On the Nature of Mixed Cultures of *Chlorella pyrenoidosa* TX 71105 and Various Bacteria

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SUMMARY

The growth of several selected micro-organisms in rapidly dividing cultures of *Chlorella pyrenoidosa* TX 71105 was studied. Bacterial proliferation was a function of algal growth and bacterial growth occurred, at least in part, as a result of the excretion of organic substances into the culture medium by rapidly dividing algae. These substances capable of supporting bacterial oxidation and growth were varied in kind and were utilized selectively by the different bacteria. Only a small fraction of the soil and air bacteria grew in the algal cultures. The majority of soil and air bacteria survived in mixed culture for several days but did not increase in numbers. On the other hand, 6 out of 8 bacteria pathogenic for man died promptly in cultures of *Chlorella pyrenoidosa*; but *Salmonella typhi* and *S. paratyphi* grew well for extended periods of time. Fungi capable of producing macrocolonies on potato glucose agar at pH 3.5 did not increase in numbers during 8 days. Yeasts and actinomycetes were not detected by the methods used; bacteriophages were observed with some regularity.

INTRODUCTION

Recent reports proposing the use of microalgae in 'closed ecological systems' suitable for space vehicles have described the inherent advantages of these organisms as gas exchangers, transformers of human wastes, and as food material (Eley & Myers, 1964; Myers, 1964; Brown, Kennedy & Tischer, 1964; McDowell & Leveille, 1964; Krauss, 1962). It is well recognized that the utilization of algae as symbionts with man in a closed space will result not in a two-organism system but rather in a very complex biological universe. Because of the impracticality of maintaining pure cultures of algae for long periods of time, even in the laboratory, it is assumed that algal populations mixed with selected bacteria will be used. In the opinion of the authors, the feasibility of using mass cultures of algae with or without bacterial contaminants is at present precluded by a dearth of knowledge of the effects of microbial contamination on the algal culture systems. The problem of bacterial contamination of algal cultures has been noted (Krauss & Thomas, 1954; Eley & Myers, 1964; Ward, Moyer & Vela, 1964; Blasco, 1965) but, unfortunately, there are few studies which elucidate the ecological and physiological relationships that must exist in cultures of algae and other micro-organisms living together. A search of the literature showed that previous investigations of the microbiology of algal cultures were concerned primarily with population

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densities and with the gross effects of bacterial contamination on the algal populations. Myers, Phillips, & Graham (1951) stated that the presence of bacteria had little effect on the growth rates of *Chlorella pyrenoidosa*. Myers (1957) reported that bacterial and mould contaminants usually have no effect on algae in rapidly growing steady-state cultures. Mayer, Zuir, Shain & Ginzburg (1964) stated that bacterial contamination of mass cultures (2000 l.) of algae was not a serious complicating factor and apparently had little or no effect on the productivity of the culture. Ward *et al.* (1964) presented data that indicated that the yield of mass of *C. pyrenoidosa* tx 71105 in steady-state cultures was substantially decreased as a result of bacterial contamination, while Nakamura (1963) proved that selected bacterial species enhanced the growth of algae in nutritionally deficient cultures. Nakamura postulated that mixed cultures of *Chlorella* and bacteria form a true symbiotic relationship in nature and that this is also the case in laboratory cultures. To the contrary, Vladimirova (1960) showed that *C. pyrenoidosa* Pringh. suppressed bacterial growth. Further studies on the bacteriology of algal cultures were clearly indicated. The purpose of the present report is to present data which may help to characterize the relationships that exist in mixed cultures of algae and other micro-organisms and to evaluate the feasibility of using these in closed systems for the support of man.

METHODS

Organisms. All work was done with the strain of *Chlorella pyrenoidosa* designated tx 71105 by Sorokin & Myers (1953). Bacteria utilized included *Bacterium anitratum* (BAT), *B. anitratum* atypical (BAA), *Mima polymorpha* (MP), and a Gram-negative organism tentatively assigned to the genus *Flavobacterium* (GNB). Cultures of fourteen other species of bacteria capable of growing in algal cultures were obtained from mass cultures of algae growing in open non-sterile systems. In addition to the above, a collection of soil and air bacteria was made in locales far removed from laboratory algal cultures. All organisms were selected on the basis of colony morphology and were probably all different one from the other and from BAT, BAA, MP and GNB.

