A NON-DILUTION BOD TEST BASED ON ANALYSIS USING A GAS CHROMATOGRAPH: 
THE GC-HBOD test

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ABSTRACT

The 5-day biochemical oxygen demand (BOD₅) 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 (Logan and Wagenseller, 1993, Wat. Env. Res., 65, 862). Since oxygen in the headspace of a sealed tube was used to replenish dissolved oxygen in the liquid no sample dilutions were necessary. The disadvantage of the original HBOD was that the sample had to be transferred from the HBOD tube to another vessel for a dissolved oxygen measurement. We report here that it is now 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 BOD₅. HBOD₅ values measured for primary and secondary clarifier effluents from an activated sludge plant of 114 and 23 mg/L were essentially identical to BOD₅ measurements of 116 and 24 mg/L. Changes in headspace volumes did not significantly change the HBODs. Secondary settled wastewater had a HBOD₅ = 83 ± 3 mg/l (±SD) for headspace volumes of 10 to 20 ml (36% to 71%) in a 28-ml tube. The HBOD₅ was also constant (84 ± 6 mg/l) when samples were diluted 20 to 60% using BOD dilution water for a fixed headspace volume of 8 ml in HBOD tubes. Nitrification increased the HBOD of non-diluted 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 HBOD₅ values similar to those obtained in the analogous BOD₅ 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.

Keywords: bioreactor, BOD, HBOD, oxygen demand, wastewater treatment

INTRODUCTION

A substantial amount of time and expense is devoted to measuring biochemical oxygen demands (BOD₅) at wastewater treatment plants. When compared to modern analytical techniques the BOD method appears quite crude due to many wet handling steps and chemical techniques. Oxygen demands have been measured using a variety of respirometric techniques (O'Brien and Clark, 1962) based on either calculating oxygen utilization 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. In general, a BOD₅ will be exerted in a respirometric BOD (RBOD) test in about 2 to 3 d (Young and Baumann, 1976) providing an estimate of the BOD₅ in less than 5 days. The high per-sample cost and relatively sophisticated operation has limited the use of respirometers in wastewater treatment plants.

A few years ago a non-dilution 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 liquid DO concentrations 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 prior to DO measurement. In addition, the HBOD₅ of 300 mg/l solutions of glucose and glutamic acid averaged only 144±20 mg/l, substantially lower than typically measured in BOD tests of 204±10 (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 headspace of the sealed HBOD tube. This gas-based HBOD method has the same advantages as the original HBOD test since it is a non-dilution 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 an inexpensive (<$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 total oxygen in the tube, and the potential for easy automation if the GC is connected to an auto sampler 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 HBOD₅ values consistent with BOD₅ test data.

METHODOLOGY

The mass of oxygen consumed during HBOD tests was calculated based on the fraction of oxygen utilized in the tube headspace during the incubation period.

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

(1)

where the HBODₙ = headspace BOD on day n [mg/L]; P₀ = total pressure of laboratory air on day 0 recorded from barometer [mmHg]; p₀,ₜ = vapor pressure of water at temperature of sample on day 0 from table of water vapor pressures (mmHg); rᵢ = relative humidity of air on day 0 read from relative humidity gauge[%]; Aₙ = oxygen peak area of sample on day n [mV-sec]; A₀ₙ = oxygen peak area from the GC in air from the day 0-tube analyzed on day n [mV-sec]; Tᵢ = 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 T₀ from reference table [mg/L]; Vₙ = total volume of empty HBOD tube [mL]; Vₙ = volume of liquid wastewater sample put into HBOD tube [mL].

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, Arizona as described in Patnaik (1996). Samples were collected in 1-L Nalgene bottles (pre-rinsed several times with the wastewater sample to be collected), placed in ice in a ice-chest, and taken to the Environmental Engineering laboratories at the University of Arizona. For one experiment 24-h composite samples from the primary and secondary clarifier overflow at the Ina Road Wastewater Treatment Plant in Tucson, AZ were analyzed using both the BOD and HBOD tests. All BOD₅ measurements reported here were made by the respective plant technicians using standard procedures (Standard Methods, 1995).

All measurements of gas phase oxygen concentrations were carried out using 8610B GC (SRI Instruments) equipped with a thermal conductivity detector (TCD) and a 3-foot long 1/8" molecular sieve 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) and a 22-gauge side-port needle. PEAKSIMPLE-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-standard (10% oxygen in helium, Aldrich) and laboratory air based on triplicate injections.

HBOD tests were conducted in triplicate using 28-ml gas-tight anaerobic culture tubes (Bellco Glass Inc.) Headspace volumes were selected to keep the final 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
\[ \text{DO}_1 = \frac{A_t}{A_o} c_{\text{sat}} \]  

(2)

where \( c_{\text{sat}} \) 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. For 20-mL or more of wastewater in a 28-mL tube over 90% of the oxygen is in the gas phase assuming typical laboratory conditions and the ideal gas law \( r=50\%, T_c=20^\circ C, y_o=0.209, p_o=17.54 \text{ mmHg}, \) and \( P_r=700 \text{ mmHg}, c_{\text{sat}} =9.09 \text{ mg/L} \).

