AN INVESTIGATION OF TECHNIQUES IN ISOLATION AND CULTURE OF THE ALGAL FLORA OF SOILS

by

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"By soil algae I mean the algae growing on and in the earth." - Petersen (1935)

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Historical Note

Although taxonomic and ecological interest in the soil algae did not arise until centuries later, Lyngbye, according to Petersen (1935), states that in Paracelsus is to be found the word Nostoc, presumably Nostoc commune. The work of Feher (1948) shows this species of Cyanophyta to be of world-wide distribution.

Real interest in the taxonomy and ecology of soil algae did not come until early in the twentieth century, however, and the efforts of Esmark in 1911 and 1914 (Petersen, 1935) are probably the first worthy of consideration in connection with the present investigation. Esmark placed soil samples in glass dishes, pressed sterile paper covers over the soil surface, and wetted the whole with sterile water. The dishes were then incubated in the chance conditions of window light and room temperature. The 1911 effort yielded 24 species of algae, according to Petersen, while in 1914, 45 species were obtained, all members of the phylum Cyanophyta.

Robbins (1912), investigating the nitrogen fixing capacity of Colorado soils, obtained results qualitatively similar to those of Esmark. Soil samples were introduced into flasks containing pure quartz crystals, after which sterile water was added, thus affording the growing cells no nutriment other than that leached from the soil sample itself. Incubation was carried out in window

light and at room temperature. Robbins obtained a list of 12 Cyanophyta and 1 diatom.

Soils of the Missouri Botanical Gardens were investigated for the distribution of surface and subsurface forms by Moore and Karrer (1919) and Moore and Carter (1926). In both instances the culture medium consisted of an aqueous solution of inorganic salts and incubation was accomplished in window light and at room temperature. Moore and Karrer obtained 10 Cyanophyta, 4 Chlorophyta, and 3 diatoms; Moore and Carter identified 7 Cyanophyta, 10 Chlorophyta, and 4 diatoms.

The vitality of soil inhabiting cells was investigated by Bristol (1920) in England. Samples from soils stored in a desiccated condition were placed in an aqueous solution of inorganic salts and incubated in window light and at room temperature. Twenty-two Cyanophyta, 19 Chlorophyta, and 16 diatoms were reported. The algal flora of Australian soils was examined under the same cultural conditions by Phillipson (1934), who secured 9 Cyanophyta, 18 Chlorophyta, 5 Heterokontae, and 2 diatoms.

James (1935), in England, performed experiments directed toward ascertaining the distribution of algae in uncultivated soils. Samples were placed in liquid and agarized inorganic salts solutions and incubated in window light and at room

temperature. Thirteen Cyanophyta, and 5 Heterokontae are reported as having appeared in these cultures.

In Denmark, Petersen (1935) published the results of an extended series of physiological, ecological, and taxonomic investigations of soil algae. Utilizing sometimes the moist soil culture of Esmark and sometimes liquid cultures containing inorganic salts, and incubating in window light or in darkness (according to the dictates of the experiment), Petersen obtained and identified 36 Cyanophyta, 38 Chlorophyta, 49 diatoms, and 12 other Chrysophyta.

Interested in the source of certain aquatic algae, Fenton (1938) examined appropriate Scottish soils by establishing moist soil cultures and incubating them in window light and at room temperature. Nine species of algae were identified.

Fritsch and John (1942) performed an ecological investigation of English soils in an attempt to establish algal distribution in relation to certain soil factors. Twenty-one Cyanophyta, 30 Chlorophyta, 20 diatoms, 1 Rhodophyta, 1 Euglenophyta, and 12 Chrysophyta were obtained from liquid and moist soil cultures containing inorganic salts in some cases and distilled water in other cases. Incubation was carried out in window light and at room temperature.

From Florida soil samples Smith and Ellis (1943) obtained 25 Cyanophyta and 11 Chrysophyta, utilizing liquid cultures containing inorganic salts and incubating in the ordinary manner. Smith (1944), however, in a subsequent investigation, incubated liquid and moist soil cultures under entirely artificial conditions. Growth was obtained in 14-18 days from cultures maintained at 25-30 °C., and in 200 foot candles of illumination. Smith identified in this instance 29 Cyanophyta, and 5 Chlorophyta.

The algal distribution in relation to certain chemical characteristics of the soil was investigated in England by Lund (1947). Little culturing of the soil samples was done, however, for most identification proceeded along the lines of what Lund termed "direct observation." A few moist soil cultures and agarized cultures were established as checks and incubated in window light and at room temperature. Lund reported 16 Cyanophyta, 29 Chlorophyta, 7 Xanthophyceae, 3 Chrysophyceae, and 2 Englenophyta.

The latest and by far most extensive survey of soil algae was completed by Feher (1948). One hundred and twenty-two soil samples were collected from all parts of the world and placed in flasks of an aqueous solution of inorganic salts. Incubation was carried out in window light and at room temperature. An extensive list of 685 species resulted; 282 Cyanophyta, 236 Chlorophyta; 142 "Zygophyceae", and 25 "Flagellata".

Concurrent investigations contributed much, on an indicative scale, to the physiology of the soil algae. For the sake of completeness the more important of these may well be mentioned here. Fritsch (1922) investigated the moisture relationships of several aquatic and terrestrial algae. Bristol (1926, 1927) characterized a small group of species isolated from the soil by demonstrating their responses to various soluble carbon compounds. Skinner and Gardner (1930) demonstrated the characterizing responses of several soil algae to certain nitrogen containing organic compounds. Allison and Morris (1930), De (1939), and Stokes (1940) investigated nitrogen fixation by certain soil algae.

Objectives

Figure 1 compares several factors common to the fifteen surveys just reviewed. The lack of repeatable cultural conditions is outstanding. Similarly, although isolation was accomplished in several instances, in no reported case was the method adequately discussed. In view of these omissions, the following objectives were established for this investigation:

1- To investigate equipment, methods, and conditions encouraging the rapid growth of soil algae under artificial, controlled conditions.

2- To investigate equipment, methods, and conditions allowing the easy derivation of unialgal cultures from a mixed microflora such as that found in the soil.

As a corollary to these it is desirable, even necessary, to at least tentatively identify the species thus isolated and cultured.

Materials and Methods

Three composite samples were formed, one each of the following soil situations:

1- Fertilised garden soil.

2- Fertilised, cultivated field soil.

3- Unfertilised, fallow field soil.

Each composite was formed of the contents of 20 borings made with a 1 inch, boring type, soil sampler. Each boring was made to a depth of 12 inches unless limited by a mantle of less depth. After the completion of each set of 20 borings, the sampler was washed and sterilised with 70 % alcohol. The 20 borings of a given composite were placed in a clean, glass, gallon jar fitted with a screw type lid and taken to the laboratory for immediate introduction into culture media.

Since no ecological results were being sought in this investigation, no attempt was made to discover the history of any sampling site. Only the appearance of a given site was used in determining which of the three classes each sample fitted.

A series of primary enrichment cultures was set up, featuring a variety not only of media, but of culture type. Figure 2 shows the organic and inorganic media selected, the culture types, and the combinations resulting. One such series was set up for each of the three composite samples.

Media formulae were taken directly from Bold (1942). In the preparation of media, chemicals of the reagent level and hard glass distilled water were used. The following list shows the media selected, and the way each was prepared for the present investigation.

Inorganic Media:

1- Knop's solution (1865). To avoid precipitation the stock solution was prepared in two parts, which combined, represent a concentration of 0.7% by weight volume.

Part A		Part B	
$Ca(NO_3)_2 \cdot 4H_2O$	4.0 gram	KNO_3	1.0 gram
Distilled water	500.0 cc.	KH_2PO_4	1.0 gram
		$MgSO_4 \cdot 7H_2O$	1.0 gram
		$FeCl_3 \cdot 6H_2O$ (1.0% aq. sol.)	1 drop
		Distilled water	500.0 cc.

In the present investigation this medium was used in a concentration of 0.35% by weight volume, or, 1/2 part A, 1/2 part B, and 1 part distilled water. The pH was adjusted to values of 5.6 and 6.4 with KOH, thus producing two media.

