DETECTION OF BIOLOGICAL AEROSOLS

BY LUMINESCENCE TECHNIQUES

Peter J. Stopa[1]*, Darlene Tieman[2,3] Philip A. Coon[1], Maurice M. Milton[2], and Dorothea Paterno[1]

[1] US Army Edgewood Chemical/Biological Center, Research and Technology Directorate, AMSSB-RRT-DD, 5183 Blackhawk Road, E3160, Aberdeen Proving Ground, MD 21010-5424

[2]Science and Technology Corporation, 10 Basil Sawyer Drive, Hampton, Virginia VA 23666

***Corresponding author:** **USA SBCCOM, AMSSB-RRT-DD, Bldg E3160, 5183 Blackhawk Road, APG, MD 21010-5424**

[3]Present Address: **University of Arkansas at Pine Bluff**
Aquaculture/Fisheries Center
Department of Aquaculture and Fisheries
P.O. Box 4912
Pine Bluff, AR 71611

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ABSTRACT

Luciferin-Luciferase (L-L) luminescence techniques were used to successfully measure the adenosine triphosphate (ATP) content (pg/ml) in aerosol samples containing either vegetative bacterial cells or spores. Aerosols were collected with wet and dry sampling devices. Evaluation for the presence of total bio-mass from both bacterial and non-bacterial sources of ATP was achieved by suspending the collected aerosol samples in phosphate buffered saline (PBS), pipeting a 50-µl aliquot of the PBS suspension into a Filtravette™, and then adding bacterial releasing agent (BRA). The sample was then reacted with L-L, and the resulting Relative Luminescence Units (RLU's), indicative of the total ATP content, were measured. Identical techniques were used to prepare the bacterial cells for analysis with one additional step: a wash with somatic cell releasing agent (SRA) before adding BRA. This step removes interfering substances or non-bacterial sources of ATP. For spore analysis, an equi-volume sample of the PBS suspension was added to an equi-volume of trypticase soy broth (TSB), incubated at 37 C for 15 minutes, and processed using methods identical to bacterial cell analysis. Using these techniques we were able to detect *Bacillus subtilis* variation *niger*, formerly known as *Bacillus globigii* (BG), in aerosol samples at concentrations $\geq 10^5$ colony forming units (CFU) per ml. Results of field and chamber trials show that one can detect the presence of bacterial and non-bacterial sources of ATP. These techniques may be appropriate to situations where the measurement of bacterial aerosols is needed.

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INTRODUCTION

Luminescence is the emission of light that does not derive energy from the temperature of the emitting body and is caused by chemical, biochemical, or physical changes of a material in a sample. Luminescence techniques utilize this phenomenon to measure the presence of an analyte in a sample that exhibits this characteristic. This typically achieved by the reaction of the analyte with a substrate that produces light.

There are two types of luminescence: chemiluminescence and bio-luminescence. Chemiluminescence measures the light generated by the products of a chemical reaction, for example the reaction of peroxide with luminol or oxetane esters. Bioluminescence, on the other hand, measures the light generated by the products of a biological reaction, typically involving an enzyme and a substrate. Examples of this include luciferin and luciferase (L-L) in the presence of ATP and reactions involving the cofactor nicotinamide adenine dinucleotide (NADH). Adenylate kinase is another reaction that has recently been described (1), but it is essentially a variation on the Luciferin-Luciferase reaction.

Chemiluminescence was used to detect biological aerosols through the use of luminol which measures the presence of heme groups present in the sample. This was the basis of Biological Detection and Warning System (BDWS) under development by the US Army in the late 1970's. It had a limited capability to detect bacteria and exhibited interferences from heavy metals.

Bioluminescence has been used to measure NADH redox reactions in bacteria and ATP levels in various types of cells using the reaction of Luciferin with the enzyme, Luciferase. This reaction is typically referred to as the Firefly Reaction. Recent advances in the technology allow one to select cell types by using various kinds of additives. For example, strong vs. mild detergents can be used to select for somatic cells, mold, pollens, and vegetative bacteria (2). The

use of enzymes that are specific for lysing cell type, e..g., staphylolysin for *Staphylococcus aureus*, has also been described (3).

