5210 BIOCHEMICAL OXYGEN DEMAND (BOD)*

5210 A. Introduction

1. General Discussion

Biochemical oxygen demand (BOD) testing is used to determine the relative oxygen requirements of wastewaters, effluents, and polluted waters; its widest application is in measuring waste loadings to treatment plants and in evaluating the plants' BOD-removal efficiency. BOD testing measures the molecular oxygen used during a specified incubation period to

- biochemically degrade organic material (carbonaceous demand),
- oxidize inorganic material (e.g., sulfides and ferrous iron), and/or
- measure the amount of oxygen used to oxidize reduced forms of nitrogen (nitrogenous demand) unless an inhibitor is added to prevent such reduction.

The seeding and dilution procedures provide an estimate of BOD at pH 6 to 8.

The methods below measure oxygen consumed in a 5-d period (5210B), oxygen consumed after 60 to 90 d of incubation (5210C), and continuous oxygen uptake (5210D). Other BOD methods published elsewhere may use shorter or longer incubation periods; tests to determine oxygen-uptake rates; and/or alternative seeding, dilution, and incubation conditions to mimic receiving-water conditions, thereby estimating the environmental effects of wastewaters and effluents.

The ultimate BOD (UBOD) test measures the oxygen required to totally degrade organic material (ultimate carbonaceous demand) and/or to oxidize reduced nitrogen compounds (ultimate nitrogenous demand). UBOD values and appropriate kinetic descriptions are needed in water-quality modeling studies [e.g., 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].

A number of factors (e.g., 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 are no effective adjustments or corrections to compensate for these factors.

2. Carbonaceous Versus Nitrogenous BOD

Microorganisms can oxidize reduced forms of nitrogen, such as ammonia and organic nitrogen, thus exerting nitrogenous demand. Nitrogenous demand historically has been considered an interference in BOD testing; adding ammonia to dilution water contributes an external source of nitrogenous demand. The interference from nitrogenous demand can now be prevented by an inhibitory chemical,[1] but if it isn't used, the measured oxygen demand 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, 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 subject to considerable error. Chemical inhibition of nitrogenous demand provides a more direct, reliable measure of carbonaceous demand.

How much nitrogenous compounds oxidize during the 5-d incubation period depends on the concentration and type of microorganisms that can carry out this oxidation. Such organisms quite often are present in raw or settled primary sewage in adequate numbers to oxidize enough reduced nitrogen forms to contribute oxygen demand in the 5-d BOD test. Most biological treatment plant effluents contain enough nitrifying organisms to cause nitrification in BOD tests. Because nitrogenous compounds can oxidize in such samples, nitrification inhibition (as directed in 5210B.5*e*) is recommended for secondary-effluent samples, samples seeded with secondary effluent, and polluted-water samples.

3. Reference

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

* Approved by Standard Methods Committee, 2016.
Joint Task Group: James C. Young (chair), Victor D. Hahn, Robert V. Menegotto, Devon A. Morgan, Robin S. Parnell, Lisa M. Ramirez, Debra A. Waller.

5210 B. 5-Day BOD Test

1. General Discussion

The BOD test is an indirect measurement of organic matter; it measures the change in DO concentration caused by microorganisms as they degrade organic matter in a sample held in a stoppered bottle incubated for 5 d in the dark at 20°C. Analysts measure DO before and after incubation, and compute BOD using the difference between DO measurements. Because initial DO is determined shortly after dilution, 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 60-mL glass bottles or larger (300-mL bottles with a flared mouth and ground-glass stopper are preferred). Clean bottles with a detergent, rinse thoroughly,

and drain before use. Alternatively, use disposable plastic BOD bottles that are capable of meeting all method quality-control (QC) checks.

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

c. Oxygen-sensitive membrane electrode, polarographic or galvanic, or *oxygen-sensitive optical probe* with appropriate meter.

