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INTERLABORATORY STUDIES OF A THIXOTROPIC GEL CULTURE METHOD FOR FIELD AND LABORATORY TESTING FOR MICROBIOLOGICAL CONTAMINATION IN FUELS

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The fuels industry has seen an increasing focus on use of regular microbiological testing to establish whether microbiological growth is occurring in distribution facilities and end user tanks and thus provide assurances that fuel is free of microbiological contamination. A number of laboratory and field methods are available, reliant on both conventional culturing techniques and non-conventional assessment methods. However, only limited studies have been conducted to establish the reliability of these methods. This presentation will discuss some of the challenges in developing and conducting an Inter-Laboratory Study (ILS) to establish repeatability and reproducibility of microbiological test methods for fuels. It will describe the approach taken and results obtained in an ILS of a thixotropic gel culture method which is widely used in the petroleum industry both in the field and in laboratories. The ILS was undertaken with a view to publishing the technique as a new IP standard method.

KEYWORDS

bacteria; biocontamination; colony forming units (CFU); fuel; fungi; Inter-Laboratory Study; IP613; microbial contamination; microbial growth; microbiology; MicrobMonitor2; thixotropic gel culture; viable aerobic microorganisms.

1. INTRODUCTION

Microbiological growth and contamination in fuel tanks and systems has been documented for many years and it still leads to sporadic but costly fuel quality and operational problems associated with use and distribution of aviation kerosene, marine diesel and gas oil, automotive fuels and other middle distillate fuels used for power generation, home heating and machinery [1, 2]. These problems may be caused by increased particulate levels due to microbial biomass, water entrainment in fuel due to microbially generated surfactants, increased acidity, sulphide contamination due to activity of sulphide generating bacteria. They typically manifest as fuel filter clogging and corrosion of fuel tanks and fuel system components. Some evidence points to an increased incidence of problems on account of the more widespread use of fuel containing readily biodegradable fuel blend components such as Fatty Acid Methyl Esters [3 – 6].

Over the past decade or so, numerous industry guidance documents addressing fuel quality have been published and include procedures to monitor, control and remediate microbial growth and contamination in fuels [7, 8]. Notably the IATA *Guidance Material on Microbiological Contamination in Aircraft Fuel Tanks* [9] first published in 2002, was one of the first guidance documents to formalise procedures for routine sampling and microbiological monitoring of fuel tanks and clearly laid out the procedures to be followed in response to microbiological test results. This guidance has been adopted by all leading aircraft manufacturers as recommended practice for aircraft operators and is incorporated in Aircraft Maintenance Manuals. Subsequently, various guidance documents for fuel suppliers and distributors have also highlighted the importance of microbiological monitoring as a measure for assuring supply of fuel of acceptable quality [10]. Usually the frequency of sampling and testing will depend on a risk assessment of the facility concerned based on prevalence of known risk factors, such as high humidity and water ingress, operational experience and historical microbiological test data.

Whilst the tests conducted may be published standard laboratory methods (e.g. IP 385 and ASTM D-6974) it is often more convenient to use field test kits [11]. A few of these are specifically recommended in various industry guidance documents, including that published by IATA. However, to date very little data is available to establish the accuracy and precision of either the laboratory methods or the field test kits. Consequently, it is difficult for fuel users and fuel facility operators to be assured of the reliability of results of microbiological analysis they obtain and for them to justify potentially costly preventative and remedial measures when contamination is indicated.

Even before testing is conducted, numerous sources of error are introduced in sampling procedures because microbiological contamination does not show homogenous distribution in fuel systems (or samples) and it is typically in a dynamic state; changes occur due to microbial growth or death, changes in dominance of different species and changes in the distribution of contamination depending on whether the fuel system is quiescent or turbulent. This has been previously discussed in more detail by Hill [12, 13].

Additionally, there are numerous inherent difficulties in establishing the accuracy and precision of microbiological methods. These will be discussed further in this paper. The paper will also describe an Inter Laboratory Study (ILS) conducted to establish the repeatability and

reproducibility of a widely used commercially available field (and laboratory) test method due to be published as a standard method, IP 613.

