THE BACTERIOLOGY OF ACTIVATED SLUDGE

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The flora of sewage, from which that of activated sludge is derived, is very heterogeneous. From faeces a large number of intestinal bacteria, including coliforms, staphylococci, streptococci, lactobacilli, and spore-bearing aerobes and anaerobes, enter the sewage, while drainage water carries with it the common bacteria of water, soil, and plant surfaces including, amongst many other types, non-faecal coliforms, chromogenic organisms, and nitrogen-fixing and nitrite- and nitrate-forming bacteria. Of this varied flora the species which would be expected to predominate in activated sludge would be those able to oxidize rapidly, under conditions of continuous aeration, the various carbonaceous constituents of sewage and to utilize the nitrogen sources, which consist very largely of ammonium salts arising from the hydrolysis of urea.

Comparatively little work appears to have been carried out to determine the characteristic bacterial flora of activated sludge or to isolate and identify those species which are normally predominant in the sludge floc. Russel & Bartow (1916) isolated thirteen varieties of non-nitrifying bacteria from activated sludge. All but four of these belonged to the *B. subtilis* group of aerobic spore-formers, while most species formed slight acid but no gas in sugar broths, hydrolysed starches, and attacked casein. Species of *Nitrosomonas* and *Nitrobacter* were also isolated. When sterile sewage was aerated with a mixed culture of the non-nitrifying bacteria, appreciable clarification was obtained but only traces of nitrite and no nitrate were formed, while the nitrifying bacteria alone had no appreciable action on the sewage. A mixture of the non-nitrifying and the nitrifying bacteria produced complete oxidation of the ammonia after aeration for 6 hr., with the formation of 18 parts per million of nitrate and a trace of nitrite. The authors concluded that bacteria other than nitrifiers play an important part in the purification of sewage. Kamm (1917) carried out similar experiments and obtained substantially the same results. Buswell & Long (1923) concluded, as a result of microscopic, examination that activated sludge consisted of zoogloeal masses of bacteria intermixed with filamentous forms. *Crenothrix polyspora*, *Sphaerotilus dichotomus*, and *Zoogloea ramigera* appeared to be present in large numbers. Harris, Cockburn & Anderson (1927) found that 61 % of organisms in activated sludge were of the *Bact. aerogenes* type and 38 % were of the *Proteus* type. The persistent predominance of these organisms, combined with the fact that many species were proteolytic in character, led the authors to conclude that the intestinal group of bacteria plays an important part in the purification of sewage by activated sludge. Heukelekian (1934) determined the total plate counts of different types of activated sludge and found that in the samples examined they varied from 9·5 to 48·5 millions per ml. of settled sludge, or from 3·7 to 73·5 millions per mg. of suspended solids. Aeration of mixtures of sewage and activated sludge containing a low concentration of suspended solids generally resulted in an increase in bacterial numbers while in mixtures with a high content of suspended solids the bacterial numbers tended to decrease as a result of aeration. The most interesting of recent contributions to the subject have been made by Butterfield (1935, 1937), Butterfield, Ruchhoft & McNamee (1937), and Heukelekian & Littman (1939). Flocs of activated sludge were picked out with capillary pipettes, washed in sterile water and teased with fine glass needles to free them as far as possible from adherent material. The floc then appeared to consist entirely of a mass of bacterial cells, and all the cells were of the same morphological type. Pure cultures of these organisms were obtained by inoculating dilutions of the dispersed floc into nutrient broth, subculturing several times, and finally plating on nutrient agar, when pin-point colonies were obtained. The bacteria were Gram-negative, non-sporing, motile, capsulated rods, which gave good growth in nutrient broth with the formation of zoogloeal masses, but produced no reaction in sugar broths, no hydrolysis of starch, and no liquefaction of gelatin. When submitted to conditions of continuous aeration in sterile sewage, to which various test substances were added, however, these organisms oxidized carbohydrates and produced ammonia from gelatin, casein, and peptone. Aeration of the organisms with a sterile synthetic solution approximating in composition to the soluble fraction of sewage resulted in the development of a well-organized floc in 48 hr. Aeration of sewage with the pure culture sludge produced in this manner resulted in rapid oxidation of the carbonaceous constituents of the sewage though no nitrification took place. The species isolated was tentatively assumed to be a strain of *Zoogloea ramigera*. Heukelekian & Schulhoff (1938) isolated pure cultures of bacteria from activated sludge and found that some of them were capable, when aerated with sterile sewage, of effecting considerable clarification without producing a marked reduction in the oxygen consumption of the effluent. These authors did not identify the organisms they used nor describe their characters.

