Review

A Review on the Radioisotopic Methods for Measuring Bacterial Production in Aquatic Environments

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Advantages and disadvantages of four radioisotopic methods, ^{3}H-thymidine, ^{3}H-adenine, ^{3}H-leucine, and ^{35}S-sulfate, for measuring bacterial production were reviewed. The major issues discussed in production methods were: (1) whether all the actively growing bacteria take up the radiolabeled (organic) tracers; (2) how each target molecule should be purified (nonspecific labeling); and (3) how important the determination of the precursor pool specific activity is (internal isotope dilution). Since all the radioisotoic methods have their own advantages and disadvantages, careful consideration must be paid in choosing the radioisotope according to the conditions of each environment investigated.

INTRODUCTION

In the last two decades, there has been an outburst of information regarding marine microbial ecology, indicating that microorganisms are a significant component in marine food web processes. Pomeroy and Johannes (1966) showed that respiration by microorganisms that passed through a 366 μm pore-sized screen accounted for 94 to 99% of the total respiration. Williams (1970) reported that almost 50% of the glucose and amino acids added to water samples were utilized by microorganisms less than 1.2 μm in size, which supported the report of Hobbie *et al.* (1972) that indicated microorganisms consumed a major fraction of the total primary production in the ocean.

In a similar fashion, bacterial biomass is known to be a significant portion of the total microbial biomass in the sea, especially in the oligotrophic ocean (Cho and Azam, 1990; Dortch and Packard, 1989; Fuhrman *et al.*, 1989), suggesting a system mostly maintained by heterotrophs. Fenchel (1982) demonstrated that the fluctuations of the biomass of nanoflagellates followed those of bacteria, which suggested that heterotrophic nanoflagellates were a major consumer of bacteria, and played an important role in linking bacteria and metazoan zooplankton (Sherr *et al.*, 1986; Sherr and Sherr, 1988).

All of these results suggested that energy flow through microorganisms may be a significant pathway in addition to the conventional grazing food chain, i.e., phytoplankton to zooplankton and subsequently to higher trophic levels (Pomeroy, 1974; Williams, 1981). Consequently, combining the concepts of autotrophic and heterotrophic flow of energy in marine food webs, Azam *et al.* (1983) have suggested the microbial loop to explain nutrient cycling through bacteria, protozoa (microflagellate and ciliates), and microzooplankton, which channels substantial fractions of the primary production. Cole *et al.* (1988), through regression analysis, found that bacterial production was approximately 20% to 30% of the primary production in oceanic environment. The results indicated that bacterial production accounted for approximately 50% of total primary production when a bacterial growth efficiency of 50% was used.

Bacteria also play an important role in biogeochemical cycling of elements below the euphotic zone (mesopelagic zone) by releasing dissolved or-

ganic matters (DOMs) from non-sinking particles (Cho and Azam, 1988b; Karl *et al.*, 1988). The high enzyme activity of bacteria on the aggregates gives rise to the release of DOM which are directly utilized by free-living bacteria, and then returned to the water column, preventing the loss of photosynthetic energy via sedimentation (Hoppe *et al.*, 1993; Smith *et al.*, 1992). It has been proposed, therefore, that bacteria are important not only as remineralizers of organic matter but also as trophic intermediates by repackaging released photosynthetically fixed organic matter which, otherwise, would be lost from the food chain.

With increased interest in studying energy flow in marine microbial food webs, several radioisotopic methods have been independently developed to measure bacterial growth rates and production. Such determinations have been essential in quantifying the role of bacteria or the efficacy of microbial loop in microbial food webs. Although radioisotopic methods provide extremely sensitive results, each method has its advantages and some limitations as well when applied to natural environments. In this paper, we critically reviewed various radioisotopic methods for measuring bacterial production. Special consideration was paid in discussing the advantages and disadvantages of each method in order to provide a useful information and to encourage any venture to improve methods in the future.

