ECOLOGIC RELATIONSHIPS

BETWEEN BACTERIA AND ALGAE IN MASS CULTURE

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Abstract

Mass cultures of algae grown without strict maintenance of axenic conditions readily become contaminated with heterotrophic bacteria. Contaminant populations from 10^6 to 10^9 viable cells per ml of algal culture are frequently encountered. Growth of bacteria in algal cultures is a function of and closely follows algal growth. Only a limited number of bacterial forms occur in large numbers in mass cultures of Chlorella. Soil, air, and putrefactive bacteria generally do not survive in algal cultures; however, two species of an enteric pathogen grow well for prolonged periods. Several bacteria significantly decrease the growth of Chlorella, but their effects are non-additive and apparently non-parasitic in nature. Filtrates of axenic algal cultures support prolific growth of contaminant bacteria. Dialyzable organic materials excreted by algae during growth serve as the major source of nutrients for bacterial growth. Chromatographic analyses of axenic algal filtrates reveal a variety of organic acids, amino acids, and compounds of higher molecular weight. Growth dynamics of selected bacteria in algal cultures is related to differential utilization of algal excretory products.

Introduction

Interactions that occur between bacteria and algae undoubtedly affect the physiology and productivity of aquatic communities (Fogg, 1962; Fogg, 1965). Bacteria have been reported to enhance algal growth (McLachlan and Yentsch, 1959; Nakamura, 1959), but are generally believed to have little or no effect on algal productivity under conditions otherwise optimum for growth (Mayer et al., 1964; Myers et al., 1951; Myers, 1957). In contrast, algae have been shown to both stimulate (Fogg, 1965) and inhibit (Pratt, 1940) growth of associated bacteria. The diverse relationships observed appear to be species contingent and influenced to a large degree by the chemical and physical environment. Soluble organic compounds, as found in lakes and streams, support the growth of heterotropic bacteria and serve to complicate the ecological and biochemical relationships involved. Seasonal fluctuations in temperature and rainfall are probably modifying parameters.

Our interest in algal-bacterial interactions was prompted by the knowledge that laboratory cultures of unicellular green algae, grown autotrophically in liquid inorganic media, frequently become heavily contaminated with bacteria. Contaminant populations often reach levels of 10^6 to 10^9 viable bacteria per ml of culture, yet little significance has been attributed to contamination and it is frequently ignored. Krauss and Thomas (1954) have suggested that contaminant bacteria grow on algal cell wall debris and possibly algal excretory products.

Our studies were initiated to elucidate the role of contaminant bacteria in mass cultures of algae used for photosynthetic gas exchange. Although quantitative data are lacking, it is believed by some investigators that bacterial contaminants are responsible for or are associated with a variety of algal mass culture maladies variously described as foaming, fouling, and sticking.

This paper presents results of NASA supported (NASA-Defense PR No. R-99) research on algal-bacterial relationships conducted at the USAF School of Aerospace Medicine, Brooks Air Force Base, Texas. The early phases of this investigation have been reported (Ward et al., 1964; Vela and Guerra, 1966a; Vela and Guerra, 1966b). A final report and manuscripts dealing with identification and bacterial utilization of algal excretory products are in preparation (Ward and Moyer, 1966; Smith et al., 1967a; Smith et al., 1967b). Hence, detailed experimental procedures will not be given in this paper.

All experimentation was done using the thermotolerant (39°C) alga Chlorella pyrenoidosa TX71105 (Sorokin and Myers, 1953) which has been recently renamed Chlorella sorokiniana (Shihira and Krauss, 1963). Algal cultures were grown on inorganic media (Knop's) in illuminated water baths, annular chambers, thin-panel mass culture devices, and constant temperature incubator-shakers. Cultures were illuminated with fluorescent lamps and aerated with carbon dioxide enriched air (1 to 5 per cent). All experiments were performed with axenic algal cultures unless deliberately contaminated with selected bacteria. Bacteria were isolated and enumerated using standard bacteriological procedures.

Bacterial Contaminants of Algal Cultures

Krauss and Thomas (1954) found that a bacterium probably belonging to the genus Flavobacterium was most common in cultures of Scenedesmus obliquus. A Flavobacterium sp. was also found to be prevvalent in cultures of Chlorella vulgaris (Levinson and Tew, 1961).

Isolations from our algal mass cultures revealed a variety of bacterial forms; several occurred in large numbers. In an effort to obtain a better representative sampling of the types of bacteria that inhabit algal cultures, we obtained samples of TX71105 cultures from five other laboratories. Results of our isolations are presented in Table 1. Only bacteria occurring in large numbers were identified. All species isolated were heterotrophic. It is evident that only a limited number of bacteria reach large populations in algal cultures. It is also significant that Pseudomonas aeruginosa and Mima polymorpha were isolated from all cultures examined.

In other experiments in this series (Vela and Guerra, 1966a) it was shown that most soil and air bacteria do not survive when inoculated into axenic algal cultures. However, two enteric pathogens, Salmonella typhi and S. paratyphi, grew well in algal cultures for prolonged periods. Thus, a highly selective mechanism appears to be operative and the unexpected growth of human pathogens suggests that it may be necessary to separate the biological components of regenerative life support systems.

Growth of Bacteria in Algal Cultures

Earlier work with large (8 liter) batch algal cultures by Ward et al. (1963) showed what appeared to be a direct relationship between growth of algae and contaminant bacteria. Experiments performed with smaller, more manageable cultures, deliberately contaminated with selected bacteria, have served to clarify this relationship.

When dilute algal cultures are exposed to light of saturating intensity (about 2000 ft-c) growth is exponential. Figure 1 shows that contaminants in light saturated cultures also grow exponentially. Algal cultures grown under light limited conditions demonstrate linear growth where the increment of cell increase is constant with time. Contaminants in light limited algal cultures also increase at a linear rate (Figure 2). These data confirm and extend the earlier work (Ward et al., 1963) and clearly demonstrate the dependence of bacterial growth on algal growth. These data also suggest, but do not demonstrate, a nutritional relationship between bacteria and algae in mixed culture as proposed by Krauss and Thomas (1954). A nutritional or pathogenic relationship would appear necessary because, with the exception of EDTA (ethylenediamine tetraacetic acid) used for chelation of trace elements, the algal medium contained only inorganic salts and distilled-deionized water. Only heterotrophic bacteria were present in the algal cultures and hence, required a source of fixed carbon for growth. Blasco (1963) has proposed a pathogenic relationship to explain growth of bacteria in algal cultures.

