Impacts of labile organic carbon concentration on organic and inorganic nitrogen utilization by a stream biofilm bacterial community

Running title: Carbon availability affects bacterial nitrogen use

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Abstract:
In aquatic ecosystems, carbon (C) availability strongly influences nitrogen (N) dynamics. One manifestation of this linkage is the importance, in the dissolved organic matter (DOM) pool, of dissolved organic nitrogen (DON) that can serve as both a C and a N source, yet, our knowledge about how specific properties of DOM influence N dynamics are limited. To empirically examine the impact of labile DOM on responses of bacteria to DON and dissolved inorganic nitrogen (DIN), bacterial abundance and community composition were examined in controlled, laboratory microcosms subjected to various combinations of dissolved organic carbon (DOC), DON and DIN treatments. Bacterial communities that had colonized glass beads incubated in a stream were treated with varying glucose concentrations and combinations of inorganic and organic N (derived from algal exudate, bacterial protein, and humic matter). Results revealed a strong influence of C availability on bacterial utilization of DON and DIN, with preferential uptake of DON under low C concentrations. Bacterial DON uptake was affected by concentration and by its chemical nature (labile vs. recalcitrant). Labile organic N sources (algal exudate and bacterial protein) were utilized equally well as a N source as DIN, this was not the case for the recalcitrant, humic matter DON treatment. Clear differences in bacterial community composition among treatments were observed based on T-RFLP (terminal restriction fragment length polymorphisms) of 16S rRNA genes. C, DIN, and DON treatments likely drove changes in bacterial community composition that, in turn, affected rates of DON and DIN utilization under various C concentrations.
Introduction:

Dissolved organic matter (DOM) is the largest component of the organic matter pool in lotic ecosystems (1, 2). Concentration and composition of DOM varies spatially and temporally (3 – 5) which, in turn, affects productivity of stream food webs (6, 7). Heterotrophic bacteria are major consumers of DOM (8, 9) and thus, DOM influences bacterial community composition (i.e., structure) and abundance (10 – 12).

Processing and fate of organic carbon (C) is fundamentally and synergistically linked to the nitrogen (N) cycle (13, 14). Despite this linkage between C and N cycles, many studies focus solely on C-dynamics (15 – 17) or N-dynamics (18 – 20) rather than their inter-relationship. In streams, specifically, C and N are tightly linked (21) and C availability strongly influences N dynamics (22, 23); yet, our knowledge about how specific properties of DOM influence N dynamics are limited.

Nitrogen is an important component of DOM (24) and a major fraction (often more than 50%) of the total dissolved N pool is dissolved organic nitrogen (DON) (25 – 27). The DON pool is composed of a continuum of compounds ranging from high molecular weight polymers, like polypeptides, to low molecular weight monomers, like amino acids and urea (28, 29). DON is also derived from different sources (e.g., allochthonous versus autochthonous) which influences composition and lability (30, 31).

Allochthonous sources contribute the majority of refractory DON to streams (32), whereas algal derived DON is more labile (33, 34). DON utilization by heterotrophic bacteria varies; the labile fraction is readily utilized (35 – 38) while the recalcitrant fraction is utilized with the aide of extracellular enzymes (39).

Reliance on organic versus inorganic forms of N depends on N availability. The ability to take up dissolved inorganic nitrogen (DIN) in the form of nitrate or ammonia is widespread among bacteria; ammonia uptake is energetically favorable but often nitrate is more available (40, 41). Although DON serves as a potential N source (and C) for
microbial communities (28, 29, 42), bacterial metabolism of DON is influenced by
inorganic N concentrations (43). High DIN concentrations inhibit production of enzymes
that scavenge N from DON (44) and reduce extracellular hydrolysis of refractory DON
(44, 45).

DOM serves as a substrate for bacterial growth (46) which in turn increases the
demand for assimilation of nitrogen (21). DON serves, potentially, as both a C and N
source. The ability of DON to meet the metabolic demand for organic C likely influences
the assimilative demand for N and whether, energetically, that demand is best met by N
from DON or DIN. To empirically examine the impact of labile DOM on responses of
bacteria to DON and DIN, bacterial abundance and community composition (based on
T-RFLP of 16S rRNA genes) (47) were examined in controlled, laboratory microcosms
subjected to various dissolved organic carbon (DOC), DON and DIN treatments.

Bacterial communities that had colonized glass beads incubated in a stream were
treated with three labile C (glucose) concentrations and four combinations of inorganic
and organic N. Because DON is diverse and compounds vary in bioavailability, three
DON sources were examined: humic matter, bacterial protein, and algal exudate.
Additional energy expenditure is required for utilization of humic matter and protein
because extracellular enzyme production is required for their assimilation (44, 48, 49). In
contrast, algal exudates are typically low molecular weight, more labile, and readily
utilized by bacteria (50 – 52).

Methods

Bacterial communities used in the experiments were grown on etched soda lime
glass beads (4mm in diameter; Fisher Scientific) that were incubated in the West Branch
of the Mahoning River, Portage County, Ohio, USA (12, 53). Beads were autoclaved,
packed in mesh bags (80 µm mesh size; WildCo- Wild Life Supply, Yulee, FL), and incubated for 45 days in a riffle in the stream.

