

Standard Operating Procedure for:

Total Dissolved Phosphorus

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1 Identification of the method

- 1.1 Total dissolved phosphorus analysis via spectrophotometer using an Ascorbic Acid Method (Standard Methods 4500-P E) following Persulfate Digestion (Standard Methods 4500-P B 5).

2 Applicable matrix or matrices

- 2.1 This method is suitable for the analysis of [environmental samples](#).

3 Detection limit

- 3.1 [Method Detection Limit](#): 0.001 mg P/L
- 3.2 This [Method Detection Limit](#) was determined by taking the calculated concentrations of the lowest calibration standard from multiple [analytical runs](#) (using seven or more individual values taken from at least three [analytical runs](#)) and calculating a standard deviation from those concentrations. This standard deviation is then multiplied by the one-sided t-statistic at the 99% confidence level for the appropriate degrees of freedom ($n - 1$).

4 Scope of the method

- 4.1 This standard operating procedure is intended to provide MU Limnology [operators](#), [technicians](#), and [analysts](#) with guidance on the analysis of total dissolved phosphorus. This document is not intended to replace individual training in this method by an experienced MU Limnology [technician](#).

5 Summary of the method

- 5.1 10 mL aliquots of well mixed filtrate are placed into acid washed culture tubes. Ammonium peroxydisulfate is then added and the tubes are autoclaved to digest/transform all forms of phosphorus into orthophosphate (PO₄). A color reagent is added to digested tubes to form a blue color. The intensity of the color is related to the concentration of phosphorus within the sample. The sample is placed in a spectrophotometer and light (880nm) is passed through the sample.

- Operating Range: 0.001 to 0.150 mg P/L
- Sample Volume: 10 ml
- Run Time: 2–3 hours
- Digest Time: 1 hour
- Samples per run: 28–42

6 Interferences

- 6.1 Arsenates can interfere with the method at concentrations of 0.1 mg As/L by forming a blue color similar to that formed by phosphate.

- 6.2 Hexavalent chromium and NO_2^- can interfere to give results that are 3% low when concentrations are 1 mg/L and 10 to 15 % low when concentrations are 10 mg/L.
- 6.3 Inorganic turbidity can interfere with the method via light absorption, resulting in higher estimates of phosphate. Removal of turbidity by centrifugation after the addition of color reagent can reduce/eliminate this interference.

7 Health and Safety

- 7.1 This method involves handling freshwater samples that may contain live microorganisms and therefore pose some threat of infection. Laboratory personnel who are routinely exposed to such water samples are encouraged to protect themselves from water borne illnesses by wearing clean disposable gloves and washing hands frequently.
- 7.2 Wear protective gloves, lab coats, and other appropriate [PPE](#) when handling all chemical substances used in this method. All [operators](#) and [technicians](#) performing this method should review the [MSDS](#) for additional information and safety concerns regarding the chemical substances used throughout these procedures.
- 7.3 The following chemicals used in this method are considered especially hazardous and should be handled with extra care:
- Sulfuric Acid
- 7.4 The digestion process of the method uses an autoclave that heats samples to >250 °F (121 °C). Care should be taken when operating the autoclave to avoid burns. Autoclaves should be allowed to cool completely before removing the samples within. Opening the autoclave while it is still hot could result in steam burns.

8 Personnel qualifications

- 8.1 Samples will be analyzed by MU Limnology staff who have been trained to the [operators](#) or [technician](#) level in this method and who are familiar with all of the MU Limnology sampling handling and labeling procedures and appropriate [SOPs](#).
- 8.2 The [Calibration Standards](#), Secondary [QC](#) Standards, Environmental [Reference](#), Spiked [Reference](#), and color reagent will be prepared by MU Limnology [technicians](#).

