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Review Article

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REVIEW

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NORMAN guidance on suspect and non-target screening in environmental monitoring

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Abstract

Increasing production and use of chemicals and awareness of their impact on ecosystems and humans has led to large interest for broadening the knowledge on the chemical status of the environment and human health by suspect and non-target screening (NTS). To facilitate effective implementation of NTS in scientific, commercial and governmental laboratories, as well as acceptance by managers, regulators and risk assessors, more harmonisation in NTS is required. To address this, NORMAN Association members involved in NTS activities have prepared this guidance document, based on the current state of knowledge. The document is intended to provide guidance on performing high quality NTS studies and data interpretation while increasing awareness of the promise but also pitfalls and challenges associated with these techniques. Guidance is provided for all steps; from sampling and sample preparation to analysis by chromatography (liquid and gas—LC and GC) coupled via various ionisation techniques to high-resolution tandem mass spectrometry (HRMS/MS), through to data evaluation and reporting in the context of NTS. Although most experience within the NORMAN network still involves water analysis of polar compounds using LC–HRMS/MS, other matrices (sediment, soil, biota, dust, air) and instrumentation (GC, ion mobility) are covered, reflecting the rapid development and extension of the field. Due to the ongoing developments, the different questions addressed with NTS and manifold techniques in use, NORMAN members feel that no standard operation process can be provided at this stage. However, appropriate analytical methods, data processing techniques and databases commonly compiled in NTS workflows are introduced, their limitations are discussed and recommendations for different cases are provided. Proper quality assurance, quantification without reference standards and reporting results with clear confidence of identification assignment complete the guidance together with a glossary of definitions. The NORMAN community greatly supports the sharing of experiences and data via open science and hopes that this guideline supports this effort.

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Motivation for this guidance

A large and increasing number of chemicals are produced and used by modern society, leading to potentially harmful exposures of ecosystems and humans. A recent global inventory tallied > 350,000 chemicals and substances [1], while > 204 million chemicals are now in the largest registries [2]. Current monitoring approaches are capable of detecting only a small set of these chemicals (e.g., tens to hundreds) and are often defined by monitoring requirements related to regulatory frameworks or other chemical management approaches. However, improvements in the sensitivity, selectivity, and operation of analytical instruments, along with advancements in software development for data treatment and data evaluation in recent years have increased the interest to go beyond the target analysis of a few dozen pre-defined chemicals. Suspect and non-target screening (NTS) of a broad range of organic compounds, including transformation products (TPs) and certain organometallic compounds, have become a popular addition to target analysis not only in the scientific community, but also for authorities and regulators [3, 4]. Note that in this article the abbreviation NTS covers the collective term "suspect and non-target screening", because many aspects and methods are the same for both.

Going beyond target screening broadens the knowledge about the chemical status of the environment and human exposure, plus it allows for retrospective screening and an early warning about emerging contaminants without upfront selection and purchase of standards. NTS also allows the screening of chemicals that are either too expensive, restricted, or not commercially available. Purchase or synthesis of standards for full confirmation can be decided subsequently based on the relevance of the identification, e.g., frequency of detection, potential ecological or toxic effect, or peak intensity. Table 1 includes several examples of applications in the field of environmental monitoring. The sampling, sample preparation, analysis and data evaluation should be tailored according to the study question, as discussed in later sections, which can limit the applicability of retrospective screening in some cases.

First studies on the detection of unknown compounds were already reported in the early 1970s, with the introduction of gas chromatography coupled to mass spectrometry with electron ionisation (GC–EI–MS) [5]. The early harmonisation and reproducible fragmentation by EI led to the inclusion of standard spectra in libraries, such as the National Institute of Standards and Technology (NIST) mass spectral library, which have been used for identification via spectral match since then. The NIST spectral library is still widely used and contains 350,704 spectra of 306,643 compounds for GC–EI–MS as of February 2023 [6]. However, the chemical coverage of GC–MS is limited to volatile compounds unless derivatisation of non-volatiles is performed. In addition, the determination of molecular structures is challenged by low

Table 1 Fields of application of suspect and non-target screening in environmental monitoring

Suspect screening	Non-target screening
Identifying expected contaminants in the environment for future monitoring	Identifying unknown or unexpected contaminants in the environment for future monitoring
Obtaining big picture of pollution by monitoring of large numbers of suspects, e.g., REACH chemicals, NORMAN SusDat	Identifying unknown chemicals from spills, specific emission sources and other events, such as stormwater run-off, industry outflow
Specific view on substance class related to specific chemistry, origins or usage, e.g., bisphenols, per- and polyfluorinated compounds, pesticides	Identifying causative chemicals for adverse effects observed through bioassays by effect-based tools including effect-directed analysis
Detecting exposure-relevant chemicals based on modelling approaches (emission, fate) in combination with chemical databases	Identifying causative chemicals for adverse effects observed in ecosystems by ecological monitoring
Detecting TPs or (disinfection) byproducts reported in literature or predicted by QSAR	Identifying new persistent and bioaccumulative compounds from multimedia and biota monitoring
Spatial monitoring along, e.g., river stretches	Spatial monitoring along, e.g., river stretches
Trend monitoring over time at one site	Trend monitoring over time at one site
Retrospective screening of emerging suspects in stored raw data to determine extent of previous contamination	Retrospective screening of unknown chemicals / masses of interest in stored raw data to determine extent of previous contamination

Note that some applications apply to both types of screening

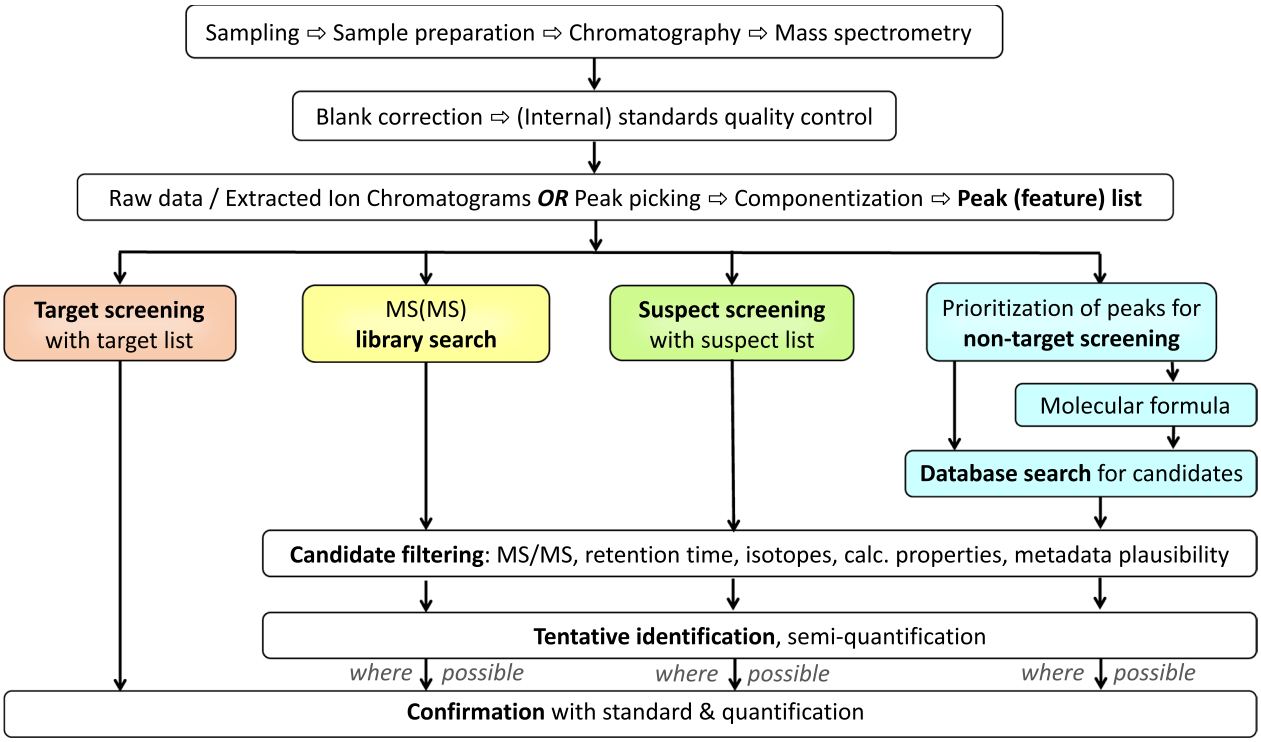

Fig. 1 Generic workflow of target, library, suspect and non-target screening of mass spectrometric data acquired using non-target methods

intensity or absence of a molecular ion in approximately 40% of GC–EI–MS spectra [7]. Chemical ionisation (CI) and atmospheric pressure chemical ionisation (APCI), as softer ionisation techniques, can increase the abundance of the molecular ion when coupled to GC separation.

Electrospray ionisation (ESI), along with APCI—both compatible with liquid chromatography (LC)—have extended the chemical space in two ways; by including more polar, water-soluble and larger molecules and by providing more accurate and detailed data on the ionised molecule. High-resolution mass spectrometry (HRMS), now available as benchtop instruments, enables the simultaneous and sensitive detection of ions in full scan mode with high mass resolution (ratio of mass to mass difference ≥ 20,000) and high mass accuracy (≤ 5 ppm mass deviation), improving possibilities for compound identification. Increasing resolution allows the separation of interferences and can reduce the need for sample preparation in some cases, while increasing mass accuracy reduces the number of candidates possible for a mass of interest. Tandem mass spectrometry (MS/MS) provides additional fragment information. Today, MS2 libraries have grown, but are not yet comparable to EI spectra libraries due to lower reproducibility and variabilities in fragmentation with different instruments, techniques and energies applied.

Given the rapid development and increasing use of NTS approaches, data quality has become an important topic. This includes procedures for quality assurance/quality control (QA/QC) as well as assessments of what level of data quality is currently achievable [8]. Several collaborative trials have been organised by the NORMAN network [9, 10], the US EPA [11] and national communities documenting an ongoing need for more harmonisation. Conferences and workshops have been arranged to exchange and evaluate best practice for preparation, acquisition, and data evaluation of samples for HRMS analysis and subsequent suspect and non-target screening workflows. First drafts of national guidelines for NTS are available in Germany (German Chemical Society) and the Netherlands (Royal Netherlands Standardization Institute), with a specific focus on water monitoring. The NTS community in the US (BP4NTA) [12] proposed an NTS study reporting tool for quality assessment of publications in the field [13].

In response to the increasing interest in NTS from regulators, risk assessors and scientists, and continuous comments on the need for more harmonisation in this field, NORMAN members involved in NTS activities have prepared this guidance document, based on the current state of knowledge. This document is intended to support scientific, commercial and governmental laboratories in conducting high quality NTS studies, and help those using NTS data to evaluate the pitfalls and challenges with these techniques. The aim is to provide guidance for all steps

(Fig. 1): from sampling and sample preparation to analysis, through to data acquisition, data evaluation and reporting in the context of NTS analyses. While most experience within the NORMAN Network still involves water analysis using LC–ESI–HRMS/MS, other matrices and instrumentations will also be covered, reflecting the rapid development and extension of the field.

Overview on analytical methods for NTS

While target methods are typically optimised for a small set of compounds with rather similar physico-chemical properties, screening methods are generally more generic. Typically, they involve limited sample processing (if any) and separation over a wide hydrophobicity range to minimise compound losses and ensure performance for as many compounds as possible. For liquid samples with sufficiently high concentrations, direct injection is often recommended where possible, while solid samples have to be extracted, usually with organic solvents, such as methanol or acetonitrile (for LC) and hexane or acetone (for GC) or solvent mixtures. For lower concentrated liquid samples, vacuum-assisted evaporative concentration [14], freeze-drying [15] or solid phase extraction (SPE) can be applied. SPE materials capable of different interactions (e.g., ion exchange, Van der Waals interactions, electrostatic interactions) can be combined to broaden the range of enrichable compounds [14, 16]. Chromatographic methods tend to use generic gradients ranging over a broad range of organic solvent content (e.g., 0–100% methanol) with reversed phase (RP, typically C_{18}) columns in LC or temperature gradient (e.g., 40–300 °C) and phenylmethylpolysiloxane columns in GC. For LC, ionisation with ESI covers the widest range of polar compounds, while EI is most common for non-polar compounds separated by GC, although APCI allows better transfer of data evaluation methods from LC–ESI–HRMS/MS workflows.

In NTS, the range of compounds covered by the method is not known a priori, but has to be inferred from knowledge about the performance of the individual method steps and processing of reference compounds. Overall, for NTS methods it is essential to have a good idea about the coverage of the compound domain, particularly about what is not covered. This is one reason why it is recommended starting with a target screening on the data before moving to NTS. The final compound domain of a screening method is the intersection of the domain of each method step, as conceptualised in Fig. 2. There are some attempts to predict the chemical domain of screening methods using quantitative structure-activity relationships (QSARs) based on physico-chemical properties [17] and an on-going activity of NORMAN, while the BP4NTA group recently released the ChemSpace approach [18].

While generic suspect and non-target screening methods strive to cover the largest possible compound domain, they are usually not suitable for compound groups with highly specific properties. Examples are small hydrophilic ionic or neutral compounds such as the pesticide glyphosate, N-nitrosodimethylamine, and

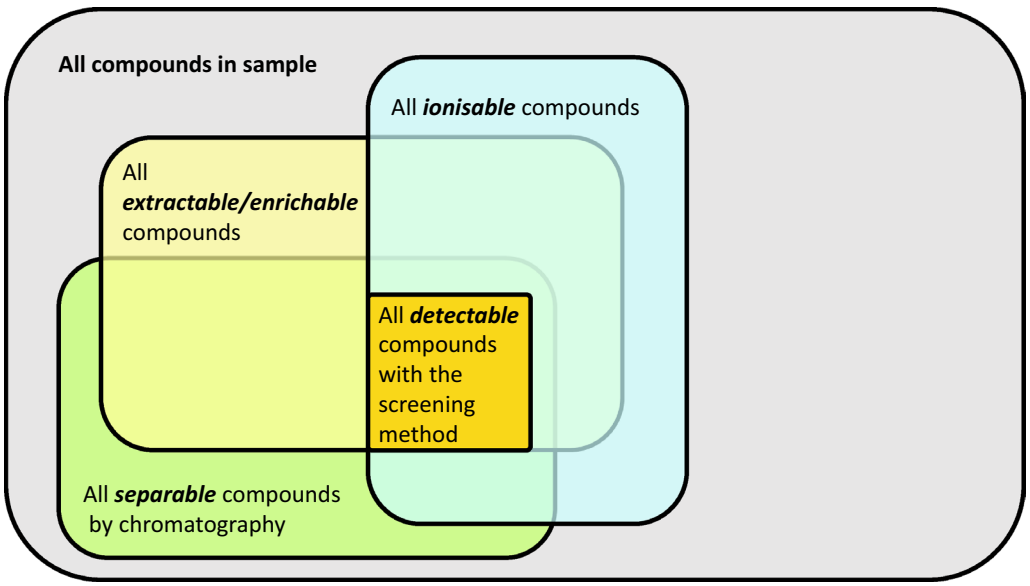

Fig. 2 Compound domain that can be analysed with a screening method consisting of a sample preparation/extraction step, a chromatographic separation and ionisation before detection by mass spectrometry. Note that the size of the area is not necessarily representative of the number of chemicals

ultra-short chain per- and polyfluoroalkyl substances (PFAS) or very non-polar high-molecular weight compounds such as > 6-ring polycyclic aromatic hydrocarbons (PAHs) for which the commonly used enrichment and separation methods are not appropriate. Furthermore, sensitivity is generally lower for screening methods compared to specific methods, which often contain purification steps to eliminate interferences, specific enrichment and separation as well as optimised ionisation conditions. This is especially relevant for compounds with low environmental effect thresholds for which very low detection limits are essential, such as pyrethroids and steroid hormones. If such specific substance classes are of interest, it is usually preferred to use a more specific method or at least evaluate with standards whether the screening method is suitable for the substance class. To address specific chemical domains, the generic methods mentioned above can often be adapted. For example, very hydrophilic compounds can be separated with hydrophilic interaction liquid chromatography (HILIC), supercritical fluid chromatography (SFC) or mixed-mode LC (MMLC) instead of RP (C_{18})-based chromatography before ESI–HRMS. The next sections address these analytical considerations step by step in greater detail and refer to LC–MS unless stated otherwise.

Sampling and sample preparation for NTS

Sampling is an integral part of a holistic approach and the beginning of the analytical chain [19], since the analytical result is no better than the selected sampling method. In NTS, sampling is typically not tailored to specific chemicals or groups of substances, although specific compound domains might be of interest. The sampling procedure should ensure maximal representation of the environmental chemical patterns, consider spatial and temporal variations and minimise both contamination and loss of compounds. Sampling should be performed by trained personnel who are aware of the risk of contamination of samples and/or losses of compounds posed by incorrect handling. Samples for NTS should not be preserved using any chemical additives (e.g., sodium azide or acids) due to potential sample contamination or sample alteration (i.e., transformation of compounds). Instead they should at least be refrigerated at 4 °C (core temperature according to ISO 5667-3) or better frozen at − 20 °C as soon as possible after sampling. The samples should be transported to the receiving laboratory under these conditions and processed as soon as possible.

To minimise contamination from the sample equipment, high quality solvents should be used for cleaning. As far as possible, sampling devices that come into contact with the sample, tubing, and sampling containers should be made from inert materials that do not sorb or release compounds. While this is in most cases true for borosilicate glass or stainless steel, some compounds also sorb to these materials (e.g., PFAS, phosphonates or complexing agents). In some cases, if the use of plastic or elastic polymers cannot be avoided (e.g., for flexible tubing or sealings), plasticiser-free polymers (e.g., high density polypropylene) and high quality silicone (for seals) should be used. In general, the most appropriate material should be selected depending on the substance domain of interest (hydrophobic or hydrophilic) and potential interference with the sampling material. The appropriate cleaning of the sampling equipment and all other laboratory (glass)ware that is in contact with the sample is critical. After cleaning with laboratory detergents, the equipment must be rinsed with ultrapure water and high-quality solvents. All materials can be baked out at the highest possible temperature (follow manufacturer instructions); for example, borosilicate glassware beakers and bottles up to 410 °C in a furnace. In addition, working in positive air pressure laboratories with air filtration systems is highly recommended for NTS to reduce the contamination of samples via air as much as possible.

Field, laboratory and procedural blanks are necessary to capture potential contamination of the samples by environmental or laboratory background, leaching of materials in contact with the sample or inherent content in the water and solvents used. Detailed examples of how to obtain blank samples for each sample type are described in the following subsections. Disposable gloves should be worn while sampling to protect the sampling person from possible toxic contaminants and to avoid any cross contamination of the samples, containers and equipment. Whenever possible, the use of cosmetics, sunscreens, soaps, medical creams, drinks with caffeine, tobacco and insect repellents should be avoided when sampling or processing samples. Such products often contain high levels of compounds of potential interest. For instance, insect repellents contain up to 50% of diethyltoluamide (DEET), such that even minimal contact will easily contaminate an environmental sample with typically 10^6 to 10^9-fold lower levels. If usage is unavoidable for human health protection, it should be noted in the sampling protocol.

The following subsections contain some basics and guiding principles for sample collection and preparation relevant for NTS of various matrices. Details on sampling strategies and methods are given, for example, in the ISO 5667 standard series and in the European Water Framework Guideline Documents [20].

Water

The sampling location, method, season and sampling time should be chosen carefully depending on the study

question. The two general approaches for water are (1) spot or grab and (2) composite sampling. Passive sampling, which can be viewed as a specific case of composite sampling, is covered in Sect. "Passive sampling". Spot sampling involves taking a single or discrete sample at a given location, time, and/or depth of a water body or groundwater aquifer that is representative only of the composition of the matrix during the time of sampling, which is usually seconds to minutes [19]. Composite or integrative samples consist of pooled portions of discrete samples or are collected using continuous automated sampling devices and combined time- or flow-proportionally in one sample, which are representative for the average conditions during the sampling period [21]. The sampling type and the volumes of water to be collected depend on the goals of the study and / or other requirements, such as the storage of backup samples or combination with, e.g., effect-based analysis.

Water samples can either be collected in the field and transported to the laboratory for further processing, or directly extracted on-site. For the latter, different types of mobile SPE devices have been developed, often designed for obtaining large water volumes (20–1000 L) for combined chemical and biological analysis that are otherwise difficult to transport to the laboratory [22, 23]. The time-integrating, microflow, inline extraction (TIMFIE) sampler [24] provides a low-volume system for continuous SPE using a syringe pump and a small SPE cartridge. At some larger monitoring stations, the use of SPE combined with LC–(HR)MS/MS and NTS is daily practice [3]. The "MS2field" online–SPE–LC–HRMS/MS system in a trailer allows in situ automated analysis of samples with a high temporal frequency and minimal lag time in the field [25].

The high sensitivity of the current generations of LC–HRMS equipment allows for direct injection (DI) of water samples without any enrichment steps. The advantages of DI are the small water volumes required, low efforts with sample processing and less risk of background contamination during sample preparation. Minimising the sample processing results in negligible losses of compounds, as each manipulation step may discriminate against substances (e.g., by evaporation, precipitation or degradation). To obtain a sufficient sensitivity, typically large volume injections are used for DI, with volumes of 100 [26], 250 [27] or up to 650 µL [28], as no further enrichment of the sample takes place. In such cases, an adjustment of the sample composition before injection by adjusting pH and solvent addition is necessary to avoid phase dewetting or injection solvent mismatch (see Sect. "Choice of separation method"). A direct preparation of sub-samples for analysis is possible in the field by transferring individual aliquots of 1 mL into autosampler vials from a larger sampling vessel. Depending on the load of suspended particulate matter, settling of particles before aliquoting alone may be sufficient; alternatively, a filtration or centrifugation step might be necessary before analysis, with the accompanying risk of compound losses. A drawback of DI is the potential contamination of the ion source with inorganic salts that would be removed by SPE or liquid–liquid extraction (LLE). This is particularly critical for samples from estuarine or marine environments, for which even a diversion of the eluent flow away from the ion source at early retention times (RT) might not be sufficient.

Performing SPE in the laboratory is still the most commonly used sample preparation method for water samples. SPE often requires a filtration step before extraction to separate particles from the water phase. In general, glass fibre filters with a nominal pore size of 0.7 µm are used, or membrane filters with 0.45 µm pore size. The given pore sizes for the separation between the solid and the dissolved fraction are primarily operational. The freely dissolved fraction can only be sampled by kinetic samplers (i.e., passive samplers) and not separated by a membrane [19]. For screening methods with LC covering medium polar to non-polar compounds, C_{18} or mixed-mode materials are often used, such as Oasis HLB (Waters) or Chromabond HR-X (Macherey Nagel). Combinations of C_{18} material with ion exchange material, other polymers and potentially even activated carbon enlarge the compound range to ionised and very polar compounds [14, 16, 29]. To avoid contamination, sufficient cleaning by organic solvents and water within the so-called conditioning step is important. Method blanks with ultrapure water to check for contamination are indispensable. Elution of the enriched chemicals from the SPE material is usually achieved by methanol or acetonitrile. Adjusting the pH of the sample and the eluent is critical to achieve optimal retention and elution. SPE can also be carried out online before the LC to save time and material [30]. Dilution of the organic SPE eluate with water before RP chromatography is recommended to achieve refocusing on the column.

For enrichment of very polar compounds that may not bind to SPE material, both vacuum-assisted evaporation [14] and freeze-drying [15] approaches have been applied successfully. However, the simultaneous enrichment of salts can lead to high ion suppression in the downstream analysis and volatile compounds may be lost.

For screening methods with GC covering non-polar compounds of sufficient volatility, either SPE with C_{18} material or LLE can be used. Both approaches involve elution or extraction with less polar solvents, such as ethyl acetate, hexane or toluene. Although purification with silica gel before GC analysis is common for specific substance class methods, this may lead to loss of

compounds and should be avoided if possible, unless it will not influence the chemical domain of interest in the given sampling campaign. Evaporation to dryness should be avoided to ensure retention of volatile compounds.

Sediment, suspended particulate matter and soil

Soil and sediment samples are well-suited to study chemical contamination throughout time and space as usually no historical water samples (apart from ice cores in some areas) are available, and many contaminants have not been recorded and studied in the past. However, the analysis of organic pollutants in soil, sediment, sludge, and suspended particulate matter can be challenging due to potential interference from natural organic matter (NOM), and the spatial variability observed from site to site. The latter makes representative sampling very important. In addition, some compounds can occur in low concentrations or be strongly bound to the matrix. Sampling for non-target screening does not differ widely from target analysis. The international standards ISO 18400-101:2017 [31], ISO 5667-12:2017 [32], ISO 5667-13:2011 [33] and ISO 5667-17:2008 [34] provide general guidance on the sampling of soil, sediment, sludge and suspended solids, respectively. For NTS, it is especially important to include reference sites from remote areas with (relatively) minor contamination, field blanks and procedural blanks to avoid detection of false positives. Together with replicates this can help to eliminate peaks resulting from the extraction procedure and instrumental analysis, similar to water samples.

In general, extraction procedures for NTS in soil, sediment, sludge and suspended solid samples are similar to procedures for target analysis and involve liquid (shaking), pressurised liquid extraction (PLE, also known as accelerated solvent extraction (ASE)), Soxhlet, ultrasonic, microwave, or supercritical fluid extractions. However, to eliminate the usual heavy matrix in soil, sediment and sludge, very specific extraction procedures (e.g., specific pH, narrow polarity of solvent) are often applied for target analysis of specific substance classes, such as polychlorinated biphenyls (PCBs), PAHs and PFAS. Further clean-up procedures or additional extraction steps are used to eliminate matrix components interfering with the analysis. As NTS aims to cover as many compounds as possible in principle, the extraction and clean-up procedures should be much less specific. For example, solvent mixtures allowing for different interactions are used to cover substances with various functional groups and physico-chemical properties. However, this can be at the expense of selectivity and sensitivity for specific substances, as matrix components will also be extracted with less specific extraction and clean-up steps. Using aprotic solvents (e.g., ethyl acetate, dichloromethane, acetone, hexane) extracts fewer natural compounds with acidic or phenolic groups. For polar compounds, PLE with in-cell clean-up employing either Florisil or neutral alumina as a sorbing phase [35, 36] or QuEChERS (quick, easy, cheap, effective, rugged, and safe)-like LLE [37] with salts to enable separation of water and acetonitrile phase has been successfully used for broad target, suspect or non-target screening in soil and sediment [38]. For non-polar compounds, Soxhlet extraction and ultrasonication with a subsequent fractionation and clean-up step using silica gel were used in soil and sediment, respectively [39] and PLE with in-cell clean-up and PLE with gel permeation chromatography (GPC) clean-up has been applied for NTS in sewage sludge [40]. Elemental sulphur (S_8) is often co-extracted from soils and should be removed to protect GC columns. Copper is commonly used to eliminate sulphur and can be added directly to the extraction process, for example, in the PLE cells [41, 42].

Air and dust

Airborne chemical pollution is usually caused by a dynamic complex mixture proportioning in gases and particles. Air samples are typically collected using a variety of commercial and self-designed samplers with filters and sorbent materials. Quartz fibre filters are most commonly used in the samplers for collecting airborne fine particles. Volatile organic compounds (VOCs) in the gas phase can be collected by adsorption on polyurethane foam (PUF) in the samplers. These sampling methods were developed in the past to enable long-term monitoring of regulated legacy contaminants [43, 44]. Typically, the extracts are further subjected to an extensive clean-up, for example, with concentrated sulphuric acid that removes the interfering matrix, but also any compounds that are not stable in the acid, which reduces their applicability to NTS methods. Analysis of raw air sample extracts without clean-up would generate contamination of the analytical system, especially with PUF matrix-based compounds, leading to mass spectra with high interferences and detection limits for contaminants that are often only present at trace levels in air samples. To address the problem of interferences originating from PUF, a new extraction and cleanup method was developed [45] and applied for NTS of emerging contaminants in Arctic air [46]. Although the authors reported detection of over 700 compounds of interest in the particle phase and over 1200 compounds in the gaseous phase, the method has its limitations. It is very time-consuming and expensive, and while the authors reported that several compounds exhibited poor recoveries (e.g., chlorfenvinphos, chlorobenzilate, dichlorvos, endrine aldehyde

and etridiazole), it is likely that this also applies to other compounds not tested in the study.

