

RECLAMATION

Managing Water in the West

Development of Methods for the Spectrophotometric Analysis of Water Samples

Research and Development Office
Science and Technology Program
(Final Report) ST-2018-1747-02



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Development of Methods for the Spectrophotometric Analysis of Water Samples

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Executive Summary

The purchase of a new spectrophotometer was a major upgrade for Reclamation Detection Laboratory for Exotic Species (RDLES) from an older instrument. This instrument will extend the capabilities of the RDLES laboratory and gives our researchers a new tool for their research. The goal of this project was to look at the methods that could be used with the spectrophotometer to analyze water samples. The primary focus of this project was to familiarize RDLES staff with this instrument and its accessories. This report has two parts: first, a short literature review to look at possible methods for the spectrophotometer, and second, analysis of chlorophyll and bovine serum albumin (BSA) to test out the instrument and Tray Cell for measuring small volumes. From this research two standard operating procedures (SOP) were created. Appendix 1 contains a general operating SOP for the instrument. Appendix 2 contains an instrument specific SOP for the analysis of chlorophyll. The next step will be to continue to use this instrument and to expand the number of different assays that can be performed. Also, to work with other researchers to identify additional potential uses for this instrument.

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Main Report

In 2016, a new spectrophotometer was purchased for the Reclamations Detection Laboratory for Exotic Species (RDLES) laboratory, which replaced an older model. The new instrument has been able to expand the capabilities of the laboratory. Tray cell microcuvette (to analyze small volumes) and dip probe (to analyze liquids) accessories were also purchased to allow for the analysis of a wider range of sample types. To better understand both software and the microcuvette accessory, two different assays were chosen (chlorophyll and bovine serum albumin [BSA]) to test out the instrument.

This research proposal contains two parts: first, a short literature review to identify the types of assays that could be performed on the spectrophotometer, and the second part of this report will focus on using the instrument to measure chlorophyll and BSA as ways to test out and learn how to use the instrument. Finally, there are two appendixes. Appendix 1 is a general SOP for the instrument. Appendix 2 is a SOP for chlorophyll analysis that is specific to the new spectrophotometer.

Part I: Literature Review

A literature and internet review showed that there were many diverse methods available that could be performed on the spectrophotometer for the analysis of a wide range of chemicals in water. The focus of this search was on water because the most common type of sample that the RDLES laboratory receives is water samples for the analysis of invasive mussels. Understanding additional assays that could be performed to enhance this work has been a driving factor for the literature search. Two major sources of information were identified: Standard Methods [1] and also the Environmental Protection Agency (EPA) library of assays.

Standard Methods [1] is a major source of published and reviewed methods for a wide range of chemicals. The methods presented in this book are peer reviewed. The EPA has a large library of assays (<https://www.epa.gov/dwanalyticalmethods>) that can also be used as a source for information about different assays. Finally, a general internet search for specific chemicals can turn up both published methods and laboratory SOPs. For example, an internet search for a chlorophyll assay turns up several SOPs that are all based on the method in the Standard Methods 10200 H book [1].

Published methods [1] for the analysis of a wide range of substances including: arsenic, aluminum, hydrazine, copper, iron, lead, chromium, pentachlorophenol (PCP), nitrate/nitrite, and biological compounds were identified. In addition, analysis for DNA concentration and purity[2], and protein concentrations with the Bradford method [3] were found. There is a wide range of substances that can be analyzed using the spectrophotometer. In addition to published methods, there are a wide range of kits available that can be used with a spectrophotometer to analyze chemicals. The advantage these kits is that the reagents and method has been standardized and tested by others.

The main lesson learned during this process is that there is a large body of literature available that can assist researchers in determining the best method to use for the analysis of their chemical of interest.

Part II: Assays

Chlorophyll Analysis

Introduction

The analysis of chlorophyll was a major reason for the purchase of the new spectrophotometer. This assay is valuable because it allows for the determination chlorophyll which is an important because it can be used estimate the algal biomass' spatial and temporal variability in a waterbodies [4]. The growth of algae is dependent on the season, nutrients, water velocity, days of accrual, shading, water temperature, and other factors. Having an understanding of the concentrations of algae and phytoplankton can help researchers understand how healthy a body of water is, its composition, and ecological status [5]. Knowing the concentration of chlorophyll *a* allows water managers to classify a lake as eutrophic (5-140 mg/m³), mesotrophic (1-14 mg/m³), or oligotrophic (0.3-2.5 mg/m³) [4][6].

Standard Methods [1] contains three methods for measuring chlorophyll *a*: spectrophotometric, fluorometric, and high performance liquid chromatographic (HPCL). The method that will be used in the RDLES laboratory will be spectrophotometric. This method is for the determination of chlorophyll in the presence of pheophytin. Standard Methods also contains a second spectrophotometric method for the determination of chlorophyll *a*, *b*, and *c* (trichromatic method). An internet search showed that there were chlorophyll SOPs from several different sources but they all used the Standard Methods 10200 H method [1] as their source for a method. Thus, both methods were adapted, and an SOP was created for the spectrophotometer that the RDLES laboratory has. The main changes were to add details on how to use the spectrophotometer and which programs to use for the analysis.

Both assays use the same sample preparation and share many of the same wavelengths for the analysis. The major difference is that for the chlorophyll/pheophytin method, hydrochloric acid is used to acidify and degrade all the chlorophyll *a* in the sample into pheophytin. Acidifying the chlorophyll sample results in the loss of the magnesium ion in the porphyrin ring, which makes pheophytin. The trichromatic method does not require the acidification step. It is important when reporting chlorophyll results to be very clear on which of these two tests were performed.

To better understand these two assays, chlorophyll assays were performed on “mock” samples. Chlorophyll was purchased, spiked into deionized (DI) water, and filtered onto filter paper for analysis. This experiment was performed several times to gain knowledge and practice in performing this assay. For example, determining where issues could arise, such as not having the samples in the dark or not grinding the samples long enough, could lead to unexpected results, or overloading the assay with too much chlorophyll on the filter. The resulting data

shown here is of several different volumes of chlorophyll being filtered and then analyzed by the Standard Methods test.

Materials and Method

Chlorophyll (World Organic), which contains alfalfa leaves (*Medicago sativa*), was purchased for use in the experiments. Known volumes of chlorophyll (0, 1, 3, 5, and 10 mL) was added to 500 mL of DI water and then filtered [1]. Once the filtering was completed the filters were folded in half, gently blotted to remove any excess liquid, placed in a pre-labeled envelope, and stored at -20°C until analyzed. Within 3 days of being prepared the filters were taken from the freezer one at a time and the chlorophyll was extracted (see Appendix 2 for more details on this process). Once the extraction was completed the samples were analyzed by both the chlorophyll/pheophytin method and the trichromatic method. Each sample was analyzed three times. The resulting data was then analyzed in an Excel spreadsheet using the formulas in Standard Methods[1].

Results and Discussion

There are two different spectrophotometric methods to analyze chlorophyll: chlorophyll/pheophytin, and trichromatic method. It is possible to see differences in the two methods. In Figure 1, chlorophyll was added from 0 to 10 mL to 0.5 L of DI water, filtered onto filter paper, extracted, and then analyzed. As expected, as increasing amounts of chlorophyll was added the concentration of chlorophyll and pheophytin increased. This shows that the extraction method was working (Figure 1). At the same time, the trichromatic method was used to analyze for chlorophyll *a*, *b*, and *c* in the pre-acidified samples (Figure 2). Again, it is possible to see that as increasing amounts of chlorophyll was added, the increase is almost a linear response. The biggest difference is that the chlorophyll *a* concentration between these two methods is different. The chlorophyll/pheophytin method can remove the interference of the chlorophyll degradation products. The trichromatic method does not have this correction in it.

