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The Microbial Community of *Ophrydium versatile* Colonies: Endosymbionts, Residents, and Tenants

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Abstract

Ophrydium versatile is a sessile peritrichous ciliate (Kingdom Protoctista, class Oligohymenophora, order Peritrichida, suborder Sessilina) that forms green, gelatinous colonies. Chlorophyll *a* and *b* impart a green color to *Ophrydium* masses due to 400–500 *Chlorella*-like endosymbionts in each peritrich. *Ophrydium* colonies, collected from two bog wetlands (Hawley and Leverett, Massachusetts) were analyzed for their gel inhabitants. Other protists include ciliates, mastigotes, euglenids, chlorophytes, and heliozoa. Routine constituents include from 50–100,000 *Nitzschia* per ml of gel and at least four other diatom genera (*Navicula, Pinnularia, Gyrosigma, Cymbella*) that may participate in synthesis of the gel matrix. Among the prokaryotes are filamentous and coccoid cyanobacteria, large rod-shaped bacteria, at least three types of spirochetes and one unidentified *Saprospira*-like organism. Endosymbiotic methanogenic bacteria, observed using fluorescence microscopy, were present in unidentified hypotrichous ciliates. Animals found inside the gel include rotifers, nematodes, and occasional copepods. The latter were observed in the water reservoir of larger *Ophrydium* masses. From 30–46% of incident visible radiation could be attenuated by *Ophrydium* green jelly

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†We dedicate this paper to the memory of Kenneth W. Estep (December 7, 1952–January 19, 1995), protoctistologist extraordinaire, discoverer of dasmotrophy and dear colleague who represented some 250,000 species like *Ophrydium versatile* at the Expert Center for Taxonomic Identification in Amsterdam, The Netherlands.

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masses in laboratory observations. Protargol staining was used to visualize the elongate macronuclei and small micronucleus of *O. versatile* zooids and symbiotic algal nuclei. Electron microscopic analysis of the wall of the *Chlorella*-like symbiont suggests that although the *Ophrydium* zooids from British Columbia harbor *Chlorella vulgaris*, those from Hawley Bog contain *Graesiella* sp. The growth habit in the photic zone and loose level of individuation of macroscopic *Ophrydium* masses are interpretable as extant analogs of certain Ediacaran biota: colonial protists in the Vendian fossil record.

Keywords: bog green jelly masses, *Graesiella* symbionts, *Navicula* symbionts, *Nitzschia* symbionts, gel matrix symbionts, Ediacaran analogues, protargol technique.

1. Introduction

Ophrydium zooids are spindle-shaped myoneme ("muscle thread")-bearing peritrichous ciliates (1826 by de St. Vincent, and 1838 by Ehrenberg in Foissner et al., 1992). Large colonies of this sessile peritrich were first described as *Ophrydium versatile* in 1786 by Müller (in Brightwell, 1848). This colonial protist forms green, gelatinous masses up to 30 cm in diameter (in Quebec, Pierre Couillard, personal communication, and in Germany, Buecker and Hausmann, 1994). The green color is imparted by endosymbiotic *Chlorella*-like algae contained within each *Ophrydium* zooid, as well as by the other cyanobacteria and algae living within the gel. Individual zooids are most often found centripetally embedded in the peripheral layers of the gel, with their buccal cilia oriented toward the surface of the gel matrix from which they protrude for feeding (Goff and Stein, 1981). The gelatinous masses are usually seen attached to aquatic plants such as *Myriophyllum* (water milfoil) and *Potamogeton* (pondweed), or unattached in shallow littoral waters (Hollowday, 1975).

Embedded in the gelatinous matrix, which consists primarily of carbohydrates (Buecker and Hausmann, 1994), live a variety of regularly-associated microbial inhabitants whose presence and distribution throughout the season was investigated. Like all peritrichs, *Ophrydium versatile* zooids reproduce by transverse fission, the division occurs while the ciliate is contracted (Hollowday, 1975). New colonies are founded by swimming, settling telotrochs that attach to aquatic substrates. The colonies probably decay by the start of winter (Eaton and Carr, 1980). Telotrochs differentiate when the axonemes of the trochal band kinetosomes grow out to form swimming organelles. Nothing is known about sexual life histories in this group of peritrichs. Whether telotrochs conjugate or settle in preexisting colonies is not

known. Neither is the role, if any, of diatoms in forming the cohesive gel mass understood.

Chlorella-like algae found within *Ophrydium* are permanent symbionts. All zooids collected contained these symbionts from the buccal apparatus to the holdfast region. Their presence in *Ophrydium* habitat may be maintained in part by their acid tolerance, in fact these green algae excrete maltose at low pH (Huss et al., 1993). Yet little is known about the functional aspects of nutrient exchange between the algal symbionts and their ciliate hosts. *O. versatile* is predaceous; it draws in bacteria, fungi, algae and other small protists through its buccal apparatus. The ability of the zooid to digest its own green algal symbiont has been demonstrated (Goff and Stein, 1981).

Winkler and Corliss (1965) described the "typical" *Ophrydium* as follows: "The gelatinous mass of an intact ophrydial colony almost defies precise verbal description". Simply stated, the masses come in many shapes and sizes. European 19th century naturalists studied the curious green gelatinous masses noting that the alignment of each zooid within the gel resembles a small eyebrow (from the Greek "ophrys", or "ophrydion", dim.). From the notes of *Fauna Infusoria*, observations from 1846–1847, Ehrenberg writes "Among the tendrils of the roots ... which grew into the water, and partly adhering to them, and partly free, were an abundance of small and large masses of this *Ophrydium*, varying in size from a pea to an egg." In this work, Ehrenberg is quoted as seeing *Ophrydium* masses during all seasons of the year and under winter ice: "balls of them, from four to five inches in diameter, raised periodically to the surface of the water, by the development of the gas of the interior...". Eaton and Carr (1980) comprehensively review the relation of *Ophrydium* to water quality. *Ophrydium* masses from Arctic brackish, coastal ponds were described by Lowe (1923).

Here the constituents of the *Ophrydium* microbial community were studied from May 1992 through November of 1994 at two Massachusetts locations. Field measurements were complemented by sample collection, microscopic observation and enumeration. Light transmittance through *Ophrydium* colonies was measured to estimate the ability of these green masses to shield the habitat by light absorption.

2. Materials and Methods

Field collections

Colonies varying in size from fewer than 1 to 15 cm diameter were collected from Hawley Bog at Hawley in the Berkshire Hills of western Massachusetts

and from a small, spring-fed pond in Leverett, Massachusetts. Both wetlands are sphagnum-rich environments that maintain acidic conditions (pH 4.5–5.5). *Ophrydium* masses were collected in the summer and fall months with the aid of a small inflatable raft or by wading. In spite of at least three autumn, winter and spring attempts, no *Ophrydium* masses were found after November 30th or before May 1st, suggesting that zooids overwinter in some inconspicuous form and that colonies are reconstituted each spring. Specimens were kept at ambient temperature in wide-mouthed glass jars until examination, usually within a few hours after removal from the field. A submerged holding pen (25L capacity) kept at Hawley Bog was used to contain *Ophrydium* specimens and examine community changes throughout the field season. Environmental measurements included temperature, dissolved oxygen, methane and pH (Duval et al., 1994b). The nitrate, sulfate and phosphorus measurements were made by the University of Massachusetts Environmental Analysis Laboratory. Some *Ophrydium* specimens were kept under incandescent light in an aerated tank at 4°C.

