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ALGAE SYMBIOSIS IN OXIDATION PONDS

II. Growth Characteristics of *Chlorella pyrenoidosa* Cultured in Sewage

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Oxidation ponds are widely used in the west and southwest United States as a simple and economical means of secondary sewage treatment. In the warmer climates, characteristic of this region, sewage in ponds decomposes rapidly, liberating principally ammonia and carbon dioxide. These compounds, together with adequate light, are the principal factors involved in the development of the green algae generally found in oxidation ponds. Among such green algae is *Chlorella pyrenoidosa,* a species frequently found near the pond outlet. *C. pyrenoidosa* is a well-known alga, widely used for laboratory experiments in photosynthesis and plant nutrition.

This paper is one of a series concerned with algae symbiosis in oxidation ponds. The first paper of the series (1) outlined the growth characteristics of a polysaprobic protozoan-like green alga, *Euglena gracilis,* when cultured in sewage. It introduced a number of relationships among the many variables influencing the complex biology of oxidation ponds. The application of these relationships to other algal species was unknown, and for this reason it was decided to repeat a major part of these experiments, utilizing a radically different alga. This paper deals with the growth characteristics of *C. pyrenoidosa* when cultured in sewage, and with the effects of the organism upon the sewage.

In the case of *E. gracilis,* properties of the cells (such as form, shape, size, density, color, rate of growth and photosynthesis, rate of respiration, vitality, and content of protein, carbohydrate, fat, ash, and various mineral ions) depend upon the environment to which the cells are exposed.

Cells exposed to favorable conditions (abundant nutrients and adequate light) are small, dark green in color, highly motile, rapidly growing and reproducing, contain a minimum of lipid, and photosynthesize much more oxygen than they respire. Cells of this type were designated as "young" cells. "Old" cells were described as those growing in an unfavorable environment; they are mostly in the palmellar state, are large and fat, yellowish in color, reproduce slowly, and may produce less oxygen than they require for respiration.

The studies with *Chlorella* were conducted in the same culture tubes as used for studying *Euglena.* These are 57-mm. pyrex tubes having a liquid volume of 1,200 ml., equipped with an internal cooling tube and peripheral 30-w. daylight fluorescent lights. Appurtenances permit feeding and withdrawal of liquid on a continuous basis and provide for bubbling air through the culture to maintain homogeneity. The assembly is designed to facilitate aseptic techniques in handling of the liquids.

During the series of tests herein reported the following factors were maintained at the following constant values: light, 1,200 fc.; temperature, 25° C.; air bubbling rate, 500 ml. per minute; B.O.D. of influent (5-day, 25° C., Warburg), 110 p.p.m. The single independent variable was the rate of application of sewage and equivalent withdrawal of the algal cell suspension. Since the total culture volume was held constant, the period that sewage was retained in the system was determined by the daily volume of sewage applied. This period has been designated as the retention period, R, defined as $R = V/v$, in which V is the culture volume in ml., v is the volume of feed or withdrawal in ml. per day, and R is expressed in days.

In performing any single test the retention period was held constant over the entire testing period, usually from 20 to 30 days. At the start of the test the 1,200-ml. tube was filled with sewage that had been inoculated with a pure culture of *C. pyrenoidosa* previously developed in sterile sewage in a separate culture flask. The content of the tube was then inoculated with a 10-ml. suspension containing approximately equal amounts of inoculum of 22 strains of sewage bacteria.* *Chlorella* populations in the growth units at the start were then about 0.1×10^6 cells per ml. and bacterial populations were about 1×10^6 colonies per ml. Daily tests showed that with $R > 3$, the algae entered a logarithmic phase of multiplication and increased to an equilibrium population of approximately 35×10^6 cells per ml. With $R < 3$, smaller equilibrium populations resulted. Under the regimen of continuous feeding and withdrawal, the equilibrium population for any condition may be maintained indefinitely. When daily control tests indicated that such equilibrium had been attained, the entire culture was sacrificed in order to have sufficient material for the numerous tests to be performed.

The retention periods investigated for *C. pyrenoidosa* varied from 0.5 to 8 days, and correspondingly the environment to which the algae were exposed varied progressively from very favorable (abundant nutrients) to very unfavorable (a shortage of one or more nutrients). Because the B.O.D. of the sewage was held constant at 110 p.p.m.† throughout the series, the B.O.D. loading is inversely proportional to the retention period. For example, at a retention period of 0.5 day, the B.O.D. loading is 220 mg. per liter per day; whereas at a retention period of 8 days the B.O.D. loading is about 14 mg. per liter per day.

Characteristics of *Chlorella* Cells

The several criteria previously developed (1), for evaluating the characteristic properties of *Euglena* cells in equilibrium cultures, were found to be applicable to *Chlorella* cells with certain exceptions.