2- Beijerinck's solution (1898). This is frequently misnamed "Beneke solution" (Bold, 1942). As given below the concentration is 0.1% by weight volume and has a pH of 7.2. It was used here in a concentration of 0.05%. The formula is as follows:

$NH_4 NO_3$	0.5 gram
K_2HPO_4	0.2 gram
$MgSO_4 \cdot 7H_2O$	0.2 gram
$CaCl_2 \cdot 2H_2O$	0.1 gram
$FeCl_3$ (1.0% aq. sol.)	1 drop
Distilled water	1000.0 cc.

3- Detmer's solution (1888). The stock solution was prepared as follows:

$Ca(NO_3)_2$	1.0 gram
KCl	0.25 gram
$MgSO_4 \cdot 7H_2O$	0.25 gram
KH_2PO_4	0.25 gram
$FeCl_3$ (1.0% aq. sol.)	1 drop
Distilled water	1000.0 cc.

This stock was used in a concentration of 33.3%, or, 1 volume of the stock to 2 volumes of distilled water. The pH of such a concentration was 6.2.

Organic Media:

1- Bold's Soil Extract (1942). Two kilograms of garden soil were placed in a 5 liter pyrex flask and 2 liters of distilled water were added. This mixture was autoclaved at 15 lbs. for 2 hours, after which it was cooled and decanted, and filtered until the supernatant was clear, but not colorless. This stock solution was then resterilised at 15 lbs. for 15 min.

The medium used is defined as a 15.0% solution of the stock in distilled water, to which is added 1.0% of a 0.5% aqueous solution of KNO_3. A liter of soil extract as used in this investigation is thus composed of 150.0 cc of soil extract stock, 10.0 cc of 0.5% aqueous KNO_3, and 840 cc of distilled water. The pH was 7.2.

2- Egg Albumen "Faulkulture". Cultures were formed individually as follows:

Seven grams of fresh egg albumen were placed in the bottom of a sterile, 1/2 pint milk bottle and then covered with about 1 inch of sterile garden soil. To this was added a surface of sterile, washed sand, on top of which was placed 15.0 grams of the soil sample. Finally, 150.0 cc of sterile, distilled water was added carefully, so as not to disturb the layering.

3- "Fortified" Detmer's solution (1888). For this medium 0.5% glucose was added to a 1/3 dilution of the Detmer stock solution described above.

The following culture types were selected:

1- Liquid. These consisted of 150 cc of liquid medium placed in 1/2 pint, square milk bottles. The tops were plugged with cotton at the start, but later the cotton plugs were replaced with cardboard milk bottle caps pierced with three small holes. Six such cultures, varying as to medium (Figure 2) were set up for each of the composite samples. After the introduction of the medium, all bottles except those containing the "Faulkulture" were autoclaved at 15 lbs. for 15 min.

2 - Agar. To the Detmer and "Fortified" Detmer solutions, 1.5% agar was added and the resulting media used in the preparation of serially diluted pour plates of the bacteriological type. To combat excessive drying of these cultures the tops of the dishes were fitted with sterile filter paper disks which were kept moist with sterile distilled water. Later, these disks were removed and the plates were grouped in 14 inch moist chambers as in Figure 3.

3 - Moist Soil. Petri dishes were filled loosely to the top with portions of the composite soil sample. Sterile filter paper disks were then placed on the soil surface and pressed firmly down until the volume of the soil was reduced to approximately one-half of the petri dish. The filter paper disks and the soil were wetted with the appropriate medium (Figure 2) and the glass petri dish lids placed on top. Later, the rims of the bottom halves of the dishes were coated with vaseline and the tops replaced as before.

In the preparation of inocula, each composite soil sample was treated separately. The large lumps of a composite were broken up by screening the sample through ordinary wire house screen. One hundred grams of this raw sample were then placed in a two liter flask containing a liter of sterile, distilled water and shaken vigorously by hand for 10 minutes. Five, 10 cc aliquots of this

suspension were drawn off in sterile pipettes and used to inoculate each of five of the liquid cultures. The sixth liquid culture, the "Faulkulture", was inoculated with 15 grams of the pure soil sample. A 10 cc portion of the soil suspension was also used to inoculate the first water blank of a series of 13 serial dilutions destined to be used in the preparation of the agarized pour plates. The remainder of the raw sample was used *per se*, as the inoculum for the three moist soil cultures of a series.

In the inoculation of the serially diluted pour plates, the method of Bold (1942) was followed. Dilutions by tenths, ranging upward from the previously established 1:1, to 1:10M were prepared according to accepted bacteriological procedures. Duplicates of each dilution past 1:1T were also prepared. One of each of the dilutions was used to inoculate a pour plate to which 15 cc of the 1.5% agarized Detmer solution was added. The duplicates of each dilution above 1:10T were used to inoculate pour plates to which the 1.5% agarized "Fortified" Detmer medium was added. All plates were agitated gently but thoroughly to insure the dispersion of the inoculum throughout the medium. In this manner a group of 13 pour plates was accumulated for each composite sample.

To facilitate the incubation of these cultures and to allow for the control of light and temperature, the incubator diagrammed in Figure 4 was constructed. Figure 5 shows the areas of illumination intensity furnished by the 5 fluorescent tubes used as the light source. The particular tubes used in this investigation are characterized by Sylvania Electric Products, Inc., of Salem, Massachusetts, as "white, 20 watt". Temperature was maintained at 25-30 °C. by means of periodic adjustment of the air circulating system shown in Figures 4 and 15.

Figure 6 shows the equipment used in the separation of the mixed algal flora furnished by the primary enrichment cultures. The dimensions are given in Figure 7. The bit, shank and head of the agitator were made from a single length of nickel-alloy wire. The head size was made to fit loosely into the small vial shown in Figure 6. The metal medicine bottle cap, through which the shank of the agitator is threaded, was of such diameter as to fit the top of the glass vial snugly. In general operation, algal material was placed in the glass vial, which contained sterile medium, and macerated by agitation. The resulting inoculum was used to seed fresh cultures.

Separation of the mixed algal flora appearing in the primary enrichment cultures was not attempted until growth was well established. In the case of the liquid and the moist soil primary enrichment culture types, the following technique was employed:

A suitable amount of fresh medium of the kind used in the alga-containing primary enrichment culture was prepared and 0.7% agar added. After sterilization at 15 lbs. for 15 min. the medium was cooled to approximately 45 °C. and stored in a paraffin oven until needed. Two cc of hard glass distilled water were pipetted into each of a suitable number of the small glass vials, and 9 cc of the same into a duplicate set of the vials. These were corked, sterilized, and cooled. A similar number of petri dishes were wrapped in paper and sterilized.

Other equipment required for the operation were: a binocular dissecting microscope with 10x oculars and 4.8x objectives, a substage microscope lamp fitted with a flat stage arranged as in Figure 8, a bunsen burner, and several ordinary dissecting needles hammered and ground as in Figure 9.

The mixed algal material that was to be separated was collected according to the culture type from which it came. In the case of the liquid primary enrichment cultures, small bits of algal material were cut from the algal pellicle in as many

places as there were observed macroscopic differences, and placed in the macerating vial. Portions of the algal film lining the sides of the culture bottle were also removed and placed in the vial. As a final precaution, the culture bottle was shaken vigorously and then 1 cc of the medium withdrawn and placed in the vial. In the case of the moist soil primary enrichment cultures, algal material was taken from the surface of the soil in the culture dish and from subsurface portions as well. Surface portions were cut from the gelatinous matrix sheathing the soil surface wherever microscopic and macroscopic differences were observed. Subsurface sampling was confined to the edges of the dish. All portions of the material were placed in one of the macerating vials.

The agitator portion of the macerator was sterilized by flaming. When the agitator was cool, the cork was withdrawn from the maceration vial and the agitator inserted. The agitator cap was held snugly over the top of the vial, and the algal material macerated. From time to time the maceration process was stopped momentarily to measure the completeness of maceration. When all macroscopically visible clumps had disappeared, maceration was discontinued.

