ORIGINAL ARTICLE

Diverse, uncultivated bacteria and archaea underlying the cycling of dissolved protein in the ocean

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Dissolved organic nitrogen (DON) supports a significant amount of heterotrophic production in the ocean. Yet, to date, the identity and diversity of microbial groups that transform DON are not well understood. To better understand the organisms responsible for transforming high molecular weight (HMW)-DON in the upper ocean, isotopically labeled protein extract from *Micromonas pusilla*, a eukaryotic member of the resident phytoplankton community, was added as substrate to euphotic zone water from the central California Current system. Carbon and nitrogen remineralization rates from the added proteins ranged from 0.002 to 0.35 µmol C l^{-1} per day and 0.03 to 0.27 nmol N l^{-1} per day. DNA stable-isotope probing (DNA-SIP) coupled with high-throughput sequencing of 16S rRNA genes linked the activity of 77 uncultivated free-living and particle-associated bacterial and archaeal taxa to the utilization of *Micromonas* protein extract. The high-throughput DNA-SIP method was sensitive in detecting isotopic assimilation by individual operational taxonomic units (OTUs), as substrate assimilation was observed after only 24 h. Many uncultivated free-living microbial taxa are newly implicated in the cycling of dissolved proteins affiliated with the Verrucomicrobia, Planctomycetes, Actinobacteria and Marine Group II (MGII) Euryarchaeota. In addition, a particle-associated community actively cycling DON was discovered, dominated by uncultivated organisms affiliated with MGII, Flavobacteria, Planctomycetes, Verrucomicrobia and Bdellovibrionaceae. The number of taxa assimilating protein correlated with genomic representation of TonB-dependent receptor (TBDR)-encoding genes, suggesting a possible role of TBDR in utilization of dissolved proteins by marine microbes. Our results significantly expand the known microbial diversity mediating the cycling of dissolved proteins in the ocean.

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Introduction

Dissolved organic nitrogen (DON) is the second most abundant form of fixed nitrogen in the ocean, with a pool size of 7.7 ($\pm$2.3) $\times 10^4$ Tg N (Karl *et al.*, 2001; Bronk, 2002; Aluwihare and Meador, 2009). The total DON pool consists of numerous low and high molecular weight (LMW, HMW) compounds and compound classes of varying concentration and bioavailability including amino acids, *N*-acetyl amino polysaccharides (for example, chitin and peptidoglycan), dissolved proteins and uncharacterizable proteinaceous matter (Tanoue *et al.*, 1995; McCarthy *et al.*, 1997, 1998; Aluwihare *et al.*, 2005). Microbial heterotrophy can control the flux and composition of DON (Carlson and Ducklow, 1995), yet the specific organisms responsible for DON transformations and their biochemical mechanisms are poorly understood (Aluwihare and Meador, 2009).

Phytoplankton are a major DON source (Nguyen and Harvey, 1997; Bronk and Steinberg, 2009). Release of DON results from a variety of forms of phytoplankton cell death including viral lysis (Fuhrman, 1999) and grazing (Worden *et al.*, 2015), and can also be released via passive diffusion

(Fogg, 1971). The liberated dissolved proteins and oligopeptides are initially regarded as labile HMW-DON compounds (Keil and Kirchman, 1993; Keil *et al.*, 2000), but become more refractory because of processes such as abiotic complexation with existing dissolved organic matter (DOM) (Keil and Kirchman, 1994). Dissolved proteins are recycled faster relative to the bulk HMW-DON pool, as evidenced by their lower $\delta^{15}N$ values (Meador *et al.*, 2007) and more modern $\Delta^{14}C$ values (Loh *et al.*, 2004) relative to bulk HMW-DON.

Protein in dissolved and particulate fractions can be readily degraded in seawater by microbial activity (Sizemore and Stevenson, 1974; Smith *et al.*, 1982), and turnover rates of proteins can be the same order of magnitude as turnover rates of dissolved free amino acids (DFAA) (Hollibaugh and Azam, 1983). LMW-DON such as DFAA released by protein hydrolysis (Billen and Fontigny, 1987) are readily assimilated by bacteria in aquatic systems (Hobbie *et al.*, 1968) and can support up to ~ 50% of bacterial production in the oceans (Kirchman, 2000).

Studies linking some microbial groups to LMW-DON (Alderkamp *et al.*, 2006; Nikrad *et al.*, 2012; Liu *et al.*, 2013) and HMW-DON (Nagata *et al.*, 1998; Cottrell and Kirchman, 2000) uptake have demonstrated important spatiotemporal variability in DON utilization, but have been limited in their ability to understand the full diversity of bacteria and archaea involved in DON cycling by the need for *a priori* selection of target groups. Nevertheless, a relatively large number of bacterial taxa in the ocean have been implicated in HWM-DOM turnover, either through experimental additions or following phytoplankton blooms, and some overlap should be expected between these taxa and those involved in HWM-DON cycling. These include the Alteromonads (for example, McCarren *et al.*, 2010; Sharma *et al.*, 2014; Mayali *et al.*, 2015), Flavobacteria (for example, Kirchman, 2002; Rinta-Kanto *et al.*, 2012; Teeling *et al.*, 2012; Sharma *et al.*, 2014; Mayali *et al.*, 2015) and the gammaproteobacterial clades NOR5/OM60, SAR92 (Teeling *et al.*, 2012; Sharma *et al.*, 2014) and SAR86 (Dupont *et al.*, 2012). Bacterial groups specifically implicated in the cycling of HMW-DON and proteins include the Flavobacteria (Cottrell and Kirchman, 2000; Kirchman, 2002), the γ-Proteobacteria subgroups Arctic96B16, Ant4D3 and SAR86 (Nikrad *et al.*, 2014), and the α-Proteobacteria clade SAR11 (Malmstrom *et al.*, 2005).

The uncultivated Marine Group II Euryarchaeaota (MGII) are also predicted to have a role in DON transformations, based on the recovery of protein degradation pathways in metagenomic data (Iverson *et al.*, 2012; Orsi *et al.,* 2015). MGII are thought to be heterotrophs, through their utilization of LMW-DON (Alderkamp *et al.*, 2006) and available metagenomic data (Frigaard *et al.*, 2006; Zhang *et al.*, 2015). However, direct evidence of their role in utilizing dissolved proteins has not been demonstrated, and interactions between archaea and HWM organic matter are largely unexplored. Several of the aforementioned groups, including the MGII, have been found in association with particulate organic matter (Delong *et al.*, 1993), where they have an enhanced genomic capacity for HMW substrate utilization (Orsi *et al.*, 2015).

We sought to determine whether microbial taxa previously implicated in HWM-DOM turnover might specifically be involved in HMW-DON (protein) cycling. We further hypothesized that MGII archaea are actively involved in protein turnover, and that this activity would be enhanced in association with particles (Orsi *et al.*, 2015). To this end, we performed DNA stable-isotope probing (SIP) using isotopically labeled (^{13}C and ^{15}N) proteins from the picoeukaryotic phytoplankter *Micromonas pusilla*. We coupled SIP with high-throughput Illumina sequencing of 16S small subunit ribosomal RNA (rRNA) genes (Hungate *et al.*, 2015; Morando and Capone, in review) in three different size fractions to identify protein assimilation by individual bacterial and archaeal operational taxonomic units (OTUs). We performed the incubations at a station within the coastal transition zone of the Central California Current, where there is a rich contextual background on biogeochemistry (for example, Collins *et al.*, 2003), and where *M. pusilla* is a resident of the phytoplankton community (Thomsen and Buck, 1998). Free-living (0.2–0.8 μm) and particle-associated (> 3 μm) fractions contained a diversity of newly implicated, uncultivated bacterial and archaeal taxa mediating the cycling of dissolved protein. Our results suggest that when dissolved proteins become available, highly diverse microbial populations with the genomic capacity to transport HMW-DON can acquire it rapidly. These results refine our understanding of DOM turnover in the ocean and indicate a strong affinity of several uncultivated clades for dissolved protein, and suggest that diverse particle-attached microbial communities can act as a DON sink.

