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A novel method for the measurement of dissolved deoxyribonucleic acid in seawater

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Abstract

A novel method was developed for the quantification of dissolved deoxyribonucleic acid (D-DNA) in seawater. This method includes addition of tetrasodium ethylenediamine tetraacetic acid (tetrasodium EDTA) to 0.22 m-filtered seawater, concentration of > 10 kDa material in the filtrate with a Centricon centrifugal concentration unit, and quantification of the concentrated D-DNA with the fluorescent double-stranded DNA stain SYBR Green I. This method requires less than 15 mL of seawater per sample even in oligotrophic environments, and samples can be analyzed in approximately 3 h. The recovery of D-DNA with this method is 75% to 85% and can be determined for each sample by measuring recovery of ^{35}S-labeled DNA added at trace amounts. This method can be used to quantify D-DNA concentrations as low as 0.01 ng mL^{-1} with high precision (standard deviation < 5% of the mean). Deoxyribonuclease (DNase) treatment of samples and virus enumeration can be used in conjunction with this method to determine the three major components of D-DNA: free or enzymatically hydrolyzable DNA (ehD-DNA), DNA within viruses, and uncharacterized bound DNA.

Deoxyribonucleic acid (DNA) is a component of the dissolved organic matter (DOM) pool in aquatic environments. Of the known DOM compounds, DNA is unique in that it is the common information molecule of all living organisms. With a C:N:P ratio of approximately 10:4:1, it is also rich in nitrogen and phosphorus compared to bulk marine organic matter, which has an average ratio of 106:16:1 (Redfield 1958). These characteristics make DNA a potentially important source of nitrogen and phosphorus, nucleotides, or genetic information for aquatic microorganisms.

Dissolved DNA (D-DNA) is operationally defined as DNA that passes through a 0.2 or 0.22 m pore-size filter (DeFlaun et al. 1986; Karl and Bailiff 1989). The two most common methods of measuring D-DNA are ethanol precipitation followed by Hoechst 33258 staining of DNA (DeFlaun et al. 1986) or by CTAB precipitation followed by reaction with 3,5-diaminobenzoic acid (Karl and Bailiff 1989). Two major disadvantages of these methods are lengthy processing times (several days) and low sensitivity resulting in the requirement of large volumes of sample (≥1 L) for determinations in oligotrophic environments. In response to these limitations, a novel method has been developed for quantifying D-DNA in seawater that requires only three hours of processing time and 13.5 mL of sample even in oligotrophic environments.

In this method (Fig. 1), tetrasodium ethylenediamine tetraacetic acid (tetrasodium EDTA) is added to 0.22 m-filtered seawater, and samples are stored refrigerated until analyzed. The addition of tetrasodium EDTA has three purposes: (1) to chelate divalent cations (Mg^{2+}, Mn^{2+}) that are cofactors for deoxyribonuclease (DNase), (2) to destabilize viral capsids thereby exposing viral DNA for analysis (Söderlund et al. 1975, Steward 2001), and (3) to buffer the pH of the sample at 10.5, which reduces adsorption of DNA to containers during storage and analysis. The sample is then concentrated with a 10 kDa Centricon centrifugal concentration unit, which retains double-stranded DNA larger than 20 nucleotides (product literature, Millipore). The concentrate is then rinsed with Tris-EDTA buffer to remove residual seawater salts and to adjust the pH to 7.5 to optimize conditions for the subsequent fluorescence assay. DNA in the recovered concentrate is quantified by measuring the fluorescence of the sample after binding with SYBR Green I, a fluorescent stain for double-stranded DNA. The recovery of DNA from each sample can be assessed by addition of trace amounts of ^{35}S-labeled DNA to the initial sample, which is compared to the ^{35}S radioactivity in the final sample. Alternatively, the recovery of a non-radioactive double-stranded DNA (dsDNA) internal standard can be measured from selected samples with the correction applied to all samples.

