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Molecular-level transformation of dissolved organic matter during oxidation by ozone and hydroxyl radical

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Abstract

Ozonation of drinking and wastewater relies on ozone (O_3) and hydroxyl radical ($\cdot OH$) as oxidants. Both oxidants react with dissolved organic matter (DOM) and alter its composition, but the selectivity of the two oxidants and mechanisms of reactivity with DOM moieties are largely unknown. The reactions of O_3 and $\cdot OH$ with two DOM isolates were studied by varying specific ozone doses ($0.1 - 1.3 \text{ mg-}O_3/\text{mg-C}$) at pH 7. Additionally, conditions that favor O_3 (i.e., addition of an $\cdot OH$ scavenger) or $\cdot OH$ (i.e., pH 11) were investigated. Ozonation decreases aromaticity, apparent molecular weight, and electron donating capacity (EDC) of DOM, with large changes observed when O_3 is the main oxidant (e.g., EDC decreases 63 – 77% for $1.3 \text{ mg-}O_3/\text{mg-C}$). Both O_3 and $\cdot OH$ react with highly aromatic, reduced formulas detected using high-resolution mass spectrometry ($O:C = 0.48 \pm 0.12$; $H:C = 1.06 \pm 0.23$), while $\cdot OH$ also oxidizes more saturated formulas ($H:C = 1.64 \pm 0.26$). Established reactions between model compounds and O_3 (e.g., addition of 1-2 oxygen atoms) or $\cdot OH$ (e.g., addition of one oxygen atom and decarboxylation) are observed and produce highly oxidized DOM ($O:C > 1.0$). This study provides molecular-level evidence for the selectivity of O_3 as an oxidant within DOM.

Introduction

Ozone (O_3) is applied during drinking and wastewater treatment for disinfection and organic contaminant oxidation.¹⁻⁴ O_3 is highly selective and reacts mainly with olefins, activated aromatic systems, and neutral amines with second-order rate constants that range 10 orders of magnitude.⁵ Additionally, hydroxyl radical ($\cdot OH$) is produced through reactions of O_3 with inorganic and organic compounds (e.g., phenols), often leading to radical chain decomposition reactions.^{6, 7} $\cdot OH$ is a less selective oxidant and reacts with most water constituents with nearly diffusion-controlled second order rate constants.⁵

The fate of dissolved organic matter (DOM) during ozonation has implications for the efficacy of ozonation and for water quality. DOM scavenges O_3 and $\cdot OH$, reducing the lifetime of both oxidants and limiting the extent of disinfection and target compound oxidation.⁸ DOM reactivity depends on both its concentration and composition.⁹⁻¹¹ Ozonation of DOM results in limited mineralization of dissolved organic carbon (DOC),¹²⁻¹⁸ but can dramatically alter DOM composition. For example, ozonation changes DOM photoreactivity¹⁹⁻²¹ and produces highly bioavailable assimilable organic carbon (AOC).²²⁻²⁵ Low molecular weight organic by-products associated with AOC include carboxylic acids, aldehydes, and ketones (**Supporting Information Figure S1**).²⁴⁻³⁰ However, identified ozonation-induced transformation products account for a fraction of the oxidized DOM^{23, 29} and do not include polar, non-volatile compounds that are not amenable to gas chromatography.²

DOM analysis using bulk measurements provides insight into how ozonation alters DOM composition. Spectroscopic measurements of DOM after reaction with ozone consistently show decreases in fluorescence^{12, 13, 31} and specific UV absorbance.^{12, 13, 16-18, 21} Decreasing molecular weight is reported by size exclusion chromatography (SEC) coupled with organic carbon

detection^{13, 32} and by sequential ultrafiltration,¹⁴ while XAD fractionation shows a shift from hydrophobic to polar hydrophilic material.^{12-14, 29, 33} Finally, electron donating capacity (EDC) of DOM decreases after oxidation, with large changes observed at low specific ozone doses.^{31, 34, 35} EDC is an operationally-defined measurement of phenolic moieties in DOM that show antioxidant properties.³⁶⁻³⁸ Phenols are highly reactive with ozone,^{25, 28, 39} suggesting that these moieties are important targets for O₃ oxidation.