There were several lessons learned while performing this assay. First, is the importance of understanding the calculations and method that is being used. The two different chlorophyll assays give different results for chlorophyll *a*, and in reporting these results it is important to indicate which assay is being used. Second, using store bought chlorophyll was an effective way of working through the method multiple times. However, consistency with pipetting the chlorophyll stock led to differences in the amount of chlorophyll in samples of the same volume. This assay is multistep and has several places where if a mistake is made then the results will be off. For example, if the acidification is not performed for the full 90 seconds, then all the chlorophyll will not be completely degraded to pheophytin and the reading will be incorrect. Based on this work it was possible to create an SOP for the analysis of chlorophyll for the RDLES laboratory.

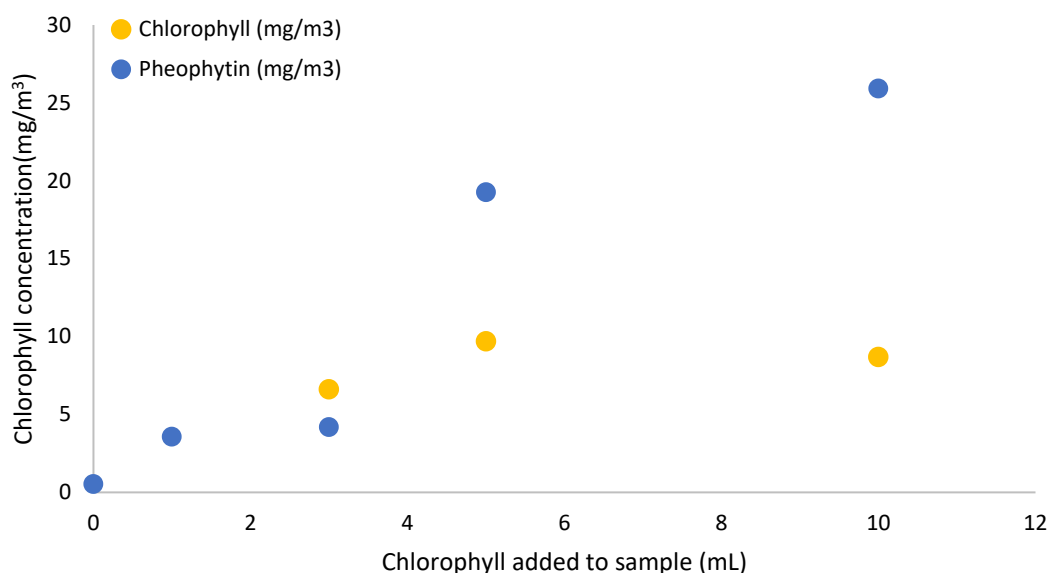


Figure 1: Chlorophyll and pheophytin analysis by acidification with different volumes of chlorophyll added

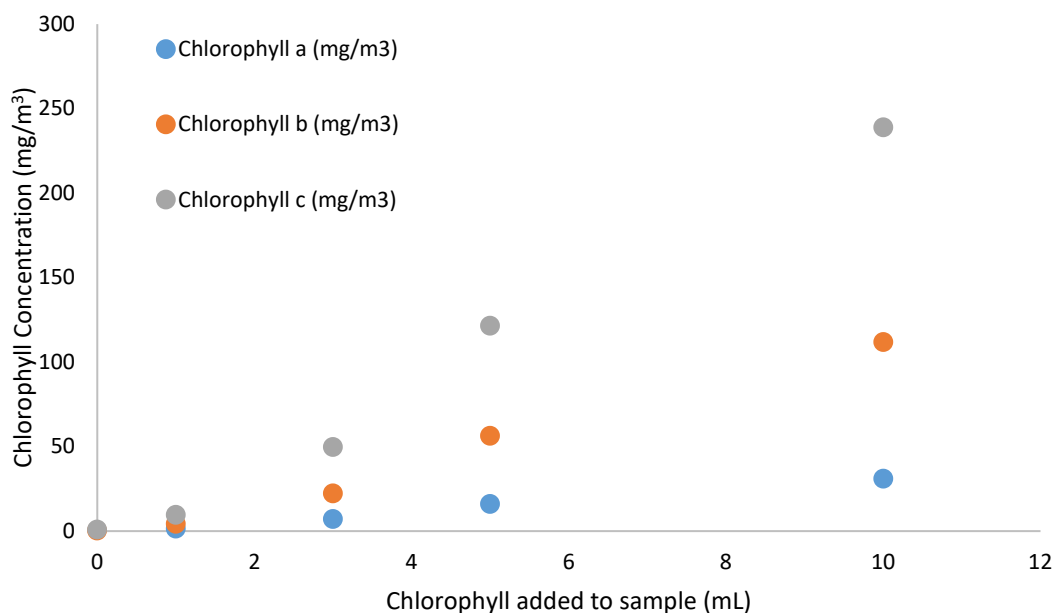


Figure 2: Trichromatic method of analysis for chlorophyll a, b, and c

BSA Assay

Introduction

To test the ultra-microvolume microcuvettes (Tray Cell) the measurement of bovine serum albumin (BSA) assay was performed. BSA is used as protein concentration standard and in this case acts as a known standard for comparing two different caps for the Tray Cell. RDLES purchased two caps (1.0 mm, factor 10 and 0.2 mm, factor 50) which have different dynamic ranges for the concentrations that they can measure. Figure 3 shows the schematic of Tray Cell

and how light can pass through the sample to take a measurement. To use the Tray Cells the sample is pipetted onto the mirror and the cap is gently placed over the sample. Once the readings have been taken, a Q-tip is used to gently remove the sample from the mirror. A Q-tip soaked in DI water is used to clean the mirror between samples. The Tray Cell is used for the analysis of small volumes (5-10 μL). In the RDLES Laboratory the Tray Cell will mainly be used for determining the concentration of DNA from DNA extractions. To gain experience with the use of the Tray Cell serial dilutions of BSA were used to compare the two different caps.

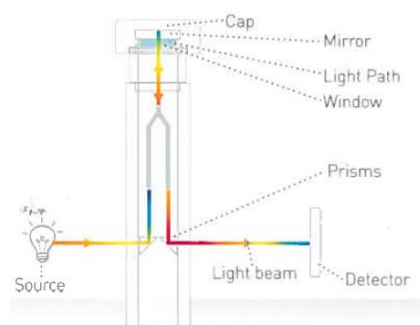


Figure 3: Tray Cell set up [7].

Materials and Methods

A stock solution of BSA (Sigma A4503-10G) was made by dissolving 0.5 grams into 10 mL of DI water. Serial dilutions were made from this stock. For each analysis at 280 nm, a blank was zeroed and then analyzed three times. Each sample was analyzed three times. Both the factor 10 and 50 caps were used to analyze each of the dilutions. The BSA standards were also analyzed over 3 days to determine the stability of the analysis. Once the analysis was performed, the resulting data was then analyzed in Excel. The absorbances were converted to concentrations by using the formula below. The resulting concentrations were averaged before being put into graphs. The equation of the line and linear regression was calculated and placed on the graph.

The calculation to determine concentration was as follows:

Concentration (mg/mL) = (1/sample specific factor) X absorbance (280 nm) x dilution factor.

