Vol. 19: 57–66, 1999 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published September 6

Bromodeoxyuridine as an alternative to ^{3}H-thymidine for measuring bacterial productivity in aquatic samples

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ABSTRACT: Measuring bacterial productivity with radiolabeled substrates such as tritiated thymidine (^{3}H-TdR) poses logistical difficulties and has high associated costs due to strict regulations on the transport, use, and disposal of radioactivity. The TdR analog 5-bromo-2'-deoxyuridine (BrdU) can be detected immunochemically and has been used for many years as a non-radioactive alternative for measuring DNA synthesis in cultures. The goal of this study was to determine whether a non-radioactive immunoassay for BrdU could be used to quantitatively measure bacterial productivity in natural aquatic samples. The first step was to determine the relative reliability of BrdU incorporation as an indicator of DNA synthesis in natural communities. Incorporation rates of ^{3}H-BrdU and ^{3}H-TdR in samples of coastal seawater and a freshwater lake were found to be highly correlated ($r = 0.98$, $n = 50$, $p < 0.0001$) with an average BrdU:TdR incorporation ratio of 0.71 ± 0.24 (mean ± SD). The results indicated that, despite an apparent kinetic discrimination, BrdU could accurately predict TdR incorporation over a wide range of bacterial productivity (0.45 to 349 pmol TdR l^{-1} h^{-1}). A filter-based chemiluminescent immunoassay was then developed and used to estimate BrdU incorporation in natural seawater and freshwater samples non-radioactively. Estimated rates of BrdU incorporation were within 0.5 to 30% of ^{3}H-TdR incorporation rates. The assay showed a linear chemiluminescent response spanning at least 1.5 orders of magnitude and a detection limit of ≤7 fmol of incorporated BrdU. These results suggest that a BrdU-based immunoassay has the potential to serve as a simple, sensitive, and quantitative non-radioactive alternative to ^{3}H-TdR for routine measurements of bacterial productivity in the field or laboratory.

KEY WORDS: Bacterial production · Tritiated thymidine · Bromodeoxyuridine · Non-radioactive · Immunoassay · Method

INTRODUCTION

The importance of bacteria in carbon and nutrient fluxes in the ocean is now well established (Fuhrman 1992, Ducklow & Carlson 1993, Azam 1998) and 2 of the most fundamental and commonly measured variables in aquatic microbial ecology are bacterial abundance and production. Incorporation of ^{3}H-thymidine (Fuhrman & Azam 1982) and ^{3}H-leucine (Kirchman et al. 1985, Simon & Azam 1989) are by far the most widely used method for measuring bacterial productivity. However, because these methods employ radioisotopes, there are significant, and occasionally insurmountable, logistical difficulties and constraints on their application in field studies. For example, shipboard use of ^{3}H often requires a separate, specialized laboratory, elaborate precautions for isotope use and disposal, and constant monitoring to ensure that the ship remains free of contamination. In addition, any personnel handling the isotopes are required to have specialized training in radiation safety. The relatively large quantities of ^{3}H typically used for bacterial productivity measurements also pose a significant contamination risk for other scientific studies which rely on sensitive and accurate measurements of the extremely low levels of ^{3}H naturally occurring in seawater. For cruises deploying from foreign ports, transport of radioisotopes through foreign countries is often neces-

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sary, but logistically difficult due to variable and sometimes very stringent regulations. In some cases, the use of radioisotopes may simply be prohibited, for example at field stations or on vessels (especially ships of opportunity) not licensed, designed, or equipped for radioactive work. Due to strict regulation, the costs of working with radioisotopes can also be substantial.

Despite the difficulties of working with radioactivity, this method has remained popular because it is direct, quantitative, sensitive and relatively simple in application. Non-radioactive alternatives matching these criteria have been lacking. Two approaches which have been explored are the frequency of dividing cells (FDC; Hagström et al. 1979) and, more recently, bacterial rRNA content, determined either in bulk as an RNA:DNA ratio (Kerkhof & Ward 1993) or at the single cell level (DeLong et al. 1989, Kemp et al. 1993, Poulsen et al. 1993). The FDC approach requires microscopic examination of large numbers of individual cells in each sample to determine the percentage which are in the process of dividing. FDC has been found to be roughly correlated with ^{3}H-TdR incorporation (Riemann et al. 1984), but this method has not found widespread use because it is somewhat more difficult and time consuming and not always readily interpretable in terms of growth rates for mixed assemblages (Moriarty 1986). Approaches based on RNA: DNA ratios or rRNA content have shown promise. The latter even has the potential to provide species specific growth rates. However, questions still remain about quantitatively relating RNA content to growth rates in natural mixed assemblages.

There are compelling reasons, therefore, for exploring and developing other non-radioactive methods for measuring bacterial productivity. One alternative which has not yet been exploited is the use of the TdR analog 5-bromo-2'-deoxyuridine (BrdU) which can be detected non-radioactively by a wide range of immunochemical techniques (Mazzotti et al. 1990, Dover & Patel 1994, Dakhama & Hegele 1996). BrdU incorporation into bacterial DNA has been used in numerous physical, biochemical, and molecular biological studies in a limited number of species (e.g. Hanawalt 1967, Binnie & Coote 1986, Yamamoto & Fujiwara 1990, Lewis & Errington 1997) and more recently as a qualitative indicator of DNA synthesis in natural communities (Borneman 1999, Urbach et al. 1999), but has not been reported previously as a quantitative measure of bacterial productivity.

