Nitrogen-Sparged Media Extends Life Span of Symbiotic Protists Found in Subterranean Termites (Isoptera: Rhinotermitidae), Providing More Time for Microscopic Examination

J. L. LEWIS AND B. T. FORSCHLER

Department of Entomology, University of Georgia, 413 Biological Sciences Building, Athens, GA 30602

Environ. Entomol. 33(5): 1145–1150 (2004)

ABSTRACT Protist communities found in the hindgut of subterranean termites (Rhinotermitidae) historically have been quantified and identified using a variety of methods, thus making comparisons between studies difficult. We examined four saline solutions and compared protist estimates by using two different cell-counting platforms. Estimates of protist populations were greater using a hemocytometer counting chamber than sealed coverslip mounts. Estimates of the protist population per *Reticulitermes flavipes* (Kollar) worker averaged 90,000 in Trager U, 79,000 in Ritter, 68,000 in Mannesmann, and 50,000 in 0.6% NaCl saline solutions. The percentage of protist survivorship significantly decreased after 5 min under microscopic examination in untreated media. Sparging one of the saline solutions (Trager U) with nitrogen gas provided a solution where protist populations averaged 91,000 and percentage of protist survivorship did not significantly decrease for 15 min. Identification and quantification of protists require time, and extending cell life provides more time to make accurate counts. We therefore propose the technique described in this study be adopted for its ease of use and improved accuracy.

KEY WORDS *Reticulitermes flavipes*, anaerobic protists, symbiotic protists, physiological saline solution, protist counting techniques

LESPES (1856) WAS THE FIRST to mention living cells in the hindgut of termites (Rhinotermitidae). The anaerobic protists he observed are now recognized to represent three orders: Trichomonadida Kirby, Oxymonadida Grassé, and Hypermastigida Grassi & Foà (Yamin 1979). These insect gut protists represent a unique symbiosis specific to certain insect families and are found only in lower termites and a wood-feeding cockroach, *Cryptocercus* sp. (Koidzumi 1921, Kirby 1937, Honigberg 1970). Molecular techniques can be used to investigate the microbial fauna found in termite hindguts (Gunderson et al. 1995, Ohkuma et al. 2000). However, morphometric identification to species is required so that specific molecular markers for the protist community can be developed (Kudo et al. 1998, Ohkuma et al. 1998). Many termite hindgut protists are difficult to culture outside of the host, leaving identification of species to two established techniques: staining and high-definition microscopy, and light microscopy by using live mounts (Inoue et al. 2000). Identification and enumeration of the protist communities require a physiological solution and a counting platform that can accommodate the time frame needed to complete observations before cell death.

In the genus *Reticulitermes*, there are several thousand anaerobic protists in each subterranean termite hindgut (Mannesmann 1969). Identifying these cells outside of the insect host requires keeping them alive in an osmotically balanced and buffered saline solution long enough to observe movement patterns that distinguish similar species. In the literature, there are three salt solutions that have been used to study *Reticulitermes* protist communities, and one for those of a wood-feeding cockroach, *Cryptocercus* sp. (Mannesmann 1969, 1970, 1972; Ritter et al. 1978; Mauldin et al. 1981; Yamaoka et al. 1983; Howard 1984; Azuma et al. 1993; Yoshimura et al. 1994; Yoshimura 1995; Inoue et al. 1997; Cook and Gold 1998, 2000). These solutions vary in proportions of sodium, potassium, calcium, and magnesium, as well as pH. Yet, aside from sealing the coverslip to reduce exposure to atmospheric oxygen, no one has examined an anoxic medium as a means of extending cell life for termite protist identification and enumeration.