The sample specific factor of BSA is 0.64 which is the absorbance of 1 mg/mL at 280 nm. For the dilution factor each of the caps is different (factor 10 and factor 50).

Results and Discussion

The purchase of two different caps (1.0 mm factor 10 and 0.2 mm factor 50) enable the analysis of two different ranges of volumes and concentrations (Appendix 1, Table 2). The Tray Cells were able to consistently detect the BSA over the dilution range. Over 3 days both caps were able to get comparable results (Figure 4 and 5). Calculating the slope of the line showed that for the 1.0 mm factor 10 cap the slopes are all very similar (0.86, 0.85, and 0.89) and that the R^2 for all three regressions are close to 1. For the 0.2 mm factor 50 cap there, day 1 and 2 has similar slopes (0.93 and 0.94), however the day 3 slope (0.80) was lower than the other two days. This difference could be due to pipetting. The R^2 values for these lines were all above 0.94.

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When compared to each other the caps also were able to produce comparable results (Figure 6). The linear regression, R^2 for both lines was 0.99. Differences in pipetting could account for any differences in the days. It is important to record which cap is being used to ensure that the dilution factor that is applied is the correct one. The factor 50 and factor 10 are applied to the formula to account for the virtual dilution factor.

In general, the Tray Cell is easy to use and produces consistent results over several days of analysis. One potential issue with the Tray Cell is that any air bubbles introduced during pipetting can interfere with the analysis and lead to inconsistent results. Another issue is that the Tray Cell needs to be checked to ensure that it is in the correct position. If it is not in correctly the readings could be lower or higher than expected. It is important to make sure gently clean the Tray Cell after each analysis. This piece of equipment will be useful for measuring small volumes of samples.

