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Fluorometric Determination of DNA in Aquatic Microorganisms by Use of Hoechst 33258

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A method for the determination of microbial DNA in aquatic environments by the use of Hoechst 33258 has been developed. With unsophisticated instrumentation and simple extraction procedures, it is possible to detect from 0.05 to 10 μg of DNA in bacterial cultures or natural water samples. The method is specific for DNA; DNase I treatment of extracts of natural microbial populations removed 95 to 100% of the observed fluorescence. DNA content ranged from 165 ng ml^{-1} for relatively eutrophic Potomac River water to 27 ng ml^{-1} for coastal Atlantic Ocean water and was correlated to an acridine orange direct count (r = 0.90).

Classically, DNA in biological material has been determined by the diphenylamine method (for a review see reference 19). Based on the reaction of diphenylamine with deoxyribose, this method requires 17 h for color development, is interfered with by a variety of substances, and has a lower limit of detection of 5 μg of DNA (3). Kissane and Robins (14) developed a fluorometric method for DNA determinations based on the reaction of diaminobenzoic acid with aldehydes that have an unsubstituted α-carbon, and many modifications of this technique have appeared recently (1, 7, 8, 20). Holm-Hansen et al. (12, 13) have employed this method to estimate DNA in marine phytoplankton. The complex methodology and extreme conditions of extraction are the major drawbacks of this method.

Recently, dyes more specific for DNA which do not require extraction or separation of DNA from cellular constituents have been employed in DNA estimations. These include adriamycin, mithramycin, ethidium bromide, 4',6-diamidino-2-phenylindole (DAPI), and Hoechst 33258. Adriamycin combines with DNA to yield a yellow product that absorbs light at 475 nm (10). As a colorimetric method, the minimum sensitivity is only 1 μg of DNA, and RNA interferes with the determination (6). The ethidium bromide technique (17) is extremely sensitive for DNA but requires RNase digestion since this fluorochrome reacts with both DNA and RNA. The ethidium dimer technique (18) suffers from the same mixed specificity. Mithramycin, although extremely specific for guanine-plus-cytosine portions of DNA, does not bind DNA effectively in the presence of nucleoproteins (9), and solubilization with heparin is required. Blanks due to unbound stain are also quite high with the mithramycin technique (22).

Recently, two structurally related fluorochromes with similar specificities for DNA, Hoechst 33258 and DAPI, have been employed for DNA determinations in crude cell homogenates (2, 4, 15). Hoechst 33258 dye, which is weakly fluorescent, increases in fluorescence in the presence of DNA, binding specifically and quantitatively (16). The greatest fluorescence occurs in portions of DNA rich in adenine plus thymine (A+T); the regions that contain guanine plus cytosine possess only 50% of the fluorescence of the A+T regions (21). Hoechst 33258 dye is thought to bind without intercalation in the major groove of the double helix of A+T-rich regions, perhaps by hydrophobic interaction with the methyl of thymidine (5). The interaction of the dye with DNA is rapid, coming to completion in the time required for the simple mixing of Hoechst 33258 and DNA solutions (15). The fluorescence of the Hoechst 33258-DNA complex is hardly affected by common laboratory reagents or low concentrations of detergents (4).

We have employed Hoechst 33258 before for direct counts of bacteria in natural water samples (19a). In this paper we report the development of a method for the determination of DNA in aquatic microorganisms by the use of Hoechst 33258. The method, based on the collection of cells on membrane filters and extraction by sonication, is simple, rapid, and easily adapted to shipboard procedures.

MATERIALS AND METHODS

Materials. Hoechst 33258 {bisbenzimide; 2-[2-(4-hydroxyphenyl)-6-benzimidazolyl]-6-(1-methyl-4-piperazyl)benzimidazole, trihydrochloride, pentahydrate}, DNase I (bovine pancreas), and RNA (wheat germ) were obtained from Calbiochem, La Jolla, Calif. DAPI

was obtained from Aldrich Chemical Co., Milwaukee, Wis. Acridine orange and DNA (calf thymus, type I) were obtained from Sigma Chemical Co., St. Louis, Mo. Scintillation-grade Triton X-100 was obtained from J. T. Baker Co., Phillipsburg, N.J. All other chemicals were reagent grade and were obtained from J. T. Baker Chemical Co. or Fisher Scientific Co., Fairlawn, N.J.