Generic non-selective extraction methods applied for targeted analysis of air are also suggested for NTS of air samples, including microwave assisted extraction (MAE), ultrasonic solvent extraction, and PLE. For LC separations, the most common solvents are ethyl acetate and methanol, while hexane and dichloromethane are used to extract GC-amenable compounds. However, due to environmental safety, dichloromethane is increasingly faced-out for laboratory usage and replaced by non-chlorinated solvents. Clean-up to eliminate interferences has to be balanced against loss of chemicals of interest in air samples. Direct analysis of VOCs in the gas phase by thermal desorption from the sorbent without any sample preparation procedure can be conducted on GC–HRMS or GCxGC–HRMS [47]. Passive sampling of air and air particles is increasingly used for NTS of airborne chemical pollution, as discussed further in Sect. "Passive sampling".

Dust is a complex mixture of settled particulate matter of both natural and anthropogenic origin, with particle sizes from nanometers to millimetres. According to the literature, there are no consistent conclusions on particle size distribution of various environmental pollution, although concentrations of some pollutants were documented to increase with decreasing particle size [48–50]. It is thus important to limit the fractionation of the sampled dust to ensure coverage of a broad spectrum of contaminants. In the literature published so far, sieved samples from vacuum cleaners are most commonly used in NTS [9, 51], although the use of the high-volume small surface sampler (HVS3) [52] and a proprietary dust collector attached to a vacuum cleaner is also being reported [53].

A combination of non-polar and polar solvents is used to ensure extraction of a wide range of compounds from the dust. Acetone was recommended as one of the solvents of choice due to its ability to dissolve plastic particles and fibres, which enabled the detection of bisphenol A and plastic additives [54]. In other studies, indoor dust was extracted by sonication with different solvents: hexane:acetone (3:1) and acetone [52], hexane:acetone:toluene [55], methanol:dichloromethane (9:1), dichloromethane [9, 56], acetonitrile:methanol (1:1) [53]. To avoid losses of contaminants, limited cleanup (for example, fractionation on a SPE column) is recommended, which should be balanced against the need for matrix removal in the analysis. The complex dust matrix is likely to interfere with the chromatography, causing a risk of high detection limits and uninterpretable results.

Biota and biofluids

The sample collection and pre-treatment methods for NTS of biota and biofluids are similar to those used in the target analysis of biota, including dissection or particle size reduction. Biota samples should be kept at − 20 °C or below for short-term storage, but kept at − 80 °C or below for long-term storage. Biota samples are sometimes freeze-dried, but this might carry risks of losing volatile compounds and/or cross-contaminating samples. Samples need to be homogenised before extraction. For fresh samples, the water content is typically determined before homogenisation and extraction [57]. A larger sample amount is recommended for NTS of biota compared with target analysis, along with replicates and QA/QC samples, to obtain consistent and high-quality HRMS/MS data for the characterisation and identification of unknown organic contaminants. Details on sampling strategies and pre-treatment for biota are given, for example, in the European Commission (EC) Guidance Document No 32 on the implementation of biota monitoring under the Water Framework Directive [58] and the Helcom monitoring guidelines [59].

Biota and biofluid samples often contain complex matrix materials, such as proteins, lipids, endogenous metabolites, and/or salts, resulting in interference with the NTS of pollutants. Therefore, a balanced approach is required to extract a wide range of chemicals while minimising the matrix effects. The selection of extraction and clean-up procedures of biota and biofluids mainly depends on (1) the polarity of the analytes of interest, (2) applied chromatography and MS techniques, (3) types and contents of the matrix interferences. Extraction of non-polar compounds from biota samples is traditionally performed using a combination of non-polar and moderately polar solvents (e.g., hexane, dichloromethane, and acetone). The resulting extracts contain varying types and amounts of matrix, such as lipids. It is difficult to remove matrix components (e.g., lipids) completely, but it is often possible to reduce the matrix: xenobiotic contaminants ratio sufficiently for the detection of the xenobiotics. For NTS, non-destructive lipid removal techniques are recommended, for example, GPC [60], dialysis [61], or adsorption chromatography [62]. Sometimes, size separation using GPC or dialysis is combined with adsorption chromatography [62] to further reduce the sample complexity and increase the probability to detect and identify new and emerging contaminants. Subsequently, the final non-polar solvent extracts are commonly analysed with GC–EI/APCI–HRMS for screening non-polar unknown

compounds. A recently developed alternative approach is equilibrium passive sampling performed by placing a passive sampler in biota tissue. [63, 64] However, this is limited to more hydrophobic compounds and has not yet been widely applied in NTS.

For polar compounds, LLE and solid–liquid extraction [65] or QuEChERS methods have become increasingly popular for extraction and purification before analysis [37]. Acetonitrile is used for the QuEChERS extraction in combination with salts (e.g., $MgSO_4$, NaCl) and sometimes buffers (e.g., citrate) for phase separation. QuEChERS extraction reduces the amount of extracted lipids, proteins, and salts compared to traditional extraction methods. Further matrix removal is achieved using freezing out [66] and/or dispersive solid-phase extraction (dSPE), e.g., using primary secondary amine (PSA) or online-mixed-mode-SPE [65]. dSPE using PSA removes acidic components (e.g., fatty acids), certain pigments (e.g., anthocyanidins) and to some extent sugars, while freezing-out removes lipids, waxes and sugars and other components with low solubility in acetonitrile that may cause matrix effects and ion source contamination in GC and LC analysis [66]. A range of sorbents has been developed for selective lipid removal using conventional or dSPE, e.g., Z-Sep (Supelco, Bellefonte, PA, USA) and EMR-Lipids (Agilent, St. Clara, CA, USA) as well as hexane/heptane clean-up [57]. Even if these have been designed to remove lipids they do also (partially) remove anthropogenic compounds with similar chemical structure or properties. As a result, such sorbents should be used with caution in NTS studies. LC–ESI–HRMS/MS is commonly used to screen polar unknown contaminants in the resulting final extracts.

The sample and matrix type play an important role in the selection of extraction solvents and clean-up strategies. For example, biofluid samples (e.g., urine, blood, serum, bile) require a relatively simple extraction and purification approach due to lower lipid contents. Organic solvents (e.g., methanol or acetonitrile) are often used to precipitate proteins in biofluid samples, followed by centrifugation or filtration. Similarly, muscle tissue usually contains less lipids than other tissues, such that less lipid removal is necessary. For screening polar compounds, the supernatant can be analysed directly with LC–HRMS [67], while non-polar solvent exchange is required to screen non-polar compounds by GC–HRMS.

Passive sampling

Passive samplers employ a receiving phase (e.g., sorbents, materials with sorption properties) to collect chemicals of interest in situ from environmental compartments (e.g., surface water and wastewater, soil, air, biological matrices) [68, 69]. Passive sampling has been established in legislative frameworks, such as the Water Framework Directive [70], international monitoring/regulatory networks (i.e., Global atmospheric passive sampling (GAPS), [71] and Aqua-GAPS [72]) and international standards [73]. A clear advantage of passive sampling techniques is the generation of time-integrated data along with high enrichment factors, which are beneficial for identification of low-level pollutants [68]. Furthermore, they allow for more direct comparisons of different matrices in terms of the compound range and the chromatographic signature, for example, sediment vs. water, which is more challenging than comparing different matrix extracts (e.g., from SPE and PLE). Thus, these techniques are increasingly used to complement more traditional monitoring of contaminants that may be difficult to analyse by spot or bottle sampling, as well as providing important spatial and temporal trend information [74].

The use of NTS with passive sampling has so far been applied to water/air analysis of samples collected with polydimethylsiloxane (PDMS), polyethylene (PE), Polar Organic Chemical Integrative Sampler (POCIS) and Chemcatchers (typically polystyrene–divinylbenzene) [75–80]. In these studies, authors reported the tentative identification of a range of halogenated, organophosphate and musk compounds, synthetic steroids, pharmaceuticals, food additives, plasticisers and pesticides. Screening studies with PDMS wristband passive samplers have provided information on exposure to a wide range of atmospheric chemicals including pesticides, legacy pollutants, consumer products and industrial compounds [81–87]. The physico-chemical properties of compounds and environmental matrix dictate the type of passive sampler and the analytical methods employed. Typically, many polar chemicals are sampled from water matrices with POCIS or Chemcatcher samplers capable of extracting up to three litres of water over deployments periods of $\leq$30 days [68]. Sample preparation and extraction of passive samplers involves similar protocols as reported for SPE and/or LLE [88], followed by reverse phase liquid chromatography (RPLC)–ESI–HRMS [76, 89]. Non-polar and moderately polar compounds are enriched from water or air using PDMS/PE samplers. Hydrophobic contaminants accumulate in these phases via diffusion. Depending on the surface area of the PDMS/PE used, they have the potential to extract hundreds of litres of water or m^3 of air. Sample preparation involves pre-extraction and cleaning of the polymers (i.e., via Soxhlet or LLE) before deployment and compound extraction using the same techniques post deployment. Analyses are typically conducted by GC–MS (or GCxGC) using EI or chemical ionisation (CI) as standard methods for the assessment of persistent and bioaccumulative compounds [75, 77, 81, 85, 90]. To cover polar and non-polar compounds together in a study, a number of different passive samplers can be deployed next to each other.

Extracts from passive samplers typically also contain monomers or oligomers from polymeric media or the sorbent itself. This leads to interferences, ion suppression and/or high background levels (reduced sensitivity), as well as analytical variation [74] and uncertainty in the identification of chemicals. A specific and thorough pre-cleaning of the passive sampler medium is required to minimise the presence of interferences. Similar to limitations observed with SPE and LLE techniques, the passive sampling media enrich many compounds, including matrix components, and therefore, drawbacks can include low recoveries and high ion suppression caused by chemical background. In such cases, a sample clean-up step is sometimes employed before analysis, but it needs to be chosen with care to minimise the number of chemicals of interest that are also removed with the matrix interferences [91]. The additional sample processing step also carries risks of sample contamination during processing. For this reason, blanks are a requirement for NTS. Appropriate extraction and field blanks to assess contamination from sample preparation, storage, processing and analysis in the laboratory and from the passive sampling medium itself are critical to minimise false positive identifications. While the background may be a burden, it can present an advantage, too, because it produces a similar level of ion suppression independently of the matrix, which can allow for better comparisons between different samples or matrices.

Specifically for passive sampling, the variation of sampling rates for different compounds and for different site conditions (flow rate, temperature) pose a problem in (semi-)quantitative analysis (i.e., comparison of peak areas) in the data. While in targeted analysis sampling rates can be determined experimentally or estimated based on chemicals with similar physico-chemical properties or performance reference compounds, this is not directly possible for compounds with unknown structure. The uncertainty can be especially significant for polar chemicals [92]. For this reason, NTS with passive sampling is best suited to determining spatial and temporal trends among sites of comparable conditions. The (semi-) quantification approaches discussed in Sect. "Quantification and semi-quantification of suspects and unknowns" are further hampered by these specific limitations for passive sampling.

LC–HRMS/MS analysis

Choice of separation method

The best LC method for NTS should ideally separate all (at the time of analysis still unknown) isobaric and isomeric compounds that cannot be distinguished by the HRMS detection while showing decent chromatographic peak shapes for all of them. As a proxy, the optimisation is usually done using a large set of reference standards covering a broad compound domain (i.e., $\log K_{ow}$, RT, structural variety). However, optimising in a particular direction will usually negatively affect the performance of other compounds of this mixture. Thus, LC methods for NTS will aim at a reasonably good performance for many compounds, rather than maximising it for a small set. However, they will likely show a bad performance for a part of the compounds in a sample, and thus complementary LC methods would be necessary to cover the whole compound inventory.

In this section, we will highlight some guiding principles for choice and optimisation of LC and other liquid-phase separation methods for NTS, addressing mainly stationary phase chemistry, column dimensions, gradient conditions, as well as eluents and eluent modifiers. The choice of the latter two is intimately linked to the choice of the ionisation method, as particularly eluent modifiers will severely impact the ionisation behaviour of molecules. A range of textbooks and numerous journal articles have been published on the proper choice and optimisation of LC methods and the underlying theoretical concepts [93–95], some websites also provide invaluable practical information [96].

In general, a LC method for screening should provide a high peak capacity, which is defined as the maximum number of peaks (of uniform width) that can be separated in an elution time window with a fixed resolution [97]. For the complex compound mixtures that are encountered in environmental or biological samples, a high peak capacity can only be achieved by gradient rather than isocratic separations. The peak capacity increases with column length and with decreasing particle sizes of the stationary phase [98] and is larger for shallow than for steep gradients. Thus, long and shallow gradient runs covering a wide range of mobile phase fractions on long columns with small particles would be the best choice for NTS, but this is limited by some practical constraints. The limit for the run time is defined mainly by the desired sample throughput, and in most methods applied in screening, LC method run times do not exceed 30 min. The peak capacity also depends on the flow rate, but this relationship is more complex: for very short gradient runs, high flow rates on short columns are better than on long columns, while for longer gradients, longer columns and lower flow rates perform better [97]. A long column with small particles will result in a high back pressure during the separation. For such cases, columns with superficially porous particles (also termed core–shell particles) are an alternative, as they offer the same peak capacities at particle sizes > 2 µm as sub-2 µm fully porous particles, thus allowing comparable performance at lower back pressures [99].

Although modern (ultra) high-performance liquid chromatography ((U)HPLC) pumps and autosamplers of all vendors can deal with back pressures of 100 MPa or above, working at high pressures decreases the robustness of the methods. Even a small deposition of insoluble matrix constituents or particles in the flow path from environmental samples bearing a significant matrix load will result in stronger pressure increases than for systems run at lower pressures and increase the risk of an excess pressure failure. Therefore, elimination of particles by filtration or (ultra)centrifugation is indispensable. Furthermore, a desired small peak width to obtain a high peak capacity through the use of short columns, high flow rates and small particle sizes might not be compatible with the HRMS detection if the cycle time is too long to provide adequate coverage of the chromatographic peak shape, i.e., less than 8–10 scans across a peak (see Sect. "Choice of mass spectrometry settings"). Moreover, high flow rates above 400 µL/min are often not well-suited for ESI, which is the most widely used ionisation technique for semi-/non-volatile species. Apart from this theoretical concept of peak capacity (further detailed in literature [97, 100]), the actual chromatographic resolution in real environmental samples is lower, as peaks are not evenly distributed in the gradient time continuum, and thus an experimental optimisation using representative samples might be necessary. While in targeted LC–MS methods the column temperature can be adjusted to change the selectivity of the separation, the main purpose in screening methods is to maintain a constant column temperature over time and to lower the viscosity of the eluents and thus backpressure in UHPLC separations.

Reversed-phase separation

In general, reverse phase (RP) separations are most widely used in NTS, employing a rather hydrophobic stationary phase chemistry (mostly C_{18}-, occasionally C_8-modified silica gel), or less often more polar columns (biphenyl, pentafluorophenyl (PFP), or phenyl-hexyl modified silica gel) as evident from overviews of methods applied in collaborative trials by the participating laboratories [9–11]. On C_{18} columns, a large fraction of typical environmental contaminants shows a good retention factor and good peak shapes, and the retention stems mainly from hydrophobic interactions. Columns with aromatic ligands (biphenyl-, PFP-, phenyl-hexyl) allow for dipole–dipole and π–π interactions with the analytes, which results in a different selectivity and an increased retention of polar compounds. All silica-based columns have a certain activity of free, acidic silanol groups which are acidic with predominant pK_a values in ranges from 3.5 to 4.6 and from 6.2 to 6.8 [101]. These also contribute to the retention of analytes through dipole–dipole (if neutral) or ion exchange (if ionised) interactions, the latter affecting particularly basic analytes, often resulting in poor peak shapes [102]. Vendors continuously expand their portfolio of columns with low free silanol group activity based on advanced synthesis and many such low-activity columns are available, but a complete elimination of free silanol groups is not possible. Polymer columns are an alternative avoiding the drawbacks of silica, but at the cost of lower peak capacities and are hardly used in screening methods. On the other hand, stationary phases associating C_{18} or C_8 ligands with a modified polar particle surface can reduce free silanol group activity while simultaneously allowing hydrophobic interactions and an enhanced retention of polar and hydrophilic compounds [103].

Further considerations in terms of column chemistry are the pH stability and the possibility to use high aqueous eluent fractions. While most silica-based columns allow pH values between 2 (hydrolysis of the bonded phase) and 8 (dissolution of the silica particle), specifically stabilised silica columns allow eluent pH between 1 and 11 or 12, allowing the use of basic eluents. Many C_{18} (and to a lesser extent other hydrophobic stationary phases) show so-called phase de-wetting [104] when used at high aqueous eluent fractions (typically > 97%) to increase the retention of hydrophilic compounds. The reason is a partial exclusion of the mobile phase from the hydrophobic pores of the bonded phase, which results in irreproducible RTs. The incorporation of polar groups in the bonded phase prevents de-wetting and allows the use of 100% aqueous mobile phases.

The preferred organic eluents for RPLC are methanol, a protic, acidic solvent acting as an H-bond donor, and acetonitrile, an aprotic solvent exhibiting dipole character. Both solvents are well-suited for polar compounds, with a range of eluent additives and exhibit a low background due to solvent clusters in ESI. Methanol is often preferred instead of acetonitrile due to the lower price, while acetonitrile has the advantage of lower viscosity and thus a lower LC back pressure. Acetonitrile also has a higher elution strength compared with methanol and often less peak broadening (e.g., for alcohol compounds). Thus, for hydrophilic compounds better retention can be achieved with methanol, while for hydrophobic compounds, the retention factors are lower and a faster elution can be achieved with acetonitrile. The addition of eluent modifiers in RPLC has two main goals, (i) improving chromatographic retention of ionisable compounds by adjusting the pH of the eluent, and (ii) improving ionisation of compounds. Both must be carefully considered and are in some cases divergent, for example, the RPLC retention of acidic compounds is improved at low pH, yielding the neutral molecule, while the presence of excess protons is not always favourable for deprotonation of these

acidic compounds in negative ion mode ESI (Sect. "Choice of ionisation technique"). The choice is typically limited to a few eluent modifiers which are volatile enough to prevent the precipitation of salts in the ion source. These are formic and acetic acid, their ammonium salts, ammonium hydrogen carbonate and ammonia or combinations thereof (Table 2), which might be used as additives or as buffers.

Buffering of eluents is a common practice to obtain reproducible RTs of ionisable compounds with pK_a values close to that pH. Small pH changes may be caused by the acidic silanol activity of the column or injection of extracts with a different pH than that of the eluent. With modern LC columns based on high purity silica with a low silanol group activity, the use of unbuffered solutions has become a more common practice. Particularly, formic or acetic acid at 0.1% concentration provide a good pH stability due to the relatively high proton concentrations, which results in a protonation of acidic compounds and of acidic silanol groups to reduce electrostatic interactions, which would cause poor peak shapes of basic compounds. Many other volatile eluent additives/buffers such as small aliphatic amines (e.g., triethylamine) or trifluoroacetic acid used in conventional LC are problematic for MS analysis. They cause severe ion suppression due to competition for charge in positive (amines) or negative mode (trifluoroacetic acid), may form stable ion pairs with other compounds preventing their detection, and can only be removed from the ion source (and LC instrumentation including tubing) after extensive cleaning procedures. The non-volatile ammonium fluoride gained some popularity as an eluent additive [52, 105]. It considerably improves the ionisation efficiency of phenolic and other compounds in ESI-mode as compared to ammonia or ammonium formate/acetate due to the high proton affinity of the fluoride anion. However, the concentration should be 1 mM or below.

Typical LC gradients comprise the whole range of organic eluent/water mixtures, starting at 0 (in case of appropriate columns) or 5% of organic eluent, increasing linearly up to 95–100%, followed by an isocratic phase of varying duration (typically < 10 min) at this level to elute hydrophobic constituents before re-equilibration. Depending on the extent of hydrophobic matrix constituents, an additional rinsing step with a solvent of higher elution strength (e.g., isopropanol) may be included before re-equilibration (e.g., for sediment extracts [106]). While it is good practice in RPLC to use the same composition of the injection solvent as the initial eluent composition (i.e., typically a high aqueous eluent fraction) to ensure good chromatographic peak shape, this approach is often difficult for screening analyses. As discussed in Sect. "Sampling and sample preparation for NTS", sample preparation is usually limited to keep as many compounds as possible, thus the final extract for analysis often contains compounds and matrices with a large hydrophobicity range. Diluting such extracts with water to match the initial eluent composition of the LC will often result in precipitation of poorly soluble matrix constituents. A subsequent filtration might result in a loss of more hydrophobic compounds along with the filtered precipitates. On the other hand, the LC gradient should start from a low organic eluent fraction (typically 5%) to allow for a retention also of the more hydrophilic compounds. As a result, sample extracts (particularly of biota or sediments obtained with less polar solvents) have to be injected with a significantly higher fraction of methanol or acetonitrile as the initial eluent composition. Such a mismatch in solvent strength and viscosity will result in a deterioration of peak shapes or split peaks of early eluting compounds [107], which becomes more severe with increasing difference in the solvent fraction and larger injection volumes. Thus, a compromise must be found during method development weighing up which solvent

Table 2 Common eluent additives for RPLC–ESI–HRMS screening methods

Additive	pK_a (at 25 °C in water)	Buffer region	Typical concentrations
Formic acid	3.75	–	2.6–26 mM (0.01–0.1%)
Ammonium formate	3.75 9.25	2.8–4.8 8.3–10.3	1–5 (10) mM
Acetic acid	4.76	–	1.8–18 mM (0.01–0.1%)
Ammonium acetate	4.76 9.25	3.8–5.8 8.3–10.3	1–5 (10) mM
Ammonium hydrogen carbonate	6.35 (H_2CO_3/HCO_3^-) 9.25 (NH_4^+/NH_3) 10.33 (HCO_3^-/CO_3^{2-})	5.4–7.4 8.3–11.3	1–5 mM
Ammonia	9.25	–	5–10 mM
Ammonium fluoride	–	–	0.1–1 mM

fraction and injection volume is feasible with still-acceptable deterioration of peak shapes for early eluting compounds. Figure 3 shows the effect of injection solvent composition on peak shapes, which ranges from a slight deterioration to a complete splitting of the compound peak, with a major portion of the compound eluting at the column dead time. Peak splitting also has an impact on the peak detection in the subsequent data processing steps (Sect. "Data (pre-)processing and prioritisation for NTS"), as badly shaped peaks might not be detected, whereas split/double peaks may suggest the presence of two isomeric compounds, like in the case of atenolol at 70% or 100% methanol in Fig. 3. For large volume injections, a mismatch between the pH of the mobile phase and the injected sample can also result in a deterioration of peak shapes for ionic compounds if their pK_a falls between these two pH values [108].

Separation of hydrophilic and ionic compounds

Highly hydrophilic compounds (in terms of environmental concern often referred to as persistent mobile organic chemicals, PMOC) [109] are often not retained on typical RP columns and elute at or close to the column dead time, where typically strong ion suppression and interferences are observed, impeding reliable peak detection, identification and quantification. Several approaches are available to achieve a separation of highly hydrophilic, neutral and ionic compounds [110, 111], including HILIC, SFC, capillary electrophoresis, ion chromatography (IC) and MMLC. Typically, the $logK_{OW}$ (or $logD_{OW}$ in case of ionisable compounds) is used as an approximation to assess mobility and also chromatographic behaviour, although the LC retention of compounds is more complex and $logD_{OW}$ alone is insufficient to predict whether a compound is actually retained on a RPLC column or not [112].

Capillary electrophoresis and ion chromatography have so far been mainly used for the analysis of inorganic ions, but allow also a separation of ionic organic compounds with a wide polarity range. However, both techniques require a specific interfacing when coupled to MS and have some methodological restrictions, which so far limited a more widespread application in environmental analysis [111, 112], although quite a few studies employed capillary electrophoresis in non-targeted metabolomics [113]. Various types of capillary electrophoresis separations exist, of which capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC) and capillary electrochromatography (CEC) are most widely used [114]. In all cases, separations are carried out in small fused silica capillaries (20–200 µm diameter), thus sample volumes and flow rates are rather small (typically < 20 nL, and 1–20 nL/min, respectively). Due to the small sample volume, capillary electrophoresis is not very sensitive. However, the use of capillary electrophoresis–MS with a nanoflow sheath liquid interface for target and suspect screening analysis of drinking water samples demonstrated sensitivity down to < 100 ng/L for some analytes [115].

Ion chromatography typically utilises salt solutions, acids or bases with relatively high ionic strengths as eluents. Thus, a coupling to MS requires a reduction of the high ion concentration by a so-called suppressor containing ion exchange membranes or resins before the ion source [116]. Such an approach was successfully used for the suspect screening of haloacetic acids in drinking water after pre-concentration by SPE [117] as well as pesticide TPs in groundwater [118]. A limiting factor for screening applications is the sorption of more hydrophobic compounds to suppressor parts, which can be reduced by higher fractions of solvents [119]. The

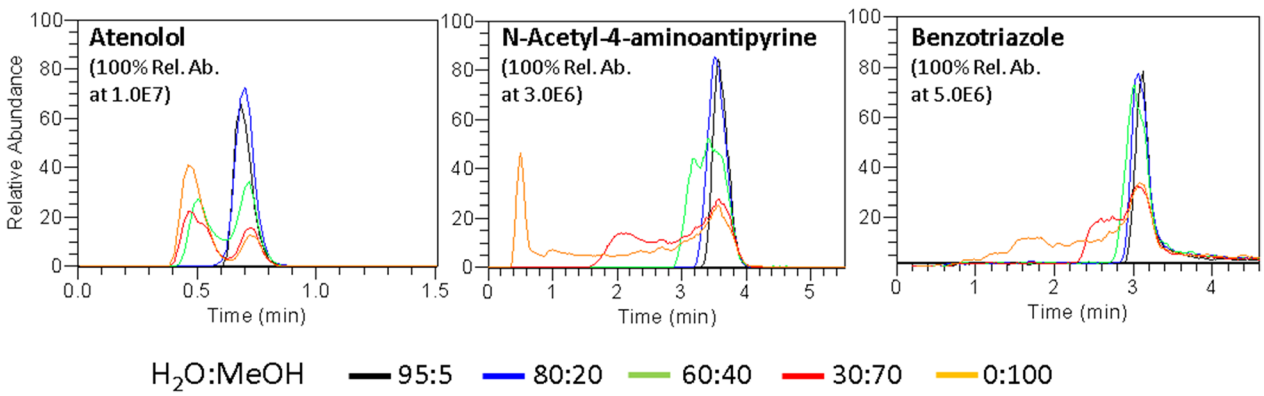

Fig. 3 Extracted ion chromatograms of three different hydrophilic compounds at 50 ng/mL depending on the injection solvent composition for a RPLC separation (10 µL injection volume into 300 µL/min water:methanol 95:5 both with 0.1% formic acid at gradient start, on a Phenomenex Kinetex C_{18} EVO, 50 × 2.1 mm, 2.6 µm particle size; column dead time is 0.5 min)

use of volatile buffers at lower concentrations in non-suppressed IC allows for a direct coupling to a MS ion source. However, the sensitivity is often lower and not all ions can be sufficiently well-separated, making this approach less suitable for screening applications.