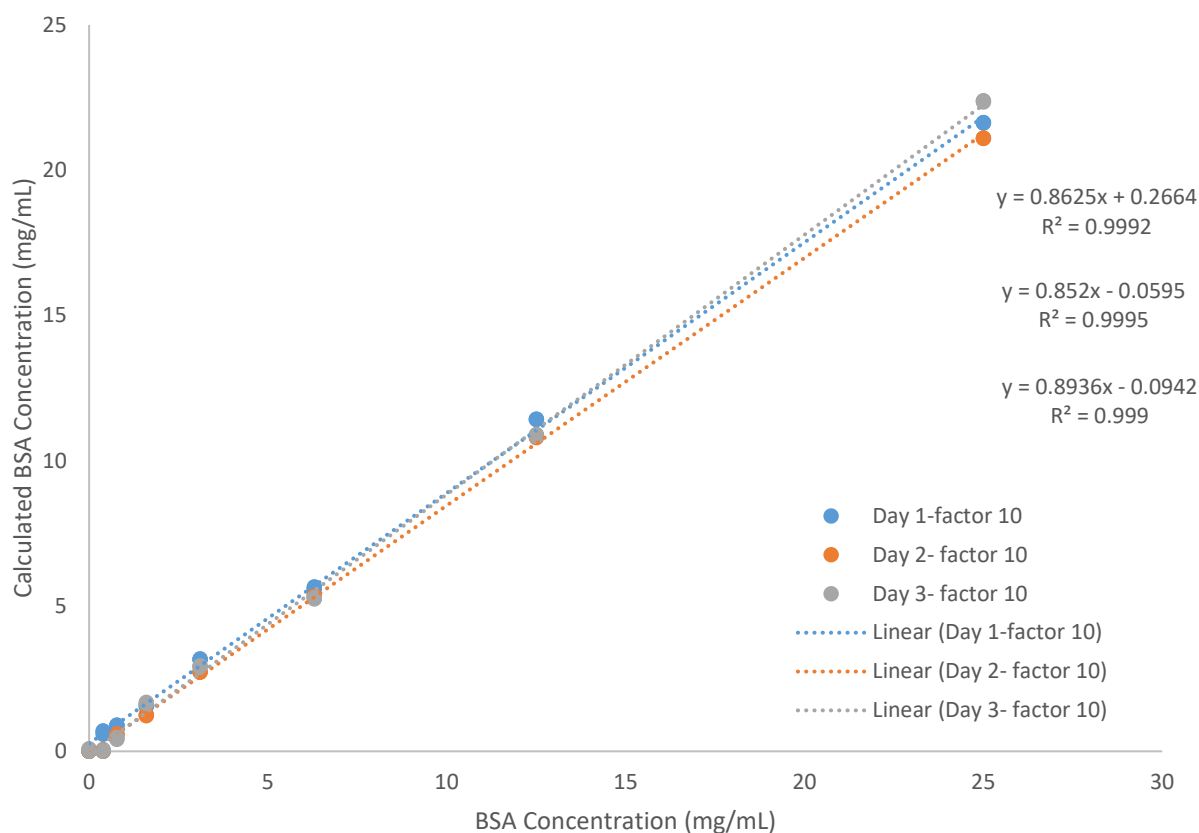


Figure 4: BSA analysis with factor 10 cap

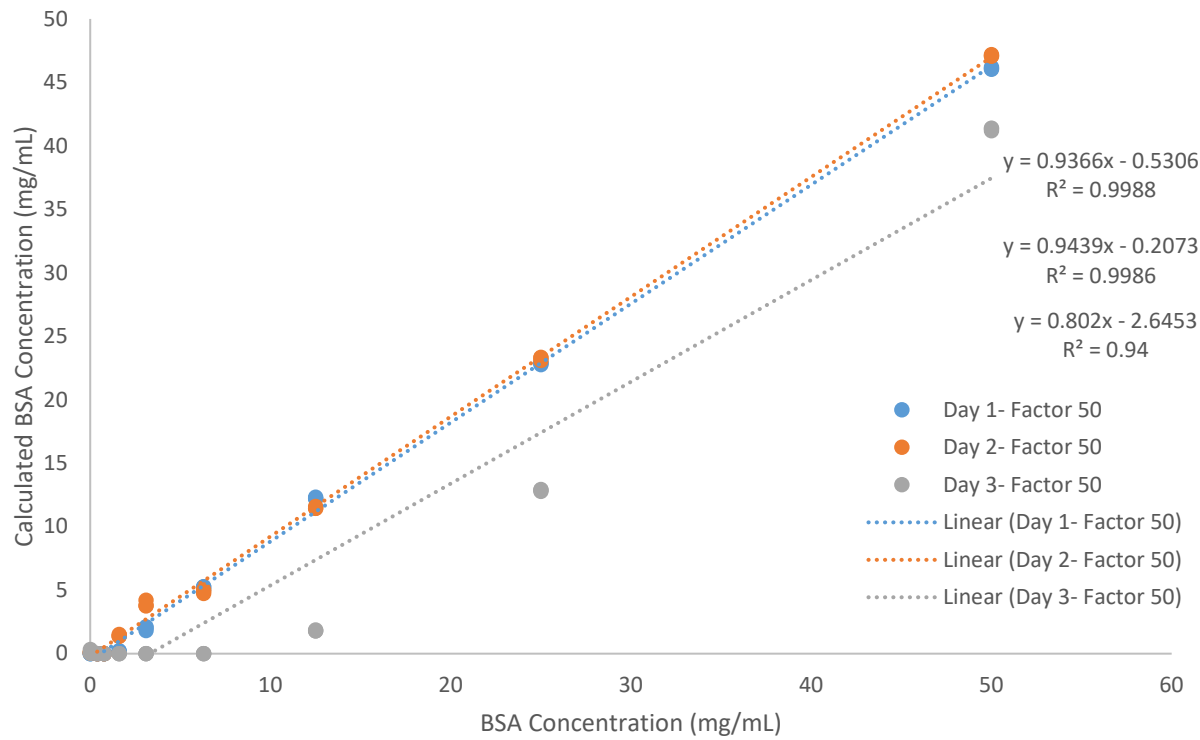


Figure 5: BSA analysis with factor 50 cap

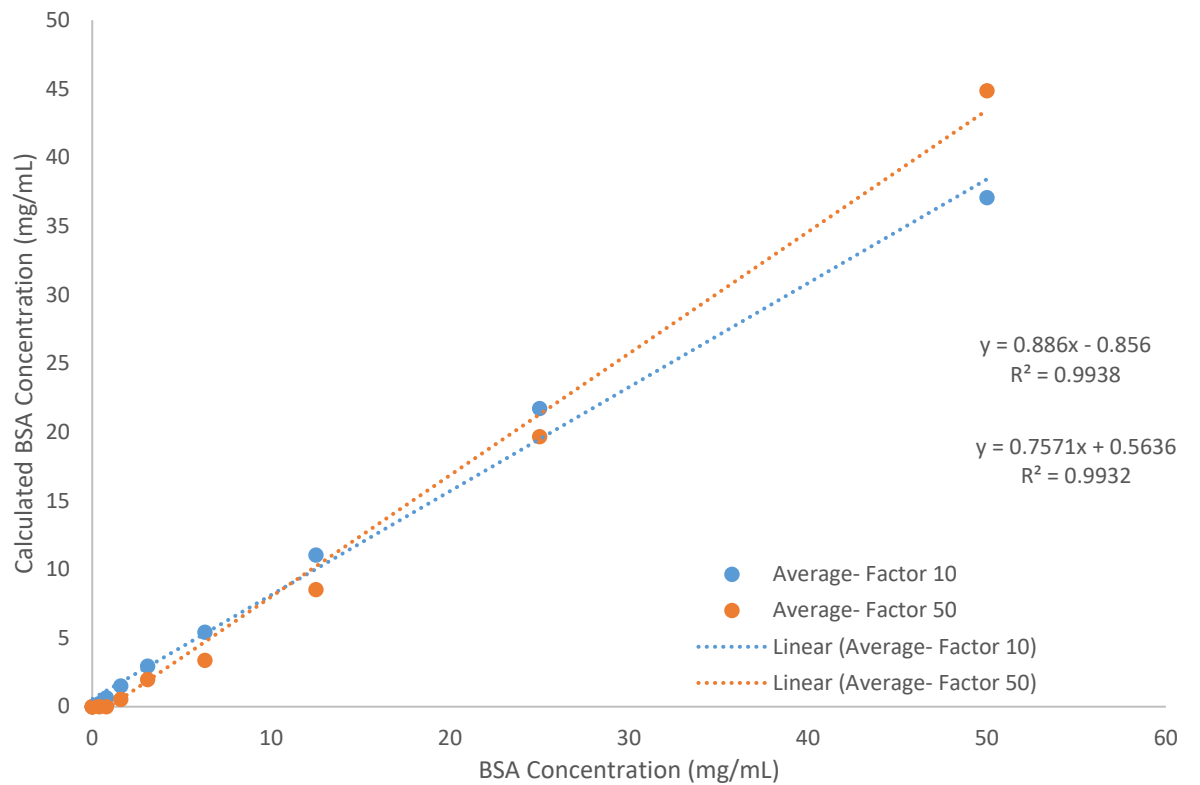


Figure 6: Comparison of two different caps over time

Conclusions

The new spectrophotometer that the RDLES Laboratory has will enable researchers to perform a variety of assays for a wide range of analytes. While the focus of this work has been on water quality, there are additional areas that this instrument could be used with different probes, such as paint analysis. A SOP for the routine use of the instrument was produced (Appendix 1). Also, an SOP for the analysis of chlorophyll (Appendix 2) was made that is specific to the instrument. The Tray Cells will be useful for the measurement of small volumes. The instrument will be a valuable tool from many years to the RDLES Laboratory.

References

- [1] APHA/AWWA/WEF, “Standard Methods for the Examination of Water and Wastewater,” *Stand. Methods*, p. 541, 2012.
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- [4] Z. Latif, M. A. Tasneem, T. Javed, and S. Butt, “Evaluation of water-quality by chlorophyll and dissolved oxygen,” *Sci. Vis.*, vol. 7, pp. 160–168, 2002.
- [5] YSI, “The Basics of Chlorophyll Measurement, Tech Note.”
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Appendix A – Standard Operating Procedure for the Spectrophotometer

Appendix A contains a general standard operating procedure for operating the spectrophotometer. This SOP is not meant to replace training with a more experienced user on the instrument.

Standard Operating Procedure for the Spectrophotometer

Reclamation Detection Laboratory for Exotic Species (RDLES) has a Cary-60 spectrophotometer. The goal of this document is to provide general information on this instrument's operation and use. This standard operating procedure is not meant to replace training with RDLES staff, but to give some a general overview of the instrument.

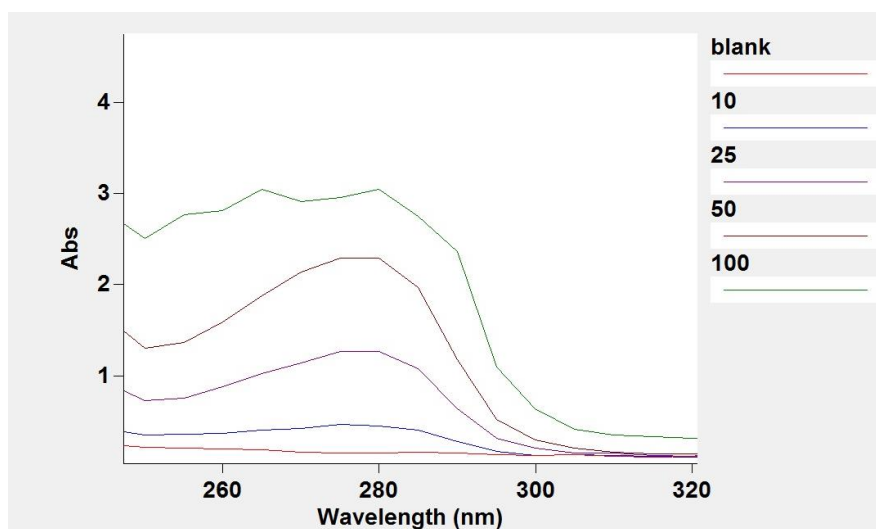


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1. SCOPE AND APPLICABILITY

This Standard Operating Procedure (SOP) is used to establish some general guidelines in the operation of the spectrophotometer. It is not meant to replace in person training or reading the operation information that Agilent has provide on the Cary-60 computer. Because of the range of different assays that can be performed this SOP is mainly focused on the general operation of the instrument.