Water temperature, pH, conductivity, dissolved oxygen (DO) were measured using a Qd/IntelliCAL Rugged Field Kit (Hach Company, Loveland, CO) and turbidity was measured with a Hach turbidimeter, model 2100P, when substrates were deployed and retrieved. DOC and total nitrogen (TN) concentration were determined using a Shimadzu TOC5000 analyzer with TNM-1 (Shimadzu Corporation, Columbia, MD).

Upon retrieval, beads from bags were pooled together and divided into subsamples. A portion of the beads was frozen at -80ºC for DNA extraction from the initial bacterial community, another was preserved with 8% paraformaldehyde in phosphate-buffered saline (pH 7.2) for bacterial enumeration, and the remainder was used in experiments as described below.

In the laboratory, each treatment and control was carried out in triplicate. Experiments were performed in flasks containing 10 g of beads in 150 ml of artificial stream water (ASW; composition per liter: 12 mg NaHCO₃, 7.5 mg CaSO₄ 2H₂O, 7.5 mg MgSO₄, 0.5 mg KCl, 10 mg CaCO₃, 10 mg K₂HPO₄, pH 6.4). Treatments consisted of different N sources (and concentrations) and different organic C concentrations (Table 1). Organic N sources were bacterial protein, algal exudate, and humic matter (obtained as described below) and inorganic N was NaNO₃. For each N treatment, there were three glucose concentrations (low; medium; high). N and DOC concentrations were selected based on average concentrations in the study stream (12). Positive controls (without N but amended with low, medium or high concentrations of glucose) were used to examine the effect of glucose amendment on bacterial communities. Negative controls (without C and N amendments) were also set-up. Flasks were incubated in the dark to prevent photochemical degradation with shaking at 25ºC and were sampled after 5 or 10 days.
Soluble bacterial proteins were obtained from cultures of *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* incubated at 27 °C for 24 hours. Proteins were extracted using the Qproteome Bacterial Protein Prep Kit (Qiagen, MD) as per the manufacturer’s protocol and proteins from the three bacteria were pooled. Algal exudates were prepared by growing *Chlamydomonas*, *Chlorella* and *Synedra* (Carolina Biological Supplies, Burlington, NC) in ASW with 20mg/L of NaNO₃. Cultures were grown under constant light for 35 days and processed as described below. Humic matter was derived from senescent red oak (*Quercus rubra*), witch hazel (*Hamamelis virginiana*) and corn leaves (*Zea mays*) extracted overnight, in the dark, in 0.027% NaCl and pooled together. Algae exudates and leaf leachates were filtered through GF/F filters (Whatman, Maidstone, UK) and filter sterilized with 0.02 µm Anodisc filters (Whatman, Maidstone, UK). Non-ionic DAX-8 resin (Supelco, Sigma-Aldrich, MO) was used to separate the humic fraction of the leaf leachate from the non-humic fraction. Total DON and DOC concentrations of the bacterial protein, algal exudate and humic matter were measured with the Shimadzu TNM-1 and TOC-5000 analyzer respectively (Shimadzu Corporation, Columbia, MD).

After 5 or 10 days, ASW from the experimental units was filtered through 0.22-µm pore-size polycarbonate filters (Poretics, Livermore, CA) before total dissolved organic carbon (TDOC) and total nitrogen concentration (TN) were determined as mentioned above. Nitrate (NO₃⁻) concentrations were measured via Ion Chromatography (Dionex chromatography system, Thermo Fisher Scientific Inc, CA). pH was measured with a Delta 320 pH meter (Mettler-Toledo, OH).

At each of the two sampling points, 5 g of beads and 5 ml of ASW from a given microcosm was preserved in 8% paraformaldehyde for bacterial enumeration. Subsequently, samples were treated with 0.1% tetrasodium pyrophosphate and sonicated at 40 KHz for 5 min (Ultrasonic cleaner, model 2210; Branson Ultrasonics Co.,
Danbury, CT) to detach bacterial cells (54). Samples were then filtered through 0.2 µm-size black polycarbonate filters (Livermore, CA) and stained with DAPI (4,6-diamidino-2-phenylindole) (55). Bacteria in 10 fields per filter were enumerated via epifluorescence microscopy and used to calculate the total number of bacteria in each flask.

For DNA extraction, 5 g of beads from each microcosm were sonicated as above and bacteria were concentrated by filtration (0.22-µm pore-size polycarbonate filters; Poretics). Filters were frozen at -80 ºC until DNA was extracted using Power-Soil DNA extraction kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer’s instructions with minor modifications as in Feinstein et al., (56).

