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APPENDIX 4 TO SUBPART A OF PART 435—PROTOCOL FOR THE DETERMINATION OF DEGRADATION OF NON-AQUEOUS BASE FLUIDS IN A MARINE CLOSED BOTTLE BIODEGRADATION TEST SYSTEM: MODIFIED ISO 11734:1995 (EPA METHOD 1647)

1.0. SUMMARY OF EPA METHOD 1647

a. This method determines the anaerobic degradation potential of mineral oils, paraffin oils and non-aqueous fluids (NAF) in sediments. These substrates are base fluids for formulating offshore drilling fluids. The test evaluates base fluid biodegradation rates by monitoring gas production due to microbial degradation of the test fluid in natural marine sediment.

b. The test procedure places a mixture of marine/estuarine sediment, test substrate (hydrocarbon or controls) and seawater into clean 120 mL (150 mL actual volume) Wheaton serum bottles. The test is run using four replicate serum bottles containing 2,000 mg carbon/kg dry weight concentration of test substrate in sediment. The use of resazurin dye solution (1 ppm) evaluates the anaerobic (redox) condition of the bottles (dye is blue when oxygen is present, reddish in low oxygen conditions and colorless if oxygen free). After capping the bottles, a nitrogen sparge removes air in the headspace before incubation begins. During the incubation period, the sample should be kept at a constant temperature of  $29 \pm 1$  °C. Gas production and composition is measured approximately every two weeks. The samples need to be brought to ambient temperature before making the measurements. Measure gas production using a pressure gauge. Barometric pressure is measured at the time of testing to make necessary volume adjustments.

c. ISO 11734:1995 specifies that total gas is the standard measure of biodegradation. While modifying this test for evaluating biodegradation of NAFs, methane was also monitored and found to be an acceptable method of evaluating biodegradation. Section 7 contains the procedures used to follow biodegradation by methane production. Measurement of either total gas or methane production is permitted. If methane is followed, determine the composition of the gas by using gas chromatography (GC) analysis at each sampling. At the end of the test when gas production stops, or at around 275 days, an analysis of sediment for substrate content is possible. Common methods which have been successfully used for analyzing NAFs from sediments are listed in Section 8.

2.0 SYSTEM REQUIREMENTS

This environmental test system has three phases, spiked sediment, overlying seawater, and a gas headspace. The sediment/test compound mixture is combined with synthetic sea water and transferred into 120-mL serum bottles. The total volume of sediment/sea water mixture in the bottles is 75 mL. The volume of the sediment layer will be approximately 50 mL, but the exact volume of the sediment will depend on sediment characteristics (wet:dry ratio and density). The amount of synthetic sea water will be calculated to bring the total volume in the bottles to 75 mL. The test systems are maintained at a temperature of  $29 \pm 1$  °C during incubation. The test systems are brought to ambient temperatures prior to measuring pressure or gas volume.

2.1 SAMPLE REQUIREMENTS

a. The concentration of base fluids are at least 2,000 mg carbon test material/kg dry sediment. Carbon concentration is determined by theoretical composition based on the chemical formula or by chemical analysis by ASTM D5291-96. Sediments with positive, intermediate and negative control substances as well as a C<sub>16</sub>-C<sub>18</sub> internal olefin type base fluid will be run in conjunction with test materials under the same conditions. The positive control is ethyl oleate (CAS 111-62-6), the intermediate control is 1-hexadecene (CAS 629-73-2), and the negative control is squalane (CAS 111-01-3). Controls must be of analytical grade or the highest grade available. Each test control concentration should be prepared according to the mixing procedure described in Section 3.1.

b. Product names will be used for examples or clarification in the following text. Any use of trade or product names in this publication is for descriptive use only, and does not constitute endorsement by EPA or the authors.

2.2. SEAWATER REQUIREMENTS

Synthetic seawater at a salinity of  $25 \pm 1$  ppt should be used for the test. The synthetic seawater should be prepared by mixing a commercially available artificial seawater mix, into high purity distilled or de-ionized water. The seawater should be aerated and allowed to age for approximately one month prior to use.

2.3. SEDIMENT REQUIREMENTS

a. The dilution sediment must be from a natural estuarine or marine environment and be free of the compounds of interest. The collection location, date and time will be documented and reported. The sediment is prepared by press-sieving through a 2,000-micron mesh sieve to remove large debris, then press-sieving through a 500-micron sieve to

remove indigenous organisms that may confound test results. The water content of the sediment should be less than 60% (w/w) or a wet to dry ratio of 2.5. The sediment should have a minimum organic matter content of 3% (w/w) as determined by ASTM D2974-07a (Method A and D and calculate organic matter as in Section 8.3 of method ASTM D2974-07a).

b. To reduce the osmotic shock to the microorganisms in the sediment the salinity of the sediment's pore water should be between 20-30 ppt. Sediment should be used for testing as soon as possible after field collection. If required, sediment can be stored in the dark at 4 °C with 3-6 inches of overlying water in a sealed container for a maximum period of 2 months prior to use.

