

## Estimating bacterial DNA synthesis from [<sup>3</sup>H]thymidine incorporation: Discrepancies among macromolecular extraction procedures

**Abstract**—The estimation of bacterial DNA synthesis in trophic studies with [<sup>3</sup>H]thymidine requires quantitative extraction of labeled DNA. To determine the DNA contribution to total macromolecular labeling in a eutrophic ecosystem, we tested three extraction procedures currently used to estimate DNA labeling from thymidine incorporation: enzymatic fractionation, acid-base hydrolysis, and phenol-chloroform extraction. Because labeled macromolecular fractions are generally defined as DNA, RNA, and proteins, we used incorporation of tritiated thymidine, uridine, and leucine to preferentially label DNA, RNA, and proteins. Our data showed that each fractionation method yielded different apparent macromolecular distributions of the same radiolabeled substrates. Enzymatic digestions of the fractions obtained by acid-base hydrolysis and phenol-chloroform extraction showed that these two procedures are inadequate for estimating bacterial DNA labeling in our ecosystem. Finally, using the enzymatic procedure at different sites, DNA labeling appeared to represent a nearly constant proportion of the labeled macromolecules (20.1%,  $r = 0.952$ ,  $n = 101$ ) over a wide range of incorporation rates.

The estimation of bacterial production via [*methyl*-<sup>3</sup>H]thymidine incorporation into macromolecules is now in wide use in continental aquatic systems (e.g. estuaries: Bjørnson et al. 1989; Torrèton et al. 1989; lakes: Nagata 1987; Simon 1987). Interpreting thymidine incorporation data requires several assumptions (see Moriarty 1986). Recent work has shown that one of these postulates, preferential labeling of DNA by [*methyl*-<sup>3</sup>H]thymidine, may not be tenable in some ecosystems (Riemann et al.

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1982; Robarts et al. 1986; Hollibaugh 1988). Moreover the ratio of labeled DNA to total insoluble precipitate can be highly variable in a given ecosystem (Moriarty 1986; Robarts et al. 1986; Smits and Riemann 1988); researchers have turned to specific extraction and purification of labeled DNA to circumvent this problem. At present, the methods used include acid-base hydrolysis (Fuhrman and Azam 1982; Riemann and Søndergaard 1984; Hollibaugh 1988), enzymatic fractionation (Riemann 1984; Servais et al. 1987; Wicks and Robarts 1987), and phenol-chloroform extraction (Wicks and Robarts 1987; Bell and Riemann 1989).

To determine the best procedure to estimate bacterioplankton productivity in the eutrophic Ebrié Lagoon, Ivory Coast, we compared the results of macromolecular fractionation by these three methods, which are easily applied in the field. In addition to the three generally differentiated macromolecular fractions, a significant amount of <sup>3</sup>H from [*methyl*-<sup>3</sup>H]thymidine was recently found in an ethanol-soluble fraction (Robarts et al. 1986).

To compare the apparent specificity of labeling of the labeled fractions as determined by the three procedures, we used tritiated thymidine (Fuhrman and Azam 1982), uridine (Karl 1982; Witzel and Graf 1984), and leucine (Kirchman et al. 1985) to preferentially label DNA, RNA, and proteins.

Water samples were collected from the subsurface (0.50-m depth) of Biétri Bay in Ebrié Lagoon. The hydrological, physico-chemical, and biological properties of this eutrophic and monomictic bay were described by Arfi et al. (1989), Guiral (1984), and Torrèton et al. (1989). [*Methyl*-



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$^3\text{H}$ ]thymidine (45 Ci mmol $^{-1}$ ), [5,6- $^3\text{H}$ ]uridine (49 Ci mmol $^{-1}$ ), and [4,5- $^3\text{H}$ ]leucine (60 Ci mmol $^{-1}$ ) were purchased from CEA (France). Samples from Biétri Bay received radiolabeled substrates at a final concentration of 20 nM and were incubated for 30 min in the dark at 30°C. On one occasion, coastal seawater was sampled in the Gulf of Guinea, 2 km offshore at 5-m depth, and assayed for thymidine incorporation (final concn, 5 nM; 1 h of incubation). Previous experiments have shown that this concentration gave maximal incorporation rates and that the incorporation was linear over this period. All incubations were stopped by chilling samples in ice. A zero-time blank was made by adding the label in a prechilled duplicate sample. Subsamples were then dispensed into several sterile test tubes.

