**Standard Operating Procedure: Excitation-Emission Matrices (EEMs)**

(Following SOP developed by Jenae Pinney on 07/05/11)

Data post-processing SOP included at the end of the document!

***Method Reference:*** *excellent general reference for EEMs in aquatic systems!*

Coble, P., Lead, J., Baker, A., Reynolds, D., & Spencer, R. (Eds.). (2014). Aquatic Organic Matter Fluorescence (Cambridge Environmental Chemistry Series). Cambridge: Cambridge University Press. doi:10.1017/CBO9781139045452

*\*\* Note: unfortunately, VT does not have access to this book, but a pdf copy of book can be requested via the Inter-Library Loan – highly recommend!*

***Calibration Reference:***

Lawaetz, A.J., Stedmon, C.A. (2009). Fluorescence intensity calibration using the Raman scatter peak of water. Applied Spectroscopy. 63: 936-940.

***Quinine Sulfate Standard Reference:***

Velapoldi, R.A., Mielenz, K.D. (1980). A fluorescence standard reference material: Quinine sulfate dehydrate. National Bureau of Standards Special Publication 260-64.

Cory, R.M., Miller, M.P., McKnight, D.M., Guerard, J.J., Miller, P.L. (2010). Effect of instrument-specific response on the analysis of fulvic acid fluorescence spectra. Limnology and Oceanography: Methods. 8: 67-78.

***Data processing and PARAFAC modeling reference:***

Murphy, K.R., Stedmon, C.A., Graeber, D., Bro, R. (2013). Fluorescence spectroscopy and multi-way techniques PARAFAC. Analytical Methods 5: 6557.

*\*\* Note: This reference includes a GREAT tutorial on PARAFAC!*

*Most up-to-date scripts (Matlab!) for running a PARAFAC model on EEMs data (following the Murphy et al. 2013 paper):*

<http://dreem.openfluor.org/>

*Link to OpenFluor: the on-line database that you can compare your PARAFAC model to in order to identify the chemical composition of different PARAFAC components.*

<https://openfluor.lablicate.com/> (note: you have to register for an account before you can upload data – it’s free!)

*Finally, a good general resource (especially if/when thinking about publishing the data! Also includes a script for applying a PARAFAC model):*

<https://portal.edirepository.org/nis/mapbrowse?packageid=edi.841.1>

*This SOP and all additional information/scripts needed to analyze samples and conduct post-processing can be found on the Carey Lab GitHub:*

<https://github.com/CareyLabVT/Reservoirs/tree/master/Data/DataNotYetUploadedToEDI/Raw_EEMs>

**Sample collection and filtration:**

1. Filter collected water through an acid-washed filter cartridge fitted with a pre-combusted (550oC for 4 hrs) Whatman GF/F 25 mm filter.
2. Sample should be collected into a pre-combusted (550oC for 4 hrs) 20 mL glass scintillation vial and capped with an acid-washed Teflon lined cap. ONLY fill sample vial ¾ full to avoid cracking.
3. Keep samples cool (preferably in a cooler w/ ice) and out of direct sunlight until storage.
4. Freeze samples at -20oC until analysis (typically within 6 months). Freezing vials on their side helps minimize vial breakage.

**Prior to analysis: the afternoon/evening before analysis**

1. Remove samples from the freezer and check for cracking.
2. Place cracked vials into acid washed disposable beakers and cover with parafilm or aluminum foil.
3. Place all samples in the fridge to thaw overnight.
4. The morning of analysis, remove samples from fridge and transfer cracked samples to new, pre-labeled, and pre-combusted scintillation vials.
5. Transport to HABB1 in a cooler to protect against sunlight and to keep cool.

**Analysis: CDOM (absorbance) and FDOM (EEMs)**

Ideally, samples will be analyzed for both absorption and fluorescence on the same day by first analyzing absorption then immediately analyzing sample for fluorescence on the spectrofluorometer. Both the spectrophotometer (CDOM, absorbance) and the spectrofluorometer (FDOM, EEMs) are located in the same location (HABB1, Lab 352) and are controlled by the same computer.

