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Green Endosymbiont of *Coleps* from Lake Cisó Identified as *Chlorella vulgaris*

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Abstract

At the metalimnion (between the oxic epilimnion and anoxic hypolimnion) of the sulfurous Lake Cisó (northeastern Spain) resides the green prostomatid ciliate *Coleps hirtus*. The population of ciliates develops up to 10^4 individuals per milliliter. Along with the cryptomonad *Cryptomonas phaseolus*, these protists form a thin layer just above the thick dominant layer of purple and green phototrophic bacteria (Chromatiaceae and Chlorobiaceae).

The form-genus *Chlorella*, on the basis of ultrastructural features, has recently been split into the following genera: *Auxenochlorella, Chlorella, Coelastrella, Graesiella, Halochlorella, Kermatia, Mychonastes* and *Scotiellopsis*. Our ultrastructural studies identify the chlorophyte (chloroprotist) endosymbionts of the Lake Cisó *Coleps* as *Chlorella vulgaris* var. *vulgaris*. These studies also revealed ectosymbiotic bacteria, possibly sulfide oxidizers, on the surface of this *Coleps hirtus*.

Keywords: *Chlorella* genera, *Chlorella*, *Coleps*, ciliate ecology, Lake Cisó, stratified sulfurous lakes, intracellular calcium carbonate precipitation

Abbreviations: LM light microscope, TEM transmission electron microscope, SEM scanning electron microscope

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1. Introduction

A series of studies of a"red anaerobic lake" (Lake Cisó) in northeastern Spain have been underway since 1976 with the goal of characterizing the microbial community. Vertical profiles throughout the annual season have been taken (Pedrós-Alió et al., 1983, 1987; Guerrero et al., 1985; Guerrero and Mas, 1988) which reveal a thick bacterial layer of Chromatiaceae and Chlorobiaceae that undergoes periodic changes in its position in the water column. During winter mixing, the red layer of phototrophic bacteria expands to the whole lake, reaching its surface. In this situation, dissolved oxygen is absent from the water. At other times, especially during summer, the bacterial layer is found at 1.5 m, where dissolved oxygen is absent (anoxic water). The concentration of hydrogen sulfide in the water column is relatively elevated due to the fact that in the sediments of the lake, which rests in karstic terrain, very active bacterial sulfate reduction takes place. Sulfate is abundant in the water (about 1 g/l) due to the dissolution of Miocene gypsum ($CaSO_4 \cdot 2H_2O$). The hydrogen sulfide during the period of mixing ranges from 35 to 50 mg/l. It is as high as 70 mg/l in the hypolimnion during the summer vertical stratification.

Large population densities of a green ciliate have been seen for several years at the top of the bacterial layer and identified as *Coleps* with chlorella-like symbionts by Dyer et al., 1986, based on Christopher and Patterson, 1983. Recognizing recent changes in the classification of both immotile spherical green algae and all ciliates, this paper identifies the host and its chlorophyte endosymbionts on the basis of an electron microscopic study.

2. Materials and Methods

Samples from the metalimnion were taken with weighted tubing connected to a pump. Samples for SEM were concentrated by filtration through a 0.2 μm pore size Nucleopore filter. Filters were re-suspended in 2.5% glutaraldehyde buffered with cacodylate and dried by critical point drying (Esteve et al., 1983). Samples for TEM were fixed with osmium tetroxide according to the method of Ryter-Kellenberger (Ryter et al., 1958). Samples were embedded in Spurr's resin and sectioned with a LKB microtome. They were placed on formvar-coated grids and examined with a Hitachi 2A transmission electron microscope at 20 kV. Autofluorescence was also observed by fluorescence microscopy, using the microscope filters.

3. Results and Discussion

Light microscope observations coupled with electron microscopy led us to estimate the presence of 30–50 algae per ciliate (Fig. 1). Most *Coleps* observed contained intracellular algae.

Ciliate ultrathin sections showed the algae to be *Chlorella*-like on the basis of autospore formation (four cells retained inside the parental wall) produced by two mitotic cell divisions (Fig. 2). Each alga is surrounded by its own plasma membrane and smooth cell wall; each, alone as a parental cell or as two or four autospores derived from a parental cell, is inside a ciliate vacuolar membrane. The algae have a conspicuous nucleus and mitochondria with tubular cristae. The algal thylakoids are well developed with pyrenoids and starch grains. The healthy ultrastructural appearance of the growing algae led us to conclude that they are functionally photosynthetic inside the *Coleps* (Figs. 3–4), consistent with observations by Christopher and Patterson (1983).

