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Inactivation of Viruses and Bacteria by Ozone, With and Without Sonication

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Selected organisms with public health significance were placed in a reaction chamber for treatment by ozonation, by ozonation and sonication, by sonication, or by sonication during oxygenation. Vesicular stomatitis virus, encephalomyocarditis virus, GDVII virus, *Staphylococcus aureus*, *Pseudomonas fluorescens*, *Salmonella typhimurium*, enteropathogenic *Escherichia coli*, *Vibrio cholerae*, and *Shigella flexneri* were inactivated by treatment with ozone. When microorganisms were suspended in phosphate-buffered saline, they were inactivated rapidly by treatment with ozone. However, microorganisms suspended in secondary effluent from a wastewater treatment plant required longer contact times with ozone for complete inactivation. Simultaneous treatments by ozonation and sonication reduced the contact time for complete inactivation of microorganisms in secondary effluent. Treatment by sonication alone or sonication and oxygenation did not inactivate microorganisms. Therefore, the simultaneous treatment of microorganisms in secondary effluent with ozone and sonication resulted in a synergistic effect.

The oxidizing ability of ozone, an allotropic form of oxygen, has long been known to be effective in the inactivation of pathogenic microorganisms (9). Several investigators (2, 4, 8) have reported that the use of low concentrations of ozone for short periods of time will disinfect water containing various bacteria. However, extrapolation of results derived from the examination of suspensions of bacteria in water, free of all other organic matter, cannot be applied directly to the treatment of water which has a high content of organic material. For example, secondary effluent from the wastewater treatment process would require longer contact time and the use of higher concentrations of ozone for effective inactivation of pathogenic microorganisms. We have utilized a method by which decontamination of secondary effluent can be obtained with short treatment times and low concentrations of ozone by the simultaneous application of ultrasonication and ozone. By using this system, microorganisms in secondary effluent can be effectively inactivated by the synergistic effect resulting from the combination of sonication with ozone treatment.

MATERIALS AND METHODS

Staphylococcus aureus, *Salmonella typhimurium*, enteropathogenic *Escherichia coli* O126:B16, *Shigella flexneri*, and *Pseudomonas fluorescens* were obtained from the departmental collection of bacteria at the Lobund Laboratory (Notre Dame, Ind.). *Vibrio cholerae* (569B Inaba strain) was obtained from R. A. Finkelstein, University of Texas Southwestern Medical School, Dallas, Tex. *S. aureus*, *S. typhimurium*, enteropathogenic *E. coli* O126:B16, and *S. flexneri* were propagated in Trypticase soy broth at 37 C for 18 h, in a shaking water bath. *P. fluorescens* was propagated at 20 C and *V. cholerae* (569B Inaba strain) propagated in Penassay broth (Difco) at 37 C. Cultures were centrifuged for 15 minutes at 1,000 × *g*, and the resulting pellet of organisms was suspended in phosphate-buffered saline (PBS) to an optical density of 0.350 at 550 nm when stock culture of bacteria was diluted 1:10 in PBS. Microorganisms (10 ml) were then added to either PBS or secondary effluent (990 ml) for inactivation studies in the treatment column at 25 C. Determinations of viable bacteria were made on spread and pour plates of Trypticase soy agar. Viable counts of *V. cholerae* were made on thiosulfate-citrate-bile-sucrose agar (7) (Difco). All samples were tested in duplicate, and plotted values represent the mean value derived from three experiments. Vesicular stomatitis virus, encephalomyocarditis virus, and GDVII virus were propagated and assayed in BHK-21 cells. BHK-21 cells were grown in Eagle minimal essential medium with fetal bovine serum (5%), Tryptose phosphate broth (5%), penicillin (100 U/ml) and streptomycin (100 μg/ml). Eagle minimum essential medium with fetal bovine serum (1%) was used for propagation and assay of viruses. Viruses were concentrated by centrifugation at 100,000 × *g* for 3 h and suspended in PBS for inactiva-

tion studies. Assays for infectious virus were performed by the 50% tissue culture infective dose method, and titers were calculated by the method of Reed and Muench (10). All microorganisms were suspended in diluent, PBS, or secondary effluent, to a final volume of 1,000 ml for inactivation studies with static conditions in the contact chamber.

