

Exploring Molecular Dynamics and Structure Change of Dissolved Organic Nitrogen under Nitrate and Phosphate Pulses in a Cascade Upflow Biofiltration System

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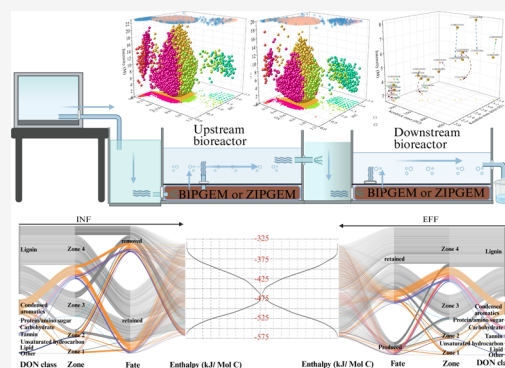
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ABSTRACT: Dissolved organic nitrogen (DON) remains a large, reactive, and under-managed fraction of stormwater nitrogen. We investigated how influent N:P stoichiometry and porous media chemistry jointly shape DON fate in a cascade upflow bioreactor packed with two specialty adsorbents, including BIPGEM (sand–clay–ZVI–perlite–biochar) and ZIPGEM (sand–clay–ZVI–perlite). Reactors received short pulses of nitrate alone (AC1) or nitrate plus phosphate simultaneously (AC2). Paired influent–effluent samples were analyzed using a 21 T Fourier transform ion cyclotron resonance mass spectrometer. Molecular formulas containing nitrogen (CHON formulas) were then classified as removed, produced, or retained. AC2 reprograms produced DON chemistry in a media-specific manner. BIPGEM shifted toward heavier, unsaturation rich products with lower inferred lability, whereas ZIPGEM shifted toward more oxidized, aliphatic products with higher inferred lability and preferential removal of unsaturated formulas. Bulk measurements showed moderate nitrate removal in both ZIPGEM and BIPGEM. However, phosphate removal was strongest in ZIPGEM under AC2, while BIPGEM released phosphate under AC1. These findings highlight influent N:P and media selection/ordering as levers to control nutrient removal and residual DON quality, with implications for downstream nitrogen cycling and stormwater treatment performance.

KEYWORDS: molecular dynamics, structure change, cascade upflow biofiltration system, dissolved organic nitrogen, nitrate and phosphate pulses



1. INTRODUCTION

Nutrient pollution and eutrophication represent typical human-natural system dilemmas, where the interplay of land utilization, infrastructure, and policy choices with hydrological and biogeochemical dynamics leads to nonlinear, feedback-oriented results.^{1,2} Traditionally, species of nutrients have been compartmentalized, giving precedence to phosphorus in freshwater and nitrogen in estuaries.³ However, increasing research indicates that successful mitigation necessitates the combined management of various nutrients.^{2,3} In the context of multiple nutrients, dissolved organic nitrogen (DON) plays a crucial, yet often overlooked role. It forms a significant portion of the total nitrogen found in both inland and coastal waters,^{4–6} and increasing research indicates that DON is not inactive⁷ but can transform into forms that support the growth of microbes and algae.⁸ Consequently, ignorance of DON fate, transport, and transformation results in a large, possibly bioavailable nitrogen reserve that eludes traditional control measures and hampers comprehensive nutrient management.⁹

From a stormwater engineering perspective, this knowledge gap is consequential because biofilters/bioretention systems are widely implemented to reduce nitrogen and phosphorus loads, yet DON removal remains variable and difficult to

predict. Long-term field and laboratory studies increasingly identify DON as an important component of the dissolved N pool in stormwater and a persistent challenge to treatment performance. For example, dissolved nitrogen management in bioretention is often framed around nitrate and ammonium, but DON can represent a large share of stormwater nitrogen and can remain biodegradable as runoff is routed through stormwater infrastructure, potentially exporting a downstream-reactive N fraction even when inorganic N is attenuated.^{10–14} In addition, storm events deliver nutrients as short pulses (often involving both N and P), which can shift competitive sorption, electron acceptor availability, and redox micro-environments within filter media. These dynamics motivate a design-relevant question: how do influent N:P forcing and porous media chemistry jointly control not only how much

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nitrogen is removed but also the molecular characteristics and transformation pathways of DON that is exported?

The primary obstacle lies in the chemical diversity of the DON. Instead of being a singular compound category, DON encompasses a vast array of organic molecules, varying in size, functionality, and redox conditions.^{15,16} Progress in ultrahigh-resolution mass spectrometry, especially FT-ICR MS (21 T Fourier transform ion cyclotron resonance mass spectrometer), has facilitated the molecular identification of dissolved organic substances, enabling the resolution of specific formulas on a large scale¹⁷ and translating formula information into chemical descriptors that proxy oxidation state, unsaturation, aromaticity, and inferred lability.^{18–21} Nevertheless, even with these advancements in chemical analysis, there remains a significant gap in knowledge, specifically the absence of a molecularly deciphered comprehension of the connection between descriptor space and fate (removed/produced/retained) during biofiltration treatment, particularly in the context of single or simultaneous nutrient pulses, which is a typical case in urban stormwater runoff in a real-world environment.

In this study, we address this research gap by employing a cascading upflow bioreactor^{22,23} filled with two types of green sorption media, including BIPGEM (biochar, zero-valent iron (ZVI) and perlite-based green environmental media) and ZIPGEM (zero-valent iron (ZVI) and perlite-based green environmental media), and subjected to regulated nitrate (designated as AC1 hereafter) and a combination of nitrate and phosphate (designated as AC2 hereafter) pulses mimicking intermittent nutrient contributions in the field conditions.²⁴ By sequentially incorporating filtration, sorption/complexation, and biotransformation, the upflow setup ensures extended interaction in designed cascade durations with consistent hydraulic properties, facilitating detailed diagnostic processes from combined influent–effluent samples. Expanding upon our previous research,²⁵ which connected DON fractional removal to the diversity of microbial species in a similar cascade bioreactor structure, this study transitions from bulk removal patterns to molecular pathways determined by fate, offering practical design perspectives for enduring stormwater treatment. The study highlights a framework focused on organic molecules, utilizing 21 T FT-ICR MS,²⁶ calculating chemical descriptors, and employing Kendrick mass defect (KMD) mapping and reaction-difference analysis to deduce pathway networks in varied nutrient conditions.

Following such a systems-thinking framework, we pose three research questions: (1) How does influent nutrient stoichiometry (i.e., AC1 versus AC2) in stormwater modulate chemical-descriptor-based thermodynamic and stoichiometric controls on the fractional fate of DON across a cascade upflow biofilter, expressed as the proportions removed, produced, and retained? (2) Do BIPGEM and ZIPGEM produce distinct formula-resolved DON fate signatures (removed, produced, retained) and mass-difference patterns under identical influent stoichiometric pulses, consistent with different balances between retention/complexation and in-filter processing? (3) Does the proposed cascade upflow configuration impart a descriptor-resolved transformation imprint on DON that can be inferred from paired influent–effluent measurements via KMD mapping? By linking DON's molecular characteristics to its observed fate under controlled N:P forcing and contrasting green sorption media recipes, the study advances a scalable and resilient intervention paradigm for stormwater treatment, which integrates sorption chemistry, microbial processing,

and molecular analytics to deliver a holistic nutrient control profile in an aquatic environment.

2. MATERIALS AND METHODS

2.1. Experimental Setup

We created and operated two parallel cascade upflow biofiltration treatment trains at the laboratory scale, treating stormwater collected from a wet-detention pond on campus (UCF). Each system comprised two packed bioreactors in series from upstream to downstream and was filled with one of two green sorption media, namely, BIPGEM or ZIPGEM. Green sorption media are mixtures of recycled and natural media as specialty adsorbents. They are designed to provide cost-effective, scalable, flexible, and sustainable features for water filtration that may be configured to fit in any landscape for the treatment of stormwater runoff, wastewater effluent, and agriculture discharge in the field. In this study, BIPGEM was composed of 80% sand, 5% clay, 5% ZVI, 5% perlite, and 5% biochar, and ZIPGEM contained 85% sand, 5% clay, 5% ZVI, and 5% perlite. The physical characteristics of ZIPGEM and BIPGEM can be seen in Table S1, and the chemical element composition can be seen in Table S2. Selected physical properties of the biochar, including density, particle size distribution, and BET-specific surface area, are summarized in Table S3.

A peristaltic pump delivered influent at a constant flow, creating upflow through shallow media beds with gravel capping to prevent resuspension, and such upflow settings can avoid media clogging and settlement after long-term operation. The reactors were connected by staggered channels to maintain a cascade (Figure 1, the dimensions

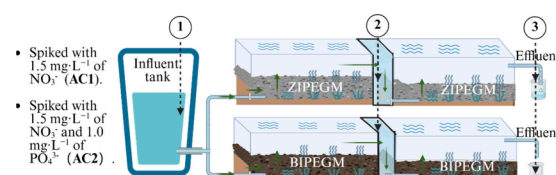


Figure 1. Experimental setup.

can be seen in the Supporting Information as Method S1). After a preconditioning period, both treatment trains were subjected to the same two influent pulse scenarios to emulate short-duration stormwater nutrient loadings (Table S4). Specifically, AC1 and AC2 denote influent conditions (not media-specific treatments): AC1 (nitrate spike, 1.5 mg L^{−1}, 2.5 h) and AC2 (nitrate + phosphate spikes, 1.5 mg L^{−1} NO₃[−] and 1.0 mg L^{−1} PO₄^{3−}, 2.5 h). For each pulse and medium, we collected paired influent and final effluent samples (500 mL) for 21 T FT-ICR MS to resolve the DON molecular composition and assign fate (removed, produced, retained) from occurrence patterns.

