SUBMERGEDEEP SD-110: EVALUATION OF READY BIODEGRADABILITY FOLLOWING OECD GUIDELINE 301B CO_2 EVOLUTION TEST

FINAL REPORT

STUDY NUMBER: 2023E-00401

TEST ITEM:

SUBMERGEDEEP SD-110

AUTHOR:

Sergio Dominguez, Ph.D.

EXPERIMENTAL STARTING DATE: November 15, 2023

EXPERIMENTAL COMPLETION DATE: December 13, 2023

SUBMITTED TO:

Engineered Fluids, Inc.

4917 Profit Drive

Tyler, TX 75707, USA

TESTING FACILITY:

Aropha Inc.

21564 Alexander Rd

Bedford, OH 44146, USA

December 21, 2023

GOOD LABORATORY PRACTICE AND ISO/IEC 17025:2017 COMPLIANCE STATEMENT

SPONSOR: Engineered Fluids, Inc.

TEST ITEM: SUBMERGEDEEP SD-110

REFERENCE ITEM: Sodium benzoate

STUDY NUMBER: 2023E-00401

This study was performed in compliance with OECD Principles on Good Laboratory Practice (ENV /MC/CHEM (98) 17), which is compatible with the Good Laboratory Practice Standards published by the U.S. Environmental Protection Agency (40 CFR Part 792) (1989), with the following exceptions:

1. Periodic analyses of municipal water for potential contaminants were not performed according to Good Laboratory Practice Standards.
2. The characterization, stability, and homogeneity of the test item under conditions of storage were not determined.

Above exceptions are not believed to have an adverse effect on the study results.

The testing also complies with ISO/IEC 17025:2017.

STUDY DIRECTOR

Sergio Dominguez, Ph.D.
Head of Laboratory

December 19, 2023
DATE

QUALITY ASSURANCE STATEMENT

This study was performed in compliance with ISO/IEC 17025:2017 and OECD Principles on Good Laboratory Practice (ENV /MC/CHEM (98) 17), which is compatible with the Good Laboratory Practice Standards published by the U.S. Environmental Protection Agency (40 CFR Part 792) (1989). The dates of all inspections and audits and the dates that any findings were reported to the Laboratory Management were as follows:

Study Phase Inspected	Date Conducted	Date Reported to Study Director	Date Reported to Management
Protocol	November 14, 2023	November 14, 2023	November 14, 2023
Stock preparation	November 15, 2023	November 15, 2023	November 15, 2023
Data and Draft Report	December 19, 2023	December 19, 2023	December 19, 2023
Final Report	December 21, 2023	December 21, 2023	December 21, 2023

The final report accurately reflects the methods, procedures and observations documented in the raw data. All inspections were study based unless otherwise noted.

Kuan Huang, Ph.D.
Quality Assurance Unit Coordinator

December 19, 2023
DATE

REPORT APPROVAL

SPONSOR: Engineered Fluids, Inc.

TITLE: "SUBMERGEDEEP SD-110: EVALUATION OF READY BIODEGRADABILITY FOLLOWING OECD GUIDELINE 301B CO_2 EVOLUTION TEST"

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STUDY INFORMATION

EXPERIMENTAL STARTING DATE	November 15, 2023
EXPERIMENTAL COMPLETION DATE	December 13, 2023
SPONSOR	Engineered Fluids, Inc. 4917 Profit Drive Tyler, TX 75707, USA
SPONSOR'S REPRESENTATIVE	David W. Sundin, Ph.D. david.sundin@engineeredfluids.com
STUDY DIRECTOR	Sergio Dominguez, Ph.D. Head of Laboratory

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1. SUMMARY

The ready biodegradability of sample "SUBMERGEDEEP SD-110" was determined following the OECD Guideline 301B CO_2 Evolution Method. As the first tier of tests (301), they are stringent and provide limited opportunity for acclimation and biodegradation to occur. As comparisons, the higher tiers of 302 and 303 increase such opportunities significantly by increasing the inoculum concentrations, lowering the test item to biomass ratios, and/or extending the test time period.

In the CO_2 evolution test, inoculated mineral medium was dosed with a known amount of test item as the nominal sole source of organic carbon and aerated with CO_2 free air at 50-100 mL/min at room temperature. The solutions were mixed continuously using magnetic stirring bars. The biodegradation of the test item was followed over 28 days by determining the CO_2 produced. The CO_2 was trapped in barium hydroxide solution and was measured by titration of the residual hydroxide.

The amount of the formed CO_2 from the test item (corrected for that derived from the blank control) was expressed as a percentage of theoretical CO_2 formation ($ThCO_2$). $ThCO_2$ is defined as the CO_2 formation when all the carbon contents are converted to CO_2. The test contained a blank control group, a reference group, and a test group. The blank control and test groups each was conducted with two replicates and the reference group contained only one replicate. The blank control was used to measure the background CO_2 evolution by the inoculum alone and was not dosed with any carbon source. The reference group was dosed with sodium benzoate, a substance known to be biodegradable, at a nominal concentration of 20 mg total organic carbon (TOC)/L. The test item group had a nominal concentration of 20 mg TOC/L for each replicate.

The results show that the degradation of the reference item passed the 60% threshold within the 10-day window (10% by day 1 and 60% by day 11), and reached 100.6% on day 28. This indicates that the activated sludge inoculum was active, and the system worked properly. The 10-day window is defined as beginning when 10% of the degradation is reached and ends after 10 days from this point (but before the 28th day). The degradation of the test item reached 10% by day 13, 60% by day 26 and 66.2% by day 28, indicating it did not meet the 10-day window requirement. However, OECD 301 is intended for pure chemicals. Since the test item is a mixture with anticipated sequential degradation of individual components, the 10-day window will not be applied to interpret the results of the test. Therefore, the test item may still be considered readily biodegradable.