Depending upon the amount of algal material in the resulting inoculum, dilution was, or was not, carried out before the inoculation of the pour plate. If the inoculum was relatively heavy, it was first diluted by the addition of 1 cc of the inoculum to one of the 9 cc water blanks. If the inoculum was relatively light, it was used *per se*. In either case, 1 cc of the final inoculum was pipetted into a sterile petri dish for the preparation of a bacteriological type pour plate. Approximately 20 cc of the still liquid, 0.7% agarized medium was added. The medium and inoculum were then well mixed by a gentle and continued swirling motion of the covered plate, and the plate set aside to cool and harden.

When eight such plates were accumulated, they were placed together in a moist chamber as shown in Figure 3. Incubation was carried out in the more brightly lighted areas of the incubator, and at 25-30 °C.

The progress of growth in the separation plates was followed closely. Plates were examined on the third or fourth day of incubation, and if not at the proper point of development, every two days thereafter. When the isolated colonies were large, but not overlapping, the plate was withdrawn and the "fishing" process executed.

At this point an additional number of sterile petri dishes were plated with 20 cc of 0.7% agarized medium of the same kind as that used in the separation plate, and set aside to harden. These were used to receive "fished" colonies from the separation plates.

For the "fishing" process, a separation plate that had reached the desired point of development was removed from its moist chamber, uncovered, and placed on the stage of the binocular dissecting microscope lamp. A spatulate dissecting needle was flamed, cooled, and an isolated colony removed from the separation plate and deposited on the surface of the medium of one of the new, transfer plates. Care was taken to make good contact between the transferred colony and the fresh medium, but at the same time "burying" of the colony was avoided. If the "fished" colony on the separation plate was sufficiently large several transfers were made from it to the same transfer plate. By this method "fishing" was continued until transfers of all microscopically different colonies had been secured. The transfer plates thus accumulated were grouped in moist chambers as before and incubated in the areas of brighter light and at 25-30 °C.

During the incubation period the final medium was prepared. This was done by placing 100.0 cc of the appropriate liquid medium in 1/2 pint, square milk bottles fitted with perforated, cardboard milk bottle caps and sterilising them at 15 lbs. for 15 min.

Incubation of the transfer plates was continued until the colonies reached a diameter size of one centimeter or better, in order to produce sufficient algal material for the remaining step. When a plate had developed to this point it was removed, placed on the stage of the binocular dissecting microscope, and uncovered for examination. Areas of the particular colony were located that were free of gross bacterial and fungal contamination. A portion of this area was removed and used in the preparation of a 0.3% agar mount on a glass microscope slide. The cells were examined under magnifications of 48x, 100x, 400x, and 950x to determine whether the colony was unialgal. At the same time sketches, descriptions of colonial growth, and tentative identification were made.

If the colony was unialgal, a second portion of the transfer colony was removed, placed in a maceration vial, and macerated. The entire inoculum thus produced was used to seed one of the previously prepared liquid culture bottles. In the event that microscopic examination revealed algal contamination in the transfer colony, the material was macerated and the resulting inoculum used in the preparation of a new separation plate, thus repeating the process.

The new separation plates were grouped in moist chambers and placed in the incubator as before. The liquid culture bottles

were racked on their sides at an angle producing the greatest liquid surface, in lines running across the incubator, thus exposing them to a range of light intensities.

In an attempt to glean some additional information on the effect of various agar concentrations and various light intensities on the growth of algal cells, the following experiments were set up.

1 - Nine pairs of bottled media were prepared and sterilized, one bottle containing 100.0 cc of Detmer's solution (1888)in 1/5 dilution, the other bottle of a pair containing the same medium with 0.3% agar added. Nine sets of inocula were prepared by the maceration of material from nine randomly selected cultures. Each pair of culture bottles was then seeded by the introduction of 2 cc each of inoculum from one of the macerating vials. Incubation was carried out at 25-30 °C., with the bottled racked in pairs on their side, in a line across the incubator to furnish light intensities ranging from 4 foot candles to 20 foot candles. The number of days elapsing before the macroscopic appearance of growth was noted and also which of each pair of bottles gave visual evidence of the heaviest growth at the end of one month.

2 - Ten transfer plates showing well established growth were selected in an otherwise random manner. One loopful of

cells was taken from each of these plates and streaked on the surface of a plate of 0.7% agarized Detmer's solution (1888) in 1/3 dilution. The ten resulting bacteriological type streak plates were then placed in moist chambers and incubated at 25-30 °C. and in approximately 30 foot candles of light. The first appearance of growth was noted for each plate.

3- Eight inocula were prepared from eight randomly selected cultures by taking one loopful of cells from one of the cultures, placing it in a macerating vial containing 2 cc of sterilized hard glass distilled water and agitating it to secure thorough mixing. Quadruplicate transfers were made from each of these eight inocula by transferring one loopful of the inoculum to each of thirty-two tubes, producing eight series of the following groups of four:

1- A tube containing 10.0 cc of Detmer's solution (1888) in 1/3 dilution.

2- A tube of the same, 0.15% agar added.

3- A tube of the same, 0.3% agar added.

4- A slant of the same, 0.7% agar added.

Incubation was carried out at 25-30 °C., and in 30-40 foot candles of light. The first appearance of growth was recorded for each. At the end of 11 days each series of four was inspected visually to determine which tube showed the heaviest growth.

To facilitate the tentative identification of the organisms isolated into unialgal culture, several established staining techniques that might be fitted into a rapid staining schedule were investigated. All killing and fixing fluids, stains, and staining schedules were taken from Johansen (1940). The following techniques were tried:

1- Simultaneous killing and fixing and mounting on slides by the osmium tetroxide technique, followed by extraction of chlorophyll in ether, in acetone, and in alcohol, followed by staining with Harris' hematoxylin, with Johansen's methyl violet-erythrosin, and with Flemming's triple stain.

2- Killing and fixing in Schaudinn's hot fluid, followed by chlorophyll extraction in ether, followed by staining with Harris' hematoxylin, all carried out in a single centrifuge tube according to the method of Dr. R. H. Rhodes of Emory University.

Results and Discussion

Although much has been accomplished in the taxonomy, ecology, and physiology of soil algae, the work has progressed slowly. An examination of the reported investigations reveals certain trends in methodology that are worthy of criticism. Figure 1 compares these factors as they are reported in the literature since 1910. Two of these factors are discussed in the following paragraphs.

There is a lack of established, general, cultural conditions leading to the rapid growth of soil algae. Most frequently window light and room temperature were sought, conditions which are highly variable at best, and hardly repeatable. Such seasonal and diurnal variations lead not only to slow growth, but the inevitable periods of darkness met in such a system encourage bacterial growth. Figure 1 shows the conditions for incubation employed in each of the sixteen surveys reviewed here, and the time before the appearance of growth whenever it was reported. It is notable that in only one - Smith (1944) - was there any attempt at controlled incubation. Even here, however, there is little chance for repetition, for the cultures were maintained in an open box and therefore subject to the fluctuations of sunlight and room temperature. By means of this system, Smith reported growth in 14-18 days.

Gerloff, Fitzgerald, and Skoog (1950), although working on an entirely different problem, grew aquatic algae in artificial conditions. What they termed "maximum growth" was obtained in 10-50 days at 25 °C. and in 40 foot candles of continuous, fluorescent light. It is indeed regrettable that this success was not reported more precisely. Such considerations as wave length of light, lighting arrangement, and growth in terms of hours or days before its appearance would have been of value in the present investigation.

There is also a lack of a facile isolation technique designed for the derivation of rapidly growing unialgal cultures. The procurement of unialgal (one species of alga, bacterial and fungal contamination allowed) cultures, or pure (one species of alga, no bacterial or fungal contamination allowed) cultures, is by no means as simple a task for the phycologist as it is for the bacteriologist or the mycologist. Gelatinous sheaths often coat the outer surfaces of algal cells, forming a matrix from which it is often difficult to wash embedded bacteria (Schramm, 1922; Bold, 1942; Gerloff, Fitzgerald, and Skoog, 1950). Furthermore, the cells of algal colonies often exhibit a tendency to cling together, so that the simple platinum needle technique of the bacteriologist is useless, the needle remaining free of cells upon withdrawal (Schramm, 1922). There are also many filamentous forms, which exist both with and without gelatinous sheaths. These sheaths are often confluent and extremely tough.