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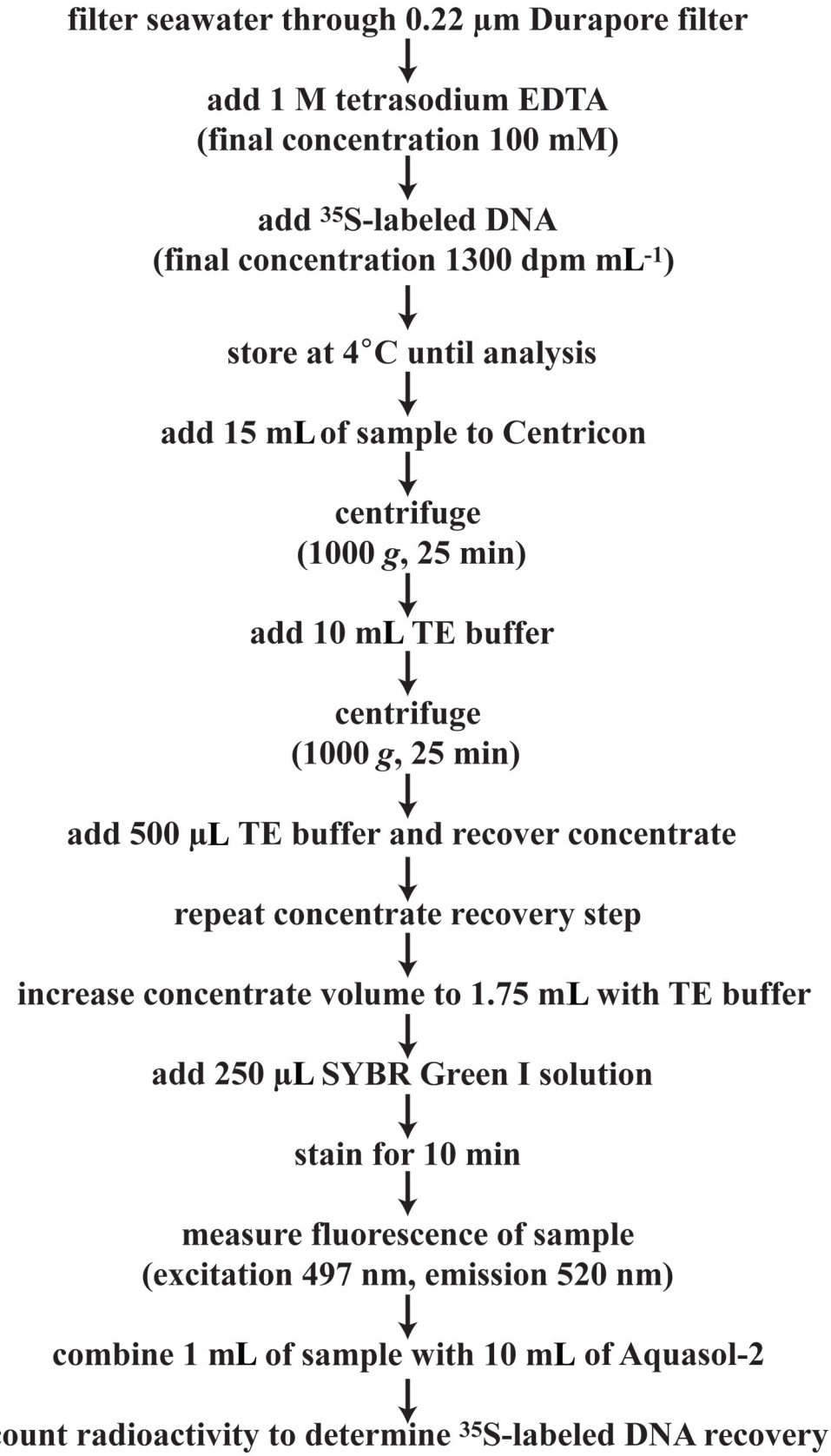

Fig. 1. Flow diagram of the developed method for measuring D-DNA in seawater

The D-DNA pool includes free or enzymatically hydrolyzable DNA (ehD-DNA; Siuda and Chróst 2000), DNA within viruses, and an as-yet-uncharacterized form of bound DNA (Jiang and Paul 1995). As these three major components of D-DNA are likely to differ in their bioavailability, it is desirable from an ecological perspective to measure them individually (Weinbauer et al. 1993; Jiang and Paul 1995; Siuda and Chróst 2000). Free D-DNA is directly hydrolyzable by DNase to low molecular weight byproducts that are readily transported by microorganisms (Paul et al. 1988; Siuda and Chróst 2000). DNA within viruses is resistant to DNase digestion (Jiang and Paul 1995) but is cycled via infection pathways and may be grazed by protozoa (reviewed by Wommack and Colwell 2000). The nature and fate of uncharacterized bound D-DNA remains unclear but is expected to be less accessible to microorganisms because it is DNase-resistant (Jiang and Paul 1995). Because of the potential ecological relevance of these differences in reactivity, we also describe in this report how the D-DNA quantification method can be used in conjunction with DNase treatment and virus enumeration to estimate the distribution of DNA among the three major components of the D-DNA pool. The concentration of ehD-DNA is calculated as the difference between untreated and DNase-digested samples. The concentration of D-DNA contained within viruses is estimated by multiplying the concentration of dsDNA viruses (determined using the method of Noble and Fuhrman 1998) by the average mass of DNA per virion (Steward et al. 2000). The amount of D-DNA in the total D-DNA sample that is neither ehD-DNA nor DNA within viruses is considered to be equivalent to the uncharacterized bound DNA described by Jiang and Paul (1995).