3. Reagents

Discard reagents 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 reagent-grade water (see Section 1080) to make all solutions.

a. Phosphate buffer solution: Dissolve 8.5 g monopotassium phosphate (KH_2PO_4), 21.75 g dipotassium phosphate (K_2HPO_4), 33.4 g disodium phosphate ($Na_2HPO_4 \cdot 7H_2O$, and 1.7 g ammonium chloride (NH_4Cl) in about 500 mL reagent-grade 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 reagent-grade water. Adjust pH to 7.2 with 30% sodium hydroxide (NaOH) and dilute to 1 L.

b. Magnesium sulfate ($MgSO_4$) solution: Dissolve 22.5 g $MgSO_4 \cdot 7H_2O$ in reagent-grade water and dilute to 1 L.

c. Calcium chloride ($CaCl_2$) solution: Dissolve 27.5 g $CaCl_2$ in reagent-grade water and dilute to 1 L.

d. Ferric chloride ($FeCl_3$) solution: Dissolve 0.25 g $FeCl_3 \cdot 6H_2O$ in reagent-grade water and dilute to 1 L.

e. Acid and alkali solutions, 1N, to neutralize caustic or acidic waste samples.

1) Acid—Slowly and while stirring, add 28 mL conc sulfuric acid (H_2SO_4) to reagent-grade water. Dilute to 1 L.

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

f. Sodium sulfite (Na_2SO_3) solution: Dissolve 1.575 g Na_2SO_3 in 1000 mL reagent-grade water. This solution is unstable; prepare daily.

g. Nitrification inhibitor:

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

2) Allylthiourea (ATU) solution—Dissolve 2.0 g allylthiourea ($C_4H_8N_2S$) in about 500 mL reagent-grade water and dilute to 1 L. Store at 4°C. The solution is stable for 2 weeks when stored at ≤6°C without freezing.

h. Glucose–glutamic acid (GGA) 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 reagent-grade water and dilute to 1 L. Prepare fresh immediately before use unless solution is maintained in a sterile container. Store all GGA mixtures at ≤6°C without freezing unless manufacturer recommendations state otherwise. Commercial preparations may be used but concentrations may vary. Discard solutions if evidence of contamination is indicated (e.g., growth occurs in the stock bottle or GGA test results are consistently low).

i. Ammonium chloride solution: Dissolve 1.15 g NH_4Cl in about 500 mL reagent-grade 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, or equivalent reagent-grade water, tap, or natural water to make 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 begins within 2 h of collection, cold storage is unnecessary. Otherwise, keep sample at ≤6°C between collection and analysis. Ideally, begin analysis within 6 h of sample collection; if impossible due to distance between sampling site and laboratory, then begin analysis within 24 h of collection. The recommended holding time is 24 h; however, the U.S. Environmental Protection Agency (EPA) allows for a 48-h holding time.

2) Composite samples—Limit compositing period to 24 h, and keep samples at ≤6°C during process. Store for the same time and temperature as grab samples, although in this case, holding time begins when the compositing period ends.

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 between 6.5 and 7.5 using an H_2SO_4 or NaOH solution strong enough not to dilute sample by >0.5%. Exceptions may be justified with natural waters when BOD will be measured at *in situ* pH values. Dilution-water pH 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. Sometimes chlorine will dissipate from sample within 1 to 2 h of standing in the light; this often occurs during transport and handling. If the chlorine residual does not dissipate in a reasonably short time, destroy it 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 and 10 mL potassium iodide (KI) solution (10 g/100 mL) per 1000 mL sample, and then titrating with Na_2SO_3 solution to the starch-iodine endpoint for residual. Add to neutralized sample the proportional volume of Na_2SO_3 solution determined by the above test, mix, and check sample for residual chlorine after 10 to 20 min. (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 (e.g., plating wastes) contain toxic metals. Such samples often require special study and treatment.

