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Identification of persistent substructures in transformation products with zebrafish embryos using cheminformatics and a suspect screening approach

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Substances transform in environmental and biological matrices to produce diverse transformation products (TPs). Some chemical moieties, termed persistent substructures, are retained in these TPs. This study used literature TPs, literature-derived analogous TPs (*i.e.*, TPs inferred by applying literature-reported transformations from structurally similar precursors) and predicted TPs to identify known and novel TPs of five selected data-poor compounds using zebrafish embryos. The workflow was then used to identify persistent substructures in a further 36 persistent, mobile and toxic (PMT) compounds from the triazine, triazole and PFAS classes. The suspect screening workflow in patRoon was applied to liquid chromatography high-resolution mass spectrometry data. This study identified 91 TPs at confidence levels 1 (confirmed) or 3 (tentative): 34 from data-poor parents and 57 from the PMT compounds, including 13 Level 1s. Among data-poor compounds, 17 TPs (52%) were analogous TPs, whereas 17% were exclusively predicted. Among PMT compounds, most TPs (63%) were predicted exclusively using BioTransformer, while 12.3% were solely literature TPs. The combined approach yielded a better TP coverage compared to any single source, revealing extensive biotransformation even for PMT substances – indicating that persistence does not exclude downstream transformation. The transformations included phase I and II metabolic reactions, as well as non-enzymatic processes. The 1,3,5-triazine ring, benzotriazole ring and CF_3 group were conserved in all TPs of their respective parent compounds, while the 1,2,4-triazole ring was found in most, but not all, triazole TPs. QSAR modelling predictions indicated that several TPs were potentially more persistent, mobile and toxic than their parents. The study shows the significance of including the concept of persistent TP substructures in chemical risk assessments.

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Environmental significance

Compounds can create transformation products (TPs) that may persist, remain mobile, and potentially harm humans and the environment. This study used a zebrafish embryo workflow with liquid chromatography-high resolution mass spectrometry (LC-HRMS) to identify 91 TPs from 41 PMT (persistent, mobile, and toxic) substances and investigate potentially persistent substructures. The 1,3,5-triazine, 1,2,4-triazole and CF_3 substructures generally resisted transformation, with many TPs predicted to be potentially more persistent and toxic than their parent compounds. Several conjugation reactions were observed, indicating potential biological reservoirs of persistent substructures. The newly-identified structures and transformation reactions have been uploaded to open resources to expand public knowledge. These findings offer important markers for monitoring and early hazard identification in regulatory assessments and water management.

1. Introduction

Persistent, Mobile and Toxic (PMT) or very Persistent and very Mobile (vPvM) substances are organic contaminants of particular concern for water resources, especially drinking water sources.[1] These substances do not easily degrade (persistent), are transported over long distances (mobile) and may cause harm to human health and the environment (toxic).[2,3] Recently, they were established as a hazard class of substances at the European level.[2] Within the European project ZeroPM, particular attention has been paid to certain classes of potential PMT/vPvM compounds, including per- and polyfluoroalkyl substances (PFAS), triazines, and triazoles,[4,5] found in the S90 ZEROPMBOX1 suspect list.[6] This is a curated list of

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potential PMT/vPvM candidate substances developed within ZeroPM, which was uploaded to the NORMAN Suspect List Exchange (NORMAN-SLE),[7] a community platform for sharing suspect lists and reference information to support non-target screening. Carlier *et al.* recently profiled the *in vitro* endocrine-disrupting properties of 36 representative compounds from these three structural classes based on the S90 ZEROPMBOX1 suspect list and Arp & Hale,[8] highlighting their potential toxicity to humans.[9] These contaminants have varied applications, ranging from processing aids in industry to pesticides and herbicides used in consumer and household products.[10,11] Since the substructures contained within these compounds may contribute to their P, M, or T properties, ZeroPM has hypothesised that understanding the structural elements contributing to their hazard is crucial for assessing their risks.[12]

The relevance of certain recurring substructures becomes particularly evident during the transformation of compounds. As compounds undergo various transformation processes in environmental and biological matrices, they generate diverse transformation products (TPs). These TPs are generally more polar and water-soluble than their precursors.[13,14] Transformation processes may eventually result in a more stable structure that is resistant to degradation prior to complete mineralisation.[15,16] These stable forms are referred to in this context as "dead-end TPs". Reported examples include trifluoroacetic acid (TFA), from fluorinated precursor degradation pathways such as fluoxetine,[17] and melamine, reported from the transformation of triazines and related compounds, including hexa-methoxy-methyl-melamine (HMMM).[18] The increased stability and polarity enhance the persistence and mobility of these TPs in the environment, potentially posing risks to wildlife, human health, and the environment.[19]

During the chemical transformation process, specific moieties or substructures, such as the CF_2 and CF_3 groups in perfluorooctanoic acid (PFOA) TPs,[20] the 1,3,5-triazine ring in terbutryn TPs,[21] and the 1,2,4-triazole ring in tebuconazole TPs,[22] may be retained in the TP. These are henceforth referred to as "persistent substructures" in this article. Where persistent substructures carry the toxicophore, *i.e.*, the structural portion associated with the chemical's toxicity,[23] the TP may have similar or even more toxic properties than the parent compound. Thus, identifying persistent substructures in TPs may be helpful for (i) identifying and predicting potentially hazardous substances, (ii) grouping and prioritising substances for chemical regulation,[5] and (iii) providing a basis for safer chemical design by designing more biodegradable substances.

Obtaining data about known and potential TPs is crucial to fully characterise persistent substructures in TPs. One way to achieve this is to curate existing TP data from the literature to make it Findable, Accessible, Interoperable, and Reusable (FAIR).[24] Efforts have been made to improve the FAIRness of existing TP data, including the use of cheminformatics tools and text-mining applications such as ShinyTPs,[25] to curate TP data from text-mining results, and strategic inclusion of reference data into the NORMAN-SLE.[26] Collectively, over 9400 reactions documenting more than 6400 TPs have been added to the PubChem database[27] through the NORMAN-SLE.[7,28] Despite these efforts, a significant gap remains between the number of compounds with TP data available and the total number of compounds. There are currently over 9400 compounds with transformation data in PubChem out of 122 million compounds (less than 0.01%), highlighting the knowledge gap. The transformation processes of some compounds have been studied extensively in biological and/or environmental matrices (including treatment studies), while other structurally similar compounds have not. For instance, terbutryn, a triazine compound,[21] and tebuconazole, a triazole compound,[22] have 10 and 12 unique TPs in PubChem,[29] respectively. This could provide some insight into the transformation processes for compounds with similar substructures, such as simetryn and metconazole for the triazine and triazoles, respectively. This information could be used to curate potential TPs for these structurally similar compounds with limited TP data. However, the formation of these TPs still needs to be investigated and validated in various matrices. Another option is to use predictive models of transformation, such as BioTransformer,[30] although prediction is prone to several challenges such as absence of novel reaction rules, limited methods for recognising when biotransformation reactions should be terminated,[14,30] and combinatorial explosion, where predicting transformations over multiple reaction steps is computationally demanding and leads to drastic over-prediction.[31,32]

Zebrafish (*Danio rerio*) embryos are a common model for studying the metabolism of organic substances. Their rapid development, with a metabolic activity beginning as early as 4 hours post-fertilisation (hpf), is ideal for investigating the metabolism of organic contaminants.[33,34] Zebrafish are freshwater vertebrate organisms, making them an environmentally relevant species and a valuable model for studying PMT compounds. Additionally, they share genetic and physiological similarities with humans, facilitating the translation of research findings into contexts related to environmental and human health issues.[35–37] Previous studies have examined the metabolism of compounds such as clofibric acid,[33] 74 PFAS,[38] and tributyltin[39] using zebrafish embryos.

Suspect screening using LC-HRMS is widely used to identify more polar compounds and TPs in biological and environmental matrices,[40] while complementary GC-HRMS workflows are typically used for volatile chemicals.[41] This approach allows for the tentative identification of substances without the need for reference standards by relying on known information such as exact masses, isotopic patterns, and fragmentation spectra to identify compounds from a predefined suspect list.[40,42] However, HRMS analysis often generates large volumes of data that require expert knowledge and multiple software tools for accurate feature extraction and annotation. To facilitate this task, the open source software patRoon integrates several different approaches to provide a complete data processing workflow.[43] PatRoon integrates *in silico* fragmentation algorithms and libraries for spectral and compound annotation, as well as TP-specific workflows to support TP identification in complex matrices.[43,44]

This study has two main objectives: to expand existing knowledge on TPs through suspect screening and identify the role of persistent substructures in 41 PMT compounds. To achieve these objectives, two experiments were conducted: (i) a workflow evaluation on five compounds with limited TP data (two triazines: simetryn and chlorsulfuron; one triazole: metconazole; and two PFAS compounds according to the OECD definition[45] bisphenol AF – also known for endocrine disrupting properties – and ADONA) to investigate the formation of TPs in zebrafish embryos. The workflow involved the use of LC-HRMS analysis combined with suspect screening using patRoon, integrating predicted TPs, curated analogous TPs from literature (where applicable), and literature TPs to identify suspect TPs; and (ii) an application of this workflow to 36 potential PMT/vPvM candidate compounds from Carlier *et al.*,[9] (nine triazines, 16 triazoles, and 11 PFAS) to characterise persistent substructures and discuss their potential implications for chemical risk assessments.

