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Comparing chromophoric dissolved organic matter methodologies

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Chromophoric dissolved organic matter (CDOM) is a combination of plant and animal decomposition byproducts and the optically active component of dissolved organic matter (DOM) in water. CDOM measurements have been a focus in the literature related to aqueous environments since the 1980s. Current CDOM analysis is conducted using spectrophotometers that are large, bulky, and expensive (most upwards of $50,000 USD). In this study, the accuracy of a more compact, less expensive (~ $5,500 USD) field spectrometer (StellarNet) was tested against a traditional spectrophotometer (Photon Technologies International (PTI)). Thirty-six samples were collected from the Neponset River Salt Marsh in Boston, Massachusetts and analyzed on both instruments with the same set of standards for comparison. The correlation between measurements taken by the two instruments was strongly linear ($R^2 = 0.9278$) and the two sets of data (StellarNet and PTI) were not statistically different ($p\text{-value} > 0.05$), indicating that the less expensive, smaller StellarNet spectrometer is reliable in addition to field appropriate. The StellarNet spectrometer requires additional analysis (compared to the PTI) to convert the output of the instrument (photons) to a concentration (QSU). Highly concentrated sample concentrations (3-fold dilutions required) were not as well-correlated between instruments ($R^2 = 0.5027$). However, this dilution error can be attributed to the length of time (1 year) between sample analysis (i.e., freezing/thawing effects) and/or sampling errors between analysts on the different instruments.

Keywords: CDOM, methodology, fluorescence

Chromophoric dissolved organic matter (CDOM), a combination of animal and plant decomposition byproducts, is produced from both marine and terrestrial sources (Coble *et al*., 2004). It is a measurement of the optically active component of dissolved organic matter (DOM) in water and the most abundant fraction of DOM in natural waters bodies, particularly in forested watersheds with wetlands (Thurman, 1983; Davies-Colley and Vant, 1987). When present in high concentrations, CDOM conveys a yellow or brownish color in solution (Coble *et al*., 2004). In coastal areas, most CDOM enters from rivers containing organic materials commonly leached from soils (Coble *et al*., 2004). Besides rivers, CDOM is additionally produced in the ocean via the release of organic molecules from organisms during processes such as bacterial and viral lysis (cell breakage), grazing, and excretion (Coble *et al*., 1998; Nelson *et al*., 1998; Steinberg *et al*., 2000).

Since the 1980s, CDOM measurements have been a focus in the literature. CDOM interferences must be accounted for before the measurements of chlorophyll, suspended sediment and phytoplankton could accurately be made from reflectance remote sensing data (Tassan, 1988; Ferrari and Tassan, 1992; Karabashev *et al*., 1993). CDOM reflectance occurs in the ultraviolet (UV)-blue section of the electromagnetic spectrum but has no exclusive identifying spectral absorbance or reflectance features (Slonecker *et al*., 2016). CDOM displays a gradually decreasing slope from the ultraviolet through the blue regions and is commonly derived using absorbance or fluorescence techniques (Slonecker *et al*., 2016).

CDOM has a significant impact on aquatic ecosystems. CDOM can affect water quality through the mobilization of metals and hydrophobic chemicals, which serves as a dominant source of reactive photochemical intermediates controlling the photolysis of natural DOM and trace organic contaminants (i.e., pharmaceuticals and personal care products; Olmanson *et al*., 2016). CDOM has been observed to act as a sunscreen by strongly absorbing UV light, in turn protecting fragile benthic habitats such as those in the Florida National Marine Sanctuary (Williams, 2002). Some species of phytoplankton flourish in increased CDOM concentrations as CDOM inputs to coastal waters, whether from river runoff or the upwelling of deep water, are frequently accompanied by large inputs of nutrient concentrations that promote phytoplankton growth (Coble *et al*., 2004). CDOM can further impact organisms via the decrease of toxicity of heavy metals (Wright and Mason, 1999), and providing organic carbon to phytoplankton as an energy source via mixotrophic growth (Lewitus *et al*., 1999; Doblin *et al*., 1999; Lomas *et al*., 2001).