2. DEFINITIONS

- 2.1. *Absorbance: A*, Defined as $A = \log (P_0/P)$, where P_0 is the radiant light striking the sample on one side and P is the radiant power emerging from the other side (From Daniel C. Harris, quantitative chemical analysis)
 - 2.2. *Bandwidth*: The width of radiation emerging from the exit slot of a monochromator
 - 2.3. *Cuvette*: A cell used to hold samples for spectrophotometric measurements
 - 2.4. *Monochromator*: A device (usually a prism, grating, or filter) for selecting a single wavelength of light
 - 2.5. *Path length*: The overall length of the path followed by a ray of light
 - 2.6. *Spectrophotometer*: A device used to measure absorption of light. It includes a source of light, a wavelength selector (monochromator), and an electrical means of detecting light
 - 2.7. *Spectrophotometry*: Any method using light to measure chemical concentrations
 - 2.8. *Transmittance: T*, Defined as $T = P/P_0$, where P_0 is the radiant power of light striking the sample on one side and P is the radiant power of light emerging from the other side of the sample
 - 2.9. *Wavelength: λ* , the distance between consecutive crests of a wave.
- Definitions are from Harris, 1997

3. GENERAL BACKGROUND

- 3.1. Anyone who wants to use this instrument must first be given training by a member of Reclamations Detection Laboratory for Exotic Species (RDLES) staff
- 3.2. The computer with the Cary-60 has tutorials for installing accessories and applications
- 3.3. The Cary Help file is very useful for any questions about the software or how to set up an experiment
- 3.4. Use the Agilent Cary 60 User's guide for general questions on setting up instrument.
- 3.5. Tutorials on the computer can take you through different things (such as alignment of the beam, different methods, etc.)
- 3.6. If there is an issue call Agilent, they will most likely be able to answer the question and provide advice on how to proceed
- 3.7. The computer is not on the internet because there could be issues with the software and network interacting
 - 3.7.1. All data needs to be saved on the computer, and saved to a memory stick to be transferred to other computers

4. SUMMARY OF METHOD

Spectrophotometry can be used for a wide range of assays. These assays involve different methods of sample preparation and analysis. The general operation of the instrument (turning on/off, program selection, saving files, etc.) are standard to all the assays that can be ran on the instrument.

5. INTERFERENCES

Keep the area around the instrument tidy and spill free. Always clean up the area after using the instrument. Make sure the dispose of any chemicals with the proper disposal methods. Make sure to align the lamp and check the instrument before using it. Make sure to validate the instrument before using it. The lamp is contained in its own module that have components that operation at high voltage. To avoid electric shock, never disassemble the module. The Cary-60 manual and Cary Help contains many solutions for troubleshooting any issues that might emerge.

6. CHEMICAL HAZARDS

The chemical hazards will vary depending on the assay that is being performed. Study the assay that is being performed to determine the chemical hazard risk and consult the MSDS forms for specific hazards and concerns in handling and disposing of chemicals. Consult with the chemical safety officer to determine the best ways of disposing of chemical wastes and excess reagents.

7. MATERIALS

- 7.1. The materials used will depend on the assay being performed. General laboratory materials of pipettes and other glassware can be used. Disposable plastic consumables can be used.
- 7.2. Plastic disposable cuvettes (catalog number: Agilent Part No 6610018800)
- 7.3. Microcuvettes Tray cells two different types:
 - 7.3.1. Factor 10, 1 mm
 - 7.3.2. Factor 40, 0.2 mm
- 7.4. Dip Probe, Standard Torlon Probe (part number 7910035100)
- 7.5. Chemical wipes
- 7.6. Squirt bottle for ethanol and DIH₂O
- 7.7. Pipettes (assorted sizes from 10 to 1000 µL)
- 7.8. Pipette tips

8. TURNING THE INSTRUMENT ON AND OFF

- 8.1. Press the green button on the spectrophotometer to turn the instrument on
- 8.2. Turn on the computer
- 8.3. Use the password taped to the computer to login
 - 8.3.1. Login: Admin
 - 8.3.2. Password: 3000hanover
- 8.4. Make sure to save all work and put the file on a memory stick
- 8.5. Turn off the computer
- 8.6. Turn off the spectrophotometer by pressing the green button
- 8.7. Make sure to clean up around the instrument
 - 8.7.1. Put away all reagents and dispose of any chemical wastes in the proper way
- 8.10. Table 1 shows some of the common issues that might arise with the instrument. If the indicator lamp is showing these conditions, make sure to inform RDLES laboratory staff.
- 8.11. If an issue arises please consult with a RDLES laboratory member. If the problem persists, contact the company for technical support.



I

Figure 1: Cary 60 sample compartment lid (1), sample compartment front door (2), and power button (3). From Cary-60 manual.

Table 1: Indicator Lamp conditions. From the Cary-60 User's Manual.

Color	State	Description
Green	Static On	Powered on; instrument is calibrated/initialized and not scanning
	Flashing	Scanning (no fault condition exists)
Orange (yellow)	Static on	Instrument initializing (not scanning)
	Flashing	Scan in progress while initializing/calibrating
Red	Static On	Initialization or calibration failed. Instrument is still allowed to operate
	Flashing	Instrument is scanning after initialization or calibration has failed

9. INSTRUMENT VALIDATION

- 9.1. Instrument performance tests: Xenon line scan test, photometric noise test, and baseline flatness
- 9.2. Open Cary WinUV
- 9.3. Open Validate
- 9.4. Press Start
 - 9.4.1. Instrument performance testing cannot be run with the dip probe accessory because of the loss of light in the system. Perform the validation tests using the microcell holder or normal cuvette holder.
- 9.5. Wait several minutes as the tests are performed
- 9.6. Cary validation report is produced
- 9.7. Save validation data as a batch file in the validation instrument file
- 9.8. If the instrument fails, the validation tests then it is necessary to troubleshoot the issue. Start by using the Cary-60 user guide to determine the best way forward.
- 9.9. Once done close validation program
- 9.10. Below are images of what the test results look like:

Wavelength Accuracy Test Xenon Line Scan Test

5/3/2018 10:19:26 PM
Instrument Settings
Ave Time : 2.000 sec
UV/Vis Interval : 0.150 nm
Tolerance for 541.9 Line ± 0.50 nm

541.920 line found at 542.047 PASSED

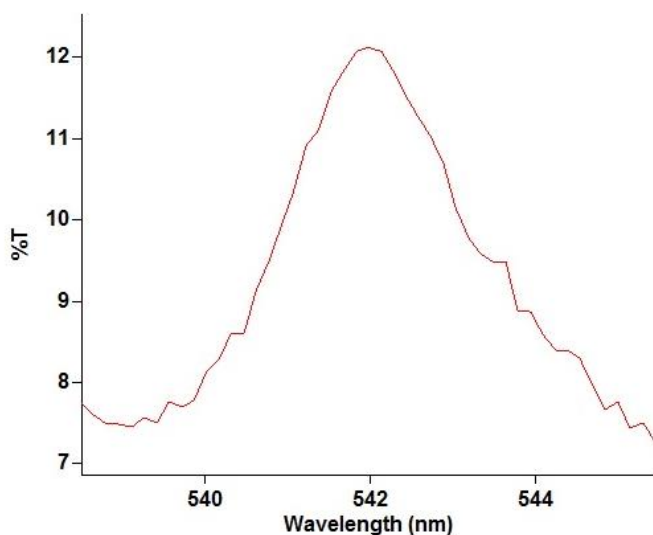


Figure 2: Wavelength accuracy test xenon line scan test outcome

Photometric Noise Test

5/3/2018 10:21:05 PM

Instrument Settings

Ave Time : 1.000 sec

UV Wavelength : 500.00 nm
0.0 Abs Test: Mean = 0.000032 Abs
Tolerance : < 0.00010000