Unidentified membrane-associated bacteria associated with the cilia were seen on each *Coleps* (Fig. 5). Intracellular biomineralization processes were also revealed in thin sections. Active precipitation of calcium carbonate surface layers occurs by intracellular production of calcite crystals (arrow) and subsequent deposition of these membrane-associated crystals at the cell surface. Both a vacuole-limited crystal (arrow) and a surface bacterium with its two points of attachment can be seen in Fig. 6.

The scanning electron micrographs show files of 12–15 longitudinal plates by a maximum of 13 horizontal ones at the cell equator. They form the basis for the drawing of Fig. 7. *Cryptomonas*, the protist, and small phototrophic bacteria are also associated with the larger *Coleps* (Fig. 8).

The classification of *Coleps* is shown in Table 1. The genus *Chlorella* has recently been split into at least 8 genera (Kalina and Punčochářová, 1987; Komarek, 1987). We summarize the salient features of these genera in Table 2; in Table 3 we detail comparisons of species of the genus *Chlorella*.

We conclude that the algal symbionts are *Chlorella vulgaris* on the basis of the criteria shown in the tables. These small green, non-motile, coccoid algae formerly were all classified as *Chlorella* spp. Nevertheless, our coccoid endosymbiont clearly can be classified as *Chlorella vulgaris* var. *vulgaris* because of its (1) smooth thin wall, (2) spherical to ellipsoidal shape, (3) size (5 μm in diameter), (4) saucer-shaped starch grains and (5) 2–4 autospores.

The physico-chemical parameters of Lake Cisó that determine the distribution of *Coleps* and its *Chlorella* are shown in Fig. 9, which also plots the distri-

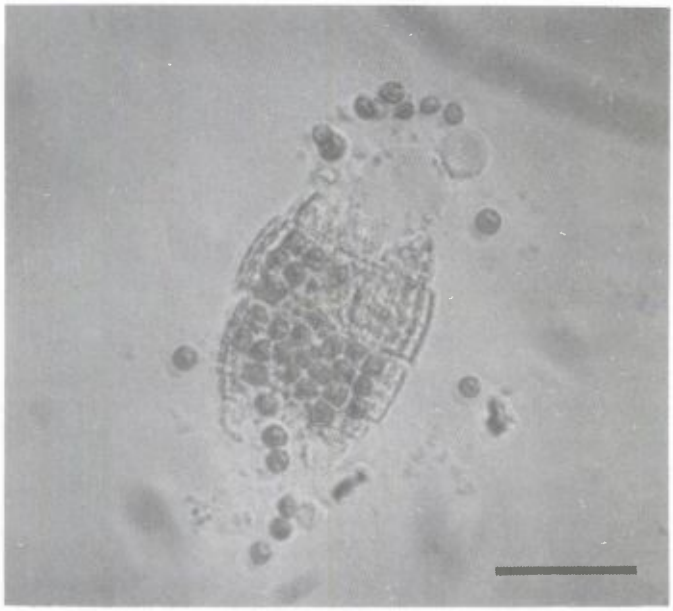

Figure 1. The rhabdophoran ciliate *Coleps hirtus* showing presence of intracellular green algal symbionts. The cell is partially disrupted, allowing release of the symbionts. (LM, bar = 10 μm). (Courtesy of J.M. Gasol).

