**Headspace Biochemical Oxygen Demand (HBOD)**

**1. General Discussion**

*a. Principle:*The HBOD test is a variation of a respirometric test in that it provides direct measurement of oxygen consumption by microorganisms in a closed-vessel under conditions of constant temperature and agitation.1 The main difference between the respirometric HBOD tests is the frequency of analysis: respirometric tests can provide essentially continuous measurements of oxygen utilization, while the HBOD test is a batch test. In the HBOD test, oxygen is continuously resupplied from a large, but finite, supply in the air in the headspace of a sealed tube through oxygen transfer from the air into the liquid. Samples must be continuously agitated in order for oxygen uptake not to be limited by mass transfer of oxygen from the air into the water.  
  
*b. Uses:*The HBOD test is useful for many of the same reasons a respirometer is useful: measuring the oxygen consumption by microorganisms during biodegradation of solutions containing specific chemicals or mixed wastes; and assessing the effect of toxic chemicals, pH, or nutrient additions on oxygen uptake.  
  
*c. Relationship to dilution BOD:* Because water and wastewater samples need not be diluted when analyzed in the HBOD test, the oxygen demand of a sample will be exerted more rapidly than in the dilution BOD test. In general, a three-day HBOD (HBOD3) is equal to the 5-day BOD (BOD5).1 Valid correlations between the BOD and HBOD tests should be established for specific wastewaters at each new site. Based on respirometric data, a BOD5 is typically exerted after 2 to 3 days for municipal wastewaters.2,3  
  
*d. Interferences:* The pressure in the tube may change during the test due to the evolution of gases and the consumption of oxygen, but there is no evidence that pressure changes affect the consumption rate of oxygen. Carbon dioxide is not stripped out of the sample tube, and high concentrations of CO2 have been noted to slow down or inhibit biological reactions.4 However, dilution tests suggest that within the recommended limits of the test for oxygen consumption, CO2 does not interfere with the test.  
  
*e. Minimum detectable concentration:*The test is best suited to HBODs slightly larger than the solubility of oxygen, or HBOD>10 mg/L. For HBODs smaller than this, the concentration of dissolved oxygen in the aqueous sample become important, and must be measured and included in oxygen depletion calculations.  
  
*f. Sampling and Storage:*

1) Grab Samples— If analysis is begun within 2 h of sample collection, cold storage is unnecessary. For longer storage periods, store samples at 0 to #4oC from the time of collection. Analysis within 6 h is preferred; do not analyze samples if stored for more than 24 h, and state storage time if stored between 6 and 24 h.  
  
2) Composite Samples— Keep samples at 0 to #4oC from the time of collection and during compositing. Use the same criteria for storage as for 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. Incubation bottles.* Any gas-tight crimp-seal bottles can potentially be used, but mass transport coefficients must be measured for new bottles. Currently, the HBOD test has only been conducted using 28-mL anaerobic test tubes (BellCo) with 25 mm Teflon-lined caps and aluminum crimp seals.1 Clean bottles with a detergent, rinse thoroughly, and dry completely before use.  
  
*b. Incubator and water bath.*All sample tubes must be shaken, on their side, on a shaker table. Agitation rates typical of shaker tables are sufficient for domestic wastewater samples. Place shaker table in an incubator, or using a shaker table with a temperature-controlled water bath. Exclude light from samples by an appropriate method, such as placing tubes in a sealed box or covering tubes (or water bath) with foil.  
  
*c. Instrument to measure oxygen in the headspace of a HBOD tube.*There are two currently proven methods to measure oxygen in tube heaspaces: an HBOD probe (Ocean Optics, Inc.) or a gas chromatograph (many manufacturers). If using the HBOD probe, follow the manufacturer’s instructinos for calibration. If Using a Gas Chromatograph (GC) it should be equipped with a thermal conductivity detector (TCD). Use an appropriate column and temperature to analyzed for oxygen gas, such as a molecular sieve column and column temperatures of 30 to 100 oC.1 Follow the manufacturer’s instructions on operating the GC. Ensure that nitrogen and oxygen peaks are adequately separated under the selected operating conditions. Pressure will change inside an HBOD tube during an HBOD test. A leur-lock gas-tight syringe must be used to maintain a constant pressure in the syringe; otherwise, laboratory air will be drawn into the syringe when it is removed from the tube. A 50 or 100 µL syringe is recommended.  
  