Macromolecules were allowed to precipitate for 15 min at 2°C in TCA at 5% final concentration (Fuhrman and Azam 1982). Cold 5% TCA-insoluble material was collected on cellulose nitrate filters (Whatman, 0.2- $\mu\text{m}$  pore size), rinsed with ice-cold 5% TCA, and placed in scintillation vials. Nucleic acids were then hydrolyzed with 0.5 ml of 0.5 N HCl at 100°C for 30 min to minimize self-absorption. Indeed, repeated experiments showed that this HCl treatment increased counts by 70%. Ethylacetate (1 ml) was added to dissolve the filters, and, after 30 min of shaking, 6 ml of scintillation cocktail was added. Radioactivity in the zero-time blank was subtracted from radioactivity in the incubated sample. Average range of all duplicate determinations was 6.4% of their respective means ( $n = 15$ ).

Two enzymatic digestion procedures were tested with [*methyl*- $^3\text{H}$ ]thymidine and three enzymes (DNase I, grade I 3,000 Kunitz units mg $^{-1}$ ; RNase A 40 Kunitz units mg $^{-1}$ ; proteinase K, nuclease-free, 20 units mg $^{-1}$ ; all from Boehringer Mannheim GmbH). The enzymatic extraction protocol developed by Servais et al. (1987) based on cell disruption by sonication with Triton X100 and liquid-phase digestion gave results very similar to those obtained by the procedure of Robarts et al. (1986) (unpubl. data). For convenience, further enzymatic hydrolyses were performed according to Robarts et al. (1986).

Filters retaining cold TCA precipitate were washed with 3 ml of ice-cold 10 mM KHCO $_3$  to remove excess acidity. The rinse removed <5% of the total radioactivity as compared to filters without rinsing. Filters were then placed in scintillation vials and stored at -20°C until processing. Storage of the filters at -20°C up to 1 week did not affect final partitioning of the label (data not shown). Enzymatic digestions were performed by adding 2.5 ml of enzyme solutions to the vials containing the filters according to the procedure of Robarts et al. with the minor modifications that follow.

Final enzyme concentrations in the tests were 80  $\mu\text{g ml}^{-1}$ . Previous experiments showed that the enzymes were not a limiting factor at that concentration. Rinse controls for DNase, RNase, and proteinase were made without enzyme in the solutions. After 1 h of incubation at 37°C on a rotary shaker, the incubation solutions were filtered onto cellulose acetate filters (Millipore GS, 0.22- $\mu\text{m}$  pore size) and radioassayed. Radioactivity in the rinse controls was low with this procedure and was subtracted from that in the corresponding enzyme treatments to obtain the radioactivity in each fraction. Radioactivities in rinse controls and enzyme treatments were very similar for the zero-time blank. For uridine experiments, the rinse control for labeled DNA determination was made by boiling the DNase solution for 30 min to eliminate DNase activity. Residual enzyme activity was thus assumed to be due to contaminating RNase and was subtracted from unboiled DNase activity. Average range of all duplicate determinations was 7.6% of their means ( $n = 30$ ).

One set of samples was subjected to the phenol-chloroform extraction by the procedure of Wicks and Robarts (1987). The chilled duplicate subsamples were hydrolyzed with NaOH (0.5 N final concn) for 15 min at 30°C and then acidified with ice-cold 100% TCA (1.4 ml per 5 ml of sample) for 15 min. Precipitated macromolecules were collected on cellulose nitrate filters (Whatman, 0.2- $\mu\text{m}$  pore size). The filters were washed three times with 3 ml of phenol-chloroform (50% wt/vol) to remove proteins and then twice with 5 ml of ice-cold

80% ethanol (vol/vol). The precipitate remaining on the filter after this treatment was defined as "bacterial DNA" by Wicks and Robarts. Filters were radioassayed as described above. Blank values from zero-time subsamples were subtracted. Average range of all duplicate determinations was 7.1% of their means ( $n = 12$ ).

