Sterilization Ability by Microwave UV-ozone Generator

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Abstract: - The 253.7 nm UV light emitted from mercury lamps is known as germicidal radiation. Additionally, it emits UV light with a shorter wavelength of 184.9 nm. This UV wavelength can generate ozone (O_3) from oxygen molecules (O_2) in the air. Ozone has been shown to effectively inactivate various pathogenic microorganisms. However, they must decompose quickly because they are harmful to the human body. Methods for decomposing ozone include a thermal decomposition method, a chemical cleaning method, an activated carbon method, and the like. However, there is no practical or efficient treatment method. Ozone generation methods include the silent discharge method, electrolytic method, photochemical reaction method, high-frequency discharge method, and radiation method. The most efficient method is silent discharge, which is concerned with nitrogen oxide (NO_X) generation caused by nitrogen molecules in the air, and deterioration of the electrodes during use is problematic. Furthermore, nitrogen oxides react with water in the gaseous phase to produce nitric acid. Nitric acid reacts with several metals to form nitrates. A sterilization device using high-concentration ozone has not been put to practical use because of the lack of an efficient method for decomposing ozone and the corrosion of metals. The UV-ozone generation method using microwave plasma used in this study can generate UV light by applying 2.45 GHz microwaves to a quartz electrodeless bulb filled with mercury. By changing the glass material and emitting UV light at different wavelengths, it is possible to develop a UV-ozone generator that can selectively generate and decompose ozone. Furthermore, since 184.9 nm UV specifically acts on oxygen molecules, NO_X is not generated. Additionally, 253.7 nm UV, which is emitted at the same time as 184.9 nm UV, can easily decompose ozone into O_2.

Key-Words: - microwave, ozone, UV, sterilization, sterile, mercury bulb, active oxygen, spore bacteria.

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1 Introduction

Ethylene oxide gas (EOG) sterilization is frequently used in medical settings along with high-pressure steam sterilization. The EOG has high permeability and is suitable for sterilizing small spaces. Furthermore, it is possible to achieve sterilization at much lower temperatures than heat sterilization methods. Therefore, it is used to sterilize optical instruments with complex structures and nonheat-resistant materials. Generally, EOG sterilization requires a sterilization time of 2–4 hours under high EOG concentrations of 450–1200 mg/L. Furthermore, because the ethylene oxide (EO) used

for EOG sterilization is toxic, a long period of 8–12 hours of aeration is required after sterilization, [1].

EOG sterilization requires over half a day for the entire process from the start to the end of aeration. Furthermore, even when aeration is performed, high EO concentrations may remain in sterilized items, and the EO may be adsorbed by plastic, which takes several days to completely disappear. Therefore, the carcinogenicity and irritation of the skin and mucous membranes caused by EO remaining in sterilized products have become a problem.

Ozone has a strong bactericidal effect and can be used to construct automatic sterilization systems in clean rooms. Additionally, it is used as a disinfectant in various fields, including food and medical fields, [2], [3]. Additionally, ozone, similar to EO, is highly toxic and can be easily decomposed by UV at 253.7 nm. However, ozone sterilization has not yet been recognized as a sterilizing agent by the International Organization for Standardization (ISO), and no sterilization indicator bacteria have been established. Bacillus atrophaeus (ATCC 9372) is a spore-forming bacterium. Spore-forming bacteria continue to proliferate as a non-spore-forming type when there is a nutrient source in the medium; however, when the nutrient source is depleted, they stop proliferating and start forming spores. As shown in Figure 1, spores are formed inside bacteria, and spore-forming cells comprise a mother cell and a precursor spore that will become spores in the future. In the late spore formation stage, the mother cell lyses, and the mature precursor spores are released from the mother cell and become spores. The spores have a morphology that is different from that of the non-spore-forming type. The morphology and structure of a spore are the basic structures common to all spores and comprise a spore shell, cortex, and core from the outside.

