DISTRIBUTION AND POPULATION DYNAMICS OF *RHIZOBIUM* SP. INTRODUCED INTO SOIL

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DISTRIBUTION AND POPULATION DYNAMICS OF *RHIZOBIUM* SP. INTRODUCED INTO SOIL

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aan Ton,
mijn ouders
en grootouders

Het drukken van dit proefschrift werd mede mogelijk gemaakt door het LEB-fonds

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STELLINGEN

1. De afwezigheid van dode cellen in natuurlijke grond wordt niet aangetoond door de waarneming van Bohlool en Schmidt, dat dode rhizobium cellen, die zijn aangehecht aan objectglaasjes, verdwijnen na ingraven van deze objectglaasjes in grond (Bottomley en Dughri).

   B.B. Bohlool en E.L. Schmidt. 1973. Soil Sci. Soc. Am. Proc. 37:561-564.
   P.J. Bottomley en M.H. Dughri. 1989. Appl. Environ. Microbiol. 55:959-964.

2. Het vochtgehalte van de grond voor inoculatie is van wezenlijk belang voor de overleving van geïntroduceerde bacteriën.

   Dit proefschrift.

3. Indien op de Y-as van Figuur 3 uit het artikel van Vargas en Hattori geen log aantal cellen/g grond maar 10^5 cellen/g grond had gestaan, dan hadden de punten in de grafiek goed overeengekomen met de aantallen genoemd in de tekst.

   R. Vargas en T. Hattori. 1986. FEMS Microbiol. Ecol. 38:233-242.

4. De verminderde afname van het aantal geïntroduceerde bacteriën in natuurlijke grond na toevoeging van een protozoëremmer en de conclusie dat protozoën de belangrijkste oorzaak zijn van de teruggang van het aantal geïntroduceerde bacteriën (Habte en Alexander), is prematuur gezien het in dit proefschrift gevonden synergetisch effect van de combinatie van predatie en concurrentie met andere microorganismen.

   M. Habte en M. Alexander. 1975. Appl. Microbiol. 29:159-164.
   M. Habte en M. Alexander. 1977. Arch. Microbiol. 113:181-183.

5. De uitspraken van Alexander geven geen eenduidig uitsluitsel over het al dan niet voorkomen van selectieve predatie door protozoën van autochtone, dan wel geïntroduceerde bacteriën.

   W.L. Chao en M. Alexander. 1981. Soil Sci. Soc. Am. J. 45:48-50.
   J.J. Pena-Cabriales en M. Alexander. 1983. Soil Sci. Soc. Am. J. 47:241-245.

6. De waarneming van Kilbertus dat poriën in verschillende gronden gemiddeld een drie maal zo grote diameter hebben als de zich erin bevindende bacteriën of bacteriekolonies, impliceert wel een heel letterlijke betekenis van survival of the "fittest".

   G. Kilbertus. 1980. Rev. Ecol. Biol. Sol 17:543-557.

7. In grond is ruimte geen beperkende factor voor de overleving van bacteriën, ook al biedt maar een deel van de totale porie-ruimte in grond mogelijkheden voor overleving van bacteriën ('habitable pore space') of bescherming tegen predatie door protozoën ('protective pore space').

Dit proefschrift.

8. Dat Applied and Environmental Microbiology zijn kolombreedte aangeeft met 3 $^{5}/_{16}$ inch, is weinig consequent met de expliciete eis dat, ook in de aan dit tijdschrift aangeboden artikelen, SI-eenheden gebruikt moeten worden.

9. Als vervolg op de studie van Kircz naar het profiel van de vrouw van de promovendus, rechtvaardigt de achterstand van het aantal vrouwelijke promovendi een vervolgstudie naar het profiel van de partner van de promovenda.

J. Kircz. NRC Handelsblad 16-2-1984.

10. Het invoeren van een dalurenkaart voor museumbezoek tijdens stille uren (Westermann) biedt in de toekomst mogelijkheden om verder te bezuinigen op de studiefinanciering.

M. Westermann. 1989. Quote 4 p. 25.

11. Er zijn geen woorden voor de blindheid voor dyslexie.

12. Hoewel het begrip biologische controle in veel gevallen toepasselijker is dan het begrip biologische bestrijding, verraadt de gebruiker hiermee zijn niet-planteziektenkundige achtergrond.

Stellingen behorende bij het proefschrift 'Distribution and population dynamics of *Rhizobium* sp. introduced into soil', J. Postma.
Wageningen, 22 september 1989.

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CHAPTER 1

INTRODUCTION

1.1 GENERAL SCOPE

In the past decades, bacteria have frequently been introduced into the soil to improve crop production. Direct beneficial effects arise when the bacteria provide the plant with useful products. This is the case with free-living and symbiotic bacteria (e.g. *Rhizobium* spp.) [50,51] which are able to fix atmospheric nitrogen. Moreover, soil bacteria may enhance the mineral uptake of plants, for example by solubilization of phosphate in soil, and the production of auxins, gibberelin and cytokinin-like substances may stimulate root development [14]. Beneficial effects of rhizosphere-colonizing bacteria also occur from the suppression of soil-borne pathogens. Mechanisms such as the production of antibiotics, myco-parasitism, competition for nutrients and cell wall degrading enzymes are suggested to be responsible for this suppression [41]. Bacteria have also been applied to soil for the degradation of xenobiotics [38,44]. It is clear that the introduction of bacteria in soil, as biofertilizers or as biological control agents, can diminish the application of chemical fertilizers and pesticides, whereas xenobiotic-degrading bacteria offer possibilities to reduce environmental problems with soil pollution. Due to recent advances in molecular biology, opportunities are created to improve bacterial strains by genetic engineering [14,35,50] and the introduction into soil of beneficial bacteria has become an important issue.

For a successful application of bacteria, the establishment and survival of the newly introduced bacterial population is necessary. However, numbers of bacteria have been found to decrease drastically after their introduction [18], so that heavy inocula were applied to increase their chances of survival. Introduced bacteria are thought to be less adapted to the soil than the autochthonous population [22] and soil usually reacts as a biological buffer against nonresident bacteria [6]. Abiotic factors, such as temperature, pH, salinity, moisture content, substrate availability and presence of oxygen are important for the survival of

bacteria. In general, high population levels of introduced bacteria can exist in soil when sterilized [33,52]. Thus, abiotic factors are not the only factors responsible for the low survival rates of the introduced bacteria. The decline of bacteria introduced into natural soil has been attributed mainly to predation by protozoa and to competition with other microorganisms [25,48,52]. Other possibilities such as microorganisms capable of producing antibiotics or lytic enzymes, bacteriophages and *Bdellovibrio* were suggested to be less important [1,24,48].

Soil type was found to influence the survival of introduced bacteria [18], as well as the numbers of indigenous bacteria [7] and the activity of the soil microflora [60]. Differences between the soil types in their pore size distribution and water relationships affect the habitable space for soil organisms [17]. Therefore, texture and structure are recognized more and more as key factors in the control of biological interactions in soil [17,59,61].

Since soil is a heterogeneously structured system, composed of a diversity of microhabitats [22,53], knowledge about bulk parameters is not sufficient to understand the processes in soil. It is the microenvironment of the bacteria that determines their survival and activity. Although the occurrence of favourable microhabitats for bacteria in soil is generally accepted [22,31,32,39,43], precise data about the distribution of introduced bacteria over such microhabitats in soil are scarce. Knowledge about the spatial distribution of bacteria in soil and methods applicable for such studies, are discussed in the following paragraph.

For a better understanding of the ecology of bacteria introduced into soil, the research done in this thesis focussed on the spatial distribution of introduced bacteria through the soil matrix. This thesis work was part of an exchange programme between Germany and the Netherlands. In the German counterpart research project the role of reserve materials on survival of bacteria introduced into soil was examined by U. Hoff (Institute for Soil Biology, FAL, Braunschweig, FRG).

1.2 DISTRIBUTION OF BACTERIA IN SOIL

The possibility of studying the distribution of bacteria on a microscale in a complex system like soil is limited by the availability of techniques. Therefore, possibilities and problems of different approaches to study the bacterial distribution in soil are discussed here first.

Microscopic techniques are hampered by the large proportion of opaque

solid particles compared to the small size of bacteria. In situ observation of bacteria in soil is not possible, but soil samples embedded in resin or plastic can be used so that the soil is not disturbed during the preparation of sections. Ultrathin sections, prepared using ultramicrotomy, can be observed with transmission electron microscopy (TEM). However, ultrathin sections of large areas of the soil fabric are technically difficult to produce [19] and sand grains damage the diamond knives. Scanning electron microscopy (SEM) has the advantage that polished blocks and even non-embedded frozen soil can be viewed ("cryoscan") [11]. Bacteria have been seen by SEM in systems with only clay minerals [15] and in clayey soils [26], but it was found to be difficult to distinguish bacterial cells from other soil components. Cells embedded in soil particles are invisible with SEM and the contrast between cells and their background is relatively poor [56]. According to Hagen *et al.* [26], the minimal number of microorganisms required for detection with SEM was between 10^7 and 10^9 cells per gram of soil.

Bacteria in thin sections prepared from embedded soil have also been observed with light microscopy after the bacteria were stained with a diachrome [2,36]. Fluorochromes would appear more suitable to stain and observe bacteria on opaque particles, since incident illumination can be used. Fluorochromes have been applied to thin soil sections [3-5,54,55], however, a reliable technique for staining bacteria in situ has not been described yet.

Microscopic observations of disturbed soil samples can give information about, for example, clustering of cells, occurrence of bacteria on organic or mineral particles, but not on the type and size of pores in which bacteria occur. In order to minimize the disturbance between bacteria and their natural surrounding Casida [10] used a special soil incubation chamber, whereas also microscope slides [40,57] and glass capillaries [9,47] have been buried in soil and recovered from time to time to be monitored. Others studied individual soil particles or aggregates [34,42] and surface films of soil crumps [8,27,62] with various microscopic techniques. However, with most of these techniques, only the surface of aggregates and soil particles could be examined.

One of the major problems of the present microscopical techniques is their inability to distinguish between viable and dead bacteria. However, the detection of specific bacteria is possible when antisera conjugated with a fluorochrome are applied.

Another approach to study the bacterial distribution in soil is the

preparation of different fractions by washing, wet sieving, dry sieving or density gradient centrifugation. In this way, soil particles of different sizes and densities with their associated bacteria are separated. Enumeration of the bacteria in the obtained fractions may provide indirect information about the distribution of bacteria over different microhabitats in soil. The advantage of such an approach is that bacteria can easily be quantified and that only viable cells are enumerated by using plate counts.

Hattori [28,29,30] proposed a 'washing-sonication method' which is based on the idea that water-stable aggregates can be washed gently to remove microbial cells from the outer part, whereas those in the inner part can be dispersed by sonic oscillation. The reliability of this hypothesis was assessed in several experiments. Interesting information about soil aggregates as microhabitats of bacteria was obtained with these experiments.

The distribution of bacteria in soil is neither random nor uniform. Their distribution echoed the distribution of organic matter in soil [12]. Using TEM, colonies or single cells associated with cellular remnants, faecal materials or amorphous organic matter have been observed [20]. Microscopic observations of the abundancy of cells on different types of soil particles, showed that the organic matter, which represented only 15% of all particles in soil, contained approximately 60% of the attached bacterial population [34]. Moreover, it was estimated that only 0.02% of the sand grain and 0.17% of the organic surfaces were occupied by bacteria [34]. The relative higher occupation of organic matter by bacteria was also detected with wet sieving: 2.2% organic matter, 83.8% sand and 14.0% silt and clay particles contained 41.7, 12.0 and 46.3%, respectively, of the culturable bacteria in soil [37]. The use of a nutrient-poor medium for the enumeration of bacteria, resulted in higher percentages of bacteria isolated from the sand particles [37].

Aggregates are also expected to be favourable habitats for bacteria. Hattori [31,32] suggested a distinct distribution of bacteria over the inner part of aggregates, containing capillary pores, and the outer part of aggregates, containing wider pores and the surface of aggregates. The inner part provides protection against water fluctuations and toxic compounds such as ethylene dibromide and $HgCl_2$ [30,32]. Gram-negative bacteria were relatively more abundant in the inner part than in the outer part, whereas most bacteria in the outer part were in sporeform

[31]. Moreover, anaerobic bacteria were present in the inner part of soil aggregates of paddy soil [63]. The detected importance of small pores agreed with TEM observations in which bacteria were most frequently observed in pores of 1-2 μm in diameter, whereas 80-90% of the cells occurred in pores of 0.8-3 μm in diameter [39]. Interestingly, in three soils a consistent ratio of 1:3 between the mean diameter of bacteria or microcolonies and the mean diameter of pores was detected [39]. The pores in which bacteria occurred, had multiple or single entrances and seemed sometimes completely closed [39]. Moreover, bacteria in interiors of micropores and mucigel deposits were found to be protected against chloroform treatment [20]. Other microscopic results showed that the numbers of colonies consisting of $\geq$6 bacterial cells were constant or decreased from the periphery to the centre of aggregates in two different soils assessed [36].

Bacteria can occur as single cells or as microcolonies [20,21]. Large groups of cells were observed on the surfaces of soil particles and aggregates [21,27,62]. The occurrence of microcolonies decreased with depth [6] and varied with soil particle type [49]. *Bacillus subtilis* occurred generally in small colonies (4-5 cells) on organic particles, but on mineral particles only 1-2 cells were grouped together [49].

The presented information clearly shows the existence of microhabitats which are favourable for survival of indigenous bacteria. In contrast to indigenous bacteria, which are thought to be mostly associated with soil particles [16,45,46], introduced bacteria are suggested to be located in relatively open areas, more exposed to soil stress factors [59]. After the introduction of *Bradyrhizobium japonicum*, the number of free bacteria decreased faster than those associated with soil particles [46]. Many authors speculated about the presence of protective niches for introduced bacteria [13,24,33,52,59], but only Vargas and Hattori [58] presented evidence for such a phenomenon. They found that after introduction of protozoa and bacteria into sterilized soil, mainly the bacteria in the outer part of aggregates were predated [58].

1.3 OUTLINE OF THIS THESIS

In this thesis, two soil types were used: a loamy sand and a silt loam. Both were Dutch arable soils, but differed in their particle size distribution, organic matter content, pH, $CaCO_3$, as well as their cation exchange capacity (CEC) [18]. As a result pore diameter distribution and

soil water retention functions were also different (Fig. 1 in Chapter 4). *Rhizobium leguminosarum* biovar *trifolii* strain R62, which is a symbiotic nitrogen-fixing bacterium, was used as a model organism to investigate the effect of the distribution in soil on the population dynamics of bacteria introduced into soil. Specific antisera and reliable antibiotic resistance markers could be obtained with this bacterium. Moreover, it was able to produce substantial amounts of reserve material. This was a prerequisit for the counterpart research project where the influence of such storage materials on the survival of introduced bacteria had to be assessed.

Two methods for the specific enumeration of bacteria after introduction into soil have been compared (Chapter 2): selective plating using an antibiotic-resistant strain and quantitative immunofluorescence. To be able to study the spatial distribution of the organism in soil, different fluorochromes (including FITC-conjugated antiserum) were tested for their ability to stain bacteria in thin soil sections (Chapter 3). In addition, a method which separated freely occurring bacteria from bacteria associated with soil particles or aggregates was developed (Chapter 4).

Emphasis on techniques was followed by studies on the influence of the spatial distribution in soil on the population dynamics of the introduced bacteria. The effect of inoculating soil at different initial moisture contents on the distribution as well as on the population dynamics of rhizobial cells, was first assessed in natural soil (Chapter 4). For a better understanding of the basic processes that control the survival of introduced bacteria in natural soil, the role of competition and predation was studied (Chapter 5). The influence of attachment to soil particle surfaces was studied with a *R. leguminosarum* strain and three mutants which were altered in their cell surface properties (Chapter 6).

The capacity of soils to contain bacterial populations was investigated (Chapter 7 and 8). The occurrence of final population levels of introduced bacteria in sterilized and natural soil was studied in more detail by using different inoculum densities (Chapter 7), whereas the magnitude and importance of the habitable pore space in supporting these population levels was discussed in Chapter 8.

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CHAPTER 2

THE DYNAMICS OF *RHIZOBIUM LEGUMINOSARUM* BIOVAR *TRIFOLII* INTRODUCED INTO SOIL AS DETERMINED BY IMMUNOFLUORESCENCE AND SELECTIVE PLATING TECHNIQUES

ABSTRACT

After the introduction of *Rhizobium leguminosarum* biovar *trifolii* into a loamy sand and a silt loam, high recovery percentages were determined using quantitative immunofluorescence. Soil type, but not inoculum density between 10^4 and 10^8 cells per gram of soil, significantly influenced the recovery percentage of the immunofluorescence technique. Recovery percentages determined using selective plating were independent of either soil type or inoculum density and exceeded those determined by immunofluorescence.

The serological and genetic markers used for detection were stable during 55 days of incubation in phosphate-buffered saline and soil extract solution. After the introduction of *R. leguminosarum* biovar *trifolii* into both sterilized soil types, the population increased to 0.5-1 x 10^9 cells per gram of soil, but a decline was demonstrated in non-sterile loamy sand and silt loam during incubation of 90 days at 15°C. Starvation of rhizobial cells in the phosphate-buffered saline and soil extract solution, as well as incubation in both soil types, resulted in a significant decrease in mean cell size.

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2.1 INTRODUCTION

Studies on the dynamics of microorganisms after their introduction into soil have recently gained more interest. Often inconsistent results were obtained in field trials and survival and establishment of microorganisms introduced in soil ecosystems are poorly understood [15].

So far, two different methods for the specific enumeration of strains introduced in soil have been used: selective plating (SP) using antibiotic-resistant strains, and quantitative immunofluorescence (IF). Both methods have their limitations. In general, plate counts are considered to underestimate the number of autochthonous soil bacteria [28]. Underestimation of introduced bacteria can be caused by aggregation of bacteria and by the presence of cells which have lost the ability to form colonies on solid media. Furthermore, strains marked with antibiotic resistance, either by spontaneous or transposon-induced mutations or by plasmid transfer, may have a weakened ecological competence. Serological techniques do not have these disadvantages, since no genetic modification is needed. Further, all cells are, in principle, stainable and can be counted, independent of their physiological condition, provided that cell walls with the antigens are present. Moreover, direct microscopical observation provides the opportunity to obtain additional information on, for example, cell size. Nevertheless, problems still exist on the use of quantitative IF for the enumeration of soil microorganisms [5,6,8,9,11, 13,20,24-26]. Uncertainties remain about the optimal dispersing and flocculation of different soil types, the stability of bacterial antigens in soil, the sensitivity and efficiency of the technique and the influence of the inoculum density on the recovery percentage.

This paper deals with the population dynamics and the changes in cell sizes of *Rhizobium leguminosarum* biovar *trifolii* by comparing the behaviour of the cells in low nutrient suspensions and in two soil types, as determined by the immunofluorescence and plate counting techniques.

2.2 MATERIALS AND METHODS

Soils. Samples from two Dutch soils, a loamy sand and a silt loam were air-dried to 8 and 20% moisture content, respectively, sieved through a 4 mm sieve and stored at 4°C. When needed, soil was sterilized by γ-irradiation (4 Mrad). To part of the loamy sand portions, 10% (w/w) of Na-saturated bentonite was added. The main characteristics of the two soils

have been described by van Elsas *et al.* [15].

Bacterial strain and antiserum. *R. leguminosarum* biovar *trifolii* R62 was isolated from Dutch arable soil. Using a spontaneous rifampicin (Rp)-resistant mutant, a clone resistant to kanamycin (Km) was produced by transposon Tn5 mutagenesis using the suicide plasmid method of Simon *et al.* [27]. Growth rates of the resistant mutant and wild type in yeast extract mannitol broth (YMB) did not differ substantially [16]. Bacterial suspensions used for inoculations, were grown in YMB [16] for 2 days at 29°C on a rotary shaker, washed twice by centrifugation (6000 x *g*, 15 min), and resuspended in demineralized water.

An antiserum against whole *R. leguminosarum* biovar *trifolii* R62 cells was prepared by four subcutaneous injections of rabbits using bacterial suspensions resuspended in phosphate-buffered saline (PBS). The first injection was without adjuvant, whereas Freund's incomplete adjuvant was used in the following injections. Blood obtained from the marginal ear vein of the rabbits was fractionated according to Allan and Kelman [1] and stored at -20°C. Part of the serum was conjugated with fluorescein-isothiocyanate (FITC) [1]. Antiserum dilutions were made in PBS supplemented with 0.05% sodium azide. The optimal dilution of the conjugated antiserum was 1:100 for staining cells in glass-slide preparations and 1:50 for soil suspensions on filters.

The conjugated antiserum did not show cross-reaction with 80 random bacterial isolates from both soil types at the optimal dilution. Only one *R. leguminosarum* biovar *trifolii* strain was stained by the unconjugated antiserum (titre 1:100), and 13 strains of nine different *Rhizobium* spp., including three other *R. leguminosarum* biovar *trifolii* strains, remained unstained.

Selective plating technique. To determine the number of colony-forming units (cfu), soil portions corresponding to 10 g dry weight were transferred to 250 ml Erlenmeyer flasks containing 95 ml of 0.1% sodium pyrophosphate and 10 g gravel (diameter 2-4 mm), and shaken on a rotary shaker for 10 min at 280 rpm (room temperature). Suspensions were serially diluted in 0.1% sodium pyrophosphate, and 0.1 ml of the appropriate dilution was plated on yeast extract mannitol agar [16] containing 50 mg/l Km, 20 mg/l Rp and 100 mg/l cycloheximide (C). Plates with 20-300 colonies were counted.

Immunofluorescence technique. In order to prepare filters with bacterial cells which could be stained and enumerated, soil suspensions were allowed to flocculate, whereafter appropriate volumes of the supernatant

could be filtered. Different combinations of dispersing solutions (demineralized water, 0.1% partially hydrolyzed gelatin diluted in 0.1 M ammonium phosphate, 0.1% sodium pyrophosphate) and flocculation agents (1 g $CaCl_2.2H_2O$, 0.7 g $Ca(OH)_2/MgCO_3$) [2] were tested for both soil types. Results indicated that for each soil type a different flocculation procedure was optimal. Hence, the silt loam (10 g) was dispersed in 195 ml of 0.1% partially hydrolyzed gelatin diluted in 0.1 M ammonium phosphate [11,20]. After blending twice with a Braun's blender two times for 20 s at maximum speed with a 5 s interval, the suspension was allowed to flocculate for 15 min. This method also appeared to be suitable for the loamy sand amended with 10% bentonite clay. In contrast, the loamy sand (10 g) was dispersed in 195 ml demineralized water and blended as described. 5 ml 20% $CaCl_2.2H_2O$ was added to 95 ml of the suspension. After shaking, the suspension was allowed to flocculate for 30 min. The supernatants of the flocculated suspensions were used for IF counting.

Polycarbonate membrane filters (Nuclepore, 0.4 μm, 25 mm diameter) stained with Irgalan black were used to filter the supernatants [4,18]. After washing with 10-20 ml sterile saline (0.85% NaCl), 0.05-0.1 ml of a gelatin-rhodamine isothiocyanate (RhITC) solution prepared according to Bohlool and Schmidt [3], was diluted in 1 ml saline and was applied on the filter to reduce non-specific staining. The filters were stained with four drops of the optimal antiserum dilution and kept 30 min in the dark and under humid conditions. Excess antiserum was removed by filtration using 10 ml saline. The use of PBS instead of saline for washing the filters resulted often in a higher background fluorescence. The stained cells were enumerated using a Zeiss epifluorescence microscope equipped with incident illumination from a HBO-50 mercury light source and a Zeiss FITC-filterpack (excitation filter BP450-490, beam splitter FT510, barrier filter LP520). Either 50, 30 or 20 microscopic fields were counted, when 0-1, 1-5 or more than 5 cells, respectively, were present per microscopic field. The cells on the filters showed a Poisson distribution ($P<0.05$). This was determined by enumerating the cell numbers per microscopic field separated 0.5 or 1 mm from the next field along perpendicular lines.

Efficiency of the detection techniques. Soil portions corresponding to 10 g dry weight were inoculated using bacterial cultures grown as described. Sterile demineralized water was added to control soil portions. The inoculum density was determined using IF and SP. Soil samples were analysed after 3 h of incubation at 12°C. For both techniques, the

recovery percentage was defined as the difference between the bacterial numbers detected in the inoculated soil and those in the control, divided by the bacterial numbers in the inoculum, determined by the same technique. The influence of the inoculum density on the recovery percentage was assayed by inoculating soil portions with different inoculum densities between 10^4 and 10^8 cells/g dry soil.

In four different experiments, the variation of the recovery percentage was studied in duplicate portions of the loamy sand inoculated with 10^7 cells/g dry soil and incubated 3 h at 12°C. For every soil portion two filters were analysed.

To compare the efficiency of both techniques, *R. leguminosarum* biovar *trifolii* cells were incubated for 90 days in the loamy sand and the silt loam. Plate counts were made of thoroughly shaken suspensions and in flocculated and non-flocculated blended suspensions. In addition, the cells in the flocculated suspension were enumerated by IF.

Population dynamics, stability of markers and cell length during incubation in low-nutrient solutions. Bacterial suspensions were prepared as described and introduced into either 100 ml PBS or 100 ml soil extract solution (SES) (pH 7.2) prepared from the loamy sand according to Parkinson *et al.* [23]. The initial cell concentrations were approximately 10^6 cells/ml. Duplicate flasks were incubated 55 days at 20°C on a rotary shaker (180 rpm). Bacterial numbers were determined by plating on non-selective media and by IF using glass-slide preparations. These were made by spreading 0.01 ml suspension on a 1 cm^2 surface area. After drying and heat fixing, these preparations were stained with antiserum. The stability of the antibiotic resistance was checked by replica plating on the selective media. The stability of the antigenic determinants was determined through the intensity of the staining reaction on the glass-slide preparations. For that purpose all cells detected by transmitted light were controlled on their reaction with the antiserum. The lengths of 20 randomly chosen stained cells were measured using an ocular micrometer in order to determine the cell sizes during the incubation.

Population dynamics and cell length during incubation in soil. Dynamics of rhizobial cells introduced in natural and sterilized soil was determined during incubation for 90 days. Loamy sand or silt loam samples corresponding to 10 g dry weight, were inoculated with approximately 4.5 x 10^7 cells/g dry soil in sterile demineralized water establishing the final moisture contents of the loamy sand and the silt loam of 18% and 42%, respectively, corresponding to pF 2.0 (-10 kPa). Control soils

received sterile demineralized water. The soil portions were incubated at 15°C in a humid chamber. After different incubation periods, bacterial numbers in duplicate soil portions and a control of each soil type were assayed using both IF and SP. In addition, the lengths of 30 IF-stained cells were measured in the non-sterile soil portions and compared with the length of the cells in the inoculum.

2.3 RESULTS

Testing of different combinations of dispersing and flocculation agents. After inoculation of bacterial cells into the loamy sand, frayed cells were detected when $Ca(OH)_2/MgCO_3$ was used as a flocculation agent. No cfu were detected in the suspension, whereas the pH of the suspension exceeded 9.5. Dispersion of the loamy sand in demineralized water followed by flocculation with $CaCl_2$ was considerably better, showing clearly stained bacterial cell walls. The combination of sodium pyrophosphate with $CaCl_2$ improved the flocculation of soil particles. However, the clear supernatant contained only about one-tenth of the number of cells obtained when using demineralized water. Therefore, it was decided to use demineralized water in combination with $CaCl_2$.

The silt loam showed a slow, but complete flocculation when demineralized water and $CaCl_2$ were used, and no cells could be detected in the supernatant. Partially hydrolyzed gelatin in ammonium phosphate resulted in a fast flocculation of the silt loam and considerable amounts (>50%) of cells were recovered in the supernatant. The loamy sand did not flocculate with the gelatin solution when combined with either $CaCl_2$ or $Ca(OH)_2/MgCO_3$.

Sensitivity, variability and efficiency of the immunofluorescence technique. The calculated detection limit of the used IF procedure was 10^3-10^4 cells/g dry soil, depending on the dilution rate and the enumerated filter surface. But the detection limit was influenced by the background population. Despite the specificity of the antiserum, the number of stained cells varied from 0 to 0.3 per microscopic field in control soil portions, corresponding to 0-10^5 cells/g dry soil. The mean background populations in both soil types were approximately 8 x 10^4 cells/g dry soil.

The main sources of variation in the IF technique were determined in four experiments in which the recovery percentage was studied each time in two loamy sand portions using duplicate filters. The greatest variation, 45% of standard error, occurred between the experiments. However,

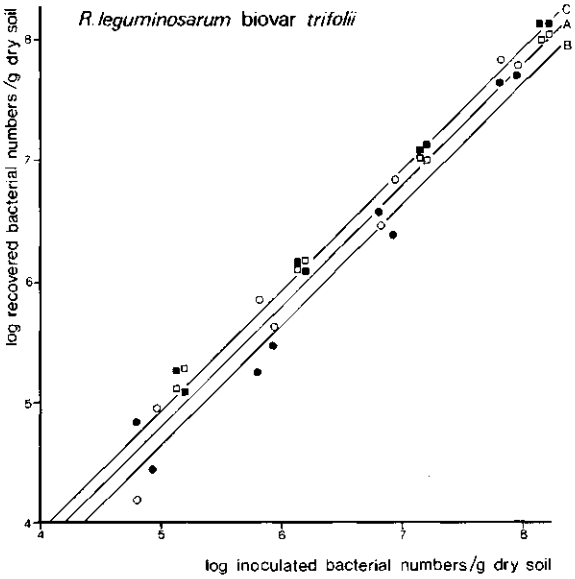

Fig. 1. Recovery of *R. leguminosarum* biovar *trifolii* from loamy sand and silt loam using different inoculum densities. O, IF counts in loamy sand; □, SP counts in loamy sand; ●, IF counts in silt loam; ■, SP counts in silt loam; A, mean recovery % of IF counts in loamy sand; B, mean recovery % of IF counts in silt loam; C, mean recovery % of SP counts in loamy sand and silt loam.

there was a variation of 36% between the duplicate soil portions and 19% between the filters.