High-resolution mass spectrometry provides molecular-level insight into DOM transformation. This semi-quantitative technique has been used to assess DOM reactivity during other drinking and wastewater treatment processes,⁴⁰⁻⁴⁴ but has only been applied to ozonated DOM in a few cases. The most mechanistic study analyzes a high [DOC] sample by QTOF-MS, revealing shifts from reduced formulas to highly oxidized formulas and providing evidence of decarboxylation.¹⁵ Compared to biological activated carbon treatment and chlorination, ozonation results in large changes in DOM composition as observed using Orbitrap and FT-ICR MS, such as formation of saturated, oxidized formulas¹⁶⁻¹⁸ and polar carbonyl species.⁴⁵ Thus, high-resolution mass spectrometry shows promise for investigating changes in DOM and specific reaction mechanisms, but it has not been applied to study the impact of ozone dose on DOM or to distinguish between reactions of O₃ and [•]OH.

The aim of this study is to test the hypotheses that phenolic moieties within DOM are susceptible to oxidation by O₃ and that [•]OH also reacts less selectively with other formulas. We compare the impact of the oxidants on two DOM isolates by using an [•]OH-scavenger to isolate the direct impact of O₃ and by conducting experiments at elevated pH that favor [•]OH. Since bulk-level studies show that large changes are observed at low ozone doses, we vary the specific ozone dose to investigate the dose needed to result in molecular-level changes in DOM. Mass spectrometry

analysis is combined with UV-vis spectroscopy and EDC measurements, which are shown to be highly complementary. This study provides molecular-level insight into the reactions of O_3 and $\cdot OH$ with DOM.

Materials and Methods

Materials. Suwannee River natural organic matter (SRNOM; 2R101N) and Upper Mississippi River natural organic matter (UMRNOM; 1R110N) isolates were obtained from the International Humic Substances Society (Denver, CO). We used DOM isolates to avoid solid phase extraction (SPE), which is used to remove inorganic ions that lead to ion suppression. SPE recoveries of DOM are low (30 – 60%),¹⁶ with poor retention of low molecular weight compounds that we hypothesize are generated during ozonation. SRNOM and UMRNOM represent DOM that is more terrestrial and more microbially-derived, respectively.⁴⁶ Details on other materials are available in **Section S1**.

Ozone Exposure Experiments. DOM stock solutions (40 mg-C/L DOM, 3 mM $NaHCO_3$) were sparged with 2% CO_2 in air for ~30 minutes to achieve a pH of 7.0, which was verified using a Metrohm 632 pH meter. The solutions were adjusted with appropriate volumes of an ozone stock solution, ultrapure water, and *tertiary*-butanol (*t*-BuOH), as discussed below. This approach maintained a pH of 7.00 ± 0.14 over the 20-hour experiment. Bicarbonate was selected because other inorganic buffers (e.g., phosphate) cause ion suppression during electrospray ionization, whereas organic buffers react much more rapidly with $\cdot OH$ and interfere with DOC analysis. DOM reaction with carbonate radical anion is negligible compared to $\cdot OH$ under the experimental conditions (**Section S2**).

DOM solutions (20 mg-C/L) were ozonated in triplicate in 16-mL glass vials with no headspace in the absence and presence of *t*-BuOH (50 mM) as an $\cdot OH$ scavenger. The ozone stock

solution was added under vigorous mixing (15 sec) to yield specific ozone doses of 0, 0.1, 0.2, 0.5, 0.75, and 1.3 mg-O₃/mg-C. The vials were sealed and stored in the dark at room temperature for 20 hours, resulting in complete ozone consumption. Mass spectrometry analyses were conducted within 8 hours.

Additional experiments with 0 and 0.5 mg-O₃/mg-C were conducted using 20 mg-C/L SRNOM in unbuffered ultrapure water adjusted to pH 11 using NaOH. This pH results in rapid decomposition of ozone ($t_{1/2} \sim 9$ seconds), yielding $\cdot\text{OH}$.^{5, 47} The final pH after the 20-hour experiment was 10.5 ± 0.2 .

Bulk measurements. DOC concentrations were measured in samples without *t*-BuOH using a Shimadzu TOC-L CSH total organic carbon analyzer. Absorbance of all samples was measured using a Cary 100 UV-vis spectrophotometer. SUVA₂₅₄ is calculated by dividing the absorbance at 254 nm by [DOC]⁴⁸ and E₂:E₃ is the ratio of absorbance at 250 nm to absorbance at 365 nm.⁴⁹ EDC was measured photometrically as described previously⁵⁰ (**Section S4**). Previous studies demonstrate that *t*-BuOH does not interfere with UV-vis or EDC measurements.^{19, 34} Bulk measurements were performed in triplicate experimental replicates.