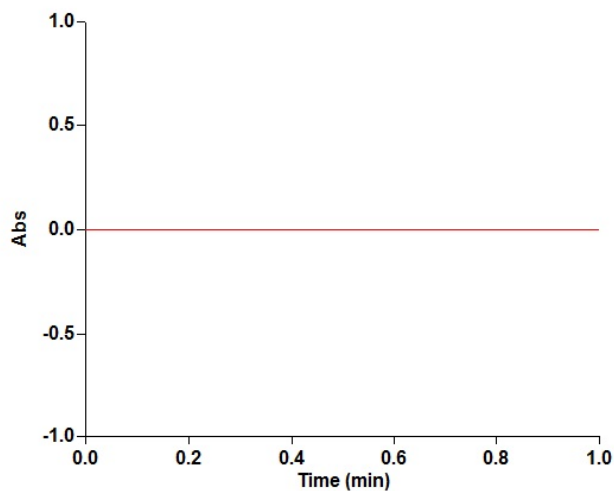


Figure 3: Photometric noise test outcome

Baseline Flatness Test

5/3/2018 10:22:13 PM

Instrument Settings

Ave Time : 0.100 sec
Interval : 1.00 nm
Scan range : 200.00 - 900.00 nm
Corrected Baseline : 200.00 - 850.00 nm, Tolerance ± 0.0010 Abs

Corrected Baseline : 200.00 - 850.00 nm, Abs from -0.0001 to 0.0002 PASSED
Baseline Correction in Abs : 0.0002

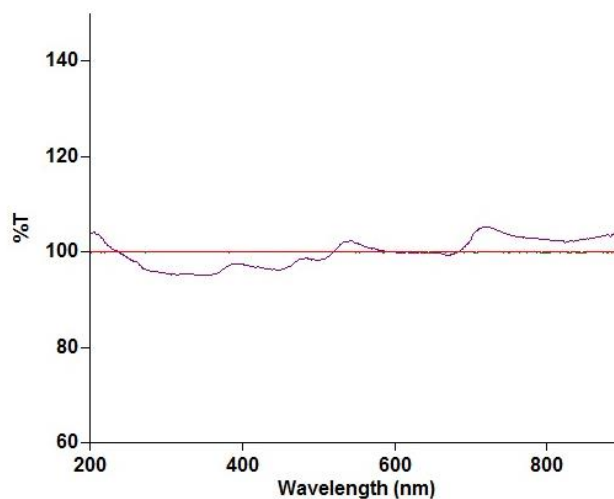


Figure 4: Baseline flatness test outcome

10. TUTORIALS FOR THE INSTRUMENT

- 10.1. The Cary WinUV window contains both the Cary Help catalog help and tutorials. It also contains the different user programs for the instrument.
- 10.2. To access the tutorials on installing accessories and application tutorials on the instrument:
 - 10.2.1. Start > programs > Agilent > Cary WinUV > Cary Tutorial
- 10.3. The application tutorials include the following:
 - 10.3.1. Cary WinUV overview
 - 10.3.2. Running a wavelength scan
 - 10.3.3. Measuring a sample at one or more wavelengths
 - 10.3.4. Running a concentration experiment using a fiber optic dip probe
 - 10.3.5. Measuring multiple samples using the multicell holder
 - 10.3.6. Running a temperature-controlled sing cell Kinetics experiment
- 10.4. To access Cary Help
 - 10.4.1. Start > Programs > Agilent > Cary WinUV > Cary Help
- 10.5. The database can be searched two different ways:
 - 10.5.1. Look through contents
 - 10.5.2. Some of the topics: safety, about your Cary, software applications, tips and tricks, troubleshooting, maintenance, accessories, contact information
 - 10.5.3. Search for specific words

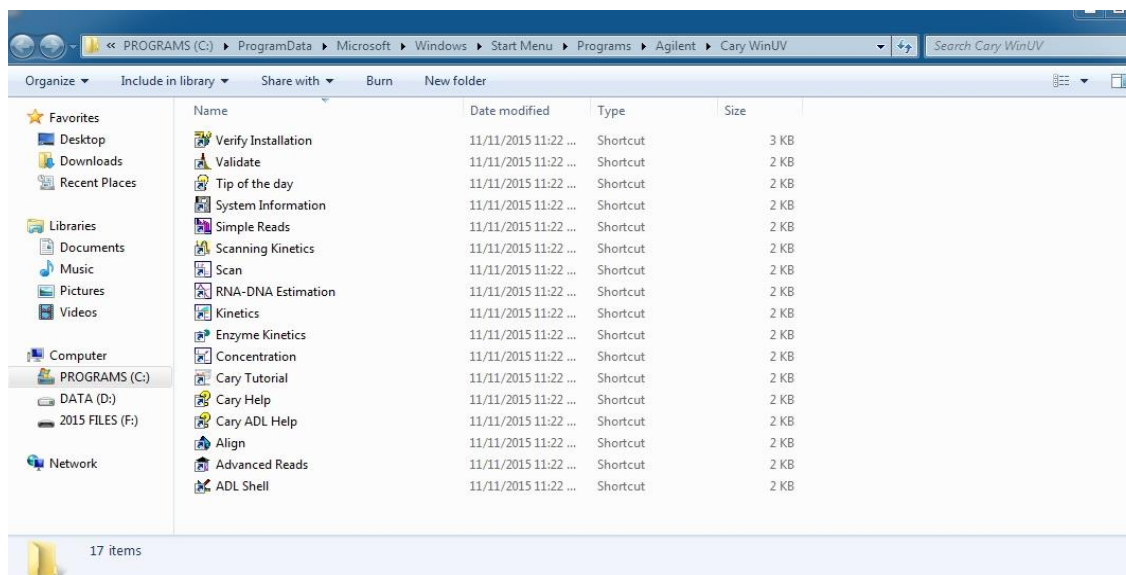


Figure 5: Cary WinUV file



Figure 6: Installing accessories in the tutorial folder

10.6. On the Cary-60 tutorial window there is information in installing the different accessories for the instrument. In particular, the fiber optic dip probe and coupler accessory are useful for setting up this accessory.

11. ALIGNMENT

- 11.1. To align the instrument to go Cary WinUV > Align
- 11.2. Rescale the current signal, zero the instrument
- 11.3. Follow the instructions in the Help window for how to best align the instrument

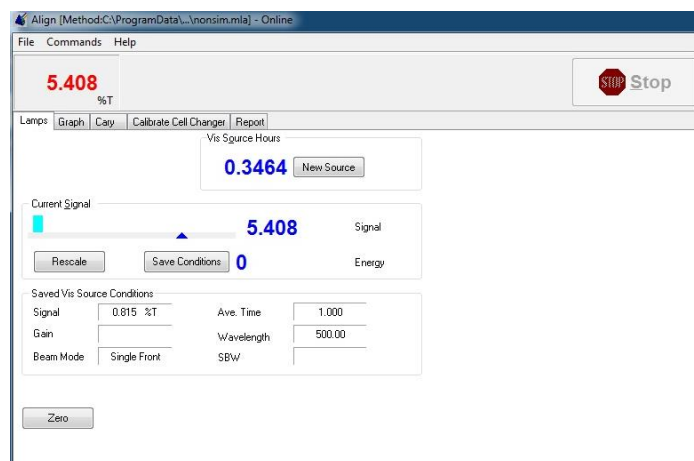


Figure 7: Alignment window

12. OPERATING THE DIP PROBE

- 12.1. Cary Help has information on how the change to the dip probe:
 - 12.1.1. Start > Programs > Agilent > Cary WinUV > Cary Help > accessories
 - 12.1.2. The dip probe does not work with the validation tests. It loses too much % transmission light
- 12.2. Open the Align program
- 12.3. Set at 500 nm
- 12.4. Put in the optical coupler (the black holder of the dip probe)
- 12.5. Turn on align
- 12.6. The instrument should register 100% transmission
- 12.7. Plug in the orange coupler cord into the optical coupler
- 12.8. It should be fastened in finger tight
- 12.9. The instrument should register 50-100% transmission
- 12.10. Plug in the dip probe
- 12.11. It should be fastened in finger tight
 - 12.11.1. The instrument should register 3-5% transmission
- 12.12. Adjust the screws on the front of the optical coupler to increase the possible transmission
- 12.13. The dip probe is ready for use
- 12.14. Determine which of the programs will be used
- 12.15. Set up the wavelength and other parameters for the assay
- 12.16. Place the dip probe in the blank sample to zero the instrument
- 12.17. Read the sample blank
- 12.18. Start to read samples
- 12.19. Once the analysis is complete save the results
- 12.20. Rinse the dip probe with DI water between each use
- 12.21. Rinse the dip probe with DI water once the assay is complete

