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Investigation of Susceptibility of Alternative Jet Fuels to Microbiological Growth; ALFA-BIRD Project

FINAL REPORT

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ISSUE/REVISION SHEET

Issue	Description / amendment	Issued /Amended By	Approved By	Issue Date
1.01	Interim report	GCH	GW	05.04.2012
1.02	Final report Inclusion of test data from assessment after 49 days Some minor corrections to fungal data at 14 days (reported incorrectly in Interim Report 1.01)	GCH	GW	30.04.2012
1.03	Corrected batch details for samples in section 2.3 (ref e-mail A Curtain 9/5/2012). Some minor revision to section 3. (Results) to clarify some observations. Addition of new section 4. (Discussion) which includes recommendations for possible further work.	GCH	GW	17.05.2012
1.04	Further discussion on relationship between CFU counts and biomass added	GCH	GW	07.06.2012

1. INTRODUCTION

1.1 Scope

ECHA Microbiology Ltd. were instructed by Airbus Operations Ltd. UK to investigate the susceptibility of a range of alternative jet fuels to microbial growth. The investigation forms part of the ALFA-BIRD *Alternative Fuels and Biofuels for Aircraft Development* Project, a multi-partner, partially EU funded project coordinated by the The European Virtual Institute for Integrated Risk Management (EEIG). The ALFA-BIRD project has short listed four fuels for broad investigation of suitability as alternative aviation fuel;

- CTL (Sasol) FSJF
- GTL (Shell) FT-SPK
- GTL (Shell) + 50% naphthenic cut
- GTL (Shell) + 20% hexanol

CTL = Coal to liquids Fischer-Tropsch
GTL = Gas to liquids Fischer-Tropsch
FT-SPK = Fischer Tropsch Synthetic Paraffinic Kerosene

We understand the CTL (Sasol) FSJF is considered to be the reference fuel for the study, the reasoning being that this fuel will have less source/process dependent variations, will place the study in a long term view and will also enable the project to have coherence and be complementary to other international initiatives (SWAFEA, CAAFI).

We understand that Airbus's deliverables for the ALFA-BIRD project state “the assessment of the impact of microbiological effects will be limited to a visual inspection of the fuel filters, fuel colour, potential erratic/inaccurate fuel level readings”; however, the scope of Airbus testing may vary from this deliverable. Consequently, we made a proposal for laboratory scale investigation of the susceptibility of the fuels to microbial growth (ECHA Ref Q3848 Ver 0.1 of 25 January 2012). Given that so little is known about the relative susceptibility of synthetic fuels to microbial degradation compared to conventional aviation fuels, we proposed that a conventional MEROX treated Jet A-1 was also investigated. Previous studies undertaken by ECHA have indicated MEROX treated Jet A-1 is more susceptible to microbial growth than hydro-treated Jet A-1 and thus it would represent a worst case for comparison.

Microbial growth was assessed in laboratory microcosms which simulate a tank containing fuel and a very small amount of water over a c. 2 month period. This report describes the findings of the laboratory study.

1.2 Technical background to microbial contamination in aviation fuels

Microbial growth in aircraft fuel tanks and aviation fuel distribution can cause problems of filter clogging, malfunction of fuel quantity indicator systems and airframe corrosion and can have a dramatic impact on fuel quality and operating safety. The implications of microbial growth are therefore potentially serious, although operational incidents are relatively rare, primarily on account of rigorous control over ingress and accumulation of water in fuel tanks both by fuel suppliers and aircraft operators. Although fuel degrading micro-organisms can utilise fuels as a nutrient source, the presence of free water is prerequisite for microbial growth. Where there are lapses in fuel housekeeping best practice, water may accumulate and even small quantities of water significantly increase the opportunity for microbial growth. Any changes in chemical composition of fuels will influence their nutrient status and this may influence the rate and extent of microbial growth which occurs. To date very little research has been published on the susceptibility of synthetic and alternative fuels to microbial growth.

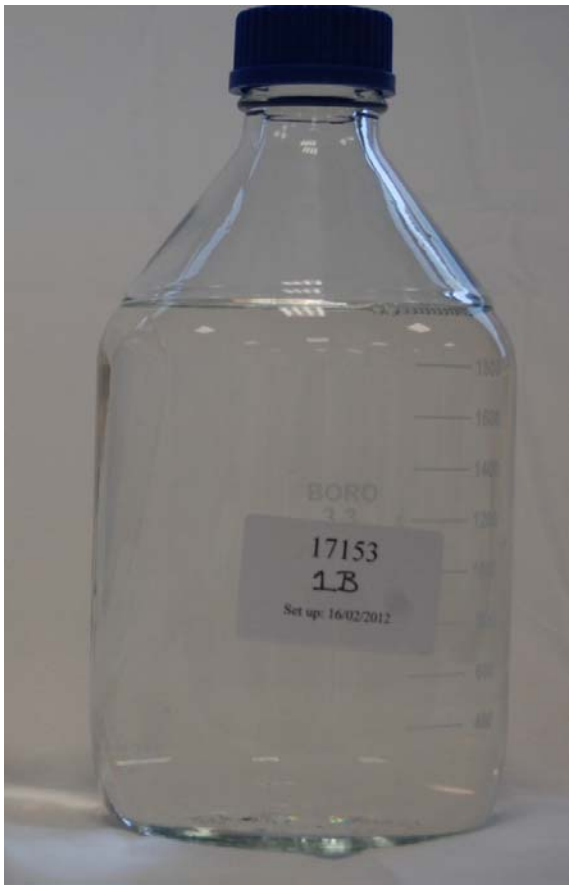
Further information on microbial growth in aviation fuel tanks is available in the IATA *Guidance Material on Microbial Contamination in Aircraft Fuel Tanks*, 4th Ed, International Air Transport Association, Dec 2011.

2 TECHNICAL PROCEDURE

2.1 Overview

The investigation was conducted by setting up glass bottle "microcosms" of 2 litres of each fuel type to which 2 ml of purified water (>10mΩ) containing a defined, mixed inoculum of bacteria, yeasts and moulds was added. (See Figure 1). The rate and extent of subsequent microbial growth was then assessed by visual assessment, examination of any observed particulates using phase contrast light microscopy and by pH measurement, total viable counts and ATP photometry of the aqueous phase after 1 week, 2 weeks, 4 weeks and 7 weeks.

Figure 1. Aviation Kerosene Microcosm (2 litre)



2 litres Fuel

2 ml water containing microorganisms

2.2 Considerations in study design

2.2.1 Fuel : Aqueous Phase Ratio

The ratio of fuel to aqueous phase used in the microcosms can dramatically influence the concentration at which any water soluble components of the fuel accumulate in aqueous phase and thus can have a significant impact on results obtained. Water soluble fuel components might be stimulatory (e.g. nutrients) or antagonistic (e.g. anti-microbial components) to microbial growth. They will migrate to water phase at a concentration determined by their partition coefficient. If the volume of fuel relative to water is not high, the water soluble components will be excessively leached from the fuel and diluted in the water phase; thus water phase concentrations will not reflect the concentrations which might be encountered in real fuel tanks. In real fuel tanks fuel:water ratio will usually be 10,000:1 or more. The investigation utilised a fuel:water ratio of 1000:1 because this is the highest ratio which is practical on a laboratory scale; it provides sufficient water phase for assessment and analysis and is a reasonable simulation of field scenario (N.B. at fuel:water ratios of 1000:1 and greater, the influence of fuel:water ratio on water phase concentration of fuel components migrating to water phase becomes minimal).

2.2.2 Selection of Test Microorganisms

When evaluating susceptibility of materials to microbial growth it is desirable to use as wide a range of micro-organisms as possible to ensure a wide range of microbial degradative capabilities are evaluated. The simplest way to do this is to use an undefined mixture of microorganisms from spoiled and contaminated samples but this approach has a disadvantage in that the conditions of the study are more difficult to control and cannot be repeated. For this reason a defined inoculum of 22 known microbial strains, (9 bacteria, 6 yeasts and 7 moulds) were used. The micro-organisms included strains from reference culture collections, which had either known hydrocarbon degrading capability and/or were known to have been isolated from fuel systems, and also microorganisms isolated in our own laboratory from a variety of contaminated fuel samples.

A full list of the micro-organisms used with details of their source and, where known, ability to degrade hydrocarbons, is provided in Tables A.1 to A.3 in Annex A.

2.2.3 Nutrient Availability

In real fuel tanks in the fuel supply chain and in aircraft, nutrients for microbial growth may be provided both by the fuel and any contaminating water. Availability of inorganic nutrients in aqueous phase, particularly nitrogen and phosphorus compounds, can significantly influence the extent of microbial growth. However, in this study, to enable comparison solely on the basis of the fuel's nutritive status it was decided that no additional supplemental nutrients would be added to the aqueous phase. Where appropriate, test microorganism suspensions were washed before addition to each microcosm to minimise transfer of nutrients from previous culture (see section 2.4 below). This ensured that only nutrients derived from the fuels under evaluation were available for microbial growth.

(Provision was made to consider supplementing aqueous phase with low levels of inorganic nutrients after 1 month in the event that none of the microcosms showed growth but this was not done.)

2.2.4 Temperature

Whilst in real life, microorganisms growing in aircraft fuel tanks will be exposed to extremes of temperature based on flight / ground time cycles, attempting to replicate this

in the laboratory would introduce considerable complexity to the study. As significant microbial growth only occurs when temperatures are above 0°C the microcosms were held at our laboratory ambient room temperature (nominally 19ºC ± 2ºC).

2.2.5 Oxygen

Our understanding is that expected levels of oxygen in fuel tank headspace on a typical flight profile will be 5 - 12 %. This will provide sufficient oxygen for aerobic microbial growth in any water phase or condensate films in fuel tanks. Thus, in this study, no specific attempts were made to investigate microbial growth under anaerobic conditions although we cannot exclude the possibility that such growth is possible in real fuel tanks. In our experience anaerobic growth is only rarely detected in aircraft fuel tanks (when fuel turnover is low, for example aircraft in storage) but it is occasionally found in storage tanks in the fuel supply chain.

2.3 Fuel samples

4 x 1 litre samples of each of the ALFA-BIRD alternative fuels were supplied by Airbus as follows;

- CTL (Sasol) FSJF (Batch Ref; 8040)
  (samples labelled SYNTHETIC JET FUEL, AVIATION FUEL)
- GTL (Shell) FT-SPK (Batch Ref; 8069)
  (samples labelled SHELL MDS (M), KEROSINE, AVIATION FUEL*)
- GTL (Shell) and 50% naphthenic cut (Batch Ref; 8271)
  (samples labelled SHELL MDS (M), KEROSINE, AVIATION FUEL*)
- GTL (Shell) + 20% hexanol (Batch Ref; 8270)
  (samples labelled 1- HEXANOL, AVIATION FUEL*)

* Shell samples (Batch references 8069, 8271 and 8270), as received, were marked with reference numbers 8426, 8293 and 8292, respectively; the batch reference numbers quoted above are those which we understand to be correct (ref; e-mail correspondence A. Curtin, Airbus 9/5/2012).

MEROX Treated Aviation Turbine Kerosene (Jet A-1) was obtained by ECHA from Shell Global Solutions (Shell sample reference S115649).

Fuel samples were not sterilised prior to use because it was considered heat sterilisation or sterilisation by filtration might influence their stability and/or nutritive status. MicrobMonitor2® tests were conducted on the fuels prior to commencing the study and these indicated no detectable microbial contamination present (<2000 cfu/litre).

2.4 Test microorganism (inoculum) preparation

Test microorganisms were either obtained as freeze dried cultures from standard culture collections and/or had been maintained in our laboratory culture collection on frozen beads (Microbank™, Pro-Lab Diagnostics).

To ensure that the isolates were adapted to growth conditions close to field conditions, each isolate was maintained as a pure culture by incubating at 25°C ±2°C in a preparation of 10 ml of ¼ strength Bushnell-Haas Mineral Salts Solution (BHMSS) over-layered with 10 ml of filter sterilised conventional Jet A-1.

Where appropriate, the identification of isolates was confirmed using API 20NE and ID32C kits (bioMérieux) for bacteria and yeasts respectively, and by microscope examination of conidiophores and conidia characteristics of moulds.

