MINERALIZATION OF NITROGEN

BY PROTOZOAN ACTIVITY IN SOIL

Promotor:	dr. A.J.B. Zehnder hoogleraar in de microbiologie
Co-promotor:	dr. J.A. van Veen hoofd afdeling bodembiologie, Stichting Ital en Instituut voor Bodemvruchtbaarheid

PETER KUIKMAN

MINERALIZATION OF NITROGEN BY PROTOZOAN ACTIVITY IN SOIL

Proefschrift
ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op woensdag 17 januari 1990
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

ISN: 5180332

"Now the number of mice is largely dependent,
as everyone knows, on the number of cats"

Charles Darwin (1859)

The work for this thesis has been accomplished at Research Institute Ital, P.O. Box 48, 6700 AA Wageningen (NL). Part of the work reported in this thesis (chapters 2, 4, 5 and 6) was carried out within the Dutch Programme on Soil Ecology of Arable Farming Systems. The author of the thesis is currently attached to the Institute for Soil Fertility, Haren (NL).

NN08201, 1331

STELLINGEN

1. Het tellen van protozoen in grond met behulp van de Most Probable Number techniek levert meer werk dan resultaten op.

2. Alle bodem organische stof is in chemische zin gemakkelijk afbreekbaar en alleen de chemische organisatie en de distributie ten opzichte van bodemorganismen voorkomt dat dit plaatsvindt.
Duxbury et al. (1989). Biogeochemistry (in press)

3. Microörganismen en protozoën zijn samen verantwoordelijk voor ruim 90% van de mineralisatie van stikstof in grond.

4. Het oplossen van problemen in de bodembiologie met behulp van een chemostat getuigt van eenzelfde naiviteit als het oplossen van het wereldvoedselprobleem door te stellen dat de aarde voor 40 tot 50 X 10^9 mensen voedsel kan produceren.
Stout, JD (1973). Amer. Zool. 13: 193-201

5. Om het functioneren van bodem-oecosystemen via introductie van genetisch gemodificeerde microörganismen succesvol te beïnvloeden, moet men ook de bodem manipuleren.

6. Water beweging in de bodem, mits voldoende begrepen, is het meest effectieve instrument om de verdeling van microorganismen na introductie in grond te reguleren.
Parke JL et al. 1986. Soil Biol Biochem 18: 583-588

7. Theoretische inzichten worden niet verkregen door 'goodness of fit' sec, maar uitsluitend door de afwijkingen in de uitkomsten van modellen, die volgen op de manipulatie van parameterwaarden, te verklaren.

8. Een combinatie van technieken uit de microbiele oecologie en de moleculaire biologie biedt veel potentiele mogelijkheden om inzicht te verkrijgen in de oecologie van microörganismen.

9. Het grootste deel van natuurlijke grond is steriel.

10. Protozoen zijn predatoren die uitsluitend planten eten.

11. Het belang van een weinig selectieve werking van chemische bestrijdingsmiddelen, toegepast in de bodem, wordt onderschat.

12. De indeling van de biologie in de klassieke subdisciplines microbiologie en zoologie belemmert het onderzoek naar voedselrelaties in grond tot op de dag van vandaag.

13. Het ontbreken van de zegswijze 'je kunt hier van de grond eten' als uitgangspunt in het Nationaal Milieu Beleidsplan is niet een van de zorgen voor morgen maar een zorg voor vandaag.

Stellingen behorende bij het proefschrift 'Mineralization of nitrogen by protozoan activity in soil' van Peter Kuikman. Wageningen, 17 januari 1990.

NNO 201, 1331

ERRATA

Chapter 3, on *page 34*:

in Figure 3 and 4 the graphs do not correspond to the legends. The graph presented in Figure 3 should be placed in Figure 4 while the graph presented in Figure 4 should be placed in Figure 3, respectively.

Chapter 4, on *page 53 and 54*:

in figure 4 and 5 the graphs do not correspond to the legends. The graph presented in Figure 4 on page 53 should be placed in Figure 5 on page 54 while the graph presented in Figure 5 on page 54 should be placed in Figure 4 on page 53, respectively.

Chapter 5, on *page 71*:

in the legend to Figure 5, replace "Recovery of bacterial ^{15}N in plant nitrogen as percentage of inoculated amount of ^{15}N" by "Recovery of bacterial ^{15}N in plant nitrogen as mg ^{15}N per microcosm."

Chapter 6, on *page 84 and 85*:

in Figure 1 and 2, the symbols used refer to: (+) no protozoa, (x) 1:10 diluted protozoan inoculum and (*) non diluted protozoan inoculum.

CONTENTS

CHAPTER 1

INTRODUCTION

Nitrogen is essential for plant growth. Many soils do not have sufficient nitrogen in available forms to support the levels of crop production that man requires for sufficient food and fibre production. Therefor, nitrogen is often applied to arable soils as a fertilizer (Newbould 1989). However, the application of nitrogen fertilizer causes environmental problems such as leaching of nitrate into groundwater which is used as drinking water and ammonia volatilization into the atmosphere. This ammonia is deposited on the surface of the earth. Nitrification then results in acidification of soils and damage to plants.

To improve efficient use of nitrogen in agriculture, more precise knowledge is needed of the dynamics of nitrogen turnover in soils and the internal cycle of nitrogen in the soil (Newbould 1989). Plants, even in heavily fertilized conditions that are found in modern agriculture, obtain part of their nitrogen requirements through microbial mineralization from various organic sources in soil (Schnurer and Rosswall 1987) including microbial biomass (Lethbridge and Davidson 1983). The release of nitrogen and other nutrients from soil organic matter or microbial biomass for plant uptake is determined primarily by the balance between mineralization and immobilization processes. In soil, microbial activity is the 'motor' driving soil nutrient cycles (Van Veen *et al.* 1989). A continuous turnover of nitrogen occurs as a result of cell lysis, the formation of new microbial biomass and the turnover of other nitrogenous cell compounds (Woldendorp 1981). Microorganisms and the organic detritus decomposer pathway account for a majority of the energy flow and nutrient turnover in most ecosystems (Wiegert and Owen 1971). Apart from their activity, microorganisms in soil are also important through their potential to act as a source and sink for nutrients.

In decomposition studies, the activities of the soil microflora are rarely separated from the activities of micro- and mesofauna elements. Therefore, the specific role of soil animals in soil nutrient transformations is often not considered (Coleman *et*

al. 1983). However, evidence is accumulating that interactions between microflora and fauna, in particular protozoa, are responsible for a significant portion of the mineralization of nitrogen in soil.

Protozoa

Protozoa are unicellular, eukaryote organisms, which were first surveyed and described by Anthonie van Leeuwenhoek (1632-1723) who developed a primitive microscope and called them animalicules or 'little animals' (Van Leeuwenhoek 1677). These microorganisms are the smallest but most numerous of all animals. Several tenthousands of species have been described although the taxonomy of the protozoa still is subject to debate. Their size may range from 2 μm (nanoflagellates) to over 6000 μm (some sarcodina species). Free-living heterotrophic protozoa are to be found in virtually all ecosystems in the world. Corliss (1973) attributes their wide geographical distribution to their tremendous range of adaptability to environmental conditions. With respect to temperature, pH and osmotic pressure as found in soils, no clear limits to protozoan life have been described in literature other than a wide tolerance by protozoa. For example, protozoa are found to be active at a range of temperatures from 0°C up to 50°C. Water though, is an absolute requirement for protozoan life. Most soil protozoa have the ability to form cysts to survive dry conditions (Bryant *et al.* 1982).

The protozoa that are generally found in arable soils include flagellates, naked amoebae, and ciliates. Heterotrophic free-living flagellates and naked amoebae are the most numerous in arable soils (Stout and Heal, 1972) and populations of 10^3 to $10^6 \times g^{-1}$ dry soil are commonly found (Singh, 1946, 1949; Singh and Crump 1953). In arable soil, Darbyshire and (1967) reported approximately 6×10^4 flagellates g^{-1} dry soil and this is about an order of magnitude lower than the numbers of small amoebae found by Clarholm (1981). The average sizes for the three main taxonomical groups of protozoa in soil, i.e. flagellates, amoebae and ciliates, are reported to be 50 μm^3, 400 μm^3 and 3000 μm^3 (Stout and Heal 1972). These figures suggest that, based on their numbers and sizes, amoebae are the most important group within the protozoan population. The protozoan biomass is estimated to be only about $2g \times m^{-2}$ (Stout and Heal 1967) which is approximately 5-10 $\mu g\ C \times g^{-1}$ dry soil. This figure should then be compared with estimates of total microbial biomass in agricultural soils, ranging from 300-500 $\mu g\ C \times g^{-1}$ dry soil (Schnurer *et al.* 1986). Even though protozoa constitute a relatively insignificant part of the total microbial biomass (Stout 1973), the importance of protozoa in soil nutrient economy should not be overlooked simply because of their small size or biomass. The importancy is related to the rate of biomass production and not to the biomass itself (Gray and Williams 1971). The protozoan biomass is of a similar order as the biomass estimated for earthworms under arable fields. Together,

protozoa and earthworms make up approximately 90% of the total animal standing crop in soils (Golebiowska and Ryskowski 1977).

Enumeration of protozoa

The methods to count protozoan numbers in soil available to date are:

(1) direct observation which is useful for testate amoebae but is considered inappropriate for ciliates, naked amoebae or flagellates because of their small size and intimite association with soil particles (Foissner 1987). Direct observation rarely provides any indication of protozoan activity for the population is often encysted (Stout and Heal 1972) ;

(2) extraction which is confined to mostly testacea (Stout and Heal 1972) where remains such as the tests can be collected and ciliates (Wagener *et al.* 1986) which migrate in an electric field;

(3) indirect enumeration using culture techniques which essentially is a modification of the dilution method used in soil bacteriology.

The latter method is based on the most probable number (MPN) technique and is the one most extensively used. A series of replicated dilutions of a soil suspension is incubated during a period of approximately 4 weeks. Bacteria are added and serve as the food source for protozoa. At regular time intervals, the presence or absence of protozoa in a series of successive dilutions until extinction, is recorded. The method was started by Killer (1913) and Cutler (1920), and further refined by Singh (1946), Darbyshire (1967), Darbyshire (1974) and Clarholm (1981). This method provides an estimate of the total number of protozoa, both active and inactive forms, in a soil suspension. Several prerequisites should be met: i) protozoa must be easily recognizable in the medium upon multiplication, ii) a single organism must be able to grow and multiply without interference or inhibition (Cunningham 1915) by other organisms including protozoa and iii) the protozoa should be randomly distributed in the soil suspension and upon dilution follow a Poisson distribution (Cochran 1950).

Although the MPN method is the most often used method, it has several disadvantages: i) the MPN estimates have a low order of precision (De Man 1975), ii) at regular time intervals, a large number of samples must be checked for presence or absence of protozoa which makes the method very time consuming and iii) the method does not descriminate between active and inactive (cystic) protozoans. When calculations and assumptions relating to protozoan numbers and biomass are made, it is essential to consider that protozoa in soil are, in general, found as resting and inactive, cystic stages (Singh and Crump 1953). Cutler (1920) developed a method to assess the number of active protozoa. Upon killing the active protozoa by exposure of soil to 2% HCl, the number of active protozoa is calculated as the difference between total number in untreated soil and the number of

cystic protozoa in soil treated with 2% HCl treated. The precision of this method is subject to debate since also a part of the cysts might die.

However, numbers of protozoa in soil are necessary for comparison with other groups of organisms. Numbers of protozoa provide the basic information to estimate biomass and assess their role in element cycling. Until now, no major breakthrough has been achieved in counting the numbers of protozoa in soil. The methods used are basically the same as the ones used by Cutler and others in the twenties and Singh and co-workers in the fourties. Improved microcopes and other machinery have enabled protozoologist to examine more samples more precisely.

Protozoa and their food

The most important food source for free living heterotrophic protozoa are bacteria (Fenchel 1987). In addition they consume algae, yeasts, fungi, nematodes, other protozoa and possibly detritus (Heal and Felton 1970). Protozoa feed by phagocytosis. This way of feeding consists of the enclosure of a food particle in a vacuole where digestion takes place. The remains are excreted into the environment. Amoebae and flagellates probably differ with respect to whether they feed on attached or on suspended bacteria and also on the basis of the size of the food particles (Fenchel 1987).

Extensive studies of the relationship between soil protozoa and bacteria (Singh 1941, 1942, Casida 1989) revealed that soil protozoa can feed on a wide range of bacteria but that they also show food preferences and select bacteria from mixed populations. Especially *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* have been recorded toxic to soil protozoa probably because of a high content of pigments (Groscop and Brent 1964).

Fenchel (1982) estimated that flagellates can ingest (and digest) a total volume of particles per hour that equals their own cell volume. This is consistent with minimum doubling times of such flagellates of about three hours. From the data by Cutler *et al.* (1922), Stout and Heal (1972) calculated the standing crop and annual production of protozoa and estimated that the generation time was from 1 to 3 days. They concluded that the annual turnover was 50-300 times the standing crop of protozoa. Elliott and Coleman (1977) calculated a mean generation time for amoebae of 2.4 days. The yield, reported for amoebae and flagellates, ranges from 40% to 50% (Calow 1977, Coleman *et al.* 1978).

Initially, the views regarding the protozoan control of bacteria in soil remained rather conflicting. Russell and Hutchinson (1909) called attention to the protozoa as a factor prejudicial to the productivity of soil by grazing on beneficial microflora. An increased formation of ammonia was observed, coinciding with an increased number of bacteria immediately following the killing of the protozoa by partial sterilization of the soil. They concluded that the bacterial numbers decreased when conditions become favorable for protozoan development. However, Waksman

(1916) did not observe any influence of protozoa on the ammonification by bacteria. He concluded that either the bacteria destroyed did not take any active part in the ammonification or that the protozoa, destroying some bacteria, influenced beneficially the decomposition of organic matter. Culture solutions containing protozoa had a more pleasant odor than those containing bacteria alone; it appeared as if the protozoa either destroyed the decomposition products or the putrefactive organisms. These observations lead Waksman (1916) to the question; "Are not the protozoa natural and necessary factors in the fertility of the soil?"

A close inter-relationship between populations of amoebae and bacteria in nature was first established by Cutler *et al.* (1922), who enumerated protozoa and bacteria on 365 successive days. They found that the numbers of active amoebae were inversely related to those of bacteria on 86% of the days. This was attributed to protozoan predation on the bacteria. Cutler (1923) found that *Dimastigramoeba gruberi* multiplied within fifteen days from 25000 to 230000 organisms per gram of soil. This result as well as similar results by other authors (Martin and Lewin 1915), proved that amoebae are active in soil. At end of the twenties, there was no doubt that their main food is represented by (soil) bacteria (Severtzova, 1928).

In 1953, Singh and Crump reported that, although partial steam sterilization of soils is generally followed by increasing numbers of bacteria, it is also followed by a marked increase in the size of the protozoan population. In a review on soil protozoa, Russell and Russell (1962) stated: "There must presumably be a relation between the numbers of protozoa and bacteria present in the soil since certain species of bacteria are the principal source of food for the protozoa". They considered the results of the older work unreliable as the techniques used for counting were inadequate. "Hence no reliable estimates can yet be made of the numbers of bacteria that are consumed daily by the soil protozoan population".

Soil from a protozoan point of view

Although high numbers of protozoa are found in soil, they still are aquatic organisms. Hence their mobility, feeding and growth in terrestrial ecosystems will depend upon their ability to cope with fluctuating moisture conditions (Nikitin 1973). Ecologically succesfull species must posses a wide tolerance to moisture fluctuations. However, Cutler *et al.* (1922) found no relation between soil moisture content and numbers of active amoebae.

In soil, protozoa and their bacterial prey inhabit the capillary water in pores between and within the mineral particle aggregates that make up the soil system (Bamforth and Bennett 1985). Hattori (1988) studied the distribution of microorganisms and their behaviour in relation to aggregate structure. He distinguished microbial cells living at the outer part of water stable aggregates form those living at the inner part by using a washing-sonication method as described by Hattori (1967). It was concluded that the majority of bacteria were found inside the

aggregates whereas the protozoa inhabited the outer parts of the aggregates. Vargas and Hattori (1986) showed that the increase in numbers of predators (the ciliate *Colpoda* sp.) was proportional to the initial prey densities in the outer zone of the aggregates. Thus, protozoan predation was restricted to the outer zone. The critical size of the pore neck, that divides pores into outer and inner parts of soil aggregates, was estimated to be 2.5μm and equals a pore diameter of 3 - 6 μm. These data confirmed observations on the activity of protozoa by Darbyshire and Greaves (1967), Darbyshire (1976) and Alabouvette *et al.* (1981). They described that in soils where pores with sizes larger than 6 μm are devoid of water, protozoan activity is halted.

Postma *et al.* (1989) showed that by introducing microorganisms into dryer soils, the distribution of those microorganims could be manipulated. The introduced bacteria tended to be safer from predators when they were inoculated into dryer soils and were expected to reach smaller pores (Postma 1989). She assumed that pores < 3μm are not accessible to protozoa. Hence, only part of the habitable pore space for bacteria in soil (pores with pore-necks > 0.8μm) offers protection from protozoan predation. The habitable and protective pore space was shown certainly not to be a limiting factor for the survival of bacterial cells. Less than 0.5% of this habitable and protective pore space was occupied by bacteria. However, one cannot exclude that locally, the available space and not the availability of substrates limits bacterial growth (Postma and Altemuller 1989, Foster 1988).

Reports on the migration of protozoa under well defined soil moisture conditions are scarce. It has been shown that protozoa do move centimeters per day if adequate water is present (Biczok 1959, Losina-Losinsky and Martinov 1930). However, Vargas and Hattori (1986) hypothesized that the outer zone of aggregates is divided into compartments. The absence of waterfilms connecting these compartments was shown to limit protozoan migration. This facilitates prey persistence in the outer zone which is closely related to the moisture condition of the soil. Since not all of the soil pore space is accessible to protozoa, soil structure is expected to strongly influence the interactions between protozoa and their food.

The activity of protozoa in the soil matrix is limited to conditions with a sufficient soil moisture content as shown for ciliates (Darbyshire 1976, Vargas and Hattori 1986) and amoebae (Bryant *et al.* 1982). Protozoa can endure dry conditions by the formation of cysts (Bryant *et al.* 1982) and so survive for decades. The estimated generation times for protozoa (2 to 48 hours) indicate that protozoa can potentially react very fast and immediate to favourable conditions in soil (Alabouvette *et al.* 1981). Protozoan populations have been shown to respond markedly to the addition of water to dry soils with an increase in total numbers as well as in numbers of active, trophic individuals (Elliott and Coleman 1977; Hunt *et al.* 1989). By using ^{15}N and ^{14}C labelled bacterial cells, Van Veen *et al.* (1985) have found that the turnover of bacterial biomass is significantly affected by soil moisture

fluctuations. Remoistening of dried soils caused a temporary (4 days) flush in carbon and nitrogen mineralization rates. However, no data exist on the specific interactions between bacteria and protozoa and the turnover of bacterial biomass in relation to soil moisture regimes.

Soil furnishes a wide variety of stress factors on protozoan life. Apparently, the most important factors are soil structure and soil moisture limiting the ability of protozoa to meet prey. Thus, the ecology of protozoa seems to be characterized by two important features: the activity is confined to small spaces and consequently small populations and activity is restricted to brief periods when moisture conditions are favourable (Stout, 1973).

Function of protozoa in soil

Since predation by protozoa removes bacteria, one might expect a decrease in bacterial activity and consequently in the decomposition of organic matter and mineralization of nutrients. However, the opposite has been observed many times. A stimulating effect of protozoan grazing on bacterial metabolism was demonstrated for ciliates in marine habitats (Johannes 1965) and for flagellates in freshwater habitats (Barsdate *et al.* 1974). Hunt *et al.* (1977) have developed a simulation model for the effect of protozoan predation on bacteria in continuous culture. The results of the model suggested that upon predation by protozoa, the growth rate of bacteria increased, even though the bacterial biomass was reduced. Bacteria were thought to respond to a higher level of available carbon, nitrogen and phosphorus upon predation. Sofar, a relationship between protozoan grazing on bacteria and nutrient mineralization was established.

However, to establish such a relationship in soils proved to be far more difficult. In sand culture, amoebae decreased the numbers of bacteria but increased the rate of ammonium and carbon dioxide production (Meiklejohn 1930, Telegdy-Kovats 1932). Coleman and co-workers expanded the ideas from observations in marine and freshwater habitats to soil and carried out a series of soil microcosm experiments. The addition of bacteria to sterilized soil microcosms resulted in an initial immobilization of ammonium nitrogen. The introduction of amoebae (*Acanthamoeba polyphaga*) always reduced bacterial numbers (Anderson *et al.* 1978), increased respiration (Coleman *et al.* 1978) and increased nitrogen mineralization (Woods *et al.* 1982). Griffiths (1986) showed an increased nitrogen mineralization in the presence of a ciliate. These results also indicated that nitrogen mineralization in terrestrial systems is not strictly microfloral. Bacterial grazers, such as amoebae, are necessary for mineralization of microbially immobilized nutrients as was shown earlier for aquatic ecosystems by Johannes (1965). By using radiocarbon-labelled glucose, Anderson *et al.* (1981) found that microbial grazing by nematodes increased substrate utilization and mineralization of nitrogen. They hypothesized that, as a result of the grazing activity of microfauna, excretion of

ammonium nitrogen in the soil solution maintained a high metabolic activity in decomposing organisms. Thus, despite reduced numbers of microorganisms, their total activity increased.

Information on the impact of protozoan grazing on bacteria cells and nutrient transformations in planted soils is limited (Stout 1980). Elliott *et al.* (1979) have presented results that indicated a significant role of soil protozoa at the soil-root interface by accelerating the mineralization of microbially immobilized nutrients. In the presence of protozoa more mineral nitrogen was found in soil. Also, plant shoot nitrogen concentration was higher as compared with soils without protozoa. They hypothesized that the effect of protozoa on the mineralization of nitrogen would be greatest under the most N limiting conditions, i.e. without the addition of mineral N fertilizer (Elliott 1978). However, only in case mineral N was added, protozoa accelerated the mineralization of microbially immobilized nitrogen. Clarholm (1985a) presented results that indicated that bacteria can mineralize nitrogen from soil organic matter. In the presence of protozoa, more nitrogen was made available to plants. Based on her experiments in planted microcosms, Clarholm (1985b) suggested that bacteria utilized nitrogen from soil organic matter when supplied with a suitable source of energy, i.e. root exudates. The nitrogen immobilized in microbial cells would then become available to plants when predators such as protozoa consumed these microorganisms and excreted excess ammonium nitrogen.

The rhizosphere is a zone of intense microbiological activity as judged from higher numbers of microorganisms in the vicinity of roots (Katznelson *et al.* 1948; Rovira 1965) presumably caused by the rhizo-deposition of carbon at the root tip (Trofymow *et al.* 1987). Katznelson (1946), Biczok (1956), Geltzer (1963) and Darbyshire and Greaves (1967, 1973) had already shown that amoebae are more numerous in rhizosphere soil where an active development of bacteria is found, than in non rhizosphere soil. Ritz and Griffiths (1987) tested the hypothesis of Clarholm (1985) by adding both glucose and nitrate to soils. They suggested that the addition of glucose would facilitate the immobilization of nitrate in microbial biomass and reduce the leaching of nitrate from soil. The re-mineralization of nitrogen and uptake by plants was stimulated by protozoan activity as indicated by an increased number of amoebae. In unplanted soils, the nitrogen that was mineralized by predator activity was rapidly immobilized again, presumably by microorganisms.

The effect of protozoa, when they are predating on bacteria in soil, could then be threefold:

(1) by grazing bacteria, protozoa do excrete excess ammonium nitrogen (Stout 1973, Fenchel 1986) which is available for plant uptake or

(2) by grazing bacteria, waste products such as cell wall material and other nutrients are deposited which may enhance microbial activity (Hunt *et al.* 1977) and by stimulating bacterial flocculation (Bamforth 1973) or

(3) whilst moving through the soil matrix searching for food particles, protozoa might (re)inoculate (new) substrates with bacteria that adhere to their cell surface or by bacteria that are not digestable and therefore egested (Finlay and Fenchel 1989).

Objectives and approaches

The turnover of nitrogen through the microbial biomass is the key process in nitrogen cycling in soil. Turnover is the process of uptake, transformation and mineralization. Quantitative relations have been established for uptake and intracellular transformation of nutrients such as nitrogen in microbial cells. However, data on the mechanisms of the release of nitrogen from microbial cells, which closes the cycle of the turnover process, are scarce. Nitrogen is essential for plant growth and other processes in soil. The main objective of this study was to determine the impact of protozoan predation of bacteria on the mineralization of nitrogen from bacterial cells in soil.

The relative importance of specific microbial populations in nutrient cycling is difficult to ascertain under field circumstances because of the myriad of interactions among organisms. In order to study the specific interactions between bacteria and protozoa, a system with limited relational complexity was used (Figure 1). The system comprised soil organic nitrogen, microbial nitrogen, mineral nitrogen and plant nitrogen. Thus, a limited number of transformations of nitrogen was studied.

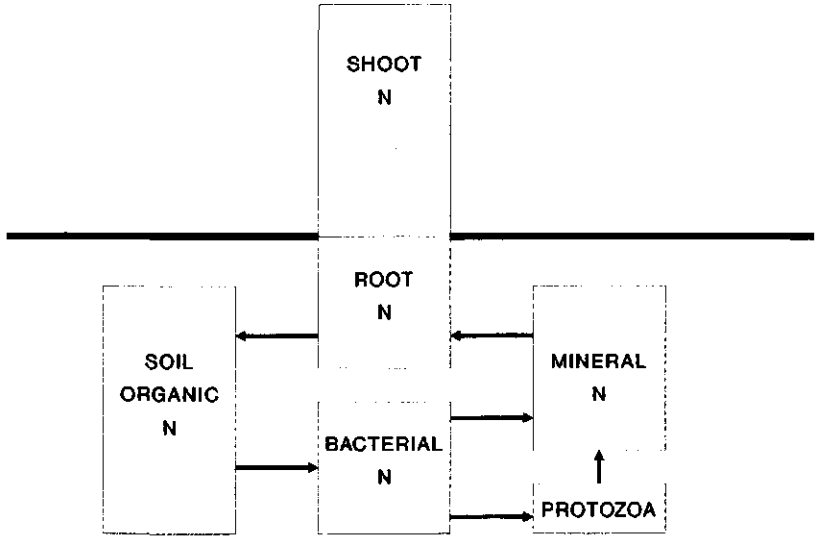

Figure 1 Relational diagram

Throughout the experiments, soil was sterilized by radiation and subsequently specific microorganisms were introduced. The gnotobiotic (presence of kwown organisms) soil microcosms were planted to wheat. The plants served as a nitrogen sink where information on the turnover of nitrogen in soil was accumulated. No mineral nitrogen was added as to ascertain that nitrogen was the factor limiting plant growth and to maximize the sink function of plants, as shown by previous experiments. To determine the role of protozoa in nitrogen transformations, soils were incubated both in the absence and in the presence of protozoa. Representatives of the most abundant taxonomical groups of protozoa in soil, flagellates and amoebae, were used. In all experiments, a loamy sand soil was used as described in chapter 2.

It is well established that soil furnishes a wide variety of constraints on the inhabiting biota. The particular structure of soil makes that the distribution of both protozoa and their prey is patchy (Foster 1988, Postma *et al.* 1989). Protozoa need a surrounding waterfilm for activity and movement. Since the availability of water is highly variable, water most likely is the factor that regulates protozoan activity by determining the mobility and the chances to meet prey organisms in the soil matrix. The transpiration of water by plants could be used to define soil moisture regimes. It is clear that the laboratory microcosm approach has obvious limitations when one aims at relating results to field conditions in nature. However, the microcosm approach does allow to manipulate and measure factors that are difficult to control under field conditions: i) maintaining a specific gnotobiotic composition of the microbial community and ii) the use of (radio)isotopes such as ^{15}N and ^{14}C to trace specific nitrogen and carbon transformations and to follow the fate of specific components such as bacterial nitrogen or carbon or soil organic material (Jansson 1958).

Outline of this thesis

The predation by protozoa on bacteria and the mineralization of bacterial nitrogen was studied in soils containing the basic food chain with bacteria and protozoa. Additionally, the impact of protozoa was studied in relation to the size of the bacterial population (chapter 2). The influence of the distribution and migration of protozoa on the turnover of specific bacterial populations and the transfer of genetic material between bacteria was examined in chapter 3. The inoculum density of protozoa was varied and spatial separation between predator and prey organisms was established in soil microcosms. In chapter 4 and 5, the impact of the soil moisture regime on protozoan predation of bacteria and on the mineralization of nitrogen was investigated both in the presence and in the absence of plants. Stable soil moisture regimes versus fluctuating soil moisture regimes were imposed on soils with bacterial-protozoan communities as to monitor the reaction of protozoa to moisture stresses. Finally, the grazing activity of protozoa and the

impact on the turnover of soil organic carbon and the uptake of nitrogen from soil organic material was studied. The continuous monitoring of carbon dioxide production and the soil moisture fluctuations created by growing plants, enabled the study of bacterial-protozoan interactions in more detail (Chapter 6).

