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ABSTRACT

The direct viable count was successfully applied to ozonated raw water and ozonated anthracite taken from an active dual media rapid sand filter. Increased incubation time resulted in greater numbers of nutrient responsive cells. The direct viable count was greater than the heterotrophic bacterial plate count. Certain cells of the natural flora could be confused with nutrient responsive cells in some instances.

INTRODUCTION

Ozonation in drinking water treatment is now receiving attention in the United States (12). Much work has been done concerning the use of ozone to kill indicator bacteria, particularly Escherichia coli (1,3,4,6,7,8,9,10,14,22). Also, there have been studies which have been concerned with the resistance to ozone of nonpathogenic bacteria commonly found in water (1,3,4,10). In these studies, death has been assessed by standard bacteriological plating procedures. Recently, it has been determined that enteric bacteria released to the environment go through a viable nonculturable phase before the cells are truly dead (5,15,18,19,20). The viable nonculturable phase is defined as a phase in which the bacterial cell will metabolize and increase in length but will not divide so that the cell can't form a colony on any laboratory medium (5,20). The viable nonculturable cell may recover full function once it has passed through the intestinal tract of an animal (20). Certain pathogenic enteric bacteria have been shown to go through this phase (5,18,19). Since ozone residuals are maintained for only short periods of time, the concept of viable nonculturable bacteria might be important when standard bacterial plating procedures are used to determine the efficiency of ozonation in killing bacteria in drinking water.

It has been proposed that the addition of yeast extract and nalidixic acid to water samples followed by 6 hours incubation, then fixing in formalin, staining with acridine orange and

examination by epifluorescence microscopy could eliminate the problem of differentiating live from dead cells in the acridine orange direct count (11,16). This procedure is now called the direct viable count (23). The theory is that in many bacteria DNA replication is inhibited thus the cell can elongate but not divide. It has been suggested that only cells which are at least three times normal size be enumerated (23). This procedure has been applied to following the viability of Escherichia coli and Vibrio cholerae in microcosms (5). The determination of viable but nonculturable cells of Salmonella enteritidis (19), and Campylobacter jejuni (18) and the enumeration of arsenic resistant bacteria in groundwater (23). We have applied this methodology to the effect of chlorine upon biofilms on rapid sand filter support material (13). This procedure will determine both viable culturable and viable nonculturable bacterial cells.

We report here the use of the direct viable count on the determination of viable cells in ozonated raw water and in biofilms on anthracite coal from a dual media rapid sand filter exposed to ozone.

MATERIALS AND METHODS

Anthracite was removed from an active dual media rapid sand filter at the Laramie surface water treatment plant, rinsed in sterile water to remove flocculated material and aliquots were ozonated for 1, 5 and 10 minutes. The ozonated coal for each time was further split into aliquots which were covered with 0.02% yeast extract and 0.025% nalidixic acid. These aliquots were incubated at 25 C for time intervals of 6, 21, and 28 hours. After the appropriate incubation the coal was fixed in 3.7% final concentration of formalin, stained for 3 minutes with acridine orange, embedded in play-doh (Kenner Parker Toys, Cincinnati, Ohio) and observed by epifluorescent microscopy. Cells which were at least three times normal length were counted as being nutrient responsive or viable. At least 100 cells on each of 3 different pieces of coal were counted. The experiment was done twice.

Another pair of experiments were done in which the ozonation of the coal was carried out for 5 minutes and the direct viable count was incubated for intervals of 6,12,21,24 and 30 hours. At least 100 cells were counted on each of 5 pieces of coal per experiment. Two types of cells were differentiated. The cell type called normal means that there was no question that the cell had elongated. The cell type called abnormal refers to a cell that most likely was a typical cell of the genus Bacillus which had not elongated but which was three times the size of a number of the cells usually seen in the biofilm.

The condition of ozonation of the coal was to place approximately a 2 mm layer of coal in the bottom of a 500 ml glass beaker and to cover this with 300 mls of ozone demand free water. The beaker

was then placed upon a magnetic stirrer which was turned on and stirred such that the coal was suspended to facilitate exposure to ozone. Ozone was then applied through a fritted glass diffuser stone. Ozone was generated by a ozonator (model 035P19-0; Ozone Research And Equipment Corporation, Phoenix, Arizona). Ozonation was done at a constant gas flow rate and ampere setting with residual ozone being determined by the DPD method of the Hach Co. (Procedures for water and wastewater analysis, P 2-85, Hach Co., Loveland, Colorado). Exposure to ozone was terminated by pouring the ozonated water off the coal and adding back ozone free water. Samples were processed to yeast extract and nalidixic acid within 20 minutes after ozonation.

