A gas chromatographic-based headspace biochemical oxygen demand test

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ABSTRACT: The 5-day biochemical oxygen demand (BOD_5) test is an established tool for measuring the concentration of biodegradable organic matter in wastewater effluents. Unfortunately, the BOD test is time consuming, labor intensive, and, due to the dilution of the wastewater, takes a relatively long time to complete. A few years ago a headspace BOD (HBOD) test was developed that avoided the need to dilute wastewater samples. A disadvantage of the original HBOD test was that the sample had to be transferred from the HBOD tube to another vessel for a dissolved oxygen measurement. This study demonstrates that it is possible to conduct HBOD tests using a gas chromatograph to measure oxygen utilization in the sealed tube and that a 3-day HBOD provides a reliable estimate of the BOD_5. The $HBOD_3$ values measured for primary and secondary clarifier effluents from an activated sludge plant were 114 and 23 mg/L; BOD_5 measurements were 116 and 24 mg/L. Changes in headspace volumes did not significantly change the HBODs. Secondary settled wastewater had a $HBOD_5$ of 83 ± 3 mg/L (mean ± SD) for headspace volumes of 10–20 mL (36–71%) in a 28-mL tube. The $HBOD_5$ was also constant (84 ± 6 mg/L) when samples were diluted 20–60% using BOD dilution water at a fixed headspace volume of 8 mL in HBOD tubes. Nitrification increased the HBOD of nondiluted wastewaters after 4 days, although nitrification appeared to be sufficiently inhibited using a standard nitrification inhibitor. A calibration test for the HBOD was developed using a 300-mg/L glucose and glutamic acid (GGA, 50% each) solution and secondary clarifier wastewater that provided $HBOD_3$ values similar to those obtained in the analogous BOD_5 test. It is argued that the simpler procedures, added precision of GC-based protocols, and more rapid exertion of oxygen demand make the HBOD test superior to the conventional BOD test. *Water Environ. Res.*, **69**, 206 (1997).

KEYWORDS: analysis, biochemical oxygen demand, effluents, gas chromatography, headspace.

Virtually all wastewater treatment plants in the U.S. carry out the conventional biochemical oxygen demand (BOD) procedures to demonstrate that they meet EPA discharge requirements, and some plants conduct additional tests to help optimize plant performance. As a result, a substantial amount of time and expense is devoted to measuring BODs at wastewater treatment plants. When compared with modern analytical techniques, the standard BOD method appears quite crude due to many wet handling steps and chemical techniques. Wastewater must be diluted with large amounts of a nutrient-rich, buffered water that is saturated with oxygen to achieve an overall amount of oxygen use by the sample that is in a range smaller than the range of oxygen solubility in water. Samples must be placed in specially designed ground glass-stoppered bottles, and many of these large bottles must be used and stored in an incubator. The only relatively modern part of the test consists of measuring the dissolved oxygen (DO) of the sample using a DO probe, but this probe often is calibrated daily with a wet chemical procedure (the Winker test) to ensure probe accuracy.

Many tests have been proposed over the years to replace the BOD test but all have suffered from some limitation. Among these tests were 8-hr (Busch, 1958; Hiser and Busch, 1964; Mullis and Schroeder, 1971) and two-day (Zehnpfennig and Nicholas, 1953) tests based on observations of plateaus in oxygen and substrate use, but these tests required additional measurements of chemical oxygen demand (COD) or dissolved organic carbon (DOC) concentrations. The BOD test has been partially automated since commercially available systems will open and sample the DO in the bottles. However, these systems are quite expensive, and there can be frequent operational problems associated with the operation of the DO probe (Hill et al., 1995).

Oxygen demands have also been measured using a variety of respirometric techniques (O'Brien and Clark, 1962) based on either calculating oxygen use from pressure changes in a sealed vessel containing air and a stirred wastewater sample or from the mass of oxygen that must be generated to maintain a constant oxygen concentration in the gas phase. Respirometers are being increasingly used for specialized applications, and the newest edition of *Standard Methods* (1995) contains a proposed respirometric method. In general, a BOD_5 will be exerted in a respirometric BOD (RBOD) test in ~2–3 days (Young and Baumann, 1976) providing an estimate of the BOD_5 in <5 days. Although many modern respirometers are reliable, their high per sample cost and relatively sophisticated operation have limited their routine use in wastewater treatment plants.

Recently, a nondilution RBOD-type test, called the headspace BOD (HBOD) test, was proposed (Logan and Wagenseller, 1993). The HBOD and RBOD tests are similar in that both are based on replenishing the DO in the liquid sample from a gas phase, or headspace, sealed in with the liquid sample. In the HBOD test, however, wastewater is sealed in small gas-tight test tubes, and the whole tube is agitated on a laboratory shaker. In the original HBOD test, oxygen consumption was evaluated by measuring the DO of a sample by pouring it into a small (10 mL) sample holder. This transfer procedure was messy and risked re-aeration of the sample before DO measurement. In addition, the $HBOD_5$ of 300-mg/L solutions of glucose and glutamic acid averaged only 144 ± 20 mg/L, substantially lower than the 204 ± 10 mg/L typically measured in BOD calibration tests (*Standard Methods,* 1995).

We report here a new method to measure HBODs based on calculating oxygen demand from the decrease in oxygen concentrations in the gas phase, or headspace, of the sealed

HBOD tube. This gas-based HBOD method has the same advantages as the original HBOD test since it is a nondilution technique, but it has the added advantage of being a dry measurement technique. Oxygen in the sealed headspace of a HBOD tube is measured using a relatively inexpensive (less than $6 000) gas chromatograph (GC) that comes equipped with column, detector, and peak measurement software. This gas chromatograph HBOD, or GC-HBOD test, allows for repeated sampling of a tube, greater accuracy in measuring oxygen in the tube, and the potential for easy automation if the GC is connected to an autosampler headspace analyzer. The accuracy and limits of the HBOD test are demonstrated by investigating the effects of sample dilution, variations in headspace volume, and the effects of nitrification on the magnitude of the HBODs. It is also shown that using high biomass concentrations the glucose-glutamic acid calibration procedure produces $HBOD_3$ values consistent with BOD_5 test data.