The continuing advances in sensitivity and simplicity of non-radioactive immunoassays together with the persistent difficulties of radioisotope use in the field have made the development of a BrdU-based assay for aquatic bacterial productivity a realistic and appealing prospect. The objectives of this study were to (1) determine whether BrdU incorporation could serve as a proxy for TdR incorporation in natural aquatic bacterial communities and, if so, (2) develop a non-radioactive assay to quantify BrdU incorporation in those communities. To accomplish these objectives, the incorporation kinetics of BrdU and TdR were first compared using radiolabeled substrates. A non-radioactive assay was then developed by combining and adapting common techniques in molecular biology and immunochemistry for use with aquatic microbial communities.

MATERIALS AND METHODS

Reagents and solutions. Specialized reagents for the chemiluminescent immunoassay, which included BrdU, monoclonal anti-BrdU Fab fragments conjugated to alkaline phosphatase (anti-BrdU-AP), blocking reagent (powder), Nucleases, Incubation Buffer, and the chemiluminescent substrate CDP-Star®, were obtained from Boehringer-Mannheim. Blocking reagent was stored at room temperature (19 to 24°C), the nuclease stock as aliquots at –20°C, and all other reagents at 4°C. Nuclease stock and Incubation Buffer were obtained as components of the BrdU Labeling and Detection Kit III and their exact formulations are considered proprietary. However, the nuclease stock is known to contain exonuclease III and 1 or more restriction endonucleases, similar to original reports on the use of nucleases in BrdU detection techniques (Dolbeare & Gray 1988, Bayer et al. 1990, Dinjens et al. 1992).

Other solutions were prepared as follows: SET (20% Sucrose, 50 mM EDTA, 50 mM Tris; pH 8), Lysis Solution (1.5 M NaCl and 0.5 M NaOH), Neutralization Buffer (1.5 M NaCl, 0.5 M Tris Cl; pH 7.8), Detection Buffer (0.1 M NaCl, 0.1 M Tris Cl; pH 9.5), Maleic Acid Buffer (100 mM maleic acid, 150 mM NaCl; pH 7.5), TE (10 mM Tris, 1 mM EDTA; pH 8.0), and 10× Blocking Buffer (10% w/v Blocking Reagent in Maleic Acid Buffer). Blocking Buffer was prepared by adding Blocking Reagent to Maleic Acid Buffer, heating to 65°C in a microwave, then stirring on a hot plate to dissolve. All solutions except the Lysis Solution were autoclaved. Blocking Buffer was stored at 4°C and all others at room temperature.

Field sampling. Coastal seawater samples were collected by submersion of a weighted polycarbonate flask on a nylon line from the end of the pier at Scripps Institution of Oceanography between November 1996 and August 1998. *In situ* temperatures ranged from 16 to 22°C. Scripps Pier samples were transported directly to the laboratory and maintained at *in situ* temperature (±2°C). Samples from Mission Bay (San Diego) and

Lake Hodges (Escondido, CA) were collected by hand submersion of polycarbonate bottles at approximately 3 to 5 m from shore in August 1998. *In situ* temperature of both the bay and lake was 29°C. Bay and lake samples were transported to the laboratory in an insulated chest at ambient temperature (ca 24°C) and used in labeling experiment within 3 h of collection. All samples were obtained from 0 to 0.5 m depth.

Productivity measurements with radiolabeled substrates. ^{3}H-TdR (DuPont NEN) and ^{3}H-BrdU (Moravek Biochemicals) incorporation assays were performed according to the microcentrifugation protocol of Smith & Azam (1992). Substrates were used at 20 nM final concentration except in saturation experiments where concentrations varied as illustrated in the results. Samples were kept in the dark as much as practical during incubations. Incubation temperatures were within 2°C of *in situ* temperature with the exception of the samples from Mission Bay and Lake Hodges which were incubated at ca 5 to 6°C below *in situ* temperature. For endpoint assays, triplicate samples and 1 blank (1 or 1.7 ml each) were incubated for approximately 1 h in microcentrifuge tubes. Blanks had trichloroacetic acid (TCA) added at T_0 (5% final). For time course assays, samples (5 to 10 ml) were incubated in duplicate or triplicate in polystyrene snap-cap tubes. At 4 or 5 time points, 1 ml subsamples were transferred to microcentrifuge tubes containing excess unlabeled thymidine (100 µM final) and stored on ice until the last time point had been sampled. Subsamples were then processed as usual by the microcentrifugation protocol.