9 Equipment and supplies

- 9.1 Glass culture tubes: Tubes are 16mL in volume, 15 mm x 125 mm in size.
- 9.2 10ml micropipette
- 9.3 Autoclave
- 9.4 [Repipette](#)
- 9.5 Centrifuge
- 9.6 Spectrophotometer
- 9.7 Ammonium Molybdate Tetrahydrate, Fisher Chemical, A647

- 9.8 Antimony Potassium Tartrate, Fisher Scientific, A-865
- 9.9 L-Ascorbic Acid, Fisher Chemical, A61
- 9.10 Sulfuric Acid
- 9.11 Ammonium Peroxydisulfate, Fisher Chemical, A682
- 9.12 Phosphate Phosphorus Standard (50ppm P), RICCA, 5830-4
- 9.13 Sulfuric Acid
- 9.14 Whatman GF/F Glass Microfiber Filters, 47mm, CAT No. 1825-047
- 9.15 47 mm Magnetic Filter Flask, PALL
- 9.16 Internal Vacuum System or Portable Vacuum Pump

10 Reagents and standards

- 10.1 Prepare standards as shown below in Table 1 in clean volumetric flasks. All stock solutions should be added quantitatively using a calibrated micropipette. Rinse all glassware three times with [UPDI](#) and then fill ~75% of the way to the line. Then add appropriate stock solution, fill to the line with [UPDI](#), cover with parafilm and invert three times to mix. Standards may be stored in glass at 4°C.

Table 1: Nominal Concentration of Phosphorus Standards

Standard Concentration ($\mu\text{g P/L}$)	150	100	50	25	0
Total Standard Volume (ml)	1000	1000	1000	1000	1000
Volume 50 mg P/L phosphate (ml)	3	2	1	0.5	-

10.2 Phosphorus Color Reagent

To a light proof, glass container and add 2100 ml of [UPDI](#) and 140ml of sulfuric acid. While the solution is still warm, add 15.0 g of ammonium molybdate and swirl for 3 minutes. Add 0.34 g of antimony potassium tartrate and swirl for another 3 minutes. This solution may be stored for up to 2 months at 4 °C.

11 Quality Control

11.1 Reagent Coloring

Prior to beginning analysis, all reagents should be visually expected for discoloring.

11.2 [Check Standards](#)

[Check standards](#) must be analyzed with every run. [Check standards](#) will be identical to the [calibration standards](#) used in the run and will be included at the end of the run.

11.3 Secondary [QC](#) standards

Secondary [QC](#) standards must be analyzed with every run. These standards should be identical in concentration to the highest calibration standard but prepared from a different stock solution.

11.4 Environmental [Reference](#) and Spiked [Reference](#)

Three tubes of the environmental [reference](#) and three tubes of the spiked [reference](#) will be included in every run and analyzed identically to the samples.

12 Analysis

12.1 Calibration and standardization

- Each [analytical run](#) will contain [Calibration Standards](#) of known phosphorus concentrations. The absorbency values from the [Calibration Standards](#) are used, along with their phosphorus concentrations, to generate a linear equation in an Excel Spreadsheet. This linear equation is then used to determine the phosphorus concentrations of the [Environmental Samples](#) based on their absorbency values.

12.2 Set-up of Phosphorus [Analytical Run](#)

- Ten mL aliquots of filtered [Environmental Samples](#) should be pipetted into clean 16 mL (125 mm x 16 mm) glass culture tubes in either duplicate or triplicate. [Environmental Samples](#) that are known to have phosphorus concentrations that exceed 0.150 mg P/L may be pre-diluted with [UPDI](#) water in order to bring the concentration down into the method's range (<0.150 mg P/L).

- Each phosphorus [Analytical Run](#) will include 24 [QA/QC](#) tubes out of the rack of 108. This includes five different [Calibration Standards](#), Secondary Standards, References, and [Spiked References](#) (triplicates of all, with one of each [Calibration Standards](#) at the back of the rack) This leaves 84 slots for [Environmental Samples](#), which allows for analysis of 42 [Environmental Samples](#) if run in duplicate or 28 [Environmental Samples](#) if run in triplicate.
- A phosphorus data sheet will be filled out with each [analytical run](#). The sheet will identify the [environmental samples](#) within the [analytical run](#), including site name, sample depth/type, date of collection, etc. Other information recorded on the analytical sheet includes information about pre-dilution of samples suspected of exceeding the analytical range (>150 μ P/L).

