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Field and Laboratory Studies of Encysted and Trophic Stages of Naked Amoebae: Including a Perspective on Population Life Cycle Dynamics

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Summary. Naked amoebae are among the most abundant soil protists, especially in highly productive soils. Their capacity to produce cysts during unfavorable growth periods, especially drying, enhances their survivability as resting stages and promotes dispersal by wind and air currents. However, the dynamics of their cycles of encystment and active growth are poorly documented. Using a recently developed culture observation method, including a dried preparation stage to detect encysted amoebae, data are presented on the ratios of active and encysted stages of naked amoebae based on field samples from diverse terrestrial sites differing in plant cover and moisture content during spring and summer months 2008 at a location in northeastern U.S.A. Percentage of encysted amoebae varied between 32% and 100% depending on locale and moisture content. Carbon content of the cysts (estimated from recently excysted individuals) relative to trophic stages varied between 22% and 100% at these same locales. Laboratory experimental studies of winter soil samples, that were cultured at 25°C to promote amoeba community growth, indicated that a dynamic relationship exists between active and encysted stages during proliferation with varying ratios depending on the moisture content and qualities of the soil at the collection site, thus suggesting a revised model as presented here of the encystment-excystment cycle for populations during a growth succession.

Key words: Eukaryotic microbial communities, microbial ecology, microbial survival strategies, protistan community succession.

INTRODUCTION

A substantial amount of biogeographic research has been done on the abundance and diversity of terrestrial naked amoebae at diverse locales spanning deserts to moist tropical regions (e.g. Anderson 2000, 2006b; Bass and Bischoff 2001; Bamforth 2007; Bamforth *et al*. 2005; Clairholm 1985; Darby *et al*. 2006; Robinson *et al*. 2002; Zaragoza *et al*. 2005). More recently, research on their ecology has gained increasing attention as evidence accumulates of their substantial abundance (Anderson 2000, 2002; Brown and Smirnov 2004; Esteban *et al*. 2006; Ning and Shen, 1998) and potential carbon content relative to other protists (Anderson 2008). However, one of their most significant attributes, shared with other terrestrial protists, is the capacity to encyst, often remaining viable for many months to years (e.g. Biddick, Rogers and Brown 1984, Pens and Rott 2008) with rapid excystment, sometimes within a few hours during favorable periods for growth (e.g. Page 1988, p. 24), or within 24 hours (e.g. Stratford and Griffiths 1978); thus favoring their survival and adaptive success

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in highly diverse environments. The proportion of encysted naked amoebae has been investigated relative to soil moisture in field studies (e.g. Anderson 2000) and in microcosm studies examining environmental variables in relation to the feeding rate of the amoebae on bacteria. For example, Bryant *et al*. (1982) subjected amoebae in soil laboratory microcosm cultures to cycles of drying and moistening over a period of 83 days. They found that although the amoebae populations were entirely encysted during drying, the total viable amoebae was unaffected by the repeated drying. They concluded that encystment was an efficient survival mechanism for the soil amoebae. Furthermore, de Moraes and Alfieri (2008) examined the growth response and encystment of *Acanthamoeba castellanii* in laboratory culture when fed with seven different bacterial species. While there was some difference in growth response, a major finding was that the presence of suitable prey bacteria delayed the onset of encystment and substantially improved the percent of viable cysts recovered compared to control preparations without the bacteria. More substantial research on encystment has been done with other protists, especially ciliates, including experimental studies of the dynamics of active and encysted stages using quantitative modeling (Elkelund *et al*. 2002). However, there has been less research attention to the dynamics of encystment and excystment in naked amoebae, especially in relation to varying environmental conditions using freshly collected soil from diverse habitats. This may be attributed in part to prior difficulties in accurately assessing the number of encysted individuals relative to the total naked amoebae at a sample site. The use of HCl or elevated temperatures to kill the active stages, while presumably preserving the viability of cysts, has not proven to be reliable (e.g. Pussard and Delay 1985). More recently, a newer method of assessing naked amoeba abundances based on a culture observation method that includes a rapid drying step to kill trophonts while preserving viable cysts has been used to more reliably estimate the proportion of cysts to total viable amoebae (e.g. Anderson 2000).

