



CMFRI SPECIAL PUBLICATION  
Number 42

**MANUAL OF TECHNIQUES FOR  
ESTIMATING BACTERIAL GROWTH RATES,  
PRODUCTIVITY AND NUMBERS IN  
AQUACULTURE PONDS**

ISSUED ON THE OCCASION OF THE WORKSHOP ON  
BACTERIAL GROWTH RATES AND PRODUCTIVITY  
ORGANISED BY

THE CENTRE OF ADVANCED STUDIES IN MARICULTURE,  
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE, COCHIN  
Indian Council of Agricultural Research  
HELD AT COCHIN FROM 2ND TO 15TH AUGUST 1986

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**MANUAL OF TECHNIQUES FOR  
ESTIMATING BACTERIAL GROWTH RATES,  
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AQUACULTURE PONDS**

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## CONTENTS

PREFACE	...	...	...	...	iii
INTRODUCTION	...	...	...	...	1
MEASUREMENT OF GROWTH RATES OF HETEROTROPHIC BACTERIA IN AQUATIC SYSTEMS	...	...	...	...	5
ISOTOPE DILUTION	...	...	...	...	11
DETAILED PROCEDURES	...	...	...	...	13
DIRECT COUNTING OF BACTERIA BY FLUORESCENCE MICROSCOPY	...	...	...	...	20
CALCULATION OF RESULTS	...	...	...	...	22
EQUIPMENT LIST	...	...	...	...	25
REFERENCES	...	...	...	...	26

## PREFACE

Heterotrophic microorganisms, especially bacteria, play an important part in decomposition processes, nutrient cycling and food chains in aquatic systems. A number of different methods have been proposed for measuring microbial growth rates, but many are not specific for bacteria or do not include the whole population. It is evident that the ideal method should involve minimal handling of the bacterial population and be applied quickly so as not to alter natural or *in situ* growth rates or to be influenced by bacterial grazers. The use of radioactive nucleic acid precursors especially thymidine, to measure the rate of DNA synthesis has many of the prerequisites of the ideal method. As with all other techniques, there are disadvantages as well as advantages in using measurements of nucleic acid synthesis.

Dr. D. J. W. Moriarty, Principal Research Scientist, CSIRO Marine Laboratories, Division of Fisheries Research, Cleveland, Australia, an authority on bacterial growth rates and productivity in aquatic systems, visited the Centre of Advanced Studies in Mariculture at CMFRI, Cochin for a brief period in August 1986 as an expert consultant to afford advice and suggestions on the subject to upgrade research, especially on bacterial growth rates and productivity in aquaculture ponds and formulation of suitable course on the subject. During this period, a six day workshop on 'Bacterial Growth Rates and Productivity' was organised and conducted by Dr. Moriarty, the course programme of which covered in depth the bacterial growth rates and productivity. Selected aspects pertaining to sampling techniques, laboratory processing of water and sediment samples, techniques in fluorescent microscopy were examined in the course of the workshop. Emphasis was laid on planning experiments for study of pond dynamics, isotope dilution and time courses for rates of labelling of macromolecules and liquid scintillation counting technique in samples taken from aquaculture ponds.

This manual was prepared in connection with the workshop conducted by Dr. Moriarty. It considers the measurement of growth rates calculated from the rate of tritiated thymidine incorporation into DNA. In India no work has been carried out on such aspects as measuring bacterial growth rates and

productivity by radio-tracer method in aquaculture systems. The techniques employed require a minimum of prior professional training. Methods requiring the use of very sophisticated equipment have also been avoided. As such, it is intended that the manual will be useful to students, scientists, environmentalists as well as aquaculture entrepreneurs.

I express my sincere thanks to Dr. D. J. W. Moriarty for preparing this manual. I also thank Dr. V. Chandrika, Scientist and counterpart to Dr. Moriarty for the assistance given in the preparation of the manual and for the keen interest shown in the conduct of the workshop.

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**Dr. P. S. B. R. James**  
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## INTRODUCTION

Microbial food webs are an integral part of all aquaculture ponds and have a direct impact on productivity, even where intensive, artificial feeding is practised. There are three major reasons for this. Firstly, in most or perhaps all ponds, respiration is predominantly due to bacteria. Hence the oxygen content of the water is governed by bacterial activity. Secondly, in all extensive, semi-intensive and some intensive aquaculture systems, bacteria also contribute significantly to the food web. They may be eaten directly by the target species (e.g. *Tilapia* or mullet) or by small animals on which the target species feed (e.g. larvae and juveniles of penaeid prawns). Thirdly, through the activity of the heterotrophic decomposers, nitrogen and phosphorus are recycled to stimulate primary production.

If we can quantify the productivity of bacteria, we can then make informed judgements about the above three functional roles of bacteria, and thus improve pond management to optimise productivity.

It is now possible to determine reasonably accurate growth rates for the heterotrophic bacteria, and thus estimate their productivity. The thymidine method for doing this is described in this manual. A brief discussion on microbial ecology in general is included, to show how such studies can be integrated with a broader study of aquaculture pond productivity.

### MODELLING TROPHIC PATHWAYS

An ultimate goal for studies on microbial productivity (which includes microalgal productivity) is to be able to predict the size of the harvest from rates of production. A model is useful in helping design experiments to achieve this goal; a simple example is shown in Fig. 1.



The example is for a marine or brackishwater pond with primary production being the only source of organic matter. It will be necessary to measure inputs and losses to the pond during water exchange each day. It is also necessary to determine how much primary production is eaten by herbivorous animals and thus how much enters the detritus pool. This will not be easy to do, so an alternative procedure is to determine bacterial productivity, and estimate the amount of organic matter needed to sustain it. The rates of other processes such as ammonia release, sulphate reduction, oxygen uptake or carbon dioxide output will provide alternative estimates to check rates of transfer between compartments. From the quantitative model, it will be possible to examine the relative importance of different pathways, and to estimate the rates for pathways where no data are available.

