Biol Fertil Soils (1999) 28:352–355

ORIGINAL PAPER

A. Berthold · A. Bruckner · C. Kampichler

Improved quantification of active soil microfauna by a "counting crew"

Received: 19 December 1997

Abstract Recently, a comparative study revealed that the predominately used most probable number (MPN) method yields seriously biased estimates for active soil ciliates (Protozoa). The direct counting method, in contrast, gave reliable and reproducible data with a high level of discrimination between the invstigated sites between the investigated sites. However, this method requires that fresh soil samples are used, limiting its versatility. In order to overcome this problem a team ("counting crew") of 13 people was trained to carry out direct counts of soil microfauna simultaneously. The densities of active ciliates, rotifers, nematodes and tardigrades in the litter layer of a spruce stand were assessed by the direct counting technique. It could be shown that there was no significant influence of individual crew members on ciliate, rotifer or nematode numbers. The ciliate abundance did not vary significantly in the bulk sample with time of day. A significant increase in active ciliates was observed when counts took longer than 90 min. Comparing our results to literature data obtained by diverse extraction methods revealed that the direct counting method is very efficient for microscopic soil metazoa as well. If a counting crew is available, more precise and realistic data on active soil microfauna can be obtained.

Key words Ciliophora · Nematoda · Rotifera · Tardigrada · Quantification methods

A. Berthold (✉)
Institute of Zoology, University of Salzburg,
Hellbrunnerstrasse 34, A-5020 Salzburg, Austria
e-mail: aline.berthold@univie.ac.at
Tel./Fax: +43-1-7890677

A. Bruckner
Institute of Zoology, University of Agriculture, A-1180 Vienna, Austria

C. Kampichler
Institute of Zoology, Free University Berlin

Introduction

The density of active microfauna is of major importance for soil ecology. Unfortunately, due to a variety of methodological shortcomings, reliable estimates of the active organisms are very scarce. On the one hand, the problem of preservation and storage of the active, and often fragile, microfauna remains unsolved. An exception are testate amoebae, which are protected by their shell during fixation and staining, and thus discrimination between active and cystic organisms is possible (Aescht and Foissner 1992). On the other hand, extraction and cultivation methods bear the risk of losing or overestimating organisms. The extensively used most probable number (MPN) method for estimating protozoan numbers includes dilution and culture steps. Its main disadvantage is that active and cystic Protozoa cannot be separated properly. Recently, a comparative study revealed that the MPN method is prone to either overestimating or underestimating active ciliates by orders of magnitude (Berthold and Palzenberger 1995). A comparative study of extraction methods for nematodes showed that an increasing number of preparative steps can lead to an increasing loss of organisms (Decker 1989).

Counting the active ciliates directly under the microscope in a soil suspension was shown to be a suitable alternative, yielding reproducible results with a high level of discrimination (Berthold and Palzenberger 1995). This method was standardised and the recovery rate of ciliates, nematodes, rotifers and testate amoebae assessed (Lüftenegger et al. 1988). However, the number of direct counts one can achieve on a single counting day limits the versatility of this method. We attempted to overcome this problem by training a team of people to perform direct counts of ciliate, nematode, rotifer and tardigrade numbers simultaneously. Thus, sufficient data were obtained to address certain methodological uncertainties. In this paper we try to answer: (1) whether and how individual counting efficiency af-

fects the numbers of microfauna, (2) whether time of day affects the results of counting ciliate numbers, and (3) how long the time spent counting a subsample can be without incurring the risk of including freshly excysted ciliates. A condensed literature overview is provided to compare numbers of microfauna in mainly coniferous litters obtained by different quantification methods.

Materials and methods

The sampling site was a 45-year-old spruce stand (*Picea abies*) located in Stanglwald forest, Styria (Austria) at 1040 m above the sea level (47°13′N, 14°59′E, Gleinalm region, National Grid reference: BMN 6705-4830-1b). The site is bare of ground vegetation and the humus form is moor humus with distinct L, F and H layers with variable thickness up to 6 cm.