The L-L reaction is the most widely used bioluminescent reaction, both clinically and environmentally. Its uses include: detection of bacteria in urine (4); sanitation/cleanliness monitoring; method for determining levels of bacteria (contamination) in water, milk, gasoline, and reagents for process streams; and screening for the contamination of meat carcasses (5). But its use to detect bacteria in aerosols has been limited, probably because most of the techniques currently in use today measure total biomass and do not have the capability to differentiate among different cell types.

The measurement of viable biomaterial in an aerosol requires a liquid sample. This can be achieved by sampling directly on a filter and extracting the filter with a liquid medium, or by sampling directly into a liquid. There are a variety of filter and liquid samplers presently available that operate in a similar fashion. Examples of sampler designs currently being used are cyclones, impactors, virtual impactors, and impingers.

A cyclone sampler draws the aerosol through a tube and which initiates a centrifugal swirling motion of the particles. They then impact onto the wall of the sampler. The wall of the sampler can be either dry or wetted through the injection of a liquid spray into the aerosol stream. Wetted-wall cyclones generally show better collection efficiency for smaller particles, such as bacteria.

In an impactor design, the sample is drawn through an orifice and particles are impacted directly onto the surface of a collection medium, such as a filter, liquid, tape, or Petri dish. Examples of this include the Anderson cascade impactor that separates the sample by particle size through the use of different size screens or slit samplers in which the air stream is impacted onto a slowly rotating Petri dish. The former design allows one to obtain viable particle size

distributions while the latter design allows one to obtain particle concentration information as a function of time.

The virtual impactor is a variation of the impactor design and allows one to sample large volumes of air and concentrate the sample. In this design, the aerosol sample is drawn through an orifice and particles are diverted into a tube where they are decelerated, "virtually impacting" into another air stream of a lesser flow rate. Ultimately the sample can be collected either onto a tape or into a liquid medium.

Currently, the impinger design is perhaps the most widely used sampler. In this design, the air sample is collected through an orifice and bubbled or impinged directly into a liquid collection medium. These designs include the All-Glass Impinger (AGI) which are in wide use. A newer design has recently been introduced in which the air sample is injected tangentially into the liquid stream, resulting in a better collection efficiency (6).

In this paper, we will show that simple and inexpensive luminescence instruments can be interfaced with these sampler devices in an effort to estimate the concentration and, in some cases, the types of bacterial cells present in the sample, such as spore vs. vegetative. We will also show that there is correlation with the classical plate count methods.

Materials and Methods

A. The Model 3550 Luminometer

The New Horizons Diagnostics, Inc., Columbia , MD, Model 3550 luminometer was chosen for this application. This instrument is small, compact, and is easy to use. Unlike other luminometers, it has a protocol utilizing selective chemistry and filtration that allows one to differentiate between bacterial and non-bacterial sources of ATP. A protocol was also developed to detect the presence of ATP-deficient bacterial spores in samples by incubation at 37 C in trypticase soy broth (TSB) to activate the spores.

The system is prepared for use by reconstituting the Luciferin-Luciferase (L-L) reagent at least one-half hour before use. The activity of the enzyme is then measured by assaying it with the ATP standard solution supplied with the kit. The resulting value is then compared to the ATP and the relative luminescence units should be within the range specified on the vial. If not, then a new vial of enzyme should be used.

For measurement of vegetative bacterial cells, the following protocol is used. Sample (50 ul to 1 ml) is added to a Filtravette(tm) and is washed with 5 drops of Somatic- Cell Releasing Agent (SRA) included in the kit. The liquid is then expressed by the use of a pressurizing device to force the liquid through the membrane. The wash with SRA is repeated. This step removes non-bacterial sources of ATP and any interfering substances. The Filtravette (TM) is removed and placed into the sample drawer of the instrument. Two drops of Bacterial Cell Releasing Agent (BRA) is added to the Filtravette (TM) to lyse the bacterial cells. It is immediately followed by 50 ul of the L-L reagent. The sample is then mixed quickly by re-expressing the liquid 4 times with the pipet. The sample drawer is then closed and the luminescence (relative luminescence unit - RLU) is recorded. Tables are then used to convert the RLU's to either ATP or bacterial concentration.

