HRES LABOATORY ANALYSES

***Sample Analysis Methods***

**SESTON-**

* Materials: 25mm 934-AH glass microfiber filters, filtering manifold, analytical balance, muffle furnace, forceps
* Ash 25mm 934-AH filters in muffle furnace 450°C for 4 hours
* Place each filter needed into numbered tins
* Pre weigh filters out to 5 decimal places record mass in milligrams
* Filter sample until filter is at maximum capacity and record volume filtered
* Remove filter from manifold using forceps and place on edge of its tin
* Let filter air dry for at least an hour before placing filter on bottom of tin
* Dry filters in drying oven for 12 hours
* Weigh dried filters out to 5 decimal places record mass in milligrams
* Ash filters in muffle furnace at 450°C for 4 hours, allow an extra hour for muffle furnace to come to temperature
* Weigh filters again out to 5 decimal places record mass in milligrams

 ***See excel WorkBook for calculations***

**Dissolved Inorganic Carbon samples**

* Materials: Shimadzu TOC analyzer vials with new septa, 934-AH glass microfiber filters
* Filter sample water through 934-AH microfiber filters (can use filtrate from seston filtering)
* Fill Shimadzu TOC analyzer vial up to the brim with filtrate and add cap with new septa
* Check that there is no air in the vial
* Immediately place sample in refrigerator

**Dissolved Organic Carbon samples**

* + Materials: 60mL Nalgene sample bottles, 934-AH glass microfiber filters
* Filter sample water through 934-AH microfiber filters (can use filtrate from seston filtering)
* Fill 60mL Nalgene sample bottles with filtrate and cap
* Immediately place sample in refrigerator

**Non Purgeable Organic Carbon samples**

* Materials: sample collection bottles, 25mm 934-AH glass microfiber filters, 60mL syringe, syringe filter housing, 300 μL pipette, 1N H2SO4
	+ - * Pipette 300 μL of 1N H2SO4 into 60 ml sample bottle
			* Using 60mL filtering syringe filter sample into sample bottle to fix NPOC sample in the field
			* Refrigerate samples on return from the field

**CHLOROPHYLL-A (NO GRIND ACIDIFICATION METHOD)**

* Materials: 25mm GF/F glass microfiber filters, methanol, 0.5M NaOH, 0.3M HCl, filter manifold, 15mL centrifuge tubes w/ stoppers, fluorometer tubes, fluorometer, freezer, forceps, 1-5mL pipette, 50 μL pipette
* Prepare basic methanol for extraction, 2mL 0.5MNaOH/ 1L methanol
* Filter 150mL of sample onto 25mm GF/F filter
* Remove filter from manifold fold in quarters using forceps and place in 15mL centrifuge tube
* Place centrifuge tubes in freezer for at least one hour
* To begin extracting chlorophyll remove tubes from freezer and pipette 5.0 mL basic methanol into each tube leave in the dark and allow approx 24 hours for extraction
* After 24 hours, shake sample and pipette 3.0 mL into fluorometer tubes
* Read samples on a fluorometer record fluorescence
* If samples are OVER you must dilute and reread (**TD-700**)
* To dilute, pipette 5 mL of basic methanol into fluorometer tube
* Pipette 1mL of OVER sample into fluorometer tube with fresh methanol
* Mark in data notebook that sample was diluted
* Reread sample and record fluorescence
* Add 50 μL of 0.3M HCl to each sample, and leave for one hour
* Reread samples on fluorometer record fluorescence
* Remove filters from fluorometer tubes, methanol can be dumped down sink with water running
* Rinse tubes three times with deionized water then fill with deionized water and let soak for 24 hours

***See excel WorkBook for calculations***

**pH**

* Materials: water sample in glass BOD bottle, pH meter
	+ Turn pH meter on and remove probe from KCl solution
	+ Place probe in first replicate and allow 10 minutes for meter to stabilize
	+ Once stable make a mental note of pH and place probe in second pH replicate
	+ Allow 10 minutes for meter to stabilize
	+ Once stable again make a mental not of pH
	+ Place probe back in first pH replicate and allow 5 minutes to stabilize and record pH
	+ Repeat for the second pH replicate
	+ pH of site is the average of the two pH replicates
	+ all results are recorded in field notebook

**Conductivity**

* Materials: Conductivity meter, water sample, deionized water
* Currently conductivity is measured in the field, but can be measured in the analytical lab
* If necessary use extra water samples or pH sample
* Check with analytical lab for current method
* Conductivity and temperature probes should be used
* Wait until conductivity reading is stable
* Record conductivity and temperature of sample

**Turbidity – In Vivo Chlorophyll - Phycocyanin**

* Materials: trilogy fluorometer, fluorometer tubes, water sample
	+ - Take the same replicates that were used for pH and pour them back and forth between clean lab beaker and glass BOD bottle to mix sample
		- Once sample has been mixed pour into fluorometer tube and cap
		- Repeat with second replicate
		- Insert turbidity module into fluorometer, read standards and samples
		- Insert chl in vivo module and read samples
		- Insert phycocyanin module and read samples
		- Phycoerythrin module may also be used if appropriate
		- All results are recorded in field notebook

\*data from field notebook can be recorded in excel WorkBook HUDSONyyyy.xlxs

**DISSOLVED ORGANIC AND INORGANIC CARBON**

Dissolved organic carbon analyses are currently performed using a Shimadzu TOC-V analyzer