*d. Barometer, hydrometer, thermometer.*The pressure, relative humidity and temperature of laboratory air must be know for accurate calculation of the oxygen in each tube. Completely digital units measuring all three parameters are commercially available. Make sure that the absolute pressure is used. (Many weather stations will adjust pressure to be sea level.)

**3. Reagents**

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 filter (through a 0.2 polycarbonate membrane) to remove excess bacteria if present. The water must contain <0.01 mg/L of heavy metals, and be free of chlorine, chloramines, caustic alkalinity, organic material, and acids. When other waters are required for special-purpose testing, state their source and quality.  
  
b. Potassium hydroxide (KOH) solution, 6 N: (CAUTION: If preparing your own solution, add KOH to water slowly and use constant mixing to prevent excessive heat accumulation.) Add 336 g KOH in 700 mL water and dilute to 1 L. Commercials solutions containing 30 to 50% KOH (by weight) may also be used.  
  
c. Nitrification Inhibitor. Use pure reagent-grade 2-chloro-6-(trichloro-methyl) pyridine (TCMP) or equivalent. If TCMP is not pure, adjust required doses accordingly. A final concentration of 10 mg/L is recommended to inhibit nitrification.  
  
d. Glucose-glutamic acid (GGA) solution. Dry reagent-grade glucose and glutamic acid powders at 103oC for 1 h. Add 0.3 g of glucose and 0.3 g of glutamic acid to distilled water, and dilute to 1 L for a final concentration of 600 mg/L. Neutralize to pH 7.0 using 6 N potassium hydroxide. The solution may be stored for <1 week at 4oC.  
  
e. Sodium sulfite (NasSO3). Use reagent grade sodium sulfite.  
  
f. Cobalt chloride (CoCl2) solution: Cobalt chloride is used as a catalyst for measuring oxygen transfer rates with sodium sulfite. Add 1.5 mg CoCl2 to 1 L of distilled water.

**4. Procedure.**

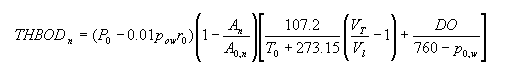
*a. Laboratory conditions:*Record the laboratory air temperature, pressure, and relative humidity at the time HBOD samples are prepared.  
  
*b. Sample volume:*The proper sample volume for a tube depends on the expected HBOD. Using Table 1, choose an appropriate liquid volume.  
  
c. Sample preparation:

1) Homogenization— If a sample contains large settleable or floatable solids, homogenize it with a blender and transfer a representative portion of the appropriate volume into an HBOD tube. Skip this step if there is a concern about changing sample characteristics.  
  
2) pH adjustment— Neutralize samples to pH 7 using H2SO4 or NaOH (if desired) if the sample will not be diluted >0.5%.  
  
3) Dechlorination— If possible, avoid analyzing samples containing residual chlorine by collecting samples prior to chlorination processes. If samples contain residual chlorine, aerate samples as described below (¶ 5) or let sample stand in the light for 1 to 2 h. If a chlorine residual is still present, Na2SO3 must be added to react with the chlorine, although doing this will remove oxygen from the sample and can injure microbes. The required volume of Na2SO3 is 10 mL 1 + 50 H2SO4, and 10 mL potassium iodide solution (10 g/100 mL) to a portion of the sample. Titrate with a 0.05 M Na2SO3 solution, and after 10 to 20 min check for residual chlorine. If this dechlorination process is followed, the sample will need to be seeded as described below.  
  
4) Toxic substances in samples— If samples contain toxic substances, they will require special treatment beyond the scope of the development of the present procedure.  
  
5) Initial oxygen concentration— For very low HBODs, it may be necessary to know the initial DO of the sample. The sample can either be aerated, or a dissolved oxygen microprobe must be used to sample the DO in the sample after it has been transferred into a tube. Samples can be aerated by agitation (using a shaker table or stir bar) or by using a diffuser connect to either a tank of clean air or to a filtered air source. Samples should not be aerated for more than 1 h; aeration will decrease the HBOD of the sample depending on the strength of the sample and the concentration of microorganisms in the sample.  
  
