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Research Paper

Novel prioritisation strategies for evaluation of temporal trends in archived white-tailed sea eagle muscle tissue in non-target screening

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ABSTRACT

Environmental monitoring studies based on target analysis capture only a small fraction of contaminants of emerging concern (CECs) and miss pollutants potentially harmful to wildlife. Environmental specimen banks, with their archived samples, provide opportunities to identify new CECs by temporal trend analysis and non-target screening. In this study, archived white-tailed sea eagle (*Haliaeetus albicilla*) muscle tissue was analysed by non-targeted high-resolution mass spectrometry. Univariate statistical tests (Mann-Kendall and Spearman rank) for temporal trend analysis were applied as prioritisation methods. A workflow for non-target data was developed and validated using an artificial time series spiked at five levels with gradient concentrations of selected CECs ($n = 243$). Pooled eagle muscle tissues collected 1965–2017 were then investigated with an eight-point time series using the validated screening workflow. Following peak detection, peak alignment, and blank subtraction, 14 409 features were considered for statistical analysis. Prioritisation by time-trend analysis detected 207 features with increasing trends. Following unequivocal molecular formula assignment to prioritised features and further elucidation with MetFrag and EU Massbank, 13 compounds were tentatively identified, of which four were of anthropogenic origin. These results show that it is possible to prioritise new CECs in archived biological samples using univariate statistical approaches.

1. Introduction

Environmental monitoring campaigns commonly apply target analysis and focus on a small number of contaminants of emerging concern (CECs), while pollutants potentially harmful to wildlife are overlooked (Sonne et al., 2020). Detecting unknown CECs in wildlife by analytical chemistry is challenging, as large numbers of naturally occurring compounds such as lipids and proteins co-exist with anthropogenic compounds in wildlife tissues. Previous studies have shown that non-target high-resolution mass spectrometry (HRMS) analysis can be used to prioritise and elucidate previously unknown CECs in wildlife (Heffernan et al., 2017; Millow et al., 2015; Myers et al., 2014; Shaul et al., 2015; Du et al., 2017). These studies highlighted the importance of minimising sample pre-treatment and clean-up for non-target screening (NTS) methods to be non-specific and extract a broad range of compounds (Heffernan et al., 2017; Dürig et al., 2020; Plassmann et al., 2016). However, the drawback, in particular for biota samples, is that this can lead to interferences by sample matrices, which may reduce the sensitivity and increase the number of peaks in the chromatogram. This in turn complicates identification of compounds using NTS. Thus, it is challenging to find the optimal compromise between extensive sample preparation to reduce matrix interferences and minimal sample preparation to avoid losing NTS compounds. Prioritisation of HRMS features (retention times (RT), mass-to-charge ratios, and peak intensities) is a key requirement in non-target screening, since it reduces the amount of data produced and allows a focus on the most relevant features of interest for further structural elucidation. Different prioritisation strategies can be used, e.g., prioritization based on regulatory databases, (Gago-Ferrero et al., 2018) effect-directed analysis (Weiss et al., 2011), case-control studies (Heffernan et al., 2017; Shaul et al., 2015; Du et al., 2017), spatial and temporal trend analysis (Beckers et al., 2020; Albergamo et al., 2019; Alygizakis et al., 2019a; Anliker et al., 2020;

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Chiaia-Hernández et al., 2017), and analysis for specific compound groups (e.g., organohalogens) (Cariou et al., 2016; Fernando et al., 2018). 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 (e.g., Fernando et al., 2018). However, biota is also used as an indicator for water quality and therefore polar and mid-polar compounds such as endocrine disrupting chemicals, pharmaceuticals, current used pesticides and per- and polyfluoroalkyl substances (PFASs) are typically separated using liquid chromatography (LC) approaches. Polar and mid-polar compounds can be bioccumulative and harmful to biota, and are often mobile in the aquatic environment (Ahrens and Bundschuh, 2014; de Wit et al., 2020). Thus, GC and LC are complementary approaches to separate organic compounds for subsequent detection in biota.