PCR of the 16S rRNA genes followed by terminal restriction fragment length polymorphism (T-RFLP) was used to examine bacterial community structure. An equimolar mixture of 5'-ACTCCTACGGAGGGTGC-3' (Eub338F-0-III) and 5'-ACACCTACGGGTGTC-3' (Eub338F-I-II), labeled with 6- carboxyfluorescein, as forward primers and 5'-ACGGGCGGTGTATAC-3' (1392R) (W= A or T) (57) as reverse primer were used. Each 25µl reaction mixture contained GoTaq Flexi DNA polymerase (2.5 U), buffer (1X), MgCl₂ (0.5 mM), bovine serum albumin (0.64 mg ml⁻¹), dideoxynucleoside triphosphates (0.2 mM each), forward and reverse primers (0.2 µM each) along with 2 µl of template DNA. Positive controls (Pseudomonas aerugi nosa genomic DNA) and negative controls (sterile deionized water) were run with each set of PCR reactions. Five PCR reactions per sample were carried out in a PTC 200 DNA Engine Cycler (Biorad, Hercules, CA) with a thermal profile of 94°C for 3 min and 40 cycles of 94°C for 30s, 57°C for 30s and 72°C for 90s followed by a final extension of 72°C for 7 min. Success of the PCR amplification was verified by pooling the five reactions per sample and performing gel electrophoresis. PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA) and digested with endonuclease Haell (2U) at 37°C for 18 to 24 hours followed by another round of clean
up with the Qiaquick PCR purification kit (Qiagen, Valencia, CA). Analysis of PCR products was performed at The Ohio State Plant Microbe Genomics Facility using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Results were analyzed via Gene Mapper 4.0 (Applied Biosystems, Foster City, CA).

To identify bacterial sequences in microcosms treated with algal exudate and amended with a high glucose concentration, PCR products of 16S rRNA genes were cloned and sequenced. PCR was carried out with the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1552R (5'-AAGGAGGTGATCCARCCGCA-3') (58, 59) in a PTC 200 DNA Engine Cycler (Biorad, Hercules, CA) with a thermal profile of 94°C for 3 min and 35 cycles of 94°C for 30s, 58°C for 30s and 72°C for 90s followed by a final extension of 72°C for 5 min. Amplified PCR products were purified using Qiaquick PCR purification kit (Qiagen, Valencia, CA) before it was ligated into the pGEM-T vector followed by overnight incubation and transformation of competent E. coli cells according to the manufacturer’s protocol (Promega, Madison, WI). Plasmids were isolated from transformants as in Ausubel et al. (60). Sequencing was performed at the Advanced Genetic Technologies Center at University of Kentucky in Lexington, KY using M13 primers. Plasmid sequence was removed and amplicon sequences trimmed for quality in Sequencher (Gene Codes Corporation, Ann Arbor, MI) using default settings. Sequences were analyzed using BLAST via the National Center for Biotechnology Information website (61).

Statistical analysis

Bacterial abundance, DOC, organic N, nitrate concentration and T-RF (terminal restriction fragment) numbers were compared among treatments via three-way ANOVA using JMP statistical software (version 10, SAS Institute Inc., Cary, NC) followed by student’s t test and Tukey’s test for Post-hoc analysis, p values < 0.05 were considered
significant. To determine the relationship between growth rate and chemical variables (DOC, inorganic and organic N used), multiple linear regression, with stepwise forward and backward selection, was used. The values to enter and leave the analyses were 0.10 and 0.05, respectively.

The contribution of N treatments, glucose concentrations and the interaction of these factors to variation in T-RFLP profiles were determined using redundancy analysis (62). R statistical software (version 2.15.1 for Windows) was used to determine differences in T-RFLP profiles. Variation in relative peak heights (Hellinger distance) and absence and presence of peaks (Jaccard’s distance) were considered for such analysis. Prior to analysis, the T-RFLP relative peak heights were square root transformed.

In addition, composition of the bacterial community was examined by calculating the relative abundance of dominant T-RF peaks (63, 64). Smaller T-RF peaks (that contributed less than 2% to the total) in a pattern were lumped together as ‘other T-RF’s’. Three-way analysis of variance (ANOVA) followed by Tukey post-hoc tests were used to identify differences in T-RF patterns among treatments and time (5 and 10 days); percent relative abundance of T-RFs were normalized by log transformation.

Results:

**Bacterial abundance**

Growth of bacteria was determined based on changes in abundance of both the attached and planktonic cells. For each DON source separately, three-way ANOVAs were used to test for significant differences in bacterial abundance among treatments (Fig. 1). Growth of bacteria in microcosms treated with DON derived from humic matter was relatively modest and, at each of the three glucose concentrations, abundance at the end of the experiment (10 days) was highest in microcosms amended with the highest concentrations of both inorganic and organic N (Fig. 1 A-C). Under high and
medium glucose concentrations, bacterial growth in high DIN and low DON treatment was significantly lower than in treatments with both high DIN and DON, revealing bacterial growth on DON. After 5 days of incubation, under all three glucose concentrations, growth in treatments with high DIN and DON was more similar to growth in high DIN and low DON; which changed after 10 days.

When protein was the DON source, bacterial growth was ten-fold higher than in microcosms with humic matter. However, like humic matter, after 10 days of incubation, bacterial abundance was highest in the high organic and inorganic N treatment under high and low glucose concentrations (Fig. 1 D-F). In contrast, under medium glucose concentration, responses to this treatment were not significantly different from the high inorganic and low organic N treatment. Like humic matter, after 5 days of incubation, bacterial growth under high DIN and high DON was similar to growth in high DIN and low DON, except under high glucose concentration.

