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Simplified Method for Dissolved DNA Determination in Aquatic Environments

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A method was developed for the determination of dissolved DNA in aquatic environments. The method is based upon the concentration of dissolved DNA by ethanol precipitation of 0.2-μm-pore-size filtered water. The DNA in concentrated extracts was quantified by the fluorescence of Hoechst 33258-DNA complexes. Fluorescence not attributable to DNA was corrected for by DNase I digestion of the extracts and averaged 25% of the total fluorescence for all samples. The effectiveness of the procedure for concentrating dissolved DNA was demonstrated by the efficient (>90%) recovery of internal standards. Concentrations of dissolved DNA from a variety of marine and freshwater environments ranged from 0.2 to 44 μg/liter, with the highest values being obtained for estuarine and river environments. The method is simple, specific for DNA, and more sensitive than previously described methods for the determination of extracellular DNA.

Approximately 90% of the organic substances in seawater exist as dissolved compounds. Although many of the low-molecular-weight compounds of the dissolved organic matter in seawater have been identified and quantified (4), only about 15% have been characterized (14). Uptake studies of the simpler dissolved organic compounds (amino acids, monosaccharides) in different aquatic environments indicate that free-living heterotrophic bacteria are the most active and efficient consumers of these compounds (1, 5, 8, 10, 17). However, few measurements of the concentrations of dissolved macromolecules have been made, and even less is known about the utilization of the molecules (18). The problems inherent in measuring the concentration of a complex macromolecule are greater than measuring that of a simple monomer. Yet, these measurements are crucial to the study of the cycling of these compounds.

As a constituent common to all living cells, DNA is a potential component of the dissolved macromolecular fraction in aquatic environments. Using the diaminobenzoic acid method of Kissane and Robins (11), Minear (16) found appreciable amounts of dissolved DNA in lyophilized lakewater. In the study by Minear, 19.6 to 57.1% of the high-molecular-weight phosphorus compounds were DNA, corresponding to 4 to 30 μg of dissolved DNA liter^{-1}. These are minimum values, since they were not corrected for recovery, which was less than 100%. This technique cannot be applied to marine samples, because seawater cannot be concentrated by lyophilization.

Pillai and Ganguly (22) used barium sulfate precipitation to concentrate nucleic acids from filtered seawater. After hydrolysis of the precipitate at 100°C for 3 h in 0.02 N HCl, the hydrolysate was compared with calf thymus DNA standards by UV spectra at 260 nm. By their analysis, the dissolved DNA concentration in Bombay Harbor varied between 13 and 24 μg liter^{-1} over 6 months. Although these numbers are comparable to those found by Minear (16) for lakewater, the specificity of this UV absorbance technique for DNA quantification is questionable.

Breter et al. (2) developed a high-pressure liquid chromatography technique for the measurement of thymine in seawater by using a cetyltrimethylammonium bromide precipitation of polyanions. The dissolved thymine concentrations measured at four depths from the surface to 30 m, ranged from 25 to 75 ng liter^{-1} or 250 to 750 ng of DNA liter^{-1}, considering DNA to be 10% thymine by weight. However, this tedious method is unsuited for routine analysis of DNA in seawater.

We have developed a technique for the measurement of particulate (cellular plus detrital) DNA in aquatic environments (21). Using this technique, we found the bacterial contribution to particulate DNA to be significant (70 to 95%) in oceanic environments (19, 20). As an extension of this research, we initiated studies on the methodology for determining extracellular or dissolved DNA in aquatic environments. This method is based on the specificity of Hoechst 33258 dye for the A+T-rich portions of native double-stranded DNA (12) and is well suited to routine analysis of environmental samples.

The persistence of measurable concentrations of extracellular DNA in aquatic environments is important for several reasons. As a compound rich in nitrogen and phosphorus, DNA could be an important source of microbial nutrition. Dissolved DNA could also be a source of nucleic acid precursors which are energetically expensive for microorganisms to synthesize de novo. The measurement of extracellular DNA may also be important in light of the environmental use of genetically engineered microorganisms, as a means to monitor DNA in aquatic environments.

