Limitations of the [^{3}H]thymidine method for estimating bacterial productivity due to thymidine metabolism

James T. Hollibaugh

Tiburon Center for Environmental Studies, San Francisco State University, Tiburon, California 94920, USA

ABSTRACT: Thymidine (TdR) was extensively metabolized by bacterioplankton from South San Francisco Bay and Elkhorn Slough, California, USA. Total ^{3}H incorporation rate was linear for 2 to 4 h, but macromolecular ^{3}H distributions differed depending on whether TdR was supplied as methyl- or 6-[^{3}H]-TdR. Methyl-[^{3}H] was incorporated at rates greater than or equal to 6-[^{3}H]. Methyl-[^{3}H] incorporation by South San Francisco Bay bacterioplankton was: 0 to 35 % into DNA, 34 to 67 % into RNA and 20 to 59 % into protein. In the same samples, 6-[^{3}H] incorporation was: 33 to 58 % into DNA, 17 to 52 % into RNA and 13 to 34 % into protein. Tritiated water was produced from both substrates. At high TdR concentrations, methyl-[^{3}H] incorporation into DNA was blocked while incorporation into protein continued. Mitomycin C blocked the incorporation of both labels into DNA and reduced uptake and incorporation into other fractions. Similar experiments indicated that TdR was not extensively metabolized by Monterey Bay, California, bacterioplankton. There, ^{3}H from both substrates was distributed similarly among macromolecular fractions and most was found in DNA. The incorporation rate of ^{3}H into DNA plateaued at a TdR concentration of 10 nM and did not decrease at higher concentrations.

INTRODUCTION

Among the numerous methods recently proposed to estimate in situ growth rates of mixed microbial assemblages, use of tritiated thymidine (thymine deoxyribonucleoside, TdR) to estimate the rate of DNA synthesis in a sample has achieved wide popularity (Tobin & Anthony 1978, Fuhrman & Azam 1980, 1982, Moriarty & Pollard 1981, Moriarty 1984, 1986). Although the concept and methodology are simple, conversion of incorporation rates to population growth rates relies on the validity of a number of assumptions about the uptake and utilization of TdR by mixed microbial assemblages.

One key assumption is that exogenously supplied, radioactively labeled TdR is incorporated into DNA without metabolic alteration other than phosphorylation (Fuhrman & Azam 1980, 1982). Metabolism of radiolabeled TdR could pose uncertainty about DNA labeling: tritium could be incorporated into DNA through other precursors or the specific activity of the thymidine triphosphate (dTTP) incorporated into DNA might differ from that of the added TdR. TdR metabol-

ism could also result in tritium incorporation in macromolecules indistinguishable from DNA by the simple extraction and hydrolysis procedures typically used to separate incorporated from unincorporated TdR.

In most environments, methyl-[^{3}H]-thymidine appears to be incorporated specifically into DNA by microbial assemblages (Fuhrman & Azam 1980, 1982, Hollibaugh et al. 1980, Moriarty & Pollard 1981, 1982, Kirchman et al. 1982, Riemann et al. 1982, Bell et al. 1983, Ducklow & Kirchman 1983, Bell 1984, 1986, Pollard & Moriarty 1984, Riemann 1984, Ducklow & Hill 1985, Ducklow et al. 1985, Lovell & Konopka 1985, Scavia et al. 1986, and others). However, a number of authors have reported that methyl-[^{3}H]-thymidine labels other macromolecules as well (Karl 1982, Reimann et al. 1982, Hanson & Lowery 1983, Ducklow et al. 1985, Lovell & Konopka 1985, Fallon & Newell 1986, McDonough et al. 1986, Robarts et al. 1986). These reports cover a wide range of aquatic habitats. If generalities can be drawn from them, it would seem that non-specific labeling patterns (incorporation of ^{3}H from ^{3}H-TdR into macromolecules other than DNA) are associated with lotic hypolimnia, especially when oxy-

gen tension is reduced (Riemann et al. 1982, Lovell & Konopka 1985, McDonough et al. 1986, Robarts et al. 1986), large particles (Ducklow et al. 1985, Fallon & Newell 1986) and meso- or bathypelagic marine samples (Hanson & Lowery 1983). Karl (1982) reported non-specific labeling in samples from oligotrophic waters off Hawaii, in Hawaiian coastal waters and in samples from an eutrophic pond. Jeffrey & Paul (1988) have reported similar non-specific labeling in South Florida coastal waters.

An initial survey of thymidine incorporation patterns by South San Francisco Bay microbial communities revealed that an unexpectedly large proportion of the radiolabel (32 to 96 %, mean 53 %) was incorporated into protein. This observation prompted an investigation focusing on TdR metabolism in these communities and the macromolecular distribution of tritium from radiolabeled thymidine. Uptake, incorporation and the macromolecular distribution of tritium supplied as either methyl-[^{3}H]- or 6-[^{3}H]-TdR was examined to differentiate between TdR metabolism and possible artifacts of macromolecular fractionation protocols. The relationships between concentration of added TdR and radiolabel distribution, and between incubation duration and radiolabel distribution, were examined. South San Francisco Bay microbial assemblages were compared with assemblages from Elkhorn Slough, a nearby estuary, and Monterey Bay, California, USA.

South San Francisco Bay and Elkhorn Slough microbial assemblages extensively metabolized TdR, while Monterey Bay assemblages did not. Macromolecular distribution and tritium incorporation rate depended on position of the label and on the concentration of added TdR. Distributions did not vary significantly during time course experiments lasting up to 4 h.

MATERIALS AND METHODS

Sampling and incubations. Most of the water samples used in these experiments were collected at 2 stations in South San Francisco Bay, California, USA (SSFB, Fig. 1a). Stn 36 is in the channel at the southern end of SSFB and is surrounded by intertidal mudflats. It is strongly influenced by freshwater inflow from local surface runoff during the rainy season (November through May). During summer the main source of freshwater at this location is treated sewage (Conomos 1979). Typical summer salinities are on the order of 25 to 23 ‰. Stn 21 is located at the northern end of SSFB southeast of Yerba Buena Island. The water at this station is a mixture of coastal, North San Francisco Bay and SSFB water. Salinity can be <10 ‰ during major floods. The physical, chemical and biological properties of water at these stations are described in Cloern & Nichols (1985).
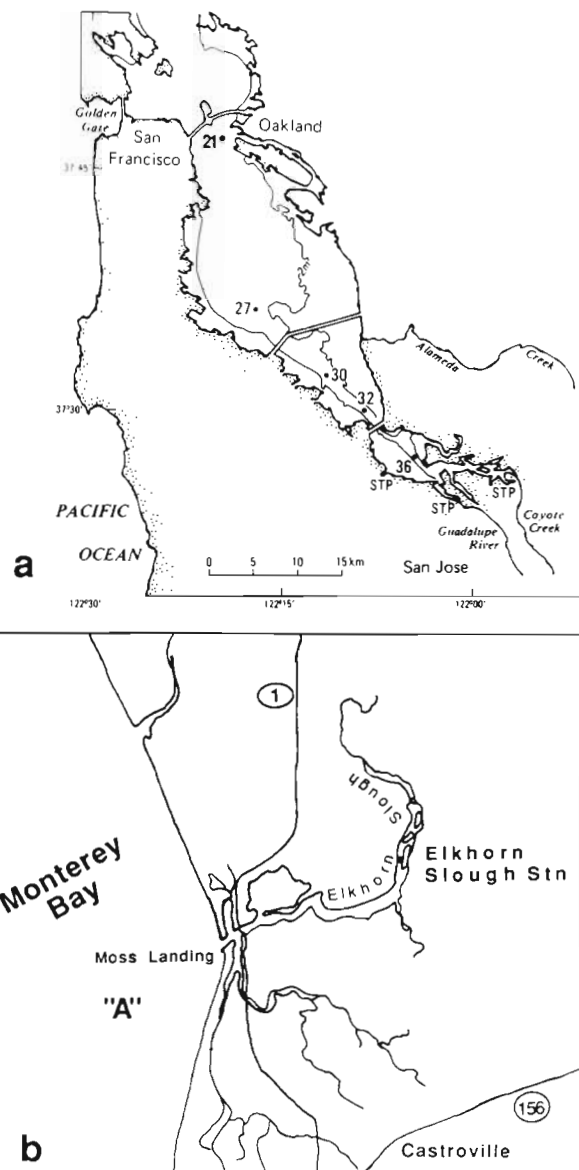

