HYDROGEN PEROXIDE METHOD

For the Orion Luminometer

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I. Reagent Preparation (Cooper et al. 2000; King et al. 2007).

pH 3 Buffer for Acridinium Ester Reagent

- In a clean glass beaker on a stir plate, add 1.5 g KH_2PO_4 to about 200 mL milli-Q water to dissolve.
- Add 1.05 mL phosphoric acid (H_3PO_4).
- Titrate to pH 3.
- Bring to 250 mL with milli-Q water in a volumetric flask.
- Store at room temperature in the dark.

10X Acridinium Ester (AE) Stock

- Add 25 mL pH 3 buffer to about 175 mL milli-Q water in a clean glass beaker on a stir plate.
- Add 5.47 mg solid acridinium ester to beaker. Cover beaker with parafilm and stir overnight in the dark.
- Bring solution to 250 mL with milli-Q water in a volumetric flask.
- Store at 4° C in the dark. This solution is stable for at least a year under these conditions.
- It is very difficult to accurately weigh out 5.47 mg of AE stock. If you significantly deviate from this, make sure to adjust the final volume of the solution accordingly.

AE Reagent

- Add 500 µL pH 3 buffer to about 10 mL milli-Q water in a fresh 50 mL falcon tube.
- Add 5 mL 10X AE stock to tube.
- Fill to 50 mL mark with milli-Q water. Seal tight and store in the dark at room temperature.
- Allow at least 24 hr to equilibrate prior to first use.
- The concentration of AE may be manipulated to provide higher sensitivity (more reagent) or broader range of linear response (less reagent). This concentration is ideal for measuring standard levels found in lake and ocean water (0 to 250 nM) but works well up to 500 nM (or even 1 µM with curve fitting for the standards).

2M $NaCO_3$ Buffer

- Place approximately 40 mL milli-Q water in a clean glass beaker on a stir plate. Slowly add 10.6 g $NaCO_3$ solid (not $NaHCO_3$!). Allow to dissolve.
- Titrate to pH 11.3 using 1N HCl.
- Pour solution into a fresh 50 mL falcon tube and bring volume to 50 mL. Seal tight and store in the dark at room temperature.

Standardized Hydrogen Peroxide Stock

- Add 23 μL 30% HOOH to 10 mL milli-Q water.
- Add 1 mL of this solution to a UV-transparent assay cuvette and measure absorbance at 240 nm.
- Wait 10 seconds and repeat this procedure. Repeat until you have obtained 6 measurements of the sample. Use the average of these samples as the A_{240} reading.
- The precise concentration of HOOH in this stock may be calculated using the molar extinction coefficient for HOOH of $\varepsilon_{HOOH} = 38.1$ L mol^{-1} cm^{-1} (Miller and Kester 1988). Path length for DU800 spectrophotometer = 1 cm; for Nanodrop = 0.1cm.
- Filter sterilize the standardized stock using a 0.2 μm nylon syringe filter.
- This stock is quite stable even on a multi-year time scale, as long as it remains sterile and protected from light. However, it is a good idea to make a fresh stock every 3 months or so.

50 mM KatAss Phosphate Buffer

- Add 1.7 g KH_2PO_4 to about 200 mL milli-Q water in a clean glass beaker on a stir plate.
- Titrate to pH 7.0 using 1N NaOH.
- Bring to 250 mL with milli-Q water in a volumetric flask.
- Store at room temperature in the dark.

0.1% Catalase Working Stock

- Weight out 10 mg catalase crystals onto dust-free weigh paper.
- Add 200 μL KatAss Buffer to a 10 mL volumetric flask. Fill about ¾ full with 30-37^{o} C milli-Q water.
- Add catalase crystals to flask and mix gently until dissolved. The solution should be a pale golden color.
- Fill to line with milli-Q water.
- Filter sterilize using a 0.2 μm PVDF (Millex GV) syringe filter.

Abyssal Blank Water (ABW)

Quantitative analysis methods are limited by the quality of the blank. Because of the ubiquity of HOOH in even the purest of waters, due to a number of factors both known and unknown, this is the limiting factor in the sensitivity of the acridinium ester method. The best blank is ABW. This is natural seawater taken from great depth (below 500 m) in oligotrophic waters away from the continental shelf. After collection, this water should be passed through a 0.2 μm polycarbonate membrane filter and stored in an airtight bottle shielded from light.