The literature is not without reported isolation techniques. Most of these revolve around the single cell method and as such are too tedious for general laboratory utility. Moreover, most are directed toward the development of pure cultures, and thus involve manipulations of considerable complexity (Schramm, 1922) which may be by-passed by the worker interested in the development of unialgal cultures.

Skinner (1932) and Bold (1942) outline essentially similar methods for the development of pure cultures. Since the unialgal culture is the first step in the derivation of the pure culture (Waksman, 1932), these methods are of service when the worker stops short of the final considerations. The two techniques employ the bacteriological assumption that a group of cells sufficiently dispersed by dilution in an agarized medium will result in the development of isolated clones, which upon the attainment of sufficient size may be transferred bodily to fresh media. The resulting cultures are supposedly unialgal, at least. If free from bacteria and fungi, they are pure cultures. If only for mechanical reasons, Bold's method seems the better, although under the conditions for incubation and the method of transfer outlined in the technique, one to several weeks are necessary for the development of colonies.

Figure 2 shows the ten primary enrichment cultures that were set up in triplicate to furnish algal material for the investigation. These ten cultures varied not only as to media, but as to culture type. Figure 10 shows how the selected media varied in inorganic and organic content, concentration, and pH. It was attempted as nearly as possible to cover the soil pH range potential to the Blacksburg area. The organic media were included for the sake of completeness even though their utilization for algal

culture other than pure culture has been shown impractical (Bold, 1942; Stokes, 1940). The three culture types selected are those used by various workers in the past. They were included to offer test material for the isolation technique developed in this investigation.

Figure 11 is a listing of the thirty primary enrichment cultures, showing the code number of the culture, the culture type, the culture medium, and the elapsed time in days before the microscopic appearance of growth. For quickness in producing algal growth, the outstanding medium would appear to be the egg albumen "Faulkulture", but it is well to note that only two out of the three cultures developed, and even here, but a single species was isolated. Not only was the algal flora singularly poor, but the protein hydrolysates offered by the bacterial breakdown of the egg albumen appear to have offered such an excellent medium for bacterial growth that it was found quite impossible to develop separation plates with the maceration technique. The single species mentioned was isolated with the aid of a micropipette. Other protein hydrolysates were used in the transfer medium because of the impracticability of autoclaving fresh egg albumen. Five tenths peptone (Difco) was added to 15.0% soil extract and 0.5% Casamino Acids (Difco) was added to 15.0% soil extract. Neither medium was successful, for the bacterial

flora developed on the separation plates was too heavy to allow algal growth.

The second most rapidly developing culture group was found to be the 1.5% agarized Detmer's solution (1888) in 1/3 dilution. These were the serially diluted, bacteriological type pour plates prepared according to the method of Bold (1942). Not only did they develop rapidly, but a rich algal flora was produced. Contrary to the report of Bold, however, in this investigation the plates without glucose were found to be more satisfactory than those with glucose added. This agrees with the findings of Stokes (1940), who observed that algal populations were held in check in organic media as long as bacterial populations flourished.

Between the remaining liquid primary enrichment cultures there seems little choice. All were found satisfactory, and although the taxonomic results are far from complete at this writing, there is sufficient evidence to show that they produce a rich, and when compared with one another, a selected flora.

Growth appeared in the most soil primary enrichment cultures in 14-24 days as compared to 11-25 days for the liquid primary enrichment cultures. These figures give a misleading impression, for the continued development in the moist soil cultures was very slow, and it was several months before they were judged ready for the separation procedure. In spite of this slowness, the moist

soil type of primary enrichment culture has proved to be very satisfactory, requiring a minimum of care and producing a rich algal flora.

The incubator diagrammed in Figure 4 was so successful it deserves considerable description. For sheathing, scrap pieces of corrugated cardboard were used. No internal bracing was found necessary. The edges and corners were bound with gummed tape, and the flanged bottom was tacked and taped to the top of an ordinary laboratory table with a light colored surface.

Figure 12 is a photograph of the completed structure, front view, showing the exhaust vent at the top, the two thermometers on the top, the cool air intake duct in the rear, and the rope hoist running from the lower front edge to the ceiling directly above the incubator. The entire front half is hinged and may be lifted like a hood by the rope hoist to provide working space and access to the cultures inside.

Figure 13 shows the same view, but with the front raised. Everything but the interior has been blacked out. Continuous illumination is provided by five, 20 watt, white fluorescent tubes. These produce more foot candles of light per unit of heat energy liberated than do incandescent bulbs. Figure 14 is a reproduction of the energy distribution curve submitted by the

Sylvania Electric Products, Inc., of Salem, Massachusetts, as characterizing the particular tubes utilized in this investigation. With the curve there is included a table showing the relative energy output in per cent. As explained by the Sylvania Electric Products, Inc., the continuous portion of the curve represents the visible light emission, while the discontinuous part of the curve, that part shown as rectangular blocks, represents the emission of the invisible mercury lines.

Figures 4 and 13 show the disposition of the five tubes within the incubator. There are two tubes in the left side and three tubes in the right side. This uneven distribution is nothing more than a reflection of expansion and a coincident attempt to secure more even illumination. Incubation was started in the left side where the two tubes are located. At a later date more space was needed and the right side of the incubator was added. Mere visual inspection, coupled with the observation that cultures located in the outer limits of the left side of the incubator were expressing considerably slower growth, suggested the need for more intense illumination. For these reasons three fluorescent tubes were mounted in the right side and positioned in an eccentric manner. Even here, however, illumination varied grossly, as Figure 4 shows. Illumination measurements were made with a Weston Illumination Meter, model 500.

The control of temperature was based at first upon thermal convection currents set up within the incubator by the heat energy emission from the five fluorescent tubes. Without a ventilating system the internal temperature ranged above 40 °C. According to Smith (1944), this is too high for algal growth. In order to lower the temperature and to afford a measure of control, an air duct was constructed to pipe cool air from a window into the incubator. This duct is shown in Figure 15, which is a rear view of the incubator. At the window end of the duct there is an air gate by which the entire duct may be opened or closed to the outside air. Similar gates are located at each of two internal openings in the rear wall of the incubator (Figure 13). The first internal gate is positioned in such a fashion that its operation has no effect upon the flow of air through the duct to the second internal gate. With this arrangement air may be admitted or excluded from either side of the incubator independently, according to the dictates of the particular situation. Such an arrangement was necessary because of the uneven distribution of the five fluorescent tubes.

As long as the outside temperature remained below that maintained within the incubator the control mechanism just described was entirely adequate. In late Spring it was found necessary to supply artificially cooled air. This was done by installing a

10 inch electric fan and an excelsior pad at the outer end of the air duct. Water dripped over the excelsior pad cooled the air drawn through it by the fan.

Both cooling systems require constant observation and adjustment by the operator to offset diurnal changes in the outside temperature. In this investigation the internal temperature was checked and recorded three times daily, at 8 AM, 4 PM, and 11 PM. This regularity was found sufficient to maintain an internal temperature of 25-30 °C.

The final measures taken to combat the excessive evaporation of culture media produced by the air circulating system of the incubator were successful. These measures were, the cardboard milk bottle caps pierced with three small holes used for the liquid cultures, the sealing of the moist soil cultures with vaseline, and the utilization of moist chambers for the agarized culture plates. Each of the three culture types, when guarded from evaporation in its respective manner, has produced actively growing cultures in excess of 3 months without additional attention.

Eighty unialgal cultures were established by means of the maceration technique. Although maceration was not complete in many instances, particularly when tough, gelatinous sheathed, filamentous forms were present, early inspection of the separation plates revealed the presence of numerous clones developing from single cells or filament fragments. The completeness of maceration, the thoroughness of dispersion of the inoculum throughout the separation plate medium, and the selection of separation plates at the proper stage of development seem to be crucial points in the technique. If maceration is so incomplete that insufficient unialgal growth foci are not produced in the inoculum, if the inoculum is insufficiently dispersed throughout the new medium, then isolated clones have little chance of developing. Likewise, progress of growth on the separation plates must be followed with care, particularly if there are motile or filamentous forms present. If development of the plate is allowed to progress too far, many of the clones that could have been "fished" with success are lost through contamination, and the process must be repeated. At the same time it is desirable to allow the clones to become as large as possible, for the larger colonies are transferred with considerably greater success than are the smaller colonies. In the transfer of clones from a separation plate to a transfer plate, only a

single species should be placed on a single transfer plate, for experience has shown that the practice of including several species on a single transfer plate leads to the contamination of otherwise useful clones.

