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Algal Culture Studies Related to a
Closed Ecological Life Support System (CELSS)

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I. INTRGDUCTION

In many respects, algae would be ideal plant components for a biologically-based closed life support system, since they are eminently suited to the closely coupled functions of food production and atmosphere regeneration. This idea was clearly recognized by an earlier generation of scientists (see, e.g., Bioregenerative Systems, NASA SP-165, 1968). A similar program is being carried out in the USSR (Gitel'son, I., et al., Problems of Space Biology, Vol. 28, Experimental Ecological Systems Including Man, NASA Technical Translation F-16993, 1975).

Work carried out during the course of our CELSS project was devoted to several aspects of the (steady-state) continuous culture of algae. Our efforts have been primarily devoted to the culture of Scenedesmus obliquus, a physiologically well-characterized green alga with good growth characteristics, and Spirulina platensis, a nutritionally well-characterized blue-green alga with less favorable growth characteristics.* Because of the differences in culturability of these two organisms, we have been using Scenedesmus as a model and control for the culture of the (possibly) more valuable Spirulina. In the following sections, we describe the results obtained in our CELSS-related studies. A formal description of some of this work has been prepared (see Appendix II).

*We have also successfully maintained several other organisms of interest in continuous culture, namely:

1. Chlorella vulgaris, an alga closely related to Scenedesmus, but which does not display rapid light-driven O_2 reduction, and thus may dissipate NO_3^- as N_2O .
2. Chlorella sorokiniana, an alga studied earlier under NASA auspices by R. Krauss et al. This alga has a very high growth rate, but is reported to volatilize large amounts of NO_3^- .
3. Anacystis nidulans, the only efficiently transformable alga currently available. (Genetic transformation, i.e., molecular gene transfer, provides an efficient means to incorporate desirable traits into an organism.)

II. DESCRIPTION OF CULTURE APPARATUSES

The three systems currently used to maintain continuous cultures of algae are shown in Figs. 1-3. All of these systems operate as turbidostats. Thus, in order for these systems to function properly, the algal culture must be homogeneous, so that a constant beam of light reaches the light detector. The three systems differ in geometry as well as in various other parameters. The characteristics of each apparatus with respect to light, gas dispersion, contamination, volume, density, temperature, and filament aggregation are shown in Table I.

Each of these systems has its advantages. The reaction vessel seems to be the most effective system for growing cultures of Spirulina platensis (see below). The large airlift is by far the best apparatus for measuring light efficiency.

Because of its unusual and distinct morphology, Spirulina has proven more difficult to culture than algae that are unicellular or form very small colonies. Spirulina tends to clump during culture; the antidote, rapid stirring and high gas feed, results in foaming. The design and construction of the culture system shown in Fig. 3 was a direct response to problems encountered during our Spirulina culture experiments. We have found that the most effective means of maintaining homogeneous cultures with this organism is a combination of adequate agitation and a NaCl concentration of > 2 g/L. (The most widely used medium contains a concentration of 1 g/L NaCl, which allows aggregation to occur.) We should note that the tendency of Spirulina to clump is not without its advantages; this alga can be readily harvested from a culture by merely lowering the ionic strength by dilution.

Our earlier studies used the culture system shown in Fig. 4a (exploded) and Fig. 4b (assembled). As is evident from Fig. 4a, the system is of modular construction for easy maintenance and repair. Components in contact with the algae are made of machinable polycarbonate plastic or Viton, both of which can be sterilized by autoclaving or by rinsing with ethanol. According to the results of tests using Scenedesmus and Chlorella, neither material caused toxic or other undesirable effects (e.g., cell adhesion). These results

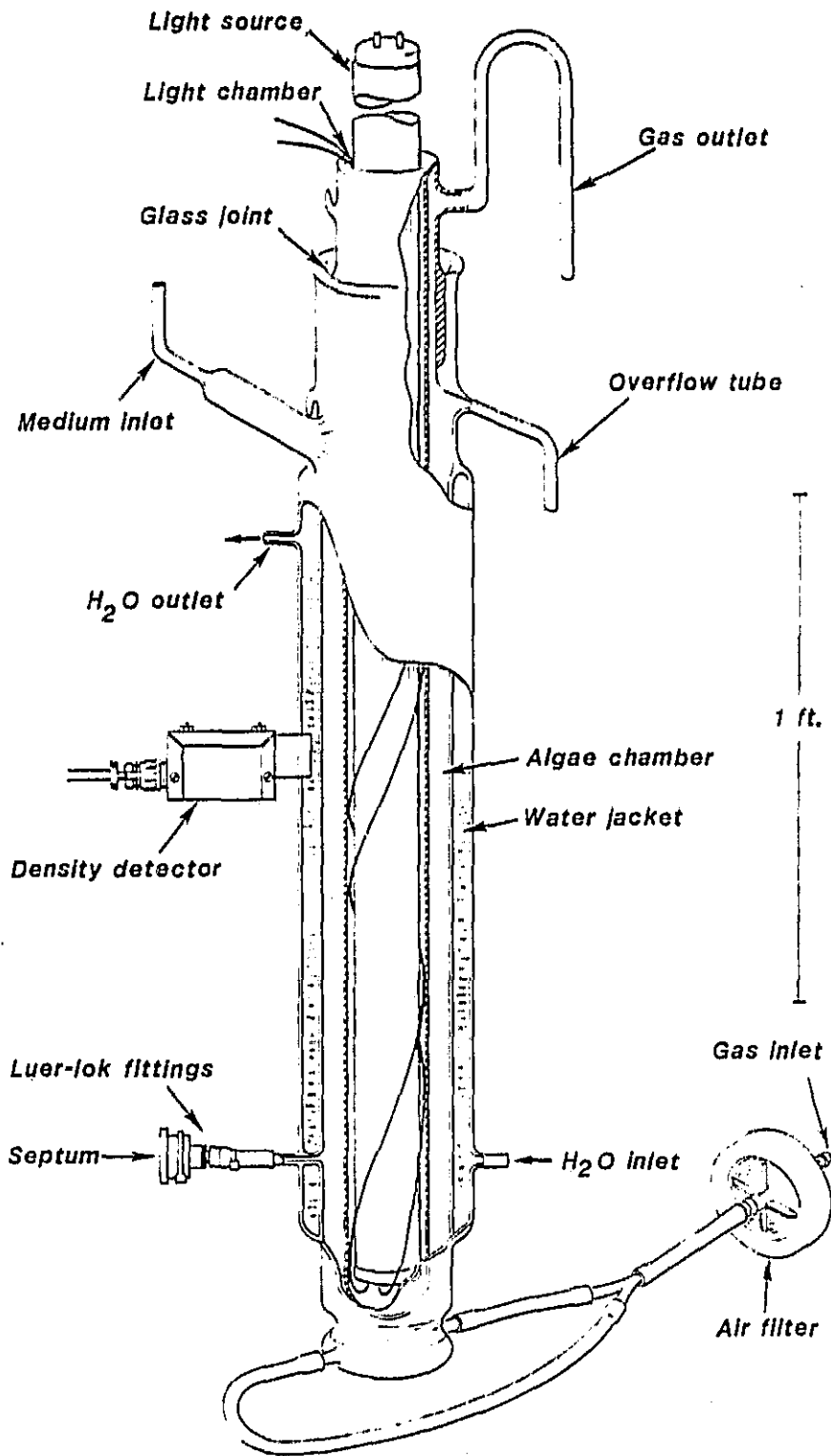


Figure 1. "Large Airlift" continuous culture apparatus

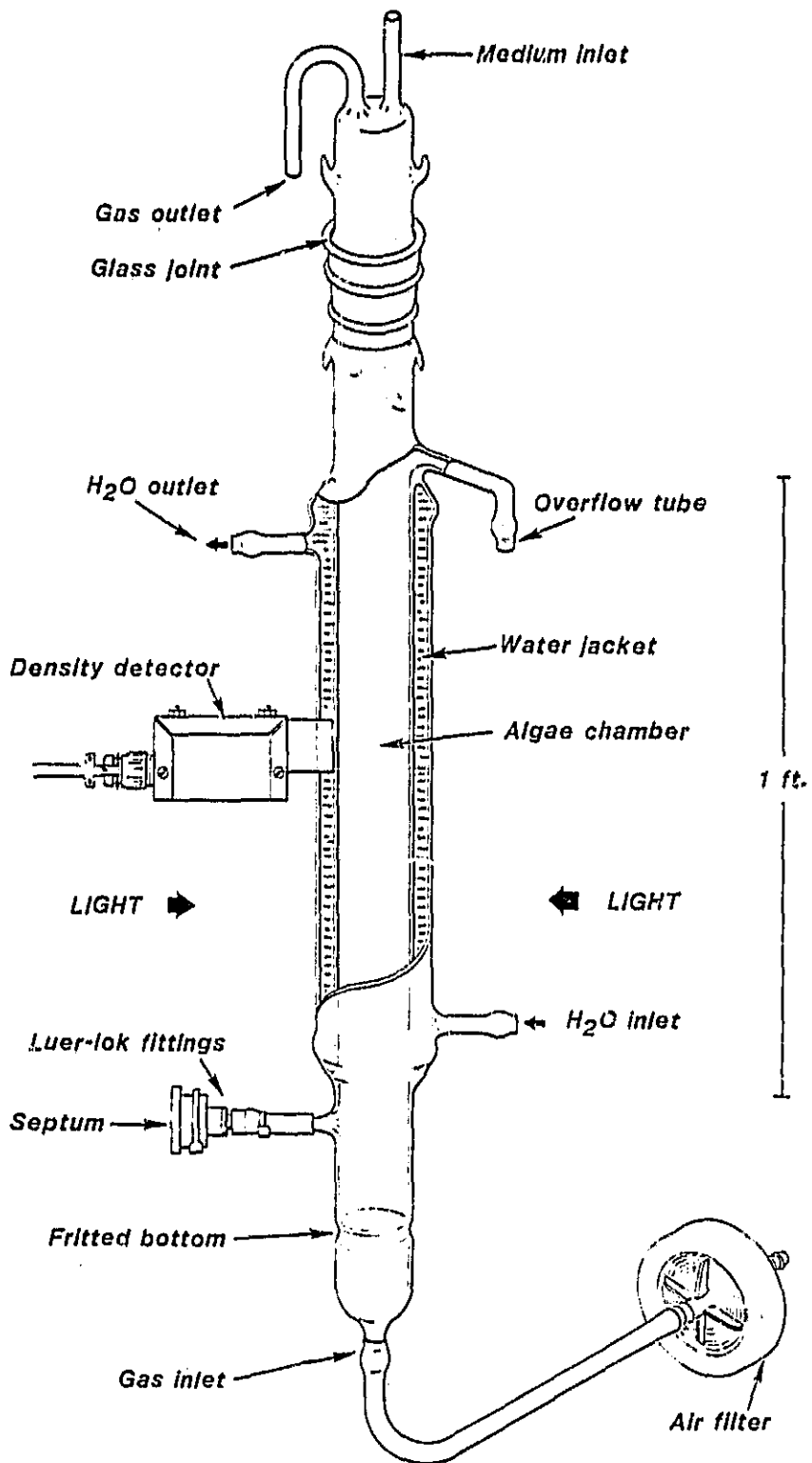


Figure 2. "Small Airlift" continuous culture apparatus

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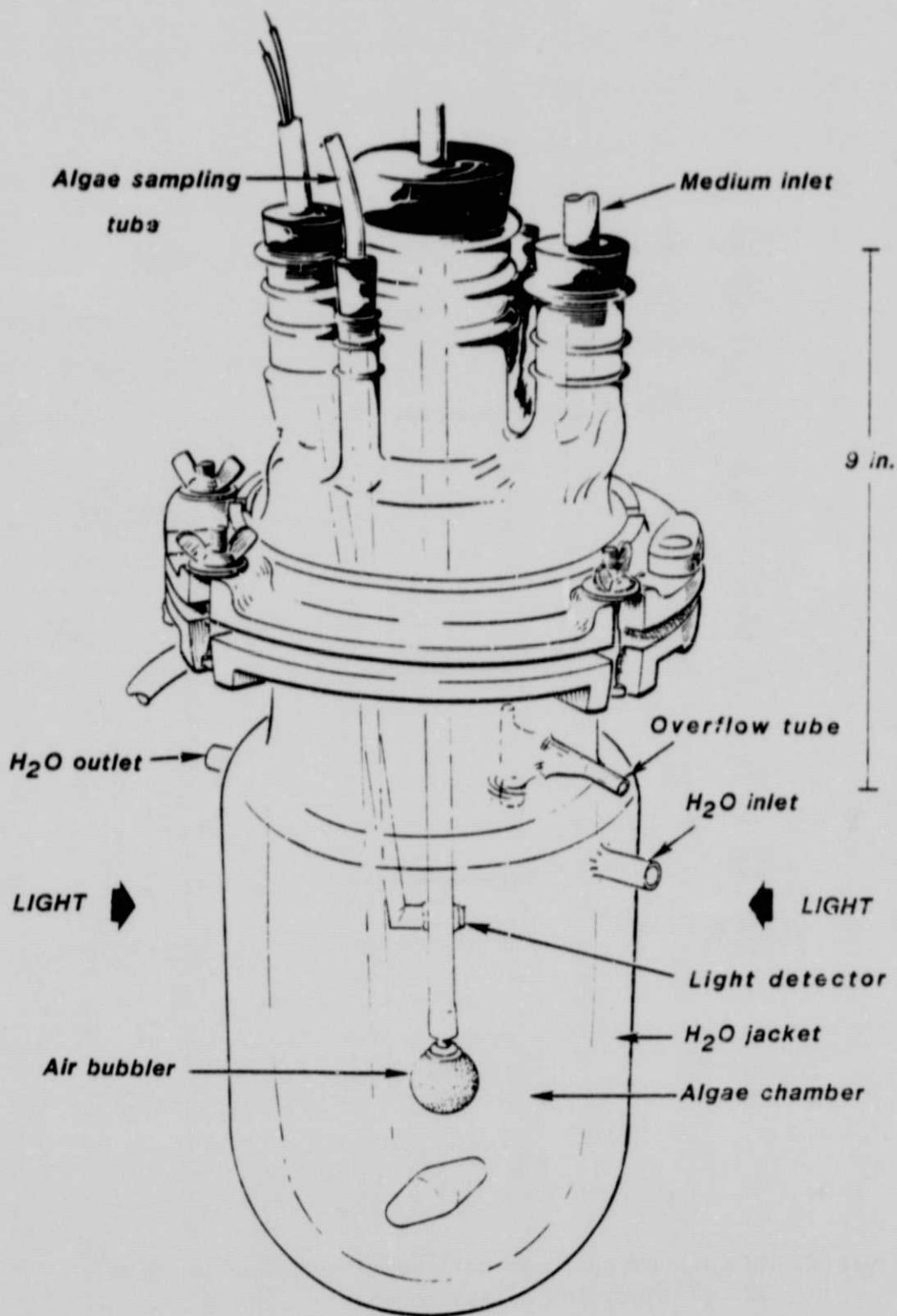