12.3 Digestion of Phosphorus

- To each tube ([calibration standards](#) and [check standards](#) as well as [environmental samples](#)) add a small scoop (approximately 0.2 g) of potassium peroxydisulfate using the scoop located in the desiccator in room 326.
- Prior to use, the water level in the autoclave needs to be checked. It should be near the top of the heating element where it connects to the autoclave wall. If the autoclave needs more water, add [UPDI](#).
- A small strip of autoclave tape will be placed on the rack before it is put into the autoclave. The autoclave is closed and the lid tightened. The six wingnuts need to be tightened evenly so the gap between the lid and the autoclave is fairly even. If the lid is tightened unevenly there will be steam leaks. Make sure the emergency release valve is in the down position.
- Turn the autoclave on, once it reaches 250 °F set a timer for 1 hour. You will want to check the autoclave throughout the digestion period to make sure the temperature remains between 250 – 260 °F.
- Once the digestion is completed, the autoclave is turned off and left to cool on its own. After it has cooled, the tubes can be removed and placed into the refrigerator if not being analyzed right away.

12.4 Color Reagent

- Rinse the re-pipette bottle with [UPDI](#) water three times to ensure it is clean.
- You will need 1.5 mL of color reagent per tube in the [analytical run](#), plus a little extra to insure you do not run out of color reagent before dosing up all tubes in the run. For full runs (108 tubes) pour 200 mL of color reagent into the [repipette](#) bottle. To this, add ascorbic acid (located in desiccator) to the rate of ~1.5 g per 100 mL of color reagent (use three large scoops of ascorbic acid per 100 mL of color reagent. Once you have added the ascorbic acid to the color reagent you have about 3.5 hours to complete the analysis before the color reagent starts to degrade.

- Swirl until the ascorbic acid has dissolved. Operate the re-pipette to flush air out of the plumbing and rinse out any remaining [UPDI](#) water. Add 1.5 mL of color reagent to each tube in the [analytical run](#). There is a 15 minute wait before you start analyzing samples to allow for color development.
- After the color reagent has been added to the tubes you can centrifuge the tubes while color development is occurring. Centrifuging will help remove any inorganic sediment from suspension, which is desirable as these materials will absorb light and lead to an overestimation of phosphorus.