On the commencement of the study, each culture in Jet A-1 and BHMSS was vigorously shaken and then a small aliquot of aqueous phase of each added to purified water to provide 100 ml of a mixed bacteria, yeast and mould inoculated aqueous phase. The volume of aqueous phase from each culture added was such that it would give an estimated concentration of 10^3 to 10^4 Colony Forming Units (CFU) per ml in the purified water (0.01 ml for bacteria, 0.1 ml for yeasts and 1 ml for moulds). Because the volume required to get the desired cell/spore concentration of moulds was relatively large, to avoid transfer of inorganic nutrients from the BHMSS, the cells/spores were first washed by centrifuging and re-suspending in purified water. Washing was not considered necessary for bacteria and yeast cultures as the volume of each culture required was small and inorganic salts would be diluted out.

To confirm the initial contamination levels, the Total Viable Count (TVC) counts of bacteria, yeasts and moulds in the mixed bacteria, yeast and mould inoculated aqueous phase were assessed on Tryptone Soy Agar (for assay of bacteria) incubated at 30°C for 3 days and Malt Extract Agar (for assay of yeast and moulds) incubated at 22°C for 5 days. It transpired that the initial bacteria counts were actually higher than expected but this did not impair the ability to assess the extent of subsequent growth.

2.5 Inoculation and incubation of microcosms

Duplicate 2 litre microcosms of each fuel type were prepared in 2 litre borosilicate glass bottles.

2 ml of the freshly prepared inoculated aqueous phase was added to each fuel microcosm which was then shaken vigorously for 10 seconds. Microcosms were then held in the dark at room temperature (nominally 19ºC ± 2ºC). Microcosms were agitated manually daily (except weekends), sufficiently to disrupt the fuel / water interface (this simulates field scenario and encourages microbial growth as it renews the fuel exposed to aqueous phase interface, which is where most microbial growth occurs) .

2.6 Assessment of Test Microcosms

An assessment of the extent of microbial contamination in each test microcosm was made using the procedures described below after 7, 14, 28 and 49 days.

The sampling procedure for routine assessment of microcosms was standardized. Prior to assessment, each microcosm was swirled gently. After visual assessment (see 2.6.1 below) all visible aqueous phase (and visible material at the interface) was removed with a pipette to a sterile container and whirl-mixed for 10 seconds. 0.5 ml sub samples of aqueous phase were then removed for assessments as described below in sections 2.6.2 to 2.6.5. The 0.5 ml was replaced with an equivalent volume of sterile purified water and the aqueous phase was then returned to the test microcosms and incubation continued as described in 2.5.

2.6.1 Visual assessment

Microcosms were assessed visually for;

- Aqueous phase clarity
- Fuel phase clarity
- Fuel / water interface sharpness & cleanliness
- Presence of particulates (specifically particulate typical of microbial biomass)

2.6.2 Phase Contrast Light Microscopy

If present, particulates at the fuel water interface were examined by phase contrast light microscopy at x100 and x400 magnification to establish whether they consisted of microbial biomass.

2.6.3 pH

pH of aqueous phase was measured using indicator strips. Because microbes produce organic acids when they degrade fuel hydrocarbons, microbial growth can be accompanied by a drop in aqueous phase pH and this can be a useful and simple indicator of microbial activity. Assessing pH also enables a better understanding of how the aqueous environment under the test fuels influences the types of microbes which will grow. Fungi prefer and are more tolerant of acidic conditions. In some cases microbial activity results in aqueous phase becoming too acidic to support the growth of many species of fuel degrading bacteria and consequently fungi will become the only or predominant active microbes.

2.6.4 Total Viable Count (TVC) of Bacteria, Yeast and Mould CFU in Aqueous Phase

The Total Viable Count of bacteria, yeast and mould Colony Forming Units (CFU) per ml in aqueous phase was assessed by standard plate count on Tryptone Soy Agar and Malt Extract Agar incubated at 30°C for 3 days and 22°C for 5 days respectively. This is a conventional microbiological technique which quantifies the number of viable (live) microbial particles able to grow on the defined culture media and can be considered the principle indicator of the extent of microbial growth in each test microcosm; reductions in TVC compared to initial starting counts will indicate the fuel provides an environment inhibitory to microbial growth whilst increases will indicate an environment stimulatory to microbial growth. Rates of increase in count and the total counts achieved provide further indication of the fuels susceptibility.

2.6.5 Adenosine Triphosphate (ATP) in water phase (ASTM D7463)

The water phase in each test microcosm was tested for the amount of Adenosine Triphosphate (ATP) using the HY-LiTE® Jet A-1 test (Merck KGaA) and HY-LiTE® 2 meter (ASTM D7463). This is a non-conventional technique which assesses the amount of ATP, a substance produced by all active biological cells, by bioluminescence and results are expressed as Relative Light Units (RLU). It can provide an alternative indication of microbial activity although there are not always direct correlations between ATP and Total Viable Counts. RLU are not an SI unit but a meter specific reading. Although the test is designed to test fuel samples with or without water, the volume required to do this was too large to enable regular assessments of both fuel and water in this study. Thus, a 0.01 ml aliquot of water phase was assessed directly and results were adjusted to express them as equivalent RLU values for 1 litre of fuel/water, assuming a fuel water ratio of 1000:1.

2.6.6 Filtration and assessment of microbial biomass at the end of the trial

It was originally intended to conduct a gravimetric assessment of the amount of particulate (i.e. microbial biomass) present in each microcosm at the end of the study; this was not done as it was estimated that the amount of microbial biomass which had developed within the timescales available for study completion was insufficient for measurement.

The bottom 10 ml of each microcosm, including fuel and any interfacial particulate and aqueous phase present, was filtered through a 0.8 µm mixed cellulose ester membrane filter. This was then dried and observed and photographed.

3. RESULTS

3.1 Visual Appearance and Microscope Examination of Microcosms

Photographs of the fuel microcosms at 7 days, 28 days and 49 days, including observation of interfacial particulate by phase contrast light microscopy at x400 magnification are provided in Annex B. Also shown are the membrane filters used to filter the bottom 10 ml of each microcosm (including fuel, interfacial particulate and water) 19 days after completion of other assessments (at 68 days).

Some very small amounts of floccose white particulate, which had the typical appearance of fungal mycelium, were observed in all microcosms after 7 days and subesequent assessment times. However, we attribute much of this particulate to carry over of fungal material from the inoculum rather than active fungal growth in the microcsoms. The only microcosms to show significant **visual** evidence of microbial growth were those containing MEROX treated Jet A-1. The interface of these microcosms had a brown, organic particulate, which had the typical appearance of fungal mycelium and bacterial extracellular polysaccharide (EPS). The water in the MEROX treated Jet A-1 microcosms had a brown discolouration.

In the GTL (Shell) + 20% hexanol microcosms, the water phase progressively dissapearred; by 1 month there was no visual evidence of a separate free water phase. This was presumably because the polarity of the hexanol enabled dispersion of the water into the fuel phase. The fuel in the GTL (Shell) + 20% hexanol microcosms was also noted to have a pale yellow-brown tinge whearas other fuels were colourless.

Microscope examination of the fuel:water interface and visual examination of filters used to filter the bottom 10 ml of the microcsoms at the end of the trial enabled further discrimination between the extent of microbial growth in each fuel type. Microscopy confirmed that the largest amount of fungal material, including large clumps of intertwining fungal hyphae and yeast cells, were observed in the MEROX treated Jet A-1 microcosms. Significant amounts of fungal material were also observed in the GTL (Shell) + 50% naphthenic cut microcosms although overall amounts of microbial biomass were much less than in the MEROX treated Jet A-1 microcosms. A few particles of fungal material (mould fragments and yeast cells) were observed in the CTL (Sasol) FSJF and GTL (Shell) FT-SPK microcosms. Only a few small isolated particles of microbial material were observed in the GTL (Shell) + 20% hexanol microcosm; we attribute this to dead remnants of microbial particulate carried over in the inoculum rather than biomass produced by active growth in the microcosms. Examination of filters used to filter the bottom 10 ml of the microcsoms at the end of the trial clearly showed that most particulate (soft brown material with typical appearance of microbial biomass) was present in MEROX treated Jet A-1 microcosms; some particulate, although far less, was observed on the GTL (Shell) + 50% naphthenic filters. Very little particulate was observed on filters used to filter other microcosms.

On the basis of visual examination, microscope examination and examination of filters used to filter the bottom 10 ml of each microcosm, the fuels can be ranked in terms of their susceptibility to microbial growth as follows (least susceptible first);

- GTL (Shell) + 20% hexanol
- CTL (Sasol) FSJF and GTL (Shell) FT-SPK
- GTL (Shell) + 50% naphthenic cut
- MEROX treated Jet A-1

3.2 pH of Aqueous Phase of Microcosms

Because the aqueous phase was purified water without any inorganic salts there was no pH buffering; purified water can be expected to be weakly acidic (due to CO_2 absorption from air). The pH of aqueous phase in all microcosms remained at pH 5.5 for the first 28 days; this pH would be expected to favour fungal growth over bacterial growth but would not be expected to inhibit bacterial growth. However, by 49 days the pH of aqueous phase in the MEROX treated Jet A-1 microcosms had dropped to pH 3.5. We attrubute this to the significant fungal activity in the MEROX treated Jet A-1 microcosms. pH in other microcosms remained at 5.5 throughout the trial.

3.3 Total Viable Counts of Aqueous Phase of Microcosms

The total viable counts of bacteria, yeasts and mould CFU per mL in aqueous phase are shown in Annex C. The Log_{10} of the mean of the values for the duplicate microcosms for each fuel type are plotted below for bacteria in Figure 1 a) and yeast in Figure 1b) and moulds in Figure 1c). Generally there was close agreement between the CFU counts in the duplicate microcosms (within the accuracy and precision expected for the TVC method).

There was an initial increase in mean bacterial count in the CTL (Sasol) FSJF, GTL (Shell) FT-SPK and MEROX treated Jet A-1. Bacterial counts remained at high levels for these three fuel types over the course of the study.

In GTL (Shell) + 50% naphthenic cut, the mean bacterial count remained at similar levels to the starting level until 14 days, indicating no significant bacterial growth or kill; after 28 days the mean bacteria count decreased below the initial level and remained at this level for the duration of the study.

In GTL (Shell) + 20% hexanol, the bacterial counts decreased to below detectable levels by 7 days and remained below detection levels for the duration of the study.

At 7 days the greatest increase in fungal counts (both yeasts and moulds) was observed in the GTL (Shell) FT-SPK microcosms. At 14 and 28 days the fungal counts for this fuel decreased slightly but then increased again at 49 days. GTL (Shell) FT-SPK and GTL (Shell) + 50% naphthenic cut showed the highest numbers of yeasts at the end of the trial, although overall levels of fungal contamination were not as high as in MEROX treated Jet A-1. At the end of the trial fungal counts were higher in both GTL (Shell) FT-SPK and GTL (Shell) + 50% naphthenic cut than they were in in the CTL (Sasol) FSJF, although observations of fuel:water interface by microscopy suggested only GTL (Shell) + 50% naphthenic cut was significantly more contaminated with fungal biomass.

Mould counts increased progressively in MEROX treated Jet A-1 microcosms; by 28 days and again at 49 days, MEROX treated Jet A-1 showed the overall highest level of fungal activity. This was predominantly mould activity, yeast counts remaining relatively low. This correlates with the observation of significant amounts microbial material at the fuel:water interface in MEROX treated Jet A-1 microcosms.