Table 1. Substance degradation summary

Substance	Biodegradation percentage
Sodium benzoate (reference)	100.6%
SUBMERGEDEEP SD-110	66.2 ± 3.8%

2. INTRODUCTION

Biodegradation is a process by which organic substances are decomposed by microorganisms into simpler and inorganic substances such as carbon dioxide, water, and ammonia. It is an essential process of the elimination of organic substances from the environment. The term "biodegradable" indicates that an organic substance can be decomposed by microorganisms in a relatively short time span in the environment it normally ends up in (e.g., freshwater, seawater, and/or soil). Tests of ready biodegradability, by definition, provide limited opportunity for acclimation and biodegradation to occur. A positive result in a test of ready biodegradability is an indication that the test item will undergo rapid and ultimate biodegradation in the environment. A negative result in a test of ready biodegradability does not necessarily mean that the test item will not be biodegraded under relevant environmental conditions but that additional testing may be needed, such as 302 and 303 series of tests.

The protocol, final report, raw data and other computer-generated information will be archived by customer number, batches, and study number at the testing facility and/or uploaded in the cloud for safer storage and easier access.

3. OBJECTIVE

The objective of the study was to measure the CO_2 evolution by a microbial population to determine the biodegradation of the test item and express it as a percentage of the $ThCO_2$, representing the biodegradation percentage.

4. COMPLIANCE

This study was performed in agreement with the OECD Guideline 301B.

This study was conducted in compliance with ISO/IEC 17025:2017 and the OECD and USEPA Good Laboratory Practice (GLP) standards.

5. JUSTIFICATION FOR SELECTION OF TEST SYSTEM

Selection of the aerobic aquatic biodegradation test was based upon the sponsor's request and the OECD guidelines. The test method determines ready biodegradability by measuring CO_2 evolution in a test system consisting of inoculum, test substance and mineral medium. The inoculum activated sludge has historically been used to evaluate the persistence of chemicals in the environment.

6. EXPERIMENTAL DESIGN

The test contained a blank control group, a reference group, and a test item group. The blank control group and reference item group each contained two replicates while the test group contained three replicates. The blank control was used to measure the background CO_2 evolution by the inoculum alone. The reference group was dosed with sodium benzoate, a substance known to be biodegradable, at a concentration of 20 mg TOC/L. The test item group was dosed with the test substance at a concentration of 20 mg TOC/L.

7. MATERIALS AND METHODS

This study was conducted according to the procedures outlined in the standard method OECD Guideline 301B CO_2 Evolution Test.

7.1 Test item

The test item "SUBMERGEDEEP SD-110" was received from Engineered Fluids, Inc. on November 10, 2023.

Figure 1. Photo of the test item

Table 2. Summary of the test item

Item	Description
Name	SUBMERGEDEEP SD-110
Lot #	20231013-SD110
Facility sample ID	111411
Physical description	Liquid
Total organic carbon content (determined by catalytic combustion)	84.94%
Storage conditions	Ambient
Receive date	November 10, 2023

7.2 Reference item

The reference item sodium benzoate was purchased from Fisher Scientific and was stored under ambient condition.

Table 3. Summary of the reference item sodium benzoate

Item	Description
Name	Sodium benzoate
CAS number	532-32-1
Facility sample ID	110900
Physical description	Solid
Purity / total solids	> 99%
C% in pure sample	58.29%
Storage conditions	Ambient
Receive date	October 05, 2021
Expiry date	October 05, 2024

7.3 Mineral medium

The mineral medium was a mixture of various anions and cations, which provides essential elements for the growth of microorganisms and maintains the pH of the system at a constant level during the biodegradation processes. The preparation procedures are as follows.

Prepare the following stock solutions using reagent grade reagents:

(a) Potassium dihydrogen orthophosphate, KH_2PO_4 . . . . . 8.50 g
Dipotassium hydrogen orthophosphate, K_2HPO_4 . . . . . 21.75 g
Disodium hydrogen orthophosphate dihydrate, $Na_2HPO_4 \cdot 2H_2O$ . . . . . 33.40 g
Ammonium chloride, NH_4Cl . . . . . 0.50 g
Dissolve in water and make up to 1 L. The pH of the solution should be 7.4.

(b) Calcium chloride, anhydrous, $CaCl_2$ . . . . . 27.50 g
or
Calcium chloride dihydrate, $CaCl_2 \cdot 2H_2O$ . . . . . 36.40 g
Dissolve in water and make up to 1 L.

(c) Magnesium sulphate heptahydrate, $MgSO_4 \cdot 7H_2O$ . . . . . 22.50 g
Dissolve in water and make up to 1 L.

(d) Iron (III) chloride hexahydrate, $FeCl_3 \cdot 6H_2O$ . . . . . 0.25 g
Dissolve in water and make up to 1 L.
Note: In order to avoid having to prepare this solution immediately before use, add one drop of concentrated HCl or 0.4 g ethylene-diaminetetra-acetic acid (EDTA disodium salt) per L. If a precipitate forms in a stock solution replace with a freshly made solution.

To prepare the mineral medium, mix 10 mL of solution (a) with 800 mL water, then add 1 mL of solutions (b), (c) and (d) and make up to 1 L with water.

Table 4. Chemical composition of the mineral medium

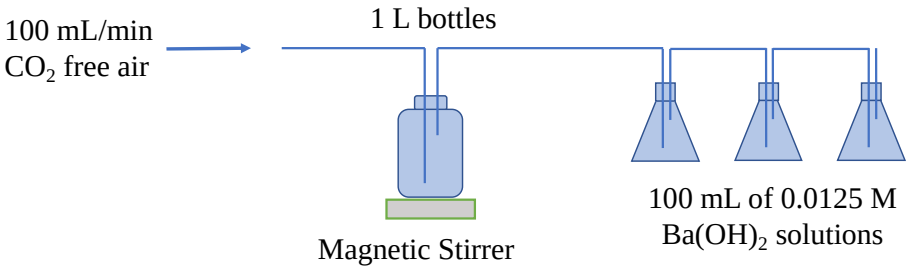
Chemical name	Formula	Mass concentration (mg/L)	Molar concentration (mM)
Potassium dihydrogen orthophosphate	KH_2PO_4	85.0	0.625
Dipotassium hydrogen orthophosphate	K_2HPO_4	217.5	1.250
Disodium hydrogen orthophosphate dihydrate	$Na_2HPO_4 \cdot 2H_2O$	334.0	1.876
Ammonium chloride	NH_4Cl	5.0	0.093
Calcium chloride	$CaCl_2$	27.5	0.248
Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	22.5	0.091
Iron (III) chloride hexahydrate	$FeCl_3 \cdot 6H_2O$	0.25	0.001

7.4 Inoculum

The inoculum was the activated sludge collected from the end of aeration tank of Twinsburg Wastewater Treatment Plant (Twinsburg, OH). The activated sludge was sieved using a 2 mm screen and settled for 15 min followed by decanting the supernatant. It was then resuspended with mineral medium to yield a total suspended solid of 3-5 g/L. The mixture solution was aerated for 5 days at room temperature (commonly known as a pre-conditioning process), and was then added into working solutions to achieve 30 mg/L of total suspended solids to initiate the biodegradation.

