Integrating Contaminant Source Indicators, Water Quality Measures, and Ecotoxicity to Characterize Contaminant Mixtures and Per- and Polyfluoroalkyl Substances (PFAS) Variability in an Urban Watershed

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ABSTRACT

Thousands of chemical contaminants threaten watersheds but are time and cost prohibitive to monitor. Identifying their sources, transport, and ecological risk is limited in heterogenous urban watersheds. We present an integrative watershed approach using source-specific indicator compounds, common water quality measures, and ecotoxicity assays to examine the distribution of contaminant mixtures in an urbanized watershed. Indicator compound concentrations were temporally and spatially distributed for treated/untreated sewage (sucralose, artificial sweetener), road runoff (diphenyl-guanidine [DPG] and 6PPD-quinone [6PPD-Q], automobile tire additives), and lawncare runoff (aminomethanephosphonic acid (AMPA), major degradant of the herbicide glyphosate). Sucralose was predominately sourced from treated wastewater; measurable concentrations in tributaries indicated raw sewage inputs. DPG and 6PPD-Q concentrations correlated to road density during base flow and were elevated during stormflow. AMPA was measurable spring through fall, especially where lawns were dense. When specific sources dominated flow, water quality measures correlated with wastewater (sulfate, potassium, chloride, and sodium) and road runoff (chromium and lead) indicators. The limited behavioral toxicity observed in exposed zebrafish (*Danio rerio*) (18%) was not well explained by source-indicators. PFAS concentrations were highly variable spatially but not well explained by our source-specific indicator compounds. More costly compound-specific monitoring may be necessary when multiple sources exist or when unexpected toxicity trends occur.

SYNOPSIS

Thousands of chemicals persist in urban waterways. We target a set of compounds to assess the presence and ecotoxicity of these mixtures and PFAS, which vary across space, season, and water level.

INTRODUCTION

Thousands of chemical contaminants compromise drinking water resources and threaten sensitive aquatic biota.[1–3] Contaminants enter waterways through sources such as stormwater, leaky sewers, treated wastewater, agriculture, and industrial waste.[4–8] Despite improved analytical approaches to detect legacy and novel contaminants at lower concentrations,[9] monitoring contaminant mixtures in urban waterways is cost prohibitive. Large monitoring efforts have characterized complex chemical mixtures in urban watersheds,[2,5,10,11] yet are constrained to specific locations and time periods. Thus, understanding of the environmental transport and fate of these complex chemical contaminant mixtures is not well understood.[12] Further, many local water monitoring efforts in the United States are limited to regulated or lower-cost water quality measures (major ions, nutrients, and trace metals).

The monitoring of specific synthetic compounds[13] or mixtures[14,15] can help identify the presence and source of contaminant mixtures. These "indicator" compounds are pollutants released by specific sources, and persist at measurable levels in waterways after release.[13] When an indicator is detected, a broad mixture of compounds associated with the pollutant source likely co-occur. For example, the detection of a wastewater indicator indicates the potential presence of pharmaceuticals, personal care products, and surfactants. To assess the biotic stress associated with urban mixtures the use of a light-dark behavioral assay with zebrafish (*Danio rerio*) larvae has been demonstrated to be an effective, low-cost, high-throughput approach (for wastewater effluent,[16,17] stormwater contaminants,[18] and metals[19]). The ecotoxicity assays assess the toxicity of contaminant mixtures that co-occur in a water sample, not the toxicity of a specific source-indicator.

We targeted three groups of indicators associated with common pollutant sources in urban watersheds. Our wastewater indicator, sucralose, is a widely used artificial sweetener under the trade name Splenda. Elevated concentrations of the compound have been measured in treated and untreated municipal wastewater.[13] Our road runoff indicators were diphenyl-guanidine (DPG) and 6PPD quinone (6PPD-Q). These two synthetic chemicals are added to automobile tires and are both detected in road dust[20] and stormwater runoff from roads and parking lots.[21] Aminomethanephosphonic acid (AMPA), the major degradation product of glyphosate, was targeted as an indicator of lawncare runoff. Glyphosate is the active compound in Roundup, a widely used synthetic herbicide for household lawns. Glyphosate is transported via runoff from urban lawns[22,23] and readily breaks down into AMPA through microbial metabolism.[24–26] We expected AMPA to serve as a better indicator given its longer half-life relative to glyphosate.[27] While AMPA and glyphosate are also found in agriculture runoff,[28] the watershed investigated in this study had minimal agricultural landcover (<2% crop and hay landcover[29]).

PFAS are emerging contaminants previously measured in North Carolina surface waters downstream from various sources: wastewater treatment plants, urban development, and fields where municipal biosolids were applied.[30] Consequently, we targeted 27 PFAS to investigate, with our integrative approach, to identify potential point and non-point sources of the compounds. The targeted compounds are commonly found in textiles, firefighting foams, or consumer products,[30] and have been considered for state and federal United States drinking water regulations.[31]

We conducted sampling in the heterogenous urban watershed of Ellerbe Creek (Durham, NC) with synoptic sampling (base flow) and temporally across seasons and flow regimes with biweekly sampling. Prior work, limited in spatial and temporal scope, documented hundreds of contaminants accumulating in the creek's outlet,[6,32] likely from stormwater, treated wastewater, and raw sewage.

The primary objective of this study was to analyze how contaminant mixtures from treated and untreated sewage, road runoff, and lawn care runoff vary spatially and temporally within a heterogeneous urban watershed. We employed an integrated approach that combined three sets of source-specific indicator compounds with standard water quality measures and a low-cost ecotoxicity assay. We had four aims: (1) How do source-specific chemical indicators change in concentration spatially and temporally across an urbanized watershed? (2) Do traditional water quality measures capture this variation? (3) Do the observed mixtures have lethal or sublethal toxicity? (4) Do PFAS concentrations track source-specific chemical indicators, implying possible attribution to a source?

For our first aim, we anticipated sucralose concentrations would be highest during base flows due to sewer leaks or wastewater effluent.[33] We expected road indicator concentrations to be highest during stormflow due to impervious surface runoff.[20,21] We anticipated elevated concentrations of AMPA during the growing season (spring through fall) when household lawns and parks are managed with lawncare products.

For our second aim, we expected source-specific indicators to correlate with other lower-cost measures, when specific contaminant sources predominated. For our third aim, we expected to observe sublethal effects in zebrafish embryos exposed to stream samples with high amounts of wastewater, road runoff, or lawncare runoff. For our fourth aim, we expected PFAS concentrations to be variable throughout the watershed, given multiple sources,[34,35] and indicator compounds to identify sources of PFAS, if any.

MATERIALS AND METHODS

Site Selection

An urbanized watershed (Ellerbe Creek) and neighboring forested watershed (New Hope Creek) were sampled during 2021–2022 (Figure 1). Water sampling took place over two regimes: biweekly sampling at two sites in Ellerbe Creek and one site in New Hope Creek, and seasonal synoptic sampling at up to 35 sites across Ellerbe Creek.