SFC utilises supercritical CO_2 as the main eluent, which might be modified by the addition of polar solvents (e.g., methanol) or aqueous salt solutions (e.g., ammonium hydroxide) to increase its low polarity in the pure state. It can be used with both hydrophobic stationary phases similar to RPLC and polar stationary phases similar to normal phase LC (NPLC) or HILIC. Thus, offering a large flexibility for adjusting the selectivity and compound domain of the separation [120, 121]. Furthermore, due to the low viscosity originally seen as a "green" substitute for NPLC, SFC gained particular interest in environmental analysis as a complementary technique to RPLC to separate hydrophilic contaminants on polar stationary phases [122, 123]. For certain substances with $logD_{ow}$ values close to 0, SFC can exhibit better sensitivity compared to RPLC due to high CO_2 content and low water content in the mobile phase which can improve ionisation in ESI [124]

Although different strategies exist for coupling NPLC to mass spectrometry, this remains a challenging issue [125] and no application for the screening of environmental samples has been published so far. Classical NPLC employing aprotic solvents is incompatible with ESI, but could be coupled with APCI or atmospheric pressure photoionisation (APPI) sources. So-called aqueous NPLC uses specific silica hydride-based stationary phases, which bear no silanol groups and can be operated in RP and NP mode, but have not found a widespread application so far [126].

Although the retention behaviour in HILIC separations is not entirely understood, the general idea is that it is caused by a partitioning of an analyte between an acetonitrile-rich mobile phase and a water-enriched layer partially immobilised onto a polar stationary phase [127]. Consequently, gradient separations start with a high fraction of acetonitrile and hydrophilic compounds are retained. The polar analytes are then eluted upon increasing the composition of the aqueous eluent. However, the aqueous fraction of the eluent must not exceed a certain level (typically around 30% [128]), otherwise the water-enriched layer at the surface will disappear, and the column will change into another separation mechanism. Sometimes buffer components (e.g., ammonium acetate) are required to minimise ionic interactions, which can lead to a decrease in ESI response. In analogy to RPLC, where the hydrophilic compounds are affected, in HILIC a large part of the more hydrophobic compounds of an environmental sample elute at the column dead time, which impairs their detection and identification. Thus, HILIC methods provide a complementary approach to RPLC and have so far been used for the screening of environmental samples together with a RPLC method [129]. Drawbacks of HILIC methods are the relatively long equilibration times when operating in gradient mode (it is recommended a post gradient re-equilibration of approximately 20 column volumes) as compared to RP separations and the need to inject the samples in a high solvent fraction [130]. For aqueous samples, which are the most relevant types of samples for HILIC separations, a solvent exchange is, therefore, required, either by a SPE method capable of retaining very hydrophilic analytes or evaporative concentration (see Sect. "Water"). In addition, highly hydrophilic compounds might not dissolve well in the injection solvent [131]. Compared to RPLC, HILIC shows broader chromatographic peaks due to slower and less uniform kinetics and mass transfer. A considerable variety of stationary phases are available for HILIC, which range from bare silica, diol, and amide to multifunctional bonded phases, also including anionic, cationic and zwitterionic functionalities. These show a widely different selectivity and retention behaviour of compounds. In particular, columns with ionic functionalities also show a strong retention of more hydrophobic, ionic analytes of the opposite charge.

In the literature, approaches combining columns with functionalities allowing multimodal interactions have also been termed MMLC separations. This term summarises stationary phases that may combine hydrophobic, ionic and/or polar functionalities, which may be operated in RP and/or HILIC mode [132]. Such mixed-mode columns have been used extensively for the separation of peptides and proteins [133], but rarely in environmental screening methods so far [15, 134]. They hold some promise to allow for the retention and separation of compounds with a wide range of physico-chemical properties in one single separation, particularly extending the RP amendable compound range towards more hydrophilic compounds. In one study, the retention of hydrophilic (ionic and non-ionic) model compounds was compared among RP, HILIC and MMLC columns, which showed a widely different selectivity [135]. The authors particularly noted that some bonded phases showed a significant column bleed from the ionic functional groups, decreasing linear dynamic range and sensitivity in LC–HRMS screening methods. Furthermore, inorganic anions and cations present in samples might cause ion suppression over a considerable RT range, as they are retained by the ion exchange functionalities as well [136].

Bieber et al. employed a direct sequential coupling of a RP with a HILIC column [122]. The poorly retained fraction of the RP-separation (*i.e.*, the hydrophilic

compounds) are transferred directly to the HILIC column, along with the HILIC eluent acetonitrile, via a mixing tee. Afterwards, the compounds retained on the RP column are eluted with a gradient with increasing acetonitrile fraction. This technique allowed covering a broad hydrophobicity range while allowing for the direct injection of aqueous samples. The combination of RP and HILIC columns is also promising for comprehensive two-dimensional LC (LCxLC), as both use compatible eluents and provide highly orthogonal separations [137, 138]. LCxLC applications in environmental screening also combined two RP columns [53, 139]. A limitation especially for screening methods, is that there is almost no software which can handle the data of the second separation dimension automatically.

Practical considerations for separation method selection

From the vast number of possible separation techniques and methods, most NTS studies so far only make use of LC, with a clear predominance of reversed phases, and laboratories have established their own routine methods and applied them in different larger scale screening studies [129, 140, 141]. The application of other techniques (especially MMLC, IC, EC, SFC) is still limited to individual, often exploratory studies, in which different setups are tested and the general usefulness of application is demonstrated. Table 3 provides a brief summary of the separation techniques discussed above, their compound domains and potential advantages and disadvantages as a starting point for the choice of the appropriate technique.

Regarding method parameters, Table 4 provides a brief overview of the main considerations for selecting the appropriate conditions. It is essential to evaluate the performance of a chosen method for the given compound domain and matrix using both representative standard compound mixtures and spiked matrix samples. For the latter, observation of the total ion chromatograms can already give an indication how well the matrix is spread along the chromatographic run time. A key question is whether to use the same or two different separation methods for both ionisation polarities. While two different separation methods can be tailored for a good retention and ionisation of respective compound types in each mode individually, using the same method for both runs allows for a direct comparison of positive and negative mode data. This is beneficial for compound identification but may reduce the coverage of some compound types. In addition, it must be considered whether a primary wide-scope screening method should be complemented with one or more additional methods for expanding the compound domain, which means an increased time and financial commitment.

Additional points to consider include background contamination and changes to the system over time. These

Table 3 Overview of liquid-based separation technologies for NTS: substance domains, advantages and potential disadvantages

Method	Domain	Advantages	(Potential) disadvantages
RPLC	Polar to non-polar	Straightforward, rather easy to understand separation	(very) hydrophilic compounds not retained Injection solvent mismatch
MM–RPLC	Polar ionic to non-polar compounds	Expanding the RP compound domain towards ions	Neutral hydrophilic compounds not be well-retained More complex optimisation
MM–HILIC	Very polar/hydrophilic and ionic	Complementary to RPLC	Hydrophobic compounds not retained More complex optimisation Solvent exchange required for aqueous samples
HILIC	Very polar/hydrophilic	Complementary to RPLC	Solvent exchange required for aqueous samples Hydrophobic compounds not retained
IC	Large range of ions, incl. inorganics	Broad domain of ionic compounds	Only for ionic compounds Removal of high salt load from eluents or samples necessary
Capillary electrophoresis	Large range of ions, incl. inorganics	Broad domain of ionic compounds	Only for ionic compounds Low flow rates/injection volumes Often lower sensitivity as compared to LC
SFC	Very hydrophilic to non-polar compounds	Green: less organic solvent consumption Likely rather versatile tuning of method possible	Additional hardware required Solvent exchange required for aqueous samples Elution not yet very predictable
LCxLC	Depending on combination of columns	Combination of different separation strategies Very high peak capacity and selectivity	Additional hardware required Data size and high complexity for evaluation

Table 4 Main considerations for the selection of LC method parameters for NTS

Selection	Consideration
Separation approach(es)	Compound domains "One fits all" compromise or different complementary methods Same or different methods for positive and negative mode ionisation
Stationary phase chemistry	Compound domains of interest Compatibility with mobile phase (pH, high aqueous fraction)
Column dimensions / particle size & flow rate	Peak width to match temporal resolution of MS (Sect. "Choice of mass spectrometry settings") Flow rate compatible with ionisation source (Sect. "Choice of ionisation technique") Overall analysis time Robustness of analysis
Gradient time	Peak capacity vs. overall analysis time
Use of inline-filter and pre-column	Protection of (more expensive) main column and robustness vs. increased dead volumes
Eluents: Methanol or acetonitrile, additional solvents	Cost (acetonitrile is more expensive) Preferred (or only possible) choice for particular stationary phase Lower viscosity of acetonitrile Protic or aprotic eluent Matrix load of samples
Eluent additives / eluent pH	Retention and peak shape of ionisable compounds Compatibility with stationary phase Compatibility with ion source MS ionisation behaviour (see Sect. "Choice of ionisation technique")
Column oven temperature	Decreased viscosity and back pressure vs. column stability (dissolution of silica gel at higher temperature)
Purity of eluents and additives	Cost vs. background noise/contamination
Injections solvent and volume	Solubility of compounds and matrix constituents vs. peak shape deterioration for high solvent fractions

can be assessed with appropriate QA/QC procedures (see Sect. "Quality assurance and quality control in NTS methods") and are only mentioned briefly here. Background contamination can arise from numerous factors, including solvents, pumps and degassers. The background present in solvents can vary among suppliers, in general, commercial LC–MS grade solvents are recommended over bi-distilled solvents or Millipore water. Carry-over and other factors should be assessed with blanks during the sample runs, while other factors such as column age, RT shifts over time and loss of separation power can be assessed with internal standards (IS) to ensure a timely replacement.

Choice of ionisation technique

The amenability of a compound to MS detection is foremost governed by the conversion to its ionised form, since this is imperative for this analysis technique. The choice of ionisation technique or interface used in MS systems both defines and restricts the analysable chemical domain. For LC–(HR)MS-based analysis of organic compounds, mainly electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), and atmospheric pressure photoionisation (APPI) are employed and will be discussed briefly in the following. The decision on which ionisation technique to use depends mainly on the mass as well as the polarity of the analytes of interest, as shown in Fig. 4.

For non-polar to moderately non-polar analytes, such as compounds without any polar functional groups or steroids, APPI and APCI are useful techniques, whereas ESI is best for molecules with polar functional groups as discussed further below. In general, it is difficult to predict ionisation efficiencies in NTS, since without analytical standards no exact quantification is possible. The need to provide some form of quantitative information in NTS has led to the development of several strategies to predict ionisation efficiencies in recent years (Sect. "Quantification and semi-quantification of suspects and unknowns").

Electrospray ionisation

ESI, first introduced by Fenn et al. in 1984 [142], is most widely employed when targeting medium-polar-to-polar compounds ranging from small molecules up to "molecular elephants" of over 100,000 Da as mentioned in Fenn's Nobel Prize speech. Typically, molecules with polar functional groups, such as alcohols, carboxylic groups, or amines ionise well. ESI is a soft ionisation technique, which allows the production of intact gas-phase ions from a liquid sample, allowing to easily hyphenate LC with (HR) MS instruments. Strictly speaking, ESI is not an ionisation source, but is rather based on ion transfer, i.e., ions must be previously present in the solution (molecules forming

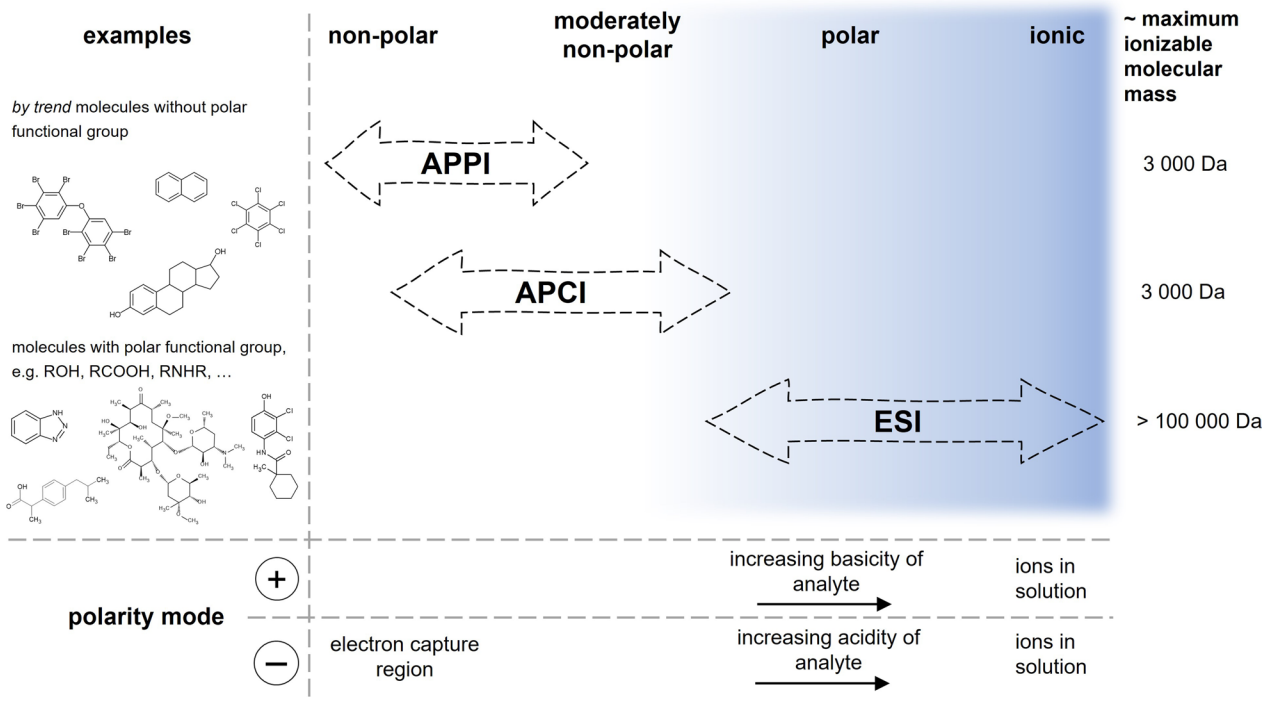

Fig. 4 Ionisation techniques and their range of applicable polarity characteristics of the respective analytes, including examples and the approximate maximum molecular mass of the analyte to be ionised

adducts, protonated or deprotonated). In short, an electrostatically charged aerosol consisting of µm-sized droplets is formed from the mobile phase (containing the analytes), supported by a nebuliser gas (N_2) under an electric field. Due to rapid solvent evaporation, the size is reduced until ions are liberated into the gas phase. Advantageously, ESI is operable in positive or negative ionisation mode, i.e., generating positively or negatively charged ions that are accelerated into the mass spectrometer. One characteristic of ESI is the formation of adduct ions (see "Glossary") depending on the sample matrix and the presence of ions in the mobile phase. Modifiers can be added to the eluent to improve ionisation efficiency of certain compounds (see Sect. "Reversed-phase (RP) separation"). If more than one ion species is formed from the native molecule, this can facilitate identification as multiple adduct species provide extra information to define the mass of the molecule of interest. However, this phenomenon renders additional steps in data analysis necessary, namely, to group/merge these different features into a single compound (e.g., $[M+H]^+$, $[M+Na]^+$, $[M+NH_4]^+$ and $[M+K]^+$ in positive mode, or $[M-H]^-$, $[M+CH_3COO]^-$, $[M+Cl]^-$ in negative mode), a process termed componentisation (see Sect. "Data pre-processing"). Furthermore, the intensity is spread over several *m/z*, leading to lower limit of quantifications (LOQs). ESI generally results in singly charged ions for small molecules, but multiply charged ion species are observed especially for larger molecules, such as proteins and other large biomolecules. For environmental cases, large molecules such as water-soluble polymeric substances in wastewater have more than one charge state [143]. Charge states can be identified by calculating the *m/z* differences between the adjacent isotopologues, e.g., $[M+2H]^{2+}$ differs ≈ 0.504 in its isotopologue pattern. ESI is an excellent choice when dealing with medium polar analytes in polar samples, such as water. However, when analysing less hydrophilic compounds in extracts of soil, sediments or biota samples, other ionisation techniques such as APCI and APPI might be more suitable.

Atmospheric pressure chemical ionisation and photoionisation

APCI and APPI are both soft ionisation techniques, producing mass spectra similar to those of ESI in terms of (low) in-source fragmentation. In contrast to ESI, however, both APCI and APPI are restricted to molecules below 2000–3000 Da, since above this limit either ion formation is not effective or in-source fragmentation increases significantly [144, 145]. APCI has been coupled with both LC and GC, whereas APPI is typically coupled with LC.

In APCI, a series of chemical reactions with mobile phase and nitrogen sheath gas molecules leads to the formation of reagent ions (e.g., NH_3, CH_4), which

consecutively react with sample molecules and generate sample ions in the gas phase. The ion species formed are primarily (de)protonated molecules and molecular ions, which should be considered when generating molecular formulas [106, 143]. Since vaporisation of the LC stream in APCI is performed by high temperatures, analysis of thermally labile molecules can be difficult [146]. Typically, lower ion suppression and/or matrix effects are observed in APCI compared with ESI. Depending on the sample matrix and the ionisation efficiency of the compound in the respective ion source, this can result in better sensitivity, e.g., for certain flame retardants [147]. Within the US EPA's Non-Targeted Analysis Collaborative Trial (ENTACT), the complementarity of ESI and APCI in expanding the chemical space coverage was highlighted, including a detailed inspection of how well diverse groups of chemicals ionise in APCI [148].

APPI can be used in LC–MS-based analysis to measure compounds of low polarity [149]. Here, ions are formed either directly (APPI) or indirectly via dopant assisted photoionisation (DA-APPI). In the case of direct photoionisation, the analyte molecule has a lower ionisation potential than the energy of the photon emitted by the light source (Ar lamp 11.2 eV; Kr lamp 10.03 eV: 10.64 eV = 4:1). In case of DA-APPI, the dopant/solvent employed is amenable to direct photoionisation, produces reagent ions and subsequently ionises the analyte. For the latter approach, care must be taken regarding the miscibility of the dopant (solvents with ionisation potential < 11.2 eV or 10.03 eV, depending on the light source) and the mobile phase, especially when using typical RPLC solvents [145]. Isopropanol (ionisation potential: 10.22 eV) and to a lesser extent methanol can serve as dopant compatible with RPLC separation [143]. As in APCI, prevalent ion species also include molecular ions. APPI is also known to be less affected by matrix effects or ion suppression [143, 150].

A limited number of publications are available comparing sensitivity using different ion sources. A study investigating 40 pesticides in garlic and tomato extracts demonstrated that ESI results in lower limit of detections (LODs) in most cases compared with APCI or APPI [150]. Another study performed a detailed comparison of ESI and APCI for polyaromatic compounds [151] showing that, since ESI yields poor (or no) detection for some compound classes, both APPI and APCI can open the analytical window to compounds of interest in the low, medium to non-polar chemical space for LC-based methods. Clearly, if a more comprehensive view of the sample is desired, combining different ionisation sources would expand the compound coverage in a sample, at the cost of increasing analysis time and effort and additional data analysis steps.

Choice of mass spectrometry settings

The choice of mass spectrometry settings is primarily guided by the HRMS instrument available, the purpose of measurement as well as the separation method(s) chosen. A minimum number of mass spectrometry detection points are necessary to describe a chromatographic peak (*i.e.,* a chromatographically separated compound) to facilitate peak finding. Although quantitative analysis generally aims for 12 to 20 data points per peak, since NTS is not necessarily quantitative, a reasonable number of points, i.e., a minimum of 7, is highly recommended to improve peak detection and thus reduce the inclusion of noise in the final results (see Sect. "Data (pre-)processing and prioritisation for NTS"). Depending on the chromatographic peak width and the corresponding cycle time/acquisition speed (see Sect. "Glossary and definitions") to provide a certain number of points, the mass spectrometry settings should be adjusted accordingly (e.g., resolution, number of MS2 experiments). In the following, the two most common HRMS instruments will be discussed in more detail, namely, time of flight MS (TOF–MS) and Orbitrap MS. These mass spectrometers are typically coupled with lower resolution mass spectrometers (resolution < 5000) such as quadrupoles or ion traps to provide MS2 or MSn capability. Fourier Transform Ion Cyclotron Resonance (FT–ICR) MS with highest resolution up to 10 million and mass accuracy < 0.2 ppm provides even greater identification capabilities but has so far mainly been used in studies on organic matter characterisation [152]. Due to higher price and longer cycle times required for ultrahigh resolution, it has only been used a few times for NTS of small molecules [153].

Full scan data, mass accuracy and resolution

TOF–MS instruments are characterised by their fast acquisition rates (easily 50 Hz, depending on the type of instrument), which does not affect its resolution. The highest achievable resolutions for state-of-the-art instrumentation are approximately 60,000 for *m/z* 300 (resolution increases for higher masses). In TOF–MS the continuous ion beam is chopped into ion packets before the flight tube, such that a certain number of ion packets (also called transients) will be combined into one mass spectrum. As a result, the higher the acquisition rate, the fewer ion packets will be combined, yielding a lower absolute signal intensity (and subsequently, lower sensitivity).

Orbitrap instruments feature resolving powers up to 1,000,000 at *m/z* 200, with isotopic fidelity up to 240,000. However, this is currently restricted to an acquisition speed of approximately 1 s, leading to a cycle time that prevents coupling with fast chromatographic front-end separation (e.g., UHPLC). However, Orbitraps still outcompete TOF

instrumentation when comparing dynamic range and resolution. Appropriate cycle times for typical LC- or GC-based separations can be reached with Orbitraps, but more data points per chromatographic peak can be acquired with TOF–MS-based detection. Figure 5 shows the required *m/z*-dependent resolving power to separate isotopologues, e.g., heavy isotope ^{37}Cl from ^{81}Br, or $^{15}N_2$ from ^{18}O. This figure also includes the *m/z*-dependent resolving power over *m/z* 100–1000 for four selected HRMS instruments (ThermoFisher Orbitrap Exploris 480, Agilent 6545 QTOF–MS, Bruker Impact II QTOF–MS and a Waters Xevo G3 QTOF–MS). The resolving powers depicted in Fig. 5 are based on empirical values from single laboratories. For Orbitrap, resolving power can be set (up to a resolution of 1,000,000) and affects the acquisition speed directly; in Fig. 5, the resolution was set to 120,000 as this is compatible with HPLC separation. While the resolving power of Orbitrap MS decreases with increasing *m/z*, it increases with increasing *m/z* for TOF–MS instruments. Importantly, with very high-resolving power, resolution of the isotopic fine structure is possible, i.e., the isotope pattern of a given molecule is further resolved and the separate contributions of heavy stable isotopes, e.g., ^{13}C, ^{15}N, ^{34}S, ^{18}O or D become apparent. This is highly beneficial for elucidating the molecular formula (see Sect. "Compound identification / confirmation").

Both mass analysers exhibit excellent mass accuracy, typically < 3 ppm (specifications range from 2 to 5 ppm mass accuracy). The interplay between resolution and mass accuracy greatly reduces the number of possible elemental composition combinations and is showcased (Fig. 6) with mecoprop, clofibric acid (both $[M-H]^- = C_{10}H_{10}ClO_3^-$) and diuron-4-hydroxy ($[M-H]^- = C_9H_{10}ClN_2O_2^-$). Figure 6a shows the mass spectra for the two isobaric ions (same nominal mass, see glossary), $C_{10}H_{10}ClO_3^-$ and $C_9H_{10}ClN_2O_2^-$, in the range of *m/z* 213.0–213.1 at different resolving powers (10,000 and 30,000). Since the two isobars are not separated with a resolving power of 10,000, a $\Delta m/z$ of 26 ppm between 213.0380 (R = 10,000) and 213.0324 (R = 30,000) is observed. Figure 6b displays the top 3 molecular formula hits for the ions using the accurate monoisotopic mass as input *m/z* and defining the ion species as $[M-H]^-$. The true ion formulas cannot be found for the mass acquired at lower resolving power, whereas the top formula is correct for both peaks acquired at R = 30,000.

