

The Oldest Fossil Endophytic Alga and Its Unusual Habitat

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Received February 16, 2003; Accepted April 4, 2003

Abstract

Lycophyte megaspores from the Lower Carboniferous of France sometimes contain a colonial (volvocacean) alga as an endophyte. This peculiar plant-plant association was briefly described more than 100 years ago and the name *Lageniastrum macrospora* introduced for the alga, but the biological significance of the discovery was never fully appreciated. Here we present a reappraisal of the original material, which to date provides the oldest unequivocal fossil evidence for endophytic algae and the only example of an alga residing in the interior of spores of vascular cryptogams.

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Keywords: Chlorophyta, coenobium, colony, endophyte, *Lageniastrum*, *Lepidodendron*, Lower Carboniferous, permineralization, megaspore, *Volvox*

1. Introduction

Algae occur in a wide variety of habitats (Tiffany, 1935) with the vast majority living in aquatic (fresh, brackish, or ocean water) environments (Oltmanns, 1923; Smith, 1955; Graham and Wilcox, 2000). Other forms thrive in terrestrial habitats that range from moist soil to desert sands (Fritsch, 1922; Hoffmann, 1989). Still others (predominantly chlorophytes) live as epiphytes and/or endophytes on/in plants, animals, and fungi, and have evolved a remarkably wide array of temporary or permanent asymptomatic, parasitic, and/or symbiotic associations with their hosts (Oltmanns, 1923; Reisser, 1992 and literature cited therein).

The fossil record of algae is extensive and dates back to the Late (perhaps even Middle) Proterozoic (e.g., Schopf, 1970; Butterfield et al., 1990; Knoll, 1992; Xiao et al., 1998). Encouraged by the copious fossil record, intensive research has focused on fossil algae. As a result, many ancient forms are increasingly well-understood relative to geographical distribution, stratigraphical range, paleobiology and -ecology; some can even be utilized as index-fossils or proxy markers for paleoenvironmental conditions (Tappan, 1980).

However, the fossil record has produced little evidence to date for algae residing on or in (and interacting with) other organisms, especially land plants. One of the very few fossil examples described of an alga-land plant association is *Phycopeltis* (Trentepohliales, Trentepohliaceae) from the Eocene (Tertiary), a form that resides as an epiphyte on leaves (Köck, 1939; Kirchheimer, 1942; Dilcher, 1962), although some believe that the fossils assigned to *Phycopeltis* represent microthyriaceous fungi (e.g., Dilcher, 1965). Pre-Tertiary evidence for interactions between algae and land plants is virtually absent, with the exception of a single report from the Carboniferous, i.e. a colonial alga that resides in the interior of lycophyte megaspores. This peculiar association was discovered more than 100 years ago by the French palaeobotanist B. Renault (1896), who provided a brief description and introduced the name *Lageniastrum macrosporae* for the alga. Since that time, the discovery has remained largely unnoticed, and thus its significance was never fully appreciated. In this paper, utilizing Renault's original thin-section preparations, we provide a more detailed account of this extraordinary biological association.

2. Material and Methods

Lycophyte megaspores containing *Lageniastrum macrospora*e were obtained from the upper Viséan (Lower Carboniferous) of Combres and Lay (two localities approximately 12 km south of Roanne) and Esnost (10 km north of Autun), Massif Central, France. For information on the geological settings of these localities, refer to Scott et al. (1984). The megaspores are three-dimensionally preserved in a silicious matrix, resulting from acid volcanism during the Viséan. Details on the preservation of fossils and a paleoecological reconstruction of the Viséan wetland ecosystem at Esnost can be found in Rex (1986 and literature cited therein); conditions at Combres/Lay have been interpreted as analogous by Galtier (1971). The isolated megaspores are assignable to *Sublagenicula nuda* (Nowak et Zerndt) Dybová-Jachowicz et al., and thought to have been produced by *Lepidodendron esnostense* Renault and *L. rhodumnense* Renault, the only lycophytes present at Combres/Lay and Esnost (Hemsley and Galtier, 1991). Other floral elements that occur with the lycophytes were detailed by Renault (1896) and Galtier (e.g., 1970b, 1980); the most spectacular fossils are exceptionally well-preserved lycophyte megagametophytes (Galtier, 1964, 1970a). A variety of microorganisms (i.e. bacteria, algae, fungi), many of which are associated with decaying or living plant materials, were also documented by Renault (1896, pp. 421-442, 472-477). Coprolites substantiating plant-animal interactions in the ecosystems at Combres/Lay and Esnost have been reported by Galtier and Rex (1986).