**Before getting started:**

1. Turn on the spectrofluorometer using the rocker switch on the right side and allow to warm-up for 30 minutes. Record lamp hours in the FluoroMax-4 log book along with the day of analysis and your initials.
2. Turn on the spectrophotometer with the rocker switch on the right side (I recommend turning the spectrophotometer on about 10-15 minutes after turning on the spectrofluorometer). Allow the lamp to warm-up 30 minutes prior to use.
   1. The spectrophotometer will run through a series of initializations. Once complete, the instrument will ask for log-in information. The User is: Administrator. There is no password, just hit enter.
3. Turn-on the computer. The Computer user name is: Administrator. There is no password.
4. Fill a squirt bottle with FRESH MilliQ DI water from the lab next door.
5. Make sure all standards (as necessary) and samples are at room temperature.
6. Make sure you have two clean quartz cuvette (located in the Fluorescence Manuals and Cells drawer). ALWAYS wear gloves when handling the cuvette (located in the ‘Fluorometer Gloves’ cabinet; please use the BLUE gloves!). Try to touch only the corners and top of the cuvette to minimize potential contamination. You will need one reference cuvette (for the spectrophotometer) and a sample cuvette that is used for both the spectrophotometer and spectrofluorometer.
7. Note: Each absorbance + fluorescence scan takes approximately 40 minutes to complete. I would suggest running about 12 samples/8 hour day.

**Daily instrument checks:**

Once both lamps have been allowed to warm-up for 30 minutes, you may begin the daily instrument checks. I recommend starting with the spectrofluorometer.

*Spectrofluorometer (EEMs, FDOM):*

1. Start by connecting the spectrofluorometer to the computer:
   1. Open the ‘FluorEssence’ program on the desktop. The fluorometer should be on before you start the software.
2. **Xenon Lamp check (excitation check):**
   1. Click “Collect” then “Experiment Setup”. This will bring up a dialogue window.
   2. The computer will need a few minutes to connect to the instrument (it will indicate it is “initializing”).
   3. Load the ‘LampCheck.xml’ file (Desktop -> ExpFiles). This should come up with the following parameters:
      * + Under ‘Monos’:

Excitation: 200 – 600 nm at 1 nm increments and a 1 nm slit width

Emission: 350 nm with a 1 nm slit width

* + - * Under ‘Detectors’:

Integration time = 0.1 s

R1 is enabled

Correction, Blank subtract, and dark offset are NOT selected

Within the “Signal Algebra” box, the Formulas should be R1

* 1. Make sure the sample chamber of the Fluoromax is empty and completely closed. Click “Run” at the bottom of the dialogue box to begin the scan.
  2. After the check is complete, the program will ask you to save the project.
     1. At the start of each day, you will want to make a new folder to save all the day’s scans in. In the pop-up window navigate to Data -> Alex. Right click to create a new folder named ‘YYYYMMDD\_IN’ where IN indicates your initials. Save the .opj file in this folder as ‘YYYYMMDD.opj’.
  3. Right click on the new file located underneath the ‘data’ folder. Re-name to: LampScan.
  4. Use the Screen Reader button to find the location of the highest peak. The peak should be **467 +/- 0.5 nm.** Record this information in the log book and on the bench sheet.
  5. Save the project under My Computer -> Local Disk (C:) -> Program Files\fluorometer\data\lamp as YYYYMMDD.
  6. Now, export the data as a .csv file. Under File -> Export click ‘ASCII Data…’. A window will pop-up asking you to choose the location to save the file. Navigate to Data -> Alex. Save file as YYYYMMDD\_LampScan.csv.

1. **Cuvette check**
   1. Rinse the quartz cuvette with fresh MilliQ water (again, ALWAYS wear gloves when handling the cuvette). Fill the cuvette with MilliQ water, wipe clean with a Kimwipe, insert the cuvette into the Fluoromax chamber and close the lid completely.
   2. Click “Collect” then “Experiment Setup”. This will bring up the same dialogue box as for the lamp check.
   3. Load the ‘cuvettecheck.xml’ (Desktop -> expFiles). This should come up with the following parameters:
      * + Under ‘Monos’:

Excitation: 240 nm with a 5 nm slit width

Emission: 270-430 nm with 2 nm increments and a 5 nm slit width

* + - * Under ‘Detectors’:

Integration time is 0.25 seconds

S1 and R1 are enabled

Correction, blank subtract, and dark offset are NOT selected

Within the “Signal Algebra” box, the Formulas should be S1 and S1/R1

* 1. Click “Run” at the bottom of the dialogue box.
  2. After the sample is finished running, right click on the new graph now located underneath the ‘data’ folder. Re-name to: CuvetteCheck.
  3. Save the project under My Computer -> Local Disk (C:) -> Program Files\fluorometer\data\cuvette’ as YYYYMMDD.opj.
  4. Export a .csv file by clicking on the newly created (and renamed) graph. Under File -> Export click ‘ASCII Data…’. A window will pop-up. Navigate to Data -> Alex -> YYYYMMDD\_IN (the folder you created above). Save file as ‘YYYYMMDD\_CuvetteCheck.csv’.
  5. Record the location of the maximum intensity and the maximum intensity in the log book and on the bench sheet. The maximum intensity should be ~50,000 CPS or less.

