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Ultrasonic Disinfection of Water Suspensions of *Escherichia Coli* and *Legionella Pneumophila*

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PREFACE

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13. ABSTRACT (Maximum 200 words)

This report describes an experiment in which water samples inoculated with *Escherichia (E.) coli* and *Legionella (L.) pneumophila* organisms were exposed to ultrasonic energy. It was found that treating these samples with pulses of ultrasound for at least 15 minutes at a 50% amplitude virtually eliminated the *E. coli* colonies. Longer exposure times were required to completely destroy *L. pneumophila*. Although the water samples experienced slight warming during the course of the ultrasonification, control experiments (in which the test samples were not exposed to ultrasound but simply warmed) have supported the conclusion that the test organisms were destroyed by acoustic energy alone. The use of ultrasound as an applied and commercial technology for disinfecting water supplies is also discussed in the report. However, the mechanisms of cell destruction, not yet fully understood, are left for the subject of a future publication.

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1. INTRODUCTION

BACKGROUND

This report describes an experiment in which ultrasound was used to destroy two pathogenic organisms known to contaminate water supplies. One of these organisms is *Escherichia (E.) coli*. Water contaminated by human waste may contain *E. coli*, several strains of which may invade the epithelial cells lining the colon, giving rise to fever and serious diarrhea in humans.[1] Certain strains are classified as enterotoxicogenic (i. e., they produce toxic substances within the intestines), whereas other strains may cause such serious conditions as hemorrhagic colitis (i.e., an inflammation of the large bowel accompanied by blood loss).

The second pathogenic organism is *Legionella (L.) pneumophila*, which results in legionellosis. Legionellosis has two distinct clinical syndromes: Pontiac fever and Legionnaires' disease. Pontiac fever is a nonpneumonic, influenza-like illness. The initial symptom of Legionnaires' disease, a pneumonia that was first observed in 1976 at an American Legion convention in Philadelphia, is a fever accompanied by cough, shortness of breath, chest pains, abdominal pain, and diarrhea. Typically, 15% to 20% of its victims die if they do not receive appropriate treatment with antibiotics. An unknown proportion of these cases may be caused by contaminated drinking water.

The *L. pneumophila* bacterium thrives in damp and wet environments. Warm temperatures, as well as environments where water stagnates and sediment accumulates, also contribute to the proliferation of this organism. Cooling towers, plumbing components, faucet aerators, shower heads, humidifiers, and hot water storage tanks in hospitals are the types of environments that may harbor *L. pneumophila,*[2] which likely gains entrance to the human body by inhalation of contaminated water. Studies have shown that this organism can exist for a long time in hospital water systems, where it can lead to outbreaks of legionellosis.

Currently, chlorination is the method of choice to disinfect water contaminated with either *E. coli* or *L. pneumophila.*[3] However, in 1974 it was reported that chlorination of water supplies can lead to the formation of volatile organic compounds known as trihalomethanes (THMs).[4] These compounds are formed by the reaction of natural organic compounds found in water with chlorine. Because certain THMs, such as chloroform, are carcinogenic,[5] several alternatives to chlorination have emerged, including the ultraviolet treatment of water, ozonation, and ultrasonic treatment. Although there are few, if any, applications using "scaled-up" ultrasonication at this time, its use as a safe, reliable alternative to chlorination appears to hold considerable promise, as discussed next.

SCOPE OF PRESENT ULTRASONICS STUDY

Ultrasonics, or ultrasonic engineering, refers to the use of acoustic energy to bring about a physical or chemical change.[6] In a fluid, this phenomenon is known as "cavitation," or the rapid formation and destruction of "bubbles."[7] When an intense ultrasonic wave passes through a fluid, bubbles may form at various defect sites, somewhat analogous to the growth of crystals by nucleation. These bubbles may collapse very suddenly, releasing intense bursts of energy. The resulting high temperatures can lead to the rupture of chemical bonds in molecular species present in the fluid. In addition to the "transient" bubbles, "stable" bubbles may be produced. The latter release less energy on collapse and may result in mechanical changes to molecular species present in the fluid.

There is a growing list of applications in the field of ultrasonic engineering that underscores the practical importance of this technology. For example, several experimental studies carried out at the Naval Undersea Warfare Center (NUWC) Division in Newport, RI, have clearly demonstrated that ultrasound generated by acoustic transducers can enhance and accelerate certain chemical and physical processes. In particular, it has been shown that ultrasonic energy can induce changes on nickel surfaces for electroplating, remove paint, and emulsify oils.

