Washington State Department of Ecology

Environmental Assessments Program

Lab Accreditation Section

Supplemental Guidance for the Determination of

BIOCHEMICAL OXYGEN DEMAND (BOD_5)

and

CARBONACEOUS BOD ($CBOD_5$)

in

Water and Wastewater

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Table of Contents

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1 Background

a. The purpose of the biochemical oxygen demand (BOD_5) test is to measure the potential of wastewater and other waters to deplete the oxygen level of receiving waters. Wastewater treatment plant operators also use the BOD_5 test to determine the efficiency of their plant by measuring BOD_5 of the influent, and of the effluent, and calculating the percent removal. The BOD_5 test may also be used to test waters other than wastewaters to determine their oxygen-depleting potential.

b. Oxygen is required (used up, depleted, consumed, assimilated) by microorganisms as they assimilate various organic and inorganic materials in water. Some of those materials contain reduced forms of nitrogen. Collectively these materials are called nitrogenous materials, and they are consumed by nitrifying bacteria. If those nitrifying bacteria are inhibited, usually using a pyridine compound, the test measures only carbonaceous organic material, and is referred to as carbonaceous BOD_5, or $CBOD_5$.[11] Carbonaceous material is that consumable material which does not contain nitrogen.

b. While the term "BOD" technically refers to the oxygen uptake demand of contaminated water, the term is **commonly** used to refer to the organic and inorganic materials consumed by bacteria, as if "BOD" is a contaminant in the water. For example, wastewater treatment plants report "percent removal of BOD" as an indication of how well the plant is performing. This document occasionally uses BOD and CBOD in such a manner.

c. Many biological treatment plant effluents contain enough nitrifying bacteria that their consumption of nitrogenous material (material containing nitrogen) is significant. Because nitrogenous demand has historically been considered an interference (i.e., the primary purpose of the BOD_5 test is to measure carbonaceous material ... nitrogenous material can be measured by other tests such as ammonia, nitrate-nitrite, and total Kjeldahl nitrogen), the $CBOD_5$ test is preferred for samples of secondary effluent. For regulatory programs, however, the bottom line is that the test specified by the regulator must be run. In some cases, the regulator may require both BOD_5 and $CBOD_5$ data

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[1] Reduced forms of other inorganic materials such as sulfides and ferrous iron are also oxidized during both BOD_5 and $CBOD_5$ tests and would create a positive bias if present in the sample or dilution water. Other inorganic materials, such as hexavalent chromium, copper, and free chlorine may create a toxic environment for the bacteria and cause a negative bias. If these types of interferrents cannot be eliminated prior to running the BOD_5 or $CBOD_5$ test, their presence can be mentioned in test reports.

d. It would take the bacteria in a wastewater sample 20-plus days to assimilate all the consumable material in a water sample. Because it is impractical to spend 20 days analyzing a BOD sample, a 5-day test has been established as the standard (hence, BOD_5). Depending on the specific waste stream, 60-70% of the available material is consumed in a 5-day period. Another test that measures the oxygen requirements of water is chemical oxygen demand (COD) which, in a relatively short time, measures essentially 100% of the chemically oxidizable material. One can expect BOD_5 and COD results for the same sample to be related, the BOD_5 value being perhaps 60-70% of the COD value depending on the nature of the sample. Also, because BOD_5 is a measure of nitrogenous **and** carbonaceous material, and $CBOD_5$ is a measure of only the carbonaceous material, one would also expect a correlation for BOD_5 and $CBOD_5$ for a given sampling site. Indeed, Table 1 shows there is a very consistent ratio of BOD_5 to COD, and BOD_5 to $CBOD_5$ for a given type of sample (in this case, performance evaluation, or PE samples from EPA's Water Pollution, or WP Studies). One should be able to determine BOD_5/COD_5 and $BOD_5/CBOD_5$ ratios for a given waste stream, although the ratios may not be as constant, or of the same magnitude as those shown in Table 1. Table 1 also shows a consistent ratio between BOD_5 and total organic carbon (TOC) for a given sampling site. If a relatively consistent BOD_5/COD ratio can be established for a given sampling point, the COD test can be used as a screening process for estimating the BOD_5 of the same sample. When used for this purpose, the Hach Company's recently developed (but not yet EPA-approved) manganese-III COD method can be used rather than an approved method, all of which generate considerable contaminated waste.

Table 1 - Comparison of BOD_5, $CBOD_5$, COD, and TOC Values for WP Studies*

Study	BOD_5 mg/L	$CBOD_5$ mg/L	$BOD_5/CBOD_5$ Ratio	COD mg/L	BOD_5/COD Ratio	TOC mg/L	BOD_5/TOC Ratio
WP040	119	100	1.19	19.2	0.62	76	1.57
WP039	37.6	31.9	1.18	60.7	0.62	24	1.57
WP038	50.3	43	1.17	81	0.62	32	1.57
WP037	93.1	80	1.16	152	0.61	60	1.55
WP036	13	11.3	1.15	20.8	0.63	8.2	1.59
WP035-1	141	117	1.21	235	0.60	93.1	1.51
WP035-2	62.5	51.6	1.21	101	0.62	40	1.56
WP034-1	30.2	26.2	1.15	48.1	0.63	19	1.59
WP034-2	9.99	8.7	1.15	15.9	0.63	6.3	1.59
WP033-1	12.1	10.7	1.13	19.5	0.62	7.7	1.57
WP033-2	54.7	46.7	1.17	88.6	0.62	35	1.56
WP032-1	70.9	64.7	1.10	111	0.64	44	1.61
WP032-2	15.2	13.7	1.11	24.3	0.63	9.6	1.58
WP031-1	44.9	38.5	1.17	70.8	0.63	28.1	1.60
WP031-2	131	112	1.17	207	0.63	82	1.60
WP030-1	14	12	1.17	21.8	0.64	8.61	1.63
WP030-2	21.8	19.5	1.12	35.4	0.62	14	1.56
Average			1.16		0.62		1.58

* Demands samples in EPA's discontinued Water Pollution Studies were a 50:50 mixture of glucose/glutamic acid.

e. The BOD_5 test is widely used to determine the degree to which a waste stream will contribute to pollution of receiving waters by depriving organisms in those waters (e.g., fish) of their source of oxygen. The BOD_5 test is of prime importance in regulatory programs and in determining the overall health of receiving waters. On the other hand, of all the tests done by and for wastewater dischargers, BOD_5 is arguably the most likely to result in invalid data if adequate precautions are not taken to assure quality of test results. The purpose of this document is to provide supplemental guidance to assist labs in following quality control procedures in published analytical methods. If used properly, those methods and procedures will provide evidence that the measurement of BOD_5 is in control, and that the results meet established data quality objectives.

f. Several technical terms used in this document may not be familiar to some readers. When in doubt about the meaning of a technical term for the purposes of this document, check Appendix I of Ecology's *Procedural Manual for the Environmental Laboratory Accreditation Program (Glossary of QA/QC Terms),* Reference F to this document.

2. Approved Methods

a. The BOD_5 test is normally required by a regulatory program that is governed either directly or indirectly by Chapter 40, Code of Federal Regulations, Part 136 (40 CFR 136) which concerns the National Pollutant Discharge Elimination System (NPDES). 40 CFR 136 allows the use of four analytical methods for reporting BOD_5 and/or $CBOD_5$: EPA Method 405.1; *Standard Methods* 5210B; USGS Method I-1578; and AOAC Method 973.44. EPA Method 405.1 refers the user to *Standard Methods* 5210B for specifics on conducting the test. It is *Standard Methods* 5210B or its derivative that is most commonly used in environmental laboratories. It must be emphasized that **this** guidance document is **not** an analytical method, and that use of this guidance document or any part of it is not required by Ecology. This supplement was written with the intent that implementation of its suggestions would meet requirements for NPDES and other discharge monitoring programs. It is not intended to be used as a stand-alone procedure, but rather as a supplement to one of the EPA-approved BOD_5 methods. If a simplified BOD_5 method is needed, the reader is encouraged to review References 11c and 11d on page 28 of this document. Both satisfy the requirements of 40 CFR 136 since they follow *Standard Methods* 5210B.

b. All BOD_5 methods approved by EPA are based on determination of oxygen depleted by bacteria in samples during a 5-day[2] period, in the dark, at 20 ± 1° C as those bacteria consume organic materials in water samples. BOD_5 samples are normally incubated in 300-mL BOD bottles, and are diluted if necessary to allow at least 1.0 mg/L of dissolved oxygen to remain at the end of the 5-day incubation. To be a valid test, at least 2.0 mg/L of oxygen must be depleted during the incubation, and at least 1.0 mg/L must be retained at the end of the incubation period. If necessary, samples must be pretreated to assure proper pH, temperature, and absence of toxic materials (e.g., chlorine) thus creating a suitable environment for survival of the BOD-consuming bacteria. The foregoing are requirements of all EPA-approved BOD_5 methods.

[2] The incubation period is generally considered to be 5 days ± **2 hours**, but the method does not specify the tolerance

3. **Sampling, Sample Preservation, Holding Times**

a. **Sampling Location.** Discharge permits or other regulatory documents usually specify sampling locations. Care must be taken to make sure the sample is representative of the water body from which it was taken. Whether taking a grab or composited sample, it is very important to thoroughly mix the sample before withdrawing an aliquot (sub-sample) for analysis. Failure to do this for a wastewater treatment plant influent, for example, can easily double BOD (and total suspended solids) values for that sample.

b. **Sample Size.** Samples should be taken in a clean plastic or glass container of sufficient size to provide enough sample for the number of BOD bottles that will be incubated for that sampling site. If the sampling site normally results in a low and predictable BOD value (e.g., many secondary effluents consistently have BOD_5's less than 10 mg/L), it may be necessary to incubate only one bottle, but it would be primarily sample (i.e., little if any dilution water).[3] Some analysts prefer to always use at least two dilutions regardless of how predictable the BOD_5 value is. The required sample could therefore be as small as 300 milliliters (if only one 300-mL bottle is to be incubated). If the sampling site normally results in high and varied BOD_5 values (e.g., raw influent) it may be necessary to incubate as many as three or four bottles, but each would contain only a fraction of sample, with dilution water added to fill the BOD bottle. Again, the total required sample might be as small as 300 mL. Another consideration in determining sample size, however, is that the sample must be representative of the waste stream, and the smaller the sample, the more difficult it is to make sure that it is representative. Also, if samples must be dechlorinated (see paragraph 6c below), or if lab standard operating procedures (SOPs) require analyzing duplicate samples, or analyzing all samples at more than one dilution, extra sample must be taken.

Table 2 - Common BOD_5 Ranges for Various Waste Samples[4]

Sample	Common BOD Range (mg/L)
Influent, domestic waste	150 - 400
Primary effluent	60 - 160
Secondary Effluent	5 - 60
Digester supernatant	1000 - 4000+
Industrial waste	100 - 3000+

[3] The question often comes up, "What is the maximum volume of sample can be used in a 300-mL bottle." The 20th Edition of *Standard Methods* finally clears this up by stating can add almost 300 mL, but leaving room for seed, and 0.33 mL of phosphate buffer, and 0.33 mL each of MgSO4, CaCl2, and FeCl3 solution, or, if the buffer and nutrients are already mixed, 1.3 mL of the mixture. Any remaining volume is taken up by adding **source** water, not dilution water. The purpose for doing this is to make sure the bottle is properly buffered, and has sufficient seed and nutrients to support the support the 5-day incubation.

[4] These BOD ranges are from *Operation of Wastewater Treatment Plants*, Vol II, 3rd Edition, EPA, 1991. Actual BODs may be outside these ranges (e.g., some industrial dischargers of BOD_5 values of <2.0 mg/L).

All things considered, **one liter** of sample is a good planning size for most sampling sites. Experience may show that some other size is better. For example, Ecology's Manchester Lab and some commercial labs require larger samples (one gallon for Manchester Lab).

c. **Preservation and Holding Times**

(1) In most environmental samples, bacteria naturally present in a waste stream are consuming BOD, and therefore depleting oxygen levels, before, during, and after the sample is taken. It is therefore very important to begin the analysis as soon as possible after the sample is taken. If storage of samples before analysis cannot be avoided, they must be kept at 4°C or lower (but not freezing) to slow the oxygen-depleting activity of the bacteria. 40 CFR 136 establishes the maximum storage (holding) time as 48 hours. *Standard Methods* 5210B recommends that the holding time not exceed six hours, and if analysis cannot start within 6 hours, the sample may be held at 4°C for up to 24 hours. *Standard Methods* also says that if analysis can start on a **grab** sample within two hours of sampling, preservation at 4°C is not necessary. **Composite** samples must be kept at 4°C during the entire compositing period, which should not exceed 24 hours. After removal from the compositor, samples should be stored at 4°C if analysis cannot start within two hours, and just like grab samples, *Standard Methods* says they should not be held longer than six hours after retrieval from the compositor. Discharge permits specify whether samples should be grab or composite.

(2) Some labs operated by permitted wastewater dischargers have difficulty meeting even the 48-hour holding time requirement, especially when required to do BOD_5s all five working days of the week or more. Relief from the requirement is allowed in 40 CFR 136 (Note 4, Table II) which states that "samples may be held for longer periods only if the permittee, or monitoring laboratory, has data on file to show that the specific types of samples under study are stable for the longer time, and has received a variance." That variance would come from the data user. Most often, for the users of this document, the data user would be the Department of Ecology. It is unlikely that Ecology would allow a variance exceeding 24 hours (72 hours total). That period should allow sufficient flexibility for the situation at any sampling site. The study supporting a request for variance should include analysis of several BOD_5 samples run within the regulatory 48-hour period compared to splits of the same samples stored at 4°C and analyzed just after 72 hours. Ecology's Lab Accreditation Section (360 895-6149) can assist in determining if there is a statistical difference between the two sets of data.

4. **Apparatus**

a. **Measurement of Dissolved Oxygen (DO).** 40 CFR 136 allows measurement of DO using either an electrode and meter, or the azide modification of a Winkler titration. Applicable methods are EPA Method 360.1 or *Standard Methods* 4500-O G (electrode), or 360.2/4500-O C (Winkler).

(1) **Electrode**. An oxygen-sensitive membrane electrode, polarographic or galvanic, with appropriate meter meets EPA-approved method requirements. The YSI 50-series, and Orion 800-series instruments are examples of commercial meters meeting those requirements

for measuring DO. The method calls for calibrating the meter using oxygen-saturated water, water-saturated air, or a Winkler titration before each use. If DO is routinely measured using an electrode and meter, checking calibration of the meter periodically with a Winkler titration will help make sure the meter is functioning properly. Standard Methods 4500-O G is a commonly-used electrode method for measuring DO.