1. Cell count, C. The number or population of cells per ml. of culture at equilibrium. A hyperbolic curve results when the cell counts are plotted as ordinates against elapsed time as abscissas, following the initial filling of the tube. Equilibrium is attained when the cell count, after increasing from its initial seed value, levels off at or near its maximum value. Equilibrium is usually reached, in the case of *Chlorella*, within a period of less than 10 days. A longer time is required to reach equilibrium when the retention period is shorter.

* These bacteria were originally isolated from sewage from three different cities—San Francisco, Richmond, and Concord, Calif. They are maintained in the laboratory by transfer to fresh sewage agar at two-week intervals. They include the following tentative and incomplete identifications: Nine strains of *Pseudomonas* sp., five strains of *Micrococcus* sp., and one strain each of *Bacillus subtilis*, *Alcaligenes faecalis*, *Escherichia coli*, *Flavobacterium* sp., *Streptococcus* sp., *Aerobacter* sp., *Proteus* sp., and *Neiserria* sp.

† The technique of preparing and maintaining a constant natural sewage is described elsewhere (2).

2. Cell shape. *C. pyrenoidosa* cells do not vary in shape but remain approximately spherical, whereas the *Euglena* cells alter from elongate to spherical. However, size changes and gross morphological variations do occur, as noted later.

3. Cell volume, V_m. The real volume, in cu. mm. of a million cells as determined by direct microscopic measurement. This measurement was undertaken for *E. gracilis* because cell shape had a decisive effect upon packing characteristics and altered the centrifuged volume. In the case of *Chlorella*, the measured volume and packed volume were found to be closely parallel, presumably due to the spherical nature of the cells. For this reason, real cell volume is not reported for *Chlorella*.

4. Packed volume, V_c. The centrifuged volume, in ml., of the cells contained in 1 l. of culture, after centrifuging at 500 *g*. The result is a function of the cell count and the cell volume.

5. Cell weight. The dry weight of cells in μg. (10^{-6}g.) per million cells.

6. Cell density. The dry weight of the cells, in mg. per cu. mm. of real volume.

7. Cell index, *I*. The packed volume, in cu. mm., of a million cells.

8. Cell color. This varies from dark green through yellow-green to yellow and brown, depending upon cell types and availability of nutrients. The intensity of green color depends primarily upon the chlorophyll content of the cells.

9. Chlorophyll content, Ch. The amount of chlorophyll, in mg. per mg. of dry cell material, or in μg. per million cells. Chlorophyll is measured spectrophotometrically in methanol solution by the methods of Mackinney (3) and calculated on the basis of the methods of Comar and Zscheile (4). Values reported are the sum of chlorophyll A and chlorophyll B. This measurement, not previously undertaken for *Euglena*, is now in routine use so that cell color changes may be systematically evaluated.

10. Cell types. Various cell types or forms of *Chlorella* cells are found, depending primarily upon cell "age." These forms, when plotted with ordinates superimposed, develop what may be termed a "morphological phase diagram." A similar diagram was developed for *Euglena* (1). The morphological phases for *Chlorella* are arbitrarily described as follows:

1. Unicell—Typical non-divided cell.
2. Giant cell—A "young" cell undivided.
3. Tetra cell—A giant cell showing four daughter cells either (a) within the old cell wall, or (b) newly separated from the old cell wall.
4. Diplo cell—A cell smaller than the giant cell and showing two daughter cells, either (a) within the old cell wall, or (b) newly separated from the old cell wall.
5. Degenerate cell—An old cell showing little chlorophyll content, large vacuoles, and many dark inclusion bodies.
6. Clumping cells—Cells showing a tendency to form palmellae.

11. Cell concentration, *D*. The oven-dry weight of the washed algal cells, expressed in mg. per liter of culture.

12. Yield, *Y*. The oven-dry weight of washed algal cells obtained daily, expressed in mg. per liter per day.

Growth Curves

Two growth curves for *C. pyrenoidosa* are shown in Figure 1. Diagram A shows the number of cells and the size of the cells in a batch culture as a function of time. Diagram B shows a cross-plot of the equilibrium populations and cell sizes in the continuous cultures as a function of the retention period.