### 3.0 TEST SET UP

The test is set up by first mixing the test or control substrates into the sediment inoculum, then mixing in seawater to make a pourable slurry. The slurry is then poured into serum bottles, which are then flushed with nitrogen and sealed.

### 3.1. MIXING PROCEDURE

Because base fluids are strongly hydrophobic and do not readily mix with sediments, care must be taken to ensure base fluids are thoroughly homogenized within the sediment. All concentrations are weight-to-weight comparisons (mg of base fluid to kg of dry control sediment). Sediment and base fluid mixing will be accomplished by using the following method.

3.1.1. Determine the wet to dry weight ratio for the control sediment by weighing approximately 10 sub-samples of approximately 1 g each of the screened and homogenized wet sediment into tared aluminum weigh pans. Dry sediment at 105 °C for 18-24 h. Remove the dried sediments and cool in a desiccator. Repeat the drying, cooling, and weighing cycle until a constant weight is achieved (within 4% of previous weight). Re-weigh the samples to determine the dry weight. Calculate the mean wet and dry weights of the 10 sub samples and determine the wet/dry ratio by dividing the mean wet weight by the mean dry weight using Equation 5-1. This is required to determine the weight of wet sediment needed to prepare the test samples.

$$\frac{\text{Mean Wet Sediment Weight (g)}}{\text{Mean Dry Sediment Weight (g)}} = \text{Wet to Dry Ratio} \quad [\text{Eq.1}]$$

3.1.2. Determine the density (g/ml) of the wet sediment. This will be used to determine total volume of wet sediment needed for the various test treatments. One method is to tare a 5 ml graduated cylinder and add about 5 ml of homogenized sediment. Carefully

record the volume then weigh this volume of sediment. Repeat this a total of three times. To determine the wet sediment density, divide the weight by volume per the following formula:

$$\frac{\text{Mean Wet Sediment Weight (g)}}{\text{Mean Wet Sediment Volume (mL)}} = \text{Wet Sediment Density (g/mL)} \quad [\text{Eq. 2}]$$

3.1.3. Determine the amount of base fluid to be spiked into wet sediment in order to obtain the desired initial base fluid concentration of 2,000 mg carbon/kg dry weight. An amount of wet sediment that is the equivalent of 30 g of dry sediment will be added to each bottle. A typical procedure is to prepare enough sediment for 8 serum bottles (3 bottles to be sacrificed at the start of the test, 4 bottles incubated for headspace analysis, and enough extra sediment for 2 extra bottles). Extra sediment is needed be-

cause some of the sediment will remain coated onto the mixing bowl and utensils. Experience with this test may indicate that preparing larger volumes of spiked sediment is a useful practice, then the following calculations should be adjusted accordingly.

a. Determine the total weight of dry sediment needed to add 30 g dry sediment to 8 bottles. If more bottles are used then the calculations should be modified accordingly. For example:

$$30 \text{ g dry sediment per bottle} \times 8 = 240 \text{ g dry sediment} \quad [\text{Eq. 3}]$$

b. Determine the weight of base fluid, in terms of carbon, needed to obtain a final base fluid concentration of 2,000 mg carbon/kg dry weight. For example:

$$\frac{2,000 \text{ mg carbon}}{\text{Per kg dry sediment}} \times \frac{240 \text{ g}}{1,000} = 480 \text{ mg carbon} \quad [\text{Eq. 4}]$$

c. i. Convert from mg of carbon to mg of base fluid. This calculation will depend on the % fraction of carbon present in the molecular structure of each base fluid. For the control fluids, ethyl oleate is composed of 77.3% carbon, hexadecene is composed of 85.7% carbon, and squalane is composed of 85.3% carbon. The carbon fraction of each base fluid should be supplied by the manufacturer or determined before use. ASTM D5291-

96 or equivalent will be used to determine composition of fluid.

ii. To calculate the amount of base fluid to add to the sediment, divide the amount of carbon (480 mg) by the percent fraction of carbon in the fluid.

iii. For example, the amount of ethyl oleate added to 240 g dry weight sediment can be calculated from the following equation:

$$\frac{480 \text{ mg carbon}}{(77.3 \div 100)} = 621 \text{ mg ethyl oleate} \quad [\text{Eq. 5}]$$

iv. Therefore, add 621 mg of ethyl oleate to 240 g dry weight sediment for a final concentration of 2,000 mg carbon/kg sediment dry weight.

