Presented at 7th International Colloquium Fuels, Technische Akadamie Esslingen, 14-15 January 2009, Ostfildern, Germany.

Using Adenosine Triphosphate to Quantify Bioburdens in Various Liquid Fuels

F. J. Passman
Biodeterioration Control Associates, Inc., Princeton, NJ, USA

Summary

Adenosine Triphosphate (ATP) is an excellent biomarker present in all living cells. During the past several years, several ATP test methods have been developed to overcome interferences that have historically made ATP testing of fuels impractical. This paper compares two of the new ATP test methods, presenting sensitivity and precision data for each method. Additionally, for one of the methods it examines the effect of fuel type on ATP test results. This method has a lower detection limit of 20 pg ATP/mL (approximately 100 bacterial cells/mL). Conventional gasoline, ethanol-gasoline blends, ultra-low-sulfur diesel and biodiesel blends carrying a range of bioburdens are examined.

1 Introduction

At the 2007 TAE Fuels Colloquium [1] Passman and Eachus reviewed the importance of effective, real-time microbial contamination condition monitoring for fuels, and described a new adenosine triphosphate (ATP) test protocol that provided reliable ATP data from fuel and fuel-associated bottom-water samples. Subsequent field work using that method revealed that a key component of the test kit was incompatible with ethanol-blended fuels. An alternate method – currently in ballot as a new ASTM standard test method [2] circumvents the material compatibility issue with conventional fuels by extracting the fuel-phase biomass and other hydrophilic particles into an aqueous capture solution. However the protocol depends on extraction of fuel-phase biomass into an aqueous capture solution. This works well in conventional fuels, but adequate phase separation does not occur in high water-retaining fuels such as ethanol-blended gasoline. This paper describes an ATP test method that addresses the limitations of the two previous protocols.

2 Materials and Methods

2.1 Microcosms

Four 3,600 mL microcosms were set up in 3.8 L glass jars. The fuels used were: conventional 87 octane gasoline (87UNL), 87 octane gasoline with 10% ($^{v}/_{v}$) ETOH (87E10), ultra low sulfur diesel (ULSD) and ULSD with 20% ($^{v}/_{v}$) soy-derived B-100 biodiesel base stock (B-20). The fuel blends were prepared in-microcosm so that each microcosm contained 3,400 mL of the required blend. After preparing the fuel blends, 200 mL of contaminated-water was added to each microcosm. Microcosms were maintained at room temperature (19±2 °C). All tests were run within two-days after the microcosms were set up.

2.2 Contaminated-water inoculum

The contaminated-water inoculum (CWI) was prepared by inoculating commercial spring water with an uncharacterized commercial preparation of freeze-dried microbes (Rid-X, Reckitt Benckiser, Inc., Parsippany, NJ, USA) used to stimulate septic tank activity. To start a CWI preparation, 1 g of freeze dried material was dispersed into 1L of spring water. This preparation incubated at room temperature in the dark for two-weeks before being used to inoculate the microcosms.

2.3 ATP

To test ATP concentration, either a bottom-water (1.0 mL) or a fuel sample (25 mL) was drawn into a disposable syringe (5 mL for water samples; 60 mL for fuel samples). The sample was then filtered through an in-line 0.7 μm filter. The filter is removed from the syringe, the plunger is removed from the syringe's barrel, the filter is replaced and 5.0 mL of a proprietary cleaning agent (all reagents are from LuminUltra Technologies, Ltd., Fredericton, NB, Canada) is dispensed into the syringe barrel. The plunger is then reinserted into the syringe barrel and the cleaning agent is pressed through the filter.

A clean 60 mL syringe is then used to air dry the filter. The barrel is removed from the syringe, the in-line filter affixed to the syringe, the barrel is replaced and air is passed through the filter. This step is repeated two or three times, until the filter is dry. The air-dried filter is reaffixed to the original syringe, from which the barrel has again been removed. Then 1.0 mL of a proprietary lysing agent is dispensed into the syringe, the barrel is replaced and the fluid is pressure-filtered into a 17 x 100 mm culture tube. Next, 9.0 mL of dilution buffer is added to the culture tube. The tube is capped and shaken to mix its contents. A 100 µL portion of the diluted sample is transferred to a 12 x 55 mm culture tube to which 100 µL luciferase enzyme reagent has been added previously. The culture tube is swirled gently for 10 sec and placed into a luminometer. Data are in relative light units (RLU) which are converted to pg ATP/mL by comparison against data from an ATP standard.

