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Application of a novel prioritisation strategy using non-target screening for evaluation of temporal trends (1969–2017) of contaminants of emerging concern (CECs) in archived lynx muscle tissue samples

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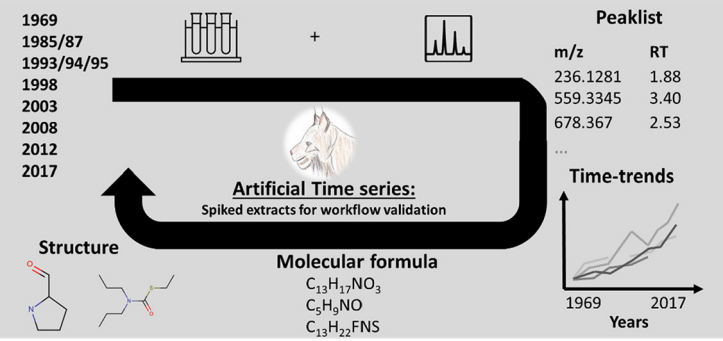
HIGHLIGHTS

- Prioritisation strategy for non-target screening with LC-HRMS using time-trend analysis
- Validation based on spiked extracts with known contaminants (n = 272)
- Analysis of Eurasian lynx (*Lynx lynx*) muscle tissue from 1969 to 2017
- Tentatively identification of 2 compounds with increasing and 1 with decreasing trend

GRAPHICAL ABSTRACT



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ABSTRACT

Most environmental monitoring studies of contaminants of emerging concern (CECs) focus on aquatic species and target specific classes of CECs. Even with wide-scope target screening methods, relevant CECs may be missed. In this study, non-target screening (NTS) was used for tentative identification of potential CECs in muscle tissue of the terrestrial top predator Eurasian lynx (*Lynx lynx*). Temporal trend analysis was applied as a prioritisation tool for archived samples, using univariate statistical tests (Mann-Kendall and Spearman rank). Pooled lynx muscle tissue collected from 1969 to 2017 was analysed with an eight-point time series using a previously validated screening workflow. Following peak detection, peak alignment, and blank subtraction, 12,941 features were considered for statistical analysis. Prioritisation by time-trend analysis detected 104 and 61 features with statistically significant increasing and decreasing trends, respectively. Following probable molecular formula assignment and elucidation with MetFrag, two compounds with increasing trends, and one with a decreasing trend, were tentatively identified. These results show that, despite low expected concentration levels and high matrix effects in terrestrial species, it is possible to prioritise CECs in archived lynx samples using NTS and univariate statistical approaches.

1. Introduction

Contaminants of emerging concern (CECs) are anthropogenic chemicals such as pharmaceuticals, per- and polyfluoroalkyl substances (PFASs), flame retardants, personal care products and industrial chemicals (Menger et al., 2020). Most ecotoxicological studies of CECs have focused

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on aquatic species (e.g., Huerta et al., 2012; Wang et al., 2021; Álvarez-Ruiz and Picó, 2020; Diamanti et al., 2020), while terrestrial species are rarely studied (e.g., Bao et al., 2015; Gómez-Ramos et al., 2019; Tue et al., 2021). Terrestrial species are often less exposed to CECs than aquatic species, which makes CEC exposure studies of terrestrial ecosystems more challenging (Moore, 1966; Mateo et al., 2012). However, data on CECs from the terrestrial abiotic environment and its wildlife may provide additional information regarding contaminant levels, patterns, and trends, and thus give further insights into the environmental transport, deposition patterns and fate of CECs (Mariussen et al., 2008). A majority of studies focusing on anthropogenic organic pollutants in the terrestrial environment have targeted regulated persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs) and pesticides (e.g., insecticides, rodenticides, fungicides) (e.g., Mateo et al., 2012; Gómez-Ramírez et al., 2014), while a few studies have also considered CECs (e.g., Mariussen et al., 2008; Gómez-Ramírez et al., 2014; Falk et al., 2019; Badry et al., 2020; González-Rubio et al., 2021).

Time-trend analyses of ecotoxicologically relevant pollutants in biota commonly apply target analysis and focus on a rather limited number of well-known pollutants (Bjurlid et al., 2018; Sun et al., 2020), while other potentially harmful substances are overlooked (Sonne et al., 2020). However, advances in environmental analytical chemistry have opened the way for novel approaches (Menger et al., 2020; Hollender et al., 2017). Wide-scope target screening is well established, while suspect screening approaches have become more sophisticated (Diamanti et al., 2020; Alygizakis et al., 2018). Methods and different approaches for nontarget screening (NTS) workflows of biota have also seen substantial recent progress (Heffernan et al., 2017; Millow et al., 2015; Myers et al., 2014; Shaul et al., 2015; Du et al., 2017; Baduel et al., 2015; Dürig et al., 2020; Dürig et al., 2022; Rebryk and Haglund, 2021). Together, wide-scope target screening, suspect screening and NTS can identify predominant chemical mixtures in biota, helping scientists and risk assessors prioritise substances for toxicity assessment and risk management measures (Alygizakis et al., 2019a; Hollender et al., 2019). In this study, NTS was applied for identification of CECs without any prior knowledge of their occurrence in terrestrial biota.