4) Samples supersaturated with DO (Table 4500-O:I)—Samples with DO concentrations above saturation at 20°C may be collected in cold waters or in water where photosynthesis occurs. To prevent oxygen loss when incubating such samples, reduce

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

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

5) Samples containing hydrogen peroxide—Hydrogen peroxide remaining in samples from some industrial bleaching processes (e.g., 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 long enough to allow hydrogen peroxide to dissipate before setting up BOD tests. Check adequacy of peroxide removal by observing DO 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 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 (i.e., distilled, tap, or reagent-grade water). Make sure water is free of heavy metals, specifically copper (<0.05 mg/L) and toxic substances [e.g., chlorine (<0.10 mg/L)] that can interfere with BOD measurements. Protect source-water quality by using clean glassware, tubing, and bottles. Deionized (DI) water often contains enough organics and microorganisms to cause the dilution-water QC check to fail (5210B.6*c*). DI water is not recommended unless dilution-water blanks consistently meet QC limits. Source water may be stored before use as long as the prepared dilution water (5210B.5*a*) meets QC 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 deteriorate others. Storing prepared dilution water (5210B.5*h*) for >24 h after adding nutrients, minerals, and buffer is not recommended unless dilution-water blanks consistently meet QC limits. Discard stored source water if dilution-water blank shows >0.2 mg/L DO depletion in 5 d (5210B.6*c*).

d. Preparation of seed suspension: Each BOD bottle must contain a microorganism population that can oxidize biodegradable organic matter in the sample. Domestic wastewater, unchlorinated or other undisinfected effluents from biological wastewater treatment plants, and surface waters receiving wastewater discharges usually contain satisfactory microbial populations. Some samples (e.g., some untreated industrial wastes, disinfected wastes, high-temperature wastes, wastes with pH values <6 or >8, or wastes stored >6 h after collection) do not contain a sufficient microbial population. Seed such samples by adding a population of suitable microorganisms; the preferred seed comes from a sample-related biological wastewater treatment system or receiving water. 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 discharge point. If using effluent or mixed liquor from a biological treatment process as a seed source, nitrification inhibition is recommended. Do not use seed from effluents that have been disinfected by chlorine or other means. Commercial seed sources may be used according to manufacturer's preparation instructions but are more likely to be unadapted to the wastewater constituents. Do not filter seed sources; filtering removes the seed microorganisms.

If adapted seed sources are unavailable, 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 seed's performance in BOD tests on the sample. BOD values that increase during 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 DO concentration is at least 7.5 mg/L before using water for BOD tests. If not, add DO by shaking bottle or aerating it 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 dilution-water blanks show a DO depletion >0.2 mg/L, then improve purification or use water from another source. Do not add oxidizing agents or expose dilution water to ultraviolet light to try to bring the dilution blank into range.

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

c. Preparation of dilutions: Using dilution water prepared as in ¶ *a* above, make at least three dilutions of prepared sample estimated to produce, at the end of the test, at least one dilution that would result in a residual DO of ≥1.0 mg/L and a DO uptake of ≥2.0 mg/L after a 5-d incubation. Two dilutions are allowed if experience with a particular sample source produces at least one bottle with acceptable minimum DO depletions and residual limits (5210B.6*a*). Individual laboratories should evaluate the need for more than three dilutions when historical sample data are unavailable. 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 DO technique and number of replicates desired. Prepare dilutions in volumetric containers (Class A glass or equivalent) and then transfer to BOD bottles, or else prepare directly in BOD bottles. Either dilution method can be used to transfer sample to respective BOD bottles.

1) Dilutions prepared in volumetric containers—Using a wide-tipped pipet or graduated cylinder, add desired amount of prepared sample to individual volumetric cylinders or flasks. Mix sample well immediately before pipetting to avoid solids loss via settling. For dilutions greater than 1:300, make a primary dilution before making final dilution in volumetric cylinders or flasks. Fill cylinders or flasks at least two-thirds full with dilution water and sample 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 cylinder or flask during transfer. When a cylinder or flask contains >67% of 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–d*) 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 container size.