Recently isolated pathogenic organisms of proven virulence used in several experiments were: *Salmonella typhi*, *S. paratyphi*, *Shigella flexneri*, *S. dysenteriae*, *Proteus vulgaris*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Corynebacterium diphtheriae*.

Cultures and analytical methods. The medium used in all flask culture experiments was Knop's medium (Phillips & Myers, 1954). It was prepared by adding the following, in order, to 950 ml. water: 50 ml. solution I, 10 ml. solution II, 0.05 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 10 ml. solution III, 2.5 g. KNO_3 , adjusted to pH 6.0 before autoclaving.

Solution I: 25.0 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20.0 g. KNO_3 , 10.0 g. KH_2PO_4 , 2.1 g. CaCl_2 , 2.85 g. H_3BO_3 , and water to 1 l.

Solution II: 8.82 g. $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.44 g. $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.924 g. H_2MoO_4 (85%), 1.57 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.49 g. $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, and water to 1 l.

Solution III: 50 g. ethylenediamine tetra-acetic acid (EDTA), 31 g. KOH, and water to 1 l.

The same Knop medium was used in mass culture experiments except that

KNO₃ was replaced by urea in a final concentration of 1.5 g./l. (1.1 times equivalent nitrogen). This substitution was made since it is felt that mass cultures of algae in space vehicles would probably use urine urea as nitrogen source.

Small flask cultures were grown in an incubator shaker fitted with fluorescent lights. The gaseous atmosphere of the incubator was maintained at 2% (v/v) CO₂ by introducing a mixture of CO₂ and air at the rate of 1 l./min.; incubation temperature was 37°, shaker excursion 5 cm. and fast enough to cause a 'splash' against the flask side but not a 'spray' up to the stoppers. One-litre Erlenmeyer flasks containing 100 ml. medium and capped with beakers were used as culture vessels.

Studies on the nature of microbial populations in mass cultures (32 l.) were done in a photosynthetic gas-exchange culture apparatus of the type described by Ward, Wilks & Craft (1963). 5% (v/v) CO₂ in air was introduced into the culture and the temperature maintained at 39°.

Algal populations in all experiments were monitored by replicate counts with a haemocytometer. Bacterial numbers were estimated by triplicate plate counts on Trypticase Soy agar or, in the case of the pathogenic bacteria, Difco brain heart infusion agar. Yeasts and fungi were enumerated on Difco potato glucose agar (pH 3.5) according to the American Public Health Association Manual on Standard Methods for the Examination of Dairy Products (11th ed. 1960).

Actinomycetes and certain pigmented bacteria were grown in a solution containing the following ingredients (g./l.): 2.0 g. NaNO₃, 1.0 g. K₂HPO₄, 0.5 g. MgSO₄·7H₂O, 0.5 g. FeSO₄·5H₂O, 20 g. reagent grade agar, and 10 g. glucose added as a sterile 10% (w/v) aqueous solution to the sterile basal medium.

Culture filtrates. Soluble algal excretion products were separated from organisms by centrifugation and filtration through 0.45 μ washed (Winneberger, Austin & Klett, 1963) filter membranes. Further separation was obtained by dialysis and extraction as outlined in Fig. 3. Warburg manometry was used to determine which of the fractions obtained were capable of supporting bacterial oxidation. Substances isolated by extraction with organic solvents were taken to dryness, redissolved in water, and taken to dryness again for three complete cycles in an effort to avoid bacterial oxidation of residual organic solvents. Further analyses of the fractions containing oxidizable substances were done by paper and thin-layer chromatography.

