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The efficiency of a direct counting method in estimating the numbers of microscopic soil organisms

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With 4 figures

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1. Introduction

Soil protozoa represent an important group in the soil animal community with regard to biomass and production (Foissner 1987). The culture techniques of Cutler (1920) and Singh (1946, 1955) have been widely used to estimate their individual abundances (Stout & Heal 1967; Stout et al. 1982). Recently, some authors have expressed doubts about the efficiency of those methods, especially concerning the abundance of the active protozoa (Stout et al. 1982; Schönborn 1989; Foissner 1988). Thus, they recommend direct methods of investigation. Soil nematologists are also at variance whether to use funnel methods or direct counting of a soil suspension (Zell 1985).

Although both methodological ways have been known for a long time, the efficiency of the various counting methods has never been tested thoroughly. There exist only a few preliminary recovery experiments (Singh 1946; Ramsay & Ball 1983; Foissner 1983). However, such basic methodological questions must be clarified, for they could strongly influence our view of the ecological significance of soil microorganisms. Thus, we performed recovery experiments on soil ciliates, testacea, nematodes and rotatorians. This kind of experiment seems to be the best way to test the suitability of a counting method (Foissner 1987).

2. Material and methods

2.1. The direct counting method used

A certain amount of fresh soil — normally 0.4 g for ciliates, nematodes and rotatorians and 0.1 g for testacea — is diluted (see sect. 2.3.) with soil extract medium[1]) or tap water (for testacea). The soil extract medium prevents the delicate ciliates, especially those from cultures, from bursting. The testate amoebae are stained with about 5 ml aniline blue[2]) overnight to distinguish full and empty tests. In this condition the samples can be stored for months. Ciliates must be counted on the day of sampling. The suspension gained is put dropwise on a slide and the various faunal groups are examined immediately.

Most ciliates, nematodes and rotatorians can be counted at 1×40 (objective 4: 1, ocular 10: 1), because their motility and size make them visible even at this low magnification. Testacea are investigated at a magnification of 1×100 (objective 10: 1, ocular 10: 1). Before counting them, the aniline blue is washed out with water by centrifugation. The pellet is thoroughly macerated by a glass-stick. To gain a homogenous distribution of the soil particles on the slide, 0.5 ml albumin-glycerin (as used in histological techniques to stick sections on a slide) are added to 5 ml of the soil suspension which is immediately dropwise examined. Identification of organisms must be performed at higher magnifications.

To get comparable results from a certain soil and person it is crucial to have an uniform dilution factor and an approximately identical examination time. An experienced worker needs, for instance, about 2 hours for the microscopical examination (counting) of a 0.4 g soil sample with ciliates and about 8 hours for a 0.1 g soil sample with testaceans. The dilution needed depends mainly on the

[1]) Add 300 g soil to 1 l distilled water, boil for 10 minutes, filter, sterilize.
[2]) 450 ml 5% aqueous phenolic solution + 30 ml 1% aqueous aniline blue + 120 ml concentrated acetic acid, filter, use it undiluted.

soil type. Soils with a considerable content of clay or with very high numbers of protozoa should be examined with higher dilutions than humic soils or soils with low individual numbers. In the course of time, experience will help in the choice of an appropriate dilution factor. Examples are shown in section 2.3.

A sample mass of 0.1 g fresh soil is usually enough for the testate amoebae, which often occur in high numbers (FOISSNER & ADAM 1981). Concerning the ciliates, some results indicate that a sample mass of 0.1 g is sufficient for soils with moderately high numbers of individuals ($300 \times g^{-1}$ wet mass). Concerning the number of species, 0.4 g might be too little for weakly populated samples (20—50 individuals $\times g^{-1}$ wet mass) (see explanation to fig. 1 and comp. LÜFTENEGGER *et al.* 1986).

2.2. Recovery experiments

The principle is to add a known number of individuals to a certain amount of soil in a certain dilution and to count how many organisms can be recovered.

In our experiments the individuals were added by micropipettes. To avoid excystation of organisms, the soils were sterilized by oven-drying (2 hours at about 120 °C) before use. Control experiments showed that ciliate cysts did not survive this treatment. Since the shells of the testacea can not be destroyed by this procedure, either soil 3, which is free of testate amoebae, was used, or full (means active and cystic individuals) and empty tests were stained with aniline blue and added to an uncoloured sample of soil 1. The plasma bodies of the full tests are dark blue, whereas the shell walls are of a faint blue. The ratio of full to empty tests was about 1 : 1. Nematodes and rotatorians were added as active, unstained individuals.