Samples can be assayed for the presence of bacterial spores by the following protocol. The sample (50 ul to 1 ml) is incubated in an equi-volume of Trypticase Soy Broth (TSB) (Remel Laboratories or equivalent) in a heating block for 15 minutes at 37 C. The entire sample is removed with an appropriately sized syringe and carefully filtered through a modified Swinnex (TM) device containing a Filtravette (TM). The Filtravette (TM) is then removed and processed according to the protocol for vegetative bacterial cells.

B. Validation Studies

The detection limit of the luminometer for ATP was determined by titrating known amounts

of ATP with a fixed amount of L-L and measuring the response. 50 ul of the ATP solution was placed into the Filtravette (TM) and 50 ul of the L-L reagent was added. The reactants were mixed and the relative luminescence units (RLU) were recorded.

The luminometer was then evaluated for sensitivity to bacteria. Bacterial strains were obtained from the American Type Culture Collection (ATCC) or other sources. They were grown according to their recommended nutritional requirements on plates or in broth. The bacteria grown on plates were then harvested by flooding the plate with 0.85% sterile saline and gently swabbing the plate to loosen the bacteria into the fluid. The wash was then placed into a centrifuge tube and the cells pelleted by centrifugation at 2000 x g. The pellets were resuspended and washed several times in this manner.

Bacteria grown in broth cultures were pelleted by centrifugation as described above, and then washed 3 times with 0.85% sterile saline.

Bacterial concentration was determined by either the plate count method or by turbidity. Bacteria suspensions were adjusted so that stock solutions of 10^8 bacteria/ml were obtained.

Log-fold serial dilutions of the bacteria were made in saline and assayed according to the above protocol. The bacteria used were representative of environmental isolates, and also varied by cell shape and Gram reaction.

C. Aerosol Studies

Natural and man-made aerosols were collected using several different types of samplers, under various conditions, for varying sampling times, and by personnel with different skill levels. The types of samplers used included All Glass Impingers (AGI); Cyclones; Virtual Impactors (Model 1021-R, SCP Dynamics, St. Paul, MN; or the XMX sampler, Dycor, Ltd, Edmonton, Alberta, CA); Filter Samplers (Personal Asbestos Filter, 5 um, MSA, Inc. Pittsburgh, PA) and prototype systems using conventional heating, ventilation, and air-conditioning (HVAC)

filters. Samplers were used according to the manufacturers' instructions without modification.

The conditions under which samples were collected included controlled aerosol chamber; outdoor field trials with intentional releases; and ad hoc background aerosols. Sampling times varied as per the exercise protocol, typically 1 minute to 1 hour.

Field trials also used personnel with varying skill levels and normally were not laboratory personnel. Exact conditions of these trials will not be included here for brevity's sake.

RESULTS

The determination of the detection limit of ATP is presented in **Figure 1.** As can be seen by the data, the nominal detection limit is about 100 pg/ml of ATP. This was determined by using 50 ul of the concentration of enzyme supplied by the manufacturer and 50 ul of sample. Note that the reaction of the L-L with ATP exhibits linear behavior over a wide concentration range. The detection limit of the technique can easily be improved by increasing the enzyme concentration, increasing the sample volume, or both. The use of the Filtravette™ facilitates this by enabling one to increase the volume by concentrating the cells on the filter portion while allowing the excess liquid to be removed.

Figure 2 shows the results of the log-fold dilution series of various types of bacteria. Using 50 ul of sample and a 1x concentration of enzyme, the detection limit is about 10^5 CFU/ml of bacteria. However, when the concentration of enzyme is doubled and the sample volume is increased by a factor of 10, detection limits of less than 10^4 CFU/ml have been achieved (data not shown). The data also shows that there is no real significant difference between the activity of gram-positive and gram-negative bacteria with respect to the extraction protocol.

