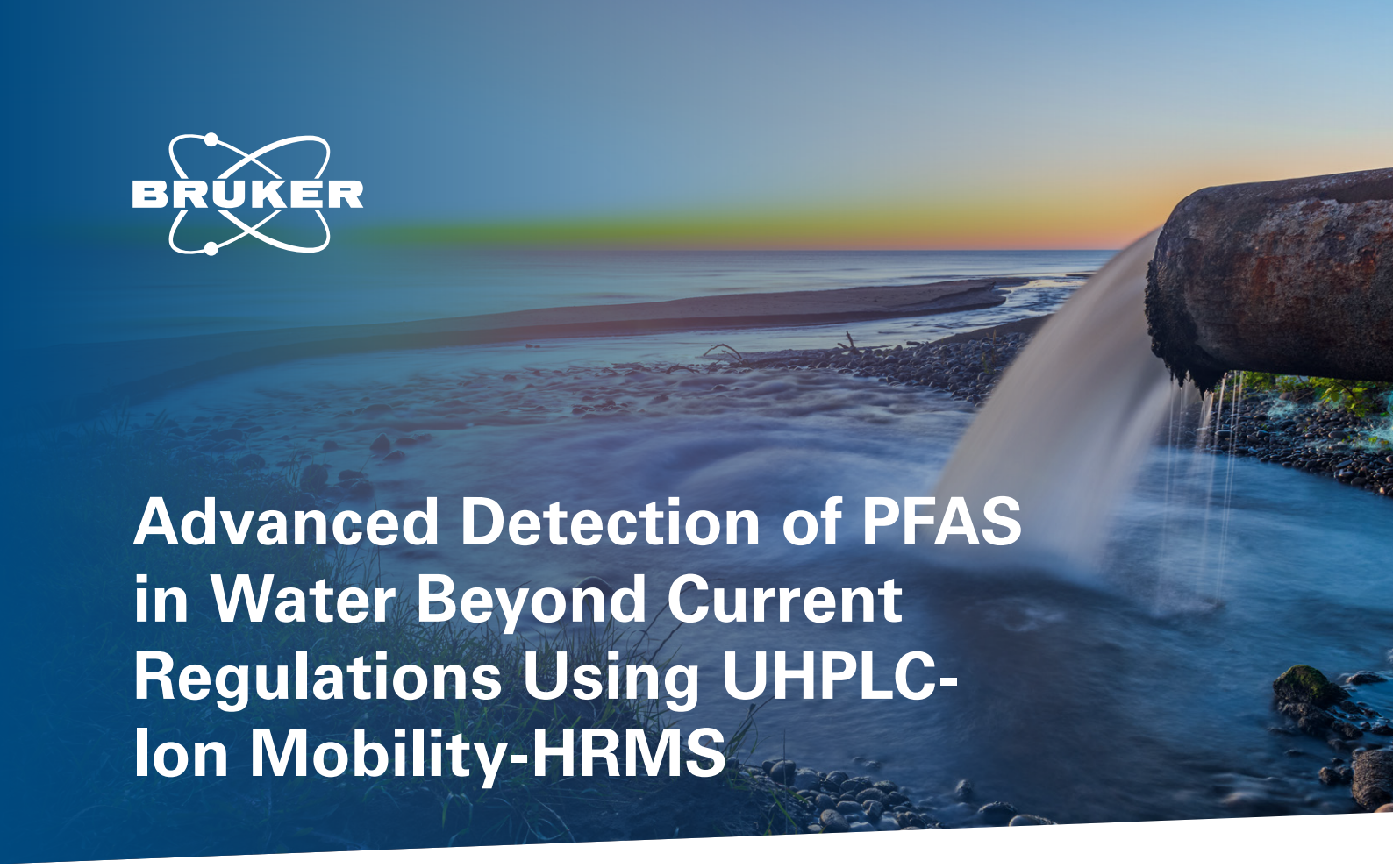

Advanced Detection of PFAS in Water Beyond Current Regulations Using UHPLC-Ion Mobility-HRMS

Abstract

The US EPA lists 14,735 PFAS species as manufactured compounds, precursors, and degradation products [1]. This makes their systematic and comprehensive monitoring an extremely challenging task. This study introduces a novel non-targeted and suspect approach. for a comprehensive analysis of PFAS, incorporating trapped ion mobility spectrometry (TIMS) alongside LC-HRMS/MS data.

