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Application News

Non-Targeted Environmental Toxicology Profiling Applied to LC/QTOF River Water Analysis

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User Benefits

- River water samples were measured by direct injection by LC/QTOF mass spectrometry with minimal pretreatment using a non-targeted data independent acquisition (DIA) method.
- Non-targeted analysis (NTA) software, LabSolutions Insight™ Profiler, was applied to perform feature detection, data alignment, statistical analysis and compound identification, successfully detecting contaminants of environmental concern (CECs).
- CECs included pharmaceuticals, antibiotics, restricted substances and wider pollutants including HMMM and phthalates.

■ Introduction

Wastewater-based epidemiological studies have provided valuable insights into wastewater treatment processes and, in recent years, have supported the identification and monitoring of contaminants of environmental concern (CECs). Both targeted and non-targeted analysis (NTA) using full-scan high-resolution mass spectrometry with data-independent acquisition (DIA-MS/MS) have been applied to detect CECs associated with treated and untreated wastewater, including those potentially discharged into receiving waters during storm overflow events.

In this study, river water grab samples were collected across tidal and freshwater environmental systems (Fig. 1). High-resolution LC/QTOF analysis was performed using a direct-injection reversed-phase method with DIA-MS/MS acquisition. Data were processed using non-targeted profiling software to characterise the detected chemical features and identify differences in their distribution both between and within predefined sample groups.

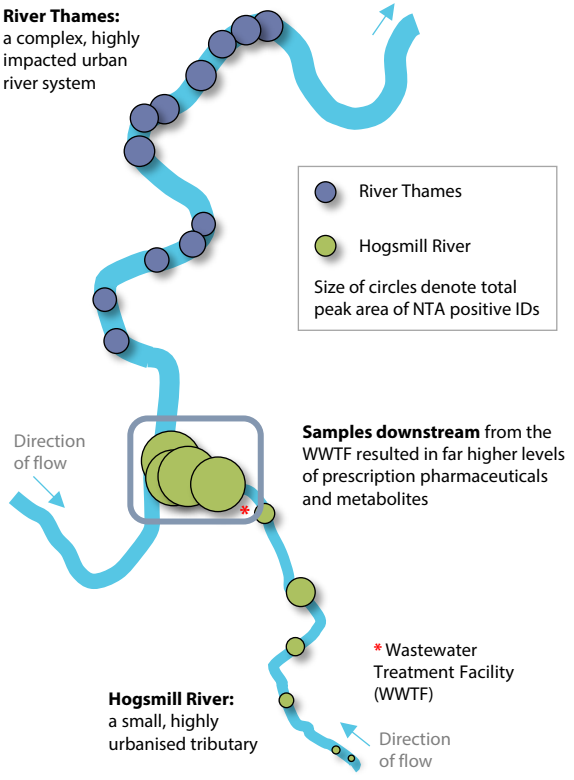


Fig. 1 Summed peak areas of positively identified compounds plotted at sampling locations. Samples taken downstream from a Wastewater Treatment Facility* on the small, highly urbanised tributary. Prescription pharmaceuticals and metabolites accounted for ~70% of all detected compounds.

■ Materials and Methods

River water samples were taken from two river systems in London, England. The River Thames represents a complex, highly impacted urban river system that integrates treated effluent, storm-driven sewer overflows, and diffuse catchment inputs, making it a critical sentinel site for monitoring CECs. It is also tidal, therefore samples were taken during high-to-low tide. The River Hogsmill is a small, highly urbanised tributary that functions as a sensitive sentinel sub-catchment, reflecting localised wastewater inputs, surface runoff, and episodic discharges.

Samples (500 µL) were prepared using Millipore centrifugal filters (Ultrafree - MC – GV, Dispose - PVDF 0.22 µm) for 4 min at 12,000 g. Aliquots from each sample were taken to create a pooled quality control (QC) sample to run throughout the analysis. Samples were measured by direct injection (40 µL) and separated using a generic reversed-phase forensic toxicology gradient. Analysis was performed by electrospray ionisation DIA-MS/MS (LCMS-9050, Shimadzu Corporation) in positive and negative ion modes. Data were processed using LabSolutions Insight Profiler software which performed feature detection, data alignment and compound identification.