For both TOF and Orbitrap, different calibration and tuning settings are applied when dealing with *m/z* < 150. Some vendors offer calibration solutions especially for the lower mass range. For certain applications, tailor-made calibration solutions specifically for the expected mass range of the analytes of interest could be useful. For TOF–MS pre-set scan ranges are typically differentiated between standard (min *m/z* 50–100 to max *m/z* 1000–1700, depending on the vendor) and extended mass range (up to *m/z* 3200), which does not directly affect the acquisition. However, for Orbitraps, the scan range should be set with more care. Especially if the minimum *m/z* is between 50 and 150, the max *m/z* should not exceed this

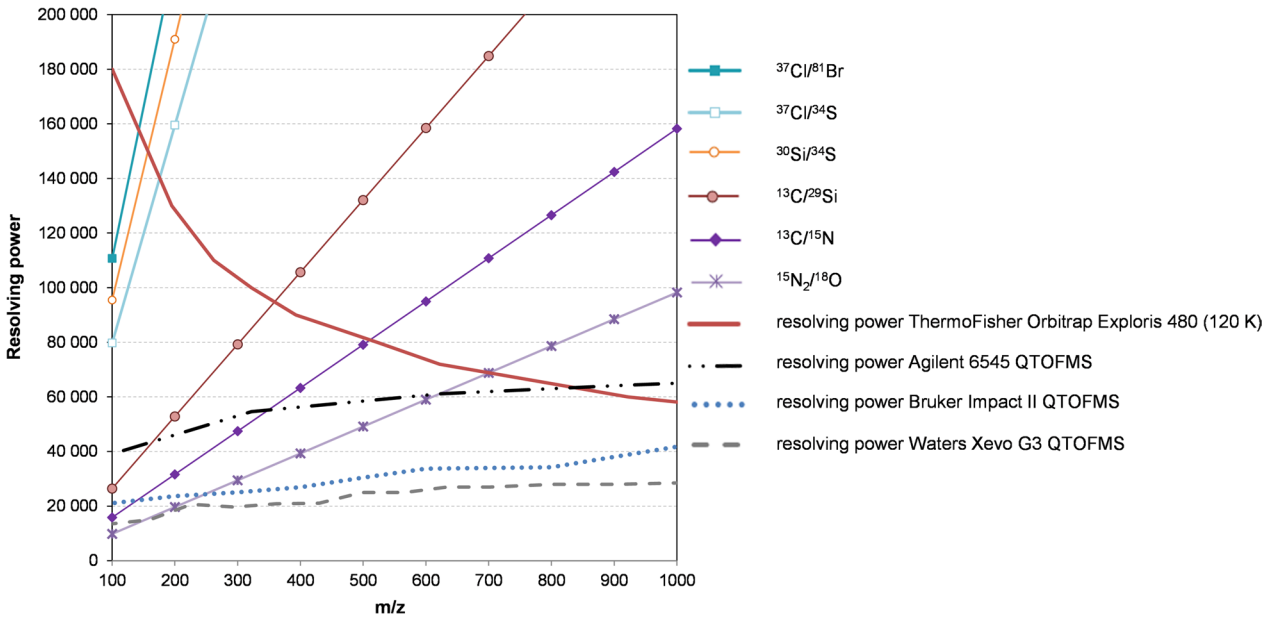

Fig. 5 Required resolving power to separate given isotopologue pairs. Empirical assessment on one Orbitrap and three QTOF–MS (see legend)

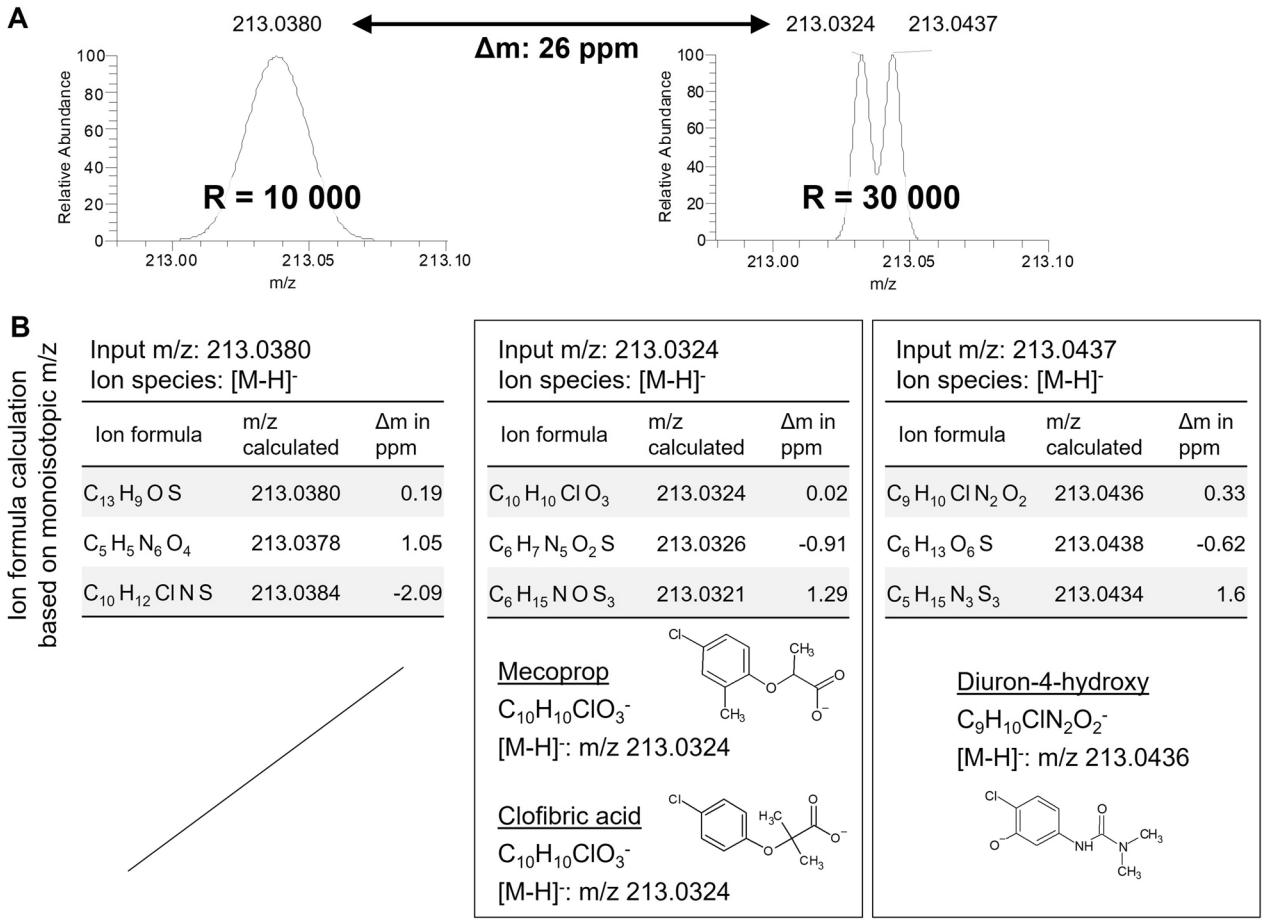

Fig. 6 Interplay between resolution (R) and mass accuracy exemplified by the two isomers mecoprop, clofibric acid and the isobar diuron-4-hydroxy. **a** Mass spectra for the isobaric ions acquired at a resolving power of 10,000 and 30,000. **b** Ion formula calculation based on the accurate monoisotopic mass and defining the ion species as $[M - H]^-$

by a factor of 10. This can render an additional scan differing in the *m/z* range necessary.

Another decision that can affect the number of acquisitions is whether to perform multiple ionisation modes in two separate runs or using polarity switching. Since some molecules are only ionisable in one polarity, running both will expand the coverage. Here, the user needs to decide if the samples should be run in two separate sequences or if polarity switching is available, considering the cycle time (consisting typically of a MS1 scan followed by a certain number of MS2 experiments) and the chromatographic peak width. If two separate sequences are run, care must be taken in terms of sample stability—ideally two sets of samples are prepared and treated the same. Otherwise, it is also possible to alternate within the sequence between positive and negative ionisation modes. However, the mass axis stability needs to be observed carefully.

Finally, there are two different modes to save mass spectrometry data, namely, centroid and profile mode. In the case of profile mode, the Gaussian peak distribution of each MS peak is recorded and saved to the data file, whereas for centroid acquisition only the mass spectral peak apex is saved. The corresponding data files differ significantly in terms of size, with profile data files roughly a factor of 10 larger. Since profile data can be converted post-acquisition to centroid but not vice versa, if storage space is not an issue, it is generally recommended to acquire in profile data and perform the conversion afterwards (see Sect. "Data (pre-) processing and prioritisation for NTS") using either vendor-specific proprietary software or open software, such as MSConvert within ProteoWizard [154].

Fragmentation settings

Fragmentation of the intact compound is essential for identification purposes and is typically performed in the collision cell of a hybrid mass spectrometer. Different types of fragmentation strategies are possible, the most commonly applied are collision induced dissociation (CID) or higher energy collisional dissociation (HCD). Different collision energies (CEs) can be selected, where

higher CEs lead to greater fragmentation (since more chemical bonds break with higher energy inputs). There is typically a rather broad range of CE values (break down curves leading to specific fragments) that lead to the same peaks in the MS2 spectrum, i.e., the same fragments of the intact ionised molecule (precursor), but at varying intensities. Depending on the compound and its functional groups, some fragments are very specific and relevant (even diagnostic) for the identification process, whereas others are rather generic. As a rule of thumb, if the precursor shows approximately 10% intensity of the base peak within a MS2 spectrum, it is considered to be reasonably well-fragmented.

Since the compound-specific optimisation of CE is not possible within NTS, there are different concepts on how to deal with this setting. Options include setting a fixed collision energy, a *m/z*-dependent single collision energy by setting a CE ramp, or multiple fixed CE for different scans. Setting a fixed CE leads to a single spectrum that can yield poor fragmentation information for specific compounds if the selected collision energy is not optimal (low values lead to too little fragmentation, whereas high CEs can result in many small and potentially unspecific fragments). Usually, single CEs between 40 and 60 generally produce reasonable spectra for many contaminant molecules, although in metabolomics slightly lower values are often used (CE 10–40). Although TOFs typically measure CE in eV, whereas Orbitraps use nominal collision energies (NCE), the values and spectra are reasonably comparable over the range 10–60 [155, 156]. While the second strategy adapts the collision energy depending on the *m/z* (increasing energy with increasing *m/z*), this can mean that non-standard CEs are generated, reducing comparability with library spectra acquired at fixed CE intervals. The third strategy, acquiring multiple MS2 spectra with varying CEs, will yield more comprehensive information, but requires more (acquisition) time and reduces the number of points per chromatographic peak. Newer instruments offer some built-in ramped or stepped CE settings that form a good compromise, producing more information-rich spectra over a reasonable CE range (e.g., CE 20–60), which can be used to create library spectra and acquire NTS data.

Precursor selection: data dependent vs. data independent acquisition

The selection of the precursor ions has a significant impact on NTS identification efforts. In general, MS2 data can be acquired either in data dependent acquisition (DDA) or data independent acquisition (DIA), although other strategies can also be used—as clarified in the following.

In the case of *data dependent acquisition (DDA)*, the fragmentation of a precursor ion is triggered and the respective *m/z* will then be isolated (via the quadrupole) and fragmented. Typically, narrow isolation windows (< 1 Da) are used to avoid the presence of isotopes in the spectra, although some TOF instruments are run with isolation windows of 2–4 Da. Fragmentation can be triggered in various ways. In "Top X" and "Top Speed" experiments, the highest intensity X *m/z* will be selected in a MS1 spectrum, then isolated and fragmented in X separate MS2 scans or for the number of scans that fit in X s, respectively. However, selection only according to intensity alone is not ideal in environmental applications, due to the lower concentration of contaminants compared with other naturally occurring substances, such as lipids, which can hamper identification efforts. Inclusion lists of given *m/z* of interest (potentially with RT) can be used to select certain *m/z* of interest at given times, which can also be extended to *m/z* values that are not of interest (exclusion lists) that are then excluded from MS2 experiments. Dynamic exclusion lists can also be used, where an ion that has already been isolated is excluded for a period of time (typically 5–20 s) to avoid isolation of the same ion in consecutive MS2 experiments. This enables acquisition of MS2 spectra for possible isomers appearing later in the chromatographic analysis. There are also combinations of these strategies (e.g., either a *m/z* value for a given RT is found in the list or if none match, the most intense X *m/z* will be triggered). It is also possible to perform a selection on certain compound groups, e.g., halogenated compounds based on their distinctive isotopologue pattern.

Since isolation in DDA is performed using narrow isolation windows that are nominal mass ranges (unlike the high accuracy precursor mass value), isobaric compounds co-eluting at the same time will also be isolated, potentially causing interferences and mixed fragmentation spectra. This should be considered in data interpretation, especially for complex matrices, such as wastewater. Some MS acquisition software check for this when selecting a precursor ion from an MS1 spectrum, while there are also several data processing techniques available to clean up such interferences (see Sect. "Data (pre-)processing and prioritisation for NTS").

Recently, a more sophisticated form of DDA, i.e., iterative exclusion, aiming at more comprehensive sampling for MS2 spectra was implemented in vendor acquisition software, termed AcquireX in Orbitrap systems, or Iterative MS2 in Agilent TOF systems. The basic principle is that highly resolved MS1 acquisition of a representative sample as well as a representative blank is acquired as first runs of a sequence. In the next step, post-acquisition, the MS logic compiles a list with all monoisotopic

m/z values that are not present in the blank or above a set threshold. This list will be then used to trigger for MS2 in a time optimised form. For groundwater samples, AcquireX increased MS2 coverage by 73% using three sample injections [157]. Similar results were obtained when using iterative exclusion on agricultural soil samples [158]. The decision on how many iterative exclusions are performed needs to be considered carefully, since multiple injections significantly prolong the required measurement time. However, these multiple injections can also act as technical replicates and assist in applying feature reduction approaches (see Sect. "Data (pre-)processing and prioritisation for NTS").

Data independent acquisition (DIA) is a more comprehensive approach, fragmenting all detectable molecules eluting from the chromatographic column, without any preselection. Typically, this is performed by alternating between MS1 only acquisition and a MS2 fragment spectra acquisition. The deconvolution of such spectra and assignment of fragments to a specific precursor (e.g., by comparing the chromatographic peak shape between MS1 and MS2 data points) is not trivial and prone to errors. In the so-called SWATH approach (Sequential Window Acquisition of all Theoretical Mass Spectra) of SCIEX, acquisition is slightly different from the other DIA modes mentioned above in terms of specificity. In this mode, the quadrupole is set to a wider isolation window (of roughly *m/z* 50) and is stepped across the entire mass detection range [159]. Thus, an MS2 spectrum of every detectable compound is acquired and the chimeric spectra are reduced. However, it complicates data treatment, since more than one high energy function is acquired for each MS1, and because a slight overlap (typically 5–10 Da) is needed between MS2 SWATH functions to avoid potential ion losses. In addition to SWATH, other manufacturers such as Waters and Thermo have developed acquisition modes that work in a similar way. Another interesting approach to "clean up" DIA spectra is using ion mobility to achieve a more confident assignment of DIA MS2 spectrum to the respective MS1 peak [160, 161], since fragment ions generated inside the instrument will share the exact drift time, providing pseudo-MS2 spectra (similar to the ones in DDA) in DIA acquisitions after filtering extracted ion chromatograms by drift time of the precursor species. This is discussed in more detail in Sect. "Ion mobility separation".

An important difference to the DDA approach is the presence of an isotopologue pattern for each fragment ion, since in DDA the precursor ion is selected using a narrow isolation window. The isotopologue pattern can be particularly useful when working with compounds containing halogens, since their isotopic pattern provides information on the fragmentation of a halogen-containing precursor compound and also the number of halogens that are present in the fragment structure. This can help with the elucidation process.

Comparing these two approaches, a compromise must be made based on selectivity of spectra (DDA) vs. the coverage of precursor ions (DIA). DDA enables much simpler data processing and cleaner spectra for identification, and careful design of inclusion/exclusion lists or approaches such as AcquireX can improve the coverage considerably. Although DIA improves coverage, the data processing is still very complex, and it remains difficult to recover fragmentation information for low intensity precursors. DIA coupled with IMS can help clean up complex spectra. Should runtimes and sample amounts allow, acquiring data in both modes (one run with DIA, one with DDA) can offer the "best of both worlds"; these can also be combined in one run if peak width allows [25]. Finally, it still often comes down to operator preference, and which data are most suitable for the downstream data analysis and questions at hand. Should instrument or sample availability be a problem, additional strategies such as DDA analysis for pooled samples could help further.

For compound class specific screening, neutral loss scanning can be of use. Here, specific fragments are selectively scanned after the collision cell. An illustrative example are glucuronide metabolites, that typically lose the glucuronide moiety ($C_6H_8O_6$), leading to a neutral loss of *m/z* 176.0321 [162]. This neutral loss can be searched after acquisition, providing important information, since an identification of metabolites can be made without introducing deconjugation steps in the sample treatment. As an example, some chemicals (e.g., bisphenol A) are constantly found in human fluids, but the presence of its metabolite is observed at nine times higher concentration in urine [163], so an underestimation will occur unless the presence of bisphenol A-glucuronide is not addressed. In addition, methods based on an additional MS2 event triggered by specific fragments or fragmentation patterns can be used to yield additional MS2 spectra of compounds of interest, for instance potentially toxic compounds [164].

GC–(HR)MS analysis

Choice of chromatography

To extend the chemical domain, LC-based NTS studies are often combined with GC approaches. The NORMAN collaborative trials demonstrated that LC–HRMS could only cover part of the chemical domain of organic contaminants and highlighted an urgent need for development of GC–HRMS workflows [9, 10]. Screening studies may also focus on compounds that are best analysed with GC–HRMS, for example, addressing bioaccumulation in lipid-rich tissues

[165] and volatile compounds in air [166, 167]. The general considerations for optimised separation are similar to those for LC-based analyses (Sect. "Choice of separation method"). Maximising peak capacity can include trade-offs with, e.g., analytical run time, since baseline separation will not be fully achievable and, given the powerful HRMS information, not always required. However, generic GC–HRMS methods, typically including 30 m GC columns and EI techniques, will not be universally applicable and might lead to high detection limits or even non-detections for some compounds, with a risk of producing false negatives. For example, the flame retardant polybrominated diphenyl ether (PBDE)-209, an important compound in, e.g., dust and sediment, is usually analysed with a shorter column and Electron Capture Negative Ionisation (ECNI). Despite these caveats, general purpose non-polar columns, such as 30 m × 0.25 mm columns coated with 0.25 µm of 100% methylpolysiloxane or 5%-phenyl-methylpolysiloxane (or equivalent arylene phases), are well-suited for NTS studies. These are stable and inert columns with low bleed and high maximum operating temperature. In addition, most retention index (RI) data, such as Kovats' [168] or van den Dool and Kratz [169] alkane RI, for isothermal and temperature programmed GC, respectively, or Lee's PAH RI [170], are generated using such columns. An overview of columns is given in Table 5. Splitless, programmable temperature vaporisation (PTV), and other non-split injectors are recommended, possibly using large solvent injection techniques to alleviate some problems associated with sample concentration to low volume. For NTS applications, helium is preferred over hydrogen as carrier gas due to the reactive nature of hydrogen. In addition, a linear temperature gradient is recommended to fully benefit from RI database information.

Additional complementary techniques are worth considering for NTS studies. Comprehensive two-dimensional gas chromatography (GC × GC) allows dispersion of the sample constituents in two orthogonal dimensions, resulting in exceptional separation power and peak capacity [171, 172]. This enhances the separation of contaminants and matrix constituents, which is particularly valuable when analysing raw or sparsely purified sample extracts. GC × GC also provides useful information on contaminant properties, such as volatility and polarity, which may be used to confirm or reject tentative candidate structures. However, evaluation of the two-dimensional data is more complex and not yet included in typical NTS workflows.

Choice of ionisation

Electron ionisation (EI) is the most commonly used ionisation technique for GC–MS. It generates reproducible fragmentation patterns at standard conditions, which allows the efficient use of commercial or self-created EI mass spectra libraries. There are large libraries with low resolution EI spectra that can be automatically queried using any vendor or third-party GC–MS software. NIST 2020 alone contains, for example, spectral information on more than 300,000 compounds and RI information on almost 140,000 compounds [6]. While the "all in one" EI-based fragmentation of the molecule is informative and acts as a fingerprint, it can only be used in workflows if the compound (spectrum) is present in a spectral database, such as NIST. Unknown identification based on low resolution EI–MS spectra is not yet suitable for routine use in NTS workflows [173]. Unfortunately, high-resolution EI databases are currently limited to a few thousand compounds and, therefore, are likewise currently insufficient for NTS. Moreover, the intensity of the molecular ion measured in EI mode (70 eV) is relatively low, if present at all. About 40% of the compounds in the NIST 08 library have a molecular ion abundance below 5% [7]. Without the molecular ion, it is challenging to perform a chemical database search to look for potential molecules that match the exact mass of the unknown feature (see Sect. "Glossary and definitions").

Various vacuum soft ionisation techniques (see Fig. 4), such as chemical ionisation (CI), field ionisation (FI), photoionisation (PI), low energy EI, and cold-EI [174, 175], as well as atmospheric pressure ionisation techniques [APCI, APPI and atmospheric pressure laser ionisation (APLI)] [176] may provide valuable information on the ionised molecule for compounds that do not display a clear molecular ion in EI. Combining CI and EI (70 eV) [177] or low and high energy EI [178], respectively, can support structure elucidation of small molecules. Finally, ECNI may be used to selectively detect halogenated compounds, using halogen

Table 5 GC column types

Column type	Examples	Domain	Advantages	Disadvantages
Non-polar column	HP-5, DB-5, and Rxi-5	Non-polar	High column efficiency and reproducibility Wide temperature range	Limited selectivity
Polar column	DB-WAX, HP-FFAP, and Rtx-1701	Polar and semi-polar	Good reproducibility	Sensitive to column bleed Lower maximum temperature

ion (Cl or Br) detection [179]. In this case, ECNI and EI data are often used in combination for characterisation and identification.

In recent years, APCI has been demonstrated to efficiently ionise EI amenable compounds with less in-source fragmentation, which translates to improved sensitivity [180–182]. Several HRMS instruments enable interfaces with either LC or GC using APCI as the ionisation source, which only requires changing the front end [183]. In contrast to hard ionisation techniques, such as EI, APCI prevents the complete fragmentation of the compounds and allows for detection of different ion species of the intact molecule, such as $[M+H]^+$ or $[M]^+$ and $[M–H]^-$ or $[M]^-$ for positive and negative ionisation, respectively (see Sect. "Atmospheric Pressure Chemical Ionisation and Photoionisation" for more details). The ionised substances can then be fragmented using DDA or DIA methods (see Sect. "Precursor selection: Data dependent versus data independent acquisition"). By having access to the intact molecular ion information, workflows that have been demonstrated to work well for LC–HRMS, where database matching usually begins with a search for molecules that match the exact mass, can also be employed for GC–APCI–HRMS/MS (Sects. "Compound identification/confirmation" and "Quantification and semi-quantification of suspects and unknowns"). In addition, accurate mass and isotopologue information improve the molecular formula prediction and thus also structure elucidation. Indeed, wide-scope target screening of several hundred non-polar compounds by GC–APCI–HRMS/MS has already been successfully applied [184, 185] and a suspect list was compiled *(NORMAN S65)*, whereas NTS workflows are currently being established. The similarity of the obtained mass spectral data structure in LC–ESI–HRMS/MS and GC–APCI–HRMS/MS permits an application of existing software tools developed for LC–HRMS/MS to GC–APCI–HRMS/MS data, either "as it is" or with only minor modifications [184]. Although not adequately assessed yet in the field of environmental chemistry, the combination of GC–HRMS/MS data acquired under soft and hard ionisation methods are complementary and will increase the confidence in identification of suspects and unknowns. Further increase of the identification can be achieved using calibrant mixture for the derivation of RI (see Sect. "GC–(HR)MS analysis").

Various demonstrations of the capability of GC–APCI–HRMS/MS include the determination of halogenated flame retardants [186, 187], unknown PFAS [188] and dioxins [189], including technologies, such as ion mobility (discussed further in the next section).

Ion mobility separation

The hyphenation of ion mobility spectrometry (IMS) with HRMS has arisen as a technique for enhanced targeted and non-targeted screening of small molecules in complex samples [190, 191]. In the screening workflows, IMS adds an extra dimension to the chromatographic separation of compounds and their mass to charge ratio. Although data sets inherently become more complex and more comprehensive, IMS provides several benefits, including an increasing peak capacity and selectivity that can further improve the identification process and the confidence level on the reported results [200]. IMS makes use of the drift time of an ion (*i.e.,* the time an ion needs to travel through the mobility cell), which depends on the size, shape and charge of the ion, as well as the drift gas used (normally N_2 or He) and the temperature as well as pressure. Ion mobility techniques can be broken down into dispersive and selective techniques [192]. Drift tube IMS (DTIMS), travelling wave IMS (TWIMS) and trapped IMS (TIMS) are three different types of dispersive forms and pass through all ions and their mobilities for later analysis. Field asymmetric IMS (FAIMS) and differential IMS (DIMS or DMS) belong to selective techniques, which filter selected ions by mobility. Thus, the former is more appropriate for NTS and coupling with HRMS. Ion separation occurs on the millisecond timescale, making it compatible with fast TOF MS acquisitions [191, 193]. Hence, several IMS–QTOF–MS systems have been developed and released, i.e., TWIMS MS from Waters, DTIMS MS from Agilent, and TIMS–TOF from Bruker [194]. Although coupling with the slower Orbitrap MS is challenging and currently commercially unavailable, it may be expected in the future [195].

When performing NTS using conventional QTOF–MS instruments, DIA is increasingly used to obtain simultaneous information on intact molecules as well as their fragment ions in a fully non-targeted way [190, 196]. However, multiple co-eluting and interfering ions may contribute to the resulting fragment spectra, especially in complex samples, which can make interpretation of spectra very difficult. In these DIA approaches, IMS enables background filtering of interfering signals. The alignment of precursor and fragment ions by the IMS drift time results in the reduction of co-eluting spectral peaks and much cleaner, higher quality mass spectra, which strongly facilitates the detection and identification process of known and unknown chemicals [191]. This can be especially helpful in complex matrices [197]. In theory, drift time obtained under conventional IMS conditions has the potential to separate isobaric or isomeric compounds that cannot be resolved by chromatography. However, especially for small molecules with only minor structural differences,

the resolution of most current IMS instruments is not yet sufficient to make this distinction. Nonetheless, current technological development will provide a higher IMS resolution on the next generation of instruments, for instance through the implementation of a cyclic IMS cell, i.e., with a longer travelling path. This is, however, accompanied by lower ion transmission and longer IMS acquisitions times, affecting the overall cycle time of the screening.

A further advantage of dispersive IMS is that drift times can be converted into collision cross-sectional (CCS) values, whereas selective techniques cannot provide CCS values due to the application of an asymmetric waveform [194]. Unlike drift time, CCS is an instrument independent value, provided that the same drift gas and ion mobility calibration standards are used [191, 198, 199]. Moreover, CCS is independent of the chromatographic conditions applied and is not affected by the sample matrix. In addition to RT, isotope pattern, precursor and fragment ion *m/z*, CCS values have shown potential as a further criterion for reliable identification of compounds [200]. Furthermore, it has shown clear improvements in targeted screening of complex feed samples by reducing the number of false positives during automated analyte detection while keeping a high detection rate [190]. The creation of empirical CCS databases is, therefore, of interest for an enhanced screening strategy. In general, empirical CCS has been shown to be robust (± 2% tolerance) across multiple platforms [201], and the role of external calibration strategies has been critically evaluated [202]. At this stage, the occasional high deviations between expected and experimental CCS values indicates that these databases must be used with care and that the instrument type should be specified and considered when using them [194, 198, 203]. Although CCS databases are being created, their utility is limited by the number of experimental CCS values available—one of the largest merged collections on PubChem currently hosts CCS values for multiple adducts of 5699 compounds from a range of open CCS databases [204]. As a result, the prediction of CCS using machine learning tools has become an area of interest [205–207]. In silico prediction of CCS facilitates the screening of many compounds for which no empirical values are available and improves the confidence of tentative identifications [197, 205, 207]. Furthermore, it helps to refine non-target data processing by reducing the potential positive matches to be further investigated, i.e., assisting with prioritisation of features of interest [196].

IMS coupled to HRMS instruments is not yet widely implemented into environmental research laboratories and the number of publications applying IMS to the analysis of complex environmental samples is still limited [200, 208, 209]. This is possibly related to their relatively recent emergence, the associated costs, and the limited options to handle the data obtained. The 4D data generated (RT, drift time, accurate *m/z* and peak intensity) is barely supported by open formats such as mzML and thus difficult to integrate into and process with open algorithms/software tools (e.g., MZmine 3.5, MS-DIAL and Skyline). Hence, research laboratories have few options other than using the software provided by the manufacturer for data processing and interpretation, i.e., UNIFI® (Waters), MassHunter® (Agilent) or Data Analysis® (Bruker). Nevertheless, the potential shown by IMS–QTOF–HRMS for target and NTS strategies opens new perspectives and possibilities in environmental analysis. The development of open tools and exchange file formats which can handle IMS data as well as the incorporation of CCS predictive tools into screening workflows would be highly beneficial and interesting, since it will stimulate and increase the number of NTS applications.

Data (pre-)processing and prioritisation for NTS

Data pre-processing

The increasing use of NTS fostered the development of a growing number of workflows for data processing. These range from general workflows and solutions embedded in commercial (vendor) software (e.g., Compound Discoverer®, MetaboScape®, MassHunter®, UNIFI®) or open access software (e.g., patRoon [210, 211], MS–DIAL [212, 213], enviMass [214], MZmine [215, 216], SLAW [217]), or InSpectra [218] that can be used for any kind of application, to more specific tools for instance for the analysis of particular classes of contaminants (e.g., FluoroMatch [219]). A generic NTS workflow consists of five main steps, namely, (i) sampling, (ii) data acquisition, (iii) preprocessing, (iv) prioritisation and (v) identification, see Fig. 1 within [3].