Preparation of unlabeled and BrdU-labeled DNA standards. Two carboys containing coastal seawater were incubated for 16 h at 18°C with either no addition or with addition of 20 nM BrdU and 0.2 nM ^{3}H-BrdU. After incubation, seawater from the carboys was filtered through separate 0.22 µm Sterivex filters (Millipore) via peristaltic pump. DNA was extracted from the filters following a procedure modified from Sommerville et al. (1989). Briefly, 1.8 ml of SET buffer containing 5 mg ml^{-1} lysozyme was added to each filter. After sealing the ends, filters were incubated at 37°C for 1.5 to 2 h. Filters were then supplemented with 180 µl of Proteinase K (20 mg ml^{-1} stock solution in water) and 100 µl SDS (10% stock) and incubated at 60°C for 2.5 h. The extraction buffer was drawn from the filters using a syringe and transferred to 15 ml Corex tubes. Proteins were precipitated by addition of 0.5 vol ammonium acetate (7.5 M stock) and centrifugation at 10000 × *g* for 8 min. The supernatant was transferred to fresh Corex tubes and purified by ethanol precipitation, phenol:chloroform extraction, chloroform extraction, and finally concentrated by a second ethanol precipitation all following standard procedures (Sambrook et al. 1989). The DNA pellets were resuspended in TE and stored at 4°C. Total DNA was measured by fluorometry using picogreen (Molecular Probes) and BrdU incorporation was determined by counting aliquots in a scintillation counter. The concentration of incorporated BrdU was calculated using the measured incorporated activity and the known specific activity of the BrdU used for labeling.

For experiments conducted 2 mo after the initial preparation of BrdU-labeled DNA, the standard was processed by diafiltration on a Microcon 100000 molecular weight cut-off spin ultrafiltration unit (Millipore) to remove any degradation products. A 30 µl aliquot of BrdU-labeled DNA was diluted to 500 µl in TE and concentrated to about 20 µl. The retentate was then diluted again to 200 µl and reconcentrated. Dilution and reconcentration steps were repeated once more and the final retentate of about 5 to 20 µl was recovered. The centricon unit was rinsed twice with 140 µl of TE and the rinses pooled with the original retentate (final volume ca 300 µl). The new concentration of incorporated BrdU was determined by scintillation counting of triplicate 10 µl aliquots. Standard curves were prepared by diluting BrdU-labeled standard DNA in a background of unlabeled DNA. Total DNA content in each standard was 1 ng with BrdU content varying between 0.18 to 0 pmol.

General blotting procedures. Nylon membranes (0.22 µm pore size; MagnaGraph, MSI) and a single backing sheet of chromatography paper were wetted with water then 6 × SSC and mounted in a slot blotter (BRL) or dot blotter (Schleicher and Schuell). Samples were filtered at ≤200 mm Hg vacuum. Except for nuclease digestion, all post-filtration treatments of blots were accomplished by incubating the membranes face up on 3 sheets of chromatography paper (Whatman 3MM) saturated with the indicated solutions. For nuclease treatment only 1 sheet of paper was used to conserve reagents. In addition, the membrane was first briefly laid face down on the paper (to ensure access of the nucleases to the upper surface of the membrane) then incubated face up in the usual manner. Following filtration and post-filtration treatments, blots were placed between 2 sheets of chromatography paper and baked for 1 to 2 h at 80 to 90°C under vacuum. Blots were then stored in a dessicator to await further processing.

DNA denaturation test. To allow antibody access to the incorporated BrdU, the DNA must first be rendered single-stranded. Methods employing denaturation by heat, acid, or alkali as well as partial digestion with nucleases were tested. Extracted DNA standard (prepared as described above) containing 2 pmol of BrdU was slot blotted in 7 wells with an equal mass of unlabeled DNA blotted in parallel as blanks. After filtering,

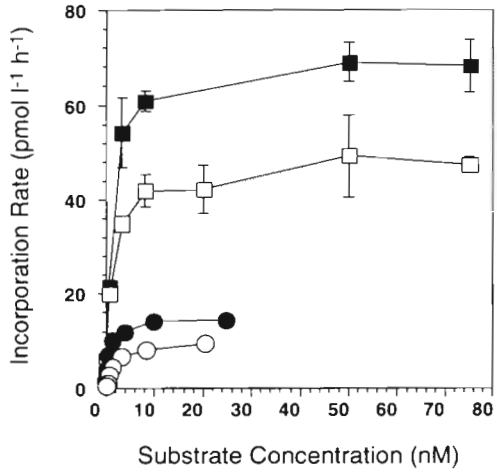

Fig. 1. Saturation curves for incorporation of TdR (filled symbols) and BrdU (open symbols) in coastal seawater tested on 17 March 1997 (circles) and 24 April 1997 (squares)

the blot was cut into pieces for separate treatments. Methods A to C were done in duplicate and D with no replication. Membrane incubations were performed at room temperature (ca 20 to 24°C). The methods were: (A) Heat and Alkali: DNA was boiled 10 min, then chilled on ice prior to blotting. After filtering, the blot was incubated on Lysis Solution for 10 min, then on Neutralization Buffer for 10 min. (B) Nuclease Digestion: The blot was equilibrated for 10 min on Incubation Buffer, then on Incubation Buffer plus Nucleases (100:1) for 30 min, then on TE. (C) Acid: The blot was incubated on 4 N HCl for 1 h, then on Neutralization Buffer for 10 min. (D) Alkali: The blot was incubated on Lysis Solution for 10 min, then on Neutralization Buffer for 10 min.