Even more recent experiments at the Division have demonstrated that ultrasonic energy can produce significant changes in the particle size and distribution of waste sewage samples.[8] It was the work in Evora[8] that prompted the present study, which shows that ultrasound can destroy certain bacteria in contaminated water samples. In fact, compared with other methods, the use of ultrasonics appears to have the best potential for the decontamination of water supplies.*

Even though it has been shown that high-power ultrasound can enhance certain chemical and physical processes, its commercial uses at this time still remain quite limited. For example, there are very few full-scale applications of this technology for water and wastewater treatment currently in use. This situation is due, in part, to an incomplete understanding of

- The conditions required to destroy bacterial organisms;
- The importance of a systematic classification of the organisms most likely to be affected by ultrasound;
- The efficiency of converting electrical energy into sound;

*Based on the above research, it follows that the transduction technology developed in the Navy for undersea high-power sonar applications could potentially be used to ultrasonify decontaminated water. With this in mind, it would be of considerable interest for the Navy to demonstrate such a capability with its high-power transducers utilizing piezoelectric lead zirconate titanate (PZT), electrostrictive lead magnesium niobate (PMN), or magnetostrictive Terfenol-D as the active material.

- The efficiency of delivering the sound energy into the liquid; and
- The cost of the technology relative to other remediation procedures.

The present study was undertaken to address the first issue, that is, to identify the laboratory conditions whereby ultrasound can be used to destroy *E. coli* and *L. pneumophila*. Water samples containing suspensions of *E. coli* and *L. pneumophila* were prepared and subjected to ultrasonic energy. After treatment, colony enumeration was performed by standard microbiological counting techniques. The colony counts of samples exposed to ultrasound were then compared to the colony counts of unexposed or untreated samples.

2. EXPERIMENT

TEST ORGANISMS

Test organisms of *E. coli* were obtained commercially as impregnated discs from Difco, Inc. A disc was dissolved in 1 mL of trypticase soy broth (a buffered broth containing tryptone, soybean peptone, dextrose, and sodium chloride obtained in dehydrated form from Difco). The inoculated broth was incubated at 35 ± 0.5°C for approximately 6 hours before becoming turbid. A platinum-iridium wire loop was used to swab a trypticase soy agar (tryptone, soybean peptone, sodium chloride, and agar from Difco) plate with the infected broth. The agar plate was incubated at 35 ± 0.5°C for 24 hours to grow clearly recognizable colonies of *E. coli*. The culture was stored under refrigeration at 1 to 5°C. A culture of *L. pneumophila* was obtained as a growth on an agar slant from the State of Connecticut Department of Public Health Microbiology Laboratory. This culture was also stored under refrigeration at 1 to 5°C.

DILUTION WATER

Laboratory-prepared dilution water was used for the experiments. This water was prepared by diluting 1.25 mL of a potassium dihydrogen phosphate solution (34 grams/500 mL) and 5.0 mL of a magnesium chloride solution (81.1 grams/1 L) to a final volume of 1 L. Measured portions were dispensed into culture bottles and then sterilized by autoclaving at 121° to 122°C under 19 psi of pressure for 15 minutes.

BACTERIAL SUSPENSIONS

The sterilized bottles of dilution water were subsequently inoculated with test organisms of *E. coli* or *L. pneumophila* using the platinum loop. The bottle was then capped and shaken vigorously. Appropriate dilutions of the suspensions were used in the testing protocols, with the volume of the final dilution being 300 mL. This dilution was divided into three equal 100-mL portions so that each would contain the same bacterial density. The first 100-mL dilution was the control; the second was sonicated; and the third was a control subjected to mild heat to raise its temperature to the temperature of the sonicated dilution.

CULTURE MEDIA

After 4.2 grams of m-Endo medium (a product containing lactose obtained in dehydrated form from Difco) were rehydrated with 50 mL of distilled water, 1 mL of ethanol was added to the suspension. The solution was carefully heated to near boiling to dissolve the agar and then cooled and stored under refrigeration at 1 to 5°C for no longer than 96 hours. This medium was used to grow and identify *E. coli*.