Microscopy

Microscopic observations and cytological analysis of the *Ophrydium* and its associated microbial community were made using a dissecting binocular microscope. Optiphot Nikon phase contrast and fluorophot microscopes were equipped with Nomarski differential interference contrast optics (DIC), a 35 mm camera back and a Sony DXC-107A color video camera. Protargol staining (see Appendix) was used to visualize *Ophrydium* colony structure. The green algal endosymbionts seen in all zooids were enumerated by direct count of optical sections. The long rod-shaped bacteria were counted using a Petroff Hausser counting chamber at 400×. Bacteria, stained with acridine orange (AO) were also counted by fluorescence microscopy at 1,000× (Hobbie et al., 1977). The viscous gel which required dilution of the sample with sterile water, was gently vortexed for several minutes to permit both the passage of the material through a 0.2 μm filter under vacuum and the even dispersal of the bacterial population for counting purposes. Direct counts of *Ophrydium versatile* zooids and *Nitzschia* diatoms were obtained at 100× magnification using a Palmer counting cell. A total of at least ten randomly chosen fields were averaged. Colonies fresh from the field were videographed using a Sony DXC-3000 video camera mounted on a Nikon Microphot (combined phase contrast, Nomarski DIC and fluorescence) microscope connected to a Sony U-Matic 7600 video recording deck. The original 3.5 hour footage was edited down to 9 minutes on a Sony U-Matic 5500-5800 editing system.

Diatom frustule preparation

Permanent preparations of the diatom population were made using methods of Batterbee (1986), with the aid of Marianne Douglas of the Department of Geology and Geography, University of Massachusetts, Amherst. Approximately 1 ml of *Ophrydium* gel was soaked in a 50:50 mixture of concentrated sulfuric and nitric acid over a two-day period to digest organic material. The remaining siliceous material was resuspended in distilled water and centrifuged at 7,000 rpm for 10 min. Samples removed by Pasteur pipette were placed on glass coverslips and allowed to air dry on a slide warmer at low heat. Naphrax® permanent mounting solution heated to 35–45°C was added dropwise. The coverslips were inverted onto glass microscope slides to render the preparations permanent.

Protargol staining

Protargol is a silver proteinate complex primarily used for analysis of ciliate structure which stains basophilic proteins and cortical kineties, including buccal ciliature (Montagnes and Lynn, 1987). Our procedure is detailed in the Appendix (Olendzenski, 1993), page 203 below.

Light transmittance

Light transmittance through *Ophrydium* masses was measured using a novel device consisting of a photodetector housed in a light excluding chamber (McMenamin and Bennett, 1994). *Ophrydium* masses in bog water were placed over the light detector (United Detector Technology Optometer 40A). The light source was a fiberoptic Nikon MKII with a quartz-halogen lamp powered by 110 AC. The removable cover, light source and external walls of the chamber were sealed with black tape.

This apparatus was used to estimate the percent of white light attenuated by 1–2 cm of *Ophrydium* gel (Fig. 1). Experimental trials measured light transmittance through a variety of different media including clear bog water, *Ophrydium* masses suspended in 2 cm of bog water, and *Ophrydium* masses positioned above the detector with no water. Incident light was held constant at 5,000 μW from a fiberoptic light source utilizing a quartz-halogen lamp with a dichroic heat dissipating reflector (McMenamin and Bennett, 1994). Values were not corrected against background attenuation by water. Water attenuation accounts for blockage of <2% of the light.

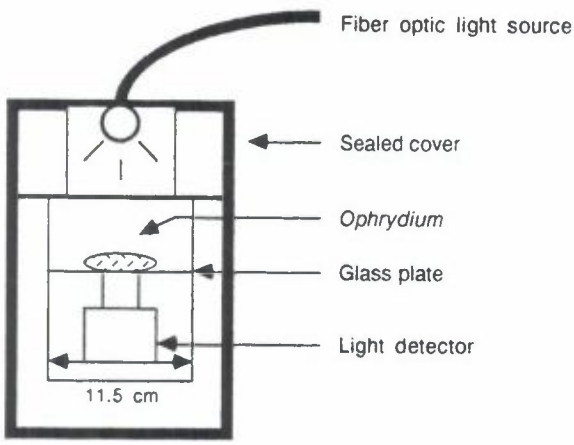


Figure 1. Schematic of the device used to measure light transmittance through live *Ophrydium* colonies.

3. Results

Field study

Hawley Bog, best described as an acidified fen with partial drainage, probably developed from an old glacial lake (Moizuk and Livingstone, 1966; Johnson, 1985). The wetland, surrounded by typical New England upland vegetation, is designated as "Cranberry Swamp" on the U.S.G.S., Plainfield 7.5 min quadrangle. A beaver pond and narrow stream channel flow through a peat bog, developing a maximum water depth of 1.5 m, dominated by *Sphagnum* moss. The waters are acidic, pH 4.5–5.5, and slightly darkened by humic materials. The bog and its watershed surrounded by a mixed conifer and deciduous hardwood forest, are removed from human influence and sources of pollution. Oxic conditions prevail throughout most of the surface waters (Duval et al., 1994b). Most *Ophrydium* masses were collected within five meters of the beaver dam from slow moving but well-oxygenated waters. The dissolved oxygen concentrations ranged from 6.5–8.6 mg/l, as determined by titration. The ionic chemistry is typical of weakly ombrotrophic bog waters, i.e., bogs in which water table and nutrient input is primarily derived from meteoric water rather than from ground water (Larsen, 1982; Drever, 1982): NO_3 = 0.138 mg/l, SO_4 = 4.24 mg/l, and total phosphorus = 1.9 μg/l. Aquatic plants common to the bog include *Potamogeton, Myriophyllum* and *Numphar*

species. Shallow waters freeze solid to a depth >0.7 m during the winter. Ice forms in late November and lasts until the melt in April. Numerous holes were chopped through the ice during the winters of 1991–1994. Only the deeper pools of the central beaver pond and deeper sections of the stream channel did not freeze solid.

A small (less than one acre), spring-fed pond in Leverett, Massachusetts was also investigated. *Ophrydium* masses collected from the Leverett site were found primarily near the shoreline along a sphagnum bank (pH = 5.0).

Seasonal abundance and colony growth rates

Peak numbers of large, well-developed *Ophrydium* colonies were observed and collected, July through August, during the summers of 1992–1994 (Fig. 2). Very few *Ophrydium* colonies were found in Hawley Bog by late October, perhaps due to colony decay. Specimens kept in the holding pen until the time of ice formation in late November became emaciated, and some even disintegrated. Whether the late season deterioration of the gel is due to increased bacterial activity, grazing by opportunistic microbes, a photoperiodic response to shorter daylength, or a combination of these and other factors is not yet known. *Ophrydium* colonies kept under incandescent light appeared healthy after several weeks in a cold room at 4°C. During May–June one *Ophrydium* mass kept in the pen at Hawley Bog doubled in size, from 4 to 8 cm in diameter, within 26 days. Outside the pen the 25 ophrydia masses that were measured, all of them attached to aquatic plants, grew most rapidly in June. In only 15 days they doubled in size from approximately 1–2 cm in diameter.

Survival of the colonies in the field and the laboratory

Ophrydium colonies were never observed in winter. Since the green gelatinous masses appeared to degenerate by autumn and most water freezes at Hawley Bog, we hypothesize that the *Ophrydium* community disperses by winter, surviving in some frozen form in the water column or sediments. We failed to see the telotroch formation, or other dispersal activities after 48 hours of darkness as described by Couillard (personal communication).

An aerated tank with *Ophrydium* was kept at 4°C under incandescent light for maintaining specimens. This tank was later moved to a windowsill at room temperature, where loose gelatinous colonies developed and persisted from July to September. By September 15th, the microbial assemblage in these colonies contained no *Ophrydium* zooids. Apparently *Ophrydium* telotrochs that abandoned the gel mass were replaced by *Paramecium bursaria*, filamentous

algae and cyanobacteria. After the zooids disappeared the *Nitzschia* diatom population in the degenerated mass declined as well.

Ophrydium *colony structure*

Ophrydium masses ("green jelly balls") were found unattached, attached to plant surfaces or laying on bottom sediments. Fine sediment was often noted in bottom-settled *Ophrydium* specimens and in others not connected to plants. *Ophrydium* masses between 2–5 cm in diameter were generally flattened spheres, mostly kidney-shaped. Larger "individuals" (10–15 cm) were more irregular and slightly depressed in the center, i.e., doughnut shaped. The central region, although it appeared hollow, contained air bubbles or other gases which seemed to add buoyancy.

All *Ophrydium versatile* colonies were composed of regularly aligned zooids containing green endosymbionts in a gel of proportionately distributed microbes: certain diatoms and large rod-shaped bacteria.

