5210 BIOCHEMICAL OXYGEN DEMAND (BOD)*

5210 A. Introduction

1. General Discussion

The biochemical oxygen demand (BOD) determination is an empirical test in which standardized laboratory procedures are used to determine the relative oxygen requirements of wastewaters, effluents, and polluted waters. The test has its widest application in measuring waste loadings to treatment plants and in evaluating the BOD-removal efficiency of such treatment systems. The test measures the molecular oxygen utilized during a specified incubation period for the biochemical degradation of organic material (carbonaceous demand) and the oxygen used to oxidize inorganic material such as sulfides and ferrous iron. It also may measure the amount of oxygen used to oxidize reduced forms of nitrogen (nitrogenous demand) unless their oxidation is prevented by an inhibitor. The seeding and dilution procedures provide an estimate of the BOD at pH 6.5 to 7.5.

Measurements of oxygen consumed in a 5-d test period (5-d BOD or BOD_5, 5210B), oxygen consumed after 60 to 90 d of incubation (ultimate BOD or UBOD, 5210C), and continuous oxygen uptake (respirometric method, 5210D) are described here. Many other variations of oxygen demand measurements exist, including using shorter and longer incubation periods and tests to determine rates of oxygen uptake. Alternative seeding, dilution, and incubation conditions can be chosen to mimic receiving-water conditions, thereby providing an estimate of the environmental effects of wastewaters and effluents.

The UBOD measures the oxygen required for the total degradation of organic material (ultimate carbonaceous demand) and/or the oxygen to oxidize reduced nitrogen compounds (ultimate nitrogenous demand). UBOD values and appropriate kinetic descriptions are needed in water quality modeling studies such as UBOD:BOD_5 ratios for relating stream assimilative capacity to regulatory requirements; definition of river, estuary, or lake deoxygenation kinetics; and instream ultimate carbonaceous BOD (UCBOD) values for model calibration.

2. Carbonaceous Versus Nitrogenous BOD

A number of factors, for example, soluble versus particulate organics, settleable and floatable solids, oxidation of reduced iron and sulfur compounds, or lack of mixing may affect the accuracy and precision of BOD measurements. Presently, there is no way to include adjustments or corrections to account for the effect of these factors.

Oxidation of reduced forms of nitrogen, such as ammonia and organic nitrogen, can be mediated by microorganisms and exert nitrogenous demand. Nitrogenous demand historically has been considered an interference in the determination of BOD, and the inclusion of ammonia in the dilution water contributes an external source of nitrogenous demand. The interference from nitrogenous demand can now be prevented by an inhibitory chemical.[1] If an inhibiting chemical is not used, the oxygen demand measured is the sum of carbonaceous and nitrogenous demands.

Measurements that include nitrogenous demand generally are not useful for assessing the oxygen demand associated with organic material. Nitrogenous demand can be estimated directly from ammonia nitrogen (Section 4500-NH_3); and carbonaceous demand can be estimated by subtracting the theoretical equivalent of the nitrite and nitrate produced in uninhibited test results. However, this method is cumbersome and is subject to considerable error. Chemical inhibition of nitrogenous demand provides a more direct and more reliable measure of carbonaceous demand.

The extent of oxidation of nitrogenous compounds during the 5-d incubation period depends on the concentration and type of microorganisms capable of carrying out this oxidation. Such organisms usually are not present in raw or settled primary sewage in sufficient numbers to oxidize sufficient quantities of reduced nitrogen forms in the 5-d BOD test. Many biological treatment plant effluents contain sufficient numbers of nitrifying organisms to cause nitrification in BOD tests. Because oxidation of nitrogenous compounds can occur in such samples, inhibition of nitrification as directed in 5210B.5*e*) is recommended for samples of secondary effluent, for samples seeded with secondary effluent, and for samples of polluted waters.

3. Reference

1. YOUNG, J.C. 1973. Chemical methods for nitrification control. *J. Water Pollut. Control Fed.* 45:637.

* Approved by Standard Methods Committee, 2001. Editorial revisions, 2011. Joint Task Group: 21st Edition—James C. Young (chair), George T. Bowman, Sabry M. Kamhawy, Terry G. Mills, Marlene Patillo, Ray C. Whittemore.

5210 B. 5-Day BOD Test

1. General Discussion

The method consists of filling with diluted and seeded sample, to overflowing, an airtight bottle of specified size and incubating it at the specified temperature for 5 d. Dissolved oxygen is measured initially and after incubation, and the BOD is computed from the difference between initial and final DO. Because the initial DO is determined shortly after the dilution is made, all oxygen uptake occurring after this measurement is included in the BOD measurement.

For sampling and storage procedures, see 5210B.4*a*.

2. Apparatus

a. Incubation bottles: Use glass bottles having 60 mL or greater capacity (300-mL bottles having a ground-glass stopper and a flared mouth are preferred). Clean bottles with a detergent, rinse thoroughly, and drain before use.

b. Air incubator or water bath, thermostatically controlled at 20 ±1°C. Exclude all light to prevent possibility of photosynthetic production of DO.

3. Reagents

Prepare reagents in advance but discard if there is any sign of precipitation or biological growth in the stock bottles. Commercial equivalents of these reagents are acceptable and different stock concentrations may be used if doses are adjusted proportionally. Use reagent grade or better for all chemicals and use distilled or equivalent water, preferably sterilized, for making all solutions.

a. Phosphate buffer solution: Dissolve 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4 \cdot 7H_2O$, and 1.7 g NH_4Cl in about 500 mL distilled water and dilute to 1 L. The pH should be 7.2 without further adjustment. Alternatively, dissolve 42.5 g KH_2PO_4 and 1.7 g NH_4Cl in about 700 mL distilled water. Adjust pH to 7.2 with 30% NaOH and dilute to 1 L.

b. Magnesium sulfate solution: Dissolve 22.5 g $MgSO_4 \cdot 7H_2O$ in distilled water and dilute to 1 L.

c. Calcium chloride solution: Dissolve 27.5 g $CaCl_2$ in distilled water and dilute to 1 L.

d. Ferric chloride solution: Dissolve 0.25 g $FeCl_3 \cdot 6H_2O$ in distilled water and dilute to 1 L.

e. Acid and alkali solutions, 1*N*, for neutralization of caustic or acidic waste samples.

1) Acid—Slowly and while stirring, add 28 mL conc sulfuric acid to distilled water. Dilute to 1 L.

2) Alkali—Dissolve 40 g sodium hydroxide in distilled water. Dilute to 1 L.

f. Sodium sulfite solution: Dissolve 1.575 g Na_2SO_3 in 1000 mL distilled water. This solution is not stable; prepare daily.

g. Nitrification inhibitor:

1) 2-chloro-6-(trichloromethyl) pyridine—Use pure TCMP or commercial preparations.*

2) Allylthiourea (ATU) solution—Dissolve 2.0 g allylthiourea ($C_4H_8N_2S$) in about 500 mL water and dilute to 1 L. Store at 4°C. The solution is stable for not more than 2 weeks.

h. Glucose-glutamic acid solution: Dry reagent-grade glucose and reagent-grade glutamic acid at 103°C for 1 h. Add 150 mg glucose and 150 mg glutamic acid to distilled water and dilute to 1 L. Prepare fresh immediately before use unless solution is maintained in a sterile condition. Store all glucose-glutamic acid mixtures at 4°C or lower. Commercial preparations may be used but concentrations may vary.

i. Ammonium chloride solution: Dissolve 1.15 g NH_4Cl in about 500 mL distilled water, adjust pH to 7.2 with NaOH solution, and dilute to 1 L. Solution contains 0.3 mg N/mL.

j. Source water for preparing BOD dilution water: Use demineralized, distilled, tap, or natural water for making sample dilutions (see 5210B.4*c*).

