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Distribution of *Bdellovibrio bacteriovorus* in Sewage Works, River Water, and Sediments

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Bdellovibrio was found in all liquid phases of the sewage works examined. The predator was also found in all the river sediments and sewage-polluted river waters examined but could not be found in some unpolluted river waters. *Bdellovibrio* was able to multiply on the high numbers of bacteria present in the aerobic percolating filter film but could not survive in anaerobic sludge. Similarly, the predator was present in the aerobic surface layers of river sediments but not in the anaerobic bottom layers. The major source of *Bdellovibrio* in the polluted rivers examined were sewage works effluents, and numbers in both river water and sediment were correlated with river water quality. It was unlikely that *Bdellovibrio* was important in reducing numbers of other bacteria in either sewage or river sediment.

Bdellovibrio bacteriovorus, the small bacterial predator of gram-negative bacteria (17), has been found to occur widely in soil, seawater, fresh water, and sewage (16). Despite these records, its role in the reduction of bacterial numbers in natural environments has not been established. It has been suggested that the predator may reduce bacterial populations in seawater (11) and soil (9). However, although it is claimed to be important in the removal of *Salmonella* spp. from polluted rivers in France (8, 10), later work has demonstrated that it does not either multiply in or control the bacterial population of a polluted British river (6). There is also a lack of evidence showing whether *Bdellovibrio* is a natural component of the freshwater microflora. Consequently the aim of the work presented here was to establish the extent and source of *Bdellovibrio* in British rivers and to find out whether it was capable of either growth on or the control of bacterial populations of sewage or river sediments that have higher bacterial numbers than river water.

MATERIALS AND METHODS

Sites and sampling. The work presented here was carried out between January 1970 and December 1972. River sites sampled in South Wales were on the Clun, Cynon, Ely, Ewenny, Kenfig, Kenson, Nant Craig-yr-aber, Nant Gadlys, Ogmore, Rhymney, Sirhowy, Taff, Thaw, and Waycock; those in Yorkshire were on the Aire, Kelk Beck, Ouse, and Went, and the River Nene was sampled in Northamptonshire. The precise location of each sampling site is unimportant but was recorded so that river water quality could be found by reference to the Report of a River Pollution Survey of England and Wales 1970 (3). In this report, British rivers were divided into four classes according to the occurrence of polluting discharges, biological oxygen demand, dissolved oxygen, turbidity, absence of fish, and frequency of complaints. These classes are referred to as follows: class 1, unpolluted; class 2, slightly polluted; class 3, badly polluted; and class 4, grossly polluted.

River water and liquid sewage samples were taken from surface water in sterile 250-ml glass-stoppered bottles; at each sampling site six samples were bulked before enumerations were carried out. Samples of final settlement sludge were taken with a long-handled grab, and percolating filter film was sampled at least 250 cm below the surface of the bed. River sediment was sampled by pushing a tubular aluminum corer (25 by 4.5 cm, inner diameter) into the sediment and sealing the bottom with a rubber bung while the tube was in the sediment. Appropriate lengths of core were extruded with a wooden plunger, and sections were cut to sample the mud at various depths. Normally the top 2 cm of sediment was used from duplicate cores.

Enumeration of *Bdellovibrio* and its potential prey. Enumerations of *Bdellovibrio* were performed in triplicate by a double-layer viable counting procedure (14) using NB-10 or NB-500 medium (6) with 0.6% agar in the top layer, 1.2% agar in the bottom layer, and *Escherichia coli* B ATCC 15144 or *Achromobacter* sp. NC1B 8250 as host. Only the slow-growing round plaques without central colonies were counted after 6 days of incubation at 30 C. These plaques were almost always due to *Bdellovibrio*, and this was periodically checked by examination of squashed plaques with phase-contrast microscopy for the typical small (0.8 by 0.25 μm), actively motile, curved, rod-shaped *Bdellovibrio*.

It should be noted that previous work has shown (14) that variations in the medium and bacterium in

the overlying agar can give differences in the *Bdellovibrio* count. From over 52 comparisons of counts obtained in this laboratory with NB-10 and *E. coli* B, NB-500 and *E. coli B*, and NB-500 and *Achromobacter* sp., the average ratio of counts for these counting conditions was 1.00:4.17:5.67.