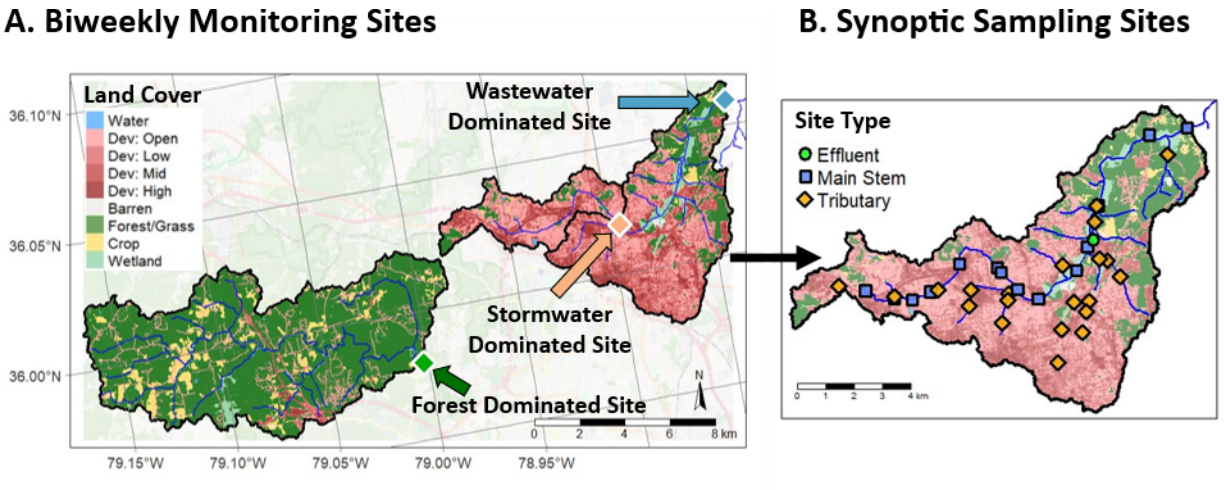

Figure 1: Sampling Sites for Temporal and Spatial Sampling. *(A) The Forest dominated site is in New Hope Creek (green), the Stormwater and Wastewater dominated sites (orange and blue, respectively) are in Ellerbe Creek (Dev, developed). (B) Within Ellerbe Creek, synoptic sampling was conducted across tributaries (yellow diamonds) and along the Ellerbe Creek (EC) main stem (blue squares). The municipal wastewater effluent outfall channel was also sampled (green circle); it releases directly into the EC main stem. Landcover data are grouped from the 2021 National Landcover Database, basemap from OpenStreetMap.*[29,36]

Biweekly sampling assessed temporal variability in concentrations, especially during different discharge regimes (Figure 1A). The wastewater dominated site ("WD") was at U.S. Geological Survey (USGS) streamgage 02086849 (Ellerbe Creek near Gorman, North Carolina) near Ellerbe Creek's outlet and downstream from a wastewater treatment plant (WWTP) effluent outfall. The stormwater dominated site ("SD") was on Ellerbe Creek's (EC) main stem at USGS streamgage 0208675010 (Ellerbe Creek at Club Boulevard at Durham, North Carolina), upstream from the WD and the WWTP. Most water inputs are from residential and road runoff. The forest dominated site ("FD") was in New Hope Creek,[37,38] which drains a forested watershed. Base-flow biweekly

samples were collected every 2 weeks (Figure 2). When biweekly sampling coincided with stormflow, samples were collected on the falling limb of the storm hydrograph.

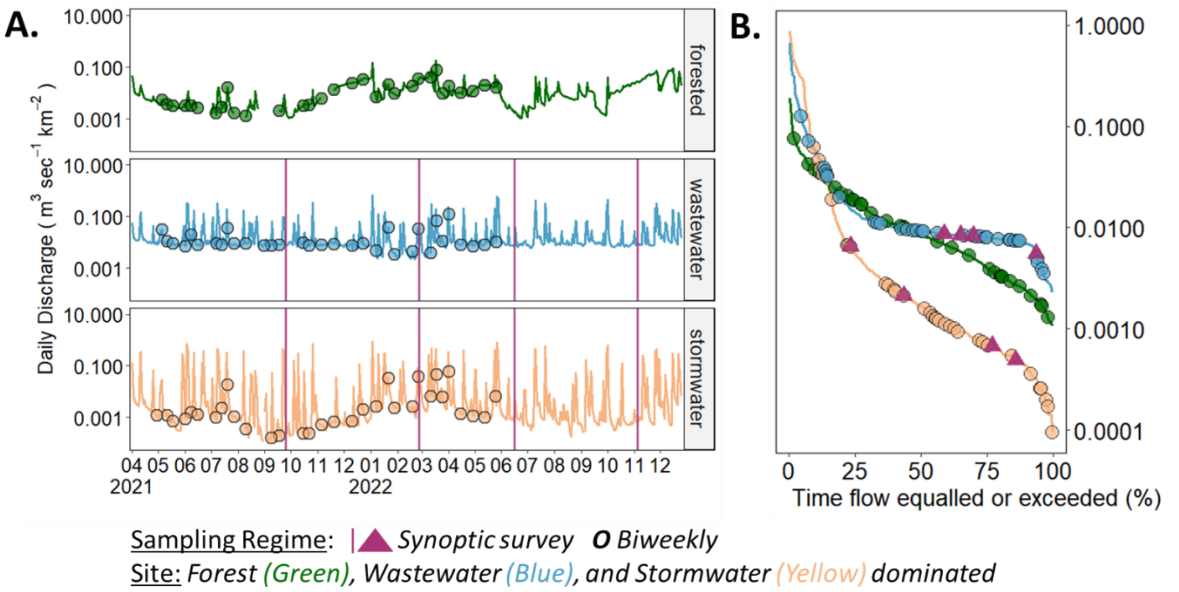

Figure 2: Discharge Regime During Biweekly and Synoptic Sampling. *(A) Biweekly sampling was conducted across a multitude of flow regimes. A circle represents when biweekly samples were collected, and purple lines represent synoptic survey dates. Y axis is daily discharge in cubic meters per second per square kilometer (m^3 sec^{-1} km^{-2}). (B) Each sampling date is marked on each site's flow-duration diagram; synoptic surveys are shown with purple triangles. Discharge is normalized to watershed area (forest dominated: 81.1 square kilometers (km^2), wastewater dominated: 56.7 km^2, and stormwater dominated: 15 km^2).*

Seasonal synoptic sampling assessed spatial variation in concentrations across the Ellerbe Creek watershed. The watershed comprises low to high density residential and commercial development across its 33 urban/suburban sub-catchments (Figure 1). Samples were collected at up to 34 sites throughout the Ellerbe watershed during relatively steady-flow conditions (Figure 1B and Tables S1, 2). We sampled up to 19 EC tributaries, draining a sub-set of these catchments, 14 EC main-stem sites, and the effluent outfall. Eleven EC main-stem sites are located upstream from the WWTP outfall, and 3 are downstream from the WWTP outfall. Synoptic sampling was conducted

during base flow on 09-25-2021 (late summer), 02-26-2022 (winter), 06-16-2022 (late spring), 11-05-2022 (fall) (Figure 2).

Water Collection

We collected water samples with a modified centroid-of-flow sampling method[39] by wading into the midpoint of stream flow. Care was taken to minimize sediment disturbance. Separate acid washed polypropylene Nalgene bottles were rinsed twice and filled with unfiltered water or acid-washed syringe filtered water, per established methods,[6] at each site. Filters were pre-combusted Whatman glass microfiber filters (GF/F) (nominal pore size = 0.7 micrometer [μm]). A separate unfiltered sample was collected in a sterile vial for *Escherichia coli* (*E. coli*) analysis. During collection, a YSI proDSS multi-probe was used to measure stream properties, including pH, water temperature, dissolved oxygen, and specific conductance. Samples were collected within a few hours of each other. Samples analyzed for *E. coli* were processed within 3 hours. Remaining samples were frozen until analysis.

A field blank was collected in the first three synoptics to verify minimal background contamination. The method quantification limit (MQL) was calculated based on the lowest analytical standard accepted for each analysis. Instrument blanks were less than MQL for analyses.

Discharge

Discharge (cubic feet per second [$ft^3\ sec^{-1}$]) was measured on a 15-minute interval for the three sites sampled biweekly. Discharge data were downloaded from the USGS National Water Information System database for the two gaged urban sites (SD and WD).[40] Discharge values ($ft^3\ sec^{-1}$) were converted to cubic meters per second ($m^3\ sec^{-1}$) by dividing by 35.315. We deployed water and air pressure sensors at the FD site because no USGS streamgage was nearby. We

166 calculated height (feet, ft) from sensor data and discharge (ft^3 sec^{-1}) by using a rating curve and
167 Manning's equation specific to the FD site.

