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Role of Microniches in Protecting Introduced *Rhizobium leguminosarum* biovar *trifolii* against Competition and Predation in Soil

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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 samples was inoculated with the flagellate *Bodo saltans*, precultured on rhizobial cells. The introduced organisms were enumerated in different soil fractions by washing the soil, using a standardized washing procedure. With this method, free organisms and organisms associated with soil particles or aggregates >50 μm were separated. The total number of rhizobia was influenced slightly (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 rhizobia decreased drastically. This decrease was more than the sum of both effects separately. Nevertheless, populations of rhizobia were still higher than in natural soil. In the presence of flagellates, higher percentages of rhizobia 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 rhizobia were lower than in soils not recolonized previous to inoculation. Thus, the presence of other microorganisms hindered rhizobial colonization of sites where they are normally associated with soil particles or aggregates.

Survival of rhizobia introduced into natural soil was shown to be influenced by their spatial distribution in soil (20). Upon introduction into relatively dry soils, higher numbers of cells were found to be associated with soil particles or 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, when smaller pores are not yet water filled, the inoculated rhizobia will reach narrower pores when they are transported passively by the water flow. Thus, more cells will reach the inner part of aggregates (20, 26). Capillary pores up to 6 μm in diameter are suggested to be the most favorable microhabitats for bacteria (11). One reason may be that bacteria in smaller pores may be protected from predation by protozoa (4, 20). Also, abiotic conditions may be more favorable 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, nontarget groups might also be affected, and specific protozoacides are not yet available (14, 15). Cycloheximide, an inhibitor of protein synthesis in eucaryotes, has only a limited effect on protozoa when applied to soil (9, 15). 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 analyzed in this way.

In the present study, the dynamics of a model organism, *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 rhizobia. The influence of competition and predation on the association of rhizobia with soil and on their population dynamics 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 we recognized that interactions other than competition and predation might occur, the added components will be referred to as competitors and predators, respectively.

MATERIALS AND METHODS

Soils. Two Dutch arable soils, a loamy sand and a silt loam (19, 20, 25), 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 (3.25 g of Oxoid nutrient broth and 13 g of agar in 1,000 ml of water, pH 7.2).

Recolonization 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 nutrient agar. These microorganisms were transferred into a soil extract solution without further identification. The loamy sand isolates were grown for 3 days as a mixed population in loamy sand extract (17) and were used to inoculate the

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sterilized loamy sand. A similar procedure was followed for the silt loam, by growing the isolates obtained from silt loam in silt loam extract. The inoculated loamy sand and silt loam were moistened up to 15 and 40% moisture content, respectively, which corresponded to a water potential of approximately −10 kPa. The soil was then incubated for at least 4 weeks at room temperature and dried again to 1.3 and 4% moisture content. Numbers of microorganisms in these recolonized dried soils, enumerated by dilution plating on nutrient agar, were between 8×10^7 and 3×10^8 CFU per g of dry soil. The absence of protozoa was demonstrated with the most probable number method described below.

Culturing and enumerating rhizobia. *R. leguminosarum* biovar *trifolii* R62::Tn*5* resistant to kanamycin and rifampin (12, 19) was used. The bacterium was cultured in yeast extract mannitol broth (12) supplemented with 25 mg of kanamycin per liter. After growing for 2 days at 29°C on a rotary shaker, the cells were washed by centrifugation (7,000 × *g*, 15 min) and suspended in sterile deionized water.

Numbers of rhizobia were determined on plates containing yeast extract mannitol agar supplemented with 50 mg of kanamycin per liter, 20 mg of rifampin per liter, and 100 mg of cycloheximide per liter. These concentrations of antibiotics were sufficient to enumerate rhizobial numbers up to 10^4 CFU/g of dry soil.