Two methods have been used to quantify termite protist populations. One involves suspending the contents of a termite hindgut in a known volume of saline solution, preparing a wet mount, and sealing the coverslip (Yoshimura et al. 1994; Yoshimura 1995; Cook and Gold 1998, 2000). The other method uses a hemocytometer cell counting chamber (Mannesmann 1969, 1970, 1972; Mauldin et al. 1981; Howard 1984; Azuma et al. 1993; Inoue et al. 1997). The use of different saline solutions and counting techniques makes comparison between studies difficult and ac-

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Table 1. Composition of physiological saline solutions used for studies of subterranean termite protists in grams of salt ingredient per 50 ml of distilled water

Ingredient	Trager U (g) (1934)	Ritter (g) (1959)	Mannesmann (g) (1969)	0.60% NaCl (g) (Kirby 1932)
	pH 7.1	pH 7.1	pH 7.8	pH 7.0
NaCl	0.1082		0.400	0.300
KCl		0.1247	0.010	
$CaCL_2$	0.0041	0.041	0.010	
$NaHCO_3$	0.0386		0.005	
NaH_2PO_4 H_2O		0.0145		
KH_2PO_4	0.0892			
$MgSO_4$ $7H_2O$	0.0024	0.0376		
$Na_3C_6H_5O_7$ $2H_2O$	0.0754			
$KHCO_3$		0.0333		

counting for the variation reported in the literature impossible.

In this article, we compared protist estimates from the hindgut of *Reticulitermes flavipes* (Kollar) by using a hemocytometer with those from a sealed wet mount. We also describe experiments using hemocytometer protist estimates to compare different physiological saline solutions. Our objective was to review and consolidate the literature and to determine whether any of the previously described saline solutions affect protist survivorship over time. In light of our findings, we propose a standardized technique that is time efficient, simple, and accurate. We hope these studies stimulate discussion on standardization of methods used to quantify termite protists, which will be important in gaining a better understanding of this important symbiosis.

Materials and Methods

Insects. One pine (*Pinus* sp.) log infested with *R. flavipes* was collected from Whitehall Forest in Athens, GA, and returned intact to the laboratory. The termites were identified to species by using published keys to the soldier caste (Scheffrahn and Su 1994) and extracted from the log as described by Forschler and Townsend (1996). Once collected, termites were kept in plastic boxes (26.99 by 19.37 by 9.52 cm) with pine slats (≈12.5 by 2.54 by 0.2 cm) and placed inside an environmental chamber where they were maintained in complete darkness at 24°C. Voucher specimens were preserved in 100% ethanol and deposited at the University of Georgia Museum of Natural History.

Preparation of Sample Unit for All Protist Counts. Protist population estimates were obtained by counting all living cells from individual termites collected from a single termite colony. The contents of one termite alimentary tract were placed in 80 μl of a selected saline solution. This constituted one replicate in all of these experiments. Protists were determined to be alive if the flagella and/or axosyle were moving and the cell membrane was intact. Dead cells were defined as those that exhibited no movement in any of the aforementioned structures. Solutions (Table 1) were prepared on a weight-to-weight basis in a 200-ml glass sample bottle (Wheaton Science Products, Millville, NJ), sterilized in a steam autoclave for 20 min at 130°C and pipetted from that reservoir by using disposable tips.

Only fourth instar or older worker termites with dark brown abdomens were selected for protist counts to ensure they had not recently molted. The last two abdominal segments were removed from a termite with forceps, thus extracting the alimentary canal that was then placed in a microcentrifuge tube containing the appropriate saline solution. The alimentary tract was gently homogenized in the microcentrifuge tube for 10 s by using a disposable pestle. We also examined the technique of gently squeezing the hindgut contents into the saline solution, but found it had no affect on protist estimates ($F = 0.0032$, df = 8, $P = 0.9559$) compared with the homogenizing technique. We therefore chose to conduct these experiments by using the homogenizing technique because it was efficient and time-effective (unpublished data). All protists were counted at 400× magnification using a Nikon compound microscope.

The numbers of protists per individual termite were determined using the following formula: (number of protists counted × volume of saline solution in the original suspension)/volume of saline solution from which protists were counted. Percent protist survivorship was calculated by taking cell counts in 5-min increments.

