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Protocol for assessing YSI EXO Total Algae sensor performance via comparisons with extracted chlorophyll *a* concentrations and identifying possible sources of interference

See https://nerrssciencecollaborative.org/project/Dix20 for project background.

A. Sampling design for comparisons between *in situ* chlorophyll *a* fluorescence (RFU) and extracted chlorophyll *a* fluorescence (µg/L) with natural water samples

The sampling design described here strives to ensure that the same water mass and phytoplankton assemblage is being sampled. The following sampling options are not mutually exclusive. For both sampling options, sondes are to be equipped with a full sensor suite (including conductivity/temperature, dissolved oxygen, pH, turbidity, and FDOM) to provide ancillary data potentially necessary for chlorophyll fluorescence *a posteriori* corrections and standardization. Field-based sampling has the advantage of sampling diel variability in realistic environmental conditions. Lab-based sampling minimizes variability in factors that may affect the observed relationship. Sampling should be conducted to capture a wide variety of environmental conditions. The number of samples collected and the sampling interval are not standardized, but one should aim to produce ≥ 30 comparison samples over at least four seasons and multiple tidal stages.

1. Field-based: Collect grab or ISCO (automated water sampler) samples *immediately adjacent* to the EXO sensors while they are actively recording. Efforts should be made to collect sample water as spatially and temporally aligned to sensors as possible.
2. Laboratory-based: Collect a large volume (10L-20L) of natural water from the site of interest, return to lab, carefully transfer sample to darkened mixing tank sitting atop a magnetic stir plate to homogenize sample volume (RPM of stir bar set to avoid a vortex), submerse EXO sensors, then continuously record at the highest possible sampling interval (~1-2 sec). After ~5 min acclimation, withdraw an aliquot for extracted chlorophyll determination. Average the *in situ* fluorescence value over the course of mixing to compare with extracted chlorophyll.

Example of a lab-based comparison set-up

B. EXO Total Algae sensor calibration

Perform a 2-point calibration of the Total Algae (TAL) sensor using Rhodamine WT dye per YSI manual, except run the calibration using RFU rather than (or in addition to) µg/L. Using RFU will allow for conversion to estimated µg/L based on empirical calibration curves developed through this assessment.

The commercially available 2.5% concentrated rhodamine solution (Kingscote Chemicals, item #106023) used for sensor calibration is stable and does not need to be stored cold. The 125 mg/L solution can be refrigerated and stored for approximately one year. The 0.625 mg/L solution is only stable for 24 hours and should be discarded following use. Check local waste guidelines for rhodamine disposal.

Note that assessment of TAL Blue Green Algae sensor output is not outlined in this protocol. An assessment of this measurement requires paired sampling with cultures or analyzing natural grab sample extractions with HPLC to quantify phycoerythrin or phycocyanin pigment concentrations.

C. Extracted chlorophyll determination

Generally, the approach outlined here is EPA method 445.0 (Arar and Collins, 1997), avoiding the use of grinding to achieve cell disruption and shortened extraction time.

- Filter and begin extraction within 12 hours of sample collection (or sample retrieval in the case of automated water sampler deployment). Store water samples at 4 °C in the dark until filtration. Perform processing in a darkened lab area.
- Prior to drawing a subsample from the water sample container, thoroughly but gently agitate the container to suspend the particulates. Use a hand or vacuum pump to filter samples on 25- or 47-mm GF/F filters with 0.7-µm pore size. If using vacuum, do not exceed 6 in Hg to avoid cell lysis. Using a graduated cylinder to measure sample volume, filter enough sample volume to show color on the filter pad. Rinse filter tower and graduated cylinder with a small amount of filtrate from the same sample or a filtered sample with similar salinity to ensure all particles are captured on the filter.
- Remove the filters with tweezers, fold and put filters in labeled scini vials or test tubes with screw tops (to avoid evaporation). Freeze at -20 °C or below for 24 hours to fracture cells. Filters may be stored for up to 3 weeks without significant loss of chlorophyll *a*.
- Add a known volume of 90% aqueous reagent-grade acetone to vials/tubes. Following the addition of acetone, shake each sample. Shake each tube again before placing it in the freezer for the steeping period. Freeze samples at -20 °C or below for 24-48 hours. Shake again once during the steeping period.
- Following the removal of samples from the freezer, invert the vials/tubes to resuspend any chlorophyll that may have settled. Prior to fluorometric analysis, allow samples to return to ambient temperature before drawing the supernatant into the sample cuvette (~15-30 minutes).
- Quantify *in vitro* chlorophyll *a* fluorescence on a fluorometer equipped with narrow band-pass filters using the non-acidified technique and the direct concentration mode of a bench-top fluorometer.