Dissolved inorganic carbon analyses are also performed using the TOC analyzer with occasional replicate sample runs performed using a Shimadzu GC

Three basic types of analyses can be performed with the TOC-V

Dissolved Inorganic Carbon (DIC) can be measured by acidification with 25% phosphoric acid

Dissolved Organic Carbon (DOC) can be measured by measuring total carbon (TC) and subtracting dissolved inorganic carbon (DIC)

Non Purgeable Organic Carbon (NPOC) can be measured by an acidification and sparge method

The TC and NPOC analyses use a high temperature combustion method

DIC samples for the GC are whole water samples collected in 300 ml BOD bottles

DIC and DOC samples are filtered water samples (934 AH or GFF filters) kept refrigerated until analysis

NPOC samples are filtered water samples preserved with 1.0 N sulfuric acid (100 µl per 20 ml)

The TOC-V can run with a standard sensitivity catalyst or a high sensitivity catalyst

Calibration curves must be created on the TOC-V before running samples

TC calibration curves are typically created using potassium hydrogen phthalate as a standard. A typical calibration curve for river or lake work would include a zero standard and 5 standards up to 30 ppm (mg/l), i.e. zero 2.5 ppm, 5.0 ppm, 10.0 ppm, 20.0 ppm, and 30.0 ppm. Higher standards can be used if appropriate. An NPOC calibration curve also uses the TC standards.

IC calibration curves are typically created using a sodium carbonate / sodium bicarbonate standard solution. Similar to the TC curve a typical IC standard curve would include 4 or 5 standards, i.e. zero, 5.0 ppm, 10.0 ppm, 20.0 ppm, and 30.0 ppm.

These standards are usually created weekly from a concentrated 1000 ppm stock standard solution. The concentrated stock solution is usually good for one field season if kept refrigerated and air tight.

A typical sample run will include running standards as samples in order to check the operation of the instrument and to perform a post run standard correction to apply to the field samples.

CREATING CALIBRATION CURVES

Choose the calibration curve type: TC, IC, NPOC.

Choose calculation method: linear regression

Uncheck zero shift

Check multiple injections

Create name for calibration curve

No. of determinations: 1

No. of injections: 2 of 3

No. of wash: 2

SD max: 0.1000

CV max: 2.00%

Auto dilution: 1

Injection volume: 50 µl

For NPOC; acid add: 1.5%; sparge time: 1:30

Peak time parameters: use default settings and multiple injections

STANDARD STOCK SOLUTIONS

TC STANDARD: 1000 ppm C, potassium hydrogen phthalate

1. Accurately weigh 2.125 g of reagent grade potassium hydrogen phthalate that was previously dried at 105 – 120 degrees C for about 1 hour and cooled in a desiccator.
2. Transfer to a 1 liter volumetric flask and dissolve in zero water to the one liter mark

IC STANDARD: 1000 ppm C, sodium carbonate – sodium bicarbonate

1. Accurately weigh 3.50 g of reagent grade sodium hydrogen carbonate that was previously dried for 2 hours in a silica gel desiccator, and 4.41 g of sodium carbonate previously dried for 1 hour at 280 -290 degrees C and cooled in a desiccator
2. Transfer to a 1 liter volumetric flask and dissolve in zero water to the 1 liter mark

POC STANDARD: IC standard is used for measuring purgeable (volatile) organic carbon

Some instruments have the optional POC analysis kit installed which contains a CO2 absorber packed with lithium hydroxide

TN STANDARD: 1000 ppm N potassium nitrate

1. Accurately weigh 7.219 g of special reagent grade potassium nitrate dried for 3 hours at 105 – 110 degrees C and cooled in a desiccator
2. Transfer to a 1 liter flask and dissolve in zero water to the 1 liter mark

Some instruments have the optional TN unit installed which contains an NOx absorber (soda lime, the same as the main unit CO2 absorber)

**Total Carbon/ Non Purgeable Organic Carbon standards**

* Materials: 1 1000mL volumetric flask, Potassium Hydrogen Pthalate, analytical balance, 5 200mL volumetric flasks, 1mL volumetric pipette, 10mL volumetric pipette, Nanopure zero carbon water

**Preparation of standards**

* To make the 5 different standards, using the 1mL and 10mL volumetric pipettes add the following amounts on the table below into designated **200mL** volumetric flasks

|  |  |
| --- | --- |
| ppm of standard | Stock solution added (mL) |
| 2.5 | .5 |
| 5.0 | 1 |
| 10 | 2 |
| 20 | 4 |
| 30 | 6 |

* + Fill each volumetric flask to 200mL line with Nanopure zero carbon water and swirl to mix
	+ Standards should be kept in refrigerator and generally new standards are made for each new sample run

**Inorganic Carbon standards**

* + - Materials: 1 1000ml volumetric flask, Sodium Carbonate, Sodium Bicarbonate, analytical balance, 4 200mL volumetric flasks, 1mL volumetric pipette, 10mL volumetric pipette, Nanopure zero carbon water