Orbitrap Mass Spectrometry. DOM samples were diluted 40:60 with acetonitrile and injected into a QExactive Plus mass spectrometer (Thermo Fisher Scientific) using negative mode electrospray ionization. Data processing was performed in *R* as described previously and considered C₁₋₈₀¹³C₀₋₁H₁₋₁₄₀O₀₋₈₀N₀₋₁S₀₋₁ formulas (<0.5 ppm error).⁵¹⁻⁵⁵ Bray-Curtis dissimilarity analysis considered the presence and absence of formulas.⁵³⁻⁵⁵ Principal components analysis (PCA) relied on the relative intensity of all identified formulas. Weighted averages of elemental ratios (H:C_w and O:C_w), double bond equivalents (DBE_w), and molecular weight (MW_w) were calculated using the relative intensity of each formula within a sample.^{43, 55} Relative intensities of

common formulas were related to ozone doses using Spearman rank correlations.⁵³ Details on instrumental settings and calculations are provided in **Section S5**.

Results and Discussion

Bulk changes in DOM composition. Ozonation experiments were conducted in the presence of an $\bullet\text{OH}$ scavenger to investigate the expected preferential reactions of O_3 with phenolic moieties within DOM. While these reactions occur at individual DOM moieties, changes in bulk DOM properties such as UV absorption or EDC provide insight into the relative reactivity of O_3 and $\bullet\text{OH}$ and are complementary to high-resolution mass spectrometry. Furthermore, analyses of bulk properties enable comparison with existing literature. The agreement in bulk analyses across studies suggests that the molecular-level changes discussed below may be widely applicable.

Upon ozonation, similar changes in UV-vis absorption properties are observed for Suwannee River NOM and Upper Mississippi River NOM. SRNOM is lower in $\text{E}_2:\text{E}_3$ than UMRNOM (i.e., 4.69 vs. 5.22, respectively; **Table S3**) and higher in SUVA_{254} (i.e., 3.34 vs. 2.92 $\text{L mg-C}^{-1} \text{ m}^{-1}$, respectively). Because $\text{E}_2:\text{E}_3$ is inversely correlated with molecular weight^{49, 54} and SUVA_{254} correlates with aromaticity,⁴⁸ this indicates that SRNOM has higher molecular weight and aromaticity. The higher measured EDC of SRNOM compared to UMRNOM (i.e., 3.29 vs. 2.47 $\text{mmol}_\text{e}/\text{g-C}$; **Table S3**) indicates that SRNOM contains more phenolic groups, in agreement with titrated phenol content⁴⁶ and flow-injection EDC measurements³⁷ of these isolates.

DOM can react with both O_3 and $\bullet\text{OH}$ during ozonation at circumneutral pH in the absence of *t*-BuOH.¹ Under these conditions, [DOC] decreases by up to 13% for SRNOM and 9% for UMRNOM (**Figure S3**; **Table S3**). Limited mineralization of DOM by ozone¹³⁻¹⁸ or by $\bullet\text{OH}$ in advanced oxidation processes (AOPs)^{56, 57} is well demonstrated.

However, changes in UV-vis spectra reveal that the DOM composition is altered. Relative and absolute A_{254} decreases and $E_2:E_3$ increases with increasing specific ozone dose at pH 7 (**Figures 1** and **S4**, respectively), with similar relative changes observed for both NOM isolates. The increase in $E_2:E_3$ suggests preferential oxidation of conjugated molecules that absorb at high wavelengths, which may be correlated with a decrease in molecular weight. Similar decreases in molecular weight due to ozonation are observed using direct measurements such as SEC^{1, 13, 15} and sequential ultrafiltration,¹⁴ as well as using UV-vis spectroscopy-derived parameters.^{21, 33, 34, 58} Large decreases in $SUVA_{254}$ are observed with increasing specific O_3 doses (**Figures 1c** and **S4c**), which agrees with previous observations^{12, 13, 16-18, 34} and correlates with decreasing aromaticity.

EDC decreases significantly at 0.1 and 0.2 mg- O_3 /mg-C, with smaller decreases at higher specific ozone doses (**Figures 1d** and **S4d**), suggesting partial oxidation of phenolic moieties. Large decreases in EDC at low specific ozone doses are observed using SEC followed by spectrophotometric detection or flow injection analysis with electrochemical detection.^{1, 31, 34, 35} This is attributed to electrophilic attack on activated aromatic compounds (e.g., phenols) by ozone.

