

CHAPTER 2-6

PROTOZOA ECOLOGY

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CHAPTER 2-6

PROTOZOA ECOLOGY



Figure 1. The ciliate protozoan *Blepharisma americana* inhabits the lobules of the liverwort *Pleurozia purpurea*. Photo by Sebastian Hess, with permission.

General Ecology

Protozoa can probably be found on almost any bryophyte if one just looks carefully (Figure 1). Larger protozoa tend to occur in bog habitats (Chardez 1967; Bovee 1979). As drier habitats are examined, the species are smaller and smaller. *Diffflugia* (Figure 2) species are typical of aquatic mosses; *Cyclopyxis* species occur on terrestrial mosses. *Centropyxis* species distribution depends on the habitat, with *C. aculeata* (Figure 3, Figure 4) in wet locations and *C. platystoma* in dry ones. *Corythion dubium* (Figure 5), *Assulina muscorum* (Figure 6), and *Trinema lineare* (Figure 7) occur generally on forest mosses (Chardez 1957; Bovee 1979; Beyens *et al.* 1986), although *A. muscorum* also is known from the cells of living *Sphagnum recurvum* (Figure 8) (BioImages 1998). *Corythion pulchellum* (Figure 9) and *Trinema complanatum* (Figure 10) occur only on forest mosses (Chardez 1960; Bovee 1979). *Nebela collaris* (Figure 11), *Centropyxis aculeata*, and *Hyalosphenia papilio* (Figure 12) occur on *Sphagnum* and other bog mosses, but not on forest mosses (Chardez 1960; Chiba & Kato 1969; Bovee 1979).



Figure 2. *Diffflugia bacillifera* with diatoms in the test. Note the small desmid beside it. Photo by Yuuji Tsukii, with permission.

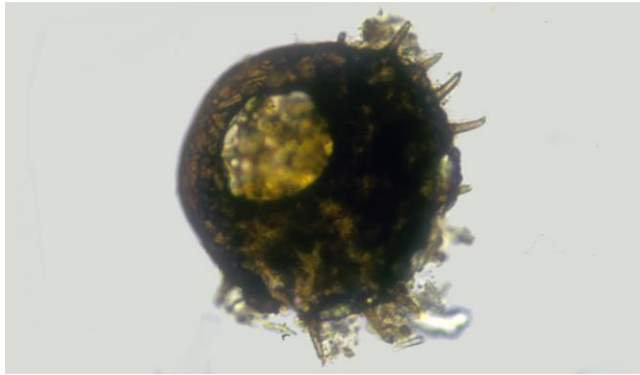


Figure 3. *Centropyxis aculeata*, a testate amoeba that commonly occurs on bryophyte leaves. Photo courtesy of Javier Martínez Abaigar, with permission.

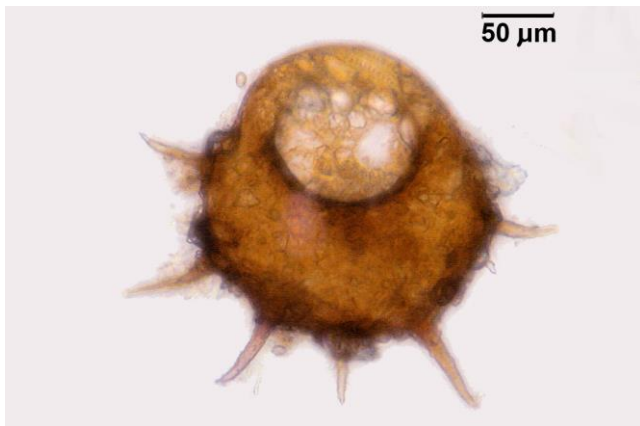


Figure 4. *Centropyxis aculeata* test. Photo by William Bourland, with permission.



Figure 5. *Corythion dubium* test. Photo by Yuuji Tsukii, with permission.

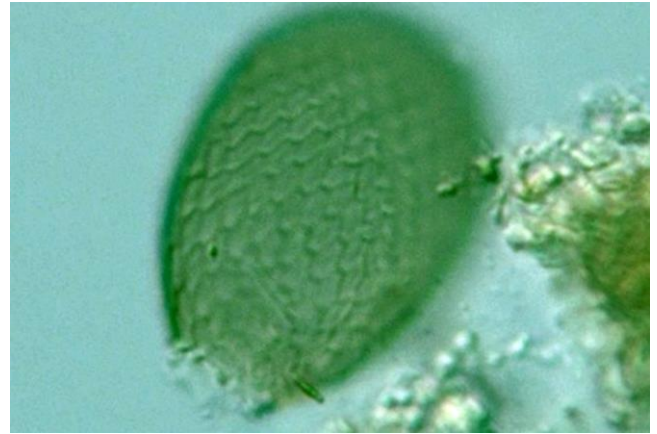


Figure 6. *Assulina muscorum*. Photo by Yuuji Tsukii, with permission.

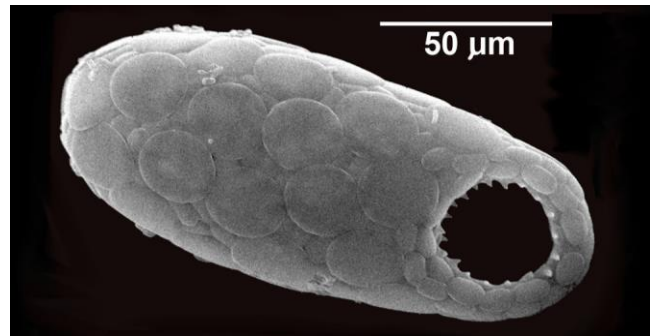


Figure 7. Test of *Trinema lineare*. Photo by Edward Mitchell, with permission.



Figure 8. *Sphagnum recurvum* var. *tenue*, a peatmoss that supports living protozoa in its hyaline cells. Photo by Jan-Peter Frahm, with permission.

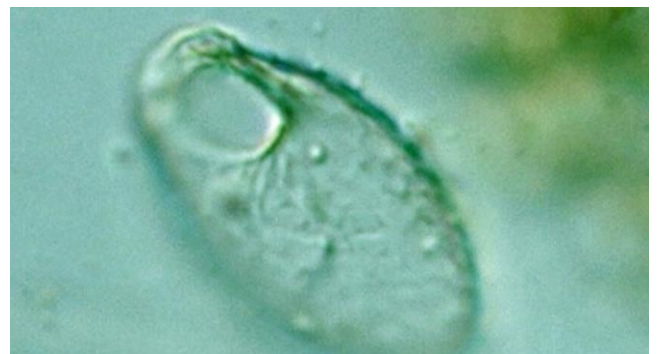


Figure 9. *Corythion pulchellum*. Photo by Yuuji Tsukii, with permission.



Figure 10. *Trinema complanatum*. Photo by Yuuji Tsukii, with permission.



Figure 11. *Nebela collaris*. Photo by Yuuji Tsukii, with permission.

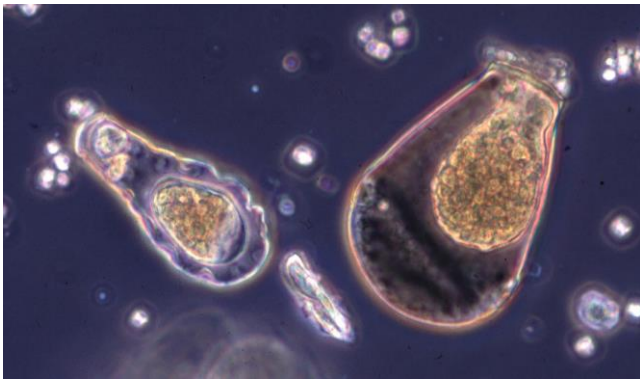


Figure 12. *Hyalosphenia papilio* and *H. elegans*. Photos by Edward Mitchell, with permission.

Protozoa are generally the most numerous invertebrates among the *Sphagnum* plants (Figure 8; ntham & Porter 1945). In a Canadian study, flagellates were the most numerous, but testate amoebae are often the most numerous.

Epiphytes

Despite the dryness of aerial habitats, protozoa are common among epiphytic bryophytes, drying and encysting as the bryophytes dry, then reviving, eating, and reproducing when the bryophytes are moist. This habitat may hold many species as yet undiscovered because it is a habitat less frequently studied by protozoologists. Nevertheless, a number of taxa are known from this unique habitat (Golemansky 1967; Casale 1967; Bonnet 1973a, b).

Antarctic

The role of protozoa is particularly important in the Antarctic. On Elephant Island of the South Shetland Islands in the Antarctic, moss carpets and turf form a major part of the habitat available to protozoa (Smith 1972). Mastigophoran (flagellate) moss inhabitants include 15 species. The Mastigophora are not unique to this habitat. Those that were in most of the moss samples also were in samples of grass/soil, clay, or **guano** (accumulation of feces). Furthermore, none of the species that was abundant in the other habitats was absent among bryophytes except *Tetramitus rostratus*, which was abundant only on guano. The Rhizopoda, including the testate amoebae, seemingly avoided the guano on Elephant Island, whereas 16 species occurred in the bryophyte habitats (Smith 1972). Several of those Rhizopoda present in the grass/soil habitat were not found among the moss samples. Fourteen species of Ciliata occurred among mosses.

