Applied and Environmental Microbiology, Feb. 1994, p. 641–653 Vol. 60, No. 2
0099-2240/94/$04.00+0
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Structure and Composition of Biological Slimes on Paper and Board Machines

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Received 6 August 1993/Accepted 3 December 1993

Biological slimes (biofilms) collected from the wet end of paper and board machines were examined by electron microscopy and analyzed for fatty acid composition, neutral sugar composition, and ATP. Electron microscopy revealed minuscule prokaryotic organisms (diameter, 0.2 to 0.4 μm). Larger cells morphologically resembling *Sphaerotilus* and *Leptothrix* spp. were found in slimes from machines using recycled fiber or unbleached pulp. The bacteria were embedded in a slimy matrix and often contained reserve materials microscopically resembling poly-β-hydroxybutyrate and glycogen. Fatty acid analysis of the slimes revealed bacterial signature fatty acids in concentrations equivalent to the presence of 2×10^{10} to 2.6×10^{12} (average, 7×10^{11}) bacterial cells (live and dead) per g (dry weight) of slime. The slimes contained several known components of bacterial polysaccharides in addition to glucose, indicating that the slime body consisted of bacterial polysaccharides. The slimes contained uronic acids equivalent to a binding capacity of 12.5 to 50 μmol of divalent cations per g (dry weight) of slime. The uronic acid-containing polysaccharides may be responsible for the accumulation of heavy metals in the slime. Calculation of the ATP contents of the slimes resulted in an estimate of 5×10^{12} cells per g (dry weight) of slime when calibrated with pure bacterial cultures isolated from the slimes. From electron micrographs, an estimate ranging from 1×10^{10} to 1.5×10^{12} (average, 4×10^{11}) cells per g (dry weight) of slime was obtained.

Biofilm growth on paper and board machines causes downtime for the machine and problems in quality of the product. The biofilms seed microbes into the process water, deteriorating the hygienic quality of the product. Biofilms also clog wires and cause discoloration of the paper. The raw materials of papermaking consist mainly of biogenic substances (cellulose, hemicellulose, starch, and wood resins) and hence are susceptible to biological growth. Slime problems in the pulp and paper industry were studied in the 1950s and 1960s (2). The problem was phased out for decades by the intensive use of biocides. The most effective biocides, such as phenyl mercuric compounds, were, however, highly toxic. The use of phenyl mercurials and other mercuric biocides in the pulp and paper industry has caused serious pollution of lake sediments (42). Therefore, their use has been banned in Finland and some other countries and is decreasing in others. The second generation of papermaking biocides was less toxic and also less effective. The use of biocides is subject to restrictions when food-quality paper products are made (5, 12). As a result, biological growth on paper and board machines is commonplace.

Wastewater discharges by paper and board mills have decreased because of environmental concern. This has been achieved by increased recycling of process water. In the 1970s, approximately 100 m^3 of water was used per metric ton of paper manufactured, but less than 10 m^3 is more usual today. As a result, the paper machine white water has become richer in nutrient salts and degradable carbon, thus contributing to microbial problems.

Although biofilm formation has been studied intensively in natural aquatic systems, wastewater treatment systems, and medical appliances (6, 7, 8, 11, 19, 21, 32), only a few studies have been made on biofilms in the paper machine environment (22, 27, 31) in spite of the fact that paper and board production represents one of the largest industries in the world. For instance, in Europe and North America, there are about 3,500 paper and board machines with an annual production of ca. 160 million metric tons (18).

Bacteria growing in a biofilm are more resistant to biocides than free-living bacteria (6, 23, 30). Thus, it is possible that the use of biocides will favor biofilm bacteria over unattached organisms. We studied paper and board machine slimes by using electron microscopy, fatty acid and carbohydrate analyses, and ATP measurement. The results contribute to understanding the ecology of biofilm bacteria as the first step towards microbial control of a paper machine with a minimum of biocides.

MATERIALS AND METHODS

Paper and board machines. The study included five different paper and board machines. Table 1 shows the raw materials used in the machines, and Table 2 describes the white water of the machines. One to three different biocides were used on the machines at any time, up to a total of 10 kg of effective substance per metric ton of paper or board produced. The active compounds of the biocides were 2,2-dibromo-3-nitrilopropionamide, methylenebisthiocyanate, 3,5-dimethyl-1,3,5 (2H)-tetrahydrothiadiazine-2-thione, 5-oxo-3,4-dichloro-1,2-dithiol, and a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one.

Collection of slime samples. Slimes were collected with a spatula from the steel surfaces of the machines during normal operation in quantities of up to 1 kg (wet weight) per sampling site. The slimes were collected during 1991 and earlier.

Isolation of bacteria. Bacteria were isolated from paper and

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TABLE 1. Raw materials used in the paper and board machines

Machine	Product	Type of pulp	Sizing agents	Mineral additive(s)[a]	Raw water treatment
1	Board	Bleached kraft pulp	Starch, AKD	None	Mechanical
2	Board	Bleached kraft pulp	Starch, resin	Kaolin, titanium dioxide[b]	Mechanical
3	Board	Bleached kraft pulp	Starch, AKD	Calcium carbonate, kaolin	Mechanical
5	Board	Unbleached and bleached kraft pulp	Starch, AKD, resin	Calcium carbonate or kaolin	Mechanical and chemical
6	Paper	Thermomechanical pulp (43%), ground wood (32%), kraft pulp (3%), recycled fiber (6%), broke (16%)[c]	None	None	Chemical

[a] Used for filling or coating. In addition, talcum powder for pitch control was used regularly.
[b] Kaolin, $Al_2O_3 \cdot 2SiO_2 \cdot 2H_2O$.
[c] Broke, reject material from the same machine.

board machine slimes as follows. The slimes were homogenized in sterile water (1 g/5 ml) and spread on plate count agar (1), starch-nutrient agar (39), and half-strength Trypticase soy agar (26). Pure cultures were obtained by repeated streaks on the respective media. The following strains, representing frequently found species, were used in the present study: *Enterobacter agglomerans* E42, *Klebsiella pneumoniae* E31, *Pseudomonas aeruginosa* E102, *Pseudomonas cepacia* E121, *Pseudomonas paucimobilis* E173, *Pseudomonas caryophylli* E217, *Flavobacterium* sp. strain E1720, *Clavibacter michiganense* E25, *Bacillus licheniformis* E2, and *Bacillus cereus* III-P4.

Electron microscopy. The samples of slimes and pure bacterial cultures were prefixed with 3% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 2 h at room temperature and washed three times in the same buffer. The specimens were postfixed for 2 h in buffered 1% (wt/vol) osmium tetroxide, dehydrated in a graded series of ethanol and propylene oxide, and embedded in Epon LX-112. Thin sections were cut with a diamond knife on a ultramicrotome and double stained with uranyl acetate and lead citrate. The grids were examined with a model JEM-1200EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) at an operating voltage of 60 kV. Morphological recognition of inorganic particles in the slimes was based on earlier work with energy-dispersive spectrometry (31).

Fatty acid analysis. Whole-cell fatty acids of bacteria were analyzed as methyl esters (29) with a Hewlett-Packard model HP5898A system, which contains a gas chromatograph, a 5% methyl phenyl silicone-fused silica capillary column, a flame ionization detector, an automatic sampler, an integrator, and a computer (Hewlett-Packard Co., Palo Alto, Calif.). The bacteria were identified on the basis of their fatty acid compositions with MIDI Microbial Identification System, library version 3.0 (Microbial ID Inc., Newark, Del.). Fatty acid compositions of 100-mg freeze-dried samples of paper and board machine slimes were analyzed by the same method as that used for bacteria but with five-times-larger reagent volumes.

Fatty acids were designated (e.g., 16:1 ω7*c*) so that the figures represent, from left to right, the total number of carbon atoms (i.e., 16), the number of double bonds (i.e., 1), the position of the double bond from the ω end of the fatty acyl chain (i.e., ω7), and the configuration of the double bond (i.e., *c* for *cis*, *t* for *trans*). Branched fatty acids were designated by the letter *i* for iso or *a* for anteiso, cyclic fatty acids were designated by *cy*, and hydroxylated fatty acids (e.g., 12:0 2OH) were designated by the position of the hydroxyl group from the carbonyl carbon (i.e., 2OH).