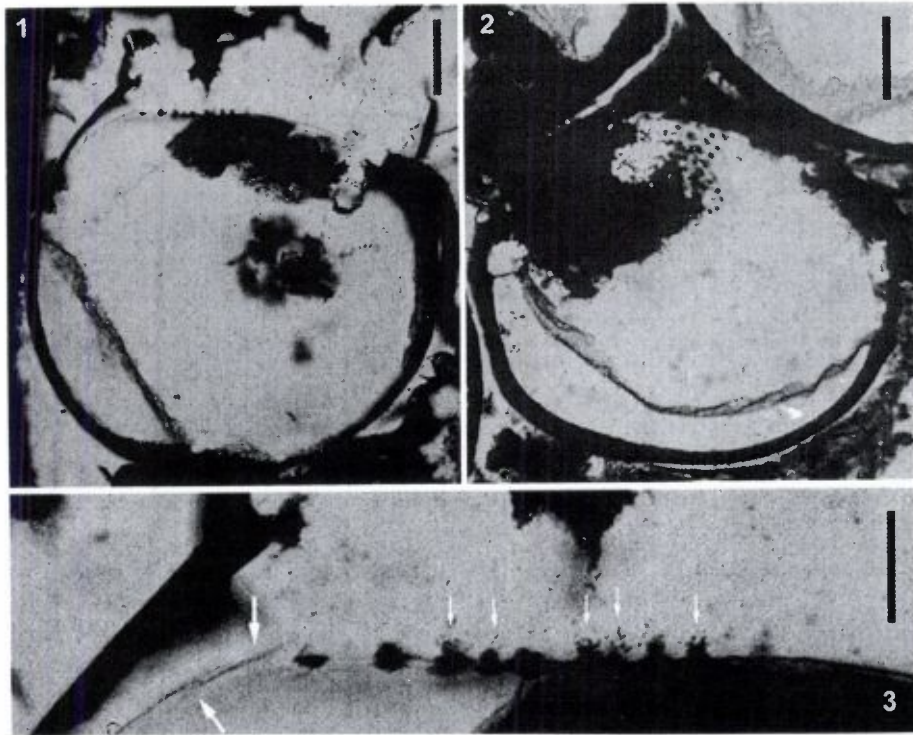
The original description and illustrations (drawings) of *Lageniastrum macrospora*e (Renault, 1896, pp. 429-434, Figs. 81-84 [Fig. 84 re-illustrated here in Fig. 6]) are based on material from Combres/Lay (Roannais). Renault reports that the alga also occurs in megaspores from Esnost, but does not detail or illustrate specimens from this locality. The material used in our study consists of the surviving original thin-sections prepared by Renault and co-workers. Today the slides are deposited at the Muséum National d'Histoire Naturelle (Laboratoire de Paléontologie) in Paris (France) and the Muséum d'Histoire Naturelle d'Autun (France). One slide (i.e. Paris: no. 1151) is from the so-called "collection Renault" that contains Renault's original material, whereas the others (i.e. Paris: no. 92, 94, 107, 108, 109; Autun: without numbers) come from the so-called "collection Roche", a selection of slides similar to those in the "collection Renault" that was prepared for loan and exchange.

3. Description

Sublagenicula nuda megaspores (cf. Dybová-Jachowicz et al., 1987, pp. 6/7) are pyriform in outline, with a circular amb, between 0.4 and 1.5 mm long and

0.4–1.3 mm wide. The most characteristic feature of the spore is a well-developed subgula with slightly constricted base and rounded apex that ranges up to 0.4 mm high and between 0.15 and 0.5 mm wide at the base. The trilete rays are straight, 0.4–0.8 mm long, with labra 20–30 μm wide. Arcuate ridges are 10–40 μm wide and 20–30 μm high. The exine is unornamented and secondary folds are common. A detailed study of the exine ultrastructure can be found in Hemsley and Galtier (1991).

