Dissolved organic matter (DOM) comprises the largest, yet least-characterized reservoir of reduced organic carbon in aquatic systems, estimated at 700 × 10^15 g C (11). DOM is important in the carbon and nitrogen cycles, the scavenging and solubilization of trace contaminants, and biogeochemical cycles of other elements (3, 14). Heterotrophic bacteria process and reprocess some of this DOM (2), channeling about one-half of oceanic primary production through the microbial loop (8).

The role of bacteria in the rate and extent of DOM mineralization and their production of (semi)refractory DOM have received less attention. Some studies indicate that bacteria produce refractory DOM that is resistant to further utilization (5, 12, 37, 39). Ogawa et al. (26) showed that a natural inoculum of marine bacteria (and undoubtedly nanoflagellates and viruses) growing on labile compounds (glucose and glutamate) produce new DOM compounds that appear to be refractory for at least a year. It was not known if a single strain of bacteria could produce similar refractory material. Bacterioplankton can also be a source of photoactive C-DOM that is refractory to a natural bacteria assemblage following photochemical alteration (18). What kinds and how many different compounds make up the refractory DOM pool are largely unknown.

In aquatic ecosystems, bacteria are consumed by protozoa and other zooplankton, which in turn release DOM as colloidal matter (17, 40) and macromolecular organic complexes (24). A substantial portion (>50%) of primary and bacterial production can be consumed by a single class of protozoa, the Ciliata (10). Therefore, ciliates can act as trophic links, nutrient regenerators, and DOM producers (38)—roles often overlooked in traditional food webs. Little is known about the effects of additional trophic levels on the production and composition of refractory DOM. Nagata and Kirchman (24) suggested that the release of DOM by protozoa is potentially important in aquatic food webs and nutrient cycles. Kujawinski et al. (19) used electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (MS) to identify 80 new DOM compounds produced when a protozoan grazed on bacteria.

Here, we analyzed the effects on DOM dynamics of a pure bacterial culture alone and when grazed on by a ciliate. We used conventional, bulk analysis to examine how trophic structure influenced dissolved organic carbon (DOC) transformations. We used ESI-MS for molecular-level characterization of compounds produced during bacterial growth and grazing. ESI-MS has been used for identification and quantification of specific compounds (13, 22, 28, 29). Terrestrial and marine DOM pools have been characterized by ESI-MS (15, 16, 20, 21). ESI-MS has also been used to gain insights into changes in DOM due to protozoan grazing (19), for characterization of DOM in rainwater (32), and to discriminate between possible refractory and labile DOM compounds in freshwater samples (30).

**MATERIALS AND METHODS**

**Experimental setup.** Production and dynamics of DOM were examined using glucose as the sole utilizable carbon source (10 mM C initial concentration). There were three treatments: sterile control, bacteria only, and bacteria plus ciliates. Experiments took place in 125-ml flasks (baked at 500°C for 4 h before use) containing 25 ml sterile, low-carbon (62.8 μM C from nitritotriacetic acid,
0.8 µM C from Na₂EDTA) modified mineral basal solution (MBS). In the modified MBS, ammonia and phosphate concentrations were 5 times lower, with a carbonate buffer concentration 50 times lower and metal concentrations 100 times lower.

All flasks (except the sterile controls) were initially inoculated with the bacterium *Pseudomonas chlororaphis*. Initial samples were taken directly after inoculation (day 0). Half of the remaining flasks were inoculated with the bacteriovorous protozoan *Uronema* sp. after 3 days, when the bacteria had reached stationary growth. Flasks were capped with aluminum foil to maintain sterility but permit air exchange. All cultures were incubated in the dark on a shaker table at 50 rpm and 20°C. Triplicate sacrificial flasks were used for each time point to prevent contamination from repeated sampling of the same flasks.

**Bacterial strain.** We used a single freshwater heterotrophic bacterial strain, *Pseudomonas chlororaphis* (ATCC 17418), a gamma proteobacterium (34). Flasks were inoculated with 10 µL of triple-washed cells harvested from cultures at the end of the log growth phase. At each time point, abundance was estimated by measuring the optical density (1-cm path length) at 550 nm of a 1-mL sample.

