Standard Operating Procedure for:

Biochemical Oxygen Demand

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1. SCOPE AND APPLICATION

1.1. This method is based on Standard Methods 5210B, Online Edition. The test measures the oxygen depletion potential of ambient, surface, or effluent waters during a 5 day incubation period. It can also help determine the BOD removal efficiency of wastewater treatment plants. During the 5 day incubation, oxygen is essentially consumed by bacteria for biochemical degradation of organic materials (carbonaceous) and oxidation of inorganic materials (sulfides, ferrous iron, and reduced forms of nitrogen). The BOD test measures both carbonaceous and nitrogenous demand for oxygen. In the CBOD test, nitrification is inhibited and therefore measures only carbonaceous demand.

1.2. This procedure is used for samples with LIMS test IDs of BOD-INHB and BOD-UNIN.

1.3. Method Detection Limits (MDL)

1.3.1. A true MDL cannot be determined for the BOD test. Therefore, MDL is set at 0.20 mg/L, since this is the maximum allowable depletion for a dilution water blank. The PQL is set at 2.0 mg/L because of the 2.0 minimum depletion method requirement.

2. SUMMARY OF METHOD

2.1. A sample is poured into an airtight 300 mL BOD bottle. It is seeded to ensure a sufficient bacterial population for the test. Nutrients and buffers may also be added to the bottle. The initial dissolved oxygen of the sample bottle is measured. It is then incubated at 20 ± 1 ºC for five days. After this, a final DO measurement is taken. The BOD is calculated using the difference between initial and final DO measurements and corrected for seed depletion.

2.2. Interferences

2.2.1. During incubation, photosynthetic production of DO can occur if samples are not kept away from light.

2.2.2. Sodium sulfite solution, which is used for dechlorination of sample, can also exert an oxygen demand. A titration for each chlorinated sample should be run, to ensure only the minimum amount of sodium sulfite is used to dechlorinate the sample.

2.2.3. Some metals and other substances in the water sample may be toxic to bacteria and can therefore cause matrix interference. Using larger dilutions can help minimize the effect of a toxic sample.

2.3. Definitions

2.3.1. IDO – Initial Dissolved Oxygen is the DO measurement taken on the first day of the test.

2.3.2. FDO – Final Dissolved Oxygen is the DO measurement taken on the final day (Day 5) of the test.

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2.3.3. 2-1 Rule - The 2-1 Rule refers to the method requirement that for a test bottle to be considered valid, it should have a minimum depletion of 2.0 mg/L after a 5 day incubation period and at least 1.0 mg/L residual DO for the final reading.

3. EQUIPMENT AND SUPPLIES – Record any equipment repairs or maintenance in LIMS Laboratory Logbook *Maintenance_Log*

3.1. 300 mL Wheaton BOD bottles

3.1.1. Wash bottles in laboratory dishwasher. When cycle is complete, immediately remove bottles from dishwasher and place in drying oven until completely dry.

3.2. Aeration manifold with Tygon tubing

3.3. BOD AutoEZ Reader w/ 4 YSI probes (S/N A0089)

3.3.1. Calibrate per SOP BB-25

3.3.2. EZ_BODReaderVSa.exe resides in \\fldep1\DEAR\Labs\Lab5\Biology\Bench Common\BOD\autoez\New AutoEZ\All Versions. To run the program from the BOD computer, create a shortcut of the most current version of the software on the BOD computer desktop.

3.3.3. Prior to first using any new version of the BOD program, make sure the program parameters are set as listed below by choosing **File/Setup/General** under the "General" tab.