Each thin-section contains numerous megaspores with a *Lageniastrum macrospora* colony present in more than 50% of the spores. In the chert that surrounds the spores, colonies of *L. macrospora*, and structures that may represent non-colonial stages of the life cycle (e.g., spermatozoids, zygotes, uni- to oligocellular germlings), were not found, although other microorganisms (e.g., bacterial colonies on plant debris, fungal hyphae and spores) are well-preserved in the matrix. It is likely that free-living colonies or stages of the life cycle existed (see below), but were simply not present when the fossilization process began, were too delicate to be preserved, or have not been detected due to their minuteness or inconspicuous appearance. The *L. macrospora* colony inside the megaspore consists of a varying number of unicellular algae arranged in a single layer; we estimate that mature colonies are constructed of up to 500 cells. The colony is bounded by a thin, transparent membrane (i.e. "membrane de gélose" *sensu* Renault, 1896) (Fig. 3 [large arrows]). It is unclear whether this membrane was produced by the algae or is a structural feature of the spore. The alga may have incorporated the plasmalemma (i.e. the membrane that separates the spore wall from the cytoplasm), but may also have synthesized its own membrane or membrane-like investment. The individual algal cells range from pyriform to lens-shaped (Fig. 3), up to 12 (sometimes 15) μm long and between 5 and 16 μm wide, with their tips (presumably the flagellar end of cell) always directed toward the spore wall. Details of the internal organization of the algal cells (e.g., chloroplast morphology) are not preserved. In one specimen (Fig. 3), the tips of the algal cells seem to bear several short filamentous structures (Fig. 3 [small arrows]), which may represent the remains of flagella. The distance between two neighboring algal cells usually varies between 2 and 35 μm , but in a few colonies, cells are much more loosely spaced. In some specimens, the individual algal cells are not interconnected, whereas in others prominent protoplasmic strands are present that connect adjacent cells (Figs. 4–6). These strands are up to 35 μm long, with a width of 2–4 μm . In still other specimens, protoplasmic strands are present, but appear to have largely disintegrated, with only a few incomplete strands still in place. The fossils provide no information as to whether dissolution of the strands occurred naturally during the development of the colony, or represents a preservational artefact.



Figs. 1-3. Longitudinal thin-sections of *Sublagenicula nuda* megaspores containing *Lageniastrum macrospora* colonies; specimens from the "collection Roche" at Autun (slides without numbers).

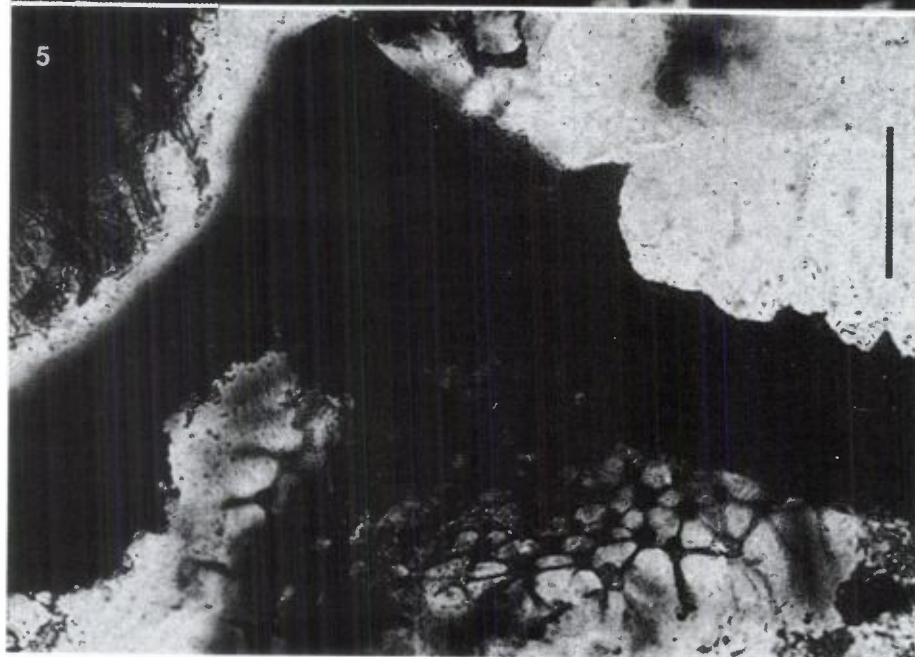
Figure 1. Spore containing a small, planar (plate-like) colony (stage 1) of *L. macrospora* located beneath the subgula; Bar = 150 μ m.

