

Bioavailability of DON from natural and anthropogenic sources to estuarine plankton

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Abstract

Utilization of dissolved organic nitrogen (DON) from natural (forests) and anthropogenic (animal pastures, urban/suburban storm water runoff) sources (three sites per source) by estuarine plankton communities was examined in spring, summer, and fall. The proportion of DON utilized ranged from 0 to 73%. Overall, urban/suburban storm water runoff had a higher proportion of bioavailable DON ($59\% \pm 11$) compared to agricultural pastures ($30\% \pm 14$) and forests ($23\% \pm 19$). DON bioavailability varied seasonally; however, the seasonal pattern differed for the three sources. Bacterial production increased linearly with the amount of DON utilized across all sources and seasons; the rate of increase was approximately five times greater per micromole of N as DON used relative to dissolved inorganic N (DIN) used. Although phytoplankton production generally increased with DON addition, the increased production was not correlated with the amount of DON utilized, suggesting that a variable portion of dissolved organic matter (DOM)-N was directly or indirectly available to the phytoplankton. This indicates that phytoplankton production is not a good measure of the amount of bioavailable DON, and measurements of the amount of bioavailable DON based on bacterial responses alone might not reflect N available to phytoplankton. Preliminary seasonal budgets of bioavailable N (DIN plus bioavailable DON) as a function of land use suggest that ~80% of the total dissolved N (TDN) from urban/suburban runoff is bioavailable, whereas a lower proportion (20–60%) of TDN is bioavailable from forests and pastures. N budgets for aquatic ecosystems based on only DIN loading underestimate bioavailable N loading, whereas total N or TDN budgets overestimate bioavailable N inputs.

Nitrogen (N) enters rivers, estuaries, and coastal marine ecosystems from natural and, in many cases, anthropogenic sources throughout their watershed and airsheds. Because N is the primary nutrient-limiting resource for plant, algal, and microbial production in many marine and some freshwater environments, increases in N inputs can markedly alter those ecosystems (e.g., Ryther and Dunstan 1971; Vitousek and Howarth 1991). To date, most measurements and models of N loading, as well as studies of its effects on ecosystems,

have focused on dissolved inorganic N (DIN = nitrate, nitrite, and ammonium). However, a considerable portion of the N inputs to aquatic ecosystems is in the form of organic N (dissolved and particulate), and there is a paucity of information on the biological availability and ecosystems effects of organic N from different sources.

Rivers, which are a major transport pathway for N from watersheds to coastal marine ecosystems, can have from 10% to over 80% of their N in the form of dissolved organic N (DON) (e.g., Meybeck 1982; Hedin et al. 1995; Seitzinger and Sanders 1997; Lewis et al. 1999). But, as recognized over 25 yr ago by Manny and Wetzel (1973), only a portion of the DON in rivers is bioavailable. For example, between 40 and 72% of the DON in two large rivers in the northeastern United States, the Delaware and Hudson rivers, was utilized by estuarine bacteria (Seitzinger and Sanders 1997). In boreal streams during a spring flood, between 20 and 55% of the DON was bioavailable to estuarine bacteria (Stepan-auskas et al. 2000).

The DON in rivers can originate from numerous natural and anthropogenic watershed sources, from atmospheric deposition, and from autochthonous production. Differences in the amount and biological availability of DON among rivers is likely due, in part, to variation in the concentration and chemical composition of DON inputs to rivers from these different sources. Between approximately 20 and 75% of the

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DON in atmospheric deposition has been reported to be bioavailable (Timperley et al. 1985; Peierls and Paerl 1997; Seitzinger and Sanders 1999). Approximately 25% of DON from two pastures and 20% of DON from a mixed hardwood forest in summer were utilized by freshwater bacteria (Wiegner and Seitzinger 2001). Conversely, only 8–15% of the bulk DON in a relatively pristine wetland in Sweden was potentially bioavailable to estuarine bacteria (Stepanuskas et al. 1999). Information on the bioavailability of DON in other sources, or over seasonal cycles, is generally lacking.

The effects of increased N loading on phytoplankton production, biomass, and species composition is of considerable interest, particularly in coastal marine waters where cultural eutrophication is increasingly apparent. The response of phytoplankton within the plankton community to N inputs can differ depending on the form of N (i.e., different organic forms, ammonium, nitrate) (Berg et al. 1997; Berman and Chava 1999). For example, additions of humic substances from river water differentially affected growth of different phytoplankton species (Carlsson and Granéli 1993), and a different community composition of phytoplankton developed in estuarine plankton bioassays to which DON from rainwater was added than with DIN additions (Seitzinger and Sanders 1999). An accurate understanding of the effects of nutrient inputs from both natural and anthropogenic sources requires development of bioavailable nutrient loading budgets and knowledge of the response of coastal ecosystems to different forms of those nutrients.

The current study addresses the dissolved component of organic N inputs to aquatic ecosystems. The major objectives were to (1) determine the proportion of DON that is bioavailable to estuarine plankton in a natural (forest runoff) and two anthropogenic (urban/suburban storm water, agricultural runoff) sources, (2) examine seasonal differences in the proportion of DON that is bioavailable from those three sources, and (3) examine the magnitude of the response of bacteria and phytoplankton production to bioavailable DON from these sources. These data are then used to explore the effect of land use on bioavailable N export from watersheds to coastal ecosystems.

Methods

Site description—Water was collected for DON bioavailability studies during spring, summer, and fall from three land use types: forest, agricultural pasture, and urban/suburban regions. All sites were located in central or northern New Jersey watersheds. Forest runoff was collected from streams draining forested watersheds, including one predominantly coniferous forest and two mixed hardwood forests. Site Pine was located in the Pinelands National Forest Reserve on the East Branch of the Bass River, a blackwater river. The river at this location (39°37'42"N, 74°26'45"W) is a second order stream (USGS topographical map scale 1:24,000). The wetland riparian area is vegetated by Atlantic white cedar (*Chamaecyparis thyoides*) and dominated by pitch pine (*Pinus rigida*) in the upland area. The soils in the cedar swamp are classified as shallow muck soils, whereas the upland soils are Lakehurst sand (USDA Soil Conversa-

tion Service maps). Both mixed hardwood sites (maple/beech/oak/hickory) were in west-central New Jersey. Hardwood-1 was located in Oxford, New Jersey (40°48'52"N, 74°58'05"W) and is a first order stream (as defined on USGS topographical map scale 1:24:000) draining a mixed hardwood forest. Hardwood-2 was located on a first order stream located near Round Mountain, Stanton, New Jersey (40°34'25"N, 74°50'17"W). The soils in the two mixed hardwood sites are stony loam (USDA Soil Conservation Service maps).

Urban/suburban storm water was collected from three sites in New Brunswick, New Jersey. Sites M and L were located on streams (Mile Run Brook and Lyell Brook) that are main conduits for residential (~0.1 ha or less lot size) New Brunswick storm water flow. Mile Run Brook is an uncovered stream and Lyell Brook is covered over and piped. Water discharge in these streams is low (generally less than 5 cm deep) during nonstorm periods compared to storm flows (generally greater than 0.5 m deep when sampled). Site G was located at a street-level storm sewer grate and received runoff from adjacent streets, small businesses (including a gas station), and residences.

Agricultural runoff was collected from swine, equine, and bovine pastures located on Cook College, Rutgers University, New Brunswick (40°28'65"N, 74°26'20"W). The density was approximately 2 to 3 animals ha⁻¹ in the swine and equine pastures and approximately 14 animals ha⁻¹ in the bovine pasture. Runoff was collected downslope of the animal pastures in a subsurface water collector consisting of a PVC pipe with drainage holes wrapped in a GeoSieve (Drainage Products, Inc.) sleeve. The collectors were buried 18–23 cm below the soil surface and above a layer of red/orange clay. The three agricultural sites are all classified as Fallsington variant loam, which is typically a surface layer (~12 cm) of gray loam over gray silt loam and a weak red clay loam. The DON from these sites was likely a combination of DON from the animals as well as DON produced by plants and soil microbes, all of which could have undergone reactions within the soils before reaching our collectors. Urea concentrations were low (<1 μM; measured in summer in swine and equine sites; Wiegner unpubl. data).