**Grazer.** We used a freshwater ciliate, *Uronema* sp. (*Scuticociliatidae*), as the bacteriovorous protozoan grazer. This ciliate is about 20 µm in its longest dimension. Prior to the experiment, ciliates were maintained in culture using protozoan pellets (Carolina Biological Supply) as a nutrient source. Before being introduced into *P. chlororaphis* cultures, ciliates were filtered through a polyester mesh (41-µm openings) to remove detritus. Ciliates in the <41-µm fraction were triple rinsed with low-carbon MBS and then concentrated onto a 5-µm Nucleopore filter and resuspended at a final density of 1,600 cells/mL. Flasks were inoculated with 25 µL of concentrated ciliate suspension (~40 cells). At each time point, abundance was determined by direct counts of a 1-mL sample using a Sedgewick-Rafter counting cell.

**Bulk chemical analysis.** At each time point, samples were taken from each flask for particulate organic carbon (POC), particulate nitrogen (PN), DOC, total dissolved nitrogen (TDN), and dissolved inorganic nitrogen (DIN).

For the POC and PN analysis, 23 mL of sample was filtered through a pre-combusted (500°C for 4 h) 25-mm-diameter Whatman GF/F filter. The filter was dried and analyzed using a Carlo Erba Instruments NA 1500 series 2 elemental analyzer (Eurovector, Italy). Results for POC and PN were corrected based on filters directly from the sterile controls. The filtrate containing the dissolved fraction was transferred to acid-washed capped polypropylene vials and stored frozen (~20°C) in the dark until needed for all other analyses.

DOC and TN were measured using a Shimadzu 5000 high-temperature combustion analyzer for DOC (4, 33) with an inline NOx detector (Antek model 410002). DOC and TN were determined as the difference between the expected medium concentration of 3,000 µM and the ammonium concentration just after day 3 samples were filtered (3,150 µM, which compared well with the overall mean value of 4.5 (standard deviation [SD], 0.9).

**Compound-level analyses.** All compound-level analyses were performed using an Agilent 1100 liquid chromatography (LC) mass spectrometer equipped with an ESI source and a quadrupole mass selective detector (30, 32). An autosampler introduced 20 µL of individual sample into the LC mobile phase that went directly to the ESI source (no LC column was used). The mobile phase was 50:50 methanol-water; the ESI ionization mode of the ESI source. The flow of the drying gas (N₂ at 350°C) in the nebulizer was 6 L/min. The quadrupole temperature was 99°C, and the quadrupole voltage was kept at 0 V (m/z 1.0). The fragmentor voltage was 40 V. The m/z openings were 1.0 to remove detritus. Ciliates in the <41-µm fraction were triple rinsed with low-carbon MBS and then concentrated onto a 5-µm Nucleopore filter and resuspended at a final density of 1,600 cells/mL. Flasks were inoculated with 25 µL of concentrated ciliate suspension (~40 cells). At each time point, abundance was determined by direct counts of a 1-mL sample using a Sedgewick-Rafter counting cell.

**Results**

**Bulk DOC, POC, and population dynamics: bacteria-only experiment.** Glucose was rapidly utilized during the exponential growth phase of the bacteria as indicated by changes in optical density and DOC concentration (Fig. 1). The initial decrease in DOC concentration from 10 mM to around 440 µM coincided with the approximate 10-fold increase in optical density by day 2. The DOC concentration decreased slightly after the initial steep DOC decline but stayed about 250 µM higher than the medium background (67 µM) for the remainder of the experiment.

The experiment results were divided into three stages based on the bacterial abundance and the DOC trends: the lag phase, where the bacterial abundance was low and the DOC was high (day 1; stage I); the exponential growth phase, where the bacterial abundance increased to a maximum and a steep decrease in DOC was observed (day 2; stage II); and the stationary growth phase, where a slight decrease in both bacterial abundance and DOC concentration occurred (days 3 to 36; stage III). If first-order kinetics were assumed for the utilization of DOC, the decay constant during day 1 (stage I) was 0.028 per day before increasing to 3.1 ± 0.1 per day in stage II. In stage III the decay constant decreased to 0.013 ± 0.002 per day, a 250-fold reduction.