Table 1. Analytical conditions

[UHPLC conditions] (Nexera X2)	
Column	Shim-pack Velox™ Biphenyl (2.1x100 mm 2.7 µm); column maintained at 40 ˚C
Flow Rate	0.3 mL/min
Mobile Phase	A: Water + 2 mM ammonium formate + 0.002% formic acid B: Methanol + 2 mM ammonium formate + 0.002% formic acid
Rinse (R0 / R1 / R2)	IPA:MeOH:MeCN:H_2O (1:1:1:1) + 0.5% acetic acid
Gradient	5% B (0-1 min) → 40% B (2 min) → 100% B (10.5-13 min) → 5% B (13-17 min)
Injection	40 µL; autosampler maintained at 4 ˚C
[MS conditions] (LCMS-9050)	
Interface	300 ˚C, 1.5 kV (ESI+) / -3.0 kV (ESI-)
Heat block, DL	400 ˚C, 250 ˚C
Gas flow rates	Nebulizing gas 2 L/min Heating gas 10 L/min Drying gas 10 L/min
MS	TOF-MS (*m/z* 100-1000)
MS/MS	DIA-MS/MS (*m/z* 40-1000; 35 events, 25 msec), CE 5-55 V (0.925 second cycle time)

■ Data Processing

Data files were loaded into LabSolutions Insight Profiler software followed by loading pre-defined processing parameters that included non-targeted feature detection, alignment, statistical analysis, identification and batch processing (Fig. 2).

■Results

Water samples were collected from multiple sites on two rivers to assess whether non-target analysis could differentiate samples based on location. The results from LabSolutions Insight Profiler data processing highlighted the need for systematic monitoring of river samples close to wastewater treatment facilities (WWTFs) as samples taken downstream of the Hogsmill River facility contained a high number of pharmaceutical compounds and metabolites, including venlafaxine, desmethylvenlafaxine, tramadol, O-desmethyltramadol, gabapentin, ketamine, clarithromycin, metronidazole, carbamazepine, and lidocaine, whilst upstream samples showed a markedly lower distribution and concentration of pharmaceuticals. Fig. 3 illustrates differences detected within the Hogsmill group showing four samples separated from the rest of the group accounting for 37% variance between all samples by principal components analysis (PCA). Significant differences identified within the PCA indicated data analysis could be improved if the Hogsmill group were separated into two groups. For example, clarithromycin although detected at very high levels in four Hogsmill samples, failed to show statistical significance due to high group variance. From 7229 aligned features there were 1925 library identifications however only 170 were statistically significant (t-test p-value < 0.05).

Feature Detection and Alignment
Detects and aligns all ion signals that behave as a peak.

Statistics
Applied to find ion signals of significance and to remove ions of high variance in QC samples when used.

Screening and Identification
Using large scale screening lists and multiple libraries to identify ion signals of interest.

Batch
A single, configurable workflow integrates feature detection, alignment, and compound identification, enabling automated batch processing for non-targeted analysis.

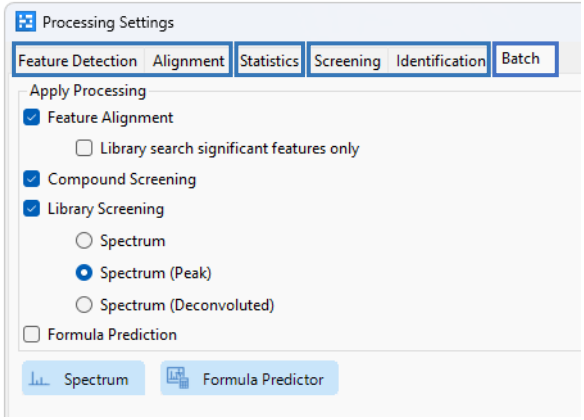


Fig. 2 Insight Profiler settings. In this example pre-defined settings are loaded before all data files are processed as a single batch from which a cascade of automated data analysis steps are performed.

