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Feasibility of the Aerosol-to-Liquid Particle Extraction System (ALPES) for collection of viable *Francisella* sp.

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I. EXECUTIVE SUMMARY

Several Biowatch monitoring sites in the Houston area have tested positive for *Francisella tularensis* and there is a need to determine whether natural occurring *Francisella*-related microorganism(s) may be responsible for these observed positive reactions. The collection, culturing and characterization of *Francisella*-related natural microorganisms will provide the knowledge base to improve the future selectivity of Biowatch monitoring for *Francisella*. The aerosol-to-liquid particle extraction system (ALPES) is a high-efficiency, dual mechanism collection system that utilizes a liquid collection medium for capture of airborne microorganisms. Since the viability of microorganisms is preserved better in liquid medium than on air filters, this project was undertaken to determine whether *Francisella philomiragia* and *Francisella tularensis* LVS maintain acceptable viability in the continuous liquid recirculation, high direct current voltage and residual ozone concentrations which occur during ALPES operation. Throughout a series of preliminary trial runs with representative gram-negative and gram-positive microorganisms, several design modifications and improvements to the ALPES optimized liquid handling, electrical stability, sampling and overall performance for biological sampling. Initial testing with *Francisella philomiragia* showed viability was preserved better in PBS buffer than HBSS buffer. Trial runs at starting cell concentrations of 1.8×10^6 and 2.5×10^4 CFU/L showed less than a 1-log decrease in viability for *F. philomiragia* after 24 h in the ALPES. *Francisella tularensis* LVS (live vaccine strain) was used as a surrogate for virulent *F. tularensis* in ALPES trial runs conducted at starting cell concentrations of 10^4, 10^5 and 10^6 CFU/L. *F. tularensis* LVS was slow-growing and required highly selective growth media to prevent overgrowth by collected airborne microorganisms. In addition, one ALPES unit intake was HEPA filtered during the final trial runs with *F. tularensis* LVS to further reduce the levels of microbial background. Results from trials with *F. tularensis* LVS showed about a 1-log loss decrease in CFUs after 24 h, but maintained final cell concentrations in the range of 10^3-10^4 CFU/L. These results indicate that the ALPES maintains acceptable viability of *Francisella* sp. in PBS buffer for up to 24 h and is a promising technology for the collection of viable airborne *Francisella* or *Francisella*-related cultures which may be observed at Biowatch monitoring sites in the Houston area and elsewhere.

II. INTRODUCTION

The Biowatch program, launched in 2003, monitors air samples in major urban cities nationwide providing early warning of a potential biological attack. The Biowatch monitoring sytem consists of dry filter units that collect airborne particles onto filter paper that are removed every 24 hours for laboratory analysis. Beginning in the fall of 2003, several Biowatch monitoring sites in the Houston area, and more recently Washington DC, tested positive by genetic analysis for *Francisella tularensis*. However, subsequent investigations showed no evidence of a malicious microbial release nor any human or animal infection with *Francisella*. These occurrences and follow-up investigations support the hypothesis that a natural occurring *Francisella*-related microorganism(s) may be responsible for these observed positive reactions.

An extensive DNA-based survey of soil and water for the presence of *Francisella tularensis* and related species was subsequently conducted in the Houston area (Barns, et. al, 2005). This survey indicated that genetic sequences related to *Francisella* were present in one water and seven soil samples. Although observed sequences were most similar to *Francisella*, they were phylogenetically distinct and suggested that the presence of a variety of previously unknown *Francisella* may be present which could be positively detected in genetic assays for *F. tularensis* (Barns, et. al., 2005). Since these genetic analyses surveyed a mixture of nucleic acids from a variety of microorganisms present in water or soil, it was not possible to match observed sequences with a specific microbial isolate. Furthermore, microorganisms collected in dry filter units may elicit positive results in genetic or immunological analyses, but these results cannot be confirmed because viability is usually rapidly lost on the filter due primarily to prolonged dessication. There is a need for isolation of viable, natural *Francisella*-related microorganisms to enable independent confirmation of observed positive reactions for *Francisella* in the Biowatch program. Therefore, this investigation was initiated to determine whether the viability of representative environmental microorganisms and *Francisella sp*. are maintained in the liquid buffer collection medium of the recently developed aerosol-to-liquid particle extraction system (ALPES).

There is need for isolation of viable, natural Francisella-related microorganisms

III. MATERIALS AND METHODS

Experimental Approach. The overall goal of this study was to determine the feasibility of using the ALPES for maintenance and recovery of viable Francisella spp. The primary reason for conducting this investigation was to determine whether selected microbial cultures and *Francisella* spp. maintain viability in the continuous liquid recirculation, high direct current voltage and residual ozone concentrations which occur during ALPES operation.

The experimental approach consisted of four key steps: 1) Demonstrate viability of selected microbial cultures for 24 hr in the ALPES; 2) Demonstrate viability of varying cell densities of *Francisella philomiragia* for 24 hr in the ALPES; 3) Demonstrate viability of varying cell densities of *Francisella tularensis LVS* for 24 hr in the ALPES; 4) Conduct iterative modifications of the ALPES to achieve reliable and uninterrupted 24 hour collection and recovery of viable microbiological samples.

Aerosol-to-Liquid Particle Aerosol Extraction System (ALPES). The ALPES is a high-efficiency atmospheric sampling device that collects airborne particles into a liquid medium that continuously recirculates within the unit (Fig. 1). The footprint of the ALPES is 18" by 12" by 12" with a power requirement of 12 Vdc and power consumption of 15 W. The air intake system provides a stable flow rate of 300 liters per minute with a minimal pressure drop across the device. The ALPES was awarded a 2003 R&D 100 Award by R&D Magazine.

Figure. 1

A schematic of the ALPES sampler is shown below. The ALPES is an electrostatic precipitation device consisting of an intermediate flow air intake system, an ionization section, a central air flow tube, a flow-through liquid cylinder, an electrostatic ground cylinder, a fluid reservoir and a fluid pump. The device circulates collection fluid from the reservoir about the charged transport tube that is energized with 8,000 V+. This flow is designed such that a continuous film of liquid is maintained on the surface of the tube. The liquid is then cycled back to the reservoir. The recirculating liquid volume provides a mechanism for continual concentration of particles collected from ambient air. In this study, collection liquids were biological buffers intended to maintain the viability and cultivability of selected microorganisms.

The liquid collection medium may be analyzed off line or integrated directly into a compatible detector for near real-time detection. Since the ALPES was operated continuously for 24 hour collection cycles in this study, the liquid reservoir volume was increased to 500 mls to minimize the effects of liquid losses due to evaporation and all analyses were conducted off-line.

