

Identifying the Molecular Signatures of Organic Matter Leached from Land-Applied Biosolids via 21 T FT-ICR Mass Spectrometry

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ABSTRACT: Intensification of wastewater treatment residual (i.e., biosolid) applications to watersheds can alter the amount and composition of organic matter (OM) mobilized into waterways. To identify novel tracers of biosolids, characterization of biosolids and their impacts on OM composition in recipient ecosystems is required. Here, water-soluble OM was leached from surface soils from Florida pastures with differing levels of biosolid amendment and an adjacent control site. The biosolid endmember was further constrained by extracting water-soluble OM from biosolids sourced from four Florida wastewater treatment facilities. Nontargeted analysis of organic molecules by negative-ion electrospray ionization 21 T Fourier transform ion cyclotron resonance mass spectrometry examined the molecular composition



of soil and biosolid leachates and identified molecular formulas unique to these biosolids and biosolid amended soils. Overall, biosolids leachates were enriched in aliphatic (+16.3% relative abundance) and heteroatomic (+42.5% RA) formulas and depleted in aromatic formulas (-33.5% RA) compared to soil leachates. A subset of 297 molecular formulas were present only in biosolids and amended soil leachates (i.e., not present in control soil leachates), the vast majority of which contained nitrogen (66%) or sulfur (27%). The identification of these molecular formulas is a key step in identifying novel tracers of biosolids movement through impacted watersheds.

KEYWORDS: biosolids, 21 T FT-ICR mass spectrometry, dissolved organic matter, negative electrospray FT-ICR MS, DOM, ultrahigh resolution mass spectrometry, water-soluble organic matter

INTRODUCTION

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Biosolids are the solid, nutrient-rich byproduct of wastewater treatment processing. Management and disposal of human waste byproducts has led to the intensifying application of biosolids to landscapes in some areas in lieu of commercially available fertilizers, despite a relatively constant biosolids production of ~6 Mmt by water resource recovery facilities in the US in 2004 and 2018.¹ Over the past 15 years, restrictions on biosolids application to some south Florida watersheds have resulted in a rapid increase in the biosolids organic matter (OM) and phosphorus load to adjacent Florida watersheds, roughly tripling land application of biosolids in the St. Johns River watershed between 2010 and 2019.² Although generally less labile than inorganic nutrients, biosolids-derived organic nutrients exhibit varying lability depending on the treatment process and source material.³ Concurrent increases in surface water nutrient concentrations and frequency and duration of harmful algal blooms in Florida waterways^{4,5} has heightened concerns regarding how biosolid land application may impact downstream aquatic environments. While previous studies have examined how the wastewater treatment process impacts

the composition of biosolids-associated OM,^{6–8} the impact of biosolids application to landscapes is not well understood.

Examining the composition of various biosolids and how this large addition of anthropogenic OM to natural systems changes the OM composition in recipient ecosystems is an important first step in understanding how this material may alter biogeochemical cycling in these and downstream systems. Furthermore, understanding the impacts of biosolids application on downstream environments requires identifying nonambiguous tracers of biosolids inputs as it moves through the environment. An inherent property of dissolved organic matter (DOM) is the extremely high structural and compositional heterogeneity.⁹ Current estimates from ultrahigh resolution mass spectrometry analysis conservatively suggest that well

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beyond 600,000 unique structures may reasonably comprise a DOM mass spectrum.¹⁰ Nontarget analysis combined with statistical approaches has recently been highlighted as a useful tool in identifying contaminants of concern for environmental health generally,¹¹ as well as in rapid response scenarios.¹² While linking nontarget analyses with known hazard databases allow for the simultaneous detection of multiple biosolids-associated contaminants,¹³ biosolids-specific indicators have yet to be described.

