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Effect of Bacterial Polysaccharide Accumulation on Infiltration of Water Through Sand

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ABSTRACT

MITCHELL, R. (Weizmann Institute of Science, Rehovot, Israel), AND Z. NEVO. Effect of bacterial polysaccharide accumulation on infiltration of water through sand. Appl. Microbiol. 12:219-223. 1964.—A study was carried out of the mechanisms of biological clogging of sand during prolonged percolation of water containing high levels of organic matter. It was found that polysaccharide-producing microorganisms predominated in clogged layers of sand. A positive correlation was observed between accumulation in the profile of polysaccharides and clogging of columns of sand in permeameters. The level of oxygen in the system appears to determine the equilibrium between production of clogging materials and their decomposition.

The possibility of reclaiming waste water or of disposing of urban sewage by percolation of sewage effluent through sand or soil to the ground water aquifer below has been under investigation for a number of years (Amramy, Caspi, and Melamed, 1962; McGauhey and Winneberger, 1963). A serious obstacle encountered in these investigations results from a rapid clogging of the surface layer of sand or soil in the infiltration ponds. The percolation failure is similar to that observed in septic tank systems and has been associated with an accumulation of ferrous sulfide in the clogged layer (McGauhey and Winneberger, 1963). Percolation failure in infiltration ponds has been associated with biological activity and has been prevented by treatment of the system with microbial inhibitors (Allison, 1947; McCalla, 1950). The current investigation is concerned with the mechanism of biological clogging of sand and with the environmental conditions governing accumulation of clogging materials during infiltration.

MATERIALS AND METHODS

Percolation experiments were carried out in glass permeameters (lysimeters) with an internal diameter of 5 cm and a length of 45 cm. A constant head was maintained by means of a float and overflow. The height of the sand column was 30 cm, and the water outlet was 5 cm below the sand surface. The hydraulic gradient was 0.35. The sand used in this study was taken from dunes near Ashkelon Beach. The dune sand contained less than 0.03% organic matter. Mechanical analysis showed 93% coarse sand and 7% fine sand.

The permeameters were packed with sand to maximal uniformity, the bulk density of which was 1.6 g/cm^3 after compaction. The added casein, sulfur, and iron were mixed with the upper 5-cm layer of sand to simulate the situation in infiltration ponds. Four replicate permeameters were used for each treatment. To rid the system of entrapped air, water was percolated through the sand for 4 days before treatment.

To obtain microbial products from decomposing casein in sand, casein and sulfur were added in porous plastic bags which were placed in the water above the surface of the sand. The microbial products settled on the sand surface or were leached into the top 5 cm of sand from which they were obtained. Tap water was used in all percolation studies. The system was maintained at a constant temperature of 25 ± 2 C. Hydraulic conductivity (rate of percolation) was determined by measuring the quantity of water passing through the permeameter during a period of 2 hr, and was calculated according to Darcy's law (Baver, 1956). Decline in hydraulic gradient was determined for each part of the column by measuring the water head in the piezometers above and below each section of the column. Percolation rate and hydraulic gradient were measured at each observation time. After these measurements were made, samples of sand were taken from the permeameters for microbial analysis. Fungal counts were made on Martin's (1950) medium. Bacteria and actinomycetes were enumerated on soil extract agar (Allen, 1957).

For polysaccharide analysis, 2-g samples of sand were taken from permeameters. The samples were extracted with 10 ml of 0.25% aqueous $Na_6(PO_4)_2$. This solution acts as a surface-active agent, and it extracted all of the polysaccharide in samples of sand. The extract contained some microbial cells. Residual microbial cells were precipitated by differential centrifugation. Total polysaccharide was determined quantitatively by anthrone reagent (Brink, Dubach, and Lynch, 1960). The standard curve was determined from polysaccharide produced by *Flavobacterium* in liquid soil extract medium amended with 2% casein.

Polysaccharide with residues of glucuronic acid (polyuronide) was determined by the method of Lynch, Hearns, and Cotnoir (1957). Hydrolysates were compared with a standard curve of glucuronic acid. In studies with different substrates, polysaccharides were collected from liquid

basal medium amended by different carbon sources at a concentration of 2% after growth on a rotary shaker for 14 days. The biological activity was stopped by autoclaving or by adding 10% phenol. NaOH (0.5 N) was added to the solution, which was then centrifuged. The precipitate was washed with 0.5 N HCl to extract soluble polysaccharide. Both acid and alkaline extracts of polysaccharide were precipitated with acetone.

