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Removal of *Escherichia coli* in Wastewater by Activated Sludge

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Removal of bacteria from wastewater treated with activated sludge was studied by the use of a streptomycin-resistant *Escherichia coli* strain. The removal appeared to be a biphasic process. A rapid sorption of bacteria to the sludge flocs took place in the first hour after seeding mixed liquor with *E. coli*. Thereafter, slower elimination of *E. coli* was observed. The latter process was due to predation on *E. coli* by ciliated protozoa. This was shown by: (i) appearance of fluorescent food vacuoles of ciliates when fluorescent *E. coli* cells were added to mixed liquor; (ii) inhibition of predation either in the presence of cycloheximide or under anaerobic conditions; and (iii) absence of predation in bulking and washed sludge.

The number of bacteria present in wastewater has been reported to be reduced 90 to 99% during activated-sludge treatment (6, 7). For coliforms, this reduction was due neither to coliphages nor to the presence of *Bdellovibrio* species (3). The principal mechanism involved in removal of bacteria from wastewater treated with activated sludge was suggested to be predation by ciliated protozoa (1, 2, 8). A secondary mechanism might be the lytic action of certain bacteria on other bacteria (2).

In this study, we show that the removal of *Escherichia coli* from wastewater treated with activated sludge is a biphasic process: at first a rapid sorption of bacteria to the sludge flocs takes place, followed by a slower elimination of bacteria, which presumably is due to predation by ciliated protozoa.

MATERIALS AND METHODS

Microorganisms. *E. coli* V2086 was isolated from a sewage plant. It was identified as *E. coli* by growth and gas production in 24 h in McConkey broth (Oxoid Ltd., London) at 44.5°C and by its characteristic indole, methyl red, Voges-Proskauer, and citrate (IMVC [++--]) reactions. In this study a mutant of this strain resistant to streptomycin (400 μg/ml) was used. Cells were inoculated from a tryptone soy agar (Oxoid) slant into peptone water (Oxoid) and incubated for 16 h at 37°C on a rotary shaker (200 rpm). The cells were harvested by centrifugation for 10 min at 8 000 × *g*, washed with a 0.9% NaCl solution, and finally resuspended in this solution to yield a viable count of about 3×10^9 cells per ml.

Activated sludge. A 3-liter sample of mixed liquor was collected in a 5-liter flask at a distance of 5 m behind the aerator of an activated-sludge plant that received predominantly domestic sewage. The plant is located at Valburg, The Netherlands. After arrival at the laboratory (about 20 min after sampling), mixed-liquor samples (500 ml; dry weight ca. 5.4 mg/ml) were aerated (100 liters/h) for 1 h at 17°C in sterile 1-liter flasks before starting the experiments.

Enumeration of *E. coli* in mixed liquor and wastewater. A 1-ml portion of *E. coli* suspension was used to inoculate 500 ml of mixed liquor. If higher or lower bacterial concentrations were tested, the concentration of the starting suspension was adjusted accordingly. At appropriate time intervals, samples from the seeded mixed liquor were taken to determine the number of streptomycin-resistant *E. coli* present either in free state or bound to the sludge flocs.

To obtain wastewater, flocs were removed from the mixed liquor either by centrifugation for 10 min at 650 × *g* or by settlement in measuring cylinders. A 500-ml sample of wastewater was aerated (100 liters/h) for 1 h at 17°C and subsequently seeded with 1 ml of *E. coli* suspension.

