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Bacteria on Intertidal Sand Grains

by

J. G. Anderson & P. S. Meadows

Department of Zoology,
University of Glasgow,
Scotland

(with 5 figs.)

Recent studies on larval and adult substrate selection by marine invertebrates (Wilson 1955, 1958, Meadows & Williams 1963, Meadows 1964, Gray 1966) have underlined our lack of knowledge about bacterial populations which might exist on the surface of marine sand grains.

ZoBell (1938) and ZoBell & Rittenberg (1938) quote figures for total numbers of bacteria per gram of sand, but give no details of their methods, and apparently do not differentiate between bacteria in the interstitial water and bacteria on the surfaces of sand grains. Pearse, Humm & Wharton (1942) appreciated that bacteria may be attached to the surfaces of individual sand grains; these authors, however, stated that their results were preliminary.

In the present investigation, experiments were conducted on sand samples from the surface of intertidal beaches. They were designed to give estimates of the total and viable numbers of bacteria per unit surface area of sand grain. Experiments are also reported on the possible importance of rain water and wave action in dislodging bacteria from sand grains.

Material and Methods

All sands were collected from sandy intertidal beaches between Wemyss Bay and Troon on the Ayrshire coast, Scotland. Samples of

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surface sand were collected between extreme low water springs and high water neaps. Sampling was conducted during the period January 1964 to May 1965.

The methods used differed between experiments, but generally included the following steps. 20 ml of sand was measured out, and washed six times in sterile seawater to remove the original interstitial water. The sea-water was poured off, and the sand quickly rinsed three times in a test solution. These rinses were disgarded. The sand was then shaken for a standard time (5 to 10 minutes for different experimental series) on a Griffin and Tatlock flask shaker at 400—600 shakes/min., with the test solutions. The shaking was repeated three times with 60 ml aliquots of the test solution, and the aliquots finally pooled. A note is made when our experiments differed from this procedure. In different experiments the pooled volume of washings was the basis for optical density measurements, and for total and viable counts.

The test solutions were usually sea-water, de-ionized water, and 0.1 % teepol in de-ionized water.

Our initial experiments were concerned with techniques for estimating the number of viable bacteria in washings.

It was found necessary to incubate plates for three weeks at 22°C, as the number of colonies visible did not reach a maximum before 15 to 20 days.

Experiments were also conducted on pour and spread plate methods. In all cases spread plates gave the higher counts. In the spread plate method, 15 to 20 ml molten agar were poured into a petri dish, allowed to solidify, and then the petri dish dried at 75°C for 1 hour. We found that this rather high temperature gave good absorption of the 0.5 ml of washings which were subsequently pipetted onto the agar.

The nutrient agar used was one based on a medium recommended by Torry Research Station, Aberdeen, Scotland, for cultivating many species in their National Collection of Marine Bacteria. It consisted of Oxoid L 30 Lab Lemco meat extract, 10 g; Oxoid L 37 peptone, 5 g; Oxoid CM 49 agar no. 3, 15 g; filtered sea-water to 1000 ml. The sea-water had been stored in the laboratory for 3 months before use.

Apart from preliminary experiments, all plating was done by the spread plate method. Irrespective of the solution used to wash sands, serial dilutions of the washings were made into successive volumes of sterile sea-water.

Total numbers of bacteria were counted under phase using a haemocytometer, and optical densities were measured with an E.E.L. nephelometer.

During preliminary experiments on the use of surface active agents in removing bacteria from sand grains, Teepol L and Lissapol MND (both manufactured by I.C.I. Ltd.) were tested at different concentrations. A solution of 0.1 % teepol in de-ionized water was found to be suitable. It had low foam characteristics, was not significantly opaque, and removed the largest number of bacteria from sand grains.