Methods

HBOD tests were conducted by measuring the changes in the oxygen concentrations in the headspace of gas-tight tubes containing wastewater or wastewater with additional substrate (glucose and glutamic acid). Oxygen concentrations were measured using a GC equipped with a molecular sieve column and thermal conductivity detector (TCD). The equations used to calculate the HBOD are summarized below.

Theoretical. The mass of oxygen consumed during HBOD tests was calculated based on the fraction of oxygen used in the tube headspace during the incubation period. The moles of oxygen in the tube were calculated from oxygen gas standards. The total moles of oxygen, m_s, injected into the GC for a calibration standard containing oxygen at a mole fraction y_s can be calculated using the ideal gas law as

$$m_s = \frac{1.316 \times 10^{-9} y_s V_i (P_T - 0.01 r p_w)}{R(T_o + 273.15)} \quad (1)$$

where V_i (mL) is the volume injected into the GC for all samples, P_T (mmHg), the total pressure; T_o (°C), the temperature; p_w, the vapor pressure of water (mmHg) at temperature T_o; r, the relative humidity (percent water saturation) of air on the day the tube was sealed (day 0); and R is the gas constant (0.0821 l-atm/mol°K). The moles of oxygen taken from the sealed HBOD tube (on day 0) in the same injection is

$$m_0 = \frac{A_0 m_s}{A_{s,0}} \quad (2)$$

where A_0 is the area (mv-s) from the GC chromatogram for oxygen in the sample on day 0, and A_{s0} is the area of the oxygen standard peak on day 0. The fraction of oxygen used after a time t is

$$O_c = \frac{A_t A_{s,0}}{A_0 A_{s,t}} \quad (3)$$

where A_t is the chromatogram area for oxygen in the sample at time t, and $A_{s,t}$ is area of the oxygen gas standard measured at time t. The moles of oxygen remaining at time t is therefore

$$m_t = O_c m_0 \quad (4)$$

The HBOD (mg/L) can then be calculated based on the disappearance of oxygen from the headspace as

$$HBOD = 3.2 \times 10^8 \frac{m_0 (V_T - V_l)(1 - O_c)}{V_i V_l} \quad (5)$$

where V_T is the total volume of the tube (mL), and V_l is the volume of liquid (mL). Combining the above equations in terms of all the known variables produces

$$HBOD = \frac{513 y_s (V_T - V_l)(P_T - 0.01 r p_w)}{V_l (T_o + 273.15)} \left(1 - \frac{A_t A_{s,0}}{A_0 A_{s,t}}\right) \quad (6)$$

This derivation neglects the change in oxygen in the liquid phase since most of the oxygen in the tube is contained in the gas phase (see below). For the calculations reported in this study, neglecting the liquid phase concentration of oxygen resulted in errors of <2% in the final HBOD.

Recent experiments in our laboratory have shown that oxygen standard gases offer no advantage to calibrating the GC using air in the laboratory and assuming a mole fraction in air of 0.209. A modified procedure based on laboratory air that includes the DO of the sample in the calculation of the HBOD is therefore included in Appendix A. It has also become obvious that decreases in oxygen in the liquid phase after samples are removed from the shaker are unimportant to the final HBOD since oxygen transport from the gas into the liquid phase is quite slow (see Discussion). In the new method in Appendix A, we do not recommend shaking or vortexing samples once they are removed from the shaker table as long as the incubation period is calculated based on the time samples were shaken.

Experimental. *Wastewater samples.* Unless otherwise noted, all wastewater samples used in HBOD tests were grab samples collected from the secondary clarifier overflow at the Roger Road Treatment facility located in Tucson, Ariz. Samples were collected in 1-L Nalgene bottles (prerinsed several times with the wastewater sample to be collected), placed on ice in an ice chest, and taken to the Environmental Engineering laboratories at the University of Arizona. For one experiment, 24-hr composite samples from the primary and secondary clarifier overflow at the Ina Road Wastewater Treatment Plant in Tucson, Ariz., were analyzed using both the BOD and HBOD tests. All BOD_5 measurements reported here were made by the respective plant technicians using standard procedures (*Standard Methods,* 1995).

Measurement of headspace-oxygen by gas chromatography. All measurements of gas phase oxygen concentrations were carried out using 8610B GC (SRI Instruments, Torrance, Calif.) equipped with a thermal conductivity detector (TCD) and a 0.9-m (3-ft) long 3.175-mm (1/8-in) packed silica (molecular sieve, SRI Instruments) column with helium as the carrier gas. The oven temperature and carrier-gas flow rate of the GC were fixed at 100°C and 10 mL/min. Samples were injected with a gas-tight syringe equipped with a pressure-lock (Alltech Associates, Inc., Deerfield, Ill.) and a 22-gauge side-port needle. PEAK-SIMPLE-II chromatography software (SRI Instruments) loaded on an IBM PC- compatible computer was used to operate the GC, collect data, and analyze chromatograms. Area counts were based on 100-μl injections with the TCD set at high gain.

An oxygen-calibration curve was developed using an oxygen-

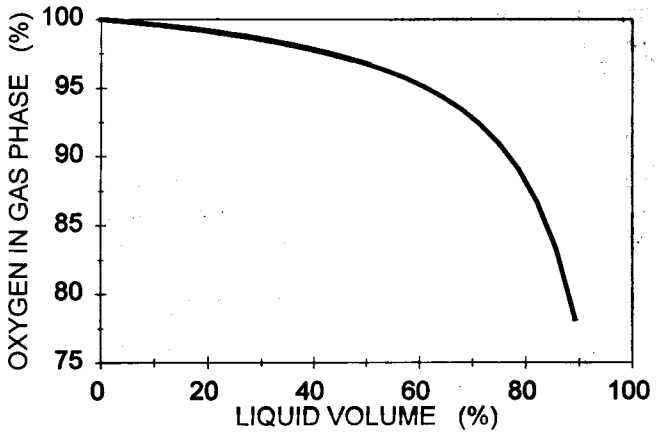

Figure 1—Percent of oxygen in the gas phase (headspace) of the percent of the volume of liquid in the HBOD tube (r = 50%, T_o = 20°C, y_s = 0.209, p_w = 17.54 mmHg, and P_T = 700 mmHg, corresponding to a DO saturation concentration of 9.09 mg/L).

standard (10% oxygen in helium, Aldrich Chemical Company, Milwaukee, Wis.) and laboratory air based on triplicate injections. The average peak areas of oxygen in laboratory air varied from 344 ± 0.55 to 357 ± 7.4 mV-s depending on daily variations in laboratory conditions of pressure, temperature, and relative humidity.