Organisms. Two flagellated estuarine bacteria (*Alteromonas citrea* and strain NRL 1280:8) were isolated from the Chesapeake Bay at Chesapeake Beach, Md. The identification of the former was provided by the American Type Culture Collection, Rockville, Md.

Collection of natural water samples. Natural populations of bacteria were collected from surface waters (0.5 to 2 m) from the Potomac River (Ft. Washington, Md.; 23 September 1981 and 6 November 1981), the Chesapeake Bay (Chesapeake Beach, Md.; 29 September 1981), the Atlantic Ocean (8 miles [ca. 15 km] southeast of Ocean City, Md.; 74°58'10" W, 38°12'05" N; 15 October 1981) and the Severn River (U.S. Naval Academy, Annapolis, Md.; 11 November 1981). Samples were collected with Teflon tubing (inner diameter, 6.35 mm) with a vacuum supplied by a Guzzler hand pump (T. H. Berge and Co., Plainfield, N.J.). Water samples were stored on ice in plastic bottles until filtered (usually <1 h). Water samples were first passed through a 110-μm mesh to reduce variability caused by zooplankton and other large particulates. Samples of 2 to 30 ml of estuarine water and 25 to 100 ml of oceanic water were filtered on 25-mm, 0.2-μm cellulose triacetate filters (GA-8; Gelman Instrument Co., Ann Arbor, Mich.). Filters were processed immediately or stored at −20°C overnight. Water samples were fixed and prepared for acridine orange direct counts (AODC) as described by Hobbie et al. (11).

Growth of bacterial cultures. *A. citrea* and NRL 1280:8 were maintained on an artificial seawater medium (ASWJP) based on a 1:3 dilution (with distilled water) of the formulation of 2216 (24) at 15°C. Cultures (45 ml each) were grown to a Klett reading of 260, washed once in ASWJP lacking the peptone and yeast extract (ASWJP − PY), and suspended in 10 ml of ASWJP − PY. Serial dilutions were made up to 1:200; 0.2-ml samples were taken in triplicate at each dilution, and the DNA content was determined (see below). Samples were taken at each dilution, diluted further with ASWJP-PY, fixed, and counted by AODC (11).

Extraction of DNA. All solutions and materials were kept at 2 to 4°C throughout the extraction procedure. For extraction of DNA from bacterial cultures, a 0.2-ml sample of culture was placed in a test tube (17 by 100 mm) on ice. A 5% Triton X-100 solution (3 μl) was added, followed by the addition of 1.8 ml of SSC (0.154 M NaCl, 0.015 M trisodium citrate, pH 7.0). The solution was sonicated for 30 s with a Biosonik III sonicator (Bronwill Scientific Inc., Rochester, N.Y.) equipped with a 3/8-in. (ca. .95-cm) probe at the 30 setting (~100 W; hearing protection required).

For the extraction of DNA from cultures or natural microbial populations retained on cellulose triacetate filters, frozen filters were placed in 30-ml beakers in ice, and 4 μl of 5% Triton X-100 and 3.0 ml of SSC were added. The filters were sonicated as described above; care was taken to keep the probe tip centered over the filter. The extract liquid containing filter particles was transferred to a 15-ml Corex centrifuge tube. Large residual filter pieces and beaker walls were washed with an additional 1 ml of SSC, which was added to the centrifuge tube. After centrifugation of the extract at 10,000 × *g* for 10 min in a refrigerated centrifuge, 2 ml of the supernatant was taken for assay.

Determination of DNA: standard method. The standard method for the determination of DNA is essentially an adaptation of the method described by Ceasarone et al. (4). A 1.5×10^{-4} M stock solution of Hoechst 33258 was prepared weekly in distilled water and stored at 4°C in a dark glass bottle wrapped in aluminum foil. The stock solution can be checked by the absorbance at 338 nm (molar extinction coefficient, 4.2×10^{-4} M^{-1} cm^{-1} at pH 7.0). Working dye solutions were prepared by dilution of the stock with SSC to a final concentration of 1.5×10^{-6} M (DNA content of unknown material in the range of 0.5 to 10 μg of DNA [high range]) or 1.5×10^{-7} M (DNA content of unknown material in the range of 50 to 750 ng of DNA [low range]; i.e., all environmental samples). A 1-ml amount of the working dye solution was added to 2 ml of the extract (brought to room temperature), and the fluorescence was measured after 10 min, with the tubes kept in low light. The fluorescence was measured with an Aminco Fluoro/Colorimeter (model J4-7439) fitted with a GE F4T4/BL UV lamp and an R-136 phototube. The excitation filter was an interference filter combination (8% transmission at λ_{max} = 367 nm; half bandwidth, 15 nm), and the emission filter combination was comprised of Corning 5-60 and 3-73 glass filters. For low-scale standards and unknown material, photomultiplier settings of 1 and 3 were employed; settings of 100, 30, and 10 were employed for high-scale samples. Equivalent results were obtained with a Perkin-Elmer MPF-2A fluorescence spectrophotometer (excitation, 350 nm; emission, 450 nm; slit width, 10 nm for both monochromats) fitted with a UV D-25 filter in the excitation beam and run in the ratio mode.

