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Testate Amoebae and Nutrient Cycling with Particular Reference to Soils

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We asked the following question: Is the lack of attention given to testate amoebae, and other protists, in studies of nutrient cycling justified by their relative unimportance or are we ignoring key players in nutrient cycling and other ecological processes? We review various aspects of the ecology of testates relevant to their role in nutrient cycling. These include their food sources, their population sizes and production ecology, the rate of test breakdown (and hence recycling of material from testates to other organisms) and non-feeding interactions with other organisms (e.g., mychorrhizae). Much of the relevant published literature dates from the late 1960s to the early 1980s, presumably due to the interest in production ecology and other aspects of ecosystem ecology at this time. There was a reduction in relevant research during the 1980s and 1990s, but there has recently been signs of renewed interest in this area. In addition to reviewing the past literature we suggest new speculations about the role of the evolution of grasses and the rise of the euglyphid testates—mediated by the silica cycle. Our main conclusion is that we currently do not know enough to answer our question about their potential importance! However, there are hints in these data which suggest that testates may be important and should be targeted by future research. Some of the main questions that should be targeted are outlined.

Keywords biogeochemical cycling, biomineralization, testate amoebae

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'It makes no sense to study evolution or ecosystems, be it in our garden soil or the bottom of the Atlantic Ocean, without recognizing the keystone activities of our microscopic cousins'. Wakeford (2001).

INTRODUCTION

As our epigraph makes clear microorganisms dominate both global biodiversity and many key ecological processes (Wilkinson 2006). Over the past decade there have been significant advances in studying the diversity and ecology of free-living microorganisms—with the widespread application of molecular methods. These studies, however, have mainly focused on prokaryotes and to a lesser extent fungi and viruses, while protists have attracted much less attention (Caron et al. 2009).

One at the few groups of protists that has received attention are aquatic algae—which play a large role in many processes such as global photosynthesis. For examples of the lack of attention the other protists have received; in a 365-page book on the biogeochemistry of the Amazon basin (McClain et al. 2001) protozoa only receive a passing reference on one page and McArthur's (2006) textbook on the evolutionary aspects of microbial ecology almost exclusively confines itself to prokaryotes! This is typical of the limited attention given to protists in many studies of biogeochemical cycling and other areas of microbial ecology, it raises the question; is this lack of attention justified by the relative unimportance of protists or are we ignoring key players in nutrient cycling and other ecological processes?

In this article we focus on one ecological grouping of protists and outline what is currently know about the role of testate amoebae in nutrient cycling and suggest some key targets for research in this area over the next decade. Much of the relevant literature is on testates in soils and mosses, and we largely confine ourselves to these terrestrial habitats—although we make

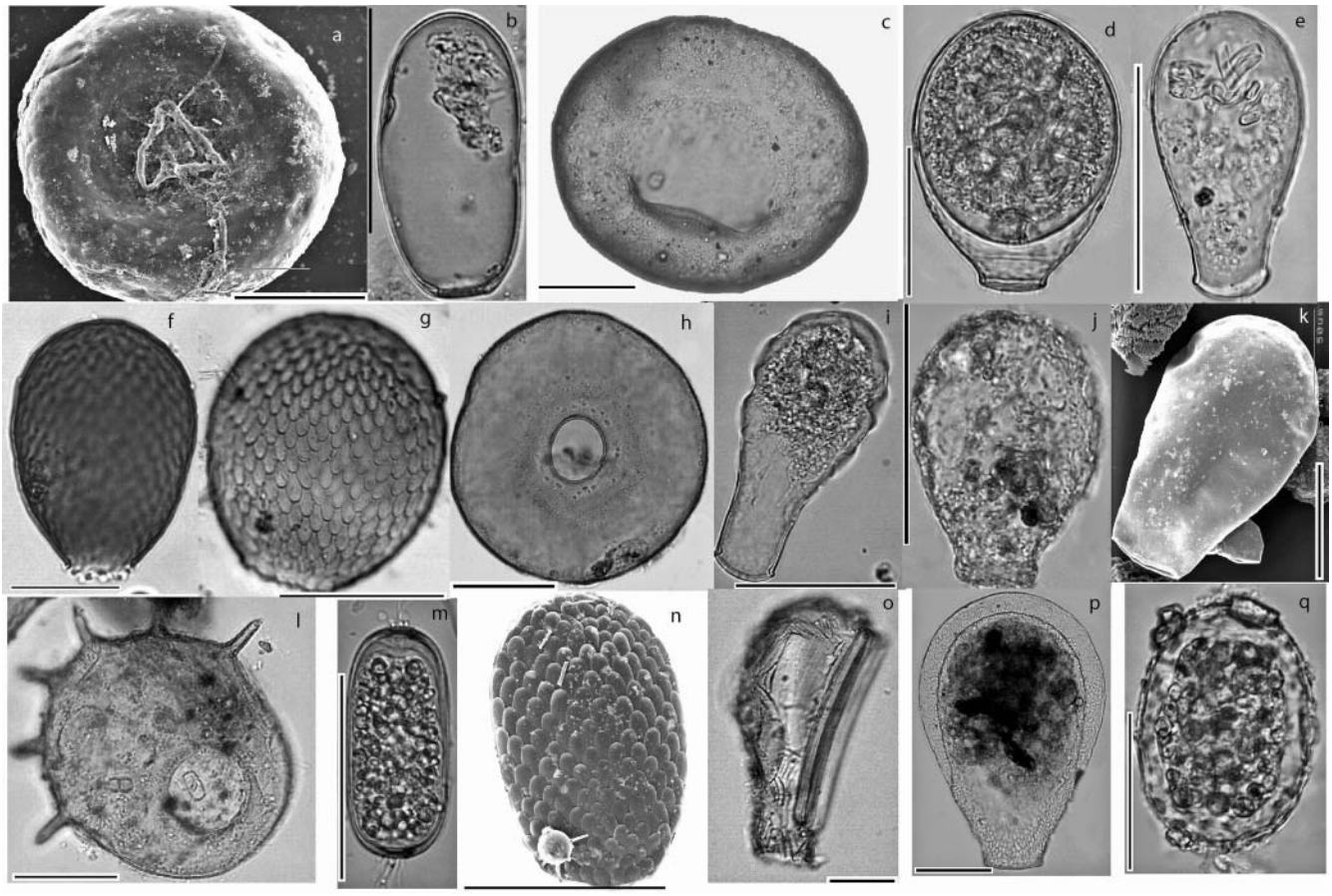

FIG. 1. Examples of the diversity of testate amoebae from soils and mosses: a) *Trigonopyxis arcula,* b) *Hyalosphenia subflava,* c) *Bullinularia indica,* d) *Nebela tincta,* e) *Nebela militaris,* f) *Assulina muscorum,* g) *Assulina seminulum,* h) *Arcella arenaria,* i) *Hyalosphenia elegans,* j) *Physochila (Nebela) griseola,* k) *Hyalosphenia papilio,* l) *Centropyxis aculaeta,* m) *Archerella (Amphitrema) flavum,* n) *Placocista spinosa,* o) *Difflugia bacillifera,* p) *Nebela carinata,* q) *Amphitrema wrightianum.* Scale bars indicate approximately 50 μm except for *A. muscorum:* 20 μm.

some comparisons with data on the role of testates in freshwater systems.