The number of *E. coli* added to mixed liquor or wastewater was determined by spread plating 0.25 ml of the streptomycin-resistant *E. coli* suspension on McConkey agar (E. Merck AG, Darmstadt, Germany) containing 400 μg of streptomycin per ml. Appropriate dilutions were made in sterile 0.1% peptone water. Plates were incubated at 37°C for 20 h before counting. To determine the number of streptomycin-resistant *E. coli* present in the mixed liquor, 7-ml samples were taken with a calibrated beaker and diluted 100-fold in a sterile solution of 0.9% NaCl containing 0.01% each of sodium pyrophosphate (Baker Deventer, The Netherlands) and Lubrol W (ICI Rotterdam, The Netherlands). This solution aids the dispersion of activated-sludge flocs (5). The solution was vigorously stirred for 30 min at room temperature, after which the viable count was determined by spread plating as described above. Validity of the method was proven with fluorescent

E. coli cells. If a sample of mixed liquor to which fluorescent *E. coli* cells were added was diluted in the pyrophosphate-Lubrol W solution, almost all fluorescent cells were present in a free state after 30 min at room temperature, and only a few remained adsorbed to the flocs. Moreover, the viable count did not change as a result of the treatment with pyrophosphate and Lubrol W. To determine the number of free streptomycin-resistant *E. coli* cells present in the mixed liquor, a 5-ml sample of mixed liquor was centrifuged for 10 s at maximal speed in a hand-driven centrifuge, and the viable count in the supernatant was determined as described above. This centrifugation did not result in pelletation of free bacteria, as was determined by treating *E. coli* suspensions in an identical manner. The number of streptomycin-resistant *E. coli* present in wastewater was determined by spread plating.

At the streptomycin concentration used (400 μg/ml), indigenous bacteria present in unseeded mixed liquor or wastewater did not grow on the agar plates. Experiments were done at least in duplicate. Enumerations were carried out in triplicate.

Fluorescence microscopy. To cells of the streptomycin-resistant mutant of *E. coli* V2086, grown overnight as described before, acridine orange (52 μg/ml; Merck) was added to obtain fluorescent staining. After 2 to 3 h of incubation at 37°C, cells were harvested by centrifugation for 10 min at 8 000 × *g* and then washed with and suspended in 0.9% NaCl solution (ca. 10^{10} cells per ml). Under the fluorescence microscope (Leitz Ortholux microscope with transmitted fluorescence; excitation and stopping filters, BG 12 and K510, respectively), cells were colored green, yellow-brown, orange, and red, depending on the amount of dye taken up (10). A 1-ml sample of this suspension was added to 500 ml of aerated mixed liquor, and, at appropriate time intervals, samples were taken and examined under the fluorescence microscope.

RESULTS

Sorption of *E. coli* to activated-sludge flocs. Addition of *E. coli* cells to mixed liquor resulted in a rapid decrease in the number of free *E. coli* in the first hour, whereas the total number of *E. coli* present in mixed liquor decreased much slower in this period (Fig. 1). Analysis of data obtained within the first 40 min after addition of different concentrations (10^{-6} to 10^{-8} cells per ml of mixed liquor) of *E. coli* to mixed liquor showed that the rapid removal of *E. coli* from the wastewater followed a Langmuir adsorption isotherm (data not shown). No difference in sorption was observed if *E. coli* cells grown at 22 or 37°C were used to seed mixed liquor. If the time interval between sampling and start of aeration, which usually was about 20 min, was extended to 1 h, no change in sorption kinetics was observed. Mixed liquor that was aerated for 24 h before seeding showed a decrease in *E. coli* similar to that of mixed liquor seeded 1 h after start of the aeration.

Reduction of *E. coli* in mixed liquor. The removal of *E. coli* by activated sludge is a biphasic process (Fig. 1). After the rapid removal, a slower removal of free *E. coli* was observed that paralleled the reduction in the viable count of the mixed liquor. Since no decrease in viable count was observed during a 8-h period if wastewater was seeded with *E. coli* (Fig. 1), it also is evident that the slow-removal phase was dependent on the presence of sludge flocs. A possible role for ciliates in the reduction of *E. coli* in mixed liquor was indicated when the experiment was repeated with bulking sludge. Again, a rapid decrease in the number of free *E. coli* was observed in the first hour after seeding and to about the same extent, but the viable count in the mixed liquor did not change appreciably during a 7-h period. A similar result was obtained if mixed liquor was centrifuged and the flocs were washed twice with a 0.9% NaCl solution followed by readdition of the wastewater. Microscopic investigation showed that both bulking sludge and washed sludge contained almost no ciliates but only flagellated protozoa.

