

Bioavailability of wetland-derived DON to freshwater and marine bacterioplankton

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Abstract

Access to bioavailable nitrogen often limits primary production in marine and freshwater ecosystems. Around 70% of nitrogen transported by rivers worldwide consists of dissolved organic nitrogen (DON), but its bioavailability has been poorly investigated. To assess the potential bacterial growth on DON, we developed a bioassay employing natural DON and bacterial inocula in medium manipulated to make N the limiting nutrient. We analyzed the bacterial utilization of the high-molecular-weight fraction of DON isolated by ultrafiltration from three wetlands in South Sweden throughout the year. The bioavailability of low-molecular-weight and bulk DON was also analyzed in one of the wetlands, where inorganic nitrogen concentration was sufficiently low and did not interfere with bioassays. The bioavailability of bulk DON in the latter wetland varied from 2% to 16%, suggesting that DON is an important nitrogen source for the biota of coastal waters. DON may be the dominant input of bioavailable nitrogen during summer, when nitrate concentrations in rivers decrease and DON bioavailability increases. Marine bacterioplankton assimilated a substantially larger fraction of DON than did freshwater bacterioplankton, on average by a factor of 2.4. This finding indicates that the susceptibility of DON to bacterial mineralization increases as it is transported from freshwaters into saline environments.

Access to bioavailable nitrogen often limits primary production in marine environments (Ryther and Dunstan 1971; Granéli et al. 1990) and in humic (Jansson et al. 1996), ultraoligotrophic (Axler et al. 1982), and phosphorus-sufficient (Schindler 1977) lakes. Enhanced eutrophication of aquatic ecosystems due to nitrogen loading is a worldwide problem (Meybeck 1982). Research on the role of nitrogen in aquatic eutrophication is almost exclusively focused on dissolved inorganic nitrogen (DIN) forms. However, about 70% of the dissolved nitrogen transported by rivers worldwide (10^{12} g yr^{-1}) is dissolved organic nitrogen (DON; Meybeck 1982). DON comprises 35–50% of the total nitrogen reaching coastal waters from Sweden (Fleischer and Stibe 1989). In temperate regions, the share of DON in total nitrogen increases during summer, when nitrate concentrations decrease in many freshwater (Pardo et al. 1995) and marine (Carlsson and Granéli 1998) environments. Usually, only a small fraction of DON is in a form readily available for algal uptake, e.g., urea or free amino acids (Antia et al. 1991).

Dissolved organic matter (DOM) is mainly composed of high molecular weight (HMW) polymeric compounds, particularly fulvic and humic acids (Thurman 1985). Some of the HMW DON can be assimilated by bacterioplankton, be mineralized in the pelagic food web, and become available for phytoplankton. Several researchers (Tranvik 1988, 1990;

Amon and Benner 1996) have called into question the traditional view of HMW dissolved organic carbon (DOC) as highly resistant to microbial degradation. There is no consistent evidence that aquatic humic carbon is less available for microbial degradation than is nonhumic matter (Tranvik 1998).

Although research on HMW DOC bioavailability has proliferated, quantitative and systematic studies on the bioavailability of DON are scarce. However, researchers have demonstrated that heterotrophic bacterioplankton can utilize humic-bound DON and make it available for phytoplankton growth (Carlsson and Granéli 1993; Carlsson et al. 1993; Bushaw et al. 1996). Seitzinger and Sanders (1997) reported that 40–80% of the bulk DON was available for bacterial utilization in the Delaware and Hudson Rivers (USA).

Often the main sources of DOM in watersheds are wetlands (Clair and Ehrman 1996). In several countries, reconstructed and newly created wetlands are used as nitrogen sinks for nonpoint agricultural sources (Leonardson 1994). To assess the performance of these wetlands, only nitrate reduction or denitrification rates usually are measured. When total nitrogen is analyzed, many wetlands appear to be net sources of DON (Jacks et al. 1994; Leonardson et al. 1994). Therefore, data on concentrations and bioavailability of DON are needed for planning and evaluation of watershed remediation projects.

DOM imported from the terrestrial environment may be an important source of nitrogen for bacteria. Upon microbial mineralization into inorganic forms, it may possibly promote the growth of phytoplankton. To gauge the potential importance of this role of DON, we made a preliminary survey of the bioavailability of the HMW fraction of DON isolated from three wetlands in South Sweden in winter, summer, and autumn. The bioavailability of low-molecular-weight (LMW) and bulk DON was also analyzed in one of the wet-

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lands. Wetlands with diverse soil types and hydrographic regimes were chosen for the survey. To assess the potential for bacterial growth on DON, we developed a bioassay employing natural DON and bacterial inocula in artificial freshwater and seawater media manipulated to make nitrogen the limiting nutrient.

Materials and methods

Experimental design—To analyze DON bioavailability, water was sampled from three freshwater wetlands in winter, summer, and autumn 1996. Tangential flow ultrafiltration was used to separate HMW DON from DIN because bacterial utilization of DIN would obscure the assay of bacterial DON bioavailability. The LMW and bulk DON bioavailability was assayed only when DIN concentration in the collected wetland water did not exceed 20% of the total nitrogen. Artificial freshwater and marine growth media were prepared with the isolated DON as the only source of nitrogen and manipulated to make nitrogen the limiting nutrient. The media were inoculated with natural bacterial assemblages, and cell density was followed until a stationary phase was reached, i.e., until all bioavailable nitrogen was incorporated into the cells. Then, the nitrogen incorporated into bacterial biomass, i.e., bioavailable nitrogen, was estimated.

