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Application of ATP-based bioluminescence for bioaerosol quantification: Effect of sampling method

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ABSTRACT

An adenosine triphosphate (ATP)-based bioluminescence has potential to offer a quick and affordable method for quantifying bioaerosol samples. Here we report on our investigation into how different bioaerosol aerosolization parameters and sampling methods affect bioluminescence output per bacterium, and implications of that effect for bioaerosol research. *Bacillus atrophaeus* and *Pseudomonas fluorescens* bacteria were aerosolized by using a Collison nebulizer (BGI Inc., Waltham, MA) with a glass or polycarbonate jar and then collected for 15 and 60 min with: (1) Button Aerosol Sampler (SKC Inc., Eighty Four, PA) with polycarbonate, PTFE, and cellulose nitrate filters, (2) BioSampler (SKC Inc.) with 5 and 20 mL of collection liquid, and (3) our newly developed Electrostatic Precipitator with Superhydrophobic Surface (EPSS). For all aerosolization and sampling parameters we compared the ATP bioluminescence output per bacterium relative to that before aerosolization and sampling. In addition, we also determined the ATP reagent storage and preparation conditions that do not affect the bioluminescence signal intensity.

Our results show that aerosolization by a Collison nebulizer with a polycarbonate jar yields higher bioluminescence output per bacterium compared to the glass jar. Interestingly enough, the bioluminescence output by *P. fluorescens* increased substantially after its aerosolization compared to the fresh liquid suspension. For both test microorganisms, the bioluminescence intensity per bacterium after sampling was significantly lower than that before sampling suggesting negative effect of sampling stress on bioluminescence output. The decrease in bioluminescence intensity was more pronounces for longer sampling times and significantly and substantially depended on the sampling method. Among the investigated method, the EPSS was the least injurious for both microorganisms and sampling times.

While the ATP-based bioluminescence offers a quick bioaerosol sample analysis method, this works demonstrates that the method output depends on bioaerosol generation and sampling methods, as well as reagent storage.

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1. Introduction

Airborne microorganisms are associated with various adverse human health effects, such as infectious and chronic respiratory diseases (Franklin, Brook, & Arden Pope Iii, 2015; Lavoie et al., 2015; Pakpour, Li, & Klironomos, 2015). Traditionally, bioaerosol samples have been analyzed using agar-based techniques (Shahamat et al., 1997); however, culture-based methods detect only the culturable bioaerosol fraction and require several days before samples can be quantified. Thus, there has been a continuous interest in the development of methods for rapid quantification of airborne biological agents sampled from laboratory and field environments (Han, An, & Mainelis, 2010; Marć, Tobiszewski, Zabiegała, Guardia, & Namieśnik, 2015; Pan, 2015; Ramji, Chiong, Hirata, Rahman, & Lim, 2014; Seshadri, Han, Krumins, Fennell, & Mainelis, 2009). As a result, numerous methods for quickly analyzing bioaerosol samples have been developed and applied, including impedimetry (Choi, Park, & Jung, 2009; Varshney & Li, 2009; Yang, Li, Griffis, & Johnson, 2004), piezoelectricity (Ebersole, Miller, Moran, & Ward, 1990; Kovář, Farka, & Skládal, 2014), and nucleic acid based assays (Berchebru et al., 2014). An adenosine triphosphate (ATP) – based bioluminescence method (Lee, Park, Im, & Jung, 2008; Mandal & Brandl, 2011; Park, Park, Lee, & Hwang, 2014; Seshadri et al., 2009) is another useful tool for simple, rapid, and effective monitoring of microbial contamination. Since ATP is present in all biologically active microorganisms as the basic energy molecule, it could be applied to detect all metabolically active cells in bacteria, fungi, and other cells (Lim, Simpson, Kearns, & Kramer, 2005). The amount of ATP per cell changes according to various environmental and physiological conditions of bacteria (Mempin et al., 2013) and also varies significantly as a function of growth rate (Schneider & Gourse, 2004). When analyzing bioaerosol samples, the amount of bioluminescence produced during the reaction between ATP and appropriate enzymes is proportional to the collected bioaerosol concentration (Karl, 1980). Thus, the method could be used as an indicator of total bioaerosol load in field or in situ studies (Han, Zhen, Fennell, & Mainelis, submitted for publication; Lin et al., 2013; Park et al., 2014; Yoon, Park, Byeon, & Hwang, 2010). Some studies are strong correlation between ATP content in indoor air and concentration of culturable bacteria (Yoon et al., 2010). It has also been shown that the exact relationship between the ATP concentration and the intensity of bioluminescence reaction depends on microorganism species (Han et al., 2010). Once a calibration curve relating ATP output of particular species with their cell counts is built, the ATP method can provide a quick and affordable solution for quantifying bioaerosol samples, including in laboratory studies to estimate the efficacy of various bioaerosol samplers (Seshadri et al., 2009).

At the same time, different bioaerosol sampling methods are known to differ in their effect on bioaerosol culturability (Zhao et al., 2011) and cell-wall integrity (Thomas et al., 2011; Zhen, Han, Fennell, & Mainelis, 2013). Since ATP-based bioluminescence is based on metabolic activity of microorganisms, the strength of ATP bioluminescence signal and accuracy of bioaerosol analysis may therefore be affected by the chosen sampling method as well. To the best of our knowledge, such information is currently not available.

