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A simple method for the quantification of amidic bioavailable dissolved organic nitrogen in seawater

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Abstract

A targeted method for the quantification of bioavailable amide N found in marine DON (bDON) is presented. The method utilizes mild acid hydrolysis to convert amide N found in proteins and *N*-acetyl amino polysaccharides to primary amine containing products that are measured using a highly sensitive (nanomolar range and precision) fluorometric technique with addition of *O*-phthaldialdehyde. We find amidic bDON concentrations ranging from 0.08 to 1.82 μM N within waters from the upper 300 m in the southern California Current, Southern California Bight, and subtropical North Pacific representing 15–33% of bulk DON concentrations. Bioassay experiments from the North Pacific revealed consumption of ~20% of the in situ bDON within 5 days. The method represents a simple and rapid tool for the quantification of bioavailable DON concentrations in seawater with improved analytical precision over traditional estimates of bulk DON concentrations.

Dissolved organic nitrogen (DON) is often the most abundant form of nitrogen in the upper ocean, of which a portion is bioavailable to microorganisms. The study of DON biogeochemical cycling in seawater has been hindered by existing analytical methods which require the separate determinations of multiple N species, total dissolved nitrogen (TDN), nitrate, nitrite, and ammonium, and then determine DON by difference, resulting in large analytical errors. This differencing approach hampers the accuracy and precision of DON measurements, especially for the deep ocean where compounding of analytical error can lead to coefficients of variation on DON determinations upwards of 30–50% when DON is <10–15% of the TDN content (Hansell 1993). A chemical method to remove all inorganic N from a sample prior to oxidation of its organic N has been needed by the scientific community for some time (Sharp et al. 2002). Reduction of nitrate plus nitrite to NO and NO_2 gas (NO_x) by reaction with transition metals such as those used in chemiluminescent methods to quantify nitrate and nitrite in natural waters, e.g., Fe(II), V(III), and Ti(III), have shown promise toward this elusive goal (Braman and Hendrix 1989; Foreman et al. 2016); however, concerns and limitations associated with preservation of marine DON molecules and analytical blanks under the reaction conditions have hindered progress to date.

However, analyses of the bulk DON pool concentration in time and space have been utilized to discern the regional (Hansell and Waterhouse 1997; Abell et al. 2000; Mahaffey et al. 2004; Landolfi et al. 2008, 2016; Torres-Valdés et al. 2009; Letscher et al. 2013*b*; Knapp et al. 2011, 2018) to global (Letscher et al. 2013*a*) scale gradients in near surface waters. The general pattern that has emerged is one in which surface ocean DON concentrations are elevated (~4.5–5.0 μM) in regions of elevated net community production such as upwelling zones and lower (~4.0–4.5 μM) within oligotrophic systems such as the subtropical mid-ocean gyres (Letscher et al. 2013*a*). This gradient is suggestive of biological removal of DON, likely as a means to satisfy N demand when inorganic N species are limiting in the oligotrophic subtropical ocean. Autotrophs are known to harbor the ability to utilize LMW DON molecules such as urea and free amino acids to satisfy N demand when nitrate and ammonium are scarce (Bronk et al. 2007); however, these DON moieties are very minor contributors to the standing stock of marine DON material (Aluwihare and Meador 2008). Remineralization of DON by heterotrophs is likely required to free inorganic N to fuel autotrophic growth in surface waters, however DON consumption has been found to preferentially occur in the shallow subsurface (Letscher et al. 2013*a*, 2015*a*). Bioassay experiments indicate upper mesopelagic (~100–200 m) zone heterotrophic microbes can consume ~0.25–0.5 μM of surface accumulated DON on the timescales of weeks to months (Letscher et al., 2013*a*, 2015*a*). This quantity of bioavailable DON revealed in bioassay experiments matches the bulk DON concentration gradient observed across the subtropical ocean and is further supported by the ^{15}N isotopic budget at the Bermuda Atlantic Time-series study (BATS) site in the subtropical North

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Atlantic (Knapp et al. 2005), with an estimate that ~0.25 μM of surface accumulated DON from the spring and summer months is remineralized upon delivery to the upper mesopelagic zone each fall/winter.

DON like dissolved organic carbon (DOC) is a complex mixture containing chemical species that span at least the biosynthetic capacity of marine microorganisms (e.g., Koester et al. 2022) but also likely includes compounds modified by biotic and abiotic processes (e.g., Wagner et al. 2020; Broek et al. 2023). Recent studies have begun to develop methods that can address this complexity and have detected 100 s or more distinct nitrogen containing compounds in surface waters (Petras et al. 2017; Koester et al. 2022; Johnson et al. 2023; Kujawinski et al. 2023; Liu et al. 2022; Sacks et al. 2022), yet many of these approaches require extraction of DOM from seawater and utilize specific analytical methods and are thus, necessarily biased as well. Thus, focusing on particular functional groups within bulk DON that have similar chemical behavior and can be traced to specific classes of parent compounds whose contributions to the DON pool are significant (e.g., Aluwihare et al. 2005; Aluwihare and Meador 2008), may reveal valuable insights into the cycling of a quantitatively significant fraction of DON.

For example, studies have shown that a significant fraction of DON is comprised of acidic hydrolysis labile and presumably enzymatically labile pool of amide molecules found as proteins and *N*-acetyl amino polysaccharides (NAAPs), and a nonhydrolyzable pool of recalcitrant amide material (McCarthy et al. 1997; Aluwihare et al. 2005; Broek et al. 2023). In the surface ocean, the high molecular weight (HMW) fraction (>1 kDa) of DON is comprised of ~40% NAAPs, ~20% hydrolysable protein, and ~30% nonhydrolyzable amide (Aluwihare et al. 2005). The remaining ~10% is found as amine material, likely dissolved amino acids and amino sugars (Aluwihare et al. 2005). The surface HMW DON pool, rich in NAAPs and hydrolysable protein, is mostly absent from deep ocean waters, leaving behind a background HMW DON pool comprised of nonhydrolyzable amide N that is present throughout the water column. Low molecular weight (LMW) DON, also present throughout the marine water column, exhibits a D-enantiomer enriched amino acid composition, suggestive of a prokaryotic source, and is enriched in heterocyclic nitrogen groups, making LMW DON distinct from the biological sources producing HMW DON in the ocean (Knapp et al. 2012; Broek et al. 2019, 2023). Thus, acid hydrolysis provides an operational method capable of separating labile and recalcitrant DON within the bulk reservoir without the need to isolate these compounds from seawater, with proteins and *N*-acetyl sugars serving as appropriate models for the widespread amide functional groups present in marine DON.

Given existing knowledge of the chemical composition of DON that is biologically labile (e.g., amines, amide found as NAAPs, and protein) and the absence of this material at depth in the ocean (Aluwihare et al. 2005), it may be possible to target this material for quantification with a direct chemical method. This approach would avoid simply relying on either the cumbersome and less precise determinations of bulk DON concentration across time and space or the more precise, molecular-level DON chemical composition assays that require specialized sampling and analytical methods and are still compositionally biased. As we obtain greater insight into the structure of DON, methods can be modified to be more inclusive of the true complexity within DON.

