Final Report to

Enumerating Bacteria in Deepwater Pipelines in Real-Time at a Negligible Marginal Cost per Analysis: A Proof of Concept Study.

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ABSTRACT

Bacterial fouling leads to inefficiencies and health, safety and environmental hazards in the oil and natural gas recovery industry. The purpose of this project was to develop a method for the real-time detection of microorganisms in bacterially contaminated substrates including flood water. The technology developed should, when deployed, be able to identify organisms 1) at the species level; 2) in real-time; 3) from real world samples; 4) quantitatively; 5) automatically.

In 2008, Livermore Instruments exclusively licensed a biodefense technology, BioAerosol Mass Spectrometry (BAMS), also known as Single Particle Aerosol Mass Spectrometry (SPAMS) from Lawrence Livermore National Laboratory (LLNL) and began development of our flagship product, the SPAMS 3.0. The SPAMS 3.0's operating principles are conceptually very simple. Particles, whether biological or not, are suspended in a carrier gas if they are not in such a gas already. The system is maintained at vacuum and particles are driven in by ambient pressure. Upon arriving at the ion source of the SPAMS' dual-polarity time-of-flight mass spectrometer, each particle is individually desorbed and ionized by a pulsed laser and the positive and negative ions are detected by their respective mass spectrometer. The mass spectra are analyzed in real-time by a two-stage pattern recognition algorithm and the microorganisms are identified accordingly. In this manner, biological organisms are identified to at least the genus and often the species level. Furthermore, because this process can be repeated many times per second, the organisms can be detected and quantified even in a background of particles at many times their own concentration. Simply put, there is no other technology that can detect biological organisms in real-time, identify them to the genus level, and report their identities and concentrations to the user at any price, and the total cost to operate a BAMS is similar to the cost to operate the slower or less precise machines. Accordingly such an instrument is an ideal candidate to be deployed to the oil and gas field.

The goal of this research was the application of LII's new SPAMS 3.0 product to bacterial identification for the analysis of microorganisms known to affect petroleum recovery. The design of the SPAMS 3.0 is significantly simplified versus the licensed system from LLNL. The system is reduced dramatically in size, weight, power consumption and cost to manufacture. It is far easier to keep in operation. In its current incarnation, it equals the LLNL design in performance and, with a minor redesign of the data system, will exceed it by a factor of 2.5.

Although the creation of the SPAMS 3.0 did not go smoothly, we were able to deploy it to our collaborator's laboratory and establish its ability to analyze microorganisms in minutes, even with the difficult sample preparation performed manually. The result was that the SPAMS 3.0 was able to distinguish organisms on the basis of general class (e.g. sulfate reducing bacteria) and genus, while species and strain were, as expected, not distinguished from one another. The ability to automatically identify microorganisms in real-time has not been demonstrated using any other technology.

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EXECUTIVE SUMMARY

Bacterial fouling leads to inefficiencies and health, safety and environmental hazards across the petroleum and natural gas recovery industry. Microbially-influenced corrosion attacks the whole system from production wellhead to refinery. Bacterially-evolved hydrogen sulfide sours the reservoir, devaluating the product and corroding extraction pipes. Bacterial iron sulfide production creates black powder accumulation, blocking pipelines. Bacteria present in the flood water are injected down hole during water flooding and become entrenched in the reservoir. Long deepwater pipelines are particularly at risk of microbial attack due to their physical size, the pressures that they bear, and the high costs of inspection, maintenance, repair and replacement.

To control microorganism contamination, the petroleum industry currently uses chemical biocides which are expensive and harmful to humans and the environment. The number and types of bacteria present impact the biocide regimen that is advised in any given scenario but bacterial identification is a slow, laborious and expensive process that cannot be used to direct, optimize or even verify the effectiveness of biocide applications. The high cost of an ineffective biocide regimen drives the overapplication of the biocides to ensure adequate bacterial control. The emerging issue of biocide resistance is further complicating these matters. An alternative technology for the identification and enumeration of bacteria, pre- and post-treatment that would return an answer within the decision cycle of the biocide application would allow far lower concentrations of biocides to be used when appropriate while warning that higher concentrations are needed in isolated cases. Such a technology would provide the added advantage of enabling emerging strategies such as targeted bacteriophage application.

BioAerosol Mass Spectrometry (BAMS) technology, originally developed for biodefense [Fergenson 2004], is the only technology capable of identifying and enumerating bacteria sampled directly from the environment in real-time. BAMS operates by collecting individual mass spectra of all particles present in a sample, bacterial or otherwise, and analyzing the spectra automatically to determine the number and types of bacteria present in real-time. Assay development and modification is far simpler for BAMS than for other "wet" microbial enumeration techniques such as PCR or immunoassays, taking only hours for nonpathogenic organisms and the theoretical precision of BAMS in bacterial enumeration is significantly higher.

As of the writing of this report, the project has been completed successfully and the SPAMS has been applied to the analysis of nine strains and species of bacteria critical to the petroleum recovery industry. Generally, strains of the same organism were indistinguishable to the SPAMS, species of the same genus were not reliably distinguishable, and different genera were distinguishable.

REPORT DETAILS

Experimental Methods:

The research program integrated an effort to allocate a prototype SPAMS 3.0 with an attempt to develop useful microbial identification libraries. Any discussion of the SPAMS 3.0 needs to consider the history of the Single Particle Aerosol Mass Spectrometry (SPAMS) programs and that will be discussed briefly here. A good background review on SPAMS and related technologies was written by Nash *et al* in 2006 [Nash 2006].

The first SPAMS (termed a BioAerosol Mass Spectrometer or BAMS) was created rapidly following the September 11 terror attacks in response to a perceived threat to national security resulting from the shipping of packages contaminated with biological agents, most significantly *B. anthracis*. Specialized, real-time analysis software, based on earlier pattern recognition work [Song 1999; Zhao 2008; Neubauer 1995], was written to operate a COTS TSI, Inc., Model 3800 ATOFMS which had been previously procured by Lawrence Livermore National Laboratory (LLNL) for biodefense research and development as a real-time identifier of microorganisms in the aerosol phase. This system was termed the BAMS 1.0 in retrospect. Unfortunately, shortly after the development of the system, the perceived threat was, in fact, encountered and the BAMS 1.0 was deployed to the Miami-Dade County Department of Health Services to Interdict it. In the following years, parallel efforts were mounted to improve the hardware and software [Steele 2008] and to develop identification libraries [Russel 2004, Srivastiva 2005].

The BAMS 1.0, while revolutionary in its speed versus specificity, had a critical limitation. It functioned by tracking particles with two lasers oriented across an aerosol beam and spaced roughly 10 cm apart. The time that it took a particle to proceed from the first laser to the second laser was used to time the firing of a third laser, 20 cm below, which was oriented through the center of the source region of a dual polarity time-of-flight mass spectrometer to ionize the particle. The ions were analyzed by the mass spectrometers and a real-time data analysis algorithm was able to identify microorganisms analyzed in this manner to the genus and, sometimes, the species. Unfortunately, because the two lasers were spaced so far from each other, when sufficient concentrations of particles would pass into the instrument it would become impossible to discern when a single particle had crossed both lasers or when two different particles had done so, and the rate at which particles were successfully ionized would decrease. As such, despite the fact that the system could theoretically analyze up to twenty particles per second, in reality its performance was capped to only two at the very maximum. Furthermore, due to timing inaccuracies, the ionization laser would miss the particle that it was aimed at up to 65% of the time.

A solution was devised at LLNL that remedied these shortcomings. Six lasers were used to track the particles, with a mathematical algorithm embedded in a field-

programmable gate array (FPGA) capable of keeping track of up to one million particles per second. Improvements to the timing precision raised the ionization rate to roughly 60% as well. A new aerosol interface was designed that was capable of introducing particles of between 0.9 and 9 micrometers in aerodynamic diameter. The improved system was termed the BAMS 1.5. This was completely reengineered into the SPAMS 2.0 (renamed because of the broadening of the application areas) which retained the performance advantages while shrinking the footprint of the system by roughly 50% but the SPAMS 2.0 cost roughly $1M to produce.