EXPERIMENTS

Before attempting to study the flora of activated sludge and to isolate predominant types it seemed essential that some means should be adopted which would liberate the bacteria from the interior of the sludge floc in which large numbers of bacteria are embedded in a gelatinous matrix. It is evident that if dilutions are prepared from this material without previous treatment and are inoculated into media for estimating bacterial numbers or isolating predominant types, the apparent count will be very much lower than the true count—for example, one colony on

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a poured plate may arise from a clump of bacteria consisting of several hundred individuals. Since, too, the sludge is accompanied by interstitial fluid in which the bacteria are much less agglomerated, plates may show colonies in the highest dilution which do not, in fact, arise from bacteria present in the largest numbers, because the latter are enclosed in the flocs of sludge. It was decided for this reason to subject the sludge to homogenization before making a bacteriological examination.

Effect of homogenization on bacterial count of activated sludge

Preliminary experiments were made to see whether an ordinary household cream-making machine, in which homogenization is effected by pumping the liquid through a narrow orifice by hand, would produce a significant increase in the bacterial count of activated sludge. Samples of sludge were obtained by aeration of domestic sewage in Winchester bottles in the laboratory, operating on the fill and draw principle, and a 1 in 10 dilution of the settled sludge in sterile water was passed through the cream-making machine (previously sterilized in the autoclave) several times. Counts of the untreated and of the homogenized sludge were obtained by plating each sample in duplicate on nutrient agar, incubating the plates at 20° C. and counting the colonies after incubation for 10 days. The average counts for each sample are shown in Table 1, from which it is evident that the counts of homogenized samples were 10–100 times greater than those of untreated samples.

Table 1. *Effect of homogenization on the plate count of activated sludge (millions per ml. of settled sludge)*

Sample	Untreated	Homogenized	Ratio homogenized: untreated
1	54	707	11·2:1
2	204	2200	10·8:1
3	114	1490	13·1:1
4	6·7	680	101·5:1

A second series of experiments was designed to compare the efficiency of two different homogenizers in breaking up the bacterial clumps in activated sludge and to find the effect on the bacterial count of passing the sludge an increasing number of times through an homogenizer. One homogenizer was the cream-making machine used in the previous experiment. The other was a specially designed apparatus,* consisting of a stainless steel cylinder with a capacity of 50 ml., the lower end terminating in a fine jet; the upper end was attached by a bronze pipe to a nitrogen cylinder. Liquid contained in the apparatus was forced through the fine orifice by the pressure of the nitrogen. Layers of stainless steel gauze (100 mesh), supported by metal washers, could be inserted in the homogenizer, when desired, in order to assist still further in disintegrating bacterial clumps. Both homogenizers were sterilized in the autoclave before use. A sample of activated sludge (not concentrated by settling) from a sewage works was passed through the cream-making machine 3, 5, and 7 times. A sample of the same sludge was then passed 5 times through the second apparatus without the use of gauzes, and a further sample was passed the same number of times through the same apparatus containing four layers of gauze. Bacterial counts of the untreated sludge and of each homogenized sample were obtained by plating in triplicate on nutrient agar, incubating the plates at 20° C., and counting the colonies after 12 days. The results are shown in Table 2. It is evident that the count of the activated sludge tended to increase with the number of times it was passed through the cream-making machine, though the count after seven passages was only slightly higher than that after five passages. The pressure homogenizer without gauzes gave results comparable with those of the cream-making machine. The use of four gauzes in the pressure homogenizer resulted in a bacterial count appreciably lower than that obtained with the same apparatus without the use of gauzes. This was probably due to the vigorous treatment disintegrating some of the bacterial cells themselves. Evidently the method adopted for homogenization must be somewhat carefully chosen so as to give the maximum disintegration while avoiding destruction of the micro-organisms. Considering the simplicity of the cream-making machine it appears that, for the purpose of isolating the predominant bacteria in the flocs of activated sludge, it is satisfactory to use this apparatus and to pass the sludge through it 5–7 times before plating.