Testate amoebae (also known as testate rhizopods, thecamoebians or arcellaceans) are protozoa in which the single cell is enclosed within a shell, usually referred to as a 'test' (Figs. 1 and 2). These tests are usually composed of either self-secreted material (which can be proteinaceous, calcite or siliceous) or so-called agglutinated tests, which incorporate material from the environment (such as sand grains, diatoms or the scales of smaller siliceous testates which have been consumed as prey). The size range of the tests is some 5–300 μm—making them large by the standards of most microorganisms (Wilkinson 2008).

The testate amoebae form a polyphyletic group traditionally placed in the phylum Rhizopoda (Margulis and Chapman 2009), but now separated in two different Eukaryote supergroups, the Amoebozoa (for the Arcellinida) and the Rhizaria (for the Euglpyhida and other testate amoebae with filose pseudopodia) (Meisterfeld 2002a, 2002b; Adl et al. 2005). However testates seem to form a reasonably uniform ecological grouping, occurring in a range of terrestrial, freshwater and occasionally brackish habitats. Studying them together is an approach similar to the study of "microalgae" (also belonging to at least two eukaryotic supergroups), naked phagotrophic free-living amoeboid protists (also belonging to both Amoebozoa and Rhizaria), plant pathogens such as oomycetes and fungi (belonging to Opistokonta and Stramenopiles), or including lichens in studies of vegetation ecology.

Testates are especially common in habitats with a high organic matter content, such as organic rich soils, peats and mosses (Sandon 1927; Ogden and Hedley 1980), but can also be found in lower numbers in arid habitats, with low levels of organic matter (e.g., Bamforth 2008; Wilkinson and Smith 2006). As such they are often the dominant microorganisms in soils with low pH and high organic matter: such as those with molder or mor humus. These humus types tend to have a lower diversity of many other microbial groups (Sandon 1927; Ponge 2003), so it is possible that testates have a particularly important role in nutrient cycling in such soils. Many, but not all, of the identified morphospecies are cosmopolitan in their distribution (Wilkinson 2001a, Smith and Wilkinson 2007).

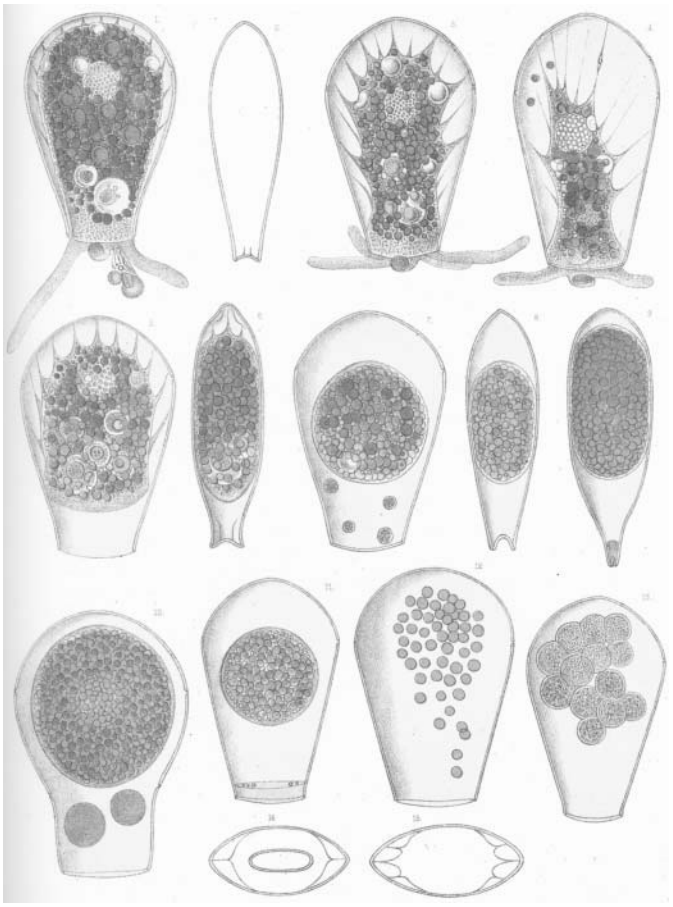

FIG. 2. *Hyalosphinia papilo* lithograph from Leidy (1897) showing 'constituent chlorophyl corpuscles'—that is endosymbiotic algae.

The presence of tests means that testate taxa can be identified by morphology and their populations can be enumerated by direct counting (without having to resort to culturing). Testates therefore are a microbial group whose ecology can be studied by approaches analogous to those used in the study of macroscopic organisms. Their evolutionary history is poorly known with only limited fossil evidence (often tests preserved in amber). The earliest uncontroversial testate fossils come from the Cretaceous (Schmidt et al. 2004); although there are fossils from the Neoproterozoic (late Precambrian), which look very like modern testates—albeit from marine sediments (Porter et al. 2003).

In this article we review what is known about the importance of testate amoebae in nutrient cycling and attempt to outline the key questions for the future. In reviewing past work we have mainly used a chronological structure. There were several significant studies during the 1970s and 1980s, following this there was a relative shortage of work until a resurgence in interest during the last few years. However, before this chronologically structured account, we first briefly review what is known of the food sources of testate amoebae.

Feeding Ecology

In assessing its role in nutrient cycling a crucial aspect of an organism's natural history is the question "from where does it get its supply of energy and nutrients"? The food sources of testate amoebae still require much more research; however, it is clear that they can utilize a range of foods, including bacteria, fungi, algae and other protozoa (Ogden and Hedley 1980). However they can also sometimes feed on quite large organisms—for example puncturing the cells of filamentous algae (Stump 1935) or, in aquatic systems, even catching nematodes (Yeates and Foissner 1995) and planktonic rotifers (Han et al. 2008). More terrestrial testates will also consume rotifers; for example the pharynx 'jaws' (trohpi) of rotifers have been seen in the cell contents of a *Nebela tincta* from terrestrial moss samples from the Swiss Alps—although in this case it is impossible to know if this was a case of predation or scavenging on the part of the testate (unpublished data; D.M. Wilkinson). Gilbert et al. (2000) also observed predation on a rotifer by *Hyalosphenia papilio* and *Nebela tincta*. Interestingly several individuals may simultaneously attack the same prey—sometimes even individuals from two different species (Gilbert et al. 2000, 2003).

Information on food for some reasonably well-studied testate amoebae are given in Table 1. It is sometimes assumed that size mainly drives testate diet (Ogden and Hedley 1980); with smaller taxa feeding on bacteria and other smaller microbes and the larger taxa taking a greater range of food. However, Table 1 suggests this may be an over simplification; for example, the largest taxon included in this table is *Trigonopyxis arcula,* which appears to be a fungal specialist. However, it is not clear if all testate taxa that have been described as fungal specialists actually directly consume hyphae, or if they actually feed on exudates from the hyphae, or bacteria feeding on such exudates (Martin Vohník, pers. comm..). It is also probably a mistake to consider all bacteria *sensu lato* as a single food category—given the enormous diversity found within the prokaryotes.