A stock solution of DNA was prepared by dissolving 5 mg of DNA in 5 ml of SSC, sonicating for 10 s, and magnetically stirring for ~30 min. Working stock solutions were made by dilution to 0.1 mg ml^{-1} (high-range standards) or 0.01 mg ml^{-1} (low-range standards), and the concentration was checked by the absorbance at 260 nm with an $A_{1\%}$ (absorbance of a 1% [wt/vol] solution in a 1-cm cell) of 174. Blanks for determinations with filters were made by sonicating a cellulose triacetate filter in SSC–Triton X-100 as described above. Checks of extraction efficiency can be made by adding known quantities of DNA to a filter prior to sonication.

Determination of DNA; increment method. A method for DNA determination based on the incremental addition of unknown or standard material to SSC as described for DAPI by Brunk et al. (2) was also investigated. This method accounts for quenching of extract solutions since all standards are internal. Filter or cell extracts were prepared as described above. Three milliliters of SSC containing 0.5×10^{-7} M Hoechst 33258 was placed in a fluorescence cuvette in the Perkin-Elmer MPF-2A fluorescence spectrophotometer, and the sensitivity was set on the 5× scale. Three or four 20 to 50-μl samples of blank or unknown material were added, and the fluorescence was mea-

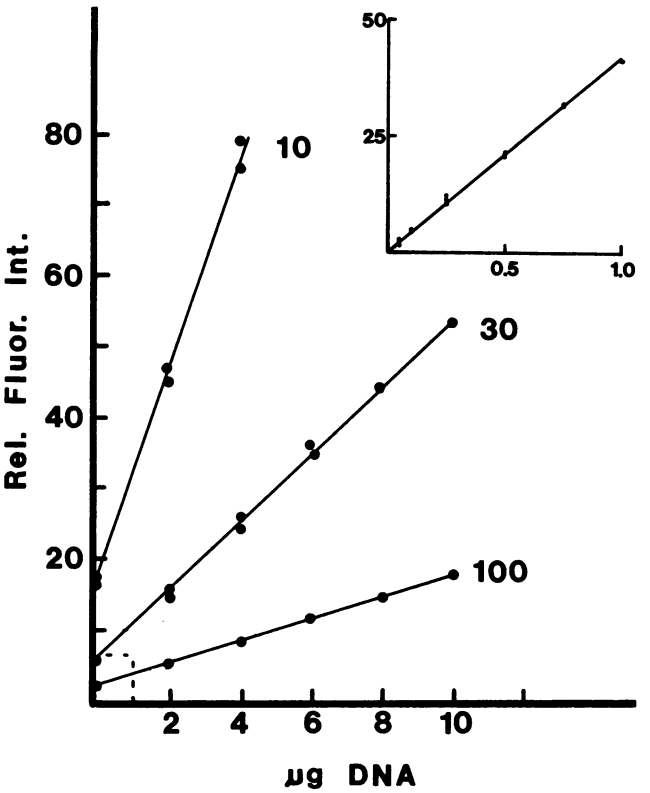

FIG. 1. Fluorescence of 0.5×10^{-6} M Hoechst 33258 as a function of DNA concentration (high range). Numbers refer to photomultiplier settings of fluorometer. (Inset) Fluorescence of low-range concentration of standards with 0.5×10^{-7} M dye and the photomultiplier set at 3.

sured after each increment; then three or four samples of a standard in an equal volume were added. A regression line (relative fluorescence intensity versus volume added) was generated for the unknown and standard material. The slope of the regression line for unknown material divided by the slope of the regression line for standard material yielded a ratio, which yielded the concentration of the unknown when multiplied by the concentration of the standard.