* Kindly lent by Dr A. T. R. Mattick of the National Institute for Research in Dairying.

Table 2. *Comparison of the effects of two homogenizers on the plate count of activated sludge (millions per ml. of sludge)*

Untreated	Homogenized				
	Passed through cream-making machine			Passed through pressure homogenizer (5 times)	
	3 times	5 times	7 times	Without gauzes	With gauzes
15·7	231	358	381	385	190

Bacterial changes during the aeration of sewage and the formation of activated sludge

Some experiments were carried out to discover the nature of the bacteriological changes which occur when sewage is aerated continuously for a long period, resulting in the formation of a small quantity of sludge. The aeration tank consisted of a 10-gal. glass cylinder, open at the top and fitted at the bottom with a diffuser plate, through which a rapid current of diffused air was passed by means of an electrically driven compressor. This tank was filled with domestic sewage which had been passed through a fine-mesh sieve to remove coarse particles, and at intervals during the period of aeration samples of the

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liquid were withdrawn under aseptic conditions. When a sample of the supernatant fluid was required the aerated liquor was allowed to settle in a sterile measuring cylinder and the resulting supernatant fluid was withdrawn by pipette.

The first period of aeration continued for 14 days. At the end of that time the current of air through the tank was stopped and the liquor allowed to settle for 2 hr. A small quantity of sludge appeared at the bottom of the tank and the supernatant fluid was siphoned off and replaced by a fresh quantity of sewage. The second period of aeration was continued for 17 days, and samples were withdrawn at intervals as before for bacteriological investigation.

In order to disintegrate bacterial clumps, particularly liable to form after aeration, which would reduce the apparent count of the aerated material compared with that of the original sewage, each sample was homogenized in a sterile cream-making machine as described above.

Enumeration of the bacteria in the aerated liquor, and in the supernatant fluid after the small amount of sludge had been allowed to settle, enabled an estimate to be made of the effect of aeration on the total count of bacteria and on the proportion in which they were present in the sludge and in the supernatant fluid respectively. Various media were inoculated in duplicate with the dilutions of each sample so as to obtain the count of different groups of bacteria. The media used and the counts obtained are shown in Table 3. The counts on solid media are the average of duplicates. In liquid media, where the highest dilution showing growth was the same in both series of duplicates, only one figure is given, but where this differed in the two duplicates both dilution figures are given. Counts of aerobic spore-formers on nutrient agar and of anaerobic spore-formers in Robertson's cooked meat were obtained by pasteurizing dilutions of the sample in Ringer's solution at 80° C. for 20 min. before inoculating into the respective media. The cooked-meat medium was heated in boiling water for 10 min. to expel oxygen and was then cooled before inoculation, and each tube was sealed with a layer of sterile vaseline after inoculation. The medium described in the table as 'litmus-dextrose-milk synthetic sewage' contained substances similar in character to some of the constituents of sewage together with small quantities of dextrose and of milk. Its composition was as follows: peptone 1 g., meat extract 1 g., dextrose 2·5 g., $(NH_4)_2SO_4$ 0·2 g., Na_2HPO_4 0·1 g., NaCl 0·03 g., KCl 0·01 g., $CaCl_2$ 0·02 g., $MgSO_4$ 0·1 g., separated milk 50 ml., distilled water 950 ml.; sufficient litmus was added to produce an adequate colour. This medium enabled acid-forming and clot-producing types, as well as total numbers of bacteria, to be estimated. Nutrient agar contained 0·3 % Lemco, 0·25 % sodium chloride, 1·0 % peptone, and 2·0 % agar-agar, made up with tap water. The malt agar consisted of 3 % malt extract made up with tap water and 2 % agar-agar.

