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Use of Direct Viable Count Methodology With Ozonation in Drinking Water

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Abstract

Viable cells were detected by direct viable count methodology on ozonated anthracite taken from an active dual media rapid sand filter. Also, at least a thousand fold more cells were determined to be viable by direct viable count methodology than by the heterotrophic plate count in raw water which had been exposed to ozone. This suggests that bacterial cells were not killed as quickly by ozone as previously thought, and that the direct viable count may have a place in assessing the effectiveness of killing bacteria by ozone. Problems were encountered in determining nutrient responsive cells which could result in errors in the direct viable count.

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 (1,3,4,6-10,14,22). 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 (5,15,18-20). 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 cannot 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 six 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 experiment was further split into aliquots for the direct viable count which was accomplished by covering the coal 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 epifluorescence microscopy. Cells which were at least three times normal length were counted as being nutrient responsive or viable. Three different pieces of coal were counted. All cells in a field were counted. The fields were chosen at random and enough fields were counted to have counted at least 100 cells on each piece of coal. The experiment was conducted twice.

Another pair of experiments was conducted in which the ozonation of the coal was carried out for five minutes and the direct viable count determined at intervals of 6, 12, 21, 24 and 30 hours. At least 100 cells were counted on each of five 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 Bacillus-like 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.

Ozonation of the coal was carried out by placing approximately a 2-cm layer of coal in the bottom of a 500-mL glass beaker and covering this with 300 mL of sterile ozone demand-free water. The beaker then was placed upon a magnetic stirrer which was turned on and stirred such that the coal was suspended to facilitate exposure to ozone. Ozone then was applied through a fritted glass diffuser stone. Ozone was generated by an ozone generator (model 035P190; Ozone Research And Equipment Corporation, Phoenix, Arizona). Ozonation was conducted 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, 1984, p. 2-85, Hach Co., Loveland, Colorado).

Exposure to ozone was terminated by pouring the ozonated water off the coal and adding back sterile ozone-free water. Water used in these experiments was glass distilled water which had been passed through a Water I Instrument (Gelman Sciences, Ann Arbor, Michigan) and had been autoclaved at 121°C for 20 minutes. 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 ozone generator 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 addition of excess sodium thiosulfate. These samples were processed by the acridine orange direct count method (Nuclepore Corporation, Pleasanton, California), by direct viable count with incubation at 20°C for 10 and 20 hours, and determination of viable cells by plating on R2A agar (17).

The heterotrophic plate count on R2A agar (17) was determined by processing 0.01 mL, 0.1 mL, 1 mL, 10 mL and 100 mL at each ozone treatment time. The 0.01 mL and 0.1 mL samples were processed by the spread plate procedure, whereas the other amounts were processed by membrane filtration (2) using Millipore HC filters. Incubation was carried out at 20°C for 7 days. The direct viable count was performed by adding 0.02% yeast extract and 0.025% nalidixic acid final concentrations to the water samples before incubation. The yeast extract had been autoclaved, while the nalidixic acid had been filter-sterilized. The experiment was repeated.

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

Results And Discussion

Presented in Table I are the results of direct viable counts (DVC) from coal incubated for various times after ozonation. Three to six percent of the cells

were nutrient-responsive when the direct viable count was incubated for six hours. In contrast, when the DVC was incubated for 21 or 28 hours, the percent responsive cells increased to 28 - 61 percent. This was an increase in responsive cells of at least four times with increased length of incubation. There was large variation in the percent responsive cells between pieces of coal. For example, on coal ozonated for five 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 previously has been shown to occur on rapid sand filter support gravel (13).

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

Time		Percent Responsive Cells			
Exposure to Ozone, min	Incubation of Direct Viable Count, hrs	High Value	Low Value	Mean	95% Confidence Limit, 1 df
1	6	7	1	2.8[a]	± 11.9
	21	82	32	61.0	± 120.8
	28	56	2	28.7	± 140.5
5	6	12	0	6.2	± 23.4
	21	62	22	45.0	± 93.4
	28	89	29	55.3	± 116.6
10	6	9	0	3.3	± 16.0
	21	75	10	36.2	± 135.3
	28	76	32	52.5	± 97.5
a = means based upon six observations					

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

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 probably were normal Bacillus cells were called normal Bacillus. The Bacillus type cells were not seen after six hours incubation,

and only occasionally seen after 12 hours incubation. However, these cells were quite evident after 21 hours incubation. This indicated that although increased time of incubation would lead to a larger DVC and possibly a better estimate of viable cells, it also could lead to the error of incorporating dividing cells into the DVC. It is almost certain that the vast majority of the Bacillus type cells were due to reproduction of one or a few Bacillus cells surviving ozonation. The percentage of Bacillus type cells was relatively constant at 14-18 percent between 21 and 30 hours of incubation of the DVC, whereas the normal sized cells that were nutrient-responsive increased from 17 percent to 41 percent of the total cells during this time.