National and international contaminant monitoring programmes, such as the National Swedish Contaminant Monitoring Programme (Odsjö, 2006) and the Artic Monitoring and Assessment Programme (AMAP), consider raptors and other predators especially suitable for monitoring of persistent, bioaccumulative and toxic (PBT) chemicals (Sergio et al., 2005). Two recent studies highlight the importance of broad chemical monitoring of raptors in relation to chemical regulations (Movalli et al., 2019; Movalli et al., 2017). Raptors and other predators have measurable responses to PBT chemicals, ranging from residue accumulation to population decline (Gómez-Ramírez et al., 2014; Sergio et al., 2005; Vos et al., 2000; Olsson et al., 1992). This is particularly the case for predators in aquatic food chains, but has also been suspected of terrestrial species, e.g., a decrease in population size for reintroduced Eurasian lynx (*Lynx lynx*) in Europe has been observed (Sindičić et al., 2013). Exposure to legacy POPs, such as pesticides and PCBs, has been demonstrated for Iberian lynx (*Lynx pardinus*) in a study in Spain (Mateo et al., 2012). In addition, conventional flame retardants have been found in liver tissues of Eurasian lynx in Norway (Mariussen et al., 2008). However, only few studies have investigated CECs in terrestrial top predators such as lynx (González-Rubio et al., 2021).

In this study, NTS is applied for the first time to the top predator Eurasian lynx, using high-resolution mass spectrometry (HRMS). Archived lynx tissue samples (1969–2017) were analysed with a previously developed NTS prioritisation strategy, using time trend analysis for prioritisation. Specific objectives of the study were to: i) assess temporal trends for selected target compounds, ii) apply a recently developed prioritisation and NTS workflow to lynx muscle tissues, and iii) tentatively identify CECs with increasing and decreasing temporal intensity trends in lynx muscle tissue over the past 50 years.

2. Materials and methods

2.1. Biota samples and storage

Muscle tissue samples from Eurasian lynx were obtained from the Environmental Specimen Bank (ESB) at the Swedish Museum of Natural History (SMNH). The tissue samples were collected during 1969, 1985/1987, 1993/1994/1995, 1998, 2003, 2008, 2012, and 2017, mainly from animals killed by hunting and traffic (Table SI1 in Supplementary Information (SI)). The ESB applied standardised protocols for sampling and storage at −80 °C (Odsjö, 2006). After arrival at the laboratory, the wet samples obtained for the analysis were stored at −20 °C until extraction, which occurred within a couple of days of arrival at the laboratory.

Selection criteria for the sample pools were: (i) availability of individual samples per sampling year, (ii) close proximity of sample locations, (iii) similar sex ratio per sampling year, and (iv) preferably adult animals. This resulted in the selection of animals collected from Jämtland, Häredalen and Dalarna, which are Swedish regions bordering to each other. Due to limited sample availability, lynx tissue samples from 3 to 10 individual animals per year or for a span of a few years were pooled, except for 1969, which was represented by one individual only. Individual samples from 1985 to 1987 and 1993 to 1995 were combined (Movalli et al., 2019). Thus, eight pooled samples were prepared for analysis, representing the years 1969 (1 individual), 1985–1987 (3 individuals), 1993–1995 (6 individuals), 1998 (11 individuals), 2003 (5 individuals), 2008 (10 individuals), 2012 (10 individuals) and 2017 (10 individuals) (Table SI1 in SI).

2.2. Chemicals

A total of 272 target CECs and 65 isotopically labelled compounds (IS) were used for spiking and quality control experiments (Tables SI2 and SI3 in SI). The selected target CECs represented pharmaceuticals (n = 107), pesticides (n = 92), flame retardants (n = 15), PFASs (n = 14), industrial chemicals (n = 12), personal care products (n = 8), phthalates (n = 6), food additives (n = 3), isoflavones (n = 3), fatty acids (n = 3), benzotriazoles (n = 2), siloxane (n = 2), surfactants (n = 2), stimulants (n = 2) and contrast media (n = 1). The reference compounds were obtained from Sigma-Aldrich (Steinheim, Germany), European Pharmacopeia Reference Standard (Strasbourg, France), Teknolab Sorbent (Kungsbacka, Sweden), USP Reference standard (USA), BOC Sciences (Shirley, NY) and Supelco (Bellefonte, PA), and were of high purity (>85%).

