OCEANOGRAPHY

Special delivery of proteinaceous matter to deep-sea microbes

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Earth's deep ocean holds a vast reservoir of dissolved organic carbon, traditionally considered old and resistant to microbial degradation. Radiocarbon analyses indicate the hidden occurrence of younger dissolved organic carbon components, assumed to be accessible to deep-sea microorganisms but not yet demonstrated. Using compound-class radiocarbon analysis, molecular characterization, and bioassay experiments, we provide direct evidence for rapid microbial utilization of young, labile, high-molecular weight proteinaceous material in bathypelagic waters. The abundance of labile proteinaceous material diminishes from epipelagic to mesopelagic waters but notably increases in bathypelagic waters, where it exhibits a short turnover time (days) and resembles surface plankton in molecular composition. This observation coincides with peak zooplankton biomass recorded over the year. The nonmonotonic depth trend suggests a deep-sea replenishment of organic particles from mesopelagic migrating zooplankton. Our results indicate the presence of labile organic molecules at bathypelagic depths and reveal a nonlinear supply of plankton-derived substrates that support microbial metabolism and carbon sequestration in the deep ocean.

INTRODUCTION

Phytoplankton and its sinking biomass fuel pelagic and benthic marine food webs. Most organic matter derived from phytoplankton is swiftly remineralized to carbon dioxide and nutrients in the upper ocean. About 10 to 20% of this organic matter escapes microbial utilization in surface waters and is transported as particulate and dissolved organic matter to mesopelagic waters (200 to 1000 m) (1, 2). The meager leftovers of fresh organic matter from the upper ocean enter the dark and cold waters of the deep ocean (>1000 m), where radiocarbon (14 C) analysis of dissolved organic carbon (DOC) indicates its persistence for thousands of years (3, 4). Chemical characterizations of deep-sea DOC reveal a paucity of common biochemicals that can be readily used by microbes (5, 6), and incubation experiments commonly fail to detect measurable microbial utilization of deep-sea DOC (7–9), indicating its refractory nature.

Despite these observations of refractory organic matter, a diverse community of biota persists in the deep ocean (10, 11). Addressing the apparent imbalance between deep-sea microbial carbon demand and supply has been a major focus of research for decades (12–15). A critical insight toward resolving this conundrum has been the observation from radiocarbon analyses of variability in deep-sea DOC ¹⁴C content, suggesting the occurrence of young DOC fractions hidden within the bulk DOC reservoir (16–19). These young fractions were hypothesized to originate from the solubilization of surface-derived sinking particles and assumed to be available to deep-sea microbial communities. Here, we use bulk and compound-class radiocarbon analysis, molecular characterization,

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and bioassay experiments to provide direct evidence for the rapid microbial utilization of labile proteinaceous material in bathypelagic waters. The depth pattern of microbial utilization suggests a replenishment of substrates to bathypelagic waters from mesopelagic zooplankton.

RESULTS AND DISCUSSION

Biological dynamics in the upper ocean

We conducted field sampling at the Bermuda Atlantic Time-series Study (BATS) site in the Northwest Atlantic Ocean in May 2016. This region exhibits pronounced seasonal variability in physical mixing, nutrient availability, and plankton dynamics (20). Chlorophyll a concentrations showed clear seasonal and interannual patterns, with maxima typically occurring in early spring (March to May) at depths of 50 to 100 m (Fig. 1A). Zooplankton biomass in the upper 200 m followed similar temporal trends and reached its highest levels during spring chlorophyll maxima (Fig. 1B). Zooplankton size distribution varied with total biomass (Spearman's r = 0.43, P < 0.05), with larger individuals (>1000 µm) being more prominent during periods of elevated biomass (Fig. 1C). Our survey occurred shortly after the spring bloom in 2016 and coincided with the maximum in zooplankton biomass (red dashed box in Fig. 1).

Variable aging of DOC in the ocean

Water samples for the determination of radiocarbon content of DOC were collected from epipelagic (2 m), mesopelagic (400 m), and bathypelagic (2500 m) waters. Major water masses identified in these layers included the North Atlantic Central Water (samples at 2 and 400 m) and the North Atlantic Deep Water (NADW; samples at 2500 m; fig. S1). Bulk DOC exhibited decreasing radiocarbon content (¹⁴C) with increasing depth (Fig. 2) (21), with the average age doubling from epipelagic ($-186 \pm 4\%$; 1590 ± 20 ¹⁴C years) to mesopelagic ($-326 \pm 4\%$; 3105 ± 20 ¹⁴C years) and bathypelagic ($-392 \pm 4\%$; 3930 ± 25 ¹⁴C years) waters. These ¹⁴C trends are comparable to previous observations in the North Atlantic Ocean (3, 22), suggesting the aging of DOC with depth and the abundance of "old"

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Fig. 1. Overview of chlorophyll a concentrations and zooplankton biomass at the BATS site during 2015–2016. The reported data include chlorophyll a (Chl-a) concentrations in the upper 300 m of the water column (**A**), total zooplankton dry weight integrated from 0 to 200 m (**B**), and the size distribution of zooplankton in the upper 200 m (**C**). Zooplankton data shown in (B) and (C) included samples collected during both day and night. The red solid line represents the moving average of zooplankton biomass. The data were obtained from http://bats.bios.edu/bats-data/. This study was conducted between 3 and 11 May 2016, as highlighted in the red dashed box.