- Company Name – FL DEP
- Bottle Volume 60 or 300 mL – 300
- Default Seed Volume m./Btl Volume – 1
- Seed Method Number: 1) Direct; 2) Seeded Dil Water; 3)Both – 1
- Both Seed Method Option – Max Vol ml – 0
- Both Seed Method Option – Vol Seed Added ml – 0
- IDO/FDO Requirement: 1)2-1 Rule; 2)25-75% Rule – 1
- Exclude Seed Controls from above Rule – FALSE
- MDL/Reporting Limit Option # 1)None; 2)Depltn Rule; 3)Seed Corrected Depltn – 1
- GGA Upper Control Limits – 228.5
- GGA Lower Control Limits – 167.5
- Outlier Rejection Criteria – Relative % i.e. 25 or(0 to turn OFF) – 30 Flag Only
- Outlier Rejection Criteria – Absolute Conc mg/L i.e. 1 or (0 to turn OFF) - 0
- Outlier Rejection Criteria – Cutoff Conc for Absolute Criteria mg/L i.e. 30 or(0 to turn OFF) – 0
- Slope(%)used to flag a sliding BOD or (0 to turn OFF) – 20
- Significant figures – 3
- Import path - \\fldep1\DEAR\Labs\Lab3\Biology\BOD\Import Files
- Worksheet path - \\fldep1\DEAR\Labs\Lab3\Biology\BOD\Worksheet
- Export Path - \\fldep1\DEAR\Labs\Lab3\Biology\BOD\Worksheet
- Archive Path - \\fldep1\DEAR\Labs\Lab3\Biology\BOD\Archive
- Site Elevation (Feet) – 876
- Theoretical D.O. Allowable Exceedance +/-(mg/L) – 0.4
- Default Super Saturation Warning Limit 9.1 (Set to Zero to use TDO+Exceedance Above – 9.1
- Default Temperature Warning Limit (20 +/-) – 3
- Date Format ie MM-dd-yy – MM-dd-yy
- External Import Path\Program (ie:'C:\EZ_BODReader1\Support\jnkProj1.exe' or leave blank) – (leave blank)

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- External Data Transfer (Path\Program or leave blank) – (leave blank)
- Include Calibration History with Final Report – TRUE
- Test IDs (separate with semicolons) and Display Order ie BOD5;CBOD5 – BOD5;CBOD5;SolBOD5;SolCBOD
- Seed Option Display Order ie S1;S2;DS1;DS2;S3;S4….. – "S1;S2;S3;S4;S5;S6;DS1;DS2;DS3;DS4;DS5;DS6)"
- Set IDs and Display Order ie PLT;QA;IW – PLT;QA;IW
- Comm Port BODAutoEZ ie COM1 – COM4
- External Meter Present TRUE/FALSE – FALSE
- Maximum Bottle Number (before wrapping back to 1) – 490
- Maximum Allowable Blank Depletion Range +/- (ie 0.24) – 0.24
- Export Entire Worksheet – FALSE
- Track Setup Date_Time of each sample - FALSE

3.4. YSI Model 50B DO Meter (S/N 96D49137)

3.4.1. Calibrate per SOP BB-25

3.5. BOD AutoEZ Trays

3.6. Disposable pipettes, 10 mL – volumes of each new lot are checked per SOP MB-03.3

3.7. Graduated cylinders, 10-250 mL

3.8. Chlorine Test Strips

3.9. Accumet Excel XL 25 Dual Channel pH/Ion meter (S/N XL94005930) – calibrate per SOP MB-03.4

4. REAGENTS AND STANDARDS

4.1. Deionized water

4.2. Nitrification Inhibitor, Formula 2533 (2% TCMP)

4.3. Phosphate-buffer solution, commercially prepared

4.4. Magnesium sulfate solution, commercially prepared

4.5. Calcium chloride solution, commercially prepared

4.6. Ferric chloride solution, commercially prepared

4.7. Sodium sulfite solution

4.7.1. In a 1 L volumetric flask, add 1.575g Na_2SO_3 to a small volume of DI water. Bring volume up to 1 L with DI water. Prepare on day of use.

4.7.2. Proper dechlorination of the sample serves as verification of the concentration of the sodium sulfite solution.

4.8. Potassium iodide solution

4.8.1. In a 100 mL volumetric flask, add 10 g of potassium iodide to a small volume of DI water. Bring volume up to 100 mL with DI water.

4.9. Sulfuric acid, H_2SO_4, 1N

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4.9.1. Slowly add 28 mL of concentrated sulfuric acid to deionized water and dilute to 1 L in a class A volumetric flask.

4.10. Sodium hydroxide, NaOH, 1N

4.10.1. Dissolve 40 g NaOH in deionized water and dilute to 1 L in a class A volumetric flask.

4.11. Glucose glutamic acid solution (GGA)

4.11.1. In a Class A volumetric flask add 0.15g of dried reagent grade glucose and 0.15g dried reagent grade glutamic acid to a small volume of DI water and bring volume up to 1L with deionized water.

4.11.2. Prepare on the day of use. Log preparation into LIMS **Standard Preparation Tracker** with a holding time of 24 hours.

