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Molecular-level chemical characterization and bioavailability of dissolved organic matter in stream water using electrospray-ionization mass spectrometry

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Abstract

We used electrospray-ionization mass spectrometry (ESI-MS) to characterize, at the compound level, dissolved organic matter (DOM) composition and bioavailability in two streams. There was considerable consistency in the composition of the DOM between the two streams (unit mass resolution): >70% of the masses detected occurred in both streams. Approximately 40–50% of the bulk dissolved organic carbon in the stream water was bioavailable during a 12-d microbial decomposition experiment. ESI-MS compound level analysis identified which masses were used, which were not, and their patterns of utilization. In both streams, ~40% of the masses decreased in concentration, ~55% did not change, and <5% increased. Despite the complex system (>1,500 DOM compounds and a natural consortia of bacteria), there was a high degree of similarity in which masses were used and the amount of each mass used between replicate flasks for a stream. There was also good agreement between the two streams in which masses were used and the amount of each mass used. This suggests that the selection by the microbial consortia of organic compounds in the complex and heretofore largely uncharacterized DOM pool is repeatable and, therefore, ultimately predictable.

Dissolved organic matter (DOM) in freshwater and seawater is a major global reservoir of carbon (C); it exceeds the amount of organic matter in all aquatic organisms and is equal to the amount of C in atmospheric CO_2 (e.g., Siegenthaler and Sarmiento 1993; Hedges and Keil 1995). Traditionally, DOM in aquatic systems was considered to be refractory (biologically unavailable) over weeks to months. We now know that a considerable amount of DOM can be rapidly used by microbial communities and that it has many important ecological roles. For example, DOM attenuates light and chelates toxic metals. It is an important energy and nutrient source in aquatic ecosystems (Benner 2002; Bronk 2002; Carlson 2002; del Giorgio and Davis 2003). DOM contains a major portion of nitrogen (N) exported by rivers to coastal systems, and this dissolved organic N (DON) contributes to coastal plankton production (Seitzinger et al. 2002; Stepanauskas et al. 2002).

Despite the importance of DOM in aquatic ecosystems, >75% of the DOM pool remains chemically uncharacterized at the compound or molecular level (Hedges et al. 2000; Bronk 2002; Benner 2003). Past analyses have been primarily limited to bulk elemental analysis (dissolved organic C [DOC] DON) or to broad structural features from bulk elemental ratios (e.g., CHO) or spectral (infrared [IR], nuclear magnetic resonance [NMR] spectroscopy, and fluorescence) signatures. Ultrafiltration techniques have been used to characterize DOM by molecular weight fraction (e.g., total DOC > or <1,000 Da; Benner et al. 1997; Hopkinson et al. 1998). Analyses of compound classes (e.g., total carbohydrates, proteins, lipids, and lignins) or a few individual compounds (e.g., dissolved free amino acids) generally ac-

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count for <25% of the total DOM. New analytical approaches are needed to characterize the complex pool of DOM compounds.

There is considerable variability in the bioavailability and ecosystem effects of DOM. For example, the proportion of DOM that is bioavailable varies in time and space (Carlson et al. 1998; Stepanauskas et al. 2002; del Georgio and Davis 2003). The response of coastal plankton communities to DOM inputs depends on the source (e.g., forests or urban or agricultural runoff) of the DOM (Seitzinger et al. 2002). Although certain harmful algal bloom species prefer DON as their N source, the occurrence of these species is sometimes correlated with DOM, but not always (Anderson et al. 2002). This ecological variability cannot be explained by the bulk level measures of DOM alone. Compound level information on the distribution and reactivity of the molecularly uncharacterized DOM is fundamental to understanding its origin, bioavailability, and ecosystem effects.

Earlier attempts to chemically characterize the molecular composition of DOM by mass spectrometry (MS) were hampered by the complex fragmentation patterns from the heterogeneous mixture of DOM compounds. Electrospray-ionization MS (ESI-MS) is a relatively new analytical application for characterizing and quantifying polar organic compounds (e.g., McIntyre et al. 1997; Poon 1997; Shang et al. 1999; Pi and Leary 2004). ESI-MS allows the characterization of intact polar macromolecules (50 to >100,000 amu) because of its low ionization voltage, thus providing compound-level information on mass, abundance, and functional groups (Cole 1997; Poon 1997; Pi and Leary 2004). Because DOM compounds in water (i.e., a polar solvent) are generally polar, ESI-MS has potential as a direct method for detecting a large fraction of the DOM compounds in aqueous environmental samples. Recently, various components of DOM from the Suwannee River (in Georgia), a black-water stream (in New Jersey), and a rainfall-fed mountain stream (in Costa Rica) were chemically characterized using ESI with quadrupole time-of-flight and Fourier transform ion cyclotron resonance (FT-ICR) MS (Kujawinski et al. 2002*a*,*b;* Kim et al. 2003; Stenson et al. 2003). DOM in 11 rainwater samples was chemically characterized using ESI-MS with a quadrupole mass spectrometer (Seitzinger et al. 2003). However, ESI analyses of aquatic environmental samples have been limited in number and have not been used to examine the dynamics of DOM cycling.

In the current study we use ESI-MS analyses of DOM compounds in two streams to begin to address three basic questions. What is the uncharacterized DOM in streams and how similar is it between streams? Which compounds are microbial communities using (or not using) in these streams? How repeatable is the compound level utilization of the DOM pool by the microbial community? In addition, we compared the total DOC estimated from ESI-MS data to direct measurements of bulk DOC.

Materials and methods

Site descriptions—Water was collected from two streams in New Brunswick, New Jersey, for chemical characterization and bioavailability studies. The sites were the same as those used previously for bulk DON bioavailability measurements (Seitzinger et al. 2002; Lyell Brook and Mile Run Brook, hereafter referred to as streams L and M, respectively). The streams drain primarily residential land (~0.1 ha. or less lot size) and are <3 km apart. Samples were collected in spring (April) during a rain event. Water was filtered on site through a prerinsed 0.5-μm string-wound polypropylene canister filter into acid-washed 20-liter cubitainers. Samples were stored on ice during transport to the lab. The water was then sterile filtered (0.1 μm filter), concentrated approximately eightfold by low-temperature vacuum evaporation (Seitzinger et al. 2002), and immediately frozen and stored until it was used in the bioavailability experiment.

Bioavailability experimental methods—The term ''bioavailable'' DOM, as used in this study, refers to that portion of the DOM that was utilized by the microbial community within the 12-d incubation period, under the experimental conditions. The overall experimental approach to measure the biological degradation of DOM in stream water was similar to that used previously (Amon and Benner 1996; Seitzinger and Sanders 1997, 1999). The natural assemblage of bacteria from a nearby pond (Weston Mills Pond) that is not connected to either study stream was added to 200 ml of sterile-filtered water from the two streams and to the control water (E-Pure water; Barnstead). The bacterial inoculum was added in the ratio of 1 ml pond water : 28 ml stream or control water. The bacterial inoculum was sonicated briefly before use, to eliminate protozoa (Seitzinger and Sanders 1997). The E-pure controls (hereafter referred to as ''procedural controls'') were conducted to check for contamination during sample processing and analysis.

