Two Chemically Distinct Pools of Organic Nitrogen Accumulate in the Ocean

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The chemical dynamics of marine dissolved organic nitrogen (DON), a reservoir featuring surface accumulations even in areas where nitrogen limits productivity, have yet to be resolved. We exploited differences in the acid lability of amide bonds within high-molecular-weight (HMW) DON to show that vertical DON profiles result in part from the presence of two chemically distinct pools of amide. Half of HMWDON in surface waters is present as *N*-acetyl amino polysaccharides. In contrast, nearly all deep-sea HMWDON, and therefore, most HMWDON, is present in amides that resist both chemical hydrolysis and biological degradation.

Low concentrations of inorganic nitrogen (such as nitrates and ammonia) are assumed to limit primary production over wide expanses of the surface ocean. However, in many of these areas, dissolved organic nitrogen (DON) accumulates to measurable quantities (*1*–*3*) despite a demonstrated role in fueling both primary and secondary production (*4*). Given the importance of nitrogen for limiting ocean productivity, mechanisms that regulate DON production and removal could help control both the ocean's N balance and, consequently, the sequestration of atmospheric carbon dioxide.

Processes that lead to DON accumulation in seawater are unclear, but vertical profiles show that upper ocean DON concentrations are enhanced by 30 to 50% over deep water values (*5*). This observation suggests a major source for DON in the upper ocean and is consistent with findings that a large fraction of inorganic N assimilated by marine phytoplankton can be returned to seawater as DON (*6*). However, an important step for explaining DON profiles in the ocean is to identify compositional features that differentiate DON fractions with diverse biological reactivity. Here we report evidence for major structural differences between the DON pools of the surface and the deep ocean.

About 30% of DON occurs as a high-molecular-weight fraction (HMWDON) that can be sampled by ultrafiltration (*6*, *7*). The depth profile of HMWDON is similar to that of total DON, with high near-surface concentrations. Despite a decline in HMWDON concentrations below the mixed layer, nuclear magnetic resonance (NMR) spectra and amino acid analyses imply a homogenous chemical composition throughout the water column (*8*, *9*). Nitrogen-15 NMR (^{15}N-NMR) spectroscopy shows that nearly all HMWDON in the ocean is chemically bound as amide functional groups (*8*). Amides are commonly found in living marine organisms within proteins and biopolymers of *N*-acetyl amino polysaccharides (*N*-AAPs, e.g., chitin and peptidoglycan). However, acid hydrolysis of HMWDON yields only small amounts of amino acids and amino sugars, suggesting that HMWDON is deficient in these polymers (*9*, *10*). However, previous studies have also demonstrated that it is difficult to draw quantitative conclusions from analyses that require depolymerization of HMWDON compounds (*10*).

Rather than relying on a hydrolysis method that efficiently depolymerizes polysaccharides and proteins, we exploited differences in the amide bonds of proteins and *N*-AAPs to quantify the contribution of these biochemicals to marine DON. Amide bonds in proteins are an integral part of the peptide linkage, and amide hydrolysis depolymerizes proteins to yield amino acids (Fig. 1). Amide bonds in *N*-AAPs are not an integral part of the glycosidic linkage, and amide hydrolysis of *N*-AAPs is not directly coupled to depolymerization. Instead, amide hydrolysis de-acetylates the polysaccharide, releasing 1 mole of acetic acid for each mole of amide-N that is hydrolyzed (Fig. 1). Therefore, proteins can be quantified and distinguished from *N*-AAPs by the products of acid hydrolysis (amino acids and acetic acid, respectively). In both cases (proteins and *N*-AAPs), the hydrolysis of the amide bond produces amine-N. We used solid-state (cross-polarization magic-angle spinning) ^{15}N-NMR spectroscopy to follow the hydrolysis of HMWDON amide and quantify the conversion of amide-N to amine-N. Concurrent measurements of acetic and amino acid generation were used to partition HMWDON into proteins and *N*-AAPs. These experiments allowed us to construct a budget of nitrogen-containing biopolymers in marine HMWDON.