Viable counts of potential prey were made in triplicate by the spread plate technique; dilutions were made in sterile river water. Heterotrophic bacteria were counted on casein-peptone-starch (CPS) agar (2, 15), and gram-negative bacteria were counted on this agar with the addition of 0.0002% crystal violet from a filter-sterilized stock (0.2 g/liter); both cultures were incubated for 10 days at 20 C. Coliform bacteria were counted on Endo agar (Oxoid Ltd.) and incubated for 48 h at 37 C; only colonies with a metallic sheen and surrounded by a red coloration of the medium were counted.

Enumerations from river water and sediment were made from water that was shaken 20 times by hand and from 7.5 g of sediment homogenized with 100 ml of sterile river water for 10 min at about 14,000 rpm. All sewage samples were homogenized for 10 min at about 14,000 rpm with the addition of 0.01% Lubrol W and 0.01% sodium pyrophosphate as deflocculants (14). Sewage sludge and percolating filter film were subsequently filtered through glass-fiber filter paper (Whatman GF/C), followed by centrifugation at 27,000 × *g* for 20 min at 4 C and filtration of the resuspended pellet through a 1.2-μm membrane filter (20). Bacterial numbers in river sediment, sewage sludge, and percolating filter film were expressed per gram of dry weight.

Analysis of results. Differences between mean counts were assessed by the sum-of-squares simultaneous test procedure (7), and all counts were subjected to a $\log x + 1$ transformation, the efficiency of which was checked by Bartlett's test and the variance ratio F_{max} test. Pearson product moment correlation coefficients were calculated by the Biomedical Computer Program package BMDO5R (5), and Kendall correlation coefficients were calculated by the Statistical Package for the Social Sciences (12).

RESULTS AND DISCUSSION

Distribution in sewage works. A number of sewage works in South Wales were examined for the presence of *Bdellovibrio* in the raw inflow, filter, and final effluents. The results (Table 1) show that *Bdellovibrio* was found in all the works examined. In all cases the numbers increased between inflow and effluent. This was probably due to growth of *Bdellovibrio* in percolating filters, since in every works there was an increase between the inflow and filter effluent but in most cases a decrease between the filter and final effluents. To test this, the predator distribution in Miskin sewage works was examined in more detail. In this case *Bdellovibrio* was enumerated by using NB-500 medium and *Achromobacter* sp. as host instead of the NB-10 medium and *E. coli* B used in the initial survey; this resulted (Table 2) in much higher numbers being observed in the raw inflow and filter effluents, which was to be expected due to the change of enumeration medium and host (14). In these cases, although no significant increase was observed between the raw inflow and filter effluent, numbers increased between filter inflow and effluent; from five of the seven samplings these increases were significant ($P < 0.05$). The drop in numbers between raw inflow and filter inflow may have been due to settlement of the *Bdellovibrio* attached to large particles in the primary settlement tanks. The increases during filtration were almost certainly due to growth of the predator in the percolating filters since high numbers were also found in the filter film. Predator numbers in this film were probably underestimated since some would be removed (14) by the filtration and centrifugation technique (20), which was used to remove the large numbers of protozoa present in the film which hindered *Bdellovibrio* enumeration. High numbers of

TABLE 1. *Relative abundance of Bdellovibrio in various sewage works in South Wales, estimated using NB-10 medium and E. coli B*

Sewage works	No. of samples	Mean no. of *Bdellovibrio*/ml in:		
		Raw inflow	Filter effluent	Final effluent
Duffryn	4	8	36	24
Miskin	17	18	32	32
Hensol	1	ND[a]	21	24
Rhyd lafa	1	ND	10	15
Peterston	3	20	30	29
St. Fagans	1	8	17	13
Rhiw Saeson	5	12	37	28
Cowbridge	4	2	7	5
Pyle	2	8	48	39

[a] ND, Not detectable in 1 ml of raw sewage.

TABLE 2. *Relative abundance of Bdellovibrio in Miskin sewage works, estimated using NB-500 medium with Achromobacter sp.*

Site sewage works	No. of samples	Mean no. of *Bdellovibrio*	
		Per ml	Per g (dry wt)
Raw inflow	7	222	
Filter inflow	7	135	
Filter effluent	7	226	
Filter film	9		2.7×10^4
Final settlement sludge	12		ND[a]

[a] ND, Not detectable.

the predators' potential prey were also found in the filter film, mean results from nine samplings being 2.2×10^{10} heterotrophic bacteria/g, 1.3×10^{10} gram-negative bacteria/g, and 1.4×10^{9} coliform bacteria/g. However, despite repeated sampling, no *Bdellovibrio* could be detected in the final settlement sludge (Table 2).