**Preparation of standards**

* + To make the 4 different standards, using the 1mL and 10mL volumetric pipettes add the following amounts on the table below into designated **200mL** volumetric flasks

|  |  |
| --- | --- |
| ppm of standard | Stock solution added (mL) |
| 5.0 | 1 |
| 10 | 2 |
| 20 | 4 |
| 30 | 6 |

* + Fill each volumetric flask to 200mL line with Nanopure zero carbon water and swirl to mix
	+ Standards should be kept in refrigerator and generally new standards are made for each new sample run

**pCO2/DIC/pH MEASUREMENT**

* + Materials: 1 L Polycarbonate bottle, silicone stopper with tubing and stopcocks, 5, 60 ml syringes with stopcocks, 16, 20 ml syringes with stopcocks, 7 BOD bottles, peristaltic pump and tubing, Gas Chromatograph, CO2/He gas standard canister, 2-3 coolers, 0.2 N Sulfuric Acid

**pCO2:**

* Fill a 60 ml syringe with the CO2/He gas standard.
* Inject two "replicate" 10-20 ml samples of the standard into a GC. If there is excellent replication then continue, otherwise adjust the GC (esp. check for water peaks) then repeat previous 2 steps.
* Inject one 10 ml "air" sample from each lake into the GC. If there is good replication then continue, otherwise inject one more 10 ml sample from the bad replicates and continue.
* Inject one 7-8 ml sample from each set of 4 samples (the 20 ml syringes). If there is good replication, continue with the next set of 4 samples; otherwise inject the remaining sample from each syringe.

**DIC**

* Draw 25 ml of water from the BOD bottle into a 60 ml syringe, taking care not to introduce any air.
* Add 0.2 ml of 0.2 N Sulfric Acid to the syringe.
* Draw 25 ml of Helium into the syringe.
* Shake syringe vigorously 100x
* Inject 10-15 ml of the gas into the GC, taking care not to inject any water.
* Repeat all above steps. If replication is bad repeat all above steps until sample replication is acceptable.

**WATER CHEMISTRY SAMPLE PRESERVATION**

* + - * + Materials: sample bottles, filtering manifold, GF/F glass microfiber filters, 500 μL pipette, 1N H2SO4
			* Pipette 500 μL of 1N H2SO4 into unfiltered water chemistry bottle and filtered chemistry bottle

**For unfiltered water chemistry samples**

* + - * Bottles can be filled with whole water

**For filtered water chemistry samples**

* + - * Filter whole water through 25mm GF/F glass microfiber filter and fill prepared chemistry bottles

Unfiltered chemistry samples are analyzed for Total Nitrogen and Total Phosphorous

Filtered chemistry samples are analyzed for Ammonia, Nitrate, and Phosphate (SRP)

Currently these analyses are done using a Lachat instrument; the analyses can be done by the user assisted by an analytical laboratory technician or they can be submitted to the lab to be analyzed

LACHAT METHOD

Special notes:

* all samples are acidified
* buffer is acidified
* all standards are acidified
* obtain all stock STANDARDS and QC from analytical lab

Pre-run Organization

* Search database for samples that need to be run
	+ Copy and paste to form a list in Excel
* Find sample bottles in lab
	+ Organize filtered (NO3, NH4, and PO4) and unfiltered (TN and TP) separately
* Determine which samples are run with replicates
* Assign consecutive numbers to each sample and replicate
* Create sample tables for Lachat software in Excel
	+ See Lab tech that runs the Lachat to find out how many samples for each type of run changes based on timing.
	+ For a full day’s run, 80 samples a run with standards inserted every 40 samples works well
	+ Include GAIN in first set of standards
		- GAIN is high standard
		- BLANK is acidified nano water
		- Include BLANK and QC in all standard sets
		- For TN, also include reagents for digestion checks, and reagent for column check
		- For TP, also include reagent for digestion checks
		- For NO3 include TN digestion checks on the first day of runs
	+ Include BLANK and QCA for standards inserted every 40 samples and at the end of the run
	+ In column A, consecutively number samples
	+ In column B list standards and sample names
	+ In column C label cup numbers for standards, checks and blanks (S1, S2, S3...)
		- For samples, leave this column blank

Save each day’s run to upload to Lachat software

* + - Save as comma delimited (\*.cvs) on to disk

\*See final page for Excel sheet examples

More detailed methods for making standards as well as digestion check solutions and QC samples can be found in a laboratory methods document

**MACRO and MICROZOOPLANKTON SAMPLE PREPARATION**

* + Materials: 125mL Qorpack glass bottle, 4% formaldehyde buffered with 60g/L sucrose

**Prepare formaldehyde solution**

* + - 60g sucrose + 8.4g sodium bicarbonate, 100 ml 37% formalin, 825 ml DI. Mix thoroughly.
* Measure approximately 30mL of 4%formaldehyde buffered with sucrose and sodium bicarbonate, into Qorpack glass bottles
* Qorpack bottles containing formaldehyde must remain in fume hood until time of use

**MICROZOOPLANKTON ABUNDANCE**

* + - * Materials: 10cc settling chambers, Olympus inverted microscope, 35μm mesh sieve, analytical balance, 10mL pipette
		- Microzooplankton are counted using 10 cc settling chambers and the Olympus inverted microscope.
		- To remove the formalaldehyde and concentrate the sample, pour it through a 35 um mesh sieve. Rinse the contents of the cup back into the sample jar using distilled water.
		- Weigh the sample and jar, record weight on counting sheet.
		- Mix the sample by drawing up 10 ml into an Oxford pipette several times.
		- Remove 10 ml of sample and place in settling chamber.
		- Allow sample to sit for a few minutes before placing cover on chamber.
		- The sample should be allowed to settle completely before counting (about half an hour).
		- Count the contents of the entire chamber. At least 100 of the most abundant rotifers should be counted.