L. pneumophila was isolated on commercial plates of buffered charcoal yeast extract

(BCYE) agar containing L-cysteine and antibiotics. This prepared agar was purchased from Remel, Inc., in sterilized plastic culture dishes.

STANDARD TOTAL COLIFORM MEMBRANE FILTER SETUP

E. coli was identified and counted by the standard total coliform membrane filter procedure. During this process, 100 mL of treated water samples or untreated controls were passed through a sterile 0.45-μm filter (millipore) via a sterilized assembly. A sterile absorbent pad was placed in a 60- by 15-mm disposable, presterilized plastic petri dish. Approximately 2 mL of the rehydrated m-Endo broth was added to the pad with a sterilized pipette. After filtration of the water suspensions, the 0.45-μm filter was placed on the pad with a sterilized forceps. The petri dish was then capped and incubated at 35 ± 0.5°C for 24 hours.

ULTRASONICATOR

Ultrasonication was performed with a Sonics and Materials, Inc., microprocessor-controlled, high-intensity ultrasonic processor (vibra cell, 600-watt model). The probe tip was 19 mm in diameter, capable of delivering medium-intensity ultrasonic energy to a volume between 25 and 500 mL at a frequency of 20 kHz ± 50 Hz. Prior to an experiment, the power supply was tuned to ensure maximum transfer of energy. In all experiments, the probe was first sterilized with isopropanol, dried with a sterile swab, and then immersed in a 100-mL sample contained in a presterilized 250-mL beaker. Sonication was performed within a sealed chamber to provide protection from airborne bacteria.

QUALITY CONTROL

Quality control procedures were the same as those specified in the 19th edition of *Standard Methods for the Examination of Water and Wastewater*.[9] Thermometers were National Institute of Standards and Technology (NIST) traceable. The temperature of the incubator used to grow the cultures was checked twice daily and maintained between 34.5 and 35.5°C. The filtration assemblies, bottles, and beakers used in the experiments were sterilized by autoclaving at 121 to 122°C under 19 psi of pressure. The effectiveness of the sterilization procedure was monitored with commercially available check strips, as well as with *bacillus stearothermophilus* spore suspensions. A thermometer was also placed within the autoclave to check the sterilization temperature. All waste cultures and organisms were destroyed by autoclaving at 121°C for 30 minutes.

The distilled water was monitored for suitability in microbiology testing. The conductivity was always found to be below 2 μmhos/cm at 25°C, the heavy metal concentration was measured to be below 0.05 mg/L, and the residual chlorine was not detectable. The standard plate count, which estimates the number of live bacteria in water, was measured to be below 1000 colony-forming units (CFU)/mL, a value considered acceptable for microbiological testing. The bacteriological quality of the water used in this study was determined to be acceptable for microbiology testing via the procedure specified by Eaton *et al.*[9]

3. RESULTS

Initial experiments demonstrated that a significant temperature rise accompanies the ultrasonication for exposure times that were as short as 10 minutes. To rule out possible thermal kills of the suspended bacteria, the experimental protocol included control suspensions whose temperature was adjusted to the temperature of the ultrasonicated suspensions. Table 1 summarizes the experimental protocol.

Table 1. Summary of the Experimental Protocol

Organism and Growth Medium	Protocol
E. coli (m-Endo)	Control (unexposed)
	Sample exposed to heat only
	Sample exposed to ultrasound
L. pneumophila (BCYE)	Control (unexposed)
	Sample exposed to heat only
	Sample exposed to ultrasound

ULTRASONIC PROCESSING OF WATER SAMPLES INOCULATED WITH *E. COLI*

An initial experiment was performed where 100 mL of a suspension of *E. coli* in water were continuously sonicated at 20 kHz for 20 minutes at 50% amplitude, which is related to the power delivered to the sample. At the completion of the experiment, the temperature of the water was measured to be 55°C, which is considered sufficient to kill *E. coli* organisms. As described above, a control heated to the same temperature was also included, as well as a control maintained at room temperature, for the duration of the experiment. At the completion of the ultrasonic treatment, the samples were passed through a 0.45-μm filter, which was placed on a pad soaked with m-Endo broth.