Table 3. *Bacterial counts of aerated sewage, sewage-sludge mixture and supernatant fluid*

Sample	Period of aeration days	Nutrient agar		Malt agar Total count	Bile salt broth Coliform bacteria	Cooked meat Anaerobic spore-formers	Litmus-dextrose-milk-synthetic sewage	
		Total count	Aerobic spore-formers				Total count	Acid-formers
First aeration:								
Fresh sewage	0	14,300,000	1,300	1,560,000	10^5	10^2	10^7	10^7
Aerated sewage, including sludge formed	1	—	—	17,600,000	—	—	10^8	10^8
Aerated sewage, including sludge formed	5	—	—	15,700,000	—	—	10^7	10^6
Aerated sewage, including sludge formed	11	31,600,000	600	6,080,000	10^3–10^4	10^2–10^3	10^7–10^8	10^5–10^6
Supernatant liquor after 1½ hr. settling	11	6,170,000	—	—	—	—	10^6	10^4–10^5
Second aeration:								
Fresh sewage	0	38,000,000	—	7,800,000	10^5	—	10^6–10^7	10^6
Sewage-sludge mixture	0	12,800,000	—	9,400,000	10^4	—	10^6–10^7	10^6
Sewage-sludge mixture	1	60,000,000	—	10,400,000	—	—	10^7–10^8	10^6–10^7
Sewage-sludge mixture	4	33,400,000	240	24,400,000	10^4+	10^2	10^7	10^7
Supernatant liquor after 1½ hr. settling	4	3,580,000	70	1,000,000	10^3+	—	10^6	10^4–10^5
Sewage-sludge mixture	17	28,200,000	—	—	—	—	—	—
Supernatant liquor* after 1½ hr. settling	17	662,000	—	—	—	—	—	—

* Not homogenized.

The results obtained indicate that the total count of bacteria increased as a result of aeration of the sewage and that the increase occurred soon after the beginning of aeration. A high total count was maintained for upwards of 11 days. After aeration for several days there was fairly rapid and apparently fairly complete separation of sludge and supernatant fluid when the aerated material was allowed to settle, but there were still large numbers of bacteria in the supernatant fluid. An increase in bacterial count again occurred soon after commencing the second aeration, but in this case the bacteria associated more quickly with the sludge than they did during the first aeration, thus leaving fewer in the supernatant fluid. With regard to the groups of bacteria mainly concerned in these changes the counts in the litmus-dextrose-milk synthetic sewage showed that acid-forming bacteria

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were always present in comparatively large numbers, but were not usually predominant, bacteria present in the highest dilutions in this medium often showing growth but no formation of acid. The numbers of aerobic spore-forming bacteria were so small as to indicate that this group of organisms plays little if any part in the changes accompanying aeration of sewage; the numbers of spore-forming anaerobes were very small, as might have been expected in an aerated liquid, but a count of 10–100 per ml. appeared to be maintained for some time. The counts on malt agar were consistently lower than those on nutrient agar, and moulds and yeasts never formed a large proportion of the total numbers of micro-organisms. Coliform bacteria, although present throughout the experiments, represented only a small fraction of the total flora at any stage. From the fact that the counts on nutrient agar compared favourably at all stages with the counts in the dextrose-milk-synthetic sewage it seems likely that the predominant bacteria were of the type capable of growing on nutrient agar, though further experiments would be necessary to confirm this.

The predominant flora of activated sludge

Investigations were made with two types of activated sludge, one being produced in the laboratory and the other procured in a fresh condition from a large modern sewage works. The former was prepared by the aeration of domestic sewage in Winchester bottles, the first batch of sewage being aerated for 1 week, after which fresh sewage was added daily to the settled sludge. Samples of sludge were taken for bacteriological examination at intervals during a total period of aeration of 14 weeks, in order to discover the nature of the predominant flora and to see whether this tended to alter as the sludge progressed from the stage when it was freshly formed from the sewage to the stage when it had been in use for a considerable time.

