COORDINATING RESEARCH COUNCIL, INC.
5755 NORTH POINT PARKWAY, SUITE 265
ALPHARETTA, GA 30022
TEL: 678/795-0506 FAX: 678/795-0509
WWW.CRCAO.ORG

March 10, 2023
In reply, refer to:
CRC Project No. AV-31-22

Dear Prospective Bidder:

The Coordinating Research Council (CRC) invites you to submit a written proposal to provide services for "Microbial Test Kit Evaluation" (CRC Project No. AV-31-22). A description of the project is presented in Exhibit A, "Statement of Work."

Please indicate if you or your organization intends to submit a written proposal for this research program by emailing jantucker@crcao.org by **March 31, 2023.** CRC will answer technical questions regarding the Request for Proposal if they are submitted in writing to jantucker@crcao.org. CRC will then return written answers to all of the bidders, along with a copy of the original questions.

A CRC technical group composed of industry representatives will evaluate your proposal. CRC reserves the right to accept or reject any or all proposals. Key contract language examples are presented in Exhibits B, C, D, and E. CRC must adhere to standard contract language with minor adjustments only in extraordinary circumstances. **Failure to agree to these contract clauses as written may result in the project being awarded to another contractor.**

Important selection factors are listed in Exhibit F. CRC evaluation procedures require the technical group to complete a thorough technical evaluation before considering costs. After developing a recommendation based on technical considerations, the costs are revealed and the recommendation is modified as needed.

The proposal must be submitted as two separate documents. The technical approach to the problem will be described in part one, and a cost breakdown that is priced by task will be described in part two. The cost proposal document should include all costs associated with conducting the proposed program. The technical proposal should not be longer than 10 pages in length (not including resumes). **The schedule / timeline information must be included in the technical proposal; failure to do so may result in your proposal being set aside as non-responsive.**

CRC expects to negotiate a cost-plus fixed fee or cost reimbursement contract for the research program.

The technical and cost proposals should be submitted to
Christopher Tennant Email: **ctennant@crcao.org**

The deadline for receipt of your proposal is **APRIL 14, 2023.**

Exhibit A - CRC Project Statement of Work
"Microbial Test Kit Evaluation**"**

CRC Project Number: AV-31-22

Background

Microbiological Contamination in aircraft fuel tanks has potential serious consequences for both operational efficiency and safety.

IATA publishes Guidance Material on Microbiological Contamination in Aircraft Fuel Tanks, a widely used and recognized publication in the aviation industry. IATA and leading airframe OEMs recommend routine microbiological testing of samples from fuel tanks, and list suitable microbial test kits that uses various technologies. Since the IATA aviation fuel forum in 2019, the industry presented field data on microbial test kits in various industry meetings, which indicate inconsistencies obtain by the various methods. Consequently, airlines and OEMs believe there is a requirement for an evaluation of the methods in a ***controlled studies*** *to* provide independent and objective data. It is envisaged data produced in a laboratory controlled study under defined conditions, will be used in conjunction with data produced by independent field trial(s), and existing field data received from airlines and OEMs and determine the reliability of the current IATA methods. To best capture the variety of microbial conditions seen across the industry, the laboratory study seeks to assess the performance of these test kits against in simulations of fuel tanks with fuel cultivated with fuel-associated microbial isolates.

Objective

To conduct an independent laboratory study to confirm the technical performance and reliability of proposed and existing IATA recommended methods for microbiological contamination in aviation fuel tanks under controlled defined conditions.

Scope of Work

Evaluate current and proposed IATA recommended Microbiological Test Kits and compare to reference test methods.

Test Methods to Include in Study:

- FuelStat® Resinae Plus (ASTM D8070)
- MicrobMonitor®2 (ASTM D7978)
- HY-LiTE® (ASTM D7463)
- Aidian: Easicult TTC and Easicult®M
- San-Ai Biochecker FC
- LuminUltra® ATP (ASTM D7687) (Not currently IATA recommended but proposed and in use by some airlines with their own internally developed action levels)
- IP385/ASTM D6974 (current laboratory reference method)
- qPCR ASTM method in development (as a possible reference technology)

Test evaluation will be carried out in accordance with the protocol developed by the IATA Microbiological Contamination Panel with input from IATA member airlines, Airbus, Boeing, Embraer, US Air Force, Microbiological Experts (Echa Microbiology, FQS, Merck, Conidia Bioscience, Aidian, San-Ai Biochecker)

Simulations of fuel tanks containing contaminated fuel and water, at various levels of contamination, will be developed and then tested with each of the methods.

Schedule

It is anticipated that the work program will take 6 to 12 months, allowing for resourcing materials, development of contaminated fuel, test evaluation and reporting.

Deliverables

The study will provide a research report which will include key data informing on the reliability of microbiological test kits for fuels. It will enable a better understanding of the implications of the results provided by each of the test methods listed above. Without this information, there is a risk that some methods, when used as a routine monitoring tool as currently recommended by IATA and leading OEMs, will not provide adequate indication of potentially serious fuel contamination or fuel tank corrosion.

References

IATA Microbial Test Kit Evaluation Plan (Testing Protocol) attached as Exhibit G.

EXHIBIT B

REPORTS

A. CONTRACTOR shall submit a technical progress report covering work accomplished during each month of the contract performance. The report shall contain a description of overall progress, plus a separate description for each task or other logical segment of work on which effort was expended during the reporting period. Periodic conference calls may also be requested by CRC to update the technical committee overseeing the project.