In late September 1995, the litter layer of 19 randomly chosen plots (25 × 12 cm) was sampled to a depth of up to 2 cm using a spatula. Each sample of 50 g fresh weight was thoroughly mixed. From each of these samples, three to four subsamples of 0.1 g were removed during the counting day and suspended in diluted soil extract. The subsamples were inspected immediately drop by drop under a microscope at × 40 magnification and the active ciliates, nematodes, rotifers and tardigrades were counted at room temperature (see Berthold and Palzenberger 1995 for details). The counting crew consisted of 13 participants who were individually trained for about 4 h to identify the microfaunal groups. A total of 63 subsamples were counted. Not everyone recorded tardigrades. The time required to count a subsample ranged from 50 min to 150 min.

The soil extract was prepared by boiling 100 g mixed litter from the sample site in 1 l demineralized water for 10 min. The hot supernatant was filtered, boiled for another 10 min and filtered again. This soil decoction was then diluted 1:3 with mineral water without carbon dioxide (Volvic, France). The pH of the diluted soil extract was adjusted to the pH of the site (pH 4.7 in distilled water).

All microfaunal counts were subjected to one-way ANOVA to test for significant influences of the counting personnel, after applying Cochran's C-test for the homogeneity of variances. Scheffe's multiple range test was used to locate significant differences by pairwise comparisons of the means. All counts of ciliates were subjected to regression analyses for the effect of time of day and duration of a count. Mean microfaunal numbers were obtained by calculating the means of the medians of the replicate counts for each of the 19 plots. For ciliates, only the counts made within 90 min were used. Statistical analyses were performed by Statgraphics Plus 5.2 software.

Results and discussion

To test the efficiency of the counting crew the influence of the individual crew members on microfaunal numbers was assessed by one-way ANOVA. No significant difference between members of the crew could be found with respect to the density of ciliates, rotifers or nematodes (Table 1). Therefore, we concluded that the variability in efficiency between members of the crew was lower than the variability between the samples. Concerning tardigrade numbers, however, a significant influence was detected between the two people who obtained the maximum and minimum values. Because tardigrades are usually too large to be overlooked under the microscope, this significant difference probably indicated a more heterogeneous distribution in litter compared to the other microfaunal groups. Regression analysis revealed a significant increase in ciliate numbers with increasing counting time (Fig. 1). The influence of the duration of the counts was not significant for counts lasting < 90 min (Table 2, Fig. 2). A counting time of 90 min is reasonable for the suspension of 0.1 g litter. The reason for this time-dependent increase was the excystment of mainly colpodid ciliates (personal observation). Therefore, it is necessary to split the 0.4-g sample, originally suggested by Lüftenegger et al. (1988), into 0.1-g samples. This sample mass is sufficient for sites with relatively high ciliate numbers (e.g. forest litters). With respect to the time of the day, the ciliate numbers in the bulk sample showed no significant change (Table 2). Our findings thus indicated that bulk samples may be kept at room temperature during the counting day without risk of serious bias with respect to ciliate numbers. Storing the bulk sample in a cooling room, on the other hand, bears the risk of los-

Table 1 Influence of counting crew on direct counts of microfauna in spruce litter samples assessed by one-way ANOVA

Organisms	n[a]	*F*	*P*
Ciliophora	53	1.62	0.13
Rotifera	59	0.43	0.93
Nematoda	59	1.17	0.34
Tardigrada	25	4.52	0.01

[a] Sample size

Table 2 Dependence of ciliate numbers on counting time and time of day

	Min	*F*	*P*	R^2	Slope
Counting time	50–150	16.00	< 0.05	22.08	5.78
	50–120	4.70	0.04	8.57	4.62
	50–100	3.00	0.09	6.25	4.29
	50– 90	0.17	0.69	0.43	1.27
Time of day		0.03	0.86	0.07	2.76