Both curves show that the cells, initially young and large, become smallest near the time of maximum population. With increasing age the cells, on

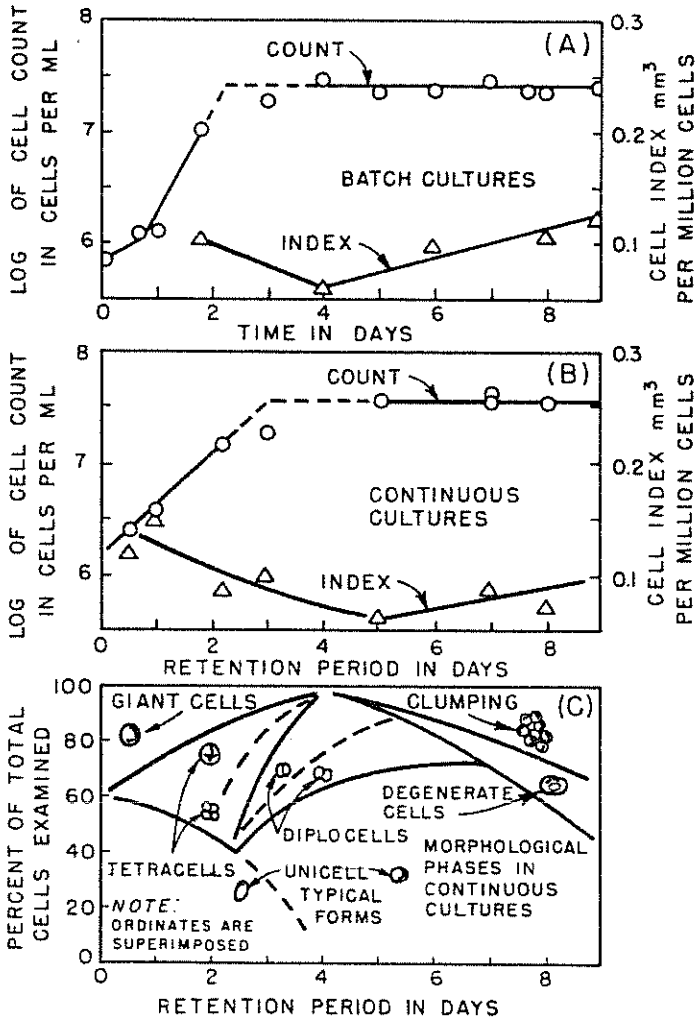

FIGURE 1.—Growth data for *C. pyrenoidosa* cultured in sewage.

the average, become larger and continue to enlarge throughout their aging period. Both curves also show a 2-day or 3-day period near the apex where cell division continues at the expense of cell size.

The similarity of the two curves is evidence that the retention period of continuous cultures represents the average age of the cells. Whether in batch or continuous culture, the individual algal cell may attain an age beyond which it can not divide, although it may continue to photosynthesize cell material and increase in size. This may come about through loss of ability to synthesize protein or through failure to obtain nitrogen in competition with younger cells. Diagram C of Figure 1 illustrates these morphological changes, which may be observed with the microscope.

Chlorophyll Content of the Cells

Figure 2A shows that chlorophyll content of the individual cell falls off rapidly as retention period increases. This indicates that the chlorophyll is broken down or inactivated as the cells become older, or else the older cells upon division contributed less chlorophyll to their daughter cells. Evidence sustaining the former possibility is indicated by the authors' recent unpublished work on the effect of light on algae growing in a sewage medium. With nutritional conditions remaining constant, the amount of chlorophyll present in individual cells is found to decrease as the amount of light avail-

able increased, whereas the rate of synthesis first increases and then decreases. Evidently chlorophyll is broken down in more highly illuminated, and older, cultures to a greater extent than in younger, less illuminated cultures. However, in even the oldest continuous cultures, as shown in Diagram C of Figure 1, a percentage of the cells remain young and retain the capacity to multiply. The burden of new cell synthesis may fall upon a diminishing percentage of cells as the average culture age increases.

The effect of the nutrition factor on chlorophyll production is shown by Figure 2B, which shows the chlorophyll content of the cell, in percentage of its total dry weight, as a function of the amount of applied food material. In the range where nutrition is limiting (that is, less than about 50 mg. per liter per day), where $R > 2.2$ days, the percentage of chlorophyll decreases as nutrition becomes more limited.

Chlorophyll Content and Synthesizing Ability

The relationship between cell chlorophyll content and cell synthesizing ability may be expressed as the ratio, mg. of cell material synthesized per day per mg. of chlorophyll present. In the range of limited nutrition (that is, where the B.O.D. loading is less than 50 mg. per liter per day), this ratio remains approximately constant at a value of 25.

At nutrient loadings greater than 50 mg. per liter per day, the ratio rapidly increases. This may be due to the greater light penetration attained at short retention periods, as a result of