The spore shell comprises two layers, an electron-dense unstructured outer shell and a lamellar inner shell. The spore shells account for approximately 50% of the total spore volume and weight, and its chemical composition is approximately 90% cysteine and keratin-like crystalline protein with a high cystine content, [4], [5], and its proportion to the total spore protein content is 38–80%, [6]. They contain carbohydrates and fats. The cortex is sandwiched between the spore shell and core, and its chemical component is peptidoglycan, similar to the cell walls of vegetative cells. Cortex peptidoglycan is composed of three types of subunits: tetrapeptide subunit, muramic acid lactam subunit, and alanine subunit, each of which accounts for 30, 55, and 15%, [7] The core, along with the surrounding spore cell membrane and germinated cell wall, is the part that grows into a vegetative cell after the spore germinates and passes through the post-germination growth period. DNA, RNA, liposomes, various enzymes, and acid-soluble low-molecular-weight proteins exist as polymeric substances, and Ca^{2+} and DPA, a component unique to spores, are especially abundant as low-molecular-weight substances; in addition, they contain many inorganic cations such as Mg^{2+}, Mn^{2+}, K^{+}, and Na^{+}, free amino acids such as glutamic acid, arginine, and lysine, and glycerate 3-phosphate, which are necessary for ATP production immediately after germination, [8].

Spores are in a dormant state with no metabolic activity and exhibit a high resistance level, not seen in other microorganisms, to chemicals such as acids and alkalis and physical environmental factors such as heat and UV radiation. When spores come into contact with an environment suitable for proliferation, they germinate, begin to multiply, and lose all their previous resistance. Because of this high resistance, it is used as an indicator bacterium for EOG sterilization, [9], in this study, we evaluated the bactericidal effect of this device by killing B. atrophaeus.

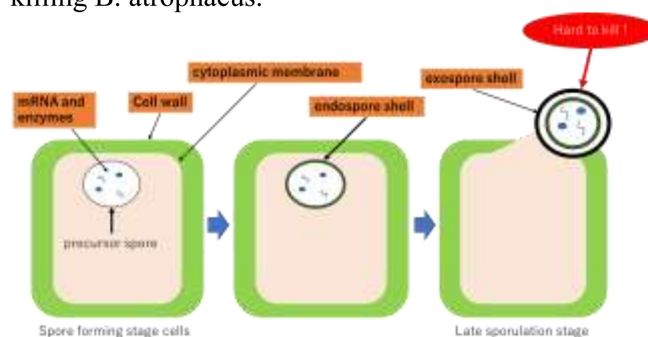


Fig. 1: Spore generation process

1.1 Ozone

Ozone is represented by the molecular formula O_3 and has a molecular structure in which three oxygen atoms are bonded in an isosceles triangular shape, are in an unstable state with one extra oxygen atom bonded to a stable oxygen, and it decomposes naturally and returns to oxygen. The single oxygen atom released when ozone decomposes has

extremely strong oxidizing power, and sterilizing and deodorizing functions. A total of 90% of ozone in nature exists in the stratosphere, approximately 10–50 km above Earth, and this layer is generally referred to as the ozone layer. The ozone layer absorbs harmful UV rays from the sun and protects the Earth's ecosystems. However, ozone, which has the second strongest oxidizing power after fluorine, is harmful to living organisms depending on its concentration. Ozone is currently used in various industrial applications. The use of ozone began with tap water sterilization; however, it was later used for decolorizing, deodorizing, and decomposing substances, such as phenol and cyanide. In addition, the application of ozone in water treatment, such as industrial wastewater treatment, has been put into practical use, and applications are being made at medical and food processing sites. Furthermore, the use of ozone has expanded, including precision cleaning for semiconductor device manufacturing and pulp bleaching. Furthermore, ozone treatment has many uses in cleaning the surfaces of materials, such as precision cleaning of substrate surfaces to produce new functional devices and surface contamination removal of optical parts for extreme ultraviolet light. Although ozone has a high utility value, its oxidizing power is extremely strong, and it is thought to reach deep into the lungs without being absorbed through the nose, throat, and trachea. The main effects of ozone on the human body include irritation of the mucous membranes of the eyes, nose, and throat as well as lung tissue damage, such as pulmonary dysfunction and pulmonary edema. In general, odors are detectable at concentrations of 0.01 ppm or higher. Exposure to 0.1 ppm can cause discomfort and nose and throat irritation. Changes in lung function can occur at 0.5–1 ppm. Exposure to 1–10 ppm can cause headaches and respiratory damage. Small animals may experience respiratory damage within 2 h of exposure to concentrations of 10 ppm or higher, [10]. The LC50 in mice and rats was approximately 6 ppm after 4 h of exposure, [11]. Therefore, for ozone to be used safely, it must be used at low concentrations or be quickly decomposed after use.