Figure 3 depicts the experiments performed to determine the presence of spores in the

sample. Spores are deficient in ATP and are virtually undetectable by the standard technique. Therefore, one needs to stimulate the production of ATP so that the cellular processes necessary to transition to a vegetative state can be initiated.

Our data show that spores are detectable after 5 minutes of incubation at a concentration of 10^6 CFU/ml; however, we routinely used a 15 minute incubation because better agreement with classical plate counting techniques was observed.

Figure 1. ATP standard curve generated with the Model 3550 Luminometer. This is a log-log plot. Note the linearity over several orders of magnitude. The detection limit of ATP is nominally 100 pg/ml.

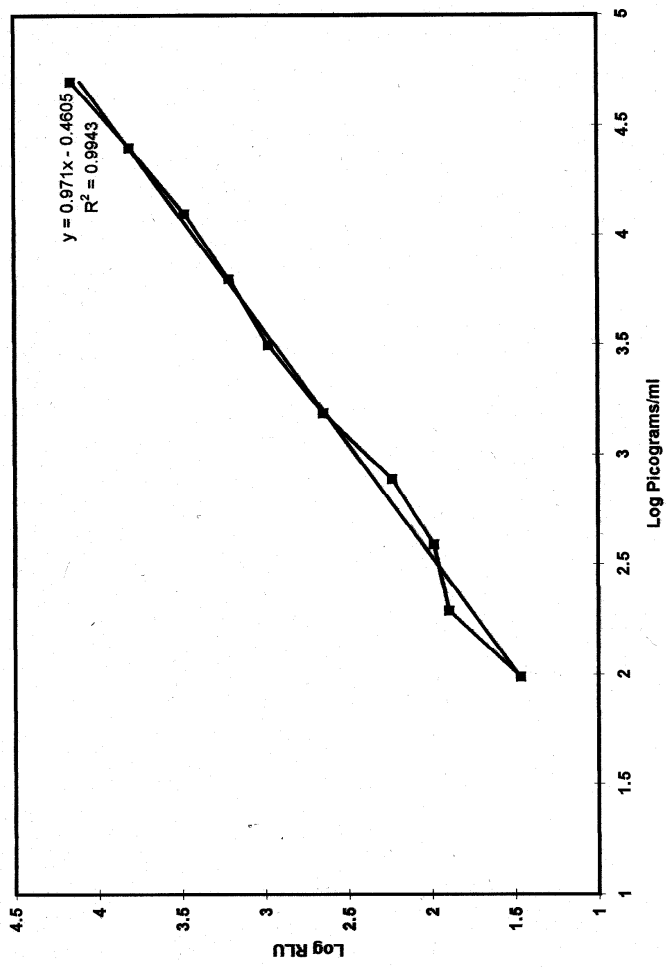


Figure 2. Correlation between bacterial concentration and relative luminescence units (RLU's) for several different species of bacteria isolated from the environment. This data was generated with a 50 ul sample. The nominal detection limit is 10^5 CFU/ml.