Figure 3 illustrates the different patterns of bacterial growth

when present as single contaminants in algal batch cultures. Bacterial growth patterns undoubtedly reflect those of algal growth to some extent since the experiments were done on different days. However, total algal growth for all control experiments was about 3.5 mg/ml, indicating that bacterial growth is dependent on both the species of bacteria involved and the amount and habit of algal growth. Concurrent growth of the five bacteria in a batch culture of TX71105 clearly indicates dominance of some bacterial species (Figure 4). B. anitratum, B. anitratum (atypical), and M. polymorpha cannot be readily distinguished on the basis of colonial form, hence, the total count for the three is given. The gram negative bacillus appears to have the greatest competitive advantage. Of special interest is that M. polymorpha grew poorly in association with other bacteria, but reproduced profusely when present as a single contaminant. These experiments suggest that interspecies antagonism may also be involved in the growth dynamics of bacteria in algal cultures.

To determine if contaminant bacteria influence the growth of algae, growth of contaminated batch cultures was compared to that of uncontaminated controls (Table 2). Data for algal growth are expressed as per cent of controls. Bacteria had a minor effect on the optical density of algal cultures. However, four of the six bacteria reduced algal cell number by about 20 per cent and caused a 5 to 13 per cent decrease in culture dry weight. The bacterial contribution to culture mass averaged one per cent or less. Ps. aeruginosa reduced algal growth even at low concentrations; however, the combined

effects of five bacteria growing in the same culture did not exceed that of any detrimental bacterium acting independently. These data quantitatively show the adverse effects of certain bacteria on algal growth. One can only speculate about the mechanism involved; however, it is significant that repeated microscopic examinations revealed no evidence of an infectious pathogenic relationship.

Growth of Bacteria in Algal Culture Filtrates

Because no evidence of parasitism or infectious disease was observed, experiments were performed to determine if algal cell walls released during cell division or products excreted during growth serve as nutrient for growth of contaminant bacteria. In addition, the possibility exists that algae excrete bacterial metabolites in response to the presence of bacteria. If algae normally excrete soluble organic materials, the effluent from bacteria-free algal cultures should contain substances oxidizable by heterotrophic bacteria.

Sterile, cell wall free, algal effluent was prepared from 24 hour axenic cultures by centrifugation and passage through 0.45 μ membrane filters. Test bacteria were grown on trypticase soy broth, centrifuged, washed twice with saline, and starved for varying periods of time depending on the experiment.

Figure 5 shows typical results obtained by Warburg respirometry. Similar results were obtained with M. polymorpha, B. anitratum, and B. anitratum (atypical). The presence of substrates in algal effluent oxidizable by the test bacteria is clearly evident. A typical growth curve is shown in Figure 6. All six of the bacteria tested

(see Table 2) increased by at least two logs in 8 hours in 20 ml portions of the algal effluent. The small increase observed in Knop's (control) proved to be due to stored food reserves. Experiments to determine if EDTA could serve as a carbon source for the test bacteria were negative.

Another experiment in this series (Vela and Guerra, 1966) was designed to determine if bacteria selectively remove certain compounds when growing in algal cultures, but leave others subject to oxidation by different bacteria. Algal cultures were grown in combination with the test bacteria and the presence of oxidizable materials in culture filtrates determined by Warburg respirometry (Table 3). It is evident that at least two or more substances are excreted by TX71105 since the gram negative bacillus oxidized substances not subject to breakdown by the other bacteria tested. The presence of several types of excretory products could explain differences in competitive advantage observed in the growth experiments.

Identification of Algal Excretory Products

Because previous experiments showed that soluble substances of algal origin serve as nutrient for growth of contaminant bacteria, it appeared important to identify these substances as a prelude to evaluating their utilization by bacteria.

Fogg (1962; 1966) has reviewed available information on the extracellular products of algae. Marine phytoplankton have recently been shown to excrete up to 25 per cent of their photoassimilated carbon

during logarithmic growth (Hellebust, 1965). The excretion of glycolic acid by C. pyrenoidosa during photosynthesis has been extensively studied by Tolbert and Zill (1956). Several organic acids have been reported as algal extracellular products. Goryunova (see Fogg, 1962) found oxalic, tartaric, succinic, and other organic acids in filtrates from cultures of Oscillatoria splendida. Appreciable quantities of glycolic, oxalic, and probably pyruvic acids were reported by Allen (1956) to be excreted by various species of Chlamydomonas. Other algal excretory products, such as amino acids and peptides, carbohydrates, vitamins and growth substances, inhibitors and antibiotics, toxins, and enzymes have been reported (Fogg, 1962). The literature concerning algal excretions is inconsistent; yet there appears to be certain species specificities with respect to the substances excreted as extracellular products.

Axenic algal cultures were grown in 400 ml of sterile Knop's medium in one liter Erlenmeyer flasks. Flasks were held at 37°C in a Model B-27 New Brunswick incubator-shaker and illuminated from beneath at about 2,000 ft-c with fluorescent lights. A gaseous atmosphere containing 4 per cent carbon dioxide was maintained. Dense cultures were transferred to sterile 200 ml centrifuge tubes and centrifuged for 40 minutes at 7,000 rpm while being maintained at 2°C. The supernatant fluid was then passed through 0.45 μ membrane filters. 100 ml samples of culture filtrate were dialyzed for 36 hours at 5°C against five changes (350 ml each) of distilled, deionized water. The dialysate was collected and stored at 5°C and subsequently concentrated to a volume of less than 100 ml in a rotary evaporator.

Fractionation schemes for the concentrated dialysates are shown in Figures 7 and 8. After dialysates were separated into various components, the fractions were evaporated to 2 ml in a rotary evaporatory. Standard paper chromatographic procedures were used for qualitative identification of substances present in the various fractions (Smith et al., 1967a; Smith et al., 1967b). Some amino acid analyses were performed using the Technicon Amino Acid Analyzer. Other standard analytical methods were used as needed. Table 4 lists the major constituents identified in axenic culture filtrates of Chlorella TX71105. Several of the compounds detected were not identified. Quantitation of organic acids was accomplished with great difficulty and with less precision than desired. The high molecular weight groups of compounds found in the non-dialyzable fraction were not further identified. Many of the substances identified are known to serve as sources of carbon and energy for the growth of heterotrophic bacteria.

