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ORIGINAL RESEARCH

Applicability assessment of ceramic microbeads coated with hydroxyapatite-binding silver/titanium dioxide ceramic composite earthplus™ to the eradication of *Legionella* in rainwater storage tanks for household use

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Abstract: Water environments appear to be the habitats of *Legionella* species. Legionellosis is considered as a preventable illness because bacterial reservoirs can be controlled and removed. Roof-harvested rainwater has attracted significant attention not only as a groundwater recharge but also as a potential alternative source of nonpotable water. We successfully developed ceramic microbeads coated with hydroxyapatite-binding silver/titanium dioxide ceramic composite earthplus™ using the thermal spraying method. The ceramic microbeads were demonstrated to have bactericidal activities against not only *Legionella* but also coliform and heterotrophic bacteria. Immersing the ceramic microbeads in household rainwater storage tanks was demonstrated to yield the favorable eradication of *Legionella* organisms. Not only rapid-acting but also long-lasting bactericidal activities of the ceramic microbead were exhibited against *Legionella pneumophila*. However, time-dependent attenuation of the bactericidal activities against *Legionella* were also noted in the sustainability appraisal experiment. Therefore, the problems to be overcome surely remain in constantly managing the *Legionella*-pollution by means of immersing the ceramic microbeads. The results of our investigation apparently indicate that the earthplus™-coated ceramic microbeads would become the favorable tool for *Legionella* measures in household rainwater storage tanks, which may become the natural reservoir for *Legionella* species. Our investigation would justify further research and data collection to obtain more reliable procedures to microbiologically regulate the *Legionella* in rainwater storage tanks.

Keywords: hydroxyapatite, silver, TiO_2, earthplus™, ceramic, *Legionella*, eradication, rainwater storage tank

Introduction

Legionellosis is an environment-related, acute respiratory infection caused by *Legionella* organisms.[1–11] Indeed, water environments appear to be the natural habitat and to serve as amplifiers for *Legionella* species, which are facultative intracellular parasitic microorganisms.[12–15] In Japan, roof-harvested rainwater has attracted significant attention not only as a water, especially in cases of gardening, watering, and car-washing. In fact, to encourage the use of roof-harvested rainwater for household purposes, not only the governmental bodies in Japan but also those in Australia,[16,17] Denmark,[18] and New Zealand,[19] have been providing subsidies to residents. However, there are insufficient data concerning the microbiological quality of roof-harvested

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rainwater and its potential health risks. We recently investigated the incidence of *Legionella* in household rainwater storage tanks in Azumino, the rural area of Nagano in Japan, and demonstrated that the indwelling of *Legionella* species in the storage tanks was observed in relation to both the presence of heterotrophic bacterial cell counts and the chemical oxygen demand values.[14]

Transmission of *Legionella* from the environment to humans occurs via inhalation or aspiration of *Legionella*-containing aerosols.[20,21] The water systems of artificial facilities, including potable water systems,[22,23] spa water,[24] and cooling towers[25,26] in large buildings, hotels, hospitals, and public baths that are contaminated with *Legionella* are the implicated sources of outbreaks and sporadic cases of Legionnaires' disease. Thus, it is important, from a public health perspective, not only to continually survey environmental water systems for the presence of *Legionella*[27–30] but also to make microbiological control of *Legionella* organisms in water environments. In particular, legionellosis occurs frequently in health care settings due to patients' susceptibility and complexity of hospital water distribution systems. Although hospitals aim to guarantee the absence of *Legionella* from their water distribution systems, there is no ideal method for ensuring total disinfection, and it is accepted that eliminating *Legionella*, once it has colonized a water supply, is extremely difficult.[31,32]

The new hydroxyapatite (HA)-binding silver (Ag)/titanium dioxide (TiO_2) ceramic composite, earthplus™, was successfully developed by Shinshu Ceramics Co., Ltd., Nagano, Japan. We have already demonstrated[33] that earthplus™-coated woven cotton and nonwoven polypropylene fabrics exhibited excellent bactericidal potentials against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* strains. The ceramic composite earthplus™, for which a Japanese patent[34] was obtained in 1998, can be coated on various materials, including fabrics, metals, and plastics commonly found in the health care environment and in welfare institutions. Several products incorporating earthplus™ are available on the market. Recently, we have successfully developed the additional product of ceramic microbeads coated with earthplus™.