Productivity measurements using BrdU. Duplicate or triplicate samples with 20 nM BrdU were incubated and subsampled as described above for the radiotracer-based, time course productivity assays. After termination of the last time point, samples were directly filtered in a blotting manifold. Blots were incubated on Lysis Solution for 10 min, on Neutralization Buffer for 10 min, on Incubation Buffer for 5 minutes, on Incubation Buffer plus Nucleases (100:1) for 30 min, and finally on TE for 5 min. Processed blots were baked and stored as described above.

Immunochemical detection. Baked blots were wetted with water, then incubated with shaking in 1× Blocking Buffer (diluted in Maleic Acid Buffer from 10× stock) for 1 h, then in 1× Blocking Buffer plus anti-BrdU-AP (100:1) for 1 h. Blots were rinsed briefly with 5 to 10 ml of Maleic Acid Buffer containing 0.3% Tween 20, followed by two 15 to 30 min washes in 10 to 20 ml of the same buffer and, finally, 2 washes in 10 to 20 ml Maleic Acid Buffer for 15 to 20 min each. Blots were equilibrated in Detection Buffer for 5 min then incubated for 10 min in Detection Buffer plus CDP-Star® (100:1). Membranes, still moist with chemiluminescent reagent, were wrapped in plastic film (Saran Wrap), allowed to sit for 5 to 10 min, then exposed to x-ray film (Hyperfilm, Amersham). Film was processed in an automatic developer.

Signal quantification. In some cases, chemiluminescent signal was quantified by scanning x-ray film images on a flat bed scanner (DuoScan, Agfa) using transmitted light to obtain a digital image. Spot intensity was then determined with image analysis software (RFLPScan; CSP, Inc.). In other cases, after exposing films for visual documentation, blots were cut into pieces and the chemiluminescent signal from individual spots determined by photometry using an ATP photometer (SAI Technology). Signal intensity was converted to pmol of incorporated BrdU based on the quantification of standard curves on each blot.

Statistics. Curve fitting for the plot of ^{3}H-BrdU versus ^{3}H-TdR incorporation rates was done by Model II linear regression. To calculate incorporation rates in time course assays, T_0 values were subtracted from each time point. Substrate incorporation rates were then calculated as the slopes of substrate incorporated versus time using Model I linear regressions forced through zero. To test for differences between incorporation rates, a 2-tailed *t*-test for comparing slopes of regression lines was employed as described by Zarr (1996).

RESULTS

Saturation curves

In 2 experiments conducted on different days with coastal seawater, incorporation of both ^{3}H-TdR and ^{3}H-BrdU showed saturation at approximately 10 nM (Fig. 1). Although saturating at the same external concentrations, the maximum rate of incorporation was lower for ^{3}H-BrdU than for ^{3}H-TdR with BrdU:TdR ratios of approximately 0.6 and 0.7 for the 2 experiments.

Correlation of TdR and BrdU incorporation rates

To test the consistency of relative incorporation rates of BrdU and TdR, measurements were made using samples collected over a period of 2 yr and a wide range of trophic conditions. During the course of the sampling period, bacterial abundance ranged from 0.9 to 6.9 × 10^9 cells l^{-1}, bacterial productivity from 0.45 to 349 pmol TdR l^{-1} h^{-1} and chl *a* from 1.4 to 166 µg l^{-1} (Fandino et al. 1998, this study). Incorporation rates of

^{3}H-BrdU and ^{3}H-TdR were found to be highly correlated (Fig. 2; r = 0.98, n = 50, p < 0.0001). Linear regression of BrdU versus TdR incorporation rates yielded a slope of 0.69 with 95% confidence limits of 0.64 and 0.72. Molar incorporation ratios (BrdU: TdR) for individual samples ranged from 0.32 to 1.89 with an average of 0.71 ± 0.24 (mean ± SD).

DNA denaturation

In developing the immunoassay protocol a variety of different methods for making the incorporated BrdU accessible to the antibody were tested. Nuclease digestion of the blotted DNA resulted in a strong chemiluminescent signal compared to denaturation by heat, acid, or alkali, which in this test yielded no detectable signal (Fig. 3A). Nuclease digestion was therefore incorporated into the complete immunoassay protocol and shown also to be effective for detecting BrdU incorporation in a natural sample (Fig. 3B).

Incorporation rates in natural samples

The non-radioactive immunoassay was compared to the radioactive microcentrifugation assay using natural seawater and freshwater samples. In the first experiment, water from Scripps Pier was labeled with either BrdU or ^{3}H-BrdU. A film image produced from the chemiluminescent immunoassay of duplicate samples collected at 5 time points and a dilution series of BrdU-labeled standard DNA is shown (Fig. 4). In this experiment, the chemiluminescent signal was quantified by direct photometry as well as by densitometry of the film image. The estimated incorporation rates (reported as mean ± SD pmol l^{-1} h^{-1}) were 32.5 ± 3.4 (photometry), 29.2 ± 1.7 (densitometry), and 32.3 ± 0.9 (radioactive microcentrifugation assay). Rates deter-