For ULSD and B-100 bottom-waters, dilution series were run by two different analysts; each in triplicate. Sampled bottom water was diluted in bottled spring water: undiluted, 1:5, 1:10, 1:50 and 1:100.

LuminUltra 1.0 ng ATP/mL standard was used to calibrate the luminometer and compute Log_{10} pg ATP/mL from raw RLU data:

(1) Log_{10} pg ATP/mL = Log_{10} [($RLU_{smpl} \div RLU_{ctrl}$) · (10,000 ÷ mL sample)]

where RLU_{smpl} was the RLU from the test sample and RLU_{ctrl} was the average of triplicate 1.0 ng ATP/mL control samples. The 10,000 value was derived from the 10-fold dilution of the extracted ATP times the conversion of ng to pg (1,000 pg/ng). To run the control test, 100 µL of 1.0 ng ATP/mL standard was dispensed into a reaction tube containing 100 µL luciferase enzyme reagent.

2.4 Statistics

Repeatability statistics were computed per ASTM E 691 [3]. Correlation coefficients for dilution curves were computed using Excel 2007 software (Microsoft, Inc. Redmond, WA, USA).

3 RESULTS

3.1 Control Data

Control RLU were determined four times during the day during which microcosm ATP testing was completed. The average reading was 9,350 ± 451 RLU (4.8% coefficient of variation). The value 9,350 RLU was used for RLU_{ctrl} in computing all Log pg ATP/mL reported in this paper.

3.2 CWI ATP

CWI was tested for ATP concentration before it was added to test microcosms. The average Log_{10} pg ATP/mL of four replicate CWI samples was 4.2 ± 0.09. Two replicates were tested using the protocol described previously by Passman *et al.* [2] and two were tested using the protocol presented in this paper. One-way analysis of variance (ANOVA) yielded F_{obs} = 6.5 where $F_{crit\ (0.95;\ 1,2)}$ = 18.5. The results from the two methods were indistinguishable.

3.3 Fuel-phase ATP

Table 1 presents the results of fuel-phase ATP repeatability testing. The averages and standard deviations were computed from duplicate tests run by each of two analysts (n = 4). The $87UNL_1$ and $87E10_2$ samples were from water-free microcosms into which 0.5 g of the freeze-dried microbe preparation had been dispersed 30 min before the samples were collected. Note that less than 1% of the challenge ATP burden is recovered from fuel-phase samples.

3.4 Water-phase ATP

Two test series were run on bottom-water samples. First, samples were collected from each microcosm to compare bottom-water ATP recoveries as a function of overlying fuel grade. The second was ULSD and B-20 dilution.

Table 2 summarizes the results of the bottom-water ATP tests. Unsurprisingly, variability increases at low ATP concentrations. ATP in gasoline

Table 1. Fuel-phase ATP data

Fuel	Log_{10} pg ATP/mL		
	AVG	S.D.	C.V. %
$87UNL_1$	2.5	0.25	10
$87UNL_2$	1.0	-	-
$87E10_1$	2.8	0.26	9
$87E10_2$	1.1	0.03	2
ULSD	1.6	0.24	15
B-20	2.2	0.01	0.7

microcosm bottom-water was several logs lower than it was in the diesel fuel microcosm bottom-water. The ATP recovered from diesel fuel bottom-water was nearly the same at the concentration in the CIW. The ATP concentration in gasoline bottom-water was near the method's lower detection limit (1.0 Log_{10} pg ATP/mL). Consequently, no attempt was made to run ATP dilutions on either the 87UNL or 87E10 bottom water. Dilutions were prepared of ULSD and B-20 bottom-water as described above. Figure 1 compares the dilution curves for Log_{10} pg ATP/mL in ULSD and B-20 bottom-water. Each data point represents the average of four analyses (duplicate analyses by each of two analysts).