DOC in the deep Atlantic Ocean. Old apparent ¹⁴C ages have been commonly measured in DOC at depths greater than 1000 m throughout the global ocean (3, 4, 16, 22). These old ages, coupled with the observed lack of microbial utilization of bulk DOC in deep waters (7–9), contribute to the prevailing view of a vast, slow-cycling DOC reservoir in the deep ocean (23).

We used tangential-flow ultrafiltration to collect high–molecular weight (HMW; >2.5 kDa) DOC, which is known to be more bioavailable than bulk DOC (24). The Δ^{14} C values of HMW DOC ranged from -64 ± 3 to -265 ± 3 per mil (‰) and were consistently more enriched (younger; by 1060 to 1600 ¹⁴C years) than those of bulk DOC at all depths (paired *t* test, *P* < 0.01, *n* = 3; Fig. 2 and table S1) (21), indicating the presence of younger HMW DOC within the total DOC pool throughout the water column. This observation is consistent with previous radiocarbon analyses of different chemical and size fractions of the DOC reservoir, indicating age diversity within the DOC reservoir (17–19, 25–28). Overall, these radiocarbon characteristics of seawater DOC suggest that the DOC pool comprises diverse organic components with variable ages and turnover times.

To gain further insights into the molecular level variability of DOC cycling, we isolated an amino acid fraction from HMW DOC using hydrolysis and liquid chromatography. The Δ^{14} C values of the HMW amino acid fraction in epipelagic ($-34 \pm 9\%$; 210 ± 80^{14} C years), mesopelagic ($-117 \pm 11\%$; 940 ± 100^{14} C years), and bathypelagic ($-142 \pm 15\%$; 1170 ± 140^{14} C years) waters were in general more enriched (i.e., younger) compared to their respective HMW DOC fractions (paired *t* test, *P* = 0.13, *n* = 3), on average 320 to 1300¹⁴C years younger (Fig. 2 and table S1). In comparison, an HMW protein-like fraction extracted previously in mesopelagic



Fig. 2. Changes in Δ^{14} C values and ¹⁴C ages of different fractions of dissolved organic matter with depth at BATS. Bulk DOC, HMW DOC (>2.5 kDa), and HMW amino acids were collected in epipelagic (2 m), mesopelagic (400 m), and bathypelagic (2500 m) waters at BATS in May 2016. One sample was collected from each depth for analysis of bulk and compound-class ¹⁴C contents due to large sample volume requirements (1000 to 4000 liters). The error bar represents the propagated total analytical error.

 $(\Delta^{14}C, -190\% \text{ at } 850 \text{ m})$ and bathypelagic $(\Delta^{14}C, -215\% \text{ at } 1500 \text{ m})$ waters in the Sargasso Sea (*17*) were 750 to 780 ¹⁴C years older than the HMW amino acid fraction extracted in the present study. This age difference, spanning several centuries, was unlikely attributable to the time of sampling between studies. Instead, the more targeted purification and recovery of amino acids via liquid chromatography could account for the younger ages in this study compared to the operationally defined protein-like material isolated previous-ly. Differences in the molecular weight cutoff of the ultrafiltration membranes used in the present (2.5 kDa) versus previous (1.0 kDa) studies could also play a role, as larger molecules are generally considered more bioavailable (*6*, *29*).

An important feature of the compound-class ¹⁴C data is that HMW amino acids did not demonstrate a consistent aging trend with depth, unlike bulk DOC or HMW DOC (Fig. 2). Specifically, ¹⁴C ages in bulk DOC increased significantly by 825 ¹⁴C years from 400 to 2500 m (*Z* test, *Z* = 25.8, *P* < 0.001), while HMW DOC ¹⁴C ages increased by 970 ¹⁴C years over the same depth range (*Z* test, *Z* = 22.9, *P* < 0.001). In contrast, the ¹⁴C ages of HMW amino acids showed no significant difference between 400 m (940 ± 100 ¹⁴C years) and 2500 m (1170 ± 140 ¹⁴C years) (*Z* test, *Z* = 1.34, *P* > 0.1). This minimal depth variation in HMW amino acid ¹⁴C ages from mesopelagic to bathypelagic waters sharply contrasts with the substantial aging observed in both bulk DOC and HMW DOC (Fig. 2 and table S1).