Utilization of Algal Excretory Products by Selected Bacteria

Utilization of algal excretory products was studied using four bacteria growing singly and in combination in axenic culture filtrates. Culture filtrates were prepared as previously described from algal cultures containing approximately 10^8 cells/ml. Test bacteria were grown on trypticase soy broth as previously described and starved for two hours in 0.9 per cent saline solution. Inocula of 10^3 viable cells of each bacterium were added singly and in combination to separate 200 ml portions of axenic culture filtrate and incubated with

agitation for 24 hours at 37°C. Bacteria were separated by centrifugation and filtration. Metabolized filtrates were fractionated for analyses using procedures shown in Figures 7 and 8.

Organic acids were assayed spectrophotometrically. Glycolic acid was measured using 2,7-naphthalenediol for color development. Precision of the technique was severely limited by interferences caused by the presence of other organic acids. The standard addition technique was only partially effective, depending on the types of organic acids present. As a consequence, only data for lactic acid and glycolic acid will be presented.

Ninhydrin-positive substances were assayed using the Technicon Amino Acid Analyzer. Several substances which appeared to be peptides could not be definitely identified. One peptide-like substance occurred in large quantities and was only slightly metabolized by the test bacteria. Other unknowns occurred in trace amounts only. Quantitation of the amino acids was relatively good. Summary data on eleven amino acids and two organic acids are presented (Table 5).

With the exception of the organic acids, B. anitratum (typical) and B. anitratum (atypical) utilized algal excretory products to about the same extent. The atypical strain utilized both lactic acid and glycolic acids; however, these acids were not metabolized by the closely related typical strain. M. polymorpha failed to use either organic acid, but the gram negative bacillus utilized all of the lactic acid and apparently excreted glycolic acid. The gram negative bacillus also failed to utilize leucine and isoleucine, but was the only bacterium

that removed the trace of lysine present in the culture filtrates. Although data are incomplete, assays for total organic acids indicated that utilization of amino acids by the test bacteria was remarkably greater than utilization of organic acids. The ecological significance of this finding is not readily apparent. However, it should be noted that the combined activities of the four bacteria served to essentially eliminate the products excreted during algal growth. This finding was previously demonstrated in the manometry studies (Table 3) and may explain the tendency of algal cultures to support only a few types of bacteria when unprotected from outside contaminants. If the contaminant flora removes excretory products as rapidly as they are released by growing algae, contamination by other heterotropic bacteria would seem unlikely. Thus it may be possible to establish a known bacterial flora in algal cultures that would effectively prevent invasion by less desirable (more harmful) forms and hence improve stability. It now appears that the observed detrimental effects of bacteria on algal growth may be due only to their utilization of the products excreted. This would represent loss of carbon and efficiency if algae normally reabsorb extracellular materials. Bacterial products may also be toxic to algae. However, we found urea, a known nitrogen source for Chlorella TX71105, to be a major excretory product of three of the four test bacteria. Other bacterial products identified were methionine, tryptophan, and glucosamine.

Contaminated algal mass cultures represent complex ecologies that can be subjected to quantitative investigation. Investigations of the

type reported should be extended to the study of natural systems and to the complex interactions that undoubtedly occur in mass cultures of other organisms being considered for bioregeneration, e.g., Hydrogenomonas spp.

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Table 1. Bacteria isolated from mass cultures of *Chlorella pyrenoidosa* TX71105 (Ward et al., 1964)

Source	Organisms isolated
USAF School of Aerospace Medicine Brooks AFB, Texas	1. *Ps. aeruginosa* 2. *Mima polymorpha* 3. Gram negative bacillus (yellow pigment) 4. *Bacillus* sp.
Martin Company Denver, Colorado	1. *Ps. aeruginosa* 2. *Mima polymorpha* 3. *Bacterium anitratum* (typical strain) 4. *Staph. epidermidis* 5. *Serratia marcescens*
University of Maryland College Park	1. *Ps. aeruginosa* 2. *Mima polymorpha* 3. *Bacterium anitratum* (atypical strain) 4. *Aerobacter cloacae*
Armed Forces Food & Container Institute, Chicago, Illinois	1. *Ps. aeruginosa* 2. *Mima polymorpha* 3. *Bacterium anitratum* (typical strain) 4. *Bacterium anitratum* (atypical strain) 5. Gram negative bacillus (yellow pigment)
General Dynamics/Electric Boat, Groton, Connecticut	1. *Ps. aeruginosa* 2. *Mima polymorpha* 3. *Bacterium anitratum* (typical strain) 4. *Bacillus* sp. 5. *Aerobacter cloacae* 6. *Aerobacter aerogenes*
U.S. Naval Research Lab. Washington, D.C.	1. *Ps. aeruginosa* 2. *Mima polymorpha* 3. Gram negative bacillus (yellow pigment) 4. *Aerobacter cloacae* 5. *Aerobacter aerogenes*

Table 2. Effects of bacteria on the growth of *Chlorella pyrenoidosa* TX71105, bacterial growth in algal cultures, and bacterial contribution to culture mass (Ward et al., 1964)

Bacterium	Algal growth with bacteria[a]			Viable bacteria	
	O.D.	Cell No.	Dry Wt.[b]	No.X10^6/ml	% Dry Wt.
Mima polymorpha	104	80	95	311.3	1.03
Bacillus, gram neg.	-	80	91	13.6	0.71
Bacterium anitratum	89	77	87	14.5	0.98
Bacterium anitratum (atypical strain)	98	100	102	1.2	0.56
Pseudomonas aeruginosa	101	80	89	0.4	0.01
Aerobacter cloacae	103	101	103	22.2	0.25
Combined bacteria[c]	91	85	88	16.1	0.93

Data are means of six or more replicates corrected for initials and

[a]expressed as per cent of bacteria-free controls,
[b]corrected for contribution of bacteria,
[c]first five bacteria listed.

Table 3. Selective utilization of oxidizable substances by bacteria growing in algal cultures (Vela and Guerra, 1966)

Filtrates from 4-day cultures of the following:	Ratio of oxygen consumed by these test bacteria[a]			
	BAT[b]	MP[b]	BAA[b]	GNB[b]
Axenic Chlorella TX71105	0.60	0.47	0.58	0.68
TX71105 + BAT	0	0	0	0.16
TX71105 + MP	0	0.03	0.03	0.16
TX71105 + BAA	0	0.02	0	0.37
TX71105 + GNB	0.12	0.07	0.15	0
TX71105 + all 4	0	0	0.01	0

[a] Ratio of μl 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.;

$$\frac{\text{Culture filtrate-Knop's medium}}{\text{0.025 M-glucose-Knop's medium}}$$

[b] BAT, Bacterium anitratum (typical); MP, Mima polymorpha; BAA, Bacterium anitratum (atypical); GNB, gram negative bacillus.

