

Appendix K: Sludge Characterization and Microbial Identification

Sludge Characterization and Microbial Identification

Washington State University (WSU) focused on investigation of the excess sludge formation in the purifiers of the vessels. Several methods, such as pyrolysis-GC/MS (Py-GC/MS), thermogravimetric analysis (TGA), ion chromatography (IC), and microbial identification were used to characterize the sludge samples obtained during the pilot test. A main cause responsible for the sludge formation was identified in order to make recommendations for preventing the excess sludge formation.

1. Organic materials in the sludge

Organic materials in the sludge were characterized using two methods, pyrolysis-GC/MS (Py-GC/MS) and thermogravimetric analysis (TGA).

1.1 Py-GC/MS analysis

Py-GC/MS analysis was carried out using a CDS pyroprobe 5000 with an Agilent GC-MS. Samples were loaded into a quartz tube and kept the oven (210 °C) to ensure adequate removal of oxygen prior to pyrolysis. Samples were pyrolyzed by heating to 500 °C, and the resulting pyrolysis vapors were separated by a (5% phenyl)-methylpolysiloxane non-polar column. The gas flow rate was 1 ml/min and helium was used as the carrier gas. The gas was then sent into a mass spectrometer (Agilent Technologies Inert XL MSD). The mass spectrometer conditions were as follows: transfer line at 150 °C, ion source 230 °C, and electron energy 70 eV. The mass spectra of predominant peaks were then compared to a mass spectra library to determine the compounds in a given peak.

Figure 1 shows the result of the sludge sample. The highest peak in the figure was at 31.5 min. Figure 2 suggests that the MS pattern of this peak was 8-Octadecenoic acid methyl ester (C₁₉H₃₆O₂), based on data from the library of standard chemicals. Thus, the sludge contains an 8-Octadecenoic acid methyl ester fraction.

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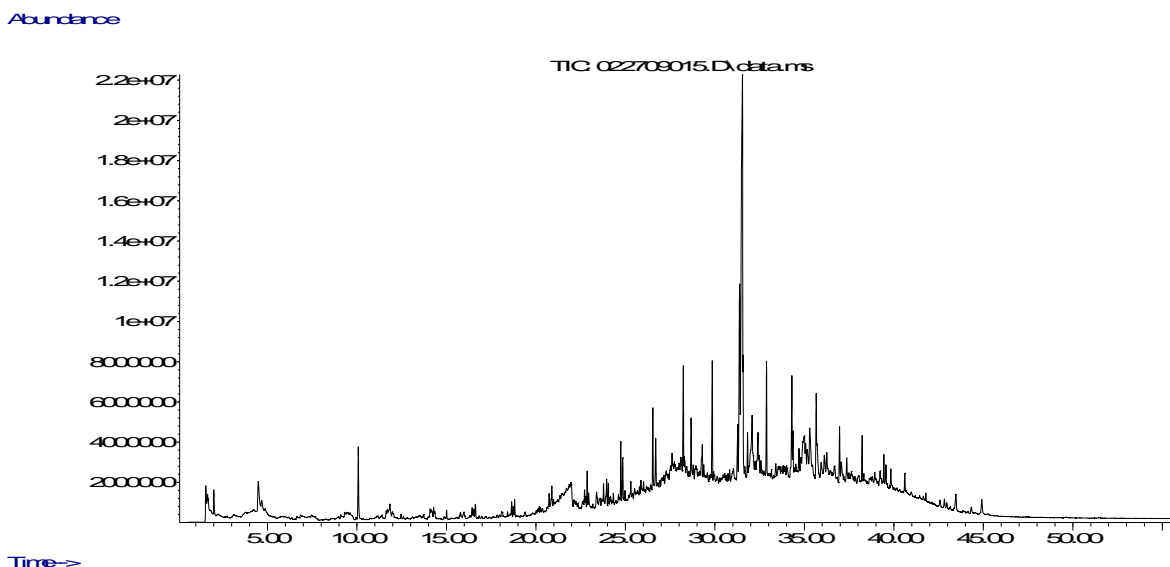


Figure 1. Py-GC/MS chromatograph of the sludge sample (May 30, 2008)