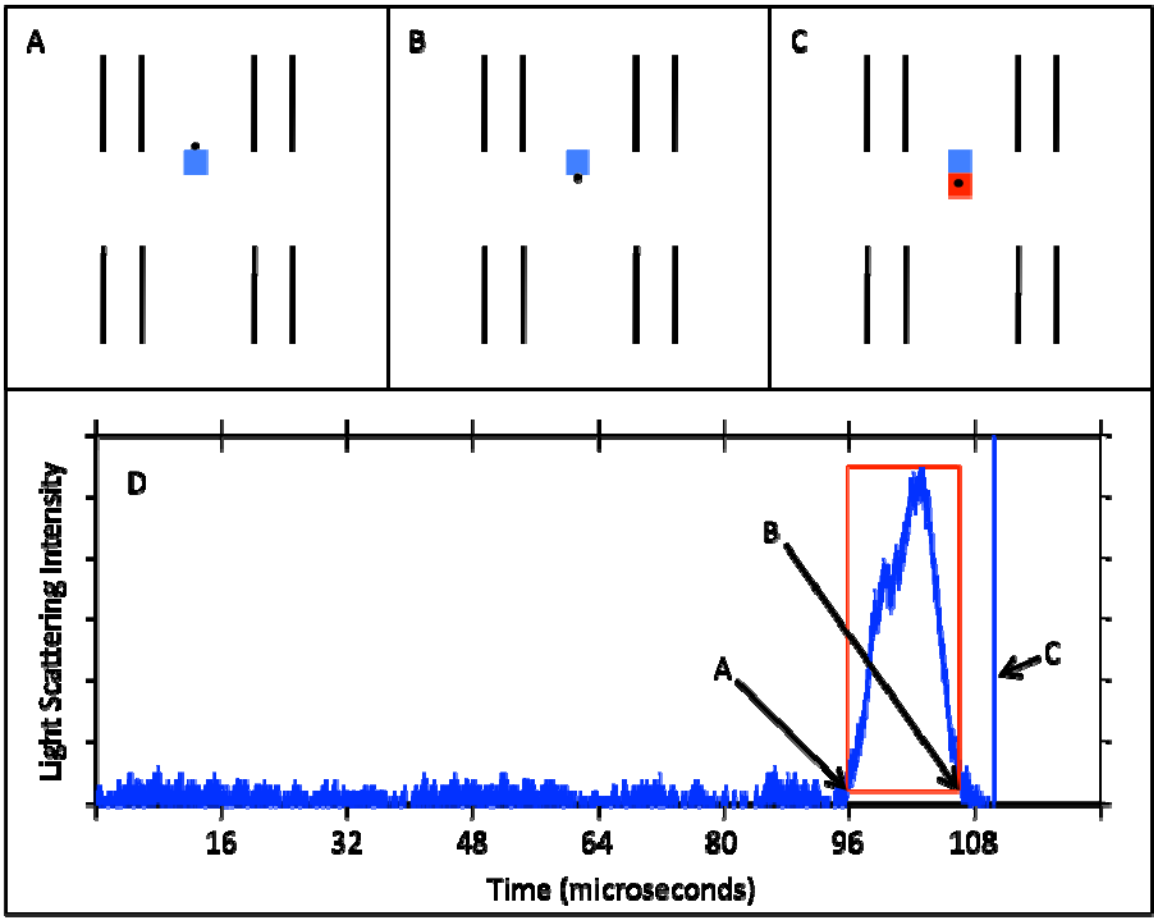

Figure 1: An illustration of the SPAMS 3.0's operating principles. Subfigures A, B and C are illustrations while subfigure D shows the actual light scattering trace from a particle crossing the square tracking laser. A particle enters the square tracking laser in frame A, proceeds through the tracking laser, and exits in frame B. Upon exiting, the particle is ionized by a pulsed high energy laser. The mound between locations A and B results from the light scattering while the ionization pulse generates the feature at location C.

Livermore Instruments licensed the SPAMS 2.0 technology from Lawrence Livermore National Laboratory in 2008 and set about designing a system that would equal the performance of the SPAMS 2.0 but which could be manufactured at a much reduced cost and which had fewer tunable parameters in the field. That design was

realized starting beginning before this project began but construction was begun at the beginning of the project.

The SPAMS 3.0 operates off of a very different principle than the earlier SPAMS devices. Rather than track the location of the particles and attempt to predict their arrival at a laser to be fired hundreds of microseconds later, particles are tracked and then fired upon immediately. Rather than measuring the velocity, and thus the aerodynamic diameter, of the particles based upon the time that it takes them to transit between multiple lasers, the SPAMS 3.0 determines their velocity by the time that it takes them to cross a single laser. Figure 1 shows this process in action. In Figure 1A, the particle has just entered the laser. It is exiting it in Figure 1B and is ionized in Figure 1C. An actual light scattering trace from a real particle is shown below in Figure 1D with the three points indicated.

There are three practical advantages to this system. The first is extreme design simplicity, from the standpoint of the optical and electronic systems. Essentially, seven lasers have been replaced with two and a truly brilliant but very expensive real-time data processing board was replaced with a COTS oscilloscope with simple triggering logic capable of actuating the ionization laser as the particle exits the tracking laser. Second is a dramatic improvement in performance. Because the SPAMS 3.0 makes no attempt to predict the time of arrival of an aerosol particle in the future but rather determines its location in the present, it never misses the particle with its ionization laser. The third is the reduction in the number of failure modes and tunable parameters. If the inlet nozzle is clean and the one laser is aligned with the other, then the system cannot fail to operate unless a component has failed.

Figure 2: Photographs of two different SPAMS 3.0 devices, one (left) installed in a customer's laboratory and the other (right) in front of LII's laboratory.

Despite the challenges faced, and despite the fact that the systems could not be deployed prior to the termination of the project, the SPAMS 3.0 was successfully prototyped, test data was obtained, and a SPAMS 3.0 was allocated to this project. Photographs of the SPAMS 3.0 appear in Figure 2. The data is described below.

RESULTS AND DISCUSSION

The following critical aspects of the SPAMS 3.0 were measured and validated:

- The ability of the square laser system to determine the sizes of the particles
- The dynamic range of the aerosol interface
- The ability of the system to acquire mass spectra

Figure 3 contains a plot of the aerodynamic diameter of aerosol particles analyzed versus the time, in seconds, required for them to transit the approximately 2.4 mm wide square laser beam. It also gives an idea of the range of particle sizes that are able to be analyzed by the SPAMS 3.0, though the curve.