Figure 2 shows the effect of gassing of mixed liquor with N_2 on the reduction of *E. coli*. Part of the adsorbed *E. coli* cells were released from the flocs, and a new equilibrium between adsorbed and free *E. coli* was established; no reduction in viable count was observed during gassing with N_2 (data not shown). If gassing with N_2 was stopped and aeration was resumed, a rapid resorption of cells took place followed by a slow reduction in the viable count paralleling that in mixed liquor aerated continuously (Fig. 2). However, when mixed liquor was gassed

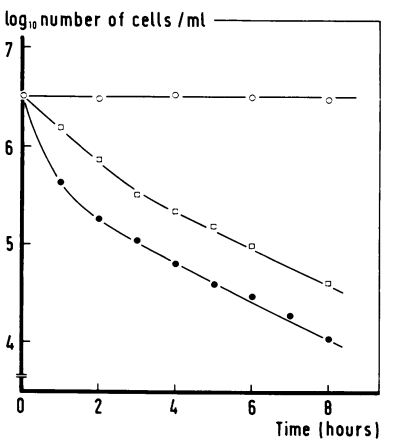

FIG. 1. *Removal of E. coli in activated sludge. Symbols:* (□) *E. coli in mixed liquor;* (●) *free E. coli in mixed liquor;* (○) *E. coli in wastewater.*

with N_2 for 5 h, a shift to aeration did not result in sorption or in a reduction of the viable count. A microscopic study of this mixed liquor showed that protozoa were not present. Furthermore, no further sorption of bacterial cells was observed.

Addition of cycloheximide, a specific inhibitor of eucaryotic protein synthesis, to mixed liquor had no effect on the extent of sorption of *E. coli* to the flocs in the first hour after seeding, but decreased the rate of removal of *E. coli* in the subsequent hours to 70, 45, and 30% of the original value when 100, 200, and 500 μg of cycloheximide were used, respectively. High concentrations of cycloheximide had to be used in this experiment, presumably since this compound was adsorbed to the flocs too; however, these antibiotic concentrations did not decrease the viability of *E. coli*.

Addition of fluorescent *E. coli* cells to mixed liquor resulted in sorption of the majority of these cells to the flocs within 30 min (Fig. 3A). Predation by ciliated protozoa was demonstrated by fluorescence microscopy. After 30 min, *Vorticella* species with fluorescent food vacuoles were observed, whereas after 2 h the vacuoles of *Aspidisca costata* were fluorescent. Although *Trachelophyllum pusillum* was present in the sludge, no fluorescent vacuoles were observed, which is in accordance with the fact that this protozoan does not feed on bacteria (8). About 6 h after addition of fluorescent *E. coli* cells, protozoa with many fluorescent food vacuoles were observed (Fig. 3B). After 21 h, almost all fluorescent bacteria had disappeared from the mixed liquor, and the food vacuoles from *A. costata* were still fluorescent in contrast to those of *Vorticella* species.

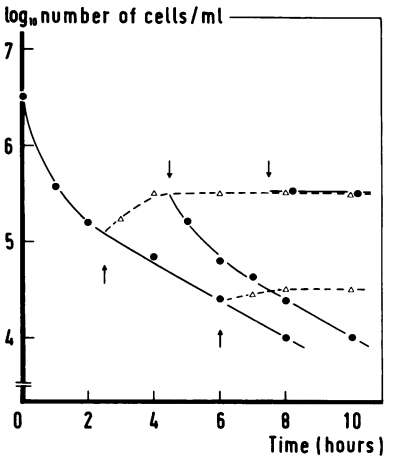

FIG. 2. *Effect of gassing with N_2 on the removal of free E. coli cells in activated sludge. Upward arrows indicate that aeration of mixed liquor with air is replaced by gassing with N_2, whereas downward arrows indicate the reverse. Unbroken lines represent the changes in the number of cells during aeration; dashed lines indicate the changes in the number of cells during gassing with N_2.*

DISCUSSION

It has been known for many years that the number of bacteria present in the effluent of an activated-sludge plant is reduced 90 to 99% as compared with the influent (6, 7). This reduction can be achieved by sorption of bacteria to the sludge flocs, or by destruction during sewage treatment, or a combination of both. If sorption were the major process, this would mean a possible hazard for public health due to the presence of pathogens in wastewater, since in many cases sludge flocs are used, e.g., for agricultural purposes.