³H-Thymidine Method

Thymidine method has been extensively used since Fuhrman and Azam (1980, 1982) first developed the technique, primarily because of its relative ease of application and its high specificity for bacteria. The rate of mole thymidine incorporated into DNA is measured directly in the field, and is converted into bacterial cell production using a conversion factor derived either theoretically or emperically. Eventually carbon production is determined using cellular carbon content (Lee and Fuhrman, 1987).

The basic steps in calculating cell production rate

can be expressed by the following equations:

$$C = A \times CF$$
$$A = (dpm \times 4.5 \times 10^{-13}) / (SA \times t \times v)$$

where,

C = cells produced (cells L^{-1} h^{-1})

A = mole thymidine incorporated into DNA (mole T L^{-1} h^{-1})

CF = conversion factor indicating cells produced per mole thymidine incorporated (cells mole T^{-1})

dpm = activity of ^3H in DNA extracted

SA = specific activity of ^3H-thymidine (Ci $mmole^{-1}$)

4.5×10^{-13} = conversion of dpm to Ci (Ci dpm^{-1})

t = time incubated (h)

v = volume filtered (L)

This relatively simple method depends on numerous assumptions such as; (1) nonspecific labeling of thymidine into TCA insoluble macromolecules, (2) internal isotope dilution by *de novo* synthesis of dTTP which is a direct precursor of DNA, (3) uptake of thymidine by all the actively growing bacteria, and (4) a conversion factor indicating cell production per mole thymidine incorporated. All of these assumptions have been tested and discussed by numerous authors.

Fuhrman and Azam (1980) assumed that the fraction of incorporated label in DNA is 80% of the total cold TCA insoluble macromolecules. Rieman (1984), using DNase and RNase hydrolysis, demonstrated that the major fraction of ^3H activity in the cold TCA insoluble macromolecules was found in DNA. Moriarty (1986) stated that the short time incubation represented mostly DNA labeling. However, Karl (1982), using an acid-base hydrolysis in separating the DNA fraction, demonstrated the nonspecific labeling of thymidine by showing the ratio of ^3H-thymidine incorporation to H_2O : RNA : DNA was 86 : 11 : 3. Hollibaugh (1988) reported that 0-35% of the ^3H-thymidine was incorporated into DNA, 34-67% into RNA and 20-59% into protein. Working with sediment samples, Carman *et al.* (1988) found that only 2% of incorporated ^3H-thymidine activity was re-

covered in the DNA fraction. Brittain and Karl (1990) generalized that the percentage of ^{3}H-thymidine activity in DNA is 25 to 30% in the oligotrophic euphotic zone, 18 to 26% in oligotrophic aphotic zone, 5 to 15% in eutrophic waters, and less than 5% in sediment samples. Robarts et al. (1986) demonstrated that macromolecules labeled with ^{3}H-thymidine included lipids which were removed by chloroform-methanol extraction. Cho and Azam (1988a) found that nitrogen starvation caused certain bacterial assemblage to utilize thymidine as a nitrogen source for protein synthesis, and resulted in nonspecific labeling in the cell by ^{3}H-thymidine. Servais et al. (1987), using DNase treatment, found that the percentage of ^{3}H incorporation into DNA was 21 to 83%, and suggested that organic carbon limitation may induce a utilization of thymidine as a carbon source rather than as a DNA precursor. Even though certain arguments regarding the methods to purify labeled DNA remain to be proven (Robarts et al., 1986; Moriarty, 1986), it is generally accepted that the purification of DNA from TCA insoluble macromolecules is essential because of nonspecific labeling of other macromolecules by ^{3}H-thymidine, resulting in overestimstion of bacterial production (Cho and Azam, 1988b; Karl, 1986; Robarts et al., 1986). The purification of ^{3}H labeled DNA from the cold TCA insoluble materials is time consuming and not applicable to natural samples. Wicks and Robarts (1987) reported a convenient method purifying ^{3}H-labeled DNA using mild alkaline treatment (0.25 N NaOH), which had 100% recovery efficiency up to 24 h when samples were kept at 4°C after alkaline treatment.