Preparation of extracellular polysaccharides. The bacteria were grown on skim milk agar (5% skim milk, 1% agar) or B-medium (28) with glucose or sucrose as the carbohydrate. The bacteria were grown at room temperature for 3 to 30 days until they had produced slime. The cells were collected from the plates, suspended in sterile, distilled water, and homogenized in a blender (Atomix; MSE, London, England) to separate capsular material or shaken for 5 min when only loose slime was present. The cells were removed by centrifugation, and the polysaccharide was precipitated from the supernatant with 2 to 3 volumes of ice-cold acetone. The precipitate was collected with a glass rod or by centrifugation and resuspended in water. Pronase was added to 0.5 to 1 mg/ml, and then the mixture was incubated for 1 h at 37°C. Acetone precipitation was repeated twice, and the final crude preparation was freeze-dried.

Analysis of neutral sugars. Freeze-dried machine slimes (10 mg) and extracellular polysaccharides (1 mg) were hydrolyzed in 0.1 M HCl at 100°C for 48 h and then neutralized with anion-exchange resin (Amberlite IRA-410; HCO_3^- form). Xylose (150 μg) was added as an internal standard before hydrolysis. The neutralized sample was filtered through glass wool and evaporated to dryness. The dry residue was dissolved in water, and one-half volume of 5% (wt/vol) $NaBH_4$ in 10 mM

TABLE 2. Description of the white water of paper and board machines

Machine	pH in headbox	Temp (°C)	TOC[a] (mg/liter)	Total N (mg/liter)	Total P (mg/liter)	Fe (mg/liter)	Mn (mg/liter)	Cl^- (mg/liter)	Suspended solids (mg/liter)
1	9–10	39–47	60–80	1.5	0.13	0.05	<0.02	31.3	1,250
2	4–5	35–41	30–50	0.7	0.2	0.1–0.2	<0.03	15–19	50–140
3	8–9	40–52	70–75	1.1	0.25	0.05–0.2	<0.02	15–23	140
5	7	45–52	40–80	0.6–0.8	0.4–0.5	0.3	0.03–0.09	9–10	250–380
6	5	48–55	600–800	13–18	4–6	2.8[b]	ND[c]	ND	6,000[d]

[a] TOC, total organic carbon measured from filtered (glass fiber) white water.
[b] Measured from acidified and filtered (glass fiber) samples.
[c] ND, not determined.
[d] Total solids.

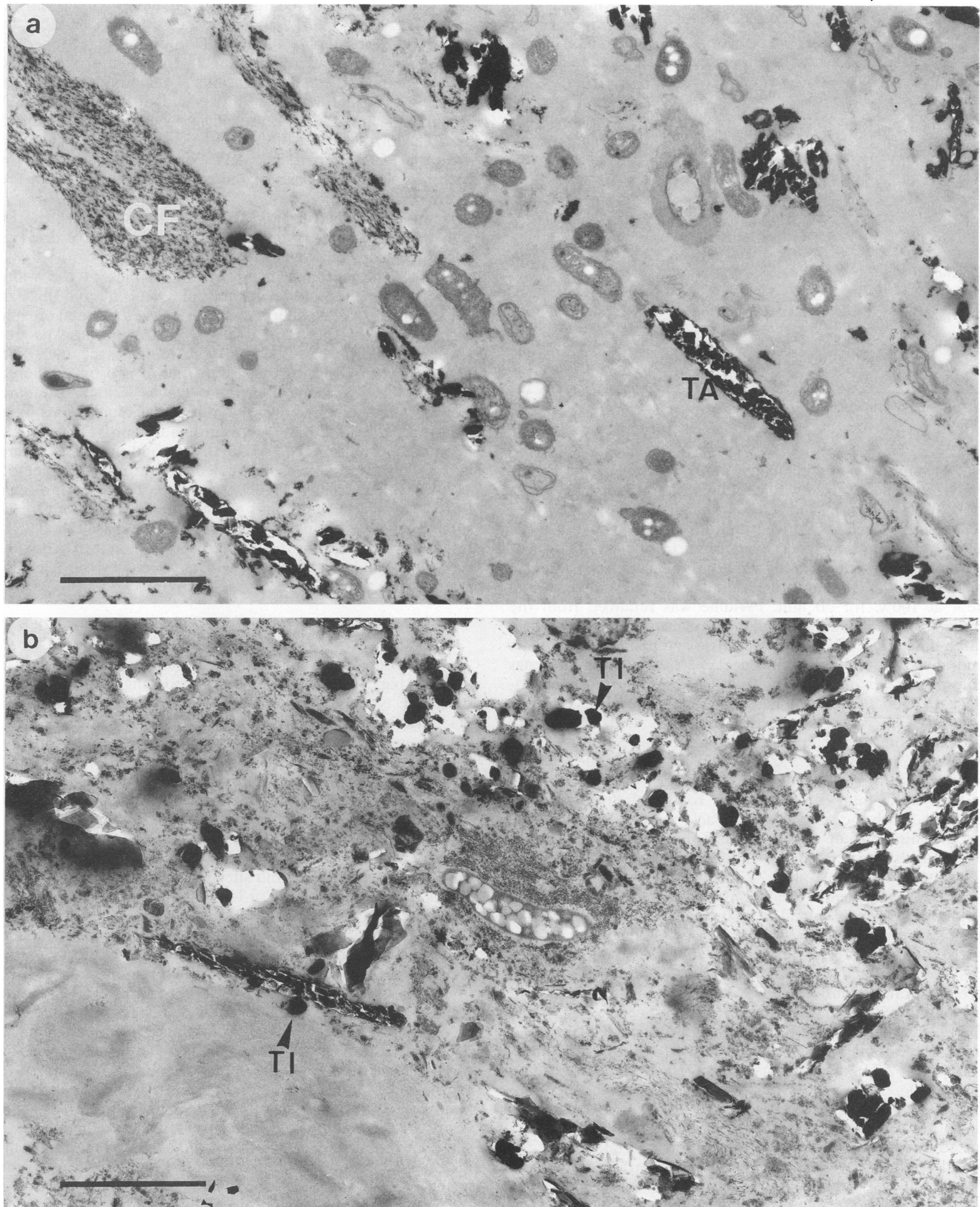

FIG. 1. Thin sections of slimes from machine 5 (a) and machine 2 (b). Bacterial cells are embedded in the slime matrix and lie in a streamlined orientation. Electron-dense shell-like structures (talcum powder [TA]), single particles (titanium dioxide [TI]), and remnants of degraded cellulosic fibers (CF) are visible. Bars, 2 μm.

TABLE 3. Slimes collected from paper and board machines

Slime	Dry wt (%)	pH	Color	Site of collection
Machine 1				
A	5.3	6.5	Pink	Wire section
B	3.5	4.9	Pink	Wire section
C	1.9	6.7	White	Wire section
Machine 2				
D	6.4	4.4	Grayish	Wire section
E	4.1	6.4	Brownish	Wire roll
Machine 3				
F	2.8	7.2	White	Under the wire rolls
G	6.1	ND[a]	Dark brown	Bearing of the returning wire
Machine 5				
J	9.7	6.9	White	Top wire
K	7.3	5.6	Green	Section were starch is sprayed on the web
Machine 6				
L	4.4	5.2	Gray	Wire roll
M	6.4	5.2	Gray	Press section

[a] ND, not determined.

NaOH was added and incubated in the dark overnight. Excess $NaBH_4$ was neutralized with cation-exchange resin (Amberlite IR-120, H^+ form), and the boric acid thus formed was removed by three repetitive additions of 5% (vol/vol) acetic acid in methanol and then evaporated to dryness. The sugar alcohols were peracetylated to give alditol acetates by adding a mixture of equal amounts of pyridine and acetic anhydride and kept at 100°C for 30 min. Pyridine was removed from the cooled sample by adding methanol and evaporating to dryness three times. The dry residue was dissolved in dichloromethane and analyzed, and the alditol acetates were identified by gas chromatography-mass spectrometry (GC-MS) with a Hewlett-Packard model HP5890A gas chromatograph, HP-5 capillary column, HP5970A mass selective detector, and HP59940A MS ChemStation. The temperature program was 3 min at 180°C and then increases of 3°C per min to 260°C, the injector temperature was 260°C, and the carrier gas was helium.

