Short Communication

Inactivation of Bacteria in Freshwater by Momentary Decompression Following High Pressurization

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Rapid and continuous pressure treatment was realized using a hydraulic pump and the momentary decompression following high pressurization was used to inactivate bacteria. The number of colony-forming E. coli decreased to 1/1000 in response to 10 cycles of pressure treatment. In groundwater samples, repeated pressure treatment led to a two-log decrease in the number of colony-forming bacteria. These findings suggest that repeated cycles of momentary decompression following high pressurization enabled a marked decrease in bacterial growth activity. The results presented herein may contribute to microbiological quality control and the safety of freshwater.

Key words: aquatic environment, bacteria, decompression, high pressure, hydraulic pump, inactivation

High pressurization in combination with heating has commonly been used for disinfection in microbiology. For example, autoclaving has long been used for the sterilization of samples and equipment used in microbiology laboratories, and retort treatment is widely applied for the preservation of food and drink (6, 7, 11). Moreover, high pressurization is combined with cooling for inactivation of microbes in heat-intolerant samples (3, 8). However, these methods may denaturalize samples as a result of the change in temperature and a great deal of energy is often required to heat or cool the samples. In addition, these treatments are difficult to apply to large volumes of samples because they require large tanks for the treatment to be effective.

In this study, we focused on the use of decompression following high pressurization for inactivation of bacteria in aquatic environments. Some studies reported that high pressurization of samples followed by decompression (e.g., French press cell) was able to decrease the number of bacterial spores (2, 4, 13). However, the efficiency of repeated pressurization and decompression for inactivation of bacteria has not been studied in detail, despite the importance of continuous treatment for high volumes of liquid samples. Here, we describe a newly developed system that employs a hydraulic pump for simple, rapid, and continuous pressure treatment to inactivate bacteria in freshwater samples.

Escherichia coli W3110 was grown in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl [pH 7.0]) at 37°C for one night. The cultured cells were then harvested by centrifugation (3,500 × g, 3 min), washed twice with phosphate-buffered saline (PBS; 130 mM NaCl, 10 mM NaH₂PO₄, 10 mM Na₂HPO₄ [pH 7.2]), and diluted in PBS to give an initial concentration of 10⁶ cells mL⁻¹. Groundwater samples were collected at Osaka University, Osaka, Japan (34°49′N, 135°31′E). These samples were used immediately after sampling. Cultured E. coli cells or groundwater samples were poured into a steel container (10 liter volume) in the fabricated system (Fig. 1) and then treated continuously by circulating with a hydraulic pump (Takako Industries, Kyoto, Japan). Pressurization was set to 0, 14, or 34 MPa. After the pressure control bulb, the pressure was momentarily decreased to standard atmospheric pressure, and samples were collected for further analysis.

Single momentary decompression followed by high pressurization was defined as one cycle, and treatment consisted of up to 10 cycles. The time required for each treatment was regulated by controlling the flow rate of the liquid sample. In this study, the time for pressurization ranged from 4 (short-term treatment) to 12 (long-term treatment) seconds (flow rate: 6.6 to 2.1 L min⁻¹).

The number of colony-forming-units (CFU) was determined by spreading diluted samples on nutrient-rich media.

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Fig. 1. The bacterial inactivation system with a hydraulic pump. Water in the system is continuously circulated and pressurized by the hydraulic pump. The pressurization is followed by momentary decompression. The high-pressure treatment occurs between the hydraulic pump and the pressure control bulb. The solid line shows the highly pressurized parts and the dotted line, the non-pressurized parts.
Fig. 2. Disruption of Escherichia coli by continuous pressure treatments (34 MPa). (a) SEM images of E. coli cells before pressure treatment, (b) E. coli cells following a single pressure treatment, (c) E. coli cells following 10 cycles of pressure treatment.

Table 1. Bacterial numbers of Escherichia coli following pressure treatments

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>Number of cycles</th>
<th>TDC$^a$ (cells mL$^{-1}$)</th>
<th>PI(+)$^b$ (cells mL$^{-1}$)</th>
<th>CFU$^c$ (CFU mL$^{-1}$)</th>
<th>PI(+)/TDC$^d$ (%)</th>
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<tr>
<td>0</td>
<td>0</td>
<td>8.2 ± 0.8 × 10$^3$</td>
<td>2.5 ± 0.6 × 10$^5$</td>
<td>6.7 ± 1.0 × 10$^5$</td>
<td>31</td>
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<td>8.8 ± 0.8 × 10$^3$</td>
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<td>6.9 ± 0.7 × 10$^5$</td>
<td>25</td>
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</table>

*a* Each value is the mean with standard deviation (n=3).

*b* Number of cycles: Number of repeated cycles of momentary decompression following 10 sec of pressurization.

*c* TDC: Total direct count of bacterial cells (DAPI-staining).

