Real-Time Testing of Bioburdens in Metalworking Fluids Using Adenosine Triphosphate as a Biomass Indicator

FREDERICK J. PASSMAN,[1] GEORGE L. EGGER II,[2] SARAH HALLAHAN,[2] BRENT W. SKINNER[2] and MARK DESCHEPPER[2]
[1]Biodeterioration Control Associates Inc., Princeton, N.J., USA
[2]Caterpillar, East Peoria, Ill, USA

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Editor's Note: This month's cover story on MWF microbicides really begins with measuring bioburdens, without which proper dosing could not be possible. My personal experience measuring biomass in quenching fluids was very time-intensive and somewhat subjective, so the concept of a highly accurate determination in less than 10 minutes is very appealing. I was further enticed by the caliber of one of the authors. This month's Editor's Choice paper is co-authored by Dr. Fred Passman, an STLE fellow known globally as an expert in metalworking fluids with decades of experience in environmental-industrial microbiology, and a pioneer of STLE's Certified Metalworking Fluids Specialist certification program. Having enjoyed some of Dr. Passman's previous presentations at the society's annual meetings, I was rewarded once again with this synoptic study.

Evan Zabawski, CLS
Editor

KEY WORDS

Adenosine Triphosphate; Condition Monitoring; Fluid Management; Metalworking Fluids; Metal Removal; Microbiology

ABSTRACT

Adenosine triphosphate (ATP) assays have been used to quantify bioburdens (biomass) in low-organic-compoundcontent fluids (freshwater, seawater, cooling tower water, and similar fluids) since the early 1950s. The original methodology was labor intensive and required considerable laboratory skill. Over the past half-century, the protocol has been simplified substantially, but until recently, chemical interferences made it impractical to use the ATP test in metalworking fluids (MWF). This article presents precision and bias statistics for a new test protocol for ATP in emulsifiable oil, semisynthetic and synthetic MWF at end-use dilutions. Additionally it presents the results of field tests in which ATP data are compared with other MWF condition monitoring data. The field evaluation demonstrates the applicability of the new protocol to MWF bioburden condition monitoring.

INTRODUCTION

Recirculating water-miscible metalworking fluids (MWF) provide an excellent environment for the growth and proliferation of microbes (Passman[1]). These organisms, primarily bacteria and fungi, can cause two types of problems in the metalworking environment. Uncontrolled microbial activity can degrade fluid performance, by changing the fluid properties (Passman[2]), selectively attacking MWF constituents, or a combination of both. Moreover, this growth can cause system fouling as evidenced by biomass accumulation on sluice and machine surfaces, filter plugging, or fluid-flow restriction (Passman[2]). Even in so-called bioresistant MWF, microbe proliferation can pose a health risk (Passman and Rossmoore[3]).

The key to successful microbial contamination control is effective condition monitoring. Historically, culture methods have been the most commonly used tool for quantifying microbial loads in MWF (Passman[1]). Much of the time, culture methods are adequate. However, the delay between culturing and data availability is typically 36 h to 48 h and may be as long as 30 d for some microbes (Todar[4]). Passman[5] has previously reported the use of catalase activity as a real-time parameter for measuring microbial loads in MWF. The principal limitation of catalase activity testing is that anaerobic bacteria (those that grow only in the absence of oxygen) are catalase-negative (they lack the catalase enzyme). Consequently, the catalase test does not detect sulfate-reducing bacteria and other anaerobic bacteria that can act as biodeteriogens in MWF.

The ATP test was initially developed for quantifying biomass in aqueous systems (Strehler and McElroy[6]; Holm-Hansen and Booth[7]). In the original protocol, ATP was extracted from cells by boiling the sample in a buffer. The extracted ATP was reacted with the enzyme-substrate complex: luciferin-luciferase. In this reaction, the luciferase enzyme catalyzes the ATP-mediated oxidation of luciferin. In the process, ATP is cleaved to yield adenosine monophoshpate (AMP) and pyrophosphate, concurrently releasing a photon of light. The amount of light generated is proportional to the ATP concentration, which is proportional to the biomass.