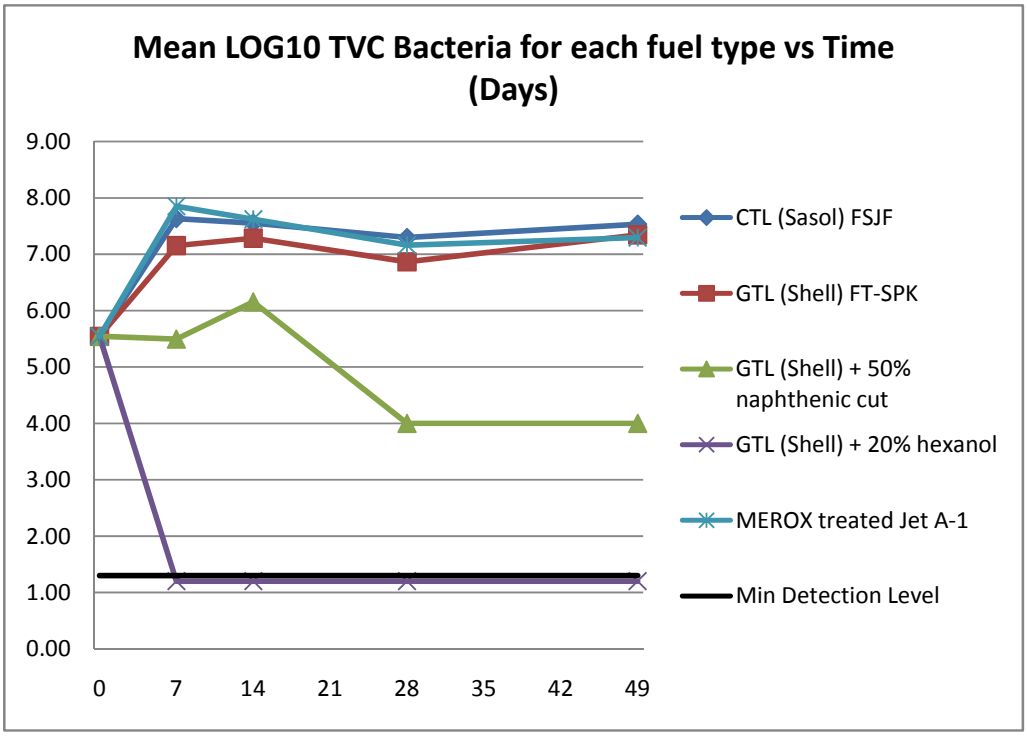
It is noteworthy that whilst GTL (Shell) + 50% naphthenic cut did not support bacterial growth, it did promote relatively high levels of fungal (particularly yeast) growth. As noted in section 3.1, visual examination and observation of membrane filters used to filter the bottom 10 ml of microcosms suggests that this fuel was the most susceptible of the four ALFA-BIRD fuels tested. The implication is that the addition of naphthenic cut increases susceptibility to fungal growth; the inhibition of bacteria may be due directly to anti-bacterial components in the naphthenic cut but alternatively could be an indirect effect due to competive inhibition of bacterial growth by more prolific fungal growth.

In GTL (Shell) + 20% hexanol, yeast and mould counts decreased to below detectable levels by 7 days and remained below detection levels over the first month of the study.

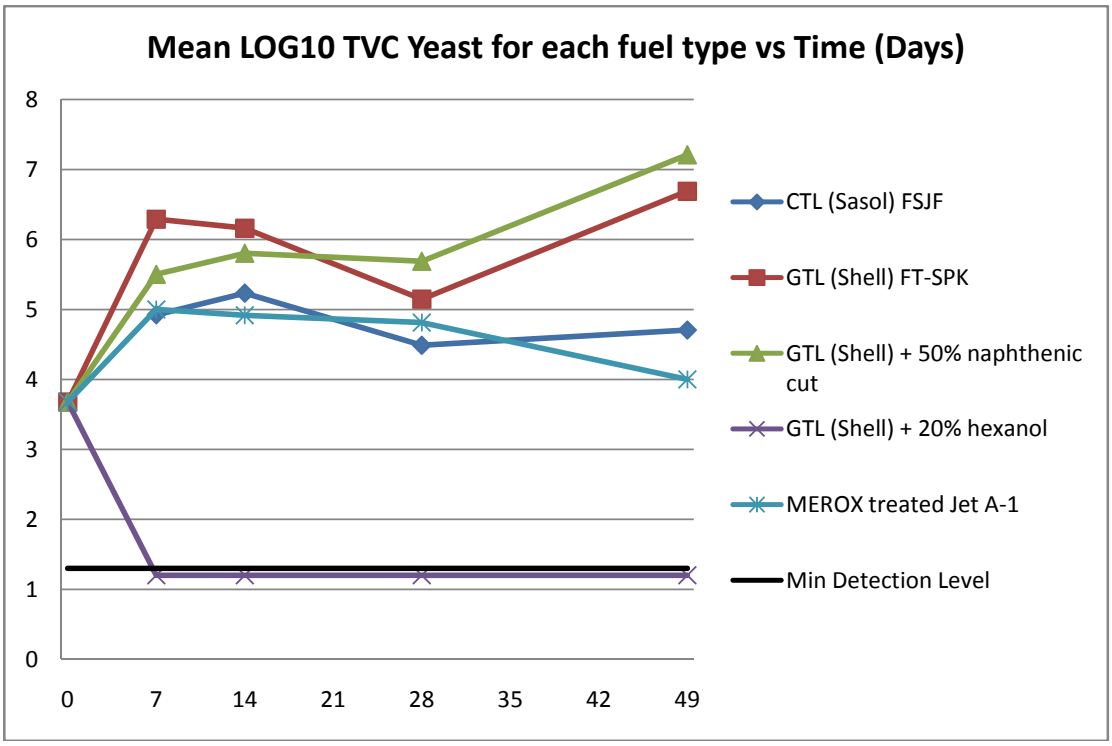
Overall, data provided by Total Viable Counts of aqueous phase correlates with observations made by visual microscope examinations. It confirms that whilst CTL (Sasol) FSJF, GTL (Shell) FT-SPK and GTL (Shell) + 50% naphthenic cut support some microbial growth, more microbial growth was detected in MEROX treated Jet A-1. Conversely, GTL (Shell) + 20% hexanol appears to be anti-microbial; this is not surprising as it is well known that alcohols have anti-microbial properties when present in water phase at concentrations exceeding about 15%. Aside from direct anti-microbial activity of the hexanol, the ability of hexanol to scavenge the free water phase from the fuel microcosms would further be expected to result in inhibition of microbial growth.

Figure 1. Log_{10} of Total Viable Counts of aqueous phase of microcosms plotted against time.

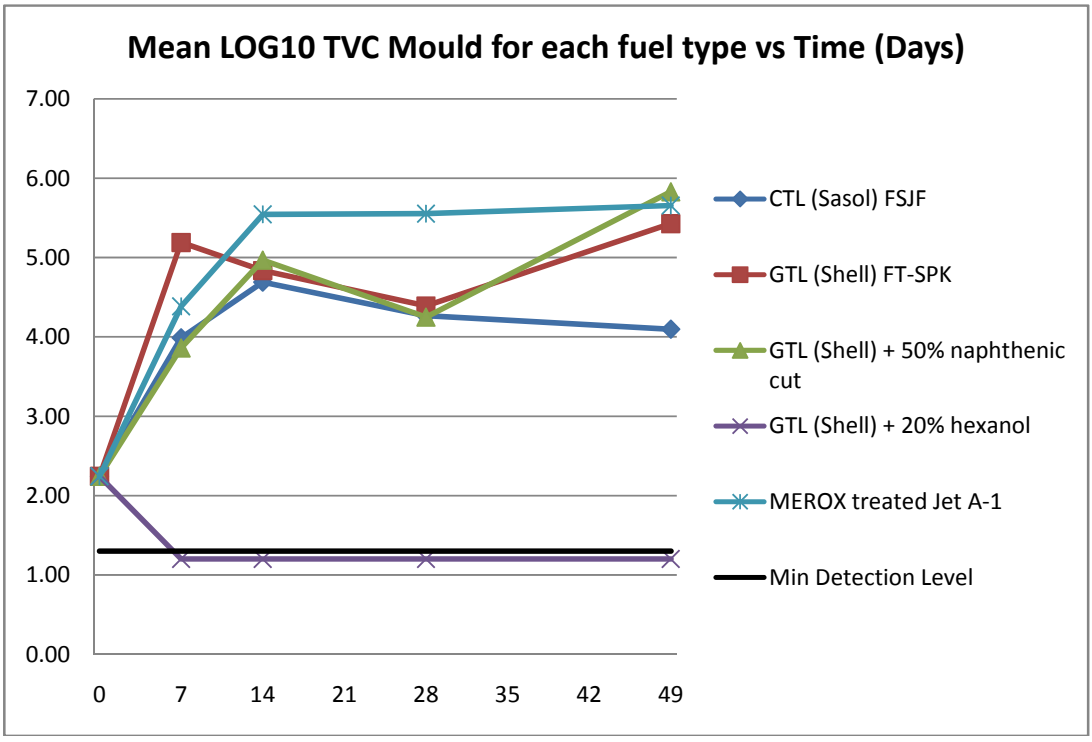
1a) Bacteria



1b) Yeasts



1c) Moulds



3.4 ATP Measurements of Aqueous Phase of Microcosms (ASTM D7463)

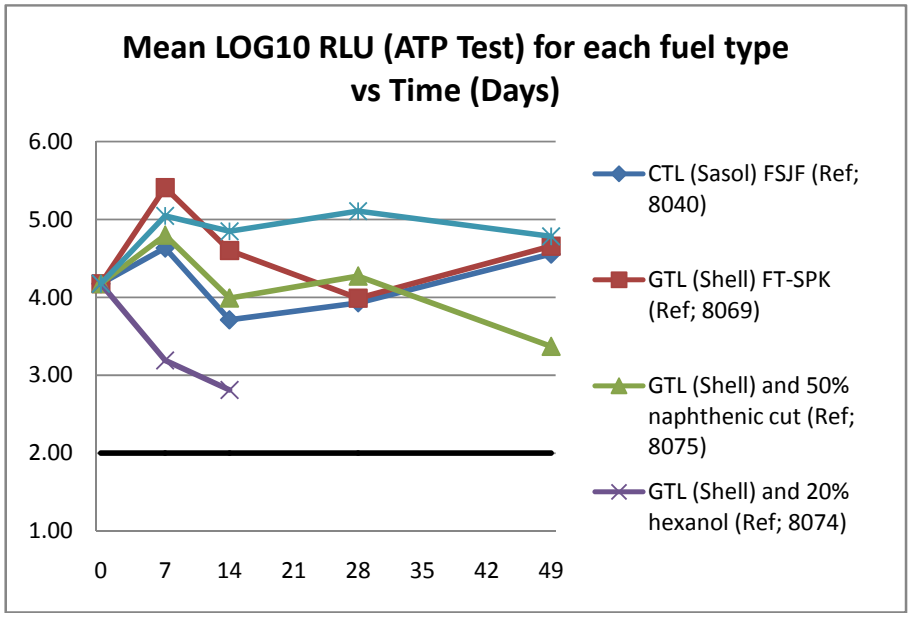
The results of ATP assessment (ASTM D 7463) of the aqueous phase in microcosms are shown in Annex C. Although in fact only a small volume (0.01 ml) of aqueous phase was tested, to enable comparison to results which might be obtained testing a fuel/water sample in the field, the results are converted to equivalent values of RLU per litre of fuel/water sample, assuming a fuel water ratio of 1000:1. The Log_{10} of the RLU values is plotted against time in Figure 2.

ATP results did not always correlate well with observations made by visual and microscope examination nor with data provided by Total Viable Counts. Considerable variability was recorded in RLU readings of duplicate samples from the same microcosm and duplicate microcosms of the same fuel type and consequently repeat tests needed to be conducted on several occasions to clarify inconsistent results.

ATP measurements did suggest a high level of initial activity in the GTL (Shell) FT-SPK microcosms, which corresponds with the high Total Viable Counts of fungi detected at 7 days in the microcosms. However, we cannot explain why three of four ATP readings showed no significant ATP in GTL (Shell) FT-SPK microcsoms at 28 days (see Table in Annex C5.2) whereas all other indicators showed clear evidence of microbial activity. Nevertheless, the trend established by averaging ATP readings, reflects the trend observed for fungal TVC data for GTL (Shell) FT-SPK microcosms; after an intial increase there was a decrease and then subsequent increase at 49 days.

ATP data was consistent with other indicators in showing that, overall, highest levels of microbial activity occurred in the MEROX treated Jet A-1 microcosms and that no microbial activity occurred in the GTL (Shell) + 20% hexanol microcosms; we were unable to take ATP readings of the latter at 28 and 49 days as no free water was available for analysis (and there was insufficient volume of fuel to sacrafice fuel for analysis).

Figure 2. ATP in aqueous phase of microcosms (expressed as Log_{10} equivalent values of RLU per litre of fuel/water sample, assuming fuel:water ratio of 1000:1).



4. DISCUSSION

The study provided a reasonable laboratory simulation of the relative extent of microbial growth which would occur in aircraft fuel tanks containing each of the fuel types under investigation. It gave clear evidence that none of the ALFA-BIRD fuels were as susceptible to microbial growth as conventional MEROX treated Jet A-1. It also demonstrated that GTL (Shell) + 20% hexanol is inherently resistant to microbial growth. Of the ALFA-BIRD fuels the GTL (Shell) + 50% naphthenic cut appeared to be most susceptible to microbial growth, although this is not demonstrated by all the indicators for microbial activity assessed.