RESULTS

Flask culture studies

In previous reports (Ward *et al.* 1963; Ward *et al.* 1964) workers at this laboratory had noted that the growth of bacteria in algal cultures closely paralleled algal growth. The data presented in the present report confirm and extend those findings. Small flask cultures of *chlorella* tx 71105 contaminated with pure cultures of selected bacteria were sampled repeatedly during the algal growth phase and well into the stationary phase. Fig. 1 represents an experiment typical of a large number of studies designed to reveal the kinetics of growth of mixed cultures of *chlorella* tx 71105 and various bacteria. It was evident that the bacterial populations increased only while the algal component of the culture was actively dividing. It was also noted that bacterial populations at stationary

phase of algal growth were always of about the same numerical magnitude as the algal populations. This equilibrium was reported previously by Mayer *et al.* (1962) in large open culture tanks. Since other investigations (Fogg, 1952) have shown

Fig. 1

Fig. 2

Fig. 1. Growth curves of *Chlorella pyrenoidosa* tx 71105 and the Gram-negative bacterium (GNB) added to the culture as a bacterial contaminant. Algal cells, ○; viable bacterium GNB, ●.

Fig. 2. Excretion of oxidizable metabolites by axenic *Chlorella pyrenoidosa* tx 71105 (lower) as a function of growth (upper). Oxidizable metabolites were measured by classical Warburg manometry.

Table 1. *Selective utilization of oxidizable substances by bacteria growing in algal cultures*

	Ratio of oxygen consumed by these test bacteria*			
	BAT†	MP†	BAA†	GNB†
Filtrates from 4-day cultures of the following:				
Axenic chlorella tx 71105	0	0.47	0.58	0.68
tx 71105 + BAT	0	0	0	0.16
tx 71105 + MP	0	0.03	0.03	0.16
tx 71105 + BAA	0	0.02	0	0.37
tx 71105 + GNB	0.12	0.07	0.15	0
tx 71105 + all 4	0	0	0.01	0

* Ratio of $\mu\text{l. O}_2$ consumed by 10^9 starved bacteria in 20 min. Ratio represents the amount of oxygen utilized by test bacteria using culture filtrate as substrate and 0.025 M-glucose as substrate. The results were corrected for endogenous respiration as measured by using Knop's medium i.e.;

Culture filtrate—Knop's medium
0.025 M-glucose—Knop's medium

† BAT, *Bacterium anitratum*; MP, *Mima polymorpha*; BAA, *Bacterium anitratum* atypical; GNB, Gram-negative rod possibly *Flavobacterium* sp.

that some algae excrete larger amounts of organic material during active growth periods than they do at resting stages, it was assumed that the same phenomenon occurred in cultures of chlorella tx 71105. To prove that rapidly dividing tx 71105 excreted large amounts of substances capable of supporting bacterial respiration and growth, axenic cultures of the chlorella were sampled daily and assayed for these materials. Figure 2 shows that the excretion of water-soluble substances capable of supporting bacterial respiration was a function of algal growth. These data were confirmed by studies which showed that substances capable of supporting bacterial growth were also excreted in the same fashion. The data (Fig. 2) also indicated that these substances accumulated in the culture fluid and were not removed completely by algal activities or autolytic reactions.

Fig. 3. Shifts in bacterial populations as a function of culture age. Algal cells, $\circ$; 'white colonies', $\triangle$; 'brown colonies', $\square$; 'green colonies', $\bullet$; and 'red colonies', $\blacktriangle$. Each colony type listed is plotted as a fraction of the total viable bacterial count.

It was then reasoned that if bacteria grew on the excreted metabolites, filtrates from contaminated algal cultures should be free from these metabolites. The data in Table 1 verify this and show that more than one substance was excreted by the chlorella since bacterium GNB could utilize substances not oxidizable by bacteria BAT, BAA and MP.

The products of algal metabolism that can be oxidized by bacteria were dialysable in distilled water when placed in Visking membranes (Union Carbide Corporation, 6733 West 65th Street, Chicago, Illinois). Fractionation of the dialysate (Fig. 4) proved that various classes of chemical compounds capable of supporting bacterial respiration were excreted by chlorella tx 71105. The progressive increase in capacity to support respiration of each fraction as purification proceeded cannot be explained at this time. It can, however, be stated that the phenomenon was reproducible. Several experiments have shown that it was not a matter of

antagonism since mixing of the separated fractions did not result in lowered oxidative capacity of the purer fractions. That is, mixing equal parts of the non-dialysable residue and the final extract (or any other combination) gave oxygen uptakes proportional to the quantity of ether-extractable material present, and no inhibition was observed. Also, mixtures of, say, non-dialysable residue and glucose did not result in lowered rates of oxygen consumption by bacteria utilizing the glucose.