When sea-water washings were left to stand for more than about 30 minutes, a flocculent precipitate formed. Less precipitate appeared in the de-ionized water washings, and least of all in the 0.1 % teepol washings. The visible differences in flocculation were paralleled at a microscopic level by differences in aggregation or clumping of micro-organisms. Because of this, measurements of optical density, total counts and viable counts were made within minutes of the end of an experiment.

RESULTS

The nature of the material washed from the surface of sand grains

When sea-water, de-ionized water or 0.1 % teepol were shaken with intertidal sand, they became cloudy. The cloudiness varied with the

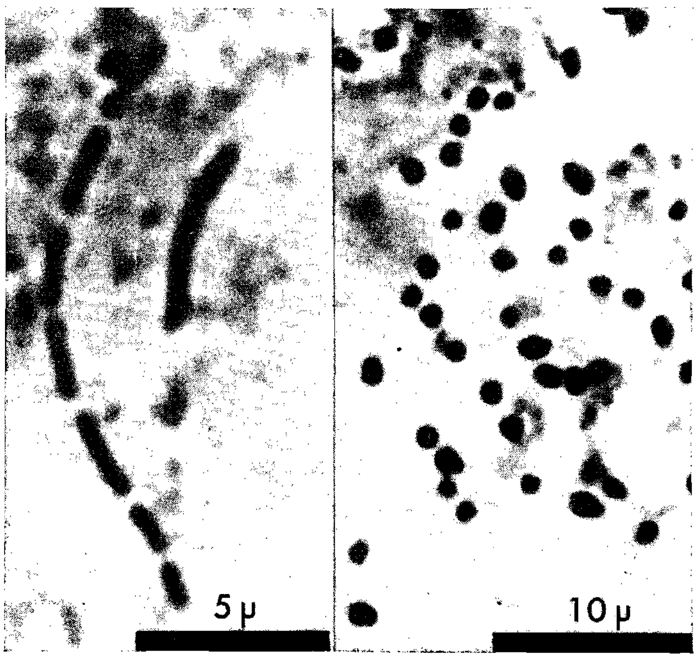

Fig. 1. Bacteria in washings. Left, a chain of rods, and right, cocci.

solution, but allowing for this, all solutions had a very similar appearance.

Using phase contrast and dark ground microscopy it was apparent that bacteria were present in large numbers (Fig. 1). We also observed a few diatoms and small amounts of unidentifiable debris (ANDERSON & MEADOWS, 1965). It was suggested to us, however, that some of the cloudiness might be due to sand grains knocking against each other and dislodging small chips of quartz, for instance. But the following experiment showed that self abrasion of this sort does not occur in our experiments.

A sample of sand was divided into two. One half was boiled in a 50/50 mixture of concentrated nitric acid and concentrated sulphuric acid for 24 hours. This treatment would remove all traces of organic matter and micro-organisms from the surface of sand grains. Both halves were then washed well with sea-water and divided into three. One third from each half was shaken for 5 minutes with sea-water, one with de-ionized water, and one with 0.1 % teepol. The acid cleaned sand imparted no detectable cloudiness to the washings (Fig. 2). The washings from the control sand were cloudy in the ratio of

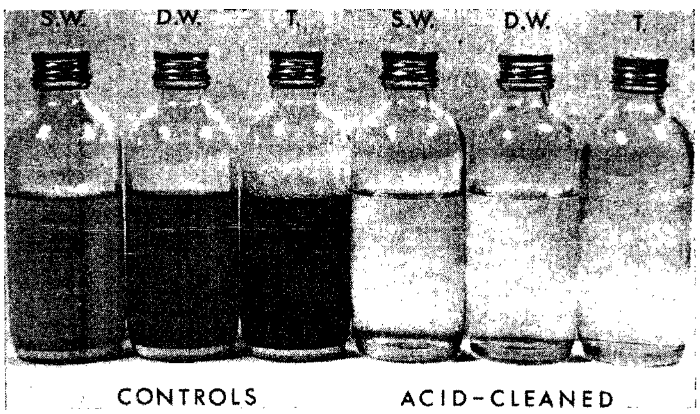

Fig. 2. Solutions which have washed acid-cleaned sand, compared with solutions which have washed untreated (control) sand.