Comparing Protist Counting Platforms. We compared protist population estimates by using a wet mount, with those using a hemocytometer cell counting chamber. All counts were taken from a single termite alimentary canal suspended in 80 μl of Trager U saline as described previously and replicated three times per counting platform. Wet mounts were prepared by placing a 4-μl aliquot, from the aforementioned suspension, onto a glass slide and sealing the coverslip with petroleum jelly. All cells were counted according to the wet mount technique described in Cook and Gold (1998). For protist estimates using a hemocytometer, 10 μl was loaded into a hemacytometer (Neubauer, Brightline, Horsham, PA), and counts were made from five small squares (0.0125 mm^2 equivalent to 0.02 μl).

Saline Solution Comparison. Total protist populations were estimated from workers belonging to one *R. flavipes* colony and were compared using four different saline solutions. Each replicate consisted of the

Table 2. Mean (±SD) estimates of total protist populations per termite during microscopic examination in a hemocytometer by using the time 0 counts as the starting point in 5 min increments for four physiological saline solutions

Min	Mean protist population estimate by physiological saline solution[a]			
	Trager U	Ritter	Mannesmann	0.60% NaCl
0	90,400 ± 13,446aA	79,200 ± 14,255aAB	68,000 ± 10,198aB	50,400 ± 6,066aC
5	64,000 ± 16,000abA	64,000 ± 10,198abA	48,800 ± 4,382abB	32,000 ± 6,325aC
10	55,200 ± 14,805abcA	51,200 ± 12,133bcAB	35,200 ± 10,733abBC	24,000 ± 4,899aC
15	45,600 ± 13,145bcA	48,000 ± 9,381bcA	29,600 ± 6,066abB	17,600 ± 3,578aC
20	33,600 ± 11,865cdA	41,600 ± 10,440cA	24,800 ± 7,155abA	8,800 ± 8,672bB
25	24,800 ± 12,133dA	27,200 ± 9,960dA	15,200 ± 7,694abA	4,000 ± 4,899bcB
30	14,400 ± 9,209eA	16,000 ± 2,828eA	8,000 ± 5,657bA	1,600 ± 3,578cdB
35	10,400 ± 7,266eA	8,800 ± 4,382fA	1,600 ± 2,191cB	0.00 ± 0.0dC
40	0.00 ± 0.0fA	0.00 ± 0.0gA	0.00 ± 0.0dA	0.00 ± 0.0dA

[a] Means ± SD within columns with the same lowercase letter, and within rows with the same uppercase letter, were not significantly different using Duncan's multiple range test ($P < 0.05$).

contents of a single termite gut suspended in 80 μl of the appropriate solution and was counted using a hemocytometer. The solutions tested were 0.60% NaCl (Kirby 1932, Trager (1934), Mannesmann 1969, Ritter et al. (1978). Five replicates per saline solution were performed.

In our experiments, protist samples were prepared and initial cell counts made within 3 min of sample preparation (time 0). Thereafter, protist counts were taken every 5 min for 40 min from the same hemocytometer mount. The four saline solutions were compared using total protist population estimates and percentage of protist survivorship over time.

Anoxic Saline Solution. One saline solution was examined to test the utility of sparging the solution of oxygen. Trager U was used in these experiments because it is the most often cited saline solution (Azuma et al. 1993; Yoshimura et al. 1994; Yoshimura 1995; Inoue et al. 1997; Cook and Gold 1998, 2000), has a stable pH, and does not precipitate out of solution when sterilized. One termite gut was homogenized, as described previously, in 80 μl of the appropriate solution (standard or anoxic), and counts were made on a hemocytometer every 5 min for 30 min, replicated five times. All termites were worker nestmates as in the other experiments. The anoxic solution was prepared by bubbling a nitrogen gas mixture (92.5% N, 5.0% CO_2, and 2.5% H) at 1 liter/min for 5 min into 6 ml of standard Trager U. For the sake of discussion, the nitrogen-sparged solutions will be termed anoxic solutions. Data used for analysis were total protist population estimates and percentage of protist survivorship from anoxic and oxic solutions compared over time.

