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Standard Test Method for Inhibition of Respiration in Microbial Cultures in the Activated Sludge Process[1]

This standard is issued under the fixed designation D5120; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method covers a batch procedure that evaluates the impact of selected wastewaters, materials, or specific compounds on the respiration rate of an aqueous microbial culture, such as activated sludge.

1.2 Alternative procedures for measurement of microbial activity, such as adenosine 5' triphosphate (ATP), specific substrate utilization, etc. are not within the scope of this test method.

1.3 The results obtained are based on comparisons in a specific test series that examines a range of concentrations of the potentially inhibitory test candidate using batch methods in a laboratory. Results are completed in a short time frame (a few hours).

1.4 The test results are specific to the microbial culture used. Microbial culture from different wastewater treatment plants will differ in kinds and numbers of organisms, and performance capability. Thus, there is no basis for comparing results for microbial cultures from different treatment facilities.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D4478 Test Methods for Oxygen Uptake (Withdrawn 1994)[3]

3. Terminology

3.1 *Definitions:*

3.1.1 *respiration rate*—the quantitative consumption of oxygen by an aqueous microbial system. The consumption is generally expressed as mg O_2/L/h.

3.1.2 EC_{50}—the concentration of the test candidate in this procedure (volume percent or mg/L) that results in a reduction of respiration rate to 50 % of that observed for the control.

4. Summary of Test Method

4.1 This test method utilizes respiration rate as the indicator of microbial activity.

4.2 A batch system that contains a microbial culture (returned activated sludge from the process or a culture maintained in the laboratory), selected nutrient dose, and a dilution of a compound, substance, wastewater, etc. (test candidate) is prepared in a container in the laboratory. The batch system is called a "cell suspension."

4.3 The nutrient dose introduces a large excess of biodegradable substrate thereby putting the culture at a high metabolic rate. Inhibition of respiration by the test candidate is observed under these conditions.

[1] This Test Method is under the jurisdiction of ASTM Committee D34 on Waste Management and is the direct responsibility of Subcommittee D34.03 on Treatment, Recovery and Reuse.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The last approved version of this historical standard is referenced on www.astm.org.

4.4 The prepared cell suspension is aerated for a 2-h period. At the end of the period, the respiration rate is determined using a respirometric or an oxygen uptake technique.

4.5 A lower respiration rate for a cell suspension that has received the test candidate compared to the respiration rate of a control cell suspension indicates inhibition of respiration.

5. Significance and Use

5.1 The objectives of the respiration inhibition tests may be defined by the interests of the user, but the test method is designed primarily for examination of the inhibition response with operating microbial systems such as an activated sludge process treating domestic or industrial wastes.

5.2 Different apparatus exist that facilitate continuous or continual measurement of respiration in microbial systems and each may be used as the tool to observe respiration in this test method.

5.3 Respirometry may utilize any apparatus and technique that will achieve the determination of respiration rate. A number of devices are presented in Appendix X1. Equivalency in the experimental capability of each device is not implied. The analyst should select the respirometric approach that best suits his needs.

5.4 The inhibitory effect of a test candidate is identified more completely by examining inhibition over a range of concentrations, such as determining the EC_{50}. The use of aerated containers permits concurrent management of a series of cell suspensions. A respirometer for each cell suspension might also be used.

6. Interferences

6.1 This test method is most readily applied to substances which, due to water solubility and low volatility, are likely to remain in the aqueous system.

6.2 Results have been observed where cell suspensions containing the test candidate had a respiration rate greater than the blank, particularly at shorter aeration periods of the cell suspensions (less than 1 h). Thus, a minimum aeration period for the cell suspensions before determinations of respiration rate is 2 h.

6.2.1 One reason for increased oxygen uptake rate in an experimental cell suspension may be that severe physical or chemical reactions with the test candidate cause a fraction of the microbial culture to be lysed. The release of very readily biodegradable soluble organic material from the lysed cells may support a higher oxygen uptake rate by the cell suspension.

6.2.2 An alternate reason for increased oxygen uptake rate is that certain test candidates (2,4-dichlorophenol for example) may uncouple the transfer of electrons involved in the process called oxidative phosphorylation in which adenosine 5' triphosphate (ATP) is formed by the phosphorylation of adenosine 5' diphosphate (ADP). The result of the uncoupling is an increase in the rate of oxygen consumption that is not related to substrate stabilization.