The organic compounds in CDOM are natural and have a high reactivity in water that can lead to both positive and negative environmental effects (Coble *et al*., 2004). The reactivity of organic compounds may decrease dissolved oxygen concentrations in waterways, resulting in a release of nutrients from the sediment (Bushaw *et al*., 1996). Eutrophication can occur from this nutrient release (Coble *et al*., 2004). Conversely, CDOM extracts specific trace metals (i.e., copper; Kieber *et al*., 2004) and polyaromatic hydrocarbons, which decreases their toxicity to surrounding organisms (Coble *et al*., 2004). CDOM reactivity is enhanced by sunlight and can act as a catalyst for the disruption of non-colored compounds, such as organic pollutants (Coble *et al*., 2004). Large amounts of ultraviolet radiation can deteriorate CDOM, releasing organic compounds in the process that are necessary for phytoplankton and bacterial growth (Miller and Moran, 1997) such as essential elements (i.e., nitrogen) and trace metals (Coble *et al*., 2004).

CDOM can be measured via absorbance or fluorescence techniques. Absorbance is a measurement of the common logarithm of the ratio of incident to transmitted spectral radiant power through a material (IUPAC, 2006). The understanding of CDOM distributions, the processes controlling CDOM, and its optical properties influences are hindered by methods used for absorbance measurement (D’sa *et al*., 1999).

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Spectrophotometers with 5- and 10-cm optical cells can measure CDOM absorption with satisfactory sensitivity in the UV and visible wavebands for copious coastal and shelf waters. However, in oligotrophic (low-nutrient) waters, the levels of CDOM absorption reach the detection limit of these instruments. Measuring the absorption spectra of CDOM in waters like this requires long pathlength cells (Bricaud *et al*., 1981; Peacock *et al*., 1994) or sample concentration (Carder *et al*., 1989). These approaches are tedious and time consuming.

Fluorescence is a measurement of the different wavelengths of light emitted when a solution is exposed to short wavelength light. This is dependent on the amount of absorbing material, the absorbance characteristics of the solution contents, and the optical path-length the light must travel through the sample (Slonecker *et al*., 2016). CDOM sensors usually use fluorescence to characterize CDOM *in situ* as opposed to absorbance due to the lower cost and higher sensitivity of field fluorometers (Slonecker *et al*., 2016).

Fluorescent dissolved organic matter (FDOM) is the part of CDOM that fluoresces and is often accepted as a proxy for CDOM (Slonecker *et al*., 2016). Measurements of CDOM fluorescence have a long history in oceanography as a gauge for terrestrial humic substances in the coastal ocean (Coble, 2007). In the past twenty years, fluorescence spectroscopy techniques have been utilized to determine the composition and dynamics of DOM in aquatic environments (Coble, 1996; Stedmon *et al*., 2003; Chen *et al*., 2004; Spencer *et al*., 2007a; Jiang *et al*., 2008; Kowalczuk *et al*., 2009; Yamashita *et al*., 2010). However, modern laboratory fluorescence spectrofluorometers (e.g., Shimadzu RF5301 spectrofluorometer, Shimadzu Inc.; Zhang *et al*., 2007; Foden *et al*., 2008; Singh *et al*., 2010) are too bulky for frequent use in the field and the measurement scans are time consuming.

There are three different forms of spectral data involved in the calculations of CDOM and comparing among these different units and equivalencies remains a significant obstacle (Slonecker *et al*., 2016). Laboratory methods typically involve measurements of absorbance at a precise wavelength between 250 and 440 nm (Slonecker *et al*., 2016). *In situ* sensors are based on fluorescence measurements where the excitation wavelength is roughly 350 nm and the emission wavelength is about 450 nm (Brezonik *et al*., 2015). Successful overhead remote sensing applications are established on reflectance measurements commonly at wavelengths greater than 500 nm (Kutser *et al*., 2005; Menken *et al*., 2006; Brezonik *et al*., 2015).

