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The measurement and distribution of dissolved nucleic acids in aquatic environments

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Abstract

Nucleic acids (DNA and RNA) are ubiquitous components of the dissolved organic matter (DOM) pool of all oceanic, neritic, estuarine, and freshwater habitats studied to date. A new method for the quantitative determination of dissolved nucleic acids (DNA and RNA) in water and sediment samples was developed, evaluated, and utilized in a study of various marine and freshwater ecosystems. Under appropriate reaction conditions, dissolved DNA (D-DNA) and dissolved RNA (D-RNA) are efficiently removed from solution with the addition of cetyltrimethylammonium bromide (CTAB) and subsequent formation of insoluble CTA-nucleic acid salts. The insoluble salts are collected, by filtration, onto glass-fiber filters and analyzed for DNA and RNA with fluorometric and colorimetric procedures, respectively. The performance of this CTAB method is simple, reliable, and reproducible for measuring dissolved nucleic acids in natural aquatic environments. For the ecosystems investigated herein, D-DNA and D-RNA concentrations ranged from 0.56 to 88 μg liter^{-1} and 4.03 to 871 μg liter^{-1}; the ratio of D-RNA to D-DNA ranged from 4.1 to 11.5.

In most aquatic environments, the organic carbon inventory is dominated by nonliving materials, present both in dissolved and particulate states. This nonliving, or detrital carbon is derived from nonpredatory losses of organic matter at all trophic levels (e.g. egestion, excretion, secretion, etc.) and from allochthonous sources (Wetzel et al. 1972). Detrital carbon food webs, initiated by the activities of heterotrophic bacteria (Pomeroy 1974), provide a mechanism for the utilization of the chemical energy contained in the nonliving carbon pool. The potential importance of this putative "microbial loop" carbon flux pathway has focused attention on studies of the concentration, molecular composition, and cycling rates of dissolved organic matter (DOM; Azam et al. 1983).

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Much of the bio-organic chemical research to date has evaluated the distribution and reaction rates of low-molecular-weight compounds, especially monomers (e.g. free amino acids, volatile fatty acids, nucleotides, vitamins, and monosaccharides). As early as 1972, however, deoxyribonucleic acid (DNA), the fundamental molecule of heredity for all self-replicating organisms, was reported as a constituent of the DOM pool of both marine (Pillai and Ganguly 1972) and freshwater (Minear 1972) environments. More recently, the presence of dissolved DNA (D-DNA) has been confirmed by the comprehensive investigations of DeFlaun et al. (1986, 1987) and Paul et al. (1986, 1987). Regional studies performed by the above-mentioned workers in the NE Gulf of Mexico and in coastal Florida environments indicate that D-DNA concentrations are highest in nearshore surface waters and that the steady state levels decrease with increasing distance from land and with increasing water depth. Statistical analyses suggest a significant correlation of D-DNA to both particulate DNA (P-DNA) and bacterial counts, the latter determined by epifluorescence microscopy (DeFlaun et al. 1987). The production and turnover of

D-DNA has also been investigated with [^{3}H]thymidine and [^{3}H]DNA radiotracers (Paul et al. 1987). In the marine environments studied, D-DNA was shown to be produced by actively growing bacteria and removed through the metabolic activities of heterotrophic bacteria and hydrolysis mediated by cell-associated and extracellular nucleases. D-DNA concentrations up to 20 μg liter^{-1} and pool turnover times as short as 6.5 h (Paul et al. 1987) suggest a significant C, N, and P flux through the D-DNA pool.

Continued study of D-DNA pool dynamics has at least two foci (DeFlaun et al. 1987). The first is to study D-DNA concentrations and fluxes to gain a better general understanding of DOM dynamics. The specialized role and distribution of DNA in viable cells, the importance of its monomeric constituents for cellular metabolism and biosynthesis, and its low C : N and C : P ratios make it an attractive macromolecule for studying planktonic rate processes. The second focus is the potential for detritus-to-viable cell genetic exchange via transformation. In the course of our initial investigations of D-DNA, we developed a new method that offers several advantages over previously described techniques. The most significant advantage is that our procedure allows one to obtain simultaneous estimates of both D-DNA and dissolved ribonucleic acid (D-RNA)—an improvement that could potentially provide ecological information not available from estimates of D-DNA alone. Herein we report the first data on total (DNA and RNA) dissolved nucleic acids in marine and freshwater habitats and provide an initial discussion of their potential ecological significance.

Materials and methods

Sample collection—Samples analyzed in this study were collected from various aquatic habitats including the oligotrophic North Pacific Ocean gyre (VERTEX-SEA station; 33°N, 139°W), Bransfield Strait (64°14′S, 61°17′W), coastal seawaters near Oahu, Hawaii (Mamala Bay, Ala Moana Beach, Magic Island, Kaneohe Bay, Kahana Bay), and two freshwater habitats located on the University of Hawaii campus (Krauss Pond, Quarry Pond). Water samples were filtered through 47-mm-diameter, 0.22-μm pore-size membrane filters (Millipore Corp., type GS), in certain cases following prefiltration through 1-μm-mesh (Nitex) or glass-fiber filters (Whatman, type GF/F) to remove larger particles. On occasion, we compared the concentrations of "dissolved" DNA and RNA resulting from the sequential filtration of seawater through a series of filters with decreasing pore sizes, ranging from 0.7 to 0.05 μm.

A typical environmental determination of D-DNA and D-RNA (samples plus internal standards) requires 1–4 liters of water depending upon sample source (greater volumes are required for oligotrophic oceanic habitats). At this stage, the filtered water samples can be either processed for D-DNA and D-RNA (as below) or stored frozen for subsequent analysis. Based on the results of long-term storage experiments that will be presented later, we recommend immediate processing. All water samples analyzed in our study (except those used in the storage experiments) were processed without storage except for the water-column samples collected at 33°N, 139°W which were filtered and stored frozen (−20°C) for 2 weeks before further processing.

CTAB-nucleic acid precipitation method—For seawater samples, the 0.22-μm filtrate is diluted with an equal volume of glass-distilled water; freshwater samples can be processed without dilution (Fig. 1). Typically, 0.5–1 liter of sample is used for a single determination, and it is recommended that five subsamples be prepared for each analysis. Three are used for replicate determination of the concentrations of D-DNA and D-RNA (both nucleic acids can be measured in a single sample); the two remaining subsamples are used to evaluate the recovery of internal DNA and RNA standards. Both DNA and RNA can be added to a single sample as neither nucleic acid significantly affects the recovery or measurement of the other (*see results*). We routinely use 5–10 μg of DNA and 50–100 μg of RNA as our internal standard concentrations. In our experience it is imperative to evaluate the recovery of added DNA and RNA for each separate habitat or environment investigat-