**To calculate abundance when only a portion of the sample was counted:**

* The weight of concentrated sample is the weight of the concentrated sample and the jar (g) - empty jar weight (g) (should be written on the label).
* This is the volume (in ml) if you assume sample density = 1.
* From that, calculate the fraction of sample counted and abundance of each species per sample. Finally the total number of organisms per sample must be corrected for the initial volume sampled and concentrated.

**MACROZOOPLANKTON ABUNDANCE**

* + Materials: Dissecting microscope, counting plates, 73μm sieve, analytical balance, 5mL pipette
* Macrozooplankton are counted using a dissecting microscope, preferably with dark field transmitted light.
* We have counting plates that have four wells which hold 5 ml each.
* To remove the formalaldehyde and concentrate the sample, pour it through a 73 um mesh sieve. Rinse the contents of the cup back into the sample jar using distilled water.
* Weigh the sample and jar, record weight on counting sheet.
* Mix the sample by drawing up 5 ml into an Oxford pipette several times.
* Remove 5 ml of sample and place it in one of the counting plate wells.
* 20 ml of sample can be counted at a time.
* Count the entire sample or subsample until 400 or more of the dominant zooplankton have been counted.
* Occasionally it is necessary to subsample for nauplii but to count the entire sample for other organisms. Make note of quantity of sample counted.

**To calculate macrozooplankton abundance**

* For subsamples the correction factor may be calculated by subtracting the bottle weight from the total weight to give sample weight and then dividing by the volume counted. This conversion factor is applied to any organism counts that were subsampled. Finally the total number of organisms per sample can be calculated and then corrected for the initial volume which is typically ~100 Liters

**PHYTOPLANKTON SAMPLES**

* + Materials: 1L amber bottle, 10mL pipette, acidic Lugols, sample

Prepare acid Lugol’s Iodine:

20 g I2; 40 g KI

40 mL glacial acetic acid + 400 mL nanopure water

* Pipette 10mL acidic Lugols into 1L amber bottle
* Cap and leave in fume hood until time of use
* Once sample is collected fill 1L amber bottle with even proportion from 3 different sample bottles from the same site

**Phytoplankton Cell Concentration Method**

Materials: small pump, pump speed controller, beaker, graduated cylinder

* Set up small pump in Caraco Lab
* Thread tubing into pump head
* Allow output to empty into a beaker

*Draw down the liquid in the sample bottle*

* Measure the Original Volume
* Use the second bottle of the same size
* Fill with DI water equal to the total volume of liquid
* Measure the DI water
* Record the Original Volume of liquid
* Set the pump speed to ~1
* Hold small intake tube at surface of the liquid
* When desired amount is removed, pump air through to empty tube

*Transfer sample to new bottle*

* + Mix sample in old bottle
	+ Pour into new bottle

*Quantitatively rinse for residual cells*

* Rinse old bottle
* Squeeze a small amount of DI water to old bottle
* Mix water
* Empty into new bottle
* Repeat

*Measure the Final Volume*

* Use the second bottle of the same size
* Fill with DI water equal to the total volume of liquid
* Measure the DI water
* Record the Final Volume of liquid

**Method for Making Semi-permanent Microscope Slides of Phytoplankton**

**Prepare Taft’s Syrup Medium (30% syrup)**

* 63 ml DI Water
* 7 ml 37% Formaldehyde Solution (substitute Lugol’s)
* 30 ml Karo Light Corn Syrup

**Preparing Microscope Slides**

* Set hotplate to LO
* Allow 15 min to warm up
* Microscope slide
* Add 100 l Sample
* Add 10 l Taft’s Syrup Medium
* Cover slip
* Add 100 l Taft’s Syrup Medium Warm both microscope slide and cover slip for ~ 3 min
* Edges of the sample should remain sticky
* Flip cover over onto slide
* Heat for ~ 3 min
* Gently press cover to remove air bubbles without pressing out liquid
* Repeat heating until no liquid can be pressed out
* Allow slide to cool for 5 min
* Make sure cover slip is firmly attached to slide
* Line cover slip with clear fingernail polish
* Allow polish to dry for 5 min
* Buff off smudges with a Kimwipe

**MEASUREMENT OF BACTERIAL ABUNDANCE**

* + Materials: 0.8 (or .45) µm x 25 mm Millipore filters (backing filters), 0.2 µm x 25 mm irgalan black stained Nuclepore polycarbonate filters or prestained poretics filters, Slides and cover slips, syringe (≥10 cc), 0.2 µm x 25 mm Millipore filters and Swinnex , or 0.2 µm x 25 mm Gelman sterile Acrodiscs, Filtration manifold, Filtration towers, Vacuum pump with tubing, 100-1000 µl pipette; **REAGENTS**:2% formaldehyde, 0.2 µm filtered (5 ml 37% formaldehyde + 90 ml distilled water), 0.05% acridine orange, Cargille type A immersion oil, Distilled water, 20% formaldehyde buffered with borax @ 30 g /l

**Preparation of unstained polycarbonate filters (Only necessary with filters not pre-stained)**

* Add 0.2 g Irgalan black to 2% acetic acid (2 ml glacial acetic acid + 98 ml distilled water).
* This does not need to be filtered. Immerse filters in Irgalan black solution for 1-2 hours.
* Carefully pour off solution (save and re-use).
* Rinse filters several times with distilled water.
* Filters may be stored in distilled water or air-dried on paper towels and then stored.