1 : 2 : 2.5 for sea-water: de-ionized water: 0.1 % teepol (these ratios are as expected from other observations – table I). If chippings contributed to the optical density of the washings, the washings from the acid cleaned sands should have been opaque.

As bacteria were the dominant part of the visible material in washings, it was of interest to relate cloudiness (optical density) with total and viable counts. Our results (fig. 3) showed a marked correlation between total counts and optical density. However the results

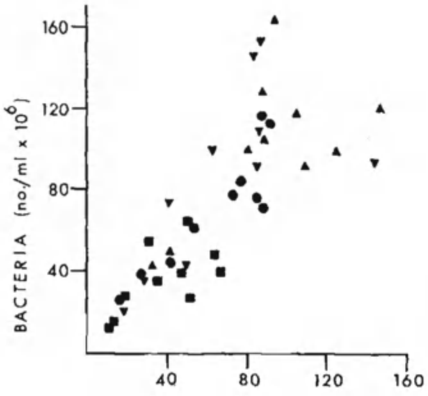

Fig. 3. Relation between total counts and optical density. Results of 10 experiments. ■, de-ionized water: ●, sea-water; ▲, 0.1% teepol; and ▼, 0.1% lissapol, washings.

from viable counts did not give such an unambiguous picture (Table I). Here the ratio $\frac{\text{viable counts}}{\text{optical density}}$ varies from 16 to 4480. If there had

TABLE I.

Relationship between viable counts and optical density for de-ionized water, sea-water, and 0.1% teepol washings.

no. exp.		(A) de-ionized water washings	A/B	(B) sea water washings	(C) 0.1% teepol washings	C/B
	viable count (a)	23800	(2.05)	11600	840	(0.07)
1	optical density (b)	31.0	(1.17)	26.4	57.0	(2.16
	ratio a/b	767.7		439.4	14.74	
	viable count (a)	26800	(2.39)	11200	1480	(0.13)
2	optical density (b)	7.0	(2.80)	2.5	16.3	(6.50)
	ratio a/b	3829		4480	90.80	
	viable count (a)	2320	(2.97)	780	560	(0.72)
3	optical density (b)	20.3	(2.90)	6.9	34.5	(5.0)
	ratio a/b	114.3		113.0	16.23	

been a close correlation between the two variables, the ratio would have been virtually constant. The variation in the ratio may be ascribed to changes in the percentage of the total numbers which are viable and can form colonies on nutrient agar. The ratios of de-ionized water and 0.1 % teepol washings in terms of both viable counts and optical densities, are in comparison remarkably constant. This means that the relative efficiency of de-ionized water and 0.1 % teepol in inducing bacteria to detach from the surfaces of sand grains, is very consistent between experiments.

From these results it seems feasible to use the optical density of a solution obtained from washing marine sand under standard conditions as a quick estimate of the total abundance of bacteria. Any estimate of viable bacteria, however, would be approximate.

Total and viable estimates of bacterial numbers on the surfaces of sand grains

One of the objects of our research has been to obtain some estimate of the total and viable numbers of bacteria on the surfaces of sand grains, expressed as numbers per g, and numbers per mm^2 of sand grain surface. To do this, it was necessary to obtain particle size distributions and weights of all our samples; these measurements were included as routine in our experiments. The usual experimental method (see material and methods section) was also designed to replace the original interstitial water, and so any possibility of obtaining information on the interstitial bacterial flora was immediately removed.

The results of 12 experiments are shown in table II. The large variation in both viable and total counts is the most immediately obvious characteristic of the data. Viable counts range from 0.2 to $40/mm^2$ of sand grain surface and total counts from 25×10^3 to $259 \times 10^3/mm^2$. There is similar variation on soil samples (BURGESS 1958, p. 47), so it is probably a common characteristic of either the techniques used or of the terrestrial and aquatic sediments themselves.

