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Habitable Pore Space and Survival of *Rhizobium leguminosarum* biovar *trifolii* Introduced into Soil

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Abstract. The hypothesis that the population size of introduced bacteria is affected by habitable pore space was studied by varying moisture content and 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 −5 and −20 kPa (pF 1.7 and 2.3). Rhizobial cells were enumerated when population sizes were expected to be more or less stable. In sterilized soils, the rhizobial numbers were not affected or decreased only slightly when water potentials increased from −20 to −5 kPa. In natural soils, the decrease in rhizobial numbers with increasing water potentials was more pronounced. Bulk density had only minor effects on the population sizes of rhizobia or total bacteria. Soil water retention curves 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 rhizobial numbers showed that only 0.37 and 0.44% of the habitable pore space was occupied in the sterilized loamy sand and silt loam, respectively. The situation in natural soil is more complicated, since a whole variety of microorganisms is present. Nevertheless, it was suggested that, in general, pore space does not limit proliferation and growth of soil microorganisms.

Introduction

Each soil system has its own distinctive "biological space" with regards to the level of microbial biomass and enzyme activity [28], and bacteria introduced into sterilized soil reach a certain population level independent of inoculum density [25, 34, 40]. Availability of substrates, moisture, pore space [16, 26, 28], and lack of migration to new colonizing sites [25] have been suggested as determining these population levels. Also in natural soils, introduced bacteria often reach a certain survival level [7, 8, 34, 40], which is different for each soil system.

In previous studies on the population dynamics of *Rhizobium leguminosa-*

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

	Loamy sand	Silt loam
pH-KCl[a]	5.4	7.2
Organic matter[b]	3.3	3.5
$CaCO_3$[b]	0.1	8.2
Texture[b]		
<2 μm	4.4	35.0
2–16 μm	0.5	20.5
16–50 μm	7.6	26.7
50–105 μm	19.8	12.8
>105 μm	67.7	5.0

[a] In $-\log(H^+)$.
[b] In g/100 g dry soil.

rum 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 [33–35]. At the water potential used (−10 kPa), the finer textured silt loam contained 40 to 45% moisture, whereas the loamy sand contained only 16 to 20% moisture. A better survival in finer textured soils was also observed for other introduced bacteria [13, 27]. A similar relation was also found for the number of indigenous bacteria in different textured soils [3]. Although many soil factors may differ among 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 the difference in pore space and pore architecture might explain at least some of the observations mentioned above.

Pore size distribution, as well as the absolute volume of water, might influence the pore volume or surface area which is suitable for the survival and establishment of bacteria (= habitable pore space), as well as the part of the habitable pore space that protects bacteria from predation by protozoa (= protective pore space). Data about habitable and protective pore space in different soils, and the implications for population dynamics of bacteria are scarce, but it seems logical to suggest that habitable and protective pore space influences the population size of introduced bacteria in those cases in which 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 −50 and −300 kPa (pF 2.7 and 3.5) [6, 15].

In the present study *R. leguminosarum* biovar *trifolii* was used as a model organism to test the hypothesis that population size of introduced bacteria is affected by habitable pore space. Rhizobial cells were introduced into sterilized and natural (= nonsterilized) 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.

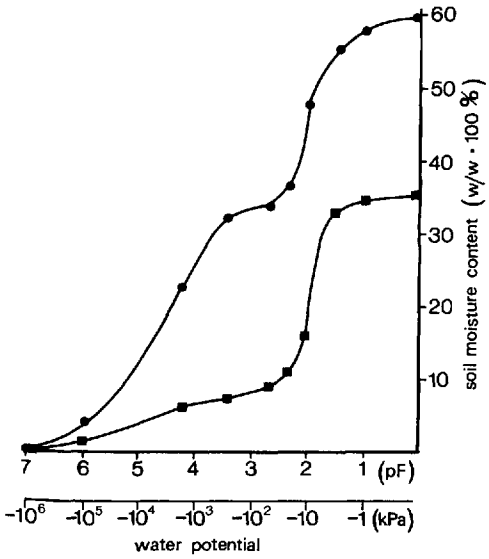

Fig. 1. Relationship between soil water potential and moisture content of repacked samples of the loamy sand (■) and silt loam (●).