Results of studies on the recovery of rhizobial cells from both soils after 3 h of incubation at 12°C with inoculum densities between 10^4 and 10^8 cells/g dry soil are shown in Table 1 and Fig. 1. Regression analysis showed no significant influence of inoculum density on the recovery percentage of both the IF and SP technique ($P>0.05$). However, fluctuations in recovery values seemed to be greater at lower inoculum densities. The mean recovery from the loamy sand determined by IF was significantly higher ($P<0.05$) than that from the silt loam and the loamy sand amended with bentonite. The recovery percentage determined by SP was not significantly influenced by soil type and was 100% ($P>0.05$).

During the IF procedure, around 40% of cfu were lost with flocculation of the soil portions which were incubated for 3 h. Longer incubation periods resulted in significantly increasing losses during flocculation

Table 1. Recovery percentages of *R. leguminosarum* biovar *trifolii* 3 h after the introduction into loamy sand, silt loam and loamy sand with bentonite, determined by immunofluorescence (IF) and selective plating (SP). Results are expressed as the mean, with standard error between parenthesis.

	loamy sand	silt loam	loamy sand + 10% bentonite
recovery % IF	67 (5)	50 (5)	48 (6)
recovery % SP	101 (7)	99 (7)	101 (9)

(P<0.05). Losses of cfu during flocculation were around 80% after 28 and 56 days of incubation. There was no significant influence of soil type on the flocculation (P>0.05). 80% of the cfu in the flocculated suspension were enumerated by IF in 3 h-incubated soil portions. In both soil types longer incubation resulted in significantly increasing numbers of IF-stained cells as compared to cfu in the same flocculated suspension (P<0.05). After 28 and 56 days the number of IF-stained cells was higher than the number of cfu in the same flocculated suspension.

Population dynamics, stability of markers and cell length during incubation in low nutrient solutions. Plate counts of *R. leguminosarum* biovar *trifolii* in PBS decreased markedly with time in contrast to IF counts which remained stable (Fig. 2). In SES, both plate and IF counts increased during the first days and than remained constant. All bacterial cells remained resistant to Km during the experiment. Also, the antigenic determinants were stable during the incubation period.

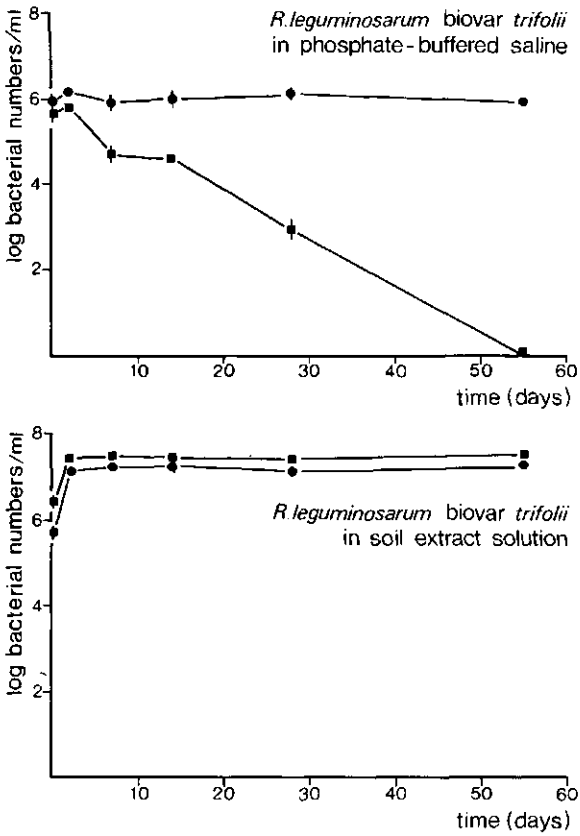

Fig. 2. Dynamics of *R. leguminosarum* biovar *trifolii* in phophate-buffered saline and soil extract solution at 20°C as determined by IF (●) and SP (■). Standard deviations are indicated by bars or are within the dimension of the symbol.

Mean cell lengths (Table 2), measured in IF preparations, decreased significantly during incubation in PBS and SES (P<0.05). This decrease could be fitted with non-linear regression by an exponential function and the final mean cell length in this model was 1.99 μm for PBS and 1.78 μm for SES. Cell diameter did not change visibly.

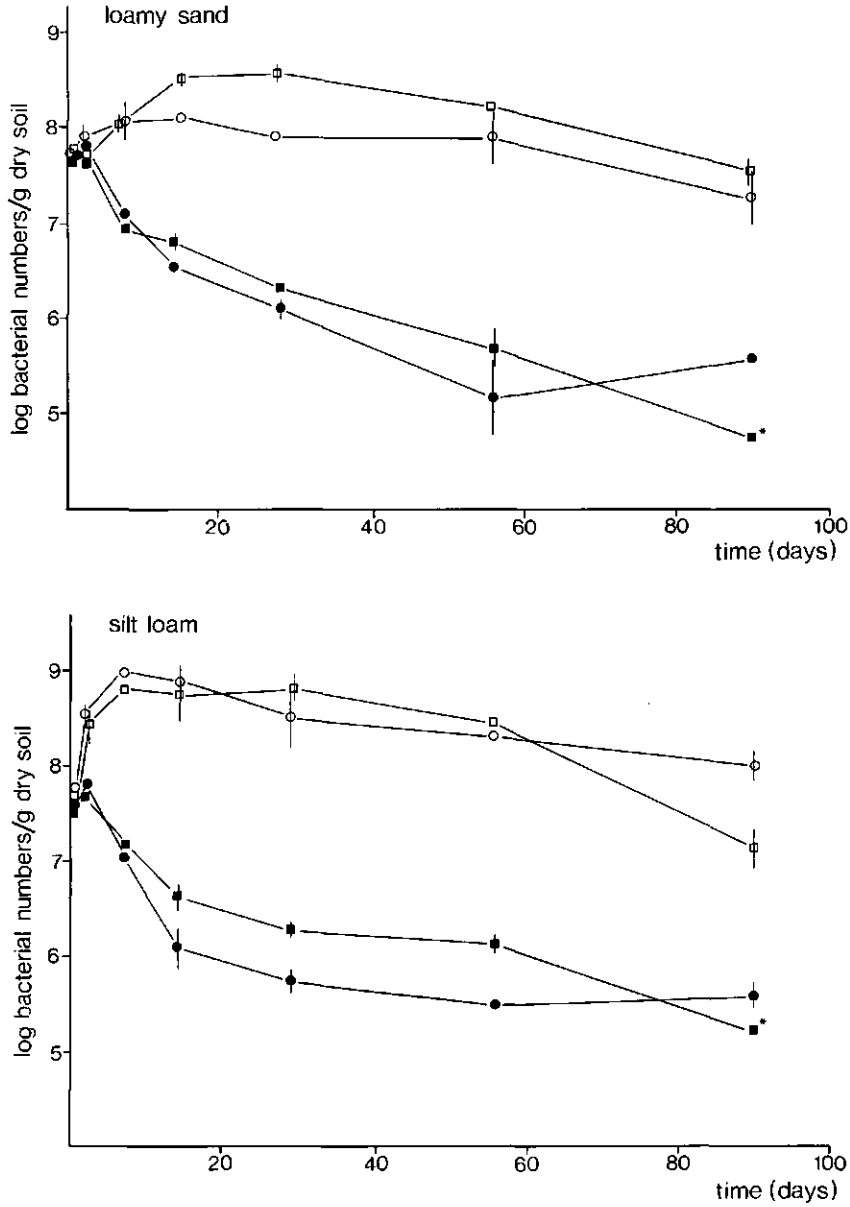

Fig. 3. Dynamics of *R. leguminosarum* biovar *trifolii* after introduction in loamy sand and silt loam (15°C, moisture contents 18 and 42%, resp.) as determined by IF (○,●) and SP (□,■). Enumeration were corrected for the recovery percentages given in Table 1. Open and closed symbols represent sterile and non-sterile soil, respectively. Standard deviations are indicated by bars or are within the dimension of the symbol. *, no duplicate present.

Population dynamics and cell length during incubation in soil. After the introduction of *R. leguminosarum* biovar *trifolii* in non-sterile loamy sand and silt loam, cell counts were increased little after 2 days of incubation, but thereafter they decreased steadily. Under sterile conditions, counts increased up to 0.5-1 x 10^9 cells per gram of soil and stayed at a high level (Fig. 3). Comparing both techniques, the IF counts corrected for the recovery percentage were similar to the plate counts during the first 7 days of incubation, thereafter the IF counts were mostly lower than the plate counts except for the last sampling date.

After the introduction in the non-sterile loamy sand and silt loam, the mean cell length of the rhizobial cells (Table 2) decreased significantly ($P<0.05$). Non-linear regression analysis showed that the ultimate mean cell length was 1.72 μm for the loamy sand and 1.62 μm for the silt loam and that the decrease in cell length was faster in the silt loam than in the loamy sand.

Table 2. Length of IF-stained *R. leguminosarum* biovar *trifolii* cells starved in phosphate-buffered saline (PBS) and soil extract solution (SES) at 20°C, and incubated in loamy sand and silt loam at 15°C.

incu-bation period	PBS *	SES *	incu-bation period	loamy sand **	silt loam **
3 h	2.66 (0.62)	2.61 (0.55)	0 h***	2.05 (0.51)	2.05 (0.51)
1 d	2.55 (0.52)	2.64 (0.51)	1 h	2.09 (0.52)	2.15 (0.54)
2 d	2.19 (0.52)	1.94 (0.38)	3 h	2.05 (0.44)	2.09 (0.37)
7 d	2.22 (0.70)	1.55 (0.36)	6 h	1.92 (0.57)	1.98 (0.55)
14 d	1.89 (0.46)	1.67 (0.40)	12 h	1.81 (0.47)	1.87 (0.36)
28 d	1.94 (0.42)	1.89 (0.46)	1 d	1.91 (0.35)	1.76 (0.44)
55 d	2.03 (0.46)	2.03 (0.58)	2 d	1.85 (0.44)	1.61 (0.42)
			8 d	2.00 (0.68)	1.76 (0.41)
			14 d	1.87 (0.42)	1.78 (0.42)
			28 d	1.79 (0.51)	1.50 (0.42)
			56 d	1.66 (0.50)	1.61 (0.37)
				1.65 (0.42)	1.63 (0.41)
			90 d	1.85 (0.53)	1.61 (0.37)

* mean cell length (μm) and standard deviation of 20 randomly chosen cells. ** mean cell length (μm) and standard deviation of 30 randomly chosen cells. *** just before inoculation.

2.4 DISCUSSION

A prerequisite for succesful use of the IF and SP technique is stability of the serological and genetic markers. In this work we showed stability of the serological and genetic markers of *R. leguminosarum* biovar *trifolii* upon incubation in low nutrient solutions during 55 days. The stability of the serological markers of a *Lotononis* strain during 12 years [14] and more than 95% stability of serological and genetic markers of *R. leguminosarum* biovar *trifolii* during 3 years [7] is described. Nevertheless, there is still some doubt as to long-term stability [29].

The IF technique developed, permitted high recoveries of the introduced *R. leguminosarum* biovar *trifolii* cells from the loamy sand and the silt loam. Variations in recovery, depending on soil type, dispersing and flocculation agent, have been reported in the literature [2,11,20]. The use of different inoculum densities (10^4-10^8 cells/g dry soil) did not produce significant differences in recovery percentage. Wollum and Miller [31] also did not detect a significant effect of inoculum density (10^6-10^9 cells/g soil) on the recovery of rhizobial cells. Crozat *et al.* [12] found a higher recovery percentage of *Bradyrhizobium japonicum* at 10^3 cells/g soil as compared to higher inoculum densities. However, at 10^4 cells/g soil or more, recovery percentages were not related to the inoculum densities.

An advantage of the IF technique for bacterial counting is that harsher and more effective disruption procedures can be used than when using viable counting methods, since microbial cell integrity and not viability is required [24]. In our hands, similar plate counts of introduced *R. leguminosarum* biovar *trifolii* were obtained by the extraction methods used for the IF and SP techniques. A draw-back for general applicability of the IF technique is that the optimal combination of dispersing and flocculation agent is dependent on the soil type, suggesting that this combination should be adjusted for each new soil studied. Since flocculation may cause, as shown, a complete loss of bacteria, a compromise should be sought between clearance of supernatants and recovery of bacteria. Bezdicek and Donaldson [2] also found that the flocculation agent which produced highest recovery did not produce the clearest supernatants.

The recovery percentages determined with IF were higher than those measured by Crozat *et al.* [12]. Nevertheless, not all rhizobial cells introduced into soil were recovered with IF. The observed lower counts

with IF than with SP in soil-less cell suspensions must be due to losses of cells during preparation and counting of the IF filters. The greater discrepancy between IF and SP counts found in soils may be due to additional losses in the desorption and flocculation procedure of the IF technique, or to masking of cells by soil particles. When the successive procedures of the IF technique were compared at prolonged incubation periods, increasing numbers of cfu were lost from the supernatant during flocculation. These observations might be explained by assuming that during the incubation period more cells were increasingly tightly attached to soil particles. Thus, for more realistic counts the recovery percentage should actually be adjusted during the incubation period.

A striking phenomenon observed was the increasing number of IF-stained cells compared to the number of cfu in the same flocculated supernatants after prolonged incubation periods. Similarly, Bohlool and Schmidt reported an increasing IF/plate count ratio upon prolonged incubation in sterile soil [20,26]. The occurrence of higher IF counts than plate counts in the same supernatant can be explained by the staining of IF-detectable non-colony-forming units. When *R. leguminosarum* biovar *trifolii* was starved in PBS, IF counts were found to be constant as compared to a decrease in plate counts. Similar results in aquatic systems were reported by Kurath and Morita [22] and Colwell *et al.* [10] who in addition, demonstrated that part of the cells which had lost the ability to reproduce on plates could still grow in the suspension after addition of nutrients. That the decrease in plate counts was not observed in soil extract solution was perhaps caused by persistence of the viability of cells due to the presence of organic substrates.

Two important questions arise when IF is used for enumeration: how long do dead cells remain detectable by IF and do dead cells accumulate in soil? Bohlool and Schmidt [4] and Cleyet-Marel and Crozat [9] showed that the majority of heat-killed rhizobial cells introduced into non-sterile soils disintegrated within 2 weeks. In addition, heat-killed *Bacillus thuringiensis* cells were not detectable by IF after a short incubation in soil, and viable cells disappeared even faster [30]. Based on such data, together with the common-sense realization that dead cells do not pile up in most natural envirnoments, Bohlool and Schmidt [6] suggested that inclusion of dead cells in counts is not likely to be a major error in IF enumerations.

A significant decrease of the mean length of *R. leguminosarum* biovar *trifolii* cells upon starvation in low nutrient solutions and in both soil

types was observed. The dynamics of cell sizes in relation to starvation in natural environments has mostly been studied in aquatic environments. Upon starvation, *Pseudomonas* cells rapidly turned into small spheroids via fragmentation, without losing their viability, followed by a continuous size reduction accompanied by a slow loss of viability [21]. Jensen and Woolfolk [19] studied cell sizes of *Pseudomonas putida* in a vigorously shaken culture. After 336 days, most cells were relatively small, non-viable and almost spherically shaped. Crozat *et al.* [13] also reported a decrease in the length of rhizobial cells after introduction into soil. On the other hand, Hill and Gray [17] showed that *B. subtilis* cells present on organic matter in soil were longer than those in culture which may, however, be due to the inhibition of septum formation by compounds of the organic substrate (Gray, T.R.G., personal communication).

IF was reliable for enumerating bacteria introduced into soil, although at a lower recovery efficiency than SP. The most important advantages of the IF technique are the possibility to enumerate non-colony-forming units which are not necessarily dead, and the possibility to determine cell sizes or other qualitative values. In addition, IF may be used as an alternative for SP when antibiotic-resistant strains are not available. In contrast, the advantages of SP are that the method is easy to apply and does not depend on flocculation processes which were shown to change within an experiment. Only cells able to divide are counted and, important for survival studies, where low numbers of cells have to be enumerated, the detection limit of SP is lower than that of the IF technique.

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CHAPTER 3

BACTERIA IN THIN SOIL SECTIONS STAINED WITH THE FLUORESCENT BRIGHTENER CALCOFLUOR WHITE M2R

ABSTRACT

Bacteria in thin soil sections were stained with the fluorescent brightener calcofluor white M2R. The fluorochrome was applied to the soil sample after fixation and before embedding in a polyester resin. Thin soil sections were prepared from the hardened blocks. Acridine orange, applied to the polished thin soil sections, was useful to counterstain the soil matrix. Best results were obtained with rhizobial cells grown in a culture medium and introduced into the test soils. After their introduction into the soil, most rhizobial cells were detected in combination with clay particles and organic matter surrounding the quartz particles. Surfaces of larger pores, especially in 'Beekeerd' loamy sand, were covered with the introduced cells. Indigenous soil bacteria were also stained, but most at a lower intensity. Comparison to observations on stained soil smears suggested that some of smaller coccoids, starving cells, and bacterial spores remained unstained.

Calcofluor white proved to be an excellent fluorochrome to study fungal hyphae and plant roots in thin soil sections. However, these tissues stained to such an extent that bacterial cells on these tissues were more difficult to detect.

J. Postma and H.-J. Altemüller. Soil Biology and Biochemistry (in press)

3.1 INTRODUCTION

One of the major problems in soil microbiology is the examination of the distribution of microorganisms in natural soil and their relation to the mineral and organic fraction of soil. The technique of preparing sections from soil embedded in a resin provides the opportunity to study relatively undisturbed soil samples. Alexander and Jackson [1,2] were the first to apply this technique to study soil microorganisms in their natural environment. With this technique larger microorganisms such as fungi have been observed [2,9,12]. There have been relatively few studies on bacteria stained with diachromes (cotton blue and soluble blue) [2,14]. To observe bacteria on opaque soil particles, fluorochromes would appear to be more suitable as incident illumination can be used.

The fluorochrome acridine orange was used to stain thin soil sections to observe roots in undisturbed soil contact [4,5,7]. Altemüller and Vorbach [6] were able to improve the fluorescence of fungal mycelia slightly with the use of trypaflavine (acriflavine). In this kind of section bacteria could only be detected under favourable conditions, for example colonies in voids.

Tippkötter *et al.* [19] also presented staining techniques for resin-embedded soil samples. The fluorochromes Mg-ANS, auramine O, calcofluor white M2R, lucifer yellow CH and uvitex OB were recommended to stain biological features [18], but the details provided were not sufficient for subsequent replication.

A reliable technique for the staining of bacteria in thin soil sections is, therefore, not available. With respect to our aim to examine the spatial distribution of a certain bacterial strain in different soil types, it was inevitable that new dyes would have to be tested. In view of their favourable properties (high contrast, low concentration and the possibility to use incident illumination) only fluorochromes were considered.

3.2 MATERIALS AND METHODS

Soils and bacterial inoculum. Three soil types were used: a 'Beekeerd' loamy sand common in the eastern part of the Netherlands, a Dutch polder silt loam and a German Parabraunerde silt loam derived from löess near Braunschweig. The soil samples were air dried and sieved <2 mm. The Parabraunerde was fractionated into aggregates of 0.5 to 1 mm size.

A strain of *Rhizobium leguminosarum* biovar *trifolii* (R62) [13,16] was used as inoculum. The rhizobial cells were grown in yeast extract mannitol broth [16].

Preparation and staining of soil smears. Small volumes (0.1 ml) of soil suspensions with rhizobial cells (10^8-10^9 cells/g soil) were spread on glass slides, dried and heat fixed. Soil smears were stained with different fluorochromes using the following procedures. In order to test the possibility to stain a specific strain, preparations were stained for 30 min under dark and humid conditions with conjugated antiserum in the concentrations 1:10, 1:25, 1:50 and 1:100. The antiserum was prepared against *R. leguminosarum* biovar *trifolii* R62 and conjugated with fluorescein iso-thiocyanate (FITC) [16]. Non-specific fluorochromes were also tested. Acridine orange (AO) was applied for 2 min and tested at the of concentrations 1.0, 0.2, 0.1, 0.07 or 0.05 g/l diluted in demineralized water. Calcofluor white M2R (Polysciences, Inc., Warrington, U.S.A.) (CFW), now named cellufluor, was applied for 5 min using a concentration of 1 g/l. A mixture of 1.74 g/l europium(III) thenoyltrifluoroacetonate (designated $Eu(TTA)_3$) and 24 mg/l CFW in 50% (v/v) ethanol [8] was also used. Soil smears were stained for 1 h. The excess of the fluorochromes was removed by rinsing with their dilution solutions. Some soil smears were also rinsed with acetone to test the influence of this solution on the staining effect.

The soil smears were mounted with a cover slip using either water or glycerol for direct control. Others were mounted with catalized Vestopal 160, a polyester resin [3], to test the durability of the dyes under conditions of resin embedding. The samples hardened within 1 day.

Preparation and staining of bulk soil portions. Glass cores (30 mm diam.), closed at the bottom with a rough cotton cloth, were filled with the three soil types and inoculated with 10^8-10^9 rhizobial cells/g soil. The bulk density was approximately 1.0 g/cm^3. The soil portions were fixed for 6 h with 2.5% (v/v) glutaraldehyde [19] and then washed twice with demineralized water. The soil portions were treated with a solution of CFW (1 g/l) in demineralized water for 12 h followed by new washing procedures. After this, the soil portions were dehydrated 4 times with 100% acetone, with each stage lasting for about 12 h.

Preparation and staining of rhizosphere soil portions. Planar glass vessels of 9 x 12 cm size and 0.8 cm wide were filled with Parabraunerde aggregates following the technique of Altemüller and Vorbach [7]. Seeds of *Lepidium sativum* were applied and a root system developed throughout

the soil under moist conditions. After 1 week the units were fixed and stained with CFW as described above. The dehydration was started with diluted acetone (50% v/v) and the strength was increased to 100% in 4 steps until the samples were free of water.

Preparation and counterstaining of thin soil sections. Both cores and planar vessels were impregnated with Vestopal 160 by displacement of the acetone with a slow rising level of undiluted resin [7]. Cores and planar vessels were catalyzed for 7 days and 3 weeks hardening, respectively. The concentration of catalyst used ranged from 0.6 to 1.8 ml/l, whereas 0.3 to 0.9 ml/l accelerator was used. These concentrations were tested for each charge of resin. After hardening, sections of 15 μm thickness were prepared [3,7].

To counterstain the soil matrix, AO (1.0 and 0.2 g/l 10% HCl) was applied to the thin section surface for 2 min and the sections were carefully rinsed with 10% HCL and demineralised water.

Microscopy and photography. The soil smears and thin soil sections were examined with a Leitz epifluorescence microscope equipped with incident illumination from a HBO-50 mercury light source and appropriate filter combinations for the different fluorochromes. Figures 1, 2 and 3, which originally were colour slides, are transformed to black and white photographs for this thesis.

3.3 RESULTS

Effects of the thin soil section procedure on the fluorochromes. The different steps which are important for sucessful staining of bacteria in thin soil sections were examined. Fluorochromes were tested on soil smears to see if they could withstand mounting in polyester resin and rinsing with acetone. Stained soil smears mounted in water or glycerol were used as a reference. Those fluorochromes which provided good results were tested further in the thin soil section technique.

When water or glycerol were used as mounting fluid, soil smears stained with FITC-conjugated antiserum resulted in clear and bright stained rhizobial cells. With polyester resin, however, the intensity of the stain was diminished and bacterial cells adhering to soil particles could no longer be detected.

The colour and intensity of bacteria treated with AO in water was found to depend on the fluorochrome concentration. Bacteria stained from an intensive red (1.0 g/l) to a weak green (0.05 g/l) and the soil colour

varied from red to orange red. The occurrence of weakly-green coloured bacteria seen on red soil particles was also reported by Rouschal and Strugger [17]. Microcolonies and bacteria varying considerably in size and shape were detected in the soil smears. Much of the staining effect disappeared after mounting in polyester resin, as the resin adsorbed some of the stain and fluoresced green. Under such circumstances the bacterial cells were poorly visible.

Bacterial cells were stained bright blue with CFW (1 g/l), whereas the soil did not adsorb the fluorochrome. The polyester resin had no negative influence on the staining intensity. The intensity of CFW stained bacterial cells did not diminish during the 9 months they were stored at room temperature.

$Eu(TTA)_3$ in combination with CFW resulted in highly intense red staining of the bacterial cells, whether mounted in water or resin. The red colour remained intensive in resin embedded preparations during the 6 months of storage. The intense red colour disappeared, however, when the soil smears were rinsed with acetone. Since acetone was used to dehydrate soil portions before embedding with Vestopal, $Eu(TTA)_3$ with CFW was not considered to be of use in the thin sectioning technique.

As only CFW was found to withstand the described procedures, CFW stained soil portions were embedded with resin for thin sectioning. Large numbers of bacteria could be detected in the thin soil sections after the soil portions were stained with CFW (Figs 1, 2, 3 and 4). Counterstaining the thin section surfaces with AO (0.2 g/l 10% HCl) resulted in a higher contrast between soil matrix and bacteria (compare Figs 1 and 2).
Bacteria in relation to the soil matrix were, therefore, studied in thin soil sections stained with CFW, mostly in combination with AO. Details about the fluorescence properties of CFW can be found in Table 1.

Detection of bacteria in undisturbed soil systems. Large numbers of uniform bacterial cells of approx. 0.7 x 2.0 μm were found together with the clay particles and organic matter surrounding the quartz particles in the three test soils. The surfaces of larger pores (mostly in the 'Beekeerd' loamy sand) were also covered with these cells (Fig. 3). The great difference in density of the cells over short distances was striking (Figs 2 and 3). The bacterial numbers also differed between different locations in the thin soil sections. Assuming that bacteria in thin soil sections could be detected up to a maximum of 3 μm depth, the number of stained bacteria in Figure 3 (approx. 100) would correspond to 2×10^9 cells/g soil at the given bulk density and magnification. In

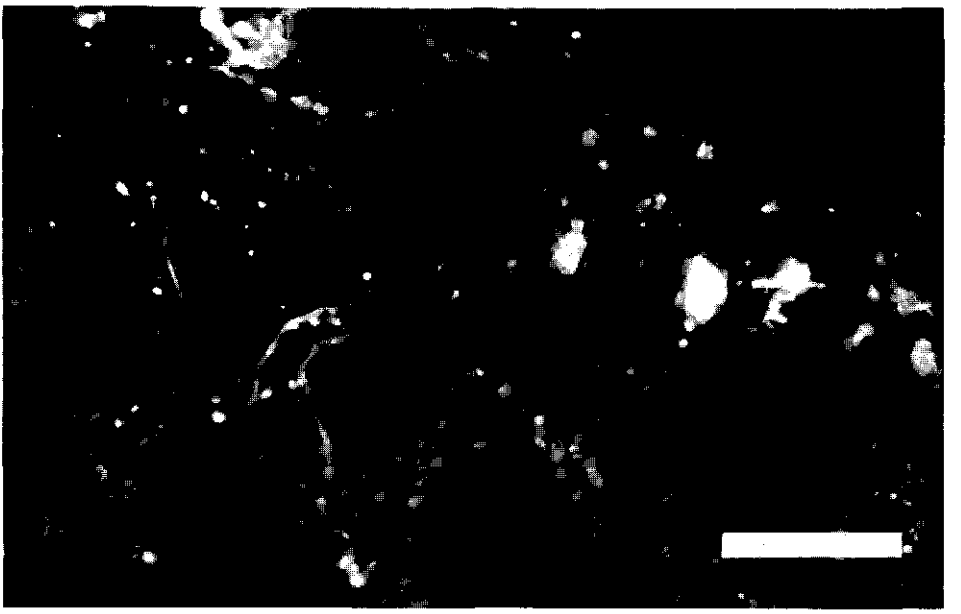

Fig. 1. Bacterial cells in Parabraunerde stained with CFW without counterstaining (UV excitation, bar=30 μm).

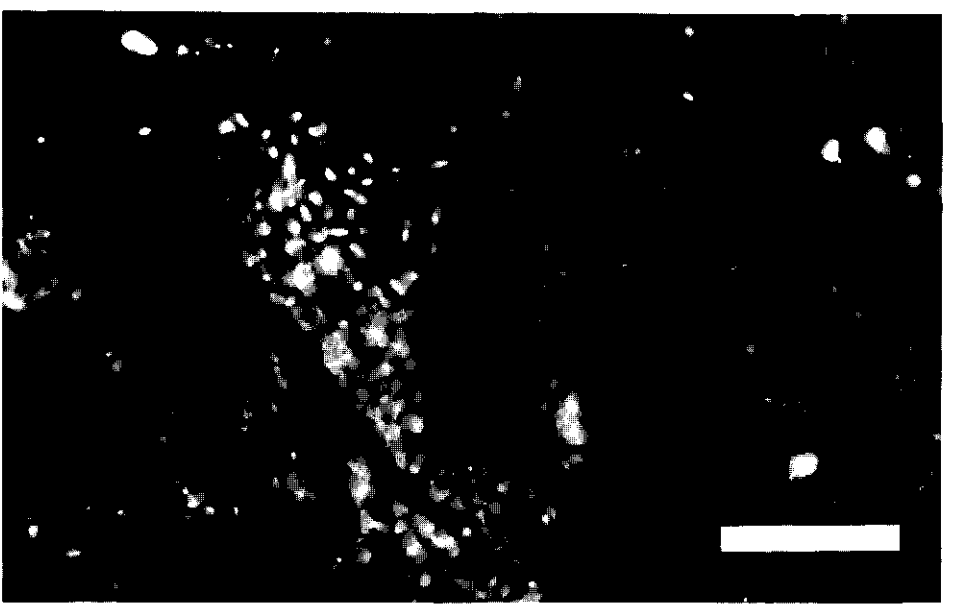

Fig. 2. Bacterial cells in Parabraunerde stained with CFW and counterstained with AO on the thin section surface (UV excitation, bar=30 μm).

