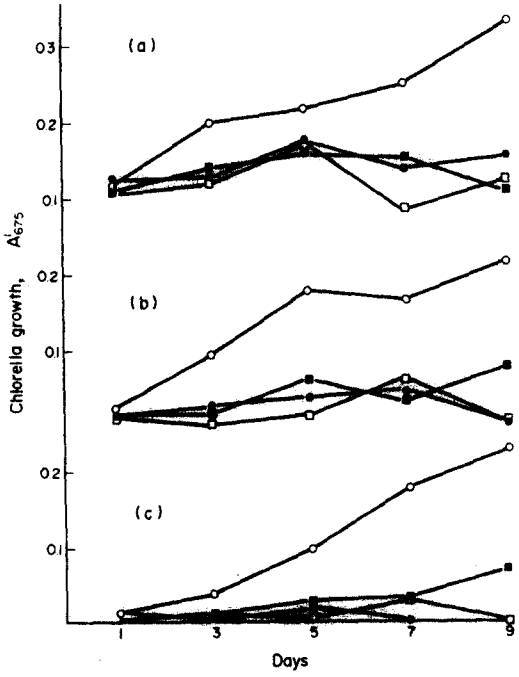

Fig. 3. Growth of *Chlorella* in mixed culture with *Anabaena*, in ASM shaken at 125 rev/min under 2500 lux continuous fluorescent light, at 28°C. Growth assayed every two days by relative absorbance at 675 nm (A'_{675}). Initial concentration of *Anabaena* (cells per ml): zero ○; 5.0×10^5 ●; 5.0×10^4 □; 5.0×10^3 ■. Initial concentration of *Chlorella* (cells per ml): 5.0×10^5 Fig. 3a; 5.0×10^4 Fig. 3b; 5.0×10^3 Fig. 3c.

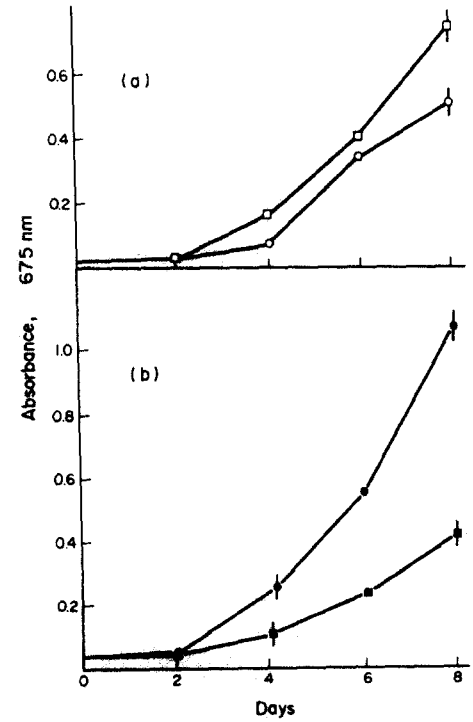

Fig. 4. Growth of *Chlorella* and *Anabaena* in interaction tubes. Shaken at 70 rev/min, under 2500 lux fluorescent light at 28°C on ASM. Growth assayed by '*in vivo* absorbance' at 675 nm. Each point represents the mean of four replicates, ± one S.E.M. (a): Growth of *Anabaena*: opposite *Chlorella* □; opposite uninoculated compartment ○. (b): Growth of *Chlorella*: opposite *Anabaena* ■; opposite uninoculated compartment ●.

Table 4. Initial and final cell concentration data for growth of *Anabaena* and *Chlorella* for 8 days in interaction tubes, shaken at 70 rev/min under 2500 lux fluorescent light at 28°C in ASM

	Cell No. 10^5 ml^{-1}
Chlorella	
Initial	1.0
Control	119 ± 8.1
Opposite *Anabaena*	53 ± 2.4
Anabaena	
Initial	1.0
Control	15 ± 1.8
Opposite *Chlorella*	24 ± 1.6

Each figure represents the mean for 4 replicates ± 1 S.E.M.

Growth of *Chlorella* (Fig. 6c) was significantly enhanced by addition of *Chlorella* filtrate and completely suppressed by filtrates of both blue-green algae. It is significant that addition of 30 ml deionized water as one of the controls did not alter growth rate over the ASM only control and thus any suppression or stimulation effect of added filtrate is not due to increase or dilution of inorganic nutrient.