2.3. Sample preparation

Sample preparation was performed in triplicate at room temperature in a fume hood using a previously validated method (Dürig et al., 2020; Dürig et al., 2022). In brief, for pooling, 0.1 to 1 g portions of muscle tissue from each individual lynx collected in the same year or span of years were weighed into 15 mL homogenisation tubes with ceramic beads, to yield approximately 1 g total wet weight (ww). The pooled samples were homogenised without solvent in a Precellys tissue homogeniser (Bertin Technologies, France). Before extraction, 50 ng of each IS were added and the solvent was left to evaporate at room temperature for 30 min. Then, 1 mL acetonitrile + 0.1% formic acid was added to the homogenisation tubes and the samples were extracted twice for 40 s at 5000 rpm in the Precellys tissue homogeniser. After centrifugation and filtration through a 0.2 μm regenerated cellulose syringe filter (Thermo Scientific, Rockwood, USA) into 2 mL Eppendorf safe-lock tubes (Eppendorf AG, Hamburg, Germany), aliquots were frozen at −20 °C for at least 16 h to denature the proteins as well as to remove lipids, waxes, sugars, and other compounds with low solubility in acetonitrile (Payá et al., 2007). Following centrifugation for 3 min at −10 °C, aliquots of 200 μL were transferred to auto-injector vials and used for analysis. As IS were added to all samples, the data could also be used for target analysis and quantification based on the biota weight up to the lower ng g^{-1} ww concentration range (Dürig et al., 2020; Grabicova et al., 2015; Grabicova et al., 2018).

An artificial time series (ATS) was prepared from a homogenised pool of 0.3 g ± 0.1 g portions of each individual lynx muscle tissue collected for the study (except 2017). The homogenised tissue was split between 15 homogenisation tubes (15 mL each), each containing 0.8 g ww on average, and spiked in triplicate with IS mixture (50 ng) and 272 target compounds at a level of 5, 10, 15, 25, 50 and 75 ng. Extraction was performed in the same way as for the other samples. This ATS was used to evaluate losses of target analytes during each step of the workflow and therefore the performance of the NTS workflow.

2.4. Instrumental analysis by UPLC-QTOF-MS

Instrumental analysis was performed using ultra-performance liquid chromatography quadrupole-time-of-flight mass spectrometry (UPLC-qToF-MS) (Xevo G2-S, Waters Corporation, Milford, MA), as reported in previous studies (Dürig et al., 2020; Dürig et al., 2022; Tröger et al., 2018). In brief, analytes were separated using a Waters Acquity I-Class UPLC system equipped with a quaternary pump. For chromatographic separation in positive ionisation mode, a reversed-phase Acquity UPLC HSS T3 column (2.1 mm × 100 mm and 1.8 μm; Waters Corporation, Milford, MA) was used, while for negative ionisation mode an Acquity UPLC BEH column (2.1 mm × 100 mm and 1.7 μm; Waters Corporation, Milford, MA) was used. Detailed information about the UPLC gradient can be found in Table SI4 in SI. The UPLC was coupled to a Xevo G2-S QToF-MS (Waters Corporation, Manchester, UK) with an electrospray ionisation (ESI) interface working in positive and negative ionisation modes. All data were collected in separate injections for positive and negative ionisation mode using data-independent resolution mode (MS^E-resolution) with low collision energy at 4 eV and a high collision energy ramp from 10 to 45 eV at a mass range of 100–1200 *m/z*. Leucine encephalin was continuously infused for lock mass correction (for details, see Table SI5 in SI). The software UNIFI Waters Scientific Information System (v 1.9.4) was used for instrument control and for identification of compounds during the target analysis step, by searching for $[M + H]^+$ and $[M - H]^-$ adducts with one absolute charge for adduct combinations and 3 mDa mass tolerance.

A solvent calibration curve containing the IS mixture (50 ng mL^{-1}) and the 272 target compounds was prepared in acetonitrile at concentrations of 0.5, 5, 10, 25, 50 and 75 ng mL^{-1} and was injected multiple times throughout the analytical run.

2.5. Quality assurance and quality control (QA/QC)

For calculation of matrix effects and quality control, a quality control sample was prepared by pooling 50 μL extract from each (pooled) year or span of years. This quality control sample was injected multiple times throughout the chromatographic sequence to monitor the performance of the LC-qToF system. For matrix effect calculations, matrix-matched standards were prepared from the quality control sample by adding the 272 target compounds in three different concentrations (10, 50, 100 ng mL^{-1}) after extraction (Fig. SI1 in SI). For absolute recovery calculations, the matrix-matched standard (50 ng mL^{-1}) was compared with a sample fortified with the 272 target compounds (50 ng mL^{-1}) before extraction (Fig. SI1 in SI). Solvent blanks consisted of pure solvent injections as well as procedural blanks (acetonitrile).