Thus the main goal of this study was to further investigate the applicability of ATP-based bioluminescence for bioaerosol analysis by analyzing how bioaerosol sampling methods (filtration, impingement, and electrostatic precipitation) and aerosolization parameters affect the strength of ATP bioluminescence signal. In addition, we also investigated how the ATP reagent storage time and the natural degradation of a bioaerosol sample over time affects the ATP bioluminescence signal. The results of this work will aid researchers in adapting ATP-based bioluminescence methods for bioaerosol research.

2. Materials and methods

The study was structured in three steps to investigate how the following factors affect the strength of the luminescence signal: (1) effect of the ATP reagent age, (2) effect of bioaerosol sample age, and (3) effect of bioaerosol aerosolization and sampling parameters. The experiments were performed with gram-positive *Bacillus atrophaeus* bacterial cells and gram-negative *Pseudomonas fluorescens*. Table 1 summarizes all the variables that were investigated in this study.

2.1. General procedure to measure ATP bioluminescence

To determine ATP content in bioaerosol samples, we used BacTiter-Glo™ microbial cell viability assay (Promega Corp., Madison, WI) and followed the manufacturer's instructions as described elsewhere (Seshadri et al., 2009). Briefly, the lyophilized BacTiter-Glo substrate and 10 mL of buffer included in the assay kit were thawed out and reconstituted in equal parts. For measurement of ATP, 100 μL of the resulting reagent (BacTiter-Glo substrate plus buffer) was then mixed with 100 μL of bioaerosol sample. The ensuing reaction lysed the cells and produced a luminescence signal, which was measured by a luminometer (model 20/20^n, Turner Biosystems Inc., Sunnyvale, CA). The strength of the signal was measured as Relative Luminescence Units (*RLU*) and is proportional to the amount of ATP that participated in the reaction.

(1) **Investigation of the luminescence signal strength as a function of the ATP reagent age**
This part had two goals: (a) to determine if storing ATP reagents in a freezer (−20 °C) affects intensity of the bioluminescence signal, and (b) to determine if storing thawed out ATP reagents in a refrigerator (4 °C) affects intensity of the bioluminescence signal. The first part was designed to determine how long reconstituted reagents could be stored before they affect bioaerosol quantification. The latter part was designed to simulate bioaerosol experiments when ATP

Table 1
Summary of the variables tested in this research.

Variables	*Variable values*		*Tested effects on bioluminescence intensity*
Bacterial aerosol species	1. *B. atrophaeus* 2. *P. fluorescence*		Gram positive vs. gram negative bacterial species
Collison nebulizer jar material and aerosolizing time	1. Glass jar 2. Polycarbonate jar	1, 5, 15, and 60 min	
Bioaerosol sampling method and sampling time	1. Button sampler (with 0.6 μm pore size polycarbonate filter, 0.5 μm pore size PTFE membrane filter, and 0.45 μm pore size cellulose nitrate filter) 2. BioSampler (5 and 20 mL of collection liquid) 3. Electrostatic precipitator with superhydrophobic surface (EPSS)	15 and 60 min	Aerosolization stress Bioaerosol sampling stress
ATP reagent storage time at −20 °C	1, 3, 5, 7, 9, and 14 days		Protocol development and quality control
Storage time of thawed out ATP reagent at 4 °C	1, 2, 4, and 6 h		
Storage time of bacterial suspension	0.08, 0.25, 1, 2, 3, 4, 5, and 6 h		

reagents are ready (thawed out) but cannot be applied immediately because bioaerosol samples are acquired over several hours. Both of these steps were necessary to develop a reliable protocol for bioaerosol quantification by ATP bioluminescence.

A bottle of fresh BacTiter-Glo buffer was completely thawed out (about 1 h), equilibrated to room temperature (23–25 °C) and then reconstituted with the included reaction substrate. The buffer and substrate were mixed by gentle vortexing to obtain a homogenous solution (i.e., reagent). Ten milliliter of the resulting reagent (buffer plus substrate) were subdivided into 20 microcentrifuge vials in equal volumes (500 μL) and stored in a freezer at −20 °C for up to 14 days. A 10 μM ATP standard (Lonza Inc., Walkersville, MD), a specialized aqueous preparation of adenosine triphosphate with a known amount of ATP, was also subdivided into 20 microcentrifuge vials in equal volumes (500 μL) and placed at −20 °C.

Experiments were performed with ATP standards and ATP reagents (BacTiter-Glo buffer and substrate) of different age (1, 3, 5, 7, 9, and 14 days) to investigate a potential decay in reagent activity with storage time. During each predetermined day, six vials of reconstituted ATP reagents and six vials ATP standard (500 μL each) were removed from the freezer and completely thawed out (approximately 1 h). Once completely thawed, the vials were placed in a refrigerator (4 °C) and removed at predetermined time intervals (0, 1, 2, 4, and 6 h) to measure bioluminescence intensity of the ATP standard. Then, 100 μL of ATP reagent was mixed with 100 μL of ATP standard, the contents were briefly vortexed, left at room temperature for 1 min, and measured for the resulting bioluminescence reaction by a luminometer (model 20/20^n, Turner Biosystems Inc., Sunnyvale, CA). The mixing and bioluminescence measurements were performed in triplicate for each investigated time period.

