

## A method for characterizing dissolved DNA and its application to the North Pacific Subtropical Gyre

Morgan D. Linney<sup>1,2\*</sup> , Christopher R. Schvarcz,<sup>1,2</sup> Grieg F. Steward<sup>1,2</sup> , Edward F. DeLong,<sup>1,2</sup> David M. Karl<sup>1,2</sup>

<sup>1</sup>Daniel K. Inouye Center for Microbial Oceanography: Research and Education (C-MORE), Honolulu, Hawaii

<sup>2</sup>Department of Oceanography, University of Hawaii at Manoa, Honolulu, Hawaii

### Abstract

Dissolved DNA (D-DNA) is a ubiquitous component of dissolved organic matter in aquatic systems. It is operationally defined as the DNA that passes a membrane filter and thus includes pools of truly dissolved “free” DNA (F-DNA), virion encapsidated DNA, DNA within membrane vesicles, and possibly other bound forms, each with different sources and lability. We investigated whether filtration ( $< 0.1 \mu\text{m}$ ), concentration by tangential flow ultrafiltration ( $> 30 \text{ kDa}$ ), and fractionation in an equilibrium buoyant density gradient could be used to discriminate the mass contributions of the different pools of filterable DNA in seawater. Spike-in experiments with a known range of DNA standards (75–20,000 bp) indicated that this method results in high recoveries of F-DNA (68–86%) with minimal degradation. Application of the fractionation method to seawater samples collected from the oligotrophic North Pacific Ocean followed by analysis of fractions (epifluorescence and electron microscopy, DNase digestion) suggested that the low-density fractions ( $1.30\text{--}1.35 \text{ g mL}^{-1}$ ) were dominated by vesicle-like particles, mid-density fractions ( $1.45\text{--}1.55 \text{ g mL}^{-1}$ ) by virus-like particles, and high-density fractions ( $1.60\text{--}1.70 \text{ g mL}^{-1}$ ) by F-DNA. The estimated concentration of DNA that is either F-DNA, in viruses, or in vesicles was  $0.13$ ,  $0.14$ , and  $0.08 \mu\text{g L}^{-1}$ , respectively in the euphotic zone and  $0.09$ ,  $0.04$ , and  $0.03 \mu\text{g L}^{-1}$ , respectively in the mesopelagic zone. The approach described should be useful for more detailed investigations of the abundance, dynamics, and sources of DNA in the distinct pools that comprise filterable DNA in aquatic environments.

Dissolved DNA (D-DNA)—a highly labile and nutrient-rich component of dissolved organic material (DOM)—is ubiquitous in all aquatic habitats investigated to date, including freshwater rivers and lakes (Pillai and Ganguly 1972), coastal marine and estuarine systems (DeFlaun et al. 1986), sediments (Dell’Anno et al. 2002), and the open ocean (Karl and Bailiff 1989; Brum 2005). Concentrations of D-DNA often represent a substantial fraction of the total DNA (DeFlaun et al. 1987; Karl and Bailiff 1989), especially in the open ocean. Spatial patterns of D-DNA concentrations in the ocean are similar to those of total microbial biomass and total DOM, decreasing with distance from shore and with depth (DeFlaun et al. 1987; Karl and Bailiff 1989; Weinbauer et al. 1993, 1995). The

concentration and bioavailability of D-DNA has both evolutionary and ecological significance. Double stranded D-DNA has the potential to alter the genetic make-up of microbial cells by the process of natural transformation (Hermansson and Linberg 1994) or, if hydrolyzed, can serve as a source of nitrogen and phosphorus (Jørgensen and Jacobsen 1996), macronutrients that are often limiting to microorganisms in open ocean ecosystems (Karl and Church 2014). As a consequence, there has been considerable interest in developing methods to investigate the nature and dynamics of this component of the larger DOM pool.

Methods to study D-DNA have usually employed  $0.2 \mu\text{m}$  pore size filters to separate the particulate from the dissolved fractions (DeFlaun et al. 1986; Karl and Bailiff 1989; Beebe 1991; Brum et al. 2004). This operational definition means that D-DNA is a heterogeneous mixture of truly dissolved or “free” DNA (F-DNA), membrane-enclosed DNA in the form of filterable bacteria (Ghuneim et al. 2018) and extracellular vesicles (Billar et al. 2014), and encapsidated DNA in the form of the many small viruses that dominate aquatic ecosystems (Wommack and Colwell 2000). Depending on the relative contributions of the

\*Correspondence: mlinney@hawaii.edu

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various D-DNA constituents and their size spectra, the total D-DNA measured could vary as a function of the filter pore size used to remove particulates. In prior investigations, estimates of D-DNA concentration were relatively insensitive to the choice of pre-filter. Negligible differences were seen, for example, between samples filtered through 0.1  $\mu\text{m}$  vs. 0.2  $\mu\text{m}$  membranes (DeFlaun et al. 1986) or among samples filtered through membranes or glass-fiber filters with pore sizes that ranged from 0.05 to 0.7  $\mu\text{m}$  (Karl and Bailiff 1989). This suggests that bacteria capable of passing a 0.2  $\mu\text{m}$  filter (e.g., MacDonell and Hood 1982) were not a major contributor to the D-DNA measurements. However, these comparisons were conducted using coastal seawater. The relative importance of 0.2  $\mu\text{m}$ -filterable bacteria in lower productivity open ocean waters could be greater. To ensure the complete exclusion of small cells and the clean isolation of F-DNA, a 0.1  $\mu\text{m}$  filter was adopted for this study. Other filter sizes may be considered in more productive ecosystems.

Previous studies have separated D-DNA by molecular weight (DeFlaun et al. 1987) or by using 0.05  $\mu\text{m}$  pore size filters (Karl and Bailiff 1989), but they did not isolate F-DNA or distinguish viral DNA from vesicle DNA. Brum (2005) found that different components of the D-DNA pool have varying turnover times in the surface ocean. Enzymatically hydrolyzable DNA (by 80 units  $\text{mL}^{-1}$  bovine pancreas DNase I) turned over within 0.1–6.2 h while viral D-DNA took 9.6–24 h, suggesting that these different components of the D-DNA pool may vary in their bioavailability, turnover, and source-sink pathways. A recent report of abundant, small (50–250 nm) DNA-containing membrane vesicles in the ocean (Biller et al. 2014) revealed the presence of yet another constituent of the D-DNA pool that is distinct in its source and composition, further demonstrating the importance in making separate quantification and turnover measurements of D-DNA components.