2. CHALLENGES TO DEVELOPING AN ILS TO ASSESS PRECISION OF A MICROBIOLOGICAL METHOD FOR FUELS

The conventional technique for quantitatively assessing microbial contamination involves counting the number of microbial colonies which form when a known volume of sample is added to a nutrient gel based culture media and incubated. The technique provides a result as Total Viable Count (TVC) of Colony Forming Units (CFU) and is the basis of the standard methods used in a wide variety of industries, for example for assessing microbial contamination levels in potable water, pharmaceuticals, food, cosmetics, industrial and consumer products and raw materials, and environmental and clinical samples. Different culture media may be used to assess different types of microorganisms although it is acknowledged that no single culture media can grow all microorganisms which may be present in a sample. In this respect, culture based microbiological tests are indicative of contamination status; whilst they may not detect all of the diverse range of microbial species which may be present in a given sample, they have a good track history of reliably indicating when levels of microbial contamination are unacceptably high and, when conducted at regular time intervals, can establish that microbial growth is occurring in a system. In the fuels industry, laboratory standard methods IP385 and ASTM D-6974 have been widely used for many years, although neither has undergone a robust Inter Laboratory Study (ILS). Other techniques, such as ATP photometry, have been more recently adapted for use in the fuels industry, usually to provide benefits of quicker analysis time (e.g. ASTM D-7463 and ASTM D-7687). Inter Laboratory Studies for some of these methods are currently planned, although initial attempts have highlighted the difficulties in conducting an ILS for microbiological test methods for fuel [14] and recently prompted publication of new guidance (D-7847-12) by ASTM [15].

The accuracy of all methods for the enumeration of microorganisms is generally poor when compared to methods used to assess chemical or physical properties of materials. Microbiological test methods are affected by both determinable and indeterminable factors. For culture methods, differences in counts by a factor of 2 or 3 are generally not considered to be significant. Microbiologists generally consider microbial numbers on a logarithmic scale and, for example, the difference in the contamination status of a fuel sample containing 2000 CFU per Litre and another containing 4,500 CFU per Litre would be largely inconsequential. Fuels chemists, engineers and quality personnel may have far higher expectations regarding the accuracy and reliability of test data than microbiological tests are able to deliver. This can present a challenge for microbiologists working in the fuels industry when trying to convince their non-microbiologically trained colleagues of the usefulness of microbiological test data. Even in industries where microbiological methods are more widely used, it has only been relatively recently that the concept of establishing accuracy and precision for microbiological methods has been seriously addressed [16]. It may be pertinent to consider whether the criteria traditionally applied when establishing the reliability of fuels test methods, such as defined in IP 367/ ISO 4259 *Petroleum products - determination and application of precision data in relation*

to methods of test, are relevant and appropriate for microbiological tests used to test fuel samples.

Microbial contamination in fuel is not a single type of analyte. Three broad types of microorganisms affect fuel; bacteria yeasts and moulds. The latter two may collectively be termed fungi. However, within these types there are hundreds, if not thousands, of species and sub-species each with a range of degradative capabilities, physical characteristics and different impact on fuel quality and fuel system operations. Different test methods may be selective to different species, types or forms of microorganism and some types or forms of microbes may not be detected by some methods. For example, culture methods only detect the microorganisms capable of growth on the nutrients present in the culture medium provided in the test. ATP methods will detect the presence of fungal activity but will not detect the presence of fungal spores, which are a dormant form of micro-organism which readily disperse in fuel. Although spores are so small that they will have little overall impact on fuel quality or filterability, the ability of a test to detect spores may be important if samples of fuel are drawn from a point slightly distant from a localised site of microbial growth in a system; in such samples spores may be the only indicator that microbial growth is occurring.