2.2. Water Sample Analysis and DOM Extraction for FT-ICR MS

Following the flowchart of Figure 1, water samples were collected at three different locations, including the effluent ports of the initial reservoir (location ①), the effluent ports of the upstream bioreactors (location ②), and downstream bioreactors (location ③). Water samples under AC1 and AC2 were analyzed in triplicate in-house using the Method 10206 of TNT 835 Nitrate Kit from HACH for nitrate and the Method 10209/10210 of TNT 843 Phosphorus Kit for phosphate.^{27,28} For each medium (BIPGEM, ZIPGEM) and influent condition (AC1, AC2), we collected paired influent and effluent water samples (500 mL) at locations ① and ③ of Figure 1. Water samples collected during AC1 and AC2 were prepared for ultrahigh-resolution mass spectrometry by solid-phase extraction of dissolved organic matter (SPE-DOM) following established protocols.²⁹ DOM was isolated by priority pollutant liquid (PPL) solid phase extraction (Bond Elut PPL, 200 mg, 3 mL) and eluted with methanol.³⁰ The detailed operation of solid phase extraction can be seen as method S2

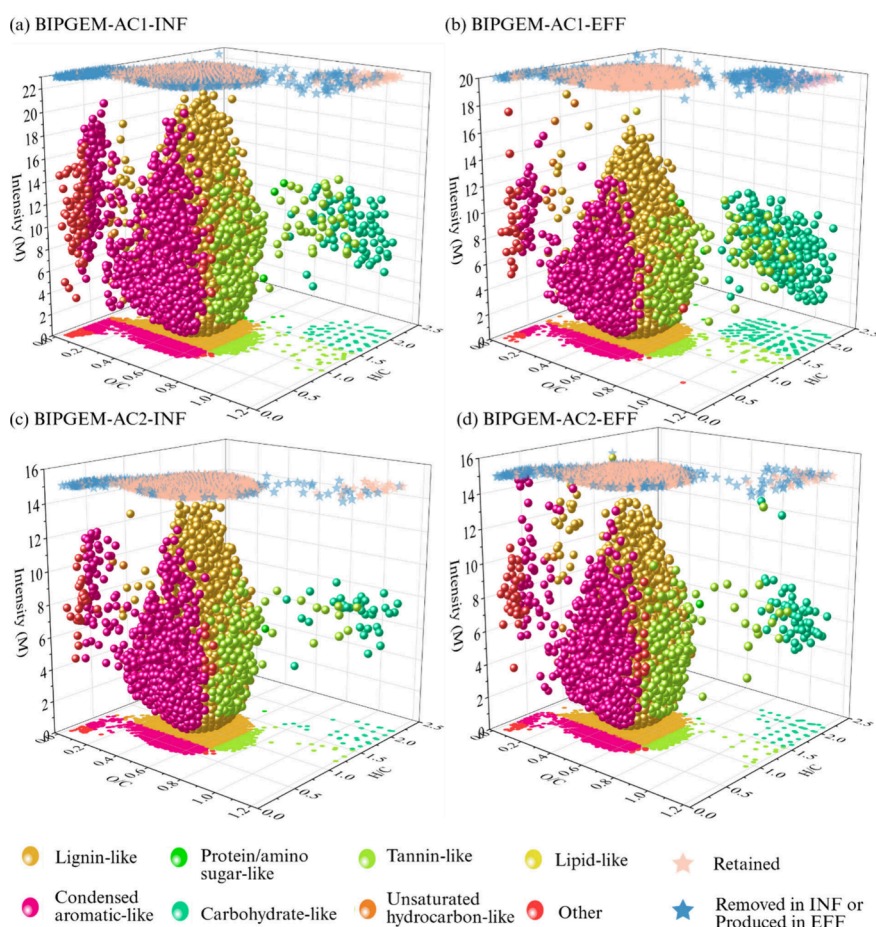


Figure 2. Three-dimensional, class-resolved O/C–H/C–intensity distributions of dissolved organic nitrogen (DON) for BIPGEM under stoichiometric pulses: (a) influent-AC1, (b) effluent-AC1, (c) influent-AC2, and (d) effluent-AC2. Points are colored by DON class (legend). Orange stars denote retained formulas (present in both influent and effluent) and blue stars denote formulas present only in the influent (removed) or only in the effluent (produced). Axes: $x = \text{O/C}$, $y = \text{H/C}$, $z = \text{normalized ion intensity (a.u.)}$; base-plane projections are shown to guide comparison across panels. It is noted that Van Krevelen diagrams show CHON formulas only.

in the supplementary file. Extracts were diluted in MeOH to 100 mg L^{-1} and analyzed at the National High Magnetic Field Laboratory (NHMFL, Tallahassee, FL) by negative-ion ESI FT-ICR MS (21 T). Mass spectra were phase-corrected and internally calibrated to abundant oxygenated homologous series using the walking calibration approach to achieve high mass accuracy.^{31,32} Because SPE does not selectively isolate DON, DON was operationally resolved during data processing by restricting molecular-formula interpretation to CHON compositions (DON proxy), while other heteroatom classes were used only for contextual reporting where noted. Elemental formulas $\text{C}_x\text{H}_y\text{N}_z\text{O}_w\text{S}_v$ were assigned, grouped by heteroatom class,³³ and visualized with van Krevelen diagrams³⁴ and Kendrick mass-defect (KMD) mapping.^{35,36} Data processing and relative-abundance calculations were performed in PetroOrg.^{36,37} The operation of 21T FT-ICR MS can be seen in the supplementary file as method S3.

2.3. Molecular Fingerprint Analysis

Unless otherwise noted, throughout the whole paper, all molecular fate/chemical descriptor analyses were performed on CHON formulas as a proxy for DON. Note that the van Krevelen classes based on FT-ICR MS exact-mass measurements and descriptor-based interpretations used in this study should be understood as operational, formula-based classifications rather than confirmed molecular structures. To reveal the intricate breakdown of DON, 9 chemical descriptors (i.e., H/C, N/C, O/C, DBE, NOSC, (DBE–O)/C, Almod,¹⁸ Xc ,¹⁹ MLBL ²⁰) were employed and isolated according to each specific DON pool.²⁵ Additionally, DON denotes compounds with only four elements: carbon, nitrogen, hydrogen, and oxygen,

operationally defined as CHON formulas within $\text{H/C} \leq 2.2$ and $\text{O/C} \leq 1.2$. Utilizing formula designations from FT-ICR MS, the molecular formulas assigned from FT-ICR MS were classified into (1) carbohydrate-like groups: $0.67 < \text{O/C} \leq 1.2$, $1.5 < \text{H/C} \leq 2.2$; (2) Proteins/amino sugars-like: $0.3 < \text{O/C} \leq 0.67$, $1.5 < \text{H/C} \leq 2.2$; (3) lipid-like: $0 < \text{O/C} \leq 0.3$, $1.5 < \text{H/C} \leq 2.2$; (4) unsaturated hydrocarbons-like: $0 \leq \text{O/C} < 0.1$, $0.67 < \text{H/C} \leq 1.5$; (5) lignin-like: $0.1 \leq \text{O/C} < 0.67$, $0.67 < \text{H/C} \leq 1.5$; (6) tannins-like: $0.67 \leq \text{O/C} \leq 1.2$, $0.67 < \text{H/C} \leq 1.5$; and (7) condensed aromatic-like: $0 \leq \text{O/C} < 0.67$, $0.3 \leq \text{H/C} \leq 0.67$.⁸ Formulas that satisfied the overall CHON van Krevelen bounds ($\text{H/C} \leq 2.2$ and $\text{O/C} \leq 1.2$) but did not fall within any of the classes listed above were grouped as ‘Other’ (unclassified CHON within the DON domain). For each medium and influent pulse scenario, CHON molecular formulas (used here as a proxy for DON) were classified from paired influent–effluent measurements as removed, produced, or retained (Method S4). Removed formulas were detected only in the influent, produced formulas only in the effluent, and retained formulas in both. These are operational detection categories within the SPE–FT-ICR MS analytical window and do not imply absolute removal, de novo synthesis, or a unique transformation mechanism in the bulk DON pool. The comprehensive computation of the chemical descriptors is also detailed in the Supporting Information file under method S4.

2.4. Molecular Reaction Network Analysis

A KMD^{38,39} analysis was used to provide an overview of the composition of the identified formulas in samples. The KMD calculations are shown in eqs 1 and (2).