Sampling of wetland water—Water was sampled from three wetlands situated in South Sweden: Amböke (56°47'N, 13°03'E), Vomb (55°40'N, 13°30'E), and Isgrannatorp (56°03'N, 14°05'E). Amböke Creek watershed is dominated by spruce forest, but its lower part contains two fens of 2.7 ha area, through which a broad water circulation occurs (Jacks et al. 1994). At the sampling point, catchment area is 42 km² with an average discharge of 0.0065 m³ s⁻¹ (annual value from 1991). The Vomb and Isgrannatorp artificially flooded meadows with subsurface water flow were described by Leonardson et al. (1994). Sandy soils are characteristic of Vomb meadows, and freshwater marsh peat comprises Isgrannatorp soils. Because of the subsurface water flow, groundwater was sampled at Vomb and Isgrannatorp from 1-m-deep bores. Surface water was taken from Amböke Creek. Winter sampling was performed on 16 January, 6 March, and 16 March, summer sampling was performed on 14, 27, and 21 June, and autumn sampling was performed on 10, 12, and 17 November in Amböke, Vomb, and Isgrannatorp, respectively. Winter sampling represented frozen surface soil and high groundwater levels, summer sampling represented active growth of vegetation and low groundwater levels, and autumn sampling represented the end of the growth season and high groundwater levels. The pH of the water was 4.5, 8.2, and 6.9 in Amböke, Vomb, and Isgrannatorp, respectively, with no significant seasonal variation.

DON isolation—Within 2 d after sampling and storage at 4° C, water was filtered through a 25- μ m pore size glass-fiber filter (Gamma-12, Whatman) and a 0.2- μ m pore size Supor filter (SuporCap 100, Gelman Sciences). The HMW fraction of DON (nominal M_r > 1,000) was concentrated sevenfold using a Millipore tangential flow ultrafilter (Prep/

Scale[®]-TFF 6 ft² Cartridge with PLAC 1-kD regenerated cellulose membrane), applying 140 kPa pressure differential and 6 liters min⁻¹ tangential flow rate. This generated a membrane permeation rate of about 30 ml min⁻¹.

After the initial concentration step, the obtained retentate was purified from the remaining DIN and LMW DON. For this reason, ultrafiltration was continued as above, while the retentate volume was kept constant by continuous additions of a fivefold volume of 1 M KCl followed by the same volume of Milli-Q water. Over 99% of the molecules not retained by the ultrafilter were washed out, including DIN and LMW DON. This calculation is based on the formula:

$$C_v = C_0 e^{-v}, \quad (1)$$

where v is the number of volumes and C_v and C_0 are the concentrations after flushing v and 0 volumes, respectively (Tranvik 1994). KCl was applied to remove ammonium adsorbed to DOM. The resulting retentate is hereinafter referred to as Retentate_{KCl}. Between 10% and 45% of bulk DON was recovered as Retentate_{KCl} (Table 1).

Another part of the retentate was washed only by a fivefold volume of Milli-Q water, omitting the KCl step. The retentate obtained after this procedure is hereinafter referred to as Retentate_w. Between 24% and 65% of bulk DON was recovered as Retentate_w (Table 1).

The application of KCl resulted in a substantial loss of DOM through the ultrafilter, possibly as a result of several processes. The fraction of DON that penetrated the ultrafilter in the presence of KCl but was retained without KCl was operationally defined as interjacent-molecular-weight (IMW) DON. The concentration of IMW DON was estimated by subtracting the DON recoveries in Retentate_w and Retentate_{KCl}. The IMW fraction comprised between 8% and 32% of the bulk DON (Table 1).

Bioavailability of the HMW DON was analyzed in all three wetlands on all three sampling occasions. The IMW DON was analyzed in summer and autumn. Bioavailability of the LMW DON and bulk DON was analyzed only in Amböke water during summer and autumn samplings when DIN concentrations were sufficiently low and did not interfere with bioassays.

Cultivation of bacteria—To prepare media for freshwater cultures, twofold concentrated artificial nitrogen-free lake water (Lehman 1980) was mixed 1:1 with the following solutions: (1) Milli-Q water (blank), (2) DON solution (ultrafilter retentate or permeate or sterile filtered wetland water), and (3) DON solution with a nitrate or ammonium spike (3–6 μ M). Seawater media were prepared by mixing 34‰ salinity artificial nitrogen-free seawater (Guillard 1975) 1:1 with the same solutions.

To ensure that no phosphorus or carbon limitation occurred in the bacterial cultures, 20 μ M Na₂HPO₄ and 138 μ M glucose were added (final concentration). Freshwater media were inoculated by adding 1% (by volume) of filtered (Whatman GF/F glass fiber filter, to remove bacterivores) water from the respective wetlands. The small volumes of inoculum ensured that no significant amounts of bioavailable nitrogen were added. Marine inocula were filtered in the

Table 1. Concentration of bulk DON and different molecular weight fractions, and the bioavailability of these fractions to freshwater (FW) and seawater (SW) heterotrophic bacteria, analyzed at three wetlands on three occasions (winter, summer, and autumn).