The observation of relatively young HMW amino acids at 2500 m suggests the presence of recently produced proteinaceous material in the deep ocean. Previous radiocarbon analyses conducted in major ocean basins showed temporal and spatial variability in deepsea bulk DOC ¹⁴C content that suggests the occurrence of younger DOC fractions (16, 19, 22, 30). Our compound-class ¹⁴C data offer more nuanced compositional evidence supporting these earlier radiocarbon measurements. Notably, while younger amino acids were detected at depth, they were not reflected in the bulk DOC or HMW DOC ¹⁴C data. This is because amino acids constitute only a small fraction (~1%) of the bulk or HMW DOC, which, in turn, makes amino acid-based assessments much more sensitive than those of bulk analysis. Overall, the radiocarbon isotopic characterizations provide direct evidence of young, fast-cycling DOC fractions in the deep ocean. However, they also raise an important question: Are these younger DOC components bioavailable to deep-sea microorganisms?

Evidence of DOC utilization in bathypelagic waters

We conducted bioassay experiments to directly measure the microbial utilization of HMW DOC with microbial inocula collected from the corresponding depths. Microbial utilization of HMW DOC was evident across all depths (Fig. 3, D to F, and table S2). The percentage of HMW DOC used during 390-day incubations decreased from $10 \pm 1\%$ in epipelagic waters to $5 \pm 1\%$ in mesopelagic waters (two-sample *t* test, P < 0.01, n = 3) and to $3 \pm 1\%$ in bathypelagic waters (two-sample *t* test, P < 0.05, n = 3). For comparison, the percentage utilization of bulk DOC decreased from 3 to 6% in epipelagic and mesopelagic waters to an undetectable level (~0%) in bathypelagic waters (Fig. 3, A to C, and table S2). Despite being relatively modest, the observed microbial utilization of HMW DOC at a depth of 2500 m demonstrates the variable bioavailability of DOC fractions in bathypelagic waters.

In contrast, hydrolyzable combined amino acids released from HMW DOC exhibited pronounced microbial utilization throughout the water column (Fig. 3, G to I). About half of this utilization occurred during the first week of incubation, with the remainder being degraded at lower rates over the course of a year (Fig. 3, G to I, and table S2). Components that undergo utilization over timescales from days to weeks and from months to years are typically classified as labile and semilabile, respectively (*31, 32*). Consequently, the HMW DOC from epipelagic to bathypelagic waters contained both labile and semilabile combined amino acids.

The relative abundance of HMW labile and semilabile combined amino acids showed an unexpected depth-related trend (Fig. 4), decreasing from $24 \pm 2\%$ at 2 m to $8 \pm 1\%$ at 400 m (two-sample *t* test, P < 0.01, n = 3) and then increasing to $13 \pm 2\%$ at 2500 m (twosample *t* test, P < 0.05, n = 3) (Fig. 4). To account for the potential effects of prolonged incubation periods (390 days) on microbial activity, we also evaluated the early stage of incubation (i.e., 9 days). The results indicated a similar depth trend in HMW amino acid utilization, increasing from $5 \pm 1\%$ at 400 m to $8 \pm 1\%$ at 2500 m (two-sample *t* test, P < 0.05, n = 3; fig. S2 and table S2). The elevated bioavailability of HMW combined amino acids in bathypelagic waters is an interesting observation, and it is consistent with the relatively young ¹⁴C ages of HMW amino acids at 2500 m (Fig. 2).

During the same cruise, we set up a separate group of bioassays using raw seawater collected from five other depths (50, 100, 300, 750, and 1500 m) to examine the depth trend of bulk amino acid



Fig. 3. Microbial utilization of dissolved organic matter at various depths of BATS. Bulk DOC (**A** to **C**), HMW DOC (**D** to **F**), and HMW amino acids (**G** to **I**) were collected in epipelagic (2 m), mesopelagic (400 m), and bathypelagic (2500 m) waters and incubated in the dark for 180 days (bulk DOC) and 390 days (HMW DOC and HMW amino acids). Data are reported as the average \pm SD (n = 3). Note that the incubations amended with HMW DOC [(D) to (I)] contained higher initial concentrations of DOC and amino acids than those in raw seawater [(A) to (C)] (refer to Materials and Methods for details).

utilization (Fig. 5). These bioassays were conducted under the same environmental conditions as those amended with HMW fractions, but they involved more frequent sampling during incubation (days 0, 1, 3, 7, 14, 28, 57, and 180; see Materials and Methods) (Fig. 5, A to C). The percentage utilization of amino acids in raw seawater exhibited a remarkably similar depth trend to that observed for HMW amino acid utilization (Figs. 4 and 5D), decreasing over the 180-day period from $12.3 \pm 1.0\%$ (n = 5) at 50 to 100 m to $7.5 \pm 1.3\%$ (n = 3) at 300 m (two-sample *t* test, P < 0.01) and then increasing to $11.0 \pm 1.2\%$ (n = 6) at 750 to 1500 m (two-sample *t* test, P < 0.01). This depth pattern remained consistent regardless of incubation duration (see Fig. 5D for utilization over 1-, 7-, and 180-day periods). These independent results support the observation of enhanced HMW amino acid utilization in bathypelagic waters.