7.5 Apparatus and system setup

The CO_2 evolution test was performed in a system shown in Scheme 1. 1 L amber bottles were used as the biodegradation reactors, each containing 0.75 L of working solutions. Magnetic stirrers were employed to mix the contents. The CO_2 free air was obtained by sending air into three washing bottles in series, each containing 0.5 M NaOH solution to remove any CO_2 originally present in the air.



Scheme 1. OECD 301B setup (TSS = total suspended solids)

7.6 Preparation of the working solutions and flasks

The working solutions were prepared based on the following steps:

(1) Add an appropriate amount of pre-conditioned activated sludge solution into 500 mL of mineral medium in order to achieve 30 mg/L of total suspended solids in the final 0.75 L of working solution.
(2) Aerate the above solution with CO_2 free air overnight at 100 mL/min to remove the original CO_2 from the system.
(3) Add an appropriate amount of test item or reference item to achieve 20.5 or 20 mg/L of TOC (ignore this step for blank control group).
(4) Add more mineral medium previously aerated with CO_2 free air to bring the total volume up to 0.75 L.

7.7 Preparation of the CO_2 absorption bottles

Three 150 mL flasks each containing 100 mL 0.0125 M $Ba(OH)_2$ solution were connected in series to each reaction bottle as CO_2 absorption bottles to recover the generated CO_2 during the biodegradation process, as illustrated in Scheme 1. The solution was free of precipitated sulphate or carbonate and its strength was determined immediately before use by the acid-base titration using 0.05 M of HCl solution with phenolphthalein as the indicator.

7.8 Test initiation

Once the working bottles (with stir bars) and CO_2 absorption bottles were prepared, they were connected in series as illustrated in Scheme 1. CO_2 free air was then pumped into all the series at a rate of 50-100 mL/min for each to initiate the reactions.

7.9 Test termination

On the last day of study, once all CO_2 evolution measurements were taken, stop the pumping of the CO_2 free air and disassemble the working flasks and CO_2 absorption bottles to terminate the tests.

7.10 CO_2 and degradation percentage determinations

During the first ten days, the analyses of CO_2 were generally made every three to four days, and then every five to seven days until the end of the test. On the days of CO_2 measurement, disconnect the $Ba(OH)_2$ absorber closest to the test vessel and titrate the hydroxide solution with 0.05 M HCl using phenolphthalein as the indicator. Move the remaining absorbers one place closer to the test vessel and place a new absorber containing 100 ml fresh 0.0125 M $Ba(OH)_2$ at the far end of the series. Titrations were made as needed, for example, when substantial precipitation was seen in the first trap and before any was evident in the second, or at least weekly.

The amount of CO_2 produced was calculated from the amount of base remaining in the absorption bottle. Since every mmol of produced CO_2 consumed 1 mmol of $Ba(OH)_2$ to form $BaCO_3$ and 2 mmol of HCl was needed for the titration of the remaining each mmol of $Ba(OH)_2$, and given that the molecular weight of CO_2 is 44 g, the weight of CO_2 produced (mg) was calculated by:

$$\frac{0.05 \times (50 - mL\ HCl\ titrated) \times 44}{2} = 1.1 \times (50 - mL\ HCl\ titrated)$$

The weights of CO_2 produced from the inoculum alone and from the inoculum plus reference/test item were first obtained, then the difference between them was the weight of CO_2 produced by the reference/test item alone. The percentage biodegradation is calculated from:

$$\%\ degradation = \frac{mg\ CO_2\ produced}{mg\ TOC\ added\ in\ test \times 3.67} \times 100$$

where 3.67 is the conversion factor (44/12) for carbon to CO_2.

7.11 Difference of extremes for the replicates of a test item

Difference of extremes at the plateau or at the end of the test was calculated using the highest replicate value subtracting the lowest replicate value, divided by the mean of the high and low replicate, multiplied by 100%:

$$\text{Difference of extremes (\%)} = \frac{\text{highest replicate value} - \text{lowest replicate value}}{\text{mean of the highest and lowest values}} \times 100\%$$

8. RESULTS AND DISCUSSION

The CO_2 evolution (mg/L) is presented in Table 6 and Figure 2, while the biodegradation percentages are shown in Table 7 and Figure 3. The results show that the degradation of the reference item passed the 60% threshold within the 10-day window (10% by day 1 and 60% by day 11), and reached 100.6% on day 28. This indicates that the activated sludge inoculum was active, and the system worked properly. The 10-day window is defined as beginning when 10% of the degradation is reached and ends after 10 days from this point (but before the 28th day). The degradation of the test item reached 10% by day 13, 60% by day 26 and 66.2% by day 28, indicating it did not meet the 10-day window requirement. However, OECD 301 is intended for pure chemicals. Since the test item is a mixture with anticipated sequential degradation of individual components, the 10-day window will not be applied to interpret the results of the test. Therefore, the test item may still be considered readily biodegradable.

