**Center for Microbial Oceanography: Research and Education (CMORE)**

**Summer Course “Genomes to Biomes”**

**Bacterial Production Protocol**

***Objectives:*** Learn techniques, assumptions, and limitations of the 3H-leucine incorporation method used to estimate heterotrophic bacterial production in aquatic ecosystems.

***Introduction:***

 Bacterial synthesis of cell biomass (termed bacterial production) forms a dominant term in the flux of organic matter through oceanic ecosystems. The term bacterial production (BP) is typically used to describe the rate of heterotrophic bacterial production and thus BP forms an important component of ecosystem secondary production. Dissolved organic matter (DOM) is the primary growth substrate for heterotrophic bacteria, so by constraining BP, we gain information on rate that organic matter is transferred from a non-living source (DOM) to a living pool of material (bacterial cell biomass). In addition, when combined with information about bacterial biomass (B), the determination of BP provides an estimate of the population growth rate (= BP/B; units are hr-1). Rates of BP are typically reported as g C L-1 hr-1; however, there are few direct methods for determining rates of carbon production by heterotrophic bacteria.

 Two commonly used methods of determining BP rely on measuring the rate at which radiolabeled precursor substrates (amino acids, nucleosides, or nucleotides) are incorporated into bacterioplankton biomass (protein or nucleic acids). In aquatic ecosystems, bacterioplankton incorporation of 3H-thymidine (TdR) and 3H-Leu are two common methods used to estimate BP. These assays share many common methodological features, although the methods measure fundamentally different intracellular processes. There are several important assumptions that should be considered when evaluating the results of the both 3H-Leu and 3H-TdR incorporation assays. Both methods rely on the addition of 3H organic precursors to whole seawater samples and then measuring the rate that these substrates are incorporated into bacterial protein (Leu) and DNA (TdR), respectively. The resulting incorporation rates are in units of mol Leu L-1 hr-1 or mol TdR L-1 hr-1; conversion of these incorporation rates into more ecologically and biogeochemically meaningful measures of BP requires the use of cellular and carbon conversion factors.

The conversion of Leu incorporation rates to carbon production requires knowledge of the amount of Leu contained in bacterial protein. Leu appears to be a relatively constant proportion of protein content, thereby simplifying the assumptions required to convert Leu incorporation to carbon production. The conversion factors used for this conversion generally range between 1.5 and 3.0 kg C mol-1 Leu incorporated. To convert TdR incorporation rates into carbon production relies on two conversion factors; one to convert TdR incorporation to cellular production (cells produced per mole TdR incorporated) and the second to convert cell production to carbon production (which depends on knowledge of the carbon content of marine bacteria; usually in fgC per cells produced). One important but often untested assumption with both TdR and Leu incorporation assays is that isotope dilution, or incorporation of unlabeled leucine and thymidine, does not substantially contribute to protein or nucleic acid production. If isotope dilution were important, measurements of Tdr and Leu could underestimate bacterial production because the cells would be actively incorporating unlabeled substrates to produce new protein or DNA. To minimize potential isotope dilution, concentrations of TdR and Leu are added to seawater samples at concentrations empirically determined to saturate the *de novo* synthesis pathways, thereby maximizing the likelihood that TdR and Leu will be assimilated from the labeled extracellular pools.

There are various approaches for measuring Leu incorporation. The most common methods rely on incubating whole seawater samples amended with the radiolabeled substrates, terminating the incubations by filtration onto 0.2 m filters or concentrating labeled cell macromolecules by centrifugation. In many cases, the incubations are terminated (i.e. the bacteria are killed) prior to filtration by the addition of either formalin or trichloroacetic acid (TCA) to the seawater sample. Both protein and nucleic acids are insoluble in cold TCA, resulting in precipitation of these macromolecules onto the filter matrix. After filtration, the sample filters are rinsed several times with 5% ice-cold TCA, followed by several rinses with ice-cold 80% ethanol (to remove hydrophobic cellular constituents such as lipid membranes). The radioactivity remaining on the filter is assumed to be associated with proteins and nucleic acids, and the filter activity is determined by scintillation counting. There are several more detailed methods of extracting cellular macromolecular constituents (for example separating RNA, DNA, and protein) and determining the radioactivity associated with each of these macromolecular fractions.

We will use a method of measuring Leu incorporation that does not require filtration; rather, we will employ a method that relies on concentration of nucleic acids and proteins by centrifugation. Incubations will be terminated by the addition of cold TCA, and the proteins and nucleic acids will be purified, and concentrated by centrifugation. The radioactive associated with the macromolecular pellet will be determined by liquid scintillation counting. Rates of Leu incorporation will be converted to carbon-based determinations of BP.