References

Alabouvette C, Couteaux MM, Old KM, Pussard M, Reisinger O and Toutain F (1981) Les Protozoaires du sol: aspects ecologiques et methodologiques Ann Biol 20: 255-303

Anderson RV, Elliott ET, McClellan JF, Coleman DC, Cole CV and Hunt HW (1978) Trophic interactions in soils as they affect energy and nutrient dynamics. III. Biotic interactions of bacteria, amoebae and nematodes Microb Ecol 4: 362-371

Anderson RV, Elliott ET, Coleman DC and Cole CV (1981b) Effect of the nematodes *Acrobeloides* sp and *Mesodiplogaster lheritiere* on substrate utilization and nitrogen and phosphorus mineralization in soil Ecology 62: 549-555

Bamforth SS (1973) Population dynamics of soil and vegetation protozoa. Amer Zool 13: 171-176

Bamforth SS and Bennett LW (1985) Soil protozoa of two Utah cool deserts. Pedobiologia 28: 423-426

Barsdate RJ, Prentki RT and Fenchel T (1974) Phosphorus cycle of model ecosystems: significance for decomposer food chains and effect of bacterial grazers Oikos 25: 239-251

Biczok F (1956) Contributions to the protozoa of the rhizosphere of wheat Acta Zool Acad Sci Hung 2: 115-147

Biczok F (1959) Experimentelle Untersuchingen uber die Wanderung der Protozoen im erdboden Acta Biol Szeged 5: 98-108

Bryant RJ, Woods LE, Coleman DC, Fairbanks BD, McClellan JF and Cole CV (1982) Interactions of bacterial and amoebal populations in soil microcosms with fluctuating moisture content Appl Environm Microbiol 43: 747-752

Casida LE Jr (1989) Protozoan response to the addition of bacterial predators and other bacteria to soil Appl Environm Microbiol 55: 1857-1859

Calow P (1977) Conversion efficiences in heterotrophic organisms Biol Rev 52: 385-409

Clarholm M (1981) Protozoan grazing of bacteria in soil - impact and importance Microb Ecol 7: 343-350

Clarholm M (1985a) Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen Soil Biol Biochem 17: 181-187

Clarholm M (1985b) Possible roles for roots, bacteria, protozoa and fungi in supplying nitrogen to plants In: (AH Fitter, ed) Ecological interactions in soil Blackwell Sci Publ, Oxford, pp 355-365

Cochran WG (1950) Estimation of bacterial densities by means of the 'most probable number' Biometrics 6: 105-116

Coleman DC, Anderson RV, Cole CV, Elliott ET, Woods L and Campion MK (1978) Trophic interactions in soils as they affect energy and nutrient dynamics. IV. Flows of metabolic and biomass carbon Microb Ecol 4: 373-380

Coleman DC, Reid CPP and Cole CV (1983) Biological strategies of nutrient cycling in soil systems Adv Ecol Res 13: 1-55

Corliss JO (1973) Protozoan ecology: a note on its current status Amer Zool 13: 145-148

Cunningham A (1915) Studies on soil protozoa J Agric Sci 7: 49-74

Cutler DW (1920) A method for estimating the number of active protozoa in the soil J Agric Sci 10: 135-143

Cutler DW (1923) The action of protozoa on bacteria when inoculated into sterile soil. Ann Appl Boil 10: 137-141

Culter DW, Lettice MA, Crump M and Sandon H (1922) A quantitative investigation of the bacterial and protozoan populations of the soil, with an account for the protozoan fauna Phil Trans Royal Soc, London, B 211: 317-350

Cutler DW and Crump LM (1929) Carbon dioxide production in sands and soils in the presence and absence of amoebae Ann Appl Biol 16: 472-482

Darbyshire JF (1976) Effect of water suctions on the growth in soil of the ciliate *Colpoda steinii* and the bacterium *Azotobacter chroococcum* J Soil Sci 27: 369-376

Darbyshire JF and Greaves MP (1967) Protozoa and bacteria in the rhizosphere of *Sinapis alba* L., *Trifolium repens* L., and *Lolium perenne* L. Can J Microbiol 13: 1057-1068

Darbyshire JF and Greaves MP (1973) Bacteria and protozoa in the rhizosphere Pestic Sci 4: 349-360

Darbyshire JF, Wheatley RF, Greaves MP and Inkson RHE (1974) A rapid micromethod for estimating bacterial and protozoan populations in soil. Rev Ecol Biol Sol 11: 465-475

De Man JC (1975) The probability of most probable numbers Eur J Appl Microbiol 1: 67-78

De Telegdy-Kovats L (1932) The growth and respiration of bacteria in sand cultures in the presence and absence of protozoa Ann Appl Biol 19: 65-86

Elliott ET and Coleman DC (1977) Soil protozoan dynamics in a shortgras prairie Soil Biol Biochem 9: 113-118

Elliott ET (1978) Carbon, nitrogen, and phosphorus transformations in gnotobiotic soil microcosms Thesis Colorado State University, pp 77

Elliott ET, Coleman DC and Cole CV (1979) The influence of amoebae on the uptake of nitrogen by plants in gnotobiotic soil In: The soil-root interface (JL Harley and R Scott Russell, eds) Acad Pr, London, pp 221-229

Fenchel T (1982) Ecology of heterotrophic microglagellates II Bioenergetics and growth Mar Ecol Progr Ser 8: 225-231

Fenchel T (1987) Ecology of protozoa Science Tech, Inc, Madison WI USA, pp 197

Finlay B and Fenchel T (1989) Everlasting picnic for protozoa New Scientist: 66-69

Foissner (1987) Soil protozoa: fundamental problems, ecological significance, adaptations in ciliates and testaceans, bioindicators and guide to the literature Progr Protistology 2: 69-212

Foster RC (1988) Microenvironments of soil microorganisms Biol Fertil Soils 6: 189-203

Geltzer (1963) On the behaviour of soil amoebae in the rhizospherees of plants Pedobiologia 2: 249

Golebiowska J and Ryskowski L (1977) Energy and carbon fluxes in soil compartments of agro-ecosystems Ecol Bull 25: 274-283

Gray TR and Williams ST (1971) Soil microorganisms Oliver and Boyd, Edinburgh

Griffiths BS (1986) Mineralization of nitrogen and phosphorus by mixed cultures of the ciliate protozoan *Colpoda steinii*, the nematode *Rhabditis* sp. and the bacterium *Pseudomonas fluorescens* Soil Biol Biochem 18: 637-641

Groscop JA and Brent MM (1964) The effects of selected strains of pigmented microorganisms on small free-living amoebae Can J Microbiol 10: 579-584

Hattori T (1967) Microbial distribution and its variation in soil aggregates Jpn J Soil Sci Plant Nutr 37: 302-304

Hattori T (1988) Soil aggregates as microhabitats of microorganisms Bull Inst Agric Res Tohoku Univ 37: 23-36

Heal OW and Felton MJ (1970) Soil amoebae: their food and their reaction to microflora exudates In: A Watson (ed) Animal populations in relation to their food resources Blackwell Sc Publ, Oxford and Edinburgh, pp 145-162

Hunt HW, Cole CV, Klein DA and Coleman DC (1977) A simulation model for the effect of predation on bacteria in continuous culture Microb Ecol 3: 259-278
Hunt HW, Elliott ET and Walter DE (1989) Inferring trophic transfers from pulse-dynamics in detrital food webs Plant Soil 115: 247-259
Ingham ER and Coleman DC (1984) Effects of streptomycin, cycloheximide, fungizone, captan, captofuran, cygon and PCNB on soil microorganisms Microb Ecol 10: 345-358
Jansson SL (1958) Tracer studies on nitrogen transformations in soil with special attention to mineralization-immobilization relationships Kungl. Lantbrukshogskolans Ann 24: 101-361
Johannes RE (1965) Influence of marine protozoa on nutrient regeneration Lymnol Oceanogr 10: 434-442
Katznelson H (1946) The rhizosphere effect of mangels on certain groups of soil microorganisms Soil Sci 62: 343-354
Katznelson H, Lochhead AG and Timonin MI (1948) Soil microorganisms and the rhizosphere Bot Rev 14: 543-587
Killer J (1913) Die Zahlung der Protozoen im Bodem Zentrbl. Backt. ParasitKde, Abt II, 36: 419
Lethbridge G and Davindson MS (1983) Microbial biomass as a source of nitrogen for cereals Soil Biol Biochem 15: 375-376
Losina-Losinsky L and Martinov PF (1930) A method of sudying the activity and rate of diffusion of protozoa and bacteria in soil Soil Sc 29: 349-362
Martin CH and Lewin KR (1915) Notes on some methods for the examination of soil protozoa J Agric Sc 7: 106
Meiklejohn J (1930) The relationship between the numbers of soil bacterium and the ammonia produced by it in peptone solution, with some reference to the effect of this process in the presence of amoebae Ann Appl Biol 17: 614-637
Newbould P (1989) The use of nitrogen fertilizer in agriculture: where do we go practically and ecologically? Plant Soil 115: 297-311
Nikitin (1973) The microstructure of the elementary microbial ecosystems In: Rosswall T (ed) Modern methods in the study of microbial ecology Bull Ecol Res Comm 17: 357-365
Postma J (1989) Distibution and population dynamics of *Rhizobium* sp introduced into soil Agricultural University Wageningen Thesis pp 122
Postma J and Altemuller HJ (1989) Bacteria in thin soil sections stained with the fluorescent brightener calcofluor white M2R Soil Biol Biochem (in press)
Postma J, Walter S and Van Veen JA (1989) Influence of different initial soil moisture contents on the distribution and population dynamics of introduced *Rhizobium leguminosarum* biovar *trifolii* Soil Biol Biochem 21: 437-442
Ritz K and Griffiths BS (1987) Effects of carbon and nitroate additions to soil upon leaching of nitrate, microbial predators and nitrogen uptake by plants Plant Soil 102: 229-237
Rovira AD (1965) Interactions between plant roots and soil microorganisms Ann Rev Microbiol 19: 241-266
Russell EJ and Hutchinson HB (1909) The effect of partial sterilization of soil on the production of plant food J Agric Sci Camb 3; 111
Russell EW and Russell EJ (1962) Soil conditions and plant growth Longmans Green and Co London
Schnurer J, Clarholm M, Bostrom S and Rosswall T (1986) Effects of moisture on soil microorganisms and nematodes: a field experiment Microb Ecol 12: 217-230
Schnurer J and Rosswall T (1987) Mineralization of nitrogen from ^{15}N labelled fungi, soil microbial biomass and roots and its uptake by barley plants Plant Soil 102: 71-78
Severtzova LB (1928) The food requirement of soil amoeboe with reference to their interrelation with soil bacteria and fungi Centralblatt fur Bakteriologie Abbt 2 73: 162-179
Singh BN (1941) Selectivity in bacterial food by soil amoebae in pure mixid culture and in sterilized soil Ann Appl Biol 28: 52-64

Singh BN (1942) Selection of bacterial food by soil flagellates and amoebae Ann Appl Biol 29: 18-22

Singh BN (1946) A method of estimating the numbers of soil protozoa especially amoebae based on their differential feeding on bacteria Ann Appl Biol 33: 112-119

Singh BN (1949) The effect of artificial fertilizers and dung on the numbers of amoebae in Rothamsted soils J Gen Microbiol 3: 204-210

Singh BN and Crump L (1953) The effect of partial sterilization by steam and formalin on the numbers of amoebae in field soil J Gen Microbiol 8: 421-426

Stout JD (1973) The relationship between protozoan populations and biological activity in soils Amer Zool 13: 193-201

Stout JD and Heal OW (1972) Protozoa In: A Burges and F Raw (eds) Soil Biology Acac Press London and New York pp: 149-189

Stout JD (1980) The role of protozoa in nutrient cycling and energy flow. Adv Microb Ecol 4: 1-50

Trofymow JA, Coleman DC and Cambardella C (1987) Rates of rhizodeposition and ammonium depletion in the rhizosphere of axenic oat roots Plant Soil 97: 333-344

Van leeuwenhoek A (1677) Phil Trans R Soc 12: 821

Van Veen JA, Ladd JN and Amato M (1985) Turnover of carbon and nitrogen through the microbial biomass in a sandy loam and a clay soil incubated with (^{14}C[U])Glucose and [^{15}N]$(NH_4)_2SO_4$ under different moisture regimes Soil Biol Biochem 17: 747-756

Van Veen JA, Merckx R and Van De Geyn SC (1989) Plant and soil related controls of the flow of carbon from roots through the soil microbial biomass Plant Soil 115: 1179-188

Vargas R and Hattori T (1986) Protozoan predation of bacterial cells in soil aggregates FEMS Microb Ecol 38: 233-242

Wagener S, Stumm CK and Vogels GD (1986) Electromigration, a tool for studies on anaerobic ciliates FEMS Microbiol Ecol 38: 197-203

Waksman SA (1916) Studies on soil protozoa Soil Sc 1: 135-152

Wiegert RG and Owen DF (1971) Trophic structure, available resources and population densities in terrestrial vs. aquatic ecosystems J Theoret Biol 30: 69-81

Woldendorp JW (1981) Nutrients in the rhizosphere In: Agricultural yield potentials in continental climates Proc 16th Coll Int Potash Inst, Bern, pp 99-125

Woods LE, Cole CV, Elliott ET, Anderson RV and Coleman DC (1982) Nitrogen transformations in soil as affected by bacterial-microfaunal interactions Soil Biol Biochem 14: 93-98

CHAPTER 2

THE IMPACT OF PROTOZOA ON THE AVAILABILITY OF BACTERIAL NITROGEN TO PLANTS [1]

Abstract

Microbial N from ^{15}N-labelled bacterial biomass was investigated in a microcosm experiment, in order to determine its availability to wheat plants. Sterilized soil was inoculated with either bacteria (*Pseudomonas aeruginosa* alone or with a suspension of a natural bacterial population from the soil) or bacteria and protozoa to examine the impact of protozoa. Plant biomass, plant N, soil inorganic N and bacterial and protozoan numbers were determined after 14 and 35 days of incubation. The protozoa reduced bacterial numbers in soil by a factor of 8, and higher contents of soil inorganic N in soil were found in their presence. Plant uptake of N increased by 20% in the presence of protozoa. Even though the total plant biomass production was not affected, the shoot:root ratios increased in the presence of protozoa, which is considered to indicate an improved plant nutrient supply. The presence of protozoa resulted in a 65% increase in mineralization and uptake of bacterial ^{15}N by plants. This effect was more pronounced than the protozoan effect on N derived from soil organic matter. It is concluded that grazing by protozoa strongly stimulates the mineralization and turnover of bacterial N. The mineralization of soil organic N was also shown to be promoted by protozoa.

[1] PJ Kuikman and JA Van Veen, Biology Fertility of Soils (1989) 8: 13-18

Introduction

Predation by protozoa and nematodes strongly determines the size and activity of microbial populations in soils and other ecosystems (Darbyshire and Greaves 1967, Ingham *et al.* 1985). Since microbial activity is a key factor in nutrient cycling in soil and the microbial biomass is an important sink and source of nutrients, the grazing activity of protozoa and nematodes may have an essential impact on the nutrient availability to plants (Duxbury *et al.* 1989). Coleman and co-workers (Coleman *et al.* 1978, Bryant *et al.* 1982, Elliott *et al.* 1979) studied the effect of predation by protozoa on N mineralization. They observed a significant enhancement of ammonium-N mineralization and subsequent uptake by plants when protozoa were added to microcosms with sterilized soil inoculated with bacteria. Data from Clarholm (1985) gave further evidence for the role of protozoa in N mineralization and uptake by plants. Predation by protozoa stimulated N mineralization. Protozoa excrete bacterial N as ammonium (Stout 1973) when grazing bacteria (Woods *et al.* 1982).

The activity of protozoa also increased the plant N concentration and uptake as well as the growth of crops (Elliott *et al.* 1979). Shoot:root ratios increased in the presence of protozoa (Clarholm 1985) which indicated an improved N supply to plants (Davidson 1969).

The present study was designed to gain further insight into the role of protozoa in the availability of microbial N to plants using ^{15}N-bacteria. We also examined the hypothesis that protozoan predation increases the mineralization of non-microbial soil organic N. We added a soil suspension containing several species of bacteria in order to immobilize more N in the soil microbial biomass and provide more serious competition for plant available mineralized N in the soil. We hypothesized that protozoa exert a more pronounced effect in soils with higher numbers of microorganisms, in terms of N mineralization and subsequent N uptake by plants.

Materials and methods

Soils

The soil used was a loamy sand and was collected from a A-horizon on arable land near Ede (NL) in 1983 (Table 1). The soil had not been fertilized for 2 years before this experiment. Two weeks before the start of the incubation the soil was sampled and air-dried until the moisture content was approximately 60% of the field capacity (12% v/w, pF approximately 2.8). The soil was then sieved (4 mm) and 1840g (1645g dry soil) samples were weighed into plastic bags. The soil was sterilized by exposion to a ^{60}Co-γ-radiation source, receiving 4 Mrad in 29 h, 2 days before the incubation was started. The sterility of the soil was tested by preparing

a dilution series of suspended soil and checking for bacterial growth on 1:2 Tryptone Soya Agar (Oxoid, U.K.) plates; no bacteria were found. These soil samples contained 28.6 mg (17.4 ppm) inorganic N at the start of the incubation; no fertilizer was added.

Table 1. Physical and chemical properties of the soil.

Origin	Ede, Netherlands
Texture class	loamy sand
Particle size distribution (%)	
Clay < 2 μm	3
Silt 2-50 μm	12
Sand > 50 μm	85
Cation exchange capacity (meq $\times$ 100 g^{-1})	9
Organic C (%)	2.0
Total N (%)	0.13
$CaCO_3$ (%)	0.10
pH (KCl)	6.2

Experimental design

Four treatments were established: (1) Inoculation of ^{15}N-labelled *Pseudomonas aeruginosa*; (2) ^{15}N *Pseudomonas* inoculation plus inoculation of a soil suspension containing a mixed, undefined bacterial population from the soil; (3) ^{15}N *Pseudomonas* inoculation plus inoculation of protozoa; and (4) ^{15}N *Pseudomonas* inoculation plus bacterial suspension plus protozoa inoculation. The *Pseudomonas aeruginosa* inoculum was prepared by growing the bacterium on a mineral medium containing glucose-C as a C source and $^{15}NH_4Cl$ as the sole N source at 29°C for 30 h. Before it was added to the microcosms, the ^{15}N-labelled bacterial suspension was centrifuged down (10000 rpm, 10 min) and washed twice in 0.1% Na-pyrophosphate. The soil suspension was prepared by blending 20g fresh soil with 200 ml 0.1% Na-pyrophosphate and filtering this suspension twice through a 1.2 μm Millipore filter in order to exclude protozoa and larger organisms. The filtrate was grown in 1:10 Tryptone Soya Broth (29°C) and checked over 48 h for contamination by protozoa, and none were detected. Before the inoculation, the soil suspension was washed in 0.1% Na-pyrophosphate. Several species of ciliates, flagellates and amoebae originating from the soil investigated were incubated and fed with *Pseudomonas aeruginosa* for 6 weeks in Neff's Amoeba Saline (Page 1967).

Wheat seeds (*Triticum aestivum* cv *Sicco*) were surface sterilized using 1.5% Na-hypochlorite and sterile deionized water. They were placed individually on 1:6

Tryptone Soya Agar in sterile test tubes in order to detect any contamination (Ø 2.2 cm, 20 cm long), and incubated at room temperature for 9 days to germinate. Only sterile seeds were used.

Incubation

The experimental unit consisted of a sterile PVC (polyvinyl chloride) plastic container (Ø 15 cm, height 9.5 cm), which was closed with a air-tight lid enclosing five tubes (Ø 1.6 cm, length 5 cm, two sides open). Sterilized soil was amended with a ^{15}N-labelled bacterial suspension of *Pseudomonas aeruginosa*, soil suspension and/or protozoa and thoroughly mixed. The soil core in the microcosms was brought to a bulk-density of 1.3g cm^{-3}. In the tubes five wheat seedlings were placed on top of the soil. The size of the inoculum was 7.4×10^7 colony forming units of *Pseudomonas aeruginosa*, 0.9×10^7 colony forming units for the soil suspension, and 5.0×10^3 protozoa, respectively, per gram of dry soil. With the inoculation of the soil, the soil moisture content was raised from 12% up to 15% (v/w).

The incubation was carried out under controlled conditions in a climate room (relative humidity 60%, day/night temperature 21/16°C and a light period of 16 hours). Additional openings were provided to add water and to allow air circulation. Soil water losses due to evapo-transpiration were determined by weighing the microcosms and estimating plant production. Moisture content was kept at 15% (v/w) or pF 2.2 by daily additions on top of the soil with sterile deionized water. The microcosms were aerated by flushing filtered (0.22 μm) air through the microcosms for 15 min at 2 h intervals.

Sampling procedure and techniques

On the sampling days, 14 and 35 days after the start of the incubation, the shoots were clipped at the base of the stems. Duplicate subsamples were taken from the soil core for analyses of N content, soil moisture content, and bacterial and protozoan numbers. The roots were sampled by washing the remaining soil on a sieve. The plant material was dried at 70°C for 48 h and weighed. Total plant N was determined in ground samples (0.5 mm) which were analysed according to a Kjeldahl procedure (Bremner 1965). After a wet oxidation-digestion procedure, the digest was distilled with 50% NaOH into boric acid and titrated to a pH of 5.1.

The soil moisture content was determined by loss of weight after drying the soil at 105°C for 24 h. Inorganic N, i.e. ammonium and nitrate, was determined by autoanalyser (van Ginkel and Sinnaeve 1980) in extracts of 10g fresh soil with 50 ml 0.5 *M* K_2SO_4 (1 h, 150 rpm on a rotary shaker), filtered over a Whatman glass-fiber (GF/C) filter. Total N in these extracts was determined by a Kjeldahl digestion method. Soluble organic N was calculated as the difference between total organic and inorganic N in the extracts. Samples that were to be analysed for ^{15}N content

were acidified to pH 3 after steam distillation and concentrated by evaporation of excess water at 60°C. Samples were stored in 0.1 *N* HCL. The ^{15}N content was determined by mass spectrometry (Finnegan MAT 25 mass-spectrometer) according to Bremner (1965) using hypobromite oxidation.

Bacterial numbers were determined in extracts of 10g fresh soil in 95 ml of 0.1% Na-pyrophosphate with 10g of gravel after 1 h on a rotary shaker. A dilution series was prepared and 0.1 ml of adequate dilutions were plated (in triplicate) on 1:2 Tryptone Soya Agar. The plates were incubated for 48 hours at 29°C. Protozoan numbers were determined by a most probable number method (Darbyshire *et al.* 1974) as modified by Clarholm (1985) using *Pseudomonas aeruginosa* as a food source. Microtiter plates were incubated at 12-15°C and scanned for protozoa, i.e. flagellates, amoebae and ciliates after approximately 1 and 4 weeks.

Statistics

The experiment was carried out in a completely randomized design which allowed analysis of variance between two factors, the addition of protozoa and of a soil suspension. Since no significant ($P<0.05$) interaction was found between these two experimental factors, only the main effect of either addition of protozoa or addition of a soil suspension or both effects are reported where significant at $P<0.05$. Only averaged values for the main effects are reported in the text (*Pseudomonas* inoculation and *Pseudomonas* with soil suspension versus *Pseudomonas* with protozoa and the combination of all three additions for the effect of protozoa, or *Pseudomonas* and *Pseudomonas* with protozoa versus *Pseudomonas* with soil suspension and the combination of all three additions for the effect of the soil suspension.

All treatments consisted of five replicated microcosms. Two and three replicated microcosms were destructively sampled after 14 and 35 days of incubation, respectively.

Results

Microbial population and soil N

After 14 days of incubation, the size of the bacterial population was 5 to 10 times larger ($P<0.05$) in soils amended with a soil suspension, compared to soils without the soil suspension. With respect to protozoan populations, only amoebae showed slightly higher numbers after the addition of the soil suspension to soils (table 2).

The bacterial populations continued to grow up to 14 and 35 days of incubation, particularly in soils without protozoa. The number of bacteria was approximately 20 times higher ($P<0.05$) in the treatments *Pseudomonas* and *Pseudomonas* with soil suspension treatment after 35 days compared to 14 days while in the *Pseudomonas* with protozoa and the combination of all three additions, the increase was only a

factor of 2 (Table 2). Also, flagellate and amoebal populations increased between day 14 and day 35 in both treatments with protozoa (Table 2). After 35 days of incubation, both protozoa and a soil suspension significantly affected ($P<0.05$) the number of bacteria in soil. The presence of protozoa reduced the bacterial populations by a factor of 8 (protozoan treatments versus those without) while the amendment of soil with a soil suspension resulted in an eight times larger bacterial population (soil suspension treatments versus those without) (Table 2).

Table 2. Numbers of bacteria and protozoa (flagellates, amoebae, and ciliates) per gram of dry soil after 14 and 35 days (mean values of two and three replicates, respectively).

treatment[a]	Bacteria ($\times 10^8$)		Flagellates[b] ($\times 10^4$)		Amoebae[b] ($\times 10^4$)		Ciliates[b] ($\times 10^4$)	
	14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d
B	0.24	4.70	ND	ND	ND	ND	ND	ND
BP	0.46	0.62	1.50	5.80	3.90	8.60	0.10	ND
BS	2.40	38.0	ND	ND	ND	ND	ND	ND
BSP	2.40	5.20	2.00	6.20	6.60	10.6	0.20	ND
$LSD^c_{0.05}$	1.86	18.35	0.74	2.70	0.35	2.78	-	-

a) B, ^{15}N- *Pseudomonas aeruginosa;*
BP, as B with protozoa;
BS, as B with soil suspension;
BSP, as B with protozoa and soil suspension

b) ND, not detectable

c) Least Significant Differences (LSD), at significance level $P=0.05$, are given for comparison of main effects, i.e. addition of a soil suspension or of protozoa.

After 14 days of incubation the presence of protozoa had raised ($P<0.01$) the inorganic N level by 37% [difference based on main effect; average for *Pseudomonas* and *Pseudomonas* with soil suspension (32.2 mg N) compared with average for *Pseudomonas* with protozoa and all three additions (44.2 mg N)], both in soils amended with a soil suspension and in soils not amended with a soil suspension; 90% of this inorganic N was in the ammonium form. The amount of total extractable N (inorganic and organic) in the K_2SO_4 extracts, however, showed no

significant differences between any of the treatments (Table 3). At the end of the incubation, no K_2SO_4 extractable inorganic N could be detected in any of the soils (Table 3).

Table 3. Inorganic and total 0.5 *M* K_2SO_4 extractable N in soil after 14 and 35 days (mean values of two and three replicates, respectively).

	N (mg N × microcosm^{-1})					
	Inorganic N[b]			Total N[d]		
treatment[a)]	0 d	14 d	35 d	0 d	14 d	35 d
B	30.6	34.4	ND	*	90.4	50.7
BP	30.6	41.5	ND	*	89.4	46.6
BS	30.6	30.0	ND	*	81.3	51.8
BS	30.6	46.9	ND	*	91.4	58.0
$LSD_{0.05}$[c]	-	3.30	-		3.2	4.9

a,b,c See notes to Table 2
d * = not determined

Plant biomass and N

After 14 days of incubation, the amount of plant biomass production (Table 4) and the fraction of ^{15}N, originally inoculated as bacterial N, recovered as plant N (Table 5) were similar for all four treatments. Both shoot and root N concentrations were affected ($P<0.05$) by the presence of protozoa: the N concentration was higher in the shoots but lower in the roots and this effect was most pronounced in soils amended with a soil suspension (Table 4).

After 35 days, the total plant biomass production was significantly ($P<0.01$) reduced by 25% from 5.42 to 4.04 g dry weight microcosm^{-1} [main effect, average of *Pseudomonas* (5.64) and *Pseudomonas* with protozoa (5.19) versus *Pseudomonas* with soil suspension treatment (4.08) and all three additions (4.00)] by the addition of a soil suspension (Table 4). In particular a reduction in root growth contributed to these differences. Both the addition of protozoa and the addition of the soil suspension reduced root biomass production by 22% ($P<0.05$) and 44% ($P<0.01$), respectively, compared to soils without protozoa or soil suspension. Also, shoot biomass production was reduced ($P<0.01$) by the addition of the soil suspension, while in the presence of protozoa shoot biomass production was elevated ($P<0.01$) from 2.60 to 2.84 g dry weight microcosm^{-1}. In those soils

where protozoa were added, the N concentration in both shoots and roots was higher ($P<0.05$) than in the shoots and the roots grown without protozoa (Table 4).

Table 4. Plant mass and N concentration after 14 and 35 days (mean values of two and three replicates, respectively).

	Plant mass (g dry wt × microcosm^{-1})						percentage N (%N)			
	Shoot		Root		Total		Shoot		Root	
treatment[a)]	14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d	14 d	35 d
B	0.45	2.65	0.13	2.99	0.59	5.64	3.93	1.92	1.55	0.69
BP	0.45	3.04	0.12	2.15	0.58	5.19	4.04	2.02	1.47	0.89
BS	0.46	2.54	0.13	1.53	0.59	4.08	3.68	1.82	1.67	0.82
BSP	0.47	2.63	0.15	1.37	0.62	4.00	4.31	2.14	1.32	0.98
$LSD^{c}_{0.05}$	0.05	0.17	0.05	0.49	0.10	0.53	0.23	0.20	0.18	0.15

a,c See notes to Table 2

Table 5. N uptake by plants, concentration of excess ^{15}N in total plant N and the fraction of inoculated ^{15}N recovered in total plant N at 14 and 35 days after the start of incubation (values are means of 2 and 3 replicates, respectively).

	Plant N uptake (mg N . microcosm^{-1})		^{15}N concentration in plant N uptake (%)		^{15}N in plants as percentage of inoculated ^{15}N (%)	
treatment[a)]	14 d	35 d	14 d	35 d	14 d	35 d
B	15.6	66.5	0.95	0.86	7.39	28.60
BP	15.9	76.2	0.88	1.25	7.03	47.95
BS	15.0	54.7	0.91	0.88	6.83	24.06
BSP	18.2	65.3	1.00	1.17	9.10	38.25
$LSD_{0.05}{}^{c}$	2.70	4.7	0.26	0.06	2.35	3.33

a,c See notes to Table 2

The total plant N uptake was significantly enhanced ($P<0.01$) by 17% by the presence of protozoa, while it was significantly reduced ($P<0.01$) by 16% after the addition of a soil suspension (Table 5). In the presence of protozoa the plants took up 65% more ($P<0.01$) bacterial ^{15}N, while the addition of a soil suspension significantly reduced ($P<0.01$) the recovery of ^{15}N by 19% as compared to soils that were not amended with protozoa or those not amended with the soil suspension, respectively (Table 5).

Discussion

A strong reduction in the size of the bacterial population in soils with protozoa coincided with protozoan growth in the second half of this experiment. These results are in agreement with those of Elliott *et al.* (1979), Clarholm (1981), and Heynen *et al.* (1988), who have also reported a reduction in bacterial numbers when grazed by protozoa. From the differences between the numbers of bacteria in soils with and soils without protozoa after 35 days of incubation, the average consumption of bacterial cells for each protozoan produced can be calculated. In soils with only *Pseudomonas aeruginosa* approximately 4000 bacteria were consumed for each protozoan. This figure corresponds well with the 3000 - 4000 bacteria calculated by Clarholm (1981). However, when the soil suspension was added, the apparent consumption was approximately 10 times higher, comparing the differences between *Pseudomonas* - *Pseudomonas* with protozoa and the two treatments with the soil suspension. This is hard to explain, in particular when one assumes that some of the bacterial species in the soil suspension are less edible for the inoculated protozoa, which were precultured on *Pseudomonas aeruginosa*.

During the first 14 days no reduction in bacterial numbers due to protozoan growth was observed. Yet, significantly more organic N was mineralized when the protozoa were active in the soil, suggesting that the predation by protozoa stimulates N turnover through the bacterial population. This may well be caused by improved conditions for bacterial activity, for instance by an enhanced availability of substrate through spreading of bacteria through the soil by protozoa when they move and eat.

The present findings also confirm earlier results reported by Elliott *et al.* (1979) and Clarholm (1985) on the stimulation by protozoa of shoot biomass production, N concentration in plants and total N uptake in plants. However, the present results do not confirm their observations of an increased total plant biomass production in the presence of protozoa. The present observed shift from root biomass to shoot biomass, indicated by a higher ($P<0.01$) shoot:root ratio as observed upon the addition of protozoa to soil, reflects the improved plant N availability. According to Davidson (1969) root growth is inversely related to N availability. The shoot:root

ratio was higher ($P<0.01$) in soils where the soil suspension was added but in this case, no positive effect on N uptake was observed. It is possible that among the microorganisms added with the soil suspension, some restricted root growth.

Net mineralization of soil organic N can be calculated from the difference between the initial inorganic N (30.6 mg N on day 0) and the sum of total plant N uptake and inorganic N at the end of our experiment. In all soils, whether inoculated with bacteria only or inoculated with both bacteria and protozoa, net mineralization of soil organic N was observed. In the presence of protozoa approximately 34% more soil organic N was mineralized, comparing protozoan treatments versus *Pseudomonas* and *Pseudomonas* with soil suspension treatments.

Protozoan grazing stimulated net mineralization by 26% in the absence and 44% in the presence of the soil suspension. These figures correspond with the 75% and the 50-100% increase in plant uptake of N, reported by Clarholm (1985) and Elliott *et al.* (1979), assuming that increased plant N uptake is due only to increased mineralization of soil organic N. The present experimental design allowed discrimination between the indirect effect of protozoan grazing on N availability to plants by stimulation of turnover processes and the direct effect by release of bacterially immobilized N. Protozoan grazing does not affect all bacteria present in the soil, since net N mineralization was reduced by 25% when sterilized soil was amended with a soil suspension compared to soil not amended with a soil suspension. This must be caused by the better immobilizing capacity of a larger soil microbial population.

Predation by protozoa indeed increases the release of bacterial N as shown by the data on the ^{15}N recovery (Table 5). This confirms the concepts presented by Clarholm (1985). The data show that ^{15}N from bacterial cells accounted for 0.88.-1.00% of the N taken up by plants (Table 5) during the first 14 days. No effect of the presence of protozoa or a soil suspension was detected. However, during the second part of the incubation period of 21 days, the ^{15}N released from bacteria contributed significantly ($P<0.01$) more to the total N uptake in soils where protozoa were present than in soils without protozoa: 1.25% and 1.17% for bacteria with protozoa and bacteria-protozoa-soil suspension versus 0.86% and 0.88% for bacteria and bacteria with soil suspension, respectively (Table 5). These data illustrate the close relationship between bacteria and protozoa with regard to N mineralization.

The delay in the release of ^{15}N from bacterial cells due to grazing by protozoa might be explained if, initially, internal cycling within the introduced bacterial population occurred to a larger extent than in the second phase of the incubation. This is supported by the observation that grazing by protozoa in the presence of a soil suspension resulted in a lower ($P<0.05$) concentration of ^{15}N since part of the ^{15}N was immobilized more strongly as the result of ^{15}N cycling within the enlarged bacterial population.