Materials and Methods

Soils

Two Dutch arable soils were used: a "beekeerd" loamy sand common in the eastern part of the Netherlands and a silt loam from the Flevo polder. The soils were air dried to 8 and 20% moisture content, respectively, sieved to collect the <2 mm fraction, 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 1,000 ml water, pH 7.2). Soil characteristics are presented in Table 1. Soil water retention curves were determined according to Klute [23] by desorption, starting with initially saturated soils. Figure 1 shows the relationship between soil water potential and moisture content of repacked samples of the loamy sand and the silt loam with bulk densities of approximately 1.4 and 1.0 g cm^{-3}, respectively.

Soil Water Potential

Glass filters with a fine porous plate made of sintered glass with a nominal maximum pore size of 1.0 to 1.6 μm were used (all glass bacteria filter, porosity 5, Schott). The glass filters were connected to a water reservoir by a continuous water column (Fig. 2). Glass cylinders, 40 mm high, 30 mm in diameter, and closed at the bottom with nylon netting, were filled with soil (10 g dry weight). After saturation of the soil, the glass cylinders were placed on the porous plate, and the desired water potential was obtained by varying the height of the hanging water column. The soil portions were protected against extensive evaporation with an aluminum 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 Fig. 2, exclusive of the glass cylinder, was sterilized in separate plastic bags by γ-irradiation (2.5 Mrad). Glass cylinders 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) [17, 33] was used as a model organism. Bacterial suspensions used for inoculations were cultured in yeast extract mannitol broth [17] supplemented with 25 mg/liter Km. After growing for 2 days at 29°C on a rotary shaker, the bacterial suspension was washed by centrifugation (7,000 × *g*, 15 min) and resuspended in sterile demineralized water.

Natural Soil Experiment

Glass cylinders were filled with the loamy sand and the silt loam (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 enough sterile demineralized water to give approximately 4 to 6 × 10^7 colony-forming units (cfu) g^{-1} dry soil. Glass cylinders were weighed and placed on the porous plates with hanging water columns of 50, 100, and 200 cm corresponding to water potentials of −5, −10, and −20 kPa, respectively, and incubated at 15°C in the dark.

Moisture contents and bulk densities of the soil portions were determined after 14 days. Approximately 70 days later, when population sizes were expected to be more or less stabilized [34], numbers of bacteria were determined by dilution plating [33]. Rhizobial cells were enumerated on plates containing yeast extract mannitol agar (YMA) [17] supplemented with 50 mg/liter Km, 20 mg/liter Rp, 100 mg/liter cycloheximide, and 50 mg/liter 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 to 3 × 10^8 cfu g^{-1} dry soil. Rhizobial cells were enumerated after 14 days on YMA when populations were expected to have stabilized [34]. The absence 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, 1,000 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 $\alpha = 0.05$. The effect of moisture content, sterility, and bulk density on the logarithmic number of rhizobial cells was analyzed with linear regression analysis. Total bacterial population size was analyzed separately with linear regression analysis.

Estimation of Pore Volume and Surface Area

The matric potential (ψ_m) of water in a capillary is related to the radius of the curvature r (μm) of the meniscus: $\psi_m = 2\sigma/r$, where σ is the surface tension (73.5 kPa μm at 15°C) [32, 39]. The effective pore neck diameter d (μm) can therefore be estimated by d = 300/water potential in kPa.