**Preparation of acridine orange**

* Make 0.05% solution by adding 50 mg acridine orange to 100 ml distilled water.
* Filter using a syringe and 0.2 µm Acrodisc or swinnex filter holder with a 0.2 µm Millipore filter.
* Freeze 10 ml aliquots in plastic scintillation vials.

**Slide preparation**

**NOTE:** Slides should be made as soon as possible after sample collection. Take care not to cross-contaminate reagents and samples with pipette tips.-or preserve for 1% final HCHO -

* Place wetted backing filters and stained Nuclepore filters on filtration towers.
* Gently stir the sample and remove 1 ml with a pipette. Add the sample to the filtration tower and dilute up to 2 ml with filtered 2% formaldehyde.
* Add 0.2 ml acridine orange and stain for 2 minutes.
* Filter samples at <200 mm Hg vacuum pressure.
* Remove filters, allow to air dry, place on a labeled (lake, date, depth, volume filtered, filter tower used) slide between two drops of immersion oil and cover with a cover slip.
* Store finished slides in freezer.

**Preparation of blank slides**

* Blanks should be made WEEKLY to check for contamination of reagents.
* Make slides following the above procedure but excluding an actual sample.
* If there are many cells in a blank, filter or mix new reagents.
* The number of cells in the blank will have to be calculated and subtracted from the total for the samples for that week.

**COUNTING PROCEDURE For BACTERIAL ABUNDANCE**

* + Materials: Olympus BH2 microscope equipped with 100 watt Hg burner, oil immersion 100x S-plan objective, and reticle with Whipple grid, Cargille type A immersion oil
* Allow frozen slides to thaw before counting.
* Be sure slides are dry; condensed moisture can be removed by pressing slide between paper towels.
* Count 10 grids from each slide for a total of approximately 400 cells per slide (300 cells minimum).
* If bacteria are abundant, count a fraction of the grid.
* Randomize selection of fields by adjusting stage to next position with several turns while not looking at the slide.

**Calculations**

***To calculate cells per grid per ml (TGM):***

 Total cells counted/# grids counted/volume filtered (ml)

***To calculate cells per liter:***

 TGM \* tower-specific conversion factor \* 1000

**Note:** The tower-specific conversion factor is the number of grids in the stained area of a filter at a given magnification and can vary from one filter tower to another. Determine this number using stage micrometer to measure diameter of stained portion of several filters. Calculate area of stained portion and divide by area of grid to determine number of grids/filter.

***To calculate bacterial biomass:***

* biomass (ug C/L) = (cells/L) \* (8.2x10-9 ug C /cell)
* The average volume of an UNDERC bacterial cell is 0.0215 µm3. Assuming 0.38 g of C per cm3 results in a conversion factor of 8.2 fg C per cell (Pace 1992).

**REFERENCES:**

Hobbie, J.E., R.J. Daley, and S. Jasper. 1977. Use of Nuclepore filters for counting bacteria by fluorescent microscopy. Applied and Environmental Microbiology 33: 1225-1228.

Pace, Michael L. 1992. Heterotrophic microbial processes. In: S.R. Carpenter and J.F. Kitchell, eds. The Trophic Cascade in Lake Ecosystems. Cambridge University Press. Cambridge, England.

**MEASUREMENT OF BACTERIAL GROWTH -- THYMIDINE INCORPORATION**

* Materials: 15 ml disposable polypropylene test tubes with caps, 0.2 µm x 47 mm Nuclepore polycarbonate filters, 47 mm Gelman AE filters (backing filters), Filtration manifold, Filtration towers, Vacuum pump with tubing, 10-100 µl pipette, 100-1000 µl pipette, 5-10 ml pipette, Incubators set at in-situ temperatures; **REAGENTS:** Methyl-3H-Thymidine (Concentration: 1 mCi/ml, (diluted to 20µCi/100µl for Cascade Lakes), 5% trichloroacetic acid (TCA) solution (500 g crystalline TCA + 1000 ml distilled water makes 50%; 100 ml 50% TCA + 900 ml distilled water to make 5%; refrigerate), 50% TCA, 5% formaldehyde solution

**Preparation of 3H-thymidine (Tdr) working stock and voucher \*(for Cascade Lakes: Hudson uses 1mCi/ml stock)**

* + Dilute 3H-Tdr to 20µCi/100µl. This is not sterile, so prepare only what you will use
	+ immediately.
	+ 3880 µl distilled water (0.2 µm filtered) + 970 µCi 3H-Tdr makes enough for about 48 samples at 20 µCi per sample.
	+ Check isotope stock by preparing a voucher each time a new batch of working 3H-Tdr stock is made: 10 µl working stock + 990 µl (0.2 µm filtered) distilled water (makes 2µCi/1000µl solution).
	+ Place 100 µl of this solution in a labeled scintillation vial.
	+ Count the voucher on the same scintillation counter that is used for counting samples.