Generally, the various parameters for assessing microbial activity (visual assessment, microscopy, TVC's, ATP Photometery and examination of solids on filter papers) were in agreement in respect of the indication of the relative susceptibilities of the different fuels to microbial growth. However, some indicators enabled better distinction of the differences in the susceptibilities of the various fuels. Total Viable Counts (TVC) provide a good estimate of numbers of viable (live) cells (Colony Forming Units or CFU) of the three broad types of microorganisms which can grow and cause quality and operational issues in fuels; bacteria, yeasts and moulds. However, there is not always a direct correlation between the numbers of microbial cells (CFU/ml) and the overall amount of biomass produced and the relationship is variable for different types and species. For example, some bacteria can produce slimes (Extracellular Polysaccharide or EPS) which forms a significantly larger percentage of the amount of overall biomass than the actual bacterial cells. Moulds, produce aggregates of growth as intertwined filaments (hyphae); these aggregates may be several mm across, but moulds also produce spores which are only a few microns across; yet both a spore or an aggregate of growth will give a CFU count of "1". So the correlation between amount of biomass and Total Viable Count (CFU/ml) is not always precise or consistent. Data provided by Total Viable Counts and ATP measurements enables quantitative assessment of microbial activity. Total Viable Counts showed all the fuels except GTL (Shell) + 20% hexanol showed moderate or heavy growth to slightly varying degrees. However, the most convincing evidence on relative susceptibilities of these fuels is provided by direct visual examination, phase contrast microscopy and by visual examination of solids collected on filters; this data provided the best means to discern differences between fuel types which showed growth. The evidence provided by examination of filters is particularly convincing as it clearly shows more microbial biomass developed in MEROX treated Jet A-1 microcosms (see Annex B, picture B.5 d). This assessent is also pertinent because it will directly relate to the propensity of microbial growth to cause engine fuel filter clogging and general fuel system fouling.

We have also considered the possibility that we did not detect some microorganisms using the TVC method. This is unlikely because we used a defined inoculum of microorganisms which we know grow on the culture medium (agar plates) we employed. Occasionally, you can get translucent biomass (e.g. bacterial EPS) which is more difficult to observe but we are very experienced in recognising it by direct visual examination and microscopy.

Some limitations of the study could be addressed in future work. It was originally intended that the extent of microbial growth which developed in each microcosm would be assessed gravimetrically (i.e. fuel, water and interface would be filtered and the dry weight of particulate collected measured). In the event, it was considered that within the limited timescale of the study, insufficient biomass for gravimetric assessment had developed in most microcosms (the MEROX treated Jet A-1 microcosm being a possible exception). Therefore gravimetric assessment was not conducted. Were the study to be repeated, it could be informative to perform gravimetric assesment after a prolonged time period, say 3 or 6 months. Prolonging, the study might also enable better discernment of

any differences in susceptibilty of the the CTL (Sasol) FSJF, the GTL (Shell) FT-SPK and the GTL (Shell) FT-SPK + 50% naphthenic cut.

In order that the inherent susceptibility of the fuel could be assessed, the study only examined microbial growth under conditions where there was no inorganic nutrient supplementation. However, in real fuel tanks, inorganic nutrients are likely to be present and these will have an impact on the susceptibility of the fuel to microbial growth (see section 2.2.3). For example, a fuel might be very susceptible to microbial degradation but only if there is an extraneous source of nitrogen and/or phosphorous available for microbial growth. In a real fuel tank, nitrogen and phosphorus could be provided by dirt or contaminated water entering the tank or from fuel tank sealants, coatings or other materials. In our study this fuel would support very little growth but in an environment where other inorganic nutrients were present, substantial microbial growth could occur. To investigate this we believe that it would be informative to repeat the study, but supplementing the water phase with an inorganic salts solution (e.g. Bushnell-Haas Mineral Salts Solution).

Consideration might also be given to scaling up the study to enable lower water ratios, similar to those encountered in real fuel tanks, to be investigated. The influence of fuel:water ratio is discussed in section 2.2.1. However, we suspect that reducing the water ratio in microcosms would in fact have minimal impact on the partitioning of any nutritive or inhibitory fuel compents to water and, thus, the relative extent of microbial growth for each fuel would possibly remain similar. A larger scale study would, however, provide more fuel, water and biomass material for analysis and enable additional testing to be conducted (e.g. particulate counting, water content analysis, ATP and TVC of fuel phase). Larger volumes of water in a larger microcosm might also be less susceptible to the pH drop caused by fungal activity; this could influence relative proportions of fungal versus bacterial growth.

Another consideration could be to consider temperature cycling of microcosms to reflect temperature fluctuations in aircraft fuel tanks. This would preferentially select for the growth of microorganisms capable of surviving the temperature extremes and would also more realistically reflect the timescales for microbial growth in real tanks. In practice growth only occurs during aircraft ground time when temperature is conducive.

5. SUMMARY

The study has enabled a clear distinction in the relative susceptibility to microbial growth of the ALFA-BIRD fuels, and comparison to conventional MEROX treated Jet A-1. In summary;

- MEROX treated Jet A-1 was by far the most susceptible of the fuels tested and showed significantly more microbial growth than the ALFA-BIRD fuels.

- GTL (Shell) + 50% naphthenic cut showed some inhibition of bacterial growth but increased susceptibility to fungal growth (predominantly yeast) compared to other ALFA-BIRD fuels. This fuel appears to be the most susceptible of the ALFA-BIRD alternative fuels but showed significantly less microbial growth than MEROX treated Jet A-1. The implication is that addition of naphthenic cut increases the susceptibility to fungal growth.

- CTL (Sasol) FSJF and GTL (Shell) FT-SPK supported some microbial growth but to a significantly less degree than MEROX treated Jet A-1. GTL (Shell) FT-SPK showed higher fungal counts than CTL (Sasol) FSJF at the end of the trial, but other indications, notably visual examination and examination of deposits on filters used to filter microcosms, suggest there was no significant difference in the extent of microbial growth in these two fuel types.

- GTL (Shell) + 20% hexanol was resistant to microbial growth; this is expected given the known anti-microbial properties of alcohols and their ability to scavenge free water phase.

ANNEX A List of Test Microorganisms used in the Inoculum for the Study

A.1 Bacteria

Reference.	Genus/species/strain	Source	Comment
ECHA 1027	*Acinetobacter* sp.	Retail site (WW) ULSD tank	Provisionally shown to degrade alkanes and aromatics; sequenced at 16S rRNA
ECHA 1025	*Burkholderia vietnamiensis*	Refinery (WW) ULSD Storage Tank	Shown to degrade phenol. Sequenced at 16S rRNA
ECHA 1026	*Kocuria* sp.	Refinery (WW) Kerosene Storage Tank	Shown to degrade Jet A-1, octane, dodecane. Sequenced at 16S rRNA
ECHA 1036	*Ochrobactrum anthropi*	Terminal (NES) ULSD (B5) Storage Tank	
ATCC 33988	*Pseudomonas aeruginosa*	Culture collection; Fuel storage tank, Ponca City, OK, USA	Reference strain cited in ASTM Standard Test Method E1259 for evaluation of fuel biocides. Also referenced in draft Airbus test methods for evaluating growth on fuel tank surfaces.
ECHA 1021	*Pseudomonas aeruginosa*	Jet fuel sample (Details not known)	ECHA strain used in Jet Fuel studies. Sequenced at 16S rRNA
ECHA 1031	*Pseudomonas oleovorans (putida)* strain Gpo1	UWCC Culture collection	Cited ability to degrade alkanes. Sequenced at 16S rRNA
DSM 6899	*Pseudomonas putida* strain F1	Culture collection	Cited ability to degrade benzene, toluene, ethylbenzene, p-cymene. Sequenced at 16S rRNA
ECHA 1024	*Pseudomonas spp.*	Terminal (EW) Gas Oil Storage Tank	Degrades some aromatics, e.g. naphthalene, toluene, phenol. Sequenced at 16S rRNA.

A.2 Yeasts

Reference.	Genus/species/strain	Source	Comment
ECHA 1040	*Candida famata (possible C. guilliermondii)*	Terminal (WE) Jet Fuel Storage Tank	
ECHA 1029	*Candida* spp.	Terminal (EE) ULSD (B5) Storage Tank	Sequenced at 18S rRNA
ECHA 1039	*Rhodotorula glutinis*	Terminal (WE) Jet Fuel Storage Tank	
ECHA 1030	*Yarrowia lipolytica*	Terminal (EE) ULSD (B5) Storage Tank	Sequenced at 18S rRNA.
ATCC 20177	*Yarrowia lipolytica*	Jet Fuel (kerosene)	Referenced in draft Airbus test methods for evaluating growth on fuel tank surfaces.
ATCC 48138	*Yarrowia tropicalis*	Culture collection; Fuel filter of M51 vehicle, Washington, USA	Reference strain cited in ASTM Standard Test Method E1259 for evaluation of fuel biocides

A.3 Moulds

Reference.	Genus/species/strain	Source	Comment
ATCC 34063 (IMI 178506)	*Acremonium strictum*	Aircraft Jet Fuel	Referenced in draft Airbus test methods for evaluating growth on fuel tank surfaces.
IMI 321985	*Acremonium strictum*	Metal Working Fluid	
ATCC 22711	*Hormoconis resinae*	Culture collection; JP-4	Reference strain cited in ASTM Standard Test Method E1259 for evaluation of fuel biocides
ATTC 20495	*Hormoconis resinae*	Aircraft Fuel Tank	Referenced in draft Airbus test methods for evaluating growth on fuel tank surfaces.
ECHA 1037	*Paecilomyces* spp.	Terminal (WE) Jet Fuel Storage Tank	
ECHA 1008	*Penicillium* spp.	UK Airport (EE) Jet A-1 Fuelling System	
ECHA 1028	*Galactomyces / Geotrichum* spp.*	Terminal (EE) ULSD (B5) Storage Tank	Teleomorph of Geotrichum; ascomycetous yeast. Sequenced at 18S rRNA

ANNEX B: PHOTOGRAPHS OF TEST MICROCOSMS

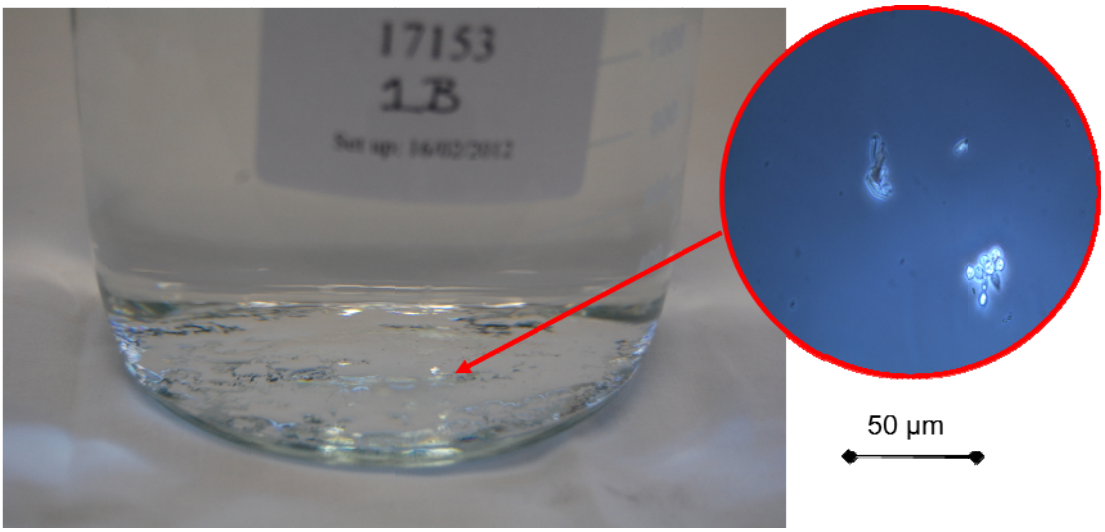
Figures B.1 to B.5 a) to c) are photographs of the fuel microcosms at a) 7 days, b) 28 days and c) 49 days, including observation of interfacial particulate by phase contrast light microscopy at x400 magnification.

Also shown (d) are the membrane filters used to filter the bottom 10 ml of each microcosm (including fuel, interfacial particulate and water) at the end of the trial (68 days).