Methods

Sampling

Seawater was collected aboard the R/V *Western Flyer* during Monterey Bay Aquarium Research Institute cruise CN13ID, 7–17 October 2013, along California Cooperative Fisheries Investigations (CalCOFI) Line 67 (Collins *et al.*, 2003; Pennington *et al.*, 2010). Sampling and hydrographic profiling were conducted using a conductivity-temperature-depth rosette sampler (Sea-Bird Electronics) equipped with (12) 10 l Niskin sample bottles. In addition to the standard conductivity-temperature-depth measurements, the profiling rosette was equipped with the following sensors: WETstar fluorometer (Wetlabs, Philomath, OR, USA), transmissometer (SeaTech, Bellevue, WA, USA), SBE43 dissolved oxygen sensor (Sea-Bird Electronics, Bellevue, WA, USA) and a radiometer (Biospherical, Inc., San Diego, CA, USA). Chlorophyll *a* (Chl *a*) pigment

concentrations were determined by fluorometry using established protocols (Pennington and Chavez, 2000). Major phytoplankton groups (*Prochlorococcus*, *Synechococcus* and picoeukaryotic phytoplankton) were quantified at sea by flow cytometry as described previously (Cuvelier *et al.*, 2010).

Stable isotope probing incubation
M. pusilla CCMP1545 was grown for 10 days at 21 °C under a light:dark cycle of 14 h:10 h using cool white light (150 µmol Q m^{-2} s^{-1}) in L1 medium (Guillard and Hargraves, 1993) based on artificial seawater amended with 2.5 mM 99 atm% ^{13}C-labeled sodium bicarbonate and 882 µM 98 atm% ^{15}N-labeled sodium nitrate (Cambridge Isotope Laboratories, Andover, MA, USA). The growth rate was 0.47 per day (s.d.: 0.09). Isotopic enrichment of the cells was obtained by mixing the culture in unlabeled media 1:1 with labeled medium, incubating for 2 days, followed by another 1:1 mixture with labeled media. This was repeated five times. The isotopic composition of whole cells was measured at the UC Davis Stable Isotope Facility using online combustion coupled to a mass spectrometer; values were 16 atm% for ^{13}C and 42 atm% for ^{15}N. It should be noted, however, that particulate C isotopic composition was determined for cells that had been in stationary phase for several days relative to the cells harvested for protein extraction, potentially leading to an underestimation of the starting atm% ^{13}C.

Proteins from *M. pusilla* were extracted according to the method by Santoro *et al.*, 2015. In brief, cell pellets were incubated at room temperature for 15 min in an extraction buffer, followed by heating at 95 °C for 10 min and shaking at 350 r.p.m. for 1 h. Protein extract was removed into a new sterile tube and centrifuged for 30 min at 4000 r.p.m. at room temperature, followed by filtration through a 5-µm pore size sterile filter. Filtered samples were then concentrated using 6 ml 5000 MWCO Vivaspin columns (Sartorius, Göttingen, Germany) down to 400 µl, split in half, and precipitated with 1.6 ml of cold (4 °C) solution of 1:1 MeOH and acetone, and 0.5 mM HCl for 3 days at −20 °C. Precipitated samples were spun down for 30 min at 4 °C at 14 500 r.p.m., air dried in a laminar flow hood and resuspended in 1 ml extraction buffer prior to quantification. Protein extracts were quantified fluorometrically. No DNA was measureable in the extracts using fluorometric measurements (detection limit = 2 ng µl^{-1}) suggesting that co-extraction of nucleic acids with the proteins was minor. However, we cannot exclude the possibility that a small amount of labeled nucleic acids were co-extracted with the proteins. There were no visible particles in the extracts suggesting that the protein was mostly dissolved.

Incubation experiments (SIP and remineralization rates) were conducted at CalCOFI station 67–70 (36.74 °N, 122.02 °W), approximately 170 km from shore, within the coastal transition zone between the coastal upwelling and oligotrophic waters offshore (Collins *et al.*, 2003). The site was selected because of the historic presence of a significant picoeukaryote population *M. pusilla* (Thomsen and Buck, 1998), and previous characterization of particle-associated taxa (Orsi *et al.*, 2015). Water for the SIP incubation was acquired from 20 m depth. *M. pusilla* protein extract was added to 4 l of seawater to a final concentration of 28 µg l^{-1} in trace metal-clean polycarbonate bottles. Bottles were amended with dual isotope-labeled proteins (^{13}C and ^{15}N), unlabeled proteins or no proteins, in triplicate. Bottles were incubated for 24 h in the dark in coolers that were halfway filled with surface seawater to maintain *in situ* temperature. The bottles were subsampled every 4 h (except for the 16 h timepoint) to determine the concentration of ammonium and DFAA, and the nitrogen isotopic composition of $NO_3^- + NO_2^-$ ($\delta^{15}N_{NOx}$). The carbon isotopic composition of dissolved inorganic carbon (DIC) was determined in samples taken at 24 h (see details below). At the end of the incubation 1–2 l of seawater was filtered sequentially through 3 µm (Pall Versapor-3000 T, Port Washington, NY, USA), 0.8 µm (Pall Supor-800, Port Washington, NY, USA) and 0.2 µm (Pall Supor-200, Port Washington, NY, USA) filters using a peristaltic pump, and DNA was extracted as previously described (Santoro *et al.*, 2010). Catalyzed reporter deposition—fluorescence *in situ* hybridization for Archaea and Euryarchaeota, DAPI staining and epifluorescence microscopy followed the protocol of Orsi *et al.* (2015). This protocol utilized the Eury806 probe that is specific for Euryarchaeota (Tiera *et al.*, 2004) and the Arch915 probe that targets the domain Archaea (Stahl and Amann, 1991), but also binds non-specifically to some pelagic marine bacteria (Pernthaler *et al.*, 2002).

Ammonium, DFAA and DCAA measurements
Water column [NH_4^+] samples were collected in 125 ml acid-cleaned polyethylene bottles and incubation samples were collected in 27 ml acid-cleaned polyethylene vials and refrigerated. Samples were analyzed within hours using a fluorometric method with a single working reagent (orthophthaldialdehyde, sodium sulfite and sodium borate) (Kerouel and Aminot, 1997; Holmes *et al.*, 1999). The system was automated with a peristaltic pump-based flow injection analysis. Detection limits, estimated as 1% of full scale, gave +/− 3 nM with the high sensitivity injection loop. An estimate of the precision from a cast where samples were analyzed from 12 bottles tripped at the same location gave 25 +/− 1 nM.

Depth profiles of DFAA and total dissolved amino acids (TDAA) were collected from the rosette into pre-combusted (450 °C, 5 h) glass scintillation vials after filtering through a sterile 0.2-µm pore size filter and analyzed as described below. DFAA were analyzed by high performance liquid chromatography (Shimadzu Prominence, Kyoto, Japan) with

fluorescence detection after pre-column *o*-phthaldialdehyde derivatization (Lee *et al.*, 2000). TDAA were analyzed in the same way as DFAA but after hydrolysis by 6 N HCl under nitrogen at 110 °C for 20 h (Kuznetsova and Lee, 2002). Dissolved combined amino acids (DCAA) were calculated as the difference between TDAA and DFAA. The coefficient of variation for replicate DFAA and DCAA samples was 10–20%. The concentrations of individual amino acids were calculated on the basis of an amino acid external standard mixture (Amino Acid Standard H, Thermo Scientific, Waltham, MA, USA).

In situ array
Water for measurements of ^{13}C, ^{15}N-labeled protein remineralization and ^{15}NH$_4^+$ oxidation rates was collected from depths of 2, 5, 10, 20, 30 and 50 m using the conductivity-temperature-depth rosette sampling system described above. Upon return to deck, water from each depth was drained directly into clear, acid-cleaned, 280 ml polycarbonate bottles. Following addition of isotopically labeled substrates to a final concentration of 50 nmol l^{-1} and 28 µg l^{-1} for ^{15}NH$_4^+$ and ^{13}C, ^{15}N-proteins, respectively, the incubation bottles were loaded into hand-made nylon mesh bags, hung at their depth of origin on a 6.3 mm double-braided line attached to a custom GPS-enabled Surface Temperature Experimental Longitude Latitude Asset (STELLA) drifter (http://www.mbari.org/bog/drifterdata/) with a 60 cm surface float, and deployed *in situ* from dusk to dawn (ca. 12 h). Following recovery of the array, samples were immediately filtered through combusted GF/F filters housed in Swinnex filter holders (EMD Millipore, Billerica, MA, USA), into acid-washed 60 ml HDPE bottles and kept frozen until analysis. The δ^{15}N$_{NOx}$ was determined at the University of Connecticut Avery Point campus using the denitrifier method (Sigman *et al.*, 2001) and a GasBench and PreCon trace gas concentration system interfaced to a Delta V^{PLUS} mass spectrometer (ThermoFinnigan, Bremen, Germany). Nitrate isotope reference materials USGS32, USGS34 and USGS35 were analyzed in parallel to calibrate δ^{15}N values. δ^{15}N precision, determined by repeat analysis of the reference materials, was found to be 0.15‰ for the entire data set. Resultant δ^{15}N$_{NOx}$ data were used to calculate rates of NH$_4^+$ oxidation or labeled protein remineralization using previously described methods (Smith *et al.*, 2014) derived from the original equations laid out by Dugdale and Goering (1967).