1.2 UV

UV radiation can be classified into three regions based on its wavelength: UVA (320–400 nm), UVB (320–280 nm), and UVC (280–200 nm). Among these, UVC radiation has the greatest impact on humans. give. The sun emits various electromagnetic waves, including UV light; however, because the ozone layer in the stratosphere absorbs UV light, UV light with wavelengths less than 280 nm does not reach the Earth's surface. Natural UV rays comprise only UVA and UVB radiation. However, artificial UV light sources contain UVC light, which has serious effects on the human body, especially the skin and eyes.

UV lamps are a technologically mature field, with many products already in use. Many types of UV lamps are used in industrial applications because of their strong emission intensity over a wide wavelength range. Examples include photochemical reactions such as photo-cleaning and photopolymerization, and representative markets include the manufacturing processes of liquid crystal panels and functional films for touch panels.

DNA is damaged by short-wavelength UV light (254 nm) emitted by germicidal lamps, [12]. This was reported by Setlow and his wife's research on Escherichia coli cell death and thymidine dimers (1962), and Cleaver's research on xeroderma pigmentosum and abnormal DNA excision repair ability (1968).

Furthermore, depending on the degree of damage caused by UV exposure, denatured lipids (lipid peroxides, free fatty acids, and small lipid fragments), which can serve as oxidative stress indicators, can be detected in the skin. These modified lipids are indicator markers and are cytotoxic.

UV is used in a variety of fields, and it must be used in a way that prevents it from directly irradiating the human body.

1.3 Mercury Excitation by Microwaves

The atoms have the lowest normal-state energy and K level (quantum number n=1). This state is known as the ground state. A state with a quantum number $n \geqq 2$ that has higher energy is called an excited state. Atoms become excited when they receive external energy such as light, heat, electric, or magnetic fields. The excited atom transitions to a lower energy level after a short time and emits light with a photon.

The energy is the energy difference between the two levels.

The mercury atoms in this study were excited by 2.45 GHz microwaves. As shown in Figure 2, [13], the emitted electromagnetic wave is caused by an excited electron transitioning from the 7d orbit to the ground state 6s orbit, emitting a dominant wavelength of 184.957 nm, and an excited electron transitioning from the 6p orbit to the ground state 6s orbit, emitting a wavelength of 253.652 nm.

Fig. 2: Mercury atomic energy level diagram

1.4 Principles of Ozone Generation and Decomposition

In this study, ozone was generated using a photochemical reaction, as shown in Figure 3. UV light with a wavelength of 184.9 nm decomposes oxygen molecules into atomic oxygen. When it combines with surrounding oxygen molecules, ozone is generated. In this study, a glass low-pressure mercury-sealed bulb that generates ozone is called a UV ozone-generating bulb.

$O_2 \xrightarrow{h\nu (\lambda = 184.9\ nm)} 2O$
Oxygen molecule — Atomic oxygen

$O + O_2 \rightarrow O_3$
Atomic oxygen — Oxygen molecule — Ozone

$O_3 \xrightarrow{h\nu (\lambda = 253.7\ nm)} O + O_2$
Ozone — Excited atomic oxygen — Oxygen molecule

Fig. 3: Ozone generation and decomposition process

In contrast, ozone decomposition requires a wavelength of 253.7 nm. Ozone is excited by 253.7 nm UV and separates into atomic oxygen and oxygen molecules. Two oxygen atoms react with each other and transform into an oxygen molecule. Additionally, as shown in Figure 4, atomic oxygen reacts with water molecules in the gas phase to generate OH radicals. In this study, the glass low-pressure mercury-sealed bulb that decomposes ozone is referred to as the UV ozone decomposition bulb.

Quartz was used as the UV-transmitting glass; however, impurities were added to the UV-ozone decomposition bulb to block 184.9 nm UV. Therefore, ozone was not generated and only UV light at a decomposition wavelength of 254.7 nm was emitted.

$O_3 \xrightarrow{h\nu (\lambda = 253.7\ nm)} O + O_2$
Ozone — Excited atomic oxygen — Oxygen molecule

$O + H_2O \rightarrow 2OH$
Excited atomic oxygen — Water molecule — OH radical

Fig. 4: Products during ozone decomposition

2 Experiments

2.1 Strain

B. atrophaeus is a spore-forming bacterium that normally exists as an actively proliferating type; however, in environments where it is difficult to survive, it forms spores and becomes dormant, stopping its growth. In general, the spore-forming state exhibits high heat and chemical resistance and is difficult to kill. In this study, we used proliferative and dormant forms of *B. atrophaeus*.