Culturing and enumerating protozoa. A flagellate, *Bodo saltans* (23, 27), approximately 3 by 8 μm, was used. During one year, the flagellates were regularly diluted in sterile deionized water with freshly grown rhizobial cells. After growing for several days at room temperature, the highest dilution containing flagellates was diluted again. In this way a uniprotozoan population with a minimum of microorganisms other than *R. leguminosarum* biovar *trifolii* was obtained. We did not succeed in eliminating the other microorganisms completely, although antibiotics were used. Nonrhizobial cells could be enumerated in the presence of high densities of rhizobial cells by dilution plating on tryptone soya agar (3.75 g of tryptone, 1.25 g of soya peptone, 1.25 g of NaCl, 13 g of agar, 1,000 ml of water), since rhizobia did not grow on this medium. To inoculate the soil, flagellates were cultured in sterile deionized water with rhizobial cells. Part of this suspension was added to the rhizobial inoculum just before inoculation of the soils.

Protozoa in suspensions were enumerated in microdilution plates by using a most probable number method (5). With a microdiluter (Titertek), fourfold dilutions with eight replicates were made in amoeba saline (120 mg of NaCl, 4 mg of $MgSO_4 \cdot 7H_2O$, 4 mg of $CaCl_2 \cdot 2H_2O$, 142 mg of Na_2HPO_4, 360 mg of KH_2PO_4, 1,000 ml of water) containing approximately 10^6 rhizobia per ml. The presence of protozoa was determined microscopically after an incubation period of 14 to 28 days at 15°C.

Experimental design. Soil samples of sterile (S) and recolonized sterilized soil (SC), corresponding to 10 g (dry weight), were incubated in glass cylinders (diameter, 30 mm), which were closed by autoclavable plastic (polypropylene, bottom) and aluminum caps (top). Deionized water was added to the soil samples 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 (20). After moistening, the soil samples were incubated in a moisture chamber for 2 days at 4°C to allow moisture equilibration. The added water in the silt loam samples spread by capillary forces only, whereas the loamy sand samples were mixed with a spatula prior to incubation. The soil samples were then inoculated with rhizobia or with a mixture of rhizobia and flagellates (+F), resulting in four treatments: S, SC, S + F and SC + F. The inocula were added in sufficient deionized water so that after inoculation the loamy sand and the silt loam contained 16 and 45% moisture, respectively, which corresponded with the field capacity (−10 kPa). Numbers of inoculated rhizobia and flagellates were 0.6×10^8 to 1×10^8 and 7×10^3/g of dry soil, respectively. The number of nonrhizobial cells added with the flagellates was 4×10^5/g of dry soil. Again, only the loamy sand samples were mixed with a spatula, and both loamy sand and silt loam samples 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 also.

Sampling procedure. Duplicate soil samples were transferred into 250-ml Erlenmeyer flasks and shaken lightly (3 min, 150 rpm at a diameter of 32 mm) in sterile deionized water as described by Postma et al. (20). Thereafter, the suspensions were decanted carefully. This procedure was repeated four times and resulted in a 500-ml washing suspension which contained soil particles approximately <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 soil remaining after the entire washing procedure (>50 μm) was shaken thoroughly (10 min, 280 rpm) with gravel (diameter, 2 to 4 mm) in 95 ml of 0.1% sodium pyrophosphate in order to suspend the particle-associated organisms. Numbers of rhizobia, other microorganisms, and flagellates in the different fractions were determined by dilution plating on yeast extract mannitol agar with appropriate antibiotics, by dilution plating on nutrient agar, and by the most probable number method, respectively. The amount of the remaining soil was weighed after filtration and drying at 105°C for one day.

Statistical analyses. The effects of initial moisture content and incubation period were studied separately, per treatment, with analysis of variance. A total analysis of variance was then carried out to analyze the effects of competition, predation, and initial moisture content by 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, the least significant quotient (LSQ) values are given ($LSQ = 10^{LSD}$).

RESULTS

Effects of initial moisture content on rhizobial numbers. One day after inoculation, the total numbers of rhizobia were similar within each treatment (Fig. 1). After 28 and 56 days of incubation, total numbers of rhizobia were unaffected by the three 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, significant differences ($P < 0.05$) due to moisture contents were present but the differences were most pronounced in treatment SC.