Analysis of total carbohydrates. Total carbohydrates were measured with the phenol-sulfuric acid method (15).

Determination of uronic acids. Quantitative analysis of uronic acids was performed by the *m*-hydroxydiphenyl method (3).

ATP measurement. ATP was measured with a luminometer (Bio-Orbit, Turku, Finland). For the measurement, fresh slime samples were diluted 100-fold in sterile distilled water. Bacteria were grown on Trypticase soy agar for 24 to 30 h, and the cells were suspended in water to a concentration of 1 mg/ml. We tried other solutions (Trypticase soy agar broth, 0.067 M phosphate buffer [pH 7.8]; or 0.1 M Tris-acetate buffer [pH 7.7]) for suspending the bacteria, with no significant improvement in the ATP yield. For the analysis, 100 μl of the sample, 100 μl of ATP monitoring reagent (luciferin-luciferase of firefly; Bio-Orbit), 100 μl of ATP releasing reagent (Bio-Orbit), and 100 μl of ATP standard solution (internal standard; 20 to 200 ng/ml in sterile distilled water; Bio-Orbit) were mixed in the order given, and the reading was taken after the addition of each reagent. ATP was also extracted from the bacteria, by two other methods. In the perchloric acid (PCA) method, 400 μl of cell suspension (1 mg/ml, in water) and 150 μl of ice-cold 3 M PCA were vortexed, incubated on ice for 60 min, and neutralized with 80 μl of TE buffer (0.4 M Tris, 0.1 M EDTA [pH 7.4]) and 80 μl of saturated K_2CO_3. The mixtures were centrifuged, diluted 1:50 with TE buffer (0.1 M Tris, 2 mM EDTA [pH 7.75]), and measured for ATP content by using 100 μl of sample, 50 μl of ATP monitoring reagent, and 100 μl of ATP standard solution. In the trichloroacetic acid (TCA) method, the cell suspensions (1 mg/ml; in water) were mixed with equal volumes of ice-cold 10% (wt/vol) TCA containing 4 mM EDTA, vortexed, and incubated on ice for 15 min. The mixtures were diluted and measured for ATP content as in the PCA method. The ATP content was calculated for each sample on the basis of the response obtained from the internal standard.

Dry weight measurements. The machine slimes and bacterial cells collected from Trypticase soy agar plates were dried at 105°C to a constant weight.

Analysis of inorganic compounds. Samples of the white water of the paper and board machines were filtered through Whatman GF/A glass fiber filters and analyzed for total organic carbon with a TOC 5050 Total Organic Carbon Analyzer (Shimadzu Corp., Kyoto, Japan) according to the ISO 8245 Standard (17). Other analyses were performed on unfiltered white waters by standard methods (1) as follows (standard method numbers are shown parentheses). Chloride ions were measured with a DX-100 ion chromatograph (Dionex Inc., Sunnyvale, Calif.; method 4110 B), iron and manganese were measured by an inductively coupled plasma method (method 3120 B) with a Plasma 40 ICP spectrometer (The Perkin-Elmer Corp., Norwalk, Conn.), and the suspended solids were measured by using Whatman GF/A glass fiber filters (method 2540 D). Total phosphorus was measured by digestion with peroxodisulfate (9), and total nitrogen was measured by the Kjeldahl method (10).

RESULTS

Description of the slimes. Eleven different slimes from five different paper and board machines producing both coated and uncoated types of paper and board were examined. The machines operated at a wide range of pH (i.e., pH 4 to 10), with temperatures from 35 to 55°C and raw materials ranging from highly purified bleached pulp to recycled fiber. Biocides were used on all machines. The slimes were collected from the wet-end wire area, the press section, and the dispersing system for spray starch.

Table 3 describes the slimes collected from the machines. The dry weight of the slimes ranged from 2 to 10%, depending on the type of machine and site of collection. The pH of the slimes was about the same or lower than that measured in the headboxes of the machines (a headbox is the container feeding the pulp slurry to the wire). Lower pH values may be related to acid production in anaerobic parts of the slime.

The slime deposits on the paper and board machines varied in thickness from barely visible to several centimeters. The slimes were usually white to grayish-opaque, but some had colored tints. Since none of the machines used any chemical dyes, the color of the slimes was likely of biological origin. The physical constitution of the slimes ranged from a slurry to viscous or pasty heterogeneous material. The slimes appeared mainly on surfaces which remained wet because of splashing water. In machine 6 using recycled fiber, the accumulation of slime into the wet-end area of the machine was massive. This is explained by the high amount of degradable organic carbon in the process water (Table 2). This result implies increasing difficulty of microbial control of the wet-end process when recycled fiber is used.

Light microscopy. Light microscopic inspection revealed eukaryotic organisms (protozoa and fungi) in slimes from

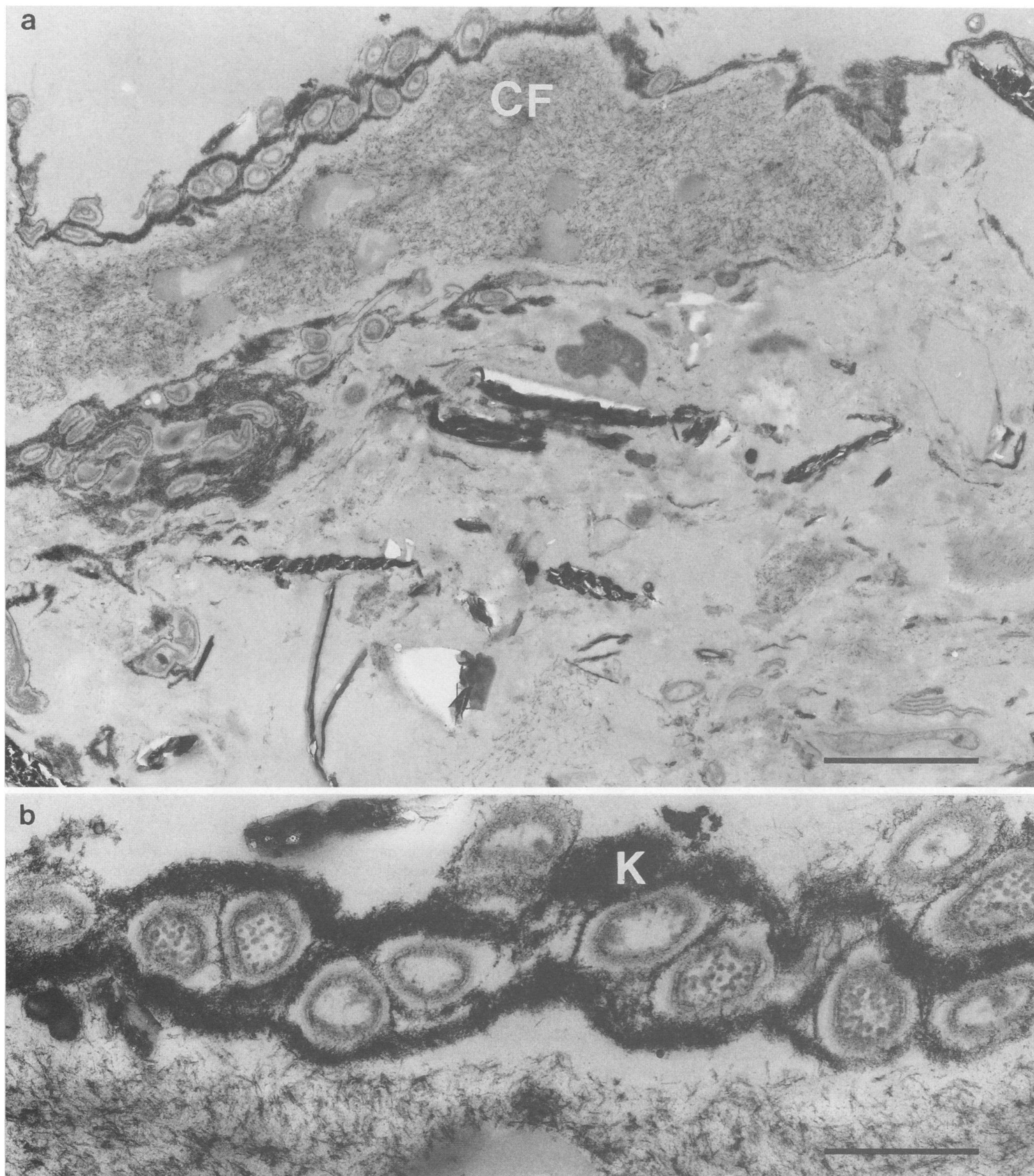

FIG. 2. (a) Thin section of slime from machine 3. The bacteria occurring in patches are small. (b) Detail of panel a showing bacterial cells surrounded by electron-dense amorphously fibrous material (kaolin [K]) and disintegrating cellulosic fiber (CF). Electron-loose space is visible between bacteria and cellulosic fiber. Bars, 2 (a) and 0.5 (b) μm.

machine 6 but not in the slimes from other machines. This may relate to the use of recycled fiber on machine 6. Needle-like shapes resembling *Sphaerotilus* spp. were always observed in some of the slimes.