We also want to emphasize that currently data on the feeding behaviour comes from a very limited number of mainly observational studies; this is an area that would benefit from more work. This is especially the case now molecular techniques mean that work in this area is not just restricted to the difficult task of attempting to see what an amoeba is eating using direct observation with a microscope (although there is still much scope for this traditional approach to the problem). In addition to ingesting food particles some testates contain endosymbiotic algae which may be a source of energy to the protists. The occurrence of these symbionts has long been known—for example Leidy (1879) remarked on the abundance of 'constituent chlorophyl corpuscles' in testate taxa such as *Hyalosphenia papilio* (Fig. 2).

Unfortunately, there are few studies that attempt to quantify the energetic benefits of such symbionts to any testate taxon. However, in one case Schönborn (1965a) was able to show that some of these testate taxa die if they are deprived of light—strongly suggesting these endosymbionts are very

TABLE 1
Food sources of some testate amoebae. B—bacteria, fu- fungi, Cb—cyanobacteria and micro algae, Fl—flagellates, Ci—ciliates, T—testate amoebae, M—metazoan, O—unspecified organic matter, P—plant material

Taxon	Size (μm)	B	Fu	Cb	Fl	Ci	T	M	O	P
Centropyxis aerophila	53–85		*	*						
Corythiom dubium	23–65	*			*					
Euglypha rotundat	22–54	*	*						*	
Hyalosphenia papilio	90–140		*	*		*		*		
Nebela tincta	76–110		*	*	*	*	*	*	*	*
Phryganella acropodia	30–85		*							
Trigonopyxis arcula	90–168		*						*	
Trinema enchelys	32–103	*	*	*			*			
Trinema lineare	18–35	*								

Data on food sources from Gilbert et al. (2000) and Schröter (2001). Data on size from Ogden and Hedley (1980) and Clarke (2003).

important to some testate amoebae. With very limited published studies of these symbiotic relationships, in this article we use the term "symbiont" in its original meaning, which makes no assumption about the cost or benefit of the interaction (c.f. Wilkinson 2001b). Finally, we note that the aquatic Euglyphid testate amoeba genus *Paulinella* is the only known eukaryote outside of the group of plants and related algae to have acquired by primary endosysmbiosis a cyanobacterium (Yoon et al. 2006).

Another obvious approach to elucidating the feeding preferences of testates is to attempt to rear them in captivity on a range of different potential foods. There are few experimental—culture based—studies on the feeding ecology of testates and most of these were conducted by Coûteaux (1985; Coûteaux and Devaux 1983). She tested the effect of the addition of fungi (Coûteaux and Devaux 1983) and also malt extract (Coûteaux 1985) on testate amoebae from soil humus. In the first experiment, of the 31 species present in the mesocosms, only *Phryganella acropodia* responded clearly by increasing its populations. Although not a direct proof of mycophagy this result strongly suggests that this species preferentially feeds on soil fungi. In the second experiment, *Phyganella acropodia* reached a plateau after three weeks and then the population of *Tracheleuglypha dentata* and to a lesser extent *Centropyxis aerophila sphagnicola* also strongly increased, but only after 4 weeks of experiment. Clearly, there is scope for more laboratory-based feeding studies.

Testate amoebae are most abundant relative to other groups of protozoa in raw humus with high organic matter content and accumulation of partly decomposed organic matter. This led Volz (1929, 1951, in Schönborn 1965) and Schönborn (1965) to believe that, unlike flagellates, ciliates and naked amoebae, testate amoebae may not be bacterial feeders but rather detritus feeders. In support of this hypothesis, Schönborn observed lignin-containing particles in food vacuoles of soil testate amoebae and conducted experiments to discover if testate amoebae could grow on sterilised humus (Schönborn 1965). Schönborn first stained living *Euglypha denticulata* individuals with phloroglucinol (which reacts with lignin to produce a red color) and observed abundant lignin-like particles inside food vacuoles. He then cultured testate amoebae on sterile *Fagus* and *Pinus* forest soils. The experiment was started in summer 1963 and observations were carried out in both November 1963 and June 1964. For November in the *Fagus* samples a total of 12 species of testate were observed as living individuals. In the *Pinus* samples 6 species were observed alive; the commonest being *Trinema complanatum*. In June, in the *Fagus* samples a total of 15 species were observed alive (but for 3 taxa there was some uncertainty about the 'live' status), once again *T. complanatum* was the most common—it was also the most common in the June *Pinus* samples.

These results suggest that some soil testate amoebae may be able to feed on humus particles. However, it is notoriously difficult to keep cultures sterile over such a long period and it is therefore very possible that the amoebae were feeding on bacteria or fungi that had contaminated the culture and developed on the humus particles. Thus it is possible testate amoebae may ingest humus particles partly, or only, for the microbes growing on and inside them. Clearly more experiments are needed to determine if testate amoebae and other soil protozoa are able to feed directly on humus particles.

Early Work Up to the 1960s

A key requirement for understanding the role of testate amoebae in nutrient cycling is quantitative data on their occurrence in different habitats. The first quantitative study of soil testate amoebae which we have been able to find was conducted by Volz (1934) who studied the communities of different groups of soil organisms in a *Carpinus-Fraxinus* (hornbeam-ash) forest and a *Pinus—Fagus* (pine-beech) mixed forest on acidic soil. Volz observed a higher density of testate amoebae (20.7×10^3 individuals g dry soil $^{-1}$) in the top centimetre of soil of the mixed hornbeam and ash forest than in the pine-beach mixed forest (3.6×10^3 ind g dry soil $^{-1}$). He also observed a sharp

decline in density with depth in the hornbeam-ash forest (3.4×10^3 ind g dry soil $^{-1}$ between 3 and 4 cm depth).

However, Volz (1934) mentions that Müller had studied soil testate amoebae 50 years previously, so this may not be the very first study on soil testates. Varga (1933, in Schönborn 1965) also reported data on testates from a range of terrestrial habitats, with a clear increase in testate amoeba species richness and density from cultivated fields, to forests. Varga also reported lower densities in summer and maximal densities in late autumn. Following this pioneer work, over 20 years elapsed until the next detailed study on soil testate amoeba was published by Bonnet and Thomas (1955), who noted existence of a specific fauna of testate amoebae in soil which was more similar to that of mosses, including *Sphagnum*, than of aquatic ecosystems.

Reviewing the known facts on soil testate amoebae in 1960, Didier Chardez (1960) noted that each soil type and horizon is characterized by a specific community and that forest soils are especially rich in testate amoebae. Chardez listed a total of 67 testate amoeba taxa that he considered as characteristic soil fauna, plus 8 additional taxa that were relatively frequent in soils. He further noted that the role of testate amoebae on chemical transformations in soil are not well-known, but since they can be very numerous, they are likely to be playing a role in humification processes. Despite all the work done to understand the functioning of soils, 50 years later these observations still stand!

