Dissolved Organic Nitrogen in Urban Streams: *Biodegradability and Molecular Composition*
Studies

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ABSTRACT

A portion of the dissolved organic nitrogen (DON) is biodegradable in water bodies, yet our knowledge of the molecular composition and controls on biological reactivity of DON is limited. Our objective was to investigate the biodegradability and molecular composition of DON in streams that drain a gradient of 19 to 83% urban land use. Weekly sampling over 21 weeks suggested no significant relationship between urban land use and DON concentration. We then selected two streams that drain 28% and 83% urban land use to determine the biodegradability and molecular composition of the DON by coupling 5-day bioassay experiments with high resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). Both urban streams contained a wide range of N-bearing biomolecular formulas and had >80% DON in lignin-like compounds, with only 5 to 7% labile DON. The labile DON consisted mostly of lipid-and protein-like structures with high H/C and low O/C values. Comparison of reactive formulas and formed counterparts during the bioassay experiments indicated a shift toward more oxygenated and less saturated N-bearing DON formulas due to the microbial degradation. Although there was a little net removal (5-7%) of organic-bound N over the 5-day bioassay, there was some change to the carbon skeleton of DON compounds. These results suggest that DON in urban streams contains a complex mixture of compounds such as lipids, proteins, and lignins of variable chemical structures and biodegradability.

Keywords: urban streams; dissolved organic nitrogen; high resolution mass spectrometry; bioassay

1. Introduction

Dissolved organic nitrogen (DON) is commonly the dominant dissolved N form in surface waters in both forested and human dominated watersheds (Pellerin et al. 2006, Pellerin et al. 2004, Stanley and Maxted 2008). Thus, DON is a potential source of reactive N in aquatic ecosystems (Berman and Bronk 2003, Bradley et al. 2010, Jørgensen et al. 2014, Killberg-Thoreson et al. 2013, Seitzinger et al. 2002, Wiegner et al. 2009). Researchers have hypothesized that DON export in streams is affected by land use patterns; however, reports in the literature are mixed. For example, Pellerin et al. (2006) reported that DON concentrations were 2.5 to 4 times higher in the urban than forested watersheds. Kroeger et al. (2006) reported that DON export increased in coastal watersheds with increase in human population, and they argued that anthropogenic N added to watersheds can be exported as DON. Other researchers found no significant effect of urbanization on DON export (Aitkenhead-Peterson et al. 2009, Stanley and Maxted 2008). When they observed no significant effect of urbanization, Stanley and Maxted (2008) suggested that increases in anthropogenic DON associated with urbanization (sewage wastes, fertilizers) were being offset by decreased influence of pre-development wetland DON, such that there was no net change in the stream DON concentrations after post development.

A growing number of studies have addressed the bioavailability and ecological significance of DON in the freshwater (Fellman et al. 2009, Petrone et al. 2011, Ylla et al. 2010) and marine ecosystems (Berman and Bronk 2003, McCarthy et al. 1998, Mozdzer et al. 2010, Petrone et al. 2009). In these studies, connecting the extent of DON bioavailability to specific DON characteristics (e.g., size fraction) is seldom straightforward because DON is not comprised of a single molecule but is a structurally complex mixture of compounds that vary in chemical structure and bioavailability. These include simple compounds (e.g., sugars, proteins, and amino

acids) that are readily used by plants and microbes, as well as more complex compounds like polyphenols and tannins that are not readily used (Neff et al. 2003).

Recently, high resolution mass spectrometry using high magnetic fields has been used to characterize DON in the marine and stream samples. The very high resolving power of high magnetic field mass spectrometry allows to make compound level assessments of DON and identify individual DON components based on the molecular formulas (Kellerman et al. 2015, Osborne et al. 2013, Lusk and Toor 2016). This technique has shown promise when some kind of comparison is desirable to evaluate DON trends in time or space, or to elucidate what makes DON biodegradable or refractory. For example, Osborne et al. (2013) and Lusk and Toor (2016) used ultrahigh resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) to assess changes at the compound level in DON in the water samples before and after bioassay experiments.

