Application of Bacteriophages to Reduce Biofilms Formed on Surfaces in a Rendering Plant by Hydrogen Sulfide-Producing Bacteria

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Application of Bacteriophages to Reduce Biofilms Formed on Surfaces in a Rendering Plant by Hydrogen Sulfide-Producing Bacteria

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Abstract

Hydrogen sulfide producing-bacteria (SPB) in raw animal by-products are likely to grow and form biofilms in the rendering processing environments resulting in the release of harmful hydrogen sulfide (H₂S) gas. The objective of this study was to reduce SPB biofilms formed on different surfaces typically found in rendering plants by applying a bacteriophage cocktail. Using a 96-well microplate method, three SPB strains of *Citrobacter freundii* and *Hafnia alvei* were separately determined as strong biofilm formers. Application of nine bacteriophages (10⁷ PFU/mL) from families of *Siphoviridae* and *Myoviridae* resulted in 33-70% reduction of biofilm formation by each SPB strain. On stainless steel and plastic templates, phage treatment (10⁸ PFU/mL) reduced the attached cells of a mixed SPB culture (no biofilm) by 2.3 and 2.7 log CFU/cm² within 6 h at 30°C, respectively, as compared to 2 and 1.5 log CFU/cm² reductions of SPB biofilms within 6 h at 30°C. Phage treatment was also applied to indigenous SPB biofilms formed on the environmental surface, stainless steel, HDPE plastic, and rubber templates in a rendering plant. With phage treatment (10⁹ PFU/mL), SPB biofilms were reduced by 0.7-1.4, 0.3-0.6 and 0.2-0.6 log CFU/cm² in spring, summer and fall trials, respectively. Our study demonstrated that bacteriophages could reduce the selected SPB strains either attached to or in formed biofilms on various surfaces effectively and indigenous SPB biofilms on the surfaces in the rendering environment to some extent.

Key words: Bacteriophage, Hydrogen Sulfide-Producing Bacteria, Biofilm, Rendering Plant.
Introduction

Hydrogen sulfide producing bacteria (SPB) are a group of microorganisms such as *Pseudomonas* and *Citrobacter* that are able to utilize sulfur and sulfur-containing compounds as electron acceptor and produce hydrogen sulfide (H$_2$S) gas. These microorganisms dwell in animal hide/skin and the gastrointestinal tracts and are also known to cause spoilage of raw animal by-products at ambient temperature (Gram and Huss 1996). H$_2$S produced by SPB can be harmful to workers’ health and corrode processing equipment with iron containing surfaces due to the reaction between H$_2$S and Fe$^{2+}$ (Werner et al., 1998; USDL-OSHA, 2003; Sheng et al., 2007; Xu et al., 2008; Sun et al., 2011). SPB readily form biofilms in spite of being able to exist in their planktonic form on animal carcasses. For example, SPB biofilm formation has been documented on tongues which are capable of generating volatile sulfur compounds and other malodor gasses (Washio et al. 2005; Taylor and Greenman 2010). These biofilms may increase health risks by corroding water mains via H$_2$S production (Seth and Edyvean 2006). In a rendering facility, raw animal by-products are the source of SPB, which can easily contaminate processing equipment, such as storage tanks and grinders, and may form biofilms on the surfaces. However, SPB biofilm formation within the rendering environment has yet to be published.

Rendering facilities desire non-corrosive agents that can reduce or eliminate biofilms. Bacteriophages, specific viruses to bacteria, have been applied to foods, food contact surfaces and animals in order to control harmful bacteria (Whichard et al. 2003; Atterbury et al. 2007; Patel et al., 2011). Although many factors such as temperature, phage level, and incident of phage attachment to bacterial cells can affect the effectiveness of phage treatment, some researchers have successfully applied bacteriophages to control the biofilms formed by *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and
Pseudomonas aeruginosa (Verma et al. 2009; Ahiwale et al. 2011; Pires et al. 2011; Kay et al. 2011; Kelly et al. 2012; Montanez-Izquierdo et al. 2012). Phage treatment could also significantly reduce biofilms on different surface types such as glass, stainless steel, plastic, and silicone rubber (Tait et al. 2002; Sharma et al. 2005; Kay et al. 2011; Ahiwale et al. 2011; Montanez-Izquierdo et al. 2012). However, there is no research in regards to SPB biofilm reduction via bacteriophage within the rendering environment.