The aim of the present study was to investigate the bactericidal properties of ceramic composite earthplus™-coated ceramic microbeads against *Legionella* species in rainwater, the causative pathogens of legionellosis, and also to assess the applicability of the ceramic microbeads to control *Legionella* pollution in household rainwater storage tanks for household use.

Materials and methods

Preparation of earthplus™-coated ceramic microbeads

Ceramic microbeads with the diameter of 3 mm, mainly composed of silica (SiO_2) and alumina (Al_2O_3), were successfully coated with HA-binding silver (Ag)/titanium dioxide (TiO_2) ceramic composite earthplus™ with the thermal spraying method and were developed by Shinshu Ceramics. The earthplus™-coated ceramic microbeads were loaded into mesh-polyethylene tubes (Φ34 mm) or mesh-polyester tubes (Φ11 mm) in cases of investigating rainwater in household roof-harvested rainwater storage tanks.

Bacterial strain used

Legionella pneumophila ATCC33215 strain, stored in Microbank vials (Pro-Lab Diagnostics, ON, Canada) at −80°C in a deep freezer, was used for the evaluation study.

Collection of rainwater samples investigated

Prior to initiating the evaluation study, we asked all of the household rainwater tank users who received subsidies from Azumino city, Nagano prefecture, Japan, to participate in the project. Among them, six users, designated as A, B, C, D, E, and F, agreed to participate in the present study, and gave us informed consents for the enrollment in the study.

Water samples from the six rainwater tanks, with internal capacities ranging from 100 L to 230 L, were collected in 1,000 mL sterile polypropylene containers, maintained at 4°C during transport to the laboratory, and tested within 6 hours. The weather, amounts of precipitation, and the atmospheric temperatures were also recorded during the days of this study.

Bactericidal activity of earthplus™-coated ceramic microbeads input into the rainwater against *L. pneumophila* ATCC33215

In order to obtain the optimal quantities of the earthplus™-coated ceramic microbeads input into rainwater, bactericidal activities of the ceramic microbeads (1 g, 0.5 g, 0.2 g, 0.1 g, and 0 g as control) were investigated, respectively, in 100 mL of rainwater from household storage tanks by adding *Legionella* cells. The preparation of *L. pneumophila* cell suspensions added into 100 mL of the rainwater was performed following the procedures recommended by the Japanese Industrial Standards L1902.2627. Prior to the examinations, *L. pneumophila* ATCC33215 strain was grown on

Glycin-Vancomycin-Polymyxin-Cycloheximide (GVPC) agar (Sysmex-Biomerieux Co., Tokyo, Japan) plates at 35°C for 72 hours. The colonies on the plates were then transplanted onto GVPC agar plates again and incubated at 35°C. After incubation for 72 hours, bacterial suspensions were made with sterilized physiological saline solution. The *Legionella* suspensions (1 mL), prepared to a McFarland No 1 standard, were put into 100 mL of the rainwater, incubated at room temperature, both with standing and with shaking (110 times min^{-1}), under both normal light and light-shielded conditions. Moreover, sustainability assessment of bactericidal activities on *Legionella* cells was made by prolonging incubation times with periodic addition of the *Legionella* suspension (1 mL) at the density of McFarland No 1 standard into 100 mL of rainwater.

Application trials of earthplus™-coated ceramic microbeads to the eradication of *Legionella*, coliform bacteria, and heterotrophic bacteria in household rainwater storage tanks

Prior to the trial studies, either 250 g or 500 g of earthplus™-coated ceramic microbeads were loaded into both the mesh-polyethylene-tubes (Φ34 mm) and the mesh-polyester (Φ11 mm) tubes, respectively. The six rainwater storage tanks of users A–F were used to assess the bactericidal activities of the ceramic microbeads on *Legionella* organisms, coliform bacteria, and heterotrophic bacteria in tanks. In evaluating the bactericidal activities against *Legionella*, the user D's rainwater storage tank with around 100 L of rainwater accumulated, into which the 250 g-loaded-mesh-polyethylene-tubes (Φ34 mm) was immersed, was examined by adding 3 mL of *Legionella* suspension adjusted to McFarland No 3 standard. On a different day, the same experiment was carried out by submerging the 500 g-loaded-mesh-polyethylene-tubes (Φ34 mm) in the tanks. In assessing the activities against coliform bacteria and heterotrophic bacteria, the storage tanks of users A–C, E, and F were subjected to the evaluation studies by putting either the 250 g- and/or 500 g-loaded-mesh-tubes (Φ11 mm or Φ34 mm) into each tank with around 100 L rainwater accumulated.