MATERIALS AND METHODS

Sampling sites. Preliminary studies on the development of the method were performed on samples from Bayboro Harbor, a eutrophic embayment of Tampa Bay, St. Petersburg, Fla. Tampa Bay was sampled at a series of stations located along a salinity gradient that extended from the mouth of the bay to the Alafia River. Sampling sites in the Gulf of Mexico included offshore, coastal, and coral reef environments in the Dry Tortugas; these were sampled on four cruises during the summers of 1984 and 1985. Freshwater samples were taken at several sites in southwestern Florida, including Crystal River, a relatively pristine, spring-fed river; the Medard Reservoir, a eutrophic body of water collecting runoff from agricultural and phosphate-mining

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regions of the Alafia River Basin; and Boyd Hill Nature Park, a thickly vegetated swamp adjoining Lake Maggiore in St. Petersburg.

Materials. Hoechst 33258 (bisbenzimide; 2-[2-(4-hydroxyphenyl)-2-benzimidazolyl]-6-[1-methyl-4-piperazyl] benzimidazole trihydrochloride), DNase I (bovine pancreas), mithramycin, and DNA (calf thymus, type I) were obtained from Sigma Chemical Co., St. Louis, Mo. DABA (3,5-diaminobenzoic acid dihydrochloride) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Bacteriophage λ DNA (0.5 μg/μl) was obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Elutip-d columns were purchased from Schleicher and Schuell, Inc., Keene, N.H., and NENsorb 20 nucleic acid purification cartridges were from DuPont-New England Nuclear Corp., Boston, Mass. Spectrapor cellulose dialysis tubing (molecular weight cutoff, 12,000 to 14,000) was from American Scientific Products, McGaw Park, Ill.

Concentration and measurement of DNA. Seawater (100 to 1,000 ml) was passed through a filter combination consisting of a GF/D filter (Whatman, Inc., Clifton, N.J.) and a 0.2-μm-pore-size (Nuclepore Corp., Pleasanton, Calif.) filter under a vacuum of ≤150 mm Hg (≤20 kPa), with the filtration flask immersed in an ice bath. The DNA in the filtrate was precipitated by the addition of 2 volumes of 200-proof (100%) ethanol. After 48 h at −20°C, the precipitate was collected by centrifugation with 500-ml centrifuge tubes in a GS 3 rotor (Ivan Sorvall, Inc., Norwalk, Conn.) at 6,800 × *g* for 20 min. The precipitate was then dialyzed at 4°C for 48 h against deionized water and then for 24 h against 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). Triplicate samples of the dialysate, ranging from 0.5 to 2.0 ml, were brought up to 2.0 ml with SSC, and 1 ml of 6×10^{-7} M Hoechst 33258 in SSC was added. The samples were warmed to room temperature, and the fluorescence was measured as previously described (21).

A 0.1-mg/ml DNA stock solution (A_{260} = 2.0 [6]) was prepared with calf thymus DNA. DNA standards ranging from 0 to 2,000 ng were brought to a final volume of 2 ml with SSC. Blanks were prepared by filtering artificial seawater (ASWJP [21]) (pore size, 0.2 μm), and then dissolved DNA was determined by the protocol outlined above for seawater samples.

Freshwater samples were treated similarly, except that 0.13 M NaCl, 5.4×10^{-3} M $CaCl_2 \cdot 2H_2O$, and 1.35×10^{-2} M $MgSO_4$ were added to the filtrate prior to the addition of ethanol to aid the precipitation of dissolved DNA.

All measurements were corrected for fluorescence from material other than DNA by DNase I treatment (21). Samples were also corrected for losses in recovery by the addition of an internal calf thymus DNA standard to replicate samples. From 5 to 10 μg of calf thymus DNA standard (spike) per 100 ml of filtrate was sufficient to assess recovery.