DNase treatment. To verify that the fluorescence observed was due to DNA, DNase I (80,000 dornase units mg of protein^{-1}) was employed to specifically degrade DNA in extracts. DNase I was dissolved in 0.02 M sodium acetate buffer, pH 5.0, containing 5 mM $MgSO_4$ for a final concentration of 2 mg ml^{-1}. To a 2-ml DNA extract in SSC (pH 7.0), 30 µl of 1 M acetic acid, 5 µl of 2 M $MgSO_4$, and 100 µl of the DNase solution described above were added (final pH, 5.3). After 2 h at room temperature, 35 µl of 1 M Tris-hydrochloride, pH 9.0, was added to return the pH to 7.0 to 7.1. Controls were cellulose triacetate filter extracts treated similarly and sample filter extracts lacking DNase. DNA was determined by either the standard method or the increment method.

DNA determinations by diphenylamine. The method of Burton (3) was employed to determine DNA in bacterial extracts. DNA was extracted either by sonication or by heating for 20 min at 70°C in 0.5 N perchloric acid (3).

RESULTS

Figure 1 shows the linearity of fluorescence obtained with DNA standards bound with Hoechst 33258 by the standard method. The correlation coefficient (23) for the linear regression was 0.999. Note also the higher fluorescence value of the blank (as indicated by the *y*-intercept) obtained in the high concentration range due to the 10-fold-greater concentration of Hoechst 33258 employed in this range.

Figure 2 shows the results of sonication for various lengths of time on the extraction of DNA from whole bacterial cells and natural microbial populations, as well as the effects on pure DNA. Near-optimal extraction of DNA occurred by 15 s and complete extraction occurred by 30 s of sonication. Sonication for periods up to 1 min had little effect on the fluorescence of the DNA-dye complex or the absorbance at 260 nm of pure DNA (Fig. 2).

A comparison of extraction methods for bac-

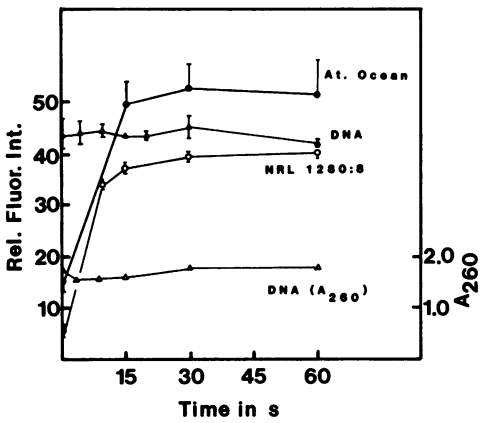

FIG. 2. Fluorescence of pure calf thymus DNA (▲), DNA of an estuarine bacterium (NRL 1280:8) (○), or DNA from a natural microbial population (●) as a function of length of time of sonication. (△) Absorbance of calf thymus DNA at 260 nm (A_{260}). Vertical bars represent ±1 standard deviation when the standard deviation was larger than the symbol.

terial DNA appears in Table 1. Cells filtered onto cellulose triacetate filters yielded an amount of DNA equivalent to that of cells sonicated in solution. Grinding cells on glass fiber or cellulose triacetate filters for 1 min yielded only 84 and 45%, respectively, of the DNA obtained with sonication.

A comparison of extraction of bacterial DNA by perchloric acid treatment or sonication was investigated by the diphenylamine method of Burton (3). Cells were either extracted for 15 min at 70°C in 0.5 N perchloric acid or sonicated for 20 s. Perchloric acid extracts contained only 78% of the DNA of sonicated extracts. When the amount of DNA cell^{-1} was determined in a bacterial culture by both the diphenylamine and Hoechst 33258 methods, the diphenylamine method yielded only 80 to 82% of the DNA yielded by the Hoechst 33258 method.

The excitation and emission spectra of Hoechst-33258-bound calf thymus DNA, Hoechst-33258-bound DNA in a bacterial cell extract, and unbound dye appear in Fig. 3. The excitation and emission maxima for the Hoechst 33258-DNA complex were 352 and 448 nm, respectively. The crude bacterial cell extract gave virtually identical spectra, suggesting that the observed fluorescence was due to DNA. For the free dye, the excitation and emission wavelengths were shifted to 346 and 470 nm, respectively.