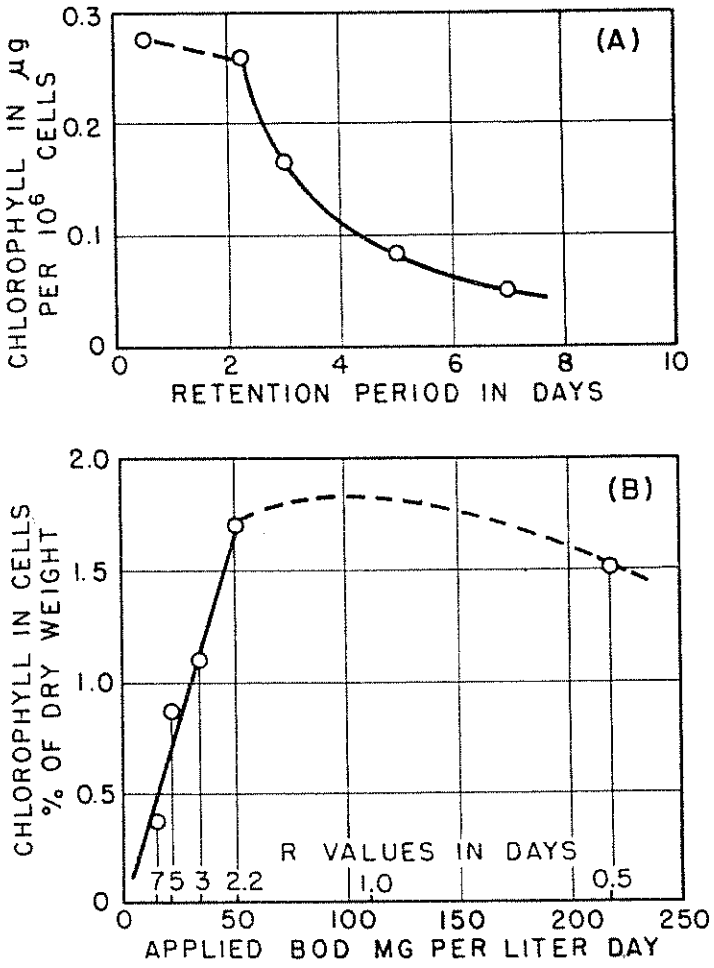

FIGURE 2.—Production of chlorophyll A + B at lighting intensity of 1,200 foot-candles.

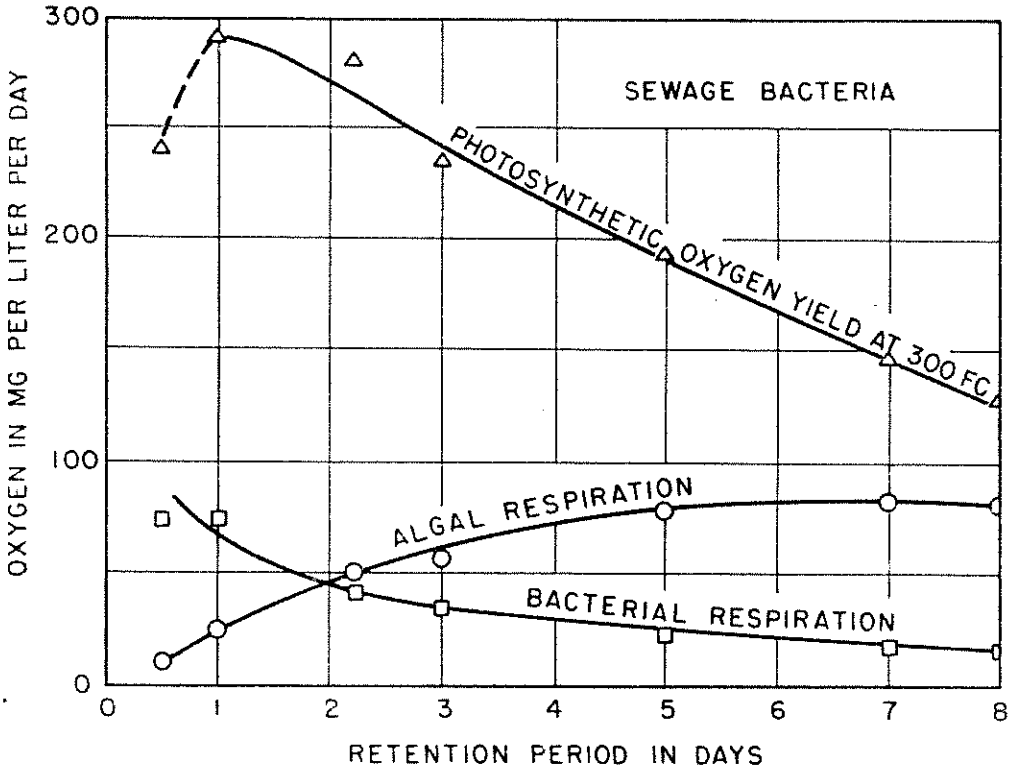

FIGURE 3.—Oxygen relationships for *Chlorella* cultured in sewage.

the marked decrease in density and turbidity of the culture.