Catalase-treated Milli-Q Blank Water (Kat-Q)

For freshwater applications, it may be desirable to produce HOOH-free milli-Q water. It is not possible to make standards in milli-Q water for the assay of seawater samples because the precipitation of carbonate salts at high pH from seawater reduces light transmission from samples in comparison to deionized water. To make Kat-Q, follow this recipe:

- Add 100 μL catalase working stock to 1L water.
- If desired, filter sterilize using a 0.2 μm PVDF filter (this is not normally necessary).
- 24 hr incubation with the enzyme is sufficient to reduce HOOH levels to the sub-nanomolar range. Prolonged incubation is not advisable, however, as degraded catalase can actually *produce* HOOH.

Hydrogen Peroxide Standards

- Make standards in 1.5 mL autoclaved black Eppendorf tubes.
- Prepare 100 μM and 10 μM stocks in **sterile** milli-Q water (**without** catalase). The exact amount of HOOH standard to add to make 100 μM will vary based on the assayed concentration of the standard (usually around 5 μL). Add 100 μL of 100 μM stock to 900 μL milli-Q to make the 10 μM stock.
- Always mix and aerate samples thoroughly, either by vortexing (preferred) or vigorous shaking.
- Always treat the blank water to be added to assay plates the same as the HOOH-added standards. Don't just add it straight out of the blank water bottle.
- The required standards differ based on the analysis protocol (see sections III and IV below).

Wash solutions

- DI Water wash: for everyday cleaning. Add 40 mL of Milli-Q water to each of two 50 mL falcon tubes. Clearly label the tubes (and their caps) "DI CO3" and "DI AE".
- 1N HCl: For cleaning after long periods of inactivity or after field work. Add 4 mL concentrated HCl to 44 mL milli-Q water in a 50 mL falcon tube clearly labeled "1N HCl".
- 1N NaOH: For cleaning after long periods of inactivity or after field work. Add 1.6 g NaOH solid to 50 mL milli-Q water in a 50 mL falcon tube clearly labeled "1N NaOH". Dissolve by gentle inversion.

II. Luminometer Method.

1) Turn on luminometer. Allow PMT to warm up for at least 1 hr prior to analysis. The acridinium reaction as well as PMT response is temperature sensitive, so it is important for the temperature inside the machine to equilibrate prior to reading.
2) Prepare ABW **blanks** and milli-Q **stocks** for standards.
3) Shake 2M $NaCO_3$ reagent to oxygenate. Strap onto **left** magnetic metal support inside luminometer bay. Make sure intake tube extends to bottom of reagent tube.
4) Repeat step 3, with AE reagent. Strap onto **right** magnetic metal support inside luminometer bay.
5) Turn on computer and open Simplicity program. Double-click "Acridinium Injection" Protocol to open.
    0. Load samples onto white, flat-bottom 96-well plates (Costar 3912 or equivalent) fitted with lids (purchased separately if necessary, may be reused). Loading scheme will be determined by the analysis protocol chosen for the samples (see subsequent sections).
6) Add HOOH to standard dilution blanks. Add standards to plates according to analysis protocol chosen. Make sure to re-cover the plate.
    - "Aging" of standards causes them to behave strangely. I am not sure what causes this problem – perhaps slow reactions with trace metals in the ABW – but it is easily remedied by not adding HOOH to the dilution blanks until just before reading the plate.
7) "Age" finished plate. If the plate is instantly read, there will be a noticeable increase in the luminescence of the blank from one side of the plate to the other due to an unknown reaction (perhaps with the atmosphere, or due to equilibrating temperatures between the solutions and the plate). If the plate is aged for 10 minutes after addition of the last sample *in the same room as the luminometer*, this effect is largely eliminated.
8) "Prime" luminometer. This involves pulling some solution through the tubes to make sure reagent is available for injection into first assay well.
    - Click the "Starburst" icon in the toolbar of the Simplicity program.
    - Check both "Injector" and "Measurement" position boxes.
    - Select "10" pumps in the boxes on the left.
    - Click "OK."
    - The machine will request you to insert the priming tray, which is a clearly labeled plastic reservoir the size of a 96-well plate. Put the plate in and click OK. The machine will then prime itself.
9) Insert Assay plate and click the "Green Dot" icon to read. If you are only reading a subset of wells, make sure to select the target wells and click the "select wells" icon prior to clicking the green dot. The luminometer will now read the plate by injecting first 50 μL $NaCO_3$ reagent and then 50 μL AE reagent, followed by monitoring luminescence for 1 s.
10) Click the "XL" icon afterward to transfer the data to Microsoft Excel for subsequent processing (see analytical protocols below for data handling methods). Save the file as "experiment ddmmyy.xls" where "experiment" is the name of your experiment and "ddmmyy" is a 6-digit representation of the date. This standardized naming scheme will help us all keep track of who owns what file.