Materials and procedures

Here, we present a chemical method that targets and quantifies the marine amidic bioavailable DON (bDON) pool. The amidic bDON pool is defined as that which is recoverable as primary amine following mild acidic hydrolysis to depolymerize proteins and NAAP material. Primary amines found as dissolved free amino acids in seawater are also quantified by the method and included in the estimate of bDON. The method employs mild acid hydrolysis (6 M HCl) to break the amide linkage in NAAPs and proteins, which results in the production of amino polysaccharides and amino acids or peptides terminating in primary amine groups after hydrolysis (Aluwihare et al. 2005). The method then employs a high sensitivity (nanomolar range) fluorometric technique with *O*-phthaldialdehyde (OPA) for the quantification of dissolved primary amines (Josefsson et al. 1977). This new method quantifies the amidic bDON concentration in marine samples with a precision of 100 nM and limit of detection of 80 nM, obtains a high daily rate of sample throughput (>40 samples/d) using manual analysis protocols, and could be employed at-sea.

Preparation of reagents and standard solutions

All reagents and standard DON solutions were prepared using purified water with a resistivity of >18.2 MΩ•cm from a water purifier system (Milli-Q, EMD Millipore, USA). A 0.4 M borate buffer solution was prepared by dissolving 9.88 g boric acid (Fisher Scientific, USA) in 400 mL of purified water using stirring to dissolve. The pH was then adjusted to ~9.5 using a ~9 mL addition of 8 M NaOH (Fisher Scientific, USA). To this buffer was added OPA, after first dissolving 100 mg OPA (Acros Organics, Fisher Scientific, USA) in 10 mL of ethanol (Molecular Biology Grade, Fisher Scientific, USA). This OPA reagent was stored in a refrigerator (4°C) in the dark for up to 6 months. Each analytical day a working OPA reagent was prepared by adding 100 μL of 2-mercaptoethanol (Fisher Scientific, USA) to a 100 mL aliquot of the OPA in borate buffer solution and stored at room temperature in the dark for at least 1 h before use. Hydrochloric acid additions were performed using 12.1 M HCl (Suprapur®, MilliporeSigma, USA, CAS #7647-01-0). Standard DON containing solutions were prepared in 0.1 M bicarbonate buffered water (pH = 8.3); 12.8 g $NaHCO_3$ (Fisher Scientific, USA) dissolved in 2 L purified water. A 10 mM N primary glycine stock solution was prepared by dissolving 0.1877 g glycine (Alfa Aesar, Fisher Scientific, USA) in 250 mL bicarbonate buffered water. Secondary glycine stock solutions (2 μM N) were prepared fresh each analytical day by diluting 50 μL of the primary stock in 250 mL of bicarbonate buffered water. A 10 mM N primary stock solution of *N*-acetyl glucosamine (NAG) was prepared by dissolving 0.553 g NAG (Alfa Aesar, Fisher Scientific, USA) in 250 mL of bicarbonate buffered water. Final concentration 1.0 μM N NAG solutions were made by diluting 10 μL of the primary NAG stock solution in 100 mL bicarbonate buffered water and stored in a refrigerator in the dark for up to 1 yr. Standard solutions of a tri-peptide (glycine–proline–glutamine; Sigma-Aldrich, USA) were made by first dissolving 5.022 mg of the peptide in 250 mL of bicarbonate buffered water. Final concentration 1.0 μM N peptide standard solutions were made by diluting 1.5 mL of the primary stock solution in 100 mL bicarbonate buffered water and stored in a refrigerator in the dark for up to 1 yr. Nitrate additions to the standard DON solutions were made from a solution of potassium nitrate (Fisher Scientific, USA) dissolved in bicarbonate buffered water. An ascorbic acid (AA) solution (12 mM concentration) was prepared fresh daily by dissolving 0.2113 g AA (Alfa Aesar, Fisher Scientific, USA) in 100 mL bicarbonate buffered water.

Acid hydrolysis

Samples of known standards or seawater unknowns were prepared for acid hydrolysis by pipetting 2 mL into precombusted 5 mL glass ampoules (Wheaton, USA). Each sample was then acidified to final concentration 6 M HCl by adding 2 mL of concentrated 12.1 M HCl. Next, each sample ampoule was bubbled with N_2 gas (Ultra High Purity Grade, Airgas, USA) for 30 s to provide an inert gas atmosphere for hydrolysis. Each ampoule was then flame sealed using a propane torch. The samples were placed in an oven set to 80°C overnight for a total of 18 consecutive hours to facilitate complete hydrolysis. Method blanks were prepared by adding 2 mL of 12.1 M HCl to 2 mL of bicarbonate buffered water and carried through the hydrolysis protocol analogous to environmental samples.

Sample evaporation

Upon completion of the acid hydrolysis step, liquid evaporation is necessary to remove sample acidity as the fluorometric determination of primary amine content requires an alkaline (pH = 9.5) solution. Sample volumes were transferred to 20 mL precombusted glass centrifuge tubes (Fisher Scientific, USA) and placed within an Acid Resistant CentriVap Centrifugal Vacuum Concentrator and Cold Trap (– 50°C) system (Labconco, USA). Evaporation was achieved after ~5 h of centrifugation at 1725 rpm, a temperature of 50°C, and vacuum pressure of <1 Torr. After complete dryness, the samples were covered with precombusted aluminum foil and analyzed immediately or stored overnight within the laboratory fume hood until analysis the following day.

Analysis of primary amine content

The dried samples were returned to the original sample volume by the addition of 2 mL of bicarbonate buffered water. Seven glycine standard solutions at 2 mL volume were prepared by dilution of the 2 μM glycine secondary stock solution with bicarbonate buffered water in the range 25 nM to 2 μM. Primary amine content of each sample was assessed by fluorometric detection using a FluoroMax 4 spectrofluorometer (Horiba Scientific, USA) with a 1 cm quartz cuvette for the Scripps Institution of Oceanography (SIO) Pier and Southern California Bight samples and a Turner Trilogy Fluorometer (Turner Designs, USA) for the Station ALOHA samples. Excitation was at 350 nm and emission recorded at 450 nm using FluorEssence software (Horiba Scientific, USA) after the addition of 1 mL of the OPA reagent to each sample. Sample fluorescence was recorded between 2 and 10 min after addition of the OPA reagent with lab room lights off due to the photosensitivity of OPA. Drift in the fluorometer's baseline signal over time was assessed with periodic measurements of bicarbonate buffered water (without OPA reagent added) and removed from the total sample fluorescence recorded at 450 nm for each sample in the analytical day's run. Sample primary amine content was computed using the slope and intercept of the glycine standard curve followed by subtraction of the hydrolysis method blank and reported in micromoles N per liter (μM N).

Seawater samples

Seawater samples were collected from the SIO Pier (32°52′N, 117°15′W) from a depth of 2 m on three separate sampling days (Jan 26, 2015; May 26, 2015; April 17, 2017), filtered through a 0.7 μm GF/F filter (Whatman, USA), and acidified with HCl to pH ~2. These samples were stored at room temperature in the dark in 2 L Pyrex glass bottles to be used as internal standards when assessing the day-to-day analytical performance of the method during the development of the protocol. Bulk DON content was assessed by measuring TDN via the high temperature combustion method (Hansell 1993) and subtracting concentrations of dissolved ammonia and nitrate as measured by the Southern California Coastal Ocean Observing System (www.sccoos.org).