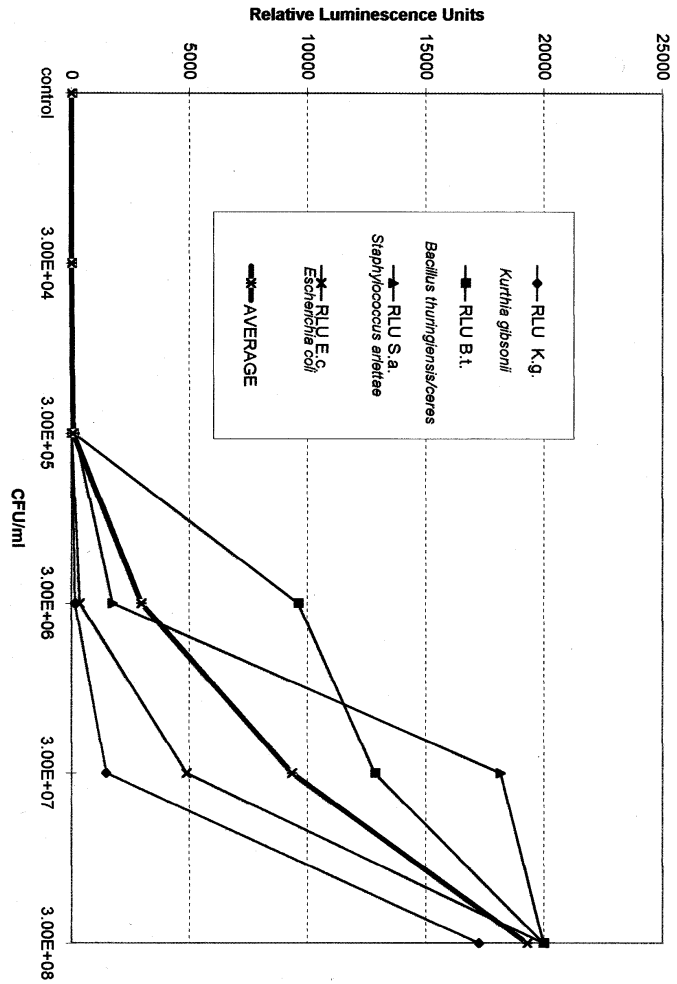
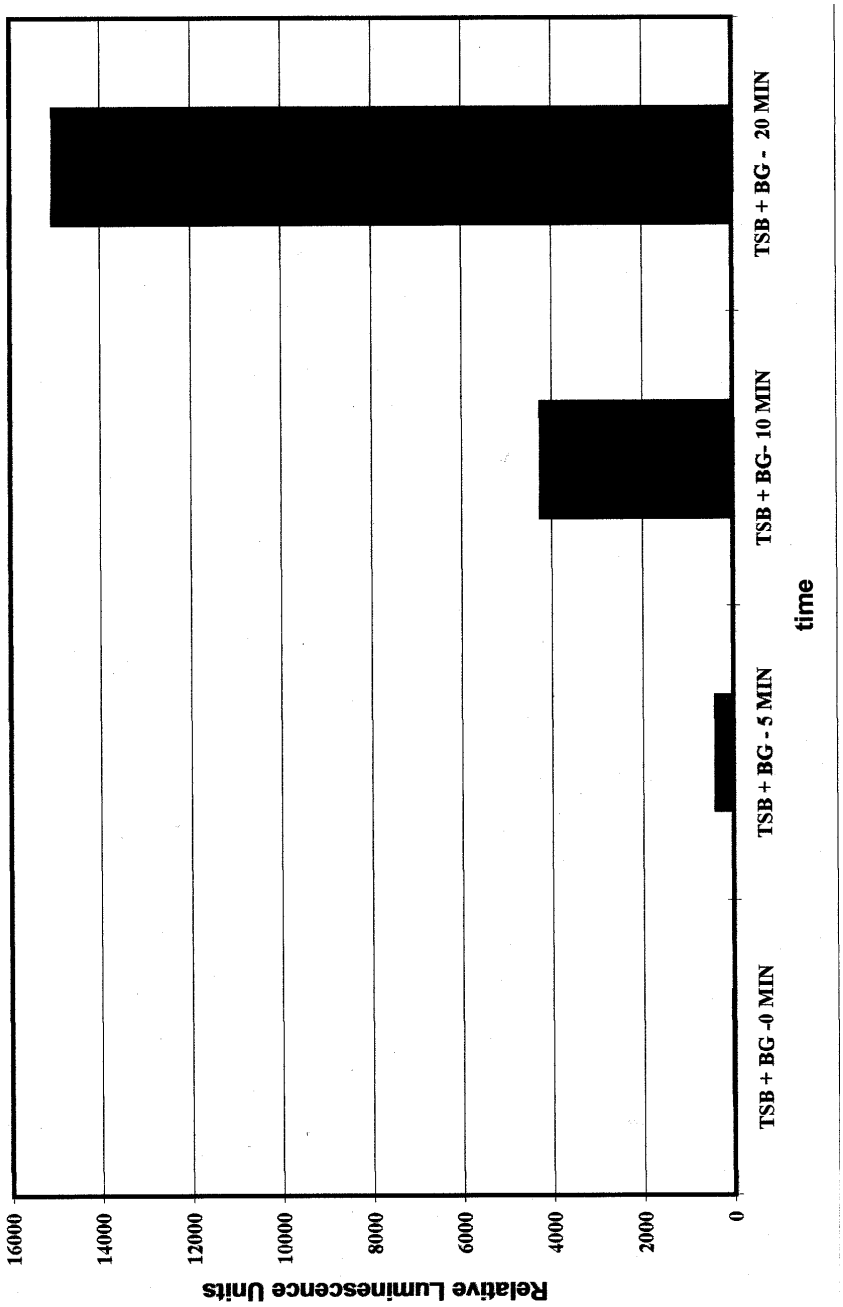