13. USING THE MICROCELL HOLDER

- 13.1. Micro volume / normal cuvette holder
- 13.2. Place the holder cell into the instrument
- 13.3. Tighten down the screws
- 13.4. Open the align program
- 13.5. Cary WinUV > Align
- 13.6. Set at 500 nm
- 13.7. Adjust the two screws on the front of the holder cell to ensure that the percent transmission is as high as possible



Figure 8: Microcell holder. Cell holder base and microcell holder. Locating holes (1), cell holder base thumbscrew location (2), Adjustment screw (3), Microcell holder thumbscrew (4), Cell lifter (5), Hexagonal ball driver (6, not shown) (From Cary User's Manual)

- 13.8. More details on Installing and alignment of the Microcell holder can be found in the user manual Page 27-29
- 13.9. See also the microcell holder information packet (TrayCell Manual)
- 13.10. Once the microcell holder is in place it is possible to use the microcuvettes
- 13.11. The instrument has two different microcuvettes
 - 13.11.1. Factor 10, LP 1 mm
 - 13.11.2. Factor 40, LP 0.2 mm
 - 13.11.3. Differences between each of the cuvettes and the volumes and concentrations that they can read.
- 13.12. Select which of these cuvettes will work best for the assay. The RDLES laboratory has the 1.0 and 0.2 mm caps for the microcuvette's.

Table 2: Tray Cell Measuring Range (from Tray Cell manual)

Nucleic Acids	Sample Specific factor**	1.0 mm (factor 10) [ng/μL]*	0.2 mm cap (factor 50) [ng/μL]*	Total measuring range [ng/μL]*
dsDNA	50	13 - 850	63 - 4250	6 - 8500
ssDNA	37	9 - 629	46 - 1345	5 - 6290
ssRNA	40	10 - 680	50 - 3400	5 - 6800
Oligomers	30	8 - 510	38 - 2550	4 - 5100
Sample quantity required		3 - 5 μL	0.7 - 4 μL	

*typical concentration values that can be measured with the spectrophotometer

**sample-specific factor of nucleic acid samples = concentration (ng/μL) at an absorbance of 1

- 13.13. Once the microcuvette is selected place it into the microcell holder
- 13.14. Select which program works best for the assay
- 13.15. Set up the wavelength and parameters for the assay
- 13.16. Gently pipette the small volume of a blank onto the microcuvette
- 13.17. Place the cap over the sample
- 13.18. Read the blank
- 13.19. Take a Q-tip to remove the liquid after each sample is read
- 13.20. Pipet each sample and analyze it
- 13.21. Once completed save the data
- 13.22. Gently wipe the microcell off with the Q-tip
- 13.23. Make sure that the microcell is clean

14. PROGRAMS AVAILABLE ON INSTRUMENT

There are several different programs available. Each program is user friendly and can be modified to fit the needs of the assay being performed. For example, parameters such as wavelength and number of wavelengths being analyzed can be adjusted. It is possible to modify the program and then save the settings for future use. The following are the programs available.

- 14.1. Simple Reads
- 14.2. Scanning kinetics
- 14.3. Scan
- 14.4. RNA-DNA Estimation
- 14.5. Kinetics
- 14.6. Enzyme Kinetics
- 14.7. Concentration
- 14.8. Advance Reads

15. SAVING DATA

- 15.1. If using simple reads program, save data as a batch file
- 15.2. Close the simple reads program
- 15.3. Open the advance reads program
- 15.4. Open the batch file that was saved
- 15.5. Save the file with .CSV format
- 15.6. This file can then be opened in Excel and graphs made from the data
- 15.7. Open the file on the computer that has excel
- 15.8. Highlight the data
- 15.9. Go text to columns to get the data into individual cells
- 15.10. Save the file as an excel spreadsheet

16. REFERENCES

Agilent Cary-60 User Manual:
<https://www.agilent.com/cs/library/usermanuals/Public/G6860-90001.pdf>

17. CONTACT INFORMATION

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Appendix B – Chlorophyll *a* and Pheophytin Analysis

Appendix B contains a standard operating procedure for the analysis of chlorophyll *a* and pheophytin. There are several SOP's available for the analysis of these two organic molecules. This SOP is meant to be a working document for how RDLES performs this analysis.

Chlorophyll Analysis by Spectrophotometry

There are many standard operating procedures available for the analysis of chlorophyll *a* and pheophytin. This document is meant to be specific to the spectrophotometer that Reclamations Detection Laboratory for Exotic Species (RDLES) has in the lab. It is not meant to replace the method presented in “Standard Methods for the Examination of Water and Wastewater”[1]. Chlorophyll *a* and pheophytin amounts are an indication of the abundance of photosynthetic organisms in a water body. Using visible wavelength spectrophotometry is one of the methods that can be used to determine this abundance. There are two assays that can be carried out at the same time when performing chlorophyll analysis. First, chlorophyll *a* and pheophytin assay where hydrochloric acid (HCl) is used to acidify the sample. Second, the trichromatic method for analyzing chlorophyll *a*, *b*, and *c*. In this assay the sample does not get acidified. Both methods use several of the same wavelengths thus making it possible to analyze the pre-acidified samples using both methods. It is important when reporting results that the method that was used and being reported is very clear to the client. Because these two different methods can give divergent results for chlorophyll *a*.

CHLOROPHYLL A AND PHEOPHYTIN ASSAY

Safety

- Always wear gloves.
- Remember: never pour anything down the drain without checking the waste disposal procedures first.
- The remaining acetone/water mixture left after the analysis can be evaporated away in a fume hood.
- Review the Material Safety Data Sheets (MSDS) for the chemicals used in this assay.
- Acetone is a chemical hazard and may be toxic to biological systems if ingested or inhaled.
- HCl's: a chemical hazard and care should be taken when preparing its solution.
- Use the fume hood to prepare all acid solutions to avoid inhalation.

Supplies

- Spectrophotometer (Cary-60)
- Volumetric flask, 10 L
- Aluminum Foil
- Tweezers
- Centrifuge, capable of 675 x g
- Rack for holding centrifuge tubes
- 15 mL centrifuge tubes
- Plastic cuvettes

Reagents to Prepare

- HCL, 0.1 N: Add 8.5 mL of concentrated HCl to approximately 500 mL water and dilute to 1 Liter.
- Aqueous acetone solution with saturated magnesium carbonate: Weigh out one gram of magnesium carbonate and transfer into a 100 mL volumetric flask. Bring up to 100 mL with deionized (DI) water. Then transfer this solution to the storage bottle with an auto-

pipette that will dispel 10 mL of the solution. Add 900 mL of acetone to bring the final volume up to 1 L and mix. Store this solution in the refrigerator.

