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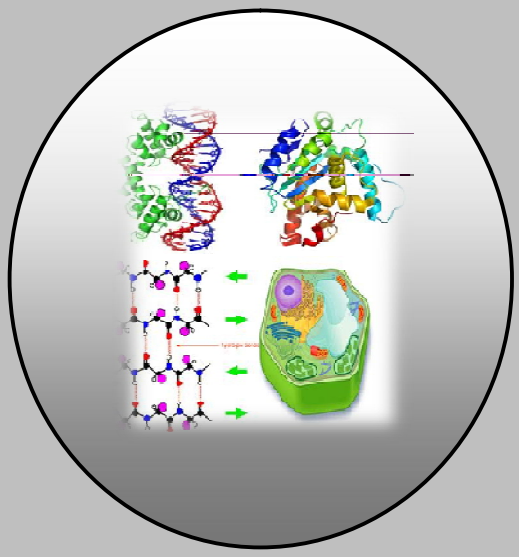

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A Study of DNA Extraction and Quantification Protocols for Soil Microflora

***Hamid Kheyrodin and **Khosro Ghazvinian**

***Faculty of Desert Science, Semnan University, Iran**
****Faculty of Veterinary and Animal Science, Semnan University, Iran**

ABSTRACT

Protozoa are abundant in the upper layer (15 cm) of soil and organic manures. Soil moisture, aeration, temperature and pH are the important factors affecting soil protozoa. Heterotrophic flagellates and naked amoebae in soil have traditionally relied on dilution culturing techniques, followed by most-probable-number calculations. Such methods are biased by differences in the culturability of soil protozoa and are unable to quantify specific taxonomic groups, and the results are highly dependent on the choice of media and the skills of the microscopists. This work describes the development of an MPN-PCR assay for detection of the common soil flagellate Heteromita globosa, using primers targeting a 700-bp sequence of the small-subunit rRNA gene. The method was tested by use of gnotobiotic laboratory microcosms with sterile tar-contaminated soil inoculated with the bacterium Pseudomonas putida OUS82 UCB55 as prey. There was satisfactory overall agreement between H. globosa population estimates obtained by the PCR assay and a conventional most-probable-number assay in the three soils.
Key words: PCR, DNA, Soil, Gene and Protozoa.

INTRODUCTION

These are unicellular, eukaryotic, colourless, and animal like organisms. They are larger than bacteria and size varying from few microns to a few centimeters. Their population in arable soil ranges from 10, 000 to 1, 00,000 per gram of soil and are abundant in surface soil. They can withstand adverse soil conditions as they are characterized by "cyst stage" in their life cycle. Except few genera which reproduce sexually by fusion of cells, rest of them reproduces asexually by fission / binary fission. Most of the soil protozoa are motile by flagella or cilia or pseudopodia as locomotors organs. Soil protozoa are generally small and more or less amoeboid or flexible (14). These characteristics make direct counting by microscopy very difficult, as especially heterotrophic flagellates and naked amoebae adhere to and are masked by the soil particles.

Therefore, quantification of flagellates and naked amoebae in soil has traditionally relied on dilution culturing techniques and subsequent most-probable-number (MPN) calculations (9, 10, 45). These laborious methods may yield misleading results, as the culturable fraction of total protozoan populations is unknown.

The application of MPN dilution culturing techniques makes it possible to identify the various culturable taxa by microscopy and to estimate total protozoan numbers in environmental samples. However, identification beyond the genus level by light microscopy is difficult, and the procedure is most likely to underestimate total numbers of protozoa (14). Some genera, such as *Spumella, Bodo,* and *Heteromita,* are easily cultivated, whereas others are clearly underrepresented by this method (14). Also, the frequency distribution of different culturable taxa in the wells may not reflect that of the original sample (19), and it is not possible specifically to quantify taxonomic groups. The results depend on the choice of media for culturing (45) and the skills of the microscopists. Thus, MPN dilution culturing techniques are not likely to provide reliable estimates of subpopulations and of the true diversity within a sample. Hence, new detection methods need to be developed for naked amoebae and flagellates in soil (16, 20).

MPN-PCR assays for prokaryote nucleic acid detection have successfully been applied in both laboratory and field soil and sediment experiments (11, 35, 37, 40). Moreover, molecular detection of human pathogenic protozoa has been successful in clinical research (24, 28). To our knowledge, however, enumeration of soil protozoa by a molecular technique has not been reported. A major reason is the scarcity of DNA sequences from soil protozoa. Without such data, it is impossible to construct specific primers for molecular detection. Biases relating to soil DNA extraction and PCR amplification further complicate the development and application of a successful molecular tool for the detection of soil protozoa (7, 18, 44).