All samples in experiments 1 to 12 were taken from intertidal sand, and looked very similar to the naked eye, consisting of fairly clean fine sand. The mean particle size of the samples fell between 0.1 and 0.6 mm diam. There was no obvious relationship between particle size and bacterial numbers, but the particle sizes of samples did not vary widely, so our results cannot be looked upon as confirming or disagreeing with those of ZOBELL (1938). ZOBELL studied the numbers of bacteria in sediments having a wide range of particle size, and showed that finer sediments contained more bacteria.

We could also find no correlation between tidal level and numbers of bacteria. Our sampling programme, however, was not geared to detecting small differences between tidal levels, as each of the samples for experiments 1 to 12 was collected from a different beach as well as from a different tidal level.

Our results (Table II) suggested that viable counts varied more than total counts, but comparison between standard deviations was difficult as the means themselves were set at very different levels.

TABLE II.

Total and viable bacteria on sand grain surfaces. V_a, V_b = *coefficient of variation for experiments 1 – 6, and 7 – 12 respectively. s.d. = standard deviation.*

exp.	substrate particle size: mean diameter (mm)	substrate particle size: s.d. (mm)	no./mm² of sand grain surface, sand agitated with: sea water	de-ionized water	0.1% teepol	no./g of sand, sand agitated with: sea water	de-ionized water	0.1% teepol
			total counts x 10³			total counts x 10⁶		
1	0.3170	0.0858	45.98	100.6	114.4	347.2	759.4	863.7
2	0.3455	0.1268	41.27	64.91	138.5	283.7	446.3	952.2
3	0.4278	0.1388	25.02	47.05	75.01	140.3	263.9	420.7
4	0.4088	0.1445	26.16	64.45	81.82	153.7	378.6	480.7
5	0.2183	0.0958	49.27	53.47	91.49	546.1	592.6	1014
6	0.5248	0.2365	109.6	245.3	258.7	501.0	1112	1183
mean	0.3737		49.55	95.96	126.7			
s.d.	0.1051		31.10	75.47	68.76			
V_a	28.12		62.76	78.65	54.29			
			viable counts			viable counts x 10³		
7	0.2315	0.1115	11.60	23.80	0.840	117.5	241.0	8.506
8	0.4303	0.1120	1.376	4.088	0.9882	7.672	22.79	5.508
9	0.1803	0.060	1.725	4.522	0.2087	21.64	56.73	2.618
10	0.4055	0.1288	5.973	14.27	0.7135	34.67	82.82	4.141
11	0.3433	0.1748	20.0	34.0	8.571	137.7	234.1	59.02
12	0.3713	0.1023	16.80	40.20	2.220		no figures available	
mean	0.3725		9.579	20.15	2.257			
s.d.	0.0989		7.833	15.13	3.164			
V_b	30.20		81.77	75.09	140.2			
$V_b - V_a$	+2.08		+19.01	−3.56	+85.91			

Calculation of coefficients of variation, however, allows comparison of variability in groups of data set at widely different orders of magnitude (SIMPSON, ROE & LEWONTIN, 1960). Such is the case for the total and viable counts in table II, where the largest difference is between the total and viable counts from 0.1 % teepol washings (means of 126.7 ± 68.76/mm² and 2.257 ± 3.164/mm² respectively). Here, although it is not obvious from the standard deviations, the viable counts vary more widely than do the total counts; coefficients of variation are 140.2 and 54.29 respectively. The coefficients of variation (V_a and V_b) and their differences (V_b—V_a), for the data in table II are recorded in rows 9, 18 and 19. It can be seen from these that although total counts are always higher than viable counts by about three orders of magnitude, the variation in the viable counts is the same (de-ionised water) or higher (sea-water and 0.1 % teepol) than the variation in the total counts. The only obvious explanation is that the three solutions used to wash the sands are very consistent within themselves in the total numbers of bacteria they remove, but are more variable in the percentage of bacteria which they kill.

