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An evaluation of fluorescence techniques for measuring DNA and RNA in marine microorganisms

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ABSTRACT: The DNA-specific fluorochrome Hoechst 33258 (H33258) and the DNA- and RNA-sensitive fluorochrome ethidium homodimer (EthDi) were used to detect ng ml^{-1} concentrations of nucleic acids in marine microorganisms. These fluorochromes were 4 to 5 times more sensitive to DNA than ethidium bromide, and their use significantly reduced the amount of sample necessary to measure nucleic acids in seawater. Both fluorochromes responded in a linear fashion to standard DNA, but the response of EthDi to RNA was non-linear for the majority of RNA types tested. Chlorophyll *a* quenched fluorescence of both fluorochromes, especially of EthDi, at concentrations that could be encountered in sample homogenates. Two techniques were used to measure DNA and RNA on cultures of marine bacteria and phytoplankton; H33258 and EthDi together (double fluorochrome technique), and EthDi with and without RNase (RNase digestion method). In general, DNA and RNA were measured equally well using either technique; discrepancies between techniques were attributed to EthDi quenching or the specificity of H33258 for DNA rich in dA-dT base pairs. The double fluorochrome technique was used to determine depth profiles of DNA and RNA/DNA in size-fractionated natural seawater These properties showed significant changes between the surface and 100 m and may be related to changes in biomass or metabolic activity. Although consideration must be given to the RNA standard used and the presence of naturally occuring pigments, the combination of H33258 and EthDi can provide a rapid and sensitive measure of nucleic acids in seawater without the use of nucleases.

INTRODUCTION

To understand plankton abundance and dynamics, some investigators have used fluorescent dyes to measure particulate DNA (Takahashi et al. 1974, Paul & Carlson 1984, Paul et al. 1985). To obtain additional information, especially about metabolic activity, fluorometric and absorbance measurements of RNA have also been made (Takahashi et al. 1974, Dortch et al. 1983, Moyer & Morita 1989). However, previous methods to measure RNA were relatively insensitive, or too elaborate for rapid analysis (Randerath & Randerath 1969, Thoresen et al. 1983, Iturriaga et al. 1984). Some fluorochromes, such as ethidium bromide (EthBr), react with nucleic acids generally and thus require a digestion step to distinguish RNA from DNA. A dimer of EthBr, ethidium homodimer (EthDi), has been developed (Gaugain et al. 1978a) with much improved sensitivity for DNA and RNA (Markovits et al. 1979, Reinhardt et al. 1982, Moyer et al. 1990). We investigated the use of this fluorochrome with a digestion step or in conjunction with the DNA-specific fluorochrome Hoechst 33258 (H33258) to measure DNA and RNA in marine microorganisms. The results suggest that the combination of H33258 and EthDi provides a sensitive method to measure nucleic acids in marine microorganisms.

MATERIALS AND METHODS

Emission scans with DNA were measured for each fluorochrome separately and with the 2 fluorochromes combined to determine if significant interference occured between the fluorochromes. The DNA response of H33258 and EthDi was also compared to the response of EthBr, a fluorochrome often used to measure nucleic acids. Several types and preparations of RNA were measured with EthDi to determine an appropriate RNA standard. The response of H33258 and EthDi with DNA was measured in the presence of standard chlorophyll *a* (chl *a*), which is known to com-

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plicate EthBr measurements (Thoresen et al. 1983). The double fluorochrome method was used to measure nucleic acids in cultures of marine bacteria at different stages of starvation-survival and in batch cultures of phytoplankton. The results were compared to measurements made using an RNase digestion method which used EthDi on subsamples with and without RNase (Thoresen et al. 1983). Natural seawater samples were also measured for DNA and RNA using the double fluorochrome method. A technique similar to the double fluorochrome method but using H33258 with EthBr to measure DNA and RNA in fish larvae has been described by Clemmesen (1988).

Chemicals. The nucleic acid stains used were Hoechst 33258 (H33258-Calbiochem, San Diego, CA, USA), ethidium homodimer (EthDi-Molecular Probes, Eugene, OR, USA) and ethidium bromide (EthBr). The standard nucleic acids used were calf thymus DNA type I (Sigma, St. Louis, MO, USA), bakers yeast RNA type III and type XI (Sigma), *Escherichia coli* strain B RNA (Calbiochem), *E. coli* strain W transfer RNA (tRNA) and ribosomal RNA (rRNA) (Sigma), and bovine liver rRNA (Sigma). *Anacystis nidulans* chl *a* was from Sigma, and RNase (DNase-free), heparin and Triton X-100 were from Calbiochem.

Solutions and standards. All glassware used was precombusted at 480 °C to remove any nuclease contamination. The buffer solutions used were 0.05 M Tris, 0.05 M NaCl, pH 7.3 (TN buffer) or 0.05 M Tris, 0.05 M NaCl, 0.001 M EDTA, 1 % NaN_3, pH 7.3 (TNEA buffer). Except where noted, TNEA buffer was used with cultures and TN buffer for natural samples. Primary stock solutions of H33258 (0.19 mM) were made in distilled, deionized water. Working stock solutions of H33258 (3.8 µM) were made fresh daily by diluting the primary stock solution in buffer. Primary stock solutions of EthDi (47 µM) and EthBr (74 µM) were made in buffer containing 1 % methanol. Concentrations of EthDi and EthBr working stock solutions were 12 µM. All solutions were stored in the dark at 4 °C. Under these conditions, EthDi was stable for months.

The concentrations of all nucleic acid standard stock solutions were determined gravimetrically, except for the rRNAs in which the concentrations were determined from the absorbance to weight conversions provided by Sigma. Stock solutions of standard DNA and *Escherichia coli* strain B RNA were made in TN buffer and portions of these stock solutions were stored frozen in sterile scintillation vials. Stock solutions of other RNA standards were made in TNEA buffer and used immediately. Standards were measured by diluting the standard stock solutions with buffer to concentrations representative of the culture and sample homogenates. Each day that samples or cultures were measured for nucleic acids, the fluorescence response of 3 standards was also determined; standard DNA with H33258, standard DNA with EthDi, and *E. coli* strain B RNA with EthDi. Previous experiments showed that these standards had fluorescence responses independent of sonication and of treatments with Triton X-100 or heparin. Stock solutions of Triton X-100 were 0.5 % (v/v) in buffer. RNase and heparin stock solutions were made fresh daily in buffer at 10 and 5 mg ml^{-1} respectively. Solutions of chl *a* were made by dissolving and diluting chl *a* in methanol, and were used immediately.