Figure 3. "Reaction Vessel" continuous culture apparatus

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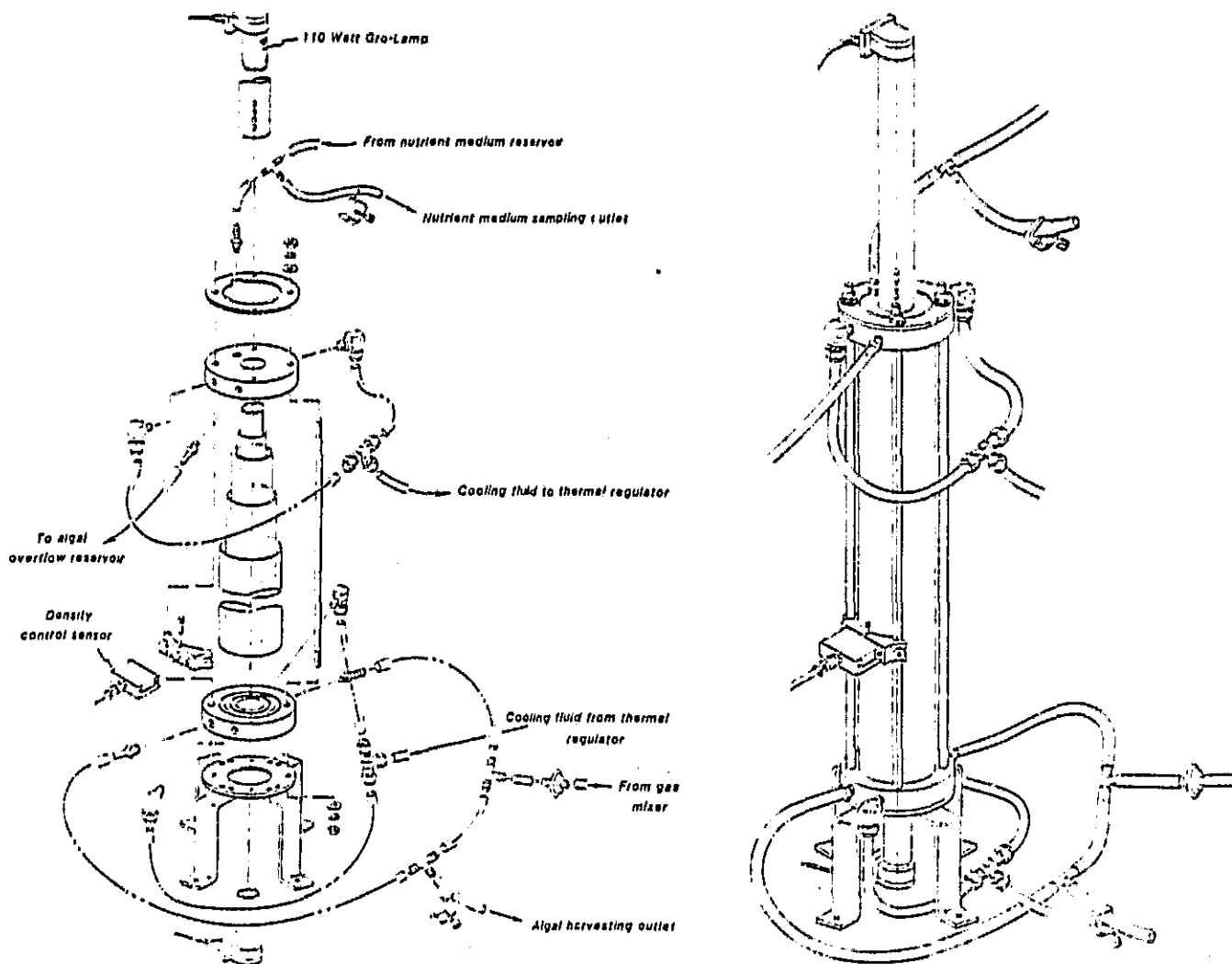


Figure 4. Diagram of constant cell density apparatus (CCDA):
a) exploded, and b) assembled.

Table I

Culture Maintenance Characteristics of
the Three Continuous-Culture Apparatuses

| | Large Airlift (Fig. 1) | Small Airlift (Fig. 2) | Reaction Vessel (Fig. 3) |
|-------------------------|--|--|--------------------------------|
| Light | Easily measurable | Difficult to measure | Difficult to measure |
| Gases and Frit Clogging | Good dispersion but some clogging on CO ₂ may occur | Good dispersion but clogging on CO ₂ occurs | Poor dispersion No clogging |
| Filament Aggregation | Some clumping | Continual | Minimal |
| Contamination | None Autoclaved due to detector | None | Cannot be |
| Volume | ≈ 11 with stir bar ≈ 895 ml with modification | ≈ 400 ml | 2 liters |
| Effective Cell Density | Algae other than <u>Spirulina</u> can be grown very densely | < Large airlift | < Small airlift |

agreed with earlier findings. However, during the course of our studies with this system, we encountered problems with maintaining sterility (when desired) and structural integrity. We therefore began using the glass systems described above.

III. LIGHT UTILIZATION EFFICIENCY

CHARACTERISTICS OF CULTURED SCENEDESMUS CELLS

Figure 5 illustrates the linear relationship between the dry weight of the cultured cells (mg/ml), their cell number (cells/ml), and their chlorophyll content (g chl/ml) obtained when the cell culture system of Fig. 1 was operated at varying cell densities. These data indicate that the relationships between cell mass, cell population, and chlorophyll/cell are constant over the range of culture conditions tested. Thus, the cells do not appear to be changing or adapting to differences in growth rate or light intensity during these experiments (see, e.g., Myers, Proceedings of the IBP/PP Technical Meeting, Trebon, 1970). The slopes of the two lines (computed by standard statistical techniques) are 2.8×10^7 cells/mg dry wt and 48.3 g chl/mg dry wt, respectively. This corresponds to 1.73×10^{-6} g chl and 3.6×10^{-5} g dry cell mass per cell.

PRODUCTIVITY AND LIGHT EFFICIENCY

Figure 6 illustrates the relationships between culture productivity (lower panel) and light utilization efficiency (upper panel) vs dry weight observed in a series of experiments in which Scenedesmus was maintained in the continuous culture system shown in Fig. 1. (See also Table II.) The productivity vs dry weight curve rises linearly until the cell density reaches a level at which light becomes limiting (1.4 mg ml^{-1} or 48 g chl ml^{-1}). At this point, 89% of the photosynthetically active radiation (PAR) is being absorbed. In the initial linear portion of the curve, productivity is limited by cell growth at the given light intensity. The slope of this initial portion reflects the maximum dilution rate of the system (dimensions of ml hr^{-1}). In the present instance, this rate is 65 ml hr^{-1} , which corresponds to a doubling time of 13.8 hr. This fairly low growth rate (about half the maximum growth rate generally observed at this temperature) reflects the rather low intensity of the light source (10% of full sunlight at the inner wall of the algal culture chamber). These low incident intensities are a main

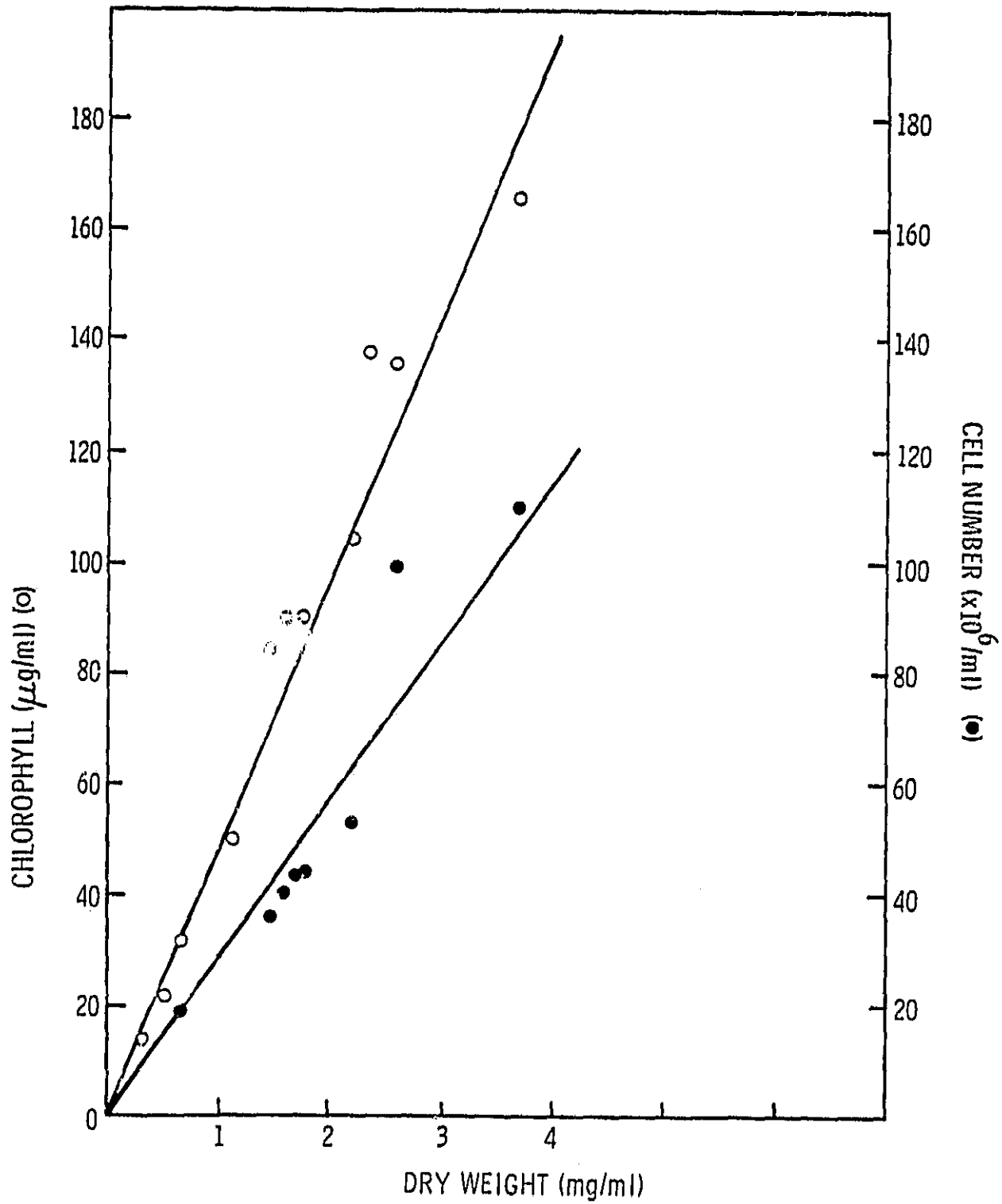


Figure 5. Relationship between dry weight of cultured cells, their cell number, and their chlorophyll content.

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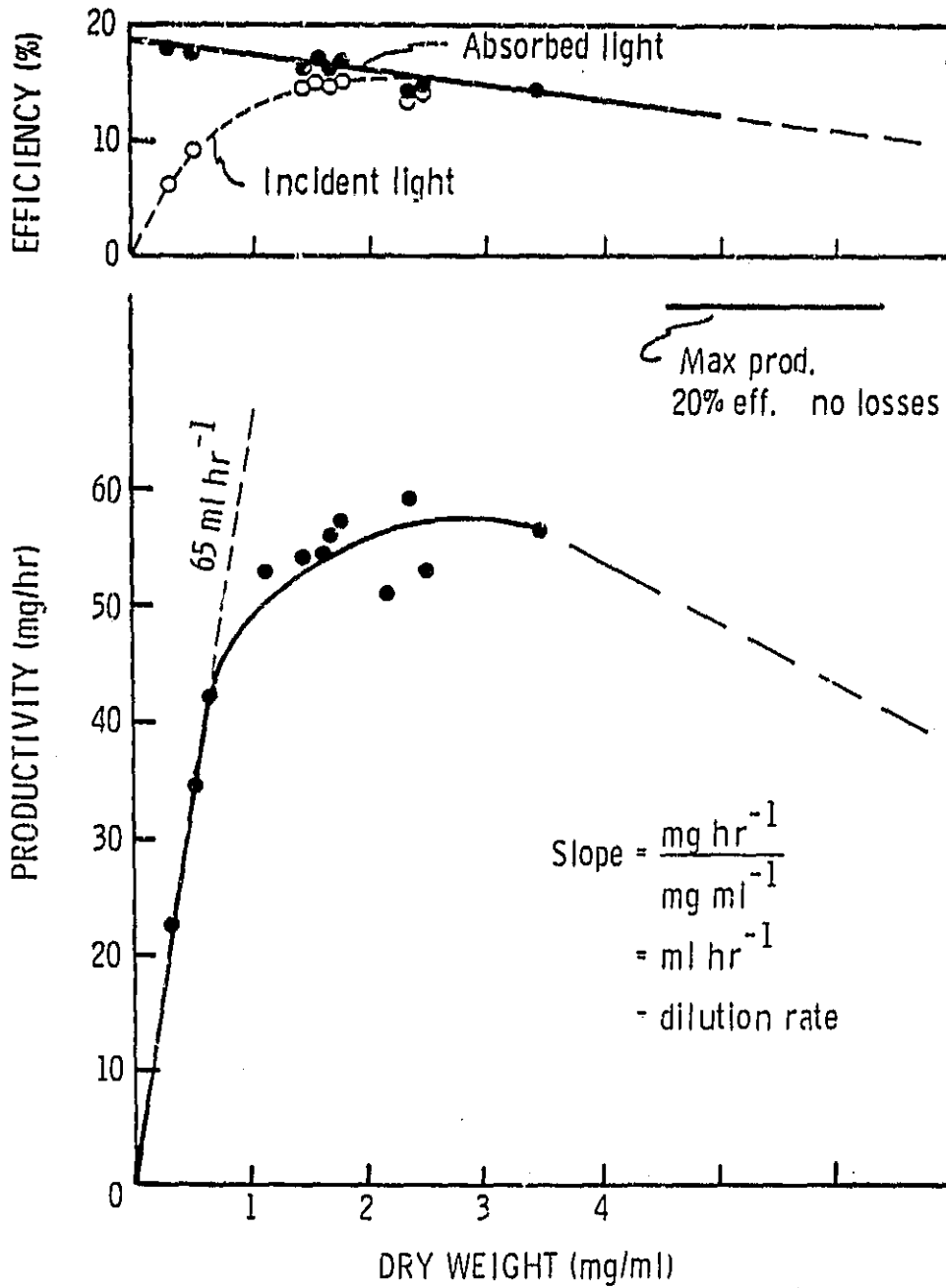


Figure 6. Relationship between culture productivity (lower panel) and light utilization efficiency (upper panel) vs dry weight (cell density).