Seawater samples for the quantification of bDON were collected aboard the R/V Oceanus during the Oc15017b student cruise of the California Current Ecosystem-Long Term Ecological Research program on July 28, 2015–Aug 2, 2015 near point concepcion within the California Current and Southern California Bight (Fig. 3). A total of 10 stations were sampled to a maximum depth of 200 m with a CTD/Rosette system. Samples were gravity filtered from a Niskin bottle through a precombusted 0.7 μm GF/F (Whatman, USA) filter into precombusted 40 mL EPA glass sample vials. Samples were then acidified with HCl to pH ∼2 and stored in the dark at room temperature until subsequent analysis ashore.

Additional seawater samples for the quantification of bDON and DON in the upper 300 m of the water column were collected aboard the R/V Kilo Moana on Hawaiian Ocean Time-series (HOT) cruise 319 from Station ALOHA (22.75° N 158° W) on Jan 30, 2020. TDN was measured by the persulfate oxidation method (Solórzano and Sharp 1980), with the resulting nitrate measured by the chemiluminescent method using an acidic vanadium (III) solution (Braman and Hendrix 1989). DON was calculated by subtracting the separately analyzed nitrate + nitrite concentration of the sample as determined by the same chemiluminescent method from TDN, with a mean precision on [DON] of ± 0.3 μM or 5–10%.

Seawater incubation experiments

Seawater incubation experiments were performed to investigate the heterotrophic consumption of bDON with waters collected at Station ALOHA on Jun 6–7, 2021, aboard the R/V Kilo Moana. Waters were collected from 5 m (5 m experiment) and 125 m (125 m experiment) via Niskin bottles and stored unfiltered (whole seawater) in 60 L HDPE carboys for <3 h or gravity filtered through a 0.2 μm Supor membrane filter (filtrate) with an Acropak 1000 capsule filter (Pall, USA). Three experimental treatments were performed for each experiment: 100% whole seawater (*Whole*), 100% filtrate (*Filtrate*), and 80% filtrate with 20% whole seawater as an in situ microbial community inoculum (*Mixed*). Dilution of the microbial community with the *Mixed* treatment releases heterotrophic bacteria and archaea from grazing pressure, enhancing the observation of microbial consumption of DOM substrates (Carlson et al. 2004), in this case bDON. The *Filtrate* and *Whole* treatments serve as abiotic and biotic controls, respectively. The seawater incubation experiments were carried out in 9 L amber HDPE carboys, incubated in the dark to isolate heterotrophic activity, at in situ temperature (25.5°C at 5 m; 22.9°C at 125 m) for a period of 113–132 h (4.7–5.5 d). Subsamples from the carboys were collected every ∼20–50 h and measured for microbial cell abundance and bDON, reported here. Microbial cell abundance was determined on unfiltered subsamples fixed with 1% glutaraldehyde and frozen at − 80°C until analysis by flow cytometry at the University of New Hampshire, stained with Sybr Green at 1 : 10,000 dilution. Cell abundance is converted to bacterial organic N concentration using a bacterial biomass content of 12.4 fg C/cell (Fukuda et al. 1998) and C : N stoichiometry of 5 : 1 (Gundersen et al. 2002).

Assessment

Linearity of standardization

The averaged standard curve computed by compiling the relative fluorescence unit (RFU) responses to glycine additions within bicarbonate buffered water performed over a dozen analytical days is shown in Fig. 1. We observed a linear response of RFU vs. standard additions of glycine within the tested range of 25–2000 nM glycine-N. Regression statistics for the averaged standard curve were: slope = 620.25 ± 24.0 (SE); intercept = 221,205 ± 20,944; regression coefficient (R^2) = 0.993. A test in the range 1–100 μM glycine-N demonstrated linearity for the standardization ($R^2 = 0.997$; data not shown) beyond that presented in Fig. 1. Standard glycine additions were performed each analytical day from a 10 μM stock solution of glycine-N, with the variability in day-to-day RFU response likely representative of the variability introduced from investigator pipetting.

Method blank

The method blank was determined by addition of 6 M HCl (final concentration) to bicarbonate buffered water carried through the acid hydrolysis, sample drying, and sample analysis with OPA reagent protocol. The value of the blank was determined each analytical day in triplicate. The average concentration of the blank was 0.30 ± 0.11 μM-N ($n = 36$). The major source of amine-N to the method blank arises from the HCl addition, as evidenced from determinations of the method blank spanning four HCl molarities in bicarbonate buffered water: 0 M HCl = 0.0 μM-N; 1 M HCl = 0.08 ± 0.05 μM-N; 4 M HCl = 0.19 ± 0.08 μM-N; 6 M HCl = 0.30 ± 0.11 μM-N, resulting in a linear dependence of blank amine-N with HCl addition (blank μM-N = 0.0738 ± 0.006 M HCl + 0.0169 ± 0.0225; $R^2 = 0.987$). The averaged triplicate value of the method blank amine-N concentration determined each analytical day was subtracted from the sample computed concentrations when reporting final sample amidic bDON concentrations.

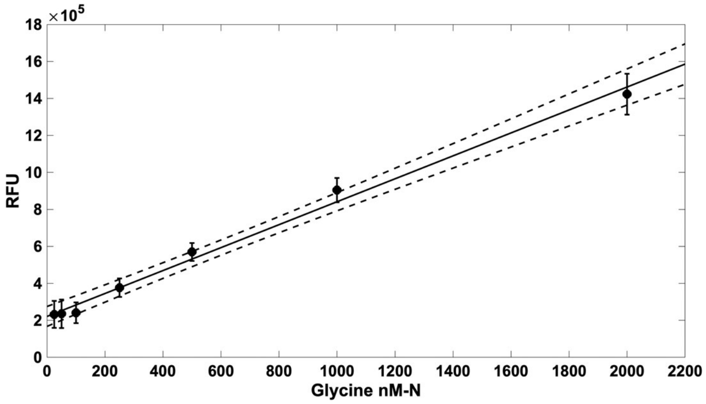

Fig. 1. Plot of RFU vs. standard additions of glycine (nanomolar N concentration, nM-N) in bicarbonate buffered (pH = 8.3) water. The average measured RFU and standard error are plotted from 12 analytical days. Regression fit (solid line) statistics are slope = 620.25 ± 24.0 (SE); intercept = 221,205 ± 20,944; regression coefficient (R^2) = 0.993. Dashed lines indicate 95% confidence intervals of the regression fit.

Table 1. bDON recoveries of standard DON molecules.

	Expected (μM N)	Measured (μM N)
Glycine	1.00	0.97 ± 0.02
NAG	1.00	0.96 ± 0.12
Gly–Pro–Glu	1.00	0.63 ± 0.10

Detection limit and precision

The method detection limit was calculated using the student *t*-test value for $p = 0.99$ multiplied by the standard deviation of measured glycine standards approximating the limit of detection. The standard deviation RFU for the 25, 50, and 100 nM glycine standards was computed ($n = 36$), as these three standard concentrations were consistently measured to have similar RFU on multiple analytical days (Fig. 1). The one-tailed student *t*-test value for 35° of freedom at the 99% confidence level is 2.44. The method detection limit in RFU units was converted to bDON μM units using the slope of the standard curve in Fig. 1, yielding a value of 0.08 μM. The analytical day precision estimated from the average standard deviation of triplicate analyses is ±0.1 μM-N.