B. CONTRACTOR shall submit to CRC a draft final report on or before DRAFT FINAL REPORT DUE DATE. The *Draft Final Report* shall be reviewed and returned to CONTRACTOR with comments no later than forty-five (45) days thereafter. The report shall document, in detail, all of the work performed under the contract including data, analyses, and interpretations, as well as recommendations and conclusions based upon results obtained. The report shall include tables, graphs, diagrams, curves, sketches, photographs, and drawings in sufficient detail to comprehensively explain the results achieved under the contract. The report shall be complete in itself and contain no reference, directly or indirectly, to the monthly progress reports and should be suitable for publication in the peer-review literature. Additional rounds of review may be required prior to acceptance of the Final Report. If applicable, data from the research shall be provided in a format suitable for releasing to the public along with the final report.

The draft report must have appropriate editorial review corrections made by the contractor prior to submission to CRC to avoid obvious formatting, grammar, and spelling errors. The report should be written in a formal technical style employing a format that best communicates the work conducted, results observed, and conclusions derived. Standard practice typically calls for a report structure that includes:

- CRC Title Page and Disclaimer Statement (both provided by CRC)
- Table of Contents
- List of Figures
- List of Tables
- List of Acronyms and Abbreviations
- Executive Summary
- Background
- Approach (including a full description of all experimental materials and methods)
- Results
- Conclusions (may also include Recommendations if CRC requests them)
- List of References
- Appendices as appropriate for the scope of the study.

Incomplete draft reports or reports of poor quality requiring additional outside editorial review may have outside editorial services charged back to the project budget.

EXHIBIT C

INTELLECTUAL PROPERTY RIGHTS

Title to all inventions, improvements, and data, hereinafter, collectively referred to as ("Inventions"), whether or not patentable, resulting from the performance of work under this Agreement shall be assigned to CRC. CONTRACTOR shall promptly disclose to CRC any Invention which is made or conceived by CONTRACTOR, its employees, agents, or representatives, either alone or jointly with others, during the term of this agreement, which result from the performance of work under this agreement, or are a result of confidential information provided to CONTRACTOR by CRC or its Participants. CONTRACTOR agrees to assign to CRC the entire right, title, and interest in and to any and all such Inventions, and to execute and cause its employees or representatives to execute such documents as may be required to file applications and to obtain patents covering such Inventions in CRC's name or in the name of CRC's Participants or nominees. At CRC's expense, CONTRACTOR shall provide reasonable assistance to CRC or its designee in obtaining patents on such Inventions.

To the extent that a CRC member makes available any of its intellectual property (including but not limited to patents, patent applications, copyrighted material, trade secrets, or trademarks) to CONTRACTOR, CONTRACTOR shall have only a limited license to such intellectual property for the sole purpose of performing work pursuant to this Agreement and shall have no other right or license, express or implied, or by estoppel. To the extent a CRC member contributes materials, tangible items, or information for use in the project, CONTRACTOR acknowledges that it obtains only the right to use the materials, items, or information supplied for the purposes of performing the work provided for in this Agreement, and obtains no rights to copy, distribute, disclose, make, use, sell or offer to sell such materials or items outside of the performance of this Agreement.

EXHIBIT E

KEY PERSONNEL REQUIREMENTS

Certain skilled experienced professional and/or technical personnel are essential for successful performance by CONTRACTOR of its obligations and work under this Agreement. These personnel are persons whose resumes were submitted for evaluation of the Proposal and are identified by CRC as "Key Personnel". CRC awards contracts based on several requirements and the reputation and experience of Key Personnel are a significant requirement. CONTRACTOR agrees that CONTRACTOR will not remove or replace any Key Personnel from the contract work without compliance with paragraphs (a) and (b) hereof.

(a) If any Key Personnel for whatever reason becomes, or is expected to become, unavailable for work under this Agreement (or any specific Project) for a continuous period exceeding thirty (30) work days, or is not expected to perform the work hours and volume of work indicated in the proposal or initially anticipated, the CONTRACTOR shall immediately notify CRC and shall, subject to the concurrence of CRC, promptly replace such Key Personnel with personnel of at least substantially equal ability and qualifications acceptable to CRC.

(b) All requests for approval of substitutions of Key Personnel hereunder must be in writing to CRC and provide a detailed explanation of the circumstances necessitating the proposed substitutions. Requests for substitution must contain a complete resume for the proposed substitute Key Personnel, and any other information requested by CRC needed to approve or disapprove the proposed substitution. CRC will evaluate such requests and notify CONTRACTOR of approval or disapproval thereof in writing. CRC is not responsible for, and shall not be charged, any fees or other costs related to such replacement Key Personnel's performance of the services until the replacement Key Consultant has obtained the same proficiency and knowledge regarding the services as the former Key Personnel.

(c) If CRC determines that suitable and timely replacement of Key Personnel who have been reassigned, terminated or have otherwise become unavailable for the contract work is not reasonably forthcoming or that the proposed replacement Key Personnel would impair the successful completion of the contract or the services ordered, at the option of CRC, (i) the Agreement (in whole or in part related to the applicable contract work) may be terminated by CRC or (ii) the contract price or fixed fee may be equitably adjusted downward to compensate CRC for any resultant delay, loss, or damage, in an amount acceptable to CRC

EXHIBIT D

RELATIONSHIP OF PARTIES

It is agreed and understood that CONTRACTOR is acting as an independent contractor in the performance of any and all work hereunder, and to the extent caused by CONTRACTOR, CONTRACTOR shall be solely liable and responsible for the payment of all legal claims for damages made by its employees or agents, or by another person or persons, on account of any property damage or on account of personal injury sustained or suffered by, or on account of the death, of any person or persons, or on account of any other legal claims arising or growing out of CONTRACTOR's negligence in the performance of the agreement; and CONTRACTOR undertakes to indemnify CRC against any such liability.