Oxygen Production

In accordance with the simplified photosynthetic equation—

$$2\ CO_2 + 2\ H_2O \xrightarrow{\text{Light}} 2\ (CH_2O) + O_2$$

—the production of cell material is accompanied by a stoichiometric production of oxygen. The generally accepted pattern of this reaction is that oxygen is derived from the photochemical decomposition of the water molecule, rather than from a splitting of the CO_2 molecule. The CO_2 molecule serves as a hydrogen acceptor within the algal cell, resulting in production of a substance of the general formula CH_2O, which may be termed "primary cell material." The oxygen is liberated outside the cell and may then serve as the ultimate hydrogen acceptor for bacterial respiration in the aerobic decomposition of sewage. Thus, water serves as a hydrogen donor for the reduction of CO_2 in algal photosynthesis, and the liberation of oxygen is incidental to the process.

The oxygen liberated by *Chlorella* under varying B.O.D. loadings, under the experimental conditions, is shown in Figure 3. The curve of oxygen production reaches a maximum at a retention period between one and two days, and is similar in pattern to the curve showing cell yield (Figure 5). These oxygen production values were calculated on the basis of Warburg respirometer tests of *Chlorella* cells under illumination of approximately 300 foot-candles, as previously outlined (1). From these data the gross oxygen yield is computed, allowing for respiration both of the algae and bacteria in the system. This large excess of oxygen is in agreement with earlier findings for *E. gracilis*, which, under continuous lighting, was found to liberate oxygen in excess of the respiratory requirement of all organisms in the system up to a retention period of 16 days. However, due to the higher rate of growth, and perhaps due to its smaller size, *Chlorella* produces an excess of

oxygen greater than that produced by *Euglena*.

On the basis of chemical analysis of the cell material, using the method described by Myers (5), the ratio of CO_2/O_2 for *Chlorella* grown under continuous lighting in sewage is found to be -0.75. This value is in agreement with that reported by Myers for similar conditions. Utilizing this ratio and the previously noted chlorophyll determinations, calculations show that 66 mg. of oxygen per day may be produced by that weight of *Chlorella* which contains 1 mg. of chlorophyll at a light intensity of 1,200 foot-candles. This finding agrees with initial rates obtained in the Warburg respirometer at 300 foot-candles, and indicates that *Chlorella* is an excellent oxygen producer when grown in sewage under continuous lighting, particularly when an abundance of nutrients is supplied. It may also indicate that little added yield is obtained by increasing light intensity from 300 fc. to 1,200 fc.

Symbiotic Sewage Treatment

The liberation of large amounts of oxygen by *Chlorella* over the entire range of B.O.D. loadings studied, suggests that a high degree of sewage treatment (that is, bacteriological stabilization) may be induced by this liberated oxygen. Figure 4 compares the B.O.D. of the influent sewage with that of the culture, and of the algae-free culture supernatant. Despite the availability of oxygen in the culture, the B.O.D. of the clear supernatant is high compared to that obtained for *Euglena* supernatants. The B.O.D. of the whole effluent is similarly higher than for *Euglena*. It is much higher than that of the influent sewage for all but the shortest retention periods.

In support of these findings, bacteriological examination of the mixed liquor from the cultures showed a low bacterial population compared to bacterial populations attained in similar systems in the absence of algae and in systems containing *E. gracilis*, as follows:

Bacteria Recovered at Equilibrium Conditions

System	Colonies per ml.
Sewage plus bacteria	1×10^8
Sewage plus bacteria plus *Euglena*	5×10^7
Sewage plus bacteria plus *Chlorella*	1×10^6