Samples from all sites were collected during or immediately following a rain event. Water was filtered on-site through a prerinsed 0.5-μm string-wound polypropylene canister filter into acid-washed 20-L cubitainers. Samples were stored on ice during transport to the lab. Subsamples for chemical analyses (ammonium, nitrate plus nitrite, total dissolved N, phosphate, and dissolved organic carbon) were filtered on-site using precombusted glass fiber (GF/F Whatman) syringe filters and frozen until analysis. Dissolved organic carbon (DOC) was determined by high-temperature combustion using a Shimadzu 5000 total organic carbon analyzer (Sharp et al. 1993); methods for other analytes are described below. The remaining water was immediately frozen and stored until the concentration step (see below).

Experimental setup and analytical methods—The overall objective of our experimental design was to test how much of the DON that enters the estuary from various sources is bioavailable to estuarine microbes during the season in

which it enters the estuary. For example, when DON released in spring from forests enters the estuary in spring, how much of the DON is bioavailable to the plankton in the estuary at that time?

The basic experimental protocol was to add concentrates of source water from a site to estuarine water (same season as source water collection) and then measure changes in DON and DIN concentrations and changes in bacteria and phytoplankton production over time. The experimental procedures were similar to those previously described for examining bioavailability of DON in rainwater (Seitzinger and Sanders 1999). Nine experiments were conducted: one for each of the three sources in spring, summer, and fall. Three sites per source were tested in an experiment, with duplicate incubations per site. Concentrates of the source water were prepared by filtering the water from the three sites for each source through a 1×10^6 -Da ultrafilter (Filtron, Inc., tangential flow ultrafiltration) to remove any remaining particles, including bacteria, then the water was concentrated by low-temperature (60°C) vacuum evaporation (Timperley et al. 1985). The concentration factors ranged from 1.1 to 134 (average 33); the higher concentration factors applied to the sources with low initial DON concentrations (e.g., forests). Recovery of DON and nitrate was generally greater than 80%; however as expected, most of the ammonium was lost during the vacuum evaporation process (final NH_4^+ concentration $0.8 \pm 0.7 \mu\text{M}$). The final concentrate was divided into aliquots and frozen until use.

For each experiment, freshly collected Barnegat Bay (New Jersey) water was filtered through a $160\text{-}\mu\text{m}$ sieve to remove macrozooplankton. Ten 1.5-L volumes of bay water were incubated in Pyrex flasks at near ambient bay water temperatures on a light:dark cycle (see Table 2 for light cycle listing). Teflon-coated stir bars stirred the water slowly. Light levels were approximately $100 \mu\text{E m}^{-2} \text{ s}^{-1}$ (photosynthetically active radiation [PAR]; Aquasun4/VHO-1 fluorescent lamps, Ultraviolet Resources International). The experiments were not subject to ultraviolet (UV) radiation because the fluorescent lamps used do not have emissions $<370 \text{ nm}$ (Ultraviolet Resources International pers. comm.). Land use and land cover in the Barnegat Bay watershed is 28% developed, 5% cultivated/grassland, 37% woody land, 20% freshwater wetland, and 10% other (Lathrop and Bogner 2001).

Duplicate flasks of estuarine water (1.5 L) received daily additions of concentrate from each of the three sites for 10 to 12 d (2 flasks \times 3 sites = 6 flasks). Two additional flasks received daily additions of DIN for the same period. The DON source water and, consequently, the concentrates contained DON as well as DIN. These DIN treatment flasks were used as a control to separate the effects of the DIN added with the DON source water on bacteria and phytoplankton production. The ratio of DON to DIN in the concentrates differed among sites. Therefore, the amount of DIN added with the concentrates was adjusted (using solutions of KNO_3 and NH_4Cl) so that all sites within an experiment had the same amount of NH_4^+ and $\text{NO}_3^- + \text{NO}_2^-$ added. The duplicate DIN control flasks received the same amount of NH_4^+ and $\text{NO}_3^- + \text{NO}_2^-$ as the DON treatments. The amount of DON-N added across sites was kept similar to the extent possible and ranged from 4.4 to $16.6 \mu\text{mol N flask}^{-1} \text{ d}^{-1}$

(volume of concentrate added was approximately 25 ml d^{-1}). A second set of controls consisted of bay water without nitrogen (DIN or DON) additions. All flasks received daily additions of phosphate (as KH_2PO_4 solution) so that the plankton would not become phosphorus limited (total addition ratio of N:P $\leq 16:1$). Axenic controls were not run as part of the current study. However, in a related study, sterile filtered incubations were conducted with summer-collected source water from one of our forest (Hardwood-2) and two of our agricultural (bovine and swine) sites; no changes in DON concentrations were observed (Wiegner and Seitzinger, 2001).

Direct comparison of the DON addition rates in our experiments to estuarine loading rates is difficult, not only because of the large differences in scale between our experiments and estuaries, but also because few estimates of DON loading rates to estuaries are available. However, if we extrapolate our experimental DON daily addition rates to an annual rate ($1,095\text{--}4,015 \text{ mmol DON-N m}^{-3} \text{ yr}^{-1}$), they are higher than total N (TN) loading rates to Barnegat Bay ($\sim 225 \text{ mmol TN m}^{-3} \text{ yr}^{-1}$; Moser 1997) and similar to N loading rates to a number of other nutrient-enriched estuaries, including New York Bay ($4,550 \text{ mmol DIN m}^{-3} \text{ yr}^{-1}$; Nixon and Pilson 1983), Little Assawoman Bay, Delaware ($2,500 \text{ mmol TN m}^{-3} \text{ yr}^{-1}$; Horsley and Whitton 1998), Newport Bay and Turville Creek in the Maryland coastal lagoon system ($1,025$ and $1,670 \text{ mmol TN m}^{-3} \text{ yr}^{-1}$, respectively; Boynton et al. 1996), and the Childs River and Quashnet River estuaries in the Waquoit Bay, Massachusetts, system ($4,290$ and $5,000 \text{ mmol TN m}^{-3} \text{ yr}^{-1}$, respectively; Short et al. 1993; Valiela et al. 1997a,b; Harrison et al. 1994; assuming average depth of 1 m for Childs River).

Time series samples (approximately 125 ml) of the flask water were collected approximately every 2 to 3 d throughout the period of N additions (10–12 d). We continued to sample each of the flasks for 3 to 7 d after the additions stopped to determine whether the 10–12-d experimental period was sufficient to quantify most of the easily degraded DON. Samples were analyzed for ammonium (Lachat, Inc. QuickChem 31-107-06-1-A), nitrate plus nitrite (Lachat, Inc. QuickChem 31-107-04-1-A), total dissolved N (TDN, Antek, Inc.), phosphate (Lachat, Inc., QuickChem 31-115-01-3-A), rates of primary production (^{14}C method modified from Strickland and Parsons 1972), and bacterial production (^3H -thymidine incorporation for agricultural experiments [Bell 1993]; ^3H -leucine incorporation for forest and urban/suburban storm water experiments [Smith and Azam 1992]).

DON was determined by the difference between TDN and DIN. TDN was analyzed by high-temperature combustion followed by chemiluminescent detection of nitric oxide using an Antek Model 7000 Total N Analyzer (Antek, Inc.) equipped with a quartz combustion tube ($1,000 \pm 10^\circ\text{C}$) and a ceramic insert (Seitzinger and Sanders, 1997). TDN samples were preserved in capped autosampler vials with 3 N HCl ($7.5 \mu\text{l}$ acid per 1.5 ml sample). Urea, ammonium, and nitrate standards (range of concentrations $10\text{--}300 \mu\text{M N}$ prepared in deionized water; Barnstead, Inc. E-pure; $7.5 \mu\text{l}$ acid per 1.5 ml) were analyzed in triplicate at the beginning and end of each analytical run. Additional organic N standards (e.g., 3-(4-morpholino)propanesulfonic acid; tris(dihydroxymethyl)

aminomethane; niacinamide; imidazole) were analyzed occasionally and were found to have N recoveries relative to urea of 95% or better. Blanks consisted of acidified deionized water.

