Stratus Eliminate

Product Analysis Report

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Overview of Project

Stratus Energy Ltd. market a number of fuel additives, one of which is the biodiesel biocide Stratus Eliminate. Microbial contamination of biodiesel storage tanks and pipework is a widespread and potentially costly issue, prevention (and intervention) of infection can be achieved by routine use of a fuel biocide.

Pseudomonas aeruginosa ATCC 33988, originally isolated from a fuel storage tank (Ponca City, Oklahoma) was chosen to test the efficacy of Stratus Eliminate, as *P. aeruginosa* is one of the microorganisms most often implicated in founding the multi-species biofilms associated with severe biodiesel contamination (Rossmoore, *et al.,* 1988). The normal pattern of fuel tank colonisation begins with a founder species such as *P. aeruginosa,* an aerobic bacteria, that alters the pH and oxygen level of the microenvironment to such an extent that other species (such as anaerobic sulphate reducing bacteria or pH sensitive fungi) may flourish. Additionally *P. aeruginosa* ATCC 33988 is utilised by the US Department of Defence as the organism of choice for biocide challenge testing (Interim Report MIL-S-53021, 1993).

Stratus Energy Ltd. were aware that Stratus Eliminate worked but did not have the test data to confirm:

- The concentration of Stratus Eliminate required to exterminate *P. aeruginosa* contamination in biodiesel.
- The time Stratus Eliminate takes to reduce / eliminate viable *P. aeruginosa* in biodiesel.

Obtaining this information would enable product validation, consumer confidence in the product and product development.

Assessment of the background microflora in biodiesel

Preceding biocide efficacy testing, an assessment of the background microflora present in the un-sterile biodiesel sample was conducted. This assessment was done to ascertain the degree of background microflora and whether a sterilisation pretreatment was required to ensure consistent results.

A selection of agar plates: Nutrient agar (NA), Malt extract agar (MEA), Clostridial agar (CA) and Pseudomonas agar (PA) were prepared according to the manufacturer's (Oxoid, Hampshire, UK) instructions, and sterilised by autoclave at 121°C for 15 minutes.

Neat biodiesel (200 µl) was spread onto five of each variety of agar plate (NA, MEA, CA & PA). The sets of plates were then incubated under five different conditions and checked periodically over 14 days to observe if any microbial growth was present. The conditions the plates were incubated under were: aerobically at 17°C, ambient room temperature, 37°C and 50°C and anaerobically at 30°C.

Table 1: Assessment of the background microflora in biodiesel

	NA	MEA	CA	PA
aerobic 17°C	1 small yellow/white circular colony, raised elevation, entire margin	1 large white fungi (black in the centre), dark underside, filiform margin	No visible growth	1 small white / pink small circular colony, raised elevation, entire margin
aerobic Room	2 small white circular colonies, raised elevation, entire margin	1 white fungi (green / brown underside), filiform margin. 2 white / yellow colonies	No visible growth	1 small white / pink small circular colony, raised elevation, entire margin
aerobic 37°C	1 white fungi (green / brown underside), filiform margin	1 large white fungi (green / brown underside), filiform margin	No visible growth	2 small white / pink small circular colonies, raised elevation, entire margin
aerobic 50°C	No visible growth	No visible growth	No visible growth	No visible growth
anaerobic 30°C	No visible growth	No visible growth	No visible growth	No visible growth

Conclusion:

Due to the negligible background microbial contamination of the biodiesel sample, it was decided that the biodiesel did not require any pre-treatment to remove background organisms, as the minimal microflora present would not significantly interfere with the biocide efficacy testing. Preceding the efficacy testing, all glassware was sterilised prior to the addition of biodiesel to ensure any contamination was introduced to the test via the biodiesel can or biodiesel.

Determination of the efficacy of Stratus Eliminate against *Pseudomonas aeruginosa*

The recommended dosage of Stratus Eliminate is 1:4000. An overnight culture of *P. aeruginosa* was prepared and washed as described below and used to inoculate eight test bottles of biodiesel (100 ml biodiesel or 80 ml biodiesel and 20 ml H H_2O).

Materials

- Glass bottles (250 ml with PTFE-lined screw caps)
- Magnetic stirrers *1 for each bottle*
- Nutrient agar (NA) plates *8 per test bottle/per sample point*
- Nutrient broth (NB)
- Buffer
- Microcentrifuge tubes
- Beaker (250 ml)
- Measuring cylinder (250 ml)
- Vortex mixer
- Magnetic stirrer plate
- Plate counter

Method

0. Prepare media and materials.
   A) Make up buffer, NB and NA plates.

B) Sterilise beaker, buffer, microcentrifuge tubes, measuring cylinder, glass bottles (containing magnetic stirrer).
C) Aseptically aliquot 900 µl of buffer into individual centrifuge tubes (for dilution series).
D) Aseptically add 100 ml of biodiesel to sterile glass bottles.

