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Microbial Ecology of Activated Sludge

II. Bacteriophages, *Bdellovibrio*, Coliforms, and Other Organisms

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ABSTRACT

DIAS, F. F. (Indian Institute of Science, Bangalore, India), AND J. V. BHAT. Microbial ecology of activated sludge. II. Bacteriophages, *Bdellovibrio*, coliforms, and other organisms. *Appl. Microbiol.* **13**:257-261. 1965.—A comparative estimation of the coliform population of raw sewage, activated sludge, and the effluent derived therefrom revealed that raw sewage had a preponderance of *Escherichia coli* (75%), as compared with 25 and 30%, respectively, in sludge and effluent. Nitrogen-free mannitol-sucrose enrichments of activated sludge resulted in the isolation of *Azotobacter agilis*, *Aerobacter aerogenes*, *Corynebacterium laevaniformans*, and an *Achromabacter* species. Sludge had a large population of *C. laevaniformans* and *A. aerogenes* but not of *Azotobacter*. The bacterial parasites, *Bdellovibrio* and bacteriophages, were not active during activated-sludge treatment. A 10-fold reduction in phage content occurred after 2 hr of aeration, but the *Bdellovibrio* population was unaffected.

In discussing the nature and function of the dominant activated-sludge bacteria, Dias and Bhat (1964) pointed out that the less dominant species associated therewith might have a role in the stabilization of sewage. In this paper are described the attributes of some of the less dominant bacteria of activated sludge, as well as the phages and *Bdellovibrio* that parasitize them. An attempt has also been made to assess their functional capacity during activated-sludge treatment.

MATERIALS AND METHODS

Media. MacConkey's agar was prepared either from a dehydrated preparation (Oxo Ltd., London) or according to the formulation given by Mackie and McCartney (1956).

Nitrogen-free mannitol-sucrose enrichments were as described earlier (Dias, 1963).

For the enumeration of *Bdellovibrio* and phages, the YP medium of Stolp and Starr (1963) was used. The bottom layer contained 1.5% agar, and the overlay contained 0.5% agar. The host bacteria were grown on slants of TGY agar (Haynes, Wickerham, and Hesseltine, 1955).

The identification of bacteria was achieved by the methods recommended by the Society of American Bacteriologists (1957). The formulation given by the American Public Health Association (1955) was followed for the coliforms.

Activated-sludge samples. Samples were obtained either from laboratory units built up on a rotary shaker (Dias and Bhat, 1964) or from the final settling tank of the plant at the Institute sewage works.

For studying coliforms, the sludges were serially

diluted in distilled water, effective dispersion being achieved by shaking on a reciprocal shaker. Dilutions expected to yield 30 to 300 colonies on MacConkey's agar were used for isolating coliforms. The nitrogen-free enrichments were seeded with 1 ml of undiluted sludge. For enumerating the bacterial parasites (phages and *Bdellovibrio*), the sample was prepared as follows. The sludge was diluted to a known volume and homogenized. The homogenate was centrifuged at about 2,000 $\times g$ for 30 min in 12-ml tubes with conical bottoms. About 6 ml of the supernatant fluid were pipetted off and re-centrifuged. This process was repeated once more, and the supernatant fluid, after suitable dilution, was used for enumerating the parasites. For enumerating the parasite content of sewage itself, the homogenization step was omitted. It may be mentioned that this centrifugation technique gave higher counts of both phages and *Bdellovibrio* than filtration through a 0.45- μ Millipore filter.

Isolation procedures. Colonies from MacConkey's-agar plates were picked after 24 hr of incubation at 37 C. All other experiments were done at room temperature (15 to 27 C). Three serial passages were made in the liquid enrichment before plating on a solid medium (agar) of the same composition.