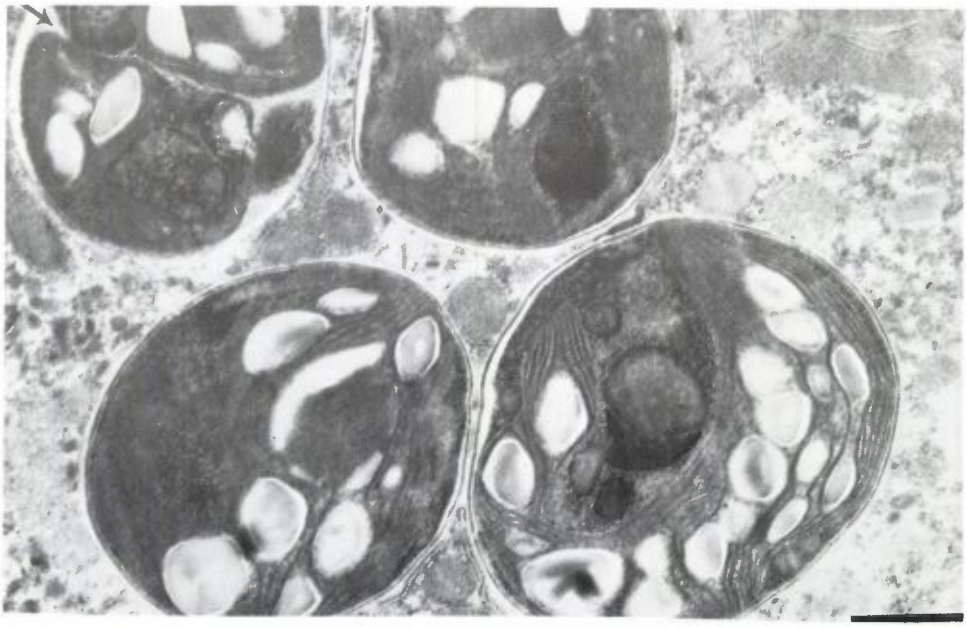

Figure 2. Formation of autospores (arrow) by algae within the *Coleps* cytoplasm. (TEM, bar = 1 μm).

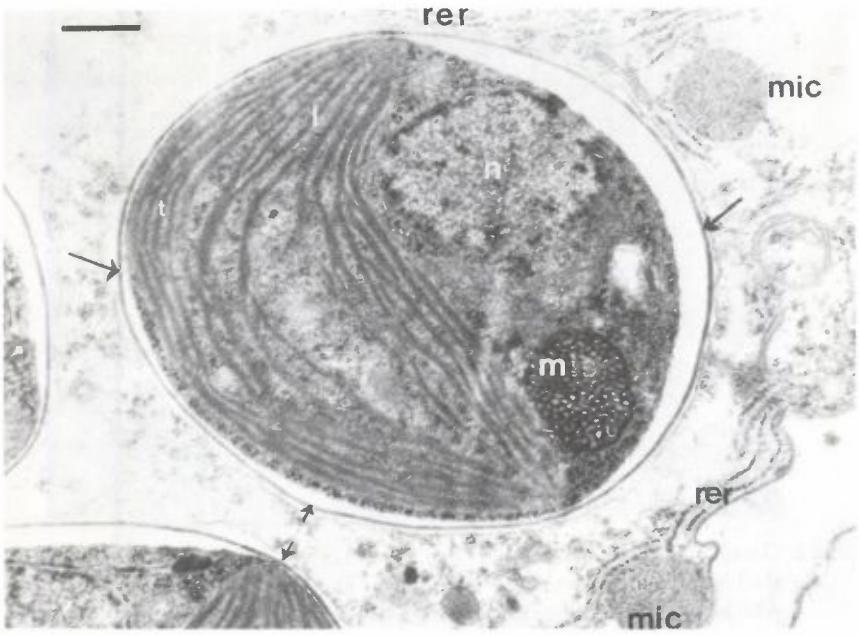

Figure 3. Algal cell in *Coleps*. Thylakoid membranes (t), nucleus (n) and a mitochondrion with tubular cristae (m) are visible. Note the smooth cell wall (arrows). Rough endoplasmic reticulum (rer) and two mitochondria (mic) of the *Coleps* can also be seen. (TEM, bar = 0.5 μm).