(2) **Winkler.** Burettes capable of measuring accurately to 0.1 mL are sufficient for the Winkler titration. Standard Methods 4500-O D is a commonly-used method for measurement of DO using a modified Winkler titration.

b. **Thermometers.** Temperature in the BOD incubator is measured to ± 1°C accuracy using a thermometer that is either NIST-certified (National Institute of Standards and Technology, formerly NBS), or has a calibration certificate or other documented evidence showing it is traceable to a NIST-certified thermometer. Ecology's Lab Accreditation Section can assist Ecology-accredited labs having a good-quality thermometer by calibrating it against a NIST-certified thermometer (call the Lab Accreditation Section at 360 895-6149). If using an air incubator, the thermometer should be immersed to the immersion line in a suitable container (e.g., beaker, Ehrlenmeyer flask) filled with water or glycol solution which acts as a heat sink. If using a water bath, simply immerse the thermometer to the immersion line. If the lab has an expensive NIST-certified thermometer, to minimize risk of breakage it should not be used in the BOD incubator. Rather, it should be used to calibrate a less expensive thermometer for use in the incubator.

c. **Incubator or Water Bath.** Either an air incubator or water bath may be used to incubate BOD bottles. The apparatus must be able to maintain a temperature of 20 ± 1°C for the entire 5-day incubation period. It must be of sufficient size to hold all BOD bottles for a given batch (i.e., environmental samples, blanks, seed controls, glucose/glutamic acid standards). For reasons explained later, it is also advantageous for an air incubator to be large enough to hold the dilution water container used for the BOD_5 determination. The incubator/water bath must exclude all light to prevent photosynthesis (which would result in a positive contribution to dissolved oxygen in the incubation bottles, resulting in a negative bias in the BOD test). Incubators specifically designed for BOD_5 use can be purchased from scientific supply vendors (such as Hach Company's Incutrol®). Less expensive are household refrigerators which have been modified to meet BOD_5 test needs. One required modification is installation of a small fan to create an airflow and ensure an even temperature throughout the refrigerator. The thermometer used to monitor temperature in the incubator should be placed in the vicinity of the majority of the BOD bottles.

d. **Dilution water container.** A glass or plastic container of laboratory grade (e.g., not an old bleach bottle), large enough to supply all dilution water to be used in a given batch of samples should be used as the dilution water container. Although not required, a convenient way to introduce dilution water into BOD bottles without creating air bubbles is to siphon the water from the dilution water container. If this technique is used, the siphon hose should terminate with a 6-inch section of glass tube such that a BOD bottle can be filled from the bottom without submerging the hose. The dilution water container and all associated equipment must be kept clean (washing with detergent and rinsing with distilled water should be sufficient . . . see next

subparagraph). If using a siphon tube, the first few milliliters of dilution water should be discarded down the drain.

e. **Incubation (BOD) Bottles.** Bottles used for BOD_5 tests should never be used for any test other than BOD_5. They can be either 75-, 250-, or 300-mL (300-mL is the most widely used). *Standard Methods* does not require the bottles to be made of any specific material. As this manual is being written (spring 2003), glass is the most common material, but disposable, plastic bottles are beginning to show up in the market. Just like the dilution water container, BOD bottles must be kept clean. Wash after each use with detergent, rinse with distilled water, drain, and store such that the bottles are not exposed to dust or other contaminants in the lab. If blanks unexpectedly become higher than normal, it may mean the BOD bottles need acid washing. This can be done by first washing, rinsing, and draining as above, and then rinsing with 1:1 mineral acid (purchased ready-made or prepared by slowly adding concentrated hydrochloric or nitric acid to an equal volume of distilled water). Rinsing is accomplished by carefully swirling 10 to 20 mL of the dilute acid until all inner surfaces of the bottle are wetted. Allow the bottle to sit for a few minutes before properly discarding the acid. Then rinse the bottle with distilled water and drain. Acid rinsing is not necessary every time the BOD bottles are used and might never be required as long as blanks are less than 0.1 mg/L. BOD bottles used for blanks should be chosen randomly to avoid checking only the "cleanest" bottle.

5. **Reagents**

a. **Buffer.** To provide the optimum environment for survival of bacteria in the incubated sample, it is necessary to buffer the sample such that it maintains a pH of 6.5 to 7.5. The buffer can be prepared with various phosphate compounds as described in Appendix A, or it can be purchased ready-made (see Appendix B). If stored, the buffer solution should be refrigerated at 4° C to preclude biological growth. It must be discarded if such growth appears because the growth has an oxygen demand which would introduce a positive bias into all BOD_5 measurements. The pH of the buffer solution should be 7.2.

b. **Nutrients.** In addition to the nutrient value of the phosphate buffer, nutrients in the form of ammonium chloride, and trace metals in the form of ferric chloride, magnesium sulfate, and calcium chloride are added to the dilution water. These solutions can be purchased ready made (all combined in one packet), or they can be prepared individually as described in Appendix A. Hach Company, North Central Labs, and perhaps others sell packets (pillows) containing both buffers and nutrients (see Appendix B).

c. **Standards.** To provide a check on efficiency of the seed and effectiveness of dilution water, *Standard Methods* 5210B implies that a standard solution should be analyzed with each batch of BOD_5 samples. The most common standard is a solution containing 150 mg/L glucose (dextrose) and 150 mg/L of glutamic acid, commonly referred to as the "G/GA" solution. This solution can be prepared as described in Appendix A, or purchased as a solution from a commercial

vendor such as Hach Company[5] or North Central Labs. In an interlaboratory study, several labs reported results for the G/GA test that averaged 198 mg/L BOD_5, with a interlaboratory standard deviation of 30.5 mg/L (see Appendix E for a discussion of statistical terms). As discussed in *Standard Methods*, a single lab should expect to achieve an average over several tests of the G/GA standard in the vicinity of 198 mg/L (results from at least 20 tests, spread out over as many batches, provide statistically significant data). If a lab's average is considerably less than 198, a stronger seed should be tried...*vice versa* if the average is considerably more than 198. A single lab should be able to achieve a standard deviation much lower than 30.5 mg/L. A typical single lab standard deviation would be in the mid- to low-teens. A standard deviation of less than 10 mg/L would indicate extraordinary precision. A standard deviation approaching or exceeding 30.5 mg/L indicates excessive random error caused by differences in the procedure from test to test. The 20th Edition of *Standard Methods* says the 30.5 mg/L should be considered to be an action limit, or three standard deviations. This would mean they expect the standard deviation for a single lab to be 10 mg/L or less. Ecology considers this goal to be overly optimistic (although achievable) and suggests that a lab should strive to get a standard deviation in the mid-teens or lower.

6. Pretreatment of Samples

a. **Sample Preparation.** After removing any items that are obviously not representative of the sampled water (e.g., sticks, other non-representative solids), the sample can be homogenized if necessary to make sure that a representative aliquot is used for the BOD_5 test. This can be done with a food blender on a slow speed, an aggressive stirring bar, or other device that provides thorough mixing without being overly disruptive to microorganisms in the sample as might occur if, for example, a high-speed blender is used. As previously mentioned, it is very important to make sure the sample is thoroughly mixed before withdrawing aliquots (sub-samples) for testing. If BOD_5 is expected to be so high that a very small aliquot must be taken (see Table 3, next page), the entire sample can be diluted such that a larger, more readily measured, aliquot may be taken.[6] If a pipette is used to measure the sample aliquot, use of a wide-tip variety is beneficial (i.e., inside diameter of tip 1/16" to 1/8").

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[5] Hach's standard, prepared according to their instructions, has a concentration of 300 mg/L each of glucose and glutamic acid, twice the concentration of the standard referred to in *Standard Methods*. Hach implies that repeated analyses of this solution should result in a mean value near 396 mg/L, with a standard deviation of 61.0 mg/L or less (i.e., twice the 198 mg/L mean and 30.5 mg/L standard deviation reported in *Standard Methods*). Without supporting analytical data, these statistics should be used with care since accuracy is concentration dependent. When using Hach Company's standard, it is best to dilute the sample to half the strength specified in the instructions (e.g., by withdrawing half the volume from the Hach vial, or by diluting it to twice the volume specified by Hach). This will result in a solution that is 150 mg/L of each ingredient as suggested in *Standard Methods*.

[6] For example, if BOD_5 is expected to be 1200 mg/L or higher, as shown in Table 3 (next page) the aliquot taken would be 0.5 mL, a difficult volume to measure accurately when working with samples containing solids. If the sample is diluted to one-tenth its original concentration (e.g., 10 mL of sample diluted to 100 mL) the aliquot taken would be 5.0 mL, a volume which can be measured with sufficient accuracy. Care must be taken to thoroughly mix such dilutions.

Table 3 - BOD_5 Dilutions

Sample (mL) added to 300-mL BOD Bottle	Expected BOD_5 Range (mg/L) Minimum	Maximum	Dilution Factor
0.5	1200	3400	600
1	630	1800	300
3	210	560	100
6	105	280	50
9	70	187	33.3
12	53	140	25
15	42	112	20
18	35	94	16.7
24	26	70	12.5
30	21	56	10
45	14	37	6.67
60	11	28	5
75	8	22	4
150	4	12	2
300	2	6	1

b. **Temperature.** Samples should be at 20 ± 1°C before initial DO is read. This can be done by placing sample containers in cold water in a sink if they are too warm, or in warm water if they are too cold. If only a small volume of sample is going to be diluted, it is not necessary to cool or warm the sample because, when diluted, it will not significantly affect the temperature of the dilution water which should already be 20 ± 1°C if source water is stored in the incubator.

c. **Adjustment of pH.** Sample pH must be in the range of 6.5 to 7.5 pH units. Add sulfuric acid (H_2SO_4) or sodium hydroxide (NaOH) of sufficient concentration that the quantity of acid or base added does not dilute the sample by more than 0.5%. For example, if the sample is one liter (1,000 mL), the acid or base should be strong enough that no more than 5 mL would be added to the sample to bring it into the range of 6.5 - 7.5 pH units. **(CAUTION! Use extreme care when using strong acids and bases!)**

d. **Dechlorination.** If allowed by the discharge permit and if possible given the design of the treatment plant, BOD_5 samples taken at wastewater treatment plants using chlorine to disinfect the final effluent should be taken ahead of the chlorination point. If this is not possible, dechlorination is required, and following dechlorination, samples must be seeded (because the chlorination process kills the bacteria that otherwise would consume the BOD in the waste sample).

(1) If the sample is not highly chlorinated, dechlorination may occur naturally if samples are allowed to sit in the light for one to two hours. Samples taken from waste streams where the final effluent is dechlorinated usually do not need further dechlorination in the lab.

(2) If necessary, residual chlorine is destroyed by adding sodium sulfite (Na_2SO_3) solution prepared according to the instructions in Appendix A. Determining how much sodium sulfite is required to dechlorinate a given amount of sample requires acidification of the sample, addition of potassium iodide, and titration with standard sodium sulfite. Since this entire procedure cannot be done on the samples that are later incubated, it must be done on a sample dedicated to that purpose. This can be done as follows:

> To a measured amount of sample neutralized to pH 6.5 - 7.5, add 10 mL of 1:1 acetic acid or 1:50 sulfuric acid, and 10 mL of 10% potassium iodide (KI) solution (10 g KI/100 mL as indicated in Appendix A) per 1,000 mL of sample. For example, if 500 mL of sample is to be dechlorinated, use 5.0 mL of acid, and 5.0 mL of KI solution. Add 0.5 mL of starch indicator solution and titrate with standard sodium sulfite to the starch-iodine end point for residual chlorine. To the neutralized sample to be incubated later, add an equivalent amount[76] of sodium sulfite as determined by the above test, wait for 20 minutes, and check for chlorine using, for example, a DPD colorimetric technique. Care must be taken not to add excess sodium sulfite. Having its own oxygen demand, excess sodium sulfite would result in a bias on the high side of the actual BOD concentration (i.e., a positive bias).

e. **Other Toxic Substances.** Some wastes, particularly industrial wastes, contain metals which are toxic to the organisms responsible for oxygen depletion during the BOD_5 incubation. Such toxic materials would result in a negative bias (i.e., lower than the actual BOD_5 concentration). The presence of toxic substances can be confirmed by testing a set of serial dilutions. If the measured BOD_5 for a given sample increases significantly as the sample is increasingly diluted, a toxic substance in the sample (i.e., matrix interference) is the most likely cause. If that toxic substance cannot be avoided, its presence should be reported with results submitted by the lab. Commercial labs may know nothing about possible toxicity of samples received from most clients. In such cases, the lab might consider doing a set of serial dilutions. Although it will be too late to do anything about toxicity at the end of the five-day incubation period, the presence of a matrix interference can at least be reported to the lab client. If a series of dilutions leaves no doubt that interference is occurring, the **highest** BOD value in the series....usually the value for the most dilute sample....should be reported.

f. **Supersaturated Samples.** If initial dissolved oxygen (DO) readings with a properly calibrated DO meter (or as measured with a Winkler titration) indicate the sample contains more DO than it should for the barometric pressure and sample temperature at the time (see Table 4 on the next page), the sample is **super**saturated with DO. Supersaturation might result when the sample has been vigorously agitated just prior to the DO reading without giving air bubbles in the sample a chance to escape, or when the sample is undergoing photosynthesis. Supersaturation at

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[7] For example, if a 100 mL sample is used for determining how much sodium sulfite is required, and it required 2.30 mL of standard KI to neutralize the residual chlorine, a 1,000 mL sample from which aliquots are to be taken for incubation would be dechlorinated with 23.0 mL of the standard KI (i.e., 1000/100 * 2.30 = 23.0).

Table 4 - Solubility of Oxygen in Water Exposed to Water-Saturated Air

(in mg/L at various atmospheric pressures and temperatures)

Pressure (mm/inches Hg)	Temperature (°C/°F)							
	17.0° C 62.6° F	18.0° C 64.4° F	19.0° C 66.2° F	**20.0° C 68.0° F**	21.0° C 69.8° F	22.0° C 71.6° F	23.0° C 73.4° F	24.0° C 75.2° F
710/28.0	9.03	8.84	8.67	**8.49**	8.33	8.17	8.01	7.86
715/28.1	**9.09**	**8.91**	**8.73**	**8.55**	**8.39**	**8.23**	**8.07**	**7.92**
720/28.3	9.16	8.97	8.79	**8.61**	8.45	8.28	8.13	7.97
725/28.5	**9.22**	**9.03**	**8.85**	**8.67**	**8.50**	**8.34**	**8.18**	**8.03**
730/28.7	9.28	9.09	8.91	8.73	8.56	8.40	8.24	8.09
735/28.9	**9.35**	**9.16**	**8.97**	**8.79**	**8.62**	**8.46**	**8.30**	**8.14**
740/29.1	9.41	9.22	9.03	**8.85**	8.68	8.51	8.35	8.20
745/29.3	**9.47**	**9.28**	**9.09**	**8.91**	**8.74**	**8.57**	**8.41**	**8.25**
750/29.5	9.54	9.34	9.15	**8.97**	8.80	8.63	8.47	8.31
755/29.7	**9.60**	**9.41**	**9.21**	**9.03**	**8.86**	**8.69**	**8.52**	**8.36**
760/29.9	9.67	9.47	9.28	9.09	8.92	8.74	8.58	8.42
765/30.1	**9.73**	**9.53**	**9.34**	**9.15**	**8.97**	**8.80**	**8.63**	**8.47**
770/30.3	9.79	9.59	9.40	**9.21**	9.03	8.86	8.69	8.53
775/30.5	**9.86**	**9.66**	**9.46**	**9.27**	**9.09**	**8.92**	**8.75**	**8.58**
780/30.7	9.92	9.72	9.52	**9.33**	9.15	8.97	8.80	8.64
785/30.9	**9.98**	**9.78**	**9.58**	**9.39**	**9.21**	**9.03**	**8.86**	**8.69**
790/31.1	10.05	9.84	9.64	**9.45**	9.27	9.09	8.92	8.75
795/31.3	**10.11**	**9.90**	**9.70**	**9.51**	**9.33**	**9.15**	**8.97**	**8.81**

the time of initial DO reading would result in a positive bias. Supersaturation can be avoided by taking the initial DO reading only on samples that are very close to 20°C, and by vigorously agitating the sample and then allowing it to settle for at least 30 minutes before taking the DO reading. A problem with supersaturation is usually indicated by high blank results (e.g., blanks exceeding DO depletion of 0.2 mg/L might be due to supersaturation) It is also possible to have supersaturated samples without the dilution water being supersaturated, in which case the supersaturation would not be revealed by blanks exceeding a depletion of 0.2 mg/L.

g. **Nitrification Inhibition.** Most waste streams contain bacteria that consume nitrogen-containing organic and inorganic materials. Glutamic acid in the G/GA standard is an example of a nitrogen-containing organic material. Ammonia is an example of an inorganic material consumed by nitrifying bacteria (i.e., bacteria that convert nitrogen-containing materials to nitrite

and nitrate) The materials they consume are called nitrogenous materials, or nitrogenous BOD_5. Carbonaceous BOD_5 ($CBOD_5$) is that part of the total BOD_5 that does not include nitrification. If $CBOD_5$ is to be determined rather than BOD_5, it is necessary to inhibit the nitrifying bacteria present in the sample and/or seed. This is done by adding 2-chloro-6-(trichloro methyl) pyridine (TCMP). If the lab is using the bottle method, 3 milligrams of TCMP should be added to each bottle. If the lab is using the graduated cylinder method, sufficient TCMP should be added to make the final concentration in the dilution water 10 milligrams TCMP per liter of dilution water. Pure TCMP dissolves very slowly in water. It may be advantageous to use commercially prepared reagent (see Appendix B).