Keywords:
PFAS; non-targeted; MetaboScape; timsTOF

Introduction

PFAS: Persistent threats to health and environment

Per- and polyfluoroalkyl substances (PFAS) are highly present in the public news as a true threat to mankind and nature. They are defined by the OECD in 2020 as "fluorinated substances that contain at least one fully fluorinated methyl or methylene carbon atom (without any H/Cl/Br/I atom attached to it)". PFAS have been manufactured and utilized since the 1940s, playing a crucial role in everyday products by serving as heat, water, and grease repellents. PFAS are considered as "forever chemicals" due to their persistent, bioaccumulative, toxic (PBT) properties and ubiquitous presence in the environment and living organisms. The associated health risks are not fully understood for all PFAS, but there is evidence that exposure to PFAS may generate adverse health effects, e.g., on reproduction and development, liver and kidney, the immune system, and may cause tumors. Today, PFAS are found in surface and groundwater due to sources such as landfill sites, agrochemical applications, manufacturing processes, firefighting activities, and more.

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Regulating PFAS: Detection and evolving limits

PFAS are routinely quantified via liquid chromatography (HPLC) coupled to triple quadrupole (TQ) mass spectrometers. Several regulations and legal limits for PFAS concentrations were established in recent years and are constantly evolving. The US EPA has released the final National Primary Drinking Water Regulation (NPDWR) addressing six PFAS compounds. Health advisories are currently in effect for these compounds: PFOA and PFOS (4 ng/L interim), PFHxS, PFNA and HFPO-DA (10 ng/L interim). The EU Drinking Water Directive 2020/2184 includes a requirement to meet a limit of 100 ng/L for a list of 20 different PFAS or 500 ng/L for all PFAS.

Challenges in comprehensive PFAS monitoring

In 2023, five EU national authorities (DE, DK, NL, NO, SE) submitted a proposal for the total restriction on the manufacturing, placing on the market and using of PFAS. Monitoring this restriction means the requirement for the detection and identification of the entity of all PFAS, far beyond the current regulatory rules. The US EPA estimates almost 15,000 species in this group as manufactured compounds, precursors, and degradation products. This makes their systematic and comprehensive monitoring an extremely challenging task. Given the vast number of PFAS compounds, only a small subset is accessible through reference standards or spectral libraries, with many existing as isomers. As a result, traditional targeted analysis using TQ or high-resolution mass spectrometry (HRMS) faces significant challenges in comprehensively covering this extensive chemical group. To address the scarcity of standards and references, several initiatives have been launched to compile PFAS data into suspect lists. The NORMAN network and NIST have generated suspect lists of nearly 5,000 PFAS entries [2,3].

Novel non-targeted approach for comprehensive PFAS analysis

Proposed here is a novel non-targeted approach to a comprehensive overview of PFAS which adds trapped ion mobility spectrometry (TIMS) to mass spectral information from UHPLC-HRMS. The data is screened against existing libraries but as well against the NIST and NORMAN suspect lists, allowing their identification without using reference libraries. The demonstrated approach shows superior performance relative to conventional alternatives in terms of time, reliability, and effectiveness.

Methods

Sampling and sample preparation

Wastewater and surface water samples were collected from 30 different sites in the Netherlands representing various infrastructures like industrial, city, or nature/recreation. Solid phase extraction was used for sample purification and pre-concentration. Sample preparation was performed according to the EPA 1633 regulation. Samples were pre-concentrated by SPE using 25 mL effluent from 200 mL water. 50 µL of internal standards (IS) were added to the samples as well as to blanks. 4 mL 0.1% NH_4OH buffer in MeOH was used for conditioning, and 4 mL 0.1% NH_4OH in MeOH for elution and reconstitution. This resulted in a 2,000-fold pre-concentration for surface water and 500-fold pre-concentration for effluents.