Table 4. Extracellular products of Chlorella pyrenoidosa TX71105 present in axenic culture filtrates

Organic acids	Amino acids	Other
Fumaric	Aspartic	Polysaccharides
Lactic	Glutamic	Short peptides
Glycolic	Serine	Nucleic acids
Oxalic	Threonine	Ammonia
Pyruvic	Isoleucine	
α-ketoglutaric	Leucine	
Oxalacetic	Tyrosine	
Ascorbic	Phenylalanine	
Gluconic	Lysine	
Galacturonic	Proline	
	Alanine	
	Glycine	
	Cystine	
	Valine	
	Histidine	
	Ornithine	

Table 5. Utilization of excretory products of Chlorella pyrenoidosa TX71105 by selected bacteria.

Compound	conc. in algal filtrate μg/ml	Relative Utilization[a] BAT	BAA	MP	GNB	Combined
Aspartic acid	13.3	+++	+++	+	+++	+++
Threonine	4.8	+++	+++	++	+++	++
Serine	5.3	+++	+++	++	+++	+++
Glutamic acid	11.8	+	+	++	-[b]	+++
Proline	3.7	+++	+++	+++	++	+++
Alanine	5.3	+++	+++	++	+++	+++
Isoleucine	2.1	+++	+++	++	-	+++
Leucine	1.9	+++	+++	+++	-	+++
Tyrosine	trace	+++	+++	-	-	+++
Phenylalanine	1.2	+++	+++	+++	++	++
Lysine	trace	-	-	-	+++	+++
Lactic acid	1.1	-	+++	-	+++	+++
Glycolic acid	1.3	-	+	-	-[b]	+

[a]Data are relative: +++, indicates complete removal; ++, trace remaining; +, one half or more remaining; - , no utilization. [b]GNB apparently excreted small quantities of these compounds.

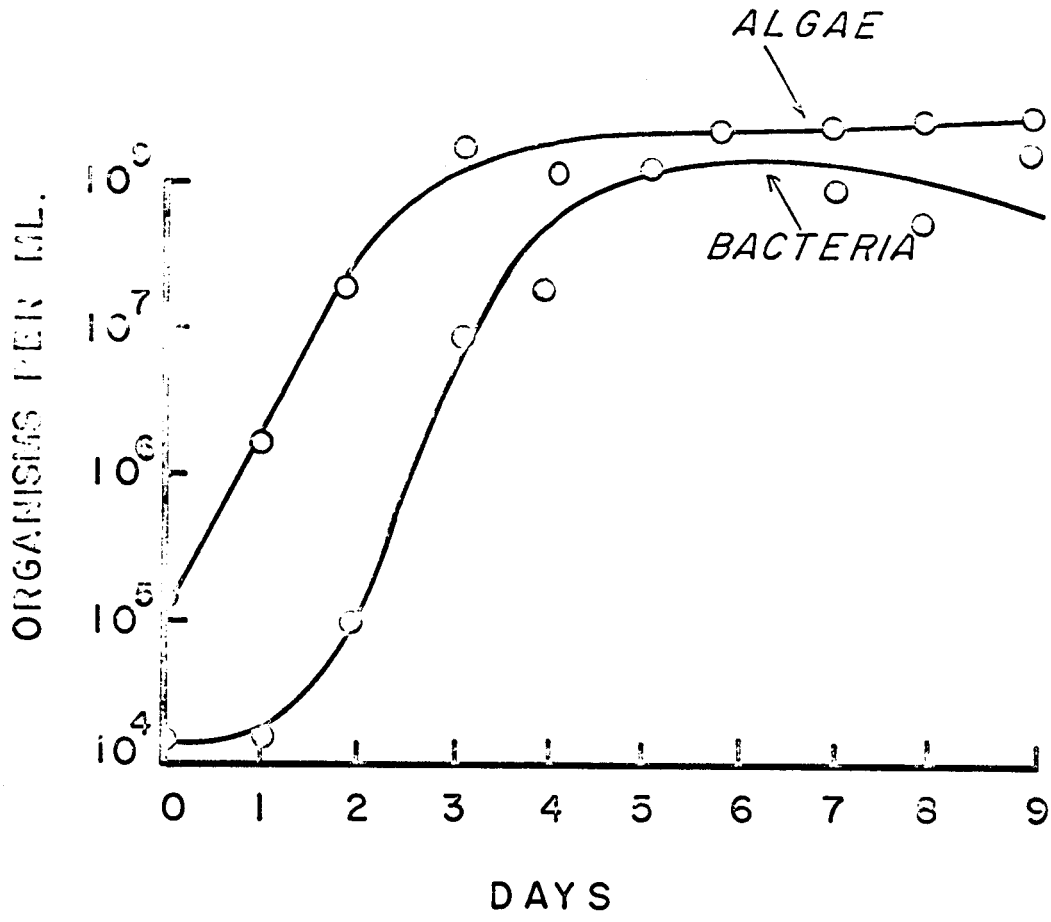

Figure 1. Growth curves of Chlorella pyrenoidosa TX71105 and a gram-negative bacillus added to the culture as a bacterial contaminant (Vela and Guerra, 1966).