Wetland	Season	Bulk DON				HMW DON			IMW DON			LMW DON		
		Concentration (μM)	% of total	Bioavailability (%)		% of DON	Bioavailability (%)		% of DON	Bioavailability (%)		% of DON	Bioavailability (%)	
				FW	SW		FW	SW		FW	SW		FW	SW
Amböke	Winter	16	57	$\geq 0.0^\dagger$	$\geq 1.4^\dagger$	35	0.1	3.2	NA	NA	NA	NA	NA	NA
	Summer	32	95	6.1	15.5	33	2.5	9.4	32	5.3	12.6	35	10.4	23.9
	Autumn	31	84	2.2	7.8	45	1.2	9.2	8	2.4	4.0	47	3.5	5.7
Isgrannatorp	Winter	176	47	$\geq 1.2^\dagger$	$\geq 0.4^\dagger$	20	5.9	2.0	NA	NA	NA	NA	NA	NA
	Summer	177	42	$\geq 1.4^\dagger$	$\geq 1.9^\dagger$	24	1.3	6.8	26	4.3	0.7	50	NA	NA
	Autumn	180	30	$\geq 0.7^\dagger$	$\geq 1.4^\dagger$	16	5.5	9.9	11	1.7	5.6	73	NA	NA
Vomb	Winter	13	46	$\geq 0.0^\dagger$	$\geq 0.7^\dagger$	12	0	5.8	NA	NA	NA	NA	NA	NA
	Summer	79	27	$\geq 0.8^\dagger$	$\geq 1.7^\dagger$	13	1.5	3.5	20	3.0	6.4	67	NA	NA
	Autumn	109	10	$\geq 1.5^\dagger$	$\geq 1.0^\dagger$	10	5.8	9.9	14	6.5	0	76	NA	NA
Summary														
Mean		90	49	4.2	11.7	23	2.6	6.6	19	3.9	4.9	5.8	7.0	16.3
SD		68	25	2.0	3.9	11	2.3	3.0	8	1.7	4.2	15	3.5	7.6
No. observations		9	9	2	2	9	9	9	6	6	6	6	2	2

NA, not analyzed.

* HMW, high molecular weight; IMW, interjacent molecular weight; LMW, low molecular weight.

\dagger Not all DON MW fractions analyzed.

same way and were derived from Öresund Strait locations where the salinity at the time of sampling was $17\text{‰} \pm 2\text{‰}$.

The pH of the cultures was adjusted by titration with HCl and NaOH to achieve pH 6.5 for the freshwater cultures and pH 8.5 for marine cultures. Cultures were distributed into four 200-ml replicate polystyrene flasks, using only acid-washed glassware and plastic ware. Bacteria were incubated at room temperature in the dark for 6–14 days, until stationary phase was reached. Samples for determination of cell density were taken at 24-h intervals, fixed with 4% borax-buffered particle-free formaldehyde (final concentration),

and stored under refrigeration. Cell counts and volume determinations were performed within 1 month after sampling.

During the summer experiment with water from the Isgrannatorp wetland, we tested whether nitrogen limitation affected bacterial growth in the cultures. When bacteria reached stationary phase (the 6th day of incubation), nitrate ($90 \mu\text{M}$, final concentration) was added to half of the replicates of each treatment. After an additional 3 days, bacterial density was compared in cultures with and without the additions (Fig. 1).

Measurements of bacterial numbers and biovolume—Bacterial density was measured using a flow-cytometric method (del Giorgio et al. 1996). Syto 13 stain ($50 \mu\text{M}$, Molecular Probes) and Fluoresbrite Carboxy YG microspheres ($1.58 \mu\text{m}$ diameter, $3 \times 10^5 \text{ ml}^{-1}$, Polysciences) were added to 1-ml subsamples and analyzed with a Becton Dickinson FacSort flow cytometer at a low sample flow rate ($12 \mu\text{l min}^{-1}$). The cytometer was controlled with the CELLQUEST 1.2 software. Bacterial cells and microspheres were separated in a log-log scattergram of green fluorescence intensity (FL1) and side scattering (SSC). Voltages for these parameters were set to 560 and 400, respectively. Samples were run for 1 min or until 10,000 cells were counted. Bacterial density in the samples was calculated using microspheres as an internal standard. The density of the microspheres in a stock solution was analyzed by epifluorescence microscopy on a weekly basis.

The flow-cytometric cell density measurements were compared with manual microscopic counts using several randomly selected samples. Microscopic slides were prepared by staining 2-ml subsamples with 10 mg liter^{-1} 4',6'-diamidino-2-phenylindole (DAPI) for 10 min and filtering onto $0.2\text{-}\mu\text{m}$ pore size black polycarbonate membranes (Poretics;

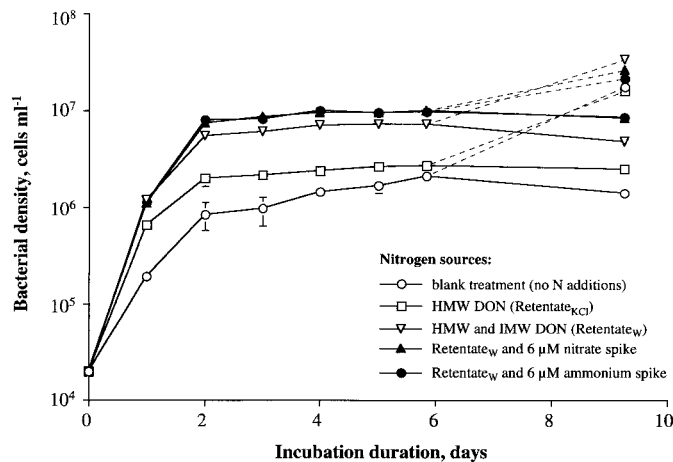


Fig. 1. Regrowth of bacteria in freshwater media on different nitrogen sources. During day 6, nitrate ($90 \mu\text{M}$, final concentration) was added to half of the cultures from each treatment (striped lines). Isgrannatorp summer experiment data. Mean \pm SD; $n = 4$ (days 0–6) and $n = 2$ (day 9).