Fig. 1. Study areas in California, USA: (a) South San Francisco Bay (SSFB); (b) Elkhorn Slough and Monterey Bay

For comparison with SSFB data, experiments were performed with water collected in Elkhorn Slough about 4 km inland of its mouth at Moss Landing, California, and in Monterey Bay near the Moss Landing 'A' buoy about 2 km offshore (Fig. 1b). The Elkhorn Slough station was in a channel 2 m deep surrounded by shallow or intertidal mudflats. The salinity at this station was 22 ‰.

San Francisco Bay samples were collected with a clean polyethylene bucket or weighted bottle attached to a line. Subsamples were immediately dispensed into containers for the experiment, treated as described below, then incubated in a flowing seawater bath. The samples were exposed to indirect sunlight during incubations.

Elkhorn Slough and Monterey Bay samples were collected from a small boat by immersing polyethylene bottles to a depth of 10 cm. The samples were returned to the laboratory where they were placed in a constant temperature room at 18 °C illuminated by cool white fluorescent lights at 100 μE m^{-2} s^{-1} PAR. Subsamples were then dispensed into containers for the experiments. All experiments were begun (i.e. the radiotracers were added) within 2 h of sample collection.

Macromolecular fractionation. Assimilated radiotracer was assigned to functionally defined macromolecular fractions (all macromolecules, DNA, RNA, and protein) by the protocols of Schmidt & Thannhauser (1945) and Schneider (1945) as modified by Fuhrman & Azam (1980, 1982). Briefly, subsamples were either extracted with 5 % trichloroacetic acid at 0 °C (cold TCA), 5 % TCA at 95 to 100 °C (hot TCA), or 0.5 N NaOH at 60 °C (NaOH). Details of the extraction procedures are presented below.

Cold TCA extractions were performed on 5.00 ml samples collected on 0.45 μm pore size Millipore filters (HAMF) held in a 12-place filtration manifold ($\leq$10 cm Hg pressure differential). The filter and retained particulate material were rinsed 3× with 2 ml of chilled, unlabeled sample, then 3× with 2 ml of cold 5 % TCA. The vacuum was broken, 2 ml of cold 5 % TCA was added to each sample cup and the filters were extracted for 5 min. The manifold was reconnected to the vacuum, the filters were rinsed 3× with 2 ml of cold 5 % TCA, then removed from the manifold and placed in scintillation vials. No difference in ^{3}H retention was observed when samples processed on the filter manifold were compared with samples extracted in test tubes.

Samples (5.00 ml) for hot TCA extractions were placed in polypropylene culture tubes with 50 μl of a carrier solution containing calf thymus DNA, bovine serum albumin, and *Torula* yeast RNA (all at 1 mg ml^{-1}), 1 mM thymidine, and 0.1 mM NaN$_3$. Five ml of chilled 10 % TCA was then added. The samples were usually extracted immediately but on occasion they were stored briefly (never more than 24 h) in a refrigerator at 2 °C prior to extraction. The samples were extracted by placing them in a 95 to 100 °C water bath for 1.0 h. Next, the samples were chilled in an ice bath, filtered through an HAMF, rinsed 3× with 2 ml of chilled 5 % TCA and placed in scintillation vials.

Tritium retained by the filter appeared to stabilize after 1 h of hot 5 % TCA extraction; however, longer extractions might have resulted in slightly more hydrolysis. The effect of higher TCA concentrations on hydrolysis (Riemann 1984) was not tested. No significant difference in fractionation was observed when samples extracted immediately were compared with samples stored in 5 % TCA at 2 °C for 48 h prior to

extraction. Although carrier solution was routinely added to these samples and to NaOH extractions (see below), comparison of samples extracted with and without carrier revealed no difference in extraction efficiencies, nor did using 0.22 μm pore size Millipore filters (GSMF) in place of the HAMF used routinely.

Samples for NaOH extraction were collected on 0.2 μm pore size Nuclepore filters (NF), rinsed 3× with 2 ml of chilled, unlabeled sample, placed in disposable polypropylene culture tubes with 50 μl of carrier solution and immediately frozen on dry ice. Sample processing was completed within 1 wk, usually within 48 h, of freezing. Comparison of samples (NaOH extractions and uptake measurements, see below) frozen first with samples processed immediately showed that freezing did not alter ^{3}H fractionation. In the laboratory, 3 ml of 0.5 N NaOH was added to each test tube, then they were placed in a 60 $\pm$ 2 °C water bath for 1.0 h. Next, they were chilled in an ice-water bath and 0.39 ml of 100 % and 5 ml of cold 5 % TCA was added to neutralize the NaOH and precipitate unhydrolyzed macromolecules (DNA and protein; RNA is hydrolyzed). The precipitate was collected by filtration through either HAMF or GSMF. Comparisons of filter types indicated no difference in retention of the precipitate. Both filters (MF and NF) from a given replicate were rinsed 3× with 2 ml of chilled 5 % TCA and placed in the same scintillation vial.

In some experiments, TdR uptake plus incorporation and tritiated water production were measured. Uptake was measured by collecting 5.00 ml samples on HAMF, rinsing the filters 3× with 2 ml of chilled sample, then immediately freezing the filter in a scintillation vial on dry ice. Tritiated water production was assessed by collecting the filtrate from the uptake sample and radioassaying 1.00 ml subsamples before and after lyophilization and reconstitution with distilled water. Radioactivity in water was calculated as the difference between the 2 numbers.