Table 2. Water-phase ATP data

Fuel	Log_{10} pg ATP/mL		
	AVG	S.D.	C.V. %
87UNL	0.6	0.16	25
87E10	1.3	-	-
ULSD	4.0	0.07	2
B-20	4.0	0.01	0

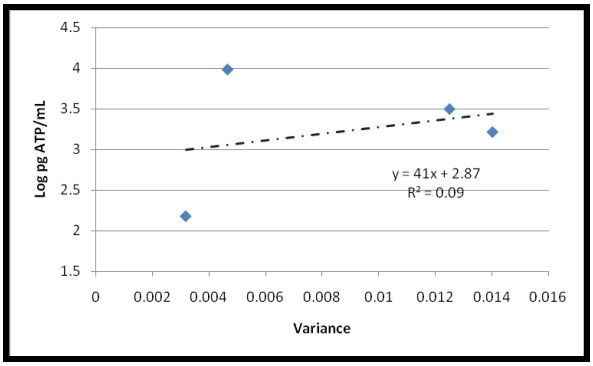
The results in the two types of bottom-water appeared to be very similar. In order to evaluate whether fuel-type affected the ATP bottom-water data, the parameter z was computed for the ULSD and B-20 bottom-water data sets (Table 3). The computed values for z were less than the critical value for z, supporting the hypothesis that the test results were not affected significantly by ULSD relative to B-10. Two-way ANOVA (dilution factor x fuel-grade) yielded F_{obs} = 11.5 ($F_{critical\ (0.95;\ 1,3)}$ = 4.2); suggesting that the minor differences in ATP results in the two media were, in fact, statistically significant.

Table 3. Z-Test between ATP in ULSD bottom-water and B-20 bottom-water.

Metric	ULSD	B-20
Mean	3.267	3.176
Known Variance	0.459	0.482
Observations	16	16
Hypothesized Mean Difference	5.000	
z	-	
P(Z<=z) one-tail	0.354	
z Critical one-tail	1.645	
P(Z<=z) two-tail	0.708	
z Critical two-tail	1.960	

Figure 2 shows that data variability is not affected significantly by ATP concentration between 2.2 and 4.0 Log_{10} pg ATP/mL (correlation coefficient, R^2 = 0.09).

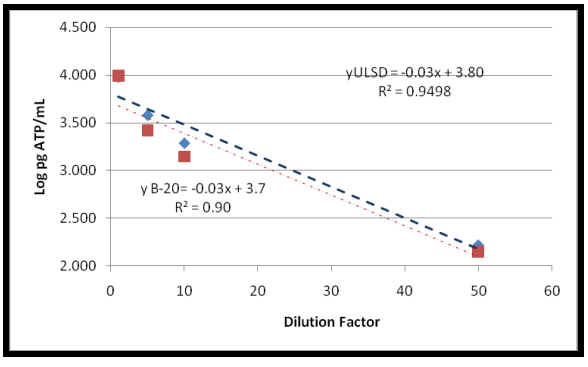
Figure 2. Variance as a function of ATP concentration.



4 DISCUSSION

The adverse economic impact of microbially influenced corrosion (MIC) in U.S. has been estimated at $138 billion U.S. per year [4]. Little and Lee did not break the impact down by industry, but noted that the principal industries affected by MIC are petroleum production, storage and transport, along with power generation and water distribution. A 2001 study by the U.S. Federal Highway Administration [5] estimated that corrosion costs associated with domestic above-ground and underground storage tanks were $8 billion U.S. This estimate did not include other components within the petroleum distribution infrastructure.

Figure 1. Dilution of ATP in fuel-microcosm bottom-water.



The author has previously discussed how uncontrolled microbial contamination can damage fuel systems and degrade fuel quality [6]. The same

publication addressed the importance of being able to obtain real-time microbiological data. As reported during the 2007 TAE Fuel Colloquium, until recently, the utility of ATP as a test parameter was limited due to unpredictable luminescence quenching and abiotic luminescence [1]. The detection of ATP in ethanol-blended fuels and biodiesel blends is particularly problematic in that different protocols must be used.