2.2 Spore Solution Preparation

A spore solution was prepared to obtain the dormant *B. atrophaeus*. After culturing the proliferative *B. atrophaeus* in TSA medium at 30 °C for over a week, colonies were collected on the medium using a disposable loop, dispersed in 10 mL of sterile distilled water, centrifuged at 4500 rpm for 10 min, and separated. At that time, given the difference in density, dormant *B. atrophaeus* was deposited in the lower layer of the sediment, and proliferative B. atrophaeus was deposited in the upper layer; therefore, only the proliferative type in the upper layer was removed using a spoon. The supernatant liquid was discarded, and the remaining dormant *B. atrophaeus* was dispersed again in 10 mL of sterile distilled water, and heat-treated at 75 °C for 30 min in a constant temperature water bath. Thereafter, centrifugation and removal of the supernatant were repeated thrice to prepare a dormant *B. atrophaeus* spore solution. After preparation, the state of spore formation was confirmed by Wiltz spore staining. In general, it is difficult to induce spore formation in

all bacterial cells. Therefore, we confirmed that over 80% of the spores were present within the field of view of the microscope and used them as dormant *B. atrophaeus* for the experiments. In addition, the prepared spore solution was dispensed into 1.5 mL tubes in 50 µL portions and stored frozen at 80 °C.

2.3 Bactericidal Effect

The prepared spore solution and 50 µL of the proliferative *B. atrophaeus* bacteria solution adjusted to McFarland No. 0.5 were diluted with 2 mL of physiological saline, filtered through a polycarbonate membrane filter (manufactured by ADVANTEC) with a pore size of 0.2 µm, and bacteria were allowed to adhere to the filter. The membrane filter was placed in a 200 L tube and exposed to ozone and UV for a predetermined period. The UV irradiance was measured using a spectroradiometer (USR-40D-14, Ushio Inc.), and the ozone concentration inside the case was measured using a gas sampling device (model GV-100, Gastech Inc.) and ozone gas. Measurements were performed using a detection tube (No. 18M, manufactured by Gastech), and the integrated value of the UV irradiance at 240–270 nm and the ozone concentration were controlled to be constant. After treatment, the membrane filter was placed in 5 mL of physiological saline and stirred for 1 min using a vortex mixer to transfer the attached bacteria to the membrane filter into saline. Physiological saline was diluted 5 times, and 10 µL was applied to the TSA medium, followed by culturing in a 30 °C incubator for 7 d, and the number of developed colonies was counted. Furthermore, the survival rate was calculated as the percentage of colonies that grew in the system exposed to ozone and UV light compared to the number of colonies that developed in the system not exposed to ozone and UV light, and the bactericidal effect was evaluated. That is, the lower the survival rate, the higher the bactericidal effect. Note that all measurements were performed at 20–25 °C and a humidity of 80% or higher.

3 Results and Discussion

3.1 Establishing Sterilization Conditions

It is generally known that the bactericidal effect of ozone increases as the humidity increases, [14]. This is because hydroxyl radicals (OH radicals) with strong oxidizing power are generated when ozone reacts with water molecules, [15]. Therefore, the humidity inside the device during sterilization was maintained at 80% or higher. The ozone concentration inside the device was measured at a humidity of 80% or higher. The ozone concentration used during sterilization was 60 ppm.

Figure 5 shows the correlation between the integrated value of UV irradiance between 240 and 270 nm from the UV ozone-generating bulb and the UV ozone-decomposing bulb, and the horizontal distance from each luminescent bulb. As is clear from Figure 5, both the luminescent spheres.

In addition, the integrated irradiance decreased with increasing horizontal distance. Based on this result, and considering workability, the horizontal distance from the luminescent bulb was adjusted to 20 cm for the UV ozone-generating bulb, and 20 cm for the UV ozone-generating bulb, so that the integrated value of UV irradiance from 240 to 270 nm was 300 µW/cm^2. The size of the ball was set at 15 cm.