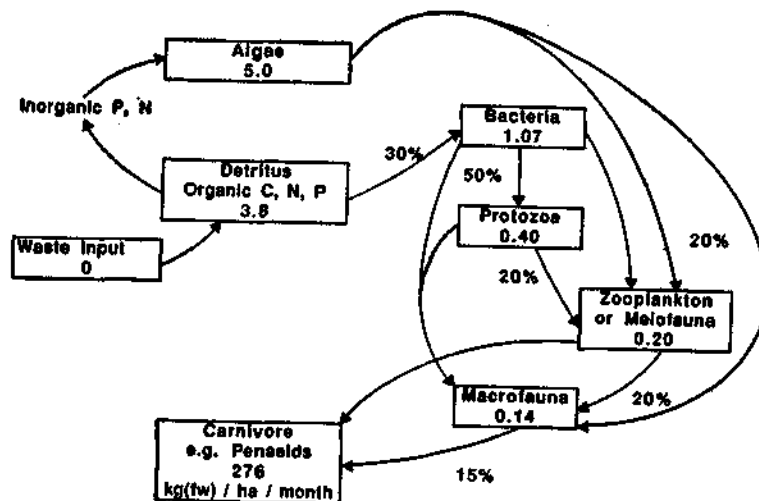


Fig. 1. A simplified conceptual model of detrital and microbial components of the food web in an aquaculture pond.

Bacteria are shown as only 1 compartment (with a central role to play) in Fig. 1, but this is a simplified concept. There are many different functional groups of bacteria, all interacting together. For a better understanding of their role in a pond, the activities and productivities of the major groups need to be studied. These groups are shown in Fig. 2 for a marine pond. Even this is a simplification of the complexities of the bacterial community, but it will be adequate at this stage.

Much organic matter is decomposed by aerobic heterotrophic bacteria in the water column and sediment surface. In the anoxic zone of the sediment, fermentation is the major process by which organic matter is broken down. Productivities of both these major groups of bacteria are determined with the thymidine method. The short chain fatty acids that are the final products of fermentation are respired to  $\text{CO}_2$  by the sulphate-reducing bacteria.

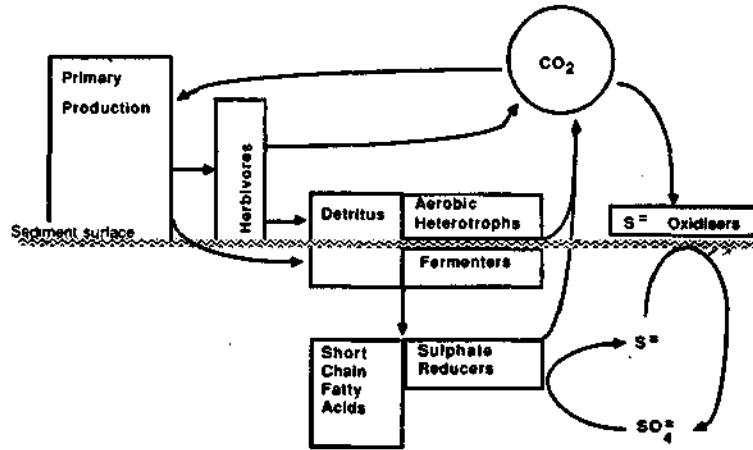


Fig. 2. Principal pathways for carbon flow and associated groups of bacteria in a marine aquaculture pond.

Most of the organic matter entering the sediment will finally be respired by these bacteria. Hydrogen sulphide is the main product of this process, and is important in the transfer of energy from lower depths of sediment to the sediment surface. Chemoautotrophic bacteria oxidise the sulphide, fixing  $\text{CO}_2$  in the process. Thus a potentially rich source of organic matter is available for animals at the sediment surface. Chemoautotrophic production of bacterial biomass could be possibly 10% to 20% of the original organic carbon entering the sediment and is, therefore, a significant process (Howarth, 1984).

#### GROWTH EFFICIENCIES

An area of microbial ecology where more research is needed is the growth efficiency of the various groups in the bacterial community. The growth efficiency is the amount of carbon converted into bacterial biomass divided by the total amount of carbon utilized by the bacteria; it is expressed as a percentage. Energy units

may also be used. Growth efficiencies may vary considerably with the species composition of the bacterial community, the composition of the organic matter and other factors such as oxygen, ammonia and phosphate concentrations.

Although the growth efficiency of any one species of bacterium may be low, the overall efficiency of a mixed community could be high. In sediments many bacteria may secrete large amounts of organic carbon in the form of low molecular weight compounds or as extracellular polymers. Their growth efficiency would, therefore, be low, but, because other species utilise that organic matter, the overall efficiency would be higher than that for individual species.

Quantitative studies are needed on the overall growth efficiency of bacterial communities in the water column and sediments, as well as in oxic and anoxic environments.

## MEASUREMENT OF GROWTH RATES OF HETEROTROPHIC BACTERIA IN AQUATIC SYSTEMS

### INTRODUCTION

At present, the most useful method for estimating growth rates of heterotrophic bacteria is the measurement of rates of DNA synthesis with tritiated thymidine. Most, but not all, heterotrophic bacteria are able to take up thymidine, so the method will underestimate growth rates. For very detailed or critical studies it would be necessary to obtain an estimate of the proportion of bacteria that do take up thymidine in the particular environment being studied.

Thymidine has been selected as a precursor, because it is used almost entirely for DNA synthesis only; it is not incorporated into other macromolecules. Microalgae and fungi cannot incorporate thymidine into DNA, because they lack a necessary enzyme; namely thymidine kinase (Fig. 3). Protozoa cannot take up thymidine at rapid rates at nanomolar concentrations and incorporate it into DNA. Thus labelling of DNA by tritiated thymidine is specific to bacteria.

