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Application of Non-Target High Resolution Mass Spectrometry Data to Quantitative Source Apportionment

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Abstract

High resolution mass spectrometry (HRMS) analyses provide expansive chemical characterizations of environmental samples. To date, most research efforts have developed tools to expedite labor- and time-intensive contaminant identification efforts. However, even without chemical identity, the richness of non-target HRMS datasets represents a significant opportunity to chemically differentiate samples and delineate source contributions. To develop this potential, we evaluated the use of unidentified HRMS detections to define sample uniqueness and provide additional statistical resolution for quantitative source apportionment, overcoming a critical limitation of existing approaches based on targeted contaminants. Using a laboratory-scale representative watershed, we assessed the fidelity of HRMS source fingerprints during dilution and mixing. This approach isolated 11-447 non-target compounds per sample for source apportionment and yielded accurate source concentration estimates (between 0.82-1.4-fold of actual values), even in multi-source systems with <1% source contributions. Furthermore, we mined the non-target data to identify five source-specific chemical end-members. While additional development studies are needed to fully evaluate the myriad factors affecting method accuracy and capabilities, this study provides a conceptual foundation for novel applications of non-target HRMS data to confidently distinguish and quantify source impacts in complex systems.

Introduction

Recent advances in computational data processing and integrated software platforms have enabled the increasing use of high-resolution mass spectrometry (HRMS) methodologies for environmental data collection.[1,2] HRMS acquires accurate mass data, supporting the detection of hundreds to thousands of unique chemicals in most environmental samples. While such detections represent only small molecule (<1000 Da) synthetic chemicals and natural products that are amenable to chromatographic separation and detection via mass spectrometry, they do reflect a substantial fraction of the analyzable chemical composition of a sample.[3] Many research efforts have exploited the richness of HRMS data to annotate and identify novel chemical contaminants without prior knowledge of their presence.[4–9] Such outcomes are exciting and insightful, especially with respect to discovery of novel and toxicologically relevant contaminants and transformation products.[10–14] However, identification typically requires substantial curation and individual effort to translate accurate mass detections to chemical formulae and subsequently confirmed identity, especially for true non-target data. Indeed, significant resources are dedicated to developing improved tools for prioritizing HRMS detections and expediting identifications.[15]

The same richness of non-target HRMS data ("metadata") that supports discovery of novel contaminants also presents a considerable opportunity to differentiate sample types, understand chemical sources, and characterize environmental fate and transport phenomena. HRMS methods essentially organize the holistic, complex organic chemical background of environmental samples into tens, hundreds, and thousands of singular and unique detections. Subsets of non-target HRMS detections could easily be grouped as "fingerprints" or "signatures", paired with complementary knowledge (e.g., legacy pollutant profiles, hydrologic

data), and correlated to specific sources or outcomes for forensic or mechanistic assessment. For example, we previously evaluated the contribution of hydrophobic sorption to engineered treatment by estimating octanol-water partition coefficients for unidentified non-target detections.[16] Farré *et al.* differentiated the NDMA formation potential of drinking water sources *via* non-target analyses, highlighting disproportionate contributions of labile DOM constituents.[17] Across a longer temporal scale, Chiaia-Hernández *et al.* reconstructed historic environmental contaminant inputs by clustering non-target detections and known anthropogenic contaminants in sediment cores.[18]

Despite this potential, few research efforts have explicitly exploited the broader characteristics of non-target HRMS data to delineate source contributions in environmental systems without spending time and effort on identifications. Such approaches are well-established in the food and herbal medicine authentication fields, where suites of unidentified non-target detections (so-called "chromatographic fingerprints") are used to validate origin or recognize possible adulteration.[19–21] The use of unidentified compounds addresses a critical constraint of efforts to track sources and differentiate samples: limited statistical power due to the co-occurrence or low abundance of targeted diagnostic compounds (also referred to as "end-members"). Soulier *et al.* (2016) demonstrated the potential to identify such end-members *via* non-target analysis by isolating ~40 specific markers for each of two groundwater sites from a complex background (12,000 detections).[22] Likewise, similarity analyses have been paired with non-target fingerprints to evaluate source-sink relationships for plastic waste[23] and diesel spills[24] in the absence of sufficient known or recalcitrant end-members. Conceptually, comparisons of sample complexity and uniqueness, especially ones based on pseudo-persistent chemical

surrogates, should be amenable to tracking and quantitatively apportioning sources in environmental systems.

We hypothesize that distinct water samples, representing and including contaminant sources, can be differentiated and quantitatively tracked in receiving waters via non-target chemical fingerprints. Our objectives were to evaluate the use of unidentified non-target HRMS data, rather than individual targeted contaminants, for quantitative source apportionment. We assessed the fidelity, retention, attenuation, and dilution of HRMS fingerprints during mixing of various representative source waters at the laboratory scale – essentially translating bulk chemical information into a physical understanding of source hydrology and sample history. Additionally, the non-target data was mined to select and identify source-specific end-members based on their dilution behavior. While additional work is needed to understand the impact of environmental phenomena on HRMS source fingerprints, we believe that this study represents the first report of a conceptual model for utilizing non-target HRMS data to differentiate *and* quantify contributions of pollutant sources in complex aqueous mixtures.

Materials and Methods

Chemicals. A complete list is provided in the SI.

Sample collection. A laboratory-scale representative watershed reflecting potential contaminant sources (roadway runoff) and subsequent hydrologic dilution was created by mixing stored water samples (**Figure 1**, **Table S1**). Roadway runoff samples were collected during storms from two urban arterials: (1) State Route 520 (SR520; Seattle, WA, USA; 47˚38´38˝N, 122˚18´25˝W) on January 18, 2018 and April 1, 2018 using a pre-cleaned 1900L stainless steel tote and composited;[25] and (2) State Route 518 (SR518; SeaTac, WA, USA; 47˚28´5˝N,

122˚18′24″W) on June 9, 2017 by a flow-weighted automatic sampler in a pre-cleaned 10L glass jar. Grab samples from Puget Sound creeks along a pristine/rural to urban/human-impacted gradient (reflecting a contamination gradient) were collected (4 L pre-cleaned amber glass bottles without headspace) during baseflow conditions from Coulter Creek (pristine; May 10, 2018; 47˚24′30″N, 122˚49′1″W), Kautz Creek (pristine; January 13, 2018; 46˚44′7.836″N, 121˚51′7.992″W), Crescent Valley Creek (low urban; December 12, 2017 and May 10, 2018; 47˚21′28″N, 122˚34′43″W), and Swan Creek (highly urban; December 13, 2017 and May 14, 2018; 47˚13′35″N, 122˚23′36″W). Samples were transported on ice to the laboratory and archived at 4˚C until extraction on May 22, 2018. Despite the extended storage time (our processing protocols typically require extraction within 24 h) and associated possible changes to sample composition (e.g., storage may implicitly select for more persistent features), these analyses only focused on the non-target composition of and differences between samples at the time of analysis and should be insensitive to changes in contaminant occurrence/concentration prior to extraction. Indeed, for certain forensic applications, such as oil spill source tracking, degradation of highly labile constituents to better isolate more recalcitrant end-member features within the sample composition may be highly beneficial.[24]

Source dilution curve & model watershed development. Here, we use the term "fingerprint" to represent the ensemble of non-target HRMS detections characteristic of a given sample or source. For quantitative applications of mass spectrometry (e.g., estimating source contributions to a complex mixture), assessment of peak area response dynamics for such fingerprints is needed. We assessed peak area response across all non-target detections in the source fingerprints via a "dilution curve" approach. We generated source-specific dilution curves for two distinct roadway runoffs (SR520 and SR518) at source concentrations of 100, 40, 16, 6,

2.5, 1, and 0.1% v/v (**Figure 1**). For SR520, two independent curves were made for comparison: 1) a "sample dilution" curve was generated prior to solid phase extraction (SPE) by diluting the source water with deionized (DI) water; and 2) an "extract dilution" curve was generated by diluting the final SPE methanolic extract from the 100% source sample with additional methanol. For SR518, a volume-limited source, the 100% source was SPE-extracted and only an extract dilution curve was generated.