The alignment of the *Ophrydium* zooids ("eyebrows") are seen in Fig. 3 and interpreted in Fig. 4. Zooids and other major and minor inhabitants of the *Ophrydium* gel mass are listed in Table 1; the most conspicuous are depicted in Fig. 5. Several morphologic forms of the contractile *Ophrydium versatile* zooid were observed. Simultaneous extension (Fig. 6) and contraction of zooids occurs without cessation. Extension of the protist is slow compared to its rapid contraction. Individual zooids measured 50 × 400 μm when fully extended, comparable to zooids measuring 300–600 μm reported by Foissner et al. (1992). Most algal symbionts were packed into the anterior two-thirds in each zooid. In deteriorated gels, clumps of the coccoid green algae maintained the outline of a former *Ophrydium* zooid. Apparently, the body of the ciliate had decayed around the surviving algae. The motile telotroch stage of this *Ophrydium* measured approximately 80 × 120 μm. Free swimming telotrochs were frequently observed and did not appear to be correlated with spatial or temporal changes in habitat. The buccal cilia generate feeding currents that sweep bacteria and other food into the infundibulum. Fine holdfasts, stalks many times longer than the zooid itself, secure their regular alignment, as they disjointedly course through the gel. Detachment of zooids from their stalks was observed (Guillemette et al., 1994).

Protargol staining revealed the ribbon-shaped macronucleus extending to about half a body length of the *Ophrydium* (Fig. 7). The single micronucleus was located near the base of the macronucleus below the trochal band, as described by Foissner et al. (1992). The trochal band, also called the locomotor fringe, easily visible in protargol stained zooids, was inferred in live zooids by a gap in the symbiotic algal distribution (Fig. 6). *Chlorella*-like symbionts

stream throughout the zooid cytoplasm. These unicellular green algae ranged in size from 3–5 μm. Each *O. versatile* zooid contained between 400–500 of these coccoids. Although ultrastructural morphology led us to confirm the identification of the Vancouver *Ophrydium* alga as the smooth-walled endosymbiont *Chlorella vulgaris* (Fig. 8), the rib-walled algal of the Hawley Bog *Ophryidum* better fits the description of *Graesiella* (Fig. 9; Komarek, 1987; Kalina and Puncochárová, 1987; for comparison criteria of *Chlorella*-like endosymbionts see Esteve et al., 1988). The diatom *Nitzschia* was common to *Ophrydium* masses at both field sites, but the alga has not yet been identified by electron microscopy for the *Ophrydium* at Leverett. *Nitzschia* made up a smaller portion (and *Gyrosigma* a larger one) of *Ophrydium* from Leverett pond compared to the Hawley Bog gel masses.

Zooid alignment

Zooids of *O. versatile*, from 6,700–9,700 individuals/ml, are spatially juxtaposed forming rows and columns within the gel matrix and are aligned approximately 500 μm apart. Each *Ophrydium* zooid occupied a surface area of approximately 0.26–0.30 mm^2. Only one kind of diatom and one morphologically-distinctive bacterium (a *Nitzschia* and the long rod) were aligned in these rows and columns in predictable proportions per zooid.

Gel inhabitants: Residents and tenants

All *Ophrydium* samples contained a diverse assemblage of organisms, some actively traverse the transparent gel which is more viscous than bog water. All were apparently in excellent health. These include cyanobacteria, spirochetes, bodonids, ciliates, diatoms, rotifers, desmids, filamentous and unicellular green algae. We interpret all those videotaped, listed in Table 1 and illustrated in Fig. 5 to be permanent and/or transient residents of the *Ohrydium* gel's nutrient-rich support system.

Bacteria

Bacterial cell counts within the gel ranged from 6.8–12.8 $\times 10^6$ cells/ml (av. 9.8 $\times 10^6$, n = 4) from masses collected in early June, to 4.6 $\times 10^7$ bacteria/ml in August. Specimens collected in late June had bacterial populations from 2.1–2.6 $\times 10^7$ cells/ml, 25–30% of which were long and rod-shaped (1 $\times$ 6–10 μm). Bacterial cell counts for the bog waters were between 4.0–4.9 $\times 10^6$ cells/ml, n = 4. Bog waters contain none or few of the long rod-shaped bacteria ubiquitous in

Table 1 Gel-associated organisms in *Ophrydium* masses[1].

Prokaryotes

Monera

Eubacteria: Spirochetes, *S. plicatilis*-type[2] (1 × 55–70 μm), an unidentified loosely coiled (1.7 × 30 μm) spirochete[2] and a tiny *Spirochaeta* sp.

Saprospira-like rigid motile helix.

Numerous rod-shaped bacteria[2] (1 × 6–10 μm).

Cyanobacteria: *Anabaena*-like chains with terminal heterocysts[2]. *Oscillatoria*-like filaments, *Gloeocapsa*[2] sp. Other unidentified filaments.

Eukaryotes

Protoctista[1,2]

Heliozoa: *Actinophrys* sp.

Desmids: *Micrasterias*; the colonial filament *Hyalotheca*[2].

Green algae: Free-living and symbiotic *Graesiella*[3], *Chlorella*[3], *Ankistrodesmus*, *Schroederia* and other unidentified unicellular and filamentous green algae.

Diatoms: *Nitzschia*[2], *Pinnularia*[2], *Navicula*[2], *Gyrosigma*[2], *Bacillaria*, *Cymbella*.

Ciliates: *Ophrydium versatile*[2], *Dileptus* sp., *Paramecium bursaria* (containing *Graesiella* or *Chlorella* symbionts), hypotrichous ciliates such as *Uroleptus musculus*[2] and others containing endosymbiotic methanogens (verified by fluorescence microscopy); a tetrahymenid hymenostome *Espejoia*-like ciliate[2], *Vorticella*, and occasional stichotrichs.

Zoomastiginids: *Rhyncomonas* (a bodonid kinetoplastid)[2].

Unidentified protists: euglenids, amoebae.

Animalia

Bosmina (cladoceran), harpacticoid copepods, rotifers, sponge spicules, nematodes, platyhelminths.

Fungi

Very few yeast or other fungi present.

[1]Classification based on Margulis, McKhann and Olendzenski, (1993). [2]Depicted in Fig. 5. [3]Depicted in Fig. 8 or 9.

the *Ophrydium* gel masses. Only these long rod-shaped bacteria (Figs. 3, 4 and 5), perhaps heterotrophs utilizing sugars excreted by algae, are aligned with *Nitzschia* throughout much of the *Ophrydium* gel. Much smaller rod-shaped bacteria (1–2 μm in length), were observed outside the gel in water samples. Spirochetes were especially numerous in *Ophrydium* gel collected in late August, September and October. At least three spirochete morphotypes,