1. **Raman check (emission check):**
   1. Keep the cuvette with MIlliQ water in the sample holder with the lid completely closed.
   2. Click “Collect” then “Experiment Setup”. This will bring up the same dialogue box as above.
   3. Load the ‘raman.xml’ file (Desktop -> expFiles). This should come up with the following parameters:
      * + Under ‘Monos’:

Excitation: 350 nm with a 5 nm slit width

Emission: 365-450 nm with 1 nm increments and a 5 nm slit width

* + - * Under ‘Detectors’:

Integration time = 0.1 seconds

S1 and R1 are enabled

Correction, blank subtract, and dark offset are NOT selected

Within the “Signal Algebra” box, the Formulas should be S1 and S1/R1

* 1. Click “Run” at the bottom of the dialogue box.
  2. Right click on the new graph now located underneath the ‘data’ folder. Re-name to: Raman.
  3. Save the project under My Computer -> Local Disk (C:) -> Program Files\fluorometer\data\raman as YYYYMMDD.opj.
  4. Export a .csv file by clicking on the newly created (and renamed) graph. Under File -> Export click ‘ASCII Data…’. A window will pop-up. Navigate to Data -> Alex -> YYYYMMDD\_IN (the folder you created above). Save file as ‘YYYYMMDD\_Raman.csv’.
  5. Record the location of the Raman peak and the maximum intensity in the log book and on the bench sheet. The peak should be located at 397 nm and have a maximum intensity ~ 400,000 CPS.

*Spectrophotometer (absorbance, CDOM):*

1. Connect the spectrophotometer to the computer:
   1. On the computer, open the program UVProbe 2.34.
   2. On the spectrophotometer “Mode Menu” screen, click F4 for “PC Control”.
   3. In the UVProbe software on the computer, click the “Connect” button near the bottom of the screen to connect with the spectrophotometer.
2. In UVProbe, check the instrument is in ‘Spectrum Mode’ located in the upper toolbar (make sure the button that looks like a rainbow coming out of a beaker is selected; if you hover the mouse over it, it should read “Spectrum”).
3. After selecting the Spectrum mode, click the “M” button located on the upper toolbar to set the parameters.
4. A dialogue window will pop-up asking for a wavelength range, scan speed, sampling interval, and scan mode. Make sure the following parameters are set:

Wavelength Range (nm): Start = **850** to End = **190**

Scan Speed: **Fast**

Sampling Interval (nm): **1.0**

Scan mode: **Single**

1. Once parameters are correct, hit okay.
2. Zero the baseline by clicking the “Baseline” button near the bottom of the screen. There should be NOTHING in the sample or reference side of the instrument and the cover should be closed.
3. Collect two quartz cuvettes from the drawer labeled “Fluorometer Manuals and Cells” located under the spectrofluorometer. YOU MUST wear gloves to handle the quartz cuvettes. Thoroughly rinse both cuvettes with MilliQ water prior to use.
4. Fill one cuvette with MilliQ water and wipe clean with a Kimwipe. Make sure there is no contamination on the outside of the cuvette. Place into the reference compartment (the cuvette holder towards the back of the instrument). This will serve as the reference and will remain in place for the rest of the analysis.
5. Fill another cuvette with MilliQ water, wipe clean with a Kimwipe, and place into the sample compartment (the cuvette holder towards the front of the instrument).
6. Close the cover and on the computer, click the “Autozero” button near the bottom of the screen.
7. Run a MilliQ sample blank by leaving the MilliQ water in the sample compartment.
8. Click the “Start” button near the bottom of the screen to start collecting the spectrum.
9. After the spectrum has been collected, the program will prompt you to save the new data set. This DOES NOT automatically save the spectrum to the hard drive.
10. To save the data, after each spectra, go to file ‘Save as’. Save the file as a .spc file (spec file). Save the file as YYYYMMDD, in the same folder you created above. All scans from the day will be saved in this .spc file. At the end of the day, you will also export the data as a .txt file.

**Sample analysis:**

Now that all instrument checks have been completed, samples can be analyzed. It’s easiest to first analyze the sample on the spectrophotometer than transfer the same cuvette to the spectrofluorometer. The first ‘sample’ will be the MilliQ water blank (this must be done at the beginning of each day!). You have already collected a blank sample on the spectrophotometer; now collect a blank sample on the spectrofluorometer as described below.