4. Preparatory Procedures

a. Sampling and storage: Samples for BOD analysis may degrade significantly during storage between collection and analysis, resulting in low BOD values.

1) Grab samples—If analysis is begun within 2 h of collection, cold storage is unnecessary. If analysis is not started within 2 h of sample collection, keep sample at or below 4°C from the time of collection. Begin analysis within 6 h of collection; when this is not possible because the sampling site is distant from the laboratory, store at or below 4°C and report length and temperature of storage with the results. In no case start analysis more than 24 h after grab sample collection. When samples are to be used for regulatory purposes make every effort to deliver samples for analysis within 6 h of collection.

2) Composite samples—Keep samples at or below 4°C during compositing. Limit compositing period to 24 h. Use the same criteria as for storage of grab samples, starting the measurement of holding time from end of compositing period. State storage time and conditions as part of the results.

b. Sample preparation and pretreatment:

1) All samples—Check pH; if it is not between 6.0 and 8.0, adjust sample temperature to 20 ± 3°C, then adjust pH to 7.0 to 7.2 using a solution of sulfuric acid (H_2SO_4) or sodium hydroxide (NaOH) of such strength that the quantity of reagent does not dilute the sample by more than 0.5%. Exceptions may be justified with natural waters when the BOD is to be measured at in-situ pH values. The pH of dilution water should not be affected by the lowest sample dilution. Always seed samples that have been pH adjusted.

2) Samples containing residual chlorine compounds—If possible, avoid samples containing residual chlorine by sampling ahead of chlorination processes. If residual chlorine is present, dechlorinate sample. In some samples chlorine will dissipate within 1 to 2 h of standing in the light. This dissipation often occurs during sample transport and handling. For samples in which chlorine residual does not dissipate in a reasonably short time, destroy chlorine residual by adding Na_2SO_3 solution. Determine required volume of Na_2SO_3 solution on a 100- to 1000-mL portion of neutralized sample by adding 10 mL 1 + 1 acetic acid or 1 + 50 H_2SO_4, 10 mL potassium iodide (KI) solution (10 g /100 mL) per 1000 mL sample and titrating with Na_2SO_3 solution to the starch-iodine end point for residual. Add to neutralized sample the proportional volume of Na_2SO_3 solution determined by the above test, mix, and after 10 to 20 min check sample for residual chlorine. (NOTE: Excess Na_2SO_3 exerts an oxygen demand and reacts slowly with certain organic chloramine compounds that may be present in chlorinated samples.) Do not test chlorinated/dechlorinated samples without seeding.

3) Samples containing other toxic substances—Certain industrial wastes, for example, plating wastes, contain toxic metals. Such samples often require special study and treatment.

4) Samples supersaturated with DO—Samples containing DO concentration above saturation at 20°C may be encountered in cold waters or in water where photosynthesis occurs. To prevent loss of oxygen during incubation of such samples, reduce DO to

* Nitrification Inhibitor Formula 2533 (2% TCMP on sodium sulfate), Hach Co., Loveland, CO, or equivalent.

saturation by bringing sample to about 20 ± 3°C in partially filled bottle while agitating by vigorous shaking or by aerating with clean, filtered compressed air.

5) Samples containing hydrogen peroxide—Hydrogen peroxide remaining in samples from some industrial bleaching processes such as those used at paper mills and textile plants can cause supersaturated oxygen levels in samples collected for BOD testing. Mix such samples vigorously in open containers for sufficient time to allow the hydrogen peroxide to dissipate before setting up BOD tests. Check adequacy of peroxide removal by observing dissolved oxygen concentrations over time during mixing or by using peroxide-specific test strips. Mixing times can vary from 1 to 2 h depending on the amount of hydrogen peroxide present. The peroxide reaction can be considered complete when the DO no longer increases during a 30-min period without mixing.

c. Selection and storage of source water for BOD sample dilution: Obtain water from suitable source—distilled, tap, or receiving water. Make sure the water is free of heavy metals, specifically copper, and toxic substances, such as chlorine, that can interfere with BOD measurements. Protect source water quality by using clean glassware, tubing, and bottles. Deionized water often contains sufficient amounts of organics and microorganisms to cause failure of the dilution water quality control check (5210B.6*c*). Source water may be stored before use as long as the prepared dilution water (5210B.5*a*) meets quality control criteria in the dilution water blank (5210B.6*c*). Such storage may improve the quality of some source waters but may allow biological growth to cause deterioration in others. Storage of prepared dilution water (5210B.5*h*) for more than 24 h after adding nutrients, minerals, and buffer is not recommended unless dilution water blanks consistently meet quality control limits. Discard stored source water if the dilution water blank shows more than 0.20 mg/L DO depletion in 5 d (5210B.6*c*).

d. Preparation of seed suspension: It is necessary to have present in each BOD bottle a population of microorganisms capable of oxidizing the biodegradable organic matter in the sample. Domestic wastewater, unchlorinated or otherwise undisinfected effluents from biological wastewater treatment plants, and surface waters receiving wastewater discharges usually contain satisfactory microbial populations. Some samples (for example, some untreated industrial wastes, disinfected wastes, high-temperature wastes, wastes having pH values less than 6 or greater than 8, or wastes stored more than 6 h after collection) do not contain a sufficient microbial population. Seed such samples by adding a population of suitable microorganisms. The preferred seed is obtained from a biological treatment system processing the waste. In this case, use supernatant from settled domestic wastewater, effluent from primary clarifiers, diluted mixed liquor from an aeration basin, undisinfected effluent, or receiving water from below the point of discharge. When effluent or mixed liquor from a biological treatment process is used as a seed source, inhibition of nitrification is recommended. Do not use seed from effluents that have been disinfected by chlorine or other means. Commercial seed sources may be used but are more likely to be unadapted to the wastewater constituents. Do not filter seed sources; filtering removes the seed microorganisms.

When acclimated seed sources are not available, develop an acclimated seed in the laboratory by continuously aerating a sample of settled domestic wastewater and adding small daily increments of sample from the waste in question. Use a soil suspension, activated sludge, or a commercial seed preparation to obtain the initial microbial population. Determine the existence of a satisfactory population by testing the performance of the seed in BOD tests on the sample. BOD values that increase with time of adaptation to a steady high value indicate successful seed acclimation.

5. Testing Procedure

a. Preparation of dilution water: Transfer desired working volume of source water (5210B.4*c*) to a suitably sized bottle (glass is preferred). Check to ensure that the dissolved oxygen concentration is at least 7.5 mg/L before using water for BOD tests. If not, add DO by shaking bottle or by aerating with organic-free filtered air. Alternatively, store the water in cotton-plugged bottles long enough for the DO concentration to approach saturation. Add 1 mL each of phosphate buffer, $MgSO_4$, $CaCl_2$, and $FeCl_3$ solution/L to prepared source water (5210B.4*c*). Mix thoroughly and bring temperature to 20 ± 3°C. Prepare dilution water immediately before use unless dilution water blanks (5210B.6*c*) show that the water is acceptable after longer storage times. If the dilution water blanks show a DO depletion greater than 0.20 mg/L, obtain a satisfactory water by improving purification or use water from another source. Do not add oxidizing agents or expose dilution water to ultraviolet light in attempts to bring the dilution blank into range.

b. Sample temperature adjustment: Bring samples to 20 ± 3°C before making dilutions.

c. Preparation of dilutions: Using the dilution water prepared as in ¶ *a* above, make at least three dilutions of prepared sample estimated to produce a residual DO of at least 1.0 mg/L and a DO uptake of at least 2.0 mg/L after a 5-d incubation. Five dilutions are recommended if experience with a particular sample does not produce at least three bottles having acceptable minimum DO depletions and residual limits (5210B.6*a*). A more rapid analysis, such as COD (Section 5220), may be correlated approximately with BOD and serve as a guide in selecting dilutions. In the absence of prior knowledge, use the following percentages of wastewater when preparing dilutions: 0.01 to 1.0% for strong industrial wastes, 1 to 5% for raw and settled wastewater, 5 to 25% for biologically treated effluent, and 25 to 100% for polluted river waters. The number of bottles to be prepared for each dilution depends on the DO technique and the number of replicates desired. Prepare dilutions in volumetric containers (Class A glass or equivalent) and then transfer to BOD bottles or prepare directly in BOD bottles. Either dilution method can be combined with any DO measurement technique.