11) You may discard the plate now. Replace the priming tray.
12) Remove reagent tubes from magnetic stands. Make sure to place the right cap back on each tube; one drop of carbonate buffer added to the acridinium ester reagent will completely inactivate it.
13) Place DI wash tubes on magnetic stands. Make sure to place the AE wash tube on the right stand and the CO3 wash tube on the left stand. Prime for 20 strokes for each injector to wash reagents from lines. Afterwards, leave lines submersed in wash tubes unless luminometer is to be inactive for an extended period.
14) Rinse priming tray with DI water and let dry outside of luminometer. Close luminometer and turn off.

Orion Maintenance.

1) It is necessary to do a more thorough cleaning after the machine has been in the field, or if it has sat for a long time inactive.
   a. Place 0.1N NaOH tubes on each injector stand. Prime for 20 strokes.
   b. Prime for 20 strokes with DI H_2O.
   c. Prime for 20 strokes with 0.1N HCl.
   d. Prime for 20 strokes with DI H_2O.
2) Occasionally the injectors will become jammed. This is often evident when the priming tray shows evidence of "spraying" after priming the injectors. If this happens, unscrew the priming tubes from the injection ports and withdraw the injector assembly. The injectors themselves are actually just disposable P10 pipettor tips. If you don't see a tip, you might need to use forceps to extract the tip from the port. When this is accomplished simply fasten a fresh tip on the injector tube, re-insert it into the port, and screw it back in. Perform the above cleaning routine prior to using the luminometer again.

III. Analytical Protocol I: The Standard Curve Method

This method is by far the simplest of the two. It is sufficient when analyzing a number of samples that are chemically and physically similar to one another. The specific properties that seem to make this method insufficient are when different samples have very different pH (≥0.5 unit) or dissolved O_2 levels. In such cases, it is necessary to use Analysis Protocol 2. However, for most applications, this protocol is sufficient.

Broadly, this method produces one master set of standards by adding standardized amounts of HOOH to blank water. These standards are added to the first and last "columns" of the assay plate and are used to construct a standard curve for interpolating the HOOH concentrations of each assay well. Two mechanical replicates are performed for each sample, and 40 samples can be assayed per 96-well plates The loading scheme for a full plate is as follows:

	Standards										**Standards**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Blank											Blank
B	15.625 nM											15.625 nM
C	31.25 nM											31.25 nM
D	62.5 nM	Samples		Samples		Samples		Samples		Samples		62.5 nM
E	Blank	1-8		9-16		17-24		25-32		33-40		Blank
F	125 nM											125 nM
G	250 nM											250 nM
H	500 nM											500 nM

STANDARDS:

Standard	[HOOH]	HOOH solution	Blank water[1]
H	500 nM	50 μL (10 μM stock)	950 μL
G	250 nM	25 μL (10 μM stock)	975 μL
F	125 nM	250 μL (H)	750 μL
D	62.5 nM	250 μL (G)	750 μL
C	31.25 nM	250 μL (F)	750 μL
B	15.625 nM	250 μL (D)	750 μL
0 (Blank)	0	0	1000 μL

[1] The choice of blank water depends on the sample background: ABW for seawater, Kat-Q for freshwater.

These standards are ideal for most ecologically-relevant applications. As the standard curve loses linearity above 500 nM, it is better to dilute more concentrated samples rather than use higher standards. The HOOH concentration of the sample is calculated by simply creating a regression line using the standards and applying it to each sample. A typical limit of detection (defined as three times the standard error of the blank) for Analysis Protocol I is less than 10 nM.

Section V gives specific details for how to calculate HOOH and standard error from Analysis Protocol I data.

IV. Analysis Protocol II: The Method of Standard Additions

This method is a classical analytical chemistry method for determining the concentration of an unknown. Basically, you run a blank along with your sample spiked either with blank water or with a known concentration of analyte. Reading are normalized based on the response of the true blank, then a linear regression is performed for the various sample + standard readings. The negative intercept of this line is the analyte concentration in the sample.

Analysis Protocol II is ideal for situations where the chemical environment is potentially variable between samples, since it does not depend on the assumption that the conditions in sample wells are identical to those in the standard curve wells.

The loading scheme for Analysis Protocol II is shown below. Typically, 180 µL of sample are added to each sample well followed by 20 µL of a 10X HOOH standard in ABW. In the following scheme, the real HOOH additions to the samples are +20 nM, +60 nM, and +180 nM. Therefore, the 10X addition stock would be 200 nM, 600 nM, and 1.8 µM, respectively. By placing these standards in the wells of a deep (1+ mL) 96 well "standard" plate, the standard additions may be rapidly added to the "sample" plate using an 8-channel pipettor.