When ^{3}H-thymidine is used in production experiments, it will be taken up by the bacteria, converted to dTMP, and then to dTTP prior to incorporation into DNA. dTMP can also be produced from dUMP by de novo synthesis (Moriarty, 1986), which will dilute the specific activity of thymidine to an unknown extent. Consequently, estimates made without a correction for isotope dilution by de novo synthesis result in a significant underestimation of production. It is essential, therefore, to measure the specific activity of dTTP, the immediate precursor of DNA, but the bacterial dTTP can not be

separated from algal dTTP in environmental samples (Fuhrman and Azam, 1980). As reported by Fuhrman and Azam (1982), there are 3 to 6 fold, and 6 to 7 fold isotope dilutions at nearshore and offshore locations, respectively. Moriarty and Pollard (1981, 1982) suggested a method to correct for the isotopic dilution of labeled thymidine by all the sources of thymidine. Using this method, they found that there was a diurnal variation in the magnitude of isotopic dilution (Moriarty and Pollard, 1982). Jeffrey and Paul (1988) reported that there may be other sources of thymine bases incorporated to DNA for which even the isotope dilution approach is not applicable.

The thymidine method also assumes that all the actively growing bacteria will take up the exogenous thymidine. This point has been also challenged by several researchers. Douglas et al. (1987) found that the average relative percentage of glutamate-active and thymidine-active cells to direct counts were 60.8% and 12.7%, respectively, which means some actively growing cells do not take up thymidine. Pedros-Alio and Newell (1988) reported that only 16% of actively growing cells incorporated thymidine in waters around Sapelo Island, Georgia. Johnstone and Jones (1989) demonstrated an inability of exogenous thymidine uptake by some chemolithotrophic bacteria such as nitrifiers and methanotrophs. Meanwhile, Gilmour et al. (1990) found that the inability of exogenous thymidine incorporation in sulfate reducing bacteria may cause considerable underestimation of bacterial production where sulfate reduction is significant. Jeffrey and Paul (1990) found that 4 of the 41 bacteria isolates collected from coastal marine environments did not incorporate thymidine, and suggested that the inability of certain bacteria to use thymidine may result from either the lack of thymidine kinase activity or the absence of a thymidine transport system.

Of all the assumptions in thymidine method, the conversion factor (CF), converting mole thymidine incorporation into DNA to bacterial cells produced, seems to be the most controversial among the authors using this method. The theoretical CF is approximately 2.5×10^{17} to 8×10^{17} cells produced per

mole thymidine incorporated, which can be derived from the amount of DNA per cell and the ratio of thymine in total DNA (Moriarty, 1988). The CF is largely affected by those previously discussed assumptions such as the abilitiy of ^{3}H-thymidine uptake by bacteria, the internal isotope dilution by *de novo* synthesis, and the degree of nonspecific labeling of ^{3}H-thymidine in the TCA insolubles. The possible inability of ^{3}H-thymidine uptake by growing bacteria and its internal isotope dilution lead to higher CFs than the theoretical CF, while the nonspecific labeling results in a lower CF. In order to correct those uncertainties, many authors have empirically derived their own CF. The CF derived by Fuhrman and Azam (1982), for example, were 1.7×10^{18} cells per mole thymidine incorporation for nearshore and 2.4×10^{18} for offshore locations. Rieman *et al.* (1987) reported a fairly consistent CF of 1.1×10^{18} cells per mole thymidine incorporated. Using the freshwater bacteria, Smits and Rieman (1988) found that the CF was varied with bacterial generation time from 2.15×10^{18} to 11.8×10^{18} when the doubling time was longer than 20 h and less than 20 h, respectively. Since the empirical CFs acquired by many researchers show substantial differences (ranging from 3×10^{17} to 6.8×10^{19}, see Table 1 of Moriarty, 1988), it should be measured carefully for each experiment. Detailed procedures for deriving an emperical conversion factor for thymidine and leucine methods are described in Kirchman and Ducklow (1993) and references therein. The thymidine method prevails for measuring bacterial production rate. Without careful considerations related to its assumptions, however, the results may lead either underestimation or overestimation with the temporal and spatial variations of bacterial community.

