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NON-CONVENTIONAL METHODS FOR ESTIMATING FUEL SYSTEM BIOBURDENS RAPIDLY

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Abstract

Three alternative, non-conventional test methods are evaluated for their ability to detect and quantify bioburdens in fuel and bottom-water samples. Two of the parameters, catalase activity and adenosine triphosphate (ATP) concentration have been used previously. This is the first report of the use of fluorescence polarization (FP) technology for fuel and fuel-associated water testing.

In this investigation, each parameter is assessed for repeatability and reproducibility. Covariance amongst the three non-conventional test method data is reported. Covariance between each of the non-conventional parameters and each of a variety of conventional parameters (viable bacteria and fungi enumeration data, fuel and bottom water chemistry) is also reported.

Although each test method has limitations, the new methods reported in this paper may contribute significantly to fuel system microbial contamination condition monitoring and biodeterioration root cause analysis efforts.

Keywords
ATP, adenosine triphosphate, biodeterioration, bottom-water, fuel, bacteria, bioburden, catalase, catalase activity, endotoxin, fluorescence polarization, fluorometer, LPS, lipopolysaccharide, microbiology, oxygen demand, viable count.

Introduction

Petroleum product biodeterioration was first reported in 1895[1]. Most recently, Passman has reviewed the fundamentals of fuel and fuel system microbiology[2]. Uncontrolled microbial activity in fuel systems can cause product degradation and system damage. Passman *et* al. demonstrated substantial loss of oxygenate and n-alkanes from gasoline stored over microbially contaminated bottom-water[3]. Microbially influenced corrosion (MIC) of steel tanks, first described in 1945[4] and has been well documented subsequently[5]. More recently, Gu[6] has described the biodeterioration of fiber reinforced polymers (FRP).

Detecting microbial contamination in fuel systems remains problematic. Samples collected routinely for fuel quality monitoring are generally ill-suited for microbiological testing[7].

Moreover the most commonly used test method – viable recovery – suffers from several considerable limitations.

Viable recovery methods depend on the ability of microbes collected from fuel or bottom-water samples to proliferate in or on a specific growth medium into or onto which they are suspended, spread or adsorbed. By definition, all growth media are selective. This means that only a fraction of viable microbes present in the original sample will proliferate in any given growth medium[8]. Although nominal time limitations are set for enumerating microbial growth in or on nutrient media (typically 48 to 72 hours for most bacteria and fungi; 1-week for sulfate reducing bacteria), substantial portions of the sampled system's indigenous population may require two or three times longer to become detectable (in broth media detection is based on fluid turbidity, color change or combination of both; on solid media detection is based on the formation of visible colonies). This time lag represents two problems. Under best conditions, there is a considerable delay between test initiation and data availability. Second, there is a substantial risk of population density underestimation. Failure to disperse individual cells from aggregates may also lead to significant population density underestimates. These limitations make a compelling case for alternative enumeration methods that reflect the total contaminant biomass, provide data speedily or accomplish both.

An ASTM document reviews the critical considerations necessarily considered when evaluating a new test method[9]. Optimally, any new method will generate data that covary with a previously accepted method. Additionally, the new microbiological method might covary with non-microbiological symptoms of biodeterioration. Finally, the new method should be reliable. It should have an adequate lower detection limit (LDL) and should not generate false-positive data.

With these considerations in mind, we evaluated three non-conventional test methods. The first method has a fifty-year history. With minor methodological improvements, adenosine triphosphate (ATP) has been used to quantify microbial biomass since the mid-1950's[10]. By the late 1960's, ATP had become an important tool for estimating microbial biomass in marine and other aquatic systems[11, 12; 13]. However, in complex fluids such as metalworking fluids, oilfield production water and fuel bottom-water, hydrocarbons and other non-ATP organic molecules interfered with the *Luciferin-Luciferase* bioluminescence reaction on which ATP quantification depended. Some molecules quenched (obscured) ATP-driven luminescence reaction. Other molecules auto-fluoresced, thereby causing positive interferences. These limitations continue to limit the ATP test's utility for fuel system biomass determination.

In 1997, Miller and Loomis were awarded a U.S. patent for a novel approach to eliminating certain ATP test interferences[14; 15]. The paper presents the results of a systematic evaluation of the applicability of the method taught my Miller and Loomis in these two patents.

Another cell constituent used to estimate bioburdens in environmental samples is lipopolysaccharide (LPS), also referred to as *endotoxin*. The LPS molecule is a characteristic component of Gram negative bacterial cell walls. In 1976, Levine and Bang[16] reported that LPS caused the lysate of horseshoe crab (*Limulus polyphemus*) hemolymph (analogous to blood) to clot. Their test was called the *Limulus lysate test* after the horseshoe crab species from which the hemolymph was harvested. Shortly thereafter, Passman *et al.* used the Limulus lysate test to

quantify microbial communities of the North Atlantic outer Continental Shelf[17]. During the past 30 years, the test has been improved and automated for use in a variety of applications[18,18;20].

Recently, Sloyer *et al.*[21] reported the use of an automated fluorescent method for estimating the numbers of viable bacteria in metalworking fluids. Based on the promising metalworking fluid data, the investigators evaluated the fluorescent method with fuel tank bottom-water samples. This paper presents the results of these tests.

Materials and Methods

Sampling - Bottom-water samples were collected from bulk storage and retail outlet underground storage tanks (UST) using a 500 ml Bacon Bomb sampler. The sample was rinsed with 70% ethanol before use. Samples were dispensed into previously unused 1-liter Boston Round bottles. Sub-samples to be tested microbiologically were transferred aseptically to sterile 50 ml high density polyethylene centrifuge tubes. Routinely, viable counts, catalase activity and ATP tests were completed within five hours after sampling. Fluorescent method (FM) and additional ATP tests were run on samples that had been shipped to the respective coauthors' laboratories.

Viable Counts – LiquiCult™ broths (MEC, Inc., Lake Placid, NY) were used to estimate bottom-water population densities. A 5.0 ml portion of bottom-water was injected into a 125 ml serum vial containing 25 ml of nutrient broth. The inoculated broth was incubated at room temperature (20 ± 3 °C) for up to five days. Population densities were estimated based on the incubation period (in days) required for the inoculated broth to develop a red tint, and the intensity of the tint on the first day it was observed. The method's lower detection limit was $\log_{10}$ 2.0 CFU/ml.

