Designation: D 5120 – 90 (Reapproved 2004)

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

This standard is issued under the fixed designation D 5120; 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 *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]

D 4478 Test Methods for Oxygen Uptake

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.

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.

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[1] This Test Method is under the jurisdiction of ASTM Committee D34 on Waste Management and is the direct responsibility of Subcommittee D34.07 on Municipal Solid Waste.

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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.

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.[3]

Ingredient	Quantity (g)
Bacto peptone[3]	32
Beef extract[3]	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.

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

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.

9.1.2.1 If the analyst elects to use an alternative nutrient feed, it should contain a biodegradable COD concentration that will establish the desired substrate saturation. Test saturation of the microbial culture with biodegradable organic material by making respiration rate observations at different concentrations of nutrient dose. Make sure that the selected minimum nutrient dose is such that an increase in dose will not result in an increase in respiration rate.

9.1.3 In the preparation of the test cell suspensions, the volume occupied by water in the control is replaced by dilutions of the test candidate. Available quantities of nitrogen and phosphorus must meet nutrients required in biochemical reactions. If nitrogen and phosphorus concentrations in the test candidate are unknown, determine the COD and add 10 mg/L of ammonium chloride (NH_4Cl) and 3 mg/L of potassium monohydrogen phosphate/100 mg/L of COD. After nutrient addition, adjust the pH of the test candidate dilutions to the normal pH for the full-scale system mixed liquor.

9.2 The number of test cell suspensions and concentrations of test candidate are selected to meet the objectives of the study. For aqueous test candidates, the concentration is represented as the volume percent of the total cell suspension volume. For dry or pure liquid test candidates, the concentration is mg/L in the total cell suspension volume.

9.2.1 For the objective of identifying possible inhibitory effects of a test candidate on a wastewater treatment process,

the minimum testing effort might be a single inhibition test in which the experimental cell suspension is at a selected concentration.

9.2.2 If a "Threshold of inhibition" or an" EC_{50}" is desired, a range of dilutions of the test candidate is prepared. A suggested minimum number of test cell suspensions is four spaced by a multiplier of not more than 3.2. For example, four systems for an EC_{50} beginning at a 4-vol % concentration and with a multiplier of 2.0 would be 4, 8, 16, and 32 %.

9.3 Provide one 2-L beaker (or other suitably sized container) for each test cell suspension and the control cell suspension.

9.3.1 Prepare the test cell suspensions. For example, to prepare a 1-L cell suspension when the microbial culture concentration is 10 000 mg/L and the objective cell microbial cell suspension concentration is 2 000 mg/L, transfer 38 mL of Marlene's mix and the desired quantity of test candidate to the 2-L beaker, bring the volume to 800 mL with tap water, transfer 200 mL of the microbial culture and initiate aeration. The rate of air flow should result in a DO concentration in the cell suspension of at least 2 to 3 mg/L.

9.3.2 The start time for each cell suspension and control in the series is staggered by a time interval required to measure respiration rate on each cell suspension at the end of the 2-h period. The staggering in start time permits the series of cell suspensions to be managed concurrently.

9.3.3 Aerate the cell suspensions for 2 h and then determine the respiration rates of the control and test candidate cell suspensions by transferring all or a portion of the cell suspension to the respiration measuring device.

9.3.4 If a dissolved oxygen probe is used, before determining the oxygen uptake rate, increase the DO of the cell suspension aliquot to the 4 to 6 mg/L range by increased aeration rate or pouring it back and forth between containers. Determine the DO of the cell suspension aliquot at 30-s intervals until the rate is established (normally a 2.5-min period). Calculate the oxygen uptake rate by plotting the DO values versus time and calculating the slope of the line.

NOTE 3—When fitting the line through the plotted DO probe data, favor the last three readings. The first two readings may not be linear and may represent the response of the DO probe as it chases the decreasing DO concentration. Calculate the slope (mg/L/min) and multiply by 60 to obtain mg/L/h.

10. Interpretation of Results

10.1 *Percent Inhibition of Respiration*— Calculate the inhibition of respiration rate as follows:

$$\text{Percent Inhibition} = (B - A)/B \times 100 \quad (1)$$

where:

A = respiration rate for the experimental cell suspension, (mg O_2/L/hr), and

B = respiration rate for the control (mg O_2/L/h).

10.2 *EC_{50}*—Determine the EC_{50} by plotting the "Percent Inhibition" employing a linear Y axis versus the concentration of the test candidate on a logarithmic X axis. Fit a straight line through the apparent slope of the data (Fig. 1). Since only two or three data points are involved in the line fitting, statistically methods are not indicated. However, if the fit is made statistically, data near 0 or 100 % inhibition should be ignored. The EC_{50} is the concentration of the test candidate that results in a 50 % inhibition of respiration.