EXHIBIT F

PROPOSAL EVALUATION CRITERIA

1) Merits of proposed technical approach.
2) Previous performance on related research studies.
3) Personnel available for proposed study – related experience.
4) Timeliness of study completion.
5) Cost.

EXHIBIT G - IATA MICROBIAL TEST KIT EVALUATION:

MODULE I; LABORATORY INVESTIGATION

Background

Microbiological contamination in aircraft fuel tanks has potential serious consequences for both operational efficiency and safety. IATA publishes Guidance Material on Microbiological Contamination in Aircraft Fuel Tanks[1], a widely used and recognized publication in the aviation industry. IATA and leading airframe original equipment manufacturers (OEMs) recommend routine microbiological testing of samples from fuel tanks, and has listed suitable microbial test kits that uses various technologies. Since the IATA aviation fuel forum in 2019, the industry presented field data on microbial test kits in various industry meetings, which indicate inconsistencies obtained by the various methods. Consequently, airlines and OEMs believe there is a requirement for an evaluation of the methods in a ***independent controlled study*** to provide objective data. This will be used to investigate the reliability and level of agreement between a range of microbiological test methods.

To best capture the variety of microbial conditions seen across the industry, this project seeks to assess the performance of these test kits through two approaches, described as Module I and Module II.

Module I – Laboratory Investigation

The performance of test methods will be assessed when used to test

a) fuel and/or water samples from laboratory microcosms in which varying degrees of microbial growth has been allowed to occur under conditions simulating fuel tanks. It is proposed that separate microcosms containing individual "type" species (1 bacteria, 1 yeast and 1 mould) and microcosms containing mixed consortia of defined microorganisms are evaluated.
b) fuel and/or water samples containing varying degrees of contamination derived by direct testing and/or appropriate dilution/combing of contaminated field samples.

Module II - Field Investigation

The performance of test methods will be assessed when used to test a wide variety of real samples from aircraft; a technical protocol for field trial evaluation will be developed and presented separately.

This document describes only the technical protocol developed by the IATA Microbial Panel for Module I, the Laboratory Investigation.

Objective Module I – Laboratory Investigation

To conduct an independent laboratory study to confirm the technical performance and reliability of the proposed methods for detection of microbiological contamination in aviation fuel tanks.

Scope of Work

Results of microbiological tests using proposed test methods will be compared to those obtained using the IP 385 reference test method. The test kits/methods to be assessed are shown in Table 1, below.

Table 1: Test Kits/Methods to be Assessed

Test Kit/Method	Manufacturer	Method Reference	Applicable sample phases to be evaluated
IP 385 (Reference Test Method)	N/A	IP 385	Fuel and Water phase
FuelStat® *resinae* Plus	Conidia Bioscience Ltd	ASTM D8070	Fuel and Water phase
MicrobMonitor®2	ECHA Microbiology Ltd	IP 613 / ASTM D7978	Fuel and Water phase
HY-LiTE®	Merck KGaA	ASTM D7463	Fuel and Water phase
Easicult®TTC	Aidian Oy	-	Water phase only
Easicult®M	Aidian Oy	-	Water phase only
San-Ai Biochecker FC	San-Ai-Oil Co., Ltd	-	Water phase only
LuminUltra® QGO-M	LuminUltra®	ASTM D7687	Fuel and Water phase
qPCR	N/A	ASTM D8412*	Fuel and Water phase

* Note: ASTM D8412 *Standard Guide for Quantification of Microbial Contamination in Liquid Fuels and Fuel-Associated Water by Quantitative Polymerase Chain Reaction (qPCR)* has been developed but is not prescriptive for example in terms of which primers and extraction procedures are used. The inclusion of qPCR is for reference purposes and to help in understanding of how this technique may be used in the future. It is suggested the qPCR method employed in this study uses at least 3 primers (e.g. for total bacteria (e.g. 16S RNA), total fungi (18S) and/or total basidiomycetes and total ascomycetes (ITS) and specific targets, as appropriate (TBD). If NGS data from the analysis of field

samples used in this study (Module I) and field trials (Module II) is available prior to conducting the full laboratory investigation, this will help inform on suitable primer selection for specific targets. The final selection can be agreed with contract laboratory at the commencement of the study.

Module I, Laboratory Investigation shall be conducted in accordance with the following protocol, which has been developed by the IATA Microbiological Contamination Panel.

Investigation Principle

Evaluating test kits against samples from laboratory microcosms simulating growth in fuel tanks

Microbiological test kits will be used to test samples from fuel/water microcosms of three pure cultures and two different mixed microbial consortia developed in laboratory microcosms simulating growth in aircraft fuel tanks.

First, individual microbial isolates will be grown in water phase over-layered with Jet A, before being used to create primary microcosms. After 8 weeks of incubation, fuel and water phase from primary microcosms will be used to inoculate fresh secondary microcosms of fuel and water. After 8 weeks of incubation, secondary microcosms will be pooled, and subsamples of water and fuel phase tested using the various microbiological test kits. Fuel and water samples derived from pooled secondary microcosms will also be diluted so that tests kits are assessed against three levels of microbiological contamination; negligible, moderate and heavy contamination according to IATA guidance.