After bacterial inoculation, the water was divided into duplicate glass bottles with foil covers and incubated in the dark at 22°C; the water was stirred gently with Teflon-coated stir bars. Time series samples (15 ml) were collected (days 0, 2, 6, and 12) from all flasks and analyzed for bulk chemical concentrations (nitrate plus nitrite, ammonium, phosphate, total DOC, and total DON) and bacterial production. Time series samples for stream L and initial and final samples from stream M were analyzed for molecular-level chemical composition by ESI-MS (Seitzinger et al. 2003). All glassware used throughout the study was cleaned by acid washing and heating to 500°C for at least 4 h.

Bulk chemical analyses—Samples were analyzed as follows for bulk chemical parameters: dissolved inorganic nitrogen (DIN; ammonium, nitrite plus nitrate; QuickChem 31-107-06-1-A and 31-107-04-1-A, respectively; Lachat), soluble reactive phosphate (QuickChem 31-115-01-3-A), DOC (Shimadzu 5000A; Sharp et al. 1993), and DON. DON was determined by the difference between total dissolved N (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; Seitzinger and Sanders 1997). Bacterial production was measured using the ^{3}H-leucine method (Smith and Azam 1992), as detailed in Seitzinger et al. (2002).

Compound-level analysis of DOM—Compound-level analysis of DOM was conducted using a quadrupole mass-selective detector equipped with an ESI source (Agilent 1100 liquid chromatography [LC]/MS; Seitzinger et al. 2003). An autosampler injected samples and standard solutions (20 μl) from individual vials into the LC system, which transported sample directly to the ESI source region of the mass spectrometer (no LC column was used in this study). The mobile phase was 50 : 50 v : v methanol : water (0.05% formic acid in E-pure water [pH 3.5]) with a flow rate of 0.22 ml min^{-1}. The drying gas was N_2 (350°C, 10 L min^{-1}, 1.76 kilograms per centimeter). The capillary voltage was 3 kV; the fragmentor voltage was 40 V. The mass range scanned was 50–2,000 (in preliminary analyses of these samples, no compounds were detected with an *m/z* >2000). Only one replicate flask for each stream for days 0 and 12 was analyzed in the 1,000–2,000 *m/z* range. Samples were analyzed in both ESI-positive and ESI-negative ion modes.

For each sample, six replicate injections were made to establish a solid statistical basis for interpreting the *m/z* and ion abundance responses generated by the ESI-MS. The mean ion abundance (±SD) for the replicate injections for each *m/z* (rounded to nearest integer) in each sample was calculated for the samples and for procedural controls. Each *m/z* with an abundance that was statistically different from zero at the 0.05 level (*t*-test; Sokal and Rohlf 1981) was retained. The samples then were corrected for procedural blanks by subtracting the ion abundance of any *m/z* found in the procedural controls from the same *m/z* in a sample. The SD of the corrected ion abundance for each *m/z* was calculated using propagation-of-error procedures (Bevington and Robinson 1992). The final data set for a sample included the average (±SD) ion abundance for each remaining *m/z* with ion abundances that were statistically different from zero. Because of the generally increased variability in detecting compounds with ion abundances <500, only *m/z* with ion abundances >500 were included in the final data set.

We tested for statistically significant differences (*t*-test; 0.05) in ion abundance between initial (day 0) and final (day 12) samples for each *m/z,* to determine whether a particular mass was bioavailable (decrease in abundance), refractory (no change in abundance), or produced (increase in abundance). These results for individual *m/z,* in some cases, were also grouped into larger amu bins (i.e., 50 amu bins, < or >600 amu, and < or >1,000 amu) for interpretation.

ESI is based on the dispersion of a liquid into small charged droplets in an electrostatic field (Van Berkel 1997). ESI is a soft ionization method and, as such, does not fragment compounds at the ionization voltages generally used. Therefore, ESI-MS provides molecular-weight information as a mass-to-charge (*m/z*) ion. In the positive ion mode, compounds with basic functional groups are preferentially ionized and gain a H^+; in the negative ion mode, compounds with acidic functional groups lose a H^+. A number of caveats apply to the interpretation of the ESI mass spectra for mixtures of unknown organic compounds, as reviewed by Kujawinski (2002). For example, three or four decimal places of mass resolution generally is required to determine the exact elemental composition of a compound. Therefore, more than one compound may be included in our unit mass bins, and we cannot be certain that the same unit mass bin in different samples contains the same set of compounds. The detected mass-to-charge ion for a compound can be affected by several factors. Some compounds form adducts (e.g., with salts and buffers) in the ESI-positive ion mode and therefore also are detected at a mass equal to their mass plus adduct. Many high-molecular-weight compounds produce multiply charged ions under ESI-MS; these multiply charged ion clusters are usually in the 500–3,000 *m/z* ion range (Crain 1997; Loo and Loo 1997). Some compounds may not be effectively ionized under the sample conditions; the molecular species detected depend on the nature of the samples (e.g., sample matrix) and the analytical conditions, including solvent composition and pH (analytical conditions were constant for the analysis of all samples in this study). In this article we sometimes refer to a detected *m/z* as a compound or mass and to *m/z* as molecular weight or amu, while recognizing, and addressing where possible, the caveats noted above.

Comparison of bulk and compound-level results—The interpretation of ESI-MS data depends, in part, on the proportion of the total DOM that the technique detects. In general, previous studies with aquatic environmental samples have not attempted a mass balance between the ESI-MS data and total DOM. Such comparisons are difficult because of the uncertainties in quantitatively estimating total DOC in a complex mixture from ESI-MS data (as discussed below). In our study, we used three approaches to provide insight into the proportion of total DOM that the ESI-MS technique is detecting.

First, for the stream water samples, we compared the total DOC concentration measured directly by high-temperature combustion (HTC; Sharp et al. 1993) to the DOC concentration estimated from the ESI total ion abundances, which we based on the average ESI-MS response to a suite of organic compound standards. ESI ion abundance is a measurement of compound concentration. The relationship between ion abundance and concentration, however, is compound specific because of differences in ionization efficiencies among compounds. A number of factors affect ionization efficiencies, including compound structure, mobile phase conditions, and the sample matrix. The calculation of the concentration of a specific compound in a sample first requires the identification of that compound on the basis of, for example, a comparison of the behavior (e.g., retention time and different mobile-phase conditions) on LC columns of authentic compound standards and the corresponding mass in the sample. Then the instrument response factor (e.g., ion abundance per μmol compound L^{-1}) determined for that compound is used to calculate compound concentration. Although we have not yet identified specific compounds in the stream samples and therefore cannot quantify specific compounds, we have determined response factors for >80 standard compounds (Seitzinger et al. 2003; unpubl. data). These compounds were analyzed under the same analytical conditions used for the stream samples (pH, solvent mixture, ionization voltage, ESI positive/negative, etc.) and at concentrations between 25 nmol L^{-1} and 50 μmol L^{-1} for

Table 1. Response of ESI-MS to standard compounds (see text for analytical conditions). Units for response factor are ion abundance per μmol compound.