Recovery of known compounds

The recovery of amine-N as measured by our protocol was determined on three known compounds: glycine (one primary amine), NAG (one secondary amine), and a peptide gly–pro–glu (one primary amine, one secondary amine, and one tertiary amine). These compounds were chosen to represent amine and amide functional groups and parent compounds that are representative of common metabolites in seawater. Solutions of each compound were made to final concentrations of 1 μM-N in bicarbonate buffered water. Model compound solutions were measured for their bDON content and the results of analyses of each compound over three analytical days is presented in Table 1. Primary and secondary amines are well-recovered by the bDON protocol, with recoveries >95%. The recovery of amine-N from the peptide gly–pro–glu was 63 ± 10%. The close correspondence between the amine-N recovery and the theoretical primary plus secondary amine content of 66.7% in the gly–pro–glu solution suggests that tertiary amines are not recovered by the bDON method, while primary and secondary amines are recovered by the HCl hydrolysis.

Interference by nitrate ions

The presence of nitrate ions in seawater may cause degradation of amines via the nitration reaction of primary amines (especially those containing aromatic rings, e.g., phenylalanine and tyrosine) to primary nitramines in acidic media such as our acid hydrolysis reactions conditions (McAteer et al. 2016). The nitration reaction proceeds with the protonation of nitric acid, catalyzed by sulfuric acid, to form the oxidative nitronium ion (Hughes et al. 1950) that may be occurring under our reaction conditions (nitrate and sulfate ions from seawater; protons from 6 M HCl addition). The oxidative power of the nitronium ion produced under these nitration reactions can be quenched in aqueous media by the addition of an antioxidant such as AA (Halliwell 1994).

Removal of nitrate interference by AA additions

We tested for the interference of nitrate ions in the sample media on the recovery of primary amines from acid hydrolysis of known amine group containing molecules and SIO pier seawater, with and without addition of 120 μM final concentration AA as an antioxidant (Table 2). No nitrate interference was observed on the recovery of glycine, which contains primary amine groups in the absence of any aromatic ring structures, up to a tested concentration of 10 μM nitrate ion. The secondary amine group containing NAG exhibited decreasing recoveries of primary amines following hydrolysis in the presence of increasing nitrate ion concentrations from 1 to 5 to 10 μM, with ~25% recovery at 10 μM nitrate ion. Addition of AA did not clearly demonstrate quenching of amine degradation under the presence of nitrate ion at 1 and 5 μM [NO_3^-], however a near doubling of recovery was observed at 10 μM [NO_3^-] with AA added. The peptide gly–pro–glu, which contains one primary, one secondary, and one tertiary amine group did not exhibit a large interference effect from nitrate

Table 2. Results from the nitrate interference tests of standard DON molecules and SIO Pier seawater. Recovered bDON concentrations are compared against the unamended value for 1, 5, and 10 μM final concentrations of nitrate [NO_3^-] in the sample media. Additions of 0.12 mM final concentration AA to the nitrate containing samples are also compared. Error bars = ±1 SD of triplicate analyses. Tests containing an asterisk are statistically different from the unamended value at the 95% confidence interval from a two-tailed *t*-test.

	Unamended (μM N)	+1 μM [NO_3^-] (μM N)	+1 μM [NO_3^-] + AA (μM N)	+5 μM [NO_3^-] (μM N)	+5 μM [NO_3^-] + AA (μM N)	+10 μM [NO_3^-] (μM N)	+10 μM [NO_3^-] + AA (μM N)
Glycine	0.94 ± 0.08	0.89 ± 0.09	0.95 ± 0.09	0.99 ± 0.02	0.92 ± 0.11	0.86 ± 0.09	0.96 ± 0.10
NAG	0.97 ± 0.05	0.85 ± 0.28	0.68 ± 0.19	0.80 ± 0.28	0.74 ± 0.21	0.26 ± 0.22*	0.90 ± 0.26
Peptide	0.68 ± 0.12	0.72 ± 0.27	0.68 ± 0.24	0.55 ± 0.22	0.43 ± 0.08*	0.44 ± 0.06*	0.64 ± 0.02
SIO pier SW	1.82 ± 0.13	1.84 ± 0.09	0.93 ± 0.24*	1.77 ± 0.03	1.50 ± 0.27	0.17 ± 0.13*	1.66 ± 0.08

Table 3. Measured bDON concentrations on SIO pier seawater and bicarbonate buffered (pH = 8.3) water following additions of 1 and 5 μM ammonium ion.

	Unamended (μM N)	+1 μM [NH_4^+] (μM N)	+5 μM [NH_4^+] (μM N)
SIO pier SW	0.57 ± 0.12	0.48 ± 0.06	0.60 ± 0.13
pH 8.3 buffered water	BDL	0.12 ± 0.01	0.08 ± 0.01

ion. Recoveries as primary amine following acid hydrolysis of the peptide were similar to nitrate free media within standard error across the three tested nitrate ion concentrations, with or without the AA additions. Nitrate concentrations up to 5 μM did not demonstrate an appreciable effect on the recovered primary amine concentration following acid hydrolysis of SIO pier seawater. At 1 and 5 μM nitrate ion concentrations, the addition of AA appeared to have a small detrimental effect on the ability to accurately measure the primary amine content of hydrolyzed seawater. However, at 10 μM [NO_3^-], the recovery of primary amine from SIO pier seawater was reduced by ~90%. This interference was nearly completely eliminated by the AA addition, producing a recovery ~10% lower than the expected primary amine concentration as measured by our protocol.

Potential interference by ammonium ions

A fluorometric method using OPA is also employed to measure dissolved ammonium ion concentrations in natural waters (Holmes et al. 1999). In the ammonium method, sodium sulfite is added to the OPA reagent in lieu of 2-mercaptoethanol, making the protocol sensitive to ammonium only, excluding sensitivity to primary amines (Holmes et al. 1999). We confirmed that the bDON protocol using the OPA and 2-mercaptoethanol reagent was insensitive to ammonium ion concentrations in additions of 1 and 5 μM final concentration of ammonium to SIO pier seawater (Table 3), with the measured concentrations following ammonium additions matching unamended SIO pier seawater within analytical error.

Potential interference by production of melanoidin compounds

Sugars and amino acids are known to condense and form polymers called melanoidins at neutral pH (Maillard 1913; Hedges 1978). As polysaccharides are ubiquitous in seawater, we tested for the potential reduction in yield of expected [bDON] from solutions containing model compounds under the reaction conditions of 6 M HCl hydrolysis which should produce sugar and amino acid monomers, albeit at acidic pH, which might mitigate melanoidin production. Multiple potential melanoidin production tests were performed: solutions containing glucose with glutamic acid (acidic amino acid), valine (neutral amino acid), or lysine (basic amino acid); a trisaccharide (maltotriose) with tripeptide (gly–pro–glu) or tripeptide (gly–gly–gly); and a dipeptide (gly–glu) with a trisaccharide (maltotriose). Solutions were made up in 0.1 M bicarbonate buffered water to a final concentration of 1 μM glucose with 1 μM amino acid, 1 μM maltotriose with 1 μM gly–pro–glu, 2 μM gly–gly–gly with and without 2 μM maltotriose, and 2 μM gly–glu with and without 2 μM maltotriose. All solutions were measured in triplicate and tested with and without the

