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# An integrated approach to monitor PFAS in the water chain

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# Colophon

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Astrid Reus, MSc.

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**Author(s)**
Dr. Frederic Béen, Dr. Bjorn Berendsen, Dr. Elvio Amato, Astrid Reus, MSc.

**Quality Assurance**
Dr. Thomas ter Laak

**Sent to**
Collaborating partners

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More information
Dr. Frederic Béen
T +31 (0)30 60 69 748
E Frederic.Been@kwrwater.nl

PO Box 1072
3430 BB Nieuwegein
The Netherlands

T +31 (0)30 60 69 511
E info@kwrwater.nl
I www.kwrwater.nl

# Samenvatting

Dit rapport presenteert een geïntegreerde aanpak voor het monitoren van per- en polyfluoralkylstoffen (PFAS) in de waterketen en biedt een uitgebreid overzicht van de uitdagingen en innovaties op het gebied van PFAS-analyse. PFAS zijn een grote groep door de mens gemaakte chemicaliën die in veel industriële en producten worden gebruikt. Hun persistentie in het milieu en potentiële gezondheidsrisico's hebben echter tot grote bezorgdheid geleid. Traditionele monitoringprogramma's richten zich meestal op een beperkt aantal bekende PFAS - tussen de 20 en 40 legacy verbindingen - maar deze aanpak omvat niet het volledige spectrum van PFAS die in het milieu aanwezig zijn. Vanwege de enorme chemische diversiteit van PFAS, gecombineerd met de variabiliteit in emissiebronnen, zijn meer alomvattende en aanpasbare monitoringstrategieën nodig.

Om deze uitdaging aan te gaan, zijn in het project verschillende analysetechnieken geëvalueerd. Een veelgebruikte methode is vloeistofchromatografie gekoppeld aan tandemmassaspectrometrie (LC-MS/MS). Deze techniek is zeer gevoelig en nauwkeurig en kan extreem lage concentraties van geselecteerde PFAS detecteren. Het grootste voordeel is de nauwkeurigheid, maar deze techniek kan alleen verbindingen identificeren die vooraf zijn geselecteerd, wat betekent dat PFAS die niet op de doellijst staan niet worden gedetecteerd. De Total Oxidizable Precursor (TOP) assay kan daarentegen PFAS-precursoren omzetten in meetbare legacy PFAS, waardoor het detectiebereik wordt vergroot. De TOP-analyse heeft echter zijn eigen beperkingen; er kunnen PFAS worden gemist die tijdens het oxidatieproces niet worden omgezet. Andere technieken, zoals extraheerbare organische fluoranalyse met behulp van verbrandingsionchromatografie (EOF-CIC) en particle-induced gamma-ray emission (PIGE), bieden een breder overzicht door het totale fluorgehalte in een monster te meten. Hoewel deze methoden een breder scala aan PFAS kunnen detecteren, hebben ze over het algemeen niet de gevoeligheid van MS-gebaseerde methoden en zijn ze mogelijk alleen effectief als de PFAS-concentraties relatief hoog zijn. De toepassing van suspect and non-target screening (SNTS) met behulp van hoge-resolutie massaspectrometrie (HRMS) werd ook onderzocht. Deze geavanceerde techniek heeft het potentieel om een breder scala aan PFAS-verbindingen te detecteren, waaronder verbindingen die gewoonlijk niet door conventionele gerichte methoden worden gedekt. Hoewel deze aanpak een belangrijke stap voorwaarts betekent, is hij niet zonder beperkingen. Detectie is nog steeds afhankelijk van de concentratie van de verbindingen en de beschikbaarheid van voldoende analytische gegevens om met zekerheid nieuwe stoffen te identificeren.

Het project erkent dat geen enkele methode het volledige spectrum van PFAS-verontreiniging kan omvatten en stelt daarom een gefaseerde monitoringaanpak voor. Deze strategie begint met conventionele gerichte analyse (met LC-MS/MS of hoge-resolutie massaspectrometrie) om bekende PFAS te kwantificeren. De resultaten worden vervolgens vergeleken met totale PFAS-metingen die verkregen zijn via de TOP-analyse of EOF-CIC. Als deze vergelijkingen discrepanties aan het licht brengen - wat duidt op de aanwezigheid van aanvullende, niet-geïdentificeerde PFAS - is de volgende stap het toepassen van suspect and non-target screening (SNTS) met hoge-resolutie massaspectrometrie. Deze gelaagde strategie helpt niet alleen om hiaten in de huidige monitoring op te sporen, maar begeleidt ook de integratie van nieuw gedetecteerde PFAS in routineanalyses, op voorwaarde dat er referentiematerialen beschikbaar zijn voor bevestiging.

Dit project biedt verschillende aanbevelingen voor toekomstig werk. Ten eerste moeten monitoringprogramma's verder gaan dan de beperkte set van legacy PFAS en aanvullende technieken opnemen om een completer beeld van de verontreiniging te krijgen. Ten tweede is er behoefte aan betere referentiedatabases en voorspellende tools om niet-doelgerichte screeningsmethoden te verbeteren en zo de identificatie van onbekende en opkomende PFAS te verbeteren. Ten slotte zal de ontwikkeling en implementatie van uitgebreide monitoringprotocollen die gerichte analyses combineren met totale PFAS-beoordelingen en non-target screening cruciaal zijn voor een nauwkeurige beoordeling van de waterkwaliteit en de bescherming van de volksgezondheid. Door deze geïntegreerde, gefaseerde aanpak kunnen toezichthouders en belanghebbenden PFAS-verontreiniging effectiever opsporen en hun inspanningen beter afstemmen op de specifieke problematiek.

# Summary

This report presents an integrated approach to monitoring per- and polyfluoroalkyl substances (PFAS) in the water chain, offering a comprehensive view of the challenges and innovations in PFAS analysis. PFAS are a large group of man-made chemicals used in many industrial and consumer applications. However, their persistence in the environment and potential health risks have raised significant concerns. Traditional monitoring programs typically focus on a limited number of well-known PFAS—usually between 20 and 40 legacy compounds—but this approach does not capture the full spectrum of PFAS present in the environment. Because of the vast chemical diversity of PFAS, combined with the variability in emission sources, more holistic and adaptative monitoring strategies are required.

To tackle this challenge, the project evaluated several analytical techniques. One widely used method is liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). This technique is highly sensitive and precise, capable of detecting extremely low concentrations of selected PFAS. Its main advantage lies in its accuracy, but it only identifies compounds that are pre-selected, meaning that any PFAS not on the target list remain undetected. In contrast, the Total Oxidizable Precursor (TOP) assay can transform PFAS precursors into measurable legacy PFAS, thereby extending the detection range. However, the TOP assay has its own limitations; it may miss PFAS that do not convert during the oxidation process. Other techniques, such as extractable organic fluorine analysis using combustion ion chromatography (EOF-CIC) and particle-induced gamma-ray emission (PIGE), provide broader overviews by measuring the total fluorine content in a sample. Although these methods can capture a wider range of PFAS, they generally lack the sensitivity of MS-based methods and may only be effective when PFAS concentrations are relatively high. The application of suspect and non-target screening (SNTS) using high-resolution mass spectrometry (HRMS) was also investigated. This advanced technique has the potential to detect a wider range of PFAS compounds, including those that are not typically covered by conventional targeted methods. Although this approach represents a significant step forward, it is not without limitations. Detection is still dependent on the concentration of the compounds and the availability of sufficient analytical data to confidently identify new substances.

Recognizing that no single method can capture the full spectrum of PFAS contamination, the project proposes a tiered monitoring approach. This strategy begins with conventional targeted analysis (using LC-MS/MS or high-resolution mass spectrometry) to quantify known PFAS. The results are then compared to total PFAS measurements obtained via the TOP assay or EOF-CIC. If these comparisons reveal discrepancies—suggesting the presence of additional, unidentified PFAS—the next step is to employ suspect and non-target screening (SNTS) with high-resolution mass spectrometry. This layered strategy not only helps to pinpoint gaps in the current monitoring but also guides the integration of newly detected PFAS into routine analyses, provided that reference materials are available for confirmation.

This project offers several recommendations for future work. First, monitoring programs should expand beyond the limited set of legacy PFAS and incorporate complementary techniques to capture a more complete picture of contamination. Second, there is a need for better reference databases and predictive tools to enhance non-target screening methods, thereby improving the identification of unknown and emerging PFAS. Finally, the development and implementation of comprehensive monitoring protocols that combine targeted analysis with total PFAS assessments and non-target screening will be crucial for accurately assessing water quality and protecting public health. By adopting this integrated, tiered approach, regulators and stakeholders can more effectively track PFAS contamination as well as tailor their monitoring efforts to the specific issues at stake.

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# 1 Problem definition

Per- and polyfluoroalkyl substances (PFAS) are a diverse group of chemicals that are ubiquitously found in the environment, including drinking water sources. Due to their potential toxic effects, these chemicals are considered a threat to human and environmental health. Besides other sources, humans are exposed through drinking water obtained from surface water, and indirectly through irrigation of crops with contaminated surface water. Some of these compounds are listed as hazardous priority substances in the European Water Framework Directive (WFD), and are also regulated by the Drinking Water Directive (DWD), which has set a threshold value of 0.1 µg/L for the sum of 20 PFAS and 0.5µg/L for total PFAS loads (van de Aa et al., 2021). The European Food Safety Authority (EFSA) has also indicated that PFAS are substances of very high concern occurring in drinking water, fish, fruit, eggs, and egg products, and has defined a maximum daily intake for 4 selected PFAS. It is important to note that there are almost 15000 compounds classified as PFAS according to the US-EPA CompTox database (https://comptox.epa.gov/dashboard/chemical-lists/PFASSTRUCT), and these can occur as complex mixtures in the environment water.