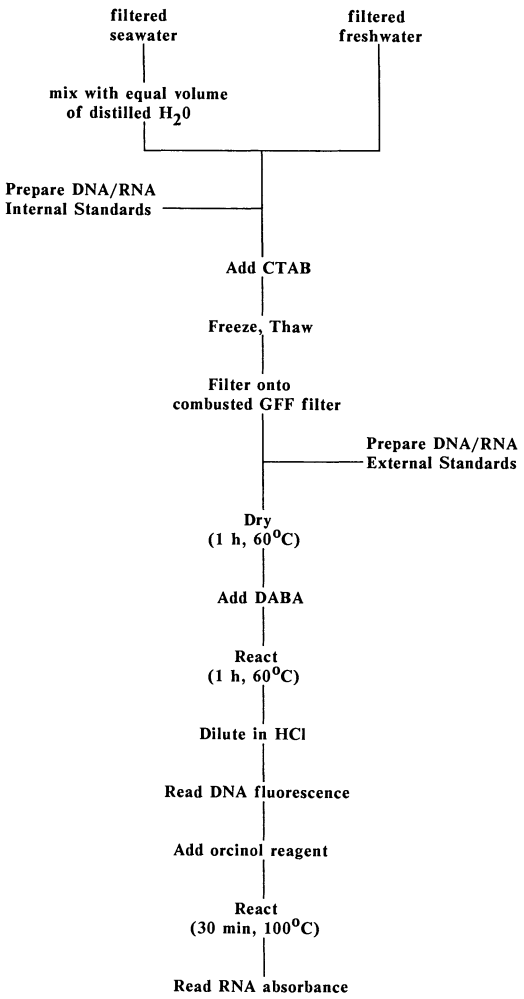


Fig. 1. Schematic representation of the CTAB-DABA-orcinol method for dissolved nucleic acids (CTAB—cetyltrimethylammonium bromide; DABA—diaminobenzoic acid).

ed. However, only one set of recovery experiments needs to be performed for a given site, provided the samples are processed simultaneously. Cetyltrimethylammonium bromide (CTAB; Sigma Chemical Co.; No. H-5882) is added in proportion to the sample volume selected (i.e. for each 100 ml of sample, add 2 ml of a stock solution of 0.5 g of CTAB dissolved in 100 ml of 0.5 M NaCl). The samples are gently mixed, then placed at −20°C until completely frozen. The frozen samples are thawed and the precipitate collected onto combusted (450°C, 4 h) glass-fiber filters (25 mm, Whatman GF/F) and placed into clean, glass liquid scintillation vials in preparation for analysis of DNA and RNA, as described below.

Table 1. Effects of charcoal treatment of DABA on the sensitivity of the DNA assay procedure.

Charcoal added (mg)	[DNA] (ng sample^{-1})	Total fluorescence at 520 nm	Net fluorescence*	Signal: noise ratio†
0	Blank	37.07(±0.06)	—‡	—
	12.5	37.83(±0.23)	0.76	0.021
	400	55.43(±0.32)	18.36	0.495
10	Blank	4.43(±0.06)	—	—
	12.5	5.30(±0.10)	0.87	0.196
	400	22.97(±0.32)	18.54	4.185
25	Blank	2.77(±0.06)	—	—
	12.5	3.20(±1.00)	0.43	0.155
	400	21.50(±0.36)	18.73	6.762
50	Blank	2.47(±0.06)	—	—
	12.5	3.07(±0.06)	0.60	0.243
	400	17.27(±0.46)	14.80	5.992

* [Total fluorescence − blank].
† Net fluorescence ÷ blank fluorescence.
‡ Not applicable.

DNA determination—DNA concentrations were measured with the 3,5-diaminobenzoic acid (DABA) fluorometric reaction (Kissane and Robins 1958; Holm-Hansen et al. 1968). The filters are dried at 60°C for 1 h. To the center of each dried filter is added 100 μl of a purified DABA solution prepared by dissolving reagent grade DABA (Sigma Chemical Co.; No. D-1891) in distilled water at a concentration of 400 mg ml^{-1}. For low level (≤0.5 μg of DNA per sample) determinations, it is necessary to purify the DABA reagent by activated charcoal treatment (Robertson and Tait 1971). We performed an experiment wherein the effects of charcoal treatment were evaluated (Table 1). Based upon these results, we routinely added 25 mg of activated charcoal per milliliter of DABA solution, followed by centrifugation and membrane filtration (Gelman Acrodisc; 0.22 μm). Subsequent to adding DABA, the samples are covered tightly and again placed at 60°C for 1 h followed by addition of 3 ml of HCl (1 M). The samples are vortexed for 1 min, quantitatively transferred to 12- × 75-mm disposable glass test tubes, and the fluorescence determined (excitation, 405 nm; emission, 520 nm). We routinely used a Perkin-Elmer dual spectral grating fluorometer (model LS-5); filter-type fluorom-

eters (e.g. Turner model 111; Gilson, Spectro-glo) can also be used. An external standard curve was prepared with commercially available DNA (Eastman Kodak Co.; No. 11453). RNA does not react with DABA, so no correction was necessary. After the DABA-DNA complex fluorescence is measured, the samples are quantitatively transferred to a clean 12- × 75-mm glass culture tube, centrifuged, and exactly 1.5 ml is added to a 16- × 150-mm test tube for subsequent determination of RNA.

RNA determination—RNA concentrations are measured with the cupric ion-catalyzed orcinol colorimetric reaction (Lin and Schjeide 1969). Each sample (1.5 ml) is diluted with an equal volume of orcinol reagent, which is prepared by dissolving 1 g of reagent grade orcinol (Sigma Chemical Co.; No. O-1875) and 0.15 g $CuCl_2 \cdot 2H_2O$ into 100 ml of concentrated HCl (11.6 M). The samples are tightly sealed and incubated at 100°C for 30 min and then allowed to cool for about 30 min before the absorbance at 666 nm is recorded. A Varian (model DMS 100S) spectrophotometer was used for most RNA determinations. An external standard curve is prepared with commercially available yeast RNA (Sigma Chemical Co.; No. R-7125). The reaction of DNA with the orcinol reagent is <10% that of an equivalent amount of RNA and is considered to be negligible in the present study. As both DNA and RNA are measured by our technique, however, it is possible to determine the precise contribution of DNA for each sample and to make the appropriate correction. In certain natural environments or laboratory samples where the concentration of DNA greatly exceeds that of RNA this correction may be necessary.

DNA- and RNA-amended seawaters—For selected experiments, reagent DNA or RNA was added to filtered (0.22 μm) coastal seawater samples (Mamala Bay and Kaneohe Bay) in order to evaluate the performance of the CTAB precipitation method with respect to sample volume and nucleic acid concentration. In the first set of experiments, exactly 10 μg of DNA was added to increasing volumes of Mamala Bay seawater to provide a set of samples with identical D-DNA mass, but at varying concentrations. The second set of recovery experiments was performed with Kaneohe Bay seawater amended with 25 μg D-DNA liter^{-1} and 250 μg D-RNA liter^{-1}. Various volumes, ranging from 0.01 to 0.2 liter, were processed for DNA and RNA content to evaluate the effect of sample volume on nucleic acid recovery. A final experiment with nucleic acid-amended seawaters was conducted to evaluate the performance of the CTAB precipitation method over D-RNA : D-DNA concentration ratios ranging from 10^{-1} to 10^2.