Figure 3. Time course of development of ATP in germinating *Bacillus* spores at 10^6 CFU/ml. The figure shows that detectable levels of ATP are achieved in as little as 5 minutes.



The correlation between culture concentration and RLU shows that a linear relationship exists when the logarithm of the RLU versus the logarithm of the concentration of cells per milliliter is plotted. An equation of the line can be calculated and a table can be generated to estimate bacterial concentration directly from the RLU's. From our data, we constructed a table by which the bacterial concentration could be estimated. This is presented in Table 1.

Table 1. Correlation between Microluminometer Response (RLU) and Bacterial Concentration.

RLU	**CFU/ml**
100-499	1x10^5
500-4999	1x10^6
>50000	1x10^7

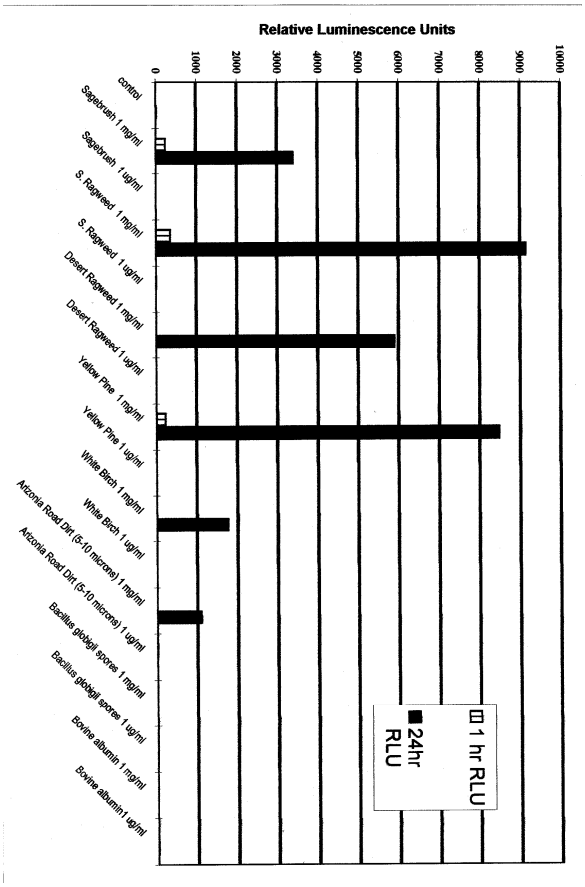
This table is based on the use of 50 ul of sample and reconstituting the L-L according to the manufacturer's directions. One can also use this table to estimate concentrations of bacteria in assays using increased sample volume by simply correcting for the volume difference. For example, if one were to use 2 ml of sample, the estimated bacterial concentration could be obtained by simply dividing the bacteria number by 40.

Before proceeding to evaluate field samples, criteria need to be developed to establish the cut-off value for detection. Our experience has shown that the Model 3550 I luminometer is not very noisy and that values of less than 30 RLU can be considered to be negative. This allows for some degree of lot to lot variability among different lots of reagents. Values between 30 and 100 are generally classified as suspicious, and should be reconfirmed if additional sample is available. A suggested protocol would be to double or triple the sample volume used in the test.

If the assay shows a corresponding increase in signal, then the sample should be considered to be positive.