12.5 Analyzing using the Genysis 2 Spectrophotometer

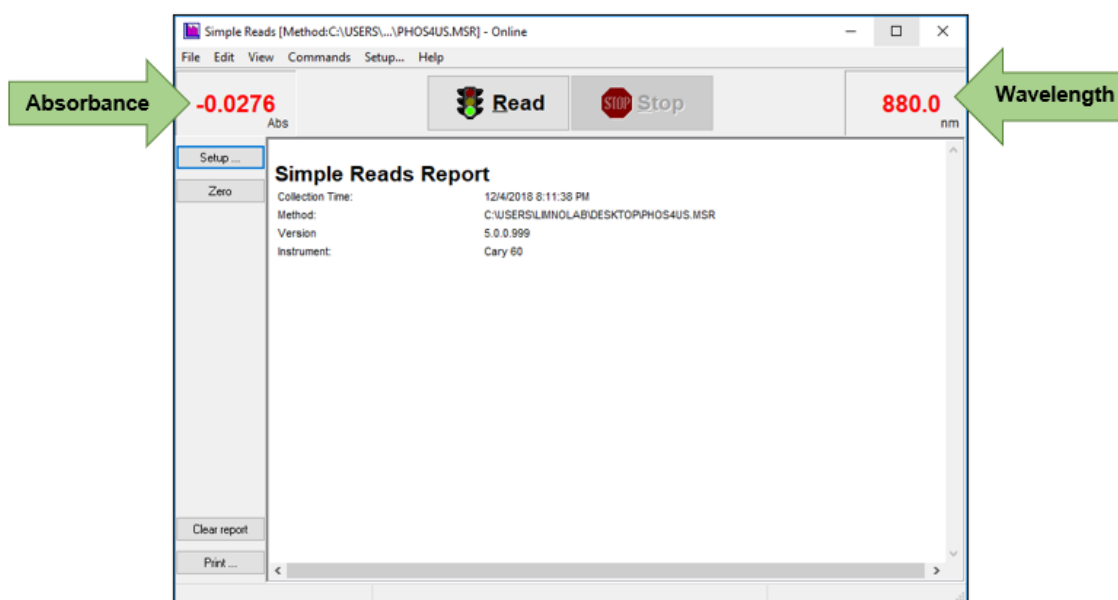
- Turn on the Genysis 2 Spectrophotometer at least 30 minutes prior to analysis.
- After the spectrophotometer has warmed up and gone through its diagnostic checks, you will need to select the Phosphorus method. From the initial screen select Simple Absorbency (#1). After the screen switches to Simple Absorbency, press the Test Types key and select Phosphorus. The spectrophotometer will adjust the location of the cell holder and set up with the appropriate wavelength (880 nm).
- Auto Zero the spectrophotometer with nothing in it (no cuvette). After rinsing out a 5 cm glass cuvette well (and wiping down the two clear sides) place the cuvette with [UPDI](#) water (2/3 full) into the spectrophotometer and close the lid. The spectrophotometer should give you a reading around 150–152.
- Turn vacuum on at wall and evacuate the [UPDI](#) water from the cuvette.
- Start with the standards, so you can check the results with previously gathered data to ensure that everything is working correctly. There should be a little more than three absorbency points for each unit of phosphorus in the standards. If there seems to be a problem, contact the lab manager. After you have completed the standards, enter appropriate information into the phosphorus logbook.
- Carefully pour the sample from the tube into the cuvette (remember, materials centrifuged to the bottom of the tubes can be re-suspended if not poured carefully, also the color reagent is acidic and will burn holes into your clothing). Make sure not to touch the clear sides of the cuvette, which will leave fingerprints that may interfere with the absorbance readings. Place cuvette into spec, close the lid, and record the reading. The spec will show the absorbance reading with a decimal you will record this number ignoring the decimal (e.g., 0.074 is recorded as 74).

- If an unknown sample has an absorbency that exceeds the high standard absorbency by >10 %, the sample will need to be analyzed using the 1 cm cuvette. Pour or pipette the sample back into the culture tube and continue with next set of tubes (assuming that all replicates are over the high standard). Once all of the tubes that can be analyzed with the 5 cm cuvette are completed, rinse and fill the 1 cm cuvette with [UPDI](#) water. You will need to re-zero the spec with this cuvette. Analyze the samples, noting in the comments column of datasheet that results from these samples need to be multiplied by 5 to compensate for the shorter light path.

12.6 Phosphorus Analysis using the CARY 60

- Turn on the Cary60 spectrometer 30 minutes prior to analysis.
- Once the Cary60 is turned on (either active or in standby mode), open the Phos4Us.MSR file on the desktop.
- Clicking the Phos4US.MSR file will launch the Simple Reads software for the Cary60.

Figure 2: Simple reads main window



- Click the Setup button on the left panel, and verify the following settings in the dialog window (pictured):
 - Read Mode set to **Read at Wavelength** with the wavelength being **880**
 - Avg time (sec) set to **1**
 - Y Mode set to **Abs**
- Press OK to close the Setup window.