Verification of the method. A series of experiments were performed to verify the following assumptions inherent in this assay: (i) that dissolved DNA was not being produced as a result of vacuum filtration or subsequent steps in the procedure; (ii) that cells were not passing through the filters, thus contributing to the observed fluorescence; and (iii) that one sample filtered (assayed in triplicate) was a reasonable estimate of the concentration of dissolved DNA.

To assess the effect of vacuum filtration on dissolved DNA values, 100-ml replicates of Bayboro Harbor water were filtered under various vacuum conditions (25 to 500 mm Hg, or 3.3 to 66.6 kPa). One replicate received an internal DNA standard, and both samples were processed for dissolved DNA measurements.

To investigate the relative importance of filterable bacteria (13) to the dissolved DNA signal, dissolved DNA was measured in both 0.1- and 0.2-μm filtrates. It is unlikely that a significant proportion of the bacteria of less than 0.2 μm would also pass through a 0.1-μm filter. Two 100-ml subsamples were filtered through a combination GF/D and 0.2-μm Nuclepore filter, and a second set of 100-ml subsamples were passed through a combination GF/D and 0.1-μm Nuclepore filter, both at 150 mm Hg (20 kPa).

The variability of the method was tested in two separate experiments. A single seawater sample was filtered by the standard procedure and precipitated in four volumes (50, 100, 150, and 200 ml). The second experiment involved filtering nine 100-ml volumes of seawater from the same sample and adding a DNA spike to one subsample to assess recovery. All of these samples were processed as described above for dissolved DNA quantification.

To determine the effect of storage on the amount of dissolved DNA measured, four 100-ml samples of Bayboro Harbor water were filtered, ethanol precipitated, and stored at −20°C. The dissolved DNA was measured in one sample each week for the following 4 weeks.

Determination of dissolved chlorophyll *a*. Dissolved chlorophyll *a* was determined by filtration of 100 ml of Bayboro Harbor water at 150 mm Hg in the dark. The chlorophyll was extracted from the filtrate by addition of 100 ml of CH_3OH and then driven into petroleum ether by the addition of sodium chloride. The petroleum ether was dried with sodium sulfate and removed by rotoevaporation. The residue was taken up in methanol, and chlorophyll *a* was determined fluorometrically. Controls included sonicated water and unfiltered water.

Fluorescence spectra. A model LS-5 spectrofluorometer (The Perkin-Elmer Corp., Norwalk, Conn.) equipped with an R928 photomultiplier tube and an R100A recorder was used to measure the fluorescence spectra of Hoechst 33258-DNA complexes. All measurements were of a 3.0-ml extract or buffer in a 1-cm fluorescence cell with a slit width of 10 nm. For excitation spectra, emission was at 472 nm; for emission spectra, excitation was at 342 nm.

Mithramycin and DABA methods. To compare the Hoechst assay with other methods of DNA quantification, further concentration of dissolved DNA was necessary. Samples were filtered, precipitated with ethanol, and dialyzed as described above, except that the SSC in the final dialysate was replaced with 0.2 M NaCl–20 mM Tris hydrochloride (pH 7.3 to 7.5)–1.0 mM EDTA (low-salt TE). The dialysate was then concentrated and purified by passage through an Elutip-d column according to the instructions provided by the manufacturer. The 400-μl eluate from the column was then precipitated with 2 volumes of ethanol at −20°C for 48 h, and the precipitate was collected by centrifugation in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.) for 10 min. The pellet was suspended in an appropriate volume of TE buffer (15).

The method used for the mithramycin assay was a modification of the method of Hill and Whatley (7), with a final concentration of 10 μg of mithramycin per ml and 10 mM $MgCl_2$. Standards were prepared (0 to 10 μg) with 0.1 mg of calf thymus DNA per ml, and TE buffer (pH 7.4) was added to a final volume of 2.0 ml. Concentrated extracts of dissolved DNA were also brought to a final volume of 2.0 ml with TE buffer. The stain was prepared by adding 12 mg of the mithramycin composite (2.5% mithramycin) to 10 ml of

TE buffer containing 30 mM $MgCl_2$. A 1-ml portion of the stain was added to each sample and to the standards, and fluorescence was measured after 5 min at an excitation of 430 nm and an emission of 570 nm.