Materials and procedures

Sampling—Seawater is vacuum-filtered through Millipore Stericup sterile vacuum filter units with Durapore polyvinylidene fluoride (PVDF) 0.22 m pore-size filters (Millipore #SCGV U01 RE), and aliquots of the filtrate are transferred to sterile 50 mL polypropylene centrifuge tubes using sterile plastic pipettes. A 1 M stock of autoclaved tetrasodium EDTA is added to the filtrate to achieve a final concentration of 100 mM. At this point, ^{35}S-labeled DNA may be added at trace concentrations (approximately 1300 dpm mL^{-1} of sample). This is an optional step that allows calculation of DNA recovery efficiency individually for each sample. Alternatively, an internal dsDNA standard for the determination of recovery efficiency may be added to replicate samples at this time. The samples are then stored at 4°C until analysis. Blanks are prepared exactly as samples using 10 kDa-filtered seawater prepared by filtering seawater through Centricon Plus-20 centrifugal concentration units with Ultracel-PL 10000 NMWL (10 kDa) membranes (Millipore #UFC2 LGC) to remove DNA.

^{35}S-labeled DNA is prepared by incorporation of ^{35}S-dCTP (ICN Biomedicals #56201H) into lambda phage dsDNA (Sigma-Aldrich #D3779) using a nick translation kit (Amersham Pharmacia Biotech #N5000). The labeled DNA obtained from nick translation is filtered using a 10 kDa Centricon centrifugal concentration unit and rinsed twice with Tris-EDTA buffer (TE buffer: 10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.5, autoclaved) to remove unincorporated ^{35}S-dCTP. The recovered ^{35}S-labeled DNA is diluted to 1000 dpm L^{-1} with TE buffer and stored frozen (–20°C) in 1 mL aliquots until use.

Analysis—Replicate 15 mL samples are pipetted into 10 kDa Centricon Plus-20 centrifugal concentration units and centrifuged for 25 min at 1000*g*, reducing the sample volume to approximately 1 mL. The filtrate is discarded and 10 mL of TE buffer is added to each of the concentrated samples. The samples are centrifuged again for 25 min at 1000*g*, reducing the sample volume to approximately 100 to 200 L. The concentrate is then recovered as directed by the Centricon Plus-20 product instructions: 500 L of TE buffer is added to the concentrate, the retentate cup is placed inverted in the sample cup, and the sample is centrifuged for 3 min at 300*g*. The recovered sample is placed in a sterile 2 mL microcentrifuge tube. The recovery process is then repeated with another 500 L of TE buffer to ensure quantitative recovery of the concentrated sample.

TE buffer is added to the pooled recovered sample to achieve a final sample volume of 1.75 mL. An external standard curve is prepared by diluting a known concentration of lambda phage dsDNA in TE buffer to a final volume of 1.75 mL for each standard. In low light, 1 L of SYBR Green I (Molecular Probes #S-7567) is diluted with 9.5 mL of TE buffer and 250 L of this SYBR Green I solution is added to each sample and standard. After 10 min of staining, the fluorescence of the samples and standards is measured in a 1 cm cuvette with a fluorometer (excitation 497 nm; emission 520 nm).

The radioactivity of the recovered sample is measured by adding 1 mL of the final sample to 10 mL of Aquasol-2 liquid scintillation cocktail in 20 mL glass scintillation vials and counting with a liquid scintillation counter configured to measure ^{35}S radioactivity. To get the percent recovery of D-DNA, the radioactivity of the final sample is compared to the radioactivity added to the sample initially. Because half of the final sample is counted, half of the volume of ^{35}S-labeled DNA added to 15 mL of the initial sample is used for this comparison. To ensure the samples are analyzed similarly, triplicate samples of ^{35}S-labeled DNA used for comparison are added to 125 L of SYBR Green I solution and brought up to 1 mL with TE buffer. After addition of 10 mL of Aquasol-2, the radioactivity of the prepared comparison sample is counted at the same time as the other samples.

The concentration of DNA in each sample is calculated by subtracting the fluorescence of the blank from the fluorescence of the sample and dividing by the slope of the regression equation from the external standard curve. After correction for concentration factor, the D-DNA concentration is divided by the fraction of recovered ^{35}S-labeled DNA, or the fraction of recovered dsDNA internal standard, to

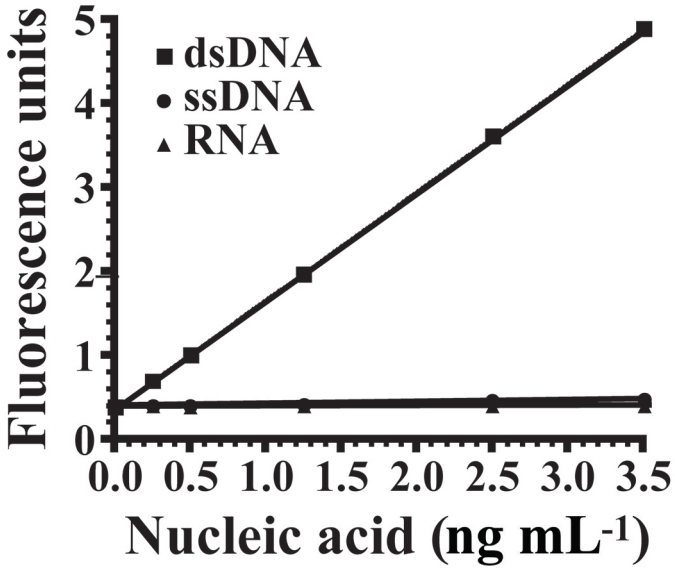

Fig. 2. Standard curves of double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), and ribonucleic acid (RNA) in TE buffer with SYBR Green I. One sample was analyzed per data point. The slopes of the regression lines of fluorescence units versus dsDNA, ssDNA, and RNA concentration were 1.29, 0.02, and 0.0005 units mL ng^{-1}, respectively.

