Major Bacterial Contribution to Marine Dissolved Organic Nitrogen

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Next to N_2 gas, the largest pool of reduced nitrogen in the ocean resides in the enormous reservoir of dissolved organic nitrogen (DON). The chemical identity of most of this material, and the mechanisms by which it is cycled, remain fundamental questions in contemporary oceanography. Amino acid enantiomeric ratios in the high molecular weight fraction of DON from surface and deep water in three ocean basins show substantial enrichment in D enantiomers of four amino acids. The magnitude and pattern of these D/L enrichments indicate that peptidoglycan remnants derived from bacterial cell walls constitute a major source of DON throughout the sea. These observations suggest that structural properties of specific bacterial biopolymers, and the mechanisms for their accumulation, are among the central controls on long-term cycling of dissolved organic nitrogen in the sea.

Most of the surface ocean is characterized by low to undetectable mineral nutrient concentrations. Such oligotrophic regions are central to global geochemical cycles, accounting for almost 40% of global primary production (*1*). Recently, the accumulation and advected export of dissolved organic matter (DOM) from such environments has been recognized as a major pathway for C flux in the upper ocean (*2*), a process likely regulated in part by the low levels of biologically available N (*3*). Yet these same waters contain substantial concentrations of fixed N in dissolved organic compounds (*4*). The apparent lower biological accessibility of this large organic N reservoir thus represents both a major control on upper ocean carbon cycles, and a corresponding "N pump" fundamental to closing oceanic N budgets (*3*, *4*).

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The chemical identity of DON is key to understanding the mechanisms by which it is formed and escapes remineralization. Although amino acids account for most N in organisms, only a small fraction of seawater DON can be identified as amino acid by common hydrolytic methods (*5*). The vast majority of this dissolved nitrogenous material appears to be contained in amide functional groups (*6*), suggesting that most long-lived DON is hydrolysis-resistant or composed of recalcitrant nonprotein amide-containing biochemicals.

Amino acid enantiomeric ratios can provide a powerful tool for characterizing nitrogenous materials (*7*). Early work identified elevated levels of D-aspartic acid and D-alanine in the open sea, indicating that D-amino acids may serve as indicators for both DOM sources (*8*) and for abiotic transformation reactions (*9*). Limitations of earlier filtration methods, however, prevented living bacteria from being excluded as a principle source of these observations (*8*), and interpretation was further complicated by substantial variability in the observed magnitude and distribution of D/L ratios with methods then available (*10*). Recent application of large-scale tangential-flow ultrafiltration to ocean waters has allowed reproducible isolation of DOM from seawater with essentially no contamination by living organisms or particulate matter (*11*, *12*), in quantities sufficient to determine a full range of amino acid enantiomeric ratios. We have used this method to examine amino acid D/L ratios in the high molecular weight fraction of oceanic DOM from the central Pacific Ocean, the Gulf of Mexico, and the North Sea (*13*). These samples include surface and abyssal depths in two oligotrophic open ocean basins, as well as a biologically dynamic coastal region.