Enumeration techniques. The MPN (most probable number) was computed from the tables given by the American Public Health Association (1955), with five tubes per dilution. The methods for the individual counts were as follows. For *Azotobacter*, serially diluted sludge was inoculated into the enrichment medium; after incubation, the medium was examined for typical

azotobacter cells. In ambiguous cases, a second passage was made and examined. Nitrogen-fixing strains of *Aerobacter aerogenes* were estimated by inoculating the enrichment medium with serially diluted sludge. After incubation for 6 days, a loopful from each tube was transferred to MR-VP medium (American Public Health Association, 1955). After 24 and 48 hr, the tubes were tested for acetylmethylcarbinol (VP test). A positive result was taken as indication of the presence of *A. aerogenes*. *Corynebacterium laevaniformans* was enumerated by plating on a nitrogen-deficient sucrose medium. The characteristic colonial form of this organism (Dias and Bhat, 1962) made enumeration feasible. Each dilution was plated on five plates. The bacterial parasites were enumerated by the conventional double-layer technique. Each dilution was plated in duplicate. In a single experiment, with five plates per dilution, the average number per milliliter of raw sewage of *Bdellovibrio* parasitic to *Salmonella paratyphi* was found to be 206, with a standard error of 5.9. The plaques produced during 36 hr of incubation were taken to be due to phages; those developing after 36 hr were regarded as being due to *Bdellovibrio* (Stolp and Starr, 1963). Clearings which were sometimes seen around bacterial colonies were not counted.

RESULTS AND DISCUSSION

Coliforms in raw sewage, activated sludge, and effluent. Totals of 178, 169, and 113 coliform cultures were isolated from sludge, sewage, and effluent (after treatment), respectively. These could be grouped as shown in Fig. 1. The most interesting finding was that activated sludge had a selective action on the different types of coliforms. The coliform population of raw sewage predominantly consisted of *Escherichia coli* or what are often termed "fecal" coliforms, whereas

in sludge and effluent the proportion of this species declined with a simultaneous increase in the proportion of *Aerobacter* and *Escherichia* other than *E. coli*. *E. coli* accounted for, respectively, 25 and 30% of the sludge and effluent coliform populations, as against 75% in raw sewage. These results are in contradistinction to those recorded for sewage filters (Allen, Brooks, and Williams, 1949; Tomlinson, Loveless, and Sear, 1962), where the proportion of *E. coli* in the effluent (obtained after filtration) was reported to be of the same order as that in raw sewage.

It is difficult to assess the role of coliforms during activated-sludge treatment because the incoming raw sewage brings its own legions. The results reported above would seem to indicate some role for the "nonfecal" types. However, when it is considered that coliforms constitute less than 0.5% of the sludge flora (Dias and Bhat, 1964) and that they are the more dominant of the aerobic forms in raw sewage (Dias, 1963), a minor function at most can be assigned to this group.

Nitrogen-free mannitol-sucrose enrichments. The results were essentially similar to those recorded previously for raw sewage (Dias, 1963). The *Azotobacter* most frequently encountered (20 isolates) was *A. agilis*. Only nine strains of *A. chroococcum*, the dominant soil form, were encountered. Other species of *Azotobacter* were not recovered, although Paranajpaye (1963) isolated *A. vinelandii* from nitrogen-free inositol enrichments. Population density studies on 20 sludge samples revealed a fluctuation from 6 to 2,800 in the MPN of *Azotobacter* per gram (dry weight), with most values falling between 150 and 2,000. These figures do not permit any conclusions regarding the status of these bacteria as constitu-

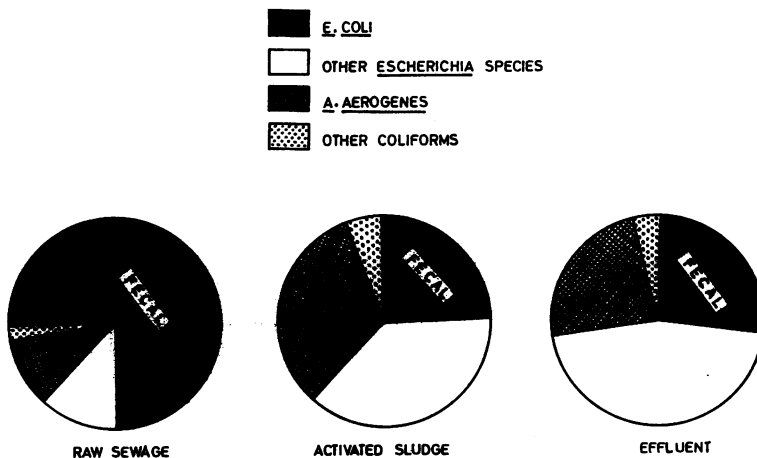


FIG. 1. Distribution of different types of coliforms in sewage, activated sludge, and effluent.