168 **Chemical Analyses**

169 Targeted chemical analyses were conducted for indicator compounds, PFAS, and water quality
170 measures. PFAS was measured only in the synoptic survey samples; the other analytes were
171 analyzed in both sampling regimes. A brief description of the analytical methodology is provided,
172 with additional information (e.g., instrument, internal standards, and compound-specific methods)
173 in the Supporting Information (Tables S3-5). All reported means were calculated with measurable
174 concentrations. Method blanks were consistently less than MQL for indicators, major ions,
175 nutrients, and *E. coli*. Concentrations were low or less than MQL for PFAS and metals.

176 Mass loading was calculated at the urban sites by multiplying the site's mean daily discharge,
177 on the sampling date, by the measured indicator concentration or ½ the MQL when unmeasurable.
178 Mass loading was not calculated for glyphosate given infrequent quantification. A full list of each
179 solute's concentration is available at the Environmental Data Initiative repository.[41]

180 ***Indicator compounds: Sucralose, DPG, 6PPD-Q, AMPA, and Glyphosate***

181 Unfiltered water was minimally processed using existing methods[26] before direct injection into
182 a liquid chromatography with tandem mass spectrometry (LC-MS/MS) for quantification of the
183 five indicator compounds.

184 ***PFAS***

185 Twenty-seven PFAS were targeted for quantification using an existing direct injection targeted
186 high-performance liquid chromatography-mass spectrometry (HPLC-MS) method deployed by the
187 North Carolina PFAS Testing Network.[42] A full compound list is provided in Tables S6-7. In one
188 field blank, 2 compounds had quantifiable concentrations; an MQL of 3 times the blank

concentration was used for these compounds (additional information in the Supporting Information). The total PFAS concentration for a given sample is the summation of quantified concentrations for each compound targeted.

Water Quality Measures: Major ions, nutrients, metals, and E. coli

Filtered water samples were assessed for major ions and nutrients. Chloride, sulfate, bromide, nitrate as nitrogen (N), sodium, potassium, magnesium, and calcium ion concentrations were quantified with ion chromatography.[43] Ammonium as N and orthophosphate as phosphorus (P) concentrations were quantified on a flow injection analyzer.[44,45] Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were quantified per U.S. Environmental Protection Agency (EPA) and ASTM methods.[46,47] Unfiltered water samples were microwave-digested[48] and quantified by inductively coupled plasma-mass spectrometry (ICP-MS)[49] for select trace metals: arsenic, cadmium, chromium, copper, cobalt, lead, nickel, and zinc. A subset of the biweekly samples, analyzed with the same methods for trace metals, have been previously published[50] and are freely available.[51]

Water samples collected in sterile vials were processed within 3 hours of collection for *E. coli* per the EPA-approved 24-hour Colilert method, with a 97-well Quanti-Tray/2000.[52]

Spatial Analyses

The watershed of each sampling site was delineated using USGS StreamStats.[53] Landcover categories were downloaded from the 2021 National Landcover Database[29] with the FedData R package.[54] Road and pipe shape layers were downloaded from the City of Durham,[55,56] and household parcel level data from InfoUSA, Inc.[57] Each measurement was divided by the total area of a site's watershed (FD: 81.1 square kilometers [km^2], WD: 56.7 km^2, SD: 15 km^2) to estimate road, pipe, and household density.

Ecotoxicity Analyses

A light-dark behavioral study was conducted on wild-type zebrafish embryos (Ekkwill) per established Danio Vision methods.[19] These behavioral assays were conducted separately for unfiltered water samples collected during the synoptic surveys (n=117). Treatment details are provided in the Supporting Information.

Statistical Analyses

We calculated non-parametric Spearman's correlation coefficients (ρ), and associated p-values, in R with the Hmisc package[58] to compare indicators and PFAS against each other and against water quality and landscape measures. For these calculations, samples with concentrations below the MQL were replaced with ½ the compound-specific MQL. We recognize different p-value thresholds are used in the fields of environmental chemistry and ecosystem ecology to assess confidence in measured correlation. Therefore, we consider a p-value of less than 0.1 as evidence of a relationship. We identify when a p-value is smaller than 0.05 or 0.01, as these values provide stronger evidence for the calculated correlations.

For zebrafish toxicity experiments, exposed larvae were compared against control groups by analysis of variance (ANOVA) with Tukey's test post-hoc. A p-value threshold of 0.05 was used to test significance.

RESULTS AND DISCUSSION

Indicators Vary Across Space, Flow Regimes, and Season

All five candidate indicator compounds were successfully quantified in at least a subset of the urban stream water samples, and the spatial and temporal variation in concentrations for each compound were consistent with our expectations for the contaminant mixtures (wastewater, road

runoff, lawncare runoff) we intended them to represent. Sucralose was measurable at about 77% of the urban synoptic sampling sites (27/35) on at least one sampling date, and the highest concentrations were consistently measured downstream from the wastewater effluent outfall channel (Figure 3). In biweekly sampling, sucralose concentrations were diluted by stormflows at the WD site downstream from the WWTP (Figure 4). Relative to the WD site, sucralose concentrations at the upstream SD site were lower or below quantification (Figure 4).

Compounds 6PPD-Q and DPG (indicators of road runoff) were quantifiable in about 94% of the urban synoptic sampling sites (34/35 and 32/35 sites, respectively) (Figure 3). In biweekly sampling of the two urban sites, concentrations of these road runoff indicators peaked during high discharge (Figure 4).

As expected, glyphosate was rarely quantifiable across the urban synoptic sites (6/35 sites) and was never quantifiable during winter months (Figures 3 and 4). Its breakdown product, AMPA, was quantifiable at about 77% (27/35) of the synoptic sites (Figure 3) and frequently across the two urban biweekly sites (Figure 4) during the growing season (fall, summer, spring). Glyphosate was not measurable in wastewater effluent whereas AMPA was near or below quantification. The distinct spatial and temporal variation between these three indicator compounds offers encouraging evidence they can be effectively used to indicate contaminant sources.