These results were to be expected since *Bdellovibrio* is aerobic and does not survive in anaerobic conditions (13, 18), so it should not be found in anaerobic sludges. Also, the fact that *Bdellovibrio* cannot grow on the natural bacteria in the River Ely (6) is due to low host numbers and not the comparatively low temperatures. In percolating filter film, bacterial numbers are much higher and conditions are aerobic, so *Bdellovibrio* growth should be possible.

Few workers have enumerated *Bdellovibrio* in sewage. However, the numbers found in this study were, although slightly lower, in the same order as those found by Dias and Bhat (4), who found that numbers on a wide variety of hosts varied between 0 and 864/ml.

Distribution in river waters. An initial survey was carried out in which *Bdellovibrio* numbers estimated on NB-500 medium with *E. coli* B as host were counted in 19 rivers in South Wales and elsewhere. Average numbers ranged from 0 to 51 *Bdellovibrio*/ml from up to 91 samplings. It was apparent that river water quality (3) influenced the numbers of *Bdellovibrio;* in general, unpolluted rivers had very low numbers (0 to 3 *Bdellovibrio*/ml), whereas grossly polluted rivers had high numbers (18 to 51 *Bdellovibrio*/ml). *Bdellovibrio* was found in all the sewage effluents examined, and the major source of pollution in the rivers studied was sewage effluent. Therefore, it was likely that sewage effluents were the major source of *Bdellovibrio* in these rivers.

Two rivers were chosen for further examination; they were the sections below Miskin sewage works on the badly polluted River Ely and below Cowbridge sewage works on the unpolluted River Thaw. The results (Fig. 1) show that there were more *Bdellovibrio* and other bacteria in the polluted river than in the unpolluted one and that predator numbers increased significantly ($P < 0.05$) at the point of entry of the sewage effluent. This increase was undoubtedly due to *Bdellovibrio* in the effluents, since other results from the River Ely (Fig. 2) showed that numbers of the predator only increased below the outfall when the effluent was flowing. The higher numbers of *Bdellovibrio* entering the river from the Miskin effluent were also reflected by the fact that, although the predator was found in all sections of the Ely

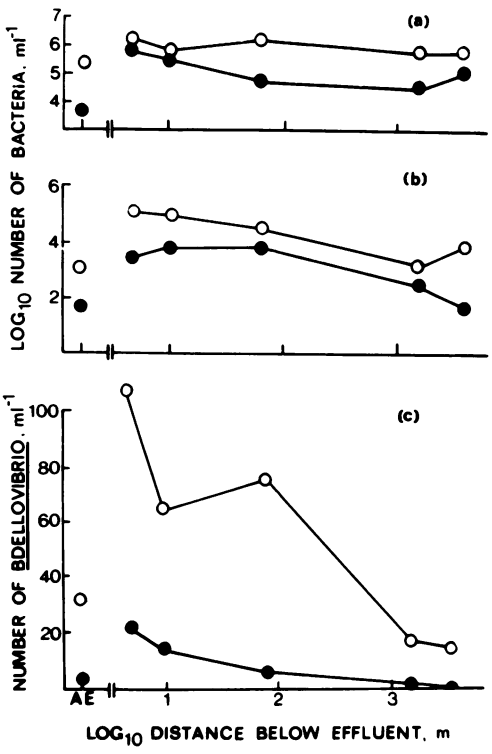

FIG. 1. *Distribution of Bdellovibrio and its potential prey in Ely river water below Miskin sewage works and in Thaw river water below Cowbridge sewage works. Bdellovibrio was estimated on NB-500 medium with E. coli B. (a) Gram-negative bacteria; (b) coliforms; (c) Bdellovibrio. Symbols:* ○, *River Ely;* ●, *River Thaw. AE, Count above effluent.*

below Miskin (6), it could not be detected in the Thaw before 1.6 km from the Cowbridge effluent. It is therefore doubtful whether *Bdellovibrio* is part of the natural microflora in this river.

Distribution in river sediments. It is likely that the rapid fall in *Bdellovibrio* numbers below sewage effluents is in part due to settlement onto the river bed (6); therefore a selection of river sediments was examined for *Bdellovibrio* to determine whether it was an important constituent of the benthic microflora. An initial survey of river sediments in seven South Wales rivers using NB-500 medium and *Achromobacter* sp. as host showed that numbers varied from 5.5×10^{1} to 2.9×10^{4} *Bdellovibrio*/g. These estimates were probably reliable since there were no protozoa present to hinder enumeration and therefore direct plating was used. Like the results for river water, unpolluted river sediments contained fewer parasites than sediments from polluted rivers.