Cultures. A gram-negative bacterial rod (OR5-88) was isolated from a seawater sample taken off the Oregon coast (44° 39.0′ N, 125° 59.6′ W) in September 1988 from 5 m using the bow-pump of the RV 'Wecoma' during cruise W8808. OR5-88 was maintained in batch culture on a rotary shaker at 11 °C using SLX medium (Moyer & Morita 1989). Two chemostats were assembled according to Moyer & Morita (1989), inoculated with log phase cells, and maintained at 11 °C with SLX medium. Constant growth was assumed after 4 l passed through the chemostats (chemostat volume = 345 ml). Doubling rates, estimated from chemostat dilution rates, were 0.5 d^{-1} and 0.0201 h^{-1} for replicate chemostats. Cells from each chemostat were harvested by centrifugation, rinsed 3 times in artificial seawater and starved separately in the dark at 11 °C in aged seawater. The aged seawater had been collected from 2000 m and stored in the dark for 2 yr before use. Replicate measurements of DNA and RNA were made on each starved culture on 9 different days of starvation up to 121 d.

Phytoplankton cultures were provided by T. Cowles and S. Neuer (Oregon State University) and included *Gymnodinium simplex, Thalassiosira weissflogii, Chlorella vulgaris, Asterionella japonica, Chaetoceros* sp., and BB1, an unclassified algae from Station P isolated by B. Booth (University of Washington). Phytoplankton cells were grown in batch culture (IMR/4 media, Eppley et al. 1967) under continuous fluorescent light from 8 Sylvania 60W bulbs at 12 °C. Replicates of each culture were measured for DNA and RNA as discussed below Because these cultures were only used to compare techniques of measuring nucleic acids, samples were used without regard to the stage of growth of the cultures.

Treatments. Cultured cells (1 to 6 ml) were pressure-filtered on 0.2 µm (bacteria cultures) or 1.0 µm (phytoplankton cultures) Nuclepore filters at < 7 psi (4.7×10^4 Pa). All filters, including filters for 0.2 µm or 1.0 µm blanks, were pre-rinsed with 15 ml of buffer. Treatments of cell homogenates and blanks were identical. The filters were placed in individual 15 ml polypropylene centrifuge tubes on ice containing 8.0 ml buffer and 40 µl of Triton X-100. Cells on Nuclepore filters were disrupted on ice with pulsed sonication for 90 s using a

Branson W-350 power source with a Heat Systems 1/8" (0.32 mm) microtip assembly at 5.0 output with 60 % duty (Paul & Myers 1982). Heparin was added (40 µl) to phytoplankton homogenates to displace nucleohistones which are known to block DNA fluorochrome binding sites in eukaryotes (Karsten & Wollenberger 1977, Bonaly et al. 1987). RNA was digested in a 2.6 ml homogenate subsample by adding 5 µl of RNase. Homogenates with heparin and subsamples with RNase were incubated at 40 °C for 20 min. Homogenate subsamples digested with RNase were measured with EthDi while homogenates without added RNase were measured with EthDi and H33258 as discussed below.

Fluorescence measurements. Fluorescence measurements of standards, blanks, and homogenates were identical and non-sequential to prevent any bias during handling. Fluorescence was measured in a 3 ml quartz cell by adding 100 µl of H33258, EthDi, or EthBr working stock solution to 2.4 ml of standard, blank, or homogenate, and mixing for 30 s to ensure complete reaction. Standard chl *a* was measured by adding 100 µl of chl *a* solution to a cuvette containing 2.4 ml of either 315 ng ml^{-1} DNA (background fluorescence), or 315 ng ml^{-1} DNA with either H33258 or EthDi. For excitation and emission scans, 100 µl of each fluorochrome, or an equivalent volume of buffer, were added to a cuvette containing 2.3 ml of either DNA or buffer. Final fluorochrome concentrations were 1.5×10^{-7} M H33258, 4.7×10^{-7} M EthDi, and 4.7×10^{-7} M EthBr.

Fluorescence measurements were made using a Perkin-Elmer MPF-66 fluorescence spectrophotometer. A rhodamine standard was used to calibrate the monochromators and normalize fluorescence. The excitation wavelengths, emission wavelengths and emission filters for H33258 were EXWL = 350 nm, EMWL = 450 nm, and EMFILT = 430 nm; for EthDi they were EXWL = 300 nm, EMWL = 620 nm, and EMFILT = 610 nm; and for EthBr they were EXWL = 290 nm, EMWL = 600 nm, and EMFILT = 430 nm. Excitation and emission slits were 20 nm. The same parameters, where appropriate, were used for excitation and emission scans, except that the emission filter was set to 390 nm for all emission scans.

Natural samples. Natural seawater samples were collected, prepared, and analyzed on July 7, 1988 about 150 km off Pt. Reyes, California (37° 24.0' N, 124° 23.3' W) on RV 'Wecoma' during cruise 8806-B. This was a region of active upwelling and mixing of nearshore and offshore surface waters (Strub et al. 1991). Samples from 4 depths (5, 20, 60, 100 m) were retrieved in 5 l Niskin bottles fitted to a Neil-Brown CTD Rosette sampler. Depth profiles of σ_t and total pigment (pig_{tot} = chl *a* and phaeopigments) were provided by Fleischbein et al. (1989). Samples were prefiltered through a 300 µm screen, and pressure filtered in duplicate through a 1.0 µm followed by a 0.2 µm Nuclepore filter. Treatments and measurements were identical to those above except that (1) filters were sonicated in 5.05 ml of buffer with 0.0025 % Triton X-100, (2) RNA and DNA measurements were made by adding 100 µl of H33258 or EthDi to 1 ml of blank, sample, or standard, (3) final fluorochrome concentrations were 1.5×10^{-7} and 5.3×10^{-7} M for H33258 and EthDi respectively.