Protein remineralization rates
Protein-derived C and N remineralization rates were determined in bottle incubations on the *in situ* array and the on-deck SIP experiment by measuring changes in stable-isotope ratios in the DIC (δ^{13}C-DIC) and inorganic nitrogen (δ^{15}N$_{NOx}$) pools after 24 h. δ^{13}C-DIC

was measured in acidified samples using a GasBench II system interfaced to a Delta V^{PLUS} isotope ratio mass spectrometer (ThermoFinnigan) at the UC Davis Stable Isotope Facility. δ^{15}N$_{NOx}$ was measured and rates of remineralization calculated as described in the previous section. A range of initial isotopic enrichment values for the starting ^{13}C atm% enrichment in the labeled proteins was used in the rate calculations, because of potential effects of the delay in measuring the starting ^{13}C atm% of the *M. pusilla* cells (see above). We used a lower value of 16 atm% ^{13}C based on the whole cell measurement and an upper value of 95 atm% ^{13}C based on the calculated atm% ^{13}C for the number of cell doublings that occurred assuming uptake of fully labeled medium. The starting ambient DCAA pool was assumed to have a δ^{13}C of -20‰ (McCarthy *et al.*, 2004) and δ^{15}N of 8‰ (McCarthy *et al.*, 2007). Limitations to this method include an incomplete characterization of both the isotopically labeled protein preparation and the ambient DCAA pool, and considerable uncertainties in the starting concentrations of both (Lipschultz, 2008).

Density gradient centrifugation and gradient fractionation
At the 24 h timepoint, one of the triplicate experimental (^{13}C, ^{15}N protein addition) and control (unlabeled protein addition) bottles (see above) was sampled for DNA-SIP. DNA was extracted from the experimental and control bottles after 24 h according to the method of Santoro *et al.* (2010). In brief, 850 µl of lysis buffer and 100 µl of 10% SDS were added to 2 ml gasketed bead-beating tubes containing the filters and 0.1 mm sterile glass beads (Biospec, Kyoto, Japan). Bead beating was performed for 1 min and samples heated for 2 min at 99 °C. After heating, 25 µl of 20 mg ml^{-1} proteinase K was added and tubes were incubated over night at 55 °C. DNA was then purified from the lysate with the DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands).

The protocol for density gradient centrifugation and gradient fractionation followed previously described methods for DNA-SIP (Neufeld *et al.*, 2007; Dunford and Neufeld, 2010), with some minor modifications. In brief, density gradient centrifugation was performed in a TV90 vertical rotor at 20 °C for 40 h at 177 000 *g* in an Optima XL-90 ultracentrifuge (Beckman Coulter, Brea, CA, USA). DNA was spun in 4 ml polyallomer Optiseal tubes (Beckman Coulter) in cesium chloride (CsCl) gradients with an average density of 1.725 g ml^{-1}. Centrifuged gradients were fractionated into 15 equal fractions via careful manual pipetting from the top of the polyallomer tube. DNA was precipitated with 2 volumes of polyethylene glycol for 2 h at room temperature and pelleted by centrifugation (30 min, 13 000 *g*). Pellets were washed once with 70% ethanol, resuspended in molecular grade water, and quantified fluorometrically using a Qubit (Life Technologies, Carlsbad, CA, USA).

PCR and qPCR

16S rRNA genes from each fraction were amplified using primers equipped for dual-indexed barcoded sequencing on an Illumina (San Diego, CA, USA) MiSeq (Kozich *et al.*, 2013), which target the V4 hypervariable region (515F/806 R; (Caparaso *et al.*, 2012)). We used a version of the 515F primer with a single-base modification (in bold) to increase coverage of the SAR11 clade (515F: 5′ − GTG**C**CAGCMGCCGCGGT AA − 3′; Apprill *et al.*, 2015). PCR reactions were carried out in 25 μl volumes with 2 μl of template added to each reaction for 35 cycles. Each gradient fraction was amplified using a different barcode combination and amplicons were gel-extracted (QIAquick Kit, Qiagen) and pooled in equimolar concentrations prior to high-throughput paired-end Illumina sequencing (MiSeq, 2×250 bp reads) at the University of Illinois Keck Center for Comparative and Functional Genomics. Each density fraction was also screened for the major subgroups of marine archaea using quantitative PCR (qPCR). qPCR for MGII Euryarchaeaota followed a previously published assay (Orsi *et al.*, 2015), while the qPCR assay for Marine Group I ammonia-oxidizing archaea using the ammonia monooxygenase subunit A (*amo*A) gene followed that of Santoro *et al.* (2010).

Bioinformatic analysis

Processing of the paired-end MiSeq data was performed according to Kozich *et al.* (2013) using MOTHUR (Schloss *et al.*, 2009), and pair-wise OTU clustering was performed in QIIME (Caporaso *et al.*, 2010). A total of 12.5 million quality-checked 16S rRNA gene sequences corresponding to 2722 OTUs were used for downstream analysis. Sequences were clustered at 97% sequence identity using UCLUST (Edgar, 2010). OTUs were identified to taxonomic groups through BLASTn searches against the SILVA database (Pruesse *et al.*, 2007). OTU tables were rarified per size fraction, to the sample with the least number of sequences ($0.2-0.8 \, \mu m = 27,327$; $0.8-3 \, \mu m = 57,110$; $>3 \, \mu m$: 34,970). Rarified counts for each OTU in the control and SIP gradients were normalized to the maximal abundance of that OTU across density fractions in unlabeled or SIP-labeled bottles. Only OTUs with >10 sequences that were detected in >50% of the fractions were used in downstream analysis. After these quality control criteria, a total of 4.1 million 16S rRNA gene sequences corresponding to 702 OTUs were used for downstream analysis. Sequence data have been deposited in the NCBI Short Read Archive under BioProject ID PRJNA287804.

We defined DNA-SIP incorporation similar to Nelson and Carlson (2012) and Hungate *et al.* (2015) on the basis of a comparison of the relative distribution of an OTU across the CsCl density gradient in a control (^{12}C and ^{14}N substrate added) versus an experimental (^{13}C and ^{15}N substrate added) incubation. OTUs incorporating the label were defined as those OTUs that exhibited a relatively unimodal distribution in the CsCl gradient and whose peak buoyant density (BD) was shifted $>0.012 \, \mathrm{g \, ml}^{-1}$ between the experiment and the control (similar to Buckley *et al.*, 2007a). In this case, a relatively higher number of sequences from an OTU retrieved in the heavy part of the gradient compared with its distribution in the control is a function of isotopically labeled protein assimilation. Thus, throughout the manuscript, we refer to OTUs meeting the criteria defined above as those assimilating protein.

Results

Cruise setting and vertical biogeochemical profiles

Oceanographic conditions during the cruise were characterized by a general decreasing gradient in phytoplankton biomass with distance from shore, with upwelling and higher concentrations of Chl *a* along the coast (Supplementary Figure S1). The site for the DNA-SIP incubations, station 67–70, had lower surface Chl *a* concentrations compared with neighboring stations to the east and west ($1.5-2$ versus $>3 \, \mu g \, l^{-1}$) (Supplementary Figure S1) and exhibited a broad subsurface chlorophyll maximum spanning the depths of 17–40 m (Figure 1a). The phytoplankton community (quantified by flow cytometry) was dominated by *Prochlorococcus* (48% of phytoplankton cells) and *Synechococcus* (37% of phytoplankton cells). Eukaryotic phytoplankton comprised 15% of total phytoplankton cells (Supplementary Figure S2). NH_4^+ concentrations ranged from below detection limits to 260 nM, with a maximum of 260 nM at 45 m (Figure 1a). Vertical profiles of DFAA and TDAA (the combination of DFAA and peptides) at 67–70 show a peak in DFAA and TDAA at 40 m (Figure 1b), which coincides with the subsurface NH_4^+ maximum at 40 m (Figure 1a). Concentrations of most individual DFAA peak at 40 m and progressively decrease in deeper water (down to 500 m), with threonine, valine, beta-alanine and methionine being exceptions to this trend (Supplementary Figure S3). The composition of DFAA between 0–40 m is significantly different from the DFAA pool found in waters 60–500 m (analysis of similarity: $P = 0.001$).