Each sample of sludge produced in the laboratory was withdrawn into a sterile measuring cylinder and allowed to settle for 1 hr. The supernatant liquor was then discarded and a 1 in 10 dilution of the settled sludge in sterile water was homogenized by passage through a cream-making machine as described previously. Serial dilutions of the homogenized material in Ringer's solution were inoculated into various media to obtain bacterial counts, the media being incubated at 20° C. for at least 10 days, with the exception of those in bile-salt broth, which were incubated at 30° C. for 3 days.

The media used and the counts obtained with each one are shown in Table 4. The synthetic sewage contained 0·3 g. peptone, 0·2 g. meat extract, 0·2 g. $(NH_4)_2SO_4$, 0·1 g. Na_2HPO_4, 0·03 g. NaCl, 0·01 g. KCl, 0·02 g. $CaCl_2$, and 0·01 g. $MgSO_4$, in 1 l. of distilled water. None of the media allowed the growth of nitrogen-fixing or nitrite- or nitrate-forming bacteria, and no attempt was made to enumerate these types.

Two samples of activated sludge taken from the same sewage works at different times were investigated, both samples being homogenized before use, without preliminary settlement. Three plates of nutrient agar and five tubes of McConkey broth were each inoculated with 1 ml. of each dilution prepared from the first sample of sludge. The average plate count at 20° C. and the most probable number of coliform bacteria (presumptive count at 37° C.) are shown in Table 5.

The second sample was plated in triplicate on nutrient agar and on sodium caseinate agar, the plates being incubated at 20° C. and counted after 15 days. The latter medium was tried because previous workers (e.g. Taylor, 1940) have found that in the bacteriological examination of water this medium yields higher counts than any of the other media commonly used, most of the increased count being due to pin-point colonies which only develop after incubation for several days. The average counts obtained are shown in Table 6.

Table 4. *Bacterial counts (millions per ml.), on various media, of homogenized activated sludge produced in the laboratory from domestic sewage*

Sample no.	Period in which sludge had been accumulating weeks	Media: Nutrient agar	Dextrose yeast extract agar	Malt agar pH 3·5	Nutrient broth	Bile salt broth (acid and gas)	Synthetic sewage
1	3	707	630	0·21	10^3	—	—
2	4	—	—	—	10^2–10^3	1–10	10^2–10^3
3	5	2200	—	—	10^2–10^3	—	10^2–10^3
4	6	1490	—	—	10^3	—	10^3
5	14	680	—	—	—	—	—

Table 5. *Comparison of total count on nutrient agar with presumptive count of coliform bacteria in homogenized activated sludge from sewage works (millions per ml.)*

Total count	Coliform bacteria
381	0·09

Table 6. *Average plate counts (millions per ml.) of homogenized activated sludge from sewage works*

Nutrient agar	Sodium caseinate agar
57·2	46·2

From the figures in Table 4–6 it may be seen that the counts on nutrient agar compare favourably with those in the other media tested. Sodium caseinate agar, when used for activated sludge, does not appear to possess the advantages which are claimed for it in the examination

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of water. It is evident also that coliform bacteria form only a small proportion of the total number of bacteria in activated sludge, thus discounting the opinion expressed by some previous workers that this group of organisms plays an important part in the activity of the sludge. Since homogenization so greatly increases the apparent count of activated sludge, and it is not at all likely that this simple process effects complete separation of the bacterial clumps into individuals, it follows that the true count will be higher than the figures given in these tables. Taking into consideration also the high moisture content of the sludge it seems likely that there are several hundred thousand million bacteria per gram of sludge calculated on the dry basis.