The addition of *t*-BuOH as an $\cdot OH$ scavenger leaves O_3 as the dominant oxidant and results in larger changes of many bulk DOM properties. The presence of *t*-BuOH has no effect on A_{254} . However, the increases in $E_2:E_3$ and decreases in EDC are much larger when DOM is exposed to only O_3 compared to both O_3 and $\cdot OH$ (**Figures 1** and **S4**). These results agree with relative differences in UV-vis spectra and EDC measurements observed in past studies that used *t*-BuOH as a scavenger.^{21, 33, 34} Part of the enhanced oxidation may be attributable to higher O_3 exposure because $\cdot OH$ -induced chain reactions promoting O_3 decay are inhibited when *t*-BuOH is added, resulting in longer O_3 lifetimes.^{5, 34} However, the results are consistent with a shift from less selective oxidation by $\cdot OH$ to more selective oxidation by ozone. For example, O_3 preferentially

reacts with DOM that absorbs at high wavelengths, whereas $\cdot\text{OH}$ reactions result in more uniform absorbance changes (**Figure S5**), as observed previously.³³ Collectively, these results suggest preferential reactivity of O_3 with DOM that is more colored and phenolic in nature. Changes in [DOC] and SUVA_{254} cannot be compared because [DOC] measurements were not possible in samples with added *t*-BuOH.

Interestingly, experiments conducted at pH 11 reveal minimal change in bulk DOM properties. Increasing the pH to 11 decreases the O_3 lifetime and enhances the rate of $\cdot\text{OH}$ formation.⁵ While normalized values of [DOC], A_{254} , and SUVA_{254} all decrease slightly, these changes are small compared to the changes observed at pH 7 (**Figures 1 and S4**). Furthermore, the increase in $E_2:E_3$ is only 10% and the decrease in EDC is within experimental error compared to the initial value. Although these results are limited to a single specific ozone dose, they are opposite the trends observed when *t*-BuOH is added and indicate that $\cdot\text{OH}$ does not decrease EDC even if it reacts with EDC-relevant moieties. For example, the reaction of $\cdot\text{OH}$ with aromatic and phenolic moieties results in hydroxylated products that also exert a high EDC.³⁹

Changes in molecular composition of DOM. Orbitrap mass spectrometry is used to assess the molecular composition of SRNOM and UMRNOM before and after ozonation. An average of 1386 $\text{C}_{1-80}\text{H}_{1-140}\text{O}_{0-80}\text{N}_{0-1}\text{S}_{0-1}$ formulas are identified in each sample (**Table S4**). A higher percentage of formulas in UMRNOM contain nitrogen ($18.8 \pm 1.4\%$) and sulfur ($8.0 \pm 1.5\%$) compared to SRNOM ($12.8 \pm 1.7\%$ and $4.2 \pm 2.2\%$, respectively) in agreement with relative bulk measurements of N and S in these isolates (**Table S1**).⁴⁶ The formulas identified in UMRNOM are slightly more saturated than those in SRNOM when comparing both H:C_w (1.27 versus 1.21) and DBE_w (6.48 versus 7.11) in agreement with SUVA_{254} measurements (**Table S3**). The number of formulas decreases with increasing ozone dose for experiments conducted in the absence *t*-BuOH

due to loss of CHO-containing formulas. Formulas are visualized in van Krevelen diagrams, which plot the ratio of H:C versus O:C for each identified formula (**Figures S6 – S8**).

Bray-Curtis dissimilarity analysis and principal components analysis are used to assess overall differences in the DOM isolates. The control samples with and without *t*-BuOH cluster together during both analyses, demonstrating that the $\cdot\text{OH}$ scavenger does not impact MS analysis (**Figure S9 – S11**). Furthermore, PCA performed using all analyzed samples shows that UMRNOM and SRNOM are distinct from each other. After exposure to ozone, the samples with *t*-BuOH cluster distinctly from samples without the scavenger. Furthermore, the samples cluster further away from each other with increasing specific ozone dose. For both isolates, the largest difference is observed between 0.2 and 0.5 mg- O_3 /mg-C (**Figure S11**), which agrees with changes in bulk measurements (**Figure 1**). These analyses reveal that there are molecular-level differences in DOM after reaction with O_3 and/or $\cdot\text{OH}$. However, they do not provide information about the nature of these differences.

Weighted averages of elemental ratios provide insight into how DOM changes when exposed to O_3 and $\cdot\text{OH}$. The largest changes are observed in relative or absolute O:C_w , which increase with increasing specific ozone doses at pH 7 (**Figures 1e and S13b**, respectively). The increase in relative O:C_w is greater in the absence of *t*-BuOH, suggesting that the combination of O_3 and $\cdot\text{OH}$ results in more oxidation. The increase in O:C_w in SRNOM at pH 11 is similar to pH 7. However, the overall oxidant exposure (i.e., $[\text{O}_3]$ and $[\cdot\text{OH}]_{ss}$) differs across the three different experimental conditions. Previous studies also report increasing O:C_w after ozonation, with large changes in SRFA with 2.5 mg- O_3 /mg-C ($\text{O:C}_w/\text{O:C}_{w,0} = 3.5$)¹⁵ and modest changes in wastewater and drinking water with ~ 1.2 mg- O_3 /mg-C ($\text{O:C}_w/\text{O:C}_{w,0} = 1.05 - 1.06$).^{17, 44}