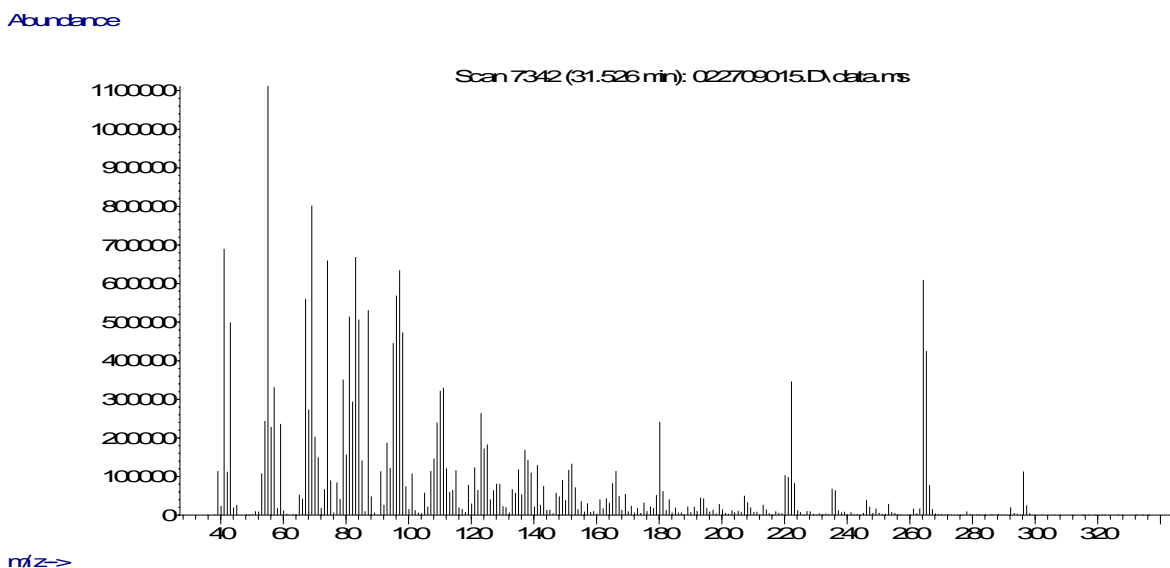


Figure 2. The MS pattern of 8-Octadecenoic acid, methyl ester (31.5 min)

1.2 TGA analysis

TGA analysis was also used to characterize the organic materials in this sludge sample. TGA analysis was conducted using a Mettler-Toledo TGA/SDTA851. Approximately 5-10 mg of sample was loaded into an aluminum pan and vaporized at a temperature range of 25-600 °C and a rate of 10 °C/min. The samples were run under nitrogen atmosphere at flow rate of 20 ml/min.

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Figure 3 shows a derivative thermogravimetric (DTG) curve converted from the TGA. DTG demonstrates the rate of weight change of the sample with temperature change. There were two distinctive peaks in the Figure 3, suggesting that the sludge consisted of two major fractions with different properties. One fraction was within temperature range between 430-490 °C. This fraction may contain heavy components. However, this was a small fraction, about 6% of the sludge. A large fraction of the sludge material evaporated within the temperature range below 250 °C. This temperature range suggests light compounds with low molecular weight including water. It is interesting that three subpeaks at 125, 130, and 136 °C were present. It is not clear what specific compounds these subpeaks relate to. Further research is required to determine specific compounds in this fraction, as it was the major part of the sludge sample.