Time-series samples are frequently used in target analysis to identify concentration trends in CECs over time (Bignert, 2002). Archived environmental samples, collected in accordance with standardised protocols and held in e.g., environmental specimen banks (ESB), provide opportunities for new prioritisation strategies through temporal trend analysis of non-target features identified in HRMS analysis. The Swedish Museum of Natural History (SMNH) has systematically collected a wide variety of environmental samples since the 1960s, providing the possibility to detect long-term trends in micropollutants in many different species (Odsjö, 2006). Tissues from top predators are commonly used for monitoring CECs, as these animals are at the top of the food web and can accumulate CECs to high concentrations as a result of biomagnification (Badry et al., 2020; de Wit et al., 2020). Previous studies have shown that prioritising features in non-target screening by means of temporal trend analysis in sediment and wastewater is possible and beneficial for identifying previously unknown CECs (Albergamo et al., 2019; Alygizakis et al., 2019a; Anliker et al., 2020; Chiaia-Hernández et al., 2017; Hollender et al., 2017). However, to our knowledge, archived biological tissues from specimen banks have not been used previously to prioritise potential polar CECs by time trends in non-target screening using LC-HRMS for detection.

In this study, a new prioritisation strategy for non-target screening was developed using time trend analysis of data from archived muscle tissue from white-tailed sea eagle (*Haliaeetus albicilla*). The samples were collected by SMNH from 1965 to 2017. Specific objectives of the work were to: i) develop and validate a non-target screening data treatment workflow based on an artificial time series (ATS) consisting of white-tailed sea eagle muscle tissue spiked with CECs ($n = 243$) at different levels, using two univariate statistical approaches (Spearman rank and Mann-Kendall), and ii) apply the validated workflow on an archived time series of white-tailed sea eagle muscle tissue collected from 1965 to 2017, to search for novel CECs.

2. Materials and methods

2.1. Biota samples and storage

Muscle tissue from white-tailed sea eagle (*Haliaeetus albicilla)* was obtained from the ESB at SMNH. The tissue samples were collected during 1965, 1983/1984, 1991, 1996, 2001, 2006, 2011, and 2017, mainly from birds killed by traffic. 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 present analysis were stored at − 20 °C until extraction, which occurred within a couple of days of arrival at the laboratory (Table SI1 in Supplementary Information (SI)).

Selection criteria for the individual samples were: (i) availability of individual samples per year, (ii) close proximity of sample locations, (iii) equal sex ratio per year (1:1, male: female), (iv) preferably adult birds, and (v) feed intake mainly from marine feed sources. To determine consumption of marine food sources for individual birds, analysis of carbon (C) and nitrogen (N) isotopes in all individual muscle tissue samples was performed at the Department of Forest Ecology and Management, Swedish University of Agricultural Sciences (Umeå, Sweden) (section II in SI). Principal component analysis (PCA) and dendrogram analysis of the $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios showed that four samples had a higher $^{15}N/^{14}N$ ratio than the others (Table SI2 and Fig. SI1 in SI), indicating that these birds had mainly consumed terrestrial organisms (Polunin et al., 2001). These four samples were therefore excluded from further analysis. The remaining eagle muscle tissue samples from approximately 10 individual birds per year were pooled (except 1983–1984, which were treated as one year) (Bignert et al., 2014). Thus, eight pooled samples were obtained for analysis (1965, 1983/1984, 1991, 1996, 2001, 2006, 2011, and 2017).