Herein, we describe the evaluation and application of a method to resolve, recover, quantify, and characterize three distinct, DNA-containing components in the < 0.1  $\mu\text{m}$  fraction of seawater. To achieve this, D-DNA in 0.1  $\mu\text{m}$ -filtered seawater was concentrated by tangential flow ultrafiltration and constituents were partitioned based on differences in their buoyant density using density gradient ultracentrifugation, a protocol commonly applied for isolating viruses and vesicles (Lawrence and Steward 2010; Brum et al. 2013a; Biller et al. 2014). DNA from individual density fractions was then quantified, as well as characterized using enzymatic and microscopic techniques to confirm the identity of each component. The molecular weight and quantity of F-DNA varied at different depths in the water column. The results suggest that the amount of DNA inside of viral particles is comparable to that found in F-DNA for seawater from the North Pacific Subtropical Gyre (NPSG), which is similar to previous reports of D-DNA pools (Jiang and Paul 1995; Brum 2005).

## Materials and procedures

A method was applied to characterize three distinct constituents of D-DNA: viral DNA, vesicular DNA, and F-DNA. Past methods employed chemical agents to precipitate DNA from seawater, but these methods are expected to lyse viral particles (DeFlaun et al. 1986; Karl and Bailiff 1989) and vesicles, thus preventing confident discrimination among the D-DNA constituents (Table 1). The main steps of our method include (1) seawater sample collection and prefiltration (0.1  $\mu\text{m}$ ) to remove particulate DNA, (2) ultrafiltration (30 kDa nominal molecular weight limit) (NMWL) to concentrate D-DNA, (3) CsCl density gradient separation of different pools of D-DNA, and (4) characterization of isolated gradient fractions.

### Collection and prefiltration

All samples for this research were collected near Station ALOHA (A Long-Term Oligotrophic Habitat Assessment; 22°45'N, 158°00'W), an open-ocean research site in the NPSG located 100 km north of Oahu, Hawaii. Since 1988, the Hawaii Ocean Time-series (HOT) program has conducted near-monthly biogeochemical and hydrographical measurements at Station ALOHA (Karl and Church 2014) and documented water mass properties (Fig. S1; Sabine et al. 1995). For this method, seawater samples were collected using a Niskin® bottle rosette equipped with a CTD and transferred to 20 L acid-washed polycarbonate carboys. Up to 50 L of seawater were collected at each sample location. Each sample was filtered through a double-layered 0.1  $\mu\text{m}$  polyethersulfone (PES) capsule filter (Supor Acropak™ 1000; Pall) by gravity, directly from the Niskin® bottle. After collection and prefiltration, samples were immediately concentrated by ultrafiltration.

### Ultrafiltration (tangential flow and/or centrifugal)

The filtrate was concentrated using a tangential flow ultrafiltration system (TFF; Millipore Pellicon 2; 0.1  $\text{m}^2$  Ultracel membrane, 30 kDa NMWL) to reduce the sample volume from 20–50 L to 100–250 mL, then further concentrated using a centrifugal ultrafilter (Centricon 70-Plus, 30 kDa NMWL; Millipore). This produced a final total sample volume of 1–2 mL. Centrifugal ultrafiltration alone may suffice for smaller volume samples collected from more productive regions.

### Density gradient separation

For all samples, vesicles, viruses, and F-DNA were separated and purified based on differences in buoyant densities using a cesium chloride (CsCl) equilibrium buoyant density gradient. The gradient was prepared by sequentially adding three density layers to the ultracentrifuge tube: 1.6, 1.45, and 1.2  $\text{g mL}^{-1}$  CsCl in SM buffer (100 mM sodium chloride, 8 mM magnesium sulfate, 50 mM Tris-HCl pH 7.5), having a homogenous CsCl density of about 1.40  $\text{g mL}^{-1}$ . The concentrated sample was gravimetrically adjusted to a density of 1.45  $\text{g mL}^{-1}$  by adding concentrated CsCl solution (1.9  $\text{g mL}^{-1}$  in SM buffer) and was added as the second layer of the density gradient

**Table 1.** Comparison of methods to extract dissolved DNA (D-DNA) from seawater.

| Reference                 | Procedure                                                                                                                                                                  | Advantages                                                                                                                                                                                                                                                                              | Comments                                                                                                                                                                                                                                                                                  |
|---------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Pillai and Ganguly (1972) | Filtration, barium sulfate precipitation, centrifugation, sodium chloride elution, hydrolysis by hydrochloric acid, UV nucleotide quantification                           | <ul style="list-style-type: none"> <li>DNA and RNA quantification</li> </ul>                                                                                                                                                                                                            | <ul style="list-style-type: none"> <li>Acid and base additions damage nucleic acids and prevent downstream analyses (e.g., microscopy, molecular weight estimates, and sequence analysis)</li> </ul>                                                                                      |
| DeFlaun et al. (1986)     | Filtration, ethanol precipitation, centrifugation, dialysis                                                                                                                | <ul style="list-style-type: none"> <li>Collects a range of low and high molecular weight DNA</li> </ul>                                                                                                                                                                                 | <ul style="list-style-type: none"> <li>Does not isolate D-DNA constituents efficiently because ethanol can lyse viral particles contributing to fluorescent quantification of F-DNA</li> </ul>                                                                                            |
| Karl and Bailiff (1989)   | Filtration, dilution of seawater 1 : 1 with EDTA, CTAB precipitation, filtration                                                                                           | <ul style="list-style-type: none"> <li>Precipitates DNA and RNA</li> <li>Simple and reproducible</li> <li>Recovery of low-molecular-weight DNA</li> </ul>                                                                                                                               | <ul style="list-style-type: none"> <li>Does not separate D-DNA constituents efficiently because EDTA can lyse viral particles, overestimating F-DNA</li> <li>Difficult to remove CTAB-DNA precipitate from GFF for downstream analyses</li> </ul>                                         |
| Beebee (1991)             | Filtration, ultracentrifugation to purify viruses and chromatography to purify free DNA, observation of pelleted viruses by transmission electron microscopy               | <ul style="list-style-type: none"> <li>Simple method to separate D-DNA pools without any chemical additions or numerous steps</li> </ul>                                                                                                                                                | <ul style="list-style-type: none"> <li>Free DNA can pellet and skew the value of viral DNA</li> <li>Vesicles and small cells are likely to contribute to the pelleted DNA</li> </ul>                                                                                                      |
| Paul et al. (1991)        | Filtration, large volume concentration by vortex flow filtration, enumeration of viruses and estimation of viral DNA contribution, estimation of F-DNA by DNase hydrolysis | <ul style="list-style-type: none"> <li>Allows for the collection and concentration of D-DNA from large volumes</li> </ul>                                                                                                                                                               | <ul style="list-style-type: none"> <li>Indirect estimate of D-DNA components (viral DNA and F-DNA)</li> <li>No separation of D-DNA components for downstream analyses</li> </ul>                                                                                                          |
| Brum et al. (2004)        | Centrifugal ultrafiltration, DNase hydrolysis to estimate F-DNA contribution                                                                                               | <ul style="list-style-type: none"> <li>Simple and reproducible</li> <li>Only requires small volumes to quantify D-DNA components</li> <li>In less oligotrophic systems, can utilize large centrifugal concentration units (e.g., Centricon 70-plus) to acquire more DNA mass</li> </ul> | <ul style="list-style-type: none"> <li>In oligotrophic systems, small volumes do not produce enough DNA mass for downstream experiments</li> <li>Indirectly quantifies D-DNA components</li> <li>Does not determine molecular weight of DNA</li> </ul>                                    |
| Brum et al. (2013a)       | Filtration, tangential flow ultrafiltration, centrifugal ultrafiltration, density gradient ultracentrifugation                                                             | <ul style="list-style-type: none"> <li>Suitable for investigating morphology and genomic data of marine viruses</li> </ul>                                                                                                                                                              | <ul style="list-style-type: none"> <li>F-DNA was not the focus of this study but could likely be isolated using the method described</li> <li>In oligotrophic systems, large volumes required</li> <li>Time-consuming</li> <li>In oligotrophic systems, large volumes required</li> </ul> |
| Biller et al. (2014)      | Filtration of large volumes >50–100 L, tangential flow ultrafiltration, ultracentrifugation, Optiprep density gradient                                                     | <ul style="list-style-type: none"> <li>Suitable for collecting vesicles</li> <li>Optiprep used for collecting membrane-bound vesicles</li> </ul>                                                                                                                                        | <ul style="list-style-type: none"> <li>In oligotrophic systems, large volumes required</li> </ul>                                                                                                                                                                                         |
| Present study             | Filtration of large volumes, tangential flow ultrafiltration (TFF), centrifugal ultrafiltration, density gradient ultracentrifugation                                      | <ul style="list-style-type: none"> <li>Directly separates D-DNA constituents (e.g., vesicles, viruses, and F-DNA)</li> <li>Recovers 75–20000 bp dsDNA using 30 kDa filter</li> </ul>                                                                                                    | <ul style="list-style-type: none"> <li>Low-molecular-weight DNA (&lt;75 bp) may be lost, depending on the nominal molecular weight limit of the ultrafilters used</li> </ul>                                                                                                              |