Unfortunately, organic chemicals dispersed or dissolved in the aqueous phase of complex solutions and emulsions such as MWF interfere with the detection of the light-emitting reaction on which the ATP test depends. Over the past 50 years, numerous improvements have been made to the ATP test protocol. However, chemical interference had remained a problem. Passman et al. have previously reported the use of a new protocol that effectively eliminates the interferences that affect ATP measurement in fuels and fuel-associated waters (Passman and Eachus[8]; Passman, et al.[9]). This article describes the validation of a new method for determining ATP in MWF.

MATERIALS AND METHODS

ATP Test Method

A 5.0-mL sample of MWF is pressure filtered though a 0.7µm, in-line, glass fiber filter. The filter retentate is then washed with a proprietary solution and air dried. Next, 1.0 mL of a proprietary extracting reagent is filtered through the filter that has the retained biomass. The extracted ATP is collected in a test tube and diluted 1:10 with a proprietary buffer solution. Finally, 100 µL of a diluted sample is mixed with 100 µL luciferin-luciferase reagent in a 12 mm × 55 mm cuvette. The cuvette is placed into a luminometer that detects emitted light at 420 nm. The luminometer readout is in relative light units (RLUs). A 100-µL, 1.00ng ATP/mL standard is used for calibration, so that the $\log_{10}$ pg ATP/mL sample can be computed from:

$$\log_{10} \text{pg ATP/mL} = \log_{10}[(\text{RLU}_{smpl} \div \text{RLU}_{ctrl}) \times (10{,}000 \div \text{mL}_{sample})] \quad (1)$$

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). A sample volume was 5.0 mL for all MWF samples. Therefore, $\log_{10}$ pg was:

$$\log_{10} \text{pgATP/mL} = \log_{10}[(\text{RLU}_{smpl} \div \text{RLU}_{ctrl}) \cdot (200 \text{ pg/mL})] \quad (2)$$

Preliminary Assessment

Initially, six used MWF samples (two emulsifiable oils [EO], two semisythetic fluids [SS], and two synthetic fluids [S]) were collected from sumps at a manufacturing facility. In order to have a moderately contaminated EO sample, the heavily contaminated EO and SS sample were diluted 1:2 in freshly prepared 5% (v/v) EO and SS, respectively. Three analysts each ran a duplicate ATP test on each of the samples. Additionally, for each fluid type, the sample with the higher ATP concentration was diluted serially in freshly diluted MWF of the same composition. The dilution curves for the three fluid types were compared to determine the effect of fluid type on ATP data.

A second preliminary assessment was run at the field evaluation site. This time, two analysts each tested eight used MWF samples (two EO, four SS, and two S) in triplicate.

Field Evaluation

Initially seven systems were included in the field evaluation (Table 1). Although it was not always possible, the objective was to collect MWF from at least one small-chip and one large-chip operation using each fluid type. Given the operations at the plant, most systems were large-chip operations. After week 4, two more synthetic systems were added to the plan in an attempt to gather data from a synthetic MWF that had detectible bioburdens. Each week, MWF samples were collected as dip samples from each sump. All tests were performed on the day that the samples were collected. The samples were tested for ATP concentration (QGO-M Test Kit, LuminUltra Technologies, Ltd., Fredericton, New Brunswick, Canada), catalase activity (Gannon and Bennett[10]), culturable bacteria and fungi (SaniCheck BF paddles, Biosan Laboratories, Warren, MI), alkalinity, the pH, the refractive index(°Brix), and the triazine concentration. The triazine concentration was determined colorimetrically using the NASH reagent (ammonium acetate, 37.5 g; glacial

Table 1 | **Metalworking Fluid Systems Sampled During Field Evaluation**

Fluid Type	Machine Types	Sump Capacity (L)
Emusifiable oil	Shaver	210
	Vertical broach	760
Semisynthetic	Hobbing	190
	Grinding	870
	Blanchard	570
Synthetic	Lathe	660
	Broach	950
	Radial drill[a]	20
	Radial drill[a]	40
	Radial drill[b]	950
	Radial drill[b]	950

Notes: a) Samples collected during last 6 weeks of field evaluation.
b) Samples collected during last 4 weeks of field evaluation.

acetic acid, 0.75 mL; acetyl acetone, 0.5 mL; demineralized water to 250 mL). For the assay, 10 mL NASH reagent is added to 20 µL sample in a test tube. After 30 min incubation at 37°C, the sample is transferred to a cuvette, the cuvette is placed into a spectrophotometer, and absorbance at 420 nm (A_{420nm}) is read. Sample A_{420nm} is compared to a calibration curve to convert absorbance results into mg triazine/L.