Analytical workflow and PFAS identification

2 µL of sample were injected into the LC/HRMS for a 20 min run time in negative ion mode. The MS method was optimized for PFAS with a parallel accumulation serial fragmentation (PASEF) acquisition workflow for non-targeted analysis for the suspect list screening. The Elute UHPLC was equipped with a kit (Bruker) to eliminate PFAS originating from the instrument and coupled to the timsTOF Pro 2 (Bruker) with a high-sensitivity VIP-HESI (Vacuum Insulated Probe-Heated Electrospray Ionization) source. MS and MS/MS analyses were performed in triplicate for each sample. The analysis workflow is described in Figure 1. Following the data acquisition and feature extraction, spectral library searches in NIST and Bruker-own PFAS libraries were performed. As next step, the data was screened against NORMAN network and NIST suspect lists. These suspect lists contain just very simple information about the name, elemental composition and InChI structure of nearly 5,000 PFAS. Finally, various MetaboScape® software (Bruker) tools are used for the identification of compounds which are not listed in any of these databases.

The **MetaboScape** software supports the ID Steps 2 and 3 by the following tools:

- **SmartFormula** generates a list of potential elemental compositions for each compound
- **CompoundCrawler** searches for corresponding structures for the proposed elemental compositions in databases and suspect lists
- **MetFrag** [4] applies comprehensive *in-silico* fragmentation based on the structural information (either InChI structures from the suspect lists or those found in public databases)
- **CCS-Predict Pro** [5] verifies the structure by comparing experimental CCS values and those predicted based on the structural information (either from InChI structures from the suspects lists or those found in public databases) The prediction of the CCS values is done with high accuracy using machine learning algorithms.

Kendrick Mass Defect (KMD) analysis for PFAS detection

Filtration and reduction of the features of interest by Kendrick Mass Defect (KMD) plots is a widely used technique for detecting and identifying chemically related compounds based on their exact mass. KMD analysis filters potential PFAS from the matrix background, based on the CF_2 repeating units. KMD is an integral part of MetaboScape. For the Kendrick mass scale, the mass of the recurring CF_2 unit in PFAS is defined as a nominal value of 50 Da. The KMD is derived from the exact mass decimals of the end group of the respective PFAS sub-species. In a diagram, the KMD is plotted against the *m/z* scale. Homologous PFAS species with the same end group have the same KMD and align horizontally in the KMD plot with the addition of more CF_2 units. They can therefore be differentiated from the remaining background signals.

