Bermuda Biological Station For Research, Inc.
Bermuda Atlantic Time-series Study

Chapter 19. Determination of Bacterial Production using Methyl-tritiated Thymidine

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Modified from: Fuhrman & Azam (1980, 1982)

1.0 Scope and field of application

This procedure describes a method for estimating bacterial production in seawater from the incorporation rate of methyl-tritiated-thymidine (^{3}H-thymidine). The technique presented here was first published by Fuhrman and Azam (1980, 1982). Since then, most aspects of the tritiated thymidine incubation technique have been thoroughly investigated. Scientists who employ this or other methods to measure bacterial production should make themselves aware of the current and historical issues that surround these techniques and make appropriate decisions about specific methodologies for their application based on the scientific requirements and constraints of their individual programs.

2.0 Definition

Bacterial production is the rate of synthesis of biomass by heterotrophic bacterioplankton, as estimated by the measurement of incorporated ^{3}H-thymidine in the cold trichloroacetic acid-insoluble and cold ethanol-insoluble cell fractions following a short term incubation, using a suitable conversion factor, F:

$$\text{Bacterial production (cells } l^{-1}h^{-1}) = F \bullet ([^3\text{H-thymidine}] \text{ pmol } l^{-1}h^{-1})$$

Where:

F = production of bacterial cells per mole ^{3}H-thymidine

3.0 Principle of analysis

The rate of bacterial production is estimated by tracing the specific incorporation of ^{3}H-thymidine into the TCA-insoluble macromolecular fraction. The incubation is terminated by adding formalin, followed by extraction of the unincorporated ^{3}H-thymidine from the bacterial cells in cold TCA and ethanol.

4.0 Apparatus

4.1 *Hoefer FH 200 Filtration Manifold with stainless steel filtration funnels.* The tritiated incubation solution can be filtered using any reliable, leak-free, acid-resistant, multi-place filtration unit.

4.2 *Packard Tri-Carb 2000 CA Liquid Scintillation Analyzer.* Samples in liquid scintillation cocktail are counted on a liquid scintillation analyzer, using the following energy window settings:

Channel A: 0-19 keV

Channel B: 2-19 keV

Samples are counted long enough to reduce the counting error to <5-10%. This is usually obtained within 10 minutes.

4.3 *Quench Corrections.* An external gamma source is used to assess quenching of individual filter samples for conversion of counts per minute (CPM) to disintegrations per minute (DPM). Quenching of the total radioactivity vials is determined by an internal standard (usually tritiated water added to a range of concentrations of toluene or chloroform as a quencher).

4.4 *Incubation bottles.* Polycarbonate centrifuge tubes (29 ml) are used for the bacterial productivity incubations. Before every cruise, the tubes are soaked in detergent (Aquet®), rinsed in Milli-Q water and soaked overnight in a 5% HCl solution. The acid is then discarded and the tubes rinsed and soaked overnight in Milli-Q water.

5.0 Reagents

5.1 Stock of *methyl-^{3}H thymidine* (60-90 Ci mmol^{-1}) is commercially available in a 0.1% ethanol solution. It can be stored in this solution if the stock is to be used within 3-4 months. However, if the stock is to be used over a longer period of time, it should be stored in 96% ethanol. Stock solution should be kept in the refrigerator, never frozen.

5.2 *Working solution from 0.1 % ethanol solution.* An aliquot of the stock solution is diluted in 0.2 µm filtered Milli-Q water (approximately 1 mCi per 5 ml Milli-Q). Working solution is made no longer than 1-2 days prior to use and is stored in the refrigerator.

Working solution from 96 % ethanol solution. An aliquot of the stock solution is transferred to a glass vial where the ethanol is evaporated. The evaporation is promoted by a vacuum pump drawing air through a Silicagel cartridge and a 0.2 µm Nuclepore filter. The tritiated Thymidine is re-dissolved in 0.2 µm filtered Milli-Q

water (approximately 1 mCI per 5 ml Milli-Q) and stored in the refrigerator for not longer than 1-2 days prior to use.

5.3 *Acid Cleaning Solution* (1 N HCl, Baker Analyzed) is prepared using Milli-Q water.

5.4 Concentrated (37%) *formaldehyde*

5.5 *Trichloroacetic Acid* (TCA) is made up in a 5% solution (weight/volume) in Milli-Q water. A pre-mixed 100% TCA solution can also be purchased and diluted to a 5% working solution. The working solution is kept at 4 °C in the refrigerator. Great care should be taken when working with TCA.