In the silt loam 1 day after inoculation, the numbers of rhizobia associated with soil particles or aggregates >50 μm (Fig. 2B) were significantly higher ($P < 0.05$) at the lowest

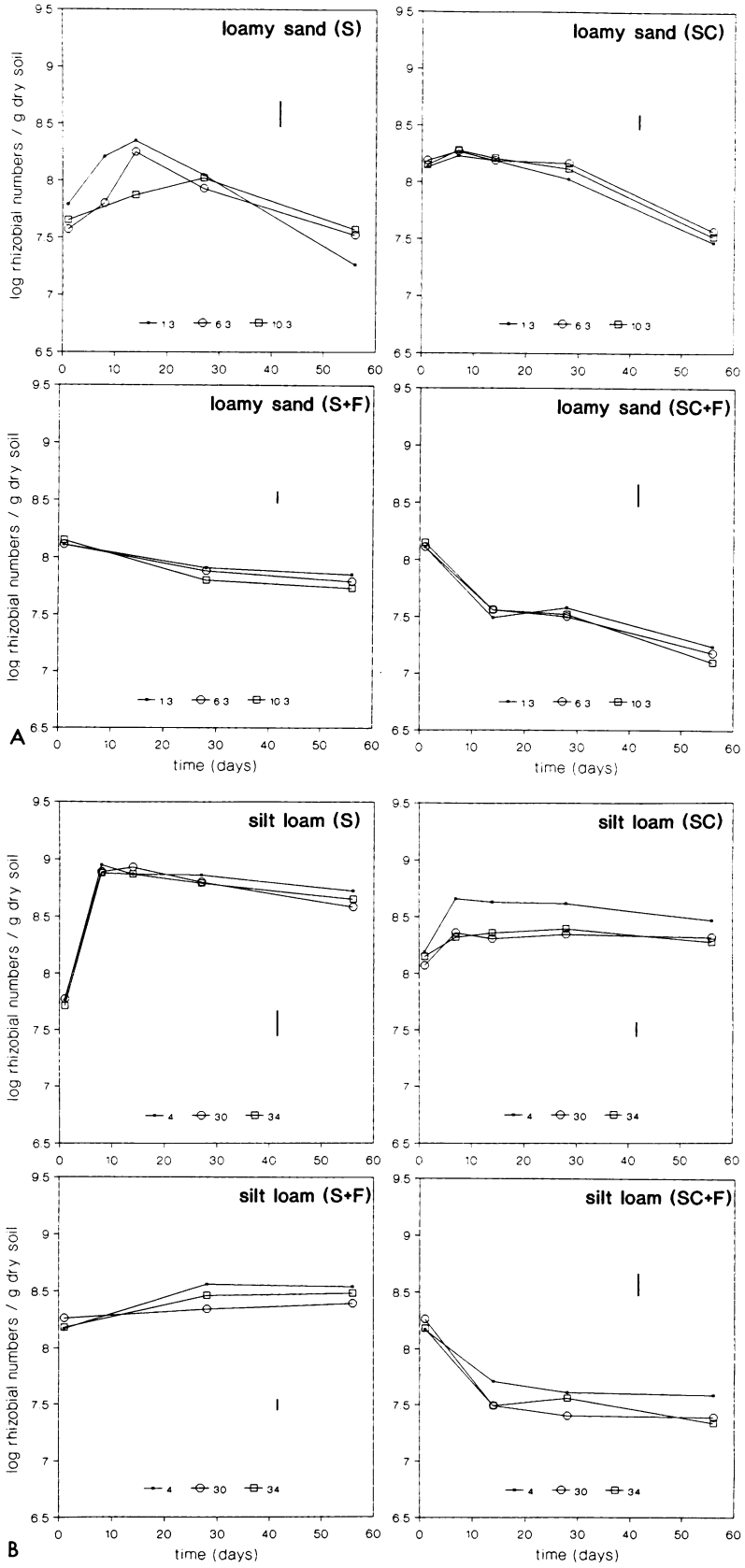

FIG. 1. Total number of rhizobia in sterilized (S) and recolonized (SC) loamy sand (A) and silt loam (B) with (+F) or without added flagellates, inoculated at different initial moisture contents (●, ○, and □ in wt/wt × 100%). Bars indicate LSD for $a = 0.05$.