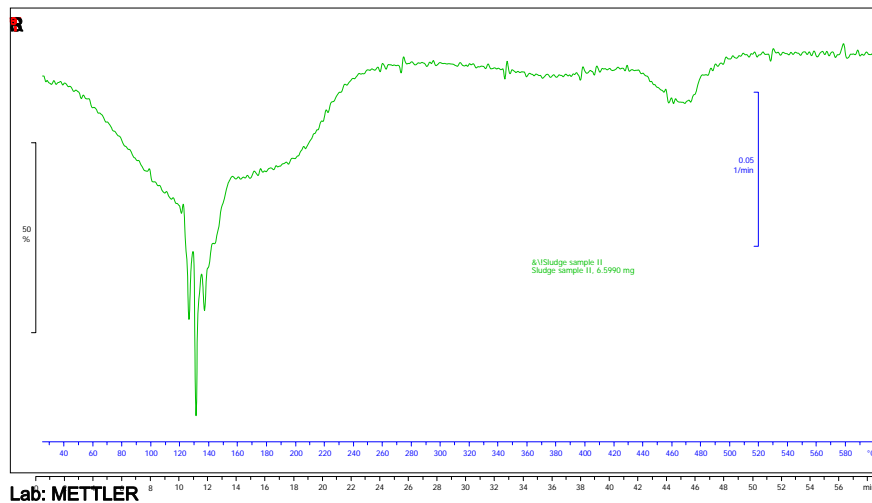


Figure 3. Derivative thermogravimetric (DTG) curve of the sludge sample (temperature change rate at 10.00°C/min within 25.0-600.0°C)

2. Water content in the sludge samples

Water content in the wet sludge was determined using the K-F titration method with a Titroline KF Titrator from Schott Instruments GmbH. Before the titration, the wet sludge samples at final concentration of 2.7% (w/v) were dispersed in organic solvents either chloroform (CHCl₃) or pyridine. These two solvents were chosen because the sludge

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appeared to be dispersed well in the solvents. The water content in the wet sludge was calculated by the content of sludge in solvents and K-F titration.

Table 1 shows the results of water content in the wet sludge samples. Water content in the solvent chloroform was 0.315 % (w/v), as obtained by K-F titration. As water solubility in chloroform was 0.795% (w/w), which is higher than the water content determined in the chloroform, water in the sludge sample did not saturate in the chloroform. Thus the K-F titration could estimate water content in the sludge from the chloroform sample. Water content in the wet sludge was approximately 11.7 % (w/w) determined from the solvent chloroform as the sludge content was 2.7 % (w/v) in the chloroform. Another solvent used was pyridine which is miscible with water. Water in the sludge could dissolve in solvent pyridine. Water content in the wet sludge was approximately 17.2 % (w/w). Therefore water is a fraction of the wet sludge in the range of 11-17 % (w/w).

Table 1. Water content in the wet sludge

Solvent	Chloroform	Pyridine
H ₂ O in wet sludge % (w/w)	11.7 ± 1.1	17.2 ± 2.2

3. Microbial role in the sludge formation

The objectives of this effort were to investigate the presence of active microbes in the sludge, isolation of microbes from the sludge, and biocide influence on microbial growth,

3.1 Observation of the sludge samples under a microscope

A sludge sample from the purifier of the *Tillikum* was collected on July 15, 2008. In order to look at the micro structure and microbial presence, this sludge sample was observed under a microscope. A typical image of the sludge is shown in Figure 4. It appears that some separated micro domains were present in this sample. Sizes of the micro domains typically ranged from 30-150 μ m. In addition, a great number of active bacteria were found in the micro domains, as shown in Figure 5. Some had a round shape and others

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had a rod shape. It appears that there were several bacteria species present in the samples. No yeast or fungi were observed in this sludge sample.

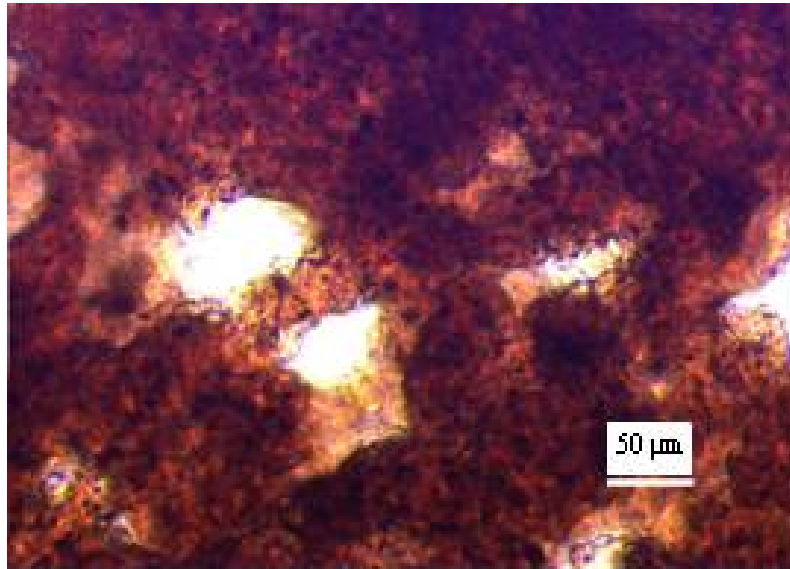


Figure 4. Image of sludge from the purifier of the *Tillikum*
(Separated micro domains appear white)