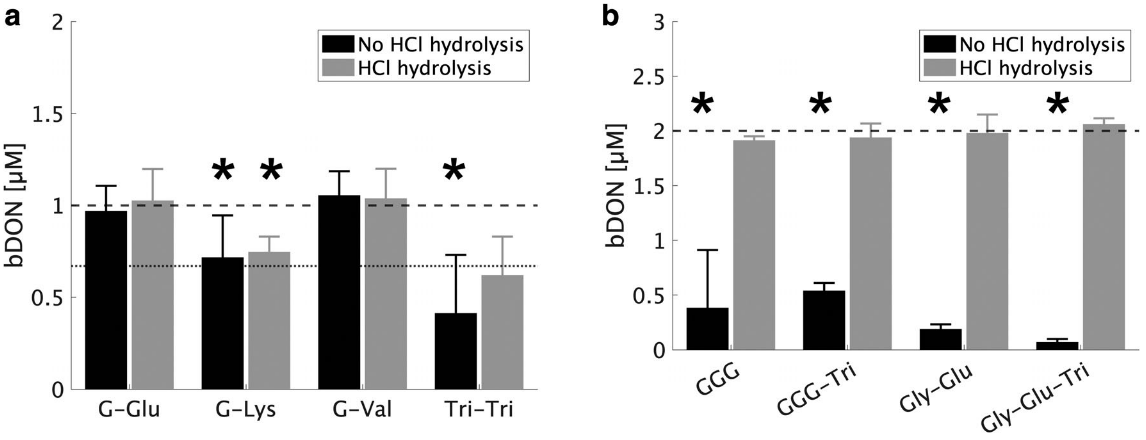

Fig. 2. Results from tests to investigate potential production of melanoidin compounds. (**a**) Glucose and amino acid tests. G–Glu = glucose + glutamic acid; G–Lys = glucose + lysine; G–Val = glucose + valine; Tri-Tri = trisaccharide (maltotriose) + tripeptide (gly–pro–glu). Dashed black line is expected [bDON] in amino acid tests. Dotted black line is expected [bDON] in tripeptide test after acid hydrolysis. Measured [bDON] are compared without (blue) and with (pale blue) 6 M HCl hydrolysis. (**b**) Trisaccharide and dipeptide and tripeptide tests. GGG = gly–gly–gly; GGG-Tri = gly–gly–gly + trisaccharide (maltotriose); Gly–Glu = gly–glu; Gly–Glu–Tri = gly–glu + trisacchride (maltotriose). Dashed black line is expected [bDON] in all peptide tests after acid hydrolysis. Error bars in (a and b) are ±1 SD of triplicate analyses. Bars containing an asterisk are statistically different from the expected value at the 95% confidence interval from a two-tailed *t*-test.

Table 4. SIO pier seawater dissolved N pools.

SIO pier SW	TDN (μM)	$NO_3^- + NO_2^-$ (μM)	NH_4^+ (μM)	DON (μM)	bDON (μM)	Frac bDON
S1 (Jan 2015)	6.27 ± 0.54	0.41 ± 0.13	0.66 ± 0.17	5.21 ± 0.58	0.82 ± 0.08	0.16 ± 0.03
S2 (May 2015)	8.99 ± 0.47	0.28 ± 0.03	0.56 ± 0.12	8.15 ± 0.49	1.63 ± 0.10	0.20 ± 0.02
S3 (Apr 2017)	12.13 ± 0.59	1.86 ± 0.15	0.63 ± 0.15	9.64 ± 0.63	1.82 ± 0.11	0.19 ± 0.02

6 M HCl hydrolysis followed by evaporation and water reconstitution protocol.

Only the glucose–lysine solutions presented evidence of reduced [bDON] yield (~73%, Fig. 2), similar to previous investigations by Hedges (1978) who found faster melanoidin production kinetics and overall yield for glucose–lysine sourced melanoidins when comparing the same three amino acids tested here. Measured [bDON] yields in the tripeptide and dipeptide solutions tested were significantly lower (~10–60% of expected) without 6 M HCl hydrolysis; however ~100% yield was recovered within measurement error following acid hydrolysis, confirming the validity of the bDON method for recovering short chain peptides as primary amines in the presence of polysaccharides.

bDON concentrations in seawater collected from the SIO pier

Seawater was collected from the SIO Pier on three separate occasions (S1 = Jan 2015; S2 = May 2015; S3 = Apr 2017) and measured over multiple analytical days to assess its bDON concentration and associated day-to-day analytical variability. The precision of [bDON] measured over multiple analytical days was similar to the within analytical day precision of triplicate analyses, i.e., ±0.1 μM-N (Table 4). In addition to [bDON], TDN, nitrate + nitrite, and ammonium concentrations were measured on S1–S3 to compute the DON concentrations. The DON concentration of SIO Pier seawater was lowest from water collected in January 2015 ($S1 = 5.21 \pm 0.58\ \mu$M), highest within water collected from April 2017 ($S3 = 9.64 \pm 0.63\ \mu$M), and intermediate in concentration within water collected from May 2015 ($S2 = 8.15 \pm 0.49\ \mu$M) (Table 4). The measured bDON concentrations followed the same pattern with ($S1 = 0.82 \pm 0.08\ \mu$M; $S3 = 1.82 \pm 0.11\ \mu$M; $S2 = 1.63 \pm 0.10\ \mu$M). Computed as a fraction, the amidic bDON content of DON fell within a narrow range of 0.16–0.20 ± 0.02 across the three samplings of SIO Pier seawater (Table 4).

bDON concentrations in seawater from the Southern California bight

The bDON concentration of seawater following 6 M HCl hydrolysis was determined on water column profiles in the upper 200 m, collected by CTD/Rosette at 10 stations aboard a cruise to the Southern California Bight in July–August 2015 (Fig. 3). The stations covered a gradient in surface ocean chlorophyll *a* (Chl *a*) concentration (Fig. 3a) within recently upwelled waters near point concepcion. Nitrate + nitrite concentrations were typically >5 μM at depths >80 m across the transect; AA at 0.12 mM final concentration was added to samples collected from ≥80 m prior to bDON analysis. bDON concentrations were highest in near surface waters (<25 m), ranging from 0.54 to 1.08 μM (Fig. 3b). The highest bDON concentration near surface waters were located toward the