Ultrahigh resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) has emerged as a powerful tool in identifying new biomarkers and contaminants of concern in the environment.^{14,15} Utilizing FT-ICR MS as a nontarget approach allows for the simultaneous characterization and assessment of more than 10,000 unique molecular formulas as potential tracers of landscape activities that do not currently have specific associated tracers, as well as providing a first step in identifying contaminants of emerging concern. FT-ICR MS has recently been used successfully to distinguish DOM composition in and identify tracers of various land-use, particularly forest and agriculture, in tropical and temperate regions as well as urban landscapes in the US Midwest.¹⁷⁻¹⁹ For example, unique CHOS₂ molecular formulas have been found in vineyard soil porewaters, which received agricultural sulfur additions as compared to nearby woodlands and grasslands.¹⁸ Several studies have also investigated the effects of wastewater treatment processes on the composition of biosolids OM.^{7,8,20–23} CHON and CHONS have been shown to be elevated in sewage sludge, with the relative abundance (RA) of CHON₄ and CHOS₂ decreasing and CHON₂ and CHOS₁ increasing with processing.²¹

The aim of this project is to characterize DOM composition from biosolid leachates and how addition of biosolids to pasture impacts the composition of water-soluble OM. Further, we aim to provide a first step in identifying geochemical tracers in the molecular-level DOM composition that reflect biosolid addition to pastures in the St. Johns River watershed (Florida, USA). Here, we analyzed the molecular-level composition of the water-soluble DOM from soils in a pasture with varying levels of biosolids application: pasture control (no biosolids application), pasture one application, pasture many applications, and a native control, which is a site with native vegetation and no previous biosolids application by negative electrospray ionization 21 T FT-ICR mass spectrometry. To constrain the biosolid endmember, material from four wastewater treatment facilities was also analyzed. As past studies have highlighted both aliphatics and heteroatomic elements are enriched in agricultural runoff^{17,19} and wastewater treatment effluent,^{24,25} we hypothesize that biosolids and sites with repeated biosolids application will exhibit enrichment in aliphatic and peptide-like containing molecular formulas as well as formulas containing the heteroatoms N and S. Additionally, we examined the molecular formulas common to biosolids and biosolids amended soils to identify potential tracers that can be investigated further in future studies of biosolids transport through landscapes.

METHODS

Study Sites and Sample Collection. Soil samples were collected from pasture field sites in the watershed of the St. Johns River, the longest river in Florida, flowing 500 km northward from Blue Cypress Lake through Jacksonville to the Atlantic Ocean. The dominant crop across the sites is

bahiagrass (*Paspalum notatum*), grown for cattle grazing. Samples were collected from two control sites: native, i.e., forested, and pasture sites without biosolids application. Other pasture sites were identified which had been amended once with biosolids within a month of sampling and those that had been amended with six applications of biosolids between 2011 and 2021. These site types are hereafter referred to as control (native), control (pasture), one application (pasture), and many applications (pasture). For each site type, surface soils were collected in triplicate as composite samples from 0 to 10 cm depth.

Water-Soluble OM Extraction from Soils and Biosolids. Soil samples were leached to extract water-soluble OM. Water extraction was used to represent what may naturally be leached from soils into waterways. Soil was passed through a 2 mm sieve and air-dried before water extraction. Air-dried soil (4 g) was mixed with 40 mL ultrapure H₂O, shaken end-to-end on a shaker for 24 h, centrifuged at 2500g for 10 min, and then filtered through precombusted (450 °C for 4 h) GF/F filters into acid washed high density polyethylene bottles. Watersoluble OM was extracted in triplicate using the same procedure as that for the soils from Class B biosolids produced at four domestic wastewater treatment facilities in Florida. The material came from a mixture of anaerobic and aerobic digestion processes. Concentrations of water-extractable organic carbon were analyzed on a Shimadzu TOC-LCSN. Nonpurgeable organic carbon concentrations were assessed after sparging at 80 mL min⁻¹ for 2.5 min using four-point calibration curves of potassium hydrogen phthalate.

Sample Preparation for 21 T FT-ICR Mass Spectrometry. The water-soluble OM was extracted with solid phase extraction (SPE) to produce concentrated, desalted extracts with a consistent dissolved organic carbon (DOC) concentration to analyze on a custom-built 21 T Fourier transform ion cyclotron resonance mass spectrometer at the National High Magnetic Field Laboratory in Tallahassee, FL. Extractions were prepared using a modified version of an established protocol.²⁶ Briefly, approximately 60 μ g of C was concentrated into 1 mL of MeOH by loading a concentration-corrected volume of sample onto a PPL cartridge that was conditioned through a series of MeOH and acidified ultrapure water (pH 2) rinses. After the sample was loaded, cartridges were rinsed with pH 2 ultrapure water, dried with N2 gas, and eluted with 1 mL of HPLC grade MeOH. Eluates were stored in precombusted $(550 \text{ }^\circ\text{C} > 5 \text{ h})$ amber glass vials at $-20 \text{ }^\circ\text{C}$ until analysis at the NHMFL.