To approximate a complex watershed, source samples (SR520 runoff) were sequentially diluted with creek water samples to create 30, 18, 10, 4, 1, and 0.16% (v/v) mixtures (Mixtures 1-6; **Figure 1**, **Table S1**). The source concentrations within these watershed mixtures were subsequently treated as forensic unknowns to be estimated from the data. To determine if the contributions of two similar sources could be differentiated, two versions of Mixtures 4-6 were created: (A) SR520 as the sole contaminant source; and (B) SR518 runoff was included as a second, similar source at concentrations of 10, 2.5, and 0.4% v/v (Mixtures 4, 5, and 6, respectively). We also note that the creek water samples, particularly those from more urban areas, were expected to have some level of roadway runoff and contaminant impact.

Sample processing and analysis. Sample processing and HRMS data analysis followed methods described previously.[26] SPE cartridges (3 mL, 100 mg Infinity cartridges with Osorb® media; ABS Materials, Wooster, OH, USA) were preconditioned with 3 mL 50% (v/v) methanol in DI water, then 25 mL DI water. Unfiltered water samples (1 L) were loaded at 10 mL/min, cartridges were rinsed with DI water (10 mL), nitrogen-dried (15 min), and eluted with methanol (2x, 2.5 mL). Extracts were concentrated with nitrogen to 1 mL and spiked with a mixture of 12 isotope-labeled internal standards (ISTDs; **Table S2**). For all surface waters, road runoff, watershed mixtures, and sample dilution curves, one 1L sample was extracted, with analytical

replicates from triplicate injections of a single undiluted extract. For extract dilutions, analytical replicates were obtained from triplicate injections of individual diluted extracts. Extracts were analyzed on an Agilent 1290 Infinity UHPLC (Santa Clara, CA, USA) with an Agilent 6530 Quadrupole Time-of-Flight LC-MS system with electrospray Jet Stream Technology. Chromatography used a reverse-phase C18 column (Agilent ZORBAX Eclipse Plus 2.1×100 mm, 1.8µm particle size) with a C18 guard column at 45°C, injection volume 5 µL, flow rate 0.4 mL/min, and a binary gradient of 5mM ammonium acetate plus 0.1% acetic acid in water (A) and 5mM ammonium acetate plus 0.1% acetic acid in methanol (B) [5% B at 0-1 min, 50% B at 4 min, 100% B at 17-20 min, 5% B at 20.1 min; stop time 22.5 min; post-time 2 min]. HRMS spectra were acquired across 100-1700 *m/z* (MS) or 50-1700 *m/z* (MS/MS) in 2 GHz Extended Dynamic Range mode with ESI+ detection.

For quality assurance and quality control (QA/QC), we monitored detector performance via check tunes before each analytical run, re-tuning if mass error exceeded 2 ppm. Every 8-12 samples, we analyzed solvent blanks (no column carryover detected), an external reference standard mixture (cotinine (retention time (RT) 3.4 min, 120 ng/mL), carbamazepine (RT 6.5 min, 50 ng/mL), and prometryn (RT 9.5 min, 50 ng/mL)), and an ISTD control (ISTD mixture in methanol). If mass error was >5ppm or area counts were >20% of initial sensitivity during the batch, the instrument was re-tuned and samples re-analyzed. All samples were analyzed within one analytical batch. Within the batch, relative standard deviation (RSD) of area counts and RT were <12% and <0.4%, respectively, in the external reference standard and ISTD controls. Method (DI water) blanks extracted via SPE were analyzed alongside samples (n=3, one injection per extract), with fold change analyses (see below) used to exclude detections in method or solvent blanks.

Data reduction and analysis. We used Agilent software (Profinder B.08.00; Mass Profiler Professional B.13.00) to extract and align detected features (exact mass-retention time pairs) across samples, group isotopes/adducts into "non-target compounds", and filter the data to avoid false positives.[25,26] Only compounds with peak area >5000, detected in 3 of 3 replicates, and present at peak area ≥3-fold that of solvent and method blanks were retained for further analysis. These, and additional, methodological and screening criteria to identify non-target compounds amenable to quantitative source estimation are summarized in **Figure 1** and described below. Due to software artifacts, some replicate detections (e.g., inaccurately identified adducts) do still exist within the dataset.[27] For compound identification efforts, suspect screening used custom databases containing ~1000 contaminants related to stormwater and vehicles, and identification confidence was assigned via criteria proposed by Schymanski *et al.*[28]

Results and Discussion

Source Complexity. To assess source complexity, we compared numbers of non-target compounds detected in the two roadway sources. Undiluted SR520 and SR518 had 314 non-target compounds in common. An additional 747 detections were unique to SR520, while only 129 were unique to SR518 (**Figure S1**). This comparison illustrated a key difference: the more chemically contaminated nature of SR520 runoff provided more potential SR520-specific end-members, making it easier to track and differentiate. In contrast, the reduced complexity of the SR518 runoff limited end-member possibilities, potentially hindering apportionment. Although such differences in mixture complexity may be partially analytical artifacts (e.g., both sources may have equivalent chemical uniqueness, but fewer SR518 compounds are amenable to LC-QTOF detection or attain detection limits), it is clear that all source tracking efforts, including

this one, require an ability to define compositional uniqueness. Sources with insufficient chemical complexity may become quite difficult to track and differentiate.

Dilution Curve Criteria. To isolate compounds appropriate for quantitative estimation, we applied a series of filtering criteria (**Figure 1**) to source dilution curves (**Table 1a**). We detected 1509, 1318, and 609 non-target compounds that met minimum data reduction criteria in the SR520 "sample dilution", SR520 "extract dilution" and SR518 "extract dilution" curves respectively (across all points along the curve). Then, we screened for compounds that were present in at least the 3 highest points and had no data gaps along their dilution curve. These criteria reduced non-target compound numbers by ~50-70% to 608, 632, and 175 compounds in the SR520 sample dilution, SR520 extract dilution, and SR518 extract dilution curves, respectively.