Porter and Feig 1980). A Nikon Labophot-2 epifluorescence microscope equipped with a Fluor $\times 100/1.30$ oil immersion lens was used. From each slide, at least 250 cells and 10 fields of view were counted. There was close agreement between the two methods, giving Pearson correlation r^2 values of 0.96 ($n = 10$) and 0.98 ($n = 6$) for freshwater and seawater cultures, respectively. Similarly, del Giorgio et al. (1996) reported close agreement between the flow-cytometric method and direct microscopic counts.

Direct image analysis was used to estimate the average biovolume of bacterial cells in stationary growth phase cultures during experiments with Amböke water. Microscopic slides were prepared as above. From each slide, three randomly selected 24-bit red-green-blue (RGB) images were acquired with an Olympus BX50 epifluorescence microscope equipped with an Olympus UPlanFI $\times 100/1.30$ oil immersion lens and connected to an Optronics VI-470 CCD video camera (768×494 active pixels, 6.4×4.8 -mm sensing area, Optronics Engineering) and an ImageGrabber-24 (Neotech). Further processing of the images was done using IPLAB SPECTRUM 3.1a software (Signal Analytics). Cell edge detection was performed by applying a Marr-Hildreth operator and then thresholding the image to zero (Ramsing et al. 1996). Only the green color fraction of the image was analyzed because it gave the best background separation. Overlapping and out-of-focus cells were not analyzed. Cell volume (V) was calculated as a cylinder with hemispherical ends:

$$V = (\pi/4) \cdot w^2 \cdot (l - w/3), \quad (2)$$

where l is the major axis of the best fitting ellipsoid, and w is the effective diameter, calculated according to the formula:

$$w = 2/(\pi - 4) \cdot \{ [l^2 + (\pi - 4) \cdot A]^{0.5} - l \}, \quad (3)$$

where A is the area of the bacterial image (Blackburn et al. 1998).

The total bacterial biovolume during stationary growth phase was calculated by multiplying cell density (determined flow cytometrically) by the average cell volume (determined by image analysis).

Estimation of DON bioavailability—The growth of bacteria was observed until their cell density reached stationary phase (Fig. 1), i.e., until all potentially bioavailable nitrogen was incorporated into biomass. From each treatment, only the highest bacterial density reached was used for DON bioavailability calculation. First, nitrogen content of an average cell (N_{cell}) was calculated:

$$N_{\text{cell}} = s / (D_{\text{DON+DIN}} - D_{\text{DON}}), \quad (4)$$

where $D_{\text{DON+DIN}}$ is cell density in cultures with DON and nitrate additions, D_{DON} is cell density in cultures with DON additions only, and s is the concentration of the nitrate spike. Concentration of bioavailable DON (b) in the media was estimated as:

$$b = N_{\text{cell}} \cdot (D_{\text{DON}} - D_b) - i/2, \quad (5)$$

where D_b is cell density in the blank treatment and i is the concentration of inorganic nitrogen in DON solution (divided by 2 because of the dilution with artificial media). Bio-

availability of HMW DON was estimated as $b(\text{Retentate}_{\text{KCl}})$, and bioavailability of IMW DON was estimated as the difference between $b(\text{Retentate}_{\text{w}})$ and $b(\text{Retentate}_{\text{KCl}})$. The bioavailability of LMW DON was estimated as $b(\text{permeate})$ as well as the difference between $b(\text{bulk DON})$ and $b(\text{Retentate}_{\text{w}})$. Both estimates gave similar results.

Chemical analyses—Before nitrogen analyses, samples were stored frozen in acid-washed plastic vials. Standard methods were applied to measure nitrate plus nitrite (Wood et al. 1967), ammonium (Chaney and Marbach 1962), and total nitrogen (Koroleff 1976) concentrations in the water. Organic nitrogen concentration was calculated by subtracting ammonium, nitrate, and nitrite from the total nitrogen. Samples for dissolved organic carbon analyses were stored frozen in precombusted glass vials with Teflon lids. Dissolved organic carbon was analyzed after acidification and purging of inorganic carbon by the Pt-catalyzed high-temperature combustion method using a Shimadzu TOC-5000 total carbon analyzer.

Results

DON comprised ca. 50% of the total dissolved nitrogen at all three studied sites during winter sampling (Table 1). During summer and autumn, the DON fraction increased in proportion in Amböke surface water whereas it decreased in Vomb and Isgrannatorp groundwaters. Ultrafiltration revealed that the HMW fraction comprised a larger part of DON in Amböke (33–45%) than in Isgrannatorp (16–24%) and Vomb (10–13%). The fraction of LMW DON was larger in Vomb and Isgrannatorp groundwaters than in Amböke surface water.