Elutip-d-concentrated extracts of dissolved DNA were also examined for DNA content by the DABA technique (11). Since the concentrated extract was already a macromolecular precipitate, perchloric acid precipitation was omitted. A DABA solution was prepared by dissolving 600 mg of DABA in 2 ml of 1 N HCl. The solution was purified by addition of 150 mg of activated charcoal (Norit A; Sigma Chemical Co., St. Louis, Mo.) and centrifugation for 4 min in an Eppendorf microcentrifuge. After three such charcoal treatments, the supernatant was filtered through a Whatman GF/F filter and used immediately. A 10-μl (or less) amount of sample or calf thymus DNA standard was added to a 9- by 75-mm test tube, and 10 μl of the purified DABA solution was then added. The tubes were capped with Parafilm and heated at 60°C for 30 min in the dark. After cooling, 1 ml of 1 N HCl was added, and the fluorescence was measured within 1 h with the LS-5 fluorescence spectrophotometer (excitation, 400 nm; emission, 500 nm).

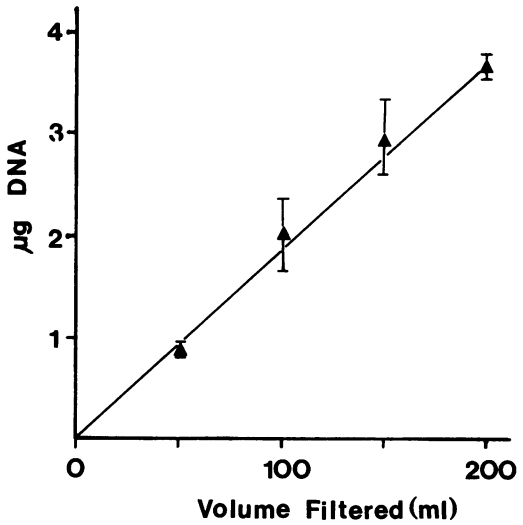

FIG. 1. Linear regression of the amount of dissolved DNA measured in four volumes of Bayboro Harbor water. The correlation coefficient was 0.997.

RESULTS AND DISCUSSION

Verification of the method. In initial studies on dissolved DNA measurement, Hoechst 33258 stain was added directly to filtered Bayboro Harbor water. Although fluorescence of such mixtures was higher than that of SSC buffer alone, this fluorescence was DNase stable and was considered to be due to material other than DNA.

Ethanol precipitation of ASWJP followed by dialysis of the precipitate provided blanks for the method. Such blanks yielded fluorescence values identical to that of SSC buffer alone. Therefore, DNA or non-DNA fluorescence was not introduced by the techniques used to isolate and concentrate dissolved DNA.

Recovery of calf thymus DNA standards added to artificial seawater was 85 to 95% (data not shown). The average recovery for DNA internal standards added to filtered natural water samples for estuarine and nearshore environments averaged 93.4 ± 6.52% standard deviation, while offshore recoveries averaged 72.3 ± 7.8%. Lower recoveries in offshore samples are probably related to the length of storage and to manipulation of large volumes of precipitate. We used percent recovery and DNase information in calculations of dissolved DNA values such that $dDNA = (C/V_s) \times (V_d/V_f) \times \%R^{-1} \times \%DNase$, where dDNA is dissolved DNA in micrograms per liter, C is the amount of DNA measured, V_s is the volume analyzed, V_f is the volume filtered, V_d is the volume of the dialysate, $\%R$ is the percent recovery of the internal standard, and %DNase is the percentage of DNase that was degradable.