The purpose of this research was to investigate the proportion of encysted naked amoebae relative to total, including carbon content of each, at three sites varying in plant cover and moisture content (pine stand, deciduous forest and *Phragmites* grass marsh margin) at a northeastern site in New York State. In addition, experimental studies were done to examine the changes in the proportion of encysted and active (trophont) stages using laboratory microcosms with soil samples taken in December and incubated at 25°C to simulate favorable growth conditions that occur during a spring succession. The succession of changes in the proportion of encysted to total naked amoebae was monitored in soil samples from the *Phragmites* moist site and drier pine stand site to establish the dynamics of growth and encystment of the naked amoebae in these two diverse locales.

MATERIALS AND METHODS

Field studies

Freshly collected soil samples (March to August 2008) were obtained from three terrestrial sites at the Torrey Cliff Reserve of Lamont-Doherty Earth Observatory, NY (white pine stand, maple/oak dominated deciduous forest and a *Phragmites* grass dominated wet marsh margin). Four sample dates were used for the pine and deciduous forest sites and two sample dates were used for the *Phragmites* site (see Table 1) depending on accessibility and available exposed, moist soil surface. Three samples from each site, thoroughly mixed and combined, were immediately returned to the laboratory where they were processed to determine the density (no./g soil dry weight) of total and encysted naked amoebae using a Culture Observation Method (COM) with a modified drying step to kill active stages and preserve the cysts for a separate analysis of their density (Anderson 2000). Only viable cysts can be detected by this procedure and it is not certain what proportion of viable cysts is capable of excysting during the culture method. However, given that we sometimes observe 100% of the dried cyst preparation, relative to the undried cultured preparation, excyst and grow, this suggests that the method is not seriously underestimating the number of cultivatable encysted forms. The COM for total amoebae is as follows. An aliquot of weighed, freshly collected soil is suspended in a known volume of micropore-filtered pond water (MFPW) and thoroughly mixed. An aliquot of the suspension is removed and further diluted with MFPW to approximately 1/50 to 1/100 of the original suspension. Sterile plastic 24-well culture dishes (PCD) are used. Each well, containing a small aliquot of malt/yeast agar as a nutrient source to support food bacteria and 2 ml of MFPW per well, is inoculated with 10 µl of the diluted soil sample suspension. After 12 to 14 days (25°C), each well of the culture dish is examined using a Nikon Diaphot inverted compound microscope (40 × phase contrast objective) and each individual naked amoeba morphotype that grew out of the soil aliquot is tallied in each well. This provides evidence of what morphotypes were present in the 10-µl aliquot deposited in the well. The total number of each morphotype in the 24 wells is summed and converted to number per ml of original soil suspension. Based on these data, the number per total ml of the original soil suspension is expressed as number per g soil (dry weight) used to make the soil suspension. Total densities were obtained by summing the densities of each of the morphotypes tallied in the 24-well dish.

The densities of encysted amoebae were determined by preparing a separate PCD where the 10 µl aliquot is deposited in each dry well before adding the 2 ml of MFPW and rapidly dried under forced air at room temperature (typically 20–30 min.). Thus, all ac-

tive stages are killed by desiccation leaving the viable cysts in the dried aliquot. An aliquot of malt/yeast agar and 2 ml of MFPW is added to each well of the PCD. After 12 to 14 days of incubation (25°C) the density of each morphotype of naked amoebae is determined as explained above for the standard COM method. The carbon content of the active amoebae and those that grew out from the dried cysts was estimated using the regression equation of Anderson (2006a) that relates total cell carbon to cell length of the locomotive amoeba. The temperature of the soil at the sampling site was measured with a thermometer inserted directly into the upper 5 cm of the soil at the time of sampling. Moisture content was determined by change in weight after drying at 109°C overnight. Organic content was assayed by change in weight of the dry soil sample after combustion at 375°C to constant weight.