These results and calculations show that grazing by protozoa brings about an improved availability of bacterial N to plants through a higher N turnover, both the N directly released from bacterial cells and the N mineralized from the soil organic matter pool. The magnitude of the protozoan grazing effect on the plant availability of organically tied N supports the concept that food web interactions, rather than microbial activity alone, are responsible for N mineralization in soil (Hendrix *et al.* 1986).

References

Bremner JM (1965) Total nitrogen. In: Black CA, Evans DD, White JL, Ensminger LE and Clark FE (eds) Methods of soil analysis. American Society of Agronomy, Wisconson USA pp 1149-1178

Bryant RJ, Woods LE, Coleman DC, Fairbanks BC, McClellan JF and Cole CV (1982) Interactions of bacterial and amoebal populations in soil microcosms with fluctuating moisture content. Appl Environ Microbiol 43: 747-752

Clarholm M (1981) Protozoan grazing of bacteria in soil: Impact and importance. Microb. Ecol. 7:343-350

Clarholm M (1985) Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. Soil Biol Biochem 17: 181-187

Coleman DC, Anderson RV, Cole CV, Elliott ET, Woods L and Campion MK (1978) Trophic interactions in soils as they affect energy and nutrient dynamics. IV. Flows of metabolic and biomass carbon. Microb. Ecol. 4: 373-380

Darbyshire JF and Greaves MP (1967) Protozoa and bacteria in the rhizosphere of *Sinapsis alba* L., *Trifolium repens* L., and *Lolium perenne* L. Can J Microbiol 13: 1057-1068

Darbyshire JF, Wheatley RE, Greaves MP and Inkson RHE (1974) A rapid micromethod for estimating bacterial and protozoan populations in soil. Rev Ecol Biol Sol 11: 465-475

Davidson RL (1969) Effects of soil nutrients and moisture on root/shoot ratios in *Lolium perenne* L. and *Trifolium repens* L. Ann Bot 33: 571-577

Duxbury JM, Smith MS and Doran JW (1989) Soil organic matter as a source and a sink of plant nutrients. Biogeochem (in press)

Elliott ET, Coleman DC and Cole CV (1979) The influence of amoebae on the uptake of nitrogen by plants in gnotobiotic soil. In: Harley JL and Scott Russell R (eds) The Soil-Root Interface. Academic Press, London, pp 221-229

Hendrix PE, Parmelee RW, Crossley jr. DA, Coleman DC, Odum EP and Groftman PM (1986). Detritus food webs in conventional and no-tillage agroecosystems. Bioscience 36: 374-380

Heynen CE, Van Elsas JD, Kuikman PJ and Van Veen JA (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

Ingham RE, Trofymow JA, Ingham ER and Coleman DC (1985) Interactions of bacteria, fungi, and their nematode grazers: effects on nutrient cycling and plant growth. Ecol Monogr 55: 119-140

Page FC (1967) Freshwater and soil amoebae. Freshwater biological association, Cumbria UK, scientific publication no. 34

Stout JD (1973) The relationship between protozoan populations and biological activity in soils. Amer Zool 13: 193-201

Van Ginkel JH and Sinnaeve J (1980) Determination of total nitrogen in plant material with Nessler's reagent by continuous-flow analysis. Analyst 105: 1199-1203

Woods LE, Cole CV, Elliott ET, Anderson RV and Coleman DC (1982) Nitrogen transformations in soil as affected by bacterial-microfaunal interactions. Soil Biol Biochem 14: 93-98

CHAPTER 3

DYNAMICS AND ACTIVITY OF BACTERIA AND PROTOZOA IN RELATION TO THEIR SPATIAL DISTRIBUTION IN SOIL [1]

Abstract

The effects of protozoan inoculum density and spatial separation of (prey)-organisms on the turnover and transport of specific microbial populations in sterilized soil were studied in soil microcosms, applying a combination of techniques. The fate of introduced microbial biomass was followed by monitoring $^{(14)}C\text{-}CO_2$ evolution. Dynamics of and genetic transfer between genetically marked bacteria were followed by selective plating using appropriate antibiotics.

Three levels of protozoan densities, i.e no protozoa, protozoa and a 1:10 diluted protozoan inoculum were established. Two methods of soil inoculation, i.e. joint introduction and separate introduction of protozoa with a donor population and of a ^{14}C labelled recipient population, respectively, were applied.

The activity of protozoa, as judged from an increased carbon dioxide evolution compared to soils not inoculated with protozoa, was larger when 100% of the soil was inoculated with protozoa (joint introduction) compared to inoculation of protozoa into only 33% of the soil which was mixed with 66% of a soil portion not inoculated with protozoa (separate introduction). The activity of protozoa accelerated the turnover of ^{14}C compared to soils without protozoa and the more protozoa were inoculated, the faster ^{14}C was respired in both soils with joint and soils with separate introduction.

The transfer of genetic material between introduced donor and recipient cells was not influenced by the presence of protozoa but was reduced when separate introduction was applied compared to joint introduction. Transfer of genetic material was only found during the first 2 days of the incubation. Substantial loss of genetic material (plasmid RP4) in the donor population was detected; the presence of

[1] PJ Kuikman, JD Van Elsas, AG Jansen, SLGE Burgers and JA Van Veen, submitted to Soil Biology Biochemistry

protozoa enhanced both donor survival and plasmid maintenance. A stimulating effect of protozoa on cellular metabolism of substrate used by the donor population is hypothesized.

These results picture soil as a system in which the distribution of micro-organisms in the absence of fluctuations in climatic conditions as in laboratory circumstances is not changing in time: protozoa hardly moved through soil and minimal translocation of bacteria was found.

Introduction

Nutrient transformations in soil such as carbon and nitrogen mineralization, are stimulated by protozoan predation of bacteria (Anderson *et al.* 1981, Coleman *et al.* 1978). Though protozoan activity generally reduced the size of the bacterial populations (Clarholm 1985, Anderson *et al.* 1978), in soils with protozoa more nitrogen is mineralized and taken up by plants as compared to soils without protozoa (Clarholm 1985, Kuikman and Van Veen 1989).

Soil contains an array of transitory habitats such as particle aggregates, air- and waterfilled pores which are inhabited by larger (macro) fauna and waterfilm organisms respectively, i.e. bacteria, fungi, protozoa and nematodes. Since the activity of soil microorganisms and so microbially mediated processes such as decomposition, mineralization, predation and conjugation are confined to the water-film, soil has to be viewed as a mosaic of microsites containing a (dis)continuous waterfilm that each may contain a bacterial-fungal-protozoan community mostly in isolation (Bamforth 1988, Stout 1973). Vargas and Hattori (1986) showed that migration of protozoa between soil aggregates was restricted by the extent to which aggregates are connected by continuous water films. Therefore, the distribution of soil microorganisms by moving actively or being allocated by other soil organisms, water flow or plant root growth is essential for the effect of protozoa on microbial activity and element turnover.

The objective of this study was to i) study the activity of protozoa in soil as a function of their movement in microsites for the search of food and of the number of protozoa that are inoculated at the start of the incubation, ii) to assess the turnover of specific bacterial cells due to protozoan activity and iii) to determine the effect of protozoa on gene transfer via conjugation between two bacterial strains introduced into soil. We hypothesized that protozoa could improve conditions for conjugation in soil either by increasing the activity of a bacterial population and its individual members or by protozoan movement and transport of bacteria through soil and so to increase the mating chance and hence conjugation.

The methodology of manipulating the positioning of inoculated bacteria and protozoa through inoculation at different soil moisture contents, originally developed by Vargas and Hattori (1986) and applied by Postma *et al.* (1989), was used in this experiment.

Materials and methods

Soils

The soil used was a loamy sand as described by Van Elsas *et al.* (1986) and Kuikman and Van Veen (1989). Several weeks before the start of the incubation, the soil was sampled from a field plot and air-dried until the moisture content was approximately 7% (pF = approx. 3.5) or 20% of the water holding capacity (WHC) of the soil. The soil was then sieved (Ø 4mm) and portions were weighed into plastic bags. The soil was sterilized by exposure to a ^{60}Co-γ-radiation source, receiving 4 Mrad in 30h, 4 days before the incubation was started. The sterility of the soil was tested by plating a dilution series of suspended soil on 1:2 Tryptone Soya Agar (Oxoid, U.K.) plates and checking for bacterial and fungal growth and for protozoa on water agar plates supplemented with a bacterial suspension. No bacteria, fungi or protozoa were found after irradiation.

Microorganisms

Pseudomonas fluorescens strain R2f was isolated from grassland soil in The Netherlands (Van Elsas *et al.* 1988). The strain was identified as a member of the fluorescent pseudomonad group with API 20E and API 50CH test strips. It forms translucent flat colonies which produce a fluorescent yellow pigment on King's B agar [proteose peptone, 20g; K_2HPO_4, 1.5g; $MgSO_4.7H_2O$, 1.5g; glycerol, 10g; agar, 15g; H_2O, 1L; pH 7.2].

A selectable chromosomal marker, transposon Tn5 (encoding resistances to kanamycin and streptomycin), was introduced into R2f by using the suicide vector pSUP2021 (Simon *et al.* 1983). The presence of a single copy of Tn5, and the absence of vector sequences, were shown by Southern hybridization analysis using plasmids pSUP202 (Tn5-less) and pSUP2021 (Tn5-loaded) as probes (Maniatis *et al.* 1982). To obtain a plasmid-containing transposon loaded R2f-derivative donor strain, plasmid RP4 was introduced into R2f by a filter mating technique (Simon *et al.* 1983) using *Escherichia coli* PC 2366(RP4) as a donor. The plasmid RP4 is self-transmissable and encodes resistances to kanamycin (Km, 75 μg/ml) and tetracycline (Tc, 75 μg/ml). A spontaneous rifampicin (Rp, 50 μg/ml) resistant mutant was selected to serve as a recipient strain for conjugation and transfer of plasmid RP4 in soil. Both bacterial strains and the methods to store and culture them have been described by Van Elsas *et al.* (1988).

Donor and recipient strain were separately grown in a mineral medium [2.0g glucose, 1.44g citric acid, 1.07g NH_4Cl, 0.5g K_2HPO_4, 0.5g KH_2PO_4, 0.2g $MgSO_4$, 0.01 g $MnSO_4$, 0.01g NaCl, 0.01g $FeSO_4$, 0.01g $CuSO_4$, 0.04mg $ZnSO_4$, 0.03mg $CoCl_2$, 0.02 mg $(NH_4)_6Mo_2O_4$, 1L H_2O, pH 6.8] in the presence of appropriate antibiotics for 30h at 29°C on a rotary shaker at 120 rpm. The medium for the recipient contained ^{14}C[U]-glucose and ^{15}N-ammonium nitrogen. Cells were harvested by centrifugation at 10.000×g for 10 minutes at 10°C and resuspended in sterile demineralized water; the centrifugation and resuspension were repeated once.

The protozoan population used consisted of one flagellated and one amoebal species. *Acanthamoeba* sp was obtained from Dr. E.T. Elliott [NREL, CO 80325 Ft Collins, USA] and has been described by Elliott and Coleman (1977); the flagellate was identified as a *Cercomonas* sp and was isolated from the loamy sand soil under investigation. Both protozoan species were cultured on water agar using *Pseudomonas* R2f::Tn5[RP4] as a food source and the appropriate antibiotics (75 μg/ml Km and 75 μg/ml Tc) to obtain monoxenic cultures. Protozoa were harvested by washing them from the agar plates with amoeba saline (Page 1976).

Experimental design and inoculation of soils

The basic experimental design included the study of the effect of two factors. Firstly, three levels of protozoa were added: no protozoa, a 1:10 diluted protozoan inoculum and a non-diluted protozoan inoculum. Secondly, the fraction of the soil into which protozoa were inoculated was either 100% (joint introduction) or 33% (separate introduction). The soil portions of the joint introduction were first inoculated with the ^{14}C-labelled recipient cells, bringing the soil moisture content from 7 to 12% (v/w). After mixing and incubation of the soil portions at 10°C for 4 hours, the non-labelled donor cells together with the protozoa were inoculated and the soil portions were mixed again. The final soil moisture content was 16% (v/w) corresponding to 45% of the WHC. In case of the separate introduction, one part (33%) of the soil was inoculated with the non-labelled donor cells together with the protozoa and the other part (66%) was inoculated with the ^{14}C labelled recipient cells, bringing the soil moisture content from 7% to 16% (v/w). After a preincubation period of 4 hours at 10°C, the portions were brought together and mixed. The non-diluted protozoan inoculum contained approximately 2000 flagellates and 300 amoebae per gram dry soil.

At day 37 of the incubation, the impact of disturbing the soil structure on the distribution of protozoa and bacteria was simulated by mixing soil again with a spatula in a sterile petri dish. Three modes of mixing were applied: [0] no mixing, [1] mixing once and [2] mixing twice; the second mixing was carried out 20 days after the first. After disturbing the soil, the incubation proceeded after bringing the soil back to a bulk density of 1.3 g/cm^3.

Incubation of the soils

Soil portions, equivalent to 100g dry soil were weighed into microcosms [sterile plastic beakers (150 ml)] and closed by a lid that allowed free air exchange. The soil portions were brought to a bulk density of 1.3 g/cm^3 on a dry weight basis. The microcosms were incubated in 1.5L glass containers together with a cup containing 10 ml 0.5 *N* NaOH to capture CO_2 evolved and 1 ml sterile demineralized water to maintain a moist atmosphere. The glass containers were incubated in the dark (temperature regime: 20°C for 16h and 15°C for 8h).

Sampling procedure and analyses

Soil microcosms were destructively sampled 0, 2, 4, 8, 14, 22, 37 and 74 days after the start of the incubation. Soil moisture content was determined gravimetrically after drying for 24h at 105°C. The numbers of bacteria were determined by extracting 10 g fresh soil in 95 ml 0.1% Na-pyrophosphate solution on a rotary shaker (10 min., 200 rpm). A 10-fold dilution series in sterile demineralized water was prepared and appropriate dilutions were plated (in triplicate) on King's B agar. The total number of bacteria was determined on plates without antibiotics, the numbers of recipient cells on plates with Rp, of donor cells on plates with Tc, Km and Sm, of plasmidless donor cells on plates with Km and Sm and of tranconjugant cells on plates with Tc, Km, Rp and Sm (50 μg/ml Rp, 80μg/ml Km, 80μg/ml Tc and 80μg/ml Sm). After incubating plates for 48-72 hours at 27°C, colony forming units were enumerated.

The number of protozoa was determined by a modified most-probable-number method (Darbyshire *et al.* 1974, Rowe *et al.* 1977), extracting 5 g fresh soil in 100 ml of Amoebae Saline, using 4-fold dilutions and eight replicated series with *Pseudomonas fluorescens* R2f as a food source. The microtiter plates were incubated during 4 weeks at 12°C in the dark and periodically scanned for the presence of protozoa.

After extraction of 25 g fresh soil in 50 ml 0.5 *M* K_2SO_4, mineral nitrogen, i.e. N-NH_4 using Nessler's reagent (van Ginkel and Sinnaeve 1980) and N-NO_3 was measured by continuous flow analysis. Carbon dioxide evolved from the soil was trapped in 10 ml 0.5 *M* NaOH. Soil ^{12}C and ^{14}C were determined in dried soil after combustion. Total ^{12}C-CO_2 released by incubation or by combustion of soils was determined by titration and ^{14}C by liquid scintillation counting (Amato 1983).

Statistics

On each sampling date, two microcosms were destructively sampled for each treatment. The data were analysed by analysis of variance over the four experimental factors, i.e. [A] the number of protozoa inoculated, [B] joint or separate inoculation of ^{14}C-labelled bacteria and protozoa, [C] time and [D] disturbance of the soil structure by mixing, respectively.

The data on ^{14}C-CO_2 and CO_2 evolved were analysed by fitting a response function with both an exponential and a linear part:

$$Y = A \times (1 - r^{day}) + B \times day \qquad (1)$$

where lim $(1 - r^{day}) = 1$ for day=infinite, r determines the moment that the function changes its exponential to a linear behaviour and A determines the y-intercept if a straight line is extrapolated. In the full model, there are separate parameter estimates for all combinations of treatments. By keeping certain parameters fixed for combinations of treatments and comparing this restricted model with the full model, it is possible to test whether the parameter estimates were influenced by the different treatments.

All differences reported are significant at the level of $P=0.05$ at least.

Results

Microbial population dynamics

The total number of bacteria in soil increased by a factor of approximately 15 during the first 2 days of the incubation (Figure 1). In the course of the incubation, the total number of bacteria recovered, was significantly less in soils that received a 1:10 diluted or a non diluted protozoan inoculum compared to soils which did not receive protozoa.

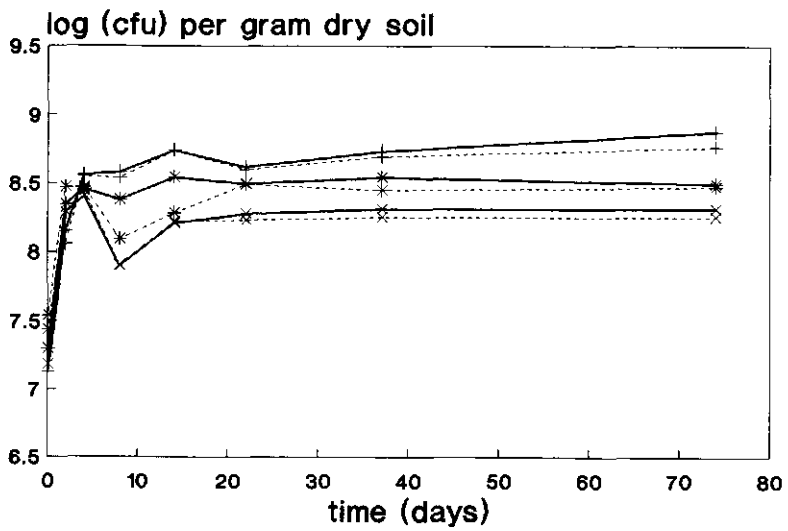

Figure 1 Dynamics of total number of bacteria on 1:10 King's B agar plates (log cfu $\times$ g^{-1}dry soil); solid lines, joint introduction; broken lines, separate introduction; + no protozoa; $\times$ 1:10 diluted protozoan inoculum added; $*$ non diluted protozoan inoculum added

The numbers of recoverable donor and recipient cells roughly stabilized after 4 and 8 days of incubation respectively (Figure 2 and 3). The population size of the recipient strain was significantly $P<0.001$) reduced by the presence of protozoa: without the addition of protozoa the population remained at a level of log 8.57 while the addition of protozoa reduced the population size in between day 2 and 8 to log 8.12 and to log 8.18 for a 1:10 diluted and a non-diluted protozoan inoculum, respectively. Joint or separate introduction of the recipient population and of protozoa did not affect the population dynamics of the recipient cells.

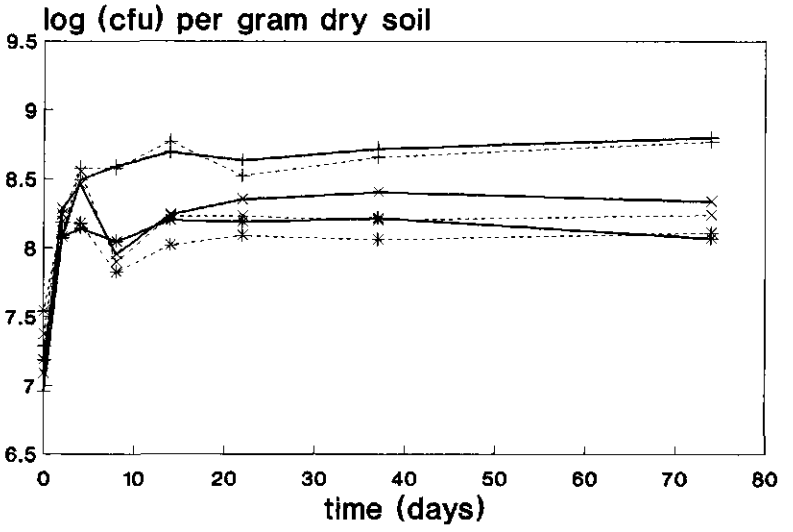

Figure 2 Dynamics of recipient populations (log cfu × g^{-1} dry soil); see legend Figure 1

The dynamics of the donor population were more complicated. Upon introduction, plasmid loss from the donor population was observed within 4 days. Roughly, 10% or less of the donor population contained the plasmid for the remaining incubation period (Figure 3 and 4). Higher numbers of donor cells with RP4, log 6.0, were found in the soils with a non diluted protozoan inoculum than in the soils without protozoa or inoculated with a 1:10 diluted protozoan inoculum. The number of plasmidless donor cells remained more or less stable in soils without protozoa. The plasmidless donor population stabilized in soils with a non diluted protozoan inoculum at a higher level and in soils with a 1:10 diluted protozoan inoculum at a lower level than in soils without protozoa.

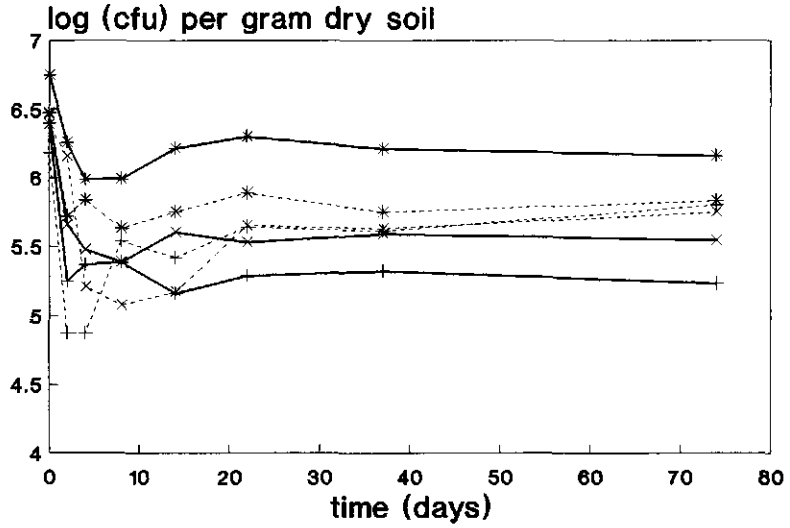

Figure 3 Dynamics of plasmidless donor populations (log cfu x g^{-1} dry soil); see legend figure 1

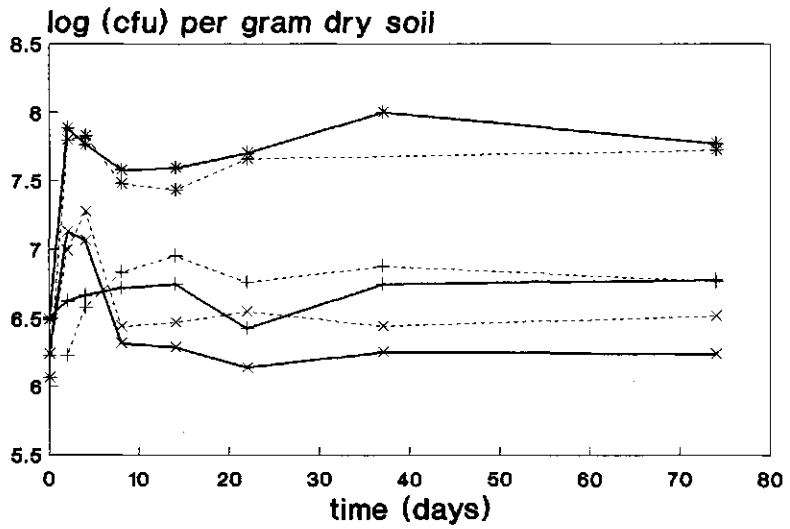

Figure 4 Dynamics of plasmid bearing donor populations (log cfu x g^{-1} dry soil); see legend fig.1

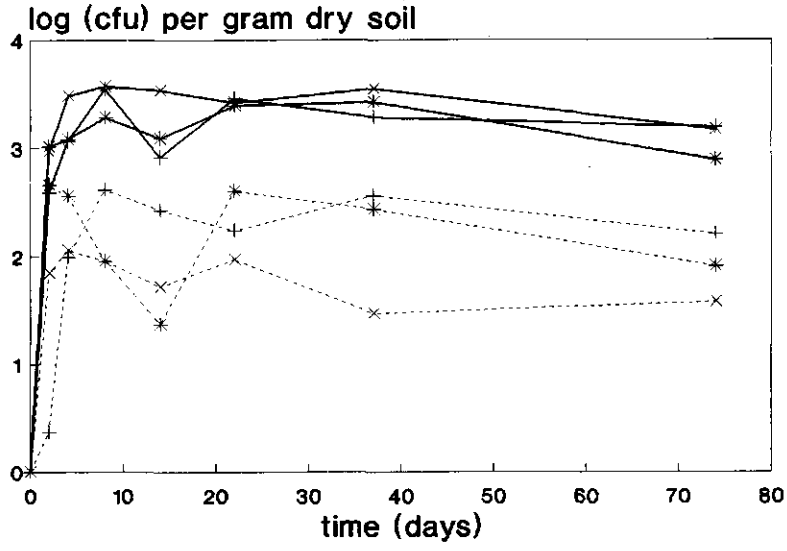

Figure 5 Dynamics of transconjugant populations (log cfu x g^{-1} dry soil); see legend figure 1

The number of transconjugant cells in soil was between 1×10^2 and 5×10^3 per gram dry soil (Figure 5). Transconjugants were already found after 2 days of incubation and their number was not affected by the length of the incubation period after day 4. A 10-fold higher($P<0.001$) number of trans-conjugant cells was found in soils with joint introduction compared to soils with separate introduction of donor and recipient populations. The presence of protozoa did not influence the number of transconjugant cells in any treatment.

The highest numbers of protozoa were found in soils inoculated with a non diluted protozoan inoculum: up to 100.000 per gram of dry soil at day 8. In soils with a 1:10 diluted protozoan inoculum up to 10.000 protozoa were re-covered after 14 days. The numbers of protozoa were not affected by either joint of separate introduction.

Transformation of carbon compounds

The rate of CO_2 evolution (mg C x g dry $soil^{-1}$ x day^{-1}) decreased as the incubation period proceeded. Analyses of the data on accumulated CO_2 evolution (Figure 6) from the soil by fitting response functions (1) accounted for 85% of the variance. The model in which the estimates for parameters r and B were kept fixed for all treatments and only parameter A was allowed to vary among treatments, was as good as the model in which none of the parameters was kept fixed. Therefore,

the CO_2 dynamics could be well described by a model in which only parameter A was significantly different for the combinations no protozoa with joint or separate introduction, joint introduction with both protozoan inoculum levels and separate introduction with both protozoan inoculum levels respectively. Whereas the accumulated CO_2 evolved differed only 10% in soils with either joint or separate introduction but without protozoa, the presence of protozoa significantly increased($P<0.01$) the CO_2 output by 150% to 490 mg C in soils where joint introduction was applied versus 40% to 322 mg C in soils where separate introduction was applied. The results from day 14 and on were not affected by joint or separate introduction or by number of introduced protozoa but only by the length of the incubation period ($P=0.015$). The rate of C-CO_2 evolution remained constant for the incubation period after day 14 and was not affected by either joint or separate introduction or the presence or absence of protozoa.

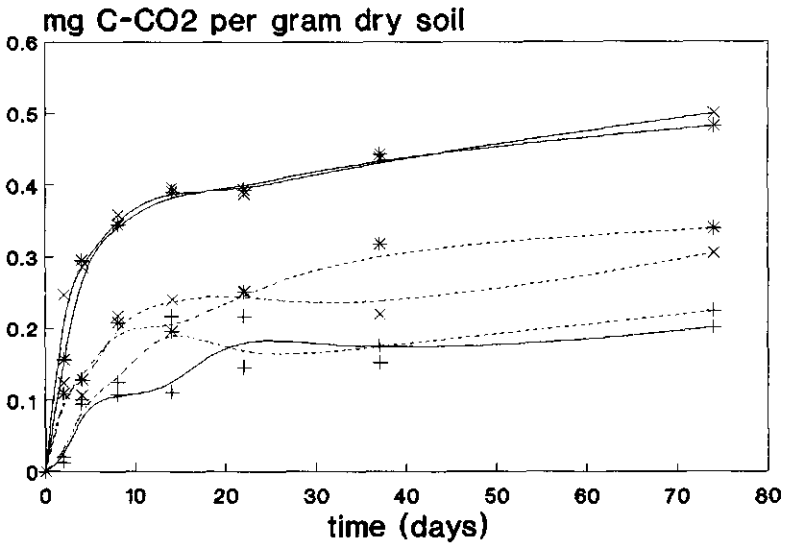

Figure 6 Accumulated carbon dioxide evolution (mg C-CO_2 × g^{-1} dry soil); see legend Figure 1

The accumulated ^{14}C evolved from the soils after 74 days of incubation was not significantly different for any of the six treatments. Approximately 40% of the ^{14}C that had been introduced as bacterial (recipient) cells was recovered as ^{14}C-CO_2 (Figure 7). To analyse the dynamics of ^{14}C-CO_2 evolution, the response function (1) was fitted and in all cases at least 97% of the variance was accounted for. Keeping r (=0.848) fixed for all treatments did not affect the fit of the model. The rate of ^{14}C evolution stabilized after approximately 22 days. This moment was not dependent

on either joint or separate introduction or on the number of inoculated protozoa.

After 14 days of incubation, from soils with protozoa 40% and 80% more($P<0.01$) ^{14}C had evolved in case a 1:10 diluted and a non diluted protozoan inoculum was applied, respectively than from soils without protozoa. Joint or separate introduction did not affect the ^{14}C dynamics. However, the more protozoa were inoculated, the faster ^{14}C was respired by the microbial community in soil. Therefore, a low estimate for parameter A was combined with a relatively high estimated value for parameter B for soils without protozoa and vica versa for soils with protozoa. This set of parameter estimates for the response function resulted in convergence for the fitted curves for ^{14}C evolution.

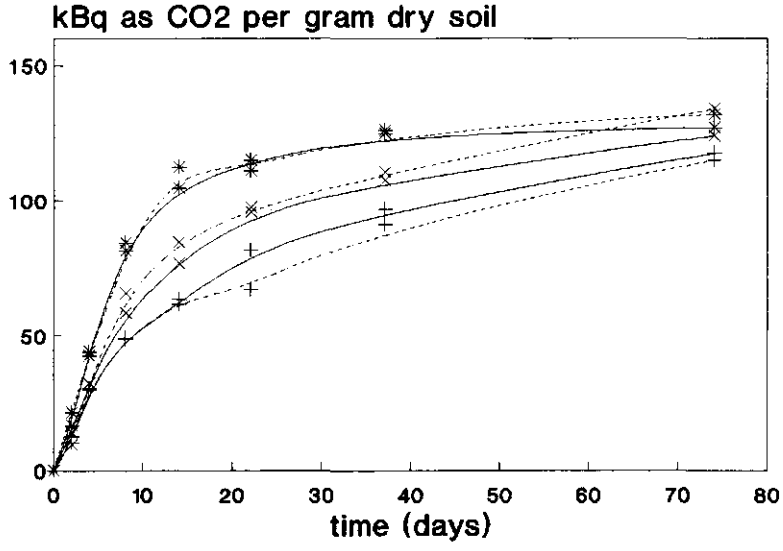

Figure 7 Accumulated ^{14}C-CO_2 evolution (kBq in $CO_2 \times g^{-1}$ dry soil); see legend figure 1

Nitrogen mineralization

At the start of the incubation 15.5 μg $N\text{-}NH_4$ per gram of soil was found. Within 2 days, approximately 30% of the ammonium nitrogen was immobilized by the microbial population (Figure 8). In all soils, ammonium nitrogen was mineralized from day 2 until the end of the incubation after 74 days. The presence of protozoa significantly increased($P<0.05$) the mineralization of ammonium; the non diluted protozoan inoculum showed the highest($P<0.05$) nitrogen mineralization, i.e. 15% more than soils inoculated with only bacteria. During the incubation period, no changes in the nitrate content of the soils were detected.