Protein remineralization rates from the *in situ* array ranged from 0.002 to 0.35 μmol C l^{-1} per day with a maximum rate at 20 m (Figure 1c). The maximum protein C remineralization rate at 20 m coincides with the subsurface chlorophyll maximum (Figure 1a) and was the depth sampled for the SIP experiment. The protein N remineralization rates (ammonification plus nitrification) measured on the *in situ* array ranged from 0.03 to 0.27 nmol N l^{-1} per day, with a maximal rate at 5 m. Protein N remineralization rates ($\pm$ s.d.) in the SIP incubation were similar to those measured on the array under *in situ* light conditions (0.11 versus 0.14 nmol N l^{-1} per day. In comparison, protein C remineralization
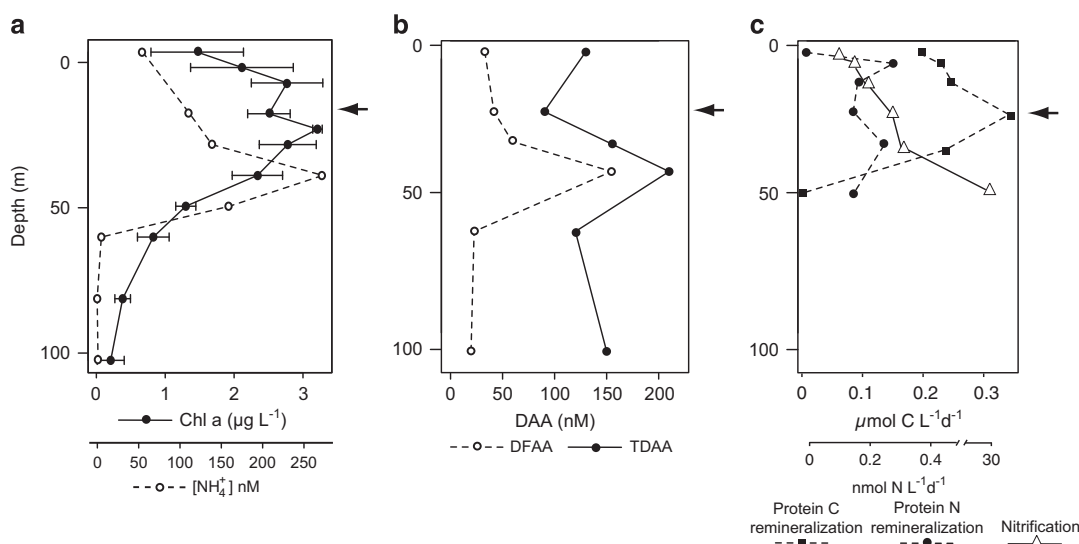
Figure 1 (**a**) Vertical profiles of Chl a and NH_4^+ at station 67–70, error bars represent s.d. of Chl a measurements over a 3-day period at station 67–70. (**b**) Vertical profiles of DFAA and TDAA at station 67–70. (**c**) *In situ* remineralization rates of protein C and N, and NH_4^+ oxidation rates. Protein N remineralization rates are the result of ammonification plus nitrification.

rates in the SIP incubation were slightly higher than the rates measured on the array under *in situ* light conditions (0.55 (±0.09) µmol C l^{-1} per day). NH_4^+ oxidation rates ranged from 0.2 nmol l^{-1} per day at the surface to a maximum of 29.8 nmol l^{-1} per day at 50 m (Figure 1c).

Timecourse measurements
There was no significant difference in net NH_4^+ production between control (no protein added) and experimental (protein added) bottles at 0 and 24 h (t test: $P > 0.05$) (Supplementary Figures S4A and B). Furthermore, there was no increase in DFAA concentration after 24 h between bottles that did, or did not, receive proteins. Depletion of aspartate, glycine, histidine, alanine, valine, serine and leucine was faster in bottles amended with proteins, relative to unamended control (Supplementary Figure S5). There were no significant differences in total free-living DAPI-stained cells between treatments after 24 h (t test: $P > 0.05$; Supplementary Figure S4C). Total DAPI-stained cells attached to particles also did not increase significantly over the course of the experiment (t test; $P > 0.05$; Supplementary Figure S4D). However, free-living euryarchaea increased significantly after 24 h (Supplementary Figure S4C). Interestingly, no archaea were observed on diatomaceaous particles, whereas a high number of bacteria were found attached to these particles (Supplementary Figure S6).

Free-living and particle-attached bacteria and archaea utilizing dissolved proteins
The microbial community in the free-living fraction was dominated by the α-Proteobacteria and γ-Proteobacteria, whereas the particle-associated fractions were

dominated by Verrucomicrobia and the Flavobacteria (Supplementary Figure S7). The most abundant free-living taxa were affiliated with the SAR11 clade followed by the SAR86 clade (Figure 2), whereas the dominant taxa in the particle-associated size fractions were affiliated with *Synechococcus*, *Roseibacillus*, *Fluviicola*, *Formosa* and uncultivated Flavobacteria (Figures 2). There was a shift in the BD of peak DNA quantity between the experimental and control bottles in all size fractions (Supplementary Figures S8A–C). Moreover, the taxonomic composition of density fractions varied significantly as a function of BD between experimental and control bottles (analysis of similarity: $P < 0.05$; Supplementary Figures S8D–F). No discernable difference in community structure was observed between control (no protein added) and experimental (protein added) incubations after 24 h (Supplementary Figure S7). Post incubation, communities in the 0.8–3 µm and > 3 µm fractions were similar to each other and contained relatively higher percentages of Flavobacteria, Planctomycetes and Verrucomicrobia compared with free-living fractions (Supplementary Figure S7).

Seventy-seven bacterial and archaeal OTUs assimilated the amended protein after 24 h (Supplementary Table S1), which exhibit high variability in relative abundance (Table 1). OTUs affiliated with γ-Proteobacteria (SAR86, KI89A, OM60 and SAR92 clades), Verrucomicrobia, Flavobacteria and MGII had the highest numbers of OTUs assimilating protein, and had the highest percentages of OTUs utilizing proteins compared with other groups (Supplementary Table S1). The dominant taxa in the free-living fraction assimilating protein were affiliated with the α-Proteobacteria (*Roseobacter*, Rhodobacteraceae), γ-Proteobacteria (SAR86 clade), and MGII (Figure 2). There was little
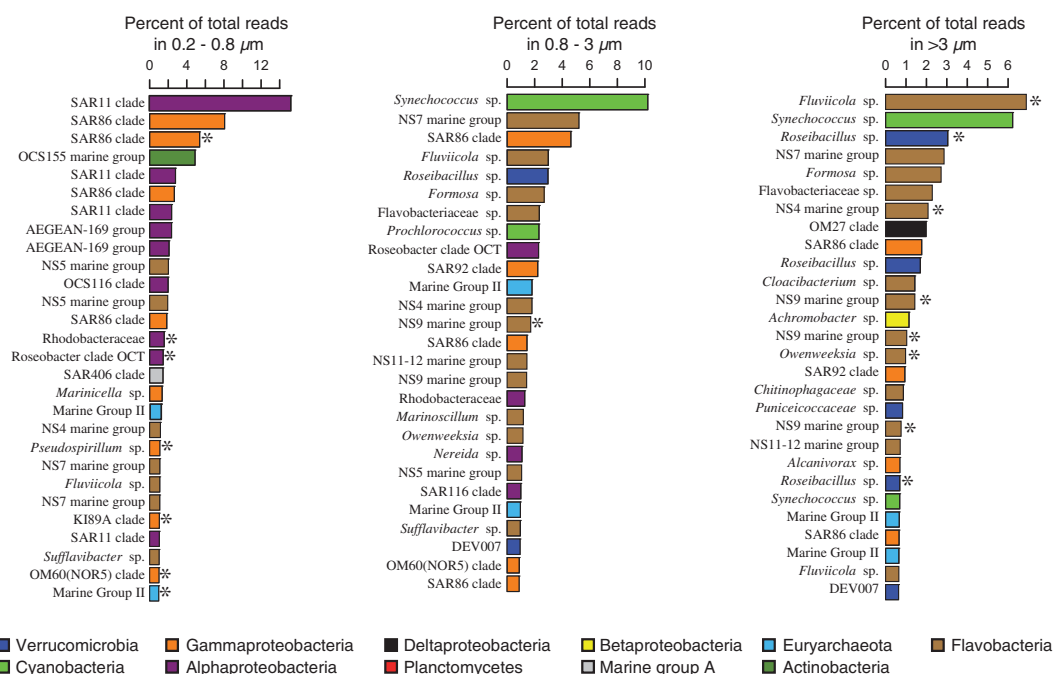
Figure 2 Relative abundance of the most abundant OTUs in each of the three size fractions after 24 h, asterisks indicate OTUs that assimilated protein.