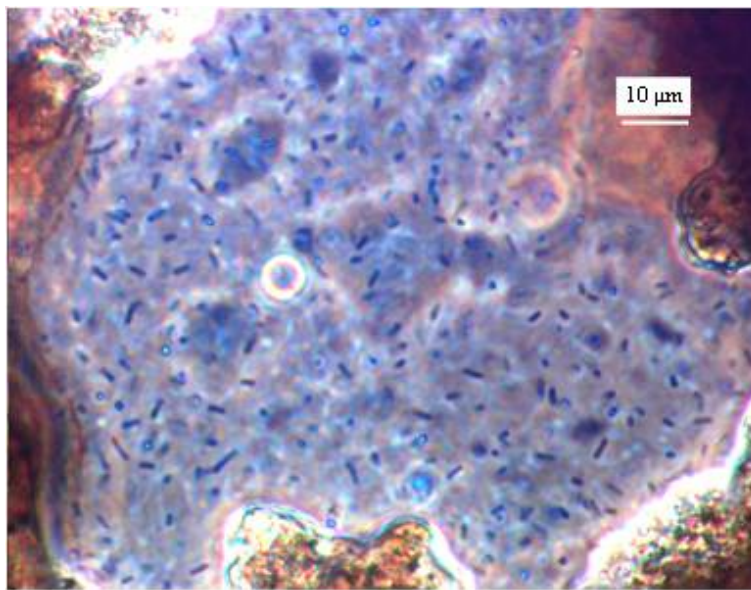


Figure 5. Images of bacteria in the sludge from the purifier of the *Tillikum*
(Active bacteria appear blue and are rod and round in shape)

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Another sludge sample from the purifier of the *Issaquah* collected July 30, 2008, was also observed under a microscope. Again, many active bacteria were found in the sludge.

The microbial tests on the sludge were conducted with test kits. The results were shown to be microbial positive, which further confirms that active microbes were present in the sludge samples. However, the B5 fuel samples tested negative, so it did not appear that the microbes were coming from the fuel supplier

3.2 Isolation of microbes from the sludge of the Tillikum purifier

To identify microbes from the sludge of the *Tillikum* purifier collected on July 15, 2008, four types of solid media for microbial growth were applied. Plate count agar (PCA) was designed for detection of bacteria, potato dextran agar (PDA) was used for cultivation of fungi possibly present in the sludge, malt extract agar (MEA) was used mainly for cultivation of potential yeasts grown in the sludge, and anaerobic agar (AA) was designated for observation of microorganisms that could grow under anaerobic conditions. PCA contained pancreatic digest of casein, yeast extract, dextrose, and agar; PDA contained potato starch, dextrose, and agar; MEA contained maltose, dextrose, glycerol, peptone, and agar; and AA contained agar with casein Peptone, sodium chloride, dextrose, sodium thioglycollate, soy Peptone, L-cystine, agar, sodium sulfoxyl formaldehyde, and methylene blue.

In order to obtain a microbial count in the sludge, 0.10 grams of wet sludge was weighed under sterile conditions and then suspended in 1.0 mL of deionized water (DI water). The samples were then mixed by vortexing for ten minutes. 10 μ L of the suspension samples were diluted into 50 mL DI water and shaken by hand for approximately two minutes. 100 μ L of the diluted samples were spread onto culture plates and incubated at 30 °C for two days.

Large numbers of bacterial colonies grew on each type of medium. However, no fungi or yeast colonies were found on the plates. Thus, bacteria were the dominant microorganisms in the sludge. Table 2 shows the results of quantitative analysis by cell

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count. The number of bacteria in the sludge from three types of culture attained a level of 10^8 per gram of wet sludge. The bacteria also grew well in both anaerobic and aerobic conditions without a significant difference.