Changing environmental conditions from epipelagic to bathypelagic waters could influence prokaryotic community composition and activity. The incubations were conducted under laboratory conditions (i.e., room temperature and atmospheric pressure), but these factors alone are unlikely to account for the observed trends. If elevated temperatures at the start of incubations were the primary driver, then amino acid utilization would be expected to increase consistently with decreasing in situ temperatures (e.g., from 750 to 1500 m). However, this pattern was not observed (Fig. 5D). Further statistical analysis of amino acid utilization across depths in relation

The remained consistent tion of labile proteinaceous molecules in the deep ocean. They also reveal a nonlinear delivery of labile substrates from epipelagic to bathypelagic waters. Sources and delivery of proteinaceous matter to the deep ocean It is intriguing to investigate the sources and mechanisms that supply relatively fresh organic matter to bathypelagic organisms. The proteinaceous matterial observed at bathypelagic depths contained an unexpectedly high proportion of labile compounds ($8 \pm 1\%$)

proteinaceous material observed at bathypelagic depths contained an unexpectedly high proportion of labile compounds ($8 \pm 1\%$ during 9-day incubations) that were used over short timescales ranging from days to weeks (Figs. 3 and 4). This rapid turnover implies that the material must have been supplied recently. It is unlikely that this material originated from the advection of DOC in deep water. The predominant water mass in this region is the

to the extent of temperature rise from in situ conditions revealed no significant relationships ($R^2 < 0.05$, P > 0.1 for 1-, 7-, and 180-day

incubation periods; fig. S3). The influence of pressure on prokary-

otic activity is known to be variable (33-35). However, much like the

effect of temperature, depressurization alone could not explain the

depth-specific trends observed, such as the nonlinear variation in

amino acid utilization from 100 to 750 m or the minimal change in utilization between 750 and 1500 m (Fig. 5D). Overall, these find-

ings from incubations with both HMW organic fractions and raw

seawater provide consistent evidence for the rapid microbial utiliza-



Fig. 4. The percentage of microbial utilization of dissolved organic matter at various depths of BATS. The utilization of bulk DOC (black circle), HMW DOC (blue circle), and HMW amino acids (brown circle) is defined as the percentage removal during the 180 to 390 days of incubation (180 days for bulk DOC and 390 days for HMW fractions). Short-term utilization (7 to 9 days) is shown in fig. S2 and table S2. Data are reported as the average \pm SD (n = 3).

NADW, which forms in subpolar regions and spreads slowly into the western North Atlantic at a rate of 1 to 2 cm/s over 2 to 3 decades (36). If NADW receives labile organic matter from the surface during its formation or subsequent southward transport, then the material would only travel a few kilometers laterally before all labile components are consumed by microorganisms. It appears that the bioavailable proteinaceous material in bathypelagic waters, particularly its labile components, was supplied recently within or near the BATS region.

To trace the origin of the bathypelagic proteinaceous material, we analyzed the chemical compositions of amino acids used in raw seawater and HMW fractions during the incubations, comparing them to surface plankton and raw seawater collected at BATS using principal components analysis (PCA). The analysis revealed three distinct clusters (Fig. 6A). Notably, the amino acids utilized at bathypelagic depth were compositionally similar to those used at epipelagic depth (paired t test, P > 0.1) and to surface plankton collected at BATS (paired *t* test, P > 0.1) (Fig. 6, A and B). In contrast, the amino acids utilized at 300 to 400 m, as well as those in raw seawater samples from various depths at BATS, formed two separate clusters that were distinct from both the bathypelagic and epipelagic samples (Fig. 6A). The compositional similarity between deep-sea bioavailable amino acids and surface plankton, combined with the observation of relatively young ¹⁴C ages in bathypelagic HMW amino acids, suggests that the proteinaceous material utilized in bathypelagic waters was recently derived from plankton. This points to the rapid transfer of organic matter from the upper water column to bathypelagic waters, rather than the slow process of lateral advection from older water masses.



Fig. 5. Microbial utilization of total hydrolysable amino acids (THAA) in raw seawater collected at BATS. Seawater collected at 50 to 100 m (A), 300 m (B), and 750 to 1500 m (C) were incubated in the dark for 180 days with subsampling on days 0, 1, 3, 7, 14, 28, 57, and 180 at room temperature (21° to 24°C). The percentage of microbial utilization of seawater amino acids over 1-, 7-, and 180-day utilization was presented in (D). Data are reported as the average \pm SD (n = 3). d, day.



Fig. 6. Amino acid compositions in different forms of organic matter at BATS. PCA for utilized bulk amino acids (utilized bulk AA; over 7 days of incubation with raw seawater), utilized HMW amino acids (utilized HMW AA; over 9 and 390 days of incubation with HMW amino acids), raw seawater samples (50 to 4300 m; no incubation), and surface plankton collected at BATS (**A**) (sample score plot); chemical composition of utilized HMW amino acids at 2 and 2500 m during 390 days of incubation (**B**); initial amino acid composition of surface plankton and raw seawater at BATS (**C**). Raw seawater samples were collected at depths of 50, 100, 150, 200, 250, 300, 400, 500, 600, 800, 1000, 1350, 2000, 3250, and 4300 m (n = 15). Error bar represents one SD.