More intensive quantitative work on testate amoebae in soils developed during the 1960s, attempted to quantify testate 'production' in a range of different soil types. For example, Heal (1963) compared the testate amoeba communities in three deciduous woodlands in the UK, a *Corylus-Fraxinus-Betula* coppice with mull humus, an Oak (*Quercus)* coppice with moder-type humus and a Oak high forest, which also had a moder-type humus. Of the three methods he tested, a culture approach, a direct count and soil section (ca. 50 μm thick slices of soil fixed in resin), the direct count method yielded the highest estimates.

Heal observed a much higher density of testate amoebae in the high forest than in the two coppices (for the top centimetre: 73×10^3 ind g dry soil $^{-1}$ in the high *Quercus* forest, 32×10^3 ind g dry soil $^{-1}$ in the *Quercus* coppice, and 13×10^3 ind g dry soil $^{-1}$ in the mixed coppice). He also observed a sharp decline in density with depth. This study suggests that humus with a higher organic matter content contain higher densities of testate amoebae.

Heal (1964) also studied upland habitats and found 50–100×10^6 ind. m^{-2} testates in a valley bog in northern England during the summer. The emphasis on 'production ecology' at this time was probably due to the focus on this and related topics in the "International Biological Programme" during the 1960s and early 1970s—an attempt to move the emphasis of community ecology from species lists to ecosystem models. This work focused on studying primary and secondary production and attempting to quantify the pathways of energy and nutrient cycling within ecosystems (Heal and Perkins 1976; H.G. Smith pers comm.). This interest in ecosystem ecology declined during the 1980s—only to increase again in recent years (see Fig 11.1 in Wilkinson 2006). This decline during the 1980s probably explains why the studies of Coûteaux, Lousier and Schöborn, described in the next section, were not immediately developed by other workers. Indeed, these papers still provide much of the relevant quantitative data for interpreting the potential role of testates in nutrient cycling and are therefore discussed in some detail.

Key Studies from the Late 1960s to the 1980s: Coûteaux, Lousier, Schönborn et al.

Although much of the work of Chardez focused on the ecological preferences of testate amoeba species he also carried out some more quantitative studies of testate populations. For example, in an Oak-Hornbeam (*Quercus-Carpinus)* forest in Belgium, Chardez and Krizelj (1970) recorded the density and biomass of testate amoebae in winter, spring and summer of two consecutive years. During the first year (1967) the samples were taken in February, April and August. The density was highest (1.1×10^3 ind g^{-1}) in August and comparable in February and April (0.8 and 0.9×10^3 ind g^{-1}, respectively). The second year (1968) sampling was done in February, May and December (not in summer). The highest density was recorded in May (2.3×10^3 ind g^{-1}), lower in December (1.4×10^3 ind g^{-1}) and lowest in February (1.1×10^3 ind g^{-1}). These results suggest that numbers can vary quite significantly from one year to the next and that densities are lowest at the end of winter, but may be high in the beginning of winter when the soil is covered by freshly fallen litter. However, although this pattern seems realistic, the numbers seem very low by comparison with the results Coûteaux obtained in her different studies conducted in the 1970 (described below). Methodological differences probably explain this discrepancy: Chardez and Krizelj analysed the top 15 cm of soil, while Coûteaux recorded high numbers in the top 1cm or samples from 4–7 cm.

Stout also studied testate amoebae communities during the early 1960s, in his case in two beechwood soils characterized by different types of soil and humus: a podsolic soil with mor humus, a brown soil with acidic mull, and a brown calcimorph soil with mull, and measured the decomposition rate of these soils by respirometry in the laboratory (Stout 1963). This was the first study combining studies of soil respiration and testate amoebae. Unfortunately, only species lists are given and no quantitative estimates of density or biomass was done.

In what seems to be the first manipulative field experiment on soil testate amoebae, Chardez et al. (1972) assessed the effects of fertilisation on the testate amoeba communities from the soils of a *Fagus* forest on acidic brown soil. The experiment consisted of addition of a high dose of N (150 kg N ha^{-1} as urea), NP (1000 kg ha^{-1} as "potassic basic slag") and NPK (combination of the 2 previous). All fertiliser addition was done in mid summer. The testate amoebae were analysed in the top 4 cm, 3 years after fertilisation. In the control plots the density of testate

amoebae was about 15×10^6 ind m^{-2} and 1.34×10^3 ind g^{-1} and the biomass 10.25 kg ha $^{-1}$. These numbers were reduced by ca. 75% in the N addition treatment. In the PK treatment a reduction of ca. 20% was observed, while in the NPK treatment the numbers more than doubled. The positive effect was attributed to the enhancement of biological activity—presumably increasing bacteria and/or root rhizosphere activity and so providing more food sources for the testates. The calcium added in the NPK slag enhances the degradation of urea and the negative effects observed in the N addition treatment were not observed. This problem was circumvented in later N addition experiments by using ammonium nitrate, which was considered to have less detrimental effects on soil organisms.

A key figure in the production ecology studies of the 1970s was Marie-Madeleine Coûteaux. Using a new method, of direct counts on a weight basis (Coûteaux 1967) she analyzed the testate amoeba communities in the humus layer of an oak forest in Belgium (Coûteaux 1969). Total densities ranged from ca. 5×10^3 ind g dry soil $^{-1}$ to ca. 40×10^3 ind g dry soil $^{-1}$. The lowest values were observed in January and February and high values in April to December, with the highest peaks in November and December. She attributed the low numbers in winter to a lowering of available moisture due to frost. The peaks in early winter are plausibly associated with an input of organic matter from leaf fall.

In a follow-up study Coûteaux (1972) compared the testate amoeba communities of the same oak forest used in her 1969 paper with that of a spruce forest. She analyzed separately the communities from the litter (L) and humus (H) layers. Interestingly, and contrary to expectation, the communities from the spruce and oak litter were very similar and the same was observed for the humus layer; by contrast in both forests the communities differed between the litter and humus layers in terms of relative abundance of different taxa (but not in terms of overall species composition). The density expressed as number of individuals per gram dry weight and the Shannon diversity were both higher in the litter than in the humus, again surprising given that the micro-environmental conditions in the litter are more variable and drought stress potentially more frequent.

These results may be explained by the lower weight per unit volume of the litter horizon when compared to the humus; even relatively low numbers of testate amoebae in leaf litter represent a high density when expressed on a weight basis. However, these results may also represent reality. As Bargett (2005) cautions, the litter layer "is often overlooked or even discarded in soil sampling regimes, but it is perhaps the most biologically active and functionally important zone of the soil profile"—although the relative importance of the litter will vary among different soil/habitat types.

However, one of the only other detailed leaf litter studies found that in a Canadian site—with a very cold winter—testates were very slow in colonizing leaf litter (Lousier 1982). Coûteaux also observed that species building compressed tests and species with an eccentric ventral aperture (plagiostomy), which are presumably better adapted for life in a thin or variable water film, were more abundant in the litter horizon while species with a terminal aperture or hemispheric species were more abundant in the humus. She also observed that in terms of number of individuals, the populations of testate amoebae of spruce and oak forests are dominated by small species (size range 25–45 μm) with a second, smaller peak around 70 μm (Coûteaux 1975c). However, she did not calculate the relative contribution of these size classes to the total biomass.