In general, the ratio of sea-water washings: de-ionised water washings: 0.1 % teepol washings, was 1 : 2 : 2.5 for total counts, and 1 : 2 : 0.25 for viable counts.

The small proportion of the total bacterial population that grew on nutrient agar was not unexpected (Table II), and is in agreement with the results of other workers in soil and aquatic microbiology (GIBSON 1957, BURGES 1958, HAYES & ANTHONY 1959, JANNASCH 1958).

So little work has been done on the microbial flora of marine sands, that there is no norm with which to compare our results. ZOBELL (1938) quotes a viable count of 22 x 10³/g of sand (median diameter 50—1000 μ), but gives no method, and states that his sand was wet when weighed. All our samples are quoted in terms of dried sand (columns 4 to 9, table II). Nevertheless, our figures are not very far from those of ZOBELL; they range from 2 to 241 x 10³/g of dry sand (viable counts). PAERSE, HUMM & WHARTON (1942), like ZOBELL, weighed sand which contained different amounts of moisture. They also outline their method for removing bacteria from the surfaces of sand grains, and it is similar to that used in the present investigation. PEARSE, HUMM & WHARTON record maximum and minimum counts of 1250 x 10³/g and 5 x 10³/g of wet sand. It is interesting that their figures show highest values from high water samples (mean 486 x 10³/g), lower values for mid tide samples (mean 110 x 10³/g), and lowest values for low water samples (mean 34 x 10³/g). ZOBELL (1938) and PEARSE, HUMM & WHARTON (1942) do not give figures for total numbers.

They also do not express their results as number per unit surface area of sand grain. We feel that this is an instructive unit of measurement, and have tended to rely on it rather than on numbers per gram, except where comparison with other work was necessary.

We found no difficulty in recording numbers in both units. Knowing the numbers of bacteria which are removed from a given weight of sand, it is simple to calculate the numbers per gram. The numbers per mm^2 of sand grain surface were calculated in the following manner. The true volume of a sample can be obtained by measuring its displacement in water. From the mean particle diameter one can calculate the mean particle volume; dividing this latter sum into the true volume of the sample gives the number of sand grains in the sample. It is then straight forward to estimate the total surface area in the sample by multiplying the surface area of one grain (obtained from the mean particle diameter) times the number of grains. In practice this can be expressed as

$$A = \frac{V.\,4\pi\,(d/2)^2}{4/3.\,\pi\,(d/2)^3} = \frac{6V}{d}$$

where A = total surface area of sand grains in the sample, V = true volume of the sample, and d = mean diameter of the sand grains in the sample.

The removal of material from intertidal sand grains by rain

Our experiments in which sand was shaken with de-ionized water, led us to consider whether a similar process might be occurring as rain fell on exposed intertidal sands. Accordingly, experiments were conducted in which de-ionized water was allowed to filter through a column of undisturbed sand.

A sample of sand was washed six times with sea-water, and allowed to settle through sea-water in a vertical glass tube, 2 cm diameter, to form a column of 30 cm. Sterile membrane-filtered sea-water was always used in this process. The mean particle diameter of the sand was 0.17 mm. The sand was retained in the tube by a plug of glass wool. The sea-water was run off to the level of the top of the sand column; sterile de-ionized water was then run through the column, and was collected over 150 minutes in 20 ml fractions at the bottom of the tube. The optical densities of the fractions were measured, and are shown as a cumulative plot in fig. 4. Sterile sea-water running through similar columns at the same time remained clear. Microscopic examination showed that the de-ionized water contained large numbers of bacteria, and the sea-water virtually none.