HBOD tests. HBOD tests were conducted in triplicate using 28-mL gas-tight anaerobic culture tubes (Bellco Glass Inc., Vineland, N.J.). Tubes were capped with Teflon septa that could be pierced with a syringe and sealed using aluminum crimp tops. Headspace volumes were selected to keep the final liquid DO >2 mg/L and to obtain a DO depletion of >1 mg/L. The DO at the end of the experiment was calculated from gas phase measurements by assuming that the wastewater and gas phases were in equilibrium using

$$DO_t = \frac{A_t}{A_o} c_{sat} \tag{7}$$

where c_{sat} is the saturation dissolved concentration of oxygen obtained from a reference table (e.g., *Standard Methods,* 1995) corrected for temperature and pressure. Most of the oxygen in the tubes is contained in the gas phase (Figure 1). For ≥20 mL wastewater in a 28-mL tube, >90% of the oxygen is in the gas phase assuming typical laboratory conditions (r = 50%, T_o = 20°C, y_s = 0.209, p_w = 17.54 mmHg, and P_T = 700 mmHg, c_{sat} = 9.09 mg/L) and the ideal gas law. In our laboratory, the air pressure is typically 700 mmHg due to the elevation of the city of Tucson at ~670 m (~2 200 ft) above sea level.

The HBODs that can be measured in a 28-mL tube are listed in Table 1 based on minimum and maximum DO criteria described above and Eq. 6 assuming typical laboratory conditions. For example, the measurable range of HBODs for headspace volumes of 5 and 15 mL of headspace volumes are 7–50 mg/L and 39–236 mg/L.

Samples were added to the HBOD tubes using a 5-mL digital dispensette (Brinkman, Westbury, N.Y.). The tubes were immediately sealed using a teflon stopper and an aluminum crimp top. The laboratory air temperature, pressure, and relative humidity were recorded while sealing the tubes. The pressure was obtained from a digital altimeter (Ultimeter Brothers Inc., Allenhurst, N.J.), while the relative humidity and temperature was measured using a hygrometer (Digital Humidiguide, Ben Meadows, Atlanta, Ga.). HBOD tubes were mixed for 30 s using a vortexor (VWR Scientific Industries, Bohemia, N.Y.), laid on their sides in a sealed box and incubated in the dark at room temperature (20°C) on a shaker table (Lab Line Instruments, Melrose Park, IL) set at 1 200–1 500 rpm.

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.

Headspace volume (V_T-V_l), mL	Liquid volume (V_l), 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

V_T = 28 mL, r = 20%, T_o = 20°C, y_s = 0.209, p_w = 17.54 mmHg, and P_T = 700 mmHg, corresponding to a DO saturation concentration of 9.09 mg/L.

Headspace oxygen concentrations were measured at various time intervals (1–15 days) depending upon the experiment. Before GC analysis of the gas in the tubes the tubes were vortexed for 30 s and set in a test-tube rack. Oxygen consumption was based the average of three injections per tube, and the three tubes averaged for calculating the final HBOD. All tubes were emptied after analysis and not reanalyzed.

To inhibit nitrification, 2-chloro-6-(trichloromethyl) pyridine (TCMP; HACH Chemical Company, Ames, Ia.) was used at a final concentration of 50 mg/L. TCMP was either added directly to the wastewater sample in the digital dispensette or, in experiments using BOD dilution water, the TCMP was added to BOD dilution water prepared using tap water and a BOD pillow (Hach). The nitrogenous HBOD (NHBOD) was calculated from the total HBOD as

$$NHBOD_n = HBOD_n - CHBOD_n \tag{8}$$

where the HBOD is measured for samples without TCMP, the carbonaceous BOD (CHBOD) is measured for samples containing TCMP, and the subscript n is used to designate the time of the analysis in days.

The production of CO_2 was not monitored during our tests since CO_2 is adsorbed by the column used in our gas chromatograph. Some studies have reported a decrease in COD removal due to concentrations of CO_2 >10% in the gas phase (Rozich and Gaudy, 1992). In our tests, the solution pH was found to remain essentially unchanged, and we therefore assumed that the build up of dissolved CO_2 was not a problem. This aspect of the HBOD test may need further investigations if CO_2 concentrations become high in the tube.

Glucose and Glutamic acid (GGA) were added in some experiments as a 50:50 mixture to samples from a stock solution of