The two taxonomically related genera *Cercomonas* and *Heteromita* are very common and abundant heterotrophic soil flagellates and are therefore considered of great ecological importance (5, 6,14, 46). Heterotrophic flagellates and naked amoebae have a major impact on soil functions because of their predation on bacteria (8, 14, 47). Protozoan predation and competition from indigenous soil bacteria seem to be major reasons why introduced bacteria usually have a low survival rate when introduced into soil in both laboratory and field experiments (1, 13, 21). Thus, for specific applications such as bioremediation of contaminated soils, protozoa may affect the survival and degradation activity of introduced bacteria.

The purpose of this study was to develop a DNA-based method for the detection and quantification of specific flagellate species in soil. The resulting MPN-PCR assay was used for examining the growth and survival of the common soil flagellate *Heteromita globosa* in a gnotobiotic laboratory soil system including polycyclic aromatic compound (PAC)-degrading *Pseudomonas putida* as prey. We compared the method with three sterilized PAC-contaminated soils. To our knowledge, this is the first report of successful enumeration of soil protozoa by an MPN-PCR assay.

SOIL MICROCOSMS

Three soils with different levels of PAC contamination were collected in September 1999 from a former asphalt and tar production plant at Ringe, Denmark. The site has been examined previously with regard to soil composition and amount of PACs (Table 1). Soil samples were taken 10 to 20 cm below the surface, air dried, and sieved through 4-mm mesh. The three soil samples were sterilized by electron irradiation (twice each at 20 kGy) at Risø National Laboratory, Roskilde, Denmark. During the irradiation procedure, the soil temperature never exceeded 55°C. The electron-irradiated samples were stored at 4°C for a maximum period of 4 weeks. Sterility was checked several times during the experiment by plate counting (bacteria and fungi) and dilution culturing in 96-well microtiter plates (protozoa), but no surviving organisms were found. Three microcosms were set up for each soil type (nine microcosms in total). Each microcosm (sterile 120-ml flasks) contained 20.0 g of soil. The microcosm soil was rewetted to 75% of water-holding capacity, except for 1.0 ml that was reserved for the bacterial inoculum. After 3 h of settling, each microcosm was inoculated with 1.0 ml of a bacterial suspension (10^8 CFU ml^{-1}), and the soil was mixed carefully with a sterile spatula. The microcosms were incubated at 20°C in the dark.

SAMPLING

We collected 1.0-g soil samples (dry weight) from each microcosm at days 7, 14, 30, 37, and 44 of the experiment. Each 1.0-g soil sample was suspended in 9.5 ml of 0.010 M phosphate buffer (pH 7.4). The resulting soil suspensions were used for dilution series for drop plating of the bacteria and as inocula for 96-well microtiter plate dilution culturing of the flagellates. Additional 0.50-g soil samples (dry weight) were collected from each microcosm at days 37 and 44 and stored at −20°C in 1.5-ml Eppendorf tubes for DNA extraction.

BACTERIA

The bacterium used for inoculation of the sterile microcosms was *P. putida* OUS82 (29, 30). The strain had been chromosomally marked with the gene for green fluorescent protein (*gfp*), and mutant *P. putida* OUS82 UCB55 was tested and found to show the same metabolic pattern as the wild type (49). *P. putida* OUS82 UCB55 mineralizes naphthalene, phenanthrene, 1-hydroxy-2-naphthoic acid, and salicylic acid (30). The bacterial strain was kept at −80°C in 50% glycerol.

The culturing procedure was as follows. A small amount of the frozen culture was spread on Luria broth (LB) agar plates (34) supplemented with 1.0 g of glucose $liter^{-1}$ and incubated for 24 h at 30°C. A single colony was transferred to 25 ml of LB in a 100-ml Erlenmeyer flask to grow for 18 h at 30°C on a rotary shaker (200 rpm). The culture was then washed three times in hydrocarbon minimal medium 2 (HCMM2) (42), resuspended in HCMM2, and incubated to starvation (24 h) at 30°C. A bacterial suspension of 10^5 CFU g of $soil^{-1}$ was made in HCMM2, and 1.0 ml was inoculated into each soil microcosm. Numbers of CFU were determined on LB agar without antibiotics by a drop plating technique (23). All colonies expressed *gfp* when examined with an epifluorescence microscope (BX50; Olympus, Hamburg, Germany).

FLAGELLATES

The *Cercomonas* sp. (similar to *Cercomonas crassicauda*) and *H. globosa* were originally isolated from a conventionally farmed agricultural soil in Foulum, Denmark (15). The cultures had been kept vivid at 10°C in 50-ml Nunc flasks by subsequent reinoculations with Neff Amoebae Saline (38) containing either 0.10 g of tryptic soy broth (Difco, Detroit, Mich.) liter^{-1} or a sterilized wheat grain as the feeding source for the bacteria that sustained the flagellate populations.