^{3}H-Adenine Method

Another approach to measure nucleic acid synthesis, and hence productivity, is the use of ^{3}H-adenine incorporation into RNA and DNA (Karl, 1979; Karl *et al.*, 1981a, 1981b; Winn and Karl, 1984). From the incorporation rate of tritiated adenine into nucleic acids and measuring the specific activity of the immediate precursor pool for DNA and RNA, the total DNA and RNA production can be calculated. The overall carbon production can then be determined from the ratio of cellular carbon to DNA. In practice, ATP is determined in an aliquot of the labeled sample, and the mean ATP pool specific activity is then calculated from the ATP concentration in the cell and activity of ^{3}H in the ATP. DNA and RNA are purified, and their respective radioactivities are determined. The basic relationship in calculating the DNA synthesis rates is as follows:

$$C = A \times B$$
$$A = (I / SA)$$

where,
C = carbon production rate (gC L^{-1} h^{-1})
A = DNA synthesis rate (g L^{-1} h^{-1})
B = C : DNA ratio (g g^{-1})
I = incorporation rate of ^{3}H-adenine into DNA (dpm L^{-1} h^{-1})
SA = specific activity of ATP purified (dpm g^{-1})

Karl (1979) indicated that there are three basic assumptions that must be met: (1) all, or most marine microorganisms can utilize exogenous adenine, (2) addition of radioactive adenine does not affect the ATP pool, ATP turnover rate, or the rate of microbial RNA and DNA synthesis, and (3) there is no intracellular compartmentalization in the ATP pool. All of these assumptions are described in detail in Karl and Winn (1984).

Karl, with the first assumption, suggested that the adenine method measures the total microbial production, and not just the bacterial production. Like thymidine, when radioactive adenine is added to a water sample, it is diluted to an unknown extent by existing extracellular pools of adenine and adenine containing compounds, and once incorporated, diluted internally by the *de novo* synthesis of adenine. Therefore, it is essential to determine the specific activity of immediate precursors of DNA and RNA, which is dATP and ATP, respectively. Once adenine is taken up, it is incorporated into a number of adenine containing derivatives, and ultimately reduc-

ed to dATP through two distinct paths: (1) AMP → ADP → dADP → dATP → DNA, or (2) AMP → ATP → dATP → DNA. Because of the rapid turnover of cellular nucleotides, the individual adenine nucleotide pools will equilibrate rapidly. Therefore, the corresponding specific activity of dATP pool can be known by measuring the specific activity of any one of the nucleotides (Karl, 1981). Finally, assuming that all the microorganisms have same metabolic response to adenine, total microbial DNA or RNA synthesis can be derived.

Karl *et al.* (1981a) found that 49 of 50 randomly selected marine bacterial isolates assimilated exogenous ^{3}H-adenine, as did algae that had been incubated with ^{3}H-adenine for 6 hours (Karl and Winn, 1984). It seems, therefore, that the adenine method overcomes both isotope dilution problem and nonspecific labeling by measuring the specific activity of ATP and by purifying the DNA and RNA separately. However, Fuhrman *et al.* (1986a, 1986b), using size fractionation and short time incubations, critically demonstrated that eukaryotic uptake of ^{3}H-adenine was negligible, and most of the ^{3}H-adenine was taken up by the bacterial fraction. Under this condition, the ATP concentration will be extracted from the entire microbial community, including photoautotrophs, but the radioactivity will be confined solely to the bacterial compartment. This fact reduces the specific activity of ATP pool, and results in an overestimation of DNA and RNA synthesis rates. Paul *et al.* (1990) also reported that ^{3}H-adenine and ^{32}P labeled DNA primarily resided in heterotrophic bacteria and not in phytoplankton. Moreover, Fuhrman *et al.* (1986a) indicated that C : DNA ratio of 50 used by Karl was too high when adenine uptake is dominated by bacteria. Despite its careful attention to detail, the adenine method also suffers limitations when measuring bacterial production in natural microbial community.