2. Materials and methods

2.1. Chemicals, reagents and standards

Analytical grade chemicals were used for all experiments. Simetryn (PubChem Compound Identifier (PCID): 13905) 98.0% was obtained from Merck Life Science BV (Hoeilaart, Belgium). Chlorsulfuron (PCID: 47491) 99.68% purity, metconazole (PCID: 86210) 99.01%, ADONA (PCID: 52915299), 90.3% purity, and bisphenol AF (PCID: 73864) with 99.9% purity, were obtained from ATCC/LCG standards (Molsheim Cedex, France). Methanol was obtained from Roti-Solv (99.95% LC-MS grade), and formic acid was purchased from Sigma-Aldrich (98% purity). The aqueous exposure solutions were all prepared in 0.3× Danieau's solution (17 mM NaCl, 2 mM KCl, 0.12 mM $MgSO_4$, 1.8 mM $Ca(NO_3)_2$, 1.5 mM Hepes, pH 7.5), and the final concentration was well below the water solubilities. Ultrapure water (Type 1), referred to here as Milli-Q H_2O, was used. Details of the parent compounds tested and TP standards are given in the SI, Tables S1 and S2, respectively.

Thirty-six compounds belonging to three structural classes, *i.e.*, 16 triazoles, 9 triazines and 11 PFAS with purity over 95% were purchased from different suppliers (SI Table S1). For most compounds stock solutions were prepared in DMSO, but some compounds were dissolved in methanol, 0.1 M NaOH, 0.5 M HCl or acetonitrile due to solubility and/or stability reasons (see SI Table S1).

2.2. Ethics statement

All practices involving zebrafish were performed in accordance with European laws, guidelines and policies for animal experimentation, housing and care (European Directive 2010/63/EU on the protection of animals used for scientific purposes). According to Article 3 of the Directive 2010/63/EU and to Annex II (Commission Implementing Decision 2012/707/EU), the present study did not involve the reporting of procedures and animals (*i.e.* zebrafish was used before 5 days post-fertilization) and as such it was not subjected to authorization by an ethics committee.

The Zebrafish Facility at the Luxembourg Centre for Systems Biomedicine (LCSB) is registered as an authorized breeder, supplier and user of zebrafish with Grand-Ducal decree of 03 April 2025, and the Zebrafish Facility at the Vrije Universiteit Amsterdam has approval number NVWA/2024/010089717.

2.3. Exposure experiment for five compounds with limited transformation data

Adult wild-type zebrafish (*Danio rerio*: AB: RRID:ZFIN_ZDB-GENO-960809-7) were housed in the LCSB Aquatic Facility (RRID: SCR_025429) according to the standard protocols.[46] Zebrafish embryos, which were obtained by natural spawning, were then sorted according to the criteria outlined by Kimmel *et al.*,[47] under a stereomicroscope (OLYMPUS SZX2-ILLT). Fertilised embryos were kept in Petri dishes with 0.3× Danieau's solution and incubated in 10 h dark–14 h light conditions at 28 °C until the start of the incubations the next day. At 24 hpf, a static exposure was performed, where zebrafish embryos (n = 35 per condition) were transferred to each well of a 6-well plate and exposed from 24 hpf to 96 hpf (*i.e.* 72 h exposure) with 5 mL of each testing solution of the five compounds (*i.e.* simetryn, chlorsulfuron, metconazole, bisphenol AF, and ADONA). Two concentrations were used per compound. Whenever the information was available, concentrations reported to be below the LC_{50} value in *Danio rerio* (life stage not always specified in the source) were used (SI, Table S1). When the LC_{50} for a compound in *Danio rerio* was unavailable, the value for another fish was used as a reference, *e.g.*, *Oncorhynchus mykiss*. In parallel, negative (unexposed embryos) and stability controls (exposure solutions without embryos) were processed in each plate. The exposure experiments were performed in triplicate to account for biological and technical variability. The plate design per exposure experiment is shown in the SI (Fig. S1). Throughout the experiments, the plates were observed every 24 hours under a stereomicroscope to remove chorions, any dead embryos, and record morphological changes. Morphological observations conducted on each tested embryo included coagulation of the embryos, absence of somite formation, non-detachment of the tail, and absence of heartbeat using the OECD fish embryonic test endpoints.[48] The overall survival rate of the embryos in all the conditions (controls and exposure concentrations) was ≥90%. At the end of the exposure experiments, each replicate containing about 32 viable larvae were washed twice with 5 mL of Milli-Q H_2O and transferred into pre-weighed and labelled 2.0 mL Precellys® tubes (the weight of the tubes with embryos was approximately 100 mg). The larvae were then snap-frozen in liquid nitrogen and transferred to a −80 °C freezer until extraction.

2.4. Exposure experiment for persistent substructure characterisation

The exposure experiment for the 36 PM compounds was conducted at the Vrije Universiteit Amsterdam (VUA), and the zebrafish embryo samples were shipped to LCSB for extraction

and measurement. Zebrafish from the transgenic line tg(tg:mCherry) were kept in tank systems with recirculating water at the Amsterdam Animal Research Centre of the VUA. The water was maintained at 27 °C and a pH of 7.2, with a 10 h dark and 14 h light cycle. The stock solutions of each of the 36 test chemicals (solvents described in SI Table S1) were diluted 1000 times in demineralised water containing 64.7 mg L^{-1} $NaHCO_3$, 5.7 mg L^{-1} KCl, 294 mg L^{-1} $CaCl_2 \cdot 2H_2O$ and 123.3 mg L^{-1} $MgSO_4 \cdot 7H_2O$. They were aerated overnight to obtain the final concentrations stated in SI Table S1.

The exposure process involved approximately 10 fertilised eggs per well, exposed to each of the 36 compounds individually in 24-well plates. No stability controls were performed in this experiment. The exposure lasted from 4 hpf to 96 hpf over a span of 4 days at 26 °C, with a 10 h dark and 14 h light cycle. After the exposure period, the embryos per compound were harvested, washed, and placed into Eppendorf tubes, which were then frozen at −20 °C for transport. The frozen samples were packed in a Styrofoam box filled with dry ice and shipped to LCSB, with a shipping duration of 24 hours. Upon arrival at LCSB, the frozen samples were immediately transferred to a −80 °C freezer and were extracted and measured as soon as possible.

2.5. Sample extraction

The sample preparation and extraction protocols were adapted from the LCSB Metabolomics Platform (RRID: SCR_024769) protocols previously reported by Heins-Marroquin *et al.*[49] and Talavera Andújar *et al.*.[50] The same extraction and measurement protocol was used for both of the above-listed experiments. Before extraction, samples were briefly thawed on ice. For extraction, 600 mg of ceramic beads (size 1.4 mm; bead for Precellys homogenisers, QIAGEN, 13113-325) and 1000 μL of extraction fluid containing methanol (MeOH)/Milli-Q H_2O/internal standard (15 : 3 : 0.5 ratio, v/v/v) were added to the tubes on ice (4 °C). Details about the internal standards are given in the SI, Table S2. The mixture was homogenised at 6000 rpm for 30 seconds, then 120 μL of Milli-Q H_2O at 4 °C and 400 μL of chloroform were added, and the mixture was incubated for 10 minutes at 1400 rpm on an Eppendorf thermomixer comfort (SNr: 5355ZL847986). After centrifugation at 15 000 rpm for 5 minutes, 760 μL of the clear monophasic supernatant was transferred to a 1.5 mL Eppendorf tube containing 200 μL of Milli-Q H_2O and 200 μL of chloroform (biphasic liquid–liquid extraction). This mixture was vortexed and centrifuged again for 5 minutes. The lower phase (300 μL) was collected, evaporated to dryness using a SpeedVac, and then reconstituted with 100 μL of 0.1% formic acid (FA) in Milli-Q H_2O and MeOH solution in a 90/10 (v/v) ratio. This approach prioritises extract cleanliness and reproducibility for qualitative TP screening rather than quantitative recovery. Finally, samples were filtered through PHENEX PTFE 4 mm syringe filters (0.2 μm) into LC vials for measurement. Similarly, 100 μL of stability controls (when used) was extracted. Additional details can be found in the SI figures Section 2. For quality control, two extraction blanks were made by extracting 100 μL of Milli-Q H_2O, and two pooled quality samples were made by aliquoting 5 μL of each sample into LC vials. These samples were injected first, then after every five samples and again at the end of the measurement, as recommended by Broadhurst *et al.*.[51] In addition, Milli-Q H_2O blanks were prepared using 100 μL of Milli-Q H_2O to test system stability and measured alongside the samples.

2.6. Suspect list generation

Suspect TPs were required for the screening of potential TPs, for use as an inclusion list for guided data-dependent MS/MS acquisition, and as a customized database for TP compound annotation. Three approaches were used for generating these suspect lists:

(1) TPs from literature and compiled in PubChem, referred to as "literature TPs".[52]

(2) TPs derived from literature information available for structurally similar precursor compounds, referred to as "analogous TPs."