The amounts of ^{3}H were calculated in the fractions macromolecules, DNA, RNA and protein, as follows. Macromolecular ^{3}H is that retained by the filter after cold TCA extraction. Protein ^{3}H is that retained by the filter after hot TCA extraction. DNA ^{3}H is the difference between ^{3}H retained after NaOH extraction and ^{3}H retained after hot TCA extraction. RNA ^{3}H is the difference between ^{3}H retained after cold TCA extraction and ^{3}H retained after NaOH extraction. To normalize for differences in sampling times for each fraction, ^{3}H incorporation data were first converted to rates in pM h^{-1} of TdR assuming that the specific activity of the incorporated TdR was the same as the added ^{3}H-labeled TdR. Radioactivity retained by formalin-killed samples or samples taken shortly after ^{3}H-TdR was added was substracted in rate calculations.

Ethanol extractions. Production of radiolabeled material that could be extracted from cells with 80 % ethanol (Robarts et al. 1986) was examined in time-course experiments with samples from Stns 32 and 21. Methyl- or 6-[³H]-TdR was added to water samples as above. Tritium incorporated into macromolecules was determined by extracting duplicate subsamples with cold 5 % TCA as described above. Following this, one of the replicates was extracted with 80 % ethanol at 0 to 2 °C (cold EtOH) as follows. The filter was rinsed 3× with 2 ml of cold EtOH, the vacuum was broken, the filter was covered with 5 ml of cold EtOH for 5 min, the vacuum was reconnected and the filter was rinsed an additional 3× with 2 ml of cold EtOH.

Near the end of the time course, samples were collected for macromolecular fractionation as described above. Production of cold-EtOH-extractable ³H during the fractionation procedures was examined by extracting sets of duplicates with cold EtOH alone, with TCA and NaOH alone, or with cold EtOH before and after TCA and NaOH extractions. Samples where the extractants were cold TCA alone or cold EtOH alone, or where cold TCA or cold EtOH were the first extractants, or that were extracted sequentially with cold EtOH and cold TCA, were collected on HAMF and extracted on the filter manifold as described above. Samples where the extractants were hot TCA alone or NaOH alone, or where hot TCA or NaOH were the first extractants, were collected on NF, placed in disposable test tubes and immediately frozen on dry ice.

In the laboratory, replicate sets of filters (frozen NF and a subset of cold-EtOH-extracted HAMF) were extracted with hot TCA or NaOH as described above. Unhydrolyzed or insoluble material, including the NF, was collected on HAMF. Finally, where appropriate, the HAMF was extracted with cold EtOH on the filter manifold as described above. The filters were placed in scintillation vials following extraction.

Incorporation time-course experiments. Water samples (100 to 200 ml) were placed in 250 ml polyethylene bottles or Pyrex Erlenmeyer flasks. Methyl- or 6-[³H]-TdR was added to final concentrations of 10 nM. Killed controls received additions of borate-buffered formalin to final concentrations of 2 % 0.5 h before radiotracer addition. In some experiments, mitomycin C was added to a final concentration of 10 µg ml⁻¹ 0.5 h before radiotracer addition. Subsamples were taken periodically and processed to determine the amount of ³H incorporated into macromolecular fractions as described above.

Saturation experiments. The purpose of these experiments was to determine the effect of TdR concentration on macromolecular ³H distribution, not to assess the degree of isotope dilution (cf. Pollard & Moriarty 1984). Five ml water samples were pipeted into sterile

polypropylene culture tubes. Methyl-[³H]-thymidine was added to duplicate sets of tubes to give a range of concentrations from 0.5 to 256 nM. TdR with a specific activity of 78.0 or 70.1 µCi nmole⁻¹ was used for concentrations ≤ 16 nM. TdR with a specific activity of 20 µCi nmole⁻¹ was used for concentrations ≥16 nM unless samples were fully fractionated when high specific activity TdR was used for all concentrations. Incubations lasted from 1.5 to 4.4 h. Experiments were terminated either by filtration (cold TCA and NaOH extractions) or by the addition of 50 µl of carrier and 5 ml of chilled 10 % TCA (hot TCA extractions). Samples were then processed as described above.

Liquid scintillation counting. TCA- and EtOH-washed Millipore filters were air-dried overnight at room temperature in scintillation vials, and then 1 ml of ethyl acetate or ethoxyethanol was added to dissolve the filter. Ethyl acetate was added directly to frozen, wet filters that had not been fixed with TCA of EtOH. After the filters had dissolved, 4 ml (filters in minivials) or 10 ml (1 ml aqueous samples in standard vials) of Aquasol 2 (NEN) was added to each vial. The samples were counted in a Beckmann 3801 liquid scintillation spectrometer. Quench corrections were made using the external standard operated in the Compton edge shift mode.

Chemicals and radiotracers. Calf thymus DNA, *Torula* yeast RNA, bovine serum albumin, NaN₃, thymidine and mitomycin C were obtained from the Sigma Chemical Company. All other chemicals used were reagent grade compounds obtained from the J. T. Baker Co. Radiotracers were obtained from New England Nuclear Corp.

RESULTS

Incorporation time courses

Incorporation of ³H into macromolecules, DNA, RNA and protein was linear with time at added TdR concentrations of 10 nM, regardless of the source of the sample or whether 6-[³H]-TdR or methyl-[³H]-TdR was used (Fig. 2; nearly identical results were obtained at other stations and on other sampling dates at this station). Methyl-[³H]-TdR and 6-[³H]-TdR were incorporated into macromolecules at nearly the same rate in some of experiments, but not others (Fig. 2; Table 1).

Macromolecular fractionation of incorporated ³H

The fraction of ³H incorporated into DNA by SSFB bacterioplankton was variable but usually was much less than 50 % of the ³H incorporated into macro-
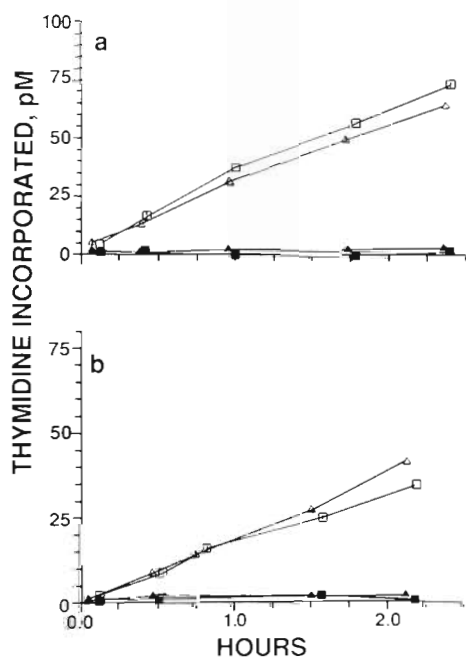
Fig. 2. Time course of ^{3}H incorporation into cold-TCA-insoluble material at (a) Stn 36; (b) Stn 21 in SSFB on 12 Jun 1985. (□) Methyl-; (△) 6-[^{3}H]-thymidine; open and solid symbols: experimental and 2 % formalin killed control, respectively. Bars give range of duplicate or triplicate determinations; range smaller than symbols when not shown.

Fig. 3. Time course of ^{3}H incorporation into material insoluble in cold and hot TCA and NaOH at Stn 36 in SSFB on 19 Feb 1986. Duplicate incubations with (□) methyl and (△) 6-[^{3}H]-thymidine. (a) Material insoluble in 5 % TCA at 100 °C; (b) material insoluble in 0.5 N NaOH at 60 °C; (c) material insoluble in 5 % TCA at 0 °C. Bars give 95 % confidence limits for triplicate determinations at each point; limits smaller than symbols when not shown

molecules (Table 1). Negative values of DNA incorporation were calculated in some experiments.