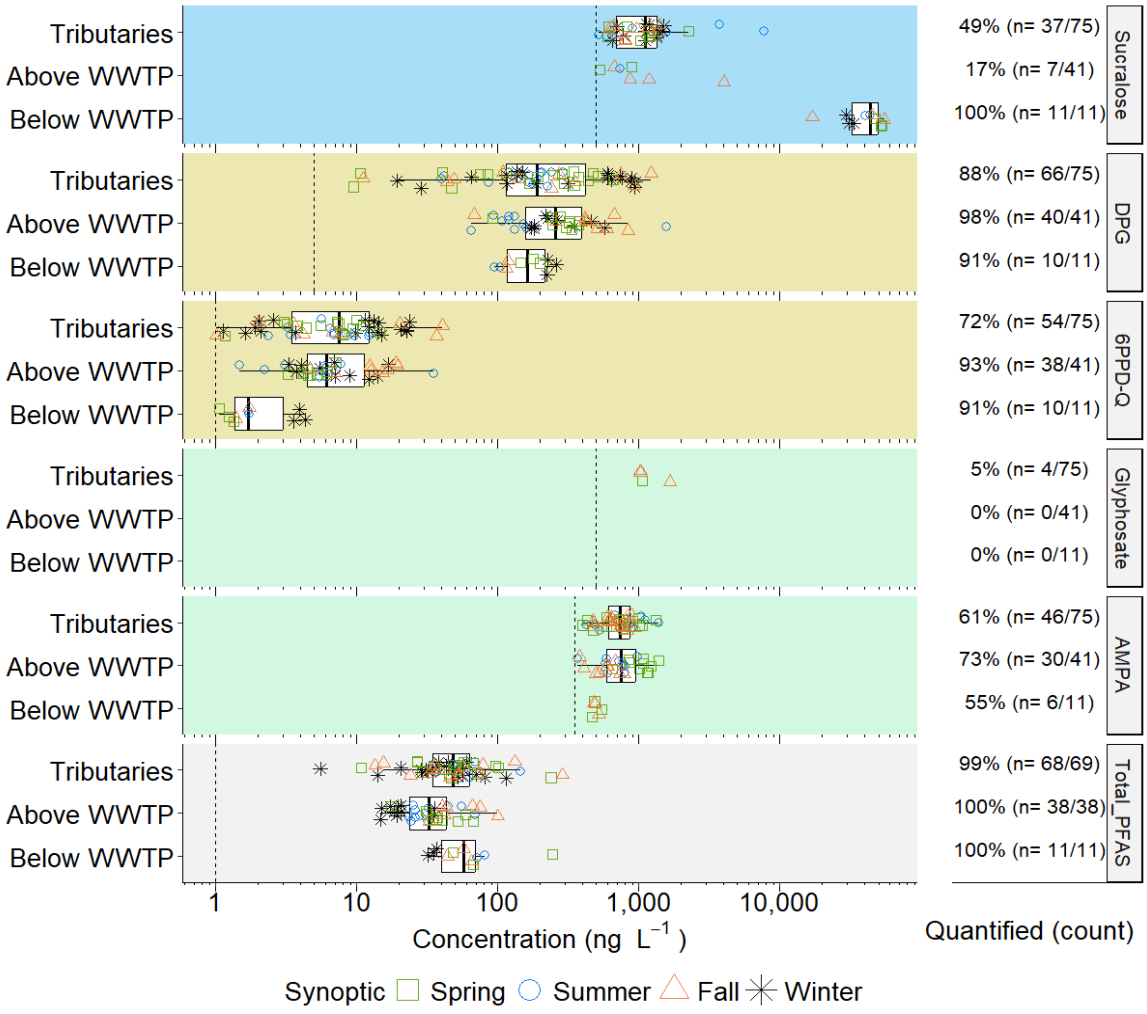

Figure 3: Indicator Compound Concentrations: Seasonal Spatial Synoptic Sampling. *Sites are grouped as Ellerbe Creek (EC) tributaries or EC main-stem sites upstream (above) or downstream (below) from the wastewater treatment plant (WWTP) effluent outfall. Each point is a site-date shaped and colored by the synoptic survey date. The compound-specific method quantification limit (MQL) is the dotted line. Values less than the MQL are not shown. Boxplots are shown when 10 or more observations are greater than the MQL and are in Tukey style (box comprised of 25th percentile, median value, and 75th percentile). The frequency of quantification and total number of samples analyzed for each group is on the right. The x-axis is log10 scaled with units of nanograms per liter (ng L^{-1}). DPG, diphenyl-guanidine; 6PPD-Q, 6PPD-quinone; AMPA, aminomethanephosphonic acid; PFAS, per- and polyfluoroalkyl substances.*

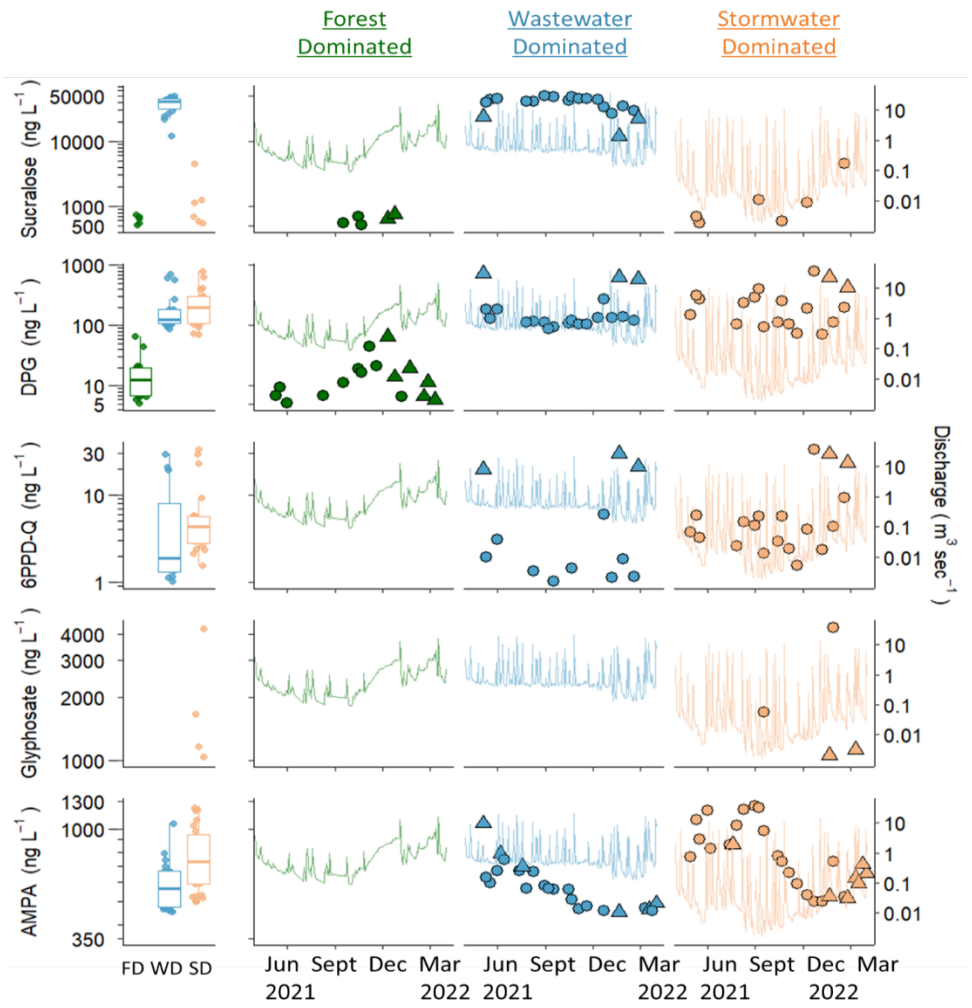

Figure 4: Indicator Compound Concentrations: Biweekly Sampling Across Flow Regimes: *Left: Compound concentrations are distributed by site: forest dominated (FD, green), wastewater dominated (WD, blue), stormwater dominated (SD, orange). Values less than the method quantification limit (MQL) are not shown. The MQL is the lowest y-axis value. Boxplots are shown when at least 10 observations are greater than the MQL. Right: a time series of water concentrations are shown for each indicator compound greater than the MQL (left y-axis labels; ng L^{-1}, nanograms per liter). Days with high daily discharge (upper 25th percentile, for a given site) are triangles; other days are circles. Daily mean discharge is shown as a line for each site (right y-axis labels; m^3 sec^{-1}, cubic meters per second). The y-axis is log10 scaled. DPG, diphenyl-guanidine; 6PPD-Q, 6PPD-quinone; AMPA, aminomethanephosphonic acid.*

On a given sampling day, the WW site had higher mass loadings of sucralose, road indicators, and AMPA relative to the SW site (**Error! Reference source not found.**). This is driven by the nearly order of magnitude higher discharge at the downstream WW site. Still, we observed higher

277 mass loading of DPG, 6PPD-Q, and AMPA during stormflow at the SW site relative to mass
278 loading of the same indicators during baseflow at the downstream WW site (**Error! Reference**
279 **source not found.**). This gives us further confidence in these three compounds as indicators of
280 road or lawncare runoff, even where wastewater effluent exists. As expected, mass loading of
281 sucralose was always higher downstream of the effluent release point (WW site) relative to
282 upstream (SW site) (**Error! Reference source not found.**).
283
284 ***Wastewater Indicator: Sucralose***
285 Spatial variation in sucralose concentrations was driven by direct wastewater effluent inputs to
286 the EC main stem. Sucralose concentrations on the EC main stem downstream from the effluent
287 outfall channel (mean: 40,654 ng L^{-1}) were similar in magnitude to concentrations measured in the
288 treated effluent (mean: 43,783 ng L^{-1}) during the synoptic surveys. Sucralose concentrations in the
289 effluent outfall were consistent with previously published concentrations of sucralose in municipal
290 wastewater effluent in the US (from 15,000 to 77,000 ng L^{-1} [13,59]). During base flow, treated
291 wastewater effluent comprises more than 95% of stream discharge at the most downstream site.
292 Released effluent is thus minimally diluted on the EC mainstem downstream of the wastewater
293 effluent outfall. Throughout the synoptics, sucralose was more frequently quantified on the EC
294 main stem downstream from the wastewater effluent outfall (100% quantified) relative to upstream
295 main-stem sites (17% quantified) and EC tributaries (49% quantified). When measurable,
296 concentrations were an order of magnitude higher at the EC main-stem sites downstream from the
297 effluent outfall channel relative to those upstream (mean: 1,272 ng L^{-1}) and in the tributaries (mean:
298 1,280 ng L^{-1}) (Figure 3).