1. **MilliQ Water 3D EEM**
   1. Before running samples, a MilliQ Water blank 3D EEM must to collected. This will serve as the blank scan used in post-processing corrections.
   2. Insert a clean cuvette filled with MilliQ water in the sample holder. Make sure the cover is securely shut.
   3. Click “Collect” then “Experiment Setup”. This will bring up the same dialogue box as above.
   4. Load the ‘3DEEM\_AH.xml’ (Desktop -> expFiles). This should come up with the following parameters:
      * + Under ‘Monos’:

Excitation: 240-450 with 5 nm increments and a 5 nm slit width

Emission: 300-600 with 2 nm increments and a 5 nm slit width

* + - * Under ‘Detectors’:

Integration time = 0.1 seconds

S1 and R1 are enabled

Correction, blank subtract, and dark offset are NOT selected

Within the “Signal Algebra” box, the Formulas should be S1 and S1/R1

* 1. Click “Run” at the bottom of the dialogue box.
  2. Re-name the three new data files (S1, R1, S1/R1). Rename each file with ‘MilliQ\_S1’, etc.
  3. Save the project under ‘My Computer -> Local Disk (C:) -> Program Files\Jobin Yvon\Data\Alex\YYYYMMDD\_IN’. All the remaining scans will be saved in this .opj.
  4. Click on the sample name with ‘\_S1/R1’, then export the data as a .csv with the naming convention ‘YYYYMMDD\_MilliQ’. Record the file name on the Bench sheet.

1. Now actual samples can be run! Remove the sample cuvette from the spectrofluorometer (using gloves!) and dump the MilliQ water. Rinse the cuvette with a pipetteful of sample, then fill the cuvette with sample.
2. Wipe cuvette clean with a Kimwipe and insert into the sample holder of the spectrophotometer and close the lid.
3. In the UVProbe software, click “Start”. This will start the scan. After the scan is complete click okay in the pop-up window. The raw data for each sample can be seen by clicking ‘Data Print’ in the toolbar.
4. Record the sample name on the Absorbance bench sheet.

**NOTE:** Sample absorbance must be above 0.02 and below 0.2 in order to run on the spectrofluorometer at 240 nm! If the sample is over 0.2, dilute the sample with MilliQ water and re-run noting the dilution used on the bench sheet. The diluted sample will also be analyzed on the spectrofluorometer!

1. Remove the cuvette and transfer to the spectrofluorometer (wearing gloves!); close the lid completely.
2. In the Fluoressence software, click Collect -> Experiment Setup. Select the same file as above (3DEEM\_AH). The settings should be the same as the MilliQ blank. Click “Run” at the bottom of the dialogue box.
3. After the sample has finished running re-name each of the three newly create files as ‘SampleName\_’. The sample name should also be recorded on the bench sheet.
4. Click on the file with ‘\_S1/R1’ in the file name and export as a .csv using the naming convention ‘YYYYMMDD\_SampleName’.
5. Repeat steps 1-8 for each sample.
6. After the last sample has been analyzed on the spectrophotometer, make sure the .spc project file is saved a final time. Then copy and paste all the data from the Data Print into Notepad. Save the .txt file as ‘YYYYMMDD\_CDOM’ in the same day’s file.
7. Close the UVProbe software. Use gloves to remove the reference cell and turn-off the spectrophotometer using the rocker switch.
8. After the last sample on the spectrofluorometer has been analyzed and saved, make sure the .opj file is saved, then close down Fluoressence. Record the final number of hours in the log book and calculate the number of hours the instrument has been on. Turn off the instrument using the rocker switch.

*Saving sample files:*

At the end of each day, sample files will need to be transferred from the instrument computer to the lab server (and then to google drive).

1. From the instrument computer, copy and paste the sample file (named YYYYMMDD\_IN) from ‘Data’ to Desktop -> Lab Water Server -> Lab-Users -> Alex.
2. Then go to the Lab Server Computer. Move files from Desktop -> Lab Water Server -> Lab-Users -> Alex to the EEMs shared google drive.
3. Turn off the instrument computer and make sure both the spectrofluorometer and spectrophotometer are turned off. Return the MilliQ squirt bottle and make sure the area is clean and tidy.

