

# Sterilizing Effects of High-Intensity Airborne Sonic and Ultrasonic Waves

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## ABSTRACT

PISANO, MICHAEL A. (St. John's University, Jamaica, N.Y.), RAYMOND M. G. BOUCHER, AND I. EDWARD ALCAMO. Sterilizing effects of high-intensity airborne sonic and ultrasonic waves. *Appl. Microbiol.* 14:732-736. 1966.—The lethal effects of high-intensity airborne sonic (9.9 kc/sec) and ultrasonic waves (30.4 kc/sec) on spores of *Bacillus subtilis* var. *niger* ATCC 9372 were determined. The spores, which were deposited on filter-paper strips, were exposed to sound waves for periods varying from 1 to 8 hr, at a temperature of 40 C and a relative humidity of 40%. Significant reductions in the viable counts of spores exposed to airborne sonic or ultrasonic irradiations were obtained. The antibacterial activity of airborne sound waves varied with the sound intensity level, the period of irradiation, and the distance of the sample from the sound source. At similar intensity levels, the amplitude of motion of the sound waves appeared to be a factor in acoustic sterilization.

The necessity for sterilizing the interiors of interplanetary spacecraft has brought about a renewed interest in cold (below 50 C) sterilization techniques (10). Previous experiments conducted by Boucher (*unpublished data*) in collaboration with the Pasteur Institute in 1953 demonstrated that the combined action of 4-hexyl-resorcinol submicroscopic aerosol with high intensity airborne sound waves (11 kc/sec) greatly enhanced the killing rate of the aforementioned compound against several types of bacteria. These results suggested the possibility of employing a sonochemical sterilization technique which would take advantage of possible synergistic effects when combining large-amplitude acoustic waves, propagated in air, with chemical or gaseous sterilants.

Prior to initiating an investigation of the antimicrobial effects of airborne sound waves applied in conjunction with other agents, however, the obvious need for a study on the response of microorganisms exposed to airborne sound alone was recognized. Accordingly, the work reported here describes the effects of sonic and ultrasonic irradiations, transmitted in air, on the viability of spores of *Bacillus subtilis* var. *niger* ATCC 9372. It is expected that the present study will serve as a basis for future research on sonochemical sterilization methods.

## MATERIALS AND METHODS

*Sound sources.* Early in this investigation, a thorough survey was made of all types of high-intensity airborne sound sources which would deliver a high acoustic energy sound beam [at least 150 db at 12 inches (30.48 cm)] at various frequencies. No existing sound sources were found which would fulfill the requirements of the experiments planned, particularly in the ultrasonic region of the frequency spectrum. At very low frequencies (i.e., up to 1,100 kc/sec), fog horns of the vibrating membrane type would give a partial answer, but each irradiating frequency would require a different exponential horn (cutoff frequency). At high frequencies, the excited quartz described by Kritz (9) would not meet all requirements, since this sound source can only be driven at specific quartz-resonant frequencies. The only promising device was the horn described by Friedman and Nowitzky (6) at the Fourth International Congress on Acoustics in Copenhagen in 1962. These authors described a hollow, exponentially shaped velocity transformer which was driven by a powerful magnetostrictive transducer. The horn was reported as producing a strong emission at 3.4 kc/sec [158 db at 14 inches (34.56 cm)] and at 23.8 kc/sec [152 db at 14 inches (34.56 cm)]. Through design modification of the velocity transformer shape, and by replacing the magnetostrictive transducer by two lead zirconate titanate crystals mounted as in a Langevin sandwich, a new powerful airborne sound source, hereafter referred to as the hollow horn, was developed. This hollow horn can be activated from a few kc/sec up

to 150 kc/sec. To achieve irradiations of high intensity over extended periods of time, the crystals were cooled at 20 C through continuous recycling of a constantly refrigerated dielectric oil (5 gal (18.925 liters)/min). The strong sonic and ultrasonic airborne waves produced by this horn are due mainly to the large amplitude excursions (20 to 200  $\mu$ ) on the walls of the velocity transformer (especially at the level of the lips) which alternatively compress and dilate the pocket of gas inside the hollow resonant cavity.

