Supplemental Methods

Sample collection

The day prior to beginning each time series, the depth of the thermocline was identified using DO and temperature with a YSI handheld probe. This determined the depth of epilimnion sampling for each lake – 4 meters for Sparkling Lake, 1.5 meters for Trout Bog, and 7 meters for Lake Mendota. A surface grab from this trip was brought back to the lab and used to determine filter clogging time by recording the volume that had passed through the filter every 30 seconds for 5 minutes. The clogging time was defined as the point where continuing to filter produced diminishing returns of collected biomass. This time was 2 minutes 30 seconds for Sparkling Lake and Trout Bog, and 1 minute 15 seconds for Lake Mendota.

# 1 hour before you go out on the boat

* Check the weather.
* Change the cheesecloth on the end of the filtering tubing

# 30 min before your timepoint

* Measure water column profiles using the sonde and the PAR meter
* Collect the water sample within 10 minutes of the official timepoint

# At your timepoint

* Collect an integrated sample of the epilimnion using the tubing
* Begin filtering for RNA and collecting environmental samples

# Back on shore

* Filter for chlorophyll
* Deliver Falcon tube of water to person running bacterial production assays

# How to collect the integrated epilimnion sample

* Dip the collection bottle in surface water and shake it to wash 3x
* Lower the weighted end of the tubing until the other end is nearly at the water surface. Hold on to the safety line while you do this!
* Pull the tubing back up to the boat. Right before the bottom of the tubing reaches the surface, reach into the water and insert the stopper at the bottom end of the tubing.
* Hold either end over the 4L sampling bottle and remove its stopper to release the water
* Shake the bottle to integrate the sample

# Filtering for RNA

* The person handling the filters should wear gloves. Make sure you have fresh cheesecloth on the end of the filtering tubing for each timepoint.
* Run 50 mL water sample through the tubing and filter holder with no filter.
* Place a 0.22 micron Supor filter grid-side facing the direction the water is coming from in the filter holder. Place the O-ring on top of the filter using the tweezers.
* Turn the pump on at ¾ speed
* Filter for a specific amount of time set that day based on the filter clogging time on that lake
* When the time is up, open the filter holder and carefully fold the filter like a hot dog 3x. Squish this rolled up filter into a 2 mL cryogenic tube labelled with the appropriate number.
* Record the time filtered and the timepoint on the sheet corresponding to that tube number
* Make sure the tube is well-sealed, then drop into liquid nitrogen.
* Repeat this process 4 times. Discard the flow through of the first filter, then keep the flowthrough of the second filter for nutrient testing. Discard the flowthrough from the remaining filters.

# Collecting filtered water samples

* While you are filtering for RNA, collect the flow-through for nutrient analysis.
* Wash the inside of the bottles by shaking with a small amount of filtered water inside, then dumping this water
* Collect two replicate bottles of 60 mL each of filtered water

# Collecting unfiltered water samples

* Collect small volumes of unfiltered water by running the pump with no filter in the filter holder. Wash all bottles with unfiltered water before filling.
  + 10 mL in a 15 mL Falcon tube for bacterial production assays. This is stored in the blue thermos filled with surface water.
  + Two replicate bottles of 60 mL each for nutrients
  + These samples include the cheesecloth pre-filtration
* Collect larger volumes by pouring water directly from the sampling bottle. Wash all bottles with unfiltered water before filling, and use the graduated cylinder to measure volumes below 250 mL.
  + 150 mL for cyanotoxin analysis (Mendota only)
  + 1 L in a dark bottle for chlorophyll analysis
  + This does not include a pre-filtration step

# Filtering for chlorophyll

* Collect unfiltered water in a tin-foil wrapped 1L bottle
* Use the same yellow pump to filter
* Put a Whatman filter in the filter holder grid side up using tweezers
* Filter 250 mL of water from the chlorophyll bottle through the glass fiber filter
* Do NOT filter through a cheesecloth
* Fold the filter up with tweezers and place in a well-labelled 2 mL cryogenic tube.
* Make sure the tube is well-sealed, and drop it into the liquid nitrogen
* Take three replicate filters for each timepoint

**Secchi Depth.** Lower the Secchi disk on the shaded side of the boat. Remove any sunglasses. Record the depth at which you can no longer see the disk. Lower the disk another half meter, then pull up, recording the depth when you first see the disk. Average the two readings. Repeat with each person on the boat. This only needs to be performed once per lake.