**Data post-processing:**

Following sample analysis, there are a series of steps that must be completed for the data post-processing. As of right now, the post-processing is being conducted in Matlab, though scripts and workflows exist for R (but have not been adapted by either the Carey or Water Quality Labs…yet!). The post-processing corrects the EEMs files following best practices in the literature (i.e., blank correction, instrument excitation/emission corrections, inner-filtering effects, and standardizes to the Raman scan, aka: RFU). The post-processing script also automatically calculates several ‘standard’ EEMs metrics (i.e., FI, HIX, BIX, Peaks A, C, T, B, M, N, various peak ratios) following scripts from C. Osburn (NCSU; i.e., ‘results\_’ files). The script also formats the EEMs files for subsequent PARAFAC modeling (i.e., ‘p\_files’). An example file used for sample correction can be found on GitHub (Example\_Correction\_Folder).

*First, make sure you have the file called ‘Correction\_Files’ saved on your computer and set a path in Matlab to this folder (see* [*here*](https://www.mathworks.com/help/matlab/matlab_env/add-remove-or-reorder-folders-on-the-search-path.html)*). Go ahead and set this path permanently so you only need to do this once! This file contains the function you will use for sample correction (EEMCorr\_Fl.m) as well as the instrument correction files (which will be used by the EEMCorr\_Fl.m script.*

* + - 1. As above, you should have already uploaded the necessary files to a google drive, which means you can access them from anywhere! The first step is to download the file from the Google Drive to a local folder on your computer.
      2. You will need the following files from the Google Drive: the CDOM text file, all of your sample EEMs (including the MilliQ blank!), and the Raman Scan.
      3. First, open up the CDOM .txt file in Excel and save as a .csv file. The first column in the CDOM file is a list of all the wavelengths at which the sample was run (190-800 nm) followed by a series of columns that contain the samples you ran (hopefully starting with the MilliQ blank!).
      4. After converting the .txt file to a .csv file, I suggest adding a row at the top and naming each column with the appropriate file name (this is where your bench sheet comes in handy!).
      5. You will then need to save each separate sample as a separate .csv file. For each of these files, you will have a column of wavelengths (i.e., 190-800 nm) followed by the absorbance values for that sample. For these files, you will need to remove the column header that name each file. I usually name these files ‘abs\_SAMPLENAME.csv’. Make sure you make one for the MilliQ absorbance scan as well! You can see examples in the Example\_Correction\_Folder.
      6. Now that you have separated out each of the MilliQ and sample absorbance scans, you can start correcting the data!
      7. Open Matlab and set your current folder to the folder you created with your absorbance scans, Raman scan, and EEMs sample files.
      8. Before running the EEMCorr\_Fl.m script, MAKE SURE YOU CHANGE THE DILUTION ON LINE 20 OF THE SCRIPT. To do this, open the script in Matlab and change the dilution to the appropriate number. If the sample was undiluted then the dilution\_factor = 1. If you diluted your sample, then you will need to change this number to reflect the appropriate dilution! If you changed the dilution, remember to save the script before running!
      9. Now that you have changed/checked the dilution, you’re ready to run the script! Type in: EEMCorr\_Fl() into the Command Window and press enter. The script will run through a series of pop-up windows that allow you the pick the appropriate files for correction. These will be the following:

‘Choose Raman Scan’ – Choose the Raman Scan from this day of analysis.

‘Choose EEM file for processing’ – This is the sample EEM that you are trying to correct!

‘Choose Blank EEM’ – This is the MilliQ blank EEM that will be used for correction.

‘Choose Absorbance Scan’ – This is the absorbance scan (separated above!) that corresponds to the EEM sample you are correcting.

‘Choose Blank Absorbance Scan’ – This is the blank MilliQ absorbance scan that you separated above.

* + - 1. From there, the script will run itself! The following files will be generated:

First, a pop-up window will appear that plots the corrected sample.

A .mat file will be saved which contains the corrected file in a Matlab data file format (note: I have yet to use these : )

A ‘p\_file’ is made – this is the corrected EEM file that you will use for subsequent PARAFAC modeling! If you \*think\* there’s even a chance you’ll run a PARAFAC model, then I suggest accumulating these p\_files in a separate folder that you can then use when you conduct apply a PARAFAC model.

A ‘results\_file’ is made – this is where all of the calculated various fluorescence indices are saved! I suggest copying and pasting each individual results file into an accumulated excl file with all the sample information and fluorescence indices for each sample.

* + - 1. And that’s it! Repeat the above steps until all EEMs samples from that day have been corrected.
      2. NOTE: Unfortunately, I don’t have a script that automatically corrects the absorbance data : ( Therefore, if you want to extract any absorbance metrices (a254, a350, Sr, etc.) from the CDOM data, you will need to do this by hand following the EDI methods.