*d* PI(+): Number of membrane-damaged cells (PI-staining).

*e* CFU: Number of colony-forming units on SCD medium (incubation: 24 hours).

*f* PI(+)/TDC: Ratio of membrane-damaged bacteria.

(soybean casein digest [SCD] agar media; incubation: 37°C for 24 h) for E. coli or nutrient-poor media (R2A agar media; incubation: 25°C for 7 days) for the groundwater samples. Damage to the cell membranes of the bacteria was determined by staining with propidium iodide (PI; Sigma) (12). Briefly, bacterial cells were trapped by vacuum onto a black polycarbonate filter (por size: 0.2 μm; Advantec, Tokyo, Japan), and stained in buffer containing 2 μg mL$^{-1}$ of PI (concentration of stock solution in sterilized water: 500 μg mL$^{-1}$) and 1 μg mL$^{-1}$ of 4’,6-diamidino-2-phenylindole (DAPI; concentration of stock solution in sterilized water: 10 μg mL$^{-1}$) for 5 min at room temperature. Bacterial cells were enumerated using an epifluorescence microscope (E400, Nikon, Tokyo, Japan) (1, 5, 9). Damage to the cell membranes of bacteria appeared as red-fluorescence and was enumerated under green-light excitation. The total bacterial concentration was determined simultaneously under ultra violet excitation.

Ten liters of the cultured E. coli suspension (diluted to 10$^6$ cells mL$^{-1}$) was poured into the container of the fabricated system (Fig. 1) and subjected to repeated high pressurization (12 sec) followed by momentary decompression (Table 1). Without pressurization (0 MPa), there was no change in the total concentration of bacteria, cell membrane-damaged bacteria, or CFU. However, repeated pressurization at 14 MPa for 12 sec followed by momentary decompression resulted in the number of cell membrane-damaged bacteria increasing and the CFU decreasing to 1/10 of the initial value, while the total number of bacteria was not changed. Treatment at higher pressure (34 MPa) led to a remarkable inactivation of E. coli cells and a decrease in the CFU to 1/1000 of the initial value.

For scanning electron microscopy, 2% (v/v) glutaraldehyde was added to each sample taken after the pressure treatment and bacterial cells in the sample were then fixed for 24 h at 4°C. Next, the fixed cells were trapped onto a polycarbonate filter (por size: 0.2 μm; Advantec) by vacuum and dehydrated successively with 50%, 70%, and 99% ethanol (for 5 min each). The filter was then air-dried, sputter-coated with gold, and observed under a scanning electron microscope (JSM 5610L, JEOL, Tokyo, Japan) in the high-vacuum mode. Scanning electron micrographs of E. coli cells following high pressurization treatment are shown in Fig. 2. Cells were damaged in response to a single
treatment and almost all of the cells collapsed after 10 cycles of the treatment.

The effects of the momentary decompression following high pressurization were examined to determine if it inactivated bacterial cells in groundwater (Fig. 3). The effects of pressurization at different levels for 12 sec and for 4 sec were compared because shortening the total treatment would be required for the application of our system to large volumes of environmental water samples. Without pressurization, the bacterial numbers were not changed, as in the experiments with *E. coli* cells (Table 1). The reduction in CFU was much greater in response to higher pressurization (34 MPa) than pressurization with 14 MPa (data not shown), and this value decreased to below the quantification limit after 10 cycles of treatment with pressurization at 34 MPa for 4 sec (short-term treatment, Fig. 3) or five cycles of treatment with pressurization at 34 MPa for 4 sec (short-term treatment, Fig. 3) or five cycles of treatment with pressurization at 34 MPa for 12 sec (long-term treatment, Fig. 3). The total time required for inactivation by long-term treatment was about 25 min, while only 15 min was required for inactivation by short-term treatment.

In previous studies, pressurization treatments over 100 MPa have been used to inactivate various types of bacteria (2, 8, 10, 11). However, our system employed lower pressure conditions (≤34 MPa) and a short pressurization time (≤12 sec per cycle), but showed highly efficient inactivation by simple continuous treatment. The system presented here does not require a large tank for pressurization and enables continuous inactivation by only circulating the sample. This inactivation technique does not use chemicals, which often cause health hazards if used in excess. As a result, this system can be used to control the microbiological quality of water circulation systems such as pools, Jacuzzis, or fountains. In addition, the inactivation efficiency of short-term treatment (pressurization for 4 sec followed by momentary decompression) may be sufficient to enable rapid inactivation of bacteria in large volume water samples by scaling-up the system. These results suggest our system to be useful for the inactivation of bacteria in ballast water, which poses a major environmental hazard because it contains various microbes thought to disrupt local ecosystems when released at foreign ports. Our new system could be used in a variety of fields in which simple treatment is required for the inactivation of bacteria in liquid samples.

Acknowledgements

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References