The chemical industry is constantly developing new PFAS, such as hexafluoropropylene oxide dimer acid (HFPO-DA) which was introduced in 2017 to replace PFOA. Scientific studies have also shown that several other PFAS (in complex mixtures) are present in Dutch surface water (Awchi et al., 2022; Hensema et al., 2020; Sadia et al., 2023). Due to the increasing number of PFAS that are found in the environment, it is not sufficient to monitor only individual substances, and a measurement of total PFAS or a PFAS-group approach has been considered by regulators to adequately identify risks to humans and the environment (Göckener et al., 2021). PFAS occurrence varies greatly from region to region, depending on the contamination sources and emissions. However, current monitoring programmes only analyse a limited set of PFAS (Joerss and Menger, 2023). As a result, the presence and distribution of all PFAS in surface and drinking water is unknown. Current monitoring programs rely on targeted methods to measure a list of 20–40 legacy PFAS. However, these compounds are not always the most relevant or the primary contributors to PFAS contamination at all locations, given the wide variety of PFAS that may be present and the variability in emission sources. As a result, conventional approaches may not provide a comprehensive assessment of water quality concerning PFAS contamination. To address this gap, more holistic monitoring strategies that incorporate complementary techniques are needed to gain a more complete understanding of the overall PFAS burden.

To design an effective and cost-efficient monitoring programme, a comprehensive picture of the occurrence of PFAS in our water (including those PFAS that are so far inadequately monitored or even unknown) and their potential risks to human health and the environment is needed. However, the chemical properties of PFAS greatly vary, and different methods are required to measure all relevant PFAS at low levels in the water cycle. To this end, we propose an integrated analytical strategy consisting of the parallel and partly sequential application of several established or developing analytical methods. This strategy strives to provide a comprehensive assessment of the occurrence of PFAS in the water cycle (river, surface, ground and drinking water), and is designed to enable (1) better mapping of the distribution of PFAS in the integrated water system, (2) effective and cost-efficient site-specific monitoring, (3) prioritization of potential risks, and (4) improve decision making for pollutant mitigation.

# 2 Consortium, objectives and project structure

This project is part of the TKI *Landbouw, Water en Voedsel* (LWV) public and private cooperation and was carried out by KWR Water Research Institute and Wageningen Food Safety Research (WFSR) in collaboration with *Evides, Oasen, Vitens, Brabant Water, Waterschap de Domme, RIWA-Rijn, Provincie Zuid-Holland, Arcadis, Witteveen + Bos and TTE Consultants*.

This project aimed to assess several approaches, including sample preparation, analytical techniques, and effect-based and in-silico methods to develop an integrated monitoring strategy to monitor PFAS contaminations in water samples. The rationale behind developing such a strategy is that several methods can be applied sequentially or in parallel as each method provides information about the (possible) presence of PFAS. The combined interpretation of the results obtained from these measurements is supposed to give a more complete picture of the presence of PFAS in water samples. Most of the techniques explored in the context of this study are available at KWR and/or Wageningen Food Safety Research (WFSR), which provide the expertise needed to execute this programme. Due to the complexity of water management, various parties including provinces, water boards, and drinking water companies were involved in this project.

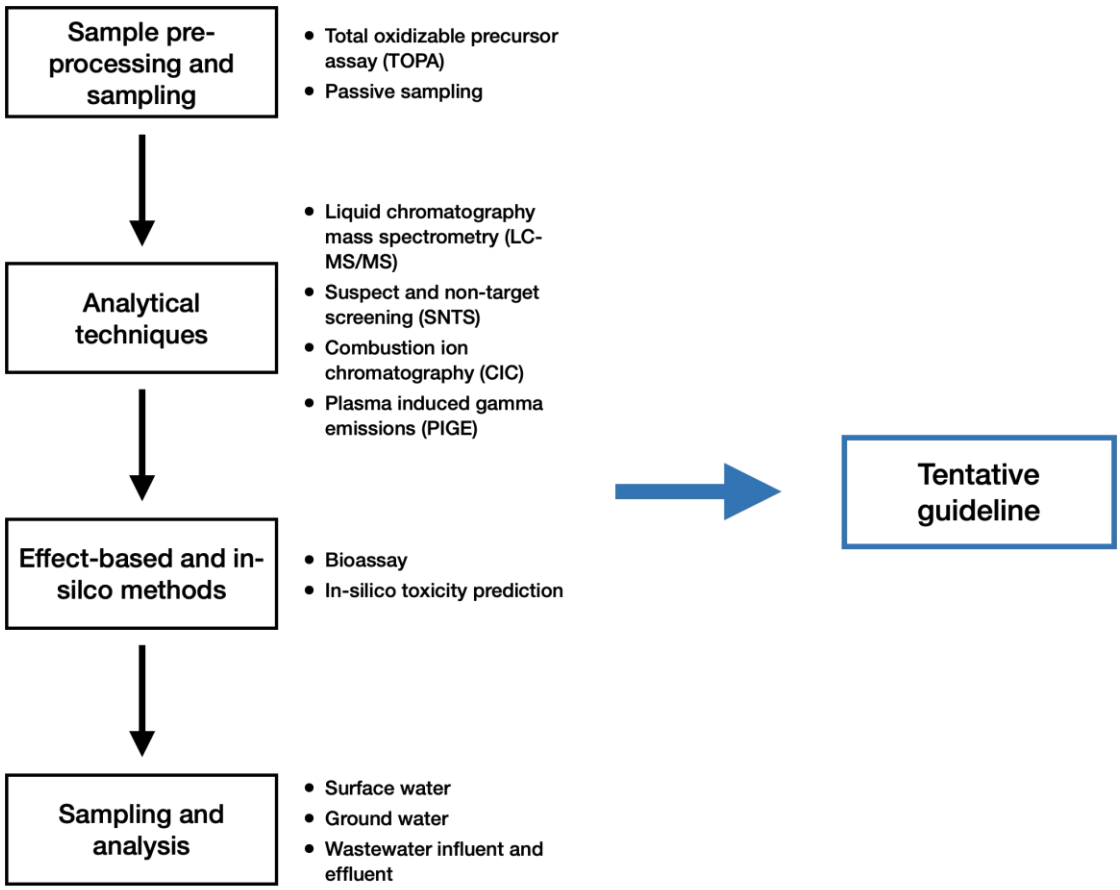


*Figure 1: Overview of the tasks/activities covered in this project.*

# 3 Developed and tested techniques

This chapter describes the methods and techniques that were tested and/or developed in the context of this project. It also provides a description of the advantages and limitations of each technique with respect to its coverage of the PFAS chemical space (see Figure 2).

PFAS analysis is a challenging task due to (1) the large number of structures potentially present, (2) their broad range of physicochemical characteristics (e.g., polar, apolar, water-soluble and volatile) and (3) the lack of a universal method which allows detecting, identifying and quantify all PFAS-like structures. The approach proposed in the context of this work involved the combination of several methods and techniques to provide a more comprehensive assessment of PFAS in water samples. We focused on different sampling methods (grab sampling and passive sampling) as well as analytical techniques that enable us to investigate the vast PFAS chemical space and identify PFAS that are typically overlooked by conventional analytical techniques. Sample pre-processing and sampling strategies

### 3.1.1 Total oxidizable precursor (TOP) assay

The Total Oxidizable Precursor (TOP) assay relies on the oxidative conversion of PFAS precursors into stable perfluoroalkyl carboxylic acids (PFCA) that are typically included in routine monitoring. This is achieved by means of hydroxyl radicals generated using thermolysis of persulfate under basic (pH >12) conditions. The difference in PFCA concentrations before and after oxidation is used to infer the presence of PFAS precursors. The occurrence of precursors may largely vary among samples, and in some cases precursors may be a dominant fraction in relation to the PFAS commonly measured by conventional methods. This technique is inherently more selective than other methods that measure general organic fluorine, such as extractable organic fluorine (EOF), adsorbable organic fluorine (AOF), and particle-induced gamma-ray emission (PIGE). The key advantage lies in the availability of structural information, including retention time and, most importantly, mass spectrometric data, which is not provided by these other techniques. However, from the data it was observed that some precursors (e.g. if modifications in the CF-tail are done) may result in the formation of other compounds than fully saturated PFCA, and thus remain non-detected by the TOP assay. As such, the overall PFAS burden in a sample may be underestimated. However, this method is typically more sensitive than those relying on the determination of total organic fluorine and is compatible with instrumentation commonly found in analytical laboratories.

### 3.1.2 Passive sampling

Passive sampling involves the in situ preconcentration of pollutants dissolved in water, followed by the extraction and analysis of the accumulated pollutants. Preconcentration is achieved using specific sorbents that are typically deployed for a few weeks, and can be used to estimate the average concentration of a given pollutant during the sampling period. The in situ preconcentration often results in higher sensitivity and lower detection limits. Passive sampling devices tested in the context of this project consisting of 4-cm long microporous polyethylene tube (MPT) filled with Strata ZT-WAX sorbent material were successfully employed to measure PFAS in groundwater (Kaserzon et al., 2019). These sorbents are also commonly used in solid-phase extraction protocols for routine analysis of anionic PFAS. Passive sampling extracts proved also suitable for performing SNTS analysis of PFAS, suggesting that these two approaches can be combined to increase the array of available sampling options and analytical capabilities. For instance, time-integrated measurements provided by passive sampling may improve the detection of chemicals that could be otherwise missed using spot sampling, which provides ‘snapshot’ concentrations that do not account for contaminants variation over time. Appropriate deployment times may also result in greater preconcentration and lower detection limits. Passive sampling devices are commercially available and their performance is typically well characterized. Depending on the type of sorbent material selected, more or less polar compounds can be retained.