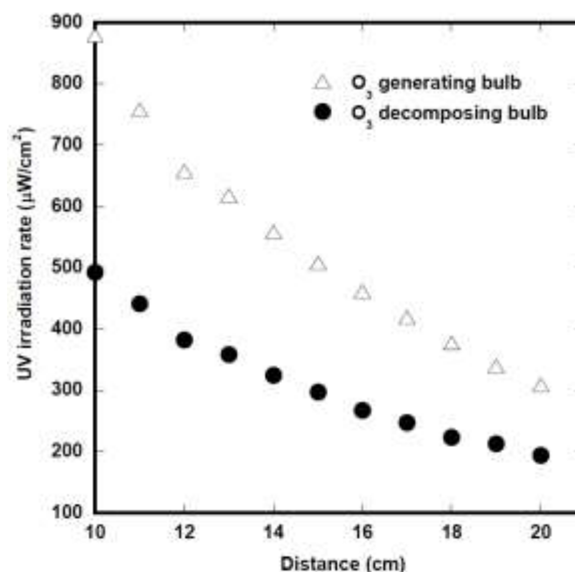


Fig. 5: Relationship between horizontal distance from each light emitting bulb and irradiance

3.2 Sterilization Effect of UV-ozone Sterilizer

Under sterilization conditions of 80% humidity or higher, ozone concentration of 60 ppm, and UV irradiance of 300 µW/cm^2 at 240–270 nm, a UV-ozone sterilizer was used to eliminate spores in UV-ozone combined treatment, UV alone treatment, and ozone alone treatment. The bactericidal effects on *B. atrophaeus* in the forming state and the dormant form in the spore-forming state were investigated. Here, a UV ozone-generating bulb that emits UV of 184.9 nm and 253.7 nm was used for the combined UV-ozone treatment. For the UV treatment alone, a UV ozone decomposition bulb that only emits 253.7 nm UV was used. For ozone treatment alone, a UV

ozone-generating bulb was used, and the UV light was blocked.

Figure 6 shows the survival rate of proliferating *B. atrophaeus* in the non-spore-forming state. The horizontal axis represents processing time. The survival rate of proliferating *B. atrophaeus* was approximately 1% after 10 min of treatment. The differential equation for the survival of *B. atrophaeus* growing in non-sporulating conditions was y = -70.58ln(x) + 190.15. These results show that the UV-ozone sterilizer has a high sterilizing effect on proliferating *B. atrophaeus*, in areas exposed to UV and in areas that are less likely to be exposed.

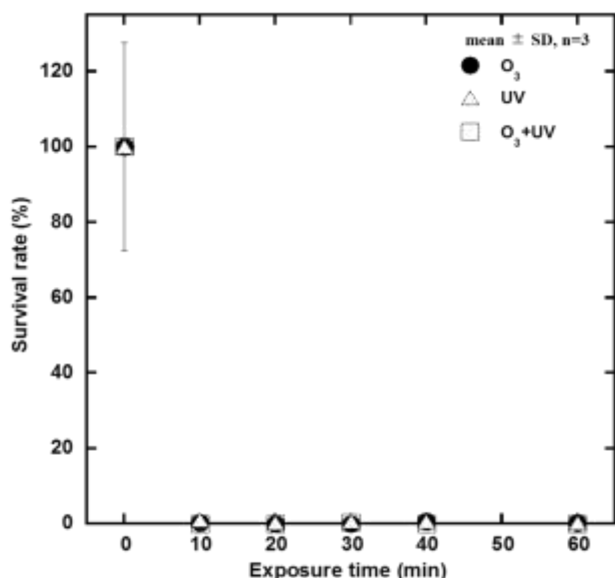
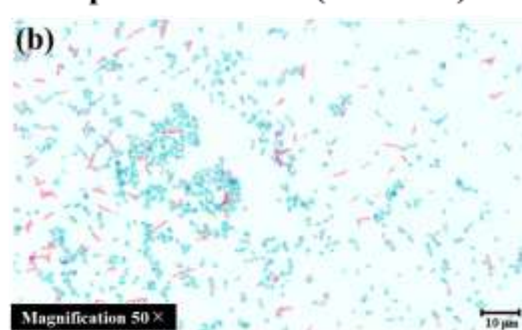


Fig. 6: The survival rate of proliferating B. atrophaeus in the non-spore-forming state

Next, we examined the survival rate of dormant *B. atrophaeus* in the spore-forming state. Figure 7 shows Wirtz spore-stained images of *B. atrophaeus* before and after the preparation of the spore fluid used as the sample. Generally, non-spore-forming bacterial cells are stained red, and spore-forming bacterial cells are stained green. As shown in Figure 7, no spores are present in the stained image before preparation (Figure 7(a)). However, spore formation was confirmed in most bacterial cells after preparation (Figure 7(b)). Therefore, we conducted measurements using dormant *B. atrophaeus*.