Figure 2. Stage 2 colony, extending downward along the inner surface of the spore wall, and, in this way, assuming three-dimensionality; Bar = 150 μ m.

Figure 3. Detail of Fig. 1, focusing on the small stage 1 colony. Large arrows indicate the membrane that holds the colony together; small arrows point toward the tips of algal cells that seem to bear short filamentous structures (possibly remains of flagella); Bar = 50 μ m.

The size of the colony, and thus the number of algal cells it contains, and the extension of the colony along the interior surface of the spore wall are variable. We hypothesize that these variations represent stages of a natural developmental sequence. Four developmental stages can be distinguished based on the specimens available:

(1) *Lageniastrum macrospora* enters the spore, settles in the area beneath the subgula, and develops into a small, planar (plate-like) colony, composed of a relatively small number of closely spaced cells (Figs. 1, 3). Colonies of this



type are always located beneath the subgula, which suggests that the alga entered the spores through the preformed suture ("micropyle" *sensu* Renault, 1896) that occurs in the center of the trilete mark.

(2) In the following stage, the colony increases in size and, as it expands, extends downward along the inner surface of the spore wall, and, in this way, assumes a three-dimensional organization (Fig. 2). Protoplasmic interconnections between adjacent algal cells are visible in many (but not all, e.g., Fig. 2) specimens at this developmental stage.

(3) The colony further increases in size and number of algal cells (Figs. 4, 5), and may eventually extend over 1/3 to 2/3 of the proximal surface of the spore body (Fig. 6). In many colonies, protoplasmic strands are recognizable, whereas in others such interconnections are either lost or more difficult to resolve. Nevertheless, in nearly every specimen, at least one or two (remains of the) strands can still be found in place, confirming that interconnections were once consistently present as a biological component of the colony.

(4) In the final stage, the colony ruptures and becomes fragmented. We do not know exactly how this was accomplished, but it is indisputable that the colony is somehow dissociated. It is possible that, at some point, the colony perishes and eventually disintegrates. On the other hand, Renault (1896) suggested that, during the life cycle, parts of the colony or individual cells separated, and that these cells exited the spore as a dispersal mechanism. However, either independently or simultaneously, diagenetic processes and/or fossilization may have also caused some colonies to rupture and become fragmented.

4. Discussion

Lageniastrum macrospora is a peculiar microfossil that has to date only been reported from the Carboniferous of central France. The assignment of *L. macrospora* to any single group of extant algae is hampered by the

Figs. 4-5. Longitudinal thin-section of a *Sublagenicula nuda* megaspore containing a *Lageniastrum macrospora* colony; specimen from the "collection Roche" at Autun (slide without number).

Figure 4. Spore containing a stage 3 colony of *L. macrospora* with prominent protoplasmic strands that interconnect adjacent cells; Bar = 75 μ m.

Figure 5. Detail of Fig. 4, focusing on the colony; Bar = 75 μ m.

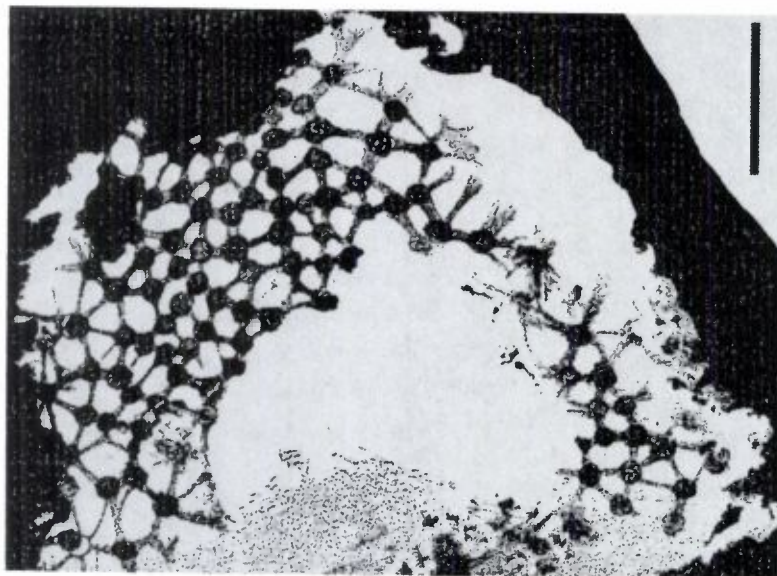


Figure 6. One of the original drawings of *Lageniastrium macrospora* published by B. Renault (1896, Fig. 84). It depicts a longitudinal thin-section of the upper part (beneath the subgula) of a *Sublagenicula nuda* megaspore that contains a mature, dome-shaped (late stage 3) colony of *L. macrospora*; Bar = 75 μm .