The success encountered through the utilization of 0.3% agar in the medium used for the preparation of temporary microscope slides deserves comment. In the method, a 0.3% agarized drop of the medium used in the alga producing culture was added to the algal material on the glass slide, and a glass cover slip added. The advantages of such an agar mount over the ordinary water mount are threefold. Not only is the increased viscosity of the agarized medium sufficient to hold the algal cells firmly in place when oil immersion is used, thus eliminating the tedious "chasing" of cells when a water mount is examined under oil, but the change in the medium from that of the 0.7% agar concentration in the transfer plate to that of 0.3% agar in the mounting medium is sufficient to induce zoospore liberation when such are present, and at the same time the change is not great enough to cause plasmolysis of the cells. In addition, the agar mount was found to last considerably longer than the water mount.

Figure 16 gives the tentative identification of the organisms established in unialgal culture, and represents only a portion of

the total flora produced in the primary enrichment cultures. In each case identification was made by microscopic examination of agar type slide mounts of organisms taken from separation plates. Descriptions were taken from Smith (1950), Fritsch (1935), and Tilden (1910). The preciseness of this effort was hindered by the lack of more complete descriptive literature, and because the organisms used in the examination were grown on an agarized medium. There are indications, at least, that gelatinous sheaths, particularly in the case of Cyanophyta, are somewhat altered and obscured by the presence of agar in the medium.

During the incubation of the eighty unialgal cultures a band of these cultures down the center of the incubator, where illumination was highest, showed considerably heavier growth than those cultures along the sides of the incubator where illumination was lower. It was this observation that led to the installation of the three fluorescent tubes in the right half of the incubator. In a search for substantiating evidence for this heavier growth, the individual elapsed times before the appearance of growth in the individual cultures were superimposed, as a grid, upon a floor plan of the incubator showing the varying areas of illumination intensities (Figure 17). This plotting did not show group responses to the illumination intensity areas. Apparently, the elapsed time before the appearance of growth is

not correlated to the heaviness of that growth at a later date. Although Algues (1951) was able to show individual responses to varying light intensities, other conditions held constant, in the present investigation there were too many variables in operation to demonstrate such a result, if present. It is noteworthy, however, that certain individuals stand forth strongly when their elapsed times for the appearance of growth is compared with that of neighboring cultures. Several of these individuals are indicated in Figure 17.

Figure 18 gives the results of experiment 1. The numbers represent the elapsed days before the appearance of growth. In all culture pairs except number 3, growth appeared in the agarized medium previous to, or at least simultaneous with, the appearance of growth in the nonagarized medium. When examined at the end of 1 month, in all cases growth was considerably heavier in the bottles containing the agarized medium. In culture pairs 3, 7, and 9, the compared elapsed times were affected by the motility of the cultured organism, since the nonagarized medium allowed an early appearance of growth due to a concentration of the organism in the lenses of light.

The results of experiment 2 are given in Figure 19. An examination of the organisms revealed the presence of motile and nonmotile unicellular forms, and filamentous forms. Since the inocula were taken from well established cultures, there was a high percentage of reproductive structures present in inocula 1, 7, 8, and 10. Presumably, the presence of a high percentage of pre-formed reproductive structures would hasten the growth of that organism in a new culture. It is indeed singular that cultures 2, 3, 4, 5, 6, and 9, all of which were found lacking in evident reproductive structures, should express growth rates parallel to those of the cultures high in reproductive structures.

The results of experiment 3 are given in Figure 20. The longer elapsed time for the appearance of growth in this experiment was at least partially due to the lightness of the inocula used to seed the cultures. An inspection of the slants revealed in two cases that growth resulted from less than 20 cells. In all cases growth appeared in the agarized media before it appeared in the nonagarized medium. The cultures were examined after the eleventh day to determine which medium produced the heaviest growth. The results were similar to those of experiment 1. In each series the heaviest growth was found in either the 0.3% or the 0.7% agarized media, while growth had

not appeared in three of the nonagarized tubes. It was not possible to differentiate further than this because of the visual method of comparison used.

Since the temperature factor was constant in all three experiments, since the higher lighting factor was constant in experiment 3, since the presence or absence of reproductive structures seems nullified by experiment 2, since in experiments 1 and 3 the presence of agar in the medium seemed beneficial to algal growth, and since the agarization factor was shown to be additive to the constant lighting factor in experiment 3, it seems reasonable to postulate that algal growth is augmented by a combination of the factors of continuous, artificial lighting in the 30-35 foot candle range and 0.3-0.7% agarization of the medium, and that these factors are additive.

Staining after fixation with osmium tetroxide gave poor results. Although the fixation was good, no method was found for the extraction of chlorophyll, nor could the various stains be cleared from the cytoplasm. For these reasons the osmium tetroxide schedule was abandoned.

The Rhodes centrifugation method, coupled with chlorophyll extraction in ether and staining with Harris' hematoxylin gave encouraging results. By this method enough cells for slides

and a generous amount for preservation were obtained by a single application of the schedule. The entire technique is carried out in a single tube of a small hand centrifuge and is completed in less than 90 minutes.

The basic schedule as finally derived is as follows:

The algal material to be stained must be in a liquid culture. Shake the culture to disperse the cells.

1- Pour the desired amount of algal material into a clean centrifuge tube. Centrifuge and decant.

2- Wash in 15 cc of distilled water. Centrifuge and decant.

3- Kill and fix for 5 min. in 10 cc of "hot" (70 °C.) Schaudinn's fluid. Centrifuge and decant.

4- Wash in 15 cc of 50% alcohol to which there has been added a little iodine solution. Centrifuge and decant.

5- Pass through 80%, 95%, and absolute alcohols and then 50-50 ether-absolute alcohol, 1 min. each, centrifuging and decanting each time.

6- Extract the chlorophyll in ether. The time required should be less than 5 minutes. Centrifuge and decant.

7- Pass back down through the same solutions to 70% alcohol, 1 min. each, centrifuging and decanting each time.

8- Stain for 20 min. in 5 cc of Harris' hematoxylin. Centrifuge and decant.

9- Wash in 15 cc of distilled water. Centrifuge and decant.

10- Destain for 5 sec. in 15 cc of distilled water to which had been added 3 drops of conc. HCl per 100 cc of water. Stop destaining action by adding 1 drop of conc. NH_4OH. Examine cells microscopically. If insufficiently destained, centrifuge and decant and add fresh acid water to repeat the process until the desired degree of destaining is obtained.

11- Pass up the chain by the same steps, 1 min. each, centrifuging and decanting each time, then into 50-50 absolute alcohol-xylol and finally into two changes of xylol, 5 min. each.

12- Mount in balsam.

If it is desired to stain only a portion of the cells and save others for different stains or for preservation, the material should be divided just after the extraction of chlorophyll in ether. The unstained cells may be taken back to absolute alcohol and then into glycerin for preservation.

Conclusions

1- Soil algae may be cultivated rapidly in a controlled environment of 25-30 °C. and 30-35 foot candles of light.

2- The presence of 0.3-0.7% agar in the medium has a hastening effect upon the growth of soil algae.

3- A mixed algal flora such as that found in the soil may be effectively separated into its component species by a technique combining mechanical maceration with bacteriological pour plating.

Summary

1- The literature concerning the taxonomy and ecology of soil algae was reviewed extensively. The lack of established, controlled cultural conditions, and the lack of facile isolation techniques in the literature was pointed out.

2- An incubator featuring a controlled environment of 2-40 foot candles of continuous, white, fluorescent light and 25-30 °C. was constructed and described.

3- Three composite soil samples were collected and inoculated into cultures of varying chemical and physical characteristics. The primary enrichment cultures thus established were incubated in the incubator at approximately 15 foot candles of light and at 25-30 °C.