In a detailed study of temporal patterns (weekly sampling) of three forest soils, Coûteaux (1975b) observed lower densities in winter for two sites in Belgium. By contrast the lowest densities were recorded in July and September in a second *Quercus* forest in France. She concluded that spring and autumn were generally the most favorable seasons for the development of testate amoeba communities. In her study sites although winter precipitation was high the soil was frequently frozen and the water was thus not available to testate amoebae.

Most of these production ecology studies of testate amoebae were conducted in the temperate northern hemisphere. In some of the few detailed studies from the tropics Coûteaux studied both savannahs and forest habitats. In two savannahs in the Ivory Coast (République de Côte d'Ivoire), west Africa, that were burned every year at the end of the dry season, she recorded a much lower density ($0.91–3.85 \times 10^3$ ind m^{-2}), biomass (0.07–0.99 kg ha^{-1}) and productivity of testate amoebae than that observed in Europe (Coûteaux 1976, 1978). The productivity was 9.28 and 12.95 kg ha^{-1} yr^{-1} in bare soil between tussocks and inside tussocks, respectively, for the *Hyparrhenia* savannah, and 3.67 and 51.67 kg ha^{-1} yr^{-1} in bare soil between tussocks and inside tussocks respectively for the *Loudetia* savannah. These values were calculated assuming an average life span of 8 days and that the observed densities are representative for the entire year cycle, 2 unverified assumptions that could greatly affect the results.

In another tropical study, of forest and clear-cut forest soils in French Guyana in South America, Coûteaux (1979) observed a density 10 times higher and a biomass 2.5–7.5 higher (1.06–1.92 kg ha^{-1}) than in the African savannahs, except where the forest was clear cut, the trees removed and the remaining branches burned (0.37 kg ha^{-1}). She also observed that testate amoebae were absent below 3–4 cm depth and attributed this absence to the lack of air (presumably oxygen?) or low organic matter content.

Despite these valuable tropical studies most of Coûteaux's work was carried out in Europe. Key production ecology studies from North America were carried out by Daniel Lousier and provide some of the main data to compare with Coûteaux's. His study sites were in poplar woodland (mainly *Populus tremuloides*) in Albert, Canada. In this system it was estimated that annual production of testate amoebae was 91×10^9 individuals

m^{-2} $year^{-1}$, and testate numbers peaked in the autumn, as with Coûteaux's European work, possibly connected to the input of nutrients from leaf fall (Lousier and Parkinson 1984). However, an obvious confounding factor is soil moisture, which may be higher in autumn than summer.

The standing crop of testates in these woodlands was estimated to be 0.4–1.2 kg ha^{-1} (Lousier 1974), so in line with the data of Coûteaux (1979) from French Guyana. Experiments that increased soil moisture lead to increased testate numbers (Lousier 1974), suggesting a role for moisture as well as nutrients in autumn peaks in testate numbers for habitats, which experience dry summers. Our own work in the European Alps also suggests a role for moisture; in a set of samples collected in late summer/autumn, limestone sites (free draining dry sites) had much lower numbers and diversity of testates than sites on less free draining granite (Wilkinson and Mitchell unpublished data).

In the context of nutrient cycling, as well as population size, the rate at which material from testates is made available to other organisms is clearly important. One mechanism by which this can happen is by predation on testates—Lousier's study was unable to provide any quantitative data on this point (indeed we are not aware of *any* quantitative studies of this topic, although Chardez recorded several anecdotal examples of predation on testate amoebae by other testate amoebae, ciliates, naked amoebae (Chardez 1985), and earthworms (Chardez 1992). However Lousier did quantify the rate at which empty testates were broken down in culture. He found a high rate of loss; with 74–94% of 'platelet' tests lost after 1 week and 42–68% of tests constructed from particles (agglutinated tests) lost over the same time period. The two types of tests exhibited different decay rates (Fig. 3), the agglutinated tests showed a linear rate of loss from the cultures, while the platelet tests showed an exponential decline—once they started to decompose, they fell apart very quickly (Lousier and Parkinson 1981).

This difference between test type is also apparent in the preservation of subfossil tests in peat bogs—where more recent work has shown that there was a clear pattern of decreasing preservation of idiosome tests ('platelet tests' in Lousier's terminology) with depth, but not for agglutinated tests (Mitchell et al. 2008). Lousier and Parkinson (1981) also, rather surprisingly, found no effect of temperature on the rate of test breakdown. As test decomposition is likely to be, at least in part, a chemical/biochemical process an increase in rate with temperature would be expected. However, they only used three relatively low temperatures in their experiments (0, 5, 10°C) and because of the large range of different treatments (and so limited replicates) in their experiments they probably had low statistical power to detect any temperature effects. Lousier's work provides one of the more detailed studies of the production ecology of testate amoebae. Unfortunately the raw data no longer survives (Lousier pers. comm.) ruling out the reanalysis of these important data using modern statistical methods.

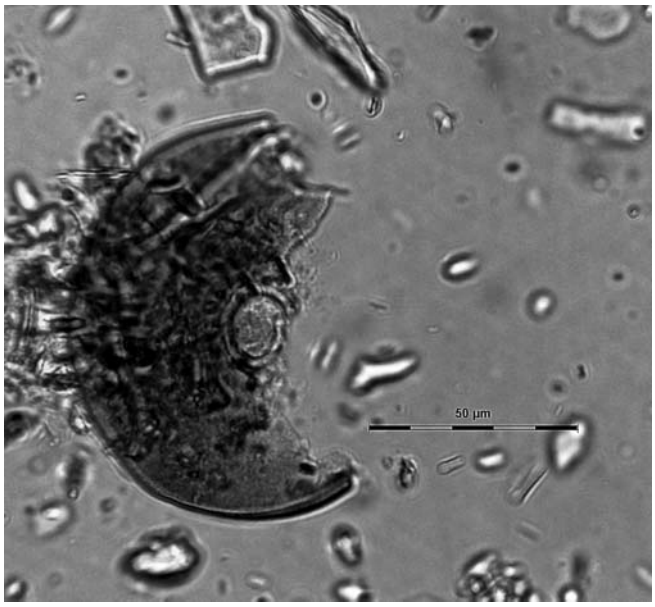

FIG. 3. The partly broken down test of *Arcella sp* from moss samples from the Swiss Alps. It is unusual to see partly broken down tests of many taxa, presumably because they break up very quickly in the soil. However broken *Arcella spp* are reasonably common, presumably because they break down more slowly than many other testates.

Based on these studies Lousier and Parkinson (1984) suggested a number of possible roles for soil testate amoebae in soil nutrient cycling. They suggested that:

1. Testates feed on other soil microbial populations and so potentially modify the composition of the soil microbial communities.
2. They accelerate the turnover of both soil microbial biomass and soil organic matter.

More tentatively they also suggested:

3. Testates may be involved in the degradation of soil plant remains (the importance of this is still unclear because of our limited knowledge of testate feeding habits—as discussed above).
4. They may be important as a prey for other organisms. This point was later expanded on by Bamforth and Lousier (1995) who suggested that the key point is that testates may form an important link in the transfer of nutrients and energy from bacteria to animals—such as various types of "worms."