The distribution of ^{3}H in macromolecular fractions differed depending on whether it was supplied as methyl- or 6-[^{3}H]-TdR. A larger fraction of 6-[^{3}H] was incorporated into DNA than methyl-[^{3}H] and more methyl-[^{3}H] was incorporated into RNA and protein than 6-[^{3}H]-TdR. The amount of cold-TCA-soluble [3]H (small molecules) in the cells was comparable to total macromolecular [3]H (data not shown). A significant amount of tritiated water or other volatile substance was produced from both substrates during the incubations (data not shown). Monterey Bay populations incorporated a larger fraction of ^{3}H into DNA than SSFB populations and there was less difference between methyl- and 6-[^{3}H]-TdR. The degree of nonspecific labeling in Elkhorn Slough populations was intermediate between SSFB and Monterey Bay populations.

The incorporation rate of methyl-[^{3}H] into hot-TCA-insoluble material may have increased slightly during time-course experiments (Fig. 3). The incorporation rate of methyl-[^{3}H] into other fractions and of 6-[^{3}H] into all fractions was constant. Similar results were obtained in time-course experiments at Stn 21 (data not shown). Extrapolation of time courses to $t = 0$ yielded blanks of 0 to 5 pM TdR apparently incorporated into
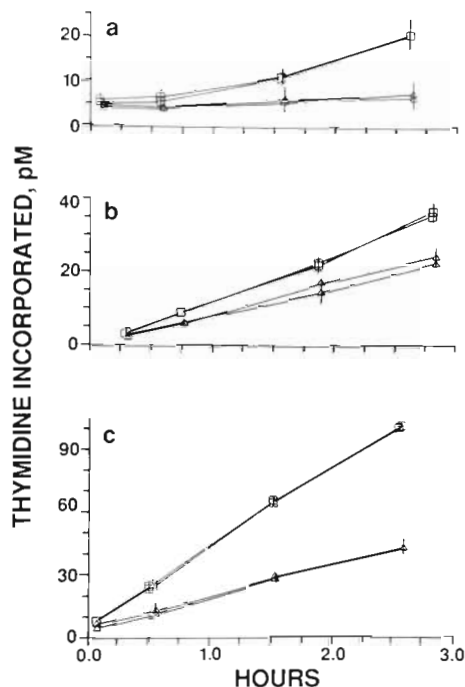
each fraction, with the greatest blank for the hot TCA extraction and the lowest for the NaOH extraction (Fig. 3). These blanks are comparable to those obtained with formalin-killed samples.

Addition of the DNA synthesis inhibitor mitomycin C completely blocked incorporation of ^{3}H into DNA. It also reduced uptake rates and ^{3}H incorporation into other macromolecules. Calculations often yielded negative rates of ^{3}H incorporation into DNA for samples incubated with mitomycin C. Mitomycin C affected uptake and metabolism of 6- slightly more than of methyl-[^{3}H]-TdR (Table 1).

Ethanol extractions

Little macromolecular 6-[^{3}H]-TdR label was removed by cold EtOH extraction, and the amount remained constant throughout time courses (Fig. 4; similar results were obtained at Stn 21; data not shown). The amount of cold-EtOH-extractable methyl-[^{3}H]-TdR label gradually increased during the incubations. The amounts of macromolecular methyl-[^{3}H] removed by cold EtOH extractions at Stn 32 after 2.7 h and at Stn 21

Table 1 Molecular distribution of ^{3}H from methyl- or 6-[^{3}H]-thymidine following uptake and catabolism by bacterioplankton. Surface water samples received additions of either methyl- or 6-[^{3}H]-thymidine. Samples were then incubated at in situ temperatures for various lengths of time. Fractionation of ^{3}H was determined in subsamples that were collected and processed as described in the text

| | | Thymidine-^{3}H uptake and incorporation rate (pM h^{-1}) | | | | | | | % of macromolecular tritium | | |
Lbl[1]	Addn[2]	Total[3]	Cold[4]	Hot[5]	NaOH[6]	DNA[7]	RNA[8]	Prot[9]	DNA	RNA	Prot
12 Jun 1985.	South San Francisco Bay Stn 36.		Incubation length 1.6–2.4 h[10]								
Me	None	50	32	18	22	2	11	19	6	34	59
6	None	75	29	10	22	12	7	10	41	24	34
Me	Mito	33	13	9.0	5.6	−3	7	9	−23	54	69
6	Mito	57	11	2.2	2.5	0	9	2	0	82	18
12 Jun 1985.	South San Francisco Bay Stn 21.		Incubation length 1.4–2.0 h								
Me	None	44	16	8.6	8.8	0	7	9	0	44	56
6	None	79	18	5.6	15	9	3	6	50	17	33
Me	Mito	44	12	7.3	3.8	−3	8	7	−25	67	58
6	Mito	56	6.1	1.8	2.2	0	4	2	0	67	33
24 Jul 1985.	South San Francisco Bay Stn 36.		Incubation length 1.7–2.8 h								
Me	None	107	79	27	26	−1	53	27	−1	67	34
6	None	41	21	3.3	15	12	6	3	57	29	16
Me	Mito	81	42	16	10	−6	32	16	−14	76	38
6	Mito	40	5.3	2.7	1.5	−2	4	3	−28	72	51
24 Jul 1985.	South San Francisco Bay Stn 21.		Incubation length 1.5–2.1 h								
Me	None	68	45	13	22	9	23	13	20	51	29
6	None	56	22	2.8	15	12	7	3	55	32	13
Me	Mito	57	19	6.7	6.0	−1	13	7	−4	68	35
6	Mito	37	6	3.7	1.8	−2	4	4	−32	70	62
20 Aug 1986.	Monterey Bay.	Incubation length 1.8–2.3 h[10]									
Me	None	102	80	5.8	57	51	24	6	64	29	7
6	None	264	129	10	102	92	27	10	71	21	8
Me	Mito	18	8.5	3.8	4.3	0	4	4	6	49	45
6	Mito	65	5.9	4.0	3.0	−1	3	4	−17	49	68
10 Sep 1985.	Monterey Bay.	Incubation length 1.8–2.5 h									
Me	None	219	168	17	96	79	72	17	47	43	10
Me	None	215	171	19	97	78	74	19	46	43	11
6	None	284	127	3.6	75	71	52	4	56	41	3
6	None	276	110	2.9	69	66	41	3	60	37	3
10 Sep 1985.	Elkhorn Slough.	Incubation length 3.0–3.7 h									
Me	None	169	118	32	81	49	37	32	42	31	27
Me	None	170	112	30	77	47	35	30	46	43	11
6	None	210	59	2.2	36	34	23	2	58	39	4
6	None	226	64	1.6	40	38	24	2	59	38	2
19 Feb 1986.	South San Francisco Bay Stn 36.		Incubation length 2.6–2.9 h								
Me	None	–	26	5.5	15	9	12	6	35	44	21
Me	None	–	26	5.3	15	9	12	5	35	45	20
6	None	–	13	2.9	15	6	4	3	46	32	22
6	None	–	15	2.5	11	9	4	2	58	25	17
19 Feb 1986.	South San Francisco Bay Stn 21.		Incubation length 2.6–2.8 h								
Me	None	–	39	7.9	13	5	26	8	12	67	20
Me	None	–	40	8.1	13	5	26	8	13	67	20
6	None	–	17	2.6	8.1	5	9	3	33	52	16
6	None	–	17	3.1	8.8	6	8	3	34	47	19