The number of contaminating bacteria introduced into the microcosms with the flagellate inocula was minimized by growing the flagellates for 2 weeks on heat-killed *Escherichia coli* K-12 (DSM 498) and subsequently on starved *P. putida* OUS82 UCB55 for 2 weeks before harvesting. The cultures were frequently inspected by microscopy to ensure vigorous flagellate activity and a small number of non-*gfp*-marked bacteria. No cysts (indicating declining activity) were observed in the cultures harvested for inoculation into the microcosms. At day 30 of the experiment, the soil microcosms were inoculated with 20 μl of each flagellate culture (*Cercomonas* sp. at 2 × 10^3 cells ml^{-1} and *H. globosa* at 2 × 10^4 cells ml^{-1}), and the soil was again mixed with a sterile spatula.

The number of flagellates in the microcosms was estimated by an MPN method on microtiter plates as described by Darbyshire et al. (10) with modifications by Rønn et al. (45). The method is based on a threefold dilution series culturing technique performed with 96-well microtiter plates, with subsequent verification of growth in single wells by examination with an inverted microscope. We used 0.10 g of tryptic soy broth liter^{-1} as the culture medium as previously described (45). The microtiter plates were placed at 10°C in the dark and examined after 1 and 3 weeks of growth.

Construction of Specific Flagellate Primers

The 18S ribosomal (rDNA) genes of the *H. globosa* and *Cercomonas sp*. clonal cultures were sequenced (L. Fredslund, F. Ekelund, and N. Daugbjerg, unpublished data). Two 700-bp 18S rDNA fragments were targeted for the MPN-PCR assay. Primer specificity was assessed by BlastN analysis (2) and by PCR results, which proved negative for DNA extracted from cultures of five related organisms (of the genera *Heteromita*, *Thaumatomonas*, and *Cercomonas*). These two tests indicated the specificity of the two sets of primers. However, when applied to DNA extracted from soil (see below), the *Cercomonas* primers produced a range of nonspecific banding patterns. These patterns were also present when the primers were used to amplify DNA from soils that had been inoculated only with *H. globosa* (data not shown). We therefore proceeded only with MPN-PCR detection of *H. globosa*. For *H. globosa,* the specific primer sequences were positioned in variable regions V4 and V7 of the 18S rRNA molecule (Heteromita-Forward: 5′ TTGTCGGCCCACGGTTTCGT 3′; Heteromita-Reverse: 5′ GAATCCTTTGTCGAACTATTTAGC 3′).

DNA Extraction from Soil Samples and PCR Amplification

DNA was extracted from 0.5 g of soil (dry weight) with a FastDNA SPIN Kit for Soil (Bio 101, Vista, Calif.) by following the manufacturer's instructions. For the bead beating step, we used a Fast Prep FP120 machine (Bio 101) (3).

PCR conditions for the specific primers were optimized with DNA extracted from the clonal cultures of the two flagellates. Optimization was achieved by varying the annealing temperature (58 to 62°C, in 1°C steps) and the number of amplification cycles (10 to 50, with steps of 10 cycles). Tenfold dilution series of DNA were used to examine the DNA detection limits with the various amplification conditions.

MPN-PCR Conditions

Primers Heteromita-Forward and Heteromita-Reverse were used to perform quantitative detection of the 18S rDNA molecules of the *H. globosa* population in the microcosms by the MPN-PCR assay. Tenfold dilution series of each soil DNA extract were made with RNA- and DNase-free H_2O, and 1 μl of each dilution was used as a template in a 25-μl PCR (26) with Ampli *Taq* Gold polymerase (Applied Biosystems, Foster City, Calif.). The second highest 10-fold dilution (10 μl into 90 μl) to produce the target 700-bp amplification product was selected as the starting point for triplicate threefold dilution series (10 μl into 20 μl) of each DNA sample to constitute the complete MPN-PCR matrix (18 tubes). The PCR program for the MPN-PCR assay was as follows: 10 min at 95°C; 50 cycles of 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C; 6 min at 72°C; and final hold at 4°C. PCR was performed on a Techne (Genius) thermal cycler. Products were run in a 1.5% agarose gel and were ethidium bromide stained before detection with digital gel documentation equipment (Gel Doc 2000; Bio-Rad Laboratories, Hercules, Calif). The numbers of positive and negative tubes that produced amplification products were scored for each sample.

Data Analysis

MPN calculations were made with the computer-assisted method developed by Briones and Reichardt (4). Values were log transformed prior to two-way repeated-measures analyses of variance of CFU counts, MPN estimates made by the dilution culturing technique, and rDNA copy number estimates made by the MPN-PCR assay.