GEODES Chlorophyll measurement protocol

*Based on NTL-LTER protocols at lter.limnology.wisc.edu*

Sample Collection

1. Save one liter of unfiltered water from integrated epilimnion sample. Make sure the collection bottle is wrapped in tin foil.
2. Back inside, filter 250 uL\* of water through a 0.3 micron nitrocellulose filter using a peristaltic pump.
3. Fold the filter with tweezers and place in a 2mL cryogenic vial. Drop the vial in liquid nitrogen.
4. Repeat with 2 more replicate filters.
5. Store filters in -80C freezer until extraction.

\*Actual volume filtered varied due to filter clogging

Extraction

1. Work in dim light conditions. Transfer filters to well-labeled 15mL Falcon tubes and add 10 mL of methanol (good quality methanol, not the stuff used for washes)\*
2. Invert to mix and incubate tubes with methanol for 20 minutes.
3. Homogenize each sample with physical disruption by inserting the tip of homogenizer into the sample and moving up and down until the filter is completely destroyed. This should take about 30 seconds to 1 minute.
4. Wash the tip of the homogenizer twice between samples using 10mL of methanol in empty Falcon tubes. Never run the homogenizer when the tip is not in liquid!
5. Incubate samples at 4C for 24 hours\*\*

\*Steps 1-5 were performed on batches of 20 samples

\*\*Actual incubation times ranged from 18 – 20 hours due to the large number of samples and time constraints

Measurement

Before starting, turn the fluorimeter on at least half an hour before the first measurement. Make sure the D lamp is in the fluorimeter, and check the calibration using both the low and high solid standards.

1. Spin the samples at 12,000 rpm for 15 minutes at room temperature.
2. Add 4 mL of each sample’s supernatant to glass vials for insertion into the fluorimeter.\*
3. Insert the vial into the fluorimeter. Record the reading (in ug/L) and the dilution factor.\*\*
4. Add 120 uL of 0.1 N HCL to the vial. Gently shake the vial and let it sit for 90 seconds before reading. Record the reading and the dilution factor.

\*This step is done in batches of 10 samples. Keep the vials next to the labelled Falcon tubes, as the glass vials cannot be labelled.

\*\* We aimed for readings between 10 – 100 ug/L. We found that samples from Sparkling Lake needed no dilution, samples from Lake Mendota needed a 1:4 dilution (1mL sample + 3 mL methanol) and Trout Bog samples needed either a 1:2 or a 1:4 dilution.

Calculations

Uncorrected chlorophyll A = Dilution factor \* reading \* (mL extraction volume/mL filtered volume)

Phaeophytin = Dilution factor \* (reading after acid – reading before acid) \* (mL extraction volume/mL filtered volume)

Measurement of TN/TDN and TP/TDP:

<https://lter.limnology.wisc.edu/protocol/total-phosphorus-and-total-nitrogen>

Samples can be frozen at -20C and kept for several months before running this protocol

**BACTERIAL PRODUCTION ASSAY**

Pre-protocol steps:

* Make working stock of C14-leucine (40 uM)
* Aliquot the amount of 100% TCA needed for each timepoint into separate tubes
* Make 5% TCA solution
* Make 80% ethanol
* All stocks/solutions should be diluted with MQ H2O

On the boat:

* Collect 10 mL of lake water in a 15 mL Falcon tube
* Fill incubation thermos with lake water
* Put Falcon tube in well-sealed plastic bag and place in thermos
* Start the protocol within 30 min of sampling

In the lab:

1. Add 1.5 mL of lake water to 6 microcentrifuge tubes. Label ONLY the lids.
2. Add 80 uL of 100% TCA to the two negative control tubes
3. Add 10 uL of C14-leucine working stock to all six tubes. Start with the experimental samples and end with the controls
4. Close lids tightly and invert all tubes 5 times to mix. Record the time on the metadata sheet.
5. Put all tubes in a plastic bag with as much air removed as possible
6. Place bag of tubes in thermos of lake water and incubate for 55 min.
7. After 55 min, remove the tubes from the thermos and arrange on bench. Exactly 5 min after removal from the thermos, begin the next step. Record the time on the metadata sheet.
8. Add 80 uL of 100% TCA to the four experimental tubes. Invert 5 times to mix.
9. Leave samples in the fridge for 1 hour.
10. After 1 hour, place samples at -20 degrees.