Figure 4 shows the linearity between DNA content and cell number (as determined by direct count) for *A. citrea* and isolate NRL 1280:8. The slope of the lines yielded DNA contents of 13.6 ng per 10^{-6} cells for *A. citrea* (r = 0.9998) and 8.4 ng per 10^{-6} cells for NRL 1280:8 (r = 0.9986). As few as 10^7 cells (100 ng of DNA) could be reliably and reproducibly detected with the method.

TABLE 1. Investigation of cell breakage techniques for the release of DNA

Treatment	% of DNA released[a] ± C.V.[b]
Sonication after suspension in SSC	100 ± 3.8
Sonication of cells on membrane filters	104 ± 2.4
Grinding for 1 min	
Cells on glass fiber filters[c]	84.1 ± 12.7
Cells on membrane filters	45.4 ± 2.4

[a] Sonication of cells after suspension in SSC taken as 100%.

[b] C.V., Coefficient of variation.

[c] A mechanized Teflon tissue grinder (Tri R Instruments, Rockville Center, N.Y.) operated at 2,000 rpm and Reeve Angel 984 H glass fiber filters were employed to extract DNA by grinding.

Similar results were obtained when DAPI replaced Hoechst 33258 on an equimolar basis. Unbound DAPI possessed a greater inherent fluorescence, resulting in higher blanks (>160% of the Hoechst 33258 blanks) and thereby reducing sensitivity. We also found that Mg^{2+} inhibited DAPI-DNA fluorescence (by 30 and 64% at 57 and 670 mM, respectively), which was not observed with Hoechst 33258.

To determine the effect of RNA on Hoechst 33258-DNA fluorescence, 10 μg of wheat germ RNA was assayed for fluorescence in the presence and absence of 10 μg of DNA. The RNA yielded a fluorescence of 2.2% of the DNA fluorescence when assayed in the absence of DNA. The fluorescence of the RNA-DNA mixture was identical (99.5%) to that of the DNA alone, suggesting little interference or fluorescence due to RNA.

Figure 5 shows the results of DNA determinations of natural waters. As little as 2 ml (100 ng of DNA) of Potomac River water was required to reliably determine DNA with this method. This may not represent the absolute sensitivity of this method; the cellulose triacetate filters released some material upon sonication that fluoresced in the presence of Hoechst 33258. This "filter blank" corresponds to 25 to 30 ng of DNA per ml of extract, which must be subtracted from values when such filters are employed. Figure 5 also shows the results of DNase treatment of microbial extracts from Potomac River, Severn River, and Atlantic Ocean waters. DNase treatment removed 95 to 100% of the DNA fluorescence, comparable to that observed for DNase treatment of bacterial cell extracts.

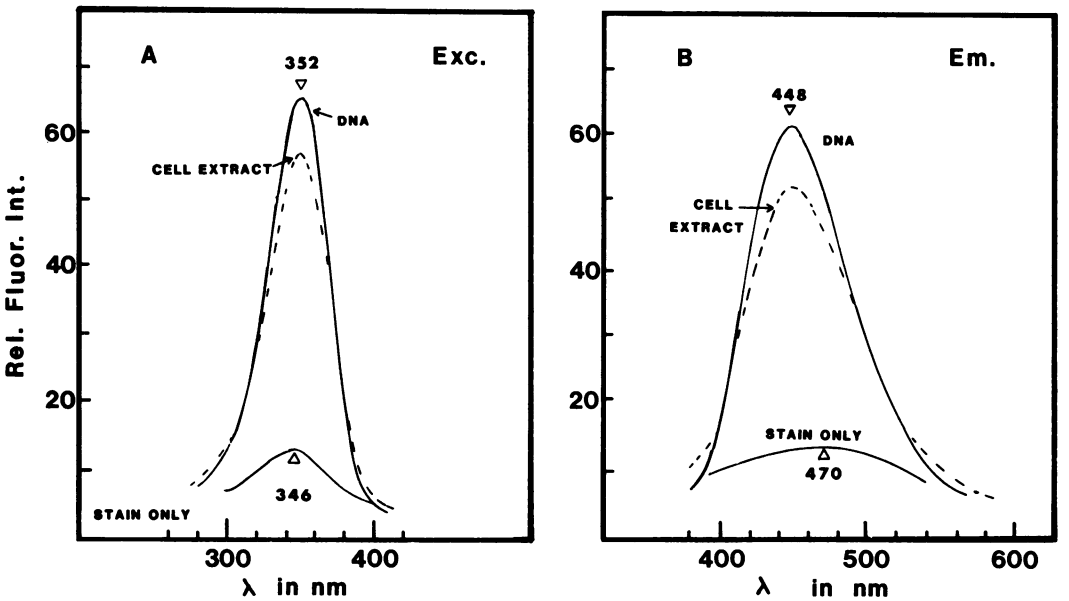

FIG. 3. Excitation (A) and emission (B) spectra of 0.5×10^{-6} M Hoechst 33258 alone, Hoechst 33258 and calf thymus DNA, and Hoechst 33258 and a cell extract of an estuarine bacterium (NRL 1280:8).