Our previous studies have isolated SPB-specific bacteriophages and demonstrated effectiveness of this biological control approach to treat SPB attached to various surfaces and animal by-products (Gong et al. 2013; Gong et al. 2014). Therefore, the objective of this study was to determine if our bacteriophage cocktail is capable of reducing biofilms formed by SPB on surfaces in a rendering facility.

Materials and Methods

Bacterial cultures and bacteriophage preparation

Both bacterial cultures and phage stocks were prepared as described previously (Gong 2014). Briefly, three selected SPB strains (Citrobacter freundii strain S12 and Hafnia alvei strains S183 and S211) isolated from meat and raw animal by-products were grown overnight in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA) at 37°C with shaking (Gong et al., 2014). Bacterial cells were collected by centrifugation, washed in 0.85% saline, and adjusted to an optical density of 0.5 at a wavelength of 600 nm (approximately 8 log CFU/mL).

SPB-specific phages were isolated from various raw animal by-products using SPB strains as hosts, and characterized through host ranges determination, restriction enzyme analysis, and transmission electron microscopy (Gong et al. 2013). Phage stock solutions were prepared
according to Heringa et al. (2010). Prior to each experiment, bacteriophage stocks were warmed at 37°C for 30 min to reduce clumping and then diluted to the desired concentrations using SM buffer [100 mM NaCl, 8 mM MgSO$_4$•7H$_2$O, 50 mM Tris-HCl (pH 7.5)]. Phage titers were determined by the double agar layer plaque assay according to Heringa et al. (2010). Based on the host range and lytic activity, nine SPB-specific bacteriophages were selected as the bacteriophage cocktail including Siphoviridae phages (211a, 214a, 214c, 217a, 218a and 12a) and Myoviridae phages (213a, 214b and 201a). All phages were confirmed as lytic phages using a mitomycin C test (Heringa et al. 2010). Among our SPB phage stock collection, Citrobacter S12 was only sensitive to phage 12a, Hafnia S183 was sensitive to phages 213a, 214b, 214c, 217a, 218a, and Hafnia S211 was sensitive to 211a, 214a, 214c, 217a and 218a. The cocktails consisted of equal amounts of phages and final titers were ca. $1 \times 10^7$, $10^8$ or $10^9$ PFU/mL for following studies.

**Bacteriophage treatment of SPB biofilms in 96-well microplate**

The efficacy of bacteriophage treatment on SPB biofilm formation was studied using a 96-well microplate as a model system (Coenye and Nelis, 2010). Three SPB strains (S12, S183 and S211) exhibiting strong biofilm formation ability were used for the following studies. In each well of 96-well microplate, SPB cultures at a concentration of ca. $1 \times 10^4$ CFU/mL were mixed separately with a nine-phage cocktail at a final titer of ca. $1 \times 10^7$ PFU/mL (treatment) or SM buffer (control). After static incubation at 30°C for 48 h, each well was washed with sterile distilled water and allowed to air-dry. Biofilms were stained by 1% crystal violet solution (Becton Dickinson, Sparks, MD) at 22°C for 45 min and then measured by a spectrometer (µQuant; Bio Tek, Winooski, VT) at 600 nm. A cut-off optical density (O.D.c) value was set as
0.042, which was determined as three standard deviations above the mean O.D. of the negative control.

**Bacteriophage treatment of SPB attached to stainless steel and high density polyethylene plastic (HDPE) templates**

Stainless steel and HDPE plastic templates (n = 16 for each; 5 × 5 × 0.3 cm; Westview plastics, Inc. Anderson, SC) were washed with detergent (Micro-90®, International Products Corporation, Burlington, NJ), and rinsed with sterile nano-pure water. Each dry template was inoculated with 100 µL mixed bacterial culture containing equal amount of those three selected SPB strains at a final concentration of ca. 1 × 10^5 CFU/mL. A sterile inoculating loop (VWR, Radnor, PA) was used to evenly spread the inoculum over entire template surface. Templates were allowed to air-dry for 6 h inside a biological hood at room temperature and washed once with 10 mL of sterile phosphate buffered saline (PBS) in order to remove any unattached cells. Bacteriophages at a final titer of ca. 1 × 10^8 PFU/mL were applied to each template which was submerged in 30 mL of 10% TSB inside a petri dish, and the SM buffer was used as control. Diluted TSB was used to simulate the limited nutrient condition observed in a rendering plant. The submerged templates were incubated at 30°C for up to 8 h. At each pre-determined sampling intervals (2, 4, 6 and 8 h), the surfaces of the templates were swabbed five times using a sterile cotton swab and then transferred into 5 mL of sterile saline in a sterile tube. The suspension was centrifuged at 5,000 g for 10 min to separate bacterial cells from remaining bacteriophage particles, and SPB population was enumerated on tryptic soy agar (TSA) plates after incubated at 37°C for 24 h (Becton Dickinson, Sparks, Maryland, USA).