Once replication of DNA has been initiated, the synthesis usually proceeds at a fixed rate until it is complete. Thus it is possible to carry out short term (usually 10 to 15 minutes) experiments with sediment or water without affecting rates of DNA synthesis. But changing the environment of bacteria will bring about changes in the rates of initiation of DNA synthesis and this will be evidenced by changed rates of thymidine incorporation after a period of time. It is necessary, therefore, to carry out experiments as soon as samples are collected, *i.e.* at the side of the pond.

## BIOCHEMISTRY OF THYMIDINE INCORPORATION INTO DNA

Thymidine is readily incorporated into DNA via a salvage pathway, but in some bacteria the incorporation stops after a short time due to breakdown of thymidine (O' Donovan and Neuhard, 1970). *De novo* synthesis proceeds via dUMP directly to dTMP (Fig. 3). Catabolism of thymidine starts with conversion to thymine and ribose-1-phosphate by the action of an inducible phosphorylase. The best radioactive label is (methyl-<sup>3</sup>H) because

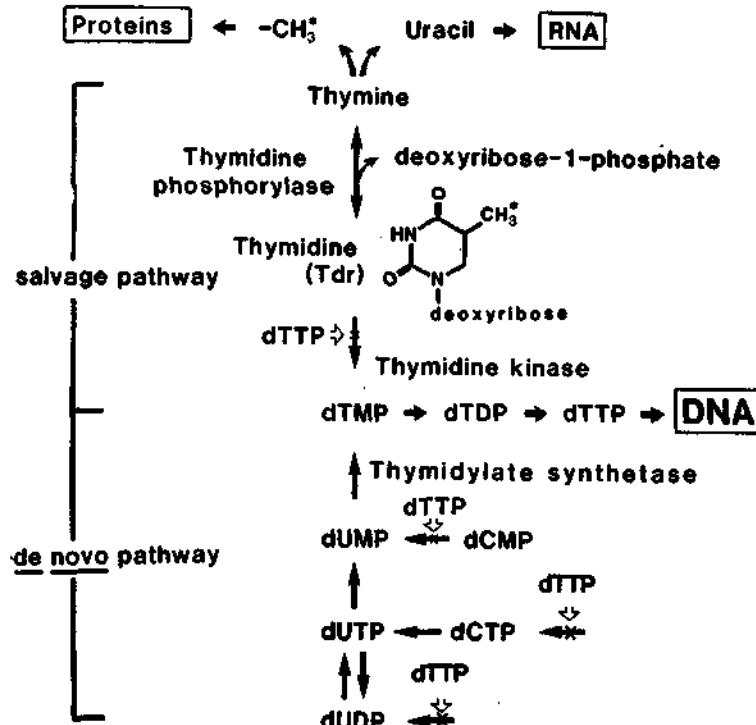


Fig. 3. Some pathways of thymidine metabolism. The asterisk shows the position of tritium labelling in thymidine. Sites of feedback inhibition by dTTP are indicated (adapted from Kornberg, 1980).

subsequent conversion to uracil removes the label. The tritiated methyl group can be transferred to a wide variety of compounds, but DNA is not labelled, as demonstrated in microorganisms that lack thymidine kinase (Fink and Fink, 1962). (2-<sup>14</sup>C) Thymidine, on the other hand, does label DNA after catabolism, because the label is retained in the resulting uracil (Grivell and Jackson, 1968).

The absence of tritium incorporation into DNA in some eukaryotic microorganisms led Grivell and Jackson (1968) to show that these organisms lacked thymidine kinase. As Kornberg (1980) pointed out, thymidine meets reasonably well the criteria for pulse labelling. These are that the precursor should be rapidly and efficiently taken up by bacteria, be stable during uptake, be converted rapidly into the nucleotides and specifically label DNA with little dilution by intracellular pools. He also outlined pitfalls in its use, of which some are particularly relevant to environmental studies and are discussed in detail below.

Thymidine is converted to dTMP by thymidine kinase (Fig. 3). This enzyme must be present for labelling of DNA to occur to a significant extent. Thymidine kinase was thought to occur in most organisms (Kornberg, 1980), but some groups of microorganisms are now known not to contain it. These include fungi (*Neurospora crassa*, *Aspergillus nidulans* and *Saccharomyces cerevisiae*) and *Euglena gracilis* (Grivell and Jackson, 1968) and a number of cyanobacteria (blue green algae) (Glaser *et al.*, 1973). It is also absent from the nuclei of various eukaryotic algae, but may be present in chloroplasts although the amount of label incorporated from tritiated thymidine into chloroplast DNA was slight and required hours or days of incubation to be shown by autoradiography (Stocking and Gifford, 1959; Sagan, 1965; Steffensen and Sheridan, 1965; Swinton and Hanawalt, 1972).

We have been unable to obtain significant incorporation of (methyl-<sup>3</sup>H) Tdr into DNA of four species of marine microalgae (*Thalassiostra*, *Isochrysis*, *Platymonas* and *Synechococcus*) which suggests that they lack thymidine kinase (Pollard and Moriarty, 1984). As there are no reports of the presence of thymidine kinase in the nuclei of small eukaryotic algae, fungi or cyanobacteria, it seems reasonable to generalize and conclude that this salvage pathway is lacking in all members of these groups of microorganisms. The lack of thymidine kinase in blue-green algae and many eukaryotic microorganisms, is a considerable advantage for studies on heterotrophic bacterial production in the marine environment. Protozoa probably do contain the enzyme (Plant and Sagan, 1958; Stone and Prescott, 1964), but as explained below their contribution to labelled DNA in short term experiments is probably small. Thus the use of thymidine provides specific information about the growth of heterotrophic bacteria that has not been available previously.