Table 2. *Growth of bacteria unrelated to algal communities in flask cultures of Chlorella pyrenoidosa tx 71105*

Isolate number	Viable bacteria/ml.	
	Day 0	Day 4
R-PD 1	1.1×10^5	1.2×10^4
C-KS 1	1.9×10^6	$<1 \times 10^3$
R-BR	4.5×10^4	1.2×10^4
C-SN 2	7.7×10^4	1.0×10^3
C-AP	4.7×10^5	$<1 \times 10^3$
R-GR 1	1.6×10^5	1.0×10^3
C-HD 2	5.2×10^5	1.8×10^4
R-YL	1.0×10^6	6.0×10^3
R-GR 4	7.8×10^5	2.1×10^4
B-RE	8.8×10^4	1.4×10^4
C-SN 1	2.4×10^5	2.0×10^3
R-GR 2	4.4×10^5	$<1 \times 10^3$
R-DD	2.8×10^5	1.0×10^4
C-FA 1	4.1×10^3	1.2×10^3
C-FA 2	4.0×10^3	1.9×10^5
C-SP 3	2.0×10^3	3.3×10^3
C-SP 4	1.6×10^3	2.1×10^4
R-PD 2	1.5×10^3	$>1 \times 10^7$
C-KS 2	4.9×10^3	6.0×10^3
R-GR 3	6.0×10^3	1.0×10^3
C-HD 1	3.1×10^3	5.5×10^3
C-SP 2	4.0×10^3	9.0×10^3

Chromatographic analyses of the two terminal fractions (Fig. 4) revealed the presence of certain organic acids and certain ninhydrin-reactive materials in pure cultures of chlorella tx 71105 that were not found in cultures mixed with bacteria.

It is inferred from these studies that bacteria grow in cultures of chlorella tx 71105 by utilizing substances excreted during the algal growth. It is further inferred that algae/bacterial population equilibria resulted from the establishment of steady-state rates of algal division and cell death at stationary phase. It has been proven that algal metabolites were utilized selectively by different bacteria (Table 1) and that various classes of chemical substances were excreted (Fig. 4).

The literature reviewed implies that algal cultures are subject to bacterial contamination but no reports were found which attempted to describe the nature of the compatible bacteria. Table 2 shows that a few select bacteria could grow well in cultures of chlorella tx 71105 but that the majority of soil and air bacteria could not proliferate in mixed culture with the algae. It was shown that those organisms incapable of proliferating in algal cultures were also incapable of multiplying in filtrates of axenic cultures or to oxidize these filtrates. Also, when filtrates of axenic cultures of chlorella were inoculated and incubated with bacteria

incapable of growing in algal cultures and the bacteria subsequently removed, it was plainly evident that the filtrates still contained substances oxidizable by bacteria BAT and GNB. These data indicate that the algae determined the kind of bacteria with which they will grow, according to the nature of the organic substances they excreted.

Table 3 shows that 6 out of 8 bacteria pathogenic for man died out rapidly in cultures of chlorella tx 71105 but that *Salmonella typhi* and *S. paratyphi* grew quite well for prolonged periods of time. It is assumed that other human pathogens might also grow in cultures of chlorella tx 71105.

Table 3. Growth and survival of some pathogens in cultures of *Chlorella pyrenoidosa* tx 71105

Organism	Days						
	0	1	2	3	5	6	7
	Viable organisms/ml.						
<i>Salmonella typhi</i>	2.0×10^5	6.9×10^6	4.7×10^6	2.9×10^3	5.4×10^4	3.5×10^4	4×10^4
<i>S. paratyphi</i>	8.9×10^4	1.0×10^6	8.0×10^2	3.9×10^2	1.8×10^4	3.0×10^2	7.9×10^2
<i>Shigella flexneri</i>	2.1×10^5	8.3×10^4	$>1 < 10$	0	—	—	—
<i>S. dysenteriae</i>	2.2×10^5	1.0×10^4	$>1 < 10$	0	—	—	—
<i>Proteus vulgaris</i>	1.5×10^5	1.3×10^6	9.4×10^3	$>1 < 10$	0	—	—
<i>Streptococcus pyogenes</i>	1.7×10^4	0	0	—	—	—	—
<i>Staphylococcus aureus</i>	1.2×10^5	0	0	—	—	—	—
<i>Corynebacterium diphtheriae</i>	1.3×10^4	0	0	—	—	—	—