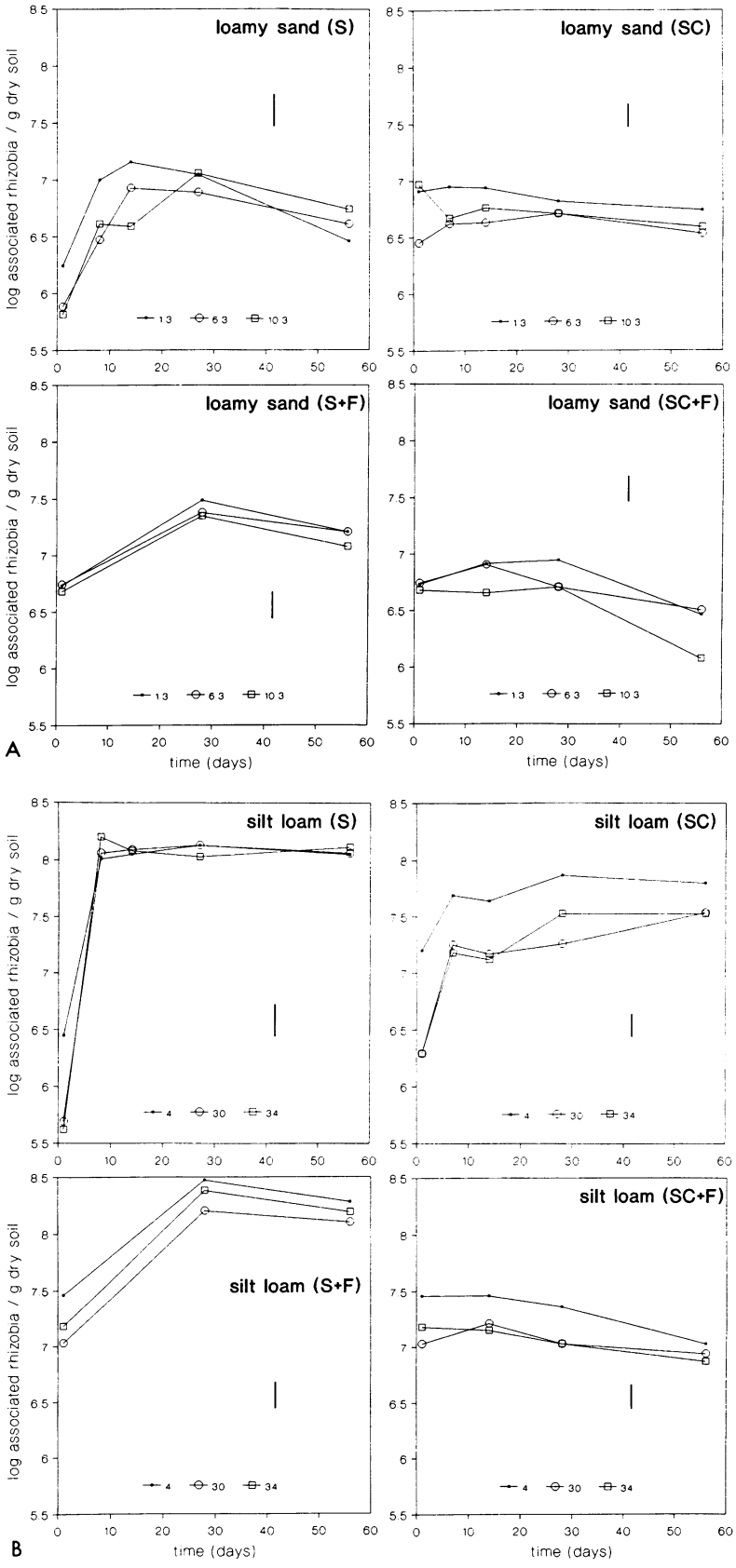

FIG. 2. Number of rhizobia associated with soil particles or aggregates >50 μm in sterilized (S) and recolonized (SC) loamy sand (A) and silt loam (B) with (+F) or without added flagellates, inoculated at different initial moisture contents (●, ○, and □ in wt/wt × 100%). Bars indicate LSD for $a = 0.05$.