Detection of *Legionella* species in rainwater samples from the storage tanks and its quantitative cultures in assessing the bactericidal activities of ceramic microbeads

All of the rainwater samples in the household storage tanks were filter concentrated in a biological safety cabinet by pouring 500 mL water samples into a sterile 47 mm filter funnel assembly containing Millipore cellulose acetate membrane filters with pore size 0.22 μm (EMD Millipore, Billerica, MA, USA), using the vacuum source and side-arm flasks necessary to operate the apparatus. When the 500 mL water samples had passed through the filters, the filters were removed aseptically from the holders with sterile filter forceps, folded outwards, and placed in sterilized screw-capped containers with 5 mL of sterile water. The screw-capped containers were then vortexed for 1 minute to free bacteria and organic material from the filter to achieve 100-fold concentrated water samples. The concentrated water samples were heat-treated by placing in a water bath at 50°C for 20 minutes, as the heating procedure was routinely adopted for effective isolation of heat-resistant *Legionella* spp. Next, each 0.1 mL of the serially 10-fold filter-concentrated water samples were inoculated onto GVPC media and incubated at 35°C for 1 week. All suspected *Legionella* colonies were spread onto GVPC media and sheep blood agar (Nippon Becton Dickinson Co., Tokyo, Japan) media incubated at 35°C, for 1 week, respectively. Colonies grown on GVPC media but not on sheep blood agar media were subjected to further polymerase chain reaction (PCR) examination[35,36] to confirm the genus. In examining the bactericidal activities of the ceramic microbeads against *Legionella* in 100 mL of rainwater with addition of *Legionella* suspensions, almost exactly similar procedures as described earlier were employed.

Total viable counts of coliform and heterotrophic bacteria in rainwater samples from household rainwater storage tanks

Each rainwater sample was immediately diluted with sterile physiological saline solution in a serial 10-fold manner. Coliform bacterial counts were carried out by pouring 1 mL of each dilution of water samples collected from the rainwater tanks into deoxycholate agars (Nissui Pharmaceutical Co., Tokyo, Japan) in triplicate, using sterile glass rods to spread the samples evenly over the agar surfaces, incubated at 35°C for 24 hours. Counts of the total viable populations of heterotrophic bacteria were carried out by pouring 1 mL of each diluent of water samples collected from the rainwater tanks into R2A agar (Kanto Chemical Co., Tokyo, Japan) plates, and incubated at 20°C for 1 week. Other procedures were just the same as those used in cases of the coliform group counts. The agar plates containing the dilution yielding the largest number of colonies sufficiently distinct to allow accurate counting were chosen, and the total numbers of heterotrophic bacterial colonies were calculated. All the measurements were performed in triplicate.

Results

Bactericidal activity of the bead particles against *Legionella* in rainwater samples

Obvious bactericidal activities of the earthplus™-coated ceramic microbeads against *L. pneumophila* ATCC33215 were observed with standing culture under normal light conditions. The strongest bactericidal activity was demonstrated in adding 1 g of the microbeads, followed by 0.5 g, and 0.2 g, respectively. When adding 0.1 g of microbeads into 100 mL of rainwater, no obvious bactericidal activities were observed. That is, the ceramic microbeads exhibited bactericidal activities against *Legionella* cells additive amount-dependently, when >0.2 g of microbeads were added to the 100 mL of rainwater, as shown in Figure 1.

Effects of normal light and light-shielded conditions on the bactericidal activities of the ceramic microbeads against *Legionella* in 100 mL of rainwater with standing culture

When adding 1 g as well as 0.2 g of the ceramic microbeads into 100 mL of rainwater, light-shielded conditions were demonstrated to result in comparatively stronger bactericidal activities against *L. pneumophila* ATCC33215, compared with normal light conditions under standing cultures, as shown in Figure 2.