Evaluating test kits against field samples

Microbiological test kits will be used to test two field samples; one sourced from a heavily contaminated aircraft fuel tank and one sourced from a heavily contaminated aviation fuel storage tank. Fuel and water phase from the contaminated field samples will be tested directly using the various test kits. Fuel and water phase from the field samples will also be diluted so that the test kits are assessed against three levels of microbiological contamination: negligible, moderate and heavy contamination according to IATA guidance.

Results obtained using the proposed and existing IATA-recommended test kits will be categorised as negligible, moderate, or heavy level contamination according to IATA guidance. Statistical analysis will then be applied to assess the degree of agreement between the results obtained using the test kits and the reference IP 385 test method.

Test Protocol

1. Evaluating Test Kits Against Samples from Laboratory Microcosms (Defined Inocula Studies)

1.1. Test Species

1.1.1. The microorganisms to be developed in laboratory microcosms separately, as pure cultures, are as follows;

- *Pseudomonas aeruginosa* (ATCC 33988)
- *Yarrowia lipolytica* (ATCC 20177)
- *Hormoconis resinae* (ATCC 20495)

Note; the strains of the three species used to evaluate test performance in assessing single species microcosms can be discussed with contract lab but should represent a bacteria, a yeast and a mould commonly encountered in jet fuel tanks.

1.1.2 The composition of the two separate consortia for use in laboratory microcosms are suggested as follows:

Consortium 1:

- *Pseudomonas aeruginosa* (ATCC 33988)
- *Acinetobacter venetianus RAG-1* (ATCC 31012)
- *Brevundimonas vesicularis* (ATCC 11426)
- *Yarrowia lipolytica* (ATCC 20177)
- *Hormoconis resinae* (ATCC 20495)

Consortium 2:

- *Bacillus licheniformis*
- *Alcaligenes* species
- *Nocardiodes luteus*
- *Candida (Meyerozyma) guilliermondii* (ATCC 6260)
- *Penicillium corylophilum*

Note; The recommendation on species above is based on the expert input and experiences of the IATA microbial panel. However it is a suggestion only, and the species used to make up each consortia can be discussed with contract lab prior to commencing the study. However, each consortia should include at least one bacteria, one yeast and one mould, and no less than 5 species in total of microorganisms commonly encountered in jet fuel tanks.

There will be three transfers of the microorganisms through fuel over the testing process (initial pure culture, primary microcosm and secondary microcosm). Microorganisms should be subcultured no more than 3 times from the initial frozen stock.

1.2. Evaluation of Pure Cultures Developed in Laboratory Microcosms

A schematic diagram depicting the experimental setup for section 1.2 is outlined in Figure A1.

1.2.1. Initial Pure Culture of Microorganisms

1.2.1.1. For each test species (1.1.1) prepare quadruplicate pure cultures by inoculating 4 x 250 mL of sterile ¼ strength Bushnell-Haas Mineral Salts Solution (BHMSS), before overlaying each with 250 mL of 0.22 µm filter sterilised Jet A fuel.

Note: Type of Jet Fuel to be used throughout the study can be agreed and discussed prior to commencing the study but it should be of a type known to be readily susceptible to microbial growth and representative of fuel widely used for commercial aviation. Generally, jet fuels with poorer water separation characteristics and higher heteroatom content have been shown to be most susceptible to microbial growth. Evaluation of performance of microbiological tests for different jet fuel types is beyond the scope of the study.

1.2.1.2. Swirl the cultures by hand for 10 seconds to disperse the fuel.

1.2.1.3. Incubate at 25°C for 4 weeks with weekly swirling for 10 seconds at sufficient force to disrupt any material at the fuel:water interface.

1.2.1.4. After 4 weeks, place ca. 40 sterile glass beads into each culture and shake vigorously by hand for 30 seconds. Allow to settle for 10 minutes before aspirating the water phase into a sterile bottle. Adjust microorganism concentrations as required in ¼ strength BHMSS and verify that the water phase contains $10^2 – 10^3$ cells or spores/ml by direct microscopic count. For bacteria, optical density of the water phase may be measured using a spectrophotometer and standard calibration curve to infer the bacterial

count. Note: These pure cultures will be used for inoculation of primary microcosms as detailed below. It is appreciated that microscopic counts of hyphae and spores associated with fungal cultures will be difficult, but the amount added can be approximate at this stage; the aim is to roughly ensure equal addition of each microorganism to primary microcosms.

1.2.2. Preparation of Primary Microcosms (Pure Culture Studies)

1.2.2.1. The following procedure is to be undertaken for **each species** under investigation (1.1.1).

1.2.2.2. Add 200 mL of fresh, sterile 0.22 µm filtered Jet A fuel to 5 x sterile 1 L HDPE containers.

1.2.2.3. Obtain 200 mL of homogenised aqueous phase for each species defined (1.1.1) and inoculate 4 of the fuel samples (1.2.2.2) to produce 4 independent primary microcosms.

1.2.2.4. To the remaining sterile fuel sample, add 200 mL sterile ¼ strength BHMSS; this will serve as a negative control.

1.2.2.5. Tighten the caps on microcosms and mix by inverting 3 times. After mixing the microcosm, the caps should not be airtight, or they should have a filtered outlet to allow air exchange.