For chromatographic analysis, the polysaccharides were hydrolyzed in boiling 2 N H_2SO_4 for 8 hr, and the hydrolysate was neutralized by $BaCO_3$. Monosaccharide units in the hydrolysates were determined by paper chromatography. The solvent used was butanol-acetic acid-water in the ratio 4:1:4. The chromatograms were immersed in 1.0% $AgNO_3$ in acetone for monosaccharide determination, and in 1% ninhydrin solution for amino groups. Uronic acids were determined in hydrolysates by Bial's reagent orcinol solution in hydrochloric acid containing $FeCl_3$.

To form ferrous sulfide chemically in the columns of sand, it was necessary to react H_2S with the soluble iron in equilibrated permeameters. A thin layer of granular FeS was placed on the sand surface, and a few drops of 2 N H_2SO_4 were added to the percolate water. The H_2SO_4 released H_2S into the percolate. The dissolved gas reacted with ferrous iron in the sand, and large quantities of colloidal FeS were formed. The hydraulic gradients in the permeameters were measured before and after precipitation of ferrous sulfide.

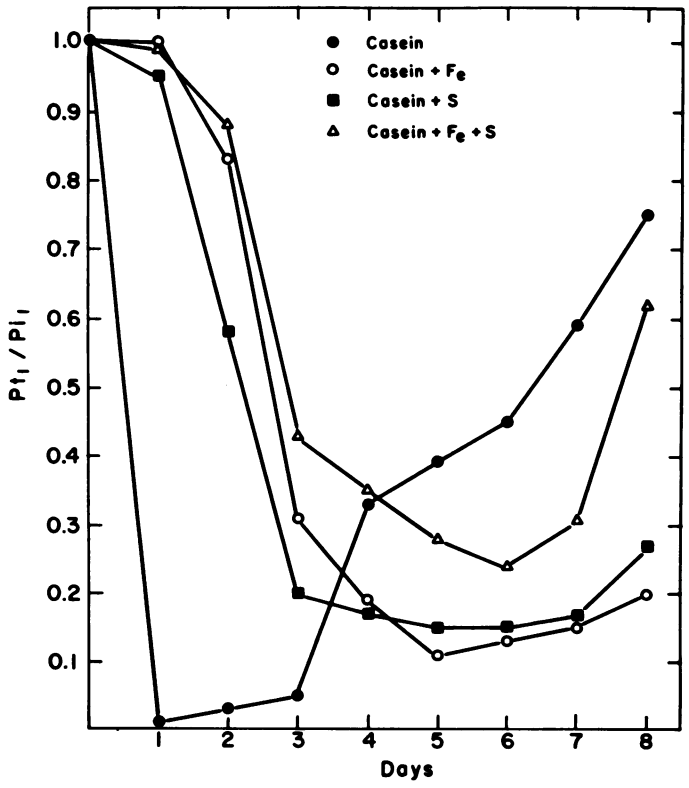

FIG. 1. *Effect of treatment with casein, iron, and sulfur on permeability of sand in permeameters.*

RESULTS

To minimize the number of variables, a mixture of casein, ferric chloride, and sulfur was used to simulate sewage effluent. Both effluent and casein were found to have a carbon-to-nitrogen ratio of six. Data obtained from percolation tests in permeameters filled with sand showed a decline of infiltration rate with time in those permeameters treated with casein alone (Fig. 1). The data indicate that clogging was independent of treatment of the sand with iron or sulfur. Columns of sand in permeameters treated with casein alone were completely clogged 24 hr after treatment. When iron or sulfur or a combination of the two was added to the sand together with casein, the clogging was not as severe and occurred 3 days later than with the casein alone. No clogging was detected without the addition of organic matter to the permeameters. Clogging continued for 10 days.

The degree of clogging was determined by measuring the percolation rate, which was calculated for the whole profile as a unit. Piezometer readings in the permeameters indicated the degree of clogging in each section of the profile by checking hydraulic gradients at different depths. An intense clogged layer was formed in the top 2 cm of sand surface in permeameters treated with casein alone (Table 1). In those permeameters treated with casein together with sulfur, the clogged layer occurred at a depth greater then 4 cm from the sand surface in the same section where the black layer of ferrous sulfide was formed.

The effect of different permeameter treatments on ferrous sulfide formation in the sand is also shown in Table 1. Ferrous sulfide was formed in all permeameters treated with casein and sulfur, with or without the addition of iron. Apparently, there is sufficient iron present in the sand for maximal ferrous sulfide formation. No ferrous sulfide was formed in sand amended with casein alone, despite the fact that clogging was most severe in permeameters treated in this way.