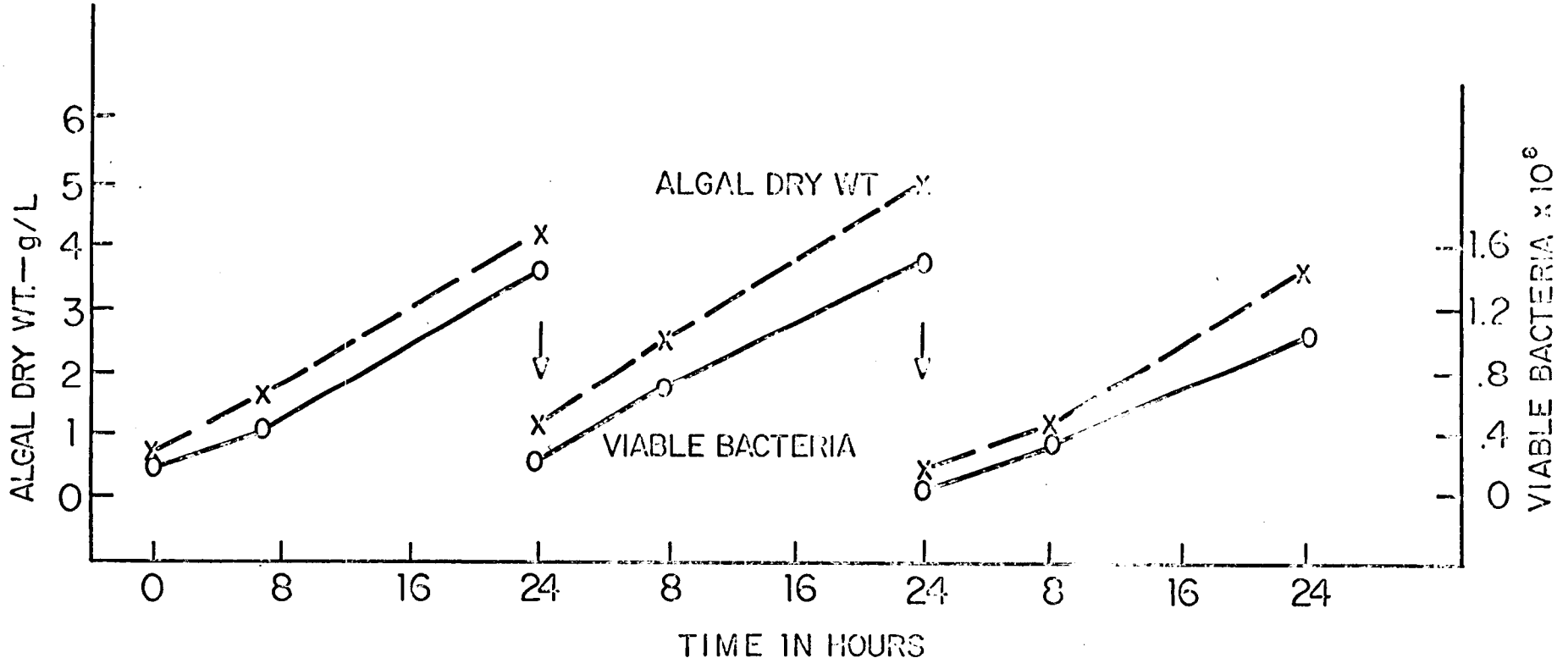

Figure 2. Growth of Chlorella pyrenoidosa TX71105 and selected bacteria in mass culture (Ward et al., 1964).

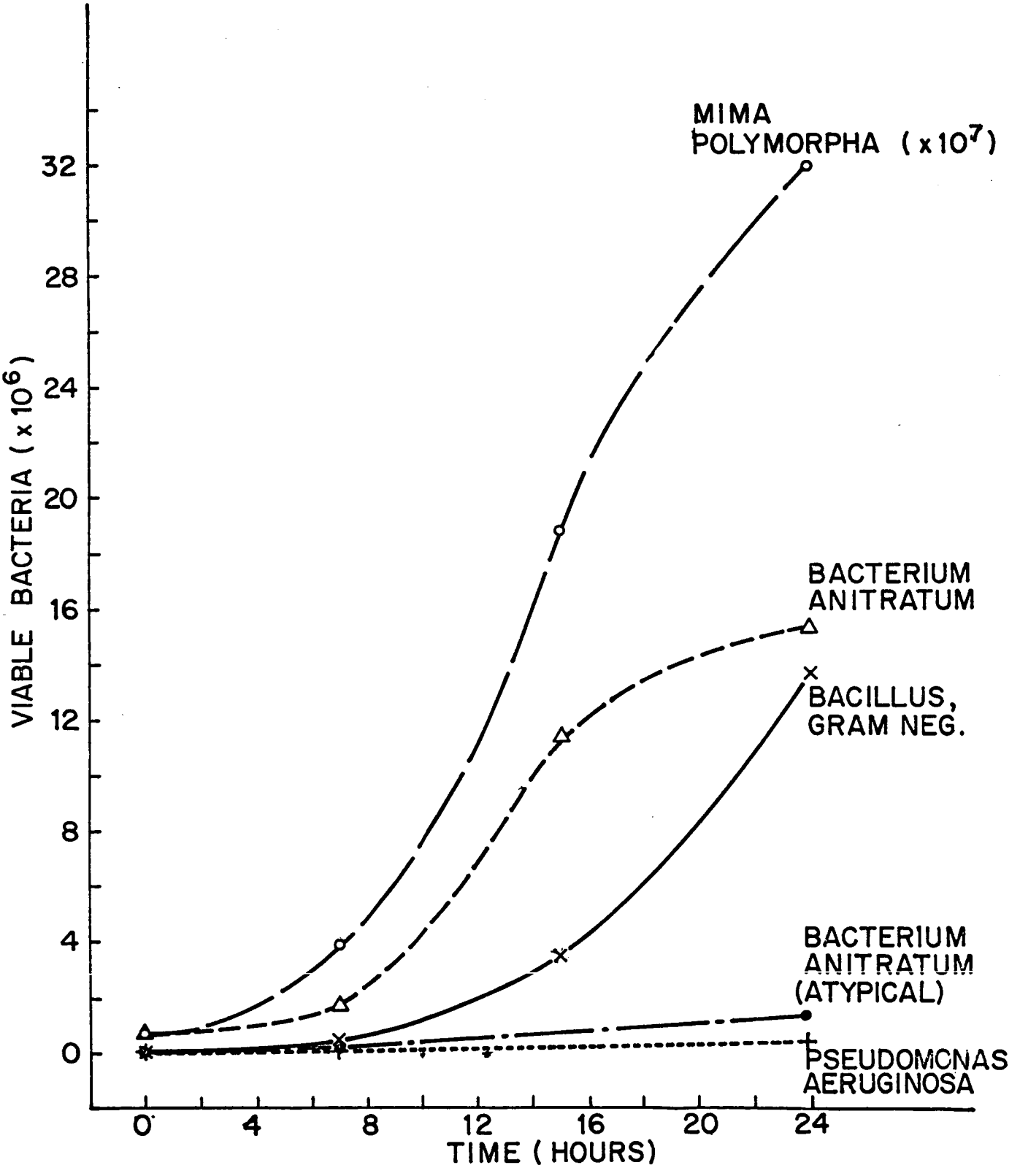

Figure 3. Growth of bacteria in algal cultures (Ward et al., 1964).