Acid-base hydrolysis was carried out as described by Fuhrman and Azam (1982) and modified by Riemann and Søndergaard (1984). Total macromolecules (here defined as DNA, RNA, and proteins) in duplicate subsamples were precipitated at 2°C with TCA (5% final concn). A second pair of subsamples was treated with 1 N NaOH (final concn) for 1 h at 60°C, then acidified with ice-cold 100% TCA (1.4 ml per 5 ml of sample) and allowed to precipitate for 15 min at 2°C (precipitate defined as DNA and proteins). A third pair of subsamples was heated at 95°C for 30 min with 20% TCA (final concn) in order to collect proteins only. Filters were radioassayed as described above. The zero-time blank was treated as described above and subtracted for each fraction. Average ranges of all seven duplicate determinations were 4.3 and 9.9% of their means (total  $n = 14$ ), following hot NaOH and hot TCA treatments, respectively. Radiolabeled DNA was calculated as the difference between the radioactivity in the NaOH precipitate and the hot TCA precipitate. Radiolabeled RNA was calculated as the difference between the radioactivity in the total precipitate and the hot NaOH precipitate.

In Biétri Bay, thymidine, uridine, and leucine incorporation rates in the cold TCA precipitate averaged 2.14 ( $n = 5$ ), 5.46 ( $n = 3$ ), and 5.10 nmol liter<sup>-1</sup> h<sup>-1</sup> ( $n = 3$ ). These high values reflect the eutrophic character of this bay (Torréton et al. 1989). The thymidine incorporation rate in coastal seawater was 17.6 pmol liter<sup>-1</sup> h<sup>-1</sup> (5 January 1990), which is close to the values given by Wicks and Robarts (1987) for coastal water from the South Indian Ocean and is in the range reported by Hollibaugh (1988) for California coastal water.

All results of the fractionation experiments are expressed as a percentage of the cold TCA-insoluble material (e.g. Table 1).

After thymidine incorporation, enzymatic digestion of TCA precipitate revealed that a small part (16.7–18.6%,  $n = 3$ ) of the label appeared in the DNA fraction, none in the RNA fraction, and a large part in the protein fraction (14.0–38.6%,  $n = 3$ ), while 43.5–68.8% (avg 57.7%,  $n = 3$ ) remained unexplained (not recovered in Table 1) by the three enzymes. It should be noted that this unexplained fraction is in part due to the rinsing controls for each fraction (avg 3.1%,  $n = 9$ ) and KHCO<sub>3</sub> rinsing (avg 2.9%,  $n = 3$ ). Thus, the remainder (avg 45.4%,  $n = 3$ ) is due either to insufficient hydrolysis by the enzymes or to another labeled fraction (*see below*). Three experiments with enzymatic fractionation showed that most of the [<sup>3</sup>H]uridine label was recovered in RNA (62.9–90.7%,  $n = 3$ , 82.6% in Table 1), while minor quantities appeared in the other fractions. Following [<sup>3</sup>H]leucine incorporation, most of the label appeared in the protein fraction (47.0 and 78.0%,  $n = 2$ ). Thus, using the enzymatic procedure, leucine and uridine achieve more specificity of labeling in the target macromolecule than does thymidine.

Following thymidine incorporation, radioactivity in total macromolecules was similar to an alkaline procedure followed by precipitation (Wicks and Robarts 1987) and standard cold TCA precipitation. A large fraction (33.0–58.5%, avg 44.3%,  $n = 4$ ) of the precipitate labeled by [<sup>3</sup>H]thymidine was solubilized by the phenol-chloroform treatment (46.0% in the example of Table 1), which is supposed to extract only proteins. Rinsing the filter with ethanol resulted in solubilization of 4.0–14.9% of the label (avg 8.4,  $n = 4$ ). The residual fraction defined as DNA by Wicks and Robarts represented 29.8–49.2% of the total label incorporated into macromolecules (avg 41.1%,  $n = 4$ ).