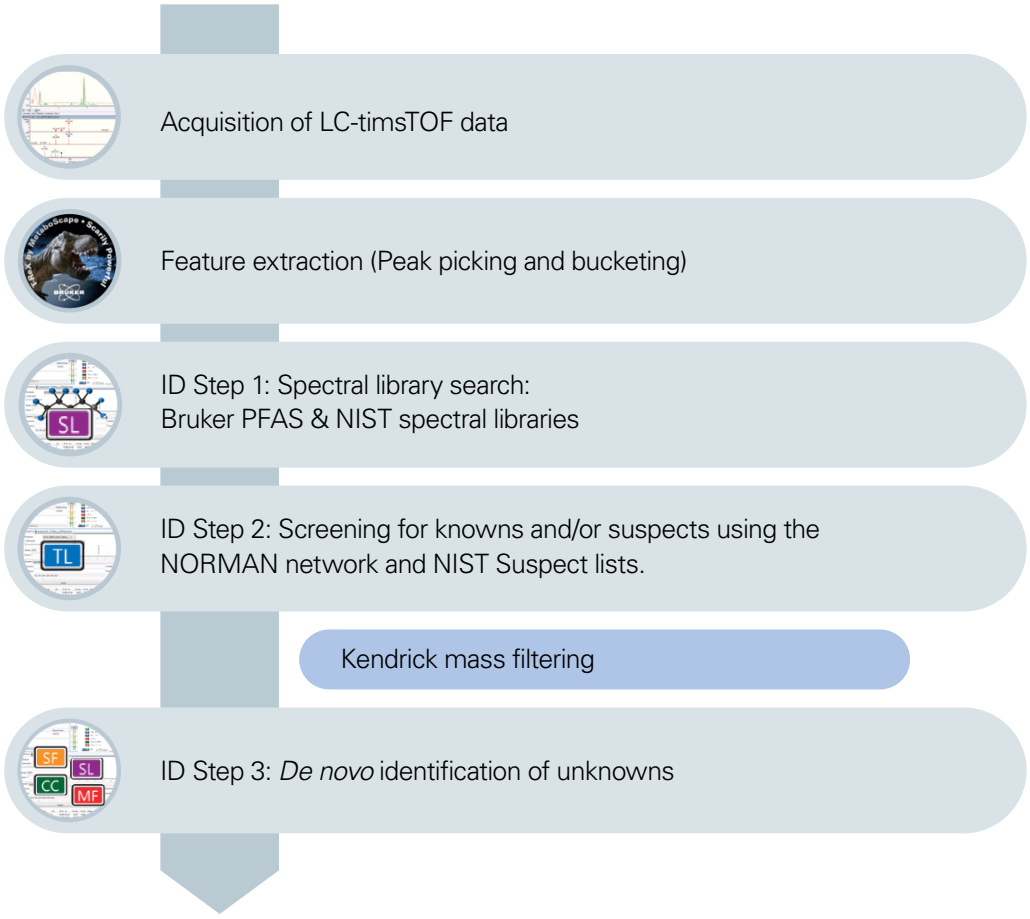

Figure 1. Workflow of the data acquisition, data treatment and identification of the potential PFAS candidates; ID Step = Identification Step.

Results

Utilizing TIMS for enhanced PFAS analysis

In recent approaches, high-resolution mass spectrometers (HRMS) have been applied to potentially differentiate PFAS compounds via small differences in the accurate molecular weights and isotopic patterns. Main issue is still the confidence in identification of the many PFAS without having access to suitable, extensive libraries. TIMS offers several ways to improve performance when monitoring thousands of PFAS in a given sample. TIMS separates ions by their collisional cross sections (CCS), which are a function of shape and size. Initially the ions are 'trapped', i.e., held stationary by the two opposing forces of a pushing gas pressure and a repelling electrical field. The electrical potential is then progressively reduced resulting in controlled release of each ion species, with those of larger cross-sectional area eluting ahead of more compact analogues (Figure 2).
Thus, collisional cross section as an parameter (CCS) as an additional, orthogonal parameter to the MS information are able to enhance the identification confidence as well the TIMS ion trapping results in higher sensitivity and lower detection limits of PFAS by background elimination. Finally, it can be used to separate coeluting isobars and isomers, providing an additional separation to UHPLC-HRMS. This is also of high biochemical interest since the physicochemical properties of for example linear and branched structures vary slightly and lead to differences in biochemical reactions/transformations, bioaccumulation and thus potential toxic exposure. Research suggests that linear PFAS sorb preferably to soil and sediments while branched isomers are more likely to remain in water due to their higher polarity. Human serum samples can contain higher levels of branched PFOS than in the ratio that is usually seen in the production of PFOS by electrochemical fluorination (70% linear) [6].

Enhanced detection and MS/MS coverage with PASEF

The heat map for a representative sample for the 10 different wastewater collection sites is shown in Figure 3. The raw MS data shows more than 8,600 detected features after extraction and highlights the complexity of the sample. Since PASEF provides an extremely high MS/MS speed of > 150 spectra/sec, a very high ratio of 58% of all features is covered by MS/MS spectra. This uniquely high MS/MS coverage is key for not missing the compounds of interest and for the subsequent comparison with *in-silico* fragments and the final identification.