Laboratory studies

To examine the dynamics of the changes in encysted and active naked amoebae, winter samples from the white pine stand and the *Phragmites* marsh site were placed in milder temperatures simulating spring growth conditions. Samples of soil from each site were collected in December 2008, when the soil community was most likely to be entering winter dormancy. A sample from each site at ambient moisture content was placed in a covered Pyrex 9-cm diameter culture dish and incubated in a non-illuminated temperature controlled chamber at 25°C to mimic the warming temperatures of spring. The rationale was to induce proliferation from the resting state and assess the changes in encysted and active forms of naked amoebae during the ensuing succession. Three samples, mixed and combined into one, were taken from each culture dish at the initial day of sampling and at subsequent five and 10 days of incubation. The densities of encysted and total amoebae for the combined sample were estimated using the modified COM as described above. The relative proportion of encysted and total amoebae was tabulated and plotted graphically as a function of time to display the dynamics of the changes in densities of encysted and active forms that occurred as the populations of naked amoebae proliferated in the laboratory-induced succession.

RESULTS

For the field studies (Table 1) the moisture content of the soil sample from the pine site was lowest overall with a mean of 20%, followed by increasing amounts at the forest site 33% and *Phragmites* site 48%. Overall, the densities of total amoebae were highest at the *Phragmites* site and least at the pine site. The percentage of cysts relative to total amoebae ranged from 81% to 100% in the soil samples from the pine site, 40% to 93% in samples from the forest site, and 32% and 36% respectively in the two samples from the *Phragmites* site. These data indicate that a substantial number of the total amoebae at these sampling sites were in an encysted stage, especially for the drier sites. Moreover, the estimated percent of carbon in the emergent encysted amoebae relative to the total amoebae varied substantially from 43% to 100% in the samples from the pine site, 11% to 100% for the forest site, and 22% and 68% respectively for the two samples from the *Phragmites* site. Because the carbon content depends on the size of the amoebae, the percentage of carbon for the encysted forms relative to the total is not exactly parallel with the proportion of the encysted forms based on densities. In some cases, the emergent encysted amoebae were larger overall than the total active amoebae, thus accounting for a higher carbon content.

Results of the laboratory studies are presented in Figs 1, 2 and Table 2. The pine stand sample (ambient 18% moisture and 6% organic content) overall had less initial total densities of naked amoebae and proliferated to

Table 1. Densities (no./g) and carbon (µg/g) of total enumerated amoebae and encysted stages in samples from the three field sites.

Date	Location	Temp. (°C)	Moisture (%)	Densities			Carbon content		
				cysts	total	% cysts	cysts	total	% in cysts
4/16	Forest	11	36	600	1,200	50	0.12	0.14	86
5/3	Forest	15	31	1,300	1,400	93	0.37	0.36	100
7/17	Forest	23	30	555	1,290	43	0.06	0.17	35
8/8	Forest	20	33	454	1,123	40	0.08	0.75	11
3/20	Pine stand	6	25	2,000	2,000	100	0.43	0.46	94
4/26	Pine stand	13	13	500	300[a]	100	0.05	0.10	100
5/30	Pine stand	16	27	1,300	1,600	81	0.25	0.71	35
8/11	Pine stand	23	13	800	858	93	0.27	0.63	43
4/19	Marsh	15	45	963	2,700	36	0.43	0.63	68
6/17	Marsh	22	50	875	2,710	32	0.38	1.70	22

[a] Note, when densities of amoebae are low and most are encysted, in some cases the number of enumerated cysts exceeds the number of enumerated active forms due to small pipetting errors, etc.