2) Dilutions prepared directly in BOD bottles—Using a wide-tip volumetric pipet or graduated cylinder, add desired sample volume to individual BOD bottles. Mix sample well immediately before pipetting to avoid solids loss via settling. For dilutions greater than 1:300, make a primary dilution before making final dilution in the bottle. Fill each BOD bottle approximately two-thirds full with dilution water and/or sample without entraining air. Add appropriate amounts of seed suspension (¶ *d* below) and nitrification inhibitor (¶ *e* below) to individual BOD bottles. Fill remainder of BOD bottle with dilution water. When a bottle contains >67% of 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–d*) 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 dilution vessels or individual BOD bottles before final dilution, as described in ¶ *c* above. Do not add seed directly to wastewater samples before dilution if they contain toxic materials. 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 enough microorganisms. Do not filter seed suspension before use. Agitate 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 added seed generally should be between 0.6 and 1.0 mg/L, but adjust seed amount as needed to provide GGA check results of 198 ± 30.5 mg/L. For example, if 1 mL seed suspension is required to achieve 198 ± 30.5 mg/L BOD in the GGA 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 nitrification inhibition and the related chemical used when reporting results. (NOTE: TCMP is the preferred nitrification inhibitor but requires handling and transfer in a solid form. ATU is not always effective in inhibiting nitrification within the 5-d incubation period, and concentrations >2 mg/L may increase carbonaceous BOD (CBOD) measurements and/or adversely affect the azide modification of the iodometric method.) Seed all samples to which nitrification inhibitor has been added.

1) Nitrification inhibition using TCMP—Add 10 mg TCMP/L to diluted sample, 3 mg TCMP to each 300-mL bottle, or proportional amounts to other sized bottles after initial sample dilution but before final filling of 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 sample if not mixed well.) Some commercial TCMP formulations are not 100% TCMP; adjust dosage appropriately.

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

f. Sealing bottles: Completely fill each bottle by adding enough dilution water so insertion of stopper leaves no bubbles in the bottle. Mix sample by turning bottle manually several times unless immediately using a DO probe with a stirrer 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 water seal during incubation.

g. Determination of initial DO: Use the azide modification of the iodometric method (Section 4500-O.C), membrane-electrode method (Section 4500-O.G), or optical-probe method (Section 4500-O.H) to determine initial DO on all sample dilutions, dilution-water blanks, and, where appropriate, seed controls. Replace any displaced contents with enough 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 using the membrane-electrode method or optical probe method, calibrate DO probe daily by following the manufacturer's calibration procedure. Make frequent calibration checks daily to ensure accurate DO readings and, ideally, perform a Winkler titration as needed to verify calibration. If using the azide modification of the titrimetric iodometric method, prepare an extra bottle for initial DO determination for each sample dilution.

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

i. Determination of final DO: After 5 d ± 6 h of incubation, determine DO in all sample dilutions, blanks, and checks as in 5210B.6*g*, using the azide modification of the titrimetric method (Section 4500-O.C), membrane-electrode method (Section 4500-O.G), or optical-probe method (Section 4500-O.H).

6. Quality Control Checks

The QC 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) whose DO depletion is ≥2.0 mg/L and residual DO is ≥1.0 mg/L after 5 d of incubation are considered to produce valid data, because ≥2.0 mg oxygen uptake/L is required to give a meaningful measure of oxygen uptake and ≥1.0 mg/L must remain to ensure that waste constituents' oxidation rates were not limited by insufficient DO. However, there are exceptions—for reporting purposes only—when testing undiluted samples and all bottles' DO depletion is <2.0 mg/L and residual DO is <1.0 mg/L (see 5210B.7).

b. Glucose–glutamic acid check: The GGA check is the primary basis for establishing the BOD test's accuracy and precision, as well as the principal measure of seed quality and set-up procedure. Together with each batch of BOD or CBOD 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 and glutamic acid solutions (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 GGA concentrations; adjust doses accordingly. Add nitrification inhibitor if seed is obtained from a source that is nitrifying, and also to all CBOD GGA checks. Evaluate data as described in 5210B.8. The resulting average BOD/CBOD for the three bottles, after correcting for dilution and seeding, must fall into the control-limit range established in 5210B.8*a*. If the average value falls outside this range, evaluate the cause and make appropriate corrections. Consistently high values can indicate too much seed suspension, contaminated dilution water, or nitrification; consistently low values can indicate poor seed quality or quantity or else the presence of a toxic material. If low values persist, prepare a new GGA mixture and check the dilution-water and seed sources.