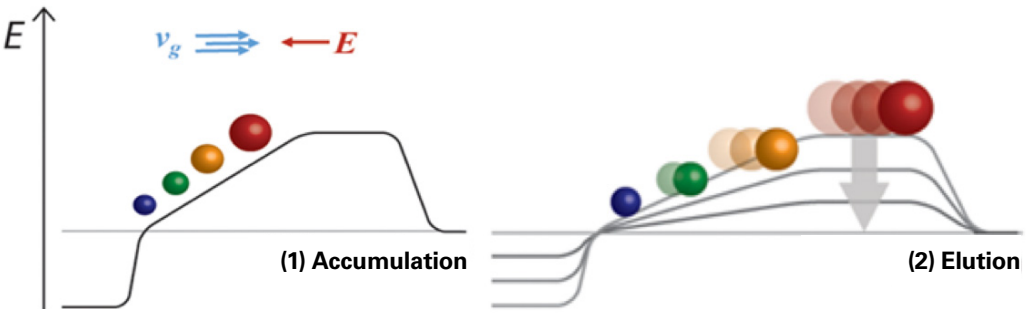

Figure 2. TIMS principle based on the parallel accumulation/trapping and serial release of compounds based on their 3-dimensional structure and size.

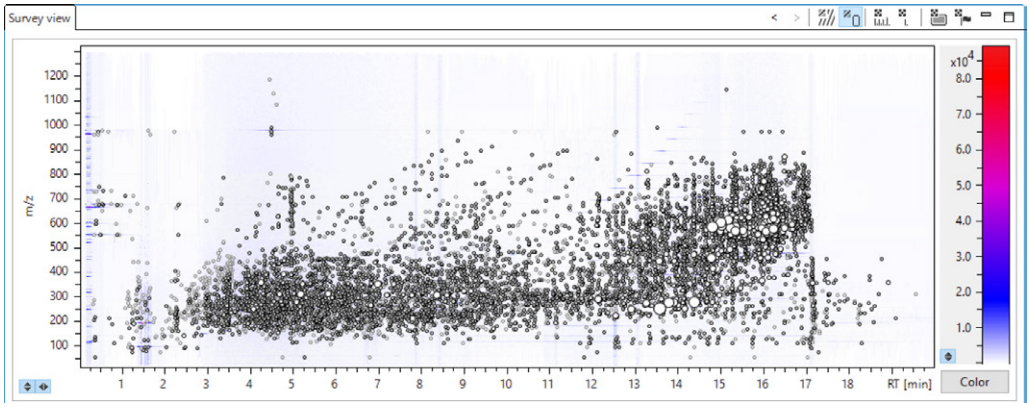

Figure 3. Heat map for an effluent sample from The Netherlands. The retention time is plotted against the detected *m/z* with a color coding for the signal intensity. 8654 features have been detected after T-Rex processing (RT alignment of multiple features, recursive extraction of different ion species for the same compound).

Simplifying data complexity with Kendrick Mass Defect

Kendrick mass defect (KMD) analysis was applied in MetaboScape to filter PFAS signals from the high number of other naturally occurring matrix compounds and to reduce the data complexity. Here, KMD filters potential PFAS from the matrix background based on the CF_2 repeating units. Candidates which may be related to PFAS show up in horizontal lines with discrete data points for the increasing CF_2 units along the *m/z* scale (Figure 4) An important condition for this efficient filtering is the high mass accuracy and precision provided by the timsTOF Pro 2. In total about 20,000 features were detected from all effluents and surface water samples of the 30 sampling sites. About 500 potential PFAS candidates were left after the KMD filtering. This very effective data reduction allows to limit the further data evaluation to the potentially relevant features only.