The range of HBODs that can be measured in a 28-mL tube is 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 38-236 mg/l.

Wastewater samples were added to the HBOD tubes using a 5-mL digital dispenseette. 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 while the relative humidity and temperature was measured using a hygrometer. HBOD tubes were mixed for 30 s using a vortexer, laid on their sides in a sealed box and incubated in the dark at room temperature \( (20^\circ C) \) on a shaker table. Headspace oxygen concentrations were measured at various time intervals \( (1-15 \text{ days}) \) depending upon the experiment. Before GC analysis samples were again 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.

Nitrification was inhibited using 2-chloro-6-(trichloromethyl) pyridine (TCMP; HACH Chemical Company, Ames, Iowa) at a final concentration of 50 mg/L. TCMP was either added directly to the wastewater sample in the digital dispenseette or, in experiments using BOD dilution water, the TCMP was added to the BOD dilution water. The nitrogenous HBOD (NHBO) was calculated from the total HBOD as

\[ \text{NHBO}_n = \text{HBOD}_n - \text{CHBO}_n \]  

(3)

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

Glucose and Glutamic acid (GGA) were added in some experiments as a 50:50 mixture to samples from a stock solution of 1000 mg/L. The HBOD due to GGA was calculated by conducting parallel experiments to determine the HBOD of the wastewater, HBOD(WW), using

\[ \text{HBOD}_n^{(GGA)} = \text{HBOD}_n^{(WW+GGA)} - \text{HBOD}_n^{(WW)} \]  

(4)

### Table 1. Range of measurable HBODs as a function of headspace and liquid volumes for a DO change of \( >1 \text{ mg/L} \) and a minimum final DO of \( >2 \text{ mg/L} \) \( (V_r=28 \text{ mL, } r=20\%, T_c=20^\circ C, y_o=0.209, p_o=17.54 \text{ mmHg}, \) and \( P_r=700 \text{ mmHg}, \) corresponding to a DO saturation concentration of 9.09 mg/L).

<table>
<thead>
<tr>
<th>Headspace volume ( V_r-V_i ) (mL)</th>
<th>Liquid Volume ( V_i ) (mL)</th>
<th>HBOD range (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>23</td>
<td>7 - 50</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>17 - 117</td>
</tr>
<tr>
<td>15</td>
<td>13</td>
<td>39 - 236</td>
</tr>
<tr>
<td>18</td>
<td>10</td>
<td>51 - 364</td>
</tr>
<tr>
<td>20</td>
<td>8</td>
<td>71 - 503</td>
</tr>
</tbody>
</table>
Figure 1. Daily HBODs and the BOD₅ of (A) primary clarifier and (B) secondary clarifier effluents from the Ina Road Wastewater Treatment Plant. Notice that the HBOD₃=BOD₅.

where the HBOD(WW) was also separated into CHBOD and NHBOD components using separate samples and eq. 3.

RESULTS

Experimental measurements indicated that the 3-day HBOD (HBOD₃) provides estimates of oxygen demand essentially identical to BOD₅ values. Wastewater samples (24-h composite) obtained from the primary and secondary clarifier overflow from the Ina Road Wastewater Treatment Plant were analyzed daily in our laboratory using the HBOD test, and compared to BOD₅ values obtained by plant personnel (Figure 1). The oxygen demands were exerted more rapidly in the HBOD sample tubes than the BODs bottles. The 3-day HBODs for the primary and secondary clarifier were 114 and 23 mg/L, values nearly identical to the BOD₅ 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. The more rapid exertion of oxygen demand observed here 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 BOD₅’s after 2 to 3 days for wastewater samples from different locations at three different treatment plants.

HBOD₃ measurements on secondary clarifier overflows from the Roger Road Trickling Filter Wastewater Treatment Plant were slightly higher, and HBOD₅ values considerably higher, than BOD₅ measurements (averaging 25±8 mg L⁻¹) due to nitrification in the samples (Figure 2). The HBOD₅ was 41 mg/L. However, the use of a nitrification inhibitor produced a lower carbonaceous HBOD₅ (CHBOD₅) value of 20±7 mg/L, which compared which was comparable to typical BOD₅ values at the plant.