Bacteria

The growth and survival of *P. putida* did not differ significantly among the three soils (Fig. (Fig.1).1). The numbers of CFU increased by the same rate (~0.65 day^{-1}) until a population level of ~10^9 CFU g of $soil^{-1}$ was reached at day 14. Inoculation of flagellates into the microcosms on day 30 caused small and insignificant fluctuations in bacterial CFU.

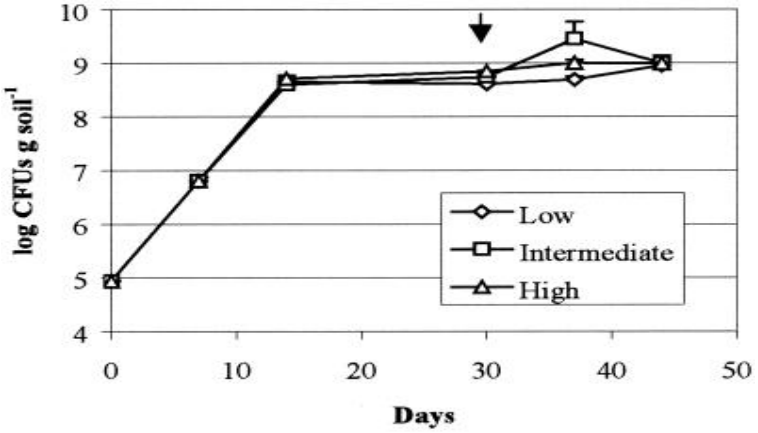

Figure. 1.
Numbers of CFU of *P. putida* OUS82 UCB55 in three sterilized soils with low, intermediate, and high levels of PAC content, as measured by drop plating. The values given are log means for three replicate microcosms; error bars represent one standard error of the mean. No significant differences were seen between growth of the inocula and final populations in the three soils. An arrow indicates inoculation with two flagellate species on day 30 of the experiment.

MPN Dilution Culturing Technique

After inoculation, both flagellate species multiplied vigorously in all soils. The numbers of *Cercomonas* cells increased to 1.1×10^3 g of soil^{-1} and 3.5×10^3 g of soil^{-1} for the low-PAC soil at days 37 and 44, respectively. The corresponding *Cercomonas* numbers in the intermediate-PAC soil were 1.7×10^3 g of soil^{-1} and 5.0×10^3 g of soil^{-1}, and those in the high-PAC soil were 5.8×10^2 g of soil^{-1}and 1.6×10^3 g of soil^{-1}. There were significantly fewer cells of both *H. globosa* (Fig.2A) and *Cercomonas* sp. in the most highly contaminated soil on day 37 ($P < 0.01$, as determined by the Tukey multiple-comparison test). On day 44, there were still significantly fewer *Cercomonas* sp. cells ($P < 0.05$) in the heavily contaminated microcosms, whereas *H. globosa* numbers were comparable in all three soils at day 44.

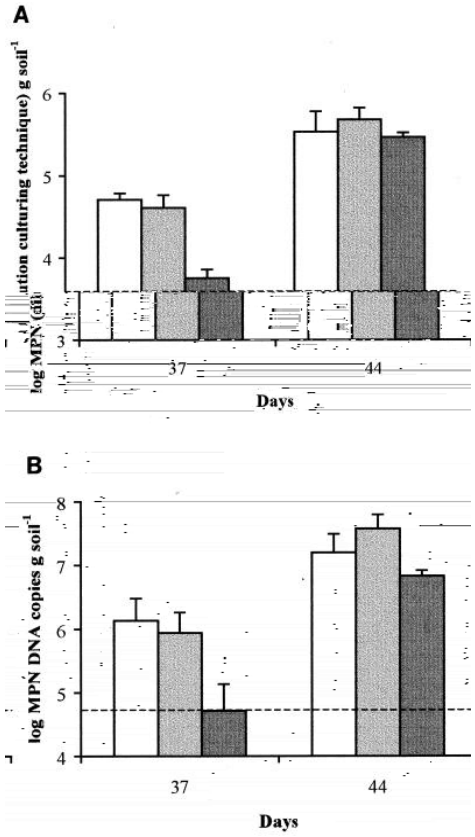

Figure. 2.
(A) MPN dilution culturing technique estimates of numbers of *H. globosa*. Values are given as log means for three replicates; error bars represent one standard error of the mean (low: white columns; intermediate: light gray columns; high: dark gray columns). On day 37, the mean estimated numbers in the soil contaminated with the highest PAC levels were significantly lower than those in the soils contaminated with the medium and low levels. This difference was no longer present at day 44 ($P < 0.01$). The broken line indicates the level of inoculation. (B) MPN-PCR estimates of *H. globosa* rDNA copy numbers. Values are given as log means for three replicates; error bars represent one standard error of the mean. Columns are as defined for panel A. On day 37, the mean estimated numbers in the soil contaminated with the highest PAC levels were significantly lower than those in the soils contaminated with the medium and low levels. This difference was no longer present at day 44 ($P < 0.05$). The broken line indicates the level of inoculation.