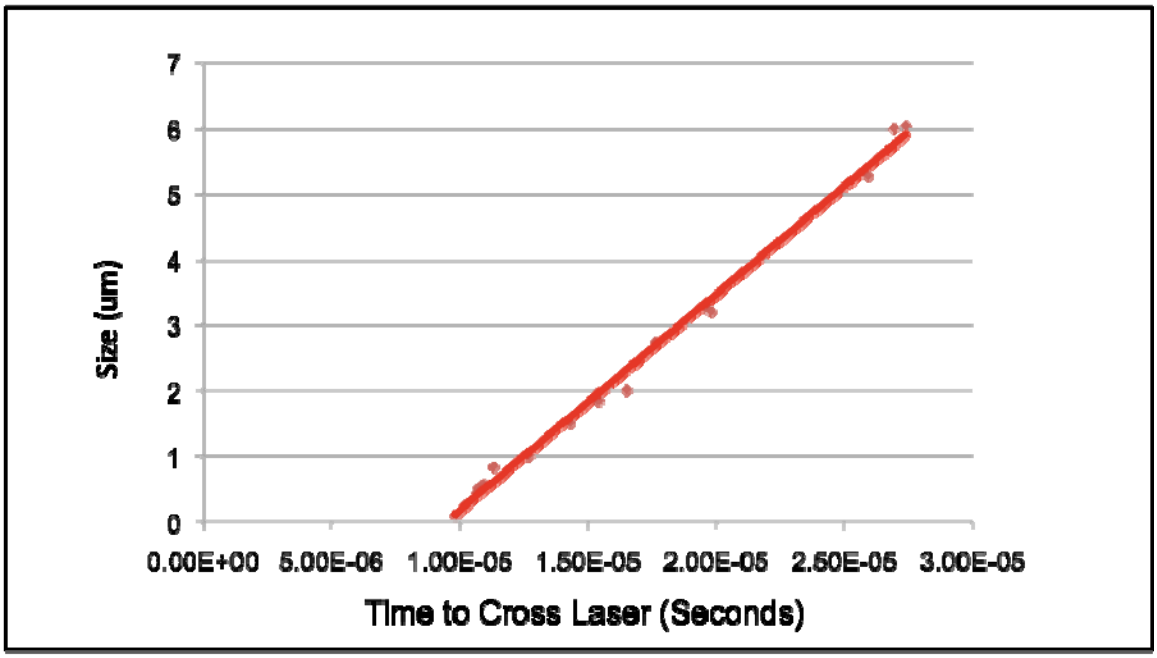

Figure 3: Transit times of 14 different sizes of particles, from 0.1 microns to 6 microns, as they cross the tracking laser. Particles as large as 9 microns in aerodynamic diameter were successfully analyzed and traces resulting from particles of roughly 30 microns were observed empirically, though aerosolizing such large particles proved challenging.

Polystyrene/latex (PSL) particles were analyzed for these experiments. 0.32, 1.0 and 3.2 micron particles were acquired from Duke Scientific (http://www.dukescientific.com) while all other sizes were acquired from Microparticles GMBH (http://www.microparticles.de). Communications with the

manufacturers indicated that the particles 4 microns and below were all purely polystyrene while those above were coated in latex to make the larger size. This may have affected the density of the particles, which would have affected their aerodynamic diameters, but as the densities were all within 5% of unity, unit density was assumed for all samples.

The PSLs were aerosolized under positive air pressure at a flow rate of roughly 5 liters per minute into a Livermore Instruments Inc. disposable nebulizer kit. The particles were dried by two Livermore Instruments Inc. diffusion dryers operated in series. The particles were transported through 3/8" tygon tubing. A T in the tubing permitted the excess aerosol to escape leaving one liter per minute for analysis. Roughly 1000 particles of each size were analyzed. The analyses typically took under two minutes. Different concentrations of the different sizes of spheres were analyzed to maintain a high rate of acquisition. Generally, between 100 and 200 microliters of 5% solids suspension was diluted in 20 mL of water. A 5 mL aliquot was placed in the nebulizer for aerosol generation.

Clearly, the size of the particles was found to be linear with transit time. This was the anticipated result but validated the approach of using a single square tracking laser to determine particle size. On the other hand, the efficiency of the focusing system over such a wide range of sizes surprised the experimenters. Conventional aerodynamic focusing lenses are able to focus particles of below one micron in aerodynamic diameter and generally can only do so over a single order of magnitude. The Livermore Instruments SPAMS 3.0, which was designed to analyze particles from 0.9 to 9 microns has proven capable of analyzing particles over a range from 0.1 microns to roughly 30 microns. The upper limit is estimated due to the difficulty of aerosolizing such large particles. Clearly the interface design succeeded beyond expectations.

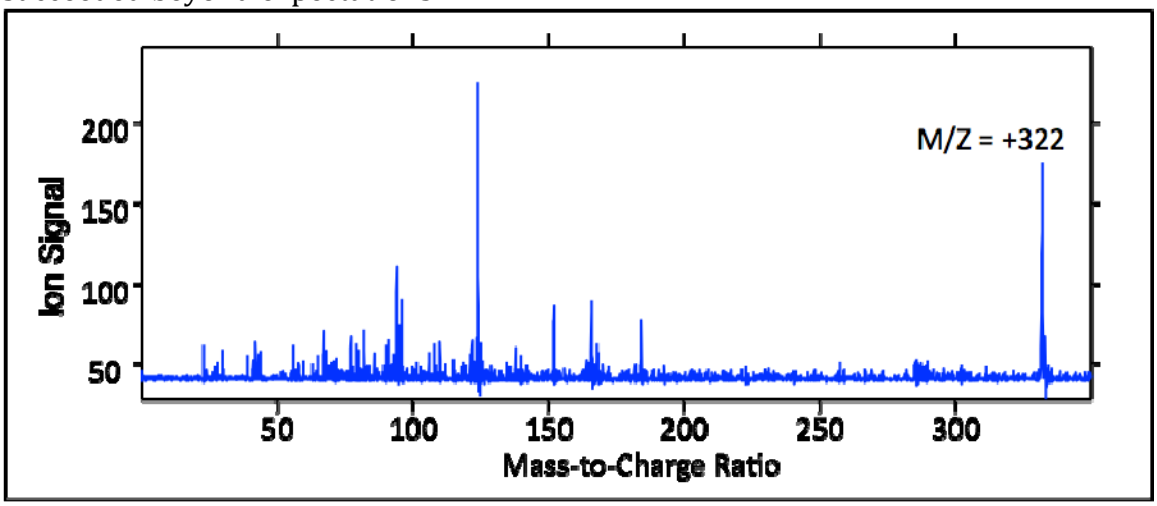

Figure 4: Mass spectrum of a single particle of a chemical standard. Notice that ions are generated into the hundreds of Daltons, the range in which most metabolites occur.

A proprietary chemical standard, supplied to us under a nondisclosure agreement with a customer, was analyzed to generate Figure 4. Obviously, however, it is possible to analyze relatively large mass ions (M/Z 322 is shown in the figure) and to do so with reasonable resolution., validating the mass spectrometer design and the method of ionization.

Figure 5 shows the numbers of particles acquired per second during these experiments. Subsequent to the completion of this testing, we redesigned our data system and are now capable of acquiring 250 mass spectra per second with the ionization laser being the limiting factor.

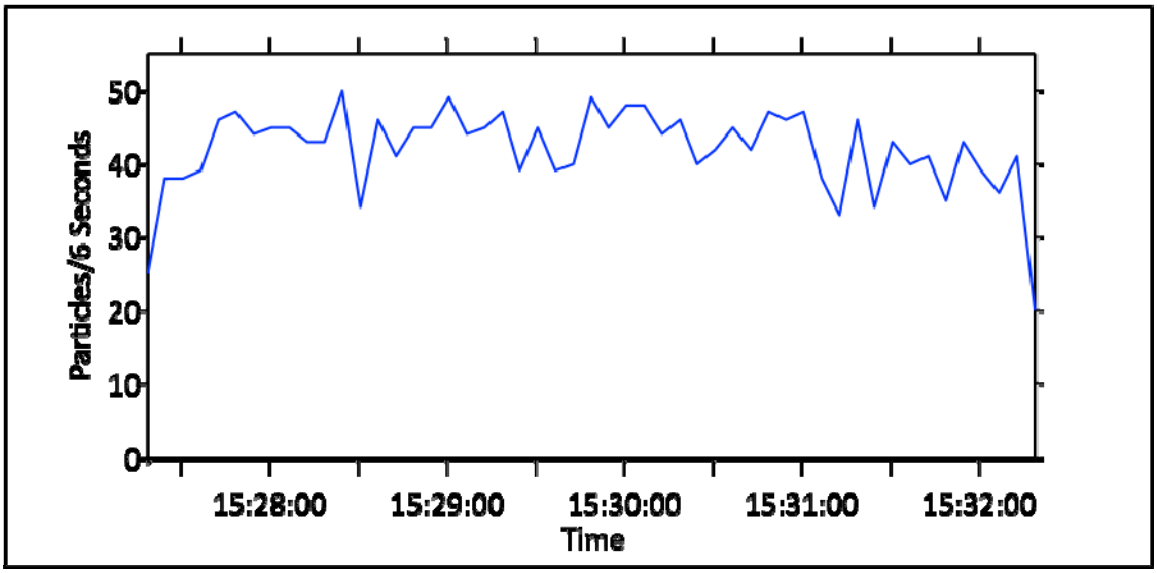

Figure 5: Particle analyses in six second periods on the SPAMS 3.0 developed for this project. Since the project's termination, we have improved this speed by a factor of 40 to 250 particles per second.