5.6 *Ethanol* (96%): stored in the refrigerator.

5.7 *Ethyl acetate* (Purified, Baker Analyzed)

5.8 *Ultima Gold liquid scintillation cocktail* (Packard)

6.0 Sampling and incubation

6.1 Shipboard sampling: A set of 8 standard depths at 20 m intervals from 0-140 m are sampled. Samples are taken from the Primary production Go-Flo cast (see Chapter 18, sections 6.11-6.12.)

6.2 *Sample dispensing.* Polyethylene gloves are worn during all stages of sampling and processing. The polycarbonate centrifuge tubes are filled directly from the Go-Flos and rinsed 3 times before filling. Three centrifuge tubes are filled from each depth and stored in the dark until all samples have been taken.

6.3 *Time zero* samples. Three additional tubes are filled with 20 ml of sample from each of several depths. 200 µl of concentrated (37%) formalin and 50 µl of tritiated thymidine working solution are added to the vials prior to sampling, in order to ensure rapid preservation. The solutions are immediately filtered and extracted, as described in section 7.1 of this chapter.

6.4 *Isotope inoculation.* Under low light conditions, 100 µl of the tritiated thymidine working solution is added to each incubation sample, to a final concentration of about 10 nM.

6.5 *Incubation procedures.* Ideally, samples should be incubated at *in situ* temperatures. This can be accomplished using temperature-controlled, refrigerated incubators and/or flowing seawater-cooled incubators. The incubation should last sufficiently long to obtain measurable uptake, but not so long as to cause uptake to depart from linearity. Incubations at the BATS site are usually for 2-3 hours.

6.6 *End of Incubation.* The incubation is ended by pouring a 20 ml aliquot from each tube into a separate reagent tube containing 200 µl concentrated (37%) formalin. The aliquots are immediately filtered and extracted, as described in section 7.1 of this chapter.

7.0 Procedures

7.1 *Filtration and extraction.* Under low light conditions, the sample aliquots are filtered onto 25 mm diameter (MFS) cellulose nitrate (0.2 µm pore size) filters, maintaining a vacuum pressure of 70 mm Hg or lower. Mixed ester filters should not be used as they bind DNA and result in insufficient counting. The Hoefer filtration manifold is connected via 1/4 inch Tygon® tubing to a filtrate collecting container and pump. The reagent tubes are rinsed with 10 ml of an ice-cold 5% TCA solution, followed by a 10 ml rinse with an ice-cold ethanol (96%) solution. After the filter funnel is removed, but with the vacuum pressure maintained, the filters are rinsed three times with ice-cold 5% TCA solution from a wash bottle. The TCA rinses are followed with 3 rinses of ice-cold ethanol from another wash bottle. The wash bottles are kept cold in an ice bucket filled with crushed ice and water during the filtration operation. Care is taken to rinse the outer edges of the filters.

7.2 *Filter processing and counting.* The filters are placed in glass scintillation vials. Caps are loosely placed on the vials, and the filters allowed to dry at room temperature overnight. If 7 ml scintillation vials are used, the filters need to be folded carefully 3 or 4 times so they are small enough to permit full immersion in the ethyl acetate. 0.5-1 ml ethyl acetate is added to dissolve the filters. Failure to dry or fully cover the filters in the ethyl acetate solution may result in incomplete dissolution and poor counting efficiency. Vortex mixing is employed to aid in dissolving the filters. Finally, when the filter solution is clear, liquid scintillation cocktail is added and mixed. The samples are counted on a liquid scintillation counter the following day.

7.3 *Total Radioactivity Sample.* Aliquots of 50 µl from three random incubation tubes are added to a set of three scintillation vials with 10 ml of scintillation cocktail to determine the total amount of label added to the samples.

8.0 Calculation and expression of results

Rate calculations. Universal factors for conversion of ^{3}H-thymidine incorporation into cell production do not exist (Kirchman *et al.* 1982; Ducklow and Carlson 1992) but there is fair consensus that the conversion factor (F) varies in the coastal and open ocean within 2± 2x10^{18} cells mol^{-1}. The rate of incorporation is reported as pmole ^{3}H-thymidine taken up per time unit after time zero blank values are subtracted.

$$[methyl\text{–}^3H\text{–}thymidine]\, pmol\, l^{-1} h^{-1} = \left(\frac{DPM}{2200}\right)\left(\frac{1000}{V}\right)\left(\frac{1}{SA}\right)\left(\frac{60}{T}\right)$$

Where:

DPM	=	disintegrations per minute of sample minus blank value
V	=	extraction volume (20 ml)
SA	=	specific activity (of added ^{3}H-thymidine)
T	=	incubation time (min)

A check on the final concentration of the tritiated incubation solution is estimated by converting the amount of the measured total activity into the final concentration of tritiated thymidine.

$$[methyl\text{–}^3H\text{–}thymidine]\, nM = \left(\frac{DPM}{2200}\right)\left(\frac{1000}{\mu l}\right)\left(\frac{1}{SA}\right)$$

Where:

µl	=	aliquot taken from incubation solution (50 µl)
SA	=	specific activity

9.0 Quality Control

9.1 *Standards and precision*. There is no absolute standard for bacterial production measurements and the accuracy is unknown. The coefficient of variation of assays performed carefully following this protocol should be 15-20% for triplicate incubations. The limit of detection will vary depending on length of incubation and the amount of sample filtered. With care, incorporation rates of 0.05-0.1 pmol l^{-1}h^{-1} should easily be detected above background.

9.2 *Non-specific incorporation of thymidine.* Much of the uncertainty with thymidine results appears due to non-specific labelling. Tritiated thymidine does not exclusively enter the bacterial DNA and several studies have demonstrated the labelling of macromolecular compounds other than DNA (Hollibaugh 1988).

Non-specific labelling makes it very important to use an extraction procedure specific for tritiated DNA (Wicks and Robarts 1987, Hollibaugh 1988, Robarts and Wicks 1989). New techniques using enzymatic digestion (Torreton and Bouvy 1991) also look promising.

10.0 Interpretation of results

A conversion factor is needed to derive bacterial production (cells or mass of C or N produced per unit time) from the incorporation rates. Conversion factors should ideally be determined experimentally for each new environment or season sampled. To determine a conversion factor, an independent measurement of bacterial production or growth rate must be made, or the relationship between thymidine incorporation and production must be determined. A variety of approaches exist for this purpose (Bjørnsen and Kuparinen, 1991; Ducklow et al., 1992; Kirchman and Ducklow, 1993). For open ocean sites the conversion factor is generally $2x10^{18}$ cells produced per mole thymidine incorporated.

11.0 References

Bjørnsen, P.K., and J. Kuparinen (1991). Determination of bacterioplankton biomass, net production and growth efficiency in the Southern Ocean. *Mar. Ecol. Prog. Ser.* **71**:185-194.

Carman, K.R., F.C. Dobbs and J.B. Guckert. (1988). Consequences of thymidine catabolism for estimates of bacterial production: An example from a coastal marine sediment. *Limnol. Oceanogr.* **33**:1595-1606.

Ducklow, H.W. and C.A. Carlson. 1992. Oceanic Bacterial Production. Advances in Microbial Ecology. K.C. Marshall ed. p. 113-181. Plenum Press.

Ducklow, H.W., D.L. Kirchman and H.L. Quinby. (1992). Bacterioplankton cell growth and macromolecular synthesis in seawater cultures during the North Atlantic spring phytoplankton bloom, May 1989. *Microb. Ecol.* **24**:125-144.

Fuhrman, J.A. and F. Azam. (1980). Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica and California. *Appl. Environ. Microbiol.* **39**:1085-1095.

Fuhrman, J.A. and F. Azam. (1982). Thymidine incorporation as a measure of heterotrophic bacterial production in marine surface waters: evaluation and field results. *Mar. Biol.* **73**:79-89.

Hollibaugh, J.T. (1988). Limitations of the [^{3}H]thymidine method for estimating bacterial productivity due to thymidine metabolism. *Mar. Ecol. Prog. Ser.* **43**:19-30.

Kirchman, D., H. Ducklow and R. Mitchell. (1982). Estimates of bacterial growth from changes in uptake rates and biomass. *Appl. Environ. Microb.* **44**:1296-1307.

Robarts, R.D. and R.J. Wicks. (1989). Methyl-^{3}H thymidine macromolecular incorporation and lipid labeling: their significance to DNA labeling during measurements of aquatic bacterial growth. *Limnol. Oceanogr.* **34**:213-222.

Torreton, J.P. and M. Bouvy. (1991). Estimating bacterial DNA synthesis from [^{3}H]thymidine incorporation: Discrepancies among macromolecular extraction procedures. *Limnol. Oceanogr.***36**:299-306.

Wicks, R.J. and R.D. Robarts. (1987). The extraction and purification of DNA labelled with methyl-^{3}H thymidine in aquatic bacterial production studies. *J. Plank.Res.* **9**:1159-1166.