The POC concentration rapidly increased to 3.1 mM by day 2 and then remained constant during the rest of the experiment (Fig. 1). The C/N ratio of the particulate matter was quite uniform throughout the experiment (data not shown), with an overall mean value of 4.5 (standard deviation [SD], 0.9).

The dissolved nitrogen concentration remained high throughout the experiment (data not shown). The ammonium concentration on day 1 was 3,150 µM, which compared well with the expected medium concentration of 3,000 µM. Ammonium decreased to about 2,800 µM on day 2 and remained there for the remainder of the experiment. Dissolved N was present essentially exclusively as ammonium; DON was below detection, and nitrite plus nitrate were about 1 µM.

(ii) **Bacteria plus bacteriovorous protozoan experiment.** Following the introduction of *Uronema* just after day 3 samples were taken, the optical density of the cultures decreased from 0.2 on day 5 to 0.006 on day 8. *Uronema* abundance increased from 6 to 5,080 per mL in the same time period (Fig. 2). From day 8 onwards, the optical density of the remaining cultures was only slightly above the sterile control values. Ciliate abundance varied cyclically from day 8 through day 20, from highs...
of 9,000 to 10,600 per ml to lows of 5,600 to 6,000 per ml (Fig. 2). Ciliates at these cell densities contribute negligibly to optical density (D. F. Gruber, S. J. Tuorto, and G. L. Taghon, unpublished data). Ciliate abundance declined sharply to 28 per ml between day 20 and day 36.

DOM was released during the increase in ciliate and decrease in bacteria abundance between day 5 and day 8 (Fig. 2). The maximum DOC increase was between day 5 and day 6, from 430 to 690 μM. During this period, Uronema increased from 8 to 500 per ml, while the OD decreased from 0.2 to 0.07. After these transient changes, the DOC concentration decreased to an average value of 315 μM for the remainder of the experiment, a level slightly lower, by 53 μM, than the DOC concentration for the bacteria-only experiment during the same time period.

POC concentration steadily decreased following Uronema addition, from 3,100 μM at the end of the exponential growth phase of P. chlororaphis, to values of 620 to 1,000 μM during the interval of greatest Uronema abundance (days 8 to 20), to a final value of 240 μM on day 36 (Fig. 2). Once Uronema was established in the cultures, the C/N ratio of the particulate matter gradually increased from its pre-Uronema value of 4.5 to 6.2 on day 36 (data not shown).

The dissolved nitrogen concentration again remained high throughout the experiment (data not shown). The ammonium concentration reached its lowest level of 2,200 μM on day 5 and then gradually increased to 2,800 μM by day 20. DON was below detection, and the nitrate plus nitrate concentration was less than 1 μM.

**ESI-MS characterization.**

(i) **Characterization of DOM produced by bacteria.** The loss of glucose ions to an undetectable level in both positive and negative ESI spectra after day 2 also indicates that glucose was rapidly utilized during the exponential growth phase of the bacteria.

The ESI spectrum changed from day 0 to day 1 and again from day 1 to day 2; it then remained relatively constant. New m/z values were detected at day 1 in both positive (118 masses) and negative (100 masses) ionization mode that were not there on day 0. Most of these new detected masses (80 in positive mode and 81 in negative mode) did not occur beyond day 1. The remaining new m/z values identified at day 1 were present throughout the experiment. On day 2 additional new masses (45 in positive mode and 27 in negative) were detected, and all of these remained present throughout the experiment (Fig. 3).

After identifying the new m/z values in the ESI spectra from the different growth stages, the molecular weight distribution of these masses was examined. The m/z values were grouped into four categories (50 to 250, 251 to 500, 501 to 750, and 751 to 1,000 m/z) to simplify the interpretation (Fig. 4). In positive ionization mode, 84% of the masses detected on day 1 only had an m/z lower than 500 (Fig. 4). This distribution is comparable to masses detected on day 1 that remained present through day 36 (Fig. 4). In contrast with day 1 results, only 38% of the new masses detected on day 2 had an m/z less than 500 (Fig. 4).