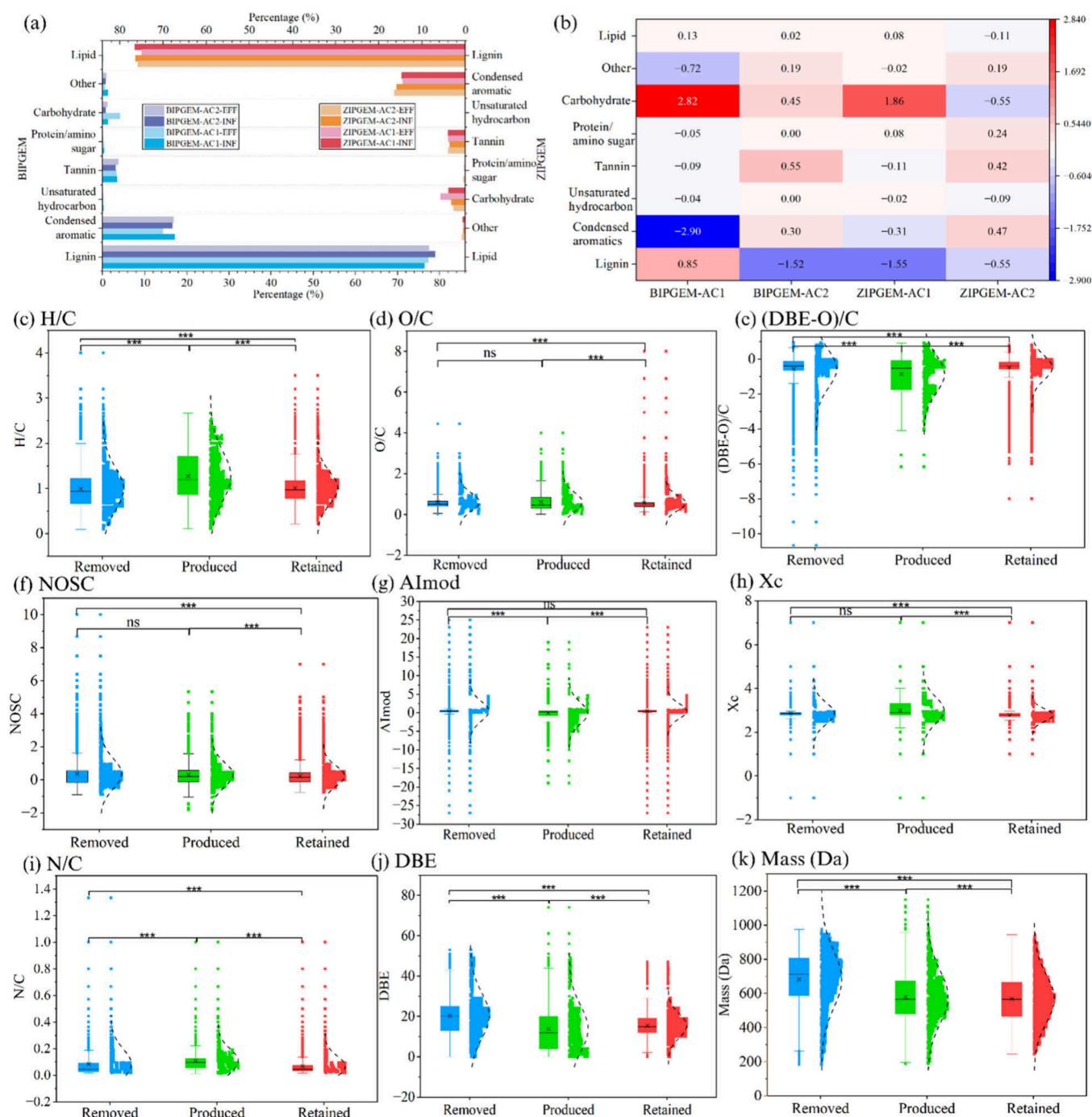


Figure 3. Class-level composition and fate-resolved descriptor distributions. (a) Stacked percentages of DON molecular classes (lignin-like, condensed aromatic, unsaturated hydrocarbon, tannin, protein/aminosugar, carbohydrate, other, lipid) for influent (INF) and effluent (EFF) of BIPGEM and ZIPGEM under AC1 and AC2. (b) Heat map of EFF – INF percentage-point change for each class (positive values indicate enrichment in the effluent). (c–k) BIPGEM–AC1: distributions of H/C, O/C, (DBE–O)/C, NOSC, Almod, Xc, N/C, DBE, and molecular mass (Da) for formulas classified by fate—removed (present only in INF), produced (present only in EFF), and retained (present in both).

$$\text{Kendrick mass}(F) = (\text{observed mass}) \times \frac{\text{nominal mass}(F)}{\text{exact mass}(F)} \quad (1)$$

$$\text{KMD} = \text{nominal mass} - \text{Kendrick mass}(F) \quad (2)$$

Based on KMD's definition, organic molecules sharing identical KMD values exhibit homology and only vary in molecular weight, with Δm representing integral multiples of the chosen mass of the functional group. If formulas in the removed DON pool and produced DON pool matched a characteristic Δm , the match was interpreted as

evidence of a potential chemical transformation consistent with that reaction class, rather than confirmation that a specific metabolic pathway occurred. Our recurrent functional groups in KMD analysis included $-\text{CH}_2$, $-\text{COO}$, $-\text{H}_2$, $-\text{OH}$, $-\text{O}$, $-\text{CO}$, $-\text{H}_2\text{O}$, and $-\text{C}_6\text{H}_5$, which were involved in methylation/demethylation, carboxylation/decarboxylation, hydrogenation/dehydrogenation, hydroxylation/dehydroxylation, oxidation/reduction, carbonylation/decarbonylation, hydration/dehydration, and aromatization/dearomatization, respectively.³⁸

Note that in chemical transformations, for example, the Δm represents a possible gain of a methyl group during methylation

reactions (e.g., conversion of cytosine to 5-methylcytosine or a possible loss of a methyl group (or its components) during demethylation reactions. Overall, Δm for the above eight chemical transformations is methylation/demethylation ($\Delta m = \pm 14.010565$ amu), carboxylation/decarboxylation ($\Delta m = \pm 43.989829$ amu), hydrogenation/dehydrogenation ($\Delta m = \pm 2.015650$ amu), hydroxylation/dehydroxylation ($\Delta m = \pm 17.002740$ amu), oxidation/reduction ($\Delta m = \pm 15.994915$ amu), carbonylation/decarbonylation ($\Delta m = \pm 27.994915$ amu), and aromatization/dearomatization ($\Delta m = \pm 77.039375$ amu).

3. RESULTS AND DISCUSSION

3.1. System Performance and Class-Resolved Organization of DON under Stoichiometric Pulses

The cascade bioreactor removed 45.94%–51.74% NO_3^- by ZIPGEM and 37.23%–47.18% NO_3^- in BIPGEM (Table S5). The upstream bioreactor accounted for most nitrate removal, while the downstream bioreactor provided additional polishing. The relative contribution varied by media and pulse scenario. For BIPGEM, the upstream bioreactor accounted for 34.02% of the 47.18% total NO_3^- removal in AC1 (72.11%) and 29.57% of 37.23% in AC2 (79.43%). For ZIPGEM, the upstream contributions were 28.54% of 45.94% (62.12%) in AC1 and 50.10% of 51.74% (96.83%) in AC2 (Table S5). Since AC1 has no spiked phosphate, the removal percentage of phosphate under AC1 is related to the natural phosphate in stormwater. Phosphate removal was modest in ZIPGEM under AC1 (15.16%) but rose to 96.06% in ZIPGEM under AC2. In contrast, BIPGEM under AC1 exhibited net PO_4^{3-} release (−20.99%), whereas BIPGEM under AC2 achieved 75.1% removal percentage. The negative PO_4^{3-} removal for BIPGEM under AC1 may reflect transient desorption and leaching⁴⁰ from BIPGEM (i.e., biochar) under nitrate-only conditions, whereas the strong gains in AC2 are consistent with rapid phosphate removal by Fe(III) oxides formed from zero-valent iron (ZVI) corrosion⁴¹ and with precipitation and sorption in both media. Operationally, the upstream bioreactor therefore experiences the steepest concentration and redox gradients,⁴² shaping the chemistry that the downstream bioreactor subsequently encounters, which is a cascading context that helps explain the fate patterns resolved in sections 3.2–3.4.