2.2. Chemicals

A total of 243 target compounds (pharmaceuticals, industrial chemicals, pesticides, flame retardants, personal care products, benzothiazoles, isoflavones, food additives, phthalates, stimulants, siloxanes, surfactants, contrast media, fatty acids, PFASs) and 65 isotopically labelled compounds (IS) were used for spiking and quality control experiments (Tables SI3 and SI4 in SI). The target compounds were selected based on environmental relevance and availability. 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). For pooling, 0.1 g portions of muscle tissue from each individual eagle collected in the same year 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). Material from the year 1965 was limited (only one bird available) and therefore 0.5 g of the individual sample was taken for analysis. 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 (2 × 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 250 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} wet weight (ww) concentration range (Polunin et al., 2001; Grabicova et al., 2015, 2018).

An artificial time series (ATS) was prepared from a homogenised pool of 0.4 g ± 0.1 g portions of each individual eagle muscle tissue collected for the study (except the years 1965 and 2017) to yield approximately 1 g total ww. The homogenised tissue was split between 15 homogenisation tubes (15 mL each), each containing 0.6–1.0 g ww, and spiked in triplicate with IS mixture (50 ng) and 243 target compounds at a level of 0.5, 5, 10, 25, and 50 ng. These levels reflect a relatively wide range in which contaminants are expected to occur in the white-tailed sea eagle samples (Sletten et al., 2016; Nordlöf et al., 2012). Extraction was performed in the same way as for the other samples. This ATS was used for the non-target workflow development to evaluate

losses of target analytes during each step of the 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) as reported previously (Dürig et al., 2020; 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 ionization 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 ionization 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 SI5 in SI. The UPLC was coupled to a Xevo G2-S QToF-MS (Waters Corporation, Manchester, UK) with an electrospray ionization (ESI) interface working in positive and negative ionization modes. All data were collected in separate injections for positive and negative ionization 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 enkephalin was continuously infused for lock mass correction (for details, see Table SI6 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 243 target compounds was prepared in acetonitrile at concentrations of 0.5, 5, 10, 25, and 50 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. 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 243 target compounds in three different concentrations (10, 50, and 100 ng mL^{-1}) after the extraction. For reaching the higher final concentrations of the target compounds in the matrix-matched standard, less extract volume was taken; however, still enough to compensate for dilution effect of the matrix by the added standards. For absolute recovery calculations, the responses of the target compounds in the matrix-matched standard (50 ng mL^{-1}) were compared with the responses of the target compounds in a sample fortified with the 243 target compounds (50 ng mL^{-1}) before extraction (Fig. SI2 in SI) as reported previously by Dürig et al. (2020).

2.6. Data handling and statistical time-trend analysis

As previously demonstrated in other studies (Hohrenk et al., 2019; Pochodylo and Helbling, 2017), proper pre-processing workflow must be applied to obtain high-quality data. The 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. The data were processed using an automated workflow described elsewhere (Alygizakis et al., 2019a). 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. SI3 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 intensity was at least 10-fold higher than that of the solvent blank injections (when present in the blank), ii) they were present in at least two of the three replicates, and iii) they had relative standard deviation (RSD) of less than 50% across the triplicates. Solvent blanks consisted of pure solvent injections as well as procedural blanks (acetonitrile). Matrix containing procedural blanks were not possible due to limited material available. Finally, time trend analysis was performed for prioritisation of increasing features using Spearman rank and Mann-Kendall correlation coefficients on the average response of each year, using R (v 4.0) software. Features with a temporal increasing intensity trend at significance level $\alpha = 0.05$ and $p > 0.8$ were prioritised (R script is provided in Section V in SI) as these features could possibly be concerning compounds even though toxicity criteria are not included. Visual inspection of the peak shapes of the prioritised features was performed, and peaks with bad peak shape were discarded. Unequivocal molecular formula for prioritised features was predicted using Waters Corporation software UNIFI (version 1.9.4). For features to be further evaluated coherent molecular formula prediction was required. Further elucidation was carried out in MetFrag using the unequivocal predicted molecular formula for candidate collection (Ruttkies et al., 2016). PubChem was used as a search database, 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 using the patent and reference counts in PubChem and the *in silico* fragment score, which was based on the experimental high-collision energy spectra. 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. Available reference standards ($n = 3$) of the tentatively identified compounds (two anthropogenic and one endogenous compound) were ordered and added to the extract at different concentrations (50 ng mL^{-1} and 200 ng mL^{-1}) for confirmation. Retention time Index (RTI) were analysed in positive and negative ionisation mode to predict retention times for the tentatively identified compounds to further support the findings. Retention time prediction models (Aalizadeh et al., 2019) were used to verify that top-ranked candidates were eluted at plausible retention times. The methodology that was followed, was also implemented in the NORMAN collaborative trial (Rostkowski et al., 2019). This method has been proven efficient in removing false positive results in suspect (Alygizakis et al., 2019b) and non-target screening (Aalizadeh et al., 2019).