When planning an ILS it is apparent that there is no single microbiological "standard" for contamination against which test methods can be evaluated. It is also very difficult to prepare samples with known levels of contamination for evaluation. In their active state, micro-organisms grow in, and have an affinity for, free water phase and it is not possible to spike fuel phase samples with known levels of evenly dispersed and stable microbial contamination. In this respect, when evaluating test methods, it is probably more valid to use contaminated field samples or samples from laboratory microcosms simulating contaminated fuel tanks. However, this presents a problem in that the exact contamination status of ILS trial samples will not be known in advance of the study.

Microorganisms are particulate, which may be of different sizes and of different degrees of aggregation, and they are denser than the fuel in which they are suspended. Their distribution will be affected by differences in sample handling and will change with time due to settling or agitation. The distribution of microorganisms in fuel will not be homogeneous. It is therefore critical when conducting an ILS to use consistent techniques for sample pre-treatment, particularly with regards to the shaking of samples and the time allowed between shaking and drawing a test aliquot for analysis. The depth at which the test aliquot is drawn should also be consistent.

Microorganisms in fuel samples may reproduce, may die or may remain unchanged with time. These changes will occur during transport between different operators or different laboratories. It is therefore not possible to determine the reproducibility of a microbiological test method between different laboratories. A compromise solution is to have different operators evaluate the test method simultaneously at the same test location.

The challenges for conducting an ILS of a microbiological test method for fuel samples are thus considerable, notably because these methods are trying to assess the presence of a dynamic, hydrophilic, particulate contaminant in a non-aqueous, hydrophobic sample. Even in industries

where microbial contamination in aqueous samples is of concern and the obstacles to conducting an ILS might be considered less challenging, it is only relatively recently that accuracy and precision studies have been a point of focus [16]. There is still much debate about how such studies can be reliably conducted. Standard microbiological methods widely used to assess microbiological quality of drinking water, environmental water and pharmaceuticals, rarely have precision data published in the context understood by most petroleum chemists. ISO/TR 13843 Technical Report *Water quality — Guidance on validation of microbiological methods* [17] describes two approaches for determining precision of microbiological colony count methods for water quality: Type A and Type B.

The Type A approach has some parallels to the approach commonly used for conventional fuels test methods for physical and chemical properties. Precision estimates are derived from statistical calculations based on parallel determinations expressed as standard deviation (standard uncertainty) or relative standard deviation on a logarithmic scale. However, such estimates have not yet made their appearance in microbiological standards. The precision of microbiological determinations is not constant even in logarithmic scale, but depends on the number of colonies counted. Consequently, Type A precision estimates are always approximate and tend to be large.

For the Type B approach, precision estimates are based on assumed probability distributions or other information, such as Poisson distribution in perfectly random suspensions. Precision statements based on the Poisson distribution are more common in microbiological test standards but the Poisson model is highly idealized and yields minimal uncertainty estimates. They are wholly inappropriate for non-aqueous samples, such as fuels, where microbes will show highly heterogeneous distribution.

3. INTER LABORATORY STUDY

3.1 Objectives

The Energy Institute, under its Microbiology Committee, instigated a work package to draft a new IP test procedure for field (and simplified laboratory) testing for microbiological contamination in fuels and associated water and to evaluate the precision of the method. The method is based on a commercially available test kit which utilises a nutrient thixotropic gel based culture medium to quantitatively estimate viable microbial contamination in samples of fuel and associated water. The method will be published as IP Standard IP 613 *Determination of the viable aerobic microbial content of fuels and associated water - Thixotropic Gel Culture Method.* The method and the precision data has also been submitted for consideration by ASTM Committee D02.14 with a view to publication as an ASTM Standard. The test is designed as a field test for the detection and enumeration of contaminating microbes in fuels, lubricants and in associated water.

Note; in the presentation of this study at the IASH conference in October 2013, the provisional method designation was given as IP 612. However, since then, the designation has been changed to IP 613.