Nucleic acid calculations. The double fluorochrome method utilized H33258 to measure DNA (Eq. 1), and EthDi to measure the fluorescence of both DNA and RNA. RNA was calculated by first determining the amount of EthDi fluorescence due to DNA (Eq. 2), and

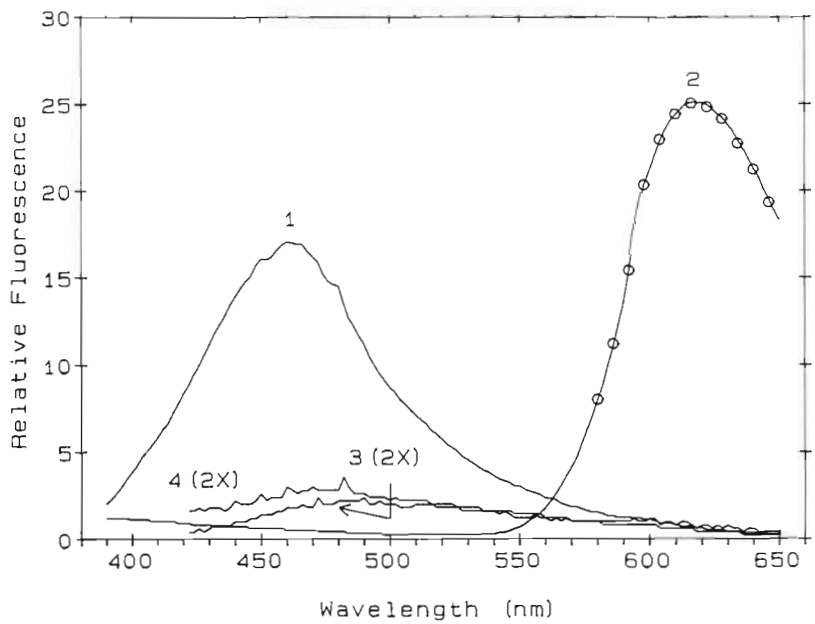

Fig. 1. Independent emission scans of H33258 at 1.5×10^{-7} M (curve 1) and EthDi at 4.7×10^{-7} M (curve 2) with 300 ng ml^{-1} of calf thymus DNA excited at 350 nm and 300 nm respectively The same scans of H33258 (curve 3) and EthDi (circles on curve 2) were also made with both fluorochromes in the same cuvette. Curve 4 is an emission scan of H33258 at 1.5×10^{-7} M without DNA. The response of curves 3 and 4 have been multiplied by a factor of 2. Curve 3 has been corrected by subtracting an emission scan of EthDi with DNA excited at 350 nm

subtracting this from the total EthDi fluorescence (Eq. 3). The RNase digestion method utilized EthDi on 2 subsamples of the homogenate, one treated with RNase and a second free of added RNase. The first was used to measure DNA (Eq. 4) and the second to measure the fluorescence of both DNA and RNA. RNA calculations also involved subtracting the EthDi fluorescence due to DNA from the total EthDi fluorescence (Eq. 5). Three daily filter blanks for each treatment were averaged and subtracted from the corresponding homogenate fluorescence. The fluorochrome-nucleic acid standard responses used in the calculations were the averages from all daily measurements. Filtration and dilution corrections used in the nucleic acid calculations are not shown. Deviations were determined by propagating the standard error of the means of the filter blanks and standards (Bevington 1969).

Double fluorochrome method using H33258 and EthDi.

$$DNA = (Sample_{H33258} - Blank_{H33258}) / \text{H33258 DNA Standard} \quad (1)$$

$$EthDi_{DNA} = DNA \times \text{EthDi DNA Standard} \quad (2)$$

$$RNA = (Sample_{EthDi} - Blank_{EthDi} - EthDi_{DNA}) / \text{EthDi RNA Standard} \quad (3)$$

RNase digestion method using EthDi.

$$DNA = (Sample_{RNase} - Blank_{RNase}) / \text{EthDi DNA Standard} \quad (4)$$

$$RNA = [(Sample - Blank) - (Sample_{RNase} - Blank_{RNase})] / \text{EthDi RNA Standard} \quad (5)$$

RESULTS

DNA emission scans

Fig. 1 shows emission scans for DNA with H33258 and EthDi. The emission peaks of H33258 (excited at 350 nm) and EthDi (excited at 300 nm) with standard DNA were at 460 nm and 617 nm respectively (curves 1 and 2). The fluorochromes were combined in a cuvette and measured independently. When combined, the EthDi DNA emission peak was unchanged (circles on curve 2), but the broad H33258 DNA emission peak was significantly reduced (curve 3). It was clear from these results that although DNA could be measured with EthDi in the presence of H33258, the converse was not true. (Curve 3 did contain an EthDi component above 550 nm, and was corrected by subtracting a scan of EthDi with DNA excited at 350 nm). Corrected or not, the 460 nm H33258 DNA emission peak (curve 1) was shifted by about 20 nm to 480 nm in the presence of EthDi (curve 3) and closely resembled the emission scan of unbound H33258 (curve 4). The reduction and shift of the H33258 fluorescence when EthDi was present may have resulted from the displacement of H33258 molecules from DNA by EthDi.

Standard responses

Three standards were regularly measured and used to calculate DNA and RNA in cultures and natural samples: DNA with EthDi, DNA with H33258, and *Escherichia coli* strain B RNA with EthDi. These standards had detection limits of 25, 35 and 70 ng ml^{-1}