Both non-target and suspect screening share several steps during pre-processing, which includes many of the data treatment steps prior to the identification workflow. These steps may include data conversion, centroiding, compression, feature detection, and componentisation. While not all pre-processing workflows include all these steps, most workflows include feature detection, componentisation and alignment over samples. Some NTS workflows use extracted ion chromatograms (XICs) instead of feature detection, which can be especially useful to find suspects that are difficult to detect as "peaks" or features (e.g., surfactants or other homologues). Features here are defined as three-dimensional entities (*i.e.,* RT, *m/z* values, and intensities), whereas components are collections of all signals related to a unique chemical constituent (e.g., isotopes, adducts, and fragments, including those derived

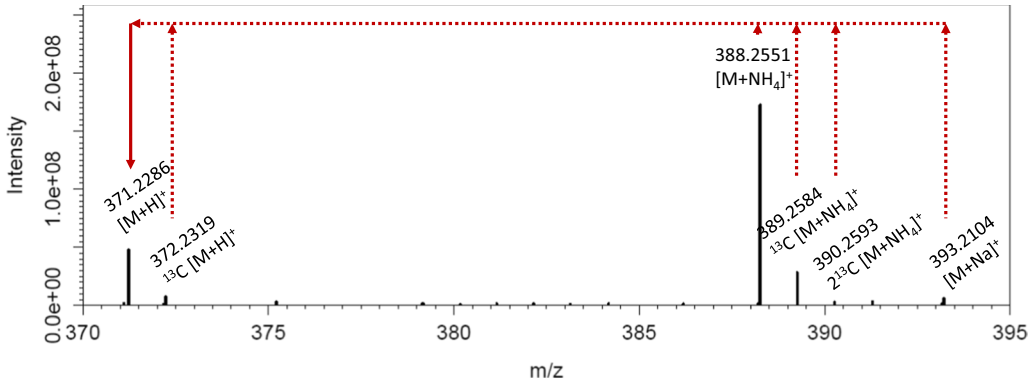

Fig. 7 Componentisation involves grouping-related adducts, isotopes (shown) and fragments (not shown) together into a group for further processing. Where available, the $[M+H]^+$ (as shown here) or $[M-H]^-$ ion is the preferred reference m/z, as these often generate the richest fragmentation patterns and are easiest for identification purposes

in-source, see Fig. 7). Typically, aligned feature lists are used during prioritisation, while the information contained within components is essential for identification.

One of the first steps in pre-processing involves recalibration of the mass spectra (if necessary, dependent on the vendor) and centroiding. When using open workflows for further processing, this step can be performed along with conversion of the vendor-specific data files into open-source formats, such as mzML or mzXML. While some vendors offer this conversion in their software, generic solutions such as MSConvert from ProteoWizard [154] offer this conversion and preprocessing options for all major vendors, using either vendor-specific or general algorithms. Almost all vendors contribute libraries for the conversion of their raw formats. Thus, using vendor-specific algorithms is generally preferable, where possible, to get the best conversion results, although the quality of results can vary depending on the vendor. Among the various steps which take place during pre-processing, centroiding is among the most relevant ones in NTS workflows, given that most feature detection algorithms use centroided data. During centroiding, signals associated with one ion are grouped together to a unique *m/z* value. This may be done during acquisition or as a part of pre-processing steps (*i.e.,* data are initially acquired in profile or continuous mode and is later converted to centroid), as discussed in Sect. "Choice of mass spectrometry settings". There are several centroiding algorithms available, ranging from simple hard-set binning to centwave algorithms applied in the mass domain [215, 220]. In addition, a recently developed algorithm takes a self-adjusting route to better fit the signal in the mass domain [221].

The centroided data are then used for feature detection in the next step of pre-processing, where signals in the time and mass domain are both grouped together to generate features. Most feature detection algorithms are focused on the time domain. One of the steps taken during the feature detection is generation of extracted ion chromatograms (XIC or EIC) with user-defined mass and retention windows. In the next step, either a Gaussian function or one of its variants is fit to the XIC to model the peak. This ultimately will result in a feature list that can be used for prioritisation and/or componentisation. There are also solutions performing feature detection on profile data or employing image analysis for the feature detection [222, 223]; the latter is particularly useful for 2-D chromatographic data, such as GCxGC or LCxLC. Other approaches based on so-called "regions of interest" (ROI) and multivariate curve resolution (MCR) have been introduced as alternative (pre-)processing strategies which operate in the mass domain and do not require alignment along the time domain [224]. However, the matrix decomposition-based methods (e.g., MCR) require multiple samples, where the features are detected at different concentration levels.

Depending on the workflow, the generated feature lists are aligned to create a master feature list across multiple samples. Currently, available tools use a combination of mass and retention windows/tolerances to align the features associated with the same chemical constituent detected in different samples. To work properly, the samples must have been analysed using the same experimental conditions, since the feature alignment tools currently available are unable to adequately align feature lists generated from data acquired using different experimental setups. Some workflows, such as recent patRoon versions [210], also merge features from positive and negative modes (when measured under the same conditions). Typically, internal standards (IS) present across all samples (see Sect. "Internal standards") are used to assess

the quality of the alignment as well as the windows/tolerances used. Alignment can also take place directly at the XIC level prior to feature detection, using IS or anchor points to calculate the shift. These approaches, even though powerful, may cause an introduction of artefacts into the signal (e.g., the use of isotopically labelled IS can interfere with componentisation if they co-elute with their non-labelled equivalent). The aligned feature lists can then be used for blank and/or noise removal, which removes features if they are present in blanks or below a certain intensity threshold. QA/QC checks can also be performed at this stage (see Sect. 'Quality assurance and quality control in NTS methods" for more details). In addition to the feature alignment algorithms, there are recursive feature detection strategies, where the master feature list over multiple samples is used for filling the gaps potentially caused by the data processing workflow. The gap filling also can take place after feature alignment. Currently, gap filling, even though very important, is not usually included as a part of the pre-processing workflows. Feature lists are eventually evaluated using various types of statistical approaches (e.g., trend and/or principal component analysis, see Sect. "Prioritisation procedures in NTS") to prioritise features of interest.

The final step of data pre-processing consists of componentisation to generate the signals associated with unique chemicals. These grouped signals at the MS1 level include isotopes, adducts, and in-source fragments (see Fig. 7), while at the MS2 level, they include collision induced dissociation fragments. For adduct detection, almost all available algorithms work with a list of most probable adducts formed for each polarity. These algorithms use the combination of these well-known adducts and user-defined mass and RT tolerances to identify the potential adducts of a precursor ion. A similar approach is used for the isolation of isotope signals, where, e.g., an *m/z* value jump of 1.0033 is used to distinguish the signal of a ^{13}C isotope from its corresponding ^{12}C peak (see Fig. 7). While this approach is used in CAMERA [225], this was originally designed on TOF data and can introduce interferences with higher resolution Orbitrap data—the nontarget algorithm [226] and enviPat approach [227, 228] can be used to assess isotope peak occurrence with varying resolutions. Another approach is based on the combination of elemental mass defect and Bayesian statistics to detect the isotope signal during the pre-processing workflow [229]. It can still be challenging to process non-standard elements and adducts (e.g., metal ions present in organometallic compounds that can be seen with LC), depending on the workflows, such that an XIC-based suspect screening is a useful alternative to deal with these special cases (see Sect. "Candidate structure search and selection"). For in-source fragment ions, a combination of RT matching and peak shape similarity assessment can be used to group the fragments with potential precursor ions, as applied, for example, in RAMClust [230]. In the case of DIA, the same strategy is used at the MS2 level to generate pseudo MS2 spectra of each potential precursor ion in the MS1 level. Additional spectral clean-up may be necessary before spectral library searching, including deconvolution (common in GC–EI–MS approaches), IMS-supported strategies (see Sect. "Ion mobility separation") or even via assignment of likely fragments based on mass defect/elemental composition using the molecular formula approach GenForm, previously MOLGEN–MSMS [231, 232], as also applied in RMassBank [233, 234]. The advantage of multi-sample XIC correlation analysis was demonstrated for spectral clean-up of DIA experiments. Screening for homologous series [235] can help group components that are related by consistent mass and RT differences (e.g., CH_2, C_2H_4O, CF_2), particularly useful for detecting related surfactants [143, 236] and PFAS series [238]. Finally, these generated components are used as inputs during the identification steps (see Sect. "Compound identification / confirmation").

Prioritisation procedures in NTS

After pre-processing, features are aligned and grouped across replicate injections and/or samples within an analytical sequence, yielding a so-called feature list or feature table for further investigation. Because environmental samples are complex chemical mixtures containing thousands of individual substances of both natural and anthropogenic origin, these feature lists can be huge. The complete elucidation of all the peaks/features present in the data is not (yet) a feasible task. Non-target approaches are laborious, time-consuming and computationally challenging, especially when the prior knowledge of the chemical compounds likely to be present is limited. Thus, prioritisation strategies are a necessary and key step in any investigation involving NTS, to focus the identification efforts on relevant signals. The optimal prioritisation strategy should be defined after a careful evaluation of the specific goals of the study (see also Table 1). Ideally, all prior steps starting from the sampling should be designed to achieve the study goal(s); however, in reality, compromises are required due to financial or time constraints. For retrospective screening, the data may have been already acquired within other monitoring campaigns, such that the existing data must be used as best possible to answer further questions. Table 6 compiles a variety of prioritisation approaches for different environmental monitoring cases and provides examples from literature. Three main principles are used: chemical signatures (exact mass of, e.g., suspected compounds, mass defect, specific isotopes, fragments, etc.), statistical methods (principal component analysis (PCA),

Table 6 NTS prioritisation approaches based on question to address and appropriate methods using chemical signature, biological activity and statistical tools

Screening for	Prioritisation approach	Methods	References
Contamination with regulated, highly used or emerging substances (suspects)	Screening with appropriate suspects lists (e.g., all registered pesticides; industrial compounds with high use; predicted TPs)	Selection or compilation of an appropriate suspect list (see Sect. "Candidate structure search and selection") and screening of exact mass in samples and blank samples	196, 240, 242, 243
Contamination spots (often local)	High intensity in few samples from specific sites but not in samples from reference sites	Statistical analysis, e.g., density estimation, probability distribution, pairwise comparisons	244
Widespread contamination	High frequency of occurrence in samples from different sites	Statistical analysis, e.g., density estimation, probability distribution	236
Contamination with compounds that produce high seasonal or intraday fluctuation	Comparison of samples over time (e.g., industrial wastewater during different production processes)	Time series analysis using, e.g., (non)linear regression, (non)-parametric, uni-/multivariate statistical approaches	245–247
Similarly or differently contaminated sites/ contamination profiles	Comparison of the MS pattern of samples from different sites	Clustering (e.g., hierarchical), unsupervised PCA or supervised PLS, selection of masses in the loading plots	26, 35
Contamination with homologues of a substance class (e.g., PFAS, surfactants)	Characteristic mass (e.g., CF_2) and RT difference	Homologue series search, Kendrick mass defect and other mass defect plots	143, 235, 238, 237, 248–250
Substance classes with specific functional groups (aldehydes, conjugates)	Neutral loss, specific fragments in MS2 spectra from compound or derivative (e.g., $-SO_3$)	Fragment search in MS2 spectra	251–253
Anthropogenic compounds with specific isotope pattern	Isotope pattern (especially relevant for Cl = Br > S > Si)	Isotope search in MS1	38, 254
Compounds causing effect	Effect-directed selection of masses in samples or fractions of samples using effect data or bioassays; Fractionation is often necessary to reduce candidate masses and chemical complexity	Comparison of masses in samples/fractions causing effect with those causing no effect using direct comparison or statistical methods	255, 256
Compounds potentially causing effects	Annotation of signals with in vivo or in vitro hazard characteristics	In silico prediction of toxicity based on chemical fingerprints produced from MS2 spectra by SIRIUS	257, 258
Persistent compounds	Comparison of samples before and after processes (e.g., water treatment) or along time scales (e.g., river stretch)	Statistical methods, such as clustering, PCA, PLS, fold change	259
Bioaccumulative compounds	Comparison of the MS pattern of samples along the trophic chain	Clustering (e.g., hierarchical), trend analysis	65
Formed TPs	Comparison of samples before and after processes (e.g., water treatment) or along time scales (e.g., along river stretch) for specific mass differences due to transformation reaction	Search for specific mass differences in mass lists of before and after samples or statistically (PCA, clustering, time series) separated groups (e.g., 15.9949 for addition of O)	241, 260–262
Formed disinfection by-products (DBPs)	Comparison of samples before and after water treatment, then isotopic pattern analysis (Cl, Br)	Isotope search in MS1 and MS2 for formed signals with specific isotope pattern	263
Isotope-supported detection of TPs, by-products	Lab experiments with isotopically labelled reagents, screening for isotope mass differences (e.g., reaction with $^{15}NO_3^-$, screening for $\Delta m = 0.9970$)	Search for specific mass differences in mass lists of lab experiments with mixtures of isotopes (e.g., $^{14}NO_3^-$ and $^{15}NO_3^-$) or of two sample series with single isotopes	264, 265

clustering) and effect-directed methods (bioassays, in silico toxicity prediction). Appropriate controls such as field and method blanks are needed to allow application of these prioritisation methods (see Sect. "Quality assurance and quality control in NTS methods"). Usually, statistical methods need a certain number of samples and/or replicates to be useful and appropriate tests have to be conducted to confirm the robustness of the statistical model [239]. In contrast, the other two prioritisation approaches can be applied even to single samples plus controls. Spiked samples or IS can be used to optimise the parameter settings for the selected method and verify the suitability of the method, i.e., that all standards are found [240, 241].

Statistical methods cover a variety of possible methodologies, from univariate to multivariate analyses, from unsupervised to supervised methods, and inclusion of additional data for prioritisation [266]. Many of these methods are implemented in various software approaches (see, e.g., Sect. "Non vendor software and algorithms for processing HRMS data") and have been used in untargeted metabolomics for a long time, but may need some adjustment for environmental studies. For example, the matrix effects are usually more different between environmental samples (e.g., influent vs. effluent) than between treated and control groups in metabolomics, which makes the group comparison more complex. For two-group comparison (e.g., sample vs. control or two different treatments) fold-change analysis, principal component analysis (PCA, supervised) or partial least squares projection to latent structures (PLS, unsupervised) are used most often. The unsupervised method supports investigating the inherent structure of the data, whereas supervised methods can help to address more specific research questions using prior knowledge to direct the interpretation. For complex data sets clustering methods and regression analysis are often applied in combination with data normalisation and data scaling to remove systematic variance and avoid focus on intense features, respectively. Unsupervised hierarchical clustering together with heat maps can help to prioritise features and visualise the results, e.g., compounds persistent in a treatment chain or formed, such as TPs (Fig. 8). However, determining the number of clusters with characteristic properties can be complex when the data are not well-known already. Top-down clustering methods such as k-means need a pre-selection of the number of clusters and less computational power but more a priori knowledge.

Structure elucidation of particular compounds might not necessarily be the final focus of each prioritisation approach, as in some cases general trends in the data may be sufficient to determine further steps. Statistical methods are often applied for such prioritisation of groups. For example, in the field of treatment (e.g., water treatment or soil remediation), approaches to assess changes in the number and characteristics of features (e.g., intensity, hydrophobicity based on retention) during treatments can be informative without knowledge of the structures. Relevant information can be gained already from assignment of probable elemental composition without full structural elucidation. This allows studying

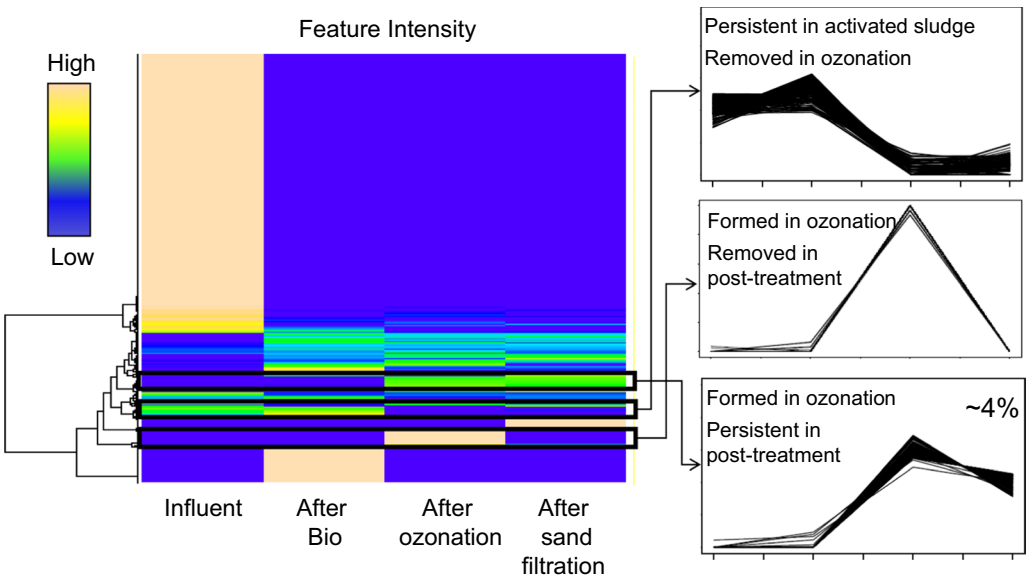

Fig. 8 Example for hierarchical clustering to prioritise features persistent or formed in wastewater treatment [259]

changes (e.g., in the overall oxidation state, the number of chlorinated or sulfonated formed TPs or in the mass profile) during treatment by measuring shifts in the molecular fingerprints and other physico-chemical changes [259, 267, 268].

Developing compound lists for suspect screening

Suspect screening has become a popular way for researchers to look for potential substances of interest in the otherwise overly complex NTS data [269]. In many ways, this could be viewed as a form of prioritisation (Table 6), as it involves selecting a certain group of chemicals to investigate, for reasons generally relevant to the study question. Suspect screening can either be performed directly on non-targeted data (e.g., via XICs based on the exact mass of the adduct species of the selected suspect(s) of interest), or following peak picking, prioritisation and other non-target data processing steps (see Sect. "Data pre-processing"). The strategy may, in the end, depend on the size of the suspect list in question. Early on, suspect screening was conceptualised to enable investigation of a relatively small list (tens to hundreds) of substances of interest, for instance pesticides [270] or pharmaceuticals [271]. Over time, larger suspect screening lists (e.g., compiled from REACH substances [272, 273] were used. In the meantime, suspect lists have become popular, although of widely varying quality, and a few platforms exist to exchange this information, including the NORMAN Suspect List Exchange (NORMAN-SLE) via https://www.norman-network.com/nds/SLE/ [269] and the CompTox Chemicals Dashboard [274] via https://comptox.epa.gov/dashboard/chemical_lists. PubChem [275] (https://pubchem.ncbi.nlm.nih.gov/) also offers functionality that can be leveraged to create suspect lists (https://pubchem.ncbi.nlm.nih.gov/classification/#hid=101). Interest has also increased in creating merged lists. For instance, the NORMAN–SLE was merged on popular request to form the combined NORMAN SusDat database [276], STOFF–IDENT combines several individual data sets [272], and the HBM4EU project compiled their own suspect list of chemicals of emerging concern for screening in human biological samples (CECscreen), which also includes simulated phase I metabolites [277, 278]. Irrespective of whether "screen big" or "screen smart" is performed, further confirmation efforts are necessary, and essentially must be in line with those required for non-target identification efforts (see Fig. 1). Screening with suspect lists of tens of thousands of suspects is effectively equivalent to non-target screening that uses a small database for assignment of candidate compounds, as several matches could be possible for each mass [269].

Many suspect lists are now supported by additional data to enhance the ability to prioritise, select or interpret potential suspect matches. Typical values such as calculated properties are either saved in the lists or can be retrieved in a number of different ways, either from large databases, such as PubChem, or via interfaces such as EPISuite [279] or ChemAxon [280, 281]. Increasingly, calculated toxicity values have also been included in, e.g., the CompTox Chemicals Dashboard and NORMAN SusDat. Exposure data are also useful and included in suspect lists in a variety of ways, from predicted exposure in the CompTox Chemicals Dashboard [274], to REACH tonnage estimates in several NORMAN–SLE contributions [269] through to exposure indices provided by the Swedish Chemicals Agency, KEMI [282, 283]. Incorporating additional data into suspect lists comes with a new set of challenges, including how to deal with various salt and mixture forms of the chemicals of interest. Exposure and hazard data may be available for products (e.g., salt or mixtures), whereas the detected form in the HRMS experiments will be an individual species without counterions or other mixture components. Some mixture components may also be visible with the chosen method (as would be the case for homologous series of surfactants), whereas metallic or small organic counterions will usually not be visible. In some cases, toxicity may even be driven by the metal counterion. Both suspect screening and toxicity prediction generally need neutral forms for screening and predictions, respectively, and neither deal systematically with organometallic compounds yet. Since MS cannot generally yield information on stereochemistry, stereoisomerism is generally disregarded, or stereoisomers are collapsed into groups by the InChIKey first block, which is the structural skeleton of a molecule, as done in MetFrag and other approaches [284]. There are several approaches to deal with salts and mixtures. For example, CompTox developed the so-called "MS-Ready" approach, linking salts and mixtures to the "MS-Ready" neutral component, which was integrated into MetFrag and allowed retrieval of additional data related to salts and mixtures [285]. PubChem has implemented a so-called "parent" compound, which can be used to find the major component in salts and mixtures, for example, via the PubChem Identifier Exchange service (ID Exchange) or programmatically [286, 287]. In the PubChemLite approach, all forms related to a given "parent compound" with annotation content of interest were totalled and collapsed into one entry (maintaining the connections to the individual entries) to form a very efficient database for screening [288]. While the handling of salts has improved recently, mixtures and organometallics remain a challenge for workflows and are areas requiring cheminformatics developments in the coming years [289, 290].

Several efforts are now investigating using predicted TPs to support suspect screening [210, 277]. Open pathway prediction software available for this includes enviPath for microbial transformation [291], or BioTransformer [292] for mammalian (specifically human) and microbial transformation. Metabolic logic can also be used to calculate the mass of potential TPs [210, 241]. In general, there is a high probability that biotransformation predictions exhibit combinatorial explosion (*i.e.,* many predicted TPs for one parent compound), increasing the number of suspects three- to more than tenfold.

Although suspect lists themselves are relatively straightforward, many users still struggle with inconsistent formats and the cheminformatics aspects of suspect lists. This is compounded by the variety of vendor and open approaches all requiring different sets of information; some vendor approaches do not require sufficient information to perform full identification following suspect screening (e.g., requiring only a mass or formula, not structure). Ideally, software supporting suspect screening should be able to accept a flexible format based on column headers, as implemented for instance in MetFrag [284], reducing user burden. A good suspect list requires high-quality information, with a clear provenance, where the data were obtained. At least one, preferably more, structural identifiers should be provided to accompany the name and additional database identifiers. The primary identifier should also be clear, such that inconsistencies can be fixed in accordance with the hierarchy of suspect list preparation. Ideally, a suspect list should contain the chemical name and one or more database identifiers, along with at least two pieces of structural information. Identifiers include the PubChem compound identifier (PubChem CID), the Distributed Structure-Searchable Toxicity (DSSTox) substance identifier (DTXSID) used in CompTox and Chemical Abstract Service (CAS) registry numbers commonly used in regulatory lists. Structural information should include at least two of the Simplified Molecular-Input Line-Entry System (SMILES), the International Chemical Identifier (InChI) or it is hashed form InChIKey (note the InChIKey can be calculated from SMILES or InChI, but not vice versa). Molecular formula and exact mass fields are useful, but can also be calculated from SMILES or InChIs. Synonyms and additional data are also common in many suspect lists. While CAS numbers are problematic due to the closed nature of the Chemical Abstracts Service, and multiple CAS numbers per compound, the reality is that these identifiers are still widely used in research and regulatory settings. Further information about the identifiers mentioned in this section and creating Findable, Accessible, Interoperable and Reusable (FAIR) suspect lists is detailed extensively in dedicated articles [293, 294].

With more and more data becoming available in online resources, it is tempting to fill suspect lists with an incredible amount of data. This comes with the danger that the information is quickly outdated, and it is often overwhelming for users, since each software requires different pieces of information. It is difficult to find the right balance between not enough, enough and too much information. Many large databases provide mapping services to obtain extra data in real-time, including the CompTox Batch Search [295] and the PubChem ID Exchange [286]. Ideally, the suspect list should contain as much specifically relevant information as possible, without overwhelming users with too many database-obtained synonyms and secondary identifiers. Similarly, many users find exact masses from all possible adduct combinations very useful in suspect lists. While this offers quick and efficient look-up opportunities, the number of possible adducts and the difficulty in predicting which adducts are likely to be detected (see Sect. "Compound identification/confirmation") can lead to an overwhelming presentation of thousands of possible masses in suspect lists, which greatly increases the risk of false positives. Simple web interfaces could be provided to support users in these cheminformatics challenges; however, development and maintenance are generally poorly supported by funding agencies, and rely on individual research groups—or on the willingness of large databases such as CompTox and PubChem to work with their users to provide the necessary functions.

Compound identification/confirmation

Confidence of identification

Currently, the environmental community uses a 5-level classification scheme proposed in 2014 widely to communicate the confidence in identification using HRMS (both GC- and LC-) in NTS [296]. Level 1, confirmed structure and the gold standard, can only be achieved with a reference standard and this is currently a prerequisite to transfer the newly identified compound to target monitoring and other regulatory processes. Level 2, probable structure, can either be achieved via high spectral match scores with a spectral library (Level 2a, see Sect. "Spectral library search") or via diagnostic evidence (Level 2b, discussed further below). Level 3, tentative candidates, is the level at which many NTS identifications remain. Level 4, unequivocal molecular formula, is possible with sufficient evidence, whereas Level 5, an exact mass of interest, is, e.g., the result of prioritisation efforts (discussed in Sect. "Prioritisation procedures in NTS") before any identification efforts have been made, or if insufficient evidence is available for higher confidence. Table 7 lists the original "Levels" text [296] with some additional context and observations made over a decade of use of the levels

Table 7 Communicating confidence in NTS via HRMS; text from original article with additional comments

Identification confidence	Minimum requirements	Comments/observations
Level 1: Confirmed structure by reference standard	MS, MS2, RT, Reference standard	For low intensity targets (no MS2), MS, RT and standard are sufficient, with lower IP
Level 2a: Probable structure by library spectrum match	MS, MS2, Library or literature MS2	High match values and sufficient fragments required, see Sect. "Spectral library search")
Level 2b: Probable structure by diagnostic evidence	MS, MS2, experimental data/context	Very rare case, often with clear parent–TP relationship
Level 3: Tentative candidate(s)	MS, MS2, experimental data/context	Most identifications remain at this level. Defining study-specific sublevels can be helpful in reports
Level 4: Unequivocal molecular formula	MS isotope/adduct (MS2 is helpful)	Many vendor approaches report formulas that are not unequivocal. See main text
Level 5: Exact mass of interest	MS	"Of interest" implies that this mass has been prioritised, see Sect. "Prioritisation procedures in NTS"

IP = identification points, RT = retention time, TP = transformation product. Further comments are made in the main text

in the community, which is discussed in more detail in the following paragraphs.

Level 5 is the common starting point for further identification efforts, where prioritisation has led to high interest in the exact mass due to criteria, such as high intensity, high frequency of occurrence etc. (see Sect. "Prioritisation procedures in NTS"). Suspect screening, on the other hand, can be considered to already start at a Level 3 (tentative candidate) if a matching mass of interest is detected. Proceeding to higher confidence does not necessarily require progression through all levels—for instance, it is not a prerequisite to define an unequivocal molecular formula (Level 4) to achieve a Level 2a or 3 match. In the first NORMAN Collaborative Trial on NTS, participants proposed a figure merging the levels with the various approaches of target and NTS, which still applies today [10].