Complementary to Lousier's work are a series of studies by Schönborn in Europe—started in the early 1970s and running throughout the 1980s. He used "production chambers," in a variety of soil and freshwater habitats in Germany, to study testate population and production ecology. These chambers were small tubes (ranging in length from 0.7–4.0 cm and diameter from 0.3–1.0 cm) with the bottom end covered by a 10 μm membrane (Schönborn 1977, 1986). These chambers were placed in the habitat under study—in the case of soil studies they

were filled with approximately 0.01–0.05 ml of soil (Schönborn, 1982, 1986). This work made use of the fact that testate amoeba shells remain after the death of the organism and thus both the change in densities of living individuals and dead empty shells can be assessed—in a declining population the proportion of empty shells should increase. His first studies were restricted to the Euglyphid testate amoebae. The production was estimated as follows:

$$P = s_t - s_0 + S_t - S_0 + d_t \quad (1)$$

where P = production, s_t = number of empty shells at time t, s_0 = amount of empty shells at time 0, S_t = number of full shells (living amoebae) at time t, S_0 = number of full shells (living amoebae) at time 0, d_t = number of shells that were destroyed (or decomposed) during the course of the experiment. These experiments were conducted using his production chambers that were left in place for a period of 14 days. At each sampling time a fresh soil sample is taken together with the production chambers and the operation is repeated. This way the d_t component of equation 1 can be ignored and the calculation of the production therefore became:

$$P = s_t - s_0 + S_t - S_0 \quad (2)$$

The biomass of individual taxa is estimated based on the biovolume of the amoeba using an ellipsoid volume. Schönborn estimated the biomass per individual of euglyphida species to range between 1.6×10^{-3} μg (*Corythion dubium*) and 10.9×10^{-3} μg (*Trinema galeata*).

The generation time was calculated as:

$$G = \log_2[(s_t + S_t - s_0)/S_0] \quad (3)$$

Mortality was estimated as:

$$M\,[\%] = 100 * (s_t - s_0)/(P + S_0) \quad (4)$$

Or if P is estimated using equation 2:

$$M\,[\%] = 100 * (s_t - s_0)/(s_t - s_0 + S_t) \quad (5)$$

Schönborn (1975) used this approach to estimate the production to biomass ratio (P/B), number of generations per year and death rate for the four dominant species. The P/B ratio of several forest soils ranged from ca. 60 to ca. 175. The shortest generation times ranged from 1.6 days for *Trinema enchelys* in the beech forest and 3.5 days for *T. complanatum* in the beech forest and *T. enchelys* in the spruce forest. The death rate of euglyphid testate amoebae was quite high. Over a 14 days period between 80% and 90% of individuals died. Thus only about 15% of individuals survive a 14-day period.

It is unclear how many of these represent the last generation produced before the experimental period or if they were older. It is therefore difficult to calculate an average life expectancy for testate amoebae. If all individuals were from the last generation before the start of the experiment then the calculated average life expectancy would be only 2–3 days, which seems too short. Therefore a fraction of the population most likely survived over a period of several generations. This interpretation agrees with the 6.1–10.8 days life expectancy estimated by Lousier (1974) in a Canadian aspen forest.

Overall this approach allowed Schönborn to determine that the production of testate amoebae was variable over time and dependant on the moisture content of the soil. The years 1973–1974 during which this study was done were relatively dry. Interestingly, *Trinema enchelys* had no measurable production for 150 days in the beech forest and 196 days in the spruce forest—results which may have some relevance to anthropogenic climate change which may increase the frequency of exceptionally hot dry summers such as the summer of 2003 in Europe (Schär et al. 2004).

Schönborn (1978) conducted further studies on production, including estimates of production expressed as biomass C (assuming organic C to account for 5% of fresh biomass). He also studied the ingestion of organic matter (particle size and duration of ingestion), fungal spores and bacteria by testate amoebae. On average each individual ingested a total volume of ca. 200 μm^3 of particles for 12 hours. On average one fungal spore was also observed in the cytoplasm as well as many bacteria (which were not quantified). The ingested particles account for ca. 9% of the total estimated testate amoebae carbon biomass production.

Schönborn's key results from his production chamber experiments in soils are summarized in Table 2. They show declining numbers and annual production of testates in the H layer of soils with increasing organic matter and decreasing pH; but substantially greater half lives for empty tests in more organic soils. In addition the litter layer of spruce *Picea abies* woodland contained high numbers of testates—dominated by *Corythion dubium* (Schönborn 1986). These data suggest higher population sizes and faster cycling of nutrients through testate populations in less acid soils. This is interesting as testate amoebae are conventionally described as characteristic microorganisms of acidic sites with high organic matter content (e.g., Sandon 1927; Smith et al. 2008); clearly there is a need to repeat and extend such studies!

Schönborn (1983) also studied the relationships between production, mortality and abundance (PMA) of testate amoebae from a mull humus soil. He recognised four PMA types:

A) Optimal type: cell division stops at high density and eventually many cells die, hence there is high abundance, production is low and the longevity of individuals is high, example: *Trinema complanatum*;
B) Productive type: production, mortality and abundance are high, example: *Trinema enchelys;*
C) Retardative type: production and mortality are relatively high but abundance is low, example. *Euglypha ciliate*;

TABLE 2
Summary of Schönborn's 'production chamber' measurements for three German soil types (data from Schönborn 1986)

Soil type	Mean testate abundance in 0.01 mL soil.	Annual testate production; individuals 0.01 mL^{-1} yr^{-1}	Half life of empty shells, days
Mull (H layer) deciduous woodland	60.8 ± 33.4	1881	6
Moder (H layer) deciduous woodland	30.0 ± 16.5	450	28
Raw humus spruce forest (H layer)	2.1 ± 2.1	254	48
Raw humus spruce forest (L layer)	142.8 ± 39.4	3620	—

D) Sporadic type: production and abundance are low and the populations die out rapidly, example: *Trigonopyxis microstoma*.

Intermediate types were described between types B and C (example: *Centropyxis plagiostoma*) and between types C and D (example: *Corythion dubium*)

Mortality was the main determining factor for these PMA types and especially the likelihood of a species surviving after a division to undergo another division. In species such as *Euglypha ciliata* in most cases one of the daughter cells dies shortly after cell division so that populations do not increase easily in size. The pattern is quite different for *Trinema enchelys* for which each daughter cell may undergo several divisions leading to larger populations. The ratio of full to empty shells was therefore considered a good indicator for changes in testate amoeba communities in soils. From type A to C mortality increased. Types C and D showed early mortality, i.e., density-independent mortality soon after cell division.

From the preceding discussion it is apparent that the occurrence of empty tests in soils was crucial to Schönborn's approach to quantifying testate production. In general the half-life of empty tests in Schönborn's studies was longer than in the work by Lousier. Both workers used different experimental approaches to measuring rates of test decomposition; Schönborn (1977 and 1986) using his 'production chambers', while Lousier and Parkinson (1981) carried out their experiments in laboratory cultures.