The objective of this study was to investigate the effect of urban land use gradient on the biodegradability and molecular composition of DON in the streams of the Alafia River watershed, which drains into Tampa Bay estuary, Florida, United States. Our preliminary data showed a weak or no significant correlation between land use and streamwater DON, similar to the findings of other researchers (Aitkenhead-Peterson et al. 2009, Stanley and Maxted 2008). Recent authors have reported that urbanization increased the lability and changed the chemical composition of bulk dissolved organic matter (DOM) in urban streams in comparison to non-urban streams (Parr et al. 2015, Williams et al. 2015). Thus, we hypothesized that urbanized streams would contain unique and more labile DON compounds. To test this hypothesis, we used 5-day bioassay experiments and positive ion mode atmospheric pressure photoionization

(+APPI) coupled with high resolution FT-ICR-MS to determine the biodegradability and molecular composition of DON in two urban streams that drain 28% and 83% of urban land use.

2. Materials and methods

2.1. Preliminary studies to assess nitrogen concentrations and forms in urban streams

The study sites included streams draining various sub-basins of the Alafia river, which is a blackwater stream, with a characteristic low gradient and surrounded by sandy soils that leach DOM into the streams. The waters of the Alafia have a characteristic golden-tan color due to the high levels of DOM, and there are low levels of sediment, which is another common trait of blackwater streams. The headwaters of the Alafia river originate from swamp and prairie lands of Polk County, Florida and extend 38.6 km westward into lower Hillsborough Bay, ultimately discharging into Tampa Bay estuary. The soils in the watershed are sandy, with moderate to slow infiltration and are dominated by spodosols, inceptisols, and alfisols in the Myakka, Winder, Zolfo, Lake, and Chandler soil groups (Doolittle et al. 1989).

First, we selected seven stream sites located in sub-basins of the Alafia river watershed and three sites located on the mainstem of the Alafia river (Figure 1). We used 2010 land use data from the Southwest Florida Water Management District and ArcGIS 10 to determine the area under urban, forest, agricultural, and wetland land uses, as well as drainage density and the number of septic systems (Supplementary Figure S1; Table 1).

Grab water samples were collected from these 10 stream sites on a weekly basis from April 15, 2013 to September 30, 2013 (n=21). This captured the 2013 summer rainy season (June to September) as well as six weeks immediately preceding the rainy season. Samples were collected in 250 mL polyethylene bottles and transported to the lab on ice and filtered through 0.45 μm

filter paper within 24 h of collection. The filtered and unfiltered samples were stored in 25-mL polyethylene scintillation vials at approximately 4 °C until analysis for N forms and concentrations, as discussed below.

2.2. *Sampling sites for DON bioavailability and molecular characterization*

The sampling sites for the biodegradability and molecular characterization of DON were selected from the subset of 10 sites used in the preliminary study. These included a highly urbanized stream (Buckhorn Creek) and a less urbanized stream (Turkey Creek). Buckhorn Creek is predominantly urban with 83% urban land use and 3% agriculture and 9% wetland land uses. Turkey Creek is characterized by mixed land uses, with 28% urban land use and 28% agriculture and 12% wetland land uses. Grab samples were collected from the most downstream points in these two streams in June 2014 in sterile 5 L polycarbonate carboys and transported to the lab on ice. The samples were immediately filtered through 0.45 µm filter paper, stored in borosilicate glass jars at approximately 4°C for <24 h, and used in the bioassay and FT-ICR-MS experiments. For the biodegradability experiment, samples were inoculated with bacteria from streamwater of the Alafia river mainstem, after methods described by Osborne et al (2013) (Figure 1; sampling point MS1). The inoculum water was collected and processed on the same day as the stream samples. The bioassay experiments were initiated by mixing 400 mL of filtered stream water sample and 125 mL of inoculum in 1 L glass Erlenmeyer flasks. The initial samples (designated as T₀) were analyzed for N forms and prepared for FT-ICR-MS analysis as described below. A second set of flasks (designated T₅) were incubated for 5 d on a 12:12 light:dark cycle. After 5 d, the incubated samples were filtered through 0.45 µm to remove microbial cells and analyzed for N forms and FT-ICR-MS analysis as described for the T₀ samples.

2.3. Nitrogen forms and concentrations analysis

For $\text{NH}_3\text{-N}$ and $\text{NO}_x\text{-N}$ analysis, filtered samples were analyzed according to EPA method 353.2 using a discrete analyzer (AQ2+, Seal Analytical Inc., Mequon, WI). For total dissolved N (TDN) analysis, a subsample of the filtered samples was oxidized with alkaline persulfate as described by Hosomi and Sudo (1986) and then analyzed for $\text{NO}_x\text{-N}$ with a discrete analyzer as above. For total N (TN) analysis, unfiltered samples were oxidized and analyzed as for TDN analysis. Particulate organic N (PON) was calculated as the difference between TN and TDN and DON was calculated as the difference between TDN and ($\text{NO}_x\text{-N} + \text{NH}_3\text{-N}$).