Bacterial abundance in microcosms with algal exudate as the DON source was similar to that achieved by the protein amendment. Also, like protein, after 10 days of incubation, highest growth occurred in high DIN and DON treatments (Fig: 1 G- I) except under medium glucose concentration. After 5 days of incubation, under high and medium glucose concentrations, growth was significantly higher in the high DIN and DON treatment, than in the other treatments.

Controls were used to examine effects of glucose concentration alone on bacterial abundance. In these glucose-only controls, cell numbers were significantly lower (10-fold lower after 5 days and 1000-fold lower after 10 days) than in glucose with N amendments (average abundance in glucose only controls= 0.16 - 0.45 [5 days] to 0.07 - 0.36 X 10^6 cells/ml [10 days]). Negative controls were not amended with DOC or N and cell abundances were low; ranging from 0.9 -1.17 (5 days) to 0.15 - 0.3 x 10^6 cells/ml (10 days).
Average decline in DOC concentration were 67.9% and 83.7% after 5 and 10 days, respectively. Final DOC concentration differed significantly among treatments. Regardless of the DON source, in positive controls with medium and high glucose amendments DOC concentration remaining was significantly higher than the respective N treatments after both 5 and 10 days of incubation (Table 2). Maximum DOC loss was observed in treatments with low glucose concentration and was lowest in treatments with high glucose, DON and DIN, irrespective of the DON source and duration of incubation. Also, microcosms with high and medium glucose had greater DOC loss (more bacterial uptake) in treatments with high DON and low DIN compared to treatments with low DON and high DIN.

Because initial differences in DOC concentration impacted final concentrations, DOC loss was expressed as DOC utilization per bacterial cell [loss of DOC (i.e. difference between initial and final DOC concentration) divided by bacterial abundance] and differed significantly among treatments (Fig. 2). Generally, experimental microcosms with higher bacterial abundance had low per cell DOC use than those with low bacterial abundance; bacterial DOC use per cell tracked patterns of bacterial abundance with some exceptions. At the end of the experiment, for all DON sources used, under low glucose concentration, bacterial abundance in amendments with high DON and low DIN was similar to that in low DON and high DIN (Fig. 1 A, D, G). However, bacterial DOC use per cell was significantly lower in the low DON and high DIN treatment than the high DON and low DIN treatment (Fig. 2 A, D, G).

Since bacterial DOC use per cell was a function of abundance, in positive controls, DOC use per cell (average= 84.35 pg/ cell ± 9) was higher than DOC use in experimental microcosms. DOC was not added to the negative controls, thus DOC use per cell was below detection.
**N utilization**

Bacterial utilization of organic and inorganic N (based on declines in DON and DIN concentrations in microcosms) differed significantly among treatments (Fig. 3). Irrespective of the DON source, under high DON, high DIN and low glucose concentration conditions, bacterial use of organic N was significantly higher than inorganic N after both 5 and 10 days. (Fig. 3: A, D, G). However, bacteria utilized organic and inorganic N equally well under medium and high glucose concentrations, when bacterial protein and algal exudate was the DON source (Fig. 3 E- F; H - I). In microcosms treated with humic matter, under both medium and high glucose concentration, bacterial use of DON was lower than DIN in amendments with high DON and high DIN (Fig. 3 B, C).

To determine if changes in pH played a role in bacterial responses, pH was measured initially, after 5 and 10 days. Initial pH values ranged from 6.50 – 7.45 and no significant differences in pH were observed; final values ranged from 6.25 – 7.20 (5 days) to 6.05 – 6.79 (10 days).

**T-RFLP**

Based on redundancy analysis (RDA) of 16S rRNA gene T-RFLP, bacterial community structure differed significantly among treatments for all DON sources (p <0.05). The largest percentage of variation in community structure was explained by the interaction between N-treatment and glucose concentration (Table 3). Generally, the percent of variation explained by this interaction was higher after 5 days (average =83%) than after 10 days (average= 60%).

For bacterial communities in humic matter treatments, composition varied among glucose concentrations and N treatments after 5 days (Fig. 4 A). Yet, after 10 days, only bacterial communities under high glucose and low DIN and DON concentrations were spatially separated on RDA axis 1, whereas, communities from other treatments were...
clustered relatively close on this axis (Fig 4 B). Other treatments varied along RDA axis 2 but, generally, those with the same glucose concentrations were the most similar.

For the humic matter amendments, number of T-RFs was highest in the treatment with low DON and high DIN under high glucose concentration but did not differ significantly among the rest of the treatments after 5 days (Fig.5 A). However, after 10 days there were significant differences in the number of T-RFs among treatments, with highest numbers recorded in microcosms treated with high DON and DIN under low glucose concentration (Fig. 5 B).