[1] Position of ^{3}H label on thymidine molecule: methyl (Me) or 6 (6); [2] Added inhibitor: nothing (none), or 10 mg l^{-1} mitomycin C (mito); [3] Total of uptake and incorporation, filter rinsed but not extracted; [4] Label insoluble in cold 5 % TCA (0 to 2 °C, 5 min), also macromolecular fraction; [5] Label insoluble in hot 5 % TCA (95 to 100 °C, 1 h), also protein fraction; [6] Label insoluble in 0.5 N NaOH (55 to 60 °C, 1 h); [7] Label in the deoxyribonucleic acid fraction: calculated as 6−5; [8] Label in the ribonucleic aid fraction: calculated as 4−6; [9] Label in the protein fraction: calculated as 5; [10] 1.0 nM methyl-[^{3}H]-thymidine addition instead of the usual 10 nM
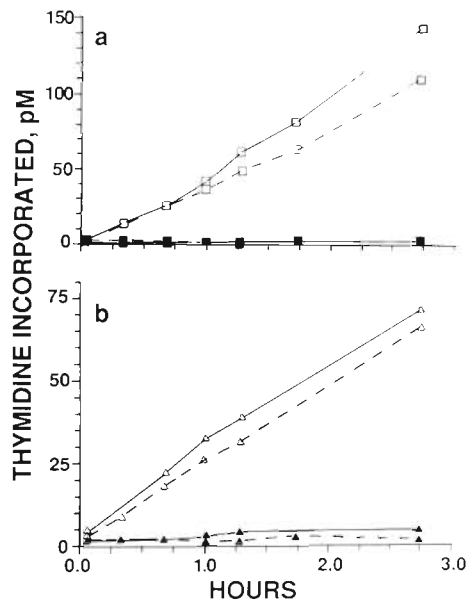
Fig. 4. Time course of incorporation of ^3H from (a) methyl-[^3H]-TdR, or (b) 6-[^3H]-TdR into (———) cold-TCA-insoluble material or (– – –) TCA and cold 80 % ethanol-insoluble material at Stn 32 in SSFB on 31 Mar 1987

after 2.3 h were 23 % and 15 %, respectively. At both stations, methyl-[^3H]-TdR was incorporated into macromolecules faster than 6-[^3H]-TdR. This difference was greater than the difference in the amount of cold-EtOH-extractable label.

Subsamples labeled for 1 to 2 h were extracted with cold EtOH before and after hot TCA or NaOH hydrolysis to determine if macromolecular fractionation procedures converted some of the label into a cold-EtOH-extractable form. The results of this experiment are presented in Table 2. There were no consistent differences between samples extracted with cold EtOH before or after TCA or NaOH extraction or hydrolysis. As observed in the time course, more of the macromolecular label was extracted by cold EtOH when supplied as methyl-[^3H]-TdR. Label from 6-[^3H]-TdR insoluble in cold EtOH alone was equivalent to label insoluble in cold TCA alone. In contrast, extraction with cold EtOH alone removed 20 % more ^3H from methyl-[^3H]-TdR than extraction by cold TCA alone. The amount of label remaining after extraction with both cold TCA and cold EtOH was the same as the amount insoluble in cold EtOH alone (Table 2).

Saturation experiments

Macromolecular ^3H incorporation rates in SSFB samples increased rapidly with added TdR concentration up to about 10 nM, then more slowly thereafter (Figs. 5, 6 and 7a; similar results were obtained in other experiments; data not shown). SSFB populations incorporated a smaller percentage of ^3H into DNA and larger percentages into RNA and protein at higher TdR concentrations. In some experiments, the rate of ^3H incorporation into DNA (or DNA + RNA in Fig. 7a) decreased at high TdR concentration and was apparently blocked at the highest concentrations tested (33 nM in Fig. 5 and 128 nM in Fig. 7a).

In Monterey Bay samples, the rate of ^3H incorporation into DNA+RNA saturated at about 8 to 16 nM added TdR and remained essentially unchanged up to 256 nM added TdR (Fig. 7b). Incorporation rate into protein increased continuously with increasing TdR concentration.

Table 2. Production of cold 80 % ethanol extractable ^3H from thymidine during productivity incubations and sample hydrolysis. Methyl- or 6-[^3H]-thymidine was incubated with SSFB bacterioplankton assemblages for 1 to 2 h. Samples were either not extracted with 0 °C 80 % ethanol (None) or were extracted after (After) or before (Before) extraction with TCA (Cold TCA, Hot TCA), NaOH (NaOH), or after a chilled sample rinse (None). Apparent thymidine incorporation rate for each replicate of a treatment is reported as $pM\,h^{-1}$

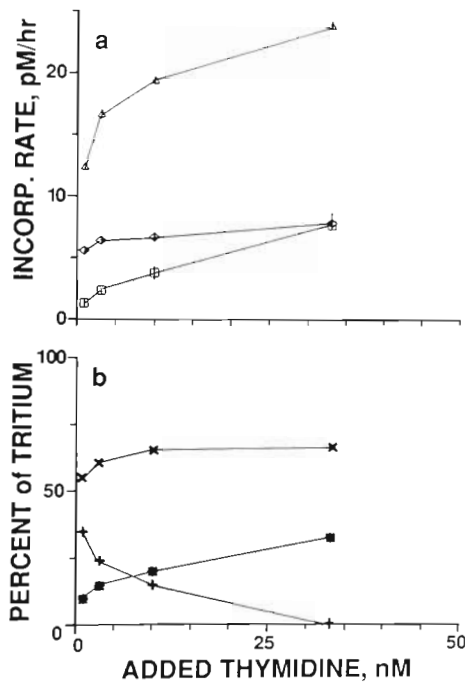| TdR position labeled:	Methyl-				6-					
Ethanol extraction:	None		After		Before		None		After		Before	
31 Mar 1987. Stn 32												
Cold TCA	48,	46	36,	36	38,	37	25,	18	20,	22	21,	20
Hot TCA	29,	31	26,	24	29,	31	10,	12	11,	11	16,	15
NaOH	22,	23	15,	16	15,	10	7,	19	19,	16	11,	12
None	61	–	38	–	–	–	63	–	24	–	–	–
31 Mar 1987. Stn 21												
Cold TCA	12,	11	9,	9	10,	9	7,	9	8,	8	8,	7
Hot TCA	3,	5	4,	4	6,	5	2,	3	2,	2	4,	3
NaOH	7,	7	6,	7	4,	5	5,	6	6,	6	4,	3
None	21,	24	10,	9	–	–	37,	36	7,	7	–	–