2.6. Data handling and statistical time-trend analysis

An appropriate pre-processing workflow must be applied to obtain high-quality data (Dürig et al., 2022; Hohrenk et al., 2019; Pochodylo and Helbling, 2017). The data acquired in this study were handled with a validated workflow (Dürig et al., 2022). In brief, raw data were recorded using the vendor software UNIFI Waters Scientific Information System (version 1.9.4) and converted and exported to *mzML* format via ProteoWizards' MSConvert (version 4.7.2) open-source software, with an intensity cut-off of 100. The data were processed using an automated workflow described elsewhere (Alygizakis et al., 2019b) to obtain a peak list of aligned features (*m/z*, retention time and intensity) across the mass ranges and retention times. From the quality control sample that was injected multiple times throughout the run (after every six to nine matrix injections), a gradual decrease in sensitivity was observed for the spiked IS. Intensities of all detected features were corrected for the average sensitivity loss of all IS, setting the average IS response detected at the first time point of the time series to 100% (Fig. SI2 in SI). When analysing complex samples with heavy matrix, the drift in the signals due to instrumental restrictions can be challenging and sometimes lead to the escape of chemicals and the generation of artefacts. Features were only considered if: i) their peak intensity was at least 10-fold higher than that of the solvent blank injections (if present in the blank), ii) they were present in at least two of the three replicates, and iii) they had relative standard deviation (RSD) in intensity of less than 50% across the triplicates. Finally, time trend analysis was performed using R (v 4.0) software (R Core Team, 2021) for prioritisation of increasing and decreasing features, with the average response of each year/span of years and two different statistical tests, Spearman rank and Mann-Kendall tests (Plassmann et al., 2018). Low sample size has significant implications for many statistical tests including the Mann Kendal statistical test. Therefore, the application of these tests must be done in a cautious way. Non-parametric tests were chosen instead of parametric tests as no knowledge or assumption of data distribution exists. Features with an increasing intensity trend at significance level $\alpha = 0.05$ and ρ (rho-value) > 0.8 were prioritised as these features could possibly be concerning compounds due to their increasing presence. Features with a decreasing intensity trend at significance level $\alpha = 0.05$ and ρ (rho-value) < -0.8 were prioritised as well for proof of concept to identify compounds with decreasing trends such as legacy pollutants (Falk et al., 2019; Sun et al., 2020; Sturm and Ahrens, 2010). Probable molecular formulas for prioritised features were predicted using Waters Corporation software UNIFI (version 1.9.4). Further elucidation was carried out in the online MetFrag Tool using the probable predicted molecular formula for candidate collection (Ruttkies et al., 2016). PubChem was used as a search database for patents and reference counts, candidates were retrieved with a mass error of 5 ppm, and $[M + H]^+$ and $[M + H]^-$ adducts were searched for features prioritised in positive and negative ionisation mode, respectively. The candidates were then scored within MetFrag using the patent and reference counts in PubChem and the *in-silico* fragment score, which was based on the experimental high-collision energy spectra (45 eV spectra from the collision energy ramp). Fragmenter score, patent counts, and PubMed reference counts were weighted equally (1:1:1), candidates with the highest MetFrag score were considered when evaluating the candidates individually. The final identification status was assigned based on all available information. A reference standard for S-ethyl dipropylthiocarbamate (EPTC) was ordered and added to the extract of the year 2017 at different concentrations (50 ng mL^{-1} and 200 ng mL^{-1}) for confirmation.

3. Results and discussion

3.1. Time trend of targeted CECs

Target screening of all 272 target compounds was performed in the lynx muscle time series. A total of 19 compounds were detected at more than six time points (Fig. 1 and Fig. SI3 in SI). These belonged to the compound classes personal care products, surfactants, fatty acids, industrial chemicals, phthalates, pharmaceuticals, pesticides, and flame retardants. Out of the 19 compounds, 7 showed a statistically significant decreasing trend (linear regression $p < 0.05$, and $R^2 > 0.8$), whereas for 12 compounds no significant trend was observed. For example, a strikingly steep decline was observed of two pesticides and two flame retardants (i.e., fenitrothion, pendimethalin, tributyl phosphate (TNBP), triphenyl phosphate (TPHP)) (Fig. 1). The decline may be a result of growing awareness of these CECs and/or implementation of risk measures (de Wit et al., 2020). This creates a need for new tools to detect CECs with increasing time trends in the terrestrial environment. NTS with time trend prioritisation in archived biota can shed light on such CECs (Dürig et al., 2022).