CDOM measurements are also not reported uniformly (Slonecker *et al*., 2016). CDOM can be conveyed as a function of color at 440 nm (C440; Brezonik *et al*., 2005). Marine chemists routinely express absorption coefficients in Naperian (spectral absorbance coefficient) units, whereas freshwater and wastewater communities typically use decadal absorption coefficients (Aiken, 2014). CDOM fluorometers report measurements in relative fluorescence units (RFUs) and several United States Geological Survey (USGS) gaging stations measure CDOM through fluorescence techniques and record CDOM concentrations as units of a Quinine Sulfate Equivalents (QSE; Slonecker *et al*., 2016). Quinine been used as a standard in fluorometric analyses due to its highly flourescent properties and has become a standard for *in situ* recording of CDOM fluorescence, which can also be reported in Quinine Sulfate Units (QSU; Slonecker *et al*., 2016).

Besides variability in measurements and reporting values, an additional difficulty with CDOM analysis is the size and cost of the instruments. In the 1990’s, these instruments cost hundreds of thousands of dollars, took up a majority of the lab’s floor space, were operated by doctoral-level staff, and were used primarily within core facilities (Okimoto and Fung, 2016). Chemists would prepare the samples, send them out for analysis, and wait for a simple spectrum to return several days later (Okimoto and Fung, 2016). With the more recent advances in materials science, microprocessors/ data storage and software languages, these factors are beginning to be less of a problem (Okimoto and Fung, 2016). For example, the current base cost of a spectrofluometer, the leading CDOM analysis tool, is $45,000 USD but can cost more than $300,000 USD depending on accessories, detectors, and ultimately the application of the end user (Figure 1).

Figure 1. Horiba PTI QuantaMaster™ 8000 Series fluorometer (computer system not shown; Horiba, 2018).

A typical fluorometer (e.g., Figure 1) measures 36 x 28 x 12 inches (WxDxH) excluding the required computer system. In recent years, portable or submersible field fluorometers have been employed to acquire rapid, real-time, high-frequency measurements of DOM in aquatic environments (Sivaprakasam and Killinger, 2003; Baker *et al*., 2004; Conmy *et al*., 2004; Killinger and Sivaprakasam, 2006; Chekalyuk and Hafez, 2008; Suping *et al*., 2010; Tedetti *et al*., 2010; Chekalyuk and Hafez, 2013; Tedetti *et al*., 2013). For example, StellarNet has created a portable UV-VIS spectrometer with 280-900 nm wavelength range that is field-appropriate and cost-effective (~ $5,500 USD; StellarNet, 2018; Figure 2).

Figure 2. StellarNet BLACK-Comet spectrometer including computer setup (StellarNet, 2018).

In this study, the accuracy of a StellarNet spectrometer was tested against a traditional spectrophotometer (Photon Technologies International (PTI)) with water samples to determine the validity as well as possible disadvantages of a smaller, cheaper spectrometer in the face of the older, bulkier technology using samples collected from an urban salt marsh.

Experimental Procedures

Study site

The second-largest tributary to Boston Harbor (Boston, MA) is the Neponset River (Gardner *et al.*, 2005). The watershed draining into the Neponset River is made up of 14 cities and towns and is home to roughly 300,000 people according to Huang and Chen (2009). The Neponset Estuary is approximately 7 km long and changes in width from about 25 to 250 m at mean lower low water (MLLW; Schiebel *et al.*, 2018). The Neponset Salt Marsh (42.277309 N, 71.045837 W) has a total area of 1.1 x 10^6 m^2 and is composed of three dominant plant species: *Spartina alterniflora* (*S. alterniflora*) which dominates the lower marsh habitat, the invasive reed *Phragmites australis* (*P. australis*) which dominates the high marsh habitat and *Spartina patens* (*S. patens*) which lies in between the two on the marsh platform (Schiebel *et al.*, 2018). Approximately 29% of the total surface area is below the MLLW mark and is usually covered by water with little to no vegetation (Schiebel *et al.*, 2018). Typical New England salt marsh sediment is made up of peat, with water content ranging from 30 to 60% (Redfield, 1972) and sediment organic carbon values ranging between 0.05 and 0.15 g cm^{-3} (Artigas *et al.*, 2015), as confirmed by observations.