7. Procedure

a. Preparation of Dilution Water

(1) **Source Water**. Water used for preparing reagent solutions and dilution water, hereafter referred to as source water, must be of the highest quality. It must contain less than 0.1 mg/L copper or other heavy metals, and must be completely free of chlorine, chloramines, organic material, acids, and bases. Distilled water prepared using a glass still in the lab is sometimes suitable, although water from some stills might contain trace metals, chlorine, ammonia, or volatile organic materials making it unsuitable. Distilled water coming from a copper still is seldom suitable. Deionized (DI) water sometimes contains organic materials leached from the resin bed. Some purchased distilled waters are suitable, and some are not. If water is purchased, many labs have found "steam distilled"[8] water to provide the best results. Distilled **drinking** water is not suitable as it generally contains chemicals (e.g., chlorine) that would adversely affect BOD_5 results. If unseeded BOD_5 blanks **always** run high (i.e., they always exceed 0.2 mg/L), the distilled water used to prepare dilution water should be one of the first things to investigate in trying to find the cause. Some labs have had success using tap water, others spring water . . . the bottom line is, if it works (i.e., if there is no evidence of toxicity, if the average result for glucose/glutamic acid analyses is not substantially less than 198 mg/L, and results for blanks are consistently below 0.2 mg/L), use it. If it does not, try another water.

(2) **CBOD and BOD Dilution Water**. Dilution water should be prepared by different procedures depending on whether it is to be used for $CBOD_5$ or for BOD_5.

(a) **Dilution Water for CBOD.** Per liter of source water, add 1.0 mL each of the phosphate buffer, magnesium sulfate, calcium chloride, and ferric chloride solutions (see Appendix A) or add the contents of pre-measured packets (see Appendix B). Aerate (i.e., saturate the solution with air) by shaking a partially filled container or by bubbling filtered air though the solution. Use a plastic or glass carboy or other container of a sufficient volume that it will provide sufficient water for an entire batch, but small enough that it can be easily shaken (if shaking is

[8] All distilled water could be thought of as steam distilled because the water goes through a steam phase. However "steam distilled" water, where that designation is advertised on the containers in large lettering, goes through a special process that results in a particularly suitable water for use in the BOD test. If the lab cannot find this type of water in local supermarkets, the Lab Accreditation Section can be contacted to determine brand names and potential sources (360 895-6149).

used to aerate). To minimize the probability that dilution water will be supersaturated with dissolved oxygen when used, store this water for at least 24 hours in the dark at 20 ± 1° C (storage in the BOD air incubator is ideal). To allow free transport of oxygen to and from the container during storage, the top of the container should be loosely fitted, or replaced with a loosely-packed wad of cotton. Check stored dilution water to determine if sufficient ammonia remains after storage. Add ammonium chloride solution (see Appendix A) if necessary to bring the ammonia level to approximately 0.45 mg/L ammonia as nitrogen.[9]

(b) **Dilution Water for BOD_5.** For BOD_5, aerate a container of source water as for $CBOD_5$ in 7a(2)1 above, but without the buffer or nutrient solutions. It is advantageous to do this no later than the day before the BOD_5 test. Place the container in the BOD incubator to make sure the water is at 20 ± 1° C when ready for use. Approximately one hour before the BOD bottles are to be set up, add the buffer and nutrient reagents to the dilution water. Do this so as to minimize introduction of air bubbles into the dilution water container. One way of doing this is to dissolve the nutrients and buffers in a small amount of water (Hach Company's and NCL's buffer/nutrient pillows are already in solution), pour the solution down the inside wall of the dilution water container, and gently swirl (not shake, as in aerating) the container to mix the solutions. The reason for not storing **BOD_5** dilution water with the nutrients/buffers already added is that a significant population of nitrifying bacteria may develop if the nutrients-buffers are present in the water. For the **$CBOD_5$** test, the nitrifying bacteria are inhibited and therefore not a problem (see paragraph 4b, *Standard Methods 5210B*).

(3) **Dilution Water Check (Blank).** Regardless of how it is made, it is necessary to check dilution water with every batch of BOD_5 samples to make sure it is not causing error in the test.

(a) One check is to make sure the buffer has done its job and that the pH is in the vicinity of 7.2. Although not mentioned in *Standard Methods,* the dilution water can be checked after buffers and nutrients have been added to determine if the buffers have stabilized pH at approximately the pH of the dilution water to 6.5 to 8.5 pH units (page 276, reference 11e). If it is not, source water is probably the cause and alternative waters should be investigated.

(b) Another check involves simply measuring the initial DO of a BOD bottle full of dilution water, incubating it for 5 days, and reading the final DO. DO depletion should not exceed 0.2 mg/L. If DO depletion of the blank **consistently** exceeds 0.2 mg/L, the cause might be the source water, as already mentioned. Or it could be that the dilution water is **super**saturated with oxygen when the initial DO is read, indicating a need to let the water stand for

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[9] Hach Company and NCL buffer-nutrient pillows contain ammonium chloride to assure 0.45 mg/L ammonia will be present. Additional NH_4Cl should not be required. Because accuracy is not critical for the test used to determine if ammonia concentration is at least 0.45 mg/L, it is not necessary to use an EPA-approved test method. Inexpensive color comparison kits (e.g., disks and cubes) made by several companies (e.g., CHEMetrics, HF Scientific, LaMotte, Orbeco-Hellige) are available for scientific equipment suppliers (e.g., VWR Scientific Products). Hach Company also provides a color disk (Cat. #2241-00) and a color cube (Cat. #22669-100) for low-range ammonia tests.

a longer period before setting up the BOD bottles. If DO depletion for the blank is excessive only **periodically**, contamination (e.g., of the BOD bottles, dilution water container, delivery hose, or other labware), failure to take both initial and final DO readings at 20 ± 1° C, or errors in DO meter calibration (e.g., not changing the membrane often enough) might be the cause. If the initial DO reading is **lower** than the final DO reading (i.e., as if oxygen was somehow generated during the 5-day incubation period), the problem could be that the initial DO is read when the dilution water temperature is considerably higher than 20 ± 1° C. Storing the dilution water in the BOD incubator at least over night should solve this problem. Higher DO levels after the 5-day incubation could also result from photosynthesis if incubation is not done in total darkness.

b. **Estimating BOD_5.** As previously mentioned (paragraph 1d), a preliminary COD test can provide a estimate of BOD_5 allowing the analyst to determine what dilutions are most likely to yield valid BOD_5 results. Being able to make an estimate is especially useful for wastewater treatment plant influents which might vary widely from week to week, and for commercial labs which might have no idea of the approximate BOD_5 of samples received in the lab. Another test that can provide an estimate of BOD_5 for wastewater samples is the total suspended solids (TSS) test. Knowing the approximate BOD_5/TSS ratio is especially useful in a domestic wastewater treatment plant where much of the BOD_5 in an influent is in the form of suspended solids. For a given waste stream, a rough BOD_5/TSS correlation can be established, although it may vary according to season, flow, or other factors. If influent TSS is used to estimate BOD_5, it is important to thoroughly homogenize the sample. This can be done with a homogenizer or high-speed blender.

c. **Seeding**

(1) **Requirement to Seed.** Samples which do not already contain enough of the proper bacteria can be analyzed for BOD_5 only after addition of "seed." Seed is nothing more than a solution containing a sufficient population of suitable bacteria. Influent to a domestic wastewater treatment plant generally contains sufficient bacteria and usually does not need to be seeded. At the other end of the plant, final effluent which has been disinfected (with chlorine or ultraviolet light, for example) usually does not have a sufficient bacteria population and must be seeded. Other waters that usually require seeding are untreated industrial or high-temperature wastes, or those of extreme pH. Effluent from a domestic wastewater treatment plant is the preferred seed for the determination of BOD_5 in samples from that plant because the treated, but not disinfected effluent, contains bacteria that are acclimated to the waste. Performance evaluation samples (PE samples, now referred to as proficiency testing, or PT samples) and standard solutions always need seeding since they contain no natural bacteria.

(2) **Sources of Seed**

(a) **Domestic Wastewater Treatment Plants.** Effluent from a domestic treatment plant also may be the best seed for waste from an industrial process or for waters not expected to contain a significant population of bacteria. If effluent from a **secondary** treatment process is determined to be too weak through either the glucose/glutamic acid test or the seed control test (see the next subparagraph), effluent from the **primary** treatment process should be

tried. In either case, use supernatant from the treated effluent (decant or withdraw using a pipette) after settling at room temperature for at least one hour but not longer that 36 hours at approximately 20°C (i.e., room or incubator temperature). Influent to a domestic wastewater treatment plant is generally not suitable because of problems with drawing homogeneous aliquots of consistent quality. If influent **is** used for seed, it should be thoroughly mixed and allowed to settle as above. Some labs have found success using settled material from a clarifier.

(b) **Commercial Labs.** Many commercial labs and some industrial discharger labs prefer to use artificial seed, such as Polybac®,Polyseed®, or BioSystems®. If these materials are used, special care must be taken in preparing the seed in accordance with the suppliers instructions. Failure to do so will result in unacceptably low results for the seed control bottle(s) (i.e., the seed control will result in a depletion of <0.6 mg/L DO per milliliter of seed) and for all samples that require seeding, including PE samples.

(c) **Other Labs.** Other labs might collect seed material periodically from a treatment plant and keep it viable by feeding it starch or some other nutrient while stored in the incubator. Trial and error will disclose how much nutrient is needed, and how long the seed can be maintained. Effectiveness of such stored seeds should be closely monitored by checking results from the G/GA and seed control tests and monitoring for trends indicating deterioration of the seed. Use of precision control charts is the best mechanism for monitoring performance of the seed and other aspects of the BOD_5 test. Accredited labs can receive a free Excel spreadsheet program the automates the control charting process by contacting the Lab Accreditation Section (360 895-6149).

(3) **Seed Check.** As already discussed, bacteria added to samples containing few viable bacteria (e.g., due to chlorination, UV treatment, extreme pH, or the action of toxicants that might be found in some industrial wastes), are called "seed" and the process is called "seeding." Seed must be of sufficient microbiological activity (i.e., must be potent enough) to properly activate a BOD bottle (or graduated cylinder if using that procedure) without adding a significant volume of seed compared to the volume of the solution to which it is added. Two tests are used to determine the effectiveness of the seeding material: (a) a check standard; and (b) a seed control check.

(a) **Check Standard.** The most widely used check standard (i.e., a solution of known concentration used to check the performance of a test) for the BOD_5 test is a solution of 150 mg/L glucose (also called dextrose) and 150 mg/L glutamic acid (i.e., the G/GA test). An alternative to G/GA is a solution of 300 mg/L potassium hydrogen phthalate (KHP). An advantage of using G/GA over KHP is that G/GA is more widely recognized as the check standard for the BOD_5 test which has resulted in a large data base for test results. Also, glutamic acid contains nitrogen which provides organic material for nitrifying bacteria to consume. An advantage of the KHP test is that the 300 mg/L solution can be used as a check standard for several other tests such as COD, TOC, pH, acidity, total solids, volatile solids, and conductivity (see Table 5). The G/GA solution could likewise be used as checks for those tests, but the expected values are not well documented. Whichever solution is used as a standard, notice that the solution is acidic and might need to be treated with sodium hydroxide (NaOH) to bring pH into the range of 6.5-7.5 pH units.

1. Glucose/Glutamic Acid (G/GA) Check Standard

a. A standard solution of 150 mg/L of glucose and 150 mg/L glutamic acid is prepared in accordance with the instructions in Appendix A. The standard can also be purchased ready-made (see Appendix B).

b. *Standard Methods* suggests a G/GA solution should be analyzed in each batch of BOD_5 samples. This is a good idea if a lab is gathering initial data on the analysis. Once the lab is confident with its ability to do the BOD_5 test with acceptable bias and good precision, frequency of the G/GA test may be reduced to perhaps one per week, or two per month. It should be remembered, however, that any set of data is more easily defended (scientifically and legally) if a check standard and other QC samples are analyzed with each batch.

c. After at least twenty (ten is sufficient if the lab is just getting started, but 20 is preferred) G/GA solutions have been analyzed over a course of several days or weeks, the mean (average) result and standard deviation of results are calculated (see Appendix E for an explanation of statistical terms). *Standard Methods* reports that in an interlaboratory study (i.e., involving many labs), the mean result was 198 mg/L, with a standard deviation of all results of 30.5 mg/L. With that in mind, the objective established by many labs is that they be able to achieve a mean of approximately 198 mg/L, with a standard deviation of 30.5 mg/L or less. One should realize, however, that the 30.5 mg/L standard deviation was calculated from data submitted by several labs, where several analysts used several different seeds, with several variations in other test parameters. This would be expected to result in a higher standard deviation (i.e., more imprecision) than would test results from a single lab. A better goal for a single lab is to expect a mean of approximately 198 mg/L, with a standard deviation in the teens (e.g., 15 mg/L) or lower. At least one lab participating in Ecology's Environmental Laboratory Accreditation Program consistently analyzed the G/GA solution with a standard deviation of approximately 4.0 mg/L. While it is not reasonable to expect every lab to achieve such precision, it should be kept in mind that such results are possible.

2. Potassium Hydrogen Phthalate (KHP) Check Standard

a. A standard solution of 300 mg/L of KHP is prepared in accordance with the instructions in Appendix A. This solution can be analyzed periodically as a supplement to the G/GA test, or it can replace the G/GA test. Before replacing the G/GA test, however, consideration should be given to the fact that G/GA results is most widely recognized as the primary indicator of performance for the BOD test.

b. If replacing the G/GA test, the KHP test should be run with the same frequency suggested for G/GA in paragraph 7b(3)(a)1b above.

c. Only limited data are available upon which to base quality objectives for the KHP test. In a series of 159 tests of the KHP solution over a period of approximately five years, Ecology's Manchester Lab achieved a mean value of 249 mg/L, with a standard deviation of 15.4 mg/L. These would be reasonable statistics to use as initial objectives

until data gathered by a lab indicates other statistics might be more appropriate. If the KHP solution is used as a standard for other tests, Table 5 shows reasonable objectives for mean values for the various tests as achieved by Ecology's Manchester Lab. Precision data (e.g., standard deviations for repeated analyses) have not been determined (except for BOD_5, as indicated above).