Electron microscopy. The slimes were examined by transmission electron microscopy. The area in thin sections occupied by bacteria in different slimes ranged from 0.3 to 7.7% (average, 3.5%) as calculated from approximately 50 electron

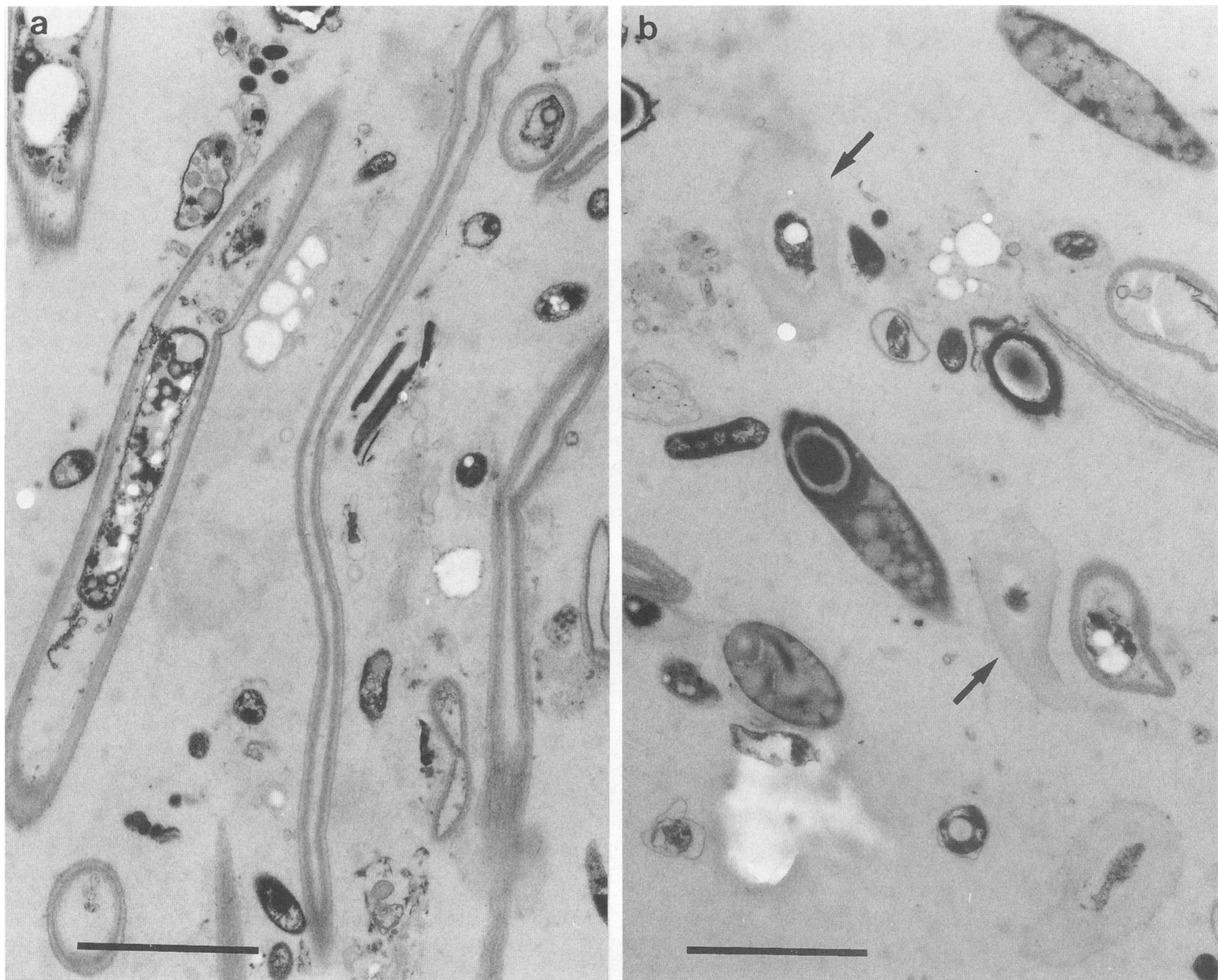

FIG. 3. Thin sections of slime from machine 5. The bacteria are held together by material with an electron-loose structure. (a) Large sheathed bacteria and empty sheaths, which were frequently observed, are visible. (b) Polarly located endospores, free spores, and numerous small bacteria with diameters of 0.25 to 0.4 μm are visible. Some cells are surrounded by lightly stained capsules (arrows). Bars, 2 μm.

micrographs. Patterns with structures resembling cell walls were interpreted as being cells. The average size of a bacterial cell was calculated to be 0.25 μm^3. On the basis of the area occupied by bacteria, we calculated a bacterial presence of 0.7×10^9 to 80×10^9 cells per g (wet weight) of slime corresponding to 0.1×10^{11} to 15×10^{11} (average, 4×10^{11}) cells per g (dry weight) of slime (dry weights shown in Table 3).

Figures 1 to 5 were chosen from about 300 electron micrographs to show special features of the slimes. Representative views of two board machine slimes with great heterogeneity are shown in Fig. 1. Bacterial cells were embedded in a slimy matrix. The diameters of the cells ranged from 0.2 to 0.45 μm. Bacteria were lying in a streamlined orientation. Some cells were densely packed with vacuoles of reserve material, and some had distinct capsules around the cell. Bacterial ghosts and cell debris were also observed. In addition, the slimes contained electron-dense particles of titanium dioxide (used as a pigment for coating the board), shell-like structures of talcum powder (added to the pulp to counteract pitch problems), and amorphous, finely dispersed material of unrecognizable origin. Remnants of degraded cellulosic fibers were also seen.

Figure 2a shows a slime from machine 3 with bacteria occurring in patches. An enlargement of Fig. 2a (Fig. 2b) shows an electron-dense, amorphously fibrous material, kaolin (used as an additive for papermaking), surrounding small (diameter, 0.2 μm) bacterial cells and disintegrating cellulosic fiber. The electron-loose space between bacteria and cellulosic fiber may result from the dissolution of the fiber by the microbes.

The bacteria in the slime from machine 5 were held together by lightly staining material (Fig. 3). No structures other than microbial cells or parts of them were seen. The amorphous material surrounding some microbes may be slime excreted by them. Some cells were surrounded by lightly stained capsules (Fig. 3b). Large, sheathed *Leptothrix*-like bacteria (0.5 by 3 μm) and empty sheaths were frequently observed. Free spores, endospores, and numerous small bacteria with diameters of 0.25 to 0.4 μm were seen.