Ultrahigh Resolution Mass Spectrometry. DOM composition for each sample (each replicate was analyzed individually) was analyzed through mass spectra produced using a custom-built 21 T hybrid linear ion trap FT-ICR MS.^{27,28} Known standards were run in triplicate with >98% of the same species observed from the same sample prep. Negatively charged ions from the SPE extracts were produced via electrospray ionization at a flow rate of 500 nL/min through a 50 μ m fused silica emitter. Conditions typically used to generate negative ions were -2.8 to -3.2 kV emitter voltage, 40% S-lens radio frequency level, and 350 °C heated metal capillary. Ions were first accumulated in an external multipole. Automatic gain control was used with an ion target of two million charges per scan. One-hundred time-domain transients of 3.1 s were acquired and coadded for all experiments with the Predator data station handling excitation and detection.²⁹ Mass spectra were phase-corrected with

Table 1. Molecular Composition of Soil and Biosolids Leachates by Site Type

	control (native) $(n = 3)$	control (pasture) $(n = 3)$	one application (pasture) $(n = 3)$	many applications (pasture) $(n = 3)$	biosolids $(n = 12)$
DOC yield $(mg C g^{-1})$	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	16.5 ± 9.2
formulas (#)	14,023 ± 1994	12,435 ± 878	$16,428 \pm 502$	$15,640 \pm 548$	11,238 ± 2602
mass (Da)	575 ± 18	580 ± 13	552 ± 13	544 ± 5	439 ± 20
AImod	0.44 ± 0.02	0.46 ± 0.01	0.44 ± 0.04	0.45 ± 0.02	0.18 ± 0.03
H/C	0.92 ± 0.04	0.87 ± 0.01	0.91 ± 0.05	0.88 ± 0.02	1.37 ± 0.06
O/C	0.51 ± 0.01	0.54 ± 0.01	0.53 ± 0.01	0.54 ± 0.01	0.44 ± 0.04
N/C	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.03 ± 0.00	0.07 ± 0.01
NOSC	0.129 ± 0.062	0.244 ± 0.008	0.201 ± 0.040	0.275 ± 0.002	-0.237 ± 0.098
СНО	84.3 ± 4.2	79.3 ± 4.0	70.1 ± 7.3	60.3 ± 1.8	31.0 ± 5.8
CHON	13.8 ± 3.5	18.5 ± 2.9	25.4 ± 7.1	35.1 ± 1.8	46.0 ± 4.3
CHOS	1.9 ± 0.8	2.1 ± 1.1	3.5 ± 0.4	3.2 ± 0.9	17.1 ± 4.3
CHONS	0.00 ± 0.00	0.01 ± 0.01	1.0 ± 0.5	1.4 ± 0.2	5.9 ± 1.4
peptide-like	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	18.2 ± 4.8
aliphatics	2.8 ± 1.6	0.5 ± 0.2	1.1 ± 0.4	1.4 ± 0.2	17.8 ± 3.7
HUP high O/C	32.4 ± 3.8	38.8 ± 5.1	39.8 ± 10.6	40.3 ± 4.4	24.2 ± 6.7
HUP low O/C	27.6 ± 3.6	20.7 ± 1.7	24.1 ± 0.6	18.6 ± 0.5	35.0 ± 5.5
polyphenolics	23.4 ± 2.4	26.9 ± 1.1	24.4 ± 5.5	27.6 ± 1.5	4.0 ± 2.7
condensed aromatics	13.7 ± 2.6	13.1 ± 2.2	10.6 ± 5.2	12.1 ± 2.3	0.5 ± 0.5