1.2.2.6. Incubate primary microcosms for 8 weeks at 25°C.

1.2.2.7. Each week, swirl the microcosms for 30 seconds with sufficient force to disrupt material at the fuel:water phase interface – do not invert.

1.2.2.8. At week 7, prior to the swirling activity, conduct the following assessments on all primary microcosms (including the control);

1.2.2.8.1. Visual examination of the fuel: water interface for evidence of microbial growth. Take photographs.

1.2.2.8.2. Aspirate 2 x 5 mL aliquots of fuel phase from just above the settled water phase and test each aliquot immediately by IP 385 procedure A (duplicate tests).

1.2.2.8.3. Sample material at the fuel:water interface and examine by light microscopy to confirm presence of microbial growth. Take representative micrographs.

1.2.2.8.4. Gently swirl primary microcosms to mix the water phase, and aspirate 2 x 1 mL aliquots of water phase from each microcosm and

test each aliquot immediately by IP 385 procedure B (duplicate tests).

1.2.2.8.5. Microcosm samples may be taken at this stage for later analysis using Next Generation Sequencing (NGS) and/or qPCR of water phase.

1.2.2.9. Proceed with the set up of secondary microcosms (1.2.3) on week 8 if IP 385 tests indicate heavy contamination according to IATA guidance (>20,000 CFU/L in fuel phase and >10,000 CFU/mL in water phase).

1.2.2.10. Do not transfer the control primary microcosm to a secondary microcosm negative control.

1.2.3. Preparation of Secondary Microcosms (Pure Culture Studies)

1.2.3.1. The following procedure is to be undertaken for each species under investigation.

1.2.3.2. Place ca. 100 sterile glass beads into each quadruplicate primary microcosm and shake vigorously by hand for 30 seconds. Allow to settle for 10 minutes.

1.2.3.3. From each quadruplicate microcosm transfer 100 mL fuel phase and 100 mL of water phase into a sterile HDPE container along with 5L of fresh, sterile 0.22 µm filtered Jet A, thereby creating 4 x secondary microcosms.

1.2.3.4. Transfer 100 mL sterile ¼ strength BHMSS to a fifth sterile HDPE container with 5 litre of fresh, sterile 0.22 µm filtered Jet A to serve as a negative control.

1.2.3.5. Invert all microcosms 3 times to mix and incubate at 25°C for 8 weeks with weekly agitation as per 1.2.2.5, 1.2.2.6 and 1.2.2.7.

1.2.3.6. At week 7, verify growth in the fuel and water phases of each secondary microcosm (including the control) as per 1.2.2.8; in addition to testing 5 mL aliquots of fuel phase (from just above interface) according to IP 385 Procedure A, additionally test 2 x 1 mL aliquots of fuel phase by this method. Ensure 'heavy' contamination according to IATA guidelines before proceeding to the Test Protocol phase (section 1.2.4 below).

1.2.3.7. Use IP 385 results to inform on the extent of dilution of fuel and water that will be required to achieve moderate level contamination (4000-

20,000 CFU/L for fuel, 1000-10,000 CFU/mL for water) or negligible level contamination (<4000 CFU/L for fuel, <1000 CFU/mL for water).

1.2.4. Test Method Assessment Protocol (Pure Culture Studies)

Note: Test kits must be used to test water and fuel samples in the manner that reflects intended use by airlines.

1.2.4.1. For each laboratory cultured species: pool two of the quadruplicate secondary microcosms (1.2.3.6) into one 10 L HDPE carboy; repeat with the remaining two secondary microcosms. This will produce two independent test samples.

1.2.4.2. Vigorously shake the carboys for 10 seconds and then allow them to settle (2 min/cm fuel height) prior to sample collection.

1.2.4.3. Carboys should have a spigot at the very bottom to enable collection of water phase. Collect the entire water phase from each carboy via the spigot into a sterile HDPE container, ensuring that any interfacial material (but no visible water) remains in the carboy with the fuel phase. It is not critical if some small amount of interfacial material is carried over to the to the water phase. Ensure that agitation is minimized during separation to limit extraction of metabolites from the fuel phase into the water phase.

1.2.4.4. Water phase analysis;

1.2.4.4.1. Shake the separated water phase vigorously by hand for 10 seconds and immediately decant 20 mL into 2 x sterile 100 mL HDPE containers and 70 mL into a third sterile 100 mL HDPE container. Water phase in these containers will be used to complete triplicate testing using the LuminUltra QGO-M, qPCR and Hy-Lite tests, respectively (sample volumes required for each test are provided in Table A3 in the Appendix).

1.2.4.4.2. The remainder of the separated water phase should then be sampled directly to conduct triplicate testing using the FuelStat *resinae* PLUS, MicrobMonitor2, and IP 385 Procedure B tests; shake

the container vigorously for 10 seconds before taking each test aliquot from the mid-point of the container.

1.2.4.4.3. Then complete triplicate testing of water phase using the dipslide-type tests (Easicult TTC, Easicult M and San-Ai Biochecker FC).

1.2.4.4.4. Subsamples collected for ATP testing (by Hy-LiTE and LuminUltra QGO-M) should be left to stand and equilibrate for 30 minutes prior to performing these tests. Subsamples taken for qPCR / NGS analysis need to be processed / preserved immediately. All other tests should be conducted on water phase immediately after it has been separated from the carboy.