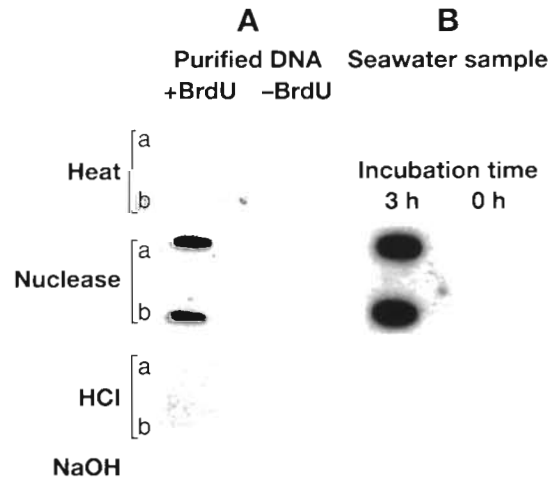

Fig. 3. Film image of chemiluminescent signal obtained in tests of the BrdU immunoassay. (A) Test of different DNA denaturation and nuclease digestion methods using unlabeled and BrdU-labeled DNA standards. DNA filtered in each well was 0.4 ng with labeled samples containing 2 pmol incorporated BrdU. (B) Test of the alkaline lysis/nuclease digestion protocol for detecting BrdU incorporation in seawater labeled for 0 and 3 h. Incubations were in duplicate with 0.5 ml filtered per well

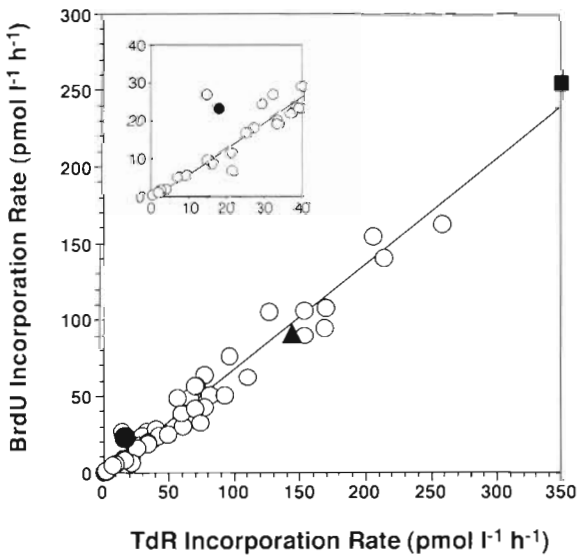

Fig. 2. Correlation of BrdU and TdR incorporation rates (r = 0.98, n = 50, p < 0.0001). Samples are coastal seawater collected at Scripps Pier (circles) and Mission Bay (triangle) and freshwater collected from Lake Hodges (square). Filled symbols indicate data from the field test in Fig. 6. The line shown is a Model II linear regression ($y = 0.69x - 0.81$). Inset shows an expanded view of the plot for 40 pmol l^{-1} h^{-1} and below

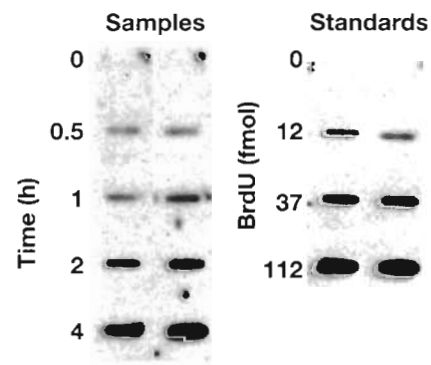

Fig. 4. Film image of chemiluminescent signal obtained in a BrdU-labeling time course of coastal seawater and a dilution series of BrdU-labeled DNA standard. Samples are from duplicate incubations and standards are duplicate blots from a single dilution series

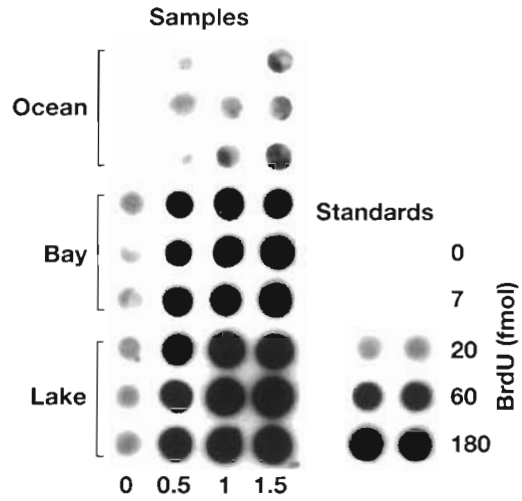

Fig. 5. Film image of chemiluminescent signal from BrdU-labeling time courses for samples collected at Scripps Pier (Ocean), Mission Bay (Bay), and Lake Hodges (Lake) and a dilution series of BrdU-labeled DNA standard

mined non-radioactively by photometry and radioactively by scintillation counting were essentially identical, but with higher variance in the former. Densitometric analysis of the film resulted in only a slightly (11%) lower estimate. None of the rates were significantly different at a 95% confidence level.