3. Results and discussion

3.1. Validation of prioritization strategy using artificial time series

The workflow developed was validated using the ATS (Section 2.3). All 243 compounds were detected in the calibration solution with the highest concentration level (50 ng mL^{-1}), whereas 233 were detected in the highest concentration level of the ATS (50 ng mL^{-1}). The failure to detect 10 compounds was due to matrix suppression (average −67% (−100% to 64%)) and/or low recovery (average 77% (16–140%)) (see Fig. SI2 in SI). A limited number of compounds ($n = 83$) was detected in the extract with the lowest concentration (0.5 ng mL^{-1}) in the ATS, and therefore this concentration was excluded from further evaluation. Temporal trend analysis of the ATS (5–50 ng mL^{-1}) was used for validation of the prioritisation of features with increasing trends (Fig. SI4 in SI). After blank subtraction, 19 272 features were detected across all injections of the ATS. A noteworthy data reduction was achieved by only considering features detected in at least two of three replicates (9 963 removed) and with RSD < 50% (12 067 removed). For a statistically significant trend to be prioritised, at least three time points needed to be

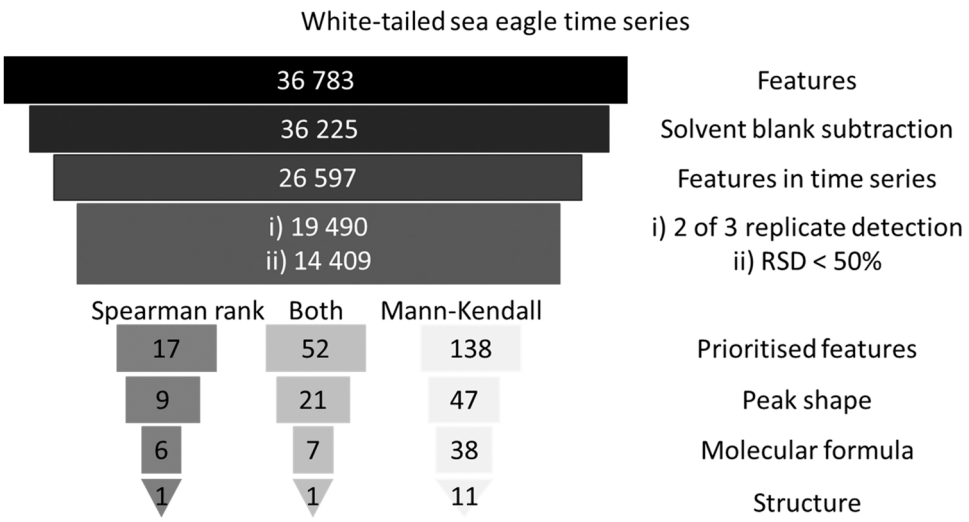


Fig. 1. Number of features in the white-tailed sea eagle time series samples during each step of the data treatment workflow.