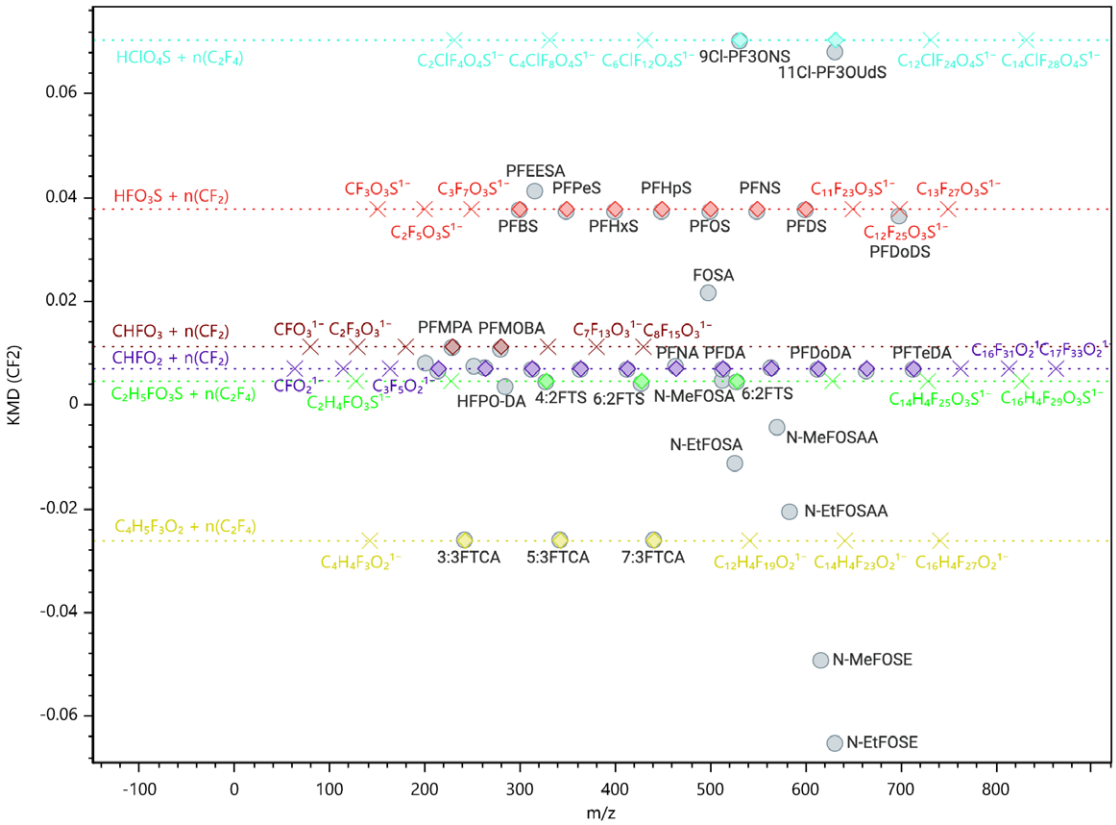

Figure 4. Part of the KMD plot for a standard mixture according to the EPA Method 1633. PFAS of the same group are aligned in horizontal chains representing the addition of CF_2 units. MetaboScape supports the visualization and extension of these homologous rows.

Multi-criteria approach for confident PFAS identification

Figure 6 shows the overview of the results in MetaboScape with an example of the experimental and library spectra match for a representative compound. For the screening of the suspect lists (ID Step 2) and the *de novo* identification (ID Step 3), the combination by four different search criteria exact mass, isotope pattern, MS/MS fragmentation and CCS value were used. The first two criteria are compared with the elemental compositions in the lists to generate a shortlist of candidates. The corresponding structures of these candidates from the suspect list (InChI) or public databases like MetLin and PubMed are *in-silico* fragmented by MetFrag and compared with the experimental MS/MS data of the features. Finally, the experimental CCS (collisional cross section) values of the remaining candidates are compared with machine learning-based predictions from the structures for the found suspects (CCS-Predict Pro). The total match of these various orthogonal criteria generates a highly confident assignment of the PFAS species without the use of reference standards.

Comprehensive PFAS annotation in wastewater

For the wastewater, in total 142 compounds in wastewater was achieved through various steps: 12 were annotated via library search (ID Step 1, dark blue), 14 through the suspect lists (ID Step 2, light blue), and 116 by *de novo* approach (ID Step 3) (Figure 5). This confirms the need to work with data of high information since a lot of PFAS were identified which are not in libraries or the suspect lists.

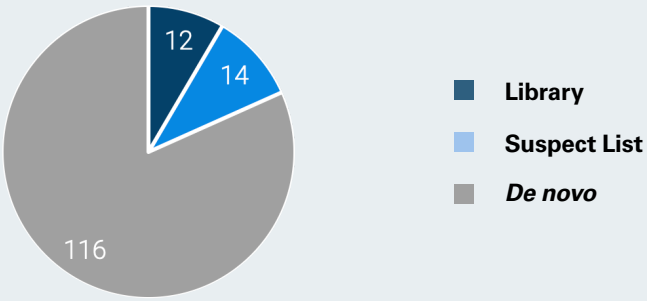

Figure 5. The identification of 142 compounds in wastewater was achieved through various steps: 12 were annotated via library search (ID Step 1, dark blue), 14 through the suspect lists (ID Step 2, light blue), and 116 using the de novo approach (ID Step 3, grey).