Effects of sample storage—To evaluate the effects of sample storage on the measurement of D-RNA and D-DNA, we compared, with time, the analysis of replicate samples that were either stored at −20°C as filtered (0.22 μm) seawater or processed immediately with the CTAB precipitation method, collected on filters, and stored at 25°C.

CTAB precipitation of dissolved nucleotides, nucleosides, and nucleic acid bases—In order to evaluate whether CTAB reacts with monomeric constituents of nucleic acids, we performed a precipitation experiment with [2,8-^{3}H]ATP (32 Ci mmol^{-1}; ICN Corp., No. 24009), [2-^{3}H]-adenine (15.8 Ci mmol^{-1}; NEN Corp., No. NET-350), [methyl-^{3}H]-thymidine (49 Ci mmol^{-1}; ICN Corp., No. 24042) and [5,6-^{3}H]uridine (55 Ci mmol^{-1}; ICN Corp., No. 24046). Each radiolabeled compound was added, individually, to a liter of filtered (0.22 μm) seawater from which triplicate subsamples were prepared. Distilled water and CTAB were added as described above. A portion of each preparation was counted for total radioactivity. The samples were frozen, thawed, and filtered (as above). The filtrates were radioassayed to provide an estimate of the percentage of the individual compounds that was removed by the CTAB precipitation procedure.

Ancillary measurements—For selected water samples, particulate ATP was collected onto a glass-fiber filter (GF/F), extracted in boiling phosphate buffer (pH 7.4, 60 mM; Karl and Craven 1980), and measured with the firefly bioluminescence procedure (Karl and Holm-Hansen 1978).

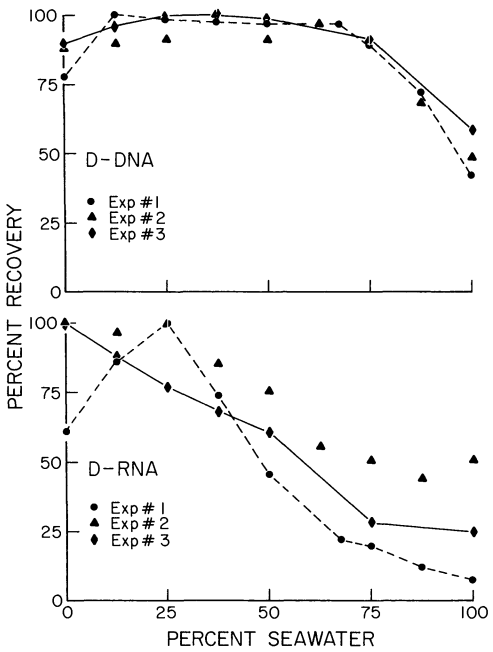


Fig. 2. Recovery of D-DNA and D-RNA as a function of salt content of the sample. The seawater samples were diluted with glass-distilled water to achieve a range of salt concentrations. The individual experiments were performed on different days with separate samples of Kaneohe Bay seawater and reagents.

Particulate DNA was collected onto GS-Millipore membrane filters (0.22 μm) and measured with the DABA assay procedure (Holm-Hansen et al. 1968). DNA synthesis, RNA synthesis, and total microbial production were measured with the [^{3}H]adenine procedure (Karl and Winn 1984). The water samples were incubated in situ for 12 h.

Results

Effects of salt on CTAB precipitation—We evaluated the performance of the CTAB-precipitation method as a function of the salt content of the sample (Fig. 2). Our results indicate that both CTA-RNA and CTA-DNA salts are more soluble in full-strength seawater (salinity, 35.5‰) than in more dilute solutions. Maximal recovery of DNA required at least 25% dilution of seawater, whereas maximal recovery of RNA did not occur until seawater had been diluted by at least 75%. These results are consistent with previous investigations of critical salt concentration (Honig et al. 1973).

Table 2. Recovery of D-DNA as a function of sample volume and D-DNA concentration.

Total sample volume (liters)	DNA concn (μg liter^{-1})	Total sample DNA (μg)	Fluorescence at 520 nm*	SD/mean (×100%)
0.02	500	10	377.9(±3.3)	0.9
0.04	250	10	400.9(±7.7)	1.9
0.10	100	10	394.0(±7.0)	1.8
0.2	50	10	395.3(±2.0)	0.5
0.5	20	10	383.8(±0.4)	0.1
1.0	10	10	385.3(±4.1)	1.1
2.0	5	10	398.5(±4.5)	1.1

* Values presented are means and standard deviations (in parentheses) for triplicate determinations. The "expected" fluorescence for a 10.0-μg-DNA sample was 390 fluorescence units as determined by the external standard curve, indicating quantitative recovery of the added D-DNA.

Based on our experimental results we recommend that seawater samples be diluted at least 1 : 1 (i.e. 50% seawater; *see Fig. 1*) with glass-distilled water before adding CTAB. Further dilution (to 10–25% seawater) is possible and will increase the efficiency of D-RNA recovery, but will also increase total sample volume and processing time.

For freshwater samples, no dilution is required before adding CTAB. We initially experimented with adding NaCl to the filtrates (to a final concn of 0.3 M) in order to eliminate the potential precipitation of acidic polysaccharides. Their presence could interfere with the orcinol measurement of RNA; no significant differences were observed, however, in the estimated concentrations of RNA from various freshwater samples.

Recovery of dissolved nucleic acids—Dissolved nucleic acids were efficiently and quantitatively recovered from seawater, regardless of absolute concentration (5–500 μg D-DNA liter^{-1}) or sample volume (0.02–2 liters) over the ranges tested (Table 2 and Fig. 3). Additional experiments, not reported here, have extended the upper limit of sample volume to 4.0 liters, and the lower limit of concentration to 0.5 μg D-DNA liter^{-1} without affecting recovery efficiency. Furthermore, recovery of RNA is not influenced by the presence of DNA, and vice versa, over a wide range of RNA : DNA concentration ratios (Fig. 4).

Sample storage—The insoluble CTA-nucleic acid salts, once concentrated onto glass-

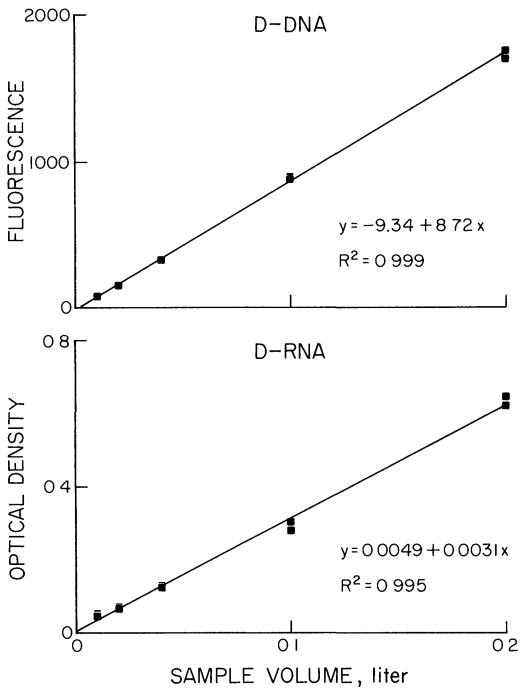


Fig. 3. Recovery of D-DNA and D-RNA by the CTAB-DABA-orcinol procedure as a function of sample volume. The seawater sample was collected from Mamala Bay, Hawaii.

fiber filters, are more stable when stored at 25°C than when the dissolved macromolecules are stored in frozen seawater (Fig. 5). A second experiment, comparing the stability of the CTA-nucleic acid salts at −20° and 25°C indicated that no significant changes occurred during long-term storage at either temperature (data not presented).