Table 6. CO_2 formation (mg/L)

Time (day)	Blank				Reference	SUBMERGEDEEP SD-110			
	Rep #1	Rep #2	Mean	Error	Rep #1	Rep #1	Rep #2	Mean	Error
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	-1.7	4.9	1.6	3.3	16.0	2.6	0.4	1.5	1.1
5	1.3	12.8	7.0	5.8	34.1	8.1	3.4	5.8	2.3
7	1.7	13.2	7.5	5.8	41.4	11.3	6.8	9.1	2.2
9	3.0	14.9	9.0	6.0	46.9	15.1	10.0	12.6	2.6
14	10.2	16.2	13.2	3.0	66.8	29.0	17.9	23.5	5.5
21	13.9	27.1	20.5	6.6	90.9	63.8	56.5	60.2	3.6
28	23.3	34.8	29.0	5.8	104.1	84.5	87.7	86.1	1.6

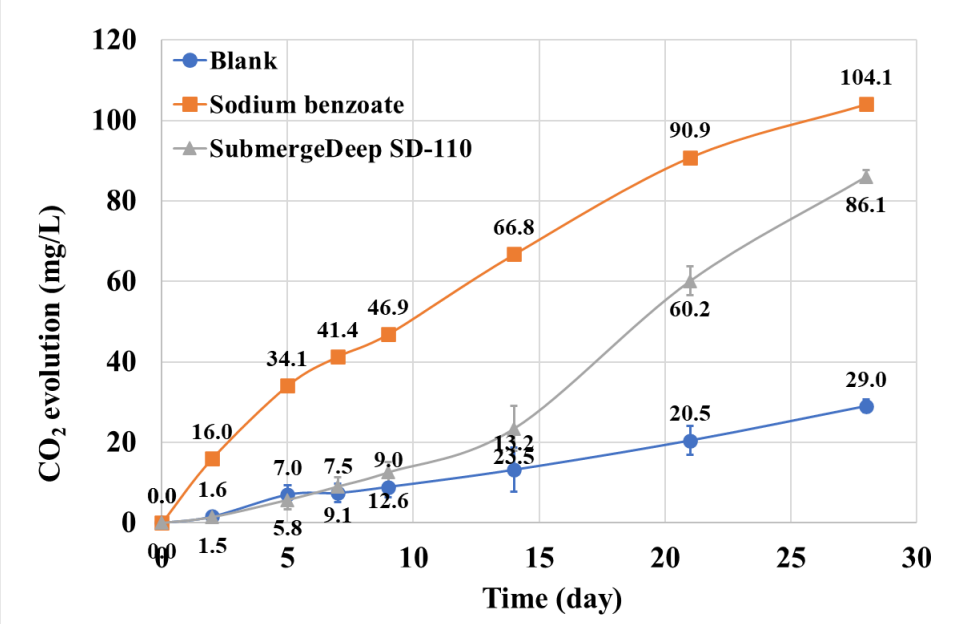


Figure 2. CO_2 evolution

Table 7. Biodegradation percentages

Time (day)	Reference	SUBMERGEDEEP SD-110			
	Rep #1	Rep #1	Rep #2	Mean	Error
0	0.0%	0.0%	0.0%	0.0%	0.0%
2	19.3%	1.1%	-1.4%	-0.2%	1.2%
5	36.3%	1.2%	-4.3%	-1.6%	2.8%
7	45.4%	4.3%	-0.8%	1.8%	2.5%
9	50.9%	7.0%	1.3%	4.1%	2.8%
14	71.7%	17.8%	5.6%	11.7%	6.1%
21	94.3%	48.7%	43.0%	45.9%	2.9%
28	100.6%	62.4%	69.9%	66.2%	3.8%

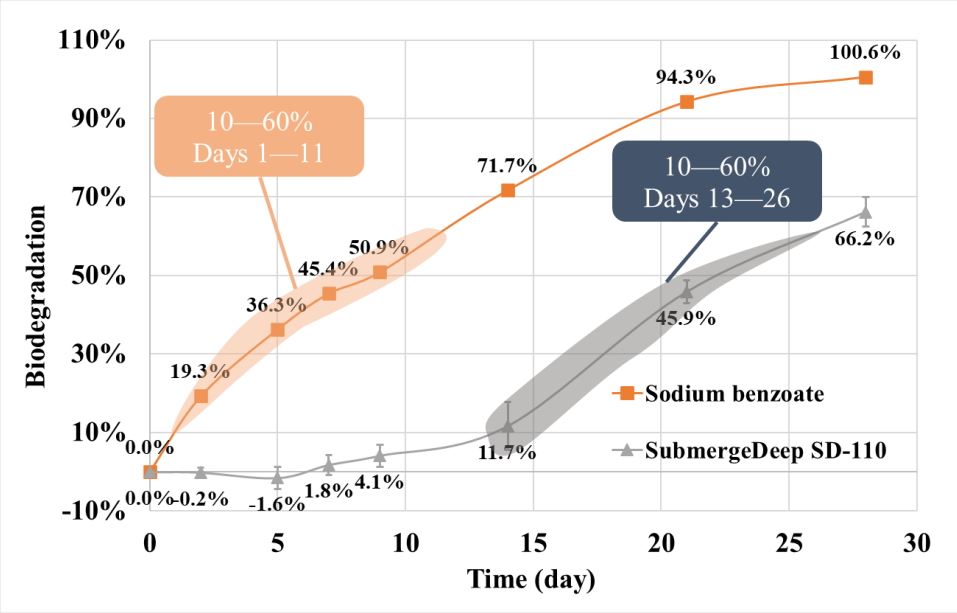


Figure 3. Biodegradation percentage

9. VALIDITY AND CONCLUSION

The test satisfied all the three requirements set by the guideline as shown below. Therefore, the test is valid.

1. The degradation of the reference item passed the 60% threshold within the 10-day window with a final value of 100.6% by day 28.
2. The CO_2 evolution in the inoculum blank at the end of the test was 29.0 ± 5.8 mg/L, within the recommended upper limit of 40 mg/L by the method.

3. The difference between the replicate extremes of the test item at the end of the test is 11.4%, less than the 20% threshold set by the method.

The degradation of the test item reached 10% by day 13, 60% by day 26, and 66.2% by day 28, indicating it did not meet the 10-day window requirement. However, OECD 301 is intended for pure chemicals. Since the test item is a mixture with anticipated sequential degradation of individual components, the 10-day window will not be applied to interpret the results of the test. Therefore, the test item may still be considered readily biodegradable.