2.4. Molecular characterization of DON by FT-ICR-MS

The DON in the 0.45 μm -filtered samples was concentrated and extracted for FT-ICR-MS analysis using 1 g, 6 mL Varian Bond Elut PPL solid phase extraction (SPE) cartridges that were first rinsed with two cartridge volumes (12 mL) of HPLC grade methanol. Flow through the SPE sorbent bed was set not to exceed 20 mL/min. After sample loading, the sorbent bed was rinsed with 12 mL of deionized water adjusted to pH ~2 with HCl and then dried by applying a light vacuum for approximately 5 min. We used 6 mL of methanol to elute the sorbent bed and collect into 20-mL borosilicate scintillation vials.

We used dopant-assisted photoionization by diluting samples (in methanol) obtained from SPE at 9:1 with toluene (JT Baker, Phillipsburg, NJ) to increase ionization efficiency. The +APPI was performed on a modified ThermoFisher APPI source (ThermoFisher Corp., San Jose, CA) (Purcell et al. 2006, Purcell et al. 2007). Samples were analyzed with a custom-built 9.4 T FT-ICR-MS (Kaiser et al. 2011) and data collection was facilitated by a modular ICR data acquisition system (PREDATOR) (Blakney et al. 2011).

Data were analyzed and peak lists generated with custom-built software (MIDAS) and processed with PetroOrg software (Corilo, Y. E. PetroOrg Software, Florida State University, All rights reserved. www.petroorg.com) (Blakney et al. 2011). Internal calibration of the spectrum was based on homologous series whose elemental compositions differ by integer multiples of 14.01565 Da (i.e., CH₂) (Kendrick 1963). Compounds with the same heteroatom content (i.e., same n, o, and s in C_cH_hN_nO_oS_s) but differing in the degree of alkylation were grouped together to allow molecular formula assignment to each homologous series (Hughey et al. 2001).

3. Results and discussion

3.1. Preliminary data on forms of nitrogen concentrations in urban streams

Our main objective was to characterize the biodegradability and molecular composition of DON in streamwaters from a highly urbanized and less urbanized sub-basins. However, to place the results in context with DON concentrations in streams draining different land uses along an urbanization gradient, we briefly report the weekly N concentrations in streamwaters collected for 21 weeks, including the summer wet season. For the 10 sampling sites, the wet season mean TN was 1.23-1.90 mg/L and DON:TDN was 0.31-0.74 (Table 2). Nitrate-N was the main dissolved inorganic N (DIN) in only two streamwaters collected from the mainstem, with mean concentrations ranging from 0.08 to 0.74 mg/L. Whereas, NH₃-N concentrations ranged from 0.10 to 0.34 mg/L across 10 stream sites.

There was no significant correlation between urban, forest, or wetland land uses and DON concentrations. A small but significant positive correlation ($r^2 = 0.07$; $P = 0.003$) between the agricultural land use and DON concentrations was observed (Supplementary Figure S2). Pellerin et al. (2006) studied 348 watersheds with varying land uses and reported a DON:TDN of 0.35 for urban streams. In our study, DON:TDN ranged from 0.31 to 0.74 across various streams, with

higher values in two most urbanized streams such as 0.59 for Buckhorn Creek and 0.62 for Poley Creek. On the other hand, DON in our study was lower than (1) Osborne et al. (2013), who observed 2.06 mg L⁻¹ TDN, of which 99% was DON, in a wet season (summer) from the Caloosahatchee River in Florida and (2) Hendrickson et al. (2007), who reported 2.2 mg L⁻¹ TDN, of which 79% (1.7 mg L⁻¹) was DON, in urban sections of Florida's St. Johns River. It is not unusual for stream TDN to be dominated by DON, in both pristine and human-impacted watersheds (Pellerin et al. 2006, Perakis and Hedin 2002). In particular, the blackwater rivers of the southeastern USA coastal plain have large fraction of N in the organic fraction (Graves et al. 2004, Hendrickson et al. 2007). Much of the organic N in blackwater streams comes from humified allochthonous organic matter derived from shallow watershed soils (Hendrickson et al. 2007). In sandy coastal plain watersheds like our study site, groundwater seepage can also be an important contributor to the organic nutrient budget of streams and estuaries. For example, Swarzenski et al. (2007) found that DON was 35% of TDN contributed by groundwater seepage to the Tampa Bay Estuary.