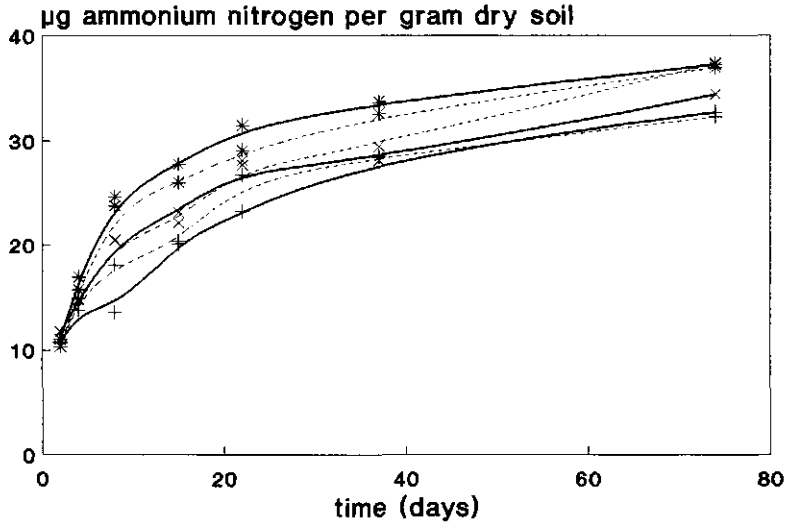

Figure 8 Ammonium nitrogen mineralization (μg N-NH_4 $\times$ g^{-1} dry soil); for legend see Figure 1

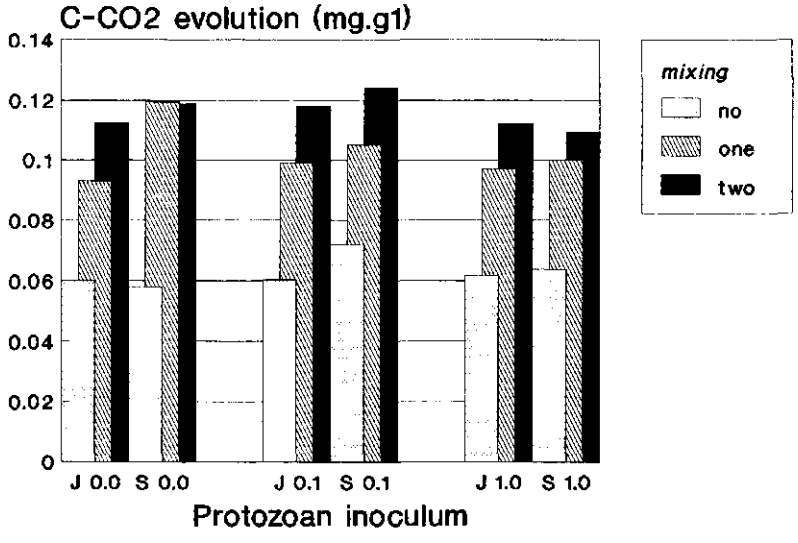

Figure 9 Accumulated carbon dioxide evolution after mixing part of the soil portions 37 days after the start of the incubation until 74 days (mg C-CO_2 $\times$ g^{-1} dry soil); [S] is separate and [J] is joint introduction; [0.0], [0.1] and [1.0] indicate no, a 1:10 diluted and a non diluted protozoan inoculum, respectively

Effects of mixing soil

The effect of disturbance, brought about by mixing the soil portions once or twice, was examined by analysing the CO_2 and the ^{14}C-CO_2 evolution over the period from day 35 until day 74. CO_2 evolution was stimulated in all soils that were mixed (Figure 9). Mixing soil once did enhance CO_2 evolution by 65% and mixing the soil twice enhanced CO_2 evolution by 85% compared to no mixing at all. The effect of mixing on CO_2 evolution was 10% higher but not significantly different in soils with separate introduction compared to soils with joint introduction. No significant effect of the inoculum level of protozoa, i.e. no protozoa, a 1:10 diluted or a non diluted protozoa inoculum could be detected.

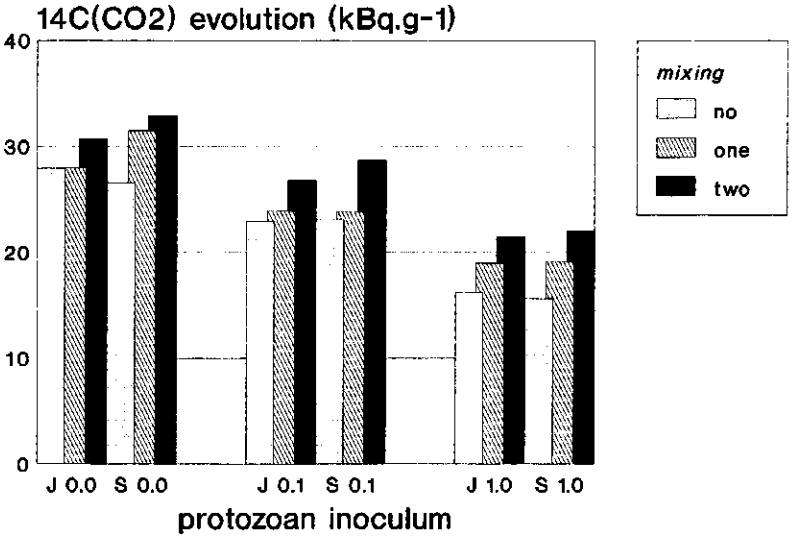

Figure 10 ^{14}C-carbon dioxide evolution after mixing part of the soil portions 37 days after the start of the incubation until day 74 (kBq in $CO_2 \times g^{-1}$ dry soil); for legend see figure 9

The ^{14}C-CO_2 evolution was stimulated($P<0.001$) by 22% and 33% after mixing the soil once and twice, respectively (Figure 10). The absence or presence of protozoa did not significantly affect the results; differences in the rate of ^{14}C evolved that existed before mixing were continued. The amount of ammonium nitrogen at the end of the incubation was reduced($P<0.01$) in soils without protozoa and with a 1:10 diluted protozoan inoculum that had been mixed once or twice while in soils with a non diluted protozoa inoculum no change in the ammonium content was observed (Figure 11).

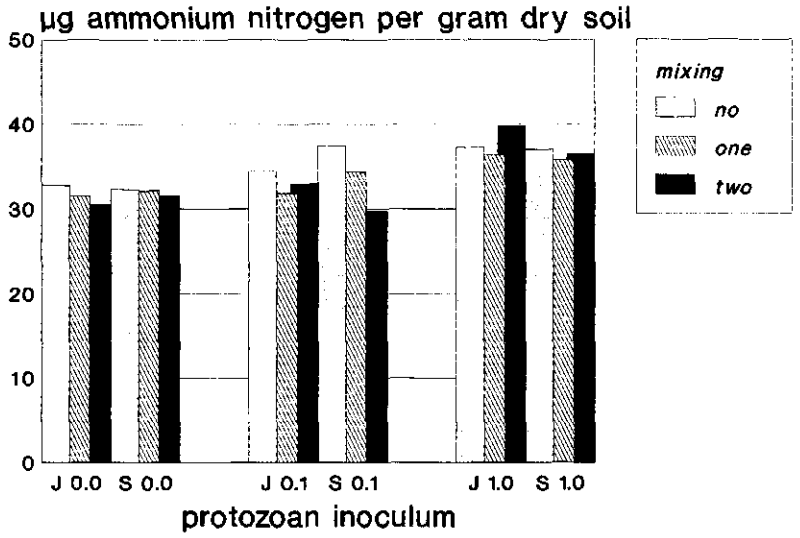

Figure 11 Ammonium nitrogen in soils after 74 days of incubation (μg N-NH_4 $\times$ g^{-1} dry soil); for legend see Figure 9

Mixing the soil did not affect the numbers of bacteria (total, donor cells with and without plasmid, recipient and transconjugant cells) that were recovered after 74 days of incubation compared to soils that were not mixed.

Discussion

The primairy goal of this experiment was to determine whether protozoa actively move through the soil. Since direct observations on amoebae and flagellates in soil are hardly feasible (Foissner 1987), indirect measurements should clarify the actual distribution of protozoa in soil over time. Carbon dioxide evolution is a suitable and easily assessable parameter to observe predatory activity of protozoa (Hunt *et al.* 1989). In general, carbon dioxide production is elevated in soils with protozoa compared to soils without protozoa containing only bacteria (Coleman *et al.* 1978, Anderson *et al.* 1981). Our experimental design included two main indicators that potentially could show to which extent the protozoa and donor population and the ^{14}C labelled recipient population were physically separated upon introduction and remained separated or were better mixed due to protozoan activity during the incubation for 74 days: i) the number of transconjugant cells and ii) the (^{14}C-)CO_2 evolution.

Upon separate introduction of recipient cells and donor cells we expected to recover none or less transconjugant cells compared to the recovery from soils with joint introduction of recipient and donor cells. Van Elsas *et al.* (1989a) did not detect the formation of transconjugant cells in soils where donor and recipient cells had been introduced into separate soil portions before these portions were mixed together. If protozoa are able to migrate and disperse donor or recipient cells or both, they might increase the chances for mating and thus the transfer of genetic material. This would then result in a higher number of transconjugant cells (Figure 5) in soils with separate introduction and with protozoa compared to such soils without protozoa.

Furthermore, it was hypothesized that if protozoa migrate freely in soil, the effects of grazing by protozoa on nitrogen and carbon mineralization, as described above, might not be dependent on the number of protozoa inoculated at the start nor on the fraction of the soil that was inoculated with protozoa. If protozoa are not mobile, either of the two factors mentioned should then affect the protozoan mediated stimulation of mineralized carbon and nitrogen.

The impact of protozoa on the CO_2 evolution, was relative to the part of the incubated soil that had been inoculated with protozoa at the start (Figure 6). This indicates that CO_2 respiration only depended on the amount of carbon substrate (=bacteria), i.e. the full amount for joint introduction or approximately 40% for separate introduction, and that the accessibility of this particular amount of substrate for the protozoa was not the limiting factor. Thus, it may be concluded that protozoa do not migrate substantially from the soil particles in which they were originally inoculated to other particles since if they did, CO_2-production would not have been dependent on the inoculation procedure. Vargas and Hattori (1986) provided evidence that only at high moisture contents of soil (more than 60% of the WHC) ciliates were mobile in the soil matrix while at relatively modest moisture contents of 45% of WHC of the soil as in our experiments, virtually no active migration of protozoa was found. Despite early observations by Losina-Losinsky and Martinov (1930) and Biczok (1959) that upon introduction of a small number of protozoa into sterilized soil, they would multiply and move, in the absence of water flow, over distances of centimeters per day at a relatively modest soil moisture (45% WHC), our results lead to the conclusion that the soil is a rather constrained system with respect to the mobility of microorganisms in the soil matrix (Hattori 1988).

The introduction of ^{14}C labelled recipient cells into soil with a low soil moisture content was assumed to result in a relative inaccessibility of these cells for protozoan predation. Postma *et al.* (1989) showed that bacteria, introduced at lower soil moisture contents were less sensitive to protozoan predation than bacteria introduced at higher soil moisture contents. In the separate introduction treatment, as judged from the number of transconjugant cells (Figure 5), donor cells were better seperated from the recipient cells than in soils with joint introduction and it

may therefore be legitimate to suppose that also protozoa, which were inoculated together with the donor population, are better spatially separated from the ^{14}C labelled recipient cells in the soils with separate introduction.

Despite separation of the protozoa and ^{14}C labelled bacteria at the start, protozoa did accelerate the ^{14}C-CO_2 evolution in the initial phase of the incubation but no effect, neither of the presence of protozoa nor of the joint or separate introduction, was detected on the total ^{14}C evolved after 74 days (Figure 7). Since the initial number of protozoa inoculated rather than the method of introducing the bacteria and protozoa, controlled the ^{14}C-CO_2 respired, the turnover of ^{14}C apparently was determined by the frequency of encounters between protozoa and labelled microorganisms in the first days after the inoculation. The number of encounters then is a function of the inoculum density of both protozoa and ^{14}C labelled bacterial cells in the soil matrix. Assuming that i) ^{14}C-labelled bacteria were to a limited extent accessible for protozoa, irrespective whether they were on the same or on other particles but also that ii) the separation of ^{14}C labelled bacterial cells and protozoa was not complete, i.e. some ^{14}C labelled cells ended up in the relatively larger pores and by mixing two soil portions upon inoculation, few ^{14}C labelled cells or protozoa may mix, it can be hypothesized that the more protozoa are inoculated the higher the chances that they meet and predate on highly labelled bacterial cells. The dynamics of ^{14}C therefore differed from the dynamics of ^{12}C. It is essential for this different behaviour between ^{12}C and ^{14}C dynamics that non labelled cells are well accessible to protozoa and that the degree of accessibility is also the limiting factor with regard to protozoan predation on ^{14}C-labelled cells. This is an additional indication that protozoa are very much restricted in their movement in soil and their access to food sources may be limited.

The analysis of data on CO_2 evolution by fitting response curves revealed that protozoa initially stimulated the carbon turnover whereas during the second part of the incubation from day 14 onwards, the CO_2 evolution rate determining parameter B was not different for soils with or without protozoa. Upon disturbance, i.e. mixing of the soil portions, protozoa and bacteria might be redistributed. This would then result in new substrates becoming better available for bacteria. In case protozoa were inoculated, microorganisms sofar not available to protozoa, would be exposed to predation. If this would have occurred, one could expect that both ^{12}C- and ^{14}C-CO_2 respiration would have increased. However, the results showed that the presence or absence of protozoa or the number of protozoa present, did not affect the ^{14}C-CO_2 and CO_2 evolution in the period following the disturbance. Therefore, significant redistribution of bacteria and protozoa upon mixing of soil is not likely.

The transfer of genes via conjugation was another, indirect indicator of studying protozoan movement. The transfer of a plasmid from cell to cell which results in a transconjugant cell requires physical contact between a donor and a recipient cell.

Conjugational transfer of plasmid RP4 occurred within 2 days following introduction of the organisms. The frequency of conjugation was significantly higher in the joint introduction treatments than when recipient and donor cells and protozoa were introduced separately. Similar results were obtained in non sterile soil by Van Elsas *et al.* (1989a). The presence of protozoa and of different initial numbers of protozoa did not affect the frequency of gene transfer by conjugation (Figure 5) even though more plasmid containing donor cells were found in soils with a non diluted protozoan inoculum. These results indicate that, movement of bacterial cells by protozoan migration does not occur. Thus, protozoa hardly influence the distribution of bacterial cells in soil and so did not affect the mating chances of donor and recipient cells. Other authors have suggested that protozoa could be important carriers for bacterial dispersion in soil (Finlay and Fenchel 1989). Our results also oppose suggestions that soil animals, such as protozoa, might be important vectors for the flow of genes through organisms in soil.

The donor population showed significant plasmid loss during the first part of the incubation (Figure 3 and 4). In previous experiments, using sterile soil, a similar loss of plasmid RP4 was noted in this loamy sand (Van Elsas *et al.* 1989b). Also the donor population size was smaller than the size of the recipient population. Possibly, the higher metabolic load posed on the donor cells by the presence of both plasmid RP4 and transposon Tn5 as compared to recipient cells, provided a competitive disadvantage. The activity of a full inoculum of protozoa stimulated donor survival as well as maintenance of the plasmid in the donor population (Figure 3 and 4). Levrat *et al.* (1989) hypothesized that, in response to predation, the turnover of the bacterial population is faster, resulting in an increase of metabolic activity of the bacteria. An increased availability of certain nutrients, i.e. phosphate (Hunt *et al.* 1977, Van Elsas *et al.* 1989b) in the presence of protozoa may explain enhanced donor survival and plasmid maintenance. During the first phase of the incubation, the 1:10 diluted protozoan inoculum was apparently too small to significantly stimulate bacterial metabolism on the level of the total donor population so that in these soils no effect on plasmid maintenance was observed.

Grazing of bacteria by protozoa enhanced nitrogen mineralization in soil as observed by several authors (Elliott and Coleman 1977, Coleman *et al.* 1978, Elliott *et al.* 1980, Clarholm 1985, Ritz and Griffiths 1987, Kuikman and Van Veen 1989). This increased nitrogen mineralization could result from the production of protozoa biomass at the expense of bacterial biomass. Based on the C:N ratio of both bacteria and protozoa of 5-10 and a reduction of the microbial biomass, nitrogen would be mineralized (Fenchel 1986) without having to assume that protozoa do affect microbial metabolism. Grazing by protozoa may increase the metabolic activity of a bacterial population probably by the provision of limiting nutrients (Hunt *et al.* 1977). Such a mechanism might contribute to the different dynamics of

carbon and nitrogen mineralization: protozoa increased the mineralization of nitrogen by only 15% whereas the mineralization of carbon more than doubled. Also, the observed immobilization of nitrogen following the disturbance of the soil structure by mixing was in agreement with the ideas presented by Hunt *et al.* (1977).

The present results on ^{12}C and ^{14}C dynamics which were each controlled either by the method of inoculation applied or by the number of protozoa inoculated respectively and the results on gene transfer via conjugation, support the hypothesis by Stout (1973) that the ecology of protozoa is characterized by two important features: their activity is confined to small spaces and consequently small populations and restricted to (brief) periods when moisture conditions are favourable. The distribution of microorganisms in soil is not changing over time in the absence of fluctuations in soil environmental conditions like in these laboratory incubations. This is caused by the very restricted mobility of both protozoa and bacteria.

References

Amato M (1983) The determination of carbon ^{12}C and ^{14}C in plant and soil. Soil Biol Biochem 15: 611-612

Anderson RV, Elliott ET, McClellan JF, Coleman DC, Cole CV and Hunt HW (1978) Trophic interactions in soils as they effect energy and nutrient dynamics. III Biotic interactions of bacteria, amoebae and nematodes. Microb Ecol 4: 361-371

Anderson RV, Coleman DC and Cole CV (1981) Effects of saprotrophic grazing on net mineralization. In: Terrestrial Nitrogen Cycles (FE Clark and T Rosswall, eds). Ecol Bull 33: 201-216

Bamforth, SS (1988) Interactions between protozoa and other organisms. Agric Ecosystems Environm 24: 229-234

Biczok F (1959) Experimentelle Untersuchungen über die Wanderung der Protozoen im Erdboden. Acta Biol Szeged 5: 98-108

Clarholm M (1985) Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. Soil Biol Biochem 17: 181-187

Coleman DC, Anderson RV, Cole CV, Elliott ET, Woods L and Campion MK (1978) Trophic interactions in soils as they affect energy and nutrient dynamics. IV Flows of metabolic and biomass carbon. Microb Ecol 4: 373-380

Darbyshire JF, Wheatley RE, Greaves MP and Inkson RHE (1974) A rapid micromethod for estimating bacterial and protozoan populations in soil. Rev Ecol Biol Sol 11: 465-475

Elliott ET and Coleman DC (1977) Soil protozoan dynamics in a shortgrass prairie. Soil Biol Biochem 9:113-118

Elliott ET, Anderson RV, DC Coleman and CV Cole (1980) Habitable pore space and microbial trophic interactions. Oikos 35: 327-335.

Fenchel T (1986) The ecology of heterotrophic microflagellates. Adv Microbiol Ecol 9: 57-97

Finlay B and Fenchel T (1989) Everlasting picnic for protozoa. New Scientist: 66-69

Foissner W (1987) Soil protozoa: fundamental problems, ecological significance, adaptations in ciliates and testaceans, bioindicators and guide to the literature. Prog Protistology 2: 69-212

Hattori T (1988) Soil aggregates as microhabitats of microorganisms. Bull. Inst. Agric Res. Tohoku Univ. 37: 23-36

Hunt HW, Cole CV, Klein DA and Coleman DC (1977) A simulation model for the effect of predation on bacteria in continuous culture. Microb Ecol 3: 259-278

Hunt HW, Elliott ET and Walter DE (1989) Inferring trophic interactions from pulse-dynamics in detrital food webs. Plant Soil 115: 247-259

Kuikman PJ and Van Veen JA (1989) The impact of protozoa on the availability of bacterial nitrogen to plants. Biol Fertil Soils 8: 13-18

Levrat P, Pussard M et Alabouvette C (1989) Action d'*Acanthamoeba castellanii* (Protozoa, Amoebida) sur la production de siderophores par la bacterie *Pseudomonas putida*. CR Acad Sci Paris t 308, Serie III: 161-164

Losina-Losinsky L and Martinov PF (1930) A method of studying the activity and rate of diffusion of protozoa and bacteria in soil. Soil Sc 29: 349-362

Maniatis, Fritsch and Saubrook (1982) Molecular cloning. A laboratory manual. Col Spring Harbor labs., USA, pp

Page F (1967) Freshwater and soil amoebae. Freshwater biological association, Cumbria UK, Sc Publ no 34

Postma J, Walter S and Van Veen JA (1989) Influence of different initial soil moisture contents on the distribution and population dynamics of introduced *Rhizobium leguminosarum* biovar *trifolii*. Soil Biol Biochem 21: 437-442

Ritz K and Griffiths BS (1987). Effects of carbon and nitrate additions to soil upon leaching of nitrate, microbial predators and nitrogen uptake by plants. Plant Soil 102: 229-237

Rowe R, Todd R and Waide J (1977) Microtechnique for Most-Probable-Number analysis. Appl Environm Microb 33: 675-680

Simon R, Priefer U and Puhler A (1983) A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology 1: 784-791

Stout JD (1973) The relationship between protozoan populations and biological activity in soils. Amer Zool 13: 193-201

Van Elsas JD, Dijkstra AF, Govaert JM and Van Veen JA (1986) Survival of *Pseudomonas Fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Microbiol Ecol 38: 151-160

Van Elsas JD, Trevors JT and Starodub ME (1988) Bacterial conjugation between pseudomonads in the rhizosphere of wheat. FEMS Microbiol Ecol 53: 299-306

Van Elsas JD, Trevors JT, Starodub ME and Van Overbeek LS (1989a) Transfer of plasmid RP4 between pseudomonads after introduction into soil: influence of spatial and temporal aspects of inoculation. FEMS microb Ecol (in press)

Van Elsas JD, Trevors JT, Van Overbeek LS and Starodub ME (1989b) Survival of plasmid-containing *Pseudomonas fluorescens* and stability of the plasmids after introduction into soils. Can J Microbiol (in press)

Van Ginkel JH and Sinnaeve J (1980) Determination of total nitrogen in plant material with Nessler's reagent by continuous-flow analysis. Analyst 105: 1199-1203

Vargas R and Hattori T (1986) Protozoan predation of bacterial cells in soil aggregates. FEMS Microb Ecol 38: 233-242

CHAPTER 4

EFFECT OF SOIL MOISTURE REGIME ON PREDATION BY PROTOZOA OF BACTERIAL BIOMASS AND THE RELEASE OF BACTERIAL NITROGEN [1]

Abstract

A soil microcosm experiment is described in which the interaction between bacteria and protozoa, as affected by soil moisture regime, was studied. Predation by protozoa led to an overall 8 % higher plant nitrogen uptake and increased turnover of bacterial biomass. The interaction between bacteria and protozoa, as measured by the availability of bacterial ^{15}N-nitrogen to plants, was significantly affected by the soil moisture regime and by the presence of protozoa. In soils with only bacteria, plant nitrogen uptake was constrained by a soil moisture regime characterized by prolonged and intensified periods of drought. In soils with both bacteria and protozoa, predation by protozoa eliminated the adverse effect of soil moisture fluctuations on bacterial nitrogen turnover. Predation by protozoa raised the uptake of bacterial nitrogen by 15 % versus 40 % at regimes with stable vs fluctuating soil moisture conditions, respectively. It was concluded that protozoa play an important role in stimulating nitrogen mineralization, which typically occurs after remoistening of a dried soil.

[1] PJ Kuikman, MMI Van Vuuren and JA Van Veen Agriculture Ecosystems and environment (1989), in press

Introduction

Interactions among soil biota play a significant role in nutrient transformations and plant nutrient availability. Elliott *et al.* (1979) postulated a significant role of protozoa in accelerating mineralization of microbially immobilized nutrients. In the presence of protozoa, nitrogen content of plants was increased. Clarholm (1985) concluded from experiments in which plants were grown in the absence or presence of protozoa in a soil system with a natural bacterial population, that grazing by protozoa increased shoot/root ratios and increased plant dry weight. Clarholm's (1985) data also showed that bacteria mineralized nitrogen from soil organic matter and that the activity of protozoa makes more nitrogen available for uptake by plants. Meanwhile, the size of the bacterial population was reduced due to protozoan activity.

A major factor regulating the dynamics of the interactions between bacteria and protozoa in soils is water (Fenchel 1987, Foissner 1987). The activity of soil protozoa is strongly reduced in dry soils as shown by Darbyshire (1976) for ciliates and by Bryant *et al.* (1982) for amoebae. The latter also showed that encystment was an efficient survival mechanism for amoebae subjected to changing moisture conditions in soils. Neither amoebal nor bacterial densities were affected by alternating dry and wet conditions of the soil in which amoebal grazing was discontinuous, as compared to constantly moist soil, where grazing was continuous. This observation was also made by Darbyshire and Greaves (1967), using planted soil that was incubated at two soil moisture tensions.

Both in natural environments and in laboratory incubation studies with soil microcosms on which plants are grown, soil moisture fluctuations are virtually inevitable. In view of the importance of protozoa for the availability of nitrogen to plants, we examined the impact of soil moisture fluctuations on protozoan activity in relation to bacterial nitrogen and its availability to plants. In this experiment we used ^{15}N-nitrogen labelled bacteria, which permitted the monitoring of the transformations of specific plant nitrogen sources.

Materials and methods

Soil

The soil used was a loamy sand, originating from an A-horizon of arable land near Ede (table 1). Four weeks prior to the start of the experiment the soil was collected and air-dried until the moisture content was 12 % (v/w). Subsequently, the soil was sieved (4 mm) and portions of approximately 1300 g (1170 g dry soil) were weighed into plastic bags. These soil portions were sterilized by exposure to a ^{60}Cobalt-γ-radiation source, receiving 4 Mrad in 29 hours, one week before the

incubation started. Sterility of the soil was tested by preparing a dilution series of suspended soil. The suspension was incubated on 1:2 Tryptone Soya Agar; no bacteria or other organisms were found. No inorganic nitrogen was added to the soil portions, that contained approximately 34 mg (29 ppm) inorganic nitrogen at the start of the incubation.

Table 1 Physical and chemical properties of the soil

Origin	Ede (NL)
Texture class	loamy sand
Particle size distribution (%)	
clay (< 2 μm)	3
silt (2-50 μm)	12
sand (> 50 μm)	85
CEC (meq/100 g)	9
Organic C (%)	2.0
Total N (%)	0.13
$CaCO_3$ (%)	0.10
pH_{KCl}	6.2

Microcosms

At the start of the incubation, the soil moisture content was adjusted to 15 % (v/w), using different inocula. Soil was inoculated with bacteria (*Pseudomonas fluorescens* and *Enterobacter cloacae*). The inoculated bacteria were pre-grown on a mineral medium containing ^{15}N-nitrogen and, before inoculation, washed two times and resuspended in sterile demineralized water. The inoculum contained approximately 3 mg bacterial nitrogen and 10^{10} bacteria per microcosm (1170 g dry soil). A protozoan inoculum, containing amoebae and flagellates isolated from the soil used in this experiment, was added to half of the soil portions while the other half received sterile demineralized water. The inoculated protozoa were precultured on *Pseudomonas fluorescens* and *Enterobacter cloacae.* The protozoan inoculum was approximately 5×10^6 organisms per microcosm.

Following the inoculation of the organisms, the soil portions in plastic bags were homogenized and transferred in to sterilized plastic pots (1.7 l) to a bulk density of 1.3 g $\times$ cm^{-3}. Each pot was planted with three 10-day old wheat (*Triticum aestivum* cv. *Sicco*) seedlings. The seeds were surface sterilized and only those that showed no bacterial of fungal contamination, after being grown on nutrient agar for 10 days, were used. The pots or microcosms were closed by a PVC-lid with a separate hole

for every wheat plant and a fourth central hole for additions of water. Infections were prevented by placing sterile cotton plugs around the stems of the wheat plants as well as in the central hole for watering. The microcosms were incubated in a climate chamber with a 14 hour light (20°C) and a 10 hour dark (16°C) period. Soil moisture content was maintained at 15 % (v/w) with sterile demineralized water by weighing the microcosms and correcting for estimated plant production.

Experimental design

The experimental design included the study of the interaction between two factors: soil moisture regime and presence of protozoa. Therefore we compared soils inoculated with bacteria only and soils inoculated with bacteria and protozoa (indicated by B and BP respectively). These soils were incubated under three different soil moisture regimes (figure 1) during a part of the overall 35 days of incubation. During the first 17 days of incubation the soil moisture content was adjusted to 15 % (v/w) every second day. During the second part of the incubation, three soil moisture regimes were imposed by varying the time between the successive adjustments of the soil moisture content to 15 % (v/w) from 1 up to 3 days (regimes indicated by 1, 2 and 3 respectively). These soil moisture regimes were characterized by respectively minimal (1), intermediate (2) and prolonged (3) periods of drought. The results in terms of soil moisture fluctuations are depicted in figure 2.

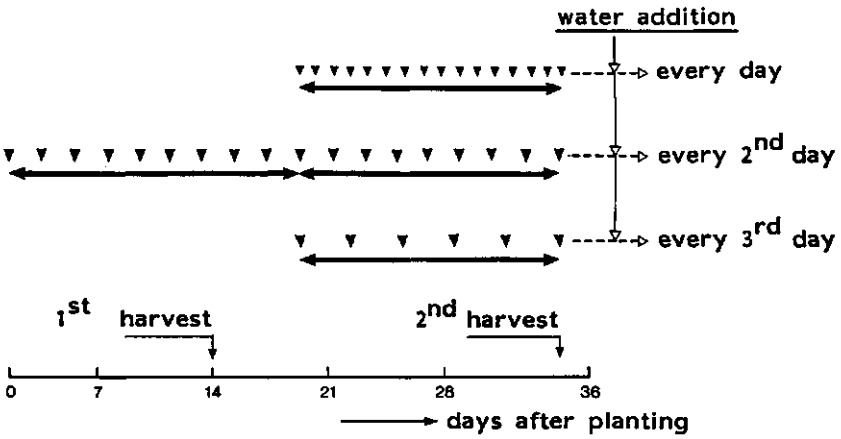

Figure 1 The experimental design, showing the three soil moisture regimes (1, 2 and 3) that were established by varying the time between water additions to the microcosms: every day/minimal, every 2nd day/intermediate and every 3rd day/prolonged soil moisture fluctuations.

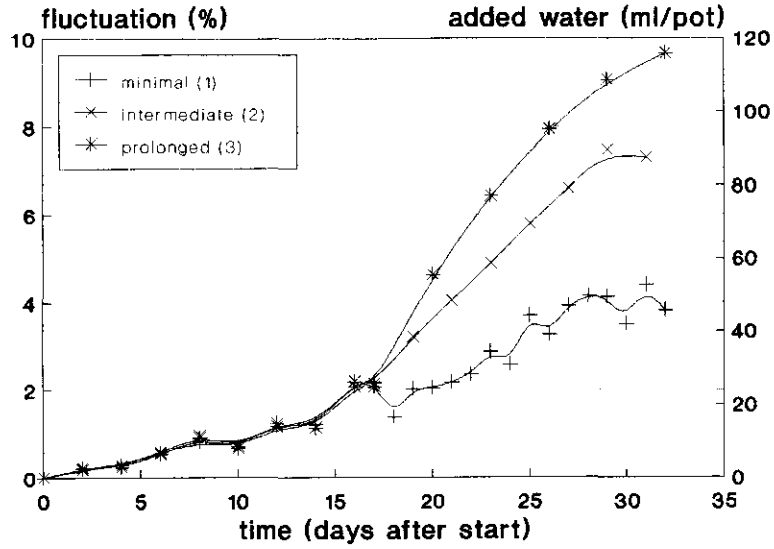

Figure 2 **The volume of water added to the microcosms (figures represent means of four replicates) and the size of the soil moisture fluctuation (percent water per g dry soil) at the moment of adding water to the microcosms, bringing the soil moisture content up to 15 %.**

Analyses

For each treatment, four replicated microcosms were destructively sampled on days 14 (B-2 and BP-2) and 35 (all six treatments). After cutting off the shoots, the roots were separated from the soil and washed on a fine (0.5 mm) sieve. Dry shoot and root yield were obtained by weighing the plant material after drying at 80°C for 48 hours. Plant nitrogen content was measured as $N\text{-}NH_4$ after Kjeldahl distillation. Preceding the total nitrogen determination the plant-material was ground on a 0.5 mm sieve in a mill and digested with sulphuric acid. Afterseparating the roots, the soil was subsampled for determination of bacterial and protozoan numbers (data not shown) and analysis of inorganic nitrogen and moisture content. No protozoa were detected in soils of the B treatment. The number of bacteria found at the end of the experiment was approximately 10^8 per gram of soil (10^{11} per microcosm). After extraction of 25 g soil in 50 ml 0.5 *M* K_2SO_4, $N\text{-}NH_4$ using Nessler's reagent (van Ginkel and Sinnaeve 1980) and $N\text{-}NO_3$ was measured by continuous flow analysis. Soil moisture content was determined after drying the soil at 105°C for 24 hours. The ^{15}N-nitrogen content of samples was determined by mass-spectrometry (Finnigan MAT 250) according to Bremner (1965).