Fig. 5. Effect of thymidine concentration on macromolecular distribution of ³H from methyl-[³H]-TdR in bacterioplankton from Stn 36 in SSFB on 19 Feb 1986. (a) Rate of incorporation into (△) cold-TCA-insoluble material, (□) hot-TCA-insoluble material, (◇) NaOH-insoluble material. (b) Percentage of macromolecular label in (+) DNA, (×) RNA, (*) protein. Bars in (a) give 95 % confidence limits for triplicate incubations at each point; limit smaller than symbol when not shown
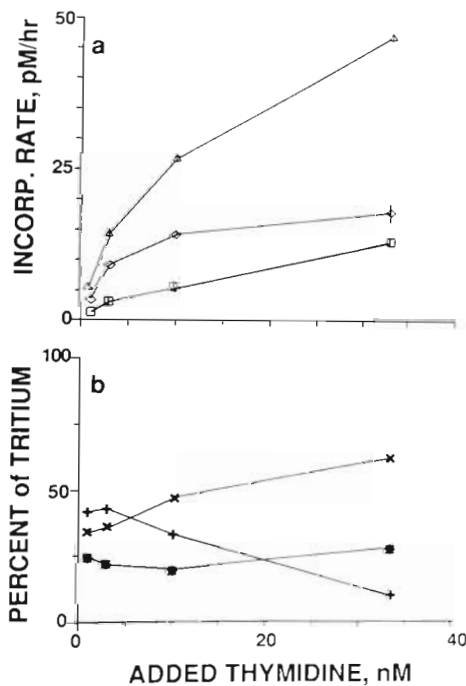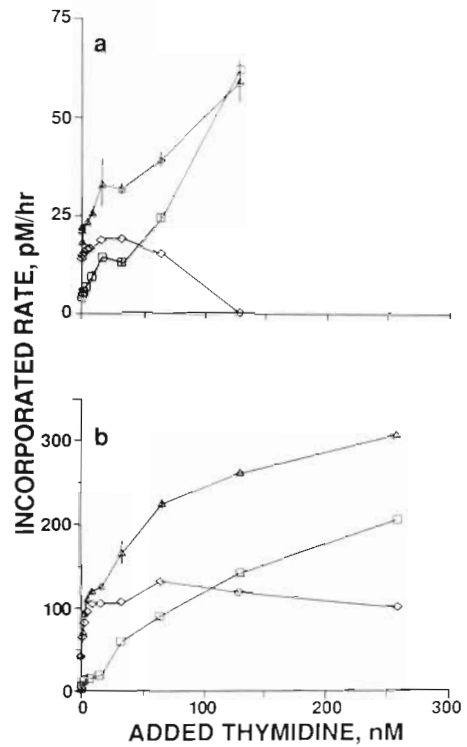

Fig. 6. As Fig. 5 for Stn 21

Fig. 7. Effect of thymidine concentration on the partitioning of ³H from methyl-[³H]-TdR between cold- and hot-TCA-insoluble material and on net incorporation into DNA+RNA (calculated as cold − hot). Water samples were collected at (a) SSFB Stn 36 on 12 Jun 1985, and (b) Monterey Bay Stn A on 20 Aug 1985, and incubated as described in text. Incorporation into (△) cold- and (□) hot-TCA-insoluble material, and (◇) net incorporation into DNA+RNA. Bars give range of duplicate incubations for cold and hot TCA extractions; range smaller than symbols when not shown

DISCUSSION

SSFB populations consistently metabolized TdR, resulting in ³H labeling all macromolecular fractions examined rather than just DNA. The labeling patterns observed with methyl-[³H]-TdR were different from those observed with 6-[³H]-TdR (Table 1). In Monterey Bay samples, labeling patterns were the same regardless of whether methyl- or 6-[³H]-TdR was used and most of the macromolecular ³H was incorporated into the DNA fraction.

A small amount of cold-EtOH-soluble ³H was produced by metabolism of the methyl-[³H]-TdR in SSFB samples. In contrast, Robarts et al. (1986) reported that populations in a hypereutrophic South African lake sequestered a significant fraction of the ³H from methyl-[³H]-TdR in a form that was not removed by TCA or NaOH extraction/hydrolysis procedures. The label could be removed by extraction with cold EtOH or chloroform-methanol. They suggested that the cold

EtOH-extractable ^{3}H was associated with lipids partitioning with the RNA fraction as determined by TCA and NaOH extraction/hydrolysis. The molecular nature of the substance(s) was not determined. SSFB populations produced EtOH-soluble ^{3}H from methyl- but not from 6-[^{3}H]-TdR, suggesting that it results from metabolism of the methyl group.

Tritium distributions that appear to indicate non-specific labeling can result from incomplete or non-specific hydrolysis of macromolecules by the Schmidt & Thannhauser (1945) and Schneider (1945) procedures. Other authors (Findlay et al. 1984, Riemann 1984, Fallon & Newell 1986, Robarts et al. 1986) have found that incomplete extraction was a source of error in their studies of TdR incorporation by microbial communities. In contrast, McDonough et al. (1986) reported that the acid-base extractions commonly employed (Fuhrman & Azam 1980) correctly estimated the amount of label in [^{3}H]-DNA added to an otherwise unlabeled plankton sample.

Riemann (1984) performed experiments to determine the optimum conditions for DNA and RNA hydrolysis. He found that DNA may be incompletely hydrolyzed by 5 % TCA and recommended increasing the concentration to 20 %. If Riemann's (1984) results are applicable to other studies where non-specific labeling was significant, some of the ^{3}H included in the protein fraction of these samples may have been unhydrolyzed DNA. However, as shown by the different labeling patterns obtained with methyl- and 6-[^{3}H]-TdR, metabolism of TdR is the most important reason for non-specific labeling of SSFB samples.

High concentrations of TdR clearly affected the macromolecular partitioning of ^{3}H from methyl-[^{3}H]-TdR by SSFB bacterioplankton assemblages and apparently blocked the flow of ^{3}H into DNA. Karl (1982) also noted that TdR concentration affected ^{3}H partitioning. In an experiment with water from Krauss Pond, the ^{3}H incorporation rate increased with increasing TdR concentration from 1.25 to 125 nM. Although he did not report incorporation into protein, a larger proportion of nucleic acid label was incorporated into DNA at high concentrations of TdR in this experiment. In a later experiment with water from the same pond, the fraction incorporated into DNA decreased as TdR concentration increased. W. H. Jeffrey (pers. comm.) found no change in the labeling pattern in South Florida coastal communities as the concentration of added label was increased from 2.5 to 500 nM.

The mechanism by which TdR concentration affects non-specific labeling is unclear. Pathways of TdR metabolism may be affected by high TdR concentrations, especially if these populations are using TdR as a growth substrate. Methyl groups from methyl-[^{3}H]-TdR might be shunted into other pathways in the

absence of any change in the rate of DNA synthesis. Alternatively, DNA synthesis may be blocked by high concentrations of TdR or its metabolites through feedback inhibition of enzymes responsible for synthesis of other DNA precursors (Moriarty 1986), or of other metabolic pathways.