## 3.2 Analytical techniques

### 3.2.1 Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is the preferred method for determining PFAS in various samples, including water. This technique is highly sensitive, allowing for the detection of very low concentrations of selected PFAS. However, a major limitation is that only pre-selected PFAS, programmed into the method beforehand, will be detected, while all other PFAS remain undetected. Furthermore, LC-MS/MS often relies on reversed phase (RP) chromatography which is not suitable for PFAS with very short carbon chains (C1 - C3, where Cn indicates the number of carbon atoms present in the molecular structure). These compounds – from here onwards referred to as 'ultrashort-chain' PFAS – are very polar and require a different chromatographic approach to ensure retention and separation. To overcome this limitation, we developed a (semiquantitative) LC-MS method based on mixed-mode (MM) chromatography to assess a selection of ultrashort-chain PFAS, i.e., pentafluoroethane sulfonamide (C2), perfluoroethane sulfonic acid (C2), perfluoropropanoic acid (C3), trifluoroacetic acid (C2 or TFA), trifluoromethanesulfonic acid (C1), and trifluoromethane sulfonamide (C1). While these compounds are less bioaccumulative compared to legacy compounds like PFOA and PFAS, they can represent a threat to human and environmental health due to their high mobility and persistence in the environment, and difficult removal from water. The MM chromatography method developed in this project was also used for suspect and non-target screening (SNTS) purposes, allowing to expand the number of ultra-short chain PFAS which can be detected (e.g., perfluoropropanesulfonic acid (PFPrS)). One limitation of LC-based methods is that these are not capable of analysing volatile PFAS (e.g., gases). In these cases, gas chromatography (GC) coupled to MS needs to be employed.

### 3.2.2 Suspect and non-targeted screening (SNTS) using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS)

Conventional monitoring approaches focus on a selection of compounds (typically 20-40) and, although extremely valuable to quantitatively assess the PFAS burden, only provide a limited view of the PFAS chemical space. Suspect and non-targeted screening (SNTS) analyses using liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) offer the possibility to investigate a broader range of chemical features present in a given sample. This approach is very promising and can be applied in multiple ways. First, it can be used with libraries to search for a large set of PFAS in a sample. Automated workflows that enable for more rapid analysis are increasingly being developed, and, in some cases, have also been incorporated in commercially available manufacturers software. Second, SNTS can be used to detect unexpected or previously unknown PFAS. This, however, requires time-consuming data processing which increases the overall cost of the analysis. Workflows developed at KWR and WFSR, as well as data analysis tools specifically designed for PFAS assessment such as FluoroMatch (Koelmel et al., 2022) or the MD/C-m/C approach (Zweigle et al., 2023), were applied to selected samples to tentatively identify PFAS that are not measured by conventional LC-MS/MS methods. These PFAS were consistently found and included fluorotelomers, sulfonamides, hydrogen-substituted polyfluoroalkyl acids, unsaturated perfluoroalkyl acids, etc. (Table 1). Several of the PFAS tentatively identified in this study were classified as 'precursors', which are fluorinated organic compounds that may undergo biochemical degradation in the environment (or during water treatment) and convert to more stable PFAS such as PFOA and PFOS, resulting in increasing concentrations of legacy PFAS. Unfortunately, for many of the detected 'unknown' PFAS, certified reference standards are lacking, which hinders the formal identification and quantification of these features. As such, the suggested structures should be regarded as tentative. A very rough estimation of the concentration of such PFAS can be made by comparing the signal intensity to PFAS for which a reference standard is available. However, such quantifications remain semi-quantitative and as such should be used as indicative estimates of concentrations. This highlights the complexity of identifying PFAS sources and managing their emissions.

### 3.2.3 Combustion ion chromatography (CIC)

Combustion Ion Chromatography (CIC) aims to determine the total concentration of organic fluorine in a sample. After a selective sample preparation to remove inorganic fluorine from a sample, a sample extract is introduced into a combustion oven. All PFAS present are first converted to HF and subsequently to F- and analysed with ion chromatography. Conventional CIC approaches show a relatively high limit of detection and fluorine background and are therefore considered not to be applicable for PFAS analysis. However, CIC was successfully used by WFSR for the analysis of total PFAS concentrations in fish samples with an instrumental limit of detection of 1.4 µg/L total fluorine. It was demonstrated in a selection of fish that the four PFAS designated by EFSA represented 10 - 25% of the total PFAS space. CIC has currently not been applied to water samples, as PFAS concentrations in water tend to be lower compared to fish. However, after the concentration of large volumes of water, CIC could be a valuable addition to the tools available for PFAS analysis. Note that such a concentration step can result in the loss of e.g. neutral or short-chain PFAS, yielding an underestimation of the total fluorine content.

### 3.2.4 Plasma induced gamma emission (PIGE)

Particle induced gamma ray emission (PIGE) spectroscopy is an emerging technique in the context of PFAS assessment. This technique uses a proton beam directed onto a carbon filter containing PFAS previously extracted from water samples. Fluorine nuclei from PFAS molecules (or any other fluorine containing compound) are excited in this process, and during de-excitation, the fluorine nuclei emit gamma-rays that are measured by a detector. The carbon filter is washed using HNO3 to remove potential inorganic fluorine interferences, although this procedure is expected to also remove ultrashort-chain PFAS (C < 4). PIGE is a promising technique for the determination of 'total' PFAS, especially because it seems more sensitive than other techniques such as extractable organic fluorine (EOF) (which may also struggle to retain ultrashort-chain PFAS). However, PIGE is not as sensitive as the TOP assay, and may require further development for applications in drinking water. In addition, PIGE is not a well-established technique and is unlikely to be available in commercial laboratories. While methods such as PIGE are very comprehensive, their lack of selectivity may result in overestimation of the PFAS burden in the sample by including organic fluorine that does not classify as PFAS.

### 3.2.5 Pros and cons of analytical techniques

Each technique evaluated in this project has its own advantages and limitations in covering the PFAS chemical space. As previously discussed, this chemical space is extremely broad, and no single technique can provide comprehensive coverage. As shown in Figure 2, none of the methods considered here fully capture the entire PFAS spectrum.

Targeted LC-MS/MS methods offer highly accurate measurements and allow detection at extremely low concentrations. However, as depicted in Figure 2, they are restricted to a limited number of legacy or well-characterized compounds, resulting in relatively narrow coverage. When combined with the TOP assay, their coverage improves, as certain PFAS precursors can be oxidized into legacy compounds detectable by targeted analysis. However, even this combination remains insufficient for PFAS that are either non-oxidizable or not included in the targeted compound list of the LC-MS/MS method used post-TOPA.

Total fluorine methods, such as EOF-CIC and PIGE, theoretically provide broader coverage than the TOP assay combined with targeted analysis. However, these techniques have significant limitations: (i) they only measure the extractable fraction of PFAS, and (ii) they lack the sensitivity of MS-based methods. As a result, their broader coverage is only meaningful when PFAS concentrations are high enough to be distinguished from background levels.

SNTS, in theory, offers slightly broader coverage of the PFAS chemical space by detecting compounds with a wider range of physicochemical properties, including both negatively and positively charged species, unlike EOF-CIC, which primarily targets anionic compounds. However, this approach is still limited by detection constraints. PFAS must be present at sufficiently high concentrations to generate adequate MS signals, and meaningful structural annotation depends on the availability of mass spectral libraries or in silico prediction tools. Additionally, SNTS methods typically rely on liquid chromatography coupled with electrospray ionization (ESI), meaning that volatile compounds, those not retained by conventional LC separation columns, or those that do not ionize well in ESI may go undetected.

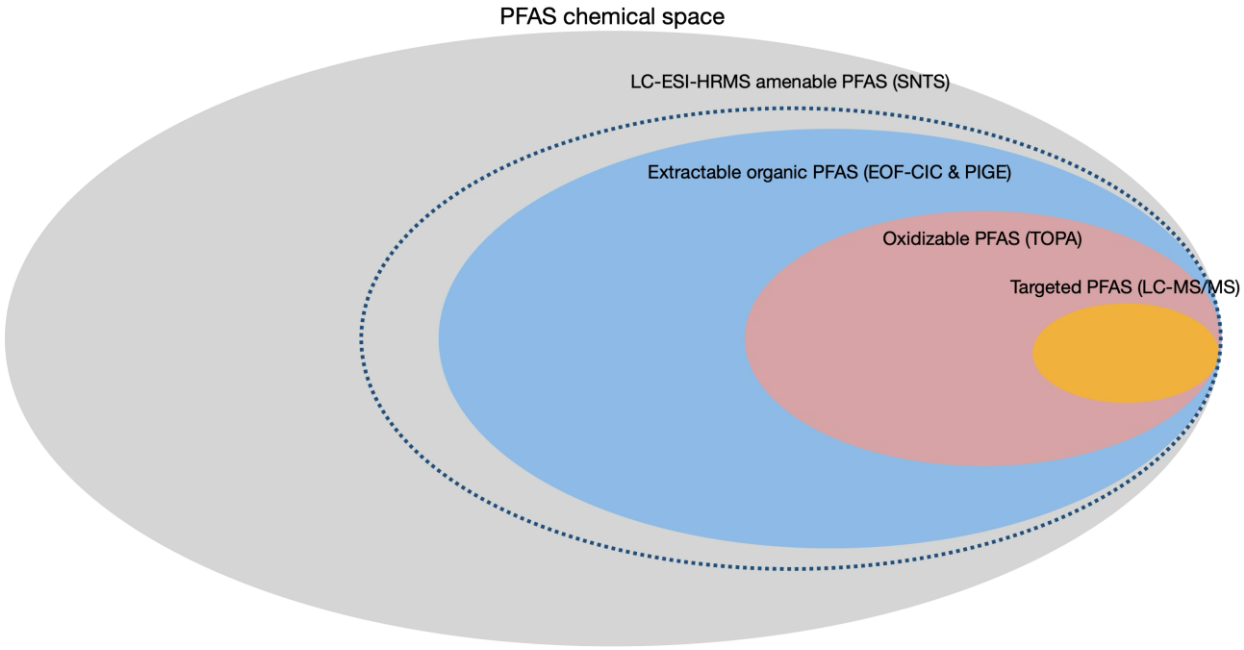


*Figure 2: Illustration of the coverage of the PFAS chemical space offered by the techniques considered in this study.* ***NB*** *proportions are not realistic but only for illustration purposes.*