overlap in protein-utilizing taxa between the size fractions (Figure 3). All of the Bacteroidetes-affiliated OTUs assimilating protein belonged to the Flavobacteria. One SAR86 OTU that assimilated protein was the third most abundant OTU (Figure 2) detected in the free-living fraction (Figure 4) and was affiliated with the SAR86 clade III (Figure 5b).

Microbial populations that assimilated protein in particle-associated size fractions are markedly different from free-living populations. For example, OTUs affiliated with Planctomycetes, *Fluviicola* (Flavobacteria), *Formosa* (Flavobacteria), *Roseibacillus* (Verrucomicrobia) and OM27 clade (δ-Proteobacteria) were the dominant populations assimilating proteins in the particle-attached fractions (Table 1, Supplementary Table S1). Most of these bacterial taxa were not observed to assimilate protein in the free-living fraction and were not abundant in the free-living fraction (Figure 2), although some were detected in relatively low abundance (data not shown). Moreover, marine Euryarchaeota affiliated with the MGII.A clade assimilated protein in both the 0.2–0.8 and 0.8–3 µm size fractions, whereas the MGII.C clade only assimilated protein in the 0.2–0.8 µm fraction (Figure 5a).

The assimilation of protein by marine euryarchaea was further validated by qPCR (Figure 6a). MGII incubated with labeled protein exhibit higher quantities of rRNA genes in gradient fractions > 1.71 g ml^{-1} in every size fraction relative to the control (Figure 6a). This assimilation of protein by heterotrophic euryarchaea was markedly larger compared with the ammonia-oxidizing thaumarchaea, which did not meet our criteria for protein assimilation (Figure 6b).

Discussion

Phytoplankton biomass supports significant secondary production by heterotrophic prokaryotes, and is central to organic matter cycling in the ocean. Phytoplankton-derived proteins likely have an important role in this process. Here, we investigated the specific microbial taxa involved in the uptake of dissolved proteins, both on and off particles, and found that although some protein-assimilating taxa were previously identified in the utilization of phytoplankton-derived organic matter, others are newly implicated.

As expected, we detected protein assimilation by free-living α-Proteobacteria, γ-Proteobacteria and Flavobacteria previously implicated in this process. The relative percentage of α-Proteobacteria assimilating protein ($<1\%$, Table 1) is markedly lower than observed in a study from coastal waters, where 13% of α-Proteobacteria cells assimilated protein (Cottrell and Kirchman, 2000). In contrast, roughly 6% of total SAR86 (γ-Proteobacteria) sequences assimilated protein in the free-living fraction (Supplementary Table S1), which is comparable with a MAR-FISH study that found 8% of all SAR86 cells assimilated protein in the surface ocean (Nikrad *et al.*, 2014). Methodological differences preclude a direct comparison between the fraction of labeled cells by MAR-FISH and the fraction of a clade with SIP-labeled OTUs (carried out here), but the indirect comparison here shows that the same groups are found to assimilate protein with both methods. The ability of SAR86 to acquire protein may be facilitated by their genomic capacity for HMW

substrate acquisition (Dupont *et al.*, 2012). An OTU affiliated with the SAR92 clade (Figure 4) assimilated more protein than any other OTU (Figure 4, BD shift; 0.036 g ml^{-1}), suggesting that this clade may be important for protein recycling in areas where the SAR92 clade reaches high abundance, such as coastal waters (Stingl *et al.*, 2007). Flavobacterial cells have a preference for HMW-DON over LMW-DON (Cottrell and Kirchman, 2000; Kirchman, 2002), but in our open ocean study, a lower percentage of free-living Flavobacteria assimilated protein compared with other groups (Table 1), which is also low compared with the percentage of Flavobacteria assimilating protein in coastal waters (Cottrell and Kirchman, 2000).

Our study also links protein utilization to several diverse and uncultivated marine bacterial taxa that previously had not been implicated in dissolved protein cycling. In the free-living size fraction, OTUs affiliated with marine Actinobacteria utilized proteins (Table 1). Within the Actinobacteria, the majority of OTUs utilizing dissolved protein were affiliated with the enigmatic actinobacterial Sva0996 clade (Supplementary Table S1), a group that has been detected in both sediment (Ravenschlag *et al.*, 1999) and water column (Bano and Hollibaugh, 2002) habitats. To our knowledge, this is the first demonstration of the substrate assimilation ability of these uncultivated actinobacteria.

We found that some taxa previously shown to respond to HMW-DOM are especially active in utilizing proteins on particles. The prevalent assimilation of protein by particle-attached Flavobacteria clades observed here (41%; Table 1, Figure 5c) is consistent with their common adhesion to particles (Fernandez-Gomez *et al.*, 2012). Particle-attached bacteria can have much higher protein degradation rates compared with free-living bacteria (Taylor, 1995), and thus particle-attached Flavobacteria may have been 'primed' for protein assimilation. Our results indicate that in addition to degrading particulate matter, particle-attached Flavobacteria also act as a sink of HMW-DOM through the utilization of dissolved proteins. An OTU affiliated with flavobacterial genus *Fluviicola* was the most abundant (7.4% of total reads) taxon assimilating protein in the >3 µm fraction, and a total of six *Fluviicola*-affiliated OTUs assimilated protein in this fraction (Supplementary Table S1). This strongly suggests that some members of the *Fluviicola*, represented by the freshwater strain *Fluviicola taffensis* RW262 (O'Sullivan *et al.*, 2005), are important degraders of proteins, and potentially particulate organic nirtrogen, in marine ecosystems.

We also found diverse, uncultivated Planctomycetes clades assimilating protein in the particle-attached size fraction (Table 1, Supplementary Table S1). This is consistent with their detection in particle-associated size fractions in previous studies (DeLong *et al.*, 1993; Crump *et al.*, 1999). A high percentage (10%) of the Planctomycetes OTUs in the >0.8 µm fractions

Table 1 A summary of taxa assimilating protein after the 24 h incubation

Microbial group	0.2–0.8 µm				0.8–3 µm				>3 µm			
	Average BD shift (g ml^{-1})	OTUs with BD shift (% total)	Sequences with % total	BD shift % total per taxon	Average BD shift (g ml^{-1})	OTUs with BD shift (% total)	Sequences with % total	BD shift % total per taxon	Average BD shift (g ml^{-1})	OTUs with BD shift (% total)	Sequences with % total	BD shift % total per taxon
Flavobacteria	0.018	2 (0.3%)	319 (0.04%)	0.3%	0.027	9 (1.2%)	29 035 (2%)	3%	0.020	16 (2.4%)	105 527 (13%)	41%
Verrucomicrobia	0.016	3 (0.4%)	2524 (0.34%)	51%	0.018	4 (0.5%)	18 835 (1.3%)	10%	0.014	8 (1.2%)	33 880 (4.3%)	46%
Euryarchaeota (MGII)	0.014	5 (0.6%)	9776 (1.3%)	52%	0.012	1 (0.2%)	704 (0.05%)	0.6%	0	0	0	0
Gammaproteobacteria	0.02	3 (0.5%)	44 354 (6%)	22%	0.017	4 (0.5%)	315 (0.02%)	0.07%	0	0	0	0
Alphaproteobacteria	0.016	2 (0.3%)	5755 (0.8%)	2%	0.014	3 (0.4%)	79 (0.005%)	0.01%	0.012	2 (0.3%)	122 (0.01%)	0.1%
Actinobacteria	0.012	1 (0.2%)	350 (0.04%)	0.7%	0	0	0	0	0.012	1 (0.2%)	357 (0.04%)	2%
Planctomycetes	0	0	0	0	0.021	2 (0.3%)	1678 (0.11%)	10%	0.01	3 (0.4%)	2,041 (0.3%)	10%
Deltaproteobacteria	0	0	0	0	0.016	6 (0.3%)	4619 (0.3%)	21%	0.012	2 (0.3%)	684 (0.1%)	2%
Cyanobacteria	0	0	0	0	0.018	1 (0.3%)	21 (0.001%)	0.006%	0	0	0	0
Total		16 (3%)	63 078 (8%)			30 (3.7%)	55 286 (3.8%)			31 (4.6%)	142 254 (18%)	