**Bacteriophage treatment of SPB biofilm formed on stainless steel and HDPE plastic templates under laboratory condition**
To produce SPB biofilm, the templates were submerged in 30 mL of 20% TSB (for biofilm formation) inoculated with the mixture of three selected SPB strains at a final concentration of ca. $1 \times 10^5$ log CFU/mL and incubated at 30°C for 2 d. Templates were washed once with 10 mL sterile phosphate buffered saline (PBS) to remove non-biofilm cells as described above. A cocktail of nine bacteriophages at a final titer of ca. $1 \times 10^8$ PFU/mL was applied to each template submerged in SM buffer inside a petri dish, and the control consisted of SM buffer only. The templates were incubated at 30°C for up to 6 h and SPB populations were enumerated as described above.

**Bacteriophage treatment of SPB biofilms formed on environmental surface, stainless steel, HDPE plastic and rubber templates in a rendering facility**

In order to investigate the efficacy of phage treatment of naturally formed SPB biofilm in real-world, field studies were performed in spring, summer and fall of 2011 in a rendering plant located in South Carolina. Templates used for laboratory studies were also employed in the rendering facility and pre-treated as described above. To produce indigenous SPB biofilm, templates were set onto raw material receiving area of the rendering facility using mounting tape. Throughout the experiment, the templates were subjected to routine processing and cleaning procedures. Templates with indigenous SPB biofilm were aseptically removed after 7 d. Environmental surfaces with the same dimensions near the mounted templates were also swabbed with cotton swabs (Puritan Medical Products, Guilford, ME) and placed in sterile tubes containing 5 mL of 0.85% saline. Upon arrival at the lab, both templates and swab samples were treated with bacteriophages at a final concentration of ca. $1 \times 10^9$ PFU/mL at room temperature (22~25°C) for 6 h. Other experimental settings and enumeration method of SPB population were the same as described above.
Statistical Analysis

Bacterial count data were converted to $\log_{10}$ CFU per mL, g or cm$^2$ for statistical analysis. An analysis of variance (ANOVA) for a completely randomized design was conducted to determine if general differences existed between treatment means using the general linear model (GLM) procedure. Specific comparisons among different phage treatments were accomplished with Tukey’s test. All statistical analyses were performed using Statistical Analysis System 9.1 (SAS; SAS Institute, Cary, NC, USA).

Results and Discussion

Bacteriophage treatment of SPB biofilms in 96-well microplate

In the control group, the biofilms formed by three selected SPB strains S12, S183 and S211 were determined as O.D. values of 0.34, 0.65 and 0.66, respectively, and the biofilm of strain S12 was ca. 46% less than other two strains (Fig. 1). With phage treatment at final titer of ca. $1 \times 10^7$ PFU/mL for 48 h, biofilms of SPB strains S12, S183 and S211 decreased to O.D. values of 0.23, 0.27 and 0.19, respectively, indicating biofilm reductions of S12, S183 and S211 by 33, 59 and 70% at 30°C, respectively.

In this 96-well microplate test, all three selected SPB strains formed biofilms having optical density (O.D.) values greater than $4 \times$ O.D.c, i.e. 0.168 (Fig. 1). This suggests that all three selected SPB strains are strong biofilm producers and may be capable of forming competent biofilms on the surfaces in the rendering plant caused by SPB contamination from raw animal materials. In agreement with our results, Hood and Zottola (1997) reported the same strong biofilm formers *C. freundii* and *H. alvei* isolated from the surfaces in a meat processing plant. In their study, the populations of these two gram-negative bacteria attached to the surfaces ranged...
from 3.7 to 4.9 log CFU/cm\(^2\). The attachment of bacterial cells and biofilm formation of bacteria occurred with a high population on the surfaces that were in contact with raw animal materials and were also observed on cleaned surfaces in the meat processing plant reaching bacterial density in a range of ca. <1-3 log CFU/cm\(^2\) (Jessen and Lammert 2003). Studies have demonstrated that biofilms could form a protective layer in meat processing plants and were capable of harboring foodborne pathogens such as \(L.\) monocytogenes, \(Salmonella\) spp. and \(Escherichia\) coli O157:H7 (Chasseignaux et al. 2002; Marouani et al. 2009, Wang et al. 2013), which may contaminate the food products and cause foodborne illness outbreaks.