We investigated microbial cell lysis during filtration in two different ways. Filtration of a seawater sample at a range of vacuum pressures from 25 to 500 mm Hg gave dissolved DNA values for samples filtered at 25, 75, and 150 mm Hg which were not significantly different, while the values for samples filtered at 250 and 500 mm Hg were significantly higher. This experiment was performed on two separate occasions. The first experiment yielded an average value of 11.75 ± 0.17 μg/liter for samples filtered at 25, 75, and 150 mm Hg and 15.30 ± 0.31 μg/liter for samples filtered at 250 and 500 mm Hg or approximately 30% higher. In the second experiment, the mean value for samples filtered at 25, 75, and 150 mm Hg was 17.10 ± 1.36 μg/liter and that for samples filtered at 250 and 500 mm Hg was 25.15 ± 1.17 μg/liter, or 47% greater. These results indicate that cell lysis was not contributing to the fluorescence at vacuum pressures of 150 mm Hg or less but may have been a factor in samples filtered at 250 and 500 mm Hg. Thus, all filtrations for dissolved DNA were performed at ≤150 mm Hg. We also looked for dissolved chlorophyll *a* production by filtration. Owing to the photolability of chlorophyll *a*, any found in the dissolved fraction can be assumed to be due to cell lysis. We found <1% of the particulate chlorophyll *a* in the dissolved fraction, while dissolved DNA constituted 11 to 226% of the particulate DNA. Therefore, it seems unlikely that dissolved DNA is an artifact of filtration under the mild vacuum used in this study.

Filterable bacteria or ultramicrobacteria have been found to pass through pore sizes of 0.45 μm (24), 0.3 μm (25) and, most recently, 0.2 μm (13). The possibility that very small cells were passing through the 0.2-μm filters was examined by filtering replicate samples through 0.1-μm Nuclepore filters. Dissolved DNA values for the 0.1-μm-filtered samples were not significantly different (Student's *t* test; significance level, 5%) and had actual values that were slightly higher than those obtained from the 0.2-μm-filtered samples. This indicates that bacteria in this size range were either not passing into the filtrate of the 0.2-μm filter or that they were not detectably contributing to the fluorescence. Cells smaller than 0.2 μm and those even smaller than 0.1 μm may have been passing into the filtrate and remaining intact. Intact cells would be resistant to DNase treatment and would not appear in our corrected estimates of dissolved DNA. Another alternative is that these cells make up such a small portion of the total amount of DNA that it is undetectable. On the basis of the concentrations of these organisms (Mary Hood, personal communication), we find this to be the more reasonable alternative.

The amount of DNA as a function of volume filtered is shown in Fig. 1. The concentration of dissolved DNA obtained in March 1986 from these four volumes collected in Bayboro Harbor was 18.32 ± 1.78 μg/liter, and the correlation coefficient for the linear regression was 0.997. These results indicate that dissolved DNA concentrations are not a function of the volume of water filtered.