4.12. Seed solution

4.12.1. Pour the entire contents of a PolySeed® capsule into a beaker containing 250 mL of dilution water. Aerate and stir the solution for one hour. Allow the bran to settle for 5 to 15 minutes. Decant into a flask through a mesh screen to remove the bran from the solution. Place a stir bar in the beaker containing the decanted seed and spin for the remainder of the test. Use within 6 hours of hydration.

5. CALIBRATION AND STANDARDIZATION

5.1. See SOP BB-25 for calibration and maintenance of DO meters.

6. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1. See SOP FS 1000 for sample collection and preservation.

6.2. Store samples at 2 – 4 ºC. Analysis should start within 48 hours of collection.

7. SAMPLE PREPARATION

7.1. Refer to SOP BB-030 for sample custody and label printing.

7.2. BOD Batch Preparation

7.2.1. Separate samples into batches, which contain a maximum of 20 samples by adding sample labels to the *BOD Run Log*. BOD-UNIN samples and BOD-INHB samples will be in different batches. Each *BOD Run Log* page will correspond to one batch and should include a dilution water blank, GGA standard and a lab duplicate.

7.3. BOD AutoEZ Worksheet Setup

7.3.1. Open LIMS. Select **General -> LIMS Browser -> Search**

- Check the following:

Search By: Job ID

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Additional Selections: Analyses
Job Status: Reported/A/C/V
Filter By Date: Received

- Click *Build List* for Job ID, select jobs, then click *Build List* for Analyses. Select all BOD analyses. Click OK. Click *Report/Files*. Click *Select All Events* and *Select All Jobs*.
- Create a BOD Worksheet template. From Available Fields, add to Selected Fields/Column Order: Field ID, Sample ID, Analysis. Sort by Sample ID. Select Save Template and save as BOD Worksheet.
- Create Delimited File and save to Desktop as "BOD*date*".

7.3.2. Open the template named SampleDEP.csv (\\fldep1\DEAR\Labs\Lab3\Biology\BOD\Import Files)

- Copy and Paste the Desktop file ("BOD*date*") into the SampleDEP.csv template (only the Sample IDs and Field IDs). Change dilution volumes in the "Vol" column and change 0 to 4 in "Qcode" column for the duplicate sample. Duplicate samples should have "_dup" added after the sample ID. Delete all unused cells.
- Save file as "BOD*date*" to Import Files.

7.3.3. Open the EZ_BOD Reader program.

- Select **File-> New**.
- Double click the blue header. Add dilution water serial #, GGA serial #, Polyseed serial #, and Tray # to the worksheet.
- Use **Worksheet-> Add Set/Batch of Samples** from the menu to add the seed control and sample sets to the worksheet. Use the file created and saved in Import Files.
- Save the file with the file name "BODMM-DD-YYYY" (MM-DD-YYYY being the current month, date, and year) in the following directory: \\fldep1\DEAR\Labs\Lab3\Biology\BOD\Worksheet in the appropriate year and month folder. Record the filename as "BODMM-DD" (MM-DD being the current month and date) in the *BOD Run Log*. Because of bugs in the program, saving the file may delete one line from the header each time. Use the enter key to add a few extra lines to the end of the header to prevent lost header info from multiple saves.

7.3.4. The BOD program keeps two temporary files. The first file is generated after the first automated reading. It is a copy of the original file and does not contain any data. The second file is generated after the next automated reading and should contain initial dissolved oxygen readings. Do not delete the temporary files.

7.4. Sample Pretreatment

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7.4.1. Check pH and record in *BOD Run Log* for all samples. If pH is outside the range of 6.0-8.0 wait until the day of the analysis and warm the sample to 20 ± 3°C. Next, adjust the pH between 7.0 and 7.2 with sulfuric acid or sodium hydroxide. Do not dilute the sample by more than 0.5%. Record the serial # for sulfuric acid and/or sodium hydroxide used in the *BOD Run Log*.

7.4.2. If samples are suspected of containing chlorine or are of an unknown source, check for chlorine with chlorine test strips. Record lot number of chlorine test strips in the *BOD Run Log*. (This can be done the day before set-up. Samples do not need to be warmed first.) If residual chlorine is present, dechlorinate sample as follows:

- Add 1 mL of concentrated sulfuric acid and 10 mL of potassium iodide (KI) solution to a 100 mL portion of sample.
- Add 5 drops of starch indicator to the sample solution.
- Titrate with sodium sulfite solution until the sample is colorless. Record the volume of sodium sulfite used in the *BOD Sample Dechlorination Log* logbook.
- Dechlorinate the original sample with the recorded amount per 100 mL volume of sample left and mix.
- Recheck the sample with a chlorine strip, to ensure that chlorine has been neutralized. If residual chlorine is still present, repeat the titration process. Record the additional volume of sodium sulfite used in the *BOD Sample Dechlorination Log* logbook.