During this series of experiments, it was noted that the flow rate of de-ionized water decreased by about 50 % of its initial value, while

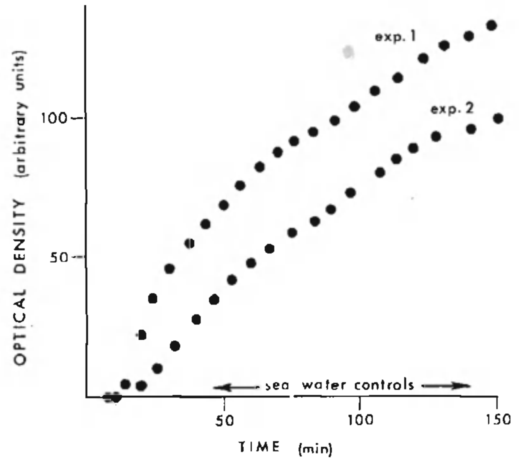

Fig. 4. Optical density of de-ionized water, and sea-water (controls), which has filtered through a column of sand. Two experiments are figured.

the sea-water flow rate decreased by about 10 %. Flow rates from a representative experiment are plotted in fig. 5.

Rain water or freshwater run-off from the land will therefore have two effects on intertidal sands. It will cause bacteria to detach from sand grains, and will reduce the movement of interstitial water.

Material dislodged from sand grains by wave action

There is no doubt that intertidal and immediately sublittoral sands are subjected to a good deal of movement (King 1959, Ingle 1966). Depending on wave force and direction, sands may move normal to the shore or along the shore, or a combination of the two. With any

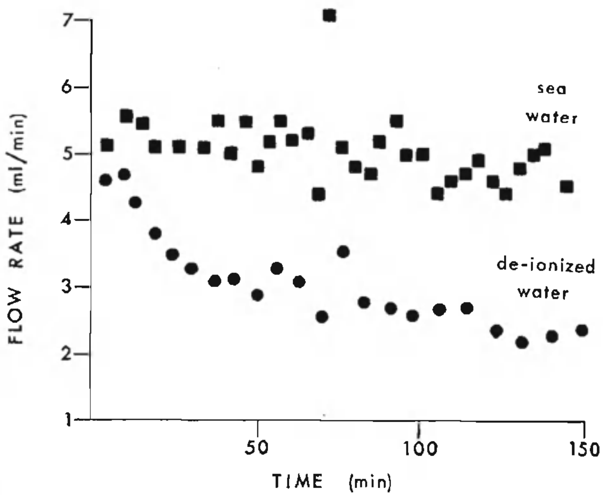

Fig. 5. Flow rates for sea-water and de-ionized water through a sand column.

significant wave action the disturbance extends to depths of between 3 and 5 cm (King 1959, p. 174).

As wave action increases so does the movement of sand, and because of this more and more particles will tend to bump against each other. Our experiments show that when sands are shaken more vigorously the optical density of the resultant washings increases (Table III), and one of the authors (P.S.M.) has observed similar

Table III.

Sands shaken with sea-water, de-ionized water, and teepol 0.1%. Influence of gentle (a) and vigorous (b) agitation for 3 minutes on optical density of washings. Unbracketed figures represent optical density in arbitrary units, and bracketed figures are the ratio b/a. The samples of sand used in each experiment were collected from different beaches.

experiment		sea-water	de-ionized water	teepol 0.1%
1	(a)	9	31	33
	(b)	23	46	60
		(2.56)	(1.48)	(1.82)
2	(a)	27	50	54
	(b)	47	64	75
		(1.74)	(1.28)	(1.39)
3	(a)	10	18	17
	(b)	18	28	43
		(1.80)	(1.56)	(2.52)

effects in heavy breakers on the Cornish coast, England. It is therefore likely that as wave action becomes heavier, bacteria and debris are progressively removed into suspension.

When wave action continues for any length of time, more and more material will be dislodged from the surfaces of sand grains. Both Pearse, Humm & Wharton (1942) and the present authors have considered this problem. We both estimated what proportion of the total bacterial population remained on sand grains after repeated periods of washing. Pearse, Humm & Wharton consider that their first wash removed between 70 and 90 % of the total bacterial population, while the present authors procedure removes a smaller proportion (Table IV).