Catalase Activity – Catalase activity was measured using the HMB™ test system (BioTech International, Inc. Sugar Land, TX). The method, described elsewhere[22], estimates bioburden by treating a sample with hydrogen peroxide. The peroxide reacts with the enzyme catalase, liberating gaseous oxygen (O_2). The pressure created by the O_2 retained in the reaction tube's head-space is proportional to the concentration of catalase in the sample. Catalase concentration reflects the bioburden. If necessary, samples were diluted with deionized water (DIW) to give pressure readings of < 20 psig (239.2 kPa).

Two-hour Oxygen Demand – A Corning Checkmate II Meter with a Checkmate II Dissolved Oxygen Sensor (Corning Life Sciences, Corning, NY) was used to measure dissolved oxygen (D.O.) in bottom-water samples. A 25 ml bottom-water sample was dispensed into a 50 ml centrifuge tube then shaken vigorously for approximately one minute. A time zero (T_0) measurement was taken immediately after agitating. After the first D.O. reading, the centrifuge tube was re-capped and allowed to stand at room temperature for 2-hours. A two-hour (T_2) reading was taken without further agitating the sample. Oxygen demand was computed as:

$$D_0\ \% = [(D.O._{T0} - D.O._{T2}) \div D.O._{T2}] \times 100 \quad (1)$$

Adenosine Triphosphate- A Profile 1 Model 4560 Bioluminometer (New Horizons, Inc., Columbia, MD), as shown in figure 1, was used to determine ATP concentration. For the test, 50 µl of sample was dispensed into a New Horizons Filtervette™ cuvette and pressure filtered

through the cuvette's base. A specially designed plunger device was used to create the pressure head over the sample. The retainate was then wash twice with three-drops of somatic cell releasing agent (SRA) by dispensing the SRA into the cuvette and pressurizing using the aforementioned plunger device. After rinsing, the Filtervette cuvette was placed over a New Horizons Profile 1 freeze-dried Luciferin-Luciferase reaction pad. A 15 µl volume of Bacterial Release Agent (BRA) was dispensed into the Filtervette cuvette and pressure filtered onto the area of the pad containing the Luciferin-Luciferase reagent. The pad was then placed immediately into the Bioluminometer. Luminescence was read as relative light units (RLU). The RLU data were transformed to $\log_{10}$ RLU to facilitate computations and correlation analysis.

The ATP test was run on ATP references standard in DIW, in 0.9% physiological saline buffer[23], a *Pseudomonas putida* isolate, bottom-water samples, bottom-water samples spiked with either ATP standard or *P. putida*, and bottom-water samples diluted in DIW. Two series of tests using ATP standard and *P. putida*, respectively to compare results from the new ATP test method against those obtained by ASTM D4012[21]. Results were reported as Log RLU where:

(1) $$\text{Log RLU} = 0.971\text{Log [ATP]} - 0.460$$

Approximately 3 pg ATP/ml creates 1 RLU.

Fluorescent Method - Bottom-water samples were tested using the method as described previously by Sloyer *et al.*[21]. Either 10 µl or 100 µl sample was added to 1 ml of endotoxin-free DIW in a 10 x 75 mm borosilicate tube. The solution was mixed using a vortex mixer, and the tube was placed into the PolarScan (Associates of Cape Cod, Inc., Falmouth, MA) fluorometer, as a blank. After blanking the fluorometer, either 1µl of BactiFluor (Associates of Cape Cod) tracer (for viable bacteria detection) or 1 µl of rENP reagent (Associates of Cape Cod) was added to the sample. The sample was vortexed once more, and placed into the fluorometer. Results were recorded as viable bacteria (VB) or endotoxin units (EU), respectively. One EU ≈ 0.1 ng lipopolysaccharide (LPS).

Results

Profile 1 Bioluminometer RLU as Function of Dilution in Water

Triplicate analysis of *P. putida* diluted in DIW were performed at 10, 100 and 1,000-fold dilutions. The results, shown in figure 2, demonstrate an excellent fit between log RLU and log dilution factor. The slope of the dilution series was 1.08 and the correlation coefficient, r^2 was 0.998.

Next, triplicate tests were run on saline solution and bottom-water samples, respectively, challenged with *P. putida.* The bottom-water samples were all from 87 Octane underground storage tanks (UST). One-way analysis of variance (ANOVA) yielded an F-ratio of 0.036 between the two data sets. $F_{\text{critical (0.95; 5, 24)}} = 2.63$. This indicates that the results in bottom-water did not differ significantly from those in normal saline. Tests were run on the initially challenged fluids and on a series of dilutions. Filter sterilized bottom-water was used for *P. putida* diluted in bottom water. Background Log RLU readings for filter-sterilized bottom-water

were 2.7 ± 0.11. For *P. putida* in saline and bottom-water, respectively, r^2 = 0.997 and 0.984. The two dilution curves shown in figure 3, Demonstrate that the relationships between RLU and pg ATP/ml do not change significantly amongst the three menstrua in which dilution series were run.

Profile 1 Bioluminometer RLU and ASTM D4012 Test Comparison

Two tests were run to compare Bioluminometer ATP determinations against those obtained using ASTM D4012. In the first series, four replicate analyses of 10 µl portions of 300 pg ATP/µl reference solution were tested by each of the two methods. The Bioluminometer data were 4.02 ± 0.017 log RLU. The D4012 results were 4.02 ± 0.021 log RLU (at 3 pg/RLU, expected results were 10,000 RLU, or 4.00 log RLU). Both methods yielded the expected log RLU results. The an F-ratio computed from a one-way ANOVA between the two data sets was 0.67 ($F_{critical\ (P=0.05,\ 3,3)}$ = 0.95), implying that the results from the two methods did not differ significantly.