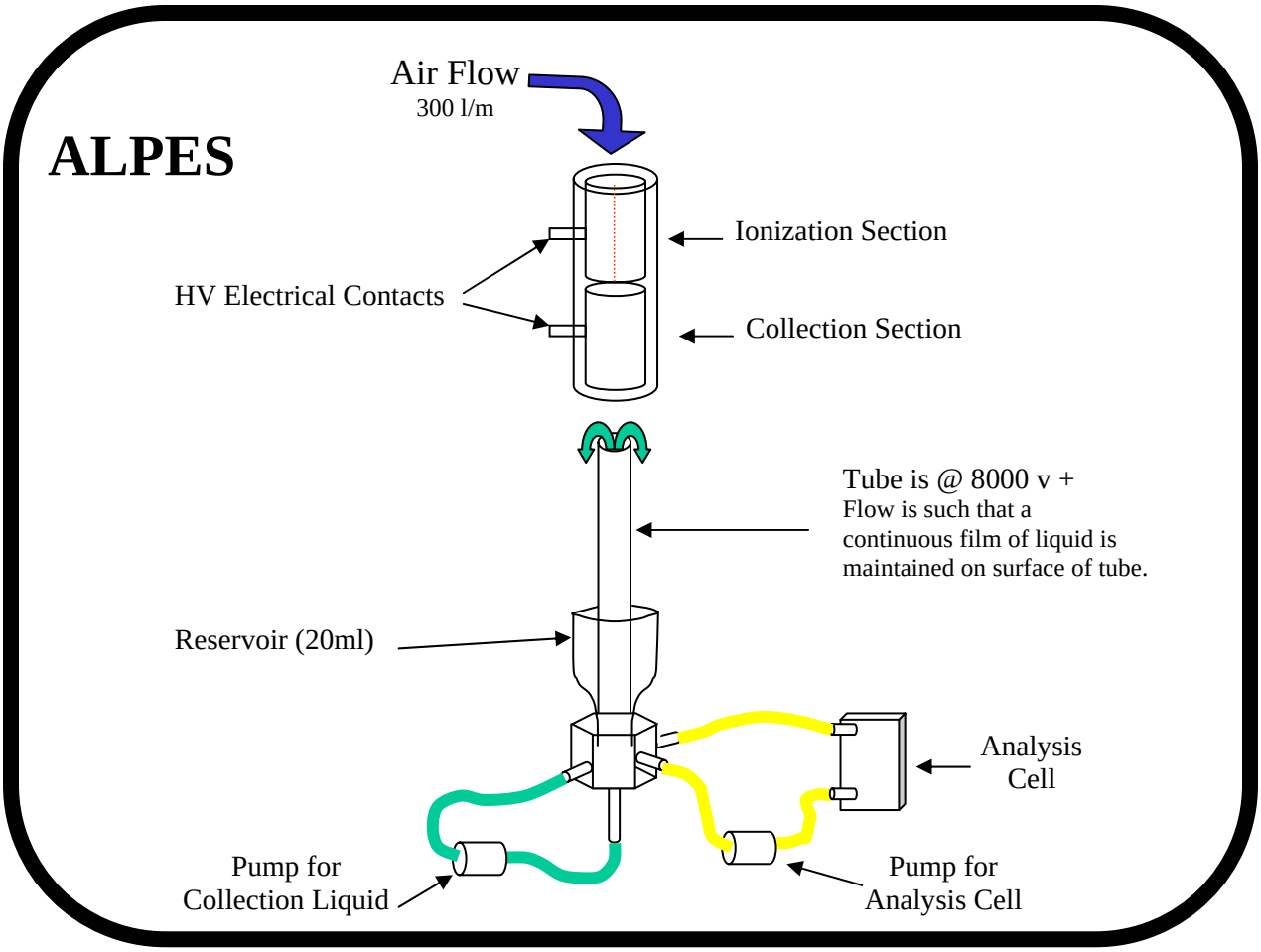

Figure 2. Schematic of the ALPES

ALPES Operation. Assembly and operation of the ALPES is described in the ALPES Operation Procedure provided in Appendix A. Prior to use, the ALPES was thoroughly cleaned by first wiping down the standpipe electrode with 70% isopropyl alcohol. The liquid reservoir and tubing was cleaned by circulating 500ml of 10% bleach for 3 min, followed by two, 3 min rinses with 70% isopropyl alcohol. The ALPES was rinsed with sterile tissue culture water (Sigma) between and after each cleaning run. The ALPES was wiped dry and air dried prior to use.

Microbiological strains. *Pseudomonas* strain #B252 was obtained from the Savannah River Site (SRS) Subsurface Microbial Culture Collection. It was isolated at SRS at a subsurface depth of 299 feet in the Congaree geological formation. Initial ID was determined by 16S and Biolog™ testing. *Aeromonas sobrina* (CB # 15-4828) culture and *Sarcina lutea* culture (CB # 15-5420) were purchased from Carolina Biological, Burlington, NC .

F. philomiragia strain GA01-2810 was isolated from water samples in Utah, USA (Versage et al., 2003). *F. tularensis* LVS (lot 12 of bottled vaccine) was obtained from the Centers for Disease Control (CDC) located in Fort Collins, CO, USA. Access to

Francisella philomiragia and *F. tularensis* LVS pure cultures was kindly provided by Drs. L. Lindler and C. Fernandez-Prada at Walter Reed Army Institute for Research (WRAIR; Silver Spring MD, USA). All experiments conducted with *Francisella* cultures were performed at WRAIR in a class II, type A biological safety cabinet.

Culture Preparation. The *Pseudomonas* culture was started from 50% Tryptic Soy Agar (TSA) plates. Stock culture purity was confirmed by microscopic and macroscopic examination. Test cultures were prepared by inoculating 50% Tryptic Soy Broth (TSB) and incubating with shaking at 25˚C for 18 hours. Cells were recovered by centrifugation (6000 RPM) and Fluorescent Antibody (FA) Buffer (Difco) washing. Washed cells were suspended in FA Buffer to achieve appropriate starting cell numbers in the cell suspension. Cell titers in final cell suspension were determined by both Acridine Orange (AO) staining with microscopic examination and by plate counting on 50% TSA. Both the Aeromonas and the Sarcina cultures were started from TSA plates. The purity of the cultures were confirmed by microscopic and macroscopic examination. Test cultures were prepared by inoculating Tryptic Soy Broth (TSB) and incubating with shaking at 25˚C for 18 hours. Cells were recovered by centrifugation (6000 RPM) and Fluorescent Antibody (FA) Buffer washing. Washed cells were suspended in FA Buffer to achieve appropriate starting cell numbers in the cell suspension. Cell titers in final cell suspension were determined by both Acridine Orange (AO) staining with microscopic examination and by plate counting on TSA. Triton-X was added to some cell suspensions before starting the ALPES unit. When used, it was added at a concentration of 0.07488 gr/liter of cell suspension.

Francisella philomiragia or *Francisella tularensis* LVS cultures were started from glycerol stocks stored at -80˚C. Stock culture purity and viability were confirmed by plating on BBL™ CHOC II (Becton Dickinson) and incubating in the dark at 37˚C and 5% CO_2. Test cultures were prepared by inoculating BBL™ Fluid Thioglycollate Medium (25ml; Becton Dickinson) supplemented with 2% (final concentration reconstituted) IsoVitaleX™ Enrichment Medium (Becton Dickinson) and incubated overnight at 37˚C, 180rpm in a 5% CO_2 incubator. Cell titers in broth culture were determined by plate counts and spectrophotometry at 600nm; OD 0.6-1.0 approximates $5x10^9$-$1x10^{10}$ cells/ml (Carmen Fernandez-Prada, personal communication). Cultures were diluted in 1L, 1x Dulbecco's Phosphate Buffered Saline (PBS; Sigma) for use in the ALPES experimental trials. All ALPES experiments were conducted in duplicate (500 mls).