Table 5 - Expected Values for a 300 mg/L KHP Standard

Parameter	Expected Value
BOD_5	249 mg/L
COD	343 mg/l
Total Organic Carbon (TOC)	141 mg/L
pH	4 4
Total Solids (TS)	300 mg/L
Volatile Solids (VS)	200 mg/L
Conductivity	169 mhos
Acidity	74 mg/L

(b) **Seed Control Check.** Another check on seed effectiveness is the seed control check. It is also used to determine what contribution the seed itself will make to the DO depletion of seeded samples. That contribution must be subtracted when calculating sample BOD_5. To do the check, set up three BOD bottles with 3, 6, and 9 milliliters of seed (or other volumes that will result in a DO depletion of at least 2.0 mg/L, and a retention of at least 1.0 mg/L after the 5-day incubation), and fill the bottles with dilution water. These are called "seed control" bottles, or sometimes "seed blank" bottles. It may be necessary to do three dilutions for the seed control until the seed material is well-characterized, at which time one bottle may give reliable results. Measure initial DO, incubate for five days, measure final DO, and calculate the DO depletion **per milliliter of seed**[10]. An effective seed results in a depletion between 0.6 and 1.0

[10] *Standard Methods* 5210B is not entirely clear on this requirement. Paragraph 5210B4d(2) states that "The DO uptake of seeded dilution water should be between 0 6 and 1.0 mg/L" but it does not associate this depletion with a volume of seed. In subparagraph 5 under "Seeding of Sample" on page 476 of *Operation of Wastewater Treatment Plants* (Reference 11d to this document), EPA says "Seed material should produce a correction of at least 0.6 mg/L **per mL of seed**." The author interprets these two statements together to indicate that not only should the seed material result in a depletion of 0 6 - 1.0 mg/L **per mL of seed**, but also that the amount of seed added to each seeded BOD bottle or to a graduated cylinder should cause a DO depletion of 0.6 - 1 0 mg/L in a seeded bottle.

mg/L for each mL of seed. Once an effective seed has been identified and it consistently results in a DO depletion of 0.6 - 1.0 mg/L per mL of seed, it is no longer necessary to incubate three bottles with each batch and only one need be done. Table 6 shows data for an effective seed. Bottle 3 did not result in valid data because the final DO was less than 1.0 mg/L.

(4) **Choice of Seed.** The bottom line in choosing a seed material is to pick one that results in G/GA tests in the vicinity of 198 mg/L, and in consistent seed control test results between 0.6 - 1.0 mg/L DO depletion per mL of seed. Paragraph 7c(2) on page 14 discusses possible sources of such a seed

Table 6 - Typical Seed Control Check

	Bottle 1	**Bottle 2**	**Bottle 3**
Sample Volume (mL)	0	0	0
Seed Volume (mL)	3	6	9
Initial DO (mg/L)	8.9	8.9	8.9
Final (5-day) DO (mg/L)	6.2	3.3	0.8
Depletion (mg/L)	2.7	5.6	8.1
Depletion per mL of seed (mg/L DO per mL)	0.9	0.93	(not valid . . . <1.0 mg/L residual DO)

d. **Dilution of Samples.** To meet the method requirement that at least 1.0 mg/L of DO remain in the sample after the 5-day incubation period, some samples must be diluted (see Table 3). They must not be diluted to such an extent, however, that DO depletion during the 5-day incubation is less than 2.0 mg/L, the method-imposed minimum DO depletion for a valid bottle. There are two alternative procedures to follow for dilution of samples prior to incubation. One involves dilution of the sample in the BOD bottle, referred to as the **bottle method**. In the other, the sample is diluted in a graduated cylinder and then an aliquot is transferred to a BOD bottle for incubation. This is referred to as the **graduated cylinder method.** Choice of a method is a matter of personal preference and either can consistently produce reliable and accurate results.

(1) **Bottle Method**

(a) Refer if necessary to Table 3 to determine how much sample to add to each BOD bottle. If the dilution would be greater than 1:100 (i.e., if less than 3 mL of sample would be added to a 300-mL BOD bottle), as a preliminary step, dilute the entire sample taken from the waste stream with reagent grade water before adding it to the BOD bottle. Do not forget to multiply the result by the additional dilution factor. If the sample being analyzed is well characterized (that is, it is well known to the lab, having been analyzed many times, with results always being within a relatively small range), and if precision for the test is very good (that is, the

standard deviation for the glucose/glutamic acid test is relatively small), it may be sufficient to set up only one BOD bottle for incubation per sampling site. (NOTE: Some discharge permit managers may not allow incubation of only one bottle, even for a well-characterized sampling site) If the water being analyzed is not well-characterized, if precision is only marginally acceptable, or if required by a regulatory program, more than one bottle, each containing sample at a different dilution, should be analyzed. The goal is that at least one of the bottles results in a DO depletion of at least 2.0 mg/L, with at least 1.0 mg/L retained at the end of 5 days.

(b) After thoroughly mixing the bulk sample, use a wide-tip pipette (i.e., 1/32 to 1/8 inch inside diameter) to transfer the desired volume of sample to individual BOD bottles. The same pipette can be used to transfer all samples if bottles are set up starting with the sample expected to result in the lowest BOD_5, proceeding eventually to that expected to have the highest BOD as the last sample. Using a different pipette, transfer into those sample bottles needing it, an amount of seed material sufficient to produce a DO depletion of 0.6 - 1.0 mg/L during the 5-day incubation (as determined by previous tests of the same or similar seed).

(c) Fill each BOD bottle with dilution water to a level where insertion of the glass stopper displaces all air, leaving none trapped in the neck of the bottle. Fill the space around the bottle stopper with distilled water to form a water seal. If using an air incubator, place a plastic cap over the stopper to prevent evaporation of the water seal while the sample is being incubated.

(2) **Graduated Cylinder Method**

(a) Seed the entire container of dilution water as necessary to result in a 5-day DO depletion in a BOD bottle of 0.6 - 1.0 mg/L. Carefully siphon dilution water into a clean graduated cylinder, usually either of 1-L or 2-L capacity depending on how many dilutions will be run for each sample. Fill the graduated cylinder approximately half full, being careful not to trap air in the water (i.e., do not form bubbles). Add the desired volume of homogenized sample, and fill to the desired level with dilution water. Mix carefully with a plunger-type mixing rod, again being careful not to trap air.

(b) Fill each BOD bottle with dilution water containing seed and sample (i.e., from the graduated cylinder) to a level where insertion of the glass stopper will displace all air, leaving none trapped in the neck of the bottle. Fill the space around the bottle stopper with distilled water to form a water seal. If using an air incubator, place a plastic cap over the stopper to prevent evaporation of the water seal while the sample is being incubated.

e. **Determination of Initial DO.** Measure initial DO using either the Winkler titration or DO probe technique. If a DO meter/probe is used to measure DO, it should be calibrated immediately prior to analysis of samples using the calibration technique recommended by the manufacturer of the meter/probe. Additionally, the DO meter/probe should be checked periodically by first taking a DO reading of dilution water in a BOD bottle using the probe, and then doing a Winkler titration on the same bottle. If the two DO readings differ by more than 0.2 mg/L, the cause of the difference should be sought and eliminated.

(1) **Winkler Method.** If the Winkler (iodometric) method is used, two bottles must be set up for each dilution, blank, standard, and seed control, one of which is dedicated solely to determination of the initial DO, the other being incubated. Initial DO of the incubated bottle is assumed to be identical to DO of the titrated bottle.

(2) **DO Probe Method.** If the DO probe method is used, at least one bottle must be set up per dilution (additional bottles at the same dilution would be replicates which are normally used only to check within-batch precision. The DO probe will displace a relatively small amount of water which can be replaced with dilution water without introducing a significant error in the process.[11] Alternatively, inserts are available which can be used to return displaced water to the bottle. Initial DO should be read starting with the blank, then the glucose/glutamic acid standard, effluent (in a wastewater treatment plant lab), seed control, and finally progressing through other samples, starting with that expected to have the lowest BOD_5 through the highest. The DO probe should be rinsed after each reading. If this procedure is followed faithfully, there is no need to fill two bottles per dilution, one for measuring initial DO and the other for incubation.

f. **Incubation.** After filling BOD bottles as described above, stopper all bottles tightly. Apply a water seal by adding source water (e.g., distilled water) around the stopper if necessary, place a plastic cap (or other device to prevent evaporation of the water seal) on each bottle, and incubate each bottle at 20 ± 1° C, in the dark, for 5 days, ± 2 hours. All bottles (blank, seed control, glucose/glutamic acid standard, environmental samples, performance evaluation samples, if any) must be incubated together as a batch.

g. **Determination of Final DO.** After 5 days, read the final DO as in paragraph 7e above.

8. **Calculations/Data Recording.** Since BOD is defined as the milligrams of dissolved oxygen consumed by bacteria per liter of sample over a 5-day incubation, the BOD_5 of an undiluted sample can be determined merely by reading the initial DO in mg/L (DO_0) and subtracting the final DO (DO_5). Thus, if an undiluted sample has a DO_0 of 8.0 mg/L, and a DO_5 of 3.0 mg/L, the BOD of the sample is 8.0 - 3.0 = 5.0 mg/L. Most samples will require dilution, however (see Table 3), which will require that a factor be applied to compensate for the fact that the incubated sample is not pure sample. In paragraph 5 of *Standard Methods* 5210B, this factor is given the designation "P" and is equal to the volume of the sample divided by the volume of the BOD bottle or graduated cylinder containing the sample. This factor, "P," is then **divided** into the DO

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[11] Some analysts prefer using two bottles per dilution even when a probe is used to measure DO. The argument for using two bottles is that insertion of the DO probe dislocates a small amount of sample from the bottle used to measure initial DO, and that replacing that displaced volume with dilution water further dilutes the sample, introducing error. It should be realized, however, that the amount of water displaced is only approximately 1% of the total volume, and that the maximum error that could be introduced is therefore only 1%. Such error is minor compared to other potential sources of error for the BOD_5 test. Another argument for using two bottles per dilution is to avoid contamination of incubated bottles by using a probe that has just been used on a bottle potentially containing BOD. If measurements are taken from the least contaminated bottle (e.g., blank) through the most contaminated (e.g., raw effluent), and the probe is rinsed properly between bottles, this potential error is also minimal. The two potential errors (slight dilution and possible contamination) tend to cancel each other, and the risk to data quality is small compared to the gain in efficiency.

depletion to determine the sample BOD_5. Some analysts find it easier to use the reciprocal of "P" (i.e., the container volume divided by the sample volume) and **multiply** the DO depletion by this factor. Both procedures come up with the same number, but for the sake of simplicity and use of most commonly used term, this document uses the latter ratio, which is identified in 8a below as the **Dilution Factor**, DF (i.e., DF = 1/P).

a. **Dilution Factor**. The dilution factor, DF, is the ratio of the final volume (e.g., for the bottle method, the volume of the BOD bottle, usually 300 mL; for the graduated cylinder method, the volume of the cylinder, usually 1,000 mL) to the volume of sample therein. DF for the bottle method = Volume of Diluted Mixture/Volume of Sample in Mixture, or . . .

Equation #1 - Dilution Factor (Bottle Method) $DF = Vol_{Mix}/Vol$

EXAMPLE #1 – Dilution Factor (Bottle Method)

The DF for a mixture of 75 mL of sample and 225 mL of dilution water in a 300-mL BOD bottle is. . .

DF = 300 mL/75 mL = 4.0

For a sample expected to have a higher BOD_5 where a small amount of sample is diluted, the DF would be higher. For a 300-mL mixture containing only 6.0 mL of sample, the DF is . . .

$$DF = 300\ mL/6.0\ mL = 50$$

Reference to Table 3 confirms that the two dilution factors above have been correctly calculated. If instead of a BOD bottle having a volume of 300 mL, a 1,000 mL (1-liter) graduated cylinder is used to prepare the sample, the DF is calculated in the same way. If 100 mL of sample is diluted in a 1,000-mL graduated cylinder, the DF is . . .

$$DF = 1000\ mL/100\ mL = 10$$

b. **BOD_5 - Not Seeded.** When samples **are not** seeded, the BOD of a sample is calculated simply as the dilution factor (DF) times the DO depletion during the 5-day incubation (DO_i - DO_5).

Equation #2 - BOD_5 (not seeded) $BOD_5 = DF\ (DO_i - DO_5)$

EXAMPLE #2

The BOD_5 for an unseeded bottle having a DF of 4.0 (as in Example #1), an initial DO of 8.4 mg/L, and a final DO of 5.4 mg/L is. . .

BOD = 4.0(8.4 mg/L – 5.4 mg/L) = 4.0(3.0 mg/L) = 12 mg/L

A question often asked is if the final DO reading needs to be "blank corrected" (i.e., should the blank contribution be subtracted from the difference between DO_1 and DO_5). If blanks are usually zero as they should be, with an occasional 0.1 mg/L depletion, and a very infrequent 0.2 mg/L, random error is causing the positive blank, and the cause of that random error may exist only in the blank bottle. Results should **not** be corrected to compensate for this random error. If blanks **usually** run 0.1 or 0.2 mg/L, or even higher, rather than blank correcting, the analyst should find the cause(s) of the high blanks and eliminate it (them). So regardless of the cause of high blanks, it is not a good scientific approach to correct for blanks. Also, *Standard Methods* 5210B, in the last subparagraph of paragraph 5 says not to blank correct.

c. **BOD - Seeded.** If samples **are** seeded, the total DO depletion over the 5-day incubation is caused by both the sample and the seed. The equation for calculating BOD of the sample becomes a little more complicated **because the DO depletion caused by the seed must be subtracted from the total DO depletion.** The seed control bottle is used to determine the contribution to DO depletion made by the seed. *Standard Methods* uses "B_1" to denote DO of the seed control bottle on Day-1, and "B_2," the DO of the seed control bottle on Day-5. To determine the total depletion caused by the seed in the sample bottles, one must also consider the ratio of volume of seed in the sample bottle, to the volume of seed in the seed control bottle. This factor is given the designation "f" by *Standard Methods* such that for the **bottle method** . . .

Equation #3 - Seed Ratio Factor (Bottle) $f = \text{Vol Seed}_{\text{Samp}} / \text{Vol Seed}_{\text{Seed Control}}$

EXAMPLE #3

The seed ratio factor, "f," if the seed control bottle contained 10.0 mL of seed, and the sample bottle contained 5.0 mL of seed is. .

$f = 5.0 \text{ mL} / 10 \text{ mL} = 0.50$

and for the **graduated cylinder method** . . .

Equation #4 - Seed Ratio Factor (Grad. Cylinder) $f = \% \text{ Seed}_{\text{Samp}} / \% \text{ Seed}_{\text{Seed Control}}$

EXAMPLE #4

The seed ratio factor, "f," if the percentage of seed in the seed control graduated cylinder is 2% (e.g., 20 mL in a 1,000 mL cylinder), and the percentage of seed in the sample graduated sample is 1% (e.g., 10 mL in a 1,000 mL cylinder) is. .

$f = 1\%/2\% = 0.50$

To determine the BOD_5 for seeded samples, the contribution of the seed to total DO depletion in the incubated sample must be taken into consideration. This is done by multiplying the difference between the initial DO of the seed control bottle (B_1 in Equation #5 below) and the final DO (B_2) by the seed ratio factor "f." BOD_5 for seeded samples is then calculated as . . .

Equation #5 - BOD_5 (seeded) $\quad BOD_5 = DF\,[(DO_1 - DO_5) - f\,(B_1 - B_2)]$

EXAMPLE #5

The BOD_5 of a bottle where the seed ratio factor, "f," is 0.50, the dilution factor, "DF," is 5, the initial DO of the seed control bottle is 8.3 mg/L, the final DO 2.3 mg/L, and the initial DO of the sample bottle is 8.4 mg/L, the final DO is 4.4 mg/L is. .

$BOD_5 = 5[(8.4\ mg/L - 4.4\ mg/L) - 0.50(8.3\ mg/L - 2.3\ mg/L)]$

$= 5[(4.0\ mg/L) - 0.50(6.0\ mg/L)] = 5[4.0 - 3.0] = 5\ mg/L$

d **Benchsheet.** Benchsheets used to record observations made during the BOD test should include space for recording the following as a minimum.