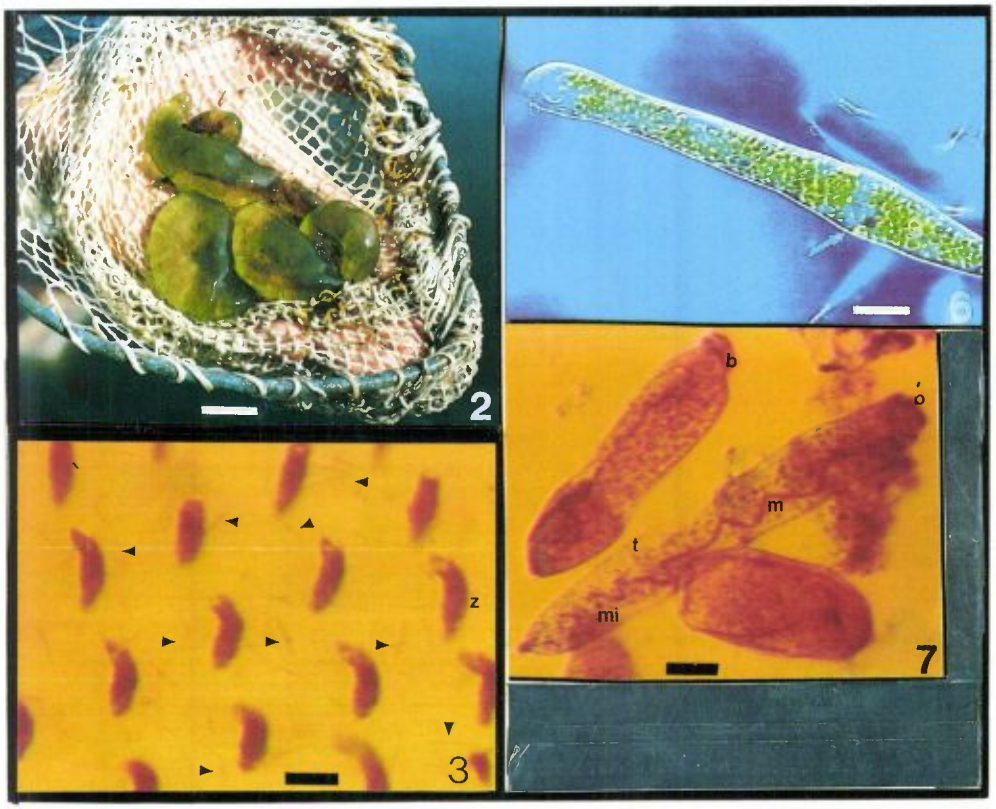


Figure 2. *Ophrydium* colonies collected in a net at Hawley Bog. Bar = 5 cm. (Courtesy of Thomas H. Teal; Duval et al., 1994a)

Figure 3. *Ophrydium* colony showing aligned zooids (z), "little eyebrows". *Nitzschia* diatoms at arrowheads. Bar = 500 μm.

Figure 6. An extended zooid of *Ophrydium versatile* with endosymbiotic *Graesiella* symbionts (G), locomotor fringe (trochal band, arrow) and buccal apparatus (b). Bar = 50 μm.

Figure 7. Protargol stained zooid, the trochal band (t), long macronucleus (m), micronucleus (mi) and buccal apparatus (b) can be seen. Bar = 50 μm.

including a thin *Spirochaeta* (<0.3 μm diameter), were videographed. The one that resembled *Spirochaeta plicatilis* measured 1 × 55–70 μm and was most abundant after late September. A loosely coiled, highly motile spirochete reached its highest population densities in August and early September. Another kind of helical bacterium, a large (1.7 × 30 μm) rigid *Saprospira*-like morphotype remains unidentified. Cyanobacteria present in *Ophrydium* gels included a coccoid *Gloeocapsa*, several *Oscillatoria*-like forms and a beaded filamentous cyanobacterium with terminal heterocysts (probably *Anabaena*).

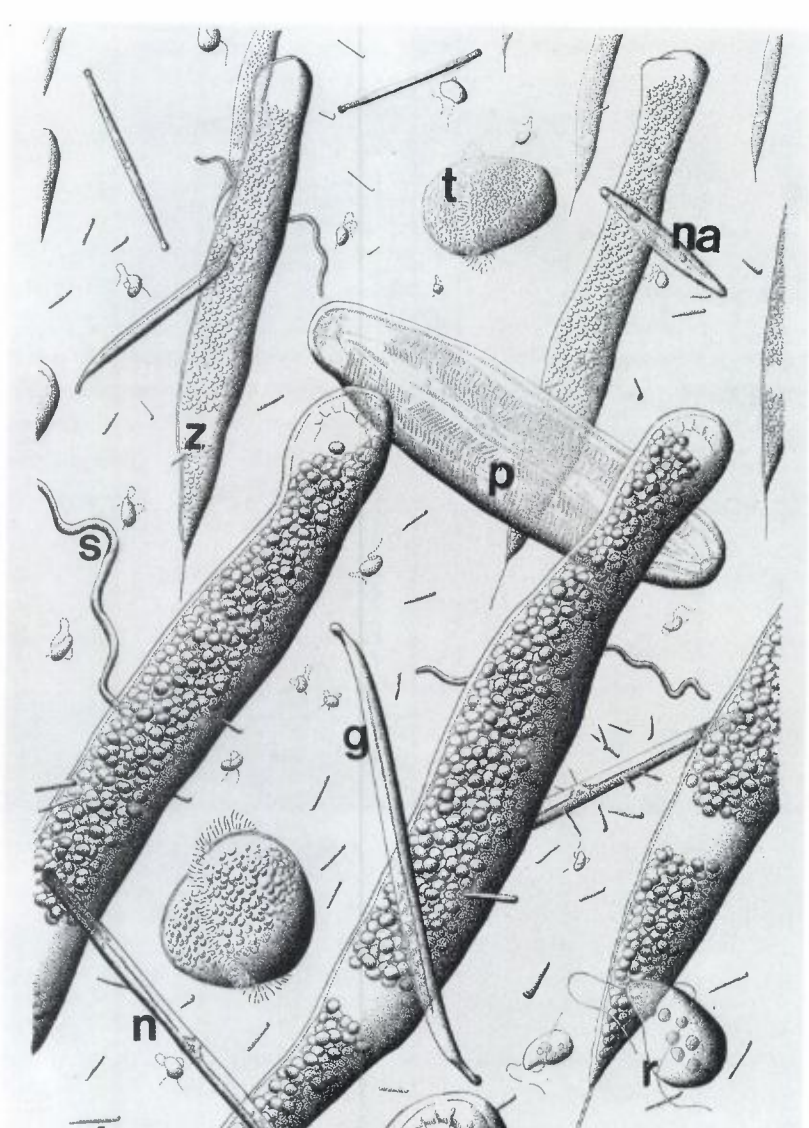


Figure 4. Structure of the *Ophrydium* colony showing zooid (z) and telotroch (t) alignment and regularly associated diatoms (g = *Gyrosigma*, n = *Nitzschia*) and bacteria (b), including spirochetes (s), in the gel. Transient gel inhabitants include *Rhyncomonas* (r), *Navicula* (na), and *Pinnularia* (p). Drawing by Christie Lyons.

Other protoctists

Large green *Dileptus* ciliates (200 μm in length) containing *Chlorella*-like algae were observed, but whether *Dileptus* feeds on or harbors these as endosymbionts is unknown. Four different ciliated protists containing internal coccoid green algae have been observed: *Ophrydium*, *Dileptus*, and *Paramecium bursaria* and a smaller unidentified rapidly swimming ciliate. Hypotrichs (*Uroleptus musculus*, etc.) and a *Espejoia*-like tetrahymenid ciliate were seen live in abundance and stained by protargol. One unidentified hypotrich contained cytoplasmic rod-shaped bacteria that, based on their intrinsic blue fluorescence at 420 nm, were tentatively determined to be methanogens.

Actinophrys-like heliozoa were observed preying upon *Ophrydium* telotrochs. A filamentous colonial desmid *Hyalotheca* sp. (Fig. 5) and the large solitary desmid *Micrasterias* were occasionally observed.

Nitzschia *diatoms*

The most common diatom noted within the gel, *Nitzschia* sp. were conspicuously present in all samples. From $5.1–7.0 \times 10^4$ individuals/ml in June and up to 2×10^5 individuals/ml by August were counted. The slender, pinnate *Nitzschia* diatoms appear to move through the gel without restriction. *Nitzschia* compose fewer than 12% of the diatom population in the Hawley Bog shallow water benthos. Since they comprise 90–95% of the diatom community within the gel, we interpret them to be intrinsic components of the *Ophrydium* mass. The ratio of *Nitzschia* to *O. versatile* increased from 8:1 in individual gels collected in early June to 20:1 for specimens collected from August–October.

Other diatoms

The diatom genera noted throughout the *Ophrydium* gel include *Navicula*, *Pinnularia*, *Gyrosigma* (all in Fig. 5) and *Cymbella*. At least five other distinct morphotypes were seen in live and protargol stained material. The *Ophrydium* masses from the Leverett site contained predominantly *Gyrosigma* diatoms.