Most bacteria that lack thymidine kinase are mutants specially selected for biochemical studies. Two wild type strains of *Pseudomonas* have been reported not to incorporate thymidine into DNA (Ramsay, 1974). The technique used to demonstrate this

was autoradiography, which is insensitive compared to liquid scintillation counting of purified DNA. Ramsay's results could mean that these bacteria lacked thymidine kinase, or that they had a deficient membrane transport mechanism. A few species of *Pseudomonas* have been found not to utilize thymidine, probably due to a deficient cell membrane transport system (Pollard and Moriarty, 1984). Fuhrman and Azam (1980) have found good agreement between bacterial growth rates in seawater measured by the incorporation of thymidine and by counting the increase in cell number. The results of an autoradiographic study on bacteria in seawater support the view that most aerobic marine heterotrophic bacteria can utilize thymidine (Fuhrman and Azam, 1982). Anaerobic bacteria with strict and limited nutrient requirements may not be able to utilize thymidine, particularly if they can transport only a limited range of metabolites. *Desulfovibrio*, for example, does not appear to be able to utilize exogenous thymidine (G.W. Skring, per comm.)

The possibility that some bacteria in seawater may be unable to incorporate thymidine into DNA means that estimates of bacterial productivity may be too low, but this disadvantage is considerably outweighed by the advantages of using thymidine to measure DNA synthesis over other techniques for estimating growth rates of bacteria in natural populations.

#### KINETICS

Bacteria, with their active transport systems, take up organic molecules much more rapidly than do algae or protozoa and can utilize nanomolar concentrations of organic molecules in their environment more effectively than algae or protozoa (Wright and Hobbie, 1966; Fuhrman and Azam, 1980). Thus in a short time period (e.g. 10 min. at 25°C, 20 min. at 15°C) tritiated thymidine should be taken up preferentially by bacteria in a mixed community.

Uptake of labelled thymidine by organisms should not be confused with incorporation into DNA, although in bacteria the latter may be the main fate of labelled thymidine assimilated intact (Hollibaugh *et al.*, 1980; Fuhrman and Azam, 1980). As mentioned above, thymidine is readily incorporated into DNA in bacteria, but thymidine phosphorylase soon converts thymidine to thymine and deoxyribose-1-phosphate (Fig. 3). Labelled thymidine concentration within cells may be rapidly depleted, so it is important to measure the rate of label incorporation into DNA and not simply label uptake. In our work with sediments we have found that label incorporation into DNA proceeds linearly for 5-8 min. at high temperatures (27°-31°C) and 20-30 min. at lower temperatures (15°-18°C) in sediments, and over 1 hr in seawater (Fig. 4).

We had interpreted the change from the linear rate to be due to adsorption of thymidine by clay in sediments, but although this undoubtedly is a factor, degradation by thymidine phosphorylase rates may also have occurred. Experimental studies to determine growth rates must be carried out in the initial linear period of incorporation of label into DNA. Uptake of thymidine by cells and incorporation into TCA-insoluble fractions are different processes, probably with different kinetics which may be uninterpretable in a mixed population.

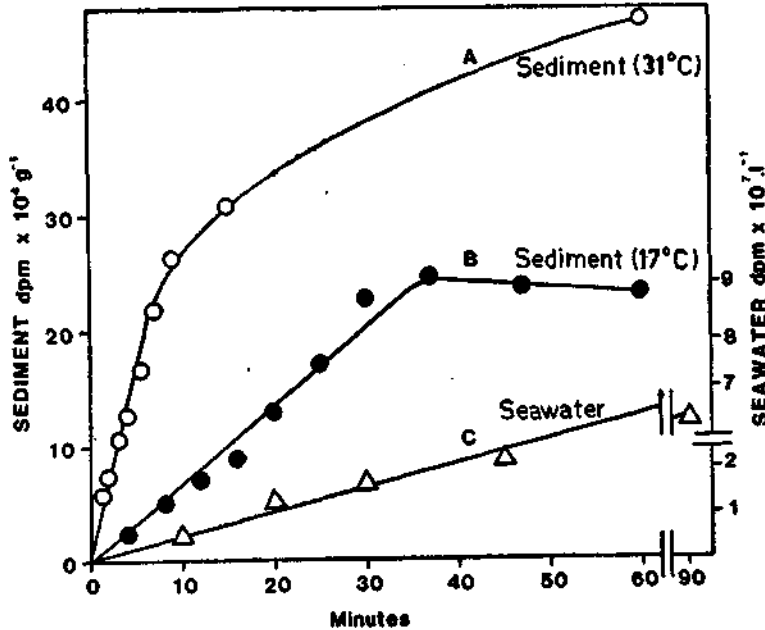


Fig. 4. Time course of incorporation of tritiated thymidine into DNA (from Moriarty and Pollard, 1981, 1982).

The kinetic studies (Fig. 4) show that thymidine is very rapidly taken up and is incorporated into DNA in less than 1 minute. We presumed that this was indicative of bacterial activity (Moriarty and Pollard, 1981), because protozoa, the other main group of microorganisms with thymidine kinase, are generally particulate feeders and probably would not have membrane transport mechanisms that are as efficient as those of bacteria.



A time course should be the first experiment carried out, as it is a good check on technique. If values are widely scattered in the first 15 minutes or so, problems in sampling, pipetting or washing may be the cause. Variable values can result from the final step with heating in 3% TCA if much water is lost by evaporation, or particles of filter or sediment are transferred to the scintillation vial. There are other reasons for carrying out a kinetic study besides the practical ones listed above. It is necessary to select an incubation period long enough to give an incorporation of tritium into DNA that is well above background adsorption; preferably at least 10 fold higher. Errors due to variable backgrounds are thus minimised.

The incubation period should be short enough to ensure that DNA is the main macromolecule that is labelled. Degradation of tritiated thymidine in the cell will eventually lead to labelling of all cellular components, but this occurs more slowly than DNA synthesis in growing bacteria. Where bacteria are not growing, degradation could be very marked. Thus a time course experiment to determine the rate of labelling of DNA itself is needed. For tropical water bodies, 15 minutes is generally a suitable time for incubation of water samples and 10 minutes for sediment.