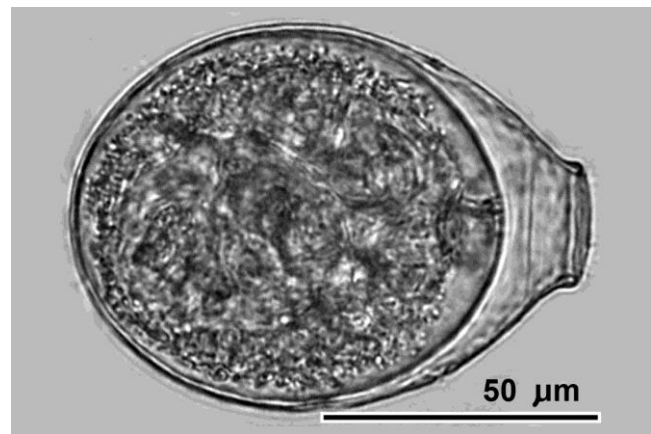


Figure 13. *Nebela tinctoria* test with living amoeba. Photo by Edward Mitchell, with permission.

The small number of Elephant Island moss samples (4 in *Polytrichum–Chorisodontium* turf & 5 in *Brachythecium–Calliergon–Drepanocladus* carpet) precludes comparison of moss preferences (Smith 1972). The most abundant ciliate, *Urotricha agilis* (see Figure 14), was abundant in both turf and carpet. In samples of turf, mean numbers per gram of fresh weight ranged 170–4,500. In carpet they ranged 250 to 7,700. On Signey Island species numbers were higher in moss turf (40), whereas on Elephant Island they were higher in moss carpet (37) than in turf.



Figure 14. *Urotricha platystoma*. Photo by Yuuji Tsukii, with permission.

Nutrient Cycling

Protozoa are common predators on bacteria and fungi (Hausmann *et al.* 2003), having the role of nutrient cyclers (Mitchell *et al.* 2008). In the Pradeaux peatland in France, the testate *Nebela tinctoria* (Figure 13) consumed mostly micro-algae, especially diatoms, associated with mosses (Gilbert *et al.* 2003). In summer they also consumed large ciliates, rotifers, and other small testate species. Micro-organisms collect between leaves and along stems of *Sphagnum*. When the system is wet, prey organisms are mostly immobile and often dead, but when conditions are drier and the water film is thin, testate fauna are able to ingest more mobile organisms than usual because these prey are slowed down by lack of sufficient free water for rapid swimming. Although we know little about their role among bryophytes, it is likely that at least in peatlands the role of moss-dwelling protozoans in nutrient cycling is significant (Gilbert *et al.* 1998a, b; Mitchell *et al.* 2008).

Habitat Effects

When protozoa and other inhabitants live on a host, they can alter the host. Insects are well known for the many forms of galls that develop on the host plant. Gradstein *et al.* (2018) discovered a white colony of protozoa, resembling gnathifers, in the swollen shoot tips of the liverwort *Herbertus sendtneri*. This resulted in cessation of the tip growth and subsequent development of innovations below the tip.

Moss Effects on Soil Habitat

The presence of mosses also affects the micro-organisms found in the underlying soil. Miroschnichenko and coworkers (1975) found that the greatest numbers of micro-organisms were under mosses (compared to other soil substrata) in a community in Russia, and Smith and Headland (1983) found similar results for testate rhizopods on the sub-Antarctic island of South Georgia. Smith (1974a, 1986) found protozoa living among the bryophytes in the South Orkney Islands and Adelaide Island of the Antarctic. Ingole and Parulekar (1990) found that the faunal density, including protozoa, was high in moss-associated sediments. These micro-organisms may account for the ability of some macrofauna to remain within the moss mat throughout a major part of their development by serving as a food source (Smith 1974a, 1986).

Epizoites

Some of the fauna, such as *Pyxidium tardigradum* (Figure 17), an **epizoite**, are hitch-hikers. This protozoan is recorded as a **symphoriont** (organism carried by and often dispersed by its host) on two species of tardigrades (Figure 15) [*Hypsibius oberhaeuseri* (Figure 16) and *Milnesium tardigradum*] that live among mosses (Land 1964; Morgan 1976). It can be so common on them (up to 35, but more typically 1-3) as to have negative effects on the tardigrade host that must expend extra energy to carry them around (Vicente *et al.* 2008). For this reason, Vicente *et al.* (2008) suggest that it should perhaps be considered a parasite.



Figure 15. **Tardigrade**. Photo courtesy of Filipe Osorio.

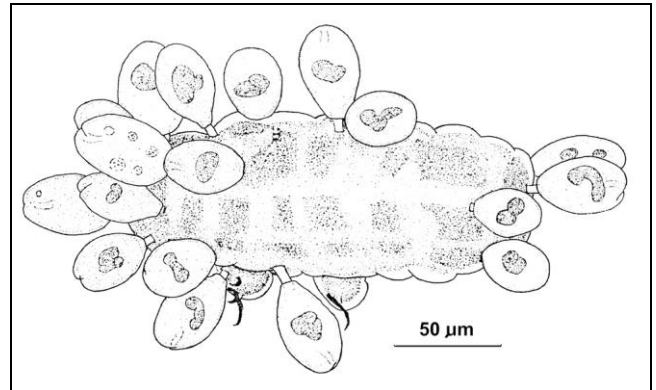


Figure 16. *Hypsibius oberhaeuseri* with *Pyxidium tardigradum* growing as a symphoriont. Redrawn from Van Der Land 1964.

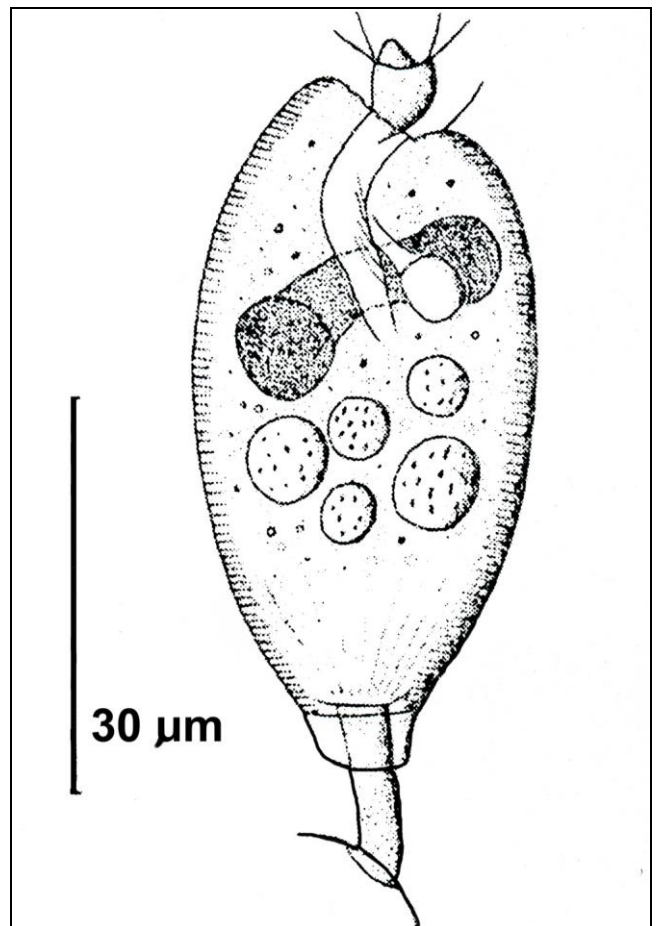


Figure 17. *Pyxidium tardigradum*, a tardigrade symphoriont. Redrawn from Van Der Land 1964.

Soil Crusts

Protozoan communities associated with cryptogamic soil crusts (Figure 18) have hardly been studied. In a study of only five crusts in southeastern Utah, Bamforth (2008) found 28 species of amoebae, 45 ciliates, and 19 testate amoebae. The number of amoebae ranged 680-2500, ciliates 20-460, and testate amoebae 2400-2500 per gram dry mass of crust. As crusts succeeded from *Microcoleus* (Cyanobacteria) to lichens to bryophytes, numbers of protozoa increased, perhaps reflecting longer periods of internal moisture in the crusts. Predominant taxa are somewhat different from cosmopolitan ones we have seen elsewhere, comprised mostly of *Acanthamoeba* (Figure 19), *Hartmanella* (Figure 20), *Vahlkampfiidae* (Figure 21), two species of *Colpoda* (Figure 22), several other colpodids, *Polyhymenophora* sp., and species of *Cryptodiffugia* (Figure 23) and *Diffugiella*.



Figure 18. Soil crust with the moss *Syntrichia ruralis*. Photo by Michael Lüth, with permission.