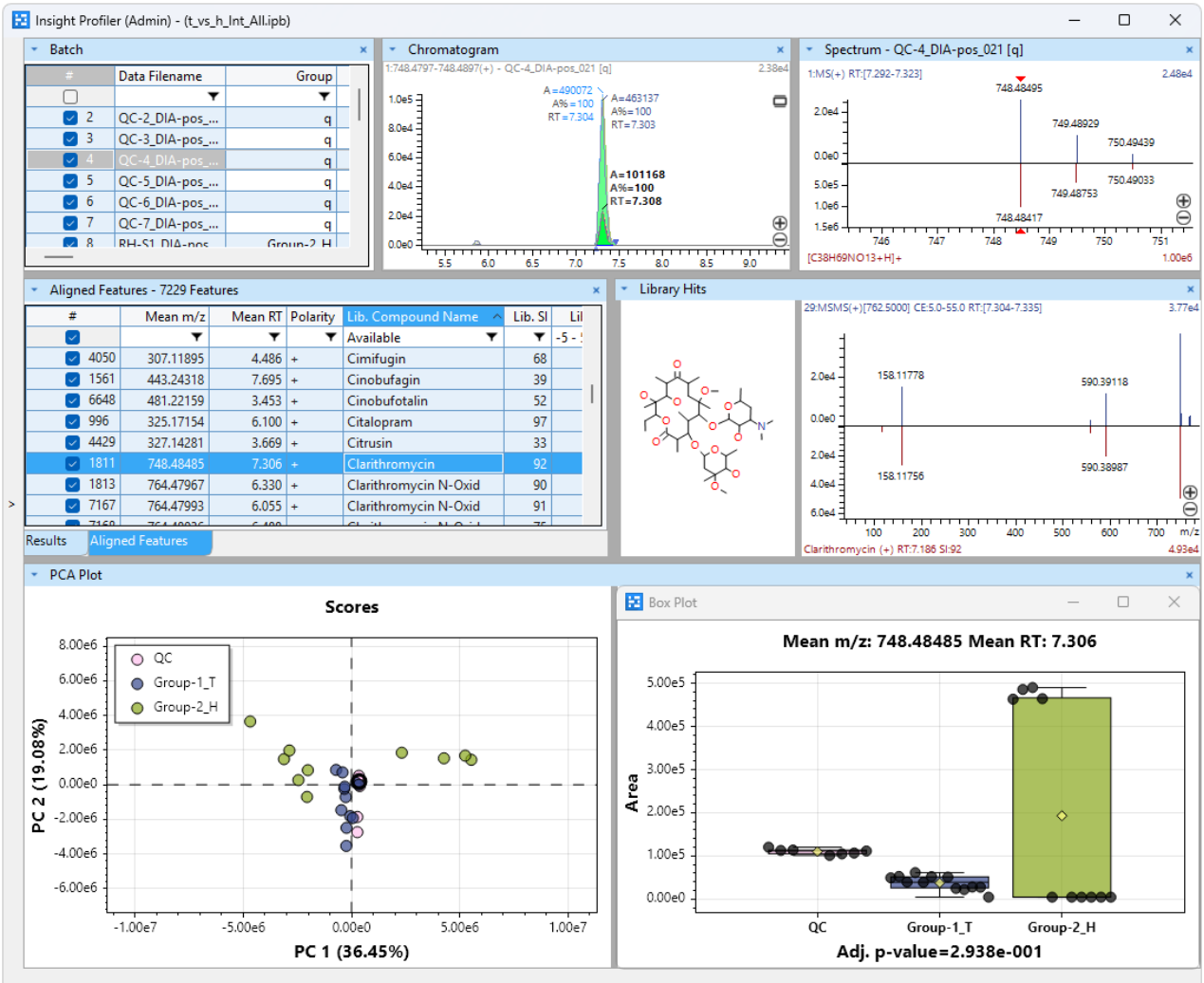
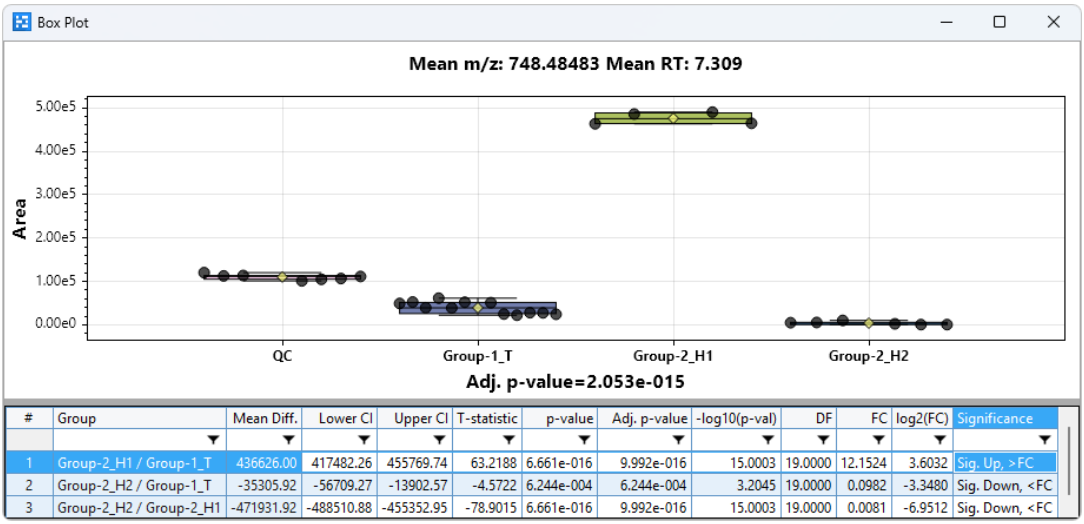