The carbon/nitrogen (C/N) ratio of all DOM MW fractions was highest in Amböke water (Fig. 2). The lowest C/N ratios were consistently found in the LMW fraction. Seasonal C/N ratio trends of the bulk DOM were different for different wetlands. Nevertheless, when separate molecular weight fractions were compared, some general patterns emerged: (1) the C/N of the HMW DOM was highest in summer, (2) the C/N of IMW DOM was lower in summer than in autumn, and (3) the C/N of LMW DOM was higher in summer than in autumn.

The bioavailability of DON was higher in seawater than in freshwater ($P < 0.001$, paired t -test, $n = 17$) by an average factor of 2.4 (Table 1). DON bioavailability did not correlate with the C/N ratio of DOM ($P > 0.05$, Pearson correlation, $n = 17$). There were no significant differences among the wetlands and seasons concerning the bioavailability of HMW and IMW DON fractions ($P > 0.05$, one-way ANOVA, $df = 2$). The bioavailability of LMW DON and bulk DON was analyzed only in Amböke water during summer and autumn experiments, when DIN concentrations were sufficiently low and did not interfere with bioassays. In these cases, LMW DON was more bioavailable than HMW and IMW in seawater ($P < 0.05$, ANOVA followed by Tukey's post hoc test, $df = 2$) and freshwater (no significant differences). On average, the bioavailable fraction of the HMW fraction was 2.6% and 6.6%, in freshwater and seawater, respectively. Corresponding values were 3.9% and

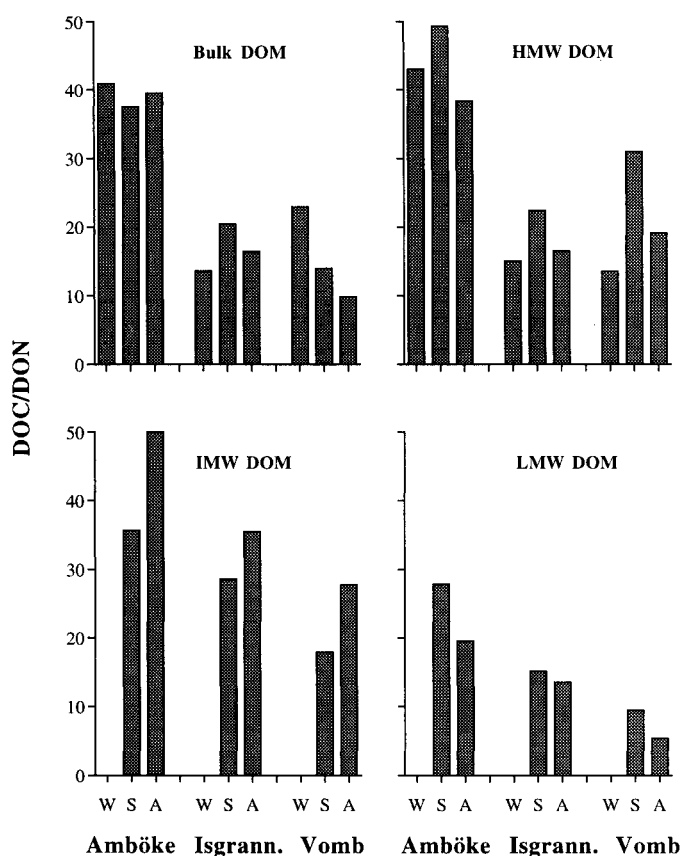


Fig. 2. DOC/DON ratios of the bulk DOM and different molecular weight fractions from Amböke, Isgrannatorp, and Vomb wetlands, winter (W), summer (S), and autumn (A) experiments.

4.9% for the IMW fraction and 7.0% and 16.3% for the LMW fraction. These averages were calculated using all available values from all wetlands and sampling occasions (Table 1).

Bioassays employing bulk DON in Amböke summer and autumn experiments revealed that 2–16% of DON was bioavailable (Table 1). High bioavailability of bulk DON during summer coincided with the lowest concentrations of DIN. Therefore, during summer, DON became the dominant pool of bioavailable nitrogen for bacteria (Fig. 3). Over 50% of the bioavailable DON was comprised of LMW compounds, except for the seawater autumn experiment, where HMW compounds comprised the major part of the bioavailable DON.

The estimated nitrogen content per bacterial cell was fairly constant at 1.89 ± 0.38 fmol (Table 2), when a single outlier (Vomb summer experiment) was excluded from calculations. Bacterial cell size in Amböke cultures averaged $0.57 \pm 0.11 \mu\text{m}^3$. There was no significant difference in cell size among cultures with different nitrogen additions ($P > 0.05$, ANOVA, $df = 3$). The calculated average nitrogen content of bacterial biomass was 3.72 ± 0.50 fmol μm^{-3} and did not significantly differ between freshwater and saltwater cultures. There was no significant difference in cell yield when nitrate and ammonium spikes were compared ($P > 0.05$, $n = 4$, ANOVA, $df = 3$; Fig. 1).

During the Isgrannatorp summer experiment, nitrate was added to half of the replicates of each treatment after bacteria reached stationary growth phase (Fig. 1). After 3 days, cell density increased in nitrate-amended flasks ($P < 0.001$, ANOVA followed by Tukey's post hoc test, $df = 1$) and decreased in unamended cultures ($P < 0.01$).