Next, a suspension of *P. putida* was prepared and triplicate portions of 50µl, 250µl and 500µl of the suspension were tested by each method. Since the D4012 method effectively dilutes the cell suspension 100-fold, Bioluminometer RLU values were consistently greater than RLU values obtained by the D4012 method. To facilitate more direct data comparison, the Bioluminometer method Filtervette retentates were suspended in 1.0 ml saline solution. The data are presented in figure 4.

The slopes of the three log RLU v. µl *P. putida* suspension curves were similar. Correlation coefficients between *P. putida* suspension volume and log RLU were 1.00 and 0.99 for the Bioluminometer and D4012 methods respectively. One-way ANOVA comparing the two data sets demonstrated that results did not differ significantly between the methods ($F_{observed}$ = 0.90; $F_{crtitcal\ (P=0.05,\ 3,\ 3)}$ = 0.95).

Profile 1 Bioluminometer ATP Data Variability

Two important sources of experimental error are variation between replicate tests run by an individual analyst (*repeatability*) and variation amongst several analysts running tests on a single sample (*reproducibility*). Given the perishablility of biological samples, it is impractical to send samples to multiple laboratories for reproducibility testing. To evaluate test variability, three UST bottom-water samples each tested by three analysts. Each analyst tested each sample five times.

Test results are presented in Table 1a and ANOVA calculations are presented in Table 1b. Data variability was comparable amongst analysts. Data from sample 3 was somewhat more variable than data from the other two samples. Neither variation amongst analysts nor samples was significant at the 95 or 99% confidence level.

A third important potential source of variation is the bioluminometer. There are currently two models of the Profile II Bioluminometer in production. Model 3560 uses the Filtervette as a

cuvette. Model 4560, the type of unit used for the studies reported in this paper uses the reagent ticket described above. To evaluate variability amongst test units, we compared test results from two Model 4560 units and one Model 3560 units. A single bottom-water sample was tested five times with each bioluminometer unit. The data, presented in Table 2a, suggest that data from the Model 3560 bioluminometer were somewhat lower than those from the two Model 4560 units. The ANOVA computation shown in Table 2b confirms that test results differed significantly amongst the three bioluminometers. However, as the Table 2c computations for ANOVA between the two Model 4560 units shows that results from same model bioluminometers are not different at the 95% confidence level. Model 4560 is more sensitive than Model 3560.

Parameter Correlation: ATP, Catalase Activity, Oxygen Demand, Viable Count

Between October 2003 and June 2004, 55 UST bottom-water samples were collected, examined for gross appearance and tested for a variety of physical chemical and biological parameters. Five test parameters were compared for the purpose of the ATP test evaluation. Since the objective was to determine whether each parameter would yield the same risk assessment, we Computed the percentage ranking agreement amongst the parameters. Risk ratings (likelihood that parameter data reflects biodeterioration) for each of the parameters is shown in Table 3. Each parameter is given a *low* (L), *medium* (M) or *high* (H).

For all of the samples, at least two of the five parameters were in the same risk category (figure 5). For 49 of the 55 samples (89%) at least three parameter ratings agreed. Tables 4a through 4e compare each of the parameters with the others. Ratings based on ATP test results either agreed with, or was more conservative than, those from gross observations and oxygen demand 84% and 89% respectively. Overall, viable recovery data (MPN) tended to give the most conservative biodeterioration risk rating.

Fluorescent Method Viable Bacteria, Fluorescent Method Endotoxin

A limited number of samples were tested using the two methods that use FP technology. Ten bottom-water samples were tested using the polarizing fluorescence method for total viable bacteria (VB), the ATP method and the catalase activity method. The data are presented in Table 5a, and covariation analyses are summarized in Table 5b. There appears to be a stronger relationship between Log VB and Log catalase activity than between any other parameter pair. Although this may be an artifact of the small sample size, it strongly suggests that further testing using the FP method is warranted.

Next, the two FP methods, VB and endotoxin concentration (EU/ml) were compared. Fifteen bottom-water samples were tested using both methods. The results, shown in Table 6 indicate that endotoxin data do not covary with VB data.

Discussion

Microbes can cause two types of damage in fuel systems. They can degrade product and system components. To minimize the risk of either type of damage, it's necessary to be able to monitor for microbial contamination easily, routinely and reliably. Several factors contribute to the

difficulty of detecting and quantifying microbial contamination in fuel systems. These have been described elsewhere[2,7;9]. Briefly, samples collected for fuel quality testing are generally in appropriate for microbiological testing. Traditional, viable cell recovery methods depend on the ability of microbes to grow on the specific nutrient media used for those methods. The time delay between sampling and data availability typically renders remedial action impractical. Contaminated product has moved downstream, contaminated tanks have received additional product. Consequently, methods that provide test results within time-frames comparable to those for other fuel quality tests provide tremendous value to the fuel management industry.

At our 1994 conference, Passman *et al.*[22] introduced the catalase activity for measuring bottom-water bioburdens. The current paper evaluates several additional rapid, non-conventional methods. Each of the methods reflects aspects of the microbial community not tested by the others. The ATP test is a measure of the concentration of a primary energy-pathway molecule. In contrast to catalase, all living cells have ATP. However, as with catalase the concentration of ATP per cell varies with both species and physiological condition. The VB method detects deoxyribonucleic acid (DNA). Unlike either ATP or catalase, DNA concentration per cell differs substantially between bacteria and fungi, but is relatively the same amongst members of the two respective kingdoms. Both DNA and ATP degrade quickly after cells die. Catalase and biodeteriogenic enzymes may persist after cells die.

Endotoxin is a component of the cell wall of Gram negative bacteria. Neither Gram positive bacteria nor fungi have this molecule. However endotoxin causes acute respiratory distress. Personnel exposed to airborne endotoxin concentrations as low as 9 EU/m^3 may show symptoms[25]. The EU concentrations detected in bottom-water samples analyzed in this study were unlikely to produce sufficient airborne EU concentrations (assume 1% of water-borne endotoxin gets aerosolized). However personnel involved in tank cleaning operations may be exposed to >9 EU/m^3.