TABLE 1. Distribution of bacteriophages and *Bdellovibrio* in sewage and activated sludge*

Sample	<i>Escherichia coli</i> NCTC 419		<i>Pseudomonas</i> <i>aeruginosa</i>	<i>P. fluores-</i> <i>cens</i> NCTC 8720	<i>P. aureo-</i> <i>faciens</i> NRRL B1543	<i>Salmonella</i> <i>typhosa</i> H 901	<i>S. para-</i> <i>typhi</i>	<i>Serratia</i> <i>marcescens</i>	<i>Proteus</i> <i>morganii</i>	<i>Aerobacter</i> <i>aerogenes</i> NCTC 418
Sewage	100	200	0	260	20	460	330	16	120	242
	18	556	0	120	0	120	120	0	410	120
	792	—†	12	114	22	320	186	0	130	230
	8,000	—	44	184	22	150	196	18	224	160
	6,400	—	0	400	22	420	56	0	490	52
	7,200	—	82	630	38	530	630	0	600	96
	9,600	—	16	184	46	96	510	0	98	82
	4,200	—	12	396	4	330	32	16	88	420
	800	—	96	356	96	86	62	4	12	634
	1,200	—	52	298	98	42	184	2	630	864
Activated sludge	220	—	0	260	0	230	110	0	98	160
	160	—	0	234	6	216	36	0	96	130
	140	—	10	86	46	48	116	2	68	52
	188	—	0	188	60	56	98	8	68	16
	398	—	16	188	70	66	64	6	66	116
	726	—	18	108	22	64	164	0	198	72

* Only phages active against *E. coli* could be detected. The first column under *E. coli* gives the phage count (per milliliter); all other columns give the *Bdellovibrio* count. No *Bdellovibrio* active against the following organisms were found: *Alcaligenes faecalis* NCIB 8158, *Bacillus megaterium*, *Corynebacterium barkeri*, and *Staphylococcus aureus* var. *citreus*.

† *Bdellovibrio* count less than phage titer, so counts could not be made.

ents of the "normal flora" or their ability to fix nitrogen during sewage stabilization (Fowler, 1920; Morgan and Gilcreas, 1960; Peck, 1923; Shibata, 1940). This technique of counting *Azotobacter*, when applied to raw sewage, never revealed the organism in dilutions higher than 1:10, and more often than not revealed its presence only in the undiluted sewage.

The nitrogen-free enrichments also resulted in the isolation of *A. aerogenes* (18 isolates) and an *Achromobacter* species (5 isolates). A strain of the former, but none of the latter, was found to fix nitrogen (Dias, 1964). Strains of these organisms possess the ability to fix nitrogen (Hamilton, Magee, and Mortenson, 1953; Jensen, 1956; Pengra and Wilson, 1958; Proctor and Wilson, 1959). Counts of *A. aerogenes* showed a MPN varying from 3×10^6 to 598×10^6 , with most values between 100×10^6 and 250×10^6 per gram of dry sludge. Another organism encountered (20 isolates) in the nitrogen-free enrichments was *C. laevaniformans*, which does not fix nitrogen. It, however, produces large amounts of levan (Dias and Bhat, 1962), which could act as storage substance during waste-water treatment, as can poly- β -hydroxybutyric acid (Dias and Bhat, 1964). The number of *C. laevaniformans* cells per gram (dry weight) varied from 0.4×10^6 to 6.8×10^6 , with an average of 2.5×10^6 . These values, taken in conjunction with the fact

that the organism can rarely be isolated from raw sewage without prior enrichment, tend to show that *C. laevaniformans* is a "normal" sludge bacterium.