(Continues)

**Table 1.** Continued

| Reference | Procedure | Advantages                                                                                                                                                                                                                                                                | Comments                                                                                          |
|-----------|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|
|           |           | <ul style="list-style-type: none"> <li>All D-DNA constituents suitable for downstream analyses (e.g., microscopy, molecular weight estimates, and sequence analysis)</li> <li>When low masses are sufficient or in eutrophic ecosystems, TFF can be eliminated</li> </ul> | <ul style="list-style-type: none"> <li>In oligotrophic systems, large volumes required</li> </ul> |

(Lawrence and Steward 2010). The samples were then spun in an ultracentrifuge (Beckman Coulter Optima XPN-80) in a swinging bucket rotor assembly (SW 41 Ti) for 72 h at 4°C at 30,000 rpm ( $154,000 \times g$ ). Samples were removed and separated into 0.5 mL fractions using a fraction collector (Auto Densi-Flow, Labconco). Marine viruses have previously been shown to migrate to CsCl densities between 1.45 and 1.55 g mL<sup>-1</sup> (Lawrence and Steward 2010), while DNA has been reported around 1.63–1.76 g mL<sup>-1</sup> (Wells and Larson 1972; Lueders et al. 2004). Marine microbial vesicles have been reported from 1.19 to 1.35 g mL<sup>-1</sup> in CsCl (Choi et al. 2015; Kwon et al. 2019), congruent with previous reports of membrane-enclosed DNA migrating around 1.30 g mL<sup>-1</sup> (Anderson et al. 1966). The density of each fraction was determined gravimetrically and the volume measured using a positive displacement pipet. The fractions corresponding to three distinct peaks in D-DNA were combined into three separate “pools” for subsequent analyses: (1) low-density DNA (1.30–1.35 g mL<sup>-1</sup>), (2) medium-density DNA (1.40–1.55 g mL<sup>-1</sup>), and (3) high-density DNA (1.60–1.70 g mL<sup>-1</sup>). CsCl was exchanged with TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA, pH 7.5) for the low- and high-density pools and with SM buffer for the medium-density viral pool, by centrifugal ultrafiltration (Amicon Ultra-0.5 Centrifugal Filter Unit, 10 kDa NMWL; Millipore). To perform the buffer exchange, 0.5 mL fractions are added to the centrifugal device and concentrated to about 0.1 mL. This concentrate is then reconstituted to 0.5 mL in the appropriate buffer and concentrated again to 0.1 mL. This is performed a total of three times to rinse the fraction of remaining salts and effectively exchange the buffer.

#### Quantification of isolated DNA

DNA in each of the three pools was quantified using a Qubit 2.0 fluorometer following the Qubit dsDNA HS Assay (ThermoFisher Scientific). While the Qubit fluorometer and proprietary buffers and fluorochromes were used in this study, other similar reagents may be used. Prior to quantification, DNA from the vesicular (low-density) and viral (medium-density) pools were extracted following the whole community and viral nucleic acid extraction methods, respectively, as described by Mueller et al. (2014). Both extraction methods

have three primary steps: (1) lysis and digestion by a lysis buffer with proteinase K, (2) salt-induced protein precipitation with ammonium acetate and, (3) nucleic acid precipitation and recovery. The whole community extraction (utilized here to extract nucleic acids from vesicles) employs a lysozyme step prior to exposure to the lysis buffer and proteinase K, which is not required for the viral nucleic acid extraction. Pools may be further investigated by additional nucleic acid assays, fluorometric protein quantification, epifluorescence microscopy, fragment analysis, or transmission electron microscopy (TEM).

#### Fragment analysis

To assess the molecular weight of recovered F-DNA, concentrated samples were analyzed by capillary electrophoresis (*Fragment Analyzer*™ Automated CE System; Advanced Analytical Technologies, Incorporated) with a 33 cm capillary using the High Sensitivity Genomic DNA Analysis Kit. Samples were run following the manufacturer's instructions (protocol DNF-488-33).