Statistics

The results were statistically analysed by analysis of variance over the two experimental factors, i.e. presence of protozoa and soil moisture regime. All differences reported are significant at the level of $P=0.05$ at least.

Results and discussion

After 14 days as well as at the end of the 35 day growth period, total plant yield varied between 1.81 and 3.01 g dry mass per microcosm and was neither affected by the presence of protozoa nor by soil moisture regime (results not shown). These results oppose results obtained by Elliott *et al.* (1979) and Clarholm (1985) which indicated that total plant yield improved in soils with protozoan activity as compared to soils without protozoa. The shoot:root ratios in this experiment varied between 2 and 3 and were not affected by protozoan presence nor by soil moisture regime. This observation contrasted with results obtained by Clarholm (1985) who showed higher shoot:root ratios in soils with protozoan activity. No explanation for these differences could be found. However, different amounts of soil per microcosm as well as different soil types were used and they were incubated under different environmental conditions (i.e. light intensity, temperature, fertilization), which all could have an effect on the shoot:root ratio.

On average, 90 % of the total plant nitrogen uptake took place during the last 20 days of the incubation. At the end of the growth period (35 days) total plant nitrogen uptake ranged from 58.3 mg (B-2) to 65.5 mg (BP-1) per microcosm (figure 3). No inorganic nitrogen could then be detected in any of the soils. In those soils where only bacteria made up the soil microbial population, significantly less ($P<0.05$) nitrogen was taken up by plants at soil moisture regimes with intermediate (2) and prolonged (3) versus a soil moisture regime with minimal (1) fluctuations. However, in the presence of protozoa, the nitrogen uptake by plants was not affected by the soil moisture regime. As compared to soils with bacteria only, in the presence of protozoa nitrogen uptake by plants increased ($P<0.01$) by 5% , 10% and 8% to 65.5, 64.9 and 63.3 mg N per microcosm at soil moisture regimes 1, 2 and 3 respectively. The activity of protozoa clearly stimulated the total nitrogen uptake by plants under all three soil moisture conditions.

When we compare the average total plant nitrogen uptake of 62.4 mg per microcosm (figure 3) with the amount of inorganic nitrogen present per microcosm at the start of the incubation (34 mg), it can also be concluded that both in soils with bacteria only and in soils with bacteria and protozoa on average 28 mg (24 ppm) nitrogen was mineralized from soil organic matter. Despite the fact that in the presence of protozoa plant nitrogen supply was improved, shoot:root ratios were not higher like it was found by Davidson (1969) and Clarholm (1985).

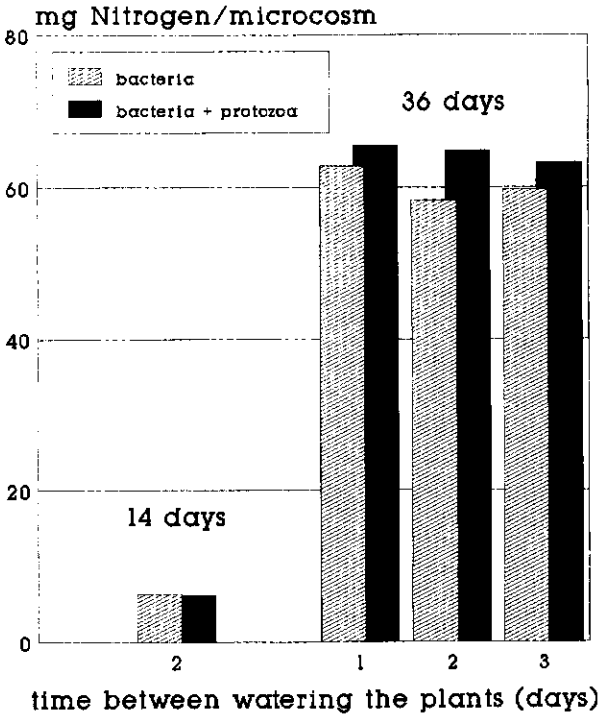

Figure 3 Plant nitrogen uptake (final plant nitrogen content minus nitrogen content of seedlings) for three soil moisture regimes at two sampling dates (figures represent means of four replicates); LSD_{water} (Least Significant Difference) = 2.1 mg N, $LSD_{protozoa}$ = 1.8 mg N.

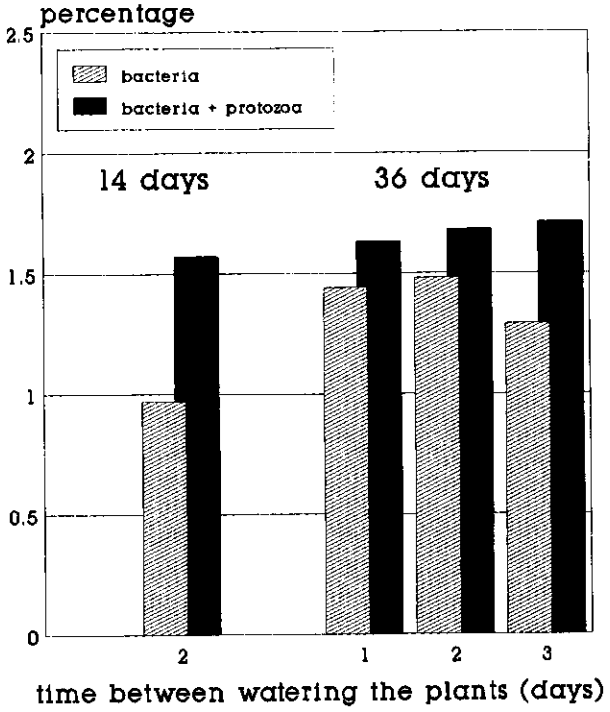

Figure 4 Recovery of bacterial ^{15}N-nitrogen in plant nitrogen as percentage of inoculated amount for three soil moisture regimes at two sampling dates (figures represent means of four replicates); LSD = 2.8 %.

In the presence of protozoa the uptake by plants of inoculated bacterial ^{15}N-nitrogen was significantly increased (figure 4). At the end of the growth period, the recovery in plants of bacterial ^{15}N-nitrogen ranged from 31 % in the B-3 treatment to 44 % in the BP-2 treatment. At this point a significant interaction between soil moisture regime and the presence of protozoa was found: grazing by protozoa stimulated the availability and uptake by plants of bacterial ^{15}N-nitrogen in general, but the extent to which this happened, was affected by the soil moisture regime. The presence of protozoa yielded 15, 30 and 40 % more bacterial ^{15}N-nitrogen in plants at soil moisture regimes with respectively minimal, intermediate and prolonged soil moisture fluctuations as compared to the B treatments.

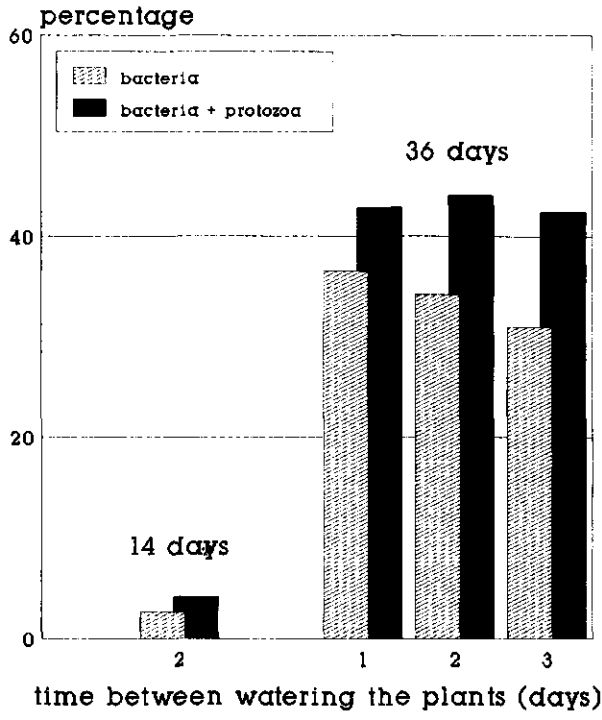

Figure 5 Relative contribution of inoculated bacterial ^{15}N-nitrogen to the plant nitrogen uptake (mg bacterial ^{15}N / mg plant N . 100 %) (figures represent means of four replicates); LSD = 0.05 %.

When the results on total nitrogen and on ^{15}N-nitrogen were combined (figure 5), it became clear that the uptake by plants of more ^{15}N-nitrogen in the presence of protozoa was not only caused by a higher total nitrogen uptake. In the presence of protozoa, but irrespective of soil moisture regime, plants took up nitrogen with a significant higher ($P<0.01$) enrichment of ^{15}N-nitrogen. In other words, the contribution of inoculated bacterial nitrogen to total plant nitrogen uptake is not affected by soil moisture fluctuations in the presence of protozoa. However, in soils with bacteria only, the mineralization of inoculated bacterial nitrogen was

severely hampered ($P<0.05$), especially when the soil moisture regime was characterized by periods of prolonged and intensified drought as in treatment B-3. This indicates that predation of bacteria by protozoa increased the net-mineralization of bacterial biomass and resulted in a higher uptake of ^{15}N-nitrogen by plants. Furthermore, protozoa are effective even under fluctuating soil moisture conditions in which prolonged and intensified periods of drought occurred during which, almost certainly, protozoan activity temporarily is stopped. The effectiveness of protozoa under these fluctuating soil moisture conditions, as judged from turnover of bacterial biomass, was even larger than that at stable soil moisture conditions which enabled continuous protozoan activity.

The higher the soil moisture tension, the more protozoa are spatially restricted when moving through the soil. Eventually, their activity is stopped and to survive protozoa have to form cysts (Bryant *et al.* 1982). Several authors (Alabouvette *et al.* 1981, Darbyshire *et al.* 1985, Vargas and Hattori 1986) have reported that pores with pore-necks smaller than approximately 3 μm are inaccessible to soil protozoa. Soils, in which pores with pore-necks larger than 3μm are devoid of water, are at pF 3. In our loamy sand this pF of 3 corresponded to a water content of 9-10 % (v/w). This would indicate that as soon as the soil lost 6 % or more water, protozoan activity was (temporary) stopped. Situations like this occurred (figure 2) in both the treatment with intermediate (2) fluctuations (day 25 and on) and the treatment with prolonged (3) fluctuations (day 20 and on).

The increased biological activity after remoistening of a dried soil, as observed by stimulated CO_2-evolution and N-mineralization (Birch 1958, Sorensen 1974) has been explained by an increase in the availability of organic matter through the disruption of soil structures and death of microbial biomass. Harada and Hayashi (1968) demonstrated that cytoplasmic material becomes easily decomposible when soils were incubated at wet-dry conditions. Presumably, these materials, mostly amino acids, originated from microbial cells that were killed upon drying the soil. Van Veen *et al.* (1985) described the increased C-and N-mineralization after remoistening of a dried soil by assuming a temporary decrease in the extent to which soil structures protect organic matter and microbial biomass. The present results indicate that protozoa rapidly recovered from the preceding drought period, so that they would utilize the increased amount of available bacterial cells and stimulated the turnover of microbial nitrogen.

References

Alabouvette C, Couteaux MM, Old KM, Pussard M, Reisinger O and Toutain E (1981) Les protozoaires du sol: aspects ecologiques et methodologiques. Ann Biol 20: 255-303

Birch HF (1958) The effect of soil drying on humus decomposition and nitrogen availability. Plant and Soil 10: 9-31

Bremner JM (1965) Isotope-ratio analysis of nitrogen in nitrogen-^{15}N tracer investigations. In: CH Black (editor) Methods of Soil Analysis Part 2 Amer Soc Agron Suc Madison WI, pp 1256-1286

Bryant RJ Woods LE Coleman DC Fairbanks BC McClellan JF and Cole CV (1982) Interactions of bacterial and amoebal populations in soil microcosms with fluctuating moisture content. Appl Environ Microbiol 43: 747-752

Clarholm M (1985) Interactions of bacteria protozoa and plants leading to mineralization of soil nitrogen. Soil Biol Biochem 17: 181-187

Darbyshire JF (1976) Effects of water suctions on the growth in soil of the ciliate *Colpoda steinii* and the bacterium *Azotobacter chroococcum*. J Soil Sci 27: 369-376

Darbyshire JF and Greaves MP (1967) Protozoa and bacteria in the rhizosphere of *Sinapsis alba* L., *Trifolium repens* L. and *Lolium perenne* L. Can J Microbiol 13: 1057-1068

Darbyshire JF Robertson L and Mackie LA (1985) A comparison of two methods of estimating the soil pore network available to protozoa. Soil Biol Biochem 17: 619-624

Davidson RL (1969) Effects of soil nutrients and moisture on root/shoot ratios in *Lolium perenne* L. and *Trifolium repens* L. Ann Bot 33: 571-577

Elliott ET Coleman DC and Cole CV (1979) The influence of amoebae on the uptake of nitrogen by plants in gnotobiotic soil In: JL Harley and R Scott Russell (editors) The Soil-Root Interface Academic Press London, pp 221-229

Fenchel T (1987) Ecology of Protozoa: The Biology of Free-living Phagotrophic Protists. Springer-Verlag Berlin/Heidelberg/New York, pp 197

Foissner W (1987) Soil Protozoa: Fundamental Problems Ecological Significance Adaptations in Ciliates and Testaceans Bioindicators and Guide to the Literature. Progress in Protistology 2: 69-212

Harada T and Hayashi R (1968) Studies on the organic nitrogen becoming decomposable through the effect of drying a soil. Soil Sc Plant Nutr 14: 13-19

Sorensen LH (1974) Rate of decomposition of organic matter in soil as influenced by repeated air drying-rewetting and repeated additions of organic material. Soil Biol Biochem 6: 287-292

Van Ginkel JH and Sinnaeve J (1980) Determination of Total Nitrogen in Plant Material with Nessler's Reagent by Continuous-flow Analysis. Analyst 105: 1199-1203

Van Veen JA Ladd JN and Amato M (1985) Turnover of carbon and nitrogen through the microbial biomass in a sandy loam and a clay soil incubated with [$^{14}C(U)$]glucose and [^{15}N]$(NH_4)_2SO_4$ under different moisture regimes. Soil Biol Biochem 17:747-756

Vargas R and Hattori T (1986) Protozoan predation of bacterial cells in soil aggregates. FEMS Microb Ecol 38: 233-242

CHAPTER 5

^{15}N-NITROGEN MINERALIZATION FROM BACTERIA BY PROTOZOAN GRAZING AT DIFFERENT SOIL MOISTURE REGIMES [1]

Abstract

The predation by protozoa of bacteria was studied in relation to soil moisture regimes. In soil microcosms, incubated at three soil moisture levels, it was shown that the activity of protozoa is halted as soon as pores with pore necks larger than 3μm are devoid of water. The activity of protozoa reduced the number of bacteria and increased the mineral nitrogen content of the soil at more favourable moisture conditions. In a series of experiments in which planted soil was subjected to soil moisture regimes with either continuous moist or fluctuating moist conditions, it was shown that protozoa generally stimulated the mineralization of nitrogen and the uptake of nitrogen in plants by 5% to 15% compared to soils without protozoa. Also, the turnover of introduced, ^{15}N labelled bacterial nitrogen was higher in soils with protozoa than in soils with only bacteria. Even though protozoa were forced to encyst upon drying of the soil, they reacted very rapidly to remoistening. Under conditions with modest fluctuating soil moisture conditions, protozoan activity resulted in an even higher plant ^{15}N-nitrogen uptake that in soils incubated under continuously moist conditions. When fluctuations in the soil moisture content were more intense and applied more frequently, protozoa still stimulated the mineralization of nitrogen. Here, their more restricted activity tended to yield less nitrogen available for plant uptake than in continuously moist soils. Protozoa did not reduce bacterial numbers in all incubations and protozoan numbers were not affected by the soil moisture regimes. An important regulation by soil moisture fluctuations on the impact of food web interactions between bacteria and protozoa to mineralization of nitrogen is hypothesized.

[1] PJ Kuikman, AG Jansen and JA Van Veen, submitted to Soil Biology Biochemistry

Introduction

Interactions among soil biota play a significant role in nutrient transformations and plant nutrient availability (Coleman *et al.* 1984). Elliott *et al.* (1979) postulated a significant role of protozoa in accelerating mineralization of microbially immobilized nutrients. In the presence of protozoa, nitrogen content of plants was increased. Clarholm (1985) concluded that grazing of bacteria by protozoa increased shoot/root ratios and increased plant dry weight. Clarholm (1985) and Kuikman and Van Veen (1989) showed that bacteria mineralized nitrogen from soil organic matter and that the activity of protozoa makes more nitrogen available for uptake by plants. Meanwhile, the size of the bacterial population was reduced due to protozoan activity. Kuikman and Van Veen (1989) also showed that protozoa increased the turnover of bacterial nitrogen in soil which resulted in an improved availability of bacterial nitrogen to plants in the presence of protozoa. Levrat (1987) supported the hypothesis that in response to predation, the turnover of the bacterial population is faster, and this resulted in an increase of the metabolic activity of the bacteria.

A major factor regulating the dynamics of the interactions between bacteria and protozoa in soils is water (Nikoljuk 1963; Fenchel 1987; Foissner 1987). Protozoa are aquatic organisms, hence their presence and activity in terrestrial ecosystems depend upon their ability to cope with fluctuating moisture conditions (Elliott and Coleman 1977) which dictate a 'stop and start' type of existence (Bamforth 1985). The activity of soil protozoa is strongly reduced in dry soils as shown by Darbyshire (1976) for ciliates and by Bryant *et al.* (1982) for amoebae. The latter also showed that encystment was an efficient survival mechanism for amoebae subjected to changing moisture conditions in soils. Darbyshire and Greaves (1967) showed that, in soils planted to *Sinapsis alba* L. and *Trifolium repens* L. and incubated at two soil moisture tensions, the (added) protozoa could not be active and reproduction was limited under relatively dry soil moisture conditions. Even if survival and reproduction did occur, relatively dry conditions could limit the effect of protozoa through the inability to move through the soil (Losina-Losinsky and Martinov 1930). The ability to meet bacterial prey cells is essential for the overall effect of protozoa (Vargas and Hattori 1986).

Both in natural environments and in laboratory incubation studies with soil microcosms on which plants are grown, soil moisture fluctuations are virtually inevitable. The use of continuously moist soil throughout an experiment, as is often the case with microcosm experiments or laboratory incubations, may lead to an incomplete understanding of how the system functions (Elliott *et al.* 1986).

The objective of this study was to find the limits to protozoan activity as they are set by the soil moisture regime. Protozoan activity was determined from the impact of protozoan grazing on bacteria and nitrogen mineralization both in fallow and in planted soils. The soil moisture regime and the intensity of soil moisture fluctuations

was varied. Bacteria or soils, labelled with ^{15}N-nitrogen, enabled the monitoring of the transformations of specific plant nitrogen sources.

Materials and methods

Soil

The soil used was a loamy sand, originating from an A-horizon of arable land in The Netherlands (organic matter 3.5 %, nitrogen 0.13 %, pH_{KCl} 6.2) as described by Kuikman and Van Veen (1989). The soil was collected from a field plot, sieved (Ø < 4 mm), and air-dried to the appropriate moisture content. Soil portions in plastic bags were sterilized by exposure to a ^{60}Cobalt-γ-radiation source, receiving 4 Mrad in 29 hours, one week before the incubation started. Sterility of the soil was tested by plating dilutions of suspended soil on 1:2 Tryptone Soya Agar; no bacteria or other organisms were found. No inorganic nitrogen was added to the soil portions.

Experimental design

The experimental design included the study of the interaction between two factors: presence of protozoa and soil moisture regime. To study the impact of protozoa soils were compared which were inoculated with bacteria only and soils inoculated with bacteria and protozoa (indicated by B and BP respectively).

To study the impact of soil moisture, four experiments were carried out. In experiment 1, fallow soils were incubated at three constant soil moisture contents: 8%, 15% and 20% (v/w). The dynamics of the bacterial and protozoan population and the mineralization of nitrogen in time was recorded.

In studying the interaction between bacteria and protozoa in planted soils, soil moisture fluctuations are inevitable. Plants will decrease the water content of soil in which they grow. When this water is replenished, drying and rewetting fluctuations are created which might affect the interaction between protozoa and bacteria and so the protozoan effect on nitrogen mineralization. In experiment 2, 3 and 4 the soils were incubated under two different soil moisture regimes during a part or all of the overall incubation period. These two soil moisture regimes were characterized as continuously moist (CM) with minimal soil moisture fluctuations and fluctuating moist (FM) with intense soil moisture fluctuations and period of drought,, respectively. In experiment 2, the soil moisture content was adjusted to 15% (v/w) every second day during the first 17 days of incubation. During the second part of the incubation, two soil moisture regimes were imposed by varying the time between the successive adjustments of the soil moisture content to 15% (v/w) from 1 to 3 days (regime CM and FM, respectively). In experiment 3, during the second part of the incubation, two soil moisture regimes were imposed by adding water to

the microcosms only if the soil moisture content had dropped to approximately 12% (regime CM) or 7% (regime FM). In experiment 4, two soil moisture regimes, as described for experiment 3, were imposed during all of the 21 days of incubation.

The soil moisture content was determined by weighing the microcosms and estimating plant weight every day. Water additions were made at the end of the light period as to enable the water to be distributed through the entire soil core in the microcosm without interference by plant uptake. For each treatment, four replicated microcosms were destructively sampled on day 10 or day 14 and day 35 in experiment 2 and 3, respectively and on day 21 in experiment 4.

Soil incubation (experiment 1)

Three days prior to the start of the incubation the soil (moisture content 6%) was inoculuted with a suspension of *Pseudomonas fluorescens* and *Enterobacter cloaceae* as well as several non-identified bacteria, isolated from a soil suspension plated and grown on 1:2 Tryptone Soya Agar. All bacteria were pregrown in a 1:2 Tryptone Soya Broth medium, harvested by centrifugation and washed twice in sterile demineralized water. The soil was incubated at room temperature for two days. Then, the soil was split into six portions and two by two the soil moisture content was adjusted to 8, 14 and 19 % (v/w), respectively by the addition of sterile demineralized water or by the addition of a protozoan inoculum one day prior to the start of the incubation. The protozoan inoculum contained both amoebae and flagellates isolated from the soil used in this experiment. The protozoa were precultured on *Pseudomonas fluorescens* and *Enterobacter cloaceae*. The size of the protozoan inoculum was 0.7×10^4 organisms per gram of dry soil.

Following inoculation of the organisms, soil portions with and without protozoa were homogenized. All microcosms, microtiter plates ($12.2 \times 7.9 \times 1.2$ cm), were filled with a mixed soil portion equivalent to 74 g dry soil and brought to a bulk density of 1.3 g $\times$ cm^{-3} and closed with a lid. The microcosms were incubated in a dark room kept a 16°C and water losses were minimized by placing them in a container with a high air humidity (R.H. 95%). Duplicate microcosms were sampled on day 0, 3, 10 and 19 after the start of the incubation.

Soil incubation with plants (experiment 2, 3 and 4)

At the start of the incubation, the soil moisture content of approximately 11% was adjusted to 15% (v/w), using different inocula. Soil was inoculated with bacteria (*Pseudomonas fluorescens* and *Enterobacter cloaceae*). The inoculated bacteria were pre-grown on a mineral medium [4.0 g glucose, 2.88 g citric acid, 1.07 g NH_4Cl, 0.5 g K_2HPO_4, 0.5 g KH_2PO_4, 0.2 g $MgSO_4$, 0.01 g $MnSO_4$, NaCl, $FeSO_4$ and $CuSO_4$, 0.04 mg $ZnSO_4$, 0.03 mg $CoCl_2$, 0.02 mg $(NH_4)_6Mo_2O_4$, 1000 g H_2O] containing ^{15}N-NH_4Cl (50% ^{15}N) for the bacterial inoculum in experiment 2 and 3 and non-labelled NH_4Cl in experiment 4. Before inoculation, the bacteria were

washed two times and resuspended in sterile demineralized water. The soil used in experiment 4, was soil that had been incubated in experiment 3 and was left after taking out subsamples for analyses. After sterilizing this soil, it contained ^{15}N in soil organic material and was inoculated with non-labelled bacteria.

A protozoan inoculum, containing amoebae and flagellates isolated from the soil used in this experiment, was added to half of the soil portions while the other half received sterile demineralized water. The inoculated protozoa were precultured on *Pseudomonas fluorescens* and *Enterobacter cloaceae*. Approximately 3×10^7 bacteria and 5×10^3 protozoa were added per gram of dry soil.

Following the inoculation of the organisms, the soil portions in plastic bags were homogenized and transferred into sterilized plastic pots (1.7 l) and brought to a bulk density of 1.3 $g \times cm^{-3}$. Portions of 1170, 1170 and 995 g dry soil were used in experiment 2, 3 and 4, respectively. Each pot was planted with three 9-day old wheat (*Triticum aestivum* cv. *Sicco*) seedlings. The seeds were surface sterilized. After being grown on nutrient agar for 10 days, only seeds that showed no bacterial of fungal contamination were used. The pots or microcosms were closed by a PVC-lid with a seperate hole for every wheat plant and a fourth central hole for additions of water. Infections were prevented by placing sterile cotton plugs around the stems of the wheat plants as well as in the central hole for watering.

The microcosms were incubated in a climate chamber with a 14 hour light (20°C) and a 10 hour dark (16°C) period. Soil moisture content was maintained at 15 % (v/w) with sterile demineralized water by weighing the microcosms and correcting for estimated plant production.

Analyses

After cutting off the shoots, the roots were separated from the soil and washed on a fine (0.5 mm) sieve. Dry shoot and root yield were obtained by weighing the plant material after drying at 80°C for 48 hours. Plant nitrogen content was measured as $N\text{-}NH_4$ after Kjeldahl destillation (Bremner 1965). Prior to the total nitrogen determination, the plant-material was ground on a 0.5 mm sieve in a mill and destructed with sulphuric acid. After removing the roots, the soil was subsampled for determination of bacterial and protozoan numbers and for analysis of inorganic nitrogen and soil moisture content.

The number of bacteria was determined by extracting 10 g fresh soil in 95 ml of 0.1% Na-pyrophosphate solution on a rotary shaker (10 min., 200 rpm). A 10-fold dilution series was prepared and appropriate dilutions were plated (in triplicate) on 1:2 Tryptone Soya Agar. After an incubation of the plates for 48 hours at 29°C, the total number of colony forming units was determined. The number of protozoa was determined by a modified, most-probable-number method (Darbyshire *et al.* 1974; Clarholm 1981) extracting 5 g fresh soil in 100 ml of Amoebae Saline (Page 1976), using 4-fold dilutions and eight replicated series with *Pseudomonas*

fluorescens and *Enterobacter cloaceae* as a food source. The microtiter plates were incubated at 12°C in the dark and scanned for the presence of protozoa after 1, 2 and 4 weeks.

After extraction of 25 g soil in 50 ml 0.5 *M* K_2SO_4, $N\text{-}NH_4$ using Nessler's reagent (van Ginkel and Sinnaeve 1980) and $N\text{-}NO_3$ was measured by continuous flow analysis. Soil moisture content was determined after drying the soil at 105°C for 24 hours. The ^{15}N-nitrogen content of samples was determined by mass-spectrometry (Finnigan MAT 250) according to Bremner (1965).

Statistics

The data from experiment 1 on bacterial numbers and ammonium nitrogen in soil were analysed with a 3-factor analysis for the factors time [day 0, 3, 10 and 19], soil moisture [7.5, 14 and 19% (v/w), respectively] and protozoa [without and with protozoa].

The data from experiment 2, 3 and 4 were statistically analysed by analysis of variance for two experimental factors, i.e. presence of protozoa and soil moisture regime.

All differences reported are significant at the level of $P=0.05$ at least.

Results

Fallow soil (experiment 1)

The average soil moisture content was 7.5, 14 and 19% (v/w), respectively in experiment 1. The soil moisture content dropped during the incubation in all cases by 1-1.5% (v/w) which was caused by condensation water formed on the lid of the microcosms.

The number of bacteria (colony forming units per gram dry soil) ranged from approximately 5×10^8 at day 0 to 9×10^8 at other days. The length of the incubation had no significant effect on the number of bacteria in soil but a significant interaction ($P<0.001$) was found between the soil moisture content and the presence of protozoa (Figure 1). At a soil moisture content of 14 and 19% (v/w), the presence of protozoa reduced the number of bacteria by a factor of 2, while at a soil moisture content of 7.5% (v/w) no effect of protozoa was found compared to soils without protozoa.

At a soil moisture content of 7.5% protozoa hardly multiplied whereas at 19% both amoebae and flagellates had grown (Table 1). At a soil moisture content of 14% amoebae were more abundant than flagellates. Preliminary experiments had indicated that protozoa were not active in soils at a moisture content of approximately 9%-11%.

Table 1 The number of amoebae and flagellates in experiment 1 (numbers per gram dry soil and standard deviation for two replicated most probable number estimates) at three average soil moisture levels.

	Amoebae ($\times 10^3$)		Flagellates ($\times 10^3$)	
time	10 days	19 days	10 days	19 days
soil moisture				
7.5%	0.15 (0.04)	0.03 (0.01)	0.00	0.03 (0.01)
14.0%	10.26 (7.09)	35.58 (9.99)	0.25 (0.17)	0.43 (0.00)
19.0%	48.30 (48.84)	26.79 (4.29)	12.75 (2.81)	19.36 (7.23)

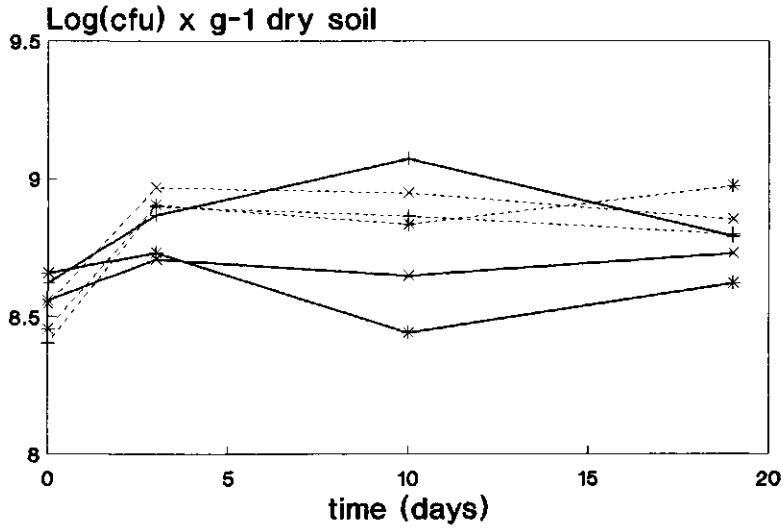

Figure 1 Dynamics of bacteria (colony forming units per gram dry soil on 1:2 Tryptone Soya Agar) in experiment 1 at three moisture contents: 7.5% (+), 14% (×) and 19% (*) and without protozoa (broken line) and with protozoa (solid line)

In all soils, net mineralization of ammonium nitrogen (approximately 0.008 mg N $\times$ g^{-1} dry soil) was found. The interaction ($P<0.001$) between soil moisture content and presence of protozoa was significant. In the presence of protozoa, more ($P<0.01$) ammonium (0.014 mg N $\times$ g^{-1} dry soil) was mineralized between day 0 and day 10 at a soil moisture content of 14% and 19% (v/w), respectively but no

effect was found at a soil moisture content of 7.5% (v/w). During the incubation period nitrate production was not found.

