

A STUDY OF SOME CHLOROPHYCEAE FROM THE  
BLACK BELT SOIL OF ALABAMA

By

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## I. INTRODUCTION

While algae have been studied in cultures since before the beginning of the present century, general acceptance of cultural techniques as an aid to algal taxonomy has been delayed. Beijerinck (1890), Artari (1902), Chodat (1913), and others grew many Chlorococcalean algae on agar supplied with mineral nutrients or in mineral solutions, but Fritsch (1935) among other phycologists, expressed doubt that such cultures were reliable for the study of form variation of algal species, inasmuch as they exhibited a high degree of polymorphism. Pringsheim (1946) however, was able to obtain pure cultures which did not possess this marked variability and made his cultures available to other workers for study.

Brunnthaler (1915) emphasized the need for detailed and prolonged study of certain algae and made this statement concerning Chlorococcum: "The genus is entirely unnatural; only a few species are known accurately to any degree. Most species do not belong here and instead may be stages of other algae." Few phycologists heeded this warning; many continued to describe new species from chance collections from nature. Gerneck (1907) and Vischer (1933) saw the need for the study of certain groups in culture but among other things, they gave little attention to zoospore type. Starr (1955) made a comprehensive study of the unicellular, spherical, zoospore-producing Chlorococcales with

the following objectives in mind; "provision of a firm foundation for the taxonomy of the spherical, zoospore-producing chlorococcaceous algae and reemphasis of the absolute necessity for prolonged and periodic study, based on unialgal cultures, before new species and genera are described or old ones identified with certainty." Starr established the following attributes as reliable in delimiting genera of this group: 1. chromatophore type, 2. presence or absence of pyrenoids, 3. zoospore type. Other investigators (Trainor and Bold 1953, Arce 1956, Herndon 1958) have also regarded these characteristics to be reliable. It is upon this foundation that the writer bases the descriptions in this thesis.

The discovery of three previously undescribed chlorophycean algae resulted from a desire to learn something about the algal flora of Alabama soil, about which little is known. Soil samples were taken from the Black Belt region because it is a relatively distinctive geographical agricultural area with an interesting diversity of macroscopic plant life; it is probable that the algal flora of the soil is equally diversified and interesting. According to the U. S. Dept. of Agriculture (1938), the Black Belt of Alabama (and Mississippi) is of the "Rendzina" soil group and is a part of the Sumpter-Vaiden soil area. Rendzina soils are regarded as immature and are developed from chalk, soft limestone, or marl. Large amounts of black organic

matter which is present were derived from a grass vegetation.

Many isolations of algae were made but only three organisms were selected for detailed study; these organisms appeared to offer the best opportunity to make a useful contribution to the knowledge of soil flora in general, and of Alabama soil algae in particular. In addition, it is hoped that the material included in this thesis will aid in delimiting these groups of algae and furnish additional evidence of the reliability of the generic criteria stated by Starr.

## II. MATERIALS AND METHODS

The algae described in this thesis were isolated from soil samples collected along the farm road from Boligee to Forkland in the Black Belt of Greene County, Alabama. More exact information regarding the location from which the soil samples were collected accompanies the descriptions of the organisms. Samples were taken in sterile screw-top test tubes, and pH determinations were made on each. Approximately 5 g. of each sample were placed in a sterile 125 ml. Erlenmeyer flask and enriched with 35 ml. of Bold's inorganic salt medium (Bold 1949). These were placed under fluorescent illumination of approximately 120 foot candles and at a temperature of about 22 degrees C. A phototactic ring was visible in the enrichment cultures within two or three weeks.

Isolations were made from the enrichment cultures by standard methods of streaking, plating, and single cell isolation using fine glass pipettes (Bold 1942, Pringsheim 1946). Bold's inorganic salt medium was the basic medium on which the organisms were grown for study. This medium was prepared as follows: six stock solutions, 400 ml. in volume were employed, each containing one of the following salts in the concentrations listed:

NaNO <sub>3</sub> .....10.0 g.	K <sub>2</sub> HPO <sub>4</sub> .....3.0 g.
CaCl <sub>2</sub> .....1.0	KH <sub>2</sub> PO <sub>4</sub> .....7.0
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....3.0	NaCl.....1.0

To 940 ml. of Pyrex distilled water was added 10 ml. of each stock solution and 1 ml. of trace elements (supplied by Dr. J. C. O'Kelley, Department of Biology, University of Alabama) from a stock, 100 ml. in volume, containing salts or acids in the concentrations listed:

H <sub>3</sub> Bo <sub>3</sub> .....0.057 g.	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O..0.036 g.
CuCl <sub>2</sub> ·4H <sub>2</sub> O.....0.107	ZnCl <sub>2</sub> .....0.312
Sequestren <sup>®</sup> - NaFe.0.895	CoCl <sub>2</sub> ·6H <sub>2</sub> O.....0.00084
MnCl <sub>2</sub> ·4H <sub>2</sub> O.....0.072	

Other media used were soil extract and soil water media. Soil extract was prepared by autoclaving a kilogram of garden soil in a liter of Pyrex-distilled water. One part of the supernatant was added to nine parts of the basic inorganic salt medium. Semi solid preparations of these media were made by the addition of 1.5 per cent agar. Soil water tubes were prepared by first placing approximately 0.004 g. of CaCO<sub>3</sub> in the bottom of a 25 ml. Pyrex test tube, then adding 1/4 inch of garden soil and filling the tube 2/3 full with tap water. The tubes were then plugged with cotton and autoclaved. All organisms described in this thesis were cultivated on each of the media described.

Fluorescent fixtures with six 18 inch tubes (General Electric 15 watt standard cool white) illuminated the cultures, the fixtures being mounted over a series of glass shelves which were separated by a distance of approximately

12 inches. Intensity of illumination was varied as desired by varying distance of cultures from the fixtures and by the insertion of diffusion screens between the fixtures and the cultures. Initial transfers received about 120 foot candles. An intensity of approximately 25 foot candles was used to maintain the cultures. An automatic timing device was attached to the lighting system to provide a fixed 12 hour period of illumination (8:00 A.M. to 8:00 P.M). Cultures were maintained in an air-conditioned room which was normally at a temperature of 22 degrees C. but which varied from 18 to 25 degrees C.