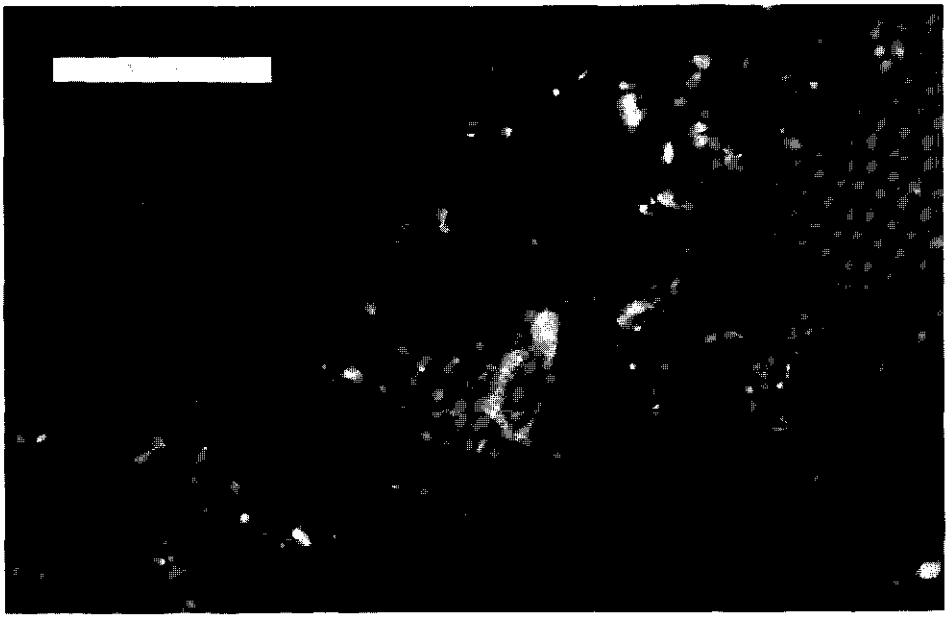

Fig. 3. Bacterial cells in 'Beekeerd' loamy sand stained with CFW and counterstained with AO on the thin section surface (UV excitation, bar=30 μm).

order to obtain an impression of the average cell density in the soil it is necessary to enumerate a large number of microscopic fields, so that microscopic fields with low numbers of bacteria will also be taken into account.

Indigenous bacteria were also stained, but at a much lower intensity. For example, in Figure 1 a large number of smaller coccoid cells can be seen in the smaller pores or enclosed by clay particles and organic matter.

The detection of bacteria on growing roots with CFW was more complicated. The brightness of the bacteria was generally found to be less than that of the root tissues (Table 1) and in most cases it was impossible to detect the bacterial cells on root surfaces.

An example of the excellent staining of root tissue with CFW is shown in Figure 5. Cell walls of *Lepidium sativum* roots in the planar vessel-system stained strong violet blue (UV excitation) or light blue (violet excitation) (Table 1). The penetration of root hairs in the surrounding soil and other features of root - soil contact were clearly visible. Cells filled with cytoplasm were well marked by the whitish fluorescence of the cytoplasm against the light blue of the cell wall (violet excita-

Fig. 4. Bacterial cells in Parabraunerde stained with CFW and counter-stained with AO on the thin section surface (UV excitation, bar=30 μm).

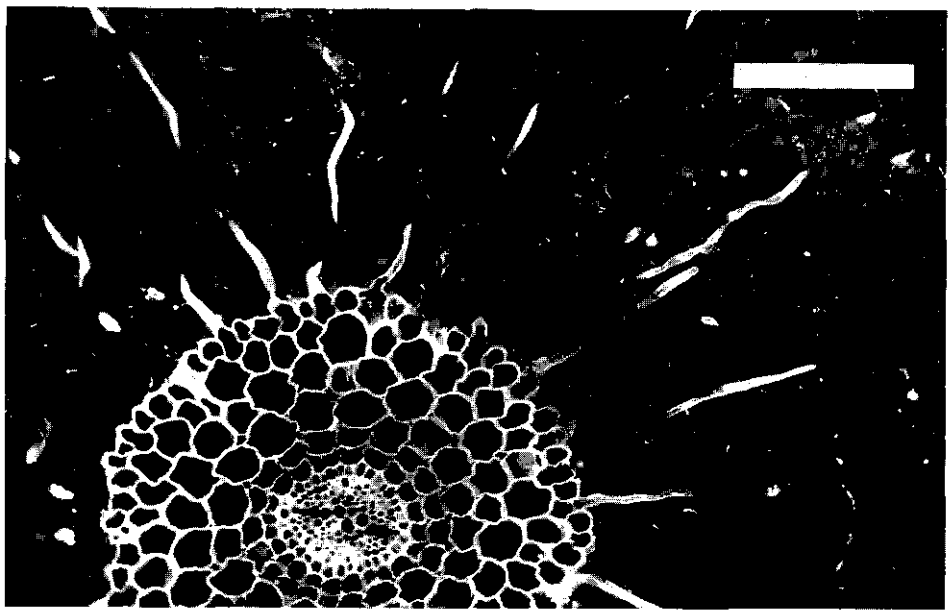

Fig. 5. Diagonal section of a *Lepidium sativum* root in the planar vessel system with Parabraunerde stained with CFW (Violet excitation, bar=200 μm, roots are light blue).

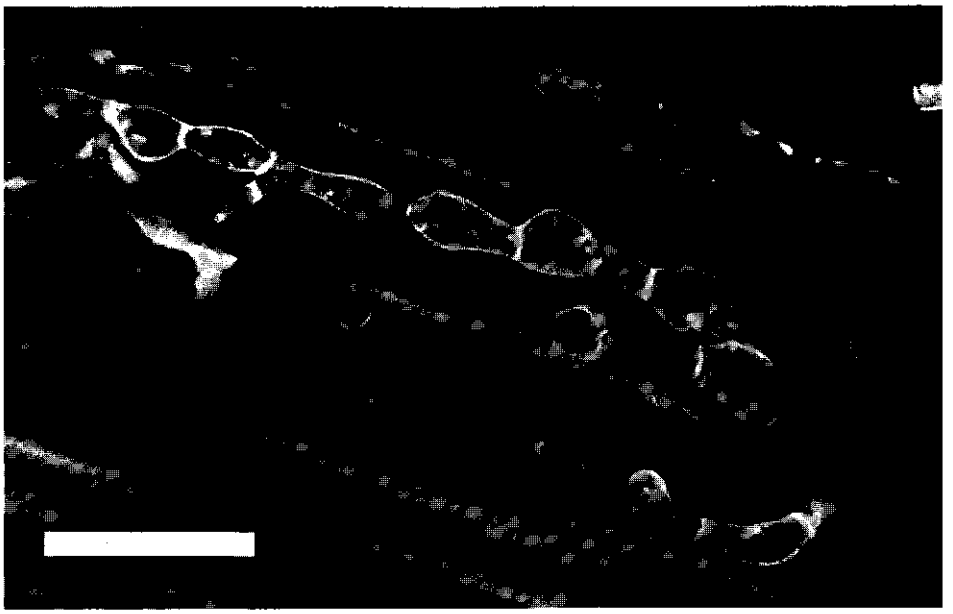

Fig. 6. Fungal hyphae in residual plant tissue in the planar vessel system with Parabraunerde stained with CFW (Violet excitation, bar=30 µm, fungal hyphae are bright blue and plant tissue is yellowish).

tion). Young hairs, root tips, early stages of side root formation and cap cells were therefore especially noticeable. In older cells a thin layer of cytoplasm remained visible for certain time.

An interesting observation was that fungal hyphae also stained well. Cell walls were light blue and only younger parts exhibit a whitish fluorescence of the internal plasma fillings (violet excitation) (Table 1). Since fungal hyphae appeared to be in some way similar to root hairs, studies of fungal mycelium around roots are restricted to such cases where the morphology is clearly visible in order to prevent any misinterpretation. On the other hand, the CFW-stained fungal tissues were easily recognizable in plant residues (Fig. 6) where the cellulose components had so far degenerated that the general fluorescence pattern was ruled by yellowish shades with occasional blue spots or fibers, representing remnants of cellulose. In such an environment the light bluish fungal hyphae formed were readily detected.

Table 1. Fluorescence properties of organic components and clay material in thin soil sections after treating the soil samples with calcofluor white M2R *).

	UV		Violet		Blue	
exitation filter	340-380		355-425		390-490	
beam splitter	400		455		510	
barrier filter	430		460		515	
Bacteria		**)				
-Rhizobium (inoculated)	light blue	2	light blue	1	light green	0
-indigenous forms	integrades to white and gray	0	integrades to white and yellowish	0	integrades to yellowish	0
Fungal tissue						
-fresh hyphal walls	violet blue	3	light blue	2	light green	1
-altered hyphal walls	whitish to yellowish	1	to light yellow	1	to yellow	0
-cytoplasma	bluish white	2	whitish	1	yellowish	0
Fresh root tissues						
-cell walls	violet blue	3	light blue	2	light green	2
-cytoplasma	bluish white	3	whitish	2	yellowish	1
Residual plant tissues						
-cell walls	yellowish to brownish	2 0	yellowish to brown	2 0	yellowish to dark yellow	2 1
Clay material	brownish	0	yellow-brown	1	yellowish	2
Clay material counterstained with acridine orange	reddish	1	red-orange	2	orange	3

*) Observation lens: Leitz NPL Fluotar 40/1.30 oil
**) Relative brightness: 0=dim, 1=moderate, 2=bright, 3=intensive

3.4 DISCUSSION

The examination of the different fluorochromes resulted in a useful staining procedure for bacteria in thin soil sections. We obtained the best results with a CFW-treatment (1 g/l) of the soil portions and counterstaining the thin section surfaces with AO (0.2 g/l 10% HCl). Other fluorochromes were unsuitable as they became ineffective at different stages of the procedure.

An attempt to stain specific bacteria in thin soil sections was unsuccessful, since FITC-conjugated antiserum lost its brightness after the resin Vestopal 160 was applied as mounting fluid. AO had to be used in low concentrations to obtain a good contrast between the soil matrix and bacterial cells. After mounting in polyester resin, however, bacterial

cells which had fluoresced green became scarcely visible, since the resin adsorbed some stain and also fluoresced green. For the soil smears $Eu(TTA)_3$ in combination with CFW proved to be excellent, even after mounting with the resin. However, acetone which is used to dehydrate the soil portions before embedding with Vestopal had a negative influence on the staining effect. Drying the soil with ethanol can not be used as an alternative, since 100% ethanol is detrimental to the staining effect [8].

Using CFW large numbers of uniform, rod-shaped bacterial cells were stained. They had the same length as freshly grown rhizobial cells (approx. 2.0 μm) [16]. Moreover, they were found along the surfaces of larger pores, whereas indigenous bacterial cells are mostly found in small pores [15]. These observations suggested that these stained cells were the introduced rhizobial cells. The indigenous bacteria were less intensively stained with CFW and were found in smaller pores. After the observations of AO-stained soil smears, where many types of microcolonies and bacteria of varying sizes and shapes were seen, we assume that not all indigenous bacteria were stained in the thin soil sections.

CFW, which has a high affinity for cellulose fibers, is active at a wide pH range (5.0 to 8.5) [11]. Since CFW is essentially non-toxic [11], it may be possible to stain bacteria before they are introduced into soil. CFW is effectively adsorbed by growing cultures of bacteria, yeasts and fungi, with active growth centers showing the greatest fluorescence [10,11]. This may explain why rhizobial cells just introduced into soil are better stained than indigenous soil bacteria, as the rhizobial cells were taken from a rich medium, whereas most indigenous bacteria are usually in a resting or starvation stage in soil which is a relatively poor medium.

Bacterial cells, but also fungal hyphae and plant roots, were clearly visible in relation to the soil matrix. This facilitates the examination of the distribution of microorganisms in natural soil. Bacteria associated with growing roots, however, were difficult to detect, since plant roots were very intensively stained. The problems encountered, as well as the staining improvements made during this study, are therefore indicative of the necessity to develop further staining procedures with fluorochromes in order to study bacteria in the rhizosphere.

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CHAPTER 4

INFLUENCE OF DIFFERENT INITIAL SOIL MOISTURE CONTENTS ON THE DISTRIBUTION AND POPULATION DYNAMICS OF INTRODUCED *RHIZOBIUM LEGUMINOSARUM* BIOVAR *TRIFOLII*

ABSTRACT

Data on bacterial distribution in soil were obtained with a method of washing and thoroughly shaking of the soil. Bacterial cells attached to or enclosed in different size groups of soil particles or aggregates were separated and enumerated on plates containing selective media. Soil portions of a loamy sand and a silt loam with different initial moisture contents were inoculated with *Rhizobium leguminosarum* biovar *trifolii*. Results of this experiment indicated that the initial moisture content influenced the distribution of the inoculated rhizobial cells. Differences in distribution were still found after prolonged incubation periods, suggesting a lack of transport and migration of the rhizobial cells. It was shown that rhizobial cells survived better in soils with a lower, than in soils with a higher initial moisture content. Rhizobial cells attached to or enclosed in soil particles or aggregates larger than approx. 50 μm had a more favourable microhabitat than unattached cells or cells attached to smaller particles.

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4.1 INTRODUCTION

Soil is a heterogeneous, discontinuous and structured environment. It is composed of a diversity of microhabitats, and because of their relatively small size and their isolation from one another, only small environmental changes are expected to be necessary to alter their microbiological composition [16]. A further assumption is that survival and activity of the microorganisms are dependent on their distribution among microhabitats. An uneven distribution of bacteria among different types of soil particles was observed by Filip and Kanazawa [4]. The numbers of bacteria decreased from organic matter to silt and clay particles and to sand particles, respectively. The distribution of bacteria among aggregates and pores of different sizes is also expected to influence their survival. Hattori and Hattori [7] suggested that capillary pores (up to 6.0 μm) are the most favourable microhabitats for bacteria. The outer part of the aggregates, containing wider pores and the surface of aggregates, is more directly exposed to influences of environmental conditions than the inner part of the aggregates with capillary pores, and the most pronounced influence of moisture, nutrients (glycine) and toxins ($HgCl_2$) was detected in the outer part of aggregates [5,6,13]. Moreover, bacteria in smaller pores may be protected from predation by, for example, protozoa [1,18]. Kilbertus [9] determined, with electron microscopy, that the smallest pores which containend bacterial cells were 0.8 μm and that most bacteria were located in pores of 1-2 μm.

We have investigated the influence of bacterial distribution among microhabitats on the dynamics of introduced bacteria in soil. A method was developed to distinguish between bacteria attached to or closed in by soil particles or aggregates of different sizes, which gives information on bacterial microdistribution in soil. *Rhizobium leguminosarum* biovar *trifolii*, used as a model organism in our experiments, was inoculated into loamy sand and silt loam with different moisture contents. On the basis of different initial moisture contents, soil portions with water-filled pores of different diameter were prepared, in order to influence the distribution of the introduced bacterial cells.

4.2 MATERIALS AND METHODS

Soils. Samples from two Dutch arable soils, a loamy sand and a silt loam, were air-dried to 8 and 20% moisture content, respectively, sieved

<2 mm and stored at 2°C. Prior to use, the soils were air-dried to moisture contents of 1.3 and 4.0%, respectively. The relationships between the soil water potentials and the moisture content of repacked samples of both soils, determinded according to Klute [10], are given in Figure 1. Particle-size distribution and other characteristics of both soils were described by van Elsas *et al.* [3].

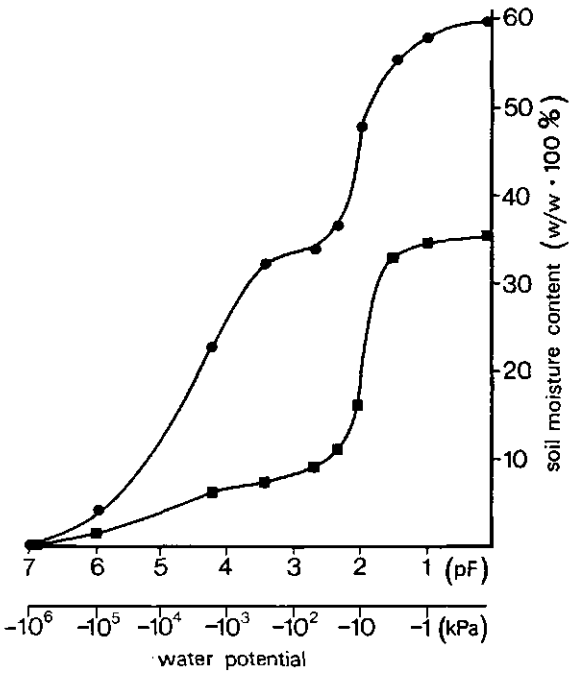

Fig. 1. Relationship between soil water potential and moisture content of repacked samples of the loamy sand (■) and the silt loam (●).

Culturing and enumerating *R. leguminosarum* biovar *trifolii*. *R. leguminosarum* biovar *trifolii* R62::Tn5 with resistance to kanamycin (Km) and rifampicin (Rp) [8,15] was used. The strain was cultured in yeast extract mannitol broth [8] supplemented with 25 mg/l Km. After 2 days at 28°C on a reciprocal shaker, the cells were washed by centrifugation (7000 x *g*, 15 min) and resuspended in demineralized water prior to inoculation.

The number of colony-forming units (cfu) in suspensions was determined on plates containing yeast extract mannitol agar supplemented with 50 mg/l Km, 20 mg/l Rp and 100 mg/l cycloheximide. These concentrations of antibiotics were sufficient to enumerate rhizobial numbers up to 10^4 cfu /g soil, without disturbance by other soil microorganisms. After dilution of the suspension in 0.1% sodium pyrophosphate, 0.1 ml was spread on duplicate plates. Plates with 20-300 colonies were counted.

Distribution of bacteria introduced into soil at various initial moisture contents. Different volumes of demineralized water were added to portions of loamy sand or silt loam corresponding to 10 g dry weight. This resulted in loamy sand portions with moisture contents of 1.3, 4, 7, 10 or 13% and in silt loam portions with moisture contents of 4, 18, 24, 30, 36 or 42%. The added water in the silt loam portions spread by

capillary forces during incubation for 2 days at 2°C, whereas the loamy sand portions were mixed well before incubation. Thus, soil portions with decreasing water potentials, and therefore with increasing water-filled pore neck diameters, were prepared. The soil portions were inoculated with approx. 3 x 10^7 washed *R. leguminosarum* biovar *trifolii* cells g/dry soil in sufficient demineralized water to ensure that the loamy sand and the silt loam contained 16 and 45% moisture after inoculation. According to Fig. 1, this should correspond to -10 kPa. Again, only the loamy sand portions were mixed and both loamy sand and silt loam portions were incubated for 1 day at 2°C. Mean bulk density of both soils was 1.0 g/cm^3. Numbers of bacteria in different soil suspensions were enumerated by selective plating after the suspensions were prepared using the following procedure.

Duplicate soil portions were transferred into 250 ml Erlenmeyer flasks. After adding 100 ml of demineralized water, the suspension was shaken lightly for 3 min on a rotary shaker (150 rpm at a diameter of 32 mm) and decanted carefully after settling for 20 s. This was repeated four times and a sample of the last washing suspension was taken, whereafter the total amount of washing syspension was increased to 500 ml. To determine the number of bacteria remaining in the washed soil (fraction A), the soil was shaken thoroughly in 95 ml 0.1% sodium pyrophosphate with 10 g gravel (2-4 mm diameter) on a rotary shaker (10 min, 280 rpm). The entire experiment was done twice in order to examine the reproducibility of the technnique. Linear regression analysis was carried out on logarithmic values.

Distribution and population dynamics of introduced bacteria. Soil portions were prepared and inoculated in essentially the same way as in the previous experiment. Different volumes of demineralized water were added to loamy sand and to silt loam portions, so that soil portions of approx. -100,000, -500 and -32 kPa were prepared. The soil portions were inoculated with 3 x 10^7 washed *R. leguminosarum* biovar *trifolii* cells/g dry soil in sufficient demineralized water to ensure that the loamy sand and the silt loam contained 16 and 45% moisture after inoculation. Mean bulk densities for the loamy sand and the silt loam were, respectively, 1.0 and 0.9 g/cm^3. Soil portions were incubated under dark, humid conditions, the first day at 2°C and thereafter at 15°C. Numbers of bacteria were determined at days 1, 28, 55 and 111 using the following procedure.

Three different soil suspensions were distinguished: fractions A, B and C, containing soil particles and aggregates larger than approx. 50 μm,

between 2 and 50 μm and <2 μm, respectively. To obtain these suspensions, duplicate soil portions were washed five times as described in the previous experiment. Precipitation speed of the soil particles in water was calculated using Stokes' law, supposing spherical particle shapes and a density of 2.65 g/cm^3. Fraction A contained the remaining soil after the entire washing procedure in which the soil particles were, each time before decanting, allowed to settle for 20 s. The bacteria in this fraction were extracted from the soil particles by shaking thoroughly in sodium pyrophosphate with gravel. The amount of remaining soil was weighed after filtration and drying at 105°C. Fraction C contained only particles which were left in the upper 1 cm of the washing suspension after 1 h of precipitation. Fraction B was the difference between numbers of bacteria in the non-precipitated washing suspension and in fraction C. Numbers of bacteria in these fractions were enumerated by selective plating, transformed to logarithmic values and analyzed by analysis of variance.

4.3 RESULTS

The washing procedure with a total of 500 ml demineralized water was sufficient to determine the number of cells which were still attached to or closed in by the soil after washing (fraction A), because the number of cells in fraction A exceeded the number of cells in the last washing suspension. The percentage of cells in fraction A decreased at increasing

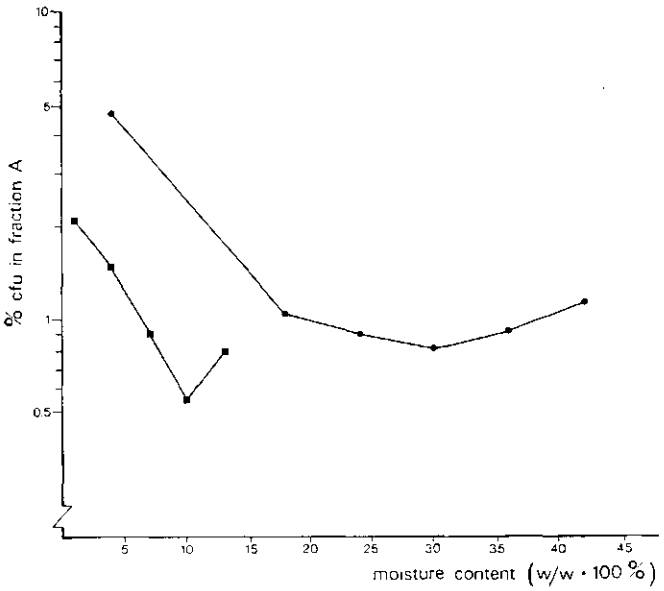

Fig. 2. Percentages of *R. leguminosarum* biovar *trifolii* cells remaining in the washed soil (fraction A) as compared to the total number of cells of this strain in the soil, 1 day incubated at 2°C after inoculating loamy sand (■) and silt loam (●) at different initial moisture contents. Standard deviations between duplicates are 0.15 and 0.21 and between replications of the experiment 0.11 and 0.24 for the loamy sand and the silt loam, respectively.

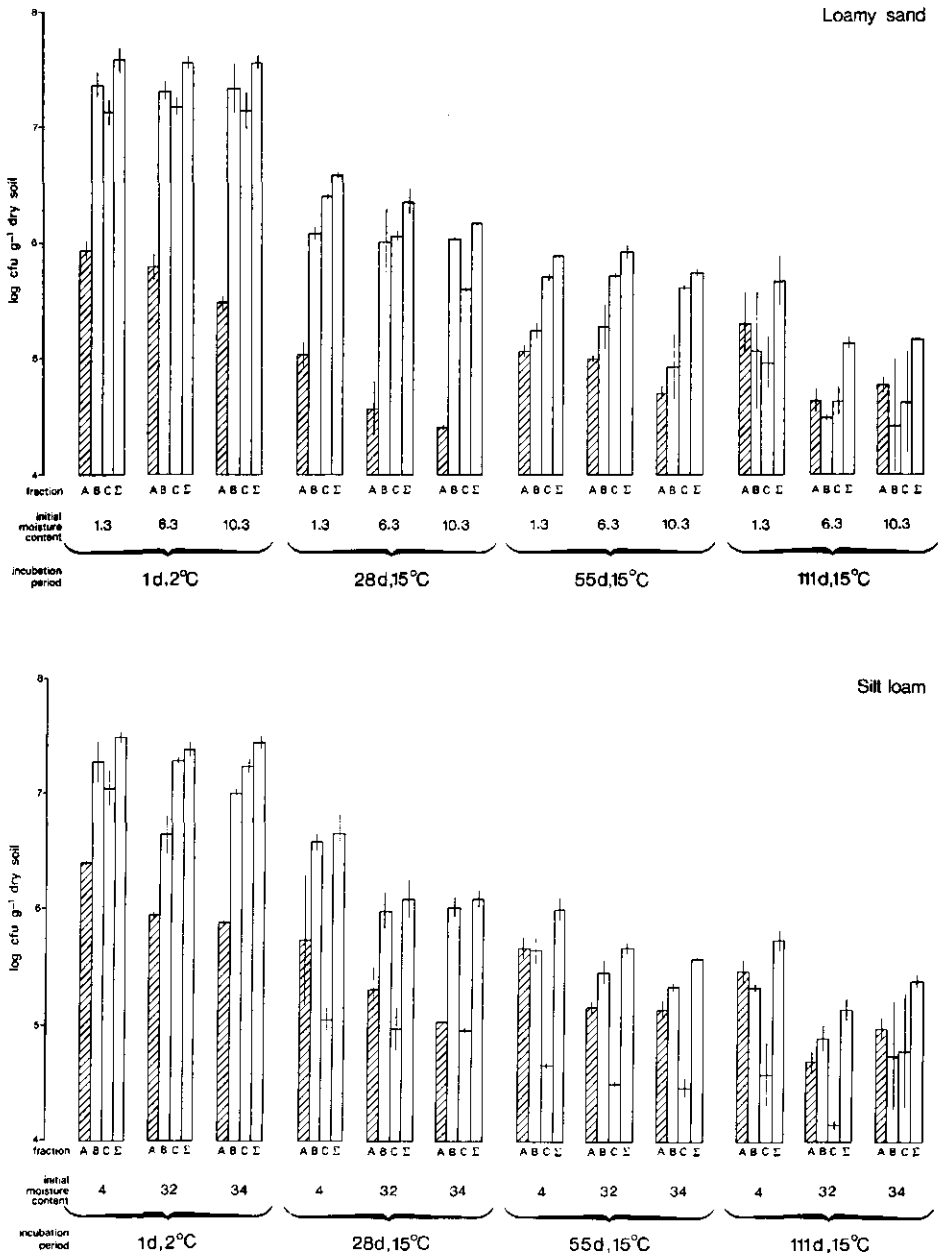

Fig. 3. Distribution and dynamics of *R. leguminosarum* biovar *trifolii* introduced into loamy sand and silt loam at different initial moisture contents. A, cfu attached to or closed in by soil particles and aggregates >50 μm; B, cfu attached to or closed in by soil particles and aggregates between 50 and 2 μm; C, unattached cfu and cfu attached to soil particles <2 μm; Σ, sum of cfu in the fractions A, B and C. Bars indicate standard deviations.

initial moisture contents (Fig. 2). Standard deviations of duplicates were small (0.15 and 0.21 for the loamy sand and the silt loam, respectively) and results were highly reproducible with the used combination of shaking speed and amplitude. The mean values of both experiments had standard deviations of 0.11 and 0.24 for the loamy sand and the silt loam, respectively. In the loamy sand, the percentages of cells in fraction A decreased up to the initial moisture content of 10%. The silt loam showed a fast decrease in the percentage of cells in fraction A up to the initial moisture content of 18%, which was followed by a slow decrease up to about 30%. At these moisture contents maximal numbers of cells were washed out.

The second experiment also showed that the first day after inoculation the number of cells in fraction A was significantly ($P<0.05$) higher at the lowest initial moisture content than at the higher moisture contents (Fig. 3). Comparing both soil types, significantly ($P<0.05$) higher numbers of cells in fraction A were found in the silt loam. After longer incubations, the pattern of decreasing numbers of cells in fraction A at increasing initial moisture contents was still present. At days 28, 55 and 111 significantly ($P<0.05$) more cells were still found in fraction A at the lowest initial moisture content compared to the two higher initial moisture contents for both soil types.