The biological pump, including the gravitational, migrant, and mixing pumps, delivers organic matter derived from phytoplankton in sunlit surface waters throughout the ocean water column (15, 37). Sinking particles are a major source of bioavailable organic matter and energy to mesopelagic and deep-sea organisms. The combined flux of organic carbon from the biological pump is estimated to be 10.2 Pg of C per year, with the gravitational and migrant pumps contributing about 81% of the total flux (38). Chemoautotrophic and mixotrophic archaea are another potential source of organic carbon and energy for deep-sea microorganisms (39). Carbon dioxide fixation by autotrophic communities (e.g., Crenarchaeota) appears to be an important source of organic matter fueling heterotrophic microbes in the deep ocean (40). However, the rates of microbial inorganic carbon fixation in the North Atlantic Ocean were found to decrease from mesopelagic to bathypelagic waters (40). Therefore, the elevated bioavailable amino acids observed in bathypelagic waters compared with mesopelagic waters (Figs. 4 and 5) were unlikely due to dark fixation by autotrophs.

The solubilization of surface-derived sinking particles via abiotic or extracellular enzymes has been suggested as an important mechanism for the delivery of modern DOC at depth (19, 41). However, while the sinking of surface particles could be a mechanism for delivering fresh carbon to the deep ocean, it often undergoes substantial degradation that results in a large loss of plankton material during transit (42-44). The bioassay experiments with upper ocean waters reflect this trend, revealing a marked decrease in the bioavailability of amino acids from surface to 300 to 400 m (Figs. 4 and 5D), indicating a diminishing supply of plankton-derived material with increasing depth in the upper ocean. However, the bioavailability of amino acids (both bulk and HMW fractions) increased significantly from mesopelagic to

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bathypelagic waters (two-sample *t* test, P < 0.05, n = 3; Figs. 4 and 5D). This reversal in depth trend cannot be explained by a continuous supply of plankton remnants settling passively from epipelagic waters. It appears that there is a replenishment of plankton-derived particles originating from mesopelagic waters.

The most plausible mechanism for delivering particles to bathypelagic waters is the active export by diel or seasonal vertical migrators residing in mesopelagic waters. Our survey occurred shortly after the spring bloom and coincided with the maximal zooplankton biomass (Fig. 1, A and B). During this period, a large portion of the zooplankton community $(63 \pm 25\%)$ was dominated by large size species (>1000 µm; Fig. 1C) that likely had enhanced swimming capabilities. Many zooplankton and fish feed near the surface at night and return to mesopelagic waters during the day, where they repackage particulate material into dense and rapidly sinking fecal pellets that traverse mesopelagic to bathypelagic waters (45-49). Fecal pellets, especially those ballasted with opal and calcite, can sink at rates of several hundred meters per day, contributing to a variable but important fraction of the sinking organic carbon at depth (46, 50-52). The solubilization (41) of rapidly sinking fecal pellets provides labile and semilabile DOC to bathypelagic microbes on short timescales. At BATS, where our study was conducted, the average contribution of fecal pellet carbon to total organic carbon flux was estimated to increase from 4% in mesopelagic waters (500 m) to 5 to 7% in bathypelagic waters (1500 to 3200 m) (53). It was also found that the contribution of fecal pellets to deep-sea organic flux increased from early spring to May (53), a trend following the temporal variation in zooplankton biomass typically observed in the upper water column at BATS (e.g., Fig. 1). The migrator-mediated processes (e.g., vertical migrations, fecal pellet production, direct excretion of DOC,

repackaging of small particles, carcasses, etc.) in mesopelagic waters establish a "special delivery" pathway that rapidly furnishes proteinaceous matter to the deep ocean. This mechanism is consistent with the presence of labile HMW amino acids observed at 2500 m in this study.

It appears from the data in this study and previous studies that the special delivery of proteinaceous matter by migrating zooplankton to the deep ocean is not restricted to specific locations. At station ALOHA (A Long-term Oligotrophic Habitat Assessment), an oligotrophic region of the subtropical North Pacific Ocean, sinking particles reaching a depth of 4000 m were found to have chemical compositions and specific energy values (joules per unit mass or organic carbon) that were distinct from those in mesopelagic waters but similar to those of surface plankton (54). The relatively high specific energy of bathypelagic particulate organic matter observed in the North Pacific Ocean aligns with our findings for the enhanced utilization of bathypelagic HMW amino acids in the North Atlantic Ocean. Both phenomena could be explained by the inputs from migrating mesopelagic zooplankton. If these processes occur in oligotrophic oceans with low plankton production, then similar mechanisms are anticipated in other regions with elevated plankton production, such as subarctic Pacific Ocean or/and seamount regions (55). A recent metaproteomic analysis of size-fractionated samples collected from surface to deep waters in the Pacific, Atlantic, and Southern Oceans revealed that zooplankton-derived proteins serve as an important substrate for microorganisms in bathypelagic waters, potentially playing a larger role there than in mesopelagic zones (56). Further field investigations on the chemical composition, energy potential, and biological availability of deep-sea organic matter across oceanic regions of varying productivity are encouraged to explore the universality of this feature.