Ozone was produced from oxygen by electrical discharge in a corona-type ozone generator. Ozone was added to bacterial cultures through a porous diffuser placed at the bottom of the plexiglass treatment column at a flow rate of 5 standard cubic ft (152.4 cm^3) per h. The plexiglass treatment column (30 cm high with a diameter of 9 cm) had a total volume of 1,700 ml. Oxygen was added at the same flow rate in control studies. Residual ozone levels were assayed in 1% potassium iodide in neutral phosphate buffer, and determined by spectrophotometric absorbance at 352 nm (12). Biochemical oxygen demand and chemical oxygen demand values were determined by *Standard Methods* (1). The ultrasonic system utilized a 40-KH_z ultrasonic generator with 150 W output energizing a 40-KH_z piezoelectric crystal transducer placed at the bottom of the plexiglass treatment column.

RESULTS AND DISCUSSION

The inactivation patterns were the same for all bacteria (Fig. 1). Bacteria, suspended in PBS, were completely inactivated after 15 s of treatment with ozone alone or simultaneous treatment with ozone and sonication. Inasmuch as wastewater is known to exert an ozone demand due to the organic material present, bacteria were suspended in a secondary effluent from a wastewater treatment plant. The level of organic material present in the secondary effluent was measured by the biochemical oxygen demand and the chemical oxygen demand. The secondary effluent used in these experiments contained 8×10^1 fecal coliforms per ml, a biological oxygen demand of 20 mg/liter and a chemical oxygen demand of 45 mg/liter. Bacteria suspended in the secondary effluent required a longer contact time with ozone for complete inactivation. Combined treatment with ozone and sonication resulted in an enhanced rate of inactivation of all bacterial strains tested (Fig. 1). Sonication at a fixed intensity for 10 min did not inactivate bacteria suspended in PBS or secondary effluent. El'piner (3) has reported a greater cavitation effect and thus a greater inactivation of microorganisms in solutions saturated with a gas. Sonication for 10 min in the presence of oxygen instead of ozone did not inactivate bacteria suspended in PBS or secondary effluent. Thus, the synergistic effect was not the result of nonspecific inactivation by sonication in a suspension saturated by a gas.

"Aftergrowth" has been reported (14) when chlorine or ozone alone was used for disinfection of wastewater (i.e., treated samples that showed apparent complete inactivation after 24 h of incubation would yield bacterial growth if further incubated for 48 h). The aftergrowth phenomenon was not observed after 72 h of incubation of the samples in broth cultures in the experiments with ozone or ozone and sonication. Therefore, the inactivation treatment was bactericidal and not bacteriostatic.

Ozonation has been reported to reduce chemical oxygen demand and biological oxygen demand values (11). Total chemical oxygen demand and total biological oxygen demand values were reduced by treatments of the secondary effluent with either ozone or sonication. Residual ozone concentrations were measured by the method of Saltzman (12) at various times in PBS and secondary effluent (Fig. 2). The difference in residual ozone concentration in PBS and in secondary effluent represents the difference in the ozone demand exerted by the organic material present in the secondary effluent and the PBS. Simultaneous ozonation and sonication of the secondary effluent resulted in higher initial ozone concentrations.

Vesicular stomatitis virus, encephalomyocarditis virus, and GDVII virus were inactivated after treatment with ozone for 15 s when suspended in sterile PBS (Fig. 3). Ozone-treated encephalomyocarditis virus (10 50% lethal doses) was not pathogenic in inoculated mice. Further studies of these and other viruses suspended in secondary effluent are in progress.

Several types of pathogenic bacteria and viruses were inactivated rapidly by ozone when suspended in fluid low in organic material. However, practical consideration in the treatment of wastewater must consider the organic material present. The ozone demand exerted by organic material in secondary effluent emphasizes the need for residual, rather than dose, concentrations when using oxidizing agents such as ozone for inactivation studies. A longer contact time was required for inactivation of bacteria in secondary effluent, due to the ozone demand exerted by the oxidizable organic material. Inasmuch as the sonication treatment did not inactivate the bacteria, the combined effect of ozonation and sonication may be interpreted as a synergistic effect. Ozone has been reported to exert an "all-or-none" bactericidal effect whereby inactivation does not occur until a critical concentration of ozone is reached (2, 4), after which total inactivation occurs. The all-or-