Next, peak area data were log-transformed and the slope, intercept, and coefficient of determination (R^2) were calculated to make a dilution regression for each non-target compound, where y = log(peak area) and x = log(dilution factor). Compounds were retained if their dilution curve exhibited a peak area decrease as source concentration was diluted (a high-level check for consistent dilution behavior and background contamination), had $R^2 \geq 0.80$ (minimum linearity) and slope ≥ 0.30 (equivalent to a minimum 2-fold change in peak area response for every 10-fold change in concentration). These criteria reduced numbers of retained compounds by another ~25-40%, qualifying 463, 535, and 103 compounds in the SR520 sample dilution, SR520 extract dilution, and SR518 extract dilution curves, respectively, for use in quantitation. Notably, few compounds were detectable at a source concentration of 0.1% (n=16 for SR520 sample dilution; n=1 for SR518 extract dilution), underscoring analytical sensitivity limitations for resolution of low-level sources.

Source Estimation. Next, we screened watershed mixture samples to isolate non-target compounds useful for estimating source concentration (**Figure 1**, **Table 1b**). Between 233-962 compounds per mixture met the minimum data filtering criteria, with fewer compounds in more dilute mixtures. We then screened for compounds that also met the source dilution curve criteria. For the SR520 source, this requirement excluded ~45-85% of the compounds in a given mixture, eliminating those not derived from the source of interest or not reliable for use in apportionment. Additionally, compounds were only retained if their mixture peak area was within the dilution curve peak area range. For SR520 source estimation, the peak area range requirement greatly impacted the number of qualified compounds in the more dilute mixtures, excluding ~35-65% of the remaining compounds in mixtures with source concentration ≤1%, while <25% of the remaining compounds in higher concentration mixtures were eliminated.

Using the remaining compounds, a source concentration (representing the magnitude of dilution the source had experienced) was independently calculated for each non-target compound from the observed peak area and the corresponding source dilution curve. Analogous to using an individual chemical calibration curve to calculate an unknown concentration, this approach yielded 11-447 individual source concentration estimates per mixture. While assessing several permutations of the method to evaluate relative performance and sensitivity to factors such as dilution curve range, sample *vs.* extract dilution, etc., the final source concentration was estimated as the median of these many individual estimates (**Figure 2**, **Tables 2, S3, S4)**.

Single-Source Estimates. Final source concentration estimates derived from the SR520 sample dilution curves and the number of contributing compounds are provided in **Table 2**. Estimates are compared to actual values in **Figure 2a**. For all single-source mixtures with concentrations ≥4%, estimates were within a factor of 1.0-1.13 of actual, indicating that non-

target HRMS data provided highly accurate estimates. Consistent with expectation, accuracy decreased with increasing source dilution and decreasing numbers of contributing compounds: for single-source mixtures with concentrations of 1% and 0.16%, respectively, estimates were 0.91- and 2.13-fold of the actual value.

In the more concentrated samples, many compounds met all screening and estimation criteria (i.e., 429 for Mixture 1 *vs.*15 for Mixture 6A). Notably, the independent estimates of source concentration yielded relatively symmetrical and smooth probability distributions, especially for mixtures with abundant detections (**Figure 3a-c**). With more dilution (dropping compounds below detection limits), detectable chemical complexity decreased and the method lost resolving power as fewer qualified compounds met statistical end-member criteria. These reduced numbers of end-members resulted in decay of the probability distribution and increased estimate error for more dilute mixtures (**Figure 3d-f**). In particular, the probability distributions for Mixtures 5A and 6A highlight a small subset of outlier end-members generating high bias to the estimates. Presumably, these high-biased outliers reflect a subset of compounds with multiple sources within the watershed, thus degrading their source specificity, or a set of compounds disproportionately affected by matrix effects or other analytical conditions. As these compounds clearly broke out of the continuous probability distribution, we recalculated source concentration estimates for Mixtures 1-3 and 4-6A after excluding outliers (i.e., estimates >1.5 times the interquartile range above the third quartile or below the first quartile).[29] After outlier exclusion, all estimates for single-source mixtures were within 0.82-1.38-fold of actual. Estimate error for Mixture 6A, the most challenging mixture, was especially reduced by outlier exclusion, with the final estimate decreasing from 2.13- to 1.38-fold of the actual concentration.

Second Source Interference. For mixtures with chemical interference from a second, similar roadway source (SR518 runoff; Mixtures 4-6B), initial estimates (outliers excluded) were 1.4 ± 0.7 times larger than those in their single-source counterparts (Mixtures 4-6A). The second source impact was clearly visible in the right-skew (high bias) of probability distributions for Mixtures 4-5B *vs.* 4-5A (**Figure 3g-h** *vs.* **3d-e**). The impact on accuracy was least noticeable in Mixture 6B *vs.* 6A (estimated 0.22 *vs.* 0.18% SR520), likely because the dilute SR518 source contributed relatively few compounds that could cause skew.

Of the 132, 44, and 12 non-outlier compounds used to estimate concentration in Mixtures 4-6B, respectively, 84, 24, and 7 were common to both sources. To account for skew resulting from multi-source contaminants, we re-filtered the data to retain only compounds unique to SR520 (**Figures 2a** and **3g-i** (green), **Table 2**). Generating estimates only from "non-outlier, source-unique" compounds (*vs.* the "non-outlier" estimate) further reduced error by 38%, 40%, and 19% for Mixtures 4-6B, respectively, indicating that screening for unique end-members improved accuracy and reduced bias. Although not always feasible in practice, these data highlighted the importance of identifying and sampling as many contributing sources as possible, particularly in systems with multiple chemically similar inputs (e.g., two roadways, cities, wastewater treatment plants, etc.).

Source Dilution Curve Range. In theory, these techniques require an effective assessment of peak area response across the entire range of possible source concentrations, thus accounting for the full extent of possible source dilution in an unknown. In practice, it may be difficult to align source dilution curve ranges with actual "field" concentrations, whether due to practical limitations or limited advance knowledge. Thus, to evaluate the sensitivity of estimates to the source dilution curve range, we repeated the analysis for Mixtures 1-3 and 4-6A using

several variants of the SR520 sample dilution curve (e.g., removing points from the top or bottom of the curve) (**Figure 4**, **Table S4**). Generally, if the actual source concentration fell within the bounds of the modified curve, the estimates were quite similar to those obtained using the full dilution curve range (average 1.1 ± 0.27 times the actual value). For mixtures with actual source concentrations below or above the bounds of the modified curve, corresponding estimates were biased low or high (e.g., 20-fold of actual for Mix 6A (0.16% SR520) with 1-100% range). These data suggest building as wide a dilution curve as possible, given possible severe inaccuracies when low-level data are missing.

For such "out of range" mixtures, we also modified the data filtering criteria described above to permit the inclusion of compounds with mixture peak areas that fell *outside* the dilution curve range. Such inclusions typically counteracted the systematic error associated with an inaccurate dilution curve range, especially for high concentration systems where the risk of skew from additional outlier values was relatively low. For example, when estimating Mix 1 (actual: 30% SR520) with dilution range 0.1-16%, the final estimate improved from 0.32- to 0.93-fold of the actual value when dilution curves were extrapolated to include 229 additional "out of range" compounds. Likewise, for estimating Mix 6A (0.16% SR520) with dilution curve range 1-100%, the final estimate improved from 20-fold to 2.5-fold of actual when including 38 additional compounds. Therefore, for systems with uncertain source concentration ranges, such iterative data analyses (with modified filtering criteria that include compound extrapolations) can assess the suitability of the source dilution curve range, as well as resulting estimate outcomes and probability distributions.