Measurements of DOM aromaticity (i.e., $H:C_w$ and DBE_w) do not follow the same trends. A decrease in aromaticity is expected based on the large decreases in $SUVA_{254}$ (**Figures 1c** and **S4c**) and DBE_w drops by 16% on average at the highest specific ozone dose. Furthermore, similar decreases are observed with and without *t*-BuOH (**Figures 1f** and **S13c**). In contrast, $H:C_w$ is constant with increasing specific ozone dose (**Figures S12a** and **S13a**). These results are unexpected because $H:C_w$ and DBE_w usually follow the same trends and agree well with $SUVA_{254}$ in whole water and size-fractionated DOM.^{43, 53-55} However, two previous studies similarly report no change in $H:C_w$ but a decrease in DBE_w in water treated with a single ozone dose, suggesting that this divergence in MS-derived aromaticity measurements may be common in ozone-treated samples.^{17, 44} These results highlight the unique information gained from molecular-level analysis, rather than bulk measurements, as well as the use of multiple metrics to assess reactivity.

The average molecular weight observed using Orbitrap MS decreases after ozonation. MW_w decreases by an average of 36 m/z at the highest ozone dose for UMRNOM and SRNOM with and without *t*-BuOH (**Figures S12b** and **S13d**). While the MW_w decrease agrees with the increase in $E_2:E_3$, these results must be interpreted cautiously. High-resolution MS is selective because only molecules with ionizable functional groups are amenable to electrospray ionization, which means that MW_w may not be representative of the overall molecular weight of DOM. For example, high-resolution MS analysis of size-fractionated DOM shows no noticeable difference in MW_w .^{54, 59}

The changes in weighted averages derived from MS data agree with the well-established oxidation mechanism of phenols. Phenol reacts with O_3 to form primary products including catechol, hydroquinone, benzoquinone, and muconic acid.^{25, 28, 39} These products undergo further oxidation to smaller carboxylic acids, such as maleic, oxalic, and formic acids. The oxidation of phenol results in large increases in $O:C$ (e.g., from 0.17 for phenol to 2.0 for formic acid), but only

a modest increase in H:C (**Figure S2a**). Furthermore, the oxidation products have lower DBE and molecular weight compared to phenol (**Figure S2b**). The known high reactivity of O₃ with phenolic compounds, as well as the agreement between trends observed in DOM isolates after ozonation and predicted trends based on phenol oxidation (compare **Figures 1e, 1f, and S2**), support the hypothesis that phenolic moieties are critical reactive functional groups in DOM.

Formulas susceptible to reaction with ozone and hydroxyl radical. High-resolution mass spectrometry data demonstrates the reactivity of O₃ and •OH with DOM at the molecular level. The data is first analyzed by identifying formulas that are only present in the control sample (i.e., denoted as “abated”) and by identifying formulas that are present in the ozonated samples but not in the control (i.e., denoted as “formed”). The number of formulas formed is nearly identical with and without *t*-BuOH for both isolates (**Figures 2a and S14a**). However, large differences are observed in the number of abated formulas. For example, 425 formulas are abated in SRNOM when both O₃ and •OH are present, but only 201 formulas are abated when O₃ is the dominant reactant (1.3 mg-O₃/mg-C). At pH 11, 741 formulas are abated compared to the formation of 57 formulas. These results indicate that both •OH and O₃ contribute to the abatement of MS-detectable formulas, but that O₃ reacts with fewer formulas. This can be qualitatively explained by the lower selectivity of •OH compared to O₃.

The formulas abated and formed during reaction with O₃ and •OH with SRNOM (**Figures 2c and 2d**) or UMRNOM (**Figure S15**) have clear differences in their composition. The group of formulas abated in presence of *t*-BuOH is more tightly clustered at lower H:C_w values (e.g., average H:C_w of abated SRNOM formulas in all five ozone doses = 0.93 ± 0.02) compared to the formulas abated in absence of *t*-BuOH (1.03 ± 0.03 ; **Figure S14d**). Formulas abated in SRNOM at pH 11 (i.e., primarily •OH as an oxidant) occupy a wide range in H:C (**Figure S16**), similar to what is

observed at pH 7 without *t*-BuOH (**Figure 2c**). For both isolates, the formed formulas are highly oxidized compared to the abated formulas (**Figures 2b** and **S14b**) and occupy a similarly wide range of H:C regardless of the presence of *t*-BuOH. Collectively, the analysis of unique formulas abated and formed during exposure to ozone indicate that O₃ preferentially consumes more aromatic DOM compared to [•]OH and that the oxidation products have similar composition under all conditions.