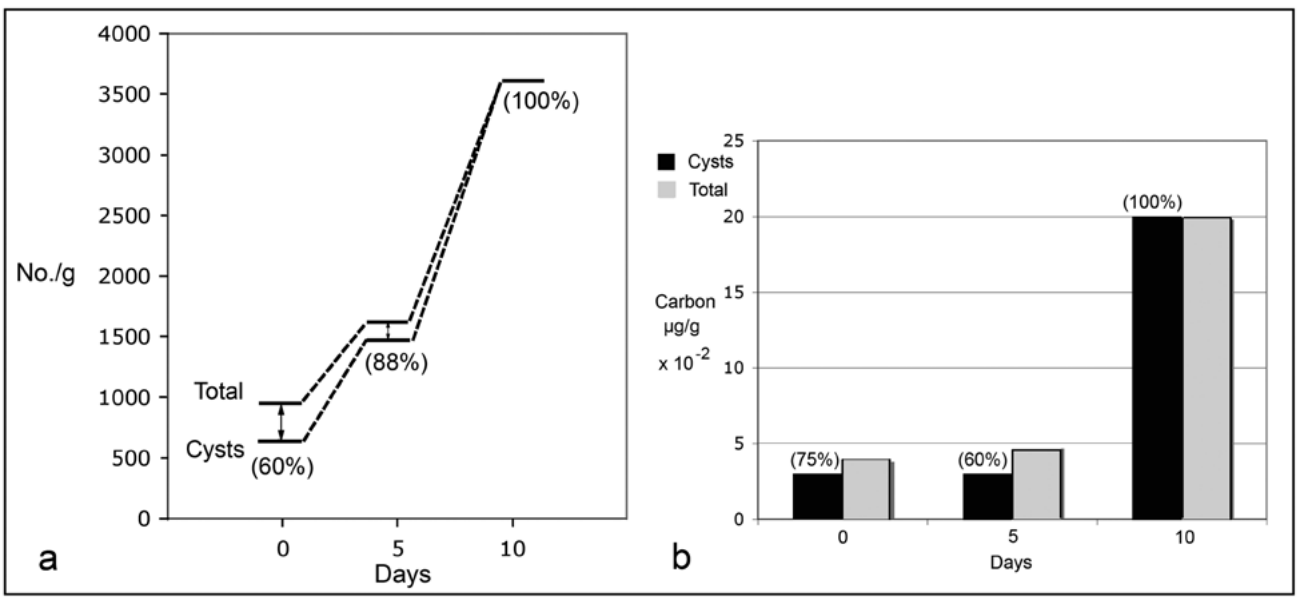

Fig. 1a, **b.** Results of the culture experiment using Pine stand soil samples. **a** – comparative plot of the densities of total amoebae (upper graph) and cysts (lower graph) including (percent encysted) at 0, 5 and 10 days; **b** – histograms of the carbon content in total amoebae (grey) and cysts (opaque) including (percent carbon in cysts) during the three sampling dates. Data are in Table 2, pine entries.