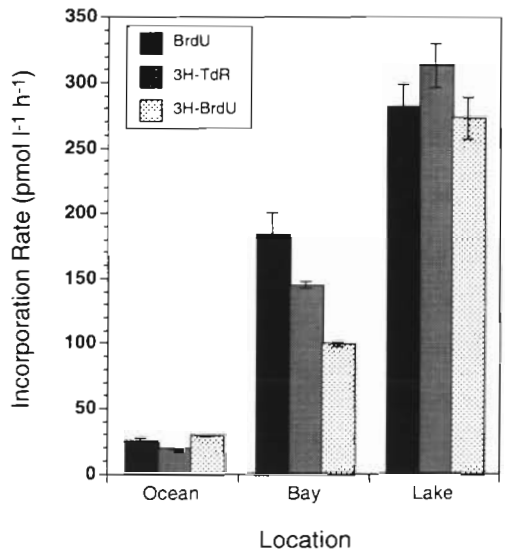

Fig. 6. Comparison of BrdU incorporation rates (measured by the chemiluminescent immunoassay) with rates for ^{3}H-TdR and ^{3}H-BrdU (measured by the microcentrifugation assay and scintillation counting). Samples are from the same environment as in Fig. 5. Rates were calculated as the slope of regression lines of pmol l^{-1} versus time for 4 time points. Time points were assayed in triplicate for each method. Error bars are the standard errors of the regressions

In a second experiment, the immunoassay was tested with samples from 3 different environments using coastal seawater collected at Scripps Pier and Mission Bay, and freshwater from Lake Hodges. Chemiluminescent signal was recorded on film (Fig. 5) and also quantified by direct photometry. Signal intensity was converted into pmol of BrdU from the standard curve. BrdU incorporation rates estimated by immunoassay were compared to measured incorporation rates of ^{3}H-TdR and ^{3}H-BrdU (Fig. 6). As in the previous field test, non-radioactive measurement of BrdU incorporation closely matched ^{3}H-BrdU estimates in the ocean and lake samples. In Mission Bay, however, the rates were significantly different (t-test, $\alpha = 0.05$). In each environment, incorporation rates of BrdU determined by immunoassay differed by ≤24% from the incorporation rates of ^{3}H-TdR and differences were not statistically significant (t-test, $\alpha = 0.05$).

DISCUSSION

Incorporation kinetics

Incorporation rates of ^{3}H-BrdU and ^{3}H-TdR saturated at similar concentrations and were strongly correlated, indicating that BrdU can serve as a reliable substitute for thymidine for DNA synthesis measurements in seawater. However, the BrdU:TdR incorporation ratio was significantly less than 1, indicating some discrimination in BrdU incorporation. One possible explanation is that this apparent discrimination was an artifact resulting from differences in substrate metabolism. The tritiated thymidine and BrdU substrates were labeled at different positions (*methyl-*, vs 6-, respectively) which could result in different levels of non-specific incorporation (Hollibaugh 1988). Non-specific incorporation was not measured in these assays so this possibility cannot be ruled out. The differences may also represent real kinetic discrimination between the substrates. Preferential incorporation of TdR over BrdU has been observed in *Escherichia coli* (Hanawalt 1967), but the effect was found to decline with increasing ratios of BrdU:TdR in the medium and with increasing temperature. In the present study, incorporation assays were carried out with saturating BrdU concentrations and no added TdR at temperatures ranging from 16 to 24°C. At least under these conditions, any effect of temperature appeared to be small relative to other sources of error in the assay, but explicit tests for possible temperature effects would be useful.

In studies with *Bacillus subtilis* (Coote & Binnie 1986), some mutant strains could apparently discriminate against BrdU uptake into the cell. In both mutant and wild type strains, there also appeared to be discrimination against BrdU by thymidine kinase, but not

DNA polymerase. Which, if any, of these mechanisms operate in natural communities under the conditions used in our experiments is unknown. From a practical standpoint, this may be a moot point given the predictabilty of BrdU incorporation over a wide range of conditions. However, direct empirical calibrations of unlabelled BrdU incorporation relative to cell proliferation in more diverse environments will ultimately be needed to properly evaluate the general applicability of this assay in field studies.

Sample processing

An important step in the BrdU immunoassay is denaturation or partial degradation of the DNA to allow the antibody access to the incorporated BrdU. In BrdU immunoassays, DNA denaturation has been accomplished by heating (Moran et al. 1985) and treatment with acid (Mazotti et al. 1990, Lewis & Errington 1997). Another typical denaturation procedure used when blotting DNA uses alkali (Anderson & Young 1985). With these procedures, renaturation of the DNA may occur once the denaturant is removed (Raap et al. 1986). To overcome this problem a nuclease treatment was adopted which irreversibly creates stretches of single-stranded DNA (Dolbeare & Gray 1988, Bayer et al. 1990, Dinjens et al. 1992). These latter reports describe the use of Exonuclease III alone or in combination with one or more endonucleases (restriction enzymes). Unfortunately, the stock of nuclease(s) used in this study was obtained as a commercially available kit component whose exact composition is considered proprietary information. Therefore, the exact enzymes and concentrations used in this study are not known. For those wishing to test the performance of known enzyme mixtures, the literature cited above will serve as a useful starting point.