There was a shift towards higher m/z values for compounds detected in negative ionization mode compared to positive mode (Fig. 4). In negative ionization mode, about 50% of the m/z values in the day 1-only and day 1 through day 36 distri-
butions fell in the lower-than-500 m/z categories (Fig. 4). This change is mostly due to the increase in the number of masses in the 751-to-1,000 m/z category. For the day 2 through day 36 distribution, 85% of the masses were greater than 500 m/z, with the majority greater than 750 m/z (Fig. 4).

(ii) Characterization of DOM following bactivorous protozoan addition. Additional new compounds were detected 3 days after Uronema addition that were not present in the bacteria-only treatment (Fig. 5). No new m/z values due to Uronema inoculation were detected in either ionization mode on day 5, 48 h after inoculation. For subsequent samples, most new masses were identified in the positive ionization mode. At day 6, 30 new masses were detected, all with a mass of >400 m/z. In the day 8 sample, 15 new compounds with masses of >400 m/z were identified. For day 9, when the transient DOC pulse decreased to the same DOC concentration as for the bacteria-only treatment, only one new compound was detected. Of the new m/z identified in the positive ionization mode on day 6, day 8, and day 9, 10 occurred on day 6 and day 8 and 1 compound remained in all three samples (Fig. 5). Thus, a total of 35 new compounds were detected in the grazer treatment in addition to the 132 compounds already present in the bacteria-only spectra, an increase of ~27%.

In the negative mode, only three new compounds were detected on day 6, and two of those had an m/z of >400. Two new compounds were identified on day 8. In the subsequent sample after the DOC increase due to the ciliate addition (day 9), no new masses were detected. A total of four new compounds were identified in day 6 and day 8 samples, of which one was present both days (Fig. 5).

**DISCUSSION**

The conversion of DOM to bacterial biomass has been studied in many natural aquatic and laboratory systems and has resulted in many published values for yield or growth efficiency.
The rate and extent of the transformation of labile DOM to refractory compounds is less studied but is at the heart of the issue of carbon storage in biologically unavailable pools. In this study we found that a pure culture of bacteria rapidly produced a complex pool of DOM from a simple labile compound. Many of the new DOM compounds (49 to 68%) were themselves used within 1 day, while the remainder formed a small refractory DOM pool (3% of the initial carbon). The addition of a bactivorous protozoan increased the extent of organic carbon remineralization from 71% to 94% but had no effect on the complexity of the DOM pool.

Changes in bulk organic carbon and nitrogen pools. The results of our bacteria-only experiment were generally similar to those of Ogawa et al. (26), although there were substantial differences in experimental conditions. For example, they used glucose as a labile DOM source, but at much lower initial concentration than we did (208 μM C versus 10,000 μM C). They also used a natural, marine bacterial assemblage as inoculum, while we used an isolated, freshwater strain. In both experiments, glucose was undetectable within 2 days. After 2 days in their experiment, bacteria converted 7% of the initial glucose carbon to POC and 15% to other forms of DOC, and the remaining 78% was respired. After 2 days in our experiment, P. chlororaphis converted 31% of glucose carbon to POC and 5% to other forms of DOC, and the remaining 64% was presumably lost as CO₂. DOC decreased only slightly over days 2 to 36, reaching a final level of 3% of the initial glucose carbon.

Introduction of a small population of a bactivorous protozoan (Uronema sp.) after the bacteria had reached stationary growth changed the distribution of carbon among the particulate, dissolved, and gaseous pools. The Uronema population rapidly increased (Fig. 2A). Although we did not directly measure the grazing rate, the size of the transient increase in DOC between days 5 and 6 (Fig. 2B) was consistent with published data on ciliate grazing rates. Assuming a bacterial carbon content of 5.8 × 10⁻⁹ μmol C/cell (70 fg C/cell [7]) and assuming between 20 and 88% of bacterial carbon is released by ciliate grazing (39), then the population of 500 ciliates/ml would have had to graze at a rate of 4,300 to 18,000 bacteria/ciliate/h to account for the 260 μM increase in DOC (also assuming no bacterial uptake of the released DOC). While high, this estimated range of grazing rates is within values reported in other studies (41).