Figure 5B compares the increment method with the standard method for DNA in the Atlantic Ocean water sample. The difference observed was not significant; mean values for the standard and increment methods were 27.6 ± 3.5 and 24 ± 3.4, respectively.

The variability of either the increment or the standard method (excluding variability due to extraction) was assessed by determining the DNA content of a standard in quintuplicate by each method. The coefficients of variation were 3.5 and 2.9% for the increment and standard methods, respectively. For natural water samples (including the variability caused by extraction), the coefficients of variation ranged from 4.7 to 22%; the larger values occurred when the volume of water filtered was small and fluorescence approached that of the blank.

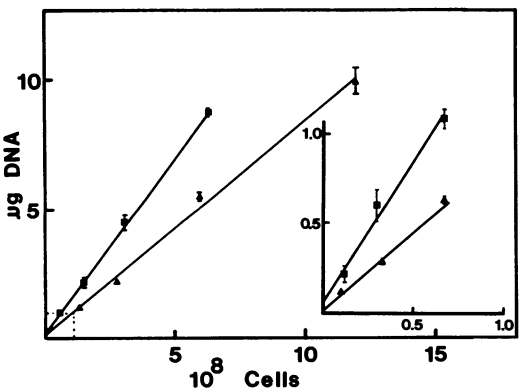

FIG. 4. Observed DNA content as a function of cell number for *A. citrea* (■) or NRL 1280:8 (▲). The inset corresponds to the hatched area in the main portion of the figure.

The relationship between DNA content and bacterial cell number as determined by AODC for the five natural microbial populations investigated is shown in Fig. 6. The regression line generated by these points has a slope of 10.0 ng of DNA per 10^{-6} cells and a correlation coefficient of 0.90. Although the aquatic ecosystems investigated were quite diverse (ranging from eutrophic Potomac River water [salinity, 2‰] to mesotrophic Atlantic Ocean water [33.25‰]), a correlation was found between bacterial cell number and DNA content.

DISCUSSION

A simple method for the determination of DNA in aquatic microorganisms has been developed. The extraction procedure has been optimized and is nondestructive to DNA. The method can be used to detect DNA in as few as 10^7 bacterial cells, and it is reliable to $>10^9$ bacterial cells. The fluorescence observed in cell extracts possessed the same excitation and emission spectra as does pure DNA and was removed by DNase digestion.

No significant difference occurred between results obtained with the increment method and the standard method. The advantage of the increment method is that it will correct for