Copepods and other animals

Harpactecoid copepods were observed at least three times swimming in the central hollow reservoirs in *Ophrydium* gels. Water trapped by the formation of the *Ophrydium* mass or flooded gas pockets may become a refugia for

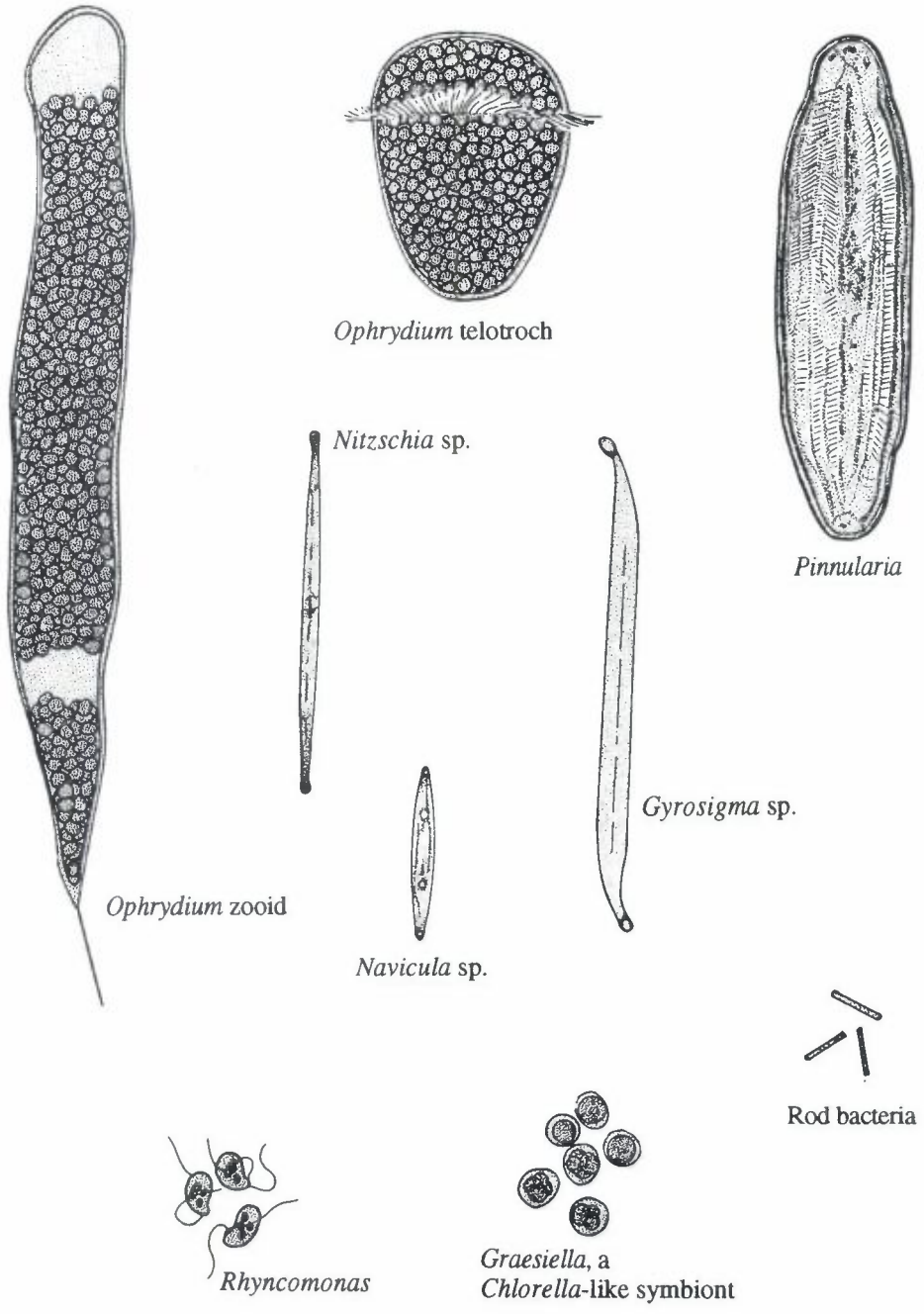


Figure 5. Major inhabitants of *Ophrydium* gel drawn to scale by Christie Lyons. All are drawn as seen at an original magnification of 400× or more.

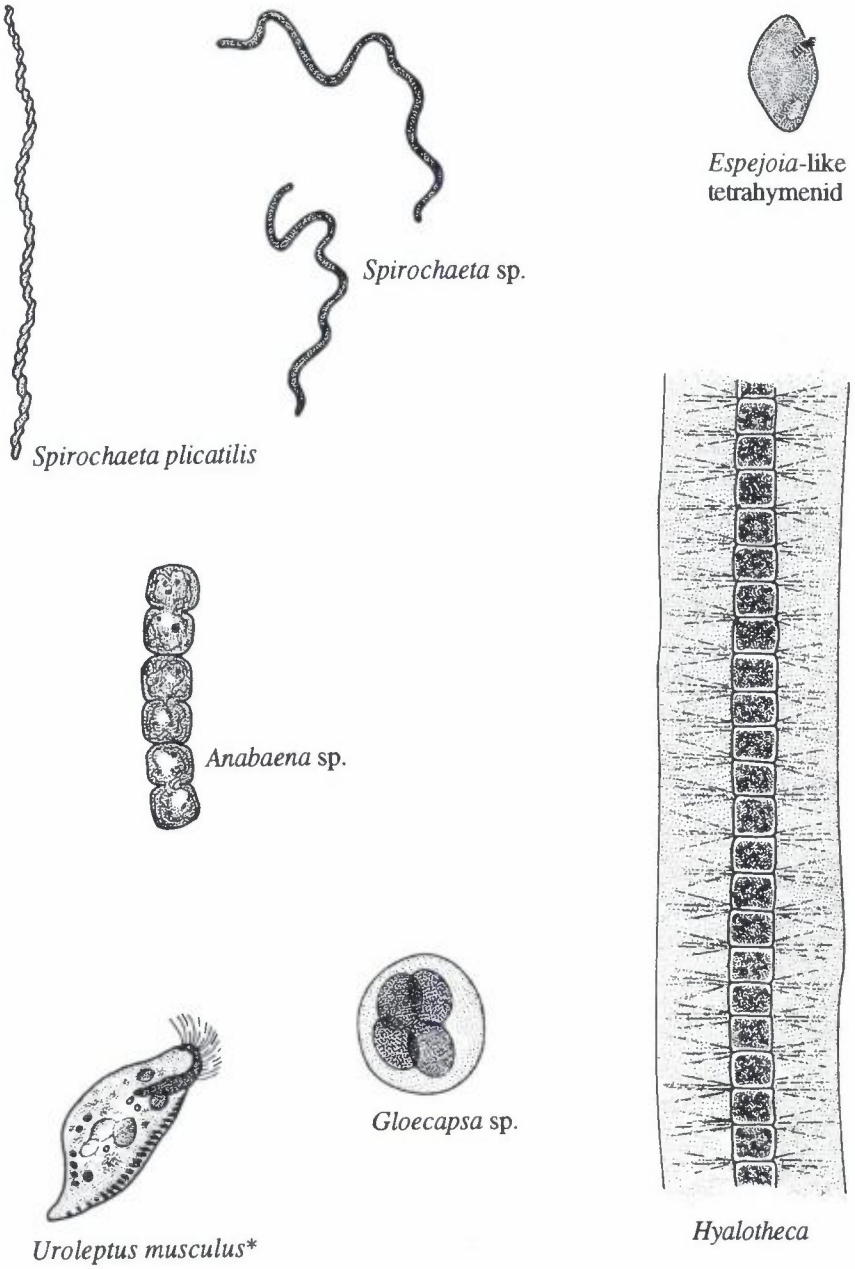
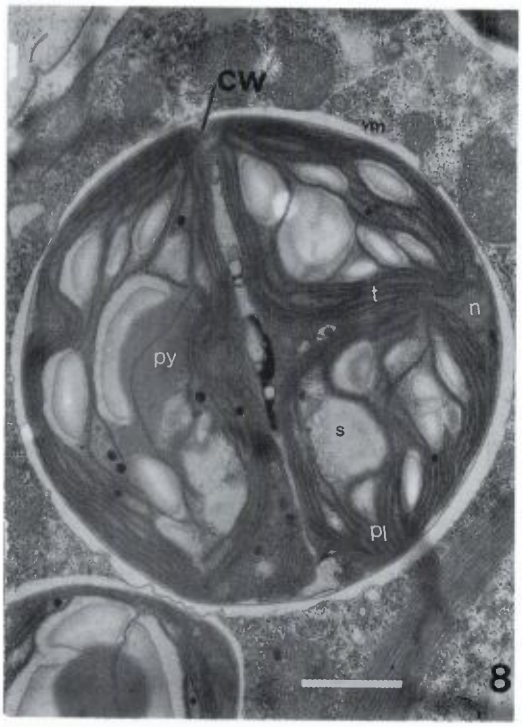


Figure 5. Continued. *Kindly identified by Dr. Arthur Borrer.