Bulk DON concentrations decreased from influent to effluent across both media and pulse scenarios, with consistently higher apparent DON removal in BIPGEM than ZIPGEM (Table S6). The O/C–H/C–intensity plots (Figure 2) show a lignin-like dominated matrix with a condensed aromatic-like shoulder and discrete clusters in the high-O/C, high-H/C portion of van Krevelen space, corresponding to the carbohydrate-like and protein/amino sugar-like regions within the CHON (DON-proxy) formulas. For BIPGEM, the effluent intensity surface under AC1 visibly builds over these carbohydrate-like and protein/amino sugar-like regions, whereas under AC2 the effluent surface expands toward the unsaturated/aromatic side while the retained cloud stays tightly bounded. The ZIPGEM landscapes (Figure S1) display the reverse stoichiometric bias. With phosphate copulsing (AC2), the effluent surface shifts the more oxidized and aliphatic (higher O/C and H/C) portion of van Krevelen space, while unsaturated features are preferentially absent from the effluent. These qualitative fingerprints anticipate the quantitative descriptor contrasts reported in section 3.2 and the saturation–redox zonation in section 3.3. Overall, FT-ICR MS assignment results are summarized in Table S7, which

reports the total number of assigned formulas and their elemental-class distributions across samples. CHON formulas constituted a substantial and relatively stable fraction of assigned formulas across all samples (18.85–21.28%), supporting their use here as an operational proxy for DON in the subsequent analyses. Across all runs, lignin-like and condensed-aromatic formulas comprise roughly 75.82%–78.96% and 14.27%–17.16% of assigned formulas, respectively, whereas formulas in the carbohydrate-like and protein/amino sugar-like regions contribute only a few percent (Table S8, Figure 3a). Although small in bulk, carbohydrate and protein/amino sugar exhibit the largest relative shifts between influent and effluent (Figure 3a,b). In BIPGEM-AC1, the fraction of CHON formulas assigned to the carbohydrate-like region increases from 1.21% (INF) to 4.03% (EFF) and condensed aromatics decrease from 17.16% to 14.27%, consistent with the AC1 effluent build-up over the carbohydrate-like and protein/amino sugar-like regions in Figure 2b and the high lability of produced formulas seen later (section 3.2). Because negative-mode ESI can underrepresent carbohydrate-like material and aromatic-rich matrices can suppress the ionization of more aliphatic/oxygen-rich components, we interpret this as an apparent enrichment that may reflect both compositional change and reduced ionization competition.^{43,44} Under BIPGEM-AC2, carbohydrate increases are smaller (from 0.65% to 1.10%), and tannin/condensed-aromatic shares are maintained or slightly higher (e.g., tannin from 3.08% to 3.64%, condensed aromatics from 16.52% to 16.81%, respectively), mirroring the shift toward unsaturation-denser products that we quantify in section 3.2. In ZIPGEM, AC1 also enriches carbohydrate (from 3.90% to 5.76%) with a slight drop in lignin and condensed aromatics, whereas AC2 reverses that trend. Carbohydrate decreases (from 3.20% to 2.64%) while condensed aromatics and protein/amino sugar increase slightly (from 15.93% to 16.40% and from 0.15% to 0.39%, respectively). The signs of these shifts agree with the media-dependent PO_4^{3-} behavior (Table S5). Under the nitrate + phosphate pulse (AC2), ZIPGEM achieves 96.06% of PO_4^{3-} removal efficiency and the produced pool shifts toward oxidized, aliphatic, more labile formulas, whereas BIPGEM under the same pulse captures PO_4^{3-} to a lesser extent (75.11%) and the produced pool becomes heavier and more unsaturation-dense. Under nitrate-only pulse (AC1), BIPGEM shows net PO_4^{3-} release (−20.99%), consistent with the relative persistence of aromatic/unsaturated features.

Taken together, the serial hydraulics (upstream bioreactor and downstream bioreactor), the media-specific nutrient handling (ZIPGEM as the stronger phosphate sink and BIPGEM exhibiting PO_4^{3-} release under AC1 but substantial removal under AC2; Table S5), and the class balances (Figure 3a,b; Table S8) define the envelope within which DON is transformed. Under nitrate-only pulses (AC1), both media tend to generate more oxygenated, aliphatic, higher-lability products that occupy carbohydrate-/protein-like regions of van Krevelen space. When phosphate is copulsed (AC2), the gating diverges by media. In BIPGEM the produced pool shifts toward heavier, unsaturation-rich and less-labile formulas, whereas in ZIPGEM the produced pool becomes more oxidized and aliphatic with preferential removal of unsaturated formulas.

3.2. Descriptor-Resolved Fate Selectivity Across Media and Conditions

Having established the class shifts (Figure 3a,b), we next asked whether chemical descriptors gate the fractional fate of DON and whether this gating depends on influent stoichiometry and media chemistry. We compared distributions of nine descriptors for formulas classified as removed (influent-only), produced (effluent-only), and retained (present in both) using BIPGEM under AC1 in the main text (Figure 3c–k) and the parallel analyses for BIPGEM under AC2 and ZIPGEM under AC1 and AC2 in the Supporting Information (Figures S2–S4). Initial molecular composition characteristics of influent and effluent DON can be seen in Table S9, while descriptor means and ranges per fate are summarized in Tables S10 & S11.

Across both media and both pulses, the retained pool collapses to a narrow and remarkably stable region of descriptor space (Tables S10 & S11, Supplementary Note 1.1). We refer to this invariant cluster as the “retained window” and use it as a physicochemical baseline against which selective shifts in the removed and produced pools can be interpreted. Importantly, “retained” denotes persistence of an elemental formula in both influent and effluent and therefore does not necessarily imply an absence of transformation, because extensive isomeric diversity and structural rearrangements can occur without changing molecular formula assignments in ultrahigh-resolution MS data sets.⁴⁵

The stoichiometry-insensitive nature of the retained window suggests a DON core that is buffered against short-term nutrient forcing, through one or more nonexclusive mechanisms. First, the retained window may reflect an intrinsically persistent/slow-cycling fraction on the pulse time scale, consistent with reports that DOM persistence can be systematically linked to molecular-level characteristics in freshwater systems.⁴⁶ Second, it may reflect reversible retention/complexation on reactive surfaces (e.g., iron (oxy)-hydroxides generated from ZVI and mineral phases), which is known to fractionate DOM and selectively partition compounds according to oxygen content and unsaturation at mineral–water interfaces.^{47,48} Third, the retained window could be compatible with persistence of oxygenated/alicyclic motifs analogous to carboxyl-rich alicyclic molecules, which have been characterized as major refractory components in DOM and are implicated in strong ligand/complexation behavior.⁴⁹ While our data set cannot disentangle these mechanisms or separate abiotic from biotic contributions, the retained window has direct environmental relevance because it represents a stable baseline DON signature exported from the cascade biofilters even when nitrate and phosphate are attenuated; iron–organic associations are also widely recognized as an important preservation pathway for organic matter in natural systems.⁵⁰

Under the nitrate pulse, the produced pool in BIPGEM systematically shifts toward higher O/C and H/C, lower DBE, and more negative (DBE–O)/C relative to both removed and retained (Figure 3c–k; significance marks on panels). Means from Table S10 illustrate the effect from removed to produced (Supplementary note 1.2). These features depict aliphatic and oxygenated products in the produced pool, which is consistent with the carbohydrate- and protein/amino sugar-like enrichment seen in Figure 3b.

Adding phosphate (AC2) does not erase this oxygenation, but it changes the balance between oxygenation and unsaturation.^{51,52} Relative to AC1, the produced pool gains

unsaturation density (i.e., DBE, (DBE – O)/C) and mass (Supplementary note 1.3) while lability (MLB_L) decreases (Tables S10–S11; Figure S5). Almod moves toward zero, indicating a greater aromatic contribution, whereas O/C and NOSC remain elevated (Also see Supplementary note 1.3). In the same runs, the removed pool becomes less oxidized/less labile than in AC1 (e.g., O/C from 0.554 to 0.491; NOSC from 0.374 to 0.25; MLB_L from 9.578 to 4.529). Thus, phosphate copulsing tilts the transformation pathway in BIPGEM away from light, labile aliphatic products toward heavier, less-labile species enriched in unsaturation, in line with the class-level drift toward aromatic families (section 3.3).

ZIPGEM shows the opposite stoichiometric response, with phosphate driving oxidation and aliphaticity.^{53,54} Without phosphate (AC1), fate separations in ZIPGEM are modest (Figure S3; Table S10). Produced is only slightly more oxygenated/aliphatic than removed (O/C (0.61 for both); H/C 1.186 (produced) vs 1.023 (removed); DBE 15.187 vs 17.858). With phosphate copulsing (AC2) (Figure S4; Table S11), produced undergoes a pronounced oxidative–aliphatic shift (Tables S10 and S11; Figure S6; Supplementary note 1.4). In parallel, removed displays the complementary pattern (lower O/C and NOSC with higher DBE), indicating preferential capture of reduced/unsaturated formulas. In short, the case of AC2 in ZIPGEM channels DON toward oxygenated, labile products while partitioning unsaturated features into the removed fraction.

Viewed together, phosphate copulsing opens two distinct gates depending on media. In BIPGEM, P elicits a saturation-dominated response, with produced formulas becoming heavier and more unsaturation-dense (i.e., DBE increases, mass increases, MLB_L decreases), whereas removed becomes more reduced. In ZIPGEM, P elicits an oxygenation-dominated response, with produced formulas becoming more oxidized and aliphatic (O/C increases, NOSC increases, DBE decreases, MLB_L increases), while removed concentrates the unsaturated endmembers. Across both media, retained persists as the stoichiometry-insensitive end point. Notably, N/C in produced increases with phosphate copulsing in both media (BIPGEM from 0.107 to 0.126; ZIPGEM from 0.103 to 0.147; Tables S10–S11), signaling greater N incorporation into effluent-only formulas under P-replete conditions.

Across runs, these descriptor displacements reveal reproducible structural edits to DON, including chain length and saturation ($\pm\text{CH}_2$, $\pm\text{H}_2$) in BIPGEM, oxygen-functionalization and deoxygenation in ZIPGEM, and modest shifts in aromaticity (Almod). The retained pool remains a stoichiometry-insensitive, oxygenated-and-saturated core, indicating limited structure change for persistent formulas, whereas produced and removed pools register the dominant edits under each stoichiometric forcing (see Figure 6a for AC2–AC1 effect sizes and Figure S9 for fate-specific deltas). These descriptor-level regularities (oxygenation vs unsaturation trade-offs, lability–mass couplings, and the stationary retained window) quantitatively substantiate research questions 1 and 2 and foreshadow the saturation–redox zonation (section 3.3) and KMD reaction networks (section 3.4) that mechanistically connect influent precursors, produced intermediates, and removed products.