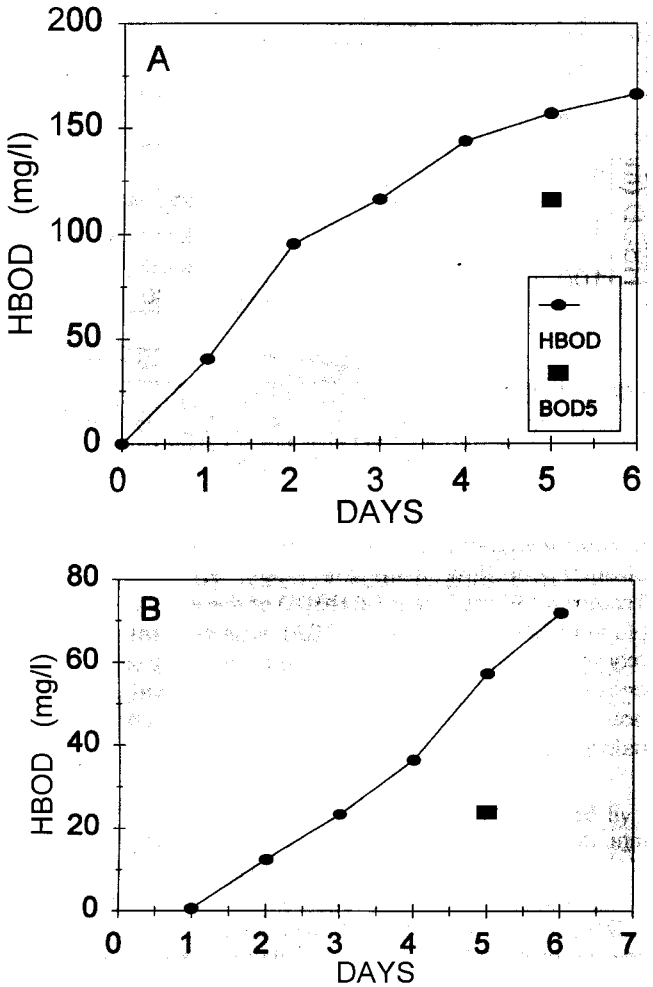

Figure 2—Daily HBODs and the BOD_5 of (A) primary clarifier and (B) secondary clarifier effluents from the Ina Road Wastewater Treatment Plant. Notice that the $HBOD_3$ = BOD_5.

1 000 mg/L. The HBOD due to GGA was calculated by conducting parallel experiments to determine the HBOD of the wastewater, HBOD(WW), using

$$HBOD_n(GGA) = HBOD_n(WW + GGA) - HBOD_n(WW) \quad (9)$$

where the HBOD(WW) was also separated into CHBOD and NHBOD components using separate samples and Eq. 8. Additional details can be found in Patnaik (1996).

Results

Experimental measurements at two different wastewater treatment plants indicated that the 3-day HBOD ($HBOD_3$) produced oxygen demands essentially identical to BOD_5 values. Daily total HBOD measurements were made on primary and secondary clarifier overflows (24-hr composites) from the Ina Road Wastewater Treatment Plant using the HBOD test and compared with BOD_5 values obtained by plant personnel (Figure 2). A nitrification inhibitor was not added to samples. The oxygen demands were exerted more rapidly in the HBOD sample tubes than the BOD_5 bottles. The 3-day HBODs for the primary and secondary clarifier were 114 and 23 mg/L, values nearly identical to the BOD_5 measurements 116 and 24 mg/L. By day 5, the HBODs had increased to 154 and 57 mg/L for the primary and secondary clarifier samples, respectively. The more rapid exertion of oxygen demand observed for the concentrated wastewater sample in the HBOD test than in the diluted BOD bottles is similar to that typically observed in respirometric tests. Young and Baumann (1976) found that RBODs were typically equal to BOD_5s after 2 to 3 days for wastewater samples from different locations at three different treatment plants.

$HBOD_3$ measurements on grab samples from the secondary clarifier overflow from the Roger Road Tricking Filter Wastewater Treatment plant were slightly higher, and $HBOD_5$ values considerably higher, than corresponding BOD_5 measurements averaging 25 ± 6 mg/L due to nitrification in the samples (Figure 3). $HBOD_5$ values for two different samples were 41 (Figure

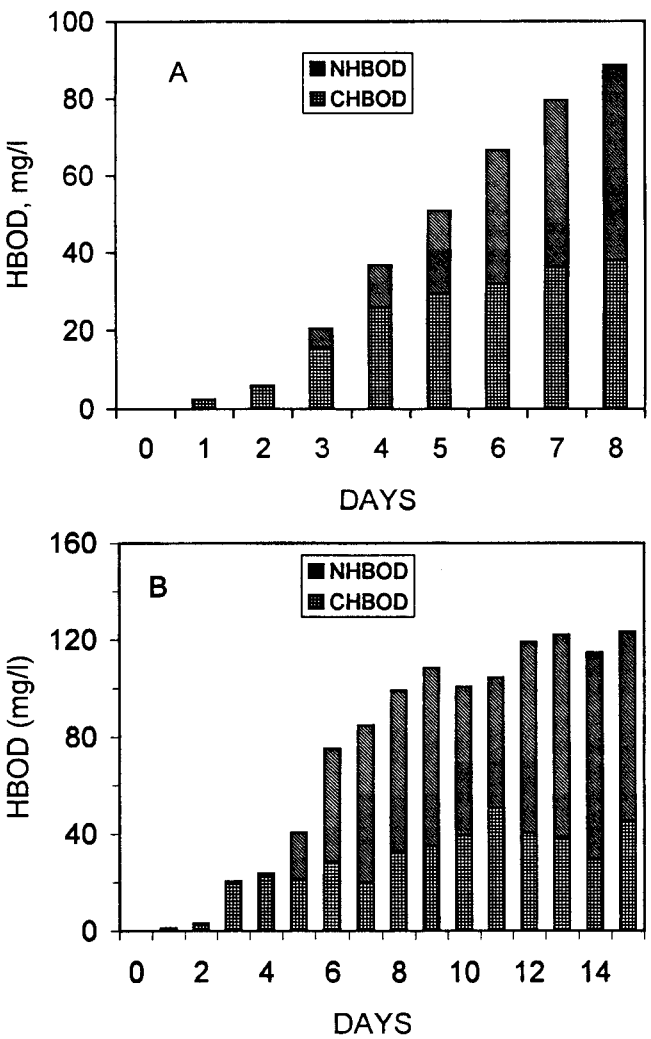

Figure 3—Daily HBODs separated into the carbonaceous (CHBOD) and nitrogenous (NHBOD) fractions from secondary clarifier effluent from the Roger Road Wastewater Treatment Plant from two different sampling days: (a) October 14, 1995; (b) April 7, 1995.