High molecular weight dissolved organic matter (HMWDOM) in surface seawater is rich in carbohydrate (60 to 80% of total C) and acetate (5 to 7% of total C), as seen in the ^{1}H-NMR spectrum for Woods Hole surface seawater (Fig. 2A) [carbohydrates, 5.2, 4.5-3.2, and 1.3 parts per million (ppm); acetate, 2 ppm]. Previous studies have shown that the chemical composition of Woods Hole HMWDOM is representative of marine HMWDOM in general (*10*, *11*). The ^{15}N-NMR for this sample (Fig. 2B) shows one major resonance at 124 ppm for amide-N (92% of total N) and a minor resonance at 35 ppm for amine-N (8% of total N). We hydrolyzed

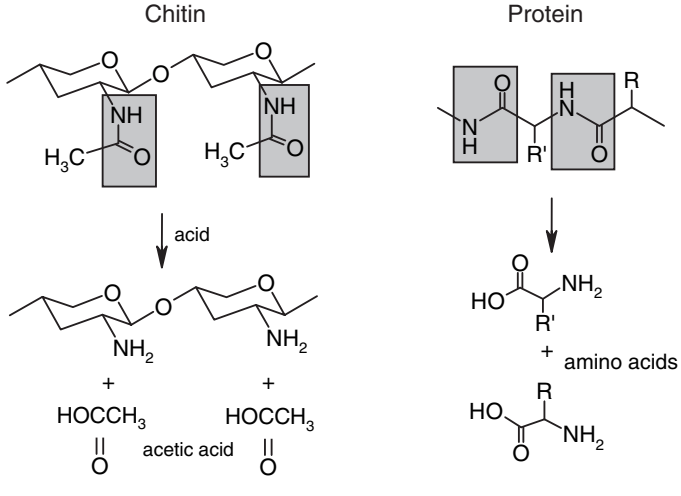

Fig. 1. Schematic showing the effect of mild acid hydrolysis on the amide linkage of proteins and *N*-AAPs (chitin). Mild acid hydrolysis (*13*) completely destroys the amide linkage (gray shaded area) in an *N*-AAP and quantitatively releases acetic acid. However, the glycosidic linkage remains unaffected, and the macromolecule is not depolymerized. Mild acid hydrolysis could likewise destroy the amide linkage in proteins (gray shaded area), thereby depolymerizing the macromolecule to release amino acids. If proteins and *N*-AAPs are the major biochemical components of HMWDON, then the sum of acetic and amino acids recovered after mild acid hydrolysis should equal the amount of amide destroyed during the reaction.

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Woods Hole HMWDON using conditions that were considerably milder than those typically employed for peptide bond hydrolysis (*12*) but that quantitatively release acetic acid from *N*-acetyl glucosamine and chitotriose (*13*). As expected in the case of *N*-AAPs, the acid hydrolysis (*13*) removed acetic acid from our samples (Fig. 2C) in quantifiable yield (Table 1) (*14*). Concurrent with the loss of acetic acid, there was a decrease in amide-N from 92% of the total N (7.1 μmol of N) to 35%, and an increase in the amount of amine-N from 8% of the total (0.6 μmol of N) to 65% (5 μmol of N) N (Fig. 2D and Table 1). Amino acid analysis showed that some proteins were hydrolyzed to amino acids that were recovered at 13% (Table 1) of the total N (*15*). The amount of amide-N converted to amine-N during acid hydrolysis, as quantified by ^{15}N-NMR spectroscopy, nearly equaled the molar sum of acetic and amino acids recovered by molecular level techniques (4.4 versus 4.3 μmol) (Table 1). The agreement between NMR spectroscopy and these molecular level analyses confirms that the hydrolyzed amide was originally present in HMWDON as proteins and *N*-AAPs. Under stronger hydrolysis conditions (*12*), we were able to recover 21% (1.6 μmol of N) of the total N in the sample as amino acids.