Table II. Scenedesmus Continuous Culture Data*

| Dry Wt. ₁ (mg ml ⁻¹) | Chlorophyll (μg ml ⁻¹) | Cell # (10 ⁶) | Dilution Rate (ml hr ⁻¹) | Productivity (mg hr ⁻¹) | Incident Light (μW cm ⁻²) | Absorbed Light (μW cm ⁻²) | Efficiency (%) | |
|--|---------------------------------------|------------------------------|---|--|--|--|----------------|----------|
| | | | | | | | Absorbed | Incident |
| 2.36 | 138 | 97.2 | 25 | 59 | 1766 | 1709 | 13.9 | 13.5 |
| 1.46 | 84 | 36.6 | 37 | 54 | 1621 | 1453 | 16.1 | 14.4 |
| 2.50 | 136 | -- | 21 | 53 | 1621 | 1577 | 14.5 | 14.1 |
| 1.60 | 90 | 40.6 | 34 | 54 | 1561 | 1393 | 16.8 | 15.0 |
| 1.69 | 104 | 43.6 | 33 | 56 | 1621 | 1482 | 16.3 | 14.9 |
| 2.21 | 50 | 53.5 | 23 | 51 | -- | -- | -- | -- |
| 1.12 | 14 | -- | 47 | 53 | -- | -- | -- | -- |
| 0.335 | 22 | -- | 68 | 23 | 1621 | 547 | 18.0 | 6.1 |
| 0.52 | 90 | 44.1 | 66.5 | 35 | 1621 | 860 | 17.4 | 9.2 |
| 1.76 | 32 | 19.6 | 32 | 57 | 1621 | 1462 | 16.8 | 15.2 |
| 0.675 | 165 | 105.0 | 62.5 | 42 | -- | -- | -- | -- |
| 3.50 | | | 15.9 | 56 | 1561 | 1538 | 15.6 | 15.4 |

*See text for details.

contributing factor to the high light-utilization efficiencies obtained in this system, since the cells are never driven into light saturation (see below).

The slope of the line drawn through zero and any point on the productivity curve corresponds to the dilution rate, and therefore the doubling time and growth rate, at this point. The productivity curve rises to a value of 58 mg hr^{-1} at 3 mg ml^{-1} , which is 80% of the maximum theoretical productivity (20% on an energy basis; see, e.g., Radmer, R. and B. Kok, in *Encyclopedia of Plant Physiology*, Vol. 5, New Series, pp. 125-135, A. Trebst and M. Avron, eds., Springer-Verlag, Berlin, 1977). One would predict that the productivity would gradually decrease at very high cell densities, since increasing amounts of biomass (with finite and significant maintenance energy) would be supported by a constant amount of absorbed incident light (97% absorption at 2.5 mg ml^{-1}). However, it is not practical to obtain such data in the present system.

The upper panel of the figure shows the efficiencies* of absorbed and incident light as a function of cell density (dry weight). The efficiency with respect to absorbed light (solid line) appears to be a steadily-decreasing (linear?) function of cell mass, reflecting 1) lack of light saturation due to the low incident intensity (see above), and 2) the significant maintenance energy required by the (increasing) biomass. If we assume the reality of the linear relationship, the maximum efficiency of absorbed light in this system is 19%. The slope of the line, $1.28\% (\text{mg/ml})^{-1}$, reflects losses due to maintenance energy, which is probably linear over the rather narrow conditions tested. Since 100% efficiency corresponds to $0.0064 \text{ W hr mg}^{-1}$, the maintenance energy is $8.19 \times 10^{-5} \text{ W hr mg}^{-1}$, and one would predict that the culture would reach light compensation at a dry wt of 14.3 mg ml^{-1} .

*These light efficiency measurements contain the following primary sources of error: 1) a small volume at the bottom of the culture (10% of the total volume) that is not significantly illuminated; 2) the overflow cell density is only 89% of the reactor density; 3) the light measurements represent the average of a somewhat asymmetrical cylindrical light field; and 4) room light was not excluded (< 3% of total incident light). All these errors are relatively small, and the more significant ones tend to cancel.

The efficiency with respect to incident light (dotted line) reflects the balance between light absorption and utilization (the initial ascending phase) and losses due to the cellular metabolism (descending phase). In the present system, the maximum light utilization efficiency occurs at 1.7 to 2.0 mg ml⁻¹ dry wt. The descending phase becomes identical to the "absorbed light" curve at high cell densities.

RELATIONSHIP OF LIGHT INTENSITY TO TURNOVER OF THE PHOTOSYNTHETIC APPARATUS

One of the primary limitations of most photosynthetic organisms is that they do not perform well in strong light (e.g., full sunlight). The photosynthetic apparatus operates somewhat like a lens; approximately 200 "light-harvesting" chlorophyll molecules transfer light energy to a reaction center, with a corresponding increase in the effective light intensity per center. Consequently, efficiency can be very high in weak light, but drops off rapidly at intensities approaching that of bright sunlight, due to the rather slow (ms) turnover of the dark reactions (see, e.g., R. Radmer and B. Kok, op. cit.).

Figure 7 is an idealized cross sectional diagram of our algal culture system. The light flux values (in units of photosynthetically-active quanta cm⁻²s⁻¹) were measured in the absence of algae. Note that they closely follow a 1/r relationship, suggesting that the total flux is conserved (except for losses due to reflection and absorption by the glass walls) during its passage through the concentric cylinders.

A rough estimate of the light flux per reaction center can be made as follows. Our data indicate that Scenedesmus has a specific absorption coefficient of ~ 0.6 l cm⁻¹ g⁻¹ over the spectral range of 400-700 nm (specific data not shown), and a chlorophyll content of 4.8% (Fig. 2; see also J. Myers, in Encyclopedia of Chemical Technology, pp. 33-51, R. Kirk and D. Othmer, eds., Interscience, NY, 1957). Note that 0.6 l cm⁻¹ g⁻¹ corresponds to (0.6)(1000 cm³) cm⁻¹ g⁻¹ or 600 cm² g⁻¹. Thus, the equivalent specific absorption is 1.2 x 10⁴ cm² (g chlorophyll)⁻¹, which is about 10% of the extinction coefficient observed at the absorption maxima. Since one g

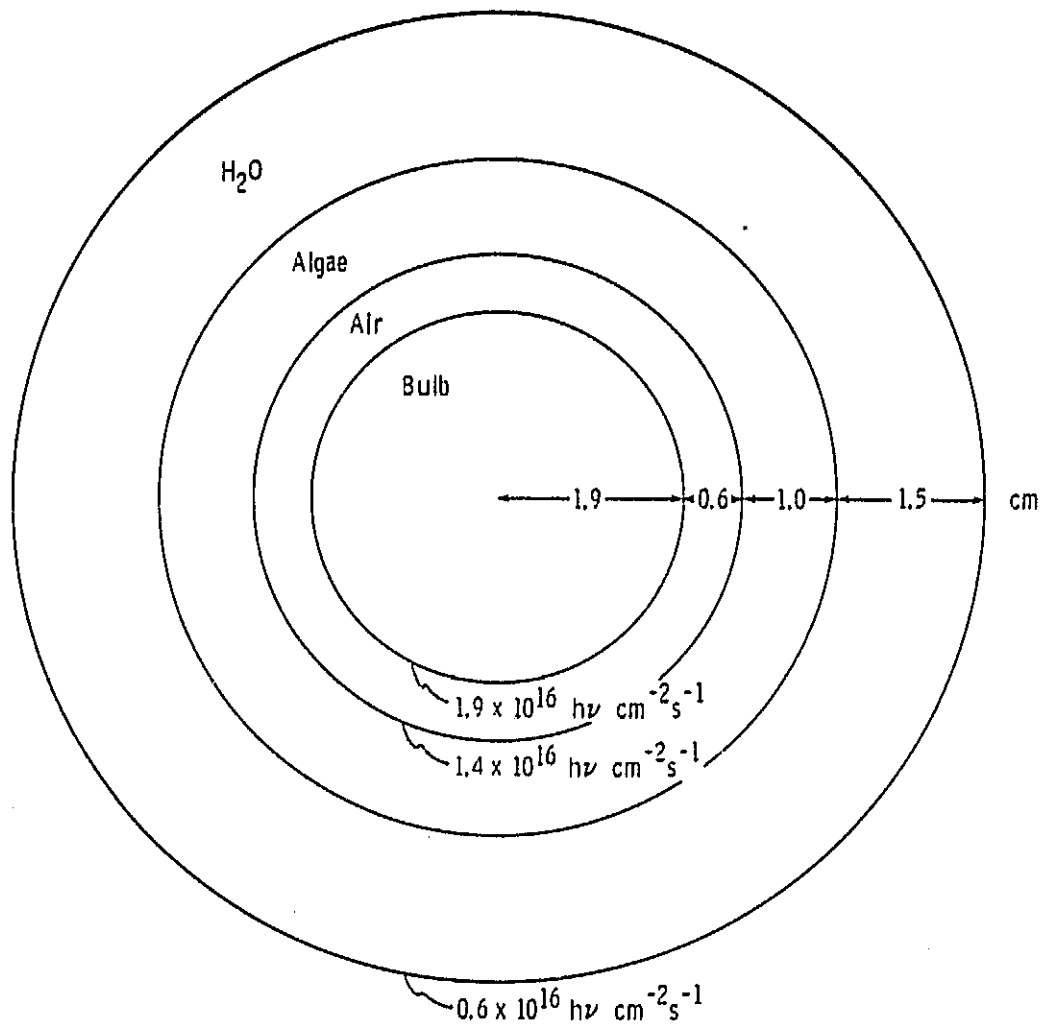


Figure 7. Schematic cross-section of "Large Airlift" continuous culture apparatus.

chlorophyll = 6×10^{20} molecules, the molecular cross section (= extinction coefficient) is $0.2 \times 10^{-16} \text{ cm}^2$ (chlorophyll molecule) $^{-1}$.

The maximum quantum flux that the algae are subjected to is $1.4 \times 10^{16} \text{ hv cm}^2 \text{ s}^{-1}$ or $0.28 \text{ hv molecule}^{-1} \text{ s}^{-1}$. Since each reaction center is connected to ~ 200 chlorophyll molecules (see above), the maximum quantum flux will result in the transfer of $\sim 60 \text{ hv s}^{-1}$ to each reaction center.

This value is well below the generally accepted maximum reaction center turnover rate of $\sim 100 \text{ s}^{-1}$ (Radmer and Kok, op. cit.). Thus the photosynthetic dark reactions are able to keep pace with the light flux, the system does not become light saturated, and high light efficiencies can be obtained.

IV. NITROGEN UTILIZATION EFFICIENCY

Krauss et al. [Proc. 21st Plenary Meeting, Committee on Space Research (COSPAR), 1978] reported that a significant fraction of the NO_3^- nitrogen provided to Chlorella cultured in a "recyclostat" was lost, probably as N_2O . The release of this gas into the atmosphere of a closed system could cause grave problems for the air regeneration system, as well as contribute to a lack of closure of the nitrogen cycle.

Because of these earlier results, one of our goals was to determine the nitrogen balance of Scenedesmus cultures, and specifically, whether compounds such as N_2O were excreted into the medium. Our initial approach was to determine the nitrogen levels of the nutrient medium, cell-free efflux, and harvested algae.

The results of these experiments (Table III) show surprisingly good agreement between added and recovered N (average 100.5% recovery) and suggest that the nitrogen entering the culture (as NO_3^-) was either incorporated in the algae or appeared as NO_3^- in the efflux supernatant. We have had no indication to date that the nitrogen is lost by the system. Although these results do not prove that there is no nitrogen loss, they do suggest that any loss must be small (e.g., < 1%), at least for NO_3^- .

Because our data obtained with Scenedesmus point to a very low production of nitrogenous by-products, we have not attempted to determine N_2O directly. (Current data indicate that the N_2O concentration in the gas stream would be too low to monitor directly.) We have observed only traces of NO_2^- (< 1 ppm) in the effluent supernatant.