Conclusions

This work focused primarily on evaluating a new ATP test method. The results demonstrated that the new method has several critical advantages over earlier ATP test methods. The new method requires fewer steps. Reagents do not need to be kept frozen during storage. ATP in the sample does not need to be extracted by boiling in a buffer solution. Interferences that have historically limited the usefulness of the ATP test for determining biomass in fuel system bottom-water have been eliminated. Laboratory testing has demonstrated that for a given sample, variation for replicate test performed by a single analyst and amongst different analysts is generally < 10%. The new method compares favorably against ASTM D4012 in terms of detection limits (the new method is nearly 100 times more sensitive), simplicity and precision.

Field studies show that the biodeterioration risk rating, based on ATP data, is generally consistent with ratings based on gross-observations, catalase activity, oxygen demand and viable counts.

Total viable cell estimates based on FP may also be useful for routine bioburdens testing.

Preliminary data presented in this report are promising. Additional testing will be needed to validate FP for VB detection. The EU data did not covary with the VB data. The low EU/ml concentrations detected suggest that this procedure may not have value for routine condition monitoring.

References

1. Myoshi, M. J. *J. Wis. Bot.* 1895. 28, 269-289.

2. Passman, F. J. *Introduction to Fuel Microbiology*. In F. J. Passman, Ed., *ASTM Manual 47: Fuel and Fuel System Microbiology – Fundamentals, Diagnosis and Contamination Control*. ASTM International, West Conshohocken, PA. 2003. p. 1-13.

3. Passman, F. J., B. L. McFarland and M. J. Hillyer. *Oxygenated Gasoline Biodeterioration and its Control in Laboratory Microcosms*. Int. Biodeg. Biodet. **47**(1): 95-106. 2001.

4. Starkey, R. L. and K. Wright. *Bull. Amer. Gas Soc. Tech. Sec.* 1945. p. 108.

5. Videla, H. A. *Manual of Biocorrosion*. CRC Press, Boca Raton. 1996. 273 pp.

6. Gu, J. D., C. Lu, R. Mitchell, K. Thorp and A. Crasto. *Fungal Degradation of Fiber-Reinforced Composite Materials*. *Material. Perform.* 1997. 36:37-41.

7. Hill, G. *Sampling Methods for Detecting Microbial Contamination in Fuel Tanks and Systems.* In F. J. Passman, Ed., *ASTM Manual 47: Fuel and Fuel System Microbiology – Fundamentals, Diagnosis and Contamination Control.* ASTM International, West Conshohocken, PA. 2003. p. .

8. Roszak, D. B. and R. R. Colwell. *Survival Strategies of Bacteria in the Natural Environment*. *Micrbiol. Rev.* **51**(3): 365-379. 1987.

9. ASTM. *E1326 Guide for Evaluating Non-conventional Microbiological Methods. Annual Book of Standards*, Vol. 11.05. ASTM International, West Conshohocken, PA.

10. Strehler, B. L. and W. D. McElroy. *Assay of Adenosine Triphosphate*. In S.P. Colwick and N. O. Kaplan, Eds., *Methods in Enzymology*. Academic Press, New York, NY. 1957. p. 871-873.

11. Holm-Hansen, O. and C. R. Booth. *The Measurement of Adenosine Triphosphate in the Ocean and its Ecological Significance*. *Limnol. Oceanog.* **11**(4) p. 510-519. 1966.

12. Hamilton, R. D. and O. Holm-Hansen. *Adenosine Triphosphate Content of Marine Bacteria*. *Limnol. Oceanog.* **12**(2): 319-324. 1967.

13. Karl, D. M. *Total Microbial Biomass Estimation Derived from the Measurement of Particulate Adenosine-5'-Triphospahte*. In Kemp, P. E., B. F. Sherr, E. B. Sherr and J. J. Cole, Eds., *Handbook of Methods in Aquatic Microbiology*. Lewis Publishers, Boca Raton, FL. p. 359-

367. 1993.

14. Miller, C. D. and L. Loomis. *Method for Detection of Surface Contaminants*. U.S. Patent No. 5,624,810. U.S. Patent Office, Washington, DC. 1997.

15. Miller, C. D. and L. Loomis. *Method for Detection of Contaminants*. U.S. Patent No. 5,736,351. U.S. Patent Office, Washington, DC. 1998.

16. Levin, J. and F. B. Bang. *The role of endotoxin in the extracellular coagulation of Limulus blood. Bull. Johns Hopkins Hosp.* **115**:265-274. 1976.

17. Passman, F.J., T.J. Novitsky and S.W. Watson. *Surface Micro-Layers of the North Atlantic: Microbial Populations, Heterotrophic and Hydrocarbonoclastic Activities*. In *Microbial-Degradation of Pollutants in Marine Environments*. U.S. Environmental Protection Agency. EPA-600/9-79-012. pp: 214-226 (1979).

18. Jorgensen, J. H., J.C. Lee and H.R. Pahren. *Rapid Detection of Bacterial Endotoxins in Drinking Water and Renovated Wastewater*. *Appl. Env. Microbiol.* **32**: 347-351. 1976.

19. Novitsky, T. J. *Bacterial Endotoxins (Pyrogens) in Purified Waters*. *In* Mittelman, M.M. and Greesey,G.G. Eds. *Biological fouling of industrial water systems: A Problem Solving Approach* San Diego: Water Micro Associates. pp: 77. 1987.

20. Pepper,I.L., K.L., Josephson, R.L. Bailey, M.D Burr, D.S. Pillai, and D.L. Tolliver. *Measuring Bacterial Contaminants in Ultrapure Water: A Rapid Analytical Method. Microcontamination* **10**:52-57. 1994.

21. Sloyer, J. L., T. J. Novitsky and S. Nugent. *Rapid Bacterial Counts in Metal Working Fluids. J. Ind. Microbiol. Biotech.* **29**(6):323-324. 2002.

22. Passman, F. J., H. L. Chesneau, and D. A. Daniels. *Catalase Measurement: A New Field Procedure for Rapidly Estimating Microbial Loads in Fuels and Water-Bottoms*. *In* Giles, H. N. Ed. *Proceedings of the Fifth International Conference on the Stability and Handling of Liquid Fuels; 03-07 October 1994, Rotterdam, The Netherlands*. U.S. Department of Energy, Washington DC; DOE/CONF-941022. p. 151-171. 1995.