Table 2. Bacteria numbers in the sludge on the cultural media

Medium type	Culture condition	Microbial number per gram of wet sludge (mean value in triplicate samples)
Anaerobic agar	anaerobic	5.28×10^7
PCA	aerobic	2.43×10^8
Two-layers of PCA	anaerobic	1.44×10^8
	aerobic	1.51×10^8

3.3 Isolation of viscous material from aqueous solution from the *Tillikum* purifier

High viscosity in the aqueous solution was observed in presence of excess sludge in the *Tillikum* purifier. It was speculated that polysaccharides were produced by the microbes and that provided high viscosity in the solution. Isolation of the viscous material from the water was attempted.

The water sample from the purifier of the *Tillikum* was centrifuged at 10,000 rpm for 20 minutes. Any insoluble materials were removed from the sample. Ethanol at a final concentration of 50% (v/v) was added into the supernatant of the aqueous solution. Some precipitates were formed and obtained after centrifugation. This procedure was repeated one more time, dissolving the precipitates with deionized water to precipitate the materials with ethanol and to obtain the materials by centrifugation. Finally, a small amount of solid material was obtained after drying at 105 °C overnight.

Ion chromatography (IC) analysis was used to identify monosaccharides from the sample. Samples were hydrolyzed in 1.0 M H₂SO₄ at 100 °C for 2 hours and diluted to obtain monosaccharide before the IC analysis. The measurement concentrations of five sugars,

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including arabinose, galactose, glucose, xylose, and fructose, were used in a standard addition protocol. All samples, including standard solutions of sugars, were filtered through 0.25 μm pore polycarbonate membranes (Nuclepore Corp., Pleasanton, CA). High-grade deionized water (18 Mohm/cm) passed through an organics removal cartridge (Unipure I system; Solution Consultants Inc., Marietta, GA) was employed throughout.

All sugar analysis was carried out using a Dionex ICS-3000 reagent free dual ion chromatography (IC) system (Sunnyvale, CA, USA), which was comprised of a DP dual gradient pump module, an EG dual eluent generator (with one KOH reservoir cartridge in use for this work) and a DC detector/chromatography module with three programmable high-pressure six-port injector valves. Briefly, the mobile phase, at a flow rate of 1.0 mL min^{-1} , consisted of ultrapure water (0.015 $\mu\text{S cm}^{-1}$; eluent A) and 250 mM NaOH (eluent B), with the following gradient: 0.0 min: 87% A, 13% B; 20.0 min: 87% A, 13% B; 40.0 min: 15% A, 85% B; 41.0 min: 100% B; 49.0 min: 100% B; 50.0 min: 87% A, 13% B; and 65.0 min: 87% A, 13% B. Due to matrix interference, quantification was carried out with standard addition.

Table 3 shows the results of the monosaccharide composition in the aqueous solution. Galactose and glucose were found in this sample. The detection of two sugars supported the presence of polysaccharides which are typically produced by bacteria. However, further research is needed to understand the role of the polysaccharides in the sludge formation

Table 3. Monosaccharide composition analysis in the aqueous solution by IC

Sample	Arabinose	Galactose	Glucose	Xylose	Fructose
Sludge	-	+	+	-	-
Liquid					

Note: “-” indicated no sugar was detected; “+” indicated sugar was detected.

3.4 Effect of biocide application on the microbial growth

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In order to simulate ferry conditions, biodiesel blend (canola based B5) was used in this batch culture. Table 4 shows the compositions of seven samples tested. The canola B5 was made by blending canola B100 from Imperium Renewable and ultra low sulfur diesel purchased on the local market. Deionized water was autoclaved at 121 °C for 15 minutes. The phase ratio of the oil to aqueous solution was 2:1 for all samples. In addition, biocide obtained from WSF at four different levels was added into some samples (samples #15 through #18). The biocide dose in sample #17 was the maintenance level used in the WSF test. Thus, sample #15 and sample #16 have higher levels of dosages, while sample #18 has a lower level of the dosage. All samples (except Sample #10) were inoculated with the WSF sludge. The seven samples described above were shaken at 190 rpm at 30 °C for four days to obtain microbial growth.