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
	95% confidence limit, 1 df	± 8.4	± 0.0	± 8.9
12	mean	3.7	0.1	3.8
	95% confidence limit, 1 df	± 7.1	± 1.3	± 6.4
21	mean	17.5	18.3	35.7
	95% confidence limit, 1 df	± 35.6	± 55.9	± 67.3
24	mean	21.3	16.7	38.0
	95% confidence limit, 1 df	± 35.6	± 21.6	± 67.3
30	mean	41.2	14.6	55.8
	95% confidence limit, 1 df	± 78.8	± 35.6	± 69.9

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. Also, the DVC incubated for 10 hours was larger than the heterotrophic plate counts (HPC) on R2A agar. In this case there were a thousand times more cells classified as being viable by the DVC method after five minutes exposure to ozone than by the HPC method. 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	10-Hour Direct Viable Count	20-Hour Direct Viable Count	Heterotrophic Plate Count on R2A Agar
0	mean	7.43[a]	6.35	6.57	5.41
	95% confidence limit, 1 df	± 0.14	± 0.10	± 0.20	± 0.22
1	mean	7.66	6.18	5.74	4.83
	95% confidence limit, 1 df	± 0.37	± 0.11	± 0.17	± 0.44
5	mean	7.40	5.13	1.39	0.93
	95% confidence limit, 1 df	± 0.20	± 0.65	± 1.54	± 0.25
10	mean	6.86	4.06	2.98	0.54
	95% confidence limit, 1 df	± 0.63	± 0.35	± 3.29	± 0.07

a - Values presented are based on two experiments and are $\log_{10}$ numbers of bacteria 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. This was done to quantify an inherent error in the DVC. There were some cells in the raw water which probably were filamentous blue-green bacteria. These cells could easily be confused with nutrient responsive cells. Therefore the AODC was done counting these cells as responsive. These results

showed that 2-3 percent of the cells in the DVC were erroneously designated as responsive.

TABLE IV. PERCENT RESPONSIVE CELLS IN OZONATED RAW WATER

Minutes of Exposure to Ozone	Parameter	Acridine Orange Direct Count	10 Hour Direct Viable Count	20 Hour Direct Viable Count
0	mean	2.0	11.5	20.0
	95% confidence limit, 1 df	± 25.3	± 44.2	± 140.5
1	mean	2.5	20.5	8.5
	95% confidence limit, 1 df	± 6.4	± 70.3	± 18.9
5	mean	2.5	15.0	1.0
	95% confidence limit, 1 df	± 18.9	± 165.8	± 12.6
10	mean	2.5	67.5	14.0
	95% confidence limit, 1 df	± 31.5	± 6.4	± 178.4

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 cannot be cultured, particularly if no other chemical disinfectant is used. Much work is needed to develop this methodology further. It is of importance to elucidate the reason for the variability in data that was seen in this study. Also, the accuracy, precision, and

universality of the method in this application needs to be determined. Other 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.

Conclusions

Viable cells were detected using the direct viable count method after ozonation. In the case of ozonated raw water, the direct viable count found a thousand fold more cells alive than did the heterotrophic plate count. Difficulties were encountered in differentiating nutrient responsive cells from some nonresponsive cells. The direct viable count methodology deserves more study to fully understand its importance and accuracy in detecting bacterial cells which have survived ozonation.

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Key Words

Ozone, Viable Cell Enumeration, Direct Viable Count Methodology, Drinking Water, Nutrient-Responsive Cells, Ozonated Anthracite Filter Medium, Ozonated Sand Filter Medium

Résumé

Les auteurs ont procédé à la détermination du nombre de cellules vivantes à l'aide de la méthode de comptage direct sur de l'anthracite ozoné issu d'un filtre de sable rapide à double couches activé. Par rapport à la numération hétérotrophique, cette méthode permit de dénombrer 1'000 fois plus de cellules vivantes dans l'eau brute ayant été traitée à l'ozone. Il semble ainsi que l'élimination des germes par l'ozone s'effectue d'une manière moins rapide qu'initialement prévu. De ce fait, le comptage direct peut pleinement se justifier pour déterminer l'efficience de l'ozone sur l'abattement des bactéries. Certains problèmes se sont posés lors de l'application de la méthode de comptage direct par la détermination des cellules sensibles aux nutrients susceptibles de conduire à des erreurs.

Zusammenfassung

Zellenkolonien wurden mittels der "Direktlebend-Zählung" an ozontem Anthrazit bestimmt, das von einem Zweistoffschnellsandfilter stammte. Es wurde festgestellt, dass mit dieser Methode etwa 1000-mal mehr Zahlen bestimmt werden können als mit der Plattenzählmethode (ozontes Rohwasser). Das kann darauf zurückzuführen sein, dass Keime durch Ozon nicht so schnell abgetötet werden wie bis anhin vermutet. Diese "Direktzählmethod" kann also durchaus ihren Platz bei der Beurteilung der Wirksamkeit von Ozon einnehmen. Probleme ergaben sich bei der Bestimmung von auf Nährstoffe ansprechende Zellen, die in Fehlzählungen resultieren konnte!