result in the final concentration of D-DNA in the original seawater sample.

Quantifying ehD-DNA—The concentration of D-DNA that can be hydrolyzed by DNase can also be measured with this method. DNase (Sigma-Aldrich #D7291) is added to replicate 0.22 m-filtered seawater samples and blank samples at a final concentration of 80 units mL^{-1}, and the samples are incubated at 23 ± 2°C for 30 min. Tetrasodium EDTA and ^{35}S-labeled DNA are then added, and these samples are stored and analyzed exactly as described for non–DNase-treated samples. The concentration of DNA that is enzymatically hydrolyzable (ehD-DNA) is calculated by subtracting the average D-DNA concentration of DNase-treated samples from the average D-DNA concentration of non–DNase-treated samples, with propagation of errors (Skoog et al. 2000).

Quantifying D-DNA within viruses and uncharacterized bound DNA—The concentration of D-DNA contained within viruses is estimated by first quantifying the concentration of dsDNA viruses within 0.22 µm-filtered seawater by staining with SYBR Green I and enumeration of viruses using epifluorescence microscopy (Noble and Fuhrman 1998). The concentration of dsDNA viruses is then multiplied by the average mass of DNA per virion for marine virus assemblages (55 attograms per virion; Steward et al. 2000) to result in the total concentration of D-DNA contained within viruses. This estimate of DNA per virion is relatively constant over many marine environments (Steward et al. 2000) but may be verified for a given environment using viral fingerprinting by pulsed field gel electrophoresis (Steward 2001). The concentration of uncharacterized bound DNA is estimated by subtracting the concentration of D-DNA within viruses from the concentration of D-DNA that is not hydrolyzable by DNase (Jiang and Paul 1995).

Assessment

The following experiments were conducted to evaluate this D-DNA quantification method. For experiments using seawater, surface samples were collected from Station ALOHA (22°45′N, 158°W), an oligotrophic site in the subtropical North Pacific gyre.

Specificity of SYBR Green I—The reactivity of SYBR Green I with dsDNA is much higher than with single-stranded DNA (ssDNA) and ribonucleic acid (RNA) in gels (Tuma et al. 1999). To ensure that this was accurate for samples in TE buffer as well, the specificity of SYBR Green I for double-stranded DNA was evaluated using standard curves of dsDNA (lambda phage, Sigma-Aldrich), ssDNA (salmon testes, Sigma-Aldrich), and RNA (ribosomal, Sigma-Aldrich) prepared in TE buffer. The molar fluorescence yield (slope of fluorescence versus nucleic acid concentration) of SYBR Green I with ssDNA and RNA was 54 and 2353 times lower, respectively, than with dsDNA (Fig. 2). The specificity of SYBR Green I for dsDNA viruses was also evaluated using cultures of the dsDNA phage T4, the ssDNA phage ϕX174, and the RNA phage MS-2. These cultures were stained with SYBR Green I and observed using epifluorescence microscopy (Noble and Fuhrman 1998). Stained phages were detected in the T4 phage cultures, but not in the ϕX174 or MS-2 cultures. Therefore, ssDNA and RNA, as free nucleic acids or within viruses, do not appear to interfere with the measurement of dsDNA in this method.

Deoxyribonuclease incubation—The effectiveness of a DNase incubation to hydrolyze free DNA and the use of tetrasodium EDTA to stop the hydrolysis in seawater samples was evaluated. A final concentration of 20 ng mL^{-1} dsDNA was added to 10 kDa-filtered seawater followed by DNase addition (final concentration 80 units mL^{-1}). Tetrasodium EDTA (final concentration 100 mM) was added prior to DNase in four replicates of the sample and four additional replicates were incubated at 23 ± 2°C for 30 min prior to tetrasodium EDTA addition. Negative controls without DNase and blank samples were also prepared for comparison. The negative controls and DNase amended samples without incubation were not significantly different (*t* test, $P < 0.01$), and samples incubated with DNase for 30 min were not significantly different from blank samples with no dsDNA added (*t* test, $P < 0.01$). This demonstrates that the addition of 100 mM tetrasodium EDTA is sufficient to stop DNase activity and that a 30 min incubation with 80 units mL^{-1} of DNase is sufficient to hydrolyze up to 20 ng mL^{-1} of dsDNA in seawater.