3.2. *Nitrogen forms and biodegradability in urban streams*

After the preliminary study, we selected two sub-basins with the most disparate urban land use (Buckhorn Creek: 83%, Turkey Creek: 28%) to investigate the biodegradability and molecular composition of DON in streamwaters during the summer wet season. Concentrations of DON in the initial samples collected in June 2014 were 1.72 mg/L (78% of TDN) in the highly urbanized stream and 1.09 mg/L (66% of TDN) in less urbanized stream (Table 3; T₀ data). Both streams had a small pool of reactive DON, as shown by DON removal during the bioassay (Table 3; T₅ data). Over the 5-day bioassay, DON removal was 5.1% (highly urbanized stream) to 7.1% (less urbanized stream) suggesting that on the time scale of 5 days at least, these streams have a small

207 reactive DON pool. Our results are in close alignment to those of Osborne et al. (2013), who
208 observed that DON was the dominant (96% of TDN) dissolved N form in the Caloosahatchee
209 River in Florida and that only 4.1% of that DON in summer wet season samples was
210 biodegradable during a 5-day bioassay. The low value of biodegradable DON in our samples and
211 those of Osborne et al. (2013) may be attributed to either an inherent refractory DON pool or
212 preferential use of DIN by biota (Bronk et al. 2007).

213 Our results and those of Osborne et al. (2013) are in contrast to Hendrickson et al. (2007),
214 who determined that 38 to 92% of summer wet season DON in an urbanized blackwater stream
215 was biodegradable. In that study, the authors attributed the large proportion of labile DON to
216 inputs from urban stormwater runoff suggesting that urbanization can shift the DON character in
217 a blackwater stream from being mostly refractory under pristine conditions to more labile under
218 developed conditions. Urban stormwater runoff has been shown to have a higher proportion of
219 biodegradable DON ($59 \pm 11\%$) compared to runoff from agricultural pastures ($30 \pm 14\%$) and
220 forests ($23 \pm 11\%$) (Seitzinger et al. 2002), and so the argument that urban stormwater runoff
221 may affect the labile DON pool of a receiving water body is plausible. However, our study
222 showed no increase in biodegradability associated with increased urbanization, though we
223 recognize the limitations of limited samples in our study, in one moment in time, so we were not
224 able to capture the temporal variability. We should also point out that like several other studies
225 of river DON (e.g., Osborne et al. 2013, Seitzinger and Sanders 1997, Wiegner et al. 2009), we
226 used a bacteria-only bioassay and did not investigate DON consumption by algae. There are
227 known algal species that can directly uptake DON molecules such as amino acids, and bacterial
228 degradation of DON may also facilitate subsequent uptake of other DON molecules (Bronk et al.

2007, Pehlivanoglu and Sedlak 2004). Hence, it is possible that we were not able to observe the full range of reactive DON because we did not include an algal growth bioassay in our study.

Furthermore, our study watershed has >120,000 septic systems, and groundwater is shallow; so it is possible that some of the DON in streamwaters may have originated from septic systems. For example, De and Toor (2015) reported that most of the organic N applied with septic tank effluent leached to shallow groundwater, pointing to the fact that this DON pool may be refractory. Another possible explanation for the low DON bioavailability in our samples could be due to the presence of 0.31 to 0.50 mg/L DIN ($\text{NO}_3 + \text{NH}_4$) in the highly urbanized and less urbanized streams, respectively. If bacteria selectively used available DIN, then DON consumption may be limited (Mulholland et al., 2000; Bernhardt and Likens, 2002; Kaushal and Lewis, 2005). Significant consumption of NH_4 in particular by riverine bacteria was observed by Wiegner et al. (2009), who pointed out that NH_4 can be preferentially taken up because its consumption requires less energy than that of NO_3 and DON.