1) Dilutions prepared in volumetric containers—Using a wide-tipped pipet, add desired amount of prepared sample to individual volumetric cylinders or flasks. Mix the sample well immediately before pipetting to avoid loss of solids by settling. For dilutions greater than 1:100 make a primary dilution before making final dilution in the bottle. Fill cylinders or flasks at least two-thirds full of dilution water without entraining air. Add appropriate amounts of seed suspension (¶ *d* below) and nitrification inhibitor (¶ *e* below). Dilute to final level with dilution water (¶ *a* above). Mix well but avoid entraining air. Siphon

mixed dilution into a suitable number of BOD bottles, taking care not to let solids settle in the cylinder or flask during transfer.

2) Dilutions prepared directly in BOD bottles—Using a wide-tip volumetric pipet, add the desired sample volume to individual BOD bottles. Fill each BOD bottle approximately two-thirds full with dilution water. Add appropriate amounts of seed suspension (¶ *d* below) and nitrification inhibitor (¶ *e* below) to the individual BOD bottles. When a bottle contains more than 67% of the sample after dilution, nutrients may be limited in the diluted sample and subsequently reduce biological activity. In such samples, add the nutrient, mineral, and buffer solutions (5210B.3*a–e*) directly to diluted sample at a rate of 1 mL/L (0.30 mL/300-mL bottle) or use commercially prepared solutions designed to dose the appropriate bottle size.

d. Addition of seed suspension: If seeding is used, add seed suspensions to the dilution vessels or to individual BOD bottles before final dilution as described in ¶ *c* above. Do not add seed directly to wastewater samples if they contain materials that are toxic before dilution. Generally, 1 to 3 mL of settled raw wastewater or primary effluent or 1 to 2 mL of a 1:10 dilution of mixed liquor/300-mL bottle will provide a suitable amount of microorganisms. Do not filter seed suspension before use. Agitate the seed suspension during transfer to ensure that the same quantity of microorganisms is added to each BOD bottle. Always record the exact volume of seed suspension added to each bottle. The DO uptake attributable to the seed added to each bottle generally should be between 0.6 and 1.0 mg/L, but the amount of seed added should be adjusted from this range to that required to provide glucose-glutamic acid (GGA) check results of 198 ± 30.5 mg/L. For example, if 1 mL of seed suspension is required to achieve 198 ± 30.5 mg/L BOD in the glucose-glutamic acid check, then use 1 mL in each BOD bottle receiving the test wastewater.

e. Addition of nitrification inhibitor: Samples that may require nitrification inhibition[1] include, but are not limited to, biologically treated effluents, samples seeded with biologically treated effluents, and river waters. Note the use of nitrogen inhibition and the chemical used when reporting results. (NOTE: TCMP is the preferred nitrification inhibitor but requires handling and transfer in a solid form. Allylthiourea is not always effective in inhibiting nitrification within the 5-d incubation period and concentrations above 2 mg/L may cause increases in carbonaceous BOD measurements. ATU concentrations above 2 mg/L also can adversely affect the azide modification of the iodometric method). Seed all samples to which nitrification inhibitor has been added. The amount of seed should be consistent with that required to achieve GGA test results in the range of 198 ± 30.5 mg/L (5210B.6*b*).

1) Nitrification inhibition using 2-chloro-6-(trichloromethyl) pyridine (TCMP)—Add 10 mg TCMP/L to diluted sample or 3 mg TCMP to each 300-mL bottle or sample dilution vessel, or proportional amounts to other sized bottles, after initial sample dilution but before final filling of the bottles with dilution water. Do not add TCMP to BOD bottles before they are at least two-thirds filled with diluted sample. (NOTE: TCMP dissolves slowly and can float on top of the sample if not mixed well). Some commercial TCMP formulations are not 100% TCMP; adjust dosage appropriately.

2) Nitrification inhibition using allylthiourea (ATU)—Add 1 mL ATU solution (5210B.3*g*2)/L diluted sample or 0.3 mL/300mL test bottle or sample dilution vessel. Do not add ATU to BOD bottles until they are at least two-thirds filled with diluted sample.

f. Sealing of bottles: Complete filling of each bottle by adding enough dilution water that insertion of the stopper leaves no bubbles in the bottle. Mix the sample by turning the bottle manually several times unless a DO probe having a stirrer is used immediately to measure initial DO concentration. As a precaution against drawing air into the dilution bottle during incubation, use a water seal. Obtain satisfactory water seals by inverting bottles in a water bath or by adding water to the flared mouth of special BOD bottles. Place a paper or plastic cup or foil cap over flared mouth of bottle to reduce evaporation of the water seal during incubation.

g. Determination of initial DO: Use the azide modification of the iodometric method (Section 4500-O.C) or the membrane electrode method (Section 4500-O.G) to determine initial DO on all sample dilutions, dilution water blanks, and, where appropriate, seed controls. Replace any displaced contents with sufficient diluted sample or dilution water to fill the bottle, stopper all bottles tightly, and water seal before beginning incubation. After preparing dilution, measure initial DO within 30 min. If the membrane electrode method is used, take care to eliminate drift in calibration between initial and final DO readings. If the azide modification of the titrimetric iodometric method is used, prepare an extra bottle for initial DO determination for each sample dilution.

h. Sample incubation: Incubate at 20°C ± 1°C the stoppered and sealed BOD bottles containing desired dilutions (¶ *a* above), seed controls (5210B,6*d*), dilution water blanks (5210B.6*c*), and glucose-glutamic acid checks (5210B.6*b*). Exclude light to avoid growth of algae in the bottles during incubation.

i. Determination of final DO: After 5 d ± 6 h of incubation, determine DO in all sample dilutions, and in all blanks and checks as in ¶ 6*b–d*, using the azide modification of the titrimetric method or the membrane electrode method.