- Validate the calibration of the fluorometer with solid standard at the beginning of every run (up to 5% drift is acceptable) and with the liquid standard 1 – 2 times per year. Calibrate to µg/L using a 6-point serial dilution (Blank + 5 known concentration standards created through serial dilution of commercially available chlorophyll standards).

Quality control steps should be completed to ensure quality and comparability.

- Lab blanks: DI water "sampled" in the lab, filtered, and analyzed to identify contamination in the laboratory environment. It is recommended to perform one lab blank for every batch analysis. This blank should be the last filter to be extracted.
- Lab duplicates: One primary sample split into two subsamples at the lab. It is recommended to perform one lab duplicate for every batch analysis or every 24 samples. Each subsample is extracted and analyzed to test precision of lab measurements.
- Method detection limit is recommended to be calculated annually or when solvent is changed (new bottle of acetone used for extractions).

D. Laboratory assessments of interferences

For all interference assessments, sondes are to be equipped with a full sensor suite (including conductivity/temperature, dissolved oxygen, pH, turbidity, and FDOM) to provide ancillary data potentially necessary for chlorophyll fluorescence a posteriori *corrections and standardization. All interference testing is to be completed without a central wiper.*

1. Assessing temperature effect

The effects of temperature on chlorophyll fluorescence quenching is conducted following the basic protocols of Watras et al. (2017), who assessed temperature compensation for *in situ* algal sensors manufactured by TurnerDesigns (Sunnyvale, CA).

Temperature quench assessments are to be conducted using natural water samples collected at the site(s) used for grab sample comparisons. Sampling is recommended to be targeted to times or locations with high anticipated chlorophyll concentrations to allow for a range of chlorophyll concentrations to be generated across a dilution series of the natural water sample. The effect of temperature is recommended to be assessed over the ambient temperature range typically observed in estuarine environments (≈ 4 – 30 °C). The EXO is recommended to be programmed at the highest possible sampling interval (~1 – 2 sec).

Collect a volume of a natural water sample (10 – 20 L) and serially dilute with filtered sample water to create four different concentrations of chlorophyll (100%, 50% 25%, 0% ambient). Filtered sample water is created by filtering the natural water sample through a 0.2-µm pore-size filter capsule (Whatman Polycap TC). Final experimental sample volumes need to be sufficient to submerge the lower third of the EXO sonde height within the sample when placed in the mixing tank. After dilution, allow samples to acclimate to initial temperature (≈ 4 °C, achieved by placing samples in an ice bath) for approximately 4 hours in a darkened lab area. Samples are then transferred to a mixing vessel with EXO sonde, placed atop a stirring hotplate and warmed to ≈ 30 °C over a period of approximately 1 hour while subject to continuous magnetic stirring

(RPM of stir bar set to avoid vortex). While warming, record EXO sonde measurements at 2-sec intervals. Collect samples for extracted chlorophyll analysis at the beginning and end of the heating treatment. Repeat for each dilution. Temperature compensation equations are to be developed following Watras et al. (2017), allowing measured fluorescence to be normalized to a reference temperature (20 °C).