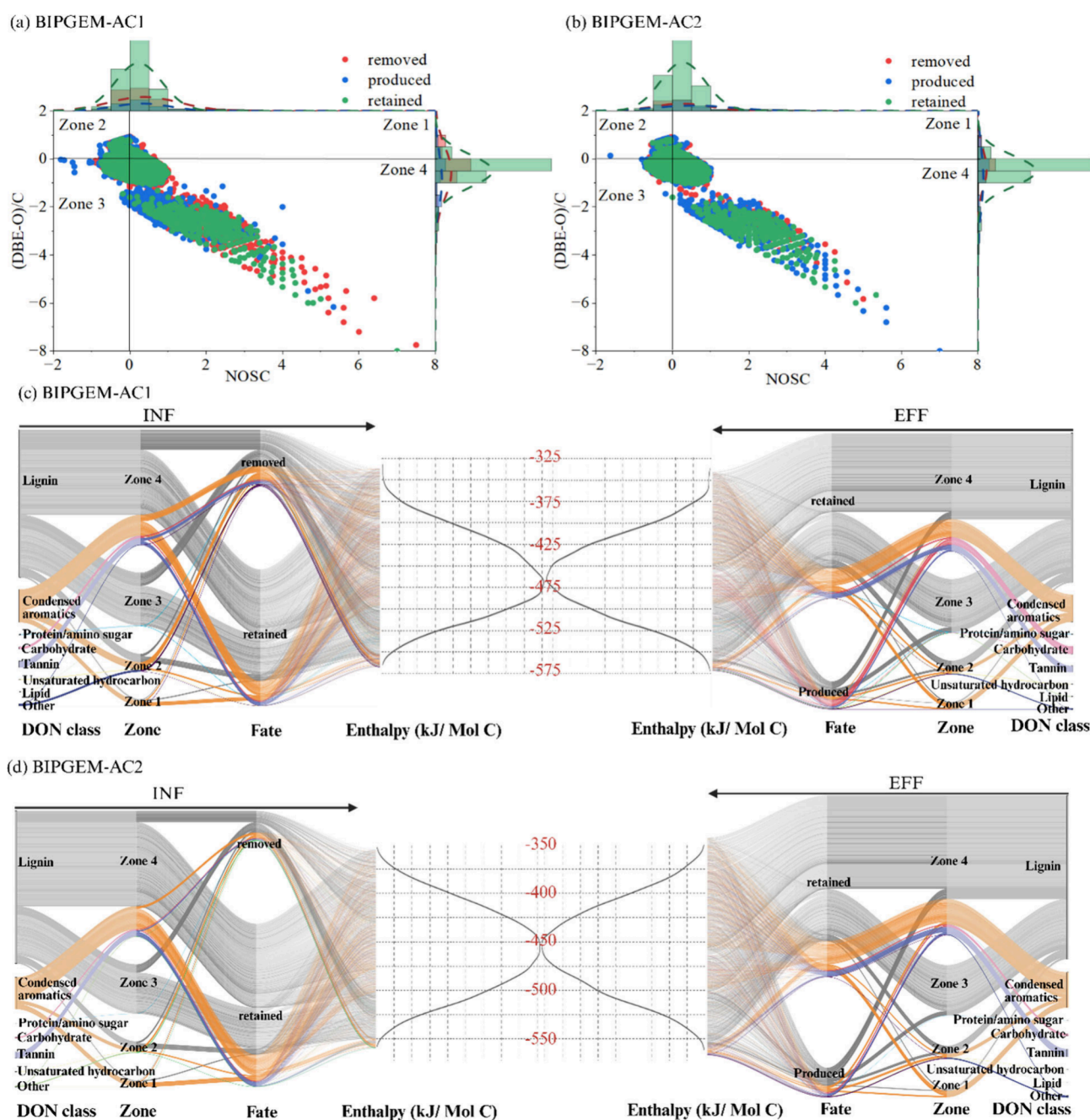


Figure 4. Saturation–redox zonation and class-to-fate flows for BIPGEM. (a–b) Saturation–redox maps for the same runs: each point is a formula colored by fate (removed = red; produced = blue; retained = green) plotted by $(\text{DBE}-\text{O})/\text{C}$ (y-axis; positive = unsaturated) and NOSC (x-axis; positive = oxidized). (c–d) Class-resolved alluvial maps for BIPGEM under AC1 (nitrate pulse) and AC2 (nitrate + phosphate pulse), linking influent (left) DON classes to fate (removed, retained, produced), a central enthalpy gauge (kJ/mol C), and the effluent (right) DON classes. Line thickness is proportional to formula counts; line colors follow class legend.

3.3. Class-Level Transformations and Saturation–Redox Zonation of DON

To connect class composition with thermodynamic controls on fate, we projected all assigned formulas onto a saturation–redox plane spanned by $(\text{DBE}-\text{O})/\text{C}$ (proxy for unsaturation density; positive = more unsaturated) and NOSC (nominal oxidation state of carbon; positive = more oxidized). The plane is partitioned into four zones (Figure 4a,b and Figure S7a,b). We then overlaid class-to-fate flows from influent to effluent and a simple enthalpy gauge, to visualize how classes feed removed, produced, and retained pools and where those pools

reside in saturation–redox space (Figure 4c,d and Figure S7c,d). Quantitative zone shares for each medium, pulse, and fate are listed in Table S12.

Zone occupancy reveals a shared backbone and media-specific differentiators. Across media and pulses, Zone 4 (oxidizing/saturated) is the backbone of the DON pool (systematically ~53.993%–72.114%; Table S12). Differences that distinguish removed from produced arise from how each system populates the unsaturated quadrants (Zones 1–2) and the reducing/saturated quadrant (Zone 3).

For BIPGEM, phosphate copulsing switches the locus of produced formulas while retaining an oxidizing signature.

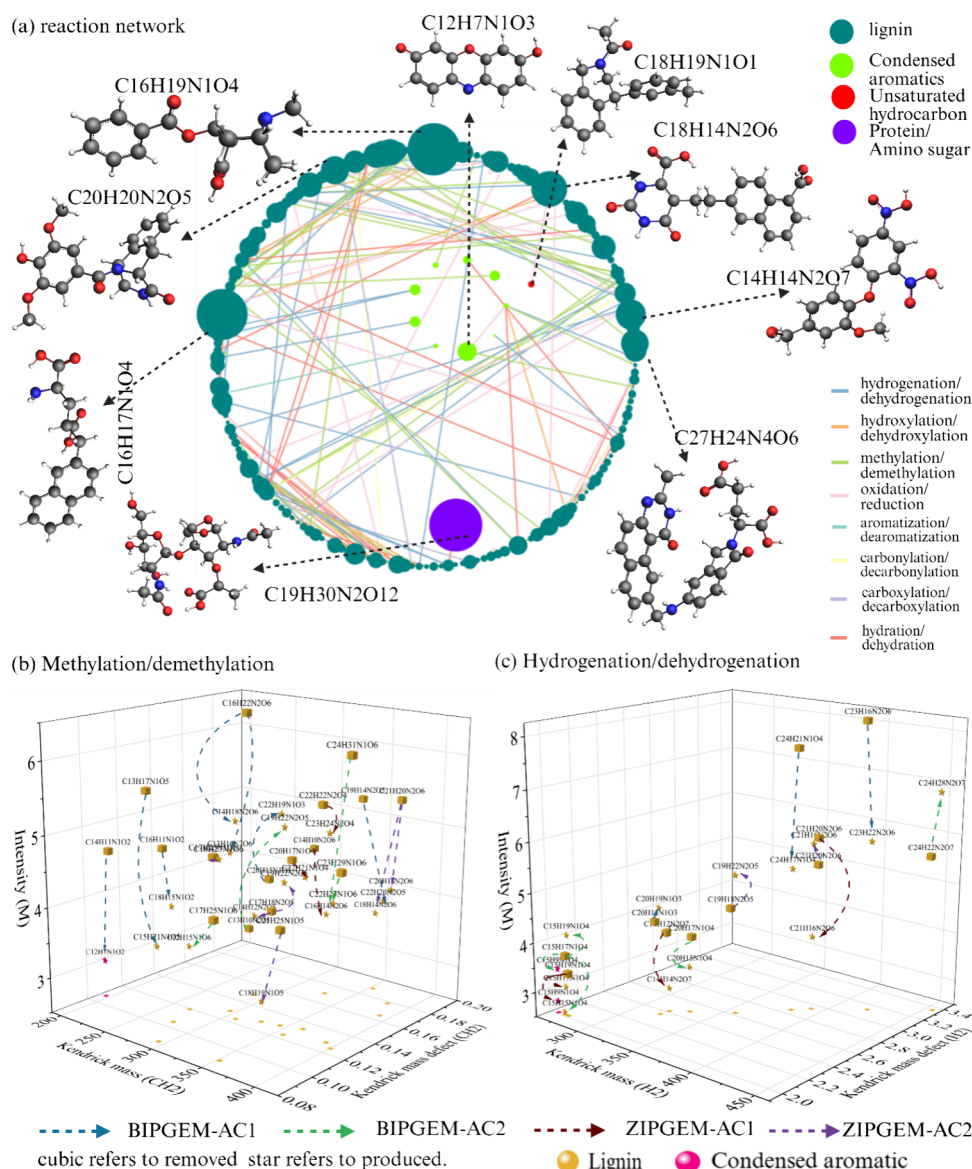


Figure 5. KMD reaction network and exemplar trajectories across paired influent–effluent formulas. (a) Circular network of mass-paired formulas colored by DON class (node size proportional to formula abundance). Representative molecular structures of linked nodes are shown around the perimeter. (b) Methylation/demethylation ($\pm\text{CH}_2$), (c) hydrogenation/dehydrogenation ($\pm\text{H}_2$).