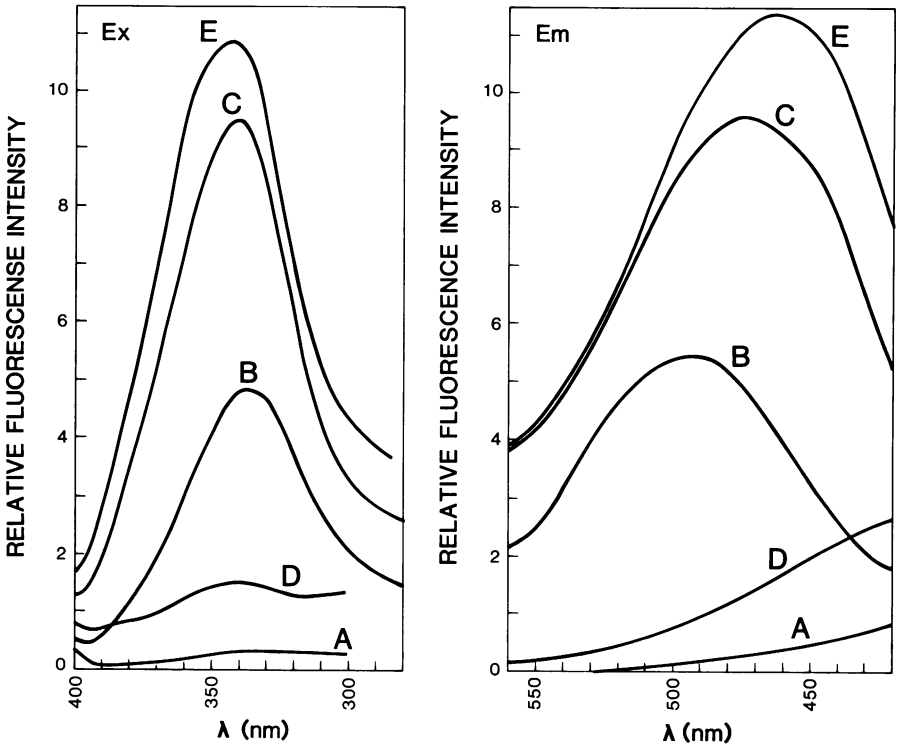

FIG. 2. Fluorescence excitation (Ex) and emission (Em) spectra of Hoechst 33258-DNA complexes. Curves: A, spectra of SSC buffer only; B, SSC containing 3×10^{-7} M Hoechst 33258; C, 200 ng of calf thymus DNA in SSC plus 3×10^{-7} M Hoechst 33258; D, SSC dialyzate of concentrated dissolved DNA from surface waters of the Gulf of Mexico; E, same as D plus 3×10^{-7} M Hoechst 33258.

The variability among samples of the same volume was calculated from the eight 100-ml subsamples of Bayboro Harbor water collected in January 1986. Means for each subsample were calculated from triplicate measurements. The overall mean for the eight samples, after correction for percent recovery and percent DNase degradable, was 6.72 ± 0.35 mg of DNA per liter (95% confidence interval), with an average coefficient of variation of 6.19%. When comparing any two subsamples, we found that the coefficient of variation ranged from 0.2 to 14%, indicating an acceptable degree of reproducibility between subsamples.

Water samples filtered and stored in ethanol at −20°C showed no loss in DNA over the first 2 weeks. Over the weeks 3 and 4, however, there was a 12% loss in DNA (12.17 versus 10.69 μg of DNA per liter). If possible, samples should be processed within 2 weeks of collection.

Fluorescence spectra. Figure 2 shows the fluorescence spectra of the dialyzed extract of dissolved DNA compared with calf thymus DNA and unbound Hoechst 33258. The free dye in solution exhibited fluorescence maxima at 340 (excitation) and 490 (emission) nm. Upon addition of either calf thymus DNA or dissolved DNA extracts, the fluorescence was greatly enhanced and there was a shift in the peak maxima toward longer wavelengths for the excitation spectra and shorter wavelengths for the emission spectra. The observed shift in spectra is characteristic of the binding of Hoechst 33258 to DNA (3, 23). The spectra of the calf thymus DNA and our dissolved DNA extracts bound to Hoechst 33258 are nearly identical, confirming that the observed fluorescence is due to double-stranded DNA.