Compound	Molecular formula	Molecular weight	Response factor
Positive mode			
9-fluorenone*	$(C_6H_4)CO(C_6H_4)$	180.2	6,912
1,6-hexadiamine	$H_2N(CH_2)_6NH_2$	116.2	5,979
urea	H_2NCONH_2	60.1	2,146
1-propanamine	$CH_3(CH_2)_2NH_2$	59.1	3,782
Negative mode			
Butenedioic acid	$HO_2C(CH_2)_2CO_2H$	116.1	11,447
Benzoic acid	$C_6H_5CO_2H$	122.1	2,083
Hexanedioic acid	$HO_2C(CH_2)_4CO_2H$	146.1	4,775

* $[M+H]^+$ and [M+Na] ion.

individual compounds in both single and multiple compound standards. Standards were prepared in deionized water; therefore, analyses do not account for streamwater matrix effects that could decrease ionization efficiencies. Standards included a wide range of compound structures and functional groups (e.g., N heterocycles, alcohols, amines, amides, carboxylic acids, simple carbohydrates, amino acids, and polycyclic aromatic hydrocarbons). For individual compounds, there was a linear relationship between ion abundance and concentration over the range of concentrations tested (generally, $r^2 > 0.95$; Seitzinger et al. 2003; unpubl. data). Among compounds, however, there was considerable variation in response factors (ion abundance per μmol compound L^{-1}; e.g., Table 1). There was no relationship between response factor and molecular weight across the range of standards tested. Therefore, as a first approximation of the DOC concentration in the stream samples on the basis of the ESI-MS data, we first applied the average response factor for our suite of standard compounds (5,370 ion abundance units per μmol compound L^{-1}) to the ion abundance in each 50 amu bin (e.g., 50–99 or 100–149 amu) across the 50–2,000 amu range. We then converted the estimated μmol compound L^{-1} to μmol C L^{-1} using a carbon weight per mole weight of 0.48 based on elemental ratios in Suwannee River DOM (Sun et al. 1997). The midpoint of a 50 amu bin (e.g., 125 amu for the 100–150 amu bin) was used as the average mole weight of the compounds in a bin. The above calculations assumed that the average response factor calculated for our standards was representative of the average response factor for the group of compounds in any one 50-amu bin; because we do not yet have specific response factors for the suite of DOM compounds in the streams, we cannot estimate the error in that assumption. Using amu for mole weight assumes that the compounds are singly charged. High-resolution ESI FT-ICR MS analysis of the Suwannee River DOM indicated that the compounds were primarily singly charged species (Kujawinski et al. 2002*b;* Stenson et al. 2002, 2003) and that selective ionization suppression of larger, intact molecules was not significant (Stenson et al. 2002). The solvent composition and capillary voltages used in those ESI studies were similar to what we used in our streamwater analyses, and the molecular-weight distribution was also similar. Therefore, the ESI detected masses should correctly represent the molecular weight of the ionizable DOM compounds [i.e., $(M + H)^+$ or M + adduct for ESI-positive and $(M - H)^-$ for ESI-negative] in their Suwannee River DOM and, by extension, in our streams. We then compared the total DOC concentration estimated from the ESI data (sum of C in each 50-amu bin) to the directly measured total DOC (HTC).

Table 2. Summary of bulk and ESI-MS chemical data for stream water used in bioavailability experiment. The mass range of ESI-MS data is 50–2000 amu; the number of *m/z* ions is based on unit mass resolution.

Characteristic	Stream L	Stream M
Bulk data		
DOC, mmol L^{-1}	8.3	9.6
DON, μmol L^{-1}	540	453
$NO_{3/2}$, μmol L^{-1}	565	570
NH_4, μmol L^{-1}	4	4
PO_4, μmol L^{-1}	9	6
Percentage decrease in DOC	51	43
ESI-MS data		
No. of *m/z* detected		
ESI-positive; basic-type compounds	605	647
ESI-negative; acidic-type compounds	830	1,104
Total	1,435	1,751
No. of *m/z* that decreased in abundance		
ESI-positive; basic-type compounds	260	298
ESI-negative; acidic-type compounds	299	309
Percent (of total)	39	35

We used samples from the bioavailability experiment to make two additional comparisons of the ESI data with directly measured DOC (HTC). The total ion abundances were compared (linear regression analysis) to the directly measured DOC concentration (HTC), using samples with a range of DOC concentrations. The total ion abundance for a sample was calculated by summing the ion abundances for each mass bin in that sample. Then the percentage of masses that decreased in abundance was compared with the percentage decrease in DOC concentration (HTC) during the bioavailability experiment.

Results and discussion

Chemical characterization of DOM in stream water—Occurrence of compounds. Approximately 1,500 masses were detected in each stream (Table 2). Despite the complexity of the DOM, there appeared to be considerable similarity between these two streams in the mixture of DOM compounds, as indicated by the number of masses, molecular weight range, and patterns in ion abundance (Fig. 1; Table 2). Many of the masses that occurred in stream L occurred in stream M; ~70% of the masses detected were common to both

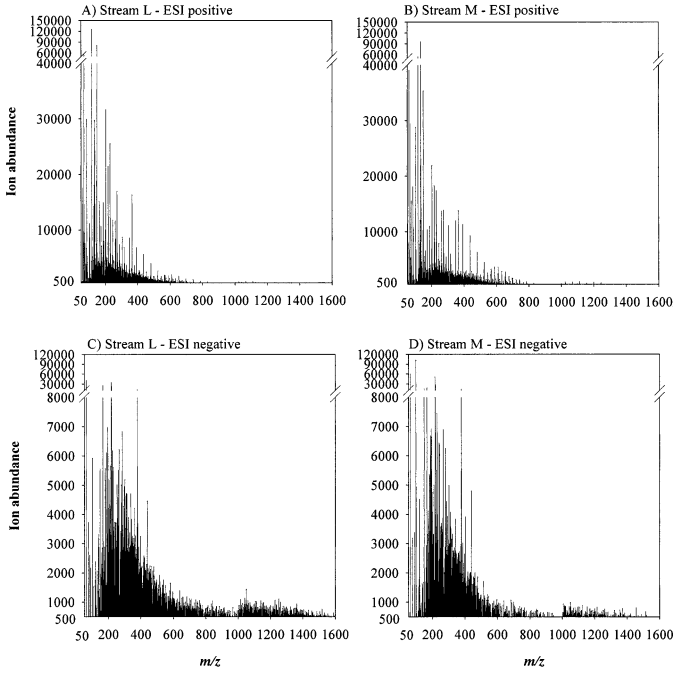

Fig. 1. Mass-to-charge ratio (*m/z*) and abundance of ions detected in positive and negative ionization modes with ESI-MS for water from streams L and M. Note the difference in the *Y*-axis scale between positive and negative mode plots.

