

Sensitivity of Three Selected Bacterial Species to Ozone

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The minimal lethal concentration of ozone in water was determined for three bacterial species: *Escherichia coli*, *Bacillus cereus*, and *Bacillus megaterium*. A contact period of 5 min was selected. The lethal threshold concentration for the cells of *B. cereus* was 0.12 mg/liter while that for *E. coli* and *B. megaterium* was 0.19 mg/liter. Low concentrations of ozone were ineffective when organic matter was present to interfere with the action of ozone on the bacterial cells. Also determined during the study was the sensitivity of spores of *B. cereus* and *B. megaterium* to ozone in water. The threshold concentration required to kill the spores of both species was 2.29 mg/liter. The cells and spores of these organisms exhibited the "all-or-none" die-away phenomenon normally associated with ozone treatment.

The treatment of domestic water to provide a microbiologically safe, aesthetic, potable end product has been normal practice since before the turn of the twentieth century. Realizing that low-level chlorination might not always be fully satisfactory, investigators have considered alternate methods of disinfection. Chlorine in low dosages is not always an effective bactericide, though it may be bacteriostatic, and regrowth often occurs. Also, low levels of chlorine usually are ineffective against protozoan cysts, worm eggs, and viruses. In addition, chlorine, even in low concentration, can enhance the capacity of certain organic compounds to cause tastes and odors in finished water supplies. Ozone has been suggested as the best available alternative to present chlorination practice (10).

Although considerable information is available concerning the resistance of vegetative cells of some bacterial species to ozone (2, 4, 7, 8, 13, 15), especially *Escherichia coli* (3-7, 14), relatively little is known about the resistance of bacterial spores (7, 12). The objective of the research was to establish, under laboratory conditions, the minimal dosages of ozone required to kill the vegetative cells of *E. coli*, *Bacillus cereus*, and *Bacillus megaterium*, and the spores of the two *Bacillus* species. The data were examined to determine whether the response of each species to ozone could be described as an "all-or-none" response (5), a phenomenon in which a threshold dose of a

bactericide must be attained before any cells die and at which the total population is killed.

MATERIALS AND METHODS

Bacterial cultures used throughout the study were *E. coli* ATCC 9677, *B. megaterium*, and *B. cereus*. No ATCC numbers were available for the latter two species, though they were typical representatives obtained from stock cultures maintained by the Microbiology Section, Virginia Polytechnic Institute and State Univ.

Ozone was generated from ultrapure air (dew point 80 F, approximately 23.5 C) by an Airox Ozonator model CZP-3C-2 (Pollution Control Industries, Inc.) and dispensed to the disinfection chamber through a porous diffuser fabricated from polyvinyl chloride plastic. Temperature in the disinfection chamber was maintained at 28 C by immersion of the flask in a water bath.

Cells used in the disinfection studies were grown in tryptic soy broth (Difco) at the appropriate temperatures (37 C for *E. coli*; 30 C for *Bacillus* species), and populations were in early stationary phase at the time the disinfection studies were conducted.

Cell concentrations in water subjected to ozonation were approximately 10⁶/ml in each experiment. The desired numbers of cells were harvested by centrifugation from appropriate samples of the tryptic soy cultures and were resuspended and washed twice with physiological saline. In one series of experiments, cells were not washed to demonstrate the protective effect of organic matter to cells subjected to ozone. Population densities were determined by plate count procedures with plate count agar (Difco). Triplicate plates were prepared for each of the serial dilutions. After contact for 5 min with a specific ozone dose (the time

being based on data presented by Meddows-Taylor [9]), the plate count procedures were repeated. Colonies were counted after 24 h of incubation at the temperature appropriate for each species. Plates with no growth after 24 h always were without colonies after 48 h, which suggests that ozone was bacteriocidal rather than bacteriostatic.

Stock suspensions of vegetative *Bacillus* cells to be subjected to ozone treatment were tested for the presence of spores by plating samples heated at 80 C for 10 min. Fewer than five colonies were observed on plates streaked with 1 ml of a 1:10 dilution and, on most plates, none were observed. Spores to be subjected to ozonation were harvested from a sporulation medium that had been agitated vigorously while incubating at least 12 h in a water bath at 30 C. The spores and remaining vegetative cells were harvested from the medium and, after washing, were resuspended in sterile, deionized water and heat-treated at 80 C for 10 min. The spores were washed three additional times to remove cellular debris, collected by centrifugation, and, finally, resuspended in 500 ml of sterile, deionized water. This suspension, containing approximately 10^7 spores per ml, was treated with ozone, and surviving spores were calculated from plate counts made before and after treatment.

Procedures for determining ozone basically were those outlined in *Standard Methods* (1). One modification was required in that ozone remaining after a specified dosing period was sparged from solution by nitrogen gas and swept into a vessel containing potassium iodide solution. The free iodine then was titrated with standard reducing agent according to the standard procedure.

RESULTS

Vegetative cells. The unwashed, vegetative cells of *B. cereus* and *E. coli* were not killed during 5 min of contact with ozone at eight concentrations between 0.04 and 0.71 mg/liter. However, when cells of *B. megaterium* and *E. coli* were washed twice in physiological saline before exposure to ozone, the populations were reduced to near zero after 5 min of contact at an ozone concentration of 0.19 mg/liter (Fig. 1). Washed, vegetative cells of *B. cereus* were killed at an ozone concentration of 0.12 mg/liter (Fig. 1). The data show clearly that destruction of vegetative cells occurs at approximately the same ozone concentration for each of the three species and that the destruction is complete once some critical concentration is attained.