6. Quality Control Checks

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

a. Minimum residual DO and minimum DO depletion: Only bottles, including seed controls, giving a minimum DO depletion of 2.0 mg/L and a residual DO of at least 1.0 mg/L after 5 d of incubation are considered to produce valid data, because at least 2.0 mg oxygen/uptake L is required to give a meaningful measure of oxygen uptake and at least 1.0 mg/L must remain throughout the test to ensure that insufficient DO does not affect the rate of oxidation of waste constituents. Exceptions occur for reporting purposes only when the depletions for tests using undiluted samples in all bottles fall below 2.0 mg/L and when the residual DO in all dilutions is less than 1.0 mg/L (5210B.7). When using membrane electrodes for measuring DO, make frequent calibration checks to ensure accurate DO readings (Section 4500-0.G.3*a*).

b. Glucose-glutamic acid check: The glucose-glutamic acid check is the primary basis for establishing accuracy and precision of the BOD test and is the principal measure of seed quality and set-up procedure. Together with each batch of samples, check seed effectiveness and analytical technique by using pro-

cedures in 5210B.5 to make BOD measurements on an equal weight mixture of glucose and glutamic acid as follows: Add sufficient amounts of standard glucose-glutamic acid solution (5210B.3*h*) to give 3.0 mg glucose/L and 3.0 mg glutamic acid/L in each of three test bottles (20 mL GGA solution/L seeded dilution water, or 6.0 mL/300-mL bottle). Commercial solutions may contain other glucose-glutamic acid concentrations; adjust doses accordingly. Add nitrification inhibitor if seed is obtained from a source that is nitrifying. Evaluate data as described in 5210B.8, Precision and Bias. The resulting average BOD for the three bottles, after correction for dilution and seeding, must fall into the range of 198 ± 30.5 mg/L. If the average value falls outside this range, evaluate the cause and make appropriate corrections. Consistently high values can indicate the use of too much seed suspension, contaminated dilution water, or the occurrence of nitrification; consistently low values can indicate poor seed quality or quantity or the presence of a toxic material. If low values persist, prepare a new mixture of glucose and glutamic acid and check the sources of dilution water and source of seed.

c. Dilution water quality check: With each batch of samples incubate one or more bottles of dilution water that contains nutrient, mineral, and buffer solutions but no seed or nitrification inhibitor. This dilution water blank serves as a check on quality of unseeded dilution water and cleanliness of incubation bottles. Determine initial and final DO as in 5210B.5*g* and *i*. The DO uptake in 5 d must not be more than 0.20 mg/L and preferably not more than 0.10 mg/L, before making seed corrections. If the dilution water blank exceeds 0.20 mg/L, discard all data for tests using this dilution water or clearly identify such samples in data records.

d. Seed control: Determine BOD of the seed suspension as for any other sample. This is the *seed control.* Ideally, make three dilutions of seed such that the smallest quantity gives at least 2.0 mg/L DO depletion and the largest quantity results in at least 1.0 mg/L DO residual after 5 d of incubation. Determine the DO uptake per milliliter of seed added to each bottle using either the slope method or the ratio method. For the slope method, plot DO depletion in milligrams per liter versus milliliters of seed for all seed control bottles having a 2.0 mg/L depletion and 1.0 minimum residual DO. The plot should present a straight line for which the slope indicates DO depletion per milliliter of seed. The DO-axis intercept is oxygen depletion caused by the dilution water and should be less than 0.20 mg/L (see ¶ *c* above). For the ratio method, divide the DO depletion by the volume of seed in milliliters for each seed control bottle having a 2.0 mg/L depletion and greater than 1.0 mg/L minimum residual DO and average the results. Seed dilutions showing widely varying depletions per milliliter of seed (±30%) suggest the presence of toxic substances or large particulates in the seed suspension. In this case, check or change the seed source.

7. Data Analysis and Reporting

a. Calculations:

1) For each test bottle having 2.0 mg/L minimum DO depletion and at least 1.0 mg/L residual DO, calculate BOD as follows:

$$\text{BOD}_5, \text{mg/L} = \frac{(D_1 - D_2) - (S)V_s}{P}$$

where:

D_1 = DO of diluted sample immediately after preparation, mg/L,
D_2 = DO of diluted sample after 5 d incubation at 20°C, mg/L,
S = oxygen uptake of seed, Δ DO/mL seed suspension added per bottle (5210B.6*d*) ($S = 0$ if samples are not seeded),
V_s = volume of seed in the respective test bottle, mL, and
P = decimal volumetric fraction of sample used; $1/P$ = dilution factor.

2) If DO depletion is less than 2.0 mg/L and sample concentration is 100% (no dilution except for seed, nutrient, mineral, and buffer solutions), actual seed-corrected, DO depletion may be reported as the BOD even if it is less than 2.0 mg/L.

3) When all dilutions result in a residual DO <1.0, select the bottle having the lowest DO concentration (greatest dilution) and report:

$$\text{BOD, mg/L} > \frac{(D_1 - D_2) - (S)V_s}{P}$$

In the above calculations, do not make corrections for DO uptake by the dilution water blank during incubation. This correction is unnecessary if dilution water meets the blank criteria stipulated in 5210B.6*c*. If the dilution water does not meet these criteria, proper corrections are difficult; do not record results or, as a minimum, mark them as not meeting quality control criteria.

b. Reporting: Average the test results for all qualified bottles within each dilution series. Report the result as BOD_5 if nitrification is not inhibited. Report results as $CBOD_5$ if nitrification is inhibited. Samples showing large differences between the computed BOD for different dilutions, for example, greater than 30%, may indicate the presence of a toxic substance or analytical problems. When the effect becomes repetitive, investigate to identify the cause. Identify results in the test reports when any of the following quality control parameters is not met:

- Dilution water blank exceeds 0.20 mg/L (5210B.6*c*),
- Glucose-glutamic acid check falls outside acceptable limits (5210B.6*b*),
- Test replicates show more than 30% difference between high and low values,
- Seed control samples do not meet the above criteria in all dilutions (5210B.6*d*), or
- Minimum DO is less than 1.0 mg/L [5210B.7*a*3)].

8. Precision and Bias

There is no measurement for establishing bias of the BOD procedure. The glucose-glutamic acid check prescribed in 5210B.6*b* is intended to be a reference point for evaluation of dilution water quality, seed effectiveness, and analytical technique. Single-laboratory tests using a 300-mg/L mixed glucose-glutamic acid solution provided the following results:

Number of months:	14	
Number of triplicates:	421	
Average monthly recovery:	204	mg/L
Average monthly standard deviation:	10.4	mg/L

In a series of interlaboratory studies,[2] each involving 2 to 112 laboratories (and as many analysts and seed sources), 5-d BOD measurements were made on synthetic water samples containing a 1:1 mixture of glucose and glutamic acid in the total concentration range of 3.3 to 231 mg/L. The regression equations for mean value, X, and standard deviation, S, from these studies were:

$$X = 0.658 \text{ (added concentration, mg/L)} + 0.280 \text{ mg/L}$$

$$S = 0.100 \text{ (added concentration, mg/L)} + 0.547 \text{ mg/L}$$

For the 300-mg/L mixed primary standard, the average 5-d BOD would be 198 mg/L with a standard deviation of 30.5 mg/L. When nitrification inhibitors are used, GGA test results falling outside the 198 ± 30.5 control limit quite often indicate use of incorrect amounts of seed. Adjust amount of seed added to the GGA test to achieve results falling within this range.

a. Control limits: Because of many factors affecting BOD tests in multilaboratory studies and the resulting extreme variability in test results, one standard deviation, as determined by interlaboratory tests, is recommended as a control limit for individual laboratories. Alternatively, each laboratory may establish its control limits by performing a minimum of 25 glucose-glutamic acid checks (5210B.6*b*) over a period of several weeks or months and calculating the mean and standard deviation. Use the mean ±3 standard deviations as the control limit for future glucose-glutamic acid checks. Compare calculated control limits to the single-laboratory tests presented above and to interlaboratory results. If the glucose-glutamic acid test results are outside the range of 198 ± 30.5, re-evaluate the control limits and investigate source of the problem. If measured BOD for a glucose-glutamic acid check is outside the accepted control limit range, reject tests made with that seed and dilution water or identify such tests clearly in all data records and reports.

b. Working range and detection limit: The working range is equal to the difference between the maximum initial DO (7 to 9 mg/L) and minimum DO residual of 1 mg/L corrected for seed, and multiplied by the dilution factor.