Abbreviations: BD, buoyant density; OTU, operational taxonomic unit. For all OTUs exhibiting a BD shift, see Supplementary Table S1 for abundance and shifts in BD. The '% total' refers to the percent of total sequences or percent of total OTUs per size fraction. '% total per taxon' refers to percent of total OTUs per size fraction. '% total per taxon' refers to percent of total sequences per taxonomic group.

assimilated protein compared with most other microbial groups, and did not assimilate protein in the free-living fraction (Table 1). Utilization of *M. pusilla* proteins by planctomycetes is consistent with their previous detection following phytoplankton blooms (Morris *et al.*, 2006) and metagenomic evidence indicating that metabolism of marine planctomycetes is linked to

phytoplankton-derived substrates, such as the diatom cell wall protein silaffin (Zeigler Allen *et al.*, 2012). The success of planctomycete cells on particles may be due to their unique holdfast structures and stalks, which allow them to attach to surfaces (Fuerst, 1995). Most Planctomycetes OTUs that assimilated protein were affiliated with the CL500–3 clade (Supplementary Table S1), an as-yet uncultured subgroup found in oligotrophic lakes (Urbach *et al.*, 2001). Another Planctomycete-affiliated OTU that assimilated protein in the >3 µm fraction was affiliated with the uncultivated Urania-1B-19 sediment group (Supplementary Table S1), reported from deep sea sediment of the Mediterranean (Heijs *et al.*, 2008).

The discovery of protein utilization by a diversity of uncultivated Verrucomicrobia taxa in all size fractions (Supplementary Table S1) suggests that Verrucomicrobia are important for DON cycling both on and off of particles. The relatively high proportion of Verrucomicrobia taxa assimilating protein in the free-living (51%) and >3 µm fractions (46%) indicates that they have a relatively strong preference for dissolved proteins compared with most other groups detected (Table 1). In the free-living fraction, Verrucomicrobia protein utilization was dominated by representatives of the Puniceicoccaceae, previously shown to assimilate exudates from *Synechococcus* in euphotic zone waters (Nelson and Carlson, 2012). This
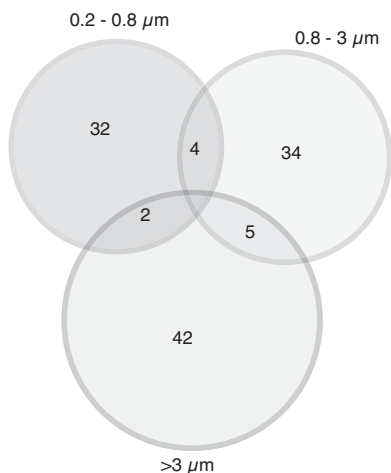

Figure 3 The number of overlapping and non-overlapping OTUs between size fractions that assimilated protein. Note that most OTUs assimilated protein in only one size fraction.
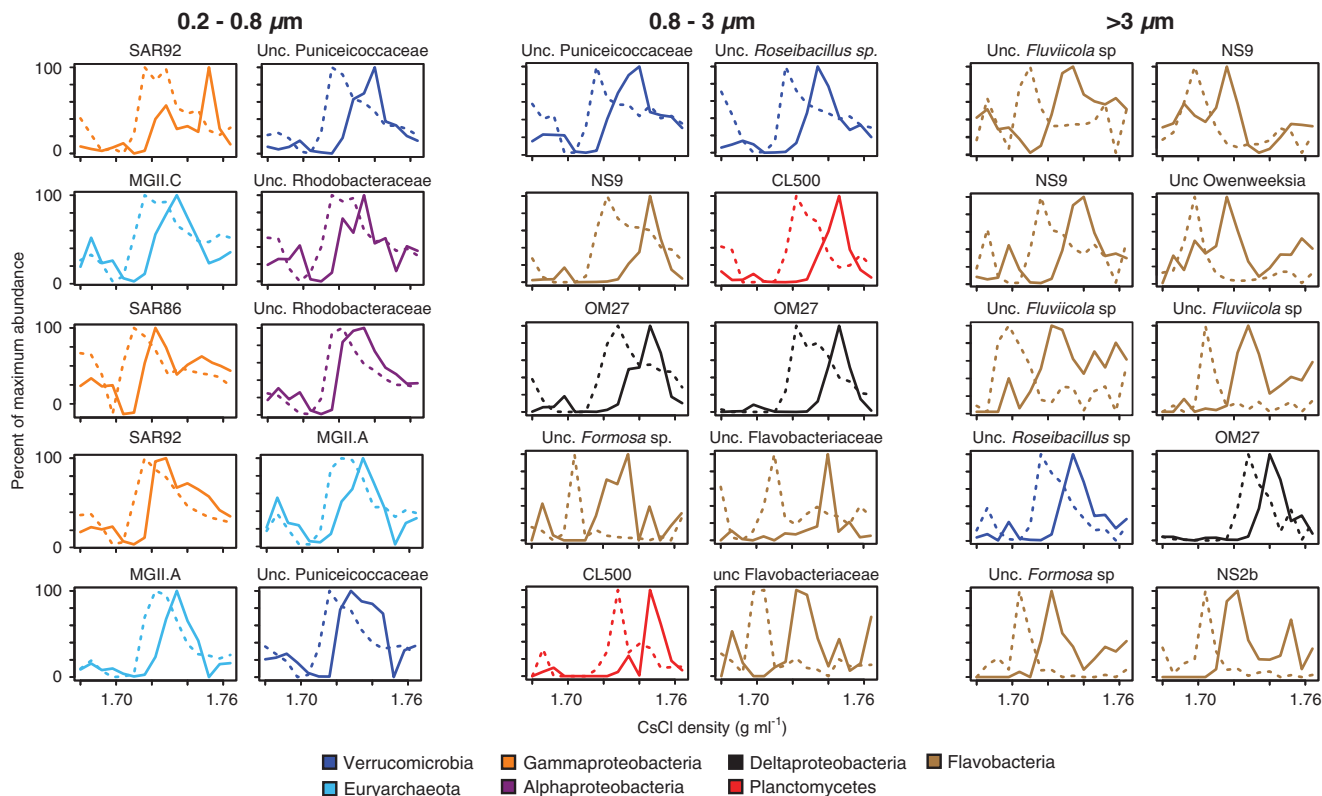