(3) TPs predicted using BioTransformer v4.0, referred to as "BioTransformer TPs".[53,54]

The literature TPs documented in PubChem (RRID:SCR_004284),[27] associated with the parent compounds of interest, were downloaded from Zenodo (RRID:SCR_004129)[55] and added to the suspect list. The analogous TPs were generated by comparing precursor compounds from literature that are structurally similar to the compound investigated, *i.e.*, simetryn, chlorsulfuron, metconazole, bisphenol AF, and ADONA. For instance, terbutryn and ametryn TPs were used to draw analogous TPs for simteryn, while metsulfuron and iodosulfuron TPs were used to draw analogous TPs for chlorsulfuron. The structures of the precursor compounds that are structurally similar to compounds that have documented TPs, used to draw analogous TPs are shown in Fig. 1 (full details in the SI, Table S3). Subsequently, transformations from these structurally similar compounds were applied to generate analogous TPs for the compound of interest (see SI figures, Section S3 for details). The BioTransformer TPs were generated using the command line version of BioTransformer v4.0[54] to predict TPs from the SMILES of the parent compounds. Two options were employed for predicting TPs over three reaction iterations: (i) the environmental microbial transformation module ("env"), and (ii) the human-related transformation option ("allHuman"), which includes four modules: CYP450, Enzyme Commission-based (EC-based), phase II, and gut microbial transformers modules.[54,56]

These TPs were then combined into a suspect list for further use. Two distinct suspect lists were created for the respective experimental setups. The first list, referred to as Zebrafish Persistent Mobile and Toxic Transformation Products (ZFPMTTPs), was created to identify TPs from the selected five data-poor compounds: simetryn, chlorsulfuron, metconazole, bisphenol AF, and ADONA. This list included literature TPs, analogous TPs and BioTransformer TPs (SI Table S4). The second suspect list, called ZeroPM S90 Transformation Products (ZPMS90TPs), was used for characterising persistent

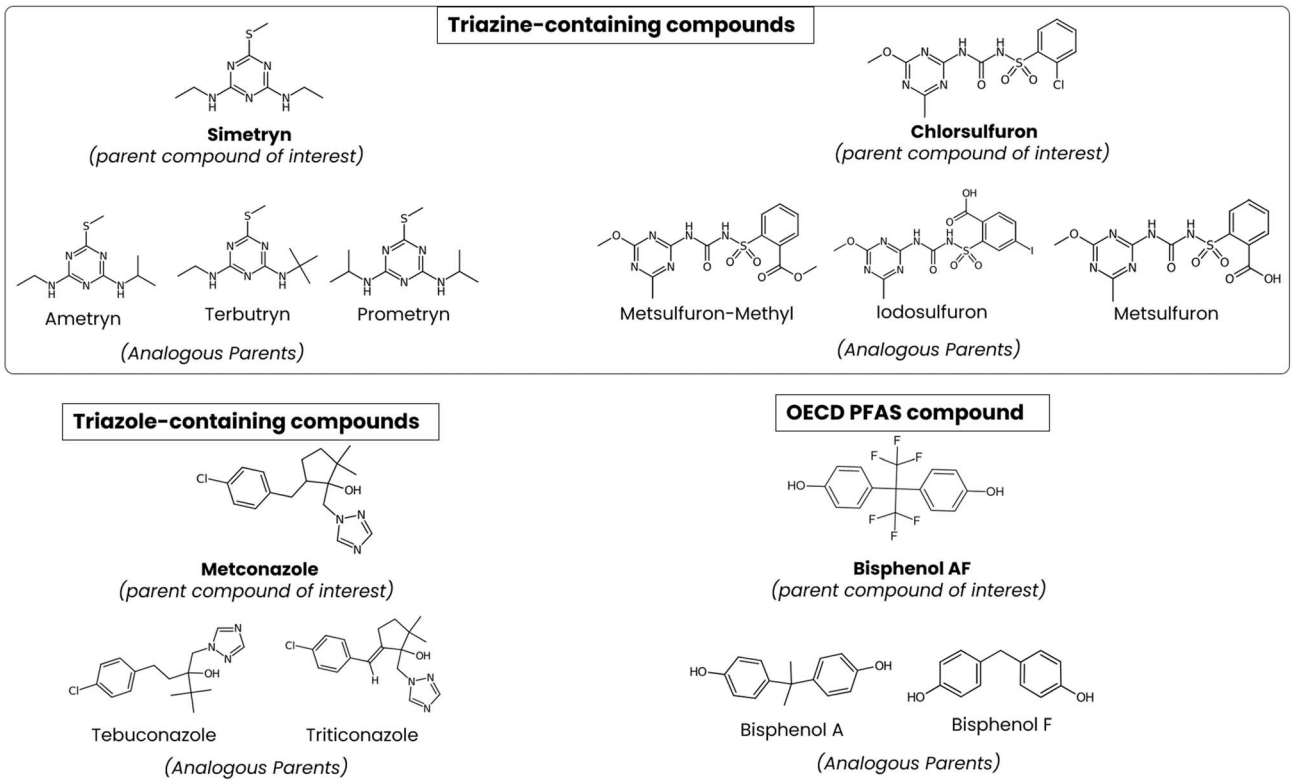

Fig. 1 Structurally similar compounds to the parent compounds of interest. Structurally similar compounds have documented TPs, used to draw analogous TPs for suspect screening.

substructures found in the TPs of the 36 PM compounds. This list contained only the literature TPs and BioTransformer TPs (SI Table S5).

2.7. LC-HRMS analysis

The LC-HRMS analysis method used was described by Talavera Andújar *et al.*.[50] A Thermo Scientific Accela LC system was used with a reverse phase (RP) method to separate the compounds of interest (non-polar and slightly polar compounds). Very polar or ionic compounds may be poorly retained and therefore under-represented using this method. With an injection volume of 5 μL, a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) BEH C_{18} column (dimensions of 1.7 μm and 2.1 × 150 mm) was used to separate the analytes. An optimised column temperature of 35 °C and a flow rate of 0.2 mL min^{-1} was used. For triazines and triazoles, the mobile phases were: 0.1% FA in Milli-Q H_2O (A) and MeOH (B). The gradient was as follows: 90A/10B at 0 min, 90/10 at 2 min, 0/100 at 15 min, 0/100 at 20 min, 90/10 at 21 min, and 90/10 at 30 min. For the PFAS samples, the mobile phases (A) and (B) were Milli-Q H_2O + 2 mM of ammonium acetate and MeOH, respectively. The gradient was 50A/50B at 2 min, 100/0 at 20 min, 50A : 50B at 10 min.

For mass spectrometry analysis, the Q Exactive™ HF (Thermo Scientific) MS was used in both positive (+) and negative (−) electrospray ionisation modes. The runtime was set to 0-30 minutes, with inclusion enabled (On). The full MS/data-dependent (dd) MS^2 settings (consistent with previous methods) were as follows: resolution (MS1 = 120 000, at m/z 200), automatic gain control (AGC) target (1.0×10^6), maximum injection time (IT) 70 ms, and scan range (m/z = 60–900). For the dd-MS^2/dd-SIM (data-dependent selected ion monitoring): resolution (MS2 = 30 000 at m/z 200), AGC target (5.0×10^5), maximum IT 70 ms, loop count,[10] Top N,[10] isolation window (1.0 Da), and (N)CE.[30] Finally, the dd settings were: minimum AGC target (8.0×10^3, intensity threshold (1.1×10^5)), apex trigger (4 to 6 s), exclude isotopes (On), and dynamic exclusion (10 s).

2.8. Data analysis

2.8.1. Suspect screening with patRoon 2.0. The workflow for suspect screening was as follows: first, features were extracted and grouped using the XCMS3 package using '*findFeatures()*' and '*groupFeatures()*'[57,58] respectively. Next, the '*screenSuspect()*' function was used for suspect screening based on the respective suspect list. Following this, a peak list with MS data was generated *via* the mzR package[59] using '*generatePeakList()*'. After filtering, molecular formulas were calculated with the '*generateFormulas()*' function and GenForm package.[43,60] Compound identification was carried out using the '*generateCompounds()*' function, where *in silico* fragmentation with MetFrag (MetFragCommandLine-2.6.1 Jar file)[61] and compound annotation with the customized database (ZFPMTTPs or ZPMS90TPs) were used. Finally, suspects were annotated with the '*annotateSuspects()*' function using the identification confidence levels integrated within patRoon.[43] The major settings for the patRoon suspect screening workflow, including the adducts used, are outlined in SI Table S6, while