\* Repeat steps 1 – 10 for each sampling timepoint. Samples can be stored at -20 for a few days\*

1. Thaw samples on benchtop.
2. Centrifuge samples at max speed for 15 min. Label the lid of each tube with the orientation of the tube in the centrifuge, as you may not be able to see a pellet after centrifugation.
3. Pour supernatant into C14 liquid waste container
4. Tap each tube on a Kimwipe to remove any remaining liquid. The Kimwipe is now solid radioactive waste.
5. Add 1 mL of 5% cold TCA to each tube. Do not shake, resuspend, pipette on, or otherwise disturb the pellet (which you may not be able to see).
6. Centrifuge samples at max speed for 5 min in the same orientation as previously. Repeat steps 13 and 14.
7. Add 1 mL of cold 80% ethanol to each tube. Do not disturb the pellet.
8. Repeat step 16.
9. Get out a new tube and add 10 uL of the C14-leucine working stock. This is a positive control.
10. Add 1 mL of scintillation cocktail to each tube.
11. Vortex all samples for approx. 3 seconds.
12. Place tubes in scintillation vials and take to the liquid scintillation counter. Label the top of the vials with the name of the sample inside.
13. Count samples on the C14 protocol
14. Report the cpmA activity of each vial on the metadata sheet.

Supplies need (per timepoint):

* 6 microcentrifuge tubes (2 mL, screw cap)
* 15 mL Falcon tube
* Thermos
* 2 Ziploc bags
* 400 uL 100% TCA
* 60 uL of C14-leucine working stock (+ 10 uL for positive control for – 1 needed per lake)
* Centrifuge
* Kimwipes
* Vortexer
* 6 mL cold 5% TCA
* 6 mL cold 80% ethanol
* 6 mL scintillation cocktail
* 6 glass scintillation vials
* Liquid scintillation counter

Internal Standards Protocol

1. Linearize the pFN18a plasmid with BamHI

|  |  |
| --- | --- |
| H2O | 7.8 uL |
| Buffer E (10x) | 2 uL |
| BSA (10 ug/uL) | 0.2 uL |
| Plasmid (100 ng/uL) | 10 uL |
| BamHI | 1 uL |

Incubate at 37\*C for 1 hour, then inactivate at 65\*C for 15 minutes

1. Purify with a phenol/chloroform extraction

* Add phenol/chloroform mix, pH = 8 for DNA
* Save the aqueous (top) layer
* Add 1mL of cold 100% ethanol and 40 uL of 3M NaAc, pH 5.2
* Precipitate for 2 hours at -80\*C, or overnight -20\*C
* Centrifuge at 12500 rpm for 30 minutes at 4C
* Add 1mL of cold 70% ethanol
* Centrifuge at 12500 rpm for 25 minutes at 4C
* Allow pellet to dry on benchtop
* Resuspend in ddH2O

1. Digest with Mung Bean nuclease – 1 hour

|  |  |
| --- | --- |
| DNA | 1 ug (30 uL) |
| Buffer (10x) | 10 uL |
| Glycerol (100%) | 5 uL |
| H2O | 54 uL |
| Mung Bean Nuclease | 1 uL |

Incubate at 37\*C for 1 hour

1. Repeat phenol chloroform step
2. Confirm complete digestion in a gel
3. Run in vitro transcription kit
4. DNase removal
5. Phenol chloroform - use RNA adjusted pH phenol/chloroform mix instead
6. Qubit quantification + run in gel

RNA extraction protocol

1. Add 400 uL of lysis solution (20 mM NaAc 3M pH 5.2, 1 mM EDTA, SDS 0,5%, all made in DEPC water) to the filters and incubate them in the water bath at 65 C for 2 min.

2. Add lysis matrix (From [FastDNA extraction kit](http://www.mpbio.com/product.php?pid=116540600) or similar) to the lysate and 1 mL of [TRIzol®](<https://www.thermofisher.com/order/catalog/product/15596026>).

3. Bead beat for 1 min at medium speed (3.5).

4. Add 1 uL of internal standard (2 ng/uL).

5. Centrifuge 5 min at 14000 x g

6. Transfer the supernatant to a new eppendorf tube.

7. Add 300 uL of chloroform, mix by gentle inversion 20 times, making an emulsion. If mixed more vigorously you can get more RNA, but the sample gets more contaminated with genomic DNA.