Statistical Analysis. Data were transformed and analyzed by one-way analysis of variance (ANOVA) with means separated by Duncan's multiple range test ($P < 0.05$) by using the Statistica for Windows package (StatSoft Inc., Tulsa, OK). The response variables were transformed by taking the base-10 logarithm of the total protist population and arcsine of the percentage of protist survivorship (Steel and Torrie 1960), but the data shown are untransformed for interpretation.

Results

Comparing Protist Counting Platforms. Protist population estimates of 86,667 ± 19,731 (SD) per termite were obtained using the hemocytometer, which was significantly greater than the estimate of 45,875 ± 4,904 protists obtained using the wet mount technique ($F = 16.057$, df = 4, $P = 0.0160$).

Saline Solution Comparison. Protist population estimates were significantly greater with the Trager U and Ritter's saline solutions over time ($F = 12.306$, df = 178, $P = 0.0006$; Table 2). The data also were analyzed using percentage of protist survivorship because of variation in the time 0 counts between saline solutions (Table 2). Protist survivorship decreased rapidly with 72.17 ± 12.52% of the protists counted in the first survey still present after 5 min, in all saline solutions combined (Table 3). There were no differences in the percentage of protist survivorship between saline solutions for the first two counts (10 min) (Table 3). At 15 min, Ritter's solution maintained cell survivorship longer than 0.60% NaCl and Mannesmann saline solutions (Table 3). Protist survivorship was significantly greater at 20 min in Trager U, Ritter, and Mannesmann saline solutions than in 0.60% NaCl. All cells were dead after 40 min on the microscope, regardless of the medium tested (Table 2).

Anoxic Saline Solution. Protists survived longer in the anoxic medium (Table 4). The percentage of protist survivorship in the standard Trager U solution decreased significantly after 5 min, with 72.72 ± 11.99% of the original number of live protists remaining (Fig. 1). In comparison, survival in the anoxic Trager solution significantly decreased (87.20 ± 11.21%) only after 20 min, by which time only 25.27 ± 6.30% of the cells were still living in Trager U (Fig. 1).

Discussion

We have attempted to synthesize the existing literature on estimating termite hindgut protist populations, and we propose a single technique using a hemocytometer for ease and accuracy. Hemocytometers are manufactured for quick and easy cell counts and consist of a counting chamber holding a known vol-

Table 3. Mean (±SD) percentage of protist survivorship during microscopic examination in a hemocytometer by using the time 0 counts as the starting point in 5-min increments for four physiological saline solutions

Min	Mean % survivorship by physiological saline solution[a]			
	Trager U (%)	Ritter (%)	Mannesmann (%)	0.60% NaCl (%)
0	100 ± 0aA	100 ± 0aA	100 ± 0aA	100 ± 0aA
5	70.46 ± 11.62bA	82.13 ± 14.25bA	72.43 ± 6.98bA	63.67 ± 11.81bA
10	60.97 ± 14.41bcA	66.50 ± 18.58cA	52.34 ± 17.45cA	48.22 ± 11.01bcA
15	50.62 ± 13.99cAB	61.37 ± 11.01cA	44.35 ± 11.59cdB	39.91 ± 10.45cB
20	36.53 ± 9.82dA	52.69 ± 8.56cA	36.68 ± 10.23dA	18.40 ± 18.16dB
25	26.34 ± 10.49deA	34.28 ± 9.71dA	22.84 ± 12.00eA	8.36 ± 10.22deB
30	15.27 ± 8.77efA	20.42 ± 3.35deA	12.37 ± 9.27eA	3.33 ± 7.45eB
35	11.01 ± 7.04fA	11.29 ± 5.95eA	2.60 ± 3.59fB	0.00 ± 0.00eB
40	0.00 ± 0.0gA	0.00 ± 0.0fA	0.00 ± 0.0fA	0.00 ± 0.0eA

[a] Means ± SD within columns with the same lowercase letter, and within rows with the same uppercase letter, were not significantly different using Duncan's multiple range test ($P < 0.05$).