3.1.4. Mix the calculated amount of base fluid with the appropriate weight of wet sediment.

a. Use the wet:dry ratio to convert from g sediment dry weight to g sediment wet weight, as follows:

$$240 \text{ g dry sediment} \times \text{wet:dry ratio} = \text{g wet sediment needed} \quad [\text{Eq. 6}]$$

b. i. Weigh the appropriate amount of base fluid (calculated in Section 3.1.3.c) into stainless mixing bowls, tare the vessel weight, then add the wet sediment calculated in Equation 5, and mix with a high shear dispersing impeller for 9 minutes.

ii. The sediment is now mixed with synthetic sea water to form a slurry that will be transferred into the bottles.

### 3.2. Creating Seawater/Sediment Slurry

Given that the total volume of sediment/sea water slurry in each bottle is to be 75 mL, determine the volume of sea water to add to the wet sediment.

3.2.1. If each bottle is to contain 30 g dry sediment, calculate the weight, and then the volume, of wet sediment to be added to each bottle.

$$30 \text{ g dry sediment} \times \text{wet:dry ratio} = \text{g wet sediment added to each bottle} \quad [\text{Eq. 7}]$$

$$\frac{\text{g wet sediment}}{\text{Density (g/mL) of wet sediment}} = \text{mL wet sediment} \quad [\text{Eq. 8}]$$

3.2.2. Calculate volume of sea water to be added to each bottle.

$$75 \text{ mL total volume} - \text{mL wet sediment (from Eq. 8)} = \text{mL of sea water} \quad [\text{Eq. 9}]$$

3.2.3. Determine the ratio of sea water to wet sediment (volume:volume) in each bottle.

$$\frac{\text{Volume sea water per bottle (Eq. 9)}}{\text{Volume sediment water per bottle (Eq. 8)}} = \text{Ratio of sea water:wet sediment} \quad [\text{Eq. 10}]$$

3.2.4. Convert the wet sediment weight from Equation 6 into a volume using the sediment density.

$$\text{g wet sediment (Eq. 6) density} = \text{volume (mL) of sediment} \quad [\text{Eq. 11}]$$

3.2.5. Determine the amount of sea water to mix with the wet sediment.

$$\text{mL wet sediment (Eq. 11)} \times \frac{\text{Sea water:sediment ratio (Eq. 10)}}{\text{Density (Eq. 11)}} = \text{mL sea water to add to wet sediment} \quad [\text{Eq. 12}]$$

Mix sea water thoroughly with wet sediment to form a sediment/sea water slurry.

3.3. Bottling the Sediment Seawater Slurry  
The total volume of sediment/sea water slurry in each bottle is to be 75 mL. Convert

the volume (mL) of sediment/sea water slurry into a weight (g) using the density of the sediment and the seawater.

3.3.1. Determine the weight of sediment to be added to each bottle.

$$\text{mL sediment (Eq. 8)} \times \text{density of wet sediment (g/mL)} = \text{g wet sediment} \quad [\text{Eq. 13}]$$

3.3.2. Determine the weight of sea water to be added to each bottle.

$$\text{mL sea water (Eq. 9)} \times \text{density of sea water (1.01 g/mL)} = \text{g sea water} \quad [\text{Eq. 14}]$$

3.3.3. Determine weight of sediment/sea water slurry to be added to each bottle.

$$\text{g wet sediment (Eq. 13)} + \text{g sea water (Eq. 14)} = \text{g sediment/sea water slurry} \quad [\text{Eq. 15}]$$

This should provide each bottle with 30 g dry sediment in a total volume of 75 mL.

3.3.4. Putting the sediment:seawater slurry in the serum bottles.

a. NOTE: The slurry will need to be constantly stirred to keep the sediment suspended.

b. Place a tared serum bottle on a balance and add the appropriate amount of slurry to the bottle using a funnel. Once the required slurry is in the bottle remove the funnel, add 2–3 drops (25  $\mu$ L) of a 1 gram/L resazurin dye stock solution. Cap the bottle with a butyl rubber stopper (Bellco Glass, Part #2048–11800) and crimp with an aluminum seal (Bellco Glass Part #2048–11020).