When evaluating a sample using luminescence techniques, one must consider the effects of interfering substances, growth of bacteria within the sample after collection, or the life cycle of the bacteria. Some limited studies were conducted in the laboratory with interfering materials that one might encounter in an aerosol sample. Pollens and mold spores were used for this evaluation. As can be seen by Figure 4, there is minimal effect from these interferences if the sample is measured within 1 hour of collection; however, evaluating the sample after longer periods of time may compromise the results. If samples can not be immediately analyzed, then one should take steps to maintain bacteriostasis within the sample.

Figure 4. Effects of selected environmental materials on the ATP reaction.



Another consideration is the bacterial life cycle. The best results are obtained when the bacteria are in the logarithmic or exponential stages of growth, but this is not ordinarily the case with environmental bacteria. The use of the assay in which the sample is incubated for 15 minutes in TSB would help to resuscitate the bacteria, but this would make the spore test indeterminate. However, the case is similar to that of the spore – the organism is stressed, has a low nutritional level, and therefore, little if any ATP.

One possible solution would be to incubate the sample in the presence of a medium that would help to resuscitate the bacteria but have little or no effect on the spore test. We currently are investigating the use of various nutrients for resuscitating bacteria that would not interfere with spore analysis protocol.

Studies were conducted with *Erwinia herbicola* that were stored in 0.85% saline for over a week and were in a starved condition. Samples of the bacteria gave negative results when assayed with the standard protocol for bacterial ATP. However, when they were assayed after being in the presence of a nutrient after 15 minutes, such as 4% sucrose or TSB, a dramatic increase in ATP activity was seen. When this same experiment was performed using the spores of *Bacillus subtilis var. niger*, formerly known as *Bacillus globigii*, an increase in activity was seen only in the TSB incubation and not in sucrose. This data suggests that one might want to consider using sugar solutions to collect the samples or use incubation with a sugar to improve detection. Further studies still need to be performed to fully validate this finding.

Studies were then performed on some *ad hoc* aerosol samples that were obtained by the XM-2 collector, which is a virtual impactor. We sampled for 30 minutes at a flow rate of 1300 liters/minute. The results are presented in Table 2.

Table 2. Results obtained with an *Ad Hoc* Background Sample.

ENVIRONMENTAL SAMPLE[1]

Assay Method		**3550i LUMINOMETER**	
	RLU	**ATP Avg pg/ml**	**BACTERIA* EQUIVALENT**
ATP Standard	2418	5000	
Control	3		
Total ATP	293		
	223	498	5.0×10^5
	203		
Bacterial ATP	22		
3550 Method	52	100	1.0×10^5
	43		

*Assumes 1 femtogram of ATPper bacteria.

1. Sample taken with xm2+ for a 2 hour period at 1300 L/min.

The table shows the result of a typical determination of an *ad hoc* background sample. The total ATP level was measured by elimination of the SRA application, which resulted in a high count. The sample was then processed as per the Model 3550 protocol and gave results consistent with plate counts or with concentrations determined by slit samplers. Interferences, such as phosphate or chloride ions, were effectively removed. In addition, we also think that the interferences from mold spores, fungal spores, and pollens are also removed.

Table 3 shows the results obtained with various types of samplers in which Bg spores were introduced into an indoor aerosol chamber under controlled conditions. The data shows that we were able to successfully detect the bacteria when the concentrations of aerosols were in excess of 15 ACPLA (Agent Containing Particles per Liter of Air). The data also shows that there is little if any correlation between the particle concentration and the resulting concentration

in various types sampling devices. However, this does demonstrate the sensitivity of the technique. The bacterial concentrations that we typically see in our area are in this range. The environment is coastal with deciduous forests.

Table 3. Determination of ATP in collection fluids from various types of Samplers and Correlated with Particle Concentration per Liter of Air).