[3]H-Leucine Method

The use of protein synthesis as an analytical tool to unravel problems of bacterial growth and production was expanded by the development of the ^{3}H-leucine method (Kirchman *et al.* 1985, 1986). Leucine is not transformed to other amino acids, and is associated with only protein (Kirchman *et al.*, 1985). The ^{3}H-leucine incorporation to protein can, therefore, be counted from hot TCA insolubles which are mainly protein. Theoretically, bacterial protein production (BPP) is calculated from the rate of leucine incorporated into protein and the fraction of leucine in protein as follows:

$$BPP = A \times 131.2 \times (100/7.3)$$
$$= A \times 1797$$

where,
A = mole of exogenous leucine incorporated to protein (mole $L^{-1} h^{-1}$)
131.2 = formula weight of leucine (g)
100/7.3 = 100/(mol % of leucine in protein)

The merit of this method is that bacterial carbon production (BCP) can be derived directly from the constant ratio of protein to cell dry weight (0.63) and carbon to cell dry weight (0.54), from which the ratio of carbon to protein (0.86) is calculated (Simon and Azam, 1989).

$$BCP = BPP \times 0.86$$

where,
BCP = bacterial carbon production (g)
0.86 = the ratio of carbon to protein

The theoretical approach, however, is affected by internal isotope dilution which leads to an underestimation of BPP. Simon and Azam (1989) found that the isotope dilution factor was approximately 2, and again an empirical conversion factor (CF) relating the bacterial cells produced per mole leucine incorporation or protein produced per mole leucine incorporated is required. Kirchman *et al.* (1986) empirically derived CFs that ranged from 1 to 84×10^{17} cells per mole of leucine incorporated, and 1.3 to 100 g protein per mole of leucine incorporated, which are generally higher than the theoretical CF (1.1×10^{17} cells per mole of leucine incorporated and 1.5 g protein per mole of leucine), indicating that there is a significant isotope dilution

by *de novo* synthesis of leucine. Because of the considerable *de novo* synthesis of leucine and the variability of the mole percent of leucine in bacterial protein, further verification of the CF is needed (Bjφrnsen and Kuparinen, 1991). It is, therefore, essential to derive an empirical CF in the leucine method as was in the thymidine method.

^{35}S-Sulfate Method

Some controversial points concerning the use of organic substrates to label actively growing populations and the isotope dilution by endogenous pools may be resolved by using an inorganic cellular element as a tracer. Since all the growing cells require inorganic nutrients and cellular inorganic pool turns over rapidly (i.e., they are formed and consumed rapidly during growth), the specific activity of cellular inorganic pool will be quickly e-quilibrated with that of the extracellular source via rapid uptake (Neidhardt *et al.*, 1990).

The incorporation of $^{35}SO_4^{2-}$ into protein has been used by Cuhel *et al.* (1982a, 1982b) to measure protein synthesis rate. The detailed experimental procedures for extracting various sulfur containing molecules and calculating the sulfate uptake or incorporation to the molecules are described in Cuhel (1993). The basic equation for calculating protein synthesis rate is:

$$S = A \times SA$$

where,
S = sulfate incorporation into protein (ngS L^{-1} h^{-1}).
A = ^{35}S-SO$_4^{2-}$ incorporated to protein (dpm).
SA = specific activity of ^{35}S-SO$_4^{2-}$ in water sample assayed (dpm ngS^{-1}).

The success of this method relies on the relative constancy of the ratio of protein sulfur to total protein (1.09±0.14%). Thiosulfate, however, may be assimilated preferentially by some bacteria (Ehrlich, 1990), which causes underestimation of protein synthesis rate. Also, because of the high average sulfate concentration in sea water (28 mM), it is necessary to use extremely high levels of the radioisotope (10-20

Ci mL^{-1}) in order to get a reasonable levels of radioactivity in the cellular protein (Cuhel *et al.*, 1982b).