Cultures were observed and studied regularly by means of hanging drop preparations. Stains used to supplement the observations included the Feulgen technique, Heidenhain's iron alum hematoxylin, and acetocarmine preparations. The Feulgen procedure (Johansen, 1940) was as follows: numerous cells were placed in a 12 ml. Pyrex centrifuge tube and which contained Schaudinn's fluid (200 ml. saturated aqueous  $HgCl_2$ . 100 ml. absolute alcohol, 9 ml. glacial acetic acid) for fixing overnight. After centrifugation and decantation of the fixative the cells were resuspended in a solution of 70 per cent alcohol in which a few iodine crystals had been dissolved, and which contained a few drops of  $I_2$ -KI solution. The cells were then rinsed in 70 per cent alcohol and hydrated through 50 and 30 per cent alcohol to distilled water. After a rinse in 1N HCl, the cells were

resuspended in a fresh solution of 1N HCl, brought rapidly to a temperature of 60 degrees C. and hydrolyzed for 10 minutes; then the cells were rinsed in cool HCl, distilled water, and placed in the Feulgen stain for approximately 5 hours. The Feulgen stain was prepared as follows: 100 ml. of boiling Pyrex-distilled water was poured over 0.5 g. Basic Fuchsin (Stain Commission Certified No. NF 66) and the mixture shaken thoroughly to dissolve the dye. When the solution had cooled to 50 degrees C., it was filtered. To the filtrate were added 10 ml. of 1N HCl and 0.5 g. of potassium metabisulfite. The mixture was then shaken thoroughly, stoppered tightly, and placed in the dark for 18 hours. After the staining period the cells were fixed to slides by smearing a small drop of albumen fixative on one end of clean glass slides, then spreading a loopful of cells on the albumen. Slides were rinsed for 10 minutes in each of three closed Coplin jars containing a differentiating solution of the following composition: 100 ml. of distilled water, 5 ml. of 1N HCl, and 5 ml. of 10 per cent potassium metabisulfite solution. Next the slides were rinsed in distilled water made alkaline by a pinch of sodium bicarbonate, and finally dehydrated through absolute alcohol and cleared in xylol for 30 minutes. Permount was used for mounting.

Heidenhain's hematoxylin was prepared by making up a 10 per cent solution of hematoxylin in absolute alcohol and

diluting it with distilled water to a 0.5 per cent aqueous solution which was allowed to age for approximately two weeks. Cells were fixed to clean glass slides with albumen, placed in closed Coplin jars containing Schaudinn's fluid, and left overnight. The slides were placed in a 70 per cent solution containing a few drops of iodine in  $I_2$ -KI and allowed to remain for several minutes, after which they were rinsed in 70 per cent alcohol, hydrated through 50 and 30 per cent alcohol to distilled water and placed in a 4 per cent iron alum (ferric ammonium sulfate) solution for mordanting. After one hour in the iron alum, the cells were rinsed in tap water and placed in the 0.5 per cent hematoxylin to remain overnight. Destaining was accomplished with a fresh iron alum solution, after which the cells were dehydrated, cleared in xylol, and mounted in Permount.

Fresh preparations with aqueous iodine ( $I_2$ -KI), methylene blue, india ink, sudan IV, and ruthenium red were made to aid in determining certain characteristics of each alga studied. Iodine preparations were used to indicate the presence or absence of starch; they were also helpful in determining nuclear condition, and in addition, made it possible to see the flagella of motile cells, which are difficult to observe in the natural condition. Methylene blue and india ink preparations aided in delimiting gelatinous or pectinaceous materials surrounding algal cells.

Sudan IV preparations were used to indicate the presence or absence of fats. Ruthenium red solutions aided in determining the presence and extent of pectin in the cell wall. Ruthenium red tests (Shaw, 1929) were made as follows: a Coplin jar was half filled with distilled water and enough ruthenium red added to yield a solution of grape-juice color. Algal cells, fixed to clean glass slides with egg albumen, were placed in Coplin jars containing the stain and allowed to remain for 12 hours. They were then removed from the solution, rinsed with water, and examined. The presence of pectin was indicated by a reddish color.

The Post-Laudermilk test for cellulose (Post and Laudermilk, 1942) was used to determine the presence and extent of cellulose layers in the cell wall of the organisms studied. Two solutions are required: (a) 2 per cent solution of iodine in a 5 per cent solution of KI, diluted 9 parts of water containing 0.28 per cent glycerine. (b) saturated LiCl. A loopful of the cells was fixed on a slide and two drops of solution (a) were added and allowed to stand for at least 30 seconds. The slide was then blotted with filter paper and a drop of solution (b) added. After 5 minutes the slides were ready for examination. A light blue color indicated the presence of cellulose.

Drawings were prepared with the aid of a Bausch and Lomb camera lucida. Final magnification of the drawings after reduction is approximately 1250. Photomicrographs

were taken at a magnification of approximately 950 with a Leitz Micam camera.

Cultures of algae used for comparative observations were obtained from the Culture Collection of Algae, Indiana University, Bloomington, Indiana.

### III. OBSERVATIONS

#### A. A New Chlorococcalean Genus, Reticulochloris.

##### 1. Genus description

##### Reticulochloris gen. nov.

Cellulae vegetativae singulares aut in coenobiis non confertis aggregatae. Cellulae singulares iuvenes sphaericae aut ellipsoideae; cellulae ellipsoideae, maturatae, sphaericae factae; cellulae cum cellulis adiacentibus compressae. Tegumentum leve. Chromatophorus reticulum ligamentorum anastomosantium, unum vel plura pyrenoidea habens. Amylum praesens.

Propogatio asexualis per aplanosporas et zoosporas. Zoosporae duo flagella longitudine aequa habentes, cum tegumentis definitis, et formam ellipsoideam, mobilitate finita, retinentes.

Propagatio sexualis non visa.

Species typica, Reticulochloris vacuolatum.

Vegetative cells solitary or in loose coenobia. Solitary young cells spherical or ellipsoidal, becoming spherical at maturity, or compressed with adjacent cells. Cell wall smooth. Chromatophore a net-work of anastomosing strands with at least one pyrenoid. Reserve photosynthate starch.

Asexual reproduction by aplanospores and zoospores.

Zoospores with two flagella of equal length, with well defined walls, and retaining an ellipsoidal shape at the end of the motility period (Chlamydomonas type).

Sexual reproduction not observed.

Type species Reticulochloris vacuolatum.

## 2. Reticulochloris vacuolatum sp. nov.

Cellulae vegetativae ellipsoideae ad sphericas secundum aetatem; cellulae vegetativae iuvenes aut sphaericae aut ellipsoideae, cellulae ellipsoideae, maturatae, sphaericae factae; diametrum paene 20  $\mu$ . attingentes. Tegumentum leve, aetatae non perspicue incrassatum. Cellulae singulares, sed saepius in numero 4 aplanosporum permanentium inter parentis tegumentum; plures cellulae interdum breviter aggregatae, materie externa pectinacea aut gelatinosa non praesente. Chromatophorus, pyrenoideum unicum excentricum habens, reticulum grossum ligamentorum implexorum. Vacuolae pulsantes praesentes. Cellulae semper uninucleatae. Amylum praesens.

