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Use of Nutrient Response Techniques To Assess the Effectiveness of Chlorination of Rapid Sand Filter Gravel†

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A direct viable counting method was used to rapidly assess the effectiveness of chlorination of biofilms on rapid sand filter gravel. A total of 50% of the cells were nutrient responsive after exposure to 0.5 mg of chlorine per liter, while this value was 25% after exposure to 25 mg of chlorine per liter. A large variation was seen in the numbers of nutrient-responsive cells on different rocks. More cells attached to the sandblasted side of marbles than to the smooth side, but there was no difference in eight of nine cases in the proportion of survival to chlorination between the two different sides. The effectiveness of chlorination appeared to be influenced by the species of bacterium in the biofilm.

There have been a number of reports of bacteria being found in water distribution systems in which residual chlorine has been maintained or of bacteria which have shown resistance to chlorination (4, 6–8, 10, 12, 13, 16–18, 19, 22, 26, 29, 30, 32, 33). Studies have shown that bacteria can have increased resistance to chlorine when the cells are in aggregates or clumps (22), in contact with or embedded in suspended inanimate particles (9, 14, 27, 31) or activated carbon particles (2, 3, 15), and attached to surfaces (8, 13, 22). Other factors which affect the resistance of bacteria to chlorine include nutrition, age of the biofilm, and production of a capsule (13). Also, the genus of bacterium involved is important (34). All of these results have been determined by plate count procedures, which only enumerate viable culturable cells, and these procedures require relatively long incubation periods.

The acridine orange direct count method has been used to enumerate bacteria in water samples; however, there has been a debate concerning the colors of the cells that should be counted (11). It was proposed that the addition of yeast extract and nalidixic acid to seawater samples followed by acridine orange staining and epifluorescence microscopy could be used to determine the number of viable cells in these samples (11). This procedure was labeled the direct viable count method and was based on the fact that viable cells would enlarge but not divide in the presence of nalidixic acid. There would be no increase in the cell size of nonviable cells. More recent studies have involved the use of this technique to follow the viability of *Escherichia coli* and *Vibrio cholerae* in microcosms (5), the determination of the effect of low concentrations of toxic chemicals on *E. coli* placed in microcosms of Chesapeake Bay water (20), the determination of viable but nonculturable cells of *Salmonella enteritidis* (24) and *Campylobacter jejuni* (23), and the determination of arsenic-resistant bacteria in well water samples (35). The effects of various nutrients and various concentrations of these nutrients on the effectiveness of the technique have been studied (21, 25).

We report here the application of the direct viable count method to the quick assessment of the efficiency of chlorination of rapid sand filter support gravel. Furthermore, the effects of surface texture of marbles and species of bacterium on chlorination efficiency were studied.

MATERIALS AND METHODS

Rocks were obtained from the bottom of an active filter which had been on-line at the Laramie, Wyo., surface water treatment plant for 24 years. These rocks were placed into Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and were incubated with shaking at 15°C, which is representative of the summer water temperature in Laramie. New rocks were obtained from a gravel supply company and were cleaned overnight in 50% HCl, rinsed well, covered with 0.1% Trypticase soy broth, inoculated with 0.2 ml of the culture described above, and incubated with shaking at 15°C. The medium was changed each day, until the culture became visibly turbid (3 to 5 days), at which time the medium was taken off the rocks and the rocks were chlorinated. After chlorination the rocks were rinsed in sterile chlorinefree distilled water. Six to eight rocks were removed and placed into 0.02% yeast extract plus 0.025% nalidixic acid and incubated at 15°C. Formalin (final concentration, 3.7%) was added after 6 h. The rocks were then stained with acridine orange, carefuly embedded in modeling clay, and observed directly by epifluorescence microscopy (Nikon Labophot). The surface roughness of the rocks was accounted for by focusing up and down through the field. Swollen or elongated cells were considered to be substrate responsive.

The conditions of chlorination were as follows: (i) one exposure to 0.5 mg of total chlorine per liter, (ii) two exposures to 0.5 mg of total chlorine per liter, (iii) three exposures to 0.5 mg of total chlorine per liter followed by one exposure to 2 mg of total chlorine per liter, (iv) two exposures to 0.5 mg of total chlorine per liter followed by one exposure to 25 mg of total chlorine per liter, and (v) three exposures to 0.5 mg of total chlorine per liter followed by two exposures to 25 mg of total chlorine per liter. Chlorination was for 5 min, and the chlorine concentration was determined by using Hach *N,N*-diethyl-*p*-phenylenediamine (DPD) pillows for total chlorine (Hach Co., Loveland, Colo.).