Detection limits are established by the minimum DO depletion and minimum DO residuals as follows:

- The lower detection limit for unseeded samples that require dilution ($S = 0$; $P < 1.0$) is 2 mg/L multiplied by the dilution factor as established by the requirement for a minimum DO depletion of 2 mg/L.
- The lower limit for seeded samples that require dilution ($S > 0$; $P < 1.0$) is approximately 1 mg/L as established by the minimum depletion of 2.0 mg/L minus the maximum seed correction, which should be less than about 1 mg/L.
- The lower limit for unseeded samples that require no dilution ($S = 0$; $P = 1.0$) is equal to the detection limit of the DO measurement method (~0.1 mg/L).
- The lower detection limit for seeded samples that require no dilution ($S > 0$; $P = 1.0$) is 0 mg/L, as established by the difference between the sample DO depletion and the seed correction.

9. References

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5210 C. Ultimate BOD Test

1. General Discussion

The ultimate BOD test is an extension of the 5-d dilution BOD test (5210B) but with a number of specific test requirements and differences in application. The user should be familar with the 5210B procedure before conducting tests for UBOD.

a. Principle: The method consists of placing a single sample dilution in full, airtight bottles and incubating under specified conditions for an extended period depending on wastewater, effluent, river, or estuary quality.[1] Dissolved oxygen (DO) is measured (with probes) initially and intermittently during the test. From the DO versus time series, UBOD is calculated by an appropriate statistical technique. For improved accuracy, run tests in triplicate.

Bottle size and incubation time are flexible to accommodate individual sample characteristics and laboratory limitations. Incubation temperature, however, is 20°C. Most effluents and some naturally occurring surface waters contain materials with oxygen demands exceeding the DO available in air-saturated water. Therefore, it is necessary either to dilute the sample or to monitor DO frequently to ensure that low DO or anaerobic conditions do not occur. When DO concentrations approach 2 mg/L, the sample should be reaerated.

Because bacterial growth requires nutrients such as nitrogen, phosphorus, and trace metals, the necessary amounts may be added to the dilution water together with buffer to ensure that pH remains in a range suitable for bacterial growth and seed to

provide an adequate bacterial population. However, if the result is being used to estimate the rate of oxidation of naturally occurring surface waters, addition of nutrients and seed probably accelerates the decay rate and produces misleading results. If only UBOD is desired, it may be advantageous to add supplemental nutrients that accelerate decay and reduce the test duration. When nutrients are used, they also should be used in the dilution water blank. Because of the wide range of water and wastewater characteristics and varied applications of UBOD data, no specific nutrient or buffer formulations are included.

The extent of oxidation of nitrogenous compounds during the prescribed incubation period depends on the presence of microorganisms capable of carrying out this oxidation. Such organisms may not be present in wastewaters in sufficient numbers to oxidize significant quantities of reduced nitrogen. This situation may be reversed in naturally occurring surface waters. Erratic results may be obtained when a nitrification inhibitor is used;[2] therefore, the specified method precludes use of a nitrogen inhibitor unless prior experimental evidence on the particular sample suggests that it is acceptable.* Monitor NO_2^--N and NO_3^--N to compute the oxygen equivalency of the nitrification reaction. When these values are subtracted from the DO vs. time series, the carbonaceous BOD time series can be constructed.[3]

b. Sampling and storage: See 5210B.4*a*.

c. Quality control (QC): The QC practices considered to be an integral part of each method are summarized in Table 5020:I.

2. Apparatus

a. Incubation bottles: Glass bottles with ground-glass stoppers,† 2-L (or larger) capacity. Glass serum bottles of 4- to 10-L capacity are available. Alternatively use nonground-glass bottles with nonbiodegradable plastic caps as a plug insert. Do not reuse the plugs because discoloration occurs with continued use. Replace plugs every 7 to 14 d. Do not use rubber stoppers that may exert an oxygen demand. Clean bottles with a detergent and wash with dilute HCl (3*N*) to remove surface films and precipitated inorganic salts; rinse thoroughly with DI water before use. Cover top of bottles with paper after rinsing to prevent dust from collecting. To prevent drawing air into the sample bottle during incubation, use a water seal. If the bottle does not have a flared mouth, construct a water seal by making a watertight dam around the stopper (or plug) and fill with water from the reservoir as necessary. Cover dam with clean aluminum foil to retard evaporation. If a 2-L BOD bottle is used, fill reservoir with sample and cover with a polyethylene cap before incubation.

Place a clean magnetic stirring bar in each bottle to mix contents before making DO measurement or taking a subsample. Do not remove the magnets until the test is complete.

Alternatively, use a series of 300-mL BOD bottles (5210B.2*a*) if larger bottles are not available or incubation space is limited.

b. Reservoir bottle: 4-L or larger glass bottle. Close with screw plastic cap or non-rubber plug.

c. Incubator or water bath, thermostatically controlled at 20 ± 1°C. Exclude all light to prevent the possibility of photosynthetic production of DO.

d. Oxygen-sensitive membrane electrode: See Section 4500-O.G.2.

3. Procedure

a. River water samples: Preferably fill large BOD bottle (>2 L, or alternatively 6 or more 300-mL BOD bottles) with sample at 20°C. Add no nutrients, seed, or nitrification inhibitor if in-bottle decay rates will be used to estimate in-stream rates. Do not dilute sample unless it is known by pretesting or by experience to have a high ultimate BOD (>20 mg/L).

Measure DO in each bottle, stopper, and make an airtight seal. Incubate at 20°C in the dark.

Measure DO in each bottle at intervals of at least 2 to 5 d over a period of 30 to 60 d (minimum of 6 to 8 readings) or longer under special circumstances. To avoid oxygen depletion in samples containing NH_3-N, measure DO more frequently until nitrification has taken place. If DO falls to about 2 mg/L, reaerate as directed below. Replace sample lost by the cap and DO probe displacement by adding 1 to 2 mL sample from the reservoir bottle.

When DO approaches 2 mg/L, reaerate. Pour a small amount of sample into a clean vessel and reaerate the remainder directly in the bottle by vigorous shaking or bubbling with purified air (medical grade). Refill bottle from the storage reservoir and measure DO. This concentration becomes the initial DO for the next measurement. If using 300-mL BOD bottles, pour all of the sample from the several bottles used into a clean vessel, reaerate, and refill the small bottles.

Analyze for nitrate plus nitrite nitrogen (NO_3^--N + NO_2^--N) (see Sections 4500-NO_2^- and 4500-NO_3^-) on Days 0, 5, 10, 15, 20, and 30. Alternatively, determine NO_2^--N and NO_3^--N each time DO is determined, thereby producing corresponding BOD and nitrogen determinations. If the ultimate demand occurs at a time greater than 30 d, make additional analyses at 30-d intervals. Remove 10 to 20 mL from the bottle for these analyses. Refill bottle as necessary from the reservoir bottle. Preserve NO_2^--N + NO_3^--N subsample with H_2SO_4 to pH <2 and refrigerate. If the purpose of the UBOD test is to assess the UBOD and not to provide data for rate calculations, measure nitrate nitrogen concentration only at Day 0 and on the last day of the test (kinetic rate estimates are not useful when the nitrification reaction is not followed).

Calculate oxygen consumption during each time interval and make appropriate corrections for nitrogenous oxygen demand. Correct by using 3.43 × the NH_3-N to NO_2^--N conversion plus 1.14 × the NO_2^--N to NO_3^--N conversion to reflect the stoichiometry of the oxidation of NH_4^+ to NO_2^- or NO_3^-.

When using a dilution water blank, subtract DO uptake of the blank from the total DO consumed. High-quality reagent water without nutrients typically will consume a maximum of 1 mg DO/L in a 30- to 90-d period. If DO uptake of the dilution water is greater than 0.5 mg/L for a 20-d period, or 1 mg/L for a 90-d period, report the magnitude of the correction and try to obtain higher-quality dilution water for use with subsequent UBOD tests.