Comparison with mithramycin and DABA. Purified DNA and dissolved DNA in concentrated extracts were quantified by the mithramycin and Hoechst 33258 methods. A commercially available preparation of phage λ DNA (0.5 μg/μl) yielded values of 0.48 and 0.44 μg/μl) by the Hoechst 33258 and the mithramycin assay, respectively. For dissolved DNA extracts from Bayboro Harbor and Tampa Bay, however, results of the mithramycin assay indicated the presence of 4.5 to 11 times (data not shown) the amount meaured by the Hoechst assay. The extract was further purified by passing the dialysate through a NENsorb 20 cartridge, which removes interfering substances (protein, salt, nucleotides, low-molecular-weight materials) not removed by Elutip-d columns. This cartridge can be used in place of a phenol-chloroform extraction step in the purification of DNA. Even after this purification, values for dissolved DNA in concentrates of Bayboro Harbor water by the mithramycin assay were still 3 times higher than those obtained by the Hoechst 33258 method (120 versus 40 ng/μl). It is possible that the differing specificities of the two fluorochromes cause some differences (i.e., mithramycin preferentially binds to GC-rich regions of double-stranded DNA [26] and Hoechst 33258 preferentially binds to AT-rich regions of double-stranded DNA [12]). It seems unlikely, however, that dissolved DNA base composition would vary that much from those of calf thymus and phage λ DNA standard. It seems more likely that there were substances in the seawater concentrates that were not removed by our purification techniques and that increased in fluorescence in the presence of mithramycin but not Hoechst 33258.

Analysis of Elutip-d-concentrated extracts from Tampa Bay water by DABA also yielded values greater than those obtained by Hoechst 33258 (average, 1.75× greater; range 1.12 to 3.14, $n = 7$). Although the DABA method is a good technique for DNA analysis in tissues (11) and environmental particulate material (9), it is a less specific method for

TABLE 1. Concentration of dissolved DNA in various aquatic environments

Environment	Avg DNA concn (range) (μg/liter)	n
Marine		
Estuarine	14.52 (6–44)	46
Coastal	10.8 (5–15)	10
Offshore		
surface	1.75 (0.5–5.0)	21
500 to 1,500 m	0.41 (0.2–0.5)	5
Coral Reef		
above	1.2 (0.63–2.0)	10
mucus	5.16 (2.45–10.45)	7
Freshwater		
Medard Reservoir	6.97	1
Crystal River	1.74	1
Boyd Hill	7.8	1

DNA than the Hoechst 33258 technique. It is based on the reaction of DABA with aldehydes possessing an unsubstituted α-carbon (11), a property of deoxyribose. However, the method will also detect single-stranded DNA, which is not detected by the Hoechst 33258 method. Another possible explanation is that the Hoechst 33258 technique, compared with the other methods, underestimates dissolved DNA for some unknown reason. We find this unlikely, since >90% recovery of added internal standards routinely occurs in the Hoechst 33258 analysis.

Concentrations of dissolved DNA in the environment. We have measured dissolved DNA in a wide variety of aquatic environments. The volume of water filtered depended upon the concentration of dissolved DNA present. Estuarine and coastal samples required only 100 ml of filtered water. For surface samples at offshore oligotrophic stations we filtered 300 ml, while for deep-water (≥ 300 m) samples, we filtered as much as 1 liter. The assay required at least 100 ng of DNA in the 2 ml of dialyzate measured (50 ng/ml) for a reliable measurement. All samples were checked for fluorescence before the addition of stain. Although this fluorescence was not normally found in our estuarine samples, it was present in about 25% of the Gulf of Mexico samples. We treated this fluorescence as a blank value and subtracted it from the fluorescence value after the stain was added. The percentage of the fluorescence that was degradable by DNase I treatment ranged (n = 101) from 24 to 100%, with an average value for all samples of 75 ± 19%. The portion of the fluorescence not degraded may be attributable to encapsulation of DNA in viruses, binding of DNA in some way which makes it unavailable to the enzyme, or simply a nonspecific binding of Hoechst 33258 dye which increases its fluorescence. We believe the last explanation is the most feasible, since attempts to "free" this DNA by sonication were unsuccessful. Sonicated extracts (30 s at 70 W) contained the same amount of DNase-resistant fluorescence as did unsonicated extracts.