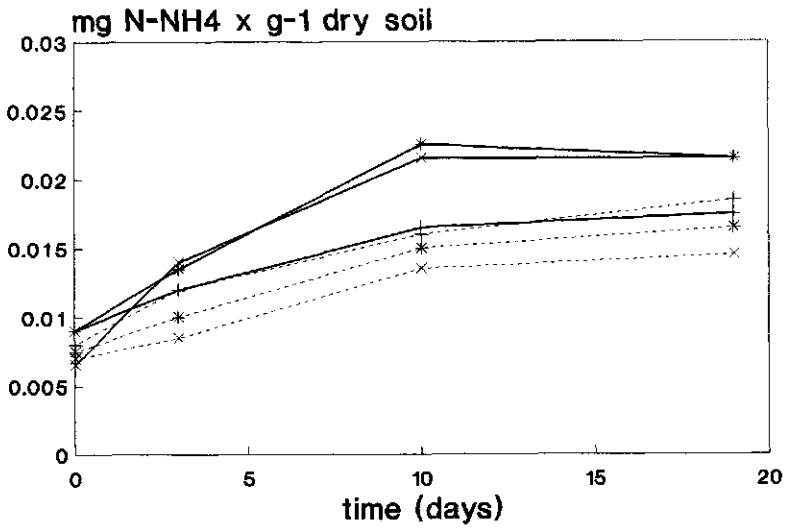

Figure 2 Dynamics of ammonium-nitrogen (mg N per gram dry soil) in experiment 1 at three moisture contents: 7.5% (+), 14% (×) and 19% (∗) and without protozoa (broken line) and with protozoa (solid line)

Planted soil (experiment 2, 3 and 4)

Soil moisture regime

The continuous moist (CM) regimes were almost identical. The three sets of fluctuating moist (FM) regimes differed in the lenght of the period during which the soil moisture content dropped to values between 6 and 8% (v/w) (Figure 3). In experiment 2, the fluctuations gradually intensified as plants transpired more water when they became older and larger. The size of the fluctuations was 6 and 8% for the fluctuating regimes only from day 23 until the end of the incubation. In experiment 3, from day 12 until the end of the incubation, soil moisture fluctuations as large as 8% were established. In experiment 4, from day 0 until day 21 soil moisture fluctuations from 6-9% were established.

The total accumulated water added in the course of the incubation was affected by the soil moisture regime: plants grown under a fluctuating moist regime transpired less water ($P<0.05$). The total accumulated water added was not affected by the absence or presence of protozoa (data not shown).

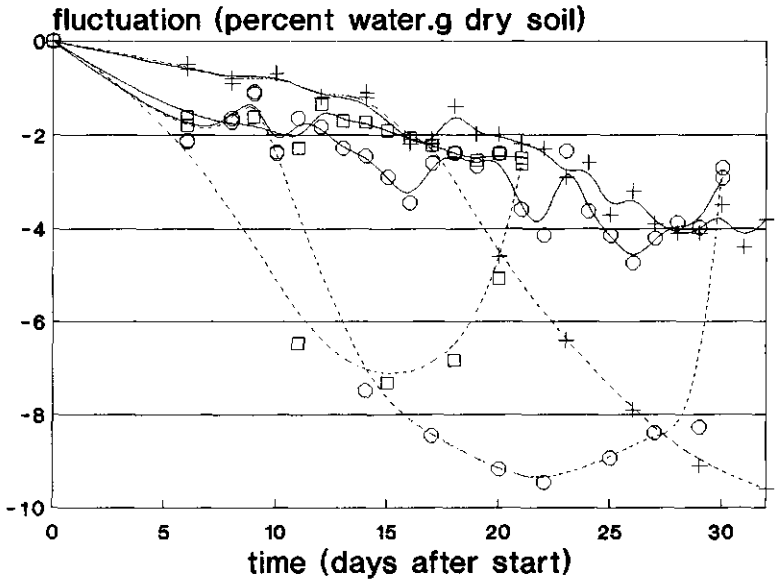

Figure 3 Soil moisture regimes imposed in experiment 2 (+), 3 (o) and 4 (□); wateradditions are indicated by the markers on the lines representing continuously moist (solid line) and fluctuating moist (broken line), respectively

Microbial populations

The number of bacteria was not affected by the sampling date (10 or 14 days and 35 days, respectively) nor by the soil moisture regime in experiment 2 and 3. Therefore, averaged numbers of bacteria for soils kept under both soil moisture regimes, are presented (Table 2). In experiment 2, the number of bacteria was significantly reduced in the presence of protozoa as compared to soils without protozoa. The number of colony forming units (cfu) recovered after 14 and 35 days was reduced ($P<0.05$) from 11.8×10^8 to 3.7×10^8 and from 5.6×10^8 to 2.9×10^8 per gram of soil, respectively (Table 2). In experiment 4, the number of cfu varied from 2.8×10^8 to 5.6×10^8 per gram of dry soil and the lowest number of cfu were recovered from soils with protozoa after both 0 and 35 days of incubation (Table 2). However, no statistical differences were detected. In experiment 4, protozoa reduced the number of cfu in soils kept under a continuous moist regime by a factor 2 from 8×10^8 to 4×10^8 ($P<0.05$). In soils kept under a fluctuating moist regime, no effect of protozoa on the number of bacteria was detected (Table 2).

Table 2 Numbers of bacteria and protozoa (flagellates, amoebae) per gram of dry soil (figures represent mean values of four replicates microcosms) in experiment 2, 3 and 4. Least Significant Differences (LSD), at significance level p=0.05, are given for comparison of the main effects, i.e. addition of protozoa.

treatment[1)]	Bacteria ($\times 10^8$)		Flagellates ($\times 10^4$)		Amoebae ($\times 10^4$)	
experiment 2	14 d	35 days	14 d	35 days	14 d	35 days
Bacteria only	11.8	9.31	n.d.	n.d.	n.d.	n.d.
Bacteria + Protozoa	3.7	4.59	1.50	5.80	3.90	8.60
$LSD_{0.05}$	1.8	0.78	0.74	2.70	0.35	2.78
experiment 3	10 d	35 days	10 d	35 days	10 d	35 days
Bacteria only	5.6	3.05	n.d.	n.d.	n.d.	n.d.
Bacteria + Protozoa	2.9	2.68	3.12	11.97	0.10	1.22
$LSD_{0.05}$	3.8	1.10	0.74	2.70	0.35	2.78
experiment 4		21 days		21 days		21 days
Bacteria only		5.7		n.d.		n.d.
Bacteria + Protozoa		4.2		3.3		0.15
$LSD_{0.05}$		2.4		-.-		-.-

note: n.d. = not detectable

Plant production

In experiment 2 and 4, total plant mass (Table 3) was neither affected by the presence of protozoa nor by the soil moisture regime at any time. Total plant mass varied between 1.81g and 3.01g dry mass per microcosm after 35 days in experiment 2, between 5.77g and 7.21g dry mass per microcosm after 35 days in experiment 3 and between 1.38g and 2.04g after 21 days in experiment 4. In experiment 3, more ($P<0.01$) shoot mass (30%) and total plant mass (16%) was found in the presence of protozoa, whereas in experiment 4, only shoot mass was 26% higher ($P<0.01$).

Table 3 Plant mass and nitrogen concentration at the end of the incubation of 35 days and 21 days for experiment 2 and 3 and experiment 4, respectively (figures represent mean values of 4 replicates) Least Significant Differences (LSD) for $P=0.05$ are given for comparison of main effects, i.e. addition of protozoa or soil moisture regime.

treatment[1)]	plant mass (g dry wt × microcosm^{-1}) shoot	root	total	percentage N (%N) shoot	root
experiment 2					
B CM	1.68	0.75	2.43	3.27	1.36
B FM	1.61	0.68	2.29	3.30	1.37
BP CM	1.87	0.83	2.21	2.31	1.37
BP FM	1.69	0.68	2.37	3.33	1.52
$LSD_{0.05}$	0.18	0.20	0.52	0.41	0.26
experiment 3					
B CM	2.93	3.06	5.99	1.19	0.56
B FM	3.13	2.64	5.76	1.18	0.60
BP CM	3.99	3.23	7.21	1.03	0.54
BP FM	3.82	2.61	6.43	1.10	0.56
$LSD_{0.05}$	0.20	0.63	0.69	0.05	0.05
experiment 4					
B CM	0.70	1.09	1.80	1.83	0.72
B FM	0.73	1.22	1.95	1.91	0.76
BP CM	0.91	1.04	1.95	1.94	0.77
BP FM	0.90	1.08	1.98	1.89	0.75
$LSD_{0.05}$	0.07	0.13	0.16	0.07	0.05

1) B and BP indicate bacteria only and bacteria and protozoa respectively and CM and FM indicate continuously moist and fluctuating moist regimes, respectively

Even though plant yield was not affected by the soil moisture regime, plants that were grown under continuously moist regimes transpired more ($P<0.05$) water than the plants grown under fluctuating moist regimes with periods of relative drought (data on water transpiration not shown). In soils with protozoa in experiment 3 and

4, plant production per unit water transpired was higher ($P<0.05$) than in soils without protozoa.

Plant nitrogen

Plant nitrogen concentration (Table 3) at 14 and 10 days in experiment 2 and 3, respectively was higher ($P<0.05$) in soils with protozoa than in soils without protozoa. At the end of the incubation period, the plant nitrogen concentration was reduced ($P<0.05$) in soils by the addition of protozoa [1.07% for soils with protozoa versus 1.19% for soils without protozoa] only in experiment 4. No inorganic nitrogen could be detected in any of the soils in neither experiment 2, 3 or 4 at the end of the incubation (Table 4).

Table 4 Inorganic 0.5 *N* K_2SO_4 extractable nitrate and ammonium nitrogen in soil in experiment 2, 3 and 4 (figures represent mean values of four replicates, respectively).

	Nitrogen (mg N × microcosm^{-1})					
	Nitrate-nitrogen			Ammonium-nitrogen		
treatment[1)]						
exp 2	0 d	14 d	35 days	0 d	14 d	35 days
B	29.5	12.0	n.d.	n.d.	23.3	n.d.
BP	29.5	19.0	n.d.	n.d.	25.7	n.d.
exp 3	0 d	10 d	35 days	0 d	10 d	35 days
B	27.9	N.D.	n.d	22.6	N.D.	n.d
BP	27.9	N.D.	n.d	22.6	N.D.	n.d
exp 4	0 d		21 days	0 d		21 days
B	3.9		n.d.	6.0		n.d.
BP	3.9		n.d.	6.0		n.d.

note: n.d. = not detectable
N.D. = not determined
1 B and BP indicate bacteria only and bacteria + protozoa, respectively

On average, almost 90% and 65% of the total plant nitrogen uptake took place during the last 20 days of the incubation in experiment 2 and 3, respectively (Table 5). At the end of the growth period (35 days) total plant nitrogen uptake ranged from 59.8 mg N to 65.5 mg per microcosm in experiment 2. In soils with only

bacteria, less ($P<0.05$) nitrogen was taken up by plants at fluctuating moist regimes versus continuously moist regimes. No effect of the soil moisture regimes on plant nitrogen uptake was found in experiment 3 and 4 (Figure 4). The presence of protozoa positively ($P<0.01$) affected the uptake of nitrogen by plants (Table 5 and Figure 4) both in experiment 2 [60.3 mg N in soils without versus 64.5 mg N in soils with protozoa], experiment 3 [48.2 mg N in soils without versus 53.2 mg N in soils with protozoa] and experiment 4 [22.0 mg N in soils without versus 25.3 mg N in soils with protozoa].

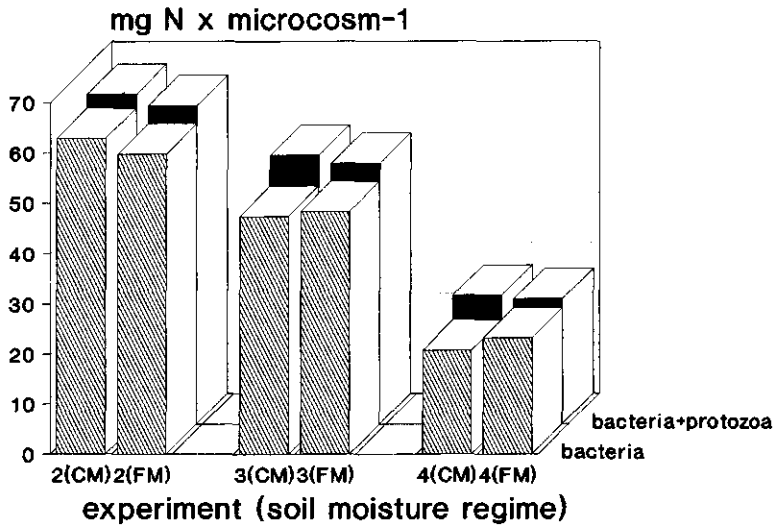

Figure 4 Plant nitrogen uptake in mg N per microcosm (final plant nitrogen content minus nitrogen content of seedlings); figures represent means of four replicates.

In the presence of protozoa the uptake by plants of inoculated bacterial ^{15}N-nitrogen in experiment 2 and 3 was significantly ($P<0.01$) increased (Table 5 and Figure 5). In experiment 2, a significant interaction ($P<0.01$) between soil moisture regime and the presence of protozoa was found: grazing by protozoa stimulated the availability and uptake by plants of bacterial ^{15}N-nitrogen in general, but the extent to which this happened, was affected by the soil moisture regime. The presence of protozoa yielded 17% and 37% more bacterial ^{15}N-nitrogen in plants in soils with continuously moist regimes and fluctuating moist regimes, respectively compared to soils with bacteria only. In experiment 3 and 4, the presence of protozoa stimulated ($P<0.01$) the uptake of bacterial or soil organic ^{15}N-nitrogen

(Table 5 and Figure 5) by 95% or 15%, respectively but no statistically significant interaction between the presence of protozoa and soil moisture regime was found.

The ratio ^{15}N indicated that in the presence of protozoa relatively more bacterial ^{15}N-nitrogen was taken up by plants compared to soils without protozoa both in experiment 2 and 3 (Figure 6). Again, the interaction between presence of protozoa and soil moisture regime was significant and mimicked the results on ^{15}N-nitrogen uptake by plants (Figure 5). However, this ratio was not affected by the presence of protozoa or by different soil moisture regimes in experiment 4 (Figure 6).

Table 5 Nitrogen uptake by plants, the fraction of inoculated ^{15}N-nitrogen recovered in total plant nitrogen and concentration ^{15}N-nitrogen in excess in total plant nitrogen (values are means of four replicates) in experiment 2, 3 and 4. Least Significant Differences (LSD), at significance level $P=0.05$, are given for comparison of main effects, i.e. presence of protozoa for experiment 3 and 4 and for comparison of interactions for experiment 2.

treatment[1]	plant nitrogen uptake (mg N × microcosm^{-1})		^{15}N in plants as percentage of inoculated ^{15}N (%)		^{15}N concentration in plant N uptake (%)	
experiment 2	14 days	35 days	14 days	35 days	14 days	35 days
B CM	8.70	63.08	4.88	36.56	0.651	0.719
B FM		59.75		30.98		0.643
BP CM	7.60	65.49	12.51	42.90	1.383	0.813
BP FM		63.25		42.46		0.833
$LSD_{0.05}$	2.86	1.81	1.41	1.69	0.104	0.030
experiment 3	10 days	35 days	10 days	35 days	10 days	35 days
B	15.86	47.85	4.88	21.90	0.651	0.970
BP	19.19	52.72	12.51	42.80	1.383	1.712
$LSD_{0.05}$	2.06	3.07	1.11	6.50	0.080	0.228
experiment 4		21 days		21 days		21 days
B		21.93		8.05		0.778
BP		25.26		9.23		0.775
$LSD_{0.05}$		1.53		0.72		0.015

1) see footnote Table 3

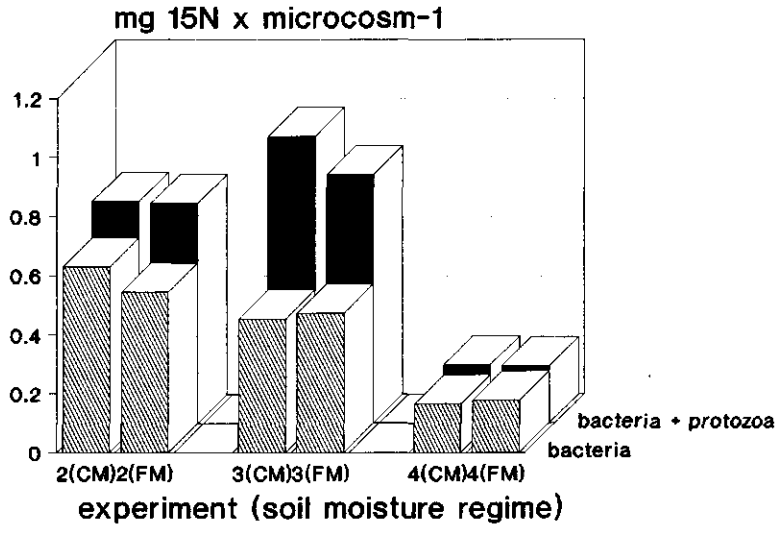

Figure 5 Recovery of bacterial ^{15}N in plant nitrogen as percentage of inoculated amount of ^{15}N. The recovery in experiment 4 is related to the inoculated amount of bacterial ^{15}N in experiment 3. Figures represent means of four replicates.

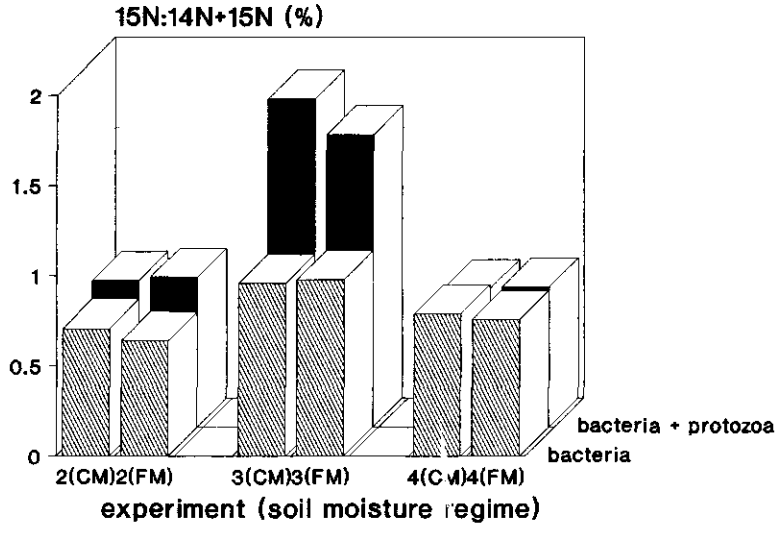

Figure 6 Relative contribution of inoculated bacterial ^{15}N to the plant total nitrogen uptake (mg bacterial $^{15}N \times mg^{-1}$ plant N $\times$ 100%). Figures represent means of four replicates.

Discussion and conclusions.

The incubation of fallow soil (experiment 2) at three soil moisture contents both in the presence and in the absence of protozoa showed that protozoan activity was found only at a soil moisture content of 14 and 19% (v/w) but not at 7.5% (v/w). Protozoan activity was indicated by a reduction of the number of bacteria (Figure 1) and an increased mineralization of ammonium-nitrogen (Figure 2). The protozoan activity was not different for the soil moisture contents 14 and 19% (v/w). These consequences of protozoan predation agree with earlier observations (Bryant *et al.* 1982, Coleman *et al.* 1984).

Two possible mechanisms for the effect of water on the population dynamics of protozoa might be i) a direct effect of the water popential on the cellular metabolism and/or ii) an indirect effect through the (in)accessibility of food(bacteria) in pores filled or pores devoid of water. The soil kept at a moisture content of 14% was at pF 2.2, based on results by Postma *et al.* (1989) for the same soil and pores with a pore neck diameter of 20 μm are filled with water. A reduction of the soil moisture content to 7.5% (pF 3.5) with waterfilled pores having neck diameters of less than 3 μm inhibited protozoan activity.

In our experiment both amoebae and flagellates were inoculated. The amoebae were more abundant than flagellates at a moisture content of 14% which leads to the conclusion that the amoebae are active at higher moisture tensions than are the flagellates. Soil amoebae are best adapted to life on surfaces and in waterfilms since they adhere with their cell, which is only a few micrometers thick, to surfaces whereas flagellates are swimming in the 'free' water (Fenchel 1987). Therefore, flagellates are expected to be restricted in their movement through the soil matrix before amoebae are. Alabouvette *et al.* (1981), Darbyshire (1976) and Darbyshire *et al.* (1985) reported that pores with pore necks less than 3-6 μm are inaccessible to ciliated protozoa. Our results showed that pores with pore necks less than approximately 3 μm are inaccessible for amoebae and flagellates, which both are considerably smaller than ciliates.

The incubation of planted soil (experiment 2, 3 and 4) enabled us to control soil moisture and at the same time create defined regimes with soil moisture fluctuations that were expected to force protozoa, temporarily, into inactive stages (Bryant *et al.* 1982). By applying the inoculation of ^{15}N-nitrogen labelled bacteria and ^{15}N-nitrogen labelled soil organic material together with wheat plants as a nitrogen sink, the ^{15}N accumulation in the wheat plants is indicative for the importance of the bacterial-protozoan interactions with respect to the transformations of specific bacterial nitrogen or soil organic nitrogen.

The activity of protozoa clearly stimulated the total nitrogen uptake by plants under all three soil moisture conditions and in both experiment 2, 3 and 4 by

approximately 5%, 10% and 15%, respectively as could be judged from the increased nitrogen uptake by plants in soils with protozoa as compared to soils without protozoa (Figure 4). Kuikman and Van Veen (1989) reported a 17% increase of the nitrogen uptake by plants, using the same soil as in these experiment and the similar incubation conditions as in experiment 2.

When we compare the average total plant nitrogen uptake of 63 mg N, 50 mg N and 24 mg N per microcosm (figure 4) in experiment 2, 3 and 4, respectively with the amount of inorganic nitrogen present per microcosm at the start of the incubation, it can also be concluded that in both soils with bacteria only and in soils with bacteria and protozoa nitrogen was mineralized from soil organic matter which agrees with results by Kuikman and Van Veen (1989).

When comparing the results on total nitrogen and on ^{15}N-nitrogen in experiment 2 and 3, it is obvious that the uptake by plants of more ^{15}N-nitrogen in the presence of protozoa was not only caused by a higher total nitrogen uptake (Figure 6). In the presence of protozoa, but irrespective of soil moisture regime, plants took up nitrogen with a significant higher ($P<0.01$) enrichment of ^{15}N-nitrogen. Thus, in the presence of protozoa the contribution of inoculated bacterial nitrogen to the total plant nitrogen uptake was not affected by different soil moisture regimes. However, in soils with bacteria only in experiment 3, the mineralization of inoculated bacterial nitrogen was severely hampered ($P<0.05$) when the soil moisture regime was characterized by periods of prolonged and intensified drought as in soils with a fluctuating moist regime (Figure 6).

As concluded before, protozoa are not active in our loamy sand at a moisture content of 8%, which corresponded to pF 3.5 and pores with pore necks less than 3 μm that are devoid of water. This would indicate that as soon as the soil lost 7% or more water, protozoan activity is (temporarily) stopped. Even though situations like this occurred (figure 3) in soils with fluctuating moist regimes in experiment 3 during the larger part of the overall incubation period of 35 days, protozoa did positivily affect the plant total nitrogen and ^{15}N-nitrogen uptake. These present results still indicate that protozoa rapidly recovered from the preceeding drought period and could stimulate the turnover of microbial nitrogen whereas, in the absence of protozoa, immobilization of plant nutrients in the microbial biomass tended to be dominating.

However, in experiment 4, where the soils with fluctuating moist regimes were characterized by more cycles of drought forcing protozoa to first encyst and excyst on remoistening, no interaction between soil moisture regime and protozoan activity with respect to uptake of bacterial ^{15}N-nitrogen was found. Thus, it seems more appropriate to hypothesize that under the moisture conditions in experiment 2, the reduced availability of bacterial ^{15}N-nitrogen at the fluctuating moist regime was a result of the immobilization of non labelled nitrogen in microbial biomass following the enhanced availability of organic matter due to disruption of structure (Sorensen

1974, Adu and Oades 1978, Van Veen *et al.* 1985). Here, protozoa were able to utilize immediately after remoistening ^{15}N-nitrogen bacterial biomass. The protozoa were probably not forced into cysts or encystment and excystment took place rapidly.

The limits of this rapid response by protozoa upon remoistening of a dried soil seemed to be limited in experiment 4. Here, periods of drought were more frequent and more intense. In experiment 4, the impact of protozoa on nitrogen mineralization as well as on the ^{15}N-)nitrogen uptake was reduced under a soil moisture regime characterized by periods of drought. Here, the different soil moisture regimes were imposed from the start of the incubation and on: so, the impact of soil moisture fluctuations on protozoan activity could be expected to be even more pronounced.

The results from experiment 4 support the observation that protozoa preferentially mineralized introduced bacterial ^{15}N-nitrogen by Kuikman and Van Veen (1989). In this experiment 4, where ^{15}N-nitrogen labelled soil organic matter was used, protozoa quantitatively stimulated the nitrogen mineralization and total plant nitrogen uptake without affecting the quality, i.e. ^{14}N versus ^{15}N, of the nitrogen that was mineralized.

In conclusion, protozoan activity is restricted by limited soil moisture. Even though the soil moisture content of planted soil dropped to levels at which protozoa were shown not to be active any longer, protozoa immediately responded to the addition of water and restoration of favourable soil moisture conditions for 2 or 3 days as shown by a stimulated nitrogen and ^{15}N-nitrogen uptake by plants. And so, the consequences of drying and rewetting cycles in terms of nitrogen mineralization are minimized by the protozoan activity. Definitely, the moisture content of the soil is the factor which primarily determines the opportunities of protozoa to move through the soil and find their bacterial food. Since protozoan grazing of bacteria is reported to be one of the most important processes of the soil food web with regard to the flux of nutrients such as nitrogen (Elliott *et al.* 1988), the results of this study may have important consequences for the trophic interactions and thus for the dynamics of nitrogen mineralization as shown by Hendrix *et al.* (1986) and Kuikman and Van Veen (1989).

References

Adu JK and Oades JM (1978) Physical factors influencing decomposition of organics materials in soil aggregates. Soil Biol Biochem 10: 109-115

Alabouvette C Couteaux MM Old KM Pussard M Reisinger O and Toutain E (1981) Les protozoaires du sol: aspects ecologiques et methodologiques. Ann Biol 20: 255-303

Bamforth SS (1985) Symposium on 'protozoan ecology': the role of protozoa in litters and soils. J Protozool 32: 404-409

Birch HF (1958) The effect of soil drying on humus decomposition and nitrogen availability. Plant and Soil 10: 9-31

Bremner JM (1965) Total nitrogen In: CA Black DD Evans JL White LE Ensminger and FE Clark (editors) Methods of soil analysis. Amer Soc Agron Wisconsin USA pp 1149-1178

Bryant RJ Woods LE Coleman DC Fairbanks BC McClellan JF and Cole CV (1982) Interactions of bacterial and amoebal populations in soil microcosms with fluctuating moisture content. Appl Environ Microbiol 43: 747-752

Clarholm M (1981) Protozoan grazing of bacteria in soil - impact and importance. Microb Ecol 7: 343-350

Clarholm M (1985) Interactions of bacteria protozoa and plants leading to mineralization of soil nitrogen. Soil Biol Biochem 17: 181-187

Coleman DC RV Anderson CV Cole JF McClellan LE Woods JA Trofymow and ET Elliott (1984) Roles of protozoa and nematodes in nutrient cycling. In: Microbial-Plant interactions. ASA Special Publication No 47 Madison pp 17-28

Darbyshire JF and Greaves MP (1967) Protozoa and bacteria in the rhizosphere of *Sinapsis alba* L. *Trifolium repens* L. and *Lolium perenne* L. Can J Microbiol 13: 1057-1068

Darbyshire JF (1976) Effects of water suctions on the growth in soil of the ciliate *Colpoda steinii* and the bacterium *Azotobacter chroococcum.* J Soil Sci 27: 369-376

Darbyshire JF Wheatley RE Greaves MP and Inkson RHE (1974) A rapid micromethod for estimating bacterial and protozoan populations in soil. Rev Ecol Biol Sol 11: 465-475

Darbyshire JF Robertson L and Mackie LA (1985) A comparison of two methods of estimating the soil pore network available to protozoa. Soil Biol Biochem 17: 619-624

Elliott ET and DC Coleman (1977) Soil protozoan dynamics in a shortgrass prairie. Soil Biol Biochem 9: 113-118

Elliott ET Coleman DC and Cole CV (1979) The influence of amoebae on the uptake of nitrogen by plants in gnotobiotic soil. In: JL Harley and R Scott Russell (eds). The Soil-Root Interface Academic Press London pp 221-229

Elliott ET Hunt HW Walter DE and Moore JC (1986) Microcosms mesocosms and ecosystems: linking the laboratory to the field. Proc 4th Int Symp Microb Ecol: 472-480

Fenchel T (1987) Ecology of Protozoa: The Biology of Free-living Phagotrophic Protists. Springer-Verlag Berlin/Heidelberg/New York 197 pp

Foissner W (1987) Soil Protozoa: Fundamental Problems Ecological Significance Adaptations in Ciliates and Testaceans Bioindicators and Guide to the Literature. Progress in Protistology 2: 69-212

Hendrix PE Parmelee RW Crossley Jr DA Coleman DC Odum EP and Groftman PM (1986) Detritus food webs in conventional and no-tillage agroecosystems Bioscience 36: 473-380

Kuikman PJ and JA van Veen (1989) The impact of protozoa on the availability of bacterial nitrogen to plants. Biol Fertil Soils 8: 13-18

Levrat P C Alabouvette and M Pussard (1987) Rôle des protozoaires dans les systèmes interactifs: cas des relations bactéries-champignons. Rev Ecol Biol Sol 24: 503-514

Losina-Losinsky L and PF Martinov (1930) A method of studying the activity and rate of diffusion of protozoa and bacteria in the soil. Soil Sci 29: 349-362

Nikoljuk VF (1963) Soil protozoa and their biological significance (in russian). Pedobiologia 3: 259-273

Page FC (1976) An illustrated key to freshwater and soil amoebae. Freshwater Biological Association Cumbria UK Sci Publ 34

Postma J Walter S and Van Veen JA (1989) Influence of different initial soil moisture contents on the distribution and populations dynamics of introduced *Rhizobium leguminosarum* biovar *trifolii*. Soil Biol Biochem 21: 437-442

Sorensen LH (1974) Rate of decomposition of organic matter in soil as influenced by repeated air drying-rewetting and repeated additions of organic material. Soil Biol Biochem 6: 287-292

Vargas R and Hattori T (1986) Protozoan predation of bacterial cells in soil aggregates. FEMS Microb Ecol 38: 233-242

Van Ginkel JH and Sinnaeve J (1980) Determination of total nitrogen in plant material with nessler's reagent by continuous-flow analysis. Analyst 105: 1199-1203

Van Veen JA Ladd JN and Amato M (1985) Turnover of carbon and nitrogen through the microbial biomass in a sandy loam and a clay soil incubated with [^{14}C(U)]glucose and $^{[15N]}(NH_4)_2SO_4$ under different moisture regimes. Soil Biol Biochem 17: 747-756

CHAPTER 6

PROTOZOAN PREDATION AND THE TURNOVER OF SOIL ORGANIC CARBON AND NITROGEN IN THE PRESENCE OF PLANTS [1]

Abstract

The impact of protozoan grazing on the dynamics and mineralization of ^{14}C and ^{15}N labelled soil organic material was investigated in a microcosm experiment. Sterilized soil was planted with wheat and either inoculated with bacteria alone or with bacteria and protozoa or with bacteria and a 1:10 diluted protozoan inoculum. ^{14}C-CO_2 formation was continuously monitored. It served as an indicator of microbial activity and the respiration of soil organic carbon. The activity of protozoa increased the turnover of ^{14}C labelled substrates compared to soils without protozoa. The accumulated ^{14}C-CO_2 evolved from soils with protozoa was 36% and 53% higher for a 1:10 and for a 1:1 protozoan inoculum respectively. Protozoa reduced the number of bacteria by a factor of 2. In the presence of protozoa nitrogen uptake by plants increased by 9% and 17% in case of a 1:10 and of a 1:1 inoculum of protozoa respectively. The constant ratio of ^{15}N : $^{14+15}N$ in plants in all treatments indicated that in the presence of protozoa more soil organic matter was mineralized. Both plant dry matter production and shoot:root ratio's were higher in the presence of protozoa. Bacteria and protozoa responded very rapidly to the addition of water to the microcosms. The rewetting response in terms of ^{14}C-CO_2 respiration rate was significantly higher during 1 day in the absence and during 2 days in the presence of protozoa after watering the microcosms. It was concluded that protozoa improved the mineralization of nitrogen from soil organic matter by stimulating the turnover of bacterial biomass. Pulsed events like the addition of water seem to have a significant impact on the dynamics of food-chain reactions in soil in terms of carbon and nitrogen mineralization.