In attempting to analyze the mass spectra that were acquired, we found that, while individual mass spectra tended to have high resolutions, when a single calibration was applied to all mass spectra, the mass accuracy could be off by as much as a full Dalton for peaks above 100 Daltons, depending on the spectrum. We realized that the particles had been struck in different locations of the ionization laser (in red in Figure 1) which had led to different amounts of kinetic energy being imparted into their resulting ions. A parametric time warping algorithm was written to adjust the calibration of each individual mass spectrum. Figure 6 shows the results of that algorithm. The upper spectrum shows an average of 1000 mass spectra that were not subjected to the autocalibration algorithm. The lower spectrum shows the same spectra once they had been autocalibrated. Note that the mass accuracy was restored when the autocalibration algorithm was successfully applied.

On October 8, 2012, Livermore Instruments Inc. deployed a SPAMS 3.0 Single Particle Aerosol Mass Spectrometer to the research laboratories of our collaborators

at Ecolyse, Inc. in College Station, TX. Ecolyse, Inc. staff scientists cultured and provided at least three independent cultures of each of the organisms noted in Table 1. The organisms fell into three categories: sulfate-reducing bacteria, thiosulfate reducing bacteria and acid-producing bacteria. They were also selected because they ranged in similarity to DD1 from being the same species but a different strain (DD2), a different species (DV), a different genus (DH), and different categories of bacteria (FP, AF TT and LAB). Each culture was grown to a concentration of roughly 10^9 organisms per mL as determined by hemacytometry.

Species	Relevance to Oilfield
Desulfovibrio desulfuricans strain Desulfuricans (DD1)	SRB
Desulfovibrio desulfuricans strain Desulfuricans (DD2)	SRB
Desulfovibrio vulgaris strain Hildenborough (DV)	SRB
Desulfotomaculum halophium (DH)	SRB
Fusilbacter paucivorans (FP)	TRB
Acidthiobacillus ferroosidans (AF)	APB
Thiobacillus thioparus (TT)	APB
Lactobacillus mucosae (LAB)	APB

Table 1: Organisms analyzed during the experiments. SRB are sulfate-reducing bacteria, APB are acid-producing bacteria and TRB are thiosulfate-reducing bacteria.

The analysis of the each culture was accomplished over a matter of minutes by a relatively simple procedure. When a given culture was to be analyzed, it was delivered to Livermore Instruments personnel by Ecolyse scientists. The culture including the organisms and their growth medium were decanted into five 1 mL centrifuge tubes and were centrifuged at full speed for 60 seconds. The supernatant was decanted and replaced with deionized water. The pellets were vortexed to resuspend the organisms. The process was repeated three times, replacing essentially all of the growth medium with deionized water. The 5 mL of suspended, cleaned organisms were decanted into a Livermore Instruments' disposable neubulizer and aerosolized under 5 liters/minute of positive pressure. The nebulizer was maintained under negative pressure during the aerosolization process. The SPAMS 3.0 draws air at 1.15 liters per minute and the excess aerosol was vented through a HEPA filter. The low to the SPAMS passed through a Livermore Instruments diffusion dryer to remove moisture prior to analysis.

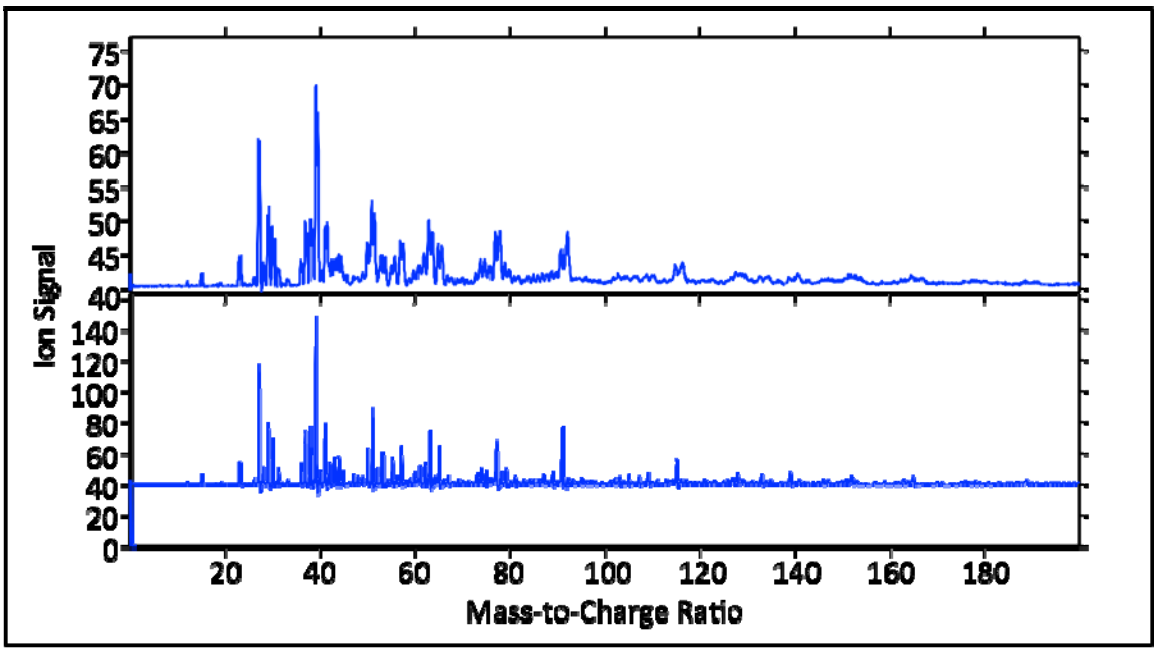

Figure 6: Averages of 1000 mass spectra. In subfigure A, they are averaged as collected while in subfigure B they are pre-calibrated by our parametric time warping algorithm and then the calibrated spectra are superimposed. The higher resolution is responsible for the taller peaks. Proper calibration of the mass spectra is critical for marker peak assignment.

In practice, the analysis of a 5 mL culture tube took roughly 30 minutes from breach of tube to completion of analysis. Subsequent to these experiments, we have succeeded in analyzing organisms directly from their own growth medium. Under these circumstances, roughly 85% of the mass spectra collected result from the growth medium but those mass spectra are clearly distinguishable from the microorganism mass spectra. The data system operated for this study limited the number of mass spectra that could be collected per second to roughly 6 as shown in figure 5. Our revised data system can now process up to 250 particles per second, more than offsetting the decrease in sensitivity due to the growth medium particles occupying the instrument's duty cycle. By using the new data system to remove the washing step while simultaneously decreasing the analysis time by five sixths, the overall throughput would increase roughly tenfold.