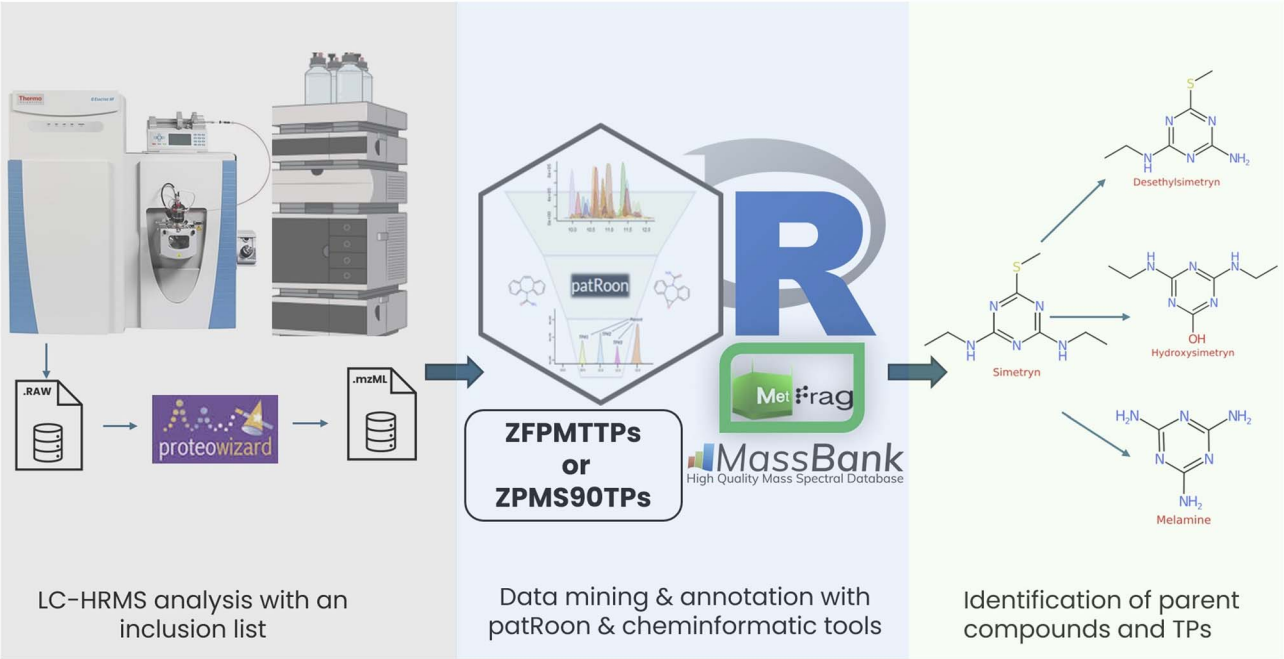

Fig. 2 General workflow for LC-HRMS and data analysis with patRoon 2.0 in R.

the scripts used for screening, including filtering details, are available in the GitLab repository.[62] Fig. 2 shows the summary of the LC-HRMS workflow and data analysis using patRoon.

2.8.2. Validation and PMT property prediction of suspect screening results. To validate the suspect screening results, each feature was cross-checked with the raw file using the Qual Browser app in Xcalibur (RRID:SCR_014593) to assess the peak shape, peak intensity (threshold, 1.0×10^5), and retention time (RT). The spectral data were then extracted and used to perform *in silico* fragmentation with the MetFrag web interface,[61] using the ZFPMTTPs or ZPMS90TPs lists in a .csv format as a customised database for annotation. Additional details on how each feature was manually validated are shown in the SI figures Section S4. A quantitative mass balance was not performed, as a full mass balance would require validated quantification and recovery correction, which was out of the scope of this study. The identification confidence level, based on[63] and adapted from the patRoon identification confidence level assignment,[43] was then assigned as follows: Level 1 is classified as a target match (confirmed structure/compound), where the retention time deviation was $\leq \pm 12$ seconds from the RT of the standard measured in-house. Level 2a indicates a good MS/MS library match, with the individualMoNAscore (calculated within MetFrag) ≥ 0.9. Level 3 represents a tentative candidate, which was determined by the following criteria: Level 3a, the individualMoNAScore is between 0.9 and 0.7; Level 3b, at least three fragments from the experimental data match those obtained from the MetFrag *in silico* fragmentation; Level 3c, the candidate compound is listed in either of the ZFPMTTPs or ZPMS90 TPs. The ± 12 seconds window from the RT of the standard measured in-house was used to account for potential RT differences between matrices and sample runs.

2.8.3 PMT property prediction. VEGA QSAR[64] was used to predict the PMT properties of parent compounds and their TPs. For persistence screening, the VEGA Ready Biodegradability (IRFMN) model v1.0.10[65] was used. This rule-based structure–activity relationship (SAR) model predicts ready biodegradability with four output classes based on OECD 301C (MITI I) data:[66] Readily Biodegradable (RB); Possible Readily Biodegradable (Possible RB); Possible Non-Readily Biodegradable (Possible Non-RB); Non-Readily Biodegradable (Non-RB). In this study, "Possible" classes are interpreted as their definitive counterparts (*e.g.*, Possible RB becomes RB). Non-RB predictions (including Possible Non-RB) were classified as persistent, and RB predictions (including Possible RB) as not persistent. Predictions marked as "Not classifiable/Not assigned" were excluded from further analysis.

For mobility screening, the VEGA KOC (OPERA) model version 1.0.1[64] was used to predict matrix-independent soil organic carbon partitioning ($\log K_{oc}$) values. Substances were classified according to EU CLP Commission Regulation 2023/707,[2] except for ionizable substances with errors, which were flagged as not assessed. The classification was as follows: For $\log K_{oc} < 2$, the compound is classified as very mobile (vM); For $2 \leq \log K_{oc} < 3$, the compound is classified as mobile (M); For $\log K_{oc} \geq 3$, the compound is classified as not mobile (Not M).

Predicted 96 hours fish LC_{50} values (mg L^{-1}) were obtained using the VEGA Fish Acute (LC_{50}) Toxicity (IRFMN) model v1.0.2[67] for assessing toxicity. The predicted LC_{50} values were classified according to the EU CLP Commission Regulation 2018/669[68] as follows: for $LC_{50} \leq 1$ mg L^{-1}: aquatic acute 1/chronic 1; for $1 < LC_{50} \leq 10$ mg L^{-1}: aquatic chronic 2; for $10 < LC_{50} \leq 100$ mg L^{-1}: aquatic chronic 3; for $LC_{50} > 100$ mg L^{-1}: aquatic chronic 4.

For all predictions, the VEGA's Applicability Domain Index (ADI) for prediction reliability was used, categorized as good (ADI ≥ 0.80), moderate (0.65 to <0.80), or low (ADI < 0.65).

3. Results and discussion

3.1. Transformation pathways of compounds with limited transformation data

After suspect screening of all five compounds with limited data, a total of 34 unique TPs were identified for the tested compounds: simetryn,[16] chlorsulfuron,[13] metconazole,[3] bisphenol AF[1] and ADONA.[1] Most of the TPs[23] were detected only in positive mode, four in negative mode, and seven in both positive and negative modes, highlighting the importance of dual ionisation mode in TP screening. Most TPs[17] originated from analogously drawn TPs from the literature, while 6 TPs were from BioTransformer predictions only, and 10 from both approaches. One of the identified TPs was from all three approaches, *i.e.*, BioTransformer, analogous and literature TP. Some TPs were also present in the stability controls (exposure medium without embryos): 13 TPs were identified in the samples only, 9 in both the samples and the stability controls, and 12 were identified exclusively in the stability controls. Out of the 13 TPs detected in samples, two TPs were detected in exposure concentration C1 only, while four TPs were from exposure concentration C2 (exposure concentrations C1 and C2 for each compound are outlined in SI Table S1). The remaining seven TPs annotated in both concentrations were mainly dealkylation, hydrolysis and oxidation reactions. Details of the variability of detected TPs across tested concentrations and transformation pathways are in SI Table S7, while MS/MS details are in SI Table S8.

3.1.1. Simetryn (triazine). Out of the 16 TPs identified for simetryn, two TPs (melamine and hydroxy-simetryn) were confirmed at Level 1, the remaining 14 were Level 3s, *i.e.* five were Level 3b and nine were Level 3c (Fig. 3). Most TPs (14 of 16) were detected exclusively in positive mode. The majority were literature analogues alone,[9] followed by both BioTransformer predicted and literature-based analogues[5] and BioTransformer predicted only.[2] These observations indicate that analogous TPs from literature increased TP coverage for simetryn, which is as expected since there are several well-studied triazine pesticides with many TPs documented in open resources.