In summary, this study provides unique field and experimental data integrating radiocarbon analyses, chemical characterizations, and bioassay experiments that broaden current understanding of the biological pump. Our observations reveal a nonlinear supply of plankton-derived labile and semilabile organic material to the cold, dark depths of the ocean. Quantifying the input of zooplankton fecal pellets and particulate material to depth and determining the extent to which this organic matter subsidizes deep-sea microbes remain challenging. Recent advances in compound-specific isotope analysis of amino acids show promise in quantitatively distinguishing zooplankton fecal pellets from bulk particles (57), offering important insights into these processes. Despite these challenges, given the versatility of microbes to convert labile substrates into longlasting refractory organic molecules (58, 59), this special delivery of proteinaceous material to deep-sea microbes, occurring either as regular or episodic events, represents an important mechanism for marine carbon sequestration via the biological and microbial carbon pumps (37, 60).

MATERIALS AND METHODS

Sample collection

Seawater samples were collected at the BATS (31°40′N, 64°10′W) site in May 2016. The depth profile of water temperature revealed relatively warm surface waters (21° to 22°C), transitioning into a mixed layer extending to approximately 30 m, followed by a steady decrease to 6° to 7°C at a depth of 1000 m (fig. S1A). Below 1000 m, the water temperature declined gradually with depth, reaching a

minimum of 2.2°C below 4000 m. Dissolved oxygen levels exhibited a pronounced decline in the upper mixed layer, with high concentrations of 219 μ mol/kg at the surface, reaching a minimum of 147 μ mol/kg at around 800 m and then increasing to a maximum of 268 μ mol/kg at bathypelagic depths (below 3000 m). Major water masses identified in this region included the North Atlantic Central Water (surface to ~1000 m), the Upper Labrador Sea Water (1000 to 2000 m), and the NADW (below 2000 m) (fig. S1B).

Large-volume samples (1000 to 4000 liters) for ultrafiltration were collected from 2, 400, and 2500 m. A pump sampling system was used to collect surface water (2 m), and a rosette sampler was used to collect mesopelagic and bathypelagic waters (400 and 2500 m). Samples for radiocarbon analysis were transferred into precombusted 1-liter amber glass Boston round bottles and immediately frozen (-20°C) for radiocarbon analysis (see below). Additional seawater samples were collected from the same depths (i.e., 2, 400, and 2500 m) for microbial inocula used in the incubation experiments (see below). Seawater samples were also collected at depths of 50, 100, 150, 200, 250, 300, 400, 500, 600, 800, 1000, 1350, 2000, 3250, and 4300 m using a rosette sampler equipped with 24×12 -liter Niskin bottles. Seawater was drained directly from Niskin bottles into acid-cleaned high-density polyethylene bottles and frozen at -20°C for analyses of DOC and amino acids. Plankton samples were collected during a previous cruise at BATS using a plankton net with a 30-µm mesh screen (5).

HMW DOC isolation

HMW DOC (>2.5 kDa) was isolated using a large-volume, tangentialflow ultrafiltration system (21, 28). Seawater samples (1000 to 4000 liters) were prefiltered through 53-µm Nitex mesh and pumped through 0.2-µm polyethersulfone cartridge filters before ultrafiltration. Ultrafiltration was performed using a custom-built system equipped with four spiral-wound polyethersulfone membranes with a molecular weight cutoff of 2.5 kDa (GE Osmonics). The concentrated HMW DOC samples were desalted via diafiltration with Milli-Q water and dried via rotovap and centrifugal evaporation. Samples were stored as a dry powder for radiocarbon analysis.

Bioassay experiments

Two sets of bioassay experiments were conducted to measure the microbial utilization of bulk DOC and HMW DOC. In the first bioassay experiments, raw seawater from depths of 50 m (20.7°C), 100 m (20.3°C), 300 m (18.8°C), 750 m (11.4°C), and 1500 m (4.5°C) were drained directly into precombusted glass vials and immediately incubated onboard in the dark. Subsamples were taken on days 0, 7, and 180 for analysis of bulk DOC concentrations. Additional subsamples were taken at a higher frequency for analysis of total hydrolyzable amino acids concentrations, on days 0, 1, 3, 7, 14, 28, 57, and 180. In the second set of bioassay experiments, raw seawater containing natural microbial communities from depths of 2 m (21.6°C), 400 m (17.9°C), and 2500 m (3.2°C) were incubated with HMW DOC isolated from the same depths, with initial DOC concentrations of 1183 \pm 3, 580 \pm 3, and 595 \pm 1 μ M, respectively. The incubations were conducted in precombusted Kimax glass bottles in the dark. Subsamples were collected on days 0, 9, 22, 51, 99, 170, and 390 for analysis of DOC and amino acids. Both sets of experiments were incubated at room temperature (21° to 24°C) inside coolers, and subsamples were collected aboard ship during days 0 to 9 and stored frozen. Following the cruise, the incubation bottles were

transported to the home laboratory and kept under the same temperature conditions. All experiments were conducted in triplicate.