Sample vs. Extract Source Dilution. In practice, it is easier to evaluate peak area response by diluting existing SPE extracts *vs.* diluting and extracting lots of whole water

samples. Generating extract dilution curves avoids the need for additional sample volume and *a priori* knowledge about the appropriate source concentration range. However, due to matrix dilution in the final extracts,[30] extract dilution may yield different source dilution curve slopes relative to sample dilution, impacting the accuracy of the resulting estimates. To evaluate the feasibility of SPE dilution curves, we compared results from SR520 curves generated via sample *vs.* extract dilution (**Figures 2a, 3; Tables 2**, **S3).**

Overall, both dilution curve approaches yielded similar median estimates for nearly all mixtures with similar numbers of qualified compounds. For all single-source mixtures (1-3, 4-6A), extract dilution estimates were within a factor of 0.87-1.4 of the actual value versus 0.82-1.4 for sample dilution. Like outcomes observed for sample dilutions, extract dilution estimates that relied only on SR520-unique compounds were more accurate for multi-source mixtures (4-6B). These data again supported the isolation of source-specific compounds to improve accuracy whenever feasible, although we note that accuracy also derives from including sufficient qualified compound numbers to populate a well-defined probability distribution.

Notably, extract dilution estimates for the highest concentration mixtures (concentration ≥4%; mixtures 1-3, 4A) were -11 ± 1% lower than sample dilution estimates, indicating that higher concentration samples contained compounds that were saturated on the SPE cartridges or strongly matrix-suppressed.[30,31] This effect would yield extract dilution curves with steeper slopes relative to sample dilutions and bias concentration estimates toward lower values for saturated features. ISTD peak area data for sample dilutions indicated some matrix suppression, as median ISTD peak area response (a proxy for matrix effects)[32] decreased with increasing source concentration along the sample dilution curve (from 97% relative response for 0.1% SR520 to 50% response for 100% SR520; **Table S5**).

We observed a surprising degree of uniqueness when comparing sample and extract dilutions: 402 non-target compounds met the screening criteria in both curves, with 61 unique to sample dilution and 133 unique to extract dilution. This difference again suggested impacts from matrix effects. For example, as matrix was diluted away in extract dilutions, we expect increasing relative peak area response for compounds that were matrix-suppressed in the lower concentration points of sample dilutions. We recognize the substantial implications of both SPE cartridge capacity and matrix effects on apportionment accuracy and quantitative applications. Indeed, many approaches to correct non-target peak area data for matrix effects are described in the literature, ranging from single standard normalization to more sophisticated strategies such as "best-matched internal standard", although studies have not converged on a consensus strategy.[33,34] Subsequent studies to explicitly evaluate best practices (e.g., high-capacity SPE cartridges, quantitative use of isotopically-labeled surrogates and internal standards) for these analytical variables in the context of source apportionment are needed. Based on the data presented here, and in the absence of established best practices for non-target data, we currently recommend the use of source sample dilution curves and raw peak area data (from a single analytical batch).

Extension to Second Source Apportionment. Methodologies for source apportionment typically require the resolution and quantitative apportionment of multiple chemical sources within a system.[35] Therefore, we also analyzed the data to estimate concentrations for both the primary SR520 and secondary SR518 sources in Mixtures 4-6B. SR518 source concentrations were estimated using the above methodologies with an extract dilution curve (**Figure 2a**, **Table 2**). When using all qualified compounds, the resulting SR518 concentration estimates were biased high by 2.5, 2.3, and 72-fold for Mixtures 4-6B, respectively. However, estimate

accuracies were greatly improved by focusing analysis on source-specific compounds, although at the cost of statistical resolution. By excluding common compounds (both sources were multilane roadway runoff), all SR518 estimates improved to 0.93-1.39-fold of actual (**Table 2**). However, these estimates used only 5, 1, and 1 qualified compounds in Mixtures 4-6B, respectively, thus losing much of the statistical power inherent to a distribution of estimates and greatly increasing the potential for gross error outcomes. For sources that lack chemical complexity and/or significant uniqueness, different separation or detection methods (e.g., GC vs. LC, APPI instead of ESI) could be applied to generate additional unique detections. Ultimately, such chemically similar sources represent the most challenging case, whether for approaches that use individual targeted contaminants or on non-target data.

Comparison with Targeted Chemical Apportionment. Quantifying source contributions typically relies on analysis of a subset of individual, known compounds (i.e., targeted chemicals) that are pre-selected based on prior knowledge (e.g., the toxic pollutant or regulated parameter driving apportionment efforts, co-occurring contaminants as surrogates).[36,37] However, it is often not possible to know *a priori* which targeted chemicals will be present or which compounds provide the best co-occurring surrogates across multiple sources. We compared the non-target methodologies to an approach using individual identified urban stormwater contaminants as possible SR520 end-members to estimate source concentrations.[25] The contaminants were: 1,3-diphenylguanidine (DPG; S1), hexa(methoxymethyl)melamine (HMMM; S1), di-formylated HMMM (Di-F HMMM; S2b), hexylamine (HA; S1), N-methyl-dicyclohexylamine (DCMA; S1), 1-cyclohexyl-3-phenylurea (CPU; S1), and N-cyclohexyl-1,3-benzothiazolamine (NCBA; S1). Estimates made using these chemicals (data from the SR520 sample dilutions) are provided in **Figure 2b** and **Tables 2**, **S3** (for calibration curves, see **Figure**

363 **S2**). All seven chemicals were detected in Mixtures 1-3 and 4A, with their corresponding
364 estimates for the SR520 source concentration between 0.9-1.6-fold of actual. However, the
365 performance of these chemical surrogates degraded with increasing dilution as their peak area
366 responses declined to non-detect levels. Only HMMM and di-formylated HMMM were detected
367 in at least eight of nine mixtures, although HMMM was present at a peak area below the source
368 dilution curve range in Mixtures 6A and 5B. Three contaminants (HA, DCMA, and CPU) were
369 not detected at SR520 concentrations <1% and two (DPG, NCBA) were not detected at SR520
370 concentrations <4%. Further, the five non-HMMM family contaminants were all detected in both
371 SR520 and SR518; thus, the generally higher estimates in Mixtures 4-6B (0.5-3.4-fold of actual)
372 relative to Mixtures 4-6A (0.38-1.8-fold of actual) indicated a lack of statistical resolution and
373 source specificity.

374 Although non-target estimate accuracy decreased as source concentrations decreased
375 (resulting in ever lower numbers of compounds to support estimates), non-target methodologies
376 will enable quantification of source contributions to relatively dilute systems where most known
377 contaminants fall below detection limits, even where nothing is known about water quality
378 composition, contaminant occurrence or uniqueness. In fact, many of the detected non-target
379 features are likely to be source and watershed specific natural products and
380 biologically/geologically derived molecules, thus also enabling source resolution and
381 quantification for natural systems and sources without clear anthropogenic contamination.