Fig. 3 LabSolutions Insight Profiler screen shot. In this example data analysis compares a small, highly urbanised tributary (Group-2_H) compared to a complex, highly impacted urban river system (Group-1_T); pooled QC samples (QC). The antibiotic clarithromycin is highlighted in the data review software with four sites in Group-2_H showing far higher concentrations at sample points in the small, highly urbanised tributary (green).



#	Group	Mean Diff.	Lower CI	Upper CI	T-statistic	p-value	Adj. p-value	-log10(p-val)	DF	FC	log2(FC)	Significance
1	Group-2_H1 / Group-1_T	436626.00	417482.26	455769.74	63.2188	6.661e-016	9.992e-016	15.0003	19.0000	12.1524	3.6032	Sig. Up, >FC
2	Group-2_H2 / Group-1_T	-35305.92	-56709.27	-13902.57	-4.5722	6.244e-004	6.244e-004	3.2045	19.0000	0.0982	-3.3480	Sig. Down, <FC
3	Group-2_H2 / Group-2_H1	-471931.92	-488510.88	-455352.95	-78.9015	6.661e-016	9.992e-016	15.0003	19.0000	0.0081	-6.9512	Sig. Down, <FC

Fig. 4 Box Plot of clarithromycin in Insight Profiler. Because such large differences were found within the Hogsmill group samples data from this river were split into two groups, the first group containing samples downstream of the WWTF (Group-2_H1) and the second group upstream of the WWTF (Group-2_H2).

The design of LabSolutions Insight Profiler is such that only minimal reprocessing is required if changes are made to the sample group identifiers. Dividing the small highly urbanised tributary into two groups: H1 – downstream of the WWTF, H2 – upstream of WWTF and including the main complex river system enabled a one-way ANOVA. Clarithromycin, previously not statistically significant (Adj. p-value 2.938e-1) was found to be highly significant following reprocessing (Adj. p-value 2.053e-15, see Fig. 4). In this analysis, 477 library identified features were found to be statistically significant between the three sampling areas compared to 170 identified by pairwise analysis. Tukey's HSD and inter group p-values were also calculated. Lamotrigine, a prescription medication used to treat epilepsy and bipolar disorder, was detected at very high levels. Desmethylvenlafaxine, the primary metabolite of the antidepressant venlafaxine was also very high.

Data processing was configured to simultaneously search five libraries including precursor ion screening by formulae and/or *m/z*. Additional libraries included MassBank, Wiley and the Collective Spectral Library (CSL)[1] for small molecules in aqueous environmental samples.

Several antibiotics including azithromycin, clarithromycin, clindamycin and trimethoprim illustrate the concern for antibiotic resistance which may arise through accidental release in treated wastewater. The most significant compound, however, was identified as dimethyldiallylammonium, a chemical commonly used in wastewater treatment as a flocculant to remove suspended solids (Fig. 5).