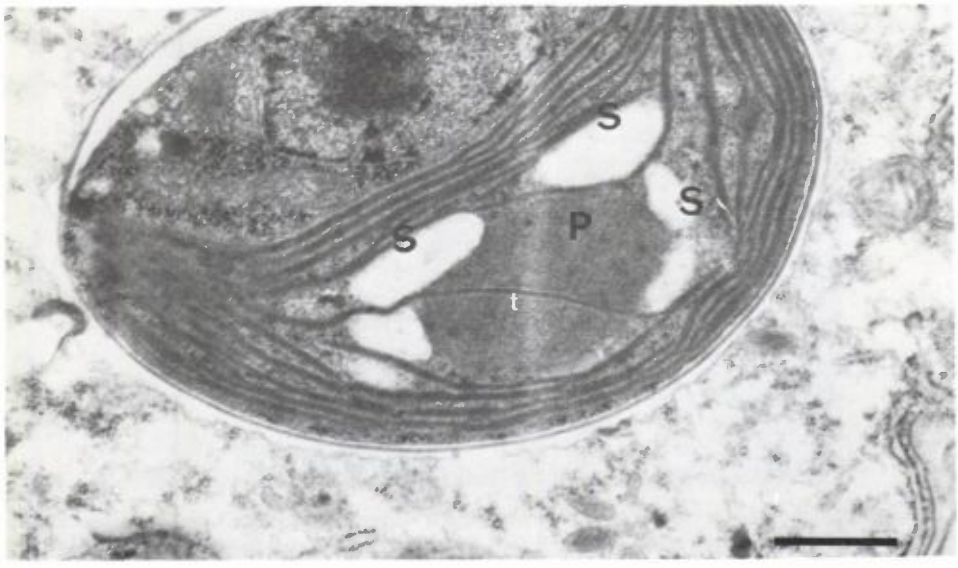

Figure 4. A *Chlorella* cell, in which the starch grains (S) are visible. A thykaloid membrane (t) can be seen traversing the pyrenoid (P). (TEM, bar = 0.5 μm).

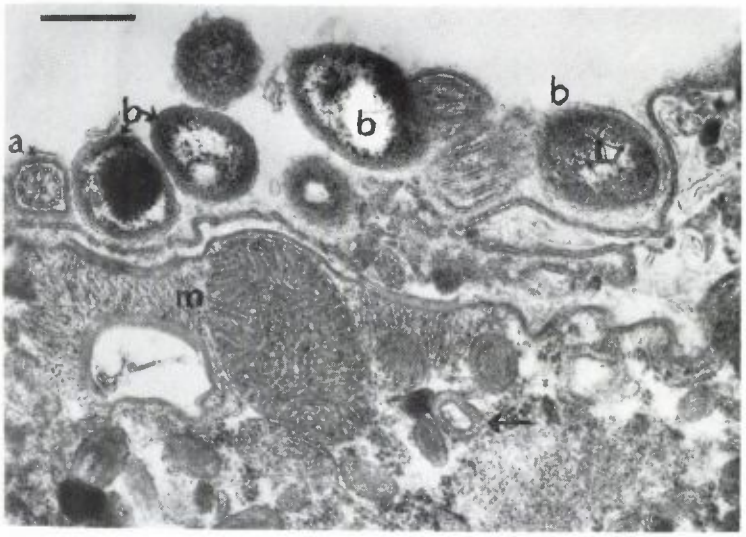

Figure 5. Cortex of *Coleps*. Associated with the outer membrane of the *Coleps* are unidentified bacteria (b). A mitochondrion (m) and a calcite crystal in a vacuole (arrow) which will be deposited on the surface layer, can be seen in the *Coleps* cytoplasm. The [9(2)+2] microtubules of the ciliary axoneme (a) are seen embedded in the same extracortical material as that which embeds the bacteria. (TEM, bar = 0.5 μm).