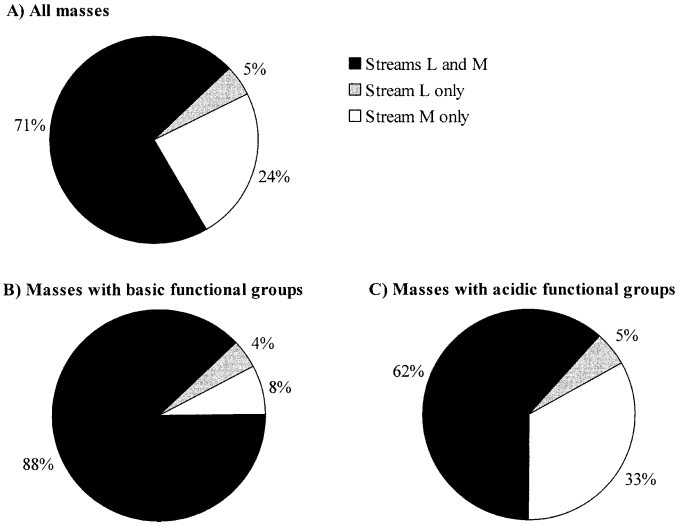

Fig. 2. Proportion of masses that were found in both streams L and M and that were unique to either stream L or M, for (A) all masses, (B) masses with basic functional groups, and (C) masses with acidic functional groups.

streams (Fig. 2A). To our knowledge, this is the first comparison at the compound level of the suite of compounds in the DOM pool between aquatic systems. As was noted above, given the unit mass resolution of our analyses, we cannot be certain, however, that the same mass in one stream represents the same compound (or set of compounds) in the other stream. The apparent similarity between the two streams at the compound level is consistent with the similarity in broad structural features of DOM in rivers. For example, the composition of dissolved total neutral sugars (free monosaccharides and combined saccharides) in rivers from geographically diverse locations is similar (Gremm and Kaplan 1997). The composition of neutral sugars as well as the ratio of total carbohydrates : acetate : lipid in the high-molecular-weight fraction of DOM from three rivers and two lakes were similar (Repeta et al. 2002).

Functional group characteristics. Analyses of samples by ESI-positive and ESI-negative provide information on the functional group properties of compounds. Compounds detected by ESI-positive contain basic functional groups and include aromatic compounds and highly conjugated systems that might also contain heteroatoms with lone-pair electrons and/or electron donator groups, such as $-OH$, $-CH_3$, $-NH_2$, $-OCH_3$, $=O$, and $-N(CH_3)_2$ (van Berkel 1997). Thus, N heterocycles, alcohols, amines, and amides are detected by positive ESI. Compounds detected by ESI-negative have acidic functional groups and include aromatic or highly conjugated systems with electron accepting groups, such as $-NO_2$, $-COOH$, halides, and CN. Such compounds include organonitrates, carboxylic acids, and inorganic ions such as nitrate and sulfate. ESI-positive and -negative detection of authentic standards that comprise this range of chemical structures and functional groups is consistent with our expectations based on their functional groups (Seitzinger et al. 2003; unpubl. data) (Table 1). Therefore, unknown DOM compounds in the stream water detected by ESI-negative are likely to have acidic functional groups, and those detected by ESI-positive are likely to have basic functional groups.

The DOM in the stream water had more masses with acidic functional groups (1.3–1.7 times more) than basic functional groups (Table 2). In addition, masses with acidic functional groups were detected at *m/z* up to ~1,600 (approximate molecular weight for singly charged compounds), whereas masses with basic functional groups generally had a *m/z* <800 (Fig. 1). The differences in chemical composition between the two streams were primarily masses with acidic functional groups (Fig. 2C). Almost all (~90%) masses with basic functional groups were found in both streams (Fig. 2B). The dominance of organic compounds with acidic functional groups in our streams is consistent with previous compound class-level analyses of river DOM. For example, humic substances, which can comprise a major portion of the DOC in rivers, contain a large portion of fatty acid, aliphatic, and carboxyl groups (Aitkenhead-Peterson et al. 2003). Identifiable carbohydrates, amino acids, and carboxylic acids are also components of river DOM.

Comparison of bulk DOC and molecular level results. The DOC concentration in streams L and M estimated from the ESI-MS data and directly measured were similar (ESI estimated and directly measured: 8.7 and 6.7 and 9.7 and 8.9 mmol C L^{-1} for streams L and M, respectively). Estimating DOC concentrations from the ESI-MS data involved a number of assumptions, as was discussed above; as such, interpretation of our DOC concentrations from the ESI-MS data must be viewed with caution. However, the reasonably good

agreement with direct measurements of DOC concentrations suggests that the ESI-MS approach is not missing a large component of the complex mixture of organic compounds in the stream water. In a similar comparison with rain, DOC concentrations measured directly (HTC) and estimated from ESI-MS data were not statistically different (Seitzinger et al. 2003).

Bioavailability of DOM in streams—A considerable body of knowledge exists concerning resource utilization in higher organisms (e.g., resource partitioning in fishes and size-selective feeding by zooplankton), selective grazing of bacteria by protozoa, and differences in uptake kinetics of inorganic nutrients by phytoplankton. This has led to the development and testing of ecological theories to explain the spatial and temporal dynamics of community composition and energy flow. In sharp contrast to higher organisms, little is known about the potentially complex dynamics of bacterial utilization of the large reservoir of uncharacterized organic carbon (Cottrell and Kirchman 2000; Bouvier and del Giorgio 2002).

A considerable proportion (10% to >80%) of DOM in freshwater and marine systems is bioavailable to microbial communities (Bronk 2002; del Giorgio and Davis 2003). This has been documented primarily by decreases in bulk DOC or DON concentrations over time with simultaneous increases in total bacterial production. However, aquatic microbial communities are highly diverse (Hiorns et al. 1997; Crump et al. 1999; Rappe et al. 2000), and there are >1,000 different DOM compounds in each milliliter of water (e.g., Fig. 1; Kujawinski et al. 2002*a*). Therefore, bulk-level measurements are not adequate to provide insight into the utilization patterns of the heterogeneous pool of DOM compounds by the highly diverse microbial community.

Recent advances in techniques to characterize microbial communities are providing new insights into the diversity of these communities, their variation in space and time, and which bacteria or groups of bacteria are active (Hiorns et al. 1997; Crump et al. 1999; Cottrell and Kirchman 2000; Bouvier and del Giorgio 2002). Moving beyond the utilization of simple organic monomers or model compounds (e.g., chitin or specific proteins) has been hampered by the lack of analytical methods to measure the majority of DOM compounds in fresh or marine waters. Using ESI-MS analyses of the stream bioavailability experiment, we begin to address the following fundamental issues: whether all DOM compounds are used equally or whether only some compounds are used and others are not, patterns of utilization with molecular weight, and repeatability in the pattern of which compounds are used or not used.