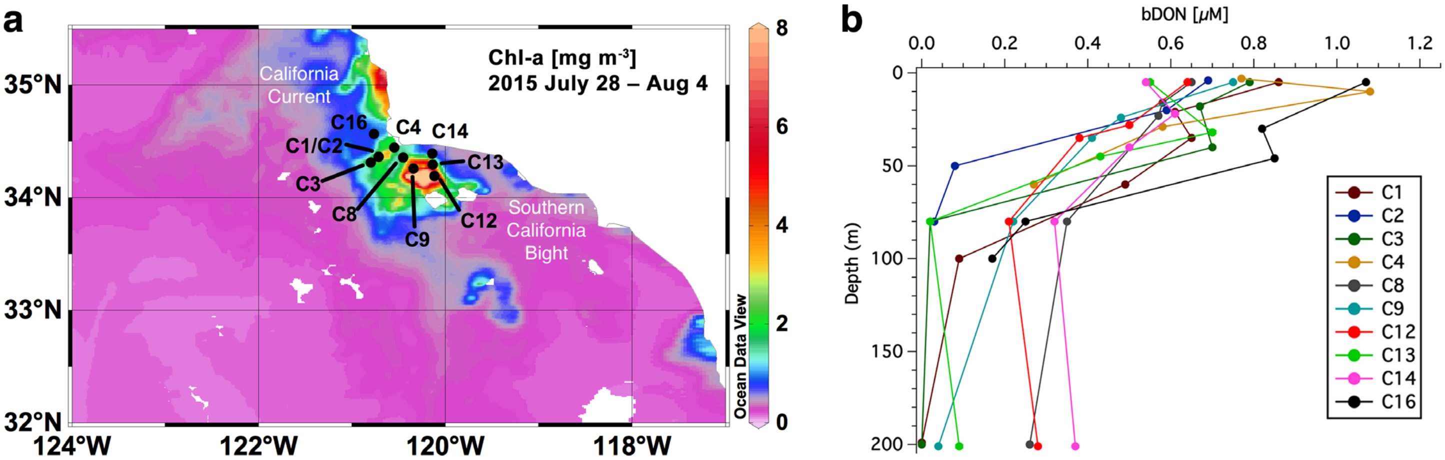

Fig. 3. (**a**) Map of the sampling stations in the California Current and Southern California Bight. Chl *a* concentration (mg m^{-3}) at the time of sampling from the MODIS-Aqua ocean color satellite is shown. (**b**) Depth profiles of bDON (μM) for 10 stations in the California Current during July–Aug 2015. bDON error bars are ± 0.1 μM and are omitted for clarity.

north (Sta. C1 and C16), near point concepcion, with the lowest near surface bDON concentrations located to the south and east (Sta. C12–C14), moving from California Current waters into the Southern California Bight. bDON concentrations decreased with depth and were lowest below the euphotic zone depth (~70 m), ranging from at or slightly below the detection limit (~20–80 nM) to 0.37 μM within the 100–200 m depth horizon.

bDON concentrations from the 10 stations in the Southern California Bight are plotted vs. in situ measured Chl *a* (Fig. 4a) and [DOC] (Fig. 4b). [bDON] and Chl *a* are positively correlated between chlorophyll concentrations of 0–1 mg m^{-3} (Fig. 4a). In general, higher bDON concentrations (>0.5 μM) are observed at Chl *a* concentrations >1 mg m^{-3}; however, a positive correlation is lacking. Similarly, bDON and DOC concentrations are positively correlated between [DOC] of 46 and 78 μM, with the highest bDON concentrations (~1.1 μM) found at [DOC] between 72 and 77 μM (Fig. 4b). This positive correlation breaks down at [DOC] above ~80 μM, however [bDON] at these higher DOC concentrations is still elevated, ranging from 0.55 to 0.75 μM bDON.

bDON concentrations in seawater from station ALOHA

The amidic bDON, DON, and nitrate + nitrite (NO_3^-) concentrations of seawater were determined on a water column profile collected from the upper 300 m at Station ALOHA (22.75°N 158°W) on Jan 30, 2020 (Fig. 5). NO_3^- was below the detection limit of 100 nM within the upper 100 m, which coincided with the depth of the surface wind-mixed layer at the time of sampling. NO_3^- concentrations increased from 0.36 μM at 125 m to 11.25 μM at 300 m (Fig. 5a). DON concentrations and its depth profile were analogous to others in the Hawaiian Ocean Time-series program (Karl et al. 2001), with concentrations of ~5.2 μM observed in the upper 75 m that decreased with depth to values of ~3.5 μM by 175 m and ~3.1 μM at 300 m (Fig. 5a). Similar to DON, the bDON depth

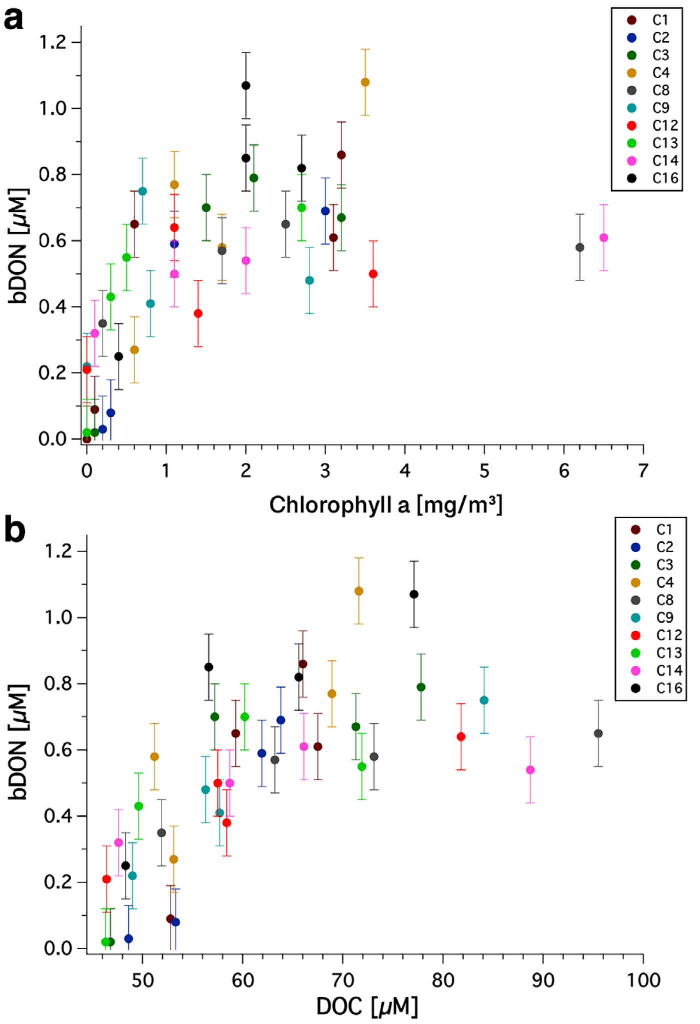

Fig. 4. bDON (μM) plotted against (**a**) bottle measured Chl *a* (mg m^{-3}) and (**b**) DOC (μM) for the 10 stations in the California Current and Southern California Bight from July to Aug 2015.

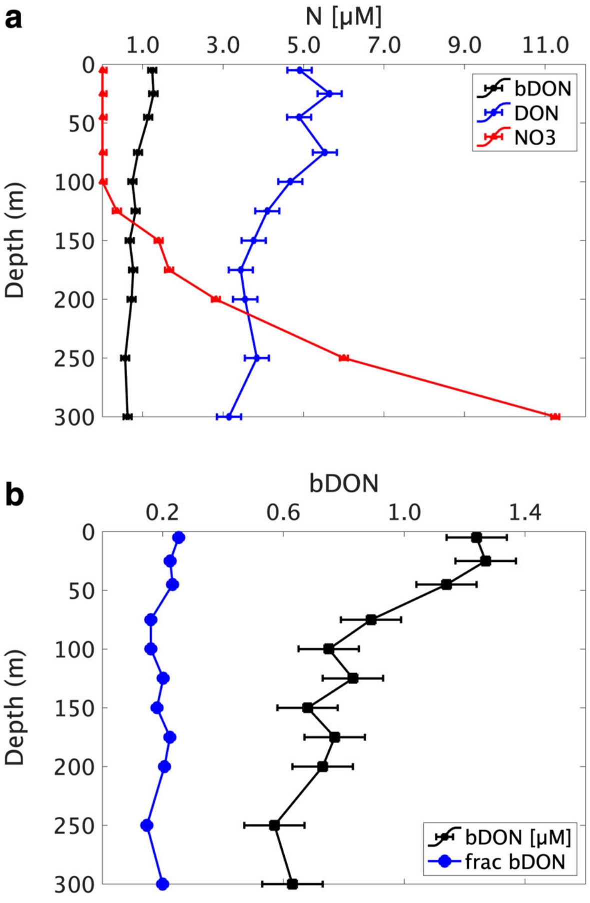

Fig. 5. (**a**) Depth profiles for nitrate + nitrite (NO_3) μM (red), DON μM (blue), and bDON μM (black) within the upper 300 m at Station ALOHA. (**b**) Depth profile of bDON μM (black) as in (a) with a rescaled x-axis to better illustrate bDON variability. The fraction of DON measured as bDON is plotted in blue.