The amount of soil, the dilution and the numbers of individuals added in the experiments were in rough proportion to the abundances of individuals in nature. The higher the abundances the smaller the investigated amount of soil and the higher the dilution and number of individuals added. To reduce counting time, most experiments were performed with only about half the soil quantity which we normally use in ecological studies.

To evaluate whether the results are influenced when several species are counted together, 2 different kinds of recovery experiments were realized: one set in which only individuals of a single species were added and experiments in which individuals of many species were added to the soil suspension. In sum, 52 experiments were carried out, 32 with ciliates, 9 with testaceans, 7 with nematodes and 4 with rotatorians.

The experiments are a résumé of the works of different persons, who have performed the investigations within the scope of ecological work during the last 10 years (FOISSNER 1985; FOISSNER & ADAM 1981; LÜFTENEGGER *et al.* 1986; PETZ *et al.* unpubl.). This explains some slight non-uniformities of the experimental design.

2.3. Soils used and methodological details

Soil 1, pine forest. Soil type: Brown earth, loamy sand. 3 different strata were investigated: 0—1 cm soil depth, pine needles; mineral content 4.9 %. 1—3 cm, mouldered pine needles; mineral content 10.2 %. 3—9 cm, raw humus; mineral content 52.1 %. Soil suspensions: To 0.1 g soil dry mass (dm) for each layer 2 ml soil extract medium are added for ciliates, nematodes and rotatorians; $0.005 \text{ g} \times 5 \text{ ml}^{-1}$ tap water for testacea.

Soil 2, alpine pasture (0—3/5 cm). Soil type: Alpine brown earth, loamy sand. Mineral content: 85 %. Soil suspension: 0.1 g soil (dm) $\times 2 \text{ ml}^{-1}$ soil extract medium for ciliates.

Soil 3, levelled ski slope (0—3/5 cm). Soil type: Raw soil, loamy sand. Mineral content: 96.5 %. Soil suspensions: 0.1 g soil (dm) $\times 2 \text{ ml}^{-1}$ soil extract medium for ciliates; $0.05 \text{ g} \times 5 \text{ ml}^{-1}$ tap water for testacea; $0.2 \text{ g} \times 4 \text{ ml}^{-1}$ tap water for nematodes.

Soil 4, oak-beech forest (5—10 cm). Soil type: Decalcified brown earth, clayey loam. Mineral content: 88 %. Soil suspensions: 0.1 g and 0.4 g soil (dm) $\times 2 \text{ ml}^{-1}$ and 8 ml tap water for nematodes.

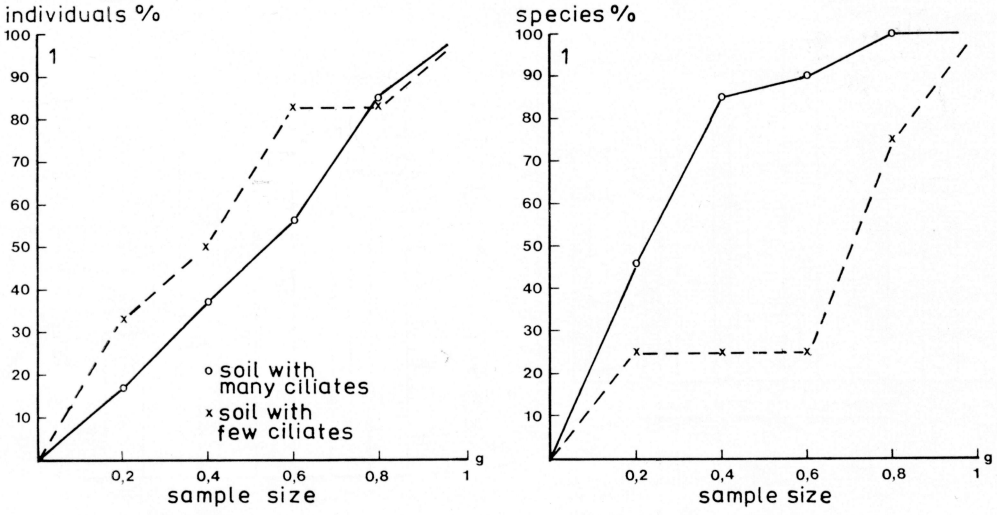
2.4. Species used

All biometrical data are based on the measurement of 10 individuals of each species.