The ranges of dissolved DNA concentrations for different environments are summarized in Table 1. Dissolved DNA concentrations decreased with distance offshore and depth below the surface. Estuarine values were the highest measured; the 44-μg/liter value was measured in May at a station on the Alafia River, which drains an agricultural region with an active phosphate-mining industry. Offshore oligotrophic stations had values for dissolved DNA in surface waters of 5 μg/liter or less, with samples deeper than 100 m having values below 1 μg/liter. The Dry Tortugas is similar to an oligotrophic environment, and microbial activity and biomass values for water sampled above the reefs were comparable to those found at other oligotrophic stations. However, samples of the coral mucus had dissolved DNA concentrations which were 2 to 12 times higher than those in the overlying water. Mucus values were more characteristic of a nearshore than of an offshore environment. Other microbial parameters measured in the coral mucus were also much higher than values in the water above the reef (J. H. Paul, M. F. DeFlaun, and W. H. Jeffrey, Mar. Ecol. Prog. Ser., in press).

Freshwater samples. Ethanol precipitation of DNA from solution requires the presence of some inorganic salts (15). The ambient levels of inorganic salts in freshwater environments (salinity, ≤1 to 2‰) were insufficient to allow recovery of internal DNA standards. When sodium chloride was added at concentrations of 0.2 or 0.5 M, the recoveries of the internal calf thymus DNA spike were low (57 and 46%, respectively). To determine the optimal concentration of salts for the precipitation of DNA, we added 10 μg of calf thymus DNA and three different salt concentrations to 100-ml volumes of deionized water. The lowest concentration of salts consisted of 0.13 M NaCl, 5.4×10^{-3} M $CaCl_2 \cdot 2H_2O$, and 1.35×10^{-2} M $MgSO_4$ (concentration of salts in 30% ASWJP). This concentration was doubled and tripled for the other two spiked deionized water samples. The best recovery of the DNA spike (90%) was obtained at the lowest salt concentration; therefore, we adopted this protocol for our freshwater samples. Of the freshwater samples, those from the eutrophic Medard Reservoir and Boyd Hill had values comparable to those of coastal and estuarine marine samples, while the Crystal River sample value was similar to those for samples found at offshore marine stations (Table 1). The dissolved DNA concentrations estimated by Pillai and Ganguly (22) in Bombay Harbor are similar to those that we found in our estuarine samples. Their values were obtained by measuring the A_{260} of a concentrated seawater sample. This technique is not recommended, because there may be RNA, protein, and other compounds in seawater that absorb at this wavelength.

We have also investigated concentrating samples by freeze-drying (16) for measuring dissolved DNA in freshwater environments. We found this method inappropriate for several reasons: (i) recovery of the added DNA spike was lower than with ethanol precipitation (64 versus 90%), (ii) there was a high fluorescence blank signal associated with the lyophilized samples before the stain was added, and (iii) ethanol precipitation was faster than freeze-drying. In comparison with the concentrations that we measured, the values of Minear (16) seem reasonable, even though they were not corrected for recovery. Recovery of total phosphorus compounds ranged from 60 to 90% and was probably even less for the DNA-containing, high-molecular-weight fraction. Correcting the values of Minear (16) for recovery yielded values higher than ours. There are several possible reasons for these higher values. Minear (16) used 0.45-μm-pore size filters, which may have allowed more filterable bacteria to be measured as dissolved DNA (24). Also, DABA measures single-stranded as well as double-stranded DNA (see above).

Estimates made by Breter et al. (2) of dissolved DNA in seawater based on nonparticulate polyanionic thymine concentrations are low compared with our values for surface water. The extensive manipulations required to concentrate samples to a final volume of 25 μl for high-pressure liquid

chromatography analysis makes this an extremely tedious method. Also, the measurement of thymine is not a direct measurement of native double-stranded DNA.

The method that we have developed for the determination of dissolved DNA in aquatic environments is simple and well suited to routine analysis. Hoechst 33258 is much more sensitive than other DNA-specific fluorochromes, allowing us to measure DNA with a minimal amount of concentration.

As well as enabling us to study the cycling of an important dissolved macromolecule, the methods that we have developed for the isolation and concentration of dissolved DNA are being used to determine its molecular weight in various environments, and are the foundation for a study involving the tracking of recombinant gene sequences in natural waters.

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