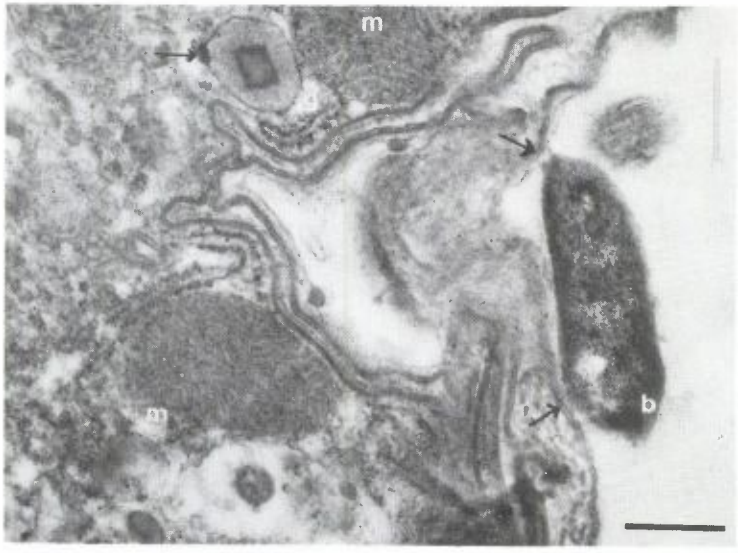

Figure 6. Section of the cortex of *Coleps* showing a vacuole which contains an intracellular calcite crystal (arrow), which will eventually participate in the formation of a surface layer. Also, two mitochondria (m) and extensive Golgi (g) are visible in the cytoplasm. An extracellular bacterium (b) is attached at two places at least (arrows) to the *Coleps*. (TEM, bar = 0.5 μm).

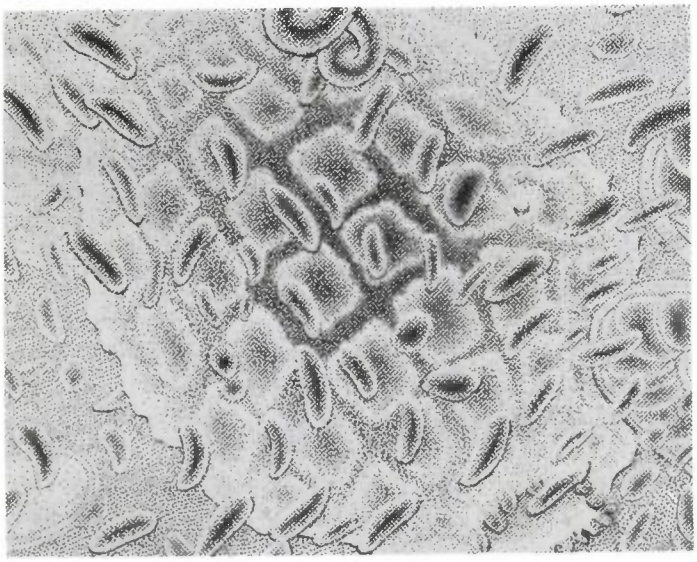

Figure 7. Drawing of exterior of *Coleps* showing the plates on the ciliate surface and relation to other microbes. In the lake, *Coleps* are surrounded by both attached and unattached bacteria. (Illustration by Christie Lyons).

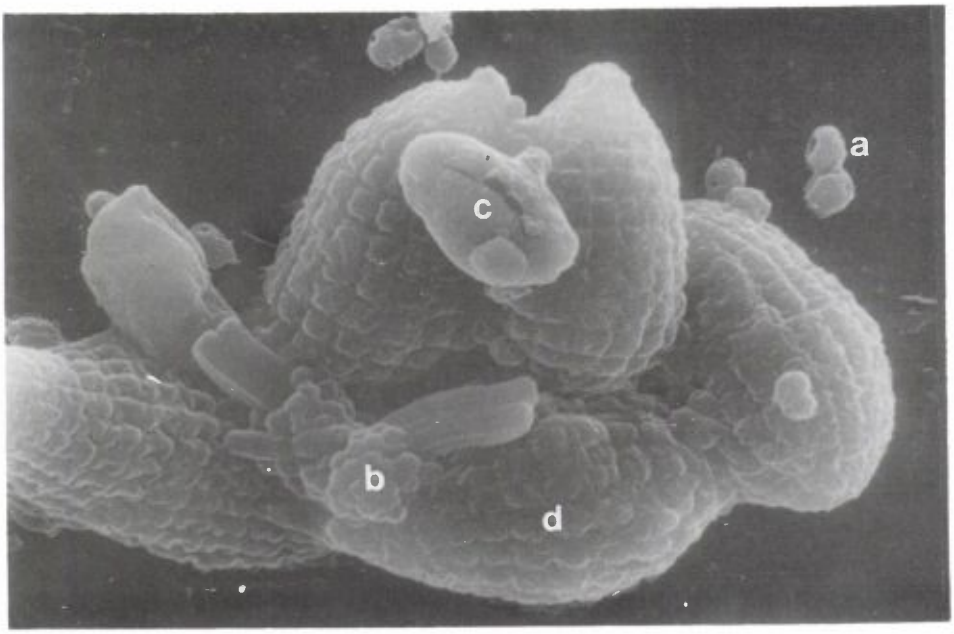

Figure 8. Clump of five *Coleps* ciliates (d) in which the rugose surface with pattern of $CaCO_3$ plates is seen. At least two different types of bacteria, *Amoebobacter* (b) and *Chromatium* (a), as well as *Cryptomonas* (c), are found associated with the *Coleps* (d). (SEM, bar = 10 μm). (From Dyer et al., 1986).