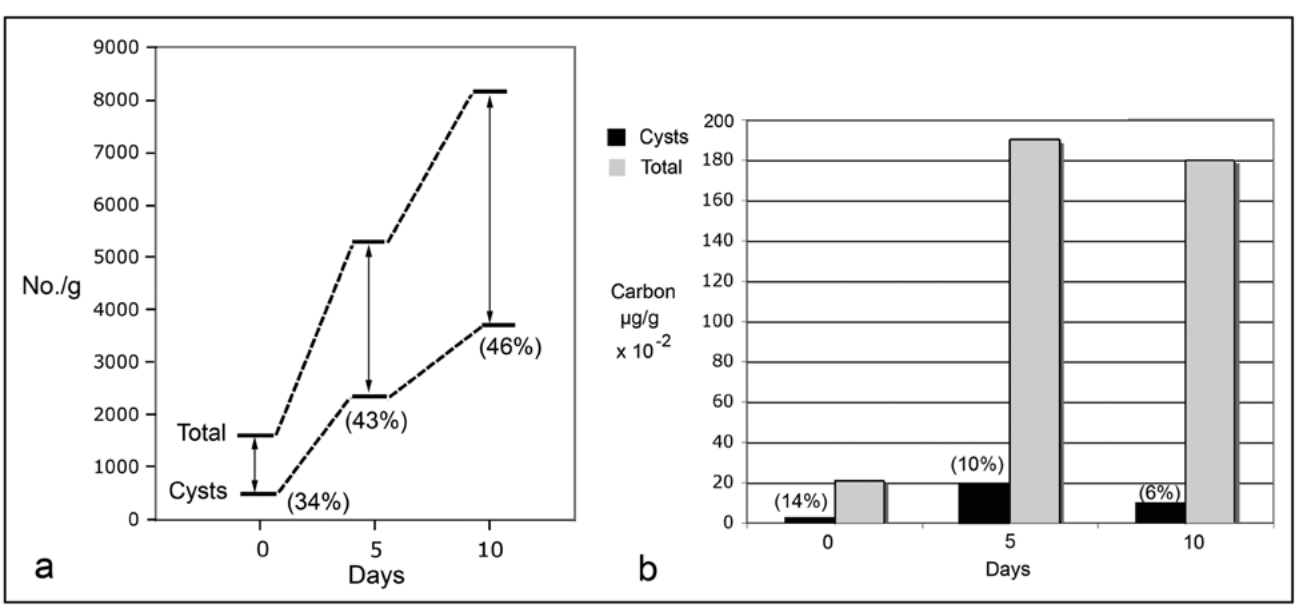

Fig. 2 a, **b.** Results of the culture experiment using *Phragmites* marsh soil samples. Graph data are plotted the same as in Fig. 1a, b using data from Table 2 for the marsh sample entries.

lower total levels after ten days compared to the *Phragmites* sample (47% moisture and 15% organic content) as is consistent with the field-based results. However, the proportion of encysted to total amoebae is more informative. In the drier pine stand sample, the proportion of encysted relative to total amoebae increased mark-

Table 2. Densities (no./g) and carbon content (µg/g) of total enumerated amoebae and of the encysted stages for samples from the experimental studies.

Date	Location	Temp. (day) (°C)	Moisture (%)	Densities			Carbon content		
				cysts	total	%	cysts	total	% in cysts
12/31	Pine	25 (t = 0)	18	530	900	60	0.03	0.04	75
1/5	Pine	25 (t = 5 d)	18	1,400	1,600	88	0.03	0.05	60
1/10	Pine	25 (t = 10 d)	18	3,700	3,700	100	0.20	0.20	100
12/31	Marsh	25 (t = 0 h)	47	532	1,580	34	0.03	0.21	14
1/5	Marsh	25 (t = 5 d)	47	2,300	5,400	43	0.20	1.90	10
1/10	Marsh	25 (t = 10 d)	47	3,800	8,200	46	0.10	1.76	6

edly during the ten days rising from an initial 60% to a final 100% (Fig. 1); whereas, the proportion of encysted to total amoebae in the *Phragmites* sample, though increasing moderately from 34% to 46% over ten days, was much more steady state (Fig. 2). The increase between day 5 and day 10 for the *Phragmites* sample was 43% to 46%, which is probably near a constant carrying state value. Furthermore, the *Phragmites* sample at peak densities had higher species richness (16 morphospecies) compared to the pine sample (seven morphospecies). Overall, in both samples there is clearly a dynamic relationship between active and encysted stages with evidence of substantial reversion of active amoebae into encysted stages during the succession. That is, as the succession proceeded, a proportion of the active amoebae reverted to an encysted stage indicating a dynamic flux between trophont and encysted forms of the amoebae during community proliferation. However, the more moist and organic rich *Phragmites* sample appeared to maintain a more consistent ratio of encysted to active forms compared to the pine stand sample during the incubation experiment. Also, as may be expected based on the higher amoeba densities in the *Phragmites* sample compared to the pine, the estimated total carbon content was much higher after ten days in the *Phragmites* sample (c. 1.8 µg/g soil) compared to the pine sample (c. 0.2 µg/g soil). The mean size ± SE of the total amoebae in the pine sample (16 ± 1.6 µm) was smaller than in the *Phragmites* sample (21 ± 1.5 µm). This further contributed to the higher carbon content of the amoebae in the *Phragmites* sample.