No known intentional untreated sewage releases occurred during sampling, yet sucralose was measured on at least one synoptic sampling date at about 71% of EC tributaries and upstream EC main-stem sites (22/31 sites); three sites had measurable concentrations across all four synoptic sampling dates. When measured in EC tributaries, sucralose concentrations were considerably variable (520 – 7,681 ng L^{-1}). The highest concentrations were routinely measured in a developed tributary to Ellerbe Creek where sewage leaks have regularly been reported (per oral communications with Durham Stormwater Division, 2021). If we assume leaking sewers were contributing sucralose concentrations at similar levels to what was measured in the WWTP effluent (mean: 43,783 ng L^{-1}, 100% quantified) in the worst-case scenario, untreated sewage may contribute up to 18% of the base flow in this tributary. Taking this assumption further, anytime sucralose was measured at concentrations greater than the MQL of 500 ng L^{-1}, leaking sewage contributed at least 1% of flow, a situation observed in 49% of EC tributary samples. Across each synoptic survey, sucralose was not consistently correlated ($p>0.1$) to two proxies of sewage usage (household and stormwater pipe density) (Table 1). In the EC tributaries sampled, septic tanks are uncommon given municipal sewer lines. We presume leaky sewer pipes rather than sewage production drove variation in wastewater inputs to the creek. Given the stochasticity of leaks, sampling of sites across time is critical for monitoring efforts and source attribution. Similar magnitudes of sucralose concentrations have been measured in urban estuaries (18-3,180 ng L^{-1})[59] and surface waters with and without known wastewater inputs (<MDL-10,000 ng L^{-1})[13,60].

In biweekly sampling, sucralose concentrations were consistently elevated at the WD site (12,031 – 55,459 ng L^{-1}) relative to the SD site (<MQL – 4,552 ng L^{-1}) and FD site (<MQL – 751 ng L^{-1}) (Figure 4). After high precipitation, sucralose concentrations at the WD site decreased as stormwater diluted wastewater effluent inputs (Figure 4). The lowest concentration of sucralose at

322 the WD site was measured (16,911 ng L^{-1}; January 21, 2022) when daily mean discharge was
323 elevated (2.21 m^3 sec^{-1}) after precipitation. In contrast, sucralose concentrations were likely
324 sourced from a diffuse set of sewer leaks at the SD site and septic fields in the FD site. However,
325 samples frequently had concentrations less than MQL for sucralose at the SD site (37% quantified),
326 making temporal dynamics difficult to assess.

327 ***Road Runoff Indicators: DPG and 6PPD-Q***

328 Both road runoff indicators, DPG and 6PPD-Q, were quantifiable in the synoptic surveys (100%
329 and 85% quantified, respectively); DPG was observed at higher concentrations (9 – 1,570 ng L^{-1},
330 mean: 299 ng L^{-1}) relative to 6PPD-Q (<MQL – 35 ng L^{-1}; mean, when measurable: 8 ng L^{-1})
331 (Figure 3). Across EC tributaries sampled in the last three synoptic surveys, road density, storm
332 pipe density, and percent developed landcover (LC21DEV: developed landcover per the 2021
333 National Landcover Database[29]) positively correlated with DPG ($\rho > +0.48$, $p < 0.05$) and 6PPD-
334 Q ($\rho > +0.59$, $p < 0.01$) (Table 1). As expected, forested landcover negatively correlated with both
335 indicators for these three synoptics ($\rho < -0.62$, $p < 0.01$) (Table 1). In the first synoptic survey, the
336 subset of EC tributaries sampled were more similar in landcover, which likely explained the
337 absence of correlations.

338 The highest concentrations of DPG (1,570 ng L^{-1}) and 6PPD-Q (35 ng L^{-1}) were observed in a
339 synoptic site that received direct stormwater drainage from an interstate highway. Treated
340 wastewater effluent had low (DPG, 106 – 351 ng L^{-1}) or unmeasurable (6PPD-Q, <MQL)
341 concentrations. Concentrations of both road indicators were elevated across EC tributaries (DPG:
342 299 ng L^{-1}, 6PPD-Q: 9 ng L^{-1}) and upstream EC main-stem sites (DPG: 324 ng L^{-1}, 6PPD-Q: 8 ng
343 L^{-1}) relative to EC main-stem sites downstream from the WWTP outfall (DPG: 169 ng L^{-1}, 6PPD-
344 Q: 2 ng L^{-1}). Overall, these relations give us confidence in the ability for both compounds to

indicate road runoff inputs, particularly during base flow across EC tributaries that range in developed land cover (LC21Dev, 14 – 100%) and storm pipe density (0.05 – 12.3 km^2 km^{-2}).

Table 1: Indicator and Landscape Correlation Coefficients in Synoptic Ellerbe Creek Tributaries

Indicator	Synoptic	Road Density	Storm pipe Density	Developed (LC21)	Forested (LC21)	House Density
Sucralose	Summer	N.S.	N.S.	N.S.	N.S.	N.S.
	Winter	N.S.	N.S.	N.S.	N.S.	N.S.
	Spring	*0.4^*	*0.52**	N.S.	N.S.	*0.46**
	Fall	N.S.	N.S.	N.S.	N.S.	N.S.
6PPD-Q	Summer	N.S.	N.S.	N.S.	N.S.	N.S.
	Winter	*0.69***	*0.8***	*0.78***	*-0.78***	*0.69***
	Spring	*0.59***	*0.65***	*0.68***	*-0.66***	*0.59***
	Fall	*0.7***	*0.64***	*0.74***	*-0.78***	*0.72***
DPG	Summer	N.S.	N.S.	N.S.	N.S.	N.S.
	Winter	*0.71***	*0.83***	*0.74***	*-0.75***	*0.71***
	Spring	*0.58***	*0.55**	*0.65***	*-0.62***	*0.48**
	Fall	*0.58***	*0.54**	*0.62***	*-0.65***	*0.59***
AMPA	Summer	*-0.52**	N.S.	*-0.49^*	*0.51^*	N.S.
	Winter[ii]	*AMPA below MQL*				
	Spring	*0.49**	*0.67***	*0.59***	*-0.58***	*0.45**
	Fall	*0.47**	*0.54**	*0.55**	*-0.49**	*0.44^*

[I] Spearman coefficients, calculated from the Ellerbe Creek tributary sites, are marked based on p-value: p = 0.1 – 0.05(^), p = 0.05 – 0.01 (*), p < 0.01 (**). Non-significant values (p>0.1) are marked as "N.S." Units are: road and storm pipe density (square kilometers per square kilometers [km^2 km^{-2}]), developed (LC21Dev, percent), forested (LC21FOREST, percent), and house density (houses km^{-2}). LC21: landcover categories from the 2021 National Landcover Database[29]

[ii] AMPA concentrations were less than the MQL across the tributaries in the second synoptic survey.

In biweekly sampling, road indicator concentrations were consistently elevated during stormflow at the WD and SD sites (Figure 4). The highest concentrations of DPG (803 ng L^{-1}) and 6PPD-Q (26 ng L^{-1}) were observed on the falling limb of a storm hydrograph at the SD site (December 23, 2021). Concentrations of both compounds were similar at the WD and SD sites during stormflow (Figure 4), when stormwater comprises >95% of discharge relative to effluent.

When the WD site returned to base flow, DPG and 6PPD-Q concentrations were lower at the WD site relative to the SD site due to dilution from wastewater effluent (Figure 4). Across biweekly sampling, 6PPD-Q was only quantifiable in 60% of the samples collected at the WW site, whereas it was quantified in all SW site samples. At the FD site, which is only 8.2% developed and 1.3% impervious cover, 6PPD-Q concentrations were not measurable. DPG was frequently quantifiable at the urban sites (100% of samples) and at the FD site (87% of samples). The maximum concentration of DPG measured (65 ng L^{-1}) was lower than the minimum concentration measured at the SD site (72 ng L^{-1}) and WD site (89 ng L^{-1}) (Figure 4).