incompleteness of the fossil record, which does not permit analysis of the complete suite of features required today in order to accurately establish the systematic position of an alga (e.g., chloroplast morphology, ultrastructure, pigments, stored photosynthates, cell-wall chemistry, life cycle, cf. Lee, 1999; Graham and Wilcox, 2000). However, *L. macrospora* displays a striking suite of morphological characters found in members of the extant chlorophyte genus *Volvox* (Volvocales, Volvocaceae), especially *V. globator* (Fig. 7A and B). *Volvox* colonies (= coenobia) are specialized cell systems in the form of hollow, mucilage-filled spherules, consisting of a single layer of up to several thousand *Chlamydomonas*-type cells surrounded by a thin mucilage investment (Janet, 1912; Mignot, 1985; Kirk, 1998). Within the coenobium, two types of cells are present, i.e. small somatic and larger reproductive cells termed gonidia. In *Volvox globator*, the somatic cells are lens-shaped to pyriform (Fig. 7A), up to 14 μm long (without flagella) and between 3 and 10 (up to 12) μm wide. The range in size and shape of the somatic cells of *V. globator* is similar to that recorded for the cells of *L. macrospora*.

In contrast to *Volvox*, however, *L. macrospora* colonies are not composed of two morphologically different types of cells; rather, all cells appear to have

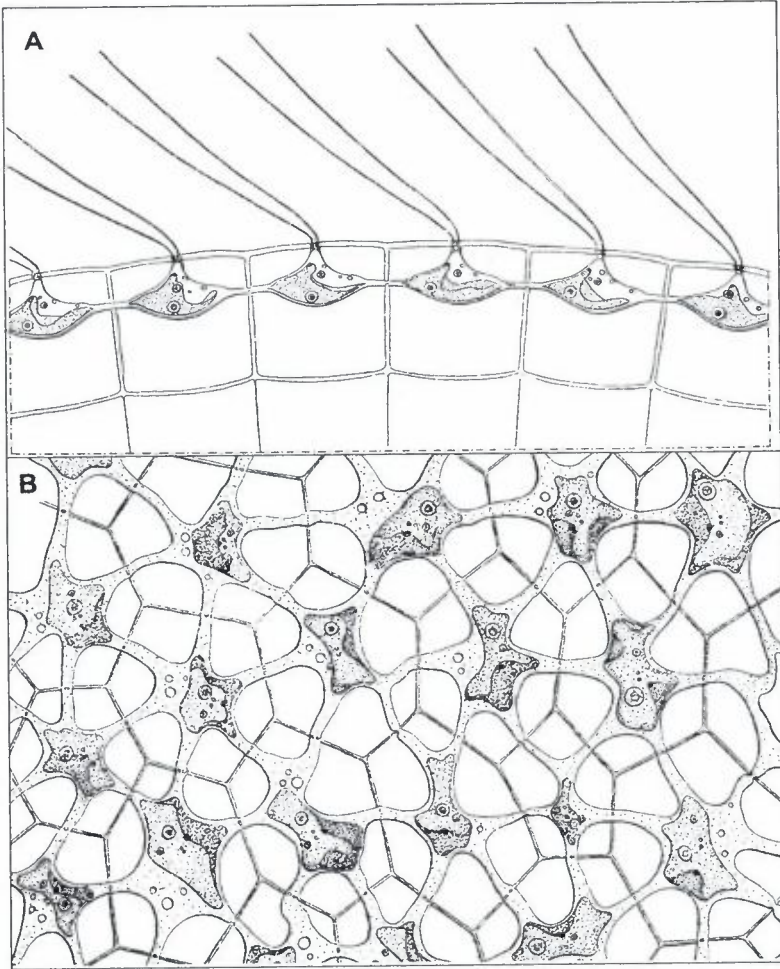


Figure 7. *Volvox globator*. A: Section through the peripheral region of a coenobium, composed of a single layer of *Chlamydomonas*-type cells, each of which is embedded in its own (non-confluent) mucilaginous envelop, the whole held together by a thin investment. B: Surface view, showing the protoplasmic strands that interconnect adjacent cells (redrawn from Janet, 1912, Figs. 3 and 6).