Recovery efficiencies of ultrafiltered DOM

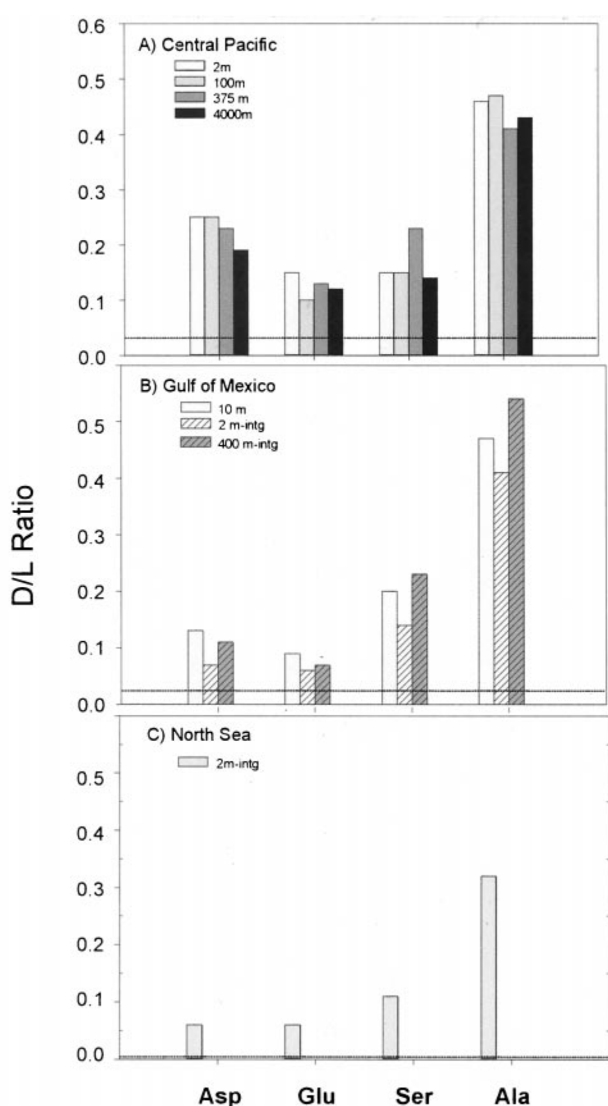
Table 1. Bulk properties and D/L–amino acid ratios from a marine cyanobacterium and for UDOM from the Central Pacific, Gulf of Mexico, and North Sea (*13*). Bulk properties include ultrafiltered dissolved organic carbon (UDOC) and nitrogen (UDON) concentrations, and atomic carbon/nitrogen ratios (C/N)a. UDOC and UDON concentrations are derived from C/N data, the dry weight recovered, and the volume filtered. D–amino acids for UDOM-hydrolyzable amino acids (*14*) are expressed as simple enantiomeric ratios, not corrected for the racemization blanks, which are shown for comparison. Sample collection depth is indicated in meters. Asterisks indicate samples in which the D/L ratio could not be quantified reliably due to chromatographic mixtures (as indicated by GC-MS); ND (not detected), samples in which the value was less than 1.5 × racemization blank. *S. bacillaris*, *Synechococcus bacillaris* (*25*).

Bulk properties and D/L–amino acid ratios	Blank	*S. bacillaris*	Central Pacific				Gulf of Mexico			N. Sea
			2	100	375	4000 m	10	2	400 m	2 m
DOC (μM)	–	–	82	85	53	45	95	97	58	76
UDOC (μM)	–	–	22.2	22.2	10.6	8.08	21.1	9.73	1.26	10.1
UDON (μM)	–	–	1.33	1.42	0.63	0.45	1.19	0.63	0.09	0.71
(C/N)a	–	–	16.8	15.6	16.9	18.4	17.8	15.4	14.2	14.2
Asp	0.09	0.14	0.39	0.39	0.36	0.33	0.28	0.18	0.22	0.17
Glu	0.06	0.09	0.23	0.17	0.20	0.19	0.16	0.13	0.14	0.12
Ser	0.03	ND	0.19	0.19	0.28	0.18	0.21	0.18	0.28	0.15
Thr	0.00	ND	ND	ND	ND	0.15	**	ND	0.05	ND
Ala	0.03	0.38	0.52	0.54	0.47	0.49	0.53	0.47	0.61	0.37
Tyr	0.04	ND	ND	0.10	**	**	ND	ND	0.10	ND
Met	0.08	ND	ND	ND	**	ND	ND	ND	ND	ND
Val	0.02	ND	ND	0.07	ND	0.06	ND	ND	0.13	ND
Phe	0.02	0.04	0.04	0.05	0.04	0.04	**	0.11	0.04	**
Leu	0.07	ND	0.12	0.13	0.12	**	ND	0.14	**	ND
Lys	0.04	0.07	ND	0.09	0.12	0.09	ND	0.08	ND	ND

(UDOM) are typically ~20% in deep waters and ~30% in surface waters (Table 1) (*12*), although very large sample sizes decrease relative recoveries (*13*). Average C/N ratios for this sample set were 17.0 for surface and 16.5 for deep water UDOM, respectively (Table 1). Hydrolyzable amino acids in these, as well as similar samples, constitute 10 to 20% of total UDOM N (*5*, *6*). Surface and deep UDOM samples show substantial and highly characteristic D/L–amino acid ratios for all the sampled ocean basins (Table 1 and Fig. 1). Alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), and serine (Ser) consistently have D/L ratios far above blank levels (Table 1 and Fig. 2) (*14*). Ala shows the most pronounced enrichment, with D/L ratios near 0.5, whereas Asp, Glu, and Ser ratios generally fall between 0.2 and 0.4. No other D–amino acids could be repeatably detected above blank levels (Fig. 1). The D/L ratios of these four were remarkably consistent between widely separate locations, sampling times, and ocean depths (Fig. 1) and appear to represent a fundamental signature of UDOM throughout the ocean.