10. REFERENCES

1. OECD, *Test No. 301: Ready Biodegradability*. 1992.
2. OECD, *OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1, OECD Principles of Good Laboratory Practice*. In Environment Directorate, OECD Paris: 1998.
3. ISO/IEC 17025:2017, *Testing and calibration laboratories.*
4. OECD, *Guidelines for the Testing of Chemicals, Section 3, Introduction*. (Adopted 23 March 2006).
5. U.S. Environmental Protection Agency, *Series 835-Fate, Transport and Transformation Test Guidelines. January 1998, OPPTS Number 835.3110: Ready Biodegradability*. 1998.
6. *Compliance PT4 webinar - 05 Biodegradation II - ECHA - Europa*. (n.d.). Retrieved July 21, 2022, from https://echa.europa.eu/documents/10162/22840246/compliance_pt4_webinar_05_biodegradationII_en.pdf/0f1d56dd-91ab-484d-a2ba-9384471c87ee

Appendix I
Reference & Test Substance Information

Reference Substance

Certificate of Analysis **Page 1 of 1**

ACROS ORGANICS

Version	00
Molecular weight	144.11
Quality Test / Release Date	02/24/2020
Molecular Formula	C7 H5 Na O2
CAS No	532-32-1
Linear Formula	C6H5CO2Na
Flash Point (°C)	

Certificate of Analysis

This is to certify that units of the lot number below were tested and found to comply with the specifications of the grade listed. Certain data have been supplied by third parties. Acros Organics expressly disclaims all warranties,expressed or implied, including the implied warranties of merchantability and fitness for a particular purpose. Products are for research use or further manufacturing. Not for direct administration to human or animals. It is the responsiblity of the purchaser, formulator or those performing further manufacturing to determine suitability based upon the intended use of the end product. Products are tested to meet the analytical requirements of the noted grade. The following information is the actual analytical results obtained.

Catalog Number	44780	**Quality Test / Release Date** 02/24/2020
Lot Number	A0416839	
Description	Sodium benzoate,99%,for biochemistry	
Country of Origin	INDIA	
Declaration of Origin	synthetic	

BSE/TSE comment	
Chemical Comment	

Result name	Units	Specifications	Test Value
Appearance (Color)		White to off-white	White
Appearance (Form)		Crystalline or granular powder	granular powder
Infrared spectrum		Conforms	Conforms
Titration with HClO4		>=98.5 %	99.8 %
Loss on drying		=<1 %	0.49 %
Solubility		(1 M in water) Clear colorless to light yellow	(1 M in water) Clear colorless
Heavy metals (as Pb)		=<0.001 %	=<0.0005 %
Chloride (Cl)		=<0.05 %	0.035 %
Sulfate (SO4)		=<0.05 %	0.01 %
Copper (Cu)		=<0.001 %	0.00017 %
Mercury (Hg)		=<0.0001 %	=<0.0001 %
Arsenic (As)		=<0.0002 %	=<0.0002 %
Zinc (Zn)		=<0.001 %	0.000033 %

L. Van den Broek, QA Manager Issued: 02-25-2020

Acros Organics
ENA23, zone1, nr 1350, Janssen Pharmaceuticalaan 3a, B-2440 Geel, Belgium
Tel +32 14/57.52.11 - Fax +32 14/59.34.34 Internet: http://www.acros.com
1 Regent Lane, Fair Lawn, NJ 07410, USA Fax 201-796-1329

Test Substance

Robertson Microlit LABORATORIES

1705 US Highway 46, Suite 1D
Ledgewood, NJ 07852
(973) 966-6668
results@robertson-microlit.com
www.robertson-microlit.com

ARP002

Kuan Huang
Aropha Inc
21564 Alexander Rd
Oakwood Village, Ohio 44146

[redacted]

Sample #: 111411 Test #: 1 Received: 11/27/2023 Completed: 11/27/2023

Carbon : 84.94 %

[redacted]

• Where speed and accuracy are elemental •

Appendix II
Study Protocol

SUBMERGEDEEP SD-110: EVALUATION OF READY BIODEGRADABILITY FOLLOWING OECD GUIDELINE 301B CO_2 EVOLUTION TEST

SUBMITTED TO:

Engineered Fluids Inc.

4917 Profit Drive

Tyler, TX 75707, USA

TESTING FACILITY:

Aropha Inc.

21564 Alexander Rd

Bedford, OH 44146, USA

November 14, 2023

PROPOSAL APPROVAL

SPONSOR	Engineered Fluids Inc. 4917 Profit Drive Tyler, TX 75707, USA
SPONSOR'S REPRESENTATIVE	David Sundin david.sundin@engineeredfluids.com
TESTING FACILITY	Aropha Inc. 21564 Alexander Rd Bedford, OH 44146, USA
STUDY DIRECTOR	Sergio Dominguez, Ph.D. Head of the Lab

PROPOSED KEY DATES:

Experimental Start Date	November 14, 2023
Experimental Termination Date	December 12, 2023
Draft Report Completion Date	December 13, 2023
Final Report Completion Date	December 14, 2023

STUDY NO.	2023E-00401
TEST CONCENTRATION	20 mg/L of total organic carbon (TOC)
TEST SUBSTANCE NO.	111411
REFERENCE SUBSTANCE NO.	110900

PROPOSAL APPROVAL

STUDY DIRECTOR	11/14/2023 DATE
SPONSOR'S REPRESENTATIVE	11/14/2023 DATE

OECD 301B CO_2 EVOLUTION TEST
STUDY PROTOCOL: 2023E-00401

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AROPHA

OECD 301B CO_2 EVOLUTION TEST
STUDY PROTOCOL: 2023E-00401

1. INTRODUCTION

Tests of ready biodegradability, by definition, provide limited opportunity for acclimation and biodegradation to occur. A positive result in a test of ready biodegradability is an indication that the test item will undergo rapid and ultimate biodegradation in the environment. A negative result in a test of ready biodegradability does not necessarily mean that the test item will not be biodegraded under relevant environmental conditions but that additional testing may be needed, such as 302 and 303 series of tests.

This study will be conducted for the sponsor in order to evaluate the potential of the test substance to biodegrade in an aerobic, aqueous environment.

2. OBJECTIVE

The objective of the study was to measure the CO_2 evolution by a microbial population to determine the biodegradation of the test item and express it as a percentage of the theoretical CO_2 ($ThCO_2$), representing the biodegradation percentage. $ThCO_2$ is defined as the CO_2 formation when all the carbon contents are converted to CO_2.

3. COMPLIANCE

This study will be performed in agreement with the OECD guideline 301B.

This study will be conducted in compliance with ISO/IEC 17025:2017, and the OECD and USEPA Good Laboratory Practice (GLP) standards.