Propagatio asexualis per aplanosporas et zoosporas, bipartitione successive protoplasti factas. Zoosporae ellipsoideae, quamquam 9.5  $\mu$ . longitudine mediocri, 5.0  $\mu$ . latitudine mediocri, 8.0  $\mu$ . ad 10.5  $\mu$ . longitudine, 4.0  $\mu$ . ad 5.5  $\mu$ . latitudine crescentes. Zoosporae habentes stigma anterior eminens, duas vacuolas pulsantes anteriores, duo flagella longitudine aequa, recurvata, zoospora quiescente.

Zoosporae, cum tegumentis, formam ellipsoideam, mobilitate finita, retinentes.

Propagatio sexualis non visa.

Vegetative cells ellipsoidal to spherical depending on age; young vegetative cells spherical or ellipsoidal becoming spherical at maturity, reaching a diameter of approximately 20 microns. Cell wall smooth, not thickening appreciably with age. Cells solitary, but characteristically in groups of 4 persistent aplanospores within a parent cell wall; occasionally forming temporary aggregations of larger numbers of cells, without extensive external pectinaceous or gelatinous material. Chromatophore a coarse net of interconnecting strands, with a single excentric pyrenoid. Contractile vacuoles present. Cells uninucleate throughout development. Starch is the reserve photosynthate.

Asexual reproduction by aplanospores and zoospores formed by successive bipartition of the protoplast. Zoospores ellipsoidal, 9.5 microns in average length, 5.0 in average width, but ranging from 8.0 microns to 10.5 microns in length and from 4.0 microns to 5.5 microns in width. Zoospores with prominent anterior stigma, two anterior contractile vacuoles, and two flagella of equal length held recurved when not in motion. Zoospores walled, retaining an ellipsoidal shape at the end of the motility period

(Chlamydomonas type).

Sexual reproduction not observed.

Type species: Reticulochloris vacuolatum.

Reticulochloris vacuolatum sp. nov. (figs. 1-12) was isolated from a soil sample collected from a cotton field located on Greene County road No. 4, 10.25 miles from U. S. Highway No. 11 near Boligee, Alabama on 2 July, 1957. A test of the soil at this location revealed a pH of 5 to 6. This organism grows well in Bold's inorganic salt medium, liquid and agar.

Young vegetative cells originating from zoospores are ellipsoidal (fig. 11) and have a reticulate chromatophore with a single excentric, almost parietal pyrenoid. As maturation occurs the stigma soon disappears but the contractile vacuoles persist, usually in a lateral position in the young ellipsoidal cell. When present in mature cells contractile vacuoles are peripheral and ordinarily two in number. Older cells may contain both contractile and static vacuoles. The static vacuoles (fig. 10) increase in size as cell size increases and are bubble-like in appearance.

Mature cells (fig. 10) average about 20 microns in diameter but larger single cells may be formed. The net-like chromatophore (figs. 2,3,5,6) coarsens with age and becomes obscured with starch. The single excentric pyrenoid (figs. 3-8) is surrounded by starch plates. The cellulose

wall layer of the cell wall is thin (approximately 0.5 microns) and the outer wall layer (Arce 1956) is also thin (approximately 0.5 microns). The outer wall layer may thicken slightly with age. No individual matrix was observed under any of the conditions of culture. The single large nucleus is sometimes visible in the living state but observations on nuclear condition were supplemented with the Feulgen technique and Haidenhain's iron alum hematoxylin (fig. 4).

Asexual reproduction is effected by formation of aplanospores and zoospores. Cleavage (figs. 5,7,12) occurs by the successive bipartition of the protoplast with karyokinesis preceding each cytokinesis. Up to 16, or occasionally more, aplanospores may be formed and released soon after formation, or, 4 large (ca. 14 microns in diameter) persistent aplanospores (figs. 8,12) may be formed and remain in a vegetative state within the parent cell wall for some time. In cultures on Bold's inorganic salt medium (2-3 weeks old) a large proportion of the cells are in tetrads of maturing aplanospores. While still within the parent cell wall these aplanospores eventually divide to form either zoospores (fig. 9) or produce additional aplanospores as if they were dissociated individual cells. Release of the products of reproduction is usually accomplished by dissolution of first the common parent cell wall, then the individual parent cell wall.

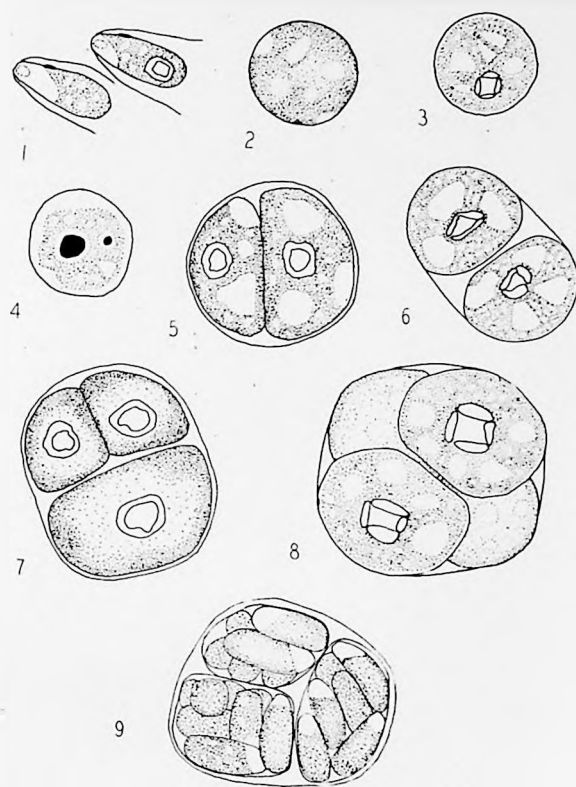
Zoospores (fig. 1) are ellipsoidal in shape but narrow anteriorly and average 9.5 microns in length, and 5.0 microns in width. Variations of 1 micron above or below these averages are common. As many as 32 or sometimes more zoospores are produced by single vegetative cells in actively growing cultures. These may remain motile for several hours after release from the parent cell. Occasionally zoospores, though active within the parent wall, are not released from the parent. These lose their flagella and stigmata and undergo cell growth within the parent wall. Zoospores possess two flagella of equal length held recurved when not in motion an anterior, slightly protuberant stigma; and two anterior contractile vacuoles. The chromatophore is generally parietal but dissections are often observed. A pyrenoid may or may not be visible. In the transition from zoospore to young vegetative cell, the ellipsoidal shape is retained. The zoospores are of the Chlamydomonas type.

Sexual reproduction has not been observed.