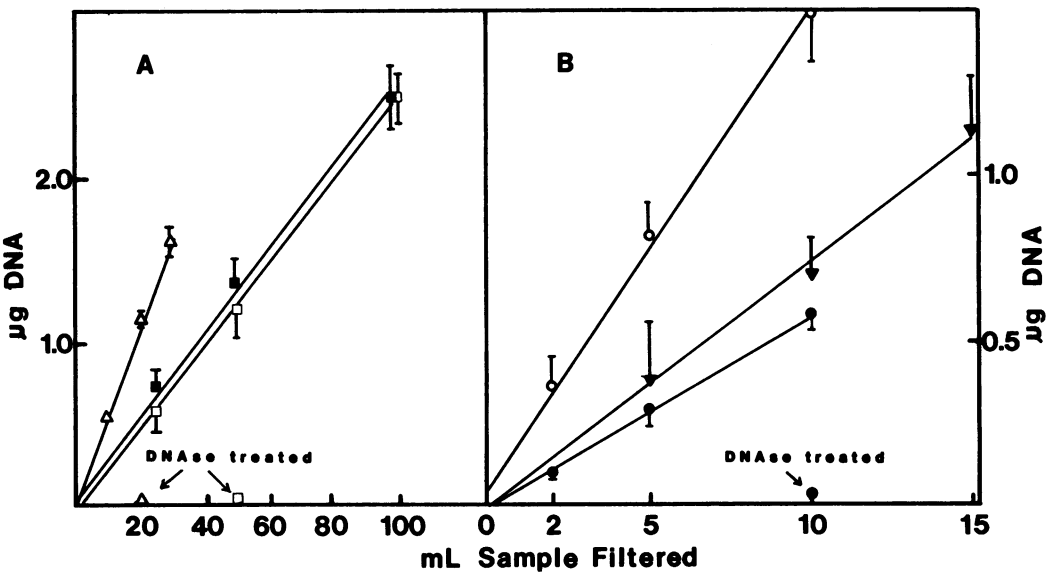

FIG. 5. DNA content as a function of sample volume for waters from various aquatic ecosystems. (A) Severn River water (△) and Atlantic Ocean water determined by the standard (■) or increment (□) method. (B) Potomac River water from 23 September 1981 (○) and 6 November 1981 (●), and Chesapeake Bay water (▼). DNase-treated samples are labeled.

quenching in cell extracts. However, the addition of eight increments to each sample greatly increases assay time, and no corrections for losses during extraction are made. To correct for any DNA underestimation due to quenching or sample extraction, an internal DNA standard can be added to replicate filters before extraction.

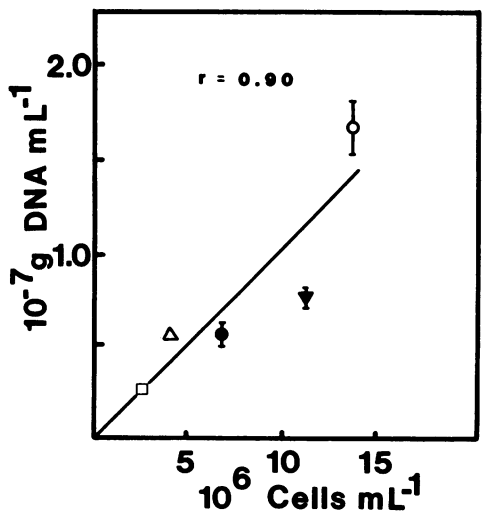

FIG. 6. Relationship between DNA concentration and bacterial cell number as determined by AODC. No estimation of the proportion of DNA attributable to microeucaryotes was made. Symbols are the same as those in Fig. 5. Vertical bars represent ±1 standard deviation when the standard deviation was larger than the symbol.

The diphenylamine method yielded only 80 to 82% of the DNA obtained with the Hoechst 33258 method. This may have been due to the partial destruction of DNA in the hot perchloric acid extraction or to a difference in the base composition between the calf thymus DNA standard and the bacterial DNA. Ceasarone et al. (4) found good agreement between the diphenylamine and Hoechst 33258 methods for various mammalian cells with calf thymus DNA as a standard. Ceasarone et al. and Labarca and Paigen (15) have emphasized the importance of employing a standard with a base composition similar to that of the unknown material. Although this presupposes a knowledge of A+T content of the unknown material and may be important with a particular bacterial species, it may be that the DNA found in heterogeneous mixtures of bacteria (as in environmental samples) more closely resembles the near-50% A+T content of calf thymus DNA.

The DNA in environmental samples ranged from 165 ng ml^{-1} for the September Potomac River water to 27 ng ml^{-1} for Atlantic Ocean water. Holm-Hansen et al. (12, 13) found DNA values ranging from ~2 to 7 µg $liter^{-1}$ for surface oceanic water in the Gulf Stream off North Carolina and off southern California. The coastal waters off La Jolla, Calif., contained 4 to 30 µg of DNA $liter^{-1}$ (12). We investigated

mostly estuarine water and freshwater; however, the results obtained with the Atlantic Ocean water were in rough agreement with those for the coastal waters investigated by Holm-Hansen et al. (12). However, discrepancies due to differences in methodology cannot be discounted.

Surprisingly, DNA content was correlated with bacterial cell number as determined by AODC. The meaning of this correlation is unclear, but it suggests that DNA content was related to bacterial cell number in these environmental samples. However, no parameters for the microeucaryotic population were measured in this study. Future investigations will include size fractionation of microbial populations and measurement of chlorophyll *a* and bicarbonate fixation to assess the magnitude of the microeucaryotic DNA signal.