Standard Plate		Sample Plate 1	2	3	4	5	6	7	8	9	10	11	12
ABW	A	Blank	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Blank
200 nM	B												
600 nM	C												
1800 nM	D												
ABW	E												
200 nM	F												
600 nM	G												
1800 nM	H												

STANDARDS (for a full plate)

Standard	10 µM HOOH stock	Blank water[1]
Blank	0	4000 µL
200 nM	10 µL	490 µL
600 nM	30 µL	470 µL
1800 nM	90 µL	410 µL

[1] The choice of blank water depends on the sample background: ABW for seawater, Kat-Q for freshwater. Approximately 4 mL of blank are required per plate; these should be prepared in 4 separate Eppendorf tubes.

Calculating HOOH concentrations is reasonably straightforward. Conceptually, if the HOOH concentration of the sample was the same as the blank, the regression line of the standard additions (y) vs. luminometer response (x) would pass very close to the origin. However, if the HOOH is elevated, the x-axis reading will be increased, leading to a decrease in the y-intercept equal to the amount of HOOH added (assuming a linear response of the system). Thus, the negative y-intercept for this regression is equal to the HOOH concentration of the sample. Section V gives specific details for how to calculate HOOH and standard error from Analysis Protocol II data.

V. Calculations.

Analysis Protocol I:

1. Average mechanical (and biological if possible) replicates (x) and calculate their standard deviation (σ_x).
2. Calculate Standard Curve using "linest" function in Excel, gathering the standard deviations of the slope and y-intercept. Note: HOOH values are "y" and luminometer readings are "x." Excel Syntax: Select a 2x3 cell array, type in the formula **=linest(y-values, x-values,1,1)**, and hit **ctrl+shift+enter**. The output should look like this:

SLOPE (m)	Y-INTERCEPT (b)
StDev of m (σ_m)	StDev of b (σ_b)
R^2	StDev of y estimate

3. Compute the HOOH value, correcting for dilution factor (d):
$$[\mathrm{HOOH}] = d(mx + b)$$
4. Calculate σ_{mx}:
$$\sigma_{mx} = mx\sqrt{\left(\frac{\sigma_m}{m}\right)^2 + \left(\frac{\sigma_x}{x}\right)^2}$$
5. Calculate σ_{HOOH}:
$$\sigma_{HOOH} = d\sqrt{\sigma_{mx}{}^2 + \sigma_b{}^2}$$

Analysis Protocol II:

1. Calculate the average (s) and standard deviation (σ_s) of the blank.
2. For each sample, use the "linest" function in Excel to produce a regression line for the added standards (y values) vs. the luminometer readings for the corresponding wells (x values). See Analysis Protocol 1 for an explication of the readout for the linest function. Excel syntax: **=linest(standards,luminometer readings,1,1)**
3. Solve for HOOH, correcting for dilution factor (d) and dilution due to standard addtions:
$$\mathrm{HOOH} = \frac{-10d(ms + b)}{9}$$
4. Calculate σ_{ms}:
$$\sigma_{ms} = ms\sqrt{\left(\frac{\sigma_s}{s}\right)^2 + \left(\frac{\sigma_m}{m}\right)^2}$$
5. Calculate σ_{HOOH}, correcting for dilutions:
$$\sigma_{HOOH} = \frac{10d\sqrt{\sigma_{ms}{}^2 + \sigma_b{}^2}}{9}$$

V. Rules of thumb for loading plates:

1. Each sample should be loaded twice to provide mechanical replicates that will be averaged later.
2. The total sample volume must be 200 μL, including dilution water and/or standard additions.
3. For samples exceeding 500 nM HOOH (the approximate limit of linearity for the AE response), dilution is required. A good way of doing this is to add sample to an amount of ABW sufficient to achieve the desired dilution. This may be done directly in each sample well, using a multichannel pipettor to add the dilution blank. If more dilution than this is required, it may be done using a similar method on a separate 96-well plate to facilitate sequential 10-fold dilutions.
4. Try to avoid bubbles and splashing (don't go to the second plunger stop on the pipettor) and try not to get sample stuck on the edges of the wells.
5. If reading samples in freshwater or milli-Q water with ABW as a blank, diluting the sample 10:1 in ABW will minimize the error associated with the different ionic strengths of the samples and the blanks while still providing results that agree well with assays done using Kat-Q standards.
6. If the samples need to remain sterile (typically the case), make sure not to take both replicate aliquots using the same tip, as the 96-well plate **is not sterile and you *will* contaminate your samples** otherwise! My usual experimental method is to withdraw a 500+ μL aliquot into a separate sterile tube (or well in a deep 96-well plate) from which I do subsequent HOOH analysis (and whatever else), thus minimally contacting the experimental solution and reducing the risk of contamination. Note that a single bacterium producing catalase can completely destroy 1 μM HOOH in a few days, so cleanliness is of utmost importance in studying HOOH over longer periods.

Works Cited.

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