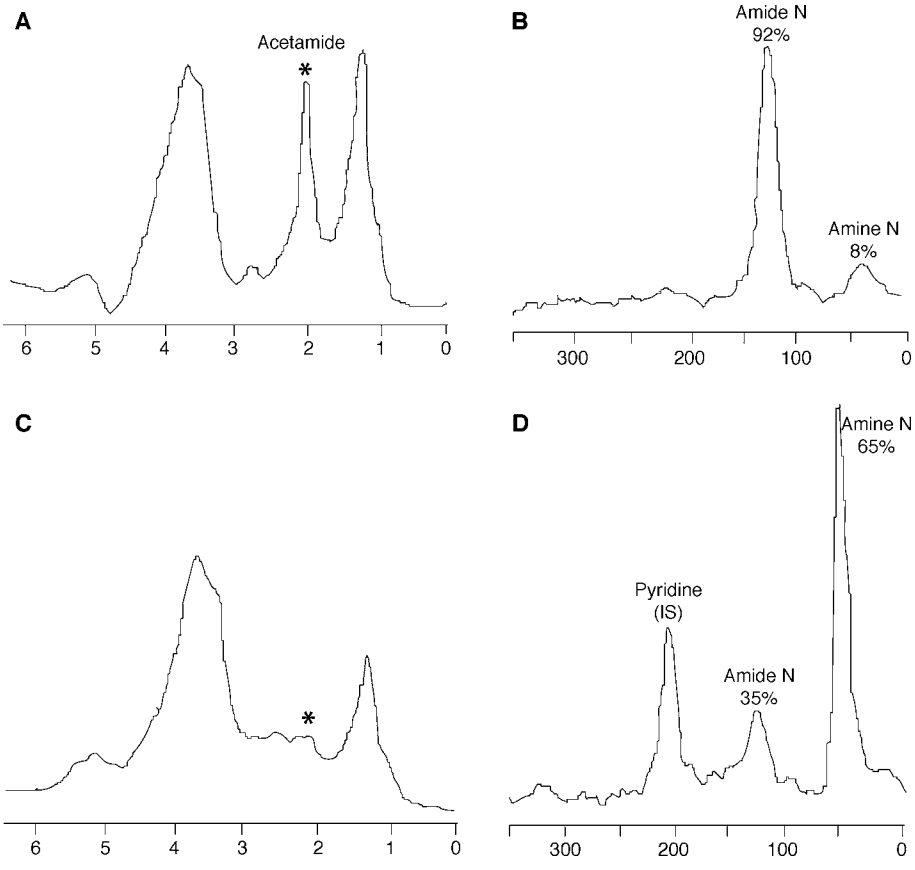

Fig. 2. The effect of mild acid hydrolysis (*13*) on the amide linkages of HMWDON isolated from Woods Hole surface seawater. (**A**) The ^{1}H-NMR spectrum [300 MHz, D_2O, δ (chemical shift in ppm downfield from tetramethylsilane)] shows bound acetic acid (*, 2.0 ppm). (**B**) The ^{15}N-NMR spectrum [400 MHz, solid-state, δ (chemical shift in ppm downfield from liquid NH_3)] before acid hydrolysis shows most nitrogen (92%) bound as amide. (**C**) Mild acid hydrolysis and organic extraction removes acetic acid, demonstrating complete de-acetylation. (**D**) This hydrolysis converts 57% of the amide-N into amine, commensurate with the amount of acetic and amino acids recovered by molecular level analyses (56%) (Table 1) and increases the total amine-N to 65%. Pyridine added to quantify losses during sample processing showed 98% recovery of HMWDON. IS, internal standard.