4. JUSTIFICATION FOR SELECTION OF TEST SYSTEM

Selection of the aerobic aquatic biodegradation test is based upon sponsor's request and various OECD guidelines. The test method determines ready biodegradability by measuring CO_2 evolution in a test system consisting of inoculum, test substance and mineral medium. The inoculum activated sludge has historically been used to evaluate the persistence of chemicals in the environment.

5. EXPERIMENTAL DESIGN

Overall, the tests will contain a blank, a reference, and a test item group. The blank control will contain mineral medium and inoculum. The reference group will contain mineral medium, inoculum, and the reference compound. The test item group will contain mineral medium, inoculum, and test item. The blank control and test item groups each will contain two replicates

OECD 301B CO_2 EVOLUTION TEST
STUDY PROTOCOL: 2023E-00401

and the reference group will only contain one test without replicates. The blank control will be used to measure the background CO_2 evolution by the inoculum. The reference group will be dosed with sodium benzoate, a substance known to be biodegradable, at a concentration of 20 mg total organic carbon (TOC)/L. The test item group will be dosed with the test substance, also at a concentration of 20 mg TOC/L. More details about the experimental setup can be found in Table 1.

Table 1. Experimental design *

#	Group	Number of replicates	Composition
1	Blank	2	a. Mineral medium. b. Inoculum.
2	Sodium benzoate (reference)	1	a. Mineral medium. b. Inoculum. c. Reference (20 mg TOC/L).
3	Test item	2	a. Mineral medium. b. Inoculum. c. Test item (20 mg TOC/L).

* Other conditions: inoculum – 30 mg/L of the total suspended solids of activated sludge; air flow rate – 100 mL/min.

6. MATERIALS AND METHODS

This study will be conducted according to the procedures outlined in the standard method OECD Guideline 301B CO_2 Evolution Test.

6.1 Test item

Information on the characterization of test, control or reference substances is required. The Sponsor is responsible for all information related to the test substance and agrees to accept, or give the testing facility authorization to dispose of, any unused test substance and/or test substance containers remaining at the end of the study.

If the test substance is highly water soluble, a stock solution of the test substance may be prepared at a nominal concentration of 1,000 mg/L in test medium. Volumetric addition of a test substance stock solution is the most appropriate route of administration for water-soluble materials. The dosing volume of the test substance stock solution will be calculated based on the nominal

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concentration of the test substance stock solution. The pH value of the stock solution will be determined and adjusted to 7.4 + 0.2 if necessary. The stock solution may be stored in a refrigerator for a maximum of three days.

For poorly soluble materials, the test substance will be administered by direct weight addition, followed by further treatment procedures to facilitate the dispersion of the sample, such as ultrasonication and/or dispersion with surfactants. Direct weight addition of a test substance that is relatively insoluble in water is the most appropriate route of administration.

The organic carbon content (C%) of the test substance will be used for the dosage calculation.

6.2 Reference item

A stock solution of sodium benzoate will be prepared at a nominal concentration of 1,000 mg/L in test medium. Volumetric addition of a stock solution is the most appropriate route of administration for water-soluble materials. The dosing volume of the reference substance stock solution will be calculated based on the nominal value of the stock solution. The pH value of the stock solution will be determined and adjusted to 7.4 + 0.2 if necessary. The stock solution may be stored in a refrigerator for a maximum of three days.

6.3 Mineral medium

The mineral medium is a mixture of various anions and cations, which provides essential elements for the growth of microorganisms and maintains the pH of the system at a constant level during the biodegradation processes. The preparation procedures are as follows.

Prepare the following stock solutions using reagent grade reagents:

(a) Potassium dihydrogen orthophosphate, KH_2PO_4 . . . . . 8.50 g
Dipotassium hydrogen orthophosphate, K_2HPO_4 . . . . . 21.75 g
Disodium hydrogen orthophosphate dihydrate, $Na_2HPO_4 \cdot 2H_2O$ . . . . . 33.40 g
Ammonium chloride, NH_4Cl . . . . . 0.50 g
Dissolve in water and make up to 1 L. The pH of the solution should be 7.4.

(b) Calcium chloride, anhydrous, $CaCl_2$ . . . . . 27.50 g
or
Calcium chloride dihydrate, $CaCl_2 \cdot 2H_2O$ . . . . . 36.40 g
Dissolve in water and make up to 1 L.

(c) Magnesium sulphate heptahydrate, $MgSO_4 \cdot 7H_2O$ . . . . . 22.50 g
Dissolve in water and make up to 1 L.

(d) Iron (III) chloride hexahydrate, $FeCl_3 \cdot 6H_2O$ . . . . . 0.25 g
Dissolve in water and make up to 1 L.

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Note: In order to avoid having to prepare this solution immediately before use, add one drop of concentrated HCl or 0.4 g ethylene-diaminetetra-acetic acid (EDTA disodium salt) per L. If a precipitate forms in a stock solution replace with a freshly made solution.

To prepare the mineral medium, mix 10 mL of solution (a) with 800 mL water, then add 1 mL of solutions (b), (c) and (d) and make up to 1 L with water.

Table 2. Chemical composition of the mineral medium

Chemical name	Formula	Mass concentration (mg/L)	Molar concentration (mM)
Potassium dihydrogen orthophosphate	KH_2PO_4	85.0	0.625
Dipotassium hydrogen orthophosphate	K_2HPO_4	217.5	1.250
Disodium hydrogen orthophosphate dihydrate	$Na_2HPO_4 \cdot 2H_2O$	334.0	1.876
Ammonium chloride	NH_4Cl	5.0	0.093
Calcium chloride	$CaCl_2$	27.5	0.248
Magnesium sulphate heptahydrate	$MgSO_4 \cdot 7H_2O$	22.5	0.091
Iron (III) chloride hexahydrate	$FeCl_3 \cdot 6H_2O$	0.25	0.001

6.4 Inoculum

The inoculum will be the activated sludge collected from the end of the aeration tank of Twinsburg Wastewater Treatment Plant (Twinsburg, OH). The activated sludge will be sieved using a 2 mm screen and settled to yield a total suspended solid of 3-5 g/L with mineral medium. The resulted solution may be aerated for no more than 7 days at room temperature if required (commonly known as a pre-conditioning process), and then added into working solutions to achieve 30 mg/L of total suspended solids to initiate the biodegradation.