1.2.4.5. Fuel phase analysis;

1.2.4.5.1. Shake the carboy containing the fuel phase vigorously by hand for 10 seconds and immediately decant a 1 litre volume into a 1 Litre HDPE container; repeat this process until 7 x 1 litre containers have been filled.

Note: it is critical to obtain an even distribution of any residual interface particulate in the fuel between the 7 replicate fuel samples.

1.2.4.5.2. Conduct triplicate FuelStat *resinae* PLUS, MicrobMonitor2, and IP 385 Procedure A tests immediately; shake one of the 1 Litre fuel subsamples vigorously for 10 seconds before taking each test aliquot from the mid-point of the container. Then leave this container to stand for 30 minutes before taking further subsamples for LuminUltra QGO-M ATP analysis (sample volumes required for each test are provided in Table A3 in the appendix).

1.2.4.5.3. Three of the 1 Litre fuel subsamples are to be used for HY-LiTE testing; these subsamples should be left to stand for 30 minutes before conducting this analysis.

1.2.4.5.4. Three of the 1 Litre subsamples are to be used for qPCR / NGS analysis and need to be processed and preserved immediately.Note: preservation procedure can be determined and agreed with the contract laboratory at time of commencement of the study.

1.2.4.5.5. Make dilutions of the separated water phase sample (using sterile ¼ strength BHMSS as diluent) and the remaining fuel phase in the carboy stock (using 0.22 µm filter sterilised Jet A as diluent) such that samples containing moderate and negligible level contamination can be tested. Use previously obtained IP 385 results (1.2.3.7) to inform on the extent of dilution that will be required.

Note: it is critical to obtain a similar even distribution of any residual interface particulate in the fuel used to make dilutions.

1.2.4.6. Conduct tests on the water phase and fuel phase dilutions as detailed in steps 1.2.4.4 and 1.2.4.5.

1.3.

1.3. Evaluation of Defined Microbial Consortia Developed in Laboratory Microcosms

A schematic diagram depicting the experimental setup for section 1.3 is outlined in Figure A2.

1.3.1. Initial Pure Culture Of Microorganisms

1.3.1.1. For each test species (1.1.2) prepare quadruplicate pure cultures by inoculating 4 x 50 mL of sterile ¼ strength Bushnell-Haas Mineral Salts Solution (BHMSS), before overlaying each with 250 mL of 0.22 µm filter sterilised Jet A fuel.

1.3.1.2. Swirl the cultures by hand for 10 seconds to disperse the fuel.

1.3.1.3. Incubate at 25°C for 4 weeks with weekly swirling for 10 seconds at sufficient force to disrupt any material at the fuel:water interface.

1.3.1.4. After 4 weeks, place ca. 40 sterile glass beads into each of the 4 replicates of each culture and shake vigorously by hand for 30 seconds. Allow to settle for 10 minutes before aspirating the water phase into a sterile bottle. Adjust microorganism concentrations as required in ¼ strength BHMSS andverify that the water phase contains $10^2 – 10^3$ cells or spores/ml by direct microscopic count. For bacteria, optical density of the water phase may be measured using a spectrophotometer and standard calibration curve to infer the bacterial count.

Note: These pure cultures will be mixed and used for inoculation of primary microcosms of defined consortia as detailed below. It is appreciated that microscopic counts of hyphae and spores associated with fungal cultures will be difficult, but the amount added can be approximate at this stage; the aim is to roughly ensure equal addition of each microorganism to primary microcosms.

1.3.2. Preparation of Primary Microcosms (Mixed Consortia Studies)

1.3.2.1. The following procedure is to be undertaken for **each microbial consortia** (1 and 2) under investigation as defined in 1.1.2.

1.3.2.2. Add 200 mL of fresh, sterile 0.22 µm filtered Jet A fuel to 5 x sterile 1 L HDPE containers.

1.3.2.3. Obtain 40 mL of homogenised aqueous phase for each of the 5 species specified in each test consortia (1.1.2) and pool together in a sterile bottle (total volume 200 mL). Repeat in quadruplicate to produce 4 replicate inocula (for each consortia).

1.3.2.4. Inoculate 4 of the fuel samples (1.3.2.2) with the 200 mL of prepared inocula (1.3.2.3) to produce 4 independent primary microcosms.

1.3.2.5. To the remaining sterile fuel sample, add 200 mL sterile ¼ strength BHMSS; this will serve as a negative control.

1.3.2.6. Tighten the caps on microcosms and mix by inverting 3 times. After mixing the microcosm caps should not be airtight, or they should have a filtered outlet to allow air exchange.

1.3.2.7. Incubate primary microcosms for 8 weeks at 25°C.

1.3.2.8. Each week, swirl the microcosms for 30 seconds with sufficient force to disrupt material at the fuel:water phase interface – do not invert.

1.3.2.9. At week 7, verify growth in the fuel and water phases of primary microcosms (including the control) as per 1.2.2.8 prior to the swirling activity, conduct the following assessments on all primary microcosms (including the control);

1.3.2.10. Proceed with the set up of secondary microcosms (1.3.3) on week 8 if IP 385 tests indicate heavy contamination according to IATA guidance (>20,000 CFU/L in fuel phase and >10,000 CFU/mL in water phase).

1.3.2.11. Do not transfer the control primary microcosm to a secondary microcosm negative control.

1.3.3. Preparation of Secondary Microcosms (Mixed Consortia Studies)

1.3.3.1. Undertake the procedure detailed in 1.2.3.2 – 1.2.3.4 to set up 4 x secondary microcosms for each microbial consortia (1 and 2) under investigation.