For primary production measurements, ^{14}C -bicarbonate (specific activity 57 mCi/mmol; Amersham) was added to 7.5 or 15 ml of sample (duplicate light and duplicate dark controls) to achieve a final concentration of approximately $0.05 \mu\text{Ci ml}^{-1}$ of sample and incubated for approximately 3 hr at the experimental light intensity and temperature. Samples were then filtered (0.45- μm Millipore HA) and fumed over concentrated HCl.

For bacterial production using the ^3H -thymidine method, four 10-ml samples were incubated for 30 min in the dark at experimental temperatures with ^3H -thymidine ($0.8 \mu\text{Ci ml}^{-1}$ sample; Amersham), with duplicate samples extracted with either hot or cold trichloroacetic acid (TCA) (Bell 1993). A thymidine conversion factor (TCF) of 3.41×10^{18} cells mol^{-1} and a carbon conversion factor (CCF) of 25 fg C cell^{-1} (Bell 1993) were used to convert thymidine incorporation into bacterial C biomass production. For bacterial production using the ^3H -leucine method, 1.7 ml of sample water were incubated with 5 μl of L-4, 5 ^3H -leucine (1 mCi ml^{-1} ; specific activity 153 Ci/mmol; Amersham) in the dark at experimental temperatures for 30 min. Blanks consisted of 90 μl of 100% TCA, 1.7 ml sample water, and 5 μl ^3H -leucine. The centrifugation, vortex, and wash sequence described in Smith and Azam (1992) was followed with the addition of a final 80% ethanol wash. A conversion factor of $3.1 \text{ kg C mol}^{-1}$ was used to convert leucine incorporation rate to bacterial C biomass production (Kirchman 1993). Radioactivity of samples for ^{14}C -bicarbonate, ^3H -leucine, and ^3H -thymidine incorporation was determined by liquid scintillation counting (Beckman LS6000IC) using Cytoscint (ICN) as scintillation cocktail.

The amount of DON utilized over the course of the experiment was calculated as the difference between the measured DON and the initial bay water plus added source water DON. The proportion of DON utilized was calculated as the total amount utilized by the last day of additions divided by the total amount added in the source water. Rates of bacteria and phytoplankton production measured every 1 to 3 d were integrated (SigmaPlot[®] v.4.01 software) over the course of the experiment. The effects of DON addition on bacteria and phytoplankton production rates were calculated as the difference between rates in treatment flasks (which received DON and DIN) and in DIN control flasks (which received the same amount of DIN as the treatment flasks). The effects of DIN addition on bacteria and phytoplankton production rates were calculated as the difference between rates in DIN control flasks and in control flasks with no N additions.

Statistical analysis—Source water chemical composition data (DON and DOC concentration, percent DON, and DOC:DON ratio) and DON bioavailability data were examined by analysis of variance (ANOVA; Systat[®] v.6.0 software) to test for effects of source, season, and sites within a source. Source water concentrations of DON were +1 log transformed and of DOC were log transformed in order to satisfy the requirements for the analysis of variance. Post

hoc tests (Tukey's) were run to test for significant differences between sources, between seasons, and between sites.

Results

Source water composition—Concentrations of DON, ammonium, nitrate plus nitrite (hereafter referred to as nitrate), and phosphate each varied by approximately two orders of magnitude across all sources, sites, and times sampled in this study (Table 1). DON concentrations ranged from $2.5 \mu\text{M}$ at the forest Hardwood-1 site in spring to $260 \mu\text{M}$ at the agricultural bovine pasture site in summer. Overall, there was a significant effect of source ($P < 0.00001$) on DON concentrations; average concentrations were lowest in the forest sites ($10 \pm 6 \mu\text{M}$), intermediate in the urban/suburban storm water sites ($61 \pm 44 \mu\text{M}$), and highest in the agricultural sites ($129 \pm 75 \mu\text{M}$). The percentage of TDN that was DON differed by source ($P < 0.00001$). The percent DON was lowest in the urban/suburban storm water ($45\% \pm 9$) and higher and similar in the agricultural ($77\% \pm 13$) and forest ($72\% \pm 19$) sources. The percent DON differed by season ($P = 0.002$) across the three agricultural sites based on the limited number of samples in this study, with percent DON highest in the fall and lowest in the spring. During summer, percent DON generally was between and not significantly different from the fall or spring. For the forest sites and urban storm water sites, percent DON did not differ across seasons ($P = 0.216$ and 0.073 , respectively).

DOC concentrations varied by more than one order of magnitude across all sources, sites, and times (Table 1). Concentrations ranged from $172 \mu\text{M}$ at the Hardwood-1 forest site in spring to $3,366 \mu\text{M}$ in summer at the agricultural bovine site. Overall, there was a significant effect of source ($P < 0.001$) on DOC concentrations, with lowest concentrations in the forest sites ($403 \pm 129 \mu\text{M}$) and higher concentrations in the agricultural ($1,305 \pm 829 \mu\text{M}$) and urban/suburban storm water ($816 \pm 225 \mu\text{M}$) sites.

The C:N ratio of the bulk dissolved organic matter differed by source ($P < 0.001$). The forest sites had the highest C:N ratios overall (53 ± 36) compared to the urban/suburban storm water (18 ± 12) and agricultural (10 ± 2) sites.

Bioavailable DON and response of the plankton community—The proportion of DON utilized by the plankton community likely depends on a number of factors, including differences in the chemical composition of the source material and the organisms present to utilize the DON, as well as physical conditions and exposure time. We use the term bioavailable DON in this study to refer to the DON that was utilized by the plankton community over the ~ 12 -d time period under our experimental conditions. Incubation temperatures ranged from 12 to 27°C (Table 2), which reflected in situ Barnegat Bay temperatures for the season during which the plankton communities were collected and the experiments conducted. Light exposure ranged from 85 to $120 \mu\text{E m}^{-2} \text{ s}^{-1}$, which is similar to light levels at middepth in Barnegat Bay (Moser 1997). Initial nutrient concentrations in the Barnegat Bay water across the nine experiments were (average \pm SD): DIN, $3 \pm 2.5 \mu\text{M}$; DIP, $<0.1 \mu\text{M}$; DON,

Table 2. Experimental conditions and amount of DIN and DON added in bioavailability studies. DIN and DON additions are reported as ranges among the three sites for each source and season.

Source type	Season	Lights (h on)	Light level ($\mu\text{E m}^{-2} \text{s}^{-1}$)	Incubation temperature ($^{\circ}\text{C}$)	Salinity	DIN* ($\mu\text{mol N flask}^{-1} \text{d}^{-1}$)	DON* ($\mu\text{mol N flask}^{-1} \text{d}^{-1}$)
Agricultural pasture	Spring	12	105	12	11	6.2	5.0–7.6
	Summer	12	120	23	16	1.1	7.4–7.7
	Fall	8	105	16	15	0.9	7.4–7.6
Forest	Spring	12	100	15	11	7.5	5.0–16.6
	Summer	12	95	27	21	2.8	4.4–15.1
	Fall	10	95	10	20	2.8	7.4–7.6
Urban/suburban storm water	Spring	12	85	12	14	14.7	7.5–11.2
	Summer	12	95	25	17	17.0	6.1–12.0
	Fall	10	95	10	20	5.2	4.7–11.7

* There were approximately 1.5 L of bay water per flask; however, the exact volume varied from day to day due to removal for analysis and addition of concentrate.

Table 3. Proportion of DON added that was utilized during the 10–12-d time course of the bioavailability experiments. Averages (SD) for replicate flasks and by source and season are shown.

Source type	Season	Site	% DON bioavailable (by site)		% DON bioavailable (by season)	
			Mean	SD	Mean	SD
Agricultural	Spring	Bovine	38	8.0		
		Equine	46	1.6	44	4.7
		Swine	47	1.1		
	Summer	Bovine	26	0.7		
		Equine	43	2.4	32	9.7
		Swine	27	1.4		
	Fall	Bovine	10	0.4		
		Equine	12	2.9	14	5.5
		Swine	21	6.1		
Mean			30	14		
Forest	Spring	Pine	28	7.5		
		Hardwood-1	8	5.6	12	14
		Hardwood-2	0	0		
	Summer	Pine	44	9.8		
		Hardwood-1	48	2.7	35	19
		Hardwood-2	13	18.2		
	Fall	Pine	31	2.8		
		Hardwood-1	13	2.1	26	12
		Hardwood-2	34	6.9		
Mean			24	17		
Urban/suburban storm water	Spring	G	73	5.7		
		L	72	0.4	68	7.3
		M	59	1.6		
	Summer	G	51	0.1		
		L	57	9.4	50	7.4
		M	42	7.1		
	Fall	G	51	0.1		
		L	72	8.5	59	11
		M	55	3.3		
Mean			59	11		

$17 \pm 5 \mu\text{M}$. During the experiments, concentrations of DIN generally remained low ($<1 \mu\text{M}$), and DIP concentrations generally were greater than $0.5 \mu\text{M}$.