TABLE III.
Nitrogen Utilization Efficiency of Scenedesmus

| <u>Sample</u> | <u>Nitrogen (mg/l)</u> | | | <u>Recovery (%)</u> |
|---------------|------------------------|-------------|-------|---------------------|
| | Medium | Supernatant | Algae | |
| 1 | 339 | 216 | 127 | 101.2 |
| 2 | 293 | 120 | 171 | 99.3 |
| 3 | 290 | 123 | 171 | 101.4 |
| 4 | 290 | 122 | 168 | 100.0 |

V. LONG-TERM CULTURE

We maintained continuous Scenedesmus cultures in the apparatus shown in Figs. 4a and 4b for about 4 months before voluntary shutdown. The cultures were monitored daily for packed cell volume (PCV), chlorophyll, dry weight, reproduction rate, and pH, and intermittently for glycolate, total N, and microbial contamination. Representative data from these long-term experiments is shown in Fig. 8. Note that the culture system displays good long-term stability. The complete data record for these experiments is given in Tables IV and V.

We addressed the interrelated topics of algal by-product excretion and microbial contamination by periodically assaying the culture supernatant. To date, we have detected no significant glycolate (< 1 ppm of this primary algal excretory product), or excreted carbon (< 25 ppm) while our cultures were in the steady state. Parallel microbial assays in some cases indicated a low (0.1 - 0.01%) non-algal biomass that did not change appreciably (with time) with respect to amount or species composition (see also Tables IV and V). These findings suggest that microbial contamination should not be a significant problem in such cultures because 1) the algae seem to excrete little or no organic compounds; and 2) microbial populations, even when present, do not take over the culture.

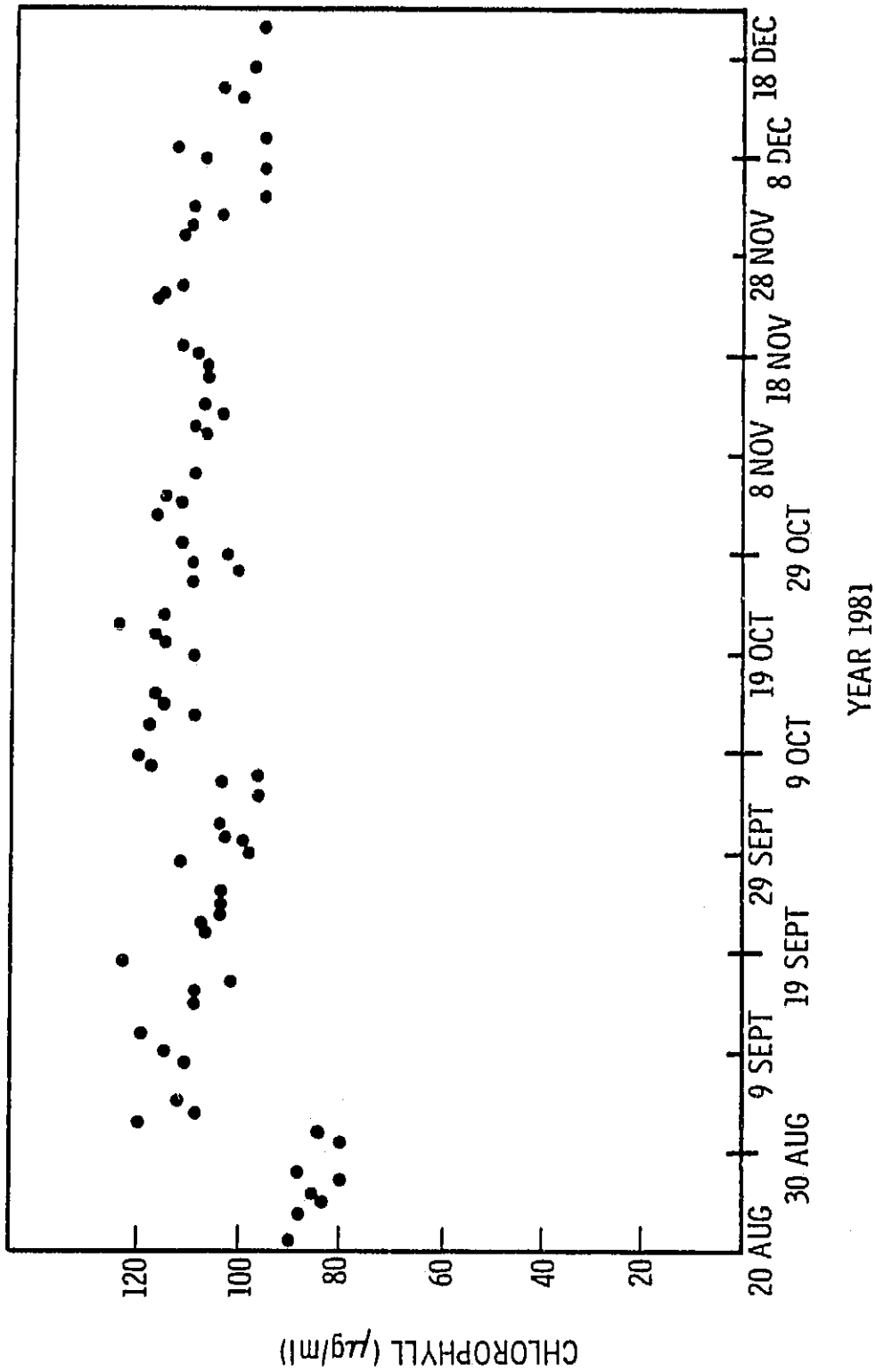


Figure 8. Data obtained during continuous culture of Scenedesmus with urea.

TABLE IV.

Data Obtained During Continuous Culture of Scenedesmus with Urea

| Date (1981) | pH | PCV ($\mu\text{l/ml}$) | Chl. ($\mu\text{g/ml}$) | Reproduction (ml/hr) | Dry Weight (mg/ml) | Cell Counts (number cells/ml) | Contam- ination (number bact/ml) | Density Reference and Other Notes |
|----------------|------|-----------------------------|------------------------------|-------------------------|--------------------------|--|---|--|
| Aug 20 | 6.25 | 9.0 | 88 | 52.4 | 2.4 | | | 1.965 |
| 21 | 6.25 | 9.0 | 90 | 54.8 | | | | |
| 24 | 6.25 | 8.5 | 88 | | 2.28 | 8.0×10^7 | | |
| 25 | 6.15 | 9.0 | 84 | 55.8 | 2.38 | | | |
| 26 | 6.15 | 8.5 | 85 | 58.2 | 2.32 | | | |
| 27 | 5.9 | 8.0 | 79 | 59.4 | 2.32 | | | |
| 28 | 6.0 | 8.0 | 88 | 55.4 | 2.4 | | | |
| 31 | 5.9 | 8.0 | 80 | | 2.16 | 8.0×10^7 | | |
| Sep 1 | 5.9 | 8.0 | 84 | 57.5 | 2.21 | | | |
| 2 | 5.95 | 9.5 | 120 | 50.0 | 2.63 | | | |
| 3 | 5.95 | 9.0 | 109 | 57.5 | 2.43 | 9.5×10^7 | | |
| 4 | 5.9 | 10.0 | 112 | 49.4 | 2.76 | | | |
| 8 | 6.0 | 9.5 | 111 | | 2.54 | | 2.92×10^6 | |
| 9 | 6.0 | 10 | 115 | | 2.72 | | | |
| 10 | 6.8 | 9.5 | | 49.1 | 2.55 | 8.95×10^7 | | |
| 11 | 6.0 | 10 | 120 | | 2.85 | | | |
| 14 | 6.1 | 10 | 109 | | 2.64 | | 2.25×10^6 | |
| 15 | 6.1 | 10 | 109 | 46.5 | 2.57 | | | |
| 16 | 6.0 | 10 | 102 | | 2.55 | | | |
| 17 | 5.95 | 13.5 | 133 | | 2.98 | | | |
| 18 | 6.0 | 11.0 | 123 | 49.4 | 2.79 | | | |
| 21 | 5.5 | 12.0 | 107 | | 2.69 | 7.26×10^7 | 2.6×10^6 | |
| 22 | 5.7 | 11.0 | 107 | 40.0 | 2.92 | | | |
| 23 | 5.7 | 10 | 104 | | | | | |
| 24 | 5.55 | 11.0 | 104 | 50.7 | 2.72 | | | |
| 25 | 5.65 | 10.0 | 104 | 46.5 | 2.86 | | | |
| 28 | 5.65 | 8.5 | 112 | | 2.48 | 8.65×10^7 | | |
| 29 | 5.4 | 8.5 | 98 | 50 | 2.46 | | | |
| 30 | 5.3 | 8.5 | 99 | 51.9 | | | | |

Table IV. (Continued)

| Date | pH | PCV | Chl. | Reproduction | Dry Weight | Cell Counts | Contamination | Density Reference and Other Notes |
|--------|------|----------------------|----------------------|--------------|------------|----------------------|----------------------|-----------------------------------|
| (1981) | | ($\mu\text{l/ml}$) | ($\mu\text{g/ml}$) | (ml/hr) | (mg/ml) | (number cells/ml) | (number bact/ml) | |
| Oct 1 | 5.4 | 9.0 | 103 | 51.9 | | | | |
| 2 | 5.3 | 8.0 | 104 | | | | | |
| 5 | 5.5 | 7.5 | 96 | | 2.28 | | | |
| 6 | 5.45 | 7.5 | 104 | 50 | 2.25 | | 1.2x10 ⁶ | |
| 7 | 5.3 | 7.5 | 96 | 52.5 | 2.29 | 6.75x10 ⁷ | | 2.110 |
| 8 | 5.4 | 8.0 | 118 | 53.8 | 2.36 | | | |
| 9 | 5.25 | 8.0 | 120 | 47.9 | 2.21 | | | |
| 12 | 5.8 | 8.5 | 118 | | 2.23 | | | |
| 13 | 5.7 | 8.0 | 104 | 52.2 | 2.23 | | | |
| 14 | 5.7 | 8.0 | 115 | 50.0 | 2.23 | | | |
| 15 | 5.8 | 8.5 | 117 | 45.6 | 2.21 | | | |
| 16 | | | | | 2.30 | | | |
| 19 | 5.7 | 8.0 | 109 | | 2.28 | | | |
| 20 | 5.5 | 8.5 | 115 | 42.5 | 2.27 | | | |
| 21 | 5.6 | 9.5 | 117 | 39.4 | 2.20 | | | |
| 22 | 5.8 | 9.5 | 124 | 45.0 | 2.30 | | | |
| 23 | 5.7 | 9.0 | 115 | 46.9 | | | | |
| 26 | 5.75 | 8.0 | 110 | | 2.40 | | | |
| 27 | 5.7 | 8.0 | 101 | 47.2 | 2.15 | | | |
| 28 | 5.5 | 7.5 | 110 | 48.8 | 2.25 | | | |
| 29 | 5.2 | 7.5 | 103 | 48.8 | 2.15 | | | |
| 30 | 5.3 | 7.5 | 112 | 48.1 | 2.24 | 6.09x10 ⁷ | | |
| Nov 2 | 5.6 | 8.0 | 117 | | 2.13 | | 8.47x10 ⁵ | |
| 3 | 5.65 | 8.0 | 112 | 48.1 | 2.22 | | | |
| 4 | 5.75 | 7.5 | 115 | 50.0 | 2.14 | | | |
| 5 | 5.8 | | | | 2.17 | | | |
| 6 | | 8.0 | 109 | | 2.23 | | 7.3x10 ⁵ | |
| 9 | 5.8 | 12.5 | 190 | | 3.05 | | | |
| 10 | 5.85 | 7.0 | 107 | 46.9 | 2.02 | | | |
| 11 | 6.0 | 7.5 | 109 | 41.2 | 2.05 | | | |
| 12 | 5.85 | 7.0 | 104 | 46.5 | 2.07 | | | |
| 13 | 5.75 | 8.0 | 107 | 45.2 | 2.16 | | 2.47x10 ⁵ | |

Table IV. (Continued)

| Date (1981) | pH | PCV (μ l/ml) | Chl. (μ g/ml) | Reproduction (ml/hr) | Dry Weight (mg/ml) | Cell Counts (number cells/ml) | Contam- ination (number bact/ml) | Density Reference and Other Notes |
|----------------|-----|----------------------|-----------------------|-------------------------|--------------------------|--|---|--|
| Nov 16 | 6.1 | 7.0 | 107 | | 2.01 | | | |
| 17 | 6.0 | 7.0 | 107 | 46.7 | 1.98 | | | |
| 18 | 5.8 | 7.0 | 109 | 50.3 | 2.05 | | | |
| 19 | 5.7 | 7.5 | 112 | 48.5 | 2.14 | | | |
| 20 | | | | | 2.05 | 7.9×10^7 | | |
| 23 | | | | | | | | |
| 24 | 5.7 | 7.5 | 116 | 43.8 | 2.21 | | | |
| 25 | 5.7 | 7.5 | 112 | 44.4 | 2.12 | | | |
| 30 | 5.5 | 7.0 | 112 | | 2.11 | | 1.85×10^6 | |
| Dec 1 | 5.4 | 7.0 | 110 | 45.5 | 2.09 | 7.78×10^7 | | |
| 2 | 5.5 | 7.0 | 104 | 48.1 | 2.00 | | | |
| 3 | 5.4 | 7.0 | 110 | 42.5 | 2.01 | | | |
| 4 | 5.4 | 6.0 | 96 | 45.3 | 1.92 | | | |
| 7 | 5.6 | 6.5 | 96 | | 1.92 | | | |
| 8 | 5.6 | 7.0 | 107 | 43 | 2.01 | | 2.53×10^6 | |
| 9 | 5.6 | 7.0 | 113 | 37.5 | 2.09 | | | |
| 10 | 6.0 | 6.5 | 96 | 43.9 | 1.93 | | 2.06×10^6 | |
| 14 | 5.8 | 6.5 | 100 | | 1.85 | | | |
| 15 | 5.9 | 6.5 | 104 | 50 | 1.85 | | | |
| 16 | 5.8 | 6.5 | 104 | 44.2 | 1.83 | | | |
| 17 | 5.7 | 6.0 | 98 | 46.9 | | | | |
| 21 | 6.1 | 6.5 | 96 | | | | | |