Table 1. Classification of *Coleps**

		Criteria
Kingdom	Protoctista (Margulis et al., 1989)	non-fungal spora, non-embryo-forming eukaryotes
Phylum	Ciliophora (Doflein, 1901)	dimorphic nuclei, characteristic kinetids
Subphylum	Rhapdophora (Lynn and Small, 1981)	ciliates with somatic monokinetids and a crown of dikinetids around mouth
Class	Prostomatea (Schewiakoff, 1896)	mouth apical to subapical
Suborder	Prorodontida (Corliss, 1974)	mouth apical to subapical
Family	Colepidae (Ehrenberg, 1838)	body often spiny, $CaCO_3$ plates usualiy in cortex
Genus	*Coleps* (Nitzsch, 1827)	body ovoid to barrel-shaped, spiny
Species	*hirtus*	60 μm long, two spines, freshwater. (Identified in B.D. Dyer et al., 1986)

*After Small and Lynn, 1985 and Margulis et al., 1989. Earlier references in Corliss, 1979.

bution data for phototrophic bacteria, members of the family Chromatiaceae. In a previous study (Dyer et al., 1986) the ciliate *Coleps* was identified by protargol silver staining (Small and Lynn, 1985) and the calcium carbonate nature of the surface plates determined by cytochemical examination.

Coleps, well-described by Ehrenberg (1838), is restricted to the surface of the anaerobic phototrophic bacterial plate where it is simultaneously in

Table 2. Genera presently and formerly *Chlorella*[*]

Genus and species (former name)	New genus	*Major basis for re-classification* Wall	Sporopollenin	Pyrenoid	Thylakoids in pyrenoid
Chlorella vulgaris vulgaris	unchanged	see Table 3	+	+	+
C. fusca v. *vacuolata*	*Graesiella*	wavy, wall without thickenings, ribs of wall form network, ribs visible only in EM[*]	+	+	–
C. fusca v. *rubescens*	*Halochlorella rubescens*	ribs of wall form network, visible only in EM, cell wall with polar thickenings	+	+	–
C. cf. *minutissima*	*Kermatia*	ribs form irregular network, 1–2 thickenings	+	+	–
Chlorella minutissima	*Mychonastes*	irregular network of ribs, visible only in EM; cysts[**]	+	+	–
Chlorella protothecoides	*Auxenochlorella*	irregular network of ribs, visible only in EM	+	–	–
Chlorella	*Scotiellopsis*	meridional ribs visible in LM, converging at poles, polar thickenings	+	+	–

[*]After Kalina and Punčochářová, 1987 and Komarek, 1987. [**]Margulis et al., 1988.
LM = light microscope EM = electron microscope

Table 3. *Chlorella*: comparison of species*

	Cell shape	Cell wall	Chloroplast
C. vulgaris var. *vulgaris* cosmopolitan, known as symbiont in *Stentor*, *Hydra*	ellipsoidal to spherical	smooth, thin	in one piece and undivided, cup-shaped or girdle-shaped filling 3/4 of cell periphery
C. vulgaris var. *autotrophica*	always spherical	smooth, thin	filling almost all of cell cavity, cup-shaped
C. kessleri known only in cultures	spherical	smooth, thin	mantle-shaped
C. fusca var. *fusca* known only in cultures	ellipsoidal to spherical	thin, well defined, smooth	parietal, forming hollow sphere, irregularly thickened, perforated by narrow incisions
C. fusca var. *vacuolata* tree bark	ellipsoidal to spherical	relatively thin, sharply defined	same as in var. *fusca*, vacuoles always present, numerous
C. luteoviridis probably cosmopolitan	spherical	thick	thin, filling barely 1/4 of cell periphery, saucer-shaped to irregularly band-shaped
C. saccharophila var. *saccharophila* aerial localities	cylindrical-ellipsoidal with broadly rounded ends	thin	thin, trough-like or band-shaped, filling 1/2 cell periphery
C. saccharophila var. *ellipsoidea* pools, soil and aerial localities	narrow or broadly ellipsoidal	relatively thick	saucer or band-shaped in younger cells, in adult cells, concavely discoid, lobed at margin