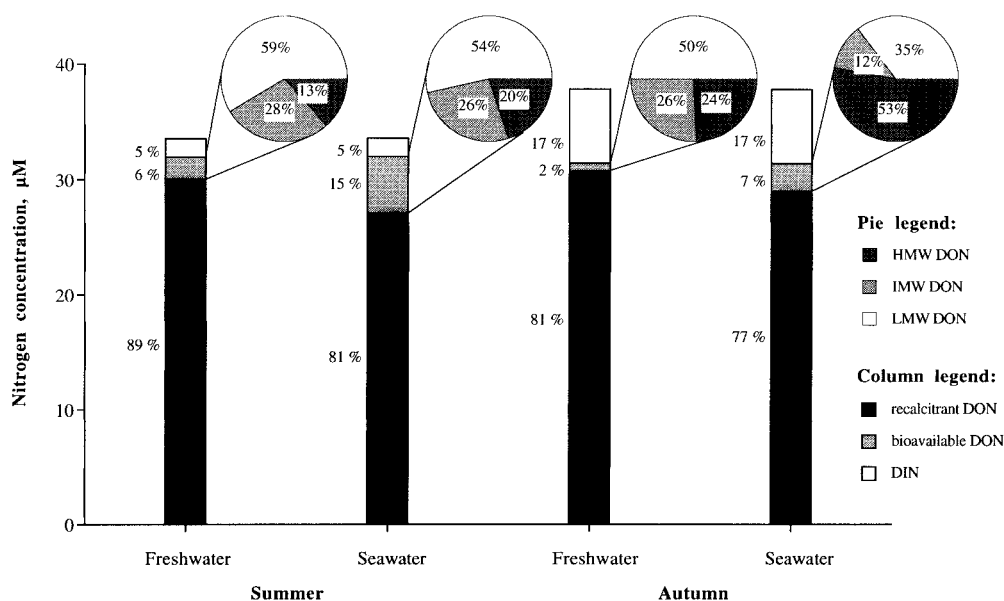


Fig. 3. Total dissolved nitrogen from Amböke summer and autumn experiments divided into DIN, bioavailable DON, and recalcitrant DON for both freshwater and seawater conditions (column diagram). Pie diagrams indicate the molecular weight composition of the bioavailable DON.

Table 2. The cell nitrogen (N) content, biovolume, and biovolume N content of average bacterial cells during stationary growth phase. Cell N content was estimated using nitrate internal standard treatments, and cell biovolume was measured by direct image analysis. Biovolume N content was estimated by dividing the first two parameters.

Wetland	Season	Seawater cultures			Freshwater cultures		
		Cell N (fmol)	Cell biovolume (μm^3)	Biovolume N (fmol μm^{-3})	Cell N (fmol)	Cell biovolume (μm^3)	Biovolume N (fmol μm^{-3})
Amböke	Winter	2.09	0.76 \pm 0.08 (12)	2.75	1.84	0.53 \pm 0.09 (12)	3.47
	Summer	2.23	0.55 \pm 0.09 (20)	4.05	1.79	0.48 \pm 0.09 (20)	3.81
	Autumn	1.89	0.45 \pm 0.16 (24)	4.20	2.66	0.65 \pm 0.28 (24)	4.09
Isgrannatorp	Winter	2.05	NA	NA	1.79	NA	NA
	Summer	2.15	NA	NA	1.55	NA	NA
	Autumn	1.39	NA	NA	2.44	NA	NA
Vomb	Winter	2.02	NA	NA	1.75	NA	NA
	Summer	7.23	NA	NA	5.11	NA	NA
	Autumn	1.30	NA	NA	1.28	NA	NA
Average		2.48 \pm 1.71 (9) 1.89 \pm 0.33 (8)*	0.59 \pm 0.13 (3)	3.67 \pm 0.65 (3)	2.24 \pm 1.09 (9) 1.89 \pm 0.42 (8)*	0.55 \pm 0.07 (3)	3.79 \pm 0.25 (3)

Values are mean \pm SD (No. replicates). NA, not analyzed.

* Data from the Vomb summer experiment not included.

Discussion

DOM as a nitrogen source—The potential bioavailability of bulk DON (2–16%, Amböke summer and autumn experiments; Table 1) was similar to reported DOC bioavailability values in rivers (19% \pm 16%; Søndergaard and Middleboe 1995). However, the 2–16% bioavailability is likely an underestimate for the Isgrannatorp and Vomb study sites. Given the high LMW proportion of DON in these two wetlands and the supposedly high relative bioavailability of this fraction, as suggested by results from the Amböke wetland (Table 1), bulk DON bioavailability is likely to be much higher than 16% at Vomb and Isgrannatorp. These estimates represent the potential bioavailability and are derived from experiments with surplus amounts of carbon and phosphorus. The momentarily bioavailable DON in situ may be lower because of limited rates of carbon and phosphorus uptake.

Assuming an average DON bioavailability of 12% in seawater (Table 1) and DON constituting 70% of total nitrogen in world rivers (Meybeck 1982), ca. 20% of the bioavailable nitrogen loading to coastal areas would be DON. However, Seitzinger and Sanders (1997) reported 40–80% bioavailability of DON in the Hudson and Delaware Rivers (USA). The most likely sources of this very labile DON are sewage inputs and phytoplankton production in these large urban rivers. In contrast, the DOM derived from wetlands is often dominated by humic compounds, generally considered less amenable to microbial degradation (Wetzel 1992). Nevertheless, DON seems to be an important nitrogen source in coastal areas receiving water from rivers with both forested and urban watersheds. The availability of DON from humic watersheds may be further promoted by photochemical transformations induced by solar radiation, as suggested by Bushaw et al. (1996).