ALPES Sampling for Representative Environmental Isolates. Prior to sampling the ALPES liquid collection medium for *Pseudomonas*, *Aeromonas*, and *Sarcina*, the high voltage was deactivated, liquid levels were returned to volume (500ml; accounting for evaporative losses) with sterile deionized water, and reservoir liquid was mixed thoroughly. A 5.5 ml sample was removed and ml was analyzed for colony forming units and the remaining sample was fixed with Sodium Azide for cold archiving. CFU was determined with 50% TSA for the Pseudomonas isolate and with TSA for the Aeromonas and Sarcina isolates.

ALPES Sampling for *Francisella* Experiments. Prior to sampling the ALPES liquid collection medium, the high voltage was deactivated, liquid levels were returned to volume (500ml; accounting for evaporative losses) with sterile tissue culture water (Sigma; maintaining original PBS concentration), and reservoir liquid was mixed thoroughly. A 12.5ml sample was removed and 2ml was analyzed for colony forming units (CFU) and 10.5ml was archived at -20˚C. Depending on the cell concentration, the 2 ml sample was either concentrated by centrifugation (3,000 rpm for 10 min at room temperature) and the cell pellet suspended in an equal volume of 1xPBS (100-200µl) prior to spread plating, or serially diluted in 1x PBS.

CFU was determined using 3 different medium formulations; BBL™ CHOC II (Becton Dickinson), BBL™ Modified Thayer Martin (MTM II; Becton Dickinson), and CHAB-A (Petersen *et al*., 2004). For the latter, an antibiotic cocktail (final concentration; 7.5 mg/L Polymyxin E, 2.5 mg/L Amphotericin B, 0.5 mg/L Lincomycin, 4.0 mg/L Trimethoprim, and 10 mg/L Ampicillin) was prepared fresh daily (stored in the dark at 4˚C) and applied evenly to CHOC II plates and air dried prior to sample plating. All plates were prepared in duplicate and incubated in the dark at 37˚C in a 5% CO_2 atmosphere. Colonies were counted on days 2 and 3 for all plating media.

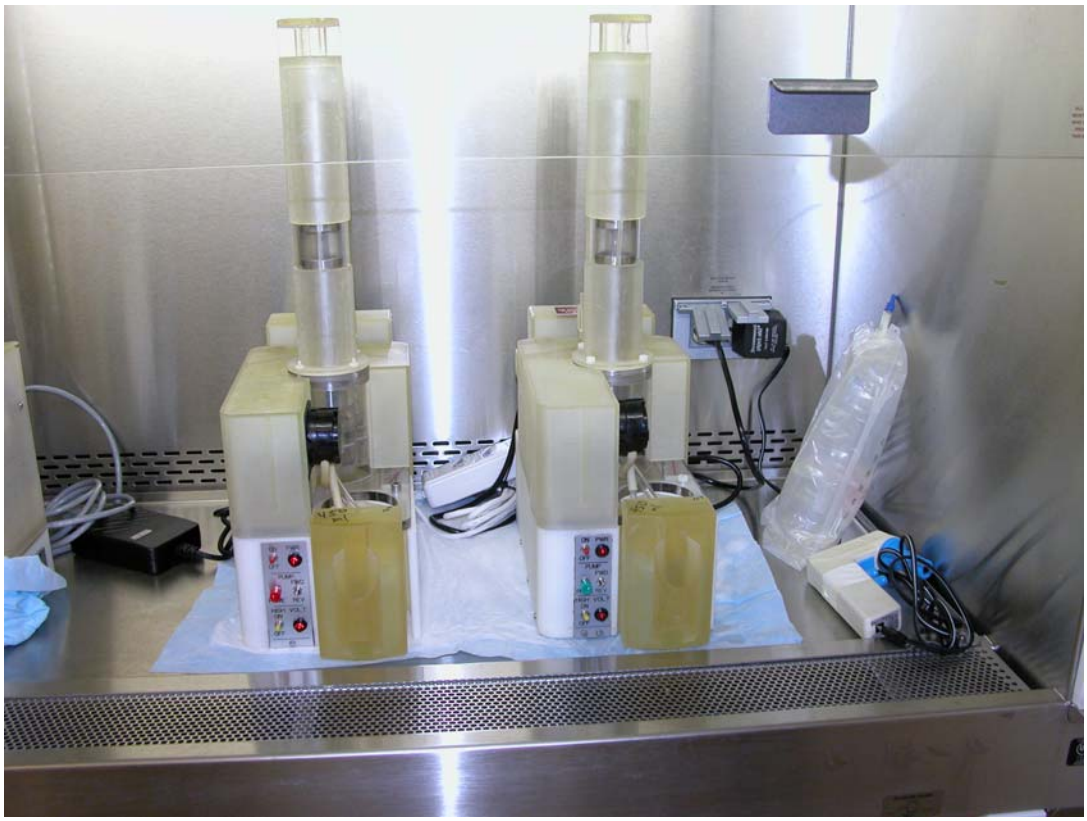

Figure 3.

ALPES units spiked with *Francisella tularensis* LVS at WRAIR.

IV. RESULTS OF EXPERIMENTAL RUNS

Survival of selected microbial cultures in the ALPES

Experimental Series 1 (January 11 – February 22, 2005)

Objectives

Examine viability of selected microbial cultures over a 24 hr period in ALPES trials conducted at SRNL. Determine whether viability is enhanced or inhibited by Triton X wetting agent.

Results

The *Pseudomonas* and *Aeromonas* cultures maintained viability for 24 hours in both ALPES and control flasks (Figure 4).

The gram positive *Sarcina* culture maintained viability through 6 h in both the control flasks and ALPES unit, but showed a decrease in viability at 22 h.

Triton X resulted in significant loss of viability for *Aeromonas* cultures in the ALPES.

Conclusions.

- The viability of selected gram negative microbial cultures were not effected by the continuous liquid recirculation, high direct current voltage and residual ozone concentrations which occur in the ALPES.
- Preliminary results shows additional testing and development may be required for gram positive target microorganisms.
- Since Triton X significantly reduced viability of *Aeromonas* in the ALPES, it should not be added as a wetting agent to the collection liquid.
- ALPES units achieved uninterrupted 24 hr operation

Actions

- Proceed to viability testing with *Francisella philomiragia* at WRAIR.