c. Using a plastic tube with a (23-gauge, 1-inch long) needle attached to one side and a nitrogen source to the other, puncture the serum cap with the needle. Puncture the serum cap again with a second needle to sparge the bottle's headspace of residual air for two minutes. The nitrogen should be flowing at no more than 100 mL/min to encourage gentle displacement of oxygenated air with nitrogen. Faster nitrogen flow rates would cause mixing and complete oxygen removal would take much longer. Remove the nitrogen needle first to avoid any initial pressure problems. The second (vent) needle should be removed within 30 seconds of removing the nitrogen needle.

d. Triplicate blank test systems are prepared, with similar quantities of sediment and seawater without any base fluid. Incubate in the dark at a constant temperature of  $29 \pm 1$  °C.

e. Record the test temperature. The test duration is dependent on base fluid performance, but at a maximum should be no more than 275 days. Stop the test after all base fluids have achieved a plateau of gas production. At termination, base fluid concentrations can be verified in the terminated samples by extraction and GC analysis according to Section 8.

#### 4.0. CONCENTRATION VERIFICATION CHEMICAL ANALYSES

a. Because of the difficulty of homogeneously mixing base fluid with sediment, it is important to demonstrate that the base fluid is evenly mixed within the sediment sea water slurry that was added to each bottle. Of the seven serum bottles set up for each test or control condition, three are randomly selected for concentration verification analyses. These should be immediately placed at 4 °C and a sample of sediment from each bottle should be analyzed for base fluid content as soon as possible. The coefficient of variation (CV) for the replicate samples must be less than 20%. The results should show recovery of at least 70% of the spiked base fluid. Use an appropriate analytical procedure described in Section 8 to per-

form the extractions and analyses. If any set of sediments fail the criteria for concentration verification, then the corrective action for that set of sediments is also outlined in Section 8.

b. The nominal concentrations and the measured concentrations from the three bottles selected for concentration verification should be reported for the initial test concentrations. The coefficient of variation (CV) for the replicate samples must be less than 20%. If base fluid content results are not within the 20% CV limit, the test must be stopped and restarted with adequately mixed sediment.

#### 5.0. GAS MONITORING PROCEDURES

Biodegradation is measured by total gas as specified in ISO 11734:1995. Methane production can also be tracked and is described in Section 7.

##### 5.1. TOTAL GAS MONITORING PROCEDURES

*Bottles should be brought to room temperature before readings are taken.* a. The bottles are observed to confirm that the resazurin has not oxidized to pink or blue. Total gas production in the culture bottles should be measured using a pressure transducer (one source is Biotech International). The pressure readings from test and control cultures are evaluated against a calibration curve created by analyzing the pressure created by known additions of gas to bottles established identically to the culture bottles. Bottles used for the standard curve contain 75 mL of water, and are sealed with the same rubber septa and crimp cap seals used for the bottles containing sediment. After the bottles used in the standard curve have been sealed, a syringe needle inserted through the septa is used to equilibrate the pressure inside the bottles to the outside atmosphere. The syringe needle is removed and known volumes of air are injected into the headspace of the bottles. Pressure readings provide a standard curve relating the volume of gas injected into the bottles and headspace pressure. No less than three points may be used to generate the standard curve. A typical standard curve may use 0, 1, 5, 10, 20 and 40 mL of gas added to the standard curve bottles.

b. The room temperature and barometric pressure (to two digits) should be recorded at the time of sampling. One option for the barometer is Fisher Part #02–400 or 02–401. Gas production by the sediment is expressed in terms of the volume (mL) of gas at standard temperature (0 °C = 273°K) and pressure (1 atm = 30 inches of Hg) using Eq. 16.

$$V_2 = \frac{P_1 \times V_1 \times T_2}{T_1 \times P_2} \quad [\text{Eq. 16}]$$

Where:

$V_2$  = Volume of gas production at standard temperature and pressure

$P_1$  = Barometric pressure on day of sampling (inches of Hg)

$V_1$  = Volume of gas measured on day of sampling (mL)

$T_2$  = Standard temperature = 273 °K

$T_1$  = Temperature on day of sampling ( °C + 273 = °K)

$P_2$  = Standard pressure = 30 inches Hg

c. An estimate can be made of the total volume of anaerobic gas that will be produced in the bottles. The gas production measured for each base fluid can be expressed as a percent of predicted total anaerobic gas production.