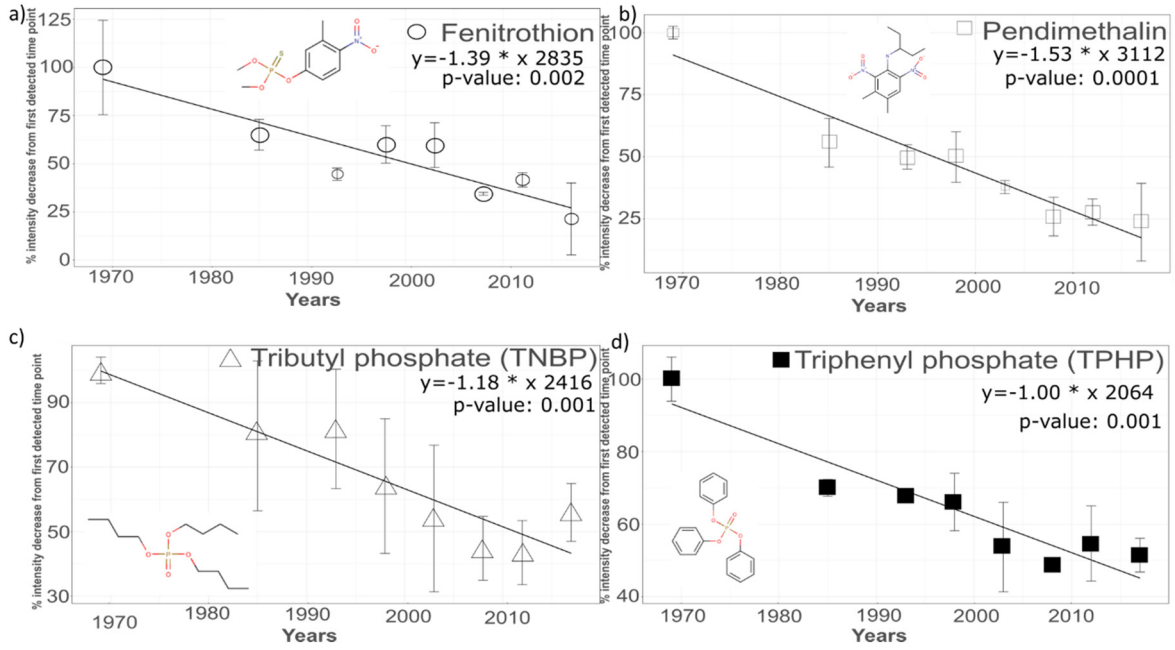


Fig. 1. Time trends in identified target compounds displaying statistically significant (linear regression $p < 0.05$, $R^2 > 0.8$) decreasing trends in lynx muscle samples from 1969 to 2017, given as average % peak intensity decrease from the first detected time point. Error bars represent standard deviation of triplicate samples. a) Fenitrothion (pesticide), b) pendimethalin (pesticide), c) tributyl phosphate (TNBP) (flame retardant), and d) triphenyl phosphate (TPHP) (flame retardant).

3.2. Evaluation of prioritisation strategy using an artificial time series

Of the 272 spiked target compounds in the artificial time series (ATS), 260 targets were detectable at concentration 75 ng mL^{-1}. All spiked target compounds were treated as non-target features, and retrospective checking was performed to determine which features belonged to the spiked target compounds. Detection failure of 12 of the spiked compounds could be due to matrix suppression and/or low recovery. A limited number (n = 157) of the compounds were detectable in the extract with the lowest concentration (5 ng mL^{-1}). Temporal trend analysis of the ATS (5 to 75 ng mL^{-1}) was used for validation of the prioritisation of features with increasing trends (Fig. SI4 in SI). A total of 24,013 features were detected in the ATS. After blank subtraction and considering data handling criteria features present in at least two of three replicates and with RSD <50% in peak intensity, 5994 features were used for trend analysis with univariate statistics (Mann-Kendall and Spearman rank). For a statistically significant trend to be prioritised, at least three time points needed to be detected of the ATS (i.e., 10, 15, and 25 ng mL^{-1}), which was fulfilled by 182 target compounds. A significant positive correlation ($R^2 > 0.8$, $p < 0.08$) was observed for 128 features (28 targets) with both statistical methods. The p-value was set to <0.08 instead of <0.05 as otherwise just one feature would have been prioritised. A noteworthy feature reduction at this step of the workflow agrees with results from previous studies applying strict selection criteria on data acquired from matrix-rich extracts (e.g., Dürig et al., 2022; Hohrenk et al., 2019; Purschke et al., 2020). Interestingly, all 128 prioritised compounds were detected in positive ionisation mode, indicating less sensitivity in the negative ionisation mode using UPLC-qToF-MS (Xevo G2-S) (Cech and Enke, 2001). This observation agrees with results from a previous study of white-tailed sea eagle samples, where 82% of 126 prioritised features were detected in positive ionisation mode using the same instrumental set-up (Dürig et al., 2022). A probable l molecular formula with iFit value > 50% using Waters Corporation software UNIFI (version 1.9.4), was assignable to 38 features (15 targets). The loss of nearly half of the target compounds (from 28 to 15 targets) during this step was possibly attributable to low intensity and high influence of matrix on the mass spectra, which resulted in unreliable molecular formula prediction by UNIFI. The loss was improved by considering a cut-off with iFit >33%, resulting in 52 features (19 targets) with a probable molecular formula. MetFrag was then used to match experimental data and predicted fragment patterns of highly cited and patented chemicals, which allowed tentative identification of 40 structures (14 targets). The loss of only a few target and non-target compounds during this step compared with previous steps indicates that after molecular formula assignment, relatively clean mass spectra remained, which could in turn be used for fragment matches in MetFrag. Combining the prioritisation and elucidation workflow described above demonstrated that 14 of the 182 theoretically possible detectable compounds in the ATS could be tentatively identified when treated as non-targets. The low detection rate of target compounds can be explained by matrix effects and low sensitivity of the target compounds. The number of targets identified has been deemed previously to be acceptable for a NTS identification workflow in biota (Dürig et al., 2022; Pochodylo and Helbling, 2017) and our results here are comparable to previous studies (Dürig et al., 2022; Alygizakis et al., 2019b). However, the major challenge with matrix effects and their implications for detecting rather small changes for individual CECs in time series remain. Recent research has addressed the challenges and potential solutions for minimizing and/or compensating for the matrix effects in the analysis of organic micropollutants of different environmental samples (Svan et al., 2018) with supercritical fluid chromatography as primary suggestion for improvements. Future studies should identify interferences sources as even supercritical fluid chromatography showed matrix suppressions and enhancements (Svan et al., 2018).