Reticulochloris vacuolatum sp. nov.

Plate No. 1 (Figs. 1-9, x 1250)

- Figure 1. Walled zoospores (Chlamydomonas type).
- Figure 2. Vegetative cell, surface view. Note net-like plastid.
- Figure 3. Vegetative cell, optical section. Note excentric pyrenoid.
- Figure 4. Vegetative cell stained with Haidenhain's iron alum hematoxylin, showing large pyrenoid and single nucleus.
- Figure 5. Late stage of bipartition (optical section).
- Figure 6. Two recently divided cells showing the net-like chromatophores with excentric pyrenoid (optical section).
- Figure 7. Cleavage of cells within the parent wall.
- Figure 8. Characteristic tetrad of aplanospores.
- Figure 9. Zoospores formed from the cells of a tetrad.



Reticulochloris vacuolatum sp. nov.

Plate No. 2 (Figs. 10-12, x 950)

- Figure 10. Mature vegetative cells showing plastids, pyrenoids, and bubble-like vacuoles.
- Figure 11. Young ellipsoidal vegetative cells which have recently originated from zoospores. Note dissected plastid and pyrenoids visible in some cells.
- Figure 12. Oil immersion view of group of cells from actively growing culture showing solitary cells as well as characteristic tetrads. Also visible are several stages in cleavage.

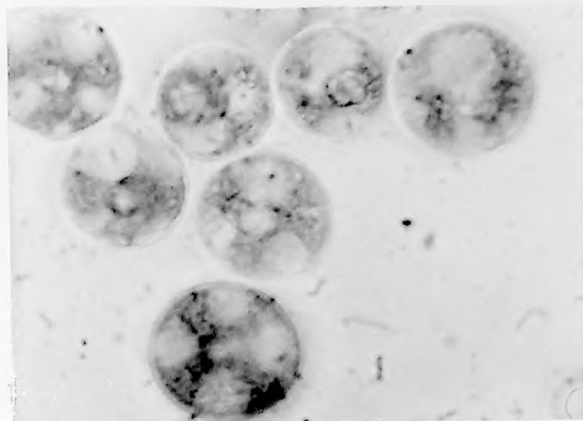


Figure 10

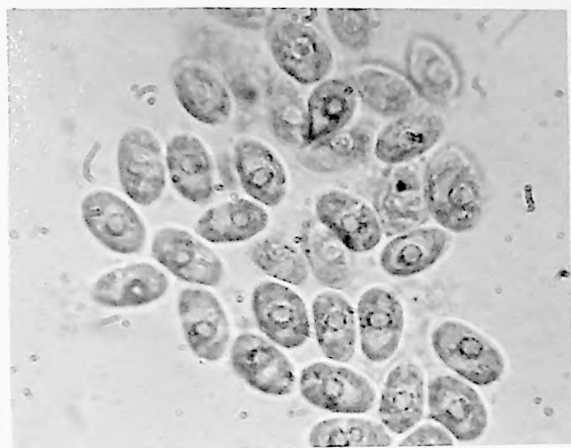


Figure 11

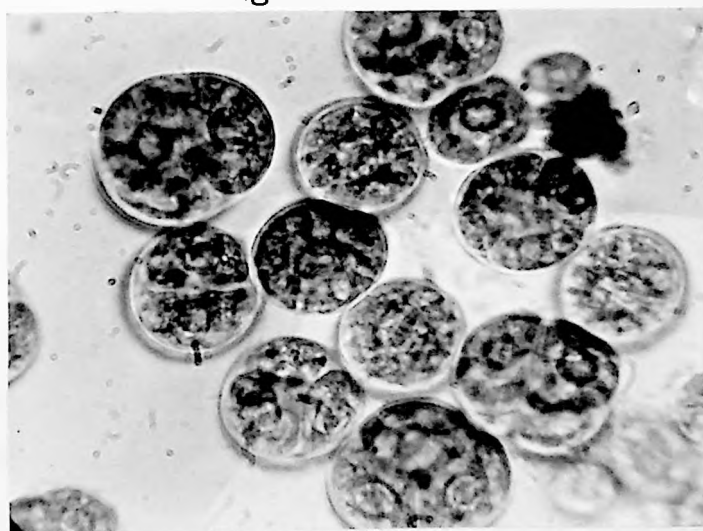


Figure 12

3. Reticulochloris alabamensis sp. nov.

Cellulae vegetativae iuvenes sphaericae aut ellipsoideae cellulae ellipsoideae, maturatae, sphaericae factae. Cellulae singulares aut in coenobiis temporaris forma indefinitis aggregatae, matricibus propriis saepe adhaerentibus, diametrum paene 30 u. attingentes. Tegumentum leve, lamina exterior tegumenti aetate incrassata, paene 4 u. attingens, cum vel sine matrice propria. Chromatophorus, pyrenoideum unicum excentricum habens, reticulum grossum ligamentorum implexorum. Nullae vacuolae pulsantes. Cellulae semper uninucleatae. Amylum praesens.

Propagatio asexualis per zoosporas et aplanosporas, bipartitione successive protoplasti factas. Zoosporae ellipsoideae, quamquam 8.0 u. longitudine mediocre, 3.0 u. latitudine, 7.0 u. as 9.5 u. longitudine et 2.5 u. ad 3.5 u. latitudine crescentes. Zoosporae habentes stigma anterior eminens, duas vacuolas pulsantes anteriores, duo flagella longitudine aequa, recurvata, zoospora quiescente. Zoosporae cum tegumentis, formam ellipsoideam, mobilitate finita, retinentes.

Propagatio sexualis non visa.

Young vegetative cells spherical or ellipsoidal, becoming spherical at maturity (or compressed with adjacent cells). Cells solitary, or in temporary groups of indefinite form, often adhering by their individual matrices,

attaining a diameter of approximately 30 microns. Cell wall smooth, outer wall layer thickening with age, attaining a thickness of about 4 microns, with or without an individual matrix. Chromatophore of coarse interconnecting net-like strands with a single excentric pyrenoid. No contractile vacuoles. Uninucleate throughout development. Starch is the reserve photosynthate.

Asexual reproduction by zoospores and aplanospores, formed by the successive bipartition of the protoplast. Zoospores ellipsoidal, 8.0 microns in average length, 3.0 microns in average width, but ranging from 7.0 microns to 9.5 microns in length and 2.5 microns to 3.5 microns in width; with prominent anterior stigma, two anterior contractile vacuoles, and two flagella of equal length held recurved when not in motion; zoospores with walls, retaining an ellipsoidal shape at the end of the motility period (Chlamydomonas type).

Sexual reproduction not observed.