approximately the same dimensions. Extant members of the Volvocaceae are characterized by the formation of a plakea (i.e. a planar stage) during early development of the coenobium (e.g., Kuschakewitsch, 1931; Smith, 1955, Figs. 12A–D; Lee, 1999, p. 240). In *L. macrospora*, early development of the colony also appears to include a planar, plakea-like stage, which is located beneath

the subgula (Figs. 1, 3). From this point, the colony expands downward (Fig. 2) along the interior surface of the spore wall and, as a result, ultimately assumes a three-dimensional, dome-like configuration. The early developmental stages of asexually and sexually produced *Volvox* coenobia are morphologically similar (Oltmanns, 1923, v. 1, p. 236). However, asexual reproduction involves the formation of daughter coenobia inside the parent spherule, whereas sexual reproduction results in the formation of new coenobia outside the confines of a parent. The fact that the endophytic *L. macrospora*e colonies develop outside the confines of a parent may suggest that they represent products of a sexual reproduction. The most compelling evidence for the volvocacean affinities of *L. macrospora*e is the presence of protoplasmic strands that interconnect the protoplasts of adjacent cells (Figs. 4–6). In modern *Volvox*, as a result of incomplete cytokinesis, the protoplasts of adjacent cells are interconnected by protoplasmic strands (Fig. 7B). In some species, these strands persist in the adult, and thus the individual cells of mature coenobia appear stellate when viewed from above (Fig. 7B), whereas in others, the strands break down at the end of embryogenesis, and mature coenobia lack any protoplasmic interconnection between adjacent cells (Kirk, 1998, p. 34). In several specimens of *L. macrospora*e, there is evidence for disintegration of the protoplasmic strands that mimics that in the *Volvox* coenobia. However, it is unclear as to whether this occurs naturally at some point during the life cycle (e.g., as the colony matures), or is the result of diagenetic alterations and/or fossilization. Based on the preceding considerations we believe that the Volvocaceae is the best modern analogue for *L. macrospora*e. The most prominent fundamental difference, however, between all extant members of the Volvocaceae and *L. macrospora*e is that the former are free-living, highly motile organisms, whereas the latter, during at least one stage of the life history, abandoned the motile life style in order to occupy the interior of megaspores. Thus, if *L. macrospora*e indeed belongs to the Volvocaceae, it were to be regarded as a derived, highly specialized form.

The fossil record of Volvocaceae is virtually nonexistent. This is probably the result of the fact that the colonies/coenobia (i.e. unicellular algae embedded in delicate mucilage investments) are not easily preserved (Tappan, 1980). One of the rare microfossils that has been interpreted as a coenobial volvocacean alga is *Eovolvox silesiensis* from the Devonian of Poland (Kazmierczak, 1975, 1981), although the affinities of this taxon remain unresolved (cf. Tappan, 1980; Kirk, 1998). Affinities with the Volvocaceae have also been suggested for a few other Paleozoic and Mesozoic microfossils (see review in Kazmierczak, 1981), but none of these records is persuasive. As a result, we believe that *Lageniastrum macrospora*e appears to represent the most convincing fossil representative of the Volvocaceae to date. Furthermore, *L. macrospora*e is unique among fossil and extant algae because no other alga is

known to date that resides in the interior of spores of vascular cryptogams. Questions remain as to the nature of the association between this alga and spore, and the biological adaptations provided in this peculiar habitat. Since experiments and/or (long-term) observations of living material are not possible, hypotheses with regard to the benefits for *L. macrospora*e residing in the interior of a megaspore can be offered only by comparing the fossils with modern free-living and endophytic algae.