[1] PJ Kuikman, AG Jansen, JA Van Veen and AJB Zehnder, submitted to Biology Fertility of Soils

Introduction

The role of protozoan predation on bacteria in soil is significant for nutrient transformations in the soil-plant ecosystem (Elliott *et al.* 1984). Increased mineralization of carbon and nitrogen upon the (pulsed) addition of glucose and ammonium nitrogen to soil microcosms in the presence of amoebae has been demonstrated (Coleman *et al.* 1978, Woods *et al.* 1982, Bryant *et al.* 1982). A significant enhancement of ammonium nitrogen uptake by plants grown in soil microcosms with bacteria and protozoa compared to those with only plants and bacteria was shown by Elliott *et al.* (1979) and Clarholm (1985) upon addition of fertilizer nitrogen and by Kuikman and Van Veen (1989) without addition of nitrogen. The latter showed that grazing by protozoa resulted in an improved availability of specific bacterial nitrogen to plants and postulated an increased turnover of nitrogen mineralization from soil organic matter.

In general, the activity of amoebae reduced the number of bacteria in soil. If protozoan grazing would only negatively affect bacterial numbers, microbially mediated processes such as decomposition of soil organic matter, would eventually be negatively affected as well and protozoa would only accelerate the cycling of nutrients temporarily. Since several reports in literature (Barsdate *et al.* 1974, Stout 1980) as well as our own preliminary experiments showed the opposite, i.e. grazing of bacteria by protozoa seemed to lead to a continuous increased turnover of organic matter, two mechanisms may be possible: a) protozoa utilize organic material other than bacterial cells or b) protozoan grazing stimulates the overall activity of the bacterial population.

Soil provides a very heterogeneous environment to both bacteria and protozoa. Their possibilities for movement are very much restricted by discontinuities in the waterfilm both in time and space (Stout 1980, Vargas and Hattori 1986, Bamforth 1988). Several authors (Postma and Altemuller 1989, Foster 1988) have shown the occurence of isolated communities of either bacteria or protozoa or both. The overall microbial activity is the summation of activity in each of these communities. Protozoa will have to actively move through the soil to reach bacterial communities and so to exert their effect on bacteria and their metabolism. Water has been shown to be the main regulating vector (Vargas and Hattori 1986). Therefore, the frequency of bacterial-protozoan interactions is not only a function of the number and the distribution of protozoa and bacteria but also of the soil moisture content. The flow of water then could contibute to the redistribution of both protozoa and bacteria through the soil.

The objectives of this study were to: (1) determine the impact of protozoan predation on bacteria on the rate of carbon and nitrogen mineralization from soil organic matter in the presence of plants at a fluctuating soil moisture regime and (2) study the effect of the number and distribution of protozoa in soil. Soils, labelled

with ^{15}N-^{14}C-organic material, were planted with wheat which served as a nitrogen sink to accumulate information on nitrogen mineralization. The continuously monitoring of ^{14}C-CO_2 yielded information on the dynamics of microbial activity.

Materials and methods

Soils

The soil used was a loamy sand as described by Kuikman and Van Veen (1989). The soil was sampled from a field plot four months prior to the start of this incubation and sieved (Ø 4mm). The soil was sterilized by exposure to a ^{60}Co-γ-radiation source, receiving 4 Mrad in 30h. An incubation experiment was carried out (described in Chapter 3) to assess the impact of protozoan grazing on bacteria as a function of protozoan movement and the number of protozoa inoculated. Therefore, the soil was amended with bacteria which were grown on a mineral medium containing ^{14}C[U]-glucose and ^{15}N-ammonium nitrogen and part of the soil was also inoculated with protozoa. The soil that was left after taking out sub-samples for several analyses, was stored at 4°C.
This ^{14}C-^{15}N-labelled soil (349 Bq $\times$ g^{-1} dry soil) was then mixed and air dried to a moisture content of 7.7% (v/w). Nine portions of each 900 g dry soil were weighed into double plastic bags and again sterilized as described before. The sterility of the soil was tested by preparing a dilution series of suspended soil and checking for bacterial growth on 1:2 Tryptone Soya Agar [Oxoid, U.K.] plates and for protozoa on water agar plates supplemented with a bacterial suspension; no bacteria or protozoa were found. The soil contained 45.5 μg N-NH_4 and 20.2 μg N-NO_3 per gram dry soil at the start.

Bacteria and protozoa

Pseudomonas fluorescens R2f was isolated from grassland soil in The Netherlands (Van Elsas 1988) and *Enterobacter cloacea* was isolated from soil in South Australia. Both bacteria were grown in a mineral medium [4.0g/l glucose, 2.88g/l citric acid, 1.07g/l NH_4Cl, 0.5g/l K_2HPO_4, 0.5g/l KH_2PO_4, 0.2g/l $MgSO_4$, 0.01g/l $MnSO_4$, NaCl, $FeSO_4$ and $CuSO_4$, 0.04 mg/l $ZnSO_4$, 0.03 mg/l $CoCl_2$, 0.02mg/l $(NH_4)_6Mo_2O_4$] on a rotary shaker at 29°C. Bacterial cells were harvested by centrifugation at 10.000xg for 10 minutes at 10°C and resuspended in sterile demineralized water. The centrifugation and resuspendation was repeated once.

The protozoan population that was used consisted of one flagellated and one amoebal species. *Acanthamoeba* sp was obtained from dr. E.T. Elliott [NREL, CO 80523 Ft Collins, USA] and has been described by Elliott and Coleman (1977), the flagellate was identified as a *Cercomonas* sp and was isolated from the loamy sand

soil which was used in this investigation. Both protozoan species were cultured on water agar and *Pseudomonas* R2f as a food source. Protozoa were harvested by washing them from the agar plates with amoeba saline (Page 1967).

Experimental design and inoculation

The experiment was carried out in a completely randomized design with nine experimental units randomized over three treatments, i.e. no protozoa, a 1:10 diluted and a non diluted protozoan inoculum. The soil portions, each 900 g (7.7% water) were inoculated with a suspension introducing 1.7 $x10^7$ bacteria and either 36.8$x10^3$ flagellates and 0.7$x10^3$ amoeba or a 1:10 diluted protozoan inoculum or sterile demineralized water without protozoa. After inoculation of the soil portions in plastic bags, they were thoroughly mixed by hand and aseptically transferred to the sterile microcosms. This experimental unit [PVC column, 1.5 l, Ø 9cm, length 24 cm] was half filled with glass beads and half with soil. The soil core was brought to a bulk density of 1.3 g/cm^3 (dry weight). Each soil microcosm was planted with one 10-day old wheat (*Triticum aestifum* cv. *Ralle*) seedling. The seeds were surface sterilized with 1.5% sodiumhypochlorite and individually germinated on 1:10 Tryptone Soya Agar (Oxoid, UK) in 20 cm tubes. Only those seedlings were used that were not contaminated as evidenced by bacterial or fungal growth on the medium.

The final soil moisture content at the start of the incubation was 15% (v/w). Losses of water due to evapo-transpiration by plants were made up by regular additions of sterile demineralized water on top of the soil cores. Weight increase due to plant growth was taken into account. The soils were incubated in a climate chamber [day/night regime: light 16h at 21°C and dark 8h at 15°C] for 34 days.

Separation between the soil in the column and the atmosphere was accomplished by a sealing of plastic silicone rubber at the stem-base of the plants after 4 days of growth on the microcosms. Necessary openings were provided for the addition of water and flushing the columns with sterile, CO_2-free air (inlet at the top, outlet at the basis of the column).

Sampling procedure and analyses

After cutting off the shoots, the roots were separated from the soil and washed on a fine (Ø 0.5 mm) sieve. Dry shoot and root mass was obtained by weighing the plant material after drying at 80°C for 48 h. Plant nitrogen content was measured as ammonium nitrogen after digestion with sulphuric acid and salicylic acid on an autoanalyser using ground (Ø 0.5 mm) plant material. After separating roots from the soil, the soil was subsampled for determination of bacterial and protozoan numbers, mineral ($N\text{-}NH_4$ and $N\text{-}NO_3$) nitrogen, ^{14}C in soil organic matter and soil moisture content.

The number of bacteria was determined by extracting 10 g fresh soil in 95 ml of 0.1% sodiumpyrophosphate solution containing 10g of gravel on a rotary shaker (10 min., 200 rpm). A series of 10-fold dilutions in sterile demineralized water was prepared and appropriate dilutions were plated (in triplicate) on King's B agar [proteose peptone, 20g; K_2HPO_4, 1.5g; $MgSO_4.7H_2O$, 1.5g; glycerol, 10g; agar, 15g; H_2O, 1000g; pH 7.2]. After an incubation of the plates for 48 hours at 29°C, the total number of colony forming units on each plate was determined.

The number of protozoa was determined by a modified, most-probable-number method (Darbyshire *et al.* 1974, Rowe *et al.* 1977), extracting 5 g fresh soil in 100 ml of Amoebae Saline, using 4-fold dilutions and eight replicated series with *Pseudomonas fluorescens* as a food source. The microtiter plates were incubated at 12°C in the dark and scanned for the presence of protozoa several times during a 4 week period.

After extraction of 25 g fresh soil in 50 ml 0.5 *M* K_2SO_4, mineral nitrogen, i.e. N-NH_4 using Nessler's reagent (van Ginkel and Sinnaeve, 1980) and N-NO_3 was measured by continuous flow analysis.

Carbon dioxide evolved from the soil was trapped in 0.5 *M* NaOH. The soil columns were flushed with CO_2 free air (10 l h^{-1}) during 15 min and 4 times day^{-1}. The evolution of ^{14}C in CO_2 was measured every day. Total carbon and ^{14}carbon content in dry soil and plant material were determined after combustion (Amato 1983). Total C-CO_2 released by incubation or by combustion of soils was determined by titration (Tinsley *et al.* 1951) and ^{14}C-CO_2 by liquid scintillation counting (Amato 1983) using Ultima Gold as a scintillation liquid (Packard, UK).

Statistics

For each treatment, three replicated microcosms were destructively sampled after 33 days of incubation. Analyses for determination of bacterial and protozoan numbers, soil mineral nitrogen, ^{14}C in soil organic matter and soil moisture content were made in duplo. The results were analysed by analysis of variance over the experimental factor, i.e. the number of protozoa inoculated at the start. The accumulated ^{14}C and total CO_2 evolution was analysed by analysis of variance. These results were also analysed by fitting a response function for each microcosm. The model used was a line plus exponential curve:

$$Y = a + (b \times r^{day}) + c \times day \quad (1)$$

where $r \leq 1$ and if [a=-b], the curve passes through the origin (0.0). Parameter r determined when the behaviour of the curve becomes linear and independent of a, b and r for the $\lim(b \times r^{day}) = 0$. Parameter c determined the slope of the linear part of the curve. The estimated parameter values were analysed by analyses of variance over the experimental factor. All differences reported are significant at the

level of $P<0.05$ at least. Statistical analyses were performed with the statistical package GENSTAT 5, release 1.3 (Genstat 5 Committee, 1987) on a VAX VMS main frame.

Results

Microbial numbers

At the end of the incubation, significantly less bacteria were found in soils with protozoa (0.76×10^8) compared to the soils without protozoa (1.30×10^8) (Table 1). The number of bacteria increased approximately 5 and 10 times in soils inoculated with protozoa and in soils without protozoa, respectively. The different sizes of the protozoan inoculum did not affect the number of bacteria nor the number of protozoa that were recovered after 33 days. Approximately 3000 amoebae per g dry soil were recovered (Table 1), which is 4 to 40 times more amoebae that were inoculated. No flagellates were recovered. Minimal numbers around the detection limit of <200 protozoa per g dry soil could be detected in samples from 2 out of 3 microcosms that had not been inoculated with protozoa.

Table 1 Numbers of bacteria and protozoa per gram dry soil (means of three replicated microcosm)

	Bacteria	Protozoa
Treatments[1]		
B	1.30×10^8	-
Bp	0.76×10^8	2.77×10^3
BP	0.76×10^8	3.01×10^3
LSD ($P=0.05$)	0.47×10^8	1.75×10^3

1 B is bacteria only, Bp is bacteria plus a 1:10 diluted protozoan inoculum and BP is bacteria plus a non diluted protozoan inoculum

Plants

The total plant dry mass production was 2.37 g dry weight per microcosms in soils with no protozoa added and significantly increased by 40% and 47% in case protozoa had been added to the soils (Table 2). Shoot mass but not root mass was positively ($P<0.01$) affected by the presence of protozoa: 65% more shoot mass in soils with protozoa compared to soils not inoculated with protozoa (Table 2). The consequences for the shoot:root ratio then are obvious: higher in the presence of protozoa than in the absence of protozoa (Table 2).

The final nitrogen concentration of plants, grown in soils without protozoa, was 33% (shoots) and 45% (roots) higher compared to soils with protozoa (Table 2). In none of the soil microcosms any mineral nitrogen, either ammonium of nitrate, could be detected at the end of the incubation. The total plant nitrogen uptake was found lowest in soils without protozoa (47.95 mg N) and significantly improved in soils inoculated with protozoa: 52.24 mg N and 55.96 mg N in soils with a diluted and a non-diluted protozoan inoculum, respectively (Table 3). The ^{15}N-nitrogen uptake in plants followed the results on total nitrogen (Table 3). The ratio ^{15}N : $^{14+15}N$ indicated that in soils with a non diluted protozoan inoculum relatively less labelled nitrogen was taken up by the plants compared to the other soils (Table 3).

Table 2 Plant dry mass (shoots, roots, total), shoot:root ratio and plant nitrogen concentration (means of three replicated microcosms) and least significant differences (P=0.05)

	Plant dry mass (g)				Plant N (%)	
	shoot	root	total	shoot:root	shoot	root
treatment[1]						
B	1.41	0.96	2.37	1.54	2.49	1.45
Bp	2.28	1.05	3.33	2.20	1.83	1.02
BP	2.39	1.09	3.48	2.21	1.91	0.97
LSD	0.33	0.34	0.58	0.56	0.43	0.27

[1] see footnote table 1

Table 3 Total plant nitrogen, plant ^{15}N-nitrogen uptake, ^{15}N-nitrogen concentration in plant nitrogen and the ratio ^{14}C-CO_2:^{15}N-plant (means of three replicated microcosms)

	Plant N (mg)	Plant ^{15}N (mg)	$^{15}N/^{15+14}N$ (%)	$^{14}C/^{15}N$ (Bq × mg^{-1})
treatment[1]				
B	47.95	0.557	1.161	17.8
Bp	52.24	0.613	1.173	22.0
BP	55.96	0.627	1.119	24.2
LSD (P=0.05)	4.19	0.059	0.042	

1 see footnote table 1

Soil organic carbon dynamics

Unfortunately, the microbial metabolization of ^{14}C, the (^{14}C)-CO_2 evolution and the response of protozoa could not be monitored during the first 4 days of the incubation since plants were too fragile to be sealed at the stem base. The rate of carbon dioxide evolution was not significantly affected by the presence of protozoa or by the number of protozoa inoculated at the beginning of the incubation and was highly variable during the overall incubation period (Figure 1). The differences in accumulated carbon dioxide respiration varied from 450-480 $\mu g\ C \times g^{-1}$ dry soil and were not significantly different at any specific time during the incubation period (Figure 1).

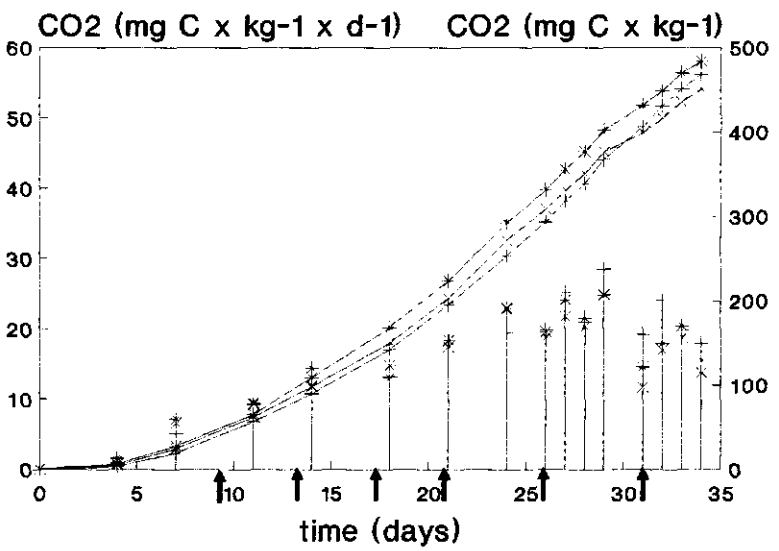

Figure 1 Rate (bars) of soil respiration (mg $C\text{-}CO_2 \times day^{-1} \times kg^{-1}$ dry soil) and accumulated (lines) carbon dioxide evolution (mg $C\text{-}CO_2 \times microcosm^{-1}$) from the soil (means of three replicated microcosms). Water added on day 9, 13, 17, 21, 26 and 31 as indicated by the arrows: 20, 30, 40, 50, 60 and 60 ml respectively

The rate of $^{14}C\text{-}CO_2$ evolution in all treatments decreased from day 5 until the end of the incubation (Figure 2). The rate of $^{14}C\text{-}CO_2$ evolution from soils which were inoculated with protozoa was consistently higher than the rate of $^{14}C\text{-}CO_2$ evolution from soil inoculated with only bacteria. However, only at a few dates, statistical differences in the $^{14}C\text{-}CO_2$ evolution rate could be detected between soils with and soils without protozoa. If differences occured, then the rate of $^{14}C\text{-}CO_2$

evolution in soils with protozoa was significantly higher than in soils without protozoa (Figure 2).

The accumulated ^{14}C-CO_2 that evolved from soil without protozoa was 9.9 Bq × g^{-1} dry soil whereas in soils with protozoa 13.5 Bq × g^{-1} and 15.2 Bq × g^{-1} dry soil evolved from soils with a 1:10 diluted protozoan inoculum and a non diluted protozoan inoculum respectively (Figure 2). Protozoa therefore significantly stimulated the ^{14}C-CO_2 evolution.

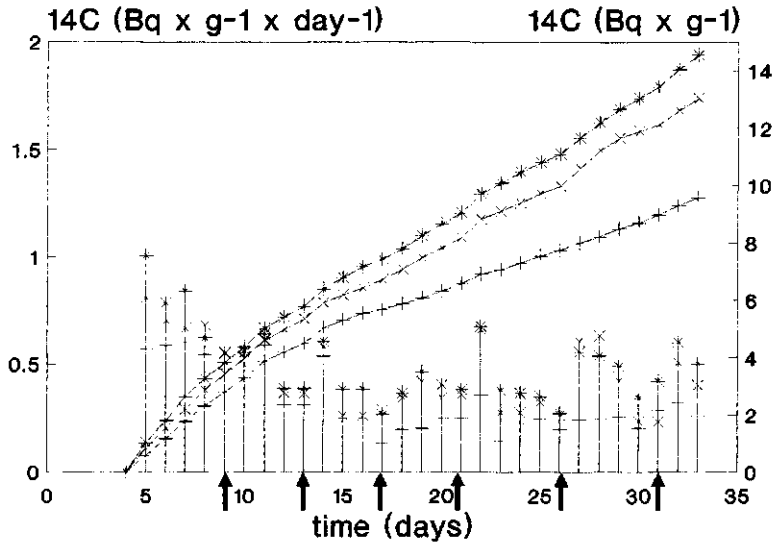

Figure 2 Rate (bars) of ^{14}C-carbon dioxide evolution (Bq × day^{-1} × g^{-1} dry soil) and accumulated (lines) ^{14}C-carbon dioxide evolution (kBq × $microcosm^{-1}$) from the soil (means of three replicated soil microcosms). Water added as indicated in the legend of Figure 1

At least 99% of the variance is accounted for in case all parameter estimates were free to be chosen by the fitted response function per microcosm for accumulated ^{14}C-CO_2 evolution . Only the estimates for parameter c (equation 1) were significantly different: 0.21 for soil without, 0.40 for soil with protozoa and 0.38 for soil with a 1:10 diluted protozoan inoculum respectively. This parameter c determines the slope of the linear part of the curve during the last 20 days of the incubation and was approximately twice as high for soils with protozoa.

The fitted response functions of total carbon respiration of individual microcosms accounted for at least 98% of the variance. Analyses of variance on the parameter estimates for the curves showed no statistical difference with respect to the

inoculation of protozoa applied. Since the estimate for r was almost 1.0, the dynamics of the carbon dioxide evolution could be best described by a linear relationship.

The rate of ^{14}C evolution peaked directly after addition of water (Figure 2) to the soil columns. The rewetting response was defined as the difference in ^{14}C-CO_2 evolution rate (Bq $\times$ day^{-1} $\times$ g^{-1}) between 5 days, immediately following the addition of water and the remaining 21 days during the incubation period when the level of ^{14}C-CO_2 evolution had stabilized (Figure 3).

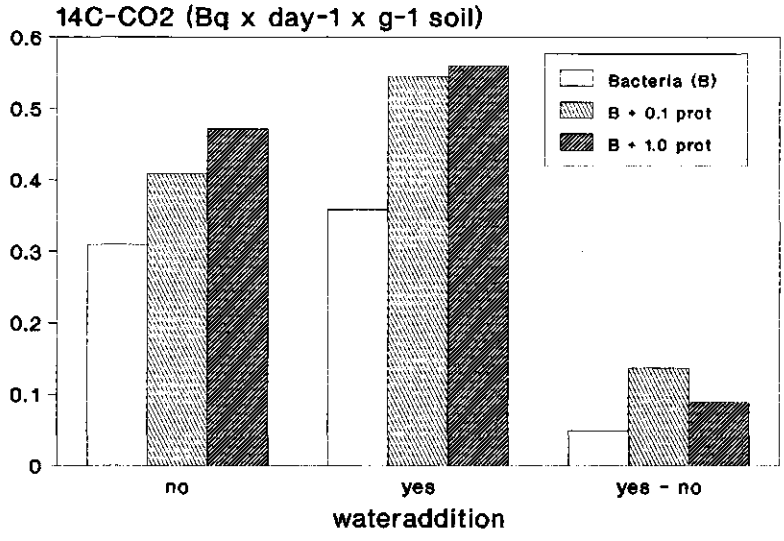

Figure 3 Averaged rate of ^{14}C-carbon dioxide evolution (Bq $\times$ day^{-1}) on 21 days not immediately following the addition of water [yes] and on 5 days immediately following the addition of water (no) respectively and the rewetting respons (difference between [yes] and [no] water addition) (means of three replicated microcosms)

The rewetting response was tested by analysis of variance. The 'averaged' rate of ^{14}C-CO_2 evolution was stimulated ($P<0.01$) by the addition of water. The rewetting response was significantly different among soil treatments and increased by 60% from 0.049 to 0.078 ($P<0.01$) in case a non diluted protozoan inoculum and by 180% to 0.137 ($P<0.01$) in case only a 1:10 diluted protozoan inoculum was applied compared to soils without protozoa added. The rewetting response was also investigated by comparing 2 days following the addition of water versus the

remaining days respectively. Based on this calculation, the rewetting response dropped to 0.002 in soils without protozoa whereas this figure was significantly ($P<0.05$) increased to 0.090 in soils with a 1:10 diluted and to 0.055 in soils with a non diluted protozoan inoculum respectively.

Discussion

By conducting an experiment in which plants were grown on soil that contained ^{14}C- and ^{15}N-labelled organic matter, both in the absence and in the presence of protozoa, we were able to simultaniously determine the turnover of carbon and nitrogen from soil organic material as affected by protozoan predation of bacteria. The activity of protozoa increased the turnover of soil organic ^{14}C-carbon as evidenced by a higher accumulated ^{14}C respiration as well as an ongoing higher ^{14}C respiration rate in soils with protozoa. During the last 15 days of the incubation, the presence of protozoa almost doubled the microbial respiration rate. The overall respired soil organic carbon was 36% and 53% higher in the soils with a 1:10 diluted and a non-diluted protozoan inoculum, respectively. These results agree with observations by Coleman *et al.* (1978) that soils with food chains that included bacterial grazers showed higher respiration rates compared to soils without bacterial grazers, i.e. amoebae and/or nematodes. Total soil respiration, i.e. from roots and microorganisms, was not affected by the presence or absence of protozoa and neither was the root production.

The reduction of the number of bacteria in soils with protozoa compared to soils without protozoa, even though smaller, confirmed earlier results on the predatory activity of protozoa (Kuikman and Van Veen 1989, Bryant *et al.* 1982, Clarholm 1981).

An increased plant mass production as well as the relative larger shoot production, indicated by an increased shoot:root ratio, in the presence of protozoa pointed to an improved plant nutrient supply as shown by Davidson (1969). Plant nitrogen availability was stimulated in the presence of protozoa, as evidenced by a higher uptake of nitrogen of 9% and 17% in soils with a 1:10 diluted and with a non-diluted protozoan inoculum density, respectively. These results confirm reports by Elliott *et al.* (1979), Clarholm (1985), Kuikman and Van Veen (1989) and Kuikman *et al.* (1989).

The uptake of ^{15}N-nitrogen by plants corresponded to the uptake of total nitrogen by plants as was shown by the similarity of the ratio between both nitrogen sources, i.e. ^{15}N and ^{14}N, in plants. Therefore it is concluded that the presence of protozoa affected the quantity and not the quality of the soil organic nitrogen that is mineralized and (made) available to plants. This is in contrast to results by

Kuikman and Van Veen (1989) who studied the turnover of ^{15}N labelled bacterial cells. In their study, relatively more bacterial ^{15}N than ^{14}N was mineralized and recovered in plants in the presence of protozoa. The relatively low ratio of $^{15}N:^{14+15}N$ in case soils received a non-diluted protozoan inoculum, might be explained by a relatively large contribution of non labelled, introduced bacterial biomass nitrogen to plant uptake due to immediate action of protozoa upon their introduction. In case less protozoa are introduced with a 1:10 diluted protozoan inoculum, a less rapid turnover of non labelled introduced bacterial nitrogen is found. This reasoning could also explain the relatively large contribution of labelled nitrogen from introduced labelled bacterial cells in the presence of protozoa as found by Kuikman and Van Veen (1989).

The impact of protozoa on the mineralization of ^{14}C was relatively larger than the impact on mineralization of (^{15}N)-nitrogen and plant nitrogen uptake as indicated by the ratio $^{14}C\text{-}CO_2$: ^{15}N-plants (Bq $\times$ mg^{-1} ^{15}N) (Table 3). As a result of the predatory activity of protozoa, nutrients are returned to the soil solution, i.e. carbon as a waste product of protozoan metabolism and excess ammonium nitrogen (Anderson *et al.* 1981). Re-circulation of this carbon and nitrogen within the bacterial-protozoan population is expected and after being respired carbon diffuses entirely from the soil whereas nitrogen can be re-used. In this case, protozoa would reduce bacterial numbers and thereby accelerate the turnover of bacterial carbon.

The rate of $^{14}C\text{-}CO_2$ respiration responded markedly to the addition of water. Several authors have pointed to the potential impact of a fluctuating soil moisture content on the microbial activity in soils (Stout 1973, Elliott *et al.* 1988). After remoistening of dried soils, an increased biological activity, as expressed by CO_2 production and N mineralization, has been generally accepted. The observed flushes are attributed to an increased availability of water-soluble substrates to the microorganisms, the disruption of aggregates and rearrangement of soil components in soils (Birch 1960, Orchard and Cook 1983, Adu and Oades 1978 and Van Veen *et al.* 1985).

In our system, soil moisture fluctuations are created by the evapo-transpiration of plants. The intensity of these fluctuations increases during the incubation period. Following the first three additions of rather modest amounts of water, no significant changes in the rate of ^{14}C respiration were detected. The final three additions of water on days 21, 26 and 31 increased the soil volumetric water content by 6%, 7.2% and 7.2%, respectively. These pulsed events can be considered as rainfall events. They provoked a clear and significant increase in the ^{14}C respiration rate by the microbial population which lasted 1 day in soils without and 2 days in soils with protozoa, as can be concluded from the calculated rewetting respons. The very rapid reaction of protozoa to the addition of water within 1 day confirms results presented by Elliott *et al.* (1988) and Hunt *et al.* (1989) who showed that protozoa responded quickly to pulsed events, i.e. rainfall, with a 5-fold increase in numbers

within 1 or 2 days. Kuikman *et al.* (1989) showed that protozoa increase the availability of bacterial nitrogen to plants even under conditions with strong fluctuations of the soil moisture content compared to conditions with a rather stable soil moisture regime. Thus, pulsed events indeed have a significant influence on the dynamics of food-chain interactions in soil in terms of carbon and nitrogen dynamics.

The number of protozoa that was inoculated did not significantly affect the nitrogen availability to plants nor did it affect the final number of protozoa recovered from soils with protozoa. Previous results have shown that in unplanted soil, protozoa hardly move actively through the soil matrix. Vargas and Hattori (1986) concluded from experiments in which the number of inoculated protozoa was varied that migration of protozoa among aggregates in soil was determined by the continuity of the water film connecting adjacent aggregates. However, the rewetting res-ponse (Figure 3) was more pronounced in soils that received a 1:10 diluted protozoan inoculum compared to soils with a non diluted protozoan inoculum. It can be hypothesized that flow of water strongly affect the distribution of protozoa such that in the 1:10 diluted protozoan inoculum treatment, relatively longer and more efficiently ^{14}C labelled bacteria were available for protozoa as compared to the non diluted protozoan ino-culum treatment in which the high initial number of protozoa reduced the number of bacteria and their activity initially to a higher extent. Regrowth of bacteria then occurred at places already inhabited by protozoa, whereas at the 1:10 diluted protozoan inoculum treatment, protozoa could inhabit places, not reached by them before the induced waterflow.

Kuikman and Van Veen (1989) have postulated that the mechanism through which the protozoa operate in stimulating nitrogen mineralization from soil organic matter, is an increased turnover of bacterial biomass. These data confirm this hypothesis by showing that protozoa do simultaneously stimulate the turnover of soil organic carbon and improve nitrogen mineralization and nitrogen availability to plants. It has been discussed whether such a stimulation by protozoa must be attributed to direct consumption of bacteria and the subsequent release of excess nutrients or indirectly by increaqsed bacterial activity or both (Barsdate *et al.* 1974, Anderson *et al.* 1981). Our results have shown that, in the presence of protozoa, upto 50% more ^{14}C and 20% more ^{15}N was mineralized than in soils with twice as many bacteria but without protozoa. So, bacterial cells would have to respire twice as much ^{14}C-CO_2 in the presence of protozoa than bacterial cells in soils without protozoa. On the other hand, if the elevated ^{14}C-CO_2 respiration must be attributed to direct consumption of 50% of the bacterial cells by protozoa, theoretically protozoa would have to respire all of the ^{14}C ingested to increase the ^{14}C-CO_2 respiration by 50%, which is unlikely (Anderson *et al.* 1981). Thus, it appears that a bacterial population, even though reduced in numbers, is more active in terms of

the amount of carbon metabolized per unit biomass when being predated upon by protozoa.

In conclusion, protozoa not only accelerate the turnover of carbon and nitrogen by reducing the size of the bacterial population but also seem to increase the activity of the bacterial population in soil.