## ISOTOPE DILUTION

The specific radioactivity of exogenous thymidine is diluted during incorporation into DNA, primarily by *de novo* synthesis of dTMP (Fig. 3). A technique for measuring the dilution of label from an exogenous precursor during synthesis of a macromolecule is to add different quantities of unlabelled precursor as well and to measure the effect on the amount of label actually incorporated into the macromolecule (Forsdyke, 1968). This technique worked well with bacterial populations in sediments (Moriarty and Pollard, 1981). A plot of the reciprocal of isotope incorporated into DNA against total amount of thymidine added is extrapolated to give the amount of dilution of isotope in DNA itself (Fig. 5). The negative intercept on the abscissa is used to determine the specific activity of tritiated thymidine actually incorporated into DNA.

This technique measures the dilution of labelled thymidine in dTTP (the final precursor for DNA synthesis) by all other precursors of dTTP, including *de novo* synthesis. Provided DNA is purified before counting radioactivity that is incorporated, it doesn't matter if only a small proportion of the thymidine that is taken up by cells is used for DNA synthesis, because only the dilution of isotope in thymidine pools that are actually being used for DNA synthesis in growing cells is measured. Thus this method is not subject to errors inherent in trying to extract and quantify nucleotides from cells.

These problems with measuring the amount of isotope dilution by *de novo* synthesis of dTMP can be avoided by using a high enough concentration of thymidine to supply all the thymine required for DNA synthesis, in which case *de novo* synthesis is switched off by feedback inhibition (Moriarty, 1986).

Isotope dilution should be minimised or eliminated. This can usually be done by having a final thymidine concentration of

20 nM in water or by using 2 nmol/ml of sediment (dry weight 0.5 g). For aquaculture ponds, 0.5 nmol thymidine in 0.1 ml of a diluted slurry is satisfactory. The slurry is made by taking the upper 0.5 cm from a 25 mm diameter core and diluting it with 10 ml of filtered pond water.

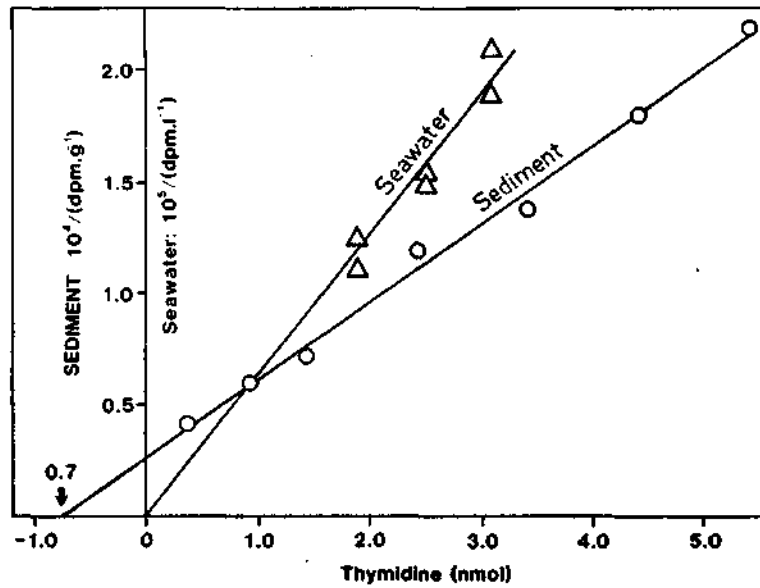


Fig. 5. Isotope dilution plots for incorporation of tritiated thymidine into DNA (from Moriarty and Pollard, 1981).

If the rate-limiting step for incorporation of tritiated thymidine into DNA is the activity of DNA polymerase, then isotope dilution can be prevented. If, however, the rate limiting step is prior to TMP synthesis, e.g. uptake into the cell, isotope dilution will occur, but will not be detectable. At present, it seems that there are not many heterotrophic bacteria in which uptake is limiting. It would be worthwhile carrying out experiments similar to those of Riemann *et al.* (1986) to check the conversion factor and thus indirectly to establish whether isotope dilution is likely to be a problem.

# 4

## DETAILED PROCEDURES

### *a. Storage of tritiated thymidine*

For 3 to 4 months: store as aqueous solution (sterile) with 2% ethanol. (Decomposition rate : 4% per month). For longer times: store as 70% ethanol solution. In this case, remove ethanol before use by evaporation and reconstitute thymidine in distilled water (sterile). Ethanol helps prevent the decomposition of thymidine that is caused by free radicals generated during radioactive decay.

### *b. Calculating amount of tritiated thymidine*

For water : use 20 nM = 0.1 nmol in 5 ml pond water

For sediment : use 0.5 nmol in 0.1 ml of diluted slurry

Thymidine will be supplied at given specific activity and concentration:

e.g. 18.4 Ci/mmol; 1 mCi/ml

thus 18.4  $\mu$ Ci = 1 nmol; 1  $\mu$ Ci/ $\mu$ l

therefore 0.1 nmol = 1.84  $\mu$ l

Shortly before experiments are undertaken, withdraw total amount needed and dilute it 20 times with filtered distilled water.

### *c. Labelling*

Draw up a working sheet, showing tube numbers, volumes of solutions to use, incubation time periods, etc. Plan at least 2 or 3 blanks for each type of sample. Collect extra samples for direct counting with acridine orange (AODC no).

Example

Tube No.	Sample	Time	Comments	AODC No.
1	Surface water	Blank	e. g. time of	1
2	"	Blank	day temperature	
3	"	15 min.		
4	"	15 min.		
5	"	15 min.		
6	Bottom water	Blank		
7	"	"		
8	"	15 min.		
9	"	15 min.		
10	"	15 min.		

d. Field kit

All experiments must be carried out at the pond site. Prepare a kit containing all necessary sampling equipment for water and sediment; pipettes, water bath, test-tube racks, ice container, solutions and dispensers for stopping bacterial activity or preservation.