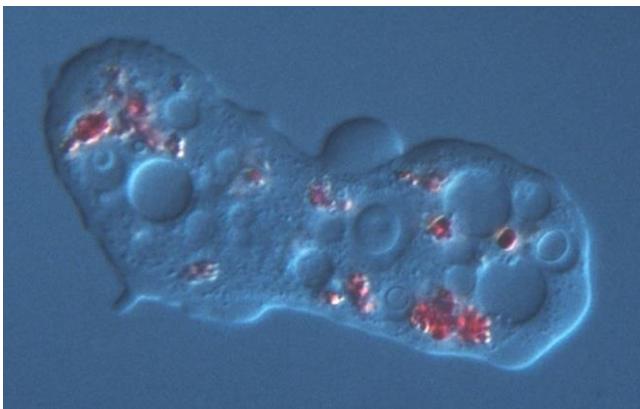


Figure 19. *Acanthamoeba* showing ingested carmine particles. Photo by Akira Kihara, with permission.



Figure 20. *Hartmanella*. Photo by Yuuji Tsukii, with permission.

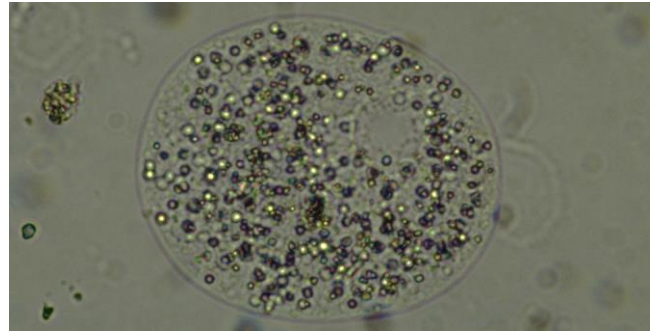


Figure 21. *Vahlkampfiidae*. Photo by Yuuji Tsukii, with permission.

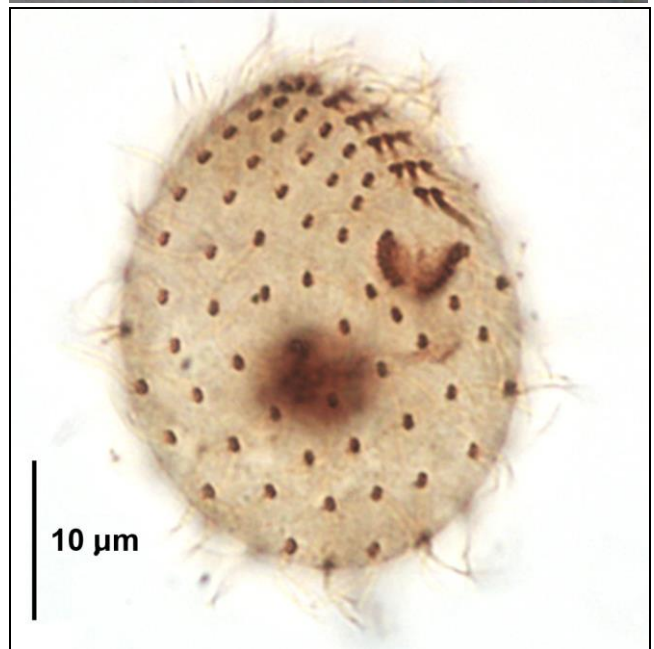
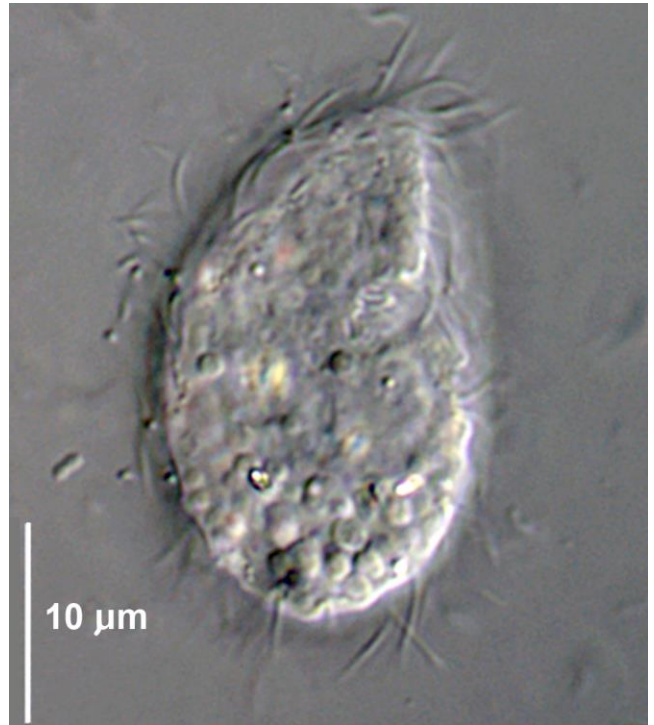


Figure 22. *Colpoda aspera*. Photos by William Bourland, with permission.



Figure 23. *Cryptodifflugia ovaliformis* on an alga filament. Photo by Yuuji Tsukii, with permission.

Vertical Zonation

Bryophyte suitability as a protozoan habitat differs in both time and space. Bryophytes offer a vertical series of habitats (Figure 24) that differ in temperature, moisture, and light, and presumably food quality and quantity. Horizontally, the substrate or height above the water table can differ, causing species differences. Hence, the micro-organisms distribute themselves in different communities both seasonally and spatially, particularly in the *Sphagnum* peatlands (Schönborn 1963; Heal 1964; Meisterfeld 1977; Mazei and Tsyganov 2007).



Figure 24. *Sphagnum subnitens* showing tips and lower branches that create habitat zones for protozoa. Photo by Michael Lüth, with permission.

Spaces: Several studies indicate that the sizes of spaces within the bryophyte habitat influence the sizes of organisms and influence the available food (Dalenius 1962; Corbet 1973; Bovee 1979; Robson *et al.* 2001). Capillary spaces among branches and leaves hold water. Gilbert *et al.* (2003) suggested that as the *Sphagnum* becomes drier, ciliate protozoa are easier to catch for food because the thin film of water slows them down. As the moss becomes too dry, rather than migrating to lower, moister areas, many of these taxa, like several invertebrate groups, can encyst, permitting them to survive desiccation (Heal 1962; Gerson 1982). And when the moss resumes activity under the stimulation of rain (or fog), the rhizopods do likewise.

Nitrogen: Nitrogen from guano seemingly deterred all the testate amoebae on Elephant Island (Smith 1972).

Nitrogen distribution affects the vertical distribution of at least some testate amoebae in *Sphagnum* communities, but nitrogen availability does not seem important for most testate amoebae in the upper centimeters of *Sphagnum* mats in the Swiss Jura Mountains (Mitchell & Gilbert 2004). There were 22 testate taxa among these mosses, although mean diversity of a typical sample was only 6.6. The species richness increased with depth. The moss-dwelling *Assulina muscorum* (Figure 25) was most abundant in the top 0-1 cm; *Phryganella acropodia*, *Heleopera rosea* (see Figure 26), and *Nebela militaris* (Figure 27) were the most abundant taxa at 3-5 cm depth. In this case, species richness increased with depth in the mat. Only *Bullinularia indica* (Figure 28) appeared to be more abundant in plots fertilized with nitrogen.



Figure 25. *Assulina muscorum*. Photo by Yuuji Tsukii, with permission.



Figure 26. *Heleopera sylvatica* showing pseudopods. Photo by Yuuji Tsukii, with permission.



Figure 27. *Nebela militaris*. Photo by Yuuji Tsukii, with permission.

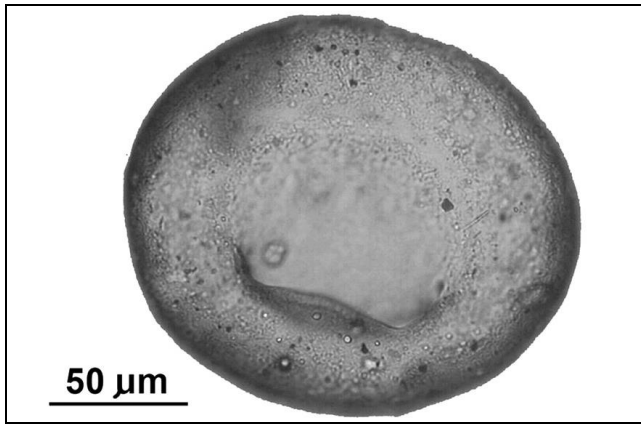


Figure 28. Test of *Bullinularia indica*. Photo by Edward Mitchell, with permission.

Temperature: The Antarctic fauna is dominated by moss-dwelling micro-organisms, including protozoa, rotifers, nematodes, and tardigrades (Schwarz *et al.* 1993). Here, temperature may play a role as important as that of moisture. This need for adequate heat results in a vertical zonation of the fauna. For example, at the Canada Glacier, in southern Victoria Land, the majority of moss-dwelling organisms were in the top 5 mm in the post-melt samples, rather than in the pre-melt samples. However, while temperatures differed, so did the available moisture, making it difficult to determine controlling factors.

Light: As one might expect, light determines the absence of protozoa with chlorophyllous symbionts in the lower strata (Chacharonis 1956). Only those surface species contain chlorophyll, either as symbiotic algae or that of their own possession. However, some with chlorophyllous symbionts may occur as deep as 6-10 cm in *Sphagnum* mats (Richardson 1981). Of the 27 species lacking symbionts in a *Sphagnum* mat, all but two exhibited maximum abundance below 6 cm. But even within the first 5 cm, vertical zonation exists. Mitchell and Gilbert (2004) demonstrated a significant difference in number of species between the first 3 cm and the 3-5 cm depth in *Polytrichum strictum* (Figure 29) of a Swiss peatland (Figure 30).