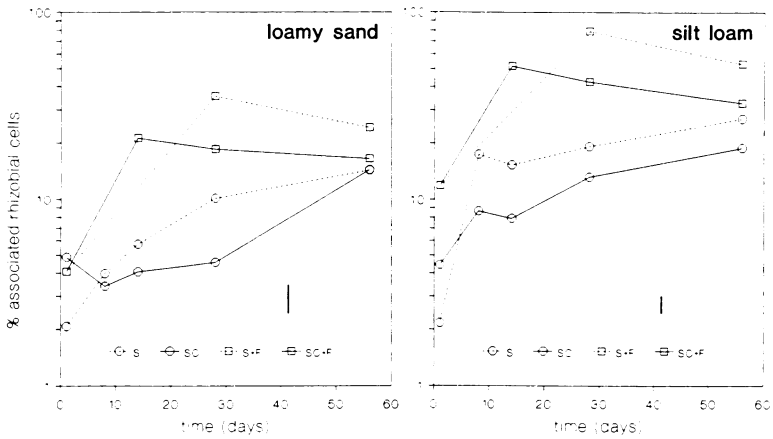

FIG. 3. Percentages of rhizobia associated with soil particles or aggregates >50 μm in sterilized (S) and recolonized (SC) loamy sand and silt loam with (+F) or without added flagellates. Mean values for the different moisture contents. – – – –, S; ——, SC; ○, no flagellates added; □, +F. Bars indicate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

initial moisture content than at the two higher initial moisture contents. The differences in numbers of particle-associated rhizobia 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, until the end of the incubation in treatment SC.

In the loamy sand, the dynamics of the number of rhizobia associated with larger soil particles did not show such a consistent pattern (Fig. 2A). Significant differences upon initial moisture content in the loamy sand occurred in treatments SC and SC + F, although in the latter treatment the initial moisture content of 6.3% did not always result in a lower number of particle-associated rhizobia as compared with 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 rhizobia after 28 and 56 days of incubation were approximately 5×10^7 and 5×10^8/g of dry soil, respectively (Fig. 1). In the loamy sand, total numbers of rhizobia were significantly reduced when both competitors and predators were added (treatment SC + F). 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 lower numbers of rhizobia as compared with nonreinoculated sterilized soil but the addition of both resulted in an even larger reduction of the rhizobial population. Rhizobial numbers decreased during the overall incubation period only when both competitors and predators were added.

The numbers of rhizobia in the washing suspension (particles <50 μm) and in the suspension with soil particles <2 μm showed a pattern similar to that of the total numbers of rhizobia (data not shown).

The numbers of rhizobia associated with soil particles or aggregates >50 μm showed a different pattern. In the loamy sand as well as in the silt loam, the highest numbers of particle-associated rhizobia 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 the treatments, SC + F, SC, S, and S + F, increasing numbers of particle-associated rhizobia were found on days 28 and 56 (Fig. 2B).

The number of particle-associated rhizobia as a proportion of the total number of rhizobia increased during the incubation period and was 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 addition of competitors resulted in a significant reduction of the percentages of particle-associated rhizobia by a factor 0.60 and 0.62 in the loamy sand and the silt loam, respectively. When flagellates were added, relatively more rhizobia were particle-associated as compared with the treatments without predators. Differences were a factor of 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 of 2 in the recolonized soils. The numbers of introduced nonrhizobial cells in 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 three initial moisture contents are presented in Table 1.

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

Dynamics of the predators. No significant effect of initial moisture content on the number of flagellates was detected. Therefore, only mean numbers are presented in Table 2. After inoculation with 7×10^3 flagellates per g of 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 too low for reliable counts, and 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 rhizobia (Fig. 3).