7.4.3. On day of set-up, place samples in 44.5 ± 0.2°C water bath for 3.5 minutes to warm to 20 ± 3°C, then aerate for 10 minutes. Record the date/time samples are placed into the water bath. This is considered the start of analysis.

Note: Do not allow samples to sit between warming and aeration as this may affect the oxygen depletion of the sample and cause results to be inaccurate.

8. SAMPLE ANALYSIS

8.1. Dilution Water Preparation

8.1.1. Determine amount of deionized water needed. Multiply 300 mL by the number of GGA, blank, seed, and dilution bottles needed and round up to the nearest liter. Add this amount to clean, autoclaved large flasks or carboys and lightly aerate overnight.

8.1.2. Immediately before use, add 1 mL of the following solutions per liter of DI water:

- $MgSO_4$ solution
- $CaCl_2$ solution
- $FeCl_3$ solution

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- Phosphate buffer solution

8.1.3. Record preparation into LIMS **Standard Preparation Tracker** with a holding time of 24 hours.

8.2. Sample Setup

8.2.1. Pull out number of trays needed for the run (12 bottles per tray) and record tray numbers in *BOD Run Log* in order of use. Record bottles numbers in the *BOD Run Log*, starting with the first position, Probe 1 Row 1 (see Figure 1), then Probe 2 Row 1 and moving through each row in this manner in *BOD Run Log*, starting with the seed controls then each batch (blank, GGA, samples).

8.2.2. Add 1 pump of nitrification inhibitor to all CBOD samples, seed, and inhibited GGA bottles. Add 6 mL of GGA solution to each GGA control bottle.

8.2.3. In the following order, add 10, 12, and 15 mL of seed to the seed bottles. Add 2 mL of seed to all GGA and sample bottles.

8.2.4. Samples with no dilutions (300 mL) can be poured directly into the BOD bottles. For dilutions, use a clean graduated cylinder to measure and pour the appropriate volume of sample. Vigorously shake sample container before pouring out sample to mix well and aid in removing excess oxygen.

8.2.5. Top off any unfilled bottles with dilution water. This should include, blank, seed control, GGA, and sample dilution bottles.

8.3. IDO readings

8.3.1. CalibrateBOD AutoEZ per SOP BB-25.

8.3.2. Place up to 2 trays in the BOD AutoEZ analyzer in *BOD Run Log* order. The tray label should be on the left side, when facing the front of the analyzer (power button side). Make sure the trays are pushed all the way to the back of the analyzer.

8.3.3. In the EZ_BOD Reader program make sure the appropriate worksheet is open and select **Read-> Automated Reader** from the menu. Click the **Start** button, check **IDO**, and enter the number of rows to read.

8.3.4. The analyzer will measure DO readings for the number of selected rows and will pause if trays need to be switched out. When the analyzer is finished, click **Exit**. If readings are questionable, Select **Read-> Manual Reader**, reread samples and write DOs. A script bug exists during this process. If an error message box appears, save file per section 7.3.3. Close and reopen the program and resume normal operation.

8.3.5. IDO readings should be between 7.5 and 9.5 mg/L. See section 10.4.1 if readings are outside this range.

8.3.6. Check the calibration of the Auto EZ after the run has completed. Enter the DO readings and standard deviations in the calibration excel workbook.

8.3.7. Select **File-> Save As** from the EZ_BOD Reader program menu to save the updated worksheet. Save in

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\\fldep1\DEAR\Labs\Lab3\Biology\BOD\Worksheet in the appropriate year and month folder.

8.3.8. Use a glass stopper to make a tight water seal on each bottle. Make sure there is no air (bubbles) in the bottles. If necessary, add dilution water to eliminate any air space. Add a plastic cover to prevent evaporation of the water seal during incubation. Place the trays on the shelf and cover to prevent exposure to light during incubation which can cause photosynthetic production of oxygen.