3.2 Thixotropic Gel Culture Method

The patented test technology has been widely used in the petroleum industry for about 15 years and was first presented at the 6th IASH conference in Vancouver in 1997 [18]. Full details of the method including the procedure for sample preparation and the test itself, will be available in the published IP 613 test standard but, in brief, the test procedure is as follows;

- Shake the sample for 30 s then allow to stand for 12 minutes to allow any suspended free water to settle.
- Draw an aliquot of fuel for test from 3 cm below the surface using a sterile syringe.
- Add 0.5 ml (aviation fuel) or 0.25 ml (other fuels) to the test bottle containing the thixotropic gel culture medium. The volume of sample tested can be adjusted in some cases to suit the detection level requirements of the user.
- If sample contains free water, 0.01 ml or 0.1 ml aliquots of water can be tested separately if required.
- Shake the gel in bottle vigorously for 30 seconds.
- The thixotropic gel becomes liquid and fuel and any microorganisms in it are dispersed throughout the gel.
- Tap the gel into a flat layer and then incubate the test at 25°C ± 3°C for up to 4 days. This is most easily achieved using simple small incubators but, if they are not available and a consistent temperature cannot be achieved, there is provision in the method to enable incubation in a warm location (e.g. warm office drawer).
- The thixotropic gel will set into a firm flat layer and with incubation, any viable microbes in the sample will grow utilising both the nutrients in the gel and the dispersed fuel sample and form visible colonies. A sensitive growth indicating dye stains active microbial growth a dark red / purple colour. Each single microbial particle produces a visible dark red / purple "colony".
- The number of colonies is then counted and a calculation performed to obtain the number of Colony Forming Units (CFU) per litre of fuel sample (or per ml of water sample).
- e.g. For an aviation fuel sample (0.5 ml tested) showing 13 colonies (e.g. see Figure 1), the CFU per litre

  = (Colony count / Vol. Tested (ml)) x 1000

  = (13 / 0.5) x 1000

  = 26,000 CFU/Litre

- If the colonies are too numerous to count (e.g. Figure 3) the test can be compared to a colour chart provided and the number of colonies and number of CFU per litre of fuel (or per ml of water) can be estimated. This estimation procedure is only utilised when very

heavy contamination is present in fuel samples or for water phase samples and it is outside the range of contamination which could be statistically evaluated in the ILS.

3.3 Ruggedness trial

As microbial contaminant levels are particularly sensitive to time and temperature it was recognised that the ILS for the test would need to be conducted simultaneously at a single site (Intertek Farnborough, UK). An initial ruggedness trial took place in March 2012 as a pilot exercise to optimise/standardise sample selection, preparation and sub-sampling and to ensure that the test method and instructions to participants were unambiguous and technically sound for the ILS.

In the ruggedness trial three participants tested 10 "field" samples at a single laboratory. The samples were 3 Jet A1, 2 Automotive diesels, 1 Marine Gas Oil & 1 water phase from the Jet A-1 sample plus 100:1 dilutions of the two automotive diesels and one of the Jet A-1 samples prepared using filter sterilised fuel sample.

Results of the ruggedness trial gave sufficient confidence to proceed to a full ILS.

3.4. ILS

The full ILS to evaluate the precision (repeatability, r, and reproducibility, R) of the proposed IP 613 test method was also conducted at one site (Intertek Farnborough, UK). Seven participants from three different organisations performed duplicate tests of 10 samples.

3.4.1 Sample details

Samples were "freshly" taken (within a few days prior to ILS commencement) from the field or from laboratory microcosms. As far as was possible, samples were selected to provide a range of contamination levels. All samples were blind coded. The participants were only informed as to whether the sample was "Jet", "Diesel" or "Water". This information was provided so that the participants could determine the volume of sample required according to the sample type. The sample details are shown in Table 1.

Fuel samples RR1 and RR4 which had been stored over aqueous nutrient solutions until the day of testing were gently swirled and then decanted from the aqueous phase. From that point onwards all fuel samples were treated in the same manner prior to sub-sampling by inverting ten times and then immediately pouring into the 125 ml Nalgene bottles that were used for testing. The water sample was derived by taking 20 ml aliquots by pipette from the water bottoms of the three jet fuel samples (RR4) and diluting with distilled water.