6) Sample temperature— All samples must be brought to the desired incubation temperature (±1oC) before adding samples to the HBOD tubes.

*d. Dilution:*It is not necessary to dilute most samples to conduct a HBOD test. If dilution is desired, for example to examine for potential toxic effects, to lower the final HBOD to avoid oxygen transport limitations, or as a result of adding nitrification inhibitor, dilute samples only with distilled or other water free from organic and chemical contamination. Dilution water can be added directly to the HBOD tubes as necessary.  
  
*e. Nutrients, minerals and buffer:* It is important to remember that if addition of nutrients, minerals or buffers are necessary to obtain an estimate of the HBOD, then these a lack of any of these components will interfere with optimal operation of a biological wastewater treatment process. The appropriate concentrations of nutrients should be added to obtain a final ratio of COD:N:P or 100:5:1, or a TOC:N:P ratio of 30:5:1. If minerals or buffering capacity is suspected to be lacking, add to distilled water and aerate overnight.  
  
*f. Nitrification inhibition:*Many domestic wastewaters at full strength and river waters will exert an oxygen demand due to nitrification unless a nitrification inhibitor is added. Prepare a concentrated solution of the nitrification inhibitor TCMP at a concentration of 100 mg/L. Dilute wastewater samples in a ratio of 1:10 to obtain a final concentration of 10 mg/L of TCMP in the HBOD tube. To determine the nitrogenous HBOD (NHBOD), also prepare samples without any nitrification inhibitor.  
  
*g. Seeding:*High concentrations of microorganisms are necessary to ensure that an HBOD will be exerted in about three days that is comparable to a BOD5. See section 5210B.4d1) for seed preparation. If available, use secondary clarifier overflow prior to chlorination. In any case, it is important to use sufficient amounts of seed to prevent major lags in oxygen utilization. When seeded samples are used, it is necessary to run parallel tests using full-strength seed to correct the sample HBOD for the seed HBOD.  
  
*h. Sample Preparation:*Add to each sample tube, in the following order: (1) any water containing nutrients, minerals, buffers or nitrification inhibitor (if necessary) ; (2) seed solution containing microorganisms (if necessary); (3) wastewater sample; (4) immediately cap bottles, and crimp seal aluminum cap on top with a crimp sealer.  
  
*i. Seed Preparation:*If a seed control is performed, repeat steps (1) and (2) and (4) in step j above for the same number of bottles used for each sample.  
  
*j. Air blanks:* Seal 3 tubes containing one or two mL of distilled water.  
  
*k. Incubation.* Incubate all tubes containing samples and seed on their sides, oriented in a direction that gives the maximum mixing of the sample. Keep samples in the dark and at 20±1 oC.  
  
*l. Oxygen Analysis in Tubes.*After an appropriate incubation time, take tubes off of shaker table, place them in an upright position near the oxygen-measuring instrument. If using the HBOD probe, calibrate the probe in air from the Air Blank tubes. Then, sample tubes. With this probe oxygen measurements are complete in a few seconds. If using a GC, using a gas-tight syringe (50 or 100 µL) with a Luer-lock type adaptor and side port needle, calibrate the GC first by measuring oxygen in laboratory air until a stable and repeatable result is obtained (at least 5 injections). Then, measure oxygen concentrations first in the Air Blank tubes and samples. Record the areas of both the oxygen and nitrogen peaks. If nitrogen peaks areas vary by more than >10% for samples versus those for the air blanks, repeat an injection.

**5. Calculations**

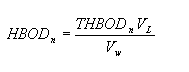
The HBOD of each tube on day n (THBOD) is used to calculate the HBOD of the water sample (HBODn = WHBODn) after correction for dilution and seed addition. Depending on the experimental objectives, it is also possible to calculate the carbonaceous HBOD (CHBOD), nitrogenous HBOD (NHBOD), and the HBOD of a glucose-glutamic acid calibration solution (GGHBOD).  
  
Calculate the HBOD for each tube using:

  
  
where: THBODn= Headspace BOD on day n of sample in HBOD tube [mg/L];  
  
P0= Total pressure of laboratory air on day 0 recorded from barometer [mmHg];  
  
p0,w= Vapor pressure of water at temperature of sample on day 0 from table of water vapor pressures [mmHg];  
  
r0 = Relative humidity of air on day 0 read from relative humidity gauge [%].  
  