TABLE V

Data Obtained During Continuous Culture of Scenedesmus with NO_3^-

| Data (1981) | pH | PCV ($\mu\text{l}/\text{ml}$) | Chl. ($\mu\text{g}/\text{ml}$) | Reproduction (ml/hr) | Dry Weight (mg/ml) | Cell Counts (number cells/ml) | Contam- ination (number bact/ml) | Density Reference and Other Notes |
|----------------|------|------------------------------------|-------------------------------------|---|--|--|---|--|
| Aug 20 | 7.7 | 8.0 | 57 | 58.9 | 1.93 | | | 2.14 |
| 21 | 7.75 | 7.0 | 62 | 67.9 | | | | |
| 24 | 7.7 | 7.0 | 54 | | 1.35 | 4.15×10^7 | | |
| 25 | 7.65 | 7.0 | 56 | 62.4 | 1.78 | | | |
| 26 | 7.7 | 7.0 | 57 | 61.8 | 1.74 | | | |
| 27 | 7.7 | 7.0 | 59 | 58.7 | 1.57 | | | KNO_3 in- creased to 20 mM |
| 28 | 7.7 | 6.0 | 61 | 65.8 | 1.44 | | | |
| 31 | 7.6 | 5.0 | 52 | | 1.2 | 2.3×10^7 | | |
| Sep 1 | 7.5 | 5.0 | 49 | 71.9 | 1.23 | | | |
| 2 | 7.7 | 8.0 | 91 | 58.1 | 1.81 | | | |
| 3 | 7.8 | 9.0 | 109 | 51.3 | 2.02 | 3.35×10^7 | | |
| 4 | 7.65 | 7.0 | 79 | 61.3 | 1.69 | | | |
| 8 | 7.7 | 6.5 | 86 | | 1.66 | | 4.05×10^6 | |
| 9 | 7.65 | 7.0 | 86 | | 1.66 | | | |
| 10 | 7.5 | 6.5 | | 55.4 | 1.61 | 3.49×10^7 | | |
| 11 | 7.8 | 6.0 | 107 | | 1.70 | | | 2.32 |
| 14 | 7.8 | 6.0 | 90 | | 1.71 | | 3.53×10^6 | |
| 15 | 7.75 | 7.0 | 91 | 56.1 | 1.77 | | | |
| 16 | 7.7 | 7.0 | 85 | | 1.69 | | | |
| 17 | 7.8 | 7.0 | 91 | | 1.64 | | | |
| 18 | 7.75 | 7.0 | 86 | 48.2 | 1.8 | | | |
| 21 | 7.6 | 7.0 | 94 | | 1.83 | 4.73×10^7 | 5.26×10^6 | |
| 22 | 7.8 | 7.0 | 94 | 53.1 | 1.77 | | | |
| 23 | 7.65 | 7.0 | 87 | | | | | 2.48 |
| 24 | 7.65 | 7.0 | 96 | 53.3 | 1.77 | | | |
| 25 | 7.7 | 7.0 | 94 | 55.3 | 1.84 | | | |
| 28 | 7.0 | 7.0 | 101 | | 1.74 | 4.67×10^7 | | |
| 29 | 7.4 | 6.0 | 89 | 61.9 | 1.72 | | | |
| 30 | 7.3 | 6.0 | 83 | 61.3 | | | | |

Table V. (Continued)

| Date | pH | PCV | Chl. | Reproduction | Dry Weight | Cell Counts | Contamination | Density Reference and Other Notes |
|---|------|---------------|---------------|--------------|------------|----------------------|----------------------|-----------------------------------|
| (1981) | | (μ l/ml) | (μ g/ml) | (ml/hr) | (mg/ml) | (number cells/ml) | (number bact/ml) | |
| Oct 1 | 7.4 | 6.0 | 83 | 59.4 | | | 6.65x10 ⁶ | |
| 2 | 7.4 | 6.0 | 78 | | | | | |
| 5 | 7.3 | 6.0 | 78 | | 1.52 | | | |
| 6 | 7.2 | 6.0 | 78 | 54.4 | 1.50 | | 6.67x10 ⁶ | |
| 7 | 7.0 | 6.5 | 78 | 60.6 | 1.52 | 3.8x10 ⁷ | | 2.600 |
| 8 | 7.3 | 6.0 | 78 | 56.3 | 1.54 | | | |
| 9 | 6.4 | 6.0 | 78 | 60.0 | 1.42 | | | |
| 12 | 5.5 | 4.5 | 43 | | 1.20 | | | |
| 13 | 5.5 | 4.0 | 43 | 52.2 | 1.14 | | | |
| 14 | 5.25 | 3.5 | 35 | 51.3 | | | | |
| CLEANED AND RESTARTED + SAME CONDITIONS | | | | | | | | |
| 23 | 7.4 | 7.0 | 92 | 45.1 | | | | 2.70 |
| 26 | 7.5 | 7.5 | 104 | | 2.13 | | | |
| 27 | 7.3 | 8.0 | 112 | 47.9 | 2.13 | | | |
| 28 | 7.3 | 8.0 | 117 | 47.5 | 2.15 | 8.25x10 ⁷ | | |
| 29 | 7.0 | 8.0 | 103 | 48.8 | 1.99 | | | |
| 30 | 6.6 | 7.5 | 110 | 48.8 | 1.91 | 6.25x10 ⁷ | 2.73x10 ⁶ | |
| Nov 2 | 6.2 | 7.5 | 110 | | 1.90 | | | |
| 3 | 6.3 | 8.0 | 107 | 48.8 | 1.96 | | | |
| 4 | 6.4 | 9.0 | 117 | 47.5 | 1.99 | | | |
| 5 | | | | | 1.90 | | | |
| 6 | 7.8 | 9.0 | 105 | | 1.89 | | 4.2x10 ⁶ | |
| 9 | 6.6 | 9.0 | 110 | | 1.98 | | | |
| 10 | 6.4 | 9.0 | 96 | 43.1 | 1.85 | | | |
| 11 | 6.6 | 8.0 | 130 | 40.6 | 1.86 | | | |
| 12 | 6.45 | 8.0 | 101 | 49.0 | 1.74 | | | |
| 13 | 6.7 | 8.0 | 99 | 49.0 | 1.87 | | 6.33x10 ⁶ | |
| 16 | 6.55 | 8.0 | 106 | | 1.86 | | | |

Table V. (Continued)

| Date | pH | PCV | Chl. | Reproduction | Dry Weight | Cell Counts | Contamination | Density Reference and Other Notes |
|--------|-----|---------------|---------------|--------------|------------|--------------------|--------------------|-----------------------------------|
| (1981) | | (μ l/ml) | (μ g/ml) | (ml/hr) | (mg/ml) | (number cells/ml) | (number bact/ml) | |
| Nov 17 | 6.7 | 8.0 | 115 | 46.7 | 1.88 | | | |
| 18 | 6.7 | 8.0 | 112 | 47.9 | 1.96 | | | |
| 19 | 6.5 | 8.0 | 96 | 50.9 | 1.82 | | | |
| 20 | | | | | 1.85 | 5.52×10^7 | | |
| 23 | 6.4 | 8.0 | 114 | | 1.84 | | | |
| 24 | 6.4 | 8.0 | 116 | 50.0 | 1.95 | | | |
| 25 | 6.3 | 8.0 | 120 | 46.9 | 1.93 | | | |
| 30 | 6.4 | 8.0 | 115 | | 1.94 | | 2.17×10^6 | |
| Dec 1 | 6.3 | 8.0 | 113 | 47.3 | 1.85 | 5.62×10^7 | | |
| 2 | 6.3 | 7.0 | 114 | 48.8 | 1.84 | | | |
| 3 | 6.2 | 8.0 | 104 | 49.4 | 1.84 | | | |
| 4 | 6.1 | 7.5 | 98 | 48.5 | 1.75 | | | |
| 7 | 6.0 | 7.0 | 100 | | 1.75 | | | |
| 8 | 6.2 | 7.5 | 107 | 45.5 | 1.77 | | 5.51×10^6 | |
| 9 | 6.3 | 7.5 | 113 | 43.5 | 1.81 | | | |
| 10 | 6.0 | 6.5 | 93 | 49.0 | 1.75 | | 1.01×10^7 | |
| 14 | 5.7 | 7.0 | 98 | | 1.77 | | | |
| 15 | 6.2 | 7.0 | 100 | 46.3 | 1.74 | | | |
| 16 | 6.3 | 7.0 | 107 | 44.8 | 1.76 | | | |
| 17 | 5.6 | 6.5 | 98 | 46.3 | 1.76 | | | |
| 21 | 6.3 | 7.5 | 99 | 49.5 | | | | |

VI. SEARCH FOR PLASMIDS IN SPIRULINA

Although it is a good source of protein, Spirulina platensis is deficient in the amino acids lysine and methionine. The intent of this project was to locate a plasmid in Spirulina which could be used as a vector to clone genes for amino acids, thus making Spirulina a more valuable food source. Plasmids have been isolated from other filamentous blue-green algae such as Anabaena variabilis, Nostoc strain MAC, and Plectonema boranum (Lambert and Carr, 1982, Arch. Microbiol. 133, 122-125).

GENERAL PLASMID PREPARATION

Axenic cultures of Spirulina platensis were obtained by the methods described in Appendix A.

The general approach for the preparation of plasmids is to 1) break open the cells, 2) remove cellular debris and chromosomal DNA, and 3) isolate the extrachromosomal plasmid DNA, which can then be visualized on an agarose gel. Below is a flow chart outlining the steps of the Spirulina plasmid preparation described by Lambert and Carr.

| <u>Procedure</u> | <u>Purpose</u> |
|--|--|
| 100 ml <u>Spirulina</u> cells grown in 2% CO ₂ in air | |
| + | |
| Incubate with 5 mg/ml lysozyme | Cell lysis (breaks cell wall) |
| + | + |
| Incubate with 2% SDS | Completes cell lysis (bursting) |
| + | + |
| NaCl precipitation and centrifugation for 30 min | Precipitates cell walls, organelles and most of chromosomal |
| + | + |
| Extraction with phenol Extraction with chloroform/ isoamyl alcohol | Removal of proteins |
| + | + |
| Volume reduction with sec-butanol | Concentrate DNA |
| + | + |
| Ammonium acetate and Ethanol precipitation | Precipitate DNA |
| + | + |
| Dry and resuspend DNA in buffer Run on gel | Visualize DNA |

Samples of DNA were run on horizontal agarose gels using Tris-borate buffer and a voltage gradient of 3-5 V/cm. When the DNA reached the opposite edge of the gel (visualized by an added dye), the entire gel was stained with ethidium bromide (a substance which intercollates into double stranded nucleic acids and fluoresces under ultraviolet light) and photographed.

Figure 9 shows a gel of a sample of DNA from the plasmid preparation described above (lane 2). The gel showed a smear of chromosomal DNA and some RNA (the large smear at the bottom of the gel), but no plasmid bands. (On a gel, a plasmid will appear as a bright band, often against a background of chromosomal DNA which is usually a smear running the length of the gel.) In this gel, there are plasmids from a different alga in lanes 8 and 9, approximately 2.5 cm from the top of the gel.

TREATMENT WITH RESTRICTION ENZYMES

Often, in order to visualize a plasmid on a gel, it is necessary to digest a sample of the DNA preparation with a restriction enzyme. These enzymes cut the DNA strands, forming many fragments which migrate to different regions of the gel according to their size. Restriction enzymes cut both plasmid and chromosomal DNA, but since a plasmid is small, it will only be cut in a few places, thus forming several bright bands against a background of chromosomal DNA fragments (laddering effect). (It is important to digest chromosomal DNA, because a chromosomal smear could mask a plasmid band.)

We attempted to digest the DNA from the Lambert and Carr preparation with the restriction enzyme Xho I after incubation with RNase and proteinase. (Both RNA and some proteins will inhibit the action of the restriction enzymes.) However, the digestion of the DNA was unsuccessful; i.e., there was no laddering. Most likely, the restriction enzyme was inhibited by something in the preparation -- e.g., proteins, algal pigments, or polysaccharides.

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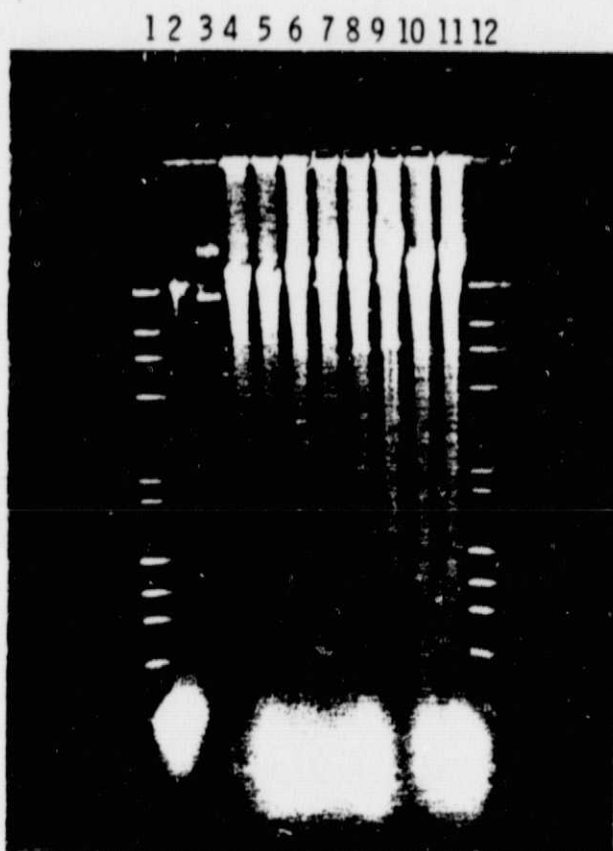


Figure 9. Electrophoretic separation of a plasmid preparation (0.7% agarose gel, run at 50V for 2 hr). Spirulina DNA is in lane 2. Lanes 3 through 11 contain DNA from different strains of Anacystis, a glue-green alga. Note the plasmid bands in lanes 8 and 9. Lane 1 and 12 contain λ DNA fragments of known sizes to serve as molecular weight markers.