The relationship assumes that the contact angle between water and soil solids is zero and that

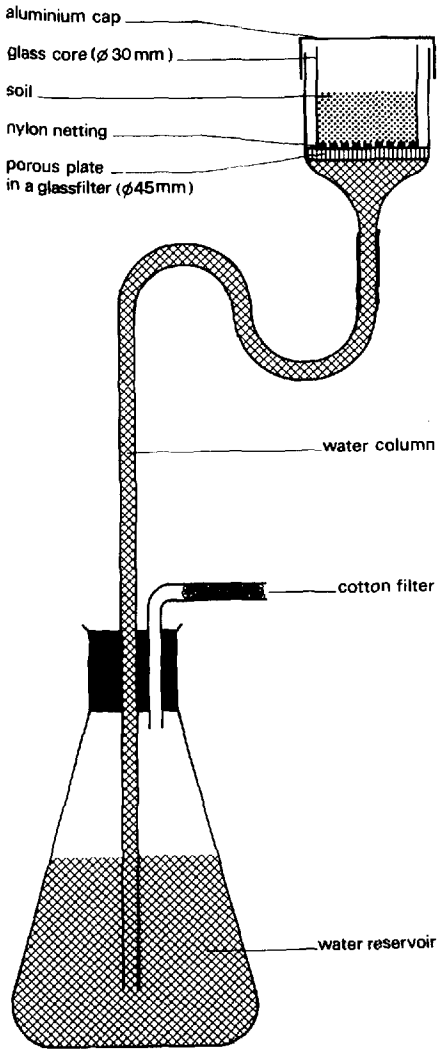

Fig. 2. Schematic diagram of apparatus used to generate soil portions with a given soil water potential.

pores are cylindrical [32]. 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 water potential decreases. Pore volume corresponding to different pore neck diameters can then be calculated from the retention curve (Fig. 1).

For estimation of the pore surface area, a distribution in pore classes of equal diameter was made by dividing the entire water retention curve into steps of 0.1 on its logarithmic scale. Pores were assumed to be cylindrical with length l (μm) and radius r (μm), thus having a volume of $l \times \pi r^2$ and a surface area of $l \times 2\pi r$. Then, the surface area of each pore class can be calculated by surface area = 2 × volume × r^{-1}.

Pore volume and surface area were also expressed in numbers of rhizobial cells that, theoretically, could occupy the pore space. The pore space needed for 1 rhizobial cell was assumed to be 1 μm^3 and 1.5 μm^2.

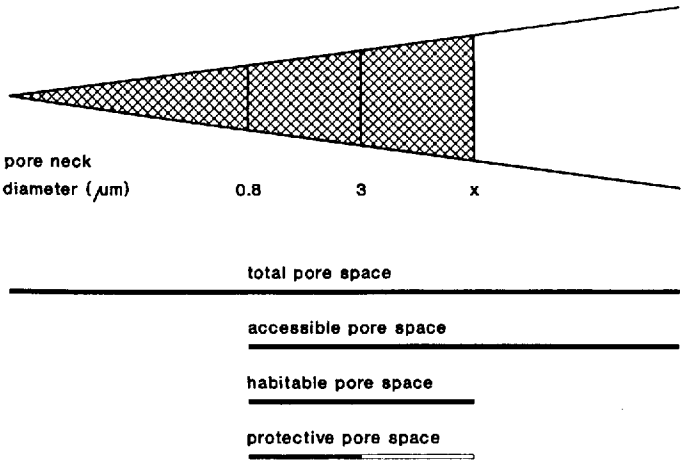

Fig. 3. Schematic presentation of total, accessible, habitable, and protective pore space. Hatched areas represent pores filled with water, and x is the pore neck diameter that is still water-filled at the water potential used (x is 30 μm at −10 kPa). ▬ and ▭ indicate if pore volume or pore surface area, respectively, of a certain pore diameter class are expected to be important.

Table 2. Moisture content of the loamy sand and the silt loam at two bulk densities and at different water potentials

	Bulk density (g/cm³)	Moisture content (wt/wt) of soil portions			
		Saturated	−5 kPa	−10 kPa	−20 kPa
Loamy sand	1.33	41.5	28.0	15.9	11.9
Loamy sand p[a]	1.42	39.0	27.7	15.8	12.1
LSD (0.05)[b]	0.02	0.9		1.0	
Silt loam	0.89	80.1	39.6	35.6	32.9
Silt loam p[a]	1.11	60.7	41.7	36.7	34.0
LSD (0.05)[b]	0.02	1.1		1.0	

[a] p, pressed soil.
[b] Least significant difference for significance level $\alpha = 0.05$.