There were also significant differences in relative abundance of T-RFs (Fig. 6 A-B). For example, after 5 days, under low and medium glucose concentrations T-RF -2 and T-RF-4 had low relative abundance but were more dominant after 10 days. T-RF-3 had high relative abundance in all treatments except under high DON, low DIN and low glucose. T-RF-12 and T-RF-19 was absent in all treatments after 5 days but together contributed 60% of the community in the treatment with low DON and high DIN under high glucose concentration after 10 days.

When bacterial protein was the DON source, after 5 days, bacterial community structure differed among glucose and N treatments with no discernible pattern based on treatment (Fig 4 C). Yet, after 10 days, communities became more similar with varying degrees of overlap. Under high glucose concentration, two N-treatments (low DON + high DIN and low DON + low DIN) had distinct communities (Fig 4 D). Number of T-RFS differed significantly among treatments after 5 days with maximum numbers under high glucose concentrations (Fig. 5 C). However, after 10 days the number of T-RFS decreased in all treatments and there were few significant differences in number of peaks (Fig. 5 D).
Significant differences among treatments were also observed in relative abundance of T-RFs (Fig. 6 C-D). Bacterial communities became more uniform in all the treatments as they were dominated mostly by T-RF-2, 6 and 7 after 10 days. Composition of bacterial communities treated with algal exudate differed significantly among treatments after 5 days; high glucose concentration with high DON and low DIN as well as low DON and high DIN conditions had the most distinct communities (Fig 4 E). However, after 10 days, communities in the high glucose treatments clustered together regardless of N treatment; this was also somewhat the case for the medium and low glucose concentrations (Fig 4 F). As observed in the humic matter amendments, the total number of T-RFs did not differ significantly between 5 and 10 days. Irrespective of incubation period, no significant differences in number of peaks were observed among treatments except for: low glucose, high DON and low DIN treatment after 5 days and high glucose, high DON and high DIN treatment after 10 days, which had relatively low number of peaks (Fig. 5 E-F). Relative abundance of specific T-RFs differed significantly among treatments (Fig. 6 E-F). T-RF-1 was dominant in all treatments after 5 days. It continued to be the dominant T-RF at the end of 10 days in treatments with low and medium glucose amendment but was absent under high glucose concentration. T-RF-2 was absent in all treatments with low glucose amendments after 5 days. However, it showed a striking increase (accounting for 72%-88% of the community) in treatments with high glucose after 10 days. Cloning and 16S rRNA sequencing followed by BLAST comparisons to GenBank performed on samples from this treatment revealed that 75% of the clones had a high degree of sequence similarity to *Pseudomonas* spp. while, the other 25% had more similarity with members of the Enterobacteriaceae (Serratia sp., Klebsiella sp., and Proteus sp., as nearest neighbors) (Table 4). Out of 12 clones, 7 demonstrated maximum sequence similarity (95-97%) to strains of *Pseudomonas aeruginosa*. 
Discussion:
Carbon availability exerts strong controls on bacterial N dynamics (23, 65, 66). In aquatic ecosystems, heterotrophic bacteria meet their N demand via utilization of inorganic and organic N, but the interplay between DIN, DON, and labile DOC are not well understood. In this study, we manipulated the supply of labile C to the bacterial community and then followed loss of N, C, and shifts in bacterial community abundance and composition. We found that the C supply impacted bacterial N utilization, and reliance on organic versus inorganic N was determined by changes in labile C concentration and quality of organic N. Likewise, availability of N can also affect C dynamics. Low N availability can result in bacterial sustenance by utilizing DOC for respiration and converting it to CO$_2$, whereas increased N availability would allow bacteria to use DOC not only for respiration but also for growth.

In freshwater, more than 50% of total dissolved N is DON (25, 67, 68), which varies in composition and bioavailability (48). DON is an important N source for heterotrophic bacteria (69, 70). Since DON-derived N can be either used directly (71, 72) or indirectly via extracellular enzymes (73, 74), bacterial uptake depends on the nature of the DON pool. The stoichiometric ratio of C: N varies among different DON compounds and plays a critical role in nutrient mineralization by heterotrophic bacteria (75). While high C:N ratio implies low N availability as found in recalcitrant compounds like humic acids, low C:N ratio implies increased availability of N as found in labile compounds, such as proteins. In our study, different DON sources elicited different bacterial responses. For example, bacteria preferentially used DIN over humic-DON under medium and high glucose concentrations since humic derived DON is more resistant to biological degradation (52) and more energy is required (39). However, bacterial uptake of humic-DON was higher than DIN under low glucose concentration.
suggesting that the humic compounds served as sources of both N and C (76) under carbon limiting conditions. High C:N ratio in humic matter coupled with the refractory nature of humic compounds accounts for the low bacterial abundance in microcosms treated with humic matter. Like humic DON, algal derived DON and bacterial protein were used more than DIN under low glucose concentrations and were more labile (50), yielding greater bacterial abundances. In contrast, under medium and high glucose concentrations, DIN and DON were used equally suggesting that even under conditions where C is less limited these DON mixtures are useful as C and N sources.