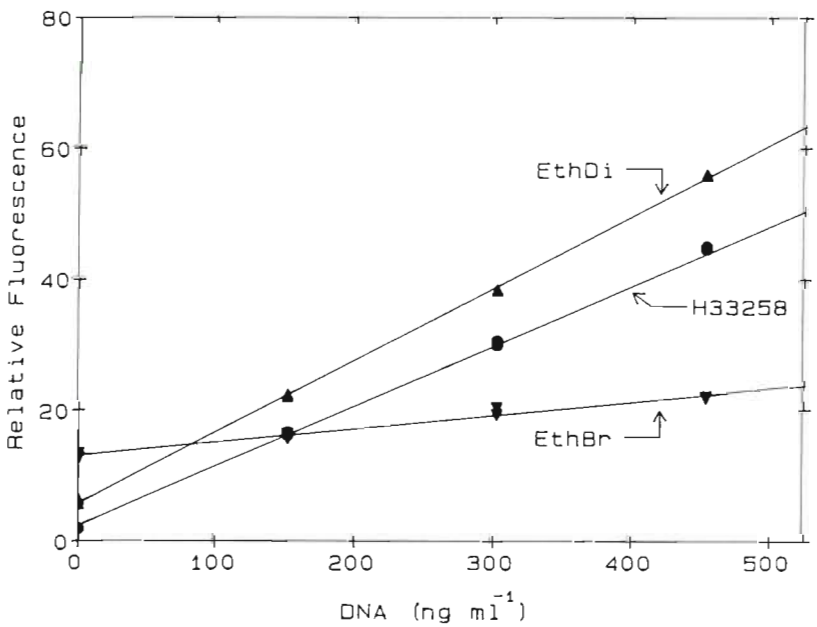

Fig. 2. Response of (▲) EthDi at 4.7×10^{-7} M, (●) H33258 at 1.5×10^{-7} M, and (▼) EthBr at 4.7×10^{-7} M with calf thymus DNA. Replicate measurements were made at each concentration. The excitation wavelengths, emission wavelengths and emission filters were 350, 450 and 430 nm for H33258; 300, 620 and 610 nm for EthDi; and 290, 600 and 430 nm for EthBr, respectively. Excitation and emission slits were 20 nm

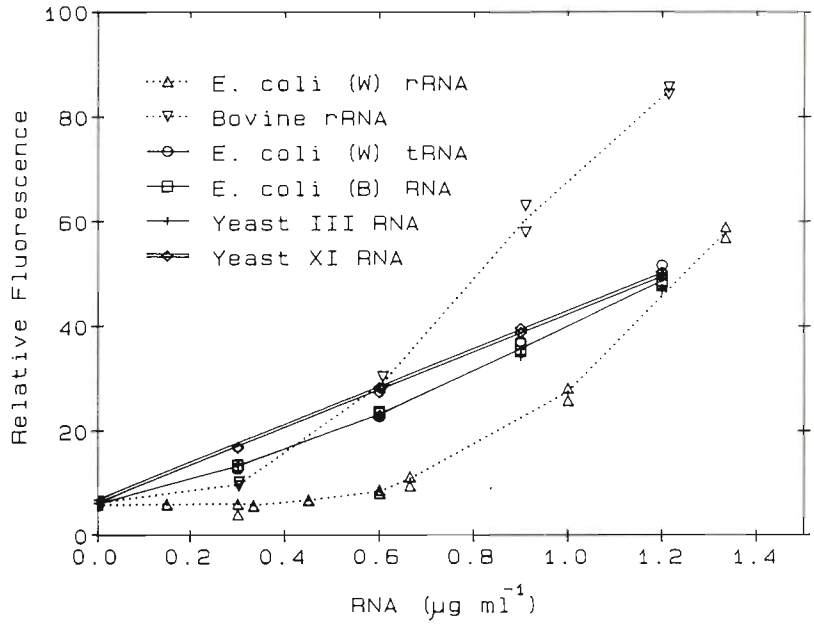

Fig. 3. Response of EthDi at 4.7×10^{-7} M with different types and preparations of RNA. The dotted lines connect (△) average *Escherichia coli* strain W rRNA and (▽) average bovine rRNA measurements. Single line connects average measurements of (○) *E. coli* strain W tRNA, (□) *E. coli* strain B RNA and (+) yeast type III RNA. Symbols are indistinct due to similarities of RNA responses. Double line is a least squares fit of yeast type XI RNA measurements (◇). Optical parameters for EthDi were the same as in Fig. 2

respectively (based on the minimal detectable fluorescence change). Typical H33258 and EthDi measurements with standard DNA are shown in Fig. 2 and a typical *E. coli* strain B RNA response with EthDi is included in Fig. 3. All the DNA standards had linear responses ($r^2 > 0.998$) while the responses of *E. coli* strain B RNA were consistently non-linear. Although EthDi and H33258 were both highly sensitive for DNA, the sensitivity of EthDi for RNA was about 3 times lower.

The sensitivity of EthDi was compared to EthBr. The detection limit of EthBr with standard DNA was about 145 ng ml^{-1} (Fig. 2), the lowest sensitivity observed for any fluorochrome-nucleic acid combination tested. (The RNA response of EthBr has been shown to be smaller than for DNA; Boer 1975.) Under our conditions, EthDi had a much lower blank and was more than 5 times more sensitive than EthBr. Therefore, replacing EthBr with EthDi significantly reduced the sample volume needed for nucleic acid determinations.

The response of several RNA standards with EthDi were also compared. EthDi had a linear response to yeast RNA type XI, a slightly non-linear response to several uncharacterized RNAs and tRNA, and a concave response to several rRNAs (Fig. 3). *Escherichia coli* strain B RNA and yeast RNA type III both showed

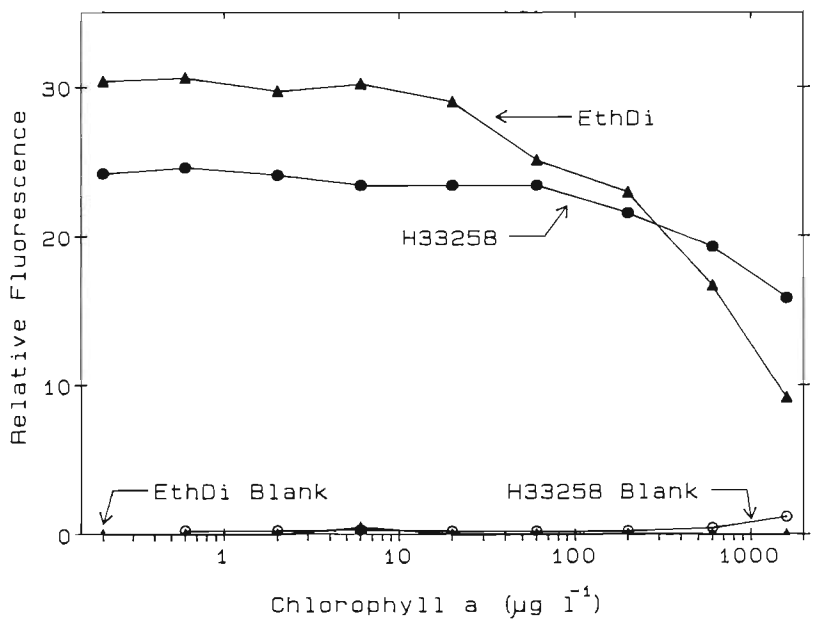

Fig. 4. Effect of chl *a* on the fluorescence of (▲) EthDi at 4.7×10^{-7} M and (●) H33258 at 1.5×10^{-7} M with 302 ng ml^{-1} of calf thymus DNA. Background fluorescence of chl *a* (without fluorochromes but with DNA) was also measured at the same wavelengths used for (△) EthDi and (○) H33258. The majority of EthDi blanks are concealed in the abscissa