(2) **Measurement of the luminescence signal as a function of bioaerosol sample age**

These tests were designed to investigate if the intensity of luminescence changes when there is time lag between when the bioaerosol sample is collected and eventually analyzed. The tests were conducted with gram-positive *B. atrophaeus* bacterial cells (ATCC 49337, American Type Culture Collection, MD) and gram-negative *P. fluorescens* (ATCC 13525). Both microorganisms are commonly used as test microorganisms (Hill et al., 1999; Horner, Helbling, Salvaggio, & Lehrer, 1995; Johnson, Martin, & Resnick, 1994; Madelin, 1994) and their mean aerodynamic diameters are 0.82 and 0.89 μm (Han et al., 2010), respectively. For each experiment, fresh bacterial cells were grown in trypticase soy broth (Becton Dickinson Microbiology Systems, Cockeysville, MD) at 26 °C and 30 °C, respectively, for 18 h in a gyratory shaking incubator (Amerex Instruments Inc., Lafayette, CA) (Han et al., 2010). The used growth time yielded optimum ATP signal (data not shown). The bacterial cells were harvested by centrifugation at 7000 rpm (6140 g) for 5 min at 4 °C (BR4; Jouan, Winchester, VA) and then washed 4 times with sterile deionized water. Prior to experiments, the resulting cell pellets were resuspended in sterile deionized water.

To simulate bioaerosol samples of different age, the final liquid suspension of each bacteria was subdivided into centrifuge tubes with equal volume (10 mL), 8 tubes in total, and stored at room temperature for 0.08, 0.25, 1, 2, 3, 4, 5, and 6 h. The luminescence signal was measured immediately after each aging time by combining 100 μL of bacterial suspension with 100 μL of Bactiter-Glo reagent and measuring the resulting signal with a luminometer. For each aging time, the luminescent signal at the time $t=0$ (as soon as the suspension was prepared) was measured as a reference. The effect of aging of bioaerosol sample was determined by calculating the ratio of the luminescence value (i.e., RLU_i) at each time period (i.e., 0.08, 0.25, 1, 2, 3, 4, 5, and 6 h) to the initial luminescence value (i.e., RLU_0) at $t=0$.

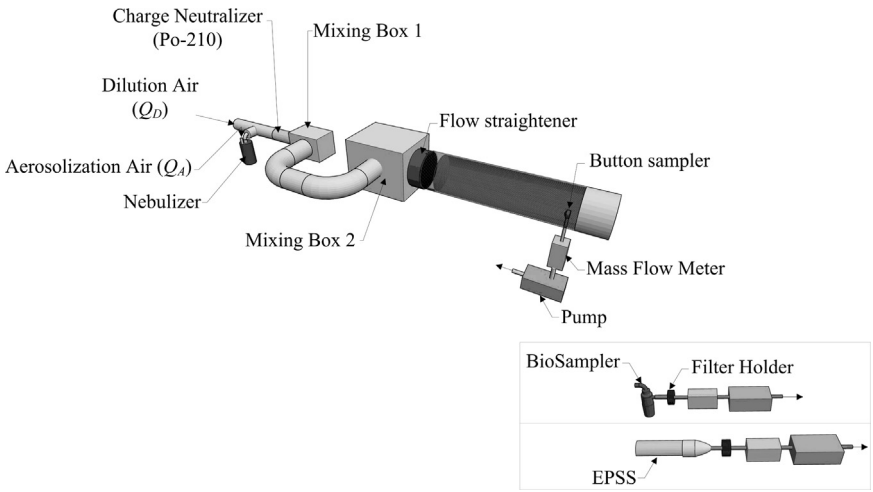

Fig. 1. Schematic diagram of the experimental setup showing testing with the Button aerosol sampler. Insert in the right bottom corner shows connections when testing with the BioSampler and the electrostatic precipitator with superhydrophobic surface (EPSS).

2.2. *Effect of bioaerosol aerosolization and sampling parameters on luminescence signal*

(a) **Experimental setup**

Figure 1 illustrates the experimental setup used to investigate how different bioaerosol sampling methods and sampling times (i.e., 15 and 60 min) affect the measured bioluminescence intensity. The test system consists of a flow control system, a particle generation system, an air–particle mixing system, and a particle monitoring system. The system was housed inside a Class II Biosafety cabinet (NUAIRE Inc., Plymouth, MN). The relative humidity during the experiments was approximately 40–50%.

A three-jet Collison nebulizer (BGI Inc., Waltham, MA) with either a glass or polycarbonate jar was used to aerosolize test particles (i.e., *B. atrophaeus* and *P. fluorescens*) from a liquid suspension at a flow rate (Q_A) of 5 L/min (pressure of 12 psi). A HEPA-filtered dilution air flow, Q_D (105 L/min), provided by an in-house compressor, was controlled by a pressure regulator and monitored by a mass flowmeter (TSI Inc., Shoreview, MN). The aerosolized particle stream and dilution air were combined and passed through a 2-mCi Po-210 charge neutralizer to reduce aerosolization-induced particle charges to Boltzmann equilibrium. The electrically neutralized particles then passed through the first mixing box which improved the uniformity of particle distribution across the flow cross-section (Han, O'Neal, McFarland, Haglund, & Ortiz, 2005). A second mixing box and a U-type duct connector enhanced turbulence further and improved particle mixing. A flow straightener was placed at the exit of the second mixing box to eliminate large scale turbulence and flow swirl generated by the mixing boxes. Finally, the well-mixed aerosol was introduced into a 0.152 m (6 in.) diameter test duct. Each test sampler was placed 6 duct diameters downstream of the exit of the flow straightener in order to provide a uniform cross-sectional concentration of the test particles. The aerosol concentration was monitored by an optical particle counter (model 1.108, Grimm Technologies Inc., Douglasville, GA) (the counter is not shown in Fig. 1).