The bioavailability to Barnegat Bay plankton of DON from three different sites was studied for each source (forest, urban/suburban storm water, agricultural) during spring, summer, and fall. In general, there was good agreement in the proportion of DON utilized between duplicate incubation flasks for a site (Table 3). Regardless of the source or season, some portion of the DON added was utilized in all experiments, with the exception of the forest Hardwood-2 site in spring. Bioavailable DON ranged from 0 to 73% of the DON added (average of duplicate flasks). Within each source, the three sites generally had similar proportions of bioavailable DON, although variability among sites was greater for forested sources (Table 3; Fig. 1). Overall, there was no statistically significant difference in the proportion of bioavailable DON among the three agricultural sites ($P = 0.75$), the three urban/suburban storm water sites ($P = 0.28$), or the three forest sites ($P = 0.41$). There was no statistically significant relationship across all experiments between the amount of DON added (Table 2) and the percentage of DON utilized ($r^2 = 0.05$; $P = 0.1$). Temperature, which varied among the experiments from 10 to 27°C (Table 2), was not related to the proportion of DON that was bioavailable.

It is possible that there was some production of DON in treatment flasks (Bronk and Glibert 1991; Collos et al. 1992; Bronk et al. 1998). However, there was no obvious indication of net in situ production, in that measured DON was less than predicted based on mass balance calculations. Although the plankton could have released some DON, it apparently was used as rapidly as it was produced. In a few cases, there was a small but measurable net change in DON in the DIN control flasks. We accounted for this in our calculation of source water DON utilization rates in the associated treatment flasks by adding or subtracting, as appropriate, this amount to/from the change in DON in the treatment flasks. If there was net DON production that was not accounted for by our DIN controls, our calculated percentages of bioavailable DON were underestimates. There was little or no net DON utilization or production ($<2 \mu\text{mol}$

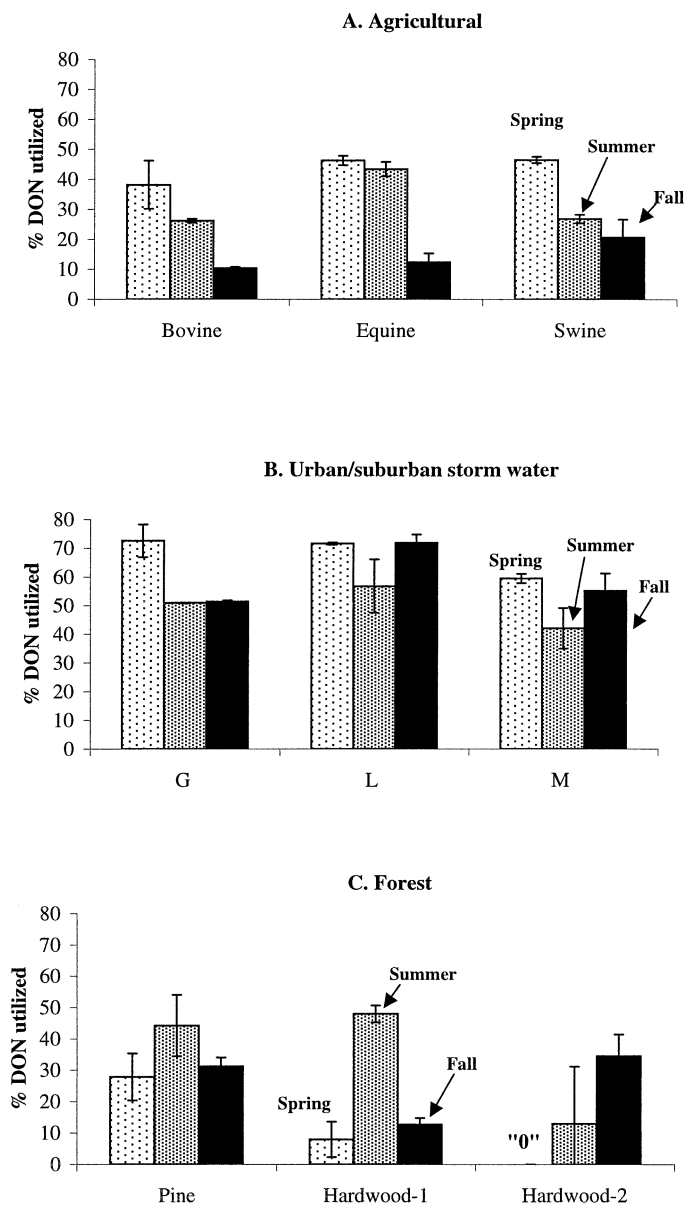


Fig. 1. Proportion (as a percentage) of DON from (A) agricultural (bovine, equine, and swine pastures), (B) urban/suburban storm water, and (C) forested sources that were utilized by estuarine plankton during spring, summer, and fall experiments. Average and standard deviation for duplicate incubation flasks for three sites per source are shown.

L^{-1} over the 10–12-d experiments) in the controls with no N addition.

Daily additions of concentrated source water to estuarine plankton communities resulted in increases in bacteria production and phytoplankton production within the first 1–3 d (e.g., representative time course data are shown in Fig. 2A,B). In almost all cases, bacterial production rates (Fig. 3B), and in most cases phytoplankton production rates (Fig. 4B), in treatment flasks receiving source water were enhanced relative to their rates in the DIN controls, which received the same amount of DIN as the treatment flasks. Note that the bacterial and phytoplankton production rates plotted

in Figures 3B, 4B, respectively, are the production rates after correcting for production in the DIN controls.

After additions stopped, there was little or no detectable additional utilization of DON (Fig. 2C), and bacteria production and phytoplankton production usually decreased, although rates generally remained higher than in the DIN controls (e.g., Fig. 2A,B). This suggests that during the period of additions, the increased bacterial and phytoplankton production in the treatment flasks relative to the DIN controls was a response to the DOM additions. The little additional utilization of DON after additions stopped suggests that most of the readily utilizable DON was degraded within the approximately 10–12-d experimental period.

Discussion

Understanding the effects of N loading on coastal ecosystems requires knowledge of the amount of bioavailable N inputs and the effect of different forms of bioavailable N on ecosystem processes. Nitrogen budgets for coastal ecosystems have generally been based on inputs of DIN or TN (e.g., Boynton et al. 1996; Nixon et al. 1996), although some studies have partitioned N inputs into DIN, DON, and PN (Nixon et al. 1995). Relationships between N loading to coastal marine ecosystems and production of phytoplankton or higher trophic levels have either been based on DIN (e.g., Boynton et al. 1982; Nixon 1992; Nixon et al. 1996) or TN inputs (e.g., Boynton et al. 1996). Similarly, experimental approaches examining the effects of nutrient inputs on coastal ecosystems (e.g., microcosms, mesocosms) generally are based on inorganic nutrients only (Oviatt et al. 1995; Taylor et al. 1995). The development of relationships that explicitly account for effects of DON (or PN) inputs has been hampered by the lack of information on how much of the organic N is bioavailable and what the responses of organisms are to the organic N entering coastal systems. The response of organisms to a given amount of N in the form of ammonium, nitrate, and/or nitrite inputs does not depend on the source of the DIN. However, DON is a complex mixture of primarily uncharacterized compounds. The results of the studies presented here demonstrate that only a portion is biologically available on the time scale of water residence time in many estuarine ecosystems and that the proportion of DON that is bioavailable to estuarine plankton communities varies by source and season.