In addition to impacts caused by differences in the type of DON, the effects of addition of labile DOC were also examined. DOC is an important driver of bacterial abundance (77) and, as expected, bacterial growth was stimulated by the addition of labile DOC (78). In microcosms with high bacterial abundance, rapid decline in DOC from the water column, along with low DOC use per cell, indicate strong competition for carbon within the bacterial community (79). In most treatments it was also observed that DOC use per cell was lower after 10 days than after 5 days. This may result from a relatively slow response of bacterial biofilms to nutrient amendments compared to free-living bacteria in the water column. However, low bacteria abundance in treatments with low N availability and in positive controls without N amendment, suggests that more of the DOC is utilized for bacterial respiration than for growth.

Heterotrophic bacteria differ in their utilization of C and N based on their metabolic capabilities and thus bacterial community composition plays a critical role in nutrient uptake (80). Bacterial community structure differed among DON sources and treatments. Previous research reveals that while differences in bacterial community composition can influence metabolism of organic N (81, 82), in freshwater ecosystems, changes in resources (nutrient concentration and quality) often trigger responses in community metabolism (83). Therefore, community composition is both a driver of...
differences in C and N utilization as well as an attribute that is influenced by C and N
treatments.

Variation in community metabolism can result from physiological acclimation,
changes in community composition, or a combination of both (78, 84 – 86). In the
experimental microcosms, differences in utilization of N under varying C concentrations
is possibly due to changes in abundance of bacteria with different nutritional needs
resulting in shifts in community composition. Community composition and functional
response are highly correlated (87) and shifts in community composition can be
accompanied by changes in the hydrolytic ectoenzyme activity (88).

We observed maximum differences in community structure among treatments
after 5 days which diminished after 10 days when bacterial protein and algal exudate
were the DON source. It is likely that as resources were depleted, lower bioavailability of
C and N led to slow growing, stable communities which are dominated by fewer T-RFs,
with similar capabilities, to utilize the left over resources (79). However, this was not
observed in microcosms treated with humic matter. In this case, lower bioavailability of
recalcitrant compounds possibly led to more diverse communities composed of different
T-RFs because enzymatic breakdown of complex compounds by some members allows
for uptake of the products by others in the community (39, 89). This possibility is
supported by the more balanced contributions of multiple T- RFs in the communities of
humic treatments relative to other DON sources. Competition is another potential
contributor to these differences in bacterial community composition among treatments as
bacteria interact to obtain C and N required for their growth (79).

The shift to dominance by a limited subset of T-RFs was most obvious in
microcosms treated with high concentrations of glucose with algal exudate as the DON
source. Regardless of N treatment, one T-RF accounted for approximately 80% of the
community. Based on 16S rRNA gene sequencing, these dominant organisms were
**Pseudomonas**, a genus that is widely distributed in aquatic ecosystems (90, 91). Being genetically and metabolically versatile, *Pseudomonas* are ubiquitous and occupy multiple ecological niches (92). Several sequences had strong similarity to *Pseudomonas aeruginosa*, which is an efficient competitor for resources; they produce antibiotics (79) and deploy toxins to attack cell walls of other bacterial competitors in a community (93). In our experimental microcosms treated with algal exudate and amended with high glucose, *Pseudomonas* sp. possibly scavenged C and N, limiting their acquisition by other members of the bacterial community. Also, bead method used may be selective or enriching for specific taxa as in this lab study with the biofilm community in a closed system, which possibly contributed to lower bacterial diversity by selecting for a limited subset of taxa such as *Pseudomonas* and members of *Enterobacteriaceae*.

Overall, there was a strong linkage between C availability and bacterial utilization of DON over DIN. Bacterial utilization of DON was not only related to availability of inorganic N but also to the nature of the DON (labile versus recalcitrant). Difference in use of organic N was strongly associated with differences in community composition. Likely, C, DIN and DON treatments drove changes in bacterial community composition that, in turn, affected rates of DON and DIN utilization under various C concentrations.
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**Figure legends:**

**Figure 1:** Increase in bacterial abundance after 5 and 10 days of incubation; A-C: humic matter, D – F bacterial protein, G- I algal exudate as DON source. C-Low, Medium, and high refer to glucose concentrations. O=organic N, I=inorganic N and H=high concentration, L= low concentration. Values are means and standard errors. Different letters above bars indicate significant differences at p <0.05.

**Figure 2:** Utilization of dissolved organic carbon (DOC) per bacterial cell in picograms (pg), after 5 and 10 days of incubation; A-C: humic matter, D - F bacterial protein, G- I algal exudate as DON source. C-Low, Medium, and high refer to glucose concentrations. O=organic N, I=inorganic N and H=high concentration, L= low concentration. Values are means and standard errors. Different letters above bars indicate significant differences at p <0.05.

**Figure 3:** Utilization organic and inorganic nitrogen per bacterial cell, in picograms (pg), after 5 and 10 days of incubation. A-C: humic matter, D - F bacterial protein, G- I algal exudate as DON source. C-Low, Medium, and high refer to glucose concentrations. O=organic N, I=inorganic N and H=high concentration, L= low concentration. Values are means and standard errors. Different letters above bars indicate significant differences at p <0.05.