c. Dilution-water-quality check: With each batch of dilution water, incubate two or more bottles of dilution water containing nutrient, mineral, and buffer solutions but no seed or nitrification inhibitor. Dilution water checks must be analyzed with each batch of samples; the dilution-water blank serves as a check on the quality of unseeded dilution water and cleanliness of incubation bottles. Determine initial and final DO for each bottle (5210B.5*e* and *i*), and average results. The average DO uptake in 5 d must not be >0.2 mg/L and preferably ≤0.1 mg/L before making seed corrections. If average dilution-water blank is >0.2 mg/L, record the data and clearly identify such samples in data records.

d. Seed control: Determine the seed suspension's BOD as for any other sample. This is the *seed control.* Ideally, make three dilutions of seed so the smallest quantity depletes ≥2.0 mg/L DO and the largest quantity leaves ≥1.0 mg/L DO residual after 5 d of incubation. Determine DO uptake per milliliter of seed by dividing the DO depletion by the volume of seed in milliliters for each seed control bottle with a 2.0 mg/L depletion and >1.0 mg/L minimum residual DO, and averaging 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; check or change the seed source.

7. Data Analysis and Reporting

a. Calculations:

1) For each test bottle with at least 2.0 mg/L DO depletion and at least 1.0 mg/L residual DO—before seed correction, 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 unseeded)],
V_s = volume of seed in respective test bottle, mL, and
P = decimal volumetric fraction of sample used; $1/P$ = dilution factor.

2) If DO depletion is <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 <2.0 mg/L.

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

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

4) If all dilutions result in DO depletion <2.0 mg/L and the sample was diluted, select the bottle with the largest volume of sample (the least dilution) and calculate the report as if the dilution had depleted 2.0 mg/L:

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

In the above calculations, do not make corrections for DO uptake by the dilution-water blank during incubation.

b. Reporting: Average test results for all qualified bottles in each dilution series. Report the result as BOD_5 if nitrification is not inhibited; report it as CBOD_5 if nitrification is inhibited. Samples with large differences between the computed BOD for different dilutions (e.g., the highest value is >30% larger than the lowest value) may indicate a toxic substance or analytical problems. When the effect becomes repetitive, investigate to identify the cause. Toxicity should be claimed only after thorough investigation using respirometric (5210D) or equivalent methods. Identify results in the test reports when any of the following QC conditions occur:

- dilution-water blank average is >0.2 mg/L (5210B.6*c*),
- GGA check falls outside acceptable limits (5210B.6*b*),
- test replicates show >30% difference between highest and lowest values,
- none of the seed control samples meet the above criteria (5210B.6*d*), or
- all dilutions result in a residual DO <1.0 mg/L [5210B.7*a*3)].

8. Precision and Bias

There is no measurement for establishing the BOD test's bias. The GGA check prescribed in 5210B.6*b* is intended to be a reference point for evaluating dilution-water quality, seed effectiveness, and analytical technique. Single-laboratory tests using a 300-mg/L mixed GGA 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 GGA ranging from 3.3 to 231 mg/L total concentration. The regression equations for mean value, X, and standard deviation, S, from these studies were:

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

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

a. Control limits: Applying the above equations to the 300-mg/L GGA primary standard yields an average 5-d BOD of 198 mg/L with a standard deviation of 30.5 mg/L. Because many factors affect BOD tests in multi-laboratory studies, resulting in extremely variable 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 own control limits by performing at least 25 GGA checks (5210B.6*b*) over several weeks or months and calculating the mean and standard deviation. Use the mean ±3 standard deviations as the control limit for future GGA checks. Compare calculated control limits to the single-laboratory tests presented above and to interlaboratory results. If any GGA test results are outside the acceptable control-limit range, identify them clearly in all data records, investigate source of the problem, and make appropriate corrections.