Table 4. Effect of biocide addition on bacteria growth

Sample ID	#10	#11	#13	#15	#16	#17	#18
Canola B5 (mL)	60	60	60	60	60	60	60
Water (mL)	30	30	30	30	30	30	30
Biocide concentration based on B5 (ml/L)	0	0	0	0.5	0.2	0.1	0.05
Sludge inoculation	NO	YES ^a	YES ^b	YES ^b	YES ^b	YES ^b	YES ^b

a: Sludge collected on May 30, 2008

b: Sludge used for all other samples collected on July 15, 2008

Figure 6 shows a photo of the seven samples after the liquid culture. Microbial growth appeared in all of the samples, except sample #10, which was a blank. Biofilm at the interface between oil and water phases was formed in the samples inoculated with the sludge. Bacteria in the biofilm were observed under a microscope. There appeared to be less biofilm at the higher levels of biocide in samples #15 and #16. However, microbial growth occurred in the four samples from samples #15 through #18 in the presence of the biocide. Thus, the biocide used here could not stop microbial growth under certain conditions, although it might inhibit microbial growth to some extent.

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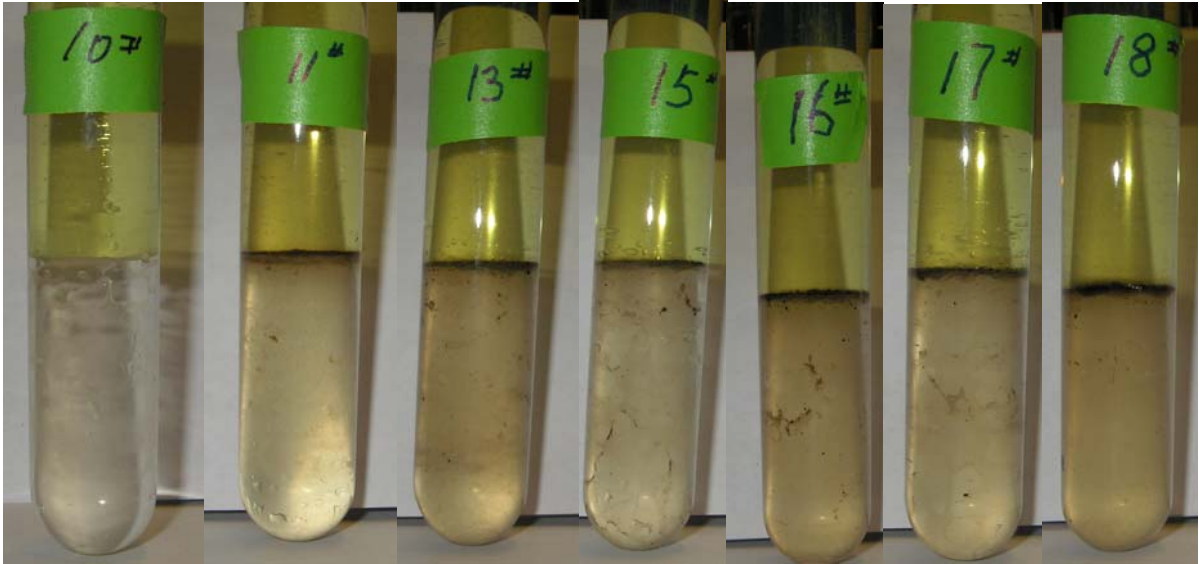


Figure 6. Microbial culture inoculated with sludge

In summary, lab research results showed that the sludge samples contained metal, microbes, water, and oil fractions (such as 8-Octadecenoic acid methyl ester) from canola biodiesel and light compounds possibly from diesel. Active bacteria were present in the sludge samples from the purifiers. The bacteria can grow in the presence of the B5 fuel and water. The bacterial contamination is one of major causes of the excess sludge formation. Thus, biocide application was recommended for inhibition of microbial growth in the pilot test. The biocide studied inhibited bacterial growth but did not stop bacterial growth in laboratory conditions. Thus, this biocide might not be the optimal choice for this ferry application.

4. Microbial identification

It is noted that the bacteria in the sludge could contain several types of strains. Isolation of the bacteria was conducted using plate streaking and gradient dilution methods. Five types of bacterial strains which could be dominant in the sludge sample were obtained after the isolation. They are designated as P1, P2, P4, P8, and P8-2. Identification of these five strains was done using molecular biological methods, including DNA extraction, 16s rRNA amplification, 16s rRNA gene clone, and DNA sequencing and sequence analysis. While three of the strains were identified, the rest of two are still being investigated.