Reticulochloris alabamensis sp. nov. (figs. 13-25) was isolated from a soil sample collected from a cotton field located on Greene County road No. 9, 3.5 miles from U. S. Highway No. 11 near Boligee, Alabama on 2 July, 1957. The pH of the soil at this location was 5 to 6. This organism grows well in Bold's inorganic salt medium, liquid and agar.

Young vegetative cells, recently formed from zoospores,

are ellipsoidal in shape (figs. 14,23). The ontogeny of the chromatophore is similar to that of Spongiochloris Starr (Starr 1955), in that the chromatophore of zoospores and young vegetative cells is generally parietal and cup-like and later becomes reticulate; but even in the zoospores of Reticulochloris the net-like nature of the chromatophore can sometimes be detected. As the cells mature, they become spherical (figs. 16,18,22). The cellulose wall layer of the smooth cell does not appreciably thicken with age, but the outer wall layer (fig. 22) sometimes attains a thickness of 4 microns. In cells from actively growing cultures in liquid soil extract medium, the outer wall layer is surrounded by an individual matrix (figs. 18,25). This matrix may also be present with cells grown in Bold's inorganic salt medium, but in this case the matrices are not as extensive. The average diameter of mature vegetative cells is approximately 20 microns, but cells up to 30 microns in diameter have been observed. These cells are often grouped in masses of variable form, apparently adhering by their individual matrices, and under these conditions are usually compressed one against another. In mature cells the net-like anastomosing strands become increasingly coarse and the strands become filled with starch. Even in cells grown in low light intensities (25 foot candles) the nature of the chromatophore may be obscured by the abundant starch. A single pyrenoid (fig. 18) is present in the net-like

chromatophore. In young cells the pyrenoid is almost parietal (fig. 14), in older cells the pyrenoid may be more nearly central but remains excentric. Contractile vacuoles have been observed in mature vegetative cells. A single large nucleus may sometimes be observed in the living condition in the cytoplasm near the pyrenoid. Stained preparations (fig. 17) (Feulgen technique, Haidenhain's iron alum hematoxylin) demonstrate that the organism is uninucleate throughout development. Starch is present as the reserve photosynthate.

Zoospores and aplanospores are produced by a successive bipartitioning of the protoplast (figs. 19,20,24). Under the conditions of culture routinely used, cleavage usually occurs during the night with the release of zoospores late the following morning. Zoosporogenesis may be induced by a transfer of mature cells to a fresh medium. Zoospores are often released from the parent cell in a vesicle following rupture of the parent cell wall. They are active within the vesicle which eventually dissolves to release them. Occasionally the zoospores are not released from the vesicle and form spherical or irregular masses of aplanospores (fig. 24). As few as 2 or as many as 32 or even more zoospores may be released and remain motile for several hours. Up to 64 or more small aplanospores may be formed. The zoospores (figs. 13,21), which are generally ellipsoidal in shape, taper slightly anteriorly. Zoospores average 8.0 microns

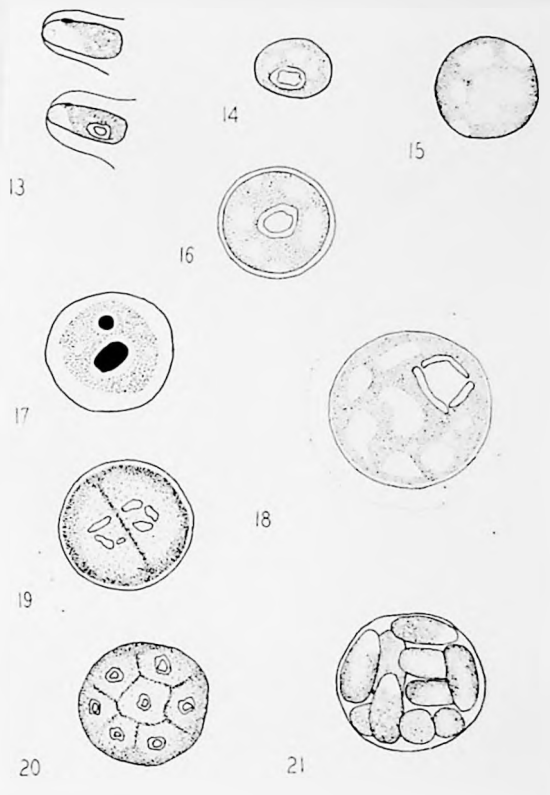
in length and 3.0 microns in width. Variations of 1 micron above or below these averages are common. A prominent anterior stigma is present, as are two anterior contractile vacuoles. At the end of the motility period the zoospores, which are walled (Chlamydomonas type), lose their flagella and stigmata, retain their ellipsoidal shape and enter a period of vegetative growth.

Sexual reproduction has not been observed.

Reticulochloris alabamensis sp. nov.

Plate No. 3 (Figs. 13-21, x 1250)

- Figure 13. Walled zoospores of the Chlamydomonas type, one with pyrenoid.
- Figure 14. Young vegetative cell recently formed from zoospore, with parietal pyrenoid.
- Figure 15. Vegetative cell showing net-like chromatophore, surface view.
- Figure 16. Vegetative cell, optical section, showing slight thickening of the outer wall layer.
- Figure 17. Vegetative cell stained with Haidenhain's iron alum hematoxylin, showing pyrenoid and single nucleus.
- Figure 18. Mature vegetative cell showing net-like chromatophore with excentric pyrenoid, and individual matrix.
- Figure 19. Early stage of cleavage (successive bipartition).
- Figure 20. Late stage of cleavage.
- Figure 21. Zoospores of the Chlamydomonas type prior to release from the parent cell.



Reticulochloris alabamensis sp. nov.

Plate No. 4 (Figs. 22-25, x 950)

- Figure 22. Mature vegetative cells. Note the thickened outer wall layer.
- Figure 23. Young vegetative cells. Note ellipsoidal shape indicating recent origin from zoospores of the Chlamydomonas type.
- Figure 24. Large cells undergoing cleavage and a group of aplanospores recently released from a parent cell.
- Figure 25. An india ink preparation of a group of vegetative cells, showing the pectinaceous material which surrounds the outer wall layer.