23. Ringer, S. *Concerning the influence exerted by each of the constituents of the blood on the contraction of the ventricle. J. Physiol.* **3**: 380-393. 1880-1882.

24. ASTM. *D4012 Standard Test Method for Adenosine Triphosphate (ATP) Content of Microorganisms in Water*. *Annual Book of Standards*, Vol. 11.02. ASTM International, West Conshohocken, PA.

25. Castellan, R. M., Olenchock, S. A., Kinsley, K. B. and Hankinson, J. L. *Inhaled Endotoxin and Decreased Spirometric Values. New Eng. J. Med*., **317**, pp 605-610. 1987.

Table 1a. ATP Precision and bias data for Log RLU data from three UST bottom-water samples analyzed by three analysts.

Sample ID	Analyst	ATP							
		LOG_{10} RLU							
		REP 1	REP 2	REP 3	REP 4	REP 5	AVG	SD	CV%
1	A	4.22	3.99	3.87	3.73	4.09	3.98	0.19	4.8%
	B	3.88	3.36	3.81	3.45	3.60	3.62	0.23	6.2%
	C	3.83	3.72	3.56	3.74	3.83	3.74	0.11	2.9%
2	A	3.48	3.25	3.85	3.16	3.12	3.37	0.30	8.9%
	B	2.82	2.87	2.70	2.62	2.75	2.75	0.10	3.6%
	C	2.89	3.09	3.29	3.35	2.97	3.12	0.20	6.3%
3	A	2.63	2.33	2.71	2.23	2.36	2.45	0.21	8%
	B	1.88	1.41	1.38	0.90	2.15	1.54	0.48	31%
	C	2.14	3.38	2.05	2.01	2.56	2.43	0.58	24%

Table 1b. ANOVA Computation summary for data presented in Table 1a.

Source of Variation	df	SS	MS	F
Among Analysits	2	3.159	1.579	2.055
Among Samples	12	1.395	0.116	0.151
Among replitcate tests	30	23.06	0.769	
Total	44	27.61		

$F_{0.5[2,12}$	3.89
$F_{0.1[2,12}$	6.93

Table 2a. ATP data variability amongst test units.

Analyst	ATP							
	LOG_{10} LUMENS							
	REP 1	REP 2	REP 3	REP 4	REP 5	AVG	SD	CV%
Model 4560 # 007	4.49	4.39	4.61	4.48	4.63	4.52	0.10	2.2%
Model 4560 # 009	4.67	4.45	4.46	4.75	4.77	4.62	0.15	3.4%
Model 3560 # 1001	4.41	4.29	4.33	4.27	4.40	4.34	0.06	1.5%

Table 2b. ANOVA Computation summary for data presented in Table 2a.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*F crit*
Between Bioluminometer	0.213813	2	0.106907	8.81341	3.88529
Error	0.14556	12	0.01213		
Total	0.359373	14			

Table 2c. ANOVA Computation summary for Model 4560 Bioluminometer data presented in Table 2a.

Source of Variation	*SS*	*df*	*MS*	*F*	*F crit*
Between Bioluminometer	0.025	1	0.025	1.503	5.318
Error	0.133	8	0.017		
Total	0.158	9			

Table 3. Bottom-water sample risk rating criteria.

PARAMETER	RISK RATING		
	L	M	H
G.O.	No Rag; Haze ≤ 2	No Rag; Haze > 2	Rag
D.O. (%)	<10	10 to 50	>50
CAT (psig)	< 5	5 to 20	>20
Log MPN	<2	2 to 4	>4
ATP (Log RLU)	<2.5	2.5 to 3.5	>3.5

Table 4a. Agreement: ATP vs Other parameters

	Numbers				Percentages				
	Same	ATP >	ATP <	Total	Same	ATP >	ATP <	ATP./=	Total
Gross Obs.	29	18	9	56	52%	32%	16%	84%	100%
DO Demand	30	18	6	54	56%	33%	11%	89%	100%
Catalase	26	6	22	54	48%	11%	41%	59%	100%
MPN	25	7	20	52	48%	13%	38%	62%	100%

Table 4b. Gross Observation vs. Other Parameters

	Numbers				Percentages			
	Same	G.O. >	G.O. <	Total	Same	G.O. >	G.O. <	Total
ATP	29	9	18	56	52%	16%	32%	100%
DO Demand	29	14	11	54	54%	26%	20%	100%
Catalase	13	6	35	54	24%	11%	65%	100%
MPN	19	5	30	54	35%	9%	56%	100%

Table 4c. D.O. Demand vs. Other Parameters

	Numbers				Percentages			
	Same	D.O. >	D.O. <	Total	Same	D.O. >	D.O. <	Total
Gross Obs.	29	11	14	54	54%	20%	26%	100%
ATP	30	6	18	54	56%	11%	33%	100%
Catalase	15	6	31	52	29%	12%	60%	100%
MPN	13	6	33	52	25%	12%	63%	100%

Table 4d. Catalase vs. Other Parameters

	Numbers				Percentages			
	Same	Cat >	Cat <	Total	Same	Cat >	Cat <	Total
Gross Obs.	15	31	6	52	29%	60%	12%	100%
DO Demand	30	6	18	54	56%	11%	33%	100%
ATP	26	22	6	54	48%	41%	11%	100%
MPN	39	8	4	51	76%	16%	8%	100%

Table 4e. MPN vs. Other Parameters

	Numbers				Percentages			
	Same	MPN >	MPN <	Total	Same	MPN >	MPN<	Total
Gross Obs.	19	30	5	54	35%	56%	9%	100%
DO Demand	13	33	6	52	25%	63%	12%	100%
ATP	25	20	7	52	48%	38%	13%	100%
Catalase	39	4	8	51	76%	8%	16%	100%

Table 5a. Comparison of polar fluorescence (VB), adenosine triphosphate (ATP) and catalase activity (catalase) data from ten bottom-water samples.

Log ATP	Log VB	Log Catalase
3.48	2.80	2.50
3.27	3.98	3.44
3.22	4.03	3.77
3.4	4.04	2.55
4.49	4.28	4.62
4.93	4.47	4.15
5.32	4.60	4.89
4.09	4.67	5.53
4.65	5.03	5.18
2.84	5.18	4.24

Table 5b. Covariance matrix for Log ATP, Log VB and Log Catalase data from Table 5a.