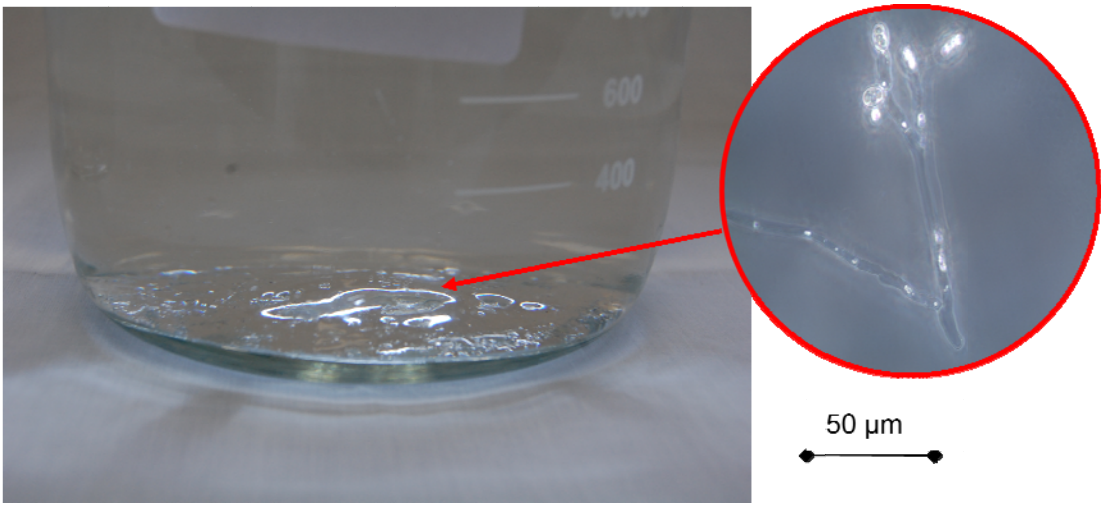
For each fuel type, both replicate microcosms showed very similar appearance so only one replicate for each fuel type is shown.

Figure B.1 Visual appearance of CTL (Sasol) FSJF microcosms.

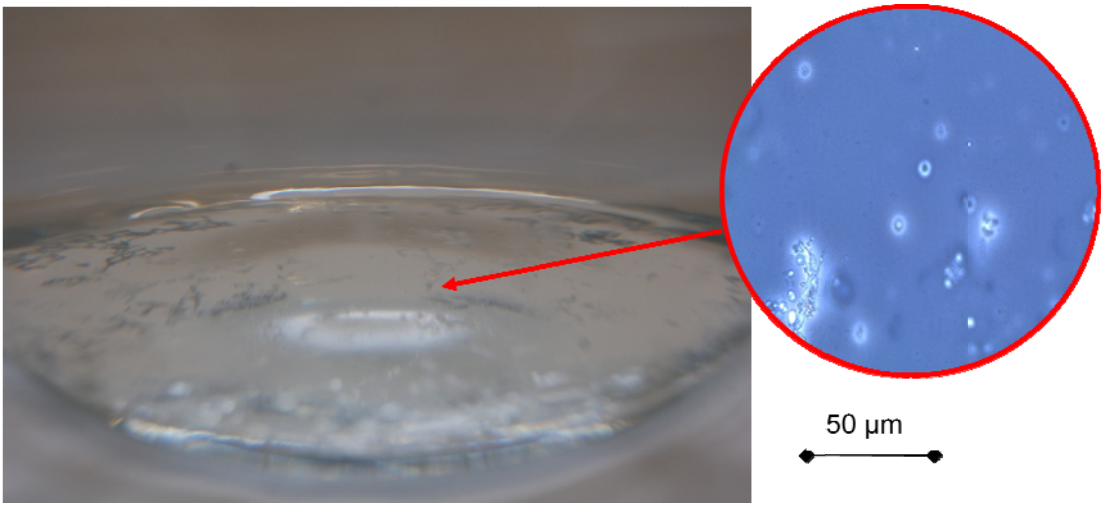
B.1 a) CTL (Sasol) FSJF at 7 days



B.1 b) CTL (Sasol) FSJF at 28 days



B.1 c) CTL (Sasol) FSJF at 49 days



B.1 d) Filter membrane used to filter bottom of CTL (Sasol) FSJF microcosm at end of trial

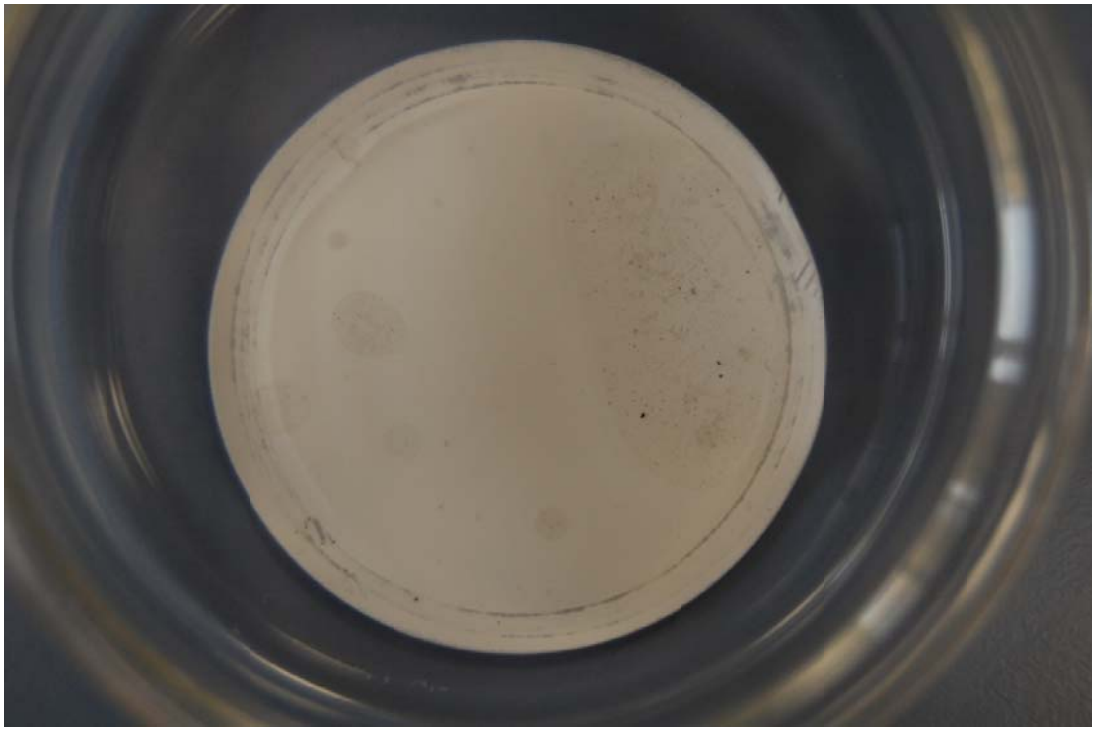
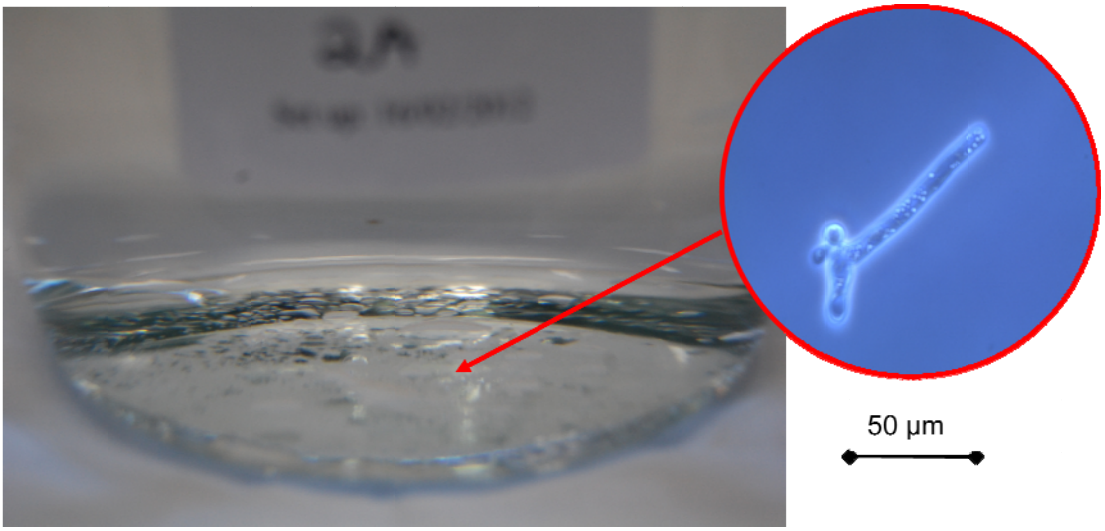
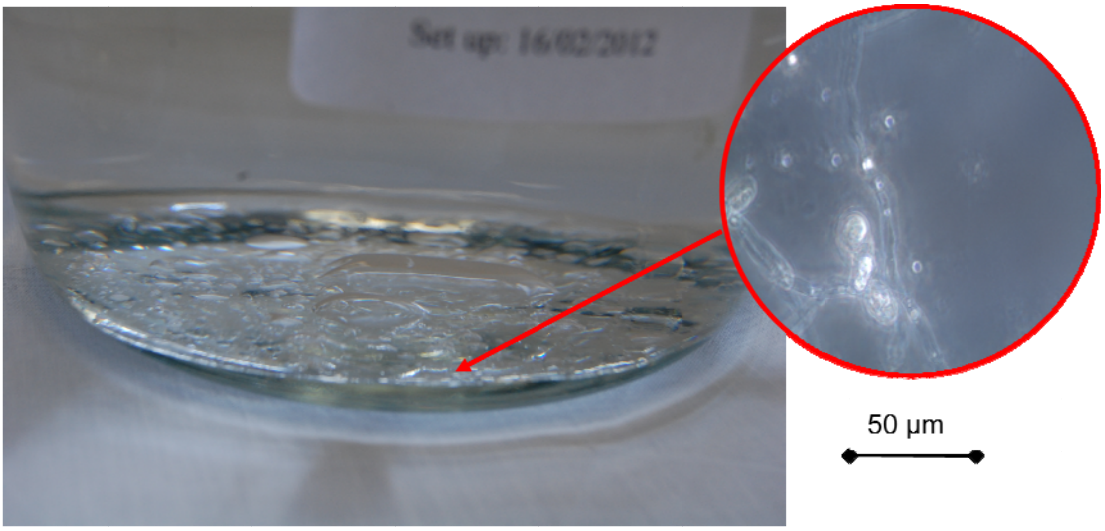


Figure B.2 Visual appearance of GTL (Shell) FT-SPK microcosms.