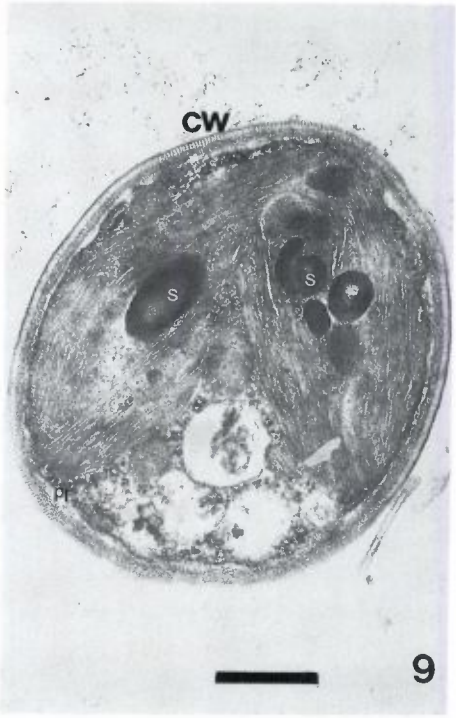


Figure 8. *Chlorella vulgaris* "autospore". Ultrastructure of green algal symbiont of *Ophrydium* from Vancouver, British Columbia: (py) pyrenoid, (s) starch, (pl) plastid, (cw) cell wall, (n) nucleus, (t) thylakoids, (vm) symbiont vesicle membrane. Bar = 1 μm. (Courtesy of Lynda J. Goff)

Figure 9. *Graesiella* sp. ultrastructure of green algal symbiont of *Ophrydium* from Hawley Bog, Massachusetts. Ribbed cell wall (cw) is a distinguishing feature of the genus. Bar = 1 μm. Photo by Floyd Craft. Abbreviations as in Fig. 8.

copepods and other small invertebrates. One particular *Ophrydium* colony, measuring 5 × 7 cm and containing 4–5 ml of water, held approximately 20–30 of these unidentified copepods. Bacteria scavenged among decaying copepod bodies. Nematodes and platyhelminths were also observed, probably feeding along the *Ophrydium* gel surface.

Light attenuation

Ophrydium masses 2–3 cm in diameter and 1–2 cm in depth were suspended in 2 cm of clear pond water over a photodetection device. These masses attenuated

between 30–46% of the incident light from a 5,000 μW light source (av. = 40%, n = 14). A similar experiment measured light attenuation (i.e., inverse of transmittance) through individual *Ophrydium* specimens 2–3 cm in diameter. The colonies were placed directly over the photodetector in the absence of water. Light attenuation for these samples averaged 26% (n = 12), i.e., lower values due to less refraction at the water/mass surface, or slight flattening of the gel mass in the absence of water.

4. Discussion

The consortia

From the diversity of tenants within the *Ophrydium* microbial community, it seems apparent that many organisms from surrounding waters can enter the gel. Predation of bacteria and small protists by larger ciliates, the presence of heliozoa and replacement of *Ophrydium* zooids by *P. bursaria,* suggest a trophically complex community resides within the viscous mass. A color 9 min videotape of live *Ophrydium* colonies is on exhibit at the New England Science Center, 222 Harrington Way, Worcester, MA 01604, USA.

While bacteria were densely aligned in certain regions of the gel, their distribution was not uniform. High bacterial counts may be partially attributed to the abundant fine sediment in certain ophryidia gels. The large rod-shaped bacterium in the Hawley *Ophrydium* gel resembles bacteria found in desmid mucilage collected from Wisconsin bogs that also contain *Ophrydium* (L. Graham, personal communication). Similar rod-shaped bacteria were present in *Ophrydium* masses collected from British Columbia (Goff and Stein, 1981). We conclude that this bacterium is a consistent component of gel communities, i.e., of *Ophrydium* and desmid masses.

Ophrydium colonies are reported to contain a diversity of diatoms both on and within the gel (Eaton and Carr, 1980). The assemblage of diatoms varied between Hawley Bog, Leverett and those described by Eaton and Carr. While *Nitzschia* was the most common diatom throughout the mucilage, its numbers were disproportionally large when compared to the *Nitzschia* population in Hawley Bog water and surface sediments. Although *Nitzschia* and other diatoms were described to adhere to the *Ophrydium* mass surface (Eaton and Carr, 1980), in Hawley Bog the diatom population in the *Ophrydium* is a living part of the community and not incidental to the gel surface. The slender *Nitzschia* penetrates and easily colonizes the gel. Along with other diatoms *Nitzschia* may populate the *Ophrydium* gel matrix after its construction or their presence and mucilage-forming ability may be required to produce the gel.

Nitzschia are known to occur in gelatinous tube-like strands (Prescott, 1978). Diatoms have been described from nearly every aquatic environment including snow and sea ice, but their association within a living complex, an *Ophrydium* mass, distinguishes this novel habitat. Association of various diatom genera with ophrydia must vary at least with season and geographical location. In our observations, *Ophrydium* colonies with their diatom populations virtually disappeared in the winter months, yet in aquatic systems that do not freeze solid, overwintering *Ophrydium* colonies have been reported (Hollowday, 1975; Buecker and Hausmann, 1994). The seasonal changes in colony cohesiveness warrant further investigation.

Free-living algae in *Ophrydium* masses may help buffer the gelatinous system by removing carbonic acid from the interstitial waters during photosynthesis. Tolerance of low pH is reported in some species of *Chlorella*. Conditions of pH within the *Ophrydium* gel are probably acidic as noted in other symbiotic green algae such as *Hydra* (Huss et al., 1993).

Since all living systems produce gases such as O_2, CO_2, CH_4, and H_2S, the unidentified gas bubbles within the *Ophrydium* mass are in essence a "municipal" byproduct. Buoyant qualities attributed to O_2 production have been suggested (Habeeb and Caldwell, 1949; Hollowday, 1975). The gas bubbles we observed are most likely oxygen. Gas production within the gel may form pockets that later fill with water to become reservoirs for larger inhabitants, such as copepods. Unique occurrences of copepods in damp moss, in the brachial chambers of certain land crabs and in rain water cups saddled in tropical trees were described by Wilson (1932). We found no mention of the presence of copepods in *Ophrydium* masses.

Other ophrydia and gelatinous masses

Ophrydium sp. are the only genus of peritrichous ciliate known to form massive gelatinous colonies (Eaton and Carr, 1980). All other members of the genus *Ophrydium* are smaller than *O. versatile*. Some, such as *O. hyalinum*, have zooids that lack symbiotic algae, or they differ in peristomal structure, (e.g., *O. eutrophicum*, Foissner et al., 1992). Other freshwater gel bodies that resemble *Ophrydium* colonies include assemblages of cyanobacteria (usually species of *Nostoc, Gloeocapsa, Gloeocystis* or *Chondrocystis*) and salamander egg masses.

Spatial constraints and algal influence

Individual *O. versatile* are regularly positioned within the gel, with limited surface area available on a given colony. To accommodate all the

zooids we measured, spacing requirements imply the presence of more than 1–2 peripheral layers of zooids. Given only a single peripheral layer of zooids a maximum of only 2,000–4,000 individuals could exist on a 1 ml colony. To increase surface the colony takes on an irregular and convoluted form (Winkler and Corliss, 1965), in which the 2–3 staggered rows of *O. versatile* align near the periphery but internal sections of the gel also harbor zooids. An average surface density of 12,500 zooids/cm^2 for smaller (200–300 μm long) *Ophrydium* zooids was reported by Buecker and Hausmann (1994).