382 **Individual Contaminants that Correspond to Dilution**. The targeted chemical end-
383 members above were selected based on prior knowledge of common roadway runoff
384 contaminants. Suspect screening efforts may yield identified contaminants that inform source
385 type,[22] but such compounds may still exhibit peak area responses that are poorly correlated to

source dilution. Although HRMS-based apportionment inherently avoids the *need* for any identification efforts, it is also possible to use the data to select and identify source or watershed specific end-members whose dilution behaviors track with that of the source. To select such indicators for the SR520 source, we identified a subset of non-target features that: 1) met SR520 sample dilutions screening criteria; 2) were detected in all six single-source mixtures and met source estimate screening criteria; and 3) were unique to SR520 relative to SR518. Five non-target compounds met all such criteria and were assigned a formula and/or identified: 282.1453@6.8 min (an in-source fragment of HMMM), 282.1732@8.6 min ($C_{18}H_{22}N_2O$, S4), 420.2349@7.2 min (di-formylated HMMM, S1), 772.5488@13.6 min (tridecapropylene glycol, S2b), and 888.6326@14.54 min (pentadecapropylene glycol, S2b). Source concentration estimates derived from these specific chemical indicators were within a factor of 1.4 ± 1.3 of actual values (**Table S6**). Much of this error was driven by two estimates for Mixture 6A made using the long-chain surfactants (tridecapropylene glycol and pentadecapropylene glycol), potentially due to an interfering source in the urban creek water used to make Mixture 6 (all other estimates were within a factor of 1.1 ± 0.32 of the actual value). Fortuitously, one of the identified surrogates (di-formylated HMMM) matched those selected from expert knowledge.

Environmental Implications. It is clear to us that substantial environmental insight can be gleaned from non-target HRMS data. Relying solely on the richness of this data and avoiding the need for individual targeted contaminants, we developed a simple method to quantitatively estimate chemical source contributions to complex mixed systems that generated relatively accurate estimates, even in multi-source systems with <1% source contributions. In contrast to the many studies that mine HRMS data to identify individual contaminants, this approach intentionally used HRMS data prior to laborious identification efforts, with the many

unidentified detections providing the statistical resolution necessary for source apportionment. These data indicate that chemical identity is not strictly necessary for source tracking and quantitative applications of mass spectrometry data. We also demonstrated that this method can identify chemical surrogates whose peak area response tracks with source dilution for systems needing targeted analytical methods. This non-target method addresses a critical limitation of existing approaches to identify and apportion sources – statistical underdetermination due to reliance on the detection of targeted contaminants that may be low abundance and/or derived from multiple sources.

Accordingly, we believe such approaches are broadly applicable to a range of systems and questions that require accurate deconvolution and quantification of source impacts, such as detecting and defining wastewater and septic impacts, remediated sediments subject to recontamination, or algal blooms derived from both agricultural and wastewater sources. Further "upstream" in the chain of anthropogenic impacts, the method could track chemical mixtures – for example, by quantifying the contribution of different vehicle fluids to stormwater. Likewise, even in natural systems without known anthropogenic impacts, non-target approaches paired with an appropriate sampling designs can even provide implicit hydraulic tracers and quantify hydrology to a reasonable degree of accuracy (e.g., our model watershed), although cost comparisons of HRMS analyses relative to stream gauges would surely come up short.

Such methodologies are likely to have many potential applications. Although basic in principle, this study suggests the substantial potential for development of novel tools that rely on unidentified HRMS detections to answer critical environmental questions. However, we also recognize the limitations of this relatively controlled experimental design and intend that this work is the first of a series of development studies and field validation efforts to more fully

characterize potential capabilities and applications for such methods. In particular, follow-up studies are needed to evaluate the impact of variables such as matrix effects, non-target compound persistence, temporal variation in source composition, and multimedia environmental partitioning of source fingerprints on apportionment accuracy.

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TOC Art

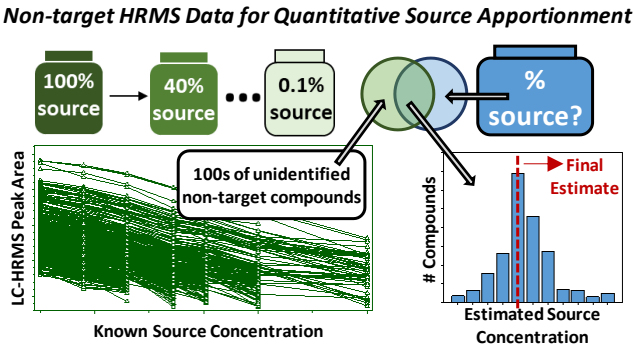
Non-target HRMS Data for Quantitative Source Apportionment
100% source
40% source
0.1% source
% source?
100s of unidentified non-target compounds
LC-HRMS Peak Area
Known Source Concentration
Compounds
Final Estimate
Estimated Source Concentration

Table 1. Number of non-target compounds detected in: (a) source dilution curves and (b) watershed mixtures that met increasingly stringent data reduction and screening criteria for subsequent use in quantitative source estimation.

(a)	**Source Dilution Curve**								
	SR520 (Sample)			SR520 (Extract)			SR518 (Extract)		
	Data Screening Criteria								
% Source	Minimum[1]	Presence[2]	Linearity[3]	Minimum[1]	Presence[2]	Linearity[3]	Minimum[1]	Presence[2]	Linearity[3]
100	1061	608	463	1061	632	535	443	175	103
40	1051	608	463	981	632	535	364	175	103
16	811	608	463	787	632	535	259	175	103
6	542	376	270	556	439	371	149	95	38
2.5	516	286	204	366	274	233	91	47	12
1	321	133	93	189	139	119	42	20	5
0.1	116	37	16	47	21	11	28	4	1
0.1-100[4]	1509	608	463	1318	632	535	609	175	103

(b)			**Data Screening Criteria**						
			Minimum[1]	Overlap[5]	PA Range[6]	Overlap[5]	PA Range[6]	Overlap[5]	PA Range[6]
			Corresponding Source Dilution Curve						
Mixture	**% SR520**	**% SR518**	--	SR520 (Sample)		SR520 (Extract)		SR518 (Extract)	
1	30	--	962	435	429	496	477	--	--
2	18	--	833	424	369	478	420	--	--
3	10	--	688	323	241	367	308	--	--
4A	4	--	532	233	177	264	203	--	--
5A	1	--	314	68	28	82	35	--	--
6A	0.16	--	263	44	15	49	20	--	--
4B	4	10	482	195	146	212	165	80	33
5B	1	2.5	617	99	54	116	77	33	13
6B	0.16	0.4	233	34	13	36	12	14	8

[1]Minimum data screening criteria met (replicate, peak area, and blank-comparison filters).

[2]See note 1; compounds also present in ≥3 highest points on dilution curve and without gaps along curve.

[3]See notes 1-2; compounds also met dilution curve linearity requirements (peak area decreased with increasing dilution, R^2 ≥0.8, slope ≥0.3).

[4]Data shown are the number of unique compounds that met screening criteria across all dilution curve points.

[5]Compounds met minimum criteria in watershed mixture **and** all criteria for corresponding source dilution curve.

[6]See note 5; compounds also with mixture peak area (PA) within PA range of corresponding source dilution curve.