detected of the ATS (i.e., 10, 25, and 50 ng mL^{-1}), which was fulfilled by 127 target compounds. For elucidation of the prioritised features, all spiked target compounds were treated as non-target features. Retrospective checking was performed to determine which features belonged to the spiked target compounds. After prioritisation using Spearman rank and Mann-Kendall, only 126 features remained (104 in positive and 22 in negative ionisation mode). As expected, the same features were prioritized using Spearman rank and Mann-Kendall, due to the similarity of these statistical approaches. High correlation should lead to a low *p*-value, however if the time series has too few data points even a high correlation would lead to non-significancy. With regards to the ATS, the spiked target compounds were detected in almost all samples which was maybe the reason why the Spearman rank correlation and Mann-Kendall *p*-value showed similar results. However, high concentration and co-elution of endogenous compounds might hamper prioritisation of exogenous compounds present in the sample. Even spiked compounds might have low response and slightly increasing intensity trends may not be prioritised in pooled matrix-rich samples with the statistical tools applied in this study. Of the 126 prioritised features (65 targets), an unequivocal molecular formula with iFit value > 50% using Waters Corporation software UNIFI (version 1.9.4) was predicted for 62 features (40 targets). The loss of 25 target compounds during this step was possibly attributable to low intensity or high influence of matrix on the mass spectra, which resulted in unreliable molecular formula prediction by UNIFI (Menger et al., 2021; Samanipour et al., 2017). Further elucidation with MetFrag and EU Massbank allowed the tentative identification of 37 structures (26 targets). The loss of 14 target compounds during this step appears to be caused by similar fragmentation patterns in MetFrag between experimental data from target compounds and predicted fragment patterns of other highly cited and patented chemicals (Ruttkies et al., 2016). For example, climbazole (PubChem ID 37907) and fuberidazole (PubChem ID 19756) were ranked lower compared to other compounds (data not shown). Combining prioritisation and the elucidation workflow described above demonstrated that 20% of the 127 target compounds could be tentatively identified in the ATS when treated as non-targets, which was considered an acceptable fraction for a non-target screening identification workflow on biota.

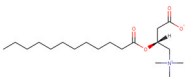
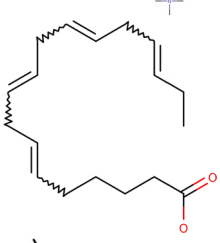
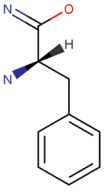
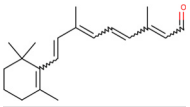
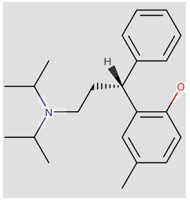
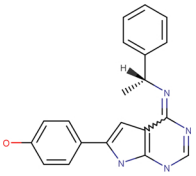
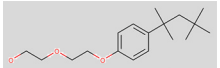
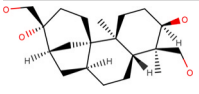
The workflow developed reduced the number of features drastically and provided a manageable number of curated features to focus upon during structural elucidation. However, the conservative approach applied led to losses of target compounds during the workflow. This indicates that during application of the workflow on real time-series samples, the number of identified compounds was likely underestimated. The advantage of using Spearman rank coefficients for prioritisation is that even with shorter time series, an increase can be detected. However, these trends might not be significant as the time series gets prolonged. The latter could be a risk if applying this statistical test in early identification. Significancy in terms of *p*-values like for Mann-Kendall gives reassurance that the increasing trend is not random, however more data points are needed which is not always possible with archived samples. The validation results for the prioritisation strategy developed here showed that temporal trends detected using univariate statistics (i.e., Spearman rank (Plassmann et al., 2016) and Mann-Kendall (Lamchin et al., 2019)) can be used for prioritisation and identification of CECs in matrix-rich samples such as biota, as previously suggested and demonstrated by Plassmann et al. (2016).