## 3.3 Effect-based and *in silico* methods

PFOS (and PFOA) have been relatively well studied and have been known to have reproductive, immunological, developmental, liver and kidney effects in animal models (Patlewicz et al., 2019). However, little toxicity information exists for the majority of PFAS. Performing toxicity tests for all these compounds is time-consuming, expensive and unethical when vertebrate animals are involved. Measuring the presence and concentrations of substances followed by comparison with available toxicological data can provide insight into possible health effects caused by the substance. Since thousands of substances may be involved and toxicological data are not available for every substance, it is time-consuming if not impossible to estimate all potential risks to human health and the environment in this way. Moreover, little is known about the potential effects of mixtures of substances. Effect-based and *in silico* methods offer solutions for this.

### 3.3.1 Effect-based methods

Effect-based methods (EBMs or bioassays) do not directly aim to detect or identify single or multiple chemical substances, but rather determine a biological effect of a single or mixture of substances. These methods use living organisms, cells or bacteria and are already being used for water quality assessment in the water sector. Different EBMs exist for different biological effects, among others, endocrine disruption, metabolism and DNA damage (Figure 3).

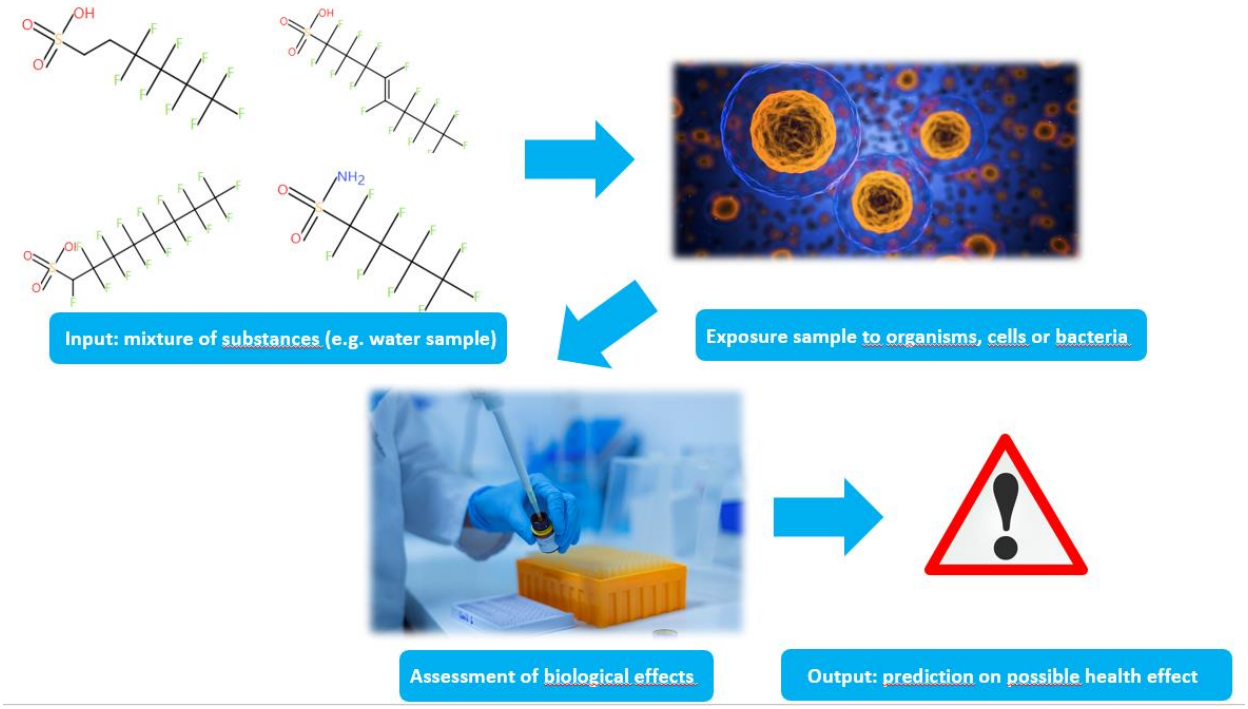


*Figure 3: Schematic overview of mixture toxicity assessment using bioassays*

EBMs can be used as effect-based screening approaches to detect total PFAS (not expressed in a concentration but as an effect) or to determine relative potency factors to express relative toxicity (Bil et al., 2021). They are especially interesting for the detection of PFASs due to the large number of different substances present within this group. Relevant factors to consider when choosing an assay for effect-based monitoring are, among others, biological reference, selectivity, and sensitivity. Evidence suggests that many PFAS are possible thyroid hormone system disrupting compounds (Weiss et al., 2009). In the past, an attempt was made to detect PFASs in water samples using the substances' ability to bind to transthyretin (TTR), a transport protein for the thyroid hormone thyroxin (T4), thereby displacing T4 from TTR. While the presence of PFASs could be detected using this effect-assay, it appeared that other substances present in the extracts from water samples also displaced T4 from TTR, and thereby this assay lacked specificity for the detection of PFASs. This could potentially be mitigated by increasing the selectivity of the extraction method for PFASs. Subsequent efforts focused on using the mechanisms underlying the current critical effect, i.e. immunotoxicity, for the effect-assay. Several potential modes of action related to the reported immunotoxicity of (certain) PFASs were tested, in particular to select effects and assays that are as sensitive as possible. Current challenges that are being addressed are the reproducibility when working with primary cells, such as peripheral blood mononuclear cells (PBMCs), and the low sensitivity, in part caused by (nonspecific) binding of PFASs to plastic and proteins in *in-vitro* culture systems, causing only a small fraction being available for biological activity. As such, the development of an applicable (sensitivity and specificity) effect-directed assay remains a challenge. Currently, EBMs are available to assess relative potency factors, but only in case pure reference standards of the PFAS are available.

### 3.3.2 Computer-based prediction of toxicity

Computer-based (*in silico*) approaches can provide information on potential characteristics of a compound, e.g. hazard. This can be interesting to determine which compounds to look for (e.g., suspect screening) or, conversely, to determine which of the compounds that have been detected (e.g., using SNTS) should be investigated further due to its potential toxicity. The *in silico* prediction is based on the chemical structure of a (tentatively identified) compound (quantitative structure-activity relationship (QSAR)) or toxicity information from structurally related data-rich compounds (read across) (Figure 4).