Figure 2. Daily HBODs separated into the carbonaceous (CHBOD) and nitrogenous (NHBOD) fractions from secondary clarifier effluent from the Roger Road Wastewater Treatment Plant.
Changing the volume of headspace in a tube did not affect HBOD measurements as long as the final DO in the liquid was >2 mg L⁻¹, and the DO drop in the liquid was >1 mg L⁻¹. The effect of headspace volume was examined using samples from the secondary clarifier at the RRTP. The average HBOD₅ of the sample was 83±3 mg/L (±SD, n=8) based on samples meeting the requirements of final DO changes. (Figure 3). Injection-to-injection (within sample) variations of area counts varied by 0.6 to 2.7%. These results indicate that the choice of the headspace 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 HBOD values can be obtained from analyzing more tubes than by multiple analyses of the same tube.

Development of a Calibration Procedure for the HBOD Test. A standard check on BOD methodology in different laboratories is to measure the BOD₅ of a 300 mg/L 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 non-diluted wastewater, the oxygen demand of a 300 mg/L (final concentration) GGA solution was combined with non-diluted wastewater (secondary clarifier overflow) without using a nitrification inhibitor. The HBOD of the wastewater, consisting of the carbonaceous and nitrogenous HBODs, were measured separately using tubes containing wastewater and wastewater with a nitrification inhibitor. The oxygen demand of a GGA solution in the HBOD test is rapidly exerted, producing three and five-day HBODs of HBOD₅= 211 mg/L and HBOD₅=228 mg/L (Figure 4). This 3-day HBOD compares favorably to the BOD₅=204±10 mg/L reported in Standard Methods (1995) for an extended series of laboratory tests, and the BOD₅=198±31 mg/L results for a multi-laboratory test of the BOD₅ of a 300 mg/L GGA solution. These results indicate that the use of a 300 mg/L concentration of a glucose and glutamic acid solution will provide a stable check on the HBOD protocol.

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. We compared the HBOD exerted over an 8 day period using a 300 mg/L GGA solution and
secondary clarifier wastewater with the HBOD produced using seeded dilution water (5 ml secondary clarifier wastewater per liter of BOD dilution water) as shown in Figure 5. The dilution water HBOD$_5$ was only 130 mg/L, while the HBOD$_5$ value for the full-strength wastewater was 228 mg/L (both values corrected for their respective seed concentrations). Thus, insufficient cell concentrations produce can lower HBODs.

**DISCUSSION**

The HBOD test results, based on measuring oxygen concentrations in the headspace of sealed tubes using a gas chromatograph, demonstrate that a GC-based HBOD test can provide rapid and reliable estimates of oxygen demands of non-diluted wastewaters. Experiments conducted using wastewaters from different wastewater treatment plants and on GGA solutions suggest that the 3-day HBOD provides a reliable estimate of the 5-day BOD. For example, wastewater HBOD$_2$ values measured for primary and secondary clarifier effluents from an activated sludge plant of 114 and 23 mg/L 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 to the BOD test, is that the HBOD test uses non-diluted 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 utilization 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 sample 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 Wagenseiler (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 essentially a dry technique. Air from the sample is withdrawn and injected into the GC without the need to pour the 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 sample with a DO probe, multiple gas injections can be used to verify the oxygen concentrations in the gas sample.

Liquid-based HBOD test results can also be erratic when samples sit still for too long prior to 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 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. Mixing the sample prior to liquid analysis is recommended to restore equilibrium conditions prior to 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 under-saturated with DO. In contrast, there is relatively little change in the final HBOD if the samples sit idle prior to analysis using gas-phase measurements. Since most of the oxygen resides in the headspace (see Figure 1), the loss of a few mg/L 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 HBOD₅ using dilution water seeded with a variety of different wastewater sources. When seeded dilution water was used here (5 ml per 1000 ml), we similarly observed a low HBOD₅=130 mg/L. However, when non-diluted secondary clarifier wastewater was used instead of BOD dilution water the HBOD₅ was 228 mg/L. This indicates that low HBOD₅ 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 to obtain higher oxygen demands. In the BOD test, for example, the 300 mg/L of GGA is diluted to approximately 5 mg/L to obtain a corresponding DO change of 3.5 mg/L (assuming a 70% exertion of BOD over 5 days). In the HBOD test it is necessary to use the more concentrated suspension of microorganisms in non-diluted 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 to the high concentration of 300 mg/L of GGA. Therefore the HBOD calibration test should be conducted by adding GGA directly to non-diluted 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, we estimate the HBOD test could reduce technician time by as much as one half compared to BOD tests. The capital costs of the HBOD test are much more easily evaluated than the labor costs. Let us assume that a typical laboratory has available a personal computer, shaker table, test tube racks and pipettors. It would cost $6000 to $8000 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 $1500 to $2000. Most of the investment costs ($4500 to $6000) 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 (three days versus five days). For some wastewater treatment plants this would allow a more rapid detection of changes in plant or discharge BOD₅ 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 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 to $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.

Finally, the authors have 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 have found it easier to work with the small sealed HBOD tubes and an easily calibrated GC than the larger BOD bottles and troublesome DO probes. We suspect that these preferences will be shared by laboratory technicians at wastewater treatment plants as well.

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