References

Adu JK and Oades JM(1978) Physical factors influencing decomposition of organic materials in soil aggregates. Soil Biol Biochem 10: 109-115

Amato M (1983) The determination of carbon ^{12}C and ^{14}C in plant and soil. Soil Biol Biochem 15: 611-612

Anderson RV, Elliott ET, Coleman DC and Cole CV (1981) Effect of the nematodes *Acrobeloides* sp. and *Mesodiplogaster lheritieri* on substrate utilization and nitrogen and phosphorus mineralization in soil. Ecology 62: 549-555

Bamforth SS (1988) Interactions between protozoa and other organisms. Agric Ecosystems Environm 24: 229-234

Barsdate RJ, Prentki RT and Fenchel T (1974) Phosphorus cycle of model ecosystems: significance for decomposer food chains and effect of bacterial grazers. Oikos 25: 239-251

Birch HF (1960) Nitrification in soils after different periods of dry-ness. Plant Soil 12: 81-96

Bremner JM (1965) Total nitrogen In: CA Black, DD Evans, JL White, LE Ensminger and FE Clark (editors). Methods of soil analysis. Amer Soc Agron WI USA, pp 1149-1178

Bryant RJ, Woods LE, Coleman DC, Fairbanks FD, McClellan JF and Cole CV (1982) Interactions of bacterial and amoebal populations in soil microcosms with fluctuating moisture content. Appl Environm Microb 43: 747-752

Clarholm M (1981) Protozoan grazing of bacteria in soil - Impact and Importance. Microb Ecol 7: 343-350

Clarholm M (1985) Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. Soil Biol Biochem 17: 181-187

Coleman DC, Anderson RV, Cole CV, Elliott ET, Woods L and MK Campion (1978) Trophic interactions in soils as they affect energy and nutrient dynamics. IV Flows of metabolic and biomass carbon. Microb Ecol 4: 373-380

Darbyshire JF, Wheatley RE, Greaves MP and Inkson RHE (1974) A rapid micromethod for estimating bacterial and protozoan populations in soil. Rev Ecol Biol Sol 11: 465-475

Davidson RL (1969) Effects of soil nutrients and moisture on root/shoot ratios in *Lolium perenne* L. and *Trifolium repens* L. Ann Bot 33: 571-577

Elliott ET and DC Coleman (1977) Soil protozoan dynamics in a shortgrass prairie. Soil Biol. Biochem. 9: 113-118.

Elliott ET, Coleman DC and Cole CV (1979) The influence of amoebae on the uptake of nitrogen by plants in gnotobiotic soil. In: JL Harley and R Scott Russell (editors). The soil-root interface Academic Press London, pp 221-229

Elliott ET, Coleman DC, Ingham RE and Trofymow JA (1984). Carbon and energy flow through microflora and microfauna in the soil subsystem of terrestrial ecosystems. In: Current

perspectives in microbial ecology (MJ Klug and CA Reddy, eds). Amer. Soc. Microb. Washington DC, pp 424-434.

Elliott ET, Hunt HW, Walter DE and Moore JC (1988) Microcosms, mesocosms and ecosystems: linking the laboratory to the field Proc 4th Int Symp Microb Ecol:

Foster RC (1988) Microenvironments of soil microorganisms Biol Fertil Soils 6: 189-203

Genstat 5 Committee (1987) Genstat 5: Reference manual. Clarendon Press, Oxford

Hunt HW, Elliott ET and Walter DE (1989). Inferring trophic transers from pulse-dynamics in detrital food webs. Plant Soil 115: 247-259.

Kuikman PJ and Van Veen JA (1989) The impact of protozoa on the availa-bility of bacterial nitrogen to plants. Biol Fertil Soils 8: 13-18

Kuikman PJ, Van Vuuren MMI and Van Veen JA (1989) Effect of soil moisture regime on predation by protozoa of bacterial biomass and the release of bacterial nitrogen. Agric Ecosystems Environm (in press)

Orchard VA and FJ Cook (1983) Relationship between soil respiration and soil moisture. Soil Biol Biochem 15: 447-453

Page F 1967 An illustrated key to freshwater and soil amoebae Freshwater Biological Association, Cumbria, UK, Sci Publ 34

Postma J and Altemuller HJ (1989) Bacteria in thin soil sections stained with the fluorescent brightener calcofluor white M2R. Soil Biol Biochem (in press)

Rowe R, Todd R and Waide J (1977) Microtechnique for Most-Probable-Number analysis. Appl Environm Microb 33: 675-680

Stout JD (1973) The relationship between protozoan populations and biological activity in soils. Amer Zool 13: 193-201

Stout JD (1980) The role of protozoa in nutrient cycling and nutrient turnover. Adv Microb Ecol 4: 1-50

Vargas R and T Hattori 1986 Protozoan predation of bacterial cells in soil aggregates. FEMS Microb Ecol 38: 233-242

Van Ginkel JH and J Sinnaeve 1980 Determination of total nitrogen in plant material with Nessler's reagent by continuous flow analysis Anlyst 105: 1199-1203

Van Veen JA, JN Ladd and M Amato 1985 Turnover of carbon and nitrogen through the microbial biomass in a sandy loam and a clay soil incubated with [^{14}C(U)]glucose abd [^{15}N]$(NH_4)_2SO_4$ under different moisture regimes. Soil Biol Biochem 17: 747-756

Woods LE, CV Cole, ET Elliott, RV Anderson and DC Coleman 1982 Nitrogen transformations in soil as affected by bacterial-microfaunal interactions Soil Biol Biochem 14: 93-98

CHAPTER 7

SUMMARY AND CONCLUDING REMARKS

In general, more than 95% of the nitrogen in soils is present in organic forms. This nitrogen is not directly available to plants unless microbial decomposition takes place with the release of mineral nitrogen. In modern agriculture, nitrogen is often applied to arable soils as a fertilizer to support high levels of crop production. Nitrogen is one of the essential nutrients and is required by plants in substantial amounts (1.5-5% $\times$ g^{-1} dry weight). Extensive application of fertilizer nitrogen causes substantial environmental problems such as leaching of nitrates into groundwater which is used as drinking water and ammonia volatilization into the atmosphere. This ammonia is deposited on the surface of the earth. Nitrification then results in acidification of soils and damage to plants.

The microbial biomass in soil is both a source and a sink for nitrogen. This renders the turnover of nitrogen through the microbial biomass a key process in nitrogen cycling in soil. Knowledge about the mechanisms involved in the mineralization of nitrogen in soils is necessary to improve control of the nitrogen cycling in arable soils. Evidence is accumulating that interactions between microflora and fauna such as protozoa are responsible for a signicant portion of the mineralization of nitrogen in soils. In this thesis, the impact of protozoan predation of bacteria on the mineralization of nitrogen from bacterial cells in soil was investigated.

Traditionally, the mineralization of nitrogen in soils has been attributed to the microflora, i.e. bacteria and fungi. Protozoa have long been recognized as the major predators of bacteria thereby regulating the size of the bacterial populations in soils. The potential impact of protozoa on the mineralization of nitrogen has only recently been recognized (Elliott *et al.* 1979). When protozoa consume bacteria, excess nitrogen is excreted as ammonium nitrogen. This relation is based on a similar carbon:nitrogen ratio for protozoa and bacteria. Additionally, it has been hypothesized that, by grazing bacteria, protozoa enhance microbial activity and eventually mineralize nitrogen from soil organic matter (Clarholm 1985).

Experiments were carried out in microcosms containing sterilized soil that was inoculated with specific microbial populations. The turnover of the ^{15}N from microbial cells was assessed by growing plants on these soils and analysing the recovery of ^{15}N in the plant. The activity of protozoa was inferred from an increased number of protozoa during the incubation period.

In Chapter 2, the impact of protozoan grazing on the mineralization of nitrogen from bacterial biomass was investigated. Soil was inoculated with the basic composition of the food chain studied, i.e. ^{15}N-labelled *Pseudomonas aeruginosa* alone or with protozoa. Protozoan grazing strongly stimulated the turnover and the mineralization of bacterial nitrogen. In the presence of protozoa, plants recovered 65% more bacterial ^{15}N than in soils without protozoa. Furthermore, the presence of protozoa resulted in a 26% higher mineralization and uptake of nitrogen from soil organic sources. Additionally, a suspension with a natural bacterial population from the soil was added to the basic composition of the food chain. An increased number of bacteria would immobilize more nitrogen. It was hypothesized that consequently protozoan grazing would exert a more pronounced effect on the mineralization of nitrogen. In this case, the presence of protozoa stimulated the mineralization and uptake by plants of nitrogen from soil organic sources by 44% compared to soils without protozoa. However, the protozoan mediated mineralization and plant uptake of ^{15}N from bacterial cells was reduced by the addition of the bacterial suspension. This effect was explained by assuming that internal cycling of nitrogen occurred to a larger extent due to an increased size of the bacterial population when both a bacterial suspension and ^{15}N-labelled Pseudomonas aeruginosa were added.

Soil provides a very heterogeneous environment with its network of pores with sizes from 0.2 μm to 2000 μm. Microorganisms have been shown to be neither randomly nor uniformly distributed through the soil fabric (Foster 1988). Part of the habitable pore space for bacteria in soil is not accessible to protozoa (Vargas and Hattori 1986). The numbers of protozoa that are found in natural soil ranges from 1×10^4 to 1×10^6 per gram dry soil. Still, protozoa have to migrate through the soil matrix to meet their prey organisms. This is especially true under the experimental conditions in our microcosms where only up to 1×10^4 protozoa per gram of dry soil have been inoculated at the start. The effect of the inoculum density of protozoa and of spatial separation of protozoa and (prey)organisms on the migration by protozoa as well as on the turnover, dynamics and transport of specific microbial populations in soil was studied (Chapter 3). The CO_2 evolution served as an indicator of microbial activity. The fate of introduced ^{14}C-labelled bacterial cells was followed by monitoring ^{14}C-CO_2 evolution. Two antibiotic resistant Pseudomonas fluorescens R2f strains were inoculated. The transfer of genes between

representatives of these strains via conjugation was a powerfull indicator of the distribution of bacteria and protozoa in soils. The activity of protozoa increased CO_2 evolution compared to soils not inoculated with protozoa. This stimulation of the CO_2 evolution was related to the percentage of a soil portion that was inoculated with protozoa. The inoculated number of protozoa did not affect the CO_2 evolution. This indicated that protozoa were only active in those particles into which they were inoculated. Protozoa accelerated the turnover of ^{14}C. The more protozoa were inoculated, the faster specific ^{14}C-labelled substrates were respired. The turnover of ^{14}C was determined by the frequency of encounters between protozoa and labelled microorganisms. It was concluded that this frequency is a function of the concentration of protozoa and ^{14}C-labelled microorganisms in the soil matrix. The results obtained in Chapter 3 picture the soil as a rather constrained system with respect to the mobility of both protozoa and bacteria in the soil matrix. Protozoa hardly influence the distribution of bacterial cells in soil through dispersion. Additionally, it was shown that protozoa did not affect the transfer of genes through organisms in soil. The results support the hypothesis by Stout (1973) that the activity of protozoa is confined to small spaces and consequently small populations.

Protozoa are in essence aquatic organisms and therefore water is essential to their functioning. Based on the average cell sizes of 10-50 μm for amoebae and 10-20 μm for flagellates, these protozoa can only enter and feed in pores with a pore neck diameter 3-20 μm and larger (Darbyshire 1976). The availability of water was shown to strongly regulate the grazing activity of protozoa (Chapter 5). In soils kept at a low soil moisture tension (3 Bar), protozoa were not active. It was estimated from a water retention curve (Postma *et al.* 1989) that at this moisture tension, pores with pore necks larger than 3 μm are devoid of water. Hence, protozoan movement and feeding was restricted because waterfilms were too thin or even absent. Only at higher soil moisture contents (0.1 Bar and 0.3 Bar), the activity of protozoa reduced the number of bacteria and increased the mineral nitrogen content in soil (Chapter 5).

Upon drying of the soil, protozoa encyst to survive dry conditions. Little information is available on the signals and the time needed to excyst and return to trophic stages when favourable soil moisture conditions are restored. In a series of three experiments, plant water transpiration was used as an experimental tool to induce soil moisture regimes with defined fluctuations (Chapter 4 and 5). Even though protozoa were forced to encyst upon drying of the soil, they reacted very rapidly to remoistening. In soils that were incubated under conditions with modest soil moisture fluctuations, protozoan activity resulted in an even higher mineralization and plant uptake of ^{15}N from bacterial cells than in soils that were incubated with a stable soil moisture regime. The activity of protozoa stimulated the mineralization of nitrogen from soil organic sources under all soil moisture regimes applied

(Chapter 4). The protozoan activity was further restricted when soil moisture regimes were characterized by more intense and more frequently induced moisture fluctuations (Chapter 5). This was shown by a reduced recovery of bacterial nitrogen in plants compared to soils that were kept continuously moist.

The effect of protozoan predation on the mineralization of carbon and nitrogen from soil organic matter was determined simultaneously in an experiment in which plants were grown in soil microcosms that contained ^{14}C-carbon and ^{15}N-nitrogen organic material both in the presence and absence of protozoa (Chapter 6). The predating activity of protozoa accelerated the turnover of microbial carbon and nitrogen by reducing the size of the bacterial population. But more important, protozoa stimulated the activity of the remaining bacterial population as judged from an ongoing higher rate of $^{14}C\text{-}CO_2$ respiration in the presence of protozoa compared to soils without protozoa. Protozoa responded immediately to the restoration of favourable moisture conditions in the soils as shown by the increased rate of $^{14}C\text{-}CO_2$ respiration. These results support the observations that protozoa numbers increased within 1 or 2 days upon the addition of water to dry soils (Hunt *et al.* 1989). Furthermore, it was hypothesized that the flow of water contributed to a (re)distribution of protozoa. In soils that were inoculated with less protozoa, the rewetting response in terms of increased rate of $^{14}C\text{-}CO_2$ respiration, was larger than in soils inoculated with more protozoa.

Several mechanisms for the action of protozoa with respect to mineralization of nitrogen have been proposed (Chapter 1):

1) by grazing bacteria, protozoan biomass is produced at the expense of bacterial biomass and excess nitrogen is excreted as ammonium
2) by grazing bacteria, protozoa produce waste products (cell wall material and other nutrients) which in turn may enhance microbial activity
3) whilst moving through the soil searching for food particles, protozoa might (re)inoculate (new) substrates by transporting bacteria that adhere to their cell surface or by bacteria that are not digestable and therefore excreted.

In all experiments, the activity of protozoa reduced the size of the bacterial populations both in planted and in unplanted soils by a factor of two (Chapter 5 and 6), five (Chapter 3) to eight (Chapter 2). As a consequence, it is concluded that consumption of bacteria is responsible for at least a part of the increased mineralization of nitrogen in the presence of protozoa. Furthermore, the activity of protozoa induced an ongoing higher microbial respiration rate in planted soils (Chapter 6). It was demonstrated that a smaller sized bacterial population was more active in terms of the amount of carbon metabolized per unit microbial biomass when being predated upon by protozoa. Also, the presence of protozoa increased maintenance of the plasmid in a plasmid containing bacterial population.

This was attributed to an improved nutrient availability in soils where protozoa grazed bacteria. With respect to the third mechanism through which protozoa could enhance mineralization of nitrogen, only limited information was available. It was shown that in unplanted soils (Chapter 3) that are incubated under stable soil moisture regimes, the migration and mobility of protozoa between aggregates is very much restricted (Vargas and Hattori 1986). Based on the observations on gene transfer, it could not be demonstrated that protozoa are important vectors in the allocation of bacteria through soil. However, it was hypothesized that water flow significantly contributes to the distribution of protozoa and probably bacteria as well through the soil matrix.

That microorganisms are responsible for mineralization of nitrogen from organic sources in soils has long been recognized. The results, presented in this thesis, showed clearly that in soils protozoan grazing of bacteria substantially improves the availability of organically bound nitrogen to plants. The mechanisms by which protozoa stimulate the mineralization of nitrogen are i) by releasing nitrogen directly from bacterial cells and ii) by stimulating the turnover of soil organic matter through the microbial biomass. The microcosm approach proved to be useful to study the mineralization of nitrogen by protozoan activity in soil. The dynamic character of planted soil was demonstrated by an ongoing respiration of soil organic carbon. In fallow soil, the protozoan activity lasted only 10 days and then, gradually, microbial activity slowed down. However, in both planted and fallow soil, protozoa exhibited their stimulating effect on carbon and nitrogen mineralization rates. Without significant water flow through the soil matrix, the mobility and migration of protozoa is low. Reallocation of bacteria in the soil matrix was virtually absent. Hence, protozoa sec do not contribute to a (re)distribution and dispersion of bacteria through soil as hypothesized by Finlay and Fenchel (1989). Meanwhile, it was demonstrated that applying a combination of techniques from the field of microbial ecology and of genetics can substantially improve our understanding of the ecology of (introduced) soil microorganisms.

Protozoa exhibited a very fast, immediate reaction to restoration of favourable moisture conditions in previously dried soils. The pulsed addition of water to dry soils have a significant impact on the dynamics of food-chain reactions in soil in terms of microbial activity and of carbon and nitrogen mineralization. Here, the effect of protozoa could be caused both by substrates made available to microorganisms through disruption of soil aggregates or through an improved distribution of the biota through the soil. The magnitude of the effect of protozoan grazing on the plant availability of organically and bacterial bound nitrogen supports the concept that food-web interactions rather than microbial activity alone, are responsible for nitrogen mineralization in soil.

References

Clarholm M (1985) Interactions of bacteria, protozoa and plants leading to mineralization of soil nitrogen. Soil Biol Biochem 17: 181-187

Darbyshire JF (1976) Effect of water suctions on the growth in soil of the ciliate *Colpoda steinii*, and the bacterium *Azotobacter chroococcum*. J Soil Sci 27: 369-376

Elliott ET, Coleman DC and Cole CV (1979) The influence of amoebae on the uptake of nitrogen by plants in gnotobiotic soil. In: The soil root interface (JL Harley and R Scott Russell, eds). Acad Pr, London, pp 221-229

Finlay B and Fechel T (1989) Everlasting picnic for protozoa. NewScientist: 66-69

Foster RC (1988) Microenvironments of soil microorganisms. Biol Fertil Soils 6: 189-203

Hunt HW, Elliott ET and Walter DE (1989) Inferring trophic transfers from pulse-dynamics in detrital food webs. Plant Soil 115: 247-259

Postma J, Walter S and Van Veen JA (1989) Influence of different initial soil moisture contents on the distribution and population dynamics of introduced *Rhizobium leguminosarum* biovar *trifolii*. Soil Biol Biochem 21: 437-442

Stout JD (1973) The relationship between protozoan populations and biological activity in soils. Amer Zool 13: 193-201

Vargas R and Hattori T (1986) Protozoan predation of bacterial cells in soil aggregates. FEMS Microb Ecol 38: 233-242

SAMENVATTING EN SLOTOPMERKINGEN

Stikstof (N) is een essentieel element in de voeding van planten. Ruim 95% van de stikstof in grond wordt aangetroffen in organische verbindingen en is niet direct beschikbaar voor opname door de plant. In de moderne landbouw wordt daarom bemesting met minerale stikstof toegepast om de noodzakelijke hoge opbrengsten van gewassen mogelijk te maken. De toepassing van stikstofbemesting kan resulteren in milieuproblemen. De vervluchtiging van ammoniak (NH_3) heeft verzuring van de grond tot gevolg omdat ammoniak via depositie weer in de grond terugkeert en door microorganismen wordt omgezet in nitraat. Nitraat (NO_3) kan uitspoelen naar dieper grondwater. Dit leidt tot een verhoogde concentratie van nitraat in het water dat voor drinkwater wordt gebruikt.

Bacterien en schimmels in grond gebruiken organische stof als voedsel/koolstofbron voor de productie van nieuwe cellen en als energiebron. Een deel van de koolstof in het organisch materiaal wordt daarbij omgezet in CO_2. Daarbij komt stikstof vrij uit organisch materiaal die, na mineralisatie in microorganismen, wordt uitgescheiden als ammonium of nitraat. Deze stikstof is beschikbaar voor opname door de plant maar kan ook door de microorganismen zelf (weer) worden opgenomen. De microbiele biomassa vormt een belangrijke bron van stikstof voor planten. Daarbij is de snelheid waarmee stikstof wordt omgezet in microbiële cellen, de turnover van stikstof, van cruciaal belang. Inzicht in de mechanismen van de mineralisatie is noodzakelijk om een efficient gebruik van stikstof in de landbouw te bevorderen.

Protozoen zijn kleine, eencellige diertjes die zich voeden met bacterien. Zij zijn derhalve predatoren op bacterien. In dit proefschrift is de invloed onderzocht van predatie op bacterien door protozoen op de mineralisatie van stikstof uit bacteriele cellen onderzocht. De predatie op bacterien door protozoen kan leiden tot een verhoging van de mineralisatie van stikstof uit bodem organische stof.

Dit zou plaats kunnen vinden doordat i) protozoen bij het consumeren van bacterien meer stikstof opnemen dan nodig is waarop deze wordt uitgescheiden als ammonium of ii) protozoen door de uitscheiding van afvalstoffen en/of ammonium de activiteit van de bacteriele populatie verhogen of iii) protozoen op hun zoektocht naar voedsel door de grond bacterien meeslepen naar organische stof in de bodem die nog niet was gekoloniseerd door bacterien.

De experimenten die worden beschreven in dit proefschrift zijn allen uitgevoerd in gesteriliseerde grond die vervolgens is beënt met specifieke bacterien en/of protozoen. De turnover van bacteriele cellen is onderzocht door de aan grond toegevoegde bacterien te labellen met isotopen. Daartoe zijn de bacterien gekweekt in een groeimedium dat een stikstofbron met een stabiele isotoop van stikstof (het ^{15}N atoom) bevat of een koolstofbron met een radioactieve isotoop van koolstof (het ^{14}C atoom) bevat. Op deze manier kan onderscheid worden gemaakt tussen respectievelijk in de grond en in bacterien aanwezige stikstof en koolstof. Dit is voor het onderzoek van groot belang. Aan de hand van de ^{15}N die door planten uit de grond wordt opgenomen kan de turnover van bacteriele stikstof in de grond worden bepaald. De turnover van bacteriele koolstof kan worden bepaald aan de hand van de ^{14}C in CO_2 die bij microbiele activiteit wordt gevormd. Het effect van protozoen is onderzocht door grond die uitsluitend was beënt met bacterien te vergelijken met grond die was beënt met zowel bacterien als protozoen.

In hoofdstuk 2 is beschreven dat in aanwezigheid van protozoen in grond, 26% meer stikstof uit bodem organische stof is gemineraliseerd en opgenomen door planten dan in grond zonder protozoen. Daarnaast bevatten de planten die zijn gekweekt op grond met protozoen lieft 65% meer ^{15}N-stikstof. De activiteit van protozoen leidt tot een reductie van het aantal bacterien zoals al veelvuldig is waargenomen.

Grond bestaat uit deeltjes met daartussen porien die varieren in grootte van enkele micrometers tot millimeters. Bacterien en protozoen leven in het water dat zich bevindt in de meeste van die porien. Deze microorganismen zijn respectievelijk 0.5-2 μm en 5-30 μm groot. De organische stof in grond maar ook de bacterien zijn noch random, noch uniform verdeeld over deze porien. Dit geldt eveneens voor de protozoen. Een deel van de bacterien leeft in porien die door een te kleine opening ontoegankelijk zijn voor protozoen. Verder kan een deel van de bacterie populatie onbereikbaar zijn voor protozoen doordat deze bacterien leven in porien die niet door water zijn verbonden met de overige porien.

In hoofdstuk 3 is de invloed van het sterk heterogene karakter van de bodem op de predatie door protozoen en de dynamiek van bacterie populaties onderzocht. Door gebruik te maken van bacterien die met behulp van genetische manipulatie resistent zijn geworden tegen specifieke antibiotica is ook informatie verkregen over i) de mate van reallocatie van bacterien door migratie van protozoen en ii) de invloed van protozoen op de overdracht van genen tussen verschillende bacterien in grond. Protozoen verhogen de mineralisatie van koolstof uit bacteriele biomassa, zoals dat ook het geval is bij stikstof. Echter, wanneer protozoen worden beënt in een beperkt gedeelte van iedere portie grond, is hun effect op de mineralisatie van koolstof uit bacterien evenredig kleiner, vergeleken met grond waarin protozoen worden beënt in alle grond van iedere portie. De migratie van protozoen in grond is zeer beperkt onder stabiele omstandigheden qua bodemvocht. Er is geen aantoonbare reallocatie van bacterien door protozoen activiteit waargenomen. Hoewel overdracht van genen tussen bacterien kon worden aangetoond, is in dit experiment vastgesteld dat protozoen daarop geen invloed hebben. De resultaten vormen aanleiding om te veronderstellen dat de activiteit van protozoen in grond beperkt blijft tot kleine, locale en onderling gescheiden oppervlakten.

Protozoen zijn in feite waterdieren en water is essentieel voor hun functioneren. Gedurende (te) droge milieuomstandigheden vormen protozoen cysten om te overleven. Wanneer water opnieuw beschikbaar komt, gaan zij weer over in actieve stadia. In de hoofdstukken 4 en 5 is onderzocht wanneer in grond de activiteit van protozoen wordt geremd door vochtgebrek. Wanneer de hoeveelheid water in grond zodanig klein is dat zich alleen in porien met een diameter minder dan 6 μm nog water bevindt, wordt geen activiteit van protozoen meer waargenomen. Opname en verdamping van water door planten leidt tot uitdroging van grond. Door de watergift aan beplante grond te manipuleren, is het effect van afwisselend (te) weinig en voldoende water op de activiteit van protozoen bepaald. Er is aangetoond dat protozoen de turnover van stikstof in dezelfde mate stimuleren bij bodemvocht regimes met sterk fluctuerende condities als bij regimes met stabiele, gunstige vochtomstandigheden. Protozoen reageren zeer snel (binnen 1 tot 2 dagen) wanneer, na uitdroging van grond, hun leefomstandigheden worden verbeterd door water toe te voegen.

In hoofdstuk 6 is de invloed van protozoen op de turnover van bodemorganische stof door microorganismen onderzocht. De turnover en de mineralisatie van stikstof en koolstof in de bodem wordt door de activiteit van protozoen gestimuleerd. In aanwezigheid van protozoen nemen planten meer stikstof op en wordt meer koolstof verademd als CO_2 dan in grond zonder protozoen. Er is aangetoond dat hierbij twee mechanismen een rol spelen. In de eerste plaats hebben protozoen een direct effect: via reductie van het aantal bacterien wordt in bacterien vastgelegde koolstof en stikstof versneld gemineraliseerd. Deze stikstof kan worden opgenomen door de plant. In de tweede plaats is er een indirect ef-

fect gevonden: de predatie door protozoen verhoogt de activiteit van de bacterie populatie, uitgedrukt in hoeveelheid gemetaboliseerde organische stof per eenheid bacteriele biomassa. Tevens geven de resultaten aanleiding te veronderstellen dat de stroming van water na watergift de verdeling van protozoen en mogelijk ook bacterien in grond via (passieve) reallocatie verbetert.

De resultaten in dit proefschrift maken duidelijk dat de beschikbaarheid voor planten van organisch gebonden en bacteriele stikstof in grond via predatie van bacterien door protozoen substantieel wordt verbeterd. Protozoen opereren via i) het beschikbaar stellen van in bacterien vastgelegde stikstof en via ii) het stimuleren van de turnover van bodemorganische stof in microorganismen door het verhogen van de activiteit per cel. In hoeverre protozoen bijdragen aan de turnover van organische stof via verplaatsing van bacterien naar nieuw substraat blijft de vraag. In grond met een beperkt watergehalte en zonder waterstroming is de beweeglijkheid en migratie van protozoen zeer beperkt. Reallocatie van bacterien door protozoen sec, in afwezigheid van waterstroming, is niet aangetoond. Er is duidelijk gemaakt dat de gecombineerde toepassing van technieken uit de genetica en de microbiële oecologie in belangrijke mate ons begrip van de oecologie van microorganismen in de bodem bevordert. Protozoen vertonen een zeer snelle, onmiddellijke reactie op het beschikbaar komen van water in droge grond. De resultaten in dit proefschrift ondersteunen het concept dat predator-prooi interacties, meer dan uitsluitend microbiele activiteit, verantwoordelijk zijn voor de mineralisatie van stikstof in grond.

NAWOORD

Het is plezierig om met dit proefschrift mijn onderzoek van de laatste jaren voorlopig af te sluiten. Als ik mijn onderzoek zeg kan dat niet zonder stil te staan bij en dank te zeggen aan een aantal mensen die mij daarbij hebben gesteund en soms onmisbaar zijn geweest.

Hans van Veen gaf de aanzet tot dit onderzoek en ik dank hem voor de grote mate van vrijheid en vertrouwen die hij me heeft gegeven in de uitvoering van het onderzoek, voor de vele felle maar nuttige diskussies over plannen en resultaten, voor zijn grenzeloze optimisme en zijn volharding ten aanzien van het schrijven.

Als ik nu meer microbioloog ben dan 5 jaar geleden dan komt die eer toe aan mijn promotor, Alex Zehnder. Hij bood voortreffelijke begeleiding: op de achtergrond maar frequent en nadrukkelijk aanwezig. Vooral in de weken voor de afronding van dit proefschrift vormde jouw optimisme een bron van inspiratie.

David Coleman introduceerde me in de fascinerende microbiologie van de bodem. I thank you, David, for letting me sit in at ZE 600 for it has lead to this thesis. You were right: "It is worth counting the little things, because it is the little things that count".

Marianne Clarholm dank ik voor de vele uren die nodig waren om mij te leren hoe protozoen te herkennen. Tack så mycket för diskussionen och gästfriheten i Sverige.

In Gerda Jansen vond ik, door haar inzet, grote doorzettingsvermogen en geduld, een geweldige steun bij mijn omvangrijke experimenten in de laatste anderhalf jaar.

Dank ben ik ook verschuldigd aan o.a. Els Nijhuis en Margarit de Klein voor het gemak waarmee zij pieken hebben opgevangen in het praktische werk en aan Jan Oude Voshaar en Saskia Burgers voor hun statistische hulp bij de interpretatie van resultaten.

De mensen in de bodembiologiegroep hebben gezorgd voor een sfeer van collegialiteit, inspiratie en saamhorigheid, voor kritiek op mijn werk en manuscripten en voor vele prettige momenten ondanks de spanning rond de overgang naar het IB. Ik denk met veel plezier terug aan de grappen en grollen omdat lachen bij werken hoort. Jullie, met name, hebben de laatste 5 jaar voor mij tot een zeer enerverende en onvergetelijke periode gemaakt.

Last but not least wil ik mijn ouders bedanken voor de mogelijkheden en de steun die zij mij gedurende de jaren van mijn studie hebben gegeven en voor hun vertrouwen in de goede afloop van dit promotie onderzoek.

Curriculum vitae

Peter Kuikman werd geboren op 12 mei 1956 in Amsterdam. Na het behalen van het Atheneum diploma aan het St. Bonifatius College in Utrecht begon hij in 1976 met de studie Biologie aan de Rijksuniversiteit te Utrecht. In 1981 werd het kandidaatsexamen biologie afgelegd. Tijdens de studie voor het doctoraalexamen bewerkte hij vakken in landschapsoecologie en natuurbeheer (dr. F. Berendse), plantenoecologie (prof. dr. G.S. Innis en prof. dr. M.J.A. Werger) en microbiologie (prof. dr. A.J.B. Zehnder, Landbouwuniversiteit Wageningen). Het bijvak plantenoecologie werd uitgevoerd aan de Colorade State University in Ft. Collins CO (USA) en het bijvak microbiologie binnen de bodembiologiegroep op onderzoeksinstituut Ital in Wageningen. Het doctoraalexamen werd afgelegd in september 1985. In 1983 wist hij het Ministerie van Defensie te overtuigen dat hem onder de wapenen roepen pure verspilling van investeringen zou zijn. Sinds januari 1985 is de auteur werkzaam als bodembioloog/protozooloog in de onderzoeksgroep bodembiologie op Ital. Inmiddels is hij getransfereerd naar het Instituut voor Bodemvruchtbaarheid (Haren) en vooralsnog gedetacheerd in Wageningen.

krips repro meppel