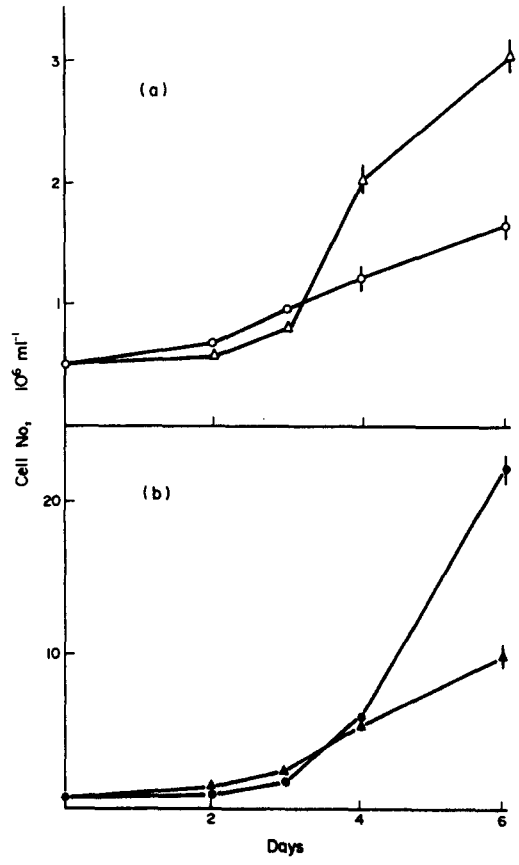

Fig. 5. Growth of *Microcystis* and *Chlorella* in interaction tubes. Shaken at 70 rev/min under 2500 lux fluorescent light at 28°C in ASM. Growth assay by cell count. Each point represents the mean of three replicates ± one S.E.M. (a). Growth of *Microcystis*: opposite *Chlorella* △; opposite uninoculated compartment ○. (b). Growth of *Chlorella*: opposite *Microcystis* ▲; opposite uninoculated compartment ●.

DISCUSSION

The *in situ* bag experiments allowed growth of the blue-green algae to be studied under physical and, in the case of the dialysis tubes, chemical conditions close to those normally experienced in the Manukau ponds. It is assumed that the dialysis membrane allowed free exchange of compounds between the cultures and the pond water; however this material has a very small pore diameter (*ca.* 3 nm) which would resist movement of higher molecular weight substances such as proteins. Various polypeptide molecules are known to be secreted by certain algae—for example the toxins produced by some blue-green algal strains—and it is therefore possible that in the dialysis tubes high molecular weight inhibitory compounds were excluded. However this is unlikely since good growth of blue-green algae on 0.45 μm filtered pond water was recorded throughout the year (Vincent & Silvester, 1979) and no inhibitory effects on

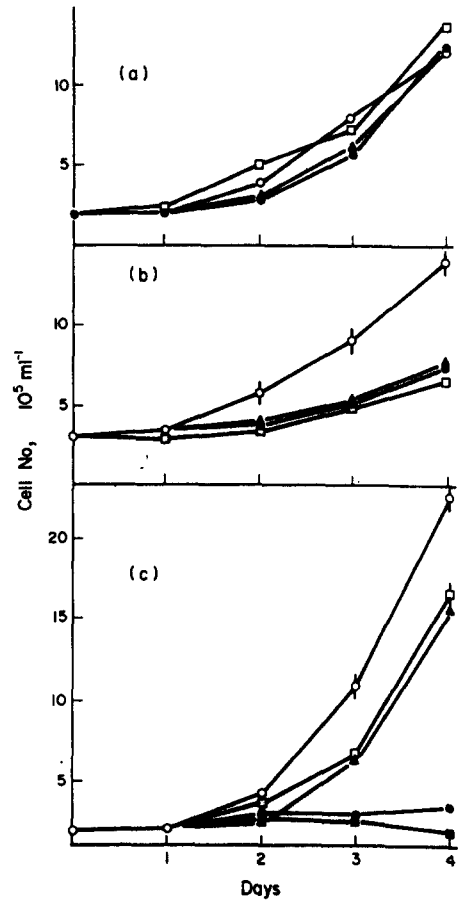

Fig. 6. Effect of filtrates on initial rates of algal growth. Algae cultured at 28°C, under 2500 lux fluorescent light, shaken at 125 rev/min. Each point represents the mean of three replicates ± one S.E.M. (a) Growth of *Anabaena* in 70 ml ASM plus 30 ml: sterile deionized water □; ASM ▲; *Anabaena* filtrate ●; *Chlorella* filtrate ○. (b) Growth of *Microcystis* in 70 ml ASM plus 30 ml: sterile deionized water □; ASM ▲; *Microcystis* filtrate ●; *Chlorella* filtrate ○. (c) Growth of *Chlorella* in 70 ml ASM plus 30 ml: sterile deionized water □; ASM ▲; *Microcystis* filtrate ■; *Anabaena* filtrate ●; *Chlorella* filtrate ○.

Chlorella or *Anabaena* could be detected in this water.