MPN-PCR

On day 37, the MPN-PCR estimate of the *H. globosa* 18S rDNA copies in the most highly contaminated soil was significantly lower ($P < 0.05$) than the estimates obtained for the soils contaminated at low and medium levels (Fig.2B). There was no significant difference among the rDNA copy numbers in the three soils at day 44. MPN-PCR was also performed on DNA extracted directly from samples of uninoculated sterile soils with the *H. globosa*-specific primers (data not shown). The presence of PCR products showed that all three irradiated soils contained a low background of intact indigenous *H. globosa* rDNA. As the copy numbers estimated by MPN-PCR did not rise above 10^3 g of soil^{-1} and as the dead cells and the free nucleic acids presumably had been degraded by the inoculated bacteria before flagellate inoculation on day 30, this background was disregarded in the calculations.

The two methods provided population estimates that agreed with respect to relative differences among the samples (Fig.3) Regression analysis revealed a significant ($P < 0.01$) linear relationship between mean dilution culturing technique values (x) and rDNA copy estimates (y): $y = 43x$.

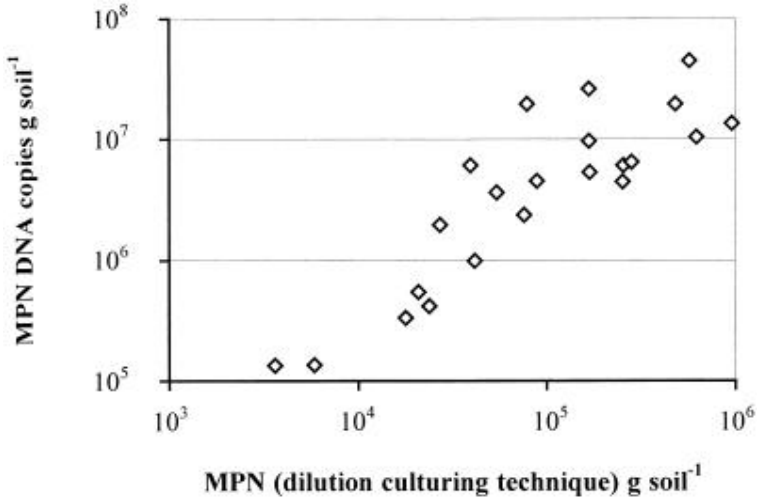

Figure. 3.
Estimates obtained by use of the MPN dilution culturing technique and the MPN-PCR assay developed for this study. Linear regression analysis between mean log population and rDNA copy number estimates revealed a significant ($P < 0.01$) linear relationship between the two, where $y = 43x$.

DISCUSSION

Population Dynamics of the Introduced Organisms

P. putida OUS82 UCB55 faced no competition from other bacteria or predators at the time of introduction to the microcosms. Dead cells and other organic matter present from the sterilization procedure provided excellent growth conditions for the inocula and allowed them to persist at high densities in the soils. High levels of PACs did not affect the numbers of *P. putida* CFU.

The two introduced flagellates were able to survive and grow on *P. putida* in all of the three soils examined. For the most highly contaminated soil, the growth of *H. globosa* at days 30 to 37 was significantly reduced (Fig.2A and B), probably because only a small fraction of the flagellate inoculum was able to adapt to the high levels of toxic compounds. These organisms, on the other hand, were able to establish a large population in the most highly contaminated soil from days 37 to 44.

The structure and moisture of the soil determine whether the flagellates gain access to the prey microhabitats in soil pores and aggregates (14, 17, 22, 41). In this study, attempts were made to provide optimal moisture conditions for the flagellate populations, but the high content of fine soil particles and the destruction of soil structure by mixing may have limited flagellate prey exploitation.

Motile *P. putida* OUS82 UCB55 was able to sustain high population densities in the presence of predatory flagellates under these very controlled conditions. We attribute this result to cryptic growth and successful bacterial colonization of the smallest micropores in the soil, where the bacteria were protected from flagellate predation (14, 31). Furthermore, single-species experiments have demonstrated that flagellate populations often form resting cysts before the prey has been fully exploited (15).

Enumeration of *H. globosa* by the MPN Dilution Culturing Technique and the MPN-PCR Assay

A comparison of Fig.2A and B shows that the MPN-PCR estimates agreed very well with the estimates obtained with the traditional MPN dilution culturing technique. Figure 3 reveals a linear correlation between the two kinds of estimates, enhancing the reliability of the molecular detection results. The linear regression analysis suggested that the number of rDNA copies for *H. globosa* was about 40 to 50 per individual genome.