Two experiments with phenol-chloroform extraction showed that the initial NaOH treatment removed 74 and 78.5% of the [<sup>3</sup>H]uridine label, suggesting significant hydrolysis of the labeled RNA. Phenol-chloroform extracted 8.4 and 11.4% of the label remaining in the precipitate. Rinsing with ethanol removed 4.5 and 0.5% of the labeled material. Finally, the labeled "DNA" remaining on the filter represented 13.1 and

Table 1. An example from one of several experiments of the partitioning of label among fractions after [<sup>3</sup>H]thymidine, leucine, and uridine incorporation (Biétri Bay, 20 December 1989). Fractions are defined in the text and expressed as percentages of 5% TCA-insoluble total material. NR—Not recovered; ND—not determined.

	5% TCA	DNA	RNA	Proteins	Lipids	NR
Enzymatic fractionation						
Thymidine	100	18.6	-0.7	38.6	ND	43.5
Uridine	100	13.6	82.6	10.1	ND	(6.3)*
Leucine	100	0.8	0.0	78.0	ND	21.2
Phenol-chloroform extraction						
Thymidine	100	36.1	ND	46.0	4.1	13.8
Uridine	100	13.1	ND	8.4	4.5	74.0
Leucine	100	55.5	ND	38.7	5.8	0.0
Acid-base hydrolysis						
Thymidine	100	15.5	21.2	63.3	ND	0.0
Uridine	100	7.5	85.8	6.7	ND	0.0
Leucine	100	20.8	29.8	49.4	ND	0.0

\* Greater than 100% recovery.

9.6% of the label present in the standard cold TCA precipitate. In three experiments with [<sup>3</sup>H]leucine, radioactivity in the cold TCA precipitate showed no difference between alkaline and standard procedures. Only 16.3–41.1% (38.7% in Table 1) of the [<sup>3</sup>H]leucine in the NaOH-TCA precipitate was solubilized by phenol-chloroform. After the ethanol extraction that removed 3.3–11.6% (5.8% in Table 1) of the labeled macromolecules, a surprisingly high amount (55.4–60.5%, 55.5% in Table 1) of the label remained in the DNA fraction.

Like many workers (Karl 1982; Riemann et al. 1982), we found a significant amount of the label (21.2–56.6%,  $n = 3$ ) from [<sup>3</sup>H]thymidine in the RNA fraction (hydrolyzed by hot 1 N NaOH). The largest fraction of the [<sup>3</sup>H]thymidine label appeared, however, in the protein fraction (38.1–63.3%,  $n = 3$ ). The smallest fraction was recovered in DNA (5.8–15.5%,  $n = 3$ ). In two experiments, the label from [<sup>3</sup>H]uridine was incorporated preferentially into the RNA fraction (84.9 and 85.8%). The remainder was equally divided between the DNA and protein fractions. The largest amount of [<sup>3</sup>H]leucine label (49.4 and 62.9%,  $n = 2$ ) was found in the protein fraction, while the RNA fraction represented 29.8 and 22.4%, and the DNA fraction was 20.8 and 14.7%.

It is clear that the three methods give contradictory results in our ecosystem. The contribution of DNA to labeled macro-

molecules varied greatly depending on the method used. We performed additional experiments to obtain more information on these discrepancies.

We subjected the final precipitate in the sequential extraction procedure of Robarts et al., referred to as DNA, to enzymatic digestion. A significant amount of the [<sup>3</sup>H]thymidine label in this fraction was hydrolyzed by the proteinase K (10.7%, Table 2) of cold TCA-insoluble material. On the other hand, the treatment of a replicate of two filters with DNase yielded the same fractionation as obtained by enzymatic hydrolysis of the cold TCA precipitate (18.9 compared to 18.6%, Tables 1 and 2). These results suggest that DNA is retained quantitatively during phenol-chloroform treatment (in agreement with Robarts et al. 1986), although the treatment did not remove all of the labeled proteins. Changing the phenol qualities (different products) and the number of washes did not significantly increase the extraction yield (data not shown). This observation is supported by the low percentage of leucine label (avg 32%,  $n = 3$ , 38.7% in Table 1) removed by phenol-chloroform extraction. Both proteinase digestion and hot TCA extraction yielded higher fractions in proteins (avg 62.6,  $n = 2$ , and 56.2%,  $n = 2$ , respectively). Moreover, nearly 50% of the [<sup>3</sup>H]leucine label remaining on the membrane after phenol-chloroform and ethanol washes was removed by proteinase K treatment (Table 2).