Statistics

Excel 2007 (Microsoft Corp., Redmond, Wa.) was used to compute the analyses of variance (ANOVAs) and slopes of the respective dilution curves among the preliminary ATP data. ATP repeatability and reproducibility were computed in accordance with ASTM E 691 (ASTM[11]).

RESULTS

Preliminary Assessment—Precision

Two-factor ANOVA (6 samples × 3 analysts) showed that the ATP concentration varied among the samples but that variation between the analysts was not significant at the 95% confidence level (*Table 2*). Interestingly, there was a small but significant interaction effect between the sample and the analyst. This interaction effect reflects the greater variability among test run on samples with <1.0 $\log_{10}$ [pg ATP/mL MWF] than on samples with ≥1.0 $\log_{10}$ [pg ATP/mL MWF]. For samples at the low end of the test method's sensitivity, the analyst technique has a significant effect on the test results. In practical terms this has two implications. When MWF have <1.0 $\log_{10}$ [pg ATP/mL MWF], microbial contamination control is adequate and the issue of greater data variability is moot. However, if precision is important, it can be improved by increasing the number of replicate tests run.

Table 2 | **ANOVA Summary; Two-Factor With Replication; ATP Concentration X Analyst**

Source of Variation	SS	df	MS	F	P-Value	F Crit
Sample	98.23008	7	14.03287	3110	6.67E-34	2.422629
Analysts	0.001996	2	0.000998	0.221191	0.80318	3.402826
Interaction	0.20565	14	0.014689	3.255474	0.005393	2.129797
Within	0.108292	24	0.004512			
Total	98.54602	47				

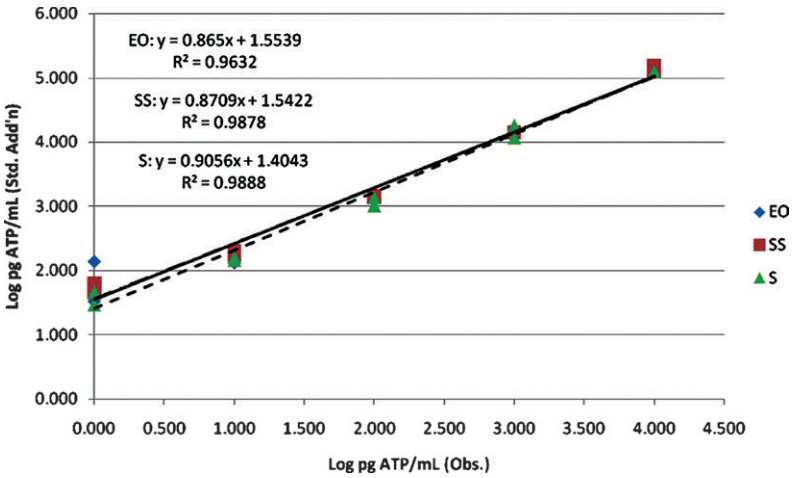

Figure 1 | **Dilution curves for ATP in emusifiable oil, semi-synthetic, and synthetic metalworking fluids.**

Subsequently, eight MWF samples were tested in accordance with ASTM E691[11] to determine the repeatability and reproducibility of the test method. Table 3 presents the data

Table 3 | **Repeatability and Reproducibility of Log [Pg ATP/Ml] in Used Emulsifiable Oil, Semi-Synthetic, and Synthetic Metalworking Fluids**

Material	Average [a] x	Repeatability Standard Deviation s_r	Reproducibility Standard Deviation s_R	Repeatability Limit r	Reproducibility Limit R
1	3.53	0.028	0.065	0.079	0.183
2	0.227	0.116	0.135	0.326	0.377
3	1.35	0.022	0.022	0.063	0.063
4	0.079	0.094	0.099	0.262	0.278
5	0.658	0.016	0.061	0.045	0.171
6	0.178	0.093	0.093	0.261	0.261
7	3.14	0.058	0.135	0.163	0.378
8	3.364	0.012	0.099	0.033	0.277
Average	1.566			0.154	0.2485

[a]The average of the laboratories' calculated averages.

from this assessment. The average repeatability limit was 10% of the overall average $\log_{10}$ [pg ATP/mL MWF]. The average reproducibility was 16%. As expected, the variability was substantially greater for ATP concentrations of <1 $\log_{10}$ [pg ATP/mL MWF]; approaching the method's lower detection limit (0 $\log_{10}$ [pg ATP/mL MWF]; 1 pg ATP/mL).