Proportion of bioavailable and refractory compounds—Bulk-level measurements demonstrated that bacterial production increased markedly during the first 2 d and remained between ~4 and 7 nmol leucine incorporation L^{-1} h^{-1} throughout the remainder of the experiment (Fig. 3). DOC concentrations decreased over the 12-d experiment by ~45–50% in both streams (Table 2; Fig. 3B). DON concentrations decreased, whereas NH_4 concentrations increased (Fig. 3C,D). Nitrate and phosphate concentrations showed no clear trends.

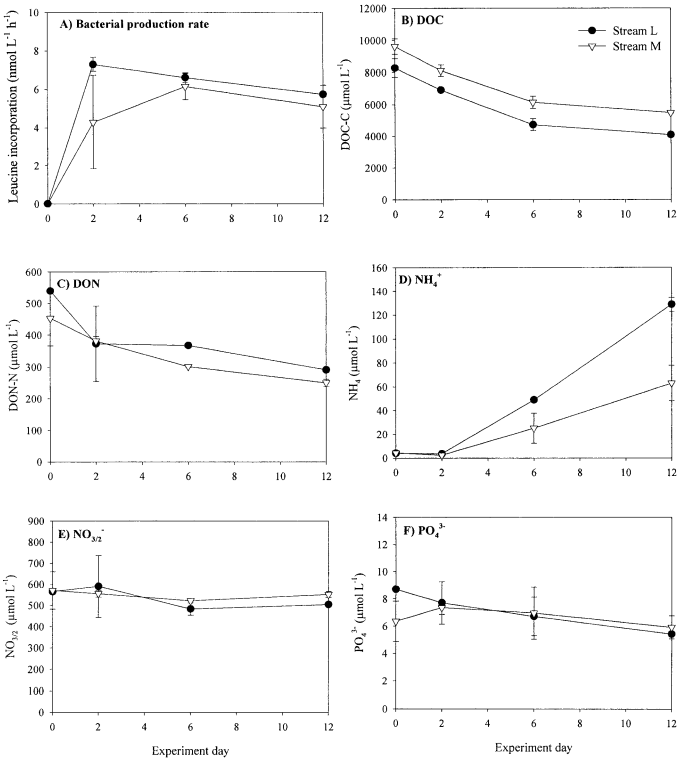

Fig. 3. Bulk-level results of 12-d stream water DOM bioavailability experiment (streams L and M): (A) bacterial production rates, and concentrations of (B) DOC, (C) DON, (D) NH_4^+, (E) $NO_{3/2}^-$ (used to designate nitrate + nitrite), and (F) PO_4^{3-}. Error bars are ±SD for replicate flasks.

Both the amount and the time course pattern of bulk DOC degradation are the result of changes in individual compounds in the mixture. Not all compounds were used, as demonstrated by the ESI-MS data. Approximately 35–40% of the masses decreased in concentration, and ~60% showed no change in concentration (Fig. 4). The proportion of the masses that were bioavailable and refractory was similar in both streams (Fig. 4A,B).

Less than 5% of the masses increased in concentration, and a number of new compounds were present on day 12 that did not occur in the initial samples (~15% of the number of masses present initially; Fig. 4). This suggests that, although most of the compounds used by the bacteria were fully degraded, some compounds were released from partial breakdown of the DOM or as new compounds from the bacteria. We are currently analyzing this group of compounds in more detail (Seitzinger et al. unpubl. data). The low percentage production of ''new'' compounds observed is consistent with ^{13}C and ^{15}N NMR and molecular-level analyses of organic matter degradation, which indicates little in situ formation of new chemical compounds (Knicker et al. 1996). In the marine environment, specifically, there is evidence that bacteria can contribute significantly to the refractory DOM pool (Ogawa et al. 2001; McCarthy et al. 1998).

We have little knowledge of utilization patterns at the

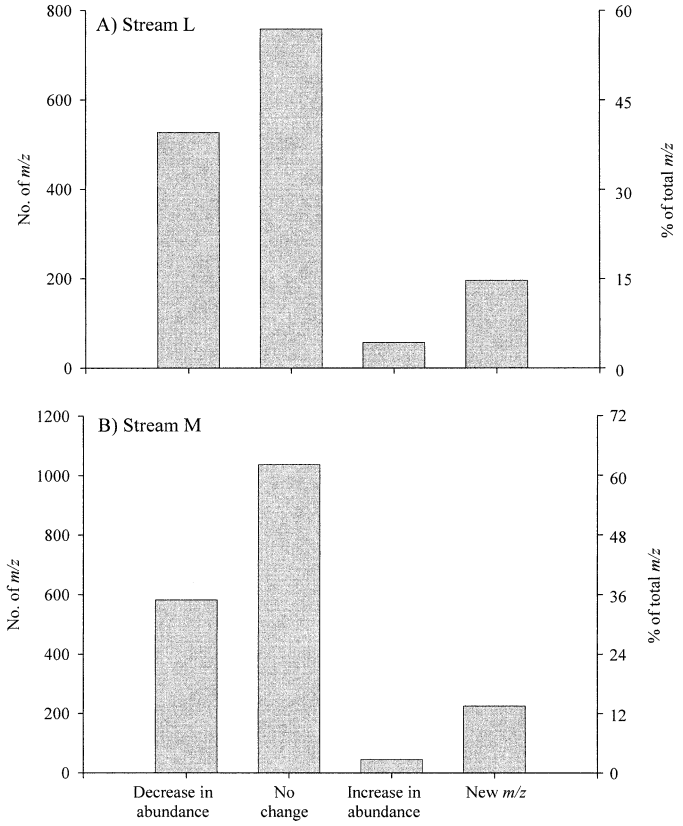

Fig. 4. Number and percentage of masses (*m/z*) in (A) stream L and (B) stream M, that decreased, did not change, or increased in concentration between days 0 and 12 of the bioavailability experiment. Also shown are number of new masses that occurred on day 12 but not on day 0.