Rivers at temperate latitudes tend to have high nitrate concentrations during high discharge periods in autumn–spring, whereas DIN concentrations are low during summer (Pardo et al. 1995). Depending on hydrological conditions in the

coastal areas, nitrogen brought by rivers in winter may be transported to the open ocean and may have less of an impact on coastal summer blooms. Because of this variation, DON can become the dominant pool of the bioavailable nitrogen in summer, whereas it is less important at other times of the year. This situation was evident in Amböke water (Table 1, Fig. 3). Coincident with low concentration of DIN, the bioavailability of DON was higher during summer than during other seasons, possibly because of the production of fresh organic matter by vegetation. Increased bioavailability of DOC during low discharge periods was observed by Leff and Meyer (1991) and Sun et al. (1997). In agreement with these findings, Ivarsson and Jansson (1994) reported an increased DOC/DON ratio in connection to high water level, promoting leaching of forest litter.

Terrestrially derived DOM may play an important role in supplying nitrogen to coastal summer algal blooms, usually dominated by cyanobacteria and flagellated algae and occasionally exhibiting toxin production (Carlsson and Granéli 1998). Additions of terrestrial DOM favor dinoflagellates (Carlsson et al. 1995) and consequently affect algal species composition. Although algae may be able to utilize some DON compounds directly (Antia et al. 1991), algal assimilation of nitrogen bound in terrestrially derived DOM probably is largely dependent on bacterial mineralization into more available inorganic forms. Our results suggest that bacterial uptake of DON followed by mineralization in the aquatic food web may contribute significantly to the supply of nitrogen to algae.

Bioavailability in freshwater versus seawater—In most cases, a substantially larger fraction of DON was available to marine bacterioplankton than to freshwater bacterioplankton (Table 1). One explanation for the observed differences could be the adaptation of marine species to low DIN. Seawater cultures exhibited a significantly higher specific aminopeptidase activity than did freshwater cultures (Stepanauskas et al. unpubl.). However, substantial chemical

changes of DOM occur when ionic strength is elevated, including coiling of humic substances (de Haan et al. 1987), aggregation of colloids (Mulholland 1981), and change in sorption mechanisms (Taylor 1995). Furthermore, cation-induced conformational changes in DOM may also reactivate extracellular enzymes inactivated by complexation with DOM (Wetzel 1992).

The salinity-dependent enhanced bioavailability of DON may occur during mixing of riverine water with marine water. Hence, a fraction of DON transported by rivers and not available for biological transformations and uptake during its residence in the freshwater environment may become a nitrogen source in coastal marine ecosystems. In contrast, inorganic nitrogen can be removed from river water by denitrification and assimilative uptake. An increased degradation of riverine DOC at elevated salinities has also been observed by others (Wikner et al. in press).

Distribution of bioavailable DON into molecular weight fractions—In the Amböke wetland, the LMW fraction of DON had the highest bioavailability in summer and autumn experiments (4–24%; Table 1). Because we were unable to separate DIN from the LMW DON, it was impossible to analyze the bioavailability of LMW DON in the Vomb and Isgrannatorp waters, where concentrations of DIN were substantial. However, the LMW fraction dominated the DON of Isgrannatorp and Vomb groundwaters, comprising 50–76% of the bulk DON (Table 1). Assuming that the LMW fraction was more available also at these sites, the bioavailability of bulk DON probably was higher in these groundwaters than in Amböke surface water.

Obviously, methods of DOM isolation from inorganic salts set limits for application of DON bioavailability assays. We used a tangential-flow ultrafiltration through a small pore size (nominal cutoff of 1,000 Daltons) membrane because it is a very gentle method applicable to large volumes. However, along with inorganic salts, the permeate contained a large portion of DOM. Several other methods reviewed by Aiken (1984b) and Abbt-Braun et al. (1991) can be used for DOM isolation, e.g., different types of chromatography, coprecipitation with salts, and solvent extraction. However, all of these methods either isolate just a certain DON fraction or involve harsh treatment, which inevitably alters the character of DON. Ion retardation chromatography (Bronk and Glibert 1991) seems to be a promising method for separation of bulk DON from nitrate and ammonium in seawater. However, this method provided a very low DON recovery from humic freshwater, with a substantial retention of organic compounds by the resin (data not shown).

Between 8% and 32% of bulk DON consisted of IMW DON (Table 1), which is DON that did not penetrate the ultrafilter at low ionic strength but that did penetrate when 1 M KCl was present in the solution. One possible mechanism for the increased penetration is desorption of LMW nitrogen-containing organic substances from the HMW matrix due to cation exchange. For example, amino acids can bind to macromolecular materials (Carlson et al. 1985). Schnitzer (1985) suggested that 75% of amino acids associated with humic matter are only loosely held by hydrogen bonds and van der Waals forces because they can be re-

moved by passage over cation-exchange resins. However, the high C/N ratio of the IMW DOM (Fig. 2) indicates that amino groups were not abundant in this fraction.

The increased penetration also could be caused by the coiling of organic polyelectrolytes, such as humic compounds and proteins (Stumm and Morgan 1996). De Haan et al. (1987) found that increased salinity facilitated DOM penetration through a dialysis membrane and enhanced DOM retardation during size-exclusion chromatography. These results were interpreted as a decrease in DOM molecular dimensions when humic substances coiled.