8.4. Before the samples are discarded, the analyst should verify that the controls and sample IDs on the worksheet match the order in the *BOD Run Log.* Initial in the *BOD Run Log* in the space provided to confirm that the controls and sample IDs have been verified.

8.5. FDO readings

8.5.1. Calibrate BOD AutoEZ per SOP BB-25.

8.5.2. Remove plastic covers and glass stoppers from bottles before placing trays in the analyzer as in Step 8.3.2.

8.5.3. Select **File-> Open** from the EZ_BOD Reader program menu and open the appropriate file from \\fldep1\DEAR\Labs\Lab3\Biology\BOD\Worksheet. Select **Read-> Automated Reader** from the menu. Click the **Start** button. Check **FDO** and click "yes" in the prompt window. Enter the number of rows to be read.

8.5.4. When the analyzer is finished, click **Exit**. If readings are questionable, select **Read-> Manual Reader**, reread samples and write DOs. A script bug exists during this process. If an error message box appears, save file per section 7.3.3. Close and reopen the program and resume normal operation.

8.5.5. Check the calibration of the BOD AutoEZ after the run has completed, and enter the DO readings and standard deviations in the calibration excel workbook.

8.5.6. Look at all BOD values to ensure that they seem logical. The difference between each BOD for all dilutions of a sample should not be greater than 30%. Check all flagged BODs. Review the flag codes in the help file in the BOD program. Reread any samples if necessary.

8.6. Before the samples are discarded, the analyst should verify that the controls and sample IDs on the worksheet match the order in the *BOD Run Log.* Initial in the *BOD Run Log* in the space provided to confirm that the controls and sample IDs have been verified.

9. DATA ARCHIVAL

9.1. Print the BOD Worksheet by selecting View-> Benchsheet, then **File-> Print** from the EZ_BOD Reader program menu.

9.2. Refer to SOP BB-031 for QC Manager data uploading and verification instructions, and SOP BB-032 for authorization instructions.

9.3. Calculations

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9.3.1. Seed Correction Factor (SCF): To determine SCF calculate the average seed result using only the seed control bottles that follow the 2-1 Rule.

$$\text{Seed Result} = \frac{(\text{IDO-FDO}) \times V_{\text{Sample}}}{V_{\text{Control}}}$$

where:

IDO = Initial Dissolved Oxygen reading on Day 1 of test
FDO = Final Dissolved Oxygen reading on Day 5 of test
VSample = Volume of seed added to sample bottles
VControl = Volume of seed added to the Seed control bottle

9.3.2. Calculate the BOD_5 for each sample dilution.

$$BOD_5 = DF \times [(IDO - FDO) - (SCF)]$$

where:

IDO = Initial Dissolved Oxygen reading on Day 1 of test
FDO = Final Dissolved Oxygen reading on Day 5 of test
DF = Dilution Factor is Bottle Volume/Sample Volume
SCF = Average Seed Result

9.3.3. The final BOD result is the average of all dilutions that follow the 2-1 rule. If no dilutions follow the 2-1 rule, use the dilution with the highest sample volume (lowest dilution) as the final result. If all the dilutions have an FDO below 1.0 mg/L, use the dilution with the smallest sample volume (highest dilution) as the final result and report with a "J" qualifier and the comment "Sample depleted to less than 1.0 mg/L for final DO measurement. Result is an estimate and may be higher than result reported". The BOD program should calculate the final BOD result accordingly; but always check the calculations to ensure that the correct BOD values are used in the calculation.

9.3.4. If any final DO readings are changed, the worksheet must be recalculated. If a flag is removed from a BOD, then the file has to be saved before recalculating "As Flagged".

10. QUALITY CONTROL

10.1. Negative Control

10.1.1. Run a dilution water quality check with every batch. The dilution water blank should contain only dilution water. Do not add seed or nitrification inhibitor.

10.1.2. The method acceptance limit for the blank is ≤ 0.20 mg/L. The laboratory historical limit for the blank is ≤ 0.50 mg/L.

10.1.3. It the blank exceeds the method limit but is within historic limits, do not qualify results. Report results with the following comment: "Dilution water blank is within laboratory historical limit of 0.50 mg/L."

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10.1.4. If the blank exceeds both limits, report batch results with a V-qualifier and add the following comment: "Dilution water blank exceeds laboratory historical limit of 0.50 mg/L."