During incubation, the number of cells in fractions B and C decreased more quickly than that in fraction A (Fig. 3) resulting in a significant ($P<0.001$) increase in the percentages of fraction A (Table 1). After 111 days, the percentage of cells in fraction C, consisting of free cells and

Table 1. Percentage of *R. leguminosarum* biovar *trifolii* cells remaining in the soil after five consecutive washings as compared to the total number of the rhizobial cells after inoculating loamy sand and silt loam at different initial moisture contents.

incubation period		1d,2°C	28d,15°C	55d,15°C	111d,15°C
soil type	initial moisture content (w/w.100%)				
loamy sand	1.3	2.2	2.8	14.6	45.0
loamy sand	6.3	1.8	1.6	12.5	37.1
loamy sand	10.3	0.9	1.4	9.3	39.8
silt loam	4.0	7.5	14.8	48.6	53.1
silt loam	32.0	3.6	16.5	32.4	34.3
silt loam	34.0	2.8	8.6	36.3	38.5

cells attached to soil particles that were <2 μm, was reduced to 10-35% of the total number of rhizobial cells in both soil types. After 28, 55 and 111 days of incubation the sum of cells in fractions A, B and C followed the same trend as the number of cells in fraction A, even at days 28 and 55, when fraction B or C contained still more cells than fraction A. So, inoculation of soil at lower moisture contents resulted in higher survival rates of rhizobial cells.

After prolonged incubation both soil types became more stable to the washing procedure since less soil came in suspension (Table 2).

Table 2. Percentage of soil that was not suspended during the washing procedure after different incubation periods.

incubation period	loamy sand*	silt loam*
1 d, 2°C	89 ± 1 (5)	78 ± 6 (5)
28 d, 15°C	93 ± 1 (6)	78 ± 6 (5)
55 d, 15°C	94 ± 1 (6)	81 ± 3 (6)
111 d, 15°C	95 ± 1 (6)	82 ± 3 (6)

* Mean value ± standard deviation (number of values)

In both experiments the method of washing and thoroughly shaking of soil suspensions showed constant recovery percentages: the sum of rhizobial cells in the different fractions was constant after 1 day at 2°C.

4.4 DISCUSSION

The results of both experiments showed that different distribution patterns of cells introduced into soil could be achieved by inoculating soils at different initial moisture contents. At lower initial moisture contents (higher water potentials), only the narrowest pores will be filled with water and the inoculum is expected to penetrate into narrower pores and more inoculated cells will penetrate aggregates than in soil with higher initial moisture contents. When water fluxes are not too large, water in pores will prevent introduced cells from penetrating these pores.

Increasing the initial moisture content, the percentage of cells attached to or closed in by soil particles or aggregates >50 μm (fraction A) decreased only up to a certain moisture content (Fig. 2), suggesting that attachment or enclosure of cells was limited to a certain initial moisture content range. The largest water-filled pore necks corresponding to these moisture contents, can be calculated when the water potential is known with pF = log 0.15 - log r [pF = log(cm H_2O) and r = radius in cm] [18]. Thus, when the moisture contents corresponded to the water poten-

tials given in Figure 1, attachment or enclosure of cells took place when pore necks up to 9 and 0.6 μm in the loamy sand and the silt loam, respectively, were not yet water filled. Similar to our results, Vargas and Hattori [18] found increasing numbers of *Aerobacter aerogenes* cells washed out of the soil when higher initial moisture contents were used, however, their recovery percentages were not constant. They calculated a critical pore neck diameter of 2.5 μm for sandy clay loam up to where cells were retained by the soil.

In the silt loam higher numbers of cells were found in fraction A than in the loamy sand, which can be due to a larger volume of capillary pores or to stronger attachment of cells to the silt and clay particles.

The second experiment showed that, at increasing initial moisture contents, the pattern of decreased numbers of cells in fraction A was still found after longer incubation periods. If appreciable transport and migration would take place, the differences would be expected to diminish, because there was no other difference between the soil portions than the initial moisture content. Thus, the persistence of these differences can only be explained by a lack of appreciable transport and migration of the cells from fraction A to places where they can be washed out. A lack of migration over short distances was also suggested by Labeda *et al.* [11].

The number of cells which were unattached or attached to smaller soil particles up to 50 μm decreased faster than the cells attached to or closed in by soil particles or aggregates >50 μm (fraction A). Moreover, the pattern of higher survival rates at lower initial moisture contents correlated with the pattern of cell numbers in fraction A. This may be interpreted by assuming that soil particles or aggregates >50 μm represent a more favourable microhabitat. Increased attachment as a reason for the change in the number of cells in fractions A, B and C can only play a minor role, because the numbers of cells in fraction A of the soil portions with different initial moisture contents were not equal. Other results affirming that the free state is less favourable for bacterial cells than attachment to or closing in by soil particles or aggregates are described by Drazkiewicz and Hattori [2], Hattori [6], Nioh and Furusaka [13] and Ozawa and Yamaguchi [14].

By sieving, rewetting and mixing the soil, we expect that aggregates have lost stability. During the incubation of the soil portions, however, we detected an increase in aggregate stability, since less soil came in suspension during the washing procedure. This increase can be a result of

biological activity of microorganisms, but also physical mechanisms can play a role [12].

The described method of washing and thoroughly shaking soil suspensions is relatively simple and the results are highly reproducible. The method gives interesting results about distribution, transport or migration and population dynamics, but being an indirect method, interpretation is sometimes difficult. Distinguishing between cells strongly attached to or closed in by soil particles or aggregates, is for example not possible using the method described. Moreover, weakly attached cells will become detached. Micro-aggregates (up to 250 μm) are suggested to be stable to rapid wetting and to agricultural practices [17], which means that the enclosure of cells in such aggregates will not be disturbed. Forces during the washing procedure influenced the size of the different fractions and the fact that fractions B and C were often showing the same pattern as fraction A, means that some disruption of soil structure during the washing procedure cannot completely be excluded.

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CHAPTER 5

THE ROLE OF MICRONICHES IN PROTECTING INTRODUCED *RHIZOBIUM LEGUMINOSARUM* BIOVAR *TRIFOLII* AGAINST COMPETITION AND PREDATION IN SOIL

ABSTRACT

The importance of microniches for the survival of introduced *Rhizobium leguminosarum* biovar *trifolii* cells was studied in sterilized and recolonized sterilized loamy sand and silt loam. The recolonized soils contained several species of soil microorganisms, but were free of protozoa. Part of these soil portions were inoculated with the flagellate *Bodo saltans*, precultured on rhizobial cells. The introduced organisms were enumerated in different soil fractions by a standardized soil washing procedure.

The total number of rhizobial cells was influenced only little (silt loam) or not at all (loamy sand) by the recolonization with microorganisms or by the addition of flagellates alone. However, when both flagellates and microorganisms were present, numbers of rhizobial cells decreased drastically. This decrease was more than the sum of both effects separately. Nevertheless, population levels were still higher than in natural soil.

In the presence of flagellates higher percentages of rhizobial cells and other microorganisms were associated with soil particles or aggregates >50 μm than in the absence of flagellates. In recolonized soils, however, the percentages of particle-associated rhizobial cells were lower than in soils not recolonized previous to inoculation. Thus, the presence of other microorganisms hampered rhizobial cells in the colonization of sites where they can be associated with soil particles or aggregates.

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5.1 INTRODUCTION

Survival of rhizobial cells introduced into natural soil was shown to be influenced by the spatial distribution of cells in soil [21]. Upon introduction into relatively dry soils, higher numbers of cells were found to be associated with soil particles and aggregates >50 μm, which resulted in a higher survival percentage than after introduction into relatively moist soils. It can be hypothesized that at lower moisture contents, smaller pores are not yet water filled, allowing the inoculum to penetrate easier into narrower pores. Thus, more cells will reach the inner part of aggregates [21,26]. Capillary pores up to 6 μm in diameter are suggested to be the most favourable microhabitats for bacteria [12]. One reason may be that bacteria in smaller pores may be protected from predation by protozoa [4,26]. Also abiotic conditions may be more favourable in these small pores, since moisture content will be more constant. Thus, these small pores might act as protective microniches for the introduced bacteria. In order to assess the role of the protective microniches in the ecology of introduced bacteria, more information is needed about the kind of protection of bacteria situated in these small pores.

The influence of particular groups or species in soil on the population dynamics of introduced bacteria can be examined by removal or reduction of these groups with biocides. However, also non-target groups might be affected and specific protozoacides are not yet available [15,16]. Cycloheximide, an inhibitor of protein synthesis in eukaryotes, has only a limited effect on protozoa when applied to soil [10,16]. The role of particular groups of microorganisms can also be studied by introducing them into sterile soil. Although this procedure does not fully cover the array of processes in natural soil, phenomena such as competition and predation might be analysed in this way.

In the present study, the dynamics of *Rhizobium leguminosarum* biovar *trifolii* introduced into sterilized loamy sand and silt loam was investigated. Soils with different initial moisture contents were inoculated in order to manipulate the spatial distribution of the rhizobial cells. The influence of competition and predation on distribution and population dynamics of rhizobial cells was studied by recolonizing the sterilized soil with a group of isolated soil microorganisms or by adding flagellates precultured on rhizobial cells, or a combination of both. Although other interactions than competition and predation might occur, the added components will be refered to as competitors and predators, respectively.

5.2 MATERIALS AND METHODS

Soils. Two Dutch arable soils, a loamy sand and a silt loam [7,20,21] were air-dried to 8 and 20% moisture content, respectively, sieved <2 mm and stored at 4°C. Prior to use, the soil was further dried to a moisture content of respectively 1.3 and 4%. The soil was sterilized by γ-irradiation (4 Mrad) and sterility was tested by dilution plating on nutrient agar (NA) (3.25 g Oxoid nutrient broth and 13 g agar in 1000 ml water, pH 7.2).

Recolonisation of sterilized soils. In order to obtain soils with different competitors but free of predators, about 80 bacterial isolates from both soils were isolated on NA. These microorganisms were not further identified. The loamy sand isolates were grown for 3 days as a mixed population in loamy sand extract [18] and used to inoculate the sterilized loamy sand. A similar procedure was followed for the silt loam, growing the isolates obtained from silt loam in silt loam extract. The inoculated loamy sand and silt loam were moistured up to approx. 15 and 40% moisture content, respectively, and were incubated for at least 4 weeks at room temperature. The soils were then dried again to 1.3 and 4% moisture content. Numbers of microorganisms in these recolonized dried soils, enumerated by dilution plating on NA, were between 8×10^7 and 3×10^8 colony forming units (cfu) /g dry soil. The absence of protozoa was tested with the most probable number method described below.

Culturing and enumerating rhizobial cells. *R. leguminosarum* biovar *trifolii* R62::Tn5 resistant to kanamycin (Km) and rifampicin (Rp) [13,20] was used. The bacterium was cultured in yeast extract mannitol broth [13] supplemented with 25 mg/l Km. After growing for 2 days at 29°C on a rotary shaker, the cells were washed by centrifugation (7000 x *g*, 15 min) and resuspended in sterile demineralized water.

Numbers of rhizobial cells were determined on plates containing yeast extract mannitol agar (YMA) supplemented with 50 mg/l Km, 20 mg/l Rp and 100 mg/l cycloheximide.

Culturing and enumerating protozoa. A flagellate, *Bodo saltans* [24], approx. 3 x 8 μm, was used. During one year, the flagellates were regularly diluted in sterile demineralized water with freshly grown rhizobial cells. After growing several days at room temperature, the highest dilution containing flagellates was diluted again. In this way an uniprotozoan population with a minimum of microorganisms other than *R. leguminosarum* biovar *trifolii* was obtained. We did not succeed in elimi-

nating the other microorganisms completely, although antibiotics were used. Numbers of non-rhizobial cells could be enumerated in the presense of high densities of rhizobial cells by dilution plating on tryptone soya agar (3.75 g tryptone, 1.25 g soya peptone, 1.25 g NaCl, 13 g agar, 1000 ml water), since rhizobial cells did not grow on this medium. To inoculate the soil, flagellates were cultured in sterile demineralized water with rhizobial cells. Part of this suspension was added to the rhizobium inoculum just before inoculation of the soils.

Protozoa in (soil)suspensions were enumerated in microtiterplates by a most probable number (MPN) method [5]. With a microdiluter (Titertek) 4-fold dilutions with 8 replicates were made in amoeba saline (120 mg NaCl, 4 mg $MgSO_4.7H_2O$, 4 mg $CaCl_2.2H_2O$, 142 mg Na_2HPO_4, 360 mg KH_2PO_4, 1000 ml water) containing approx. 10^6 rhizobial cells/ml. The presence of protozoa was determined microscopically after an incubation period of 14 to 28 days at 15°C.

Experimental design. Soil portions of sterile (S) and recolonized sterilized soil (SC), corresponding to 10 g dry weight, were incubated in glass cores (diameter 30 mm), which were closed by autoclavable plastic (polystyren) (bottom) and alluminium caps (top). Sterile demineralized water was added to the soil portions in order to obtain moisture contents of 1.3, 6.3 and 10.3% in the loamy sand and 4, 30 and 34% in the silt loam. These moisture contents corresponded in both soils with water potentials of -100,000, -500 and -32 kPa, respectively [21]. After moistening, the soil portions were incubated in a moisture chamber for 2 days at 4°C. The added water in the silt loam portions spread by capillary forces only, whereas the loamy sand portions were mixed with a spatula prior to incubation. The soil portions were then inoculated with rhizobial cells or with a mixture of rhizobial cells and flagellates (+F), resulting in 4 treatments: S, SC, S+F and SC+F. The inocula were added in as much demineralized water so that after inoculation the loamy sand and the silt loam contained 16 and 45% moisture, respectively, which corresponded with pF 2 (-10 kPa). Numbers of inoculated rhizobial cells and flagellates were 0.6-1 x 10^8 and 7 x 10^3 /g dry soil, respectively. The number of non-rhizobial cells added with the flagellates was 4 x 10^5 /g dry soil. Again, only the loamy sand portions were mixed with a spatula and both loamy sand and silt loam portions were incubated for 1 day at 4°C and thereafter at 15°C. Bulk densities of both soils varied between 0.9 and 1.1 g/cm^3. Numbers of bacteria and protozoa were determined 1, 28 and 56 days after inoculation and for some samples on days 7 and 14 too.

Sampling procedure. Duplicate soil portions were washed 5 times with 100 ml sterile demineralized water as described by Postma *et al.* [21]. This washing suspension contained soil particles approx. <50 μm. A suspension with soil particles <2 μm was obtained by taking a sample from the upper 1 cm of the washing suspension that was allowed to settle for 1 h. The remaining soil after the entire washing procedure (>50 μm) was shaken thoroughly (10 min, 280 rpm) with gravel (diameter 2-4 mm) in 95 ml 0.1% sodium pyrophosphate in order to suspend the particle-associated organisms. Numbers of rhizobial cells, other microorganisms and flagellates in the different fractions were determined by dilution plating on YMA with appropriate antibiotics, by dilution plating on NA and by the MPN method, respectively. The amount of the remaining soil was weighed after filtration and drying at 105°C for one day.

Statistical analyses. First, the effects of initial moisture content and incubation period were studied separately per treatment with analysis of variance. Then, a total analysis of variance was carried out to analyse the effects of competition, predation and initial moisture content, using only the results of days 28 and 56, when population sizes became more or less stable. In all analyses of variance the logarithm of the response variable was used, since we were interested in proportional effects on bacterial numbers or percentages and the variance of replicates appeared to be stable on the log scale. Least significant differences (LSD) were calculated for a=0.05. When percentages have been presented instead of their logarithmic values, LSQ-values (least significant quotient) are given ($LSQ=10^{LSD}$).

5.3 RESULTS

Effects of initial moisture content on rhizobial numbers. One day after inoculation, the total numbers of rhizobial cells were similar within each treatment (Fig. 1). After 28 and 56 days of incubation, total number of rhizobial cells were unaffected by the 3 initial moisture contents in all treatments of the loamy sand and in treatment S of the silt loam. In treatments SC, S+F and SC+F of the silt loam, however, total numbers of rhizobial cells introduced into the dryest soil were significantly ($P<0.05$) higher than the numbers of cells introduced into soils with higher initial moisture contents.

In the silt loam, 1 day after inoculation, the numbers of rhizobial cells associated to soil particles >50 μm (Fig. 2) were significantly

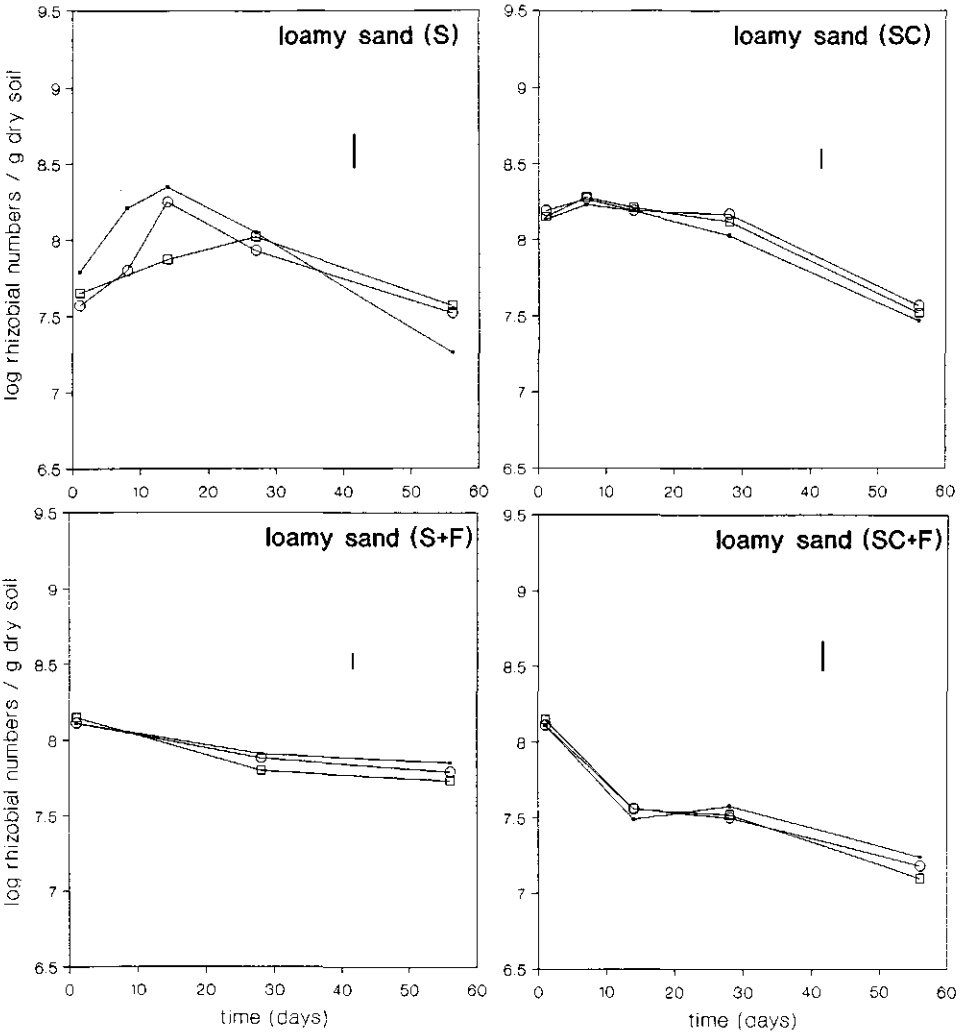
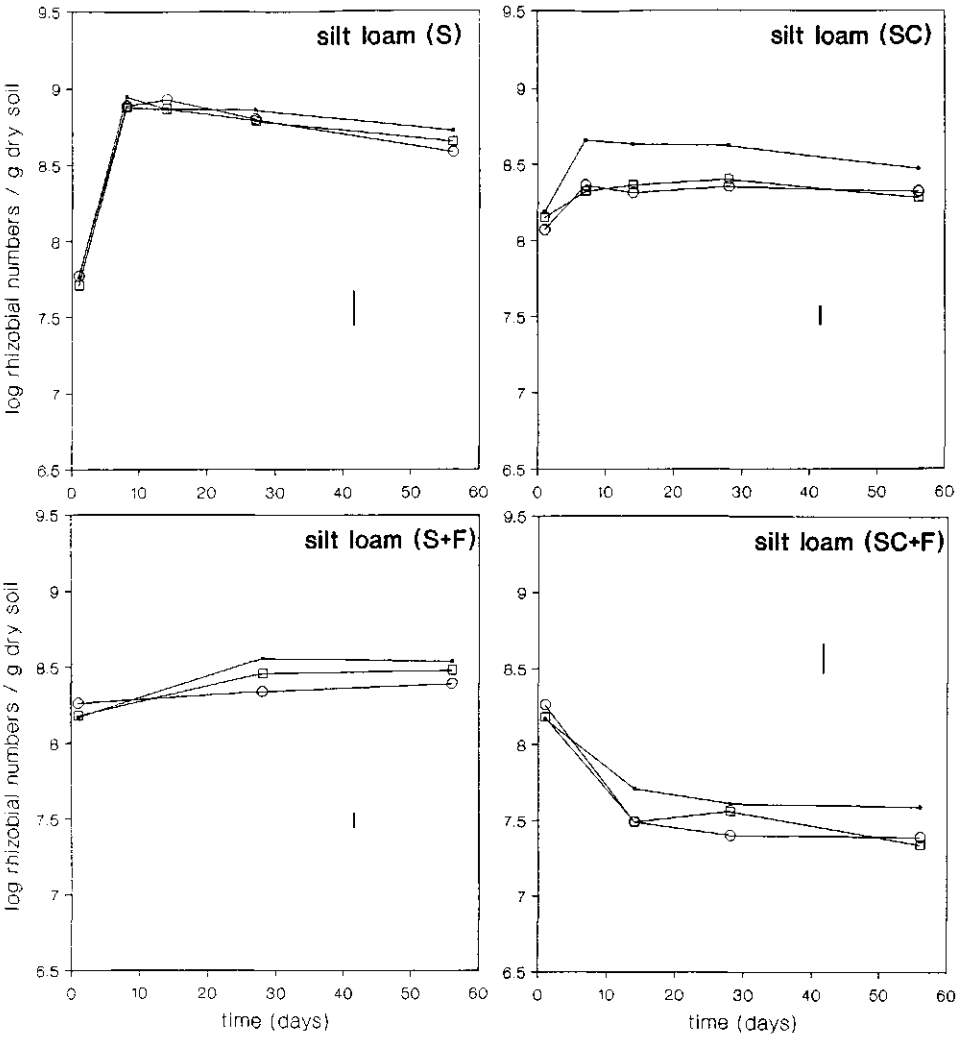


Fig. 1. (left and right page) Total number of rhizobial cells in sterilized (S) and recolonized (SC) loamy sand and silt loam with or without added flagellates (+F), inoculated at different moisture contents. •, O and □ represent an initial moisture content of 1.3, 6.3 and 10.3% in the loamy sand and 4, 30 and 34% in the silt loam. Bars indicate least significant difference (LSD) for a=0.05.

higher ($P<0.05$) at the lowest initial moisture content than at the two higher initial moisture contents. The differences in numbers of particle-associated rhizobial cells as a result of the different initial moisture contents, disappeared in treatment S within 7 days, whereas in the other treatments these differences were significant over a longer period, untill the end of the incubation in treatment SC.



In the loamy sand, the dynamics of the number of cells associated with larger soil particles did not show such a consistent pattern (Fig. 2). Significant differences upon initial moisture content in the loamy sand occured in treatments SC and SC+F, although in the latter treatment the initial moisture content of 6.3% did not always result in lower numbers of particle-associated rhizobial cells as compared to the lowest initial moisture content.

No clear differences due to different initial moisture contents were detected in rhizobial numbers in suspensions with soil particles <2 μm.

Effect of competition and/or predation on rhizobial numbers. In sterilized loamy sand and silt loam (treatment S) total numbers of rhizobial

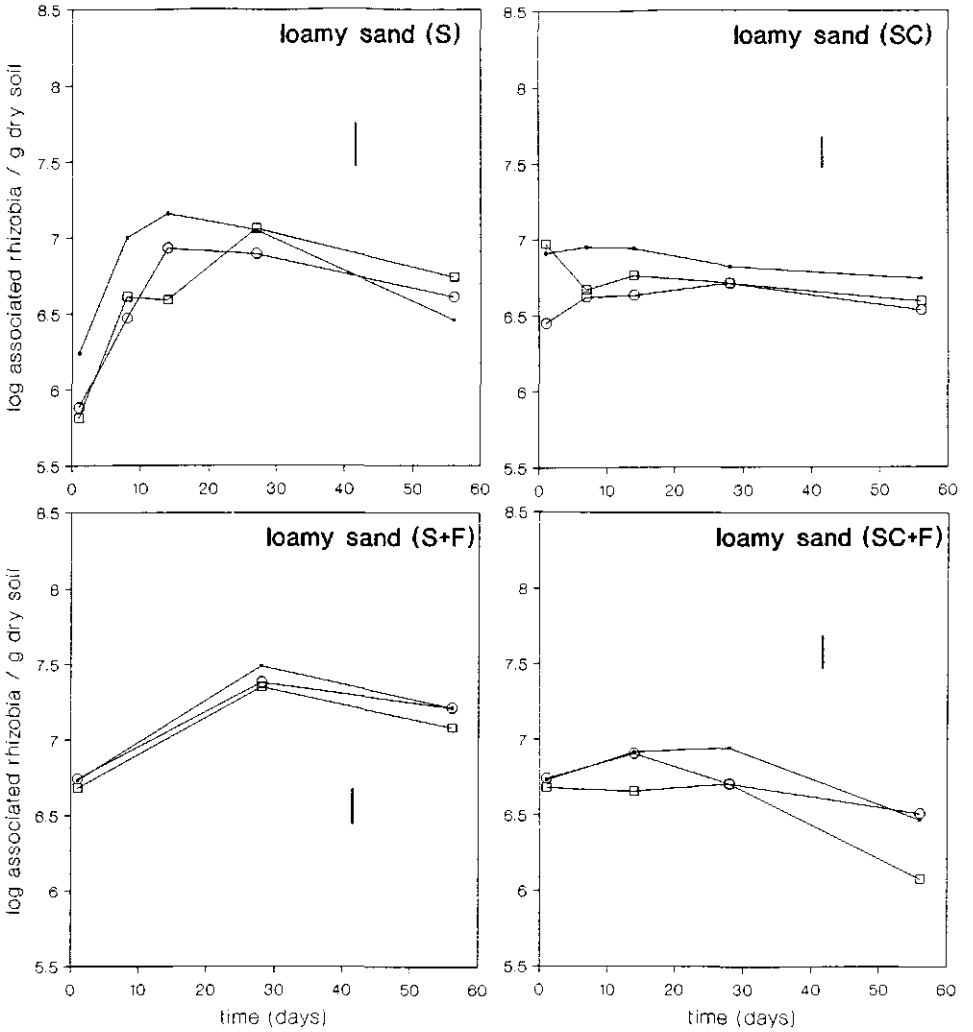
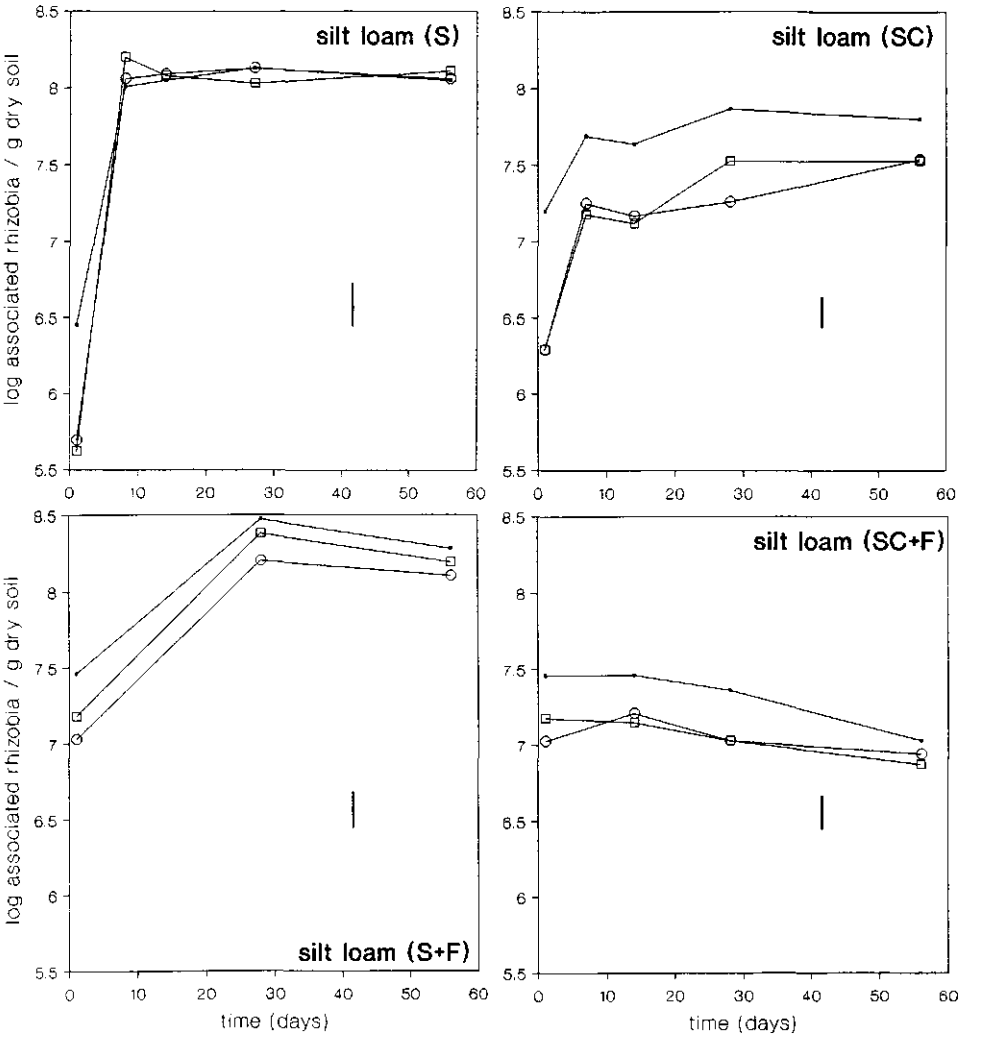


Fig. 2. (left and right page) Number of rhizobial cells associated with soil particles or aggregates >50 μm in sterilized (S) and recolonized (SC) loamy sand and silt loam with or without added flagellates (+F), inoculated at different moisture contents.
•, O and □ represent an initial moisture content of 1.3, 6.3 and 10.3% in the loamy sand and 4, 30 and 34% in the silt loam. Bars indicate least significant difference (LSD) for a=0.05.

cells after 28 and 56 days of incubation were approx. 5 x 10^7 and 5 x 10^8 /g dry soil, respectively (Fig. 1). In the loamy sand total numbers of rhizobial cells were significantly reduced when both competitors and predators were added (treatment SC+F). The addition of only one of these components had no significant effect. In the silt loam, however, the



addition of only competitors or only predators resulted in a significant reduction as compared to non-reinoculated sterilized soil, but the addition of both resulted in an even stronger reduction of rhizobial numbers. Only when both competitors and predators were added, rhizobial numbers decreased during the overall incubation period.