(1) Date and time the sample was taken

(2) Identification of the sampler and analyst

(3) Identification of the sample (e.g., raw influent, oxidation ditch, final effluent)

(4) Sample pH

(5) Sample temperature when initial DO is read

(6) Bottle Numbers

(7) Volume of seed in each BOD bottle or in the graduated cylinder

(8) Volume of sample in each BOD bottle or in the graduated cylinder

(9) Initial DO of each bottle

(10) Final DO of each bottle

(11) Space for results of calculations, such as DO depletion (drop), determination of the seed correction factor "f," dilution factor, and BOD_5

(12) An indicator of whether results are BOD_5 or $CBOD_5$

(13) Date/time the initial DO and final DO were read

(14) A space to indicate the benchsheet has been reviewed with the date and initials of the reviewer.

(15) Comments

A sample benchsheet is shown at Appendix C for the bottle method, and Appendix D for the graduated cylinder method. Note that these benchsheets indicate only one bottle is incubated for the blank, seed control, and standard. This may vary according to the needs of the lab, experience of the analyst, and desires of the supervisor. It also implies that as many as three should be run for the influent since BOD_5 strength is variable for that sample. The number of bottles incubated for effluent samples is a matter of choice, based on experience of the analyst and variability of samples with regard to BOD_5 strength. Individual wastewater discharge permits or other regulatory requirements may dictate the number of bottles that must be incubated for each sampling site.

9. Quality Assurance/Quality Control

a. **Minimum criteria.** To be a valid test for a given BOD bottle, incubation must result in a DO depletion of at least 2.0 mg/L[12], with at least 1.0 mg/L DO remaining at the end of the incubation period. The optimum depletion is half the available DO, or 3-4 mg/L for most situations. For a series of dilutions, results for all bottles meeting these criteria are averaged to come up with the final BOD_5 to be reported[13][11]. If none of the bottles for a given sample meet the criteria, the lab might report (e.g., on the Discharge Monitoring Report, DMR) a value for the bottle that comes closest to meeting the criteria and add a note to the report indicating that the value is an estimate and why. If the test is being run in a wastewater treatment plant, it might be

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[12] Some analysts interpret this requirement in *Standard Methods* as meaning that the DO depletion caused by the sample **plus** the seed contribution must be at least 2.0 mg/L. The author interprets the requirement to mean that the sample alone must contribute a depletion of at least 2.0 mg/L. If the author's interpretation is not correct, the method-imposed minimum detection limit (MDL) for BOD_5 would be 1.0 mg/L (an undiluted bottle, i.e., DF = 1, where the sample contributed a 1.0 mg/L depletion, and the seed contributed 1.0 mg/L, the maximum suggested for the seed). But the MDL is not 1.0 mg/L, it is 2.0 mg/L, meaning that the 2.0 mg/L minimum depletion applies to the sample without consideration for the depletion caused by the seed.

[13] An exception to the rule that you average all bottles meeting the depletion/retention criteria is when the sample exhibits toxicity (i.e., something in the sample inhibits activity of the bacteria). Although *Standard Methods* 5210B does not specify what is meant by "exhibits toxicity," one indication is that the DO depletion per milliliter of sample is **greater** for the more dilute bottles. In such cases, there is justification for reporting the BOD_5 value for the most dilute sample that met the depletion/retention criteria. For example, for the data in Table 7 below, where the seed was contributing a depletion of 0.6 mg/L in each bottle, the data justifies reporting 222 mg/L, BOD for the most dilute bottle.

possible to resample and still meet monitoring requirements. If certain samples never seem to deplete at least 2.0 mg/L DO, such samples must be run with less dilution. If they are already being run at full strength (e.g., 298 milliliters of sample and 2 mL of seed) and still fail to deplete at least 2.0 mg/L DO, they should be reported as "<2.0 mg/L BOD_5" (i.e., less than 2.0 mg/L, the method-imposed minimum detection limit). If certain samples never seem to leave at least 1.0 mg/L remaining after the incubation, they need to be further diluted. If necessary, the entire sample can be diluted as a preliminary step before analysis.

Table 7 – Evidence of Possible Toxicity Interference

Source	Bottle	Sample Vol.	Seed Vol.	Initial DO	Final DO	Deple-tion	Dilution Factor	BOD_5
	1	2	1	8.9	3.9	5.0	150	750
	2	5	1	8.9	4.6	4.3	60.0	222
Final Effluent	3	10	1	8.9	4.3	4.6	30.0	120
	4	20	1	8.9	3.9	5.0	15.0	66.0

b. **Blanks.** At least one unseeded dilution water blank should be run with each batch of samples. The purpose of the blank is to indicate absence of: (1) contamination or supersaturation of dilution water with DO; (2) temperature problems; (3) atmospheric pressure problems, and; (4) and other sources of error that may not be related to the samples themselves. The lab should attempt to keep all blanks below 0.1 mg/L, but action need not be taken unless they exceed 0.2 mg/L. In the final BOD_5 calculation, results should NOT be corrected for the blank value as already explained. If an isolated blank exceeds a depletion of 0.2 mg/L DO, the report to the data user (e.g., on the Discharge Monitoring Report, or DMR, to Ecology in some cases) should note the fact that the blank exceeded method allowances. If blanks typically run higher than 0.2 mg/L, the following potential causes should be investigated, preferably one at a time so as to isolate the actual problem and eliminate it.

(1) **Contamination.** Contaminated labware may contribute to BOD_5 in the blank. Try thoroughly washing and rinsing all labware, possibly using different techniques, and running a blank on several bottles in a single batch. If one technique seems to give better results (i.e., lower blanks), stick with it.

(2) **Supersaturation.** If the initial DO is read in BOD bottles when oxygen is supersaturated, a positive blank will result, and all such bottles incubated will have results that are biased high. The best way to avoid supersaturation is use dilution water which is at 20 ± 1° C, and to avoid aerating dilution water, sample, or seed immediately prior to taking the initial DO reading. Dilution water can be aerated the day before setting up the BOD bottles (or earlier), and stored in a container protected by having a cotton plug in, or a loose cap on its opening to allow escape of entrapped air, or entry of air if the water was not already saturated. When nutrients/buffers are added to the dilution water on the day of the test (i.e., when BOD_5 and not

$CBOD_5$ is being run), and when samples and dilution water are added to BOD bottles, care must be taken to avoid forming air bubbles.

(3) **Temperature.** The saturation level of DO in water depends on atmospheric pressure and temperature (as well as on other factors such as salinity which normally are not encountered by most labs doing BOD_5 testing). The colder the water, the more dissolved oxygen it can contain at the saturation point. Effluent samples (or other samples expected to have a low BOD_5, and thus a requirement for little dilution) must be warmed or cooled to within ±1° of 20°C when initial DO is read. For samples expected to have higher BOD_5 strengths and thus requiring dilution, it is more important that the dilution water be at 20 ± 1°C since the diluted sample is primarily dilution water. A good way to assure that temperature is to keep aerated source water for BOD_5, or aerated dilution water for $CBOD_5$, at least overnight in the BOD incubator. If some blanks for BOD_5 are positive, but others are negative (i.e., as if DO had **increased** during incubation), temperature might be the problem. Another problem that could cause negative blanks would be photosynthesis during incubation. Exclusion of light during incubation precludes photosynthesis.

(4) **Pressure.** If blanks are positive for some batches, and negative for others, the problem might be temperature (as indicated above), or it might be a problem with compensating for atmospheric pressure changes between the initial DO and final DO readings. DO meters are often calibrated by compensating only for the elevation of the lab, and not the actual atmospheric pressure. As any Washingtonian knows, atmospheric pressure can change dramatically during a five day period (even though elevation of the lab remains constant). Calibration based on atmospheric pressure as measured with a barometer is preferred to basing calibration on elevations. Major manufacturers of DO meters sell top-end meters that have built-in barometers and automatically correct for atmospheric pressure (as they do temperature).

(5) **Source (Distilled/Deionized) Water.** More often than not, labs having a problem with high blanks check for the problems in subparagraphs b(1) through b(4) above, and find none is causing the problem. The water used to prepare dilution water is often the culprit. Water distilled in a lab often contains ammonia, amines, and possibly other materials that contribute to BOD_5 (i.e., cause high blanks). Other contaminants such as copper might inhibit bacterial activity. Although such contaminated water would not be detected by blank results, they might cause a negative bias in the glucose/glutamic acid determination and in environmental samples. Even if distilled water is put through a deionization (DI) column, the water may still contain organic material leached from the column. In addition to keeping the distillation apparatus clean and replacing DI columns frequently, it may be necessary to add an activated charcoal column as the final scrubber in the water source. An alternative, and a much cheaper option when trying to locate the source of high blanks, is to try using distilled water purchased from a local store. If one brand still results in high blanks, try another. Steam distilled water (see paragraph 7a(1)) seems to work best (when it can be found). If it can be shown that water is the source of the high blanks, then it might be worthwhile in the long run to upgrade the distilled/deionized/scrubbed water system in the lab.

(6) **Measurement of DO.** If either the initial, final, or both DO measurements are not made properly, excessive blanks could result. When possible, the analyst who measures initial DO should also measure final DO.

c. **Check Standard.** The primary purpose of analyzing a BOD_5 check standard, whether it be glucose/glutamic acid, potassium hydrogen phthalate (KHP), or some other material, is to determine if the seed used by the lab is sufficiently potent for the BOD_5 test. If the mean (average) value following several analyses of the glucose/glutamic acid standard is significantly lower than 198 mg/L (the guidance given in *Standard Methods),* a stronger seed is needed. If the mean is considerably higher than 198 mg/L, a weaker seed is indicated. The standard deviation for results of the repeated analysis of the glucose/glutamic acid standard indicates the total precision of analysis. *Standard Methods* indicates the standard deviation should be 30.5 mg/L or lower, but, as previously mentioned, a good single lab should be able to run the test consistently such that the standard deviation is in the teens (with less than 10 mg/L indicating extraordinary precision). Many things can cause imprecision such as variable seed, temperature and pressure problems, contamination, DO measurement problems, inattention of analysts, and countless other sources of variability. It is usually much easier to discover and eliminate a problem causing a low (or high) mean value for the glucose/glutamic acid test than it is to do the same for a high standard deviation indicating excessive imprecision.

d. **Duplicates.** The purpose of running occasional duplicates is to determine if within-batch precision is a problem. Imprecision in BOD_5 testing (or in any measurement system) is caused by **within-batch imprecision**, and **between-batch imprecision** See Appendix E for a more thorough discussion on use of statistics in BOD_5 testing

10. **Method Performance Summary (Bias, Precision, Detection Limit, Working Limits)**

a. **Bias.** A good lab should be able to achieve a mean value in the vicinity of 198 mg/L for repeated analyses of the 150 mg/L glucose plus 150 mg/L glutamic acid standard.

b. **Precision (or Imprecision)**

(1) **Total Imprecision.** A good lab should be able to achieve a standard deviation of less than 20 mg/L for repeated analyses of the glucose/glutamic acid standard (remember that the 30.5 mg/L standard deviation cited in *SM 5210B* is for several labs doing the G/GA test). An MS Excel control charting file is available to accredited labs from Ecology's Lab Accreditation Section that calculates the total standard deviation of repeated results for analysis of the GGA standard. The control charting program also tracks performance on subsequent GGA analyses.

(2) **Within-batch Imprecision.** Because within-batch imprecision is expected to be the minor contributor to total imprecision, one might expect the within-batch standard deviation for several duplicate pairs to be less than the standard deviation calculated for between-batch imprecision. The same Excel program mentioned above calculates the standard deviation of the differences for duplicate measurements of similar samples and also tracks performance on

subsequent duplicates. It is important that the samples chosen to duplicate vary little in concentration from batch to batch (e.g., final effluent that generally runs in the vicinity of 10 mg/L BOD_5). Dividing the standard deviation of the difference (which is calculated by the above-mentioned Excel program) by the square root of 2 gives an estimate of the within-batch standard deviation (i.e., $s_{wi} = s_{diff}/\sqrt{2}$).

(3) **Between-batch Imprecision.** Because between-batch imprecision is expected to be the major contributor to total imprecision, one might expect the between-batch standard deviation estimate based on calculation of variance (see Appendix E, Statistics) to be somewhat more than the standard deviation for within-batch precision.

c. **Detection Limit.** Because of the method requirement that a test is valid only if 2.0 mg/L or more DO is depleted, and assuming that such a depletion was achieved for an undiluted sample (i.e., the DF = 1.0), the theoretical detection limit is 2.0 mg/L DO (i.e., 2.0 mg/L depletion times 1.0 = 2.0 mg/L).

d. **Working Limits**

(1) **Minimum.** The minimum working limit is 2 mg/L, the MDL addressed above.

(2) **Maximum.** Considering that waste samples might contain significant suspended solids, the minimum volume of a waste sample that can be measured with any degree of accuracy might be 1.0 mL. The maximum BOD_5 working range for such a sample that is actually incubated might be 2400 mg/L, assuming that initial DO was 9.0 mg/L and the final DO 1.0 mg/L, the minimum for a valid test. DO depletion of 8.0 mg/L times the dilution factor of 300 calculates to be 2400 mg/L BOD_5. However, one must also consider that the incubated sample may have been prepared by diluting the actual environmental sample. If that dilution factor were 1:100, for example, the maximum BOD_5 would 240,000 mg/L. In practice, the test itself does not limit the maximum measurable BOD_5.

11. References

a. *Standard Methods for the Examination of Water and Wastewater,* 19th Edition, 1995, APHA-AWWA-WEF, page 5-2 ff.

b. *Manual of Methods for Chemical Analysis of Water and Wastes,* EPA-600 4-79-020, 1983, Method 405.1.

c. *Basic Laboratory Procedures for Wastewater Examination,* 4th Edition, 2002, Water Environment Federation.

d. *Operation of Wastewater Treatment Plants,* Vol II, 3rd Edition, EPA, 1991.

e. *Chemistry for Sanitary Engineers,* 1967, McGraw Hill, page 394 ff. (This reference has been revised in a later edition titled *Chemistry for Environmental Engineering,* 4th Edition, 1994, McGraw Hill. The 4th Edition was not available to the author of this document.)

f. *Procedural Manual for the Environmental Laboratory Accreditation Program,* Washington Department of Ecology, Document #02-03-055, November 2002.

Appendices:

A - Preparation of Solutions
B - Sources of Reagent Grade Chemicals
C - Sample Benchsheet (Bottle Method)
D - Sample Benchsheet (Graduated Cylinder Method)
E - Statistical Terms Used in BOD Testing
F - Troubleshooting the BOD Test
G – BOD/CBOD Checklist

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Appendix A

Preparation of Solutions

1. **Acid Solution** (for neutralizing caustic samples). While stirring, slowly add **28 mL** of concentrated **sulfuric acid** (H_2SO_4) to distilled water. Dilute to **1,000 mL**. Can be stored indefinitely (CAUTION: Do not add water to acid!)

2. **Ammonium Chloride Solution** (nutrient for nitrifying and other bacteria). Dissolve **1.15 grams** of **ammonium chloride** (NH_4Cl) in approximately **500 mL** of distilled water, adjust pH to 7.2 with NaOH solution, and dilute to **1000 mL.** Can be stored indefinitely in a well-sealed container

3. **Calcium Chloride Solution.** Dissolve **27.5 grams of anhydrous calcium chloride** ($CaCl_2$) in distilled water and dilute to **1000 mL.** Can be stored indefinitely.

3. **Base (Alkaline) Solution** (for neutralizing acidic samples). Dissolve **40 grams** of **sodium hydroxide** (NaOH) in distilled water. Dilute to **1000 mL**. Can be stored indefinitely.

4. **Ferric Chloride Solution.** Dissolve **0.25 grams** of **ferric chloride (hydrated)** ($FeCl_3 \cdot 6H_2O$) in distilled water and dilute to **1000 mL**. Can be stored indefinitely.