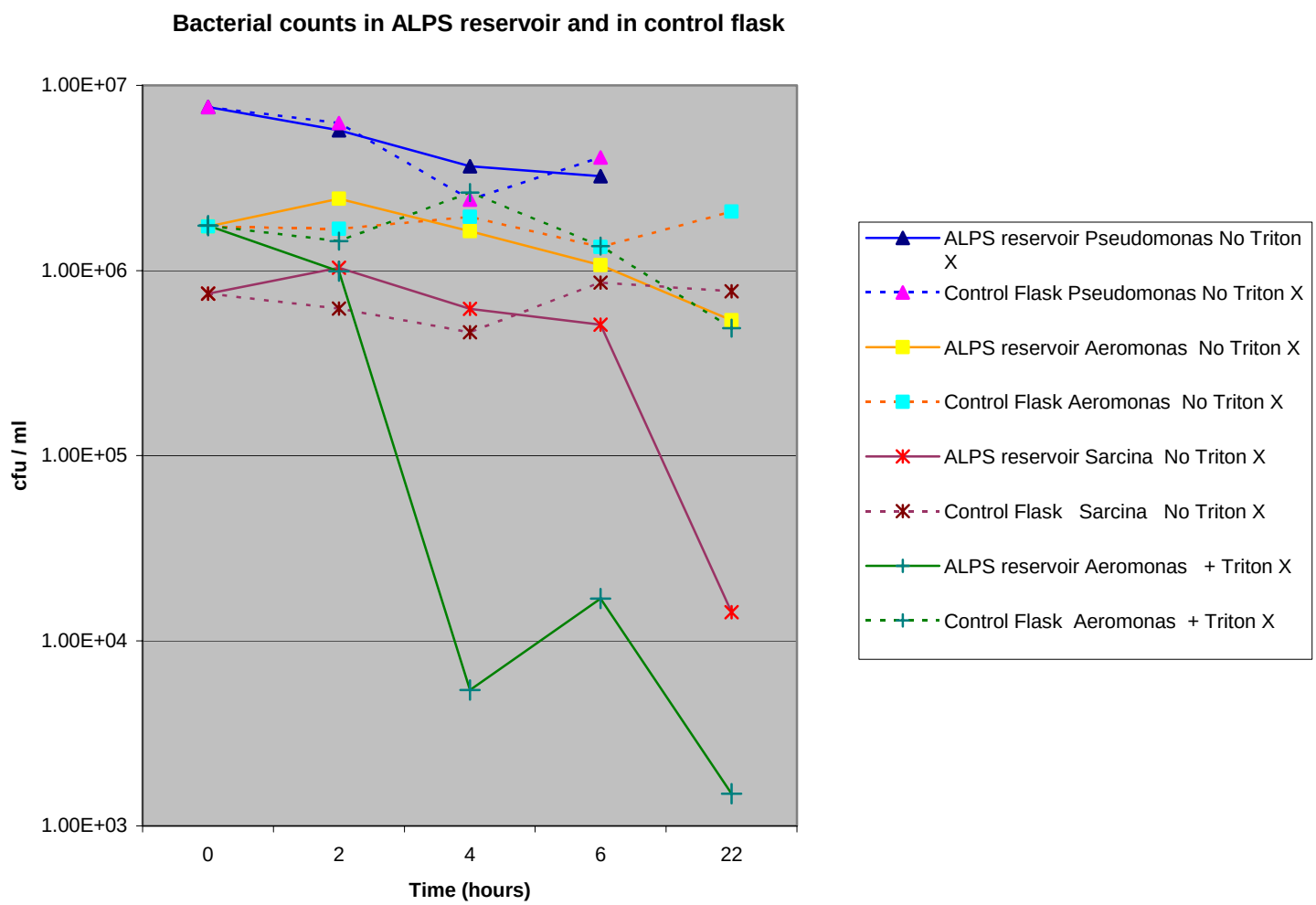

Figure 4. Viability of selected microbial cultures and ALPES and Control flask in presence or absence of Triton X.

Survival of *Francisella philomiragia* in the ALPES

Experimental Series 1 (May 16-19, 2005)

Objectives

Perform initial 24 hour trials with two ALPES units at WRAIR, spike with *Francisella philomiragia* and examine viability at multiple sample times up to 24 hr by plating onto Chocolate Agar–GC Medium plates. Additional objectives included 1) determination of an appropriate biological buffer for follow-on experiments with *Francisella* spp., and 2) provide preliminary evaluation of ALPES operation over 24hr.

Results

1st ALPES unit was spiked with *Francisella philomiragia* at 6 x 10^6 CFU/liter in Hanks Balanced Salt Solution (HBSS). No change in viable microbial cell counts observed at 2 hr, 4 hr or 6 hr run time. ALPES maintained operation for 24 hrs. However, no viable *Francisella* observed at 24 hr.

2nd ALPES unit was spiked with *Francisella philomiragia* at 6 x 10^6 CFU/liter in Phosphate Buffered Saline (PBS) buffer. No change in viable microbial cell counts observed at 2 hr, 4 hr, or 6 hr.

Electrical malfunction observed in 2nd ALPES unit at 8 hours. Problem diagnosed as liquid flow rate exceeding the capacity of the collection vessel located at the base of the collection tube, resulting in flooding of the electrical components in the bottom of the unit. No data collected beyond 6 hours.

Conclusions

- *F. philomiragia* shown to remain fully viable in ALPES for 6 hrs in PBS and HBSS.
- *F. philomiragia* not fully viable after 24 hrs. when suspended in HBSS buffer
- HBSS buffer determined unsuitable in ALPES for long-term sampling.
- ALPES liquid handling and system not optimal for volume needs when collecting microbial samples

Actions

- PBS buffer used in all subsequent ALPES trials
- ALPES liquid handling system redesigned to better accommodate larger volumes (up to 500 mls).
- Additional trial runs scheduled at WRAIR

Survival of *Francisella philomiragia* in the ALPES (Cont'd)

Experimental Series 2 (May 24-27, 2005)

Objectives

Perform 24 hour trail runs using *F. philomiragia* at two different cell concentrations with two ALPES units at WRAIR. Plate all samples on Chocolate Agar – GC Medium plates

Results

1st ALPES unit with *Francisella philomiragia* at 10^3 CFU/liter in PBS buffer. No change in counts at 2 hr, 4 hr, or 5.5 hr run time. No change in count numbers at 24 hr run time, but some colonies were of atypical appearance. Therefore, 24hr results considered to be suspect.

2nd ALPES unit with *Francisella philomiragia* at 10^6 CFU/liter in PBS buffer. No change in counts at 2 hr, 4 hr, or 5.5 hr run times. No results from 24 hours run due to electrical malfunction within unit after 6 hr run time - trial was stopped.

Conclusions

- *F. philomiragia* shown to remain fully viable in ALPES in PBS buffer for 24 hrs.
- Results are promising, but some atypical *F. philomiragia* colonies observed. Indications that background contamination must be confronted for these initial experiments.
- Further data needed to confirm 24 hr viability of *F. philomiragia* in ALPES
- ALPES requires further modification in order to address continued electrical malfunction.

Actions

- Liquid reservoir located at the base of the standpipe was expanded in size in order to accommodate high flow rate and thereby prevent liquid spillage into electrical components.
- Small but continuous internal leaking was noted within the ALPES units. Problem traced to damaged (aged) O-ring seals. All O-rings were replaced.
- ALPES modified. Electrical components reconfigured such that critical components no longer located under liquid handling components.
- ALPES housing fabricated from a different type of plastic to provide enhanced electrical insulation. This was done to prevent arcing.
- ALPES modified so that secondary containment plate installed under all liquid handling components.
- Additional trial runs scheduled at WRAIR.

Survival of *Francisella philomiragia* in the ALPES (Cont'd)

Experimental Series 3 (June 27 - 30, 2005)

Objectives

Perform 24 hour trail runs using *F. philomiragia* at two different cell concentrations with two ALPES units at WRAIR. Plate all samples on Chocolate Agar – GC Medium plates

Results

1st ALPES unit with *Francisella philomiragia* at 10^6 CFU/liter in PBS buffer. No significant loss of viable counts at 2 hr, 4 hr or 6 hr, small drop at 24 hr.