Achieving an unequivocal molecular formula (Level 4) is often quite challenging, especially for large molecular masses (> 500 Da) and in the absence of a characteristic isotope pattern. Elements in organic molecules with significant isotope abundance are Br (^{81}Br 97%), Cl (^{37}Cl 32%), Si (^{29}Si 5.1, ^{30}Si 3.3%), C (^{13}C 1.1%), S (^{33}S 0.8%, ^{34}S 4.21%), N (^{15}N 0.4%) and O (^{18}O 0.2%), while Na, P, F and I (among others) are monoisotopic. The halogens Br and Cl, with high abundance M + 2 provide the most characteristic pattern. However, the presence of multiple Cl and/or Br in the formula can shift the highest intensity isotope peak away from the lowest mass peak in the isotope pattern, which can be a problem for some workflows that assume decreasing intensity of isotope peaks. High mass accuracy and high resolution facilitate the molecular formula assignment. Only a resolution above 20,000 to 100,000 (depending on the elements) allows users to distinguish isobars (same nominal but different exact mass) and to see the isotope fine structure (e.g., ^{15}N and ^{34}S, can only be distinguished from ^{13}C peaks with a resolution > 100,000, see Fig. 5). The enviPat approach can be used to visualise isotopic patterns for given formulas and adducts under varying resolution settings [227, 228]. Seven heuristic ("Golden") rules including restrictions for the number of elements and element ratios of hydrogen/carbon and heteroatoms can be applied manually or with a freely available algorithm [297], although care must be taken when applying these rules in environmental contexts as some common contaminants of interest fall outside these rules, including highly halogenated species. While restriction of the number of elements based on prior knowledge can be useful to restrict the number of formulas, it comes at a risk. For example, inclusion of fluoride, which is low mass (19) and monoisotopic, increases the number of molecular formulas tremendously, rendering unequivocal formula assignment near impossible. However, since there are many fluorinated compounds in the environment, excluding fluorine means that the correct formula may be missed. The tools GenForm (formerly MOLGEN–MSMS), [231, 232], SIRIUS [298] and ZODIAC [299] also use the MS2 information with increasingly sophisticated algorithms for the formula annotation, with corresponding improvements in performance. While many vendors will offer molecular formula calculations in their software, it is best to treat these with caution unless the settings have been carefully selected; many formulas reported using vendor software in the NORMAN Collaborative Trial did not match the formulas of the candidates proposed by the participants based on their expert knowledge [10].

The majority of top-ranked candidates from prediction software combined with database searches have to be classified as level 3 (tentative identification), because the information available from the relatively sparse MS2 spectra usually does not allow for an unambiguous structure assignment. For example, positional isomers such as o-, m-, p-substituted aromatics cannot usually be

distinguished based on mass spectrometric and chromatographic information without reference standard, while many spectra exhibit generic losses related to hydroxyl or amino groups that could be placed in many locations (and thus apply to many candidates). In terms of environmental outcomes, if toxicity prediction is similar for the isomers and the isomers originate from the same exposure source, unambiguous identification might not always be essential, although this is often required for regulatory interventions. Candidate selection is discussed further in Sect. "Candidate structure search and selection".

The assignment of a level 2a, library spectrum match, is often included in vendor and some open software and is relatively easy to implement in workflows (see Sect. "Spectral library search"), but this typically only enables annotation of a few thousand well-known compounds compiled in libraries. It is also possible to use spectral matches from literature, although this is difficult to automate. Level 2b is less clear, as different experimental evidence can lead to the probable structure. The reality is that a Level 2b, with truly diagnostic evidence that leads to one clear structure and eliminates *all other possible candidates*, is quite a rare case (see note in Table 7) and often only possible when there is a clear parent–TP relationship evident, although many scientists seem tempted to push the evidence to upgrade Level 3 to Level 2b. In a few cases, only one structure is meaningful due to diagnostic fragments in the MS2, for example, the (hydroxy-tert-butyl)-irgarol example included in the original publication, where fragmentation clearly indicated the location of the hydroxy on the tert-butyl group and symmetry led to only one possible structure [296]. In a few cases, the atoms involved, even without clear MS2 evidence, lead only to one probable structure. More often, many MS2 fragments may exist, but selection of the true structure is impossible due to the large number of possible structures with very similar predicted MS2 spectra (e.g., steroidal structures without characteristic functional groups or heteroatoms). Such cases will remain Level 3.

Finally, not every feature can be unambiguously identified with HRMS/MS if no standard is available. If identification is essential due to high concentration or toxic effects, further efforts with orthogonal techniques could be used, or several standards for tentative matches could be purchased (if available). The former can be very time-consuming, the latter can get expensive, and availability of standards is often an issue. Combination of GC and LC using different columns and combined with different ionisation techniques (discussed in Sects. "LC-HRMS analysis" and "GC-(HR)MS analysis") or derivatisation of specific functional groups, performing hydrogen–deuterium exchange (HDX) as discussed below in Sect. "Candidate structure search and selection" can provide additional experimental information. Finally, nuclear magnetic resonance spectroscopy (NMR) can provide clear structural identity but much higher concentrations in the µg/L range are needed for the analysis, along with sufficient purity of the sample.

Since the original article in 2014, which itself was inspired by the four-level Metabolomics Standards Initiative (MSI) system [300], numerous initiatives have explored refinements to the 5 level system described above. Not all will be reviewed here, instead a few developments of most relevance for this guidance document will be mentioned. Retention time, despite its popularity in candidate selection even at the time (see Sects. "Candidate structure search and selection" and "Retention time prediction and indices") was not explicitly included in the original level scheme except for Level 1 (confirmed), but rather left more implicit under the term "experimental data" (see Table 7). Increasing interest in also using CCS for candidate selection inspired a revised set of criteria to support the five-level system, keeping the same 5 levels but including more explicit guidance for considering both RT and CCS information [200]. CCS is discussed further in Sect. "Ion mobility separation"; RT information in Sect. "Retention time prediction and indices". Given the difficulty in predicting unequivocal formulas for PFAS and other fluorine-containing compounds, but a great deal of specialised information available for identification purposes, the PFAS community published their own guidance extending the five-level system to support PFAS identification, including very detailed criteria for each level and several sublevels [301]. The metabolomics community has been discussing revisions to the MSI levels for many years. The latest proposal includes a chemistry-based, instrument-independent seven-level system ranging from A–G, where A and B distinguish stereoisomers (generally not possible with HRMS), meaning that Levels 1–5 described above would roughly translate to levels C–G in that system. These proposed levels are still under discussion.

Recently, a simplified and automated identification point (IP) system scaled from 0 to 1 and compatible with the five level system was proposed for the communication of the evidence for the identification level by NORMAN members [302]. The weighting of the parameters mass accuracy (< 5 ppm, mandatory), isotopic fit (at least one isotope, 0.2), MS2 fragmentation (0.2–0.4 dependent on the number of matching experimental or in silico fragments), and predicted retention time index (0.15) was derived from a machine learning model trained on data generated by four laboratories equipped with different instrumentation. The evidence-based scoring is intended to improve the precision and reproducibility in reporting

while being suitable for automation. In the automated system, DIA spectral acquisition is penalised compared to DDA as the spectra are less specific due to the presence of many precursor ions (see Sect. "Choice of mass spectrometry settings"). NORMAN intends to use this approach for risk assessment in combination with suspect screening using SusDat in the coming years.

Spectral library search

There are several reviews on spectral library searching coverage and quality [303, 304], as well as some European efforts in comparison and criteria for addition to libraries [155, 156]. Since much has happened in recent years, some basics for EI–MS and MS2 libraries are summarised in the sections below.

EI–MS libraries

As mentioned above, spectral libraries for GC–EI–MS are well-established due to the reproducible nature of the spectra across laboratories. The main library in use is the NIST library, which comes with free search software (for both EI–MS and MS2 data) but requires a licence for the spectral library itself. The NIST spectral format (.msp) is widely used in vendor and open software for exchanging spectral data and many vendors provide direct integration of the NIST library in their software (often including the licence for the EI–MS and MS2 libraries). NIST is also working on ways to integrate their libraries in automatic open workflows for those who have a licence. The NIST/EPA/NIH EI–MS Library, 2020 release (NIST20 for short) contains 350,704 EI–MS of 306,643 compounds, including 43,774 replicate spectra [6]. It also includes 447,289 retention index (RI) values from 139,382 compounds, where 114,629 compounds have both RI and EI–MS available [6]. The combined Wiley Registry (12th Edition) and NIST20 contains > 1 million EI–MS of > 860,000 unique compounds [305]. The contents of the NIST MS2 library are covered in the next section.

Searching a measured spectrum with the NIST MS Search generally results in three values to consider: match, reverse match and probability (see Fig. 9). In short, the match value is the match of the measured spectrum to the library spectrum, whereas the reverse match is the match of the library spectra to the measured spectrum, which ignores peaks in the measured spectrum that are not in the library spectrum, which could indicate background noise in the unknown spectrum. For EI–MS, 999 indicates a perfect match (e.g., when querying library spectrum against library, see Fig. 9a), > 900 is an excellent match, 800–900 a good match, 700–800 a fair match, < 600 is a very poor match, while 0 indicates absolutely no peaks in common. Cutoffs for "good" matches can depend on the sample, but generally values > 700 are considered good for complex samples with many interferences, > 800 or higher for samples with fewer interferences. In addition to the match values, the probability gives an estimate of how likely the match is to be the correct answer. Figure 9a shows the query of a library spectrum of atrazine, where the perfect match is shown at the top, with four replicate spectra below, with lower

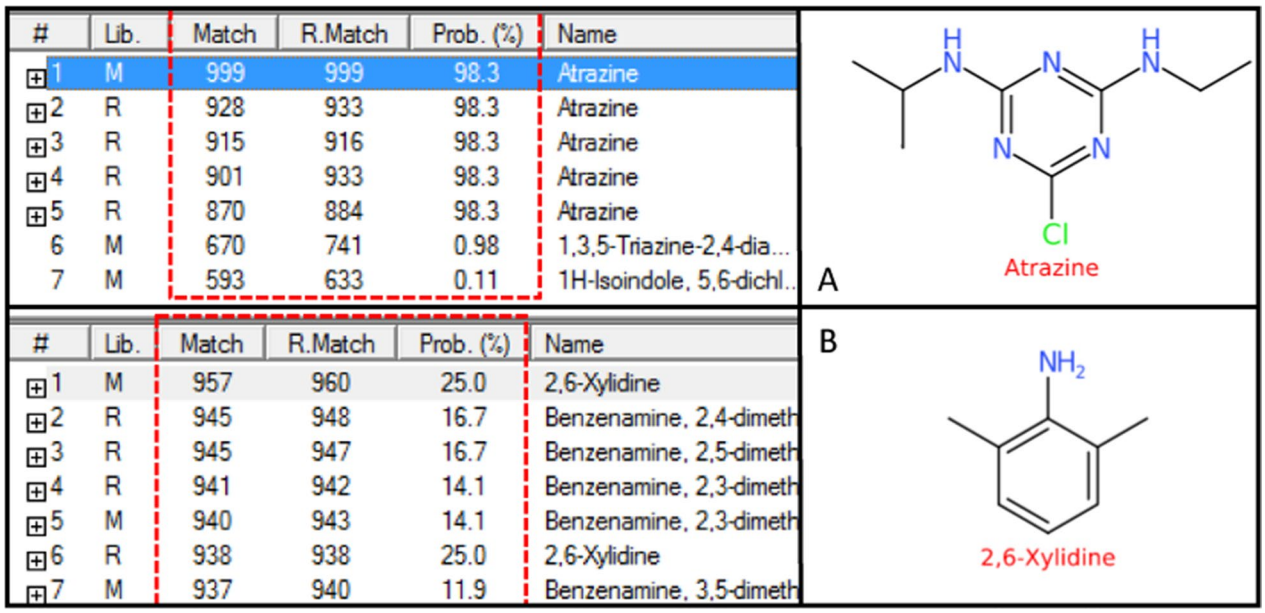

Fig. 9 Spectral match values and probabilities from NIST MS Search. **A** atrazine and **B** 2,6-xylidine (2,6-dimethylbenzenamine). Search results (left) extracted from NIST MS Search [6]; structures (right) depicted with CDK Depict [306]

match and reverse match values, but all with identical probabilities of being the correct match (since they all belong to the same compound). Figure 9b shows the case of 2,6-xylidine (2,6-dimethylbenzenamine), where several isomers in NIST have very similar spectra, such that the match values for all are very high, but the probability is low, because the spectra for all isomers are very similar. RI information would be needed to help determine the correct isomer in this case.

Further details on the algorithms behind EI–MS spectral library searching can be found elsewhere [307–309]. At this stage, the majority of EI–MS data available in libraries is still low resolution (unit mass) data, although the libraries can be used to search high-resolution EI–MS data (EI–HRMS). As EI–HRMS becomes more widespread, the proportion of accurate mass EI–MS spectra will likely increase and some of the search algorithms and strategies may need to be adjusted accordingly.

MS2 libraries

Although tandem MS libraries lagged well behind EI–MS libraries for many years in terms of compound coverage [303, 304], the METLIN library changed everything in 2020, expanding their library to 860,000 compounds as of March 2023 [310–312]. The METLIN library offers MS2 at 4 collision energies in positive/negative mode for the 860,000 compounds, although the list of compounds covered has not been released publicly (the previous reviews contained smaller METLIN collections). A licence is required for use, with a reduced price for academic subscriptions [310]. The NIST20 MS2 collection, also licensed, now contains 1.3 million spectra of 186,000 precursor ions from 31,000 compounds, with a more detailed breakdown available on their website [6]. Use of the NIST MS2 library is similar to the EI–MS library (see previous section). Thermo's mzCloud can be viewed online or accessed via Thermo software (with a licence) and includes 10,545,159 spectra of 20,944 compounds as of 12 March 2023 [313]. In terms of the open libraries (where the data are available for download and integration in open workflows), MassBank Europe (MassBankEU [314, 315]), MassBank of North America (MoNA [316]) and the Global Natural Products Social Molecular Networking (GNPS [317, 318]) are the most relevant, although the Human Metabolome Database (HMDB [319]) and related resources such as DrugBank also contain some spectra of interest. All libraries contain a mixture of natural and anthropogenic chemicals, although some allow the creation of subsets for more specific searching. PubChem now integrates many MS2 spectra, including previews and top 3 or 5 peaks for easy browsing and viewing. In terms of numbers (12 March 2023) they contain: MassBankEU 90,471 spectra of 16,881 compounds; MoNA 2,049,395 spectra (1,844,353 in silico, 205,042 experimental) of 650,263 compounds; GNPS: 587,959 spectra (~ 27,000 compounds in January 2022). Many other libraries contain mostly endogenous metabolites and narrow substance classes that are not so suitable for NTS of anthropogenic chemicals.

Several of the libraries mentioned above, especially those with licences, are not freely accessible but sometimes included in vendor software (e.g., METLIN, NIST, mzCloud). Some of the databases can only be searched online (single or batch search), whereas others can be downloaded and included in workflows (e.g., MassBank, MoNA, GNPS). Especially for the open libraries that allow user contributions (MassBank, MoNA and GNPS), the quality of the spectra can vary enormously (low resolution vs. high resolution, noisy spectra vs. spectra curated manually or by algorithms, such as RMassBank) [234] and this should be considered carefully. All three open databases mentioned also allow the creation of subsets to address this issue (so that advanced users can select the data of interest). Several software programs for MS1 and MS2 searches come with the vendor software of the instruments. Furthermore, NIST MS search (see previous section), MS–DIAL [213], OpenMS [320], mzMine 3 [216] and other free workflows are available for this purpose. For instance, the MassBank database can be downloaded in a variety of formats and used with NIST MS search, MS–DIAL, patRoon [210], MetFrag [284] and others.

Due to the variability of fragmentation behaviour between instruments and the wide range of collision energies used, caution is still needed when matching MS2 spectra and the thresholds should be adjusted accordingly (and resulting matches/reverse matches interpreted with care). When comparing spectra from the same instrument and with the same collision energy, high match thresholds of 0.9 (or 900, depending on whether scaled to 1 or 1000) should be used, as, e.g., spectra of mixed isomers can still yield match values of 0.87 as revealed in the ENTACT trial. Lower thresholds of ~ 0.7 can be used if searching libraries with a variety of instruments and collision energies, although with caution [321]. A detailed comparison of TOF and Orbitrap spectra revealed that despite the instrumental differences and different collision energy units, the produced spectra are quite comparable within the collision energy range of 10–60 eV (TOF) / NCE (Orbitrap), dropping off only at very low or very high energies—although fewer TOF spectra were run above 60 eV and fewer Orbitrap spectra below 15 NCE, limiting this comparison [155]. When not using the identical instrument or collision energy settings, merged spectra of different collision energies generally contain more fragments and offer a more

robust match and thus better match values and greater likelihood of correct identification [322]. In general, the false positive rate is reduced with increasing numbers of matching fragments, especially with larger fragments ($m/z > 100$). MS2 spectra acquired with DDA and a narrow precursor mass window (< 1 Da) are more characteristic than spectra acquired with DIA, where fragments from more compounds might be included.

Spectral similarity can also hint to structural-related compounds such as compounds of the same substance class with a diagnostic fragment (e.g., azoles [270]) or TPs [322]. Newer algorithms such as molecular networking in GNPS help to find related spectra and thus related compounds, such as TPs [323]. METLIN also includes a neutral loss search to increase the chance of a match [310], while the GNPS team have also produced a library of close matches to known spectra obtained via molecular networking [324]. This is especially relevant as MS2 spectra of many TPs are absent from the libraries due to the lack of reference standards. Researchers obtaining MS2 data on TPs are strongly encouraged to provide their MS2 spectra with their publications, preferably in a FAIR format that is easy to integrate into open libraries, to increase the coverage of these compounds (see Sect. "FAIR data management and community efforts").

Candidate structure search and selection

For features that remain without a tentative identification following either suspect screening (Sect. "Candidate structure search and selection") or spectral library search (Sect. "Spectral library search"), the next step in the process usually involves candidate structure search in a compound database, followed by candidate selection, as shown in Fig. 1 (blue boxes) and discussed further in the rest of this section.

Candidate structure search

The chemical space applicable to environmental applications is extremely large, such that the choice of database to search for candidates plays a significant role in likelihood of downstream success of tentative identification while minimising the risk of both false positives (incorrect structure identified) and false negatives (correct structure missing). The largest chemical registry, CAS, now has > 204 million structures [2] but is not accessible to open workflows. The updated Global Inventory of > 350,000 substances is a subset of regulated substances, but does not yet have structures associated with it [1]. The use of suspect lists was covered in Sect. "Candidate structure search and selection"; some large suspect lists or the consolidated NORMAN SusDat collection (> 100,000 compounds) can also be used as compound databases for candidate searching. Over the last decade, the most popular databases for NTS candidate search have been the two largest open collections, PubChem and ChemSpider, due to their comprehensive, open collections and the combination of website and programmatic access options. As of March 2023, PubChem contains 115 million chemicals [275], while ChemSpider contains 118 million chemicals [325]. While ChemSpider is still integrated in many vendor workflows, relatively recent changes to their programmatic access conditions means that user quotas are often insufficient to integrate ChemSpider in high throughput NTS workflows, such that PubChem is now the easiest of the two large databases to work with. However, queries to these databases often result in thousands to tens of thousands of matching candidates. The release of the US EPA's CompTox Chemicals Dashboard (hereafter CompTox) in 2016 introduced a new compound database of high relevance for environmental applications, with 1,200,059 chemicals and extensive environmental and toxicological information available [274]. The PubChemLite for Exposomics collection is a selection of ~ 450,000 chemicals (updated monthly) relevant for environmental, metabolomics and exposomics applications formed from major annotation categories in PubChem [288], including TPs. Candidate searches with CompTox or PubChemLite tend to return tens to hundreds of candidates per exact mass or formula, considerably less than searching the entire PubChem or ChemSpider. The additional information provided with both can help support candidate selection further (see Sect. "Candidate structure search and selection"). Although compound databases are often criticised for lacking information about TPs, there are now concerted efforts to address this via the NORMAN–SLE and PubChem [269, 288, 326], with FAIR templates available for community submission of this data [293].

Any database search is clearly restricted to the chemicals present in the database and can thus not identify any true unknowns that have not yet been documented. Although it is technically possible to generate structures of interest via structure generation approaches [173], this is not generally feasible for routine NTS applications due to the combinatorial explosion resulting in billions of candidates if generation cannot be constrained sufficiently. In LC–HRMS, structure generation has been applied to very tightly constrained cases, such as transformation of benzotriazoles, where the benzotriazole substructure helped constrain candidate numbers sufficiently for practical application [327]. Although two open structure generators have been published recently [328, 329], these do not yet offer sufficient substructure functionality for MS applications, which have so far been coupled with the MOLGEN suite of structure generators [330]. Recent developments in deep learning have opened up opportunities for autoencoders to support de

novo structure elucidation from MS, demonstrated with MSNovelist [331].

There are two main ways to search compound databases in MS-based NTS—by molecular formula or mass. If exact mass is available, it is usually most computationally efficient to search by exact mass, where the narrower the error margin in the mass range, the better the results (see Table 8). If only nominal mass is available, searching by molecular formula is preferable as too many candidates will be retrieved by nominal mass. Isotope and fragment information can be used to restrict candidates (when searching by exact mass) or possible molecular formulas (again, see Table 8). Several approaches are available to calculate molecular formulas for HRMS data, including GenForm [231, 232], the Seven Golden Rules [297], SIRIUS [298] and ZODIAC [332]. Many vendor software packages also offer molecular formula calculation; however, parameter settings are extremely important and especially the presence of fluorine can be a confounding factor in environmental NTS (see Sect. "Confidence of identification"). Inclusion of a molecular formula calculation step will often increase runtimes dramatically with the added caveat of potentially selecting the wrong formula and missing the candidate of interest, such that in high throughput workflows the recommended procedure currently is an exact mass search followed by candidate filtering, unless the molecular formula is needed, e.g., for structure generation.

Candidate selection: experimental information

Filtering of potential candidate structures can be performed using experimental data, such as the MS2 fragmentation, RT, CCS in ion mobility, and considering ionisation plausibility. Interpretation of MS2 spectra using expert knowledge is challenging and time-consuming for complex molecules, which is not feasible in high throughput NTS studies. Thus, the candidate search is often coupled directly with some form of in silico MS2 interpretation to rank the candidates, combined with other filtering approaches. Commonly used approaches for the MS2 spectral interpretation include the machine learning fingerprint-based approach of SIRIUS [298, 333], the in silico bond disconnection-based fragmenter MetFrag [284, 334] and CFM–ID [335, 336], where machine learning and fragmentation rules are applied. Several reviews and evaluations go into further details comparing the approaches [337–339]. Typically, only MS2 data are used for identification as they are generally the most present in spectral libraries and concentrations in environmental samples are generally not high enough to acquire MS^n. However, MS^n fragmentation has been shown to be very useful for identifying, e.g., novel N-heterocyclic PFAS in fish samples [340]. Retention time information is often included in candidate filtering (see Sect. "Retention time prediction

Table 8 Useful information for filtering of candidates in NTS

Filtering parameter	Recommendation	Importance	Remark
Accurate mass	Set window as small as possible (< 5 ppm or < 2 mDa) based on added standards; Check for adducts, ion-source fragments to avoid incorrect assignment of molecular ions	Very high	Higher resolution of Orbitrap helps differentiation of compounds with small mass differences
Isotope pattern	$^{12}C/^{13}C$, $^{35}Cl/^{37}Cl$, $^{79}Br/^{81}Br$ most relevant; isotopic fine structure ($^{14}N/^{15}N$, $^{32}S/^{34}S$ differentiation) only for very high-resolution measurements Less reliable at trace levels or if the intensities of isotopes fall below limit of detection (LOD)	High	QTOF is generally more accurate than Orbitrap for ion ratios but Orbitrap resolves fine structure. In DIA or DDA with isolation window > 1 Da, isotope patterns are in MS2
Characteristic fragments	e.g., $C_2F_5^-$, $C_3F_7^-$ for PFAS; SO_3^- for sulfonated surfactants Larger fragments provide more information	Very high	DDA is more specific, DIA offers more coverage. Intense peaks often exhibit more fragments with consistent ratios between them
Retention time	Accepted deviation depending on the prediction system, to be checked with standards	High	Not yet accurate enough to select candidates in isolation
Collision cross section (CCS)	< 2% deviation from predicted CCS values	High	Cleaner spectra with ion mobility but often intensity loss
Homologous series	Detection of specific mass increase (e.g., CH_2) and RT increase	Medium	Very useful for specific substance classes, such as PFAS and surfactants
Mass defect	O, F, P, S, Cl, Br have a negative mass defect and substances with these elements have a lower mass defect than CH substances	Medium	Very useful for specific substance classes, such as halogenated compounds (e.g., PFAS)
Library match	See Sect. "Spectral library search" for details. The use of merged spectra (different collision energies) can increase inter-instrument comparability	Very high	RT/RI/RTI needed to separate highly similar spectra of isomers

and indices" for a more detailed discussion); recently the combination of retention order and spectral information was shown to enhance identification performance, even improving the ranking performance of SIRIUS slightly [341]. The use of CCS in candidate selection is increasing but not yet widespread in NTS applications (see Sect. "Ion mobility separation" for further details). There is a version of PubChemLite available with predicted CCS values, which was recently applied in NTS of mussels [197]. It is likely that this will increase in the future as the increased sharing of CCS values will eventually lead to improved predictive accuracy and improve the benefits for candidate selection.

For consideration of ionisation plausibility in candidate selection, simple rules are typically applied, such as the presence of polar functional substituents that make an ionisation with ESI possible. For example, all N containing compounds can usually be ionised in the positive mode, whereas O containing hydroxyl and carboxyl substituents make a negative ionisation more plausible (see Sect. "Electrospray ionisation (ESI)"). Carboxylic acids are often observed in both modes, with diagnostic losses in each. MetFrag has a SMARTS-based filter [342] to add such functional group restrictions during candidate retrieval. Further circumstantial evidence for selection is often linked to the specific experimental study, e.g., TPs of a parent compound found in the sample or added to the experiment. For surfactants and perfluorinated compounds, which often are part of a homologous series, detection of other homologues with specific fragmentation patterns can give additional evidence [236].

For specific cases, hydrogen deuterium exchange (HDX) experiments can help determine the number of easily exchangeable hydrogens that are directly linked to function moieties, such as OH, SH, NH, NH_2 [343]. Ideally, deuterated solvents are used as mobile phases for LC and not only added post-column to achieve full exchange of hydrogens. The non-deuterated precursor masses in MS1 of an unknown compound should be matched to the deuterated precursor masses, looking for a mass difference of $X \times (2.014102 - 1.007825) = 1.006277(X)$ units within a given RT window, which could be determined using experiments with known standards. The number of deuterium groups, X, can then be deduced from the mass difference and help to filter potential candidates with different functional groups as shown successfully for environmental samples [26, 343].

LC separations at different pH values can provide further experimental evidence for certain functional groups changing their protonation state and consequently also changing their retention behaviour between different pH values. Muz et al. used separation at pH 2.6, 6.4, and 10 to differentiate alkylated and aromatic amines with different pKa values as the neutral form elutes later [252].

Table 8 summarises the main pieces of experimental information used in candidate selection with NTS.

Candidate selection: metadata/annotation information

Finally, the use of additional information, often termed metadata, expert knowledge or annotation information, can be used to support the selection of candidates. Metadata such as the number of references associated with a candidate has been used for many years; reference counts are available for the major databases PubChem, ChemSpider and CompTox. The combination of both reference and patent information was shown to be more powerful than reference information alone during evaluation of MetFrag [284]. Both patent and reference counts are available for PubChem and PubChemLite. The influence of various scores on candidate selection for various in silico approaches was evaluated in the 2016 CASMI contest [339]. During evaluation of PubChemLite, the addition of the annotation count score improved the ranking further; this can be used either as a scoring term or as a way of pre-selecting the candidates of interest according to their category, e.g., selecting pharmaceuticals or agrochemicals if these candidates are of particular interest [288]. For well-known environmental chemicals (with reference standards available and spectra in MassBank), the performance of PubChemLite was such that the correct candidate is among the top 2 in 94% of cases using MS2, patent, literature and annotation counts [288]. Furthermore, hazard, exposure, consumption and market data have also been used in candidate selection. The hazard and exposure index values provided by the Swedish Chemicals Agency (KEMI) have been included in several NORMAN–SLE lists and applied in several studies [243, 344]. CompTox also provides hazard and exposure data, although the absolute values provided (rather than the index-style approach of KEMI) have proven more difficult to interpret in the context of NTS [344].