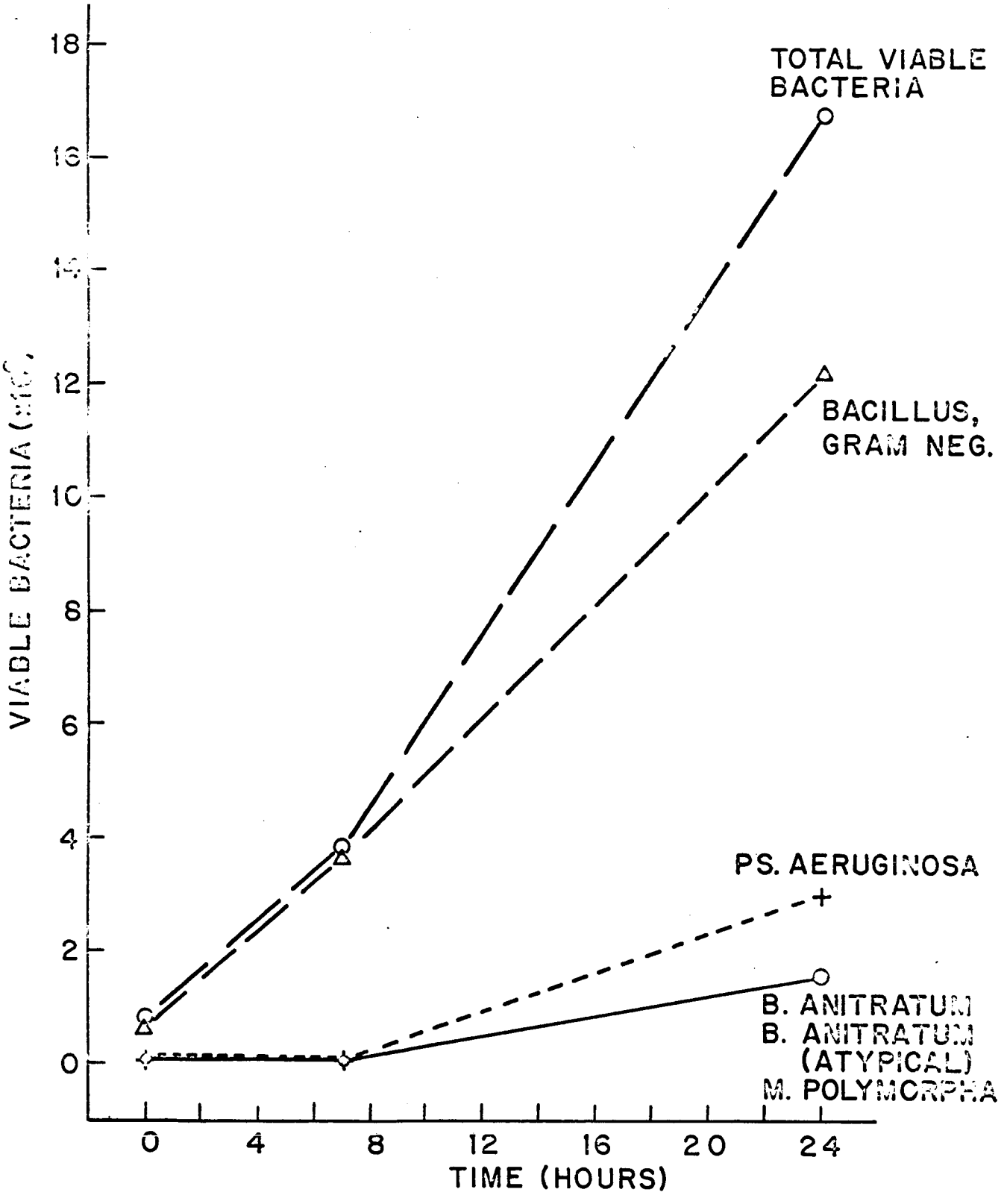

Figure 4. Concurrent growth of five bacteria in a culture of Chlorella pyrenoidosa TX71105 (Ward et al., 1964).