**Incubation procedure**

* Take 6 replicate 10 ml samples each from the epilimnion and the metalimnion and place in clean, labeled (lake, date, depth, and live or control) polypropylene test tubes.
* Add 100 µl (20 µCi) working stock 3H-Tdr to each tube.
* Stop incorporation in two tubes by adding 2ml of 5% formaldehyde for controls
* Incubate for one hour (**Hudson 30 minutes**) at in situ temperatures.
* At end of incubation, stop "live" tubes by adding HCHO.
* Place thoroughly wetted backing and Nuclepore filters in filtration towers; filter contents of each tube (200 mm Hg vacuum pressure or less).
* Rinse twice with ice cold 5% TCA (keep in ice bath) by adding 2 ml TCA to each tube and pouring onto filter.
* Remove filter tower and carefully rinse edge of filter with 2 ml 5% TCA. Place filter back in test tube, cap, and freeze.

**BACTERIAL PRODUCTION -- DNA EXTRACTION**

* + Materials: 100-1000 µl pipette, 5-10 ml pipette, refrigerated centrifuge, centrifuge tubes, ice bath, vacuum pump with aspiration apparatus, hot plate and water bath, scintillation vials; REAGENTS: alkaline extractant: ethylenediaminetetraacetic acid (EDTA), lauryl sulfate - sodium salt (SDS), sodium hydroxide, carrier DNA solution, 3 N HCl, 50% trichloroacetic acid (TCA), 5% trichloroacetic acid (TCA), scintillation cocktail

**Preparation of alkaline extractant**

* Dissolve the following in 1L distilled water:

 12 g sodium hydroxide

 7.5 g EDTA

 1 g SDS

**Preparation of carrier DNA solution**

* Prepare a solution of 5 mg carrier DNA per ml of alkaline extractant. 10 ml is enough for 84 samples (50 mg DNA, 10 ml extractant).

**Extraction**

* Add 5 ml alkaline extractant to each tube with filter; leave at room temperature for 15 minutes.

**Precipitation**

* Remove filter with forceps and transfer extractant to centrifuge tubes in ice bath.
* To each tube, add:

 0.5 ml 3 N HCl

 1.0 ml 50% TCA

 100 µl carrier DNA solution

* Turn on centrifuge and set temperature to 0°C.
* Leave tubes in ice bath for 30 minutes; add ice as necessary to keep samples cold.
* Centrifuge samples at 12000 rpm for 15 minutes.
* Aspirate supernatant, being careful not to aspirate any of the pellet. Add 3 ml 5% TCA.
* Repeat steps 5 and 6 (centrifuge, aspirate, add TCA). Load tubes for the second spin so that the pellet is facing to the outside of the centrifuge.
* Immerse tubes in 100°C water bath for 30 minutes; all of the pellet should dissolve. Cool.
* Centrifuge samples at 10000 rpm for 10 minutes.
* Place 1 ml supernatant in scintillation vial; add 10 ml scintillation cocktail; count in scintillation counter.

**CALCULATIONS:**

**Note:** When calculating bacterial production from thymidine incorporation, we assume no dilution by unlabelled thymidine pools. It is possible to measure isotope dilution; see Moriarty (1986) for a discussion.

***To calculate incorporation of 3H-thymidine into DNA (TDRi, in pmol/L/h):***

 TDRi = (CPMs) (Vc) (TDRp)

 (Vs) (T) (Ceff) (DPMt)

* where:

 CPMs = counts per minute for sample

 Vc = volume correction; only 1 of 3 ml left at end of extraction is counted, so Vc = 3 (no units)

 TDRp = amount of 3H-Tdr added to each sample (pmol)

 Vs = volume of sample used in incubation (L)

 T = length of incubation (h)

 Ceff = counting efficiency of fluor, determined from quench curve

 DPMt = DPM for 3H-Tdr added to sample

***To calculate bacterial growth in cells per liter per hour:***

 cells/L/h = (TDRi) (cf)

* where:

 TDRi = 3H-Tdr incorporated into DNA (pmol/L/h)

 cf = a conversion factor: 5.2x109 cells produced per nmol 3H-Tdr incorporated (Smits and Riemann 1988).

***To calculate bacterial production in ug C/L/h:***

 ug C/l/h = (cells/L/h) \* (8.2x10-9 ug C/cell)

* Determination of cell to biomass conversion factor: The average volume of an UNDERC bacterial cell is 0.0215 um3. Assuming 0.38 g of C per cm3 (Lee and Fuhrman 1987, Simon and Azam 1989) results in a conversion factor of 8.2 fg C per cell.