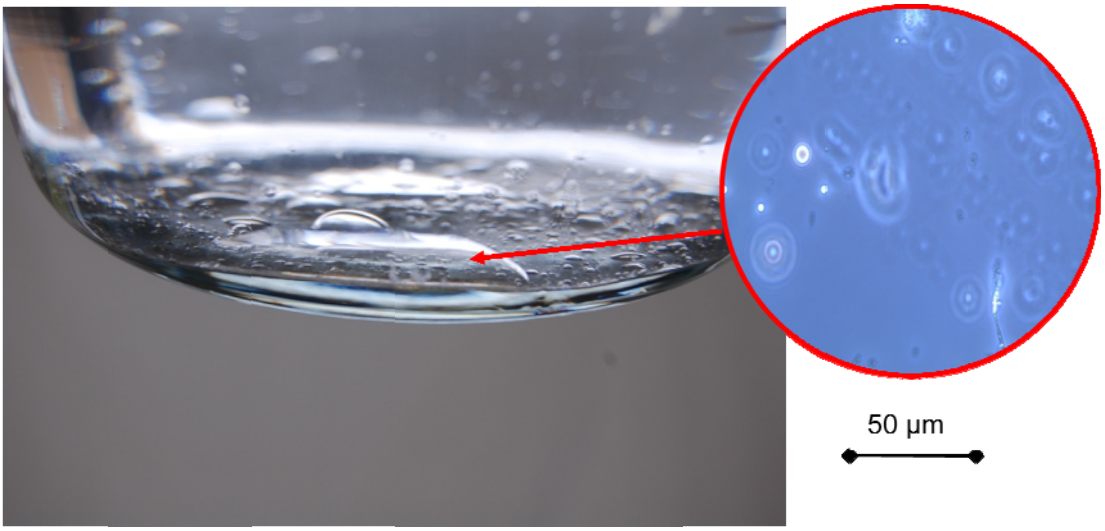
B.2 a) GTL (Shell) FT-SPK at 7 days



B.2 b) GTL (Shell) FT-SPK at 28 days



B.2 c) GTL (Shell) FT-SPK at 49 days



B.2 d) Filter membrane used to filter bottom of GTL (Shell) FT-SPK microcosm at end of trial

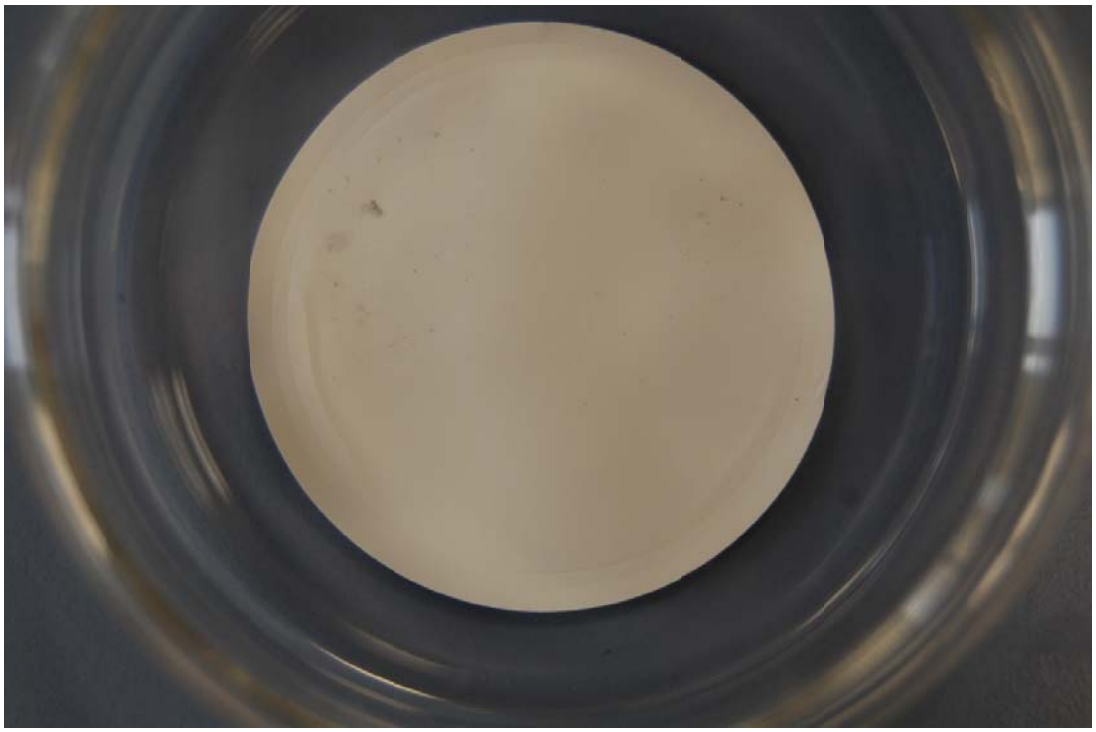
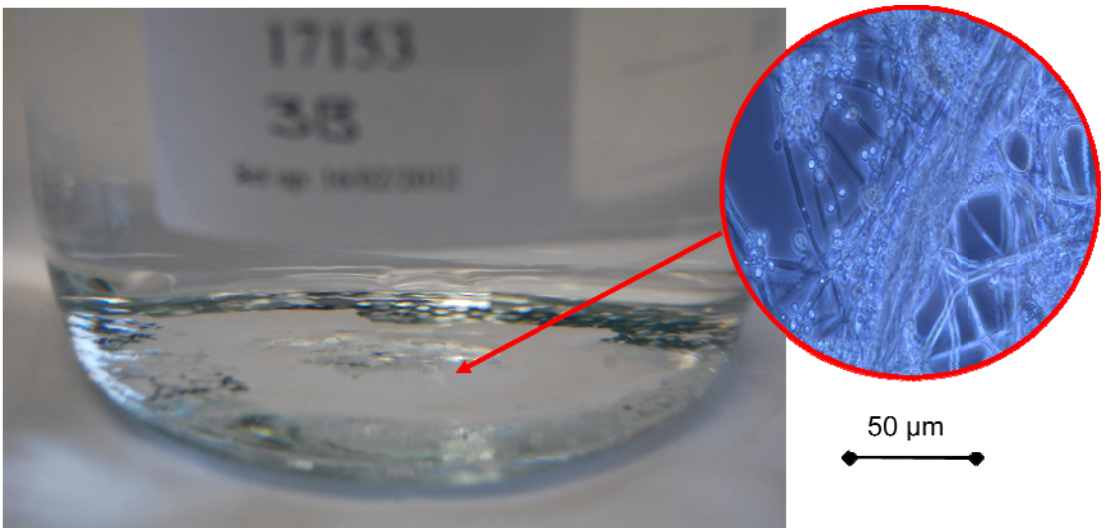
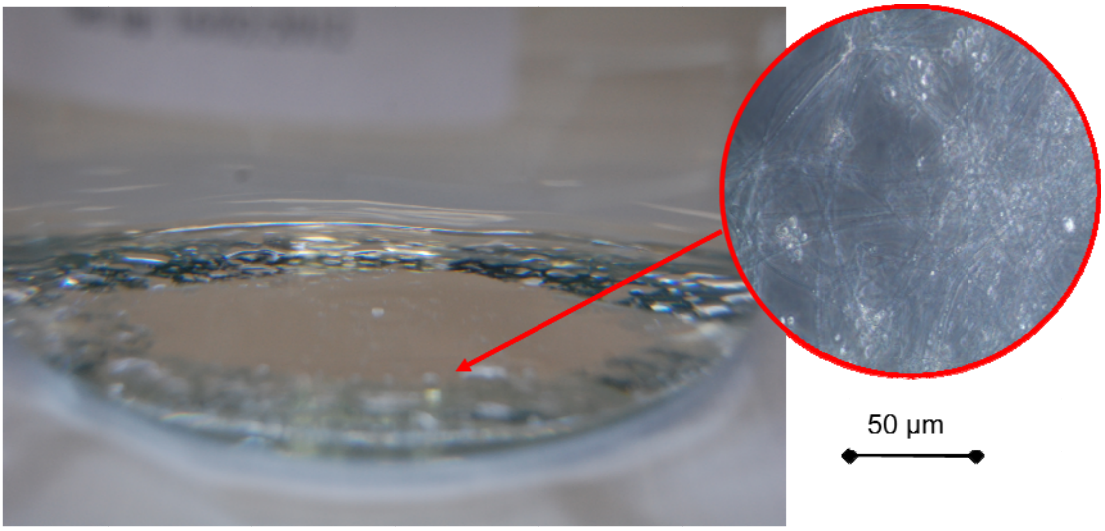


Figure B.3 Visual appearance of GTL (Shell) + 50% naphthenic cut microcosms.

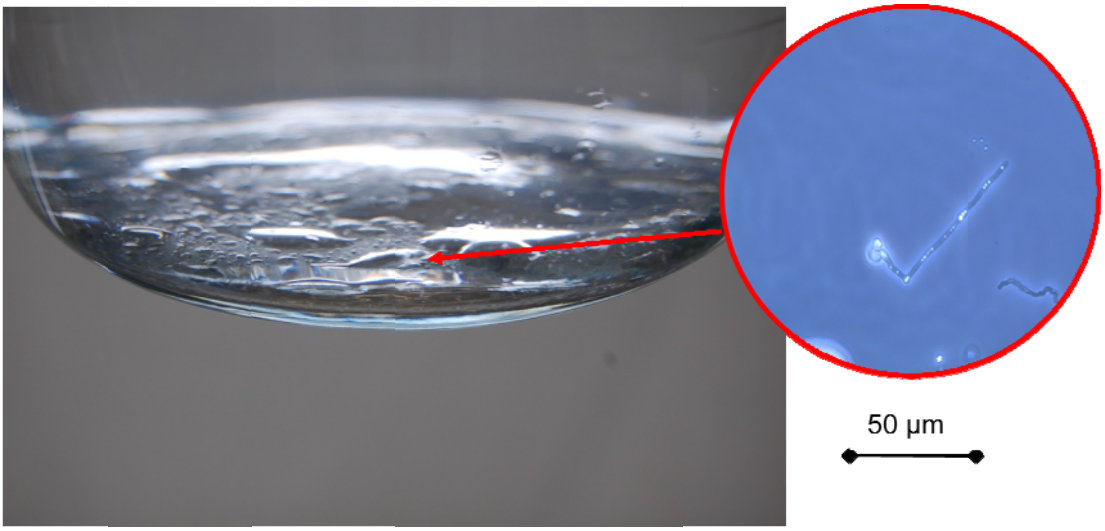
B.3 a) GTL (Shell) + 50% naphthenic cut at 7 days



B.3 b) GTL (Shell) + 50% naphthenic cut at 28 days



B.3 c) GTL (Shell) + 50% naphthenic cut at 49 days



B.3 d) Filter membrane used to filter bottom of GTL (Shell) + 50% naphthenic cut microcosm at end of trial

