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Viability of Soil Bacteria: Optimization of Plate-Counting Technique and Comparison Between Total Counts and Plate Counts Within Different Size Groups

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Abstract. Viable counts of heterotropic soil bacteria were 3–5 times higher on low-nutrient agar media compared with a series of conventional agar media. Substantial amounts of monosaccharides and amino acids were present in solid media made from distilled water and agar powder, and a salt-solution agar medium (without organic substrates added) gave practically the same colony counts as the low nutrient soil extract agar medium. MPN values were comparable to or lower than plate counts. A search for slow-growing cells in the negative MPN tubes by fluorescence microscopical examination after 3 months incubation was negative.

The viable counts were 2–4% of the total microscopical counts in different soils. Assuming that the colony-forming cells did not derive from the numerous "dwarf" cells present in soil, a calculated percent viability of the larger cells was about 10%. The ecological significance of the plate-counting technique is discussed.

Introduction

Energy available for bacteria in soil and water is largely in the form of organic carbon, and thus heterotrophic bacteria are likely to be dominant. However, only 0.1–1% of the bacteria observed with a fluorescence microscope are able to form colonies on normal nutrient agar media [9, 10, 18].

This discrepancy between microscopical counts and plate counts can be partly attributed to a high percentage of dead or moribund cells within the population of each species, as has been demonstrated in aged pure cultures of bacteria [25, 33, 34, 37]. It seems likely, however, that a large part of the discrepancy is attributable to selectivity of the agar media and incubation conditions [4, 11, 18, 19]. This implies that the species that we are able to isolate from the agar plates represent a minority of the natural bacterial flora. Thus, the plate-counting technique seems an inadequate method not only for counting all viable cells [4], but also for isolating the dominant heterotrophic species in natural environments. The use of different agar media and incubation conditions is hardly a feasible and adequate way to compensate for this shortcoming of the plate-counting method, since there is a great probability of overlap between the sections of the bacterial flora accounted for by the different agar media and

Table 1. Soil characteristics

Soil type	Code	pH[a]	Total organic C (%)[b]	Total organic N (%)[b]	Clay content (%)[b]
Clay loam	CL	5.5	3.0	0.30	23
Sandy loam	SL	5.4	1.7	0.15	8
Organic soil	OS	5.0	39.0	1.70	1
Spruce forest soil	SF	4.5	5.5	0.28	9

[a] pH measured in a slurry of soil and distilled water (2 ml per g soil)
[b] Percent of soil dry weight

Table 2. Soil extract types, stock solutions, and agar media

Soil extract type	Code	Composition/preparation
Cold extracted soil extract	CSE	Air dry soil mixed 1:1 with tap water, particles removed by setting for 2 hr and centrifugation (5,000 × *g* for 20 min), and filter sterilized (0.2 μm)
Autoclaved soil extract	ASE	Soil + water (1:1) mixed and autoclaved (121°C for 20 min), filtered through glass wool, and autoclaved
Nutrient stock solution	NS	10 g per liter of sodium citrate, sodium succinate, glucose, fructose, xylose, peptone (Difco), and yeast extract (Difco). pH adjusted to 7 (H_2SO_4 addition). Filter sterilized (0.2 μm)
Phosphate buffer stock solution	PB	64 g KH_2PO_4, 36 g Na_2HPO_4, 1,000 ml distilled water (pH 7). Filter sterilized (0.2 μm)
Cold-extracted soil extract agar	CSEA	400 ml cold-extracted soil extract (CSE), 600 ml tap water, 1 ml phosphate buffer stock solution, 1 ml nutrient stock solution (NS) (varying amounts of NS or single nutrients applied in some experiments), 20 g agar (NMD)
Winogradsky's salt solution agar	WSA	400 ml distilled water, 600 ml Winogradsky's salt solution, 0.1 g NH_4NO_3, 20 g agar (NMD)
Thornton's medium	Th	1 g K_2HPO_4, 0.5 g KNO_3, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 0.1 g NaCl, 10 mg $FeCl_3 \cdot 6H_2O$, 0.5 g asparagin, 1 g mannitol, 1,000 ml soil extract (ASE), 900 ml tap water, 20 g agar (NMD)