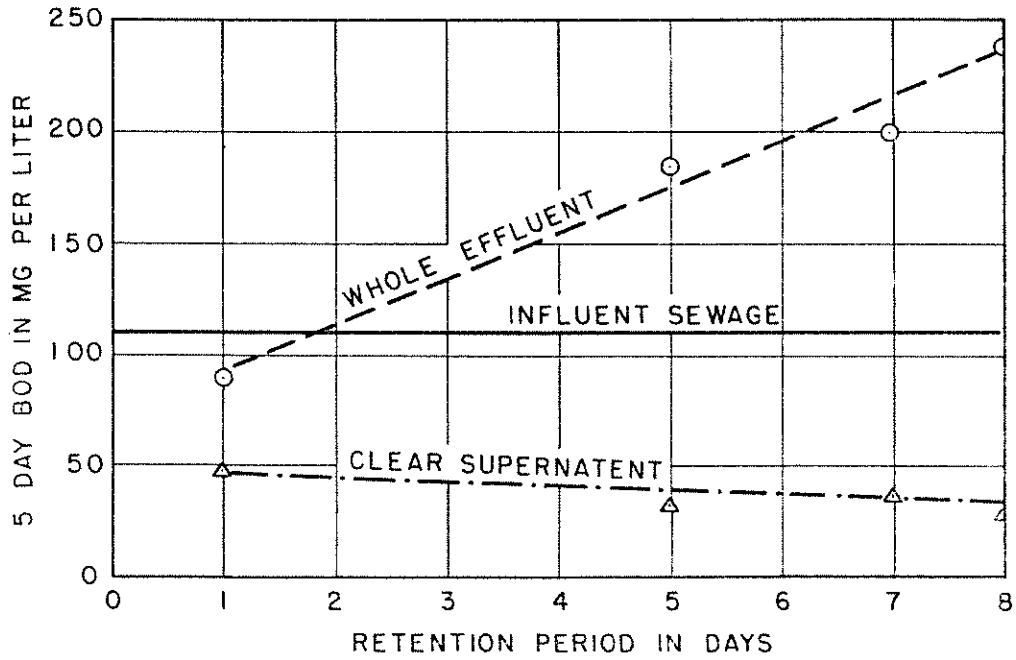

FIGURE 4.—Comparison of B.O.D. of influent sewage with B.O.D. of *Chlorella* culture and of culture supernatant.

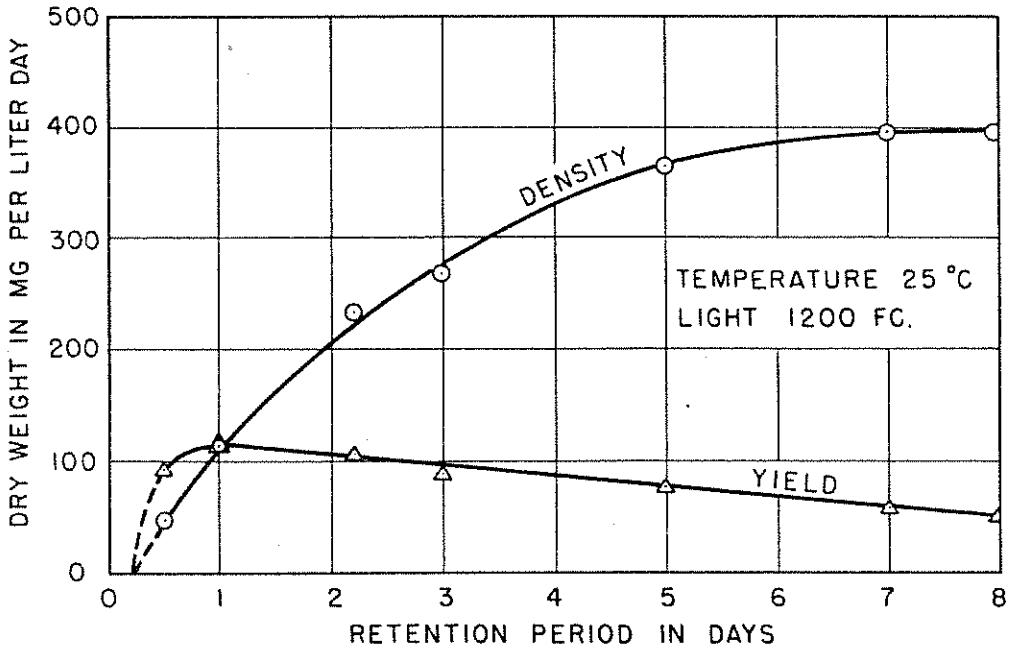

FIGURE 5.—Yield and density of *Chlorella* cultured in sewage.

Similar findings have previously been reported (6)(7). This evidence indicates that some factor inhibits bacterial action upon the sewage substrate while the *Chlorella* cells are present. The fact that the supernatant exerts a relatively high B.O.D. suggests that bacterial growth is less inhibited following the physical removal of the *Chlorella* cells. As previously noted, comparable supernatants from *Euglena* cultures had lower B.O.D. values, ranging from 10 to 20 mg. per liter.

In another series of tests, *Chlorella* was found to grow more vigorously and give higher yields of cell material in sterile sewage than it did when bacteria were present.

Yield of Algal Cell Material

Figure 5 shows the manner in which the yield of algal cell material increases with increased B.O.D. loading (reduced retention period). These data correspond closely to the data presented for *Chlorella* by Cook (8), differing only in the lower magnitude of the yield. In Cook's studies an extra supply of CO_2 was made available to the algae by fortifying the CO_2 content of the bubbling air to 5 per cent.

Calculations based upon algal cell analyses and sewage analyses indicate that the principal factor limiting increased yield at long retention periods, or low B.O.D. loadings, is a lack of carbon. The amounts of various nutrient elements made available when the B.O.D. loading is 15 mg. per liter per day are compared with the amounts required in the following:

	Nutrient (mg./l./day)					
	C	N	P	K	Ca	Mg
Available in sewage	13.2	3.00	1.00	1.90	3.00	1.10
Required for *C. pyrenoidosa*	24.0[1]	1.42	0.62	0.63	0.90	0.14

[1] 10.8 mg. supplied from bubbling air.