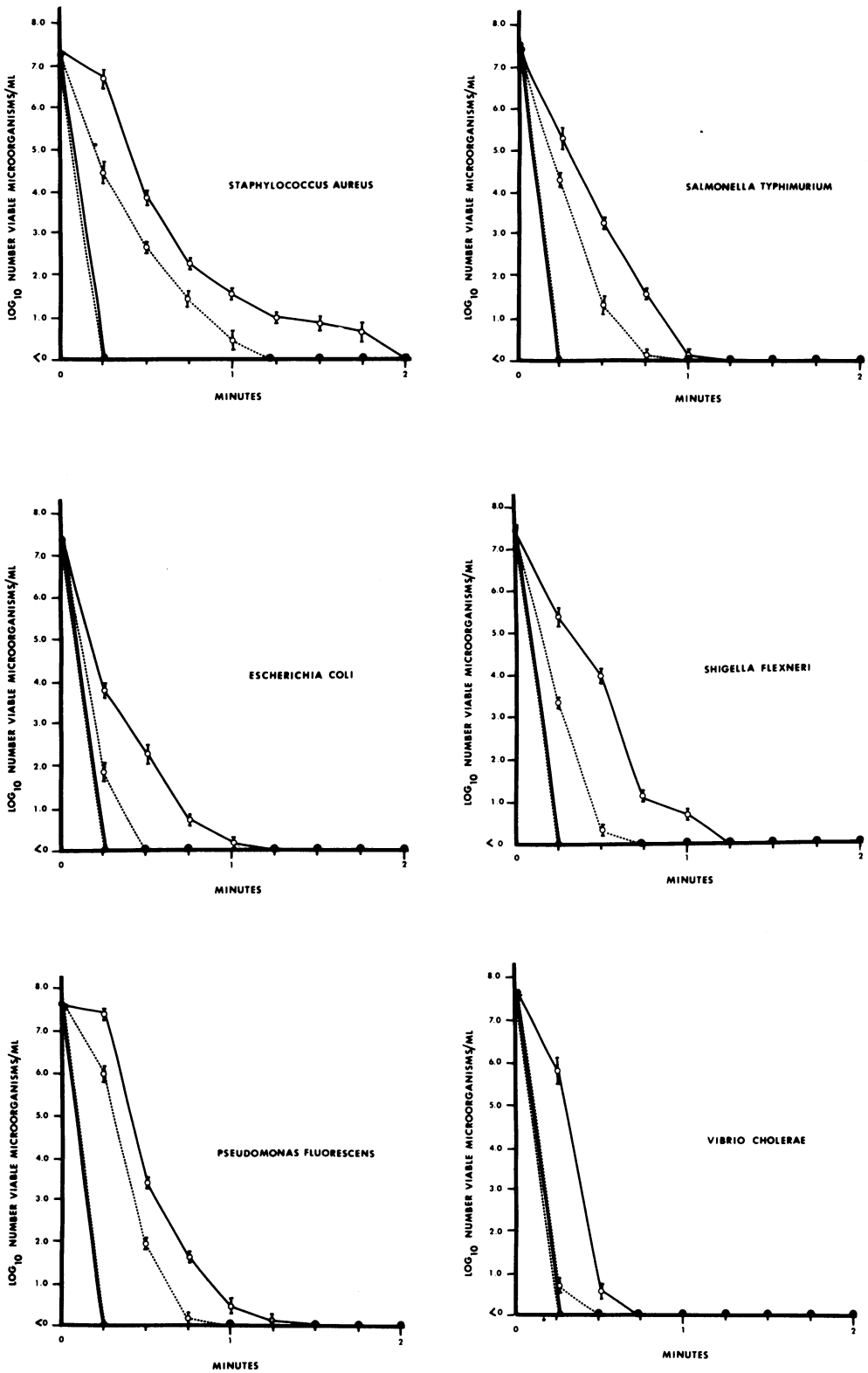


FIG. 1. *Treatment of bacteria by ozonation in PBS* (—●—), *ozonation and sonication in PBS* (----●----), *ozonation in secondary effluent* (—○—), *or ozonation and sonication in secondary effluent* (----○----).