Therefore, it is difficult to know if the different results derive from different experimental methods and/or different testate habitats—it is also hard to know how realistic these rates are of what happens under field conditions. Presumably the results of both Schönborn and Lousier are best considered as conservative estimates—field conditions will have a wider range of processes (such as the presence of larger invertebrates and a wider range in abiotic variables such as temperature or moisture), which may speed up the breakdown of empty tests and so increase the rate of nutrient cycling from tests. However, studies by Coûteaux (Coûteaux and Ogden 1988; Coûteaux 1992) failed to find any significant test breakdown in culture—even after 150 days! It is clear that the rate at which testates break down is a priority for future research to attempt to reconcile these very different results, as it is key to understanding the role of testates in nutrient cycling.

Unfortunately, the different methods of estimating testate production used by Lousier and Schönborn make it hard to directly compare their results—although both produce estimates of testate amoebae biomass in soils of the same order of magnitude. Schönborn (1977) also published more limited, estimates of testate production from freshwater habitats in German River Saale. He found a mean testate production of 79×10^3 individuals m^{-2} day^{-1} as a biomass this equated to 3.0 mg m^{-2} day^{-1}. However, as with Lousier's (1974) estimates, the biomass of the different testate taxa was calculated using a number of simplifying assumptions—we know of no direct measurements of the masses of individual testate amoebae.

To put the role of soil testates into a wider context Schöborn (1992) compared his testate data from a German beech *Fagus sylvatica* wood with data on the arthropods and 'worms' (Nematoda, "Rotatoria" = Rotifera, Enchytraeidea, Lumbricidae) and concluded that testate production was similar to that of these groups—and exceeded them in 'raw humus'. Warner (1987) presented estimates of testate abundance from *Sphagnum* peatlands in Canada. He found densities of shells to range between 8.62–46.7×10^3 individuals g dry weight $^{-1}$ and between 1.47×10^3–32×10^3 per cm^3—similar to more recent studies from the Swiss Jura (Laggoun-Défage et al. 2008). In other peatland studies Mitchell et al. (2000) found a mean of 90 individual testates on 2-cm long strands of *Sphagum magellanicum* in a Swiss bog. Unfortunately because of the different units and approach used it is very difficult to compare many of these results with each other or with the earlier work by Heal (1964) for British upland peatlands.

Post 2000; Recent Work on C and N Cycles

Following the work in the 1970s and 1980s studies relevant to the role of testates in nutrient cycling are rare during the 1990s until the PhD work of Dagmar Schröter (Schröter 2001; Schröter et al. 2003). Schröter studied the decomposer systems of four European coniferous forests (mainly *Picea abies*) on a latitudinal transect from NE France to northern Sweden. Schröder et al. (2003) estimated that testate amoebae represented on average 68.5% of the total biomass of 'animals' (no other group

of protozoa was studied and the testate amoebae were treated as animals), and that the contribution of testate amoebae to the carbon and nitrogen cycles was, respectively, 79.9% and 96.5% of that of all 'animals.'

For comparison, in a study of five European *Sphagnum*-dominated peatlands testate amoebae represented on average 59% of the biomass of animals and heterotrophic protists and 17% of the total microbial biomass (Mitchell et al. 2003). This is consistent with the suggestion by one of us (Wilkinson 1998, 2006) that textbooks often overestimate the importance of true animals in decomposition and 'to a first approximation the animals are of no importance to the functioning of the system.' In the study of Schröder et al. (2003) the species richness of testate species found ranged from 34 to 40 species, apparently showing a classic decline with increasing latitude. Such a pattern is common in macroorganisms but has only been convincingly described in a small number of microbial groups (Sherratt and Wilkinson 2009). However in Schröter's study there is a confounding, non-climatic, variable—with nitrogen deposition from atmospheric pollution also declining with increasing latitude. Indeed a principle conclusion of this work was the importance of this nitrogen pollution and that at the more northern (low nitrogen) sites mineralization rate was low and the decomposer community was dominated by fungi. Further south (higher N) mineralization rates were higher and bacteria played a prominent role. The importance of testate amoebae was thought to be in releasing nitrogen immobilized by bacteria (Schröter et al. 2003).

There may, however, be a closer link between fungi, testates and nutrient cycling than was assumed by Schröter et al. (2003). Recent work by Vohník et al. (2008) has suggested a role for testates in nutrient cycling in nutrient poor soils via mycorrhizal fungi. In the Ericaceae, plants form ericoid mycorrhizae in which the fungal partner aids the plant in the uptake of nutrients—especially nitrogen compounds (Aerts 2002). Vohník et al. (2008) examined soils from under three species of *Rhododendron* (shrubs in the Ericaceae) at a range of central European sites and showed intimate associations between the hyphae of the ericoid mycorrhizae and testate amoebae shells.

For example, in soils associated with all three of the *Rhododendron* species studied they found that over 40% of the shells of the testate genus *Trigonopyxis* were colonized by fungal mycelium. As these are large testates (Table 1) they are presumably a good source of nutrients for the plants to access via their mutualistic fungal associates (it was not clear if the testates were dead before fungal colonization). *Trigonopyxis* appears to be characteristically associated with *Rhododendron*. In a study in England of woodlands containing the introduced *R. ponticum* Sutton and Wilkinson (2007) found that *Trigonopyxis* was reliably associated with the shrub, but largely absent from soils from parts of the same woodlands which did not support *Rhododendron.* Ongoing, unpublished, work by Vohník is also finding saprophytic fungi colonizing testate shells (see Fig. 1 in Wilkinson 2008).

In their review of protozoa in tropical leaf litters Bamforth and Lousier (1995) point out that one of the key processes of nutrient cycling in many tropical forests is the 'rapid uptake [of nutrients] through an elaborate root system and mycorrhizae penetrating the rapidly decomposing litter on the floor of rainforests'. In this context it would be worth looking for a similar mechanism as that described by Vohník et al. (2008)—with testates concentrating nutrients from the leaf litters and acting as nutrient hot spots that can be colonized by mycorrhizae and so speeding the return of nutrients to the forests plants.

Post 2000: The Silica Cycle

The silica cycle does not get the same prominence as the carbon or nitrogen cycle in most textbooks but is crucial to the working of the global system. On geological time scales silicate weathering is the main sink for atmospheric carbon dioxide (so linking to the C cycle) and on shorter time scales leakage of silica from soils to aquatic systems is important for diatom primary production—another link to the C cycle (and oxygen cycle) as diatom remains sink to become preserved in ocean sediments (Berner 2004; Street-Perrott 2008).

Testate amoebae with silica rich shells may form an important part of the silica cycle in some soils, although there are very few studies relevant to this question. Recently, Aoki et al. (2007) cultured testates from soils in a pine-oak forest on Nagoya University campus in Japan. They studied two taxa *Euglypha rotunda* and *Trinema enchelys* (Table 1)—two very common taxa found from the tropics to the poles and possessing silica rich tests. They showed that, although the amount of silica in testate shells in forest soils at any one time was relatively small (0.45–1.57 kg SiO_2 ha^{-1}), the rapid turnover of testates—suggested by the majority of studies of test decomposition reviewed here—gave them a much greater annual importance (10–277 kg SiO_2 ha^{-1} yr^{-1}).