When nitrification inhibitors are used, GGA test results outside the control-limit range often indicate that incorrect amounts of seed were used. Adjust the amount of seed added to the GGA test so results fall within range (5210B.6*b*).

b. Working range and reporting 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, including any intermediate dilutions performed (5210B.5*c*).

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

- The lower reporting limit for unseeded samples that require no dilution—except for nutrient, mineral, and buffer solutions ($S = 0$; $P = 1.0$)—is equal to the DO measurement method's detection limit (~0.1 mg/L).
- The lower reporting limit for seeded samples that require no dilution—except for seed, nutrient, mineral, and buffer solutions ($S > 0$; $P = 1.0$)—is the difference between sample DO depletion and seed correction.

9. References

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2. U.S. Environmental Protection Agency, Office of Research and Development. 1986. Method-by-Method Statistics from Water Pollution (WP) Laboratory Performance Evaluation Studies. Quality Assurance Branch, Environmental Monitoring and Support Lab., Cincinnati, Ohio.

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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. Be familiar 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] 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 more 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 whose oxygen demands exceed the DO available in air-saturated water; in such cases, either dilute sample or monitor DO frequently to ensure that low DO or anaerobic conditions do not occur. Reaerate sample when DO concentrations approach 2 mg/L.

Because bacterial growth requires nutrients (e.g., nitrogen, phosphorus, and trace metals), the necessary amounts may be added to dilution water, along with a buffer to keep pH in the bacterial-growth range and enough seed for an adequate bacterial population. (No specific nutrient or buffer formulations are included here because of the wide range of water and wastewater characteristics and varied applications of UBOD data.) That said, if the result will be used to estimate the oxidation rate of naturally occurring surface waters, adding nutrients and seed probably will accelerate the decay rate and produce misleading results. If only UBOD is desired, adding supplemental nutrients that accelerate decay and shorten test duration may be advantageous. When using nutrients, also add them to the dilution-water blank.

How much nitrogenous compounds will oxidize during the prescribed incubation period depends on how many relevant oxidizing microorganisms are present. These organisms may be too scarce in wastewaters to oxidize significant quantities of reduced nitrogen, but abundant in naturally occurring surface waters. Results may be erratic when a nitrification inhibitor is used,[2] so do NOT use one unless prior experimental evidence on

the particular sample suggests that it is acceptable.* Monitor nitrite nitrogen (NO_2^--N) and nitrate nitrogen (NO_3^--N) to compute the oxygen equivalency of the nitrification reaction. When these values are subtracted from the DO versus time series, the CBOD time series can be constructed.[3]

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

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

2. Apparatus

a. Incubation bottles: 2-L or larger glass bottles with ground-glass stoppers;† 4- to 10-L glass serum bottles are available. Alternatively, use nonground-glass bottles with nonbiodegradable plastic caps as a plug insert. Do not reuse plugs because they become discolored 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 hydrochloric acid (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.

Use a water seal to avoid drawing air into sample bottle during incubation. If 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 using a 2-L BOD bottle, 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 magnets until test is complete.

Alternatively, use a series of 300-mL BOD bottles (5210B.2*a*) if larger bottles are unavailable or incubation space is limited.

b. Reservoir bottle: 4-L or larger glass bottle. Close with plastic screw 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 else 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 pretesting or experience shows that ultimate BOD will be high (>20 mg/L).

Measure DO in each bottle, stopper it, 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 30 to 60 d (minimum of 6 to 8 readings), or longer under special circumstances. To avoid oxygen depletion in samples containing ammonia nitrogen (NH_3-N), measure DO more frequently until nitrification has taken place. If DO falls to about 2 mg/L, re-aerate 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, re-aerate. Pour a small amount of sample into a clean vessel and re-aerate 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, empty all of the bottles into a clean vessel, re-aerate, and refill the small bottles.