**Figure 4:** Ordination plots from redundancy analysis of 16S rRNA gene TRFLP peak relative abundance after 5 (A, C, E) and 10 days (B, D, F). A and B= humic matter, C and D=bacterial protein, E and F=algal exudate. C-Low, Medium, and high refer to glucose concentrations. O=organic N, I=inorganic N and H=high concentration, L= low concentration. Values are means and standard errors.

**Figure 5:** Number of peaks of 16S rRNA gene TRFLP profiles, after 5 and 10 days of incubation. Graph A) after 5 days and B) after 10 days with humic matter as the organic.
nitrogen source; C) after 5 days and D) after 10 days with bacterial protein as the
organic nitrogen source; E) after 5 days and F) after 10 days with algal exudate as the
organic nitrogen source. C-Low, Medium, and high refer to glucose concentrations.
O=organic N, I=inorganic N and H=high concentration, L= low concentration. Values are
means and standard errors. Different letters above bars indicate significant differences
at p <0.05.

Figure 6: Percentage dominant TRF’s, after 5 and 10 days of incubation. Graph A) after
5 days and B) after 10 days with humic matter as the organic nitrogen source; C) after 5
days and – D) after 10 days with bacterial protein as the organic nitrogen source; E)
after 5 days and F) after 10 days with algal exudate as the organic nitrogen source. C-
Low, Medium, and high refer to glucose concentrations. O=organic N, I=inorganic N and
H=high concentration, L= low concentration.
Figure 1:

A

B

C

D

E

F

G

H

I

5 days

10 days

Increase in bacterial abundance (cells/ml × 10^9)

Increase in bacterial abundance (cells/ml × 10^9)

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Increase in bacterial abundance (cells/ml × 10^9)
Table 1: Carbon and nitrogen treatments used in the experiment. Organic nitrogen sources consisted of bacterial protein, algal exudate and humic matter, and nitrate was the source of inorganic nitrogen. Three concentrations of organic carbon, in the form of glucose, were used for each nitrogen treatment. OH - Organic nitrogen high concentration, OL – Organic nitrogen low concentration, IH - Inorganic nitrogen high concentration, IL – Inorganic nitrogen low concentration.

<table>
<thead>
<tr>
<th>Carbon Treatment</th>
<th>Nitrogen treatments</th>
<th>Glucose (mgC/L)</th>
<th>Organic Nitrogen (mgN/L)</th>
<th>Inorganic Nitrogen (mgN/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-Low</td>
<td>OH+IL</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OL+IH</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>OL+IL</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OH+IH</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C-Medium</td>
<td>OH+IL</td>
<td>10</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OL+IH</td>
<td>10</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>OL+IL</td>
<td>10</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OH+IH</td>
<td>10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C-High</td>
<td>OH+IL</td>
<td>25</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OL+IH</td>
<td>25</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>OL+IL</td>
<td>25</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>OH+IH</td>
<td>25</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2: Final DOC concentration in C and N amended microcosms after 5 and days of incubation. Three concentrations of organic carbon, in the form of glucose, were used for each nitrogen treatment. OH - Organic nitrogen high concentration, OL – Organic nitrogen low concentration, IH - Inorganic nitrogen high concentration, IL – Inorganic nitrogen low concentration.

<table>
<thead>
<tr>
<th>Carbon Treatment</th>
<th>Nitrogen treatments</th>
<th>Final DOC concentration (mg C/L)</th>
<th>Humic</th>
<th>Bacterial protein</th>
<th>Algal exudate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5 days</td>
<td>10 days</td>
<td>5 days</td>
<td>10 days</td>
</tr>
<tr>
<td>C-Low</td>
<td>OH+IL</td>
<td>0.57</td>
<td>0.40</td>
<td>0.35</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>OL+IH</td>
<td>0.50</td>
<td>0.33</td>
<td>0.35</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>OL+IL</td>
<td>0.41</td>
<td>0.14</td>
<td>0.27</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>OH+IH</td>
<td>0.51</td>
<td>0.36</td>
<td>0.38</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>+ve ctrl</td>
<td>0.29</td>
<td>0.44</td>
<td>0.17</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>-ve ctrl</td>
<td>0.21</td>
<td>0.83</td>
<td>0.25</td>
<td>0.79</td>
</tr>
<tr>
<td>C-Medium</td>
<td>OH+IL</td>
<td>6.72</td>
<td>4.35</td>
<td>5.24</td>
<td>2.73</td>
</tr>
<tr>
<td></td>
<td>OL+IH</td>
<td>5.02</td>
<td>2.62</td>
<td>5.13</td>
<td>2.96</td>
</tr>
<tr>
<td></td>
<td>OL+IL</td>
<td>8.13</td>
<td>6.01</td>
<td>5.94</td>
<td>2.86</td>
</tr>
<tr>
<td></td>
<td>OH+IH</td>
<td>5.33</td>
<td>1.80</td>
<td>3.94</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>+ve ctrl</td>
<td>9.31</td>
<td>8.60</td>
<td>8.35</td>
<td>6.51</td>
</tr>
<tr>
<td></td>
<td>-ve ctrl</td>
<td>0.21</td>
<td>0.83</td>
<td>0.25</td>
<td>0.79</td>
</tr>
<tr>
<td>C-High</td>
<td>OH+IL</td>
<td>12.42</td>
<td>9.42</td>
<td>10.33</td>
<td>4.79</td>
</tr>
<tr>
<td></td>
<td>OL+IH</td>
<td>10.54</td>
<td>5.87</td>
<td>9.75</td>
<td>3.63</td>
</tr>
<tr>
<td></td>
<td>OL+IL</td>
<td>11.76</td>
<td>5.48</td>
<td>10.52</td>
<td>4.77</td>
</tr>
<tr>
<td></td>
<td>OH+IH</td>
<td>9.36</td>
<td>3.31</td>
<td>8.92</td>
<td>3.32</td>
</tr>
<tr>
<td></td>
<td>+ve ctrl</td>
<td>22.18</td>
<td>19.07</td>
<td>19.33</td>
<td>17.16</td>
</tr>
<tr>
<td></td>
<td>-ve ctrl</td>
<td>0.21</td>
<td>0.83</td>
<td>0.25</td>
<td>0.79</td>
</tr>
</tbody>
</table>
Table 3: Percentage variation in bacterial community structure, after 5 and 10 days, explained by N treatments, glucose concentration and the interaction between N- treatments and glucose concentration, from redundancy analysis of 16S rRNA gene TRFLP profiles.