2. Assessing turbidity interference

Methods to assess the effects of turbidity on the attenuation of the chlorophyll fluorescence signal follow the recommendation of Downing et al. (2012). These authors successfully assessed turbidity interference and developed correction methods for fluorescence-based *in situ* dissolved organic matter (FDOM) sensors, which operate on the same principles as fluorescence-based *in situ* chlorophyll sensors.

Conduct turbidity experiments using natural water collected at the site(s) used for grab sample comparisons (as described above). Evaluate *in situ* chlorophyll fluorescence over a range of turbidity up to 1000 FNU by serially adding a standardized source of turbidity to the natural water sample. The recommended turbidity standard is combusted (450 °C for 4 hrs) and homogenized marsh mud with a high silt fraction.

Collect a natural water sample (10 – 20 L) that, to the extent possible, has a sufficient chlorophyll concentration and is low in turbidity (typically in high salinity estuaries this is a sample collected at high tide and dominated by ocean waters). Transfer the sample to a laboratory mixing vessel atop a magnetic stir plate, stirred at low speed to keep cells homogeneously suspended. Place an EXO sonde with a suite of calibrated sensors in the mixing tank and program to record data every 2 seconds. With constant gentle stirring to maintain a uniform sample suspension, add aliquots of combusted mud slurry serially to the mixing tank, allowing the EXO to record data for a 5-min period after each aliquot addition, until a turbidity value of approximately 1000 FNU is reached. Aliquot amounts will vary depending on the size of the mixing tank but aim to elicit a noticeable response with every addition. The relationship between chlorophyll fluorescence attenuation and turbidity can then be modeled, and correction coefficients can be formulated following recommendations of Downing et al. (2012). Collect samples for extracted chlorophyll analysis at the beginning and end of the experimental treatment to ensure phytoplankton biomass does not change over the course of the manipulation. Extracted samples will likely need to be filtered prior to fluorometric analysis. Replicate as much as practical.

3. Assessing FDOM interference

The potential effects of interference from fluorescence of dissolved organic matter (FDOM) on chlorophyll fluorescence will be done in an analogous fashion to the turbidity interference assessment (above), but serially adding small quantities of a defined source of FDOM. Collect samples for extracted chlorophyll analysis at the beginning and end of the experimental treatment to ensure phytoplankton biomass does not change over the course of the manipulation. Interpretation of results needs to account for sample dilution.

The effect of FDOM on *in situ* chlorophyll fluorescence is recommended to be evaluated over a range of FDOM concentrations up to 200 QSU using a concentrated FDOM surrogate. Create the

surrogate by filtering ambient swamp water naturally high in FDOM through a 0.2-µm filter and concentrating the filtered water 5 – 10 times using a heated stir plate (80 °C). Humic acid solution (Sigma Aldrich) may also be tested as a standard surrogate for a commercially available FDOM source.

Collect a natural water sample that, to the extent possible, has a sufficient chlorophyll concentration and is low in natural FDOM sources. Transfer the sample to a laboratory mixing vessel atop a magnetic stir plate, stirred at low speed to keep cells homogeneously suspended. Place an EXO sonde with calibrated sensors in the mixing tank and program to record data every 2 seconds. With constant gentle stirring, add aliquots of FDOM surrogate to the mixing tank, allowing the EXO to record data for a 5-min period after each aliquot addition until an FDOM value of 200 QSU is reached. The nature of the relationship between chlorophyll fluorescence (RFU) and FDOM fluorescence (QSU) can then be assessed for potential correction.

E. References

Arar, E.J., and G.B. Collins. 1997. *Method 445.0: In vitro determination of chlorophyll* a *and pheophytin* a *in marine and freshwater algae by fluorescence.* United States Environmental Protection Agency, Office of Research and Development, National Exposure Research Laboratory.

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Watras, C.J., K.A. Morrison, J.L. Rubsam, P.C. Hanson, A.J. Watras, G.D. LaLiberte, P. Milewski. 2017. A temperature compensation method for chlorophyll and phycocyanin fluorescence sensors in freshwater. *Limnology and Oceanography: Methods* 15:642-652.