Renault (1896, p. 429–434) interprets *Lageniastrum macrospora*e as a saprophyte that resides in dead or non-viable megaspores. He theorizes that many lycophte megaspores were not mature/viable at the time they were shed from the sporangium, and that those falling into water or onto moist soil eventually became colonized by various fungi and algae, including *L. macrospora*e. This interpretation suggests that no long-term real interaction existed between *L. macrospora*e and the megaspores, and that the algae may simply have been caught accidentally within the spores. However, we found that the fossil material offers compelling evidence to contradict this interpretation. Many of the infected spores (e.g., Fig. 4) are still completely closed with the exception of a narrow suture ("micropyle" *sensu* Renault, 1896) in the center of the trilete mark, and it seems very unlikely (if not impossible) that a mature *L. macrospora*e colony was accidentally transported into the interior through this suture. Perhaps juvenile colonies or flagellate unicells could accidentally have been caught within the spores, but it is not reasonable to assume that a free-living, motile colonial alga frequently (in some 50% of the spores) develops normally inside another organism where space is limited and it is deprived of mobility and sunlight. Based on these considerations, it seems more likely that a real relationship existed between *L. macrospora*e and the spores, and that the alga was a true endophyte, which "deliberately" infected mature/viable spores prior to megagametophyte development by entering the suture as a passage to the interior. Further supporting this hypothesis is the fact that young colonies, consisting of only a few cells, are always positioned in the subgula area (i.e. beneath the "micropyle") (cf. Figs. 1, 3), but are never found elsewhere in the spore. Perhaps the life cycle of *L. macrospora*e included a motile one- to few-cell state ("swarmer state"); motile cells or small cell clusters could easily enter and exit the spores through the suture in the trilete mark. The result of sexual reproduction in extant *Volvox* is a thick-walled zygote, which, after a period of dormancy, germinates (by meiosis) and produces one large viable germling and a set of three small non-viable cells ("polar bodies") (e.g., Starr, 1975; Kirk, 1998). The germling is a biflagellate unicell that swims about for a short time before settling down to cleave and form a small germling spheroid (Kirk, 1998, p. 216/217; Graham and Wilcox, 2000, p. 480). Presupposed that sexual reproduction of *L. macrospora*e was similar to that seen in extant *Volvox*, motile germlings may have been

effective as "swarmers" that entered the spores. In the interior of the spores, the *L. macrospora*-germlings then developed into multicellular colonies by heterotrophically utilizing the nutrient-rich spore contents. In this scenario, the alga would be a true endoparasite. However, this interpretation must also be regarded as problematical. Some extant algal endophytes of land plants are not parasites *sensu stricto* because they neither have significant negative effects on the host, nor are they nutritionally completely dependent (Pringsheim, 1963; Joubert and Rijkenberg, 1971; Chapman and Waters, 1992). Rather, these forms are asymptomatical space-parasites ("Raumparasiten" *sensu* Pringsheim, 1963, p. 380), which inhabit, but do not (or not significantly) adversely affect their hosts. They may utilize some of the host's resources (e.g., water, minerals), but still remain capable of photosynthesis, and thus are not (or not completely) physiologically dependent. Since it remains uncertain as to whether *L. macrospora* caused any pathological symptoms and to what extent the alga was physiologically dependent on the megaspore, a parasitic nature of the *Lageniastrum macrospora*-*Sublagenicula nuda* association cannot be established with certainty. Still, if *L. macrospora* were a space-parasite, capable of photosynthesis, how was photosynthesis accomplished, taking into consideration that the spore walls presumably excluded most of the light from the interior? It is interesting to note that most modern *Volvox* species do not tolerate extended exposure to direct sunlight (Mast, 1907; Kirk, 1998). If light intensity exceeds the optimum for the species, the chloroplasts produce toxic photooxidants, which accumulate and eventually kill the alga; as a result, *Volvox* displays negative phototaxis. *Lageniastrum macrospora* was perhaps capable of photosynthesizing with the minimum amount of light that passed through the spore wall. At the same time, the alga may have gained protection against excessive sunlight exposure from the spore wall.