1.3.3.2. At week 7, verify growth in the fuel and water phases of each secondary microcosm (including the control) as per section 1.2.2.8 . Ensure 'heavy' contamination according to IATA guidelines before proceeding to the Test Protocol phase (section 1.3.4 below).

1.3.3.3. Use IP 385 results to inform on the extent of dilution of fuel and water that will be required to achieve moderate level contamination (4000-20,000 CFU/L for fuel, 1000-10,000 CFU/mL for water) or negligible level contamination (<4000 CFU/L for fuel, <1000 CFU/mL for water).

1.3.4. Test Method Assessment Protocol (Mixed Consortia Studies)

1.3.4.1. For each laboratory cultured consortium: pool two of the quadruplicate secondary microcosms into one 10 L HDPE carboy; repeat with the remaining two secondary microcosms. This will produce two independent test samples.

1.3.4.2. Continue with the steps detailed in sections 1.2.4.2 to 1.2.4.5.

2. Evaluating Test Kits Against Contaminated Field Samples

2.1. Test methods will be assessed against two field samples; one sourced from a heavily contaminated aircraft fuel tank and one sourced from a heavily contaminated aviation fuel storage tank.

2.2. Prior to collection of field samples for use in the study, the presence of heavy contamination (according to IATA guidance limits) should be established in samples of fuel and associated water from the tanks using the IP 385 method.

2.3. A total of 10 litres of fuel phase and 200 mL of water phase is required from each system to complete the necessary testing. If sample volumes are limited, it may be possible to dilute very heavily contaminated samples to produce samples that are still heavily contaminated

according to IATA guidance. Equally, samples from similar source may be combined to improve diversity of microbial communities assessed.

2.4. The following procedure should be undertaken for each field sample under investigation.

2.4.1. Upon receipt of a field sample at the test facility, transfer it to a 10 Litre HDPE carboy which has a drainage spigot at the bottom.

2.4.2. Vigorously shake the carboy for 10 seconds and then allow them to settle (2 min/cm fuel height).

2.4.3. Then take samples to verify the level of microbial contamination in the fuel and water phase as per 1.2.2.8; ensure fuel and water phase is heavily contaminated (according to IATA guidance) before proceeding further. Use the IP 385 results to inform on the extent of dilution of fuel and water that will be required to achieve moderate level contamination (4000-20,000 CFU/L for fuel, 1000-10,000 CFU/mL for water) or negligible level contamination (<4000 CFU/L for fuel, <1000 CFU/mL for water).

2.4.4. Follow steps 1.2.4.2 to 1.2.4.5 to test water phase and fuel phase samples (and the dilutions of these phases).

4. Data Interpretation and Statistical Analysis

4.1. Through the combined testing of laboratory microcosms and field samples in this study, each microbiological test method applied will generate 36 data points when used to test fuel or water phase, at each contamination level assessed (negligible, moderate or heavy).

4.2. Data obtained using Fuelstat *resinae* PLUS, HY-Lite, Luminultra QGO-M, and the culture test methods (IP 385, MicrobMonitor2, and the Dip-slide type tests) will be categorised as negligible, moderate or heavy according to the following table;

Test Method	Sample type	Negligible Level Contamination	Moderate Level Contamination	Heavy Level Contamination
Culture methods*	Fuel	<4 000 CFU/L	4 000 – 20 000 CFU/L	>20 000 CFU/L
	Water	<1 000 CFU/mL	1 000 – 10 000 CFU/mL	>10 000 CFU/mL
HY-LiTE (ASTM D7463)	Fuel and / or Water	<1 000 RLU	1 000 – 5 000 RLU	>5 000 RLU
FuelStat *resinae* plus (ASTM D8070)	Fuel	<150 µg/L	150 – 750 µg/L	>750 µg/L

	Water	<33 µg/mL	33 – 166 µg/mL	>166 µg/mL
LuminUltra QGO-M (ASTM D7687)	Fuel	<10 pg/mL	10 – 100 pg/mL	>100 pg/mL
	Water	<100 pg/mL	100 – 1000 pg/mL	>1000 pg/mL

* Fuel and water samples: IP 385, MicrobMonitor2 (IP 613 / ASTM D7978). Water samples only: Easicult TTC, Easicult M and San-Ai Biochecker FC. For the IP 385, the total count of fungi and bacteria should be considered.

4.3. Statistical analysis using Cohens Kappa and Finn Coefficient methods will be applied to assess the agreement between the results obtained using the test methods and the IP 385 reference test method.

5. References

1. IATA (2015) Guidance Material on Microbiological Contamination in Aircraft Fuel Tanks, 5th Ed. IATA: Montreal, Canada.

Appendix 1: Experimental Matrices

Table A1: Microbial Compositions to be Tested

Sample	# Independent samples
Laboratory cultured microcosm, *P. aeruginosa*	2
Laboratory cultured microcosm, *Y. lipolytica*	2
Laboratory cultured microcosm, *H. resinae*	2
Laboratory cultured microcosm, consortium 1	2
Laboratory cultured microcosm, consortium 2	2
Field sample, aircraft fuel tank	1
Field sample, aviation fuel storage tank	1

Table A2: Final Sample Matrix for Each Microbial Composition*

Sample	# of Samples	Notes
Fuel phase - undiluted	3	Heavy contamination in fuel
Fuel phase – dilution	3	Moderate contamination in fuel
Fuel phase – dilution	3	Negligible contamination in fuel
Sterile, 0.22 µm-filtered jet fuel	3	Negative control
Water phase – undiluted	3	Heavy contamination in water
Water phase – dilution	3	Moderate contamination in water
Water phase – 1:100 dil.*	3	Negligible contamination in water
Sterile ¼ strength Bushnell Haas Mineral Salts Solution	3	Negative control

* Four microbial compositions to be tested in total (see Table A1).