Recovery of low-molecular-weight nucleic acid constituents—We evaluated the efficiency of the CTAB-precipitation method for the removal of free nucleotides (ATP) and monomeric constituents of RNA (adenine, uridine) and DNA (thymidine). Our results indicate that none of these low-molecular-weight compounds are removed from solution by CTAB addition (Table 3). For the nucleosides and purine base, there was no significant removal ($\leq 1\%$) and for ATP there appeared to be an insignificant (1–4%) loss.

Size fractionation experiments—Both the DNA and RNA measured in coastal seawaters appear to be truly dissolved and are not removed by filtration through membrane filters with a mean pore size of 0.05 μm (Fig. 6). Greater than 90% of the DNA and RNA which passes through a GF/F fil-

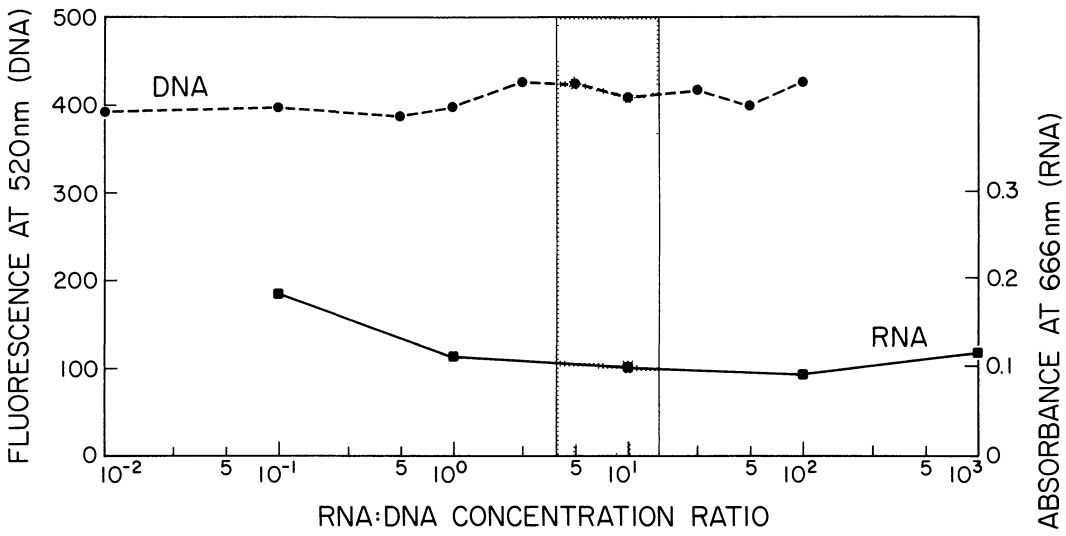


Fig. 4. Recovery of DNA (fluorescence) and RNA (absorbance) as a function of RNA : DNA concentration ratio. For the DNA recovery experiment the DNA concentration was fixed at 10 μg per sample and the ratio was varied by adding RNA. For the RNA recovery experiment the RNA concentration was fixed at 100 μg per sample and variable DNA was added to achieve a given ratio. The RNA absorbance values were corrected for DNA interference at RNA : DNA ratios ≤ 1. The stippled region indicates the range of concentration ratios observed in our field measurements.

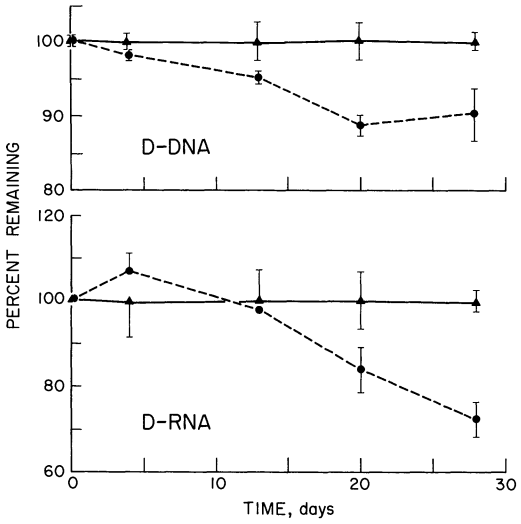


Fig. 5. Effects of sample storage on the recovery and measurement of D-DNA and D-RNA. Three sets of samples were prepared on day zero and stored frozen (dashed lines) or precipitated with CTAB and stored dry at 25°C (solid lines). With time, subsamples were removed and analyzed for DNA and RNA and expressed as percent of time-zero values. Values are means (n = 3) ±1 SD.

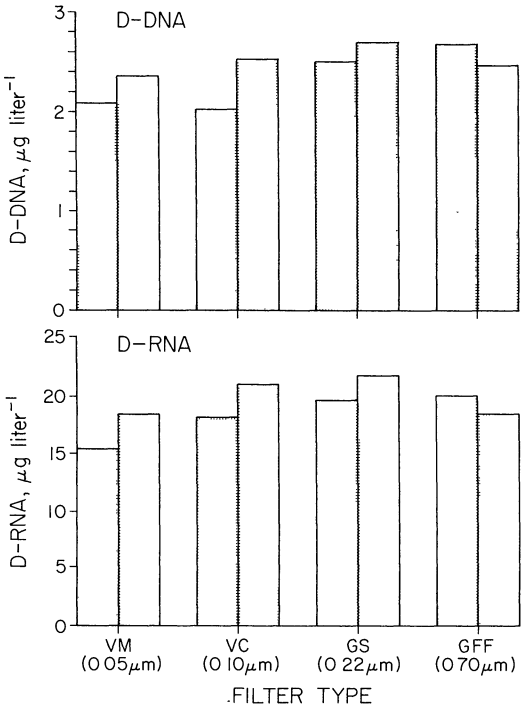


Fig. 6. D-DNA and D-RNA concentrations in Kaneohe Bay as a function of the type of filter used to isolate the "dissolved" fraction. Filter types VM, VC, and GS are all from Millipore Corporation and composed of mixed esters of cellulose acetate and cellulose nitrate. Filter type GFF is manufactured by Whatman and is a glass-fiber composite.

ter (with a 98% retention of all particles ≥0.7 μm; Whatman Inc.) also passes through a VM filter (with a 0.05 μm porosity; Millipore Corp.). There was no systematic change in the dissolved DNA : RNA ratio as a function of filter size.