A simple model to explain the non-specific labeling patterns observed in SSFB samples is that TdR is phosphorylated to thymidine monophosphate (dTMP), then the methyl group at the 5 position is lost or rapidly exchanged with methyl groups from some other methyl donor. Demethylation of dTMP produces deoxyuridine monophosphate (dUMP). Demethylation of methyl-[^{3}H]-TdR produces unlabeled dUMP and labeled methyl carrier, possibly N^5,N^{10}-methylene-[^{3}H] tetrahydrofolate (meTHF) although other carriers may be involved (see below). With 6-[^{3}H]-TdR, labeled dUMP and unlabeled methylated carrier are formed. Methylation of dUMP produces dTMP which would be labeled if the dUMP was initially derived from 6-[^{3}H]-TdR and unlabeled if it was derived from methyl-[^{3}H]-TdR or newly synthesized. It is assumed that the specific activity of me-THF (or other methyl donor) is low because TdR demethylation contributes only a small fraction of the methyl groups carried. A similar scenario can be developed if TdR is demethylated directly, but the deoxyuridine formed may have to be phosphorylated to dUMP so that it can be methylated by thymidine synthetase.

TdR degradation in *Escherichia coli* is thought to proceed via cleavage of the bond between thymine and deoxyribose mediated by thymidine phosphorylase (Kornberg 1980, Mandelstam et al. 1982, Moriarty 1986). The thymine residue produced can then be demethylated to uracil in a reaction involving THF. The uracil produced can be degraded further or incorporated into RNA fairly directly (uracil → uridine → UTP → RNA) or into DNA indirectly (uracil → uridine → UTP → CTP → CDP → dCDP → dCTP → DNA, or uridine UDP → dUDP → dUMP → dTMP → dTTP → DNA). Metabolism by this pathway should result in more label from 6- than from methyl-[^{3}H]-TdR being incorporated into RNA. This is the opposite of what was found in SSFB (Table 1) and suggests that SSFB bacterioplankton do not metabolize TdR by this pathway. Thymidine synthetase, the enzyme catalyzing the methylation of dUMP to dTMP, is not reversible in *E. coli*, suggesting that another enzyme may catalyze the proposed demethylation reaction. However, catabolic and anabolic pathways usually contain different enzymes even if they have common intermediates.

The model proposed here predicts that the label would be conserved in TdR (or dTMP, dTTP) to a greater extent with 6- than with methyl-[^{3}H]-TdR. A larger fraction of 6- than of methyl-[^{3}H] should

appear in DNA and the difference between the two is an index of de novo synthesis of dTMP from dUMP. Similar results would be expected with TdR labeled at any position other than the methyl group. Karl (1982) found that 27 to 36 % of ^{3}H from methyl-[^{3}H]-TdR was incorporated into DNA by a sample from Krauss Pond, while 51 to 62 % of the label from 2-[^{14}C]-TdR was incorporated into DNA by the same sample.

The model also suggests that a large fraction of labeled uridine or deoxyuridine should be incorporated into DNA in samples where non-specific labeling by TdR is extensive. Witzel & Graf (1984) compared the incorporation of uridine and TdR into DNA and RNA. Fifty % of the methyl-[^{3}H]-TdR and 94 % of the 5,6-[^{3}H]-uridine taken up appeared in a fraction designated as non-specific labeling (their Table 1). Calculated from their data, 84 % of methyl-[^{3}H]-TdR incorporated into nucleic acids appeared in DNA. By comparison, 19 % of the label from 5,6-[^{3}H]-uridine incorporated into nucleic acids appeared in DNA. This value is probably 50 % of the amount actually incorporated into DNA, (assuming the specific activities at the 5 and 6 positions to be equal), because ^{3}H at the 5 position would be lost upon methylation to dTMP.

Another prediction from the proposed model is that inhibiting thymidine demethylation should prevent non-specific labeling. Jeffrey & Paul (in press) report that the deoxyuridine analogue 5-fluoro deoxyuridine (FdU) blocked non-specific labeling at low concentrations of added methyl-[^{3}H]-TdR, but that the inhibition was reversed at higher concentrations of TdR. FdU is a non-competitive inhibitor of the methylating reaction of thymidine synthetase, but it probably also affects other enzymes with similar substrates or products, possibly competitively. Jeffrey & Paul (1988) also report that the dihydrofolate reductase inhibitors amethopterin and trimethoprim did not reliably prevent non-specific labeling. This enzyme is involved in the regeneration of the coenzyme tetrahydrofolate, suggesting that a methyl carrier other than me-THF is involved in non-specific labeling.

Non-specific labeling makes the interpretation of thymidine incorporation data in relation to bacterioplankton productivity problematic. On the one hand, much or most of the ^{3}H from methyl-[^{3}H]-TdR is incorporated into fractions other than DNA. Correction of this error would lower production estimates in direct proportion to the non-DNA fraction of incorporated label. On the other hand, extensive metabolism of TdR calls into question the assumption that the specific activity of dTTP incorporated into DNA is the same as that of the added TdR. Correction for isotope dilution would raise production estimates.

The application of isotope dilution analysis (Moriarty & Pollard 1981, Pollard & Moriarty 1984, Bell 1986) to correct production estimates should be treated with caution. Care must be taken to measure only incorporation into DNA in samples where non-specific labeling is extensive. In addition, the method assumes that the only route for ^{3}H incorporation into DNA is via dTTP, which may not be true in these samples. The method also assumes that added TdR does not affect growth or DNA synthesis rates, which also may not be true. Furthermore, the isotope dilution method was originally developed to study processes in uniform populations of cells (Forsdyke 1971). Its application to mixed microbial assemblages should be treated with caution.

It is evident from the results of this study and from others cited above that it is necessary to verify that methyl-[^{3}H]-TdR is being incorporated primarily into DNA when it is used to measure bacterioplankton DNA synthesis rates. In samples where non-specific labeling is extensive, a better production estimate probably can be obtained with 6-[^{3}H]-TdR than with methyl-[^{3}H]-TdR, although this must be verified experimentally. Hollibaugh et al. (1980) and Fuhrman & Azam (1980, 1982) used methyl-labeled TdR instead of ring-labeled TdR for 2 reasons. First, it can be obtained at higher specific activities than other forms. This is an advantage when working where bacterial production is low and it is necessary to maximize sensitivity, but it is unnecessary in most coastal environments and there are cost and stability advantages to using lower specific activity substrates packaged in ethanol. Secondly, they reasoned that demethylation, if it occurred, would produce (ultimately) unlabeled uridine or cytidine so that RNA would not be labeled. The methyl group was assumed to be lost to the pool of low molecular weight compounds in the cell or respired. Paradoxically, the results of this study suggest that the methyl group is the source of non-specific labeling and not the ring.

CONCLUSIONS

Incorporation of ^{3}H from methyl-[^{3}H]-thymidine into macromolecular fractions other than DNA (non-specific labeling) can be a significant source of error in bacterioplankton productivity estimates in certain environments. The degree of non-specific labeling observed in this study varied spatially both between and within the systems studied and varied temporally in South San Francisco Bay. Non-specific labeling was due to metabolism of the substrate by populations in the samples. It was significantly less with 6-[^{3}H]-TdR than with methyl-[^{3}H]-TdR. Non-specific labeling and the apparent de novo synthesis of thymidine monophosphate leading to isotope dilution may be related manifestations of the same metabolic processes. High substrate concentrations appear to affect these processes

where non-specific labeling is significant, making interpretation of isotope dilution experiments problematic.