In the light of these data, the implication of FeS in the clogging process was investigated further. Microorganisms utilizing the organic material found in sand or water

TABLE 1. *Piezometer readings showing the depth of the clogged layer in permeameters treated with casein, sulfur, and iron, and observations on the formation of the ferrous sulfide layer*

Treatment	Hydraulic gradients at different depths* (cm)				Depth of ferrous sulfide layer	Depth of the clogged layer
	0–2	2–4	4–22	22–24		
					cm	*cm*
None	0	0.37	0.34	0.35	—	—
Casein	1.5	1.00	0.42	0.50	—	0–2
Casein+Fe	—	—	—	—	—	0–2
Casein+S	0	0	0.80	0.25	8–10	4–22
Casein+S+Fe	0	0	0.64	0.15	8–10	4–22

* All depths were measured relative to the sand-water interface.

release H_2S, which is soluble in water, and moves down the profile with the percolate water, reacting with iron dissolved from the sand grains. The limiting factors for biological FeS formation are the available sulfur found in the percolate water, and microaerophilic conditions in the system. To simulate this biological process of FeS formation, an excess of granular FeS was added to the sand surface in permeameters. Dilute H_2SO_4 was then added to release H_2S, which formed a precipitate of colloidal FeS on the surface of the sand grains to a depth of approximately 20 cm. No clogging was obtained in any of the permeameters containing excess quantities of colloidal FeS in the sand profile. Granular FeS was layered on the sand surface and also mixed with the sand, but caused no reduction in percolation rate.

An attempt was made to correlate clogging with accumulation in sand during the percolation period of total polysaccharide and of polysaccharide with glucuronic acid residues (polyuronide). Data obtained (Table 2) showed a high correlation between these microbial products found in sand, and the clogging of sand as determined by percolation rate. The correlation coefficient for total polysaccharide was 0.71, and for the polyuronide fraction was 0.92. Treatment of permeameters with casein together with sulfur resulted in formation of a layer of FeS. However, no additional clogging or accumulation of additional clogging agents was observed.

A study of the microbiological changes occurring in flooded sand amended by organic matter was undertaken. As a result of these amendments, there was a decline in the abundance of fungi and actinomycetes and an increase in the bacterial population. Polysaccharide-producing microorganisms developed in treated sand and reached a maximum 10 days after amendment. The majority of these bacteria were identified as strains of *Flavobacterium*.

In pure-culture studies, the bacterium was grown on a liquid basal medium amended with different types of organic matter including glycogen, glycerol, glucose, mannitol, soil extract, and yeast extract. Polysaccharides were extracted and determined after 14 days of incubation. The results indicate that this strain of *Flavobacterium* produces basically the same products from different carbon sources (Table 3). The monosaccharide units detected were glucose, galactose, xylose, and glucuronic acid. The difference among substrates was found to be limited to the ratio between the fractions of polysaccharides with and without glucuronic acid residues.

Polysaccarides containing glucuronic acid residues were found to be more resistant to both chemical and biological decomposition than those consisting solely of simple sugars. Drastic chemical treatment is required to decompose polyuronides, whereas simple polysaccharides are easily decomposed. Similarly, 1 day of drying of clogged sand caused a decline of 85% in the total polysaccharide in the sand, and a decline of only 20% of the polyuronide fraction. Data were obtained indicating that large quantities of polysaccharides are produced in pure culture from low concentrations of organic matter. As little as 0.01% glucose was required for growth of *Flavobacterium*. Polysaccharide production was detected at a concentration of 0.25% glucose, reached a maximum at 0.50% glucose, and was shown to be correlated with oxygen tension. Under totally anaerobic conditions, no polysaccharides were produced, although substantial quantities were produced microaerophilically.

To investigate the possibility that microbial cells alone can cause clogging, the microorganisms were separated from the mass of polysaccharides produced in liquid culture. The two fractions were added separately to the sand in permeameters. It was found that 1 g (wet weight) of either fraction caused clogging. Data obtained from clogged permeameters showed a ratio of 1:10 of poly-

TABLE 2. *Accumulative concentration of total polysaccharide and polyuronide during prolonged percolation of water through sand treated with casein and sulfur in permeameters*

Time after treatment	Total polysaccharide per g of sand		Polyuronide per g of sand		Pt/Pi*	
	Casein	Casein + S	Casein	Casein + S	Casein	Casein + S
days	*mg*	*mg*	*μg*	*μg*		
1	0.0	0.0	0.0	0.0	0.97	0.98
2	7.0	10.0	369	306	1.01	0.93
4	12.5	13.5	996	1,038	0.53	0.57
7	14.5	15.0	1,381	1,807	0.11	0.08
9	15.8	12.0	2,727	2,210	0.16	0.16
11	13.8	17.5	2,775	—	0.08	0.09
15	7.0	13.0	3,155	3,325	0.06	0.08

* $\text{Pt/Pi} = \dfrac{\text{permeability after treatment}}{\text{permeability before treatment}}$.