The numbers of rhizobial cells in the washing suspension (particles <50 μm) and in the suspension with soil particles <2 μm showed a similar pattern as the total numbers of rhizobial cells.

The numbers of rhizobial cells associated with larger soil particles showed a different pattern: in the loamy sand, as well as in the silt loam, highest numbers of particle associated cells were found when only

predators were added (Fig. 2). There were no significant differences between the other treatments in the loamy sand. In the silt loam, however, all treatments differed significantly: in the order of treatments SC+F, SC, S and S+F, increasing numbers of particle-associated rhizobial cells were found on days 28 and 56.

Particle-associated cells as a proportion of the total number of rhizobial cells increased during the incubation period and were in all treatments significantly ($P<0.001$) higher in the silt loam than in the loamy sand (Fig. 3). On days 28 and 56, the presence of competitors resulted in a significant reduction of the percentages of particle-associated cells by a factor 0.60 and 0.62 in the loamy sand and the silt loam, respectively. When flagellates were added relatively more cells were particle-associated as compared to the treatments without predators. Differences were a factor 2.33 and 2.63 in the loamy sand and the silt loam, respectively.

Dynamics of the competitors. The first day after inoculation, total numbers of microorganisms had already increased by a factor 2 in the recolonized soils. The introduced numbers of non-rhizobial cells in

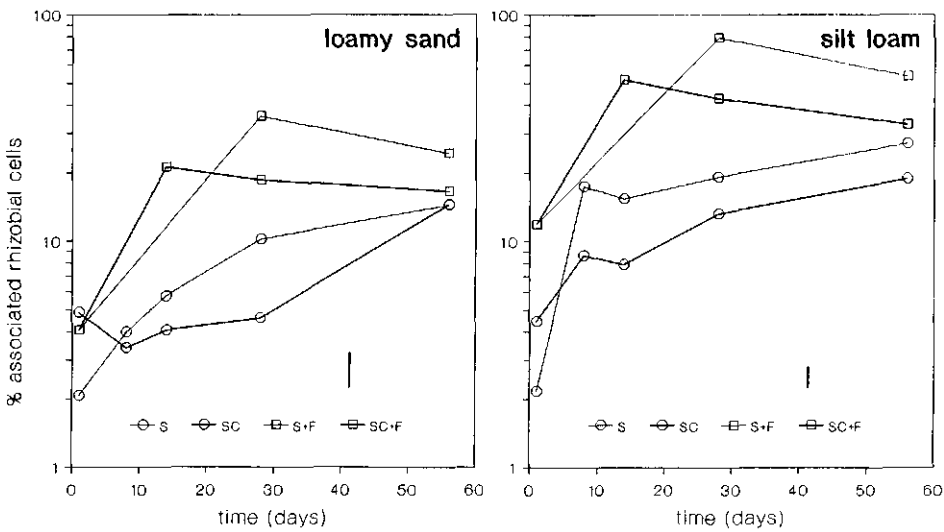


Fig. 3. Percentages of rhizobial cells associated with soil particles or aggregates >50 μm in sterilized (S) and recolonized (SC) loamy sand and silt loam with or without added flagellates (+F). Mean values for the different moisture contents are presented.
——, sterilized soil; ——, recolonised sterilized soil; ○, no flagellates added; □, flagellates added. Bars indicate least significant difference (LSD) between treatments at days 28 and 56.

treatment S+F were low, but increased drastically during further incubation (Table 1). The initial moisture content had no significant effect on the number of competitors one day after inoculation nor after prolonged incubation, so that interaction with the effect of initial moisture content on rhizobial numbers can be excluded. Mean data of the 3 initial moisture contents are presented in Table 1.

The percentages of particle-associated competitors (Table 1) resemble strongly those of particle-associated rhizobial cells (Fig. 3).

Table 1. Total numbers, numbers and percentages of particle-associated microorganisms in the sterilized soils (S) that were recolonized with microorganisms (SC) and/or amended with flagellates (+F).

	log total number		log associated		% associated	
incubation in days	28	56	28	56	28	56
loamy sand SC	8.92	8.88	8.10	7.91	17.3	17.0
loamy sand S+F	8.67	8.78	7.97	8.07	20.4	20.0
loamy sand SC+F	8.38	8.26	7.67	7.56	19.7	20.6
	LSD = 0.15		LSD = 0.27		LSQ = 1.6	
silt loam SC	9.25	9.42	8.51	8.84	19.1	28.4
silt loam S+F	9.14	9.21	9.01	8.93	72.5	53.8
silt loam SC+F	9.02	9.06	8.74	8.75	53.3	49.4
	LSD = 0.09		LSD = 0.17		LSQ = 1.3	

LSD = least significant difference (a=0.05)
LSQ = least significant quotient (a=0.05)

Table 2. Total numbers, numbers and percentages of particle-associated flagellates in sterilized soils (S) and sterilized recolonized soils (SC) where flagellates (+F) were added.

	log total number		log associated		% associated	
incubation in days	28	56	28	56	28	56
loamy sand S+F	6.32	5.06	4.30	3.37	1.0	2.4
loamy sand SC+F	4.37	3.56	3.05	ND	5.1	ND
	LSD = 0.18		LSD = 0.25		LSQ = 1.5	
silt loam S+F	5.97	4.74	5.21	3.62	20.2	7.8
silt loam SC+F	4.17	4.27	2.78	2.85	4.4	4.1
	LSD = 0.18		LSD = 0.18		LSQ = 1.9	

ND = not detectable
LSD = least significant difference (a=0.05)
LSQ = least significant quotient (a=0.05)

Dynamics of the predators. No significant effect of initial moisture content was detected on the numbers of flagellates; therefore, only mean numbers are presented in Table 2. After inoculation with 7×10^3 flagellates /g dry soil, numbers increased 10 to 300 times during the first 28 days. Thereafter, numbers decreased again.

In both soils total numbers and numbers of particle-associated predators were higher in treatment S+F than in treatment SC+F (Table 2). Numbers of particle-associated predators were sometimes to low for reliable counts, as a consequence the percentages of particle-associated predators showed relatively larger variances. The results show that the percentages of particle-associated predators are much lower than the percentages of the particle-associated competitors (Table 1) and particle-associated rhizobial cells (Fig. 3).

5.4 DISCUSSION

Distribution and population dynamics of rhizobial cells were first assessed in the absence of other organisms. By inoculating sterilized soils at increasing moisture contents, decreasing numbers of rhizobial cells were associated with soil particles, similar to results previously found in the natural soil [21]. However, in contrast with these earlier observations in natural soil, the differences in distribution disappeared rather quickly in sterilized soil and significant differences in survival were not detected. Thus, the maintenance of these differences in distribution might be the result of the presence of other organisms, rather than of abiotic factors.

The decline of bacteria introduced into natural soil has been attributed mainly to predation by protozoa [1,9,10,22] and to competition with other microorganisms [11,14,19]. Other possibilities such as microorganisms capable of producing antibiotics or lytic enzymes, bacteriophages and *Bdellovibrio* were suggested to be less important [1,9,22]. Therefore, sterilized soil was recolonized with a group of microorganisms which were assumed to act as competitors. To part of the sterilized and recolonized soil portions, flagellates pregrown on rhizobial cells, acting as predators, were added. The added flagellates and microorganisms were not sufficient to obtain the same effects as in the natural soil. Nevertheless, in the recolonized silt loam significant differences due to the initial moisture content were detected. Thus, competition with other microorganisms may be an important factor to maintain the differences in

distribution of rhizobial cells due to the initial moisture contents. The presence of predators, as a single group, did not result in similar differences.

The experimental design used in this study covered only part of the entire array of processes that determine the population dynamics of introduced bacteria in natural soil. However, it did give indications on the role of individual components such as competition and predation on the survival of introduced bacteria. Moreover, it added information to further develop the concept of the existence of protective microniches provided by physical and chemical conditions of the soil for the introduced bacteria.

The spatial distribution of rhizobial cells was influenced by the presence of competitors as well as predators. Many suitable niches will be occupied by the introduced competitors after a recolonization period of 4 weeks. Rhizobial cells then have to compete for substrates and habitable pore space with the large numbers of microorganisms already present. This could explain that in the recolonized soil lower percentages of rhizobial cells were associated with soil particles and aggregates as compared to soil that was not recolonized previous to inoculation. Although indigenous rhizobia were mentioned to be competitive enough to use readily available substrates in soil in the presence of a natural soil population [8,27], introduced rhizobia were shown to be less competitive [8,19]. Not only the competitive ability, but also the moment of inoculation is important. Inoculated bacteria have less chance to colonize suitable microniches when other competitive microorganisms are already present [3,25].

Predators caused the opposite effect on the distribution of rhizobial cells as the competitors: the percentages of particle-associated rhizobial cells were higher in treatments where predators were present than in treatments without predators. Also the percentage of particle-associated competitive microorganisms increased in the presence of predators. This agrees with the concept that particle-associated bacteria are found in smaller pores which are physically protected from predation [4,21,26]. The larger flagellates were not able to enter these sites. Evidence for this is the observation that most of the flagellates were, similar to the results of Vargas and Hattori [26], not associated with soil particles and aggregates >50 μm. Firm attachment to soil surfaces might also protect bacteria from predation by filter feeding flagellates (K.B. Zwart, pers. communication).

It is surprising, that recolonization and addition of flagellates alone had little effect on the total number of rhizobial cells. Even in the treatment where flagellates were added in combination with a low number of microorganisms which increased drastically during incubation, rhizobial numbers did not decrease to numbers comparable to the treatment where flagellates were added to the recolonized soils. This could be due to the presence of a different microbial population and/or to the sequence of introduction [3,25].

Our results indicate that rhizobial cells inoculated in non-recolonized sterilized soil were able to compensate for the loss of predated cells, since the absolute number of particle-associated rhizobial cells was higher in this treatment than in sterilized soil without flagellates. This higher number of particle-associated cells can be explained by regrowth in combination with selection on particle-associated cells, since active attachment of rhizobial cells as a direct response to the presence of predators is not likely. A compensation of predated cells by regrowth can be expected [1,6,11], since predation deminished bacterial numbers more effective when bacterial growth was inhibited [11]. In addition, protozoa have been detected to cause a higher respiration rate and N-turnover [6,17]. Substrates produced by metabolic activity or death and lysis of protozoa may allow bacterial reproduction [23,25].

The survival of rhizobial cells only decreased drastically when competitors and predators were both added to the sterilized soil. The fact that the population of rhizobial cells did not decrease down to levels found in natural soil, can be due to the larger variation in species of predators and microorganisms in natural soil. Amoebae are thought to be important bacteriophorous predators in soil based on their higher numbers [2,17] and combinations of predators increase the overall activity in soil [6].

The synergetic effect of the combination of competitors and predators may be explained in the following way. Flagellates predate mainly on accessible bacterial cells, i.e. cells outside the protective microniches, which resulted in an increased percentage of particle-associated rhizobial, as well as non-rhizobial, cells. The capability of the introduced rhizobial cells to compensate for the loss of predated cells will be dependent on competitional factors. In the recolonized sterilized soil, however, the regrowth of rhizobial cells will be limited by the presence of competitive microorganisms at many of the suitable microniches. Thus, with the increased turnover due to predation, the

competition for substrates and habitable pore space will become more important for the survival of introduced bacteria.

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CHAPTER 6

POPULATION DYNAMICS OF *RHIZOBIUM LEGUMINOSARUM* Tn5 MUTANTS WITH ALTERED CELL SURFACE PROPERTIES INTRODUCED INTO STERILE AND NON-STERILE SOILS

ABSTRACT

The influence of cell surface properties on attachment to soil particles and on population dynamics of introduced bacteria was studied in sterilized and natural (=non-sterilized) loamy sand and silt loam. *Rhizobium leguminosarum* strain RBL5523 and three Tn5 mutants with altered cell surface properties were used. Cellulose fibrils were absent in RBL5762. Both RBL5810 and RBL5811 produced 80-90% less soluble exopolysaccharides and RBL5811 had in addition an altered lipopolysaccharide composition.

In sterilized soil the total number of cells, as well as the number of particle-associated cells, of RBL5523 and RBL5810 were higher as compared to cell numbers of RBL5762 and RBL5811. Differences in percentage of particle-associated cells between strains in sterilized soil were only found at high inoculum densities, when populations increased little. In the natural silt loam final population sizes, as well as numbers of particle-associated cells, of RBL5523 were higher as compared to the other strains after 56 and 112 days of incubation. Differences between final numbers were, in both natural soils, maximal a factor 10 and none of the mutants survived systematically to a lesser extent than the other mutants. Nevertheless, RBL5810 had a lower growth rate and RBL5811 had in all cases lower percentages of particle-associated cells than the other mutants.

The importance of association with soil particles or aggregates for the survival of introduced cells was affirmed by the pronounced increase of the percentage of particle-associated cells during incubation in natural as well as sterilized soil. However, no clear relation between altered cell surface properties, particle-association and survival was found.

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6.1 INTRODUCTION

Bacteria introduced into soil have to cope with harsh conditions in the soil and to compete with well-adapted indigenous organisms [3,26]. An important aspect for survival of bacteria might be their association with soil particles or aggregates. For example, 90% of the indigenous rhizobial cells were found to be associated with soil particles [19] and extensive procedures (sonication, blending and shaking) for dislodging indigenous soil bacteria, demonstrate the strength of their association. As for introduced bacteria, the relative number of particle-associated cells increased with time after their introduction [1,19,23,24].

Association with soil particles or aggregates can be due to enclosure in pores or attachment to soil particle surfaces. Enclosure in pores can be manipulated by varying the moisture content prior to inoculation [23,24,30]. The impact of attachment, i.e. irreversible contact between bacterial cells and soil particle surfaces, might be examined by using bacteria with altered cell surface properties. Mutants with altered properties such as absence of cellulose fibrils, exopolysaccharide (EPS) or lipopolysaccharide (LPS) have been used for studying the infection proces of rhizobial cells [4,8,13,17,28]. However, the effect of these properties on attachment and on population dynamics in bulk soil are unknown. In natural soil not only the attachment, but also biotic interactions might be influenced by altered cell surface properties. Speculations have been made about the protective function of capsular and extracellular polysaccharides against predators and bacteriophages [5,9,29].

In the present paper, the effect of an altered cell surface on particle-association and population dynamics of introduced bacteria was studied in sterilized as well as in natural soil. A *Rhizobium leguminosarum* strain and three Tn5 mutants were used as model organisms. Since attachment may be influenced by growth of cells, different inoculum densities were applied to sterilized soil. In survival studies in natural soil, inoculations were executed at two different initial moisture contents in order to establish differences in distribution of the inoculated cells according to Postma *et al.* [24]. Total cell numbers and numbers of particle-associated cells were distinguished.

6.2 MATERIALS AND METHODS

Soils. Two Dutch arable soils, a loamy sand and a silt loam [6,21,24] were air dried to 8 and 20% moisture content, respectively, sieved <2 mm and stored at 4°C. Part of the soil was sterilized by γ-irradiation (4 Mrad) and sterility was tested by dilution plating on nutrient agar (3.25 g Oxoid nutrient broth and 13 g agar in 1000 ml water, pH 7.2). The non-sterilized (or natural) loamy sand and silt loam were, prior to use, further dried to a moisture content of 1.5 and 5%, respectively.

Bacterial strains. Strain RBL5523 [25] is derived from *R. leguminosarum* biovar *trifolii* strain LPR5039 which was cured from its Sym plasmid [11]. In a rifampicin (Rp)-resistant derivate of LPR5039, the *R. leguminosarum* Sym plasmid pRL1::Tn1831 was crossed by selecting for transfer of spectinomycin (Sp) [25]. Tn1831 codes for resistance against Sp, streptomycin (Sm) and mercuro chloride [20]. RBL5762, RBL5810 and RBL5811 were Tn5 mutants of this strain obtained by transposon mutagenesis as described by Smit *et al.* [28], following the method of Beringer *et al.* [2]. The strains were choosen for their altered cellulose fibril, EPS or LPS properties (Table 1). Each mutant contained only one Tn5 insertion and the location of Tn5 was not in the Sym plasmid as was determined by the method described by Smit *et al.* [28]. The impaired EPS production of RBL5810 and RBL5811 was due to different mutations, since complementation for EPS production occured with different clones from a LPR5039 cosmid bank (H.C.C. Canter Cremers, pers. communication).

Table 1. Bacterial strains

	cellulose fibrils	EPS	LPS	contact angle [4]	el.phor. mobil. [4]
RBL5523	+	+	+	40	-2.6
RBL5762	- [1]	+	+	24	-2.5
RBL5810	+	- [2]	+	40	-2.2
RBL5811	+	- [2]	- [3]	44	-0.6

1) diminished fluorescence on RMM plates with Km and 0.02% calcofluor white under UV light and no extracellular fibrils detected by electron microscopy [28]
2) colonies with non-mucoid appearence on YMA and a reduction of soluble exopolysaccharide production of 80-90% (w/w) as compared to RBL5523 (M.C.C. Canter Cremers, pers. communication)
3) colonies lacking the O-antigen-containing LPS species determined according to de Maagd [17]
4) data obtained from M.C.M. van Loosdrecht: contact angle (°) and electrophoretic mobility (10^{-8} m V^{-1} sec^{-1}) are a measure for adhesion of bacterial cells to surfaces [14,15]

Growth rate. The strains were pre-cultured in yeast extract mannitol broth (YMB) [10] supplemented with 50 mg/l Sm, whereafter 250 ml Erlenmeyer flasks containing 50 ml YMB without Sm were inoculated so that approx. 10^4 cells/ml were obtained. For each strain, two such batch cultures were incubated on a reciprocal shaker at 15°C. Cell numbers were determined daily by dilution plating on yeast extract mannitol agar (YMA) [10], supplemented with 100 mg/l cycloheximide (C) in order to suppress fungal growth.

Colony morphology. To assess the ability of the strains to produce slime, the strains were cultured on plates containing solid media with different C/N ratio's and colony morphology was monitored after 6-10 days at 28°C. YMA, trypton yeast agar (TYA) (5.0 g trypton (Oxoid), 3.0 g yeast extract (Oxoid), 1.0 g $CaCl_2.2H_2O$ and 13 g agar in 1000 ml water) and soil agar were used. Soil agar was prepared of 50 g γ-irradiated (4 Mrad) loamy sand or silt loam in 100 ml water agar (13 g agar per 1 H_2O).

Stability of antibiotic resistance markers. Inocula cultured in the presence of Sm and cells reisolated from both sterilized soils after an incubation of 54 days at 15°C were tested for their antibiotic resistance by replica plating. From each combination of strain, soil and inoculum density, at least 100 colonies from YMA without antibiotics were transferred to YMA with 200 mg/l Sm, 100 mg/l Sp and 20 mg/l Rp.

Sterile soil experiment. Inocula of the 4 strains were cultured in YMB supplemented with 50 mg/l Sm. After growing for 2 days at 29°C on a rotary shaker, the cells were washed by centrifugation (7000 x *g*, 15 min), and resuspended in sterile demineralized water. Two inoculum densities were used to inoculate glass cores containing sterilized loamy sand and silt loam, corresponding to 10 g dry weight. Inoculum densities corresponded to approx. 10^7 and 10^9 cells/g of dry soil. After inoculation, the moisture content was 16% in the loamy sand and 40% in the silt loam. The loamy sand portions were mixed with a spatula and in the silt loam portions the added moisture spread by capillary forces only. After incubation for 1, 14, 33 and 54 days at 15°C in a moisture chamber to prevent the soil from evaporation, duplicate soil portions were washed 5 times with sterile demineralized water as described by Postma *et al.* [24]. The remaining soil after the washing procedure was shaken thoroughly (10 min, 280 rpm) with gravel (diameter 2-4 mm) in 95 ml 0.1% sodium pyrophosphate in order to suspend particle-associated bacteria. Rhizobial cells in both suspensions were enumerated by dilution plating on YMA supplemented with 100 mg/l C, so that total numbers and numbers of particle-associated

cells were distinguished.

Natural soil experiment. Glass cores containing loamy sand and silt loam corresponding to 10 g dry weight were prepared. In order to manipulate the distribution of the inoculated rhizobial cells, demineralized water was added to part of the soil portions, obtaining loamy sand with 1.5 and 6.5% and silt loam with 5 and 30% moisture. The soil portions were incubated in a moisture chamber for 2 days at 4°C. The added water in the silt loam portions spread by capillary forces only, whereas the loamy sand portions were mixed with a spatula prior to incubation. The soil portions were then inoculated with rhizobial cells cultured and washed as described and added in as much demineralized water as was needed to obtain 1-4 x 10^7 cells/g dry soil and final moisture contents of 16 and 45% in the loamy sand and silt loam, respectively. Again, only the loamy sand portions were mixed with a spatula and both loamy sand and silt loam portions were incubated for 1 day at 4°C and thereafter at 15°C. Bulk density was approx. 1.0 g/cm^3 for both soils. Total rhizobial numbers and numbers of particle-associated cells were determined in duplicate soil portions with the washing procedure [24] after 1, 28, 56 and 112 days of incubation. YMA supplemented with 100 mg/l Sm, 50 mg/l Sp, 20 mg/l Rp, 100 mg/l C and 50 mg/l benomyl was used to enumerate the introduced strains. This combination of biocides was shown to have no influence on rhizobial numbers, but resulted in a sufficient suppression of indigenous microflora to enumerate 10^3 rhizobial cells/g of dry soil.

Statistical analyses. Effects of strain variation, incubation time, inoculum density or initial moisture content were studied using analysis of variance. The logarithm of the response variable was used, since proportional effects on bacterial numbers and percentages are studied, and the variance of replicates appeared to be stable on the log scale. Least significant differences (LSD) were calculated for significant levels a=0.05.

6.3 RESULTS

Growth rate. Generation times of the strains RBL5523, RBL5762 and RBL5811 were 10-11 h, whereas RBL55810 had a generation time of 19 h.

Colony morphology. RBL5523 and RBL5762 formed mucoid colonies on YMA (high C/N ratio), but not on TYA (low C/N ratio). On soil agar, which was expected to have a C/N ratio of approx. 11, the tiny colonies of RBL5523 and RBL5762 were also muciod. Colonies of RBL5810 and RBL5811 were non-mucoid on all solid media tested.

Stability of antibiotic resistance markers. All colonies of the 4 strains were resistent to the antibiotics tested when the strains were cultured in YMB supplemented with Sm, as well as after incubation for 54 days in sterilized soil. There was one exception: 2% of the colonies were not resistent to the combination of Sm, Sp and Rp in the treatment where 10^7 RBL5811 cells/g dry soil were introduced into sterilized silt loam.

Sterile soil experiment. After introduction into sterilized soil,

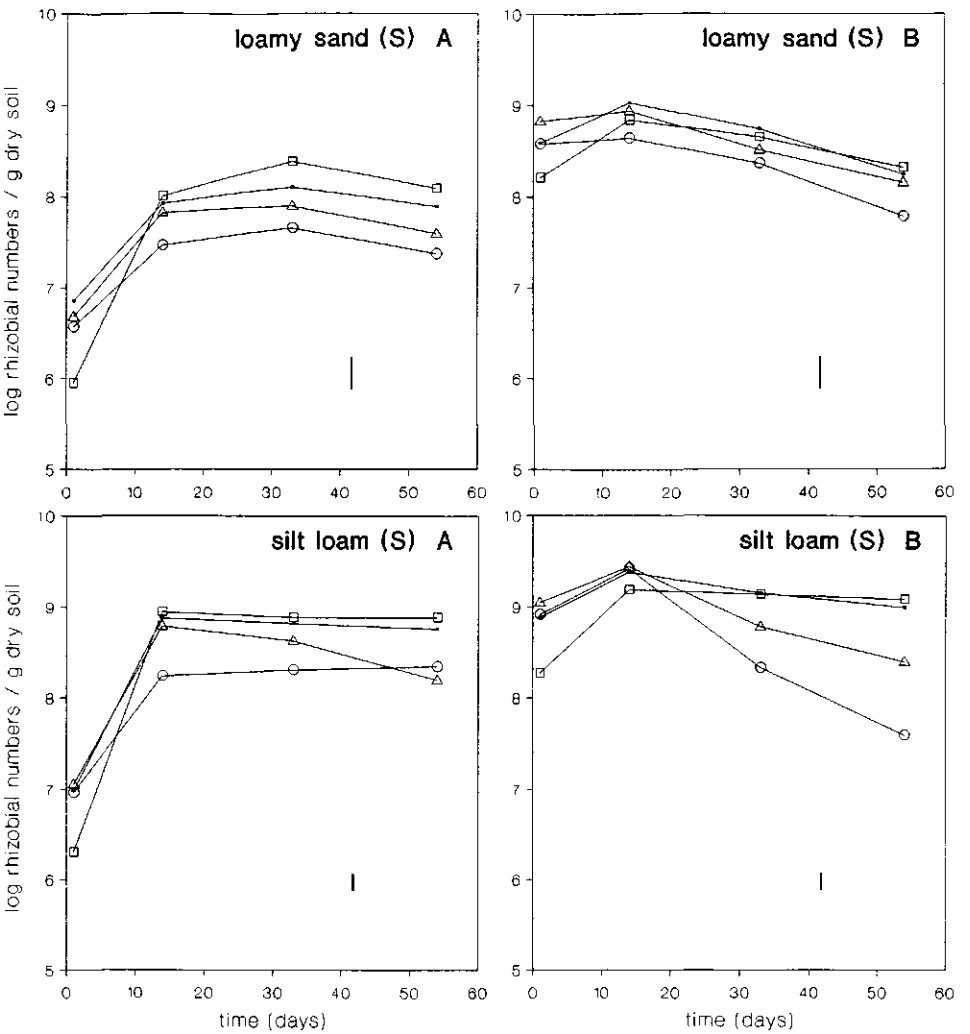

Fig. 1. Total cell numbers of 4 *R. leguminosarum* strains introduced into sterilized loamy sand and silt loam at two inoculum densities. A, low inoculum density; B, high inoculum density. •, RBL5523; ○, RBL5762; □, RBL5810; △, RBL5811. Bars indicate least significant difference (LSD) for a=0.05.

populations of the 4 strains increased during the first period of incubation (Fig. 1). When approx. 10^7 cells/g dry soil were introduced, cell numbers increased, in general, with a factor 32 and 64 in the loamy sand and the silt loam, respectively. At the higher inoculum density (10^9 cells/g dry soil) increases were only 2 and 4 fold, respectively. After 33 and 54 days of incubation, when populations were stabilized or started to decrease, differences in total numbers due to the inoculum density had

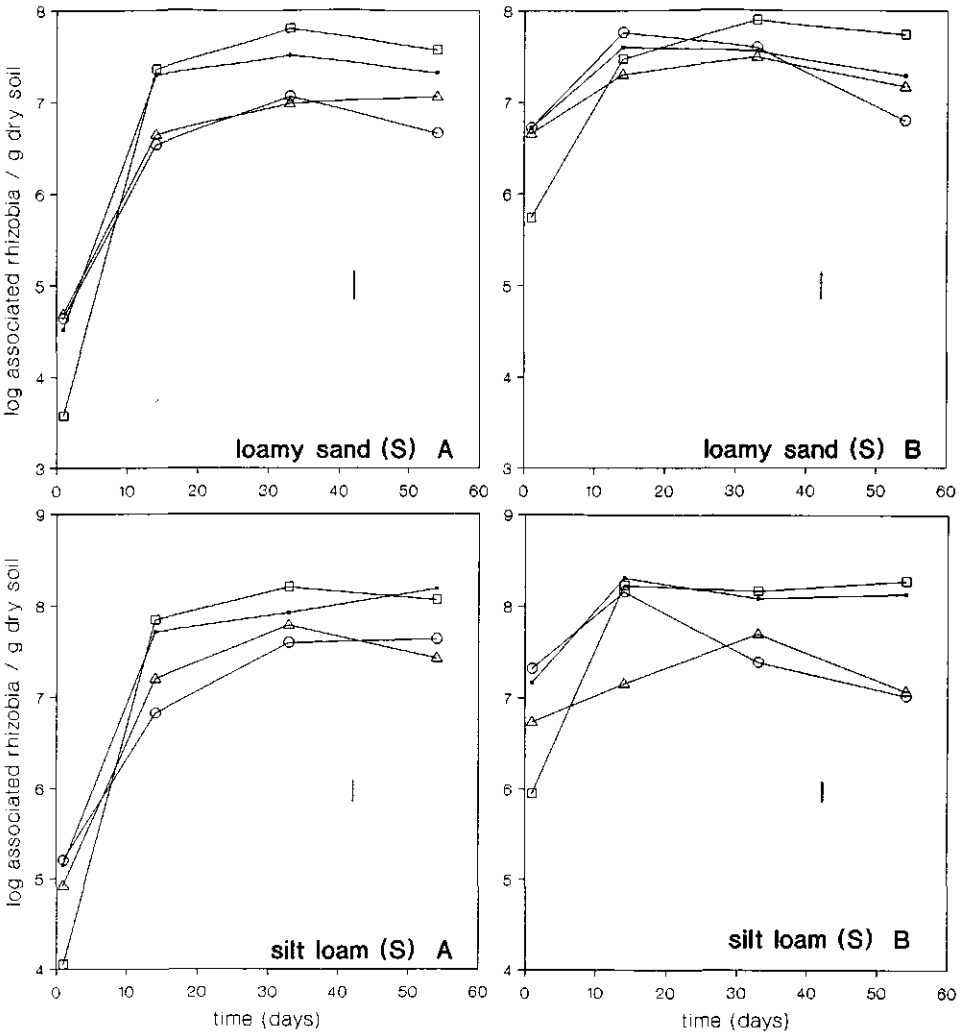

Fig. 2. Numbers of particle-associated cells of 4 *R. leguminosarum* introduced into sterilized loamy sand and silt loam at two inoculum densities. A, low inoculum density; B, high inoculum density. •, RBL5523; ○, RBL5762; □, RBL5810; △, RBL5811. Bars indicate least significant difference (LSD) for a=0.05.

disappeared in the silt loam. In the loamy sand population levels were still significantly higher at the high than at the low inoculum density; the difference was decreasing, but was still a factor 3.5 at day 33 and 2.5 at day 54. The strains RBL5523 and RBL5810 showed in most cases significantly higher final population levels (mean values at days 33 and 54) than RBL5762 and RBL5811. Often the final population level of RBL5762 was lower than RBL5811, but this difference was only significant in the silt loam when high inoculum densities were used (Fig. 1).