Under AC1 (nitrate only), produced is more present in Zone 1 than removed (5.806% vs 3.359%) and depleted in Zone 3 (from 21.38% to 17.741%), consistent with the descriptor pattern of higher O/C and H/C and lower DBE (Section 3.2). With phosphate copulsing (AC2), the produced pool exhibits dual enrichment. It remains strong in Zone 4 but increases further in Zone 1, while Zone 3 drops (Supplementary note 2.1). In contrast, removed under AC2 allocates more material to Zone 3 and less to Zone 4 than produced (Supplementary note 2.1). This reallocation toward oxidizing/unsaturated space for produced under AC2 dovetails with BIPGEM's descriptor shift toward greater unsaturation density and mass sustained oxidation (Section 3.2). The enthalpy traces add a thermodynamic nuance. In AC1 the produced formulas are, on average, more exothermic than removed (mean enthalpy ≈ -457.41 kJ/mol C vs -443.11 kJ/mol C), whereas in AC2 produced becomes less exothermic than removed (≈ -436.06 kJ/mol C vs -453.72 kJ/mol C), consistent with the

emergence of heavier, less-labile unsaturation-rich products (Tables S10–S11; Figure 4a,b).^{51,55}

For ZIPGEM, nitrate favors unsaturated DON molecules, and phosphate copulsing drives oxygenated–aliphatic DON molecules.^{56,57} Under AC1, the produced pool shifts out of Zone 4 and into the unsaturated quadrants (Zone 1 + Zone 2) (Supplementary note 2.2). Under AC2, the tendency reverses (Supplementary note 2.2), with produced in ZIPGEM dominated by Zone 4 and with smaller shares in Zones 1–2. These reversals match the descriptor-level findings (section 3.2). AC1 yields modestly unsaturated products, whereas nitrate and phosphate copulsing (AC2) channels the system toward oxygenated, aliphatic, labile products and partitions unsaturated end-members into removed. As with BIPGEM, the enthalpy contrast flips between pulses. Produced is more exothermic than removed in AC1 but less exothermic in AC2 (Tables S10–S11; Figure S7a,b).

The class-to-fate flowlines explain how these zonal shifts arise. In BIPGEM–AC1 (Figure 4c), effluent-only formulas

(produced) draw disproportionately from carbohydrate and protein/amino sugar precursors, populating Zones 1 and 4 and depleting Zone 3, which aligns with the large, positive carbohydrate change in Figure 3b. In BIPGEM–AC2 (Figure 4d), streams from condensed aromatics and lignin-like precursors contribute more strongly to produced, evident in the greater Zone 1 share and the thermodynamic inversion noted above. In ZIPGEM (Figure S7(c,d)), the AC1 and AC2 contrast maps directly onto Zone-4 dominance and decreased unsaturated quadrants in the produced pool, reflecting the media-specific role of phosphate documented in Section 3.2 and anticipated by the stage-resolved nutrient behavior (Section 3.1). Overall, the saturation–redox maps and their zonal tallies provide a compact, mechanistic synopsis. Zone 4 forms the persistent backbone of DON across systems, while phosphate toggles whether transformation favors unsaturation density (BIPGEM) or oxygenated aliphaticity (ZIPGEM). These zonal fingerprints set up the Kendrick mass-defect mapping in Section 3.4, where KMD reaction networks trace how formulas move across zones from influent precursors to effluent products.

3.4. KMD Analysis and Reaction Network

To move from descriptor patterns (Sections 3.1–3.3) to putative mass-difference families, we used KMD analyses to organize paired influent–effluent formulas into homologous series and to assign putative functional groups transformation network (Figure 5; Figure S8). We first compiled a network using formulas that matched structures in the RCSB Protein Data Bank (RCSB PDB) (Figure 5a). Across this set, the most frequent reactions were methylation/demethylation and hydrogenation/dehydrogenation, followed by carbonylation/decarbonylation, oxidation/reduction, and hydration/dehydration; carboxylation/decarboxylation, hydroxylation/dehydroxylation, and aromatization/dearomatization were comparatively rare (Table S13). Restricting to the high-confidence subset (formulas present in both influent and effluent) with 1–2 hits in RCSB PDB sharpened this hierarchy. $\pm \text{CH}_2$ and $\pm \text{H}_2$ together account for ≈ 57 –60% of assignments, oxygen-inserting official groups ($\pm \text{O}$, $\pm \text{OH}$, $\pm \text{H}_2\text{O}$) for ≈ 39 –41%, whereas $\pm \text{CO}$ / $\pm \text{CO}_2$ and phenylation/aromatization together contribute $\leq 9\%$ (Table S13). Thus, DON processing in the cascade filters is overwhelmingly described by small, modular substitutions that tune saturation and oxygenation rather than wholesale changes to the carbon skeleton. Retained formulas dominated both by count and by cumulative relative abundance across all runs, whereas removed and produced pools accounted for proportionally smaller fractions of the total CHON signal, indicating that much of the detectable turnover occurred among relatively lower-abundance formulas (Table S14).

KMD trajectories are consistent with the atomic-level structure changes underlying the descriptor and zonation patterns. We observe side-chain methylation/demethylation ($\pm \text{CH}_2$), hydrogenation/dehydrogenation ($\pm \text{H}_2$) that modulate saturation, and oxygen-inserting steps ($\pm \text{O}$, $\pm \text{OH}$, $\pm \text{H}_2\text{O}$) together with carbonyl/carboxyl edits ($\pm \text{CO}$ / $\pm \text{CO}_2$). Figures 5 and S8 show that these putative transformations account for most paired differences between influent and effluent, suggesting that DON structure change proceeds through a sparse mass-difference network shift rather than wholesale skeleton replacement.

The 3D KMD trajectories further resolve how media and influent stoichiometry select among these putative KMD transformations (Figure 5b,c; Figure S8). Two consistent features emerge. First, transitions may be occurring largely within aromatic super classes (lignin-like and condensed aromatics), indicating that the aromatic scaffold persists while side chains are incrementally modified (colored symbols in Figure 5b,c; Figure S8). Second, the direction and prevalence of official groups mirror the descriptor shifts in Section 3.2 for BIPGEM under AC1. Trajectories linking influent (cubes) to effluent (stars) are dominated by oxygen-adding and saturation-increasing official groups ($\pm \text{O}$, $\pm \text{OH}$, $\pm \text{H}_2\text{O}$, $\pm \text{H}_2$), consistent with the higher O/C, NOSC and lower DBE of the produced pool. Representative series include $\text{C}_{24}\text{H}_{17}\text{N}_1\text{O}_3 \rightarrow \text{C}_{24}\text{H}_{17}\text{N}_1\text{O}_4$ (oxidation, Figure S8c) and $\text{C}_{24}\text{H}_{21}\text{N}_1\text{O}_4 \rightarrow \text{C}_{24}\text{H}_{17}\text{N}_1\text{O}_4$ (dehydrogenation, Figure 5c), together with modest $\pm \text{CH}_2$ shifts that lengthen or shorten side chains (Figure 5b). These steps map cleanly onto the Zone 4 (oxidizing–saturated) occupancy documented in Section 3.3.

For BIPGEM under AC2, with phosphate copulsing, the dominant links pivot toward unsaturation building and carbonyl chemistry. Dehydrogenation ($-\text{H}_2$) and carbonylation ($\pm \text{CO}$) are more common, whereas net oxygen insertion appears less prevalent (Figure 5c; Figure S8e). The putative transformations are joined by sequences such as $\text{C}_{24}\text{H}_{22}\text{N}_2\text{O}_7 \rightarrow \text{C}_{24}\text{H}_{28}\text{N}_2\text{O}_7$ (hydrogenation), $\text{C}_{15}\text{H}_{17}\text{N}_1\text{O}_4 \rightarrow \text{C}_{15}\text{H}_{15}\text{N}_1\text{O}_4$ (dehydrogenation), and $\text{C}_{23}\text{H}_{20}\text{N}_4\text{O}_4 \rightarrow \text{C}_{25}\text{H}_{20}\text{N}_4\text{O}_6$ (carbonylation). These putative transformations increase DBE and decrease molecular lability, mechanistically explaining the heavier, more unsaturation-dense produced formulas and the greater residence in reducing–saturated quadrants observed for BIPGEM under AC2 (Section 3.3).