E. gracilis gave yields comparable to those of *Chlorella*, in spite of its slower growth rate.

Figure 5 shows that as the retention period is increased the density of the culture, expressed in mg. of cell material per liter of culture, increases continuously. This increase in density results in a reduction of the light entering the culture. Also, it results in a high B.O.D. load upon the system, due

TABLE I.—Comparative Analyses of *Euglena* and *Chlorella* Cultured in Sewage

Algae	Dry Weight (mg./l.)	Per Cent of Dry Weight							C/N
		C	H	N	P	K	Ca	Mg	
E. gracilis	368	49.	6.4	4.0	1.9	0.38	1.5	0.30	11
C. pyrenoidosa	390	49.	7.0	2.9	1.3	1.3	1.8	0.29	17

to the increasing respiratory requirements of the algal cells.

Composition of Cell Material

The comparative analyses of *E. gracilis* and *C. pyrenoidosa* (Table I) indicate that sewage-cultured *Chlorella* and *Euglena* are of similar composition. They differ mainly in carbon and nitrogen content; that is, in the C/N ratio. The analyses of Table I are for algae grown at a retention period of 8 days. This retention period is slightly above optimum for *Euglena,* but is greatly above optimum for *Chlorella.* The result is a higher C/N ratio for *Chlorella,* indicating storage of carbohydrate in the older cells. Since the culture density, *D,* continuously increases, whereas the population remains constant, the density of the individual cells increases with age, presumably because of storage of carbohydrates.

Carbon Balance

When grown experimentally in an inorganic medium, *Chlorella* is often supplied with air containing 5 per cent

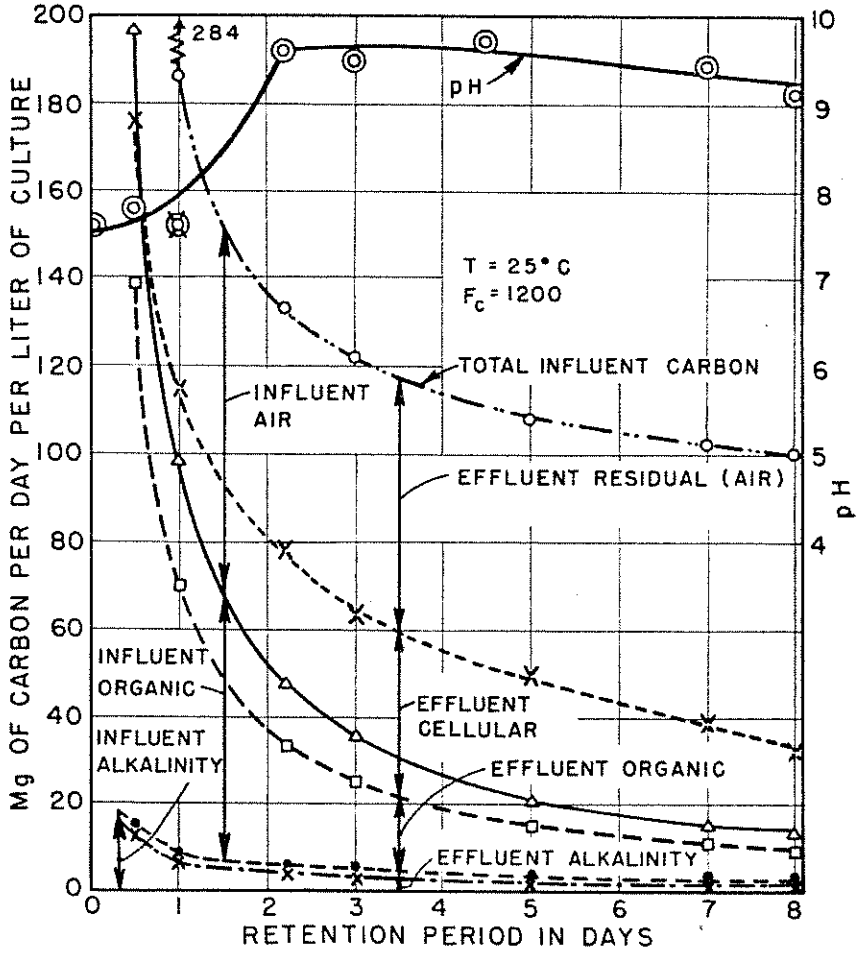

FIGURE 6.—Carbon balance for *Chlorella* cultured in sewage.