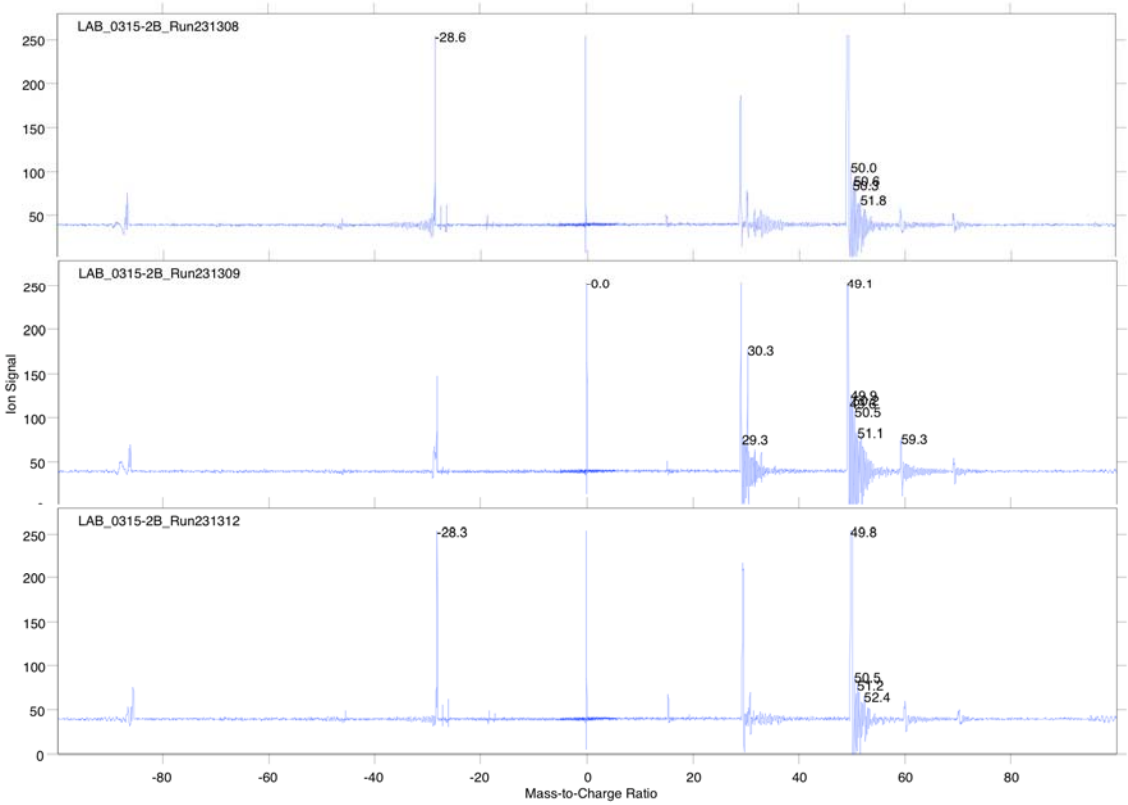

Figure 6: 3 Consecutive Lactobacillus Organism Mass Spectra Mass Spectra

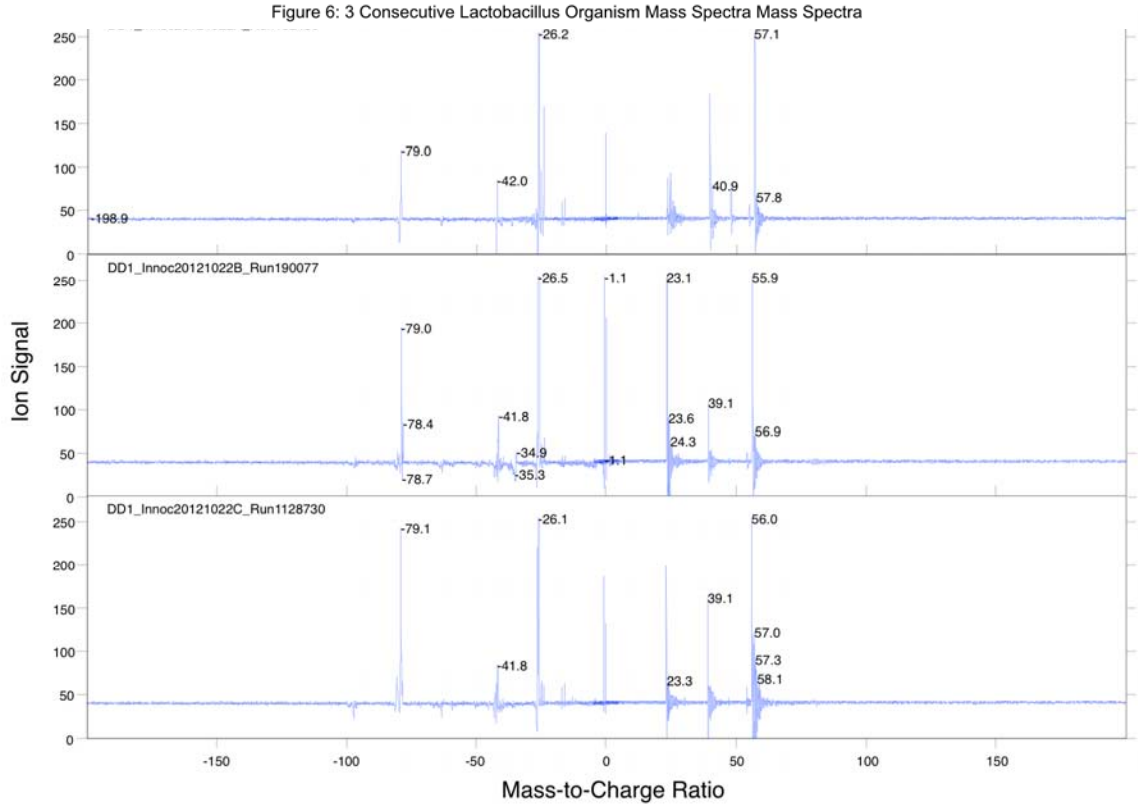

Figure 7, Individual *D. desulfuricans* microorganism mass spectra from 3 different cultures.

Figure 6 shows the stability of the microorganism mass spectra observed. Three consecutive mass spectra of *Lactobacillus mucosae* are clearly nearly identical. The serial numbers of the mass spectra (in the upper left hand corner of each mass spectrum) are not consecutive due to intermediate spectra failing checksumming across the multiple waveforms that are transferred from the data acquisition computer to the control computer. Figure 7, on the other hand, shows the stability of the microorganism between cultures. Each mass spectrum was collected from a different culture of *D. desulfuricans* (DD1) and all are clearly similar but are notably different from the *L. mucosae* mass spectra in Figure 6.

Figure 8 shows three mass spectra of individual organisms of *Acidothiobacillus ferrooxidans* (AF). Mass spectral differences between the AF and DD1 are superficially obvious. Similarly, AF and DD1 are clearly distinguishable from the three mass spectra of *Fusilbacter paucivorans* (FP) in Figure 9, the three mass spectra of *Thiobacillus thioparius* (TT) in Figure 10, and the three mass spectra of *Desulfotomaculum halophilium* in Figure 11. On the other hand, the spectra of a second isolate of *D. desulfuricans* (DD2) in Figure 12 clearly resemble the other *Desulfovibrio* organism mass spectra, both those of DD1 and of DV (Figure 13). Spectra of individual organisms of the two strains/two species of *Desulfovibrio* appear in Figure 14 and are clearly indistinguishable. So, while SPAMS is able to discern genera, it does not appear to be able, in its current incarnation, to be able to discern species, at least of *Desulfovibrio*.