A method presented in 2007 [2] circumvented the materials compatibility issue. However, because that protocol depends on aqueous extraction, the extraction efficiency suffers when the method is used in fuels that have >0.25% water solubility.

Unless the protocol is modified, stable suspensions or dispersions of the aqueous capture solution can occur in biodiesel blends and ethanol blends. Even in conventional fuels, it has been observed that the extraction procedure is only approximately 50% efficient (R. Fass, personal communication). Yields from second extractions are approximately the same as those from initial extractions. Yields from third extractions suggest that > 95% of the fuel-phase ATP is removed by the first two extractions. The 50% extraction efficiency is not a serious impediment to the procedure. For routine condition monitoring, trends rather than absolute data are most important. For diagnostic testing, doubling the sample size compensates for the 50% extraction efficiency. However, sample size can become a limiting issue. The method described by Passman *et al.* [2] requires 250 mL to 1 L of sample. Once used for ATP extraction, these samples cannot be used for other analyses. In contrast, the current method requires 25 mL of fuel sample. Recovery efficiency tests (data not shown) demonstrated >95% recovery during the first ATP extraction.

Interestingly, challenge populations behave differently from wild populations in gasoline-associated bottom-water. ATP concentrations in field-collected 87UNL underground storage tank bottom-water samples ranged from 1.98 to 4.46 Log_{10} pg ATP/mL [1]. Passman *et al.* [2] permitted their challenge populations to acclimate for several months before dosing those microcosms with microbicides. Initial ATP concentrations in the 87UNL microcosm bottom-waters were 3.9 to 4.2 Log_{10} pg ATP/mL. It may be that during the initial exposure period cell lysis occurs. Only after the population produces sufficient exopolymer to form a barrier between cells and the environment does the population begin to proliferate. When Passman *et al.* challenged fuel CARB II 87UNL, they did not observe significant fuel chemistry changes during the first 90-days of the study. However, substantial fuel deterioration had occurred between the time that the 90-day sample was collected and the time a final set of samples was collected just before the microcosms were prepared for disposal after seven-month's storage [7]. Further testing is needed to evaluate this hypothesis.

5 CONCLUSIONS

In condition monitoring, speed and accuracy are substantial benefits. The ATP test is fast from two perspectives. It can be performed in the field; minimizing delays between sampling and data acquisition. Moreover, the test can be completed in less than five minutes. This makes it possible for field operators to obtain data needed for making contamination control decisions on a real-time basis. The test method presented in this paper addresses the primary limitations of earlier ATP test methods. It is not affected by the sample matrix. Except for adjusting sample volumes (1.0 mL for water samples; 25 mL for fuel samples) the procedure is the same regardless of fuel-grade. Although data can be recorded in RLU, the protocol provides a simple means for converting RLU to Log_{10} pg ATP/mL so that data from different protocols, different instruments or different reagent batches can be compared easily. As such, this new protocol is likely to be of some value to the fuel industry.

6 REFERENCES

[1] Passman, F. J. and A. C. Eachus: Adenosine Triphosphate as a Rapid Measure of Microbial Contamination in Fuel Systems. In: Bartz, W. J. *Ed.* Fuels 2007 – 6th International Colloquium. Technische Akademie Esslingen, Ostfildern, Germany. (2007) 689-695.

[2] Passman, F. J., E. English and C. Lindhardt: Using Adenosine Triphosphate Concentration as a Measure of Fuel Treatment Microbicide Performance. In: R. E. Morris. *Ed. Proceedings of the 10th International Conference on the Stability and Handling of Liquid Fuels*; 7-11 October, 2007, Tucson, Arizona.

[3] ASTM E 691 Practice for Conducting an Interlaboratory Study to Determine the Precision of a Test Method. www.astm.org, ASTM International, West Conshohocken, PA, USA.

[4] Little, B. and J. Lee: Microbiologically Influenced Corrosion. Wiley-VHC Verlag, Weinheim, Germany. 2007.

[5] CC Technologies Laboratories, Inc.: Corrosion Costs and Preventive Strategies in the United States, Report to Federal Highway Administration (FHWA), Office of Infrastructure Research and Development, Report FHWA-RD-01-156, http://www.corrosioncost.com/home.html. 2001.

[6] 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) 1-13.

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