Synoptic and biweekly sampling results demonstrate the ease for quantifying DPG and 6PPD-Q (Figures 3, 4); however, the potential toxicity of the road runoff mixtures they represent are not understood without targeted stormflow sampling. Prior studies demonstrate contaminants associated with road runoff, including DPG, are highest in concentration during the first flush through storm peak, or rising limb, of a storm hydrograph and where road density is elevated.[63] We could only capture the falling limb of storm hydrographs in biweekly sampling; thus, our measured concentrations of DPG and 6PPD-Q were lower relative to those previously measured directly from stormwater for 6PPD-Q (210-720 ng L^{-1} in Toronto,[13] mean 593 ng L^{-1} in Saskatoon,[64] and mean 7,000 ng L^{-1} in Seattle[65]) and DPG (400-364,000 ng L^{-1} in Saskatoon[64] and 1,800 ng L^{-1} in Seattle[66]). We encourage careful interpretation of road indicators collected during base flow or the falling limb of the storm hydrograph; measurements can identify hotspots, but to quantify loading and the risk of contaminant mixture pulses to stream biota, targeted hydrograph sampling is warranted.

Lawncare Runoff Indicators: AMPA and Glyphosate

384 The main indicator of lawncare runoff, AMPA, was only measurable during the growing season
385 (spring, summer, fall) in our synoptic surveys (Figure 3). AMPA was quantifiable throughout EC
386 tributaries in 46 of the 55 samples collected (<MQL – 1,375 ng L^{-1}, mean, when measurable: 751
387 ng L^{-1}). Wastewater effluent, which had concentrations of AMPA near or less than the MQL during
388 the synoptic surveys (<MQL – 477 ng L^{-1}), dilutes EC tributary contributions. Thus, on the EC
389 main stem, AMPA concentrations were measurable upstream from the effluent outfall in 30 of the
390 31 samples collected (<MQL– 1,376 ng L^{-1}, mean, when measurable: 772 ng L^{-1}) while
391 concentrations were near or less than the MQL downstream from the outfall (<MQL – 546 ng L^{-1},
392 6/8 samples quantifiable).
393 Throughout the synoptic surveys, glyphosate was less frequently quantifiable. Glyphosate was
394 measurable in only 10% of EC tributary samples in the spring and fall synoptic surveys (<MQL –
395 1,663; mean, when measurable: 1,194 ng L^{-1}; 4/40 samples), but not anywhere on the EC main
396 stem. Our results were consistent with other studies that also found seasonality in glyphosate
397 concentrations,[67] while our work demonstrates AMPA concentrations also vary seasonally.
398 During the spring and fall synoptics, concentrations of AMPA across EC tributaries were
399 positively correlated (ρ = +0.59 and +0.55, $p < 0.05$) to a measure of lawn density (LC21DEV)
400 (Table 1). A substantial portion of the developed landcover category included lawns of suburban
401 homes and municipal parks across the sampled watersheds of EC tributaries (LC21Dev: 14 –
402 100%). The positive correlation between AMPA and storm pipe density during these two synoptics
403 (ρ = +0.67 and +0.54, $p<0.05$) indicated the potential importance of stormwater infrastructure for
404 transporting lawncare runoff across the watershed during the growing season. We expect the
405 absence of similar correlations in the summer synoptic survey (Table 1) was due to the subset of
406 tributaries sampled that were more similar in landcover.

In biweekly sampling, glyphosate was only measurable in the SD site (<MQL – 4,323 ng L^{-1}; mean, when measurable: 2,056 ng L^{-1}; 4/31 quantified) (Figure 4). Glyphosate was not consistently higher during stormflow at the SD site (Figure 4). This may be due to the proximity of the SD site to a city park immediately upstream from the sampling site (<50 m) and suburban lawns that surround the park. Application of glyphosate in the park and lawns during base flow may leach into the site.

AMPA was more frequently measured relative to glyphosate in biweekly sampling at both the SD site (100% and 13% quantified, respectively) and WD site (81% and 0% quantified, respectively). As we expected, glyphosate and AMPA concentrations were not quantifiable at the FD site, which has a low density of lawns throughout its forested watershed. AMPA at both urban sites was elevated in concentration during the growing season. AMPA concentrations were lower ($p < 0.01$) at the WD site (<MQL – 1,053 ng L^{-1}; mean, when measurable: 582 ng L^{-1}) relative to the SD site (<MQL – 1,248 ng L^{-1}; mean, when measurable: 791 ng L^{-1}). AMPA concentrations were near or less than the MQL during the winter at both sites (December and January) (Figure 4), when lawncare was limited.[68] During higher discharge, AMPA was reduced in concentration at the SD site, although a similar trend was not observed at the WD site (Figure 4). The greater frequency of quantifications for AMPA was likely due to microbial degradation of glyphosate in soils and surface waters[24] and AMPA's longer half-life relative to glyphosate in surface waters.[27,69]

We have stronger confidence in AMPA, relative to glyphosate, as a conservative and persistent indicator of lawncare runoff. We did not measure high AMPA concentrations in the wastewater effluent or downstream from the wastewater effluent outfall channel during our synoptic sampling (Figure 3). AMPA concentration increased during the growing season in our biweekly sampling of the WD site (Figure 4). This, together with the positive correlation between AMPA and lawn

measures across our spring and fall synoptic surveys, gives us confidence AMPA was sourced from lawn runoff, rather than non-landscape contaminant sources like phosphate detergent.

Although phosphorus detergents have been banned since 1988 in Ellerbe Creek's watershed,[70] in urban watersheds where phosphorus detergents remain in use, lawncare runoff may not be the only AMPA source. The detergent can degrade into AMPA during sewage treatment,[22,71] and AMPA is measurable in wastewater effluent year-round in watersheds where the detergents are used.[25,67]

PFAS Concentrations Highly Variable though not Explained by Source Indicators

The concentrations of 17 quantified PFAS compounds were highly variable throughout both the watershed and across seasons (Figures 5 and S3). Across the sites and synoptic surveys, total PFAS concentrations ranged from <MQL to 287 ng L^{-1} (Figure 3), with a median of 52.2 ng L^{-1}. We frequently quantified perfluorohexanoic acid (PFHxA, 97% quantified), perfluorooctanoic acid (PFOA, 97% quantified), perfluorooctanesulfonic acid (PFOS, 96% quantified), perfluorobutanesulfonic acid (PFBS, 92% quantified), perfluorohepanoic acid (PFHpA, 91% quantified), perfluorohexanesulfonic acid (PFHxS, 91% quantified), and perfluorobutanoic acid (PFBA, 88% quantified) across the Ellerbe Creek watershed. Variability in concentrations were likely driven by a diffuse set of PFAS sources common to urban watersheds[35] and degradation of multiple precursor PFAS into common degradation products. We did not have measurable concentrations of 10 PFAS, including precursor compounds that likely degraded in the environment (e.g., 4:2, 8:2, and 10:2 fluorotelomer sulfonic acid) (Tables S6-7).