5. **Glucose/Glutamic Acid** (standard solution for the BOD test). Dissolve **150 mg** of dried **glucose** (also called dextrose) and **150 mg** of dried **glutamic acid** in **1000 mL** of distilled water. Prepare fresh daily as needed. Can be purchased as a prepared solution from Hach (Cat. No. 14865-10), North Central Labs (Cat. No. B-12D), and perhaps others. If Hach's solution is used, their instructions call for dilution to a solution that is 300 mg/L glucose and 300 mg/L glutamic acid. This solution should be further diluted to 150 mg/L of each by adding an equal volume of distilled water to the solution prepared using the Hach instructions.[1]

6. **Magnesium Sulfate Solution.** Dissolve **22.5 grams** of **magnesium sulfate (hydrated)** ($MgSO_4 \cdot 7H_2O$) in distilled water and dilute to **1000 mL.** Can be stored indefinitely.

7. **Phosphate Buffer Solution.** Dissolve **8.5** grams of **potassium dihydrogen phosphate** (KH_2PO_4), **21.75 grams** of **dibasic potassium phosphate** (K_2HPO_4), **33.4 grams** of **dibasic**

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[1] Instructions accompanying the Hach Company standard say the 300 mg/L solution can be analyzed without further dilution and that the expected result for repeated analyses should be double the 198 ± 30.5 mg/L as suggested in *Standard Methods* 5210 B, or 396 ± 61.0 mg/L. When using Hach's standard, it is best to dilute the sample to half the strength specified in the instructions (e.g., by withdrawing half the volume from the Hach vial, or by diluting it with twice as much water). This will result in a solution that is 150 mg/L of each ingredient, the concentration suggested in *Standard Methods*. Hach plans to provide a 150mg/L solution in the future.

sodium phosphate (hydrated) ($Na_2HPO_4 \cdot H_2O$), and **1.7 grams** of **ammonium chloride** (NH_4Cl) in approximately 500 mL of distilled water and dilute to **1000 mL.** Check pH... should be 7.2. Can be stored in sample refrigerator at approximately 4°C. Discard upon any sign of biological growth.

8. **Potassium Hydrogen Phthalate** (alternative standard for BOD test). Dry a thin layer of **potassium hydrogen phthalate** crystals in a beaker for one hour at 103 - 110° C. Cooling in a desiccator for 30 minutes. Weigh out **300 milligrams** of the dried **KHP** and dissolve in **1000 mL** of distilled water. This solution is biodegradable but should be stable for approximately six months if kept well stoppered and refrigerated at 4° C.

9. **Potassium Iodide** (for titration to determine volume of sodium sulfite needed for dechlorination). Dissolve **10.0 grams** of reagent grade **potassium iodide** (KI) in **100 mL** of distilled water. Can be stored indefinitely.

10. **Sodium Sulfite** (for dechlorination). Dissolve **1.575 grams of Na_2SO_3 in 1000 mL** of distilled water. Sodium sulfite solution is not stable and must be prepared daily as needed.

11. **Starch Solution** (for iodine titration in dechlorination procedure). Make **10 grams** of soluble starch into a smooth paste with warm water. Pour into approximately **1000 mL** of boiling water. This solution does not store indefinitely and should be prepared fresh when deterioration is first noticed.

Appendix B

Sources of Reagent Grade Chemicals[1]

	VWR[2]	Fischer[3]	Sigma[4]	Curtin Matheson[5]	NCL[6]	Hach[7]
Sodium Sulfite (Na_2SO_3)	JT3922-1	S447-500	S 8018	832-151	S-67	195-0
Potassium Iodide	VW5225-1	P410-500	P 4286	831-718	P-73	167-01
Glucose (Dextrose)	EM-DX0156-1	D16-500G 7528		MDX0156-1 G-50	11251-53	
Glutamic Acid	JTM756-7	A125-100	G 6904	423-287	G-60	23409-23
Potassium Dihydrogen Phosphate (KH_2PO_4)	JT3246-1	P284-500	P 0662	M5108-1	P-92[8]	170-01[8]
Potassium Hydrogen Phthalate (KHP)	EM-PX1571-MB-2	MK-6704-125	P3792	M4876-2	P-61	315-34
Potassium Phosphate Dibasic (K_2HPO_4)	JT3254-1	P288-500	P 8281	831-761	P-91[8]	7080-34[8]
Seed Capsules						
Bioseed	52468-360	-	-	022-354	-	-
Polyseed	66130-430	13-297-200	-	-	-	24712-00
BioSystems	-	-	-	-	B-600	-
Sodium Phosphate, Dibasic (hydrated) ($Na_2HPO_4 \cdot 7H_2O$)	JT3824-1	S373-500	S 9390	423-323	[8]	[8]
Ammonium Chloride	JT0660-11	A649-500	A 5666	830-154	A-17[8]	105-01[8]
Magnesium Sulfate (hydrated) ($MgSO_4 \cdot 7H_2O$)	EM-MX0070-1	M63-500	M 1880	MMX0070-1	M-10[8]	6088-34[8]
Calcium Chloride	EM-CX0156-1	C10-500	C 4901	MCX0156-1	C-5[8]	7114-34[8]

Ferric Chloride (hydrated) ($FeCl_3 \cdot 6H_2O$)	EM-FX0210-1	I88-500	F 2877	MFX0210-1	F-10[8]	1772-26[8]
2-Chloro-(trichloro-methyl) Pyridine (TCMP), 2.2%					N-50	2579-24
Glucose/Glutamic Acid Standard					B-12D	14865-10

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[1] By including them in this list, Ecology does not endorse or discourage the purchase of chemicals from any vendor.
[2] VWR Scientific Products, P.O Box 3551, Seattle, WA 98124, (800) 932-5000
[3] Fischer Scientific, P O. Box 58056, Santa Clara, CA 95050, (800) 766-7000
[4] Sigma Chemical Company, P O Box 14508, St. Louis, MO 63178, (800) 325-3010
[5] Curtin Matheson Scientific, 822 South 333rd Street, Federal Way, WA 98003-6343, (800) 323-3987
[6] North Central Laboratories, P.O Box 8, Birnamwood, WI 54414, (800) 648-7836
[7] Hach Company, P O. Box 389, Loveland, CO 80539, (800) 227-4224
[8] Pillows containing all the chemicals marked by this note are available from NCL (H-14161) and Hach (14861-98 for 3 liters of dilution water; other sizes are available).

Date/Time Samples Taken:
Samples Taken By:

BOD BENCHSHEET (BOTTLE METHOD)

_____ BOD (Indicate which)
_____ CBOD

A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q
Sample	pH	Temp Deg C	Bottle #	In 300-mL Bottle: mLs Sample	In 300-mL Bottle: mLs Seed	All DOs below in mg/L: DO Initial	All DOs below in mg/L: DO 5-Day	All DOs below in mg/L: DO Drop G-H	All DOs below in mg/L: Seed Ratio Factor (f) ***	All DOs below in mg/L: Seed Correction (Jseed) X f	All DOs below in mg/L: Corr. DO Drop J-L	Dil. Factor 300 /E	BOD below in mg/L: Bottle BOD M x N	BOD below in mg/L: Reported BOD
Blank *														
Seed Control **														
Standard														
Influent, Dil. 1														
Influent, Dil. 2														
Influent, Dil. 3														
Effluent														

Appendix C (Page C-1)

* **Dilution Water Blank DO Depletion** (should be ≤0.2 mg/L)

** **Check of Seed Strength** (should be 0.6 - 1.0 mg/L per mL of seed)

J ÷ F for Seed Control = _____ mg/L ÷ _____ mL seed

= _____ mg/L DO per mL seed

*** **Seed Ratio Factor** = f = Vol Seed(samp)/Vol Seed(seed control)

Comments:

Samples analyzed by:

Date/Time initial DO:

Date/Time final DO

Checked by/date:

Date/Time Samples Taken:
Jan 29, 1998 7:30 a.m.
Samples Taken By:
I. M. Labber

BOD BENCHSHEET (BOTTLE METHOD)

X BOD (Indicate which)
____ CBOD

A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q
				In 300-mL Bottle		All DOs below in mg/L							BOD below in mg/L	
Sample	pH	Temp Deg C	Bottle #	mLs Sample	mLs Seed	DO Initial	DO 5-Day	DO Drop G-H	Seed Ratio Factor (f) ***	Seed Correction (Jseed) X f	Corr. DO Drop J-L	Dil. Factor 300 /E	Bottle BOD M x N	Reported BOD
Blank *	7.2	20.0	10			8.6	8.5	0.1						
Seed Control **	7.2	20.0	6		5	8.6	5.3	3.3						
Standard	6.8	20.0	12	5	1	8.6	4.7	3.9	0.20	0.66	3.24	60.0	194.4	194.4
Influent, Dil. 1	7.2	20.0	15	5	0	8.6	5.2	3.4	0.0	0.00	3.40	60.0	204.0	
Influent, Dil. 2	7.2	20.0	16	10	0	8.5	1.1	7.4	0.0	0.00	7.40	30.0	222.0	192.0
Influent, Dil. 3	7.2	20.0	17	15	0	8.5	1.0	7.5	0.0	0.00	7.50	20.0	150.0	
Effluent	7.0	20.5	3	250	1	8.5	4.1	4.4	0.20	0.66	3.74	1.2	4.5	4.5
Effluent Dupe	7.0	20.0	19	250	1	8.5	4.0	4.5	0.20	0.66	3.84	1.2	4.6	4.6

* **Dilution Water Blank DO Depletion** (should be ≤0.2 mg/L)

** **Check of Seed Strength** (should be 0.6 - 1.0 mg/L per mL of seed)

J ÷ F for Seed Control = **3.3** mg/L ÷ **5** mL seed

= **0.66** mg/L DO per mL seed

*** **Seed Ratio Factor** = f = Vol Seed(samp)/Vol Seed(seed control)

Comments:
Trying new seed - Clarifier #1
Rained all night 1/28-29

Samples analyzed by:
I. M. Labber

Date/Time initial DO:
1/29/98, 9 am

Date/Time final DO
2/3/98, 8:30 am

Checked by/date:
Iona Mercedes

Date/Time Samples Taken:

BOD BENCHSHEET (BOTTLE METHOD)
(Continuation Sheet)

A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q
				In 300-mL Bottle		All DOs below in mg/L							BOD below in mg/L	
Sample	pH	Temp Deg C	Bottle #	mLs Sample	mLs Seed	DO Initial	DO 5-Day	DO Drop G-H	Seed Ratio Factor (f) ***	Seed Correction (Jseed) X f	Corr. DO Drop J-L	Dil. Factor 300 /E	Bottle BOD M x N	Reported BOD

Comments:

Use of BOD Benchsheet (Bottle Method)

Following is an explanation of the various columns on the BOD Benchsheet on the preceding pages. Those columns which are self explanatory are not included below.

Column A...Sample. Identification of the sample being analyzed. Note there is only one line provided for the blank, seed control, and standard. The form can be modified to do replicates (e.g., duplicates) for any of these samples. Also, there are three lines provided for an "effluent" sample in recognition of the variability of such samples. Any sample for which the lab does not have a good estimate of the BOD should be run in more than one dilution.

Column B...pH. pH of the sample (before dilution, if it is a diluted sample).

Column C...Temp Deg C. The temperature of the solution in the bottle (should be 20 ± 1° C).

Column E...mLs Sample. Milliliters of sample, either from the environment, or the glucose/ glutamic acid standard, in the BOD bottle.

Column F...mLs Seed. Milliliters of seed in the BOD bottle.

Column J...DO Drop G - H. DO depletion over 5-day period. Initial DO minus final DO.

Column K...Seed Ratio Factor (f). Ratio of seed in the sample to seed in the seed control. Equals the volume of seed in the sample divided by the volume of seed in the seed control bottle.

Column L...Seed Correction (J_{seed}) x f. The DO depletion caused by the seed which must be subtracted from the overall DO depletion. Calculated by multiplying the Column J value for the seed control bottle by the seed correction factor, "f" (the Column K value) for the bottle being analyzed.

Column M...Corr. DO Drop J – L. The DO depletion caused by the sample (overall DO depletion minus the seed correction). The value in Column J minus the value in Column L for the bottle being analyzed.

Column N...Dil. Factor 300/E. The factor by which the DO depletion must be multiplied to account for dilution of the sample (called the dilution factor in this guidance document). Calculated by dividing the volume of the BOD bottle (300 in the case of the example) by the milliliters of sample in the bottle being analyzed.

Column P...Bottle BOD M x N. The calculated BOD of the bottle being analyzed, which is the corrected DO depletion (value in Column M) times the dilution factor (value in Column N).

Column Q...Reported BOD. The BOD value to be reported. If there was only one dilution, P and Q will be equal. If there was more than one dilution, this is the average of all those meeting the reporting criteria of $\geq$ 2.0 mg/L DO depletion with retention of $\geq$ 1.0 mg/L DO.

Date/Time Samples Taken:
Samples Taken By:

BOD BENCHSHEET (CYLINDER METHOD)

_____ BOD (Indicate which)
_____ CBOD

A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q
Sample	pH	Temp Deg C	Bottle #	In 1-L Grad. Cyl. mLs Sample	In 1-L Grad. Cyl. mLs Seed	All DOs below in mg/L DO Initial	All DOs below in mg/L DO 5-Day	All DOs below in mg/L DO Drop G-H	All DOs below in mg/L Seed Ratio Factor (f) ***	All DOs below in mg/L Seed Correction (Jseed) X f	All DOs below in mg/L Corr. DO Drop J-L	Dil. Factor 1000/E	BOD below in mg/L Bottle BOD MxN	BOD below in mg/L Reported BOD
Blank *														
Seed Control **														
Standard														
Influent, Dil. 1														
Influent, Dil. 2														
Influent, Dil. 3														
Effluent														

* QC Test - Blank DO drop should be <0.2 mg/L

** Seed Strength Check (should be 0.6 - 1.0 mg/L per mL seed)

J ÷ F for Seed Control = _____ mg/L ÷ _____ mL seed

= _____ mg/L DO per mL seed

*** Seed Ratio Factor = f = % Seed(samp)/% Seed(seed control)

Comments:

Samples analyzed by:

Date/Time initial DO:

Date/Time final DO

Checked by/date:

Date/Time Samples Taken:

BOD BENCHSHEET (CYLINDER METHOD)
(Continuation Sheet)

A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q
				In 1-L Grad. Cyl.		All DOs below in mg/L							BOD below in mg/L	
Sample	pH	Temp Deg C	Bottle #	mLs Sample	mLs Seed	DO Initial	DO 5-Day	DO Drop G-H	Seed Ratio Factor (f) ***	Seed Correction (Jseed) X f	Corr. DO Drop J-L	Dil. Factor 1000/E	Bottle BOD MxN	Reported BOD

Comments:

Date/Time Samples Taken:
1/29/98. 8:30 a.m.
Samples Taken By:
Anna Lyst

BOD BENCHSHEET (CYLINDER METHOD)

X BOD (Indicate which)
____ CBOD

A	B	C	D	E	F	G	H	J	K	L	M	N	P	Q
				In 1-L Grad. Cyl.		All DOs below in mg/L							BOD below in mg/L	
Sample	pH	Temp Deg C	Bottle #	mLs Sample	mLs Seed	DO Initial	DO 5-Day	DO Drop G-H	Seed Ratio Factor (f) ***	Seed Correction (Jseed) X f	Corr. DO Drop K-L	Dil. Factor 1000/E	Bottle BOD MxN	Reported BOD
Blank *	7	20.0	4			8.6	8.3	0.3						
Seed Control **	7.2	20.0	6		15	8.6	6.1	2.5						
Standard	6.9	20.0	2	15	3.0	8.6	4.8	3.8	0.2	0.50	3.30	66.7	220	220
Influent, Dil. 1	7.2	20.0	11	15	0.0	8.5	6.3	2.2	0.0	0.0	2.20	66.7	147	
Influent, Dil. 2	7.3	20.0	12	30	0.0	8.5	4.8	3.7	0.0	0.0	3.70	33.3	123	132
Influent, Dil. 3	7.3	20.0	13	50	0.0	8.4	2.1	6.3	0.0	0.0	6.30	20.0	126	
Effluent	7.1	20.5	20	800	5.0	8.6	4.5	4.1	0.33	0.83	3.27	1.3	4.1	4.1

* QC Test - Blank DO drop should be <0.2 mg/L

** Check of Seed Strength (should be 0.6-1.0 mg/L per mL seed)

J ÷ F for Seed Control = **2.5** mg/L ÷ **4.5** mL seed

= **0.6** mg/L DO per mL seed

*** Seed Ratio Factor = f = % Seed(samp)/% Seed(seed control)

Comments:

Blank exceeded criteria (<0.2 mg/L). Random contamination suspected. Does not invalidate batch. Will acid-rinse all glassware after this batch.