#### Assessment

The following experiments were conducted to evaluate the effectiveness of this method for isolating F-DNA and to characterize the distribution of different D-DNA components in the density gradient fractions. The samples subjected to these experiments were collected from the euphotic (5, 75, and 125 m), upper mesopelagic (150 and 250 m), and lower mesopelagic (350, 500, and 1000 m) zones of the water column. For all samples, DNA concentrations exhibited maxima within three distinct density ranges of the D-DNA cesium chloride gradient and were partitioned into three pools: (1) low-density DNA, 1.30–1.35 g mL<sup>-1</sup>; (2) medium-density DNA, 1.40–1.55 g mL<sup>-1</sup>; and (3) high-density F-DNA, 1.60–1.70 g mL<sup>-1</sup> (Fig. S2A,B). The amount of DNA between these three pools (1.20–1.30, 1.35–1.40, and 1.55–1.60 g mL<sup>-1</sup>) together accounted for < 10% of the sum of all density fractions (total D-DNA). All homogenized samples representing each of these density ranges were analyzed by TEM (morphology), epifluorescence microscopy (virus-like particle enumeration), DNA quantification before and after treatment with DNase I,

and protein quantification. D-DNA characterizations are reported for four separate depth profiles at Station ALOHA, collected in November 2017, April 2018, May 2018, and June 2019.

### Transmission electron microscopy

Electron microscopy of negatively stained samples was routinely used to characterize the density gradient pools. A small subsample (4  $\mu\text{L}$ ) of concentrated, buffer-exchanged fractions was applied for 45 s to carbon-coated Formvar-coated copper grids (Electron Microscopy Sciences) that were rendered hydrophilic by glow discharge. Grids were then washed with 10  $\mu\text{L}$  of ultrapure water (Barnstead, NanoPure), negatively stained with 2.5% uranyl acetate for 45 s, washed with 10  $\mu\text{L}$  of ultrapure water again, and allowed to air dry. Material on the grids was examined by TEM (Hitachi HT7700) at 65,000–100,000 $\times$  magnification at 100 kV. High-resolution images were captured with a 2048  $\times$  2048 CCD camera (AMT XR-41B). For all three D-DNA cesium chloride gradient pools (low-, medium-, and high-density), the first 50 randomly observed objects were used to assess the dominant structures in each sample.

The low-density DNA pools were dominated by filamentous (10  $\times$  500–600 nm) and coccoid (30–100 nm diameter) structures (Fig. 1). The medium-density pools contained a variety of virus-like morphotypes (tailed and nontailed). The emphasis of observations was not on characterizing viral morphologies, but on examining the F-DNA (high-density) pool to determine whether viruses or vesicles were present. No viruses were observed in the 1.60–1.70 g mL<sup>-1</sup> D-DNA pool. For all samples, this pool was dominated by narrow structures of approximately the same width (6–8 nm) but with varying length. These results suggest that the dominant structures in the low- and medium-density pools are likely encapsulated, whereas the high-density D-DNA pool is dominated by linear structures (Table 2).

### Epifluorescence microscopy

After the density and nucleic acid content of each D-DNA pool was determined, DNA-containing virus-like particles (VLPs) were enumerated by epifluorescence microscopy (Suttle and Fuhrman 2010). A volume containing at least 50 ng of DNA from each pool was diluted in ultrapure water to 1 mL. After dilution, samples were gently filtered ( $\leq$  13 kPa) onto a 0.02  $\mu\text{m}$  Anodisc (Whatman) filter. Subsequently, the filter was stained with 200-fold diluted PicoGreen reagent (ThermoFisher Scientific) which is highly specific to double-stranded DNA. Following staining, phenylenediamine was applied to a glass slide and the filter, to prevent fading. VLPs were enumerated at 1000 $\times$  magnification using a Nikon Eclipse 90i microscope. No VLPs were observed in any of the high-density pools (1.60–1.70 g mL<sup>-1</sup>). By comparison, VLPs were found in both the low-density and medium-density D-DNA pools (Table 2). These data support the TEM observations that the low- and medium-density pools may be dominated by encapsulated DNA particles

(e.g., vesicles and/or viruses), whereas these are absent from the high-density pool.

### DNase treatment

To test the effectiveness of DNase I in hydrolyzing the three D-DNA pools, subsamples ( $\sim$  50 ng DNA from each pool) were treated with 5 U mL<sup>-1</sup> DNase I (ThermoFisher Scientific from *Escherichia coli* cells, RNase-free, EN0521) for 30 min at 37°C. The reaction was terminated by the addition of 50 mM EDTA, followed by incubation at 65°C for 10 min. To assist in confirming which pools contained F-DNA, DNA concentrations were measured before and after DNase I treatment using a Qubit 2.0 fluorometer (Table 2). Buffer samples (negative controls) and triplicate controls of 50 ng dsDNA ladder (ThermoFisher Scientific GeneRuler 1 kb DNA Ladder, SM0312) were also analyzed. In low- and medium-density pools the loss of DNA was minimal following DNase I treatment (89–101% and 88–98% DNA remaining, respectively). In all F-DNA samples the fluorometrically measured DNA was reduced to 3–8%, suggesting that a major portion of this DNA pool is susceptible to DNase digestion and may not be protected by an organic (lipid or protein) or inorganic (e.g., clay particles) structure.