an EthDi response almost identical to purified tRNA, supporting the manufacturer's claims that these RNAs were predominantly tRNA. Also, the similarity between the EthDi response with yeast RNA type XI and tRNA suggested that yeast RNA type XI, thought by the manufacturer to include both tRNA and rRNA, contained a large proportion of tRNA. The EthDi response of 2 rRNA standards from different sources was similar in shape, but appeared to be offset by about 0.4 μg ml^{-1}. *E. coli* strain W rRNA showed no fluorescence response at concentrations below 0.6 μg ml^{-1}, but above 1.3 μg ml^{-1} the response was similar to that expected for tRNA. Bovine liver rRNA showed no response below 0.3 μg ml^{-1}, but above 0.6 μg ml^{-1} the strongest response of any RNA was observed. Because the response of EthDi depended on the type of RNA, the choice of an RNA standard was critical for interpreting RNA concentrations. For example, a sample homogenate with low RNA concentrations may have been overestimated if referenced to an rRNA standard or underestimated if referenced to a tRNA standard. Finally, there was no fluorescence response of *E. coli* strain B RNA treated with RNase (not shown) which indicated that RNase digestions were complete.

Chlorophyll *a* interference

In homogenates of natural samples prepared for the double fluorochrome method, chl *a* concentrations of 60 μg l^{-1} have been observed (unpubl.). The fluorescence of H33258 with standard DNA was independent of standard chl *a* concentrations below 60 μg l^{-1}, but began to decrease at higher chl *a* concentrations with a 30 % fluorescence loss at 1600 μg l^{-1} chl *a* (Fig. 4). The fluorescence of EthDi with DNA decreased when chl *a* concentrations were above 20 μg l^{-1} and was reduced 70 % at concentrations of 1600 μg l^{-1}. For homogenates with chl *a* concentrations above 20 μg l^{-1}, fluorescent quenching must be considered, especially with EthDi. The background fluorescence of chl *a* (measured with 302 ng ml^{-1} DNA at H33258 and EthDi wavelengths without the presence of fluorochromes) was minimal, even at 1600 μg l^{-1}. Therefore the influence of high pigment concentrations cannot be corrected by simply subtracting the background fluorescence of sample homogenates as suggested by Thoresen et al. (1983), but must include corrections for quenching of the sample fluorescence.

Comparison of methods

DNA measurements made with the double fluorochrome method and the RNase digestion method were compared using bacterial (Fig. 5A) and phytoplankton (Fig. 5B) cultures. Each replicate was plotted individually since much of the error between replicates was likely due to filtering small volumes (1 to 6 ml). (DNA in bacterial cultures of OR5-88 decreased over 121 d of starvation from about 1500 to 100 μg l^{-1}. Details of the bacterial results in relation to starvation-survival will

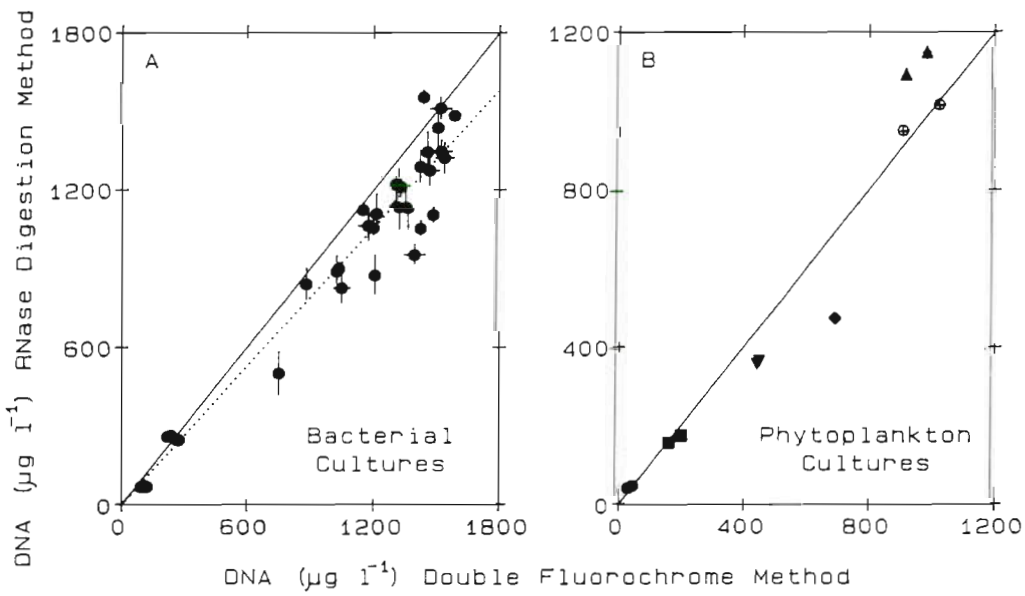

Fig. 5. Comparison of DNA measured with the double fluorochrome method and the RNase digestion method (A) on cultures of the marine bacteria OR5-88 at different stages of starvation survival, and (B) on batch cultures of phytoplankton. The phytoplankton cultures measured were (●) BB1, (■) *Chlorella vulgaris*, (▼) *Thalassiosira weissflogii*, (◆) *Asterionella japonica*, (▲) *Gymnodinium simplex*, and (⊕) *Chaetoceros* sp. Solid lines indicate ideal fits, dotted line is the best fit through the origin of the bacterial measurements. Error bars indicate propagated standard errors of the blanks and standards

be discussed in a separate publication.) On average, DNA concentrations in cultures of OR5-88 were 12 % higher when measured with the double fluorochrome method relative to the RNase digestion method. The specificity of H33258 for DNA rich in dA-dT base pairs (Weisblum & Haenssler 1974, Comings 1975) may have caused this difference. Six phytoplankton cultures were also measured for DNA using both techniques (Fig. 5B). DNA concentrations for cultures of *Chaetoceros* sp. (900 to 1000 $\mu g\ l^{-1}$), *Chlorella vulgaris* (150 to 200 $\mu g\ l^{-1}$), and BB1 (30 to 50 $\mu g\ l^{-1}$) were measured equally well by both techniques. Relative to the RNase digestion method, the double fluorochrome method resulted in higher DNA concentrations for *Thalassiosira weissflogii* (440 vs 360 $\mu g\ l^{-1}$) and *Asterionella japonica* (690 vs 470 $\mu g\ l^{-1}$), and lower concentrations for *Gymnodinium simplex* (920 to 980 vs 1100 to 1150 $\mu g\ l^{-1}$). Measurements on phytoplankton cultures were probably complicated by the base specificity of H33258 and by quenching and background fluorescence from pigments. Still, DNA was generally measured equally well for all cultures using either of the techniques, both of which were sensitive, simple, and fast, although the RNase digestion method required an extra digestion step.