4- The mixed algal flora produced in the primary enrichment cultures was separated into unialgal cultures by a technique combining mechanical maceration with bacteriological pour plating. Eighty such unialgal cultures were established. The equipment and the technique were described.

5- Three experiments utilizing small groups of organisms were set up to investigate the general effect of light intensity and agarized media upon algal growth. The results indicated that algal growth is hastened by lighting near the 30 foot candle range and the presence of 0.3-0.7% agar in the medium.

6- The Rhodes centrifuge technique for the staining of protozoa was modified to produce a rapid staining schedule for soil algae. This schedule involves killing and fixing in "hot" Schaudinn's fluid, chlorophyll extraction in ether, and staining in Harris' hematoxylin. The entire technique requires less than 90 minutes.

7- A tentative listing of 21 genera was reported as having been established in unialgal culture.

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Explanation of Figures

Figure 1. Summary of methods and results of previous surveys of soil inhabiting algae.

Figure 2. Table showing the combinations of culture media and culture types utilized in this investigation.

Figure 3. Photograph of the arrangement of separation plates in a moist chamber.

Figure 4. Scale diagram of the cardboard incubator used in this investigation.

Figure 5. Floor plan of the incubator, showing illumination intensity areas, in foot candles, produced by the five, 20 watt, white, fluorescent tubes used as the light source in the incubator.

Figure 6. Photograph of the macerator used in the isolation technique developed in this investigation.

Figure 7. Diagram and dimensions of the agitator attachment of the macerator.

Figure 8. Photograph of the dissecting microscope light and stage used in the examination of separation plates.

Figure 9. Diagram of dissecting needle shape.

Figure 10. Comparison of the primary enrichment media used in this investigation, showing their inorganic and organic content, concentration, and pH.

Figure 11. Table of elapsed times for the macroscopic appearance of growth in the 30 primary enrichment cultures established in this investigation.

Figure 12. Photograph of the exterior of the incubator, front view.

Figure 13. Photograph of the interior of the incubator, front view. Note the arrangement of the 5 fluorescent tubes, and the 2 internal openings of the air circulating system located in the rear wall of the incubator.

Figure 14. Relative energy distribution curve and energy distribution in arbitrary color bands of a 20 watt, white, fluorescent tube manufactured by Sylvania Electric Products, Inc., of Salem, Mass. The curve and data are taken from Sylvania Electric Products, Inc., Engineering Bulletin No. 0-153, by T. C. Sargent.

Figure 15. Photograph of the exterior of the incubator, rear view. Note the cool air duct which connects the 2 internal openings in the rear wall of the incubator with the window, and the three air gates located in the duct.

Figure 16. List of the tentatively identified organisms isolated into unialgal cultures by the maceration technique.

Figure 17. Floor plan of the left half of the incubator, showing the 20-30 foot candle area of illumination within dotted lines, upon which the elapsed times for the macroscopic appearance of growth in the 80 unialgal cultures has been plotted in their respective positions within the incubator.

Figure 18. Results of Experiment 1.

Figure 19. Results of Experiment 2.

Figure 20. Results of Experiment 3.

Name	Date	Locality	Culture type	Culture medium	Incubation	Time	Isolation	No. Spp.
Esmark	1911 1914	S. America	moist soil	water	room temp. window light	2-60 days	--	24 45
Robbins	1912	Colorado	liquid-sand	water	room temp. window light	30-60 days	some	13
Moore & Karrer	1919	Mo. Bot. Gard.	liquid-sand	inorganic salts	room temp. window light	21-90 days	--	17
Bristol	1920	England	liquid-sand	inorganic salts, water	room temp. window light	60 days	some	57
Moore & Carter	1926	Mo. Bot. Gard.	liquid	inorganic salts	room temp. window light	--	no	35
Phillipson	1934	Australia	liquid	inorganic salts	room temp. window light	28-35 days	yes	34
James	1935	England	liquid, agarized	inorganic salts	70-75 °C window light	--	--	18
Petersen	1935	Denmark	liquid, moist soil	inorganic salts, water	room temp. window light	--	--	136
Fenton	1938	Scotland	moist soil	water	room temp. window light	--	no	9
Fritsch & John	1942	England	liquid, moist soil	inorganic salts, water	room temp. window light	--	some	85
Smith	1943 1944	Florida	moist soil liquid	water inorganic salts	room temp. window light 25-30 °C 200 ft, cndls	18 days 14-18 days	yes yes	36 34
Lund	1947	England	agarized, moist soil	inorganic salts	room temp. window light	--	no	57
Feher	1948	world wide	liquid	inorganic salts	room temp. window light	--	no	685

Figure 1

Summary of methods and results of previous surveys of soil inhibiting algae. Note the conditions of incubation and the general lack of isolation into unialgal culture.

Culture type / Medium	Liquid	Agarised	Moist Soil
15% Soil Extract	X		X
0.35% Knop's (pH 5.6)	X		X
0.35% Knop's (pH 6.4)	X		X
0.05% Beijerinck's	X		
"Faulkulture"	X		
"Fortified" Detmer's (1/3 dilution)	X	X	
Detmer's (1/3 dilution)		X	

Figure 2

Summary of the combinations of culture media and culture types used in this investigation.
Three series of these ten cultures were set up.

Figure 3

Arrangement of petri dishes in moist chamber.

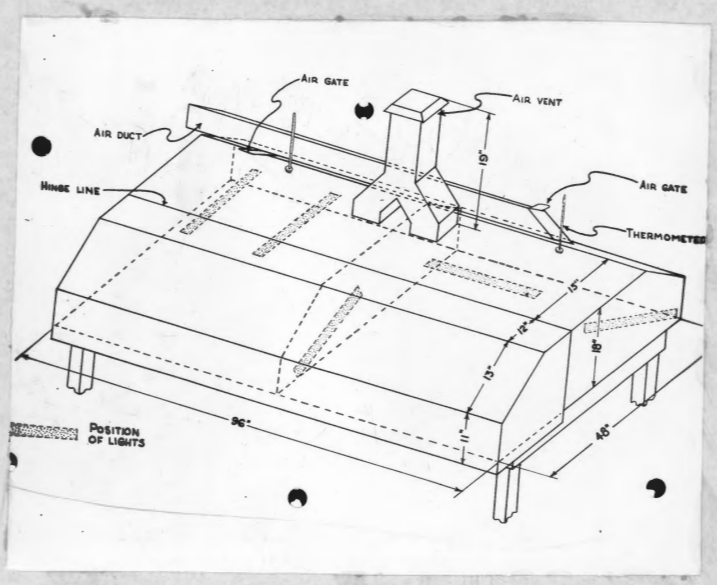
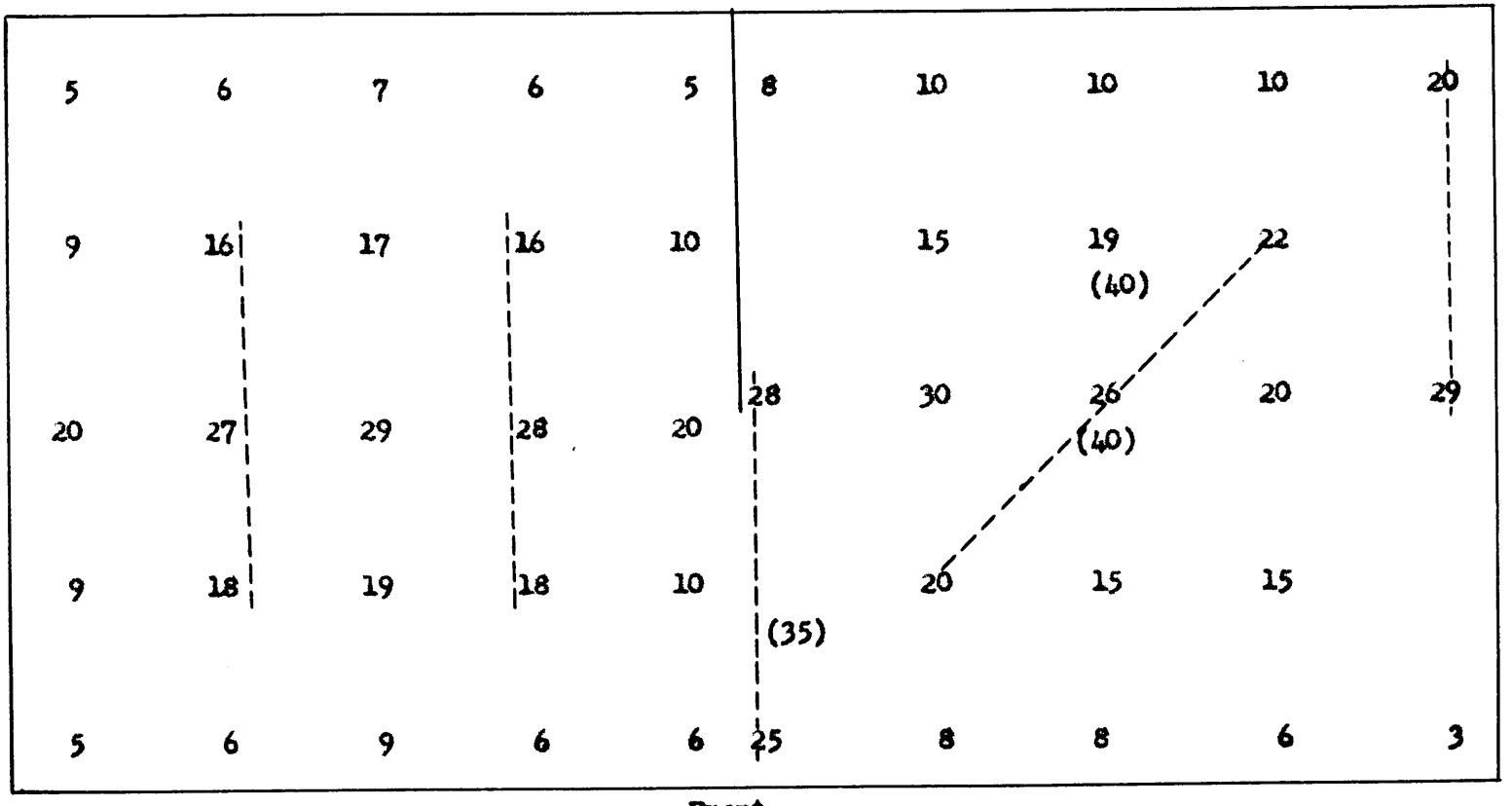