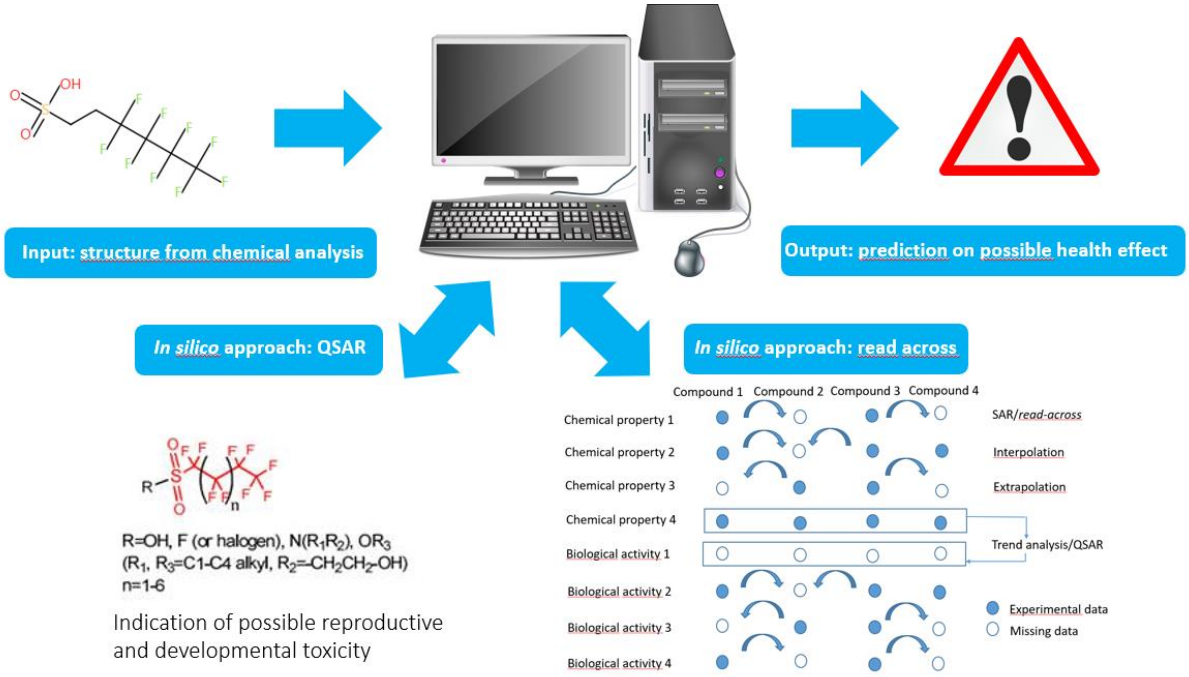


*Figure 4: Schematic overview of* in silico *prediction of toxicity*

Commonly used *in silico* tools are QSAR Toolbox, VEGA QSAR and CASE Ultra. The results from QSAR evaluation and read-across are model predictions and should as such be interpreted with caution, hence emphasizing the need for expert judgement of the reliability of the results. In addition, predicted results can be confirmed using bioassays. It is acknowledged that *in silico* predictions of bacterial mutagenicity (*i.e.* causing DNA damage in bacteria) are most robust because there is information on structure-activity relationships of mutagens and there is relatively abundant experimental data compared to other biological effects (Ashby, 1985; Benigni and Bossa, 2008). In the current project, the added value of *in silico* prediction of PFAS as part of an integrated approach for PFAS assessment was explored using eight compounds. Full hazard and risk assessment were out of scope of the current project. Consequently, underlying data points of QSAR predictions were not checked in this project. Details of the *in silico* predictions can be found in Annex 2.

From this preliminary exploration of the added value of *in silico* prediction of PFAS as part of an integrated approach for PFAS assessment, it was concluded that QSAR models can be useful to prioritise possible toxicologically relevant PFAS to be screened for using SNTS or included in routine monitoring methods. In order to use QSAR and read-across for human health hazard and risk assessment, more structurally similar data-rich compounds are needed to increase the robustness of the models in all software tested. For hazard and risk assessment, mixture effects should also be taken into account and warrant more investigation, e.g. using EBM. Custom-made *in silico* approaches specifically designed for PFAS, such as those developed by Patlewicz et al. (2019, 2022) may be preferred over commonly available tools for prioritisation, e.g. for further toxicological assessment or inclusion in a monitoring program.

# 4 Application of analytical techniques

The techniques tested for sample preparation (i.e., solid phase extraction, passive sampling and TOP assay) and analytical measurements (i.e., targeted, suspect and non-target screening, combustion ion chromatography, plasma-induced gamma emission) were applied to a broad selection of water samples (n = 50) obtained from consortium partners. From this the added value of each technique was assessed. Finally, samples of which were suspect for containing detectable concentrations of unexpected PFAS (based on the sampling location or application of the TOP assay) were studied more in depth with non-target screening approaches, aiming to detect and tentatively identify as many PFAS as possible as described in Chapter 2.

## 4.1 Sampling

Surface water and drinking water samples were obtained by the consortium. These included samples of wastewater treatment plants (influent and effluent), surface and groundwater. Sampling locations were both randomly selected and selected based on the expectation or knowledge of finding elevated PFAS concentrations due to their connection with a known PFAS source (e.g. PFAS producing industry or a history of frequent use of firefighting foams).

## 4.2 Tentative guideline for comprehensive monitoring

The wide range of analytical techniques used in this study enabled us to assess the diverse and complex occurrence of PFAS in water samples. Legacy PFAS, which are the most commonly monitored compounds, are unlikely to be the only relevant fraction of PFAS in water samples, and, in some cases, have shown to be a minor portion of the ‘total’ PFAS burden in the samples. Therefore, a comprehensive monitoring approach should include a combination of measurements that allows to assess, step by step, the complexity of PFAS contamination and the need for further investigations. This procedure is shown in the flow chart below (Figure 5).

Comparing total PFAS analysis with targeted analysis helps estimate whether routine analysis might miss a significant portion of PFAS. Each method for total PFAS analysis has its own benefits and shortcomings (see 3.2.5). Currently, the TOP assay is the most applicable in routine settings since it requires no additional equipment. However, this methodology may underestimate the total fluorine content. In the near future, CIC-EOF is expected to become a viable alternative for total PFAS analysis in routine settings. However, at present, this technique is not suitable for assessing total organic fluorine content in relatively pristine matrices, such as drinking water and groundwater, due to its high background signal, which results in elevated limits of detection and quantification. Additionally, the measured fluorine content might not be exclusively related to PFAS, as other sources can contribute to the fluorine loads. Laboratories need to purchase a CIC instrument, which is generally not part of standard equipment in routine laboratories. Despite current limitations, further technical development is expected to enhance total PFAS analysis. These techniques, although limited at present, can still be effective in indicating the presence of unknown PFAS in samples.

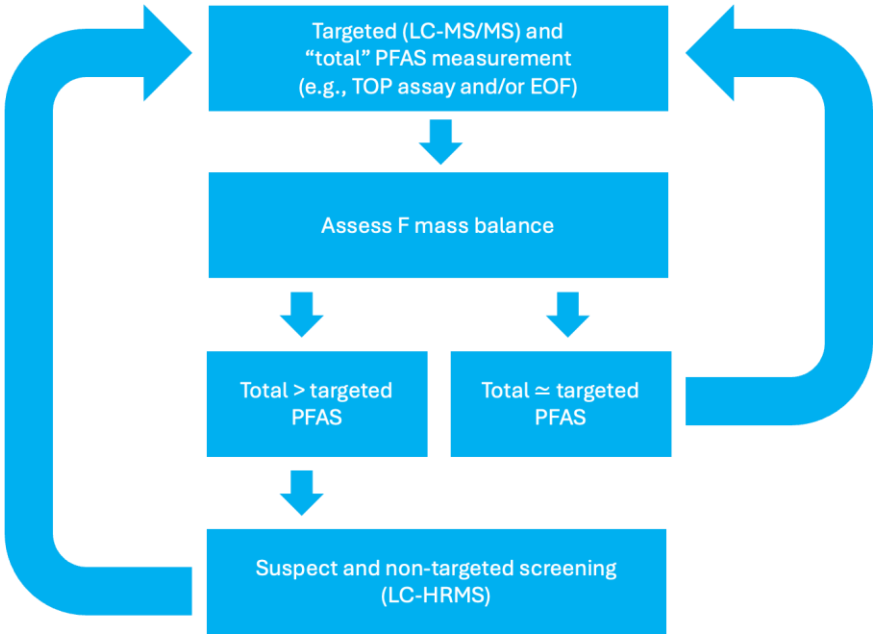


*Figure 5: The suggested mass balance evaluation workflow for assessing PFAS levels in water samples begins with a comparison between conventional targeted PFAS analysis and total PFAS analysis, using methods such as the TOP assay or EOF. The next step involves determining the fluorine mass balance to evaluate whether the total PFAS concentrations exceed those identified in targeted analysis. If a discrepancy is observed, suspect and non-target screening can be conducted to identify additional compounds. If new PFAS are detected and identified through this approach, they can be incorporated into routine targeted methods, provided reference standards are available. Alternatively, if the total and targeted PFAS concentrations are comparable, existing routine methods can be considered sufficient for the time being.*

Applying non-targeted screening approaches, either as a stand-alone option or as a follow-up of a total PFAS analysis, can allow to detect and (tentatively) identify a broader range of unexpected or previously unknown compounds. Even though they have not been formally identified, features likely corresponding to PFAS should be registered in libraries or databases so that their detection frequency can be traced. This information can then be used to prioritise compounds that require more thorough investigation (e.g., high detection frequency and/or intensity with respect to known compounds/internal standards). Ideally, such recording should be done in a fully automated way. It is expected that the application of suspect and non-target screening (SNTS) will continue to increase in future as this approach is one of the only ones allowing to cover a broad range of PFAS and at the same time provide (partial) information about their structure. Furthermore, if applied more routinely, SNTS for PFAS screening will become far more cost-effective compared to the current situation. However, SNTS requires additional instruments (different compared to those used for conventional targeted analysis) as well as specialised personnel. In parallel, quantitative and total PFAS methodologies remain important to complement these methods as they allow to detect potential changes in the overall PFAS loads which might be attributed to the introduction of novel and previously unknown compounds which have still not been detected or identified using SNTS. Furthermore, availability of reference standards, in particular of new or emerging compounds identified using SNTS will be essential to enable accurate PFAS quantification necessary for both regulatory and risk assessment purposes. Alternatively, robust semi-quantitative approaches, either based on the use of isomers/analogue standards or ionisation efficiency (Kruve et al., 2021), should be developed to overcome the limitation related to the availability of reference standards.

# 5 Experimental results

Targeted LC-MS/MS analysis (both short- and medium/long-chain PFAS) and TOPA were applied to a range of selected samples yet, due to budget constraints, only a subset was also analysed with SNTS using LC-HRMS. The sample selection was made based on:

- Discrepancy in the observed mass balance (see Figure 5);
- High contamination with legacy PFAS;
- Diversity of water types (influent, effluent, ground water, surface water);
- Diversity in sampling locations and potential sources.

In Paragraph 5.1 a summary of the results for selected samples is presented. In Paragraph 5.2 an overview of detected PFAS is presented with reference to Annex 1.