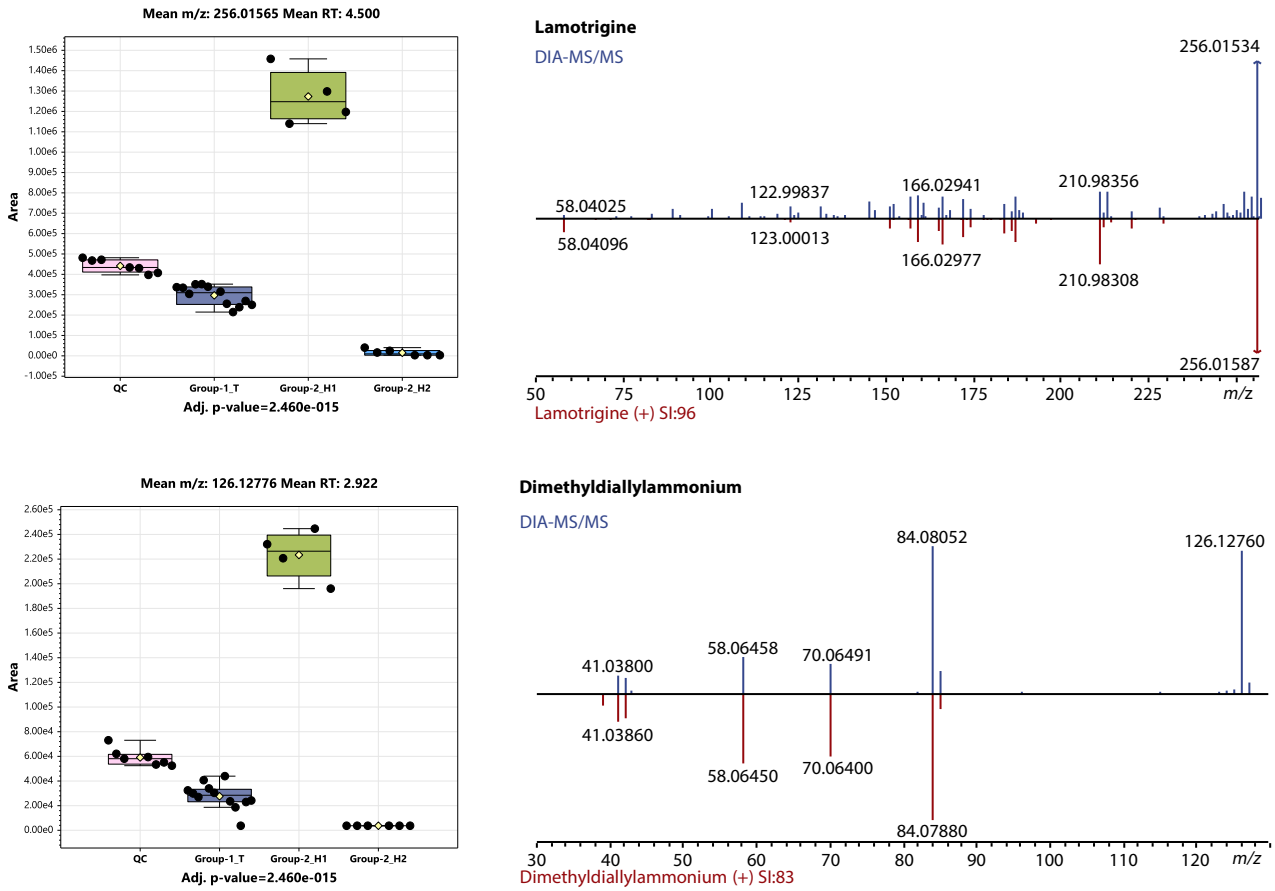


Fig. 5 Examples of statistically significant compounds. Lamotrigine was identified using Wiley LC-HR-MS/MS Library of Drugs, Poisons and Their Metabolites and dimethyldiallylammonium was identified using the Collective Spectral Library (CSL)[1]. These were detected at very high levels in Group-2_H1 downstream of the WWTF. The upper spectra show the acquired DIA-MS/MS spectra and the lower spectra are library MS/MS spectra.