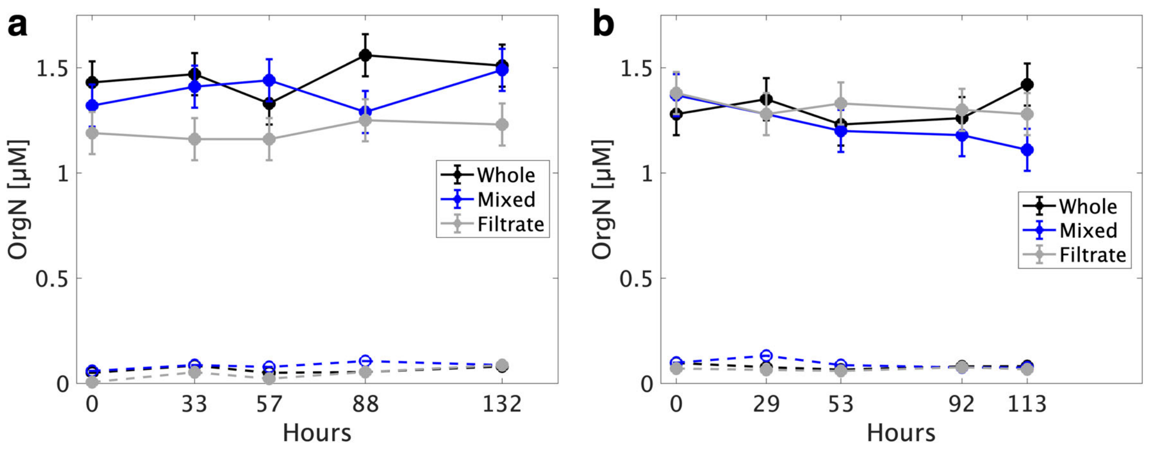

Fig. 6. bDON [μM] (solid lines) and bacterial biomass N (dashed lines) changes within seawater incubations initiated at Station ALOHA in June 2021 (whole water treatment = black lines; Mixed treatment = blue lines; Filtrate treatment = gray lines). (**a**) Incubations conducted with seawater from 5 m depth collected on June 6, 2021; in situ [DON] = 4.47 μM. (**b**) Incubations conducted with seawater from 125 m depth collected on June 7, 2021; in situ [DON] = 4.21 μM. Error bars omitted on bacterial biomass N for clarity.

profile exhibited elevated concentrations within the upper 45 m, in the narrow range 1.1–1.25 μM, that decreased with depth to values of ~0.7–0.9 μM at depths of 75–200 m, and finally to values of ~0.6 μM in the depth range 200–300 m (Fig. 5b). The fraction of bulk DON that was measured as bDON after 6 M acid hydrolysis (e.g., bDON/DON) was 0.23–0.25 in the upper 45 m and averaged 0.18 ± 0.02 below (Fig. 5b).

bDON consumption in seawater incubations conducted at station ALOHA

Initial [bDON] in the 5 m experiment *Whole* treatment was 1.46 ± 0.09 μM which represented ~33% of the in situ [DON] = 4.47 μM. Both [bDON] and bacterial N were largely invariant across all three treatments within the 5 m experiment over the course of 132 h suggestive of minimal bacterial growth and associated bDON consumption (Fig. 6a). Initial [bDON] in the 125 m experiment *Whole* treatment was 1.28 ± 0.11 μM, which represented ~30% of the in situ [DON] = 4.21 μM. Similar to the 5 m experiment, [bDON] and bacterial N were largely invariant in the *Whole* and *Filtrate* treatments of the 125 m experiment, with some evidence of bDON production between the final two time points in the *Whole* treatment (Fig. 6b). However, [bDON] is significantly lower, 1.11 ± 0.08 μM, than the initial value 1.37 ± 0.10 μM (*t*-test, $p < 0.008$) after 113 h of incubation in the *Mixed* treatment of the 125 m experiment (blue line, Fig. 6b), delta [bDON] = 0.26 ± 0.14 μM, indicating net bDON consumption over 4.7 d at a rate of 0.05 ± 0.01 $\mu\text{M d}^{-1}$ ($p < 0.007$). Bacterial N increased 34 nM (+ 35%) between the initial and second time points in the *Mixed* treatment of the 125 m experiment, which explains ~13% of the observed bDON consumption. Nitrate accumulation was not observed in any of the treatments in both experiments (not shown). The remainder of remineralized bDON likely accumulated within the ammonium pool or potentially the particulate N pool which were not measured.

Discussion

The presented amidic bDON quantification method exhibits excellent recovery of DON molecules containing primary and secondary amide N atoms representative of marine metabolites (e.g., glycine and NAG), however tertiary amide N atoms do not appear to be recovered by the method. Thus, the method may underestimate total amidic bDON concentrations of seawater if a portion of tertiary amide N containing DON molecules are bioavailable to marine microbes. We confirmed that the method is insensitive to ammonium ion concentrations present within the seawater sample. However, a potential interference from the presence of nitrate ions at concentrations >5 μM was identified, presumably via the production of the nitronium ion, which can destroy a portion of the primary amines generated under the acidic reaction conditions. Addition of the antioxidant AA appeared to inhibit this side reaction, restoring quantification of bDON concentrations within samples when comparing nitrate free and nitrate >5 μM solutions. Potential interference by production of melanoidins (sugar + amino acid polymers) under the method reaction conditions was also investigated, identifying a ~25% under-recovery when a basic amino acid (lysine) and sugar monomer (glucose) were present in the media. Some evidence for melanoidin production was also observed involving dipeptides or tripeptides with trisaccharide under neutral pH conditions, however quantitative recovery of liberated primary amine was found after the acid hydrolysis protocol was applied.

Considering these caveats, we can now focus on the observed amidic bDON trends. bDON concentrations measured within the southern California Current and Southern California Bight were highest in euphotic zone waters and decreased with depth within the mesopelagic zone in accordance with known depth patterns of the bulk DON concentration and the proteinaceous and NAAP enriched HMW DON in marine waters (Aluwihare et al. 2005; Letscher et al. 2013*a*). bDON concentrations were positively correlated with Chl *a* and DOC concentrations (Fig. 4), suggesting similar biogeochemical processes are responsible for the production and accumulation of DOC, bDON, and Chl *a*—presumably net community production of organic matter sustained by autotrophs in the upwelling regime (Hansell and Carlson 1998) investigated in our study. Disappearance of bDON with depth below 100–200 m in the Southern California Bight and at Station ALOHA suggests that under reduced or absent net community production bDON does not accumulate. This observation is consistent with rapid removal in subducted and aged water masses, presumably due to heterotrophic consumption of amidic bDON although abiotic processes cannot be ruled out by our dataset (e.g., adsorption onto particles).