Samples analyzed by:
Anna Lyst
Date/Time initial DO:
1/29/98, 9:30 a.m.
Date/Time final DO
2/3/98, 10:30 a.m.
Checked by/date:
S. Visor

Use of BOD Benchsheet (Graduated Cylinder Method)

Following is an explanation of the various columns on the BOD Benchsheet on the preceding pages. Those columns which are self explanatory are not included below.

Column A...Sample. Identification of the sample being analyzed. Note there is only one line provided for the blank, seed control, and standard. The form can be modified to do replicates (e.g., duplicates) for any of these samples. Also, there are three lines provided for an "effluent" sample in recognition of the variability of such samples. Any sample for which the lab does not have a good estimate of the BOD should be run in more than one dilution.

Column B...pH. pH of the sample (before dilution, if it is a diluted sample).

Column C...Temp Deg C. The temperature of the solution in the bottle (should be 20 ± 1° C).

Column E...mLs Sample. Milliliters of sample, either from the environment, or the glucose/ glutamic acid standard, in the graduated cylinder.

Column F...mLs Seed. Milliliters of seed in the graduated cylinder.

Column J...DO Drop G - H. DO depletion over 5-day period. Initial DO minus final DO.

Column K...Seed Ratio Factor (f). Ratio of seed in the sample to seed in the seed control. Equals the percentage seed in the sample divided by the percentage seed in the seed control.

Column L...Seed Correction (J_{seed}) x f. The DO depletion caused by the seed which must be subtracted from the overall DO depletion. Calculated by multiplying the Column J value for the seed control bottle by the seed correction factor, "f" (the Column K value) for the bottle being analyzed.

Column M...Corr. DO Drop J – L. The DO depletion caused by the sample (overall DO depletion minus the seed correction). The value in Column J minus the value in Column L for the bottle being analyzed.

Column N...Dil. Factor 1000/E. The factor by which the DO depletion must be multiplied to account for dilution of the sample (called the dilution factor in this guidance document). Calculated by dividing the volume of the graduated cylinder (1000 in the case of the example) by the milliliters of sample in the graduated cylinder used to fill the bottle being analyzed.

Column P...Bottle BOD M x N. The calculated BOD of the bottle being analyzed, which is the corrected DO depletion (value in Column M) times the dilution factor (value in Column N).

Column Q...Reported BOD. The BOD value to be reported. If there was only one dilution, P and Q will be equal. If there was more than one dilution, this is the average of all those meeting the reporting criteria of $\geq$ 2.0 mg/L DO depletion with retention of $\geq$ 1.0 mg/L DO.

Appendix E

Statistics in BOD/CBOD Testing *

1. Most analysts are very faithful about running quality control tests such as a blank, a check standard (e.g., glucose/glutamic acid), and a duplicate in every BOD test. Some analysts consider only the result from each individual test to determine if the procedure is "in control" and meeting data quality objectives. For example, an analyst might consider a glucose/glutamic acid results to be acceptable as long as it is between 167.5 mg/L (i.e., 198 – 30.5 mg/L) and 228.5 (i.e., 198 + 30.5) because their interpretation of *Standard Methods* is that the expected average result should be within 30.5 mg/L of 198. Such analysts have not used statistics properly in evaluating their performance.

2. The statistics needed to evaluate performance of the BOD/CBOD test are relatively simple. The simplest is the ***mean*** or ***average result*** of repeated analyses of the same sample or type of sample. Virtually all analysts know that an average result is simply the sum of all results divided by the number of results. If the average result is close enough to the true or accepted value, the test is ***unbiased***. Bias is one of the components of accuracy (or inaccuracy), the other being precision (or imprecision).

3. Another statistic needed to fully evaluate lab performance, a little more complicated than the average, is the ***standard deviation***. The standard deviation of repeated analyses of the same sample is an indication of precision, the other component of accuracy. Only if a procedure is unbiased AND precise can it be said it is being done accurately.

4. One other statistic might be used to assist the analyst in discovering possible sources of error, and that is the ***relative percent difference***, or RPD. For the purposes of this guidance document, RPD is simply the difference between two results for a duplicate pair, divided by the average of the two, and expressed as a percentage**. The RPD gives the analyst an indication of within-batch precision, which is influenced by differences between, for example, two bottles in a BOD batch. Contaminated BOD bottles, where some are more contaminated than others, might cause random differences between two bottles in a duplicate pair. This would contribute to within-batch imprecision.

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* Analysts need not feel committed to using all of the statistical analysis tools mentioned in this appendix. The tools are provided to allow an in-depth review of lab performance if such is desired by the analyst or lab management.

** Some statisticians prefer to give RPD a sign, positive if the first result is larger than the second, and negative if the second result is larger than the first. For the purposes of this guidance document, it is simpler just to think of the RPD as an indication of how much the two results differed without considering which was greater.

5. While there are many other statistics that can be applied to analytical results, the three above are those easiest to apply to BOD testing. The following is a discussion of how these statistics can be applied to each of the three types of QC tests mentioned above, the blank, the check standard, and the duplicate.

Blanks

6. Normally it is not necessary to apply statistics to blank results. Either the blanks meet the criterion that they be less than 0.2 mg/L, or they do not. If only an occasional blank exceeds 0.2 mg/L (say one every ten batches), there is no cause for alarm. On the other hand, if blanks are normally 0.1 or 0.2 mg/L, even though they seldom exceed 0.2 mg/L, the cause of the positive blanks should be sought and eliminated if possible.

Check Standard (Glucose/Glutamic Acid)

7. Check standards are arguably the most useful QC test in that they give the analyst information about bias AND precision, the two components of accuracy.

a. **Average, the Bias Indicator**. The statistic associated with bias is the average, or mean value for repeated analyses. To be statistically valid, the average should be calculated based on at least 20 results (*Standard Methods 5210B* suggests 25). The average, as stated above, is simply the sum of all results, divided by the number of results, or....

Equation #1 - Average

$$\bar{x} = \frac{\sum x}{n}$$

where "x" is each result, "$\sum x$" is the sum of all results, and "n" is the number of results. The average is "$\bar{x}$," pronounced "x-bar." For repeated analyses of the glucose/glutamic acid standard, the average BOD should be in the vicinity of 198 mg/L.

(1) How close should it be to 198 mg/L? If it is considerably greater than 198, say 240, all seeded samples (e.g., effluent from a wastewater treatment plant) are probably biased on the high side. If the plant is still able to meet its permitted effluent limit for BOD with the results known to be biased on the high side, there is no significant risk to the environment, and the bias could be acceptable. However, the lab may not be able to successfully analyze performance evaluation samples, which would have an adverse impact on its accreditation.

(2) If, on the other hand, the average result is considerably lower than 198, say 156 mg/L, results for seeded samples would probably be biased on the low side, meaning a plant might be contributing more BOD to the receiving water than its lab results indicate. This IS a risk to the environment, and an attempt should be made to increase the average result, usually by using an alternative seed which would then be used for all seeded samples.

b. **Standard Deviation, the Precision Indicator.** A statistic associated with precision is the standard deviation of repeated analyses. This statistic is easily calculated using a scientific calculator or computer, or if such a calculator/computer is not available, it is not-so-easily calculated using the following equations where "s" is the standard deviation...

Equation #2 – Standard Deviation

$$s = \sqrt{\frac{\sum x_i^2 - \left[\left(\sum x_i\right)^2 / n\right]}{n-1}}$$

$$s = \sqrt{\frac{\sum x_i^2 - n\left(\bar{x}\right)^2}{n-1}}$$

$$s = \sqrt{\frac{\sum \left(x_i - \bar{x}\right)^2}{n-1}}$$

where "x_i" is the ith result, "$\bar{x}$" is the mean of the "i" results, and "n" is the number of results used to calculate $\bar{x}$.

For smaller labs that do not have a scientific calculator or computer, and have no desire to work their way through the above equations to determine standard deviation, analytical results can be sent to Ecology's Quality Assurance Section where the calculation can be made by computer and reported to the lab.

8. **Duplicate Samples**

a. **Relative Percent Difference (RPD), the Within-batch Precision Indicator.** By analyzing duplicate samples, the analyst can get a feeling for the precision of analyses within a batch. One statistic used to express the degree of within-batch precision indicated by the duplicates is relative percent difference (RPD). For the purposes of this discussion, RPD is simply the absolute difference between the two results for a duplicate pair, divided by the average result, and multiplied by 100 so as to express the statistic as a percent. Thus...

Equation #3 - RPD

$$RPD = \frac{100\left|D_1 - D_2\right|}{(D_1 + D_2)/2}$$

$$= 200\left|D_1 - D_2\right| / (D_1 + D_2)$$

Where "D_1" is the first result in the duplicate pair, "D_2" the second, and "$\left|D_1 - D_2\right|$" is the absolute difference between the two ("absolute" meaning without regard to whether it is plus or

minus). For example, if the first result in a duplicate pair is 12 mg/L, and the second result is 20 mg/L, the relative percent difference is...

Example – RPD

$$RPD = 200\,|12 - 20|/(12 + 20)$$

$$= 200(8)/32 = 1600/32 = 50\%$$

In preparing this guidance, the author could not find a widely-recognized data quality objective for the RPD of duplicate BOD samples. Past experience indicates that the 50% RPD given in the example above, or lower, might be a reasonable goal for effluent samples in the range of 10 to 20 mg/L. RPD should decrease as concentration increases. For example, RPD for duplicate glucose/glutamic acid samples (accepted value of 198 mg/L) might be 5% or even lower

b. **Standard Deviation of the Difference, Another Within-batch Precision Indicator.** If samples in a narrow concentration range, perhaps effluent from a wastewater treatment plant that normally runs in the 10 – 20 mg/L range, are analyzed in duplicate over several batches, one can calculate the standard deviation of the difference by pooling the duplicate differences. This statistic is discussed in Appendix J of the *Procedural Manual for the Environmental Laboratory Accreditation Program* and will not be further addressed here. An Excel spreadsheet program is available gratis from Ecology that constructs a control chart based on the standard deviation of the difference between duplicate pairs in a relatively narrow concentration range (see the following paragraph). An advantage of using duplicates to estimate precision is that analyses are done on real samples as opposed to synthetic samples (i.e., glucose/glutamic acid) analyzed as a BOD standard.)

9. **Statistics Applied to Control Charting**

a. Control charting is a means of visually tracking performance to determine when a procedure is "out-of-control" (i.e., not meeting data quality objectives). Even better, control charts indicate when a procedure is **headed** out-of-control so the analyst can pause, eliminate the source of the problem, and prevent the out-of-control situation. In BOD/CBOD testing, there are two control charts that can be used very effectively to continuously monitor results, a control chart based on repeated analyses of the check standard, and a chart based on the difference between duplicate pairs. Both of these charts are discussed in detail in Appendix J of the *Procedural Manual for the Environmental Laboratory Accreditation Program* and will be described only briefly here.

b. A control chart can be used to monitor performance on the glucose/glutamic acid standard by analyzing results from the last 20 tests (or the last 22 tests, eliminating the highest, and the lowest, and using the 20 remaining.)

(1) The chart is constructed by drawing a center line on a graph representing the average (mean) result, and above it, a line representing the average plus two standard deviations, and another representing three standard deviations. The same is done below the mean, subtracting two and three standard deviations. Before this chart is constructed, the lab must make sure the average value is close enough to the 198 mg/L guidance of *Standard Methods*. Once constructed, this chart is used to monitor subsequent results for the GGA test. All subsequent results should lie between the outer limits of the chart (the lines at plus and minus three standard deviations, referred to as **action**

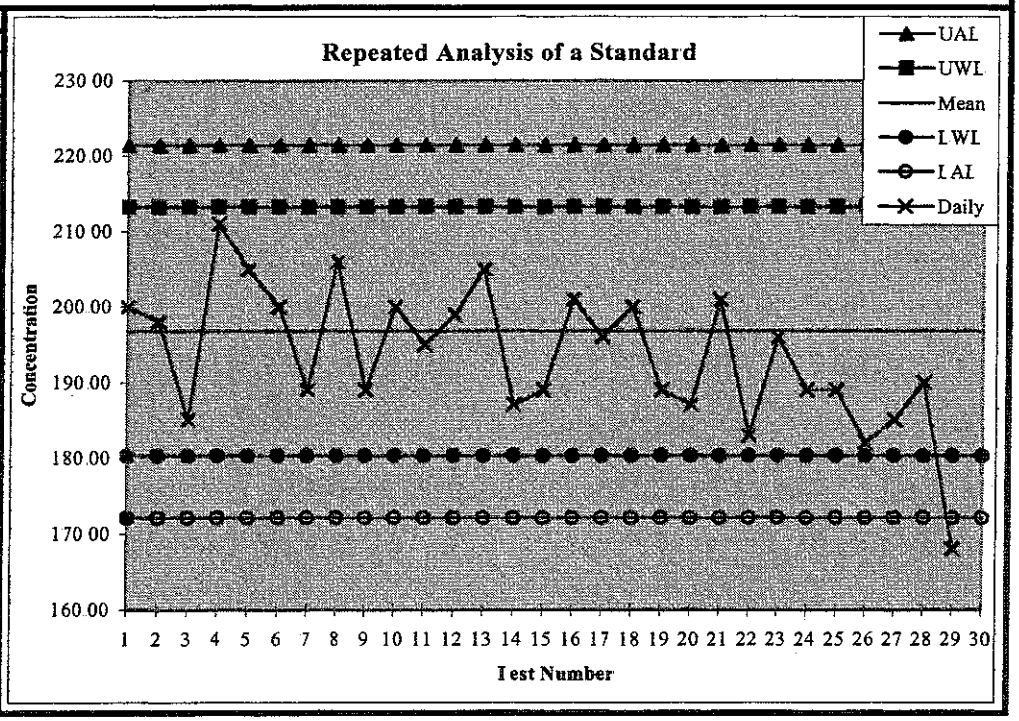
limits). Only 5% of subsequent points should lie outside the plus and minus two standard deviation limits, referred to as **warning** limits. Very importantly, there should be no noticeable trend (e.g., upward or downward) in the subsequent results.

(2) Figure E-1 is a control chart based on real data resulting from repeated analyses of a glucose/glutamic acid standard for BOD. The lab's average for 20 results was 196.8 mg/L, the central line on the chart, and the standard deviation for those same 20 results was 8.2 mg/L. This lab was obviously "in-control" at the time the chart was constructed. The 196.8 average is certainly close enough to the *Standard Methods* guidance of 198 mg/L, and the standard deviation of 8.2 mg/L indicates excellent precision. The lab knew that it had a problem where a result would be far below what could be considered acceptable. This meant to them that it was time to get fresh seed from a local wastewater treatment plant. The cycle was repeated several times in the past years. By plotting their results, the author showed the lab that, had they been using a control chart, they would have undoubtedly noticed the downward **trend** in results well before the unacceptably-low result. By using such a chart and collecting fresh seed as soon as the downward trend is noticed, the lab has since avoided out-of-control situations.