8. Incubate at RT for 3 min.

9. Centrifuge at 13000 RPM for 15 min at 4 C.

10. Take aqueous phase, about 800 uL avoiding interphase (organic phase, pink-colored can be used for DNA extraction). Do this on ice.

11. Divide the 800 uL into two 1.5-eppendorf tubes. Add 1 mL of 100% cold ethanol and 40 uL of NaAc 3M pH 5.2 to each tube. Mix the tubes by inversion to avoid freezing the sample.

12. Incubate at -20 for 2 h or at -80 for 15 min. Centrifuge at 12500 RPM for 30 min at 4 C.

13. Wash with 70% cold ethanol. Centrifuge at 12500 RPM for 25 min at 4 C.

14. Extract as much as solvent as possible and resuspend the (probably invisible) pellet in 350 uL buffer RW1 (from [QIAGEN RNeasy® Mini kit](https://www.qiagen.com/us/shop/sample-technologies/rna/rna-preparation/rneasy-mini-kit#resources))

15. Add the resuspended RNA to the spin column from the RNeasy kit. Spin for 15 seconds at 12,000 rpm. Discard flowthrough.

16. Add 10 uL DNAse 1 to 70 uL buffer RDD. Gently invert to mix and centrifuge briefly.

17. Add DNAse mix to column (80 uL) and incubate at room temp for 15 minutes.

18. Add 350 uL of buffer RW1 to the column. Spin for 15 seconds at 12,000 rpm. Discard flowthrough.

19. Add 500 uL of buffer RP1 to the column. Spin for 15 seconds at 12,000 rpm. Discard flowthrough.

20. Add 500 uL of buffer RP1 to the column. Spin for 2 minutes at 12,000 rpm. Discard flowthrough.

21. Place column in new 1.5 mL tube. Add 30 uL of RNAse free water and centrifuge for 1 minute at 12,000 rpm.

22. Quantify using the Qubit kit for RNA.

DNA extraction protocol

Note: the first steps use the same lysis method as the RNA extractions to better compare sample results. After that, we use the standard McMahon Lab phenol/chloroform prep for lake filters.

Note 2: Filters collected throughout the summer of 2016 from all three lakes for 16S rRNA gene amplicon sequencing were cut in half. Half of each sample was extracted using the phenol/chloroform prep below and the other halves were extracted using the FastDNA Spin Kit with modifications as in Linz, et al. “Bacterial community composition and dynamics spanning five years in freshwater bog lakes.” mSphere 2017.

1. Add 400 uL of lysis solution (20 mM NaAc 3M pH 5.2, 1 mM EDTA, SDS 0,5%, all made in DEPC water) to the filters and incubate them in the water bath at 65 C for 2 min.

2. Add lysis matrix (From [FastDNA extraction kit](http://www.mpbio.com/product.php?pid=116540600) or similar) to the lysate and 1 mL of phenol/chloroform/isoamyl alcohol (25:24:1)

3. Bead beat for 1 min at medium speed (3.5).

5. Centrifuge 5 min at 14000 x g

6. Transfer the supernatant to a new eppendorf tube.

1. Add 750 μl of phenol/chloroform/isoamyl alcohol, invert several times, and centrifuge at 13,000 x g for 5 minutes at room temperature.

*Phenol reads at the same wavelength as DNA in a nanodrop, so if you need extra pure DNA or a really accurate concentration measurement, you may want to consider doing an extra wash with just chloroform/isoamyl alcohol.*

1. Transfer the upper aqueous phase to a 2 ml tube.
2. Add 800 μl of ice cold isopropanol, invert several times to precipitate out DNA. Hold on ice for 10 min. then centrifuge at 13,000 x g for 15 minutes.

*Alternatively you can leave the samples in a -20 freezer for as long as a couple days. It won’t hurt your samples.*

1. Carefully remove the supernatant without disturbing the pellet.

*Pour off the supernatant into a waste beaker. The supernatant contains chloroform, so put it into halogenated waste! DNA is transparent so it’s fine if you don’t see a pellet. If you do see something, it’s probably due to impurities in your sample.*

1. Add 1 ml of room temperature 70% ethanol and invert the tube several times to mix.
2. Centrifuge at 13,000 x g for 10 minutes. Remove the supernatant carefully and set the tube upright on a paper towel to dry the DNA pellet.
3. Resuspend the DNA in 100 μl of ddH2O.