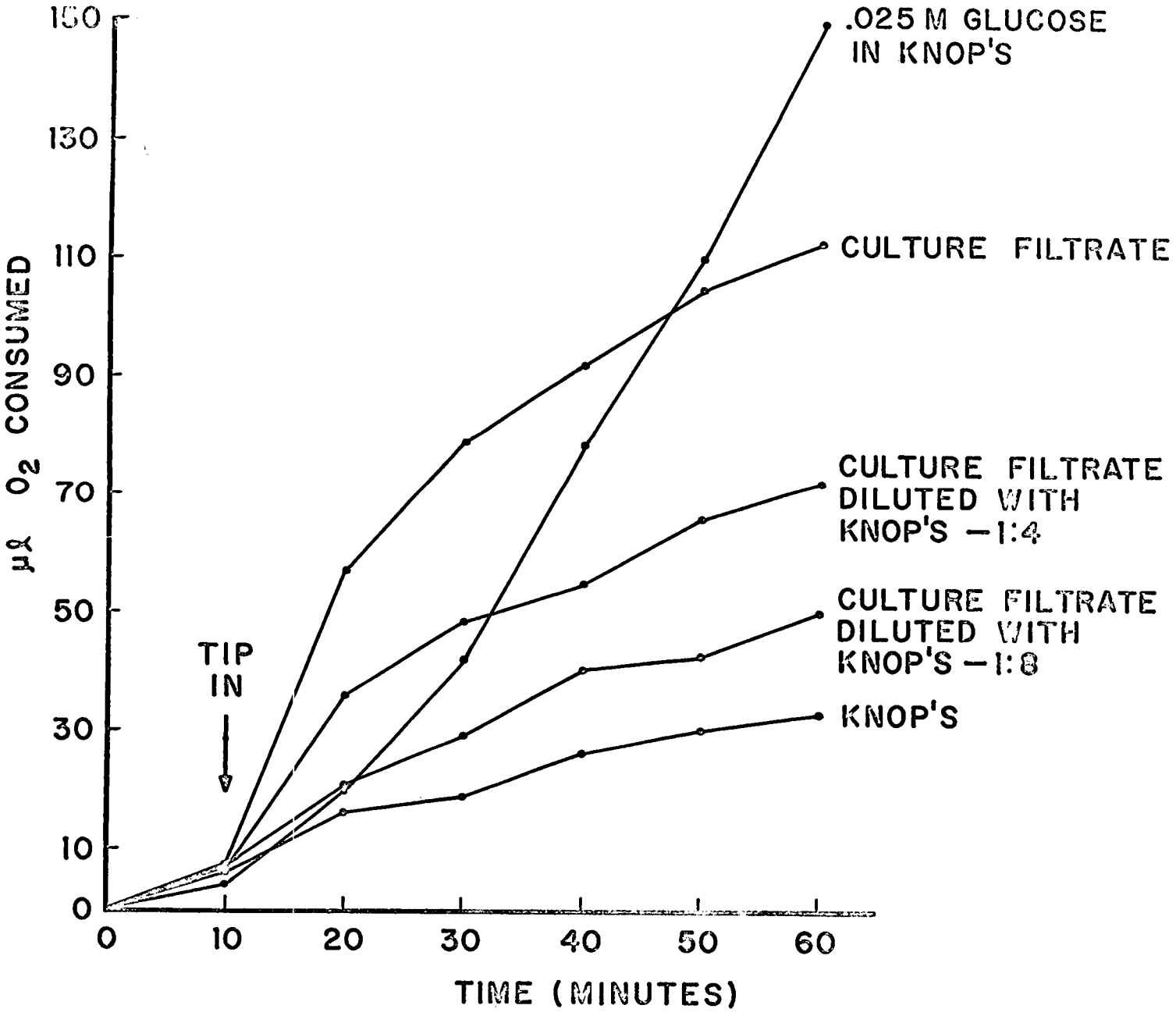

Figure 5. Oxidation of culture filtrate from axenic Chlorella pyrenoidosa TX71105 cultures by Pseudomonas aeruginosa (Ward et al., 1964).

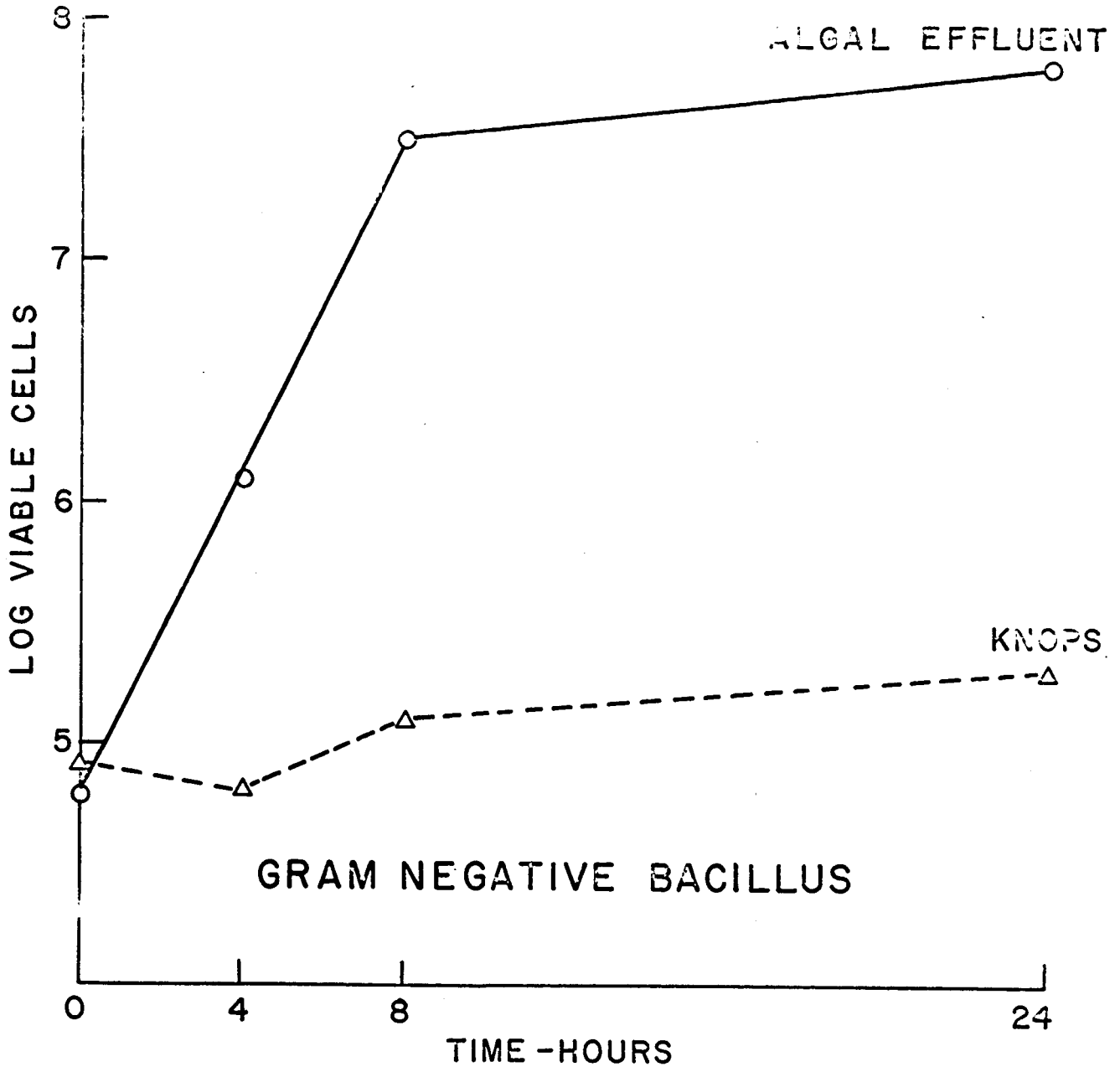

Figure 6. Growth of a gram negative bacillus in effluent Knop's solution from 24-hour axenic Chlorella pyrenoidosa TX71105 cultures.