MPN estimates obtained by the dilution culturing technique and PCR were comparable because the experimental setup provided good growth conditions for the flagellates. The growth of *H. globosa* was vigorous during the experiment, and as active *H. globosa* is easily cultivated, it is reasonable to assume a high degree of flagellate survival and proliferation in the microtiter plates used for the culturing technique.

Hence, we believe that our MPN dilution culturing technique provides good estimates of actual population sizes.

The suitability of PCR procedures for exact quantitative purposes has been questioned by Ferre (18) and Rongsen and Liren (44), who argued that MPN-PCR estimates are only semiquantitative. It is true that all MPN procedures provide estimates and not absolute quantification. The MPN-PCR quantification gives a conservative estimate bounded by the loss of target DNA during extraction and sample dilution and by the extent of PCR inhibition during amplification. Compared to quantitative competitive PCR assays, MPN-PCR assays are generally less influenced by violations of the underlying PCR assay assumptions. In competitive PCR assays, equal amplifications of template and standard nucleic acids can be difficult to ensure (33, 51). Furthermore, it is essential to run the PCR within the exponential phase of the amplification, when none of the PCR ingredients is yet depleted (12, 54). In MPN-PCR assays, the end point of DNA amplification is the terminal plateau phase of PCR, and the procedure is thus relatively robust and able to accommodate wide differences in amplification efficiency without affecting the estimation of DNA target copy numbers (7).

After the development and validation of new primers, an MPN-PCR assay can provide detailed knowledge of the dynamics of protozoan subpopulations in soil, because these primers can be designed to target various taxonomic groups. However, estimation by an MPN-PCR assay of total numbers of protozoa in an environmental sample is not possible, since soil protozoa belong to multiple and widely different taxonomic lineages, many of which have not yet been subject to sequencing studies (32, 39, 50).

The numbers of rDNA copies in protist genomes vary among different taxa. Rocio et al. (43) found that 69 isolates of the trypanosome flagellate *Leishmania* subgenus *Viannia* had between 20 and 40 rDNA copies per cell. The parasitic apicomplexan *Theileria parva* possesses only 2 copies (27), whereas the macronucleus of the free-living ciliate *Tetrahymena thermophila* contains 18,000 rDNA copies (52). These characteristics create the problem of converting estimates of rDNA copy numbers into numbers of organisms.

An MPN-PCR assay can be biased by suboptimal DNA extraction and PCR protocols. Care should therefore be taken to optimize every step of the procedure. With an MPN-PCR assay, it should be possible to detect few gene copies if they are present in a PCR tube. For multicopy genes, such as rDNA, the sensitivity of a PCR assay should therefore be improved, compared to that of a culturing assay, by a lowered detection limit. Any environmental contaminant present in a sample will be minimized as a consequence of dilution (7, 53). Hence, at medium and high template concentrations, the MPN-PCR assay is a strong tool. Environmental contaminants may significantly affect detection when only a low copy number of target DNA is present in a sample (25, 36).

Experience from the extraction of bacterial DNA suggests that no single method allows total recovery in all situations. We did not try to compare the extraction efficiencies of different ways of extracting protozoan DNA from soil. However, the FastDNA SPIN Kit for Soil has previously proven highly efficient and reproducible (3, 26) for the extraction of bacterial DNA from soil, as shown with spores from the gram-positive *Actinomycetes,* normally regarded as difficult to lyse (48).

We therefore selected the Fast DNA SPIN Kit for Soil for our extraction and purification of protozoan DNA from soil.

The application of MPN-PCR assays for soil protozoa is currently limited by the scarcity of molecular data, a factor which prevents the development of specific primers for the major soil protozoan lineages. Future construction of specific primers targeting various protozoan taxa can provide detailed knowledge about population dynamics that cannot be obtained by culturing methods or direct counting. When combined with sequencing of products from environmental samples, molecular detection also provides a method for identifying many previously unknown protozoan taxa. The present results should encourage further experimental work within the field of molecular detection of soil protozoa.

Table 1. Soil composition and content of PACs in the three soils used for this study.

Component	Soil characteristics (% of dry wt) in soils with the following level of contamination:		
	High	Medium	Low
Clay	10.4		13.0
Plain silt	7.6		8.0
Coarse silt	4.6		7.8
Fine sand	23.6		29.2
Coarse sand	44.0		33.0
Total carbon	2.37		1.59
Total nitrogen	0.07		0.08
Total (cation exchange capacity (milliequivalents/100 g)	9.6		16.4
pH ($CaCl_2$)	7.61	7.55	7.46
Total PAC (µg/g of soil)[a]	1,313.5	30.9	3.2

[a]Torben Nielsen, unpublished results.