2nd ALPES unit with *Francisella philomiragia* at 10^4 CFU/liter in PBS buffer. No significant loss of viable counts at 2 hr, 4 hr, 6 hr, small drop at 24 hr.

Table 2. Time course of *F. philomiragia* survival in ALPES

Francisella philomiragia	Initial count CFU / liter	2 hr CFU / liter	4 hr CFU / liter	6 hr CFU / liter	24 hr CFU / liter
ALPES unit 1	1.80E+06	2.00E+06	2.10E+06	2.20E+06	5.00E+05
ALPES unit 2	2.50E+04	2.50E+04	2.00E+04	2.00E+04	8.00E+03

Conclusions

- *F. philomiragia* showed acceptable viability in ALPES trials in PBS buffer for 24 hrs.
- All modified ALPES units performed well in all 24 hr runs with no problems.

Actions

- Experimental focus advanced from *Francisella philomiragia* to *Francisella tularensis* LVS
- ALPES exterior further improved so that liquid containment vessels reconfigured/remolded by SRNL rapid prototyping staff to ensure complete closure of all liquids during ALPES run. This reconfiguration increased biocontainment and ease of handling of sampling liquids.
- ALPES modified so that entire unit was molded from a single piece of plastic to further assure that unit is liquid-tight.
- Additional trial runs scheduled at WRAIR

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Survival of *Francisella tularensis LVS* in the ALPES

Experimental Series 1 (August 8 - 11, 2005)

Objectives

Perform 24 hour trail runs with three ALPES units at WRAIR with *Francisella tularensis LVS* tested at concentrations of 10^4, 10^5 and 10^6 CFU/liter. Plate all samples on Chocolate Agar – GC Medium plates

Results

ALPES Trial 1 with *Francisella tularensis* LVS strain at 10^4 CFU/liter in PBS buffer. No significant change in counts at 2 hr or 4 hr run times. However, all samples collected beyond 6 hr were contaminated. All subsequent samplings were overgrown with contaminants and *Francisella tularensis* LVS colonies could not be counted.

ALPES Trial 2 with *Francisella tularensis* LVS strain at 10^5 CFU/liter in PBS buffer. No significant change in counts at 2 hr, 4 hr, or 6 hr run times. 24 hr run was overgrown by contaminants. 24 hr run time samples contained contaminants that overgrew plates and masked and/or inhibited the *Francisella tularensis* LVS colonies.

ALPES Trial 3 with *Francisella tularensis* LVS strain at 10^6 CFU/liter in PBS buffer. No significant change in counts at 2 hr, 4 hr, or 6 hr run times. The 24 hr run time samples contained contaminants that overgrew plates and masked and/or inhibited the *Francisella tularensis* LVS colonies.

Note**:** ALPES trials 4, 5 and 6 were replicates of trials 1, 2 and 3.

ALPES trial 4 with *Francisella tularensis* LVS strain at 10^4 CFU/liter in PBS buffer. No significant change in counts at 2 hr run time. However, 4 hr, 6 hr, 24 hr, and 30 hr run time samples contained contaminants that overgrew plates and masked and/or inhibited the *Francisella tularensis* LVS colonies.

ALPES trial 5 with *Francisella tularensis* LVS strain at 10^5 CFU/liter in PBS buffer. No significant change in counts at 2 hr run time. However, 4 hr, 6 hr, 24 hr, and 30 hr run time samples contained contaminants that overgrew plates and masked and/or inhibited the *Francisella tularensis* LVS colonies.

ALPES trial 6 with *Francisella tularensis* LVS strain at 10^6 CFU/liter in PBS buffer. No counts could be made because all samples contained contaminants that overgrew plates and masked and/or inhibited the *Francisella tularensis* LVS colonies.

Survival of *Francisella tularensis LVS* in the ALPES (Cont'd)

Experimental Series 1 – Cont'd (August 8 - 11, 2005)

Reserved samples were examined by a fluorescent monoclonal antibody and flow cytometry at WRAIR. LVS counts were inconsistent due to high background (microbiological and particulates) of these samples.

Conclusions

- Several trials at several test concentrations suggest that *F. tularensis LVS* maintains viability in ALPES for up to 6 hrs.
- Since *Francisella tularensis* LVS strain requires 48-72 hours to grow, environmental microorganisms collected through the sampler from the air had ample time to grow on the plates and those colonies interfered with ability to recover and count *Francisella tularensis* LVS colonies.
- Selective media needed to accurately and reliably quantify collected *Francisella tularensis* LVS against a background of microbes present in air. This will be especially important during open air sampling.
- Flow cytometry for detection of *Francisella tularensis* LVS against a background of environmental microorganisms is promising, but requires further development. Further development of flow cytometry is worthwhile, but beyond the scope of this study
- All modified ALPES units performed well in all 24 hr trial runs with no problems. It was determined that the simultaneous operation of 3 ALPES units (approx. 900L air / min) exceeded the capacity of a single laminar flow hood, resulting in the collection of airborne contaminants from laboratory ambient air.

Actions

- Consulted with LANL and CDC – Fort Collins. CDC Ft. Collins has noted inhibited growth of *Francisella tularensis* LVS in the presence of other bacteria.
- Subsequent plating for final trials will be on three different antibiotic medium formulations: 1) Chocolate Agar – GC Medium. 2) MTM (Modified Thayer Martin) 3) CHAB media with 5 antibiotics.
- Schedule final ALPES trial runs at WRAIR
- Produce plan to modify ALPES to HEPA pre-filter air to minimize collection of non-target microorganisms allowing ready determination of viability of *Francisella tularensis* LVS after 24 hr in ALPES.

Note: HEPA pre-filtration module is solely for the purpose of completing the final ALPES trials at WRAIR to demonstrate *Francisella* viability after 24 hrs. Subsequent field deployment of the ALPES for environmental biocollection will obviously not utilize the HEPA pre-filter module.

Survival of *Francisella tularensis LVS* in the ALPES (Cont'd)

Experimental Series 2 (Mid September thru October 13, 2005)

Objectives

Design, manufacture and implement a HEPA air pre-filtration module for ALPES to enable completion of the final *F. tularensis LVS* viability trials at WRAIR.

Results

SRNL rapid prototyping produced an air pre-filtration module which performed well on the ALPES.

Trial runs of the modified ALPES were run for 24 hours at SRNL. Modified units ran and performed well.

Units with HEPA pre-filtration yielded background colonies of 10 cells/ml after 24 hours of operation.

ALPES without HEPA pre-filtration yielded up to 10^{3-4} cells/ml after 24 hours.

Obtained media formulas and purchased growth media components as recommended by CDC – Fort Collins.

Conclusions

- Modified ALPES units and improved *Francisella* growth medium should enable success in final ALPES trials of *Francisella tularensis* LVS at WRAIR.
- HEPA filtration module resulted in a three-four log reduction of background microorganisms.