A second type of horn of a different design (Fig. 1) was used in experiments requiring low frequencies (9.9 kc/sec). This horn, hereafter referred to as the focusing horn, was made of a 9.5-inch (24.13 cm) long aluminum conical velocity transformer which extended into a cup [radius of curvature = 1.5 inches (3.81 cm)] with a 2.25-inch (5.715 cm) external diameter. Through the thinning of the cup walls and the increase in radiating area, it was possible to improve the poor impedance mismatch from solid to gas, thus achieving high airborne sound intensities.

The characteristics and power output of the two horns, when driven by a Broad Band Multisons 500-w generator (Macrosonics Corp., Carteret, N.J.), were determined under conditions identical to those used in actual tests. With the hollow horn (Macrosonics FH 14), using a 100-ohm impedance, the total power output computed from series of 10-point measurements within the 4-inch (10.16-cm) diameter cross section of the irradiating chamber was 1.25 w at 12 inches (30.48 cm), with no resonant conditions. The frequency of emission was 30.4 kc/sec ( $\pm$  0.1 kc/sec). The average intensity at 12 inches (30.48 cm) on the axis within a 0.5-inch (1.27 cm) radius circle was 151 db ( $\pm$  0.5 db). With the focusing horn, using a 400-ohm impedance, the total power output computed from series of 10-point measurements within the 4-inch (10.16-cm) diameter cross section of the irradiating chamber was 2.37 w at 12 inches (30.48 cm), with no resonant conditions. The frequency of emission was 9.9 kc/sec ( $\pm$  0.1 kc/sec), the average intensity at 12 inches (30.48 cm) on the

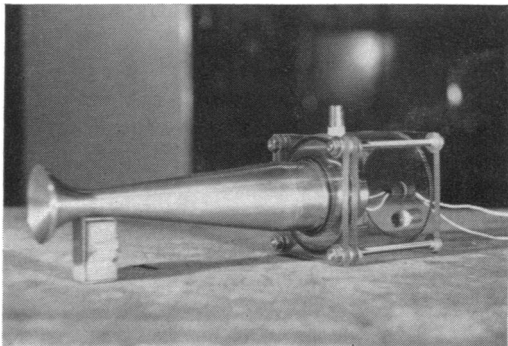


FIG. 1. Macrosonics low-frequency (9.9 kc/sec) focusing horn with attached Plexiglas cooling chamber. Visible on the extreme right are the two electrical leads which are connected to the generator.

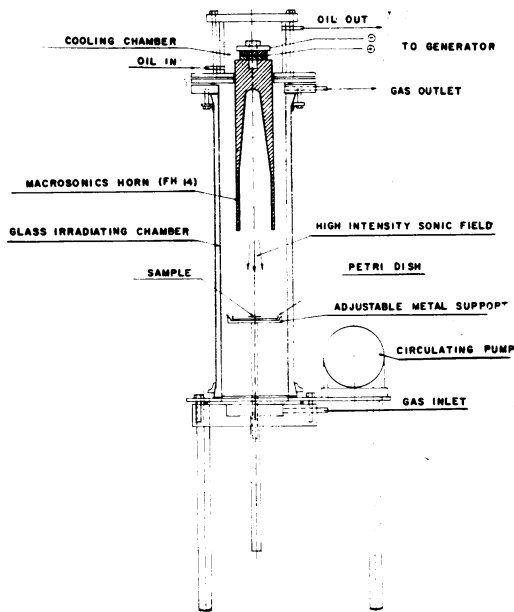


FIG. 2. Schematic representation of apparatus for airborne sound irradiation experiments. The hollow horn (Macrosonics FH 14) is shown in place.

axis within a 0.5-inch (1.27-cm) radius circle was 151 db ( $\pm$  0.5 db).