Discussion

Comparison of CTAB precipitation to previously used methods—We considered the methods used previously for isolating and measuring D-DNA from aquatic environments and evaluated their advantages and limitations for our field application (Table 4). The lyophilization procedure described by Minear (1972) cannot be used for seawater samples because of the presence of

Table 3. Precipitation of monomeric nucleic acid components by the CTAB procedure. Figures are means (±SD).

Compound	Mass added (μg liter⁻¹)	Isotope added* (μCi liter⁻¹)	Isotope recovered*† (μCi liter⁻¹)	Recovered (%)
Nucleic acid base				
adenine	0.43	50.40(±0.3%)	49.92(±1.1%)	99.0
Nucleosides				
thymidine	0.31	61.72(±0.6%)	61.37(±1.8%)	99.4
uridine	0.15	33.86(±0.9%)	34.16(±0.6%)	100.9
Nucleotide				
ATP	0.76	47.67(±0.9%)	46.16(±1.4%)	96.8

* Based on triplicate determinations.

† Isotope recovered is defined as radioactivity remaining in dissolved form after the CTAB-precipitation procedure described in materials and methods.

Table 4. Methods for isolating and detecting dissolved nucleic acids in aquatic environments.

Reference	Nucleic acid	Method	Detection	Comments
Minear 1972	DNA	Filtration, cation exchange, lyophilization, gel chromatography to isolate HMW fraction	DABA fluorescence	Extraction method cannot be used for seawater samples due to presence of salt
Pillai and Ganguly 1972	DNA	Filtration, $BaSO_4$ precipitation, hydrolysis	UV spectroscopy	Extraction method not specific for nucleic acids; assay system lacks specificity for DNA
Breter et al. 1977	DNA	Filtration, CTAB precipitation (25°C, 24–48 h), centrifugation, acid hydrolysis, evaporation, dissolution	Isocratic high-pressure liquid chromatography of thymine base	Extraction method and assay system are tedious and time-consuming
DeFlaun et al. 1986	DNA	Filtration, ethanol precipitation (−20°C, 48 h) centrifugation, dialysis against water (4°C, 48 h), dialysis against buffer (4°C, 24 h)	Hoechst 33258 fluorescence	Extraction method is time-consuming; assay system specific for double-stranded DNA
Present study	DNA, RNA	Filtration, CTAB precipitation (−20°C, 1–3 h), filtration	DABA fluorescence (for DNA), orcinol reaction (for RNA)	Extraction method can be used for D-DNA and D-RNA; DNA assay system detects both single-stranded and double-stranded DNA; overall method is simple, fast, and reproducible

$\sim 10^6$ times more dissolved salts than total dissolved nucleic acids. Furthermore, this method concentrates all dissolved constituents present in the water sample (including extracellular nucleases) and does not constitute a net "purification" of the nucleic acid fraction. The method of Pillai and Ganguly (1972), using $BaSO_4$ precipitation, offers a potential method for separating and partially purifying nucleic acids from the seawater salts. This method, however, also precipitates dissolved polysaccharides which are known to interfere with the orcinol detection of RNA and, therefore, was not further evaluated in our study.

Ethanolic precipitation has been used by DeFlaun et al. (1986, 1987) and Paul et al. (1986, 1987) to isolate D-DNA from freshwater and marine habitats. This procedure is widely used in molecular biology and seems to perform well. Although not explicitly stated in the studies of DeFlaun et al. and Paul et al., the ethanolic precipitation procedure catalyzes the removal of both D-DNA and D-RNA as well as dissolved proteins. A disadvantage of the DeFlaun et al. (1986) procedure is the time-consuming isolation period (at least 5 d) during which DNA is precipitated and desalted by dialysis. As our primary objective was to develop a procedure that was compatible with rapid shipboard analysis, ethanolic precipitation was not practical. Breter et al. (1977) used the cationic detergent CTAB as an alternative method for effectively removing D-DNA from seawater samples. As this method of DNA isolation appeared to be straightforward and amenable to rapid shipboard processing we further investigated CTAB as our method of choice (Table 4).

The use of CTAB to isolate nucleic acids and separate DNA and RNA was first re-

ported in the early 1950s (Jones 1953) and has continued extensively as an efficient precipitating agent for dissolved nucleic acid. The unique properties of CTA-nucleic acid salts and their differential solubilities in solutions of varying concentrations of NaCl provide an analytical framework: for separation of DNA and RNA from proteins and polysaccharides (Bellamy and Ralph 1968), for separation of DNA from RNA (Young 1968; Honig et al. 1973), and for differentiation of DNA based on molecular weight and conformation (Honig et al. 1973). CTA-nucleic acid precipitates can be redissolved in 1 M NaCl and have been shown to yield purified preparations of DNA and RNA in unchanged, native configuration (Bellamy and Ralph 1968). One additional advantage of the CTAB isolation procedure is that, as opposed to ethanolic or trichloroacetic acid precipitation methods, proteins do not react with the precipitating agent. Consequently, the use of CTAB eliminates potential contamination by nucleases known to occur in seawater (Maeda and Taga 1973).

It had been shown previously (Sibatani 1970) that the recovery of nucleic acids is dependent on NaCl concentration. Our results with seawater are consistent with results obtained with NaCl solutions. D-DNA and D-RNA appear, however, to have different critical salt concentrations for the optimal formation of insoluble salts (Fig. 2). Consequently, it is mandatory to use internal standards in order to obtain accurate estimates of dissolved nucleic acids in marine and freshwater habitats. Our laboratory results with nucleic acid-amended seawaters indicated that recovery of either RNA or DNA is not affected by the presence of the other nucleic acid over wide concentration ratios (Fig. 4).

We observed a loss of about 15–30% of D-DNA and D-RNA in stored seawater over a period of 30 d (Fig. 5). We presently interpret this loss to be the result of extracellular nucleases. If nucleases are suspected to be present in a given sample, one could add EDTA as recommended previously (Sibatani 1970). We have used this modification for samples collected in the eutrophic coastal area of the Antarctic peninsula (Bailiff and Karl in press). We also determined that there was no difference between the recoveries of CTA-nucleic acid salts when stored at either −20° or 25°C. We recommend that CTAB precipitation be carried out as soon as possible after sample collection to minimize inadvertent losses of dissolved nucleic acids.

Detection of isolated nucleic acids—Several methods currently exist for the quantitative determination of DNA and RNA. We selected DABA (DNA) and orcinol (RNA) on the basis of specificity, compatibility, and ease of operation. DABA is specific for aldehydes with an unsubstituted α-carbon, which is a property of deoxyribose. DABA does not react with ribose or with RNA. Other methods of analysis include diphenylamine, DAPI, Hoechst 33258, ethidium bromide, mithramycin, and adriamycin, all of which could, in theory, be used to measure the D-DNA isolated by the CTAB procedure. DAPI and Hoechst 33258 are two structurally related dyes that react specifically with DNA to produce a strongly fluorescent complex. Substantial fluorescent enhancement occurs when either dye reacts with an A-T-rich portion of double-stranded DNA. Although both dyes react only with double-stranded DNA, only DAPI seems to bind via intercalation (Comings 1975). This requirement for native DNA could be an advantage or a disadvantage, depending on the specific objectives of the field program. If one desires to measure total DNA, as was our objective, then the limited reactivity of DAPI and Hoechst 33258 is a disadvantage. Replicate sample analysis by DABA and one of the above-mentioned fluorochromes could, in theory, be used to determine the percentage of denatured DNA present in a given habitat.