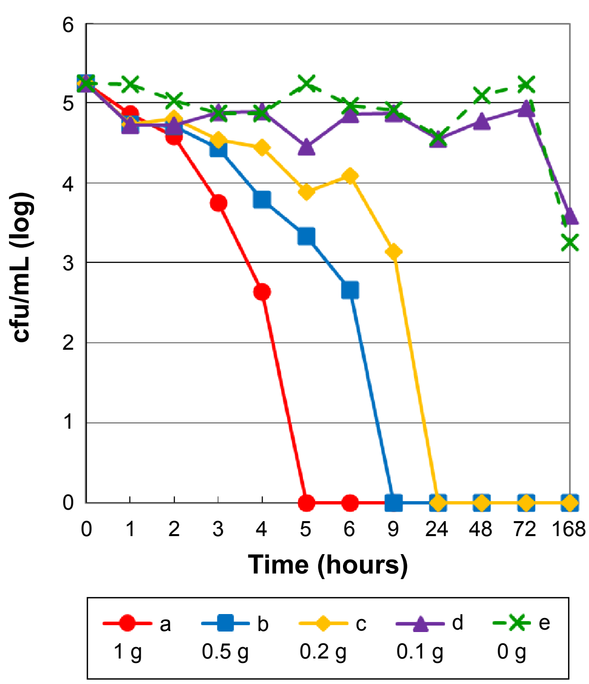

Figure 1 Bactericidal activities of earthplus™-coated ceramic microbeads against *Legionella pneumophila* ATCC33215 by the input amount into 100 mL of rainwater with standing culture under normal light conditions.

Notes: (a–e) Rainwater derived from the tank user D-owned rainwater storage tank; 1 g to 0 g: added grams of the ceramic microbeads input; Time 0: *L. pneumophila* ATCC33215 suspensions were added to the respective rainwater tank user-owned tank on the Time "0."

Abbreviation: cfu, colony-forming units.

Effects of standing and shaking cultures on the bactericidal activities of the ceramic microbeads against *Legionella* in 100 mL of rainwater under normal light conditions

As shown in Figure 3, shaking cultures yielded stronger bactericidal activities of the ceramic microbeads against *L. pneumophila* ATCC33215 in comparison with standing cultures in both the cases of 1 g and 0.2 g input into 100 mL of rainwater.

Sustainability assessment of bactericidal properties of the ceramic microbeads against *Legionella* periodically added into 100 mL of rainwater with standing culture under normal light conditions

The *L. pneumophila* ATCC33215 cell suspensions (1 mL) adjusted to McFarland No 1 standard were put into 100 mL of rainwater taken from the tank user D's rainwater storage tank, simultaneously with earthplus™-coated ceramic microbeads on the day September 3, 2013. As shown in Figure 4, the numbers of *L. pneumophila* added into 100 mL of rainwater were reduced to below the detection limit in 6 hours. Approximately 4 weeks later (October 1), additional 1 mL of *L. pneumophila* cell suspensions were put into the rainwater. The cell counts of *L. pneumophila* added were observed to decrease to undetectable levels in 48 hours. Two weeks later (October 15), additional 1 mL of the suspensions were put into the rainwater. The cell counts were reduced from >5 $\log_{10}$ colony-forming units (cfu)/mL to below 2 $\log_{10}$ cfu/mL in 192 hours. However, when examined on December 3, the numbers of the *L. pneumophila* cells were found to decrease to below the detection limit. After the additional input of *L. pneumophila* suspensions (December 3), the cell counts decreased from >5 $\log_{10}$ cfu/mL to 3 $\log_{10}$ cfu/mL in 192 hours.

Bactericidal activities of the ceramic microbeads-loaded-mesh-polyethylene (Φ34 mm)- or mesh-polyester (Φ11 mm)-input into household rainwater tanks against added *L. pneumophila* ATCC33215

As the numbers of inhabiting *Legionella* organisms in all of the household rainwater tanks were demonstrated to be