Table 2. Analysis of Variance (ANOVA) Among Soil Site Types and Biosolids Leachates for Variables Shown in Figure 1^a

		n	DOC yie	ld (mg C g	⁻¹) formu	ıla (#)	mass	AImod	H/C	O/C	N/C	NOSC	СНО
One-way ANOVA				11	5		79	157	133	14	77	71	103
significance level				**	*	*	***	***	***	***	***	***	***
control (native)		3		В		AB A		А	В	А	А	В	Α
control (pasture)		3	В		В		Α		В	А	А	AB	Α
one application (pasture)	e application (pasture) 3 B		А	L	AB	А	В	А	AB	AB	AB		
many applications (pastur	e)	3		В	А	L	В	А	В	Α	В	А	В
biosolids leachates		12		А	В	С	С	В	А	В	С	С	С
				Signific	ance Level fo	or Pairwise	Compa	arisons					
with biosolids				***	*		***	***	***	*	***	***	***
between soil types				n.s.	*		*	n.s.	n.s	n.s	**	*	**
							Н	UP, high	HUP, lo	w	poly-	conde	ensed
	п	CHON	CHOS	CHONS	peptide-like	aliphatics		O/C	O/C	1	phenolics	arom	atics
One-way ANOVA		53	26	55	32	53		7	45		84	43	3
significance level		***	***	***	***	***		***	***		***	**	**
control (native)	3	Α	Α	А	В	AB		AB	ABC		А	Α	
control (pasture)	3	Α	Α	А	В	А		А	BC		А	Α	
one application (pasture)	3	AB	Α	AB	В	AB		А	В		Α	A	В
many applications (pasture)	3	В	А	В	В	В		А	С		А	А	
biosolids leachates	12	С	В	С	А	С		В	Α		В	В	
				Signific	ance Level fo	or Pairwise	Compa	arisons					
with biosolids		**	***	***	***	***		*	***		***	*	
between soil types		**	n.s	**	n.s.	**		n.s.	***		n.s.	n.	s.

"Abbreviations: DOC: dissolved organic carbon; Almod, modified aromaticity index; NOSC: nominal oxidation state of carbon; HUP: highly unsaturated p < 05, **p < 0.01, ***p < 0.001.

absorption mode processing³⁰ to increase resolving power by ~30% and internally calibrated with 10–15 highly abundant homologous series to cover the entire molecular weight distribution based on the "walking" calibration method.³¹ All FT-ICR MS transients and elemental composition files are publicly available via the Open Science Framework at https://osf.io/74d9m/.

Elemental Composition Assignment. Singly charged ions (170-1200 Da) were assigned molecular formulas within the bounds of $C_{1-100}H_{4-200}O_{1-30}N_{0-4}S_{0-2}$ and ± 0.3 ppm error using PetroOrg software.³² Modified aromaticity index (AI_{mod}) and nominal oxidation state of carbon (NOSC) were

calculated for each formula.^{33–35} Molecular formulas were classified by heteroatomic content, specifically formulas that only contain C, H, and O (CHO) and formulas with N \geq 1 (CHON), S \geq 1 (CHOS), and N and S each \geq 1 (CHONS). Formulas were also categorized based on their elemental ratios and AI_{mod}. Formulas were classified into six groups as previously described: condensed aromatics (CA; AI_{mod} \geq 0.67), polyphenolics (PP; 0.67 > AI_{mod} > 0.50), highly unsaturated and phenolic formulas (HUP; AI_{mod} \leq 0.50, H/C < 1.5), aliphatics (H/C \geq 1.5, O/C \leq 0.9, N = 0), peptide-like formulas (H/C \geq 1.5, O/C \leq 0.9, N > 0), and sugar-like formulas (H/C \geq 1.5, O/C > 0.9).³⁶ The RA of sugar-like

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Figure 1. Molecular characteristics of soil leachates from study sites and biosolids leachates. Boxplots of (a) CHO, (b) CHON, (c) CHOS, (d) CHONS, (e) N/C, (f) aliphatics, (g) AI_{mod} , and (h) polyphenolics weighted by RA. Thick horizontal lines indicate the median value with individual data points shown by site type. Sites shown are surface soil (0–10 cm) replicates from each of the four land-use types: control (native), control (pasture), one application (pasture), and many applications (pasture); and biosolids leachate.