Figure 4a shows a section of slime from the wire area of

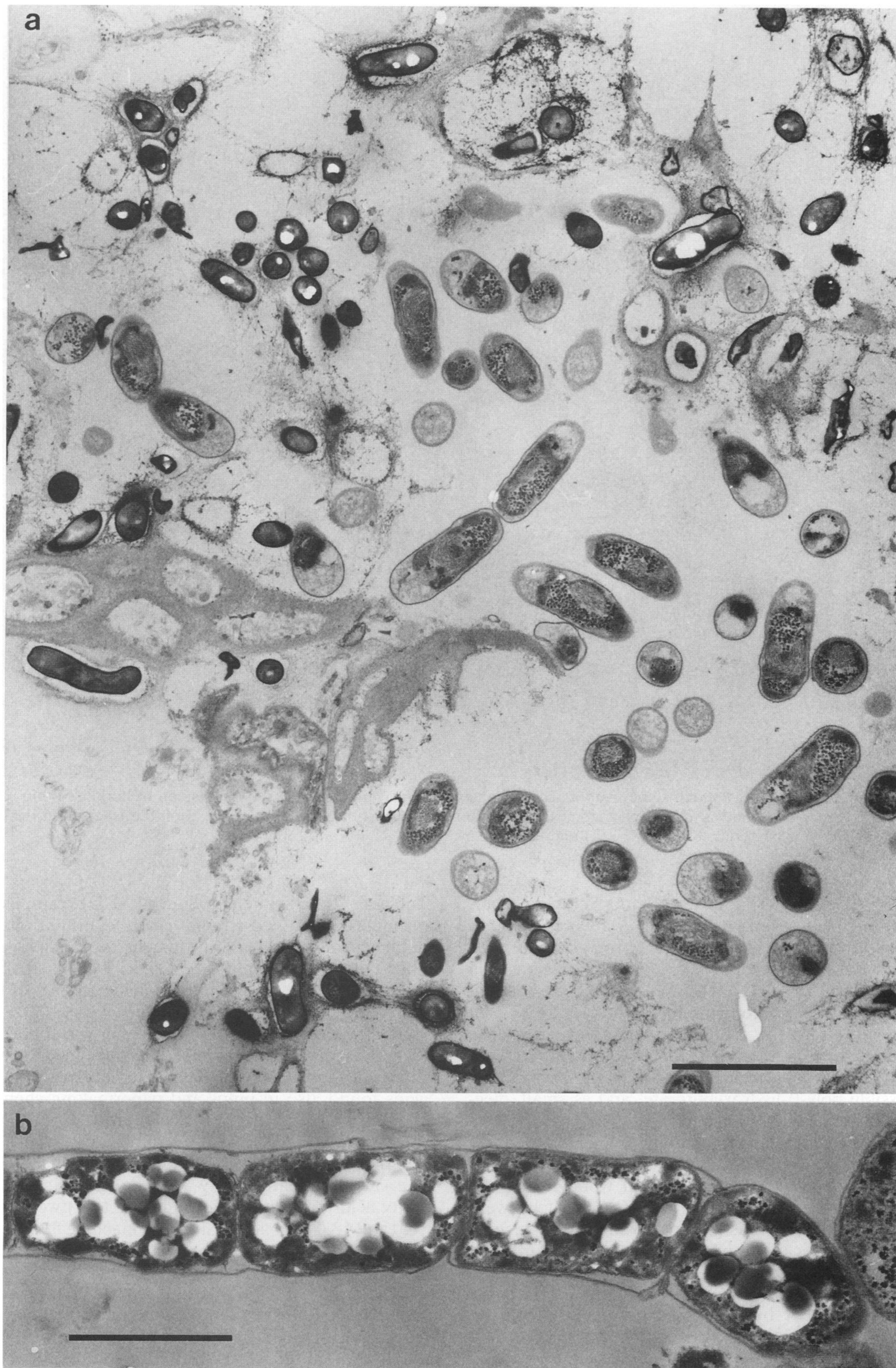

FIG. 4. (a) Thin section of slime from machine 6. Microcolonies of gram-positive bacteria are embedded in densely staining slime with a fibrous structure. Small communities of larger, gram-negative bacteria surrounded by less-electron-dense slime are visible. (b) *Sphaerotilus*-like sheathed bacteria often found in the slimes. Bars, 2 μm.

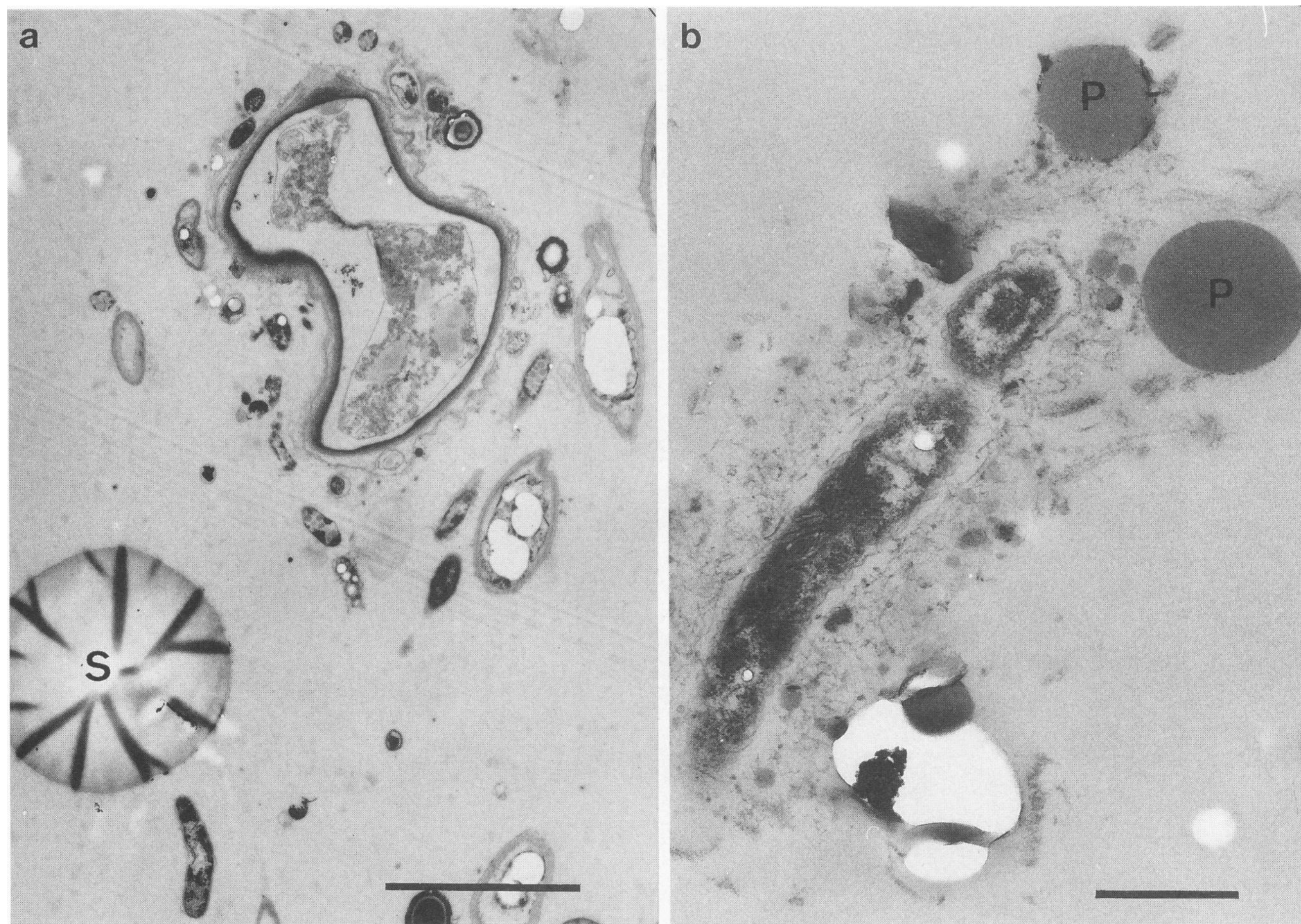

FIG. 5. (a) Thin section of spray starch slime from machine 5 showing large starch particles (S) and small bacteria; (b) thin section of slime from machine 6 (using ground wood) shows pitch particles (P) of different sizes. Both of these particles are interdispersed in the bacterial slime matrix and in the vicinity of bacterial cells. Bars, 4 (a) and 0.5 (b) μm.

paper machine 6 using ground wood and recycled fiber. Microcolonies of small, gram-positive bacteria were seen embedded in densely staining slime with a fibrous structure. In addition, small communities of larger, gram-negative bacteria were surrounded by less-electron-dense slime. The sheathed *Sphaerotilus*-like bacteria were extremely large (1.5 by 2.7 μm; Fig. 4b) compared with the other members of the bacterial community (Fig. 4a). Large amounts of reserve material were seen in the sheathed bacteria both as large, nonstaining particles, possibly poly-β-hydroxybutyrate, and as small, electron-dense particles, possibly glycogen (Fig. 4b).

The slime collected near the starch delivery system of machine 5 frequently contained starch granules (Fig. 5a). Slime from machine 6, which uses ground wood, contained pitch particles of different sizes (Fig. 5b). Both particles were interdispersed in the bacterial slime matrix and in the vicinity of bacterial cells, indicating that these substances may be utilized by the microbes.