## 5.1 Outcome of the application of the presented strategy

An overview of relevant findings in a diverse selection of water samples is presented in Table 1. Here the results of targeted LC-MS/MS, TOPA and SNTS are presented together.

## 5.2 Overview of PFAS detected using suspect and non-target screening

KWR and WFSR applied SNTS to detect and tentatively identify less common or even novel PFAS which are not included in conventional LC-MS (targeted) methods. Annex 1 provides an overview of the compounds detected by the two laboratories in a range of water samples.

*Table 1: Overview of the application of the strategy to selected water samples. [1]Compounds reported in this column were detected and quantified with the targeted LC-MS/MS method (non-detected compounds are not reported). [2]Compounds reported in this column were either detected only after TOP assay or showed an increase in concentration after TOP assay (marked with a +). [3]Compounds reported here were **tentatively** identified using suspect and non-target screening. Potential structures are presented in Annex 1.*

| # | Sample information | Potential source | [1]Targeted LC-MS/MS (ng/L) | [2]TOP Assay (ng/L) | [3]Suspect and non-target screening | Remarks |
|---|---|---|---|---|---|---|
| 1 | Surface water | PFAS manufacturing plant | PFHxA: 17<br>PFHPA: 23<br>PFOA: 1300<br>PFNA: 10<br>PFDA: 1<br>PFBS: 6<br>PFHxS: 1<br>PFHpS: 0.1<br>PFOS: 4<br>HFPO-DA: 214<br>6:2-FTS: 0.1 | No significant mass balance discrepancy. Note that low concentrations of other PFAS will not result in a significant difference in total PFAS concentration due to the high concentration of PFOA. | - 4 isomers of PFOA, potentially branched<br>- 3 isomers of PFOS, potentially branched<br>- PFPrA<br>- H-PFOA<br>- Oxa-U-PFOA<br>- Di-acids (C7-9)<br>- 6:2-FTCA | Concentrations of the non-legacy PFAS are estimated to be below 10 ng/L. Even though several 'unknown' PFAS were detected, targeted LC-MS/MS gives a good impression of the total PFAS load due to the high concentration of PFOA. |
| 2 | Ground water | Firefighting foams from near airstrip | PFOS: 0.5 | PFHxA: +16<br>PFOA: +10<br>PFPA: +2<br>PFHpA: +1<br>PFNA: +0.2 | - PFSA (C11-17)<br>- U-PFSA (C12-18)<br>- Oxa-PFSA (C12, 14-16)<br>- Oxa-U-PFSA (C12,14-16) | Concentrations of the 'unknown' PFAS were estimated (based on SNTS signals) to be below 10 ng/L, yet they were the most prominent PFAS detected in the sample. It is unknown if the detected PFAS explain the formation of the PFAS detected in TOPA. Long chain substances occur in various isomers:: potentially branched. |
| 3 | Wastewater treatment plant influent and effluent | Unknown | PFHxA: 2<br>PFHpA: 0.8<br>PFOA: 3<br>PFNA: 0.2<br>PFDA: 0.2<br>PFBS: 1<br>PFHxS: 0.3 | PFHxA: +8<br>PFHpA: +1<br>PFUnDA: +1 | -- 11:3 FTCA<br>- 6:2-FTUCA<br>- HPFPrS | Concentrations of the 'unknown' PFAS are estimated (based on SNTS signals) between 2 and 7 ng/L. They have a significant contribution to the total PFAS concentration. The presence of 11:3-FTCA and 6:2-FTUCA might explain the increase in TOPA. |

| # | Sample information | Potential source | [1]Targeted LC-MS/MS (ng/L) | [2]TOP Assay (ng/L) | [3]Suspect and non-target screening | Remarks |
|---|---|---|---|---|---|---|
| 4 | Surface water | Air deposition | PFOS: 1<br>PFHxA: 2<br>PFHpA: 4<br>PFOA: 5<br>PFNA: 0.6<br>PFBS: 10<br>PFHxS: 1<br>PFHpS: 0.1<br>PFOS: 6<br>HFPO-DA: 1 | PFOA: +5 | - 2 isomers of PFNA and PFOS, potentially branched<br>- TFA<br>- PFBA<br>- HPFPeA | The estimated concentration of TFA is 5 ng/L and of PFBA 8 ng/L. These significantly contribute to the total PFAS concentration. The estimated concentration of HPFPeA is 1 ng/L, which is a minor contribution to the total PFAS load.<br>No substance was detected that explained the PFOA increase in TOPA. |
| 5 | Surface water | Unknown | PFHxA: 2<br>PFHpA: 1<br>PFOA: 3<br>PFNA: 0.7<br>PFDA: 0.2<br>PFBS: 0.8<br>PFHxS: 0.6<br>PFOS: 2<br>6:2-FTS: 1 | PFPeA: +7<br>PFHxA: +3 | - 3 isomers of PFOS, potentially branched<br>- PFBA<br>- 3:2-FTCA<br>- HDiOxa-PFOA<br>- diMe- AmPr-FHxAd | The concentration of the PFAS of which the structure could be tentatively annotated using SNTS was estimated to be below 1 ng/L. However, additional PFAS-like features were detected, yet their structure could not be tentatively identified and hence it cannot be excluded that these might still have a significant contribution to total PFAS loads. The increase of PFPeA and PFHxA in TOPA is not yet explained by the detected PFAS. |
| 6 | Wastewater treatment plant influent and effluent | Unknown | PFPA: 15<br>PFHxA: 7<br>PFHpA: 4<br>PFOA: 2<br>PFNA: 0.5<br>PFDA: 0.5<br>PFBS: 2 | PFPA: +17<br>PFHxA: +5 | - 3:2 H-FTB<br>- 6:2 FTSO2PrAd-DiMeEtS | The estimated concentration of the ‘unknown’ PFAS is below 2 ng/L. This does not account for the observed increase in TOPA. It is expected that other, still undetected PFAS are present in the sample. |

| # | Sample information | Potential source | [1]Targeted LC-MS/MS (ng/L) | [2]TOP Assay (ng/L) | [3]Suspect and non-target screening | Remarks |
|---|---|---|---|---|---|---|
| | | | PFHxS: 0.7<br>PFOS: 1<br>HFPO-DA: 0.4<br>6:2-FTS: 3 | | | |

# 6 Discussion

In the samples selected for the in-depth study, several non-legacy PFAS were detected using SNTS. In most cases, the estimated concentrations were low and did not significantly add to the concentration of legacy PFAS. However, in more contaminated samples, 'unknown' PFAS appeared to represent the largest fraction of total PFAS or contributed significantly to overall concentrations. It is important to note that the toxicological relevance of these newly detected PFAS remains unknown. These findings highlight that limiting monitoring efforts to a small set of legacy PFAS, without considering the potential contribution of unexpected or previously unidentified PFAS, may lead to an underestimation of total PFAS contamination. This is particularly relevant for locations suspected of being impacted by localized emission sources, such as firefighting training sites and industrial activities, where additional PFAS may be released.

The application of a multi-method strategy also revealed that, in some cases, compounds detected using SNTS do not fully account for the total PFAS load observed after applying TOPA. This suggests that additional PFAS structures may be present in the data but remain undetected due to the algorithms and filtering steps used in SNTS data processing. Another possibility is that some PFAS are not retained during sample preparation or are strongly adsorbed to surfaces such as tubes or vials. Additionally, certain unknown PFAS may be non-ionizable in the mass spectrometer and are, as such, left undetectable. These limitations are not exclusive to SNTS but also apply to targeted LC-MS/MS analysis, as both methods often rely on similar sample preparation and ionization techniques.

Source profiling, i.e., linking specific PFAS to particular sources or locations, was beyond the scope of this project, as it would require a substantial number of samples. However, in the case of firefighting foams, the results suggest that long-chain perfluorosulfonic acids (PFSAs) should be expected from this specific source. In particular, both saturated long-chain PFSAs and Oxa-PFSAs should be considered for inclusion in monitoring efforts. More in-depth studies are required in future to link PFAS profiles to potential sources. Because the developed strategy proved to be able to detect a wide range of 'unknown' PFAS, this approach is beneficial for source detection. In particular, formal structural elucidation is not necessary for source identification, as the chemical fingerprint of PFAS-like structures is sufficient to investigate source identification. However, developing such an approach requires a well-thought sampling campaign to build a database of PFAS profile and source pairs that can later be used to identify potential sources.

# 7 Conclusions

Within this project, PFAS analysis techniques have been further developed and evaluated to improve the detection of 'unknown' PFAS in water. The advantages and limitations of these methods have become clearer, leading to the proposal of a tiered approach. This strategy begins with conventional targeted analysis using LC-MS/MS or LC-HRMS. The detected PFAS levels are then compared to indicative "total" PFAS concentrations obtained through either the TOP assay (TOPA) or extractable organic fluorine analysis using combustion ion chromatography (EOF-CIC), as described in Figure 5. The choice between TOPA and EOF-CIC depends on the expected PFAS levels in the samples. For relatively pristine samples, TOPA is preferred due to its higher sensitivity. In contrast, for more contaminated samples, EOF-CIC may provide a more comprehensive assessment of total PFAS contamination by capturing a broader range of compounds. However, it is important to note that TOPA may not detect PFAS that, after oxidation, do not match those covered by targeted methods, while CIC may lack the sensitivity to detect PFAS at lower concentrations. If the mass balance analysis indicates the presence of additional PFAS beyond those detected in targeted methods, suspect and non-target screening (SNTS) using HRMS is recommended. SNTS tools for data processing have advanced significantly and have proven effective in detecting 'unknown' PFAS. However, since not all PFAS can be detected with this technique, employing a tiered approach remains crucial for a more comprehensive assessment.