3.2. Workflow application to eagle muscle tissue time series

The validated data treatment workflow was applied to the real eagle muscle tissue time series (1965–2017) (Fig. 1). After blank subtraction, 26 597 features were obtained, which was a higher number of features than for the ATS, probably due to the separate analysis of the pooled samples (i.e., not combining them to one pool as for the ATS). Setting the requirements of detection in at least two out of three replicates (7 107 removed) and RSD < 50% (12 188 removed) reduced the number of features to 14 409. Finally, a total of 207 features were prioritised using univariate statistical approaches (17 by Spearman rank only, 138 with Mann-Kendall only, and 52 with both statistical approaches (Fig. 1 and Fig. SI5 in SI). Spearman rank test was able to pick up a few but linear correlations in the time series more effective than Mann-Kendall did. Interestingly, in the real time series the two statistical approaches prioritised different features, whereas in the ATS the same features were prioritised by the two statistical approaches. One likely cause for this could be that the spiked compounds in the ATS were dominant (high signal intensity) and therefore picked up by both statistical approaches as discussed above (see Section 3.1). In total, 130 prioritised features were removed due to bad peak shape. For 51 of the 207 prioritised features (25%), it was possible to predict an unequivocal molecular formula with iFit value > 50% in Waters Corporation software UNIFI. For Spearman rank only, Mann-Kendall only and both statistical approaches, 6, 38, and 7 features were assigned an unequivocal molecular formula, respectively, showing that it was possible to reduce the extensive number of curated features to a reasonable number using the approach developed in this study.

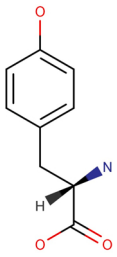
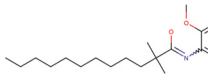
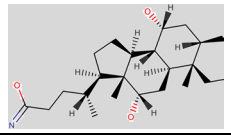
Comparison of the experimental high collision energy spectra for those features with MetFrag (Ruttkies et al., 2016) and EU MassBank resulted in 13 tentatively identified structures (1 prioritized by

Table 1
The 13 tentatively identified features in the white-tailed sea eagle time series samples (1965–2017), along with their top ranked structure (via MetFrag), x-fold increase to the max intensity compared with the first detected time point, identification status according to Schymanski et al. (2014), and detected adducts. Compounds which are most likely of anthropogenic origin are highlighted in grey.

PubChem CID	Molecular formula	Top ranked structure	Name	RT (min)	Predicted RT (min)	Plausible RT	x-fold increase	Identification status (Schymanski et al., 2014)	Adducts
168381	$C_{19}H_{37}NO_4$		Dodecanoyl-L-carnitine	9.08	8.91	Yes	5.2	Level 3	$[M+H]^+$
163841	$C_{18}H_{28}O_2$		6,9,12,15-Octadecatetraenoic acid	11.9	12.41	Yes	21	Level 3	$[M+H]^+$
10917	$C_7H_{15}NO_3$		(-)-L-Carnitine	0.97	0.01	Yes	1.3	Level 2a	$[M+H]^+$
445694	$C_9H_{12}N_2O$		L-Phenylalaninamide	4.20	1.89	Yes[a]	1.2	Level 3	$[M+H]^+$
1071	$C_{20}H_{30}O$		Retinol	12.9	11.84	Yes	3.7	Level 2a	$[M+H]^+$
443879	$C_{22}H_{31}NO$		R-(+)-Tolterodine	9.35	8.43	Yes	2.3	Level 3	$[M+H]^+$
6918403	$C_{20}H_{18}N_4O$		PKI166	9.7	8.23	Yes[a]	1.6	Level 3	$[M+H]^+$
7689	$C_{24}H_{41}NO_2$		4′-Hydroxystearanilide (EPA TSCA)	14.0	14.24	Yes	2.8	Level 1	$[M+H]^+$
24775	$C_{18}H_{30}O_3$		Octoxynol-2	6.51	10.47	Yes[b]	2.2	Level 2a	$[M-H]^-$
457964	$C_{20}H_{34}O_4$		(+)-Aphidicolin	7.68	3.42	Yes[b]	2.9	Level 3	$[M-H]^-$