A strong matrix effect (on average −41% for pharmaceuticals (n = 101) and −41% for pesticides (n = 94)) was observed for the method applied (Fig. SI1 in SI), explaining the low rate of identified target compounds in the ATS. Previous studies have highlighted the importance of minimizing sample pre-treatment and clean-up for NTS to be non-specific and extract a broad range of compounds (Myers et al., 2014; Shaul et al., 2015). However, the drawback, in particular for biota samples, is that this may lead to strong matrix effects, thus reducing the sensitivity, potentially compromising mass accuracy and increasing the number of background masses in the chromatogram, which in turn complicates identification of compounds using NTS (Heffernan et al., 2017; Dürig et al., 2020; Dürig et al., 2022). It is therefore challenging to find a compromise between extensive sample preparation to reduce matrix effects and minimal sample preparation to avoid losing non-target compounds. The results from the artificial time series and application of the NTS workflow showed that only a

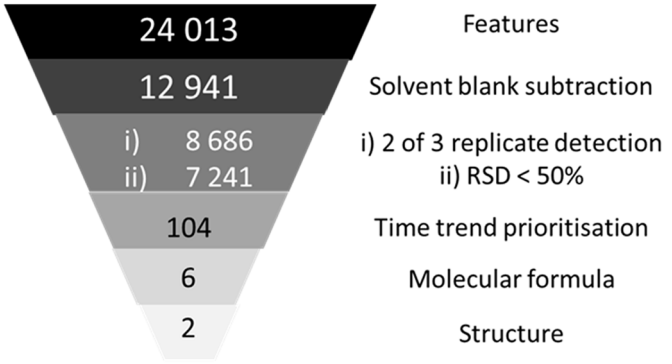


Fig. 2. Number of features in the lynx time series samples during each step of the data treatment workflow identifying contaminants of increasing concern with increasing time trends.

small fraction of target compounds (8%) was prioritised and tentatively identified, suggesting a need for improved sample preparation methods without losing compounds.

3.3. Time-trend prioritisation and elucidation

A NTS workflow using time trend prioritisation was applied (Dürig et al., 2022), with the slight modification of iFit cut-off >33%, to the real lynx time series (1969–2017) (Fig. 2). After blank subtraction, 12,941 features were obtained, which was a considerably lower number than in a previous NTS study of white-tailed sea eagle muscle tissue (26597), where the same workflow was applied (Dürig et al., 2022). This was probably due to the lower levels of CEC contamination in terrestrial lynx compared with white-tailed sea eagle. It should also be noted that the white-tailed sea eagle samples analysed in Dürig et al. (Dürig et al., 2022) were from individuals mainly feeding on marine (Baltic Sea) prey, thus reflecting biomagnification levels from a highly polluted marine environment (Purschke et al., 2020; Miller et al., 2013). Setting the requirements of detection in at least two of the three replicates (4255 removed) and RSD < 50% (5700 removed) reduced the number of features to 7241. Finally, a total of 264 features were prioritised using univariate statistical approaches (160 by Spearman rank only, none with Mann-Kendall only, 104 with both statistical approaches) (Fig. 2, Table SI6 in SI). Sen's slope can be further used to evaluate the strength of the trend (Mama et al., 2021). For elucidation, only features prioritised with both statistical approaches were considered. Of the 104 prioritised features, the majority (93%) were detected in positive ionisation mode, indicating lower sensitivity in negative ionisation mode (Dürig et al., 2022). Visual inspection of the prioritised features showed that a high incidence of split or double peaks led to difficulties in assigning molecular formula, resulting in less tentatively identified features. In addition to peak shape problems, most prioritised features had high measured molecular mass (on average 737 Da, range 100–1187 Da), hampering their elucidation. Some of these features are likely substances co-eluting with endogenous compounds such as proteins and fatty acids (Plassmann et al., 2018).