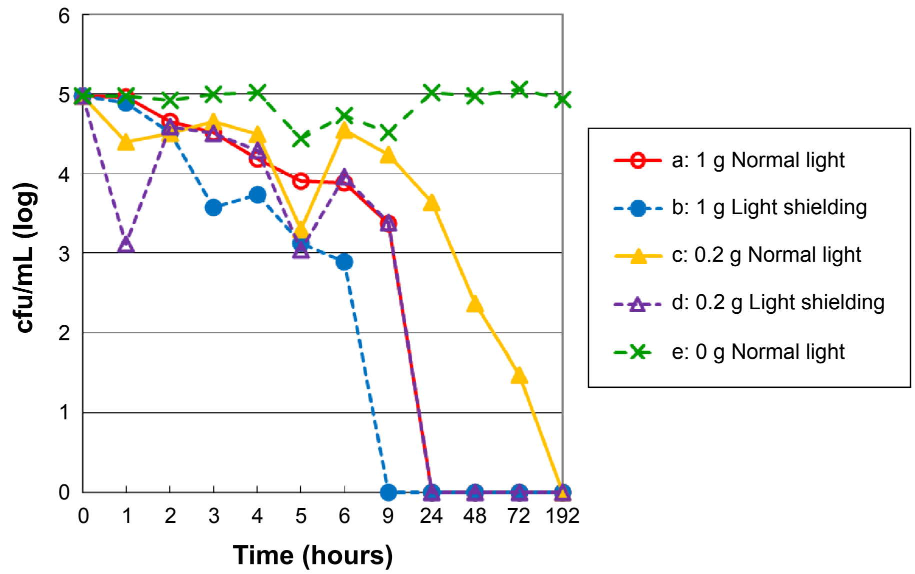

Figure 2 Effects of normal light and light shielding conditions on the bactericidal activities of the earthplus™-coated ceramic microbeads against *Legionella* in 100 mL of rainwater with standing culture.
Notes: (a–e) Rainwater derived from the tank user D-owned rainwater storage tank; 1 g to 0 g: added grams of the ceramic microbeads input; Time 0: *Legionella pneumophila* ATCC33215 suspensions were added to the respective rainwater tank user-owned tank on the Time "0."
Abbreviation: cfu, colony-forming units.

extremely small for the evaluation study, the *L. pneumophila* ATCC33215 suspensions (3 mL) adjusted to McFarland No 3 standard were put into the tank user D's rainwater storage tank, in which the 250 g- or the 500 g-loaded-mesh-polyethylene-tubes (Φ34 mm) were simultaneously immersed. As shown in Figure 5, when submerging 500 g-loaded-mesh-polyethylene-tubes (Φ34 mm) into the tank, counts of simultaneously-added *Legionella* cells were found to reduce to below the detection limit in 1 day by means of culture method, despite being PCR-positive. However, the presence of *Legionella* was not detected by either the culture and PCR methods in one additional day. The state of cell counts being below the detection limit lasted for 28 days. Almost similar reduction phenomena were observed