Figure 29. *Polytrichum strictum*. Photo by Michael Lüth, with permission.

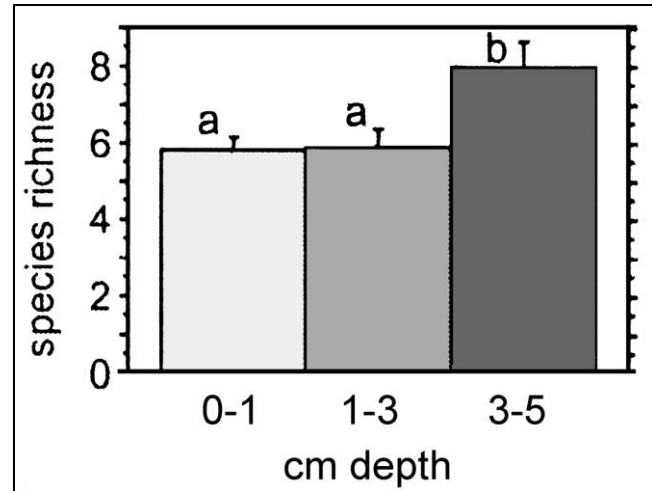


Figure 30. Vertical distribution of species richness of testate amoebae in a *Polytrichum strictum* "carpet" of a Swiss peatland. Redrawn from Mitchell & Gilbert 2004.

Community Differences: As for a number of other moss habitats, the *Sphagnum* peat mat provides vertical differences in microhabitat that are further expressed as vertical community differences (Meisterfeld 1977; Strüder-Kypke 1999; Mitchell *et al.* 2000). Strüder-Kypke found that even in the upper 30 cm of the mat, two very different protistan communities are dictated by the strong vertical zonation. Both light and nutrients differ, causing the upper region to support a denser colonization, mostly of autotrophic cryptomonads and vagile ciliates (able to move about or disperse in a given environment). On the other hand, deeper samples exhibited heterotrophic flagellates and sessile peritrich ciliates.

Presence of testate amoebae at greater depths within the moss mat does not always indicate a retreat to a location of greater moisture. Schönborn (1977) demonstrated that 15% of the shells can be transported to lower depths by 550 mm rainfall, but 400 mm generally does not seem to cause a noticeable downward loss.

Zoophagy by Liverworts?

Carnivorous plants are well known among the flowering plants, but the ability of bryophytes to attract and trap organisms has been questionable. Who would guess that these seemingly primitive organisms can attract their own prey? But one interpretation is that the leafy liverwort genera *Colura* (Figure 31, Figure 32) and *Pleurozia* (Figure 33) have **lobules** (water sacs) that do just that (Hess *et al.* 2005). And this is not an isolated example. In the Aberdare Mountains, Kenya, Chuah-Petiot and Pócs (2003) found many protozoa inhabiting the lobules of the epiphytic *Colura kilimanjarica* (Figure 31, Figure 32).



Figure 31. **Upper:** The leafy liverwort, *Colura*. **Lower:** This lobule of *Colura* houses the ciliate protozoan *Blepharisma americana*. Photos by Jan-Peter Frahm, with permission.

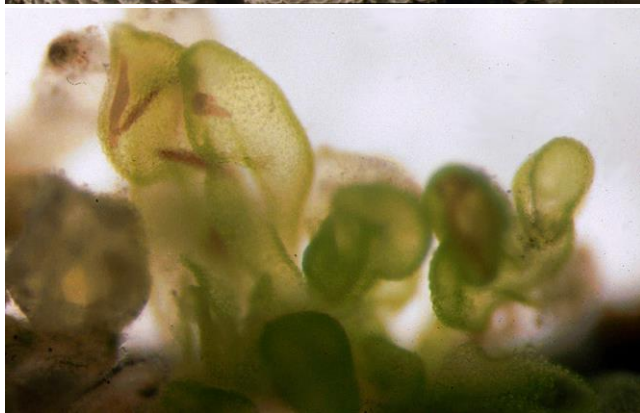


Figure 32. **Upper:** SEM of lobule of *Colura*. **Lower:** Living lobule. These lobules of *Colura* are inhabited by the reddish ciliate protozoan *Blepharisma americana*. Photos by Jan-Peter Frahm, with permission.



Figure 33. Underside of *Pleurozia purpurea* showing lobules where invertebrates often live – and die. Photo by Sebastian Hess, with permission.

Lobules are usually considered to be water storage organs. However, in these genera, they might also serve as traps. Goebel (1888, 1893, 1915) did not consider it likely that these were real traps. He argued that insectivorous plants have attractants in order to lure their prey into their traps. Although the lobule resembles the trap of the bladderwort, *Utricularia*, Goebel argued that that does not mean it is used the same way. He furthermore argued that the benefit gained by the excrement from animals (and dead animals?) would be less than that gained from the water. Since having the animals does not preclude also providing a water reservoir, it would seem that zoophagy would simply be an added benefit. Schiffner (1906) even reported chironomid larvae in the lobules, suggesting an even larger source of fecal matter. But the openings in *Pleurozia* are small, only about 300 μm , and closed by a round "lid" of hyaline cells (Hess *et al.* 2005). What causes these organisms to enter in the first place?



Figure 34. *Pleurozia purpurea*, a leafy liverwort with lobules that can house a variety of invertebrates, including the ciliate *Blepharisma americana*. Photo by Sebastian Hess, with permission.

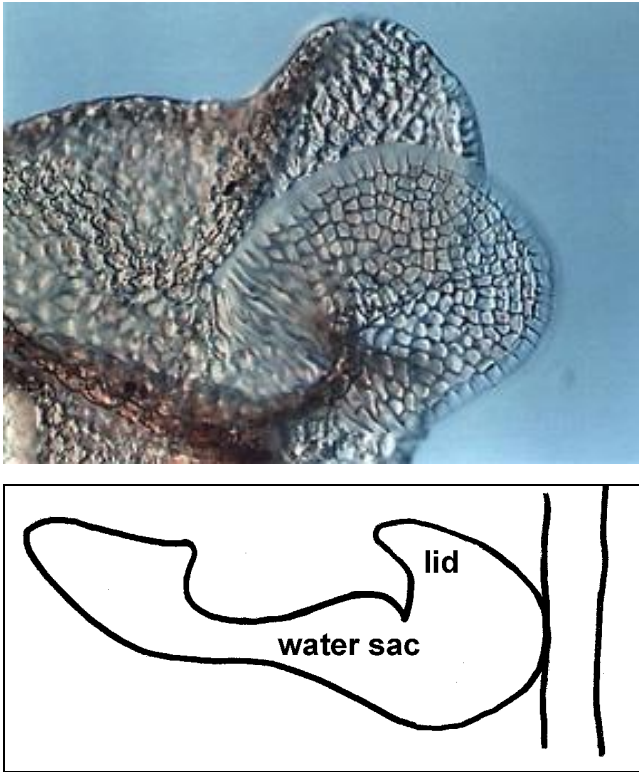


Figure 35. **Upper:** Lobule of *Pleurozia purpurea* showing lid. Photo by Sebastian Hess, with permission. **Lower:** Lobule redrawn from Hess *et al.* (2005). This lobule of *Pleurozia purpurea* serves as home and apparently ultimately as a trap for a wide range of protozoa and invertebrates.

Barthlott *et al.* (2000), using feeding experiments with the ciliate protozoan *Blepharisma americana* (Figure 1, Figure 36-Figure 38), demonstrated that *Colura* does indeed catch protozoa with its lobules. Hess and coworkers (2005) set out to determine if *Pleurozia purpurea* (Figure 33-Figure 35) is likewise carnivorous.



Figure 36. The ciliate *Blepharisma americana* that inhabits "zoophagous" liverworts. Photo by Yuuji Tsukii, with permission.

Again using *Blepharisma americana*, a cohabitant of *Sphagnum* mats with *Pleurozia purpurea*, Hess *et al.* (2005) performed dozens of experiments in Petri dishes to

see if the dispersion of the protozoan remained random. Indeed, the protozoa gradually accumulated around the *Pleurozia*! Within only 30 minutes, 86% of the lobules contained the protozoa. After several hours, up to 16 protozoans were trapped, and further observation failed to reveal any that escaped.

The mode of attraction is only speculation. Barthlott *et al.* (2000) found that older parts of *Colura* were more effective at attracting *Blepharisma americana* (Figure 37, Figure 38) than were younger parts, suggesting that concentrations of bacteria may have been a factor. In fact, in experiments on *Colura*, Barthlott *et al.* (2000) found that *B. americana* moves over the bryophyte surface "like a vacuum cleaner," devouring the bacteria.



Figure 37. A stained *Blepharisma americana*. Photo by Yuuji Tsukii, with permission.