D–Amino acids are gradually produced in ancient organic matrices by abiotic racemization and may also be formed by degradation reactions of specific protein L–amino acids (*9*). However, the amino acid D/L and molar signatures in UDOM are not consistent with either of these origins. Bada and Hoopes (*9*) suggested abiotic dehydration of Ser as the most likely explanation for the near racemic D/L-Ala ratios they observed in the deep Pacific. This mechanism should produce linked decreases in Ser and threonine (Thr) as well as a concomitant buildup of α-amino butyric acid (Aba) (*9*). UDOM isolates, however, exhibit no increased D-Ala at depth and minimal variation in mole percent Ser or Thr (*5*). Similarly, no Aba was detected by gas chromatographic–mass spectral (GC-MS) analysis of any sample.

Although abiotic equilibration of enantiomeric forms is a well-known D–amino acid source, stereochemical inversion rates at ocean temperatures should be far too slow (*8*, *15*) to account for appreciable racemization over average DOM residence times of 4000 to 6000 years (*16*, *17*). Even if D–amino acid signatures represent a far older component of UDOM, the observed highly selective pattern of D enrichment remains inconsistent with abiotic generation. Although multiple factors affect geochemical racemization rates (*18*), relative rates among different amino acids generally follow a regular succession (*19*). Ala in particular is among the slower amino acids to racemize (*20*). At the observed Ala D/L ratio of ~0.5, abiotically derived D/L ratios of both phenylalanine (Phe) and in particular Asp (*21*) should be approaching their equilibrium D/L value of 1.0 (Fig. 2). However, in UDOM values of Asp are among the lowest observed D/L ratios, and D-Phe was near blank levels (Fig. 2). Moreover, the absence of any depth trend in the D/L ratios of any amino acid (Fig. 1) argues strongly that the stereoisomeric compositions of UDOM amino acids are not generated by aging or



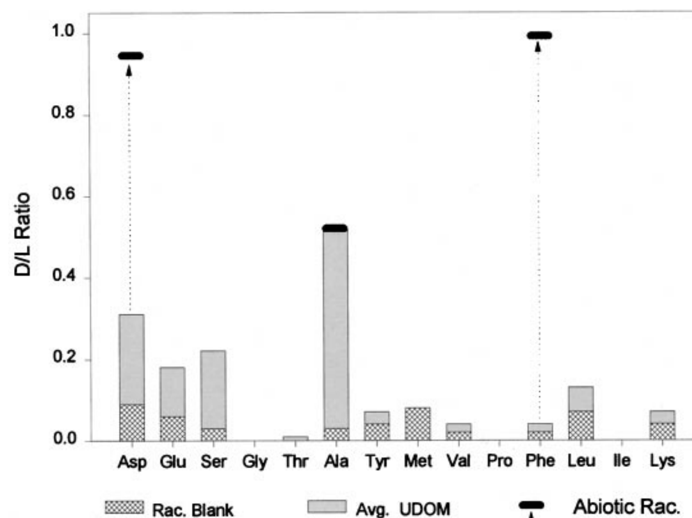


Fig. 1 (left). UDOM D/L ratios for (**A**) Central Pacific, (**B**) Gulf of Mexico, and (**C**) North Sea at various depths for the four principle amino acids. Open bar for Gulf of Mexico represents the surface sample (10 m) independently processed with 0.2-μm cartridge filters (*13*). Dashed lines reflect average values for all the other amino acids measured. D/L values in this figure have been adjusted for racemization blanks by subtracting molar quantities expected from hydrolytic racemization from total molar quantities of D-acid measured. These estimates do not account for reverse racemization of original D forms, and thus represent minimum ratios. **Fig. 2 (above).** Alternative D–amino acid sources: Comparison of the magnitudes and distributions of D–amino acid ratios encountered in UDOM with those from other potential sources. Hydrolysis: Average hydrolysis blank D/L ratios (hatched bars) are superimposed on overall average UDOM D/L ratios (solid bars). Average racemization blanks were D/L ratios produced from pure L mixtures during hydrolysis. Abiotic racemization: Relative abiotic D/L ratios (dark bars), based on relative racemization rates for natural waters (*20*), were estimated to correspond with a hypothetical average "abiotic" UDOM D/L-Ala ratio of 0.5.

selective utilization of L–amino acids over time, but instead represent an intrinsic biochemical signature of seawater DON.

The principal biochemical sources of D–amino acids are peptidoglycans, the main structural component of bacterial cell walls. Peptidoglycans are heterogeneous polymers composed of an amino-sugar backbone cross-linked with peptide bridges. The bridging peptides are characterized by a number of unusual nonprotein amino acids and D–amino acids. In particular, the D-enantiomeric forms of Ala, Glu, and Asp are prevalent (*22*). D-Ala is the most abundant D–amino acid in most peptidoglycans and the only one that is universally incorporated (*23*). The enantiomeric patterns that characterize UDOM thus closely match those characteristic of bacterial peptidoglycans.