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REFERENCES

1. Acea, J. M., Moore, C. R. and Alexander, M. 1988. Survival and growth of bacteria introduced into soil. *Soil Biol. Biochem*. 20: 509–516.
2. Altschul, S. F., Madden, T. L., Schaeffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*. 25: 3389–3402.
3. Borneman, J., Skroch, P. W., O'Sullivan, K. M., Palus, J. A., Rumjanek, N. G., Jansen, J. L., Nienhuis, J. and Triplett, E. W. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Environ. Microbiol*. 62: 1935–1943.

4. Briones, A. M. and Jr., Reichardt, W. 1996. Estimating microbial population counts by 'most probable number' using Microsoft Excel. *J Microbiol Methods*. 35: 157–161.
5. Cavalier-Smith, T. and Chao, E. E. 1996 and 1997. Sarcomonad ribosomal RNA sequences, rhizopod phylogeny, and the origin of euglyphid amoebae. *Arch. Protistenkd*. 147: 227–236.
6. Cavalier-Smith, T. 1998. A revised six-kingdom system of life. *Biol. Rev*. 73: 203–266. Chandler, D. P. 1998. Redefining relativity: quantitative PCR at low template concentrations for industrial and environmental microbiology. *J. Ind. Microbiol. Biotechnol*. 21: 128–140.
7. Clarholm, M. 1985. Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. *Soil Biol Biochem*. 17: 181–188.
8. Cutler, D. W. 1920. A method for estimating the number of active protozoa in the soil*. J. Agric. Sci.* 10: 135–143.
9. Darbyshire, J. F., Whitley, R. E., Greaves, M. P. and Inkson, R. H. E. 1974. A rapid micromethod for estimating bacterial and protozoan populations in soil. *Rev Ecol Biol Sol*. 11: 465–475.
10. Degrange, V. and Bardin, R. 1995. Detection and counting of *Nitrobacter* populations in soil by PCR. *Appl. Environ. Microbiol*. 61: 2093–2098.
11. Diaco R. 1995. Practical considerations for the design of quantitative PCR assays. In: Innis, M. A., Gelfand, D. H., Sninsky, J. J., editors; Innis, M. A., Gelfand, D. H., Sninsky, J. J., editors. PCR strategies. San Diego, Calif: *Academic Press*, Inc. pp. 84–108.
12. Eberl, L., Schulze, R., Ammendola, A., Geisenberger, O., Erhart, R., Sternberg, C., Molin, S. and Amann, R. 1997. Use of fluorescent protein as a marker for ecological studies of activated sludge communities. FEMS *Microbiol. Lett*. 149: 77–83.
13. Ekelund, F. and Ronn, R. 1994. Notes on protozoa in agricultural soil with emphasis on heterotrophic flagellates and naked amoebae and their ecology. FEMS *Microbiol. Rev*. 15: 321–353.
14. Ekelund F. 1996. Growth kinetics of five common heterotrophic soil flagellates. *Eur J. Soil Biol*. 32: 15–24.
15. Ekelund, F., Christensen, S., Ronn, R., Buhl, E. and Jacobsen, C. S. 1996. An automated technique for most-probable-number (MPN) analysis of densities of phagotrophic protists with *lux* AB labelled bacteria as growth medium. *J. Microbiol Methods*. 38: 177–182. England, L. S., Lee, H. and Trevors, J. T. 1993. Bacterial survival in soil: effect of clays and protozoa. *Soil Biol Biochem*. 5: 525–531.
16. Ferre, F. 1992. Quantitative or semi-quantitative PCR: reality versus myth. PCR *Methods Appl*. 2: 1–9.
17. Foissner, W. 1987. Soil protozoa: fundamental problems, ecological significance, adaptations in ciliates and testaceans, bioindicators, and guide to the literature. In: Corliss, J. O., Patterson, D. J., editors; Corliss, J. O., Patterson, D. J., editors. Progress in protistology. Vol. 2. Bristol, United Kingdom: *Biopress*, Ltd. pp. 69–212.
18. Foissner, W. 1999. Soil protozoa as bioindicators: pros and cons, methods, diversity, representative examples. *Agric. Ecosyst. Environ*. 74: 95–112.
19. Habte, M. and Alexander, M. 1977. Further evidence for the regulation of bacterial populations in soil by protozoa. *Arch. Microbiol*. 113: 181–183.
20. Heijnen, C. E., Van Elsas, J. D., Kuikman, P. J. and Van Veen, J., A. 1988. Dynamics of *Rhizobium leguminosarum* biovar *trifolii* introduced into soil: the effect of bentonite clay on predation by protozoa. *Soil Biol. Biochem*. 20: 483–488.