Three bacteria were isolated from the original culture, which was obtained by covering the rocks with Trypticase

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TABLE 1. Nutrient-responsive cells on inoculated rocks exposed to chlorine

Conditions of exposure to chlorine	No. of rocks	% Responsive cells when >45 cells were counted			No. of rocks with <45 cells
		High no.	Low no.	Mean ± SD	
One exposure to 0.5 mg/liter	48	79	1	46 ± 19	8
Two exposures to 0.5 mg/liter	13	57	35	51 ± 7	2
Three exposures to 0.5 mg/liter followed by one exposure to 2 mg/liter	4	65	28	50 ± 17	0
Two exposures to 0.5 mg/liter followed by one exposure to 25 mg/liter	33	77	0	24 ± 19	4
Three exposures to 0.5 mg/liter followed by two exposures to 25 mg/liter	11	45	12	25 ± 10	2

soy broth. These bacteria were identified with the API 20E system (Analytab Products, Plainview, N.Y.) as *Enterobacter cloacae*, a member of the Centers for Disease Control (CDC) group VE-1 and a fluorescent pseudomonad. To study the effect of surface texture on the attachment and protection of bacteria from chlorination, glass marbles were sandblasted on one side. The marbles were placed in pure cultures of the bacteria described above and incubated for 2 days at 15°C, to allow for growth and attachment. The marbles were then chlorinated for 5 min at total chlorine concentrations of 0.25, 2.5, and 25 mg/liter. The control was distilled water (0 mg of total chlorine per liter). After chlorination the marbles were rinsed; placed in 0.02% yeast extract and 0.025% nalidixic acid for 6 hours; and then placed in Formalin (3.7%), stained with acridine orange, and observed by epifluorescence microscopy. The percentage of nutrient-responsive cells was then determined. Fifty microscopic fields or 100 cells were counted for both the smooth and sandblasted sides of the marble.

Four models of dual media rapid sand filters were made to scale in 1.5-inch (3.81-cm)-diameter plastic tubes. An additional tube was filled with glass marbles. These tubes were put on-line for feeding with coagulated, flocculated, and settled water. The columns were backwashed in regular cycles with finished water which had a residual chlorine level of 1 mg/liter. After 6 months of use, marbles and rocks were removed and processed as described above for the sandblasted marbles.

Data were analyzed by analysis of variance and the Duncan new multiple range tests (28).

RESULTS AND DISCUSSION

The results of exposing rocks inoculated with bacteria to various conditions of chlorination are presented in Table 1. Approximately 50% of the cells in the biofilm survived exposure to 0.5 mg of chlorine per liter, whereas only 25% of the cells were nutrient responsive when they were exposed to 25 mg of chlorine per liter. Multiple exposures to chlorine did not select for more resistant cells ($P > 0.01$). Also, exposure to 25 mg of chlorine per liter caused significantly more death than exposures to 0.5 or 2.0 mg of chlorine per liter ($P > 0.01$). Much variation was seen between rocks with respect to the number of cells that were observed. Of 56 rocks, 8 had less than 45 cells attached to them in 50 microscopic fields (the area of a microscopic field was 0.016 mm^2). Also the percentage of responsive cells varied from 0 to 79 on different rocks.

The results of studies to determine whether surface texture was important in the attachment of bacteria and to see whether exposure to chlorine caused detachment are given in Table 2. Generally, there was an average of one cell per field on the smooth side of the marble, while there were four cells per field on the sandblasted side. The one exception was that there was 0.4 cell per field on the smooth side of the marble exposed to 0.25 mg of chlorine per liter. These results indicate that more cells attached to the rough side of the marble than to the smooth side and that exposure to various levels of chlorine did not cause detachment to occur in at least five of six cases. The reason for the reduced total number of cells on the smooth side exposed to 0.25 mg of chlorine per liter is unclear.

The results of the effect of surface texture studies with pure cultures of bacteria are given in Table 3. Generally, it can be seen for all three bacteria that the amount of scatter of the data was less on the sandblasted surface than on the smooth surface. In addition, when a two-way analysis of variance was run on chlorine concentration versus rough or smooth surface for each organism, the percentage of responsive cells for two of the three organisms that were not exposed to chlorine was significantly higher on the rough side compared with that on the smooth side of the marbles ($P > 0.01$). The mean of the percentage of responsive cells ranged from 34 to 47 on the smooth side, while it was 73 to 88 on the sandblasted side of the marbles. There was no significant difference in the percentage of responsive cells on the rough or smooth side of the unchlorinated marbles for

TABLE 2. Effect of surface type on attachment of bacteria

Surface	Parameter values at the following chlorine concn (mg/liter)[a]:							
	0		0.25		2.5		25	
	No. of fields counted	Total no. of cells	No. of fields counted	Total no. of cells	No. of fields counted	Total no. of cells	No. of fields counted	Total no. of cells
Smooth	46 ± 9	49 ± 38	49 ± 4	20 ± 35	48 ± 7	38 ± 39	45 ± 14	51 ± 40
Sandblasted	27 ± 18	103 ± 44	24 ± 11	101 ± 20	21 ± 18	96 ± 29	19 ± 12	111 ± 15

[a] Values are means ± standard deviations. All mean values are based on results for 12 marbles.