In general, caution must be taken when using non-experimental data to rank candidates, and the weighting of the different experimental and non-experimental parameters should be handled with care. While these "metadata" scores generally help sort interesting candidates to the top of candidate lists, the experimental evidence should be considered carefully to avoid the selection of well-known compounds with less well-matched experimental evidence above TPs with better analytical scores but fewer reference or patent counts. Figure 10 shows the importance of considering experimental scores (here the MetFrag score and the library similarity score, circled in red, which are highest for the third and correct candidate, desethylterbutylazine) above reference and patent counts (which are highest for the top ranked candidate simazine) when choosing

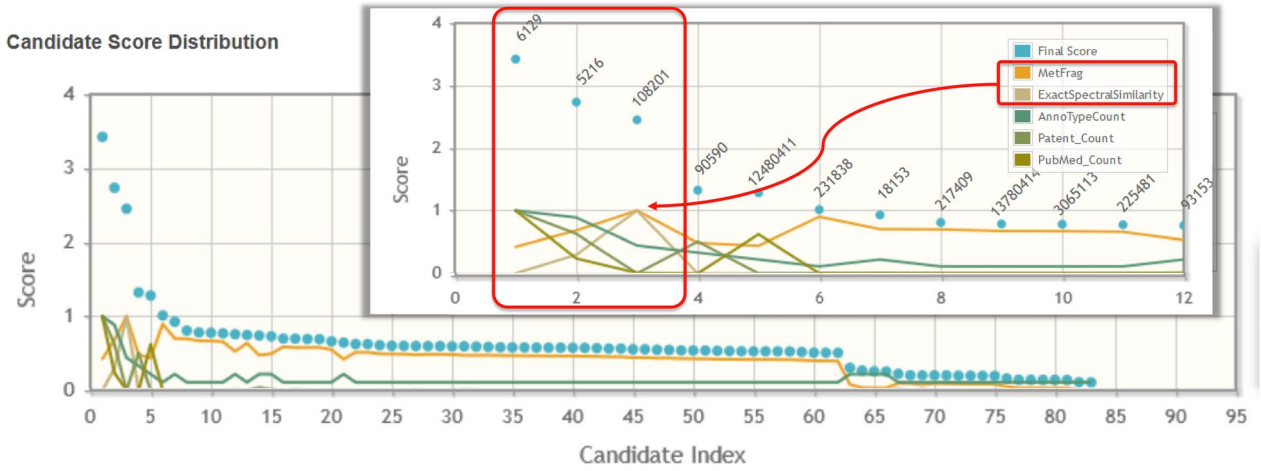

Fig. 10 Candidate selection in NTS: identifying desethylterbutylazine using MetFrag, MassBank spectrum MSBNK-Eawag-EA067112 and PubChemLite (details in [345]). The numbers in the inset are the PubChem Compound ID (CID) of the candidates

candidates for the mass spectrum MSBNK–Eawag-EA067112. This example was identified in the first NORMAN NTS Trial [10, 345]. Moving away from purely score-based ranking, the additional annotation categories offered in PubChemLite can also be used to support interpretation of candidate structures without necessarily being used in the ranking.

Retention time prediction and indices

In NTS of organic compounds, chromatography is almost always coupled to MS to ensure separation of compounds prior to detection. RT is orthogonal information to mass spectrometric information and has been successfully used as a criterion to filter potential candidate structures in LC [16, 346] and GC via both retention index (RI) and boiling point (BP) information [173, 347]. Retention on the chromatographic column is directly related to chemical structure and can thus be predicted using physico-chemical properties.

Retention index use in GC

In GC, retention indexes such as Kovats or Lee index are well-established [348] and are listed for many chemicals in the NIST database (see Sect. "EI-MS libraries"). They are based on a normalisation of the RT based on n-alkanes (Kovats) or PAHs (Lee) eluting before and after a chemical compound. Retention indices in GC exhibit excellent precision of < 0.5% under reproducible measurement conditions [349].

Retention time prediction in LC

For polar compounds separated with LC, many more interactions with the polar functional groups of the chemical can occur. The properties of the column (RP, NP, HILIC) must be considered for the prediction. Linear quantitative structure-retention relationships (QSRR) for RPLC are usually based on the n-octanol water partition coefficient $\log K_{ow}$ or $\log D_{ow}$, which accounts for the pH dependent speciation of the eluent (only the neutral fraction is used for the retention prediction). For one chromatographic method in a single laboratory, plotting of RTs of all standards vs. $\log K_{ow}$ or $\log D_{ow}$ at the pH of the eluent is a simple method to derive a linear correlation (usually r^2 in the range of 0.5–0.8, depending on the number of chemicals) [16, 106, 350]. Using this correlation, it can be determined whether candidate structures are in the 95% prediction interval (usually ± a few minutes) with their $\log K_{ow}/\log D_{ow}$ value vs. their RT. This procedure does not allow a comparison with other columns, gradients and laboratories and a sufficient number of standards is needed over the whole RT range (at least 20 compounds). The approach implemented in PredRet overcomes the restrictions of linear regressions by applying generalised additive models (GAM) to enable a robust prediction [351]. PredRet is continuously supplemented with new data and allows the prediction of the RTs of suspected or tentatively identified compounds for the custom chromatographic system by pairwise modelling compounds with known RTs against the systems included in the PredRet database (http://predret.org/). PredRet allows highly accurate predictions for a reasonable number of compounds [352].

Multivariate QSRRs use additional descriptors, such as molecular mass, polarity, etc. In newer approaches, machine learning (e.g., neural networks) and large databases are used to improve the prediction of RT as well as also collision cross section based on the physico-chemical properties [353–356]. Several machine-learning-based

QSRR methods for RP [357–360] and a few for HILIC [361, 362] have been developed. More QSRR methods are proposed in the field of metabolomics, but are not necessarily fully transferable to anthropogenic compounds with a greater range of properties and elements. Halogenated compounds, especially highly fluorinated compounds, are notorious outliers in predictive methods.

Retention time index developments for LC

Unlike GC, retention time index (RTI) use is not yet clearly established in LC, due to the greater variety of compounds covered and thus properties which influence separation. This section provides an overview of recent developments.

QSRR models for RPLC with ESI+ and ESI− were developed for application in the NORMAN network [363]. For each linear model, four important descriptors were selected, with logD_{ow} being the most important (68% contribution). For example, a unified RTI ranging from 1 to 1000 is calculated for ESI positive with the following equations:

$$\begin{aligned}\text{pred. RTI}_{(\text{ESI+})} &= -57.003(\pm 19.619)\\ &+ 70.903(\pm 1.119)\ \log D_{\text{pH=3.6}}\\ &+ 159.88(\pm 11.555)\ \text{HybRatio}\\ &+ 62.219(\pm 10.285)\text{TDB5r}\\ &+ 0.5516(\pm 0.0226)\ \text{THSA}\end{aligned} \quad (1)$$

where HybRatio = hybridisation ratio; TDB5r = 3D topological distance-based autocorrelation lag 5/weighted by covalent radius; THSA = charged partial surface area.

Two sets of 18 calibrants were selected for each of ESI+ and ESI− based on the maximum overlap with the RTs and chemical similarity indices from a total set of 2123 compounds (303 and 1820 compounds for ESI− and ESI+, respectively). The calibration set was evaluated by seven groups with their chromatographic systems including various, mostly C_{18}-columns, different mobile phases with methanol and acetonitrile water gradients and different standard mixtures. The linearity of the calibration curves were found in most cases acceptable and the uncertainty for the RTI prediction for various test compounds low, with better prediction for methanol than acetonitrile gradients and ESI+ than ESI− [363]. University of Athens provides a platform, where all RTI-related calculations with the models can be performed online (http://rti.chem.uoa.gr/). The tool also provides information about the uncertainty and whether the selected compound falls into the applicability domain. Using this tool, the RTI predictions were calculated for > 65,000 substances listed in the NORMAN Substance Database.

Two methods have been published using different homologous series of compound classes as an RT reference, in a similar approach to Kovat's and Lee RTI in GC. The first method uses N-alkylpyridinium sulfonates (NAPS), which ionise well in ESI+ and ESI− modes with $[M+H]^+$ and $[M+HCOO]^-$-ions and are also UV-active, aiding also use in non-MS–LC-based detection [364]. The two oppositely charged groups (quaternary imine and sulfonate) theoretically enhance the pH-independence and thus stable RTs. The NAPS approach has been tested on > 500 small molecules on two LC–HRMS-systems with two different RP-C_{18} columns, three gradients and four different flow rates. While RT comparison between the systems was difficult, the NAPS-normalised values were in good agreement, without systematic deviations. Some drawbacks of the NAPS are: (1) C_1–C_3-NAPS elute in or close to the void volume and thus do not provide robust predictions, (2) the behaviour under different pH scenarios is not yet investigated, and (3) difficult synthesis. NAPS are available from the Canadian National Research Council (NRC) [365]. The second class of homologous series used for RTI are cocamide diethanolamines (C(n)-DEA) [366]. This approach was developed to overcome the mentioned drawbacks of the NAPS and the previous QSRR-based model presented above [363]. The C(n)-DEA series yield acceptable peak shapes in positive and negative mode, with diagnostic masses in MS1 and MS2 spectra at even low collision energies. While a pH-dependent RT shift was observed, requiring pH-specific models to improve the prediction quality, such specific models result in good comparability between different chromatographic systems. Both homologous RTI approaches appear promising, but are not yet extensively tested in NTS. The advantage of the series-based RTI is the possibility to calculate the RTI by applying simple formulas, without the need to train QSRR-based models as in the earlier RTI model trialled by NORMAN [363].

Non vendor software and algorithms for processing HRMS data

Several non-vendor software/tools are available for processing HRMS data, with different algorithms and functionality to support the data processing based on hypotheses/goals of the research. Various overviews for software in metabolomics exist [367, 368], as well as reviews covering whole NTS workflows mentioning a range of software [369]. However, metabolomics workflows generally have somewhat different requirements compared with NTS of environmental samples, such that not all the software used for metabolomics is equally applicable to environmental NTS. The text below provides an overview of existing processing tools that are used in NTS of environmental samples, focusing on the most relevant tools and features within. This is summarised in greater detail in Additional file 1: Table S1.

FluoroMatch offers workflows specifically targeted at detection and identification of PFAS. This includes annotations using community libraries and grouping of features by CF_2 series. The software comes in a fully automated version going through the whole workflow, including peak picking and blank filtration, or a modular version, allowing users to do their own peak picking and filtering before going into the identification of compounds [219].

patRoon combines established algorithms in a single workflow tailored for environmental NTS, offering many options for different workflows within one framework. For example, it includes seven different feature detection/peak-picking algorithms including XCMS, OpenMS, enviPick and DataAnalysis from Bruker. It is freely available, uses R as its base language and has a user interface to facilitate use of the workflows [210, 211].

XCMS2 was developed for processing MS2 data and comparing these results to the METLIN database. It can also provide structural information on features that could not be identified using the library by looking for characteristic fragment ions and neutral losses, which can then be compared to the database entries of compounds with different precursor masses [370].

XCMS Online The online version of XCMS provides the same base functions as the desktop version with the added benefit of not needing a lot of computing power as data are uploaded for processing. Furthermore, it offers email notification when data are finished processing, additional visualisation and statistical options (e.g., PCAs, RT correction curves, mirror plots) and the possibility to share data [371].

SIRIUS was developed with a focus on identification of unknown compounds in non-target approaches, but recently received a "zero parameter feature detection" to simplify workflows (since version 4.4.0). SIRIUS is a collection of different separate tools that together provide molecular formula annotation (ZODIAC, [332]), prediction of compound classes without the need of databases (CANOPUS, [372]), and library search (PubChem) based on a molecular structure fingerprint predicted by fragmentation trees which are calculated from the MS2 spectrum (CSI:FingerID, [298, 333]).

KPIC2 offers a feature extraction with parameters (i.e., mass tolerance) based on the clustering of ions without the need to set them oneself, removing the arbitrary setting of parameters by the researcher. This feature extraction algorithm is integrated in patRoon. KPIC2's pattern recognition, based on Partial least squares discriminant analysis (PLS–DA) and RandomForest can be used as a tool for prioritisation of features [373].

OpenMS provides a massive range of more than 185 different tools for different steps of a processing workflow [320]. Although originally developed for proteomics and metabolomics, it is also commonly used in environmental analysis settings via the algorithms used in patRoon for data pretreatment, peak picking, componentisation and adduct annotation.

Digital Sample Freezing Platform (DSFP) The DSFP was developed by NORMAN as a collaborative effort for retrospective screening of data uploaded to the platform [374], using NORMAN SusDat [375] for annotation. Data sets can be uploaded, archived, processed and compared to publicly available data already in the database. This helps find spatial or temporal trends for chemicals of concern [374].

GNPS The Global Natural Product Social molecular networking platform (GNPS) was developed as a "big data" tool for MS2 data curation and analysis [318, 376]. Molecular networking looks for similarities between MS2 spectra in the data set, clustering them under the assumption that similar structures/compounds produce similar fragmentation patterns, which then aids in the identification. The GNPS public data repository (GNPS-MassIVE) used for annotation, includes reference spectra from the community as well as third-party data like the different MassBank libraries [376].

enviMass is one of the few non-vendor processing software that is not free of charge, although earlier open versions are still available [214]. Current versions come with complete software support, including workflow training and on-demand data processing services. In addition to standard processing, it has different tools available for data mining, such as long-term monitoring, large scale clustering and profiling and also offers cross-platform processing when working with data from different vendor instruments [377].

MZmine currently in its third version, MZmine is a modular toolbox offering a range of different options for raw data import, peak list methods (alignment, filtering, etc.), statistical analysis and visualisation [215, 216]. Custom databases and online databases such as PubChem, KEGG and HMDB can be used for candidate searching. Parts of MZmine are also integrated in other third-party software, such as SIRIUS and GNPS.

InSpectra Newly released, InSpectra offers a completely automated cloud-based processing, including a comprehensive workflow based on a range of previously published algorithms for feature detection [221], deconvolution [378], prioritisation [379], annotation using the US EPA CompTox database [274] and eventually comparison to data that others have processed before to find temporal and spatial trends of chemicals of emerging concern.

MetAlign was developed to allow the processing of GC–MS and LC–MS data in the same application, conversion to and from vendor formats, allowing to inspect

files visually in vendor software after preprocessing, and export of results to spreadsheets for further statistical analysis. Its focus is on the preprocessing of data including baseline correction, denoising and accurate mass calculation [380].

MS-DIAL Originally developed for metabolomics and lipidomics, MS–DIAL supports most MS vendor formats without the need of previous file conversion [212, 213]. It provides its own spectral data kit for annotation of unknown compounds, which also includes CCS values for ion mobility spectrometry, as well as a range of third-party libraries, such as MassBank and the GNPS repository [212]. Similar to InSpectra, it offers its own powerful deconvolution tool for DIA data [369].

SLAW SLAW is a scalable and self-optimising workflow for the non-target LC–MS data analysis [217]. It includes peak picking, parameter optimisation, sample alignment, gap filling, adducts and fragment annotation, and the extraction of consolidated MS2 information and isotopic pattern, and across all samples. SLAW runs as a command-line tool in a Docker or Singularity container and scales well to several hundreds of samples.

Quantification and semi-quantification of suspects and unknowns

The difficulty in quantifying compounds in NTS with LC–HRMS arises from vastly different ionisation efficiency of the chemicals in the electrospray ionisation source. At the same concentration, two compounds may yield LC–HRMS signals differing by several orders of magnitude [381, 382]. The response of the compound depends on the hydrophobicity of the compound [382–384], acid–base properties [385, 386], etc. Interestingly, even structural isomers may have response factors that differ by orders of magnitude, where some of the most prominent examples include dialkyl phthalates and the corresponding terephthalates. The response factor also depends on the mobile phase used in the analysis [387] and thus the gradient program and chromatographic separation. All these factors make quantification of NTS results challenging. At the same time, quantification is essential to communicate the concentration and relevance of detected chemicals to stakeholders.

To obtain quantitative results in the absence of analytical standards, different strategies have been developed. These include using peak areas either directly or with statistical data treatment, isotope dilution, radiolabelling, using the calibration curve of chemicals which are structurally or chromatographically similar to the detected chemicals. It is also possible to use predicted ionisation efficiencies for quantification [388, 389]. Although isotope dilution and radiolabelling are very accurate and applicable in other NTS applications, such as laboratory studies of transformation processes, these methods are unfeasible in the context of environmental screening, due to the impossibility of performing labelling at the scale of the environmental area of interest. This leaves approaches such as using calibration curves of structurally similar chemicals, ideally in the same sample matrix, and ionisation efficiency predictions as practical options for quantification in NTS.

Surrogate standard-based approaches

The first possibility for semi-quantification is to use the calibration curve from a chemical suggested to have similar ionisation efficiency for the quantification of the (tentatively) identified compounds [390, 391]. Choosing the chemical with similar ionisation efficiency is challenging and can be addressed with different approaches.

The 2D-based chemical similarity can be used to find the most similar analytical standard available. The 2D-linear fragment descriptors based on the atom pairs and atom sequences of a given structure can be used to calculate the Tanimoto coefficient, which is then used as a measure of similarity [392, 393]. Since the analytical standard of the parent compound of pesticides and pharmaceuticals is usually available, such a similarity-based approach can be used for quantification of the TPs [394]. However, based on the large-scale comparison for 355 micropollutants, the similarity should also be evaluated based on the structure. In cases, where transformation results in the cleavage of a functional group relevant from the ionisation perspective, the differences in the response factor of the TP and parent may reach several orders of magnitude, resulting in errors up to a factor of 50. Furthermore, this approach is generally applicable only for TPs.

Another possibility is to assume that the chemicals with most similar ionisation efficiency elute close to each other in LC–HRMS [395]. The approach is easy and can also be applied to compounds, where the structure is not fully resolved. Using a compound with a similar RT to the compound of interest has a significant advantage, since full identification of the structure is not required and thus all detected compounds can be quantified. At the same time, the compound eluting closest to the compound of interest will not necessarily have the most similar ionisation efficiency [394]. However, in the case of homologous series, this approach provides a simple and accurate solution to find chemicals with most similar properties [396].

The structural similarity and retention information can be, furthermore, combined to account for impacts of both on the ionisation efficiency. The major focus is given on the chemical similarity based on the maximum common substructure overlap (MCSO) and Jaccard index as well as the inclusion of RT data calculated from RTIs. The chemical similarity is calculated for the structurally

annotated MS2 fragmentation, where the specific substructure or ionisable moiety is dominant. These factors aim to give larger weight to the functionalities that are relevant from the ionisation efficiency point of view [397].

The structural similarity-based methods suffer from a shortcoming that a relatively large set of analytical standards is needed to cover the possible structural space of the analytes of interest (environmental pollutants). An alternative set of approaches is based on predicting the ionisation efficiency of the chemicals in LC–HRMS and to use this ionisation efficiency to estimate the concentration of tentatively identified chemicals.

Ionisation efficiency-based approaches

Ionisation efficiency prediction algorithms need to account for all factors affecting the ionisation efficiency. These are the structure of the chemical and mobile phase composition (organic modifier composition, pH, buffer type) at the RT of the chemical. It is advantageous if these effects can be "learned" based on previous knowledge across different laboratories. Recently, automated approaches based on thousands of previous ionisation efficiency measurements have been developed to predict the ionisation efficiencies of tentatively identified chemicals, based on 2D descriptors of the chemical [388, 389]. One of these approaches accounts for the mobile phase composition [389], while the other assumes the same mobile phase composition [388]. The predicted ionisation efficiency values are transferred to LC–HRMS specific response factors using a set of chemicals with known concentration and instrument-specific response factors. Such chemicals could be chemicals used for quality control or chemicals quantified with targeted methods in parallel to the non-target analysis. Quantification based on predicted ionisation efficiency has already been used for suspect screening in water [398–400].

Exploring the possibility of combining different approaches, i.e., chemical similarity analysis (chemical fingerprints and MCSO), ionisation efficiency, MS full scan spectrum, MS2 spectrum and RTIs is currently under investigation. In this exploratory approach, the RTI mixture along with one IS are required to harmonise the calibration curve parameters before establishing the ionisation scale. Consensus ionisation efficiency values from a quantitative structure-property relationship (QSPR) model based on support vector machine regression are used for quantification of the NTS data.

Factors influencing quantification

In general, the accuracy of the quantification is impacted by the accuracy of the response factor used for the quantification (either predicted or from a similar chemical) as well as by the possible errors in sample and data pretreatment.

First, the peak areas returned by different data treatment software may have different meanings. The area may correspond to (1) only to the monoisotopic peak of the parent ion, (2) the whole isotope pattern of the parent ion, or (3) all peaks (isotope peaks, fragments, adducts of the same chemical). These differences must be considered during quantification. In the case of the predicted ionisation efficiency approach, the ionisation efficiency is predicted for one species, usually protonated or deprotonated molecule. Therefore, the used peak area type needs to match the modelled ionisation efficiency.

Second, it is essential to assure that the signal of the compound is in the linear range, as all the quantification methods assume a linear relationship between the signal and the concentration of the contaminant. Ideally, this assumption should be verified by measuring the sample at several dilution factors and comparing the predicted concentrations. If measurements are performed in the linear range and no ionisation suppression occurs, the results of the dilutions should match. However, if the dilutions do not agree, the results from the more diluted sample are usually more accurate as it is more likely to be in the linear range and less impacted by the matrix effect. Thus, for more confident quantification results it is suggested to run the samples with at least two dilutions (e.g., undiluted and a tenfold dilution) and assess the results for both dilutions.

Lastly, the confidence in the quantification results needs to be communicated [401] to aid the decision making based on NTS data. Importantly the uncertainty arising both from the modelling/similarity estimation as well as from the analysis need to be combined. However, research in this field is still at its infancy and more robust solutions are likely to emerge in near future.

Quality assurance and quality control in NTS methods

The main concern regarding QA and QC of NTS methods is related to the occurrence of false positives (type I errors), i.e., erroneously detected peaks/features and identified compounds which are not present, and false negatives (type II errors), i.e., compounds which were present in the original sample, but not included in the feature list, or suspects reported erroneously as absent. Both types of error can occur at almost every step of an NTS workflow and are harder to detect in NTS than in targeted approaches due to the sheer number of chemicals of interest and the sample and data complexity. For example, in the case of type II errors, it is extremely challenging to assess whether an analyte was absent in the sample in the first instance or has been lost during the

analytical process. The latter can be caused by inadequate sampling techniques, poor extraction efficiencies [402, 403], chromatography [109] and matrix effects during mass spectrometric data acquisition [404].

Not all QA/QC procedures common to targeted applications, which rely to a large extent on the use of reference standard compounds, can be used in NTS without limitations, which warrants the need for NTS specific QA/QC methods. Challenges to be considered regarding the QA/QC in NTS methods are included in the following sections.

Internal standards

One of the most common approaches to account for several issues is the use of IS, i.e., chemicals which are not in the analytical scope and with a very high certainty absent in any of the samples. In MS-based target analysis of organic micropollutants the addition of IS, at certain points of the workflow, has become the gold standard for quantification to compensate for losses of physico-chemically similar compounds when used for internal calibration approaches [8, 405]. IS are in almost all cases isotopically labelled chemicals, where a certain portion of the C, N, or H atoms was replaced by ^{13}C, ^{15}N or ^{2}H. The set of chosen IS should ideally cover the whole range of the chemical space to be explored, spanning the whole RT and *m/z* range, the ionisation mode of interest and representative physico-chemical properties [406, 407]. However, it is virtually impossible to account for all substances potentially present in the sample, as only a limited number of compounds are available in isotope-labelled form from vendors and the high pricing typically limits the purchase of many compounds in most laboratories. Therefore, having at least one IS per expected compound group (e.g., plasticisers, pesticides, pharmaceuticals, etc.) is suggested, including those that are structurally characteristic (e.g., halogen atoms, aromatic groups etc.), and have a respective elution time that span RT window of interest [67]. Other uses of IS include, for example, intensity and RT alignment during processing [408]. Note that IS can cause interferences during data processing and identification if not considered properly.

Blanks

Blanks are important for most steps in the workflow; however, their focus is mainly on identifying false positives which can originate from different sources of contamination. Different types of blanks that can be included, covering different steps of the workflow, are according to [67]:

- Matrix blanks: A matrix sample without the contaminants that are investigated can be difficult to obtain for NTS, due to the absence of uncontaminated samples. Alternatives such as artificial matrices (e.g., for urine, wastewater, soil) can be considered.
- Laboratory blanks: Blanks that go through the same sample preparation workflow as the actual samples, for example, when conducting filtering or solid phase extraction.
- Field blanks: Blanks that go through the whole process from sampling to analysis together with the samples. To obtain field blanks, collection devices containing artificial matrices or solvents are prepared together with the samples and submitted using the same procedure. Examples are passive samplers kept in MilliQ water or pure water, which are brought to the sample collection site and treated like any sample from there on. Field and laboratory blanks can be combined if it is not relevant to know the origin of the contamination. A conservative number of field blanks is about 10–20% of the total sample number.
- Instrument blanks: Blanks that show contamination introduced by the instrument (LC, GC and MS) and mobile phase solvents. These can be MilliQ water or a vial of mobile phase at starting conditions or a solvent in case of GC analysis. Instrument blanks are recommended to be analysed within each sample batch, at the beginning and end of the batch, and before and after QC samples.

These blanks are used during the data processing workflow to eliminate features resulting from contamination or background, thus facilitating the identification of relevant features [38, 259, 379, 409]. The elimination of a feature is mostly based on a relative intensity between sample and blank, which can be set individually depending on, for example, matrix and applied methods. Commonly a 3:1 or 10:1 sample-to-blank ratio is used, but stricter limits can be applied if necessary or as a conservative approach, even exclusion if occurring in a blank.

Reproducibility/sensitivity

Some QA/QC procedures designed to measure reproducibility or repeatability can be easily adopted from targeted analysis, as they are still sufficient for the issue they address. For example, the randomisation of the sample sequence for chromatography can help to reduce systematic errors as a result of carry over, while the use of sufficient replicates of the whole process can demonstrate the repeatability of the method [38, 379]. Furthermore, it can also be shown with the use of pooled samples, as done routinely in metabolomics [410]. However, for environmental samples it is recommendable to use a pooled

sample for each sample type (e.g., wastewater influent and effluent) due to the varying matrix effects [411]. Decreasing sensitivity over the course of an analytical sequence can, for example, be investigated by injecting spiked quality control samples throughout a sequence or checking the area of IS over time [266]. This can also be used to help validate mass accuracy and precision of the system (e.g., stable RTs). To increase precision, avoid false positives and false negatives it is recommended to have two, ideally three technical replicates as verified for different matrix types [412].

Instrument calibration

To ensure acceptable mass accuracy and resolution, regular instrument calibration is necessary. Different approaches are possible, partly dictated by the type of instrument (i.e., vendor) used. In general, it can be differentiated between external and internal mass calibration [413]. While external calibrations are executed in between two chromatographic runs, using either vendor or in-house calibrant solutions, internal mass calibrations measure one or multiple compounds simultaneously during acquisition of sample data, either continuously or in fixed time intervals (e.g., "MassLock" techniques using Leucine Enkephalin (Leu-Enk) [414]). Alternatively, post-acquisition corrections based on these well-known masses, background ions or other contaminants can be applied [415–417]. While all techniques have their individual disadvantages, a common drawback is the necessary extrapolation from a few calibrants to the remainder of the investigated *m/z* range. Therefore, the use of multiple compounds spread over the whole mass range for calibration is nearly always recommended.