* Some analysts have reported satisfactory results with 2-chloro-6-(trichloromethyl) pyridine (Nitrification Inhibitor, Formula 2533, Hach Co., Loveland, CO, or equivalent).

† Wheaton 2-L BOD bottle No. 227580, 1000 North Tenth St., Millville, NJ, or equivalent.

TABLE 5210:I. UBOD RESULTS FOR WASTEWATER SAMPLE

Day	(1) Average DO* *mg/L*	(2) Average Blank DO† *mg/L*	(3) Accumulated DO Consumed by Sample‡ *mg/L*	(4) Average NO_3-N *mg/L*	(5) NBOD *mg/L*§	(6) CBOD *mg/L*‖
0	8.1	—	0	0.0	0	0
3	5.6	—	2.5	—	0	2.5
5	3.5/8.0	—	4.6	0.0	0	4.6
7	6.2	—	6.4	—	0.23	6.2
10	3.2/8.2	—	9.4	0.10	0.46	8.9
15	4.3	—	13.3	—	0.58	12.7
18	2.7/8.1	—	14.9	0.15	0.69	14.2
20	6.6	—	16.4	—	0.80	15.6
25	5.4	—	17.6	0.20	0.92	16.7
30	2.6/8.2	—	20.4	—	0.92	19.5
40	5.3	—	23.3	0.20	0.92	22.4
50	3.1/8.0	—	25.5	—	0.92	24.6
60	4.5	—	29.0	—	0.92	28.1
70	3.3/8.1	—	30.2	—	0.92	29.3
90	5.4	—	32.9	0.20	0.92	32.0

* Two readings indicate concentrations before and after reaeration.
† None was used.
‡ Column (1) – blank correction (none needed in the example).
§ Column (4) × 4.57 (linear interpolation between values).
‖ [Column (3) – Column (5)] × dilution factor.

Ultimate CBOD = 34.5 mg/L; CBOD decay rate = 0.03/d (calculated with first-order equation from 5210C.4).

When the weekly DO consumption drops below 1 to 2% of the total accumulative consumption, calculate the ultimate BOD using a nonlinear regression method.

b. Wastewater treatment plant samples: Use high-quality reagent water (see Section 1080) for dilution water. Add no nitrification inhibitors if decay rates are desired. If seed and nutrients are necessary, add the same amounts of each to the dilution water blank. Use minimal sample dilution. As a rule of thumb, the ultimate BOD of the diluted sample should be in the range of 20 to 30 mg/L. Dilution to this level probably will require two or three sample reaerations during the incubation period to avoid having dissolved oxygen concentrations fall below 2 mg/L.

Use 2-L or larger BOD bottles (alternatively, multiple 300-mL BOD bottles) for each dilution. Add desired volume of sample to each bottle and fill with dilution water.

Fill a BOD bottle with dilution water to serve as a dilution water blank. Treat blank the same as all samples. Follow procedure given in ¶ *a* above and incubate for at least as long as UBOD test.

4. Calculations

An example of results obtained for a wastewater sample, undiluted, without seed and nutrients, is given in Table 5210:I.

UBOD can be estimated by using a first-order model described as follows:

$$BOD_t = UBOD\ (1 - e^{-kt})$$

where:

BOD_t = oxygen uptake measured at time t, mg/L, and
k = first-order oxygen uptake rate.

The data in Table 5210:I were analyzed with a nonlinear regression technique applied to the above first-order model.[4] However, a first-order kinetic model may not always be the best choice. Significantly better statistical fits usually are obtained with alternative kinetic models including sum of two first-order and logistic function models.[1,3–8]

5. Precision and Bias

The precision of the ultimate BOD test was assessed with a series of replicate tests in a single laboratory. Interlaboratory studies have not been conducted.

Reference	Replicate No.	UBOD *mg/L*	Precision Summary*
2	1	154	μ = 151 mg/L
	2	154	
	3	145	CV = 3.5%
5	1	10.3	
	2	11.1	
	3	9.6	μ = 10.0 mg/L
	4	9.9	
	5	9.8	CV = 5.8%
	6	9.6	
6	1	12.8	μ = 12.4 mg/L
	2	12.6	
	3	12.6	CV = 4.4%
	4	11.6	

* μ = mean,
CV = coefficient of variation.

Bias was assessed by determining the BOD of a known concentration of glucose (150 mg/L) and glutamic acid (150 mg/L). This solution has a UBOD of 321 mg/L to 308 mg/L, depending on extent of nitrification. The results of the study conducted in triplicate were:[1]

Estimated* UBOD *mg/L*	Theoretical BOD *mg/L*	Percent Difference
276	308/321	−10/−14
310	308/321	+1/−3
303	308/321	−2/−6

* By statistical model.

6. References

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5210 D. Respirometric Method

1. General Discussion

a. Principle: Respirometric methods provide direct measurement of the oxygen consumed by microorganisms from an air or oxygen-enriched environment in a closed vessel under conditions of constant temperature and agitation.

b. Uses: Respirometry measures oxygen uptake more or less continuously over time. Respirometric methods are useful for assessing: biodegradation of specific chemicals; treatability of organic industrial wastes; the effect of known amounts of toxic compounds on the oxygen-uptake reaction of a test wastewater or organic chemical; the concentration at which a pollutant or a wastewater measurably inhibits biological degradation; the effect of various treatments such as disinfection, nutrient addition, and pH adjustment on oxidation rates; the oxygen requirement for essentially complete oxidation of biologically oxidizable matter; the need for using adapted seed in other biochemical oxygen-uptake measurements, such as the dilution BOD test; and stability of sludges.

Respirometric data typically will be used comparatively, that is, in a direct comparison between oxygen uptakes from two test samples or from a test sample and a control. Because of inherent differences among uses, among seed cultures, among applications of results, and among instruments, a single procedure for respirometric tests applicable to all cases cannot be defined. Therefore, only basic recommendations and guidelines for overall test setup and procedure are given. Follow manufacturer's instructions for operating details for specific commercial instruments.

c. Types of respirometers: Four principal types of commercial respirometers are available. Manometric respirometers relate oxygen uptake to the change in pressure caused by oxygen consumption while maintaining a constant volume. Volumetric respirometers measure oxygen uptake in incremental changes in gas volume while maintaining a constant pressure at the time of reading. Electrolytic respirometers monitor the amount of oxygen produced by electrolysis of water to maintain a constant oxygen pressure within the reaction vessel. Direct-input respirometers deliver oxygen to the sample from a pure oxygen supply through metering on demand as detected by minute pressure differences. Most respirometers have been instrumented to permit data collection and processing by computer. Reaction-vessel contents are mixed by using a magnetic or mechanical stirring device or by bubbling the gaseous phase within the reaction vessel through the liquid phase. All respirometers remove carbon dioxide produced during biological growth by suspending a concentrated adsorbent (granular or solution) within the closed reaction chamber or by recirculating the gas phase through an external scrubber.

d. Interferences: Evolution of gases other than CO_2 may introduce errors in pressure or volume measurements; this is uncommon in the presence of dissolved oxygen. Incomplete CO_2 absorption will introduce errors if appropriate amounts and concentrations of alkaline absorbent are not used. Temperature fluctuations or inadequate mixing will introduce error. Fluctuations in barometric pressure can cause errors with some respirometers. Become familiar with the limits of the instrument to be used.