5.1.1. Calculate the total amount of carbon in the form of the base fluid present in each bottle.

a. Each bottle is to contain 30 g dry weight sediment. The base fluid concentration is 2,000 mg carbon/kg dry weight sediment. Therefore:

$$2,000 \text{ mg carbon/kg sediment} \times (30 \text{ g} \div 1,000) = 60 \text{ mg carbon per bottle} \quad [\text{Eq. 17}]$$

5.1.2. Theory states that anaerobic microorganisms will convert 1 mole of carbon substrate into 1 mole of total anaerobic gas production.

a. Calculate the number of moles of carbon in each bottle.

b. The molecular weight of carbon is 12 (i.e., 1 mole of carbon = 12 g). Therefore, the number of moles of carbon in each bottle can be calculated.

$$\frac{60 \text{ mg carbon per bottle} / 1,000}{12 \text{ g/mole}} = 0.005 \text{ moles carbon} \quad [\text{Eq. 18}]$$

5.1.3. Calculate the predicted volume of anaerobic gas.

One mole of gas equals 22.4 L (at standard temperature and pressure), therefore,

$$0.005 \text{ moles} \times 22.4 \text{ L} = 0.112\text{L (or 112 mL total gas production)} \quad [\text{Eq. 19}]$$

5.2. GAS VENTING

a. If the pressure in the serum bottle is too great for the pressure transducer or syringe, some of the excess gas must be wasted. The best method to do this is to vent the excess gas right after measurement. To do this, remove the barrel from a 10-mL syringe and fill it 1/3 full with water. This is then inserted into the bottle through the stopper using a small diameter (high gauge) needle. The excess pressure is allowed to vent through the water until the bubbles stop. This allows equalization of the pressure inside the bottle to atmospheric without introducing oxygen. The amount of gas vented (which is equal to the volume determined that day) must be kept track of each time the bottles are vented. A simple way to do this in a spreadsheet format is to have a separate column in which

cumulative vented gas is tabulated. Each time the volume of gas in the cultures is analyzed, the total gas produced is equal to the gas in the culture at that time plus the total of the vented gas.

b. To keep track of the methane lost in the venting procedure, multiply the amount of gas vented each time by the corrected % methane determined on that day. The answer gives the volume of methane wasted. This must be added into the cumulative totals similarly to the total gas additions.

6.0. TEST ACCEPTABILITY AND INTERPRETATION

6.1. TEST ACCEPTABILITY

At day 275 or when gas production has plateaued, whichever is first, the controls are evaluated to confirm that the test has been performed appropriately. In order for

this modification of the closed bottle biodegradation test to be considered acceptable, all the controls must meet the biodegradation levels indicated in Table 1. The inter-

mediate control hexadecene must produce at least 30% of the theoretical gas production. This level may be reexamined after two years and more data has been generated.

TABLE 1—TEST ACCEPTABILITY CRITERIA

| Concentration            | Percent biodegradability as a function of gas measurement |                           |                                 |
|--------------------------|---|---------------------------|---------------------------------|
|                          | Positive control  | Squalane negative control | Hexadecene intermediate control |
| 2,000 mg carbon/kg ..... | ≥60% theoretical .....                                    | ≤5% theoretical .....     | ≥30% theoretical.               |

6.2 INTERPRETATION

a. In order for a fluid to pass the closed bottle test, the biodegradation of the base fluid as indicated by the total amount of total gas (or methane) generated once gas production has plateaued (or at the end of

275 days, which ever is first) must be greater than or equal to the volume of gas (or methane) produced by the reference standard (internal elefin or ester).

b. The method for evaluating the data to determine whether a fluid has passed the biodegradation test must use the equations:

$$\frac{\% \text{ Theoretical gas production of reference fluid}}{\% \text{ Theoretical gas production of NAF}} \leq 1.0 \quad [\text{Eq. 20}]$$

Where:

NAF = Stock base fluid being tested for compliance

Reference fluid = C<sub>16</sub>-C<sub>18</sub> internal olefin or C<sub>12</sub>-C<sub>14</sub> or C<sub>8</sub> ester reference fluid

7.0. METHANE MEASUREMENT

7.1. METHANE MONITORING PROCEDURES

a. The use of total gas production alone may result in an underestimation of the actual metabolism occurring since CO<sub>2</sub> is slightly soluble in water. An acceptable alternative method is to monitor methane production and total gas production. This is eas-

ily done using GC analysis. A direct injection of headspace gases can be made into a GC using almost any packed or capillary column with an FID detector. Unless volatile fuels or solvents are present in the test material or the inocula, the only component of the headspace gas that can be detected using an FID detector is methane. The percent methane in the headspace gas is determined by comparing the response of the sample injections to the response from injections of known percent methane standards. The percent methane is corrected for water vapor saturation using Eq. 21 and then converted to a volume of dry methane using Eq. 22.

$$\text{Corrected \% CH}_4 = \frac{\% \text{ CH}_4}{1 - \frac{D \times 22.4 \text{ L/mol}}{18 \text{ g/mol} \times 1,000}} \quad [\text{Eq. 21}]$$