***Laboratory Specifics***:

Water will be subsampled from the CTD rosette into darkened polyethylene bottles; use a cooler to keep samples near *in situ* temperatures. We will incubate triplicate 1.5 ml seawater samples from 25 m depth each day. After incubation, samples will be processed using the microcentrifuge method developed by Smith and Azam (1992) to determine rates of Leu incorporation into protein. The resulting incorporation rates will be converted to carbon-based estimates of bacterial production.

***PROTOCOL:***

**Things to remember:** 1 Ci = 2.22 x 1012 disintegrations per minute (DPM). We add 20 nM final concentration of each tracer to samples. You are working with a stock Leu that is 1 mCi/ml and ~108 Ci/mmol.

* **Safety First:** You will be working with a radioisotope (3H)- it is very important to be aware of what you are doing and know the risks associated with 3H exposure. Keep your work area clean and contain the isotope. If you have a spill, make sure to alert the instructors and take the appropriate measures to clean up the spill. Change gloves frequently! Each time you open fridge, touch laboratory surfaces, etc.

***Supplies:***

5 mCi 3H-3-,4-,5- Leu (MP Biomedical or ICN); typically ~110-130 Ci/mmol

2.0 ml Axygene microcentrifuge tubes + caps

gloves

lab coats

Pipettes (1-10 ul, 2-200 ul)

Repeat Pipette with tips (tip suitable for 100 ul aliquots)

Pipette tips (10-100 ul)

Microcentrifuge tube racks

Floating microcentrifuge racks

Bottle/top dispenser for 80% EtOH

Bottle/top dispenser for 5% TCA

***Equipment:***

Refrigerated microcentrifuge

Surface seawater incubator

**SET UP, INOCULATION, AND INCUBATION**

1. Lay fresh benchkote in your work area if needed and have a supply of clean paper towels at each station
2. Set up numbered tubes; use a sequential numbering scheme that clearly differentiates each sample.
3. At the CTD rosette, fill polyethylene sampling bottle with seawater. Place bottles in cooler to maintain temperature.
4. Add ~100 l of primary working stock radioisotope to each tube. Record start time of incubation in the sample log sheets.
5. Remove 1.5 ml of labeled seawater from each tube and place into prelabeled, empty 2.0 ml Axygene microcentrifuge tubes. These samples will serve as time zero blanks.
6. Add 100 ml of 100% TCA to each tube, cap, and place in refrigerator until processed.
7. Cap the 40 ml centrifuge tubes and affix to primary production array; typically one tube is incubated in the light and one tube will be placed in a dark bag which is placed inside a mesh bag for attaching to the array.

**TERMINATING INCUBATIONS AND PROCESSING SAMPLES**

1. While samples are incubating, add 100 l of 100% TCA to triplicate 2.0 ml microcentrifuges for each sample. Label caps.
2. After incubation period (typically 2-10 hours, depending on the productivity of the ecosystem), remove sample tubes from *in situ* array; add 1.5 mL of sample to triplicate sample tubes
	* pipet water carefully to avoid splashing sample and isotope out of the tubes!
3. Cap firmly, mix well, and place samples in freezer for 10 minutes. Record the time samples were killed.
4. Centrifuge tubes at 4oC at highest speed (~14,000 rpm) for 15 minutes
5. Gently pour out the sample into wide-mouth waste jar and tap lip of tube on a paper towel to get the last drops of radioactive liquid off.
6. Add 1 mL of cold 5% TCA to each tube
7. Cap and centrifuge tubes at highest speed for 5 minutes
8. Gently pour out the sample and gently tap lip of tube on a paper towel to get the

 last drops

1. Add 1 mL ice-cold 80% ethanol
2. Cap and centrifuge tubes at highest speed for 5 minutes
3. Pour out the alcohol and remove drops
4. Place tubes in fume hood until dry (overnight)
* Ethanol is a powerful quenching agent so make sure no ethanol remains in tubes!
1. Add 1 mL scintillation cocktail to each vial, vortex well

24. Put in LSC racks and run using 3H protocol

**Calculating rate of Leu or Tdr incorporation**

Leu or TdR incorporation rate =

{(dpm sample)-(dpm blank)}/(incubation time)/(2.22 x 106 dpm per Ci)\*(specific activity of 3H-leucine stock in units of nmol per Ci)/(volume of seawater incubated)

Biomass Production = (Leu incorporation) \* (1.5 Kg C/mol)

**References:**

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