Bioavailability of DON by source and season—The proportion of DON that was utilized by the estuarine plankton communities varied by source/land use. Overall, a higher proportion of DON was utilized from the urban/suburban storm water runoff ($59\% \pm 11$) compared to the agricultural ($30\% \pm 14$) or forest ($24\% \pm 17$) sources (ANOVA; $P < 0.0001$) (Table 3). The agricultural and forest sources had similar proportions of bioavailable DON ($P = 0.35$).

Season also was important in determining the proportion of DON exported from different land uses that was bioavailable to the estuarine plankton. All three sources exhibited seasonal differences in the proportion of DON that was utilized (agriculture $P < 0.001$; urban/suburban $P = 0.01$; forest $P = 0.05$) (Fig. 1; Table 3). In addition, a different sea-

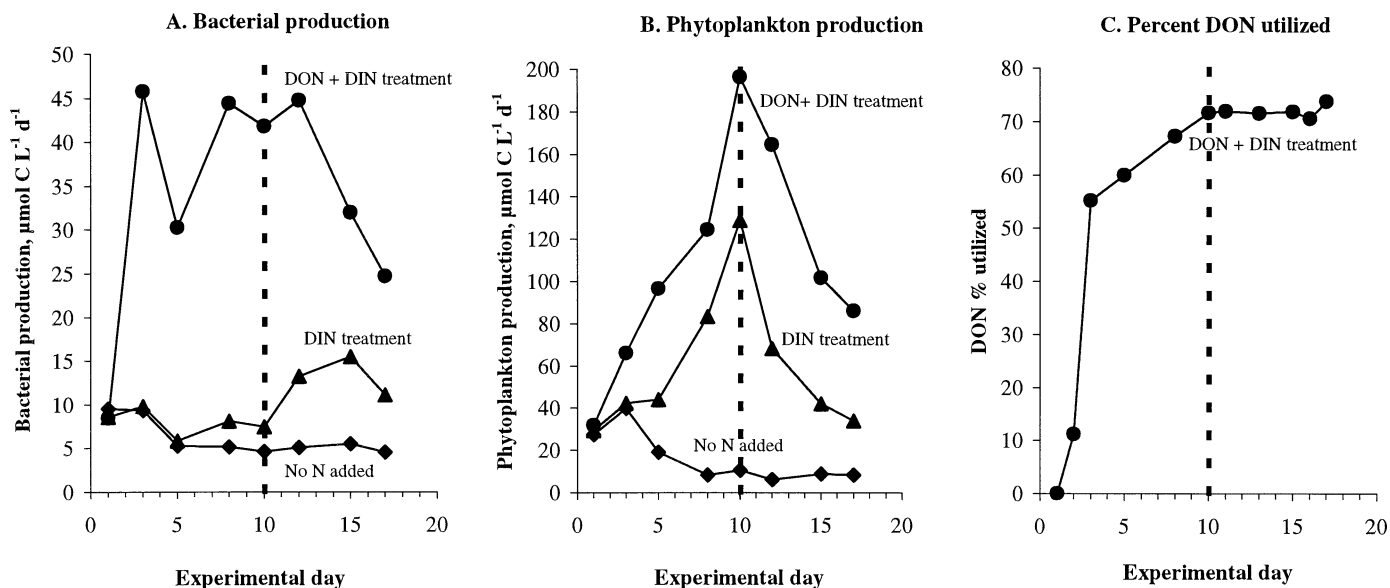


Fig. 2. Time course results from the fall urban/suburban storm water experiment (site L) show (A) rates of bacterial production, (B) rates of phytoplankton production, and (C) percentage of DON added that was used. DIN treatment received the same amount of DIN as the storm water DON + DIN treatment. Each point represents the average value of duplicate incubation flasks. The dashed vertical line indicates the last day of N additions.

sonal pattern was observed for each source in the proportion of DON that was bioavailable. In the agricultural sites, the proportion of DON that was utilized was highest in spring, lowest in fall, and generally intermediate in summer (Fig. 1A). In contrast, in the urban/suburban storm water runoff sites, the proportion of DON that was utilized was lowest in summer and higher in the spring (all three sites) and the fall (two out of three sites) (Fig. 1B). Yet a third seasonal pattern was seen in two (Pine and Hardwood-1) out of the three forest sites; in those two sites the proportion of DON that was utilized was highest in summer and lower in spring and fall (Fig. 1C). A previous study in three Swedish wetlands found no seasonal differences in the bioavailability of DON (Stepanuskas et al. 1999). However, these studies included only the high and intermediate molecular weight fractions of DON (Stepanuskas et al. 1999); the low molecular weight fraction, which can account for the majority of the DON, was not examined seasonally. Other studies of seasonal differences in the bioavailability of external inputs of DON to aquatic systems are generally lacking.

The present experiments demonstrate that the DON from different sources and seasons can vary significantly in its bioavailability to the plankton communities present during the time that it would enter the estuary. The seasonal patterns in the proportion of DON utilized, as well as within-season differences among sources, could be due to a number of factors. Differences in the chemical composition of DOM associated with its production in the forest, pastures, or urban landscapes; biological and chemical alteration of DOM within the different land uses before export; or both might account for the different patterns observed. Differences in the composition of the bay plankton community among experiments also could be responsible for the observed differences in the seasonal patterns and within-season differences.

Sorting out the contribution of these and other factors to the patterns of DOM utilization awaits further study. Measurements of the molecular level chemical composition of various DOM sources and the effect of microbial species composition are currently being conducted and could help to address these questions (Seitzinger et al. unpubl. data).

In addition to microbial processes, photochemical processes can alter DON, and in some cases release DIN (e.g., Bushaw et al. 1996). UV wavelengths are generally considered to be most important in that regard but do not penetrate deeply into the water column of a turbid estuary. The current experiments were conducted with exposure only to PAR wavelengths, so photochemical processing of DON was assumed to be minimal. Furthermore, photochemical processes (UV or PAR) did not affect the bioavailability of the N or C component of the bulk DOM in a separate study that we conducted with summer-collected DON from one of our forest (Hardwood-2) and two of our agricultural (bovine and swine) sites (Wiegner and Seitzinger 2001).

Although additional data are needed to determine whether DON is more or less bioavailable from natural or anthropogenic sources, there is a suggestion that DON released from soils might be less bioavailable than DON not originating from or subject to soil processes. An average of 24% (± 17) of DON was bioavailable to estuarine plankton communities in three experiments from each of three eastern U.S. forests in the current study; in four out of nine of those forest measurements, less than 15% of the DON was bioavailable (Table 3). The proportion of DON utilized from our Hardwood-2 site (21% ± 7) and equine (25%) and swine (25% ± 13) pastures in summer by freshwater bacteria were also similar (Wiegner and Seitzinger 2001). The bioavailability of DON in subsurface runoff from the animal pastures (30% ± 14) was not significantly different than from the

forests in the current study. Between 8 and 16% of the bulk DON was bioavailable from a relatively pristine wetland in Sweden (range of two experiments with estuarine bacteria; Stepanauskas et al. 1999). DON bioavailability was 19–28% in two forested streams in northern Sweden, although this increased to 45–55% during a spring flood (Stepanauskas et al. 2000). Bioavailability of humic-associated nitrogen in river water from a watershed dominated by coniferous forest varied between 8 and 37% (Carlsson et al. 1999). Conversely, the bioavailability of DON in urban/suburban storm water runoff from areas with considerable areas of impervious surface was consistently high ($59\% \pm 11$) (Table 3). A relatively high proportion of DON also was bioavailable in two experiments with rainwater collected from an urban/suburban site (46–75%; Seitzinger and Sanders 1999).

From a carbon perspective, aliphatic C appears more bioavailable than aromatic C (Moran and Hodson 1994; Sun et al. 1997; Hopkinson et al. 1998). Aromatic humic compounds are likely a major component of DON from forest and agricultural soil sources. Although the chemical composition of the bioavailable portion of the N-containing portion of DOM has not been characterized, the lower proportion of bioavailable DON from forest, wetland, and agricultural soil sources compared to urban/suburban runoff or rainwater may also be related to the degree of aromaticity of the N containing DOM compounds.