Characters of predominant bacteria in activated sludge produced in the laboratory

Representative strains of the predominant bacteria were isolated from the cultures on nutrient agar, in nutrient broth, and in synthetic sewage, which had been obtained from the activated sludge produced in the laboratory. Samples had been taken at intervals after the sludge had first begun to form until the process had been in operation for 14 weeks. Colonies on nutrient agar plates were subcultured first into nutrient broth and, after incubation, cultures in the latter medium were transferred to nutrient agar slopes. Cultures in synthetic sewage, obtained from the highest dilutions of activated sludge which yielded growth in this medium, were plated first on synthetic sewage agar, and the resulting colonies were subcultured into synthetic sewage again before being transferred to nutrient agar slopes. No case was found of a strain able to grow in synthetic sewage but not on nutrient agar, although cultures on the latter medium showed considerable variation in rapidity and luxuriance of growth.

The morphological and physiological characters of forty-eight cultures, thirty from homogenized sludge and eighteen from untreated sludge, obtained in this way, were determined. The results, given in Table 7, show that all the species isolated were rod forms, forty-two were slender, Gram-negative, non-sporing types, and only one was a spore-forming bacillus. Of the forty-two Gram-negative rods twenty-six had no action on sugars nor on proteins, and thirteen had no action on sugars but digested casein and liquefied gelatin. These two types, then, may be said to have constituted the main part of the flora of the sludge flocs. Species within each type differed only in the character of the growth on nutrient agar slopes, which varied from sparse to luxuriant, and in pigment, which ranged from white, through cream, to yellow. Their general characters indicate that they are water and soil types, and the majority would be classified in the genera *Achromobacterium* and *Chromobacterium* (Topley & Wilson, 1936), or if one adopts the classification of Bergey (1934), those with the yellow pigment would be placed in the genus *Flavobacterium*. The classification of many of the strains is, however, a matter of some difficulty, owing to their biochemical inactivity.

It is interesting to note that there was a marked tendency for the predominant flora of the sludge to change from a non-proteolytic to a proteolytic character as the sludge changed from the early period of formation to the period when it had been in use for some weeks. Thus, of the twenty-six non-proteolytic, Gram-negative rods, twenty were isolated during the first 4 weeks of aeration, while of the thirteen proteolytic Gram-negative rods all were isolated after the fourth week of aeration. This suggests that during the early period, when the amount of protein in the sewage-sludge mixture was small, a proteolytic flora did not develop owing to the lack of a suitable substrate for growth, but that it became established in the later stages of aeration, when the amount of protein, built up in the form of bacterial cells in the sludge, became appreciable.

Characters of predominant bacteria in activated sludge taken from a sewage works

Colonies on plates of nutrient agar and of sodium caseinate agar, poured from homogenized activated sludge, were subcultured into nutrient broth from the former medium and into sodium caseinate broth from the latter. After incubation and growth the broth cultures were all transferred to nutrient agar slopes. No strain was encountered which was able to grow on sodium caseinate agar but not on nutrient agar. A total of twenty-three cultures was obtained, nine from the former medium and fourteen from the latter, and the characters of each strain were determined. These are described in Table 8, where the cultures are divided into five groups. It may be seen that all the organisms, except two, were rod forms, and all but five were Gram-negative. As in the case of the activated sludge produced in the laboratory, Gram-negative rods appeared to constitute the main part of the flora of activated sludge from a large-scale plant. Of the eighteen Gram-negative bacteria, seventeen had no action on sugars. Four of the latter had no action on proteins either, but eleven both digested casein and liquefied gelatin, while two liquefied gelatin but had no action on casein. From the facts that only three of the cultures were able to grow well at 37° C., and the majority were unable to grow at all at that temperature, it is evident that the predominant strains in this sample of activated sludge were water and soil types and not intestinal organisms. The eleven cultures in group I produced a fluorescent green soluble pigment, the characters of seven (subgroup *a*) corresponding with those of *Pseudomonas fluorescens*, while three (subgroup *b*) were strains of *Pseudomonas pyocyanea*. The remaining culture (*c*) in this group possessed characters intermediate between these two species. The characters of the four cultures in group II correspond with those given for *Bact. metalkaligenes* by Levine & Soppeland (1926), who isolated this organism from 2 % skim milk which had been aerated for several hours with activated sludge. Group III consists of two Gram-negative rods with a yellow pigment and would be placed in the genus *Chromobacterium* according to Topley & Wilson (1936) or in the genus *Flavobacterium* according to Bergey (1934). The two cocci with a pink pigment in group IV are species of *Rhodococcus*. Group V contains four cultures which are difficult to classify from the characters which were determined, but the Gram-negative strain is very similar to *Achromobacterium liquefaciens*, isolated by Frankland (1894; quoted by Bergey, 1934) from water.