Figure 2. Molecular composition of the four soil leachates and biosolids leachates. The molecular formulas common to each leachate type are shown in van Krevelen space. Sites shown are surface soil (0-10 cm) from each of the four land-use types: (a) control (native), (b) control (pasture), (c) one application (pasture), and (d) many applications (pasture); and (e) biosolids leachate. Note: panel (e) is the combination of row 1 in Figure 4.

formulas was <1% in all samples and was thus not considered in further analysis. The percent RA of the HUP formulas was subdivided at O/C = 0.5 into high and low O/C groups (HUPs, high O/C; HUPs, low O/C, respectively). The number of structures each molecular formula may be comprised of is currently unknown and may be extremely vast.³⁷ Yet basic structural features, such as the presence of an aromatic structure, can be surmised from combinations of elemental ratios and AI_{mod} .³⁴

Statistical Analysis. Statistical analyses were performed in R.³⁸ Analysis of variance (ANOVA) was used to assess differences among the four site types and biosolids leachates. First, Bartlett's test for homogeneity of variance was applied to determine whether variance could be considered equal. Where the assumption of homogeneous variance failed, an ANOVA not assuming equal variance was applied. Pairwise comparisons were carried out in a similar fashion depending on the homogeneity of variance. A principal component analysis using R package "vegan"³⁹ was utilized to determine the compositional variability among samples and to reduce the high number of metrics deduced from FT-ICR MS analysis into major gradients in the data set. Overlap in formulas that were present in all replicates among groups is visualized using the package "ComplexUpset".^{40,41} This was done to characterize the molecular formula intersections and to identify molecular formulas that are common to both the biosolids and the sites where biosolids have been applied in order to identify potential tracers of biosolids application in the landscape.

RESULTS AND DISCUSSION

DOC Yields from Biosolids and Soil Leachates. DOC yields in the soil leachates covered a much smaller range (0.2–0.4 mg C g⁻¹ soil) and were significantly lower than in the biosolids leachates (5.5–29.6 mg C g⁻¹ biosolids; Tables 1 and 2). The DOC yield from soil sites is consistent with the global distribution of DOC yield from soil leachates, with our DOC yields similar to the average DOC yield (~0.2 mg C g⁻¹ soil) observed across soil leachate studies distributed globally.⁴² The average DOC yield observed for the biosolids leachates (mean \pm s.d.: 16.5 \pm 9.2 mg C g⁻¹ biosolids), despite a considerable range depending on the water resource recovery facility, is similar to the cutoff established elsewhere for mature composts.^{43,44}

Composition of DOM in Biosolids and Soil Leachates. DOM compositional differences were readily apparent among the soil leachates. The number of molecular formulas ranged from 6700 to 16,800 and was lowest in the biosolids leachates and highest in the amended soils (p < 0.05), with control (native) not significantly different from either biosolids or amended soil leachates and control (pasture) not significantly different from the biosolids leachates (Tables 1 and 2). Typically, the number of molecular formulas increases with contributions from heteroatom containing formulas such as N and S, potentially reflecting increasing diversity of inputs.^{17,19} However, biosolid leachates exhibited the fewest number of molecular formulas assigned, yet were significantly enriched in CHON, CHOS, and CHONS compared to all control and amended soil leachates (Figure 1a-d, Tables 1 and 2).

Molecular formulas only containing CHO were enriched in control (native) and control (pasture) compared to many applications (pasture) (Figure 1a, Table 2). Similarly, both control (native) and control (pasture) sites were significantly depleted in N-containing compounds compared to many applications (pasture), as reflected in the N/C, CHON, and CHONS (Figure 1; Tables 1 and 2). This is also apparent when looking at the molecular formulas present in all replicates of each soil and leachate type (Figure 2). CHONS molecular formulas in particular were not present in either of the control soils. Although the differences in CHOS between the four control and amended soil leachates were not significantly different, amended soils were slightly enriched in CHOS (Tables 1 and 2), suggesting that specific CHOS formulas may be potentially useful tracers of biosolids amendment. The heteroatomic composition of the DOM observed in the control (native and pasture) leachates is within the range of that previously reported for forested watersheds, and the one application site is within ranges reported for mixed agricultural usage; however, the many applications site was elevated in CHON by ~5% compared to previously reported values for mixed agricultural watersheds.¹⁹ Overall, the elevated number of molecular formulas in the amended soil leachates as well as an enrichment of heteroatoms in the amended soils compared to the control (native) and control (pasture) reflect the mixing of two sources of OM: OM from the pasture ecosystem itself and the addition of distinct biosolids material that was applied to the amended pastures.