Table 2. Continued

Soil extract type	Code	Composition/preparation
Yeast extract	YP	5 g peptone, 1 g yeast extract, 0.01 g $FePO_4 \cdot 2H_2O$, 1,000 ml tap water, 20 g agar (NMD)
Yeast extract peptone soil extract agar	YPS	0.4 g K_2HPO_4, 0.5 g $(NH_4)_2HPO_4$, 50 mg $MgSO_4 \cdot 7H_2O$, 100 mg $MgCl_2$, 10 mg $FeCl_3$, 100 mg $CaCl_2$, 1.0 g peptone, 1.0 g yeast extract, 250 ml soil extract (ASE), 750 ml tap water, 20 g agar (NMD)
Soil extract glucose agar	SEG	1 g K_2HPO_4, 1 g $CaCO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 50 mg $FeSO_4 \cdot 7H_2O$, 50 mg $Na_2MoO_4 \cdot 2H_2O$, 1.5 g glucose, 200 ml soil extract (ASE), 800 ml tap water, 20 g agar (NMD)
Plain soil extract	PS	0.5 g K_2HPO_4, 500 ml soil extract (ASE), 500 ml tap water
Water agar	WA	20 g agar (NMD or Difco), 1,000 ml distilled water
Cold-extracted soil extract liquid medium	CSEL	1,000 ml CSE, 1 ml NS, 1 ml PB

incubation conditions, and on all media so far tested, only a minor fraction of the total number of cells gives rise to colonies.

Thus, it may be argued that for the majority of heterotropic bacteria in soil, a suitable technique for isolation and cultivation under laboratory conditions has not yet been developed. The present paper is the first part of a study in which we have tried to characterize the colony-forming as well as the "non-colony-forming" bacterial cells in soil. An optimization of the agar plate-count technique has been performed, and a comparison has been made between this technique and fluorescence microscopy, with respect to cell size, morphology, and total numbers of bacteria per g of soil.

Material and Methods

Soil Types

Three different cultivated soils—a clay loam (CL), a sandy loam (SL), and an organic soil (OS)—were studied together with the mineral layer of a brown-earth soil under Norway spruce (SF). The soils were collected by taking several subsamples within a 1-m^2 plot. The subsamples were mixed and crushed to pass a 3-mm brass sieve. The soils were not allowed to dry out, and were stored at 5°C. Soil characteristics are listed in Table 1.

Dispersion and Dilution of the Soil for Bacterial Counts

Soil samples were mixed 1:10 in sterile diluent (distilled water or buffers) and homogenized for 3 × 1 min in a Waring Blendor (Waring, Connecticut, U.S.A.) with intermittent cooling in an ice bath as described by Fægri et al. [10]. From this soil homogenate, serial dilutions were prepared for fluorescence microscopy counts, plate counts, and inoculation of most probable number (MPN) tubes.

Fluorescence Microscopy

Appropriate dilutions were stained with acridine orange and counted microscopically on 0.2-μm polycarbonate membranes according to Hobbie et al. [15]. The size distribution of the cells was investigated by counting the cells within different size groups. The dimensions of the cells were determined by comparison with circles of known diameters in a New Porton G 12 eye graticule (Graticules Ltd., Kent, England). Cells observed to be in direct contact with each other or to a common soil particle were assumed to stick together as one unit during the dilution of the soil. The number of cells per such "free unit" (including single cells) were counted, and the average value was used as a correction factor for comparison of the fluorescence microscopical counts and the viable counts (plate counts or MPN values) [31].

Plate-Counting Technique

The plate-counting media were solidified by using 20 g per liter of ordinary agar powder (Norwegian Medical Depot, Oslo, Norway). The inoculum (0.1-ml samples from appropriate soil dilution) was spread on the agar surface of a 9-cm plate with a sterile bent glass rod. The plates were incubated at 21°C for 1 month, and the number of colonies were counted with a stereomicroscope. To avoid loss of water from the agar, the plates were packed in plastic bags during incubation.

Diluents and Dilution Levels

Four different diluents were compared: distilled water, Calgon (0.2% sodium hexametaphosphate buffered to pH 8.5 with $NaCO_3$) [30], Winogradsky's salt solution [8, 29], and cold-extracted soil extract (CSE, see Table 2). Two parallel dilution series of the CL were prepared in each diluent and inoculated on plates with the CSE agar medium (CSEA, Table 2). In later experiments, Winogradsky's salt solution was used. To investigate the relationship between the dilution level and the number of colonies per plate, the CL was diluted in Winogradsky's salt solution to 10^{-5} and 10^{-6} g per ml, and a series of CSEA plates was inoculated with different volumes (10–100 μl) representing 10–1,000 ng soil per plate.