The shade provided by the plants could also contribute to the higher concentrations of protozoa near the branches of *Pleurozia purpurea* (Hess *et al.* 2005), but if so, the liverwort would probably be less effective as a refuge in the field where other mosses were also present.

Hess and coworkers (2005) claim that the large number of organisms in the lobules in such a short time is too great to be attributed to chance. However, they fail to provide any statistical evidence or probability to support this claim, for example, alternative liverworts or mosses. They furthermore state that the organisms die there, but they provide no data on the deaths of the organisms. They do point out that there is no direct evidence that any nutrients provided by the organisms are used by the liverworts, but there is likewise no evidence to the contrary. In any case, the liverworts could benefit from the cleaning of bacteria that block light and compete for nutrients.

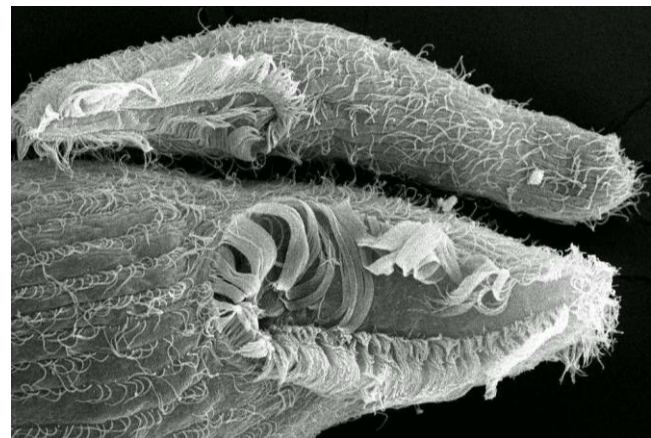


Figure 38. SEM photo of *Blepharisma* demonstrating small cell on top and large, cannibalistic cell below. Under starvation conditions, larger individuals become cannibalistic. Photo by Pauline Gould, with permission.

Zoophagy is the process of eating animals (*phag* = eat, devour; Hanson 1962; Lincoln *et al.* 1998). There is a fine distinction in what constitutes just eating compared to true carnivory, wherein living organisms are killed (or not) and digested. In this case, it seems that the animals may be trapped, but there is no real proof that they are consumed by the plant. Does admitting the animals into the trap (lobule) then make the liverworts zoophagous? Hess *et al.* (2005) argue that animals die in the traps and subsequently release their cell contents, bursting in the case of *Blepharisma americana*. These dead animals are then decomposed by bacteria. Surely some of the nutrients released are absorbed by the liverworts. Is this not a process parallel to that of the pitcher plant *Sarracenia purpurea*? Many so-called carnivorous plants, like *S. purpurea*, seem to lack enzymes to digest all or some of the parts of their prey and depend on resident bacteria to accomplish the task. With this broad definition of carnivory, could we not call the liverworts **carnivorous**? I think I want more data on whether this is a chance event or true trapping before I make that claim. Such experiments would need controls of leafy liverworts with no "traps" to see if the protozoa simply accumulate wherever there is shelter. On the other hand, I wonder how many leafy liverworts with locules provide preferred housing for protozoa.

Dispersal

For any organism to succeed, it must have a means of dispersal. Protozoans can't go very far on their own. They are too small to crawl far on pseudopods or paddle their way with a flagellum or cilia, the common means of transportation for the majority of protozoan moss dwellers. But they can travel reasonable distances as passengers on the mosses, riding on fragments that establish a new home where they land.

Sudzuki (1972) conducted experiments using electric fans to determine the success of wind as a dispersal agent, using mosses as one of the sources of invertebrate fauna. He found that the smaller organisms – micro-organisms, including protozoa, were easily dispersed by light breezes as well as wind. Larger organisms such as gastrotrichs, flatworms, rotifers, nematodes, oligochaetes, tardigrades, crustaceans, and arachnomorphs, on the other hand, rarely were dispersed at wind velocities of less than 2 m per second [tornadoes are generally 27-130 m per second (Allaby 1997)]. In the field, colonization progressed from flagellates to ciliates to rhizopods, suggesting that passive dispersal was not the only factor controlling their colonization rates.

Once an organism becomes airborne, turbulent air may take them 3,000 to even 17,000 m on thermal drafts, with winds carrying them much higher and farther (Maguire 1963). Puschkarew (1913) found that protozoan cysts average about 2.5 per cubic meter, making these organisms readily available for dispersal and colonization on suitable bryophytes.

Smith (1974b) likewise considered that the mosses themselves served as dispersal agents for the protozoa. In particular, moss invasions of volcanic tephra on Deception Island in the Antarctic greatly increased the protozoan fauna. Not only do the mosses provide a great increase in suitable niches, but since they were most likely colonized

by protozoa in their former locations, fragments arriving on the island could easily carry communities of fauna as passengers.

Rain can carry many algae and protozoa (Maguire 1963). Rain-borne organisms seem to originate predominantly from splash, typically from plants and soil, and do not travel far vertically, so that mechanism is most likely only suitable for local habitat travel.

In streams, the water movement itself serves as an effective dispersal agent, and aerial dispersal from waterfalls and rapids can carry algae and other *Aufwuchs* to new locations.

Raccoons are very effective in carrying whole communities of organisms, particularly protozoa, and can accomplish distances of at least 60 meters (Maguire 1963). Both terrestrial and aquatic birds contribute to dispersal, and other mammals contribute, but their relative role is not known.

Several scientists have discussed the dispersal of micro-organisms by insects (Maguire 1963; Parsons *et al.* 1966). Such mechanisms could easily contribute to the colonization of bryophytes by their micro-inhabitants. The many aquatic insect inhabitants will be discussed in an upcoming chapter. Consider the activity of insects among bryophytes, especially in streams, and their subsequent relocation due to swimming or stream drift. The *Aufwuchs* could easily be carried from one location to another by these mobile inhabitants (Figure 39). Emerging insects may also swipe micro-organisms trapped by the surface tension and carry them to resting locations, including bryophytes, on land.

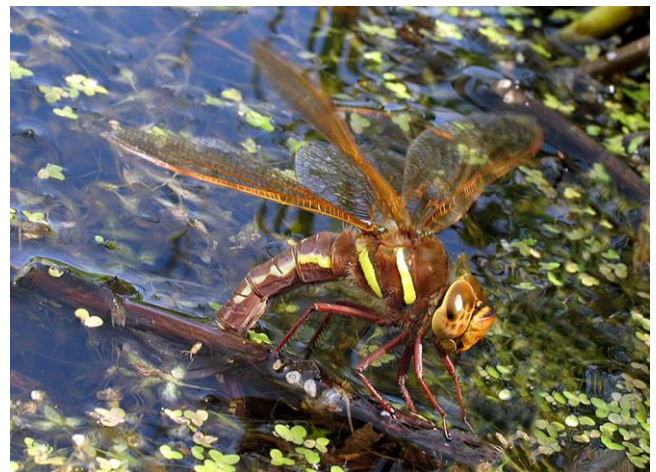


Figure 39. Dragonfly *Aeshna grandis* female ovipositing and exposing herself to possible transport of protozoa. Photo by David Kitching, with permission.

Although few studies seem to have directly addressed the dispersal of micro-organisms by insects to bryophytes, we can infer at least some possibilities from more general studies on dispersal by insects. Maguire (1963) examined the distance both horizontally and vertically to which organisms were dispersed from a pond in Texas and another in Colorado. Dragonflies (Figure 39) and wasps, in particular, carried several species of protozoa and one species of rotifer. Parsons *et al.* (1966) found amoeboid and other protozoan cysts on adult Odonata, suggesting the possibility of a relatively long dispersal range. Odonata in

a short-term experiment dispersed up to 860 m to the farthest pond in the experiment (Conrad *et al.* 1999). Michiels and Dhondt (1991) estimated that 80% of adult dragonfly *Sympetrum danae* had migrated 1.75 km or more to their study site. But more importantly, evidence suggests they can migrate 3500 km or more across the Indian Ocean (Anderson 2009). This and other long-distance migrations provide a potential yearly means of dispersal for the micro-organisms.

Cosmopolitan

'Everything is everywhere, but, the environment selects' (in Wit & Bouvier 2006; O'Malley 2008). This statement, often called the Baas Beeking Principle, has been applied to microscopic organisms that are globally distributed by high dispersal, and that lack biogeographic patterns (Fontaneto *et al.* 2008). But Wit and Bouvier made it clear that the original hypothesis "did not disregard the biogeography of free-living microorganisms." Finlay *et al.* (1996) extend the concept to suggest global species diversity is inversely related to body size. Therefore, the huge number of protist individuals makes global dispersal inevitable through normal events such as ocean circulations, groundwater connections, hurricanes, damp fur, dust storms, etc. (Weinbauer & Rassoulzadegan 2003). This argument is supported by the fact that the estimated number of free-living ciliates is about 3000, whereas there are about 10,000 species of birds and 120,000 species of Lepidoptera (butterflies and moths) (Lawton 1998).

The concept of global distribution describes well the major protozoa associated with bryophytes. This concept does not preclude, however, the presence of cryptic species that differ in less recognizable traits (Richards *et al.* 2005; Fontaneto & Hortal 2008; Fontaneto *et al.* 2008; Kooistra *et al.* 2008), and in recent detailed studies distinct genetic species have been found in disparate parts of the world (Telford *et al.* 2006; Fontaneto *et al.* 2008; Kooistra *et al.* 2008).