Biosolid leachates were characterized by an enrichment of organic markers of a high degree of biological inputs. The weighted average of the mass was significantly lower in the biosolids leachates (mean \pm s.d.: 439 \pm 20 Da) than in the soil leachates (563 \pm 19 Da; Tables 1 and 2). The lower mass observed in the biosolids leachates was mirrored by other metrics that reflect high microbial productivity and has been observed to be elevated in effluent from a wastewater treatment plant, such as high H/C, and N/C, and aliphatic, peptide-like and $HUP_{low O/C}$ formulas.^{25,45} For example, H/C was elevated in the biosolids leachates (1.37 ± 0.06) compared to the soil leachates (0.89 \pm 0.04), with the average H/C of the biosolids leachates near the values observed in wastewater effluent elsewhere and the molecular lability boundary, a cutoff for highly bioavailable DOM.^{25,46} Similarly, N/C was elevated in the biosolids leachates (0.07 ± 0.01) compared to the many applications (pasture) (0.03 ± 0.00) with the control (native and pasture) exhibiting the lowest N/C (0.01 ± 0.00 , for both; Figure 1e; Tables 1 and 2). Peptide-like and aliphatic molecular formulas were again elevated in the biosolids leachates ranging from 11.3 to 22.9% RA and 14.0-26.6% RA (mean \pm s.d.: 18.2 \pm 4.8 and 17.8 \pm 4.8% RA, respectively). This was significantly higher than any of the soil leachates, with peptide-like formulas ranging from 0.0 to 0.3% RA and aliphatic formulas ranging from 0.3 to 4.6% RA across both control and amended soils (Figure 1, Tables 1 and 2). Many applications (pasture) were enriched in aliphatics compared to the control (pasture) (Figure 1); however, there was no significant difference between many applications (pasture) and the control (native), due to the higher variability observed in control (native) (Figure 1d; Table 2). Decaying litter in Florida has been shown to both be enriched in aliphatics as well as highly bioavailable to microbial degradation.⁴⁷ Thus, the elevated RA of aliphatics in the control (native) may reflect inputs of the decaying litter layer to the surface soils.

The degree of oxygenation can also be used to determine the source and reactivity of DOM.^{35,48} For example, O/C in soil leachates exhibited an overall higher range and average (0.49–0.55) than that in the biosolid leachates (0.39–0.49; Tables 1 and 2). Exhibiting a similar trend, nominal oxidation state of carbon, an estimation of the average oxidation state of a given molecular formula, showed the soil leachates contained more oxidized DOM (0.071–0.278) than in the more reduced biosolids leachate DOM (–0.386 to –0.103; Tables 1 and 2). Similarly, all pasture soil leachates were enriched in HUP_{high O/C} (ranging from 32.6 to 51.9% RA) compared to biosolids leachates (13.2–33.9% RA; Tables 1 and 2). Control (native) HUP_{high O/C} was not significantly different from the



Figure 3. Intersections among leachate types. The (a) H/C and (b) O/C distribution and (c) number of molecular formula and heteroatomic makeup for molecular formulas within each (d) intersection among leachate types. Intersections show commonality between or among groups or formulas that are unique to a particular group. Blue rectangles indicate the intersections of molecular formulas highlighted in Figure 4.

other soil or biosolid leachates (Table 2). Alternatively, $HUP_{low O/C}$ along with parameters associated with a high degree of saturation and heteroatomic content has been shown to be elevated in systems associated with nutrient inputs and where time for microbial production of OM is length-ened.^{19,45,49–51} Here, $HUP_{low O/C}$ is significantly elevated in the biosolids leachates (29.5–43.8% RA) when compared to pasture soil leachates (18.0–24.8% RA; Tables 1 and 2), and control (native) was not significantly different from either (23.5–30.2% RA). The values of $HUP_{high O/C}$ are slightly lower and $HUP_{low O/C}$ are elevated in the soil leachates compared to previously observed for riverine DOM in agricultural watersheds; however, the general pattern of anthropogenically influenced sites being depleted in $HUP_{high O/C}$ and elevated in $HUP_{low O/C}$ compared to sites dominated by soil OM is consistent with previous findings.¹⁹

Modified aromaticity index (AI_{mod}), an indicator of aromatic content as well as the relative contribution of vascular plant derived DOM to a system,^{34,45} was particularly low in the biosolids leachates ranging from 0.14 to 0.21 (mean \pm s.d.: 0.18 \pm 0.03; Figure 1, Table 1), compared to the four control and amended soil sites (0.39–0.47; Figure 1, Tables 1 and 2) as well as aquatic sites influenced by urban and agricultural inputs or high microbial production.^{19,45,49} Polyphenolics and condensed aromatics, often elevated in organic soil horizons, were similarly significantly lower in the biosolids leachates (0.9-8.0% RA and 0.1-1.3% RA, respectively) as compared to the soil leachates (18.1-28.7% RA and 4.6-15.5% RA, respectively; Figure 1g,h, Tables 1 and 2). Despite the higher degree of anthropogenic inputs to the amended soil and biosolids leachates, the values observed here overlapped with those observed in natural aquatic systems.^{19,45}