Comparison of Agar Media

The number of colonies developing on different agar media was investigated in a series of separate experiments. In each experiment a single dilution series of each soil was used to inoculate all the media that were compared. Five parallel plates were inoculated from each dilution level.

The CSEA (Table 2) was compared with 5 different media frequently reported in the literature for plate counting of soil bacteria. These media were Thornton's medium (Th) as modified by Fægri et al. [10], yeast extract peptone agar (YP) [28], yeast extract peptone soil extract agar (YPS) [5], soil extract glucose agar (SEG) [30], and plain soil extract agar as described by Smith and Worden [32]. The composition of these media is shown in Table 2.

A series of experiments with media formulations similar to CSEA (Table 2) were carried out in order to test the effect of different nutrient components. The effect of the soil type used to prepare CSE (Table 2) was tested by making CSEA media with soil extracts from the CL, the SL, and the OS. Dilutions of the same soil types were used to inoculate the plates. The effect of the soil-extract preparation was tested by comparing the plate counts on normal CSEA medium, a CSEA medium in which autoclaved soil extract (ASE, Table 2) was used instead of CSE, and a CSEA medium containing both soil extract types (200 ml per liter of each).

The effect of different amounts of the nutrient stock solution (NS, Table 2) was investigated by making different CSEA media containing 0, 0.1, 10, and 100 ml NS per liter. The effect of each single nutrient component in NS was tested by making a series of different CSEA media (without NS), with only one of the nutrient components added in each, and at three different concentrations (0.01, 1, and 5 g per liter). The effect of eliminating both soil extract and organic nutrients was tested, first, by comparing the colony counts on CSEA media containing 0, 200, and 400 ml soil extract (CSE) per liter (the media were otherwise identical to CSEA as shown in Table 2), and, second, by comparing the colony counts on Winogradsky's salt solution agar (WSA, Table 2) with and without organic nutrients (NS) added (1 ml per liter), and on a CSEA medium without nutrients (NS) added.

Most Probable Numbers

For each dilution level (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} g/ml), 20 replicate MPN tubes were inoculated with 1-ml samples, and the numbers of positive tubes were recorded after incubation at 21°C for one month. Three different liquid media were compared: Brain Heart Infusion broth (Difco, U.S.A.), which was used by Casida [6] for growing fastidious soil bacteria; Eugon broth (BBL), which is another medium prepared for fastidious organisms; and a cold-extracted soil extract liquid medium (CSEL, Table 2). In a separate experiment with the CL, the MPN tubes (with the CSEL medium), which were negative after 1 month incubation, were incubated further for 3 months and then investigated by fluorescence microscopy in order to see if low numbers or extremely small cells were present. The whole content of each tube was stained by adding acridine orange solution and the bacteria was counted on 0.2 μ polycarbonate filters as described by Hobbie et al. [15]. Uninoculated tubes were stained as well, in order to check the background countings.

Morphological Classification of Colony-Forming Organisms

To investigate the morphology of the colony-forming organisms on the different media, all colonies on one or several agar plates and the cells in positive MPN tubes were examined by phase contrast microscopy. The organisms were grouped into 8 relatively broad groups based on size, spore formation, and cell morphology.

Measurements of Amino Acids and Carbohydrates

Free α-amino acids were measured as CO_2-evolution by the ninhydrin reaction according to Van Slyke et al. [36]. The amounts of peptides and proteins were measured as free α-amino acids after hydrolysis in 6 N HCl according to Bremner [3]. Mono- and disaccharides were determined by gas chromatography of their O-isopropyliden derivatives [26]. In order to investigate whether the agar powder itself contained significant amounts of water-soluble proteins, amino acids, and carbohydrates, a water extract from water agar was prepared as follows: Distilled water with 2% agar powder (WA, Table 2) was autoclaved and poured into petri dishes (20 ml in each). On top of the solidified agar layer, 20 ml of distilled water was added. The plates were then left at room temperature for 48 hours. The water was then decanted, concentrated under vacuum, and analyzed for proteins, amino acids, and carbohydrates as outlined above. The extraction and analysis was

Table 3. Fluorescence microscopic counts: Total numbers of cells, average number of cells per free unit, and the calculated number of free units