Dry weight of the bacteria. The dry weight of the pure bacterial cultures varied from 15 to 26% (wt/wt), with an average of 20%, which was used in calculations of bacterial presence in the slimes.

Biochemical characterization of slime biomass. (i) Fatty acid composition. The fatty acid compositions of the raw materials used on the paper and board machines are shown in Table 4. The alkyl ketene dimer (AKD) resin, used on board machines, contained straight-chain saturated fatty acids. The unbleached pulps, (thermo)mechanical pulps, and recycled fiber contained mainly 18:2 ω6,9*c*, 18:3 ω6,9,12*c*, 18:1 ω9*c*, 16:0, 17:0 *a*, and 18:0. The fatty acid contents of the bleached pulps were very low.

A much greater variety of fatty acids was found in the slimes (Table 5) than in the pulps. The fatty acids are grouped in Table 5 to show bacterial signature fatty acids (group A), fatty acids found also in pulps and papermaking chemicals (group B), and the remaining fatty acids (group C). The fatty acids in the slimes may originate from microorganisms, wood fibers, and sizing agents used on the machines, such as AKD and resin size. The dominating fatty acids in the board machine slimes (fatty acids A to J from machines 1 to 5) were straight-chain 16:0 and 18:0 fatty acids. Slime K (machine 5) differed from the other board machine slimes in having a high content of diunsaturated 18:2 ω6,9*c* fatty acid. The paper machine slimes (slimes L and M from machine 6, which uses mechanical pulp and recycled fiber) contained mainly polyunsaturated 18:2 ω6,9*c* and 18:3 ω6,9,12*c* fatty acids together with 18:1 ω9*c*, 16:0, and 17:0 *a*, all of which were also found as dominating fatty acids in the pulps used on the same machine.

Table 6 shows the whole-cell fatty acid compositions of the bacteria isolated from the machine slimes. In the gram-

TABLE 4. Fatty acid compositions of paper and board mill pulps and AKD resin

Fatty acid[a]	Fatty acid composition (mg/g [dry wt]) of[b]:						
	Bleached board mill pulps		Paper mill pulps				
	Birch	Pine	Unbleached chemical pulp	Thermomechanical pulp	Mechanical pulp	Recycled fiber	AKD resin
12:0	—[c]	—	—	—	—	—	0.1
14:0	—	—	—	—	—	—	0.7
15:0 *a**	—	—	—	0.02	—	—	—
15:0*	—	—	—	—	—	—	0.5
16:0 *a*	—	—	—	0.03	0.02	—	—
16:1 ω7*c*	—	—	0.04	0.05	0.07	0.04	—
16:0	0.08	0.02	0.2	0.4	0.3	0.4	4.5
17:1 *i*/17:1 *a*	—	—	—	0.09	—	—	—
17:0 *a*	—	—	0.2	0.3	0.3	0.2	—
17:0*	—	—	0.03	0.04	0.05	0.03	0.6
18:3 ω6,9,12*c*	—	—	1.0	1.7	1.6	0.8	—
18:2 ω6,9*c*/18:0 *a*	—	—	2.1	3.6	3.4	1.7	—
18:1 ω9*c*	—	—	1.0	1.6	1.8	0.9	—
18:0	0.07	0.02	0.1	0.1	0.1	0.5	4.9
19:0 *a*	—	—	0.02	0.02	0.03	—	—
18:0 2OH	—	—	—	—	—	—	0.1
20:2 ω6,9*c*	—	—		0.03	0.04	—	—
20:1 ω9*c*	—	—	—	0.06	0.06	—	—
20:0	0.04	—	0.04	0.03	0.04	0.03	—
Total fatty acids	0.19	0.04	4.7	8.1	7.8	4.6	11.4
Total bacterial signature fatty acids (% of total fatty acids)	—	—	0.03 (0.6)	0.06 (0.7)	0.05 (0.6)	0.03 (0.7)	1.1 (10)

[a] Bacterial signature fatty acids are indicated by an asterisk.
[b] Average of two parallel samples with a standard deviation of 5 to 50%.
[c] —, <0.02 mg/g.

negative strains, the major fatty acids were 16:0, 18:1, 16:1 ω7*c*, 17:0 *cy*, 14:0, and hydroxylated fatty acids with 10, 12, 14, or 16 carbon atoms, depending on the species. In the gram-positive strains, the major fatty acids were branched 15:0 *i*, 15:0 *a*, 16:0 *i*, and 17:0 *a*.

Table 5 shows that 4 to 26% of the fatty acids in slimes from machines processing chemical pulp (machines 1 to 5) qualified as bacterial signature fatty acids, but in the paper machine that used mechanical pulp and recycled fiber (machine 6), this figure was around 1%. In bacteria, the signature fatty acids constituted 24 to 69% of the whole-cell fatty acids (Table 6). As expected, the proportion of signature fatty acids in clean pulps and papermaking chemicals was low (Table 4). It is therefore possible to use signature fatty acid content to estimate the contribution of prokaryotic biomass in machine slimes. By using the signature fatty acid contents of the bacteria for calibration and with the average volume of one cell equal to 0.25 μm^3 (on the basis of electron microscopy), a density of 1.1 g/cm^3, and a dry weight of 20%, we calculated the slime bacterial presence to range from 2×10^{10} to 2.6×10^{12} (average, 7×10^{11}) bacteria per g (dry weight) of slime.

(ii) Carbohydrate composition. At least five different sugars in addition to glucose were detected in the slimes and extracellular polysaccharides of slime pure cultures when analyzed by GC-MS for neutral sugars (Fig. 6). Since starch and cellulose, present on the machines, are composed of glucose, sugars other than glucose can be used to estimate the contribution of microbes in the slime polysaccharide. In the board machine slimes (with the exception of slime K, collected near the starch delivery point of machine 5), 9 to 55% (wt/wt) of the neutral sugars were sugars other than glucose (Fig. 6a). In the polysaccharides isolated from the pure slime cultures, this figure was 65 to 85% (average, 74%; Fig. 6b). Therefore, it seems that 12 to 74% of the board machine slime polysaccharide may be of bacterial origin.

All slimes were also analyzed for uronic acids and total carbohydrates (Table 7). The uronic acid content of the board machine slimes ranged from 0.5 to 2%, and that of the bacterial extracellular polysaccharides ranged from 0.5 to 7% (wt/wt) of the dry weight. The total carbohydrate content of the board machine slimes was high, ranging from 35 to 97% (wt/wt) of the dry weight.

(iii) ATP measurements. Table 8 shows the ATP content of slimes collected from machine 6, nine bacteria isolated from machine slimes, and two reference strains of species occurring in the slimes. The ATP contents differed considerably between different bacteria, when the commercial ATP-releasing reagent was used. ATP recovered from *Pseudomonas* species was approximately 1% of that from gram-positive bacteria. The ATP yield obtained with two other methods for cell breaking, i.e., extractions with PCA and TCA, was much higher and less variable between different bacteria. This suggests that a species-dependent variation of lysis of the cells with the commercial reagent was the reason for the low ATP yield.