The molecular compositions of biosolids from the four water resource recovery facilities appeared broadly similar (Supporting Information Figure 1) and were predominantly represented by low aromaticity and a high content of heteroatomic molecular formulas (Supporting Information Table 2). Both ozone and UV oxidation in wastewater treatment have been shown to decrease absorbance and therefore aromaticity.^{8,52} Organic sulfur and nitrogen on the other hand are resistant to biodegradation and coagulation in particular and often remain elevated after treatment.^{7,22,23} Despite the clear compositional separation between biosolid leachate and soil leachate DOM (Figures 1 and 2), a higher degree of variability was observed among the biosolid source types than within each soil type (Figure 1, Table 1, and Supporting Information Figure 1). For example, two of the biosolids leachates were ~14% more enriched in S-containing molecular formulas than the other

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Figure 4. Molecular indicators of biosolids amended soils. Molecular formulas common to all biosolids leachates are shown in the top row (intersection A in Figure 3). Molecular formulas common to biosolids and soils amended with biosolids once and many times are shown in tan (intersection C in Figure 3). Additional molecular formulas common to biosolids and only soils amended with biosolids many times are shown in brown (intersection B in Figure 3).

two (Supporting Information Table 1 and Figure 1). This may reflect the variability in both source materials to wastewater treatment facilities as well as differences in the treatment processes, which has previously been shown to impact residual DOM composition.⁵³ This variability is also unsurprising as studies have shown differing impacts of wastewater treatment on biosolids composition.^{7,8,20,22}

Intersections Among Soil and Biosolids Leachates. To identify DOM tracers of biosolids input to landscapes for potential use in future studies of biosolids storage in and transport through landscapes, molecular intersections were identified among the leachate types (Figure 3). Intersections required the presence of a molecular formula in all replicates of each leachate type and combination of types (Figure 3). Any intersection involving biosolids required the presence of a molecular formula in all replicates of all four biosolids leachates. First, formulas common to all four biosolid leachates (n = 2884) without regard to the presence in other sample types were predominantly CHON (48%), followed by CHO (32%), CHOS (15%), and CHONS (5%; Figure 4, top row). Then, molecular formulas unique to each leachate type were identified as well as intersections, or shared commonality, between and among groups. The largest subset of molecular formulas was not common to any leachate type (n = 10,363), spanned the entire H/C and O/C ranges (Figure 3a,b), and was comprised of molecular formulas from each class (CHO, CHON, CHONS, and CHOS; Figure 3c). The intersection of all leachate types was comprised of 833 predominantly CHO and CHON molecular formulas and showed a smooth distribution of H/C and O/C with median values H/C ~ 1 and $O/C \sim 0.5$, whereas the intersection of the soil leachates was much larger (n = 7386) with the distribution of H/C skewed slightly lower and O/C skewed slightly higher (Figure 3). Molecular formulas common to biosolids leachates only (n= 1452, box labeled A in Figure 3d) were enriched in CHON, CHOS, and CHONS compared to CHO, and skewed to high H/C and low O/C (Figure 3), which is consistent with the high heteroatomic content and overall composition of the biosolids leachate DOM.