**Bacteriophage treatment of SPB attached to stainless steel and HDPE plastic templates**

Initial populations of three selected SPB strains mixture attached to stainless steel and HDPE plastic templates were approximately 3.8 and 3.2 log CFU/cm\(^2\), respectively (Fig. 2A-B). The population of three selected SPB strain mixture started to increase after 2 and 4 h in the control on stainless steel and HDPE plastic templates, respectively, followed by a rapid increase of SPB population to ca. 5.3 and 5.6 log CFU/cm\(^2\) at 8 h, respectively. For phage treatment, reductions of SPB population on stainless steel and HDPE plastic templates occurred immediately after phage treatment with the highest reductions were observed at 4 and 6 h, respectively. Overall, phage treatment with a final titer of ca. \(1 \times 10^8\) PFU/mL reduced the attached three selected SPB strain mixture on stainless steel and HDPE plastic templates up to 2.3 and 2.7 log CFU/cm\(^2\) at 30°C, respectively, as compared with the control. In agreement with these results, Sharma et al. (2005) reported that phage KH1 treatment with a titer of 7.7 log PFU/mL reduced \(E.\) coli O157:H7 attached to stainless steel surface from 2.6 to 1.4 log CFU/coupon within 1 d at 4°C. Moreover, Patel et al. (2011) applied a cocktail of 6 selected phages with a titer of 8 log PFU/mL
to reduce *E. coli* O157:H7 attached to steel harvester blade resulting in a reduction of 4.5 log CFU/blade within 2 h at 22°C.

**Bacteriophage treatment of SPB biofilm formed on stainless steel and HDPE plastic templates under laboratory condition**

As a novel biocontrol method, bacteriophage treatment has been successfully demonstrated with high effectiveness for eliminating biofilm formation on a variety of surfaces in many studies (Sharma et al., 2005; Sillankorva et al., 2010; Montanez-Izquierdo et al., 2012). In our study, initial populations of biofilms formed on stainless steel and HDPE plastic templates by a mixture of three selected SPB strains were approximately 6 and 6.2 logs CFU/cm$^2$, respectively (Fig. 3A-B). SPB biofilm population on both templates increased slightly during 6 h incubation for the control. With phage treatment, reductions of SPB population on both surfaces were observed starting from 2 h until the end of experiment, and highest reductions of three selected SPB strains were observed at 6 h. Phage treatment with a final titer of ca. $1 \times 10^8$ PFU/mL reduced the biofilms formed on stainless steel and HDPE plastic templates up to 2 and 1.5 log CFU/cm$^2$ at 30°C, respectively, as compared with the control.

**Bacteriophage treatment of SPB biofilms formed on environmental surface, stainless steel, HDPE plastic and rubber templates in a rendering facility**

Field studies of phage treatment applied to SPB biofilms were conducted in a rendering plant located in South Carolina where the average environmental temperatures of spring, summer and fall trials were 22, 30 and 14°C, respectively. In the spring trial, initial populations of indigenous SPB biofilms on environmental surface, stainless steel, HDPE plastic and rubber templates were 5.2, 4.2, 4.6 and 5.7 log CFU/cm$^2$, respectively. During 6 h incubation at room temperature (22–25°C), indigenous SPB biofilm
population in the control group increased to 6.8-8.1 log CFU/cm², whereas phage treatment with a titer of ca. $1 \times 10^9$ PFU/mL reduced population of indigenous SPB biofilm on environmental surface, stainless steel, HDPE plastic and rubber templates up to 0.7, 1.4, 1 and 0.9 log CFU/cm², respectively, as compared with control (Table 1).

During the summer trial, initial populations of indigenous SPB biofilms on environmental surface, stainless steel, HDPE plastic and rubber templates were 6.8, 6.2, 6.1 and 6.4 log CFU/cm², respectively. During the 6 h incubation at room temperature, indigenous SPB biofilm population in control group increased to 6.6-7.2 log CFU/cm², whereas phage treatment with a titer of ca. $1 \times 10^9$ PFU/mL reduced the populations of indigenous SPB biofilms on environmental surface, stainless steel, HDPE plastic and rubber templates by 0.4, 0.3, 0.6 and 0.6 log CFU/cm², respectively, as compared with control (Table 1).