The main effect of grazing on the bulk carbon pools was to increase the amount of glucose-C converted to CO₂. The POC concentration steadily declined from day 5 to day 20 at an average of 130 μM C/day (Fig. 2B). DOC concentration also declined over this time interval, although at a slower rate.
We assume that any differences between the amounts of carbon originally added as glucose and measured as DOC or POC represent carbon respired as CO₂ and lost from the system. The mass balance for nitrogen, an element that should not have been lost from the system, was excellent. We accounted for 94% of the added NH₄-N as particulate or dissolved nitrogen throughout the incubation period.

The most likely explanation for the increase in carbon conversion to CO₂ is the well-known inefficiency of energy and carbon transfer between trophic levels. Typically, 10 to 20% of food energy is transferred between successive trophic levels (25). On day 20, after the population of *Uronema* had been at 5,600 to 10,600 per ml for 13 days (Fig. 2A), the percentage of glucose-C present as POC was only 22% of the bacteria-only value. By the end of the experiment there was 2,600 µM POC in the bacteria-only flasks and only 9% of that, 240 µM POC, in the flasks with bacteria plus bactivorous protozoan. Thus, our results are quite consistent with a 10 to 20% trophic transfer efficiency.

A puzzling result is the continued, albeit slight, decline in DOC concentration in the grazer treatment. After the transient pulse had subsided by day 9, the DOC concentration continued to decline from 340 µM to 60 µM by day 20 (Fig. 2B), while DOC was constant at 370 µM in the bacteria-only treatment (Fig. 1B). If the residual DOC present after *P. chlororaphis* had depleted all the glucose were refractory, then we would expect similar residual levels of DOC in both treatments. One possibility is uptake of DOC by *Uronema*. Protozoa can actively take up low-molecular-weight (LMW) compounds across the cell membrane and larger dissolved compounds by pinocytosis (10). While such DOC uptake may be insufficient

![Graph](http://aem.asm.org/Downloaded from http://aem.asm.org/)

**FIG. 5.** New masses detected in time point samples with positive and negative mode of ESI-MS. This figure is a composite of results from the bacteria-only and bacteria plus bactivorous protozoan experiments. For comparison, the dissolved organic carbon concentrations of the bacteria plus bactivorous protozoan time points are included.
to meet the cell’s metabolic needs, it represents a sink for DOC. The increase in DOC to a level comparable to the bacteria-only treatment by day 36 (Fig. 2B), after the Uronema population had declined precipitously (Fig. 2A), is consistent with this explanation.

The presence of Uronema led to an increase in the C/N ratio of the particulate matter. The C/N ratio of the bacteria-only treatment was invariant at 4.5, indicating that N was not limiting. Protozoa have higher C/N ratios than their bacterial prey (6), and so grazing can result in a release of the excess nitrogen from the particulate to the dissolved phase. The average NH₄⁺ concentration in the grazer treatment was 2,650 μM, compared to 2,250 μM in the bacteria-only treatment, consistent with this explanation.

Formation of DOM compounds during bacterial growth. P. chlororaphis changed the DOC pool from a single labile compound (glucose) to a more complex pool. We detected ~200 labile and refractory compounds (masses) in this study. Detection of masses in the day 1 sample (118 in positive and 100 in negative ESI mode) that were not present on day 0 indicates production or release of compounds associated with the bacterial utilization of glucose. Of these new masses, a majority no longer appeared later in the experiment (Fig. 4), which could indicate complete remineralization of these labile compounds, incorporation into biomass by P. chlororaphis during the exponential growth phase, or release as new compounds on day 2. The utilization of newly released compounds suggests a dynamic cycling of organic compounds by P. chlororaphis during the lag and exponential growth phases. Increasing the sampling frequency during these phases could give additional insight into production and consumption of DOM compounds by P. chlororaphis.

A number of new masses were identified in the day 1 and day 2 samples that remained present for the remainder of the experiment (Fig. 4). These new masses appeared to be compounds that were refractory to utilization by P. chlororaphis for the duration of the experiment. These refractory compounds were likely responsible for the relatively constant DOC concentration from day 2 through day 36. A larger percentage of masses detected in negative ESI mode exhibited higher molecular weights (m/z values) compared to those detected in positive mode (Fig. 4). This indicates that P. chlororaphis produced more high-molecular-weight (HMW) compounds with acidic functional groups (e.g., carboxyl and amino groups) than compounds with basic functional groups (e.g., hydroxyl and amine groups) during day 1 and day 2.