Definition of the Different Categories of Pore Space Used

Total, accessible, habitable, and protective pore space are distinguished (Fig. 3). Only part of the total pore space is accessible for bacteria. Pores <0.8 μm in diameter are considered to be too narrow to be accessible for rhizobial cells (Fig. 3). The habitable pore space is defined as the part of the accessible pore space which is suitable for the survival and establishment of bacterial cells; the presence of water is important. In natural soil, where predators such as protozoa are present, only the surface of the habitable pore space and (assuming that pores <3 μm are not accessible for these predators) the volume of pores between 0.8 and 3 μm probably offer protection. Therefore, this is called protective pore space (Fig. 3).

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

Water potential (−kPa)	Pore neck diameter (μm)	Loamy sand		Silt loam	
		Volume (cm^3/g)	Surface (m^2/g)	Volume (cm^3/g)	Surface (m^2/g)
10^6–400	<0.8	0.071	>>>[a]	0.306	>>>[a]
400–100	0.8–3	0.020	0.055	0.026	0.092
100–10	3–30	0.067	0.021	0.114	0.027
10–0	>30	0.201	0.015	0.155	0.011

[a] Extremely large surface area.

Results

Bulk Density and Soil Moisture Content

By pressing the soil portions, bulk density was increased significantly ($P < 0.05$), resulting in significantly ($P < 0.05$) 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 had decreased, since the soil moisture content of the pressed soil had not decreased at a water potential of −5 kPa. In the pressed silt loam, the volume of larger pores had decreased, but moisture content at −20 kPa, and thus the volume of pores with pore necks <15 μm had increased. In the two experiments, the moisture contents equivalent to −5, −10, and −20 kPa corresponded well to the values of the water retention functions in Fig. 1 for the loamy sand, whereas in the silt loam the values in the two experiments were lower as compared to Fig. 1.

Population Size

Linear regression analysis explained >99% of the variance between rhizobial numbers at different soil moisture contents (Fig. 4). 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 with increasing moisture content was detected in both natural soils. Rhizobial numbers at moisture contents equivalent to −10 kPa were 2.5 to 3.2 × 10^8 and 5 to 6.3 × 10^8 cfu g^{-1} dry soil for the sterilized loamy sand and silt loam, and 2 to 4 × 10^5 and 3 to 6 × 10^5 cfu g^{-1} dry soil for the natural loamy sand and silt loam, respectively. Bulk density had an effect on rhizobial numbers only in the loamy sand ($P < 0.05$).

No significant influence of moisture content on total bacterial numbers was found in either natural soil (average number, 3 × 10^7 and 1.6 × 10^8 cfu g^{-1} dry soil in the loamy sand and the silt loam, respectively) (Fig. 4).