Analyze for NO_2^--N + NO_3^--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 after 30 d, make additional analyses at 30-d intervals. Remove 10 to 20 mL from the bottle for these analyses, and 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 UBOD test's goal is to assess UBOD and not to provide data for rate calculations, measure NO_3^--N 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 NH_4^+ oxidation to NO_2^- or NO_3^-.

When using a dilution-water blank, subtract the blank's DO uptake 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 the dilution water's DO uptake is >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 subsequent UBOD tests.

When weekly DO consumption drops below 1 to 2% of the total accumulative consumption, calculate UBOD 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 general rule, the diluted sample's UBOD should be in the range of 20 to 30 mg/L. Dilution to this level probably will require two or three sample re-aerations during incubation to prevent DO concentrations from falling 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.

* Some analysts have reported satisfactory results with 2-chloro-6-(trichloromethyl) pyridine (Nitrification Inhhibitor, 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
80	5.4	–	32.9	0.20	0.92	32.0

* Two readings indicate concentrations before and after re-aeration.
† 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.
UCBOD = 34.5 mg/L; CBOD decay rate = 0.03/d (calculated with first-order equation from 5210C.4).

4. Calculations

An example of results obtained for an undiluted wastewater sample, 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 via 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

UBOD-test precision was assessed via 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 to 308 mg/L, depending on the extent of nitrification. The results of the study, conducted in triplicate, were:

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.

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5210 D. Respirometric Method

1. General Discussion

a. Principle: Respirometric methods directly measure the oxygen consumed by microorganisms in an air- or oxygen-enriched environment in a closed vessel under constant temperature and agitation.

b. Uses: Respirometry measures oxygen uptake more or less continuously over time. Respirometric methods are useful for assessing the biodegradation of specific chemicals; the treatability of organic industrial wastes; the effect of known amounts of toxic compounds on a test wastewater's or organic chemical's oxygen-uptake reaction; the concentration at which a pollutant or a wastewater measurably inhibits biological degradation; the effect of various treatments (e.g., 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 (e.g., the dilution BOD test); or a sludge's stability.

Respirometric data typically will be used comparatively (i.e., direct comparisons of oxygen-uptake rates in two test samples or in a test sample and a control). Because of inherent differences among uses, seed cultures, instruments, and applications of results, no single procedure for respirometric tests is applicable to all cases. Therefore, only basic recommendations and guidelines for overall test setup and procedure are given. Follow manufacturer's instructions for operating specific commercial instruments.

c. Types of respirometers: Four principal types of commercial respirometers are available: manometric, volumetric, electrolytic, and direct-input. Manometric respirometers relate oxygen uptake to the pressure change due to oxygen consumption when volume is constant. Volumetric respirometers measure oxygen uptake in incremental gas-volume changes when pressure is constant (at the time of reading). Electrolytic respirometers monitor how much oxygen is produced when water electrolyzes to maintain constant oxygen pressure in the reaction vessel. Direct-input respirometers deliver oxygen from a pure-oxygen supply to a sample via on-demand metering in response to minute pressure differences.

Most respirometers have been instrumented to permit data collection and processing via computer. Reaction-vessel contents are mixed by a magnetic or mechanical stirring device or by bubbling the reaction vessel's gaseous phase through its liquid phase. All respirometers remove carbon dioxide (CO_2) produced during biological growth by suspending a concentrated adsorbent (granular or solution) in 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 DO. 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 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. The upper limits of oxygen-uptake rate are determined by the ability to transfer oxygen into solution from the gas phase, which typically is related to mixing intensity. Transfer limits typically range from $<$10 mg O_2/L/h for low-intensity mixing to $>$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 BOD at 20°C (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 BOD and respirometric oxygen uptake at any time after 2 d.[1,2] Correlations between respirometric measurements and 5-d BOD for municipal wastewaters seem to occur at about 2 to 3 d incubation; however, 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 UBOD (see 5210C). In many cases, it is reasonable to consider that the 28- to 30-d oxygen uptake is essentially equal to UBOD.[3]

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

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 oxygen-uptake measurements.

h. Sampling and storage:

1) Grab samples—If analysis is begun within 2 h of sample collection, cold storage is unnecessary. Otherwise, keep sample ≤6°C from the time of collection. Begin analysis within 6 h of collection; when this is not possible, store ≤6°C and report storage temperature and duration. Never start analysis >24 h after grab-sample collection.