Since other researchers have noted larger pools of reactive DON in human-dominated waters (Hendrickson et al. 2007, Seitzinger et al. 2002), our results for sub-basins of the Alafia watershed demonstrate that there may be large spatial and temporal variability in DON dynamics among urban watersheds located in different climatic zones or with different types of vegetative cover. Indeed, many factors other than land use may contribute to the generation and transport of DON to a given urban stream. For example, Kaushal and Lewis (2005) observed that the quality and amount of dissolved organic C (DOC) in bulk organic matter affects DON dynamics in streams. They observed that net DON consumption is low when C:N ratio is high, the C is concentrated in highly labile fractions of the bulk organic matter, and DIN is present, and they added that seasonal patterns and landscape vegetation are two factors known to indirectly affect

DON consumption by influencing the DOC character of the bulk organic matter. The extent of interaction with organic matter-rich watershed soils may also affect stream DON dynamics. The DON pool has been reported to be more labile if extensive watershed impervious cover prevents shallow groundwater inputs to streams from interacting with soil (Hendrickson et al., 2007). Several authors have made the argument that for urban watersheds, the urban form that includes type and extent of tree canopy coverage, the connectivity of impervious surfaces, and the extent of open spaces, is more predictive of water quality than the percent of a watershed under urban land use (Goonetilleke et al. 2005, Liu et al. 2012, Mitchell 2006).

3.3. Mass spectra of urban streamwaters

The mass spectra of the stream water samples included peaks ranging from about 150 to 700 m/z (mass:charge ratio) (Figure 2A-B). The spectra contained 8,482 and 6,222 ions for which formulas could be assigned for the highly urbanized and less urbanized streams, respectively. The smaller insets in the figure (representing a 0.4 Da unit spectral window isolated from the two streams) illustrate in a small window just how complex these streamwater samples are and why there is a need for ultrahigh resolution. It is important to note that these peaks represent the bulk DOM, and not just N-bearing compounds, which we place in context with the bulk DOM later in this discussion.

Since our interest is in the DON component of the bulk DOM, we focused our analysis of the FT-ICR-MS data on N-bearing formulas. Table 4 divides all identifiable bulk DOM peaks into compound classes and shows that the highly urbanized and less urbanized streams had 1,866 and 1,590 N-bearing formulas (CHON), respectively. A comparison of the two streamwaters showed a 63% overlap in identified N-bearing molecular formulas and there were 533 CHON formulas unique to the highly urbanized streamwater and 237 CHON formulas unique to the less

urbanized streamwater. In other words, each streamwater contained a subset of unique DON formulas suggesting that although DON concentrations were not significantly different, there were compositional differences in the DON pools in streamwaters.

3.4. Molecular composition and sources of DON in urban streams

We used van Krevelen diagrams, as described by Kim et al. (2003), to make comparisons of the N-bearing compounds in terms of H/C vs. O/C composition. We also compared the m/z and the mean double bond equivalency (DBE: number of rings plus double bonds expected for a given molecular formula) between the two streamwaters (Figures 3A and 3B). A compound's aromaticity is expected to increase with increase in the DBE (Klein et al. 2006). The mean m/z and DBE were higher in streamwaters from highly urbanized (353, 19) than less urbanized sub-basins (336, 18), respectively. This suggests that the DON in the less urbanized stream is characterized by slightly smaller and less aromatic compounds (means more biodegradable) than the highly urbanized stream (Sleighter and Hatcher 2008). This corresponded to the findings of our bioassay study, which showed a slightly higher DON biodegradability in the less urbanized than the highly urbanized stream. This may be due to the influence of agricultural land use (especially the presence of a cow/calf operation) in the less urbanized sub-basin that likely contributed manure with DON pools containing aliphatic amines and proteins (Pehlivanoglu-Mantas and Sedlak 2006).

We overlaid the diagrams in Figures 3A-B with sections to point out where important classes of biomolecules are known to fall on a van Krevelen diagram (Kim et al. 2003). The highly urbanized and less urbanized streams contained the large majority of the N bearing compounds in lignin-like molecules, accounting for 81% and 78% of the DON compounds, respectively (Figures 3A-B; Table 5). The lignin-like materials are likely derived from allochthonous

298 terrestrial plant sources and represent the bulk of the semi-labile and refractory DON pool
299 (Meyer 1990, Moran et al. 1999, Sleighter et al. 2014). The low DON bioavailability in both
300 streamwaters, as indicated by our bioassay results, is likely a result of dominance of lignin-like
301 compounds. Lipid-like compounds were 8 to 13% and protein-like compounds were 4 to 5%, and
302 both were higher in the less urbanized than highly urbanized streamwaters. These two compound
303 types, along with the amino sugars, are aliphatic and likely to be autochthonous and microbially
304 derived (Bhatia et al. 2010, Ohno et al. 2014). Lipids, proteins, and carbohydrates (amino sugars)
305 typically represent the bulk of the labile pool (Hendrickson et al. 2007). These labile compounds
306 were more abundant in the less urbanized than the highly urbanized stream, similar to bioassay
307 results, pointing to the slightly higher DON lability in the less urbanized stream.