### Protein quantification

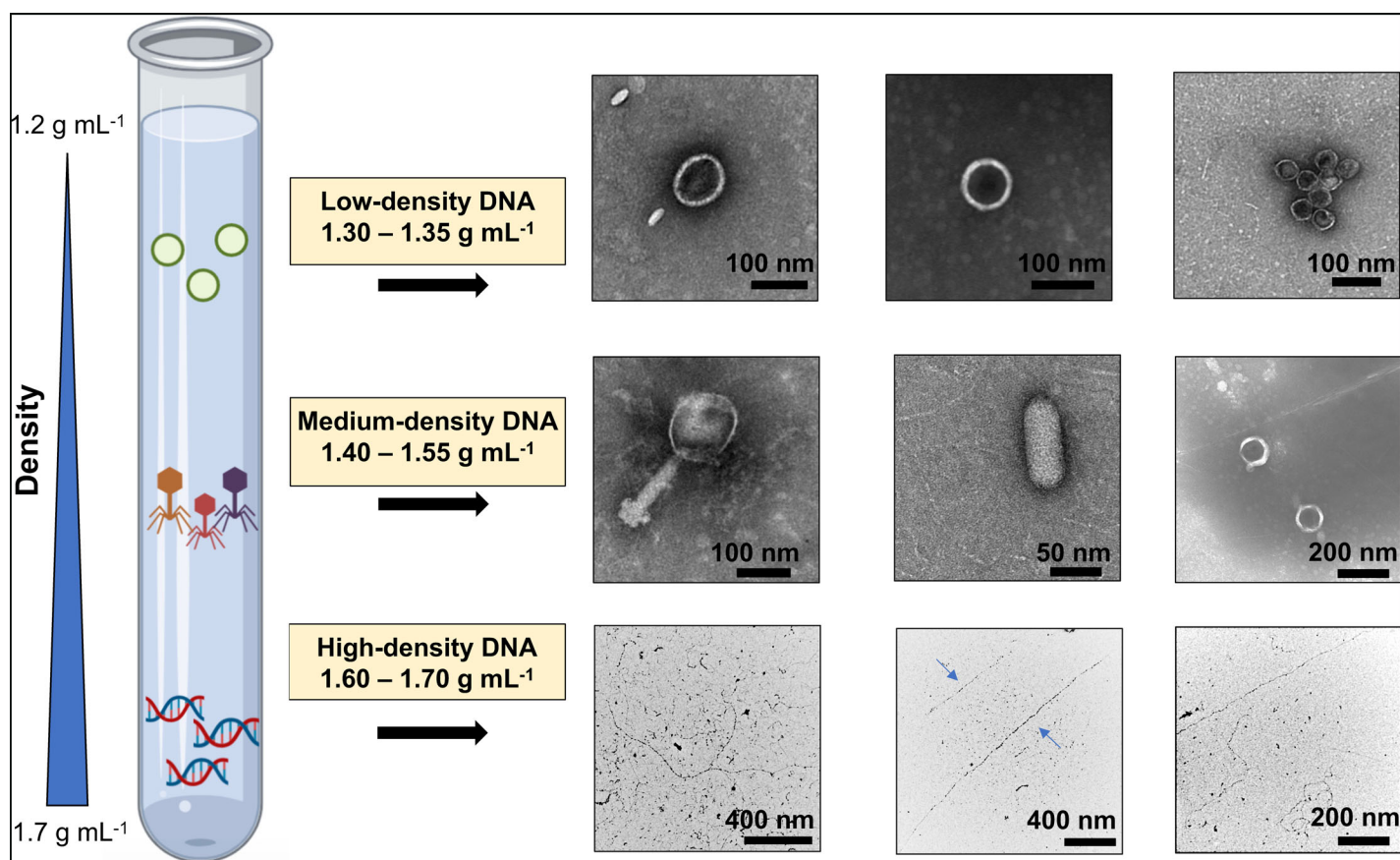
Viral particles are often encapsulated by a protein coat, and the concentration of protein associated with this coat can be quantified using fluorescent dyes. To measure the concentration of protein and DNA in separated viral and F-DNA pools, fluorometric protein and dsDNA quantifications were performed (following the Qubit Protein and the Qubit dsDNA High Sensitivity Assays, respectively). The low- and medium-density pools for all depths had a much lower average DNA : protein ratio than that for the F-DNA pools (Table 2), indicating that the contents of the low- and medium-density pools are dominated by both protein and DNA structures, whereas the F-DNA pools are proportionately higher in DNA.

### Method validation

To validate the method, we investigated the effect of prefiltration (double-layered 0.1  $\mu\text{m}$ ) on D-DNA concentrations, analyzed the recovery of DNA of varying molecular weights, and conducted a field experiment to assess the concentration of each D-DNA pool with respect to depth.

#### Prefiltration

Since it is possible that F-DNA may be an artifact of microbial cell breakage during prefiltration, concentrations were quantified before and after the prefiltration of *Prochlorococcus marinus* strain MIT9301 cells ( $2 \times 10^8$  cells mL<sup>-1</sup>) through a double-layered 0.1  $\mu\text{m}$  capsule filter. *P. marinus* is a small (0.5–0.7  $\mu\text{m}$ ) cyanobacterium that is ubiquitous in tropical and subtropical marine ecosystems (Chisholm et al. 1992; Partensky et al. 1999). These cells were carefully added to seawater collected from 1000 m at Station ALOHA to achieve a



**Fig. 1.** Transmission electron micrographs of the dominant structures observed in D-DNA pools collected from the low-density (vesicles), medium-density (viruses), and high-density (F-DNA) fractions, which correspond to distinct D-DNA peaks that were consistently observed in cesium chloride gradients.

**Table 2.** Microscopy and molecular characterizations of the cesium chloride gradient pools.

| Pool                                           | Dominant contents (examined by TEM)               | Epifluorescence VLP detection | % DNA remaining post-DNase treatment | DNA : protein range |
|------------------------------------------------|---------------------------------------------------|-------------------------------|--------------------------------------|---------------------|
| Low-density (1.30–1.35 g mL <sup>-1</sup> )    | Filamentous structures, round viruses or vesicles | VLP detected                  | 89–100                               | 1.2–2.3             |
| Medium-density (1.40–1.55 g mL <sup>-1</sup> ) | Viruses (various morphologies)                    | VLP detected                  | 88–98                                | 4.3–6.8             |
| High-density (1.60–1.70 g mL <sup>-1</sup> )   | Linear structures (DNA)                           | VLP absent                    | 3–8                                  | 30.6–42.1           |

final concentration of  $6 \times 10^5$  cells mL<sup>-1</sup>. Deep seawater was used in this experiment because it contains a minimal concentration of D-DNA. The concentration of added cells used in this experiment is comparable to total microbial surface concentrations at Station ALOHA and about two times higher than surface *P. marinus* cell counts, therefore exposing the capsule filter to a similar cell load. Prior to the addition of the *P. marinus* cells, the concentration of F-DNA in this 1000 m seawater was found to be  $0.09 (\pm 0.01) \mu\text{g L}^{-1}$ , using the previously described method. Cultured cells, containing approximately  $1 \mu\text{g L}^{-1}$

total DNA were carefully added to 10 L of seawater, then filtered at  $50 \text{ mL min}^{-1}$  through the capsule filter. The filtrate was collected, concentrated by tangential flow ultrafiltration, and F-DNA was subsequently measured using the method described herein. After the prefiltration of *P. marinus*, F-DNA was  $0.08 (\pm 0.02) \mu\text{g L}^{-1}$ , a value that was not significantly different from the initial F-DNA concentration. Viral and vesicle DNA were also measured but were undetectable before and after filtration. Therefore, the prefiltration step used to separate dissolved from particulate DNA did not result in the artifactual

production of D-DNA, a finding in accordance with previous tests using natural communities prefiltered through 0.2  $\mu\text{m}$  filters (DeFlaun et al. 1986; Beebee 1991). When sampling biomes that are known to have many delicate cell assemblages, other concentration methods (e.g., gravity, reverse filtration) may also be considered.