Figure 4

Incubator used in this investigation.



( ) raised 12" above floor of incubator.

Figure 5

Illumination intensity areas on floor of incubator. Dotted lines show position of lights.

Figure 6

Macerator used in the isolation technique.

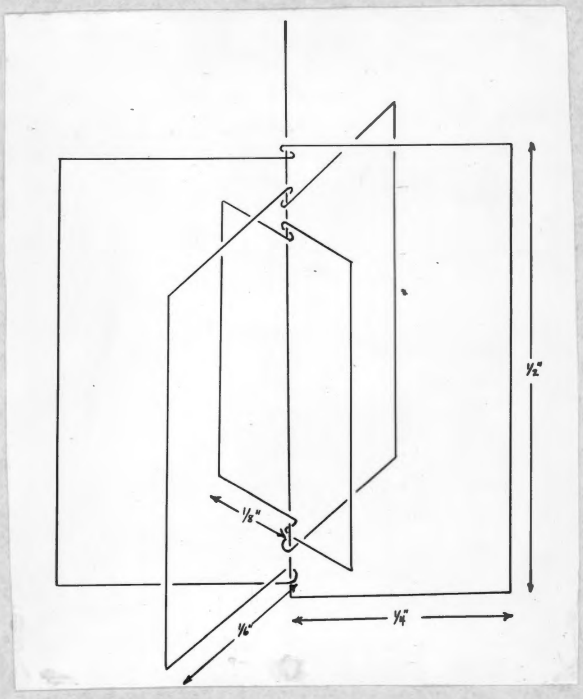


Figure 7

Dimensions of macerator head.

Figure 8
Dissecting microscope light and stage.

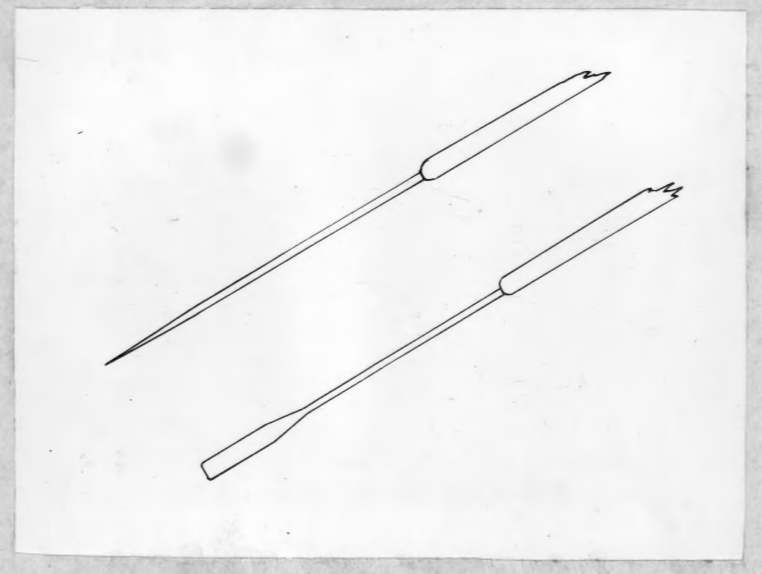


Figure 9
Dissecting needle shape, side and top views.

Inorganic Media

Name of Medium	Knop's (1865)	Detmer's (1888)	Beijerinck's (1898)
$Ca(NO_3)_2 \cdot 4H_2O$	4.0 grams	1.0 gram	
KNO_3	1.0 gram		
$NH_4(NO_3)_2$			0.5 gram
K_2HOP_4			0.2 gram
KH_2PO_4	1.0 gram	0.25 gram	
$MgSO_4 \cdot 7H_2O$	1.0 gram	0.25 gram	0.2 gram
$CaCl_3 \cdot 2H_2O$			0.1 gram
KCl 1% aq.		0.5 gram	
$FeCl_3 \cdot 6H_2O$	1 drop	1 drop	1 drop
Distilled water	1 liter	1 liter	1 liter
Conc. stock	0.7%	0.2%	0.1%
Conc. used	0.35%	0.05%	0.05%
pH	5.6 & 6.4	6.2	7.2

Organic Media

Name of Medium	Soil Extract	"Faulkulture"	"Fortified" Detmer's
Soil extract stock	150.0 cc.		
Fresh egg albumen		70.0 grams	
Detmer stock			333.0 cc.
Glucose			5.0 grams
KNO_3	10.0 cc.		
Distilled water	840.0 cc.	930.0 cc.	666.0 cc.

Figure 10
Comparison of the primary enrichment media used.

- Garden Soil -

Culture No.	Culture type	Culture medium	Elapsed time for growth
A1	Liquid	Soil Extract	13 days
A2	"	Knop's (pH 5.6)	21
A3	"	Knop's (pH 6.4)	23
A4	"	Beijerinck's	13
A5	"	"Faulkulture"	6
A6	"	Fortified Detmer's	12
A7	Moist Soil	Soil Extract	23
A8	"	Knop's (pH 5.6)	18
A9	"	Knop's (pH 6.4)	20
A10	Agarized	Detmer's	12
A10	"	Fortified Detmer's	12

- Fertilized and Cultivated Field Soil -

Culture No.	Culture type	Culture medium	Elapsed time for growth
B1	Liquid	Soil Extract	13 days
B2	"	Knop's (pH 5.6)	22
B3	"	Knop's (pH 6.4)	24
B4	"	Beijerinck's	12
B5	"	"Faulkulture"	no growth
B6	"	Fortified Detmer's	12
B7	Moist Soil	Soil Extract	21
B8	"	Knop's (pH 5.6)	14
B9	"	Knop's (pH 6.4)	18
B10	Agarized	Detmer's	12
B10	"	Fortified Detmer's	12

- Unfertilized and Fallow Field Soil -

Culture No.	Culture type	Culture medium	Elapsed time for growth
C1	Liquid	Soil Extract	11 days
C2	"	Knop's (pH 5.6)	25
C3	"	Knop's (pH 6.4)	23
C4	"	Beijerinck's	15
C5	"	"Faulkulture"	4
C6	"	Fortified Detmer's	13
C7	Moist Soil	Soil Extract	24
C8	"	Knop's (pH 5.6)	14
C9	"	Knop's (pH 6.4)	17
C10	Agarized	Detmer's	12
C10	"	Fortified Detmer's	12

Figure 11

Elapsed times for the macroscopic appearance of growth.