In mixed culture, *Anabaena* outgrew and dominated *Chlorella*, providing the concentration of the green algae was not high. Competition for a limiting nutrient is one of the many possible factors operating in mixed culture. In the interaction and mixed culture experiments it is possible that the depression of growth of *Chlorella* was due to differential rates of uptake of nutrients by the two algae. Blue-greens are known to be capable of assimilating phosphate at much faster rates than green algae, and to grow on stored reserves for extended periods (Shapiro, 1970). Therefore rapid nutrient uptake by *Anabaena* may have resulted in nutrient depletion and hence lowered growth rates of *Chlorella*. Other factors regulating growth in mixed cultures include competition for light and dissolved gases. Blue-green algae are capable of more efficient utilisation of low light intensities, as might be experienced with concentrated cultures in which light shielding is high, than green algae due to the high efficiency of energy transfer by phycobilins to chlorophyll *a* (Emerson & Lewis, 1942). These algae also exhibit more favourable CO_2-uptake kinetics (Shapiro, 1973), which would be particularly significant at low carbon dioxide tensions.

The *in vivo* absorbance assay proved to be a useful technique for following the general growth trends of *Chlorella* and *Anabaena* in mixed culture. However it is only an approximate guide since it assumes that the 615–675 nm absorbance ratios remain constant throughout the period of growth. In fact these ratios vary somewhat as nutrient conditions and the physiological state of the algae change. The final cell counts and absorbance measurements at day nine for the mixed culture experiment appear to correlate well, with the exception of certain treatments in the 5×10^5 *Chlorella* series, probably due to changes in the relative cellular concentrations of the major pigments.

The very severe depression of growth of *Chlorella* by the presence of *Anabaena* suggests that some inhibitory compound was the major responsible factor; this was verified by filtrate and interaction tube experiments. Such inhibition effects have been reported in other blue-green algal studies. Vance (1965) found both in laboratory and field experiments that *Microcystis aeruginosa* produced compounds which suppressed the growth of *Cryptomonas ovata*, *Ceratium* spp. and *Phacus longicauda*. Jakob (1961) demonstrated that filtrates from cultures of the soil alga *Nostoc muscorum* had a lethal effect on several eucaryotic algae including *Euglena* and *Cosmarium* species as well as on another blue-green algae, *Phormidium*. The toxic substance responsible was isolated and identified as a dihydroxyanthroquinone. Various inhibitory substances produced by green algae have been discovered. *Chlorococcum* was found to produce a substance which inhibited the growth of *Chlamydomonas*. This factor was located in the steam volatile fraction of *Chlorococcum* filtrates and its activity was found to be strongly dependent upon pH (Kroes, 1971, 1972). Certain strains of *Chlorella vulgaris* are well-known producers of auto-inhibiting compounds. These 'chlorellins' appear to be peroxides which under 'certain critical conditions' are produced by the photo-oxidation of fatty acids (Scult, 1964). The ecological role of these antibiotic substances remains unclear. Vance (1965) believes that such compounds are responsible for the sudden decline of blooms and are of importance in the control of algal succession. However in view of the ameliorating effect the environment would have on these substances, and the ability of many algae to adapt to various toxins and antibiotics, Sieberth (1968) has concluded that extracellular inhibitors produced by algae act as controlling factors only under exceptional conditions.

In the filtrate and interaction tube studies it appeared that the strain of *Chlorella* isolated from Mangere produces compounds which have a stimulatory effect on its own growth and on the growth of *Microcystis*. One algal product frequently reported in the literature for its stimulatory properties is glycollate. Sen & Fogg (1966) observed that a planktonic strain of *Chlorella pyrenoidosa* which liberates extracellular glycollate grows twice as fast at low light intensities in the presence of $1.0\,mg\,l^{-1}$, of glycollate than in purely mineral medium. More recently, Lord & Merrett (1971) demonstrated photoassimilation of glycollate by another strain of *C. pyrenoidosa*, while Nelson *et al.* (1969) have found that *Scenedesmus obliquus* secretes glycollate but can assimilate this compound both heterotrophically and by photoassimilation. Several other compounds which have a growth stimulation effect have been reported. Filtrates from the green alga *Hormotila blennista* stimulated the growth of itself and of a planktonic *Scenedesmus* species (Monahan & Trainor, 1970). This effect was later associated with a high molecular weight heat-stable component of the filtrate (Monahan & Trainor, 1971).

In the interaction tube experiments the stage of growth of each alga appears to be important in determining the activity of the growth compounds. A similar effect has been noted by Proctor (1957) who discovered that filtrate from a 2 day old culture of *Chlamydomonas* stimulated growth of *Haematococcus* while filtrate from 4–6 day old cultures proved toxic. The interaction experiments using the Manukau *Chlorella* isolate add further support to the conclusions of the previous paper (Vincent & Silvester, 1979) that the current absence of blue-green algae from these ponds is associated with their inability to successfully compete with high resident populations of green algae rather than to extracellular product effects. On the other hand it appears that, at least for the strains used in this study, as long as the physical conditions for growth are adequate, *Anabaena* and *Microcystis* are able to completely dominate *Chlorella* by production of some toxic or inhibitory extracellular product. Dominance by blue-green algae in oxi-

dation ponds is a common phenomenon in lightly loaded ponds during the establishment phase (Haughey, 1969) and the present study shows that this dominance will be easily initiated if the green algal population drops significantly and if this happens during the summer when temperatures favour blue-green algal growth.

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