COLLECTOR	ACPLA	PLATE COUNT (CFU/ML)	MICROLUMINOMETER RESULT
XM2^{+}	10.78	2.65E+03	NEGATIVE
XM2^{+}	11.18		NEGATIVE
XM2^{+}	14.59	3.04E+03	NEGATIVE
XM2^{+}	14.60		NEGATIVE
XM2^{+}	15.15	1.17E+03	POSITIVE
XM2^{+}	15.15	1.67E+03	NEGATIVE
XM2^{+}	15.70	2.57E+03	NEGATIVE
XM2^{+}	19.64	1.00E+02	POSITIVE
XM2^{+}	26.72	1.63E+03	POSITIVE
XM2^{+}	29.14	3.81E+03	NEGATIVE
XM2^{+}	34.72	1.50E+01	POSITIVE
XM2^{+}	41.31	6.25E+02	NEGATIVE
XM2^{+}	57.45	3.95E+02	POSITIVE
XM2^{+}	59.91	2.58E+03	POSITIVE
SASS	6.35	7.87E+00	NEGATIVE
SASS	14.59	1.05E+02	POSITIVE
SASS	29.14	2.48E+03	POSITIVE
SPINCON	11.18	2.03E+03	NEGATIVE
SPINCON	30.43	7.28E+03	NEGATIVE
SPINCON	34.72	9.53E+03	POSITIVE
SPINCON	41.31	8.03E+03	NEGATIVE
SPINCON		0.00E+00	POSITIVE
PAM4	10.78	9.83E+03	NEGATIVE
PAM5	10.78	3.26E+03	NEGATIVE
PAM4	14.59	8.30E+01	POSITIVE
PAM5	14.59	9.40E+01	POSITIVE
PAM4	14.60	7.70E+03	NEGATIVE
PAM5	14.60	4.26E+03	NEGATIVE
PAM4	15.70	8.57E+03	NEGATIVE
PAM5	15.70	1.18E+03	NEGATIVE
PAM5	29.14	9.39E+01	NEGATIVE
PAM4		2.66E+02	NEGATIVE

The data shown here suggests that this system can be used to measure fluxes in the biological content of aerosols. It shows excellent correlation with liquid samples generated in the laboratory and with field samples.

One thing to note is that the data obtained during the aerosol chamber trials was obtained using minimally skilled individuals in protective clothing under field conditions. The data is reproducible and consistent, although some anomalies exist. This suggests that relatively untrained personnel can use this equipment to obtain information about potentially hazardous situations.

The characterization of biological materials in aerosols is difficult, even under controlled conditions. Despite this, the data shown here does present a strong argument that this system can be useful to detect biological materials in aerosols.

The data presented here also suggests that this device can be interfaced with various types of air sampler equipment, in spite of differences among them. For example, the highest degree of success and consistency seems to be with the XM-2+ sampler. The cyclones seem to have more sporadic negatives. This could be due to several factors, such as the damaging the bacterium when it slams into the wall of the sampler. Effects of air samplers on bacterial viability have been reviewed by Terzieva et al. (7).

Potential applications of the technique include biological warfare agent detection by both military or civilian first responders; sterility monitoring in operating rooms, clean rooms, and food processing facilities. Presently there are no mandatory quality control procedures or guidelines that one must meet for some of these types of facilities. However, this could change in the future as we begin to appreciate the impact of biological aerosols on the health and well being of others.

Heating, ventilation, and air-conditioning systems (HVAC Systems) are another area

where this technique has opportunity. It can be used to measure filter integrity and cooling water for the presence of high counts of bacteria. This may indicate an abnormal condition and could be used to avert outbreaks of airborne diseases, such *Legionella pneumophila.* The potential also exists to replace the Anderson Impactor Samplers using culture plates that currently are used to determine mold and fungi contamination. This could provide a capability for on the spot screening of HVAC systems by industrial hygiene or other professionals; however, this still would still have to be validated and accepted by the community.

CONCLUSIONS

A device that measures ATP luminescence from biological samples was evaluated and demonstrated a detection limit of 100 pg/ml of ATP. This system was then evaluated and found that it could reliably detect 10^5 CFU/ml of bacteria that were in log phase (actively growing).

The system was evaluated with several types of air sampler systems with *ad hoc* aerosols and aerosols released under controlled conditions. It was found that the luminometer could reliably measure changes in the biomass of these samples and that the signal intensity did correlate with plate counts. Personnel with varying skill levels can use the system with good results. This suggests that it has application to various types of field applications.

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