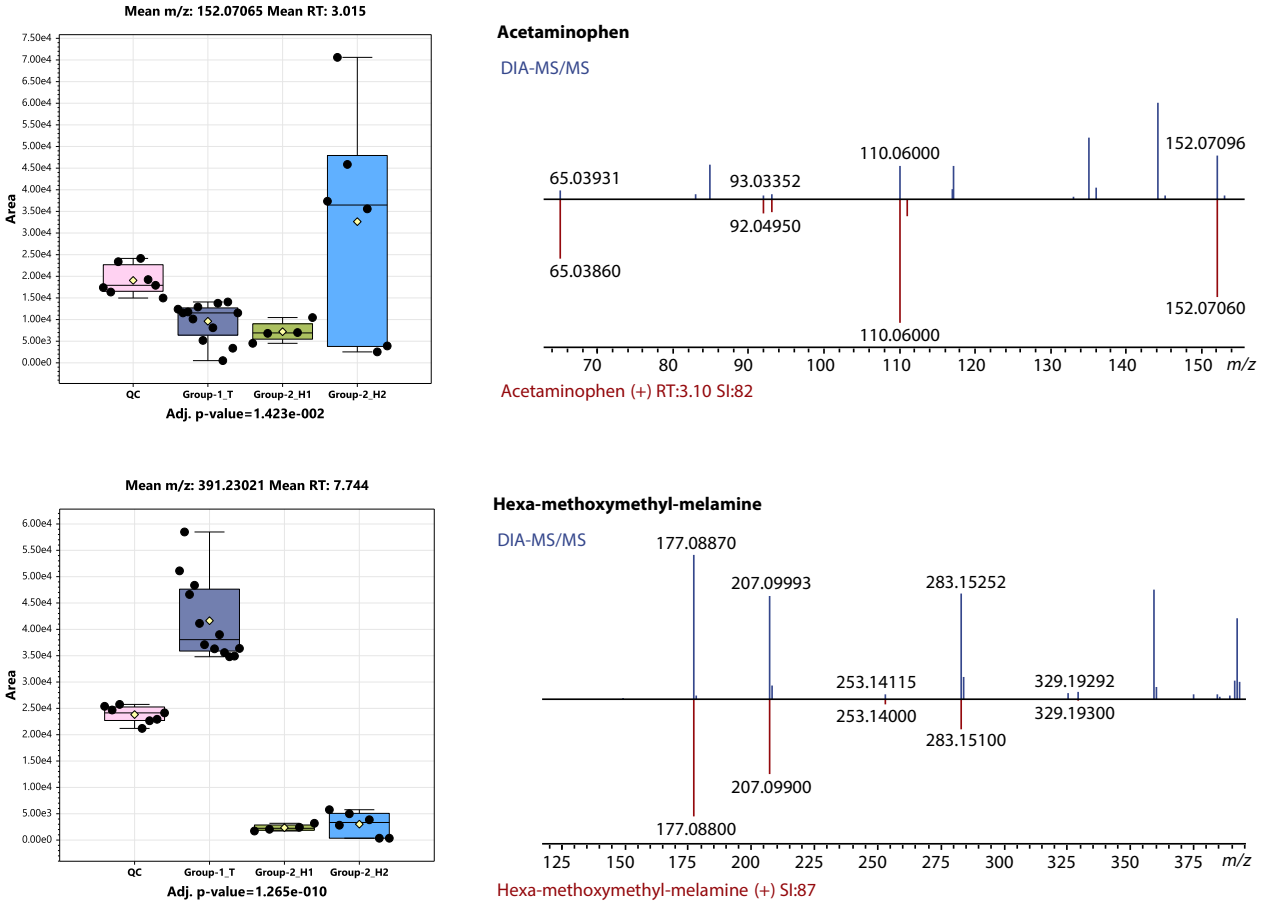


Fig. 6 Example CECs showing profiles from different sample locations. Acetaminophen (identified using the Shimadzu HRAM Library for Forensic Toxicology) was detected at low levels downstream of the WWTF indicating only treated WW entering the waterway. Identification of hexa-methoxymethyl-melamine by the CSL illustrates significant differences between the two river groups but also shows a trend of increasing concentration in downstream River Thames samples (Group-1_T). The upper spectra show the acquired DIA-MS/MS spectra and the lower spectra are library MS/MS spectra.

Fig. 1 illustrates the impact of the WWTF by plotting summed peak areas of all positively identified compounds from each sampling site (circle size proportional to summed peak area). It was notable, however, that acetaminophen (paracetamol) and caffeine, recognised as well-established tracers of wastewater influent, were detected at all sampling sites at relatively consistent levels (Fig. 6). These compounds in addition to the high levels of dimethyldiallylammonium in Group-2_H1 indicate evidence that river water samples impacted by the WWTF most likely contained treated wastewater as opposed to untreated.

It was also notable that the most significant contaminant was identified as hexa-methoxymethyl-melamine (HMMM), an additive widely used in coatings, plastics, and rubber and increasingly recognised as a road-derived emerging contaminant in urban aquatic environments.

■ Conclusion

This work has shown that direct injection of river water with minimal pretreatment can successfully identify large numbers of emerging contaminants ranging from pharmaceuticals to environmental pollutants. Non-targeted analysis was able to identify significant differences between and within sample groups demonstrating the diversity of sample types in environmental toxicology studies. The work has also highlighted the advantage of extending the scope of detection to third party repositories in NTA.

■ References

1. Lessmann *et al.* (2026) Collective Spectral Library; Federal Institute of Hydrology (Bundesanstalt für Gewässerkunde); https://doi.org/10.5281/zenodo.16901589

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