The tested bioaerosol samplers included a Button aerosol sampler (SKC Inc., Eighty Four, PA), a BioSampler (SKC Inc.) and a newly developed electrostatic precipitator with a superhydrophobic surface (EPSS) (Han, Fennell, & Mainelis, 2015; Han, Nazarenko, Lioy, & Mainelis, 2011). The Button aerosol sampler and the BioSampler were chosen for this study because they operate based on filtration and liquid impingement collection mechanisms, respectively, which are the two most commonly used bioaerosol sampling methods (Reponen, Willeke, & Grinshpun, 2011). Also, collected samples could then be analyzed using culture-independent methods and compared the newly developed EPSS (Han et al., 2010). The Button aerosol sampler was tested with three different filters: a 0.6 μm pore size polycarbonate filter (Millipore, Billerica, MA), a 0.5 μm pore size PTFE membrane filter (Millipore), and a 0.45 μm pore size cellulose nitrate filter (Millipore). It was operated at its nominal flow rate of 4 L/min. For the BioSampler, either 5 or 20 mL of collection fluid was placed in appropriate sampling cups, and the sampler was operated at its nominal flow rate of 12.5 L/min. The EPSS was operated with charging/collection voltage of −9 kV and a sampling flow rate of 10 L/min. The bacteria collected by the EPSS were removed by a 40 μL rolling water droplet for analysis.

Our earlier study showed that when bacteria are aerosolized by a Collison nebulizer, different jar material, i.e., polycarbonate vs. glass, produces different effect on the integrity of bacterial cells, likely due to higher flexibility of polycarbonate compared to glass (Zhen et al., 2013). Therefore, to assess the effect of aerosolization by the Collison

nebulizer on the physiological state of the test bacteria and the resulting bioluminescence signal, glass and polycarbonate jars were used and the resulting bioluminescence signals were compared.

(b) **Comparison metrics**

Effect of aerosolization

10 mL of freshly prepared suspension of each bacterial species was placed in the Collison nebulizer and aerosolized for 0, 1, 5, 15, and 60 min. After aerosolization, two 100 μL aliquots were transferred from the suspension remaining in the Collison nebulizer into two different 1.5 mL centrifuge tubes: one aliquot for the ATP analysis and the other for the Acridine Orange epifluorescence microscopy (AOEM) (Han et al., 2010). For each aerosolization time, the ATP bioluminescence intensity (*RLU*) was normalized to the total number of cells counted by microscopy (N_{AOEM}) and ratio R was calculated:

$$R_0 = \left[\frac{RLU}{N_{AOEM}}\right]_0 \text{ or } R_i = \left[\frac{RLU}{N_{AOEM}}\right]_i, \tag{1}$$

where R_0 is the ratio for $t=0$ min (i.e., a reference ratio) and R_i is the ratio after a certain aerosolization time (i.e., 1, 5, 15, and 60 min). These ratios indicate ATP content per cell and allow to account for day to day variations in cell concentration when performing replicate experiments. The effect of aerosolization on the physiological state of bacteria was then determined by comparing the ratio after the nebulizer operation, R_i, to the initial ratio R_0 (reference):

$$RR(Collison)_i = \frac{R_i}{R_0} = \frac{\left[\frac{RLU}{N_{AOEM}}\right]_i}{\left[\frac{RLU}{N_{AOEM}}\right]_0}, \tag{2}$$

where $RR(Collison)_i$ is a dimensionless ratio comparing the ATP output per cell before and after aerosolization for a certain time i. It can also be used to investigate the effect of different Collison jar material on the ATP output per cell.

(c) **Effect of sampling methods**

The biological particles were aerosolized and then sampled using the BioSampler (12.5 L/min), the Button aerosol sampler (4 L/min), and the EPSS (10 L/min). After sampling was completed by the BioSampler, the remaining collection liquid was transferred into a centrifuge tube. For the Button sampler, bacteria were eluted from the filter into sterile deionized water using the procedure described elsewhere (Wang, Reponen, Grinshpun, Gorny, & Willeke, 2001). We used this procedure to determine the elution efficiency for each filter type and bacterial species since the elution efficiency affects the total number of bacteria per sample and the resulting bioluminescence reaction. For *B. atrophaeus*, the elution efficiency was 97 ± 14% for polycarbonate filter, 96 ± 5% for PTFE, and 98 ± 12% for cellulose nitrate filter; for *P. fluorescens*, the elution efficiency was 102 ± 8% for polycarbonate filter, 86 ± 3% for PTFE, and 107 ± 8% for cellulose nitrate filter. For the EPSS, once sampling was completed, a 40 μL water droplet was used to remove the collected bacteria as described elsewhere (Han et al., 2010), and the droplet containing particles was transferred into a centrifuge tube, where its volume was increased to 100 μL by adding sterile deionized water. Similar to the assessment of the effects of aerosolization stress, the effect of sampling method and time on bioluminescence intensity was investigated by comparing the *RLU* output per bacterium in a sample collected by a particular sampler with the *RLU* output per bacterium before aerosolization (suspension in Collison nebulizer):

$$RR(sampler)_{k,l} = \frac{R_{k,l}}{R_0} = \frac{\left[\frac{RLU}{N_{AOEM}}\right]_{k,l}}{\left[\frac{RLU}{N_{AOEM}}\right]_0}, \tag{3}$$

where k is sampler and l is sampling time.