Where:

D = The density of water vapor at saturation (g/m<sup>3</sup>, can be found in CRC Handbook of

Chemistry and Physics) for the temperature of sampling.

$$V_{\text{CH}_4} \text{ (ml)} = (S + V) \times \frac{P - P_w}{T + 273} \times \frac{\text{CH}_4}{100} \times \frac{273}{760} \quad [\text{Eq. 22}]$$

Where:

V<sub>CH<sub>4</sub></sub> = Volume of methane in the bottle

S = Volume of excess gas production (measured with a pressure transducer)  
 V = Volume of the headspace in the culture bottle (total volume—liquid phase)  
 P = Barometric pressure (mm Hg, measured with barometer)  
 T = Temperature (°C)  
 P<sub>w</sub> = Vapor pressure of water at T (mm Hg, can be found in CRC Handbook of Chemistry and Physics)  
 CH<sub>4</sub> = % methane in headspace gas (after correction for water vapor)

b. The total volume of serum bottles sold as 125 mL bottles (Wheaton) is 154.8 mL.  
 c. The volumes of methane produced are then compared to the volumes of methane in the controls to determine if a significant inhibition of methane production or a significant increase of methane production has been observed. Effective statistical analyses are important, as variability in the results is common due to the heterogeneity of the inoculum's source. It is also common to observe that the timing of the initiation of culture activity is not equal in all of the cul-

tures. Expect a great variability over the period when the cultures are active, some replicates will start sooner than others, but all of the replicates should eventually reach similar levels of base fluid degradation and methane production.

7.2. EXPECTED METHANE PRODUCTION CALCULATIONS

a. The amount of methane expected can be calculated using the equation of Symons and Buswell (Eq. 23). In the case of complete mineralization, all of the carbon will appear as wither CO<sub>2</sub> or CH<sub>4</sub>, thus the total moles of gas produced will be equal to the total moles of carbon in the parent molecule. The use of the Buswell equation allows you to calculate the effects the redox potential will have on the distribution of the products in methanogenic cultures. More reduced electron donors will allow the production of more methane, while more oxidized electron donors will cause a production of more carbon dioxide.

$$\frac{12 \text{ mole CH}_4}{\text{mole hexadecene}} \times \frac{22.4 \text{ L}}{\text{mole CH}_4} \times \frac{1,000}{\text{L}} \times \frac{1 \text{ mole hexadecene}}{224.4 \text{ g hexadecene}} \times \frac{23 \text{ g hexadecene}}{\text{kg dry soil}} \times \frac{0.03 \text{ kg}}{\text{culture}} = 84 \text{ (ml)} \quad [\text{Eq. 24}]$$

b. An example calculation of the expected methane volume in a culture fed 2,000 mg/kg hexadecene is as follows. The application of Symons and Buswell's equation reveals that hexadecene (C<sub>16</sub>H<sub>32</sub>) will yield 4 moles of CO<sub>2</sub>

and 12 moles of CH<sub>4</sub>. Assuming 30 g of dry sediment are added to the bottles with 2,334 mg hexadecene/kg dry sediment (*i.e.*, equivalent to 2,000 mg carbon/kg dry sediment) the calculation is as follows.

$$\frac{12 \text{ mole CH}_4}{\text{mole hexadecene}} \times \frac{22.4 \text{ L}}{\text{mole CH}_4} \times \frac{1,000}{\text{L}} \times \frac{1 \text{ mole hexadecene}}{224.4 \text{ g hexadecene}} \times \frac{23 \text{ g hexadecene}}{\text{kg dry soil}} \times \frac{0.03 \text{ kg}}{\text{culture}} = 84 \text{ (ml)} \quad [\text{Eq. 24}]$$

c. By subtracting the average amount of methane in control bottles from the test bottles and then dividing by the expected volume an evaluation of the completion of the process may be conducted.

8.0. CONCENTRATION VERIFICATION ANALYSIS

The Concentration Verification analysis is required at the beginning of the test to ensure homogeneity and confirm that the required amount of fluid was delivered to the sediments at the start of the test.

8.1. Three samples per fluid need to be analyzed and achieve ≤20% Coefficient of Variability and an average of ≥70% to ≤120% of fluid delivered to sediment.

8.2. If a third party performs the analysis, then the laboratory should be capable of de-

livering the homogeneity data within seven days, in order to identify any samples that do not meet the homogeneity requirement as quickly as possible.