Soil type	Total numbers ($\times 10^{10}$) per g[a]	Average number of cells per free unit	Total numbers of free units ($\times 10^{10}$) per g[a]
CL	1.0	1.9	0.50
SL	1.2	2.3	0.50
OS	4.3	2.8	1.50
SF	0.6	2.5	0.24

[a] Soil dry weight

Table 4. Size distribution of the cells as observed by fluorescence microscopy

Volume groups	Volume limits (μm^3)	Diameter limits for cocci (μm)	Frequency of cells within each size group (percent of total)			
			Soil type			
			CL	SL	OS	SF
Cocci						
I	<0.065	<0.5	60	58	60	77
II	0.065–0.18	0.5–0.7	14	23	23	14
III	0.18–0.52	0.7–1.0	4	5	4	3
IV	0.52–1.44	1.0–1.4	0.5	1.5	0.4	0.8
V	>1.44	>1.4	0.3	0.1	0.01	0.1
Rods						
II	0.065–0.18	—	17	9.0	12	4.6
III	0.18–0.52	—	4	2.5	0.7	0.9

done with water agar prepared with two different commercial agar powders; one was delivered from the Norwegian Medical Depot (NMD, Oslo, Norway), the other was Bacto-agar (Difco, U.S.A.).

The cold-extracted soil extract was also analyzed for carbohydrates and amino acids, after being concentrated under vacuum.

Results

Fluorescence Microscopy

The total number of cells per g soil ranged from 0.6×10^{10} (SF) to 4.3×10^{10} (OS) (Table 3). The number of free units per g soil (which we assume to be the theoretical maximum number of colony-forming units) was calculated from the total cell counts and the average number of cells per free unit. These values were 0.24, 0.5, 0.5, and 1.5×10^{10} per g soil in soil SF, CL, SL, and OS, respectively.

Table 5. Effect of dilution level on colony numbers: Relationship between the amount of soil applied to the agar plate and the number of colonies

Amount of soil per plate[a] (ng dry weight)	Average number of colonies per plate[b]	Colony numbers ($\times 10^6$) per g soil dry weight
1000	107	110 (33)[c]
600	82	140 (42)
300	50	170 (52)
180	39	220 (67)
90	20	220 (67)
60	17	280 (85)
30	10	330 (100)
10	3, 2	320 (97)

[a] Calculated from dilution level (g soil per ml) and the volume applied per plate
[b] Four replicate plates at each level
[c] Percent of highest value in brackets

The size distribution of the cells in each soil was investigated (Table 4). The small cells (volume group I) dominated numerically in all the soils. The majority of the cells within this volume group were coccobacilli, but it was difficult to distinguish between cocci, coccobacilli, and rods due to the small dimensions; all morphologies within this volume range ($<0.065\ \mu m^3$) were therefore combined in one group. The combined numbers of rods and cocci within volume group II ($0.065–0.18\ \mu m^3$) represented 19–35% of the total number of cells (SF and OS, respectively), and the larger cells (volume groups III, IV, and V combined) represented between 5–10% of the total number of cells.

Diluents and Dilution Level

Distilled water as a diluent resulted in 15% fewer colonies than Calgon, Winogradsky's salt solution, and soil extract (CSE), which were all within ±6% of each other.

The relationship between the amount of soil (CL) per plate, and the number of colonies developing per plate, is shown in Table 5. The maximum number of colonies per g soil was obtained with 10 to 30 ng soil per plate. With increasing amounts of soil per plate, the number of colonies per g soil was gradually reduced to 33% of the maximum values.

Comparison of Agar Media

The colony counts on the conventional agar media and on CSE (the composition of each medium is listed in Table 2) are shown in Fig. 1. The media clearly separated into two groups, with CSEA and PS giving 3–5 times higher colony numbers than any of the other media. The differences between these two groups