Internal standard curves and detection limits—An internal standard curve (also known as a standard addition) was created by addition of varying amounts of lambda phage DNA to triplicate samples of 0.22 m-filtered seawater followed by processing with the centrifugal concentration method, includ-

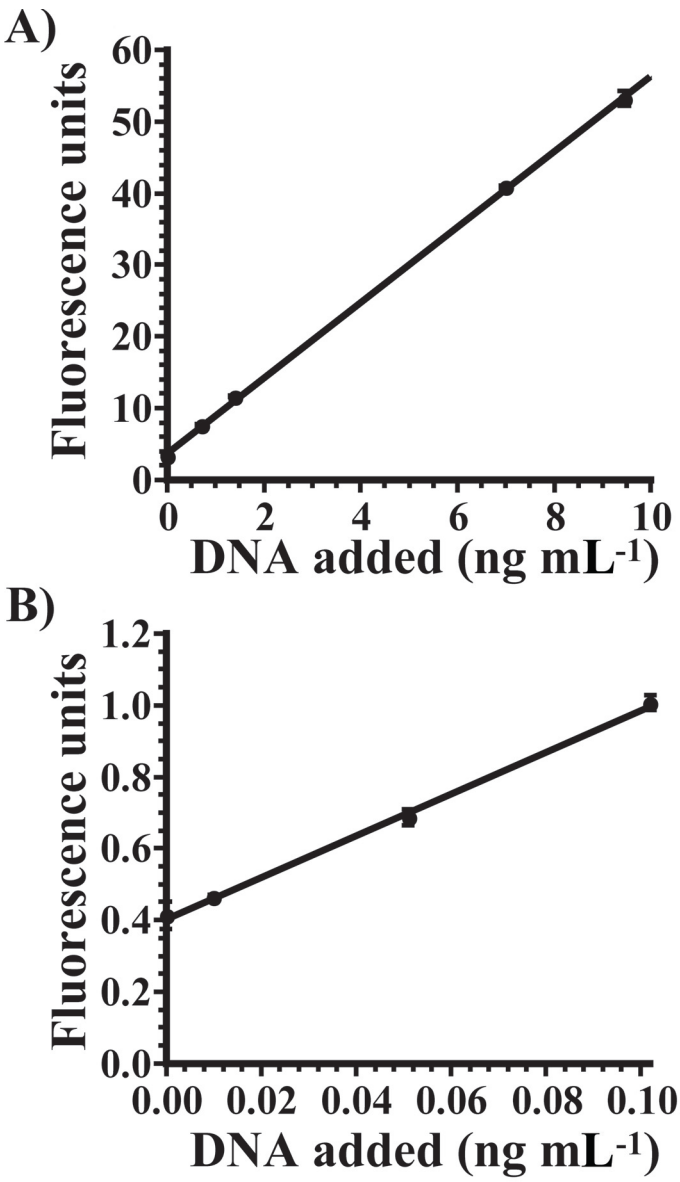

Fig. 3. Internal standard curve of lambda phage DNA added to 0.22 m-filtered seawater (A) and determination of the detection limit of the centrifugal concentration method using lambda phage DNA added to 10 kDa-filtered seawater (B). Error bars represent one standard deviation of the mean (n = 3). The r^2 values for the regression lines (A and B) are > 0.99.

ing addition of ^{35}S-labeled DNA. The fluorescence of the processed samples increased linearly with the amount of DNA added, and standard deviations were always less than 3% of the mean (Fig. 3A). The recovery of each sample was assessed by calculating the recovery of added lambda phage DNA as well as ^{35}S-labeled DNA. These recoveries were consistent over a broad range of DNA concentrations, averaging 74% (±5%). The apparent loss of DNA was most likely due to adsorption onto the Centricon filter or loss during sample manipulation. Furthermore, the recovery of ^{35}S-labeled DNA was in agreement (±2%) with the recovery of added lambda phage DNA. These results show that the method accurately measures standard dsDNA over a 10-fold range of DNA concentrations and that ^{35}S-labeled DNA recovery correctly represents sample DNA recovery.

The detection limit of the centrifugal concentration method was evaluated empirically with a very low range internal standard curve (< 0.1 ng mL^{-1}) in triplicate 10 kDa-filtered seawater samples followed by processing with this method. The fluorescence of the recovered samples had a linear relationship with added DNA through the lowest concentration tested (Fig. 3B). These results show that D-DNA can be measured with this method in seawater with concentrations as low as 0.01 ng mL^{-1} using only 13.5 mL of seawater.