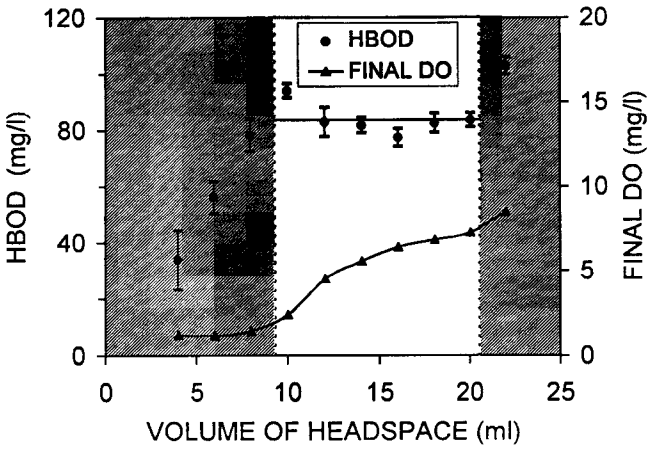

Figure 4—Final $HBOD_5$ values calculated from tubes with different headspace volumes using wastewater from the secondary clarifier of the Roger Road Wastewater Treatment Plant (no nitrification inhibitor). The shaded portions indicate areas that did not meet the requirements of a DO change of >1 mg/L and a final DO of >2 mg/L.

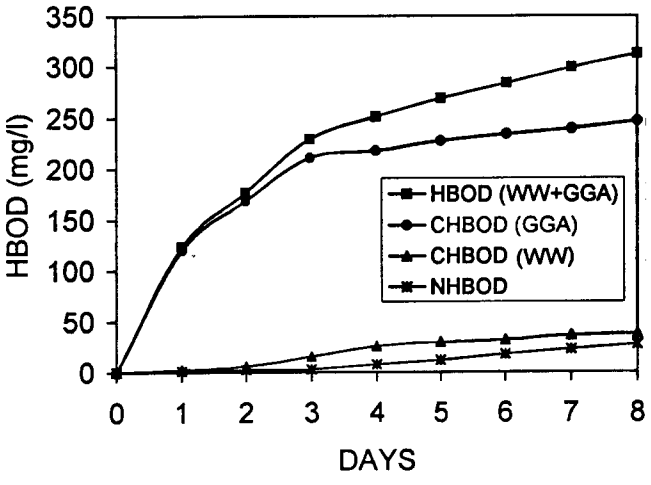

Figure 5—Daily HBOD values exerted during a GGA calibration test using a single sample from the secondary clarifier overflow from the Roger Road Wastewater Treatment Plant. The total HBOD of the wastewater sample with GGA, HBOD(WW+GGA), was separated into the oxygen demands exerted by the wastewater carbonaceous demand, CHBOD (WW), the wastewater nitrogenous demand, NHBOD (WW), and the GGA, HBOD (GGA) using separate tubes for each analysis.

3b) and 50 mg/L (Figure 3a). However, the use of a nitrification inhibitor produced lower carbonaceous $HBOD_5$ ($CHBOD_5$) values of 21 and 29 mg/L. The $CHBOD_3$ values were 16 ± 7 and 20 ± 7 mg/L, which was considered to be acceptable in comparison with the range of BOD_5 values measured by plant personnel. Slight decreases in HBOD results on days 10 and 13 (Figure 3b), compared with HBODs for previous days, reflect sample-to-sample variability resulting from individual tubes being sacrificed to produce a cumulative oxygen uptake curve over time.

Effect of headspace volume. Changing the volume of headspace in a tube did not affect $HBOD_5$ measurements as long as the final DO in the liquid was >2 mg/L, and the DO drop in the liquid was >1 mg/L. The final DO concentrations in the liquid were estimated from headspace measurements using Eq. 7. The effect of headspace volume was examined using secondary clarifier effluent at the RRTP. The average $HBOD_5$ of the sample was 83 ± 3 mg/L (n = 6) based on samples meeting the requirements of final DO changes (Figure 4). Injection-to-injection (within sample) variations of GC area counts varied by 0.6 to 2.7%.

These results indicate that the choice of the head space volume will not affect the HBOD measurement as long as the final DO concentrations are met. Since tube-to-tube variations are larger than within-sample (injection-to-injection) variations, more accurate HBODs values can be obtained from analyzing replicate tubes than by multiple analyses of the same tube.

Development of a calibration procedure for the HBOD test. A standard check on BOD methodology is to measure the BOD_5 of a 300 mg/L GGA solution using a seeded dilution water. A similar calibration procedure was developed for the HBOD test. Since the HBOD test is based on the examination of nondiluted wastewater, the oxygen demand of a 300-mg/L (final concentration) GGA solution was combined with nondiluted secondary clarifier overflow without using a nitrification inhibitor. The HBOD of the wastewater, consisting of the carbonaceous and nitrogenous HBODs, was measured separately using samples with and without nitrification inhibitor.

Daily HBOD measurements demonstrated that the oxygen demand of the GGA solution is rapidly exerted, producing an $HBOD_3$ = 211 mg/L and an $HBOD_5$ = 228 mg/L (Figure 5). This 3-day HBOD compares favorably to the BOD_5 = 204 ± 10 mg/L reported in *Standard Methods* (1995) for an extended series of laboratory tests, and the BOD_5 = 198 ± 31 mg/L results for a multilaboratory test of a 300-mg/L GGA solution.

To determine the effect of the concentration of the GGA solution on the final HBOD, tests were conducted with secondary clarifier wastewater and GGA solutions ranging from 50 to 500 mg/L. Samples were corrected for the $HBOD_5$ exerted by the wastewater and then normalized by the GGA concentration. The oxygen $HBOD_5$ exerted by the GGA solutions was nearly constant at GGA concentrations >200 mg/L (Figure 6). Values of the $HBOD_5$/GGA ratios varied from 0.63 (at 50 mg/L of GGA) to 0.76, with an average ratio of 0.73 for GGA concentrations >200 mg/L. These results indicate that the use of a 300-mg/L concentration of a glucose and glutamic acid solution should provide a stable check on the HBOD protocol.