Sample collection

S. alterniflora and *S. patens* samples were collected in October 2017 for a separate experiment and publication (Schiebel *et al.*, *in prep*). Living and detrital biomass was removed (i.e., cut from the stem of the plant) at the same 1- and 10-m distances from the creek bank in the salt marsh. Plant biomass samples were collected by hand within the same 100 m^2 plot (variations within plant species by area were not explored) from the marsh for each experiment and cleaned using deionized water to remove any sediment. Duplicates (*i.e.*, 2 separate samples of plant matter were exposed to each treatment) were taken from plants of similar height. Clean sample splits were weighed and then placed in a Fisher Isotemp 200 Series oven within two hours of sample collection for one week at 50°C to perform water content analysis and determine dry weights.

Ten grams of the remaining fresh, cleaned samples were then placed directly into 1-liter, opaque (0% transmission) Teflon bottles filled with one liter of Boston Harbor seawater with relatively low (compared to marsh concentrations) CDOM (approximately 20 QSU) concentrations. Each incubation experiment was initiated on the same day that samples were collected (Day 0). Four total sample bottles were created for each plant species for eight samples total (i.e., two *S. patens* living biomass samples and two *S. patens* detrital biomass samples with corresponding samples of *S. alterniflora*). Leaching experiments were conducted for ten days to encompass the initial, rapid release of carbon from plant matter (Chapin *et al.*, 2011; Wang *et al.*, 2014) with discrete duplicate samples per treatment taken on days 0, 1, 5, and 10 for a total of 64 samples (4 sample days in duplicate for each of 8 sample treatments).

Sample analysis

CDOM samples were filtered through a precombusted 0.7-μm glass fiber filter (Whatman GF/F), stored in precombusted (4 hours at 500°C) 40-mL amber borosilicate glass vials with Teflonlined screw caps, and frozen until initial analysis.

CDOM fluorescence was first measured on all 64 samples using a PTI QM-1 spectrofluorometer in 2017 within one week of sample collection. Single fluorescence emission scans from 350 to 650 nm were collected for an excitation wavelength of 337 nm. The fluorescence of Milli-Q water (<5 QSU) was subtracted from sample spectra before integration with the level of blank relative to the level of samples greater than 1%. Peak areas were integrated and converted to QSUs, where 1 QSU is equivalent to the fluorescence emission of 1 μg L^{-1} of quinine sulfate solution (pH 2) integrated from 350 to 650 nm at an excitation wavelength of 337 nm (Chen and Gardner, 2004). All sample data were collected using a 1-cm quartz cuvette and expressed in QSUs. With samples above 100 QSU (up to 3 m^{-1}), a controlled dilution approach (CDA) was employed (Kothawala *et al.*, 2013; Zeng *et al.*, 2016), in which samples were diluted with Milli-Q water to levels such that the inner filter effect (IFE) was negligible (Turner, 1985). The original fluorescence was then calculated based on the dilution factor.

Of the 64 total samples analyzed in 2017, only 36 samples had enough sample remaining to re-run in 2018. These 36 samples and the exact same standards were analyzed using a StellarNet Miniature Spectrometer in September 2018 (the original samples remained frozen and standards were refrigerated in between analyses). Sample wavelengths were integrated from 350 to 650 nm at an excitation wavelength of 337 nm. Emitted photons were measured at 90 degrees incident from the excitation source so that only emitted light was measured. Emitted light was split into different wavelengths via diffraction and a detector measured the number of photons at each wavelength and a computer produced a spectrum of intensity (photon count) versus wavelength. Microsoft Excel was implemented to convert the photon count from the instrument to a CDOM concentration in QSU via a trapezoidal calculation. For each data point in the spectrum, two y-axis points were averaged and divided by the x-distance between the y-axis points. In this way, 1 nm between each data point or

0.23 nm between each data point is represented appropriately and QSU values from each instrument can be compared. All parameters (wavelength range, integration time, etc.) were the same for both instruments.