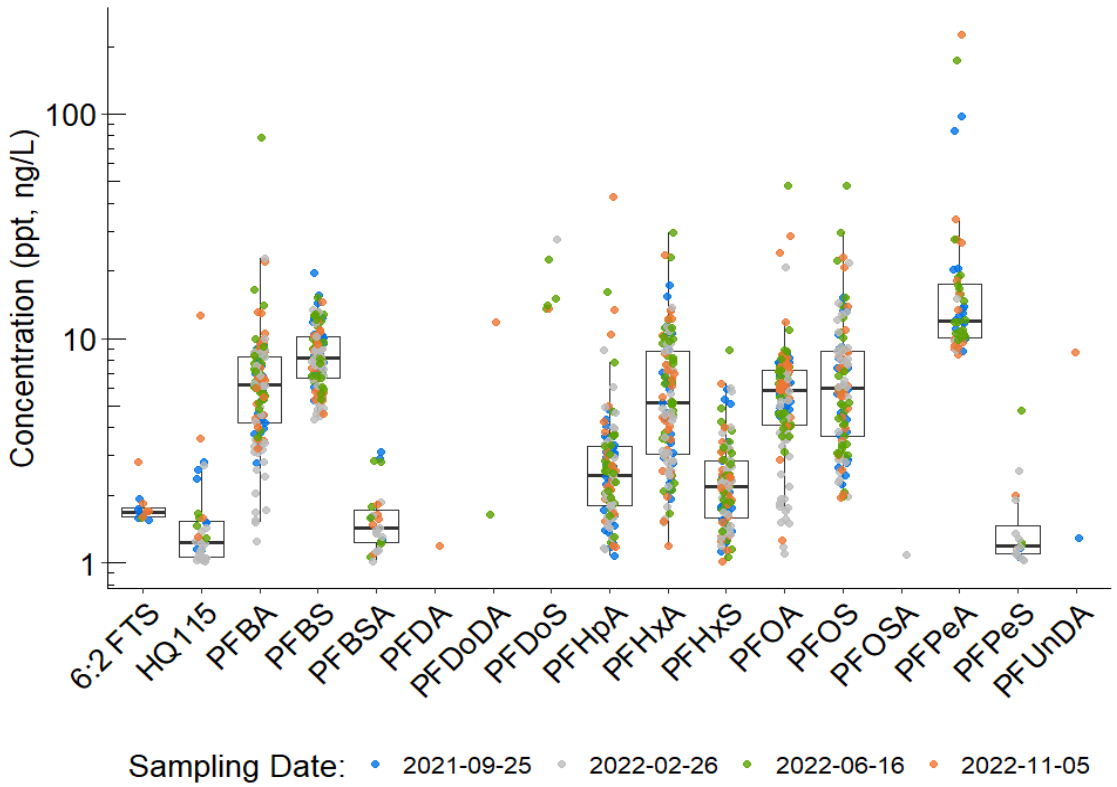

Figure 5. Per- and Polyfluoroalkyl Substances (PFAS) Concentration Range: *17 of the targeted PFAS compounds had at least 1 water sample with a measurable concentration. Samples are colored by sampling date. Samples with concentrations less than the method quantification limit (MQL) are not shown. The MQL is 4.0 and 8.3 nanograms per liter (ng L^{-1}) for perfluorobutane sulfonic acid (PFBS) and perfluorononanoic acid (PFNA); the MQL for all other PFAS is 1 ng L^{-1}. The y-axis is shown on a log-10 transformed scale. Boxplots are shown when 10 or more observations are greater than the MQL and are in Tukey style (box comprised of 25th percentile, median value, and 75th percentile). 6:2 fluorotelomer sulfonic acid (6:2 FTS), bis(trifluoromethane)sulfonimide lithium salt (HQ115), perfluorobutanoic acid (PFBA), perfluorobutane sulfonic acid (PFBS), perfluorobutane sulfonamide (PFBSA), perfluorodecanoic acid (PFDA), perfluorododecanoic acid (PFDoDA), perfluorodecane sulfonic acid (PFDoS), perfluorohexanoic acid (PFHxA), perfluorohexanesulfonic acid (PFHxS), perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), perfluorooctanesulfonamide (PFOSA), perfluoropentanoic acid (PFPeA), perfluoropentane sulfonic acid (PFPeS), perfluoroundecanoic acid (PFUnDA).*

Treated wastewater was a source of perfluorobutylsulfonamide (PFBSA) to Ellerbe Creek. PFBSA was frequently measured in the treated wastewater effluent (1.60 – 2.85 ng L^{-1}) released into Ellerbe Creek. Upstream of the wastewater effluent outfall, PFBSA was infrequently measured (7/34 quantified; <MQL – 1.48 ng L^{-1}) on the EC-stem while PFBSA was frequently

measured downstream of the effluent outfall on the EC main-stem (10/11 quantified; <MQL –3.13 ng L^{-1}) (Table S6). Prior research has demonstrated PFBSA is produced during wastewater treatment, and is not measurable in untreated sewage.[72] This likely explains why PFBSA was infrequently measured in samples collected across the EC tributaries where our wastewater indicator sucralose was measurable (2/37 samples). Given the presence of PFBSA in 6 samples collected from the EC tributaries (1.12 – 1.85 ng L^{-1}), additional unidentified sources are likely for the PFBSA.

The source of other targeted PFAS was not clear. Our source indicators did not consistently correlate and explain PFAS concentrations (Table S9) throughout the synoptic surveys. Only one PFAS compound consistently correlated to a landcover measure (storm pipe density and PFHxS; $\rho = +0.40 – 0.65$ and $p < 0.1$; Table S9).

Traditional Water Quality Measures Infrequently Predict Indicator Concentrations

The relative stability and specificity of our indicator compounds could be useful in watersheds where lower-cost water quality measures have multiple sources or stream conditions enable their degradation (e.g., nutrients) or removal (e.g., sorption and precipitation of ions/metals). The major ions, nutrients, and trace metals did not consistently correlate with our source indicators across synoptic or biweekly sampling ($p > 0.1$) (Tables 2 and S10). Reactive nitrogen and phosphorus-based nutrients were inconsistently correlated with indicators of lawncare runoff and wastewater across sampling; both likely degrade readily in-stream.[73,74] Trace metals transported from roads via stormwater (e.g., lead, copper, zinc)[75,76] did not consistently correlate with our road indicator compounds (DPG and 6PPD-Q) throughout sampling regimes.

The WD site may demonstrate the usability of lower-cost water quality measures when a dominant point source, such as wastewater effluent, outweighs other sources. Compounds elevated

in treated wastewater (sulfate, potassium, chloride, and sodium) were positively correlated with sucralose in biweekly sampling at the WD site (ρ = +0.52 to +0.78, $p < 0.05$) and negatively correlated with road indicators (ρ = -0.48 to -0.66, $p < 0.05$) (Table 2). Two trace metals common in vehicle wear and historical gasoline use, chromium and lead,[77] were positively correlated to both road indicators at the WD site (ρ = -0.48 to -0.66, $p < 0.05$) (Table 2). These trends are likely driven by dilution of effluent (sucralose enriched) with stormwater (DPG and 6PPD-Q enriched). However, these correlations were not statistically significant ($p > 0.1$) at the other two sites sampled biweekly (Table 2) and infrequently across synoptic surveys of EC tributaries (Table S10).

Table 2: Biweekly Sampling Spearman Correlation Coefficients Between Indicators and Water Quality Measures [i]

Site	Indicator	SO_4	K	Cl	Na	Cr	Pb	NO_3	Ni	Cu
WD	Sucralose	*0.55**	0.52*	0.60**	0.78**	-0.44^	-0.66**	*N.S.*	0.48*	*N.S.*
	6PPD-Q	-0.59**	-0.53*	-0.48*	-0.66**	0.45^	0.68**	*N.S.*	*N.S.*	*N.S.*
	DPG	-0.62**	-0.52*	-0.53*	-0.66**	0.82**	0.80**	*N.S.*	*N.S.*	*N.S.*
	AMPA	*N.S.*	-0.34^	*N.S.*	*N.S.*	*N.S.*	*N.S.*	-0.49**	0.38*	*N.S.*
SD	Sucralose	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	-0.49*
	6PPD-Q	-0.40^	*N.S.*	*N.S.*	*N.S.*	*N.S.*	0.47^	*N.S.*	*N.S.*	*N.S.*
	DPG	-0.48*	-0.46*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	0.45^
	AMPA	*N.S.*	*N.S.*	-0.33^	*N.S.*	-0.53**	-0.41*	*N.S.*	-0.51**	*N.S.*
FD [ii]	Sucralose	*N.S.*	0.61**	*N.S.*	*N.S.*	*N.S.*	*N.S.*	-0.48*	*N.S.*	*N.S.*
	DPG	*N.S.*	0.76**	*N.S.*	*N.S.*	*N.S.*	-0.51*	-0.56*	*N.S.*	*N.S.*