Bdellovibrio and bacteriophages. The first set of experiments was designed to survey the phage and *Bdellovibrio* populations of raw sewage and activated sludge. *E. coli* was the only organism which contained phages in detectable numbers in raw domestic sewage (Table 1). In the earlier phases of this survey, the *E. coli* phage titers were lower than that of *Bdellovibrio*; subsequently, the phage titer increased, and it became impossible to estimate *Bdellovibrio* on the *E. coli* plates. Whether this change was due to climatic conditions (the temperature being higher during the latter period) is an interesting ecological consideration that cannot be answered from the limited data recorded.

In conformity with the observations of Stolp and Starr (1963), *Bdellovibrio* active against gram-positive bacteria were not detected. Among the gram-negative bacteria tested, *Alcaligenes faecalis* was the only species against which no *Bdellovibrio* were detected; only a few parasites occurred which could lyse *Pseudomonas aeruginosa*. *Bdellovibrio* species attacking *Salmonella* were always present. This is the first time that a *Bdellovibrio* active against the enteric pathogens has been detected. To verify that the plaques were

TABLE 2. Changes in phage and *Bdellovibrio* population* of sewage during storage

Time <i>hr</i>	<i>Escherichia coli</i>	<i>Pseudomonas fluorescens</i>	<i>Salmonella paratyphi</i>
0	6,000	50	100
2	5,400	62	122
6	7,000	54	104
10	6,600	56	96
24	5,200	64	126
48	5,400	64	108

* Results for *E. coli* are expressed as number of phage per milliliter; for the other organisms, number of *Bdellovibrio* per milliliter.

in fact due to *Bdellovibrio*, a pure culture was isolated by the method of Stolp and Starr (1963) and maintained for five transfers.

The next experiment was designed to determine the effect of storage of raw sewage on the phage and *Bdellovibrio* populations. Detritus-free sewage (700 ml) was placed in a 1-liter Erlenmeyer flask; at various intervals of time up to 48 hr, samples were withdrawn and analyzed. The flask was left undisturbed during the periods between samplings. Neither the phage nor the *Bdellovibrio* populations (with respect to the hosts tested) changed appreciably during the storage period (Table 2). The coliforms, during the same period, decreased by 82%. This indicates that neither group of parasites is involved in the destruction of bacteria during storage.

An attempt was then made to test the fate of the parasites during activated-sludge treatment. An aerated rotary shaker unit (Dias and Bhat, 1964) was used. The unit functioned efficiently, in that the permanganate value (4 hr) of the effluent obtained after 6 hr was 14.8 ppm. At various times, samples were withdrawn and analyzed for parasites. The results (Table 3) support the conclusion that neither *Bdellovibrio* nor phages are functional during activated-sludge treatment. Interestingly, however, there was a 10-fold decrease in the phage population of the system (sewage plus sludge) within 2 hr of aeration, the phage density remaining fairly constant thereafter for 25 hr. There exists the possibility that the phages get adsorbed to the sludge, and are thus not enumerated. On the other hand, the *Bdellovibrio* species did not seem to be affected by activated sludge. The conclusion that phages do not function during activated-sludge treatment is consistent with the results obtained with other sewage-disposal methods (Ware and Mellon, 1956; Pretorius, 1962).

It was noted that activated sludge, in contrast with raw sewage, harbored a large number of

TABLE 3. Fate of phages and *Bdellovibrio* during activated-sludge treatment*

Time <i>hr</i>	Sample	<i>Escherichia coli</i>	<i>Pseudomonas fluorescens</i>	<i>Salmonella paratyphi</i>
0	Sewage (influent)	2,000	240	248
0	Sewage + sludge	2,460	268	230
2	Sewage + sludge	230	272	226
4	Sewage + sludge	198	288	236
6	Sewage (effluent)	190	246	206
25	Sewage (effluent)	180	240	249
25	Sewage + sludge	208	264	210

* Results for *E. coli* expressed as the number of phage per milliliter; for *P. fluorescens* and *S. paratyphi*, the number of *Bdellovibrio* per milliliter.

bacteria (other than *Bdellovibrio*) which lysed other bacteria. (The centrifugation process does not eliminate all bacteria.) This observation merits further study, and might lead to a better understanding of ecological factors in the activated-sludge complex.

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