Spores. Figure 2 shows the effects of ozone on the spores of *B. cereus* and *B. megaterium*. Much higher concentrations of residual ozone than those required for reduction of vegetative cells were necessary to decrease substantially the size of the spore populations of *B. cereus* and *B. megaterium*. A concentration of residual ozone of 2.29 mg/liter was required in both the

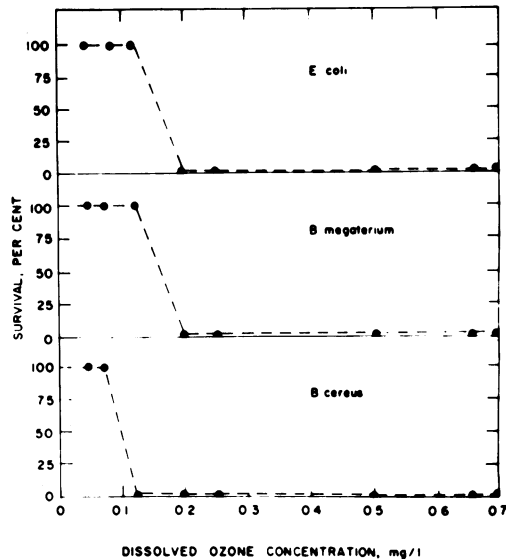


FIG. 1. Effects of ozone on washed, vegetative cells of *E. coli*, *B. megaterium*, and *B. cereus* in deionized water. Points on the figure are averages of three replicate plate counts for a single experiment at each dosage for each species.

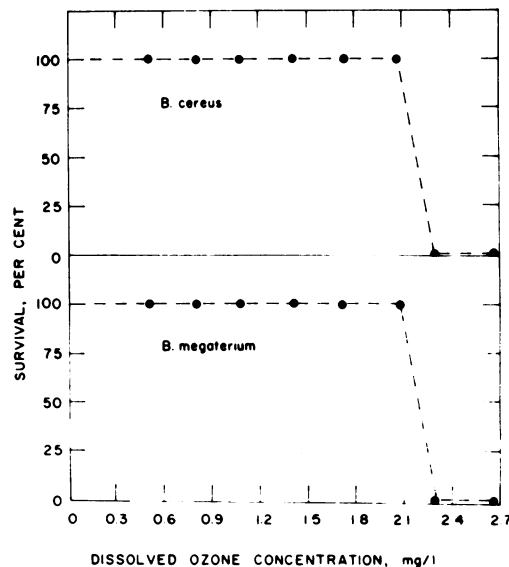


FIG. 2. Effects of ozone on washed spores of *B. cereus* and *B. megaterium* in deionized water. Points on the figure are averages of three replicate plate counts for a single experiment at each dosage for each *Bacillus* species.

spore populations, again using the 5-min contact period, and, as in the case of the experiments with vegetative cells, the destruction was complete.

DISCUSSION

Sensitivity of vegetative cells. The vegetative cells of the bacterial species investigated were extremely sensitive to low concentrations of residual ozone. The threshold of toxicity for *B. cereus* was approximately 0.12 mg/liter, whereas that for *B. megaterium* and *E. coli* was approximately 0.19 mg/liter. These values correlate well with those cited in the literature, especially those cited for *E. coli*. Whitson (15), Bringmann (3), Sykes (13), Guinvarch (6), Bean (2), and Torricelli (14) cited values ranging from 0.1 to 0.2 mg/liter of residual ozone as effective for killing *E. coli*. All organisms exhibited the frequently discussed all-or-none effect. No difference in resistance to ozone could be correlated with differences in cell wall structure of the bacterial species used.

When data on unwashed vegetative cells were compared with those in Fig. 1 it was obvious that enough organic nutrient medium was carried over on the unwashed cells to create an ozone demand in the disinfection chamber. The result was a false, initial estimate of the dosage required to kill the bacteria.

Sensitivity of spores. The spores of *B. cereus* and *B. megaterium* were from 10 to 15 times more resistant to ozone than were their vegetative cells. The greater resistance of spores to oxidation probably is a result of the protection afforded the protoplast by the spore coat. Bringmann (3) and Fetner and Ingols (5) described ozone as a general protoplasmic oxidant. The protoplasm of the vegetative cell is protected only by a cell wall, whereas the protoplasm of a spore is protected by a thick cortex, multilayered spore coat, and an exosporangium.

The spores of *B. cereus* and *B. megaterium* were equally resistant to ozone in these experiments, probably because their chemical and physical compositions are similar, and exhibited the all-or-none effect, the threshold being between 2.03 and 2.29 mg/liter.

Applications to water treatment technology. Historically, in the United States, chlorine rather than ozone has been the agent of choice for disinfecting public water supplies. However, ozone has been used for years as a disinfectant in France (6, 11). When ozone has been used in the United States, it has been primarily for the removal of iron, manganese, color, or tastes and odors (11). An often cited disadvantage of using ozone as a disinfectant is that it disappears rapidly from solution by decomposition to oxygen (9) and, hence, it is

impossible to maintain a residual in a water distribution system. O'Donovan (11) challenges this assertion and presents a rational argument in defense of ozone.

Our results indicate that ozone, in relatively low concentrations, is an effective bactericide against both vegetative cells and spores of three bacterial species. In practical applications, ozone most probably would be applied at higher dosages (0.5–10 mg/liter) and for longer contact periods (2–10 min) because, as was shown in this research, organic matter present in the water will exert an ozone demand and prevent the full utilization of the applied dose as a disinfectant (4, 6, 14).

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