Table 2. Quantitative source concentration estimates derived from various method permutations, including different source dilution curves, different groups of non-target compounds, or individual targeted compounds. Actual mixture source concentrations are provided for comparison. The number of non-target HRMS compounds used to derive each estimate are shown in parentheses and italicized, and optimal estimates (lowest observed error) for each source mixture are highlighted in bold. Mixtures in which targeted contaminants were not detected are indicated by an asterisk.

Dilution Curve Used:			**SR520 Sample Dilution**						**SR520 Extract Dilution**				**SR518 Extract Dilution**				**Targeted Contaminants, from SR520 Sample Dilution Curve[4]**						
Compound Group Used:			All Non-target Compounds[1]		All Non-target Compounds, Exclude outliers[2]		SR520 Unique Compounds, Exclude outliers[3]		All Non-target Compounds, Exclude outliers[2]		SR520 Unique Compounds, Exclude outliers[3]		All Non-target Compounds, Exclude outliers[2]		SR518 Unique Compounds, Exclude outliers[3]		DPG	HMMM	Di-F HMMM	NCBA	HA	DCMA	CPU
Mix	Actual % SR520	Actual % SR518	Estimated % SR520 (*# compounds used*)										Estimated % SR-518 (*# compounds used*)				Estimated % SR520						
1	30	--	31.3	*(429)*	**31.2**	***(401)***	--		27.8	*(447)*	--		--		--		37.1	33.6	30.3	33.3	32.9	35.3	29.4
2	18	--	20	*(369)*	**20**	***(354)***	--		17.8	*(386)*	--		--		--		22.5	20.6	21.3	21.7	21.4	19.9	20.4
3	10	--	10	*(241)*	**9.9**	***(225)***	--		8.7	*(278)*	--		--		--		11.7	10.5	11.4	9.5	11.8	11.3	11.0
4A	4	--	4.5	*(177)*	**4.5**	***(166)***	--		4.1	*(185)*	--		--		--		6.0	4.9	5.6	3.6	6.3	6.4	4.8
5A	1	--	0.91	*(28)*	**0.82**	***(23)***	--		1.2	*(32)*	--		--		--		*	0.57	0.78	*	1.2	1.8	0.66
6A	0.16	--	0.34	*(15)*	**0.22**	***(12)***	--		0.22	*(14)*	--		--		--		*	0.06	0.11	*	*	*	*
4B	4	10	6.6	*(146)*	5.7	*(132)*	**4.2**	***(50)***	6	*(150)*	4.3	*(57)*	25.4	*(31)*	**13.9**	***(5)***	7	2.7	3.3	2.7	7.9	12.3	7
5B	1	2.5	2.3	*(54)*	1.8	*(44)*	**1.4**	***(13)***	3.1	*(70)*	2.9	*(25)*	5.7	*(11)*	**2.4**	***(1)***	*	0.5	0.62	*	2.1	3.4	0.8
6B	0.16	0.4	0.2	*(13)*	0.18	*(12)*	**0.15**	***(5)***	0.18	*(10)*	0.13	*(3)*	28.8	*(12)*	**0.37**	***(1)***	*	*	0.09	*	*	*	*

[1]Estimate made with all non-target compounds that met screening criteria in source dilution curve and watershed mixture.
[2]See note 1, with outlier estimates excluded (outliers were >1.5 times the interquartile range above or below the third or first quartile, respectively).
[3]See notes 1 and 2, with compounds also detected in the secondary source excluded.
[4]Compounds are 1,3-diphenylguanidine (DPG), hexa(methoxymethyl)melamine (HMMM), Di-formylated (Di-F) HMMM, N-cyclohexylbenzothiazolamine (NCBA), hexylamine (HA), N,N-dicyclohexylmethylamine (DCMA), and 1-cyclohexyl-3-phenylurea (CPU).