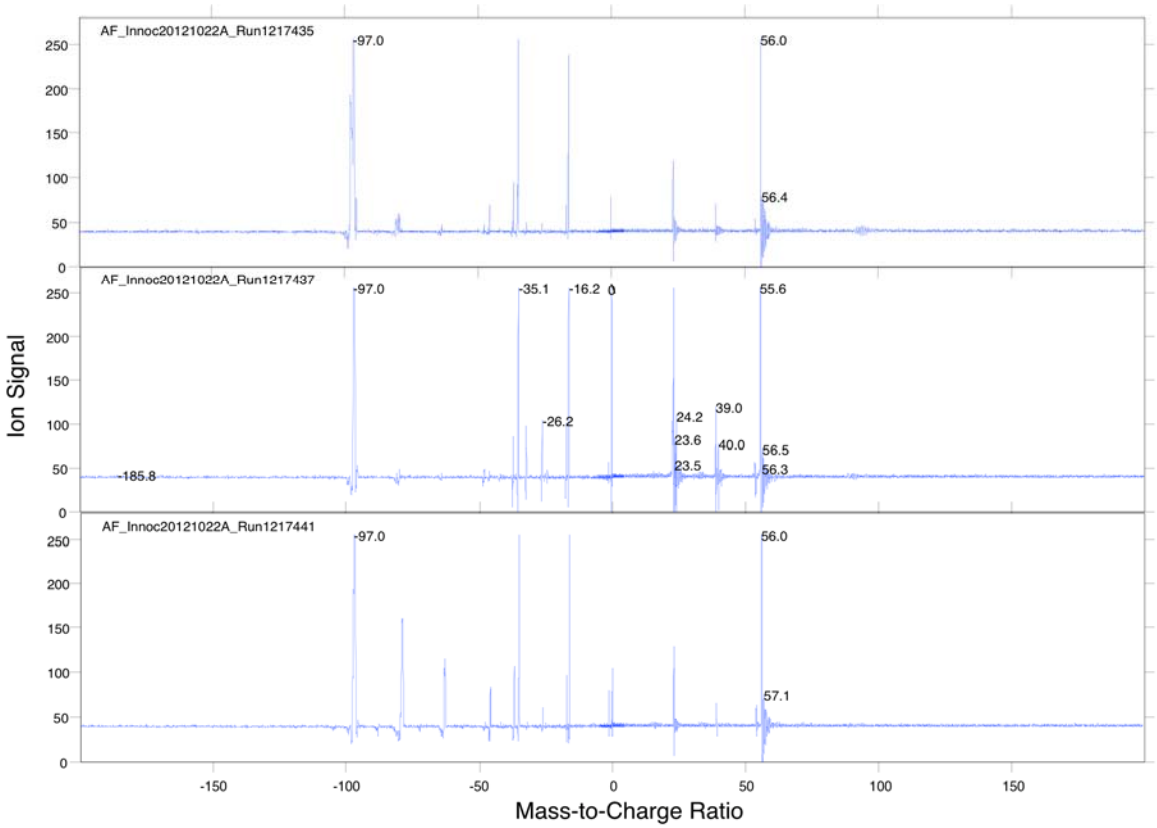

Figure 8: Mass spectra of 3 individual *Acidithiobacillus ferroxidans* organisms collected from the same culture.

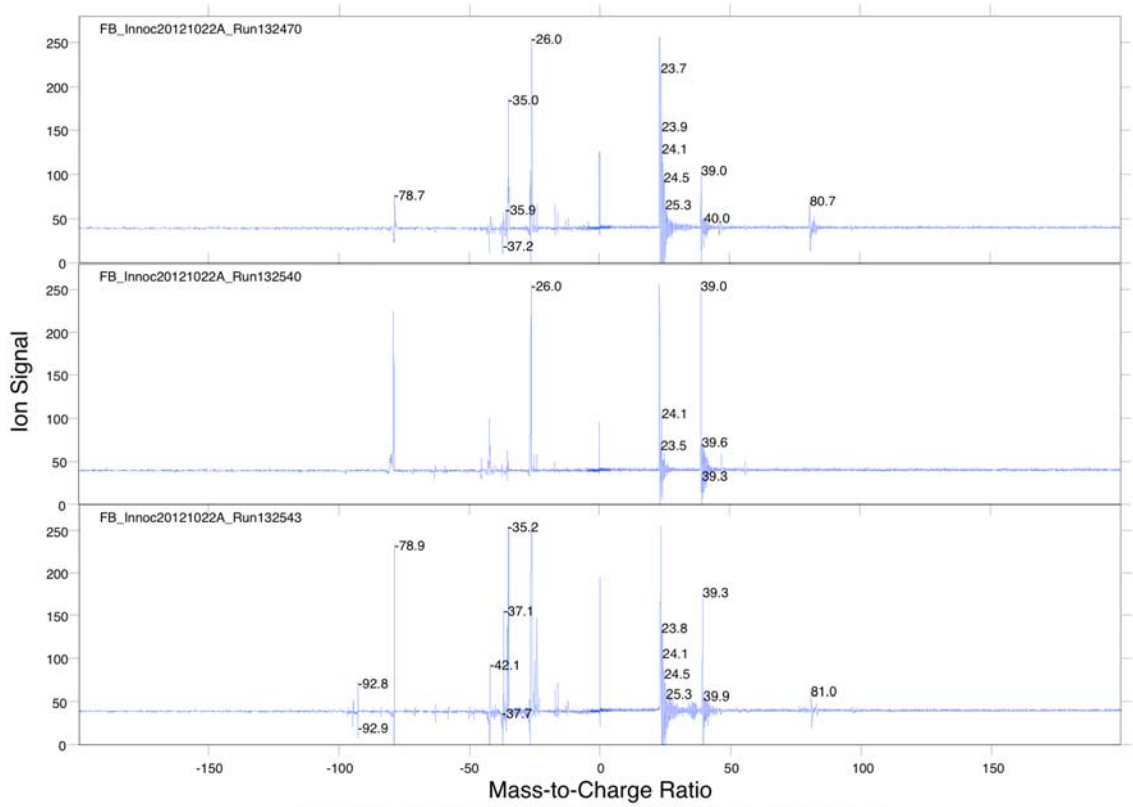

Figure 9: Mass spectra of three individual *Fusilbacter paucivorans organisms.*

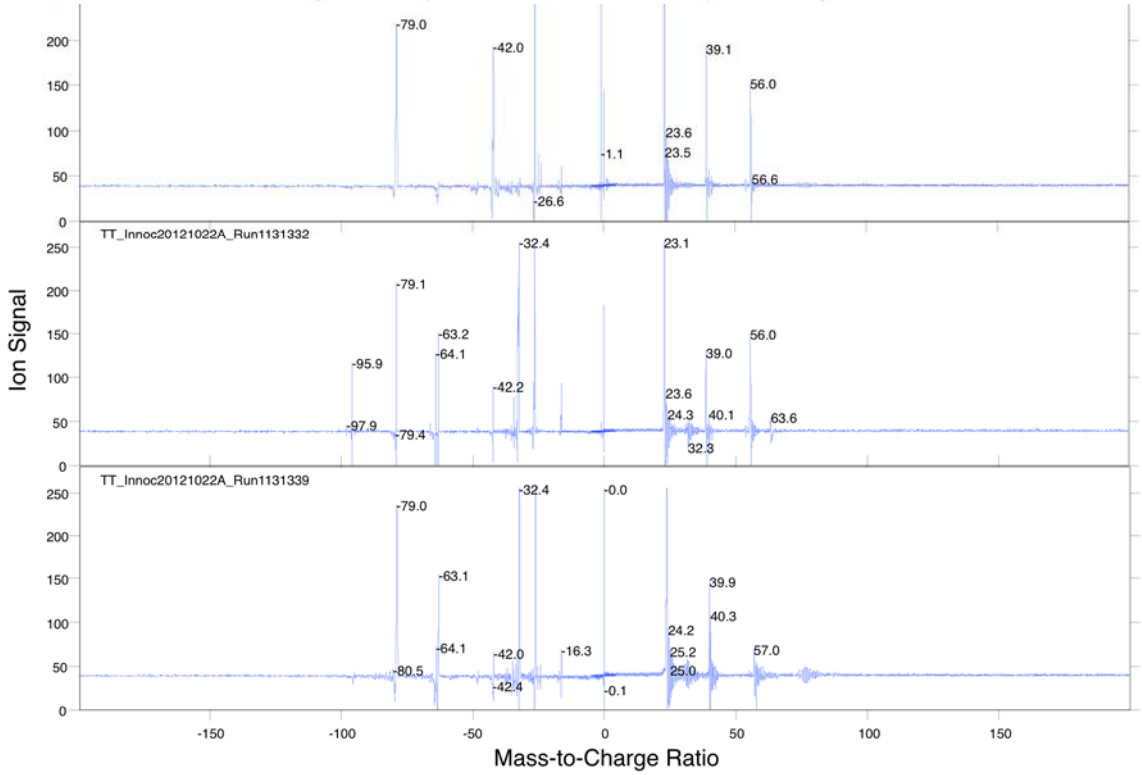

Figure 10: Mass spectra of three individual *Thiobacillus thioparus* organisms.