8.3. If one sediment/fluid set, out a multiple set batch of samples, fails these criteria, then that one set of samples must be discarded and a fresh set of spiked sediment prepared, started, and analyzed to ensure homogeneity. The same stock sediment is used to prepare the replacement set(s). The remaining sets do not need to be re-mixed or restarted.

8.4. The re-mixed set(s) will need to be run the additional days as appropriate to ensure that the total number of days is the same for all sets of bottles, even though the specific days are not aligned.

8.5. Re-mixing of bottle sets can be performed multiple times as a result of a failure of the analytical criteria, until the holding time for the stock sediment has expired (60 days). If the problem set(s) has not fallen within the acceptable analytical criteria by then, it must not be part of the batch of bottles run. If the problem batch is one of the controls, and those controls were not successfully prepared when the sediment holding time expired, then the entire test must be restarted.

#### 9.0 PROGRAM QUALITY ASSURANCE AND QUALITY CONTROL

##### 9.1 Calibration

9.1.1. All equipment/instrumentation will be calibrated in accordance with the test method or the manufacturer's instructions and may be scheduled or triggered.

9.1.2. Where possible, standards used in calibration will be traceable to a nationally recognized standard (e.g., certified standard by NIST).

9.1.3. All calibration activities will be documented and the records retained.

9.1.4. The source, lot, batch number, and expiration date of all reagents used with be documented and retained.

##### 9.2. Maintenance

9.2.1. All equipment/instrumentation will be maintained in accordance with the test method or the manufacturer's instructions and may be scheduled or triggered.

9.2.2. All maintenance activities will be documented and the records retained.

##### 9.3. Data Management and Handling

9.3.1. All primary (raw) data will be correct, complete, without selective reporting, and will be maintained.

9.3.2. Hand-written data will be recorded in lab notebooks or electronically at the time of observation.

9.3.3. All hand-written records will be legible and amenable to reproduction by electrostatic copiers.

9.3.4. All changes to data or other records will be made by:

a. Using a single line to mark-through the erroneous entry (maintaining original data legibility).

b. Write the revision.

c. Initial, date, and provide revision code (see attached or laboratory's equivalent).

9.3.5. All data entry, transcriptions, and calculations will be verified by a qualified person.

a. Verification will be documented by initials of verifier and date.

9.3.6. Procedures will be in place to address data management procedures used (at minimum):

a. Significant figures.

b. Rounding practices.

c. Identification of outliers in data series.

d. Required statistics.

##### 9.4. Document Control

9.4.1. All technical procedures, methods, work instructions, standard operating procedures must be documented and approved by laboratory management prior to the implementation.

9.4.2. All primary data will be maintained by the contractor for a minimum of five (5) years.

##### 9.5. Personnel and Training

9.5.1. Only qualified personnel shall perform laboratory activities.

9.5.2. Records of staff training and experience will be available. This will include initial and refresher training (as appropriate).

##### 9.6. Test Performance

9.6.1. All testing will done in accordance with the specified test methods.

9.6.2. Receipt, arrival condition, storage conditions, dispersal, and accountability of the test article will be documented and maintained.

9.6.3. Receipt or production, arrival or initial condition, storage conditions, dispersal, and accountability of the test matrix (e.g., sediment or artificial seawater) will be documented and maintained.

9.6.4. Source, receipt, arrival condition, storage conditions, dispersal, and accountability of the test organisms (including inoculum) will be documented and maintained.

9.6.5. Actual concentrations administered at each treatment level will be verified by appropriate methodologies.

9.6.6. Any data originating at a different laboratory will be identified and the laboratory fully referenced in the final report.

9.7. *The following references identify analytical methods that have historically been successful for achieving the analytical quality criteria.*

9.7.1. Continental Shelf Associates Report 1998. Joint EPA/Industry Screening Survey to Assess the Deposition of Drill Cuttings and Associated Synthetic Based Mud on the Seabed of the Louisiana Continental Shelf, Gulf of Mexico. Analysis by Charlie Henry Report Number IES/RCAT97-36 GC-FID and GC/MS.

9.7.2. EPA Method 3550 for extraction with EPA Method 8015 for GC-FID. EPA Method 3550C, Revision 3. February 2007. Ultrasonic Extraction. EPA Method 8015C, Revision 3. February 2007. Nonhalogenated Organics by Gas Chromatography.

9.7.3. Chandler, J.E., S.P. Rabke, and A.J.J. Leuterman. 1999. Predicting the Potential Impact of Synthetic-Based Muds With the

Use of Biodegradation Studies. Society of Petroleum Engineers SPE 52742.