Fig. 6 shows RNA measurements by both methods on the same cultures of bacteria (Fig. 6A) and phytoplankton (Fig. 6B) measured for DNA. Like bacterial DNA, the bacterial RNA concentration decreased with 121 d of starvation from 1000 to 1500 $\mu g\ l^{-1}$ down to less than 100 $\mu g\ l^{-1}$. Some values were less than zero, even when considering the range of standard errors. These measurements were assumed to be below detection limits of the techniques and were set equal to zero. The large uncertainties associated with bacterial RNA measurements were primarily due to variance in the filter blanks. RNA errors were also amplified by the number of steps necessary to propagate standard and blank SEMs. The 2 methods measured similar RNA concentrations on phytoplankton cultures of *Chaetoceros* sp. (600 to 710 $\mu g\ l^{-1}$), *Chlorella vulgaris* (320 to 430 $\mu g\ l^{-1}$), and BB1 (110 to 190 $\mu g\ l^{-1}$), the same cultures for which both techniques measured equal amounts of DNA. For both methods, RNA is calculated by subtracting DNA from the same total fluorescence value, so this result is not unexpected. *Asterionella japonica* had an RNA value of about 600 $\mu g\ l^{-1}$ with the RNase digestion method, but RNA was almost absent (60 $\mu g\ l^{-1}$) using the double fluorochrome method. The RNase method also measured higher RNA relative to the double fluorochrome method for *Thalassiosira weissflogii* (980 $\mu g\ l^{-1}$ vs 870 $\mu g\ l^{-1}$), but lower RNA for *Gymnodinium simplex* (950 to 1200 $\mu g\ l^{-1}$ vs 1400 to 1600 $\mu g\ l^{-1}$). Scatter about the ideal fit for RNA (Fig. 6) was much larger than for DNA (Fig. 5) due to the relatively lower sensitivity of EthDi for RNA.

Natural samples

Depth profiles of σ_t, pig_{tot}, DNA and RNA/DNA were measured in natural waters (Fig. 7). The nucleic acid measurements were made on size fractionated (>0.2 μm but <1.0 μm) samples using the double fluorochrome method. These results were part of a larger study to be described in a separate publication. This station had a mixed layer of about 20 m with σ_t = 24.9 and $pig_{tot} > 1.0\ \mu g\ l^{-1}$. From 25 to 100 m, σ_t increased to

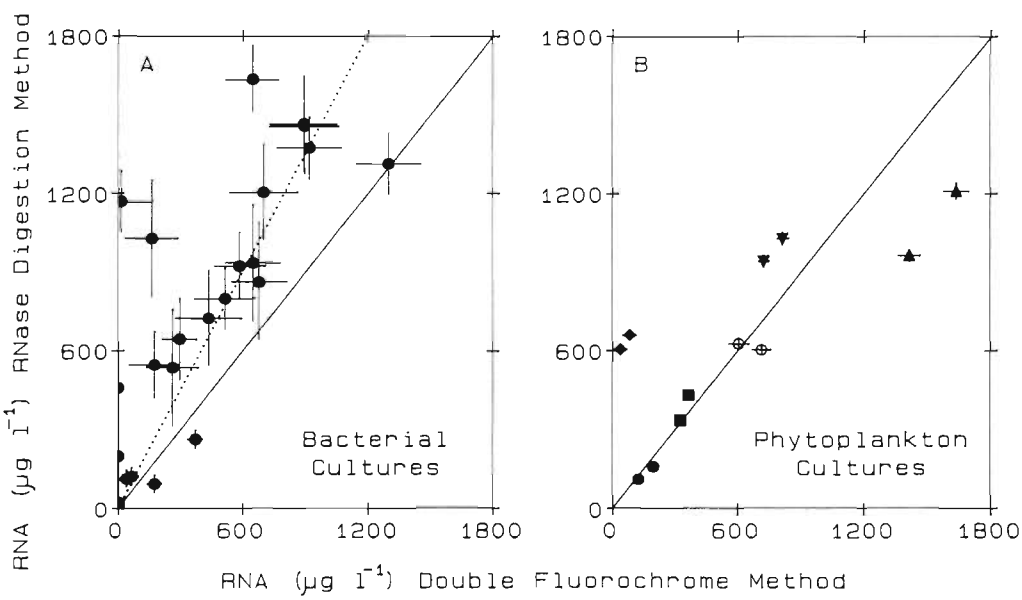

Fig. 6. RNA measurements on (A) marine bacteria and (B) phytoplankton cultures as in Fig. 5

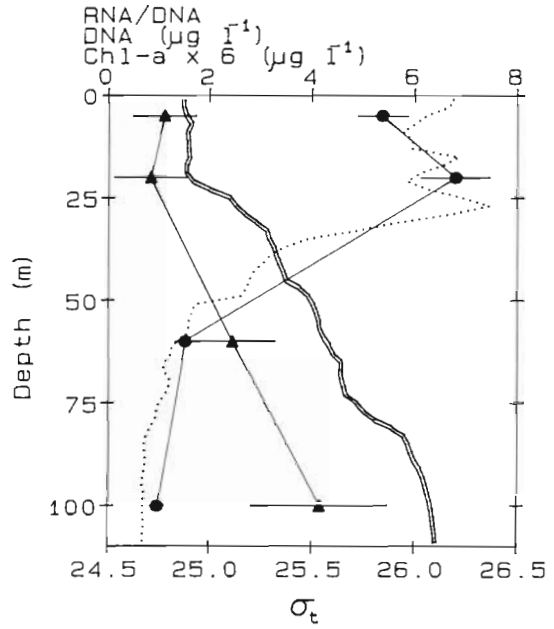

Fig. 7 Depth profiles of (●) DNA and (▲) RNA/DNA in the >0.2 µm but <1.0 µm size fraction of natural seawater samples. Also shown are profiles of σ_t (double line) and total pigment (chl *a* and phaeopigments; dotted line). Error bars indicate propagated standard errors of the standards, blanks, and replicate means

>26.0, and pig_{tot} decreased to the detection limit of the instrument. DNA concentrations were 5 to 7 µg l^{-1} in the upper 20 m but dropped to 1 to 2 µg l^{-1} at 60 to 100 m, and RNA/DNA ratios increased with depth from about 1 near the surface to about 4 at 100 m. The speed and simplicity of this technique allowed for preparation and analysis of these samples for DNA or RNA at sea in less than 10 min per replicate.