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LITERATURE CITED

1. **Barth, C. A., and B. S. Willershausen.** 1978. Improvement of DNA quantitation following proteolytic extraction. Anal. Biochem. **90**:167–173.
2. **Brunk, C. F., K. C. Jones, and T. W. James.** 1979. Assay for nanogram quantities of DNA in cellular homogenates. Anal. Biochem. **92**:497–500.
3. **Burton, K.** 1968. Determination of DNA concentration by diphenylamine. Methods Enzymol. **12B**:163–166.
4. **Ceasarone, C. F., C. Bolognesi, and L. Santi.** 1979. Improved micro-fluorometric DNA determination in biological material using 33258 Hoechst. Anal. Biochem. **100**:188–197.
5. **Comings, D. W.** 1975. Mechanisms of chromosome banding. VIII. Hoechst 33258 interaction. Chromosoma **52**:229–243.
6. **Dalbow, D. G., and B. M. Bartuska.** 1979. Modification of the quantitative DNA assay using adriamycin. Anal. Biochem. **95**:559–562.
7. **Erickson, L. C., R. Osieka, N. A. Sharkey, and K. W. Kohn.** 1980. Measurement of DNA damage in unlabeled mammalian cells analyzed by alkaline elution and a fluorometric DNA assay. Anal. Biochem. **106**:169–174.
8. **Fiszer-Szafarz, B., D. Szafarz, and A. Guevara de Murillo.** 1981. A general, fast, and sensitive micromethod for DNA determination: application to rat and mouse liver, rat hepatoma, human leukocytes, chicken fibroblasts, and yeast cells. Anal. Biochem. **110**:165–170.
9. **Groyer, A., and P. Robel.** 1980. DNA measurement by mithramycin fluorescence in chromatin solubilized by heparin. Anal. Biochem. **106**:262–268.
10. **Hill, B. T.** 1976. The use of anti-tumour antibiotics for simple quantitative assays for DNA. Anal. Biochem. **70**:635–638.
11. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
12. **Holm-Hansen, O.** 1969. Determination of microbial biomass in ocean profiles. Limnol. Oceanogr. **14**:740–747.
13. **Holm-Hansen, O., W. H. Sutcliffe, Jr., and J. Sharp.** 1968. Measurement of deoxyribonucleic acid in the ocean and its ecological significance. Limnol. Oceanogr. **13**:507–514.
14. **Kissane, J. M., and E. Robins.** 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J. Biol. Chem. **233**:184–188.
15. **Labarca, C., and K. Paigen.** 1980. A simple, rapid, and sensitive DNA assay procedure. Anal. Biochem. **102**:344–352.
16. **Latt, S. A., and G. Stetten.** 1976. Spectral studies on 33258 Hoechst and related bisbenzimidazole dyes useful for fluorescent detection of deoxyribonucleic acid synthesis. J. Histochem. Cytochem. **24**:24–29.
17. **Le Pecq, J. B., and C. Paoletti.** 1966. A new fluorometric method for RNA and DNA determination. Anal. Biochem. **17**:100–107.
18. **Markovits, J., B. R. Roques, and J. B. Le Pecq.** 1979. Ethidium dimer: a new reagent for the fluorometric determination of nucleic acids. Anal. Biochem. **94**:259–264.
19. **Munro, H. N., and A. Fleck.** 1966. The determination of nucleic acids. Methods Biochem. Anal. **14**:113–176.

19a. **Paul, J. H.** 1982. Use of Hoechst dyes 33258 and 33342 for enumeration of attached and planktonic bacteria. Appl. Environ. Microbiol. **43**:939–944.

20. **Schneider, W. C.** 1980. Simplified isolation and quantitation of cytoplasmic DNA from rat liver. Anal. Biochem. **103**:413–418.
21. **Weisblum, B., and E. Haenssler.** 1974. Fluorometric properties of the bisbenzimidazole derivative Hoechst 33258, a fluorescent probe specific for AT concentration in chromosomal DNA. Chromosoma **46**:255–260.
22. **Williams, S. K., A. W. Sasaki, M. A. Matthews, and R. Wagner.** 1980. Quantitative determination of deoxyribonucleic acid from cells collected on filters. Anal. Biochem. **107**:17–20.
23. **Zar, J. H.** 1974. Biostatistical analysis. Prentice-Hall, Inc., Englewood Cliffs, N.J.
24. **Zobell, C. E.** 1941. Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. J. Mar. Res. **4**:42–75.