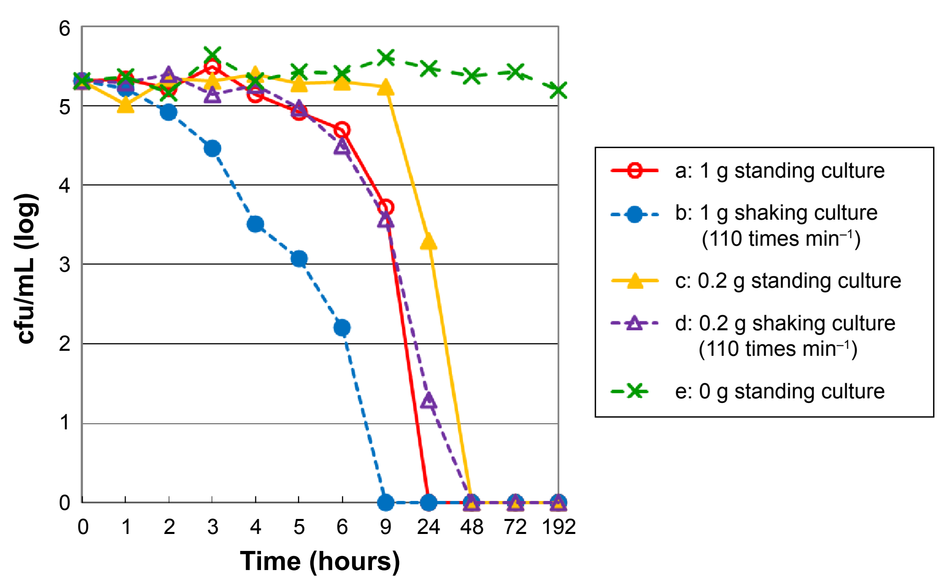

Figure 3 Effects of standing and shaking cultures on the bactericidal activities of the earthplus™-coated ceramic microbeads against *Legionella pneumophila* ATCC33215 in 100 mL of rainwater under normal light.
Notes: (a–e) Rainwater derived from the tank user D-owned rainwater storage tank; 1 g to 0 g: added grams of the ceramic microbeads input; Time 0: *L. pneumophila* ATCC33215 suspensions were added to the respective rainwater tank user-owned tank on the Time "0."
Abbreviation: cfu, colony-forming units.

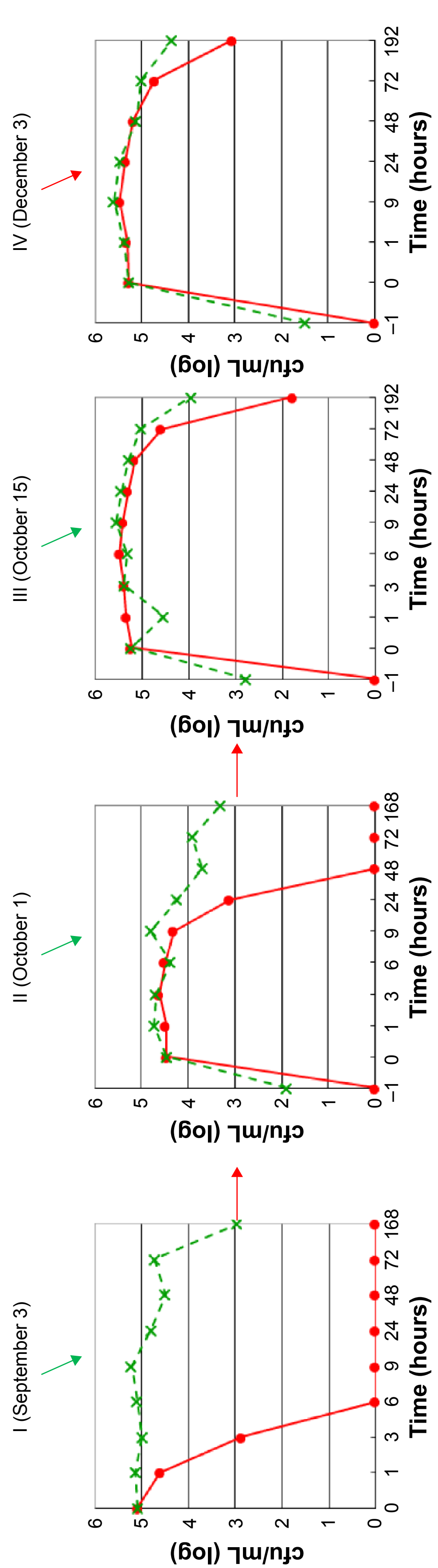

Figure 4 Sustainability appraisal of bactericidal activities of earthplus™-coated-ceramic microbeads-input into 100 mL of rainwater against periodically added *Legionella pneumophila* ATCC33215 with standing culture under normal light conditions.
Notes: At the respective time "0" on the day indicated above. —●—: 1 g of earthplus™-coated ceramic microbeads put into 100 mL of rainwater. —×—: control; no addition of earthplus™-coated ceramic microbeads into 100 mL of rainwater.
Abbreviation: cfu, colony-forming units.