Each horn was individually fastened to the top of a cylindrical glass irradiation chamber [18  $\times$  4 inches (45.72  $\times$  10.16 cm)]. The sample was placed within the bottom half of a petri dish supported by a metal plate (Fig. 2). The plate was mechanically adjusted to the desired level in the irradiating chamber. The chamber itself was located within a polyethylene enclosure which measured 41  $\times$  18  $\times$  18 inches (104.14  $\times$  45.72  $\times$  45.72 cm).

**Preparation of spore suspension.** The organism, *B. subtilis* var. *niger* ATCC 9372, was grown on slants of nutrient agar (Difco) which were supplemented with  $MnSO_4 \cdot H_2O$  in a concentration of 10 ppm (3). Maximal sporulation occurred after 5 days of incubation at 37 C, at which time the spores from each slant were suspended in 5 ml of distilled water. The spore suspensions were then combined, washed three times in distilled water, and reconstituted to the initial volume. The spores were heat-shocked at 80 C for 20 min (5). Viable counts were made by appropriate plating techniques with the use of Trypticase Soy Agar (BBL).

**Preparation of spore-treated paper strips.** Strips of filter paper (Whatman no. 1), measuring 1  $\times$  0.25 inches (2.54  $\times$  0.635 cm), were sterilized at 121 C for 15 min and then inoculated with 0.01 ml of a spore suspension of *B. subtilis* var. *niger* which contained  $1.6 \times 10^8$  spores per milliliter. The strips were dried in sterile glass petri dishes for 24 hr at 37 C and were then stored in sterile glass jars at 25 C.

**Sonic treatment of paper strips.** Three spore-treated

paper strips were secured with sterile stainless-steel pins, to the bottom half of a sterile, plastic petri dish. The dish was placed on the metal plate (adjusted to the height desired) located in the test chamber. The latter was then sealed tight by means of bolts attached to its lower extremity. Air, dried by passage through a silica gel (28 to 200 mesh) column, was recirculated through the chamber by means of a small pump, while the temperature was simultaneously raised to 40 C. After stabilizing for 15 min, the chamber was evacuated to approximately 3 inches (7.62 cm) of mercury, and an appropriate volume of sterile, distilled water, designed to achieve a relative humidity of 40%, was introduced. The paper strips were exposed to sound waves for periods of 1, 2, 4, or 8 hr. After irradiation, the three paper strips were removed from the chamber, extracted with 30 ml of sterile, distilled water for 1 min at 8,000 rev/min in a sterile blending apparatus. The number of viable cells present in the extract was determined by plating on Trypticase Soy Agar and is expressed as spores per milliliter.

**Controls.** The number of spores present on paper strips not subjected to sound waves was determined by plate counts at the initiation, midpoint, and termination of the experimental period. The control paper strips were extracted, and the number of viable cells was quantitated in the same manner employed for the irradiated paper strips.

**Statistical methods.** The means of the plate counts of irradiated paper strips were compared with the mean of the control counts to determine whether significant population differences, attributable to sound, were discernible. Student's *t* test, as applied to nonpaired experiments (1), was employed as an index of significance between sound-irradiated populations and nonirradiated controls. The *t* values were calculated from the mean of 30 plate counts for the control, and from the means of six platings for each test condition reported. A significance level of 1% was selected.

## RESULTS

As indicated previously, control counts were made throughout the period of experimentation, which was approximately 6 weeks in duration. The paper strips gave no evidence of detrimental effects, attributable to storage, with regard to cell counts. Controls plated at the initiation of the experiments, and those plated midway and at the end of the testing period, showed similar spreads between individual counts. The mean value of all paper strips was  $1.73 \times 10^5$  spores per milliliter. The latter figure served as a basis of comparison with counts obtained from paper strips exposed to sound.

Irradiation of spores of *B. subtilis* var. *niger* with sonic waves (9.9 kc/sec) resulted in significant decreases in viable counts under certain conditions (Table 1). Meaningful reductions in cell populations, at the 1% level of significance, were always obtained when spore-treated paper strips were irradiated for 8 hr, regardless of the distance of the specimens from the sound source. Reducing the period of irradiation to less than 8 hr, however, yielded values which were not significant except when the strips were irradiated at a distance of 1 inch (2.54 cm) from the transducer for 4 hr. Irradiation periods of 2 hr or less did not result in significant decreases in the viable count, regardless of the intensity level. The sharpest decrease in the viable count was observed after 8 hr at the 9.5-inch (24.13 cm) position, which corresponded to resonant conditions (a whole number of 0.5 wavelengths).