In contrast, phosphate copulsing may drive ZIPGEM toward oxygenation-dominated pathways. Produced formulas preferentially follow oxidation/hydroxylation/hydration sequences ($\pm \text{O}$, $\pm \text{OH}$, $\pm \text{H}_2\text{O}$) with accompanying hydrogenation of side chains (Figure S8a,c,d), while the removed fraction concentrates complementary deoxygenated/unsaturated partners (Figure S8e,f). These steps directly account for the higher O/C and NOSC and lower DBE of the ZIPGEM–AC2 produced pool and its stronger residence in the oxidizing–saturated zone.

Across all conditions, aromatization/dearomatization events (phenyl gain/loss) are rare ($\leq 2\%$), in line with the minimal movement along AImod reported in Section 3.2. In aggregate, the KMD analysis shows that the stoichiometric gating identified earlier operates by reweighting a small set of mass-difference networks. Phosphate copulsing may tilt BIPGEM toward dehydrogenation/carbonylation (unsaturation-dense products) but tilts ZIPGEM toward oxygen-inserting and hydrogenating steps (oxidized, labile products). This molecular transformation reconciles the class-level drifts (Figure 3b), the saturation–redox zonation (Figure 4), and the fate-resolved descriptor trends (Figure 3c–k and Figures S2–S6), providing a mechanistic bridge from influent precursors to effluent products in the cascade biofilters. Because our FT-ICR MS interpretation is formula-based and our KMD analysis is based on mass differences, our results provide compositional evidence consistent with modular edits (e.g., Δm families corresponding to H-transfer, methyl shifts, and oxygen-insertion equivalents) rather than direct confirmation of

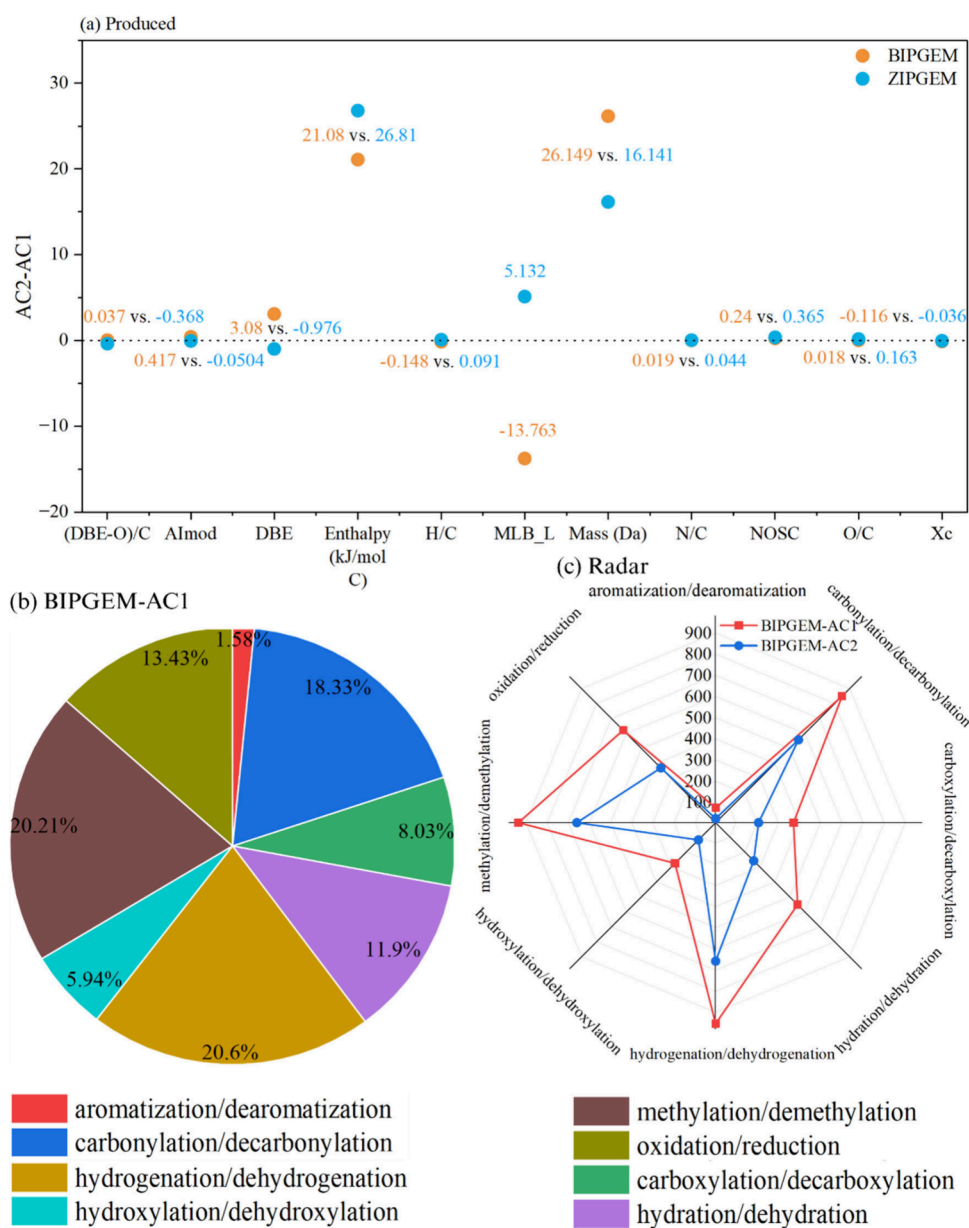


Figure 6. (a) The mean difference between AC2 and AC1 for produced across chemical descriptors, (b) the percentage of each reaction in BIPGEM at AC1, (c) the counts of reactions in BIPGEM under AC1 and AC2.

specific reaction mechanisms. Because compound-specific SPE recovery tests, dedicated media blanks, and isotopic tracing were not included, formulas in the produced pool cannot be uniquely attributed to microbial synthesis and may also reflect transformation of pre-existing DON, release from media or biofilm, and/or analytical threshold effects. Future work using isotope-labeling tracers and/or targeted MS/MS would be required to verify mechanism-specific pathways.^{58–60}

3.5. The Role of Phosphorus in DON Transformation

Phosphate availability emerges as a control lever that does not simply scale DON processing but reweights transformation pathways in a media-specific manner. Together with sections 3.2–3.4, the effect-size map in Figure 6a shows that the combined presence of nitrate and phosphate (AC2) is associated with a shift BIPGEM toward heavier, more unsaturation-dense and less-labile produced formulas (higher DBE and mass with lower MLB_L), whereas ZIPGEM shifts

toward more oxygenated and more labile produced formulas (higher O/C, H/C, NOSC with lower DBE). The stability of the retained pool across AC1–AC2 (small deltas in Figure S9b) corroborates the stoichiometry-insensitive “retained window” identified in section 3.2, implying that stoichiometric forcing gates transformation primarily by redirecting what is removed or newly produced, rather than by altering the persistent core. These AC2 responses are consistent with freshwater studies showing that relief of P limitation accelerates the consumption of reduced, high-energy substrates, leaving more oxidized residues (i.e., higher NOSC), a trajectory expected from thermodynamic selection on organic matter oxidation state.^{61,62}

Consistent with this interpretation, our KMD-based mass-difference families provide transformation fingerprints that align with the observed descriptor shifts, but they should be interpreted as compositional patterns consistent with candidate transformations rather than direct confirmation of reaction

Table 1. The Significance Comparison on Chemical Descriptors between BIPGEM and ZIPGEM

	Removed		Produced		Retained	
	AC1	AC2	AC1	AC2	AC1	AC2
H/C	ns	***	***	***	***	***
DBE	***	***	***	***	***	***
(DBE-O)/C	***	***	ns	***	***	***
Almod	***	***	ns	ns	***	ns
Xc	***	***	ns	***	***	ns
O/C	***	***	ns	***	***	***
NOSC	***	ns	ns	***	ns	***
N/C	***	***	ns	***	***	***
Mass (Da)	***	***	ns	ns	ns	***

^aOrange: The mean value of BIPGEM is significantly higher than that of ZIPGEM (at 0.05 level). Blue: the mean value of ZIPGEM is significantly higher than that of BIPGEM (at 0.05 level). ns refers to not significant. Details can be seen in Figure S12.

mechanisms. In BIPGEM, AC2 increases the relative weight of hydrogenation/dehydrogenation and methylation/demethylation, while reducing hydration and hydroxylation (see Figure 6b,c). In ZIPGEM, AC2 more strongly weights oxygen-inserting equivalents (hydration, hydroxylation) and tempers methylation (Figures S10–S11). This reweighting mirrors the saturation–redox zonation in section 3.3. BIPGEM's AC2 products populate more unsaturation-rich, lower-lability descriptor space, whereas ZIPGEM's AC2 products concentrate in oxidizing–saturated space (higher NOSC, higher O/C). The use of KMD spaces to diagnose such putative transformation fingerprints is consistent with contemporary DOM reaction-family analyses employing KMD and mass-difference approaches.^{63,64}

At the thermodynamic level, studies in lakes and experimental systems show that adding P increases bacterial mineralization rates and growth, hastening the preferential removal of reduced/labile compounds and shifting residual DOM toward higher oxidation state (higher NOSC) and often greater aromatic/saturation density, which is consistent with the same directionality captured in our AC2–AC1 deltas for produced formulas (Figure 6a).^{62,65} This behavior is predicted by thermodynamic theory linking the NOSC to energy yield during oxidation and has been repeatedly observed as DOM moves along degradation continua in freshwater systems.^{61,66} According to our data, ZIPGEM under AC2 (nitrate and phosphate copulse) follows this oxygenation-first trajectory (higher O/C, NOSC; lower DBE), whereas BIPGEM under AC2 exhibits a saturation-dominated route (strengthening $\pm$ H₂ and $\pm$ CH₂ steps and suppressing oxygen-insertion reactions), culminating in heavier but less-labile products (Figure 6a–c; section 3.4).