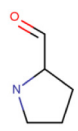
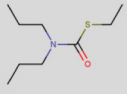
For six of the 104 features prioritised (6%), it was possible to assign a probable molecular formula with iFit value >33%. Comparison of the experimental high-collision energy spectra for those features with MetFrag (Ruttkies et al., 2016) resulted in two tentatively identified structures (pyrrolidine-2-carbaldehyde and S-ethyl dipropylthiocarbamate; Table 1), which have natural and anthropogenic origin, respectively (see Section 3.4).

The workflow was also applied to prioritise features with a decreasing intensity trend, as proof of concept to identify compounds with decreasing trends such as legacy pollutants (Falk et al., 2019; Sun et al., 2020; Sturm and Ahrens, 2010). A total of 93 features were prioritised using univariate statistical approaches at significance level $\alpha = 0.05$ and $\rho > -0.8$ (93 features by Spearman rank in total, of which 61 features were in overlap with Mann-Kendall, and no additional features were prioritised with Mann-Kendall only) (Fig. SI5 in SI). For elucidation, only features prioritised with both statistical approaches were considered. For 11 of these 61 features (18%), it was possible to predict a probable molecular formula with iFit value >33%. More probable molecular formulae were assignable to features with decreasing time trends (11 features) compared with increasing time trends (6 features). In structural elucidation, comparison of the experimental high-collision energy spectra of the 11 features with MetFrag (Ruttkies et al., 2016) resulted in one tentatively identified structure (octadecanenitrile) of anthropogenic origin (see Section 3.4).

3.4. Tentatively identified compounds

It proved possible to prioritise and tentatively identify two structures (pyrroline-2-carbaldehyde and S-ethyl dipropylthiocarbamate) with statistically significant increasing temporal trends in the lynx muscle samples, and one structure with a statistically significant decreasing temporal trend (octadecanenitrile). Pyrroline-2-carbaldehyde is an amino acid found in living organisms, while S-ethyl dipropylthiocarbamate (EPTC) is an herbicide used for pre-emergence control of weeds in cultivation of field beans and potatoes (United States Environmental Protection

Table 1
Summary of the three tentatively identified features in lynx tissue time series samples (1969–2017), and their top-ranked structure (via MetFrag), retention time (RT), x-fold increase/decrease to maximum intensity compared with the first detected time point, identification level (Schymanski et al., 2014) and detected adducts. Compounds assessed to be of anthropogenic origin are highlighted in grey.

PubChem CID	Molecular formula	Top ranked structure	Name	RT (min)	x-fold increase	x-fold decrease	Identification status (Schymanski et al., 2014)	Adducts
3323161	C_5H_9NO		Pyrrolidine-2-carbaldehyde	1.57	11		Level 3	$[M+H]^+$
12968	$C_9H_{19}NOS$		S-Ethyl dipropylthiocarbamate	6.66	2.1		Level 1	$[M+H]^+$
12532	$C_{18}H_{35}N$		Octadecanenitrile	12.56		3.1	Level 3	$[M+H]^+$

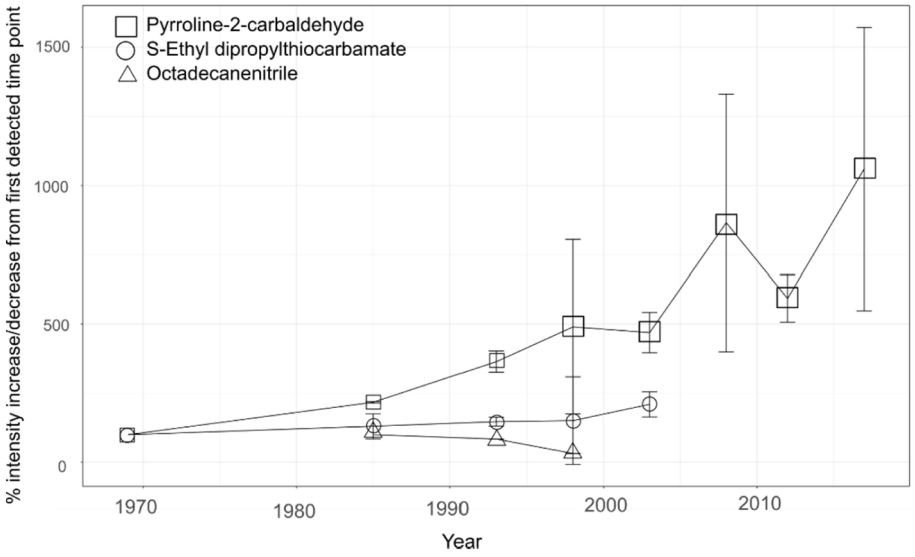


Fig. 3. Time trends for tentatively identified compounds (features) displaying statistically significant (Mann-Kendall $p < 0.08$, Spearman rank correlation coefficient (ρ) > ± 0.8) increasing and decreasing trends in lynx muscle samples from 1969 to 2017, given as average % peak intensity increasing/decreasing from the first detected time point. Error bars represent standard deviation of triplicate samples.