ume of solution upon which is etched a standardized grid of nine 1.0-mm squares. These "large squares" are divided and subdivided into smaller squares (9 mm^2 with the central square divided into 1/400-mm^2 areas). In our experiments using a hemocytometer, it took 3 min to count all the protist cells from 0.02 μl, while using the wet mount technique of Cook and Gold (1998), it took 1 h to count all the protists from the 4-μl aliquot. Protist population estimates using the hemocytometer averaged 86,667 ± 19,731 per termite and were significantly greater than the estimates of 45,875 ± 4,904 protists obtained using the wet mount technique.

The salt solutions compared in this study differ in the amount and kind of ingredients. Kirby (1932) used 0.60% NaCl solution for comparative studies and descriptions of *Trichonympha* from several species of termites. This solution was the easiest to make because it contained only sodium chloride salt dissolved in distilled water. Trager (1934) developed two media, A and U, based on preliminary experiments using protists from *Zootermopsis angusticollis* (Hagen) and *Reticulitermes flavipes*. He determined a solution containing 0.30 to 0.40% NaCl, with a ratio of 97:3 sodium to potassium, and pH 6.8–7.2 was optimal for culturing the protists found in *Z. angusticollis*. In solution A, the protist *Trichonympha sphaerica* survived for 6 wk, but in Trager U, *T. sphaerica* populations increased during the same frame (Trager 1934). Our experience with Trager U was that it was easy to prepare, remained in solution after autoclaving, and maintained a consistent pH while providing the highest protist estimates (Table 2). Mannesmann (1969, 1970) stated that a 0.90% NaCl solution was best, based on a comparison of six different salt solutions he designed, although no data were provided. Saline of Ritter (1959) has a higher ratio of potassium to sodium and higher pH value than the previous three solutions. Ritter's solution was designed using flame-spectrometry to determine the potassium concentration in the hindgut of *Cryptocercus punctulatus* (Ritter 1959). *Cryptocercus* sp. has hypermastigid and oxymonad symbionts that are closely related to those found in termites (Nalepa et al. 2001), and because Ritter et al. (1978) was able to double the size of certain protist populations in 6–8 d by using this

Table 4. Mean (±SD) estimates of total protist populations per termite during microscopic examination in a hemocytometer by using the time 0 counts as the starting point in 5 min increment for Trager U and anoxic Trager U saline solutions

Min	Mean % protist survivorship[a]	
	Trager U	Anoxic
0	81,600 ± 17,573aA	91,200 ± 9,960aA
5	62,400 ± 18,022abA	88,800 ± 10,733aB
10	49,600 ± 10,807abA	92,800 ± 9,121aB
15	39,200 ± 13,682abA	88,000 ± 10,198aB
20	20,800 ± 7,694abA	85,600 ± 12,837aB
25	16,00 ± 10,198bcA	70,400 ± 11,524aB
30	12,00 ± 8,485cA	56,800 ± 19,677bB

[a] Means ± SD within columns with the same lowercase letter, and within rows with the same uppercase letter, were not significantly different at each time interval between treatments by using Duncan's multiple range test ($P < 0.05$).

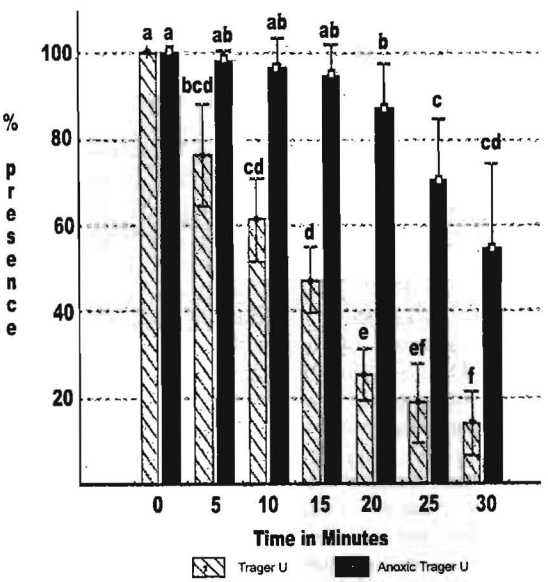

Fig. 1. Mean (±SD) percentage of protist survivorship during hemocytometer microscopic examination by using time 0 counts as the starting point in 5-min increments for Trager U and anoxic Trager U saline solution over time. Means with the same letter were not significantly different over time using Duncan's multiple range test ($P < 0.05$).