River sediments in the sections of the Ely and

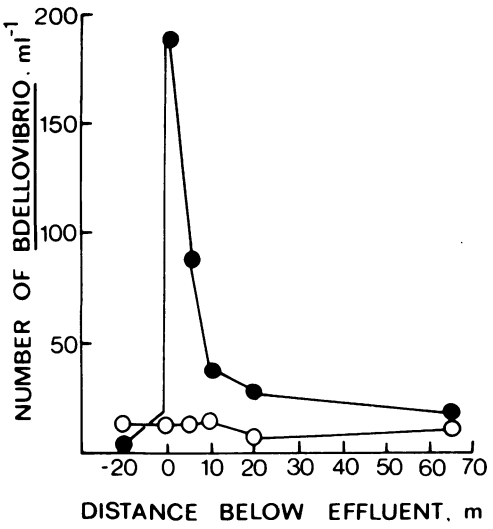

FIG. 2. *Effect of Miskin sewage effluent on Bdellovibrio in the River Ely, estimated on NB-10 medium with E. coli B. Symbols: ●, effluent flowing; ○, effluent off. All points are means of four samplings.*

Thaw below sewage works outfalls were also examined for *Bdellovibrio*. The results (Fig. 3) were similar to those for the river water and showed significant ($P < 0.05$) increases immediately after entry of the effluent. There was, however, less difference in the relative numbers of *Bdellovibrio* between the two rivers. The sediment in the Ely was examined in more detail 65 m below Miskin outfall. The results (Fig. 4) show that *Bdellovibrio* was only found in the top 5 cm of sediment, whereas heterotrophic bacteria, gram-negative bacteria, and coliforms were found down to 12 cm, with little decrease in numbers below the 2-cm level. This can be explained once again by the aerobic nature of *Bdellovibrio* (13, 18), which consequently should only be found in the uppermost aerobic region of the sediment. Its potential prey, however, are either facultative, as are the coliforms, or else are likely to be mainly facultative, as are heterotrophs in sediment (1), and so should occur in both the aerobic and anaerobic zones. This is supported by work that has found certain lake sediments to have negative Eh values below 3 m due to oxygen depletion (19).

There has been no other work in which *Bdellovibrio* was enumerated in river sediments. However, the numbers found in the work reported here agree well with the range of 1×10^3 to 9×10^4 *Bdellovibrio*/g found by Klein and Casida in a study of sewage-polluted and normal soils (9).

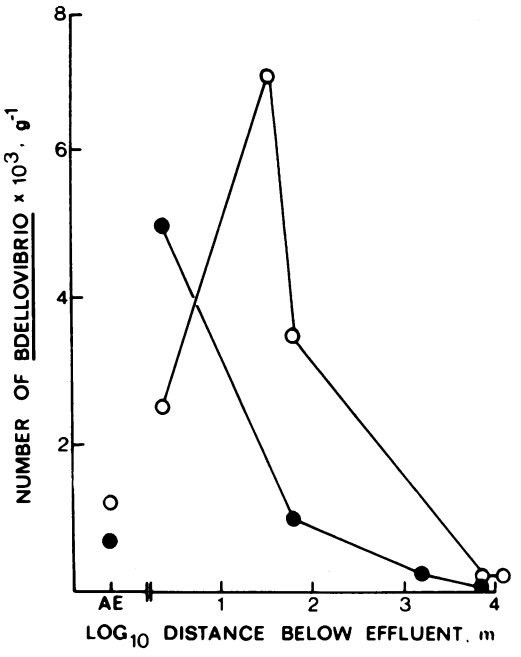

FIG. 3. *Distribution of Bdellovibrio in River Ely sediment below Miskin sewage works and in River Thaw sediment below Cowbridge sewage works. Bdellovibrio was estimated on NB-500 medium with E. coli B. Symbols: ○, River Ely; ●, River Thaw. AE, Count above effluent. All points are means from two cores.*