Statistical analysis

A two-sample assuming equal variances t-test was applied to the treatment groups (PTI and StellarNet). A two-sample assuming equal variances t-test compares whether the average difference between two groups is significantly different or not at the 95% confidence interval (Zar, 1999). When run in excel, the two-sample assuming equal variance t-test is chosen in the data analysis tool pack and the two desired rows of data are highlighted for comparison. The output is provided in a table format with a p-value, t-statistic, and a t-critical value for both one- and two-tailed analysis. In this case, the two-tailed distribution values were used because there was not a previous assumption for how the instruments would be significantly different (i.e., one instrument reads higher or lower). The two-tailed test accounts for the possibility of either result. In other words, the two-tailed tests in both directions of the average mean. There are two important outputs to consider from a two-sample assuming equal variances t-test. First, if the t-critical value is higher than the t-statistic, then the two populations are not significantly different from each other. Conversely, if the t-statistic is higher than the t-critical value, then the two populations are significantly different. The second important output of the two-sample assuming equal variances t-test is the p-value (two-tailed here). If the p-value is below 0.05, then the data is reliable and not due to chance at the 95% confidence interval (Zar, 1999).

Results

Correlation between instruments

The correlation between the two instruments for the 36 samples analyzed was average when all samples (i.e., outliers) were included in the dataset (Figure 3).

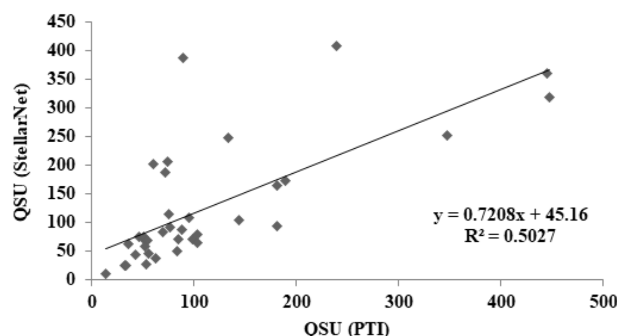

Figure 3: All samples (n=36) analyzed on a PTI QM-1 spectrofluorometer and a StellarNet Miniature Spectrometer with outliers included.

A total of 6 outliers were identified as needing strong dilutions (i.e., 3-fold dilutions or higher) and removed from the dataset, resulting in a strong linear correlation of the sample concentrations between instruments (Figure 4).

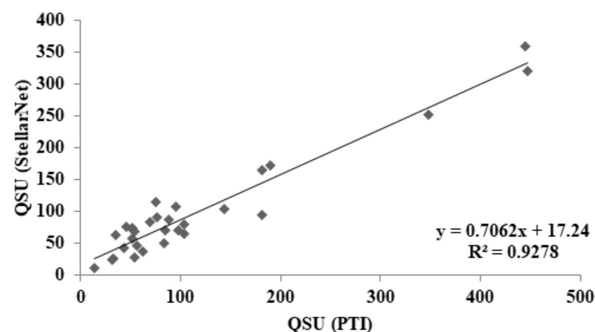

Figure 4: Dataset with outliers (3-fold dilutions or higher) removed (n=30) analyzed on a PTI QM-1 spectrofluorometer and a StellarNet Miniature Spectrometer.

Statistics

A two-sample assuming equal variances t-test was conducted to determine if the treatment groups (PTI and StellarNet) were significantly different from each other once the 6 outliers were removed (Table 1).

Table 1. Results from two-sample assuming equal variances t-test

Parameter	Value
T-statistic	0.6347
T-critical value (two-tailed)	2.0017
P-value (two-tailed)	0.5281

Because the p-value is greater than 0.05, and the t-statistic is less than the t-critical value, the two treatments are statistically the same.

The systemic dilution error only occurred in highly concentrated samples (3-fold dilutions or higher). Not all dilutions were outliers; dilutions that only required 1- or 2- fold dilutions were well correlated on both instruments (Figure 4). A total of 13 samples needed to be diluted in the total dataset (36 samples), 6 of which were highly concentrated (outliers) and 7 of which were low to moderate dilutions that were not considered outliers.

Discussion

The difference between the concentrations observed for more concentrated samples could be due to two different phenomena. First, sample handling could create a difference in the concentration of the samples between different instruments. These samples were run by two different analysts at two different times. The six intense dilutions, in turn, were also completed by two different analysts. Sampling handling and dilution calculations, if handled differently, could result in a difference in CDOM concentrations between the two instruments (Miller *et al*., 2002).