With the available and optimized tools and strategy, several 'unknown' PFAS were detected in the water samples that were collected within this project even though they were mostly present in low concentrations. Due to the lack of reference standards the tentative annotation could not be confirmed and also exact quantification is impossible. These PFAS should be added to routine monitoring programs. Some of the 'unknown' PFAS could directly be attributed to their source, e.g. the use of firefighting foams. Unfortunately, in most cases the sources of the 'unknown' PFAS are unclear and require more extensive evaluation of potential sources or coincides with other known (fluorinated)microcontaminants to suggest potential sources. Note that also the absence of a validated structure also hampers the assessment of the toxicological relevance of these PFAS.

This study clearly demonstrates that there is a multitude of PFAS in the Dutch waters, many of which are not included in standard targted methods. Although the toxicological relevance of most PFAS beyond those included in routine monitoring remains unknown, their presence cannot be ignored. Therefore, it is important to consider adapting current monitoring approaches to better capture the full range of PFAS contamination. The strategy developed in this project can assist in determining which PFAS are relevant to a specific location and the associated stakes (e.g., drinking water abstraction). Using the data obtained from applying the proposed strategy, conventional (targeted) monitoring programs can be updated to include a broader and more tailored list of PFAS structures.

# 8 Recommendations

To better understand the burden of PFAS, it is important to invest in (i) further optimisation of analytical methods to improve their coverage, sensitivity and cost-effectiveness; (ii) setting up monitoring campaigns/programs allowing to obtain a comprehensive view of PFAS pollution in water.

Firstly, additional efforts should focus on closing the PFAS mass balance. Several total PFAS measurement techniques are available to achieve this, and they will also be essential for enforcing potential total PFAS limits, which are expected to be implemented soon by the EU or its member states. A robust, sufficiently selective and sensitive effect-based assay, or alternatively a PFAS-specific sample preparation or fraction protocol which allows to remove non-PFAS-like structures, would be a highly valuable technique to add to the toolbox. With such a technique the toxicological relevance of PFAS could also be taken into account. Furthermore, to close the mass balance, an investment in PFAS reference standards is required to allow confirmation, but more importantly, quantification of the PFAS concentration in a sample to enable closing mass balances in the presented tiered approach (see Figure 5). As it is impossible to synthesize high-quality reference standards for all relevant PFAS, studies on the (semi-)quantification of PFAS without reference standards would be beneficial for this purpose as well.

Secondly, additional efforts are needed concerning sampling strategies. Currently, grab samples are mostly used for monitoring purposes. The advantage is large volumes of water can easily be collected. However, such samples only provide a snapshot of water composition and might hence not be representative for longer periods. Depending on the goal of the analysis and the type of water, automated composite samplers and/or passive sampling could be used. Furthermore potential long term trends such as seasonal dynamics related to fluctuations in emissions or variable behaviour / environmental fate processes of specific PFAS related to temperature or weather conditions, should be taken into account in monitoring strategies.

Finally, to promote mitigation measures and effectively reduce PFAS emissions, it is important to identify sources. For some PFAS the source is known, being industry or the local use of firefighting foams. For many of the ‘unknown’ PFAS found in this study, the emission source remains unknown. By developing a spatial water analysis strategy and by analyzing many potential samples for the ‘unknown’ PFAS, sources of PFAS might become more easily traceable, and their presence might be preliminarily linked to sources or activities. This might trigger further investigations of sources or mitigation strategies. As mentioned earlier, PFAS profiling would not require formal identification of the detected compounds as their chemical signature (i.e., retention time and mass spectral data) could be sufficient to trace their origin and follow their fate in the environment.

# 9 Outlooks

As outlined above, there is currently no single method that can detect all PFAS. To estimate the total burden of PFAS and identify the contributing compounds, a combination of techniques must be used. Discussions are ongoing regarding which technique should be designated as the standard for "Total PFAS" in drinking water monitoring, as indicated by the recast of the Drinking Water Framework Directive (The European Parliament and the Council of the European Union, 2020). Further developments concerning the sensitivity of total fluorine methods, such as CIC, as well as the coverage and cost-effectiveness of LC-(HR)MS methods will play an important role in improving monitoring programs. In the meantime, the TOP assay will play a crucial role in providing accurate and quantitative information about the PFAS burden of oxidisable precursors and legacy PFAS. Nevertheless, additional studies are needed to evaluate the robustness and reproducibility of this method under varying circumstances (e.g., matrix type and composition, impact of fluorinated polymers). Moreover, the outcomes of this project have underscored the importance of better understanding potential PFAS sources. Comprehensive and well-planned sampling campaigns covering a wide range of potential emission sources, combined with analytical methods that provide a broad PFAS profile, particularly using suspect and non-target screening in a semi-quantitative manner, will be crucial to achieving this goal. Furthermore, the implementation of an analytical strategy which relies on a tiered approach as presented in Figure 5 will also remain essential to ensure that monitoring and research efforts are both efficient and comprehensive.

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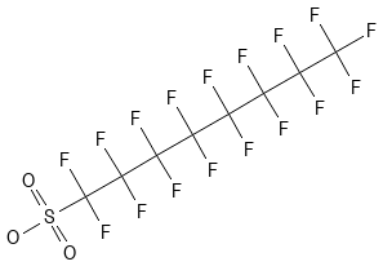
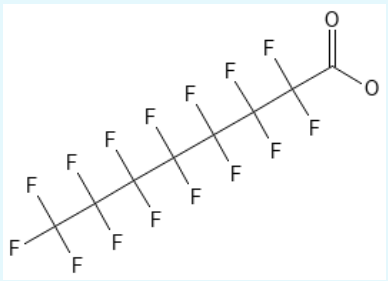
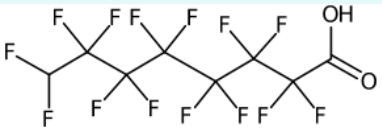
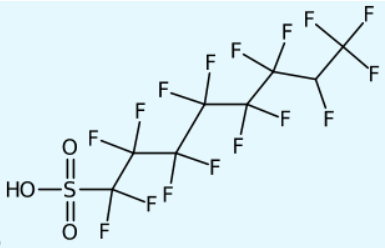
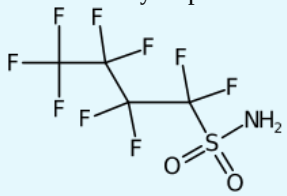
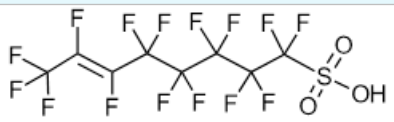
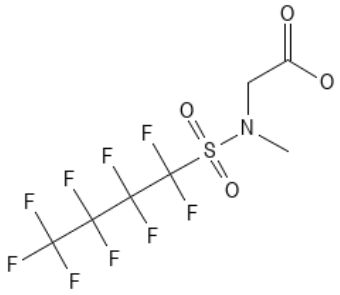
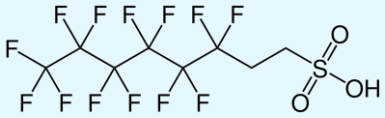
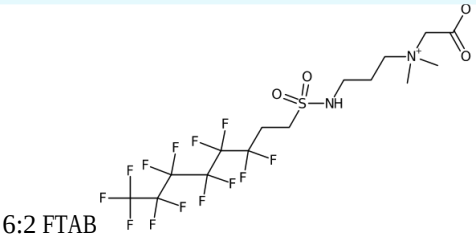
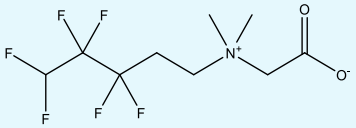
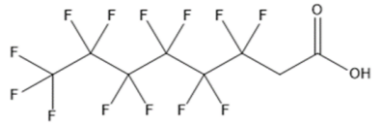
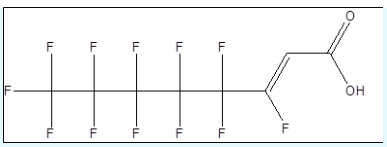
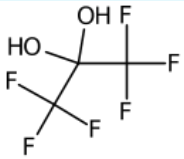
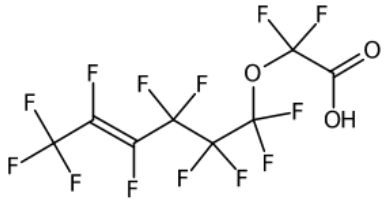
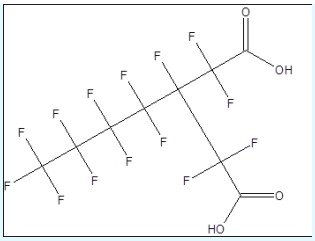
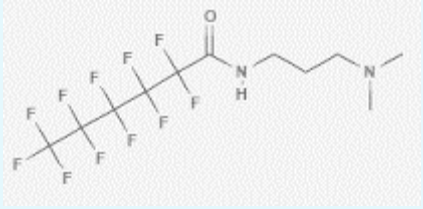
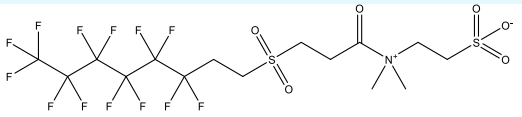
# Annex 1: PFAS structures tentatively identified in analysed samples