No spore formation(proliferative type)

Spore formation(dormant)



***B. atrophaeus* Wilts staining image**
(red: bacterial cells, green: spores)

Fig. 7: Differences in bacterial formation

The results of each process are shown in Figure 8. The horizontal axis is the same as Figure 6. The survival rate of dormant *B. atrophaeus* in the combined UV-ozone treatment and the UV treatment alone was approximately 1% at a treatment time of 10 min, which was similar to that of proliferating *B. atrophaeus*. However, when treated with ozone alone, the survival rate was approximately 50% after a treatment time of 10 min, decreased as treatment time increased, and decreased to approximately 1% after a treatment time of 60 min. From these results, it was found that the UV-ozone combination treatment showed a strong bactericidal effect against dormant *B. atrophaeus*. Furthermore, when compared with the results of UV and ozone treatments alone, it was suggested that the bactericidal effect of the combined UV-ozone treatment was mainly due to the bactericidal effect of UV. 253.7 nm UV is effective in killing spore-forming bacteria. For example, irradiation the surface of an aluminum plate (1276 cm^2) coated with *B. atrophaeus* spores with 253.7 nm UV light, and at an integrated UV light intensity of 100 mJ/cm^2, the number of viable bacteria on the plate decreased by 103–104, [16]. Thus, treatment with 253.7 nm UV is effective in killing spore-forming bacteria. However, sterilizing areas that are not easily exposed to UV, [17].

However, as mentioned above, although the bactericidal effect of ozone treatment alone was inferior to the bactericidal effect of the UV-ozone combination treatment and UV treatment alone, the survival rate decreased and the bactericidal effect increased as the treatment time increased. Therefore, long-term treatment with a UV-ozone sterilizer showed a strong sterilizing effect on dormant *B. atrophaeus*, even in areas that are not easily exposed to UV.

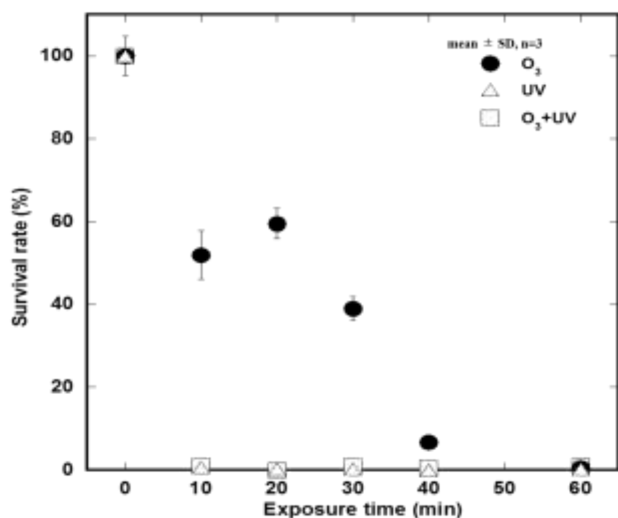


Fig. 8: The survival rate of dormant state B. atrophaeus in the spore-forming state

4 Conclusion

We prototyped a sterilization device (UV-ozone sterilization device) using the UV-ozone generation method and verified its effect on proliferative and dormant *B. atrophaeus*. The UV-ozone sterilizer showed a strong sterilizing effect on proliferating *B. atrophaeus*, regardless of the presence or absence of UV exposure. Furthermore, a strong bactericidal effect was observed against dormant *B. atrophaeus* in the UV-exposed areas. However, sufficient sterilization effects can be obtained by extending the treatment time, even in areas that are less likely to be exposed to UV radiation. These results revealed that the UV-ozone sterilizer was effective at sterilizing both proliferative and dormant *B. atrophaeus*.

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Contribution of Individual Authors to the Creation of a Scientific Article (Ghostwriting Policy)

- Naoki Kusumoto devised and created a sterilization system using ozone.
- Atsuya Watanabe sterilized the bacteria.
  Yuya Hasunuma invented the bacterial culture method
- Hiraoka created a microwave generator.
- Norimich Kawashima invented the low-pressure mercury bulb issuing system using microwaves
- Yoshikazu Tokuoka provided extensive guidance, including the adjustment and preparation of the entire experiment.
- Hitoshi Kijima supervised this study.

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