Analysis of DOC, amino acids, and radiocarbon (¹⁴C)

Concentrations of DOC were determined using a high-temperature combustion method and a Shimadzu TOC-V instrument with a nondispersive infrared detector. Water samplers were acidified to pH 2 to 3 before injection. Milli-Q waters and seawater reference standards were injected every sixth sample to determine the instrumental blanks and to monitor the performance of the analysis (*61*). The blank values were negligible, and the reference standards were within the range of reported values (41 to 44 μ mol of C per liter). The coefficient of variation among four injections of a given sample was typically <1%.

Concentrations of D- and L-enantiomers of hydrolyzable combined amino acids were determined using an Agilent 1260 ultrahighperformance liquid chromatography system equipped with equipped with a fluorescence detector (*62*). Seawater samples were hydrolyzed using a vapor-phase technique that liberates free amino acids from their combined forms (*63*). The amino acids were derivatized with *o*-phthaldialdehyde and *N*-isobutyryl-L-cysteine and separated on a Poroshell 120 EC-C18 column eluted with a mixed mobile phase of potassium dihydrogen phosphate and methanol:acetonitrile. A total of 20 hydrolysable combined amino acids were quantified with a detection limit of 0.5 nM for most amino acids. Racemization during acid hydrolysis was corrected according to Kaiser and Benner (*63*).

Natural abundance radiocarbon was determined for total seawater DOC (i.e., bulk DOC), HMW DOC, and the HMW amino acid fraction. Total seawater DOC was isolated for radiocarbon analysis using ultraviolet photochemical oxidation following Walker et al. (64) and graphitized using the sealed-tube zinc method (65). HMW DOC and the HMW amino acid fraction were combusted to carbon dioxide, cryogenically purified, and graphitized following Vogel et al. (66). The amino acid fraction was isolated from HMW DOC following liquid-state acid hydrolysis with a combination of cation exchange chromatography and high-performance liquid chromatography (HPLC). Approximately 60 mg of dry HMW DOC material was weighed into 8-ml glass vials with 5 ml of hydrochloric acid (HCl; 6 N), flushed with nitrogen gas, and heated to 110°C for 20 hours. Resulting hydrolysates were dried under N2 gas, redissolved in 2 ml of HCl (0.1 N), and purified using cation exchange chromatography (Bio-Rad, AG50W-X8; 200 to 400 mesh) following the methods of Takano et al. (67). The amino acid fraction was then isolated following the HPLC method developed for the isolation of individual amino acids described in Broek et al. (68), Broek and McCarthy (69), and Bour et al. (70). The entirety of the HPLC mobile phase eluted during the known analytical windows encompassing all proteinaceous amino acids was collected into single 200-ml glass jars and dried under vacuum via centrifugal evaporation. Dry amino acid isolates were then transferred to 6-mm guartz tubes with 100 µl of 60°C Milli-Q water and dried under vacuum. Radiocarbon analyses were performed at the Lawrence Livermore National Laboratory, Center for Accelerator Mass Spectrometry and at the UC Irvine Keck Carbon Cycle Accelerator Mass Spectrometry facility. The isotopic values were corrected to the date of collection and are reported in accordance with conventions set forth by Stuiver and Polach (71) as background and δ^{13} C corrected fraction modern, Δ^{14} C, and conventional radiocarbon age [years before present (ybp)]. Amino acid fraction radiocarbon values were further corrected for

exogenous carbon added during fraction purification by subtraction of a directly determined process blank encompassing the HPLC isolation and all subsequent processing.

Statistical analysis

Statistical differences among variables were assessed using Z test, t test, and PCA. The Z test was used to test difference in radiocarbon content and age between two depths, with one ¹⁴C measurement taken for each organic fraction at each depth. This test assumes that the variance is known, and we are confident in the reliability of the instrumental uncertainty as an estimate of the true SD for Δ^{14} C values. Paired t test and two-sample t test were used to evaluate differences between dependent variables (e.g., bulk DOC Δ^{14} C value versus HMW DOC Δ^{14} C value at a given depth) and independent variables (e.g., percentage utilization among different depths), respectively. The statistical analyses were conducted as two-tailed tests with a significant level of $\alpha = 0.05$, using IBM SPSS software. PCA was performed to evaluate compositional trends in different forms of organic matter based on the relative abundances (in mole %) of amino acids, using the R programming language.