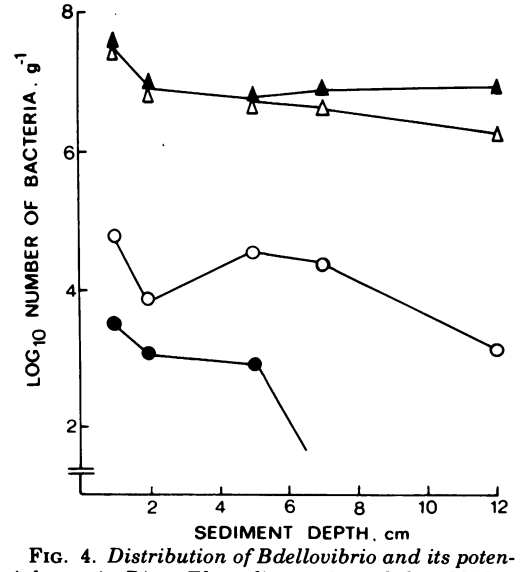

FIG. 4. *Distribution of Bdellovibrio and its potential prey in River Ely sediment 65 m below Miskin sewage works. Bdellovibrio was estimated on NB-500 medium with E. coli B. Symbols: ●, Bdellovibrio; ○, coliforms; △, gram-negative bacteria; ▲, heterotrophic bacteria. All points are means from three cores*

TABLE 3. *Correlation coefficients and their significance for Bdellovibrio, its potential hosts, and other parameters in river water, river sediment, and sewage*[a]

Sample	Variables		No. of data pairs	Correlation coefficient[b]	Significance[c]
River water	Heterotrophs/ml	*Bdellovibrio*/ml[d]	227	0.682	***
	Gram-negative bacteria/ml	*Bdellovibrio*/ml	38	0.007	NS
	Coliforms/ml	*Bdellovibrio*/ml	85	0.392	***
	Temp	*Bdellovibrio*/ml[d]	95	0.115	NS
	Water quality[e]	*Bdellovibrio*/ml[d]	22	0.749[f]	***
River sediment	Heterotrophs/g	*Bdellovibrio*/g	26	0.091	NS
	Gram-negative bacteria/g	*Bdellovibrio*/g	26	0.284	NS
	Coliforms/g	*Bdellovibrio*/g	26	0.098	NS
	Water quality[e]	*Bdellovibrio*/g	10	0.701[f]	**
	Bdellovibrio in overlying water/ml	*Bdellovibrio*/g	43	0.859	***
Sewage	Heterotrophs/ml	*Bdellovibrio*/ml[d]	72	0.171	NS
	Gram-negative bacteria/ml	*Bdellovibrio*/ml[d]	30	0.006	NS
	Coliforms/ml	*Bdellovibrio*/ml	20	0.075	NS
	Temp	*Bdellovibrio*/ml[d]	75	0.400	***

[a] *Bdellovibro* were estimated by using NB-500 medium and *E. coli* B unless otherwise stated.
[b] Pearson product moment correlation coefficients quoted unless otherwise stated.
[c] **, $P < 0.01$; ***, $P < 0.001$; NS, not significant.
[d] *Bdellovibrio* numbers estimated by using NB-10 medium and *E. coli* B.
[e] River water quality classified according to reference 3.
[f] Kendall correlation coefficient obtained from analysis of the mean *Bdellovibrio* count from a variety of sites (n = 4 to 91).

Correlations. Correlation coefficients were calculated between the numbers of *Bdellovibrio* found in river water, sediment, and sewage and its potential prey and other parameters. The results (Table 3) show that predator numbers in sewage and sediments were not correlated with heterotrophic, gram-negative, or coliform bacteria; therefore it seems unlikely that *Bdellovibrio* was controlling the numbers of any of these bacteria in these habitats. This conclusion in part supports the earlier work of Dias and Bhat (4), which showed *Bdellovibrio* to be unimportant in the activated sludge process. In river water where *Bdellovibrio* has no controlling influence on bacterial numbers (6), there were significant correlations between predator numbers and heterotrophic and coliform bacteria. This may be because effluents discharging large numbers of heterotrophs also discharge large numbers of *Bdellovibrio* (Fig. 1) and because coliform and *Bdellovibrio* numbers decline together in the River Ely (6). The highest correlation coefficient observed was between *Bdellovibrio* numbers in river sediments and those in the overlying water; this probably indicates that most parasites enter the sediment after sedimentation from the water. It is not clear from the present work whether *Bdellovibrio* subsequently grows in the sediment. However, the lack of correlation between predator numbers and temperature in water and the significant correlation between these parameters in sewage possibly reflect lack of *Bdellovibrio* growth in the water and temperature-dependent growth of *Bdellovibrio* in the percolating filters. Finally, the significant correlations observed between river water quality and predator numbers in water and sediment reflect again the fact that sewage effluents, which are the major influences on river pollution in the rivers studied, are also the source of *Bdellovibrio* in those rivers.

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