TABLE 3. Nutrient-responsive cells of different bacteria attached to marbles

Surface and bacteria	% (mean ± SD) responsive cells at the following chlorine concn (mg/liter)			
	0	0.25	2.5	25
Smooth				
Enterobacter cloacae	45 ± 29	9 ± 13	5 ± 7	4 ± 4
CDC group VE-1	47 ± 36	23 ± 23	20 ± 26	3 ± 5
Fluorescent pseudomonad	34 ± 26	14 ± 4	7 ± 7	9 ± 5
Sandblasted				
Enterobacter cloacae	76 ± 13	8 ± 9	3 ± 5	4 ± 6
CDC group VE-1	73 ± 15	5 ± 5	1 ± 1	1 ± 1
Fluorescent pseudomonad	88 ± 4	2 ± 1	7 ± 3	7 ± 3

[a] Values are based on results for 16 marbles for *Enterobacter cloacae* and 4 marbles for the other two types of bacteria.

the isolate identified as a member of CDC group VE-1. Also for this organism, the percentage of responsive cells on the smooth side exposed to 0.25 mg of chlorine per liter was significantly higher than that on either side exposed to 25 mg of chlorine per liter and the sandblasted side exposed to 2.5 mg of chlorine per liter ($P > 0.01$). For the other two bacteria, there were no significant differences between the results for any of the conditions of chlorination on either side of the marble. Thus, it appears that attachment and protection from chlorination may be affected by the specific organism involved.

There were no significant differences in nutrient-responsive cells on either marbles or rocks from model filters which were not chlorinated (Fig. 1). The mean value for the

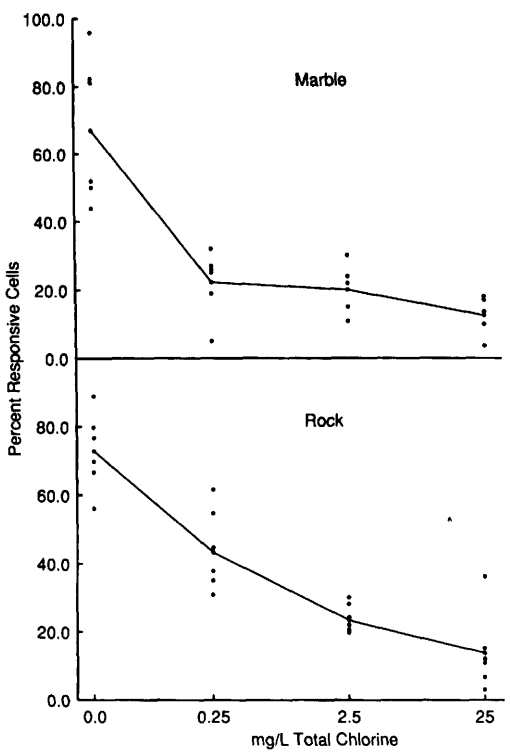

FIG. 1. Nutrient-responsive organisms on marbles and rocks in model filters. The line is drawn through the mean, with each observation presented as a point around the mean.

responsive cells on the unchlorinated marbles was 68%, while it was 73% on the unchlorinated rocks. The number of responsive cells on the rocks exposed to 0.25 mg of chlorine per liter was significantly higher than the sum of all the cells on the chlorinated marbles or the number of cells for the other two conditions of chlorination of rocks ($P > 0.01$). This could possibly reflect an inadequate application of chlorine to the rock. Alternatively, cells located in pores may have been protected.

The results suggest that the direct viable count method has a potential for assessing the effectiveness of chlorination of biofilms. This procedure can give results in as little as 6 h. Additional work is needed to refine and confirm the validity of the procedure. It needs to be determined whether yeast extract is the best substrate to use, what concentration of substrate is best, what the best incubation time and temperature are, and how the responses of different organisms are different. Also, the reasons for the large variation in the numbers of responsive cells between rocks and marbles seen in this study need to be elucidated so that the accuracy of this method can be determined.

The finding of *Enterobacter cloacae* extends the occurrence of this organism to the biofilm in an active rapid sand filter and to another state in the country. Sloughing of this organism into the distribution system could lead to coliform problems in the distribution system. Sloughing of other bacteria could lead to high plate counts of heterotrophic bacteria in the distribution system. The type of support media and all components of the rapid sand filters could be important in the establishment of a biofilm. The acid solubility of filter material could be important in the dissolving of filter material in acidic waters (1). It could also be an indication of how easily the filter material could be pitted, which could aid in biofilm production and protection from chlorination through a backwash cycle. The acid solubility of rocks depends on the origin of the rocks; therefore, the type of rock used in the filter may be important in the establishment of the biofilm. The high variability in the number of responsive cells on different rocks seen in this study may have been a reflection of this. Furthermore, the fact that some rocks had very low numbers of responsive cells and other rocks had high numbers of responsive cells could indicate that certain conditions exist which, if known, could be used to enhance or inhibit biofilm growth.

One surprising observation was that there was very little difference in the percentage of responsive cells on smooth or sandblasted sides of marbles under a given condition of chlorination. The reason for this is unclear. It is clear, however, that many more cells attached to the sandblasted side, probably because of its increased surface area. Thus, the total number of surviving cells was greater on the sandblasted side, and it would be expected that the same would apply to the surface of rocks. It could be postulated that smooth spherical support material would minimize the number of attached cells in a filter.

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