Our study demonstrates that the removal of bacteria from wastewater treated with activated sludge is a biphasic process. In the first hour after addition of bacteria to activated sludge, sorption is the predominant process, and sorption obeyed a Langmuir adsorption isotherm. However, the principal mechanism responsible for removal of bacteria is predation by ciliated protozoa. This was shown in several ways.

Addition of bacteria to bulking activated sludge not containing ciliates resulted in a normal sorption, but the viable count of the mixed liquor did not decrease. A similar result was obtained if mixed liquor was centrifuged, and the flocs were washed, after which the wastewater was resupplied. Such sludge contained no ciliates. The tendency of ciliates to rupture during centrifugation has already been shown by Proper and Garver (9). Gassing of activated sludge with N_2 resulted in cessation in the decrease of the viable count and, with prolonged treatment, also in a reduction of the protozoan population. Moreover sorption did not occur after a 5-h gassing period with N_2, which indicated that the consistency of the flocs must have changed largely. It was shown by fluorescence microscopy that ciliates present in mixed liquor fed on bacteria. Our conclusion agrees with that of Curds and Fey (2), who demonstrated that protozoa drastically reduced the survival time of *E. coli* in activated sludge and concluded that ciliates were primarily responsible. Recently it was shown that the removal of *E. coli* from estuarine water was dependent on the presence of protozoan predators (4).

It cannot be excluded that still other mechanisms for the destruction of *E. coli* in activated