Figure 22

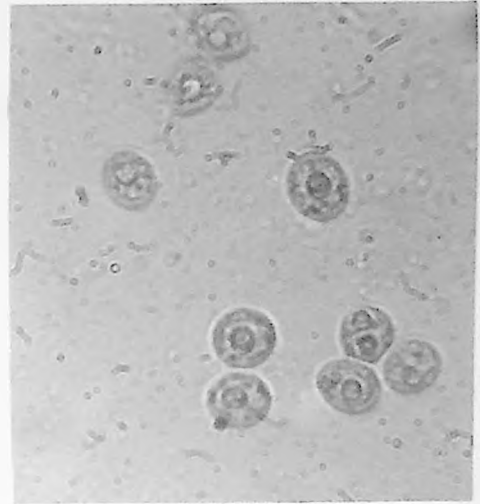


Figure 23

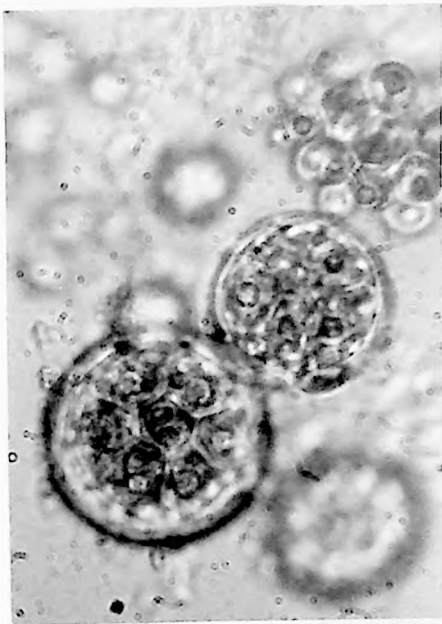


Figure 24

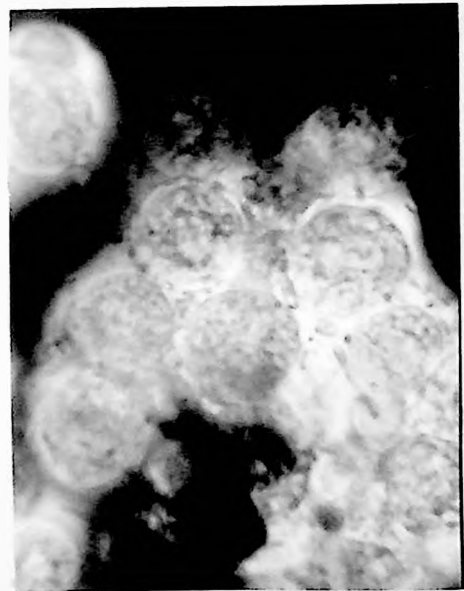


Figure 25

#### 4. Discussion

Starr (1955) distinguished the unicellular, spherical, zoospore-producing genera of the Chlorococcales primarily on the basis of: 1. chromatophore type, 2. presence or absence of pyrenoids, 3. zoospore type. In his study Starr clearly demonstrated the reliability of these attributes and other investigators (Trainor and Bold 1953, Arce 1956, Herndon 1958) have confirmed their reliability. The two chlorococcalean algae described in this investigation are characterized by: 1. vegetative cells with net-like chromatophore, 2. the presence of at least one pyrenoid in vegetative cells, and 3. production of walled (Chlamydomonas type) zoospores. The combination of generic characteristics stated above is found in no other known unicellular, spherical, zoospore-producing Chlorococcalean alga and for this reason, and since these characteristics have proved to be useful and reliable, the genus Reticulochloris has been erected.

The two species of this genus, which have been isolated from soil and described in this thesis, may be distinguished from one another by a number of attributes. Vegetative cells of Reticulochloris vacuolatum possess both static and contractile vacuoles. The cell wall does not appreciably thicken with age and no individual matrix is produced under any of the conditions of cultivation used in this investigation. Characteristically, 4 large (ca. 14 microns in

diameter) persistent aplanospores are produced and retained within the parent cell wall, forming a distinctive tetrad. Under the same conditions of culture, the outer cell wall layer of R. alabamensis thickens considerably with age, and an individual matrix may be produced. No contractile vacuoles have been observed in the vegetative cells, and the tetrads characteristic of R. vacuolatum are not produced. On the average, smaller zoospores and a greater number of smaller aplanospores are produced by R. alabamensis. Further, more photosynthate is produced by R. alabamensis as evidenced by the masking of the chromatophore. The writer regards these differences as sufficient to recognize these organisms as distinct species.

B. The Genus *Chlorosarcina* (Gerneck) Vischer

1. Genus description

*Chlorosarcina* (Gerneck) Vischer emend.

Cellulae vegetativae singulares aut in sarcinis non filamentosis aggregatae. Sarcinae divisione vegetativae cellularum factae. Cellulae sphaericae; cellulae inter se compressae, angulatae factae. Chromatophorus, sine nullis pyrenoideis, cavus sphericus. Amylum et oleum praesentia.

Propagatio asexualis, aut sarcinis, divisione vegetativae cellularum formatis, dissociatis aut zoosporis factis. Zoosporae habentes duo flagella longitudine aequa, sine tegumentis, sphaericae, motilitate finita, factae (*Protosiphon* genus).

Propagatio sexualis non visa.

Vegetative cells solitary or in non-filamentous packets resulting from vegetative cell division. Cells spherical, becoming angular when mutually compressed. Cell wall smooth. Chromatophore hollow, spherical, without pyrenoids. Reserve photosynthate starch and oil.

Asexual reproduction by dissociation of packets of cells produced by vegetative cell division, and by production of zoospores. Zoospores with two flagella of equal length, without walls, becoming spherical at the end of the motility period (*Protosiphon* type).

Sexual reproduction not observed.

2. Chlorosarcina macrovacuolatum sp. nov.

Cellulae vegetativae singulares aut in sarcinis non filamentosis aggregatae. Sarcinae divisione vegetativae cellularum factae. Cellulae sphaericae; cellulae inter se compressae, angulatae factae, diametrum paene 12 u. attingentes. Tegumentum leve; lamina interior cellulosa tenuis, lamina exterior pectinacea et matrix propter aetatem u. aliquibus incrassatae. Chromatophorus, nulla pyrenoida habens, cavus, parietalis, saepe cum apertura unilaterali. Vacuolae pulsantes praesentes. Cellulae semper uninucleatae. Oleum et amyllum in cellulis veteribus.

Propagatio asexualis aut sarcinis dissociatis aut zoosporis factis. Zoosporae saepe fusiformatae sed forma variantes; quamquam 8.0 u. longitudine mediocri, 3.5 u. latitudine mediocri, 7.0 u. ad 10.0 u. longitudine et 2.5 u. ad 4.0 u. latitudine crescentes; habentes stigma anterior, duas vacuolas pulsantes anteriores, et duo flagella longitudine aequa. Zoosporae sine tegumentis, sphaericae, motilitate finita, factae (Protosiphon genus).

Propagatio sexualis non visa.

Vegetative cells solitary or in non-filamentous packets resulting from vegetative cell division. Cells spherical, becoming angular when compressed with adjacent cells, reaching a diameter of approximately 12 microns. Cell wall smooth, inner cellulose layer thin, outer pectinaceous

layer and matrix thickening with age, attaining a thickness of several microns. Chromatophore hollow, parietal, usually with a unilateral opening, without pyrenoids. Contractile vacuoles present. Uninucleate throughout development. Oil and starch produced in old cells.