Effect of sample dilution on the HBOD tests. The magnitude of the HBOD value is relatively stable when a sample is partially diluted with BOD dilution water, but large dilutions will reduce biomass concentrations and result in low estimates of the HBOD. To demonstrate the effect of dilution on the $HBOD_5$, a series of HBOD tests were run using a fixed volume of sample (20 mL) and headspace (8 mL). Diluting the sample with 4–12 mL of BOD dilution water (20–60%) did not produce an appreciable change in the final $HBOD_5$ of secondary clarifier effluent when values with a final DO of <2 mg/L and

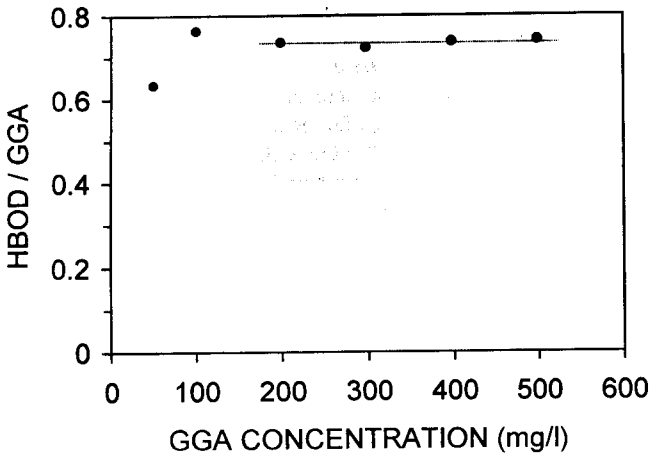

Figure 6—The $HBOD_5$ exerted at different GGA concentrations was normalized by dividing the final HBOD by the GGA concentration, producing an average ratio of 0.73 for GGA concentrations >200 mg/L (dashed line).

DO changes <1 mg/L were excluded (Figure 7). However, when the sample was diluted by 80% using 16 mL of dilution water, the $HBOD_5$ was appreciably lower than other values. Thus, excessive dilution of samples may decrease the exertion of oxygen demand in a HBOD test.

Additional evidence of reduced HBOD values produced by dilute cell suspensions is shown in Figure 8. The HBOD exerted during an 8-day period using a 300-mg/L GGA solution and secondary clarifier effluent was compared with the HBOD produced using seeded dilution water (5-mL secondary clarifier effluent per liter of BOD dilution water). The dilution water $HBOD_5$ was 130 mg/L, while the $HBOD_5$ value for the full-strength wastewater was 228 mg/L (both values corrected for

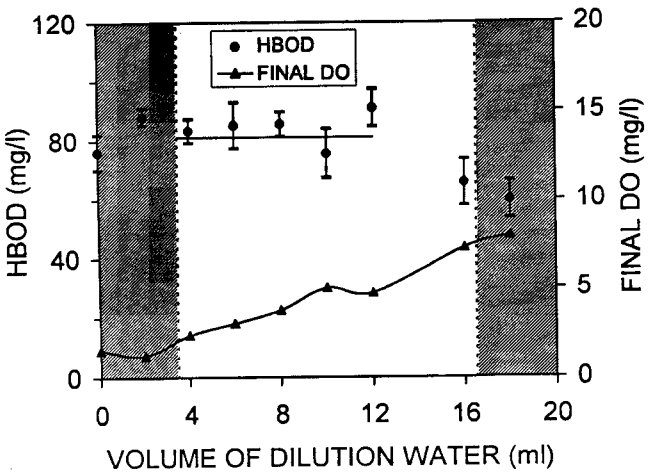

Figure 7—Final $HBOD_5$ values calculated by diluting wastewater from the secondary clarifier of the Roger Road Wastewater Treatment Plant (no nitrification inhibitor) with BOD dilution water. The shaded portions indicate areas that did not meet the requirements of a DO change of >1 mg/L and a final DO of >2 mg/L.

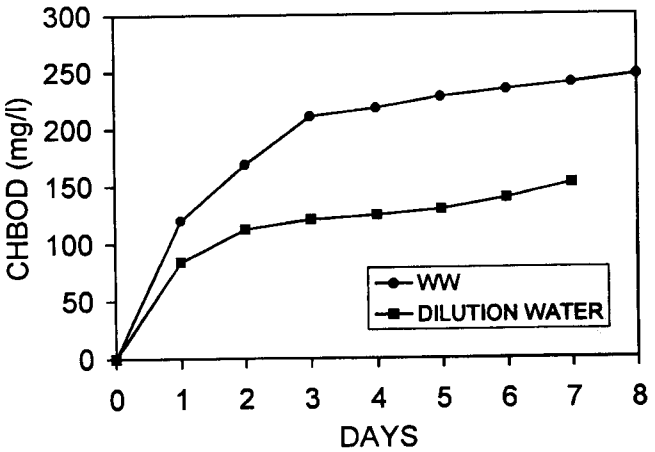

Figure 8—The daily HBOD (GGA) values exerted by samples containing 300 mg/L of GGA: samples run using nondiluted wastewater, ●, and samples using seeded BOD dilution water, ■, (5 mL seed per 1 L of dilution water). HBODs have been corrected for secondary clarifier overflow used as the inoculum.

their respective seed concentrations). Thus, insufficient cell concentrations can produce lower values of the oxygen demand in HBOD tests.

Discussion

The HBOD test results based on measuring oxygen concentrations in the headspace of sealed tubes using a gas chromatograph demonstrates that a GC-based HBOD test can provide rapid and reliable estimates of oxygen demands of nondiluted wastewaters. Experiments conducted using samples from two wastewater treatment plants and on GGA solutions suggests that the 3-day HBOD provides a reliable estimate of the 5-day BOD. For example, $HBOD_3$ values for primary and secondary clarifier effluents from an activated sludge plant of 114 and 23 mg/L, respectively, were essentially identical to BOD_5 measurements 116 and 24 mg/L. The reasons for the more rapid exertion of oxygen demand in the HBOD test, compared with the BOD test, is that the HBOD test uses nondiluted samples. By diluting organic matter and biomass in the BOD test to levels that will consume less than ~7 mg/L of oxygen in a 5-day period, the overall microbial growth and substrate use kinetics are substantially reduced. This results in the reduction of organic matter in a BOD test over periods of days, when detention times of only hours are required in the treatment system to achieve similar removals.