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4.1 DNA extraction, 16s rRNA amplification and sequencing

The isolates were cultured in liquid Luria-Bertani medium at 37 °C overnight. The cells in mid-log phase were harvested by centrifugation at 12,000 rpm for 2 min. DNA was extracted using the Wizard genomic DNA purification kit (Promega, USA).

Internal fragments of 16s rRNA were amplified from genomic DNA using universal primers 8f and 926r (based on E.coli 16s rRNA positions). Amplification was performed in 50 µl (total volume) reactions that contained 20 ng (1 µl) of sample DNA, 1 U of AmpliTaq DNA polymerase, 1× AmpliTaq reaction buffer, 1.5 mM MgCl₂, 100 mM dNTP, 5% DMSO and 0.05 mM of each primer. Initial DNA was denatured at 94 °C in a PTC-100 Programmable Thermal Controller for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min and annealing at 72 °C for 2 min, which was followed by a final extension at 72 °C for 10 min.

The recombinant plasmids contained the 16s rRNA insert were sequenced with bacterial 926r and 8f primers. The insert sequences were determined using Big Dye ver. 3 cycles of sequencing reactions, and resolved using an automatic sequencer (3100 PRISM Genetic Analyzer). Sequences were trimmed to exclude the PCR primer sites corrected with Chromas 2 (Chromas Version 2.22; <http://www.techne.lysium.com.au/chromas.html>). For identification with the closet relatives, inserted sequences were compared to those available GenBank (<http://www.ncbi.nlm.nih.gov>) databases. The CLUSTAL X program (version 1.83) was used to align the target sequences with reference sequences, and phylogenetic trees were constructed based on neighbor-joining method by the software package MEGA.

4.2 16srDNA identification and characteristics of the isolates

Phylogenetic positions of the three stains were shown in Figure 7. By analyzing the partial 16sRNA gene sequences with reference sequences of related genus, phylogenetic trees were constructed as mentioned in the methods. P1 was found to show 99% homogeneity with *Klebsiella oxytoca*. Thus it was tentatively referred to as *Klebsiella*

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oxytoca strain P1. P2 showed 98% of homogeneity with *Klebsiella pneumoniae*, and also displayed different biochemical and physiological characteristics than *Klebsiella pneumoniae*. For example, the cell surface of P2 was not as rough as the reference. Most importantly, P2 did not produce any exopolysaccharides, while *Klebsiella pneumoniae* commonly does. Thus, it was tentatively assigned as *Klebsiella* novel species strain P2. The coccus P8-2 showed 99% homogeneity with *Staphylococcus epidermidis*, therefore it was referred to as *Staphylococcus epidermidis* strain P8-2.

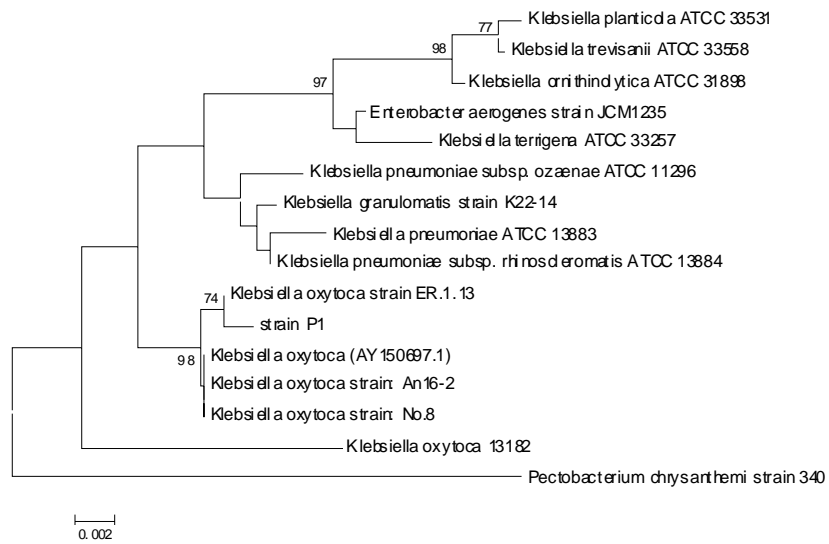
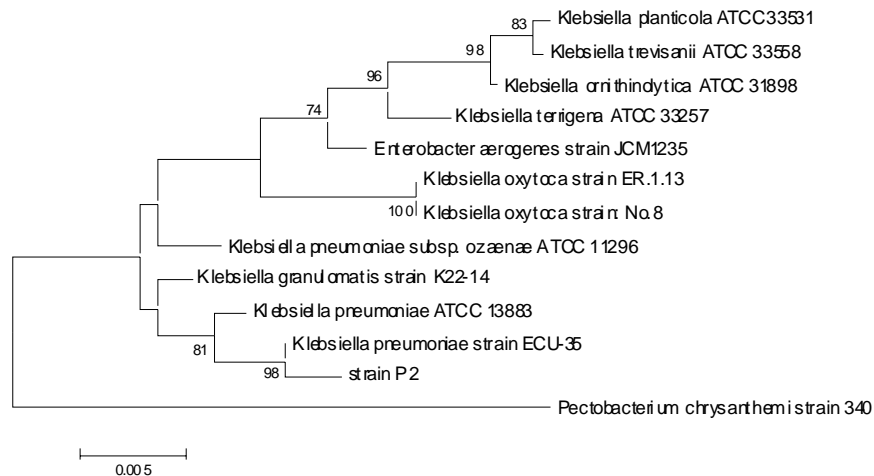