2) Composite samples—Keep samples ≤6°C during compositing. Limit compositing period to 24 h. Store using the same criteria as for grab samples; holding time begins when the compositing period ends.

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 any algae in sample from forming oxygen. Use red, actinic-coated bottles for analysis outside of a darkened incubator.

3. Reagents

The following reagent formulations produce 1-L solutions, 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) or equivalent reagent-grade water. DI water may be used but often contains high bacterial counts. The water must contain <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* potassium hydroxide (KOH) (¶ *g* below) and dilute to 1 L.

c. Ammonium chloride solution, 0.71*N:* Dissolve 38.2 g 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 ($MgSO_4$) solution, 0.41M: 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. Alternatively, 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 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* KOH (¶ *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.

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 (¶ *b* above), 0.65 mL ammonium chloride solution (¶ *c* above), 1.0 mL calcium chloride solution (¶ *d* above), 0.22 mL magnesium sulfate solution (¶ *e* above), 0.1 mL ferric chloride solution (¶ *f* above), 1 mL trace element solution (¶ *n* above), and 1 mL yeast extract solution (¶ *o* above) to about 900 mL water. Dilute to 1 L. This 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 assembling, testing, calibrating, and operating the instrument. NOTE: The manufacturer's stated maximum and minimum measurement limits are not always the same as the

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

instrument's output limits. Make sure test conditions are within measurement limits.

b. Sample volume: The sample volume or concentration of organic chemicals to be added to test vessels is a function of expected oxygen-uptake characteristics and the instrument's oxygen-transfer capability. 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 typically range from 15 min to 6 h.

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 concerned about changing sample characteristics, skip this step.

2) pH adjustment—Neutralize samples to pH 7.0 using H_2SO_4 (5210D.3*h*) or NaOH (5210D.3*i*) without diluting the sample >0.5%.

3) Dechlorination—Avoid analyzing samples containing residual chlorine by collecting them ahead of chlorination processes. If residual chlorine is present, aerate as described in ¶ *d*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 sample. Titrate with 0.025*N* Na_2SO_3 solution to the starch-iodine endpoint (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. Reseed 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 DO concentrations smaller or larger than 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 needed to prepare for each dilution depends on test objectives and number of replicates desired.

f. Nutrients, minerals, and buffer: Add enough 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 used (1 mL/50 mg/L COD or UBOD 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 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 enough seed culture to prevent major lags in the oxygen-uptake reaction but not so much that the seed's oxygen uptake exceeds about 10% of the seeded sample's oxygen uptake.

Determine the seeding material's oxygen uptake in the same way as for any other sample. This is the seed control. Typically, 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 raise sample temperature.

5. Calculations

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

Correct oxygen uptake for seed and dilution as follows:

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

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 QC 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 GGA mixture 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 GGA solution (5210D.3*k*); 6 mL phosphate buffer (5210D.3*b*); 2 mL each of ammonium chloride (5210D.3*c*), magnesium sulfate (5210D.3*e*), calcium chloride (5210D.3*d*), ferric chloride (5210D.3*f*), and trace element solution (5210D.3*n*) to approximately 800 mL water. Add 10 mg TCMP/L to inhibit nitrification. Add sufficient

seed from a suitable source (5210D.4*h*) to give a lag time <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 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 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 GGA mixture (5210D.6) with 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 with 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 a given instrument's sensitivity.

b. Control limits: To establish laboratory control limits, perform a minimum of 25 GGA checks over several weeks or months and calculate the 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 the source of error. Forother 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 each commercial instrument's limits. Refer to manufacturer's specifications.

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