Ciliates: *Colpoda inflata* (STOKES) KAHL [mean length ($\bar{x}$) = 51 µm], *Cyclidium muscicola* KAHL (20 µm), *Frontonia depressa* (STOKES) KAHL (80 µm), *Gonostomum affine* (STEIN) STERKI (76 µm), *Grossglockneria acuta* FOISSNER (45 µm), *Holosticha multistilata* KAHL (100 µm), *Keronopsis muscicola* (KAHL) FOISSNER (95 µm), *Lamtostyla perisincirra* (HEMBERGER) BERGER et FOISSNER (65 µm), *Opisthonecta minima* FOISSNER (87 µm), *Pseudocyrtolophosis alpestris* FOISSNER (24 µm), *Pseudoplatyophrya nana* (KAHL) FOISSNER (21 µm). *O. minima* and *G. acuta* were from pure cultures, the other organisms originated from the investigated soils and were cultured with the non-flooded petri dish method of FOISSNER (1987).

Fig. 1. Test for optimal sample size for ciliate counting. Detailed explanation on page 101 (Appendix).

Fig. 2. Recovery rates for the ciliate species, and correlation ($r_s = 0.7$) between body size and percentage of recovery. a: Single species experiments (*F. depressa*, *H. multistilata*, *G. acuta*: n = 1; *O. minima*: n = 3; other species: n = 2). b: Multiple species experiments (n = 2). c: Comparison of recovery rates using two different soils (soil 2 and 3).


individuals %
1
o soil with many ciliates
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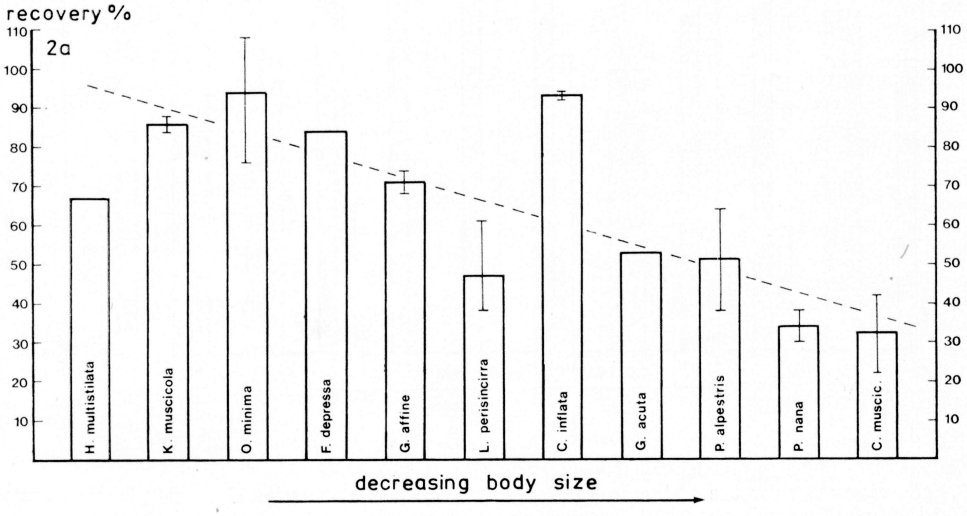
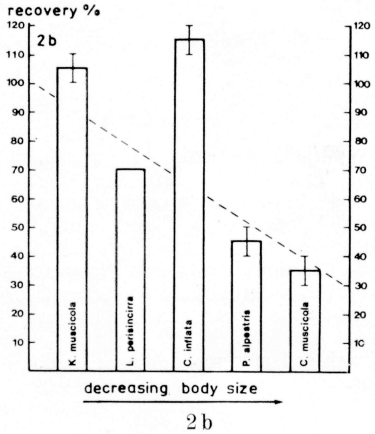
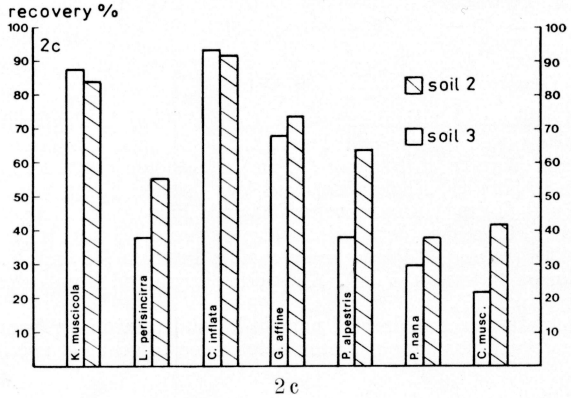
1


recovery %
2a
H. multistilata
K. muscicola
O. minima
F. depressa
G. affine
L. perisincirra
C. inflata
G. acuta
P. alpestris
P. nana
C. muscic.
decreasing body size

2a


recovery %
2b
K. muscicola
L. perisincirra
C. inflata
P. alpestris
C. muscicola
decreasing body size

2b


recovery %
2c
soil 2
soil 3
K. muscicola
L. perisincirra
C. inflata
G. affine
P. alpestris
P. nana
C. musc.