Figure E-1 – Control Chart Based on Repeated Analysis of a GGA Standard

CASCADE WTP LAB GLUCOSE/GLUTAMIC ACID BOD STANDARD

Glucose/Glutamic Acid BOD			
Preliminary Tests		Daily Tests	
Test	Result	Test	Result
1	200	1	200
2	192	2	198
3	201	3	185
4	185	4	211
5	204	5	205
6	210	6	200
7	195	7	189
8	200	8	206
9	189	9	189
10	203	10	200
11	211	11	195
12	208	12	199
13	197	13	205
14	185	14	187
15	189	15	189
16	201	16	201
17	200	17	196
18	190	18	200
19	189	19	189
20	187	20	187
STATISTICS for Preliminary tests		21	201
		22	183
		23	196
		24	189
Std Dev	8.2	25	189
Mean	196.8	26	182
UAL	221.5	27	185
UWL	213.3	28	190
LWL	180.3	29	168
LAL	172.1	30	



(3) Figure E-2 is a control chart based on fictitious but reasonable results for analysis of effluent BOD duplicates. This control chart is based on a central line of 0.0 mg/L, the expected difference between duplicates in a pair. Upper and lower warning and action limits are charted on each side of the central line based on the standard deviation of the difference between the pairs in 20 duplicates. This chart illustrates how a lab's precision had been doing well, with results #1 through #17 or so being within the warning limits. But, after that, the precision had deteriorated, indicating a change for the worse. This lab would have then paused to find the problem causing the greater imprecision (it could have been a new analyst, a new seed, a malfunctioning DO meter, or one of many other sources of random error).

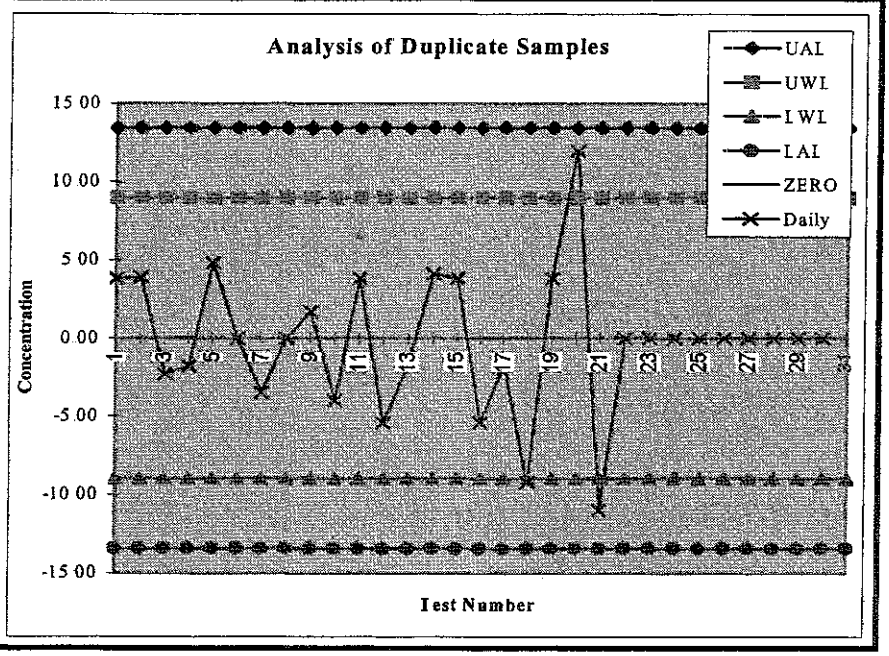
Figure E-2 – Control Chart Based on Analysis of Duplicate Samples

CASCADE WTP LAB EFFLUENT DOB DUPLICATES

BOD in mg/L

Preliminary Tests			Daily Tests			
Test	#1	#2	Test	#1	#2	Difference
1	10.4	14.2	1	18.0	14.2	3.80
2	18.0	12.6	2	16.5	12.6	3.90
3	16.5	14.8	3	12.5	14.8	-2.30
4	12.5	20.1	4	14.2	16.0	-1.80
5	14.2	10.4	5	18.0	13.2	4.80
6	12.6	18.0	6	16.5	16.5	0.00
7	14.6	16.5	7	12.5	16.0	-3.50
8	20.1	12.5	8	14.2	14.2	0.00
9	14.2	10.4	9	16.5	14.8	1.70
10	12.6	18.0	10	12.6	16.5	-4.00
11	14.8	19.5	11	14.2	10.4	3.80
12	20.1	12.6	12	12.6	18.0	-5.40
13	18.0	14.2	13	14.8	16.5	-1.70
14	16.5	12.6	14	20.1	16.0	4.10
15	12.5	14.8	15	14.2	10.4	3.80
16	14.2	20.1	16	12.6	18.0	-5.40
17	18.0	18.0	17	14.8	16.5	-1.70
18	16.5	16.5	18	12.0	21.2	-9.20
19	12.5	12.5	19	18.0	14.2	3.80
20	14.2	14.2	20	20.0	8.0	12.00
			21	9.00	20.00	-11.00
			22			
			23			
			24			
			25			
			26			
			27			
			28			
			29			
			30			

STATISTICS for Preliminary tests	
Std Deviation of the difference	4.48
UAL	13.4
UWL	9.0
LWL	-9.0
LAL	-13.4



c. An MS Excel spreadsheet file was used to generate each of the preceeding control charts. Copies of the Excel files are available to accredited labs by contacting the Department of Ecology Quality Assurance Section.

Appendix F

Troubleshooting the BOD/CBOD Test

The results of quality control (QC) tests analyzed with each BOD/CBOD batch, combined with the results for the seed control and environmental samples, can be used to tell the analyst that a problem exists. The results can also give hints on what might be causing the problem, and what to do about it. Absence of any indicators of a problem is reassuring evidence that the analytical process is "in control." This appendix addresses some of the common problems experienced in BOD/CBOD labs and possible causes, and suggests actions to eliminate the problems. The following is organized in sections according to the sample to which the results apply. Possible causes are listed in order of likelihood with the most probable first.

Sample	Indicator	Possible Cause	Possible Solution	Reference
Blank	Usually exceeds 0.2 mg/L	Source water is unsuitable	Incubate several blanks using alternate water(s); choose best	Pg 26, para 9b(5) Pg 13, para 7a(3)(b)
	Sometimes exceeds 0.2 mg/L (i.e., DO depletion exceeds 0.2 mg/L)	Dilution water is supersaturated when poured into BOD bottle	Aerate day before test, store in incubator, add nutrients/buffers day of test, swirl to mix, siphon into bottom of BOD bottles	Pg 25, para 9b(2) Pg 13, para 7a(3)(b) Pg 10, para 6f
		Labware is contaminated	More aggressive cleaning of labware including acid rinse if necessary	Pg 25, para 9b(1) Pg 13, para 7a(3)(b)
	Sometimes negative (i.e., final DO is greater than initial DO)	Temperature of bottle contents is sometimes >20 ± 1°C when initial DO is read *	Keep source water, dilution water in incubator	Pg 25, para 9b(3) Pg 13, para 7a(3)(b)
		Photosynthesis is occuring during incubation	Clean BOD bottles, incubate in dark	Pg 26, para 9b(3) Pg 13, para 7a(3)(b)

Sample	Indicator	Likely Cause	Possible Solution	Reference
Blank (Cont'd)	Sometimes negative, sometimes positive	DO is not being measured correctly	Check entire DO measurement procedure	Pg 26, para 9b(6) Pg 13, para 7a(3)(b)
		Atmospheric pressure not being accounted for when calibrating DO meter	Use barometer and Table 4 when calibrating DO meter	Pg 25, para 9b(4) Pg 13, para 7a(3)(b)
		Any combination of the above possible causes	Be more consistent at doing the entire procedure correctly	Pg 25, para 9b Pg 13, para 7a(3)(b)
Glucose/ Glutamic Acid	Standard deviation of last 20 results is less than but approaching 30.5 mg/L	Analysis not being done the same, time after time. Could be variable seed, temperature or pressure problem, faulty DO readings, inattention of analyst(s), or any of countless other things	Look for sources of variability, eliminate them	Pg 26, para 9c Pg 27, para 10b
	Standard deviation of last 20 results is > 30.5 mg/L *	Many sources of random error; BOD procedure is in big trouble	Request assistance from Ecology (e.g., technical assistance specialists or Quality Assurance Section	Pg 26, para 9c Pg 27, para 10b

(Note: Before worrying about the average result (see next two entries) for several analyses of the glucose/glutamic acid solution, the analyst must be sure that the analysis is being done consistently (i.e., that the standard deviation is acceptable, well below 30.5 mg/L).

Average result for last 20 results is >> 198.5*	Seed is too strong	Try alternate seed	Pg 26, para 9c
Average result for last 20 results is << 198.5*	Seed is too weak	Try alternate seed (seed with many bacteria of the right type is needed)	Pg 26, para 9c

* > is "greater than;" >> is "much greater than;" < is "less than;" << is "much less than"

Sample	Indicator	Likely Cause	Possible Solution	Reference
Duplicate Effluent Samples	RPDs (see App E) for duplicate pairs exceed 50% (for samples in the range of 5-20 mg/L)	Random errors within the batch; could be improperly mixed seed, faulty measurement of seed, contaminated BOD bottles, faulty DO probe, and countless other things that change from bottle to bottle	Try to eliminate possible sources of random error within a batch	Pg 27, para 9d Pg 26, para 10b
Seed Control Bottle(s)	DO depletion is <0.6 mg/L per mL of seed in bottle	Seed is too weak	Try alternate seed (seed with many bacteria of the right type is needed)	Pg 14, para 7c(2)
	DO depletion is >1.0 mg/L per mL of seed in bottle	Seed is too strong	Try alternate seed	Pg 14, para 7c(2)
	DO depletions sometimes <<0.6, sometimes >>1.0 mg/L per mL of seed in bottle	Seed is too variable	Need to find a more consistent seed (for example, raw influent is often too variable)	Pg 14, para 7c(2)
Environmental Samples	No dilutions leave at least 1.0 mg/L DO after incubation	Samples not dilute enough	Add even more-dilute samples to batch (some labs do preliminary TSS or COD to estimate BOD and proper dilution)	Pg 24, para 9a Pg 9, Table 3 Pg 13, para 7b
	No dilutions deplete at least 2.0 mg/L DO	Samples too dilute	Run essentially 100% sample (e.g., 285 mL sample, 10 mL buffer/ nutrient solution, 5 mL seed)	Pg 24, para 9a Pg 9, Table 3
	Significant BOD increase for more-dilute bottles	Matrix is interfering with the test	Report BOD of most dilute bottle	Pg 24, Note 11 and Table 7

Influent BOD (and TSS) suddenly higher than normal, or always higher when done by one analyst than when done by others	Sample is not being thoroughly mixed before taking aliquots (sub-samples)	Thoroughly mix sample by shaking, rolling, stirring, blending (at low speed)	Pg. 4, para 3a

NOTE: Knowledge can be gained by studying this document; wisdom can be gained only by observation. When trying to discover what is causing a problem with the $BOD_5/CBOD_5$ test, the wise thing to do is change one likely cause at a time…maybe two…and observe what impact the change has on the indicator being monitored. Keep good records, and if something works, use it!

Appendix G

BOD_5/$CBOD_5$ Checksheet

Ref: SM (16th ed) 507; SM (18th, 19th, 20th ed) 5210 B; EPA 405.1

	Yes	No	Comments
1. Is approved method followed?			
Method _____			
2. Is incubator adequate (i.e., clean, excludes light, circulates air/water)?	_____	_____	_____
3. Are samples stored (if necessary) at 4° C?	_____	_____	_____
4. Is sample source and type (i.e., grab or composite) recorded?	_____	_____	_____
5. Are samples analyzed within:			
_____ 48 hours?			
_____ 6 hours?			
6. If DO probe is used, is it calibrated:			
_____ against air?			
_____ against Winkler titration?			
_____ against O_2–saturated water?			
before each day's use?	_____	_____	_____
7. If DO probe is used, is it properly maintained so - -			
there are no bubbles under the membrane?	_____	_____	_____
the membrane is not allowed to dry out?	_____	_____	_____
there is no growth under the membrane?	_____	_____	_____

8. Are proper BOD bottles used (e.g., 75-, 125-, 250-, or 300-mL)? _____ _____ _____

Sealed during use? _____ _____ _____

9. Does incubator maintain temperature of 20 ± 1° C? _____ _____ _____

10. Is incubator thermometer certified to ± 1° C? _____ _____ _____

11. Is buffer added to dilution water...

only on day of use for BOD? _____ _____ _____

on day of use or earlier for CBOD? _____ _____ _____

12. Is buffer stored in refrigerator? _____ _____ _____

13. Is source water...

_____ Deionized

_____ Distilled What type of still _____

_____ Purchased Brand Name _____

14. Is dilution water protected from atmospheric contamination? _____ _____ _____

15. Are dilution water blanks analyzed? _____ _____ _____

16. Is the blank depletion...

usually zero? _____ _____ _____

sometimes 0.1 mg/L? _____ _____ _____

always less than 0.2 mg/L? _____ _____ _____

17. Are BOD bottles and glassware cleaned with non-phosphate detergent and (as necessary) acid rinsed? _____ _____ _____

18. Are samples neutralized to pH 6.5 – 7.5? _____ _____ _____

19. If doing CBOD, is nitrification inhibitor added to . . .

_____ dilution water?

_____ sample?

20. Are reagents for dilution water properly prepared - -

Ferric chloride (0.25 g/L)? _____ _____ _____

Magnesium sulfate (22.5 g/L)? _____ _____ _____

Calcium chloride (27.5 g/L)? _____ _____ _____

Phosphate Buffer (pH = 7.2)? _____ _____ _____

Sodium sulfite (1.575 g/L), prepared daily? _____ _____ _____

. . .or are they purchased pre-packaged _____ Vendor: _____

21. Are bottles brought to 20 ± 1° C before reading initial DO? _____ _____ _____

22. A samples thoroughly mixed before taking aliquots (sub-samples)? _____ _____ _____

23. Is reference solution (150 mg each of glucose & glutamic acid diluted w/distilled water to 1 L) run with each batch of samples? _____ _____ _____

24. Do repeated results for the G/GA solution average. . . .

in the vicinity of 198 mg/L for BOD? Average: _____ mg/L

in the vicinity of 184 mg/L for CBOD? Average: _____ mg/L

25. Is the standard deviation of repeated analyses of the G/GA solution . . . . . .

much less than 30.5 mg/L for BOD? _____ _____ _____

much less than 28.3 mg/L for CBOD? _____ _____ _____

26. If present, is chlorine removed with sodium sulfite and are samples properly seeded? _____ _____ _____

27 What is the source of seed...

_____ final effluent from WWTP?

_____ primary effluent from WWTP?

_____ Artificial seed (e.g., Polybac)? Which one? _____

_____ Frozen sewage?

_____ Other? What? _____

28. Are proper dilution techniques used? _____ _____ _____

29. Do dilution have depletions of at least 2 mg/L? _____ _____ _____

and retention of at least 1 mg/L? _____ _____ _____

30 Are samples incubated for 5 days ± 2 hours? _____ _____ _____

31. Are calculations completed properly? _____ _____ _____

32. Are records properly authenticated (i.e., signed/initialled by analyst and one other)? _____ _____ _____

33. Are duplicates run regularly (not a requirement)? _____ _____ _____

34. Is a control chart available and used? _____ _____ _____