Asexual reproduction by dissociation of packets and by production of zoospores. Zoospores generally spindle-shaped, but variable in form; 8.0 microns in average length, 3.5 microns in average width, but ranging from 7.0 microns to 10.0 microns in length and from 2.5 microns to 4.0 microns in width; with anterior stigma, two anterior contractile vacuoles, and two flagella of equal length. Zoospores without walls, becoming spherical at the end of the motility period (Protosiphon type).

Sexual reproduction not observed.

Chlorosarcina macrovacuolatum sp. nov. (figs. 26-39) was isolated from a soil sample collected from a pasture on Greene County road No. 19, 0.4 miles from U. S. Highway No. 11 near Boligee, Alabama on 2 July, 1957. Soil pH at this location was 6 to 7. This organism grows well on Bold's inorganic salt medium and on soil extract medium, liquid and agar.

Young vegetative cells just formed from zoospores are spherical (fig. 29), but in groups are angular as a result of compression by adjacent cells. Solitary cells occur

but the most common growth habit is that of non-filamentous packets (figs. 32,35,38,39), the products of cell division remaining in close association with one another. The chromatophore (figs. 29,30) is parietal and cup-shaped with no pyrenoids. A unilateral opening in the chromatophore is usually present in old cells or those grown in intense illumination. The single nucleus (fig. 29) may be observed in living cells through the unilateral opening. That the organism was uninucleate throughout development was confirmed by the study of stained preparations made with the Feulgen technique and with the Haidenhain iron alum hematoxylin technique previously described.

The diameter attained by mature cells in actively growing cultures is approximately 12 microns. The cellulose wall layer (as determined by the Post-Laudermilk test) of the cell wall is quite thin (less than 0.5 microns) and remains thin, but the outer wall layer, which is pectinaceous in nature, may increase in thickness with age. Single cells and groups of cells are characteristically enclosed in a pectinaceous matrix (figs. 32,35,38,39) which may become rather extensive, especially in soil extract media. The extent of the matrix and outer wall layers can be more easily seen in methylene blue preparations than in untreated cells or groups of cells. Starch occurs in fine granules and is not found in great quantities in young cells. In older cells more starch is

present but these also contain considerable quantities of oil, demonstrable with Sudan IV.

As an individual cell approaches maturity, it becomes somewhat elongate, cleavage occurs, and a cellulose wall layer is laid down approximately across the equatorial plane of the cell (fig. 31). The daughter cells increase in size and remain in close association with one another. The daughter cells may then in turn divide. Direction of division is not restricted but commonly occurs in three directions, and non-filamentous, sarcina-like packets result. While products of division are retained in close association with one another, the individual cells may separate somewhat (fig. 32) and eventually dissociation of the packets may occur if zoospore formation does not intervene. Two large contractile vacuoles (fig. 32) are often observed in vegetative cells. Stigmata are occasionally seen on vegetative cells (fig. 34). These stigmata do not appear to be the persistent stigmata of the zoospores from which they were derived; they may appear, persist for a time, and disappear. The conditions under which this occurs have not been determined.

In addition to dissociation of packets, asexual reproduction is effected by zoospore formation. In actively growing cultures, usually 4 zoospores are formed by successive bipartition of the protoplast of each cell. Under the conditions of cultivation, cleavage occurs at night

after transfer of cells to a fresh medium, with the release of zoospores occurring in the early morning, usually after less than an hour of illumination. Cleavage furrows are delicate, not nearly so distinct or broad as those found in similar stages of Reticulochloris. When zoosporogenesis occurs in hanging drop preparations, almost every cell in the preparation produces zoospores simultaneously, and liberation is complete usually within 5 to 10 minutes following the first signs of motility. First the common matrices dissolve, releasing the smaller packets, which in turn dissociate, releasing the individual cells. Finally the individual cell wall ruptures or dissolves to release the zoospores (fig. 37), usually contained in a vesicle. The period of motility is approximately an hour in duration.

Zoospores (figs. 26,37) vary considerably in form but are generally spindle-shaped. They possess two flagella of equal length, an anterior, slightly protuberant stigma very near the apex of the cell, and two anterior contractile vacuoles. The zoospores average 8.0 microns in length, and 3.5 microns in width, but variations of 1 micron from these averages are common. The chromatophore is parietal. Zoospore movement is rapid and rarely ceases, even momentarily. At the end of the motility period the zoospores lose their flagella and become spherical in shape, indicating that they are of the Protosiphon type.

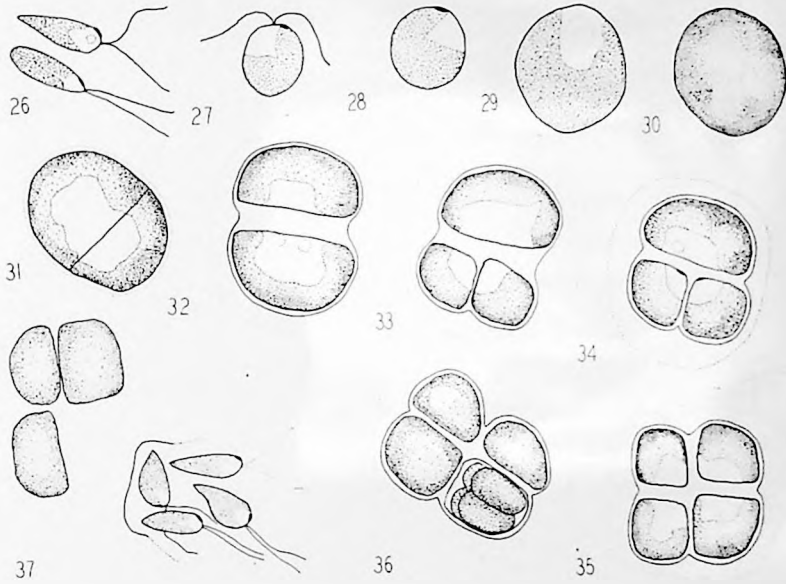
Sexual reproduction has not been observed.

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Chlorosarcina macrovacuolatum sp. nov.

Plate No. 5 (Figs. 26-37 x 1250)

- Figure 26. Zoospores of the Protosiphon type.
- Figure 27. Zoospore near the end of the motility period becoming spherical.
- Figure 28. Young spherical vegetative cell just formed from zoospore but retaining the stigma.
- Figure 29. Vegetative cell showing the nuclear condition.
- Figure 30. Vegetative cell, surface view, with unilateral opening in cup-like plastid.
- Figure 31. Cell in division.
- Figure 32. Mature cells, one with two large contractile vacuoles.
- Figure 33, 34 and 35. Packet-like groups of vegetative cells; note stigmata on cells in fig. 34.
- Figure 36. Zoospore formation.
- Figure 37. Release of Protosiphon type zoospores.