6.2.3 A respiration rate by an experimental cell suspension that is greater than the respiration rate of the control represents microbial system damage. The degree of damage is not quantified by comparison of respiration rates for the test candidate and the control. Whether the cause is due to uncoupled electron transfer or lysis of cells can be determined by comparing the filtered Dissolved Organic Carbon (DOC) of the experimental cell suspension with the sum of the DOC of the control plus that added by the test candidate. A higher DOC represents cell lysis.

6.3 Where industrial wastewaters in the sewer system are continually introducing inhibitory components to the collective wastewaters, it may not be feasible to utilize the returned sludge from the process directly as the microbial culture. The maintenance of a protected culture of organisms in the laboratory may be necessary.

7. Apparatus

7.1 *Respirometer or an Oxygen probe*— An apparatus capable of measuring the respiration rate or oxygen uptake rate of the cell suspension.

7.1.1 *Respirometer*—A device that receives the cell suspension, or an aliquot and provides a technique for measurement of oxygen utilization to be interpreted as respiration rate (see Appendix X1).

7.1.2 *Dissolved Oxygen Probe and Instrumentation*—An alternate device for the measurement of respiration rate as oxygen uptake rate.

7.2 *Culture Tank*—If it is deemed necessary to maintain a microbial culture in the laboratory, the apparatus required is a container with adequate mixing and oxygen transfer. The container should hold at least four times the volume of culture that might be used in one day.

7.2.1 The culture tank should be adequately mixed to insure that the culture remains in suspension and that sufficient mechanical or bubble aeration occurs to maintain the desired dissolved oxygen (DO) concentration.

NOTE 1—Energy input should not be such that the biological floc is sheared to sizes smaller than that which exists in the large-scale process. Mixing and aeration provided through diffused aeration in a laboratory-sized container may result in an excessive power input. Consider controlling the power input per unit volume to approximately that which exists in the large-scale process. For example, pure oxygen for aeration in combination with mechanical mixing may be utilized to achieve a balance between oxygen transfer and mixing. Determine the mixer power input by measuring the electrical power consumed at different operating speed, and adjust the mixer speed to achieve a power input that is equivalent to that which exists in the large-scale system.

NOTE 2—Cultures grown at low (0.5 to 2 mg/L) and high (>5 mg/L) DO concentrations possess different kinetic capabilities. Thus, to maintain a laboratory culture with performance capabilities similar to those of the full-scale culture, the DO concentration should be maintained at the level appropriate for the full-scale process. The probable explanation for the difference in culture performance is that higher concentrations of oxygen penetrate more completely through the floc particles.

7.3 *A pH Probe and Instrumentation.*

7.4 *Dissolved Oxygen Probe*—If utilized, the following apparatus is needed:

7.4.1 *Biochemical Oxygen Demand (BOD) bottles.*

7.4.2 *Agitation Device*, may be used with the dissolved oxygen probe in the BOD bottle. The device must provide complete mixing of the microbial culture in the BOD bottle.

7.4.3 *Magnetic Stirrer and Magnetic Stirring Bar,* alternatively, may be used to mix the BOD bottle.

7.5 *Beakers, 2-L size,* (or other containers of suitable size).

7.6 *Clean, Oil-Free Air Supply,* to provide cell suspension mixing and aeration.

7.7 *Fritted Glass Diffusers or Pasteur-Pipets,* as air diffusers.

8. Reagents

8.1 *Microbial Culture*—The microbial culture to be used is the returned sludge from the full-scale facility. For those activated sludge system where industrial contributions regularly cause microbial inhibition, direct use of the returned sludge may be impractical. For those systems where microbial inhibition is not a continuous problem, the returned sludge may be used directly if, by observed system performance, it appears to be healthy.

8.1.1 A microbial culture may be maintained in the laboratory. The culture should be maintained at the temperature of the full-scale mixed liquor and approximately at the concentration of the full-scale process returned sludge.

8.1.2 If maintenance of a microbial culture is to be practiced in the laboratory, and if the inhibition tests are to be related to a specific activated sludge wastewater treatment process, the initial microbial culture should be taken from the process returned sludge.