Data processing

QA/QC procedures for data processing steps are limited currently, as results are highly dependent on the chosen approach. For example, it has been shown that changing the algorithm and/or parameters for processing can change the obtained results drastically [418]. Therefore, the chosen procedures should be kept consistent across all batches and communicated transparently. A first step in the application of data processing workflows should always be to be able to successfully detect all added IS (in terms of correct RT and *m/z*) and potentially also some chemicals which can be reasonably expected in the respective sample, like commonly known contaminants. As IS are typically added at comparably high concentrations, their isotope peaks should be included as well to check the performance for lower intensity signals. Finally, the use of a qualitative identification scale, including full identifications using reference standards for the highest level of certainty, helps to rank the published results [296]. Nevertheless, expert knowledge and, therefore, subjectivity, still has a crucial role in processing, potentially adding more points of uncertainty. Further definition and harmonisation of expected QA/QC procedures for NTS is beyond the current document but would be an important step forward for routine application of NTS.

Recommendations

1. Inclusion of all undertaken steps to prove validity of the used method in all studies and publications (see Sect. "Reporting"), especially which IS have been used and which have been detected/not detected. This should incorporate an evaluation of how representative the chosen IS are for the chemical space which is supposed to be explored in the respective research.
2. Publication of all used raw data (if possible), (analytical) methods including sampling and sample preparation, algorithms and parameters used for processing to ensure that others can retrace and verify the findings (FAIR principles).
3. Inter-laboratory trials can help to pinpoint factors influencing the outcome of an NTS and could be done in a more regular fashion, focusing on all aspects of NTS workflows.
4. Following guidelines to standardise manual examination of results to reduce the influence of subjectivity similar to ones existing for target analysis, e.g., ISO 21253-1 [419], ISO 21253-2 [420], and SANTE 11312/2021 [421].

Reporting

Communicating an NTS study and its findings is important for further interpretation and use for, e.g., legislators and policymakers. However, many times it is difficult to evaluate the validity and quality of the study, because not all information for this evaluation is provided. The NTS community can learn from reporting experiences and best practices in the more mature and established metabolomics research field [422]. Besides communicating the data results and findings, a sound NTS study reporting should as a minimum include descriptions of; (a) study design including as much metadata as possible, (b) data acquisition, (c) data processing and analysis and (d) any used QA/QC metrics [67]. These reporting approaches (see Table 9) are generally used in the community to increase the NTS study reproducibility and transparency, and were recently formally suggested by Peter and coworkers [13]. Good practices around scientific data management and stewardship are urgently needed in the NTS community. Therefore, it is highly recommended to share data via public repositories (Sect. "FAIR data management and community efforts") and make the data FAIR

(findable, accessible, interoperable and reusable)—this will ensure transparency and maximise the impact of the research effort.

FAIR data management and community efforts

NTS studies include scientific and regulatory investigations, monitoring and cohort studies, all of which serve societal and ecological needs. These studies generate a high amount of full-scan HRMS and processed data, which is often only exploited for the specific aims of the related studies or available workflows. Typically, only several hundreds of suspect or unknown substances are reported as identified in a single sample at various levels of confidence, whereas additional thousands of detected substances (e.g., up to 3000–10,000 in wastewater samples) remain unknown [236]. Obviously, this information is of great value for retrospective screening efforts and should not be discarded, such that managing and sharing NTS data according to FAIR principles is decisive for the rapid growth of the NTS field.

The need to preserve all recorded mass spectral information led to the digital archiving of environmental HRMS data [423]. The archiving offers a possibility of retrospective suspect screening of contaminants of concern for various research and policy purposes. To achieve this goal, a common repository for HRMS chromatograms of environmental samples, accompanied by a suite of compound structure–elucidation software tools, has been developed by the NORMAN network [374]. The NORMAN Digital Sample Freezing Platform (DSFP) is part of the NORMAN Database System [424], where the substance database is built on contributions made via the NORMAN–SLE [269]. It is designed to store HRMS data and meta-data (sampling site, matrix and sample description, instrumental setup etc.), which are required for interpretation of the results. The DSFP offers wide-scope retrospective suspect screening, semi-quantification based on structural similarity of detected compounds with IS and interactive visualisation of the results. It has been used so far in numerous studies, e.g., references [425–427]. In addition, national or basin-specific repositories for NTS have been (or are being) constructed to help address privacy restrictions and various requirements, which is still compatible with the FAIR motto "as open as possible and as closed as necessary". This kind of digital archiving has also been applied in other disciplines, such as metabolomics and natural products research, where several prominent platforms have been developed including MetaboLights [428], MassIVE/GNPS [318] and Metabolomics Workbench [429]. NTS repositories such as DSFP (main focus organic contaminants), GNPS (natural products), MetaboLights and Metabolomics Workbench (metabolites), and other national repositories have their own unique approaches and purposes. They focus on different scientific communities, have distinct features and capabilities, and may have different users and stakeholders. Understanding these differences and their common points allows the researchers to choose the most suitable repository for

Table 9 Reporting recommendations in NTS studies, modified from 13

	Reporting recommendations
☑	Study design should include scope, aim and hypotheses, and if possible chemical space coverage considerations from the applied methodologies
☑	Sample information and preparation will involve possible study site descriptions, sample collection type (e.g., grab or flow proportional), sampling equipment, storage, sample preparation and any extraction or clean-up processes
☑	Quality control sample descriptions, such as any used blanks (field, process, and instrument blanks), pooled samples, as well as fortified (spiked with native and isotopic labelled standards)
☑	Analytical sequence descriptions such as randomised sample order and blocking, batches and technical replicates*
☑	Platform description and used settings, such as chromatography (e.g., ion exchange), column, injection technique and volume, mobile phases, gradients, mass spectrometer, ionisation and acquisition mode (e.g., data dependent acquisition with inclusion list)
☑	Data processing needs in-depth descriptions of applied software and workflow settings (e.g., blank filtration, algorithms and synthetic values (gaps) filling) and workflow decision diagrams, and if any data conversions are made*
☑	Chemometrics and statistical analysis should be described (e.g., adjusted *p* value, hierarchical clustering methods, scaling and differential analysis) alongside any used software
☑	Annotation and confidence level should be detailed (e.g., workflow steps and cutoff criteria can be displayed in a decision tree diagram*). Used software (scoring algorithms) and libraries should be reported, as well as mass error and RT tolerance
☑	Quantification or semi-quantification of identified substances can be determined and uncertainties reported
☑	QA/QC in data acquisition, processing and analysis should be described. System suitability checks, maintenance schedule (e.g., weekly tune of HRMS) and, e.g., RT and mass accuracy on used IS
☑	Public data repository accession number and information should be included (e.g., the NORMAN Digital Sample Freezing Platform, https://dsfp.norman-data.eu/)

*Recommended as an appendix or journal supplementary material

their data and align their practices with the FAIR principles effectively.

To meet the increasing demand for reusability and interoperability of the environmental HRMS data, reference laboratories of the NORMAN Association have placed special focus on the implementation of harmonised practices for instrumental analysis, data acquisition and data reporting. For this purpose, a series of collaborative trials have been conducted to validate applicability of harmonised approaches for digital archiving in different matrices: river water [10], indoor dust [9], and biota (in progress). Significant outcomes have been achieved over recent years in the field of retention time indexing, which allows for the inter-comparability of data acquired by different LC–HRMS systems at various chromatographic conditions through the use of a set of calibrant substances [363]. Recommendations have also been made for FAIR reporting of chemical (suspect list) and TP information (see [293, 294] and Sect. "Developing compound lists for suspect screening"). A simplified guidance document has been developed to define 'minimum requirements' for data acquisition and upload in NORMAN DSFP [430]. Additional steps towards the harmonisation are expected in the field of semi-quantification (NORMAN trial on semi-quantification ongoing; see Sect. "Quantification and semi-quantification of suspects and unknowns"). After validation within a network of NORMAN laboratories, all advancements are rapidly incorporated in DSFP. The widespread use of DSFP and similar repositories and systems has the potential to revolutionise environmental chemical monitoring in Europe as currently discussed with the Partnership for Chemical Risk assessment project (PARC, https://www.eu-parc.eu/) and beyond.

To achieve full implementation of FAIR principles, NTS data repositories will need to meet specific requirements, which may require significant time and monetary investments. Requirements include sufficiently robust, fit-for-purpose infrastructure (available over a long time frame), providing comprehensive metadata, assigning persistent identifiers, employing common vocabularies and ontologies, ensuring data accessibility and open access, using standardised data formats, and enabling interoperability. Interoperability can be achieved using common (open) formats, providing application programming interfaces (APIs) and semantic machine-interpretable metadata integration. Although aligning with FAIR principles is feasible in many ways, FAIRification requires significant effort and commitment, efforts that are not currently rewarded sufficiently, limiting wide uptake. While mandates may be effective, incentive-based rewards for data sharing (e.g., GNPS, DSFP, where additional services are offered to data contributors) are also important (compare carrot—reward, vs. stick—mandate). Implementing FAIR data management practices can present challenges, particularly in the context of funding requirements, publisher deposition policies and regulatory/privacy considerations. While it is likely that publishers will gradually develop data deposition policies for HRMS and other environmental data, these policies may conflict with funding mandates and may also differ from FAIR principles. The NTS community will need to navigate the challenge of aligning these requirements and ensuring their data meets FAIR standards while complying with publisher, funding and legal considerations.

In conclusion, the effective management and sharing of NTS data according to FAIR principles is crucial for the growth and advancement of the NTS field. While various NTS repositories exist with their own unique approaches, it is essential to understand their differences and commonalities to choose the most suitable repository and align practices with FAIR principles. The digital archiving of environmental HRMS data plays a vital role in preserving valuable information, discovering the newest emerging contaminants and enabling retrospective suspect screening for research and policy purposes. The implementation of harmonised practices for instrumental analysis, data acquisition, and data reporting, along with the continuous development of guidelines and standards, further promotes reusability and interoperability in the field. By embracing FAIR principles and leveraging collaborative efforts, the NTS community can revolutionise environmental chemical monitoring, fostering advancements in research, and promoting evidence-based decision-making for societal and ecological needs.

Glossary and definitions

Important terms for NTS in environmental analysis are compiled below, considering definitions made by the International Union of Pure and Applied Chemistry (IUPAC) [431] if available:

Adduct ion Ion resulting from the interaction of an analyte or an analyte ion with one or more other molecules or ions often within the ion source (e.g., $[M+Na]^+$, $[M+NH_4]^+$ and $[M+K]^+$ in positive mode, or $[M+CH_3COO]^-$, $[M+Cl]^-$ in negative mode).

Atmospheric pressure chemical ionisation (APCI) A soft ionisation technique based on the chemical interactions between vaporised solvent ions or gaseous molecules and analytes within the ion source under atmospheric pressure. The process may involve transfer of an electron, proton or other charged species between the reactants. The solvent or gas is ionised by corona discharge.

Atmospheric pressure photoionisation (APPI) A soft ionisation technique based on generating photons via a

vacuum ultraviolet light source to ionise (in)directly molecules in the gas phase. APPI is suitable for moderately non-polar to non-polar compounds.

Blank A type of sample, used as part of quality assurance/quality control (defined later), that ideally does not contain the analyte molecule(s). Blanks are usually used to correct or monitor for any form of contamination that may happen throughout the different stages of the work from sampling all the way to instrumental analysis. The different types of blanks include equipment/instrumental blank, field/sampling blank, extraction blank or method blank, and solvent blank.

Capillary electrochromatography (CEC) This microscale separation technique is a hybrid between capillary electrophoresis and liquid chromatography (LC). CEC can be performed in packed, monolithic and open-tubular columns.

Capillary electrophoresis An analytical technique that separates ions based on their electrophoretic mobility with the use of an applied voltage. The electrophoretic mobility is dependent upon the viscosity, the charge and the molecular weight of the molecule.

Collision cross-sectional value (CCS) The measure of the cross-sectional area of an ion in the gas phase can be obtained via different types of ion mobility technologies. It is derived from the measured mobility as a function of experimental parameters (temperature, pressure) using the Mason–Schamp equation. It is related to its chemical structure and three-dimensional conformation.

Collision-induced dissociation (CID) A mass spectrometry technique that induces fragmentation of ions in the gas phase by colliding them with neutral gas molecules (typically helium, nitrogen or argon).

Componentisation The process of grouping together different isotopologues/adducts that originate from the same molecule.

Data-Dependent Acquisition (DDA) A preselection-based tandem mass spectrometric (MS2) acquisition method for molecular structure determination (identification). In DDA, selected ions (usually within a defined *m/z* window, such as 1 Da) are subjected to fragmentation (MS2). The experimenter defines the number of precursor ions that get sent to the collision cell for fragmentation, which are usually the top N most abundant ions. DDA allows direct association of fragment ions to their respective precursor ion.

Data-Independent Acquisition (DIA) In DIA, all ions or a defined mass-to-charge (*m/z*) window (typically > > 5 m/*z*) are subjected to fragmentation (MS2) for molecular structure determination. Unlike DDA, it is difficult to connect the fragment ions to their precursor ion and thus, DIA experiments rely on deconvolution.

Deprotonated molecule Common form of an ionised molecule in negative polarity formed through the loss of a hydrogen (practically a proton). Typically, represented as $[M–H]^-$.

Electron ionisation (EI) An ionisation technique based on the interaction between high energy electrons and analyte molecules, in the gas phase to produce ions. Because of the use of high-energy electrons, it is a hard ionisation method that leads to intense fragmentation of molecules during ionisation.

Electrospray ionisation (ESI) It is a soft ionisation method, producing mainly the ionised form of intact molecules (little in-source fragmentation). This technique is based on ion transfer and is suitable for both polar small molecules and macromolecules.

Exact mass The exact mass of an ion or molecule is the calculated theoretical mass for a given isotope composition (monoisotopic mass).

Extracted ion chromatogram (EIC) The two-dimensional plot (intensity vs. *m/z*) of the detection of a specific *m/z value* (ion) over the course of a chromatographic run.

FAIR data Findable, Accessible, Interoperable and Reusable data [432].

Feature In mass spectrometry, a feature is the combination of chromatographic and mass spectral peak, and thus it is a signal including time (RT), mass (*m/z*), and intensity.

Full width at half maximum (FWHM) The width of a peak at half of its maximum height.

Gas chromatography (GC) A chromatographic technique used to separate and analyse volatile and thermally stable components of a sample, ideally without decomposition.

Gel permeation chromatography (GPC) A type of chromatography that separates analytes based on size. Often used as a clean-up step for reducing the lipid content of sample extracts for contaminant analysis as it takes advantage of the large size of many lipids, which can cause signal suppression.

Higher-energy collisional dissociation (HCD) A CID technique specific to the Orbitrap mass spectrometers and provides beam-type CID MS2. In beam-type CID MS, ions can be activated multiple times, resulting in richer spectra than resonance-type CID ("CID").

High-resolution mass spectrometry (HRMS) A technique that uses a mass spectrometer capable of high resolution (≥ 5000) and high accuracy (± 0.001 Da). The most common high-resolution mass spectrometers are TOF, Orbitrap and FT–ICR.

Internal standard (IS) Substance added to samples, blanks, and standards to correct for variations in the analytical process related to a target compound,

preferably with as similar physico-chemical properties as possible—ideally a stable isotopically labelled analogue.

Ion enhancement A form of matrix effect during the ionisation that reinforces the formation of ions.

Ion mobility spectrometry (IMS) An analytical technique used to separate ionised molecules in the gas phase based on their mobility in an inert gas (termed "buffer gas" or "drift gas") and electric field. Different types of IMS have been developed, such as drift tube IMS (DTIMS), travelling wave IMS (TWIMS), trapped IMS (TIMS), field asymmetric IMS (FAIMS) and differential IMS (DIMS or DMS).

Ion suppression A form of matrix effect during the ionisation that suppresses the formation of ions, e.g., due to ion competition.

Isobars Molecular species with the same integer/nominal mass but different exact mass (different digits after the decimal place).

Isomers Compounds with the same molecular formula and exact mass but a different structure or different spatial arrangement of the atoms within a molecule.

Isotopes Atoms of the same element that possess the same atomic number (same number of protons) but a different mass number (different numbers of neutrons). For example, the isotopes of hydrogen are ^{1}H (hydrogen), ^{2}H (deuterium) and ^{3}H (tritium), with nominal masses of 1, 2 and 3 Da, respectively.

Isotope pattern The pattern forms in the mass spectrum by the mass spectrometric separation of the various isotopes of the atoms in a molecule. The isotope pattern is dependent on the combination and frequency of the individual atoms in the molecule.

Isotope ratio The ratio between the number of atoms of one isotope and the number of atoms of another isotope of the same element in the same molecule, often expressed in relation to the naturally most frequently occurring isotope.

Isotopic fine structure The mass spectral signature arising from the combination of naturally occurring isotopes within the molecule being measured, which can aid in more accurate formula assignment but requires a certain level of mass resolving power.

Isotopologues Molecular entities that differ only in their isotopic composition (e.g., CH_4, CH_3D, CH_2D_2).

Limit of detection (LOD) The lowest concentration of an analyte that is significantly above the background. Typically, a signal-to-noise ratio of three is used.

Limit of quantification (LOQ) The lowest concentration of an analyte that can be quantified with certainty. Typically, a signal-to-noise ratio of ten is used.

Liquid chromatography (LC) A technique used to separate a mixture into its individual components. The separation takes place based on the partitioning of the analytes with the mobile phases and stationary phases (typically silicon-based particles). Different modes are depending on the mobile and stationary phases used, including reverse phase LC (RPLC), normal phase LC (NPLC), hydrophilic interaction LC (HILIC), ion-exchange chromatography (IC), and mixed-mode LC (MMLC).

Mass accuracy A measure of a mass spectrometer's ability to measure the mass of a molecule, calculated by taking the difference between the mass of an ion measured (*m/z*) and the theoretically calculated exact mass (*m/z*) of the same ion. Bias to the exact mass is typically given as relative deviation in ppm (parts per million) or as absolute deviation (in either Da or u, or the milli mass unit, mmu).

Mass defect The mass defect of an atom, molecule or ion is the difference between the nominal and the monoisotopic mass. Most organic molecules have a positive mass defect, since they are very often composed of atoms with small positive mass defects (e.g., H, N) and nearly negligible negative mass defects (e.g., O, F). Some elements such as chlorine and bromine have relatively large negative mass defects.

Mass resolution The measure of the ability of a mass resolving power of a mass spectrometer to discriminate/resolve between two measured masses. This is determined by the ratio $m/\Delta m$ at FWHM.

Mass spectrometry (MS) The branch of science dealing with all aspects of mass spectrometers and the results obtained with these instruments.

Matrix effects The phenomenon in which the ionisation efficiency of a compound is changed by the presence of (interferences in the) matrix. Typical matrix effects are ion suppression and ion enhancement.

Molecular ion Ion formed by removal or addition of one (or more) electrons to a molecule without fragmentation, e.g., $[M]^{+\bullet}$. Typically observed in EI, as well as APCI and APPI.

Monoisotopic Mass The monoisotopic mass of molecules or ions is referred to as the sum of the monoisotopic masses of the most abundant elements in its formula (e.g., C_6H_6O: $^{12}C_6\ ^{1}H_6\ ^{16}O$: 84.0419).

Non-target screening (NTS) Analytical method for detecting a broad range of compounds. Screening in full scan mass chromatograms for masses of interest based on criteria such as signal intensity or frequency of occurrence or other criteria posed by the scientific question in place, and subsequent identification using mass spectrometric information (e.g., isotope pattern, MS2 fragmentation, RT) and possibly metadata (e.g., environmental context, consumption, commercial relevance). Sometimes also called non-target

analysis (NTA), non-targeted screening or untargeted screening.

Peak (chromatographic peak or mass peak) A two-dimensional plot with a Gaussian-like shape. Chromatographic peak has intensity as the dependent dimension (i.e., "y" axis) and RT as the independent dimension (i.e., "x" axis). Mass peak has an intensity as the dependent dimension (i.e., "y" axis) and the mass to charge (*m/z*) values as the independent dimension (i.e., "x" axis).

Protonated molecule A common form of an ionised molecule in positive polarity, formed through the association of a proton (H^+) with the neutral form of a molecule. Annotated as $[M+H]^+$.

Quality assurance (QA) and Quality control (QC) QA/QC protocols and procedures implemented for sampling, sample preparation, data acquisition, data analysis, and data mining to ensure that sample analysis is consistent, comparable, precise, and accurate.

Quantitative Structure–Activity Relationship (QSAR) A computational modelling method for revealing relationships between quantitative structural properties of compounds and activities of those compounds shown in experimental systems.

Quantitative Structure–Retention Relationship (QSRR) A technique used to predict the retention time of analytes on a chromatography column based on their physico-chemical properties.

Retention index (RI) An instrument-independent descriptor of retention time on a chromatography column. Usually, calibrants such as alkanes with different chain lengths in GC analysis are used to convert retention times into system-independent constants. Often added as a descriptor in library entries.

Retention time (RT) The time it takes for a compound to pass through a chromatographic column after injection and be detected.

Retention time index (RTI) The retention time index of a compound is its chromatographic retention time in LC normalised to the retention time of selected calibration compounds or a fitted regression based on calibration compounds. The RTI is (ideally) independent of the chromatographic system, allows the comparison of values measured by different laboratories and assists in the identification of compounds by comparison with listed values.

Solid phase extraction (SPE) A sample preparation technique to extract and enrich the analytes from a complex matrix. It is based on the same principles as chromatography and uses the difference of chemical behaviour of analyte molecules and interfering compounds present in sample matrix (a sorbent or resin).

Spectral library A collection of measured fragmentation spectra. For hard ionisation (e.g., EI) mostly MS spectra are collected, whereas for soft ionisation (e.g., ESI, APCI, etc.), techniques typically MS2 (MS/MS) are included.

Supercritical fluid chromatography (SFC) The separation technique is a type of chromatography, using a supercritical fluid, usually carbon dioxide mixed with some modifiers, as the mobile phase.

Suspect screening Searching in full scan mass chromatograms for exact masses of molecular ions/adducts of compounds expected in the sample without using a reference standard. Subsequently, other mass spectral information is used for tentative identification of potential hits; unambiguous identification (confirmation) is performed by comparison to reference standards.

Abbreviations

ACN	Acetonitrile (organic solvent)
APCI	Atmospheric pressure chemical ionisation
APPI	Atmospheric pressure photoionisation
ASE	Accelerated solvent extraction (see also PLE)
BP	Boiling point
BP4NTA	Benchmarking and publications for non-target analysis, US working group on NTA (https://nontargetedanalysis.org/)
CCS	Collision cross section
CE	Collision energy
CEC	Capillary electrochromatography
CI	Chemical ionisation
CID	Collision induced dissociation
CID (PubChem)	PubChem compound identifier
CZE	Capillary zone electrophoresis
DBPs	Disinfection by-products
DEET	Diethyltoluamide
DI	Direct injection
DIMS	Differential ion mobility spectrometry (also DMS)
DMSO	Dimethyl sulfoxide (organic solvent)
dSPE	Dispersive solid phase extraction
DSSTox	Distributed structure-searchable toxicity
DTIMS	Drift tube ion mobility spectrometry
DTXSID	Distributed structure-searchable toxicity (DSSTox) substance identifier
EC	European Commission
ECNI	Electron capture negative ion chemical ionisation
EIC	Extracted ion chromatogram (see also XIC)
EI	Electron ionisation
ENTACT	EPA's non-targeted analysis collaborative trial
ESI	Electrospray ionisation
FAIMS	Field asymmetric ion mobility spectrometry
FAIR	Findable, accessible, interoperable, reusable
FI	Field ionisation
FT-ICR MS	Fourier-transform ion cyclotron resonance mass spectrometry
GAM	Generalised additive models
GAPS	Global atmospheric passive sampling or global passive sampling
GC	Gas chromatography
GCxGC	Two dimensional gas chromatography
GNPS	Global natural products social molecular networking
GPC	Gel permeation chromatography
HCD	Higher energy collisional dissociation
HDX	Hydrogen deuterium exchange
HILIC	Hydrophilic interaction chromatography or hydrophilic interaction liquid chromatography
HMDB	Human metabolome database
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry

HVS3	High volume small surface sampler
IC	Ion chromatography
ID Exchange	PubChem identifier exchange service
IMS	Ion mobility spectrometry
InChI	International chemical identifier
InChIKey	Hashed form of the international chemical identifier
IP	Identification points
IS	Internal standard(s)
LC	Liquid chromatography
LCxLC	Two dimensional liquid chromatography
LLE	Liquid–liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MAE	Microwave assisted extraction
MassBankEU	MassBank Europe
MCR	Multivariate curve regression
MEKC	Micellar electrokinetic capillary chromatography
MeOH	Methanol (organic solvent)
MMLC	Mixed-mode liquid chromatography
MoNA	MassBank of North America
MS	Mass spectrometry
MS2	Tandem mass spectrometry, also MS/MS
MSI	Metabolomics standards initiative
NAPS	N-alkylpyridinium sulfonates
NCE	Nominal collision energy
NIH	National Institutes of Health (USA)
NIST	National Institute of Standards and Technology (USA)
NIST20	NIST/EPA/NIH EI-MS Library, 2020 release
NMR	Nuclear magnetic resonance spectroscopy
NOM	Natural organic matter
NORMAN	Network of reference laboratories, research centres and related organisations for monitoring of emerging environmental substances
NORMAN-SLE	NORMAN suspect list exchange
NPLC	Normal phase liquid chromatography
NTA	Non-target analysis, alternative term for non-target screening
NTS	Non-target screening—including suspect and non-target screening
PAHs	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis
PCBs	Polychlorinated biphenyls
PDMS	Polydimethylsiloxane
PE	Polyethylene
PFAS	Per- and polyfluoroalkyl substances
PFP	Pentafluorophenyl (analytical column material)
PI	Photoionisation
PLE	Pressurised liquid extraction (see also ASE)
PLS	Partial least squares
PMOC	Persistent mobile organic chemicals
POCIS	Polar organic chemical integrative sampler
ppm	Parts per million
PSA	Primary secondary amine
PTV	Programmable temperature vaporiser
PubChem CID	PubChem compound identifier (CID). "PubChem" has been appended to the beginning to avoid confusion with collision induced dissociation
PUF	Polyurethane foam
QA	Quality assurance
QC	Quality control
QSPR	Quantitative structure-property relationship
QSAR	Quantitative structure-activity relationship
QSRR	Quantitative structure-retention relationship
QTOF	Quadrupole coupled to time of flight mass spectrometry
QuEChERS	Quick, easy, cheap, effective, rugged, and safe (sample preparation method)
REACH	Registration, evaluation, authorisation and restriction of chemicals (EU regulation)
RI	Retention index
ROI	Regions of interest
RP	Reverse phase or reversed phase
RPLC	Reversed phase liquid chromatography
RT	Retention time
RTI	Retention time index
SFC	Supercritical fluid chromatography
SMILES	Simplified molecular-input line-entry system
SPE	Solid phase extraction
SusDat	NORMAN substance database (NORMAN Network)
TIC	Total ion chromatogram
TIMFIE	Time-integrating, MicroFlow, inline extraction
TIMS	Trapped ion mobility spectrometry
TOF	Time-of-flight
TPs	Transformation products
TWIMS	Travelling wave ion mobility spectrometry
UHPLC	Ultrahigh-performance liquid chromatography
US EPA	United States Environmental Protection Agency
VOCs	Volatile organic compounds
XIC	Extracted ion chromatogram (see also EIC)
IUPAC	International Union of Pure and Applied Chemistry

Supplementary Information

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Additional file 1: Table S1. Compiles details of non-vendor software and algorithms for processing HRMS data.

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