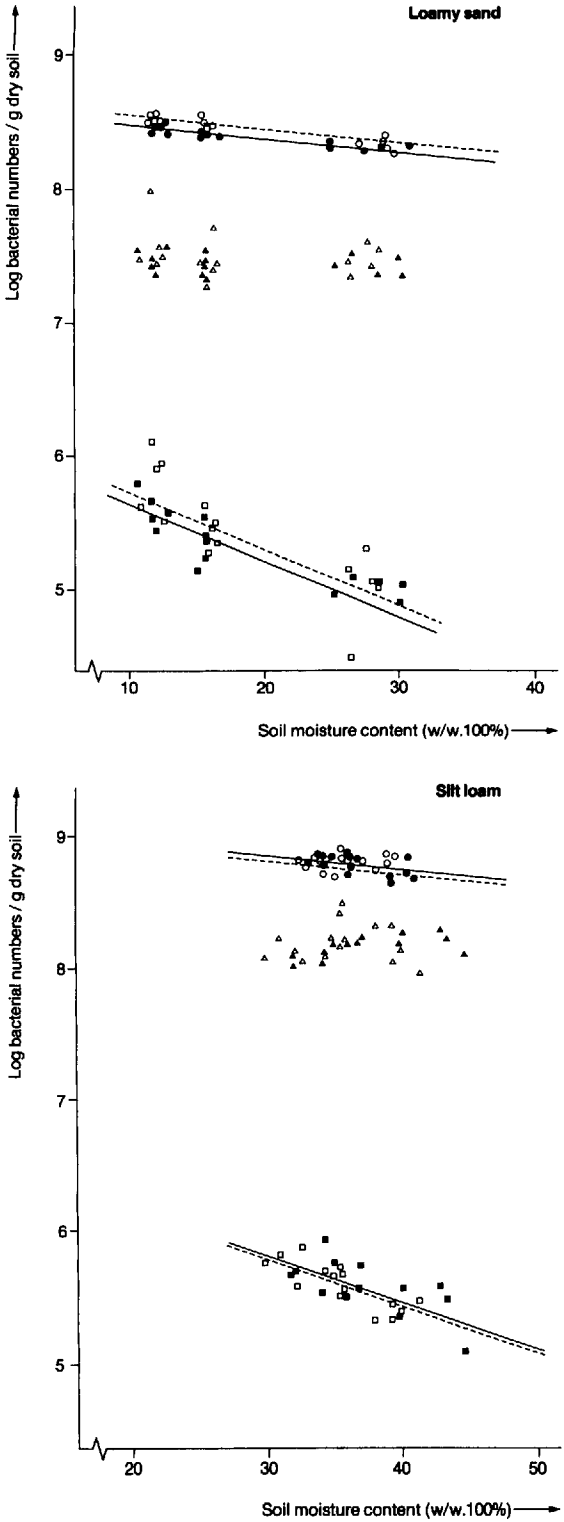

Fig. 4. Numbers of *R. leguminosarum* biovar *trifolii* and total bacterial populations in the loamy sand and the silt loam at moisture contents equivalent to water potentials of −20 to −5 kPa. Rhizobial cells in sterilized soil (○,●) and in natural soil (□,■), and total bacterial population in natural soil (△,▲). Open symbols with dotted lines represent the nonpressed soil samples, and closed symbols with solid lines represent the pressed soil samples.

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

Water potential (−kPa)	Pore neck diameter (μm)	Loamy sand		Silt loam	
		Volume[a] (cells/g) ×10[10]	Surface[a] (cells/g) ×10[10]	Volume[a] (cells/g) ×10[10]	Surface[a] (cells/g) ×10[10]
10^6–400	<0.8	0[b]	0[b]	0[b]	0[b]
400–100	0.8–3	2.0	3.7	2.6	10.2
100–10	3–30	6.7	1.4	11.4	1.8
10–0	>30	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	

[a] Pore volume and surface area needed for 1 rhizobial cell was assumed to be 1 μm^3 and 1.5 μm^2, respectively.
[b] Minimum pore neck diameter was assumed to be 0.8 μm.

Pore Volume and Surface Area

Pore volume and surface area, calculated from the water potential functions given in Fig. 1, are summarized for the pores with a pore neck diameter of <0.8, 0.8 to 3, 3 to 30, or >30 μm (Table 3). An estimation of the number of rhizobial cells that, theoretically, can occupy the pore space is given in Table 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 water potentials from −20 to −5 kPa. 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 to collect the <2 mm fraction. Thus, soil aggregates >1 mm, which are found to be partly anaerobic at −10 kPa [9], would be present. In natural (= nonsterilized) soil, biotic factors in addition to oxygen limitation are expected to play a major role, since the decrease in rhizobial numbers with increasing moisture contents was more pronounced in the natural than in the sterilized soil. It is known that predators such as protozoa are more active at higher soil moisture contents [10, 24]. A decrease of the number of introduced bacteria with increased moisture contents was previously detected [5, 19, 30, J. L. Park, A. D. Rovira, G. D. Bowen, 1984. *Phytopathology* 74:806, Abstract], and an optimum water potential between −63 and −32 kPa has been found [5, 19]. The total bacterial

population size, however, did not decrease with increasing moisture contents, similar to results of Seifert [38] and Howie [19].