Most of the TPs were found only in exposed embryo samples,[10] while some were detected in both the exposed embryo samples and the stability controls.[4] The remaining two were exclusively found in the stability controls. This indicates the impact of non-enzymatic transformation reactions. One possible explanation for this observation is that the absence of competing biotic pathways in the stability controls allows simetryn to follow slow but direct abiotic routes that lead to a more efficient accumulation of melamine. This finding is consistent with the literature that reports melamine as a product of the non-enzymatic degradation of the triazine herbicide cyromazine,[69] although its formation from methyl-*s*-triazine herbicides has not been frequently described in literature. Hydroxy-simetryn was detected with higher intensity in embryos than in the stability controls, providing evidence of phase I reactions like sulfur substitution and ring hydroxylation reported in analogues like terbutryn and ametryn.[70] Sulfur-

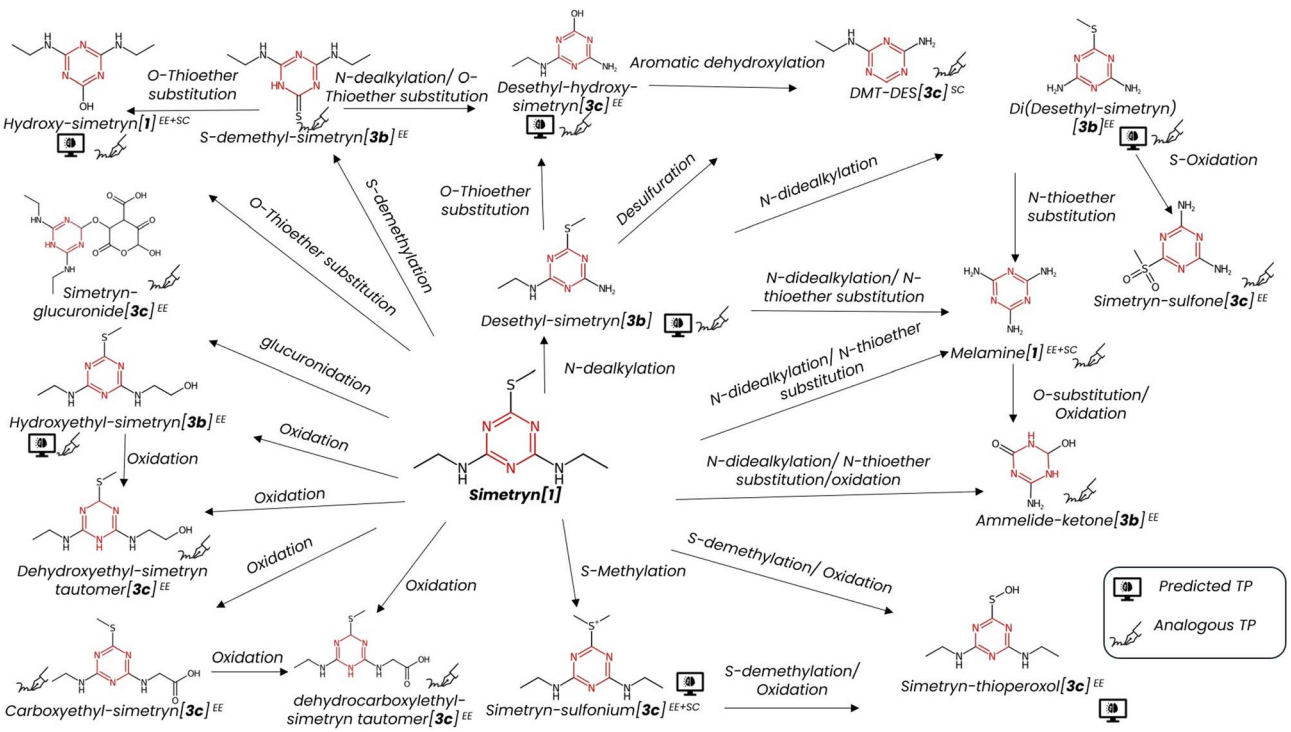

Fig. 3 Proposed simetryn transformation pathway. The persistent substructure (1,3,5-triazine ring) is highlighted in red. EE = exposed embryos; SC = stability controls. Predicted and analogous TPs are marked with symbols (see legend).

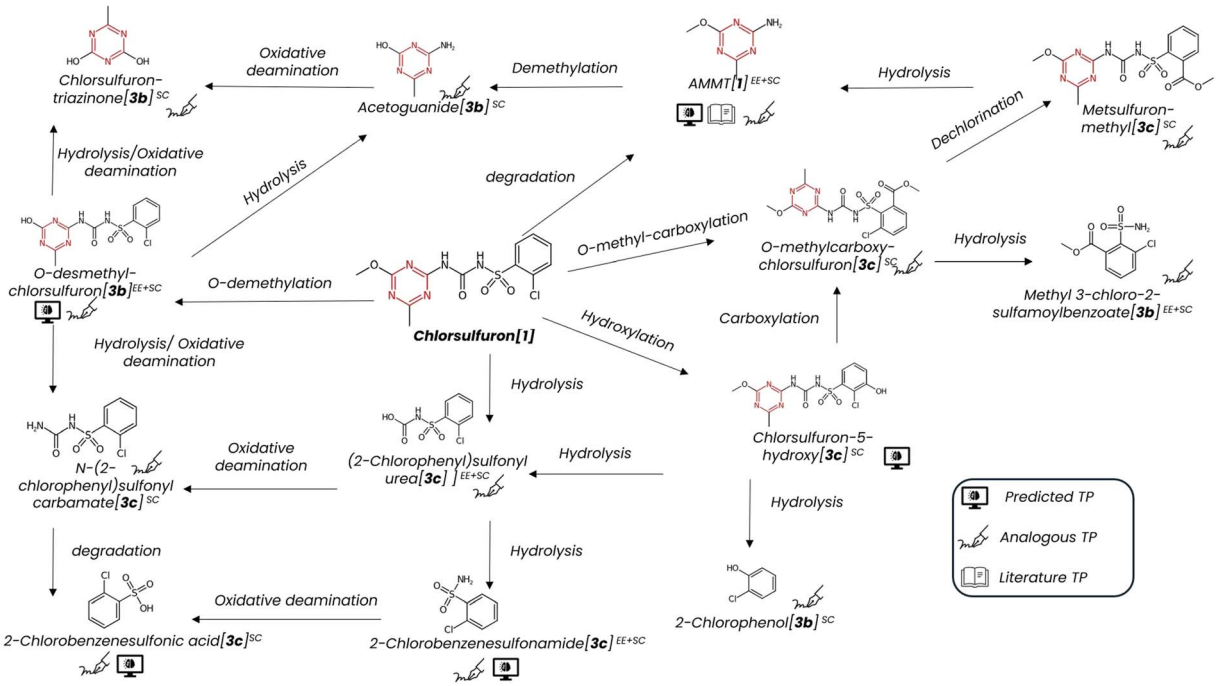

Fig. 4 Proposed chlorsulfuron transformation pathway. The persistent substructure (1,3,5-triazine ring) is highlighted in red. EE = exposed embryos; SC = stability controls. The source of the TP (prediction, literature, analogous) is indicated with symbols (see legend).

modified TPs were also annotated, including sulfoxides, sulfones, and sulfonium species. These results also align with previous reports on microbial, plant and wastewater degradation of methylthio-*s*-triazines.[70,71] *N*-Dealkylated and *N*-didealkylated-TPs were also detected, a typical metabolic reaction for many triazine pesticides including simazine, atrazine and terbutryn.[21,71,72] Evidence of phase II reactions was also seen with the detection of simetryn-glucuronide. Similar reactions have been reported with other *s*-triazines (*e.g.*, terbutryn, ametryn) in mammals and plants.[70,73] This indicates that embryos can mediate phase I and phase II reactions even at early developmental stages. Importantly, all of the TPs retained the 1,3,5-triazine ring, supporting the concept of persistent substructure in the transformation of triazines. It appears that these moieties are resistant to further degradation, suggesting environmental persistence and potential for accumulation.

3.1.2. Chlorsulfuron (triazine). Out of the 13 TPs for chlorsulfuron, one was confirmed at Level 1: 2-amino-4-methoxy-6-methyl-1,3,5-triazine (AMMT). The other 12 were identified at Level 3, with six Level 3b and six Level 3c (Fig. 4). While the majority of the TPs were analogous TPs,[7] some were derived from both literature and BioTransformer predictions[4] or predicted by BioTransformer alone.[1] In addition, the confirmed TP, AMMT, is documented as a literature TP of chlorsulfuron and other related triazine-containing compounds (iodosulfuron and metsulfuron) previously reported in surface water.[74] Most of the TPs were found in the stability controls[8] rather than in both exposed embryo samples and stability controls.[5] This suggests that the transformation of chlorsulfuron is primarily abiotic.

Approximately 50% of the TPs retained the 1,3,5-triazine ring, while the other half resulted from cleaving the sulfonylurea bridge, producing phenyl-sulfonamide-type products. This pattern supports the previously reported two degradation pathways of sulfonylureas, leading to either triazine-derived or phenyl-derived metabolites.[75–77] Such cleavage is specific to the sulfonylurea linkage, under both biotic and abiotic conditions.[78,79] Importantly, for all TPs involving the triazine-containing portion of chlorsulfuron, the triazine ring remained intact, reaffirming its potential as a persistent substructure.