Figure 12

Front, exterior view of incubator.

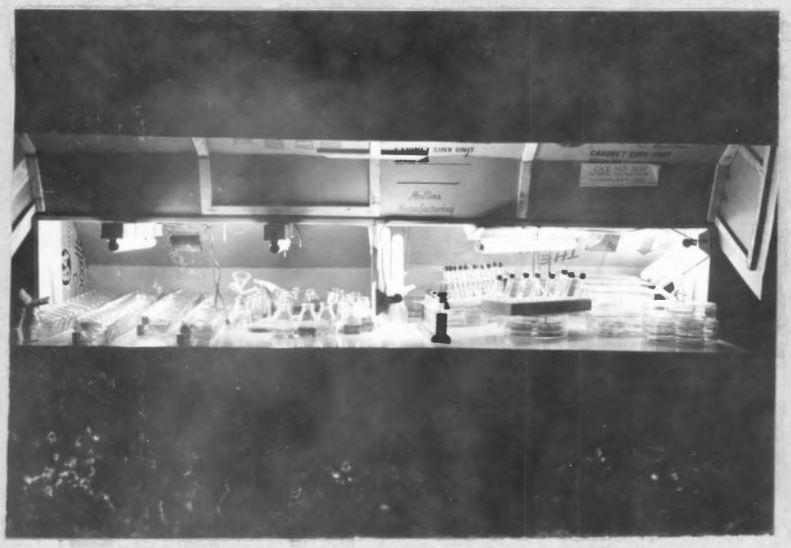
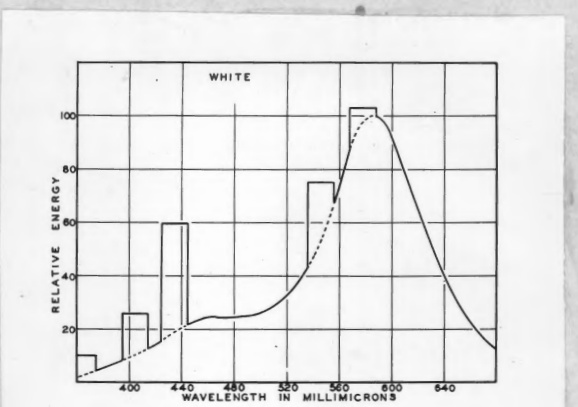


Figure 13

Front, interior view of incubator.
Note arrangement of lights
and internal openings of air ducts.



ENERGY DISTRIBUTION IN ARBITRARY COLOR BANDS

	Millimicrons	White
Violet	380-430	9.94%
Blue	430-490	12.93
Green	490-560	25.39
Yellow	560-590	21.90
Orange	590-630	20.69
Red	630-680	9.15
		100.00

Figure 14

Energy distribution curve and color bands of light used. Engineering Bulletin No. 0-153, T.C. Sargent, Sylvania Electric Products, Inc.

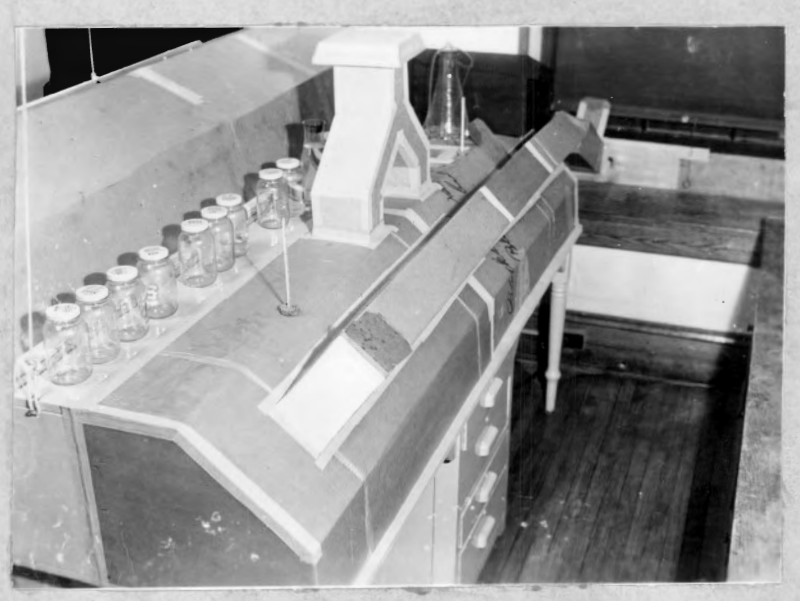


Figure 15

Back, exterior view of incubator. Note air duct.

Cyanophyta	Chlorophyta	Chrysophyta
Albrightia	Chlamydomonas	Bumilleria
Anabaena	Chlorella *	
Heterohormogonium	Chlorococcum *	
Lyngbya *	Hormidium	
Microcoleus	Microspora	
Nostoc *	Palmellococcus	
Oscillatoria *	Protococcus	
Plectonema	Stichococcus	
Pluto	Trebouxia	
Schizothrix		
Trichodesmium	* Several species	

Figure 16
Genera tentatively identified in unialgal culture.

Row No.

1	2	3	4	5	6
17	6	11			(4)
14	6	4	11		(4)
6	9	3	11		6
6	6	11	10		5
6	8	5	9		6
5	4	(10)	(10)		3
6	4	8	5		3
5	(12)	8	7	3	3
3	3	7		3	
5	(10)	8	5	3	
(12)	(12)	8	10	5	
7	4	5	13	4	
7	5	6	14	3	
7	11	6	12	5	
	11		14	7	
	(3)		14	9	
			16	13	

Figure 17
Incubator floor, with elapsed times for appearance of growth in cultures shown. Note shorter times in horizontal band across incubator floor. This corresponds with 20-30 foot candle area of illumination.

Culture No.	Elapsed time before appearance of growth	
	Agarized Medium	Nonagarized Medium
1	7 days	7 days
2	7	12
3	5	3
4	5	6
5	5	6
6	6	6
7	14	17
8	6	6
9	7	9

Figure 18

Culture No.	Elapsed time for growth	Type Organism
1	1	Chlamydomonas like; heavy palmelloid formations.
2	3-1/2	Large, filamentous Cyanophyta; few heterocysts; many akinetes.
3	1-1/2	Microspora like; no evident sporangia.
4	1	Chlorella like; few sporangia.
5	1-1/2	Stichococcus like; no evident reproductive structures.
6	1-1/2	Unicellular Cyanophyta; very small.
7	1-1/2	Chlorococcum like; many zoosporangia.
8	4	Chlorococcum like; different from culture 7.
9	1	Bumillaria like; no evident reproductive structures.
10	1	Chlorococcum like; same as culture 7.

Figure 19

Fig. 18: Results of experiment 1.
Fig. 19: Results of experiment 2.

Series No.	Elapsed time for growth in: Detmer's solution	Detmer's, 0.15% agar	Detmer's, 0.3% agar	Detmer's, 0.7% agar	Type Organism
1	11	6	6	6	Chlorococcum like; forms zoosporangia, but few present.
2	no growth at 11 days	8	6	6	Protococcus like; reproduction by simple fission.
3	6	6	5	3	Chlorella like; very few sporangia present.
4	no growth at 11 days	11	6	5	Filamentous Cyanophyta; small heterocysts and many large akinetes.
5	10	7	6	10	Chlorococcum like; wide range in cell size; large, gravid cells.
6	11	5	5	5	Chlorococcum like; less range in cell size than No. 7; no gravid cells present.
7	11	7	7	6	Chlamydomonas like; few reproductive structures present.
8	no growth at 11 days	7	6	6	Stichococcus like; no evident zoosporangia present.

Figure 20
Results of experiment 3.