DISCUSSION

The emission peaks of H33258 and EthDi with standard DNA showed little overlap suggesting that these fluorochromes could be combined to measure RNA and DNA in a single cuvette simply by changing excitation and emission wavelengths. Unfortunately H33258 fluorescence with standard DNA disappeared in the presence of EthDi. EthDi had a small excitation peak at 530 nm (not shown) which could have absorbed H33258 fluorescence. EthDi may have displaced H33258 from DNA as suggested by a 20 nm shift in the diminished H33258 emission peak which corresponded to the free fluorochrome. Hoechst 33258 binds in the major groove on the outside of DNA double helices with a binding constant of about 10^5 M^{-1} (Comings 1975, Müller & Gautier 1975) while EthDi at low ionic strength mono-intercalates with a binding constant of about 10^8 M^{-1} in 0.2 M Na^+ (Gaugain et al. 1978b, Delbarre et al. 1983). H33258 binding sites may have been blocked or disrupted by either the stronger binding of EthDi or simply from EthDi intercalation, which is known to elongate DNA (Berman & Young 1981, Waring 1981). Because EthDi fluorescence was relatively unchanged in the presence of H33258, a double fluorochrome method could be used to determine DNA and RNA in a discrete cuvette or in a flow cytometer by first measuring H33258 then adding and measuring EthDi. Cuvettes must be cleaned after every measurement as any residual EthDi will compromise subsequent H33258 measurements.

It has been suggested that EthDi is 100 times more sensitive for DNA than EthBr (Markovits et al. 1979), but under our conditions the dimer was only about 5 times more sensitive. Still, Thoresen et al. (1983) found that EthBr measurements on phytoplankton samples required filtration of 1 to 5 l of coastal seawater while the high sensitivity of H33258 and EthDi allowed us to measure DNA and RNA on as little as 25 ml of surface water. Sample homogenates prepared for EthBr measurements also had high background fluorescence (fluorescence without EthBr) resulting from high pigment concentrations (Thoresen et al. 1983). Due to our smaller sampling volumes, chl *a* concentrations in over 100 natural sample homogenates prepared for EthDi and H33258 measurements were typically less than 20 µg l^{-1}, but several were as high as 60 µg l^{-1} (unpubl.). Although minimal background fluorescence was observed for these natural sample homogenates and for standard chl *a* at H33258 and EthDi wavelengths, Fig. 4 suggests that EthDi fluorescence was quenched at chl *a* concentrations above 20 µg l^{-1}. Other cellular constituents might also interfere with the fluorescence of H33258 or EthDi. For example, in experiments similar to those in Fig. 4, phycoerythrin concentrations of 1000 µg l^{-1} resulted in quenching and background fluorescence of EthDi, but not of H33258 (Baeuerle 1990). Also, phycocyanin showed little background fluorescence or quenching of EthDi at 1000 µg l^{-1} (Baeuerle 1990). As probably true with any nucleic acid stain, accurate measurements should include standard additions of DNA to quantify any fluorescence quenching (see Saxberg & Kowalski 1979).

The phytoplankton and bacteria cultures used to compare DNA measurements made with the double fluorochrome method and the RNase digestion method represented a wide range of DNA concentrations and various stages of growth. DNA measurements by the 2 techniques were generally very similar. For some cultures significantly higher DNA values were observed for 1 of the 2 techniques. These discrepancies may have resulted from pigments or other cell constituents quenching or contributing background

fluorescence to one of the fluorochromes, or from the known dependence of H33258 on the base composition of DNA. Fluorescence of H33258 is stronger with A·T-rich DNA than with G·C-rich DNA (Weisblum & Haenssler 1974, Comings 1975). Therefore, variations in DNA measured with H33258 may have reflected changes in the base composition of the samples relative to a standard, in this case 60 % (A+T) calf thymus DNA (Comings 1975). Unlike H33258, EthDi is not sensitive to base compositions of natural DNA (Markovits et al. 1979). Therefore, if the double fluorochrome and RNase digestion techniques are both used to measure DNA of a sample, the ratio of the 2 measurements can be used to estimate the base composition of that sample. Data from Weisblum & Haenssler (1974) and Comings (1975) was used to develop a relationship between the ratio of $DNA_{Double\ Fluorochrome}$ / $DNA_{RNase\ Digestion}$ and the mole fraction (A+T). Our bacterial culture OR5-88, which should have had minimal background and quenching complications, had a $DNA_{Double\ Fluorochrome}$ / $DNA_{RNase\ Digestion}$ ratio of 1.14 which corresponded to 70 to 75 % (A+T). Similar calculations were made with *Gymnodinium simplex* (45 to 50 % A+T) and *Asterionella japonica* (100 to 125 % A+T). The DNA base compositions of the bacterial cultures and *G. simplex* were in the range observed for many types of organisms: 30 to 70 % (A+T) (Green 1974, Comings 1975, Müller & Gautier 1975). In contrast, EthDi quenching or a large background fluorescence at H33258 wavelengths must have occurred in samples of *A. japonica*. Because of the dependency of H33258 on base composition, the RNase digestion method should be used when measuring pure cultures, each of which has a specific base composition. On natural samples, accuracy of the double fluorochrome method improves, because as the variety of organisms increases, the mole fraction (A+T) should approach 50 %.