Table 1- ILS Sample Details

Sample Code	Sample identifier/type
RR1	F-76 marine gas oil ("naturally" contaminated sample previously stored over a Parberry and Thistlethwaite medium)
RR2	Marine gas oil (field sample)
RR3	Automotive diesel (composite of 2 field samples)
RR4	Jet A-1 (three samples from laboratory microcosms individually inoculated with yeast, mould and bacteria and stored over ¼ strength Bushnell Haas broth; on day of test each of the 3 microcosms was swirled, fuel phases decanted off and combined)
RR5	Jet A-1 ex airport sample
RR6	Water bottom sample from each of the 3 constituent microcosms of **RR4**, combined and then diluted approximately 10:1 with distilled water
RR7	Automotive diesel (composite of 2 field samples)
RR8	Jet A-1 (field sample)
RR9	Automotive diesel (field sample)
RR10	Automotive diesel (field sample)

3.4.2 ILS Testing protocol

Most participants were non-microbiologists and not all were previously familiar with the test. Participants were given the written method and a brief demonstration of the procedure prior to commencement of the ILS.

To ensure that samples were tested in a random sequence, participants were required to conduct testing in the order provided on their individual test Report Sheets. It is appreciated that the difference in testing order would result in different time delays between sample preparation and test for the nominally replicate samples assigned to each participant. All testing (except the incubation stage) was completed within c. 4 hours. The standardised shaking and settling procedure was used by each participant before drawing the aliquot for test from each individual sample.

All tests were incubated in the same incubator at 25ºC and inspected after four days. The test method recommends examination of the test bottles at least once in the first three days and to mark with a permanent marker on the bottle the position of any colonies that have developed at that stage (see Figure 1). This process makes the final evaluation more convenient as individual colonies can merge and streaking can occur in the gel: making it more difficult to distinguish some colonies (particularly at higher levels of contamination). For the ILS the participants were

not available to examine and mark the colonies after one or two days but did have digital photographs of all of the water sample tests after one day and the fuel tests after two days: the photographs were taken to help participants evaluate the colony counts at the completion of the test incubation (see Figures 2 and 3).

Figure 1 – Example of a test of a marked and counted test bottle (after 4 days)

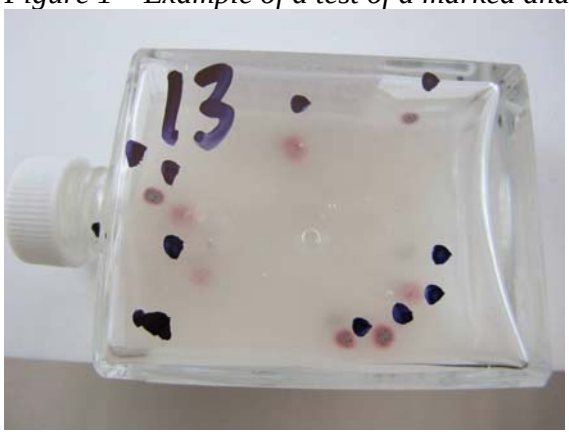


Figure 2 – photograph of a test of fuel sample taken after 2 days

Figure 3 – photograph of a test of water sample taken after 1 day (left) and 2 days (right)

3.4.3 ILS Results

The collated test results for all fuel samples are provided in Table 2. For each participant (Lab A to G) the colony count is provided on the left and the reportable result (CFU per Litre) on the right in each column.

Note, for Jet fuel samples (0.5 ml tested) colony count is multiplied by 2000 to obtain the CFU per Litre. For other fuel types (0.25 ml tested) the colony count is multiplied by 4000 to obtain the CFU per Litre. The letter "e" after the colony count indicates it was estimated by reference to the chart provided with the method rather than an actual count.

The collated test results for the water sample (RR6) are provided in Table 3. For each participant (Lab A to G) the colony count is provided on the left and the reportable result (CFU per ml) on the right in each column.

Note, for water samples (0.01 ml tested) colony count is multiplied by 100 to obtain the CFU per mL. For the water sample tests, all colony counts were estimated by reference to the chart provided with the method rather than actual counts.