2.3. Statistical analysis

The measured bioluminescence values (*RLU*), dimensionless ratios *RR*(*Collison*) and *RR*(*sampler*) were analyzed as a function of storage time, particle type, aerosolization time, Collison jar type, and sampler type using ANOVA (SigmaPlot 2011, Version 12.3, Systat Software Inc., San Jose, CA). The differences between individual pairs of variables were examined by using Holm–Sidak method, which takes into account multiple comparisons. The $p < 0.05$ was considered significant at $\alpha = 0.05$.

3. Results and discussion

Figure 2 shows the strength of bioluminescence signal (*RLU*) as a function of ATP reagent and ATP standard storage time (i.e., 0, 1, 2, 4, and 6 h) at 4 °C once they were completely thawed after having been stored at −20 °C for 1, 3, 5, 7, 9, and 14 days. The results show that within 1 h of the ATP reagent and ATP standard thawing out, the luminescence intensity of their reaction decreased on average by 36 ± 9%. However, the luminescence intensity did not change substantially over the next 5 h indicating a time window when bioaerosol quantification by this method should be performed. Storing the reagents at −20 °C for up to 14 days did not significantly affect the reaction intensity ($p > 0.05$). A two-way ANOVA indicated

statistically significant effect of both variables (storage time of frozen reagents at −20 °C (days) and storage time of thawed out reagents at 4 °C (hours)). However, pairwise comparison using Holm–Sidak method showed that there was not significant effect when thawed out reagents were kept at 4 °C for 2 h or longer. Within this time frame (2–6 h), the effect of storing frozen reagents at −20 °C was also not statistically significant with the exception of hour 2 between the 5th and 14th day. Thus, the results indicate how to design a protocol for ATP bioluminescence method to include storage conditions that do not significantly affect the intensity of the bioluminescence signal. The reagent manufacturer also indicated that to achieve maximum sensitivity, equilibration time longer than 15 min may be required.

Figure 3 presents change of bioluminescence intensity per bacterium after a certain time in suspension relative to the bioluminescence intensity at $t=0$ (ATP intensity measured immediately after preparing bacterial suspension) for *B. atrophaeus* and *P. fluorescens*. Firstly, one can observe that the RLU_i/RLU_0 ratio at $t=0.08$ h (5 min) is above >1 (approximately 1.48 for *P. fluorescens* and 1.39 for *B. atrophaeus*) and then begins to decrease as the bacterial suspensions are stored longer. Since the total number of bacteria in each sample does not change with time, the observed change in RLU_i/RLU_0 ratio could be interpreted as natural degradation of the ATP signal intensity produced by bacteria over time. Over the six hour period, the RLU_i/RLU_0 ratio for *B. atrophaeus* decreased gradually to approximately 0.97 of that at $t=0$, while at 4–5 h the RLU_i/RLU_0 ratio for *P. fluorescens* was still about 21% higher than that at $t=0$; it did decrease to approximately 1 after 6 h. One can also observe that after the initial decay over approximately 1–2 h, the observed bioluminescence intensity does not change substantially. The pairwise comparisons of RLU_i/RLU_0 ratios for *B. atrophaeus* from 2 to 6 h and for *P.*

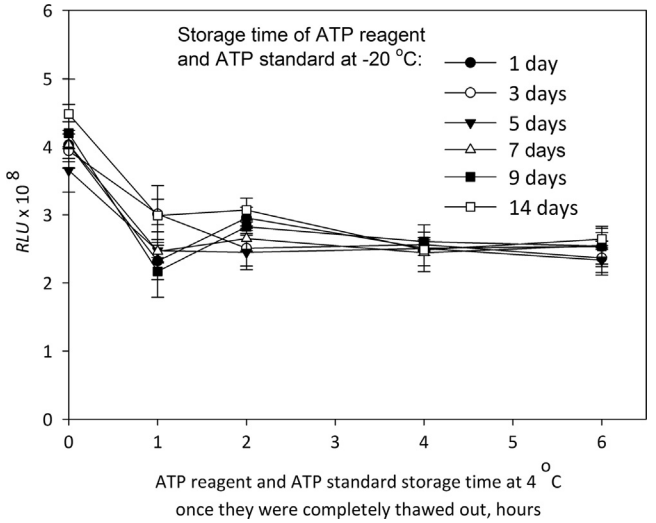

Fig. 2. Bioluminescence intensity (*RLU*) as a function of ATP reagent and ATP standard storage time at −20 °C (1, 3, 5, 7, 9, and 14 days). Error bars denote one standard deviation, based on triplicate measurements.

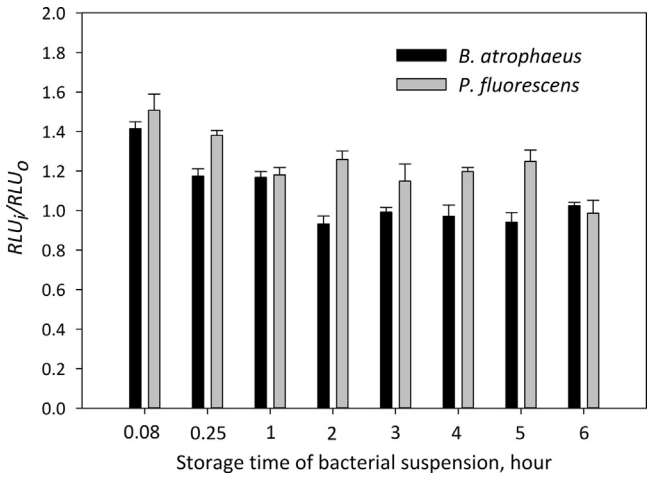

Fig. 3. Change in the amount of bioluminescence activity per bacterium (luminescence intensity per bacterium at certain time $=i$ vs. reference luminescence intensity per bacterium at time=0) as a function of *B. atrophaeus* and *P. fluorescens* suspension storage time (0.08, 0.25, 1, 2, 3, 4, 5, and 6 h). Error bars denote one standard deviation, based on triplicate measurements.