The *Chlorella*-like symbiont may be optimally situated to maximize solar absorption for photosynthesis. Algal secondary pigments may also provide a photoprotective role by screening UV. The light absorption qualities of *Ophrydium* masses which may permit photosensitive microbes to live within the gel are most likely attributed to its algal inhabitants. Their ability to absorb photosynthetically active radiation (PAR), and capacity to filter potentially harmful solar irradiation within the *Ophrydium* mass, are important attributes that influence "individual" physiology and proliferation in community structure. The resistance of the gel to ultraviolet irradiation (UV) was noted (Buecker and Hausmann, 1994). Photoprotection from UV may indeed provide an alternative habitat, the *Ophrydium* gel, for organisms that would otherwise reside in deeper pelagic or benthic environments. A phycosphere-like phenomenon in which *Ophrydium* excretes the organics may regulate bacterial density and population positioning in the gel.

Evolutionary significance

The *Ophrydium* colony provides a unique gel microenvironment inhabited by a rich aquatic microbiota. In physiological terms, the gel is both a "skeleton" and a "tissue" matrix of an individual *Ophrydium* mass. *Ophrydium* gel masses show properties of cohesion, coherence and integrity that are reminiscent of the loose individuality of certain large protoctists such as coralline algae and kelp. In eukaryotes individuality is always a complex product of the interaction of formerly independent individuals. We suggest that the loosely defined individuality of the *Ophrydium* colony is similar to that of the ancient Ediacaran biota. The "pneu" structural elements in the enigmatic late Proterozoic "garden of Ediacara" biota (e.g., *Pteridinium*, *Phyllozoan*) are possible ancient analogues to *Ophrydium* (McMenamin and McMenamin, 1990). During the Vendian Period supplemental nutrition by phototrophy via microbial symbionts was likely in translucent large protoctists and/or early animals residing in the photic zone (McMenamin, 1993). The potential for nutrient and genetic exchange within an *Ophrydium*-like gel may have been optimal. The layered structure of actively photosynthetic

Ophrydium colonies, i.e., the zooid-symbiont complex such as that described here, may be the closest analogue persisting today of the "quilted structures", soft-bodied shallow water photic zone fossils found in arenaceous substrata. These organisms dominated the late Proterozoic eon but became extinct or greatly reduced by the base of the Cambrian (Paleozoic era) about 540 million years ago (McMenamin, 1993).

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Appendix: Protargol staining technique

(after Olendzenski, 1993)

This simplified protargol method, as modified by Coats and Heinbokel (1982) and Montagnes and Lynn (1987; 1993), uses Millipore filters made from mixed cellulose esters and an agar film embedding to anchor and adhere fixed protists as they are transferred through stain solutions. Prior to staining protists are fixed in Bouin's picric acid fixative.

Timing

This procedure takes the better part of two consecutive days, i.e., 3–5 hours per day. On the first day the protists are left overnight in the stain solution. The use of filters and agar greatly reduces the length of the original protargol procedure; the traditional "enrobement" step using egg albumin and parlodian (e.g., Lee et al., 1985, p. 4) is bypassed. On the second day, development and fixation analogous to the photographic process is completed, and the preparation is rendered permanent. Silver grains from protargol silver proteinate adhere to basophilic proteins in the cell. The pattern of these proteins (as yet unidentified but not tubulin) is observable after silver is reduced by development in a hydroquinone, sodium carbonate, and sodium thiosulfite solution, "fixed" in sodium thiosulfate and "stopped" in water. Protargol preparations of H. Kirby and L.R. Cleveland have lasted more than fifty years!

Materials and solutions

Timer (minutes and seconds)
Formalin (37% formaldehyde to make 3.7% solution in dH_2O)
Calcium carbonate (powdered)
Picric acid (powdered). Caution: Dry picric acid powder is shipped in wooden boxes; it can explode when sparked. Do not store in a jar with a metal cover, and always keep the moisture level of picric acid powder at about 10%. Drops of distilled water can be added to picric acid powder that seems too dry.
Vials with non-leaking screw caps (e.g., 20 ml scintillation vials)
Filters: 1. For samples 0.45–8.0 μm pore size, 25 mm in diameter (Millipore HAWP-02500 or other cellulose acetate and cellulose nitrate or mixed cellulose ester filters); 2. For gold chloride: Whatman qualitative filter paper, e.g. DE81.
Millipore filtration apparatus (25 mm filter holder with fritted glass support)

Side-arm flask
Nalgene Mityvac hand vacuum pump (Fisher #6130)
Fine forceps
Agarose or noble agar
Single-edged razor blades
Pasteur pipettes
Glass plates (4" square)
Hot plate
Slide warmer (60°C)
Columbia jars
0.5% $KMnO_4$ in distilled water
5% oxalic acid in distilled water
Protargol silver stain (Source: ROBOZ Surgical Supply, Washington, DC or Polysciences Inc., Paul Valley Industrial Park, Warrington, PA 18976)
pH paper (to check stain solution, pH 6–10)
Copper sheet, cut into squares the size of coverslips (four for each Columbia jar used for staining)
5% sodium sulfite and 4% anhydrous sodium carbonate in distilled water
Isopropyl alcohol: previously used, 90% or 100% (for cleaning copper squares)
Hydroquinone
0.5% gold chloride in distilled water (Source: Polysciences Inc.: gold trichloride, acid trihydrate). Must be filtered after each use; 10 ml will stain 4–5 sets of filters.
2% oxalic acid in distilled water
5% sodium thiosulfate in distilled water
Isopropyl alcohol series: 30, 50, 70, 90 and 100%
Molecular sieves
Xylene
Mounting medium (e.g., "Permount")
Coverslips, #1 thickness
Slides
Weights (fishing weights, small rocks, pennies)
Paper towels

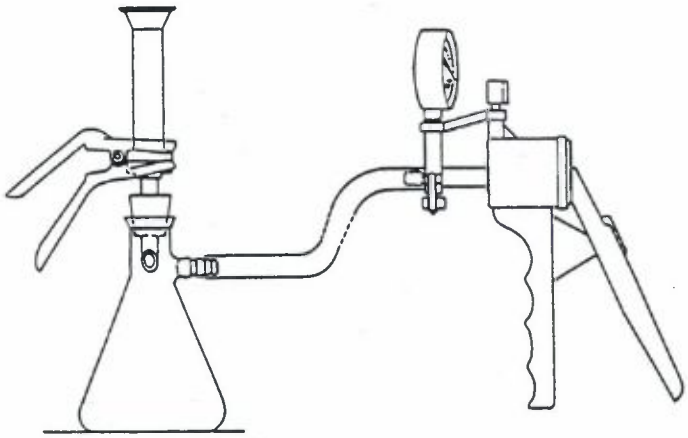
Fixation

Sample must be fixed at least one day prior to the protargol procedure; it can be done months or even years before.

Fixation with Bouin's fixative

Extreme care should be used when handling Bouin's fixative, a strong yellow solution. The stock solution, which keeps indefinitely, is made by saturating formalin with calcium carbonate and picric acid. Saturate formalin with $CaCO_3$ over 3–4 days with frequent mixing; settle and decant; then saturate with picric acid for 3–4 days, mixing frequently. Solid precipitate is allowed to settle to the bottom of the container.

In a 20 ml vial (e.g., scintillation vial), place 1 ml of Bouin's fixative. Add two drops of glacial acetic acid and 9 ml of culture medium or filtered water (sea or fresh water, but not distilled) in which the protists are growing. Protists in their medium or in their ambient water should be gently added dropwise to the fixative solution to reach a total volume of 20 ml. Cells should be fixed at least overnight, but at room temperature in a sealed vial they last indefinitely.



(Day 1)

Filtering

1. Agar

To prepare 1.5–2.5% agar, melt 0.15–0.25 g Noble agar or (preferably) agarose in 10 ml of distilled water by placing the test tube in a beaker of boiling water. Leave the tube in beaker water bath, on a hot plate set at approximately 90°C, i.e., at a temperature just above solidifying.