2c

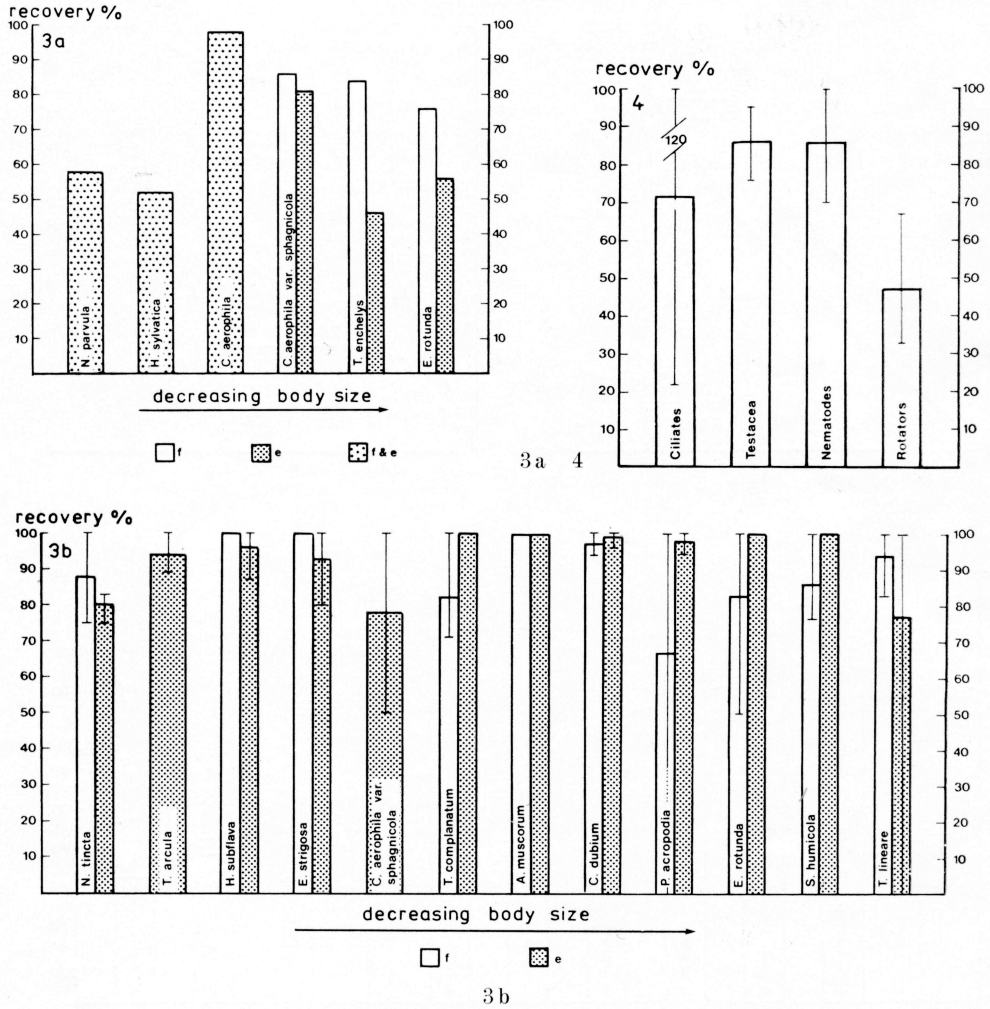


Fig. 3. Recovery rates for the testacean species. a: Single species experiments with soils 3 and 4 (n = 1; the values for *N. parvula*, *H. sylvatica* and *C. aerophila* are from Foissner 1983). b: Multiple species experiments with soil 1 (n = 3). f, full tests; e, empty tests.

Fig. 4. Percentage of recovery of the ciliates (n = 32), testacea (n = 9), nematodes (n = 7) and rotatorians (n = 4) from all experiments.