**References**

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**MEASUREMENT OF BACTERIAL PRODUCTION -- LEUCINE INCORPORATION USING FILTRATION**

* + Materials: peristaltic pump with tubing, 15 ml disposable test tubes or centrifuge tubes, with screw caps, incubators capable of maintaining in situ, temperatures and heating to 85°C, centrifuge tubes for extraction (minimum volume: 20 ml, caps should seal tightly), filtration apparatus, vacuum pump, 0.45 µm x 25 mm Whatman cellulose nitrate filters, scintillation vials; **REAGENTS**: L-[4,5-3H] Leucine (concentration: 1 mCi/ml; (diluted to 15µCi/100µl), 50% trichloroacetic acid (TCA) solution, 5% trichloroacetic acid (TCA) solution, 80% ice-cold ethanol, ethylene glycol monomethyl ether (EGME), scintillation cocktail

**Preparation of 3H leucine solution and voucher**

* + - Dilute 3H-Leu to 15µCi/ 100µl. This is not sterile, so prepare only what you will use immediately. 3.82 ml distilled water (0.2 µm filtered) + 670 µCi 3H-Leu makes enough for about 42 samples.
		- Check isotope stock by preparing a voucher each time a new batch of working 3H-Leu stock is made: 10 µl working stock + 990 µl (0.2 µm filtered) distilled water (makes 1.5µCi/1000ul solution).
		- Place 100 µl of this solution in a labeled scintillation vial.
		- Count the voucher on the same scintillation counter that is used for counting samples.

**Leucine addition**

* Carefully uncap tubes and add 100 µl 3H-leucine solution to each tube. Note time. Replace caps on 5 of the six tubes, the remaining tube will serve as a control.
* Pour the control sample into one of the extraction tubes; immediately add 2 ml 50% TCA and cap.
* Incubate each set of tubes at in situ temperature for 45 minutes.
* While samples are incubating, prepare the rest of the extraction tubes by placing 2 ml 50% TCA in each tube.
* Transfer samples to extraction tubes and cap. Heat in 75 degree C water bath for 30 minutes, then allow to cool completely.
* Filter contents of each tube through wetted (distilled water) Whatman filter.
* Rinse extraction tube with 2 ml 5% TCA and pour through filter.
* Remove filter towers and rinse each filter twice with 0.5 ml 5% TCA, once with 0.5 ml 80% ice-cold ethanol, and once with 0.5 ml distilled water. Filter until very dry.
* Place each filter in a labeled scintillation vial. Add 1 ml EGME to dissolve filter. Allow to dissolve for at least 24 hrs.
* When filter has completely dissolved, add 10 ml scintillation cocktail, allow to dissolve for at least 48 hrs, and count on scintillation counter.

**Calculations**

**Note:** When calculating bacterial production from leucine incorporation, we assume no dilution by unlabelled leucine pools. It is possible to measure isotope dilution; see Simon and Azam (1989) or Chróst (1990) for a discussion. All calculations (except part D) from Simon and Azam, 1989.

***To calculate 3H-leucine incorporation (LEUi, in moles/L/h):***

 LEUi = (CPMs) (LEU)

 (Ceff) (DPMt) (Vs) (T)

* where: CPMs = counts per minute for sample

 LEU = amount of 3H-leucine added (mol)

 Ceff = counting efficiency for fluor, calculated from quench curve

 DPMt = DPM for 3H-leucine added to sample

 Vs = sample volume (L)

 T = length of incubation (h)

***To calculate bacterial protein production (BPP, in g/L/h) from 3H-leucine incorporation:***

 BPP = (LEUi) (100/7.3) (131.2)

* where: LEUi = rate of leucine incorporation (mol/L/h)

 100/7.3 = 100/mol% of leucine in protein

 131.2 = the formula weight of leucine

***To calculate bacterial carbon production (BCP, in g/L/h) from BPP:***

 BCP = (BPP) (0.86)

***To calculate bacterial production (BPRO in cells/L/h) from bacterial carbon production***:

 BPRO = BCP

 (cell carbon content)

* where: BCP = bacterial carbon production (g/L/h)

 cell carbon content = 8.2 fg C per cell

**Note:** The average volume of an UNDERC bacterial cell is 0.0215 µm3. Assuming 0.38 g of C per cm3, cell carbon content is 8.2 fg C per cell (Pace 1992).

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**MEASUREMENT OF BACTERIAL PRODUCTION -- LEUCINE INCORPORATION USING MICROCENTRIFUGATION**

* + Materials: 2 mL Eppendorf disposable microcentrifuge tubes and caps, microcentrifuge, vortex mixer, pump with aspirating tube and pipette; REAGENTS: L-[4,5-3H] leucine (conc.: 1mCi/mL, S.A.: approx. 60 Ci/mmol), 50% TCA, 5% TCA, scintillation cocktail (Scintiverse BD)

**Preparation of 3H leucine solution and voucher**

* Dilute stock isotope such that the final concentration of the sample is around 17.5 nM. This is what has been used in the past. Smith and Azam have noted using a 20 nM concentration in their methods paper (Hudson/Baltimore 50nM).
* Use 0.2 um filtered distilled or RO water to make enough solution for samples and vouchers.
* Add 100 uL volumes of isotope solution to each sample so that accurate and replicable volumes can be attained.
* For Hudson River 50 nM solution:

 50 nM = 50 nmol per liter = 0.05 nmol per ml

 In 1.6 ml sample need 0.08 nmol

 Example; for leucine with specific activity of 60.0 Ci per mmol = 60 µCi per nmol, 0.08 nmol is 4.80 µCi per 100 µl.

* To make working stock add 48.0 µCi (µl) to 1000 µl.
* Check isotope concentration by preparing at least two vouchers for each solution made.
* Pipette 100 uL of working stock into microcentrifuge tubes.
* Add 1.5 mL of scintillation cocktail and measure on the LSC.