SARKOSYL TREATMENT

The preparation of Lambert and Carr produced less DNA than expected from the amount of cells used, probably due to incomplete lysis. Spirulina has a tough polysaccharide sheath surrounding the cell walls which may not be sensitive to the lysozyme-SDS treatment. Preliminary tests showed that a 30-minute incubation with Sarkosyl (an anionic detergent) resulted in significant cell wall disintegration. When the cells were incubated with Sarkosyl prior to treatment with lysozyme and SDS, DNA recovery was increased at least twofold, which indicated that the Sarkosyl improved cell lysis.

Figure 10 shows the results of a large scale (4-2) plasmid preparation (derived from Lambert and Carr) after treatment with RNase, proteinase, and solvent extractions to clean the DNA. Prior to running the gel, the DNA was incubated with BAM HI, another general restriction enzyme. The gel showed some DNA, but no chromosomal digestion or plasmid bands. Two more sets of solvent extractions removed most of the protein, but digestion by restriction enzymes was still inhibited (data not shown). Extended dialysis (up to 3 days) with large pore size dialysis tubing markedly improved the purity of the DNA (as determined by A_{260}/A_{280}) but did not noticeably increase restriction enzyme digestion.

CASSE PROCEDURE

Because of the difficulties encountered with the procedure of Lambert and Carr, we tested the procedure of Casse et al. (J. Gen. Micro. 113, 229-242, 1979), which is especially designed to isolate large plasmids often removed with the chromosomal DNA. This preparation does not rely on centrifugations of DNA at high speeds, as most procedures do; instead, the DNA is cleaned with a series of salt-saturated solvent extractions.

Figure 11 shows the gel obtained with samples of Spirulina DNA prepared according to the Casse procedure. Again, we observed no plasmid bands. Although no DNA was present, there was a significant amount of RNA, indicating that the cells were lysed. It is unlikely that the DNA was damaged, since this procedure is reported to be very gentle.

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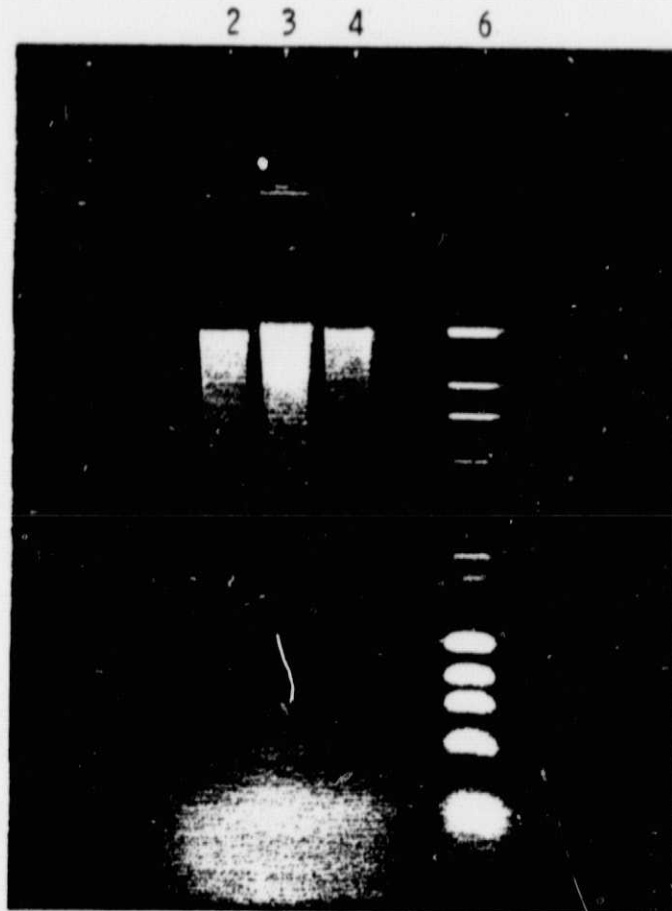


Figure 10. Electrophoretic separation of a plasmid separation (0.7% agarose gel, run at 50V, 2½ hr). Spirulina DNA from a modified Lambert and Carr preparation that included treatment with Sarkosyl to aid in cell lysis. DNA in lane 2 has been incubated with Bam HI, and in lane 4 with Xho I. Untreated DNA is in lane 3. Lane 6 shows λ DNA markers.

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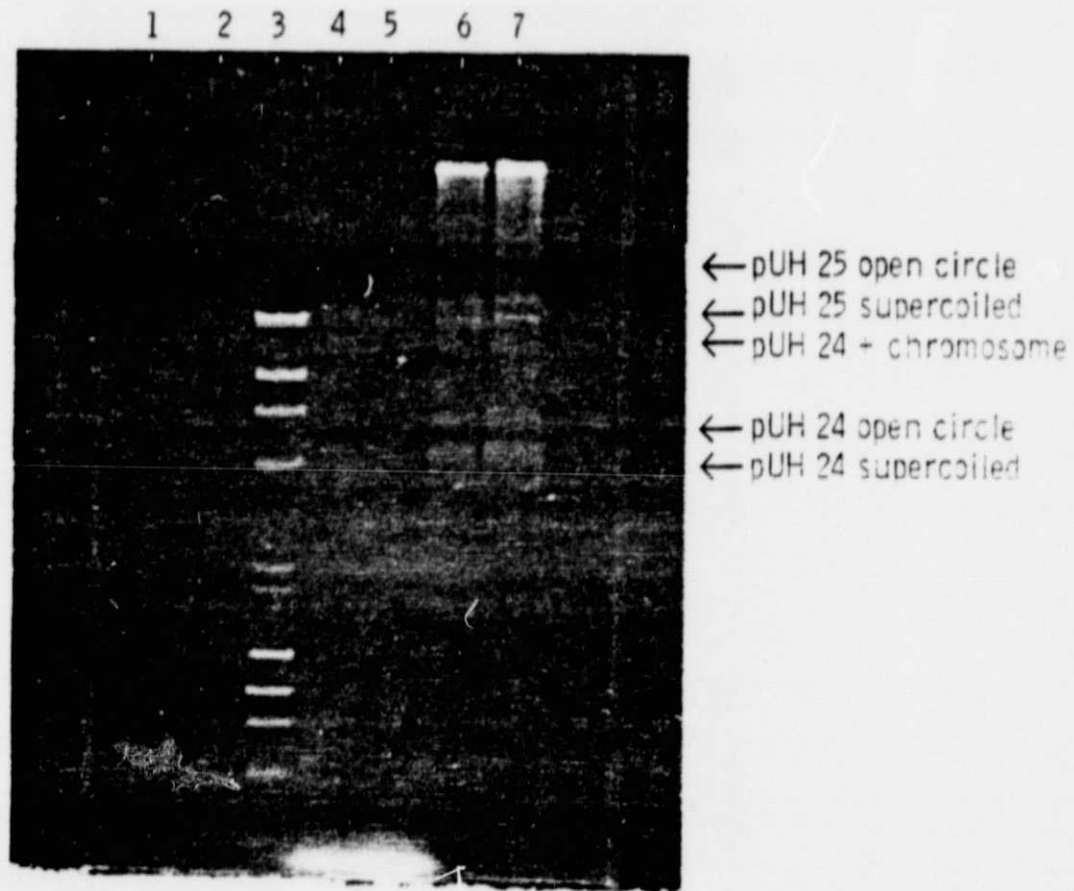


Figure 11. Electrophoretic separation of a plasmid preparation (0.7% agarose, 50V, 2½ hr). Lanes 4 and 5 contain Spirulina DNA from the Casse preparation. There is no chromosomal DNA, but the presence of RNA proves that the cells were lysed. Lanes 6 and 7 show Anacystis chromosomal DNA and plasmids isolated by the Casse procedure. The plasmids are identified by arrows. Lane 3 contains λ DNA marker.

As a positive control, the same Casse procedure was used on a 2-2 culture of Anacystis nidulans R2, a unicellular blue-green alga known to contain two plasmids, pUH24 and pUH25. As shown in Fig. 7, this preparation was very successful in isolating Anacystis plasmid DNA; large amounts of different forms of both plasmids were observed.

CONCLUSION

We performed six different plasmid preparations on our strain of Spirulina platensis over a three-month period. (This effort was not initiated in vacuo; we have extensive experience in isolating plasmids from other blue-green algae.) The fact that we have been unable to detect plasmids in Spirulina, while at the same time routinely isolating them from other blue-green algae, leads us to conclude that our Spirulina strain does not contain any significant extrachromosomal material.

Our strain of Spirulina platensis has been "in captivity" for a long time, and since it is not under any environmental pressure to maintain plasmids, it may have lost them. If deemed appropriate, we could screen Spirulina strains recently isolated from nature for endogenous plasmids. Genetic transformations do not require the use of endogenous plasmids, but are often made easier by their presence. Further work along these lines will await feedback from NASA.

APPENDIX A: METHODS

OPERATION OF GLASS CULTURE SYSTEMS (FIGS. 1-3)

These continuous culture systems are based on the earlier work of several other groups, notably Myers and Clark (J. Gen. Physiol. 28, 103-112, 1944); also, Kuhl and Lorenzen (in Methods in Cell Physiology, Vol. I, D. Prescott, ed., pp. 159-187, Academic Press, NY, 1964); and Ammann and Lynch, (Appl. Microbiol. 13, 546-551, 1965). A primary goal of the design and construction of our culture systems was to provide a means to control and monitor important physiological parameters, such as light flux, light absorption, temperature, and growth rate. Another important consideration was our goal of constructing a system that was harvestable on demand. This latter requirement precluded the use of a chemostat system; instead, we use the turbidostat system described below.

Figure 1 is a diagram of the culture apparatus that has been most frequently used in our laboratory. The culture system is made of transparent glass and consists of three concentric, cylindrical chambers.

The innermost chamber houses the illuminating source (a standard 40-watt, cool white, high-output, fluorescent bulb). The middle chamber, with a volume of 890 ml and a width of 1.0 cm, contains the algal culture. Temperature control is provided by a refrigerated bath (Neslab RTE-4; $\pm 0.01^\circ\text{C}$ temperature control from -30°C to $+100^\circ\text{C}$) which circulates water through the outermost chamber.

Cell density is maintained by monitoring the light transmission through the culture using a photoconductive cell (Clairex CL604L). The output of this photocell is amplified, integrated (to remove the ac component from the light), and digitized. This value is then compared to a preset digital reference. When the processed photocell output exceeds that of the reference for 4 s, a peristaltic pump is turned on for a preselected period of time allowing for the addition of medium to the culture. A corresponding volume of suspension is displaced from the algal chamber via the overflow tube. After a latent time of 40 s to allow for mixing of any newly added medium, the

monitoring cycle is reinitiated. Turbidity is monitored and controlled by a microcomputer (SYM-1, Synertek Corporation) and ancillary electronics built in house. With this microcomputer system, eight culture systems can be controlled simultaneously.

Either air or CO₂-enriched air is admitted through the fritted bottom of the culture module at a flow rate of about 800 cm³/min; this flow rate is sufficient to provide relatively rapid mixing and prevent cell settling.

The culture systems shown in Figs. 2 and 3 use identical control systems to that of Fig. 1. The three systems differ primarily in their geometry.

The culture systems used earlier, shown in Figs. 4 and 5, employed an analog system constructed in-house to control the cell density. In this system, the output of the photocell is amplified, integrated (to remove the ac component from the light), and compared to a reference voltage. When the processed photocell output differs sufficiently from that of the reference for several seconds (we generally used 4 s), a modified Teflon solenoid valve (Valcor 51C56T34-ID) is triggered to admit a preselected amount of nutrient (about 6 ml) to the culture. After a latent time (40 s) to allow for mixing, the monitoring cycle is reinitiated.

Measurements of Cell Characteristics

Chlorophyll was determined by adding an aliquot of algal culture to a 1:1 mixture of Triton X-100 and 5% KOH in MeOH, heating at 63°C for 3 min, and centrifuging. Optical density was measured at 645 nm. Packed cell volume (PCV) was determined by centrifuging (clinical centrifuge, high speed) 1 ml of algal culture in a modified hemocrit vessel. Cell density was determined using a Coulter Counter Model TAI1 with PCAII accessory. Dry weight determinations involved filtering a 10-ml aliquot of algal culture through glass fiber filter (approximate retention 2.6 μm), rinsing thoroughly with distilled water, and drying at 110°C. (The filter paper was dried overnight at 110°C prior to weighing; dried samples were cooled to room temperature in a dessicator before weighing.) The growth rate of the culture was determined by

measuring the volume that overflowed during a defined time period (usually 16-24 hr). The productivity (mg/hr) of an algal culture is defined as the product of the dry weight (mg/ml) of the culture and the overflow rate (ml/hr).

Light Measurements

Light intensity was measured with an ISCO Model SR spectroradiometer between 400-700 nm in increments of 25 nm; these data were integrated to yield light intensity in units of $\mu\text{W}/\text{cm}^2$. Measurements were made at eight points around the culture apparatus, and the values averaged to correct for any lack of symmetry. Light efficiency was calculated using the absorbed light intensity (cal/hr) and the biomass productivity, converted from mg/hr to cal/hr, assuming a conversion of 5.5 cal/mg. (See, e.g., R.L. Miller and C.H. Ward, in *Atmosphere in Space Cabins and Closed Environments*, pp. 186-222, K. Kammermeyer, ed., Appleton-Century-Crofts, New York, 1966).

Carbon and Nitrogen Analyses

A 10-ml volume of algal culture was centrifuged at 10,000 RPM for 10 min, and an aliquot of the supernatant acidified with 0.05 ml concentrated H_2SO_4 /ml supernatant. Glycolic acid was determined by heating (100°C for 20 min) the acidified supernatant in a 4-fold greater volume of 0.01% 2,7-dihydroxynaphthalenediol in concentrated H_2SO_4 (Calkins, *Anal. Chem.* 15:762, 1943). Absorbance was measured at 530 nm with a Cary 15 spectrophotometer. Total excreted carbon was determined by measuring the chemical oxygen demand of the supernatant (Oceanographic International Corporation's Standard Ampule C.O.D. method). The nitrate concentration of the supernatant and growth media were determined on a Dionex Model 16 ion chromatograph. Nitrogen analysis of the freeze-dried algal pellet was done by an outside laboratory (Galbraith Laboratories, Knoxville, TN).