Preliminary Assessment – Bias

Duplicate ATP dilution series were prepared in EO, SS, and S fluids, respectively. Freshly prepared (5% v/v) fluids were spiked to have final concentrations of 10,000, 1,000, 100, 10 pg ATP/mL MWF. The dilution curves are shown in Figure 1. The slopes are not significantly different for log ATP concentration versus log RLU among the three fluid types. Two-factor ANOVA (5 ATP concentrations × 3 fluid types) confirmed that the only statistically significant source of variation was the ATP concentration (95% confidence level).

Field Study

During the 12-week field evaluation, 93 of 104 MWF samples were collected and tested for all of the parameters listed in Table 1. Triazine concentrations were not tested in samples from one of the SS fluid machines. Table 4 presents the overall correlation matrix for the 93 samples for which a complete data set was obtained (including triazine concentration). The only strong correlation was between the log ATP concentration and the log CFU bacteria/mL. However, when the database was split among the fluid types, different relationship patterns appeared. The greatest number of strong correlations was observed in the EO sample set (*Table 5*). ATP still correlated strongly with bacterial culture data but showed significant inverse relationships with the refractive index (MWF concentration), the triazine concentration, the reserve alkalinity, and the pH. In the SS fluid systems (*Table 6*), ATP covaried most strongly with the bacterial culture data, the catalase activity, and the pH. The SS systems were the only ones in which the catalase activity showed a strong covariation with the ATP and

Table 4 | **Correlation Matrix; All Samples on Which All Parameters Were Tested** [a,b]

Parameter	[ATP]	Catalase Activity	Log CFU B/mL	°Brix	[Triazine]	Alkalinity	pH
[ATP]	**1.00**	0.11	**0.85**	(0.08)	**(0.33)**	**(0.27)**	**(0.38)**
Catalase activity		**1.00**	0.15	0.10	(0.05)	0.15	(0.10)
Log CFU B/mL			**1.00**	(0.05)	**(0.40)**	(0.20)	**(0.37)**
°Brix				**1.00**	**0.57**	**0.54**	(0.12)
[Triazine]					**1.00**	**0.41**	0.14
Alkalinity						**1.00**	0.27
pH							**1.00**

Notes: a) Matrix is based on data from 93 samples collected from 11 systems. b) The critical value for the correlation coefficient, |r| = 0.204 at P = 0.05 and 0.268 at P = 0.01; significant correlations (P = 0.05) are highlighted in bold font.

Table 5 | **Correlation Matrix; Emulsifiable Oil Samples** [a,b]

Parameter	[ATP]	Catalase Activity	Log CFU B/mL	°Brix	[Triazine]	Alkalinity	pH
[ATP]	**1.00**	**0.57**	**0.83**	**(0.66)**	**(0.71)**	**(0.82)**	**(0.73)**
Catalase activity		**1.00**	**0.50**	**(0.56)**	(0.43)	**(0.55)**	(0.15)
Log CFU B/mL			**1.00**	**(0.70)**	**(0.64)**	**(0.78)**	**(0.62)**
°Brix				**1.00**	**0.50**	**0.72**	**0.36**
[Triazine]					**1.00**	**0.89**	**0.52**
Alkalinity						**1.00**	**0.55**
pH							**1.00**

Notes: a) Matrix is based on data from 24 samples collected from two systems. b) The critical value for the correlation coefficient, |r| = 0.404 at P = 0.05 and 0.515 at P = 0.01; significant correlations (P = 0.05) are highlighted in bold font.