TABLE 1. Total numbers, numbers, and percentages of particle-associated microorganisms in SC, S + F, and SC + F

Type of soil and treatment	No. of microorganisms per days of incubation[a]					
	Log total		Log associated		% Associated	
	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	

[a] LSD, $\alpha = 0.05$; LSQ, $\alpha = 0.05$.

DISCUSSION

The population dynamics of rhizobia and their association with soil were first assessed in the absence of other organisms. Inoculating sterilized soils at increasing moisture contents showed that decreasing numbers of rhizobia were associated with soil particles or aggregates, similar to results previously found in the natural soil (20). However, in contrast with earlier observations in natural soil, the differences in numbers of particle-associated rhizobia in sterilized soil disappeared rather quickly during incubation and significant differences in survival were not detected. Thus, the maintenance of these differences in association with soil 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, 8, 9, 21) and to competition with other microorganisms (10, 13, 18). Other possibilities, such as microorganisms capable of producing antibiotics or lytic enzymes, bacteriophages, and *Bdellovibrio* spp., were suggested to be less important (1, 8, 21). 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 samples, flagellates (precultured 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. However, the differences due to the initial moisture content were more pronounced in the recolonized soils than in the soils inoculated with predators only. Thus, competition with other microorganisms may be an important factor in maintaining the differences in association of rhizobia with soil due to the initial moisture contents.

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 about the roles 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 association of rhizobia with soil 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. Rhizobia then have to compete for substrates and habitable pore space with the large numbers of microorganisms already present. This could explain why in the recolonized soil, lower percentages of rhizobia were associated with soil particles and aggregates as compared with 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 (7, 28), introduced rhizobia were shown to be less competitive (7, 18). 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, 24).

Predators caused the opposite effect on the association of the rhizobia with soil as did the competitors: the percentages of particle-associated rhizobia 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 preda-

TABLE 2. Total numbers, numbers, and percentages of particle-associated flagellates in S + F and SC + F

Type of soil and treatment	No. of microorganisms per days of incubation[a]					
	Log total		Log associated		% Associated	
	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	

[a] ND, Not detectable; LSD, $\alpha = 0.05$; LSQ, $\alpha = 0.05$.

tors. This agrees with the concept that particle-associated bacteria are found in smaller pores which are physically protected from predation (4, 20, 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, personal communication).

It is surprising that recolonization and addition of flagellates alone had little effect on the total number of rhizobia. 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 those in the treatment where flagellates were added to the recolonized soils. This could be due to the presence of a different microbial population or to the sequence of introduction or both (3, 24).

Our results indicate that rhizobia inoculated in nonrecolonized sterilized soil were able to compensate for the loss of ingested cells, since the absolute number of particle-associated rhizobia was higher in this treatment than in sterilized soil without flagellates. This higher number of particle-associated rhizobia can be explained by regrowth in combination with selection of particle-associated rhizobia, since active attachment of rhizobia as a direct response to the presence of predators is not likely. A compensation for the loss of cells due to predation by regrowth can be expected (1, 6, 10), since predation diminished bacterial numbers more effectively when bacterial growth was inhibited (10). In addition, protozoa have been shown to cause a higher respiration rate and N turnover (6, 16). Substrates produced by metabolic activity or death and lysis of protozoa may allow bacterial reproduction (22, 24).

The survival of rhizobia decreased drastically only when competitors and predators were both added to the sterilized soil. The fact that the population of rhizobia 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 bacteriovorous predators in soil, based on their higher numbers (2, 16), and combinations of predators increase the overall activity in soil (6).

The synergistic effect of the combination of competitors and predators may be explained in the following way. Flagellates predate mainly on accessible bacteria, i.e., bacteria outside the protective microniches, which resulted in an increased percentage of particle-associated rhizobial as well as nonrhizobial cells. The capability of the introduced rhizobia to compensate for the loss of ingested cells will be dependent on competitional factors. In the recolonized sterilized soil, however, the regrowth of rhizobia will be limited by the presence of more 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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