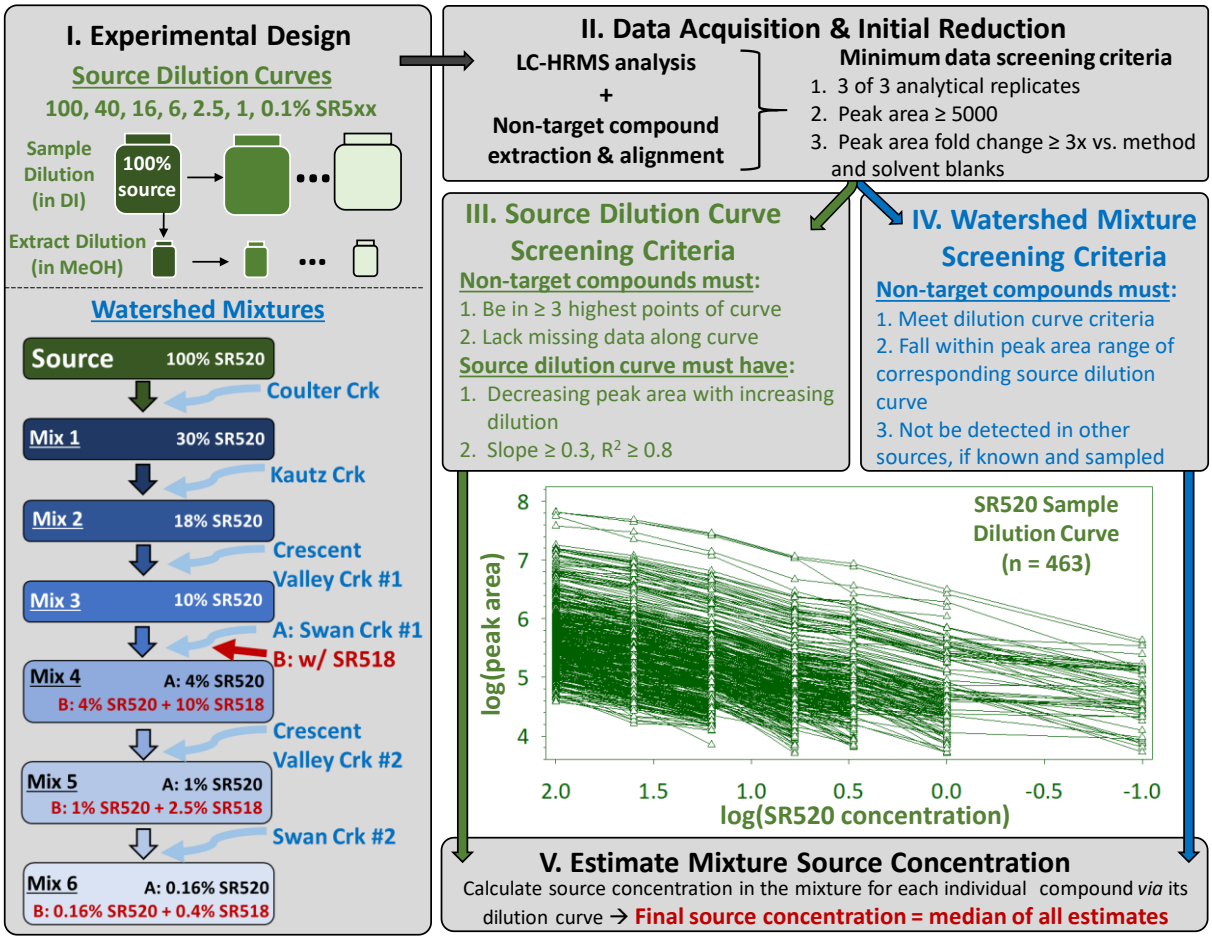

Figure 1. Schematic of experimental design and data analysis workflow. Panel I describes the experimental design for source dilutions, including generation of curves via sample dilution prior to solid phase extraction and via dilution of the SPE extract. Sources are roadway runoff from SR520 and SR518. Panel I also depicts the laboratory-generated watershed mixtures, with SR520 runoff as the primary source and SR518 runoff introduced as a secondary source (Mixtures 4-6B). Note that two sets (different sampling times) of Swan Creek and Crescent Valley Creek were used. Panel II describes data acquisition and initial data reduction steps applicable to all samples. Additional screening criteria to obtain non-target compounds amenable to quantitative estimation of source concentration are shown in Panels III and IV for the source dilution curve and watershed mixtures, respectively. The inset plot depicts an example of peak area responses for the 463 non-target compounds that met all screening criteria for the SR520 source curve generated via sample dilution. Panel V describes the final source estimation, using compounds that met all criteria in Panels II-IV.

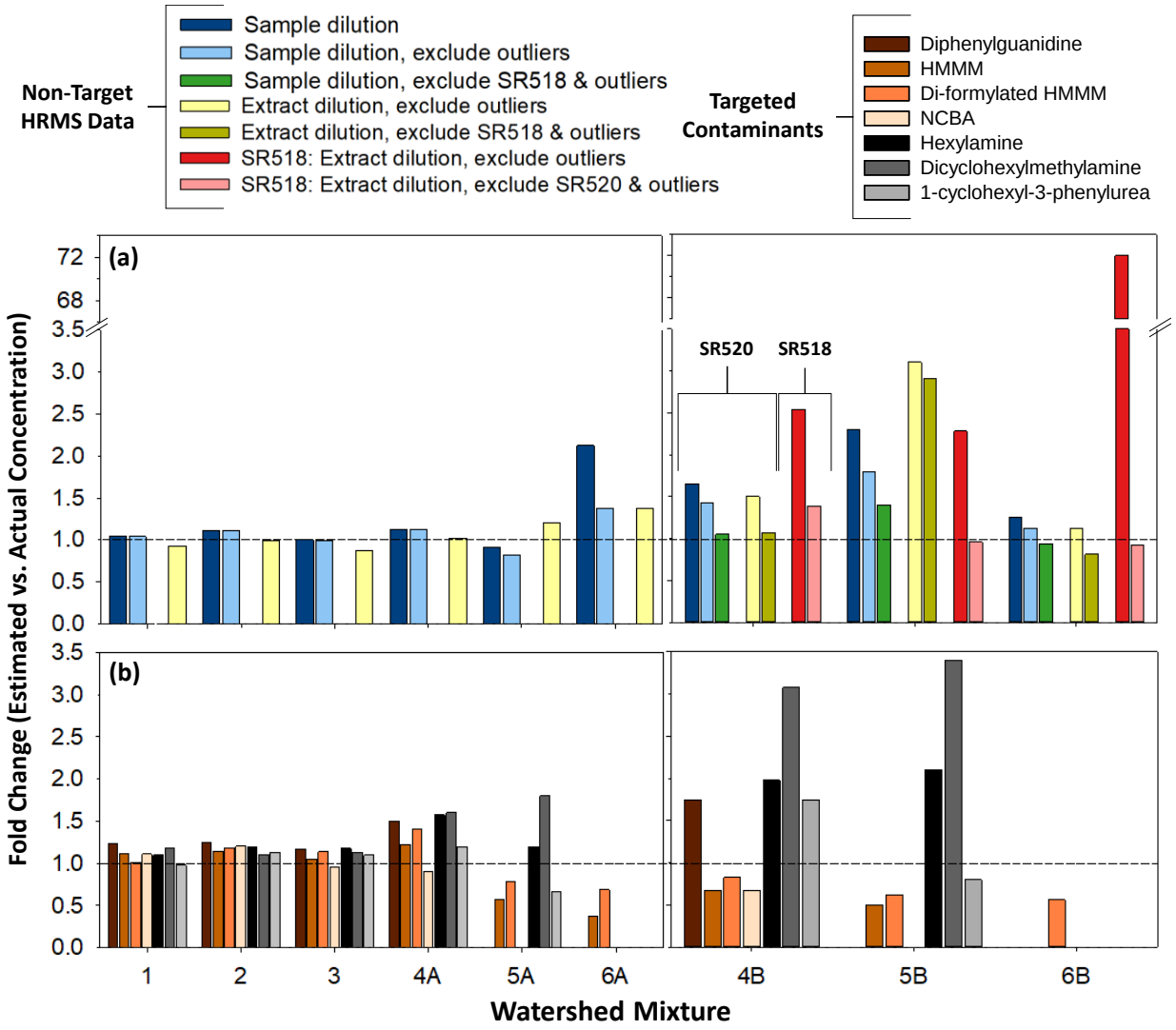

Figure 2. Estimates of roadway source concentration (as fold change of estimated vs. actual source concentration) for nine representative watershed mixtures, derived from (a) various permutations of the non-target HRMS data, and (b) targeted contaminants used as chemical surrogates. Unless noted otherwise in the legend, all source concentrations are for SR520 roadway runoff. The dashed line indicates the actual source concentration.