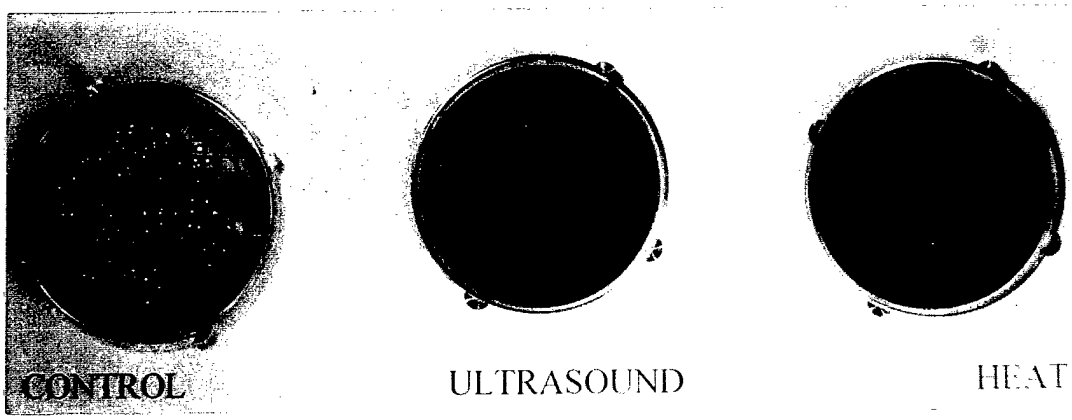
After incubation at 35 ± 0.5°C, no colonies were observed either in the ultrasonic sample or in the heated sample (table 2). The colonies in the untreated control were too numerous to

count (TNTC). It was evident that no conclusions could be drawn from this experiment concerning the efficacy of the ultrasound treatment, since it could not be determined if the heat generated by the ultrasound, rather than the ultrasound itself, was destroying the organisms. Accordingly, a second experiment was attempted during which the ultrasonication was performed in the pulse mode where the ultrasonic energy was "on" for 1 second and "off" for 1 second. Hence, for a 15-minute sonication, the entire experiment would take 30 minutes. The suspension was further diluted to reduce the number of colony counts in the control. However, here again, the temperature rise was significant in the ultrasonic sample, and no colonies were observed in either the sonicated or the heated control suspensions (table 2).

A third experiment was performed where the amplitude (i.e., power) was reduced to 40%, the pulse was changed to 1 second "on" and 3 seconds "off," and the total exposure to ultrasound was 5 minutes. As shown in table 2, the number of colonies in the ultrasonically treated samples were significantly reduced, whereas the number of colonies in most of the controls were too numerous to count, with the exception of two cases in which it was possible to enumerate and count the colonies. Note that by using a lower amplitude and by operating in the pulse mode, the temperature rise was much less pronounced. Sonicating for 10 minutes achieved an even greater kill. Finally, extending the exposure to ultrasound to 20 minutes resulted in complete destruction of the *E. coli* suspension (table 2, last entry). A photograph of the culture dishes after incubation is shown in figure 1. These data provide strong evidence that the ultrasonic energy, not the buildup of heat, destroyed the bacterial organisms.

Table 2. Colony Counts of Water Samples Inoculated with E. Coli After Various Treatments

Amplitude (%)	Total Duration (min)	Exposure to Ultra-sound (min)	Colonies Remaining After Ultrasound Treatment (col/100 mL)	Colonies Remaining After Exposure to Heat Only (col/100 mL)	Control (col/100 mL)
50	20	20	0 (T_{max} = 55°C)	0 (T_{max} = 55°C)	TNTC
50	30 (pulse mode)	15	0 (T_{max} = 50°C)	0 (T_{max} = 54°C)	52
40	20 (pulse mode)	5	312 (T_{max} = 39°C)	TNTC (T_{max} = 39°C)	TNTC
40	40 (pulse mode)	10	73 (T_{max} = 39°C)	TNTC (T_{max} = 39°C)	TNTC
40	80 (pulse mode)	20	0 (T_{max} = 41.5°C)	88 (T_{max} = 42°C)	153



Note: *E. coli* samples were planted on m-Endo media and incubated at 35 ± 0.5°C for 24 hours. The plate on the left is from a control. The plate in the middle is from contaminated water exposed to ultrasonic energy (40% amplitude) in the pulse mode for a total of 80 minutes (the actual exposure time to ultrasound was 20 minutes). The maximum temperature was recorded as 41.5°C. The plate on the right was from a water sample whose temperature was raised to 42°C for the entire duration of the ultrasound experiment. Although not clearly visible in this photograph, colonies still were present in the heated water, whereas in the ultrasonicated sample, all the colonies were destroyed.