Treatment of the test organism with ultrasonic waves (30.4 kc/sec) also resulted in significant reductions in viable counts (Table 2). As was the case with sonic waves, meaningful decreases in

TABLE 1. Means of plate counts ( $\times 10^5$ ) of spores of *Bacillus subtilis* var. *niger* irradiated with airborne sonic waves (9.9 kc/sec) and corresponding *t* values<sup>a</sup>

| Irradiation time                   | Sample position and no. of wavelengths |                                     |                                      |                                      |
|------------------------------------|--|-------------------------------------|--------------------------------------|--------------------------------------|
|                                    | 9.5 inches (24.13 cm), 7 $\lambda^b$   | 5.0 inches (12.7 cm), 3.6 $\lambda$ | 3.5 inches (8.89 cm), 2.54 $\lambda$ | 1.0 inches (2.54 cm), 0.76 $\lambda$ |
| <i>hr</i>                          |  |                                     |                                      |                                      |
| 1                                  | 1.77 (0.12) <sup>c</sup>               | 1.56 (1.17)                         | 1.69 (0.50)                          | 1.45 (2.37)                          |
| 2                                  | 1.77 (0.44)                            | 1.52 (0.81)                         | 1.51 (0.83)                          | 1.41 (2.66)                          |
| 4                                  | 1.46 (1.83)                            | 1.56 (1.00)                         | 1.37 (2.48)                          | 1.22 (3.48**)                        |
| 8                                  | 0.89 (3.59**)                          | 1.09 (6.40**)                       | 1.18 (4.90**)                        | 1.14 (6.21**)                        |
| Intensity level at sample position | 155 db                                 | 153 db                              | 153.5 db                             | 154 db                               |

<sup>a</sup> Results are expressed as spores per milliliter.

<sup>b</sup> Pressure antinodal position.

<sup>c</sup> Numbers in parentheses = *t* values.

\* Indicates significance level of 1%.

TABLE 2. Means of plate counts ( $\times 10^6$ ) of spores of *Bacillus subtilis* var. *niger* irradiated with airborne ultrasonic waves (30.4 kc/sec) and corresponding *t* values<sup>a</sup>

| Irradiation time                   | Sample position and no. of wavelengths |                                     |                                     |                                       |
|------------------------------------|--|-------------------------------------|-------------------------------------|---------------------------------------|
|                                    | 9.5 inches (24.13 cm), 13.2 $\lambda$  | 5.0 inches (12.7 cm), 7 $\lambda^b$ | 3.5 inches (8.89 cm), 5 $\lambda^b$ | 1.0 inches (2.54 cm), 1.5 $\lambda^b$ |
| <i>hr</i>                          |  |                                     |                                     |                                       |
| 1                                  | 1.85 (1.38) <sup>c</sup>               | 1.66 (0.70)                         | 1.83 (0.43)                         | 1.82 (0.76)                           |
| 2                                  | 1.53 (2.38)                            | 1.56 (2.21)                         | 1.53 (2.06)                         | 1.05 (3.54**)                         |
| 4                                  | 1.27 (1.31)                            | 1.36 (3.08**)                       | 1.29 (3.70**)                       | 0.76 (4.85**)                         |
| 8                                  | 1.11 (8.88**)                          | 1.06 (3.86**)                       | 0.91 (5.23**)                       | 0.95 (6.39**)                         |
| Intensity level at sample position | 156 db                                 | 159 db                              | 161 db                              | 161 db                                |

<sup>a</sup> Results are expressed as spores per milliliter.

<sup>b</sup> Pressure antinodal position.

<sup>c</sup> Numbers in parentheses = *t* values.