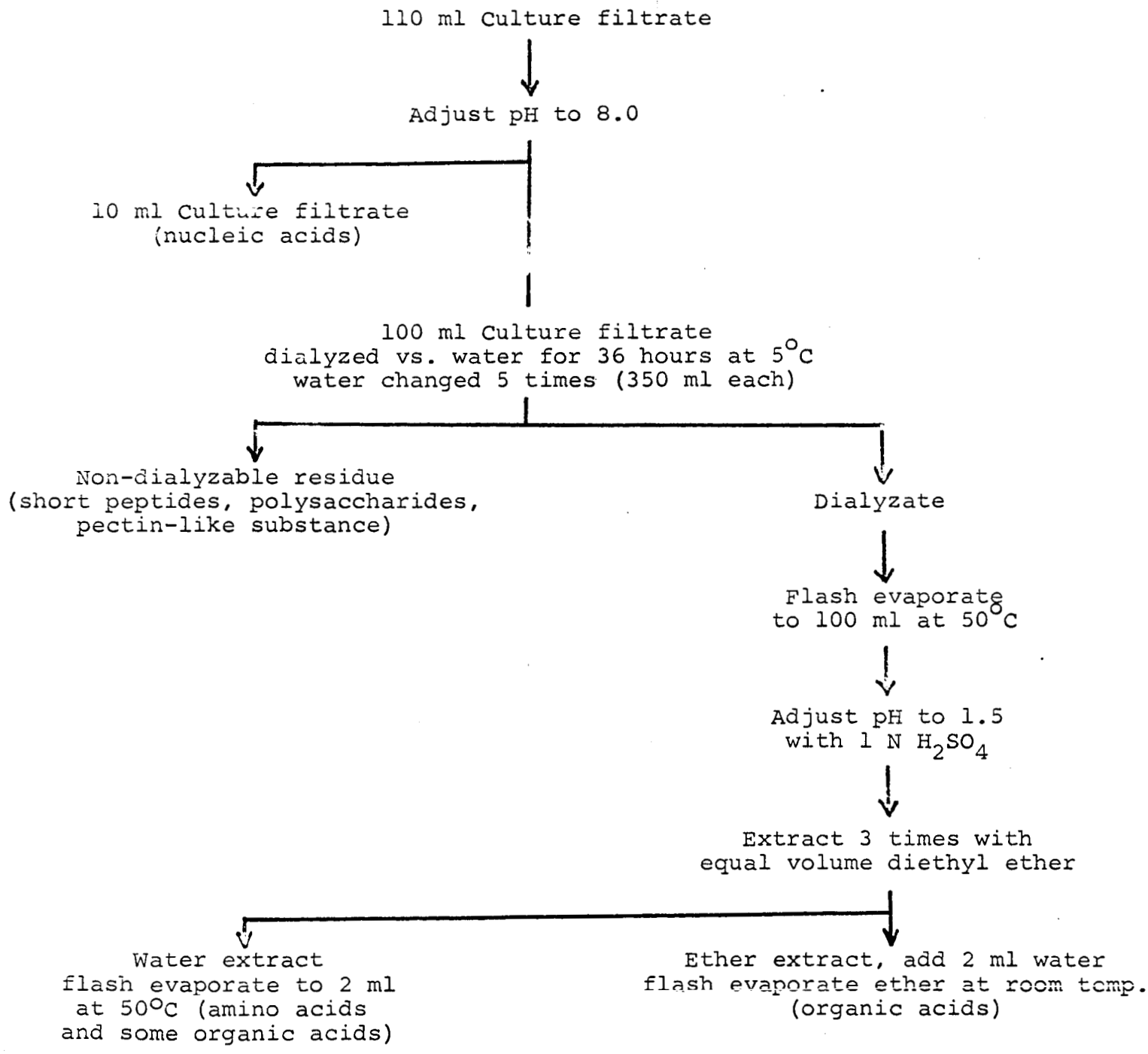

Figure 7. Fractionation scheme I.

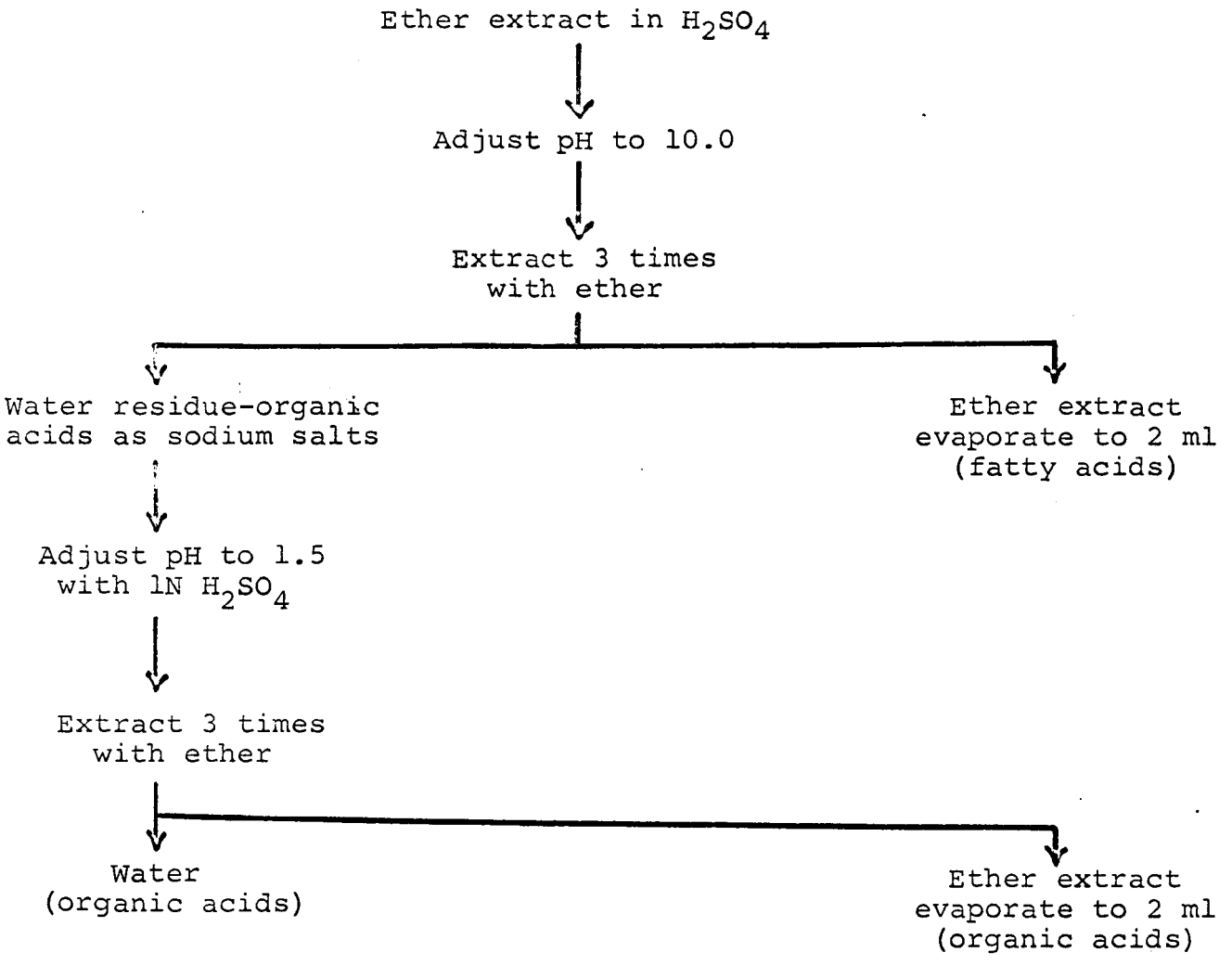

Figure 8. Fractionation scheme II.