Using acetic acid as a proxy for *N*-AAPs, our results indicate that HMWDON in surface seawater is 43% *N*-AAPs (Table 1), 21% hydrolyzable protein, and 29% (2.2 μmol of N) nonhydrolyzable amide (*16*). The remaining HMWDON in our Woods Hole sample (8%, 0.6 μmol of N) initially present as amine was not characterized by our analyses but may be present as basic or N-terminal amino acids of proteins or as amino sugars. We obtained a similar agreement between the quantity of N converted from amide to amine and the molar sum of amino acids plus acetic acid recovered by molecular level analyses for HMWDON sampled from the North Pacific Ocean (Table 1); the agreement confirms the ubiquity of *N*-AAPs. Our analyses demonstrate that soluble *N*-AAP biopolymers contribute ~26% of the carbon and 40 to 50% of the nitrogen to surface ocean HMWDOM (*16*).

Peptidoglycan, currently assumed to dominate the oceanic reservoir of HMWDON, is rich in *N*-acetyl glucosamine and *N*-acetyl muramic acid (present in a 1:1 ratio) (*9*). Both sugars contain amide-N and are potential sources for the *N*-AAPs we quantified by our NMR experiment. The presence of acetylated amino sugars in HMWDON has been inferred previously from mass spectrometric data (*17*, *18*), but recoveries of *N*-acetyl glucosamine and *N*-acetyl muramic acid, which make up the amide-rich glycan of peptidoglycan, are low (*10*, *11*, *19*). Even under conditions specific for peptidoglycan hydrolysis (*20*, *21*), we were unable to detect muramic acid in our samples. Glucosamine, though present, contributed <1% of the total carbon, far lower than the ~10% HMWDOM-carbon ex-

Table 1. Change in amide content and yields of acetic and amino acids, upon mild acid hydrolysis of HMWDON. All percentages are expressed relative to total μmol of N.

Sample	Total (μmol N)*	Amide (μmol N)	Δ Amide (μmol N)†	Acetic acid (μmol)	Amino acids (μmol)	Σ Acetic + amino acids (μmol)	Unhydrolyzed amide (μmol N)‡
Woods Hole (5 m)	7.7	7.1 (92%)	–4.4	3.3 (43%)	1.0 (13%)	4.3	2.7 (35%)
MAB (1000 m)	7.7	7.7 (100%)	–1.8	1.3 (17%)	0.8 (10%)	2.1	5.8 (76%)
Hawaii (23 m)	6.2	6.2 (100%)	–3.7	3.3 (53%)	0.5 (8%)	3.8	2.4 (39%)
Hawaii (600 m)	6.2	6.2 (100%)	–3.7	3.4 (55%)	0.4 (6%)	3.8	2.4 (39%)

*Values are expressed per 100 μmol of HMW dissolved organic carbon and calculated based on the C/N ratio of each sample. †Δ Amide is the change in the amount of amide-N after hydrolysis, as quantified by integration of the ^{15}N-NMR spectra. In all cases, hydrolysis resulted in the loss of amide-N. ‡The quantity (μmol of N) of amide remaining in the sample after mild (*13*) hydrolysis, quantified directly by ^{15}N-NMR spectroscopy. Higher concentrations of HCl (*12*) hydrolyzed a greater percentage of the amide-N, leaving slightly less (2.2 and 5.5 μmol) amide-N unhydrolyzed in the Woods Hole and MAB samples, respectively. In order to quantify the amount of amide-N remaining after strong acid hydrolysis the Σacetic acid (mild) + amino acids (strong) was first determined and then subtracted from the initial amide content of the HMWDON sample (before hydrolysis) (*16*). Here, acetic acid is being used as a proxy for *N*-AAPs.

pected if all of the acetic acid in our samples was from peptidoglycan. Possible explanations for the general discrepancy between NMR-derived estimates of *N*-AAPs and molecular-level carbohydrate analyses are incomplete depolymerization of *N*-AAPs and rapid Maillard condensation reactions of hydrolysis products (*22*).

Muramic acid also contains lactic acid, which can be quantitatively released from peptidoglycan without depolymerizing the glycan (*23*). For peptidoglycan, 1 mol of lactic acid will be released for every 2 mol of acetic acid. Concentrations of lactic acid (*14*) in our samples were <0.4% of HMWDOM-carbon, an order of magnitude less than expected if all of the acetic acid we recovered was from peptidoglycan. The low concentration of lactic acid measured in this study and the low concentration of D-amino acids previously reported for HMWDON (*9*) together suggest that peptidoglycan is at best only a minor component of HMWDON. In addition, the D-amino acids in HMWDON so far identified to be present in peptidoglycan (*9*) could be present in a number of compounds synthesized by prokaryotes and eukaryotes (*24*, *25*).