The bulk C:N ratio has been found in some studies to relate to bioavailability of DOM. Accumulation of bacterial biomass (bacterial regrowth bioassays; mg bacterial biomass mg^{-1} initial DOC) is inversely correlated with the C:N of the bulk DOM in a number of studies. These include studies with Ogeechee River water ($r^2 = 0.63$, $n = 14$; one outlier omitted from regression) and with plant/algal leachate ($r^2 = 0.79$, $n = 5$; our calculations using data in Sun et al. 1997), and across four rivers ($r^2 = 0.84$, $n = 4$; our calculations using data in Hopkinson et al. 1998). This correlation with C:N suggests that proteinaceous moieties are preferentially consumed (Sun et al. 1997). Although we cannot directly compare our results to the above studies because of differences in methodologies, we found no statistically significant relationship between the C:N ratio of the DOM added and the proportion of DON that was bioavailable either across all sources ($r^2 = 0.04$, $P = 0.12$) or within a source ($r^2 \leq 0.04$, $P \geq 0.4$). The C:N ratio was generally lowest for the agricultural sites (about 7:1 to 13:1), intermediate for the urban/suburban storm water sites (7:1 to 49:1) and highest for the forest sites (19:1 to 140:1) (Table 1). The highest proportion of bioavailable DON was in the urban/suburban storm water, which had intermediate C:N ratios of the added DOM. Further investigations of the chemical composition of the N and C moieties of the bioavailable portions of DOM across sources and seasons are needed.

Plankton response—Bacteria: Bacteria generally are considered the most likely pathway by which DON is utilized in aquatic ecosystems, with some or all of that DON mineralized by the microbial food web and thus subsequently available for phytoplankton. Bacteria can use inorganic N as well as dissolved organic N (Kirchman 1994; Kroer et al. 1994). However, most studies of organic N utilization by

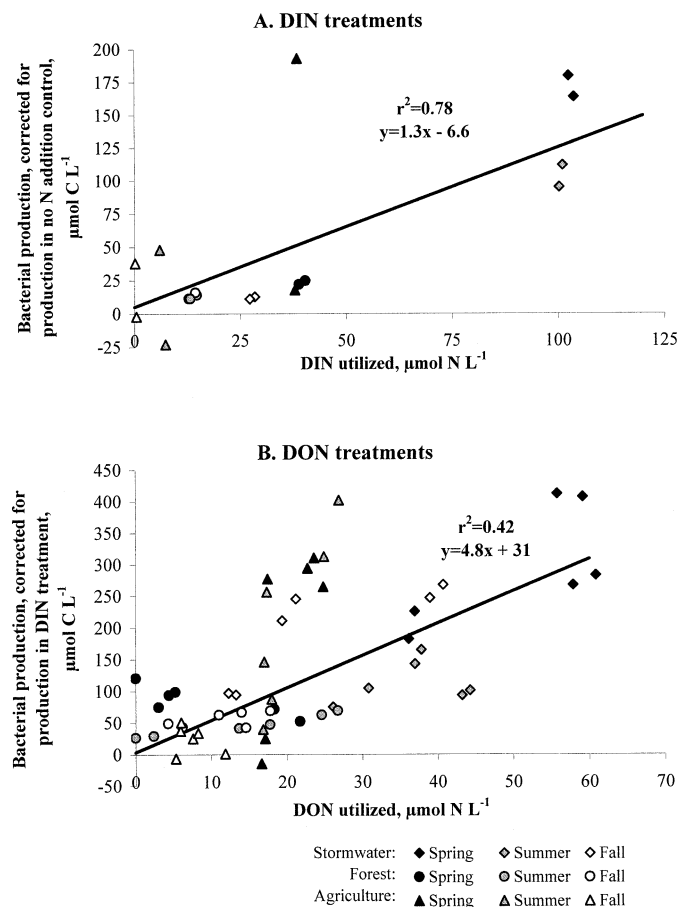


Fig. 3. Relationship between integrated bacterial production and nitrogen utilization in bioavailability experiments. (A) DIN treatments: integrated bacterial production (corrected for production in controls with no N addition) versus the amount of DIN utilized; (B) DON treatments: integrated bacterial production (corrected for bacterial production in DIN treatment to account for effect of DIN in the DON source water) versus the amount of DON utilized from additions of urban/suburban storm water, agricultural pasture, and forest runoff. Each point represents the results from an individual flask.

bacteria are based on simple organic compounds such as dissolved free and combined amino acids. Utilization by freshwater or estuarine bacteria of complex DON from natural or anthropogenic sources has received less attention (Carlsson et al. 1995, 1999; Seitzinger and Sanders 1997, 1999; Stepanauskas et al. 1999, 2000; Wiegner and Seitzinger 2001). Estuarine bacterial production in the current experiments generally increased when N was added in either the inorganic or organic form. Bacterial production was enhanced in estuarine water receiving only DIN (relative to controls with no N additions) by approximately $1 \mu\text{mol C} \mu\text{mol}^{-1}$ DIN used by the plankton ($r^2 = 0.78$; $P < 0.001$) (Fig. 3A). Bacterial production in estuarine water receiving DON from the forest, agricultural, or urban/suburban sources was further enhanced relative to that accounted for by the DIN in the added source water (Fig. 3B). The increased bacterial production was not related to the total amount of DOM as C or N added, but rather with the amount of DOM-N

utilized. Enhanced bacterial production in the DON treatments (i.e., increased production over DIN treatments) increased linearly relative to the amount of DON utilized ($P < 0.001$; Fig. 3B). The amount of DON utilized accounted for about 42% of the variation in bacterial production. The increase in bacterial production was approximately $5 \mu\text{mol C } \mu\text{mol}^{-1}$ DOM-N utilized. It is not known whether the enhanced bacterial production represents a response of the bacteria primarily to the N or C component of the DOM. In the absence of measured rates of DOC utilization, we estimated the amount of DOC utilized by multiplying the amount of DOM-N utilized (Fig. 3B) by the measured C:N ratio of the DOM in the source water (Table 1). This assumes that bacteria were utilizing the C and N component of the DOM in a ratio consistent with the C:N ratio of the DOM added, which may or may not be a correct assumption. There was no significant relationship between bacterial production and the estimated DOC utilized ($r^2 = 0.006$; $P > 0.5$). The relatively good relationship between the bacterial production and the amount of DOM-N used suggests that the bacterial response was primarily to the N, rather than the C, component of the DOM.

The increase in bacterial C production with organic N ($\sim 5 \mu\text{mol C } \mu\text{mol}^{-1}$ DOM-N used) is higher than with inorganic N ($\sim 1 \mu\text{mol C } \mu\text{mol}^{-1}$ DIN used in control flasks). The higher bacterial N-specific production with DON relative to DIN used could be due to a number of factors, including the availability of C as well as N in the DOM. In addition, phytoplankton were utilizing DIN, most likely to a considerably greater extent than the DON.

Phytoplankton: Nitrogen is the primary nutrient limiting phytoplankton production in many estuarine and coastal marine ecosystems. In addition to nitrate and/or ammonium, some phytoplankton can take up small molecular weight organic N compounds (< 600 nominal molecular mass) (Antia et al. 1991; Berg et al. 1997), as well as some larger organic compounds following exoenzyme breakdown (e.g., Pantoja and Lee 1994; Mulholland et al. 1998). Only a few studies have examined the effect of the complex mixture of DON from the environment on phytoplankton communities. Addition of DOM from rainwater can increase phytoplankton production and biomass and change community composition (Timperley et al. 1985; Peierls and Paerl 1997; Seitzinger and Sanders 1999). DON from humic substances in riverine water might have similar effects (Carlsson et al. 1995). In the current study, phytoplankton production in estuarine water increased linearly with additions of DIN (in combination with PO_4 additions) ($r^2 = 0.98$; $P < 0.001$) (Fig. 4A). There was additional enhancement of phytoplankton production in DON treatments above that which could be accounted for just by the amount of DIN added in the source water (Fig. 4B). Increases in phytoplankton production in the DON treatments (above that due to DIN) generally ranged from 20 to $500 \mu\text{mol C L}^{-1}$ (integrated over the course of the experiment). However, there was no consistent quantitative relationship between increased phytoplankton production and the amount of DON utilized in the treatments ($r^2 = 0.02$; $P > 0.2$). This suggests that a variable portion of the DON utilized was directly or indirectly (through microbial min-