An internal standard curve was also created for samples with DNase treatment. DNase was added to the samples after tetrasodium EDTA addition to prevent DNA hydrolysis. The fluorescence of the samples increased linearly with the amount of DNA added (Fig. 4A). Recovery of added lambda phage DNA was 76% (±5%) and was within 3% of ^{35}S-labeled DNA recovery. The lower limit for samples with DNase treatment was evaluated exactly as for samples without DNase addition. Again, DNase was added to the samples after tetrasodium EDTA addition. The fluorescence of the recovered samples had a linear relationship with added DNA concentration through the lowest concentration tested (Fig. 4B). Therefore, the detection limit of this method with DNase addition is ≤0.01 ng mL^{-1}. A significantly less expensive DNase (Sigma-Aldrich #D5025) can be used in this method for samples that do not require such a low detection limit. The use of this lower-grade DNase results in a linear relationship between fluorescence and added DNA concentration with a detection limit of 0.2 ng mL^{-1} (Fig. 4C).

Quantification of DNA within viruses—Durapore PVDF filters were tested to ensure that viruses did not adsorb to them during the prefiltration step. Viruses were enumerated before and after filtration through the 0.22 m pore-size Durapore filters in triplicate seawater samples. The filters allowed 101% ± 5% of the viruses in the unfiltered seawater to pass through, thus including them in the D-DNA pool.

To ensure that DNase was not hydrolyzing DNA within viruses, triplicate 0.22 µm-filtered seawater samples and cultured T4 phage added to 10 kDa-filtered seawater were treated with DNase. Viruses were stained with SYBR Green I and enumerated by epifluorescence microscopy (Noble and Fuhrman 1998) before and after DNase addition. DNase treatment did not significantly change the abundance of viruses in seawater or T4 phage added to 10 kDa-filtered seawater (t test, $P < 0.01$).

The centrifugal concentration method was then evaluated to ensure that DNA within viruses was accurately measured with this method. Cultured T4 phages were added to triplicate 10 kDa-filtered seawater samples, and the DNA in the samples was quantified with the centrifugal concentration method using ^{35}S-labeled DNA for determination of sample DNA recov-

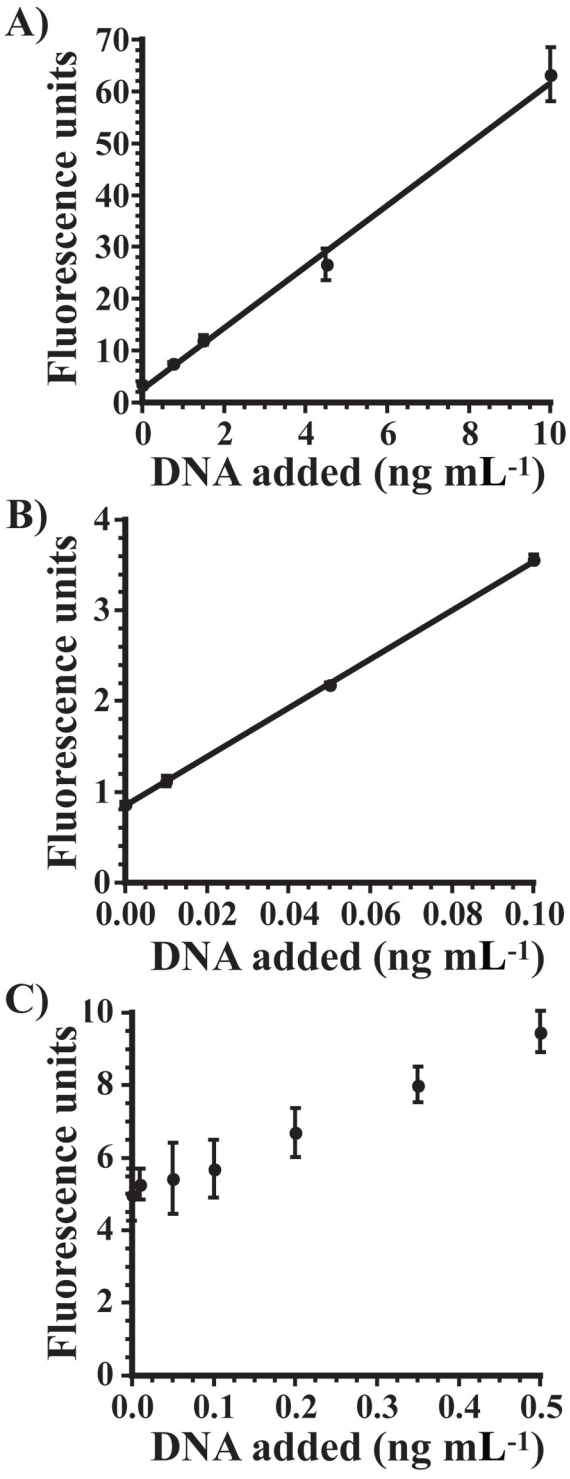

Fig. 4. Internal standard curve of lambda phage DNA added to 0.22 m-filtered seawater with DNase (Sigma-Aldrich #D7291) and analyzed using the centrifugal concentration method (A). Determination of the detection limit of the centrifugal concentration method with DNase (Sigma-Aldrich #D7291) addition (B) and lower-grade DNase (Sigma-Aldrich #D5025) addition (C) using lambda phage DNA added to 10 kDa-filtered seawater. Error bars represent one standard deviation of the mean (n = 3). The r^2 values for the regression lines (A and B) are > 0.99.