Because *N*-AAPs represent ~40 to 50% of the HMWDON in surface waters, and given that 40 to 50% of HWMDON is removed below the mixed layer (*6*, *8*), we hypothesize that the global decrease in HMWDON with depth in the oceans results from the selective removal of *N*-AAPs. To test this hypothesis, we analyzed HMWDOM collected from a depth of 1000 m in the Middle Atlantic Bight (MAB), which has radiocarbon, ^{1}H-NMR, and molecular-level properties characteristic of deep-sea HMWDOM (*10*). In agreement with previous ^{15}N-NMR spectra of deep sea HMWDON (*8*), the ^{15}N-NMR spectrum of our sample shows one major resonance characteristic of amide-N (100% of total N) (Fig. 3A). Mild acid hydrolysis (*13*) decreased amide-N from 100% to 76% of total N and increased amine-N from undetectable levels to 24% of total N (Fig. 3B and Table 1). After acid hydrolysis, acetic acid was released from HMWDOM, as were amino acids, which increased from undetectable levels to 10% of total N. The sum of acetic plus amino acids (2.1 μmol) in the hydrolysis products was similar to the increase in amine-N (1.8 μmol of N) observed with ^{15}N-NMR spectroscopy. Molecular-level analyses after strong acid hydrolysis showed deep-sea HMWDON was 17% *N*-AAPs (Table 1), 12% hydrolyzable protein, and 71% nonhydrolyzable amide (5.5 μmol of N) (*16*). Despite the surface water abundance, a large fraction of *N*-AAPs are lost during mixing into the deep ocean. Proton NMR spectra of numerous deep-sea samples showed a sharp decrease in acetate below the mixed layer, confirming the loss of *N*-AAPs with depth (Fig. 4). The loss in *N*-AAPs and the relative increase in nonhydrolyzable amides will not result in any change to the ^{15}N-NMR spectrum (*8*).

The lack of amines in marine HMWDON implies that amine-N is more labile than amide-N. Many marine microorganisms have cell surface–bound deaminases that are capable of extracting amine-N from a variety of organic compounds (*26*). These enzymes could render HMW amine-N more biologically available by allowing organisms to bypass polymer hydrolysis or uptake. The eventual depth-dependent loss of *N*-AAPs from HMWDON suggests either that *N*-AAP compounds are labile or that organisms have developed a mechanism to access the nitrogen in *N*-AAPs without complete hydrolysis of the polymer (perhaps through cell surface–bound acetamidases). Although we were unable to depolymerize *N*-AAPs using acid hydrolysis, the capacity to enzymatically degrade *N*-AAPs may be widespread among marine microbes. In particular, the ability to hydrolyze chitin [β (1→ 4)-(*poly*) *N*-acetyl D-glucosamine] is notable in members of the marine α-proteobacteria and the *Cytophaga-Flavobacter* cluster (*27*, *28*). The widespread occurrence of chitinase activity and the ubiquity of chitinase genes in marine bacteria (*29*) imply that chitin-like biopolymers are important substrates in the marine environment, consistent with the abundance of *N*-AAPs in HMWDON. The presence of specialized bacteria could explain the ultimate removal of *N*-AAPs from the marine environment.