At the enzymatic or stoichiometric level, P limitation is well-known to elicit phosphatase expression and P-scavenging strategies that reshape DOM/DON processing. Recent work showed that even humic-like DOM, which is typically considered recalcitrant, can be consumed under P stress with elevated alkaline phosphatase activity, highlighting that DOM quality and community state pivot on P availability.^{67,68} In the absence of added P, transformation is more selective and class-

specific (sections 3.1–3.2), and the retained window remains stable (Figure S9b). When P is supplied (AC2, the combined presence of nitrate and phosphate), communities can relax P-scavenging constraints, increase growth efficiency, and engage broader substrate utilization. In BIPGEM, this manifests as a shift toward side-chain modification and H-transfer (Figure 6b,c), while in ZIPGEM it manifests as oxygen-inserting reactions (Figures S10–S11). Importantly, although microbial activity was not measured in this molecular data set, qPCR results from our study on the same cascade biofilters⁶⁹ indicate substantial denitrification genetic potential (nirS, nosZ) under AC1–AC2 (Table S15), providing contextual support for biologically mediated redox/N cycling capacity during the pulse experiments. Because microbial community composition and activity were not measured concurrently with FT-ICR MS sampling, and because ZVI-containing media can also drive abiotic sorption/fractionation and iron-mediated redox transformations, the present data set cannot distinguish biotic from abiotic contributions to the observed DON transformations.

Additional lake-scale context connecting nutrient status, DOM chemodiversity, and oxidation-state trajectories is provided in Supplementary Note S3. Our findings also resonate with studies of nutrient stoichiometry and long-term DON fate in ecosystems. In P-limited lakes subject to chronic N inputs, for instance, researchers have observed an accumulation of refractory DON compounds. Goldberg et al.⁷⁰ reported that high-elevation lakes transitioning to P limitation (due to N deposition) showed buildup of protein-like DOM with multidecadal residence times and proposed that refractory proteinaceous material can act as a sink for excess reactive N in the absence of sufficient P.⁷⁰ This phenomenon directly parallels our AC1 condition, where P scarcity is consistent with curtailed DON turnover and greater persistence of N rich organic residues. Conversely, the AC2 condition (nitrate + phosphate copulsing) can be seen as preventing such accumulation by enabling microbes to mineralize or assimilate DON that would otherwise become “locked” in recalcitrant forms.

In practical terms, Figure 6 and Figures S9–S11 distill the phosphorus copulsing effect into two actionable levers for

stormwater design: (i) tuning influent N:P to select an oxygenation-dominated regime (ZIPGEM) or a saturation-dominated regime (BIPGEM), and (ii) selecting/ordering media to exploit these regimes under cascade hydraulics (upstream shock, downstream polish; section 3.1). The literature suggests such stoichiometric steering is predictable—P enrichment increases microbial use of labile substrates and pushes residual DOM/DON toward higher NOSC—while our KMD-resolved Δm fingerprints (Figure 6b,c; Figures S10–S11) show how the relative prevalence of modular edit families changes across media under AC2.^{61,62} Together, phosphorus copulsing shapes both the extent and the mode of DON transformation, with media chemistry determining which transformation fingerprints are most strongly expressed under otherwise similar nutrient pulses.

A consolidated synthesis paragraph is provided in Supplementary note S4 to avoid repeating the key implications in the main text. It is important to note that this analysis focused on CHON formulas to resolve DON-specific transformations. Phosphorus-containing molecular formulas (e.g., CHOP or CHONP) were not included in the primary interpretation. While inclusion of CHOP could provide additional insight into coupled organic N–P molecular dynamics, such analysis would extend beyond the defined scope of this study. Future work integrating CHON and CHOP transformation networks may further clarify phosphorus–organic nitrogen coupling mechanisms in biofiltration systems.

3.6. Media-Level Comparison and System Design Implications

To translate the preceding molecule-centric results into actionable design, we compare the two media as whole-system selectors of DON fate under spiked nitrate-only (AC1) and spiked nitrate+phosphate (AC2) pulses. With this systems lens in place, we reveal where the two media truly diverge and what those divergences mean for robust, scalable stormwater treatment by fate-media-resolved descriptor distributions (Figure S12), significance testing of between-media contrasts (Table 1), and class-level accounting of formulas removed, produced, and retained (Table S16).

Across both pulses, formulas removed by BIPGEM are heavier and more unsaturation-dense than those removed by ZIPGEM: DBE, (DBE–O)/C, AImod, and mass are all significantly higher for BIPGEM, whereas O/C, NOSC, N/C, and X_c are higher for ZIPGEM (Figure S12 and Table 1). This pattern is consistent with the BIPGEM recipe (biochar and ZVI) favoring π – π interactions,⁷¹ hydrophobic partitioning,⁷¹ and metal-mediated complexation⁷² of lignin-like/condensed features, while ZIPGEM preferentially takes up more oxygenated, N-enriched formulas. This is also consistent with class balances table (Table S16). The removed pool in BIPGEM is dominated by lignin-like and condensed-aromatic formulas with small carbohydrate shares (Table S16), whereas ZIPGEM removes a larger fraction of oxygenated classes (e.g., carbohydrates and protein/amino sugar). Mechanistically, this matches Sections 3.2–3.3, with BIPGEM behaving as a physical retention/complexation sink for aromatic and high-DBE DON, while ZIPGEM expresses sorption of polar, oxidized species. However, because pore structure, surface functional groups, and nitrogen content of the biochar were not characterized, the present study cannot determine whether the observed BIPGEM–ZIPGEM differences arose primarily

from adsorption, redox/electron-transfer processes, enhanced microbial attachment, or a combination thereof.

Media effects invert when we examine produced formulas. Under nitrate only (AC1), differences are modest; under nitrate + phosphate (AC2) they diverge. BIPGEM–AC2 produces formulas with higher DBE and more negative (DBE–O)/C than ZIPGEM (Table 1), consistent with the unsaturation-building mode inferred from the reaction families (Section 3.4). In contrast, ZIPGEM–AC2 produces formulas with higher O/C, NOSC, X_c , and N/C (Table 1 and Figure S12), indicating oxygenation and aliphaticity. Class data align with these descriptor shifts: in BIPGEM the carbohydrate share of the produced pool drops strongly from AC1 to AC2 (from 22.78% $\rightarrow$ 4.96%), with lignin-like features rising (Table S16), whereas in ZIPGEM the produced pool remains more carbohydrate/protein-like and becomes modestly more oxygenated under AC2. These media-specific outcomes mirror the phosphate-gated trajectories we resolved by KMD (Section 3.4): BIPGEM weights $\pm H_2$ and $\pm CH_2$ edits that build unsaturation density, while ZIPGEM weights $\pm O/\pm OH/\pm H_2O$ edits that introduce oxygen and increase nominal oxidation state.

The retained pool shows systematic media contrasts even as its spread remains tight. BIPGEM retains formulas with higher DBE and (DBE–O)/C, while ZIPGEM retains formulas with higher O/C, NOSC, N/C, and X_c (Table 1 and Figure S12). In other words, when a formula survives both influent and effluent, its structural fingerprint still remembers the media, BIPGEM-retained = more unsaturated/aromatic; ZIPGEM-retained = more oxidized/N-enriched.

In a word, ZIPGEM provides more reliable phosphate control across pulses, while also delivering stronger nitrate removal and driving the produced DON toward a more oxidized, aliphatic, and comparatively labile signature. BIPGEM's strength is compositional steering of DON rather than P capture. Under phosphate copulses it shifts the produced pool toward heavier, unsaturation-rich, less-labile structures, whereas under nitrate-only pulses it can release previously sorbed phosphate.

Framed in the system design terms, BIPGEM and ZIPGEM offer complementary levers for managing DON structure change and nutrient removal in cascade upflow biofilters. Selecting the order and areal loading of each layer allows practitioners to target either phosphate capture with oxidative polishing of residual DON (ZIPGEM-led) or molecular shaping toward unsaturation-rich, less labile products (BIPGEM-led), while the retained window remains stable and narrow. This media-aware, fate-resolved perspective turns molecular-level diagnostics (Figures S12, 3, 4, 5, and Tables 1, S16) into actionable rules for designing sustainable, scalable, and resilient stormwater treatment.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.est.5c16948>.

Media matrices and reactor dimensions; SPE procedures; 21 T FT-ICR MS acquisition, data processing, and formula assignment; chemical descriptor definitions and operational classification of removed, produced, and retained DON formulas; supplementary tables and figures summarizing elemental-class distributions, de-

scriptor statistics, biochar properties, and CHON fate-pool distributions based on formula count and cumulative relative abundance; supplementary notes on descriptor trends, saturation-redox zonation, phosphorus effects, interpretation limits, and lake-scale nutrient/DOM oxidation-state context; references (PDF)

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Notes

The authors declare no competing financial interest.

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