LITERATURE CITED

Bell, R. T. (1984). Thymidine incorporation rates and bacterioplankton dynamics during early spring in Lake Erken. Arch. Hydrobiol. Beih. Ergeb. Limnol. 19: 81–89

Bell, R. T (1986). Further verification of the isotope dilution approach for estimating the degree of participation of [^{3}H]thymidine in DNA synthesis in studies of aquatic bacterial production. Appl. environ. Microbiol. 52: 1212–1214

Bell, R. T., Ahlgren, G. M., Ahlgren, I. (1983). Estimating bacterioplankton production by measuring (^{3}H)thymidine incorporation in a eutrophic Swedish lake. Appl. environ. Microbiol. 45: 1709–1721

Cloern, J. E., Nichols, F. H. (1985). Temporal dynamics of an estuary: San Francisco Bay. Hydrobiol. 129: 1–237

Conomos, T. J. (1979). Properties and circulation of San Francisco Bay waters. In: Conomos, T. J. (ed.) San Francisco Bay: then urbanized estuary. Pac. Div., AAAS, San Francisco, p. 47–84

Ducklow, H. W., Hill, S. M. (1985). Tritiated thymidine incorporation and the growth of heterotrophic bacteria in warm core rings. Limnol. Oceanogr. 30: 260–272

Ducklow, H. W., Hill, S. M., Gardner, W. D. (1985). Bacterial growth and the decomposition of particulate organic carbon collected in sediment traps. Cont. Shelf Res. 4: 445–464

Ducklow, H. W., Kirchmann, D. L. (1983). Bacterial dynamics and distribution during a spring diatom bloom in the Hudson River plume, USA. J. Plankton Res. 5: 333–355

Fallon, R. D., Newell, S. Y (1986). Thymidine incorporation by the microbial community of standing dead *Spartina alterniflora*. Appl. environ. Microbiol. 52: 1206–1208

Findlay, S. E. G., Meyer, J. L., Edwards, R. T (1984). Measuring bacterial production via rate of incorporation of [^{3}H]thymidine into DNA. J. Microbiol. Methods 2: 57–72

Forsdyke, D. R. (1971). Application of the isotope dilution principle to the analysis of factors affecting the incorporation of [^{3}H]uridine and [^{3}H]cytidine into cultured lymphocytes. Biochem. J. 125: 721–732

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

Fuhrman, J. A., Azam, F. (1982). Thymidine incorporation as a measure of heterotrophic bacterial production in marine surface waters: evaluation and field results. Mar Biol. 66: 109–120

Hanson, R. B., Lowery, H. K. (1983). Nucleic acid synthesis in oceanic microplankton from the Drake Passage, Antarctica: evaluation of steady state growth. Mar. Biol. 73: 79–89

Hollibaugh, J. T., Fuhrman, J. A., Azam, F. (1980). Radioactively labeling natural assemblages of bacterioplankton for use in trophic studies. Limnol. Oceanogr 25: 172–181

Jeffrey, W H., Paul, J. H. (1988). The effect of 5-fluoro 2'-deoxyuridine on [^{3}H]thymidine incorporation by bacterioplankton in the waters of southwest Florida. Appl. environ. Microbiol. (in press)

Karl, D. M. (1982). Selected nucleic acid precursors in studies of aquatic microbial ecology. Appl. environ. Microbiol. 44: 891–902

Kirchman, D. L., Ducklow, H. W., Mitchell, R. (1982). Estimates of bacterial growth from changes in uptake rates and biomass. Appl. environ. Microbiol. 44: 1296–1307

Kornberg, A. (1980). DNA replication. Freeman, San Francisco

Lovell, C. R., Konopka, A. (1985). Seasonal bacterial production in a dimictic lake as measured by increases in cell numbers and thymidine incorporation. Appl. environ. Microbiol. 49: 492–500

Mandelstam, J., McQuillen, K., Dawes, I. (1982). Biochemistry of bacterial growth. Blackwell Scientific Publishers, Boston

McDonough, R. J., Sanders, R. W., Porter, K. L., Kirchman, D. L. (1986). Depth distribution of bacterial production in a stratified lake with an anoxic hypolimnion. Appl. environ. Microbiol. 52: 992–1000

Moriarty, D. J. W. (1984). Measurements of bacterial growth rates in some marine systems using the incorporation of tritiated thymidine into DNA. In: Hobbie, J. E., Williams, P. J. (eds.) Heterotrophic activity in the sea. Plenum Publishing Corp., New York, p. 217–231

Moriarty, D. J. W. (1986). Measurements of bacterial growth rates in aquatic systems from rates of nucleic acid synthesis. Adv. Microb. Ecol. 9: 245–292

Moriarty, D. J. W., Pollard, P. C. (1981). DNA synthesis as a measure of bacterial productivity in seagrass sediments. Mar. Ecol. Prog. Ser. 5: 151–156

Moriarty, D. J. W., Pollard, P. C. (1982). Diel variation of bacterial productivity in seagrass *(Zostera capricorni)* beds measured by rate of thymidine incorporation into DNA. Mar. Biol. 72: 165–173

Pollard, P. C., Moriarty, D. J. W. (1984). Validity of the tritiated thymidine method for estimating bacterial growth rates: measurement of isotope dilution during DNA synthesis. Appl. environ. Microbiol. 48: 1076–1083

Riemann, B. (1984). Determining growth rates of natural assemblages of freshwater bacteria by means of ^{3}H-thymidine incorporation into DNA: comments on methodology. Arch. Hydrobiol. Beih. Ergeb. Limnol. 19: 67–80

Riemann, B., Fuhrman, J. A., Azam, F. (1982). Bacterial secondary production in freshwater measured by ^{3}H-thymidine incorporation method. Microb. Ecol. 8: 101–114

Robarts, R. D., Wicks, R. J., Sephton, L. M. (1986). Spatial and temporal variations in bacterial macromolecule labeling with [methyl-^{3}H]thymidine in a hypertrophic lake. Appl. environ. Microbiol. 52: 1368–1373

Scavia, D., Laird, G. A., Fahnestiel, G. L. (1986). Production of planktonic bacteria in Lake Michigan. Limnol. Oceanogr. 31: 612–626

Schmidt, G., Thannhauser, S. J. (1945). A method for the determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues. J. biol. Chem. 161: 83–89

Schneider, W. C. (1945). Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. biol. Chem. 161: 293–303

Tobin, R., Anthony, D. (1978). Tritiated thymidine incorpora-

tion as a measure of microbial activity in lake sediments. Limnol. Oceanogr. 23: 161–165

Witzel, K.-P., Graf, G. (1984). On the use of different nucleic acid precursors for the measurement of microbial nucleic acid turnover. Arch. Hydrobiol. Beih. Ergeb. Limnol. 19: 59–65