Yet another explanation is based on the limitations of ultrafiltration. Membranes are known to get fouled with humic substances (Aiken 1984a). Hence, they become negatively charged and start to retard other negatively charged substances, which otherwise would penetrate the membrane. When the cation concentration in the solution is increased, the charge of the fouling is screened and penetration of negatively charged LMW substances is facilitated.

If the latter two phenomena are significant, ultrafiltration-based DOM molecular weight distribution comparisons at different salinities should be done with caution. Several authors have reported a higher proportion of LMW DOM in seawater than in freshwater, as determined by ultrafiltration (see Amon and Benner 1996). Facilitated penetration through membranes in saline water would lead to lower HMW fraction recoveries than that in freshwater, even at similar actual molecular weight distribution. However, desorption of LMW DOM may explain lower HMW DOM occurrence in the ocean than in freshwater.

Bioavailability assays—The strength of the bioassay technique applied in this study lies in its ability to account for natural chemical and metabolic diversity. Similar assays for DOC bioavailability are widely applied but generally depend on several conversion factors. Reported biovolume to biomass ratios (Fry 1988) and growth yields (del Giorgio et al. 1997) vary several fold, which severely limits the precision of estimates that depend on fixed conversion factors. To avoid these problems, we used a different approach. DIN spikes, assumed to be 100% bioavailable, were applied as internal standards to enable calculations of nitrogen content in an average cell. A similar approach is not applicable to DOC bioavailability studies because spikes of labile DOC (e.g., glucose) would not give a growth efficiency representative of natural DOC. In contrast, growth efficiency is not involved in our DON bioavailability calculation because nitrogen is not respired under oxic incubations.

Part of the nitrogen from nitrate spikes and from DON substrates might be used by bacteria for the production of extracellular material (e.g., released enzymes, extracellular polymers). This use would decrease bacterial cell yield per unit of nitrogen added. Consequently, nitrogen content of an average cell would be overestimated (N_{cell} , Eq. 4). Similar biases could also be caused by the growth of bacteria on the walls of the cultivation flasks. However, assuming that different bioavailable nitrogen compounds result in similar biomass yields, DON bioavailability estimations (b , Eq. 5) would not be affected because N_{cell} is overestimated to the same degree as ($D_{\text{DON}} - D_b$) is underestimated. Bioavailabil-

ity estimated using internal standards includes nitrogen used by bacteria not only for cell buildup but also for the production of extracellular material. However, such extracellular production did not seem to account for a large portion of nitrogen flow in our study; N_{cell} estimates did not exceed reported values of the elemental composition of bacterial cells. The estimated mean N_{cell} was $3.72 \text{ fmol N } \mu\text{m}^{-3}$ (Table 2). Assuming the C/N ratio of bacterioplankton to be 5 (Fagerbakke et al. 1996), this corresponded to $223 \text{ fg C } \mu\text{m}^{-3}$ and fell in the middle of the range of values ($130\text{--}650 \text{ fg C } \mu\text{m}^{-3}$) reviewed by Fry (1988).

Our estimates of DON bioavailability were based on several assumptions:

1. We assumed that different bioavailable nitrogen compounds yield identical increase in bacterial biomass per mol nitrogen: Otherwise the use of an internal nitrate standard would give erratic results. In support of this assumption, nitrate and ammonium additions resulted in identical cell density (Fig. 1), although ammonium is preferred over nitrate as a nitrogen source by bacteria (Kirchman 1994), and incorporation of nitrate into biomass requires higher energy inputs. The similar cell yields may have been due to the high concentration of glucose in the growth medium, providing excess energy. We used DIN spikes instead of DON (e.g., amino acids) to avoid possible binding of DON monomers to macromolecules (Carlson et al. 1985) and subsequent decrease in their bioavailability.

2. Bacteria were assumed to be nitrogen limited during stationary growth phase: The assumption is confirmed by the observed stimulation of bacterial growth by additional inputs of nitrate during stationary growth phase (Fig. 1). Furthermore, all cultures were monitored for DIN concentration at the end of the incubations, and DIN was consistently below detection limit ($<0.3 \mu\text{M}$), supporting the assumed nitrogen limitation.

3. We assumed that all bioavailable nitrogen was consumed after bacteria reached stationary growth phase: However, after the exponential growth phase, slow DON utilization could still continue undetected, causing bioavailability underestimates. Therefore, the obtained bioavailability estimates should be viewed as an operationally defined short-term DON bioavailability. On a longer time scale, various environmental factors may alter DON bioavailability in natural waters. Examples of such factors are solar radiation (Bushaw et al. 1996) and changes in water ionic strength.

4. We assumed that DON degradation potential did not depend on the source of bacterial inocula: In a previous study employing similar regrowth bioassays, Tranvik and Höfle (1987) found that bacterial growth was not inoculum dependent. However, it is unknown to which degree the apparent bioavailability may be underestimated because of unculturability of certain natural microorganisms.

Conclusions—Freshwater wetland-derived DOM may be an important nitrogen source for coastal ecosystems during summer, when concentrations of inorganic nitrogen are low

and DON bioavailability is high. Our data indicate that marine bacterioplankton can assimilate a substantially larger fraction of DON than can freshwater bacterioplankton, suggesting an enhanced DON uptake in coastal waters. In general, the bioavailable fraction of bulk DON was similar to corresponding values previously reported for DOC.

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