3.1.3. Metconazole (triazole). Three TPs were identified for metconazole. The first, 1,2,4-triazole-1-acetic acid, an analogous TP, was confirmed at Level 1. The second, 2-hydroxy-metconazole, was detected at Level 3b and was both a predicted and analogous TP. The third, 4-hydroxy-dechloro-metconazole, was tentatively identified at Level 3c and was only predicted. The proposed transformation reactions, including ring hydroxylation, hydrolysis, and dechlorination, align with findings from other triazole fungicides, such as difenoconazole and triticonazole.[74,80,81] The dechlorinated TP, 4-hydroxy-dechloro-metconazole, was exclusively detected in stability controls, suggesting that its formation is mainly non-enzymatic. This is supported by evidence of the dechlorination of a similar fungicide, tebuconazole, previously reported in aquatic media.[82] The 1,2,4-triazole ring was retained in all identified TPs (Fig. 5). This indicates structural stability and environmental persistence of the 1,2,4-triazole ring. Some 1,2,4-triazole-containing fungicides have been shown to have

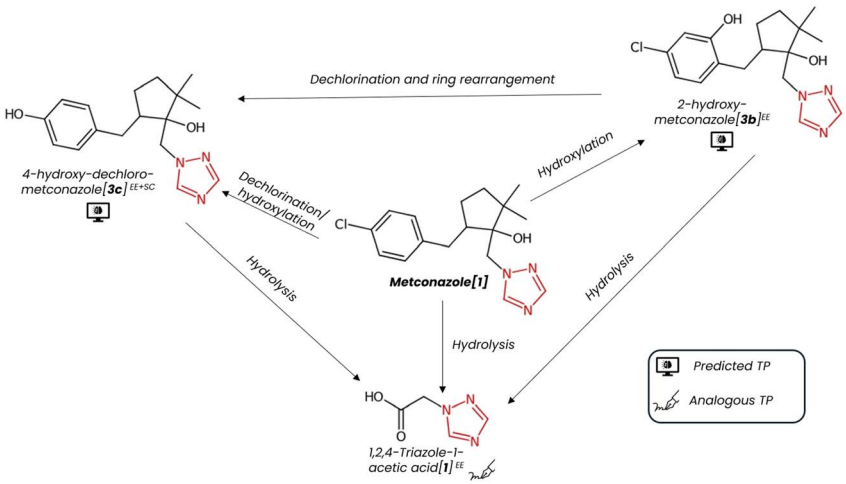

Fig. 5 Proposed metconazole transformation pathway. The persistent substructure is highlighted in red (1,2,4-triazole ring). Represent. EE = exposed embryos; SC = stability controls. Predicted and analogous TPs are marked with symbols (see legend).

endocrine-disrupting properties, showing their potential toxicity to humans and the environment.[9]

3.1.4. Bisphenol AF and ADONA (PFAS). One TP, bisphenol AF glucuronide, was detected at Level 3b for bisphenol AF. This TP was both predicted and drawn from an analogous compound in literature. The glucuronidation is proof of a phase II reaction in the elimination of bisphenol AF, also reported in other bisphenol transformation studies.[83] During this transformation, the trifluoromethyl (CF_3) group was retained in the TP (Fig. 6). For ADONA, one tentative TP was observed and annotated at Level 3c: 1,1,2,2,3,3-hexafluoro-3-(trifluoromethoxy)propan-1-ol or perfluoroether pentanol (Fig. 6). This TP is found in both exposed embryos and stability controls, indicating that it originates from both biotic and abiotic reactions. The limited number of ADONA TPs is consistent with literature describing ADONA/PFECAs as highly persistent and resistant to hydrolysis, direct photolysis, photo-oxidation and biodegradation under typical conditions.[84] In contrast, more degradation pathways for PFECAs are often observed under advanced treatment conditions, such as UV-generated hydrated electrons or strong oxidative systems[85]

3.2. Persistent substructure characterisation for 36 compounds

Of the 36 parent compounds exposed, 33 were included in suspect screening. Three parents: perfluoroethanesulfonic acid (PFES), trifluoromethanesulfonic acid (TFMSA), and 1,2,4-triazole were excluded because they had neither literature TPs nor

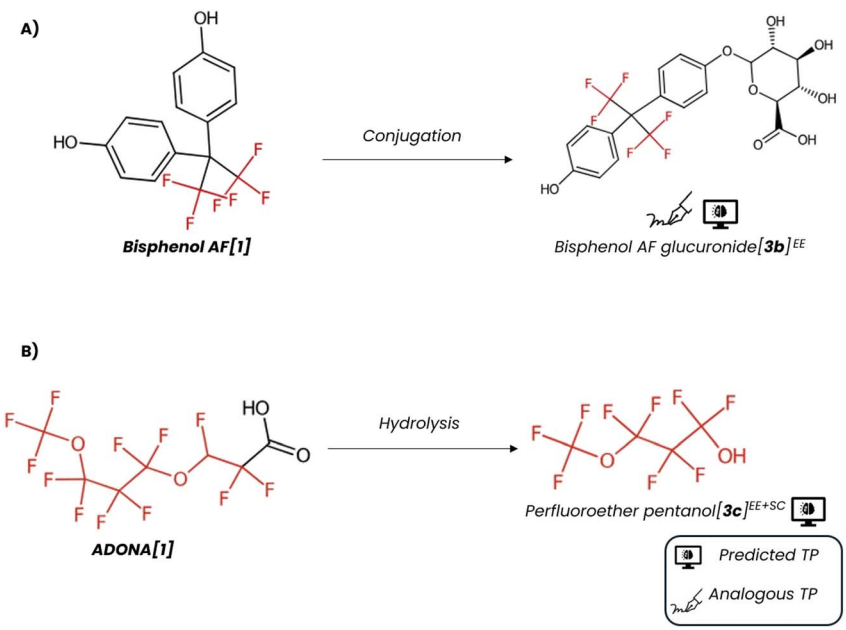

Fig. 6 Proposed transformation pathway for bisphenol AF (A) and ADONA (B). The persistent substructure (CF_2 and CF_3 groups) is highlighted in red. EE = exposed embryos; SC = stability controls. Predicted and analogous TPs are marked with symbols (see legend).

did predictions yield any human or environmental TPs. Across the 33 screened parents, TPs were not observed for 13 compounds. Among PFAS, 5 TPs were identified originating from 4 of the 9 PFAS parents screened. Among triazines, 20 TPs were identified originating from 4 of the 9 triazine-containing parents screened. In the case of triazole-containing compounds, 31 TPs were identified from 12 of the 15 triazole parents screened. In total, 56 TPs were identified from 20 of 33 compounds. The TPs had different sources, originating from literature TPs,[6] BioTransformer TPs,[36] or both,[14] showing the added value of combining both predicted and known data in TP screening experiments. Identification confidence varied from confirmed (Level 1) to tentative (Level 3) across the compound classes (PFAS, triazines, and triazoles). Details of the transformation pathways are given in the SI Table S9 and SI figures Section S5, while the MS/MS information is in SI Table S10.

3.2.1. Triazines. Twenty TPs were identified from four triazine compounds (atrazine, ametryn, deethylatrazine, cyromazine), with details shown in SI figures Section S5.1. Four TPs were confirmed at Level 1, while the rest were tentatively identified. No TPs were found for ammeline, ammelide, benzoguanamine, cyanuric acid, or melamine, which was in line with expectations since they are typically terminal or dead-end TPs for other triazine-containing compounds. For example, ammeline and cyanuric acid are commonly recognised as dead-end TPs of atrazine metabolism.[86,87] The observed transformation reactions involved phase I reactions (deisopropylation, dealkylation, hydrolysis, oxidation, thiol substitution, dechlorination, demethylation, and methylation) and a single phase II glutathione conjugation reaction. This is similar to the reactions observed in simetryn and chlorsulfuron above. Additionally, the same TPs were identified from different compounds. For instance, desmethylthio-desethyl-simetryn (DMT-DES) or dechloro-disopropyl atrazine (DDIA) was identified as a TP for both simetryn and atrazine. Similarly, desethyl-simetryn (or deisopropyl-ametryne) and di(desethyl-simetryn) (or desethyl-deisopropyl ametryn) were detected in both simetryn and ametryn. This demonstrates the consistency of zebrafish embryos in metabolising triazine-containing compounds. It also demonstrates that *N*-dealkylation is a conserved metabolic pathway for structurally related compounds. Interestingly, 100% of the triazine TPs retained the 1,3,5-triazine ring, showing the persistence of the substructure. This has also been previously reported in the degradation of methylthiol-*s*-triazines and chloro-*s*-triazines.[71,88]

3.2.2. Triazoles. In the triazole class, 31 TPs were identified from 12 of 16 parent compounds (SI Fig. S5.2). Three TPs were confirmed, while the others were tentatively detected. Difenoconazole and bitertanol produced the most TPs, with 7 and 6, respectively. No TPs were observed for 1,2,4-triazole, triazole acetic acid, cyproconazole, and tetraconazole. The absence of TPs for the first two is expected, as they are typically recognised as terminal or dead-end TPs of many triazole fungicides.[89] In contrast, cyproconazole and tetraconazole have reported TPs listed in PubChem[55] identified under specific environmental conditions, indicating that the zebrafish embryo might not have suitable metabolic pathways for their transformation, or that any TPs formed were present at concentrations below the analytical limit of detection.