308 Each point on the van Krevelen diagrams represents a unique chemical formula present in the
309 samples (Figure 3A-B). Because there are more than 1,500 points on each diagram, it is easier to
310 visualize where each sample is unique by plotting a new van Krevelen diagram that highlights
311 the *unique* formulas in each sample. A visual inspection of Figure 4 shows that many of the
312 formulas unique to the highly urbanized stream have lower H/C (1.0 to 1.5) and higher O/C (0.2
313 to 0.4) values as compared to less urbanized stream, where unique formulas cluster in a wider
314 swath of H/C (0.5 to 1.5) and O/C (0.1 to 0.8). In the highly urbanized stream, only 7.6% of the
315 unique formulas fall within the lipid-, protein-, and amino sugars-like regions, whereas 82% of
316 the formulas occupy the lignin-like region of van Krevelen space (Figure 4; Table 6). The less
317 urbanized stream had 18.4% unique formulas within the lipid-, protein-, and amino sugars-like
318 regions and 74% within the lignin-like region. In both streamwaters, the lipid-, protein-, and
319 amino sugars -like formulas are likely microbially derived, and may represent unique
320 autochthonous DON sources, although they may also be derived from fresh plant material. In

contrast, the lignin-like formulas are generally believed to originate from allochthonous DON sources such as humified soil organic matter or well-degraded plant materials in the watersheds (Meyer 1990, Ohno et al. 2014, Sleighter et al. 2014).

Of particular interest is the wide swath of lignin-like formulas with H/C values <1.5 in the highly urbanized stream. The sampling location for this site was at a point where the stream runs through an urban apartment complex and where stormwater runoff from the surrounding impervious surfaces is channeled directly into the stream. A visual observation at the site pointed to high tree canopy coverage of live oak, which sheds leaves in the spring every year. The leaves accumulate and slowly break down on impervious surfaces for 3-4 months and then become part of the summer stormwater runoff into the stream. It is possible that this is the source of unique formulas observed in the urban stream in this study. A good next step to test this possibility would be to study the degradation and molecular character of DON from the spring and summer leaf material that enters the urban stream. From this study, we can only say with certainty that there is some allochthonous source of low H/C (<1.5) and well-oxygenated organic matter in the highly urbanized stream that we do not find in the less urbanized stream. This conclusion is supported by an inspection of the relative abundance of N-bearing compound classes in each sample (Supplementary Figure S3), which shows how the highly urbanized streamwater had higher abundances of the higher oxygenated (O7-O11) compounds, whereas the less urbanized streamwater had higher abundances of the lower oxygenated (O1-O6) compounds.

3.5. *Linking DON biodegradability with molecular composition in urban streams*

To gain insights on the bioassay results, we obtained FT-ICR-MS spectra for the streamwater samples from bioassay experiment. Figures 5A-B show van Krevelen diagrams for reactive (those that were present only in the T_0 samples) and formed (those that were present only in the

T₅ samples) formulas in both streamwaters. It is clear that formulas in a wide range of H/C and O/C values remained after the bioassay, and that most formulas found at the beginning of the bioassay were still present after 5 days. The removed formulas are predominantly on the left side of the van Krevelen space, with O/C usually <0.4, whereas the formed formulas cluster more towards the right of the diagrams, with O/C values >0.4 (Figure 5A and 5B). These results are similar to Osborne et al. (2013), who conducted a 5-day bioassay of streamwater from Caloosahatchee River in Florida and observed that removed formulas had O/C values from 0.1 to 0.3 and H/C values greater than 1.5. The formed formulas in their study clustered in the center of van Krevelen space, with a full range of O/C values from 0.2 to 0.8. The clear pattern of reactive formulas is good evidence that DON biodegradability is indeed linked to chemical composition and that shifts in stream environmental factors could shift DON bioavailability. For example, anoxic conditions might prevent the reactions that shift compounds toward the right of the van Krevelen diagram, since this pathway takes place due to the addition of oxygen to the reactive formulas.

The position of the reactive (removed) formulas on the van Krevelen diagrams shows that the less oxygenated, more saturated compounds were more biodegradable in the streamwaters. Their formed counterparts were more oxygenated and less saturated but still contain N. This indicates that the microbes changed the carbon skeleton of the reactive DON fraction without concomitant consumption of N. In other words, the microbes converted the reactive compounds into the newly formed compounds through either addition of O or loss of H. Our results are in line with Lechtenfeld et al. (2014) and Koch et al. (2014), who both noted that bulk DOM compounds can be altered biologically and/or chemically in ways that lead to higher oxygenation and lower saturation without concomitant mineralization of the DOM. These changes to the carbon

skeleton of DON compounds are responsible for moving the original DON pool toward greater recalcitrance (Lechtenfeld et al. 2014).