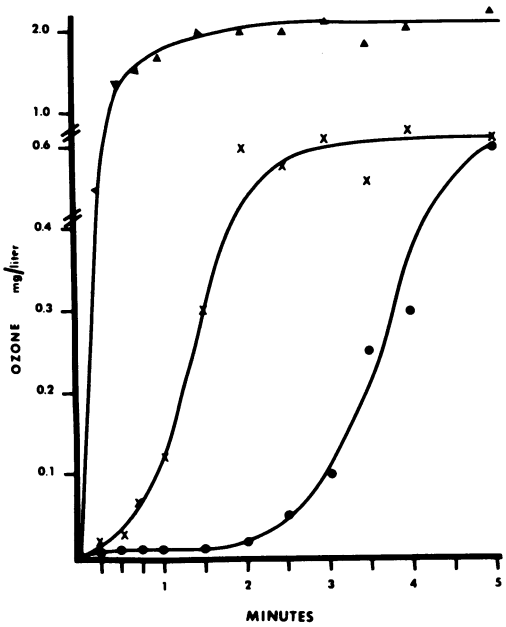
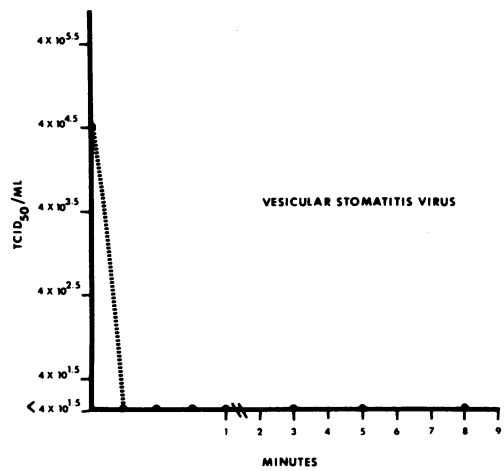
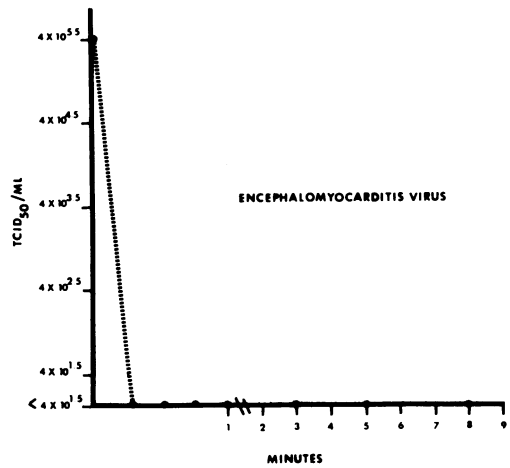


FIG. 2. *Residual ozone concentration after treatment by ozonation in PBS (▲), ozonation in secondary effluent (●), or by sonication and ozonation in secondary effluent (×).*





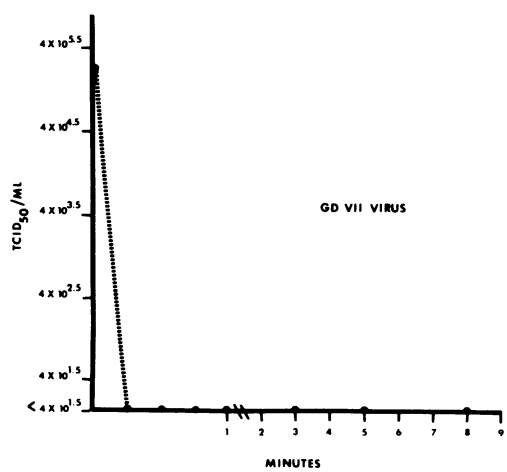


FIG. 3. *Treatment of vesicular stomatitis, encephalomyocarditis, or GDVII virus in PBS by ozonation. $TCID_{50}$, 50% tissue culture infective dose.*

none phenomenon was not observed in these studies.

Sonication reduced or altered oxidizable organic material as measured by biological oxygen demand and chemical oxygen demand determinations, and thus reduced the ozone demand of the secondary effluent. A large amount of ozone is unavailable for disinfection because of the limited solubility of the gas in water (5, 13). Simultaneous treatment of secondary effluent with ozone and sonication resulted in higher initial residual ozone concentrations. This may be due to enhanced interphase transport by sonication. Sonication may also enhance total inactivation of microorganisms by ozone by breaking up particulate organic material and clusters of bacteria so they are exposed to the oxidizing power of ozone. Cavitation, produced by sonication, may also enhance inactivation by reducing the high surface tension caused by organic material. Cavitation also makes and breaks bubbles, resulting in small bubbles and an increased surface area. Meddows-Taylor (7) reported more effective results with ozone when small-diameter bubbles were used. Ozonation of secondary effluent does not produce recognizable compounds toxic to aquatic life (J. W. Arthur, data presented at the Institute on Ozonation in Sewage Treatment, University of Wisconsin, Milwaukee, 1971). Ozone is stable only for a short period of time and breaks down to form free oxygen which is used for sustenance by aquatic life. The application of simultaneous treatment with ozone and sonication is thus an effective bactericidal treatment process in the disinfection of wastewater. (A wastewater treatment plant utilizing simultaneous sonication and ozonation is in operation in Indiantown, Fla.)

ACKNOWLEDGMENT

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