The fluorometric DABA assay system can detect 10–20 ng of DNA with a signal-to-background noise ratio of 2 : 1. Ten nanograms of DNA is about equal to that contained in 50 ml of open-ocean seawater. This level of sensitivity is conferred by the purification of DABA with activated charcoal, resulting in an order of magnitude reduction in background fluorescence (Table 1), and the use of a high quality, stable dual diffraction grating spectral fluorometer (Perkin-Elmer, model LS-5).

The detection of RNA is based on a reaction between orcinol and the pentose sug-

Table 5. Summary of D-DNA and D-RNA measurements in aquatic habitats.

Sample location	D-DNA (μg liter⁻¹)	D-RNA (μg liter⁻¹)	Dissolved RNA:DNA	Reference
Marine: coastal/estuarine				
Bombay Harbor, India (range of four stations)	13.4–80.6	—*	—	Pillai and Ganguly 1972
Northern Adriatic Sea (range of two stations; 0–30 m)	0.05–0.8†	—	—	Breter et al. 1977
Tampa Bay (Feb–May 85; range of several stations)	9–12.5	—	—	Paul et al. 1986
Loggerhead Key, Dry Tortugas (range of several stations)	0.72–1.32	—	—	Paul et al. 1986
Bayboro Harbor, Florida (Mar 86)	18.32(±1.78)	—	—	DeFlaun et al. 1986
Tampa Bay/Charlotte Harbor, Florida	10–19	—	—	DeFlaun et al. 1987
Florida Bay, Florida	10–15	—	—	DeFlaun et al. 1987
Bransfield Strait, Antarctica (Dec 86; range of 69 stations)	6–15	—	—	Bailiff and Karl 1987
Kaneohe Bay, Hawaii (Nov 86; range of eight stations)	10–16	—	—	Present study
Kaneohe Bay, Hawaii (Mar 88; range of four stations)	2.66–3.15	20.6–31.9	7.8–10.3	Present study
Ala Moana Beach, Hawaii	19–21	—	—	Present study
Magic Island, Hawaii	14–19	—	—	Present study
Mamala Bay, Hawaii (Mar 88)	1.02(±0.08)	6.67(±2.67)	6.54	Present study
Kahana Bay, Hawaii (Mar 88; water column)	4.70	51.1	10.9	Present study
Kahana Bay, Hawaii (Mar 88; sediment pore-water range of several depths, 0–15 cm)	12.2–28.4	54.4–192.8	4.5–6.8	Present study
Marine: offshore				
SE Gulf of Mexico (loop current)	0.7–4.1	—	—	Paul et al. 1986
Gulf of Mexico (~25°N, 83°W)	0.2–1.9	—	—	DeFlaun et al. 1987
Gulf of Mexico (~27°N, 85°W)	2–5	—	—	DeFlaun et al. 1987
Gulf of Mexico (~26°30′N, 85°W; 0–1,500 m)	0.3–4.1	—	—	DeFlaun et al. 1987
N. Pacific Ocean (33°N, 139°W; 0–400 m)	0.56–1.39	4.03–13.9	4.1–11.5	Present study
Freshwater				
Alafia River, Florida	44	—	—	DeFlaun et al. 1986
Medard Reservoir, Florida	6.97	—	—	DeFlaun et al. 1986
Crystal River, Florida	1.74	—	—	DeFlaun et al. 1986
Boyd Hill, Florida	7.8	—	—	DeFlaun et al. 1986
Pine Lake, Washington	7–10‡	—	—	Minear 1972
Lake Washington, Washington	4–9‡	—	—	Minear 1972
Moses Lake, Washington (Jul–Dec 69; range of several stations)	10–30‡	—	—	Minear 1972
Quarry Pond, Hawaii	3.54(±0.03)	23.0(±0.16)	6.50	Present study
Krauss Pond, Hawaii	88	871	9.9	Present study

* No data available.
† Assumes DNA is 10% thymine, by weight.
‡ Reported as "minimum" concentrations.

ar moiety of purine nucleotide bases at elevated temperature. This general colorimetric reaction is not specific for RNA, per se. In crude cell extracts or in particulate matter collected from natural ecosystems non-RNA, orcinol-reactive materials (mostly polysaccharides) exist and would be expected to affect the accuracy of orcinol-derived estimates of RNA. However, the CTAB method described here does not precipitate poly-

saccharides (Bellamy and Ralph 1968), so potential nonspecificity of the orcinol reaction is not a problem. One limitation of the spectrophotometric-based orcinol reaction for RNA is the relative lack of sensitivity compared to fluorometric methods. If one uses a high-quality spectrophotometer (such as the Varian model DMS 100S used in this study) with a low-volume 5- or 10-cm pathlength cell, total RNA as low as 250–500 ng can be detected. At present, this sensitivity sets the lower limit on the water sample volume required for combined measurement of D-DNA and D-RNA.

Nature of the "soluble" nucleic acids—The presence of detectable levels of dissolved nucleic acids in environmental samples raises the possibility of artifacts based on the methods used to separate the particulate phase from the dissolved phase. DeFlaun et al. (1986) investigated the possibility of microbial cell lysis during vacuum filtration and concluded that lysis was not contributing to D-DNA levels at vacuum pressures ≤ 150 mm Hg. It is conceivable that small, viable cells, spores, virus particles, or detrital aggregates may have contained the nucleic acids that we classify as dissolved. A representative analysis of D-DNA and D-RNA as a function of membrane filter pore size and, hence, particle size (Fig. 6) revealed, however, that an order of magnitude decrease in the mean pore size of the filter over the range of 0.05–0.7 μm had little effect on the quantity of D-DNA or D-RNA measured in the respective filtrates. This experiment was performed on four separate occasions with a variety of source materials and provided essentially identical results.

The CTAB-precipitation method and the DABA detection system used in our procedure ensure that both single-stranded and double-stranded DNA are measured. Although we did not attempt to determine the minimum size of a DNA or RNA molecule that is required to form an insoluble CTA-nucleic acid salt, we do know from direct experimentation that neither nucleotides (ATP), nucleosides (uridine and thymidine), nor nucleobases (adenine) are removed from solution by the CTAB-precipitation procedure (Table 3). In previous studies, it was determined that CTAB precipitation is capable of the quantitative recovery of DNA digests ≤ 121 base pairs long (Cockerill 1988) and of single-stranded DNA fragments as small as 200 nucleotides (Sclafani and Wechsler 1981).