The use of a GC to analyze oxygen in the headspace of sealed tubes has resulted in a substantial improvement in the HBOD protocol in comparison to the liquid-based technique originally proposed by Logan and Wagenseller (1993). In the original method, the tube was opened and the wastewater sample poured into a container. This liquid transfer risked aeration of the sample DO and underestimation of the final HBOD. The liquid-based technique still relied upon the use of a DO probe, and these probes are subject to frequent problems and require constant calibration and maintenance. In contrast, the gas-based technique is less prone to problems since it is essentially a dry

technique. Air from the sample is withdrawn through the septum and injected into the GC without the need to pour a liquid sample or to force a sample to overflow the container (as in a BOD test) to form an air-tight seal when a DO probe is inserted into a bottle.

The gas measurement approach is inherently more accurate than liquid measurements in the original HBOD test and the BOD test. The GC can easily be calibrated with air in the laboratory while a DO probe used in liquid samples must be occasionally calibrated with a Winkler test. While only one measurement can be made per bottle with a DO probe, multiple gas injections from a single tube can be used to verify the oxygen concentrations in the gas phase.

Liquid-based HBOD test results can also be erratic when samples stand still too long before DO analysis. When a sample is not mixed, the consumption of oxygen in the wastewater by the microorganisms can result in a liquid phase DO not being in equilibrium with the gas phase concentration. Thus the DO measured in the liquid may not reflect the oxygen consumption in both the liquid and gas phases. Since small changes in the DO can create large changes in the final HBOD, this additional consumption of DO could over estimate the final HBOD in a liquid-based test. Mixing the sample before liquid analysis is recommended in the liquid-based test to restore equilibrium conditions between the gas and liquid phases before DO measurement, but there is no easy method to verify that equilibrium between the gas and liquid phases has been reached resulting in a sample that could be over- or undersaturated with DO. In contrast, there is relatively little change in the final HBOD in the gas-based test if the samples sit idle before GC analysis. Since most of the oxygen resides in the headspace (see Figure 1), the loss of a few milligrams per liter of DO in the wastewater results in no measurable change in the gas phase oxygen concentration. Thus, once a HBOD tube is no longer mixed, the gas phase concentration of oxygen is constant and produces no further change in the calculated HBOD.

The different estimates of the HBOD obtained for the GGA calibration tests performed at different dilutions explains previously low values obtained by Logan and Wagenseller (1993) for GGA solutions in liquid-based HBOD tests. They measured an average value of 144 ± 20 mg/L for the $HBOD_5$ using dilution water seeded with a variety of different wastewater sources. When seeded dilution water was used here (5 per 1 000 mL), we similarly observed a low $HBOD_5$ = 130 mg/L. However, when nondiluted secondary clarifier wastewater was used instead of BOD dilution water, the $HBOD_5$ was 228 mg/L. This indicates that low $HBOD_5$ values for GGA solutions will be produced when substrate to microorganism ratios are high as observed in HBOD tubes with GGA and BOD dilution water. These so-called food to microorganism ratios need to be more evenly matched in the HBOD test to obtain accurate oxygen demands. In the BOD test, for example, the 300 mg/L of GGA is diluted to ~5 mg/L to obtain a corresponding DO change of 3.5 mg/L (assuming a 70% exertion of BOD during 5 days). In the HBOD test, it is necessary to use the more concentrated suspension of microorganisms in nondiluted secondary clarifier wastewater to obtain a sufficient substrate to microorganism ratio. The use of dilution water in the HBOD test provides too small a concentration of microorganisms compared with the high concentration of 300 mg/L of GGA. Therefore the HBOD calibration test should be conducted by adding GGA directly to nondiluted wastewaters.

Economic considerations of the BOD and HBOD tests. It is not possible to fully compare the total costs of the HBOD and BOD tests since a large factor in the cost comparison is a highly variable labor cost for the technician. Since the HBOD test is easier to prepare, run, and analyze, the HBOD test could reduce technician time by as much as one-third to one-half compared with BOD tests. The capital costs of the HBOD test are much more easily evaluated than the labor costs. Assuming that a typical laboratory has available a personal computer, shaker table, test tube racks, and pipettors, it would cost $6 000–8 000 to assemble all the other components for the HBOD test, consisting of: gas chromatograph, data acquisition system, chromatogram software for a PC and molecular sieve column; test tubes, caps, and crimper; syringes and needles; digital dispensettes (or mechanical pipettors); thermometer, barometer, and hydrometer. While this may seem like a large investment, new DO probes and associated equipment can cost upwards of $1 500–2 000. Most of the investment costs ($4 500–6 000) for the HBOD equipment are associated with the purchase of the gas chromatograph. However, some laboratories will already own a GC. If costs for BOD bottles and other glassware and chemicals are compared only on the basis of costs for HBOD bottles and minor ancillary equipment (not including the GC), the costs for the expendables and other items are similar for the HBOD and BOD tests.

Other considerations. From a practical viewpoint, one of the most obvious advantages of the HBOD test may be that it can provide more rapid estimates of wastewater oxygen demands than a BOD test (3 versus 5 days). For some wastewater treatment plants, this would allow a more rapid detection of changes in plant or discharge BOD_5 values and provide an earlier warning of violations that could lead to reduced fines. In systems where there are other operational problems, the effect of nutrient additions or combination of in-house wastewater streams to reduce toxic levels of chemicals could be easily be evaluated on full-strength wastewaters. At many sites, respirometers have been purchased to address such problems, but respirometers are quite expensive on a per-bottle basis. In contrast, the number of samples analyzed using the HBOD method can easily be increased at little or no additional expense.