Actions

- Schedule final ALPES trials at WRAIR
- Conduct trials with two ALPES units in parallel. Unit 1: HEPA filter assembly, PBS buffer, LVS. Unit 2: No filter, PBS buffer, LVS.
- Dilution plating on 3 different medium formulations: 1) Standard enriched chocolate agar, CHAB, 2) MTM, CHAB with 1 antibiotic, and 3) CDC Ft. Collins medium with 5 antibiotics (CHAB). All media are CHAB, one without antibiotic, MTM – 1 antibiotic, and CDC containing 5 antibiotics.

Survival of *Francisella tularensis LVS* in the ALPES (Cont'd)

Experimental Series 3 (May 15-19, 2006)

Objectives

Complete work to analyze *Francisella tularensis* LVS viability at 2 different cell concentrations (10^5 cells/ml and 10^5 cells/L) during 24hrs in the ALPES at WRAIR. 2 ALPES units operated in parallel. HEPA filtration module used on one unit to ensure no background contamination.

A primary focus was timing the start of these experiments to enable microbial sampling for viability at sample times approaching 24 hrs.

Plate samples onto three different medium formulations: Choc II, MTM II (contains 1 antibiotic), and CHAB-A (containing 5 different antibiotics).

Results

TIME	CHOC II	MTM II	CHAB-A
0 hr	13 x 10^5 CFU/ml	13 x 10^5 CFU/ml	13 x 10^5 CFU/ml
17.5 hr	4-5 x 10^4 CFU/ml	4-5 x 10^4 CFU/ml	3-4 x 10^4 CFU/ml
21 hr	3.5-4 x 10^4 CFU/ml	3.5-4 x 10^4 CFU/ml	3.5-4 x 10^4 CFU/ml
24 hr	3.5-4 x 10^4 CFU/ml	3.5-4 x 10^4 CFU/ml	3.5-4 x 10^4 CFU/ml

Table 2. Time course of *F. tularensis* LVS colony forming units (CFU) during 24h circulation in the ALPES analyzed on 3 different medium formulations. Starting concentration of cells was 13 x 10^5 CFU/ml. 2 ALPES units were tested, one was equipped with a HEPA filter and the other without, but all results were identical between units. Colonies were counted after 2 days of incubation at 37˚C in a 5% CO_2 atmosphere. Ranges are provided for duplicate measurements taken at each time point.

There were no observed differences between HEPA filtered and non-filtered ALPES units on any of the growth media tested. Colonies appeared to grow equally well regardless of medium type, although in general colonies on the CHAP-A medium were slightly smaller than those on either CHOC or MTM growth media.

These results, as well as the data from Experimental Series 1, indicate an approximate one-log decrease in CFU for *Francisella* spp. after 17hr in the ALPES, but viability was maintained through 24 hr of operation.

2nd Trial Run. The ALPES units were only sampled at times 0, 17 and 24 hr and the results are shown in Table 2. These results again provide clear demonstration that *F. tularensis* LVS maintains viability in the ALPES for up to 24 hr.

ALPES-Control	CHOC II	MTM II	CHAB-A
0 hr	8×10^5 CFU/L	8×10^5 CFU/L	8×10^5 CFU/L
17 hr	5×10^4 CFU/L	4×10^4 CFU/L	3×10^4 CFU/L
24 hr	2×10^4 CFU/L	1×10^4 CFU/L	2×10^4 CFU/L

ALPES-HEPA	CHOC II	MTM II	CHAB-A
0 hr	8×10^5 CFU/L	8×10^5 CFU/L	8×10^5 CFU/L
17 hr	1×10^4 CFU/L	1×10^4 CFU/L	1×10^4 CFU/L
24 hr	6×10^3 CFU/L	3×10^3 CFU/L	4×10^3 CFU/L

Table 3. Time course of *F. tularensis* LVS colony forming units (CFU) during 24h circulation in the ALPES analyzed on 3 different medium formulations. Starting concentration of cells was 8×10^5 CFU/L. 2 ALPES units were tested, one was equipped with a HEPA filter and the other without, but all results were identical between units. Colonies were counted after 2 days of incubation at 37˚C in a 5% CO_2 atmosphere.

Conclusions

- In both experimental runs (Table 2 and 3), *F. tularensis* LVS showed an initial drop in concentration, but plateaued at around 10^4 CFU/L after 17-24 hrs of operation.
- This initial drop in CFU/L is likely caused by distribution of spiked cells throughout the ALPES liquid volumes and surfaces.
- HEPA filtration module resulted in a 3-4 log reduction of background microorganisms.
- *F. tularensis* LVS shows acceptable maintenance of viability in the ALPES for 24 hrs in presence of collected natural airborne microorganisms.

V. CONCLUSIONS

The overall conclusions from this project are as follow:

- The viability of representative environmental microorganisms, *Francisella philomiragia* and *Francisella tularensis* LVS are maintained at acceptable levels in the Aerosol-to-Liquid Particle Extraction System (ALPES) and appeared to minimally impacted by continuous liquid recirculation, high direct current voltage or residual ozone concentrations which occur in the ALPES.

- The ALPES units modified for enhanced bioaerosol collection demonstrated efficient and reliable operation for 24-hour collection cycles.

- The growth medium formulations MTM II, and CHAB-A (Polymyxin E, Amphotericin B, Lincomycin, Trimethoprim, and Ampicillin) provided improved selective culturing of *Francisella* sp. in a mixture of collected airborne microorganisms.

- Preservation, culturing and recovery of *Francisella* sp. and *Francisella*-related microorganisms is likely to be better in liquid medium in the ALPES than in dry filter unit samplers currently in use.

VI. RECOMMENDATIONS

- The ALPES should advance to bioaerosol exposure testing to determine the collection efficiency for aerosolized *Francisella* sp. under controlled conditions.

- Upon successful completion of bioaerosol exposure testing, the ALPES should be deployed in parallel with Biowatch collectors in Houston to enable the collection, isolation and culturing of viable, natural Francisella-related microorganisms for further study.

- A continuing need will exist for improved selective growth medium and culturing procedures to enable the selective isolation and culturing of viable *Francisella* sp. and *Francisella*-related microorganisms occuring at low levels in the high concentration and diversity of robust background microorganisms typically collected during air sampling.

VII. ACKNOWLEDGEMENTS

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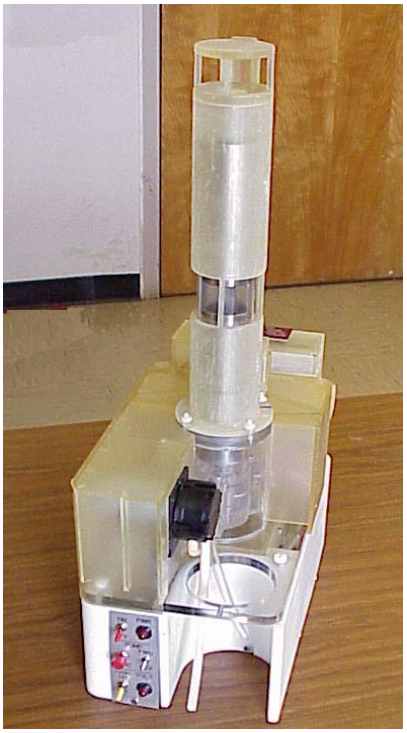
VIII. APPENDICES