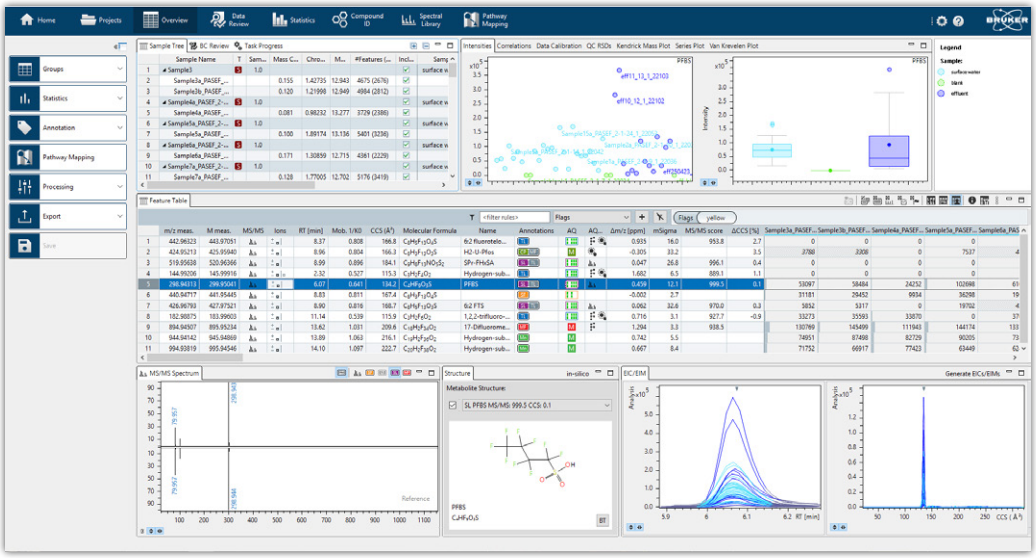

Figure 6. Overview of the results in MetaboScape with a butterfly overlay of the experimental and the spectral data for a representative compound.

PFAS distribution and detection in surface water

For the surface water samples, 137 features could be annotated as PFAS. The number of detected PFAs varies at the different sampling sites, as expected from their local conditions and infrastructure. Figure 7 compares these numbers for the 18 sites. While a higher number was observed e.g., at the sides 1, 13 and 17, lower contamination is visible e.g., at the sites 4 and 15. A couple of PFAS precursors and degradation products have been found which are not screened yet in any legal directives and would have been overlooked in a targeted approach. I.e. while the targeted compounds would be quantified at actual low levels, the degradation products may be present at high levels posing a potential hazard and health risks. An example is H2-U-PFOS which was identified in step 3. This unsaturated PFOS has already been found before in the environment, but a standard is not commercially available yet. Compounds like this one cannot be found with any current target or library search approach.