Table 2 ILS Results for Fuel Sample Tests

Sample		Lab A		Lab B		Lab C		Lab D		Lab E		Lab F		Lab G	
No.	Type	Col. count	CFU / litre	Col. count	CFU / litre	Col. count	CFU / litre	Col. count	CFU / litre	Col. count	CFU / litre	Col. count	CFU / litre	Col. count	CFU / litre
RR1	F-76	8	32,000	16	64,000	18	72,000	14	56,000	8	32,000	9	36,000	22	88,000
RR1 rpt		7	28,000	13	52,000	11	44,000	2	8,000	18	72,000	18	72,000	10	40,000
RR2	MGO	14	56,000	22	88,000	22	88,000	8	32,000	10	40,000	14	56,000	17	68,000
RR2 rpt		15	60,000	24	96,000	15	60,000	7	28,000	21	84,000	10	40,000	17	68,000
RR3	Automotive diesel	2	8,000	2	8,000	1	4,000	8	32,000	1	4,000	1	4,000	2	8,000
RR3 rpt		1	4,000	1	4,000	5	20,000	1	4,000	0	<4,000	2	8,000	6	24,000
RR4	Jet A-1	0	<2000	4	8,000	4	8,000	2	4,000	3	6,000	3	6,000	2	4,000
RR4 rpt		2	4000	1	2,000	6	12,000	0	<2,000	2	4,000	3	6,000	1	2,000
RR5	Jet A-1	3	6,000	21	42,000	78	156,000	100e	100,000	40	80,000	29	58,000	56	112,000
RR5 rpt		6	12,000	14	28,000	12	24,000	9	18,000	250e	500,000	63	126,000	44	88,000
RR7	Automotive diesel	0	<4000	0	<4,000	0	<4,000	0	<4,000	0	<4,000	0	<4,000	0	<4,000
RR7 rpt		0	<4000	0	<4,000	0	<4,000	0	<4,000	0	<4,000	0	<4,000	0	<4,000
RR8	Jet A-1	47	94,000	38	76,000	46	92,000	74	148,000	39	78,000	32	64,000	74	148,000
RR8 rpt		35	70,000	44	88,000	63	126,000	64	128,000	70	140,000	62	124,000	52	104,000
RR9	Automotive diesel	28	112,000	6	24,000	9	36,000	9	36,000	13	52,000	4	16,000	59	236,000
RR9 rpt		9	36,000	19	76,000	20	80,000	32	128,000	31	124,000	17	68,000	25	100,000
RR10	Automotive diesel	209	836,000	65	260,000	150e	600,000	84	336,000	1000e	4,000,000	74	296,000	207	828,000
RR10 rpt		230	920,000	118	472,000	70	280,000	100e	100,000	150e	600,000	88	352,000	123	492,000