Previous studies investigated abated and formed formulas in samples treated by ozone, as well as in [•]OH-based AOPs. Reaction with a combination of O₃ and [•]OH generally results in removal of unsaturated (i.e., aromatic) formulas to form formulas that are more saturated and more oxidized. Although past work is limited to a single ozone dose in each study, the trend is consistent in wastewater effluent¹⁷ and in drinking water samples.^{16, 18} These results are supported by ¹³C-NMR analysis, which reveals loss in aromaticity due to removal of phenolic content and an increase in aliphatic and carboxyl content.¹² Similarly, more highly saturated formulas are abated and a higher number of formulas are completely removed when [•]OH is the primary oxidant in a UV/H₂O₂ AOP compared to ozonation.¹⁶ Furthermore, a separate analysis of SRFA after reaction in an AOP provides additional evidence of reaction of [•]OH with aliphatic formulas.⁵⁷ Our results also suggest preferential abatement of aromatic formulas when O₃ is present; however, the differences in abated and formed formulas are much larger and the O:C range is much higher in this study. We attribute this result to the use of SPE in previous studies,^{16-18, 45, 57} which shows poor recovery of the highly oxidized transformation products observed here.

Preferential reactivity of O₃ compared to [•]OH is also observed in formulas that decrease in relative intensity during ozonation but are not completely abated. This analysis considers formulas that are present in the control sample and in the five specific ozone doses. The relative intensity of

each formula is compared to the specific ozone dose using a Spearman rank correlation (**Section S5**). Formulas that decrease in intensity with increasing ozone dose are denoted in cool colors (i.e., rho values of -0.6 to -1.0 indicate strong negative correlations),⁶⁰ suggesting that these formulas react during ozonation.

During exposure of DOM to O₃ and [•]OH, less oxidized formulas (i.e., low O:C) consistently decrease in intensity (**Figures 3a** and **S17a**), which we attribute to reaction with the oxidants. In contrast, formulas that increase in intensity (i.e., potential oxidation products) are highly oxidized (**Figures 3b** and **S17b**). The shift between consumed formulas and produced formulas at an O:C ratio of ~0.6 is also observed if only CHON formulas are considered (**Figures S17** and **S18**), which is expected because O₃ oxidizes N-containing compounds, such as amines, through partially similar mechanisms as phenols.⁵ The H:C value of formulas that undergo oxidation span a wide range when both O₃ and [•]OH are present (**Figure 3a**), with many formulas with H:C >1.5 decreasing in intensity. Similarly, the formulas that increase in relative intensity after ozonation vary widely in H:C but rarely reach >1.5.

A clear shift toward more oxidized formulas is also observed in UMRNOM and SRNOM when *t*-BuOH is present. In this case, however, a diagonal divide is observed between oxidized and produced formulas in both isolates (i.e., formulas with negative correlations vs. formulas with positive correlations with specific ozone dose; **Figures 3c, 3d**, and **S19**). The formulas that undergo oxidation by O₃ are much more tightly clustered compared to **Figure 3a**, with fewer high H:C formulas reacting. This difference in reactivity when O₃ is the primary oxidant is also observed when CHON formulas are considered separately (**Figures S18** and **S19**). In contrast, the shift in oxidized to produced formulas under conditions in which [•]OH is the primary oxidant is solely from

low O:C to high O:C; there is no preferential reactivity of low H:C formulas (i.e., no correlation with aromaticity; **Figure S20**).

The difference in reactivity between O₃ and •OH is further demonstrated by combining Spearman rank correlations determined in all pH 7 samples (**Figure 3e; Section S5**). Formulas that undergo oxidation (i.e., decrease in intensity with increasing specific ozone dose) in both isolates regardless of the presence of *t*-BuOH are denoted as O₃ and •OH reactive (**Figure 3e**). These formulas are reduced (i.e., average O:C = 0.48 ± 0.12) and aromatic in nature (i.e., H:C = 1.06 ± 0.23), corresponding to lignin- and tannin-like formulas.^{61, 62} Formulas that only decrease in intensity when *t*-BuOH is absent are only amenable to oxidation by •OH and are highly aliphatic (i.e., H:C = 1.64 ± 0.26). Formulas in the same region are also reactive at pH 11 (**Figure S20**). The same analysis for produced formulas does not provide such a clear distinction (**Figure S21**), although it is noteworthy that most of the produced formulas are associated with both O₃ and •OH.