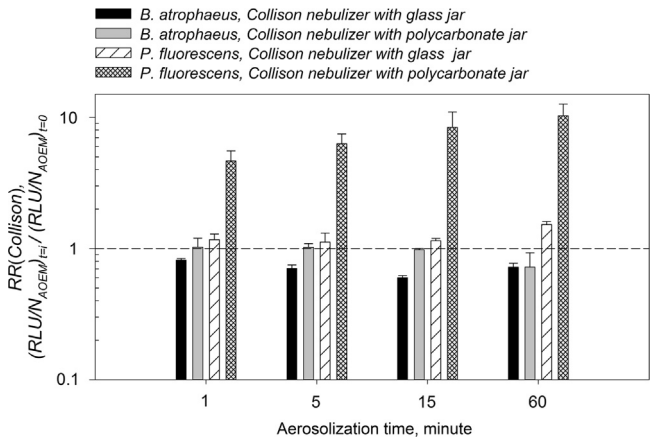

Fig. 4. Effect of aerosolization on RLU output per bacterium for *B. atrophaeus* and *P. fluorescens* as a function of aerosolization time (i.e., 1, 5, 15, and 60 min) and Collison jar material (i.e., glass and polycarbonate jars). The *RR*(*Collison*) ratio represents a dimensionless ratio of the ratio $R_i=(RLU/N_{AOEM})_i$ after a certain aerosolization $t=i$ over the reference ratio $R_0=(RLU/N_{AOEM})_0$ at $t=0$. Error bars denote one standard deviation, based on triplicate measurements.

fluorescens from 1 to 5 h were not statistically different ($p > 0.05$). The difference between the two bacteria was statistically significant ($p < 0.001$). When pair-wise comparisons of bacteria were performed for each time point, the differences were statistically significant for all time points except $t=1$ and 6 h. Thus, if bioaerosol samples are collected at different time points for analysis by ATP bioluminescence at a later time, they should be stored within the above indicated time frames to avoid bias in sample quantification.

Figure 4 presents the effect of aerosolization on *RLU* output by *B. atrophaeus* and *P. fluorescens* as a function of aerosolization time (i.e., 1, 5, 15, and 60 min). The data also compares the effects due to different jar material of the Collison nebulizer: glass vs. polycarbonate. One can see that the *RR*(*Collison*) for *B. atrophaeus* was at or below 1, depending on the Collison jar material. When aerosolizing *B. atrophaeus* with a glass jar, the *RR*(*Collison*) values were 0.81 ± 0.01, 0.71 ± 0.05, 0.60 ± 0.02, and 0.72 ± 0.05 for 1, 5, 15, and 60 min of aerosolization time, respectively. According to pair-wise comparison, the differences among *RR*(*Collison*) for all time points were not statistically significant. When a polycarbonate jar was used, the *RR*(*Collison*) values were close to unity for the first 15 min (1.03 ± 0.17, 1.02 ± 0.07, and 0.97 ± 0.02, for 1, 5, and 15 min, respectively) and then decreased to 0.72 ± 0.21 after 60 min of aerosolization time. According to the pair-wise comparison, the *RR*(*Collison*) after 60 min of aerosolization was statistically different from that at other aerosolization times ($p < 0.05$). From 1 to 15 min aerosolization time, the average *RR*(*Collison*) value for polycarbonate jar (1.01 ± 0.03) was statistically different from that for a glass jar (0.71 ± 0.11) ($p < 0.001$). However, for the 60 min nebulization time, the results between the glass and the polycarbonate jars were not statistically different ($p = 0.989$).

Conversely, the *RR*(*Collison*) values of *P. fluorescens* were above unity for both jar materials. For the glass jar, the values were 1.17 ± 0.12, 1.12 ± 0.19, 1.15 ± 0.05, and 1.52 ± 0.08 for 1, 5, 15, and 60 min of aerosolization time, respectively. The *RR* (*Collison*) values for 60 min were statistically different from those at other time points ($p < 0.05$). For the polycarbonate jar, the *RR*(*Collison*) values for the same time points were 4.65 ± 0.91, 6.29 ± 1.18, 8.37 ± 2.58, and 10.29 ± 2.33, respectively. According to pair-wise comparisons, the *RR*(*Collison*) value at 60 min was statistically different from that at 1 min ($p < 0.05$). All other comparison pairs were not statistically different. For each aerosolization time of *P. fluorescens*, the difference between the glass and the polycarbonate jars was statistically significant ($p < 0.05$).

Except when aerosolizing *P. fluorescens* with a polycarbonate jar, the *RR*(*Collison*) values averaged over time were close to unity: 0.71 ± 0.09 for *B. atrophaeus* and glass jar, 0.94 ± 0.14 for *B. atrophaeus* and polycarbonate jar, and 1.24 ± 0.19 for *P. fluorescens* and glass jar. For *P. fluorescens* and polycarbonate jar, however, the average *RR*(*Collison*) value was much higher: 7.40 ± 2.45.