The number of particle-associated cells increased strongly between day 1 and 14 (Fig. 2). RBL5523 and RBL5810, which survived best, showed at days 33 and 54 in all cases a higher number of particle-associated cells than RBL5762 and RBL5811.

Particle-associated cells as proportion of the total number of cells was, 1 day after inoculation, not significantly affected by the inoculum density and was only 0.4-2.5%. The percentage of particle-associated cells increased up to 8-28% during 33 days of incubation. Thereafter, the percentage of particle-associated cells stabilized. Interestingly, there were significant differences in percentage of particle-association between strains when high inoculum densities were applied. However, applying low inoculum densities, the percentage of particle-associated cells were not significantly different for the 4 strains and, in general, values were higher than at the high inoculum densities.

Natural soil experiment. Introduction of the 4 strains into both natural soils resulted in a decrease of cell numbers (Fig. 3). One day after inoculation, differences in initial moisture content did not influence total numbers (Fig. 3). However, after prolonged incubation survival levels of RBL5523, RBL5810 and RBL5811 were higher in the silt loam with 5% than in the silt loam with 30% moisture before inoculation. In the silt loam with 5% as well as 30% moisture before inoculation, RBL5523 survived better than the Tn5 mutants and significantly lower numbers of RBL5811 cells survived in the silt loam with an initial moisture content of 30% as compared to RBL5762 and RBL5810 (Fig. 3). Survival levels in the loamy sand differed little, except for RBL5810 which had lower numbers of cells than the other strains after 112 days in the loamy sand with an initial moisture content of 6.5%.

The number of particle-associated cells was more or less constant during the first 28 days of incubation. This was followed by a steady decline during the rest of the experiment (Fig. 4). One day after inoculation there was a significantly higher number of particle-associated

cells in both soils at lower than at higher initial moisture contents; the only exception was RBL5762 in loamy sand. After an incubation of 56 and 112 days, the numbers of particle-associated cells (Fig. 4) showed a similar pattern as the total numbers (Fig. 3): the same sequences in cell numbers of strains were found and the differences due to initial moisture content were again clearly present in the silt loam for the strains RBL5523, RBL5810 and RBL5811.

The percentage of particle-associated cells was, one day after inoculation, significantly higher in the soils inoculated at lower than at higher moisture contents. One day after inoculation, the percentage of particle-associated cells was only 0.1-4.2%, but increased up to 20-60% in 56 days. Thereafter the percentage stabilized. In the loamy sand no differences due to initial moisture content were present, whereas in the silt loam the percentage of particle-associated cells was higher for each strain at the lower than at the higher initial moisture contents. RBL5811 had in both soils significantly lower percentage of particle-associated cells as compared to the other 3 strains. RBL5523 had in the silt loam, at both initial moisture contents, significantly higher percentages than the other strains.

6.4 DISCUSSION

Association with soil particles, as a result of attachment to surfaces of soil particles or enclosure in soil pores, has been shown to influence the survival of introduced bacteria in soil. In the present study *R. leguminosarum* mutants with altered cell surface properties were compared for their possible differences in their capacity to attach to soil particles. Differences in final population sizes and numbers of particle-associated cells between the strains were detected, but it was hard to relate them to the altered cell surface properties.

Comparable population sizes were expected for the different strains within one sterilized soil, since each soil system is suggested to have its own 'biological space' [18] with a distinct capacity to maintain a certain population [12,22]. The 4 strains were expected to be more or less similar in their nutritional need, since the mutants used were all originating from the same strain and differed only by a Tn5 insertion. However, final population sizes of the strains in the sterilized soils differed for unknown reasons: RBL5523 and RBL5810 had, in general, higher final population sizes than RBL5762 and RBL5811.

In the natural soil, where more pronounced differences were expected due to the influence of biotic factors, differences between strains were, in general, not larger than a factor 10. In the natural silt loam RBL5523 had approx. 3 times higher final population sizes than the strains with altered cell surface properties. However, in the natural loamy sand differences were less clear. It was remarkable that RBL5810, which had a much slower growth rate, did establish well.

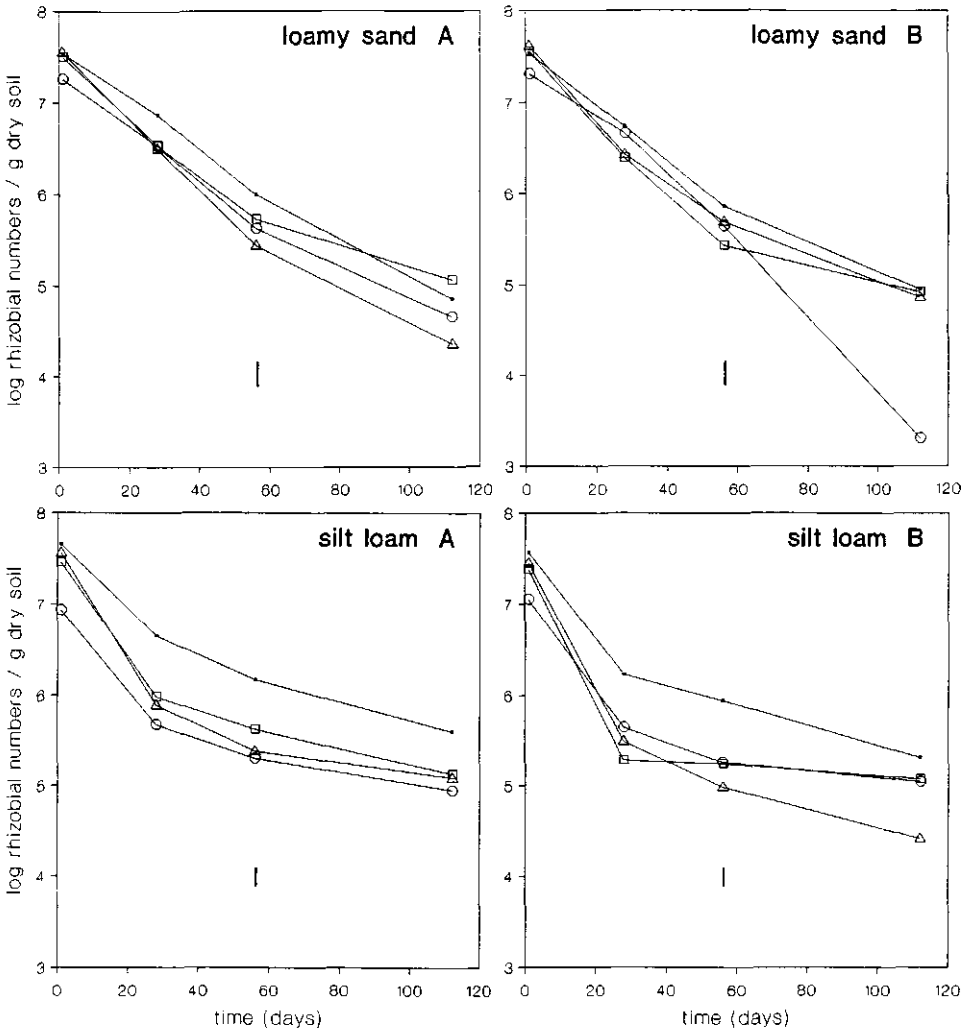

Fig. 3. Total cell numbers of 4 *R. leguminosarum* strains introduced into natural loamy sand and silt loam with two different moisture contents before inoculation. A, low initial moisture content; B, high initial moisture content. •, RBL5523; ○, RBL5762; □, RBL5810; △, RBL5811. Bars indicate least significant difference (LSD) for a=0.05.

The importance of particle-association was affirmed in several ways. (1) In both sterilized and natural soil the percentage of particle-associated cells increased drastically during incubation, similar to previous results [23,24]. (2) In sterilized and natural soil, final population sizes of the 4 strains followed the same pattern as the number of particle-associated cells. In the natural silt loam the differences in numbers of particle-associated cells were more pronounced and earlier

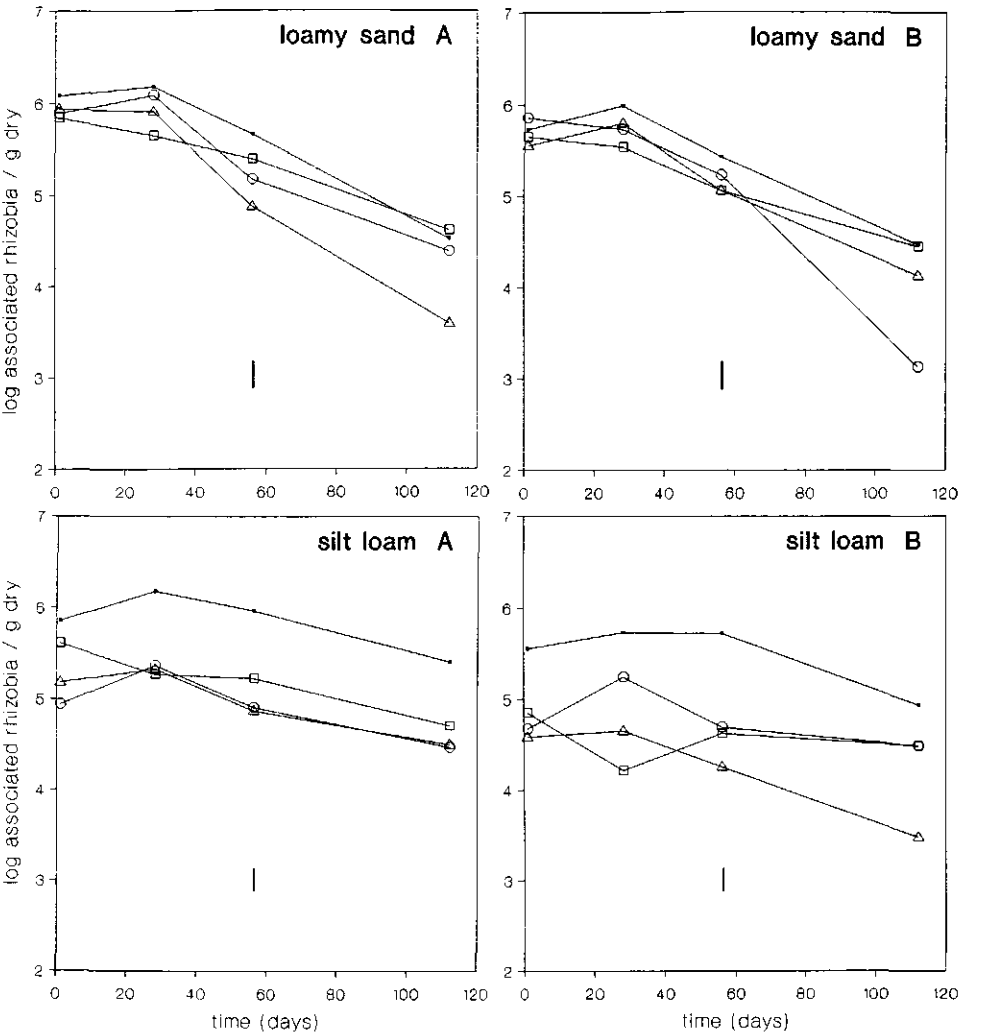

Fig. 4. Numbers of particle-associated cells of 4 *R. leguminosarum* strains introduced into natural loamy sand and silt loam with two different moisture contents before inoculation. A, low initial moisture content; B, high initial moisture content. •, RBL5523; ○, RBL5762; □, RBL5810; △, RBL5811. Bars indicate least significant difference (LSD) for a=0.05.

visible than the differences in the total population size, giving evidence that the population size was influenced by the number of particle-associated cells and not vise versa. (3) A higher percentage of particle-associated cells due to the inoculation procedure for 3 of the 4 strains in natural silt loam, resulted in a higher survival rate. (4) RBL5811 which had the lowest percentage of particle-associated cells in both soils, was one of the lesser surviving strains, whereas the better survival of RBL5523 in natural silt loam corresponded with the highest numbers and percentage of particle-associated cells. However, to what extent attachment to particle surfaces contributes to the particle-association is not clear.

Differences between strains in percentages of particle-associated cells were only found in sterilized soil when population sizes had increased 2-4 times during incubation. When population sizes increased 32-64 times, no differences between strains were found, and the percentage of particle-associated cells was in general higher than when populations increased little, suggesting that cell proliferation had more effect on association than the alterations of the cell surface of *R. leguminosarum*.

The differences between the strains in final population size and numbers of particle-associated cells could not be explained by EPS production or adhesion properties. The two EPS impaired strains, RBL5810 and RBL5811, reacted differently. EPS production has been suggested to play a role in attachment of bacteria to soil particles [1,7] and to protect bacteria from predation [5,9,29]. However, inconsistent results on attachment [1] as well as on predation [27] of EPS producing bacteria have been found. Concerning the measured contact angle and electrophoretic mobility of the 4 strains (Table 1), RBL5811 was expected to have highest adhesion values to glass, polystyrene surfaces and probably also to soil particles [15,16]. However, RBL5811 was the strain with the lowest percentage of particle-associated cells. Reasons for this might be the relative low adhesion values of all strains used and the fact that adhesion is only a first reversibel step in the attachment process to surfaces.

Thus, particle-association is found to be important for the survival of introduced bacteria, but none of the results gave evidence for the role of attachment. It might be that, in soil, enclosure in pores is of more importance than attachment to surfaces. Another possibility is that differences between the strains studied were not large enough, or that all used strains had too low adhesion values.

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CHAPTER 7

INFLUENCE OF THE INOCULUM DENSITY ON THE GROWTH AND SURVIVAL OF *RHIZOBIUM LEGUMINOSARUM* BIOVAR *TRIFOLII* INTRODUCED INTO STERILE AND NON-STERILE LOAMY SAND AND SILT LOAM

ABSTRACT

After the introduction of *Rhizobium leguminosarum* biovar *trifolii* into natural loamy sand and silt loam, bacterial numbers increased only directly after inoculation. Thereafter, bacterial numbers decreased until an equilibrium was reached. This decrease was exponential on the log scale and could be described by the function $Y = A + B \cdot R^t$, where Y is the log number of rhizobial cells at time t, A represents the log of the final population size, B is the difference between the log(initial number of bacteria) and A, R is the daily reduction factor of Y-A and t is time in days after inoculation. The final population sizes increased with increasing inoculum densities (10^4-10^8 bacteria/g soil). In sterilized soil, however, the populations increased up to an equilibrium, which was not affected by the inoculum density.

The final population sizes were higher in the silt loam than in the loamy sand in natural, as well as in sterilized soil. The final population size was reached earlier in the natural silt loam than in the loamy sand. Also the growth rate in sterilized soil was higher in the silt loam than in the loamy sand. The growth rate of low inoculum densities in the silt loam was exponential and approximately the same as in yeast extract mannitol broth. The growth rate in the loamy sand could be improved by increasing the bulk density of the soil from 1.0 to 1.4 g/cm^3.

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7.1 INTRODUCTION

The growing interest in the introduction of selected or genetically engineered bacteria into soil for beneficial effects on crop growth, makes it necessary to improve the understanding of the ecology of introduced organisms [4,9]. Introduced bacteria have to cope with the harsh conditions of the soil and to compete with well-adapted indigenous organisms [4,26]. In general, bacterial numbers tend to drop upon introduction, but proper mathematical descriptions fitting the survival curves are scarse. In a few studies on the survival of bacteria an exponential decrease was detected, which enabled the authors to calculate 'rate constants of dying' [21] or 'half-live' values [16]. Crozat *et al.* [8] and Corman *et al.* [6] found that log transformed numbers of bacteria did not show a constant decrease, but rather fitted a function reaching a non-zero equilibrium. Steinberg *et al.* [28] proposed a more complex mathematical model on the basis of a predator prey relation.

An important aspect, when introducing microorganisms into soil, is the inoculum density. A minimum inoculum level is necessary to obtain beneficial effects on crop growth [3,15,27]. Wood and Cooper [30] showed that at increasing inoculum densities, increasing numbers of *Rhizobium leguminosarum* biovar *trifolii* survived under stress conditions in a liquid medium. Also Postgate [23] mentions that dense populations are killed to a lesser extent than sparse populations. Crozat *et al.* [7,8] and Corman *et al.* [6], however, showed that the inoculum density did not affect the final population size of *Bradyrhizobium japonicum* strains in different soils.

In the present paper a strain of *R. leguminosarum* biovar *trifolii* with two antibiotic-resistance markers was used as a model organism. Different inoculum densities were introduced into two soils under sterile and non-sterile conditions, and bacterial numbers were determined by selective plating. The decrease of the introduced bacteria was mathematically described and it was tested whether or not the final population size was affected by the inoculum density. Additional experiments were carried out to explain some of the phenomena found during these experiments. The nutritional state of the inoculated bacteria was varied in order to examine its impact on the growth and survival of the cells immediately after inoculation. Furthermore, the growth rates in the sterilized soils under varying conditions were compared with the growth rate in yeast extract mannitol broth.

7.2 MATERIALS AND METHODS

Soils and bacterial inoculum. Samples from two Dutch arable soils, a loamy sand and a silt loam [9,24] were air-dried to 6-8 and 19-20% moisture content, respectively, which corresponded to approximately pF 4 (-10^3 kPa). The soil was sieved <2 mm and stored at 4°C. When needed, the soil was sterilized by γ-irradiation (4 Mrad). Sterility was tested by dilution plating on nutrient agar (3.25 g Oxoid nutrient broth and 13 g agar in 1000 ml water, pH 7.2).

R. leguminosarum biovar *trifolii* R62::Tn5 with resistance to kanamycin (Km) and rifampicin (Rp) [14,24] was used as a model organism. Bacterial suspensions used for inoculations were cultured in yeast extract mannitol broth (YMB) [14] supplemented with 25 mg/l Km. After growing for 2 days at 29°C on a rotary shaker, the bacterial suspension was washed by centrifugation (7000 x *g*, 15 min), and resuspended in sterile demineralized water (SDW).

Inoculations with different bacterial densities. Soil portions corresponding to 10 g dry weight were incubated in glass cores (diameter 30 mm), which were closed by autoclavable plastic (polystyren) (bottom) and an aluminium cap (top). The bacterial suspension prepared as described, was diluted in SDW as to obtain inocula of 7×10^7, 7×10^6 and 7×10^5 cells/g dry soil. Soil moisture content in the loamy sand and the silt loam was 8 and 19% before inoculation and 18 and 44% after inoculation, which corresponded to approximately pF 2 (-10 kPa). In the silt loam the added inoculum was spread by capillary forces only, whereas the loamy sand portions were mixed with a spatula. Bulk densities were 1.0 and 0.9 g/cm^3 for the loamy sand and the silt loam, respectively. The soil portions were incubated at 15°C in a moisture chamber to minimize evaporation. After 3 h, 2, 7, 14, 28, 56, 83, 111 and 125 days of incubation, duplicate portions were taken to determine the numbers of rhizobia by selective plating on yeast extract mannitol agar containing 50 mg/l Km, 20 mg/l Rp and 200 mg/l cycloheximide [24]. Soil portions without inoculum were used as control and indicated that the medium was sufficiently selective to enumerate rhizobial numbers up to 10^3 bacteria/g dry soil. Total numbers of bacteria in both soils after incubation for 83 days at 15°C were determined on nutrient agar.

This experiment was repeated under similar conditions in both sterilized and natural soil. A wider range of inoculum densities was applied: 3×10^8, 3×10^6 and 3×10^4 cells/g dry soil. The moisture content of

the loamy sand and the silt loam before inoculation was 6 and 20% and after inoculation 16 and 40%, respectively. Bulk densities were 1.0 and 0.9 g/cm^3. Numbers of rhizobia were determined after 3 h, 2, 7, 29, 56, 84 and 112 days of incubation.

Statistical analysis of the effect of inoculum density. The survival curves were described by the function:

$$Y = A + B \cdot R^t \qquad \text{where,}$$

Y = log number of bacteria on time t,

A = log of the final population size,

B = log of the ratio between the initial number of bacteria and the final population size, or the difference between log (initial number of bacteria) and A,

R = daily reduction factor of Y-A,

t = time in days after inoculation.

10^Y and 10^A are given in cells/g dry soil and B and R^t are dimensionless.

This exponential curve on the log scale is equivalent to the Gompertz curve on the number scale that was used by Corman *et al.* [6]. In the present paper the curves were fitted on the log scale, since the variance between the replicates appeared to be stable on the log scale. The curves were fitted with a non-linear regression algorithm of the statistical program Genstat 5 [11]. The goodness of fit of the curves was checked by comparing their residual variance with the variance between the replicates. Within each set of three curves, first the effect of the inoculum density on R was tested. Then the effect of the inoculum density on A and B was tested.

The influence of the inoculum density on the final population size in sterilized soil was tested by analysis of variance with the statistical program Genstat 5 [11].

Inoculation with starved and non-starved cells. The influence of the nutritional state on growth and survival of cells immediately after inoculation was studied in the following experiment. Two types of inoculum were prepared: a bacterial suspension which was cultured and washed as described previously and a bacterial suspension which was cultured in YMB, starved for 3 days at 15°C in phosphate-buffered saline (pH 7.2) (PBS) and then resuspended in SDW. Non-sterile soil portions were inoculated with the non-starved and starved bacterial suspensions. Bacterial numbers were determined by selective plating after 3 h, 2, 3 and 4 days of incubation in a moisture chamber at 15°C. In order to determine cell sizes, 0.1 ml of the inoculum suspensions and of suspensions of the soil

portions were spread on glass slides, dried, heat-fixed and stained with 1:100 diluted antiserum against the used rhizobial strain [24]. Bacterial numbers were determined by epifluorescence microscopy [24]. The length of 15 randomly-chosen stained cells was measured with an ocular micrometer. The effect of starved and non-starved inoculum on cell length and log transformed bacterial numbers was tested by analysis of variance.

Growth rate in sterilized soil and in YMB at 15°C. In order to compare the growth rate in sterilized soil under varying conditions with that in a liquid growth medium, low inoculum densities of bacterial suspensions cultured in YMB and resuspended in SDW were added to sterile loamy sand and silt loam. The loamy sand was also inoculated with a bacterial suspension resuspended in YMB instead of SDW. Loamy sand with a higher bulk density was prepared by pressing the soil after the inoculum was mixed through the soil with a spatula or by inoculating without mixing the soil. In the latter treatment the inoculum was spread by capillary forces only. The soil portions were incubated at 15°C in a moisture chamber. Moreover, two 300 ml Erlemeyer flasks containing 100 ml YMB were inoculated, placed on a reciprocal shaker (120 rpm) and incubated at 15°C as well. Bacterial numbers were determined by selective plating every 1 or 2 days until stationary phase. Generation times during the exponential phase were calculated.

7.3 RESULTS

Different inoculum densities in natural soil. After inoculation in natural soil, bacterial numbers increased between 3 h and 2 days of incubation in both soils at all inoculum densities (Fig. 1). From the second day on, bacterial numbers decreased to an equilibrium population size. Ommitting the observations at 3 h, the data fitted well to the equation $Y = A + B \cdot R^t$, as the residual variance did hardly exceed the variance between the replicates. The resulting values of A, B and R in the two experiments are given in Table 1. Both experiments showed in principle the same trends, although the exact values of A, B and R were sometimes different.

The daily reduction factor of Y-A (R) was not significantly ($P>0.05$) affected by the inoculum density and curves with equal values for R were fitted, in order to obtain more stable estimates of the asymptotes A. The given R values corresponded with reaching the final population size within 10% of the asymptote in 80-120 days for the loamy sand and in 50-

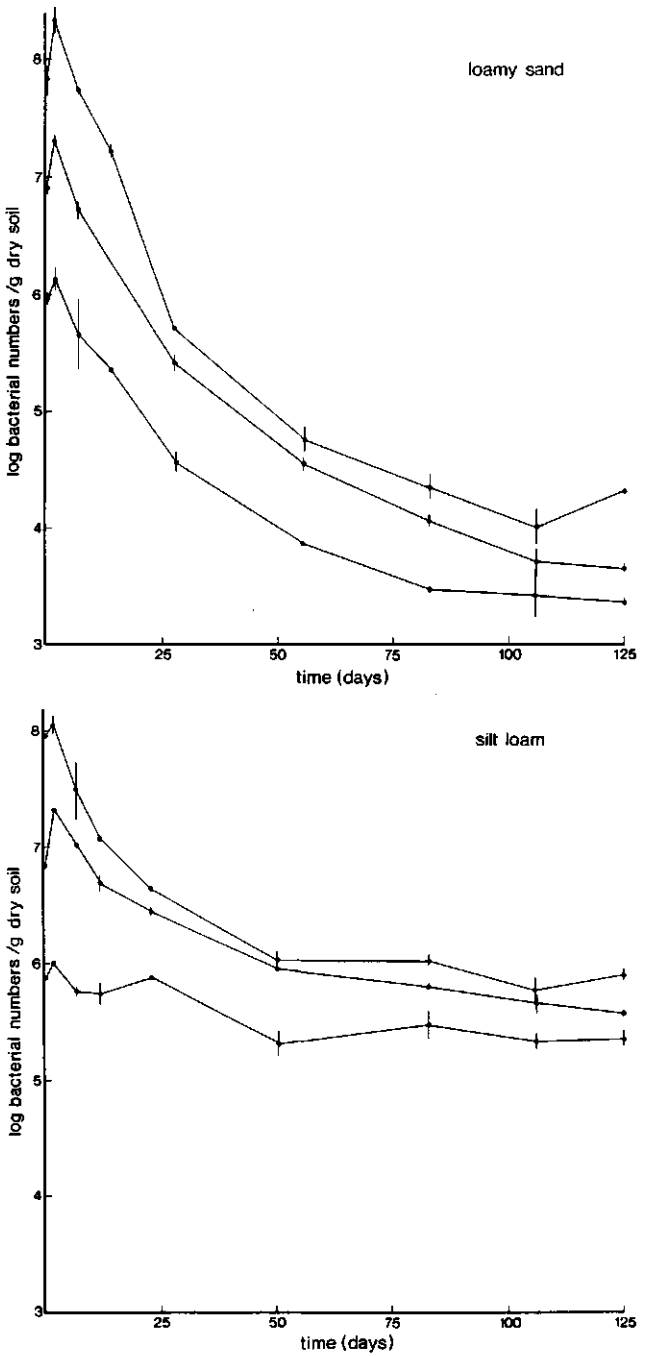

Fig. 1. Population dynamics of *R. leguminosarum* biovar *trifolii* in natural loamy sand and silt loam after inoculation with 7 x 10^7, 7 x 10^6 and 7 x 10^5 cells/g dry soil. Standard deviations are indicated by bars or are within the dimension of the symbol.

Table 1. Experimental data of *R. leguminosarum* biovar *trifolii* in natural loamy sand and silt loam fitted with non-linear regression.

soil type	inoc dens. (log)	end exp. days	$Y = A + B \cdot R^t$ * A	B	R	variance accounted for
loamy sand	7.85	125	3.94	4.68	0.972	99.2%
	6.85	125	3.62	3.88	0.972	
	5.85	125	3.24	3.01	0.972	
loamy sand	8.48	112	4.94	3.55	0.978	97.9%
	6.48	112	3.82	2.75	0.978	
	4.48	112	2.03	2.14	0.978	
silt loam	7.85	125	5.83	2.45	0.960	97.3%
	6.85	125	5.71	1.77	0.960	
	5.85	125	5.39	0.65	0.960	
silt loam	8.48	112	5.84	2.70	0.950	98.3%
	6.48	112	4.52	1.92	0.950	
	4.48	112	2.90	1.42	0.950	

* Y = log number of bacteria on time t
A = log of the final population size
B = log (initial number of bacteria) - A
R = daily reduction factor of Y-A
t = time in days after inoculation.
10^Y and 10^A are given in cells/g dry soil, B and R^t are dimensionless and t is given in days.