Collectively, Spearman rank correlations demonstrate that O₃ preferentially oxidizes highly aromatic formulas, whereas more aliphatic formulas are susceptible to oxidation by •OH (**Figure 3e**). These results agree with the differences observed in formulas completely consumed during ozonation (**Figure 2**), as well as with the reactivity of these oxidants with individual compounds.⁵ This selectivity primarily applies to formulas within DOM undergoing oxidation, with similar products produced by both O₃ and •OH. The non-selectivity of product formation is expected because O₃ and •OH can produce similar products, such as carboxylic acids, ketones, and aldehydes.^{25, 57} To our knowledge, this is the first study to delineate between O₃-reactive and •OH-reactive formulas in DOM at a molecular level.

DOM reaction mechanisms. The mechanisms of O₃ and •OH oxidation are investigated by identifying product formulas that correspond to known oxidation pathways (**Table S5**). For

example, $\cdot\text{OH}$ reacts with organic compounds through pathways such as hydroxylation (e.g., aromatic rings, olefins) or decarboxylation (e.g., carboxylic acids), resulting in products with one added oxygen or with the loss of CO_2 .^{3, 5, 63} Analogously, O_3 reacts through the addition of one (e.g., phenols, olefins, tertiary amines) or two oxygen atoms (multiple reaction sites).^{1, 3, 5, 64} For each pathway, we constructed a mass list based on the formulas in each control sample (e.g., control sample formulas after the loss of CO_2) and searched for these predicted products in the reacted samples. In the case of oxygen addition, it is possible that a product could be attributable to both +1O and +2O pathways (e.g., $\text{C}_7\text{H}_8\text{O}_4$ could result from $\text{C}_7\text{H}_8\text{O}_3 + 1\text{O}$ and/or $\text{C}_7\text{H}_8\text{O}_2 + 2\text{O}$). If both parent formulas are present in the control sample, these formulas are denoted as +1O/+2O.

There is clear evidence of oxygen addition in DOM after ozonation. For example, UMRNOM in the absence of *t*-BuOH contains 31 – 39 formulas corresponding to +1O addition, 25 – 88 formulas corresponding to +2O addition, and 56 – 110 formulas that are attributable to either transformation pathway (**Table S6**). The number of oxygen addition formulas generally increases with specific ozone dose and most product formulas have O:C ratios >0.5 (**Figures 3f and S22**). Similar trends in the number of unique oxidized products and in the type of formulas produced are observed in presence of *t*-BuOH and in both sets of SRNOM samples. We hypothesized that a higher proportion of +2O transformation formulas would be present in samples with the $\cdot\text{OH}$ scavenger. However, most of the detected formulas are denoted +1O/+2O (i.e., $58.5 \pm 10.6\%$ of formulas with added oxygen averaged across all samples). Thus, it is not possible to distinguish between preferential oxidation by O_3 or $\cdot\text{OH}$ based on the comparison of oxygen addition products.

In contrast, there is a clear distinction in the number of formulas attributable to decarboxylation with and without *t*-BuOH. For example, 88 – 186 $-\text{CO}_2$ formulas are observed in

UMRNOM exposed to both O_3 and $\cdot OH$, whereas only 18 – 57 $-CO_2$ formulas are observed in UMRNOM in the presence of the $\cdot OH$ scavenger (**Figures 3f** and **S23; Table S6**). This agrees with the ability of $\cdot OH$ to react with DOM via decarboxylation.^{63, 65, 66} The number of decarboxylated formulas in SRNOM are similar with and without the scavenger, as well as at pH 11. While this result does not follow the trend observed in UMRNOM, the number of $-CO_2$ formulas in SRNOM samples is low (i.e., 15 – 31), suggesting that SRNOM is less amenable to reacting by this mechanism. Similar transformation products via the pathways studied here are detected in non-target analysis of ozone transformation products of micropollutants³ and a QTOF-MS study found evidence of decarboxylation in SRFA.¹⁵

Implications for Water Treatment. Ozonation significantly alters DOM composition with minimal mineralization. UV-vis spectroscopy and EDC measurements reveal decreases in aromaticity, apparent molecular weight, and phenolic content, with larger changes observed when *t*-BuOH is added and O_3 is dominant. These bulk-level results agree with studies using similar techniques^{12, 13, 16-18, 21, 31, 34, 35} and, importantly, agree with molecular-level results observed by Orbitrap MS. For example, formulas that are shown to be reactive with O_3 (**Figure 3**) occupy the same region of the van Krevelen diagram as formulas positively correlated with $SUVA_{254}$ and negatively correlated with $E_2:E_3$.^{43, 53, 55} The consistency in our data set with bulk-level results suggests that the molecular-level transformations observed here may be common in ozone-treated waters.