**Leucine incubation**

* Add 1.5 mL sample volumes to each microcentrifuge tube.
* Add 100 uL of isotope solution to each sample.
* Immediately "kill" the controls with 0.3 mL 50% TCA.
* Incubate "live" samples for 0.45 minutes to 1 hour before "killing" with 0.3 mL 50% TCA.

**Centrifugation**

* + Turn on the microcentrifuge with the switch on the back of the machine near the electrical cord input.
	+ Open the microcentrifuge lid with the black lever on the right side. This only works when the microcentrifuge is on.
	+ To open the cover when the machine is off, use the metal rod and insert it in the hole at the front (upper right) of the centrifuge.
	+ Remove the clear plastic rotor cover. Make sure the rotor is on tight using the silver hex wrench. This wrench fits on the nut in the center and tightens clockwise. Load samples into the openings making sure the rotor is balanced with samples and replace the clear plastic cover. Shut the microcentrifuge lid.
	+ The centrifuge has a timer which can automatically be set using the rotating wheel on the front of the machine. Check to see that it is set for 10 minutes. The speed dial should be set at 14000 rpm. The digital readout displays the speed of the rotor while running. Adjust if not stable at 14000 rpm.
	+ Start the microcentrifuge by pushing the start/stop lever down towards "start". The machine will not run if the lid is not properly closed. Also, the lid cannot be opened while the rotor is spinning. The centrifuge will stop when it is done with its timed cycle.
	+ When the rotor stops spinning and the light stops flashing and/or you hear a click, the lid can be opened with the black lever to the right. Remove the clear plastic rotor and remove samples.

**Sample handling**

* After the samples have been "killed", centrifuge for 10 minutes at 14000 rpm.
* Aspirate the liquid from the vial, taking care to remove all liquid.
* Add 1.5 mL 5% TCA to each tube and vortex for 5-10 seconds.
* Centrifuge again for 10 minutes at 14000 rpm, and aspirate liquid.
* Add 1.5 mL scintillation cocktail(Scintiverse BD).
* Vortex tubes, and place in glass scintillation vial. Count samples using the appropriate program for 3H (0-400 window) on the scintillation counter.

**MEASUREMENT OF FLAGELLATE ABUNDANCE**

* + Materials: 0.8 µm x 25 mm Millipore filters (backing filters), 1.0 µm x 25 mm stained Nuclepore polycarbonate filters, Slides and cover slips, Filtration manifold, Filtration towers, Vacuum pump with tubing, 10-100 µl pipette, 100-1000 µl pipette, 5-10 ml pipette, Olympus BH2 microscope equipped with 100 watt Hg burner, oil immersion 60x S-plan APO objective (numerical aperture = 1.4), and reticle with Whipple grid, Cargille type A immersion oil; **REAGENTS**: 0.033% proflavine solution, 10% glutaraldehyde (100 ml 25% glutaraldehyde +150 ml distilled water; keep refrigerated), Cargille type A immersion oil, Distilled water

**Preparation of 0.033% proflavine solution**

* Add 33 mg proflavine to 100 ml distilled water and freeze in 10 ml aliquots in plastic scintillation vials.

**Slide preparation**

* Prepare two filters for each sample. Place wetted backing filters and stained Nuclepore filters on filtration towers.
* Gently stir sample and place 10 - 30 ml subsamples in filter towers.
* Add 40.0 µl proflavine for each 10 ml of sample and stain for two minutes.
* Add 1 ml cold 10% glutaraldehyde for each 10 ml of sample to make a 1% final solution; let sit for two minutes.
* Filter under as low a vacuum as possible (<100 mm Hg).
* Remove filters, allow to air dry, place on a labeled (lake, date, depth, volume filtered, filter tower used) slide between two drops of immersion oil and cover with a cover slip.
* Store finished slides in freezer.

**Counting Procedure**

* Allow frozen slides to thaw before counting. Be sure slides are dry; condensed moisture can be removed by pressing slide between paper towels.
* Flagellates are counted using a 60x objective.
* Using the stage micrometer for measurements, count 5 mm or 10 mm "strips" of the sample.
* The width of a strip is the width of the grid.
* Count at least 50 flagellates per sample (40 minimum).

**Calculations**

***To calculate the number of flagellates per liter:***

 ( totalF / #strips )\*( tower cf / vol )\*1000

* Where:

 totalF = total number of flagellates counted

 #strips = the number of 10 mm strips counted

 tower cf = tower-specific conversion factor

 vol = volume of sample filtered (ml)

**Note:** The tower-specific conversion factor is the number of 10 mm strips in the stained area of a filter at a given magnification and can vary from one filter tower to another.

**CILIATE ABUNDANCE**

* Materials: Inverted microscope, Graduated cylinders (100 ml), 10 ml plankton settling chambers, aspiration apparatus
* Pour entire sample into a 100 ml graduated cylinder; rinse sample jar into cylinder with distilled water. Let sample settle overnight.
* Aspirate all but approximately 8 ml of sample, being careful not to disturb settled sample. Pour sample into settling chamber, rinse cylinder with 2 ml distilled water and pour into settling chamber. Allow sample to settle for several hours or overnight.
* Count ciliates on inverted microscope by scanning the entire chamber at 150x.