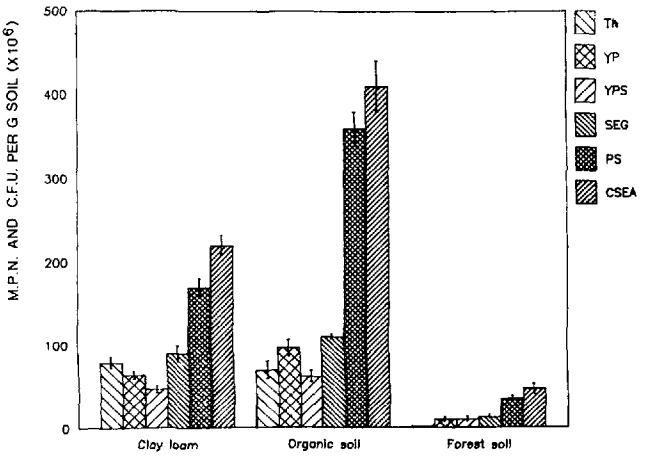

Fig. 1. Colony counts ($\times 10^6$) per g soil dry weight on several conventional agar media and on CSEA. For abbreviations, see Table 2.

were significant at the 5% level while the differences within each group were not. It is noteworthy, however, that CSEA gave consistently higher counts than PS (15–30% difference) with all three soils tested; this difference was significant at 10% level (tested with average values for each soil as paired observations). Similarly, SEG gave consistently higher colony numbers than the other media in this group (Th, YP, and YPS).

The colonies were counted at intervals during the incubation period. On the inferior agar media (Th, YP, YPS, and SEG) the numbers of colonies reached a constant level after 7–10 days, whereas on PS and CSEA they increased continuously for 3 weeks. The majority (60%) of the colonies on PS and CSEA were smaller than 1 mm in diameter, and a stereomicroscope was preferentially used for counting.

The quality of the soil extract used in the CSEA medium had little influence on the colony counts. The CSEA media prepared with soil extract from the different soils gave practically identical colony counts. The CSEA media containing 400 ml per liter of ASE, or 200 ml per liter of both ASE and CSE, gave only 15–20% lower colony counts than the normal CSEA medium.

The effect of varying amounts of the NS (Table 2) in the CSEA media is shown in Table 6. At low concentrations, (0.1 and 1 ml NS per liter) the colony numbers were equal (SF) or about 30% higher (CL and SL) than what was observed without added NS. With higher concentrations of NS, a substantial reduction in colony counts was observed for all the soils.

The effect of the different components in NS when added alone at three different concentrations (0.01, 1.0, and 5.0 g/liter) is shown in Table 7. At a concentration of 0.01 g per liter, none of the nutrients resulted in significantly higher colony counts than that obtained on the basal medium without nutrients added. Glucose and peptone did not affect the number of colonies even at the highest concentration (5 g per liter), whereas the other nutrients reduced the colony counts by 40–60% at this concentration.

Eliminating the soil extract from the CSEA medium did not reduce the colony counts (CL) significantly. Practically identical colony counts (CL) were obtained on the CSEA media with 0, 200, and 400 ml CSE per liter (data not shown).

Table 6. Colony counts on CSEA media with varying amounts of NS

Soil type	ml NS per liter of agar medium[a]	Colony counts ($\times 10^6$) per g soil dry weight
CL	0	280 (40)[b]
	0.1	380 (60)
	1	340 (30)
	10	90 (20)
	100	40 (10)
SL	0	53 (5)
	0.1	68 (7)
	1	73 (7)
	10	36 (4)
	100	—[c] —
SF	0	54 (5)
	0.1	54 (5)
	1	40 (3)
	10	55 (5)
	100	10 (3)

[a] One ml NS per liter gives 10 mg per liter of each component in NS (see Table 2)
[b] Standard deviation of the average value in brackets
[c] Not tested

Table 7. Colony counts on CSEA media with varying amounts of the different NS nutrients added alone

Nutrient compound added[a]	Average number of colonies per plate[b] nutrient concentration		
	0.01 g/liter	1 g/liter	5 g/liter
Glucose	20 (3)	18 (2)	21 (3)
Fructose	22 (4)	19 (2)	15 (3)
Xylose	18 (2)	13 (2)	11 (2)
Citric acid	14 (3)	14 (3)	8 (3)
Succinic acid	20 (2)	14 (2)	12 (1)
Yeast extract	20 (2)	17 (2)	8 (2)
Peptone	17 (1)	19 (2)	18 (2)
No additions:		19 (1)	

[a] The basal medium was CSEA without nutrient stock solution, NS, added
[b] The plates were inoculated with 0.1 ml of the 10^{-6} g/ml dilution of CL