e. Minimum detectable concentration: Most commercial respirometers can detect oxygen demand in increments as small as 0.1 mg but test precision depends on the total amount of oxygen consumed at the time of reading, the precision of pressure or volume measurement, and the effect of temperature and barometric pressure changes. Upper limits of oxygen uptake rate are

determined by the ability to transfer oxygen into the solution from the gas phase, which typically is related to mixing intensity. Transfer limits typically range from less than 10 mg O_2/L/h for low-intensity mixing to above 100 mg O_2/L/h for high-intensity mixing.

f. Relationship to dilution BOD: Variations in waste composition, substrate concentration, mixing, and oxygen concentrations from one wastewater source to another generally preclude use of a general relationship between oxygen uptake by respirometers and the 5-d, 20°C, BOD (see 5210B). Reasonably accurate correlations may be possible for a specific wastewater. The incubation period for respirometric measurements need not be 5 d because equally valid correlations can be made between the 5-d dilution BOD and respirometric oxygen uptake at any time after 2 d.[1,2] The point of common dilution and respirometric BOD seems to occur at about 2 to 3 d incubation for municipal wastewaters. Correlations between respirometric measurements and 5-d BOD for industrial wastes and specific chemicals are less certain. Respirometric measurements also can provide an indication of the ultimate biochemical oxygen demand (UBOD) (see 5210C). In many cases, it is reasonable to consider that the 28- to 30-d oxygen uptake is essentially equal to the UBOD.[3]

More commonly, respirometers are used as a diagnostic tool. The continuous readout of oxygen consumption in respirometric measurements indicates lag, toxicity, or any abnormalities in the biodegradation reaction. The change in the normal shape of an oxygen-uptake curve in the first few hours may help to identify the effect of toxic or unusual wastes entering a treatment plant in time to make operating corrections.

g. Relationship to other test methods and protocols: This method supports most of the protocols and guidelines established by the European Organization for Economic Co-operation and Development[3] (OECD) that require measurement of oxygen uptake.

h. Sampling and storage:

1) Grab samples—If analysis is begun within 2 h of sample collection, cold storage is unnecessary. Otherwise, keep sample at or below 4°C from the time of collection. Begin analysis within 6 h of collection; when this is not possible, store at or below 4°C and report length and temperature of storage. Never start analysis more than 24 h after grab sample collection.

2) Composite samples—Keep samples at or below 4°C during compositing. Limit compositing period to 24 h. Use the same criteria as for storage of grab samples, starting the measurement of holding time from the end of the compositing period. State storage time and conditions with results.

2. Apparatus

a. Respirometer system: Use commercial apparatus and check manufacturer's instructions for specific system requirements, reaction vessel type and volume, and instrument operating characteristics.

b. Incubator or water bath: Use a constant-temperature room, incubator chamber, or water bath to control temperature to ±1°C. Exclude all light to prevent oxygen formation by algae in the sample. Use red, actinic-coated bottles for analysis outside of a darkened incubator.

3. Reagents

Formulations of reagent solutions are given for 1-L volumes, but smaller or larger volumes may be prepared according to need. Discard any reagent showing signs of biological growth or chemical precipitation. Stock solutions can be sterilized by autoclaving to provide longer shelf life.

a. Distilled water: Use only high-quality water distilled from a block tin or all-glass still (see Section 1080). Deionized water may be used but often contains high bacterial counts. The water must contain less than 0.01 mg heavy metals/L and be free of chlorine, chloramines, caustic alkalinity, organic material, or acids. Make all reagents with this water. When other waters are required for special-purpose testing, state clearly their source and quality characteristics.

b. Phosphate buffer solution, 1.5*N:* Dissolve 207 g sodium dihydrogen phosphate, $NaH_2PO_4 \cdot H_2O$, in water. Neutralize to pH 7.2 with 6*N* KOH (¶ *g* below) and dilute to 1 L.

c. Ammonium chloride solution, 0.71*N:* Dissolve 38.2 g ammonium chloride, NH_4Cl, in water. Neutralize to pH 7.0 with KOH. Dilute to 1.0 L; 1 mL = 10 mg N.

d. Calcium chloride solution, 0.25*M:* Dissolve 27.7 g $CaCl_2$ in water and dilute to 1 L; 1 mL = 10 mg Ca.

e. Magnesium sulfate solution, 0.41*M:* Dissolve 101 g $MgSO_4 \cdot 7H_2O$ in water and dilute to 1 L; 1 mL = 10 mg Mg.

f. Ferric chloride solution, 0.018*M:* Dissolve 4.84 g $FeCl_3 \cdot 6H_2O$ in water and dilute to 1 L; 1 mL = 1.0 mg Fe.

g. Potassium hydroxide solution, 6*N:* Dissolve 336 g KOH in about 700 mL water and dilute to 1 L. **CAUTION: Add KOH to water slowly and use constant mixing to prevent excessive heat buildup.** Alternately, use commercial solutions containing 30 to 50% KOH by weight.

h. Acid solutions, 1*N:* Add 28 mL conc H_2SO_4 or 83 mL conc HCl to about 700 mL water. Dilute to 1 L.

i. Alkali solution, 1*N:* Add 40 g NaOH to 700 mL water. Dilute to 1 L.

j. Nitrification inhibitor: Reagent-grade 2-chloro-6-(trichloromethyl) pyridine (TCMP) or equivalent.[3]*

k. Glucose-glutamic acid solution: Dry reagent-grade glucose and reagent-grade glutamic acid at 103°C for 1 h. Add 15.0 g glucose and 15.0 g glutamic acid to distilled water and dilute to 1 L. Neutralize to pH 7.0 using 6*N* potassium hydroxide (¶ *g* above). This solution may be stored for up to 1 week at 4°C.

l. Electrolyte solution (for electrolytic respirometers): Use manufacturer's recommended solution.

m. Sodium sulfite solution, 0.025*N:* Dissolve 1.575 g Na_2SO_3 in about 800 mL water. Dilute to 1 L. This solution is not stable; prepare daily or as needed.

n. Trace element solution: Dissolve 40 mg $MnSO_4 \cdot 4H_2O$, 57 mg H_3BO_3, 43 mg $ZnSO_4 \cdot 7H_2O$, 35 mg $(NH_4)_6 Mo_7O_{24}$, and 100 mg Fe-chelate ($FeCl_3$-EDTA) in about 800 mL water. Dilute to 1 L. Sterilize at 120°C and 200 kPa (2 atm) pressure for 20 min.

* Formula 2533, Hach Chemical Co., Loveland, CO, or equivalent. NOTE: Some commercial formulations are not pure TCMP. Check with supplier to verify compound purity and adjust dosages accordingly.

o. Yeast extract solution:[3] Add 15 mg laboratory- or pharmaceutical-grade brewer's yeast extract to 100 mL water. Make this solution fresh immediately before each test in which it is used.

p. Nutrient solution:[3] Add 2.5 mL phosphate buffer solution (3*b*), 0.65 mL ammonium chloride solution (3*c*), 1.0 mL calcium chloride solution (3*d*), 0.22 mL magnesium sulfate solution (3*e*), 0.1 mL ferric chloride solution (3*f*), 1 mL trace element solution (3*n*), and 1 mL yeast extract solution (3*o*) to about 900 mL water. Dilute to 1 L. This nutrient solution and those of ¶s *n* and *o* above are specifically formulated for use with the OECD method.[3] (NOTE: A 10:1 concentrated nutrient solution can be made and diluted accordingly.)