Table 2. Enzymatic fractionation of DNA obtained by the phenol-chloroform procedure (Biétri Bay, 20 December 1989). Results are expressed as percentages of total incorporation in cold TCA-insoluble material. NR—Not recovered; ND—not determined.

Fraction	5% TCA	Purified DNA	Enzymatic procedure			
			DNA	RNA	Proteins	NR
Thymidine	100	36.1	18.9	0.0	10.7	6.5
Leucine	100	55.5	0.0	ND	25.7	29.8

DNA fractions determined with acid-base hydrolysis and DNase treatment were also systematically different.

The [*methyl*-<sup>3</sup>H]thymidine label was not incorporated into the RNA fraction according to the enzymatic approach, although the acid-base procedure suggested significant incorporation into RNA (*see above*). RNase removed a significant fraction of the uridine-labeled macromolecules, indicating that it was capable of hydrolyzing labeled RNA, if it had been present. This result suggests that [*methyl*-<sup>3</sup>H]thymidine was not incorporated into RNA and that the apparent RNA labeling observed with acid-base hydrolysis may be an artifact of that procedure. This result corroborates the observations of Riemann (1984), Moriarty (1986), and Robarts et al. (1986). They found that RNA labeling is minimal during short incubations and contrasts with the significant RNA labeling reported by Servais et al. (1987) after enzymatic digestion. The identity of the material extracted by hot NaOH in our thymidine experiments is unclear. The acid-base procedure also indicated that radiolabel from leucine appeared in this fraction, while the enzymatic procedure suggested that no leucine label appeared in nucleic acids. In one experiment, DNase digestion revealed that thymidine-labeled DNA represented 17.9% of the initial cold TCA precipitate, while it represented 7.7% after hot NaOH hydrolysis (Table 3). Thus, some of the macromolecules hydrolyzed by hot NaOH are likely to be DNA. This observation agrees with the low recovery of [<sup>32</sup>P]DNA (68%) following hot NaOH treatment reported by Robarts et al.

Determination of label from leucine appearing in protein also leads to contradictions among the three methods. Proteinase

Table 3. Enzymatic recovery of DNA after acid-base hydrolysis following [<sup>3</sup>H]thymidine incorporation (Biétri Bay, 31 January 1990).

Treatment	Cold 5% TCA	Hot NaOH	Hot 5% TCA	Hot 20% TCA
Total precipitate	100	52.0	49.0	35.3
DNA	17.9	7.7	3.5	0.0

K treatment revealed more labeled protein than either hot 20% TCA or phenol-chloroform extractions (*see above and Table 1*). In contrast, more thymidine label appeared in protein as determined by hot 20% TCA hydrolysis (avg 46.3%, *n* = 3) and by phenol-chloroform extraction (avg 44.3%, *n* = 4) than by proteinase treatment (23.8%, *n* = 3). These results may not be contradictory because leucine and thymidine are not supposed to label proteins with the same pathway. Treating the hot 20% TCA precipitate with DNase revealed no labeled DNA (Table 3). Moreover, after uridine incorporation, virtually no label was found in the hot 20% TCA precipitate (7.5 and 5.7%, *see above*). These observations suggest that hot 20% TCA treatment removes all the labeled nucleic acids. Acid-base hydrolysis indicated that only 49.4 and 62.9% of [<sup>3</sup>H]leucine in the cold TCA precipitate was incorporated in proteins with the remainder being incorporated in the DNA and RNA fractions (*see above*). Nevertheless, enzymatic digestions revealed labeling of neither DNA nor RNA from leucine incorporation.

We conclude that hot 20% TCA hydrolysis underestimates labeled protein content and that it cannot be used to determine nucleic acid labeling. Some workers use 5% TCA rather than 20% TCA to minimize the hydrolysis of protein (e.g. Simon and Azam 1989). Comparison between the two treatments showed that 5% TCA hydrolyzed less material than 20% TCA (Table 3), but DNase digestion of the hot 5% TCA precipitate revealed that DNA was incompletely hydrolyzed by this treatment (Table 3), in agreement with Riemann (1984) and Servais et al. (1987). In conclusion, none of the methods tested could be recommended for determining protein labeled by [<sup>3</sup>H]thymidine.