It is also probable that during prolonged movement, sand particles will be worn down by self abrasion. In the experiments of the present investigation, however, self abrasion does not occur to any noticeable extent (Fig. 2).

TABLE IV.

Influence of three successive periods of shaking on optical density of resultant washings. Optical densities are expressed as a percentage of the sum of the figures for sea-water, de-ionized water, and 0.1% teepol solutions from the first period of shaking.

	exp. 1	exp. 2	exp. 3
1st 4 minute period	100	100	100
2nd 4 minute period	85	50	66
3rd 4 minute period	58	51	54

SUMMARY

The number of bacteria on the surfaces of intertidal sand grains has been estimated by shaking sand with sea-water, de-ionized water and 0.1 % teepol. Total counts, viable counts, and optical densities of the resultant washings were measured. 0.1 % teepol caused the largest number of bacteria to detach from sand grains, followed by de-ionized water, and lastly by sea-water. Total counts and optical densities were strongly correlated. There was little relation between viable counts and optical densities.

The total and viable number of bacteria on the surfaces of sand grains showed great variation, but viable counts varied more than did total. Sand particle size ranged from 0.18 to 0.52 mm diam. Total counts ranged from 25 x 10^3 to 259 x 10^3/mm^2 of sand grain surface, that is from 140 x 10^6 to 1183 x 10^6/g dry sand; the respective viable counts were 0.2 to 40/mm^2 and 2.6 x 10^3 to 241 x 10^3/g. No relationship was observed between bacterial numbers and particle size.

Experiments suggest that heavy wave action will remove large numbers of bacteria from the surfaces of sand grains. It is also evident that rain water and run-off from the land will wash bacteria from the surfaces of intertidal sand grains, and at the same time will reduce the flow of interstitial water.

RÉSUMÉ

On a calculé le nombre de bactéries sur les surfaces des granules de sable, situées entre la marée haute et la marée basse. La méthode de calcul était la suivante: secouer le sable ou dans l'eau de mer, ou dans l'eau déionisée, ou dans le 'teepol' 0.1 % (détersif). On a calculé les comptes totaux, les comptes viables et les densités optiques des produits de lavage ainsi obtenus. C'était le 'teepol' 0.1 % qui a détaché

des granules de sable le plus grand nombre de bactéries, l'eau déionisée en a détaché moins, tandis que l'eau de mer en a détaché encore moins. Il est évident qu'une corrélation définie existe entre les comptes totaux et les densités optiques, mais une telle corrélation était beaucoup moins évidente entre les comptes viables et les densités optiques.

Le compte total et le compte viable de bactéries sur la surface des granules de sable démontraient de grandes variations, mais ces variations étaient plus extrêmes dans le cas du compte viable que dans le cas du compte total. Les dimensions des parcelles de sable s'échelonnaient de 0,18 à 0,52 mm en diamètre. Les comptes totaux s'échelonnaient de 25 x 10^3 à 259 x 10^3/mm^2 de surface des granules de sable, c'est à dire de 140 x 10^6 à 1183 x 10^6/g sable desséché; les comptes viables respectifs s'échelonnaient de 0,2 à 40/mm^2 et de 2,6 x 10^3 à 241 x 10^3/g. Nul rapport n'était perceptible entre le nombre de bactéries et la dimension des parcelles.

Les expériences semblent indiquer qu'une violente activité des ondes peut détacher de grands nombres des bactéries des surfaces des granules de sable. Il est évident que l'eau de pluie et l'eau qui s'écoule de la terre peuvent aussi déplacer les bactéries des surfaces de granules de sable, situées entre la marée haute et la marée basse, et qu'en même temps ces eaux diminueront le mouvement de l'eau interstitieille.

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