Similar fractions of DON concentrations measured as amidic bDON (bDON/DON) were found for waters collected at the SIO Pier (0.16–0.20) and the upper 300 m at Station ALOHA (0.15–0.25). In the SIO Pier data, bulk DON variability across seasons is $\pm 2.25\,\mu$M and that for bDON is $\pm 0.53\,\mu$M. Thus, we estimate that a comparable ~24% of the bulk DON seasonal variability is captured by amidic bDON variability as defined by our analytical protocol. In terms of variability with depth, we note that bDON fractions were elevated in the upper 45 m at Station ALOHA (0.23–0.25) and systematically lower at depth (0.15–0.20; mean 0.18) in support of the notion that the 6 M acid hydrolysis we present here as a protocol to measure amidic "bioavailable" DON does in fact capture a portion of the DON pool that is biologically remineralized with depth. The overall loss of bulk DON from near surface waters to 300 m depth measured at ALOHA in Jan 2020 was 1.7 μM N (5.2 – 3.5 μM N). The decrease in bDON concentrations over the same depth interval is 0.6 μM (1.2 – 0.6 μM). Thus, on the order of 35% (0.6/1.7) of the net DON remineralization with depth can be explained as the loss of the amidic bDON captured by our protocol with the caveat that 300 m water at ALOHA is supplied laterally to the site rather than sourced from overlying surface waters. Bacterial N demand is low in the subtropical North Pacific, on the order of ~0.1 nM N d^{-1} (Church et al. 2004), and is thus unlikely to contribute significantly to the remaining unexplained DON and bDON net remineralization quantity with depth at ALOHA. The chemical composition of the remaining ~65% of DON molecules that are not captured by our method but are remineralized between the surface and depth could include nonacid labile amides (McCarthy et al. 1997; Aluwihare et al. 2005), also tertiary amines, quaternary amines like glycine betaine and choline (Boysen et al. 2022), and aromatic N, including amino acids and pigments.

Confirmation that compounds captured by our amidic bDON assay are bioavailable was provided by seawater incubations performed with water collected from 125 m at Station ALOHA in the subtropical North Pacific when the heterotrophic microbial community was released from grazing pressure (*Mixed* treatment). However, a similar amidic bDON consumption was not observed for the analogous *Mixed* treatment performed at 5 m. Similar results have been obtained for DOM consumption incubations performed in other subtropical locations, i.e., nil DOC and/or DON consumption in near surface waters and measurable consumption within the deeper euphotic zone to upper mesopelagic depths (Carlson et al. 2004; Letscher et al. 2013*a*, 2015*a*). Genomic data from these studies demonstrated the distinctness of the heterotrophic microbial communities found between the surface and subsurface, similar to what has been found at the BATS site in the subtropical North Atlantic (Treusch et al. 2009), implicating this as a potential cause of the divergent results for the same DOM substrate supplied to microbial communities across depth. The magnitude of bDON consumption in the 125 m *Mixed* treatment, 0.26 μM N, represents consumption of 19% of the initial [bDON] present in the experiment over the course of 4.7 d. The incubations were terminated due to cruise logistic constraints so it is not known if [bDON] would have been continued to be drawn down if the experiments were carried out longer. Regardless, the ~0.25 μM quantity of bDON consumed represents ~50–100% of the observed lateral gradients in upper ocean (0 – 100 m) bulk DON (Letscher et al., 2013*a*, 2015*b*, 2016), supporting the relevance of the presented amidic bDON method as a tool to investigate the role of DON as an organic nutrient supporting net community production across ocean basin scales. In support of this, we estimate the first order kinetic rate constant for amidic bDON consumption at $k = 0.045\ d^{-1}$ from the 125 m experiment or a mean lifetime of ~22 d, allowing for its lateral export away from DON accumulation regions (e.g., upwelling systems) toward more oligotrophic systems. This is a minimum estimate of the bDON lifetime as we note that grazing pressure, absent from the *Mixed* treatment but present in situ, should slow the microbial uptake of bDON. Marine DON vertical gradients are larger, i.e., ~0.5–2 μM, and are likely explained by a combined consumption of the amidic bDON quantified by the presented method, nonacid labile amides, and LMW DON molecules, which may or may not contain proteinaceous material (Ianiri et al. 2022) and appear to cycle distinctly from the DON (Broek et al. 2019) targeted by the presented amidic bDON method. The bioavailability of the DON, which is potentially "missed" by the presented chemical method, requires further investigation.

Conclusions and recommendations

We have developed a sensitive, simple, and feasible technique for quantifying amidic bDON in natural seawater. The method is cost-effective, has high sample throughput, and is suitable for shipboard measurements. The analytical precision of the amidic bDON method reported here, ± 0.1 μM, represents a significant improvement over the reported precision of determinations of the bulk marine DON concentrations using the traditional methods of UV-peroxide, persulfate, and high temperature oxidations when determining TDN, which are typically within the range of ± 0.2–1.0 μM (Sharp et al. 2002; Knapp et al. 2011; Letscher et al. 2013*a*; Landolfi et al. 2016; Foreman et al. 2019). Due to the potential destruction of primary amines from the presence of nitrate ions, we recommend addition of an antioxidant such as AA to sample analyses in which the expected nitrate concentration exceeds 5 μM. Hydrolysis of proteinaceous and NAAP material that results in primary amine products with a basic pH such as lysine are likely underestimated by the presented technique due to the formation of melanoidin like compounds. Further improvements to the precision of the amidic bDON method may be possible by the introduction of automated steps in the protocol such as all transfers of the analyte between vessels, e.g., sample bottle, hydrolysis ampoule, centrifuge tube, fluorometric cell, etc.

Using the new technique, we estimated amidic bDON concentrations of 0.1–1.8 μM in the upper 300 m of the southern California Current and Station ALOHA in the subtropical North Pacific, representing ~15–25% of the bulk DON concentration. We are confident in assigning the bDON definition to this operationally defined fraction because we definitively show that it varies seasonally with indicators of productivity, decreases with depth, and decreases with DON consumption in incubation experiments. This measured bDON fraction and its measured consumption within days in incubation, is in close correspondence with that estimated by lateral gradients in bulk DON concentration across ocean basins (e.g. ~0.5 μM removed from a ~4.5 μM surface ocean pool; Letscher et al. 2013*a*), suggesting that the presented amidic bDON method may quantify a significant fraction of the bioavailable component of the marine DON pool.

Future applications of this assay should follow a laterally advected water mass to directly examine removal rates of bDON and correlate with parameters of bacterial N production to better understand the partitioning of the consumed N, as well as with changes in microbial metabolism to identify the pathways mediating this removal. This higher throughput bDON assay with some capacity to provide insights on the composition of reactive DON, was developed to be implemented in studies that sample at the high spatial and temporal frequencies that are necessary to capture the dynamics of the labile and semi-labile DON cycling in the upper ocean. Where possible these assays should be combined with other methods aimed at deconvoluting DON composition so that we can better identify compounds that are both visible to our amidic bDON assay and invisible to the assay but dynamic (i.e., bioavailable) over the same time scale.

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Conflict of Interest

None declared.

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