10.2. Positive Control

10.2.1. Run a glucose-glutamic acid check with every batch. Use 6 mL of GGA solution/300 mL of dilution water.

10.2.2. The method acceptance limit for the BOD GGA standard is 198 ± 30.5 mg/L, and for the CBOD GGA standard is 165 ± 26 mg/L.

10.2.3. If the GGA standard exceeds method limits, report batch results with a "J" qualifier and the following comment: "Refer to QA report for QC exceeding limits."

10.3. Sample Replicates

10.3.1. Analyze one sample duplicate per batch. The acceptance limit is ≤ 25% RPD.

10.4. Troubleshooting

10.4.1. Initial DO Readings should be within 7.5-9.5 mg/L.

- If readings too high, samples or dilution water may be supersaturated with oxygen. Make sure samples are within 20 ± 3°C and vigorously shake or aerate to reduce DO level.
- If readings are too low, samples or dilution water may need to be further aerated. Also, check to make sure probe stirrers are working properly.
- Additionally, check meter calibration.

10.4.2. Dilution water blanks are exceeding method and/or historical limits.

- Check cleanliness of BOD incubation bottles.
- Make sure deionized water is sterile and aerated overnight in a clean flask before using for dilution water.
- Ensure that the probes are rinsed thoroughly between samples, and after manual readings.
- Change rinse tubing and clean around the rinse dispensers.
- Obtain clean calibration water if contamination is suspected.

10.4.3. GGA standard exceeding method limits.

- GGA standard may be expired or ineffective. Check the glucose and glutamic acid. Dry new aliquots and prepare fresh GGA.
- High GGA values may indicate contamination of dilution water or high seed values or too much seed added.
- Low GGA values may indicate poor seed quality, not enough seed being added, or toxicity.

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10.5. Refer to QA manual for corrective action and handling out-of-control data.

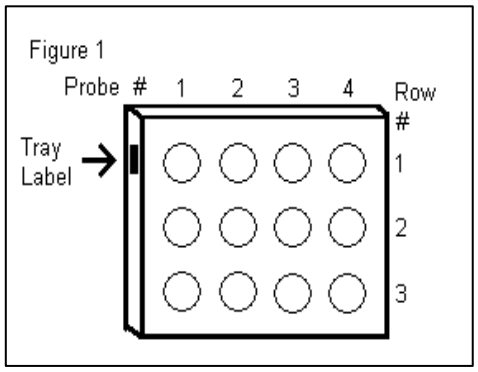
11. SAFETY/HAZARDOUS WASTE MANAGEMENT

11.1. Refer to the Laboratory Health and Safety Plan.

11.2. Read MSDS information for reagent handling and disposal.

12. TABLES, DIAGRAMS

12.1. Figure 2. Tray Diagram



13. REFERENCES

13.1. Standard Methods Online Edition, 5210B

13.2. NELAC Quality Systems Manual

13.3. FDEP Laboratory Quality Manual http://www.dep.state.fl.us/labs/library/lab_sops.htm

13.4. FDEP Laboratory Health and Safety Plan http://www.fldepnet.org/content/dear/bureau-laboratories-safety-documents

13.5. DEP SOP-001/01 FS 1000, General Sampling Procedures

13.6. DEP SOP BB-25, YSI Model 50B/AutoEZ DO Meter Calibration

13.7. DEP SOP BB-30, Sample Custody, Preparation Labels and Worksheet Instructions for Bench Biology Samples

13.8. DEP SOP BB-31, LIMS QC Manager Data Uploading Instructions for Bench Biology and Microbiology Samples

13.9. DEP SOP BB-32, Test Level Authorization of Uploaded Results for Bench Biology Samples

APPENDIX OF CHANGES

05/18/2009	Updated SOP style and updated to online edition of SM5210B.
05/18/2010	Updated file locations and reference to updated safety manual.
05/04/2011	Updated reference to QA manual and file directories.

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04/24/2013	Added instructions for preparing GGA.
04/23/2014	Changed Sample Preparation and Sample Analysis instructions to be compatible with new EZ_BOD Reader program.
01/16/2015	Formatted for 508 compliance
02/24/2015	updated to reflect new file locations and added software setup information.
05/20/2016	Edited Sample Preparation; Sample Analysis; Data Archival; Quality Control; Troubleshooting; References; and hyperlinks.
12/23/2016	Updated BOD AutoEZ program settings, added clarification for new procedures, removed “greater than 10x the blank” instructions.

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