Figure 4 OTUs exhibiting the greatest degree of protein assimilation. Lines represent the distribution of OTUs in the control (unlabeled proteins, dashed lines) and experiment (labeled proteins, solid lines). The *y* axis represents the relative abundance of each OTU normalized to its maximal abundance across all density fractions. See Supplementary Table S1 for relative abundance and BD shifts for all OTUs.
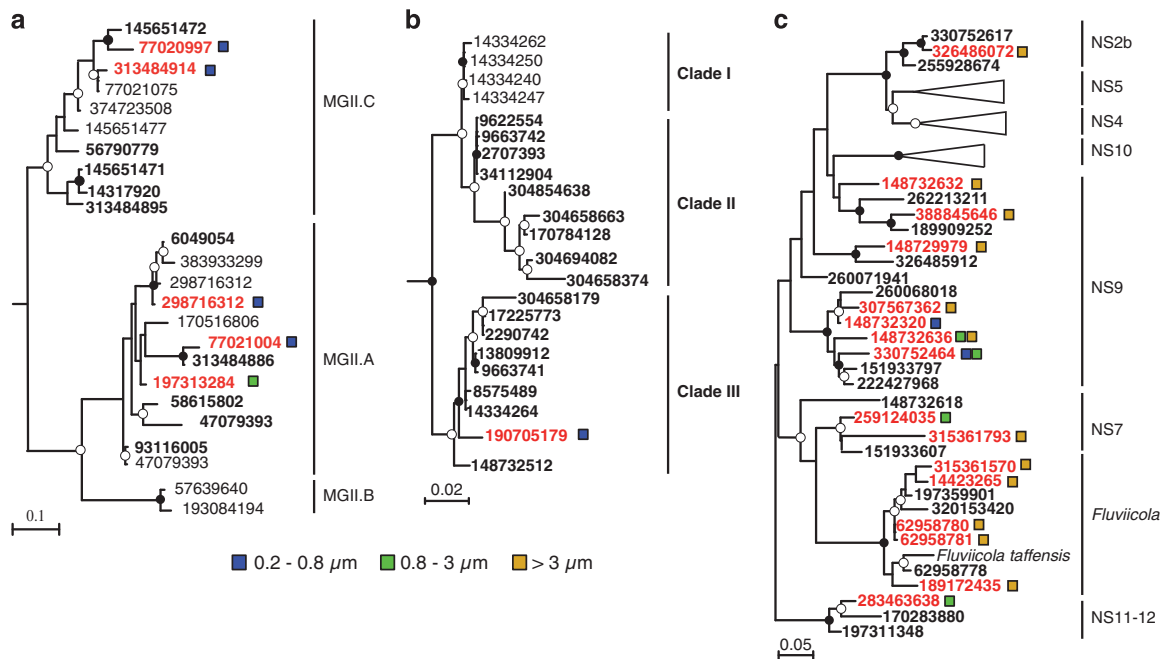
Figure 5 Maximum likelihood phylogenies (PhyML) of MGII (**a**), SAR86 (**b**) and Flavobacteria (**c**) 16S rRNA genes sharing >97% sequence identity with OTUs, which assimilated (red font), or did not assimilate (bold black font), protein after a 24 h incubation. Colored squares indicate the size fraction where protein assimilation was observed. Black circles at nodes represent bootstrap support of >90% and white circles represent bootstrap support of >50%. Numbers represent NCBI GI accession IDs.

supports biogeographic and genomic evidence suggesting that certain marine Verrucomicrobia species have a preference for phytoplankton-derived HMW-DOM (Herleman *et al.*, 2013). Verrucomicrobia have also been identified as degraders of polysaccharides in the Arctic (Cardman *et al.*, 2014) and given their ubiquity in the ocean and presence on POM (Freitas *et al.*, 2014), chemoheterotrophic activity of Verrucomicrobia taxa should contribute markedly to DOM and POM turnover. OTUs affiliated with *Roseibacillus* were the dominant verrucomicrobial group assimilating protein in the particle-associated fraction (Supplementary Table S1). The few cultivated *Roseibacillus* species do not exhibit N-acetyl-β-glucosaminidase, trypsin or chymotrypsin activity (Yoon *et al.*, 2008), enzymes which degrade HMW-DON such as chitin and protein. Thus, it seems likely that some particle-attached *Roseibacillus* cells might instead acquire protein C and N by transporting the oligopeptides released through protein hydrolysis from extracellular enzymes manufactured by other bacteria.

Protein assimilation was identified in OM27 clade organisms (Supplementary Table S1), which are most closely related to the predatory deltaproteobacterial genus *Bdellovibrio* (Fuchs *et al.*, 2005) and have a geographically wide distribution (for example, Rappe *et al.*, 1997; Fuchs *et al.*, 2005). The OM27 clade had the highest percentage of OTUs (21%) assimilating protein compared to all other groups in the 0.8–3 μm fraction suggesting their activity was relatively high in this size range (Table 1). This may indicate the formation and activity of bdelloplasts, structures that are formed as predatory *Bdellovibrio*

divide inside the periplasmic space causing the prey cell size to increase (Abram *et al.*, 1974). This cross-feeding phenomenon of predatory bacteria has been observed in previous DNA-SIP studies, whereby *Bdellovibrio* populations assimilated C after preying on methanotrophs incubated with ^{13}C methane (Morris *et al.*, 2002). We hypothesize that OM27 clade organisms correspond to an uncultivated group of predatory bacteria because (i) the closest phylogenetic relation of this clade is to the predatory *Bdellovibrio* genus (Fuchs *et al.*, 2005) and (ii) 1–2 μm-sized bdelloplasts (Fenton *et al.*, 2010) should be enriched in the 0.8–3 μm size range, which is the size range where OM27 group-specific protein assimilation (21%) was higher than all other groups (Table 1). We conclude that OM27 clade organisms acquired the label through predation on bacteria that had assimilated the dissolved protein substrate (that is, cross-feeding), and were then captured as swollen bdelloplasts in the 0.8–3 μm fraction.

We confirmed protein utilization by uncultivated taxa affiliated with marine Euryarchaeota (Figure 5a). Our study provides the first direct link between MGII cells and protein recycling, as well as identification of the specific MGII taxa utilizing dissolved proteins. Compared with the other major microbial groups, a relatively high percentage (52%) of free-living MGII OTUs assimilated protein, suggesting that they have a strong affinity for this substrate (Table 1). The physiology of planktonic MGII is hypothesized to be based on protein metabolism (Reysenbach and Flores, 2008; Iverson *et al.*, 2012), and heterotrophic euryarchaea in sediments also appear to utilize
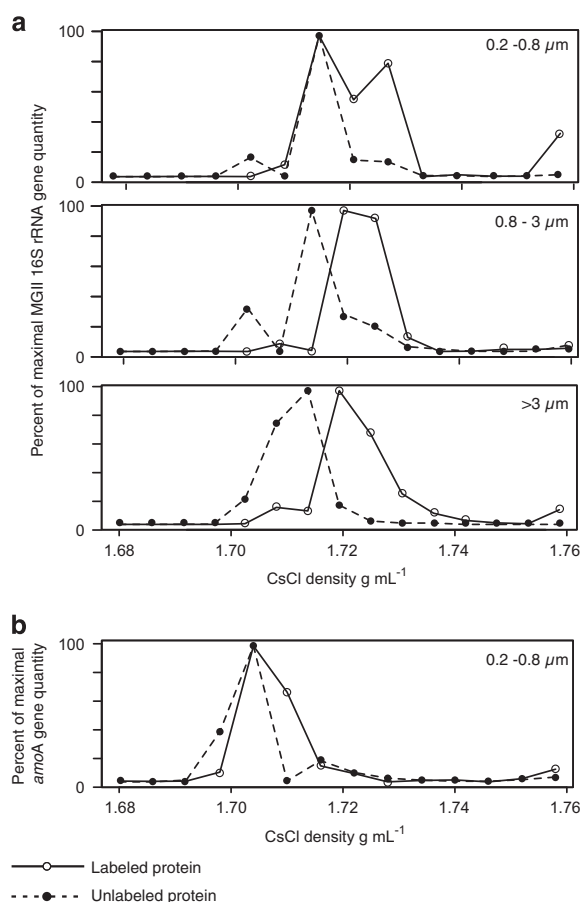
Figure 6 (**a**) Quantification (qPCR) of MGII Euryarchaeota 16S rRNA genes in density gradient fractions from the control and experiment, in the three size fractions. In all size fractions, higher quantities of MGII rRNA genes are detected in density fractions >1.71 g ml^{-1} in DNA extracted from bottles amended with labeled proteins, relative to the control. (**b**) Quantification (qPCR) of MGI (Thaumarchaeota) *amoA* genes in density gradient fractions from the control and experiment, in the 0.2–0.8 µm size fraction.

detrital proteins (Lloyd *et al.*, 2013; Orsi *et al.*, 2013). The strong affinity for protein substrates by marine euryarchaea shown here (Table 1) provides direct evidence supporting these hypotheses. Assimilation of protein by MGII (Figure 6a) was markedly larger compared with thaumarchaea (Figure 6b), which according to our criteria did not assimilate protein.

We originally hypothesized that MGII would also be active in protein assimilation on particles, because of an enrichment of MGII genes involved in HWM-DOM degradation in particle-associated metagenomes (Orsi *et al.*, 2015). Although we did not observe any MGII OTUs assimilating protein in the >3 µm fraction (Table 1), qPCR analysis with MGII-specific primers did show protein assimilation by MGII in this size fraction (Figure 6a). Thus, MGII OTUs assimilating protein in the >3 µm fraction, and potentially other size fractions, were missed using the 'universal' tag-sequencing PCR primers.