One consideration to support "everything is everywhere" is the small number of species of protozoa relative to 750,000 species of insects and 280,000 species of other animals (Papke & Ward 2004). Morphological data support the concept that dispersal is worldwide, suggesting there would be fewer than 5000 morphological protozoan species. Could this also be the explanation for the small number of bryophytes relative to other plants? In both cases, molecular evidence is starting to suggest that there may be cryptic species with genetic differences that are not expressed morphologically (Logares 2006), revealing distributions that are much more restricted.

Bryophyte protozoan communities are remarkably similar no matter where the bryophytes occur and consist primarily of cosmopolitan species. Davidova (2008) compared the testacean communities of epiphytic bryophytes to those of soil bryophytes in Strandzha Natural Park, South-Eastern Bulgaria, and found them to be quite similar in their taxonomic richness, species diversity, and community structure. The most common taxa in both habitats were *Centropyxis aerophila* var. *sphagnicola*, *C. aerophila* (Figure 40), *Phryganella hemisphaerica*, *Euglypha rotunda* (Figure 41), *Corythion dubium* (Figure 5), *Trinema enchelys* (Figure 42), and *T. lineare* (Figure 7). Among these, only *Phryganella hemisphaerica* is

missing from the sites in Switzerland, Alaska, Sweden, Finland, Netherlands, Britain, Bulgaria, and North America as summarized in Table 1 of Chapter 2-2. The epiphytic community had 34 taxa in 13 genera, whereas the soil mosses had 31 taxa in 13 genera.

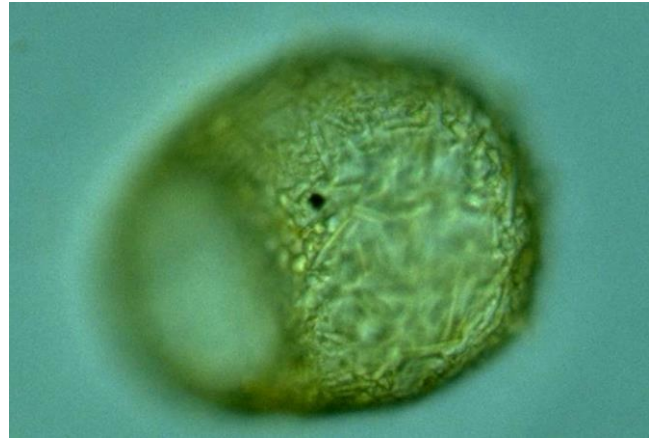


Figure 40. *Centropyxis aerophila* test. Photo by Yuuji Tsukii, with permission.

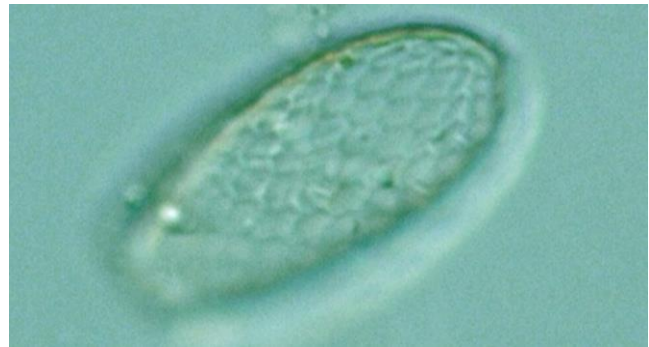


Figure 41. *Euglypha rotunda*. Photo by Yuuji Tsukii, with permission.



Figure 42. *Trinema enchelys*. Photo by Yuuji Tsukii, with permission.

The moss-dweller *Nebela* (*Apodera*) *vas* (Figure 43) has been touted to refute the Baas Beeking Principle (Mitchell & Meisterfeld 2005; Smith & Wilkinson 2007). In 89 collections, representing 25 publications, mosses represented 59% of its habitat, with *Sphagnum* being the most common (Smith & Wilkinson 2007). Its distribution

is throughout the equatorial region at high altitudes, southern cool-temperate, and sub-Antarctic zones, but it is conspicuously absent in the Holarctic northern hemisphere. Its absence from hundreds of samples from seemingly suitable habitats in the northern hemisphere support the contention that its absence is not a fluke of sampling (Mitchell & Meisterfeld 2005). This distribution is definitely not cosmopolitan, despite its wide pH range (3.8-6.5) (Smith & Wilkinson 2007). Although it has a rather defined climatic range (temperate to sub-Antarctic), its absence in this climate throughout most of the more frequently studied northern hemisphere cannot support the concept of "everything is everywhere." Evidence such as this has been used to argue that micro-organisms are dispersed following the same principles as macro-organisms (BioMed Central 2007). Genetic differences that are not detectable from morphology suggest that global diversity of micro-organisms may be greater than has been suspected (BioMed Central 2007; Fontaneto *et al.* 2008). Such evidence suggests that care is needed in assigning names to microbial/protozoan collections.

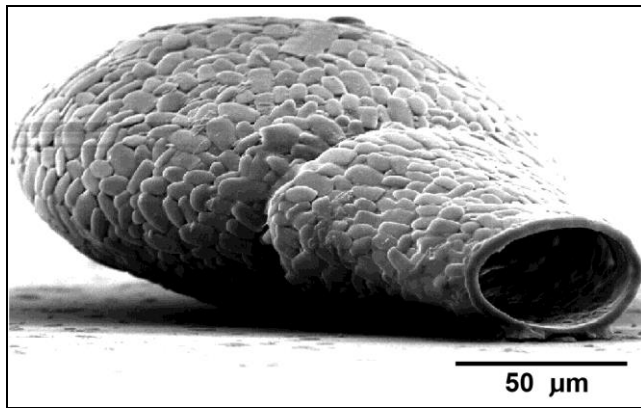


Figure 43. SEM view of *Apodera* (*Nebela*) *vas* showing test. Photo by Edward Mitchell, with permission.

Jenkins *et al.* (2008) have tested the size hypothesis, using 795 data values on dispersal units from published research. They found that active dispersal vs. passive dispersal matters greatly, with active dispersers dispersing significantly farther ($p < 0.001$) while having a significantly greater mass ($p < 0.001$). They showed that size does make a difference, but not always as predicted by the Baas Beeking Principle. Among active dispersers, it is the larger dispersers that go the greater distances, perhaps related to required energy. The principle does not even hold well for the passive dispersers. The distances travelled by these dispersal units were random with respect to mass.

How well does the size:dispersal distance relationship hold for bryophytes that travel by spores? One might argue that as a group, they are more cosmopolitan than seed plants and less cosmopolitan than the protozoa. Fortunately for the protozoa, they are not very specialized for particular bryophytes.

Communities as Biological Monitors

Ciliates living among bryophytes in Czechoslovakia are sensitive to air pollution, giving us another way to assess the effects of air pollutants (Tirjakova & Matis 1987). Testate amoebae, including *Assulina* (Figure 25),

Corythion (Figure 5, Figure 9), *Euglypha* (Figure 41), and *Heleopera* (Figure 26), as well as *Euglena* (Figure 44) and Cyanobacteria, in a *Sphagnum* bog of Tierra del Fuego, South America, were sensitive to UV-B radiation (Robson *et al.* 2001). But surprisingly the testate amoebae and rotifers were significantly more abundant and had greater species diversity under current levels of UV-B radiation than those that received reduced UV-B. The fungal component likewise had significantly greater abundance and species diversity under the current dosage than under the reduced dosage.



Figure 44. *Euglena mutabilis*, a common euglenoid among bryophytes, particularly in peatlands. Photo by Yuuji Tsukii, with permission.

Because pollution affects the entire community, moss-dwelling protozoans can often be a more efficient means of assessing pollution damage than other biological components. In a study in France, Nguyen-Viet *et al.* (2007a, b) assessed the response of the protozoan community under simulated lead pollution. Using Pb^{+2} concentrations ranging from 0 to 2500 $\mu g L^{-1}$, they found that biomass decreased significantly for bacteria, microalgae, testate amoebae, and ciliates at 625 and 2500 $\mu L^{-1} Pb^{+2}$ after six weeks. The microbial biomass decreased as the densities of testate and ciliate protozoa decreased, but the relative biomass of bacteria to that of the protozoa remained constant. The correlation between the two groups increased as the lead concentration increased. Hence, the protozoa provided an effective and relatively inexpensive means of assessing the community response.

Enhanced CO_2 had the opposite effect on the community relationships (Mitchell *et al.* 2003). Biomass of the testate amoebae decreased by 13% while the heterotrophic bacteria increased by 48% when the CO_2 was increased to 560 ppm, compared to those at an ambient CO_2 concentration of 360 ppm. Mitchell *et al.* (2003) suggest that the increase in bacterial biomass may be a response to increased exudation from *Sphagnum* under the higher CO_2 regimen.

As discussed in an earlier sub-chapter, the testate amoebae can serve as indicators of drainage in *Sphagnum* mires, as noted by Warner and Chmielewski (1992) in northern Ontario, Canada. As the water level falls, some species increase while others decrease.