More than 90% of DON is sequestered in the deep sea, and most deep-sea HMWDON

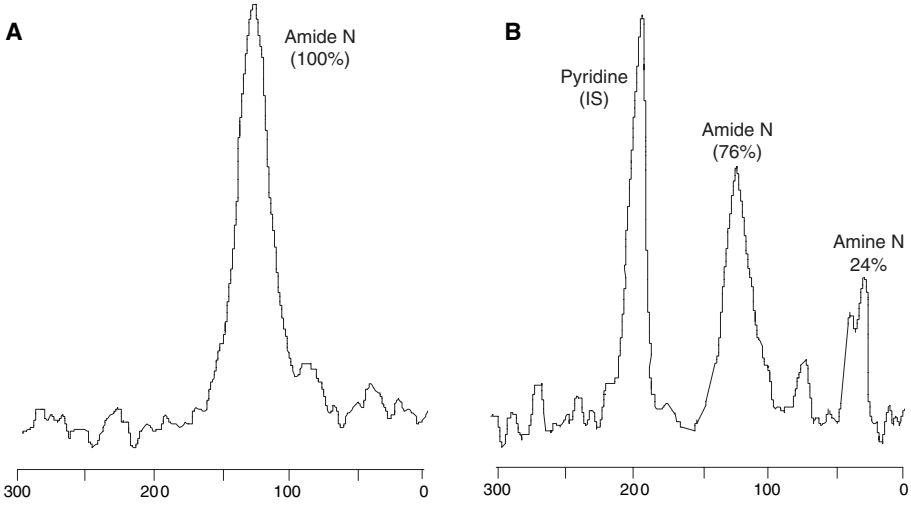

Fig. 3. The effect of mild acid (*13*) hydrolysis on the amide linkage of deep-sea HMWDON. (**A**) The ^{15}N-NMR spectrum of HMWDON shows only one resonance for amide-N [400 MHz, solid state δ (chemical shift in ppm downfield from liquid NH_3)]. (**B**) Treatment of the sample with mild acid converts 24% of the nitrogen to amine but does not affect most (76%) of the nitrogen. Pyridine (IS), added as an internal recovery standard, showed >98% N recovery through the hydrolysis procedure. Deep-sea HMWDON may also include some (<13% total N) contribution from pyrrole- and indole-N, which resonate between 130 and 215 ppm and overlap with the amide region in our ^{15}N-NMR spectrum.

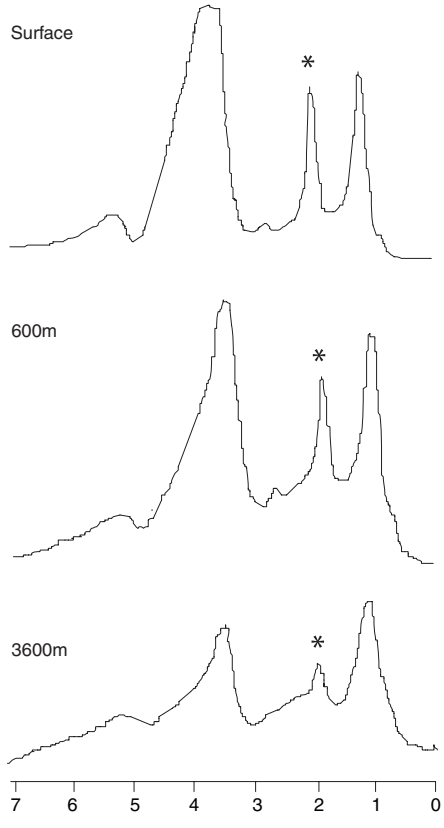

Fig. 4. The ^{1}H-NMR spectra [400 MHz, D_2O, δ(ppm)] of HMWDOM isolated from several depths (surface, 600 m, and 3600 m) in the Pacific Ocean (31°N, 159°W; the total water column depth was 5770 m). The peak at 2.0 ppm (*) arises from acetate, which is presumed to be present in *N*-AAPs. The clear decrease in the relative amount of acetate with depth is interpreted as a depth-dependent loss of *N*-AAPs.

is not hydrolyzed by treatment with strong acids (Table 1). Hydrolysis-resistant amides have been observed in marine particulate matter and sediments, where the resistance to chemical hydrolysis has been attributed to physical sorption and encapsulation (*30*). Amide-N in DON is not physically protected, but previous experiments have shown that the biodegradation rate of labile compounds such as proteins is substantially reduced by abiotic complexation within marine DOM (*31*). Long-term protein-DOM interactions may lead to structural modifications that render proteins resistant to chemical hydrolysis and unavailable to bacteria. This mechanism could lead to the sequestration of nitrogen in the dissolved phase (*32*) and give rise to the hydrolysis-resistant amide-N observed by ^{15}N-NMR.