Based on the foregoing analysis of the dynamics of the ratios of encysted and active stages of naked amoeba populations during a community succession, a revised diagram of the life cycle of encysting protists is recommended (Fig. 3). The typical circular diagram of alternating encysted and active forms that characterizes the overall life cycle of individual organisms subjected to alternations in favorable and unfavorable growing conditions, is amended to include an inner set of antiparallel arrows representing the dynamic intermediate state of encysment and excystment of the protists that

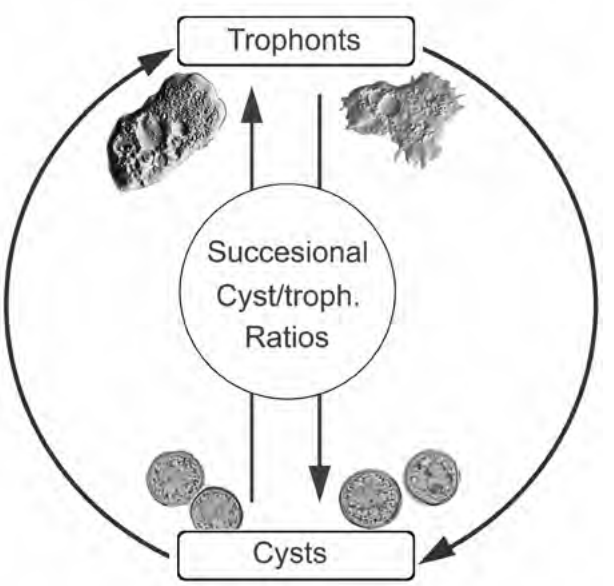

Fig. 3. A modified diagram of the classical life cycle of amoeba encystment and excystment (outer circular arrows) as represented for an individual organism during conditions promoting growth (trophont stage) or during extreme stress as during desiccation (cyst stage) is amended (internal set of antiparallel arrows) indicating the balance between ratios of encysted and active (trophont) stages that occur during a community succession; varying with factors such as time, the environmental conditions, and density dependent interactions that either promote enhanced growth or gradually favor increasing proportions of encysted forms (see Figs 1, 2).

occurs as a succession proceeds. That is, in addition to the pools of active and encysted forms that represent the end members of extremes in favorable to unfavorable growth conditions (outer circular arrows), the dynamic ratio of encysted to active forms that occur during proliferation of a community (inner antiparallel arrows) should also be indicated. This more fully represents the complexities of the protistan community encystment and excystment in response to varying environmental conditions that include periods of rapid proliferation during succession as well as more extreme end member states where the bulk of the protists are either largely active or dormant and fully encysted.

DISCUSSION

The dynamics of naked amoebae community succession are poorly understood, partially due to the difficulties in earlier studies to fully account for the densities of encysted and trophic forms. A current debate centers on how best to monitor naked amoeba abundances in terrestrial environments, where at any point in time a substantial amount of the amoebae may be in a dormant state. For example, Adl *et al*. (2008) have proposed a method of direct microscopic observation of small aliquots of soil samples deposited on agar plates as a way of documenting only the active naked amoebae, arguing that other methods that involve culturing (such as serial dilution techniques) overestimate the number of active amoebae due to excystment of dormant stages during the culturing. This is a valid criticism, if the culturing technique does not include a method of estimating the proportion of amoebae that are encysted. However, if only the active stages are enumerated, this may seriously underestimate the total potential standing stock of amoebae in a soil sample and also, from an ecological perspective, may substantially underestimate the total capacity for productivity. Moreover, it does not account for the total carbon pool in the encysted as well as trophic forms. Hence, a more systematic understanding of the percentage of encysted amoebae relative to active in a variety of habitats is needed to better estimate what proportion of amoebae at any point in time are encysted and how much of the potential active amoeba carbon is contained in the encysted as well as the active stages of amoebae. This study addresses these two aspects of naked amoeba ecology using current enumeration techniques (modified COM) that accounts for encysted and active forms. However, culture observation methods may underestimate the number of morphospecies, because some are not amenable to growth in laboratory conditions. This limitation needs to be kept in mind when interpreting amoeba densities based on laboratory culture observation methods, in general.