- Fill a [UPDI](#) squirt bottle with ultra-pure (UP) [DI](#) water. Retrieve the 5cm glass cuvette from the appropriately labeled Tupperware container, and a plastic beaker to be used as a waste container. Rinse the cuvette with [UPDI](#) water (this can be done at the sink). Dry with a Kimwipe.
- Use the squirt bottle to fill the cuvette with [UPDI](#) water. Ensure that the clear side panels (the narrow sides) are dry and free of smudges. Place the cuvette in the cuvette holder inside the Cary60.
- Slide the lid closed. Now zero the spec by pressing the Zero button on the Simple Reads user interface.
- Take a reading of your zeroed sample to verify that the zero is correct by pressing the Read button on the user interface.
- Dump the sample into the waste beaker. Triple rinse the cuvette with [UPDI](#) water out of the squirt bottle. Refill with [UPDI](#) water and place into the cuvette holder. Close the lid and press Read. Verify that the reading for this [UPDI](#) sample is +/- 0.001 of 0. Dump the contents of the cuvette into the waste beaker and triple rinse with [UPDI](#).
- It takes some time for the color reagent to develop. Typically, by the time your first set of samples finish spinning in the centrifuge, enough time has passed that you can begin running your standards. As a general rule of thumb, the absorbance for each standard is approximately 3x the concentration. Refer to the Cary60 P-Log for standard absorbances generated by the spec.
- Pour the sample into the cuvette. Ensure that the side panels are dry and free of smudges. Place the cuvette into the cuvette holder, close the lid, and press read. Once you have a reading, dump the contents of the cuvette into the waste container, triple rinse with [UPDI](#), and you are ready to repeat with your next tube.
- Once you have run your standards, you are ready to run your centrifuged samples. If your waste beaker becomes full, use the vacuum to empty your beaker into the Acid Solution vacuum bottle located alongside the spec.
- It is strongly encouraged that you save your data often. To do this, navigate to File > Save Data As. In the dialog box, navigate to the folder into which you would like to save your data.
- In the file type drop down, select Rich Text Format .RTF. This will save your read report into a Word Document. Every time you save your report, overwrite the previous version that you saved so that there is only 1 file for your run. This will keep our folders from getting cluttered up with multiple versions of the same run.

- Once you hit read the absorbance value is automatically populated into the read report, and there is no way of editing the report within Simple Reads. DO NOT PRESS CLEAR REPORT. This will erase all of the data collected in the run and you will have to start over. Take note of any readings that you will not want to include in your final report (e.g., if you accidentally took a reading without closing the lid and had to take a second reading of the sample with the lid closed, you hit the read button multiple times for one sample, etc.)
- Once you finish your run, be sure to save the data one last time before closing the Simple Reads window. Once the Simple Reads window is closed, your data will be gone. It is recommended that you open the saved file and verify that all of your information is there and that the file is not corrupt before closing the Simple Reads window.
- Copy and paste your absorbance values from the .RTF report into the P excel template. Save this file onto the computer using the same name as the .RTF file. Again, back up on Box and a USB drive.

12.7 Post Analysis Treatment of Tubes

Triple rinse your test tubes with 3 tap, 3 [DI](#), then fill the tubes with [DI](#) to soak overnight. Remove the paper labels from the caps and place caps in a CAP RINSE bottle. Triple rinse with 3 tap, 3 [DI](#), and leave the caps to soak in [DI](#) overnight.

13 Data acquisition, calculations, and reporting

- 13.1 Calculations of concentrations: Concentrations of unknown [environmental samples](#) are based on linear equation for the regression data from the [Calibration Standards](#).
- 13.2 Reporting Results: Results should be reported in milligrams phosphorus per liter (mg P/L) up to 3 decimal places (0.000).

14 Computer hardware and software

- 14.1 Windows 7 Enterprise
- 14.2 Cary Eclipse Simple Reads Application, Software Version 1.2 (146)

15 Method performance

- 15.1 [Method Detection Limit](#): 0.001 mg P/L.
- 15.2 Precision: [CV](#) <5% or range < 0.005 mg P/L.
- 15.3 Calibration $r^2 > 0.99$

16 Pollution prevention

- 16.1 All reagents and standards will be prepared in appropriate volumes to reduce waste.
- 16.2 All sample and reagents will be handled according to [MU EHS](#) policies in order to ensure proper disposal.