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Table 7. *Characters of predominant bacteria isolated from activated sludge produced in the laboratory*

No. of cultures	Morphology	Spores	Gram	Dextrose	Lactose	Sucrose	Maltose	Nitrate reduction	Indol formation	Litmus milk	Gelatin liquefaction
22	Rod	–	–	–	–	–	–	–	–	–	–
3	,,	–	–	–	–	–	–	+	–	–	–
1	,,	–	–	–	–	–	–	–	–	Alkaline	–
9	,,	–	–	–	–	–	–	+	–	Soft clot followed by digestion	Stratiform
4	,,	–	–	–	–	–	–	–	–	Soft clot followed by digestion	Stratiform
3	,,	–	–	+	+	+	+	–	–	Acid	–
2	,,	.	+	–	–	–	–	–	–	Soft clot followed by slow digestion	–
3	,,	.	+	–	–	–	–	–	–	Soft clot followed by digestion	Slow stratiform
1	,,	Central oval	+	+	–	+	+	–	–	Digested	Stratiform

Table 8. *Characters of predominant bacteria isolated from activated sludge from a sewage works*

Group	No. of cultures	Morphology	Spores	Gram	Growth on nutrient agar: 20° C.	Growth on nutrient agar: 37° C.	Pigment	Acid production in: Dextrose	Acid production in: Lactose	Acid production in: Sucrose	Acid production in: Maltose	Nitrate reduction	Indol formation	Litmus milk	Gelatin liquefaction
I *a*	7	Small rod	–	–	Good	– or sparse	Growth brownish yellow, diffusible pigment fluorescent green	–	–	–	–	–	–	Digested	Stratiform
b	3	Small rod	–	–	Good	Good	Do.	–	–	–	–	–	–	Digested	Crateriform
c	1	Small rod	–	–	Good	Sparse	Do.	–	–	–	–	–	–	Digested	Crateriform to stratiform
II *a*	3	Small rod	–	–	Good	–	Creamy white	–	–	–	–	–	–	–	–
b	1	Small rod	–	–	Good	–	White	–	–	–	–	+	–	–	–
III *a*	1	Small thin rod	–	–	Good	Sparse	Yellow	–	–	–	–	–	–	–	Crateriform
b	1	Short rod	–	–	Good	–	Yellow	+	+	–	+	Slight	–	Slight acid	–
IV	2	Coccus	–	+	Good	– or sparse	Pale pink	–	–	–	–	+	–	–	–
V *a*	2	Small rod	–	+	Sparse	–	Colourless	–	–	–	–	–	–	–	–
b	1	Very short rod	–	+ (faint)	Sparse	–	Colourless	–	–	–	–	–	–	–	Slow stratiform
c	1	Small thin rod	–	–	Good	–	Creamy white	–	–	–	–	–	–	–	Slow stratiform

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DISCUSSION OF RESULTS

The use of an homogenizer to disintegrate the flocs of activated sludge before making a bacteriological examination undoubtedly made easier the isolation of bacteria inside the flocs and their separation from the smaller number of bacteria in the interstitial fluid. It is for this reason, no doubt, that the intestinal bacteria, particularly the *Bact. coli-Bact. aerogenes* group, and the aerobic spore-forming bacteria, which have been thought by some previous workers to play an important part in the constitution and activity of activated sludge, were found in this study in negligible numbers.