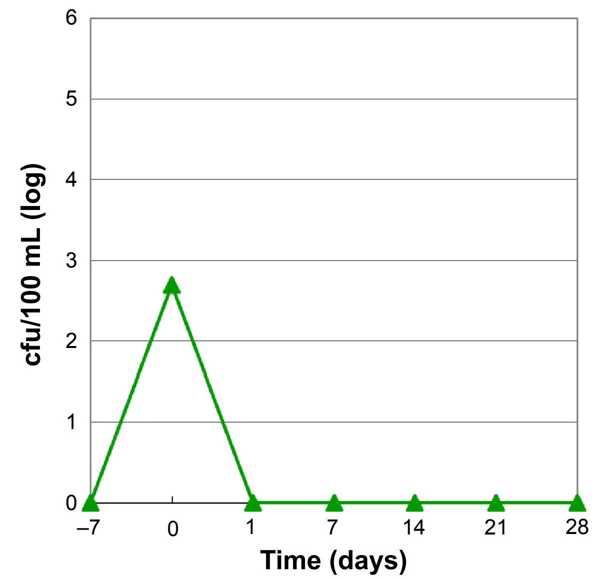

Figure 5 Bactericidal activities of earthplus™-coated ceramic microbeads (500 g)-loaded mesh-polyethylene-tube (Φ34 mm) input into household rainwater tank against added *Legionella pneumophila* ATCC33215.
Notes: Day 0: *L. pneumophila* ATCC33215 cells were added to the rainwater tank of tank-user D on the day "0".
Abbreviation: cfu, colony-forming units.

when immersing 250 g-loaded-mesh-polyethylene-tubes (Φ34 mm) in the tank (data not shown).

Bactericidal activities of the ceramic microbeads (500 g)-loaded mesh-polyethylene-tube (Φ34 mm) or mesh-polyester-tube (Φ11 mm) input into household (A–F) rainwater tanks against coliform and heterotrophic bacteria in rainwater tanks

The reduction phenomena of viable cell numbers of coliform bacteria were observed in all the household tanks except one tank (C), as shown in Figure 6. Afterward, a reducing state of the coliform bacterial cell counts persisted for about 7 weeks. Moreover, when the reducing tendencies during the interval of 14 days to 28 days of tanks A–D were compared with those of tanks E and F, bactericidal activities of mesh-polyester-tube (Φ11 mm) to coliforms were demonstrated to be superior to those of mesh-polyethylene-tube (Φ34 mm). Almost similar phenomena were observed when applying 250 g-loaded mesh-polyethylene (Φ34 mm) or mesh-polyester (Φ11 mm) tubes (data not shown). In investigating the bactericidal activities of the 250 g- and 500 g-ceramic microbeads-loaded mesh-polyethylene (Φ34 mm) or mesh-polyester (Φ11 mm) tubes against heterotrophic bacteria, almost similar reduction curves were obtained (data not shown).

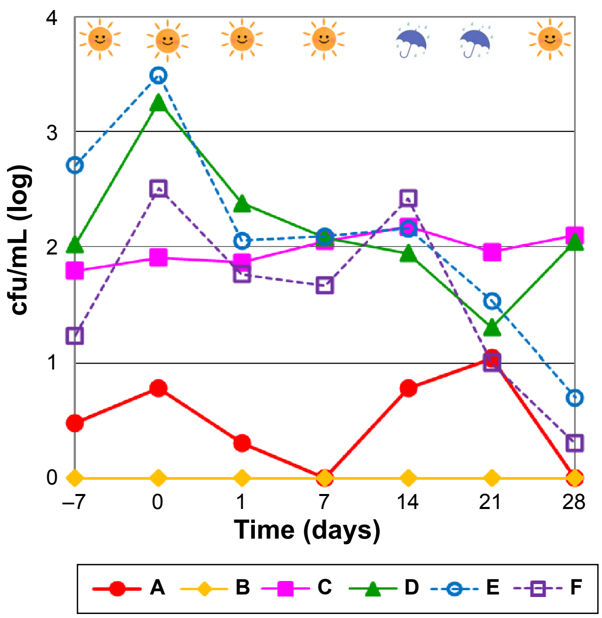

Figure 6 Bactericidal activities of earthplus™-coated ceramic microbeads (500 g) input into household (**A**–**F**) rainwater tanks against coliform bacteria in rainwater tanks.

Notes: Tank-users (**A**–**D**) earthplus™-coated ceramic-beads (500 g)-loaded mesh-polyethylene-tube (Φ34 mm). Tank-users **E** and **F**: earthplus™-coated ceramic-beads (500 g)-loaded mesh-polyester-tube (Φ11 mm). Day 0: the respective ceramic microbeads-loaded mesh tubes were put into each rainwater tank on the day "0".

Abbreviation: cfu, colony-forming units.