10.3 *Inhibition Threshold*—The intercept of the straight line in Fig. 1 with 0 % inhibition is an indication of the inhibition threshold.

11. Precision and Bias

11.1 *Precision*—It is not practical to specify the precision of this test method for measurement of inhibition of respiration of microbial cultures because the desired results are the demonstrated impact of a test candidate on the *specific* microbial culture that exists in a *specific* activated sludge facility. Microbial cultures at different activated sludge facilities will differ in kind, numbers, and overall activity of the mixed population of microorganisms, and the resultant sensitivity to a specific test candidate. Thus, microbial cultures from different activated sludge systems are not suitable as the reference culture in measurements of interlaboratory procedural precision.

11.1.1 With respect to the microbial culture at a specific activated sludge facility, the response to a particular test candidate can vary with time due to shifts in the microbial culture's performance through acclimatization or other factors. Thus, the microbial culture at a specific activated sludge facility is not a suitable reference for the measurement of single operator precision.

11.2 *Bias*—Since there is no reference microbial culture for determining the bias of this test method for measuring inhibition of respiration of microbial cultures, no statement on bias is being made.

12. Keywords

12.1 inhibition of respiration; microbial cultures; microbial cultures in the activated sludge process; respiration

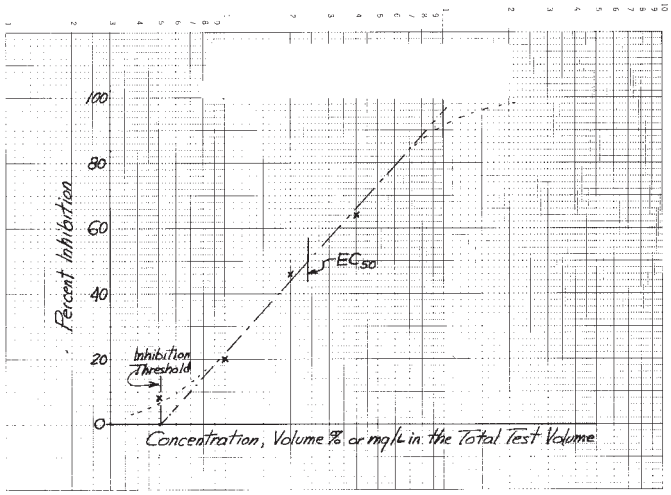


FIG. 1 Illustration of Inhibition Results

APPENDIX

(Nonmandatory Information)

X1. RESPIROMETERS FOR EXAMINATION OF MICROBIAL INHIBITION

X1.1 *Respirometers:*

X1.1.1 Respirometers are available in a variety of sizes. In some of the respirometric operations, the sample volume in the apparatus may contribute to measurement sensitivity because of the increased quantity of oxygen used per unit of time. However, smaller sized systems, such as an oxygraph[4] with 1.5 mL of sample in the measuring cell, are suitable for measurements of oxygen uptake rate in this test method.

X1.1.2 The basic principle of respirometry is the detection of oxygen removed from a gas volume that is in contact with the cell suspension. Microbial respiration consumes oxygen and releases carbon dioxide as a by-product of the biochemical reactions. If the carbon dioxide is removed from the gas volume, oxygen removal to support respiration will cause a net reduction in the gas volume. Measurement of the amount of oxygen utilized with time defines the respiration rate.

X1.1.3 The available respirometric systems reduce labor as compared to manual procedures and provide convenient electronic and computer supported procedures for interpretation and recording of results.

X1.2 *Apparatus:*

X1.2.1 The principle of all respirometers is similar. Carbon dioxide released to the gas space is removed by reaction with potassium hydroxide. The net change in pressure as oxygen is removed from the gas volume into the cell suspension is interpreted through either the pressure change (constant volume device) or the amount of oxygen required to maintain a constant pressure (constant pressure device).

X1.2.1.1 An example of a constant volume respirometer is presented by the COMPU-TOX respirometer in Fig. X1.1. A change in pressure in the gas space of the reaction bottle is referenced to a fixed pressure and oxygen is introduced to reestablish the gas space pressure. The quantity of oxygen introduced is measured and interpreted as oxygen consumption.

X1.2.1.2 A second example of a constant volume respirometer is the electrolytic biochemical oxygen demand (EBOD) respirometer in Fig. X1.2. The removal of oxygen from the gas space is sensed by the pressure change at the electrolysis cell. The cell generates oxygen to reestablish the gas space pressure.