Table 3 ILS Results for Water Sample Tests

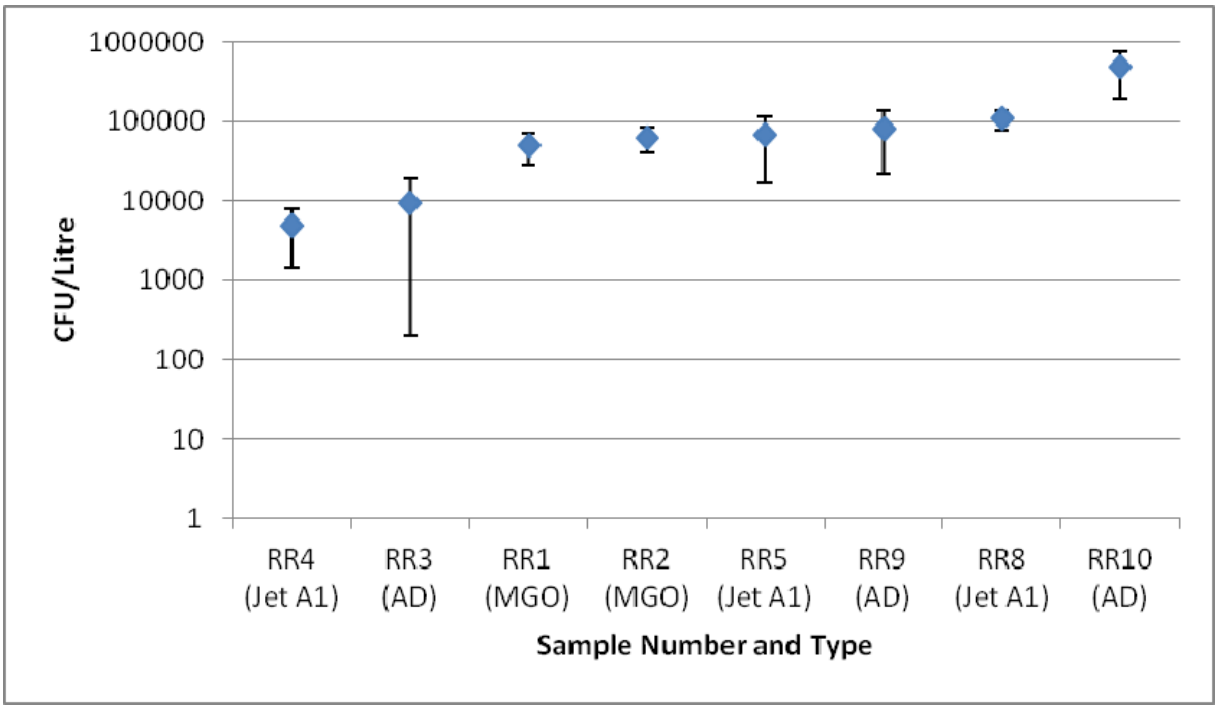
Sample		Lab A		Lab B		Lab C		Lab D		Lab E		Lab F		Lab G	
No.	**Type**	**Col. count**	**CFU / ml**	**Col. count**	**CFU / ml**	**Col. count**	**CFU / ml**	**Col. count**	**CFU / ml**	**Col. Count**	**CFU / ml**	**Col. count**	**CFU / ml**	**Col. count**	**CFU / ml**
RR6	Water from Jet A-1 sample	10,000	1,000,000	≥10,000	≥ 1,000,000	5,000	500,000	≥10,000	≥ 1,000,000	10,000	1,000,000	>10,000	1,000,000	>10,000	1,000,000
RR6 rpt		10,000	1,000,000	≥10,000	≥1,000,000	5,000	500,000	≥10,000	≥1,000,000	10,000	1,000,000	>10,000	1,000,000	>10,000	1,000,000

All colony counts estimated from chart provided

3.4.4 Statistical evaluation of the ILS data

Cursory examination of data from a microbiologist's perspective suggests reasonable correlation and consistency between replicates and participants and as good as we would have expected. This is illustrated by plotting the Standard deviation of fuel sample test results (all operators and all repeats) for each sample as shown in Figure 4. The plot does not include the results for sample RR7 which gave consistently "<4000 (i.e. none detected) for all operators and all repeats.

Figure 4 Standard deviation of all test results (all operators and all repeats) for each sample.



A statistical evaluation of the data was undertaken by the Energy institute statistician in accordance with IP 367/ ISO 4259 *Petroleum products - determination and application of precision data in relation to methods of test*, as implemented in ASTM computer program D2PP. After initial evaluation some data values were revised as they were not acceptable for statistical evaluation. Negative results reported as <2000 or <4000 cfu/litre were treated as "0". The data for sample RR7 was excluded as all results were "0" implying perfect precision. Results based on estimated colony counts were excluded; this included all results for the water sample RR6.

The revised Repeatability and Reproducibility values are given below;

Range of Results	Repeatability	Reproducibility
4700 – 470180	1.423(x+5000)	1.548(x+5000)

Where x is the average of results being compared.

For the revised values the Repeatability is less than the Reproducibility (as would normally be expected) but both values are very high. Note reproducibility, in this case, applies to nominally replicate samples tested by different operators at the same facility.