Agency, 2021a; Swedish Civil Contingencies Agency, 2021; United States Environmental Protection Agency, 2021b).

The tentatively identified endogenous compound, pyrroline-2-carbaldehyde, steadily increased by 1000% from 1969 to 2017 (Fig. 3). The high standard deviation of the intensity of pyrroline-2-carbaldehyde for 2008 and 2017 indicates that its time trend may have stagnated around 2003. No data gaps (i.e., peaks below the intensity cut-off) were observed for this feature. Changes of endogenous compounds over time are common (Xia et al., 2015; Xia et al., 2012), however, causes for this specific trend are unknown.

Pesticides are directly applied to agricultural fields, making terrestrial animals vulnerable to exposure through feed intake and inhalation (Mateo et al., 2012). Predators feeding in the terrestrial environment, such as lynx, are thus particularly vulnerable (Mateo et al., 2012; Mariussen et al., 2008; Falk et al., 2019). Therefore, it is likely that pesticide compounds accumulate in muscle tissue of lynx. The intensity of the herbicide EPTC steadily increased by 200%. However, in contrast to pyrroline-2-carbaldehyde, EPTC was not detected after 2003, indicating risk reduction measures taken resulted in a reduction in release of this compound to the environment (Swedish Chemicals Agency, 2021; Pesticide Properties Database, 2021). EPTC was sold in Sweden between 1972 and 1997, but in 1999 use of this bioactive substance was banned in Sweden (Swedish Chemicals Agency, 2021), the outcome of which is evident in the results of this study.

Octadecanenitrile, also known as stearonitrile, is a fatty nitrile used in cosmetics and chemical manufacturing, according to US EPA (United States Environmental Protection Agency, 2021b). This substance is pre-registered by REACH and meets criteria for category 1A or 1B carcinogenicity, mutagenicity, or reproductive toxicity (ECHA, 2021). Octadecanenitrile was tentatively identified with a 3-fold decreasing trend from 1985 to 1998 (Fig. 3). The lack of data (i.e., below intensity cut-off) after 1998 may indicate less production.

4. Conclusion

The targeted CECs detected in lynx muscle tissue time series showed decreasing trends, but new CECs were identified. Despite disadvantages of heavy matrix effects and low expected concentrations of CECs in the terrestrial species lynx, the prioritisation approach for NTS provided meaningful results and was able to capture anthropogenic CECs with significant temporal trends. Thus, NTS of terrestrial biological matrices is of high relevance to pinpoint unknown, potentially harmful compounds with increasing time trends. The decreasing time trends identified for some CECs can be used to evaluate risk management measures. The use of archived biological tissue provides more possibilities for successful prioritisation of CECs in biota using NTS.

5. Limitations and recommendations

Although minimizing sample pre-treatment and clean-up for NTS to be non-specific and to help extract a broad range of compounds is important, it should not be underestimated that a high matrix effect reduces the sensitivity and increases the number of background masses in the chromatogram. Non-discriminatory clean-up steps as suggested by Gil-Solsona et al. (2021) are promising.

Gas chromatography (GC) approaches are commonly applied when investigating biological samples, since hydrophobic compounds tend to bioaccumulate and thus can be expected to be detected by GC analysis (Fernando et al., 2018). However, more hydrophilic compounds such as pharmaceuticals, pesticides and per- and polyfluoroalkyl substances (PFASs), which are typically separated using liquid chromatography (LC) approaches, are often more mobile and can also be bioaccumulative and harmful to biota (Ahrens and Bundschuh, 2014). Thus, GC and LC are complementary approaches to separate organic compounds for subsequent detection in biota.

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CRediT authorship contribution statement

Wiebke Dürig: Conceptualization, Methodology, Validation, Formal analysis, Writing – original draft, Writing – review & editing. **Nikiforos A. Alygizakis:** Conceptualization, Validation, Formal analysis, Writing – review & editing. **Karin Wiberg:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing. **Lutz Ahrens:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Additional information, data, and figures are provided in Supplementary Information, which is available free of charge via the Internet at http://pubs.acs.org. Supplementary data to this article can be found online at doi:https://doi.org/10.1016/j.scitotenv.2022.153035.

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