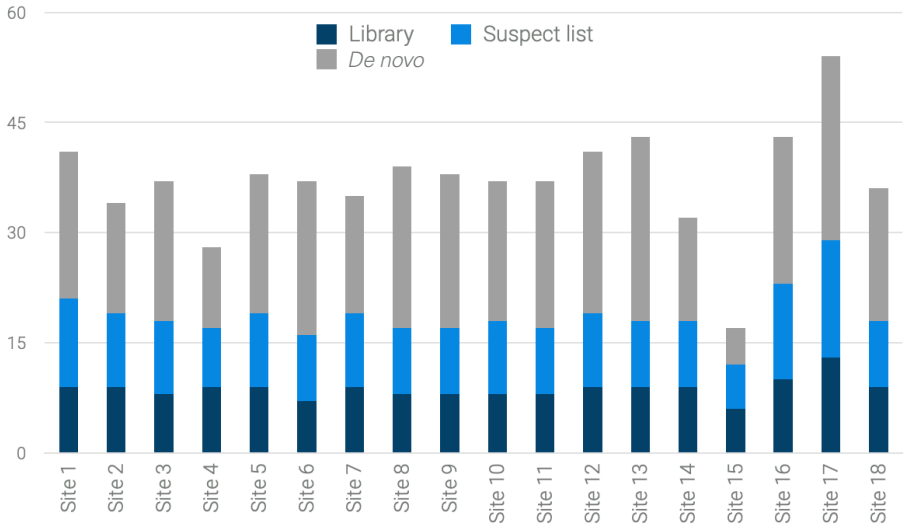

Figure 7. Number of PFAS compounds found at the different surface water sites. While a higher number was observed e.g., at the sides 1, 13 and 17, lower contamination is visible e.g., at the sites 4 and 15. This is possibly due to the local infrastructure and environmental circumstances.

Scoring framework for non-target PFAS analysis

The certainty of PFAS identifications resulting from HRMS data must be communicated through a reliable and harmonized approach. Charbonnet *et al.* [7] have recently proposed a confidence scale along with identification criteria which are specific to the suspect or nontarget analysis of PFAS by HRMS. Confidence levels range from 1a (Confirmed by Reference Standard) and 1b (Indistinguishable from Reference Standard) to 5 (Exact Masses of Interest). Adding the structural information to the identification workflow by MS/MS and CCS values results in scoring levels of 1 and 2 mostly (table 1), thus confirming the reliability and high confidence of the combined Ion Mobility-HRMS approach. As well, the KMD filtering supports higher scores with the annotation of homologues compound series (level 2c).The study enforces the need of non-target PFAS studies and proves the high sensitivity of the workflow for the successful identification of yet unknown or unexpected PFAS products, precursors and degradation compounds.

Table 1. Charbonnet scorings and respective results from the ion mobility-HRMS workflow presented here. The combination of mass accuracy, library search and structural information by MS/MS and CCS values results in scoring levels of 1 and 2.

Level	Identification confidence	ID step
1a	Confirmed by reference standard	BDAL PFAS library including in-batch analysis of CRS
1b	Indistinguishable from reference standard	BDAL PFAS library
2a	Probable by library spectral match	NIST PFAS library
2b	Probable by diagnostic fragmentation evidence	Suspect list including *in-silico* fragmentation for MS/MS and CCS prediction
2c	Probable by diagnostic homologue evidence	Homologues PFAS series from KMD plots
3a	Positional isomer candidates	
3b	Fragmentation-based candidate	
3c	Circumstantial candidate with fragmentation evidence	
3d	Circumstantial candidate with homologue evidence	
4	Unequivocal molecular formula	
5a	PFAS suspect screening exact mass match	Just MS data comparison with suspect lists
5b	Nontarget PFAS exact mass of interest	

Conclusions

Addressing analytical challenges in PFAS detection

Increasingly stringent regulatory requirements intensify the need for robust and reliable PFAS detection and identification, but the analytical challenge is significant. There are thousands of PFAS, but few standards and libraries available. Samples routinely hold compounds of interest in complex matrices.

Comprehensive 4D workflow for PFAS identification

Presented here is a novel and unique approach for matching high-quality 4D data of exact mass, isotope pattern, MS/MS and CCS with the information from libraries as well as large suspect lists. The usage of the timsTOF Pro 2 and MetaboScape in combination with the "3 ID steps" suggests that all three annotation tools were used in sequential order to annotate one feature. By library search, NIST or NORMAN network PFAS suspect list screening and *de novo* compound elucidation proved to be a comprehensive approach for a fast and efficient identification of unknown PFAS in complex environmental matrices. It represents a simple workflow with automated feature finding and consecutive annotation, direct result and information generation.

Advancing PFAS analysis beyond targeted methods

This approach extends the PFAS analysis beyond the constraints of established targeted methods and enables the detection of a virtually unlimited number of PFAS including isomers in complex matrices. This takes the PFAS analysis to a new level that is consistent with societal efforts towards greater health and environmental protection and security for the future, beyond the current regulatory requirements.

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