Preparation of Axenic Cultures of *Spirulina platensis*

S. platensis is a difficult organism to purify since bacteria can reside within the polysaccharide sheath as well as in the surrounding medium. Several attempts were made to purify Spirulina using standard methods before we found one that was successful.

The method that produced axenic Spirulina platensis was adapted from T. Ogawa and G. Terui (J. Ferment. Technol., Vol. 48, No. 6, pp. 361-367, 1970). Our procedure was as follows:

1. Filter a 10-14 day old culture through an ethanol-sterilized Whatman #540 filter (pore size 8 μ m) housed in a Millipore filter assembly.
2. Transfer algae into 30 ml sterile media and resuspend.
3. Repeat Step 1.
4. Transfer algae into \geq 150 ml sterile media and resuspend.
5. Homogenize algae filaments for 40 s in an ethanol-sterilized homogenizer assembly.
6. Repeat filtration as in Step 1.
7. Transfer and resuspend as in Step 2.
8. Filter as in Step 1.
9. Transfer and resuspend in 80 ml sterile media.
10. Expose to ultraviolet radiation (8-W bulb) for 7 min at a distance of 38 cm. Agitate culture during irradiation. After irradiation, cover and store in the dark for 3 h (to avoid photoreactivation of bacteria).
11. Dilute so that one drop contains one fragment of the algae. Inoculate 98 test tubes containing 5 ml media with one drop of the suspension and incubate in the light.

We found that Spirulina grew in 8 tubes, 4 of which were bacteria free.

APPENDIX B

"Algal Culture Studies Related to a Closed
Ecological Life Support System"

Presented at

6th Annual Meeting of the Commission on
Gravitational Physiology

Lausanne, Switzerland

September 18-21, 1984

Will be published in The Physiologist

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ALGAL CULTURE STUDIES RELATED TO A
CLOSED ECOLOGICAL LIFE SUPPORT SYSTEM

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Long-term cultures of *Scenedesmus obliquus* were maintained in an annular air-lift column operated as a turbidostat. We observed a linear relationship between the dry weight of the cultured cells, their cell number, and their chlorophyll content over a broad range of cell density at constant illumination. Thus, the cells did not appear to be adapting to differences in growth rate or light intensity during these experiments. Productivity vs dry wt rose linearly until the cell density reached a level at which light became limiting; at this point ~ 89% of the photosynthetically active radiation (PAR) was being absorbed. The maximum dilution rate of the system corresponded to a doubling time of 13.3 hr, about half the maximum growth rate generally observed at this temperature. Productivity at the maximum was ~ 80% of the maximum theoretical productivity. The rather low incident intensities (~ 10% of full sunlight) were a main contributing factor to the high light utilization efficiencies obtained in this system, since the cells were never driven into light saturation.

In many respects, algae would be ideal plant components for a biologically-based closed life support system, since they are eminently suited to the closely coupled functions of food production and atmosphere regeneration.* In this communication, we report some findings on the (steady-state) continuous culture of *Scenedesmus obliquus*, a physiologically well-characterized green alga with good growth characteristics.

METHODS

Description of Culture Apparatus

Long-term cultures were maintained in annular air-lift columns operated as turbidostats. These

* This idea was clearly recognized by an earlier generation of scientists (see, e.g., Bioregenerative Systems, NASA SP-165, 1968). A similar program is being carried out in the USSR (Gitel'son, I., et al., Problems of Space Biology, Vol. 28, Experimental Ecological Systems Including Man, NASA Technical Translation F-16993, 1975).

continuous culture systems are based on the earlier work of several other groups, notably Myers and Clark (J. Gen. Physiol. 28, 103-112, 1944); also, Kuhl and Lorenzen (In Methods in Cell Physiology, Vol. 1, O. Prescott, ed., pp. 159-187, Academic Press, NY, 1964); and Ammann and Lynch, (Appl. Microbiol. 13, 546-551, 1965). A primary goal of the design and construction of our culture systems was to provide a means to control and monitor important physiological parameters, such as light flux, light absorption, temperature, and growth rate. Another important consideration was our goal of constructing a system that was harvestable on demand. This latter requirement precluded the use of a chemostat system; instead, we use the turbidostat system described below.

Figure 1 is a diagram of the culture apparatus constructed and used in our laboratory. The culture system is made of transparent glass and consists of three concentric chambers.

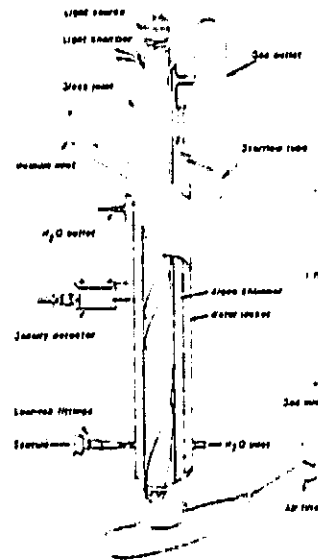


Figure 1.

The innermost chamber houses the illuminating source (a standard 40-watt, cool white, high-output, fluorescent bulb). The middle chamber, with a volume of 890 ml and a width of 1.0 cm, contains the algal culture. Temperature control is provided by a refrigerated bath (Neslab RTE-4; $\pm 0.01^\circ\text{C}$ temperature control from -30°C to $+100^\circ\text{C}$) which circulates water through the outermost chamber.

Cell density is maintained by monitoring the light transmission through the culture using a photoconductive cell (Clairex CL604L). The output of this photocell is amplified, integrated (to remove the ac component from the light), and digitized. This value is then compared to a preset digital reference. When the processed photocell output exceeds that of the reference for 4 s, a peristaltic pump is turned on for a preselected period of time allowing for the addition of medium to the culture. A corresponding volume of suspension is displaced from the algal chamber via the overflow tube. After a latent time of 40 s to

allow for mixing of any newly added medium, the monitoring cycle is reinitiated. Turbidity is monitored and controlled by a microcomputer (SYM-1, Synertek Corporation) and ancillary electronics built in house. With this microcomputer system, eight culture systems can be controlled simultaneously.

Either air or CO₂-enriched air is admitted through the fritted bottom of the culture module at a flow rate of about 300 cm³/min; this flow rate is sufficient to provide relatively rapid mixing and prevent cell settling.

Measurements of Cell Characteristics

Chlorophyll was determined by adding an aliquot of algal culture to a 1:1 mixture of Triton X-100 and 5% KOH in MeOH, heating at 53°C for 3 minutes, and centrifuging. Optical density was measured at 645 nm. Packed cell volume (PCV) was determined by centrifuging (clinical centrifuge, high speed) 1 ml of algal culture in a modified hemocrit vessel. Cell density was determined using a Coulter Counter Model TAPI with PCAII accessory. Dry weight determinations involved filtering a 10-ml aliquot of algal culture through glass fiber filter (approximate retention 2.5 μm), rinsing thoroughly with distilled water, and drying at 110°C. (The filter paper was dried overnight at 110°C prior to weighing; dried samples were cooled to room temperature in a desiccator before weighing.) The growth rate of the culture was determined by measuring the volume that overflowed during a defined time period (usually 16-24 hr). The productivity (mg/hr) of an algal culture is defined as the product of the dry weight (mg/ml) of the culture and the overflow rate (ml/hr).

Light Measurements

Light intensity was measured with an ISCO Model SR spectroradiometer between 400-700 nm in increments of 25 nm; this data was integrated to yield light intensity in units of μW/cm². Measurements were made at 8 points around the culture apparatus, and the values averaged to correct for any lack of symmetry. Light efficiency was calculated using the absorbed light intensity (cal/hr) and the biomass productivity, converted from mg/hr to cal/hr, assuming a conversion of 5.5 cal/mg. (See, e.g., R.L. Miller and C.H. Ward, in *Atmosphere in Space Cabins and Closed Environments*, pp. 186-222, K. Kammermeyer, ed., Appleton-Century-Crofts, New York, 1966.)

Carbon and Nitrogen Analyses

A 10-ml volume of algal culture was centrifuged at 10,000 RPM for 10 min, and an aliquot of the supernatant acidified with 0.05 ml concentrated H₂SO₄/ml supernatant. Glycolic acid was determined by heating (100°C for 20 min) the acidified supernatant in a 4-fold greater volume of 0.01% 2,7-dihydroxynaphthalenediol in concentrated H₂SO₄ (Calkins, Anal. Chem. 15:762, 1943). Absorbance was measured at 530 nm with a Cary 15 spectrophotometer. Total excreted carbon was determined by measuring the chemical oxygen demand of the supernatant (Oceanographic International Corporation's Standard Ampule C.O.D. method). The nitrate concentration of the supernatant and growth media were

determined on a Dionex Model 16 ion chromatograph. Nitrogen analysis of the freeze-dried algal pellet was done by an outside laboratory (Galbraith Laboratories, Knoxville, TN).

RESULTS AND DISCUSSION

Characteristics of Cultural Cells

Figure 2 illustrates the linear relationship between the dry weight of the cultured cells (mg/ml), their cell number (cells/ml), and their chlorophyll content (μg chl/ml). These data indicate that the relationships between cell mass, cell population, and chlorophyll/cell are constant over the range of culture conditions tested. Thus, the cells do not appear to be changing or adapting to differences in growth rate or light intensity

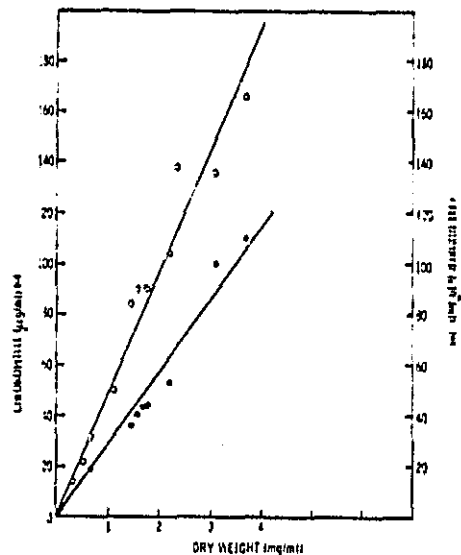


Figure 2.

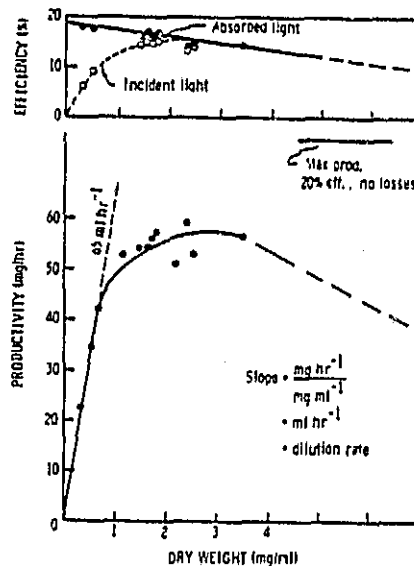


Figure 3.

during these experiments (see, e.g., Myers, Proceedings of the IBP/PP Technical Meeting, Trebon, 1970). The slopes of the two lines (computed by standard statistical techniques) are 2.8×10^7 cells/mg dry wt and $48.3 \mu\text{g chl/mg dry wt}$, respectively. This corresponds to $1.73 \times 10^{-6} \mu\text{g chl}$ and $3.6 \times 10^{-5} \mu\text{g dry cell mass per cell}$.

Productivity and Light Efficiency

Figure 3 illustrates the relationships between culture productivity (lower panel) and light utilization efficiency (upper panel) vs dry weight observed in a series of experiments in which Scenedesmus was maintained in the continuous culture system shown in Figure 1. The productivity vs dry weight curve rises linearly until the cell density reaches a level at which light becomes limiting ($\sim 1.4 \text{ mg ml}^{-1}$ or $\sim 48 \mu\text{g chl ml}^{-1}$). At this point, $\sim 89\%$ of the photosynthetically active radiation (PAR) is being absorbed. In the initial linear portion of the curve, productivity is limited by cell growth at the given light intensity. The slope of this initial portion reflects the maximum dilution rate of the system (dimensions of ml hr^{-1}). In the present instance, this rate is 65 ml hr^{-1} , which corresponds to a doubling time of 3.3 hr. This fairly low growth rate (about half the maximum growth rate generally observed at this temperature) reflects the rather low intensity of the light source ($\sim 10\%$ of full sunlight at the inner wall of the algal culture chamber). These low incident intensities are a main contributing factor to the high light-utilization efficiencies obtained in this system, since the cells are never driven into light saturation (see Appendix).

The slope of the line drawn through zero and any point on the productivity curve corresponds to the dilution rate, and therefore the doubling time and growth rate, at this point. The productivity curve rises to a value of $\sim 58 \text{ mg hr}^{-1}$ at $\sim 3 \text{ mg ml}^{-1}$, which is $\sim 80\%$ of the maximum theoretical productivity ($\sim 20\%$ on an energy basis; see, e.g., Radmer, R. and B. Kuk, in Encyclopedia of Plant Physiology, Vol. 5, New Series, pp. 125-135, A. Trebst and M. Avron, eds., Springer-Verlag, Berlin, 1977). One would predict that the productivity would gradually decrease at very high cell densities, since increasing amounts of biomass (with finite and significant maintenance energy) would be supported by a constant amount of absorbed incident light ($\sim 97\%$ absorption at 2.5 mg ml^{-1}). However, it is not practical to obtain such data in the present system.

The upper panel of the figure shows the efficiencies* of absorbed and incident light as a function of cell density (dry weight). The efficiency with respect to absorbed light (solid line) appears to be a steadily-decreasing (linear?)