Since neither soil extract or organic nutrients (NS) had been found to increase the colony numbers substantially, we tested whether both components could be excluded without reducing the colony counts (Table 8). Practically identical results were obtained on all the agar media, except for the spruce forest soil which gave 15–25% lower values with WSA without NS, compared with values

Table 8. The effect of eliminating both the soil extract and organic nutrients in an agar medium

Medium	Colony counts ($\times 10^6$) g^{-1} [a]		
	CL	SL	SF
WSA	260 (10)[b]	49 (3)	31 (3)
WSA + 1 ml NS per liter	280 (30)	48 (3)	41 (4)
CSEA without NS	250 (30)	46 (2)	36 (4)

[a] Soil dry weight
[b] Each estimate is based on six replicate plates; the standard deviation of the average in brackets

obtained with the other two media. The differences were, however, not statistically significant.

Most Probable Numbers

With CL, SL, and OS, the MPN values were lower than the plate counts (CSEA) irrespective of the MPN medium. The difference between the MPN values with CSEL medium and the plate counts were small, however, and not statistically significant. With SF, a good agreement was obtained between the plate counts and the MPN values (Fig. 2).

The detection limit of the fluorescence microscopical examination of the MPN tubes was about 10^5 cells per tube, as judged from the level and variation of the blank values (counts on uninoculated tubes). Twenty negative MPN tubes inoculated with the 10^{-8} and 10^{-9} dilutions of the CL were investigated after a 4-month incubation. None of the tubes had detectable growth.

Morphological Classification of Viable Organisms

In order to see if different media would favor different morphological types, the numbers per g soil within the different morphological groups were calculated from their relative frequency on the agar plates/MPN tubes and the total number of colonies/MPN values on each medium (Table 9). Assuming a binomial distribution, the upper confidence limits for those morphological types that were not observed has been calculated. Due to the low number of colonies investigated, each estimate is uncertain and must be treated with caution. Some patterns, however, were quite clear: The numbers of thin rods were about 10 times higher on CSEA compared with the agar media YP and SEG, both for soil CL and OS. Corresponding results were seen for the MPN technique comparing CSEL with Eugon and Bhib. CSEL and CSEA also gave somewhat higher numbers of small cocci and coccobacilli compared to the other media. For the other morphological groups, CSEA gave numbers in fairly good agreement with the other agar media. Thus, CSEA supported growth of a greater number of thin rods, small cocci, and coccobacilli than the other media, apparently without excluding the other morphological types.

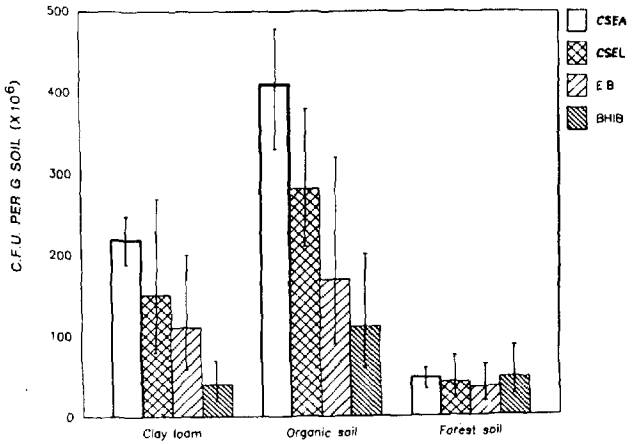

Fig. 2. PN ($\times 10^6$) per g soil dry weight with three different liquid media (cross-hatched bars) compared with colony counts on CSEA (open bars). For abbreviations, see Table 2.

The thin rods (diameter $< 0.7\ \mu$) were normally short (0.7–1.4 μ), but some very slender (0.3–0.4 $\times$ 2.5–5 μ) rods were also seen. The small cocci were normally between 0.5 and 0.7 μ in diameter. Cocci or coccobacilli with diameters below 0.5 μ were rarely seen.

Measurements of Amino Acids and Carbohydrates

The concentrations of water-soluble aldoses and α-amino acids in water agar (WA, Table 1) prepared with two different commercial agar powders is shown in Table 10, together with the corresponding data for the soil extract (CSE, Table 2). The concentrations were generally much higher in the Difco agar compared to the NMD agar, but both agars had relatively high concentrations of both aldoses and amino acids compared with that in the soil extract. Acid hydrolysis of the water extracts from the water agars resulted in very high concentrations of aldoses.