Supplementary Materials

This PDF file includes: Figs. S1 to S3 Tables S1 and S2 References

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Supplementary Materials for

Special delivery of proteinaceous matter to deep-sea microbes

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Figs. S1 to S3 Tables S1 and S2 References



Fig. S1. Profiles of temperature and dissolved oxygen (A) and salinity (B) at the Bermuda Atlantic Time-Series Study (BATS) site in May 2016. Data were obtained from <u>http://bats.bios.edu/bats-data/.</u> Major water masses identified in this region included the North Atlantic Central Water (NACW; surface to ~1000 m), the Upper Labrador Sea Water (ULSW; 1000–2000 m), and the North Atlantic Deep Water (NADW; below 2000 m) (72, 73).



Fig. S2. Short-term microbial utilization of dissolved organic fractions at BATS. The utilization of bulk dissolved organic carbon (bulk DOC; black circle), high-molecular-weight DOC (HMW DOC; blue circle), and HMW amino acids (brown circle) is defined as the percentage removal during 7-9 days of incubation (7 days for bulk DOC and 9 days for HMW fractions). Long-term utilization (180-390 days) is shown in Fig. 4. Data are reported as the average \pm standard deviation (N=3).



Fig. S3. Microbial utilization of seawater amino acids (%) versus difference (Δ T) between in situ temperature and incubation temperature. Percentage utilization (%) of seawater amino acids over 1-, 7-, and 180-day periods is shown. Δ T is calculated as the difference between in situ water temperature (20.7°C at 50 m, 20.3°C at 100 m, 18.8°C at 300 m, 11.4°C at 750 m, and 4.5°C at 1500 m) and the actual incubation temperature (i.e., at room temperature 21-24°C; 22°C is used for consistency).

Table S1. Summary of concentrations and radiocarbon (¹⁴C) content of dissolved organic fractions at the Bermuda Atlantic Time-series Study (BATS) site. Bulk dissolved organic carbon (bulk DOC), high-molecular-weight DOC (HMW DOC), and HMW amino acid fraction (HMW AA fraction) were isolated in epipelagic (2 m), mesopelagic (400 m), and bathypelagic (2500 m) waters at BATS in May 2016. UCIAMS: UC Irvine Keck Carbon Cycle Accelerator Mass Spectrometry; CAMS: Center for Accelerator Mass Spectrometry.

Depth	Sample type	Concentration (µmol C/L)	UCIAMS# or CAMS#	Fm	Δ ¹⁴ C (‰)	¹⁴ C age (ybp)
2 m	Bulk DOC	68.4 ± 0.8	180280	0.8204 ± 0.0018	-186.1 ± 4.0	1590 ± 20
400 m	Bulk DOC	49.8 ± 0.2	180269	0.6793 ± 0.0016	-326.1 ± 4.0	3105 ± 20
2500 m	Bulk DOC	44.4 ± 0.5	180276	0.6129 ± 0.0018	-392.0 ± 4.0	3930 ± 25
2 m	HMW DOC	17.0 ± 0.1	175988	0.9359 ± 0.0031	-64.1 ± 3.1	530 ± 30
400 m	HMW DOC	9.5 ± 0.2	175989	0.8291 ± 0.0026	-170.9 ± 2.6	1505 ± 30
2500 m	HMW DOC	8.1 ± 0.1	175991	0.7349 ± 0.0025	-265.1 ± 2.5	2475 ± 30
2 m	HMW AA fraction	0.22 ± 0.02	178443	0.9742 ± 0.0090	-33.8 ± 9.0	210 ± 80
400 m	HMW AA fraction	0.07 ± 0.01	180130	0.8899 ± 0.0105	-117.4 ± 10.5	940 ± 100
2500 m	HMW AA fraction	0.06 ± 0.01	178444	0.8648 ± 0.0150	-142.3 ± 15.0	1170 ± 140

Table S2. Microbial utilization of dissolved organic matter at BATS. Bulk dissolved organic carbon (bulk DOC), high-molecular-weight DOC (HMW DOC), and HMW amino acids were collected in epipelagic (2 m), mesopelagic (400 m), and bathypelagic (2500 m) waters at BATS in May 2016. Microbial utilization is reported as the percentage (%) of initial concentrations during short-term (7-9 days) and long-term incubations (180 days and 390 days for bulk and HMW fractions, respectively).

	She	ation	Long-term utilization				
	(i.e., labile)			(i.e., labile + semi-labile)			
Denth	Bulk	HMW	HMW	Bulk	HMW	HMW	
Depth	DOC	DOC	amino acids	DOC	DOC	amino acids	
2 m	1.8±0.9%	0.6±0.3%	10.2±4.6%	5.8±1.1%	10.2±0.9%	24.5±1.9%	
400 m	0.2±0.6%	0.8±0.2%	5.3±0.6%	3.3±0.2%	5.4±1.3%	8.4±0.9%	
2500 m	0.0±0.4%	0.2±0.4%	8.4±1.4%	0.0±1.0%	3.4±1.3%	13.0±2.4%	

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