	Log ATP	*Log VB*	*Log Catalase*
Log ATP	1.000		
Log VB	0.633	1.000	
Log Catalase	0.630	0.919	1.000

T**able 6. Comparison of polar fluorescence (VB) and endotoxin (EU) data from 15 bottom-water samples.**

Log VB	EU
4.140	55
4.163	18
4.168	48
4.170	76
4.172	44
4.188	55
4.378	39
4.565	0
4.839	301
4.881	67
4.899	112
5.080	124
5.130	32
5.150	85
5.188	140
	r^2 = 0.181

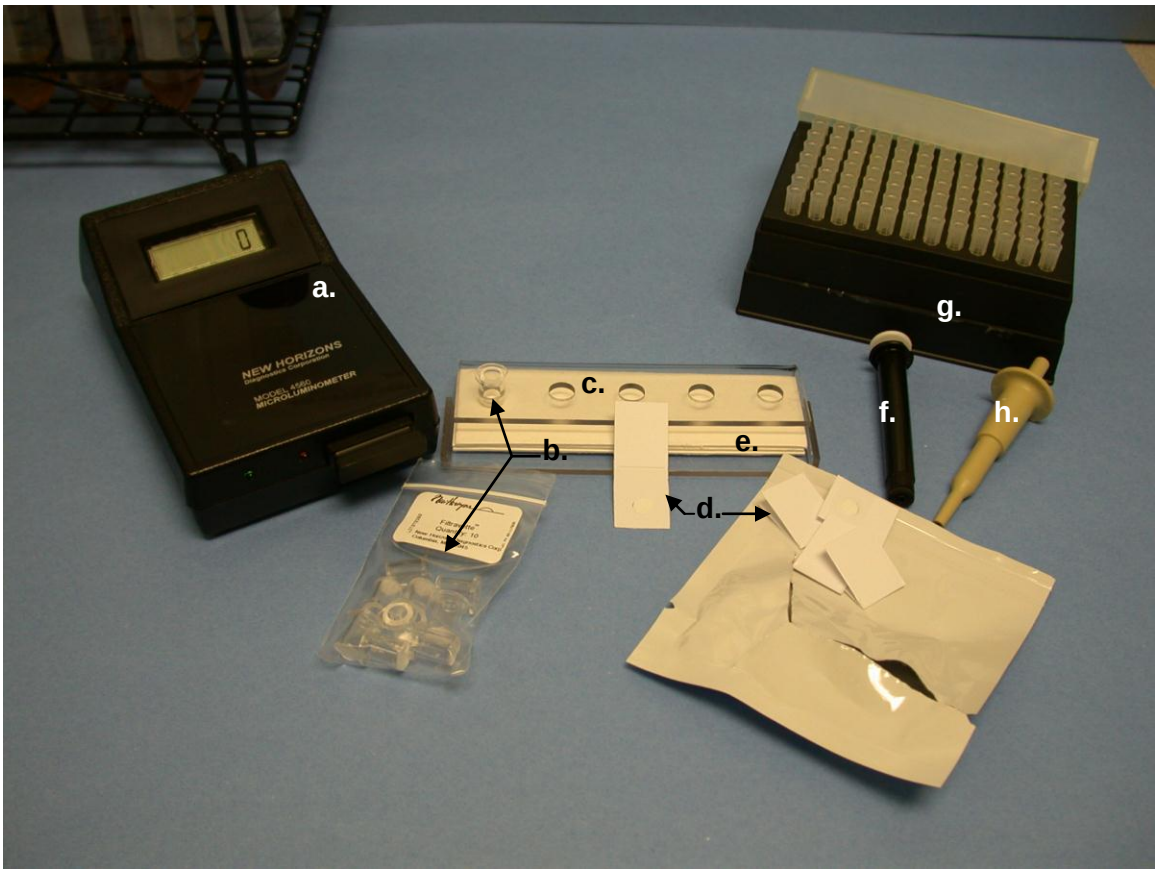

Figure 1. Profile 1 Model 4560 Biolumiometer and ATP test kit supplies. a. Bioluminometer, b. Filtervette cuvettes, c. cuvette holder, d. Luciferin-Luciferase reaction pads, e. blotter pads, f. plunger device, g. micro-pipette tips and h. micro-pipetter.