For the fall trial, initial populations of indigenous SPB biofilms on environmental surface, HDPE plastic and rubber templates were 4.6, 3.6 and 5.4 log CFU/cm², respectively. During the 6 h incubation at room temperature, SPB population in control group increased to 5.3-6.3 log CFU/cm², whereas phage treatment with a titer of ca. $1 \times 10^9$ PFU/mL reduced population of indigenous SPB biofilm on environmental surface, HDPE plastic and rubber templates up to 0.55, 0.21 and 0.25 log CFU/cm², respectively, as compared with control (Table 1).

Biofilm is a complex and protected ecosystem composed of many microbial species involved. The presence of a non-susceptible bacterial population can protect bacteriophage-susceptible strains from being attacked by the phage, possibly by creating a thick physical barrier such as exopolysaccharides (EPS) within the depths of the biofilm, which may reduce the efficacy of phage treatment against biofilms. For example, in a study investigating dual species biofilm, Sillankorva et al. (2010) observed only ca. 1 log reduction of the bacterial population in biofilm
after 4 h of phage treatment suggesting that dual species bacteria of *Pseudomonas fluorescens* and *Staphylococcus lentus* in a biofilm may be able to protect each other from phage infection. As demonstrated in our study, the populations of SPB were reduced at different rates when the cells were simply attached as compared to the biofilm (Fig. 2-3). SPB was reduced immediately after applying phages against the attached SPB, but a slow and gradual reduction of SPB population was observed in phage treatment against SPB biofilm. In our field study, lower reductions (0.3-1.4 log CFU/cm²) by phage treatment were observed in controlling indigenous SPB biofilm as compared to the reductions (1.3-2.7 log CFU/cm²) of three selected SPB biofilms under laboratory condition. This could be explained by the limited host range of phage cocktail being used in this study and protective nature from indigenous microflora. For example, only one phage (12a) specific for *Citrobacter* was included in our phage cocktail due to the limitation of our phage collection for this species. Therefore, the likelihood of selection for a phage resistant *Citrobacter* strain may be much higher than for two *Hafnia* strains which are susceptible to multiple phages in the cocktail. For enhancing the efficacy of phage treatment when attempting to eliminate multiple-species biofilm, especially for biofilms formed with high population of indigenous microflora, screening a phage or a phage cocktail with broad host range is necessary. The physical properties of a template’s surface such as hydrophobicity and roughness are important factors that may influence the initial attachment of bacterial cells, biofilm formation and survival of microorganism under phage attack (Characklis et al. 1990). In general, bacterial cells are less likely to attach to the hydrophilic surfaces such as stainless steel and glass as compared to the hydrophobic surfaces such as plastic and rubber (Shi and Zhu, 2009; Van Houdt and Michiels 2010). The extent of biofilm formation was observed to increase as the surface roughness increases due to the diminished shear forces and higher surface area on rougher
surfaces (Characklis et al. 1990). The rougher surfaces can also accumulate much more nutrients in a matrix form to support the growth of biofilm (Characklis et al. 1990). In agreement with their studies, higher initial populations (ca. 5.4-6.4 log CFU/cm², \( P < 0.05 \)) of indigenous SPB biofilm formed on rubber templates than other material templates were observed for each season in our field study (Table 1). However, in our laboratory studies, higher initial population (\( P < 0.05 \), Fig. 2) of SPB was attached to stainless steel templates than HDPE templates, and no difference was observed in the study of SPB biofilm (\( P > 0.05 \), Fig. 3). This may be explained by the fact that biofilm formation is a more complex process and depends on many other factors such as condition of bacterial cells and nutrient availability (Van Houdt and Michiels 2010). Therefore, general biofilm formation on a particular material surface may not be predicted accurately.

Temperature is another key factor affecting the effectiveness of phage treatment. Our field studies demonstrated that phage treatment was slightly better in spring as compared to other seasons. In this study, higher temperature (30°C) in summer encouraged a higher initial population of SPB biofilm as compared with those in spring (22°C) and fall (14°C). Rapid growth of bacteria in biofilms including those bacteriophage resistant mutants may occur at high temperature. As a result, the effective titer of phage treatment decreased at the beginning of the phage treatment in summer trial due to higher initial SPB population, which resulted in a reduced effectiveness of phage treatment (Gong 2013).