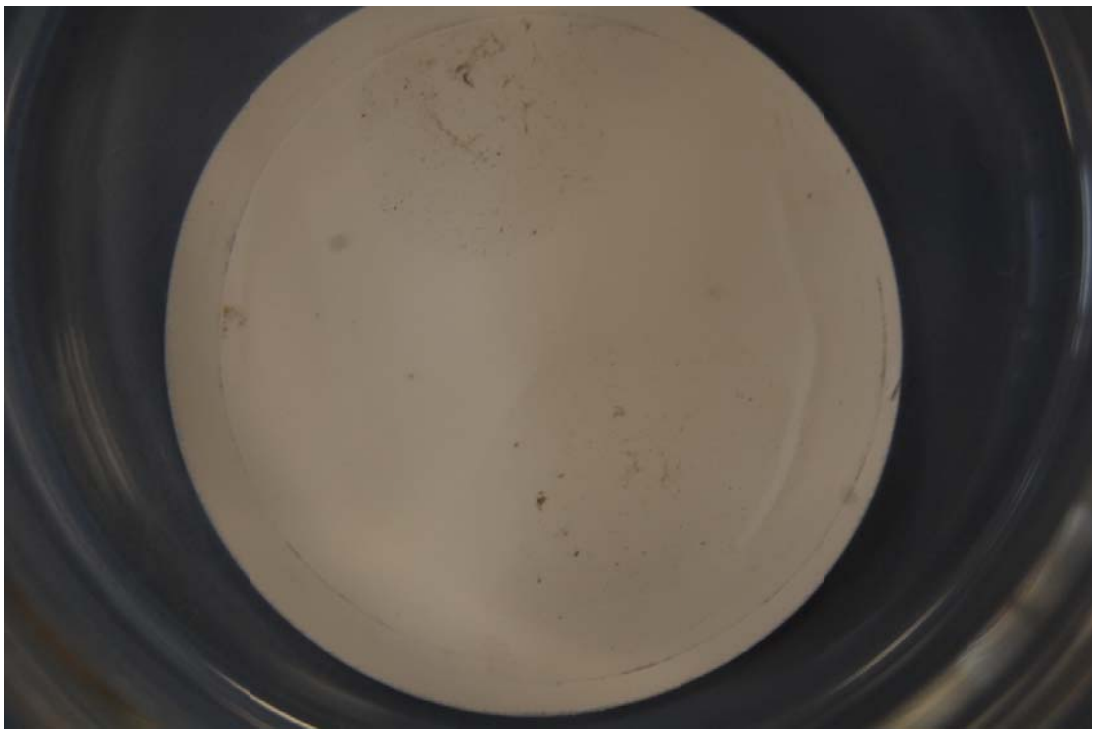
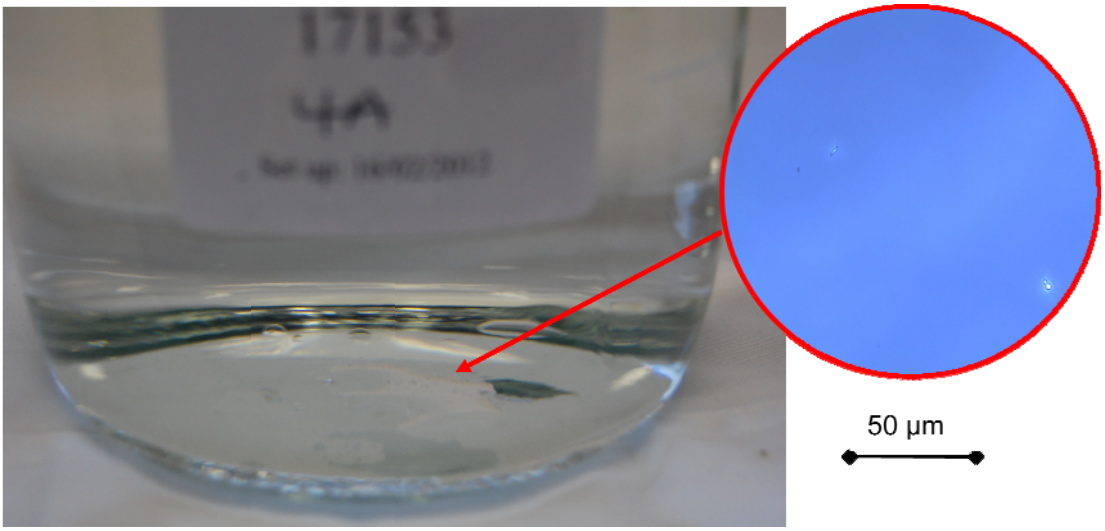
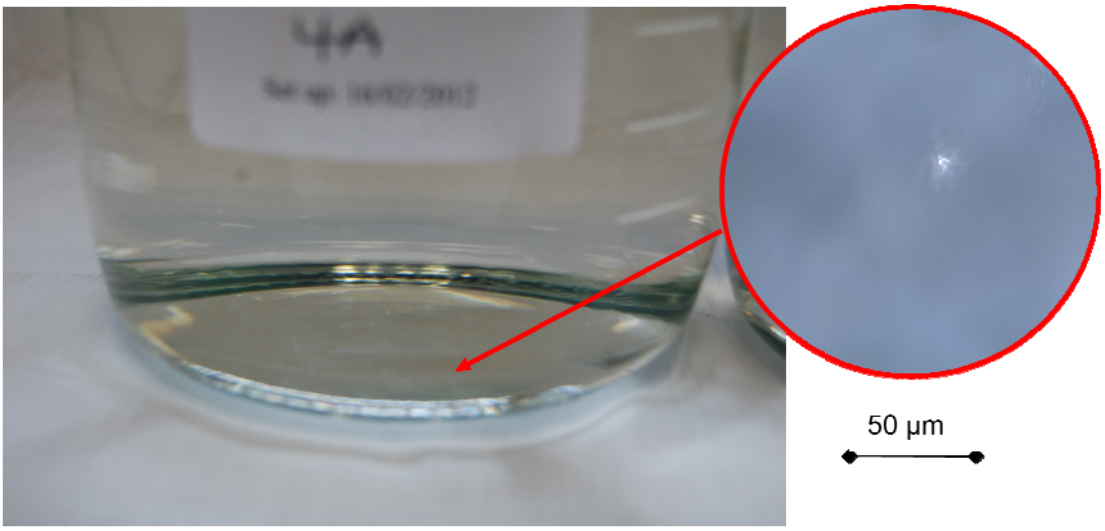


Figure B.4 Visual appearance of GTL (Shell) + 20% hexanol microcosms.

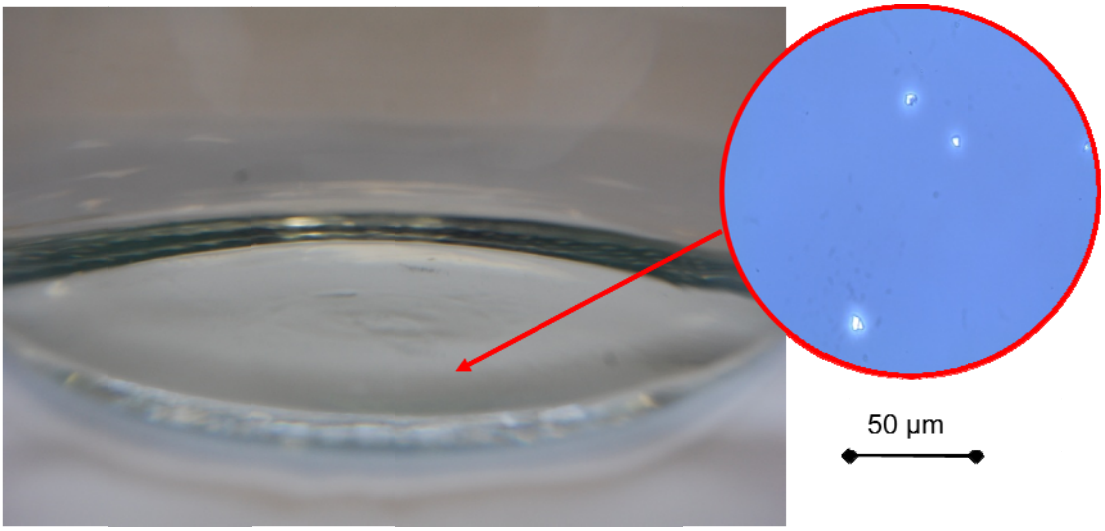
B.4 a) GTL (Shell) + 20% hexanol at 7 days



B.4 b) GTL (Shell) + 20% hexanol at 28 days



B.4 c) GTL (Shell) + 20% hexanol at 49 days



B.4 d) Filter membrane used to filter bottom of GTL (Shell) + 20% hexanol microcosm at end of trial

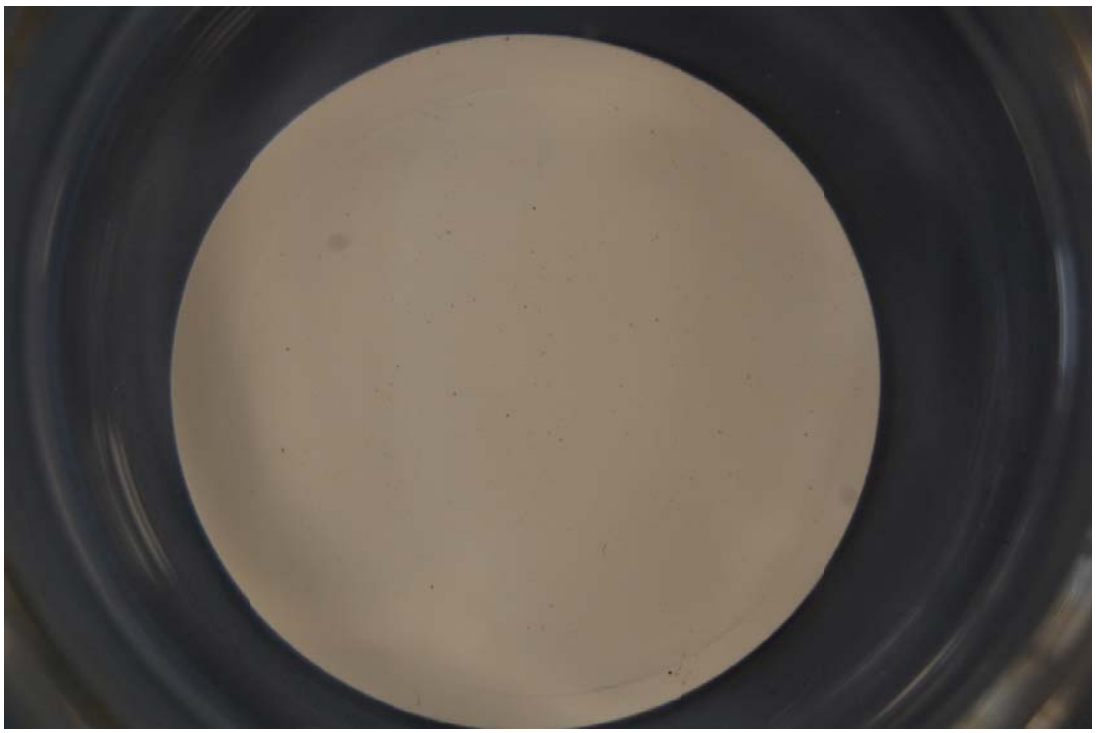
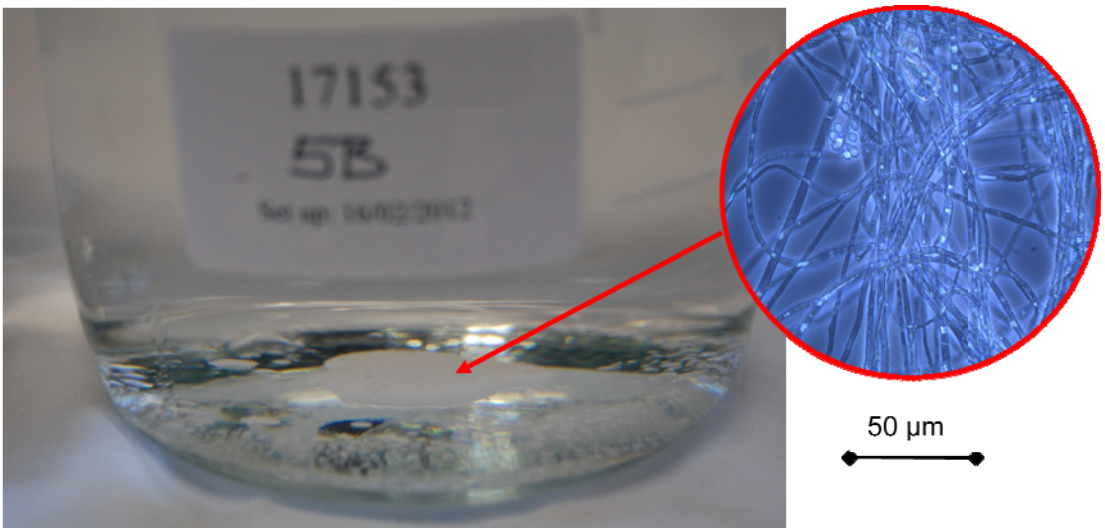
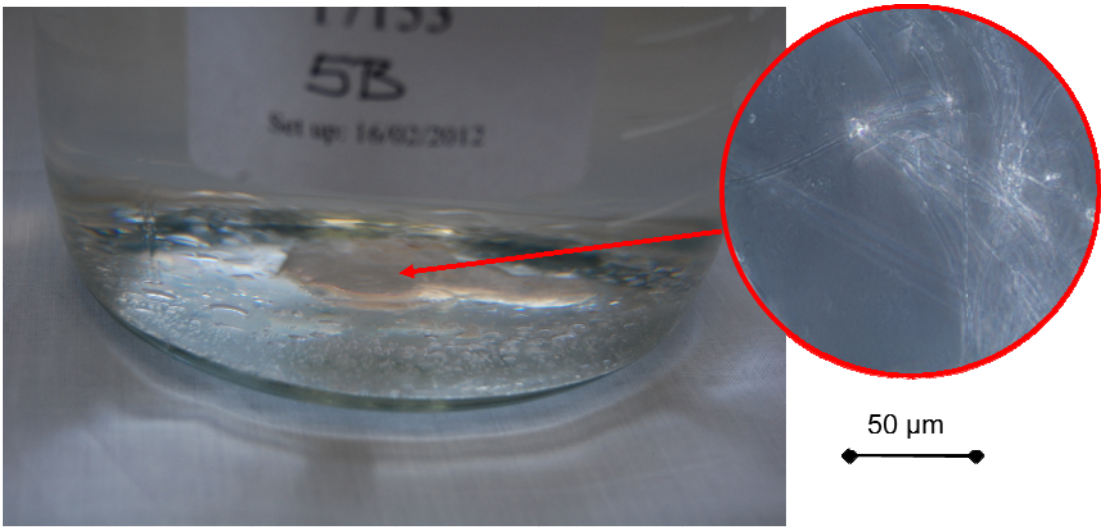


Figure B.5 Visual appearance of MEROX Treated Jet A-1 microcosms.