Samples of raw water were ozonated by bubbling ozone through the sample until the ozone residual was 1.2-1.3 mg/L which took 1-3 minutes then turning off the ozonator and allowing the ozone residual to dissipate. One tenth to 0.3 mg/L residual ozone remained at the end of the experiment. Samples of water were taken at 0,1,5 and 10 minutes of exposure to ozone. The ozone residual was eliminated by the addition of excess sodium thiosulfate. These samples were processed by the acridine orange direct count (Nuclepore Corporation, Pleasanton, California). The direct viable count with incubation at 20 C for 10 and 20 hours, and the determination of viable cells by plating on R2A agar (17). Incubation of the R2A agar plates was at 20 C for 7 days. The spread plate procedure was used at 0 and 1 minute exposure to ozone and membrane filtration (2) on Millipore HC filters was used for 5 and 10 minutes to exposure to ozone. The experiment was done twice.

Data were analyzed by analysis of variance and Duncan's new multiple range test (21).

RESULTS AND DISCUSSION

Presented in Table I are the results of direct viable counts (DVC) incubated for various times of coal from a rapid sand filter which had been ozonated for various times. Three to 6 percent of the cells were nutrient responsive when the direct viable count was incubated for 6 hours. In contrast, when the DVC was incubated for 21 or 28 hours, the percent responsive cells increased to 28 to 61 percent. These numbers were significantly higher than those for the 6 hour incubation ($P=0.05$). There was large variation in the percent responsive cells between pieces of coal. For example, on coal ozonated for 5 minutes with the DVC incubated for 21 hours one piece of coal had 75 percent responsive cells while another piece only had 10 percent of the cells responsive. The reason for this is unclear, however this has previously been shown to occur on rapid sand filter support gravel (13). An observation during these experiments was that the medium covering the coal became turbid with time. This indicated that growth of bacteria was occurring. A gram stain of the broth revealed a large gram positive rod shaped bacterium. Since this organism produced

spores and grew under aerobic conditions it most likely belongs in the genus Bacillus. This cell could easily be confused with responsive cells of some small bacteria commonly seen on the coal.

TABLE I DIRECT VIABLE COUNT OF OZONATED ANTHRACITE FROM A RAPID SAND FILTER

Time		Percent Responsive Cells			
Exposure to ozone (minutes)	Incubation of the direct viable count (hours)	high value	low value	mean	standard deviation
1	6	7	1	2.8[a]	2.3
	21	82	32	61.0	23.3
	28	56	2	28.7	27.1
5	6	12	0	6.2	4.5
	21	62	22	45.0	18.0
	28	89	29	55.3	22.5
10	6	9	0	3.3	3.1
	21	75	10	36.2	26.1
	28	76	32	52.5	18.8

a - means based upon 6 observations

Given in Table II are the results of experiments designed to relate incubation time of the DVC to the appearance of the gram positive bacterium. Any elongated cells not the size of the Bacillus were counted as normal responsive cells while those cells which were probably normal Bacillus cells were called normal Bacillus. Data were analyzed by adding both counts together to see if the inclusion of the Bacillus cells as responsive cells would change the interpretation of the results. The Bacillus type cells were not seen after 6 hours incubation and only occasionally seen after 12 hours incubation. These cells were quite evident after 21 hours incubation, however there was no significant difference in the numbers seen after 21,24 or 30 hours of incubation (P=0.05). In contrast, when the normal responsive cells were analyzed, it was found that incubation of the DVC for 30 hours gave significantly higher counts than any of the other times of incubation (P=0.05). Also the DVC after 21 and 24 hours of incubation were significantly higher than those at 6 and 12 hours incubation (P=0.05). The same conclusions were reached when both counts were added together (P=0.05), however inclusion of the Bacillus type cells would unduly inflate the number of cells considered to be responsive because it

is almost certain that the vast majority of these cells are due to reproduction of one or a few Bacillus cells surviving ozonation.