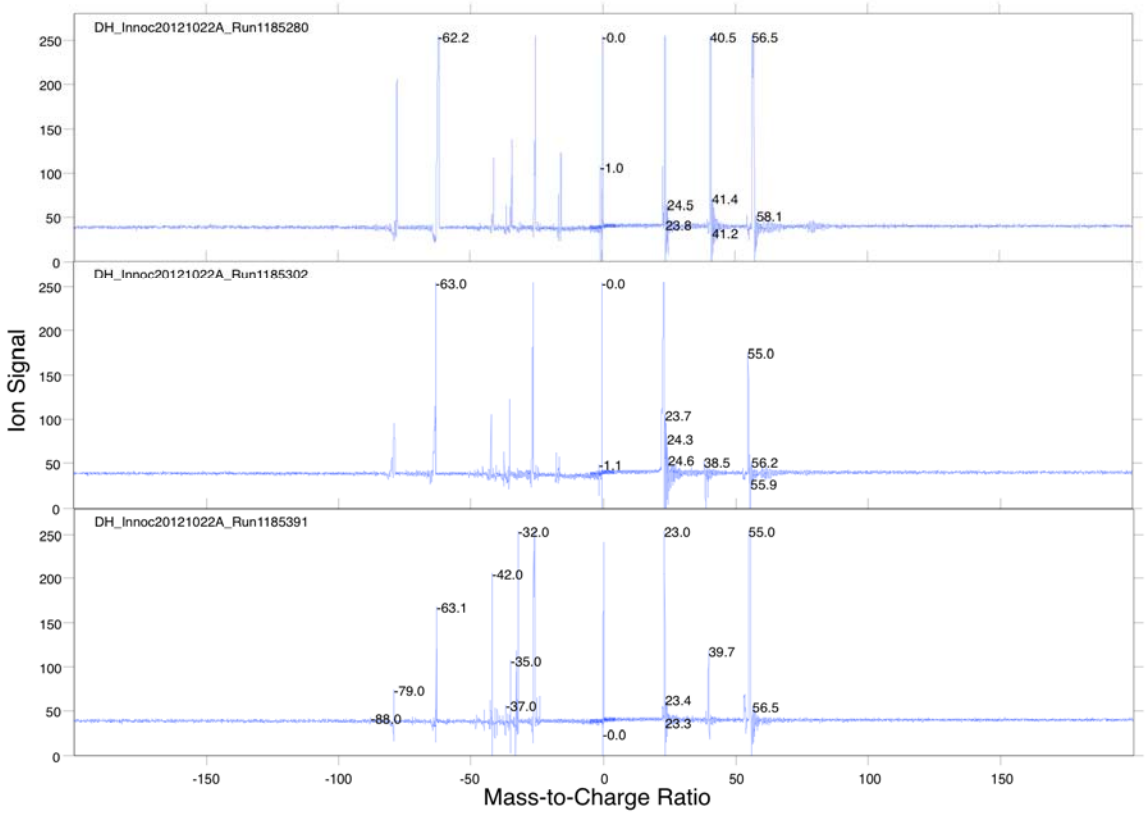

Figure 11: Mass spectra of 3 individual *Desulfotomaculum halophilium* organisms.

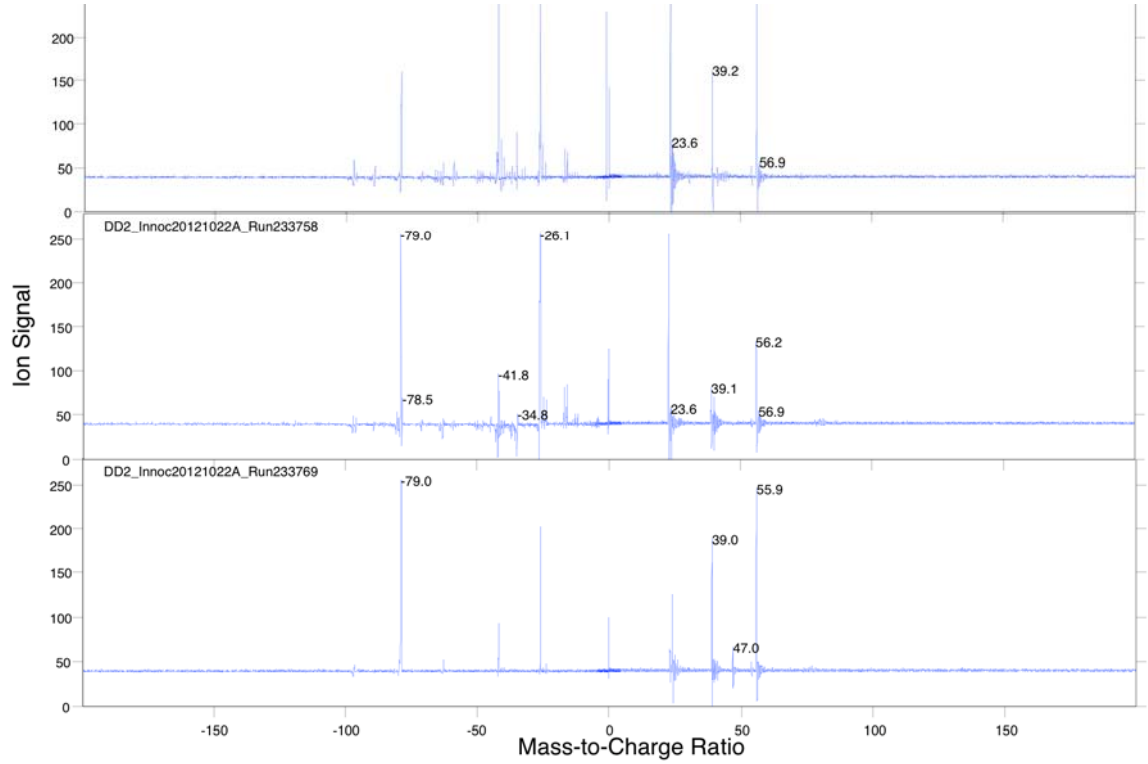

Figure 12: 3 individual *Desulfovibrio desulfuricans* (DD2) organism mass spectra. Note the similarity to the other *Desulfovibrio* strains' mass spectra.