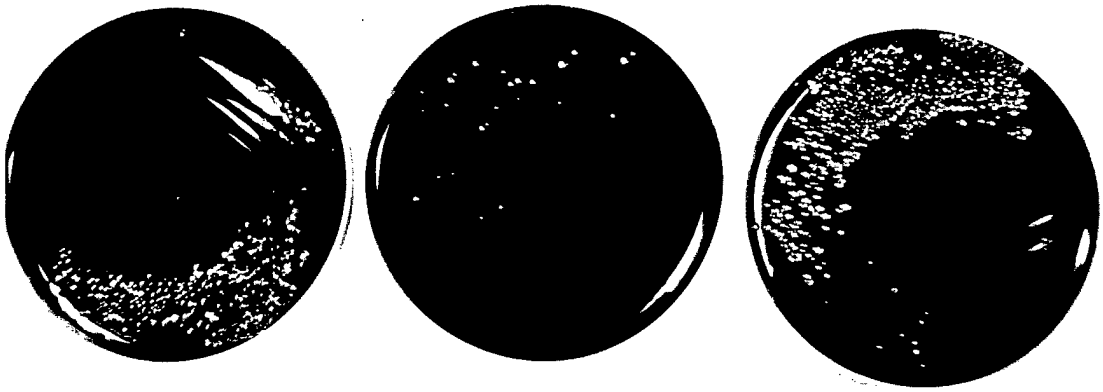
Figure 1. Effects of Ultrasound and Temperature on E. Coli Bacteria

ULTRASONIC PROCESSING OF WATER SAMPLES INOCULATED WITH *L. PNEUMOPHILA*

Table 3 summarizes the results obtained when bacterial suspensions of *L. pneumophila* were exposed to ultrasound or heat. Untreated controls were also included. As shown in the table, a significant colony kill was achieved after 20 minutes in the pulse mode. However, ultrasonicating for 30 minutes resulted in complete colony kill. Note that the *L. pneumophila* strains used in this experiment are more resistant to a temperature rise to 54° C than are the strains of *E. coli* used in the experiment described in the previous section. Nonetheless, the effectiveness of ultrasound in destroying *L. pneumophila* is underscored both by the results in table 3 and the plates in figures 2 and 3.

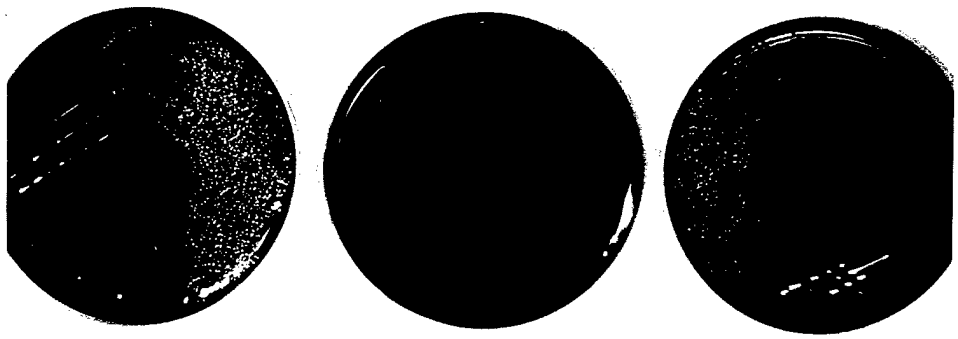
Table 3. Colony Counts of Water Samples Inoculated with L. Pneumophila After Various Treatments

Amplitude (%)	Total Duration, (min)	Exposure to Ultra-sound (min)	Colonies Remaining After Ultrasound Treatment (col/100 mL)	Colonies Remaining After Exposure to Heat Only (col/100 mL)	Control (col/100 mL)
40	80 (pulse mode)	20	25 (T_{max} = 41°C)	TNTC (T_{max} = 41°C)	TNTC
40	120 (pulse mode)	30	0 (T_{max} = 45°C)	TNTC (T_{max} = 54°C)	TNTC



Note: BCYE media containing antibiotics were swabbed with water samples inoculated with *L. pneumophila*. The plate on the left is an inoculum from the control. The plate in the middle was swabbed from an infected water sample exposed to ultrasonic energy (40% amplitude) in the pulse mode for a total time of 80 minutes (the actual exposure time to ultrasound was 20 minutes). The maximum temperature was recorded as 41.5°C. The plate on the right was from a water sample whose temperature was raised to 42°C for the entire duration of the ultrasound experiment.