Robarts et al. (1986) and Robarts and

Table 4. Percentage of label loss from precipitated macromolecules with a cold 80% ethanol wash after [ $^3\text{H}$ ]thymidine and leucine incorporation. In parentheses, percentage of total cold TCA precipitate (Biétri Bay, 20 December 1989).

Treatment	Cold TCA	Hot NaOH	Hot TCA
Thymidine	15	28 (22)	48 (31)
Leucine	1.5	32 (23)	19 (9)

Wicks (1989) found that a significant fraction of thymidine label could be removed by cold 80% ethanol (up to 76% of total cold TCA precipitate) and interpreted as labeled lipids. Hollibaugh (1988) performed the same experiments and obtained a smaller amount (15–23%). In our study (Table 4), ethanol rinsing removed an average of 14.9% (range 8.1–19.4%,  $n = 7$ ) of [ $^3\text{H}$ ]thymidine label from cold 5% TCA precipitate. In four experiments, ethanol systematically extracted more labeled material on the cold TCA precipitate (avg 15.1%) than did a subsequent phenol-chloroform extraction (avg 8.4%); this is not surprising because chloroform is also a lipid solvent.

Furthermore, Robarts and Wicks reported that up to 87% of labeled molecules appearing in precipitates following acidic or basic hydrolysis were soluble in ethanol. Hollibaugh found that 10–31% of label disappeared after these treatments. In our study, three experiments also showed that ethanol removed a substantial fraction after hot NaOH and hot TCA extraction (*see example in Table 4*). In any case, these fractions were higher than those obtained directly on cold TCA precipitate. Thus, we were unable to estimate the contribution of this ethanol-extractable material in the three labeled fractions. Finally, the presence of a labeled ethanol-soluble fraction may be another source of error in estimating the labeled DNA fraction with acid–base hydrolysis.

DNase digestion showed no difference between the untreated and ethanol-washed cold TCA precipitate on the membranes (data not shown). Thus DNA is not removed by ethanol rinsing. This ethanol-extractable fraction may explain part of the label not recovered by enzymatic diges-

tions. Thus the fraction not recovered by the enzymatic procedure would be reduced to an average of 29.3% ( $n = 3$ ), which may be due in part to insufficient hydrolysis by the enzymes used. We have shown that proteinase K incompletely hydrolyzed the labeled proteins, but this finding does not exclude insufficient hydrolysis by DNase. Underestimation of labeled DNA, even if so, may not be important. Indeed, the phenol-chloroform extraction provides an overestimate (avg 41.1%,  $n = 4$ ) of labeled DNA. The proteinase treatment of this DNA revealed that a significant amount (~10% of cold TCA precipitate, and it may also be an underestimate) of the remaining label was due to proteins. Thus, if labeled DNA is underestimated with the DNase procedure (avg 17.7%,  $n = 3$ ), it cannot exceed 30% of the cold TCA precipitate in Biétri Bay.

Some of the discrepancies between methods that we report have not been observed by others. Riemann (1984) obtained reasonable agreement between enzymatic digestion and acid–base hydrolysis of thymidine-labeled macromolecules. Wicks and Robarts (1987) showed that DNA was the only labeled component present after phenol-chloroform and ethanol washes, as attested by DNase digestion. Finally, using [ $^3\text{H}$ ]adenine, Bell and Riemann (1989) found close agreement between acid–base hydrolysis (Fuhrman and Azam 1982) and phenol-chloroform extraction (Wicks and Robarts 1987). To test whether our contradictory results were characteristic of the bacterioplankton from Ebrié Lagoon, we compared the acid–base and enzymatic procedures after thymidine incorporation in a coastal seawater sample at a station not influenced by lagoon water. Although the labeled DNA contribution rose to 51% of the total labeled macromolecules, the same discrepancies were also noted (Table 5). These results suggest that the discrepancies among the different methods can be found in other ecosystems (more oligotrophic) than Ebrié Lagoon.