In addition to hydrolysis, dechlorination, and hydroxylation as seen in the metconazole transformation reactions, other phase I reactions were observed, such as oxidation, methylation, and demethylation. Phase II reactions involved glucuronidation and sulfation. These reactions have also been previously reported in triazole degradation and benzotriazole biotransformation studies.[81,90] Furthermore, all seven benzotriazole-derived TPs retained the benzotriazole structure, and of the remaining 23 triazole TPs, 20 preserved the 1,2,4-triazine ring, showing the stability of the benzotriazole and triazole rings. The stability of the triazole ring has also been shown in previous degradation studies, a property that is valuable in the synthetic context.[91,92] 1,2,4 triazole has been reported as being very mobile, with potential toxicity to reproduction.[1]

3.2.3. PFAS. For the PFAS-exposed embryos, 5 TPs were tentatively detected across four parent compounds, including GenX (2), PFPrA (1), PFHxS (1), and TFA (1). PFBS, a documented TP of PFHxS, was the only TP confirmed at Level 1, while the rest were tentatively detected (see SI figures Section S5.3). Phase I transformation was primarily limited to hydrolysis, while Phase II involved conjugation with glycine and taurine. These findings align with the generally limited susceptibility of PFAS to biotransformation. Products conjugated with glycine and taurine have previously been observed in zebrafish.[38,93,94] Importantly, all the TPs retained the CF_3 groups, highlighting the documented persistence of the fluorinated carbon backbone in PFAS.[95,96]

3.2.4. Summary of persistent substructure characterisation. Overall, the results indicate that zebrafish embryos can metabolise diverse compounds from the triazine, triazole, and PFAS classes through phase I and II reactions, confirming their biotransformation capacity even at early developmental stages. For data-poor compounds with two tested exposure concentrations, three of the seven TPs from both concentrations were *N*-dealkylation, with one *S*-demethylation, oxidation, hydrolysis, and glucuronidation, indicating these pathways may be important for monitoring and ecotoxicology. Interestingly, TPs were identified from compounds prioritized due to persistent, mobile (PM) properties, indicating that not all may be persistent, but rather that substructures retained in their TPs seem to be persistent. Some such as deethylatrazine, a known and confirmed TP of atrazine, produced as many TPs[7] as the parent compound. While the metabolic activity varied among compound classes, all persistent substructures (the 1,3,5-triazine ring, 1,2,4-triazole/benzotriazole rings, and CF_3 groups) remained largely conserved in the TPs, highlighting their potential role in environmental persistence. Some parent compounds had no detectable TPs, consistent with them being reported as dead-end or terminal TPs in literature, such as cyanuric acid (from atrazine),[87] melamine (from HMMM),[18] and 1,2,4-triazole and triazole acetic acid (from fluconazole).[89] Similarly, TFA is generally considered a dead-end TP PFAS product, yet a taurine conjugate was also observed, suggesting a possible excretion pathway but also a reversible reservoir of human and environmental contamination. This also suggests

that TFA binding to taurine residues in proteins is a relevant "storage" or bioaccumulation mechanism of TFA. Several terminal TPs have been flagged for regulatory concern, such as 1,2,4-triazole as a PMT substance, and TFA and melamine as vPvM substances,[1] showing their environmental relevance.

3.3. The value of the multi-source TP workflow and custom database

Combining *in silico* predictions, literature-derived analogues, and known literature TPs resulted in better coverage of TPs compared with just one approach. In the initial study involving five selected compounds, 52% of the TPs were identified from analogous TPs. This highlights the importance of incorporating prior knowledge into screening strategies. In the study of 36 compounds, 65% of the identified TPs were due to BioTransformer predictions, highlighting the benefit of incorporating *in silico* predictions into screening strategies, when detailed analogous TPs creation is not feasible. Several literature TPs were also identified and confirmed, validating the workflow and demonstrating the added value of including known TPs, and contributing knowledge to open databases. Furthermore, the multi-source TP approach shows how complementary insights can be derived from different information sources. For instance, while literature TPs aided in screening known TPs, literature-derived analogues were particularly valuable for pesticides like simetryn, chlorsulfuron, and metconazole, as previous studies on similar compounds provided insights into potential transformation pathways. Conversely, BioTransformer identified novel TPs, such as sulfoxide or sulfonium-containing triazines *e.g.*, simetryn-sulfonium, which are rarely described in zebrafish or environmental systems but may form under oxidative conditions.[56,97] Because there is no zebrafish-specific model in BioTransformer, the environmental microbial module and the allHuman models were selected to represent potential environmental and human TPs. Since no MS/MS was obtained for these tentative matches, and reinjection and reanalysis were not possible due to limited remaining extract volume, further investigations in a future study will be needed to confirm the presence of such TPs. Nevertheless, a multi-source strategy enhances TP coverage for this workflow, provides validation through confirmation of known TPs, explores novel pathways and helps identify TPs that may arise under unstudied conditions. This workflow uses a single collision energy (NCE 30), but a future improvement could be to use stepped collision energies to improve MS/MS for better TP elucidation. One of the limitations of this suspect screening workflow is potentially missing unpredicted TPs. Future work could focus on complementary approaches such as full NTS and molecular networking to identify more TPs and persistent substructures, but this was beyond the scope of the current effort.

The use of a custom TP database helped reduce the candidate search space and data processing time, while also expanding the TP coverage of data-poor compounds that were not represented in public libraries. For example, when annotating Bisphenol AF glucuronide, using MetFrag with PubChem generated over 1700 candidates and took more than 1.5 minutes. In contrast, the ZFPMTTPs custom TP database returned a single candidate in a matter of seconds. A limitation of customised databases is that they rely on manual curation, which can impact scalability, especially in fast-paced and larger-scale screening projects. In the future, artificial intelligence (AI) can potentially help by automating repetitive tasks and TP discovery through the analysis of large datasets on reaction pathways, mode of action, and more. Machine learning (ML) and deep learning (DL) approaches could, in principle, predict transformation pathways more effectively than rule-based methods, prioritise TPs based on environmental context, and improve structural annotation by predicting retention times and MS/MS spectra. However, these approaches rely on extensive curated training data (which is currently lacking, as discussed above), and computational resources, while their predictions still require experimental confirmation. This highlights the complementarity of computational and experimental strategies to achieve broader TP coverage and a deeper understanding of micropollutant fate.

3.4. PMT relevance of TPs

The PMT properties of parent compounds and TPs were predicted using the VEGA QSAR toolbox, which integrates various ecotoxicological models (see Section 2.7.3). Most compounds yielded predicted values, but their reliability scores varied from strong experimental support to low-confidence predictions. While most (84%) of the mobility predictions fell within the more reliable range (experimental, good, and moderate), only 12% of persistence predictions and 2.3% of toxicity predictions were in a similar reliability range. As a result, persistence and toxicity predictions discussed below should be interpreted with greater caution. Ideally, all predicted results should be further confirmed with experimental evidence, however this is beyond the scope of the current study.

The predicted ecotoxicological and environmental behaviour of the annotated TPs showed differences in the PMT properties compared to the parents (SI Tables S7 and S9). Most of the TPs were predicted to be persistent and mobile. For instance, 68% of the TPs from the ZFPMTTPs list and 73% of the ZPMS90TPs were predicted to be non readily biodegradable/possibly non readily biodegradable (persistent), using the ready biodegradability model. Among the 36 parents on the ZPMS90 TPs list, 29 of them were predicted for persistence amongst which 26 were predicted to be persistent. All 36 compounds were predicted for mobility amongst which 31 were predicted to be at least mobile. These results align with their selection based on PM properties. Moreover, all the parent compounds that had no TPs were predicted to be persistent. According to the EU CLP criteria for hazard classification using $\log K_{oc}$ values,[2] about 90% of the annotated TPs from both lists were predicted to be at least mobile. For the mobility, the observed patterns align with expectations. For example, the prevalence of more mobile TPs demonstrates that degradation and biotransformation processes typically enhance polarity, improving solubility and elimination. While increased polarity can facilitate elimination

from organisms, it can also increase aqueous mobility and environmental transport, potentially increasing exposure and shifting risk to other organisms and compartments. This reaffirms the argument that TPs can have similar or more hazardous properties than their parent compounds,[5,98] reinforcing the need for regulatory risk assessment of both parent compounds and their TPs. In addition, the repeated emergence of certain TPs such as desethyl-simetryn and di(desethyl-simetryn) shows the importance of considering cumulative exposure from different parent compounds in mixture risk assessment.

According to the EU CLP criteria for the classification of substances hazardous to the aquatic environment,[68] 100% of TPs were predicted to meet the thresholds for classification as chronically toxic to fish (see SI Tables S7 and S9). Among these, some TPs, including *S*-desmethyl-ametryn, deethylatrazine-aldehyde, benzotriazole glucuronide-glycine conjugation, and 1,2,4 triazole-glucuronide, were all predicted to be vPvM and chronically toxic to fish. Interestingly, certain predicted toxic TPs are conjugates, but the low reliability of these predictions highlights the need for better predictive models and further confirmation with experimental data. Some TP reactions can be reversible *in vivo* and in the environment, primarily through phase II deconjugation,[99,100] acting as reservoirs that release parent compounds. This process could increase bioavailability and concentrations of deconjugated TPs, raising ecological concerns. Some *N*-glucuronides are resistant to hydrolysis and enzymatic cleavage, highlighting their potential stability.[101,102]

Two TPs, 2-dechloro-desdioxolane difenoconazole and keto-bitertanol, were predicted to have acute toxicity, whereas their parent compounds, difenoconazole and bitertanol, were predicted to have chronic toxicity 3 and acute toxicity, respectively. PFBS is predicted to have similar toxicity but greater mobility than its parent PFHxS. This supports the evidence that some TPs, including PFAS TPs, could have similar or more concerning PMT properties than their parents, as in the case with PFHxS and its TP PFBS (1). Both compounds are registered in the candidate list of Substances of Very High Concern (SVHC) by the European Chemical Agency (ECHA),[103] indicating their regulatory relevance.