The majority of strains isolated were Gram-negative rods with no action on carbohydrates, though many of them had decided proteolytic characters. Their temperature relations and general characters indicated that they were derived from water or soil rather than from the intestine. It was found by Taylor (1942) that of the cultures isolated by him from English lakes and streams the great majority were Gram-negative rods, and he concluded that the flora of water differs in this respect from the flora of soil, which contains a large proportion of Gram-positive types. Moreover, the bacteria producing a fluorescent green pigment, which constituted a large part of the flora of one sample of activated sludge studied here, are usually associated with water. It seems likely, therefore, that the bacteria which predominate in activated sludge are derived largely from the water supply or the drainage water which enters the sewage. Organisms from this habitat might be expected to find activated sludge a more suitable medium than would the intestinal group, since the temperature in an activated sludge plant is rarely much above 20° C. and is not infrequently considerably lower.

Although a variety of media were tested none showed any superiority to nutrient agar. It is, however, advisable to use several media for purposes of isolation when making a survey of a bacterial flora, since one type may prefer one medium although able to grow on another. An example of this was encountered in the study described here. Although sodium caseinate agar gave a rather lower total count than nutrient agar it appeared to be particularly suited to species of *Pseudomonas*, since eight of the eleven cultures isolated were recovered from the former medium.

The comparative uniformity of the characters of bacteria composing the flora of the sludge floc is strongly contrasted with the varied characteristics of the flora of fresh sewage. This difference, however, follows logically from a consideration of the previous history of the organisms. Sewage is merely a reservoir which brings together bacteria from different habitats, those from the intestine, for example, having been accustomed to a food supply rich in carbohydrates and proteins and showing active biochemical characters accordingly, while those from water have the inactive characters of organisms adapted to an environment lacking in carbohydrate and protein. Aeration of sewage for a considerable time would be expected to encourage a type of organism suited to a dilute fluid in which the amount of carbohydrate and protein is extremely small. It is for this reason probably that in the early stage of formation of activated sludge most of the bacteria in the flocs are neither saccharolytic nor proteolytic. When the proportion of activated sludge in the sewage-sludge mixture becomes appreciable it provides sufficient protein to encourage a proteolytic flora, but the paucity of carbohydrate still persists and the bacteria in the sludge flocs continue to show an absence of saccharolytic characters.

SUMMARY

Homogenization of activated sludge greatly increased the apparent bacterial count obtained by plating on a solid medium, and for this reason facilitated the isolation of the predominant flora by liberating the bacteria from the interior of the sludge flocs. The ordinary domestic cream-making machine was found to be quite effective for this purpose.

The high counts obtained indicated that there are probably several hundred thousand million bacteria per gram of dry matter in activated sludge.

Aeration of sewage was found to result in an increase in the total count of bacteria, and a high count was maintained for a considerable time. After aeration for several days fairly rapid and apparently fairly complete separation of the sludge occurred on allowing the liquid to stand, but the supernatant liquor contained large numbers of bacteria. When a fresh quantity of sewage was added to the small quantity of sludge which settled and a second period of aeration commenced, the bacteria were found to associate more quickly with the sludge than they had during the first aeration. Coliform bacteria and spore-forming aerobes formed only a small fraction of the total number of micro-organisms concerned in these changes, and moulds and yeasts never accounted for a large proportion. The predominant bacteria did not produce acid from glucose.

Various media were tested in investigating the bacteriology of activated sludge. None showed any superiority to nutrient agar. Coliform bacteria and aerobic spore-formers were encountered in negligible numbers. The morphological and physiological characters of seventy-one strains of the predominant bacteria were determined. The majority were Gram-negative rods with no action on carbohydrates and were members of the genera *Achromobacterium*, *Chromobacterium*, and *Pseudomonas*. Those isolated from activated sludge soon after its formation from aerated sewage were found to be non-proteolytic, but a proteolytic flora was established after the sludge had been built up for a period of 4 weeks. The characters of the predominant bacteria indicate that they are water types derived from the water supply or the drainage water which enters sewage. Intestinal bacteria appear to be unimportant.

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