More likely, the multiple freezing and thawing of these samples over the course of analysis and/or the storage techniques used could account for the variance in the strongly diluted samples (Mueller and Austin, 1995). The primary causes of sample instability over time are due to microbial and photochemical degradation and the effects of these processes on DOM absorbance and fluorescence in aquatic samples is well documented (Moran *et al*., 2000; Del Vecchio and Blough, 2004; Tzortziou *et al*., 2007; Wickland *et al*., 2007; Osburn *et*

al., 2009). Standard CDOM protocols call for sample storage in cold (approximately 4°C), dark environments for this reason. While samples were stored this way, they were removed, thawed, and run many times over the course of the year that could have led to variations in the samples with higher CDOM concentrations.

CDOM protocols call for analysis as soon as possible after collection to avoid any impacts from microbes and/or sunlight. There is little consensus on the amount of time for which it is appropriate to store CDOM samples. A National Aeronautics and Space Administration (NASA) study found no difference in CDOM absorption values for refrigerated samples re-analyzed within 24 hours (Mitchell *et al*., 2000). Similarly, Spencer *et al*. (2007b) found no difference in sample reproducibility after 7 days. However, they found that analytical reproducibility decreased after 2 months due to a shift from protein-like fluorophores in water to more fulvic-like fluorophores. Hudson *et al*. (2009) also observed a decrease in intensity over time in refrigerated samples and a great removal of protein-like fluorescence compared to fulvic-like fluorescence as well. It is therefore assumed that the length of time (1 year) that the samples were frozen (and thawed multiple times) led to the discrepancy between sample concentrations between the two instruments for highly concentrated samples.

In addition to the dilution error observed, one drawback of the StellarNet instrument is the output signal. The PTI instrument output is in counts that are directly converted to the CDOM concentration of the standards used (QSU) in Excel. The analyst is able to copy and paste the counts from the instrument into Excel and, using a simple formula, obtain QSU values. This is quick and efficient. Conversely, the StellarNet spectrometer output is photons and the software does not have an area integration function that calculates the entire emission spectra in one output. Thus, a more complicated, longer procedure is needed to convert the photons under the curve of the emission spectra to a CDOM concentration. A trapezoidal calculation must be completed in Microsoft Excel for each data point in the spectrum as noted above. This results in several open programs at once with multiple Excel sheets, is easily confusing, and can lead to errors in reporting if not done properly. For field sample analysis using the StellarNet instrument, it is recommended that samples be analyzed in the field and then the data converted at a later time (the spectra and data are saved immediately so no data is lost in the field).

Conclusion

CDOM is important for moderating aquatic processes. CDOM measurements have been a focus in the literature since the 1980s and current CDOM analysis is conducted using spectrophotometers that are large and bulky as well as expensive. In this study, the accuracy of a compact, inexpensive field spectrometer (StellarNet) was tested for accuracy against a traditional spectrophotometer (PTI). A total of 36 total samples were collected from the Neponset River Salt Marsh in Boston, Massachusetts and analyzed on both instruments with the same set of standards for comparison (R^2 = 0.5027). Highly concentrated samples (i.e., 3-fold dilutions or higher; n=6) were identified as outliers and removed from the dataset, resulting in a strong linear correlation of the sample concentrations between instruments (R^2 = 0.9278). Statistically, the two treatment groups were not different (p-value > 0.05). The systemic dilution error can be attributed to the length of time (1 year) between sample analysis (i.e., freezing/thawing effects) and/or sampling errors between analysts on the different instruments. This study shows that the less expensive, smaller StellarNet spectrometer is both reliable in addition to field appropriate. One drawback of the StellarNet instrument is the need to convert the output of the instrument (photons) to a concentration (QSU) versus the PTI instrument, although this is still amenable to the more expensive, lab-only option as data can be stored and analyzed at a later date if needed. Future studies would involve determining the time for which CDOM samples can be frozen with no difference in reproducibility.

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