Discussion

HA-binding Ag/TiO_2 ceramic composite earthplus™ was developed by Shinshu Ceramics Co., Ltd, Nagano, Japan, and can be coated on various materials, including fabrics (curtains, linen, clothes, and socks), metals, and plastics commonly found in the health care environment and in welfare institutions. Several products coated with earthplus™ are already available on the market: an air purifier with earthplus™-coated filters,[37] a water-purifier tank with earthplus™-coated plates, and a mask coated with earthplus™.[38] Additional products of the ceramic microbeads were newly devised by successfully coating earthplus™ by means of thermal spraying procedures.

TiO_2, the component of "earthplus™," is now recognized[33] to generate strongly oxidizing substances against microbes, molds, or odors under visible-light irradiation, and it degrades or decomposes them into carbon dioxide, water, and/or other smaller molecules. The energy from photons generates an electron-hole pair on the TiO_2 surface, which reacts with hydroxide ions (OH^-) in water to yield a hydroxyl radical (OH^+). In addition, the electron in the conduction band can reduce O_2 to produce superoxide anions (O_2^-). Both the OH^+ and the O_2^- are extremely reactive against organic compounds and promptly transform them into nontoxic materials. TiO_2 is one of the most promising photocatalysts, not only in water environments but also under dry conditions. TiO_2 materials only behave as an accelerator without their structure being altered, and theoretically, their catalytic activities should last indefinitely.

Our investigation, as shown in Figures 1–6, apparently demonstrated that the ceramic microbeads should have bactericidal activities against not only *L. pneumophila*, the causative agents of legionellosis, but also coliform and heterotrophic bacteria in household rainwater storage tanks. Their bactericidal activities, especially against *Legionella* cells, were comparatively stronger under light-shielded conditions than under normal light conditions, as shown in Figure 2, and the findings are favorable as the ceramic microbeads are to be utilized by immersing them in dark rainwater storage tanks.

In recent years, contamination of water systems has gradually become recognized as an important risk factor all over the world,[39–44] and many cases of Legionnaires' disease have been detected in *Legionella*-contaminated water systems.

As shown in Figure 6 of long-term trials with periodical input of *Legionella* cells, the bactericidal activities of the earthplus™-coated ceramic microbead were demonstrated to be persistently exhibited against *L. pneumophila.* The findings that the bactericidal activities against *Legionella* are long-lasting were actually favorable.

Water is indeed the major natural habitat for *Legionella* species, and colonization of water distribution systems depends on a combination of several factors, including water stagnation, sediment accumulation, commensal microflora, and water temperature. *Legionella* species can grow at temperatures of 25°C–45°C; therefore, rainwater storage tanks usually installed in the household gardens would become relatively optimal environments for inhabiting *Legionella* species.

We previously clarified that coliform and heterotrophic bacteria multiply profusely during ongoing sunny days in household rainwater storage tanks. On the other hand, with ongoing rain, coliform and heterotrophic bacterial counts decreased, probably because of dilution by the continual flow of rainwater. In the experiment of bactericidal activities of the ceramic microbeads (500 g)-loaded mesh-polyester-tube (Φ11 mm), it should be noted that no obvious coliform bacterial multiplication was observed during sunny days, as shown in Figure 6. Moreover, almost similar phenomena were observed as in the experiment of applying the ceramic microbeads (500 g)-loaded mesh-polyethylene-tubes (Φ34 mm).

Although earthplus™-coated ceramic microbeads possess bactericidal properties against *Legionella* cells, time-dependent attenuation of the bactericidal activities against *Legionella* were also noted in the sustainability appraisal experiment as shown in Figure 4. Therefore, the problems to be overcome surely remain in constantly managing the *Legionella*-pollution by means of immersing the ceramic microbeads periodically.

Legionellosis is considered as a preventable illness because bacterial reservoirs can be controlled and removed. Indeed, rainwater storage tanks may become the natural reservoir for *Legionella* species. The results of this investigation apparently indicate that earthplus™-coated ceramic microbeads would become the favorable tool for *Legionella* measures in household rainwater storage tanks. Our investigation indicates the need for further research and data collection to obtain more reliable procedures to microbiologically regulate the *Legionella* in rainwater storage tanks.

Acknowledgments
This study was conducted in co-operation with Azumino city. We would like to express our sincere appreciation to Azumino city for their support and are also grateful for the active support of the local communities of six rainwater tank users (A–F) in Azumino city during the course of this study.

Disclosure
The authors report no conflicts of interest in this work.

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