Collecting and Sorting

There are lots of references for collecting, preserving, and enumerating aquatic and soil taxa of protozoa, but few on methods for bryophyte fauna. However, many methods for soil will apply equally well to the bryophyte fauna. A thorough coverage of methods is in Adl *et al.* (2008), with methods for peatland microfauna in Gilbert and Mitchell (2006). A special method for holographic viewing of live testate amoebae is presented by Charrière *et al.* (2006).

Collecting

Collecting protozoa that live among mosses is simple and requires no special equipment. In thick cushions or mats of bryophytes, extraction can be achieved with a stainless steel corer. In some circumstances, a knife can be used to cut a core and the core then placed into a cylindrical plastic container (Lamentowicz & Mitchell 2005). Stream bryophytes should be collected in a way that avoids as much loss downstream as possible. This can be achieved by shielding the bryophyte from most of the flow and especially shielding it as it breaks through the surface. One's hands are often sufficient to achieve this, but a container might be used over the bryophyte, enclosing as much of its depth as possible while dislodging it from the substrate. For non-quantitative collections in almost any habitat, a hand-grab is usually sufficient. For diversity studies, it is important to get the moss down to its substrate because zonation often occurs.

Storage & Preservation

Bryophytes and adhering water/moisture can be kept in jars or polyethylene bags until they are returned to the lab. If the weather is warm, it is desirable to place the containers in a cooler with ice. Oxygen is a problem, so open containers or vials with loose lids will help. For aquatic collections, some free water might be needed, making it necessary to confine the water by such means as a wad of paper towel or cloth above the water level to avoid splashes out of the jar. Parafilm may suffice for short time periods, or two, separated layers of screen or mesh.

The most rewarding experience is to observe the protozoa live as they swim about in the water film, gyrate from a stalk, or engulf a food item. Some species will remain alive only a few hours after collection (Samworth 1995). If the organisms are to be kept for a few days, place them in a refrigerator (not freezer) or incubator that is set in the range of 5-15°C (Glime pers. obs.). The container should be covered to reduce evaporation, but not sealed. Jars with lids should have the lid on loosely to permit air exchange. If the jar is opened and a foul odor escapes, there has not been enough air exchange, and many of the organisms will be dead – and perhaps subsequently eaten by the more hardy ones.

Preservation

If the sample is to be kept for long in the field before returning to the lab, and the weather is hot, it might be necessary to preserve the organisms. This is fine for testate amoebae, but may make counting and identification of other protozoans difficult or impossible.

Preservation of bryophyte protozoan samples is like that of other protozoa, using 2% glutaraldehyde (final

solution) (Mitchell *et al.* 2003), formaldehyde (Fisher *et al.* 1998; Gilbert *et al.* 1998a, b), or glycerol (Hendon & Charman 1997b), but the water content of the bryophyte must be considered in calculating the dilution. For example, saturated *Sphagnum* typically has 95% water content (Gilbert & Mitchell 2006).

Long-term Storage of Cysts

One choice for long-term storage is to let the mosses and their fauna dry slowly in air for several days. This can be done in open paper bags, a method typically used for drying bryophytes, or in open jars. Cool drying is preferable for many species, but survivorship will vary depending on the climate of origin and should be tested against fresh samples if the samples will be used for quantitative or diversity work.

Once the samples are dry and the protozoa have encysted, they can be sealed in containers and stored at 4°C. Again, the effects of storage should be tested for any quantitative or diversity work. Tropical taxa may require a warmer storage temperature (Acosta-Mercado & Lynn 2003). This method will only work for species that readily encyst and for testate rhizopods.

Extraction

Organisms can be extracted from the bryophyte-water matrix with a teat pipette (*i.e.* volume is unimportant) and placed as a drop on a glass microscope slide. Bryophyte inhabitants can be squeezed into a sample bottle with little danger to them, but this may have disastrous results for larger fauna that may be of interest. Protozoa can be concentrated in a centrifuge or by running the water through a fine nylon mesh (Samworth 1995), but smaller organisms will be lost and adhering organisms will remain behind on the bryophyte.

Gilbert *et al.* (2003) reduced the negative effects of squeezing by pressing a sieve (1.5 mm mesh) on the moss surface and sucking the water up with a syringe. They were unable to solve the problem of adhering organisms, including some microbial groups. Others are missed because they live inside *Sphagnum* cells. This method creates minimal destruction of the *Sphagnum* mat, even through repeated sampling, except for the trampling by the people doing the sampling.

In their book on *Sphagnum* ponds, Kreutz and Foissner (2006) suggest a slide on slide method (Figure 45). Mosses can be washed in a small amount of suitable water, preferably rainwater or other water that won't kill the fauna. In most cases, lots of detrital matter will come off the mosses, along with many members of the fauna. Dense material will collect on the bottom of the container and can be drawn into a pipette/dropper (ca 2 mL). Material can be transferred onto a glass slide to cover most of the slide. A second slide is then used at an angle to push the flocculent detrital matter to the end of the slide. When the edge of the top slide reaches near the end of the bottom slide, the top slide is lowered onto the bottom one and used as a coverslip. A smaller version of this method (*i.e.* a smaller sample of water and detritus) can be done in the same way with a drop of the water and detritus in the middle. In this case, a coverslip of the desired size can be used in the same manner as the top slide described above. Note that both

methods will be biased toward mobile organisms. Tardigrades, rotifers, sessile protozoans, and other attached organisms will be poorly represented, if at all, by this method (and most others!). To see these, branches of moss need to be examined under the microscope.

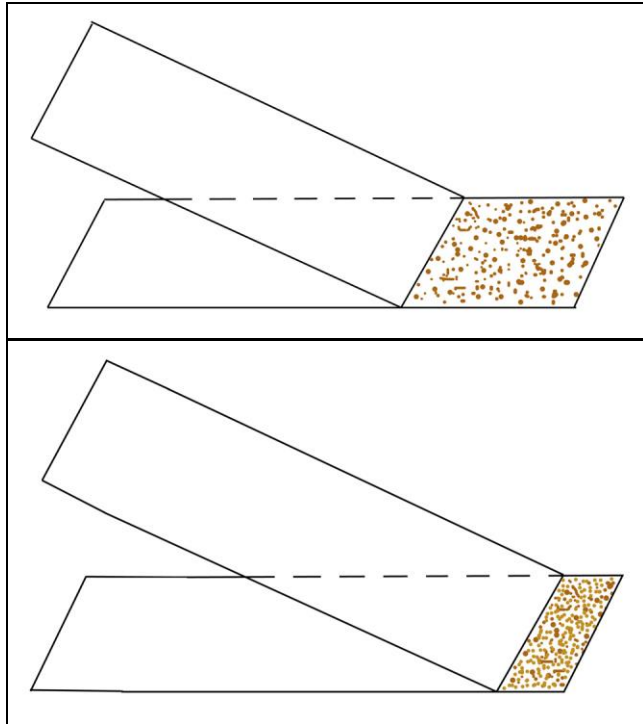


Figure 45. Slide on slide method of concentrating and extracting micro-organisms. Drawing by Janice Glime based on images in Kreutz and Foissner 2006.

Testate Amoebae

The non-flooded Petri dish method (below) can be used to culture testate amoebae as well, but a longer time may be needed to wake up the cysts (Adl *et al.* 2008).

One method to extract **testate** organisms is to dry the bryophytes at 65°C, then sieve and back-sieve them with a sieve that retains all particles in the range of 10-300 µm. The standard method seems to be that of Hendon & Charman (1997b). A standard length of moss is cut and boiled for 10 minutes to loosen the amoebae. The boiled samples are filtered first at 300 µm, then back filtered through 20 µm. The organisms retained by the 20 µm filter are stored in 5 ml vials with glycerol.

Another method for extracting testate species is to put single shoots of bryophyte samples in a vial and shake them with a vortex mixer (Nguyen-Viet *et al.* 2004). This solution can be filtered through a 40 µm mesh filter and washed with deionized water to remove larger organisms. The tiny testate species will most likely all go through the filter due to the force of the water. The filtered water can then be placed in a plankton-settling chamber for 24 hours so the testae will settle to the bottom. For this method, Nguyen-Viet *et al.* (2004) used 20 samples of approximately 0.3 g fresh weight of living moss, placed in a glass vial with 7 ml of 4% formaldehyde.

A different approach to extraction is to boil the living bryophyte stems in distilled water for 20 minutes, stirring occasionally (Lamentowicz & Mitchell 2005). This

solution with moss is then sieved through a 300 µm sieve to remove large constituents. The filtrate can then be concentrated with a centrifuge at 3000 rpm for 4-6 minutes. The tests can be stored in glycerol.