Regardless whether *Lageniastrum macrospora* was a saprophyte, true parasite, or space-parasite, occupying the interior of megaspores may have had several adaptive advantages. For example, when habitat conditions deteriorate, many modern algae produce various types of thick-walled resting cells. In the resting stage, the algae are comparatively immune to environmental stress, and thus resting cells can serve as a refuge population for re-colonization of isolated bodies of water following periods of unfavorable conditions (Sandgren, 1983). For example, thick-walled *Volvox* zygotes can resist both late summer droughts and winter freezes, and thus are essential for long-term survival in temperate environments (Kirk, 1998, p. 189). On the other hand, resting cells may also serve to sequester genotypes from selection over periods of generations within local environmental fluctuations (Coleman, 1983). In her reconstruction of the Viséan ecosystem at Esnost, Rex (1986, Fig. 3) depicts small pools and lakes scattered across the landscape. These bodies of water represent the habitat of *L. macrospora*. The environment at Combres/Lay and

Esnost was dominated by active volcanism (Rex, 1986), and thus the ecosystems were highly dynamic due to occasional lava flow.

Based on the environmental conditions it is reasonable to conclude that the small pools/lakes were unstable ecosystems, which perhaps only existed for relatively short periods of time. Megaspores are known to be highly resistant to most environmental influences and may remain viable for many years prior to germination based on, among other things, thickness and composition of the spore wall and quality and quantity of stored materials. It is therefore possible to envisage that *L. macrospora*e was perhaps not capable of forming resting cells, but may have endured periods of unfavorable conditions within the megaspores. The possibility also exists that, together with *L. macrospora*e, plankton-feeding animals were present, and that these consumed large quantities of planktonic algae. As a result, there was undoubtedly a selective advantage for organisms to become encapsulated from predators. Lycophyte megaspores might have offered protection against predators in either of two ways: 1) the spores may have been too large to be ingested, and 2) if ingested, the spore wall may have functioned as a sort of protective shell, which enabled the algae to pass unscathed through the alimentary canal of the animal.

5. Concluding Remarks

The *Lageniastrum macrospora*e-*Sublagenicula nuda* association represents a unique type of plant-plant interaction, since, as far as we are aware, no other fossil or extant alga has been reported inside the spores of vascular cryptogams. The biology and ecology of the association is poorly understood, and will likely never become fully deciphered due to the nature of the fossil record. Nevertheless, the fossils add to our general understanding of the diversity of interactions between different groups of organisms in the late Paleozoic, and thus offer still another perspective of the complexity within late Paleozoic ecosystems. Moreover, the *Lageniastrum macrospora*e-*Sublagenicula nuda* association is significant because it demonstrates that the genetic information and developmental mechanisms underlying the establishment and sustenance of complex alga-land plant interactions were in place in the Early Carboniferous. This suggests that the evolutionary origins of such bioses date much farther back in geological time. Perhaps the evolutionary history of interactions between algae and land plants parallels that between fungi and land plants. The latter is well-documented today based on a body of fossil evidence for saprophytic, parasitic, and/or symbiotic fungi throughout geological history (e.g., Stubblefield and Taylor, 1988; Taylor and Osborn, 1996).

Understanding of the paleoecology of ancient ecosystems has progressed rapidly in recent years to the point that researchers are now able to examine highly complex interactions involving previously understudied organisms. Nevertheless, our knowledge of Early Carboniferous terrestrial ecosystems is still very incomplete, especially with regard to the inventory of microorganisms (including microalgae) and the roles they play in the biology and ecology of land plants and animals. The permineralized Viséan flora from Combres/Lay and Esnost is well understood today. Renault (1896), who provided the first account on this flora, also described microorganisms, many of which were associated with living or decaying plant material. Thus, Combres/Lay and Esnost offer a rare opportunity to not only take an inventory of land plants and microorganisms in a late Early Carboniferous ecosystem, but also, at a cellular level, decipher their structure, biology, and ecology, including interactions at several levels of biological organization. The *Lageniastrum macrospora*-*Sublagenicula nuda* association outlined in this paper demonstrates the potential of the material from Combres/Lay and Esnost, and we expect that further studies will yield information that may eventually contribute to a more complete understanding of the evolutionary history of, and perhaps processes leading to, the interactions that are common in the microbiological world today.

Acknowledgments

This research was supported by the Deutsche Forschungsgemeinschaft (DFG grants Ke 584/7-1 and 584/7-2) and the National Science Foundation (NSF grant OPP-0003620). The authors thank Drs. Jean Broutin and Jean Dejax (Paris, France) for making available the original material of *Lageniastrum macrospora* and Derek W. Kellogg (Lawrence, KS, USA) for technical assistance.

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