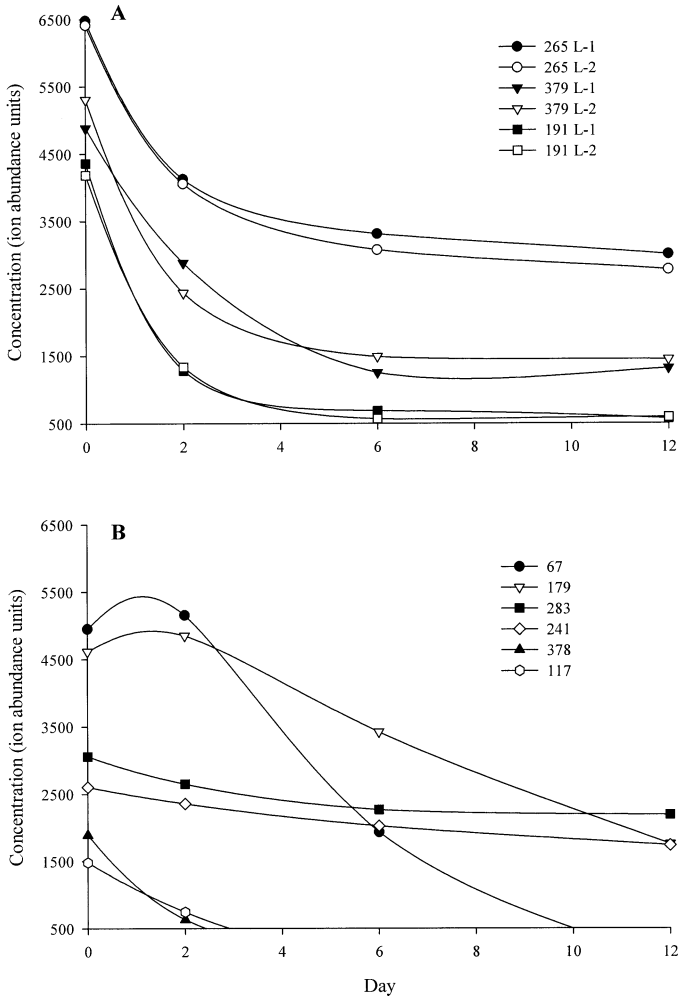

Fig. 5. Examples of time-course patterns of ion abundance for selected *m/z* during the bioavailability experiment. (A) Rapid decrease and (B) various patterns of decrease in concentration. Data for replicate flasks (L-1 and L-2) are shown in top panel.

compound level in a complex DOM mixture. This is because few studies have documented time course patterns of change at the individual compound level from natural DOM. In the current experiment, masses that decreased in concentration generally showed a progressive decrease over time. Some masses, however, decreased more rapidly (Fig. 5A) than others (Fig. 5B), which suggests differences in bioavailability. Other masses showed an initial lag before decreasing in concentration (Fig. 5B). Of the masses that decreased in concentration, fewer than half (~40%) completely disappeared, and ~60% decreased significantly ($\alpha = 0.05$) in concentration but still had detectable concentrations. The latter group could be due to some factor inhibiting utilization or to more than one compound in a mass bin where one (or more) compound is reactive and one (or more) is refractory. These patterns at the individual compound level are consistent with previous measurements at the level of compound class or total monomer. For example, hydrolyzable neutral sugars were measured during a DOM bioavailability experiment with fresh, algal-derived DOM from an Arctic ice floe (Amon et al. 2001). Although the hydrolyzable sugar monomer concentrations do not represent individual compounds (but rather moieties occurring throughout some unknown number of compounds), the patterns (e.g., rapid, gradual, lag in decrease, complete/partial decrease concentration, and no change) were similar to those that we observed for individual compounds from the ESI-MS data.

Agreement between changes in bulk DOC concentration and either the proportion of the total number of masses used or total ion abundance are consistent and suggest that the ESI-MS analyses reflect the behavior of most of the DOM compounds in the stream water. In stream L, 39% of the masses decreased in concentration, whereas the bulk DOC concentration decreased by 51% (Fig. 4; Table 2). In stream M, the percentage of masses that decreased in concentration (35%) was somewhat smaller than in stream L, as was the decrease in total DOC concentration (43%). If only a few masses had decreased in concentration, the bulk and molecular-level data would be inconsistent. In fact, the number of compounds was large, and each contributed only a small amount to the total DOC concentration. As a further comparison, the total ion abundance was linearly related ($r^2 = 0.93$; $\alpha = 0.05$) to the direct measurement of DOC (HTC) in time-series samples from the bioavailability experiment. Previous comparisons of bulk DOC and ESI-MS total ion

abundance for rainwater also were linearly correlated (r^2 = 0.72; Seitzinger et al. 2003).

Chemical characteristics of bioavailable compounds—Functional group characteristics provide additional information about the chemical characteristics of the bioavailable and refractory compounds. There were no overall differences in bioavailability of detected masses with acidic or basic functional groups. A similar number of masses with basic and acidic functional groups were bioavailable (Table 2), and the total decrease in ion abundance was similar for compounds with basic and acidic functional groups. The pattern was the same in both streams.

In addition to functional group characteristics, size may be an important factor determining the reactivity of DOM compounds. For example, most microorganisms must break down large compounds into small subunits outside the cell by extracellular enzymes or ectoenzymes before transporting them through their cell membranes (Burns and Dick 2002). Therefore, smaller molecules might be more readily used than larger molecules. In contrast, the size-reactivity continuum model for DOC predicts that a larger proportion of the high-molecular-weight DOC is labile than the low-molecular-weight DOC in both marine and freshwater systems (Amon and Benner 1996). In that model, high and low molecular weights were operationally defined by ultrafiltration as compounds $>$ and $<$1,000 Da, respectively. Monomers, oligomers, or compounds $<$600 amu are often cited as the largest molecules that are generally transported through bacterial cell membranes (Hedges et al. 2000; Williams 2000), although there are exceptions (Burns and Dick 2002).

A number of relationships between size and reactivity are indicated by the ESI data. There was no consistent trend across the 50–2,000 amu molecular-weight range in the proportion or amount of DOC utilized (Fig. 6), which indicates that there was not a continuum of change in reactivity with size, as was suggested by the size-reactivity continuum model (Amon and Benner 1996). (Initial DOC, in relative C units, was estimated from the ESI-MS data of initial samples from the bioavailability experiment by multiplying the total ion abundance in each 50-amu bin by the estimated C weight per mole weight; an instrument response factor was not applied. The DOC used was calculated based on statistically significant differences in ion abundance for individual *m/z* in each 50-amu bin. See ''Materials and methods'' section for details.) There were consistent differences, however, if the detected compounds were grouped into just two categories—$>$ and $<$600 amu (referred to as low molecular weight [LMW_{600}] and high molecular weight [HMW_{600}]). Most of the DOM in the stream water was in the LMW_{600} fraction. This is indicated by the larger proportion of masses, ion abundances, and estimated DOC in the LMW_{600} than the HMW_{600} streamwater fraction (Fig. 7A,B,C, respectively). However, the group of LMW_{600} DOM compounds was less reactive than the group of HMW_{600} compounds. A smaller proportion of the masses (35% vs. 48%; LMW_{600} vs. HMW_{600}), ion abundance (18% vs. 44%), and estimated DOC (19% vs. 46%) decreased in LMW_{600} than in HMW_{600} (Fig. 7). Therefore, although bacteria may be able to transport DOM $<$600 amu directly through their cell walls without first breaking it down into smaller moieties, this does not seem to be a major criterion for reactivity. The results do support the general idea of the size-reactivity model, in that a larger proportion of the higher molecular weight DOM was used than the the lower molecular weight DOM (Amon and Benner 1996), if the data are grouped into large bins of molecular weight (e.g., total $<$ or $>$1,000 amu). The patterns were similar if either 600 or 1,000 amu is used to define the division between high- and low-molecular-weight DOM.