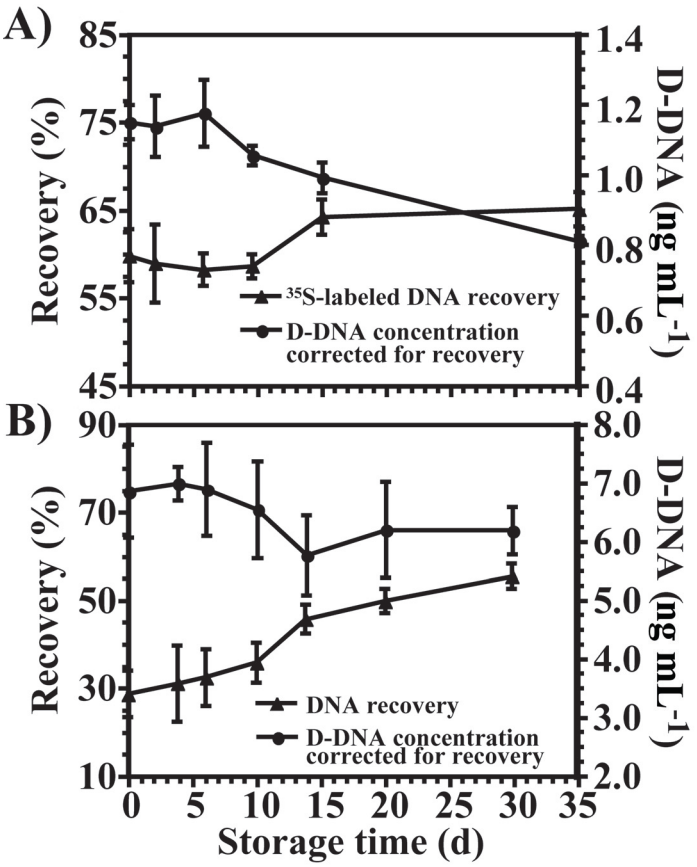

Fig. 5. Effect of storage time on recovery of ^{35}S-labeled DNA and D-DNA concentration corrected for ^{35}S-labeled DNA recovery in 0.22 µm-filtered seawater samples (A). Effect of storage time on recovery of lambda phage DNA internal standards in replicate samples and D-DNA concentrations corrected for DNA internal standard recovery in 0.22 µm-filtered seawater samples (B). Error bars represent one standard deviation of the mean (n = 4).

ery efficiency. The concentration of T4 phages was quantified by epifluorescence microscopy and converted to DNA within phages by assuming 168 kbp per phage (Mosig 1994). The calculated amount of DNA within phages in the sample (1.03 ± 0.08 ng mL^{-1}) was not significantly different from the DNA measured by the centrifugal concentration method (0.97 ± 0.07 ng mL^{-1}; t test, $P < 0.001$), showing that DNA within this model virus is accurately quantified by this method.

Sample storage—The effect of sample storage on the recovery of ^{35}S-labeled DNA was determined over the course of 35 d. Samples were collected from 0.22 m-filtered seawater and stored exactly as previously described for samples collected in the field. Four samples were analyzed immediately, and four more samples were analyzed per day at 2, 5, 10, 15, and 35 d after storage at 4°C. The recovery of ^{35}S-labeled DNA started to decrease after 5 d of sample storage, and the measured D-DNA corrected for ^{35}S-labeled DNA recovery increased by 0.1 ng mL^{-1} after 10 d (Fig. 5A). These results show that through 10 d of sample storage, recovery of ^{35}S-labeled DNA accurately cor-

Table 1. Measurements of total D-DNA, ehD-DNA, DNA within viruses, and uncharacterized bound DNA using the centrifugal concentration method*

Location	Total D-DNA (ng mL^{-1})	ehD-DNA (ng mL^{-1})	D-DNA within viruses (ng mL^{-1})	Uncharacterized bound DNA (ng mL^{-1})
Station ALOHA† (22°45′N, 158°W)				
5 to 100 m	1.08 ± 0.06 to 1.27 ± 0.06	0.46 ± 0.15 to 0.66 ± 0.18	0.62 ± 0.02 to 0.68 ± 0.06‡	–0.06 ± 0.19 to 0.03 ± 0.10
150 to 500 m	0.21 ± 0.01 to 0.61 ± 0.04	0.06 ± 0.09 to 0.35 ± 0.15	0.12 ± 0.01 to 0.33 ± 0.02‡	–0.07 ± 0.16 to 0.03 ± 0.09
Kaneohe Bay, Hawaii (21°26.6′N, 157°48.6′W)				
Surface	3.41 ± 0.09	1.91 ± 0.14	0.82 ± 0.11§	0.68 ± 0.2