<table>
<thead>
<tr>
<th>DON source</th>
<th>Variation explained by N-treatment</th>
<th>Variation explained by glucose concentration</th>
<th>Variation explained by N-treatment * glucose concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 days</td>
<td>10 days</td>
<td>5 days</td>
</tr>
<tr>
<td>Humic matter</td>
<td>11%</td>
<td>15%</td>
<td>14%</td>
</tr>
<tr>
<td>Bacterial protein</td>
<td>12%</td>
<td>3.6%</td>
<td>17%</td>
</tr>
<tr>
<td>Algal exudate</td>
<td>13 %</td>
<td>8.1%</td>
<td>20%</td>
</tr>
</tbody>
</table>
Table 4: Best matches of the different sequences obtained from the microcosms treated with Algal exudate and amended with high glucose, DON and DIN.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Length of sequence (bp)</th>
<th>Gene Bank Accession</th>
<th>Best match (BLASTN)</th>
<th>% Identity/E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLAEhc1</td>
<td>1200</td>
<td>KC211291.1</td>
<td><em>Pseudomonas aeruginosa</em> strain KLU02</td>
<td>97/0.0</td>
</tr>
<tr>
<td>CLAEhc2</td>
<td>1029</td>
<td>JQ659882.1</td>
<td><em>Pseudomonas aeruginosa</em> strain R7-521</td>
<td>96/0.0</td>
</tr>
<tr>
<td>CLAEhc3</td>
<td>1046</td>
<td>JQ659882.1</td>
<td><em>Pseudomonas aeruginosa</em> strain R7-521</td>
<td>97/0.0</td>
</tr>
<tr>
<td>CLAEhc4</td>
<td>1241</td>
<td>JQ659882.1</td>
<td><em>Pseudomonas aeruginosa</em> strain R7-521</td>
<td>96.0</td>
</tr>
<tr>
<td>CLAEhc5</td>
<td>649</td>
<td>JF708077.1</td>
<td><em>Pseudomonas aeruginosa</em> strain mpc1</td>
<td>95/0.0</td>
</tr>
<tr>
<td>CLAEhc6</td>
<td>641</td>
<td>KCS03912.1</td>
<td><em>Pseudomonas</em> sp. ZS-1</td>
<td>77/2e-10</td>
</tr>
<tr>
<td>CLAEhc7</td>
<td>694</td>
<td>JN622013.1</td>
<td><em>Pseudomonas</em> sp. WC-1</td>
<td>88/2e-23</td>
</tr>
<tr>
<td>CLAEhc8</td>
<td>1230</td>
<td>KCS03912.1</td>
<td><em>Pseudomonas aeruginosa</em> strain R7-521</td>
<td>97/0.0</td>
</tr>
<tr>
<td>CLAEhc9</td>
<td>1193</td>
<td>KC211291.1</td>
<td><em>Pseudomonas aeruginosa</em> strain KLU02</td>
<td>97/0.0</td>
</tr>
<tr>
<td>CLAEhc10</td>
<td>378</td>
<td>GU569122.1</td>
<td>Uncultured <em>Rahnella</em> sp. clone JBXB28</td>
<td>80/9e-47</td>
</tr>
<tr>
<td>CLAEhc11</td>
<td>1292</td>
<td>HQ018868.1</td>
<td><em>Klebsiella</em> sp. GX17</td>
<td>92/0.0</td>
</tr>
<tr>
<td>CLAEhc12</td>
<td>1300</td>
<td>NR_041979.1</td>
<td><em>Serratia ficaria</em> strain DSM 4569</td>
<td>92/0.0</td>
</tr>
</tbody>
</table>