Table A3: Sample Volumes required for each test

Test Kit	Analysis Time	Fuel Sample Volume (mL)	Water Sample Volume (mL)
FuelStat *resinae* Plus	10 min	200	20
Hy-LiTE	< 10 min	1000	10
MicrobMonitor2	1-4 days	0.5	0.1
IP 385	3-5 days	5 (IP 385 Procedure A for fuel phase)	1 mL (IP 385 Procedure B for water phase)
LuminUltra QGO-M	10 min	20	5
Easicult TTC	1-4 days	N/A	Dip slide method
Easicult M	3-5 days	N/A	Dip slide method
San-Ai Biochecker FC	2-5 days	N/A	Dip slide method
qPCR	24 hour	500-1000	5
NGS	Variable	500-1000	5
Total Volume (assuming the same DNA extract can be used for qPCR & NGS		**~ 2.3 L***	**~ 50 mL****

*Total volume required for triplicate testing ~7 Litres **Total volume required for triplicate tests ~150 mL

Figure A1: Evaluation of pure cultures developed in laboratory microcosms

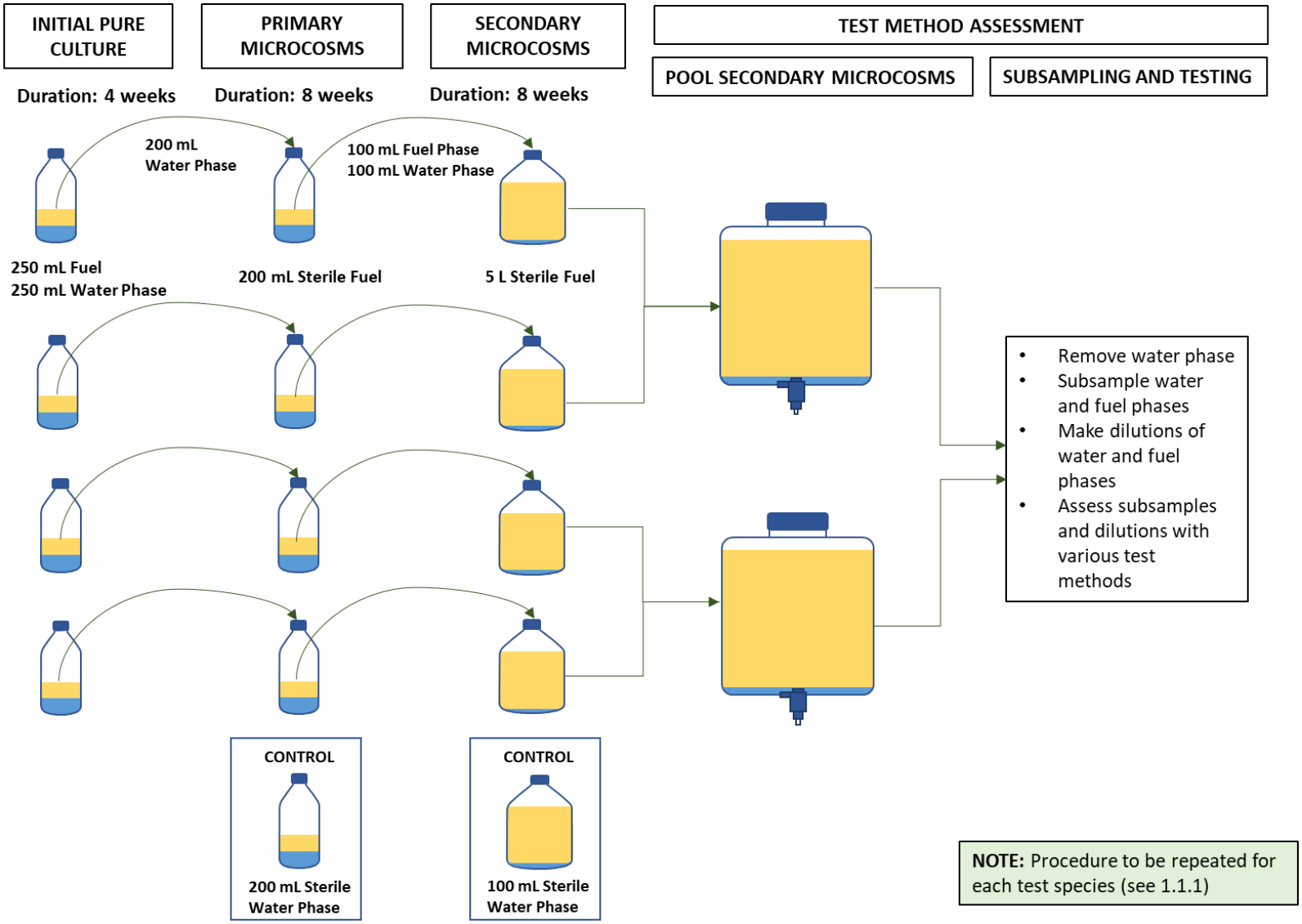

Figure A2: Evaluation of defined microbial consortia developed in laboratory microcosms