- Chlorophyll *a* certified stock standard from Turner Designs.
(<http://www.turnerdesigns.com/dyes-stds-pigments/article/507-chlorophyll-standards>)

Calibration and Standardization

- No calibration is required.
- Standardization is based on the Turner Chlorophyll *a* reporting limit check standard in an intermediate stock solution of 90% acetone.
- Cuvettes used to zero the instrument and measure sample absorbance are matched.

Sample Collection

1. Two-liter grab samples are collected in brown polyethylene bottles.
2. Mail the bottles overnight to the laboratory on ice.
3. Once the samples arrive in the lab, the water should be filtered onto filter membranes.
4. Fold the filter membrane in half, gently blot off excess liquid, and place in a pre-labeled envelope.
5. After filtration, the filters can be stored frozen for up to 28 days.
6. In addition, create a laboratory reagent blank by filtering 1 L of DI water, store the filter, and process with the field sample filters.
7. Make sure to record the volume of water filtered because this volume is needed to calculate the chlorophyll concentration.

Day 1- Sample preparation

1. Before extracting the filters in test tubes, make up a fresh batch of 90% acetone and 10% distilled water solution in an auto-pipette bottle that will dispel 10 mL of this solution into each test tube.
2. Label two sets of test tubes (15 mL conical) that will correspond to the filter samples and place in a test tube rack.
3. Perform all the extraction steps in the dark.
4. Remove the filter from the freezer. Process the samples one at a time. Keep the other filters in the freezer.
5. Open the envelope that the filter is in. Gently tear the filter in half and place into the pre-labeled conical tube.
6. Add 3 ml of 90% acetone 10% water to the filter.
7. Use narrow spatula to grind up the filter into fragments. Vortex or shake the sample to suspend the sample throughout the liquid.
8. Add up to 10 mL of 90% acetone 10% water and shake the conical tube to distribute the filter throughout the solution.
9. Cover sample in tinfoil and place the sample in the refrigerator to store at 4° C for 18-20 hours.
10. Repeat this process for all the water samples and the sample blank.
11. Make sure to use a new spatula for each sample.
12. Clean all the spatulas and the area where the extractions were performed once all the filters have been processed.

Day 2- Sample Analysis

1. Turn on the spectrophotometer and computer.
2. Run the validation program to check the instrument.
3. Open the sample reads program.
4. Under set up select the wavelengths for this analysis.
 - a. For chlorophyll *a* and pheophytin use: 664, 665, and 750 nm.
 - b. For the trichromatic method use: 647, 664, 665, and 750 nm.
 - i. For both assays the 750 nm measurement is for turbidity and is subtracted from the other wavelengths. If the 750 nm absorbance reading is greater than 0.005 that is an indication that the sample has been poorly clarified and causes a measurement error.
 - c. All these wavelengths can be selected and analyzed at the same time.
5. The centrifuge by the spectrophotometer can hold up to six samples at a time.
6. Pull out the samples for centrifugation. Keep the remaining samples in the refrigerator under tin foil.
7. Centrifuge the samples for two minutes at 2000 rpm.
 - a. This is done to get the filter debris to the bottom of the tube.
 - b. If the filter material is present when the sample is analyzed it will cause errors in the readings.
8. Place the samples under tin foil to keep them away from light.
9. Blank the spectrophotometer with a “blank” of the 90% acetone 10% water mixture.
10. Once the instrument is blanked, start analyzing samples for chlorophyll *a*.
11. Transfer 3 mL of the sample to a fresh pre-labeled 15 mL conical tube (this will be used for the pheophytin analysis).
12. Transfer ~2 mL of the sample to a plastic cuvette.
13. Analyze the sample by hitting start on the computer. Perform each analysis in triplicate with the instrument.
14. Pour the sample from the cuvette into the liquid waste container.
15. Use a fresh plastic cuvette for the next sample.
16. Continue to perform this process for all the samples.
17. Once all the samples have been analyzed, it is time to analyze the samples for pheophytin.
18. Set up a 3 mL blank sample in a 15 mL conical tube with the 90% acetone 10% water mixture and add 0.1 mL of 0.1 N HCl to the blank.
 - a. Gently invert for the blank for 90 seconds.
 - b. Use this sample to re-blank the instrument prior to running samples.
19. Perform this step on one sample at a time. Take the 3 mL sample in the 15 mL conical tube and add 0.1 mL of 0.1 N HCl to the sample.
 - a. Gently invert the sample for exactly 90 seconds.
20. Pour the sample into a fresh plastic cuvette and read on the spectrophotometer. Analyze each sample in triplicate.
21. Continue to do this until all the samples have been analyzed.
22. Save the results with the days date and the type of analysis performed on the computer. Transfer the file to a memory stick.
23. Use the chlorophyll assay spreadsheet to calculate the concentrations of chlorophyll *a* and pheophytin in the sample. This spreadsheet requires the starting volume of the sample to

be entered in one of the columns. Also, the trichromatic method analysis can be calculated. Place these values into the reporting sheet.

Calculations (see chlorophyll and pheophytin calculation spreadsheet)

Key values to record

1. Volume of water filtered (volume of sample in L/1000)
2. Volume of extract in L (volume of acetone used 10 mL or 0.01 L)
3. mg/m^3 is the same as ug/L . These two units can be interchangeable.

General notes:

Correction of the samples (subtract 664-750 and 665-750) to correct for turbidity in the sample

Chlorophyll a and Pheophytin Calculation

Chlorophyll ug/L = $[26.7 (\text{corr. } 664 - \text{corr. } 665) \times \text{volume of extract in L}] / [(\text{volume of sample in L})/1000 \times 1 \text{ cm}]$

Pheophytin, ug/L = $[26.7 (1.7(\text{corr. } 665) - \text{corr. } 664) \times \text{volume of extract in L}] / [(\text{volume of sample in L})/1000 \times 1 \text{ cm}]$

Trichromatic Method

$\text{Ca} (\text{mg}/\text{L}) = 11.85 (\text{OD}664) - 1.54 (\text{OD}647) - 0.08 (\text{OD}630)$

$\text{Cb} (\text{mg}/\text{L}) = 21.03 (\text{OD}647) - 5.43(\text{OD}664) - 2.66 (\text{OD}630)$

$\text{Cc} (\text{mg}/\text{l}) = 24.52 (\text{OD}630) - 7.60 (\text{OD}647) - 1.67 (\text{OD}664)$

The OD values for OD664, 647, and 630 are all corrected for turbidity by subtracting the OD750 from value.

After the concentration of the pigment in the extract is determined, to calculate the pigment per unit volume is done using this equation.

Chlorophyll *a*, $\text{mg}/\text{m}^3 = \text{Ca} \times \text{extract volume (L)} / \text{volume of sample (m}^3\text{)}$

One Liter equals 0.001 m^3 .

References

- [1] APHA/AWWA/WEF, "Standard Methods for the Examination of Water and Wastewater," *Stand. Methods*, p. 541, 2012.

Data Sets that Support the Final Report

If there are any data sets with your research, please note:

- Share Drive folder name and path where data are stored:
- Z:\DO\TSC\Jobs\DO_NonFeature\Science and Technology
\ 2017-PRG-Spectrophotometric Analysis of Water Samples
- Point of Contact name, email, and phone: Jacque Keele, jkeele@usbr.gov, 303-4452187
- Short description of the data: Spreadsheets, standard operating procedures, and background literature.
- Keywords: Spectrophotometer, chlorophyll analysis, absorbance, water analysis
- Approximate total size of all files: 40MB (folder size)