21. Hoben, H. J. and Somasegaran, P. 1982. Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. *Appl. Environ. Microbiol*. 44: 1246–1247.
22. Homan, W. L., Vercammen, M., De Braekeleer, J. and Verschueren, H. 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. *Int. J. Parasitol*. 30: 69–75.
23. Jacobsen, C. S. 1995. Microscale detection of specific bacterial DNA in soil with a magnetic capture-hybridization and PCR amplification assay. *Appl. Environ. Microbiol*. 61: 3347–3352.
24. Johnsen, K., Enger O., Jacobsen, C. S., Thirup, L. and Torsvik, V. Quantitative selective PCR of 16S ribosomal DNA correlates well with selective agar plating in describing population dynamics of indigenous *Pseudomonas* spp. in soil hot spots. *Appl. Environ. Microbiol*. 65: 1786–1789.
25. Kibe, K. M., Ole, M. O. K., Nene, V., Khan, B., Allsopp, B. A., Collins, N. E., Morzaria, S. P., Gobright, E. I. and Bishop, R. P. 1994. Evidence for two single copy units in *Theileria parva* ribosomal RNA genes. *Mol Biochem Parasitol*. 66:249–259.
26. Kikuta, N., Yamamoto, A. and Goto, N. 1996. Detection and identification of *Entamoeba gingivalis* by specific amplification of rRNA gene. *Can J. Microbiol*. 42: 1248–1251.
27. Kiyohara, H., Takizawa, N., Nagao, K. 1992. Natural distribution of bacteria metabolizing many kinds of polycyclic aromatic hydrocarbons. *J. Ferment. Bioeng*. 74: 49–51.
28. Kiyohara, H., Torigoe, S., Kaida, N., Asaki, T., Iida, T., Hayashi, H. and Takizawa, N. 1994. Cloning and characterization of a chromosomal gene cluster, *pah*, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. *J. Bacteriol*. 176: 2439–2443.
29. Kuikman, P. J., Van Elsas, J. D., Jansen, A. G., Burgers, S. L. G. E. and Van Veen, J. A. 1990. Population dynamics and activity of bacteria and protozoa in relation to their spatial distribution in soil. *Soil Biol. Biochem*. 22: 1063–1074.
30. Kumar, S. and Rzhetsky, A. 1996. Evolutionary relationships of eukaryotic kingdoms. *J. Mol. Evol*. 42: 183–193.
31. Lee, S. Y., Bollinger, J., Bezdicek, D. and Ogram, A. Estimation of the abundance of an uncultured soil bacterial strain by a competitive quantitative PCR method. *Appl. Environ. Microbiol*. 62: 3787–3793.
32. Maniatis, T., Fritsch, E. F. and Sambrook, J. 1982. Molecular cloning—a laboratory manual. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory.
33. Michotey, V., Mejean, V. and Bonin, P. 2000. Comparison of methods for quantification of cytochrome cd_1-denitrifying bacteria in environmental marine samples. *Appl. Environ. Microbiol*. 66: 1564–1571.
34. Miller, D. N., Bryant, J. E., Madsen, E. L. and Ghiorse, W. C. 1999. Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol*. 65: 4715–4724.
35. Murakami, K., Kanzaki, K., Okada, K., Matsumoto, S. and Oyaizu, H. 1997. Biological control of *Rhizoctonia solani* AG2–2 IIIB on creeping bentgrass using an antifungal *Pseudomonas fluorescens* HP72 and its monitoring in fields. *Ann. Phytopathol. Soc. Jpn*. 63: 437–444.

36. Page F C. 1998. A new key to freshwater and soil gymnoamoebae. Culture Collection of Algae and Protozoa. Ambleside, United Kingdom: *Freshwater Biological Association*.
37. Patterson, D. J. 1999. The diversity of eukaryotes. *Am Nat*. 154: S96–S124.
38. Picard, C., Ponsonnet, C., Paget, E., Nesme, X. and Simonet, P. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. *Appl. Environ. Microbiol*. 58: 2717–2722.
39. Postma, J., Hok-A-Hin, C. H. and Voshaar, J. H. O. 1990. Influence of the inoculum density on the growth and survival of *Rhizobium leguminosarum* biovar *trifolii* introduced into sterile and non-sterile loamy sand and silt loam. FEMS *Microbiol Ecol*. 73: 49–58.
40. Ridgway, H. F., Safarik, J., Phipps, D., Carl,

51. Wilson, I. G. 1997. Inhibition and facilitation of nucleic acid amplification. *Appl. Environ Microbiol*. 63: 3741–3751.
52. Zachar, V., Thomas, R. A. and Goustin, A. S. 1993. Absolute quantification of target DNA: a simple competitive PCR for efficient analysis of multiple samples. *Nucleic Acids Res*. 21: 2017–2018.

Corresponding author: Prof. Hamid Kheyrodin, Faculty of Desert Science, Semnan University, Iran. **Email:** hkhyrodin@yahoo.com