Because free-living bacteria are abundant in ocean water, direct contamination of DON isolates must be carefully excluded (*8*). Our UDOM isolation protocol was designed to remove essentially all natural microorganisms, and its efficacy has been verified both by filter intercomparison and direct bacterial counts. On average, >99% of oceanic bacteria are removed by the 0.1-μm pore size filters with which most samples were processed before UDOM isolation. In addition, the central Pacific samples were directly examined by epifluorescence microscopy after passage through the 0.1-μm pore size prefilter, and bacterial numbers were below detection (*12*). Typical estimates for N per cell of oceanic bacteria (*24*) indicate that even a maximal limit of 10% of original microbes passing the 0.1-μm pore size filter would contribute less than 1% to total UDOM amino acids. The previously mentioned lack of any depth trend in UDOM D–amino acid abundance, despite a corresponding 10-fold decrease in bacterial abundance (*12*), confirms that the observed D–amino acids derive not from concentrated bacteria, but from the vastly larger pool of nonliving DOM.

The observed high D/L ratios strongly suggest that peptidoglycans constitute a major component of UDOM N. Most characterized oceanic water column bacteria are Gram-negative, typified by relatively thin peptidoglycan layers (*22*). Although the specific bacterial sources of peptidoglycan in the open sea are unknown, the cell wall–enriched fraction of a cosmopolitan oceanic cyanobacterium, *Synechococcus bacillaris*, gives Ala D/L ratios of 0.38, in the same general range as UDOM values (Table 1) (*25*). This supports prior evidence that cell wall material of marine bacteria is compositionally similar to that of terrestrial bacteria (*26*). The contrasting relative amounts of other D–amino acids from *Synechococcus* suggests, as would be expected, that this bacterial species alone does not dominate UDOM sources.

Because of the inherent variability among known peptidoglycan structures (*27*) and the poorly defined species compositions of marine bacterial communities, finite percentages of peptidoglycan in UDOM isolates cannot be quantified with certainty. However, the following rough estimate of relative N contributions can be made using broad structural information. Ala represents the most useful source tracer, because it is a main component of proteins and has the most consistent D/L ratios within studied peptidoglycans (*22*, *23*). The D enantiomer makes up about 30% of total hydrolyzable Ala in UDOM [percent D = D/(D+L), thus 30% D is equivalent to a D/L ratio of ~0.5], whereas pure peptidoglycans contain ~40 to 50% D-Ala. On the basis of these D–amino acid contents and average structural features of common peptidoglycans and proteinacous materials, a calculation of the relative total N contributions from these two biochemical types can be made. Such a calculation (*28*) indicates that peptidoglycan accounts for a similar amount of the total N (45 to 80%) as is derived from hydrolyzable protein. Thus, in terms of N, this one structural biopolymer may be at least as abundant in UDOM as conventional proteinaceous material. In fact, this could be a minimal estimate, because conventional hydrolytic methods are optimized at near 100% for proteins (*29*) and might be substantially less efficient for peptidoglycan residues in an environmental matrix. Methods that define "protein" on the basis of total hydrolyzable amino acid yields from seawater DOM would also actually include a component of peptidoglycan-derived amino acid.

Such a large peptidoglycan component carries major implications regarding the chemical composition of the high molecular weight fraction of marine DOM. For example, UDOM also should be enriched in amino sugar, a major component of peptidoglycan. Such an enrichment is consistent with aldose-poor UDOM sugar composition (*5*), as well as the specific identification of an amino sugar component by thermal desorbtion–mass spectrometry (*30*) and pyrolysis (*31*). In addition, comparison of average amino acid compositions of UDOM (*5*) with those of planktonic sources (*32*) indicates that UDOM is measurably enriched in Ala, Glu, and Ser, consistent with an additional nonproteinaceous source for these specific amino acids. Finally, evidence for an important peptidoglycan component is supported by previously mentioned observations (*6*) of a predominant amide component not quantifiable by conventional amino acid analysis.

Although UDOM alone accounts for a substantial fraction of total oceanic DOM, the extent to which these results apply to all dissolved nitrogenous material will be determined by overall size-related compositional trends. The extent of such size-related differences within the oceanic DOM pool are not fully known. However, comparisons of many properties of UDOM and total DOM (for example, C/N ratios, stable isotopic ratios, radiocarbon content, amino acid yields, and molecular-level signatures) indicate a high degree of overall compositional similarity (*5*, *11*, *12*). Recent studies of filtered whole seawater from the surface Arctic Ocean has yielded almost identical D–amino acid distributions and ratios as those in our UDOM isolates, yet from a distinctly separate ocean region (*33*). Earlier identification of high D–amino acid concentrations in unfractionated seawaters (*8*, *9*), though more variable, is also consistent with the presence of peptidoglycan structural remnants in smaller size classes of dissolved material.