60 days for the silt loam. Although the differences were sometimes small, the final population size (A) was significantly ($P<0.001$) affected by the inoculum density within each set of curves (Table 1). Higher inoculum densities resulted in higher final population sizes. Also the values of B were significantly affected by the inoculum density ($P<0.001$). Higher inoculum densities resulted in higher values of B. This means that lower survival percentages were found when more bacteria were inoculated, since the survival percentage is equal to $1/10^B \cdot 100\%$. Interestingly, the survival in the silt loam was 10-100 times higher than in the loamy sand.

The size of the total bacterial population was $4.5\text{-}7.9 \times 10^7$ cells/g loamy sand and $1.3\text{-}2.7 \times 10^8$ cells/g silt loam.

Inoculation with starved and non-starved cells. Rhizobial cells cultured in YMB showed an increase of cell numbers just after inoculation in natural soil (Fig. 1 and Table 2). However, upon introduction of starved cells, bacterial numbers decreased significantly ($P<0.05$) immediately after inoculation in both soils.

The mean cell length of starved rhizobial cells was significantly

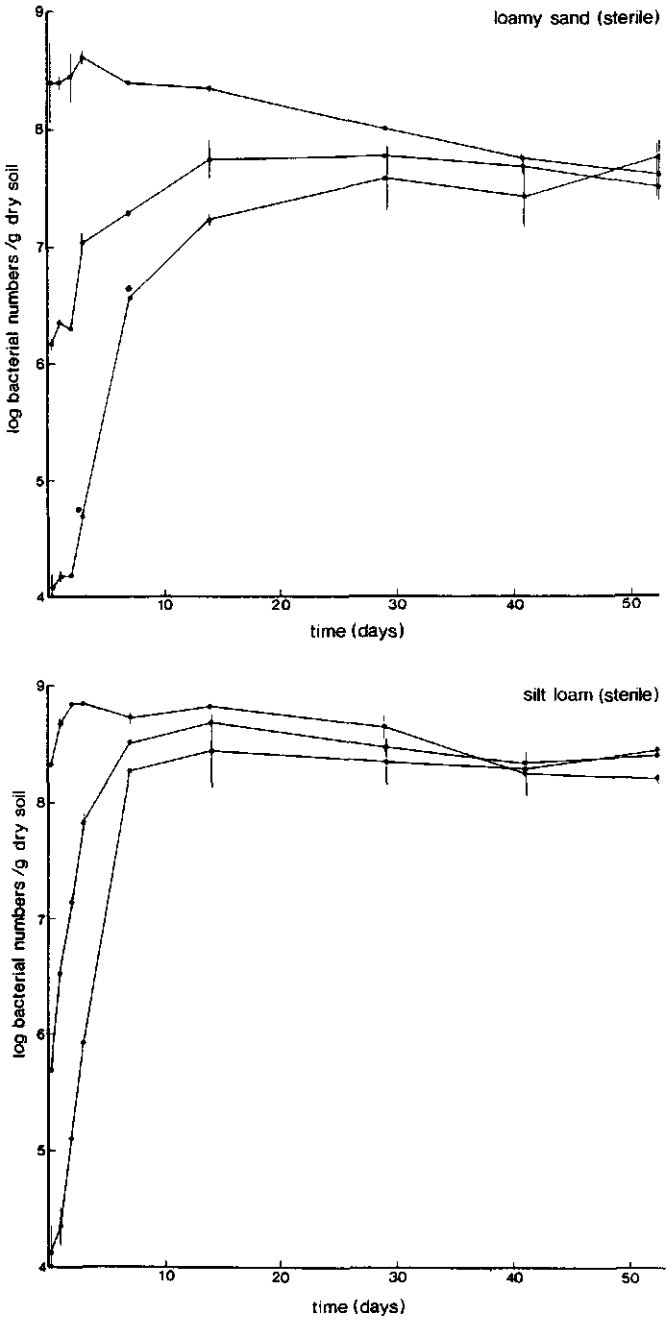

Fig. 2. Population dynamics of *R. leguminosarum* biovar *trifolii* in sterilized loamy sand and silt loam after inoculation with 3 x 10^8, 3 x 10^6 and 3 x 10^4 cells/g dry soil. Standard deviations are indicated by bars or are within the dimension of the symbol. *, replicate without bacterial growth was excluded.

shorter ($P<0.05$) than the mean length of non-starved cells (Table 3). The mean cell length of non-starved cells decreased significantly ($P<0.05$) after inoculation, while the mean cell length of starved cells just before inoculation was not significantly different from the cells 3 h, 2, 3 and 4 days after inoculation.

Different inoculum densities in sterilized soil. In sterilized soils the rhizobial numbers reached a population size of approximately 5×10^7 bacteria/g loamy sand in 30 days and 2.5×10^8 bacteria/g silt loam in 14 days (Fig. 2). During the last 20 days of the incubation, the population size was not significantly ($P>0.1$) affected by inoculum densities and remained more or less constant. In the loamy sand not only the time

Table 2. Log cell numbers of starved and non-starved inoculum of *R. leguminosarum* biovar *trifolii* after inoculation in natural loamy sand and silt loam.

	non-starved inoculum*		starved inoculum*	
	loamy sand	silt loam	loamy sand	silt loam
3 h	7.82	7.59	7.97	7.71
2 d	7.83	7.77	7.61	7.51
3 d	7.83	7.69	7.56	7.39
4 d	7.64	7.30	7.41	7.03
LSD (0.05) = 0.21				

* non-starved inoculum was grown in yeast extract mannitol broth, starved inoculum was grown in yeast extract mannitol broth and then starved in phosphate-buffered saline for 3 days at 15°C.

Table 3. Mean cell length in μm of starved and non-starved inoculum of *R. leguminosarum* biovar *trifolii* just before and up to 4 days after inoculation in natural loamy sand and silt loam.

	non-starved inoculum*		starved inoculum*	
	loamy sand	silt loam	loamy sand	silt loam
0 h**	2.42		1.65	
3 h	1.86	1.98	1.78	1.50
2 d	2.00	1.85	1.96	1.80
3 d	2.17	1.55	2.02	1.63
4 d	1.72	1.46	1.64	1.25
LSD (0.05) = 0.42				

* non-starved inoculum was grown in yeast extract mannitol broth, starved inoculum was grown in yeast extract mannitol broth and then starved in phosphate-buffered saline for 3 days at 15°C.
** just before inoculation

needed to reach the equilibrium population size was longer than in the silt loam, also the lag time was longer (Fig. 2).

Growth rate in sterilized soil and YMB at 15°C. Introduction of low inoculum densities into sterile silt loam resulted in an exponential growth of cells, starting 1 day after incubation (Fig. 2). The generation time was 11.2 hours and only slightly higher than the 10.3 hours in YMB at 15°C (Table 4). The growth rate in the loamy sand, as well as in the loamy sand amended with YMB, was rather irregular. The lag times were different for different soil portions. Using only those portions with increased bacterial numbers, the calculated growth rate in the loamy sand was still much lower than in the silt loam and in YMB. However, the growth rate in the loamy sand could be improved by increasing the bulk density from 1.0 to 1.4 g/cm^3 (Table 4). The generation time was reduced from $\geq$22.9 to 13.5 hours, which is in the same range as the generation time in silt loam and YMB.

Table 4. Generation time of *R. leguminosarum* biovar *trifolii* at 15°C in different growth media, inclusive sterilized soil.

growth medium	inoculum	bulk g/cm^3	soil treatment mixed	soil treatment pressed	generation time *
loamy sand**	in SDW	1.0	yes	no	$\geq$ 22.9
loamy sand**	in YMB	1.0	yes	no	$\geq$ 22.9
loamy sand	in SDW	1.5	yes	yes	13.5
loamy sand	in SDW	1.4	no	no	13.5
silt loam	in SDW	0.9	no	no	11.2
YMB					10.3

* generation time in hours calculated on duplicate soil portions or Erlenmeyer flasks during exponential phase

** soil portions with no growth were excluded

7.4 DISCUSSION

R. leguminosarum biovar *trifolii* introduced into sterile loamy sand and silt loam, reached a final population size, which was not affected by inoculum density. This corresponds to experiments of Bennett and Lynch [2]: independent of the inoculum size, 3 bacterial species colonized sterilized barley roots up to a final population size, representing the substrate supply in the rhizosphere. Nannipieri *et al.* [20] found that each soil system has its own distinctive 'biological space' with regards to the level of microbial biomass and enzyme activity. It is therefore suggested that the final population size in sterilized soil represents

the capacity of the soil in terms of available habitable space, moisture and substrate for maintenance of the bacteria. Comparing both soils, the 'biological space' of the silt loam is 5 times higher than of the loamy sand. In addition, our experiments showed that the total number of bacteria in natural soil corresponded well to the number of rhizobia in the previously sterilized soil.

Not only was the final population size in the sterilized silt loam higher than in the loamy sand, it was also reached earlier. The addition of low inoculum densities to the sterile silt loam resulted in an exponential growth almost equal to the growth rate in YMB. The fact that the growth rate in soil was comparable with that in a shaken liquid culture, indicates that the solid phase of the silt loam was not really a physical barrier for the growth of cells. In the loamy sand the growth rate was certainly not exponential and slower than in the silt loam even after addition of YMB. Therefore, nutritional factors did not affect the growth rate in the loamy sand. On the other hand, the growth rate in the loamy sand could be improved by raising the bulk density from 1.0 to 1.4 g/cm^3. Water retention curves of both soils [25] showed that the loamy sand had relatively fewer small pores than the silt loam. At similar bulk densities the loamy sand had therefore more larger pores which are air filled at pF 2 (-10 kPa) than the silt loam. Since growth of cells, or formation of microcolonies, is a form of movement [5,19], it is likely that large, air-filled pores function as a barrier for translocation and thus inhibit colonization and accessibility to substrate sources. Also Hamdi [12] showed that translocation of rhizobial cells through sterilized soil was sooner restricted in soil with larger soil particles, because of discontinuous water-filled pores.

In natural soil, however, a net increase of rhizobial numbers was only found the first 2 days after inoculation. This increase corresponded to 0.4-1.7 cell divisions and was accompanied by a decrease in cell length. After the inoculation of starved cells, which were already found to be shorter, no such increase of cell numbers was detected. It is therefore suggested that the increase in cell numbers just after inoculation with non-starved cells is a result of cell division, which might be necessary for survival in poor media such as soil. A decrease in cell length after introduction into soil was also detected by Crozat *et al.* [8] and Postma *et al.* [24]. In aquatic environments, such a rapid size reduction by fragmentation is suggested to play a role in the survival mechanism of bacteria in a starvation medium [18].

From the second day on, the number of rhizobia decreased until an equilibrium was reached after approximately 50 and 100 days in the silt loam and the loamy sand, respectively. This shows that part of the introduced rhizobial cells could survive over extended periods. Since the decrease could well be described by an exponential equation on the log scale, on the original scale the rate of the decrease of the finally disappearing cells, in cell numbers, became smaller after longer incubation times. Such a phenomenon might be explained assuming at least 2 key factors responsible for the decrease: (1) a fast factor such as predation and (2) a slower factor such as starvation caused by competition for nutrients with other microorganisms.

The final population size in natural soil was clearly affected by the soil type: survival of the introduced rhizobia was 10-100 times higher in the silt loam than in the loamy sand. This difference in survival could not be explained by the 'biological space' alone, since this was mentioned to be 5 times higher in the silt loam than in the loamy sand. An additional factor is probably the relative higher number of small pores in the silt loam. These small pores may provide physical protection of microorganisms against predation and harsh environmental conditions. Also indigenous bacteria occur mostly in pores smaller than 6 μm in diameter [13,17]. Van Veen *et al.* [29] mention characteristic capacities of soils to preserve organic matter, as well as microorganisms, as a result of physical protection. In our experiments the equilibrium population size was, during at least 125 days, not only influenced by soil type, but also by inoculum density. Crozat *et al.* [7,8] and Corman *et al.* [6], however, detected in experiments with *B. japonicum* a final population size which indeed depended on soil type, but was not affected by the inoculum density. Moreover, inoculum densities of *B. japonicum* below the equilibrium population size, resulted in an increase of cell numbers, which did not happen in our experiments. These differences in results on the impact of inoculum density can be explained by the fact that the bacterial species used was different; fundamental differences in the survival kinetics between fast and slow growing rhizobia have been detected [22]. Also different soil conditions or moisture regimes will have influenced the results. Moreover, in our experiments all samples were obtained from individual soil portions, whereas Crozat *et al.* [7,8] took their samples at different incubation periods from the same bottle.

The significant influence of the inoculum density on the final population sizes was not a result from a definite survival percentage of the

inoculated cells, since the survival percentage was found to be lower at higher inoculum densities. Higher final population sizes at higher inoculum densities can be explained by assuming that more cells will reach a protective niche, when more cells are introduced. In the mean time, higher numbers of rhizobia will result in increased competition, causing an increased death rate.

The existance of different population sizes during at least 125 days under similar environmental conditions, is only likely when there is no extensive translocation of bacteria. Otherwise, bacterial numbers are expected to tend to a common final population size, as was the case in sterilized soil. Translocation of rhizobia in natural soil has been observed to be limited [1,10] and could not be detected in the absence of a transporting agent [19]. In natural soil, considerable translocation associated with development of microcolonies or motility is unlikely, because carbon and energy supply is limiting and, at least for rhizobia, growth rarely occurs in the absence of added organic compounds [19,22].

In conclusion, the existence of different final population sizes of introduced bacteria incubated in natural soil under apparently the same environmental conditions, suggests that differences in distribution over protective and non-protective microniches might be extremely important for survival of introduced bacteria.

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CHAPTER 8

HABITABLE PORE SPACE AND POPULATION DYNAMICS OF *RHIZOBIUM LEGUMINOSARUM* BIOVAR *TRIFOLII* INTRODUCED INTO SOIL.

ABSTRACT

The hypothesis that the population size of introduced bacteria is affected by habitable pore space, was studied by varying moisture content or bulk density in sterilized as well as in natural loamy sand and silt loam. The soils were inoculated with *Rhizobium leguminosarum* biovar *trifolii* and established and maintained at soil water potentials between pF 1.7 and 2.3 (-5 and -20 kPa). Rhizobial cells were enumerated when population sizes were expected to be more or less stable. In sterilized soils the numbers of rhizobial cells were not affected or decreased only slightly with decreasing pF values. In natural soils the decrease in numbers of rhizobial cells with decreasing pF values was more pronounced. Bulk density had only minor effects on the population sizes of rhizobia or total bacteria.

Soil water retention functions of both soils were used to calculate volume and surface area of pores from different diameter classes, and an estimation of the habitable pore space was made. Combining these values of the theoretical habitable pore space with the measured numbers of rhizobial cells, showed that only 0.21 and 0.25% of the habitable pore space was occupied in the sterilized loamy sand and silt loam, respectively. These results showed that, in general, pore space is not limiting the proliferation and growth of soil microorganisms.

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8.1 INTRODUCTION

Each soil system has its own distinctive "biological space" with regards to the level of microbial biomass and enzym activity [30] and bacteria introduced into sterilized soil reached a certain population level independent on inoculum density [27,37,43]. Availability of substrates, moisture, pore space [18,28,30] and a lack of migration to new colonizing sites [27] have been suggested to determine these population levels. Also in natural soils, introduced bacteria often reach a certain survival level [9,10,37,43], which is different for each soil system.

In previous studies on the population dynamics of *R. leguminosarum* biovar *trifolii* introduced into different soils, similar water potentials were used during incubation and survival was higher in the silt loam than in the loamy sand; in sterilized as well as in natural soil [36,37,38]. At the water potential used (pF 2), the finer textured silt loam contained 40-45% moisture, whereas the loamy sand contained only 16-20% moisture. A better survival in finer textured soils is also observed for other introduced bacteria [15,29]. A similar relation was found for the number of indigenous bacteria in different textured soils [4]. Although many soil factors may differ between soil types, soil moisture is a major factor influencing bacterial survival and activity. Finer textured soils contain, in general, more water when kept at a similar water potential than coarse soils and a larger available pore space might explain at least part of the results.

Pore size distribution as well as the absolute volume of water might influence the pore volume or surface area which is suitable for bacteria to survive (=habitable pore space) and the part of the habitable pore space that protect bacteria from predation (=protective pore space). Data about habitable and protective pore space in different soils, and the implications for population dynamics of bacteria are scarse, but it seems logic to suggest that habitable and protective pore space influences the population size of introduced bacteria in those cases that water stress does not have a direct effect on bacterial cells. In general, activity of soil bacteria is not negatively affected up to water potentials between pF 2.7 and 3.5 (-50 and -300 kPa) [8,17].

In the present study *Rhizobium leguminosarum* biovar *trifolii* was used as a model organism. Bacterial cells were introduced into sterilized and natural (=non-sterilized) loamy sand and silt loam, and the soils were adjusted to different water potentials. Moreover, total pore volume was

varied by using two bulk densities. Numbers of rhizobial cells and the total populations were enumerated by plating techniques. In addition, pore volume and pore surface area of both soils were calculated for different pore size classes using water retention functions.

8.2 MATERIALS AND METHODS

Soils. Two Dutch arable soils, a loamy sand and a silt loam, were air dried to 8 and 20% moisture content, respectively, sieved <2 mm and stored at 4°C. Part of the soil was sterilized by γ-irradiation (4 Mrad) and sterility was tested by dilution plating on nutrient agar (3.25 g Oxoid nutrient broth and 13 g agar in 1000 ml water, pH 7.2). Soil characteristics are presented in Table 1. The relationship between soil water potential and moisture content of repacked samples of the loamy sand and the silt loam with bulk densities of approx. 1.4 and 1.0 g/cm^3, respectively (Fig. 1), was determined according to Klute [25].

Soil water potential. Glass filters with a fine-porous plate made of sintered glass with a nominal maximum pore size of 1.0-1.6 μm were used (all-glass bacteria filter, porosity 5, Schott). The glass filters were connected with a water reservoir by a continuous water column (Fig. 2). Glass cores, 40 mm high, 30 mm in diameter and closed at the bottom with a nylon

Table 1. Particle size distribution and other characteristics of the soils sieved <2 mm and stored at 4°C.

		loamy sand	silt loam
pH-KCl	1)	5.4	7.2
organic matter	2)	3.3	3.5
$CaCO_3$	2)	0.1	8.2
lutum	2)	4.3	30.9
2-16 μm	2)	0.5	18.1
16-50 μm	2)	7.3	23.6
50-105 μm	2)	19.1	11.3
>105 μm	2)	65.4	4.4

1) in $-\log(H^+)$
2) in g/100g dry soil

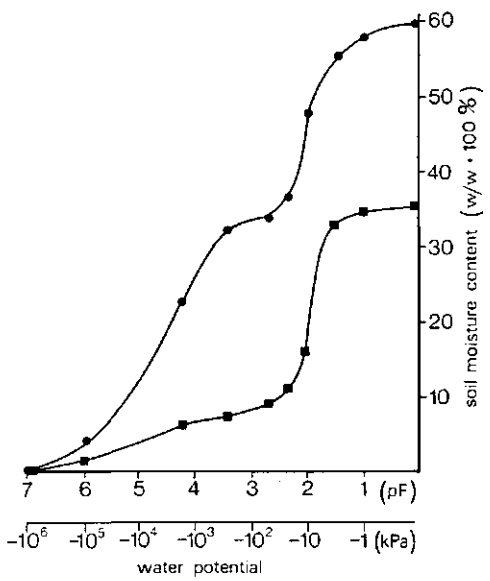

Fig. 1. Relationship between soil water potential and moisture content of repacked samples of the loamy sand (■) and the silt loam (●).

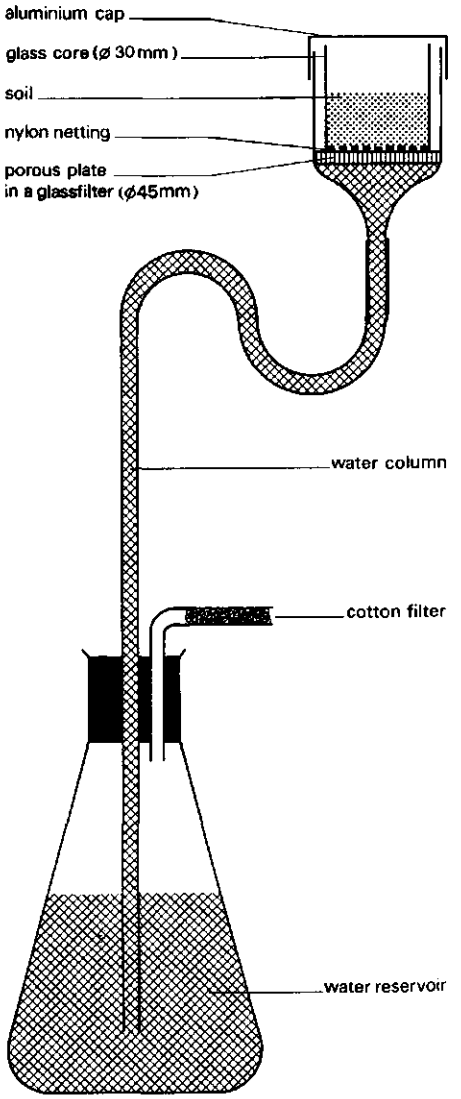

Fig. 2. Equipment to establish soil portions with a given soil water potential.

netting, were filled with soil corresponding to 10 g dry weight. After saturation of the soil, the glass cores were placed on the porous plate and the desired water potential was obtained by varying the hight of the hanging water column. The soil portions were protected against extensive evaporation with an aluminium cap. After 14 days, when the water potential was established, soil moisture contents were determined by weighing the soil portions.

With this system it was possible to establish the water potential under sterile conditions. The entire equipment as shown in Figure 2, exclusive the glass core, was sterilized in separate plastic bags by γ-irradiation (2.5 Mrad). Glass cores with nylon netting were autoclaved in glass containers and aseptically filled with irradiated soil, saturated, and placed on the porous plates, which were then closed with a large sterile plastic bag.

Bacterial strain. *R. leguminosarum* biovar *trifolii* R62::Tn5 with resistance to kanamycin (Km) and rifampicin (Rp) [19,36] was used as a model organism. Bacterial suspensions used for inoculations were cultured in yeast extract mannitol broth [19] supplemented with 25 mg/l Km. After growing for 2 days at 29°C on a rotary shaker, the bacterial suspension was washed by centrifugation (7000 x *g*, 15 min), and resuspended in sterile demineralized water.

Natural soil experiment. Glass cores were filled with the loamy sand

and the silt loam, corresponding to 10 g dry weight. Part of the soil portions were pressed by hand in order to obtain higher bulk densities. All soil portions were then saturated during 1 day with rhizobial cells in as much sterile demineralized water that approx. 4-6 x 10^7 cells/g dry soil were obtained in the saturated soil. Glass cores were weighed and placed on the porous plates with hanging water columns of 50, 100 and 200 cm corresponding to pF values of 1.7, 2.0 and 2.3 and incubated at 15°C in the dark.

Moisture contents and bulk densities of the soil portions were determined after 14 days and approximately 70 days later, when population sizes were expected to be more or less stabilized [37], numbers of bacteria were determined by dilution plating [36]. Rhizobial cells were enumerated on plates containing yeast extract mannitol agar (YMA) [19] supplemented with 50 mg/l Km, 20 mg/l Rp, 100 mg/l cycloheximide and 50 mg/l benomyl, whereas total bacterial populations were enumerated on nutrient agar.

Sterilized soil experiment. A similar experiment as in natural soil was carried out in sterilized soil under sterile conditions throughout the experiment. The inoculum density was 1-3 x 10^8 cells/g dry soil. Rhizobial cells were enumerated after 14 days on YMA when populations were expected to be stabilized [37]. The absense of other microorganisms was checked on tryptone soya agar (3.75 g tryptone, 1.25 g soya peptone, 1.25 g NaCl, 13 g agar, 1000 ml water). Rhizobial cells did not grow on this medium.

Statistical analyses. The effect of pressing soil portions on bulk density and the effect of bulk density and water potential on moisture content were examined with analysis of variance. Least significant differences (LSD) were calculated for significant levels a=0.05. The effect of moisture content, sterility and bulk density on the logarithmic number of rhizobial cells was analysed with linear regression analysis. Total bacterial population size was analysed separately with linear regression analysis.

Estimation of pore volume and surface area. The effective pore neck diameter can be approximately expressed as:

$$pF = \log 0.15 - \log r,$$

where r is the radius of curvature of the capillary pore (cm) and the water potential, pF, is the head expressed as log(cm H_2O) of the energy of water holding at 15°C [42,44]. The relationship assumes that the contact angle between water and soil solids is zero and that pores are

cylindrical [34]. Pore volume and moisture content are related if no swelling occurs during saturation of the soil and when pore water is replaced by air without shrinkage when the pF value increases. Pore volume corresponding to different pore neck diameters can then be calculated from the retention function (Fig. 1).

For estimation of the pore surface area, a distribution in pore classes of equal diameter was made by deviding the entire water retention function into steps of 0.1 on the pF scale. Pores were assumed to be cylindrical with length 1 (cm) and radius r (cm), thus having a volume of $1 \times \pi r^2$ and a surface area of $1 \times 2\pi r$. Then, the surface area of each pore class can be calculated by:

$$\text{surface area} = 2 \times \text{volume} \times r^{-1}$$

Pore volume and surface area were also expressed in numbers of rhizobial cells that, theoretically, can occupy the pore space. The pore space needed for 1 bacterial cell was assumed to be 1 μm^3 and 1.5 μm^2, and pores <0.8 μm were expected to be too narrow for the cells to entre.

8.3 RESULTS

Bulk density and soil moisture content. By pressing the soil portions, bulk density was increased significantly ($P<0.05$), resulting in significant ($P<0.05$) lower moisture contents for pressed soil at saturation (Table 2). However, in the loamy sand only the volume of pores with pore necks >60 μm (pF 1.7) had decreased, since the soil moisture content of the pressed soil had not decreased at a water potential of pF 1.7. In the pressed silt loam the volume of larger pores had decreased, but moisture

Table 2. Bulk density and moisture content of the loamy sand and the silt loam established to different water potentials.

	bulk dens. g/cm^3	moisture content (w/w) pF 0 saturated	pF 1.7 50 cm	pF 2.0 100 cm	pF 2.3 200 cm
loamy sand	1.33	41.5	28.0	15.9	11.9
loamy sand p [1)]	1.42	39.0	27.7	15.8	12.1
LSD(0.05)	0.02	0.9		1.0	
silt loam	0.89	80.1	39.6	35.6	32.9
silt loam p [1)]	1.11	60.7	41.7	36.7	34.0
LSD(0.05)	0.02	1.1		1.0	

[1)] p = pressed soil

content at pF 2.3 and thus the volume of pores with pore necks <15 μm had increased. In the two experiments the moisture contents equivalent to pF 1.7, 2.0 and 2.3 corresponded well to the values of the water retention functions in Figure 1 for the loamy sand, whereas in the silt loam the values in the two experiments were lower as compared to Figure 1.

Population size. Linear regression analysis explained >99% of the variance between numbers of rhizobial cells at different soil moisture contents (Fig. 3). Rhizobial numbers decreased significantly ($P<0.05$) in the sterilized loamy sand when moisture contents increased, but in the silt loam rhizobial numbers were unaffected by the moisture content. A more pronounced decrease ($P<0.05$) of rhizobial numbers was detected in

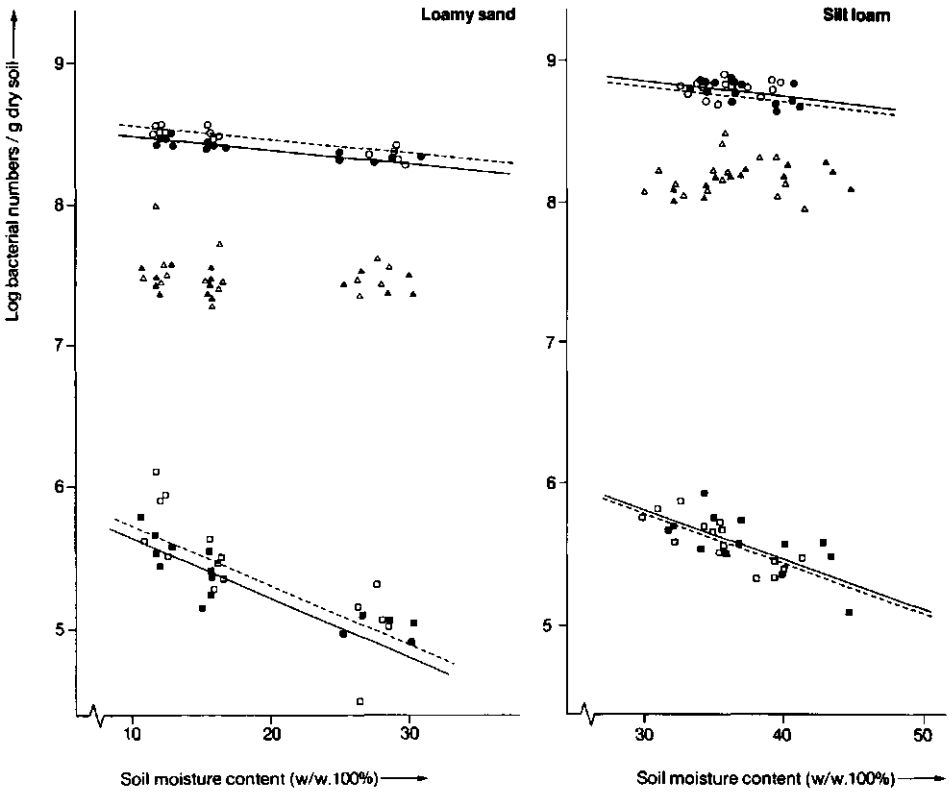

Fig. 3. Numbers of *R. leguminosarum* biovar *trifolii* and total bacterial populations in the loamy sand and the silt loam at moisture contents equivalent to pF 2.3-1.7. Rhizobial cells in sterilized soil (○,●) and in natural soil (□,■), and total bacterial population in natural soil (△,▲). Open symbols with dotted lines present the non-pressed and closed symbols with solid lines present the pressed soil samples.

both natural soils. Rhizobial numbers at moisture contents equivalent to pF 2.0 were 2.5-3.2 x 10^8 and 5-6.3 x 10^8 cells/g dry soil for the sterilized loamy sand and silt loam, and 2-4 x 10^5 and 3-6 x 10^5 cells/g dry soil for the natural loamy sand and silt loam, respectively. Bulk density had only effect on bacterial numbers in the loamy sand ($P<0.05$).