A boat may be necessary for collecting sediment and water. Syringes and a syringe filter assembly are useful for obtaining filtered pond water on the site.

e. Procedure

Carry out experiments and store samples on ice or in a refrigerator until filtering is carried out. Detailed procedures are as follows:

**Part I : Detailed procedures for standard measurement of rates of DNA synthesis in water column with TCA**

1. Collect water samples; dispense 5 ml into bottles. Bottles should be teflon, polycarbonate or polypropylene.
2. Add 5  $\mu$ Ci (5-methyl-3H) thymidine (about 25 Ci/mmol) at known time intervals (see note i below). Store tritiated thymidine in 2% (v/w) ethanol; the ethanol does not need to be removed prior to assay.
3. Incubate at *in situ* temperature for 5-30 min. (see note ii below).

- 4a. Stop by adding 0.2 ml 37% formaldehyde containing 0.1 g thymidine per 100 ml and buffered about pH 8 (sodium tetraborate). Store cold until water can be filtered.
- 4b. Filter through polycarbonate (or cellulose nitrate) membrane filters (0.3–0.45  $\mu$ m pore size)
5. As soon as filtration is complete, add 5 ml of 80% ethanol and draw it through. Repeat once, then wash with 1–2 ml of ice-cold 3% trichloroacetic acid (TCA) to the filter. Repeat cold TCA wash 4 times.
6. Remove filter, place in centrifuge tube and store if necessary. If backgrounds are high, try cutting out ring around edge that was under filter holder.
7. Add 2 ml 3% TCA, cap the tubes, heat at 90°C for 30 min. and then cool and centrifuge if particulate matter is noticeable.
8. Take 0.5 ml, place it in a mini-vial and add 4 ml of water-miscible scintillation fluid and count.

- Notes:*
- i. This amount of isotope should be adequate to overcome isotope dilution. For aquaculture ponds, 20 nM thymidine is usually adequate. If detritus is present, upto 50 nM may be needed. A preliminary isotope dilution experiment should be carried out for any new environment that is investigated.
  - ii. The incubation time should be determined by a preliminary time-course experiment. It should not exceed the time period during which the rate of DNA synthesis is linear. For tropical waters, 10 min is usually enough; colder temperate waters may require 20 – 30 min.
  - iii. Blanks should be prepared by adding formalin before tritiated thymidine.

**Part II : Detailed procedures for measurement of isotope dilution during DNA synthesis in sediments**

1. Shortly before use, set up isotope dilution series in polypropylene centrifuge tubes by adding the following amounts of a 100  $\mu$ M

thymidine solution: 0, 5, 10, 15, 20  $\mu$ l. Add 25  $\mu$ Ci of (5-methyl-<sup>3</sup>H) thymidine (approximately 25 Ci mmol<sup>-1</sup>). Keep cold, but not frozen (see note i below).

2. Collect sediment, mix and dispense even amounts (about 10–50 mg dry weight) into tubes at known time intervals. Mix, incubate in water bath at *in situ* temperature for 5–15 min. (The time is temperature dependent; see note ii below).
3. Stop reaction with 10 ml 80% ethanol (ethanol 80 ml, water 20 ml, Na Cl 2 g, thymidine 0.01 g). After storage, remove the ethanol by centrifuging.
4. Add 2–5 ml 80% ethanol, mix and filter (see Part 1, 4b); wash out tube with more ethanol.
5. Wash filter with 2 ml 3% ice-cold TCA; repeat 4 times.
6. Transfer filter to centrifuge tube; add 2 ml 3% TCA; cap tubes; heat at 90°C for 30 min. Cool and centrifuge.
7. Transfer 0.5 ml to mini-scintillation vials (or 1 to 2 ml to large vials). Add water-miscible scintillant fluid and count radioactivity. Be careful not to transfer any sediment.
8. Blanks are prepared by mixing thymidine and ethanol before adding sediment.

### **Part III : Detailed procedures for standard measurement of DNA synthesis in sediment**

1. Use stock (5-methyl-<sup>3</sup>H) thymidine with a specific activity of about 25  $\mu$ Ci mmol<sup>-1</sup>, stored in 2% (v/v) ethanol. Add 25  $\mu$ Ci of tritiated thymidine diluted to about 100  $\mu$ l. (see note i).
2. Collect sediment in small cores (e.g. 2–3 cm plastic tubing). Cores should not be stored; the assay should be carried out as soon as possible after collection.
- 3a. Make slurry of the top 0–5 mm with 10 ml of pond sediment (10–50 mg). Take 100  $\mu$ l (0.1 ml) for each assay, including 2 blanks (add ethanol first).
- 3b. Repeat for deeper sediment depths if desired.

- 3c. For sandy sediments, do not make a dilute slurry, it is too hard to dispense. Use 1 ml syringes as corers and take 2-3 mm depth intervals.

(Alternatively, collect cores in small syringes and expel individually into centrifuge tubes set up as in No. 1 and proceed).

4. Incubate at *in situ* temperature for predetermined time (see note ii below). Stop reaction by adding 10 ml ethanol : water : Na Cl: thymidine (80 ml: 20 ml: 2 g: 0.1 g).
5. Proceed as in 3, Part II above.
6. Blanks should be prepared by mixing ethanol with the thymidine before adding sediment.