Although BrdU incorporated into DNA was readily detected after heat denaturation in several initial tests (data not shown), the direct comparison with other methods (Fig. 3) suggested that nuclease digestion was superior to the other procedures tested and was thus adopted for the field studies. It should be noted, however, that no attempt was made to optimize each protocol and the other methods of denaturation might also be suitable using conditions different from those employed here. Preliminary results have suggested that the nuclease digestion could be carried out simultaneously with antibody binding (data not shown) which could decrease processing time. This strategy has been previously employed (Dinjens et al. 1992) and is the recommended protocol in some commercially available cell proliferation assays (Boehringher Mannheim).

The typical procedure for immobilizing DNA on nylon membranes is UV cross-linking, but membranes in this procedure were baked due to the possibility of UV-induced debromination (Hutchinson & Köhnlein 1981). Other researchers have used UV cross-linking to membranes in BrdU immunoassays (Dakhama & Hegele 1996, Haider et al. 1997), but possible effects of UV on the sensitivity have not been tested. If brief UV irradiation can be used without significant decreases in sensitivity, this would also significantly reduce the processing time.

Sensitivity

A conservative estimate of the detection limit of the immunoassay is provided by the standard curve in the field test (illustrated in Fig. 5). The lowest dilution in the standard curve was at least 3 to 10 times above background (determined by photometry), indicating that the detection limit using this protocol is ≤7 fmol incorporated BrdU. Indeed, with this protocol we readily detected BrdU incorporation in 0.5 ml of coastal Pacific seawater in only 30 min, which was equivalent to 6 fmol BrdU. If we assume that filtration volumes could reasonably be increased to 5 ml and that incubation times of 6 h are acceptable, this should allow detection of ca <0.2 pmol l^{-1} h^{-1}. At this level of sensitivity, productivity should be readily measurable even in oligotrophic environments. However, the practical detection limit of the assay will ultimately have to be determined empirically in the field.

Precision and accuracy

With the present protocol, the variance of triplicate measurements for any given time point was significantly higher than obtained with radiotracers. However, the error of a rate measurement is reduced when using regression of multi-point time courses rather than simple end point assays. Further improvements in methodology may overcome some of this variability. However, even with the reduced precision relative to radiotracer approaches, our results suggest that in many cases the non-radioactive BrdU immunoassay approach may provide reasonably accurate estimates of bacterial productivity relative to ^{3}H-TdR. In field tests, the BrdU immunoassay-based estimates were within 0.5 to 30% of the ^{3}H-TdR estimates. It should be noted that errors in both ^{3}H-TdR and BrdU will contribute to the observed discrepancies. Although the ^{3}H-TdR assay is clearly more precise, it is not actually possible with the current data to determine which, if either, method provided more accurate estimates of DNA synthesis.

Standardization

A reliable standard is critical to obtaining accurate estimates of productivity with the immunoassay. Chemiluminescent signal obtained from the assay can vary substantially in absolute terms due to many factors. These would include the temperature at which the chemiluminescent reaction is performed as well as varying quality of the reagents (e.g. nucleases, antibody and substrate may vary from batch to batch or decline in activity with age). Variability in these factors must be compensated by normalizing the signal to that obtained from a DNA standard with a known BrdU content.

For convenience, the DNA standard used in this study was labeled with BrdU and trace amounts of ^{3}H-BrdU. For a truly non-radioactive assay, standard DNA labeled only with BrdU can easily be prepared. At some point, however, the specific BrdU content of the standard (moles BrdU per mass of DNA) must be determined. For situations where radioisotopes can be used in the laboratory, a non-radioactive standard could be calibrated against a tracer-labeled standard such as used here. Although radioisotopes would not be completely eliminated in this scenario, this does allow isotope use to be restricted to occasional standard calibration; routine use of the assay could be carried out non-radioactively. In situations where radioisotope use must be completely eliminated, other non-radioactive assays for BrdU content such as high performance liquid chromatography (Stratford & Dennis 1992) or gas chromatography-mass spectrometry (Stetson & Maybaum 1986) might be employed.

After calibration of a BrdU-labeled DNA standard, care must be taken to avoid degradation. With degradation, a smaller fraction of the filtered standard DNA (and thus BrdU) will be immobilized on the blotting membrane. This would result in an apparent decline in the signal per BrdU in the standard and, ultimately, an overestimation of BrdU incorporation in the samples. Some degradation was observed in the DNA standard used in this study after storage for several months in TE. The degradation was detected by comparing standard curves before and after removal of degradation products (data not shown). Assuming the original specific BrdU content of a standard is known, corrections for degradation can be readily accomplished simply by re-measuring the DNA content of the standard after removal of degradation products. To minimize degradation, standard DNA solutions should be stored refrigerated in TE and shielded from light. Large batches of calibrated BrdU-labeled DNA standard could be stored for longer periods as frozen, lyophilized aliquots.

Economy

The immunoassay described here is relatively simple, inexpensive, and amenable to application at sea or in the field. The minimum processing time for the immunoassay is substantially longer than for other methods, but much of that time is consumed by incubations during which the investigator may attend to other tasks. In addition, there is economy of scale with the immunoassay, since multiple blots holding many samples can be handled simultaneously with little increase in processing time. Although some samples in this study were quantified one at a time in a simple photometer, imaging an entire blot with x-ray film also worked well and was very quick. Instruments are available which can quantify chemiluminescent signal for all samples on a blot simultaneously. Therefore, the assay does not necessarily require a significant increase in labor, and could actually save time and effort for very large numbers of samples such as generated on long oceanographic cruises with an intensive sampling program.