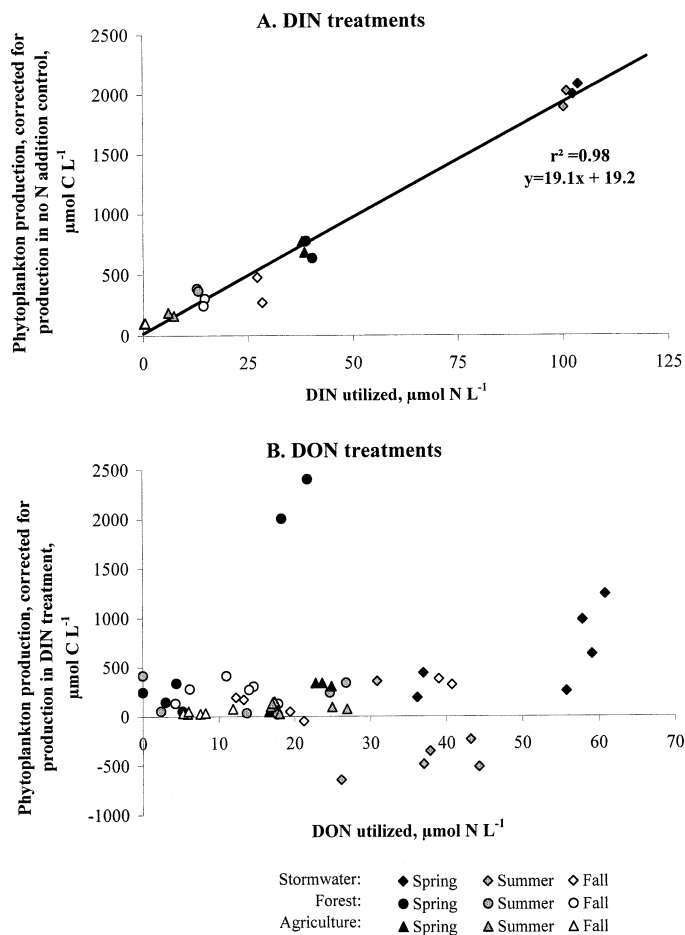


Fig. 4. Relationship between integrated phytoplankton production and nitrogen utilization in the bioavailability experiments. (A) DIN treatments: integrated phytoplankton production (corrected for production in controls with no N addition) versus the amount of DIN utilized. (B) DON treatments: integrated phytoplankton production (corrected for phytoplankton production in DIN treatment to account for effect of DIN in the DON source water) versus the amount of DON utilized from additions of urban/suburban storm water, agricultural pasture, and forest runoff. Each point represents the result from an individual flask.

eralization) reaching the phytoplankton. The duration of the experiments (approximately 2 weeks) was long compared to the generation time of bacteria or bacterial grazers and thus should have been sufficient for microbial mineralization of DON utilized by the bacteria. The results of the current study also provide insight into the interpretation of data from other approaches that have been used to measure DON bioavailability. They indicate that phytoplankton production is not a good measure of the amount of bioavailable DON (Fig. 4B) and that measurements of the amount of bioavailable DON based on bacterial responses alone may not reflect N available to phytoplankton.

In a few cases, there was evidence of inhibition of phytoplankton production by some component (organic or inorganic) of the source water. Specifically, during summer in the three urban/suburban storm water runoff treatments, phytoplankton production decreased (relative to DIN controls;

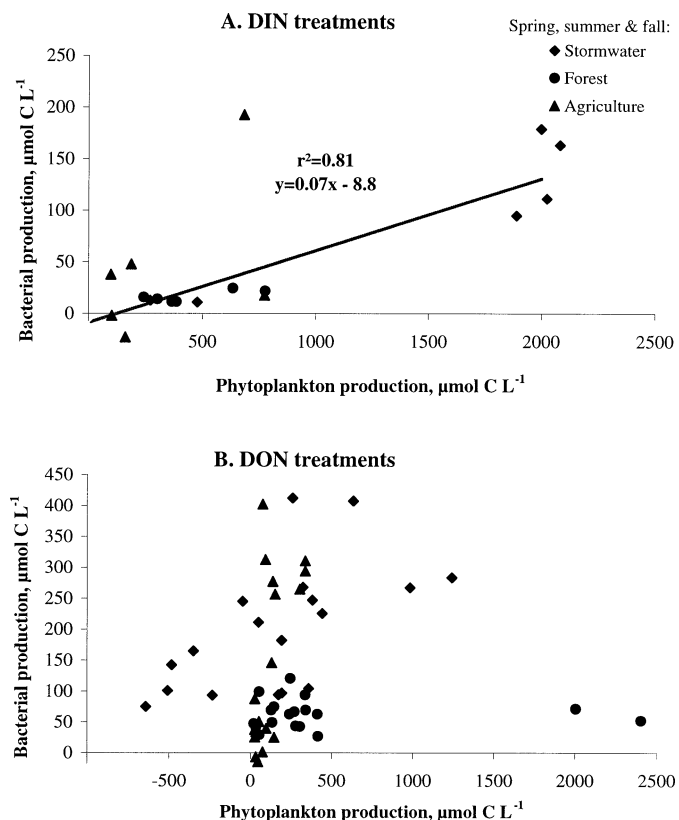


Fig. 5. Bacterial production versus phytoplankton production of estuarine plankton communities in (A) DIN treatments and (B) DON + DIN treatments receiving additions of urban/suburban storm water, agricultural pasture, or forest runoff. See captions of Figures 3, 4 for explanation of control corrections.

negative values in Fig. 4B). However, the urban/suburban storm water did not inhibit bacterial production, and in fact, bacterial production increased relative to controls (Fig. 3B) and about 50% of the storm water DON added was utilized by the plankton community (Table 3). Although we have no information on the specific compounds in the storm water, a number of compounds are known to inhibit photosynthesis that do not affect heterotrophic production (e.g., some herbicides) and could have been present in the storm water.

Bacterial production is correlated with phytoplankton production across a wide range of aquatic systems (Cole et al. 1988). In the current experiments, bacterial production was related to phytoplankton production in the treatments receiving only DIN as a N source ($P < 0.001$; Fig. 5A, although the relationship is driven strongly by the cluster of points at the high end). In the DON treatments, there was no significant relationship between bacterial production and phytoplankton production ($r^2 = 0.01$, $P > 0.2$; Fig. 5B). This supports the previous conclusion that a variable portion of the DON utilized by the bacteria is being mineralized and made available to the phytoplankton. It also indicates that the bacteria were not primarily depending on release of DOM from phytoplankton for growth, but more likely on the DOM from the source water.

Effect of land use on bioavailable N budgets—There has been considerable progress in understanding the magnitude of DIN export by rivers to coastal ecosystems as a function of watershed properties, including land use, N inputs to watersheds (e.g., Meybeck 1982; Smith et al. 1997; Caraco and Cole 1999; Arheimer and Liden 2000), or both. The controls on DON export by rivers are not as clear (Hedin et al. 1995; Arheimer and Liden 2000; Lovett et al. 2000; McHale et al. 2000). The current study illustrates the need to understand both how much DON is exported by different land uses and how much of the DON is biologically available from each source/land use in order to predict the effects of N loading on ecosystem processes. Here, we used a simplified approach, based primarily on the results of the current study, to begin to explore the effect of land use on the proportion of total dissolved N (TDN) that is exported as bioavailable N (DIN plus that portion of DON that is bioavailable) to coastal systems. These calculations do not include N transformations that occur during river transport (e.g., denitrification, DON degradation by river microbes, etc.). Therefore, they are most applicable to small coastal watersheds or to sources close to the estuary in larger watersheds, where the travel time for water runoff is short (a few days or less). Many estuaries fall into this category. For example, the estimated mean travel time for rivers draining the Barnegat Bay watershed is 0.6 d (R. Alexander pers. comm.). The mean water travel time from headwater to mouth is 3 d for 6,102 river systems out of 6,152 identified globally (Vörösmarty et al. 2000).