Chlorosarcina macrovacuolatum sp. nov.

Plate No. 6 (Figs. 38-39, x 950)

Figures 38, 39. Non filamentous packets of vegetative cells, resulting from vegetative cell division. Note packets surrounded by pectinaceous material.

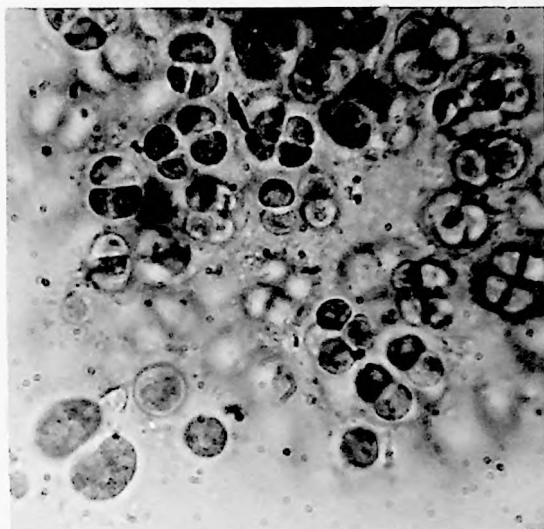


Figure 38

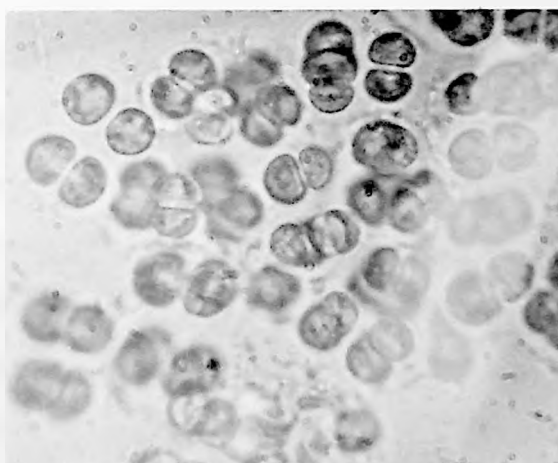


Figure 39

### 3. Discussion

Gerneck (1907) described two species, Chlorosarcina minor and Chlorosarcina elegans. Both species possessed a hollow spherical chromatophore and produced zoospores of a similar type, but Chlorosarcina elegans lacked pyrenoids. In 1933 Vischer emended the genus and placed Chlorosarcina minor in the genus Chlorosphaera. He retained Chlorosarcina elegans in the genus Chlorosarcina but described it as a genus imperfecte notum, and justifiably so, since Gerneck stated that he had studied the alga only briefly before losing the culture. However, Vischer's use of the genus name Chlorosphaera has been considered invalid by Smith (1933) since Chlorosphaera had previously been used by Henfry (1859). Herndon (1958) proposed the name Chlorosarcinopsis for members of this genus and further delimited the genus as follows: "to include only those unicellular chlorophycean algae capable of vegetative cell division, unrestricted in direction, which possess the following attributes in addition: 1. a simple parietal chromatophore, 2. at least one pyrenoid, 3. production of zoospores with 2 flagella of equal length whose bodies lack well defined walls and become spherical at the end of the motility period (Protosiphon type, Starr 1955)."

The genus Chlorosarcina, differs from Chlorosarcinopsis in the absence of pyrenoids. Vischer recognized that the presence or absence of pyrenoids was a reliable

characteristic on which to base a separation of the genera of this group when he emended Chlorosarcina in 1933. Starr (1955) used this characteristic, together with chromatophore type and zoospore type, to delimit the spherical zoospore-producing genera of the Chlorococcales. He found these attributes to be stable and dependable, as did Herndon (1958) in his work with the vegetatively dividing Chlorosphaerales. Vischer, however, apparently did not recognize the importance of zoospore type and therefore did not include this characteristic in his description. For this reason, the writer believes that it is desirable to include a further emendation of the genus Chlorosarcina, specifying zoospore type in an amplified description.

Chlorosarcina elegans, as described by Gerneck (1907), attained a maximum size of 27 microns, produced no gelatinous matrix, and produced numerous zoospores from a single cell. In contrast, Chlorosarcina macrovacuolatum attains a maximum size of approximately 12 microns. The pectinaceous material which surrounds the packets is easily observed, even without the aid of india ink or methylene blue preparations. Further, only 4 zoospores are usually produced by one cell and two large contractile vacuoles are often present in the vegetative cells of Chlorosarcina macrovacuolatum.

#### IV. SUMMARY

1. Reticulochloris gen. nov., a spherical, zoospore-producing member of the Chlorococcales, is defined as possessing the following combination of characteristics:

- a) vegetative cells with a net-like chromatophore.
- b) vegetative cells with at least one pyrenoid.
- c) walled (Chlamydomonas type) zoospores.

2. Reticulochloris vacuolatum sp. nov., is characterized by the formation of persistent aplanospore tetrads, the presence of contractile vacuoles in mature vegetative cells, and a thin cell wall which does not appreciably thicken with age.

3. Reticulochloris alabamensis sp. nov., is distinguished by the absence of tetrad formation, the absence of contractile vacuoles in mature vegetative cells, and a thin cell wall, the outer layer of which thickens with age.

4. Chlorosarcina (Gerneck) Vischer is emended to include only those unicellular or colonial Chlorophyceae capable of vegetative cell division (Chlorosphaeraceae) with the following combination of characteristics:

- a) vegetative cells with a cup-shaped, parietal chromatophore.
- b) vegetative cells with no pyrenoids.
- c) zoospores without walls (Protosiphon type).

5. Chlorosarcina macrovacuolatum sp. nov. is distinguished from other species of this genus by the presence of contractile vacuoles in mature vegetative cells, the production of an external pectinaceous matrix, the production of relatively few zoospores per cell, and the small size of the vegetative cells (approximately 12 microns in diameter).

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GRADUATE SCHOOL  
UNIVERSITY OF ALABAMA

(Thesis Approval Form)

Name of Candidate Temd Robert Deason  
Major Subject Biology  
Title of Thesis A Study of Some Chlorophyceae  
from the Black Belt Soil of Alabama.

Approved by:

Thesis Committee:

\_\_\_\_\_, Chairman  
\_\_\_\_\_  
\_\_\_\_\_

Head of Department ~~or School~~ \_\_\_\_\_

Dean of the Graduate School \_\_\_\_\_

Date May 7, 1958