\* Indicates significance level of 1%.

plate counts occurred after 8 hr of irradiation, with test strips placed at each of the four distances employed. In addition, significant results were also obtained after 4 hr of irradiation, when the specimens were located at distances of 5, 3.5, and 1 inch (12.7, 8.89, and 2.54 cm), respectively, from the transducer. Beyond this, the most striking reduction in the viable count was recorded when the strips were placed 1 inch (2.54 cm) from the sound source and exposed to ultrasonic waves for merely 2 hr.

A comparison of the data contained in both tables reveals that almost twice as many significant values were obtained at the 156 to 161 db level as at the 153 to 155 db level. Further comparison indicates that the application of sonic and ultrasonic waves of nearly equal intensity (155 and 156 db), for the same period of time (8 hr), to test strips placed 9.5 inches (24.13 cm) from the sound source, resulted in greater kill with sound waves of 9.9 kc/sec.

Consideration was also given to the possibility that the sonic chamber itself might adversely affect the viability of spores of *B. subtilis* var. *niger*. Accordingly, spore-impregnated paper strips were allowed to remain in the chamber for periods up to 8 hr under conditions identical with those employed for the experimental paper strips, except that sound energy was not applied. Plate counts of these strips revealed no significant reductions attributable to the test chamber alone.

#### DISCUSSION

Exposure of spores of *B. subtilis* var. *niger* to high-intensity airborne sound resulted in statistically significant reductions in viable counts as compared with spores not exposed to sound.

From the data collected, it appears that the antibacterial activity of airborne sound waves is directly related to the sound intensity, the period of irradiation, and the distance of the sample from the sound source. Preliminary observations at the 155 to 156 db level indicated that, under certain conditions, airborne sonic waves were more destructive than ultrasonic waves. Confirmation of this finding would mean that the amplitude of motion of the gas molecules which impinge against the microbial surface may be a factor in acoustic sterilization.

Attempts to explain the antibacterial activity of airborne sound waves on a physical basis must take into account the possible transformation of acoustic energy into heat. Greguss and Erdelyi (8), in fact, reported that the effects of ultrasonic waves in gaseous media have been explained on the basis of the energy absorbed from sound waves. These authors indicated that the temperature gradient in the medium irradiated by ultrasonics changes with the acceleration of the field. The ability of sound to kill by means of heat generation was reported by Frings, Allen, and Rudnick (7). They described the lethal effects of airborne ultrasonic waves (19 kc/sec and intensity of 1 w/cm<sup>2</sup>), produced by a rotary siren, for mice, roaches, and various insects, after an exposure of 1 min to the high-intensity beam. In the present investigation, no direct evidence was obtained which would implicate thermal phenomena in the reduction of viable counts of spores of *B. subtilis* var. *niger* exposed to sound. Based on the results of others, the possibility exists, however, that the more lethal effects noted at the higher intensity levels were due, at least in part, to the generation of greater amounts of heat.

The results obtained in the present study can also be examined from a chemical point of view. The reported lower water content of spores (11, 12), for example, might influence any lethal effects of airborne sound, since it is known that the absorption of acoustic energy is greatly affected by the nature of the medium through which it is transmitted. In addition, culture conditions have been reported to affect the lipid content of spores as well as their resistance to toxic agents (4). Thus, it is conceivable that the response of *B. subtilis* var. *niger* to airborne sound waves is governed to some extent by the chemical composition of the spores at the time of irradiation. As an example, it is known that lipoidal substances readily absorb ultrasonic frequencies (2), and any changes in the lipid level of the test organism might directly affect the amount of acoustic energy taken up by the spore.

A direct comparison of the data presented here with similar studies was not possible, because of the lack of such reports in the scientific literature. This deficiency is understandable in consideration of the relatively high impedance which air offers to the passage of sound waves. In addition to this, high power intensity sound sources, with frequency flexibility, have only recently been made available. It is, in fact, now possible to irradiate microorganisms at intensity levels one order of magnitude higher than those used in the present report. This new capability in high-intensity sonic and ultrasonic generation will enable further work in an area which requires much more experimental data before a valid evaluation can be made on the efficacy of airborne sound waves in sterilization procedures.

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