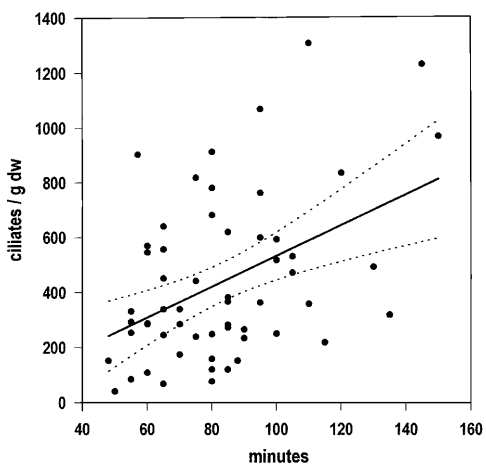

Fig. 1 Significant correlation between counting time (50–150 min) and ciliate numbers. For statistics, see Table 2

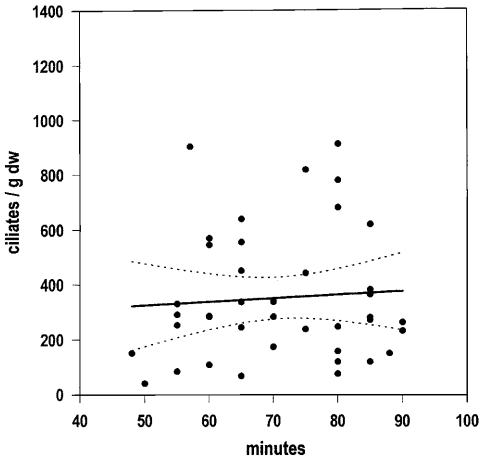

Fig. 2 No significant correlation between counting time (50–90 min) and ciliate numbers. For statistics, see Table 2

ing fragile ciliates due to a larger temperature change when they are transferred to the microscope.

Protozoan numbers are frequently estimated by serial dilutions and the MPN method. In a recent, comprehensive study this method was compared to the direct counting technique. It was shown that the MPN method leads to unreliable estimates by over- or underestimating active ciliate numbers (Berthold and Palzenberger 1995; Table 3). In contrast, counting of a fresh soil suspension assesses the number of active fauna directly. The second major advantage of the latter method is that only very few preparative steps are needed. The ciliate, rotifer and nematode densities presented in this study are in the usual range reported from litters obtained by direct counting (e.g. Petz and Foissner 1989). Adaptation of the pH of the diluted soil decoction to that of the investigated site reduces the osmotic stress of the ciliates. Thus, if the pH is adjusted and the counting time does not exceed the critical 90 min, methodological errors can be kept to a minimum.

A comparison of nematode, rotifer and tardigrade numbers yielded in this study with literature data derived from comparable biomes also emphasised the efficiency of the direct counting technique for microscopic Metazoa (Table 3). The comprehensive review of Petersen and Luxton (1982) gives data on nematode numbers obtained by extraction methods which are lower than our results. In contrast, Volz (1951) performed direct counts as well, obtaining very high values. According to Petersen and Luxton (1982), values obtained by this latter method may be more accurate. Reasons for the very high nematode numbers obtained in Volz's

Table 3 Numbers of microfauna in forest litters estimated by means of different methods. *dm* Dry matter, *Bdl* below detection limit, *Dc* direct counts, *MPN* most probable number, *Bwf* Bearman wet-funnel method, *Oea* modified Oostenbrink elutriation apparatus, *Swf* Sohlenius's wet-funel method, *Extraction* diverse extraction methods, *Filter* modified Oostenbrink cottonwool filter method, *MBwf* modified Bwf, *L* litter, *F* fermentation, *H* humus