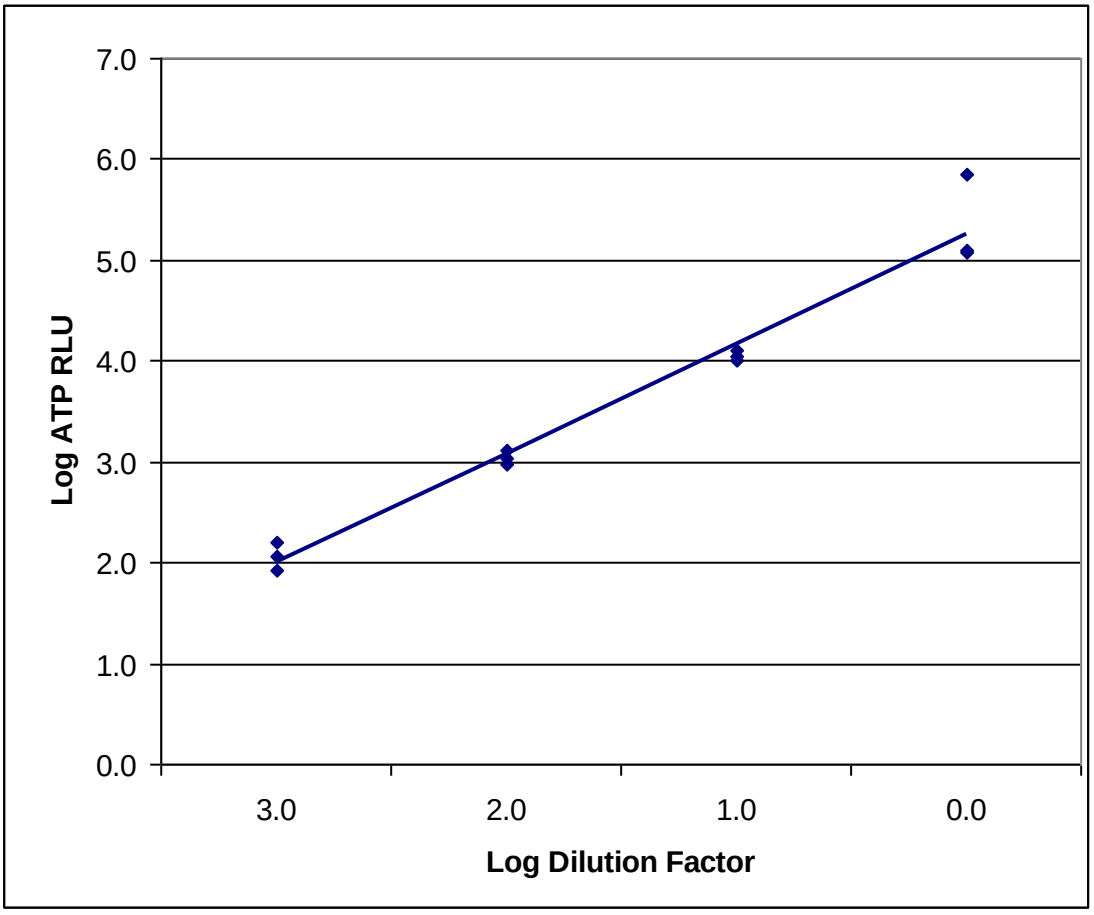

Figure 2. Log ATP RLU v. Log CFU *P. putida*/ml in DIW. Coefficient of variation, r^2 = 0.998. Data points are for each of three replicate analyses at each *P. putida* cell density. Line is from equation: Log ATP RLU = (1.08 Log CFU *P. putida*/ml) – 3.42.

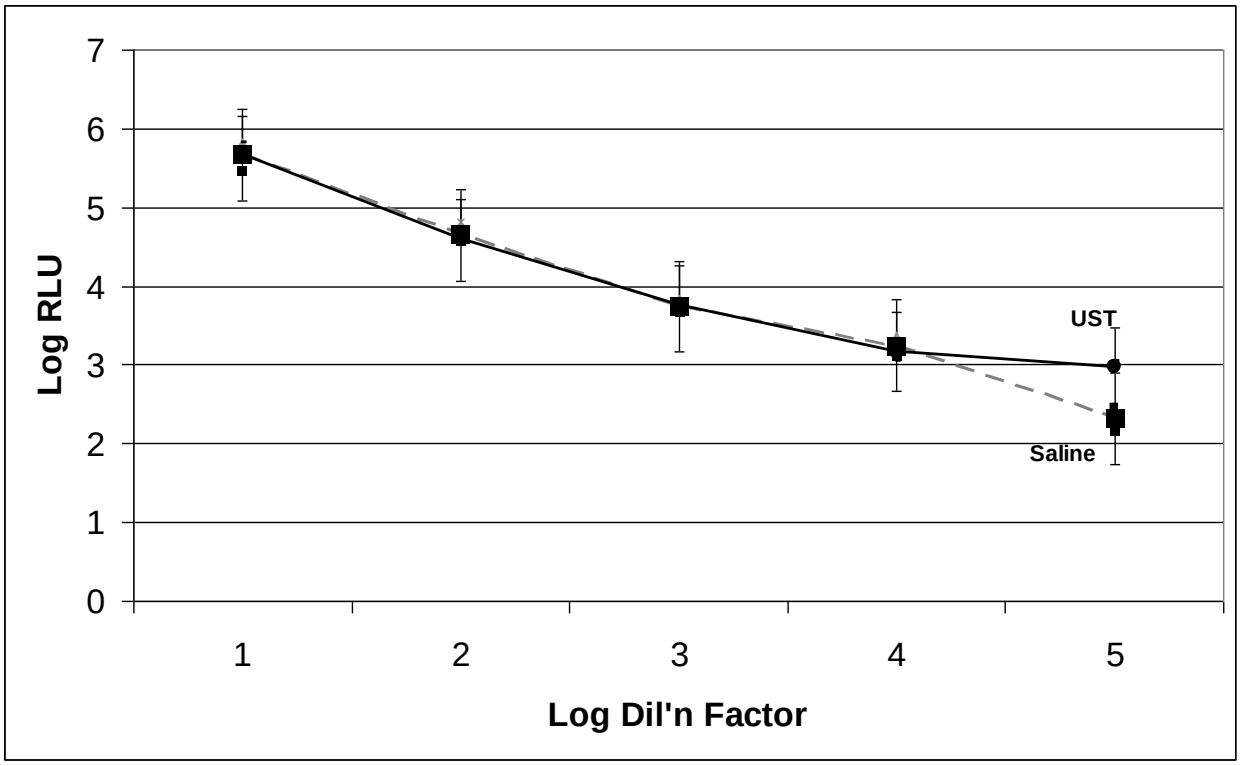

Figure 3. Log ATP RLU v. Log Dilution Factor for *P. putida*/ml in UST bottom-water or normal saline. Coefficients of variation, r^2 for *P. putida* in saline = 0.997; in bottom-water = 0.984 Data points are for each of three replicate analyses at each *P. putida* cell density.