Bulk density somewhat affected rhizobial numbers, as well as the total bacterial population size. In the loamy sand with a higher bulk density, only the volume of pores >60 μm diminished, and rhizobial numbers were somewhat 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.

To improve the understanding of these results, the pore space was estimated for various pore neck diameter classes by using the water retention curve (Fig. 1). The values of the pore volume and surface area calculated this way are only estimations, since pores are not cylindric. Moreover, at water potentials below −100 kPa the water content–water potential relationship in soil is dominated by surface area adsorption effects [32]. 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 adsorption techniques might be useful techniques for the assessment of the size distribution of such small pores, but these techniques are not yet fully explored for soil systems. With backscattered electron scanning images, which has been applied for the characterization of the soil pore network, only pores larger then 3 μm have been studied [4, 11, 20]. Therefore, the pore size distribution obtained from the water retention curve is used for a first estimation of pore space. Soil retention curves obtained by desorption of saturated soil were expected to give the most accurate information, since also the survival experiments with rhizobia were executed in soil which had been saturated during inoculation.

The pore space which is of interest for the survival of rhizobia must at least be accessible to them (Fig. 3). In natural soils, smallest pores which were reported to be colonized had a diameter of 0.8 μm [22]. Rhizobial cells measure 0.5–0.9 × 1.2–3.0 μm [21], and a pore neck diameter of >0.8 μm would be sufficient for pores to be entered. 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 −10 kPa. These percentages agree quite well with the occupied pore volumes calculated to be 0.1 [2] or 0.4% [6].

Survival and establishment of rhizobia will also be dependent on the presence of water. At −10 kPa, pores >30 μm have been drained. Therefore, the habitable pore space is estimated from only the volume of pores between 0.8 and 30 μm in diameter (Fig. 3). 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 −10 kPa occupied only 0.37 and 0.44% of the habitable pore space, respectively (Table 4).

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 [31, 36]. Nioh and Furusaka [29] detected that most bacteria in wider pores are absorbed to surfaces, whereas part of the bacteria in smaller pores occurred freely. Increased percentages of particle-associated bacteria were detected in the presence of protozoa [35]. Particle-associated

bacteria are expected to be better protected against predation, either as a result of enclosure in pores inaccessible to predators [12, 35, 41], or possibly by attachment to surface areas. Thus, in natural soil only part of the habitable pore space, the so-called protective pore space, offers protection (Fig. 3). The rhizobial cells occupied only 0.001% of the protective pore space in both natural soils (Table 4); 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 to 0.75 μm [22]. Therefore, for calculations, the same mean cell size for the total population as for rhizobial cells was used, resulting in an estimated occupation of approximately 0.09 and 0.36% of the protective pore space in the loamy sand and the silt loam by culturable bacterial cells.

Although large parts of the accessible pore space are not suitable for survival and establishment of rhizobia, and although large parts of the habitable pore space are not protected, habitable as well as protective pore space are not expected to be a limiting factor for the survival of rhizobial cells, since in all cases <0.5% of the habitable and protective pore space were occupied by bacteria. This 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. 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.

Substrate availability is known to be a major limiting factor in soil and higher numbers of introduced bacteria [1, 37], as well as of indigenous populations [16, 37], have been detected after the addition of substrates. Now that pore space was not found to be a limiting factor, the higher relative occupation of the pore space in the silt loam as compared to the loamy sand suggests 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 [18] 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 to 10% of the root surface area was covered by bacteria [14, 26].

Thus, it is concluded that habitable and protective pore space are not limiting factors. Nevertheless, their relative size might be very important for the survival of introduced bacteria, since upon introduction, cells will be distributed over the protective pore space and the nonprotected part of the habitable pore space. This distribution will influence the survival of the introduced bacterial cells.

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