O_3 reacts selectively with activated aromatic systems (e.g., phenols).⁵ This study confirms that O_3 -reactive formulas within DOM are highly aromatic in nature, occupying the same space on the van Krevelen diagram as phenols and lignin-like compounds. Additionally, transformation pathways observed in model compounds, including in non-target analysis of micropollutants,³ are

observed, suggesting that well-established oxidation mechanisms also occur with individual DOM moieties.

DOM is an important scavenger of O_3 and $\cdot OH$, competing with target compounds during ozonation. The reactivity of DOM with these oxidants depends on its composition. For example, more aromatic, high-molecular weight DOM is more reactive with O_3 ,^{11, 14} while rate constants for the reactions between $\cdot OH$ and DOM are less variable.^{1, 11, 13} Although this study was not designed to quantify reaction kinetics, it provides a mechanistic understanding for correlations between O_3 rate constants and measurements of aromaticity.⁶⁷ Furthermore, it provides molecular-level evidence for why DOM with higher phenolic content and higher EDC is a potent O_3 scavenger.³¹

The composition of oxidized DOM has implications for subsequent water treatment processes and for waters that receive ozonated effluent. Ozone produces DOM that is highly bioavailable, yet the compounds associated with AOC (e.g., carboxylic acids and aldehydes) only comprise a fraction of the organic carbon.^{2, 23, 25, 68-70} The polar, higher molecular weight formulas detected by high-resolution MS may also contribute to AOC because aromatic DOM is less bioavailable and formulas with high O:C are selectively degraded.⁷¹⁻⁷³ DOM composition also has implications for abiotic processes, such as photodegradation. Many of the oxidized product formulas observed here occupy the same van Krevelen space as formulas associated with triplet DOM and singlet oxygen production,^{53, 55} confirming observations that ozonation increases quantum yields of these species in undiluted wastewater.^{20, 21} Finally, the molecular composition of DOM observed after ozonation is highly oxidized, with many formulas with O:C values that are outside the range encountered in natural and engineered waters (i.e., >1.0).^{17, 41, 42, 53, 55, 65} Future studies should determine if these high O:C formulas are unique to ozone-treated samples, or if these

formulas are simply not well retained by SPE and therefore not routinely observed in high-resolution MS studies of DOM.

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Supporting Information

Figures S1 – S23 and Tables S1 – S6 are included in Supporting Information. The material is available free of charge via <http://pubs.acs.org>.

Figure 1. Relative changes in bulk parameters and weighted averages derived from Orbitrap MS data for UMRNOM and SRNOM as a function of the specific ozone dose. (a) Absorbance at 254 nm, (b) $E_2:E_3$, (c) specific UV absorbance ($SUVA_{254}$), (d) electron donating capacity (EDC), (e) $O:C_w$, and (f) DBE_w in UMRNOM with and without *t*-BuOH at pH 7, SRNOM with and without *t*-BuOH at pH 7, and SRNOM at pH 11. Error bars represent the standard deviation of triplicate bulk measurements.

Figure 2. (a) Number of unique formulas abated or formed in SRNOM with and without *t*-BuOH as a function of the specific ozone dose. (b) Weighted O:C of unique formulas abated or formed in SRNOM. van Krevelen diagrams for formulas present only before ozonation (blue) or present only after ozonation (red) in (c) SRNOM in 1.3 mg-O₃/mg-C without *t*-BuOH at pH 7 and (d) SRNOM in 1.3 mg-O₃/mg-C with *t*-BuOH at pH 7.

Figure 3. Formulas that (a) decrease or (b) increase in relative intensity with increasing specific ozone dose in UMRNOM samples ($n_{\text{samples}} = 6$; $n_{\text{formulas}} = 890$). Formulas that (c) decrease or (d) increase in relative intensity with increasing specific ozone dose in UMRNOM samples with added *t*-BuOH ($n_{\text{samples}} = 6$; $n_{\text{formulas}} = 1052$). Rho values determined by Spearman rank correlations are noted in the legend (negative rho values = negative correlation with ozone dose; positive rho values = positive correlation; **Section S5**). (e) Formulas that are only reactive with ·OH ($n_{\text{formulas}} = 53$) compared to formulas that are reactive with O₃ and ·OH ($n_{\text{formulas}} = 191$) in UMRNOM and SRNOM ($n_{\text{samples}} = 24$). (f) van Krevelen diagrams for transformation products with one or two added oxygen atoms and decarboxylation products in UMRNOM at 0.5 mg-O₃/mg-C.

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