4. Conclusions

We used bioassay and FT-ICR-MS to determine the biodegradability and characterize the molecular composition of DON in urban streamwaters. van Krevelen diagrams of the bulk DON showed the majority (78-81%) of DON compounds were lignin-like, and therefore likely refractory or only semi-labile in urban streams. This observation is supported by a concomitant 5- day bioassay study, which showed that DON in streamwaters had limited biodegradability. The highly urbanized stream in particular showed a unique set of lignin-like DON formulas not found in the less urbanized stream, and we suggest that one possible source of this is allochthonous organic matter from humified soil materials or highly degraded plant materials in the sub-basin. Though these streams are both highly influenced by human activity, they have only small reactive DON pools, at least during the summer. The bioassay results showed that during the 5-day of bacterial incubation only 5 to 7% of the DON was removed from streamwaters. By linking FT-ICR-MS data to the bioassay results, we showed that most reactive formulas were lipid-and protein-like structures with high H/C and low O/C values. Conversely, very few of the lignin-like compounds were removed during the 5-day bioassay, confirming that DON in the lignin-like pool is largely refractory. Comparisons of reactive formulas and formed counterparts indicate that microbes changed the reactive formulas into more oxygenated and less saturated N-bearing formulas that are also refractory. Thus, while there was very a little net removal of organic-bound N over the 5-day bioassay, there was some change to the carbon skeleton of DON compounds—with a shift towards higher oxygenation and less hydrogenation. This suggests that DON pools in urban watersheds may be compositionally dynamic even if

concentrations differences are not observed and that microbes alter the DON composition in urban streams.

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Table 1 – Land use characteristics of sub-basins of the Alafia River watershed.

	Map ID	% Urban	% Forest	% Agriculture	% Wetlands
Buckhorn Creek	BHC	83	2	3	9
Turkey Creek	TKC	28	3	28	12
Poley Creek	PC	68	9	9	10
Bell Creek	BLC	60	9	7	19
Fishhawk Creek	FHC	29	22	26	14
Little Fishhawk Creek	LFHC	56	12	16	9
Fishhawk Creek, Upstream	FHC-UP	30	40	18	5
Mainstem 1	MS1	21	5	24	13
Mainstem 2	MS2	42	7	27	14
Mainstem 3	MS3	19	15	15	13

Table 2 – Mean N concentrations ($\pm$ SD) and DON:TDN in the water samples collected from various sub-basins of the Alafia River watershed during the 2013 wet season (n=21). Concentrations are in mg/L.

	NH ₄ -N	NO ₃ -N	DON	TN	DON:TDN
Buckhorn Creek	0.14 $\pm$ 0.08	0.17 $\pm$ 0.08	0.48 $\pm$ 0.15	1.30 $\pm$ 0.39	0.59 $\pm$ 0.16
Turkey Creek	0.34 $\pm$ 0.28	0.16 $\pm$ 0.30	0.82 $\pm$ 0.43	1.90 $\pm$ 0.50	0.62 $\pm$ 0.27
Poley Creek	0.13 $\pm$ 0.13	0.21 $\pm$ 0.21	0.55 $\pm$ 0.25	1.23 $\pm$ 0.51	0.62 $\pm$ 0.24
Bell Creek	0.15 $\pm$ 0.05	0.08 $\pm$ 0.09	0.63 $\pm$ 0.28	1.29 $\pm$ 0.80	0.74 $\pm$ 0.28
Fishhawk Creek	0.11 $\pm$ 0.08	0.19 $\pm$ 0.06	0.69 $\pm$ 0.33	1.52 $\pm$ 0.51	0.67 $\pm$ 0.21
Little Fishhawk Creek	0.10 $\pm$ 0.05	0.15 $\pm$ 0.08	0.59 $\pm$ 0.26	1.27 $\pm$ 0.42	0.68 $\pm$ 0.19
Fishhawk Creek, upstream	0.21 $\pm$ 0.31	0.27 $\pm$ 0.08	0.87 $\pm$ 0.37	1.73 $\pm$ 0.57	0.64 $\pm$ 0.17
Mainstem 1	0.19 $\pm$ 0.15	0.48 $\pm$ 0.41	0.35 $\pm$ 0.35	1.44 $\pm$ 0.62	0.46 $\pm$ 0.27
Mainstem 2	0.13 $\pm$ 0.37	0.74 $\pm$ 0.14	0.37 $\pm$ 0.34	1.69 $\pm$ 0.29	0.31 $\pm$ 0.21
Mainstem 3	0.23 $\pm$ 0.12	0.41 $\pm$ 0.37	0.53 $\pm$ 0.35	1.28 $\pm$ 0.35	0.57 $\pm$ 0.29