Four land use scenarios were considered; watershed export of DIN, total DON, and bioavailable DON were estimated for each scenario. In the first three scenarios, we assumed that 100% of the watershed is either forested, urban/suburban, or pasture. DIN yield from forests ($0.3 \text{ kg DIN-N ha}^{-1} \text{ yr}^{-1}$; Correll et al. 1995), pastures ($0.8 \text{ kg DIN-N ha}^{-1} \text{ yr}^{-1}$; Correll et al. 1995), and urban/suburban areas ($3 \text{ kg DIN-N ha}^{-1} \text{ yr}^{-1}$; Wahl et al. 1997) were based on published studies. Total DON yield for each land use was estimated based on those rates of DIN yield and the ratio of DON:DIN exported from the different land uses that we measured in the current study (Table 1; average DON:DIN for each season for each source used). Bioavailable DON yield was then calculated as total DON yield times the fraction of total DON that was bioavailable for each land use (Table 3). In the fourth scenario, we estimated bioavailable N export from a theoretical, mixed land use watershed in which 50% of the watershed area is urban/suburban, 25% forested, and 25% pasture. Estimates of N contributed from urban/suburban areas do not include N inputs from wastewater treatment facilities.

DOM sources (nonpoint) to rivers during base flow are considered to be primarily from deeper groundwater and soil horizons, whereas during storm flows, considerable DOM may be contributed from more superficial soil horizons (Boyer et al. 1996). Source water used in the current study was collected during storm flows. Although there may be differences in DON bioavailability between storm and non-storm flow conditions, there is no consistent pattern of DON or DOC bioavailability with flow based on previous studies in rivers (Leff and Meyer 1991; Benner et al. 1995; Sun et

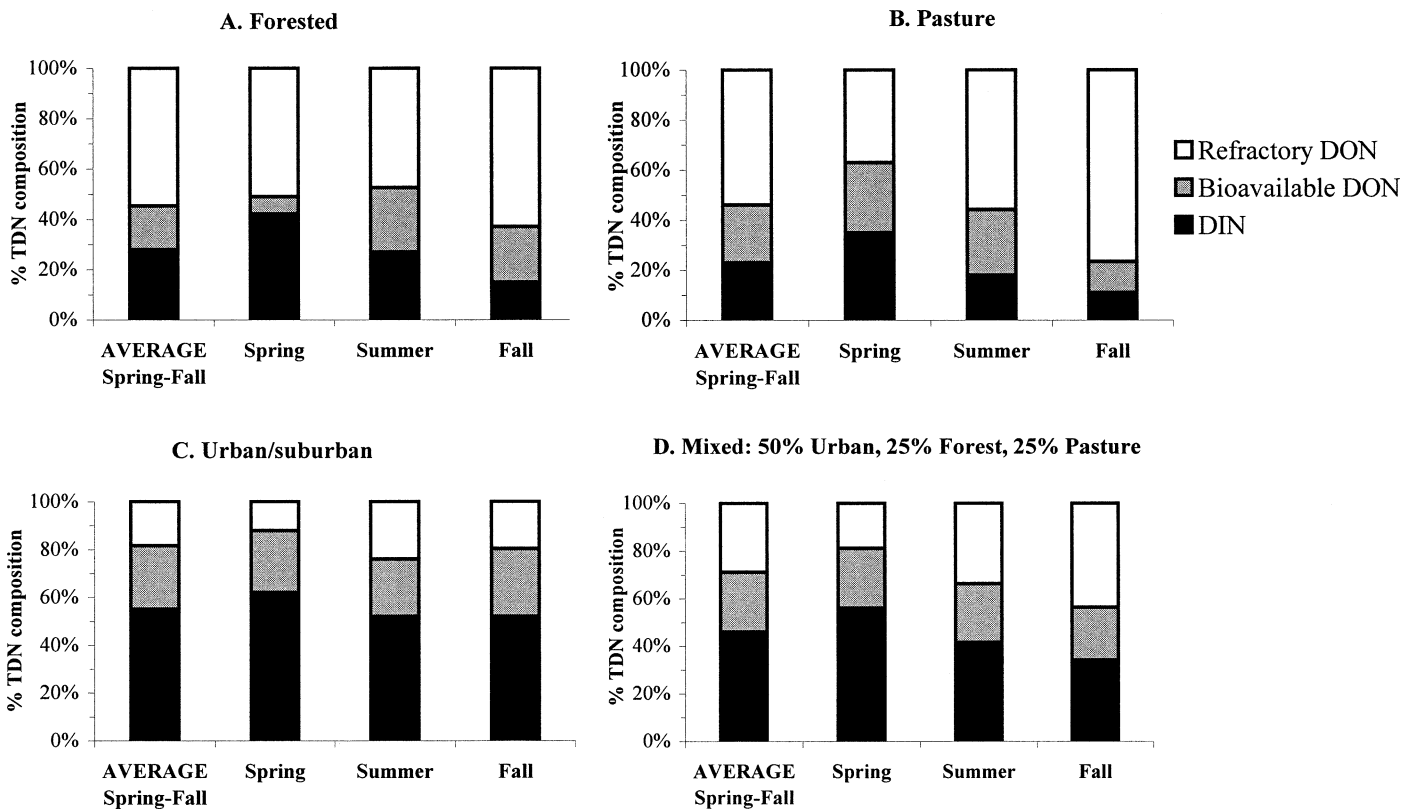


Fig. 6. Preliminary estimates of proportion of TDN that is bioavailable N (DIN + bioavailable DON) and refractory N (refractory DON) exported from (A) forests, (B) pasture, (C) urban/suburban, and (D) mixed land use watershed. Results are based on DIN and DON composition of source water (Table 1), bioavailability experiments (Table 3), and published literature. See text for details of calculations.

al. 1997; Volk et al. 1997; Stepanauskas et al. 2000). In the following calculations, we assumed that our DON bioavailability measurements were representative of both storm and nonstorm flow conditions.

These scenario calculations indicate the potential importance of land use in contributing to the variability (both among rivers and seasonally) in the proportion of TDN that is bioavailable N in rivers, exported to coastal ecosystems (Fig. 6), or both. In addition, they illustrate that land use can affect the relative amounts of DIN and DON that comprise the bioavailable N. For example, in the forested land use scenario, only ~40–50% of the TDN was bioavailable in spring through fall (Fig. 6A). In spring, most of the bioavailable N was DIN, whereas in summer and fall, DON and DIN contributed approximately equal amounts. For the pasture land use scenario, the proportion of bioavailable N decreased from spring (~60%) to summer (~45%) to fall (~25%) (Fig. 6B). DIN and DON accounted for approximately equal proportions of bioavailable N in all three seasons. The urban/suburban land use scenario differed considerably from the forested and pasture scenarios in that bioavailable N accounted for a higher proportion of TDN in all seasons (~75–90%) (Fig. 6C). At least half of the DON was bioavailable; however, bioavailable DON accounted for only approximately a third of the bioavailable N in all seasons. In the mixed land use scenario, bioavailable N accounted for 55–80% of the TDN, with DON accounting for

a substantial portion (approximately a third) of the bioavailable N in all three seasons (Fig. 6D).

Summary—Predicting the effects of nutrient inputs on coastal marine ecosystems becomes increasingly important as nutrient inputs increase because of human activities in their watersheds and airsheds. Although numerous physical and biological factors contribute to the response of coastal ecosystems to a given rate of nutrient (N and P) loading, the current study suggests that we need to know not only DIN or TN loading, but also bioavailable DON (and PN). The results of the current study indicate that both source/land use and season may be important in determining the amount of bioavailable DON. For example, urban/suburban storm water runoff had a higher proportion of bioavailable DON compared to agricultural pastures and forests, and the seasonal pattern of bioavailable DON differed for the three sources. These differences may be due to a number of factors, including the chemical composition of the DOM and the community composition of the receiving biota. The current studies also point out that the response of the plankton community to bioavailable DON is more complex than to DIN. There was a linear increase in bacterial production when either DIN or bioavailable DON was added to estuarine plankton communities. However, the increase in bacterial production per micromole N added was approximately five times greater when N was added as bioavailable DON

compared to when it was added as DIN. The effects of DON inputs on phytoplankton production were not consistent. Although phytoplankton production increased linearly with the amount of DIN added, there was no consistent relationship between the amount of bioavailable DON added and the increase in phytoplankton production. Future refinements in our understanding of the effects of human activities in watersheds on coastal ecosystems will clearly need to include a more comprehensive knowledge of the seasonal pattern of bioavailable DON contributed by different sources, as well as the ecosystem effects.

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