D-DNA and D-RNA concentrations in nature—The field results obtained with the CTAB-DABA-orcinol detection procedure suggest a ubiquitous distribution of D-DNA and D-RNA in natural ecosystems (Table 5). Concentrations varied among the environments studied with maximal values in the hypereutrophic freshwater pond (maximal D-RNA and D-DNA concentrations of 871 and 88 μg liter^{-1}) and minimal values at oligotrophic oceanic sites (minimal D-RNA and D-DNA concentrations of 4 and 0.6 μg liter^{-1}). In spite of this wide range in absolute values of D-DNA and D-RNA among the ecosystems investigated, there was a relatively constant ratio of D-RNA to D-DNA (range, 4.1–11.9). From the few data available, there does not seem to be any relationship between the concentration of either D-DNA or D-RNA and the D-RNA : D-DNA concentration ratio. The nucleic acid concentration ratios observed in these samples are not substantially different from the concentration ratios expected to occur in viable cells at the growth rates expected in nature (i.e. $\mu = 0.1$–1 d^{-1}; Karl 1981).

Irrespective of the above-mentioned relationship between dissolved nucleic acid concentrations and standing stock of microbial cells among various diverse environments, these general relationships do not hold within a given habitat (Figs. 7–10). For example, in a coastal-to-open-ocean transect beginning in Kaneohe Bay (Fig. 7), the D-DNA concentrations were relatively constant despite a decrease of nearly two orders of magnitude in total particulate ATP (Fig. 8). Clearly, in this habitat the increased standing stocks and microbial productivity within Kaneohe Bay did not appear to influence the ambient pools of D-DNA. Similarly, at two very different ocean stations (one in a eutrophic Antarctic habitat and the other in the oligotrophic North Pacific Ocean) we observed a change of, at most, a factor of twofold to threefold for the D-DNA

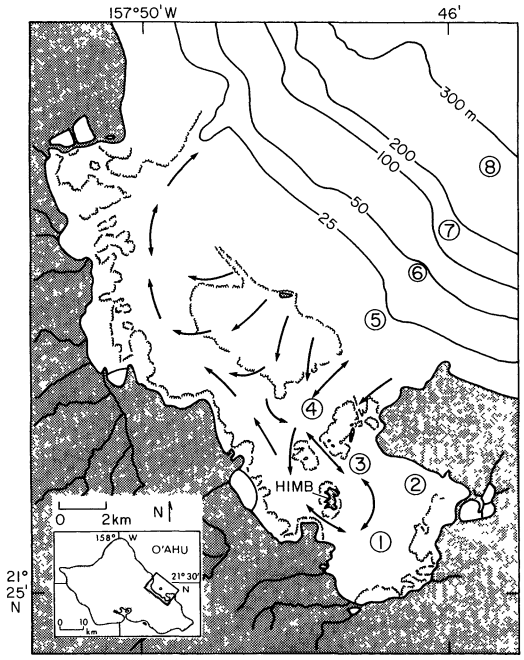


Fig. 7. Map of Kaneohe Bay, showing the mean current patterns (arrows) and location of the stations (numerals) sampled in this study. HIMB is the location of the Hawaii Institute of Marine Biology which served as our field base of operations.

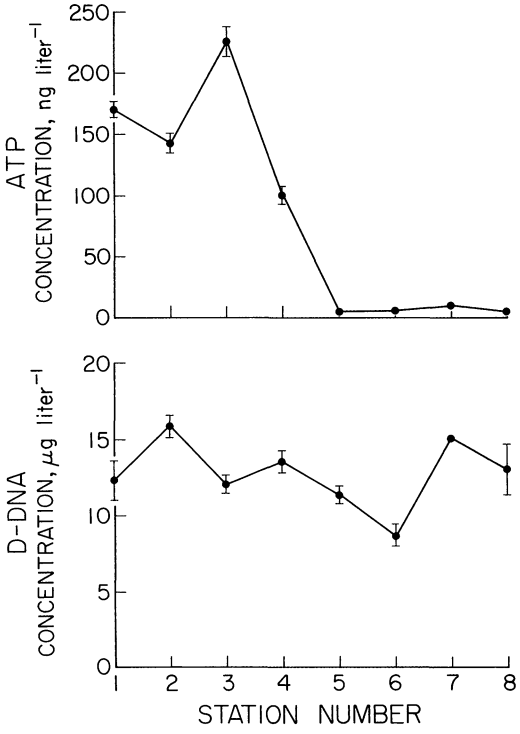


Fig. 8. Particulate ATP and D-DNA concentrations for the stations depicted in Fig. 2. Values are means of triplicate determinations ±1 SD.

and D-RNA concentrations in the upper portion of the water column (Figs. 9–10), despite order-of-magnitude, depth-dependent decreases in parameters of total microbial biomass (particulate ATP and DNA) and total microbial production (based on [^{3}H]adenine assimilation. These two sets of oceanic field data seem to be at odds with the suggestion of rapid microbial production and uptake of dissolved nucleic acids, unless the ambient concentrations are always kept at a low pool size.

Ecological significance of dissolved nucleic acids—The occurrence of dissolved DNA and RNA in aquatic environments and the reported rapid rates of uptake of D-DNA in samples collected from nature suggest a significant flux of C, N, and P through the total dissolved nucleic acid pool. Until the appearance of the recent, more comprehensive reports of the presence of D-DNA in environmental samples (beginning with DeFlaun et al. 1986), the previously published data could have been dismissed as artifactual. The convincing evidence currently available on the distribution of D-DNA and, now, D-RNA demands a serious evaluation of the potential pathways for the production and removal of dissolved nucleic acids under in situ conditions.

Detrital nucleic acids are considered to exist in dynamic equilibrium between dissolved and particulate phases. Principal pathways for the production of detrital, and in particular dissolved, nucleic acids include excretion and exudation, losses due to inefficient feeding, and death and autolysis. Of these three pathways, we know that both unicellular algae (Minear 1972) and bacteria (Catlin 1956; Demain et al. 1965; Hara and Ueda 1981; Ueda and Hara 1981) can produce extracellular nucleic acids during normal growth and metabolism under laboratory conditions. Studies of D-DNA production by exponentially growing cultures of *Bacillus subtilis* (Ephrati-Elizur 1968) concluded that DNA excretion may be the result of temporarily unbalanced