Organisms	Mean[a]		Method[b]	Forest	Layer	Reference
	g dm^{-1}	10^4 m^{-2}				
Ciliophora	328		Dc	Spruce	L	This study
	400		Dc	Spruce	L	Berthold and Palzenberger (1995)
	71 000		MPN	Spruce	L	Berthold and Palzenberger (1995)
	Bdl		MPN	Spruce		Jentschke et al. (1995)
	(2750)[b]		MPN	Pine	L	Janssen and Heijmans (1998)[c]
Nematoda	1032	670	Dc	Spruce	L	This study
		2980	Dc	Oak-ash	LFH	Volz (1951)
	225		Bwf	Pine	H	Parmelee et al. (1993)[d]
		100	Oea	Pine	L	Schouten et al. (1990)[e]
	(270)[b]		Oea	Pine	LFH	Schouten and Arp (1991)[f]
		600	Oea	Pine	LFH	Schouten et al. (1998)[g]
	440	290	Swf	Pine	H	Ohtonen et al. (1992)
		250	Extraction	Coniferous		Petersen and Luxton (1982)[h]
Rotifera	399	252	Dc	Spruce	L	This study
		68	Dc	Oak	LFH	Volz (1951)
		51	Bwf	Pine	LFH	Persson et al. (1980)
		3	Filter	Spruce	LFH	Huhta and Koskenniemi (1975)
Tardigrada	49	32	Dc	Spruce	L	This study
		15	Dc	Oak	LFH	Volz (1951)
		5	Bwf	Pine	LFH	Persson et al. (1980)
		1.3	MBwf	Pine	LFH	Hallas and Yeates (1972)
		4	Filter	Spruce	LFH	Huhta and Koskenniemi (1975)

[a] Mean number of untreated controls per gram of dry mass or per square metre of investigated layer
[b] Number per gram of fresh mass
[c] Mean number from litterbags placed in the litter layer and renewed every two months
[d] Microcosms in tubes
[e] Mean calculated from the three sites and both sampling techniques
[f] Maximum number extracted by differently modified Oostenbrink elutriation apparatus
[g] Maximum number in reference core samples
[h] Upper value of the most frequent estimates

study might be the different habitat examined, the investigation of a mixed sample of the L, F and H layers and spatial effects. Compared to optimised extraction methods as used by Schouten et al. (1998), the values per square metre seemed to approximate the direct counts. Rotifer and tardigrad densities obtained by diverse extraction methods are far below the direct counts, indicating that these organisms are more sensitive to extraction processes than nematodes (Table 3).

We consider a mixed sample volume of 0.1 g litter to be sufficient for counting ciliates, nematodes and rotifers. We suggest that the sample volume for tardigrades should be increased and that the protozoan groups should be treated separately, i.e. testacea with the aniline blue method (Aescht and Foissner 1992), and active ciliates should be counted directly to give also the possibility of assigning characteristic species to their feeding groups (Aescht and Foissner 1993; Berthold 1994). Unfortunately, the direct method is not suitable for naked amoebae and flagellates because they are either hyaline and attached to soil particles or too small to be counted in a reasonable time (Schönborn 1989). New, comparative data suggest that the density gradient centrifugation method by Griffiths and Ritz (1988) yields more accurate results for naked amoebae (Mommertz 1997).

The direct counting method has repeatedly been shown to be very precise in differentiating between sites or treatment effects (e.g. Petz and Foissner 1989; Berthold and Palzenberger 1995). These first results presented here show that direct counts obtained by a counting crew is a worthwhile alternative to the MPN and extraction methods for quantification of microfaunal densities. The training, per person, takes only a few hours and the number of crew members needed depends on the study design. If some microscopes and trained personnel are available, the counting of ciliates, rotifers, nematodes and even tardigrades can be combined effeciently with respect to time. Employing a counting crew can therefore be a very valuable approach to obtaining more reliable data of the active soil microfauna, their actual biomass and their role in the food web.

Acknowledgements We wish to thank the Austrian Federal Ministry of Science, Research and the Arts (BMWFK) for financial support as well as A. Lametschwandtner, W. Foissner and K. Treiblmayer for kindly providing working facilities at Salzburg University. We want to give our special thanks to the counting crew, namely D. Bachl, G. Buchmeier, S. Gapar, B. Hiptmair, M. Jerabek, S. Kasinger, E. Ladurner, A. Leitner, G. Pfister, U. Rathmayr, U. Riedlmair, U. Schiftner and G. Stadlbauer, not only for their help but especially for their enthusiasm. For manuscript improvement we are thankful to W. Petz, B. Jank, T. Bolger, B. Möbes-Hansen and C. Stur, and as well as to M. Stachowitsch for linguistic revision.

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