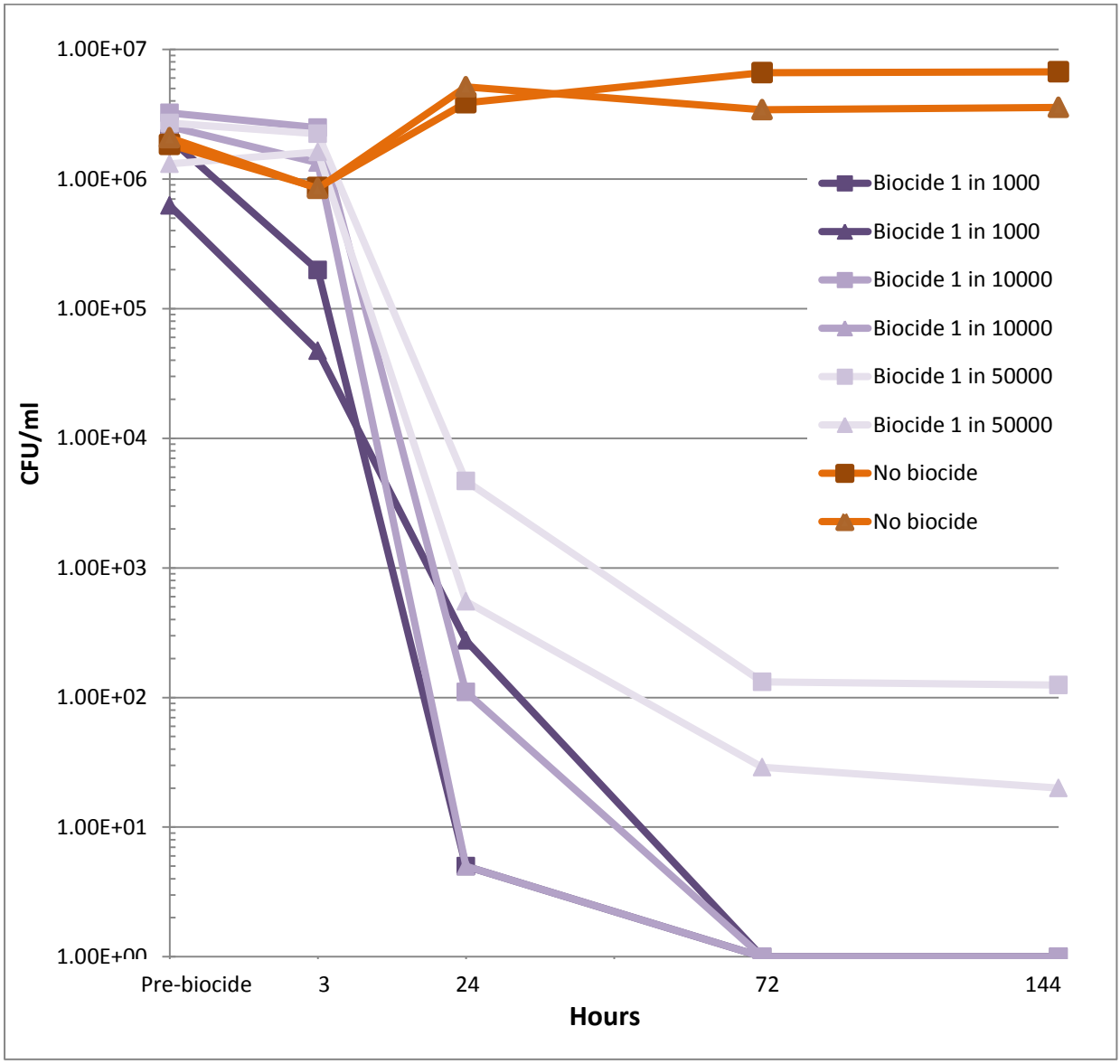
1. Revive culture and grow on NA plates.
2. Subculture isolated colony on a NA plate.
3. Subculture into NB → incubate overnight.
4. Wash cells.
   A) Aliquot 45 ml of overnight culture into sterile centrifuge tubes.
   B) Centrifuge at 12,000 rpm for 10 minutes.
   C) Discard supernatant.
   D) Add 5 ml of sterile buffer.
5. Inoculate washed cells into biodiesel.
   → Take plate count (Day 1, pre-biocide).
   A) Shake bottle vigorously for 5 seconds, place on magnetic stirrer plate.
   B) Remove a 100 µl sample and pipette into 900 µl of buffer → vortex.
   C) Perform dilution series → plate out relevant dilutions onto NA plates.
6. Add biocide to relevant bottles.
7. Take plate count (Day 1, post-biocide 3 hours later).
8. 24 hours post-biocide plate count.
9. 72 hours post-biocide plate count
10. 144 hours post-biocide plate count.
11. Record results and determine the CFU/ml.