To identify unique molecular tracers of biosolid input to the landscape, assessing the intersections between biosolid leachates and amended soils was of particular interest. The intersection between biosolids leachate, one application (pasture), and many applications (pasture) was comprised of 126 molecular formulas (labeled C in Figure 3d). However, biosolids application can be quite patchy, thus requiring the presence of a tracer after one application was determined to be overly stringent. Consequently, the intersection of the commonality between biosolid leachate and many applications (pasture) was determined to be the most conservative way to identify molecular tracers of biosolids input. The intersection between biosolids leachate and many applications (pasture) comprised an additional 171 molecular formulas (labeled B in Figure 3d). Overall, intersection B was skewed toward higher H/C and lower O/C than intersection C (Figure 3) and was less enriched in CHON (59 vs 75%) and more enriched in CHOS (31 vs 21%); however, the overall composition of the two intersections is similar (Figure 4, bottom row). Furthermore, both intersection B and C were enriched in molecular formulas containing more than 1 N compared to the data set prevalence of 10% and 11% for CHON₃ and CHON₂, respectively. For example, 17% of the molecular formulas in intersection B were CHON3 and 16% were CHON2, whereas only 6% of intersection C was CHON₃ but 42% were CHON₂. Anaerobic digestion of sewage sludge has been shown to reduce the occurrence of CHON3 and CHON4 while increasing CHON2,²¹ suggesting these residual molecular formulas have resisted degradation and are thus somewhat persistent, at least to the extent that this study design can address.

Unique Molecular Signatures of Biosolid DOM. Aliphatics were highly enriched in the biosolids leachates. We hypothesized that aliphatics would also be enriched in the sites amended with biosolids, and among the soil sites, there was a small, yet significant increase between the pasture control and many applications (Tables 1 and 2, Figure 1). However, the native control exhibited a higher yet more variable aliphatic content (Table 1, Figure 1). Thus, while biosolid amendment likely increases the RA of aliphatics in water extractable DOM from these soils, the native vegetation appears to influence aliphatic content as well as the biosolids. Aliphatics have been shown to be enriched in leachates of fresh litter; however, this material is highly bioavailable with up to 90% loss in 1 month.^{54,55} Furthermore, the molecular formulas unique to biosolid leachates exhibited an elevated H/C (Figures 3a and 4), whereas most of the molecular formulas identified as indicators of biosolids input (i.e., the intersections between biosolids and amended soils) were between an H/C of 1.0-1.5 (Figures 3a and 4). These molecular formulas have both survived the wastewater treatment process and persisted in the environment until sampling, suggesting they are stable in the environment at least on time scales of months. The lack of an aliphatic signature in the sites with biosolids application is likely due to the high bioavailability of aliphatics⁵⁶ and highlights how the composition of the intersections between biosolids and amended soil leachates must be used to identify unique tracers of biosolid movement through the watershed.

Recently, combining complex mixture analysis with statistical approaches including artificial intelligence has been proposed to protect drinking water quality from the everexpanding sources of environmental contaminants,⁵⁷ and nontargeted screening approaches have recently been shown to offer promising avenues for assessing ecosystem health, particularly when paired with machine learning and artificial intelligence algorithms.^{11,12} Linking nontarget analyses with known contaminant databases can simultaneously identify multiple hazards in biosolid-associated OM.¹³ Here, the intersections B and C (Figures 3 and 4), totaling 297 molecular formulas (Supporting Information), are unique to the biosolid leachates and amended soil leachates of this study. This molecular fingerprint shows promise as a potentially useful tracer of biosolid movement through amended watersheds, where the presence of this suite of molecular formula is indicative of biosolid input. Furthermore, these molecular formulas provide fertile ground for future studies searching for structure-specific tracers of biosolid inputs. Heteroatomic formulas emerged as an indicator of biosolid amendment in this initial study of surface soils with differing levels of biosolid application (Figure 4). This is consistent with our hypotheses that heteroatomic formulas would be enriched in DOM extracted from soils that have been amended with biosolids. Ncontaining formulas have been found enriched in rivers draining watersheds with mixed agricultural use (e.g., crops and grazing) in both tropical and temperate biomes.^{17,19} Additionally, DOM enriched in N and S have both been linked to wastewater effluent inputs across a wide range of conditions and environments,^{24,25,58} septic-impacted groundwater,⁵⁹ and anthropogenic inputs in world rivers.⁶⁰ While future studies should include testing more biosolids materials for these tracer formulas to identify potential formulas for structural analysis, biosolid application clearly impacts the composition of DOM that can be leached from surface soils, with likely impacts for downstream ecosystems. Taken together, these results highlight the potential of nontargeted ultrahigh resolution mass spectrometry for providing unambiguous tracers of anthropogenic impacts like applications of biosolids onto the landscape and its mobilization into aquatic ecosystems.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.3c06678.

Tables of number of molecular formulas by heteroatomic class for each group and van Krevelens of the four biosolids (PDF)

Molecular formulae, theoretical m/z, and biosolid indicator categories (XLSX)

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Notes

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