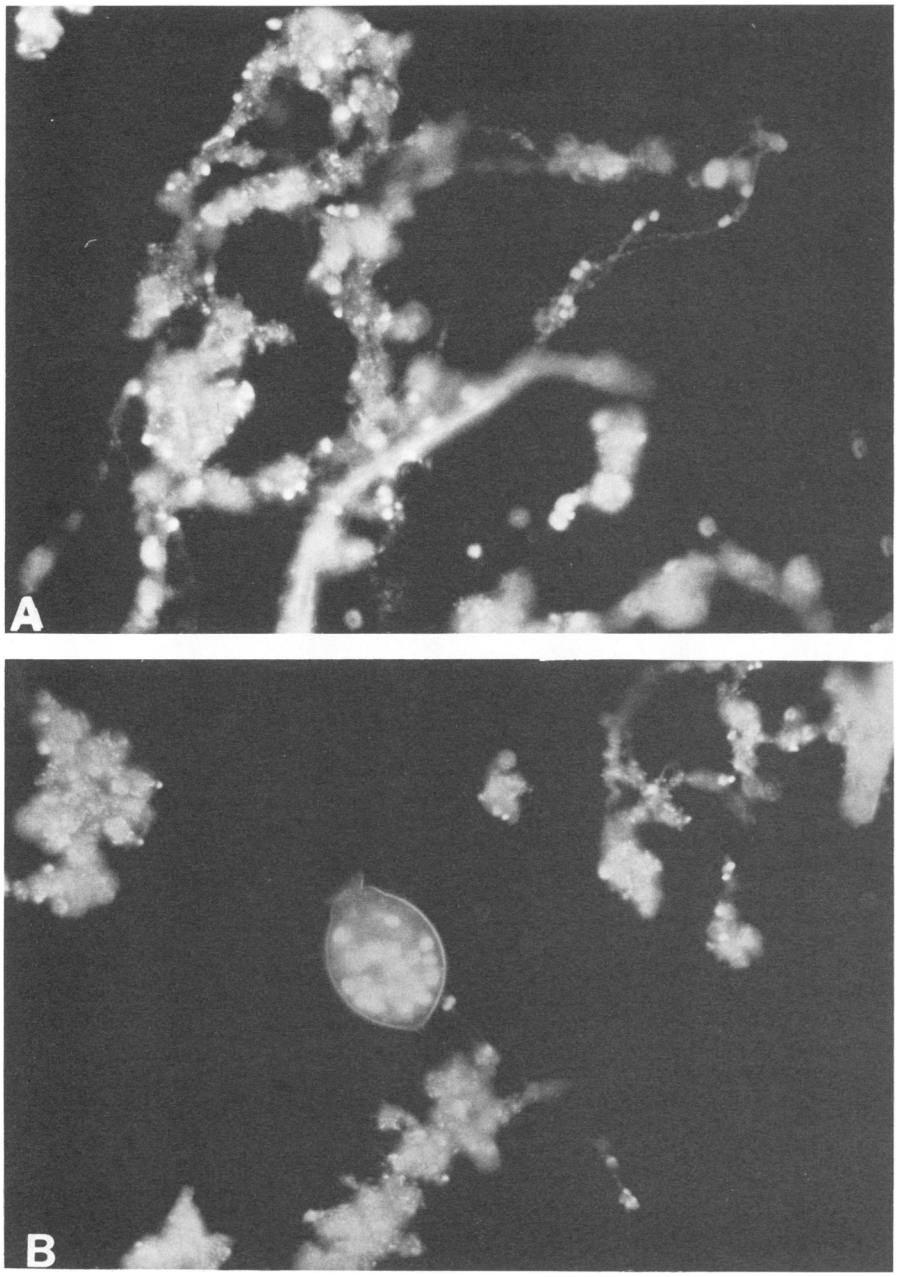

FIG. 3. *Predation of protozoa on bacteria adsorbed on sludge flocs demonstrated by fluorescence photomicroscopy. (A) Bacteria adsorbed to the flocs; (B) a Vorticella with fluorescent food vacuoles.*

sludge are operable. Clearly, wastewater itself appears to play no role, but lytic bacteria present in the sludge flocs may contribute to the removal of bacteria (2).

LITERATURE CITED

1. **Curds, C. R.** 1973. The role of protozoa in the activated-sludge process. Am. Zool. 13:161-169.
2. **Curds, C. R., and G. J. Fey.** 1969. The effect of ciliated protozoa on the fate of *Escherichia coli* in the activated-sludge process. Water Res. 3:853-867.
3. **Dias, F. F., and J. V. Bhat.** 1965. Microbial ecology of activated sludge. II. Bacteriophages, *Bdellovibrio*, coliforms, and other organisms. Appl. Microbiol. 13:257-261.
4. **Enzinger, R. M., and R. C. Cooper.** 1976. Role of bacteria and protozoa in the removal of *Escherichia coli* from estuarine waters. Appl. Environ. Microbiol.

31:758-763.
5. **Gayford, C. G., and J. P. Richards.** 1970. Isolation and enumeration of aerobic heterotrophic bacteria in activated sludge. J. Appl. Bacteriol. 33:342-350.
6. **Kabler, P.** 1959. Removal of pathogenic microorganisms by sewage treatment processes. Sewage Ind. Wastes 31:1373-1382.
7. **Kampelmacher, E. H., and L. M. van Noorle Jansen.** 1970. Salmonella – its presence in and removal from a wastewater system. J. Water Pollut. Control Fed. 42:2069-2073.
8. **Pike, E. B., and C. R. Curds.** 1971. The microbial ecology of the activated sludge process, p. 123-147. *In* G. Sykes and F. A. Skinner (ed.), Microbial aspects of pollution. Academic Press Inc., London.
9. **Proper, G., and J. C. Garver.** 1966. Mass culture of the protozoa *Colpoda steinii*. Biotechnol. Bioeng. 8:287-296.
10. **Reploh, H., G. Gängel, and A. Nehrkorn.** 1960. Forschungsberichte des Landes Nordrhein-Westfalen, no. 856, Cologne.