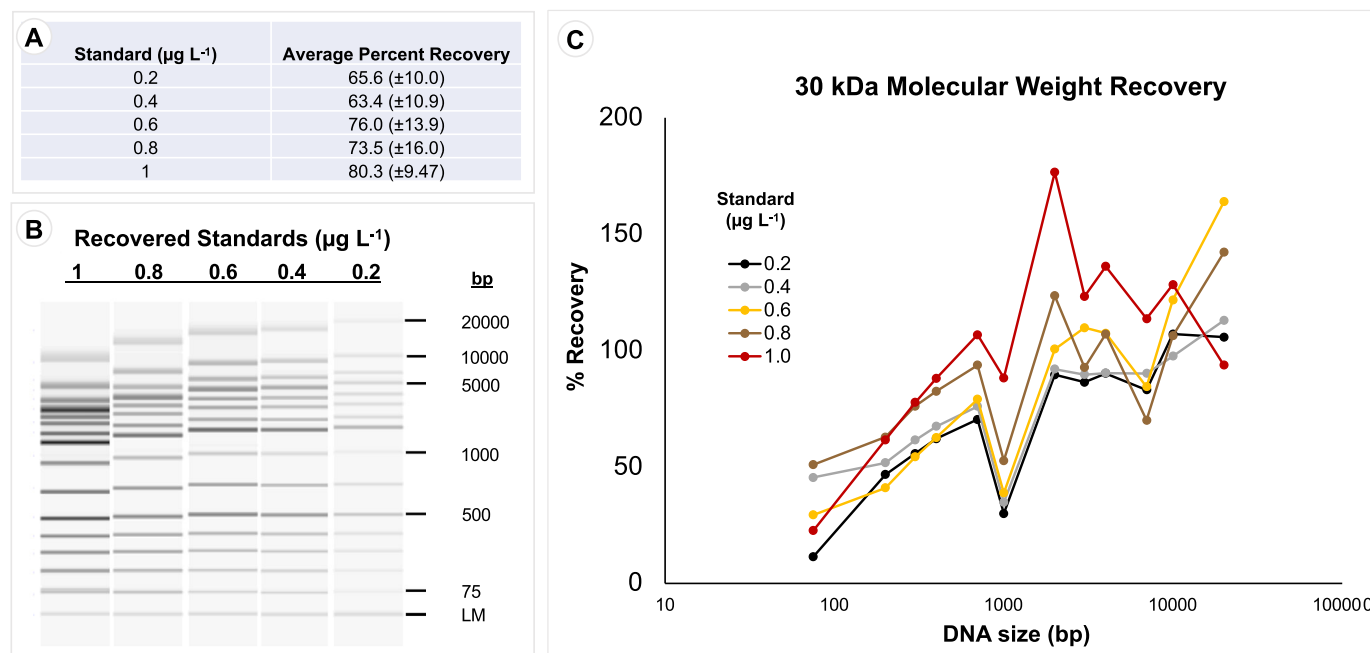
#### Internal standard curve and recovery

To assess the recovery of DNA with variable molecular weights using the method described herein, an internal standard curve (0, 0.2, 0.4, 0.6, 0.8, and 1  $\mu\text{g L}^{-1}$ ) was created in duplicate by the addition of varying amounts of 75–20,000 bp ( $\sim 4.8 \times 10^4$  to  $1.3 \times 10^7$  g mol $^{-1}$ ) DNA ladder (GeneRuler 1 kb Plus DNA Ladder, ThermoFisher Scientific) to 5 L of 0.1  $\mu\text{m}$ -filtered seawater from 1000 m at Station ALOHA, followed by processing with tangential flow ultrafiltration, centrifugal ultrafiltration, and density gradient separation. After collection of the high-density F-DNA pool (1.60–1.70 g mL $^{-1}$ ) from the CsCl density gradient, the sample was exchanged into TE buffer by centrifugal ultrafiltration. Absolute recovery was determined by measuring mass-standardized fluorescence with a Qubit 2.0 fluorometer following the Qubit dsDNA HS Assay (ThermoFisher Scientific). The recovery of the ladder DNA from 30 kDa ultrafiltration and subsequent density gradient separation was 68–86% (Fig. 2A). The size and relative recoveries of fragments of

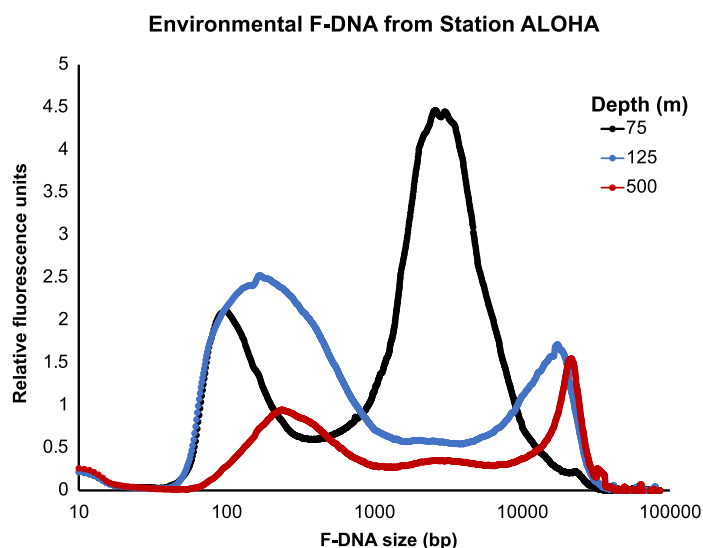
different sizes were determined from migration rate and fluorescence intensity of each band using capillary electrophoresis as described above. Because of possible nonlinearity in the relationship between mass and fluorescence, the fragment-based recoveries (Fig. 2B) can only be considered estimates of relative recovery. All molecular weights from the ladder were recovered with little to no shearing (Fig. 2C). For comparison, three environmental F-DNA samples were measured using fragment analysis (Fig. 3) and illustrate the molecular sizes of F-DNA that can be recovered from an open-ocean ecosystem.

#### Low-molecular-weight filtrate DNA

Using a tangential flow filter with a 30 kDa nominal molecular weight cutoff may lead to the loss of DNA < 50 bp. To assess whether low-molecular-weight DNA was lost in the tangential flow ultrafiltrate (permeate), the permeate was processed using the cetyltrimethylammonium bromide (CTAB)-precipitation method of Karl and Bailiff (1989). Two-liter filtrate samples from four depths (5, 75, 125, and 500 m) at Station ALOHA collected on HOT-297 in November 2017, were investigated in triplicate. Following this procedure, 0.05 ( $\pm 0.01$ ), 0.12 ( $\pm 0.03$ ), and 0.14 ( $\pm 0.02$ )  $\mu\text{g DNA L}^{-1}$  were recovered in the < 30 kDa permeate from 5, 75, and 125 m, respectively. These values account for 25, 38, and 45% of the F-DNA and 10, 17, and 27% of the total D-DNA collected from these three depths following the method



**Fig. 2.** Molecular size-dependent recovery of ladder and environmental F-DNA. **(A)** Average percent recovery for total ladder DNA, measured fluorometrically. **(B)** Recovery of ladder DNA (75, 200, 300, 400, 700, 1000, 2000, 3000, 4000, 7000, 10,000, 20,000 bp) using the method described herein. Five concentrations of ladder DNA standards (0.2, 0.4, 0.6, 0.8, and 1  $\mu\text{g L}^{-1}$ ) were added to separate samples of 0.1  $\mu\text{m}$ -filtered seawater collected from 1000 m at Station ALOHA and processed with this method using 30 kDa ultrafiltration. Capillary electrophoresis results from the fragment analyzer reveal little to no degradation of the recovered DNA ladder standards. LM (lower marker) is the low-molecular-weight standard that is added to calibrate and align all samples. **(C)** Percent recovery of the same ladder DNA. The resulting recoveries were calculated based on uncalibrated fluorescence intensity and are thus intended to indicate only relative differences in recovery among bands. Bands at 500, 1500, and 5000 were not included in the analysis because they contained higher mass than the other bands and relative recoveries would be confounded by nonlinearity in the fluorescence-mass relationship.