Figure 7. (A) Phylogenetic positions of the strain P1



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Figure 7. (B) Phylogenetic positions of the strain P2

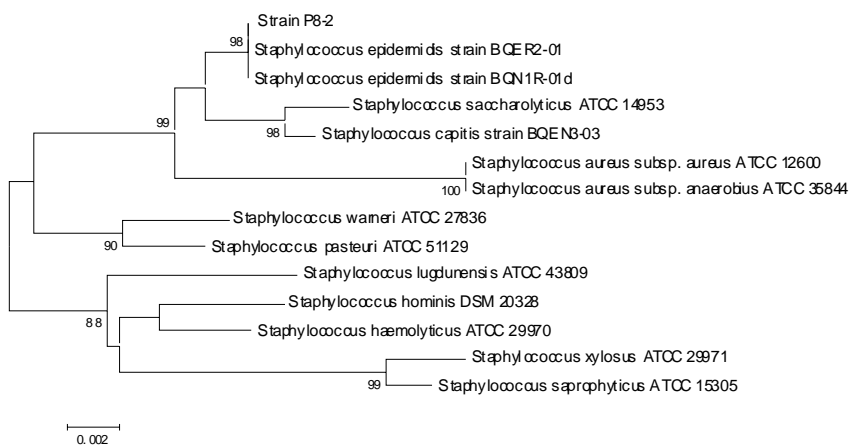


Figure 7. (C) Phylogenetic positions of the strain P8-2

In summary, three bacteria of the five strains were identified as *Staphylococcus epidermidis*, *Klebsiella oxytoca*, and a potentially novel strain of *Klebsilla*. The three identified bacteria are opportunistic disease-causing microorganisms. It appeared that none of these three microbes had been reported in contaminated diesel fuel or soil environments. Identification of the other two strains will require further research. Possible reasons that the strains could not be identified are that single colonies were not isolated from the samples and that high viscosity in these two samples made purifying the strains difficult.

5. Conclusions

Excess sludge was formed in the purifier of the *Tillikum* when the vessel burned the canola-based B5. The sludge sample studied contained metal (~11% ash), water (11-17%), major fractions of organic materials including 8-Octadecenoic acid methyl ester from canola biodiesel, and bacteria. The number of bacteria in the sludge attained a level of 10^8 per gram of wet sludge. Bacteria in the sludge could be grown in both anaerobic and aerobic conditions in the culture media. Galactose and glucose were detected in the aqueous solution from the purifier, suggesting the presence of viscous polysaccharides produced by the bacteria. Three bacteria of the five dominant strains were identified as

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Staphylococcus epidermidis, *Klebsiella oxytoca*, and a potentially novel strain of *Klebsilla*. The bacteria played a key role in the sludge formation. While microbial growth in the ferry fuel vessel tanks is one of the major causes for excessive sludge formation resulting in filter clogging, the problem of excess sludge was solved by application of biocide in the fuel during the testing period. Biocide application is strongly recommended when biodiesel blend fuels are used in marine ferry conditions.