The use of a gas chromatograph may not seem advantageous compared to a DO probe due to the need to operate and maintain the GC. The analysis of oxygen on a GC, however, is quite simple, and the GC operation relatively trouble free for many reasons: there are no liquid samples to clog or contaminate the syringe, injection port, or column; the molecular sieve column is durable and stable for gas injections; the thermal conductivity detector (TCD) is one of the most reliable and simple detectors for a GC; the volume of gas injected is relatively large, making sample injections accurate; no calibration standards are necessary since laboratory air can be used to calibrate the GC; and peak areas are linear with the concentration of oxygen so a single point calibration can be used. With the GC system we used, a personal computer can be used to both set operational parameters (such as oven temperature and so forth) and to measure peak areas so that stand alone integrators are not necessary, and the chromatography software is easy to learn and use. Thus, for oxygen analysis, the GC is a relatively simple instrument

to use. Many laboratories already own and use GCs for other applications, which might make it convenient to use a GC for a HBOD test than to learn to use a new instrument such as a respirometer.

The HBOD protocol should be amenable to automation, although the costs for this are at present quite high. The use of tubes that could fit on headspace autosamplers, produced by a number of manufacturers, would remove the need for manual GC injection. Such autosamplers are quite expensive ($20 000–25 000) and may only be justified in cases where they are already present in the laboratory or where large numbers of samples must be analyzed. Since the resistance to oxygen transport into a liquid is liquid-phase controlled, that is oxygen is transferred into the liquid phase only very slowly in the absence of agitation, samples sitting on an autosampler rack will not appreciably change in their gas phase oxygen concentrations during the time required for automated analysis. Thus, the HBOD values should be stable over sufficiently long time periods to permit the use of autosamplers.

The authors have also found the HBOD test to be a useful tool for introducing students in university classrooms to oxygen-demand measurements and as a first introduction to the operation of a gas chromatograph. The HBOD test has been incorporated into the course offered at the University of Arizona on biological wastewater treatment. The students find it easier to work with the small sealed HBOD tubes and an easily calibrated GC than the larger BOD bottles and a DO probe that must be calibrated every time with a Winkler test. We suspect that these preferences will be shared by laboratory technicians at wastewater treatment plants as well.

The conventional BOD test will continue to be a useful test to indicate the rate oxygen demand is exerted when wastewater is highly diluted by a receiving water body. The HBOD test probably offers little advantage to the BOD test when the BODs after dilution are less than oxygen saturation concentrations in water. However, there are many instances when oxygen demands of nondiluted samples are required, for example within treatment systems such as wastewater lagoons, when the HBOD test will be worth using. In addition, the faster exertion of the $HBOD_3$ versus the BOD_5 may prove to be very important to industry to provide more rapid detection of wastewater discharges to POTWs that exceed permit limits. The wider use of respirometers attests to the need for oxygen demand data for on concentrated wastewaters. The HBOD test can provide relatively affordable data on oxygen demand for a large number of samples in the same manner as more expensive respirometric systems.

Acknowledgments

Credits. This work was funded in part by a National Science Foundation (NSF) Grant (BES 94–14423). Iris Velasco provided assistance with some laboratory experiments, and Andrea Palmer, Nancy Barry and other members of the Pima County Technical Services Laboratory provided cooperation and BOD test data.

Submitted for publication May 1, 1996; revised manuscript submitted August 12, 1996; accepted for publication August 26, 1996.

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Appendix

The calculations needed to calculate a HBOD can be simplified by calibrating the GC using laboratory air. A simplified equation and HBOD protocol is presented in this Appendix. This calculation includes dissolved oxygen concentrations in the liquid and assumes that the liquid sample added by the digital dispensette becomes saturated with oxygen. To use the HBOD equation presented here, it is required that a separate tube containing only air was sealed on the same day that the HBOD samples were prepared.

1. Assemble glassware, wastewater samples, etc.
2. Record laboratory temperature, pressure, and relative humidity. Make sure wastewater sample is at room temperature.
3. Pour sample into digital dispensette bottle.
4. Add appropriate volume of sample (for example 15 mL of a wastewater for an expected HBOD in the range of 100 mg/L) to each test tube using digital dispensette. It is recommended that you use three to five tubes per sample. Repeat as necessary for each sample.
5. Cap and seal all wastewater samples.
6. Seal two additional completely empty test tubes.
7. Place all tubes in a test tube rack, place rack in box (to keep

samples in the dark), and place tubes on their sides on a shaker table in an incubator at 20°C.
8. Incubate on shaker table for appropriate number of days (typically 3 or 5 days).
9. After samples have been incubated, take tubes off of shaker table, place them in an upright position near the GC.
10. Using a gas-tight syringe (50 or 100 μl) with a Luer-lock type adaptor and side port needle, measure oxygen concentrations in the two empty tubes and in the headspace of all tubes containing samples. Record the areas of both the oxygen and nitrogen peaks. If nitrogen peak areas change by >5%, reanalyze a sample.
12. Calculate the HBOD for each tube using:

$$HBOD_n = (P_0 - 0.01 p_{0,w} r_0)\left(1 - \frac{A_n}{A_{0,n}}\right) \times \left[\frac{107.2}{(T_0 + 273.15)}\left(\frac{V_T}{V_1} - 1\right) + \frac{DO}{760 - p_{0,w}}\right] \quad (10)$$

where:

$HBOD_n$ = Headspace BOD on day n [mg/L];
P_0 = Total pressure of laboratory air on day 0 recorded from barometer [mmHg];
$p_{0,w}$ = Vapor pressure of water at temperature of sample on day 0 from table of water vapor pressures [mmHg];
r_0 = Relative humidity of air on day 0 read from relative humidity gauge [%].
A_n = Oxygen peak area of sample on day n [mV-s];
$A_{0,n}$ = Oxygen peak area from the GC in air from the day 0-tube analyzed on day n [mV-s];
T_0 = Temperature of air on day 0 [°C].
DO = Saturation dissolved oxygen concentration in water at 760 mmHg (1 atm) in water-saturated air at temperature T_0 from reference table [mg/L];
V_T = Total volume of empty HBOD tube [mL];
V_L = Volume of liquid wastewater sample put into HBOD tube [mL].