Another important point is that, although a larger proportion of the HMW_{600} DOM was reactive, the LMW_{600} DOM compounds were a major contributor to the total amount of reactive DOM in the stream water. The number of masses used, decrease in ion abundance, and the estimated DOC utilized were similar or greater in the LMW_{600} fraction compared with in the HMW_{600} (Fig. 7A,B,C). If 1,000 amu is used as the cutoff, then the LMW_{600} fraction becomes even more important. All of the above patterns were similar in both streams.

Repeatability of DOM compound utilization by microbial communities—Comparison of compound-level DOM bioavailability within a site. The replicate flasks for a stream had the same initial chemical and biological (bacterial inocula) composition, and both the chemistry (Fig. 1) and likely the biology (Hiorns et al. 1997; Crump et al. 1999) were highly complex. However, despite this seemingly complex system, there was considerable similarity in compound-level DOM degradation patterns between replicate flasks. There was a high degree of similarity between the replicate flasks in which masses were used, as well as which masses were not used. Many of the same masses decreased in concentration in replicate flasks (e.g., L-1 and L-2; Figs. 5A, 8A). This was true for both stream L and stream M.

The similarity between replicate flasks at the compound level accounts for the similarity in bulk level DOM degradation between replicate flasks for a site in this and previous experiments (*see SDs in Fig. 3B;* Seitzinger et al. 2002). It is also consistent with the aggregated compound-level data. For example, the proportion of the total number of masses that decreased in concentration was similar between replicate flasks. Thirty-nine percent and 44% of masses decreased in concentration in replicate flasks of stream L; in replicate flasks of stream M, 36% and 38% of masses decreased. (These comparisons are for *m/z* $<$1,000 because only one replicate flask was analyzed by ESI-MS for *m/z* $>$1,000).

Not only were the same masses used in each flask (unit mass resolution), but the change in concentration (decrease in ion abundance) of each of those masses was highly correlated in replicate flasks (Fig. 8A). This suggests a high degree of selectivity at the compound level by the bacteria in the consortia. The level or mechanism of selection is not known but likely relates to the enzyme systems present for organic matter degradation (Burns and Dick 2002).

There were also some masses that were used in only one flask. Many of these had low ion abundances and thus may be due to analytical uncertainty in our ability to statistically determine net changes; others are likely real differences between flasks.

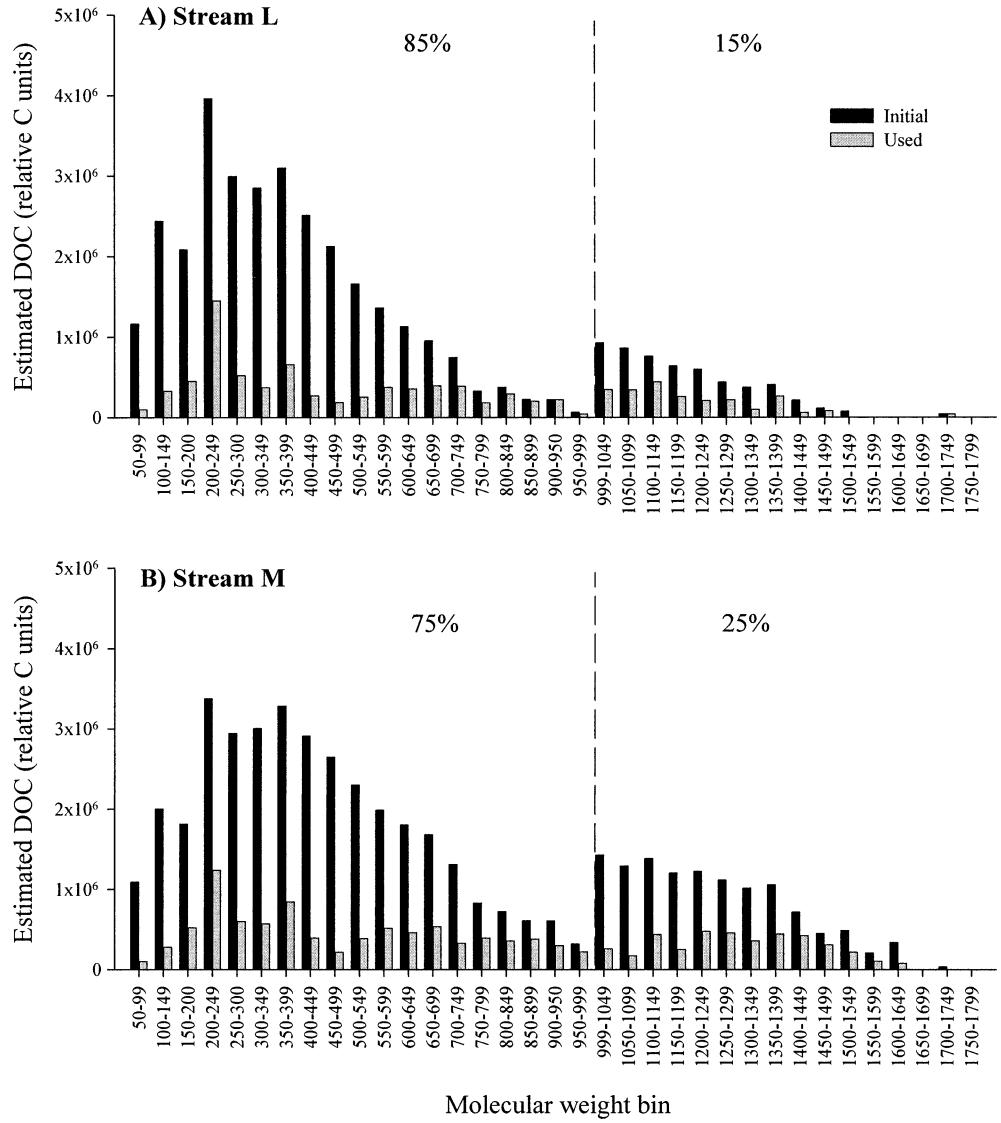

Fig. 6. Preliminary estimate of relative amount of C (unitless) in 50-amu bins, based on ESI-MS data. Estimated initial DOC and decrease in DOC during bioavailability experiment are shown for streams (A) L and (B) M. The proportion of estimated DOC that is <1,000 amu is indicated.

Comparison of compound-level bioavailability between streams—In both streams, the masses that were used (or not used) and the magnitude of decrease in concentration were similar in many cases. This is illustrated by the decrease in initial ion abundance for the same masses in streams L and M (Fig. 8B). The overall similarities between the two streams in which masses were used and the magnitude of decrease are also illustrated by the similarity in pattern of estimated DOC decrease by 50-amu bins (Fig. 6A,B). The biology also helps to inform us of the chemistry. Many of the masses that we interpreted as being the same compound in both streams were also used (or not used) by the bacteria in both streams. This supports our interpretation that many of these masses are the same compounds in both streams, which we based on their unit mass and positive/negative mode detection characteristics (Fig. 2); higher resolution ESI-MS is required to further confirm this interpretation.