TABLE 3. *Influence of carbon source on quantity and type of polysaccharide produced by Flavobacterium in liquid media*

Treatment	Yield of polysaccharide	Identification tests					
		Anthrone reaction	Bial reaction	Monosaccharide units by chromatography: Glucose + galactose	Monosaccharide units by chromatography: Xylose	Monosaccharide units by chromatography: Glucuronic acid	Amino groups
	mg/liter						
Casein (2%)	56	+	Tr*	+	+	Tr	+
Glycerol (2%)	107	+	+	+	Tr	+	+
Glycogen (2%)	153	+	+	+	Tr	+	+
Glucose (2%)	48	+	+	+	−	+	+
Glucose (1%) + NH_4Cl(1%)	70	+	Tr	+	−	Tr	+
Mannitol (2%)	107	+	+	+	Tr	+	+
Soil extract (10%) + glucose (1%)	87	+	Tr	+	−	+	−

* Trace.

saccharides to cells, indicating that polysaccharide constituted 90% of the clogging material.

Discussion

The evidence obtained suggests that the major factor causing clogging in sand amended by organic matter under prolonged percolation of water is the accumulation of polysaccharides both with and without glucuronic acid residues. High correlations were found between clogging, as measured by percolation rate, and the accumulative concentration of polysaccharides.

Treatment of the sand with casein without the addition of sulfur resulted in accumulation of polysaccharides and in clogging of the column of sand. However, no ferrous sulfide layer was formed, indicating that the clogging process is independent of ferrous sulfide accumulation. Furthermore, colloidal ferrous sulfide chemically precipitated in the permeameters failed to impair water percolation. It would appear that the formation of ferrous sulfide is an indicator of the reducing conditions which result in accumulation of polysaccharides. It was surprising to find that, in permeameters in which ferrous sulfide was formed together with polysaccharides, the clogging was not as severe as in permeameters with polysaccharide alone. These data suggest that not all of the added organic matter was available for production of polysaccharides when FeS was formed, and that some part was used for growth of a sulfur- and iron-reducing microflora.

The accumulation of polysaccharides in a clogged layer of sand was associated with an increase in numbers of bacteria, especially in the polysaccharide-producing *Flavobacterium*, which was the predominant bacterium found in clogged sand. Chromatographic studies showed that the monosaccharide units of polysaccharides produced by this bacterium in liquid medium were similar to those found in sand. Other slime-producing microorganisms, including *Bacillus cereus*, *Chromobacterium violaceum*, *Alcaligenes faecalis*, *and Zooglea ramigera*, are known to cause clogging of sand filters (Calaway, 1957), but were not found to be present in large numbers in this study. The high concentration of polysaccharide produced by *Flavobacterium* per unit cell suggests that the bacterial cells alone would not have an appreciable effect on the percolation rate of water through sand. Gupta and Swartzendruber (1962) provided evidence to show that in biologically clogged sand the bacterial count was not high enough for the cells alone to seal the pores. The role of polysaccharides in increasing aggregation was emphasized by Rennie, Truog, and Allen (1954), who demonstrated that the addition to soil of polysaccharide extracted from cultures of *Agrobacterium radiobacter* was followed by a marked increase in aggregation.

Avnimelech and Nevo (Soil Sci., *in press*) showed that treatment of sand with materials having high carbon-to-nitrogen ratios resulted in prolonged clogging, whereas treatment with casein, which has a low C–N ratio, was followed by a temporary decline in percolation rate. It would appear that amendment with high C–N ratio materials is associated with production of larger quantities of polysaccharides with uronic acid residues, and that these polysaccharides are more resistant to biological degradation than are polymers of simple sugars. This hypothesis is supported by Chesters' (1959) observation that uronic acid content of organically amended soil increased to a greater extent in the absence than in the presence of added nitrogen. Data obtained in the current study with pure cultures of *Flavobacterium* suggest that the substrate governs the ratio of formation of stable to unstable polysaccharide by the bacterium.

Addition of 0.25% organic matter was found to be sufficient to yield large quantities of polysaccharides in pure culture. It has been reported that approximately 0.5% organic matter is deposited in the surface 5 cm of sand during infiltration of sewage effluent (Amramy et al., 1962). Thus, a plentiful supply of organic matter is always present in sewage effluent infiltration ponds for maximal polysaccharide production. In addition to a supply of organic matter, the clogging of sand and soil appears to be correlated with a low oxidation-reduction potential. It is probable that prevention of the formation of a reduced layer in the sand or soil would result in continuous high percolation rates. A number of aquatic plants, including rice, are known to cause oxidation of the soil in which they are grown. It is possible that such biological oxidizing agents may be used as an alternative to the current practice of periodic drying (Amramy et al., 1962) to obtain continuous infiltration during reclamation or disposal of sewage effluent.

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