3.4.5 ILS - Discussion

This ILS was conducted under idealised conditions: participants were in one location conducting the test method simultaneously (effectively) on sub samples that been handled and stored in an identical fashion. And although the precision values derived from this Study are consistent with what might be expected for a microbiological test method they are clearly worse than would be required for petroleum testing methods commonly used for assessing physical and chemical properties of fuels and determining compliance with fuel specifications. Additionally, according to the requirements for IP 367/ ISO 4259, the lower limit of the scope for a test method should be not less than twice the reproducibility – from this Study this is not the case and variability of results within all samples is high, and fails the pooled limit of quantitation test (ASTM D 6259) – which relates to the degree of uncertainty in the results generated by a test.

However, microbiological tests are not intended to be used to determine compliance with fuel specifications. The implementation of specification limits for microbiological contamination in fuels is generally not appropriate and microbial contamination levels cannot be used alone or directly to make inferences about fuel quality or fitness for use [19]. The repeatability and reproducibility values determined by the ILS will not be included in the test method and the maximum levels of detection quoted in the test method that was used for the ILS will be removed to avoid their potential and unintended use in fuel specifications.

The primary purpose of microbiological testing is to assess whether fuel storage and distribution facilities or end user fuel tanks are subject to microbial growth and alert fuel suppliers or users to the potential for fuel quality or operational problems. Testing may be conducted on a routine basis or to investigate incidents. The ILS data indicates that the procedure is appropriate for such applications and can provide valuable information on the microbiological status of the systems sampled.

When interpreting results it must be appreciated that the test result applies only to the sample tested and not necessarily to the bulk fuel at the location where the sample was drawn. To assist in results interpretation, guidance limits are cited in various industry advisory documents including the Energy Institute *Guidelines for the investigation of the microbial content of petroleum fuels and for the implementation of avoidance and remedial strategies*. These guidance limits are usually specific to a type of fuel, facility, sampling location and fuel operations.

The precision values generated by the ILS are consistent with what would be expected for a microbiological test method and demonstrate that this test method is suitable for its intended application, namely to provide an assessment of the level of microbial contamination in fuel and associated water samples. The definition of these categories (be it absent, light, medium or

heavy) being dependant on the nature and circumstances of the sample(s) and on any agreement between customer and supplier.

4. SUMMARY

Microbiological testing is increasingly used by the fuels industry to provide assurances about the microbiological contamination status of fuel tanks and systems. There is consequently a need to better understand the reliability of the test methods employed. Approaches for assessing the precision and accuracy of microbiological test methods have only recently been considered in depth. There are inherent determinable and indeterminable sources of error which will affect microbiological testing and, in general, microbiological methods will be considerably less precise than methods used to assess physical or chemical properties of materials. The microbiological testing of fuel presents particular additional challenges when considering how to evaluate a test method by an Inter-Laboratory Study.

An Energy Institute funded Interlaboratory Study (ILS) was conducted in January 2013 to determine the precision of a thixotropic gel culture test method for the determination of the viable aerobic microbial content of fuels and associated water. 10 test samples were blind-coded and tested in duplicate in random order by 7 participants simultaneously at a single site. A statistical evaluation was performed and the resulting calculated expressions of repeatability and reproducibility were as follows:

Repeatability (r) = 1.423(x+5000)

Reproducibility (R) = 1.548(x + 5000)

Repeatability and reproducibility values derived for this test method provided worse precision than would be required for test methods in general use in the petroleum industry for determining compliance with fuel specifications and do not meet the requirements for IP 367/ ISO 4259 “Petroleum products - determination and application of precision data in relation to methods of test”. It is therefore recommended not to include precision values in the published test method.

However, microbiological tests are not intended to be used to determine compliance with absolute fuel specifications or limits and the implementation of specification limits for microbiological contamination in fuels is generally not appropriate. The precision values obtained in the ILS are consistent with what would reasonably be expected for a microbiological test method and demonstrate that this test method is suitable for its intended application, namely to provide an assessment of the level of microbial contamination in samples.

Consequently, the method will be published in 2014 as IP 613 “*Determination of the viable aerobic microbial content of fuels and associated water - Thixotropic Gel Culture Method*”. Publication as an ASTM Standard is also under current consideration.

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