Field-based study

The field-based studies document that a substantial amount of the accountable carbon content in the total standing crop of naked terrestrial amoebae may exist within the carbon pools of cysts ranging from 11% to 100% of total amoeba carbon in the soil samples analyzed here. The total amoeba carbon content (µg/g soil dry weight) varied from 0.10 to 1.70 with the amount in the cysts ranging between 0.05 to 0.43. These results suggest that adequate attention needs to be given to the densities and carbon content of encysted terrestrial naked amoebae as well as the active forms if a complete accounting is to be made of their presence and ecological roles among major ecosystems. The field samples used here, representative of a wide range of habitats characteristic of temperate woodlands and wetlands in northeastern U.S.A., included marsh margin, deciduous forest, and dry pine barren locales. While these sites may be typical for the region, the data presented here are limited to the habitats where the samples were taken, and research at other major geographic regions and with different habitats may yield other patterns of active and encysted forms of naked amoebae.

The ratio of encysted to active forms in this study is clearly moisture dependent as has been documented in a previous longitudinal, multi-year seasonal study at one grassy site on the Lamont-Doherty Reserve (Anderson 2000). In that research, a fairly well constrained correlation ($r^2 = 0.95$) was found between percentage of trophonts and percent soil moisture as follows:

$P = 2.843 \cdot M - 5.594$, where P = percentage of trophonts and M = percent of moisture content.

In the current study, when all of the data points for the field samples are used (Table 1), the correlation between the densities of active trophonts and soil moisture is 0.88 ($r^2 = 0.8$); though as may be expected, there is some scatter due to the differences in the soil composition and other site-specific factors across the three sampling sites.

Laboratory studies

The experimental studies using microcosms in controlled temperature chambers demonstrate that the ra-

tio of encysted to total naked amoebae varies during proliferation of populations when winter samples are warmed simulating spring bloom conditions. There is a substantial increase in trophonts over 10 days of incubation (25°C), but the proportion of cysts also increases, indicating a dynamic relationship with the active forms. However, the proportion of cysts relative to total amoebae varies depending on the sample site. For the more moist, marsh site, the proportion of encysted amoebae reaches a nearly constant value (43–46%) suggesting that there is perhaps a density-dependent equilibrium effect that maintains a relatively steady state balance between active and encysted forms at least during the early phases of succession monitored in this study. By contrast, the data for the drier pine sample show that the proportion of encysted amoebae relative to total continues to increase with time, eventually becoming 100%. Thus, under the drier conditions of the pine soil, and perhaps the more limited organic content, the density-dependent factors tend to shift the balance toward increasingly dormant stages as the succession proceeds. Similar density-dependent explanations have been given for the ratio of encysted to active stages of ciliates in laboratory temperature-controlled cultures under enhanced organic enrichment conditions (e.g. Ekelund *et al.* 2002). It is also possible that the amoebae in the drier pine site, prone to greater environmental pressures due to frequent wetting and drying, may be more r-selected and thus cycle more frequently between encysted and trophic stages. Based on these composite data, a modified model of the cyst/trophont life cycle is recommended to better represent the dynamics of population encystment and excystment during ecological successions (Fig. 3). The research reported here suggests that the intermediate stages of encysted and active stages of naked amoebae during a succession may be dynamically affected by both biotic (e.g. density dependent) and abiotic (e.g. moisture content, nutrients, etc.) factors that characterize particular geographic locales. Further research more broadly with naked amoebae and other protists is needed to explore the complexities of the biotic and abiotic factors that determine the dynamic balance (Fig. 3) between encysted and active stages in field-based and laboratory controlled studies for a variety of geographic regimes.

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