Comparing predicted fish LC_{50} values against available experimental fish LC_{50} endpoints for TPs with public data (n = 10; in SI Table S14) showed that predicted LC_{50} values were generally lower than experimental values (3 out of 10 within 3-fold), indicating a tendency for the QSAR predictions to overestimate toxicity. The largest discrepancies were observed for PFBS (predicted 9.42 mg L^{-1} *vs.* experimental 1938 mg L^{-1}) and 1,2,4-triazole (predicted 17.06 mg L^{-1} *vs.* experimental 498 mg L^{-1}). In contrast, some values were closer, *e.g.*, for 2-chlorophenol (predicted 9.92 mg L^{-1} *vs.* experimental range 8.1–21 mg L^{-1}) and methyl-1*H*-benzotriazole (predicted 26.1 mg L^{-1} *vs.* experimental 55 mg L^{-1}), supporting cautious interpretation of QSAR toxicity outputs as screening-level prioritisation evidence.

Although some TPs have well-documented transformation pathways and toxicological properties, such as AMMT[104] and 2-hydroxyatrazine,[105] several other TPs have little or no known information, including hydroxy-simetryn, which represents a critical knowledge gap that warrants further research. However, the availability of standards for some TPs is limited, posing a major challenge to TP studies. Nevertheless, insights into some transformation reactions of structurally similar compounds may provide prior knowledge of the TP behaviour. As a result of structural similarity between the source TPs and the analogous TPs, the toxicological profile of the source TPs can potentially be read-across to the analogous target TPs, aiding in hazard anticipation and prioritisation.[106,107] AI and ML could also potentially help fill these gaps by improving read-across methods. Similar to the case of the potential use of ML and DL to increase TP identification, the reliability of these AI and ML models depends on the availability of high-quality training data, highlighting the need for more experimental data. The complementary approach may include AI predicting potential metabolic and toxicological properties that experimental methods can later confirm. This process could provide information to facilitate substance prioritisation and regulation.

3.5. Relevance of persistent substructure in TPs

In both studies (*i.e.*, the five selected data-limited compounds and the 36 PM compound studies), the 1,3,5-triazine ring, 1,2,4-triazole ring, and CF_3 group are mostly retained during the transformation of triazines, triazoles, and PFAS, respectively, implying that these structures are persistent substructures in the TPs of these compound classes. These persistent substructures indicate the chemical stability and environmental relevance of both parent compounds and their TPs. In addition to being identified as persistent and mobile, 19 of the 36 parent compounds, including ametryn, difenoconazole, and GenX, have also been recognised as endocrine disruptors,[9] while the immunotoxicity of PFHxS and PFOA has also been shown in zebrafish embryos.[108] The TPs of some of the 36 compounds are predicted to be potentially harmful, with 2-dechloro-desdioxolane difenoconazole predicted to be acutely toxic to fish. This combination of predicted PMT properties raises concerns for the environment and for human health. Moreover, the triazine ring is known for its resistance to oxidative breakdown, while the 1,2,4-triazole ring is resilient against metabolic and photolytic degradation. These properties enable these compounds to evade conventional wastewater treatment processes, raising concerns about their potential human exposure routes through drinking water.[88,109,110] For PFAS, degradation yields shorter chain PFAS, which are often more mobile and of equal or greater concern to drinking water resources.[95,111] Thus, persistent substructures can serve as "structural alerts" or markers for persistence, mobility and potential toxicity.

The potential of persistent substructures as a cheminformatics-based grouping strategy for managing PMT substances has been previously reviewed,[5] although without experimental confirmation of these substructures, as in this study. Knowledge of persistent structures could have various purposes, including flagging compounds in regulatory dossiers for P and M assessment of parents and TPs, and guiding the

prioritisation of chemical groups under the PMT/vPvM assessment frameworks. These could also be used to improve predictive read-across for hazard assessment, assuming that TPs sharing these substructures are likely to exhibit similar modes of action, chronic effects, environmental persistence and mobility. However, over 800 000 substances contain the 1,3,5-triazine ring, more than 900 000 contain the 1,2,4-triazole ring, and more than 7 million contain CF_2 and CF_3 groups in PubChem, highlighting the sheer number of compounds that contain these substructures and the potential PMT/vPvM regulatory challenges that arise.[5] Although grouping compounds based on persistent substructures alone cannot solve this problem, it could provide a practical approach to streamline the assessment process. This method would allow for collective evaluation of compounds, which may aid in prioritising them under PMT/vPvM frameworks and facilitate consideration of mixture toxicity. In addition to persistent substructure, use cases can also be used to prioritise compounds for PMT assessment. For example, if compounds have the same persistent substructure, such as a 1,3,5-triazine ring, and are used for the same purpose, such as pesticides, they may produce TPs with similar PMT properties. Knowledge of persistent substructures can also guide the development and testing of more effective remediation methods. For instance, recognition that PFAS molecules resist oxidative methods directs the development of specialised technologies such as electrochemical or plasma degradation, which are more effective in the degradation of PFAS.[96,112] Moreover, recognising these persistent substructures could help inform safer chemical design. Avoiding the incorporation of persistent substructures into new compound manufacturing could reduce the risk of creating more persistent and mobile compounds in the future. This is in line with the Safe and Sustainable by Design framework recommended by the European Commission.[113,114]

4. Conclusion

This study uses multiple sources for TPs, including predicted TPs, analogous TPs from literature, and literature TPs, to investigate the biotransformation of PM substances using zebrafish embryos. This approach enhanced the coverage and annotation of TPs, generating new TP data for poorly characterised compounds. In addition, it confirms known TPs but also explores novel pathways and identifies new TPs that may arise under biotransformation, expanding understanding of the fate of organic contaminants in the environment. Additionally, the study highlights the importance of conducting risk assessments for both parent compounds and TPs, as many of the latter were found to be potentially more persistent, mobile, and toxic than their parent compounds.

Retained moieties in transformation products, also known as persistent substructures, include components like the 1,3,5-triazine ring, 1,2,4-triazole/benzotriazole rings, and CF_3 groups. These moieties were consistently found in identified TPs derived from triazine, (benzo)triazole, and PFAS parent compounds, respectively. This suggests their chemical stability and environmental significance. These substructures can act as structural markers for persistence, mobility, and potential toxicity. This has broader applications, including the grouping and management of PMT substances, conducting read-across for hazard determination and prioritisation under PMT/vPvM assessment frameworks, and guiding the development of effective remediation strategies and safer chemical designs.

These findings enhance the understanding of how PMT-relevant substances undergo biotransformation and demonstrate the importance of combining experimental and computational approaches to anticipate and manage risks associated with TPs in aquatic systems. This study identified 29 compounds that were not in the PubChem database at that time (SI Table S11) and 57 candidate TPs that were not listed as TPs at that time. These compounds (see SI Table S12 for transformation pathway) and MSMS of measure standards (SI Table S13) were uploaded to the NORMAN-SLE S74 REFTPS list (including confidence levels and metadata) for integration within the NORMAN-SLE and PubChem, thereby expanding the known range of TPs and sharing information for future validation, monitoring, and risk assessment.

Ethical statement

All practices involving zebrafish were performed in accordance with European laws, guidelines and policies for animal experimentation, housing and care (European Directive 2010/63/EU on the protection of animals used for scientific purposes). According to Article 3 of the Directive 2010/63/EU and to Annex II (Commission Implementing Decision 2012/707/EU), the present study did not involve the reporting of procedures and animals (*i.e.* zebrafish was used before 5 days post-fertilization) and as such it was not subjected to authorization by an ethics committee. The Zebrafish Facility at the Luxembourg Centre for Systems Biomedicine is registered as an authorized breeder, supplier and user of zebrafish with Grand-Ducal decree of 03 April 2025, and the Zebrafish Facility at the Vrije Universiteit Amsterdam has approval number NVWA/2024/010089717.

Author contributions

PC: conceptualisation, data curation, investigation (lead), methodology, visualisation, writing original draft (lead), writing review and editing, MLCM: methodology, supervision, writing review and editing, MPC: investigation (support), methodology, writing review and editing, TH: funding acquisition, supervision, writing review and editing, ELS: conceptualization, data curation, funding acquisition, investigation, supervision, writing original draft, writing review and editing.

Conflicts of interest

The authors state that there are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). The raw data is deposited

in Zenodo under DOI: 10.5281/zenodo.17234821 (https://doi.org/10.5281/zenodo.17234821). The R-scripts used in the manuscript can be found in GitLab (https://gitlab.com/uniluxembourg/lcsb/eci/Zebrafish_PMT_TPs). The S90 ZeroPMBox1 compounds are available on Zenodo under DOI: 10.5281/zenodo.5854251 (https://doi.org/10.5281/zenodo.5854251). The FAIR data can be found in S74 REFTPS version 0.25.1 under DOI: 10.5281/zenodo.19023648 (https://doi.org/10.5281/zenodo.19023648). Supplementary information: figures and text in a PDF, and all tables (Tables S1–S13) in an Excel file. See DOI: https://doi.org/10.1039/d5em00825e.

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