TABLE II EFFECT OF INCUBATION TIME UPON THE DIRECT VIABLE COUNT OF BIOFILM ON COAL OZONATED FOR FIVE MINUTES

Time of Incubation (hours)	Parameter	Type of cell: Normal Responsive	Normal Bacillus-like	Both
6	mean[a]	1.7[b]	0.0	1.7
	standard deviation	2.1	0.0	2.1
12	mean	3.7	0.1	3.8
	standard deviation	1.8	0.3	1.7
21	mean	17.5	18.3	35.7
	standard deviation	8.9	14.0	16.9
24	mean	21.3	16.7	38.0
	standard deviation	9.0	5.3	6.5
30	mean	41.2	14.6	55.8
	standard deviation	19.9	8.8	17.6

a - based upon 10 observations
b - values presented are percent responsive cells

Shown in Table III are the results of comparing methods for enumerating bacteria in ozonated raw water. The highest number of cells was seen when the acridine orange direct count (AODC) was used (P=0.05). Also, the DVC incubated for 10 hours was significantly higher than the heterotrophic plate counts on R2A agar (P=0.05). The decrease in numbers in the DVC incubated for 20 hours is unexplained.

TABLE III ENUMERATION OF BACTERIA IN OZONATED RAW WATER

Minutes of exposure to ozone	Parameter	Acridine orange direct count	Ten hour direct viable count	Twenty hour direct viable count	Heterotrophic Plate Count on R2A agar
0	mean	7.43[a]	6.35	6.57	5.41
	standard deviation	0.18	0.13	0.25	0.28
1	mean	7.66	6.18	5.74	4.83
	standard deviation	0.48	0.14	0.21	0.57
5	mean	7.40	5.13	1.39	0.93
	standard deviation	0.25	0.83	1.97	0.32
10	mean	6.86	4.06	2.98	0.54
	standard deviation	0.81	0.45	4.21	0.08

a - values presented are based on two experiments and are numbers per 100 ml.

Presented in Table IV are the results of comparing the AODC with the DVC incubated for 10 hours and 20 hours when the values were converted to percent responsive cells. There were some cells in the raw water which were probably filamentous blue green bacteria. These cells could easily be confused with nutrient responsive cells. Therefore the AODC was done counting these cells as responsive and amounted to 2-3 percent of the cells counted. The DVC incubated for 10 hours gave significantly higher results than the other two methods (P=0.05).

TABLE IV PERCENT RESPONSIVE CELLS IN OZONATED RAW WATER

Minutes of exposure to ozone	Parameter	Acridine orange direct count	Ten hour direct viable count	Twenty hour direct viable count
0	mean	2.0	11.5	20.0
	standard deviation	2.8	4.9	15.6
1	mean	2.5	20.5	8.5
	standard deviation	0.7	7.8	2.1
5	mean	2.5	15.0	1.0
	standard deviation	2.1	18.4	1.4
10	mean	2.5	67.5	14.0
	standard deviation	3.5	0.7	19.8

It was observed that some of the coal disintegrated during ozonation. Other than a consumption of filter material the significance of this observation to the methodology used is unknown.

The Bacillus sp. which grew out of the biofilm on the coal was not seen in experiments using raw water, however a different type of cell was found which could be confused with responsive cells. This suggests that the natural flora of the water or part of the water system under study will be important in the use of DVC.

The data presented show that the DVC gave positive results when applied to ozonated biofilm or raw water. Development of this methodology for application to the assessment of the efficiency of ozonation applied to drinking water may be important in finding cells which are viable but can't be cultured, particularly if no other chemical disinfectant is used. Much work is needed to further develop this methodology. Aspects of the methodology which need to be studied include temperature of incubation, determination of the best nutrient(s) and the optimum concentration to use, application of fluorescent antibody methodology to identify bacteria and the best time of incubation just to name a few.

CONCLUSION

Increased incubation time of the direct viable count led to increased numbers of nutrient responsive cells for bacteria attached to anthracite which has been ozonated. This was not seen for unattached cells which were free in raw water which was ozonated.

The direct viable count shows potential for being used to enumerate cells that have been ozonated and are still viable, however much work needs to be done to refine the procedure and to develop a standard methodology. Difficulties were encountered in differentiating responsive and nonresponsive cells.

ACKNOWLEDGMENT

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