**Fig. 3.** Molecular weight distribution for environmental F-DNA recovered from three depths at Station ALOHA (75, 125, and 500 m). Relative fluorescence units are plotted as a function of DNA fragment sizes as determined by capillary electrophoresis from 10 bp to the limit of resolution at approximately 40,000 bp.

described in this manuscript. The recovered DNA in the permeate from the 500 m sample was undetectable ( $< 0.01 \mu\text{g L}^{-1}$ ).

### Field application

To verify the efficacy of this method, we collected samples at or near Station ALOHA on four separate cruises (HOT-297 November 2017, FK180310 April 2018, HOT-302 May 2018, HOT-312 June 2019) and processed the samples using the method described above. All samples were concentrated by tangential flow ultrafiltration (30 kDa NMWL) and separated by buoyant density centrifugation. Low-density encapsulated DNA, medium-density encapsulated DNA, and high-density F-DNA were found to contribute on average 15, 46, and 39% of the total D-DNA, respectively, with ranges of 8–43%, 20–64%, and 23–66% (Fig. 4). Within these four depth profiles, medium-density viral assemblages and F-DNA are the primary contributors to total D-DNA concentrations ( $0.03$ – $0.32$  and  $0.02$ – $0.31 \mu\text{g L}^{-1}$ , respectively). On one occasion in May 2018, the total D-DNA ( $0.73 \mu\text{g L}^{-1}$ ) collected from 50 m was dominated by the low-density vesicle pool ( $0.33 \mu\text{g L}^{-1}$ ). This was not observed for any other depth or profile, so is considered to be an outlier. F-DNA, the medium-density viral pools, and total D-DNA peaked at 75 m ( $0.14$ – $0.30$ ,  $0.24$ – $0.31$ ,  $0.40$ – $72 \mu\text{g L}^{-1}$ , respectively) on two cruises when this depth was measured (November 2017 and June 2019).

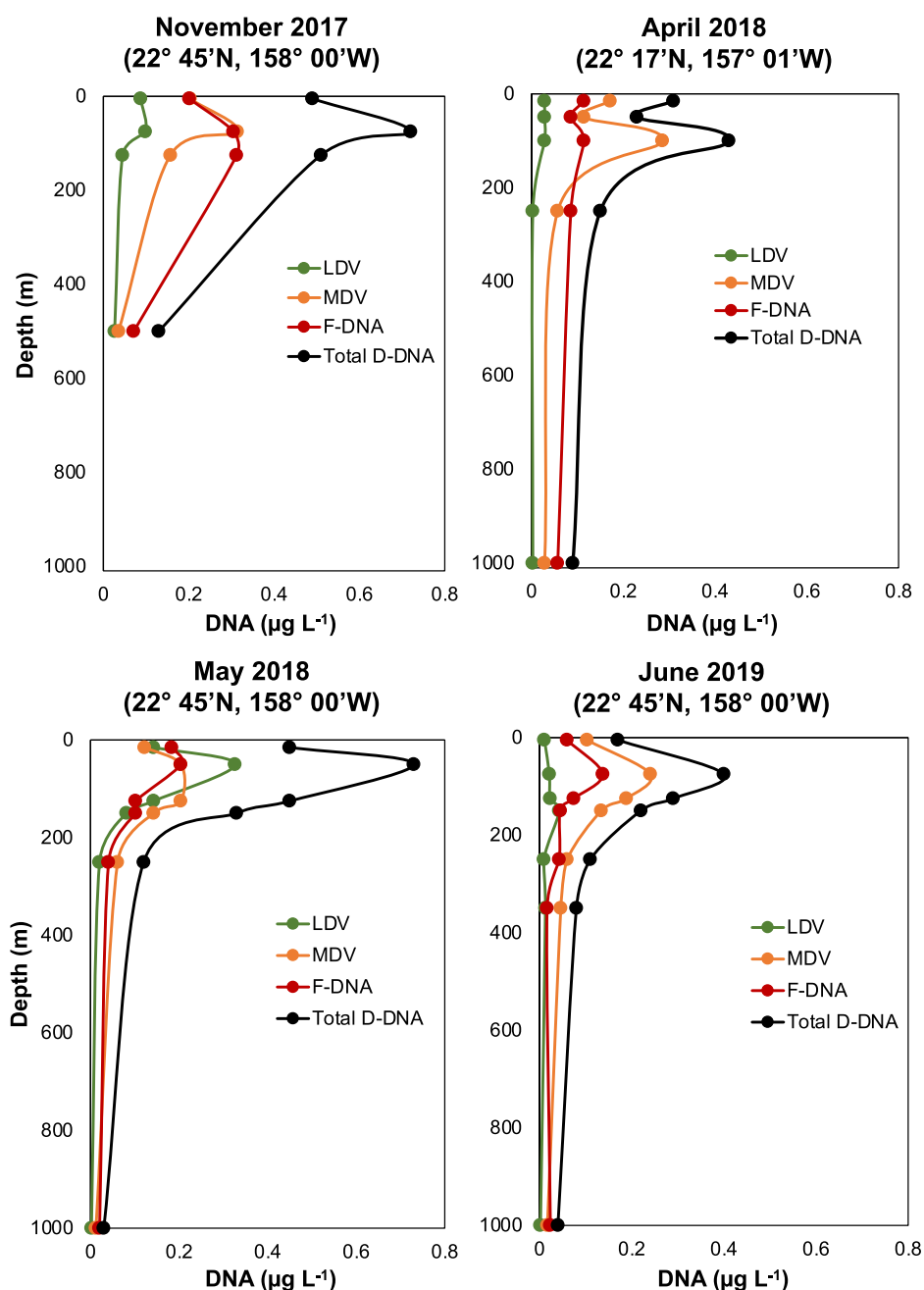
### Discussion

This method provides a means not only to discriminate among, but also to recover, distinct components of operationally defined D-DNA. The nondestructive concentration and