Table 3. Mean nitrogen concentrations () in the initial (T₀) and 5 day bioassay (T₅) samples collected in June 2014 from the highly urbanized stream (Buckhorn Creek) and less urbanized stream (Turkey Creek). Concentrations are in mg/L.

	Highly Urbanized Stream (Buckhorn Creek)			Less Urbanized Stream (Turkey Creek)		
	TDN	DIN	DON	TDN	DIN	DON
T ₀	2.20 $\pm$ 0.40	0.48 $\pm$ 0.09	1.72 $\pm$ 0.27	1.65 $\pm$ 0.22	0.56 $\pm$ 0.04	1.09 $\pm$ 0.33
T ₅	1.92 $\pm$ 0.38	0.36 $\pm$ 0.09	1.63 $\pm$ 0.14	1.34 $\pm$ 0.14	0.33 $\pm$ 0.11	1.01 $\pm$ 0.46
% Reactive DON			5.1 $\pm$ 0.16			7.1 $\pm$ 0.49

Table 4 – Number of peaks and spectral abundances in the highly urbanized stream (Buckhorn Creek) and less urbanized stream (Turkey Creek).

Compound Class	Highly Urbanized Stream (Buckhorn Creek)		Less Urbanized Stream (Turkey Creek)	
	# Peaks	Relative Spectral Abundance	# Peaks	Relative Spectral Abundance
CHO	5663	67.72	4058	63.70
CHOS	690	2.46	224	0.80
CHON	1866 (533 unique)	9.65	1590 (257 unique)	11.64
CHS	14	0.04	31	0.13
HC	249	1.54	319	3.24
Total	8482		6222	

Table 5 – Percent of assigned peaks in each biomolecular class in the highly urbanized stream (Buckhorn Creek) and less urbanized stream (Turkey Creek).

	Highly Urbanized Stream (Buckhorn Creek)	Less Urbanized Stream (Turkey Creek)
Lipids	8	13
Proteins	4	5
Lignins	81	78
Amino Sugars	0.3	0.3
Unsaturated Hydrocarbons	0.3	0.6
Condensed Aromatics	1	1
Tannins	4	2

Table 6 – Percent of assigned peaks found in unique formulas for each biomolecular class in the highly urbanized stream (Buckhorn Creek) and less urbanized stream (Turkey Creek).

	Highly Urbanized Stream (Buckhorn Creek)	Less Urbanized Stream (Turkey Creek)
Lipids	5	13
Proteins	2	5
Lignins	82	74
Amino Sugars	0.6	0.4
Unsaturated Hydrocarbons	0.6	4
Condensed Aromatics	8	3
Tannins	2	0.4

Fig. 1 – Various sampling sites on the Alafia River. Abbreviations for the sample sites are explained in Table 1. Map data source: Southwest Florida Water Management District. MS1-Mainstem 1; MS2-Mainstem 2; MS3-Mainstem 3; BHC-Buckhorn Creek; BLC-Bell Creek; FHC-Fishhawk Creek; LFHC-Little Fishhawk Creek; FHC-UP-Fishahawk Creek Upstream; TKC-Turkey Creek; PC-Poley Creek; SP-South Prong.

Fig. 2 – Mass spectra of the streamwater samples from (A) highly urbanized stream (Buckhorn Creek) and (B) less urbanized stream (Turkey Creek).

Fig. 3 – van Krevelen diagrams of the streamwater samples from (A) highly urbanized stream (Buckhorn Creek) and (B) less urbanized stream (Turkey Creek).

Fig. 4 – van Krevelen diagrams of formulas unique to highly urbanized stream (Buckhorn Creek) and less urbanized stream (Turkey Creek).

Fig. 5 – van Krevelen diagrams for reactive and formed formulas in (A) highly urbanized stream (Buckhorn Creek) and (B) less urbanized stream (Turkey Creek).

Graphical Abstract