These annual figures are approximately equal to the amount of silica entering the soil from plants and leaf litter; as land plants (especially grasses) play a key role in the biomineralization of silica (Raven and Giordano 2009) this is an especially striking result! Aoki et al. (2007) suggest that siliceous testates may be particularly important in soil silica cycling because the small silica-rich testate scales easily dissolve in soils, increasing silica mineralization. As one of us has previously pointed out: 'Given the global significance of the silica cycle in soils—such as its link to marine diatoms—the suggestion that a group of protists unknown to most ecologists may be significant players in the soil silica system, with an importance matching that of plants, illustrates how little we know about soil processes of potentially global importance' (Wilkinson 2008).

A consideration of testates in the context of the silica cycle also suggests interesting geological speculations. The earliest testate fossils with unambiguous siliceous idiosomes come from rocks of Eocene date (Schmidt et al. 2004). This makes sense in the context of the availability of silica in soils. The earliest grass fossils (mainly pollen grains) are around 65–90 million years

old, while the earliest grassland soils, associated with grass-dominated habitats are approximately 48 million years old, that is Eocene in date (Retallack 2001). The relevance of grasses is that their silica rich phytoliths weather more rapidly than abiological mineral silica (Raven and Giordano 2009).

The limited available molecular phylogenies for the origin of euglyphids (testates with silica rich shells) suggests a date of ca. 200 million years ago for the divergence between *Euglypha rotunda* and *Paulinella chromatophora*, the closest relative taxon in that study (Berney and Pawlowski 2006). The terrestrial Euglyphida (including *Euglypha, Assulina, Tracheleuglypha, Trinema, Corythion* and related taxa) are more derived than the common ancestor of *Euglypha* and *Paulinella* (Lara et al. 2007) and must therefore have appeared significantly later, possibly around 100 million years ago. Such a date would match that of the origin of grasses.

Testates with siliceous idiosomes may, therefore, have quickly become important in the cycling of silica in soils following the evolution of grasses. Indeed the expansion of grasses may have been a key trigger for the evolution of these testate taxa. This could have global implications, as it has been suggested that grasses increased the flux of silica to the oceans with implications for the radiation of marine diatoms—key players in the global carbon cycle (Falkowski et al. 2004; Raven and Giordano 2009). Testates with siliceous idiosomes (such as *Euglypha* spp) could potentially have increased the rate of silica mineralization in soils with direct implications for nutrients availability to diatoms.

So What Do We Currently Know?

Here, we briefly summarize the above discussion, highlighting what is known and some of the main gaps in our knowledge. We organize this section under the following subheadings: food, population size/production, rate of test breakdown and non-feeding interactions with other organisms.

Feeding ecology. What an organism feeds on is obviously a crucial question in assessing its potential role in nutrient cycling. It is clear that testates as a group consume a wide range of food, including bacteria, other protist and some small metazoans. It is also apparent, at least in a small number of well studied cases (e.g., *Phryganella acropodia*), that some testates specialize in certain foods types—such as fungi. However there is still much we don't know about the feeding behaviour of testates; for example it is still unclear if any species primarily feeds on humus particles (rather than ingesting humus to consume associated bacteria) and far more studies are needed of the role of endosymbiotic algae.

Population size/production. To assess the role of any group in nutrient cycling it is obviously necessary to have data on their numbers—or biomass. In general organisms with high biomass are more likely to play a significant role in ecosystem function—the "mass ratio hypothesis" (Grime 1998; Wilkinson 2006). However, note the comments on the rate of turnover in the next section. Because different authors have estimated testate numbers in different ways it very difficult to compile summary statistics on their abundance in soils. However several authors (e.g., Chardez and Krizelj 1970; Coûteaux 1969; Warner 1987; Laggoun-Défarge et al. 2008) have presented data for number of individual testates per gram of soil (usually, but not always, dry weight) and typical figures seem to be in the thousands to tens of thousands of individuals per gram.

Unfortunately, it is difficult to compare these figures with data collected in other ways—such as individuals per m^2 or individuals per ml of soil. A further problem is that in the context of the mass ratio hypothesis—that the extent to which a species affects ecosystem function shows a strong positive correlation with its biomass (Wilkinson 2006)—our methods of converting testate numbers to biomass are extremely approximate. One thing that the majority of relevant studies appear to agree on is that testates make up a particular high proportion of microbial biomass in organic rich soils, suggesting they may play a particularly important role in such soils.

Another point of agreement is that testate numbers tend to be greatest in litter and humus layers, declining as one goes down a soil profile. Several studies from temperate areas suggest a peak in testates in autumn; however, it is not clear if this is due to increased nutrients from leaf fall, and/or increased moisture or other factors. Clearly there is a need for new work, building on the classic studies of Coûteaux, Lousier, and Schönborn if we are to move beyond the point made by Chardez (1960) 50 years ago, that the role of testate amoebae on chemical transformations in soils are not well known, but that since they can be very numerous they are likely to be playing a role in humification and other processes.

Rate of breakdown of tests in soils. It is clearly important to document the rate at which testates break down in the soil. For example the work of Aoki et al. (2007) illustrates this point by showing that although the amount of silica in testate shells in their forest soils at any one time was relatively small, the rapid turnover of testates resulted in them having a much greater annual importance than suggested by their biomass in the soil at any one time. Given the important of the rate of breakdown of empty tests the very different results coming from the classic studies of the 1970s and 1980s are a cause for concern and we clearly need further work in this area. However, it does appear that idiosome tests break down more quickly than agglutinated tests.

Non-feeding interactions with other organisms. Although we know something about the organisms consumed by testate amoebae we have no good quantitative data on what preys upon them—the state of the art is currently a small number of anecdotal papers describing other protists and 'worms' eating testates (Chardez 1985, 1992). In addition, the recent work by Vohník et al. (2008) has suggested a role for testates in nutrient cycling in nutrient poor soils via interactions with mycorrhizal fungi. This opens up whole new areas of study. A wide range of other interactions may be important, for example in this article we have argued that interactions with silica rich grasses may have

been crucial in the evolution of testates with silica rich tests (the euglyphids). Studies of interactions between testates and other organisms—and the role of these interactions in nutrient cycling—have only just started and provide a rich area for future research.

CONCLUSION

At the start of this article we asked the question: is this lack of attention given to testates and other protists in studies of nutrient cycling justified by their relative unimportance or are we ignoring key players in nutrient cycling and other ecological processes? The main conclusion from our attempt to summaries what is currently known about testate amoebae and nutrient cycling is that we currently do not know enough to answer this question! However, there are hints in these data that suggest that testates may be important and should be targeted by future research. For example as Chardez (1960) pointed out 50 years ago, their biomass—especially in more organic rich soils—suggests they are likely to be playing a role.

More recent ideas on the interactions between testates and some mycorrhizea (Vohník et al. 2008) or their role in the silica cycle (Aoki et al. 2007; this paper) highlight the extent of our ignorance and the importance of future research in this area. This is particularly important given the uncertainties about the effects of climate change on soil processes (Bardgett 2005). When—as is currently the cases—we are unable to disentangle the relative effects of nutrient inputs and soil moisture on autumnal temperate zone peaks in soil testate numbers, then we are unlikely to be able to make sensible guesses about the effects of future climate change on the ecological functions of testate amoebae in soils and other habitats.

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