Using current list prices for all reagents, and excluding capital equipment expenses, the estimated cost per sample for the immunoassay is roughly half of that for radiolabeling. The largest contributor by far to the expense of radiolabeling is the cost of the ^{3}H-TdR. In contrast, the cost of BrdU is trivial and the largest expense in the immunoassay is for the chemiluminescent substrate. If a discounted contract price for ^{3}H-TdR (available to some large institutions) is applied instead of list price, then the costs of the assays are roughly comparable.

Other potential advantages and disadvantages of BrdU

Other possible advantages of BrdU are its likely greater stability and perhaps greater specificity as a tracer. With prolonged storage of ^{3}H-TdR, labeled degradation products will form over time which will incorporate with kinetics different from thymidine. Even with fresh stocks there is the potential for metabolism of ^{3}H-TdR once it has been taken up by the cell so that macromolecular pools other than DNA can also become labeled (Moriarty 1986, Hollibaugh 1988). Concern with this problem led to the development of an extraction procedure (Wicks & Robarts 1987) in an attempt to ensure measurement of ^{3}H incorporated specifically into DNA. With BrdU-based immunodetection, the specificity of the antibody for BrdU would presumably ensure that brominated degradation products, whether produced during storage or by cell metabolism, would not be detected if incorporated into

other compounds. However, to our knowledge this has not been specifically tested.

Another very promising aspect of BrdU labeling is the potential to estimate DNA synthesis rates of individual bacteria by immunocytochemical fluorescence microscopy. Recent work has clearly demonstrated the ability to visualize BrdU-labeled DNA in cultured bacteria (Lewis & Errington 1997, Urbach et al. 1999). With continuing improvements in both signal amplification (e.g. Van heusden et al. 1997) and imaging (e.g. with cooled CCD cameras and image processors), detection of BrdU incorporation even by slowly growing individual marine bacteria may ultimately be feasible.

The antigenicity of BrdU can also been used to separate BrdU-labeled from unlabeled DNA by immunoprecipitation (Haider 1997). This technique has recently been exploited to demonstrate group-specific variability in BrdU incorporation in natural bacterial assemblages (Borneman 1999, Urbach et al. 1999). In principle, BrdU-labeling could also be combined with *in situ* rRNA probing (Amann et al. 1995), immunomagnetic cell separation (Bard & Ward 1997), or reverse genome probing (Pollard 1998) as alternative means to measure group-specific incorporation in complex assemblages.

It is important to note that BrdU is not completely free of problems. Some disadvantages are: (1) BrdU, though not radioactive, is a potential mutagen via radiosensitization (Szybalski & Djordjevic 1959) or other genotoxic effects (Morris 1991). Deleterious effects, however, have only been reported at high concentrations and/or chronic exposure. Typically BrdU is considered to have low toxicity (Haider et al. 1997) and is even administered in humans *in vivo* (e.g. Marchal et al. 1997). Nevertheless, this compound should be handled with due care. (2) BrdU causes photosensitization (Hutchinson & Köhnlein 1981, Yamamoto & Fujiwara 1990) so bacterial productivity assays may need to be carried out with minimal light exposure. In most situations this would be of minor consequence, since dark incubations are typically done in bacterial productivity assays. However, light incubations are sometimes desired and investigation of the conditions under which BrdU photosensitization can occur would be valuable. (3) As noted above, the labeling kinetics of BrdU relative to TdR may be affected by temperature. Whether this is actually a problem under the conditions employed for assays of aquatic bacterial productivity has yet to be determined. However, for the temperature range covered in this study, there was relative uniformity in BrdU:TdR incorporation ratios for widely differing environments. This suggests that even if more extreme temperatures are found to significantly alter the relative kinetics of BrdU incorporation, it is likely to be a systematic, predictable and therefore correctable effect.

CONCLUSION

In order for the BrdU immunoassay to be incorporated as a routine technique for measuring bacterial productivity, the reliability and accuracy of the immunoassay must be further investigated under the wide range of environmental conditions likely to be encountered in the field. However, this study clearly demonstrates the potential for BrdU to serve as a practical, economical, non-radioactive alternative to ^{3}H-TdR for measurement of bacterial productivity in natural aquatic systems. With further development, this method should greatly ease some of the logistical constraints on field measurements of bacterial productivity.

Acknowledgments. We are grateful to J. Ray for inspiring this investigation and for sharing advice on methodology. We also thank L. B. Fandino for providing field measurements of ^{3}H-TdR and ^{3}H-BrdU and gratefully acknowledge J. T Hollibaugh, H. W. Ducklow, and 3 anonymous reviewers for suggestions to improve the manuscript. This work was supported by an NSF Small Grant for Exploratory Research (OPP 97-08153) awarded to F.A.

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Editorial responsibility: Fereidoun Rassoulzadegan, Villefranche-sur-Mer, France

Submitted: September 24, 1998; Accepted: January 8, 1999
Proofs received from author(s): August 13, 1999