Ecosystem implications—The compound-level information on chemical composition and bioavailability in these two streams provides insight into previously observed patterns in ecosystem effects of DOM. For example, there is agreement for a particular DOM land use/source type (within the same geographic region) in the proportion of total DON that is bioavailable and in the magnitude of stimulation of microbial and phytoplankton production (Seitzinger et al. 2002). This is consistent with the similarity in the compound level chemical composition and bioavailability of DOM in the two streams with similar watershed land use in the current study. Among source types (e.g., DOM from agricultural vs. forested vs. suburban land use), however, the bioavailability at the bulk DOM level and the plankton effects are variable (Seitzinger et al. 2002). The data presented here and recent ESI-MS compound level analysis of DOM in some of these other sources (e.g., rainwater, Seitzinger et al.

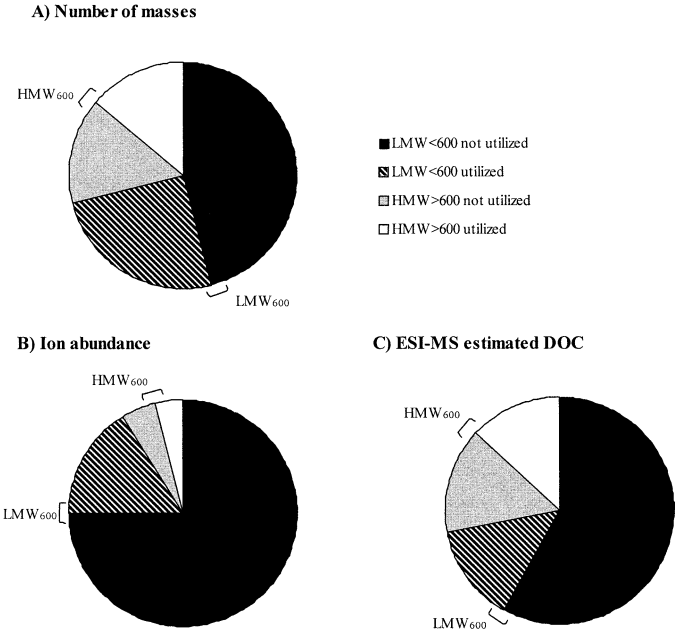

Fig. 7. Proportion of the (A) total number of masses, (B) ESI-MS total ion abundance, and (C) ESI-MS estimated DOC concentration in the LMW_{600} or HMW_{600} fraction in the stream water. Also shown is the portion of the LMW_{600} or HMW_{600} DOM that was used or not used during the bioavailability experiment.

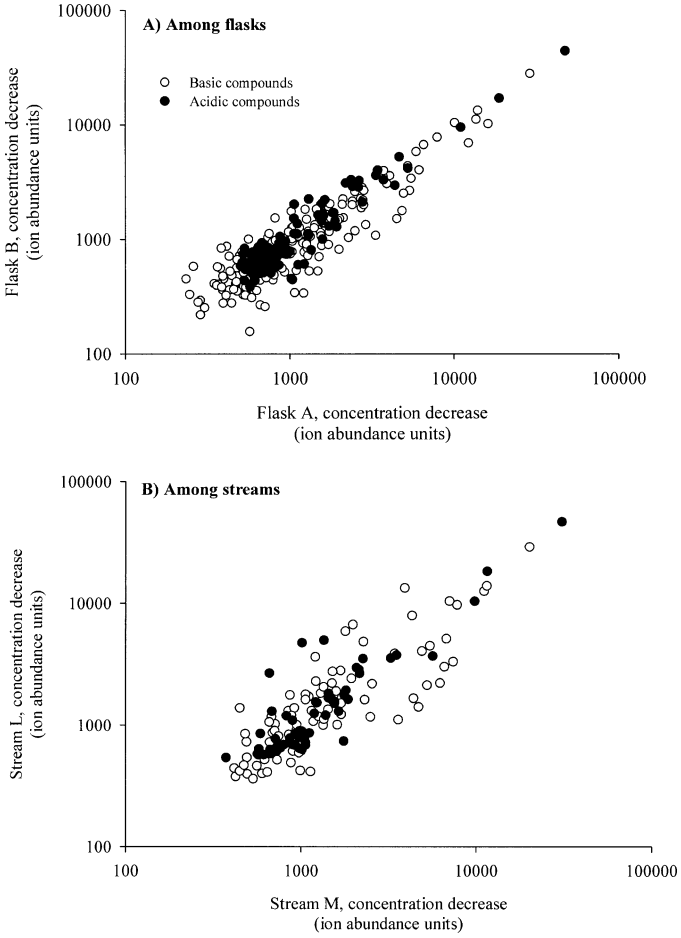

Fig. 8. Decrease in concentration (ion abundance units) of masses in (A) replicate flasks (L-1 and L-2) from stream L between days 0 and 12 of the bioavailability experiment. Only masses that decreased in both flasks are shown Linear regression $r^2 = 0.9$; $\alpha \ll$ 0.001. (B) Streams L and M between days 0 and 12 of the bioavailability experiment. Only masses that decreased in both streams are shown. Linear regression $r^2 = 0.88$; $\alpha \ll 0.001$. Decreases are plotted as positive numbers. Each point is a different mass (*m/z*). Compounds with basic functional groups and acidic functional groups are indicated.

2003; agricultural and forest runoff, Seitzinger et al. unpubl. data) demonstrate that the chemical signatures can be quite different among sources (although similar within a source type). Thus, differences in bioavailability and ecosystem effects observed among source types are likely due, at least in part, to the compound-level differences in chemical composition. Furthermore, it suggests that compound-level analyses are required to understand the effects of DOM in aquatic ecosystems.

Compound-level information on the distribution and reactivity of DOM also provides new insight into the formation of the DOM pool. The molecular level composition of DOM rapidly becomes uncharacterizable by traditional analytical methods after release (e.g., exudation, lysis, and rupture of cells) from living organisms (Hedges et al. 2000). This pool of uncharacterized compounds is the net result of transformation and utilization of DOM. Little is known about how these previously uncharacterized DOM compounds are formed, which ones are utilized (and thus disappear from the pool), or how repeatable, and therefore predictable, their formation and utilization are. The similarity in the chemical composition of the two streams (Fig. 2) suggests a similar set of biological (and/or chemical) reactions in the formation of these compounds within the watershed and stream from the parent material. The reproducibility in the compound-level biological decomposition of the stream DOM between the replicate flasks for a stream (Fig. 8A), as well as between the streams (Fig. 8B), also suggests that the microbial utilization of DOM compounds in the environment may be repeatable and, therefore, ultimately predictable. Additional studies across a wider range of sites and across seasons and the identification of specific compounds are needed.

ESI-MS provides a window into the ''black box'' of DOM compounds in the environment. It provides a novel tool that can be used to begin to chemically characterize the large fraction of previously uncharacterized DOM in aquatic and terrestrial ecosystems; understand the compound-level dynamics of DOM production, utilization, transport, and ecosystem effects; and develop and test ecological theories of microbial resource utilization.

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