recovery of these materials means that each pool can be further investigated to determine its distinctive characteristics. Downstream analysis of vesicular, viral, and F-DNA (e.g., nucleic acid base or sequence analyses) can provide insight into the microbial composition of D-DNA. Furthermore, biogeochemical analyses of low-molecular-weight D-DNA utilizing CTAB (Karl and Bailiff 1989) can yield insights into the F-DNA size distribution and molecular weight, thereby providing estimates of nitrogen and phosphorus associated with this recovered DNA, and consequences for nutrient limitation in the open ocean. Few studies have specifically isolated and characterized F-DNA, so its sources and physiochemical properties (e.g., stoichiometry, possible modifications, taxonomic affiliations, etc.) are largely unknown. Beebee (1991) used ultracentrifugation to separate viruses and soluble DNA (F-DNA). The pellet was examined by TEM, and was found to be enriched in virus-like particles. The soluble DNA in the supernatant was purified by chromatography, and the molecular weight was measured using gel electrophoresis. They reported that the pelleted “viral” pool was the primary contributor to D-DNA collected from different aquatic environments (78% of D-DNA in pondwater and 85% in seawater). Jiang and Paul (1995) utilized a similar method of ultracentrifugation to separate the bound and soluble DNA pools, and subsequently characterized the pools using hybridization with rRNA gene-targeted probes. They found that soluble DNA (F-DNA) comprised half of the D-DNA, while viral DNA and “other” bound DNA contributed the other half. While these methods provide important datasets, differential centrifugation offers a less complete separation of D-DNA components than density gradient ultracentrifugation. Additionally, the current method provides more complete recovery of F-DNA (68–86%) than Beebee (1991; 36%) and Jiang and Paul (1995; 65%). As a common component of DOM, F-DNA may play important roles in cycling labile substrates, transferring genetic information, and providing limiting nutrients to marine microorganisms.

Previous studies have identified a fraction of D-DNA that is resistant to DNase enzymes (Jiang and Paul 1995; Brum 2005). In this study, treatment with DNase was used to confirm the identity of the F-DNA constituent collected from the CsCl density gradient. The small residual after digestion (3–8%) suggests that only a minor fraction of the F-DNA pool is DNase-resistant and that, enigmatically, most of the F-DNA is in a form that should be very readily degraded or taken up by microorganisms. The dominance of this labile form of DNA must therefore be sustained by continuous production.

The DNase-resistant portion of the F-DNA may represent DNA that is modified (e.g., phosphorothioation or methylation) or associated with proteins (e.g., histones or other DNA binding proteins). One source of phosphorothioated DNA, which is resistant to many nuclease activities, is produced by *Pelagibacter ubique* (Eckstein 2000; Wang et al. 2007), one of the most abundant microorganisms in open-ocean systems (Morris et al. 2002). Protein binding may also confer DNase



**Fig. 4.** Depth profiles of various pools of dissolved DNA constituents collected from the North Pacific Subtropical Gyre. “F-DNA” represents the high-density free DNA pool (blue), “LDV” represents the low-density pool containing vesicular and viral DNA (red), “MDV” represents the medium-density DNA pool dominated by viruses (yellow), and total D-DNA is the summation of these three pools (black).

resistance. Both prokaryotic and eukaryotic cells contain genome packaging proteins which help organize and compress their genetic material. Once microbial cells are lysed or grazed and their DNA is released into the environment, these proteins may still be bound to DNA, possibly impeding degradation. Both bacterial binding proteins and histones have been shown to protect DNA from DNase I digestion (Van Holde et al. 1980; Mukherjee et al. 2008). Histones and

bacterial binding proteins have also been shown to protect DNA from environmental stressors such as ultraviolet radiation, oxidative stress, and degradation by acid (Enright et al. 1992; Ljungman and Hanawalt 1992; Oleinick et al. 1994; Wang et al. 2012; Takata et al. 2013; Almarza et al. 2014; Wang and Maier 2015). These modifications may extend the residence times of DNase-resistant F-DNA, altering its dynamics and bioavailability. However, the low concentrations of

the DNase-resistant F-DNA suggest that even this material is turning over relatively rapidly in the ecosystem.

The distribution of dissolved DNA components shown here for Station ALOHA follows a pattern consistent with DOM, in which concentrations are higher at the surface and decrease with depth. The 75 m peaks observed in November 2017 and June 2019 of F-DNA, the medium-density viral pools, and total D-DNA correspond to maximum abundances in *Prochlorococcus* (van den Engh et al. 2017), viral abundances (Brum 2005), and cell-associated cyanophage (DeLong et al. 2006). The cause of this 75 m maximum is still an area of active research. In all 1000 m samples, D-DNA components were significantly lower than at the surface, with F-DNA dominating. The dynamic concentration ranges of each of these dissolved DNA components suggest there may be different zones of net production and net utilization with depth.

The depth distribution patterns of D-DNA observed in this study are similar to those observed previously in the North Pacific Subtropical Gyre (Karl and Bailiff 1989; Brum 2005). However, absolute concentrations observed during this current study are approximately 50% lower. This may be due, in part, to the efficiency of recovery of our method, which we estimate ranged from 68–86%. One known source of loss in this method was the passage of low-molecular-weight DNA through the ultrafilter during concentration, which accounted for 10–27% of total D-DNA. Higher concentrations reported in previous studies could also have resulted from the use of larger pore size prefilters (0.2  $\mu\text{m}$ ), which may inadequately remove some of the smallest and most abundant prokaryotes (e.g., *P. ubique*). The use of double-layered 0.1  $\mu\text{m}$  polyethersulfone capsule filters in this study would have more effectively removed the cellular fraction, minimizing contributions of living cells to the D-DNA. While this would also exclude viruses > 0.1  $\mu\text{m}$  in size, their relative abundance in most oceanic regions appears to be low (Brum et al. 2013b). The concentrations quantified in this study are comparable to surface particulate DNA concentrations recovered from glass fiber or 0.2  $\mu\text{m}$  polycarbonate filters: 0.75–2.38  $\mu\text{g L}^{-1}$  in the equatorial Pacific Ocean (Winn and Karl 1986) and 0.8–5.9  $\mu\text{g L}^{-1}$  in the Eastern Mediterranean Sea (Dell'Anno et al. 1999).

### Comments and recommendations

The method described in this paper should be applicable to many different types of marine and freshwater samples. Depending on the total mass of DNA one requires for downstream analysis (e.g., sequencing or further physical or chemical characterization) and the DNA concentrations in the samples, the method might be simplified. In water with sufficiently high biomass, for example, the volume concentrated might be reduced to the point that one could omit the tangential flow ultrafiltration step and proceed directly to centrifugal ultrafiltration, thus reducing time, effort and expense.

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### Conflict of Interest

None declared.

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