9.7.4. Chandler, J.E., B. Lee, S.P. Rabke, J.M. Gelliff, R. Stauffer, and J. Hein. 2000. Modification of a Standardized Anaerobic Biodegradation Test to Discriminate Performance of Various Non-Aqueous Base Fluids. Society of Petroleum Engineers SPE 61203.

9.7.5. Munro, P.D., B. Croce, C.F. Moffet, N.A. Brown, A.D. McIntosh, S.J. Hird, and R.M. Stagg. 1998. Solid-Phase Test for Comparison for Degradation Rates of Synthetic Mud Base Fluids Used in the Off-shore Drilling Industry. *Environ. Toxicol. Chem.* 17:1951-1959.

9.7.6. Webster, L., P.R. Mackie, S.J. Hird, P.D. Munro, N.A. Brown, and C.F. Moffat. 1997. Development of Analytical Methods for the Determination of Synthetic Mud Base Fluids in Marine Sediments. *The Analyst* 122:1485-1490.

9.8 The following standards are approved for incorporation by reference by the Director of the Federal Register in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies may also be inspected at EPA's Water Docket, 1200 Pennsylvania Ave. NW., Washington, DC 20460 and at the National Archives and Records Administration (NARA). For information on the availability of this material at NARA, call 202-741-6030, or go to: [http://www.archives.gov/federal\\_register/code\\_of\\_federal\\_regulations/ibr\\_locations.html](http://www.archives.gov/federal_register/code_of_federal_regulations/ibr_locations.html).

9.8.1 ASTM International. Available from ASTM International, 100 Barr Harbor Drive, P.O. Box C700, West Conshohocken, PA 19428-2959, or online at <http://www.astm.org>.

9.8.1.1 ASTM D5291-96, Standard Test Methods for Instrumental Determination of Carbon, Hydrogen, and Nitrogen in Petroleum Products and Lubricants, approved April 10, 1996.

9.8.1.2 ASTM D2974-07a, Standard Test Methods for Moisture, Ash, and Organic Matter of Peat and Other Organic Soils, approved March 15, 2007.

[77 FR 29837, May 18, 2012]

#### APPENDIX 5 TO SUBPART A OF PART 435—DETERMINATION OF CRUDE OIL CONTAMINATION IN NON-AQUEOUS DRILLING FLUIDS BY GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS) (EPA METHOD 1655)

##### 1.0 SCOPE AND APPLICATION

1.1 This method determines crude (formation) oil contamination, or other petroleum oil contamination, in non-aqueous drilling fluids (NAFs) by comparing the gas chromatography/mass spectrometry (GC/MS) fingerprint scan and extracted ion scans of the test

sample to that of an uncontaminated sample.

1.2 This method can be used for monitoring oil contamination of NAFs or monitoring oil contamination of the base fluid used in the NAF formulations.

1.3 Any modification of this method beyond those expressly permitted shall be considered as a major modification subject to application and approval of alternative test procedures under 40 CFR 136.4 and 136.5.

1.4 The gas chromatography/mass spectrometry portions of this method are restricted to use by, or under the supervision of analysts experienced in the use of GC/MS and in the interpretation of gas chromatograms and extracted ion scans. Each laboratory that uses this method must generate acceptable results using the procedures described in Sections 7, 9.2, and 12 of this appendix.

##### 2.0 SUMMARY OF METHOD

2.1 Analysis of NAF for crude oil contamination is a step-wise process. The analyst first performs a qualitative assessment of the presence or absence of crude oil in the sample. If crude oil is detected during this qualitative assessment, the analyst must perform a quantitative analysis of the crude oil concentration.

2.2 A sample of NAF is centrifuged to obtain a solids free supernate.

2.3 The test sample is prepared by removing an aliquot of the solids free supernate, spiking it with internal standard, and analyzing it using GC/MS techniques. The components are separated by the gas chromatograph and detected by the mass spectrometer.

2.4 Qualitative identification of crude oil contamination is performed by comparing the Total Ion Chromatograph (TIC) scans and Extracted Ion Profile (EIP) scans of test sample to that of uncontaminated base fluids, and examining the profiles for chromatographic signatures diagnostic of oil contamination.

2.5 The presence or absence of crude oil contamination observed in the full scan profiles and selected extracted ion profiles determines further sample quantitation and reporting requirements.

2.6 If crude oil is detected in the qualitative analysis, quantitative analysis must be performed by calibrating the GC/MS using a designated NAF spiked with known concentrations of a designated oil.

2.7 Quality is assured through reproducible calibration and testing of GC/MS system and through analysis of quality control samples.

##### 3.0 DEFINITIONS

3.1 A NAF is one in which the continuous—phase is a water immiscible fluid such