6.5 Apparatus and system setup

The CO_2 evolution test will be performed in a system shown in Scheme 1. 1 L amber bottles will be used as the biodegradation bottles, each containing 0.75 L of working solutions typically including mineral medium, test items or reference item, and inoculum. Magnetic stirrers will be

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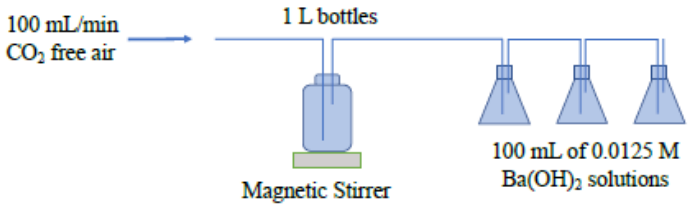
employed to mix the contents at 400 rpm. The CO_2 free air will be obtained by sending air into three washing bottles in series, each containing 0.5 M NaOH solution to remove any CO_2 originally present in the air.



Scheme 1. OECD 301B setup

6.6 Preparation of the working solutions and flasks

The working solutions will be prepared based on the following steps:

(1) Add an appropriate amount of pre-conditioned activated sludge solution into 500 mL of mineral medium in order to achieve 30 mg/L of total suspended solids in the final 0.75 L of working solution.
(2) Aerate the above solution with CO_2 free air overnight at 100 mL/min to remove the original CO_2 from the system.
(3) Add an appropriate amount of test item or reference item to achieve 20 mg/L of TOC (ignore this step for blank control group).
(4) Add more mineral medium previously aerated with CO_2 free air to bring the total volume up to 0.75 L.
(5) Determine the initial pH of the working solution.

6.7 Preparation of the CO_2 absorption bottles

Three 150 mL flasks each containing 100 mL 0.0125 M $Ba(OH)_2$ solution will be connected in series to each reaction bottle as CO_2 absorption bottles to recover the generated CO_2 during the biodegradation process, as illustrated in Scheme 1. The solution should usually be free of precipitated sulphate and carbonate and its strength should be determined immediately before use by the acid-base titration using 0.05 M of HCl solution with phenolphthalein as the indicator.

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6.8 Test initiation

Once the working bottles (with stir bars) and CO_2 absorption bottles are prepared, they are connected in series as illustrated in Scheme 1. CO_2 free air is then pumped into all the series at a rate of around 100 mL/min for each to initiate the reactions. Mix the solutions at 400 rpm throughout the incubation. The solutions are under diffused light during incubation.

6.9 Test termination

On the last day of study, measure the pH of the working solutions, add 1 mL of concentrated hydrochloric acid to each of the reactors to decompose inorganic carbonate and release trapped CO_2. Continue aerating the reactors overnight to collect the released CO_2.

6.10 CO_2 and degradation percentage determinations

During the first two weeks, the analysis of CO_2 is generally made every three to four days, and then every five to seven days until the last day. On the days of CO_2 measurement, disconnect the $Ba(OH)_2$ absorber closest to the test vessel and titrate the hydroxide solution with 0.05 M HCl using phenolphthalein as the indicator. Move the remaining absorbers one place closer to the test vessel and place a new absorber containing 100 mL fresh 0.0125 M $Ba(OH)_2$ at the far end of the series. Titrations are made as needed, for example, when substantial precipitation is seen in the first trap and before any is evident in the second, or at least weekly.

The amount of CO_2 produced will be calculated from the amount of base remaining in the absorption bottle. Since every mmol of produced CO_2 consumes 1 mmol of $Ba(OH)_2$ to form $BaCO_3$ and 2 mmol of HCl will be needed for the titration of the remaining each mmol of $Ba(OH)_2$, and given that the molecular weight of CO_2 is 44 g, the weight of CO_2 produced (mg) is calculated by:

$$\frac{0.05 \times (50 - mL\ HCl\ titrated) \times 44}{2} = 1.1 \times (50 - mL\ HCl\ titrated)$$

The weights of CO_2 produced from the inoculum alone and from the inoculum plus reference/test item are first obtained, then the difference between them is the weight of CO_2 produced by the reference/test item alone. The percentage biodegradation is calculated from:

$$\%\ degradation = \frac{mg\ CO_2\ produced}{mg\ TOC\ added\ in\ test \times 3.67} \times 100$$

where 3.67 is the conversion factor (44/12) for carbon to CO_2.

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6.11 Difference of extremes for the replicates of a test item

Difference of extremes at the plateau or at the end of the test is calculated using the highest replicate value subtracting the lowest replicate value, divided by the mean of the high and low replicate, multiplied by 100:

$$\text{Difference of extremes (\%)} = \frac{\text{highest replicate value} - \text{lowest replicate value}}{\text{mean of the highest and lowest values}} \times 100$$

7. RESULTS AND DISCUSSION

No bias is expected in this study and statistical methods will not be used in the analysis of the data. Interpretation of the results will be based on the following:

1. The reference substance must achieve 60% degradation within the 10-day window.
2. The CO_2 evolution of the inoculum blank control does not exceed 40 mg/L in 28 days.
3. The difference of the extremes of replicates should not exceed 20% at the end of the test.

8. RECORDS TO BE MAINTAINED

Records to be maintained will include, but not be limited to, the following:

1. The signed protocol.
2. Identification and characterization of the test substance as provided by Sponsor (if available).
3. Experimental start and termination dates.
4. Calculations and preparations of test concentrations.
5. Inoculum source and pretreatment data.
6. The final report.

9. FINAL REPORT

A final report of the results of the study will be prepared by the testing facility. The report is to include, but is not limited to, the following, when applicable:

1. Name and address of facility performing the study.
2. Dates on which the study was initiated and completed.
3. Objectives and procedures stated in the approved protocol, including any changes in the original protocol.

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4. Identification and characterization of the test substance as provided by Sponsor including name, CAS number, percent active, percent carbon, and other characteristics, if provided by the Sponsor.
5. A description of the transformations and calculations performed on the data.
6. A description of the test system.
7. A description of the preparation of the test solutions, the testing concentrations, the route of administration, and the duration of the test.
8. A description of all circumstances that may have affected the quality or integrity of the data.
9. The name of the study director, the names of other scientists or professionals, and the names of all supervisory personnel, involved in the study.
10. The signed and dated reports of each of the individual scientists or other professionals involved in the study, if applicable.
11. The location where the protocol, final reports, and raw data are to be stored.
12. A statement prepared by the Quality Assurance Unit listing the dates that study inspections and audits were made and the dates findings were reported to the Study Director and Management.