*Ranges are reported for when multiple depths were sampled.
†Brum (2003).
‡Calculated using the average amount of DNA per virion obtained by pulsed field gel electrophoresis for each depth using the method of Steward (2001).
§Calculated using the average amount of DNA per virion from a range of marine environments (Steward et al. 2000).

rected for losses of natural DNA. After 15 d of sample storage, the correction for recovery of ^{35}S-labeled DNA overestimated the loss of natural DNA by approximately 13%. Therefore, it is recommended that samples be analyzed within 10 d after sample collection. This storage experiment was repeated using an internal standard of lambda phage DNA in 4 replicate samples per time point to calculate D-DNA recovery (Fig. 5B). The results were similar to the storage test using ^{35}S-labeled DNA to calculate recovery except that the use of internal DNA standards in separate samples results in larger standard deviations for recovery and corrected sample D-DNA concentration measurements. An attempt to increase storage time by freezing samples was unsuccessful due to irreversible precipitation that decreased DNA recovery and increased variability.

Field application—This D-DNA quantification method has been successfully used in a study of the composition and dynamics of the D-DNA pool at Station ALOHA (Brum 2003). A summary of the results from a depth profile in that study are shown in Table 1 as well as results from a coastal location. These results are similar to total D-DNA measurements reported by Karl and Bailiff (1989) near these locations using the CTAB precipitation method.

Discussion

This novel centrifugal concentration method accurately and precisely quantifies D-DNA in seawater and offers several advantages over the current D-DNA quantification methods being used. One major advantage is that the centrifugal concentration method requires less than 15 mL of seawater per determination even in oligotrophic environments, which is significantly less than the 100 to 1000 mL of sample required for the ethanol precipitation method (DeFlaun et al. 1986) and the 500 to 1000 mL of sample required for the CTAB precipitation method (Karl and Bailiff 1989). This means that less time is spent filtering samples and the need for sample storage space is greatly reduced. In addition, time-course incubation experiments (e.g., D-DNA production or uptake experiments) can be performed on a much smaller scale.

The centrifugal concentration method also offers the advantage of rapid sample analysis, requiring approximately 3 h for a set of samples using a centrifuge with 16-sample capacity. This is a substantial improvement over the ethanol precipitation method, which requires that samples be frozen for 2 d and dialyzed for 3 d (DeFlaun et al. 1986) and the CTAB precipitation method, which requires samples to be frozen, thawed, and filtered a second time prior to analysis (Karl and Bailiff 1989).

In addition, it has been shown that this newly developed method can be used to quantify the major components of the D-DNA pool. The use of DNase treatment and enumeration of dsDNA viruses coupled with total D-DNA measurements allows ehD-DNA, DNA within viruses, and uncharacterized bound D-DNA to be quantified individually. This capability is of importance because existing evidence suggests that these major D-DNA components may be cycled through different pathways and at different rates (Weinbauer et al. 1993; Jiang and Paul 1995; Siuda and Chróst 2000; Brum 2003). Therefore, further study of D-DNA dynamics will require rapid and routine quantification of these individual D-DNA components. This novel D-DNA quantification method will facilitate the study of D-DNA composition and dynamics because of its low sample volume and processing time requirements and its ability to be used to quantify the major components of the D-DNA pool individually.

Comments and recommendations

This method can be used for field application exactly as described in this paper. Freshwater samples have also been analyzed using this method with similar recoveries to seawater samples. However, rigorous assessment of this method in freshwater including internal standard curves, detection limit determination, and DNase effectiveness have not been conducted.

DNase activity is dependent on the pH of samples. If the ehD-DNA quantification method is to be used in samples with a pH extremely higher or lower than average seawater, then the effectiveness of DNase to hydrolyze free DNA in these samples should be evaluated. In addition, if the concentration of free D-DNA is expected to exceed 20 ng mL^{-1}, then the concentration of DNase may need to be increased to greater than 80 units mL^{-1} to ensure complete hydrolysis of all free DNA.

The specific activity of ^{35}S-labeled DNA will vary depending on the amount of ^{35}S-dCTP incorporated into the lambda phage dsDNA during nick translation and the amount of ^{35}S radioactive decay that has occurred. The specific activity can be determined prior to each sampling to ensure that a sufficient amount of radioactivity is added to each sample (approximately 1300 dpm mL^{-1}) and that the corresponding amount of labeled DNA added is at trace amounts that do not affect the fluorescent measurement of D-DNA. Repeated freezing and thawing of ^{35}S-labeled DNA should be avoided to minimize shearing of DNA, which can cause incorrect recovery measurements.

The use of ^{35}S-labeled DNA to calculate sample D-DNA recovery efficiency is highly recommended. This method of recovery determination for individual samples results in much more precise D-DNA concentration measurements than measuring recovery of internal standards in separate samples and is less expensive because fewer samples are analyzed.

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