*Notes :* i. This is a suggested range of thymidine concentration. To prevent isotope dilution, about 1 nmol thymidine is needed per 0.2 g dry weight of sediment. I have found this to be satisfactory for a variety of sediments.

ii. For sediments with a surface temperature of about 27-30°C, 5 min. may be enough. For temperatures around 15-25°C, 15-20 min. should be adequate. Longer times may be needed for colder temperatures. A time course should be carried out first.

iii. The recovery of DNA may be measured by adding a known amount of bacteria labelled with <sup>3</sup>H-Tdr in culture during log phase. Centrifuge culture (6000 x g, 20 min.); resuspend in seawater and formaldehyde (1% v/v and sodium tetraborate pH 8-9 (25 mg/100 ml). Centrifuge again. Resuspend in same solution, use for recovery experiments - check total dpm by filtering and washing with cold TCA as for seawater (p. 1).

iv. For short term incubations, there is no significant difference in results between anaerobic sediments that are mixed briefly as described here and anaerobic sediments that are retained in cores and injected.

v. Check for label in protein in some samples by adding perchloric acid (12 M) to final concentration of 0.5 M; heat at 100°C for 30 min; centrifuge. Count supernatant (DNA only; protein precipitates).



## ADDENDUM

With some sandy sediments, recovery of DNA is poor with the method in Part II, procedure steps 4 and 5 on page 16.

If the recovery of DNA from cultured cells (note iii, page 17) is low, use one of the following procedures (Pollard, 1987).

### Part IV: Alternative procedures for extracting DNA from sediment

A is the best procedure, but is time-consuming. B is simpler, but may give high background counts for some sediments.

1 and 2. As in Part II or Part III

- A 3. Stop reaction with 10 ml 80% ethanol mixture (as in Part II). After storage, remove the ethanol by centrifuging. Then add 2 ml 0.6M NaOH and 10 mM thymidine. Heat at 100-105°C for 1 hr in an autoclave or 100°C for 2 hr in an oven.
- A 4. Centrifuge (3,000-5,000 x g); take half the supernatant (1 ml if no evaporation losses in the oven).
- A 5. Transfer supernatant to dialysis tubing (molecular weight cut-off : 6,000-8000). A series of samples can be kept in series in one long piece of tubing (Pollard, 1987).
- A 6. Dialyze against running water overnight. Transfer to centrifuge tubes, centrifuge if any precipitate is present. Make up to known volume.
- A 7. Transfer 0.5 ml to mini-scintillation vials (or 1 to 2 ml to large vials). Add water-miscible scintillant fluid and count radioactivity.

*Note:* For short term incubations of sediment where bacteria are growing rapidly, no protein is labelled. In oligotrophic sediments, where bacteria are starved, protein may be labelled. The procedure below, steps 4, 5, 6, should be followed to check if protein, which does not hydrolyze readily in 5% TCA, is present. If it is present, steps B 4, 5, 6 must be used for all samples.

- B 1 and 2. As above.
- B 3. Stop reaction with the 80% ethanol mixture. Centrifuge. Repeat twice to wash sediment. Then add unlabelled DNA (100  $\mu$ l, 5mg ml<sup>-1</sup>, 2 ml 0.6M NaOH. Heat and centrifuge as in 3 above.
- B 4. Take half supernatant, chill on ice to 0°C. Add concentrated HCl (approx. 3 drops) until pH of about 1 is reached. Stand on ice for about 30 minutes while DNA precipitates.
- B 5. Centrifuge at 6000 xg or filter (Whatman GF/F). Wash 4 times with ice-cold 80% ethanol.
- B 6. Transfer pellet or filter to small tube, add 1 ml 5% TCA, heat at 100°C for 30 min. Cool.
7. As in 7, above.

# 5

## DIRECT COUNTING OF BACTERIA BY FLUORESCENCE MICROSCOPY

### *Sample preparation*

1. Collect water (5 ml) or sediment (0.5 ml).
2. Fix with formaldehyde (0.2% final concentration: add 300  $\mu$ l formalin to 5 ml water).
3. Store in the refrigerator.
- 4a. Blend or homogenise water for about 0.5–1 min.
- 4b. Add 5 ml water to sediment, blend for 5 min. (or use ultrasonic probe for 0.5 min.)

### *Counting*

1. Prepare acridine orange (AO) solution: 10 mg/10 ml filtered water, add formaldehyde to final concentration of 2%. (The AO must be high quality, specially prepared for fluorescence microscopy).
2. Prepare irgalan black solution: about 100 mg in 100–200 ml 2% acetic acid, (filtered water).
3. Filter wash water (use 0.2–0.3  $\mu$ m filters for all filtration).
4. Stain filters (Polycarbonate) in irgalan black.
5. Wash filter, place in filter funnel.

6. Add 1 ml filtered water and 0.5–3 ml pond water or 0.05–0.1 ml sediment. (The amount will need to be selected by trial – about 20 to 40 bacteria per grid field).
7. Add acridine orange: 10–20  $\mu$ l. Stain for 1–10 min.
8. Turn on vacuum pump; draw water through and leave pump on while removing filter.
9. Place filter on a small drop of water on microscope slide. Add a drop of non-flourescent oil, coverslip then more oil.
10. Count about 10 grid squares (about 30 bacteria each, *i. e.* total of 10 x 30 = 300 bacteria) per filter; select grids randomly.
11. For one pond in each major experiment and each season, measure sizes of bacteria from random samples (about 200).
12. The intensity and colour of the fluorescence is dependant on the biochemical composition of the bacteria and the incubation time with the stain.
13. Calculation: calculate average ( $\bar{X}$ ) standard error for No./grid.

$$\text{No./ml} = \frac{\bar{X} \times \text{area of filter}}{\text{area of grid}} \times \frac{1}{\text{vol. filtered (ml)}}$$

$$\frac{\text{area of filter}}{\text{area of grid}} = \text{constant factor}$$

# 6

## CALCULATION OF RESULTS

i. Convert cpm to dpm.