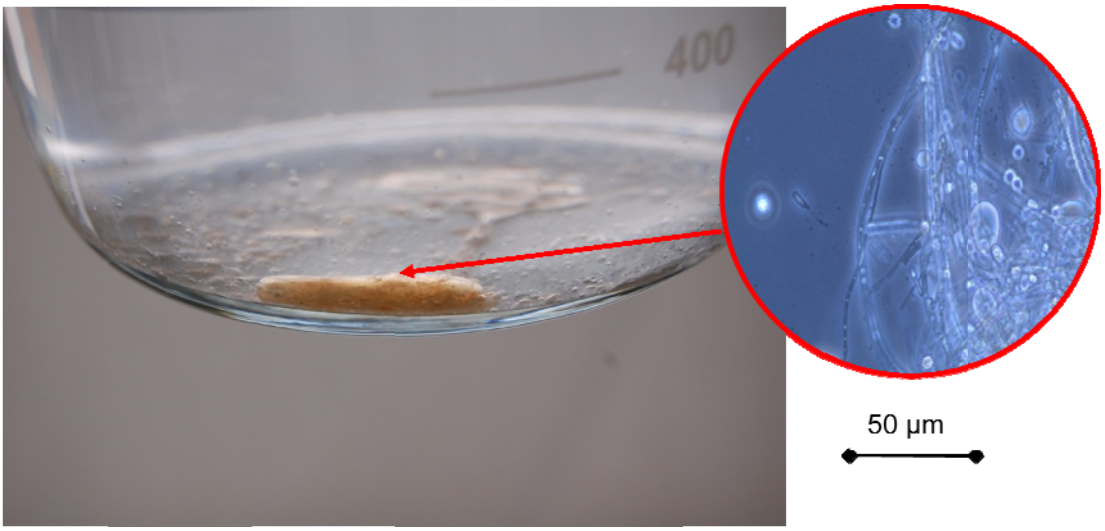
B.5 a) MEROX Treated Jet A-1 at 7 days



B.5 b) MEROX Treated Jet A-1 at 28 days



B.5 c) MEROX Treated Jet A-1 at 49 days



B.5 d) Filter membrane used to filter bottom of MEROX Treated Jet A-1 microcosm at end of trial

ANNEX C: TOTAL VIABLE COUNT AND ATP DATA FOR TEST MICROCOSMS

C.1 Total Viable Count of Bacteria, Yeasts and Moulds in Aqueous Phase used for Inoculum at Day 0 (Colony Forming Units (CFU) / ml)

Fuel	Assay Replicate	Total Viable Count (CFU / ml)
Bacteria	1	3.10×10^5
	2	3.90×10^5
	Mean	***3.50×10^5***
Yeasts	1	5.30×10^3
	2	4.20×10^3
	Mean	***4.75×10^3***
Moulds	1	200
	2	150
	Mean	***175***

N.B. Beacuse all microcosms were inoculated with the same aqueous phase, the Mean counts shown above are used as the starting (Day 0) counts for bacteria, yeasts and moulds in all microcosms

C.2 Total Viable Count of BACTERIA in Aqueous Phase of Microcosms (Colony Forming Units (CFU) / ml)

Fuel	Microcosm Replicate	Assay Replicate	Total Viable Count of Bacteria (CFU / ml)			
			Day 7	Day 14	Day 28	Day 49
CTL (Sasol) FSJF	1A	1	5.80×10^7	3.70×10^7	2.39×10^7	5.20×10^7
		2	4.40×10^7	4.70×10^7	1.85×10^7	4.20×10^7
	1B	1	3.70×10^7	2.70×10^7	1.88×10^7	2.06×10^7
		2	3.20×10^7	3.10×10^7	1.79×10^7	2.17×10^7
	Mean		***4.28×10^7***	***3.55×10^7***	***1.98×10^7***	***3.41×10^7***
GTL (Shell) FT-SPK	2A	1	1.90×10^7	2.10×10^7	6.60×10^6	1.47×10^7
		2	2.40×10^7	1.90×10^7	7.70×10^6	1.64×10^7
	2B	1	8.30×10^6	2.00×10^7	7.70×10^6	2.81×10^7
		2	5.80×10^6	1.70×10^7	7.40×10^6	2.98×10^7
	Mean		***1.43×10^7***	***1.93×10^7***	***7.35×10^6***	***2.21×10^7***
GTL (Shell) + 50% napthenic cut	3A	1	1.80×10^5	8.80×10^5	1.00×10^4 *	1.00×10^4 *
		2	1.53×10^5	9.80×10^5	1.00×10^4 *	1.00×10^4 *
	3B	1	4.30×10^5	1.92×10^6	1.00×10^4 *	1.00×10^4 *
		2	4.90×10^5	1.92×10^6	1.00×10^4 *	1.00×10^4 *
	Mean		***3.13×10^5***	***1.43×10^6***	***1.00×10^4***	***1.00×10^4***
GTL (Shell) + 20% hexanol	4A	1	<20	<20	<20	<20
		2	<20	<20	<20	<20
	4B	1	<20	<20	<20	<20
		2	<20	<20	<20	<20
	Mean		***<20***	***<20***	***<20***	***<20***
MEROX treated Jet A-1	5A	1	5.80×10^7	4.80×10^7	1.63×10^7	2.20×10^7
		2	8.30×10^7	3.70×10^7	1.40×10^7	2.22×10^7
	5B	1	7.20×10^7	3.90×10^7	1.52×10^7	1.59×10^7
		2	7.10×10^7	4.30×10^7	1.28×10^7	1.90×10^7
	Mean		***7.10×10^7***	***4.18×10^7***	***1.46×10^7***	***1.98×10^7***

* Estimated count; colonies could not be counted accurately due to overgrowth by fungi

C.3 Total Viable Count of YEASTS in Aqueous Phase of Microcosms (Colony Forming Units (CFU) / ml)

Fuel	Microcosm Replicate	Assay Replicate	Total Viable Count of Yeasts (CFU / ml)			
			Day 7	Day 14	Day 28	Day 49
CTL (Sasol) FSJF	1A	1	6.50×10^4	2.40×10^5	4.40×10^4	6.20×10^4
		2	7.00×10^4	3.10×10^5	2.80×10^4	5.10×10^4
	1B	1	1.00×10^5	1.00×10^5	2.80×10^4	4.50×10^4
		2	~	3.20×10^4	2.30×10^4	4.50×10^4
		Mean	***8.38×10^4***	***1.71×10^5***	***3.08×10^4***	***5.08×10^4***
GTL (Shell) FT-SPK	2A	1	3.20×10^6	9.70×10^5	1.20×10^5	1.94×10^6
		2	4.40×10^6	9.20×10^5	8.70×10^4	2.05×10^6
	2B	1	1.00×10^5	1.89×10^6	1.96×10^5	7.40×10^6
		2	~	2.01×10^6	1.57×10^5	8.20×10^6
		Mean	***1.95×10^6***	***1.45×10^6***	***1.40×10^5***	***4.90×10^6***
GTL (Shell) + 50% napthenic cut	3A	1	2.50×10^5	5.40×10^5	4.60×10^5	1.78×10^7
		2	3.20×10^5	8.50×10^5	4.00×10^5	1.94×10^7
	3B	1	3.50×10^5	5.90×10^5	5.60×10^5	1.35×10^7
		2	3.50×10^5	5.60×10^5	5.30×10^5	1.41×10^7
		Mean	***3.18×10^5***	***6.35×10^5***	***4.88×10^5***	***1.62×10^7***
GTL (Shell) + 20% hexanol	4A	1	<20	<20	<20	<20
		2	<20	<20	<20	<20
	4B	1	<20	<20	<20	<20
		2	<20	<20	<20	<20
		Mean	***<20***	***<20***	***<20***	***<20***
MEROX treated Jet A-1	5A	1	1.00×10^5 *	5.00×10^4	6.00×10^4	1.00×10^4 *
		2	~	4.00×10^4	3.00×10^4	1.00×10^4 *
	5B	1	1.00×10^5 *	1.70×10^5	5.00×10^5	1.00×10^4 *
		2	~	7.00×10^4	1.20×10^5	1.00×10^4 *
		Mean	***1.00×10^5***	***8.25×10^4***	***6.50×10^4***	***1.00×10^4***

~ Yeasts present but not possible to obtain count due to overgrowth by bacteria / mould

* Estimated count; colonies could not be counted accurately due to overgrowth by bacteria / mould.

C.4 Total Viable Count of MOULDS in Aqueous Phase of Microcosms (Colony Forming Units (CFU) / ml)

Fuel	Microcosm Replicate	Assay Replicate	Total Viable Count of Moulds (CFU / ml)			
			Day 7	Day 14	Day 28	Day 49
CTL (Sasol) FSJF	1A	1	1.00×10^4	8.00×10^4	2.00×10^4	1.90×10^4
		2	1.20×10^4	8.00×10^4	1.80×10^4	1.60×10^4
	1B	1	6.00×10^3	1.70×10^4	1.60×10^4	8.00×10^4
		2	1.00×10^4	1.90×10^4	2.00×10^4	7.00×10^4
	Mean		***9.75×10^3***	***4.90×10^4***	***1.85×10^4***	***1.25×10^4***
GTL (Shell) FT-SPK	2A	1	9.00×10^4	7.00×10^4	3.00×10^4	1.30×10^5
		2	8.00×10^4	6.00×10^4	2.00×10^4	1.70×10^5
	2B	1	2.30×10^5	5.40×10^4	2.10×10^4	3.30×10^5
		2	2.20×10^5	9.00×10^4	2.70×10^4	4.40×10^5
	Mean		***1.55×10^5***	***6.85×10^4***	***2.45×10^4***	***2.68×10^5***
GTL (Shell) + 50% napthenic cut	3A	1	4.00×10^3	7.00×10^4	3.50×10^4	3.80×10^5
		2	8.00×10^3	4.10×10^4	3.20×10^4	2.10×10^6
	3B	1	9.00×10^3	1.40×10^5	2.00×10^3	1.30×10^5
		2	8.00×10^3	1.20×10^5	2.00×10^3	9.00×10^4
	Mean		***7.25×10^3***	***9.28×10^4***	***1.78×10^4***	***6.75×10^5***
GTL (Shell) + 20% hexanol	4A	1	<20	<20	<20	<20
		2	<20	<20	<20	<20
	4B	1	<20	<20	<20	<20
		2	<20	<20	<20	<20
	Mean		***<20***	***<20***	***<20***	***<20***
MEROX treated Jet A-1	5A	1	2.20×10^4	4.00×10^5	3.50×10^5	5.20×10^5
		2	2.20×10^4	3.40×10^5	3.60×10^5	4.60×10^5
	5B	1	2.30×10^4	2.80×10^5	3.30×10^5	5.10×10^5
		2	3.00×10^4	3.80×10^5	3.90×10^5	3.20×10^5
	Mean		***2.43×10^4***	***3.50×10^5***	***3.58×10^5***	***4.53×10^5***

C.5 ATP Readings

ATP readings shown below were converted to an equivalent mean LOG_{10} RLU per litre of fuel/water (see Figure 2 in section 3.4 of main report) as follows;
- subtracting 20 from the actual RLU reading (the background reading of the Hy-Lite Meter)
- Multiply by 100 (Because 0.01 ml of aqueous phase was tested and Fuel:water ratio was 1000:1)
- Calculate the mean of the two microcosms for each fuel type.
- Plot the LOG_{10} of the mean value.

C.5.1 ATP in Aqueous Phase used for Inoculum at Day 0 (RLU reading for 0.1 ml).

Reading; 170

N.B. Because all microcosms were inoculated with the same aqueous phase, this RLU reading was used to calculate the starting (Day 0) LOG_{10} RLU per litre of fuel/water in all microcosms

C.5.2 ATP in aqueous phase of microcosms (RLU reading for 0.1 ml).

Fuel	Microcosm Replicate	RLU / litre			
		Day 7	Day 14	Day 28	Day 49
CTL (Sasol) FSJF	1A	650	94	100	490
	1B	250	49	110	270
GTL (Shell) FT-SPK	2A	4400	390	410 & 8*	310
	2B	780	450	11 & 11*	640
GTL (Shell) + 50% napthenic cut	3A	610	48 & 24*	16 & 270	55
	3B	690	16 & 380*	270	32
GTL (Shell) + 20% hexanol	4A	39	33	No water available for test	
	4B	32	19		
MEROX treated Jet A-1	5A	30 & 1100*	600	1100	860
	5B	1700	850	1500	15 & 770*

* test was repeated because the initial result was considered inconsistent with that expected by comparison with replicate microcosm or previous results. In calculating the RLU per litre of fuel/water the mean reading of the two tests was taken.