Given that N from algae, as well as most of that from bacteria, is in the form of proteins containing no D–amino acids, the elevated D/L ratios in UDOM indicate that the high molecular weight nitrogenous material dissolved in seawater is enriched in bacterial cell wall material. Bacteria are widely recognized as major consumers of organic matter and in some oligotrophic ocean regions may also be the major primary producers (*34*). Thus, abundant potential sources for bacterial cell-wall material likely exist throughout the water column. The accumulation and environmental persistence of these materials may also be related in part to intrinsic structural properties. Structural polymers can display long-term geochemical stability (*35*). The interwoven polysaccharide matrix of peptidoglycan, coupled with the unusual peptide substituents and structural variability, creates a heteropolymer resistant to many common hydrolytic enzymes (*36*). In addition, laboratory experiments and the identification of specific bacterial membrane proteins in seawater (*37*, *38*) suggest that more labile biochemicals may also be shielded by close association in a cell-wall matrix.

The high D/L-amino acid ratios found in UDOM indicate that a substantial fraction of dissolved organic N in the sea is of bacterial origin. This result challenges the common paradigm that the enormous reservoir of oceanic dissolved material is predominantly derived from algal sources. Central oceanic ecosystems are characterized by intensive bacterial recycling of DOM, coupled with similarly dynamic bacterial removal by protozoans and viruses (*39*). These processes represent direct pathways for introduction of bacterial cell wall structures into dissolved and colloidal seawater pools (*38*, *40*), where minor differences in bioreactivity may result in substantial accumulation. Bacterial predation, coupled with the intrinsic structural properties of bacterial cell wall material, may thus be among the major controls on the long-term cycling of organic N in the sea.

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14. UDOM amino acid hydrolysis was conducted at 150°C for 70 min, generally with the method of Cowie and Hedges (*29*). Individual D– and L–amino acids were quantified as pentafluoropropyl isopropyl esters by gas chromatography with flame ionization detection (*41*), and peak identities were verified by GC-MS. Analytical variability in D/L ratios of UDOM samples was less than 15%. Racemization blanks were determined by multiple hydrolyses of pure L–amino acid mixtures, as well as protein standards. Enantiomeric ratios in commercially available D–amino acid–containing peptides could be repeatably determined to within 5%.
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The CO_2 Balance of Unproductive Aquatic Ecosystems

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Community respiration (*R*) rates are scaled as the two-thirds power of the gross primary production (*P*) rates of aquatic ecosystems, indicating that the role of aquatic biota as carbon dioxide sources or sinks depends on its productivity. Unproductive aquatic ecosystems support a disproportionately higher respiration rate than that of productive aquatic ecosystems, tend to be heterotrophic ($R > P$), and act as carbon dioxide sources. The average *P* required for aquatic ecosystems to become autotrophic ($P > R$) is over an order of magnitude greater for marshes than for the open sea. Although four-fifths of the upper ocean is expected to be net heterotrophic, this carbon demand can be balanced by the excess production over the remaining one-fifth of the ocean.

Aquatic ecosystems cover 70% of Earth's surface (*1*) and contribute 45% of the global primary production (*2*). Yet, the role of their biota in the global CO_2 budget remains a subject of debate (*3–5*). Many freshwater ecosystems act as CO_2 sources (*6*); in contrast, oceanic ecosystems are assumed to act as CO_2 sinks (*7, 8*). This assumption has been challenged by calculations suggesting that the coastal ocean may be net heterotrophic (*9*) and by the finding that bacterial metabolism exceeds phytoplankton production in unproductive waters (*10*), which make up >30% of the ocean. These conclusions are based on indirect calculations and controversial assumptions (*3*). Here, we compare the gross primary production (*P*) and respiration (*R*) rates of aquatic communities to elucidate whether the biota of aquatic ecosystems acts as net CO_2 sources ($R > P$) or sinks ($R < P$). We compiled data obtained over the past five decades from studies in which oxygen evolution was used as a surrogate for carbon fluxes (*11*).

Community metabolism varied by over four orders of magnitude across aquatic ecosystems (Table 1). Marshes tended to be more productive than other aquatic ecosystems, whereas open sea communities showed the lowest production and respiration rates (Table 1). The

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