This study and others (Robarts et al. 1986; Servais et al. 1987) clearly show that in some habitats acid–base hydrolysis is inadequate to purify thymidine-labeled DNA. In our study, phenol-chloroform extraction also

Table 5. Enzymatic digestion and acid-base fractionation after [ $^3\text{H}$ ]thymidine incorporation in coastal seawater (Gulf of Guinea, 5 January 1990). Results are expressed as percentage of total incorporation in cold TCA material. NR—Not recovered.

Treatment	5% TCA	DNA	RNA	Proteins	NR
Enzymatic digestion	100	51.0	0.1	7.5	41.4
Acid-base procedure	100	15.3	60.9	23.8	0.0

failed to obtain pure labeled DNA in contrast with another study (Wicks and Robarts 1987). It should be noted that an otherwise efficient extraction procedure, giving DNA with a small contaminating fraction, may be inadequate for our purpose if that contaminating fraction is composed of highly labeled material. No one has demonstrated that the thymidine label is evenly distributed in the protein fraction. Proteins bound to DNA in living cells may be highly labeled and difficult to separate from DNA with standard procedures. Our study does not provide clear proof that the enzymatic extraction of DNA is really quantitative.

Although Servais et al. (1987) demonstrated complete hydrolysis of purified DNA from *Escherichia coli* by enzymatic treatment, it remains to be shown that DNase alone completely hydrolyzes the labeled DNA in a natural bacterioplankton assemblage. Nevertheless, this procedure may be considered specific, i.e. extracted material is likely to be only DNA. In this way it provides at least an underestimate of labeled DNA. The comparison with phenol-chloroform extraction shows that this underestimate, if it exists, may not be very important. We provide some evidence that treatment with proteinase K is inefficient to hydrolyze all thymidine or leucine-labeled proteins.

Thus none of the three methods tested was adequate to describe completely the partitioning of the thymidine label in bacterioplankton of Biétri Bay. The DNase procedure appears, however, to be the best available method to extract labeled bacterial DNA in this bay. This extraction procedure after thymidine incorporation was applied under a variety of environmental

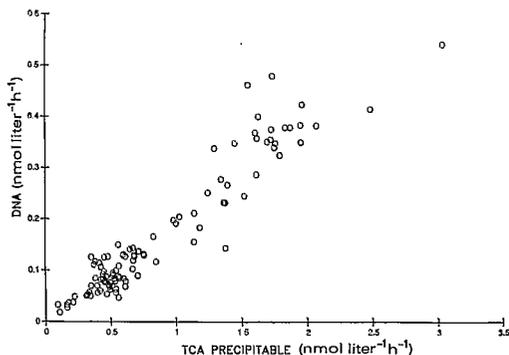


Fig. 1. Comparison of [ $^3\text{H}$ ]thymidine incorporation into DNA and into cold TCA precipitate (Ebrié Lagoon, October 1989–February 1990).

conditions in Ebrié Lagoon, with varying depth, oxygen level ( $>0$ ), salinity, trophic level, and time of day. Labeled DNA appeared to be a relatively constant fraction of the incorporated label over a wide range of total incorporation rates ( $0.1$ – $3.03$   $\text{nmol liter}^{-1} \text{h}^{-1}$ ; Fig. 1). Linear regression gives the equation

$$\text{DNA} = 0.201\text{TCA} - 0.006$$

$$(r = 0.952, n = 101).$$

The parameters DNA and TCA are expressed in  $\text{nmol liter}^{-1} \text{h}^{-1}$ .

A decrease of the DNA:TCA precipitate ratio has often been noticed when environmental conditions shift from rich to poor nutrient concentrations (Servais et al. 1987; Cho and Azam 1988); this trend has been interpreted as an adaptation to take advantage of any C or N source. We report here a labeled DNA contribution to total labeled macromolecules (20.1%) that is in the lower range of literature values for very eutrophic ecosystems. It agrees with data in mesotrophic (Riemann et al. 1982) and eutrophic (Robarts et al. 1986) limnetic ecosystems. Finally, our data suggest that, at the present time, the comparison of the DNA fractions obtained with different procedures may be problematic and that the available methods may be consistent in a given ecosystem and inadequate in others. It must be emphasized that knowledge of these discrepancies continues to be essential.

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