In conclusion, this study applied a bacteriophage cocktail to treat both attached SPB and biofilms of SPB on various surfaces typically found in rendering plants under different environmental settings. Our study demonstrated that bacteriophages could reduce the selected SPB strains either attached to or in formed biofilms on various surfaces effectively and
indigenous SPB biofilms on the surfaces in the rendering environment to some extent. Due to the host specificity of bacteriophage treatment, optimization of bacteriophage cocktail based on the indigenous SPB is critical for improving phage treatment effectiveness.

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References


**Figure Captions**

Figure 1: Phage treatment of SPB biofilm formed in 96-well microplate. Black and blank columns represent O.D. values of biofilm in control and phage treatment, respectively. “A” and “B” means statistical difference (P < 0.05) between control and phage treatment for each SPB strain. The error bars represented standard error of each data point from the average of duplicate trials.

Figure 2A-B: Bacteriophage treatment (titer of ca. 1 × 10^8 PFU/mL) of three selected SPB strains attached to stainless steel (A) and HDPE plastic (B) templates. Symbols “■” and “○” represent SPB population in control and phage treatment, respectively. The error bars represented standard error of each data point from the average of duplicate trials.

Figure 3A-B: Bacteriophage treatment (titer of ca. 1 × 10^8 PFU/mL) of three selected SPB strains biofilm formed on stainless steel (A) and HDPE plastic (B) templates. Symbols “■” and “○” represent SPB population in control and phage treatment, respectively. The error bars represented standard error of each data point from the average of duplicate trials.
Table 1. Summary of phage treatment of SPB biofilm on surfaces in the rendering plant

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<th>Season</th>
<th>Template</th>
<th>Initial SPB population (log CFU/cm²)</th>
<th>SPB population after 6 h incubation (log CFU/cm²)</th>
<th>Control</th>
<th>Phage treatment†</th>
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<tr>
<td></td>
<td>ES</td>
<td>5.2±0.28(2)‡</td>
<td>8.1±0.01A</td>
<td>7.4±0.15Ba</td>
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</tr>
<tr>
<td>Spring</td>
<td>SS</td>
<td>4.2±0.01(3)‡</td>
<td>7.6±0.15A</td>
<td>6.2±0.05Bb</td>
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<td>HP</td>
<td>4.6±0.34(2)‡</td>
<td>6.8±0.28A</td>
<td>5.8±0.05Bb</td>
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<tr>
<td></td>
<td>RB</td>
<td>5.7±0.10(1)‡</td>
<td>7.3±0.07A</td>
<td>6.4±0.21Ba</td>
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<tr>
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<td>6.8±0.01(1)‡</td>
<td>7.2±0.03A</td>
<td>6.8±0.03Bb</td>
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<tr>
<td>Summer</td>
<td>SS</td>
<td>6.2±0.08(3)‡</td>
<td>6.8±0.06A</td>
<td>6.5±0.03Bb</td>
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<tr>
<td></td>
<td>HP</td>
<td>6.1±0.21(3)‡</td>
<td>6.6±0.03A</td>
<td>6.0±0.10Ba</td>
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<tr>
<td></td>
<td>RB</td>
<td>6.4±0.06(2)‡</td>
<td>6.7±0.05A</td>
<td>6.1±0.02Bb</td>
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<tr>
<td></td>
<td>ES</td>
<td>4.6±0.05(2)‡</td>
<td>6.3±0.04A</td>
<td>5.8±0.10Bc</td>
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<td>Fall</td>
<td>HP</td>
<td>3.6±0.06(3)‡</td>
<td>5.3±0.05A</td>
<td>5.1±0.02Bc</td>
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<tr>
<td></td>
<td>RB</td>
<td>5.4±0.04(1)‡</td>
<td>5.6±0.02A</td>
<td>5.3±0.03Bc</td>
<td></td>
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* ES, Environmental swab; SS, stainless steel; HP, high density-polyethylene plastic; RB, rubber.
† Phage treatment with titer of ca. 1 × 10⁹ PFU/mL.
‡ Average population ± standard deviation; for control and phage treatment, average populations with different upper case letters in the same row are significantly different (P < 0.05); for each material in different seasons, average populations with different lower case letters in the same column are significantly different (P < 0.05); for different materials in each season, average initial populations with different bracketed numbers in the same column are significantly different (P < 0.05).
SPB (log CFU/cm²) vs. Time (h)