medium in an anaerobic chamber he designed, we included it in our study. This solution precipitates out of solution and does not maintain a consistent pH after autoclaving.

To compare saline solutions, we used total protist population estimates and the percentages of protist survivorship from individual nestmate worker termites from a single termite population to reduce the variation due to differences in colonies or castes (Grosovsky and Margulis 1982; Cook and Gold 1998, 1999). The 0.60% NaCl solution of Kirby (1932) does not seem to be a good medium for quantifying termite protists (Table 2) because it consistently provided smaller protist population estimates compared with the other solutions tested. We counted a mean protist population in *R. flavipes* of 50,400 ± 6,066 in 0.60% NaCl at time 0, compared with 79,200 ± 15,1334 per individual in Ritter, Trager U, and Mannesmann solutions by using nestmate termite workers. In our saline solution comparison, we found Trager U provided the highest protist estimates at time 0 (Table 2), but after 5 min, only 70.46 ± 11.62% of the original population remained. However, when we used the anoxic saline solution, our population estimates averaged 91,200 ± 9,960 at time 0, and percentage of protist survivorship did not decrease for 15 min.

Protist population estimates in our study (91,200 ± 9,960) were greater than those reported in the literature, which ranged from 21,000 to 40,000 (Mannesmann 1969; Mauldin et al. 1981; Howard 1984; Cook and Gold 1998, 2000). Previous counts may be smaller because they did not include smaller genera (*Monocercomonas, Microjoenia,* and *Trichomonas*) in their estimates. However, we have found populations of these protists account for 5,747 ± 2,633 flagellates per termite, which represent a small proportion of the total protist population and is less than the average standard deviation of the means obtained by Lewis (2003).

Total protist population numbers are only one reason to optimize the counting methods used in studying termite–protist communities. Identification of species and counts of protist species per termite require time. The decrease in number of live protists counted per square over time is most likely due to cell death. Extending cell life by optimizing a counting technique would theoretically provide more accurate counts over time. This point is critical because up to 15 min on the microscope might be needed to make an accurate assessment. To make accurate counts, either the time spent needs to be decreased or cell life needs to be extended.

The increased time needed to complete a protist count results from at least two factors. First is the need to count an increased number of hemocytometer squares to account for rare species (<10% of the total population) during a survey. Second is the need to identify the protists to species, which takes longer than counting cells depending on one's familiarity with species characteristics. Species identification also is simplified by observation of living cells because movement patterns can quickly distinguish similar species, thus eliminating the need for examining less obvious and therefore harder (and longer) to find characters or using time-consuming staining techniques that also require high-resolution microscopy to differentiate species (Lee et al. 1985).

These studies have demonstrated that by using nitrogen-sparged Trager U physiological saline, the time frame available for making accurate termite protist counts increases from 5 to 15 min (Fig. 1). This extension of cell life, by using the anoxic medium, provided estimates that were more than double any previously reported protist populations from subterranean termites. Further studies are needed to elucidate how these results affect our understanding of termite protist communities and how this can contribute to a better understanding of this important symbiosis. It is our hope that these studies stimulate additional research using nitrogen-sparged Trager U saline solution with a hemocytometer so comparison of results becomes commonplace.

Acknowledgments

We are grateful to Sabine Grube and Renata Radek for their helpful suggestions. We acknowledge Hope Ritter Jr. and Mark Farmer for guidance and continued interest in this research. We also thank Glen Ware for assistance with the statistical analysis.

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Received 17 March 2004; accepted 20 May 2004.