An= Oxygen in sample on day n: for HBOD probe, percent of oxygen in the air in the tube [%]; for GC, peak area [mV-sec ];  
  
A0,n= Oxygen in the day 0-tube analyzed on day n [%] or [mV-sec];  
  
T0= Temperature of air on day 0 [oC].  
  
DO= Saturation dissolved oxygen concentration in water at 760 mmHg (1 atm) in water-saturated air at temperature T0 from dissolved oxygen reference table [mg/L];  
  
VT= Total volume of empty HBOD tube [mL];  
  
VL= Volume of liquid wastewater sample put into HBOD tube [mL];

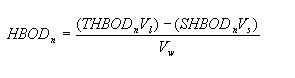
*a. Water Sample*— No dilution: If the sample was not diluted, the HBOD of the water sample is the same as the HBOD of the tube calculated using eq. 1, or

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*b. Sample HBOD with dilution (no seed):* If the sample contains any dilution water, the sample HBOD is



where VL is the total sample liquid volume and Vw is the volume of the water sample. The term VW/VL is equal to the fraction of the liquid that this the water sample, or VW/VL= P where P is the decimal volumetric fraction of sample used. The total liquid volume must be the sum of the water sample and dilution water volume, or VL=VW+VD, where VD is the volume of dilution water assumed to have an HBOD=0.  
  
*c. Sample HBOD with Seed:*The HBOD of the sample and the seed must be determined separately. Calculate the HBOD of the seed using method a) or other methods in this section, as appropriate. The HBOD of the water sample is then calculated from the HBOD of the seed (SHBOD) and the HBOD of the tube containing both sample and seed (THBOD) as

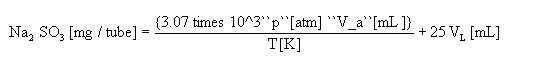


*d. Carbonaceous HBOD (CHBOD):*The CHBOD can be measured for a sample by either adding the nitrification inhibitor TCMP into the water sample, or into the dilution water. If TCMP is added to the water sample, calculate the CHBOD as in part a) or if seed is used as in part c). If TCMP is added into the dilution water, calculate the CHBOD as in part b), or if seed is used, calculate the HBOD as in part c) recognizing that TCMP must be added to both the sample and seed tubes.  
  
e. Nitrogenous HBOD (NHBOD): In order to calculate the NHBOD, samples must be analyzed with and without nitrification inhibitor. Calculate the total HBOD of just the water sample using either a) or b) depending on whether dilution of the sample is necessary. Run in parallel samples containing nitrification inhibitor. The NHBOD is calculated as the difference between the two results, as

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**6. Quality Control**

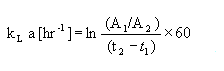
In order to test the procedures necessary for the HBOD protocol, it is necessary to ensure that samples are adequately mixed (aerated) and that the other procedures are adequately run. In order to show that samples are well mixed, measure the mass transport coefficient in tubes for typical mixing conditions. Any time a new mixing speed, shaker table, or tube-type is used, check to see that the mass transport coefficient meets the requirements as described below. The glucose-glutamic acid check is suggested to verify proper operation of the GC, gas injections, seed (if used) and general laboratory procedures.  
  
a. Mass transport coefficient: Add sodium sulfite (Na2SO3) to cobalt chloride solution at a concentration (>0.5 M Na2SO3) that will always ensure that sodium sulfite is in excess and oxygen uptake will be first order with respect to oxygen in the headspace. The mass of sodium sulfite added is calculated as the sum of the mass of chemical necessary to react with all oxygen in the air in an empty tube, and mass in the liquid at a concentration of 0.05 M. The mass of sodium sulfite Na2SO3 is therefore

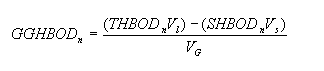
  
  
where p is the partial pressure of oxygen in air, T the temperature, Va the volume of air and VL the volume of liquid (Logan and Kohler, 1999). For typical conditions of 0.209 atm of oxygen, 20oC (293 K), this can be simplified to

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The procedure for calculating the mass transport coefficient is as follows:

1) Place the appropriate mass of sodium sulfite (calculated using eq. 6 or 7) into each of 3 tubes.  
  