4. Procedure

a. Instrument operation: Follow respirometer manufacturer's instructions for assembly, testing, calibration, and operation of the instrument. NOTE: The manufacturer's stated maximum and minimum limits of measurement are not always the same as the instrument output limits. Make sure that test conditions are within the limits of measurement.

b. Sample volume: Sample volume or concentration of organic chemicals to be added to test vessels is a function of expected oxygen uptake characteristics and oxygen transfer capability of the instrument. Small volumes or low concentrations may be required for high-strength wastes. Large volumes may be required for low-strength wastes to improve accuracy.

c. Data recording interval: Set instrument to give data readings at suitable intervals. Intervals of 15 min to 6 h typically are used.

d. Sample preparation:

1) Homogenization—If sample contains large settleable or floatable solids, homogenize it with a blender and transfer representative test portions while all solids are in suspension. If there is a concern for changing sample characteristics, skip this step.

2) pH adjustment—Neutralize samples to pH 7.0 with H_2SO_4 or NaOH of such strength (5210D.3*h* and *i*) that reagent quantity does not dilute the sample more than 0.5%.

3) Dechlorination—Avoid analyzing samples containing residual chlorine by collecting the samples ahead of chlorination processes. If residual chlorine is present, aerate as described in ¶ 5) below or let stand in light for 1 to 2 h. If a chlorine residual persists, add Na_2SO_3 solution. Determine required volume of Na_2SO_3 solution by adding 10 mL 1 + 1 acetic acid or 1 + 50 H_2SO_4 and 10 mL potassium iodide solution (10 g/100 mL) to a portion of the sample. Titrate with 0.025*N* Na_2SO_3 solution to the starch-iodine end point (see Section 4500-Cl.B). Add to the neutralized sample a proportional volume of Na_2SO_3 solution determined above, mix, and after 10 to 20 min check for residual chlorine. Re-seed the sample (see ¶ *h* below).

4) Samples containing toxic substances—Certain industrial wastes contain toxic metals or organic compounds. These often require special study and treatment.[3]

5) Initial oxygen concentration—If samples contain dissolved oxygen concentrations above or below the desired concentration, agitate or aerate with clean and filtered compressed air for about 1 h immediately before testing. Minimum and maximum actual DO concentrations will vary with test objectives. In some cases, pure oxygen may be added to respirometer vessels to increase oxygen levels above ambient.

6) Temperature adjustment—Bring samples and dilution water to desired test temperature (±1°C) before making dilutions or transferring to test vessels.

e. Sample dilution: Use distilled water or water from other appropriate sources free of organic matter. In some cases, receiving stream water may be used for dilution. Add desired sample volume to test vessels using a wide-tip volumetric pipet or other suitable volumetric glassware. Add dilution water to bring sample to about 80% of desired final volume. Add appropriate amounts of nutrients, minerals, buffer, nitrification inhibitor if desired, and seed culture as described in ¶s *f–h* below. Dilute sample to desired final volume. The number of test vessels to prepare for each dilution depends on test objectives and number of replicates desired.

f. Nutrients, minerals, and buffer: Add sufficient ammonia nitrogen to provide a COD:N:P ratio of 100:5:1 or a TOC:N:P ratio of 30:5:1. Add 2 mL each of calcium, magnesium, ferric chloride, and trace mineral solutions to each liter of diluted sample unless sufficient amounts of these minerals are present in the original sample. Phosphorus requirements will be met by the phosphate buffer if it is used (1 mL/50 mg/L COD or ultimate BOD of diluted sample usually is sufficient to maintain pH between 6.8 and 7.2). Be cautious in adding phosphate buffer to samples containing metal salts because metal phosphates may precipitate and show less toxic or beneficial effect than when phosphate is not present. For OECD-compatible tests, substitute the nutrient, mineral, and buffer amounts listed in 5210D.3*p* for the above nutrient/mineral/buffer quantities.

g. Nitrification inhibition: If nitrification inhibition is desired, add 10 mg 2-chloro-6-(trichloromethyl) pyridine (TCMP)/L sample in the test vessel. Samples that may nitrify readily include biologically treated effluents, samples seeded with biologically treated effluents, and river waters.[4]

h. Seeding: See 5210B.4*d* for seed preparation. Use sufficient amounts of seed culture to prevent major lags in the oxygen uptake reaction but not so much that the oxygen uptake of the seed exceeds about 10% of the oxygen uptake of the seeded sample.

Determine the oxygen uptake of the seeding material as for any other sample. This is the seed control. Typically, the seed volume in the seed control should be 10 times the volume used in seeded samples.

i. Incubation: Incubate samples at 20°C or other suitable temperature ±1.0°C. Take care that the stirring device does not increase the temperature of the sample.

5. Calculations

To convert instrument readings to oxygen uptake, refer to manufacturer's procedures.

Correct oxygen uptake for seed and dilution by the following equation:

$$C = [A - B(S_A/S_B)](1000/N_A)$$

where:

C = corrected oxygen uptake of sample, mg/L,
A = measured oxygen uptake in seeded sample, mg,

B = measured oxygen uptake in seed control, mg,
S_A = volume of seed in Sample A, mL,
S_B = volume of seed in Sample B, mL, and
N_A = volume of undiluted sample in Sample A, mL.

6. Quality Control

The quality control practices considered to be an integral part of each method are summarized in Table 5020:I.

Periodically use the following procedure to check distilled water quality, instrument quality, instrument function, and analytical technique by making oxygen uptake measurements using a mixture of glucose and glutamic acid as a standard check solution.

Adjust water for sample formulation to test temperature and saturate with DO by aerating with clean, organic-free filtered air. Protect water quality by using clean glassware, tubing, and bottles.

Prepare a *test solution* by adding 10 mL glucose-glutamic acid solution (3*k*); 6 mL phosphate buffer (3*b*); 2 mL each of ammonium chloride (3*c*), magnesium sulfate (3*e*), calcium chloride (3*d*), ferric chloride (3*f*), and trace element solution (3*n*) to approximately 800 mL water. Add 10 mg nitrification inhibitor (TCMP)/L. Add sufficient seed from a suitable source as described in 5210D.4*h* to give a lag time less than 6 h (usually 25 mL supernatant from settled primary effluent/L test solution is sufficient). Dilute to 1 L. Adjust temperature to 20 ± 1°C.

Prepare a *seed blank* by diluting 500 mL or more of the seed solution to 800 mL with distilled water. Add the same amount of buffer, nutrients, and TCMP as in the test solution, and dilute to 1 L. Adjust temperature to 20 ± 1°C.

Place test solution and seed blank solution in separate reaction vessels of respirometer and incubate for 5 d at 20°C. Run at least three replicates of each. The seed-corrected oxygen uptake after 5 d incubation should be 260 ± 30 mg/L. If the value of the check is outside this range, repeat the test using a fresh seed culture and seek the cause of the problem.

7. Precision and Bias

a. Precision: No standard is available to check the accuracy of respirometric oxygen uptake measurements. To obtain laboratory precision data, use a glucose-glutamic acid mixture (5210D.6) having a known theoretical maximum oxygen uptake value. Tests with this and similar organic compound mixtures have shown that the standard deviation, expressed as the coefficient of variation, C_v, is approximately 5% for samples having total oxygen uptakes of 50 to 100 mg/L and 3% for more concentrated samples.[1,2] Individual instruments have different readability limits that can affect precision. The minimum response or sensitivity of most commercial respirometers ranges from 0.05 to 1 mg oxygen. Check manufacturer's specifications for sensitivity of the instrument at hand.

b. Control limits: To establish laboratory control limits, perform a minimum of 25 glucose-glutamic acid checks over a period of several weeks or months and calculate mean and standard deviation. If measured oxygen uptake in 5 d at 20°C is outside the 260 ± 30 mg/L range, re-evaluate procedure to identify source of error. For other samples, use the mean ±3 standard deviations as the control limit.

c. Working range and detection limits: The working range and detection limits are established by the limits of each commercial instrument. Refer to manufacturer's specifications.

8. References

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