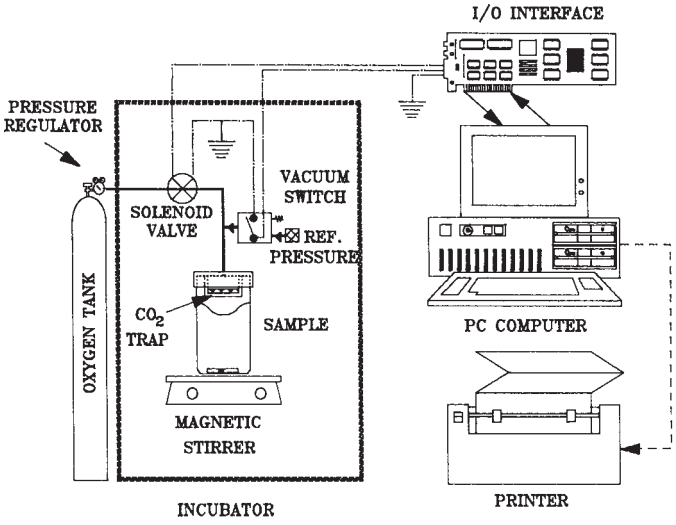


FIG. X1.1 Volumetric Respirometer

The flow of current to generate the oxygen is interpreted as weight of oxygen consumed.

X1.3 *Interferences:*

X1.3.1 Physical conditions associated with respirometric measurement of oxygen utilization can cause errors in the interpreted respiration rate. Refer to specific operating instructions or information from manufacturers for corrective procedures to minimize errors.

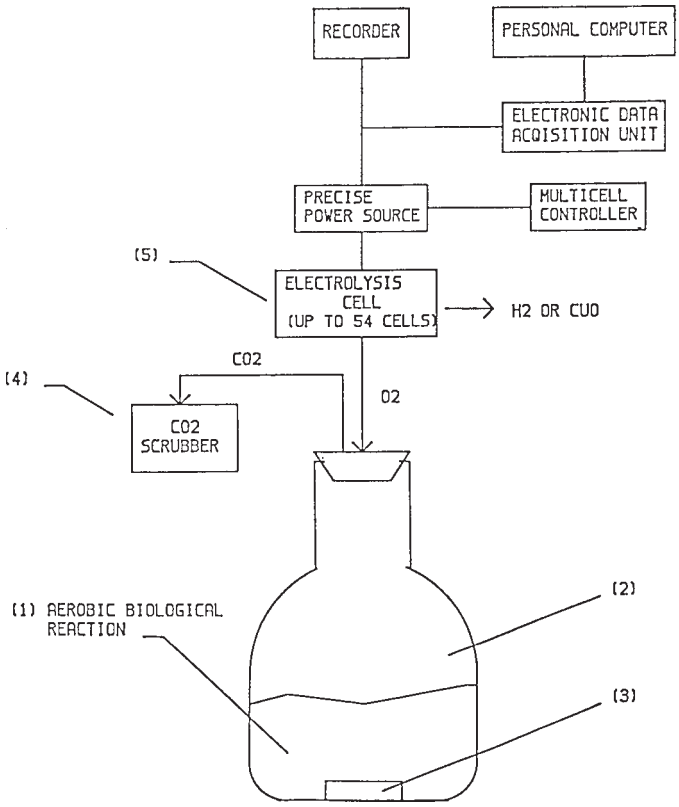
X1.3.1.1 Changes in barometric pressure during the period of respiration measurement will cause an error in the apparent utilization of oxygen for those respirometers that reference internal pressure to barometric pressure.

X1.3.1.2 Temperature imbalance due to a cell suspension temperature different from the temperature of gas in the respirometer will cause an error in apparent gas space volume or pressure (equivalent oxygen utilization) until the temperatures have had a chance to equilibrate.

X1.3.1.3 When the cell suspension, at the time of introduction to the respirometer, has a low or zero oxygen concentration, a falsely high oxygen consumption rate will exist until the operating DO concentration for the cell suspension is reached.

X1.3.1.4 Dissolved oxygen concentration in the cell suspension will vary if the partial pressure in the respirometer varies during the period of uptake rate measurement. The change in dissolved oxygen concentration will represent an error in uptake rate measurement.

[4] Gilson Oxygraph, available from Gilson Medical Electronics Inc., Box 27, 3000 W. Beltline, Middletown, WI 53562 or its equivalent, has been found suitable for this purpose.



NOTE 1—The respiration of a cell suspension (1) is measured enclosed in a sealed reactor vessel (2) under continuous mixing (3). The reactor shares gas with a carbon dioxide scrubber (4) and an electrolytic cell (5) that generates oxygen in response to oxygen consumption to support respiration by the cell suspension.

FIG. X1.2 Electrolytic Respirometer