Testacea: *Assulina muscorum* Greef (45 µm), *Centropyxis aerophila* Deflandre (64 µm), *C. aerophila* var. *sphagnicola* Deflandre (53 µm), *Corythion dubium* Taranek (40 µm), *Euglypha rotunda* Wailes et Penard (36 µm), *E. strigosa* (Ehrenberg) Leidy (72 µm), *Heleopera sylvatica* Penard (72 µm), *Hyalosphenia subflava* Cash et Hopkinson (80 µm), *Nebela parvula* Cash (91 µm), *N. tincta* (Leidy) Awerintzew (90 µm), *Phryganella acropodia* (Hertwig et Lesser) Hopkinson (37 µm), *Schoenbornia humicola* (Schoenborn) Decloitre (36 µm), *Trigonopyxis arcula* (Leidy) Penard (85 µm), *Trinema complanatum* Penard (45 µm), *T. enchelys* (Ehrenberg) Leidy (50 µm), *T. lineare* Penard (35 µm). All species originated from cultures (non-flooded petri dish method of Foissner 1987).

A natural assemblage of nematodes and rotatorians from the fresh soil of a meadow was used. These organisms have not been determined to genus or species level.

3. Results

Ciliates: The best recovery rates were obtained with *O. minima* (94 %), *C. inflata* (93 %) and *K. muscicola* (86 %) (fig. 2a). In 5 of 32 experiments slightly more than 100 % of the individuals were recovered (figs. 2a, b). A possible reason for this is the high motility of some species, e. g. *O. minima* and *C. inflata*. There is apositive correlation ($P \leq 0.01$) between the size of the organisms and the percentage of recovery (figs. 2a, b), which is in accordance with the preliminary results of FOISSNER (1983). In the experiments with 5 species together (fig. 2b), about 10 % more individuals were recovered as compared with the single species experiments (fig. 2a). Recovery rates were generally higher in the experiments with soil 2 as compared with soil 3 (fig. 2c).

Testacea: The recovery rates in the single species experiments were about 20 % lower than those in the multiple species experiments (figs. 3a, b). Especially low values were gained for *T. enchelys* and *E. rotunda* in the single species experiment (fig. 3a). With the exception of *N. parvula* and *H. sylvatica* a positive correlation ($P \leq 0.05$) between body size and recovery could be seen (fig. 3a). In the multiple species experiments 100 % of species and 92 % of individuals (full and empty tests) were recovered (fig. 3b). A positive correlation between body size and recovery rate could not be proved ($P > 0.1$).

In sum, 86 % of the testacea, 85 % of the nematodes, 72 % of the ciliates and 47 % of the rotatorians were recovered on average in all experiments (fig. 4).

4. Discussion

Although 52 experiments were performed, our data are not sufficient to solve all problems. However, the most important question about average recovery has been clarified satisfactorily.

Culture techniques can over- or underestimate individual abundances. Sometimes the error is probably very high (FOISSNER 1987). In contrast, direct methods of investigation nearly exclusively underestimate the abundances (fig. 4). The mean recovery rates show, however, that the error is not very dramatic (fig. 4), but it can be considerable with smaller species (fig. 2a). The comparison of the single species and multiple species experiments suggests that the direct method provides good results even with multiple species systems, which are found in nature.

Obviously there is some influence of the soil type on the recovery rate (fig. 2c). A high mineral content, especially of clayey material, causes non-transparent suspensions and reduced recovery (fig. 3a), whereas the humic soil 1 yields very high values even of the small testacean species (fig. 3b). In this soil the recovery rates of the 3 investigated strata show no differences, presumably because the mineral content is substantially lower than in the other soils. Further reasons for the strikingly good recovery in soil 1 are probably the high dilution [0.005 g soil (dm) $\times$ 5 ml^{-1} H_2O, comparable to the direct counting method of COÛTEAUX (1967, 1975)], and the staining of the empty tests, which normally are uncoloured.

The results obtained for the nematodes (fig. 4) correspond with those of ZELL (1985), who discussed the methodological shortcomings in soil nematology and found direct counting to be the best method.

The percentage of recovered rotatorians is not satisfactory (fig. 4). Possible reasons for that might be the tendency of rotatorians to accumulate soil particles on their cuticula, and their inactivity as a result of transfer shock. Further experiments should be performed, since the few tests performed by us are not sufficient to draw a serious conclusion.