The ATP contents of the machine slimes were in the same concentration range as those of the bacterial biomass. The average ATP content of the bacteria was 111 μg/g (dry weight) of cells. The use of this value to calculate the biomass in the slimes results in approximately 300 mg of bacterial biomass per g (dry weight) of slime. This value corresponds to 5×10^{12}

TABLE 5. Fatty acid composition of slimes collected from paper and board machines

Fatty acid or determination	Fatty acid composition (mg/g [dry wt] of slime)[a]									
	A	B	C	D	E	G	J	K	L	M
A. Signature fatty acids of bacterial origin										
15:0 *i*	0.9	0.7	0.2	—[b]	0.05	0.1	0.05	0.06	0.1	—
15:0 *a*	0.1	0.05	—	—	—	0.05	0.02	0.08	0.2	0.2
15:0	0.3	0.2	0.1	—	0.04	0.06	0.06	0.03	—	0.1
16:0 *i*	0.3	0.05	—	—	—	0.03	—	0.06	—	—
16:1 ω5*c*	0.04	—	—	—	—	0.04	—	0.04	—	—
17:0 *i*	0.2	0.08	0.04	—	—	0.04	—	—	0.09	0.09
17:0 *cy*	—	0.06	0.1	—	—	—	0.06	—	—	—
17:0	0.3	0.2	0.1	0.03	0.07	0.09	0.07	0.03	0.3	0.3
18:1 ω7*c*/ω9*t*/ω12*t*	1.0	0.3	0.2	—	0.1	0.4	0.2	0.3	—	—
19:0 *cy* ω8*c*	0.3	0.09	0.04	—	—	0.03	0.03	—	—	—
Sum of group A	3.4	1.7	0.8	0.03	0.3	0.8	0.5	0.6	0.7	0.7
B. Fatty acids found also in pulps and papermaking additives (sum of group B)[c]	7.3	5.0	1.8	0.8	2.4	2.0	1.8	6.0	46.2	59.3
C. Fatty acids of bacterial or other origin (sum of group C)[d]	2.4	1.0	0.5	0	0.1	0.3	0.1	0.1	2.1	3.4
Fatty acids in total (A+B+C)	13.1	7.7	3.1	0.8	2.8	3.1	2.4	6.7	49.0	63.4
Bacterial signature fatty acids (% of total)	26	22	26	3.8	11	26	21	9.0	1.4	1.1
Amt of bacterial cells (mg)/g of slime[e]	142	71	33	1.3	13	33	21	25	29	29
No. of bacterial cells/g of slime (10^{11})[f]	26	13	6	0.2	2	6	4	5	5	5

[a] Average of two parallel samples with a standard deviation of 5 to 50%.
[b] —, <0.02 mg/g.
[c] Group B includes fatty acids 12:0, 14:0, 16:0 *a*, 16:1 ω7*c*, 16:0, 17:0 *a*, 18:3 ω6,9,12*c*, 18:2 ω6,9*c*/18:0 *a*, 18:1 ω9*c*, 18:0, 19:0 *a*, 18:0 2OH, 20:2 ω6,9*c*, and 20:0.
[d] Group C includes fatty acids 9:0, 10:0, 11:0 *i*, 10:0 3OH, 11:0 *i* 3OH, 13:0 *i*, 13:0 *a*, 14:0 *i*, 14:0 2OH, 14:0 3OH/16:1 *i*, 15:0 *i* 2OH/16:1 ω7*t*, 15:0 *i* 3OH, 15:0 2OH, 17:1 *i* ω9*c*, 17:1 *i* ω5*c*, 17:1 *i*/17:0 *a*, 17:1 ω8*c*, 17:1 ω6*c*, 16:0 *i* 3OH, 16:0 2OH, 16:0 3OH, 18:0 *i*, 17:0 *i* 3OH, 17:0 2OH, 17:0 3OH, 19:1 *i*, 19:0 *i*, 19:0, 18:1 2OH, 20:4 ω6,9,12,15*c*, 20:3 ω6,9,12*c*, 20:1 ω9*t*.
[e] Calculated by using an average signature fatty acid content of bacterial cells of 2.4% (wt/wt; from Table 6).
[f] Calculated by assuming the dry weight of one bacterial cell as 5.5×10^{-14} g (corresponding to the average size of 0.25 μm^3 for a bacterial cell).

TABLE 6. Fatty acid composition of bacteria isolated from board machine slimes

Fatty acid or determination	Fatty acid composition (mg/g [dry wt] of bacterial cells)[a]									
	Kp	Ea	Pa	Pce	Pp	Pca	Fl	Cm	Bl	Bc
A. Bacterial signature fatty acids										
15:0 *i*	—[b]	—	—	—	—	—	12.6	0.2	4.8	4.5
15:0 *a*	—	—	—	—	—	—	0.9	22.6	8.9	0.9
15:0	—	0.1	0.3	0.2	—	0.2	—	—	—	0.4
16:0 *i*	—	—	—	—	—	—	—	12.4	0.7	1.9
16:1 ω5*c*	—	—	—	0.1	0.6	0.2	—	—	—	—
17:0 *i*	—	—	—	—	—	—	—	—	2.1	1.5
17:0 *cy*	3.0	0.9	0.5	4.7	—	3.1	—	—	—	—
17:0	—	—	0.2	0.3	—	0.3	—	—	—	—
18:1 ω7*c*/ω9*t*/ω12*t*	16.4	15.2	42.4	34.5	24.8	30.4	—	—	—	—
19:0 *cy* ω8*c*	0.2	—	0.5	3.9	—	2.5	—	—	—	—
Sum of group A	19.6	16.2	43.9	43.7	25.4	36.7	13.5	35.2	16.5	9.2
B. Other fatty acids (sum of group B)[c]	38.0	52.1	57.8	52.5	13.8	41.5	20.6	18.2	7.4	12.2
Fatty acids in total (A + B)	57.6	68.3	101.7	96.2	39.2	78.2	34.1	53.4	23.9	21.4
% of total bacterial signature fatty acids	34	24	43	45	65	47	40	66	69	43

[a] Average of two parallel samples with standard deviations of 5 to 25%. Abbreviations: Kp, *K. pneumoniae* E31; Ea, *E. agglomerans* E42; Pa, *P. aeruginosa* E102; Pce, *P. cepacia* E121; Pp, *P. paucimobilis* E173; Pca, *P. caryophylli* E217; Fl, *Flavobacterium* sp. strain E1720; Cm, *C. michiganense* E25; Bl, *B. licheniformis* E2; Bc, *B. cereus* III-P4.
[b] —, <0.1 mg/g.
[c] Group B includes fatty acids 10:0, 10:0 3OH, 12:0 *i*, 12:0, 12:0 2OH, 12:1 3OH, 12:0 3OH, 13:0 *i*, 13:0 *a*, 14:0 *i*, 14:0, 15:1 ω5*c*, 15:1 *i*, 15:1 *a*, 14:0 2OH, 14:0 3OH/16:1 *i*, 16:1 ω11*c*, 16:1 ω7*c*, 16:1 ω7*t*/15:0 *i* 2OH, 16:0, 15:0 *i* 3OH, 15:0 2OH, 17:1 *i* ω10*c*, 17:1 *i* ω5*c*, 17:1 ω8*c*, 17:1 ω6*c*, 17:1 *i*, 17:1 *a*, 17:0 *a*, 16:1 2OH, 16:0 *i* 3OH, 16:0 2OH, 16:0 3OH, 18:1 ω5*c*, 18:0, 17:0 *i* 3OH, and 18:1 2OH.

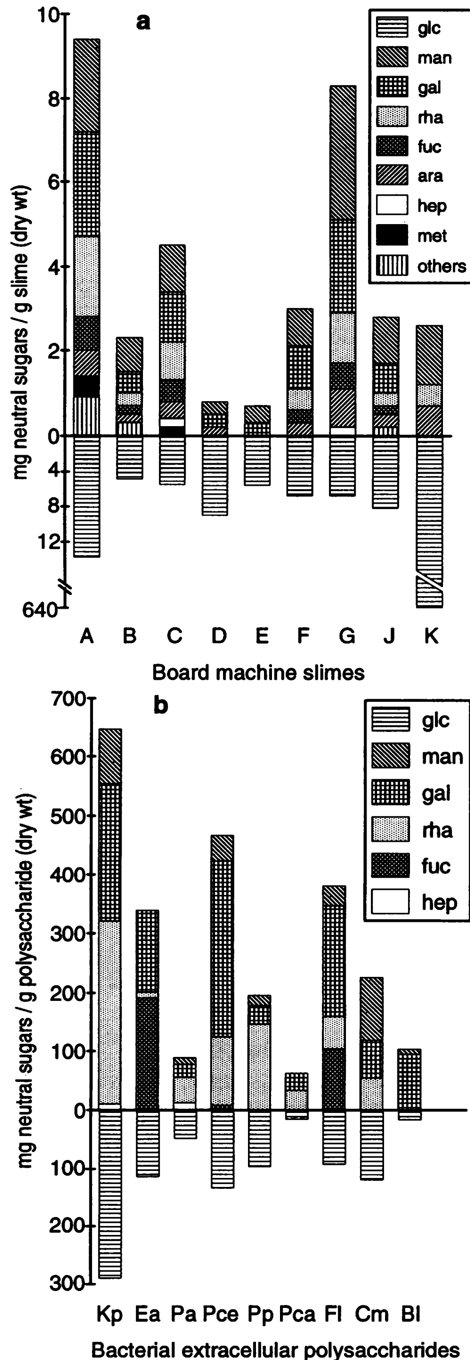

FIG. 6. Neutral sugar composition of the board machine slimes (a) and of the extracellular polysaccharides of bacteria isolated from board machine slimes (b) measured by GC-MS. Abbreviations: Glc, glucose; man, mannose; gal, galactose; rha, rhamnose; fuc, fucose; ara, arabinose; hep, heptoses; met, methoxy sugars; others, unidentified sugars; Kp, *K. pneumoniae* E31; Ea, *E. agglomerans* E42; Pa, *P. aeruginosa* E102; Pce, *P. cepacia* E121; Pp, *P. paucimobilis* E173; Pca, *P. caryophylli* E217; Fl, *Flavobacterium* sp. strain E1720; Cm, *C. michiganense* E25; Bl, *B. licheniformis* E2.