The SAR11 clade (Giovannoni *et al.*, 1990), which includes *Pelagibacter* (Rappe *et al.*, 2002), readily assimilates DFAA (Malmstrom *et al.*, 2004; Nikrad *et al.*, 2012) and was the most abundant free-living taxon (Figure 2). However, no SAR11 OTUs assimilated protein in our incubations. This led us to investigate the question: Do some groups acquire proteins via a HMW transmembrane transport mechanism that is absent in SAR11 cells? TonB-dependent receptor (TBDR) proteins transport HMW compounds that exceed the typical range of normal porins (<600 daltons) by catalyzing high affinity transport of Ni-, Cu-, Fe-chelates, proteins, siderophores and polysaccharides across the outer membrane (Schauer *et al.*, 2008), and are absent in all *Pelagibacter* genomes ($n = 19$) available in IMG (as of March 2015). TBDR are important for microbial competition in the ocean, as cells with TBDR were observed to outcompete other microbes lacking TBDR in the presence of labile HMW-DOM released after a phytoplankton bloom in the North Sea (Teeling *et al.*, 2012) and were also overexpressed in marine communities following HMW-DOM additions (McCarren *et al.*, 2010). TBDR have been identified as important components of algal glycan utilization in *Gramella forsetti*, a widespread marine flavobacterium pivotal to remineralization of complex organic matter (Kabisch *et al.*, 2014). However, it is unknown whether TBDR also assist in protein acquisition by marine microbes.

Two observations suggest that TBDR may facilitate protein uptake by certain marine microbes. First, a positive correlation (Spearman correlation: $r = 0.55$, $P = 0.02$) exists between the number of OTUs per group assimilating protein and the percent of genomes in that group that encode TBDR proteins (Figure 7). Second, the gross majority of lineages that assimilated protein encode TBDR transmembrane transporters (Supplementary Table S1), whereas SAR11-affiliated OTUs that lack these transporters did not. The dominance of SAR11 taxa in the free-living fraction (Figure 2) combined with their lack of substrate assimilation suggests that hydrolyzed amino acids from the labeled protein substrate were not readily available for assimilation during the 24 h incubation. Given the correlation between groups containing TBDR and the number of OTUs per group assimilating protein (Figure 7), it seems likely that some TBDR encoding marine microbes may be able to transport larger oligopeptides (>600 daltons) quickly, before they are hydrolyzed into smaller amino acids. As suggested for polysaccharides (Teeling *et al.*, 2012), this would help them compete against cells lacking TBDR during periods of increased protein availability (for example, during blooms). This hypothesis is consistent with experimental demonstration of TBDR transport of large proteins (>600 daltons) such as serum transferrin and hemoglobin across cell membranes (Noinaj *et al.*, 2010), suggesting that this is a mechanism of protein acquisition in the ocean as well.

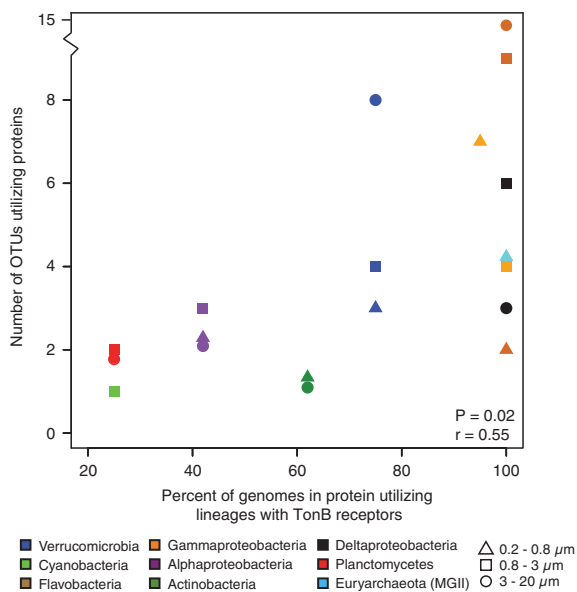Despite uptake of the labeled proteins by abundant, diverse lineages, it is not likely that cells acquired the

Figure 7 Relationship between the number of OTUs assimilating protein and the percentage of genomes per group that encode TBDR. Searches for TBDR in genomes of each group were performed using the control term search function in IMG (img.jgi.doe.gov) against all finished and draft genomes in each group (as of March 2015), using 'tonb' as a keyword. The correlation was performed on all data points (including the number of Flavobacteria OTUs in >3 μm outlier); the *y* axis break was inserted to reduce plotting space. See Supplementary Table S1 for a complete summary of values used in this plot and the number of genomes per group used to calculate percent values on the *x* axis.

label after remineralized enriched protein C and N passed through an inorganic dissolved phase (as DIC or NO_3^-). The $\delta^{13}C$ of DIC at the end of the experiment was only enriched 3‰ (~1 atm%) relative to the controls. Such a low level of enrichment would be insufficient to produce a detectable labeling of the DNA meeting our threshold of 0.012 g ml^{-1}. Similarly, the highest final $\delta^{15}N_{NOx}$ in the experimental incubations was 17‰ (< 0.4 atm%), the uptake of which would not produce a detectable shift in DNA density because 25 atm% is the minimal ^{15}N enrichment in DNA needed to detect a nitrogen-based assimilation signal with DNA-SIP (Buckley *et al.*, 2007b). For similar reasons, it is unlikely that ^{15}N was acquired via $^{15}NH_4^+$ regenerated from the labeled proteins. The maximum shift in DNA BD possible from an N source with 50 atm% enrichment is 0.01 g ml^{-1} (Buckley *et al.*, 2007b). Thus, OTUs being labeled solely through $^{15}NH_4^+$ assimilation is highly unlikely because the starting ^{15}N enrichment of our protein extract was 42 atm% and we used a DNA BD threshold of 0.012 g ml^{-1} for defining assimilation.

Protein remineralization to DIC in the *in situ* array and SIP incubation (Figure 1c) indicates that the amended proteins were not only assimilated, but also used as an energy source. The profile of protein C remineralization rates (Figure 1c) is similar in shape to previously reported hemoglobin remineralization rates in the Southern California Bight, which were also found to peak in the subsurface chlorophyll maximum (Hollibaugh and Azam, 1983).

We acknowledge that our calculated C remineralization rates (0.002–0.35 μmol l^{-1} per day) based on the assumption of 16 atm% labeling of the added proteins may be overestimates, but are similar in magnitude to previous studies (Keil and Kirchman, 1993; Nagata *et al.*, 2003). Calculations using the calculated value of the *M. pusilla* cells of 95 atm% yields rates that are much lower (0.001–0.10 μmol l^{-1} per day), but a profile that is similar in shape.

Conclusion

Our study demonstrates a direct link between phytoplankton-derived dissolved protein and several dominant, yet uncultivated, members of the bacterial and archaeal community and significantly expands the known microbial diversity responsible for mediating protein turnover. Substrate assimilation was observed after a relatively short incubation time (24 h), indicating that the high-throughput DNA-SIP method used here is sufficiently sensitive in detecting substrate assimilation. Correlative evidence suggests that TBDR receptors are a potential mechanism used by certain marine microbes for protein utilization, a finding that warrants further investigation. Although protein assimilation was widespread within certain taxonomic groups (Verrucomicrobia and MGII archaea), in others, fewer sub-clades were found to be active in protein uptake (for example, α-Proteobacteria, SAR86, and Actinobacteria). Some taxa found in particle-attached fractions were particularly active in protein assimilation (Flavobacteria, Verrucomicrobia and Planctomycetes), while their free-living counterparts were not. Microbial utilization of protein in particle-associated fractions indicates that some particle-attached microbes are active in the degradation of dissolved protein. Particle-attached bacteria are typically thought to act as sources of DOM through hydrolysis of particulate HMW organic matter (Cho and Azam, 1988). However, the particle-attached bacteria and Euryarchaeaota, utilizing dissolved HMW-DON shown here demonstrate that a diversity of particle-attached microbial communities also act as a sink of important DON substrates over relatively short timescales.

Conflict of Interest

The authors declare no conflict of interest.

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