CO_2. This results in yields high above those obtainable with sewage and plain air. Probably higher yields of *Chlorella* in sewage could occur if more carbon were available from bacterial decomposition of the organic matter; but, as previously noted, bacterial examinations and B.O.D. determinations on the supernatant indicate that *Chlorella* inhibits bacterial decomposition of the sewage, thereby imposing a self-limiting factor upon its own growth. In the tests reported, only two other sources of carbon were available to *Chlorella*—the carbon contained in the alkalinity of the sewage and in the air employed for bubbling. The utilization of either of these carbon sources does not contribute to sewage treatment. Figure 6 shows the sources of carbon and its fate during the tests, as a function of retention period. The greatest source of carbon is from bubbling air. Reduction in alkalinity accounts for only a small fraction of the total carbon utilized. It is probable that *Chlorella* utilizes carbon sources (sugars) other than CO_2. The direct use of carbon sources independent of bacterial activity may partially account for the fair degree of sewage treatment obtained even though bacterial growth is inhibited.

Figure 6 includes a curve showing the pH of the culture at a varying retention period. With increasing retention period, up to $R = 2.2$, the pH of the culture increases, to a maximum of 9.6. Evidently the amount of carbon dioxide abstracted from the sewage alkalinity increases with retention period, with a corresponding increasing release of hydroxyl ions, up to $R = 2.2$ days. The reduction in alkalinity which also occurs is not associated with the abstraction of carbon, as explained previously (1).

Conclusions

Algae cells growing together with bacteria in laboratory culture units, with temperature and lighting constant, are greatly affected by the rate of application of nutrient sewage. At high rates of sewage application, corresponding to short retention periods, the cells are young, vigorously multiply, and photosynthesize abundant quantities of oxygen and cell material. As the retention period is increased, or the rate of loading decreased, the cells tend to remain in a vigorous multiplying state at the expense of cell size, but eventually at longer retention periods an increasing percentage of the cells cease to multiply and enter a phase of cell enlargement that leads ultimately to senescence and death. Senescent algae are yellow, low in chlorophyll, multiply infrequently, and may photosynthesize less oxygen than they consume in respiration. In the case of *Chlorella*, autolysis of older cells may serve as a meager source of nutrition for new cells, but their ultimate death leads to putrefactive decomposition and to effluent B.O.D. magnitudes far exceeding those of the influent sewage.

A continuous culture at some particular retention period resembles in many ways a batch culture having an age equal to that retention period. However, in batch cultures the organisms are in a changing nutritional environment, whereas in continuous cultures at equilibrium the environment is constant. For this reason the continuous cultures furnish experimental material for study of algal physiology in quantities that can be made available in no other way. By relating such laboratory findings to the natural phenomena in lakes, reservoirs, and ponds, where the sources of nutrients vary with the seasons, the reasons for sudden occurrence and death of algae may become apparent. For example, if nutrient materials enter the water body at some particular time, but no subsequent sources of nutrient are made available, bloom may occur and then be followed by senescence, death, and putrefaction.

Both *Chlorella* and *Euglena* have

been found to photosynthesize rapidly in a rich nutritional environment. Both tend to overextend their populations at the expense of parent cell material, and both pass through a microscopically observable series of morphological phases that remain remarkably constant when nutritional conditions are constant. Both algae enter a senescent phase that is marked by reduced photosynthetic capacity, yellow color, reduced chlorophyll content, and increased tendency toward cell enlargement with accompanying higher respiration rates. In both cases, if the cells are removed from the mixed liquor after growth, the supernatant has a low B.O.D. that appears to be essentially independent of retention period.

These similarities are offset by the following differences: *Chlorella* grows more rapidly than *Euglena,* entering the various phases at lower retention periods. *Chlorella* tends to autotrophic nutrition, and may decidedly inhibit the saprophytic bacteria necessary to the first-stage decomposition of sewage. By inhibiting such bacterial action, *Chlorella* may limit the carbon and nitrogen made available for its continued growth. Also, there is some evidence indicating that certain bacteria may inhibit the *Chlorella* itself. *Euglena* on the other hand, being a facultative saprophyte, appeared to grow better in the presence of sewage bacteria than in sterile sewage. There is no evidence of specific bacterial inhibition by *Euglena gracilis* (7).

In spite of its slower rate of growth *Euglena* compares favorably with *Chlorella* in the yield of cell material when grown in sewage. Cell-free supernatants from *Euglena* cultures are lower in B.O.D. than are *Chlorella* supernatants, and the whole effluents from *Chlorella* cultures show a high B.O.D. as compared to *Euglena* whole effluents.

Summary

Chlorella pyrenoidosa has been grown in continuous culture on preserved sewage in the presence of sewage bacteria in continuous light and at constant temperature. The morphology and physiology of this alga are greatly affected by the rate of sewage application. Symbiotic activity is reduced because of bacterial inhibition by *Chlorella.* The sewage treatment afforded by *Chlorella* and by the bacteria not inhibited by its presence is satisfactory but poor when compared with treatment obtained with *Euglena gracilis.* The results of the present experiments with *Chlorella pyrenoidosa* are compared to those previously reported for *Euglena gracilis* and are found to be generally comparable.

Acknowledgments

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