*Overview of PFAS structures **tentatively identified** using SNTS. *Only class very indicative class information was obtained for these features hence no structure is reported.*

| Class of compounds | Example structure | Remark |
|---|---|---|
| Legacy PFSA (linear and branched) | PFOS  | |
| Legacy PFCA (linear and branched) | PFOA  | |
| Polyfluorinated PFCA |  8H-PFOA | |
| Polyfluorinated PFSA |  7H-PFOS | C3-C10 |
| Long-chain PFSA | C11-C17 | C11-C17 |
| Sulfonamides | Perfluorobutylsulphonamide  | C3-C8 |

| Class of compounds | Example structure | Remark |
|---|---|---|
| Unsaturated PFSA |  | C6-C8 and C12-C18 |
| PFSA ether or ketone | * | C13-C17 |
| Unsaturated PFSA ether or ketones | * | C12, C14-16 |
| PFSA carbonyl | * | C6 |
| PFSM-amine | MeFBSAA  | |
| Fluorotelomer sulfone |  | 6:2 |
| Fluorotelomer sulfonamide betaine |  6:2 FTAB | |
| Hydroxy-fluorotelomer betaine |  | 3:2-H-FTB |
| Fluorotelomer carboxylic acid |  | 3:2, 6:2, 11:3 |
| Unsaturated fluorotelomer carboxylic acid |  | 6:2 |
| Fluoro diols |  | Position of diols is unknown |

| Class of compounds | Example structure | Remark |
|---|---|---|
| Unsaturated PFCA ether | Oxa-U-PFCAs(n3)  | |
| PFCA diacids |  | C7-C9; the exact position of the acid groups is unknown |
| PFCA ether | * | |
| Dimethy-amminopropyl-FHx-amide |  | |
| 6:2 fluorotelomer sulfonyl propanoamido dimethyl ethyl sulfonate |  | 6:2 FTSO2PrAd-DiMeEtS |

# Annex 2: Detailed results of the *in silico* predictions

The following substances were selected for feasibility investigation of the added value of *in silico* tools for PFAS assessment:

| Compound | Rationale for selection |
|---|---|
| 4:2 FTS | Difference only for the number of carbon atoms from 6:2 FTS → interesting to see if QSAR predicts differences in toxicity due to chain length |
| 6:2 FTS | Structurally similar to PFOS, except that 2 F that are replaced by 2 H interesting to compare to PFOS and see if QSAR predicts differences in toxicity due to the different amount of F |
| 8:2 FTS | Difference only for the number of carbon atoms from 6:2 FTS → interesting to see if QSAR predicts differences in toxicity due to chain length |
| PFOS | Common PFAS |
| H-PFOS | Commonly found using NTS, structurally similar to PFOS, except that one F is replaced by one H → interesting to compare to PFOS and see if QSAR predicts differences in toxicity due to the different amount of F |
| U-PFOS | Found using NTS, structurally similar to PFOS except that there is a C=C in the structure → interesting to compare to PFOS and see if QSAR predicts differences in toxicity due to the presence of double bonds |
| FBSA | Same as PFBS but with different functional group (sulfonamide instead of sulfonic acid) → interesting to compare to PFBS and see if QSAR predicts differences in toxicity due to different functional group. |
| FOSA | Same as PFOS but with different functional group (sulfonamide instead of sulfonic acid) → interesting to compare to PFOS and see if QSAR predicts differences in toxicity due to different functional group |

The following profilers were applied to the selected PFAS:

| |
|---|
| DNA binding by OASIS |
| DNA binding by OECD |
| Estrogen Receptor Binding |
| Toxic hazard classification by Cramer |
| Carcinogenicity (genotox and nongenotox) |
| DART scheme |
| DNA alerts for Ames, CA and MNT by OASIS |
| In vitro mutagenicity (Ames) alerts by ISS |
| In vivo mutagenicity (micronucleus) |
| Protein binding alerts for chromosomal aberration |
| rtER Expert System – US EPA |
| Repeated dose (HESS) |

Toxic hazard was predicted as high (Cramer class III) for all selected PFAS. Yet, no structural alerts were predicted by QSAR Toolbox based on any of the profilers used, except for PFOS. This compound showed the structural alert *Alkyl carbamodi-thioic acids, alkyl sulfonates and perfluorinated compounds (PFCs)* on DART scheme. This structural alert has the following explanation regarding PFAS: *Perfluorinated compounds (PFCs) with sulfonate functional groups, were studied and showed both reproductive and developmental toxicity. The core structural features for these chemicals are indicated in Figure 1 where R is OH, F (or another halogen), N(R1R2), or OR3. R1 and R3 are alkyl groups with 1 4 carbons, and R2 is a hydroxyethyl group. The alkyl chain length can range from 4 to 10 carbons (n=1-6), and PFCs with longer chain lengths, such as perfluorooctanesulfonate (PFOS) (C8) tend to be more toxic than those with shorter chains (C4). The crystal structure of the HAS-PFOS complex indicates that the strongly polar sulfonyl group interacts with hydrophilic residues of HAS, while the perfluorinated carbon tail interacts with adjacent hydrophobic residues of HAS (information obtained from QSAR Toolbox, original reference: Shengde, W., Joan, F., Jorge N., Michael L., Cathy L., George D., and Karen B.,(2013) Framework for identifying chemicals with structural features associated with the potential to act as developmental or reproductive toxicants. Chem.Res.Toxicol. 26(12), 1840-1861).*


*Figure: The scope of structural features of perfluorinated compounds (PFCs) (figure obtained from QSAR Toolbox)*
*Indeed, for PFOS data was available in the QSAR Toolbox on developmental and reproductive toxicity. Therefore, there was no reason to perform a read across based on the structural alert on the profiler DART scheme. For the other compounds and biological effects, a read across could not be carried out since the end-point specific profilers did not reveal a structural alert. 6:2 FTS and PFOS appeared to be the most data-rich compounds based on the databases included in the QSAR Toolbox, and for FOSA also some datapoints were available.*

Although feasibility of using the QSAR Toolbox (version 4.5) for evaluating PFAS was not demonstrated in the current project, it is acknowledged that QSAR Toolbox is a robust tool as it is based on many databases. Provided that there is sufficient and reliable data available of structurally similar PFAS, the tool could be useful for QSAR and read-across of PFAS (to be confirmed by future studies with compounds from more structural subclasses).

VEGA QSAR (version 1.1.5) was regarded as not useful for evaluation of PFAS due to the low reliability provided by the model for most of the predictions in general and the frequent lack of structurally similar analogues, based on the selected subset of compounds. Nevertheless, the exercise with VEGA QSAR in this revealed interesting findings with regard to how the QSAR model dealt with small differences in chain length or composition. For the cases studied, it was confirmed that the nature of the structural subclass of the PFAS is not always taken into account in VEGA QSAR's suggestions of analogues.

CASE Ultra (version 1.9.0.8) also frequently showed low reliability of predictions for bacterial mutagenicity provided by the models related to bacterial mutagenicity, which was likely due to the frequent lack of structurally similar analogues, and a lack of identified structural alerts. In addition to the low reliability of these model results, for three compounds and two models each, the compounds were outside the applicability domain, as the compounds include structural elements that are not included in the training datasets of the models. Although CASE Ultra did include experimental results for two out of eight compounds with respect to bacterial mutagenicity, the models related to bacterial mutagenicity included in the software were regarded as not useful for evaluation of PFAS based on the selected subset of compounds.

Besides using available *in silico* tools, it is also possible to develop custom made *in silico* models. For example, custom QSAR models for different toxicity endpoints have been derived based on ToxCast data in previous research of KWR. The ToxCast database consists of 21 databases, encompassing over 3.7 million toxicity data records (US EPA 2014). ToxCast data (based on experimental results) were available for PFOS (CAS: 1763-23-1) and FOSA (CAS: 754-91-6) only for a limited set of *in vitro* assay endpoints (mainly NVS_ADME_hCYP2C9, an assay endpoint focusing on enzymatic activity related to the gene CYP2C9). Within a project concerning substances of very high concern (SVHC), these chemicals were classified as SVHC and clustered within a cluster with other PFAS. Unfortunately, QSAR models (based on structural elements of compounds) for this cluster of compounds performed poorly when predicting toxicity of PFAS outside the training dataset (the dataset on which the model was based). Performance (based on physicochemical descriptors of chemicals) of QSAR models derived within the projects focusing on persistent mobile organic compounds (PMOC) was slightly better for the training set, but still poor for PFAS outside the training dataset.

In research conducted by Patlewicz et al. in 2019 and 2022, an approach was developed to categorize PFAS for prioritization for tiered toxicity testing based on chemical structure. Chemical substructures of PFAS were determined by using sub-structural features from ToxPrint, which are different from the set of structural elements used in previous KWR research. Seventy-five PFAS substances were identified for further prioritization and testing using new approach methodologies (NAM), *i.e.* methods for hazard and risk assessment not using (vertebrate) animals. However, Patlewicz et al. also concluded that structural category alone insufficiently explained the variability in activity. This variation can help revealing structure-activity relationships, especially within narrow categories where there is a large variability. For the current project, development of a QSAR model for PFAS was out of scope. For future research, it is recommended that substances in general (but particularly PFAS) may be first categorized based on structural similarity before training of separate models. This is in line with one of the conclusions from KWR research on SVHC, stating that variation in activity within predefined clusters (based on structural similarity) for SVHC is generally lower than variation observed across the clusters.