No significant influence of moisture content was found for the total bacterial numbers in both natural soils, which had an average number of 3 x 10^7 and 1.6 x 10^8 colony forming units/g dry soil in the loamy sand and the silt loam, respectively (Fig. 3).

Pore volume and surface area. Pore volume and surface area, calculated from the water potential functions given in Figure 1, are summarized for the pores with a pore neck diameter <0.8, 0.8-3, 3-30, >30 μm (Table 3 and 4).

Table 3. Pore volume and surface area of the loamy sand and the silt loam.

pF	pore neck diameter	loamy sand volume (cm^3/g)	loamy sand surface (m^2/g)	silt loam volume (cm^3/g)	silt loam surface (m^2/g)
>3.6	<0.8 μm	0.071	>>>	0.306	>>>
3.6-3	0.8-3 μm	0.020	0.055	0.026	0.092
3-2	3-30 μm	0.067	0.021	0.114	0.027
<2	>30 μm	0.201	0.015	0.155	0.011

Table 4. Pore volume and surface area in the loamy sand and the silt loam expressed in bacterial cells that, theoretically, can occupy the pore space.

pF	pore neck diameter	loamy sand volume[1] (cells/g) x 10^{10}	loamy sand surface[1] (cells/g) x 10^{10}	silt loam volume[1] (cells/g) x 10^{10}	silt loam surface[1] (cells/g) x 10^{10}
>3.6	<0.8 μm	0[2]	0[2]	0[2]	0[2]
3.6-3	0.8-3 μm	2.0	3.7	2.6	10.2
3-2	3-30 μm	6.7	1.4	11.4	1.8
<2	>30 μm	20.1	0.8	15.5	0.7
accessible pore space		28.8		29.5	
habitable pore space		8.7		14.0	
protective pore space		3.4		4.4	

1) pore volume and surface area needed for 1 bacterial cell was assumed to be 1 μm^3 and 1.5 μm^2, respectively
2) minimum pore neck diameter was assumed to be 0.8 μm

8.4 DISCUSSION

In contrast to the hypothesized increase of bacterial numbers when more water-filled pores are present, experimental values showed constant or decreasing numbers of rhizobial cells in sterilized soil when moisture content increased equivalent to pF values from 2.3 to 1.7. The detected decrease of cell numbers in sterilized loamy sand at a higher moisture content can be explained by oxygen limitation in part of the soil. The soil was sieved <2 mm, thus soil aggregates >1 mm, which are found to be partly anaerobic at pF 2 [11] are present. In natural soil, biotic factors are expected to play a role in addition to oxygen limitation, since the decrease in rhizobial numbers with increasing moisture contents, was more pronounced in the natural than in the sterilized soil. From predators such as protozoa it is known that they are more active at higher soil moisture contents [12,26]. A decrease of introduced bacteria with increased moisture contents was previously detected [6,21,32,35] and an optimum water potential of pF 2.8-2.5 has been found [6,21]. The total bacterial population size, however, did not decrease with increasing moisture contents, similar to results of Seifert [41] and Howie [21].

Bulk density affected rhizobial numbers as well as the total bacterial population size only little. In the loamy sand with a higher bulk density, only the volume of pores >60 μm diminished and rhizobial numbers were little lower as compared to the lower bulk density. In the silt loam the volume of pores <15 μm had increased by pressing the soil, but no significant influence on rhizobial numbers was found. Since mainly larger pores diminished by pressing the soil, results might be influenced by oxygen limitation.

To improve the understanding of these results, the available pore space was estimated for various pore neck diameter classes by using the water retention function (Fig. 1). The calculated values of the pore volume and surface area are only estimations, since pores are not cylindric. Moreover, at water potentials of pF >3 the water content - water potential relationship in soil is dominated by surface area adsorbtion effects [34]. A realistic value for the surface area of pores <0.8 μm, which is expected to be extremely large, cannot be given with the method used. Mercury porosimetry and gas adsorbtion techniques might be usefull techniques for the assessment of the size distribution of such small pores, but these techniques are not yet fully explored for soil systems (L.K. Koopal, pers. communication). With backscattered electron scanning

images, which has been applied for the characterization of the soil pore network, only pores larger than 3 μm have been studied [5,13,22]. Therefore, the pore size distribution obtained from the water retention is used for a first estimation of available pore space for bacteria.

For this study, only the part of the pore space suitable for bacteria to survive is of interest. Total, accessible, habitable and protective pore space are distinguished (Fig. 4). Pores must at least be accessible for the bacterial cells. In natural soils, smallest pores which were colonized had a diameter of 0.8 μm [24]. Rhizobial cells measure 0.5-0.9 x 1.2-3.0 μm [23] and a pore neck diameter of >0.8 μm would be sufficient to enter pores. Assuming a cell volume of 1 μm^3 for the introduced cells, only 0.11 and 0.21% of the accessible pore space (Table 4) was occupied in the sterilized loamy sand and silt loam at pF 2. These percentages agree quite well with the occupied pore volumes calculated to be 0.1% [2] or 0.4% [8].

The habitable pore space, defined as the pore space suitable for the cells to survive, is additionally determined by the presence of water (Fig. 4). At pF 2, pores >30 μm have been drained, therefore the habitable pore space is estimated by using only the volume of pores between 0.8 and 30 μm in diameter (Table 4). Part of the drained pores might have a sufficient waterfilm for bacteria to survive, but the surface area of these pores is relatively small as compared to the rest of the habitable pore space. Thus, it can be calculated that the number of rhizobial cells present in the sterilized loamy sand and silt loam at pF 2, occupied only 0.37 and 0.44% of the habitable pore space, respectively.

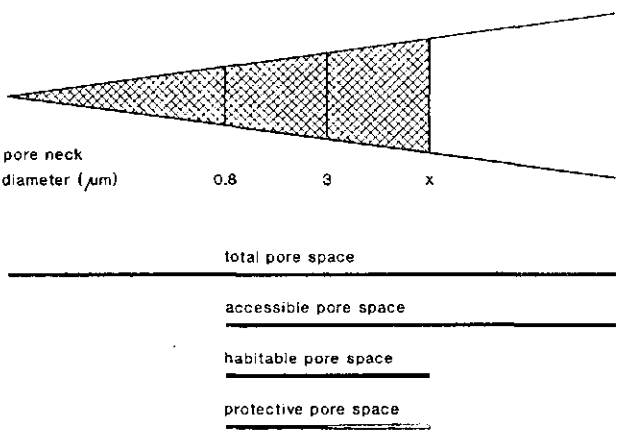

Fig. 4. Schematic presentation of total, accessible, habitable and protective pore space. XXXXX represent pores filled with water and x is the pore neck diameter that is still water-filled at the pF value used (x is 30 μm at pF 2). ▬▬ and ═══ indicate if pore volume or pore surface area of a certain pore diameter class are expected to be important.

In natural soil the situation is more complicated, since association of cells with soil particles is found to be important for the survival of introduced as well as indigenous bacteria [33,39]. Nioh and Furusaka [31] detected that most bacteria in wider pores are adsorbed to surfaces, whereas part of the bacteria in smaller pores occured freely. Increased percentages of particle-associated bacteria were detected in the presence of protozoa [38]. Particle-associated bacteria are expected to be better protected against predation, either as a result of enclosure in pores inaccessible to predators [14,38,44], or possibly by attachment to surface areas. Thus, in natural soil only part of the habitable pore space offers protection. This protective pore space might be estimated by the volume of pores between 0.8 and 3 μm and the surface area of pores between 3 and 30 μm (Fig. 4, Table 4) on the assumption that pores <3 μm are not accessible to predators. The rhizobial cells occupied only 0.001% of the protective pore space in both natural soils, however, a much larger part was occupied by other bacteria. Bacteria in different soils have been found to have a mean diameter of 0.6-0.75 μm [24]. Therefore, the same mean cell size for the total population as for rhizobial cells is used, resulting in an occupation of approx. 0.09 and 0.36% of the protective pore space in the loamy sand and the silt loam by culturable bacterial cells. Natural soils might contain large numbers of non-culturable cells, however, there are especially smaller sized cells that are non-culturable [3,7].

Although large parts of the accessible pore space are not suitable for bacteria to survive, and although large parts of the habitable pore space are not protected, habitable as well as protected pore space are not expected to be a limiting factor for the survival of bacterial cells, since in all cases less then 0.5% of the habitable and protective pore space were occupied by bacteria. The conclusion that pore space is, in general, not a factor limiting survival of bacteria in soil, explains the minor impact of increased water-filled pore volumes in sterilized soil, either as a result of increased moisture content, or, as in the silt loam, by increased bulk density. The presence of higher numbers of introduced bacteria [1,40] as well as of indigenous populations [18,40] after the addition of substrates, affirm that not pore space but substrate availability is one of the major limiting factors in soil. Nevertheless, bacteria are not evenly distributed through soil and it can not be excluded that locally, where substrate is present, pore space limits bacterial growth.

An interesting fact was that, eventhough the silt loam has a larger pore space, the relative occupance of this pore space was in all cases higher than in the loamy sand. This may be caused by a better substrate availability in the silt loam. Such an increased occupation of the pore space related to substrate, agrees with data of Hissett and Gray [20] who detected microscopically that only 0.02% of the soil mineral surface area but 0.17% of the organic matter was occupied by bacteria. Moreover, in a soil system with a continuous nutrient input through exudation by grass or wheat roots, 4-10% of the root surface area was covered by bacteria [16,28].

The fact that habitable and protective pore space are not limiting factors, does not mean, however, that they are unimportant for the survival of introduced bacteria. Upon introduction, cells will be distributed over the protective pore space and the non-protected part of the habitable pore space. If extensive translocation of bacterial cells does not occur, this spatial distribution will influence the survival of the introduced bacterial cells.

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SUMMARY AND CONCLUDING REMARKS

In this thesis the population dynamics of bacteria introduced into soil was studied. In the introduction, the existence of microhabitats favourable for the survival of indigenous bacteria is discussed. Knowledge about the distribution of introduced bacteria over such microhabitats, however, is scarse. Nevertheless, it was hypothesized that upon introduction, bacteria reach other microsites in soil than bacteria which are already present for some time, thereby influencing the survival of introduced organisms. Methods to study the distribution of introduced bacteria in soil, as well as the effect of their distribution on the population dynamics, were assessed. A model organism, *Rhizobium leguminosarum* biovar *trifolii* and two different soils, a loamy sand and a silt loam, were used for this purpose.

Two methods for the enumeration of bacteria introduced into soil were compared (Chapter 2). Although immunofluorescence was a very promissing method at the moment we started our work, selective plating proved to be more suitable for the enumeration of low numbers of introduced bacteria, since it had a lower detection limit than immunufluorescence. In addition, selective plating did not depend on flocculation processes which were shown to influence significantly the results obtained with the immunofluorescence technique. Moreover, only cells able to divide were counted. However, with the immunofluorescence technique we were able to determine cell lengths and we detected that the length of cells which were grown in a rich medium decreased after their introduction into soil.

To study the micro-distribution of bacteria in soil, different fluorochromes were tested on their ability to stain bacteria in thin sections of undisturbed soil samples. Calcofluor white M2R in combination with acridine orange was successfully applied for the detection of bacteria in thin soil sections (Chapter 3). However, specific staining of the introduced rhizobia with conjugated antiserum was not successful. Therefore, an alternative method for the assessment of the distribution of introduced bacteria, a soil washing procedure, was used in Chapters 4,

5 and 6. With this method, free occuring bacteria and bacteria associated with soil particles or aggregates >50 μm were distinguished.

The bacterial distribution through soil could be manipulated by inoculating soils at different initial moisture contents. At a lower initial moisture content, only the narrowest pores are filled with water, so that inoculated rhizobial cells will reach narrower pores when they are transported passively by the waterflow. At a higher initial moisture content, water already present in the narrower pores prevent the introduced cells from entering these pores. With such an inoculation procedure, rhizobial cells were found to be associated to a larger extent with soil particles when soils were inoculated at lower initial moisture contents. In natural soils, this resulted in an improved survival of rhizobia during at least 100 days after inoculation (Chapter 4). Moreover, the number of particle-associated cells decreased less than the number of free occuring cells in natural soil. It was concluded that rhizobial cells associated with soil particles or aggregates >50 μm occupied a more favourable microhabitat than free occuring cells. In sterilized soil, numbers of both particle-associated and free occuring cells increased and the initial differences in distribution did not result in different final population levels (Chapter 5). Therefore, it was concluded that the microhabitats in natural soil rendered protection to biotic rather than to abiotic factors.

The influence of competitors and predators on the distribution and population dynamics of rhizobium was studied by the addition of specific groups of organisms to sterilized soils (Chapter 5). Previous to inoculation with rhizobia, sterilized soils were recolonized with several bacterial isolates which were obtained from these soils and part of the soil portions were inoculated with a flagellate precultered on rhizobial cells. In the presence of flagellates, which predate on bacteria, a higher percentage of particle-associated rhizobial cells was present than in the absence of flagellates. In recolonized soils, i.e. in the presence of competitors, the percentages of particle-associated rhizobial cells were lower than in soils that were not recolonized previous to inoculation. Thus, the presence of competitors made it more difficult for rhizobial cells to colonize the microsites where they can be associated with soil particles or aggregates. The total number of rhizobial cells was influenced only little (silt loam) or not at all (loamy sand) by the competitors or by the addition of flagellates alone. However, when both competitors and predators were present, numbers of rhizobial cells

decreased drastically. This synergetic effect was explained by hypothesizing that after the predation of accessible bacterial cells by the flagellates, the regrowth of rhizobial cells will be limited by the presence of competitive microorganisms in many of the favourable microhabitats.

The association of rhizobial cells with soil particles may be the result of enclosure in pores or attachment to soil surfaces of rhizobia. The role of attachment was studied with a *R. leguminosarum* strain and three Tn5 mutants which were altered in their cell surface properties (Chapter 6). Although the importance of association with soil particles or aggregates was affirmed, the results gave no evidence that attachment to soil particle surfaces was an important factor for the survival of introduced cells.

The final population level of introduced rhizobia was studied in more detail by inoculating sterilized and natural soils with different inoculum levels (Chapter 7). In sterilized soils, populations reached, independent of the inoculum density, a final level which was suggested to represent the carrying capacity of the soils in terms of available habitable pore space, moisture and substrate for survival of the bacteria. In natural soil, however, the survival levels were dependent on the inoculum density. In this case, the chances of introduced cells to reach favourable microhabitats, determined the survival level of the entire population.

In all experiments final population levels in natural and in sterilized soils were higher in the silt loam than in the loamy sand (Chapter 2-8). Pore space which is suitable for bacteria to survive (=habitable pore space) or which protects bacteria from predation (=protective pore space) was estimated for both soils. The occupancy by bacteria was in all cases lower than 0.5%, so that no serious space limitation could be expected. Therefore, the larger water-filled pore volume at the water potential used (pF 2) in the silt loam as compared to the loamy sand, could not explain the differences in population sizes. In sterilized soil substrate availability was suggested to determine the final population level. In natural soil, however, the survival of rhizobial cells was suggested to be dependent on the number of introduced bacteria that reached the protective pore space (Chapter 4 and 7).

In this thesis it is shown that the soil washing procedure is useful for the study of the distribution of introduced bacteria. Immediately

after introduction, only few bacteria were associated with soil particles. The number of particle-associated bacteria decreased less pronounced than the number of free occuring bacteria, giving evidence that the distribution of introduced bacteria in soil is indeed an important factor influencing its survival. Moreover, the distribution could be manipulated by inoculating soil at different moisture contents. Inoculation of dryer soils, as well as the use of higher inoculum densities, resulted in higher survival levels, which could be well explained with the concept of distribution of cells over protective and non-protective pore space. The occurance of different population levels under apparently the same environmental conditions during incubation, suggests that extensive translocation in natural soil is absent.

The ability to manipulate the distribution and thereby to influence the survival of introduced bacteria, is important for the application of bacteria in soil. The availability of methods for biological control of soil-borne pathogens, nitrogen fixation and degradation of xenobiotics in soil, is depending on the possibility of introduced bacteria to establish in soil. The knowledge obtained in this research project can be used to improve the survival of bacteria introduced into soil.

The spatial distribution of bacteria through the soil matrix might also be a useful concept for other areas in soil(micro)biology. The occurance, for example, of genetransfer in different soil systems might be better understood when more details about bacterial distribution are available. Also the preservation of organic matter and the activity of predators will depend on the distribution of bacteria in the soil matrix.

SAMENVATTING EN SLOTOPMERKINGEN

Bacteriën kunnen aangewend worden ter bevordering van de plantaardige produktie, bijvoorbeeld door fixatie van atmosferische stikstof of door bestrijding van ziekten en plagen. Ook kunnen ze ingezet worden voor de afbraak van milieuvreemde stoffen. Echter, voor een succesvolle toepassing van bacteriën in de bodem is een goede overleving noodzakelijk. Het voorkomen van microhabitats die gunstig zijn voor de overleving van bacteriën, zoals organische stof en kleine poriën in aggregaten, is besproken in de inleiding van dit proefschrift. Kennis over de ruimtelijke verdeling van **geïntroduceerde** bacteriën over zulke microhabitats is echter schaars. Men mag aannemen dat bacteriën na hun introductie op andere plaatsen in de grond voorkomen dan bacteriën die reeds langere tijd aanwezig zijn en dat dit hun overleving beïnvloedt. Daarom is in eerste instantie gezocht naar een geschikte methode om de ruimtelijke verdeling van geïntroduceerde bacteriën in grond te onderzoeken. Vervolgens is de invloed van de ruimtelijke verdeling van geïntroduceerde bacteriën op hun overleving bestudeerd aan de hand van een model organisme, *Rhizobium leguminosarum* biovar *trifolii*. Dit onderzoek is uitgevoerd in twee verschillende grondsoorten, nl. zwak lemig zand en een kleigrond.

Twee methoden voor het kwantificeren van bacteriën na hun introductie in grond zijn onderling vergeleken (hoofdstuk 2). Hoewel immunofluorescentie een veel belovende techniek was op het moment dat het onderzoek startte, bleek uitplaten op selectieve media geschikter voor het tellen van lage aantallen geïntroduceerde bacteriën, ten gevolge van een lagere detectiegrens. Bovendien was de selectieve plaat methode niet afhankelijk van uitvlokprocedures zoals de gebruikte immunofluorescentie procedure en werden alleen levende cellen geteld. Met immunufluorescentie was het echter mogelijk om cellengtes te bepalen. Cellengtes bleken af te nemen nadat cellen vanuit een rijk medium in de grond gebracht waren.

Om de ruimtelijke verdeling van bacteriën in de grond te bestuderen werden verschillende fluorescerende kleurstoffen getest op hun vermogen om bacteriën in slijpplaatjes van ongestoorde grondmonsters te kleuren

(hoofdstuk 3). Bacteriën waren zichtbaar in de slijpplaatjes na kleuring met calcofluor white M2R in combinatie met acridine oranje, maar specifieke kleuring van geïntroduceerde bacteriën bleek niet mogelijk. Daarom is in hoofdstuk 4, 5 en 6 een alternatieve methode gebruikt, een gestandaardiseerde procedure van grond wassen, waarbij onderscheid gemaakt werd tussen vrij voorkomende bacteriën en bacteriën geassocieerd met grond. Geassocieerde bacteriën kunnen ofwel aangehecht zijn aan, danwel ingesloten zijn in gronddeeltjes of aggregaten.

De ruimtelijke verdeling van geïntroduceerde bacteriën in grond bleek afhankelijk te zijn van het vochtgehalte voor inoculatie. Bij een laag vochtgehalte zijn alleen de kleinste poriën met water gevuld en zullen de bacteriën tot in de kleinste poriën terecht komen als ze passief met de waterstroom meegevoerd worden. Als de grond voor inoculatie vochtiger is, is er al water aanwezig in de kleinere poriën, waardoor het binnendringen van deze watergevulde poriën door bacteriën belemmerd wordt. Inoculatie van bacteriën in droge grond resulteerde in een groter aantal met grond geassocieerde bacteriën. In niet gesteriliseerde grond had dit tot gevolg dat de overleving van bacteriën tot meer dan 100 dagen na inoculatie van droge grond beter was dan na inoculatie van natte grond (hoofdstuk 4). Bovendien nam in de niet gesteriliseerde grond het aantal met grond geassocieerde bacteriën minder sterk af dan het aantal vrij voorkomende bacteriën. Hieruit werd geconcludeerd dat associatie met grond gunstig was voor de overleving van de bacteriën. In gesteriliseerde grond namen zowel de vrij voorkomende als de geassocieerde bacteriën toe en verdwenen de verschillen in verdeling als gevolg van de inoculatie procedure na verloop van tijd (hoofdstuk 5). Daarom werd aangenomen dat het voordeel van associatie met grond, in de niet gesteriliseerde grond, het gevolg was van bescherming tegen biologische factoren.

De invloed van concurrentie en predatie op de ruimtelijke verdeling en op de populatie dynamica van geïntroduceerde bacteriën werd bestudeerd door herkolonisatie van gesteriliseerde grond met verschillende bacterie-isolaten, en door aan een deel van de grond een flagellaat voorgekweekt op rhizobium toe te voegen (hoofdstuk 5). In aanwezigheid van de flagellaat was het percentage met grond geassocieerde bacteriën hoger dan in afwezigheid van de flagellaat. In de aanwezigheid van concurrerende bacteriën was het percentage geassocieerde rhizobium cellen juist lager dan in hun afwezigheid. Geconcludeerd werd dat de aanwezigheid van concurrerende bacteriën, rhizobium belemmerde om de gunstige plaatsen, daar waar het met de grond geassocieerd kon zijn, te bereiken. Het totaal

aantal rhizobium cellen werd maar weinig beïnvloed door de aanwezigheid van concurrenten of predatoren alleen. Slechts wanneer beiden aanwezig waren, nam het aantal rhizobium cellen sterk af. Dit synergistische effect werd als volgt verklaard: na de predatie van bacteriën door de flagellaten is het vermogen van bacteriën om dit verlies te compenseren afhankelijk van hun concurrentie-vermogen. De hergroei van rhizobium cellen zal dus belemmerd worden als geschikte locaties voor groei al bezet zijn door concurrerende bacteriën.

De associatie van rhizobium cellen met grond kan het gevolg zijn van zowel aanhechting aan grondoppervlaktes als van insluiting in poriën. De rol van aanhechting werd bestudeerd met behulp van een *R. leguminosarum* stam en drie Tn5 mutanten met veranderde oppervlakte eigenschappen (hoofdstuk 6). Hoewel het belang van de associatie met grond bevestigd werd, gaven de resultaten geen aanwijzing dat aanhechting aan oppervlaktes een belangrijke rol speelde bij de overleving van geïntroduceerde bacteriën.

De uiteindelijke populatieomvang van geïntroduceerde rhizobium werd meer in detail bestudeerd door gesteriliseerde en niet gesteriliseerde grond te inoculeren met verschillende inoculumdichtheden (hoofdstuk 7). In gesteriliseerde grond werd, onafhankelijk van het inoculumniveau, een populatieomvang bereikt waarvan werd aangenomen dat het de capaciteit van de grond vertegenwoordigde ten aanzien van de combinatie van beschikbare porie-ruimte, vochtgehalte en substraat voor bacteriën. In niet gesteriliseerde grond was het overlevingsniveau echter afhankelijk van de inoculumdichtheid. In dit geval bepaalde de kans van geïntroduceerde bacteriën om gunstigere plaatsen, d.w.z. plaatsen die zowel bescherming bieden als waar substraat aanwezig is, te bereiken, de overleving van de gehele populatie.

In alle experimenten was de uiteindelijke populatieomvang, in niet gesteriliseerde en in gesteriliseerde grond, hoger in de kleigrond dan in de zandgrond (hoofdstuk 2-8). De porie-ruimte die geschikt is voor bacteriën om te overleven en de porie-ruimte die bacteriën beschermt tegen predatie in beide gronden werden geschat. De bezettingsgraad door bacteriën was in alle gevallen minder dan 0,5%, zodat ruimte alleen niet werkelijk limiterend is. Daarom kon een groter watergevuld porie-volume bij de gebruikte waterpotentiaal (pF 2) in de kleigrond t.o.v. de zandgrond, het verschil in populatieomvang niet verklaren. In gesteriliseerde grond wordt het populatieniveau zeer waarschijnlijk door de beschikbaarheid van substraat bepaald. In niet gesteriliseerde grond werd de overle-

ving bepaald door de verdeling van de geïntroduceerde bacteriën over de beschermde en de niet beschermde porie-ruimte (hoofdstuk 4) en door het absolute aantal geïntroduceerde cellen (hoofdstuk 7). Verplaatsing van rhizobium in niet gesteriliseerde grond werd gering geacht (hoofdstuk 4 en 7).

In dit proefschrift werd aangetoond dat de gestandaardiseerde procedure van grond wassen bruikbaar is voor het bestuderen van de ruimtelijke verdeling van geïntroduceerde bacteriën in grond. Direct na inoculatie waren weinig bacteriën geassocieerd met grond. Het aantal geassocieerde bacteriën nam minder sterk af dan het aantal vrij voorkomende bacteriën, wat aantoont dat de verdeling van geïntroduceerde bacteriën in grond inderdaad een belangrijke factor is die de overleving beïnvloedt. Bovendien kon de verdeling gemanipuleerd worden door bij verschillende vochtgehaltes te inoculeren. Inoculatie van drogere grond, maar ook het gebruikt van hogere inoculum-dichtheden, resulteerde in een hoger overlevingsniveau, wat verklaard kon worden met het concept van de verdeling van bacteriën over beschermde en niet beschermde porie-ruimte. De aanwezigheid van verschillende populatieniveau's tijdens gelijke incubatieomstandigheden, suggereert een geringe verplaatsing van bacteriën in niet gesteriliseerde grond.

De mogelijkheid om de verdeling van geïntroduceerde bacteriën te veranderen en daardoor ook de overleving te beïnvloeden, is belangrijk voor een succesvolle toepassing van bacteriën in grond. Het potentieel aan beschikbare methoden voor onder andere biologische bestrijding van bodemgebonden ziekten, stikstof fixatie en afbraak van milieuvreemde stoffen in de bodem, hangt in sterke mate af van de mogelijkheid van de geïntroduceerde bakteriën om zich in voldoende mate te handhaven. De kennis die in dit onderzoek is opgedaan, kan aangewend worden om de overleving van geïntroduceerde bacteriën in grond te verbeteren.

Kennis omtrent de ruimtelijke verdeling van bacterën in grond is ook belangrijk voor andere aspecten van de bodem(micro)biologie. Het optreden van bijvoorbeeld genoverdracht in verschillende grondsystemen zou beter begrepen kunnen worden indien meer bekend is omtrent de verdeling van bacteriën in grond. Ook de opbouw en afbraak van organische stof en de aktiviteit van predatoren zijn mede afhankelijk van de verdeling van bacterien in de grond.

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CURRICULUM VITAE

Joeke Postma werd op 10 oktober 1958 geboren te Leiden. Na het behalen van het Atheneum-B diploma op het Zernike College te Groningen, werd in datzelfde jaar gestart met de studie planteziektenkunde aan de Landbouwhogeschool te Wageningen. Het vakkenpakket in de doctoraal fase bestond uit fytopathologie, nematologie en bodemvruchtbaarheid en bemesting. De praktijktijd werd verricht bij Statens Plantevern te Aas in Noorwegen en bij de buitendienst van de Plantenziektenkundige Dienst te Nijmegen. Na afronding van de studie in juni 1983, heeft de auteur ca. een jaar bij de vakgroep Algemene en Regionale Landbouwkunde en vervolgens ca. een jaar bij het Instituut voor Planteziektenkundig Onderzoek gewerkt. Oktober 1985 startte de auteur met een onderzoek naar de overleving van geïntroduceerde bacteriën bij de Stichting Ital, hetgeen geresulteerd heeft in dit proefschrift. Dit onderzoek werd uitgevoerd in het kader van een Duits-Nederlands samenwerkingsproject, waarbij een deel van het onderzoek werd verricht op het Institut für Bodenbiologie van de FAL te Braunschweig. Vanaf 1 augustus zal de auteur wederom werkzaam zijn bij het Instituut voor Planteziektenkundig Onderzoek te Wageningen.