Figure 2. Effects of Ultrasound and Temperature on L. Pneumophila



Note: In this experiment, the ultrasonic treatment lasted for 30 minutes in the pulse mode for a total of 120 minutes. The maximum temperature in the ultrasonicated and heated samples was 45°C. By extending the time of ultrasonication, all *L. pneumophila* organisms were destroyed by the action of the sound alone.

Figure 3. Effects of Ultrasound and Temperature on L. Pneumophila in an Experiment Similar to That of Figure 2

4. CONCLUSIONS

The results recorded in this study provide compelling evidence that ultrasound can destroy *E. coli* and *L. pneumophila*, two potentially pathogenic organisms. That the colony kill was not due simply to the heating of the water samples during ultrasonication was confirmed by experiments where control samples were exposed to similar warm temperatures. Although this and other investigations have shown that ultrasonics can be effective in destroying certain microorganisms, the underlying mechanisms are not yet completely understood. It is believed that destruction of bacterial organisms may involve mechanical disruption of the bacterial cell membrane, as well as cell damage due to the production of free radicals.

Ultrasonics has the potential to be "engineered" into practical dual-use applications. For example, while the Navy may want to consider using this technology to disinfect water used for human consumption by the fleet, there are also potential civilian applications. For example, given that coliform bacterial contamination of private well water supplies is a national health and environmental problem, a transducer could be developed that would allow homeowners to disinfect drilled and dug wells infected with such bacteria. If this technology can be demonstrated to be effective against a broad range of pathogenic bacteria, parasites, and viruses, then its commercial appeal will be further enhanced. To achieve these goals, as well as to investigate other important issues suggested by the results of this study, additional research will have to be undertaken. The following list of experiments is suggested:

- Because the extent of bacterial damage was not considered in this study, the conditions employed during ultrasonication in these experiments may have simply "stressed" or "damaged" the bacteria rendering them incapable of forming colonies.[9,10] However, even "damaged" organisms can constitute a significant public health risk. Thus, experiments would have to be designed to enumerate injured organisms and to rule out the possibility that the ultrasonic processing is simply damaging rather than completely destroying the organisms.

- Several experiments will also have to be carried out to systematically enumerate bacterial kills as a result of changing the amplitude and other ultrasonic conditions. The power output, the frequency, and the location of the transducer required to achieve maximum destruction of the organisms will have to be carefully determined to identify the optimum conditions needed for effective disinfection. Such experiments will yield data from which colony kills can be plotted versus amplitude, time of processing, changing pulse conditions, frequency, energy output, location, etc. The transducers employed in these experiments should include high-power sonar drivers employing PZT, PMN, or Terfenol-D as the active material. These transducers are available at NUWC Division Newport.

- Since this work was focused on only two organisms, *E. coli* and *L. pneumophila*, the ability of ultrasonic processing to disinfect water samples infected with other pathogenic

organisms, such as *Klebsiella pneumoniae*, the fecal streptococcus and enterococcus groups, *Giardia lamblia*, and *Cryptosporidium,* must be determined. Viruses can also be considered for future studies.

- Experiments should be planned to explore the underlying biochemical mechanisms of colony kill. For example, it is important to understand whether the mechanism is primarily a mechanical disruption of the bacterial cell membrane or whether free-radical mechanisms are involved in colony kill.

- Ultrasonic processing technology in combination with other processes such as the use of ultraviolet or visible light to enhance the destruction of harmful organisms should be explored. One of the drawbacks to ultraviolet light is that it is not deeply penetrating, and therefore, its effectiveness is dramatically reduced when treating samples containing particulate matter. On the other hand, ultrasonication of particulate matter can reduce particulate size and may enhance the exposure of embedded organisms to ultraviolet light. Thus, the ultrasound may perform two tasks: the direct destruction of organisms, as well as the enhancement of the effectiveness of the phototoxic effects of ultraviolet radiation.

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