To measure RNA, a proper RNA standard must be chosen. Selecting an RNA standard is complicated because the response of EthDi differs for tRNA and rRNA, the 2 major components of cellular RNA. Concentrations below 300 ng ml^{-1} of rRNA could not be detected, yet, depending on the organism and growth conditions, 80 to 90 % of cellular RNA is rRNA, with the remainder being mostly tRNA (Darnell 1968, Lewin 1974). Uncharacterized *Escherichia coli* strain B RNA, thought to be mostly tRNA (see 'Results'), was the standard used for our samples, but because low concentrations of rRNA could not be measured, RNA measurements were considered as minimal concentrations. The varying standard responses may have been due to changes in excitation or emmision spectra. Berdalet & Dortch (pers. comm.) observed such changes for different RNA standards, and with different lots of the same standard. RNA measurements were further complicated because the extent of intercalation may have varied between standard RNA and cellular RNA. Proteins are bound to the bulk of cellular RNA and may alter (increase or decrease) intercalation; intercalation of several tRNAs is known to change upon the formation of a tRNA-protein complex (Ferguson & Yang 1986, Kuznetsova et al. 1987). When protein-free RNA standards are used, the proteins in natural samples should be digested or otherwise efficiently removed from RNA (see Karsten & Wollenberger 1977). Using samples untreated for proteins and whole ribosomes as an RNA standard may prove a simpler and more accurate procedure, especially with bacterial samples free of nucleohistones.

The non-linear responses observed for all but one of the RNA standards might reflect conformational changes in RNA with increasing concentration. Because the RNA helix must elongate or alter its conformation to accept an intercalator (Berman & Young 1981, Waring 1981), changes in tertiary structure which alter the flexibility of RNA also affect intercalation (Reinhardt et al. 1982, White & Draper 1989). For example, intercalation of EthBr with phenylalanine specific tRNA ($tRNA^{Phe}$) has been shown to be limited to one or several binding sites under conditions which stabilize the $tRNA^{Phe}$ tertiary structure (Tao et al. 1970, Urbanke et al. 1973, Kean et al. 1985). Therefore, if RNA tertiary structure was modified with increasing RNA concentration, either the number of EthDi binding sites in RNA or the affinity of EthDi at a particular binding site may have been altered. However any relationships between changes in RNA tertiary structure and RNA concentration remain unclear. In naked RNA, tertiary structure could have been modified by nonspecific RNA aggregation (Boedtker 1968). Whatever the causes of the non-linear RNA standard curves, yeast RNA type XI was not affected.

Response curves for the 2 rRNAs with EthDi had the same general shape but were separated by about 0.4 μg ml^{-1}. This offset may have been caused by difficulties in determining the actual concentration of rRNA, RNA base specificity of EthDi, or preferential binding of EthDi to bovine liver rRNA due to tertiary constraints as discussed above. Concentrations of rRNAs were not determined gravimetrically but by the UV absorbance to weight conversions provided by the manufacturer. The accuracy of these conversions was not determined, and deviations could explain variability in the standard curves. (The standards may have been partially digested by nucleases during commercial preparation.) Also, if EthDi binding sites are limited in certain RNA types or preparations, then comparing standards based on weight would not be valid. The units μg l^{-1} were used because neither the molecular weights nor the number of binding sites per molecule for the various RNAs was

known. Finally, both EthBr and EthDi are specific for poly(A-U) over other synthetic polyribonucleotides, especially poly(G-C) (Markovits et al. 1979, Babayan et al. 1987). Although the base specificity of a fluorochrome with synthetic polynucleotides does not necessarily imply an analogous preference in natural samples (Markovits et al. 1979), such specificity might explain the differences between rRNAs if the mole fraction (A+U) was higher for bovine liver rRNA binding sites.

Although bacterial RNA concentrations were considerably higher using the RNase digestion method (Fig. 6A), for most measurements this discrepancy disappeared if DNA values were corrected for an estimated 70 % dA-dT base composition. Several bacterial samples resulted in RNA values less than zero even when considering the range of their standard errors. Several factors may have contributed to these negative numbers. On these days the EthDi filter blanks (measured with fluorochromes but without sample) were high relative to other days and relative to H33258 and RNase treated filter blanks. Subtraction of high blanks from the total EthDi fluorescence would have resulted in low RNA measurements. Also, all but one of these samples occurred after 15 d of starvation. Cellular RNA may have been catabolized during starvation to an extent that homogenate RNA concentrations were lower than the detection limit of the method.

Phytoplankton RNA measurements were always detectable. The low RNA concentrations of *Asterionella japonica* with the double fluorochrome method were probably related to EthDi quenching relative to H33258 as mentioned above. High RNA/DNA ratios were observed for batch cultures of BB1 and *Chlorella vulgaris* and low RNA/DNA ratios were found for *Chaetoceros* sp. and *Gynodinium simplex*. Although not the focus of this study, the concentrations and ratios of nucleic acids are very likely related to the ages of the cultures. High RNA/DNA ratios may have been from young cultures with a small number of rapidly-growing cells, while low RNA/DNA ratios may have been measured in older cultures containing many slow growing cells in a nutrient-depleted medium (Dortch et al. 1983).

Depth profiles of DNA and the RNA/DNA ratio measured in the 0.2 to 1.0 µm size fraction of natural seawater using the double fluorochrome technique (Fig. 7) demonstrated the usefulness and sensitivity of this method. A simple explanation of the data could be that there were a relatively large population of bacterial size organisms (Paul & Carlson 1984) near the surface with relatively low growth rates, and fewer organisms with higher growth rates at 50 and 100 m. This assumes that DNA is a conservative measure of biomass and RNA/DNA increases with metabolic activity. Differences with depth in growth rates might be caused by changes in nutrients (DOM) whereas differences in standing stock might be a result of more grazing at 50 to 100 m. The RNA/DNA profile might also be influenced by an increasing tRNA/rRNA ratio in natural populations with depth.

We have demonstrated the use of 2 techniques to measure DNA and RNA in the marine environment, both of which are sensitive, simple and rapid, but subject to some qualifications. These techniques can provide data to help understand population dynamics of marine plankton. Using H33258 and EthDi together and without nucleases to measure nucleic acids may be especially useful because the technique could be the basis of a flow cytometric system for whole seawater. Such a system might include a flow-through filter and sonicator to isolate and disrupt desired size fractions of organisms and 2 lasers or a split-beam laser with 2 detectors to excite and measure both H33258 and EthDi.

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