The data with *B. atrophaeus* show that for up to 15 min of aerosolization, the polycarbonate Collison jar enables up to 30% stronger bioluminescence signal per bacterium compared to the glass jar. Surprisingly, for *P. fluorescens,* bioluminescence output per bacterium increased slightly when using a Collison nebulizer with a glass jar compared to the fresh suspension. Even more surprising was the substantial increase in the bioluminescence output per bacterium when the polycarbonate jar was used. We speculate that mechanical stress during the aerosolization of *P. fluorescens* with both the glass and polycarbonate jars causes damage to bacterial membranes, including the release of intercellular material (Zhen, Han, Fennell, & Mainelis, 2014); this partial lysis results in the release of intracellular ATP, which is then available to participate in bioluminescence reaction.

However, when a glass jar is used, the ATP molecules suffer substantial mechanical stress due to their multiple impacts into the rigid glass wall and become unable to participate in bioluminescence reaction. Polycarbonate material, on the other hand, is less rigid (more flexible) and absorbs part of the impact energy thus resulting in lower stress to the ATP molecules, which are now more abundant in the suspension, leading to a higher ATP signal per bacterium. An increase in ATP bioluminescence signal from gram negative *Escherichia coli* following their sonication was observed in another study

(Turner, Daugherity, Altier, & Maurer, 2010). At the same time, even if the bacterial membrane is damaged, the bacterium still retains its physical shape and can be counted under a microscope. Some fraction of bacteria might be completely damaged and not counted under a microscope thus also leading to a higher bioluminescence output per bacterium. Damage to the integrity of the bacterial membrane was also observed in our previous study of DNA release from *E. coli* (also a gram-negative organism) after aerosolization (Zhen et al., 2013). Our previous aerosolization studies with *P. fluorescens* observed damage to bacterial culturability due to prolonged aerosolization (Mainelis et al., 2005). Since *B. atrophaeus* is a gram positive bacterium with a thick outer wall, the mechanical stress of aerosolization and sampling does not result in the cell membrane rupture (Zhen et al., 2013), but apparently the stress is still sufficient to reduce *RLU* output per bacterium as illustrated with *RR*(*Collison*) values being below unity. Here again, since the polycarbonate jar is able to absorb part of the impaction energy from the bacteria, it produces lower stress and higher *RR*(*Collison*) compared to the glass jar. The above-mentioned study by Turner et al. (2010) also observed minimum change in ATP bioluminescence signal when gram-positive *Staphylococcus aureus* was sonicated.

Figure 5 presents *RR*(*sampler*) ratio for three different samplers and their sampling parameters, sampling times and two different bacteria.

3.1. Data for B. atrophaeus

For this bacterium, the *RR*(*sampler*), when sampled with the BioSampler, ranged from 0.07 to 0.21; when sampled with the Button sampler, the *RR(sampler)* ranged from 0.10 to 0.52; the EPSS showed the highest values for each time point ranging from 0.45 to 0.68. One can also observe that the *RR*(*sampler*) values for this bacterium in Fig. 5 are lower than *RR* (*Collison*) data with the polycarbonate jar in Fig. 4 where the aerosolization effects are shown. The difference was statistically significant ($p < 0.001$) for each tested sampler and for both sampling times. The lower values *RR*(*sampler*) compared to *RR*(*Collison*) indicate that *B. atrophaeus* is subject to additional stress during sampling, which leads to lower bioluminescence output per bacterium.

Overall, for *B. atrophaeus* data in Fig. 5, the effects of sampler and sampling time were statistically significant ($p < 0.001$). According to Holm–Sidak pair-wise comparison, the *RR*(*sampler*) was statistically different ($p < 0.001$) for all sampler pairs, except the following pairs: BioSampler with 5 mL vs. BioSampler with 20 mL; BioSampler with 5 and 20 mL vs. Button sampler with cellulose filter; and Button sampler with polycarbonate filter vs. Button sampler with cellulose filter. The effect of sampling time was statistically significant ($p < 0.005$) for all samplers except BioSampler with 20 mL and Button sampler with cellulose filter. Also, for each sampling time, the EPSS had the highest *RR*(*sampler*) values compared to other samplers. The differences ranged from 0.16 (Button sampler with Teflon filter) to 0.53 (Button sampler with cellulose filter) and were significant when compared with all samplers ($p < 0.001$).

3.2. Data for P. fluorescens

On the other hand, the average *RR*(*sampler*) for *P. fluorescens* were clearly above unity and ranged from 4.13 ± 0.90 (BioSampler with 5 mL) to 6.15 ± 2.32 (Button sampler with polycarbonate filter). For 15 min of sampling, the values of *RR* (*sampler*) were statistically significantly lower than *RR*(*Collison*) ($p < 0.05$) for BioSampler with 5 mL and the Button sampler