CONCLUSION

The major upshots in discussing methods for measuring bacterial production are: (1) whether actively growing bacteria will take up the radiolabeled tracer; (2) how the target molecules should be purified; (3) what is the range of external and internal isotope dilution; and (4) how important the determination of specific activity in measuring bacterial production rate using radioactively labeled compounds is (Table 1).

The choice of radioisotope should be decided carefully according to each environmental condition. For example, in the hydrocarbon cold seep where chemoautotrophic bacterial primary production is significant, organic tracer such as adenine was not taken up by the actively growing methylotrophic bacteria (LaRock *et al.*, 1994). Similar environment is the hydrothermal vent where Fe, S or Mn oxidizing bacterial processes are important (Jannasch, 1995; Karl, 1987). It is, therefore, conceivable to develop a method using essential inorganic nutrient, such as phosphate, as a redioisotopic tracer. As discussed in the sulfate method, inorganic tracer resolves the problem of label uptake by actively growing populations and the internal isotope dilution (specific activity of precursor pool). In this

Table 1. Suitability of the four radioisotopic methods for measuring bacterial production.

Controversial Points[1]:	A	B	C	D
^{3}H-thymidine:	++[2]	++	++	-
^{3}H-adenine:	+	-	+	+
^{3}H-leucine:	++	-	++	-
^{35}S-sulfate:	-	-	-	+

[1]controversial points:
A: label uptake by all the growing bacterial populations
B: extraction of target molecules
C: specific activity of precursor pool
D: specificity for heterotrophic bacteria
[2]++ controversy remains, but may be resolved when accurate empirical CFs are derived.
+ controversy remains.
- controversy seems to be resolved.

vein, the labeling of lipid-phosphate using ^{32}P was proposed (Hyun, 1994), and the method has been being developed for an application to aquatic environments by authors.

The question that how many cells are actually growing is another important issue in calculating the growth rate of actively growing bacterial populations from the bacterial production and total cell number (biomass). Since not all the bacteria are actively growing (Morita, 1982), when the production is divided by the bacterial cell number (gC produced L^{-1} hr^{-1}/gC L^{-1}), the use of total (both living and dead) cell number enumerated using epifluorescence microscopy merely yields the turnover rate of total bacterial populations, and does not account for the growth rates of actively growing bacterial populations. Recently, the report of non-nucleotide-containing bacteria (Ghosts) under the epifluorescence microscopy (Zweifel and Hagström, 1995) casts more doubt on the estimation of bacterial biomass and growth in marine environment. Direct estimation of bacterial growth by pulse labeling of target molecules, such as nucleic acids, protein or phospholipid, has been proposed for the determination of growth rates of actively growing populations (LaRock et al., 1988; LaRock and Hyun, 1993). Primary advantages of this time-course experiment is to determine: (1) the growth (or death) rates of microbial community without compensating for a lack of isotopic equilibrium; (2) the relative biomass of metabolically active bacterial populations; and (3) the shape of bacterial growth (exponential or linear).

Tremendous amount of results regarding bacterial production using radioisotopic tracer (mainly using thymidine) have been accumulated for the past two decades (Ducklow and Carlson, 1992). However, in reviewing the assumptions of each technique and the relative applicability to various environments, there seems to be no consensus among the research groups applying each method to various environments (Azam and Fuhrman, 1984; Karl, 1986). Conclusively, it is still necessary to improve and develop a method for measuring bacterial production that overcomes the difficulties of other methods.

This methodological area in order to quantify the role of bacteria in the aquatic environments is still open for the marine microbial ecologists.

LIST OF ABBREVIATIONS

AMP: adenosine 5'-monophosphate
ADP: adenosine 5'-diphosphate
ATP: adenosine 5'-triphosphate
dADP: deoxyadenosine 5'-diphosphate
dATP: deoxyadenosine 5'-triphosphate
DNA: deoxyribonucleic acid
DNase: deoxyribonuclease
dTMP: deoxythymidine 5'-monophosphate
dTTP: deoxythymidine 5'-triphosphate
dUMP: deoxyuridine 5'-monophosphate
RNA: ribonucleic acid
RNase: ribonuclease
TCA: thichloroacetic acid

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