Discussion

A common feature of the inferior agar media (Fig. 1) seems to be their high concentration of either inorganic or organic compounds compared to CSEA and PS (Table 2). Further experiments (Tables 6, 7, and 8) demonstrated that excess amounts of nutrients rather than lack of essential compounds is the main reason for the low colony numbers of the "rich" media. The negative effect of high nutrient concentrations has been observed for the quantification of bacteria both in soil [14, 22, 24, 27], and in water [2, 4, 16]. This has been attributed to the dominance of "low-nutrient" or "oligotrophic" bacteria [16] whose distinctive feature is their ability to grow at very low-nutrient concentrations (1–15 mg C/liter) at first isolation [21], or rather their inability to grow at high nutrient concentration at first isolation. The latter seems a more consistent definition, according to the characteristics given by Kuznetsov et al.

Table 9. Frequency of different morphological groups of bacteria growing on solid (agar plates) and liquid (MPN tubes) media: Numbers per g soil within each group as calculated from the total numbers of colonies (or MPN values) and the observed relative frequency on each medium

		Calculated numbers per g soil dry weight ($\times 10^6$)									
		Cocci		Cocco bacilli	Rods					Total numbers per g soil (10^6)	Number of colonies examined
Soil type	Medium	<0.7 μ	>0.7 μ	(0.5–0.7 μ)	diameter <0.7 μ	diameter >0.7 μ	Rods with endospores	Branched cells	Irregular cells		
	Solid media:										
CL	CSEA	23	6	17	118	23	−a[a,b]	11	23	220	39
	YP	11	4	−b[a,b]	4	4	15	11	15	64	17
	SEG	3	−c[a,b]	6	16	22	−c[a,b]	9	32	90	28
OS	CSEA	42	28	57	226	28	14	−d[a,b]	14	410	29
	YP	−[a]	16	−e[a,b]	22	22	11	16	11	97	18
	SEG	5	10	−f[a,b]	10	48	14	5	19	110	23
	Liquid media:										
CL	CSE-10	23	12	23	63	17	−g[a,b]	−g[a,b]	6	150	26
	Eugon broth	4	30	4	12	12	4	12	30	110	26
	Bhib	5	10	−h[a,b]	10	−h[a,b]	10	5	−h[a,b]	40	28

[a] Not detected

[b] a–h: Upper confidence limit (10%) for groups that are not detected are: a: 12, b: 8, c: 7, d: 31, e: 11, f: 10, g: 13, h: 10 (binomial distribution assumed)

Table 10. Concentrations (mg per liter) of water-soluble sugars and amino acids in WA and in CSE

	WA (NMD agar)	WA (Difco agar)	CSE
Before hydrolyses			
Glucose	12	32	8
Galactose	8	96	4.8
amino acids	1.6	4.8	3.5
After hydrolysis			
Glucose	100	380	4.8
Galactose	100	260	25
Mannose	—[a]	—	2.3
Xylose	15	2	1.8
Arabinose	—	2	1.0
amino acids	n.d.[b]	n.d.	7.3

[a] —, not detected
[b] n.d., not determined

[21], although it may be questioned whether this would justify the term "oligotrophy."

The effect of the single compounds at different concentrations was not very dramatic (Table 7). Glucose and peptone had no effect; the effect of fructose, xylose, and succinate was moderate; and citrate and yeast extract resulted in about 50% decrease in colony numbers at the highest concentration (5 g per liter). Assuming that the different compounds inhibit different parts of the microflora, the combined effect at 1 g/liter would be similar to that observed (Table 6).

The total number of colonies was not much affected by eliminating the organic compounds (NS) (Table 6), the soil extract, or both from the agar medium (Table 8). Thus, traces of soluble organic carbon in the agar, perhaps combined with volatile carbon material in the air [12], was evidently sufficient to support the development of the same number of bacteria as CSEA and PS. The values for soluble glucose and galactose in the agar used in this study (NMD, Table 10) were about 10 times higher than the limit for a selective medium for oligotrophs as defined by Ohta and Hattori [27]. The Difco agar contained much higher amounts of water-soluble carbon material. Hattori [13] demonstrated differences in colony numbers on different types of agar used to solidify a low-nutrient medium.

It has been shown that about 30% of soil bacteria growing on PS required vitamins for growth, and about 10% required soil extract [17, 22]. It is likely, therefore, that some bacterial species are excluded by omitting soil extract and organic nutrients from the agar, although this was not detected as a reduction in colony counts in the present experiments.