10. CHANGES TO FINAL REPORT

If it is necessary to make corrections or additions to the final report after it has been accepted, such changes shall be made in the form of an amendment issued by the Study Director. The amendment shall clearly identify the part of the report that is being amended and the reasons for the alteration. Amendments shall be signed and dated by the Study Director and approved by the Sponsor's Representative.

11. CHANGES TO PROTOCOL

Planned changes to the protocol will be in the form of written amendments signed by the Study Director and approved by the Sponsor's Representative after it has been accepted. Amendments will be considered as part of the protocol and will be attached to the final protocol. Any other changes will be in the form of written deviations signed by the Study Director and filed with the raw data. All changes to the protocol will be indicated in the final report.

12. ISO/IEC 17025:2017 AND GOOD LABORATORY PRACTICES

This study will be conducted and reported in compliance with ISO/IEC 17025:2017, and Good Laboratory Practice Standards as published by the U.S. Environmental Protection Agency (40 CFR Part 792) (1989), which are compatible with the Organization for Economic Cooperation

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and Development (OECD) Principles of Good Laboratory Practice (ENV/MC/CHEM (98) 17). A statement of compliance, signed by the Study Director, will be included in the final report. The Sponsor will be responsible for compliance with Good Laboratory Practices for procedures performed by other laboratories (e.g., residue analyses or pathology). Each study conducted by the testing facility is routinely examined by the testing facility Quality Assurance Unit for compliance with Good Laboratory Practices, Standard Operating Procedures and the specified protocol. The protocol, final report, raw data and other computer-generated information will be archived by customer number, batches, and study number at the testing facility and/or uploaded in the cloud for safer storage and easier access.

13. REFERENCES

1. OECD, *Test No. 301: Ready Biodegradability*. 1992.
2. OECD, *OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1, OECD Principles of Good Laboratory Practice*. In Environment Directorate, OECD Paris: 1998.
3. OECD, *Guidelines for the Testing of Chemicals, Section 3, Introduction*. (Adopted 23 March 2006).
4. U.S. Environmental Protection Agency, *Series 835-Fate, Transport and Transformation Test Guidelines. January 1998, OPPTS Number 835.3110: Ready Biodegradability*. 1998.
5. ISO/IEC 17025:2017, *Testing and calibration laboratories*.
6. *Compliance PT4 webinar - 05 Biodegradation II - ECHA - Europa*. (n.d.). Retrieved July 21, 2022, from https://echa.europa.eu/documents/10162/22840246/compliance_pt4_webinar_05_biodegradationII_en.pdf/0f1d56dd-91ab-484d-a2ba-9384471c87ee

Appendix III
ISO/IEC 17025:2017 Certificate of Accreditation

PERRY JOHNSON LABORATORY ACCREDITATION, INC.

Certificate of Accreditation

Perry Johnson Laboratory Accreditation, Inc. has assessed the Laboratory of:

Aropha Inc.
21564 Alexander Road, Bedford, OH 44146

(Hereinafter called the Organization) and hereby declares that Organization is accredited in accordance with the recognized International Standard:

ISO/IEC 17025:2017

This accreditation demonstrates technical competence for a defined scope and the operation of a laboratory quality management system
(as outlined by the joint ISO-ILAC-IAF Communiqué dated April 2017):

Environmental Testing
(As detailed in the supplement)

Accreditation claims for such testing and/or calibration services shall only be made from addresses referenced within this certificate. This Accreditation is granted subject to the system rules governing the Accreditation referred to above, and the Organization hereby covenants with the Accreditation body's duty to observe and comply with the said rules.

For PJLA:

Tracy Szerszen
President

Perry Johnson Laboratory Accreditation, Inc. (PJLA)
755 W. Big Beaver, Suite 1325
Troy, Michigan 48084

Initial Accreditation Date:	*Issue Date:*	*Expiration Date:*
May 08, 2023	May 08, 2023	July 31, 2025

Accreditation No.:	*Certificate No.:*
120195	L23-370

The validity of this certificate is maintained through ongoing assessments based on a continuous accreditation cycle. The validity of this certificate should be confirmed through the PJLA website: www.pjlabs.com

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Certificate of Accreditation: Supplement

Aropha Inc.
21564 Alexander Road, Bedford, OH 44146
Contact Name: Mr. Kuan Huang Phone: 717-877-0197

Accreditation is granted to the facility to perform the following testing:

FIELD OF TEST	ITEMS, MATERIALS OR PRODUCTS TESTED	SPECIFIC TESTS OR PROPERTIES MEASURED	SPECIFICATION, STANDARD METHOD OR TECHNIQUE USED	RANGE (WHERE APPROPRIATE) AND DETECTION LIMIT
Environmental – Biodegradation [F]	Organic Materials (e.g., Chemicals, Plastics, Oils, and Lubricants)	Aerobic Biodegradability in Freshwater	OECD 301B OECD 301D OECD 301F OECD 306 ISO 9408 ISO 9439 ISO 10707 ISO 14851 ISO 14852 ASTM D5864 ASTM D6731	Range: Up to 100 % D.L.= 0.1 %
	Organic Materials (e.g., Chemicals, Plastics, Oils, and Lubricants)	Aerobic Biodegradability in Seawater	ISO 16221 ISO 23977-1 ISO 23977-2 ASTM D6691	
	Organic Materials (Mainly Polymers, Like Plastics)	Aerobic Biodegradability in Soil	ISO 17556 ASTM D5988	
Environmental – Oxygen Demand [F]	Organic Materials or Water Samples	BOD, COD	EPA Method 405.1_BOD EPA Method 410.4_COD	D.L.= 0.000 1 mg O_2/mg substance, or 0.0001 mg O_2/L

1. The presence of a superscript F means that the laboratory performs testing of the indicated parameter at its fixed location. Example: Outside Micrometer [F] would mean that the laboratory performs this testing at its fixed location.