Earlier studies indicated that bacteria can transform labile compounds to compounds that are resistant to further mineralization. This transformation resulted in an increase of the average molecular weight of the initial substrate DOC pool (5, 12). Molecular weight was determined in these studies by size exclusion/gel filtration, which can only be used to infer a molecular weight range, based on elution time. In our study we were able to observe a shift to higher molecular weight with a mass unit resolution within the 50 to 1,000 m/z bin (the LMW-DOM pool). It is possible that the labile compounds released on day 1 were taken up, transformed into new compounds, and released on day 2 as refractory compounds. This scenario is supported by the shift towards higher molecular weights for masses detected in day 1 and day 2 samples (Fig. 4). However, the mechanisms responsible for this “transformation” are still poorly understood. Analysis of the elemental composition of the formed compounds using high-resolution mass spectrometry might help to elucidate differences in elemental composition as well as the number of functional groups (15, 19, 36).

Kujawinski et al. (19) found in their study of biological DOM that the most common mass difference could be explained by the addition of multiples of -CH₂CH₂O, although the underlying chemistry was not clear.

Impact of bactivorous protozoan grazers on DOM. The addition of a grazer increased the complexity of the DOM pool as established by P. chlororaphis, but only temporarily. The DOC and ESI results from the Uronema experiment (Fig. 2 and 5) point to an initial release of DOM compounds that were utilized within 3 days. The compounds that were released due to grazing were mostly low-molecular-weight compounds (<400 m/z). These compounds appeared, like the compounds detected on day 1 only in the P. chlororaphis experiment, to be utilized by P. chlororaphis. Thus, the addition of Uronema did not change the composition or the molecular weight distribution of the DOM pool over a long time period. The utilization of these compounds could be due to a low availability of more labile organic compounds at this time point in the experiment. Taylor et al. (39) also suggested that protozoan grazing enhanced DOC release and that the DOC released was composed of primarily low-molecular-weight compounds that were reused relatively quickly.

Most previous studies investigating DOM produced by bacteria have looked at shifts in ranges of molecular weight or overall changes in some functional groups (e.g., amino acids). With ESI-MS, we could see production and subsequent utilization of compounds in time series experiments. The next step is to focus on the elemental composition and structure of these compounds and find clues for why these compounds are labile or refractory. The similar molecular weight distributions of labile and refractory compounds within the <1,000 m/z bin identified in this study suggest that molecular weight is not the determining factor. The size-reactivity continuum model (1) suggests an overall low bioavailability (reactivity) of low-molecular-weight compounds. The degradation of reactive HMW DOM (>1,000 MW) by bacteria leads to the formation of more refractory LMW DOM (<1,000 MW) in this model. Implied in this approach is that, overall, refractory LMW DOM is older than reactive HMW DOM. In our study we found that, within the LMW DOM pool, bacteria released DOM with decreased reactivity (starting from labile glucose), even though this DOM was recently produced. The results from this study are still consistent with the size-reactivity model (1), because the refractory compounds are still part of the LMW pool, but with the addition that the refractory compounds can be formed within the LMW pool and are not necessarily all breakdown products from HMW compounds.

With tools like ESI-MS, the overall dynamics of individual compounds within the LMW DOM pool can be investigated. An important conclusion of this study is that the addition of a higher trophic level (i.e., grazer) to a single-bacteria system has a substantial effect on the rate and extent at which carbon remineralization occurs, but it has a minimal impact on the quantity and quality of DOC. It has been hypothesized (24) that ciliate grazers may also shape DOC as they release com-
pounds that are not able to be reused by bacteria. Our experi-
ment suggests that bacteria could be the primary shapers of DOC and play the prominent role in converting labile organic carbon to refractory. Therefore, while the bacteria-ciliate interaction is crucial for determining the fate of particulate organic carbon, it possibly has a smaller influence on dissolved organic carbon in aquatic systems.

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