Table 6 | **Correlation Matrix; Semi-Synthetic Metalworking Fluid Samples** [a,b]

Parameter	[ATP]	Catalase Activity	Log CFU B/mL	°Brix	[Triazine]	Alkalinity	pH
[ATP]	**1.00**	**0.82**	**0.92**	**(0.39)**	(0.03)	(0.17)	**(0.80)**
Catalase activity		**1.00**	**0.78**	(0.19)	(0.14)	(0.01)	**(0.63)**
Log CFU B/mL			**1.00**	(0.28)	**(0.46)**	(0.17)	**(0.75)**
°Brix				**1.00**	0.13	**0.72**	**0.60**
[Triazine]					**1.00**	**0.70**	**0.43**
Alkalinity						**1.00**	**0.58**
pH							**1.00**

Notes: a) Matrix is based on data from 36 samples collected from three systems. b) The critical value for the correlation coefficient, |r| = 0.321 at P = 0.05 and 0.413 at P = 0.01; significant correlations (P = 0.05) are highlighted in bold font.

the CFU bacteria/mL. In contrast to EO the relationships between the biological parameters and the refractive index, the triazine concentration, and the alkalinity were not significant in the SS fluid systems. The biological parameters in the S systems hovered at or below the lower detection limits of the methods used. Consequently, there were no significant correlations between any of the biological parameters and any of the chemical parameters. Consequently, the S correlation matrix is not shown. The only strong correlations in S fluids were among the refractive index, the triazine concentration, and the reserve alkalinity. Interestingly, the correlation between the reserve alkalinity and the triazine concentration weakened, moving from EO to SS to S fluids.

DISCUSSION

Nominally, the microbial population densities in recirculating MWF are in a dynamic steady state. If the system is not perturbed, culture recoveries will tend to fall within one order of magnitude of the mean population density (for example, in a system with an average of 1×10^3 CFU/mL, the densities will generally range from 1×10^2 to 1×10^4 CFU/mL). However, if the system is perturbed in any of a number of ways that stimulate microbial proliferation (Vahle and Passman[12]), the population densities can increase to $>1 \times 10^6$ bacteria/mL in a matter of hours (a population with a mean generation time of 0.5 h will double 10 times in 5 h; Todar[4]). Passman, et al. have discussed this previously apropos to the time it takes (1 to 14 days, depending on the organism's generation time) for a colony to become visible on a culture plate (Passman, et al.[13]). The substantial delay between the sample collection and data availability can reduce the effectiveness of the fluid management effort. Consequently, a test method that can provide reliable data rapidly represents a significant contribution to MWF management.

One available rapid method is the catalase test. This method was introduced by Gannon and Bennett in 1981[10].The test can be completed in approximately 20 min, including handling and the reaction time. However, the protocol does not detect catalase negative bacteria. The limitations of the catalase test have already been noted in the Introduction section of this article. Because ATP is present in all living cells, it is not subject to the limitations of the catalase test. Hamilton and Holm-Hansen[14] demonstrated the utility of ATP as an indicator of bacterial biomass. More recently, Christian, et al.[15] compared different methods for measuring the bacterial growth rate in mixed populations. In this study, the ATP concentration covaried better with biovolume measurements than with other growth parameters. This is consistent with earlier observations that the ATP concentration depends on the population density, the cell size, the cell activity, and the microbial species. Dormant cells will have substantially less ATP/cell than will metabolically active cells.

The current study focused on bacteria. Although CFU fungi/mL were included in the test plan, only one machine containing SS fluid yielded fungal colonies consistently. However, all weekly samples also yielded 7 $\log_{10}$ CFU bacteria/mL. The ATP concentrations in these samples ranged from 2.97 to 5.0 $\log_{10}$ pg ATP/mL. The consistently high bacterial recoveries may have obscured any relationship between the ATP concentration and the fungal colony counts. The author has previously used filtration to discriminate between bacterial and fungal biomass as determined from catalase activity[16]. Future testing will evaluate whether the same strategy is applicable to the ATP test.

Also, this article only addresses the ATP quantification in bulk fluid samples. In a separate article, the authors will present data supporting the use of ATP to determine the total biomass in surface residue samples.

CONCLUSIONS

Historically, chemical interferences rendered the ATP testing inapplicable to complex liquids such as MWF. The results presented in this article demonstrate that ATP can be successfully separated from interfering chemicals. Moreover, ATP data can be used to monitor the total biomass in the MWF system samples.

This protocol can be used in the laboratory or in the field. The training requirement is minimal and the test requires less than 10 min to perform. Based on the excellent repeatability and reproducibility results obtained, and the strong correlation between ATP and bacterial culture data, ATP can be a powerful tool for improving the microbial contamination condition monitoring in MWF systems.

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