* These light efficiency measurements contain the following primary sources of error: 1) a small volume at the bottom of the culture ($\sim 10\%$ of the total volume) that is not significantly illuminated; 2) the overflow cell density is only 89% of the reactor density; 3) the light measurements represent the average of a somewhat asymmetrical cylindrical light field; and 4) room light was not excluded ($< 3\%$ of total incident light). All these errors are relatively small, and the more significant ones tend to cancel.

function of cell mass, reflecting 1) lack of light saturation due to the low incident intensity (see above), and 2) the significant maintenance energy required by the (increasing) biomass. If we assume the reality of the linear relationship, the maximum efficiency of absorbed light in this system is 19% . The slope of the line, $1.28\% (\text{mg/ml})^{-1}$, reflects losses due to maintenance energy, which is probably linear over the rather narrow conditions tested. Since 100% efficiency corresponds to $0.3064 \text{ W hr mg}^{-1}$, the maintenance energy is $6.19 \times 10^{-5} \text{ W hr mg}^{-1}$, and one would predict that the culture would reach light compensation at a dry wt of 14.3 mg ml^{-1} .

The efficiency with respect to incident light (dotted line) reflects the balance between light absorption and utilization (the initial ascending phase) and losses due to the cellular metabolism (descending phase). In the present system, the maximum light utilization efficiency occurs at i_{\cdot} to 2.0 mg ml^{-1} dry wt. The descending phase becomes identical to the "absorbed light" curve at high cell densities.

Nitrogen Utilization Efficiency

Krauss et al. (Proc. 21st Plenary Meeting, Committee on Space Research (COSPAR), 1978) reported that a significant fraction of the NO_3^- nitrogen provided to Chlorella cultured in a "recyclostat" was lost, probably as N_2O . The release of this gas into the atmosphere of a closed system could cause grave problems for the air regeneration system, as well as contribute to a lack of closure of the nitrogen cycle.

Because of these earlier results, one of our goals was to determine the nitrogen balance of Scenedesmus cultures, and specifically, whether compounds such as N_2O were excreted into the medium. Our initial approach was to determine the nitrogen levels of the nutrient medium, cell-free efflux, and harvested algae.

The results of these experiments (Table I) show surprisingly good agreement between added and recovered N (average 100.5% recovery) and suggest that the nitrogen entering the culture (as NO_3^-) was either incorporated in the algae or appeared as NO_3^- in the efflux supernatant. We have had no indication to date that the nitrogen is lost by the system. Although these results do not prove that there is no nitrogen loss, they do suggest that any loss must be small (e.g., $< 1\%$), at least for NO_3^- .

TABLE I

| Sample | Nitrogen (mg/l) | | | Recovery (%) |
|--------|-----------------|-------------|-------|--------------|
| | Medium | Supernatant | Algae | |
| 1 | 339 | 216 | 127 | 101.2 |
| 2 | 293 | 120 | 171 | 99.3 |
| 3 | 290 | 123 | 171 | 101.4 |
| 4 | 290 | 122 | 168 | 100.0 |

Because our data obtained with Scenedesmus point to a very low production of nitrogenous by-products, we have not attempted to determine N_2O directly. (Current data indicate that the N_2O

concentration in the gas stream would be too low to monitor directly.) We have observed only traces of NO_2^- (< 1 ppm) in the effluent supernatant.

Long-Term Culture

We maintained continuous *Scenedesmus* cultures of this type for about 4 months before voluntary shut down. The cultures were monitored daily for packed cell volume (PCV), chlorophyll, dry weight, reproduction rate, and pH, and intermittently for glycolate, total N, and microbial contamination. Representative data from these long-term experiments is shown in Figure 4. Note that the culture system displays good long-term stability.

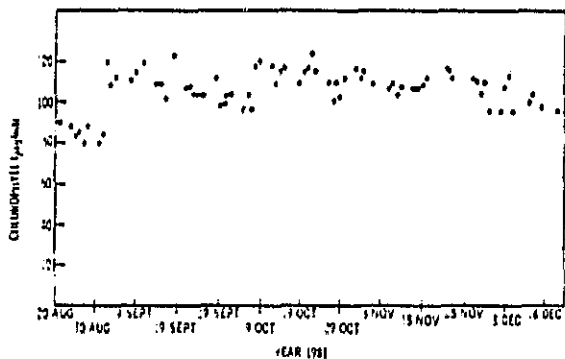


Figure 4.

We addressed the interrelated topics of algal by-product excretion and microbial contamination by periodically assaying the culture supernatant. To date, we have detected no significant glycolate (< 1 ppm of this primary algal excretory product), or excreted carbon (< 25 ppm) while our cultures were in the steady state. Parallel microbial assays in some cases indicated a low (0.1 - 0.01%) non-algal biomass that did not change appreciably (with time) with respect to amount or species composition. These findings suggest that microbial contamination should not be a significant problem in such cultures because 1) the algae seem to excrete little or no organic compounds; and 2) microbial populations, even when present, do not take over the culture.

APPENDIX

Relationship of Light Intensity to

Turnover of the Photosynthetic Apparatus

One of the primary limitations of most photosynthetic organisms is that they do not perform well in strong light (e.g., full sunlight). The photosynthetic apparatus operates somewhat like a lens; approximately 200 "light-harvesting" chlorophyll molecules transfer light energy to a reaction center, with a corresponding increase in the effective light intensity per center. Consequently, efficiency can be very high in weak light, but drops off rapidly at intensities approaching that of bright sunlight, due to the rather slow (ms) turnover of the dark reactions (see, e.g., R. Radmer and B. Kok, in *Encyclopedia of Plant Physiology*, Vol. 5, New Series, pp. 125-135, A. Trebst and M. Avron, eds., Springer-Verlag, Berlin, 1977).

Figure 5 is an idealized cross sectional diagram of our algal culture system. The light flux values (in units of photosynthetically-active quanta $\text{cm}^{-2}\text{s}^{-1}$) were measured in the absence of algae. Note that they closely follow a $1/r$ relationship, suggesting that the total flux is conserved (except for losses due to reflection and absorption by the glass walls) during its passage through the concentric cylinders.

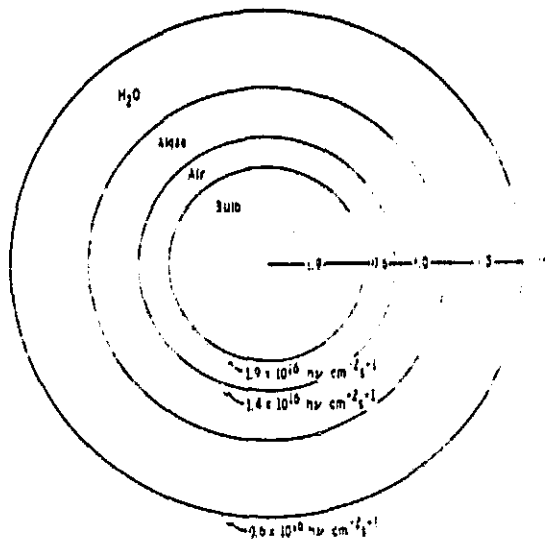


Figure 5.

A rough estimate of the light flux per reaction center can be made as follows. Our data indicate that *Scenedesmus* has a specific absorption coefficient of $\sim 0.6 \text{ l cm}^{-1} \text{ g}^{-1}$ over the spectral range of 400-700 nm (specific data not shown), and a chlorophyll content of 4.8% (Figure 2; see also J. Myers, in *Encyclopedia of Chemical Technology*, pp. 33-51, R. Kirk and D. Othmer, eds., Interscience, NY, 1957). Note that $0.6 \text{ l cm}^{-1} \text{ g}^{-1}$ corresponds to $(0.6) (1000 \text{ cm}^3) \text{ cm}^{-1} \text{ g}^{-1}$ or $600 \text{ cm}^2 \text{ g}^{-1}$. Thus, the equivalent specific absorption is $1.2 \times 10^4 \text{ cm}^2 (\text{g chlorophyll})^{-1}$, which is about 10% of the extinction coefficient observed at the absorption maxima. Since one g chlorophyll = 6×10^{20} molecules, the molecular cross-section (= extinction coefficient) is $0.2 \times 10^{-16} \text{ cm}^2 (\text{chlorophyll molecule})^{-1}$.

The maximum quantum flux that the algae are subjected to is $1.4 \times 10^{16} \text{ quanta cm}^{-2} \text{ s}^{-1}$ or $0.28 \text{ quanta molecule}^{-1} \text{ s}^{-1}$. Since each reaction center is connected to ~ 200 chlorophyll molecules (see above), the maximum quantum flux will result in the transfer of $\sim 60 \text{ quanta s}^{-1}$ to each reaction center.

This value is well below the generally accepted maximum reaction center turnover rate of $\sim 100 \text{ s}^{-1}$ (Radmer and Kok, op.cit.). Thus the photosynthetic dark reactions are able to keep pace with the light flux, the system does not become light saturated, and high light efficiencies can be obtained.

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Controlled Ecological Life Support Systems (CELSS):
A Bibliography of CELSS Documents Published as NASA Reports

1. Johnson, Emmett J.: Genetic Engineering Possibilities for CELSS: A Bibliography and Summary of Techniques. (NASA Purchase Order No. A73308B.) NASA CR-166306, March 1982.
2. Hornberger, G.M.; and Rastetter, E.B.: Sensitivity Analysis as an Aid in Modelling and Control of (Poorly-Defined) Ecological Systems. (NASA Purchase Order No. A77474.) NASA CR-166308, March 1982.
3. Tibbitts, T.W.; and Alford, D.K.: Controlled Ecological Life Support System: Use of Higher Plants. NASA CP-2231, 1982.
4. Mason, R.M.; and Carden, J.L.: Controlled Ecological Life Support System: Research and Development Guidelines. NASA CP-2232, 1982.
5. Moore, B.; and R.D. MacElroy: Controlled Ecological Life Support System: Biological Problems. NASA CP-2233, 1982.
6. Aroeste, H.: Application of Guided Inquiry System Technique (GIST) to Controlled Ecological Life Support Systems (CELSS). (NASA Purchase Order Nos. A82705B and A89697B.) NASA CR-166312, January 1982.
7. Mason, R.M.: CELSS Scenario Analysis: Breakeven Calculation. (NASA Purchase Order No. A70035B.) NASA CR-166319, April 1980.
8. Hoff, J.E.; Howe, J.M.; and Mitchell, C.A.: Nutritional and Cultural Aspects of Plant Species Selection for a Controlled Ecological Life Support System. (NASA Grant Nos. NSG-2401 and 2404.) NASA CR-166324, March 1982.
9. Averner, M.: An Approach to the Mathematical Modelling of a Controlled Ecological Life Support System. (NASA Contract No. NAS2-10133.) NASA CR-166331, August 1981.
10. Maguire, B.: Bibliography of Human Carried Microbes' Interaction with Plants. (NASA Purchase Order No. A77042.) NASA CR-16630, August 1980.
11. Howe, J.M.; and Hoff, J.E.: Plant Diversity to Support Humans in a CELSS Ground-Based Demonstrator. (NASA Grant No. NSG-2401.) NASA CR-166357, June 1982.
12. Young, G.: A Design Methodology for Nonlinear Systems Containing Parameter Uncertainty: Application to Nonlinear Controller Design. (NASA Cooperative Agreement No. NCC 2-67) NASA CR-166358, May 1982.

13. Karel, M.: Evaluation of Engineering Foods for Controlled Ecological Life Support Systems (CELSS). (NASA Contract No. NAS 9-16008.) NASA CR-166359, June 1982.
14. Stahr, J.D.; Auslander, D.M.; Spear, R.C.; and Young, G.E.: An Approach to the Preliminary Evaluation of Closed-Ecological Life Support System (CELSS) Scenarios and Control Strategies. (NASA Cooperative Agreement No. NCC 2-67) NASA CR-166368, July 1982.
15. Radmer, R.; Ollinger, O.; Venables, A.; Fernandez, E.: Algal Culture Studies Related to a Closed Ecological Life Support System (CELSS). (NASA Contract No. NAS 2-10969) NASA CR-166375, July 1982.
16. Auslander, D.M.; Spear, R.C.; and Young, G.E.: Application of Control Theory to Dynamic Systems Simulation. (NASA Cooperative Agreement No. NCC 2-67) NASA CR-166383, August 1982.
17. Fong, F. and Funkhouser, E.A.: Air Pollutant Production by Algal Cell Cultures. (NASA Cooperative Agreement No. NCC 2-102) NASA CR-166384, August 1982.
18. Ballou, E. V. : Mineral Separation and Recycle in a Controlled Ecological Life Support System (CELSS). (NASA Cooperative Agreement No. NCC 2-53) NASA CR-166388, March 1982.
19. Moore, B., III; Wharton, R. A., Jr.; and MacElroy, R. D.: Controlled Ecological Life Support System: First Principal Investigators Meeting. NASA CP-2247, 1982.
20. Carden, J. L. and Browner, R.: Preparation and Analysis of Standardized Waste Samples for Controlled Ecological Life Support Systems (CELSS). (NASA Cooperative Agreement No. NCA 2-OR260-102) NASA CR-166392, August 1982.
21. Huffaker, R. C.; Rains, D. W.; and Qualset, C. O.: Utilization of Urea, Ammonia, Nitrite, and Nitrate by Crop Plants in a Controlled Ecological Life Support System (CELSS) (NASA Cooperative Agreement No. NCC 2-99) NASA-CR 166417, October 1982.
22. Gustan, E. and Vinopal, T.: Controlled Ecological Life Support System: Transportation Analysis. (NASA Contract No. NAS2-11148) NASA CR-166420, November 1982.
23. Raper, C. David, Jr.: Plant Growth in Controlled Environments in Response to Characteristics of Nutrient Solutions. (NASA Cooperative Agreement No. NCC 2-101) NASA CR-166431, November 1982.
24. Wydeven, T.: Composition and Analysis of a Model Waste for a CELSS. NASA Technical Memorandum 84368, September 1983.

25. Averner, M., Karel, M., and Radmer, R.: Problems Associated with the use of Algae in Bioregenerative Life Support Systems. (NASA Cooperative Agreement No. NCC 2-210) NASA CR-166615, November 1984.