[i] *Spearman coefficients are marked based on p-value: p = 0.1 – 0.05(^), p = 0.05–0.01 (*), p < 0.01 (**). Non-significant values (p>0.1) are marked "N.S." Positive correlations are green and negative correlations are red. WD, Wastewater dominated site; SD, stormwater dominated site; FD, forest dominated site; SO_4, sulfate; K, potassium; Cl, chloride; Na, sodium; Cr, chromium; Pb, lead; NO_3, nitrate; Ni, nickel; Cu, copper; 6PPD-Q, 6PPD-quinone; DPG, diphenyl-guanidine; AMPA, aminomethanephosphonic acid.*

[ii] *6PPD-Q and AMPA were not quantifiable at the forest dominated site.*

Exposure to Urban Stream Water Not Acutely Toxic, Limited Behavioral Sub-lethality

Sublethal behavioral effects were observed in only 18% of the synoptic samples (23/126) (Table S8). The contaminant mixtures associated with road runoff could be contributing to sublethal behavioral toxicity (Figure S4). We observed hypoactivity in zebrafish exposed to three stream samples with the highest concentrations of our road indicators. However, we did not observe a clear trend in behavioral response when fish were exposed to stream samples containing elevated wastewater or lawncare runoff.

The use of ecotoxicity assays could help identify when a combined mixture of sources, or untracked sources, are of concern. For example, we observed sublethal toxicity at a main-branch site upstream of the effluent release point in every synoptic survey. Yet, there was no consistent trend in our indicators. In the first synoptic we observed evidence of road runoff (DPG: 15070.42 ng L-1 and 6PPD-Q: 35.08 ng L-1) and sewage inputs (sucralose: 738.95 ng L-1) but limited lawncare runoff (AMPA <MQL) at the site. In contrast, in the last synoptic sampling we did observe evidence of lawncare runoff (AMPA: 1069 ng L-1) but limited road runoff (DPG: 275.77 ng L-1 6PPD-Q: 5.19 ng L-1) or sewage inputs (sucralose <MQL). Another contaminant mixture, not captured by our indicators, could impact this site. Other sites with comparable concentrations of each indicator did not have sublethal toxicity.

Integrating Indicator Compounds into Monitoring Efforts

The integration of source-specific indicators into monitoring and management efforts could improve understanding of contaminant sources and the prevalence of their contaminant mixtures, especially when multiple sources of common water quality measures exist. When a pollutant

source predominates, non-specific water quality measures can help identify sites with high loadings of associated contaminant mixtures. Co-elevated concentrations of compounds like chloride and sodium could indicate significant wastewater or road runoff inputs. However, a follow-up analysis of wastewater and road runoff indicators are necessary to determine the source; as we observed, contaminant mixtures vary based on location in the watershed (road density) and time of year (winter vs. summer). Ecotoxicity assays can provide a complementary mechanism to identify "hotspots" within a stream network that otherwise may not stand out with water quality measures or indicator compounds. We observed sublethal toxicity across a gradient of sites impacted by wastewater and runoff from roads and lawncare, implying the potential for either other contaminant mixture sources or toxicity associated with co-exposure to multiple mixtures.

This integrated approach requires customization based on the watershed assessed and resources available for analysis. The timing of sampling must be carefully considered given temporally dynamic contaminant mixture concentrations. For example, base-flow conditions may be ideal for measuring leaky sewer infrastructure, whereas sampling during the first flush of a storm or developing a concentration-discharge relationship,[78] albeit difficult in flashy streams, could identify road and lawncare runoff sources. If resources permit, additional source-specific indicators for other sources could be targeted or non-targeted fingerprint analyses[14,15] could be included for known contaminant sources. As demonstrated with the highly variable concentrations of PFAS in Ellerbe Creek, source-specific indicators are limited in their capacity to trace emerging contaminants released by multiple point and non-point sources or that are reactive in-stream.

SUPPORTING INFORMATION AVAILABLE

Additional methods, correlations, and solute timeseries (PDF)

Table S9: Correlations between per- and polyfluoroalkyl substances (PFAS) and other measures during synoptic surveys in the Ellerbe Creek tributaries (XLSX).

Table S10: Correlations between indicators and water quality measures during synoptic surveys in the Ellerbe Creek tributaries (XLSX).

This information is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

J.R.B., E.S.B., and P.L.F designed and conceived the study. J.R.B. led field sampling. A.S.J. and J.R.B. conducted chemical analyses with support from P.L.F. N.B. conducted ecotoxicity assays with support from N.J. J.R.B wrote the first draft of the manuscript. All authors collaborated to write, edit, and approve the final version of the manuscript.

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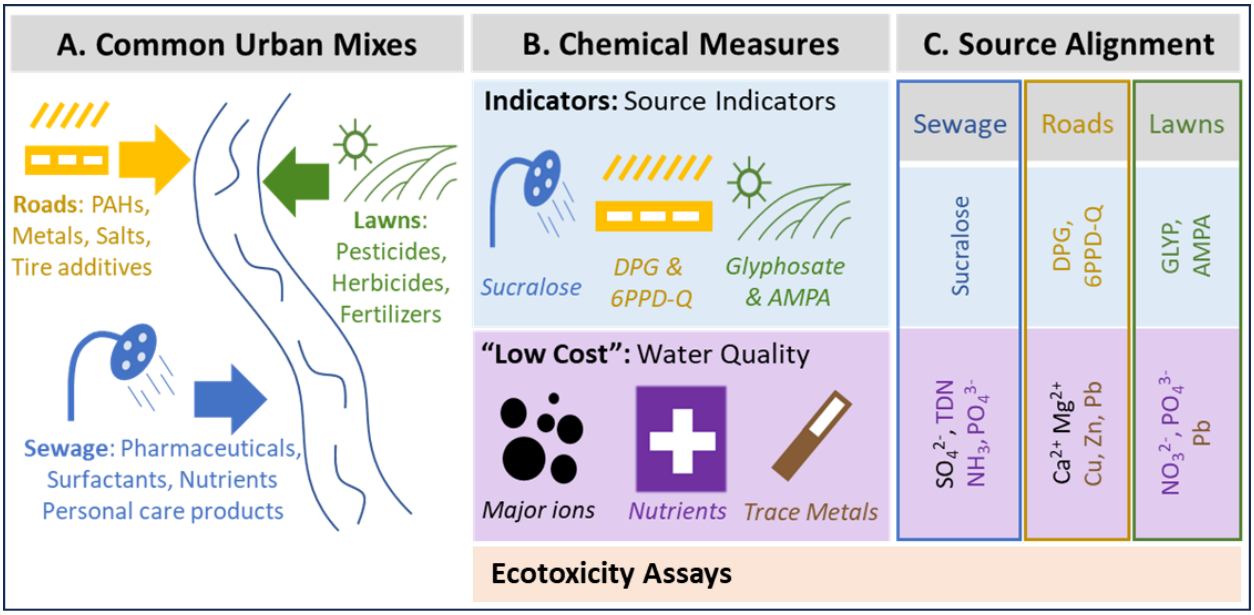

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865 Caption: Urban watersheds (A) introduce chemical contaminant mixtures from roads, lawns, and
866 treated or untreated wastewater. (B) Sources are assessed with an integrated approach of source-
867 specific indicator compounds, common water quality measures, and ecotoxicity assays. (C)
868 Solutes are mapped to potential contaminant sources. Polycyclic aromatic hydrocarbons (PAHs),
869 diphenyl-guanidine (DPG), 6PPD-quinone (6PPD-Q), aminomethanephosphonic acid (AMPA),
870 glyphosate (GLYP), calcium (Ca2+), magnesium (Mg2+), copper (Cu), zinc (Zn), lead (Pb),
871 nitrate (NO32-), phosphate (PO43-), sulfate (SO42-), total dissolved nitrogen (TDN), ammonia
872 (NH3).