APPENDIX 1

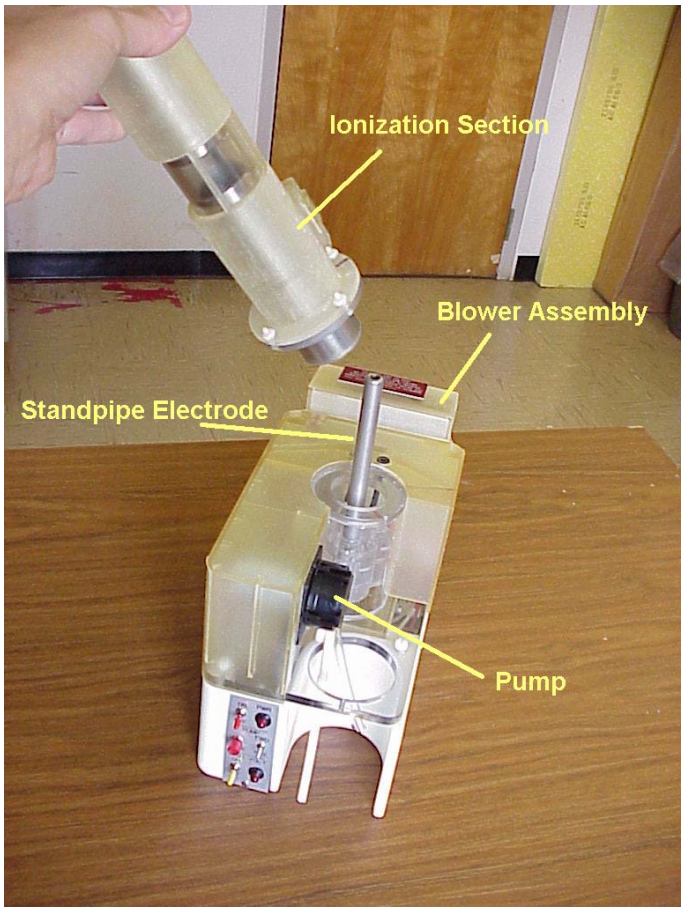
ALPES Operating Instructions

- Electrostatic particle collection system which uses liquid as collection media
- **ALPES can be an electrical shock hazard and must be used with caution**
- ALPES is an experimental laboratory instrument currently in development. It should only be used by qualified personnel using appropriate techniques. Comments and suggestions should be directed to management of the Nonproliferation Technology Section of the Savannah River National Laboratory.



Principle of ALPES Electrostatic Collection

- Four subsystems: Air blower, ionization (**shock hazard**) section, collection section (**shock hazard**), liquid pump
- Air blower pulls air though negative ionization section, past positively charged liquid, and exhausts air at left rear side of unit
- Negative ionization section is a tube in which a wire charged to -8,000 volts imparts a charge to air and particles as they pass by
- Immediately below negative ionization section, the collection section consists of an electrode charged to +8,000 volts attracts and captures particles in a water film. The electrode is in the form of a "standpipe" which has a continuous flow liquid up through and over it's surface.
- The liquid pump moves liquid (**shock hazard**) from a reservoir up through the charged standpipe to form a flowing film of liquid on the outer surface. By gravity, the liquid flows down the standpipe and drains back to the reservoir. Liquid is continuously recycled though the system in this way



Electrical Hazards of the ALPES

- Negative ionization section charged to -8,000 volts (maximum of -10,000)

  The ionization section contains a thin wire which presents a shock hazard if touched or probed with a conductive item. The wire is visible in the upper portion of the removable ionization section. Normally an air intake cover limits access to this wire in operation. Specific test configurations may not include the cover and therefore intentional contact is possible but inadvertent contact is unlikely.

- Collection section electrode charged to +8,000 volts (maximum of +10,000)

  The collection section consists of a standpipe which presents a shock hazard if touched or probed with a conductive item. The standpipe is visible by removing the ionization section or by viewing through the clear portion of the ionization

section. In normal operation liquid will flow up through and over the surface of standpipe. The liquid presents the same shock hazard as the bare standpipe.

- All liquid, including in reservoir, charged to +8,000 volts (maximum of +10,000)

  Liquid is circulated by pump from the reservoir, up through and over the standpipe and flows by gravity back to the reservoir. By flowing over the charged standpipe the liquid also becomes charged to the same voltage. Liquid and all parts of the ALPES unit will be at high voltage and presents a shock hazard.

- Use of 115VAC outlet for ALPES power supply module

  Normally a 115VAC outlet provides power to the ALPES unit via the power supply module. **115VAC outlets can provide deadly voltage and current. Water in contact with 115VAC power presents a serious electrocution hazard and should be avoided. If this situation occurs move away from the water to a dry location and have power removed from the outlet from a remote location such as a breaker panel.**

Precautions when using the ALPES

- **Never touch, pour or sample liquid in the reservoir or circulating in the ALPES system while it is operating.** The liquid is charged to a high voltage and is therefore a shock hazard. Even items such as pipettes and swabs can conduct electricity and cause a shock to the user. Any activities involving ALPES liquids should be performed with the system turned off and unplugged. This includes wiping drips, cleaning up spills or correcting flow issues.

- **Never touch, reach into, or probe any internal surface or component of the ALPES unit.** High voltages are present and even non-conductive tools may carry a shock to the user. Activities which require internal access, such as cleaning and adjustment, should only be performed with the unit unplugged and in a safe, clean area.

- **In the event of even a minor electrical shock do not continue using the ALPES.**

- **Never remove, defeat or alter covers, interlocks or electronics in or on the ALPES unit.**

- **Never use flammable or volatile liquids in or around an ALPES unit.** Electrical discharge can result in explosion, fire and shock hazard. Only NTS (Savannah River National Laboratory) approved collection media and cleaning agents should be used.

- **Inspect the ALPES unit before use.** Important items are electrical connectors, switches, wires, liquid hoses and fittings, plastic covers. If these items are missing or damaged do not use the unit. Inspect the blower assembly and unit base of signs of liquid. All liquid outside the normal flow path must be eliminated before power is connected to the unit.

- **Only use the power supply module supplied with the ALPES unit.** Using an incorrect power supply may increase shock hazard and damage the unit. Presently the power supplies convert 100-220 volts AC to 12 volts 1 AMP DC. They are specially configured to electrically isolate the ALPES unit from AC power.

- **Do not improvise by using automobile/aircraft/boat power, batteries, bench top power supplies or other alternate sources of power.** These sources may not provide necessary electrical isolation and may present a serious shock and electrostatic discharge hazard beyond the ALPES unit itself.

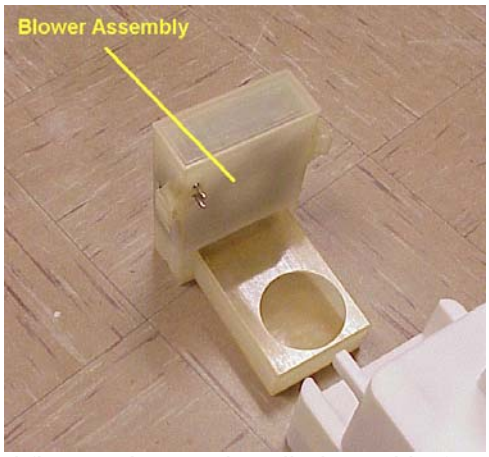
- **Operated ALPES only on a level and stable surface.** The unit will not operate safely or correctly if not level or disturbed while in operation. Resulting spills and erratic liquid flow will cause shock hazards.