In general, our results indicate a correlation between recovery rate and size and motility (*O. minima !*) of organisms, soil type, dilution and staining (testacea). However, there is the great probability of an "individual error". Therefore each worker should estimate *his* error, if the results are used for studies of energy turnover etc. For comparison of experimental sites and related investigations, our mean values seem to be a reliable basis for estimating the counting error.

By using a higher magnification, recovery rates could probably be improved, but the working time would increase so much that one could not recommend this method for practical purposes, e.g., bioindication studies. The partial loss of the smaller species, which have often very short generation times, is undoubtly a disadvantage in ecosystem studies. But on the other hand, the reduction of soil volumes and the use of increased magnifications, as is characteristical for the method of COÛTEAUX (1967, 1975), causes the loss of a part of the rare autochthonous species, which are often voluminose forms. One individual of *Centropyxis oomorpha*, for instance, is equivalent to 40 individuals of *Trinema lineare* with respect to biomass (FOISSNER 1987).

Unfortunately, a comparison of our results with those of culture techniques is difficult, because there are only a few experiments with ubiquitous, well growing species, which yielded a recovery of about 70 % (CUTLER 1920; SINGH 1946). These values are comparable to our findings. But direct methods have the great advantage that they need much less laboratory equipment and that they do not overestimate the abundances, which could be a serious problem with culture techniques (FOISSNER 1987).

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6. Zusammenfassung

[Untersuchungen zur Effektivität einer direkten Zählmethode bei der Ermittlung der Abundanz mikroskopischer Bodenorganismen]

Es wird eine direkte mikroskopische Methode zur Erfassung der Abundanzen der aktiven Ciliaten, Testaceen, Nematoden und Rotatorien beschrieben. Die Zählung erfolgt in einer wäßrigen Boden-Suspension. Die Effektivität des Verfahrens wurde mit „Wiederfang"-Experimenten geprüft. Es wurden 22—120 % der Ciliaten (durchschnittlich 72 %), 46—100 % der Testaceen (durchschnittlich 86 %), 70—100 % der Nematoden (durchschnittlich 85 %) und 33—67 % der Rotatorien (durchschnittlich 47 %) wieder gefunden. Für die Ciliaten wurde eine starke positive Korrelation zwischen Körpergröße und Wiederfangrate festgestellt. Die Resultate weisen darauf hin, daß die direkte Zählung eine brauchbare Methode für die Abschätzung von Abundanzen ist. Im Vergleich zu den bei Protozoen häufig angewandten Kulturmethoden bietet sie den Vorteil der sehr einfachen Durchführbarkeit und der sehr geringen Möglichkeit einer Überschätzung der Abundanzen aktiver Individuen, die ein ernstes Problem bei Kulturmethoden ist.

Schlüssel-Wörter: Protozoa, Testacea, Ciliata, Nematoda, Rotatoria, Abundanz, Zählmethoden.

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Appendix: Detailed explanation of fig. 1.

Fresh soil of soil 3 was used. In the left graph the individuals which were found in 1 g of the soil represent 100 %. The 1 g soil sample was counted by subsampling of 0.2 g (5 × 0.2 g). The curves are nearly straight, indicating a homogenous distribution of the organisms. That means, one could stop counting at 0.2 or 0.4 g and multiply the gained individual number 1 × 5 or 1 × 2.5 to get the individual number of 1 g soil. The error would be tolerable. In the right graph the same was done for the species number. In soils with moderately high numbers of ciliates about 85 % of the species, which are found in 1 g, occur in the 0.4 g subsample. This is certainly a good compromise between working time and winning of additional information. For soils with few ciliates, a sample mass of 0.4 g is obviously too little.

Synopsis: *Original scientific paper*

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A direct microscopic method for the estimation of the numbers of active ciliates, testacea, nematodes and rotatorians is described. Counting is performed in an aqueous soil suspension. The efficiency of the method was tested with "recovery" experiments. 22—120 % of the ciliates (mean 72 %), 46—100 % of the testacea (mean 86 %), 70—100 % of the nematodes (mean 85 %) and 33—67 % of the rotatorians (mean 47 %) were recovered. A strong positive correlation between body size and percentage of recovery could be shown for the ciliates. The results suggest that direct counting is a reliable method for the estimation of abundances. In comparison to culture techniques, which are frequently used for protozoa, this method offers the advantages of a very simple procedure and a very limited possibility of over-estimation of the abundances of active individuals, which is a serious problem with culture techniques.

Key words: Protozoa, Testacea, Ciliata, Nematoda, Rotatoria, abundance, counting methods.