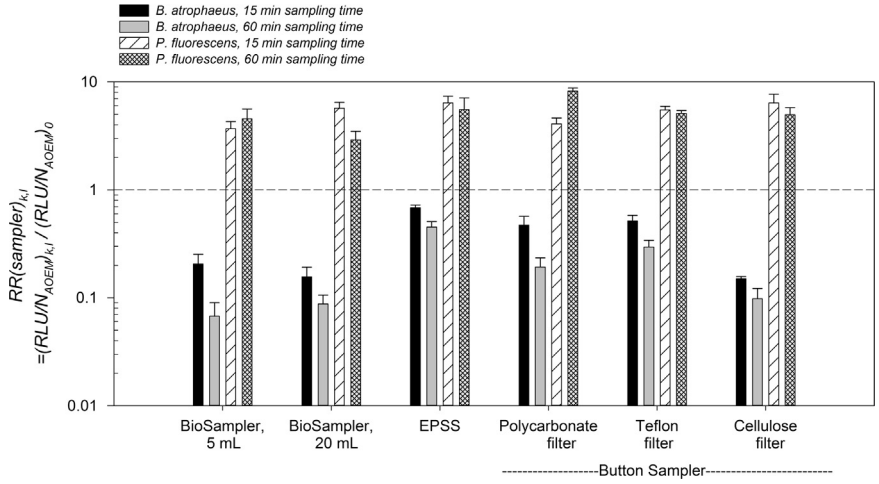

Fig. 5. Comparison of the *RR*(*sampler*) ratio for three different samplers (i.e., BioSampler, Button sampler, and EPSS), two different sampling times (i.e., 15 and 60 min) and two different bacteria (*B. atrophaeus* and *P. fluorescens*). The $RR(sampler)_{k,l}$ ratio represents a dimensionless ratio of the ratio $R_{k,l}=(RLU/N_{AOEM})_k$ for a certain sampler k after a certain sampling time l over the reference ratio $R_0=(RLU/N_{AOEM})_0$. The latter is determined in microbial suspensions before aerosolization. A Collison nebulizer with a polycarbonate jar was used in all experiments. Error bars denote one standard deviation, based on triplicate measurements.

with polycarbonate filter. For 60 min sampling, the values of *RR*(*sampler*) were statistically significantly lower than *RR* (*Collison*) for all samplers except the Button sampler with polycarbonate filter. When 15 and 60 min data were pooled together to minimize experimental variability, then *RR*(*sampler*) values were statistically significantly lower ($p < 0.005$) than *RR*(*Collison*) for all samplers.

Overall for *P. fluorescens* data set in Fig. 5, the effect of sampler on *RR*(*sampler*) was statistically significant ($p < 0.001$). According to Holm–Sidak pair-wise comparison, *RR*(*sampler*) for EPSS and Button sampler with polycarbonate filter were statistically significantly higher than that for BioSampler with either 5 or 20 mL of collection fluid ($p < 0.05$). The effect of time on the overall *P. fluorescens* data set was not statistically significant ($p = 0.80$); however, Holm–Sidak pair-wise comparison showed differences in *RR*(*sampler*) between 15 and 60 min sampling time for BioSampler with 20 mL and Button sampler with polycarbonate filter.

In general, Fig. 5 shows that, when using *B. atrophaeus* and *P. fluorescens*, the *RLU* output per bacterium decreased after sampling compared to *RLU* output per bacterium just after aerosolization. This indicates that the sampling stress and, possibly, stress during bioaerosol transport form the aerosolization point to the sampling point, results in lower bioluminescence output per bacterium. This is in agreement with other studies reporting stress to bioaerosols during sampling, including their lower culturability (Chen & Li, 2005). This study, however, focuses on the effects of sampling on bioluminescence output per bacterium. For *B. atropheus*, and both 15 and 60 min sampling times, the lowest difference between the *RR*(*Collison*) and *RR* (*sampler*) was for EPSS. For *P. fluorescens* and both 15 and 60 min sampling times, the EPSS yielded the second lowest difference between the *RR*(*Collison*) and *RR*(*sampler*). If Button sampler data with different filter materials are pooled together to minimize experimental variability, then the EPSS yields the lowest difference between the *RR*(*Collison*) and *RR*(*sampler*) among the tested sampler types. These observations suggest that of the tested samplers the EPSS is among the least stress inducing devices when it comes to bioaerosol sampling for analysis by the ATP-based bioluminescence.

Overall the data presented here show that the ATP-based bioluminescence could be used to rapidly analyze performance of bioaerosol samplers. However, one has to take into account that the *RLU* output depends on bacterial species, as well as sampling and aerosolization methods. Once the effects of aerosolization and sampling in a particular setup are known, the ATP-based bioluminescence analysis provides a quick and convenient way to analyze bioaerosol samples, especially during bioaerosol sampler development and testing.

4. Conclusion

We found that multiple factors affected ATP-based bioluminescence intensity when analyzing bioaerosols: i.e., reagent decay over time, natural degradation of the signal produced by the tested bioaerosol (i.e., *B. atrophaeus* and *P. fluorescens*) liquid suspension over time, nebulization method (Collison nebulizer with glass and polycarbonate jars) and also bioaerosol sampling method. Surprisingly, *P. fluorescens* showed higher bioluminescence intensity per bacterium after aerosolization compared to *P. fluorescens* suspension before aerosolization. An opposite effect was observed for *B. atrophaeus*: bioluminescence intensity per bacterium decreased after aerosolization compared to that before aerosolization. For both microorganisms, the EPSS affected the bioluminescence output per bacterium the least among the investigated sampling methods.

Overall, this work shows that the ATP-based bioluminescence method could be applied for rapid analysis of bioaerosol samples; however, researchers should take into account different bioluminescence output by different species as well as changes to that output depending on aerosolization and sampling techniques and parameters. Such changes might be especially important in field studies where multiple organisms aerosolized through different pathways are encountered. It is hoped that the results of this study will contribute toward wider application of this technique in bioaerosol research.

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