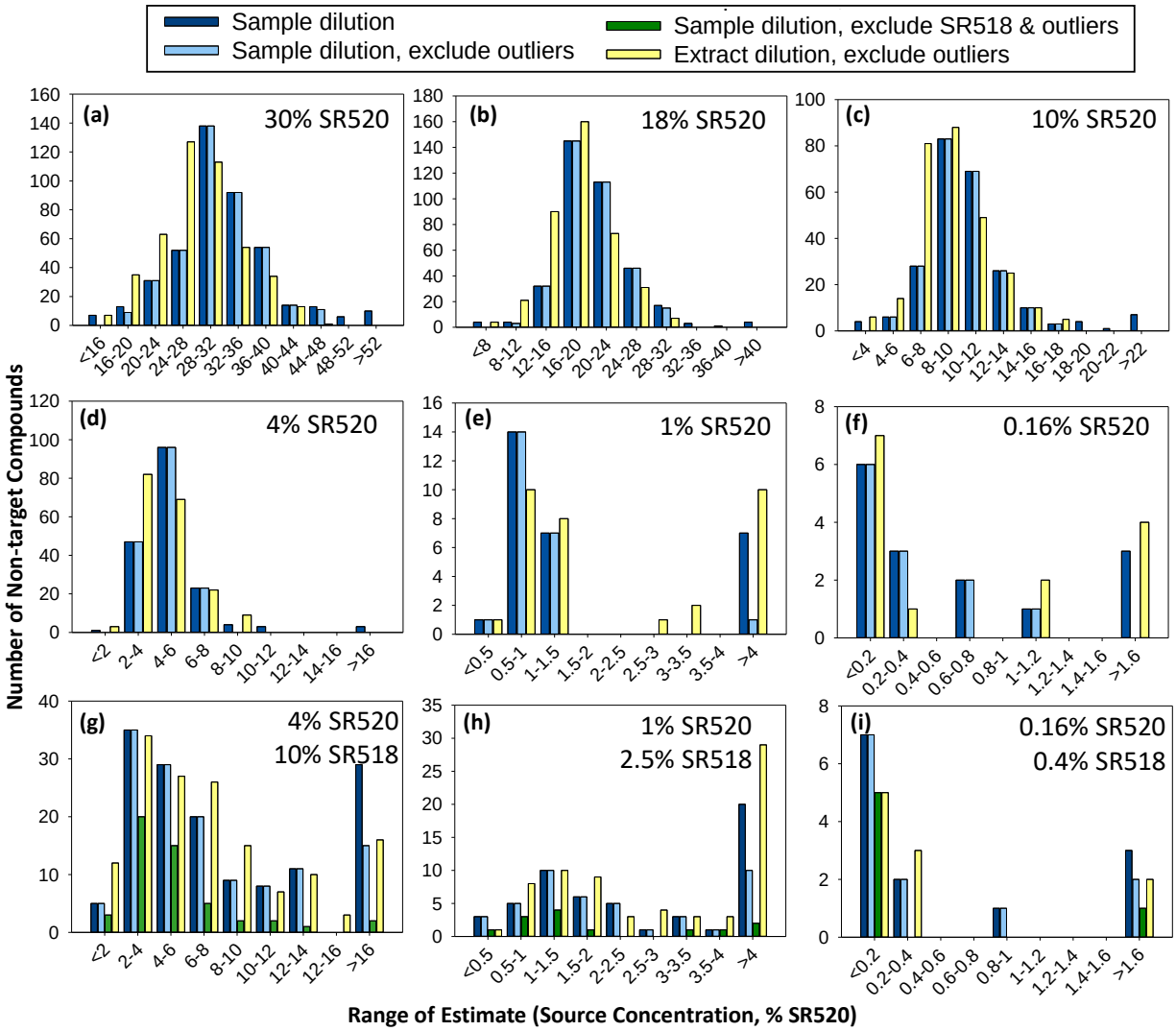

Figure 3. Histograms showing the distribution of SR520 concentration estimates and number of contributing non-target compounds for the watershed mixtures: (a) Mix 1, (b) Mix 2, (c) Mix 3, (d) Mix 4A, (e) Mix 5A, (f) Mix 6A, (g) Mix 4B, (h) Mix 5B, and (i) Mix 6B, with the actual source concentration noted on each plot. Estimates from the SR520 sample dilution (including & excluding outliers) and extract dilution (excluding outliers) curves are shown for all nine mixtures; estimates using the SR520 sample dilution curve made only with SR520-unique compounds (i.e., excluding outliers and excluding compounds also detected in SR518 runoff) are only shown for the multi-source mixtures (g-i).

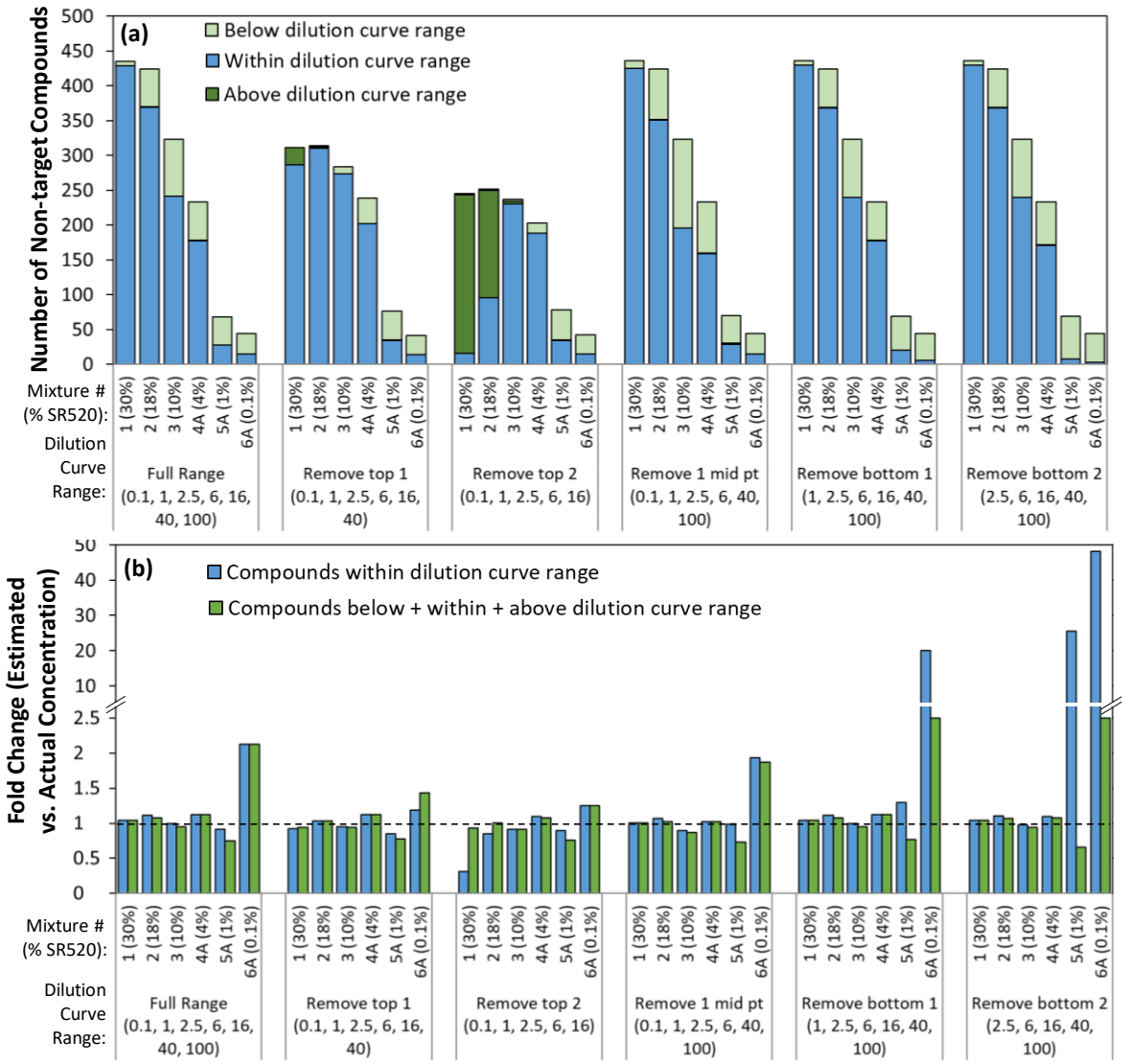

Figure 4. The impact of six iterations of dilution curve range on: (a) the number of non-target compounds in each of the six single-source mixtures that were detected at peak areas below, within, and above the range of the SR520 sample dilution curve, and (b) the resulting SR520 concentration estimates (shown as fold change relative to the actual SR520 concentration) using only non-target compounds with peak areas within the dilution curve range vs. those with peak areas above, within, and below the dilution curve range. Outlier values were included in estimates. The dashed line in (b) indicates actual source concentrations.