17 Data assessment, acceptable criteria for quality control measures and actions for out-of-control or unacceptable data

- 17.1 Individual [analytical runs](#) will be entered into an Excel spreadsheet created specifically for phosphorus analysis. The absorbency values associated with the [Calibration Standards](#) will be entered in the appropriate cells and a regression analysis will be conducted.
- The calculated concentrations of the [Calibration Standards](#) (automatically calculated by the spreadsheet) will be checked to determine if values are acceptable. Standards will be deemed acceptable if the average concentration of the two replicates are +3 µg P/L of the expected concentration or within 5% of expected concentration.
- 17.2 Absorbency values for the [environmental samples](#) and [QC](#) samples will be entered into the spreadsheet. Phosphorus concentrations will automatically be calculated by the spreadsheet.
- Replicates of [environmental samples](#) are deemed acceptable if the Coefficient of Variance ([CV](#)) of the calculated concentrations is <5% or the range of calculated concentrations is <5 µg P/L. The spreadsheet is set up to determine if these two criteria are not met, and the data will automatically be flagged as needing to be re-analyzed.
 - [Check Standards](#) placed at the end of the [analytical run](#) will be reviewed. Calculated phosphorus concentrations of these [Check Standards](#) should be +3 µg P/L or within 5% of expected values. If the [Check Standards](#) fail to meet criteria the lab manager will be notified and the [analytical run](#) will be evaluated to determine if there was “drift” during the course of the run. If there was machine drift (all [Check Standards](#) showing an upward or downward shift in results) the [analytical run](#) will be discarded.
 - Results from the [Reference](#) samples and the Spiked [Reference](#) samples will be copied into an electronic Spike Log after each run. The Spike Log will automatically calculate averages for both the [Reference](#) and Spiked [Reference](#) samples, determine recovery of the Spike, and flag any results if recovery is not +15% of the spike concentrations of 40 µg/L.

$$\frac{\%R = \text{matrix spike result} - \text{sample result}}{\text{matrix spike value}} \times 100$$

- 17.3 If the [Calibration Standards](#)/regression is deemed out-of-control (low r-square, concentrations of [Calibration Standards](#) are off, and/or slope does not meet criteria) the lab manager will evaluate the run. The Lab Manager will make decisions concerning the removal of any [Calibration Standards](#) that may be outliers. After any outliers are removed, the regression will be re-calculated. If the Calibrations Standards/Regression is still out-of-control, the run will be discarded.

- 17.4 Environmental [sample replicates](#) that fail to meet criteria will be re-analyzed. Original and re-analyzed results will be evaluated to determine cause of initial poor replication (outlier or sample with high variability). Decisions concerning outliers are made following the protocol laid out in Standard Methods Test of Discordancy for a single outlier in a normal sample.
- High outliers are dropped if: $(\text{Valuemax} - \text{Allavg}) / \text{AllStdDev} > 1.94$
Valuemax = Maximum value of replicates (suspected outlier).
 - Allavg = the average of all replicates.
 - AllStdDev = the standard deviation of all replicates.
 - 1.94 = Critical Value for 6 values and a 1% level of significance (The Critical Value will be adjusted based on the number of values being evaluated).
 - Low outliers are dropped in: $(\text{Allavg} - \text{Valuemin}) / \text{AllStdDev} > 1.94$
 - Valuemin = the minimum value of replicates (suspected outlier).
 - High variation is not considered problematic if the difference between two adjacent values, when values are ordered low to high, is multiplied by two and does not exceed the total range of values. Variable data that are considered problematic will be reviewed by the lab manager to determine appropriate steps, which may include re-analyzing the sample again if there is enough water left.

18 Waste management

- 18.1 All waste generated is considered hazardous.
- 18.2 All analyzed standards, and reagents should be treated as waste upon completion of the run.
- 18.3 Waste should be kept in an approved container with proper labeling.
- 18.4 Waste will not be held for longer than 6 months and [MU Environmental Health and Safety \(EHS\)](#) will be notified an appropriate time before this point so that waste can be collected and disposed of.

19 References

- 19.1 Standard Methods for the Examination of Water and Wastewater, 23rd Edition. 2017. Method 4500P E. Ascorbic Acid Method. American public Health Association. Washington, DC.