Our data show that two chemically distinct pools of organic nitrogen accumulate in the ocean. The higher concentration of HMWDON in the mixed layer (relative to deep ocean values) largely reflects the presence of *N*-AAPs, which degrade on time scales of upper ocean mixing. These newly added biopolymers are chemically distinct from the refractory HMWDON pool that exists throughout the water column. If we assume the proportion of *N*-AAPs, protein, and nonhydrolyzable amide measured in our samples is representative of global HMWDON, then as much as 80% of the decrease in HMWDON with depth involves the removal of *N*-AAPs. The abundance of amide-N throughout the water column suggests amides are more biologically recalcitrant than other forms of organic-N. The ubiquity of amide linkages in HMWDON is not surprising, given that most organic nitrogen in phytoplankton is protein. However, the important contribution of *N*-AAPs to upper ocean HMWDOM, and the resistance of amides in deep sea HMWDOM to chemical and biological degradation, are unexpected results that help elucidate the currency of DON in the marine nitrogen cycle.

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13. De-acetylation of HMWDON samples was performed in 1 N HCl. Samples were heated overnight at 90°C under an atmosphere of N_2. The acetic acid produced during the hydrolysis was extracted from a weighed aliquot of the hydrolysate into either ethyl ether or dichloromethane. After extraction, the presence of acetic acid in the organic fraction was confirmed by solution-state ^{1}H-NMR before low molecular weight acids (e.g., acetic acid) were quantified. The aqueous fraction of the hydrolysate was lyophilized and, as shown in the ^{1}H-NMR (Fig. 2C), no longer contained acetamide. Changes in C and N were assessed with solid-state ^{13}C- and ^{15}N-NMR spectroscopy. Under these hydrolysis conditions, no muramic acid (de-acetylated) or amino sugar monomers were detected.
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21. We modified the method provided in (*20*) and hydrolyzed samples (in 4 N HCl) overnight at 90°C. We recovered no muramic acid and only small amounts of glucosamine and galactosamine from surface samples. This modified hydrolysis method was tested on chitin oligomers, peptidoglycan (from *Bacillus subtilus*), and bacterial cells (mixed laboratory culture) to ensure the quantitative (>90%) recovery of glucosamine and de-acetylated muramic acid. We assessed amino sugar recoveries by high-performance liquid chromatography and fluorescence detection of *o*-phthaldialdehyde-derivatized samples (*15*) or gas chromatography after derivatizing to alditol acetates (*10*).
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Assessing Methane Emissions from Global Space-Borne Observations

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In the past two centuries, atmospheric methane has more than doubled and now constitutes 20% of the anthropogenic climate forcing by greenhouse gases. Yet its sources are not well quantified, introducing uncertainties in its global budget. We retrieved the global methane distribution by using space-borne near-infrared absorption spectroscopy. In addition to the expected latitudinal gradient, we detected large-scale patterns of anthropogenic and natural methane emissions. Furthermore, we observed unexpectedly high methane concentrations over tropical rainforests, revealing that emission inventories considerably underestimated methane sources in these regions during the time period of investigation (August through November 2003).

Methane (CH_4) is, after carbon dioxide (CO_2), the second most important anthropogenic greenhouse gas (*1*). It also has an indirect effect on climate through chemical feedbacks (*1*, *2*). More than 50% of present-day global methane emissions are anthropogenic, the largest contributors being fossil fuel production, ruminants, rice cultivation, and waste handling (*3*). The natural source strength of CH_4, mainly constituted by wetlands, is particularly uncertain, because these emissions vary considerably in time and space (*4*, *5*) and available ground-based measurements are sparse, albeit precise, and limitedly representative at larger scales. Better knowl-

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