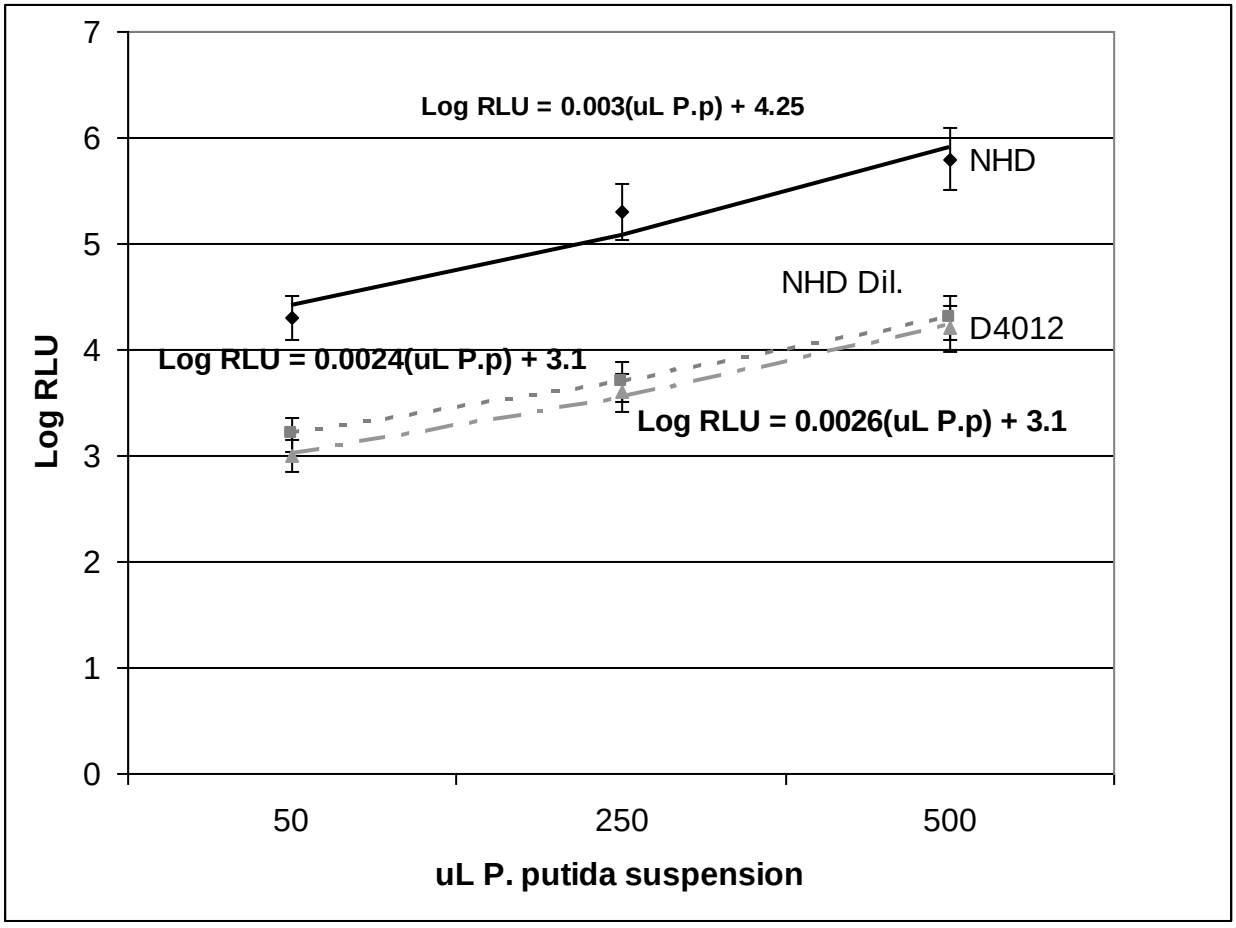

Figure 4. Comparison of Bioluminometer and ASTM D4012 data. (log 3 RLU ≈ log 6 *P. putida* cells/ml). F-ratio computation indicates that at 95% confidence level, results from the two methods are the same. Log RLU v. µl *P. putida* suspension > 0.99 for both methods. NDH curve is for Bioluminometer method unmodified. NDH Dil. curve is for Bioluminometer data for *P. Putida* suspension diluted 1:100 to reproduce effect of ASTM D4012 method.

a.

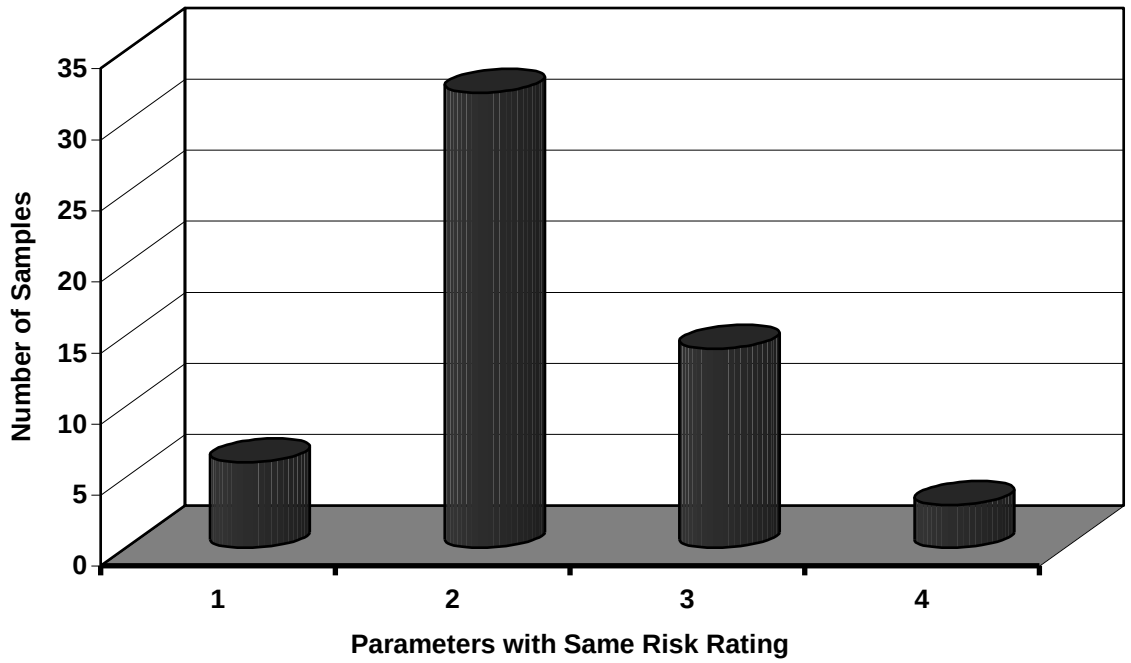

Figure 5. Agreement amongst biodeterioration risk test parameters: gross appearance, ATP, catalase activity, oxygen demand and viable counts.