Automatic scintillation counters can usually be programmed to convert counts per minute to disintegrations per minute. A set of quench standards is necessary.

ii. Subtract values for blanks.

iii. Calculate number of cells dividing (N) using formula given below:

I. *To calculate rate of bacterial division from rate of DNA synthesis or rate of tritiated thymidine incorporation.*

1. Proportion of thymine in 4 bases (A, G, C, T) average:  $p = 0.25$ .

2. Amount of DNA per cell (range 1.7 to 5 fg; average:  $w = 2 - 2.5 \text{ fg } (10^{-15}\text{g})$ ).

3. Amount of Tdr incorporated (mol Tdr x 318) (318 = molecular weight)

$$N = \frac{\text{mol Tdr} \times 318 \times 1/p}{w}$$

N = number of bacteria dividing

4. Thus  $N = \text{mol Tdr incorporated} \times 6 \times 10^{17}$ .

$6 \times 10^{17}$  is the theoretical factor; many measured values are higher, so we use  $1 \times 10^{18}$  which best fits experimental data. It is possible that mol Tdr incorporated is underestimated, due to isotope dilution and poor uptake of Tdr by some bacteria.

## II. To calculate mol T incorporated into DNA

### 1. Isotope Dilution

Need to know the specific activity (S.A.)  $^3\text{H}$  Tdr of the radioactive isotope (tritiated thymidine) after it is converted into DNA. Thus we need to determine the S.A. of TTP.

If we give the bacteria enough thymidine to stop them synthesising their own TTP, then the S.A. of TTP will be the same as that of the thymidine we add. That is, there will be no isotope dilution.

### 2. Calculate amount of radioactivity (disintegrations per minute = dpm) from counts pm (cpm).

$$3. \text{ mol T incorporated (T)} = \frac{\text{dpm}}{\text{S.A.}} = \frac{\text{dpm} \times \text{mol}}{\text{ci} \times 2.22 \times 10^{12}}$$

picomol =  $10^{-12}$  mol

Usual units: p mol Tdr incorporated

$$= \text{dpm} \times \frac{\text{mmol} \times 10^{-3}}{\text{Ci} \times 2.22} \text{ or } \times \frac{1}{1000}$$

S.A. is usually quoted as Ci/mmol.

1 Ci =  $2.22 \times 10^{12}$  disintegrations per minute (dpm).

### III. Complete calculation

No. of bacteria dividing per hour

$$= N_h - 1 = \frac{\text{dpm} \times 1 \times \frac{1.62}{t} \times 10^4}{\text{S.A.}}$$

where t = incubation time in minutes

Derivation:

$N = 6 \times 10^{17}$  x mol Tdr incorporated into DNA

$$= 6 \times 10^{17} \times \frac{\text{dpm} \times 1 \times 10^{-3}}{\text{Ci} \times 2.22 \times 10^{12}} \times \frac{60}{t}$$

where t = time (min.);  $1 \times 10^{-12} \frac{\text{mol}}{\text{Ci} \times 2.22 \times 10^{12}} = \frac{1}{\text{S.A.}}$

N.B. A liquid scintillation counter with a set of quench standards for tritium is needed to calculate dpm from cpm.

IV. *Specific growth rate* ( $\mu$ ) is obtained by dividing the values for number of cells produced in a given time (N) by the total number present ( $N_t$ ).

$$\mu = \frac{N}{N_t}$$

Generation time, or doubling time, (g) is the reciprocal of  $\mu$  times the natural log of 2:

$$g = \frac{\ln 2}{\mu}$$

V. *To obtain productivity in terms of carbon* : the average cell volume (V) should be calculated from size measurements under the microscope. Assume specific gravity is 1.1 and carbon content is 22% of wet weight (Bratbak and Dundas, 1984).

Thus carbon content (C) = V x 1.1 x 0.22

e.g. if V = 0.5  $\mu\text{m}^3$

$$C = 0.12 \times 10^{-13} \text{ g}$$

$$= 1.2 \times 10^{-13} \text{ gC per cell}$$

$N \times 1.2 \times 10^{-13} = \text{gC produced per hour (unit time)}$ .

## EQUIPMENT LIST

**Isotopes:** (methyl  $^3\text{H}$ ) thymidine (e.g. Amersham Cat. No. TRK 418 or CEA (France) Cat. No. TMM 199C).

**Chemicals:** Ethanol, NaCl, thymidine, Trichloroacetic acid, Liquid scintillation counting fluid for aqueous samples and formaldehyde.

**Other Items:** 2-3 ml disposable plastic (polypropylene) syringes (for coring sediment), 10 ml disposable plastic tubes (e.g. blood sample vials from hospitals), General lab glassware, plasticware, pH meter, oven ( $100^\circ\text{C}$ ), autoclave or large pressure cooker, refrigerator, crushed ice, centrifuge (3000 - 5000 g), centrifuge tubes to fit, 10 ml and 20 ml capacity scintillation counting vials (5 ml capacity preferably), vacuum pump, filtration apparatus 47 mm, 25 mm diameter (separate for  $^3\text{H}$ ,  $^{14}\text{C}$ ), filters 47 mm, 25 mm (membrane,  $0.45\ \mu\text{m}$  pore size), racks to hold centrifuge tubes, etc. polypropylene bottles or tubes, 20 to 50 ml capacity and liquid scintillation counter.

### Enumeration of Bacteria:

10-20 ml plastic vials or tubes, ultrasonic disruptor, acridine orange (BDH - highest quality available) irgalan black (black cloth, dye) (Ciba - Geigy), acetic acid, formalin, measuring cylinders, pipettes - 1 and 5 ml, automatic pipettes, filter apparatus (25 mm) single unit, Buchner flask, vacuum pump, filters: polycarbonate (Nuclepore)  $0.2\ \mu\text{m}$  poresize x 25 mm diameter, microscope slides, coverslips, forceps, non-fluorescent immersion oil, epifluorescence microscope (FITC filter set) (narrow band blue excitation, green emission) grid for eyepiece, eyepiece micrometer and calibration stage micrometer.



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