*After Fott and Nováková, 1969

Pyrenoid	Nucleus	Size (μm)	Autospores
spherical to ellipsoidal, 2–5 saucer-shaped starch grains	not distinct *in vivo*	2.0–7.6× 3.8–6.4	2–4 (rarely 8–16)
large, 2–3 saucer-shaped starch grains	distinct *in vivo*	4–10	4–8
broadly ellipsoidal, 2 (rarely more) cup-shaped starch grains	slightly eccentric	2.5–8.9	2,4,8, or 16
large, usually spherical, 2 or more more saucer-shaped starch grains	slightly eccentric, visible	3.3–8.9 × 3.7	2–4 (rarely 8)
same as in var. *fusca*		3–14 × 5–16.5	2,4,8 (rarely 16)
distinct, more-or-less spherical, covered with starch grains		3–14 × 5–10	various numbers of small and one large, unequal in size
spherical, not distinct, without starch-sheath		4.5–10.2 2.5 × 6.5	2,4,8 (rarely 16)
distinct, numerous starch grains at periphery		2–15 × 1.5-13	2–32, unequal size

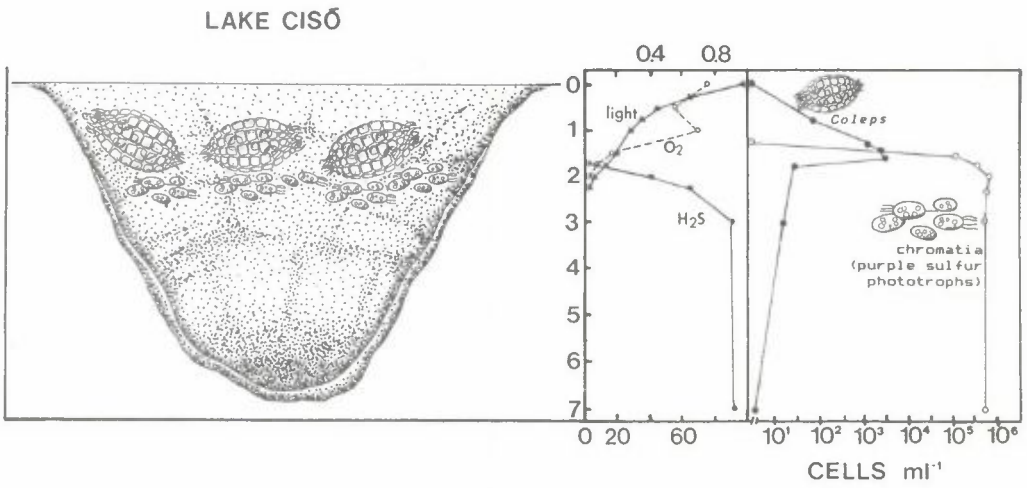

Figure 9. (Left hand panel) Diagram of Lake Cisó showing *Coleps* at 1 m depth, just above the phototrophic bacterial layer. (Central panel) Vertical distribution of light, oxygen and sulfide on June 15, 1984. Depth in meters. Values for oxygen (empty circles) correspond to the upper scale and have to be multiplied times 10 to obtain mg/l. Sulfide (full circles) is shown in mg/l in the lower scale. Light (triangles) is shown as percentage of surface light intensity in the lower scale. The second maximum in oxygen concentration is probably due to oxygenic photosynthesis by *Chlorella* and *Cryptomonas*. (Right hand panel) Numbers of *Coleps hirtus* (full circles) and purple phototrophic bacteria (empty circles) on the same date. (Dyer et al., 1986; Illustration by Rae Wallhausser).

contact with sulfide from below and oxygen generated by its algal symbionts. The bacterial surface symbionts which are regularly associated with *Coleps* therefore may be sulfide-oxidizing organisms held at the oxidized/reduced interface by the actively motile ciliates.

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