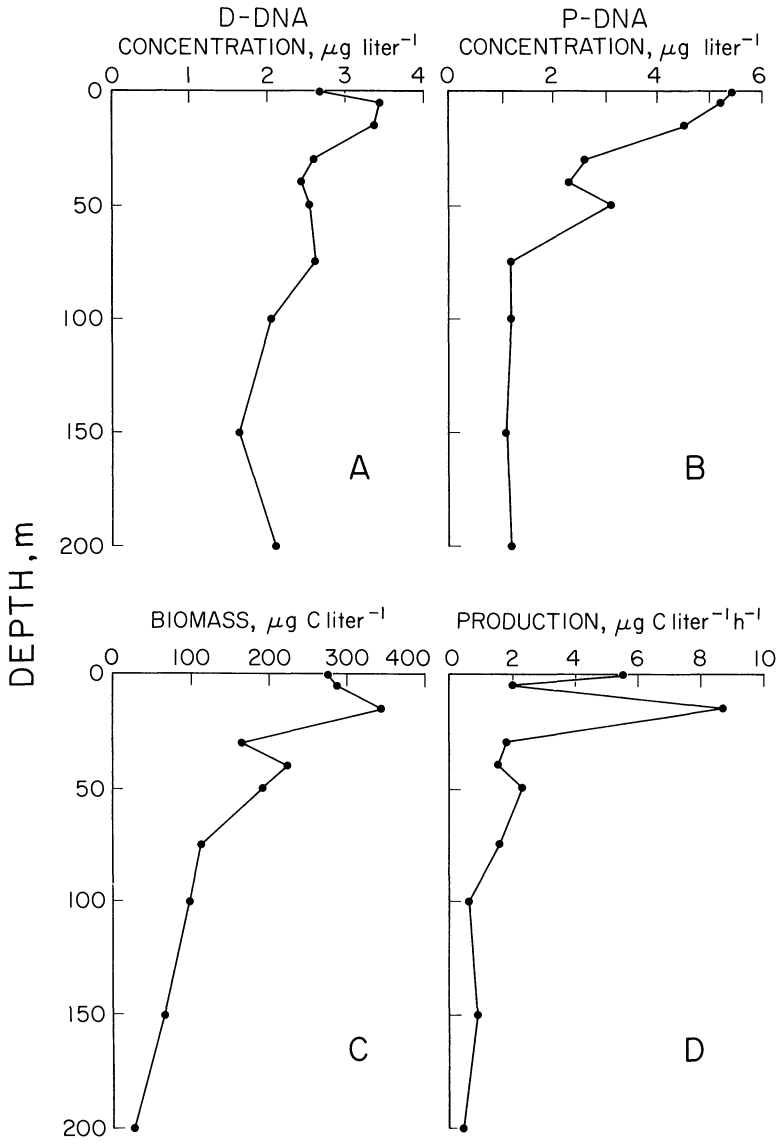


Fig. 9. Vertical distribution of D-DNA, P-DNA, biomass-C (ATP concn × 250), and production (based on [^{3}H]adenine assimilation; Karl and Winn 1984) for water collected at a station in the southern sector of Bransfield Strait. These data were obtained during the Research on Antarctic Coastal Ecosystem Rates (RACER) field program in December 1986.

growth. Sinha and Iyer (1971) suggested that the rate of release of DNA and RNA from bacteria may be accelerated when cells are in the physiological state of competence for transformation, thereby promoting efficient genetic exchange. Genetic mutants selected for transformation impairment did not release DNA or RNA into the medium. Revertant strains were positive for nucleic acid release (Sinha and Iyer 1971). Paul et al. (1987) conducted field experiments with [^{3}H]thymidine and confirmed that heterotrophic bacteria produce D-DNA under field conditions as well. We performed a similar experiment with [^{3}H]adenine which labels both algae and bacteria (Karl and Winn 1984). Our results (unpubl.) confirm those of Paul et al. (1987) and demonstrate both

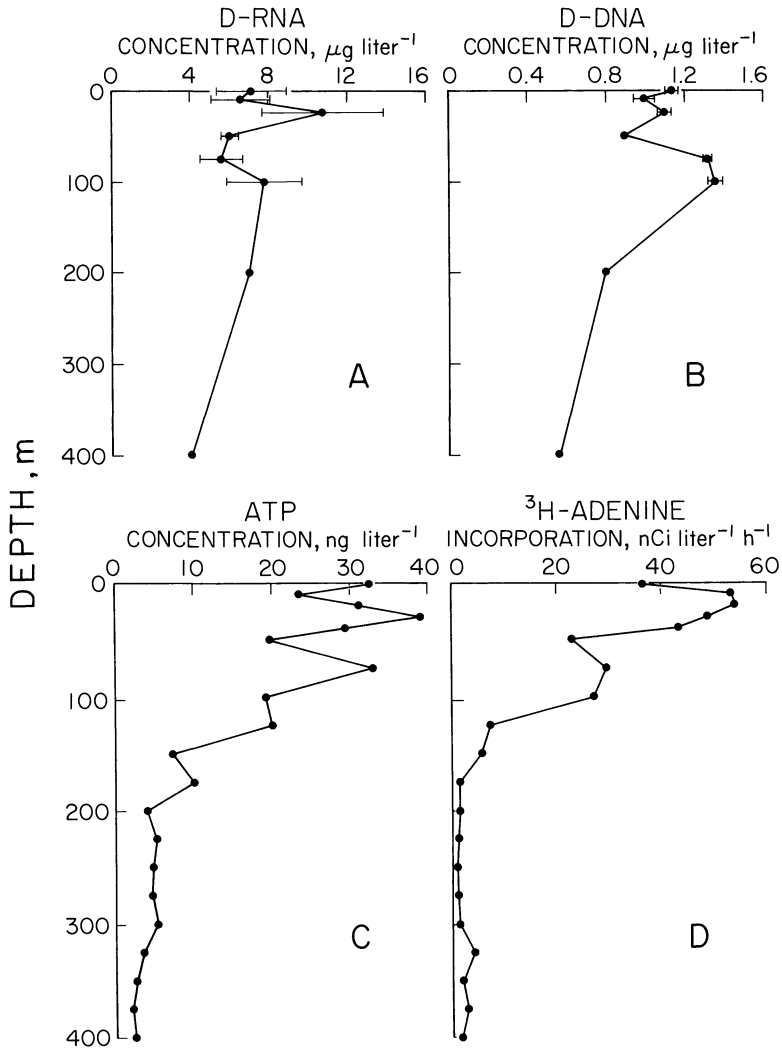


Fig. 10. Vertical distribution of D-RNA, D-DNA, ATP, and [^{3}H]adenine incorporation for water collected at a station in the North Pacific Ocean (33°N, 139°W). These data were obtained during the Vertical Transport and Exchange (VERTEX) field program in January 1988.

production of D-DNA and D-RNA by actively growing metabolizing cells and rapid turnover of the dissolved nucleic acid pools.

Once produced by the various pathways mentioned above, dissolved nucleic acids seem to be readily degraded in the water column and sediments of aquatic habitats (Bazelyan and Ayzatullin 1979; Paul et al. 1987; Novitsky 1986). The removal (uptake and assimilation) of dissolved nucleic acids is most likely mediated by viable cells or cell-associated nuclease activity, but extracellular nucleases capable of hydrolyzing detrital DNA and RNA may also be important (Maeda and Taga 1974). DNA- and RNA-degrading bacteria both are ubiquitous in the marine environment (Maeda and Taga 1974). Dissolved macromolecules, such as DNA and RNA, may also be adsorbed onto detritus and ingested directly by filter-feeding organisms. Dissolved monophosphate nucleotides, nucleosides, and nucleic acid bases may constitute intermediate pools in the dissolved nucleic acid assimilation pathway, although few measurements are available on the concen-

trations of selected pool constituents. A complete resolution of the dissolved nucleic acid cycle in nature is certain to provide insight into microbial physiology, microbial population interactions, and the mechanisms of organic matter recycling.

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