Results

Table 2: The efficacy of Stratus Eliminate in biodiesel inoculated with *P. aeruginosa* (CFU/ml)

	1 *P. aeruginosa* + H_2O + biocide **1 in 1000**	2 *P. aeruginosa* + biocide **1 in 1000**	3 *P. aeruginosa* + H_2O + biocide **1 in 10000**	4 *P. aeruginosa* + biocide **1 in 10000**	5 *P. aeruginosa* + H_2O + biocide **1 in 50000**	6 *P. aeruginosa* + biocide **1 in 50000**	7 *P. aeruginosa* + H_2O	8 *P. aeruginosa*
Pre-biocide	2.05E+06	6.25E+05	3.24E+06	2.54E+06	2.71E+06	1.31E+06	1.85E+06	2.08E+06
3 hours	1.99E+05	4.72E+04	2.49E+06	1.34E+06	2.23E+06	1.62E+06	8.60E+05	8.45E+05
24 hours	5.00E+00	2.75E+02	1.10E+02	5.00E+00	4.69E+03	5.50E+02	3.88E+06	5.13E+06
72 hours	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.32E+02	2.90E+01	6.61E+06	3.42E+06
144 hours	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.25E+02	2.00E+01	6.70E+06	3.56E+06

Chart 1: The efficacy of Stratus Eliminate on *P. aeruginosa* in biodiesel (CFU/ml)



□ – biodiesel + H_2O, Δ – 100% biodiesel

Conclusions and future work:

Stratus Eliminate achieved a complete kill of *P. aeruginosa* contamination at 1 in 1000 and 1 in 10,000 after 72 hours. The recommended dosage is 1 in 4000. Biocide concentration of 1 in 1000 began to significantly reduce the number of viable organisms within the biodiesel after only 3 hours (2.05E+06 down to 1.99E+05 and 6.25E+05 down to 4.72E+04). After 24 hours, all biocide concentrations tested demonstrated a marked reduction in viable organisms, even at 1 in 50,000 the viable organisms were reduced from 2.71E+06 down to 4.69E+03 in the biodiesel sample containing H_2O and from 1.31E+06 down to 5.50E+02 in the 100% biodiesel sample.

Future work recommended from this project would involve co-contamination of biodiesel with a consortium of biodiesel degrading organisms, to ensure that the biocide has the ability to remove established contamination consisting of a multi-species biofilm at the low concentrations which were found to be effective at against the *P. aeruginosa* contamination.

Additionally there does not appear to be a consensus standard method published for enumerating filamentous fungi, which are known to join the consortium of organisms which can infect biodiesel. Unlike with bacterial species, methods relying on direct counts of colony forming units have been found to be unacceptable, as the estimation does not correlate well to the actual contamination present (Gaylarde *et al*., 1999). A potential future project the Biofuel Research Centre could undertake would be to develop a new method for enumerating a multi-species contamination within biodiesel.

Gaylarde, C. C., F. M. Bento & J. Kelley (1999) Microbial contamination of stored hydrocarbon fuels and its control. *Revista de Microbiologia* **30**:1-10.

Interim Report MIL-S-53021 (1993) Compatibility and efficacy of biocides qualified under military specification MIL-S-53021. Interim Report BFLRF No. 282. S.R. Westbrook, Belvoir Fuels and Lubricants Research Facility (SwRI), Southwest Research Institute, San Antonio, Texas and M.L. Alexander, Southwest Research Institute, San Antonio, Texas.

Rossmoore, H. W., J. W. Wireman, L. A. Rossmoore & V. F. Riha (1988) Factors to consider in testing biocides for distillate fuels. *Distillate Fuel: Contamination, Storage and Handling. ASTM STP 1005,* H. L. Chesneau and M. M. Dorris, Eds., American Society for Testing and Materials, Philidelphia, pp.95-104.