2) Add the cobalt chloride solution of volume VL.  
  
3) Immediately cap tubes and place on shaker table. Start timing from the moment the sample is shaken.  
  
4) Analyze the concentration of oxygen in the tubes at times of 1, 3 and 5 minutes.  
  
5) Calculate mass transport coefficients, kLa [hr-1], as

  
  
where A1 and A2 are the oxygen concentrations (peak area or percent oxygen) in the tube headspaces measured at times t1 and t2. For example, if laboratory air is analyzed using the HBOD probe to have a peak area of 20.9%, after sodium sulfite is added, the sample is capped, and placed on the shaker table, after 1 min analysis of the air in the tube indicates 18% oxygen, but after 3 min the concentration is 13.1%. Using eq. 8, kLa=9.6 hr-1. Each run with three tubes produces two values of kLa.  
  
6) Repeat steps 2 through 6 at least three times. A typical value for kLa is 8±2 hr-1.  
  
The mass transport coefficient is the maximum rate of oxygen transport into liquid, and therefore, it represents the maximum rate that substrate can aerobically be consumed. Extensive BOD testing (5210 B 5) and some HBOD testing1 indicates that the DO should remain above 1 and 2 mg/L (respectively) for oxygen not to limit the rate of BOD exertion. For typical mass transport coefficients, HBODs as high as 1340 mg/L-d are possible at the start of an HBOD test, although the maximum daily HBOD declines to 192 mg/L-d at the end of the test due to oxygen depletion in the sample headspace (Logan and Kohler, 1999). If greater oxygen transfer rates are needed, the intensity of mixing can be increased or samples can be diluted.  
  
b. Glucose Glutamic Acid Calibration. Because many full-strength wastewaters will nitrify, it is important to run a calibration test with nitrification inhibitor in all samples. A seed solution is needed to supply concentrated suspension of microorganisms. Prepare a GGA solution as described above. Add TCMP to both the GGA solution and the seed at a final TCMP concentration of 10 mg/L. The HBOD of the GGA solution (GGHBOD) must be determined using separate samples containing GGA and the seed, and samples containing only seed. Calculate the HBOD of the seed using method 5a) or 5b). The HBOD of the GGA water sample is then calculated from the HBOD of the seed (SHBOD) and the HBOD of the tube containing both sample and GGA (THBOD) as

  
  
where THBOD is the HBOD of the tube containing GGA and seed filled to a volume VL and containing a volume of seed, VS, and volume of GGA, VG, and SHBOD is the HBOD of the seed determined in separate experiments. Note that VL=VG+VS.  
  
Run at least tubes in triplicate. Three-day GGHBOD’s (211 mg/L) have been reported to be within the range of BOD5 results (204±10 mg/L). If values are not within this range, make changes to the protocol such as using new reagents to ensure high quality, changing the source of the seed, checking the accuracy of the gas chromatograph, or the mixing rate of samples on the shaker table.

**7. Precision and Bias**

*a. Precision.* There is no standard available to check the accuracy of the HBOD technique, but it is assumed to compare well with respirometric techniques. The precision of the test can be examined using the glucose-glutamic acid calibration technique described above in section 6b.  
  
*b. Control limits.*Perform a minimum of 25 glucose-glutamic acid checks on the GGHBOD3 over a period of several months and calculate the mean and standard deviation. If the mean of the three-day tests is outside the range of 204±10 mg/L, re-evaluate the procedure and make changes accordingly.

**8. References**

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Table 1. Range of measurable HBODs as a function of headspace and liquid volumes for a DO change of >1 mg/L and a minimum final DO of >2 mg/L (VT=28 mL, r=20%, To=20oC, ys=0.209, pw=17.54 mmHg, and PT=700 mmHg, corresponding to a DO saturation concentration of 9.09 mg/L).

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| Headspace volume  VT-Vl(mL) | Liquid Volume  Vl (mL) | HBOD range  (mg/L) |
| 5 | 23 | 7 - 50 |
| 8 | 20 | 12 - 86 |
| 10 | 18 | 17 - 117 |
| 15 | 13 | 39 - 236 |
| 18 | 10 | 51 - 364 |
| 20 | 8 | 71 - 503 |