2. *Filtering*

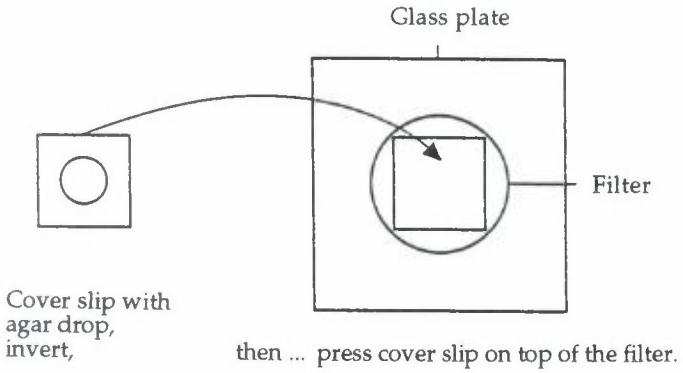
Filter Bouin's fixed cells onto filters using a hand vacuum pump. Use an appropriate amount of sample: enough to see easily but avoid clumping or clogging of filter. Cells can be spread out by resuspending. Usually a 5–10 ml volume of fixed sample is adequate. Do not let the pressure on the pump gauge go above 10 centimeters of mercury. Resuspend cells in dH_20 to wash out Bouin's fixative. Filter until just a small amount of liquid remains on surface of filter. Release the vacuum on the pump.

3. *Evaporation of water*

Remove wet filter from apparatus. Place, residue side up, on a piece of warm glass that has been stored on the slide warmer (60°C). Allow liquid to evaporate until water forms a sheen on the surface of filter. Never let filters dry out completely at any time during the entire procedure.

4. *Covering filter with agar*

Pipette a 1.8 cm diameter drop of liquid agar onto a coverslip. Quickly invert coverslip and place agar side down onto filter. Press down gently. Remove glass plate from slide warmer. Allow agar to solidify. Agar layer should be very thin. Trim three filter edges protruding from coverslip with a razor blade. Carefully peel off filter from coverslip. Place filters in distilled water in a Columbia jar to thoroughly wash off fixative. Filters can remain in water until proceeding with staining.



Staining

All steps take place in Columbia jars; four filters will fit in one jar. Solutions are best set up in a series of Columbia jars and filters moved from jar to jar but solutions may be emptied from and added to a single jar after washing.

5. Dissolving Protargol

Place 0.2 grams Protargol on surface of 10 ml of water in a foil-wrapped Columbia jar. Prepare one jar for each group of 4 filters. Let dissolve slowly; do not stir. The pH should be 8.8. If necessary, adjust with HCl or NaOH.

6. Cleaning copper squares

Copper squares must be cleaned. To clean previously used copper, hold with large tongs and flame each one in Bunsen burner until red hot. Plunge red hot squares into previously used isopropyl alcohol (95–100%). Remove clean copper squares to paper towel; let dry.

7. Bleaching

Place filters in 0.5% $KMnO_4$ for 5 minutes. Solution of 0.5% $KMnO_4$ should be refrigerated; make up fresh solution after 1–2 months storage. This strong oxidant bleaches cytoplasmic structures to allow visualization of surface patterns. Some protists (i.e., pigmented *Stentor*) may require up to 10 minutes bleaching.

8. Washing out permanganate

Dump $KMnO_4$; wash filters well with distilled water until pink color disappears. Filters can be washed by repeatedly emptying Columbia jar and adding distilled water from a squirt bottle. Organic matter on the filter will appear light brown.

9. Bleach stop

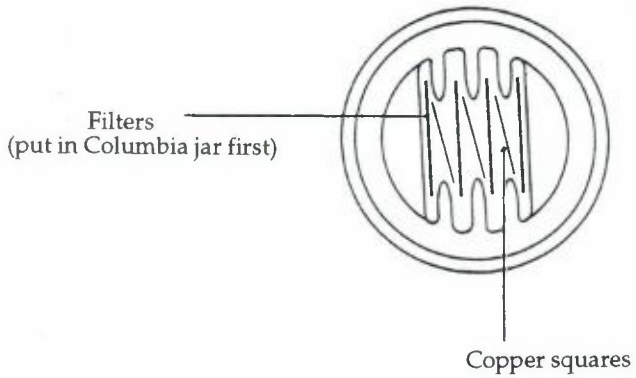
Add filters to 5% oxalic acid for 5 minutes to stop the $KMnO_4$ bleaching process. Organic material loses its color.

10. Washing out oxalic acid

Dump oxalic acid and wash well with distilled water for 5 minutes.

11. Protargol staining

Place 4 filters in jars containing 2% Protargol, which should be dissolved by now. Place 3 cleaned copper squares between the filters at an oblique angle. The fourth copper square lies flat across the top of the jar. Cover jars and put in a dark place (e.g., a drawer). Leave overnight or at least until the pH shifts to 6.8.



Columbia jar seen from the top.

(Day 2)

Developing

12. Developer preparations

The developer for Protargol is a saturated solution of hydroquinone in 5% sodium sulfite and 4% anhydrous sodium carbonate. Prepare developer by gently swirling hydroquinone crystals into a solution of 5% sodium sulfite and 4% anhydrous sodium carbonate in distilled water. This can be done in a beaker. Use a magnetic stir bar and stir for about ten minutes. A final concentration of about 1% hydroquinone forms a saturated solution. Let undissolved crystals fall to the bottom of the beaker before dispensing into clean Columbia jars.

13. Filters into developer

Transfer filters directly from Protargol to developer for 5–10 minutes. Development may be monitored under a dissecting scope and stopped when desired structures appear well stained. Be sure to check that staining is dark enough. If not, leave in developer longer (10–12 minutes).

14. Washing out developer

Transfer filters to distilled water. Wash for 5 minutes, rinsing several times. (Dump out developer).

15. Gold chloride

Place filters in 0.5% gold chloride for 1.5 minutes using Teflon-coated forceps. Watch time closely. (You may want to try Coats modification of 0.2% AgCl for 3 minutes). Filters become dark purple. Gold chloride solution should be filtered for reuse and stored in the refrigerator. Gold reacts with the silver, acting as a toner to add contrast to the stained organism.

16. Gold chloride stop

Rinse filters in dH_20 for 10 seconds. Place filters in 2% oxalic acid for 5 minutes. Oxalic acid stops the reaction of gold chloride.

17. Washing

Discard oxalic acid and wash well with distilled water for 5 minutes.

18. Sodium thiosulfate

Add 5% sodium thiosulfate for 5 minutes. Sodium thiosulfate acts as a fixer for the deposited silver grains.

19. Sodium thiosulfate stop

Discard sodium thiosulfate and wash well with several rinses of distilled water for at least 5 minutes each.

Dehydration

20. Water removal

Dehydrate filters for 5 minutes each in 30%, 50%, 70%, 90%, 100%, 100%, and 100% isopropyl alcohol. Store 100% isopropyl with "molecular sieves" to remove water. Steps involving 100% isopropyl alcohol and xylene should be done under a hood. Perform dehydrations in the same jar, emptying and refilling with the next alcohol concentration in the series. Save 90% and 100% alcohols to use in cleaning copper.

21. Filter clearing

Soak filters for 5 minutes each in 3 changes of 100% xylene. Dehydration through xylene clears the filters and allows specimens to be mounted permanently in xylene-soluble mounting medium (e.g., "Permount").

22. Preparation for mounting

Place filters in 50% xylene/50% mounting medium (e.g., "Permount") (or in 20% xylene/80% Permount) in Columbia jars in hood for 1 hour. This allows mounting fluid to permeate filters.

Mounting

23. Permanent mounts

Place one small drop of Permount on cleaned, labeled microscope slide. Position filter sample side up, on top of Permount and place another small drop of Permount on filter. Be sure filter is agar side up! Cover with #1 coverslip and weight down coverslip (fish weights, penny packs or other small weights are fine). Leave 1–2 days to dry. Do not observe slides under any oil immersion objective until completely dry.

All is not lost if filters become opaque (due to incomplete infiltration) by mounting medium. Soak the slide in xylene, remove filter and repeat steps from 100% isopropyl alcohol dehydration of step 20 above. At least pieces of most filters can be salvaged and some of the best preservation may be in these fragments.

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