TABLE 7. Carbohydrate content of board machine slimes and extracellular polysaccharides of pure cultures of bacteria isolated from the slimes

Sample	Carbohydrate content (mg/g [dry wt] of slime or polysaccharide)		
	Neutral sugars[a]	Total carbohydrates[b]	Uronic acids[c]
Board machine slimes			
A	23	560	20
B	7	640	10
C	10	ND[d]	10
D	10	550	5
E	7	870	5
F	10	ND	10
G	15	970	10
J	11	350	10
K	630	890	10
Bacterial polysaccharides			
K. pneumoniae E31	940	570	50
E. agglomerans E42	450	350	70
P. aeruginosa E102	140	170	5
P. cepacia E121	600	480	50
P. paucimobilis E173	290	230	30
P. caryophylli E217	80	100	5
Flavobacterium sp. strain E1720	470	200	5
C. michiganense E25	350	320	50
B. licheniformis E2	120	200	30

[a] Measured by GC-MS.
[b] Measured by the phenol-sulfuric acid method.
[c] Measured by the *m*-hydroxydiphenyl method.
[d] ND, not determined.

bacterial cells per g (dry weight) of slime when the average dry weight of one bacterial cell was 5.5×10^{-14} g.

DISCUSSION

The present study shows that the use of three different independent methods of biomass estimation, i.e., electron microscopy, signature fatty acid analysis, and ATP measurement, resulted in similar estimates of the biofilm bacterial content, around 10^{11} to 10^{12} cells per g (dry weight) of slime. The study expands our earlier work on the microbial composition and the contribution of inorganic elements in paper and board machine slimes (27, 31).

Microbial biomass in a certain environment can be quantified by its phospholipid fatty acid content (35, 41). A total of 58 different fatty acids were found in the paper and board machine slimes, and 19 different fatty acids were found in papermaking materials by using the same fatty acid analysis procedure. This suggests that microbes must have a major input in slime fatty acid composition. When the environment contains fatty acids from sources other than microbes (fiber and additives in the case of paper and board machine slimes), signature fatty acids, specific for bacteria, can be used for the estimation. We used a set of 10 signature fatty acids adopted from the literature (37, 38).

ATP assay has been used qualitatively as an estimation method for changes in microbial contents in different environments (13, 14), including the paper machine wet end (43). Although the commercial lysis reagent gave more variable results with pure bacterial cultures than PCA or TCA, at the mill the commercial reagent kit is the most convenient method, and satisfactory estimates of bacterial amounts were obtained by taking into account the low recovery of ATP.

TABLE 8. ATP content of paper machine slimes and bacterial pure cultures

Sample	ATP content (μg/g [dry wt] of slime or bacteria)[a]		
	Commercial reagent	PCA	TCA
Slimes (from machine 6)			
L	41 (5)		
M	31 (4)		
Board mill strains			
E. agglomerans E42	158 (2)	1,500	744
K. pneumoniae E31	165 (6)	714	405
P. aeruginosa E102	4 (6)	697	550
P. cepacia E121	2 (3)	411	470
P. caryophylli E217	2 (3)	487	215
Flavobacterium sp. strain E1720	22 (21)	277	140
C. michiganense E25	297 (2)	609	938
B. licheniformis E2	235 (13)	613	266
Avg of board mill strains[b]	111 (±118)	664 (±369)	466 (±273)
Type strains			
P. aeruginosa ATCC 10145[c]	2 (3)	614	85
C. michiganense DSM 20134[d]	600 (1)	962	381

[a] Bacterial cells were lysed with the commercial reagent, PCA, or TCA. Extracellular ATP contents (measured before cell breaking) are shown in parentheses.
[b] ±Standard deviation.
[c] ATCC, American Type Culture Collection (Rockville, Md.).
[d] DSM, Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany).

The carbohydrates present on board machines using bleached pulp include cellulosic fibers and starch from the sizing agents, both composed of glucose only. Carbohydrate analysis of the machine slimes revealed several sugars other than glucose, all known components of bacterial extracellular polysaccharides (4, 36), indicating that the slimes originated from microbial polysaccharides. The yield of total carbohydrates obtained by the phenol-sulfuric acid method was about 25 to 100 times higher than the amount of neutral sugars obtained by the GC-MS method, whereas no such difference was observed when extracellular polysaccharides from slime bacteria were analyzed. Therefore, the major part of the polysaccharide in the biofilm may not be accessible under mild conditions of hydrolysis for the preparation of alditol acetates in comparison with strong acid hydrolysis by the phenol-sulfuric acid method.

The bacteria most often found in paper and board machine slimes belonged to the genera *Pseudomonas*, *Flavobacterium*, and *Clavibacter*. The neutral sugar composition of the extracellular polysaccharides of the bacteria belonging to these genera was similar to that found in the board machine slimes. *Bacillus* species were less abundant in the slimes but dominant in the viable flora of paper and board products because their spores survive the high temperatures prevailing at the dry end of the machine (40).

Anionic groups of bacterial polysaccharides are known to bind metals (4, 24). Earlier, we observed a high accumulation of heavy metals in paper and board machine slimes, i.e., 30 to 60 μg of iron, 2 to 3 μg of chromium, and 1 μg of copper per g (wet weight) of slime, which is equivalent to 11 to 21 μmol of iron, 0.8 to 1.2 μmol of chromium, and 0.3 μmol of copper per g (dry weight) of slime (27, 31). We found in the slimes uronic acids equivalent to a binding capacity of 12.5 to 50 μmol of divalent cations per g (dry weight) of slime. The uronic acid-containing polysaccharides in the slime may thus be responsible for the accumulation of heavy metals in the slime.

The paper machine white water as a culture medium is exceptional because of its high carbon content and low nitrogen and phosphorus content (C/N ratio ranging from 40:1 to 90:1). These conditions may favor the production of excessive polysaccharides by the bacteria (36) and also the production of energy reserve polymers such as poly-β-hydroxybutyrate and glycogen (33), as was visible in almost all electron micrographs.

Machine biofilms are of major importance in the deterioration of paper and board products. Detachment of biofilms from the surfaces during the machine operation frequently leads to the appearance of patches of slime in the product. The biofilm may seed the paper product with bacteria and heavy metals, leading to discoloration and development of odors. The odorous compounds have been identified as fatty aldehydes (25) resulting from metal ion-catalyzed oxidation (16) of fatty acids and resin acids contained in the paper.

Our studies showed that paper machine biofilms had complicated biological and chemical structure. Morphologically, they resembled wastewater biofilms (7, 20, 32) and biofilms in industrial water cooling towers (34) or natural river ecosystems (8). Other biofilms, such as those in medical devices, have been described mainly by scanning electron microscopy, which shows only the surface of the biofilm (19).

ACKNOWLEDGMENTS

We thank Juha Mentu and Henry Lindell for fruitful discussions and advice, Hannu Heikkinen for cooperation with investigations at the paper mill, Eila Elomaa for providing the results of inorganic analyses, Veikko Kitunen and Jussi Uotila for advice with mass spectral analyses, Tuire Koro and Mervi Lindman for preparing the thin sections for electron microscopy, Riitta Boeck for help with fatty acid analyses, and Helmi Savolainen for media preparation. The equipment of the Department of Electron Microscopy at the University of Helsinki was kindly placed at our disposal.

This work was financially supported by the Foundation for Biotechnical and Industrial Fermentation Research and the Foundation for Research of Natural Resources in Finland.

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