Non-testate Taxa

The non-testate taxa are somewhat more difficult to work with because they are best seen while active. One alternative is to culture them, using the non-flooded Petri dish protocol described by Adl *et al.* (2008):

1. Place bryophyte sample in a 5- or 10-cm Petri dish. Several Petri plates can be set up initially and drained on different days to avoid depleting nutrients with the wash.
2. To culture, moisten sample with distilled water or wheat grass medium.
 - a. To make wheat grass medium, combine 1 g wheat grass powder and 1 L distilled or deionized water in a 2-L Erlenmeyer flask.
 - b. Boil at a gentle rolling boil for 2 minutes, then let settle and cool for 1 hour.
 - c. Filter into a new flask through several layers of cheesecloth to remove the grass residue.
 - d. Adjust the pH to appropriate level (based on sample pH) with a phosphate buffer.
 - e. Autoclave in screw top bottles for 20 minutes.
 - f. Bacteria growth can be reduced by diluting to 1/10 or 1/100 strength.
3. Alternatively, a culture can be made from a dilute solution of detritus from the moss.
4. Incubate at 15°C in the dark or at ambient field temperature. Be sure plates do not desiccate.
5. Observe every few days for signs of activity, up to about 30 days. Some testate amoebae will take several weeks or even months to leave the encysted stage and become active.
6. To observe, moisten the culture plate with a squeeze bottle of distilled or deionized water.
7. Tilt the plate until there is enough to drain the water into a new plate.
8. Observe the drained water in the new plate with a dissecting microscope and oblique transmitted illumination; capture organisms with micro-dissecting tools or a micropipette, then observe with an inverted microscope with phase contrast if possible (see observation section below). Most will require 100-400X to be seen well.
9. Note that the often abundant cercomonads form thin filopodia that explore tiny pores (<1 µm diameter). These adhere to flat surfaces and are not easily seen or dislodged. They may require staining (see below).
10. The original plate can be returned to the incubator.

Observation

Live observations can be done with a small branch, a leaf, or just a drop of adhering water on a glass slide with a compound microscope. A few larger protozoa might be observed with a dissecting microscope. A cavity slide will avoid crushing as the slide dries. Further confinement can

be achieved with this type of slide by putting a drop of water on the cover slip, then inverting it over the cavity, making a **hanging drop** slide. Alternatively, putting Vaseline at the corners of a cover slip on a standard flat slide will keep the cover slip from crushing them. More water can be added at the edge of the cover slip and will be drawn under by capillary action.

Ciliates and flagellates can be slowed down by a viscous substance such as methyl cellulose. Observing them in the interstitial water of intact bryophytes also tends to slow them down. Note that these organisms are mostly transparent and viewing may be improved by using darkfield and/or closing down the diaphragm of the microscope. An inverted microscope has the advantage of giving you a better view of those protozoa that settle on the bottom, especially testate amoebae.

Start your observations with a low magnification and move up after you have found a quiet one you want to observe, preferably surrounded by a bryophyte leaf or other confinement.

For testate amoebae, observation of dead material is not a problem, albeit not so interesting. The test is well-preserved and can be observed and identified at the convenience of the observer.

Staining

Staining can make the organisms easier to see (Figure 46), and vital stains may help to provide behavioral information. For example, neutral red can be used to follow digestion (Howey 2000). Newly formed vacuoles will stain bright red. As digestion proceeds, the vacuole will become yellowish, indicating a change in pH toward alkaline. Powdered carmine can also be used to indicate the location of the vacuole. Subsequent observation with Nomarski differential interference contrast can provide clear visibility. The observer should experiment with brightfield, darkfield, India ink in the solution, oblique illumination, phase contrast, or whatever types of optical contrast may be available. Unfortunately, all stains appear eventually to be toxic, so the viewing time is limited (Howey 2000; Table 1). **WARNING:** Read the labels carefully; many stains are also highly toxic to humans!



Figure 46. *Oxytrichia fallax* stained with Protargol. Photo by William Bourland, with permission.

Table 1. Concentrations needed to stain *Paramecium* and toxicity after one hour. Table from Howey 2000.

Stain	Min Conc to Stain	Toxicity - % dead in hour
bismarck brown	1:150,000	0
methylene blue	1:100,000	5
methylene green	1:37,500	5
neutral red	1:150,000	3
toluidine blue	1:105,000	5
basic fuchsin	1:25,000	30
safranin	1:9,000	30
aniline yellow	1:5,500	0
methyl violet	1:500,000	20
Janus green B	1:180,000	40
Nile blue	1:30,000	
Rhodamine	1:20,000	

Identification

There are some specialty keys available, and lots of pictures on the internet. However, internet pictures and keys should be used with caution and the source of information evaluated because these are unreferenced and often contain errors. A good general reference for identification is the publication by Lee *et al.* (2002), "The Illustrated Guide to the Protozoa." Its nomenclature is in places outdated, so usage should be checked in Adl *et al.* (2005). A more recent aid is a book by Kreutz and Foissner (2006). This book has wonderful color pictures, but there is no designation to tell which were on bryophytes and which were in open water.

Quantification

Adl *et al.* (2008) advised that taxa must be counted within one or two days of collection because temperature and moisture changes will shift the bacterial communities and this will, in turn, cause a change in community structure of the protozoa.

To quantify the sample size, the bryophyte can be weighed after drying. However, some amoebae will become glued to the bryophyte by the attending algae and detrital matter, thus contributing to the weight. Biovolumes can be estimated by using the geometrical shapes and an appropriate formula for that shape, then multiplying by the number obtained (Mitchell 2004).

Adl *et al.* (2008) provided a method to estimate protozoa per gram of dry soil. It could be modified for bryophyte purposes. For any quantification, the method must be consistent among those communities being used for comparison. One can use stem length, wet weight, or dry weight, but these have different biases for different bryophytes and those must be dealt with. Furthermore, different methods may favor the observations of some protozoan taxa. For example, larger organism are more easily seen, testate organisms are more likely to fall from the moss upon shaking, sessile organisms will most likely not fall at all.

Charman (1997) suggested a method for quantifying the testate amoebae and warned of its shortfalls. You may be familiar with methods of determining pollen density by including a known number of *Lycopodium* spores in the sample (for example, 200) and using the ratio of those

observed on the slide to those put in the sample. Unfortunately, in the testate samples extracted from mosses, the number of tests estimated was reduced by up to 80% and the number of taxa was reduced by 60%, probably due to differences in weight, making this a less than desirable method. Using KOH to digest the organic matter did not destroy the tests, and permitted extraction of more tests, but they were damaged and more difficult to identify. Charman concluded that a water-based preparation with sieving was the best method.

Various combinations of filtration, vortex, and centrifuge can be used to get the best results for particular circumstances. Different mesh sizes can be used with back filtration to classify the organisms into size groups (Kishaba & Mitchell 2005). The organisms collected between 15 and 350 μm are a typical size group of Testacea examined (e.g. Warner & Charman 1994; Booth & Zygmunt 2005).

Summary

Larger protozoa tend to occur in moist or bog habitats, whereas drier habitats have smaller ones. Some even occur within the hyaline cells of *Sphagnum*. Some protozoa are exclusive to *Sphagnum*; others occur only on forest mosses. Those on epiphytic bryophytes are able to dry with the mosses and encyst during periods of drought. Moisture also contributes to the vertical zonation of protozoa in peatlands. Soil crusts can have some of the highest numbers of species. Moisture is the major determining factor on species distribution and survivorship, with terrestrial species able to withstand drying more than wet habitat species can. Over 400,000 individuals can occur in one square meter of terrestrial mosses. Studies in the Antarctic suggest that temperature and moss growth form play roles in the number of species.

Drying slows the mobile organisms and permits larger protozoa to capture them. Their consumption of micro-organisms places the moss-dwelling protozoa in the role of nutrient cycling. The bryophytes further contribute to ecosystem processing by affecting the moisture and temperature, hence altering the protozoan fauna, in the underlying soil.

Some protozoa are hitch-hikers on other bryophyte inhabitants, such as those that ride around on tardigrades. Others have green algae as symbionts and are thus restricted to photic zones on the bryophytes, whereas those without these symbionts typically occur below 6 cm depth. Yet others (*Pleurozia*, *Colura*) seem to trap protozoan prey in leaf lobules. In fact, it appears that the leafy liverwort *Pleurozia purpurea* may actually attract *Blepharisma americana*.

Dispersal is likely to be as passengers on bryophyte fragments. A successional pattern from flagellates to ciliates to rhizopods suggests that other factors determine colonization rates. Some colonization comes from dormant cysts awaiting suitable conditions. Dispersal of cysts and living organisms can be facilitated by splashing raindrops. Some may even be facilitated by insects, birds, raccoons, and other mammals.

The small size of protozoans and other micro-organisms led to the assumption of cosmopolitan distribution, a concept known as the Baas Becking Principle, or "everything is everywhere." However, recent studies on distribution and genetic differences have brought this principle into question.

Bryophyte-inhabiting protozoa are sufficiently sensitive to some types of air pollution that they can be used as monitors, but not all are sensitive to the same things, so community structure is likely to change.

Collecting is relatively simple, but quantification is tricky. Testate species can be separated by physical means, but other taxa often require culturing to awaken cysts. Some may be amenable to staining to further clarify identification.

Acknowledgments

Paul Davison has been wonderful in helping me with the methods portion of this chapter, including the suggestion to include it. Edward Mitchell provided me with a large number of papers and photographs. Both of these researchers were invaluable in helping me with areas where I was often not personally familiar with the subject.

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