8.1.2.1 Care should be taken to obtain the microbial culture when, by appearance and performance, the culture is considered to be healthy.

8.1.2.2 The microbial culture should be fed daily with the actual process wastewater if the wastewater is of suitable quality and not inhibitory. Determine the wastewater quality by following the procedure in Section 9. Prepare a control cell suspension and a wastewater cell suspension. If the wastewater cell suspension does not show inhibition, it is suitable for use as feed material.

8.1.2.3 If wastewater of good quality is not available, a synthetic feed, such as Marlene's Mix (see 8.2) or other feed similar in character to the wastewater should be used (sucrose has been used successfully with domestic wastewater activated sludge). The feed application should not be excessive. For example, preferably it should be equal to about one-half of the food-to-microorganism ratio that exists in the full-scale process.

8.1.2.4 When the full-scale system is considered to be in good condition, replenish one third to one half of the volume of the microbial culture daily with the returned sludge from the full-scale system. The replenishment will aid in maintaining a microbial culture with approximately the same population dynamics as the full-scale process.

8.1.2.5 Replenishment and feeding should be done at the end of a work day so that the culture will have an overnight period to complete the synthesis of substrate. Replace any water that has evaporated over night by adding distilled or deionized water.

8.2 *Nutrient Dose Preparation (Marlene's Mix)*—A solution of the following substances is prepared for use when conducting inhibition studies. Store the prepared solution in a refrigerator at 4°C. Warm the portion of the feed to be used in tests to the operating temperature of the test before use. Replace the solution after 14 days of storage or earlier if it becomes odorous.[4]

Ingredient	Quantity (g)
Bacto peptone[4]	32
Beef extract[4]	22
Ammonium chloride	11
Sodium chloride	1.4
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.8
Potassium dihydrogen phosphate	3.5
Potassium monohydrogen phosphate	4.5
Distilled water	make up to 1 L

8.3 *Stock Inhibitor Solution*—Dissolve 0.5 g of 3,5-dichlorophenol in 10 mL of 1N NaOH, dilute to 30 mL with distilled water, add 1N H_2SO_4 to the point of incipient precipitation (approximately 8 mL of 1N H_2SO_4 will be required), bring the volume to 950 mL with distilled water, adjust the pH to the range of 7 to 8, and bring the volume to 1 L. The EC_{50} of 3,5-dichlorophenol for relatively non-acclimatized microbial cultures from domestic wastewater plants is about 10 to 30 mg/L but may be outside this range. For acclimatized microbial cultures, the EC_{50} will be higher, and values <200 mg/L has been reported. The stock solution may be used to check experimental technique and possibly the susceptivity of a microbial culture.

9. Procedure

9.1 Prepare experimental and control cell suspensions so that each is identical in its concentration of microbial culture and Marlene's mix.

9.1.1 When the test is related to an operating activated sludge process, the microbial culture concentration, and the initial pH, make sure that the temperature of the cell suspension (throughout the experimental period) is the same as that in the process mixed liquor. As an example of the control cell suspension, the following table applies if a microbial concentration of 2000 mg/L is desired and the microbial culture concentration is 10 000 mg/L.

Component	Percent of Preparation Volume
Microbial Culture (at 10 000 mg/L)	20[A]
Marlene's Mix	3.8
Tap Water	76.2 or as required to equal 100

[A] If the culture is not at 10 000 mg/L, adjust the volume percent to obtain the desired microbial concentration in the cell suspension.

9.1.2 Make sure that the concentration of readily biodegradable organic material in the nutrient dose (such as Marlene's Mix) is high enough that an additional increment of biodegradable organic material will not result in a significant increase in the rate of respiration. That is, during these tests the microbial culture is essentially saturated with substrate. Marlene's Mix has a soluble Chemical Oxygen Demand (COD) concentration of approximately 60 000 mg/L. The resulting COD introduced to a cell suspension by the nutrient dose is about 2300 mg/L.

[4] Dehydrated Bacto Nutrient Broth, available from Difco Laboratories, P.O. Box 1058A, Detroit, MI 48732 or equivalent has been found suitable for this purpose.