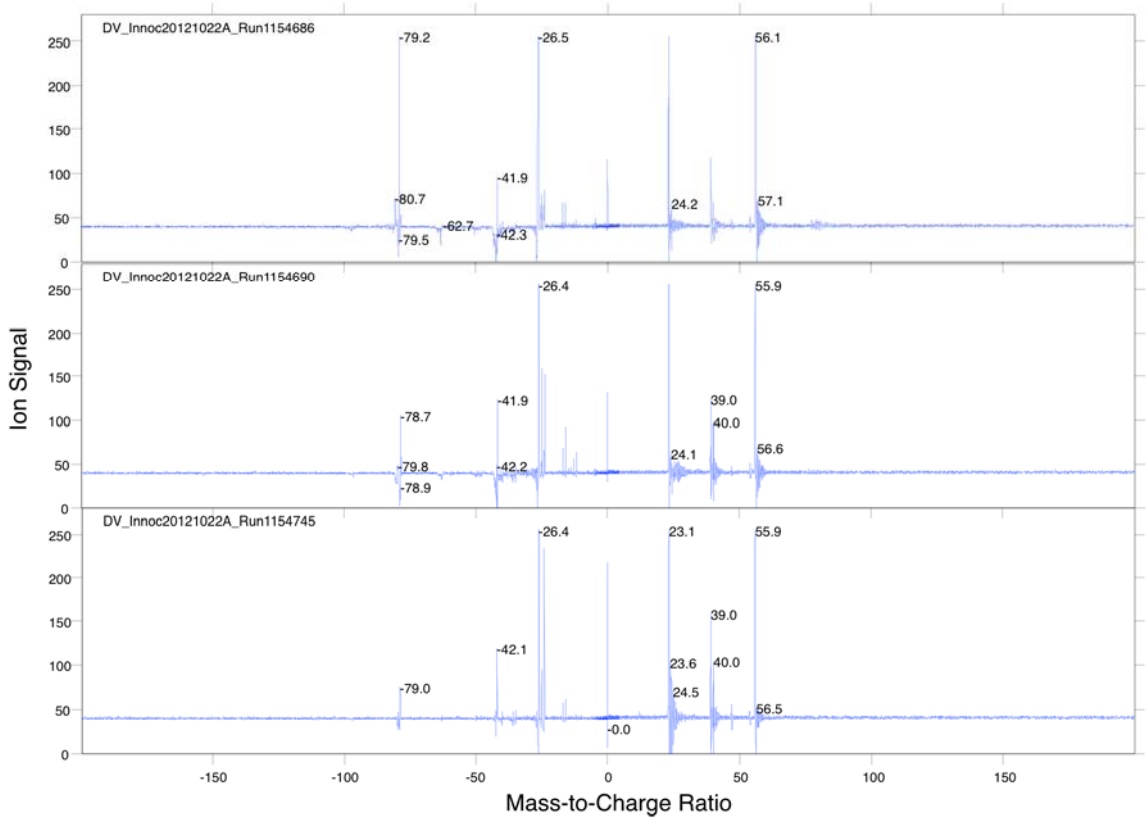

Figure 13: Mass spectra of three individual *Desulfovibrio vulgaris* microorganisms.

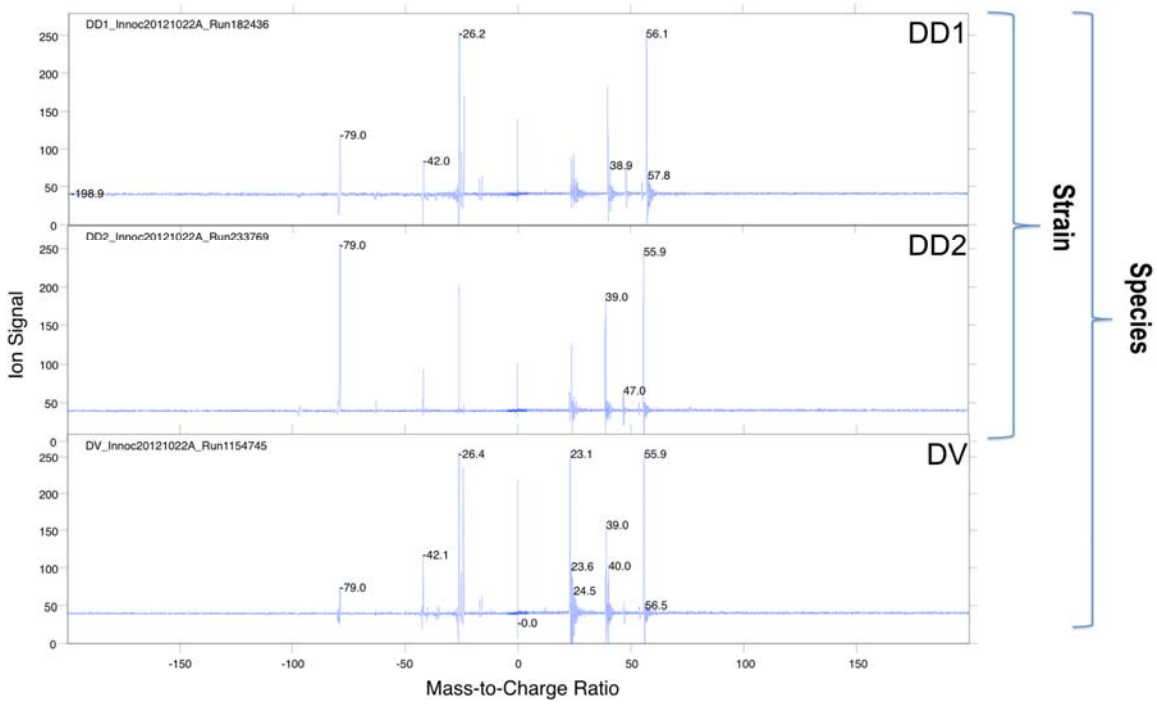

Figure 14: Mass spectra of individual organisms of two strains of *Desulfovibrio desulfuricans* and of a single *D. vulgaris* organism.

Impact to Producers

Petroleum microbiological assays are currently highly labor intensive and are very slow. The SPAMS technology has been demonstrated to dramatically reduce the

labor and time requirements while yielding most of the information of classic culture assays. When fully developed, SPAMS or a derivative technology will significantly reduce the resources currently committed to microbiological assays.

Technology Transfer Efforts

Papers concerning the application of SPAMS to petroleum microbiology were presented at several forums organized by RPSEA. The following presentations were made at the following meetings:

"Enumerating Bacteria in Deepwater Pipelines in Real-Time and at Negligible Cost"presented in-person by David Fergenson at the Ultra-Deepwater Flow Assurance TAC Quarterly Meeting on March 4, 2010

"Enumerating Bacteria in Deepwater Pipelines in Real-Time and at Negligible Cost" presented in-person by David Fergenson at the RPSEA Ultra-Deepwater Forum on June 22, 2010

"Enumerating Bacteria in Deepwater Pipelines in Real-Time and at Negligible Cost" presented in-person by David Fergenson at the Ultra-Deepwater Technology Forum on July 27, 2011

"Enumerating Bacteria in Deepwater Pipelines in Real-Time and at Negligible Cost" presented by David Fergenson telephonically at the Environmental, Safety, Regulatory and MetOcean TAC Meeting on January 26, 2012

"Enumerating Bacteria in Deepwater Pipelines in Real-Time and at Negligible Cost" presented by David Fergenson telephonically at the Environmental, Safety, Regulatory and MetOcean TAC Meeting on January 26, 2012

In addition, the SPAMS technology was discussed in general at the AAAR conference in 2009.

Conclusions

We successfully deployed a SPAMS 3.0 to a petroleum microbiology laboratory as intended. The system significantly exceeded our expectations of its performance. The fact that the system could analyze such small particles allows the hope that it could analyze not only bacteria but also viruses as well which is critical to our collaborator on the project. The ability to size the particles was validated, as was the efficiency of the SPAMS 3.0's design. Significantly, LII successfully trained unaffiliated personnel in the operation of the SPAMS 3.0 in a laboratory environment. The SPAMS system was able to perform analyses that would normally have taken days in a matter of roughly 30 minutes and with subsequent upgrades would be able to perform those experiments in roughly 5 minutes. The results are able to identify the genera, within the narrow range trained upon, of the organism.

Recommendations

The technology has been proven to work and the application area should be broadened by training across many more genera and growth conditions. In addition, instrument development efforts increasing the informative power of the mass spectra should be pursued including improvements to the ionization process.

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LIST OF ACRONYMS AND ABBREVIATIONS

APB – Acid-producing Bacteria
BAMS - BioAerosol Mass Spectrometry
SPAMS - Single Particle Aerosol Mass Spectrometry
COTS - Commercial Off The Shelf
TSI - Thermal Systems, Inc.
ATOFMS - Aerosol Time-of-Flight Mass Spectrometry
FPGA - Field-Programmable Gate Array
LLNL – Lawrence Livermore National Laboratory
LII – Livermore Instruments Inc.
PSL - Polystyrene/Latex
SRB – Sulfate-reducing Bacteria
TRB – Thiosulfate-reducing Bacteria