- **Turn off and unplug the unit if arcing or improper liquid flow is observed.** Arcing (electrical discharge resulting in sparks and popping/snapping sounds) and improper liquid flow may result in a shock hazard and damage to the unit. In some cases these problems can be corrected by minor adjustments. Turn off and unplug the unit before making adjustments. If repeated adjustments fail to correct the problem do not use the unit.

Typical Operation

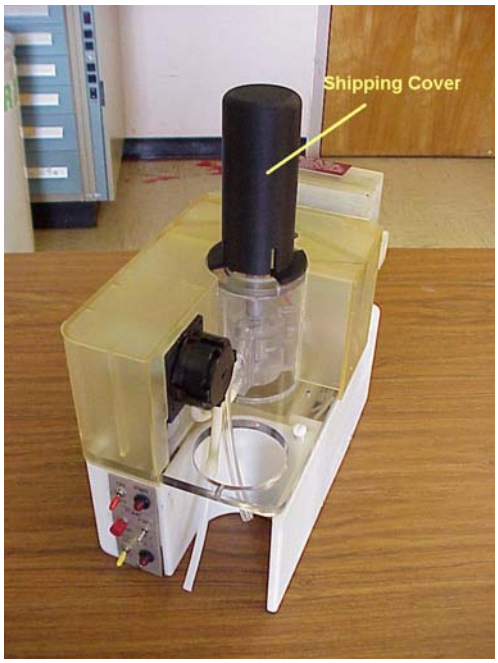
ALPES Startup

- Carefully unpack ALPES unit and components.
- Inspect unit and ensure all required components are included.
- Place unit on stable, level, clean surface for assembly
- Observe that all switches are in the OFF position and that there is no liquid in the unit base

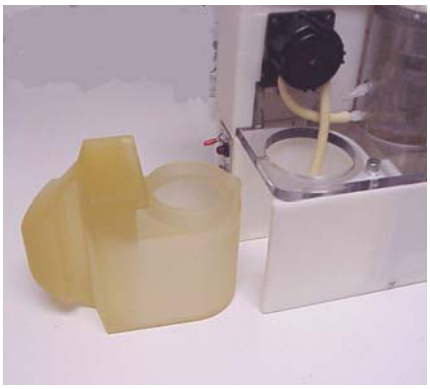


or blower assembly. All liquid outside the normal flow path must be eliminated before power is applied to the unit.

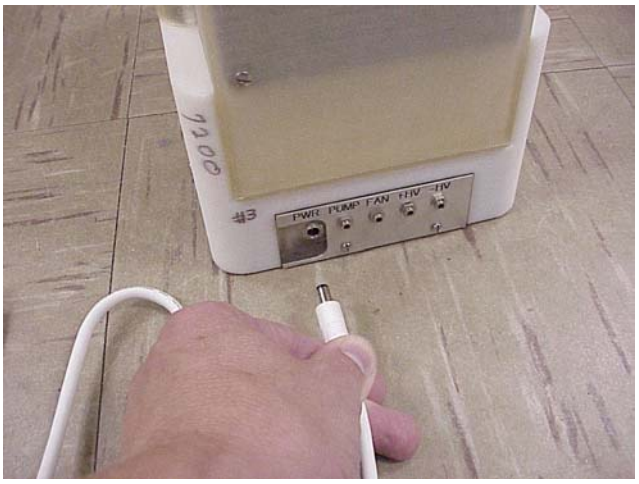
- Remove shipping cover from standpipe electrode. Do not touch standpipe with fingers as even small amounts of dirt and oil can interfere with proper liquid flow. Observe that the standpipe is clean and securely installed.



- Fill supplied ALPES reservoir with appropriate amount of liquid sampling media and insert into reservoir area. The pump intake tube should be below the surface of the liquid. The liquid return line (drain) should normally be just above the surface of the liquid for easy flow.



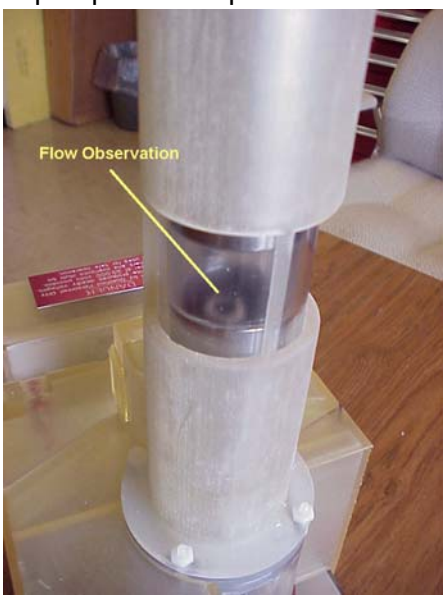
- Plug the power supply round plug into the power jack in the back panel of the unit. Check that all switches are in the OFF position (for the pump switch center is OFF). Plug the power supply module into an AC outlet.



- Move only the power switch **(HV switch remains OFF)** to the ON position and observe that the power light is illuminated and air is being exhausted from the blower assembly (left rear of unit). If not check power connections or get technical assistance.
- Move the pump switch to the FWD (forward) position. The pump should begin moving liquid from the reservoir up through the standpipe and over it's surface, then liquid should drain back to the reservoir. It is important that the liquid form a continuous film over the surface of the standpipe. If this does not happen after several seconds press the PRIME button to increase pump speed. **Do not use PRIME if high voltage is ON as this may cause arcing.** This may speed the "wetting" process. If the PRIME function does not result in an even film then cleaning of the standpipe may be required. This should only be accomplished with clean swabs and NTS (Savannah River National Laboratory) approved cleaning agents.

SRNL

- When reliable film is established move the pump switch to the center OFF position and move power switch to the OFF position. Clean up any spills and inspect blower assembly for liquid. If the blower assembly contains liquid remove it from the unit and dry out (blower top cover is removable).
- With all power off install the ionization section onto the unit, ensuring that it is fully seated and aligned.
- Move the PWR switch to the ON position then move pump switch to the FWD (forward) position to start the pump. Observe flow through the clear section of the ionization section. If flow is improper turn OFF pump and unit power and correct problem.



- If proper and stable flow is observed move the high voltage switch to the ON position. If arcing (sparks and snapping/popping sounds) is observed immediately turn high voltage to OFF, pump to OFF and power to OFF. Inspect for obvious misalignment problems, improper flow or foreign material in the unit. If problem is not obvious or correctable seek technical assistance.
- With proper flow and no arcing the unit can be operated for extended periods. Appropriate warnings and supervision should be applied to the ALPES operating area. It is recommended that the unit should be attended at all times or routinely observed for proper function.

ALPES Shutdown

- Move high voltage switch to the OFF position.
- Move and hold the pump switch in the REV (reverse) position to drain liquid from the pump tubing. Then allow the switch to return to the center (OFF) position.
- Move power switch to the OFF position and unplug power to the unit.
- Remove reservoir from unit and return power supply module to shipping case.
- Clean and dry unit and components.
- Return unit and components to shipping case.