The effect of colony density on the colony number per g soil increased gradually with increasing inoculum density (Table 5). Mutual competition is probably the most important reason for this phenomenon. The quite dramatic effect observed in this study, even at low colony density (10–20 per plate), is highly

relevant for plate-counting methods, such as the spotting method [11] and the microcolony technique [35], where colonies develop very close to each other. A similar result was obtained by Jensen [19] in a study of forest soil. Torella and Morita [35] observed growth restriction after a few generations on microslides. The MPN values should not be affected, theoretically, by such mutual competition, assuming the outcome of a competition between different bacteria within an MPN tube will always result in visible growth by at least one species, thus ensuring a positive tube. Still, the MPN values with CSEL were similar or lower than the plate counts (Fig. 2), and the negative tubes remained negative for another 3 months even when tested with fluorescence microscopy for the presence of low numbers of cells. Thus, if extremely slow-growing bacteria were numerous in the soil, the soil extract medium (CSEL) was not a suitable medium for them.

It is important to know to what extent the bacteria that form colonies on rich media will also do this on the low-nutrient media. A detailed investigation of this question would be laborious, but we can get some indications from the morphological study (Table 9). In general, the number of bacteria per g soil within each morphological group was within the same order of magnitude on all media except for the thin rods, which were 10 to 20 times as numerous on CSEA compared to the rich-media YP and SEG. Also, the small cocci (<0.7 μ) tended to occur in higher numbers on CSEA compared to other media. Similar, but less dramatic trends, were seen in the comparison between the soil extract MPN medium (CSEL) and the two rich MPN media (Table 9).

The results therefore indicate that the low-nutrient agar CSEA did not exclude the typical "zymogenous" flora [22] that grew on the rich media. Further, the difference in total colony numbers between CSEA and the rich media seems attributable mainly to special morphological types growing only at low-nutrient agars at first isolation. The maximal plate counts (CSEA) represent only 1–2% of the total number of cells counted microscopically. If corrected using the numbers of cells per free unit (Table 2) [31], this percentage is increased to 4.2, 2.7, and 2.0 for CL, OS, and SF, respectively.

The microscopical counting demonstrated that 60–80% of the cells had volumes smaller than 0.065 m^3 (Table 3). Such small cells were rarely observed among the colony forming cells. Starvation-induced cell size reduction has been demonstrated with isolated seawater bacteria [1, 20] and soil bacteria [7], but it is still open to discussion as to whether the majority of the numerous, extremely small cells in soil and water are such starvation-induced dwarfs of normally sized species, or whether they are a special population. Fry and Zia [11] investigated the percent viability of natural bacterial populations of different fresh waters, and observed a strong negative correlation with the percentage of cells with volumes <0.2 m^3 (epifluorescence total counts). The experimental results of Lund and Goksøyr [23] indicated that there exists a large population of "autochthonous bacteria" that is characterized by very small cells (1/10 of the volume of normal colony forming cells) with an inability to form colonies on agar. On this basis, one could assume that the colony-forming cells were derived only from size groups II–V and not from the numerous "dwarf" cells in group I (Table 4). Then the average percent viability for these groups (II–V) would be about 10% for CL, SF, and OS, and about 3% for SL.

These values (except for SL) would indicate that the colony-forming cells are fairly representative for the "large cells," considering that a large fraction of the cells from each species in soil are probably dead or moribund. The presence of a high percentage of nonviable but intact cells within a population has been demonstrated in pure cultures under varying conditions [25, 33, 34, 37].

The hypothesis that colony formers are representative of size groups II–V has some important implications: First, it would mean that the colony-forming cells represent a very large part of the bacterial biovolume in soil, since size groups II–V represent 80–90% of the bacterial biovolume. Thus, the ecological significance of colony-forming cells may be great despite their low numbers. Second, if the majority of the noncolony-forming species in soil belong to size group I, their total biovolume is very small, although cell numbers are high. Combined with a slow metabolism [23], this implies that their energy requirement represents only a small fraction of the energy flow through the soil ecosystem. This should make their often-alleged heterotrophy less obvious, although still quite probable. Third, a mechanical separation of the different size groups would be a useful tool, both for testing the hypothesis and possibly for obtaining information about the characteristics of the noncolony-forming cells in soil.

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