Mass culture studies

Studies of bacterial growth in non-sterilized open mass cultures of chlorella tx 71105 revealed that the same algae/bacteria population relationships existed there as were observed in flask cultures. All mass culture studies utilized the natural microbial flora normally encountered in non-sterilized algal culture systems. It was possible to monitor the growth of heterotrophic and autotrophic bacteria, fungi, yeasts and actinomycetes. Table 4 (representative of the data obtained from 5 experiments) shows that bacteria proliferated as a function of algal growth in mass cultures of chlorella tx 71105. It also shows that heterotrophic bacteria outgrew bacteria capable of utilizing KNO_3 as sole nitrogen source. This indicates that most bacterial growth resulted from the utilization of organic nitrogenous substances excreted by the algae rather than from the use of KNO_3 from the algal medium. Table 4 presents data which show that the fungi survived but did not grow in cultures of chlorella tx 71105. No significant fungal populations were detected in algal cultures maintained for 8 days and neither yeasts nor actinomycetes were found in these cultures by the methods used.

It was evident that constituents of the detectable microbial populations varied as the algal cultures aged. New cultures generally contained a low but widely varied bacterial population, while ageing cultures in the stationary phase reached equilibrium with a few bacterial species only. Figure 3 shows the shifting population balances as a function of culture age. These population changes were also observed during stationary phase; it is assumed that they were the result of a

Fig. 4. Fractionation of culture filtrate. Numbers in parentheses give $\mu\text{l. O}_2$ consumed by 10^9 viable *Bacterium anitratum* in 40 min. All fractions were reconstituted to original quantity with water.

Table 4. *The growth of various micro-organisms in mass cultures of Chlorella pyrenoidosa tx 71105*

Time (hr)	Bacteria					Fungi	
	Algae	Trypticase			KNO ₃ agar		TSA-KNO ₃ KNO ₃
		soy	agar				
		Organisms/ml.					
0	2.5 × 10 ⁶	1.5 × 10 ⁶	9.5 × 10 ⁵	0.5	1.1 × 10 ²		
8	3.1 × 10 ⁶	2.0 × 10 ⁷	1.4 × 10 ⁶	13	1.0 × 10 ²		
18	2.9 × 10 ⁷	4.7 × 10 ⁷	5.9 × 10 ⁵	77	1.2 × 10 ²		
26	1.5 × 10 ⁸	1.0 × 10 ⁸	>10 ⁶	—	3.3 × 10 ²		
42	9.4 × 10 ⁸	2.7 × 10 ⁸	3.2 × 10 ⁷	8	4.0 × 10 ²		
50	1.4 × 10 ⁹	2.7 × 10 ⁸	1.8 × 10 ⁷	14	4.0 × 10 ²		
76	1.5 × 10 ⁹	2.4 × 10 ⁸	1.4 × 10 ⁷	16	1.8 × 10 ²		
84	1.7 × 10 ⁹	1.6 × 10 ⁸	2.1 × 10 ⁷	7	2.0 × 10 ²		
104	2.2 × 10 ⁹	2.2 × 10 ⁸	2.4 × 10 ⁷	8	1.8 × 10 ²		
128	1.8 × 10 ⁹	9.7 × 10 ⁷	4.1 × 10 ⁶	23	1.1 × 10 ²		

Counts were also made of yeasts and actinomycetes but in no sample did the count of either of these organisms exceed 10 organisms/ml.

given organism's ability to utilize substances not available to a faster growing organism.

Throughout these experiments it was obvious that bacteriophages were present in some mass algal cultures. The presence of these phages was confirmed by serial passage on host bacteria isolated from the cultures.

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