(*continued on next page*)

Table 1 (*continued*)

PubChem CID	Molecular formula	Top ranked structure	Name	RT (min)	Predicted RT (min)	Plausible RT	x-fold increase	Identification status (Schymanski et al., 2014)	Adducts
6057	$C_9H_{11}NO_3$		L-(-)-Tyrosine	0.62	1.04	Yes	1.6	Level 2a	$[M-H]^-$
122327	$C_{23}H_{39}NO_4$		PD-128042	12.4	13.77	Yes	26	Level 3	$[M-H]^-$
447685	$C_{24}H_{41}NO_4$		Cholamide	12.4	4.85	Not plausible	15	Level 4	$[M-H]^-$

[a] RT plausible despite the observed difference between experimental and predicted retention time.
[b] RT plausible despite the observed difference between experimental and predicted retention time. It is suggested to use other verification tool

Spearman rank only, 11 by Mann Kendall only, and 1 by both statistical approaches) (Table 1). Structures were obtained in MetFrag from the most likely predicted molecular formulas with an iFit > 50% and using PubChem as a search database (see Section 2.6 for details). The MetFrag fragmenter results, patent count, and PubChem reference count were weighted equally (1:1:1) and considered in the final scoring. Potential hits were evaluated in depth individually. Four of the 13 structures were tentatively identified with a level 2 and had a match in EU Massbank (71 568 entries in 2020).

In this study, analysis with univariate statistical methods led to prioritisation and elucidation of potential CECs in biota. Similarly, Purschke et al. (2020) recently developed a multivariate statistics approach including principal component analysis (PCA) for temporal trend analysis of complex matrices using industrial wastewater, also resulting in prioritisation and elucidation of new CECs. This indicates that multivariate and univariate statistics are both suitable strategies for data reduction and prioritisation using temporal trend analysis of HRMS data in matrix-rich samples (Plassmann et al., 2016; Purschke et al., 2020). It remains to be determined whether these two statistical approaches are complementary to each other.

3.3. Tentatively identified compounds

With the workflow developed, it was possible to prioritise and tentatively identify 13 structures with statistically significant increasing temporal trends in the eagle muscle tissue samples. Retention time prediction excluded that the candidate with mass 406.2975 was "Cholamide". 4′-Hydroxystearanilide was confirmed by standard addition to level 1 (see Fig. SI8 in SI) (Schymanski et al., 2014). Anthropogenic compounds prioritised and elucidated in this study mainly belonged to the families pharmaceuticals (i.e., R-(+)-tolterodine, and (+)-aphidicolin) and cosmetics (i.e., octoxynol-2), while some of the structures (viz. dodecanoyl-L-carnitine, 6,9,12,15-octadecatetraenoic acid, L-phenylalaninamide, retinol, PKI166, L-(-)-tyrosine, and PD-128042) were endogenous compounds like fatty acids, vitamins, amino acids, or inhibitors previously mentioned in metabolomics papers (Pekala et al., 2011; Johnson, 2017). The prioritisation of the latter compounds could be explored as potential biomarkers for increasing exposure to external stressors, such as CECs, in wildlife over time. MassBank includes both endogenous and exogenous compounds, which permitted tentative identification of both naturally occurring and anthropogenic compounds. Identification of anthropogenic compounds (e.g., CECs) via non-target screening is desired. Filtering out anthropogenic compounds in non-target screening is challenging, as naturally occurring compounds co-exist with anthropogenic compounds. In addition, naturally occurring compounds could be released from human products. Plassmann et al. (2016) suggested comparing the original feature list against known metabolite databases to exclude endogenous compounds, which could be beneficial when dealing with many prioritised features.

The increase to the highest intensity from the first detected time point of the 13 tentatively identified compounds is shown in Fig. 2. There were steadily increasing trends in all tentatively identified features, by up to 160% from 1965 to 1996 and even up to 500% from 1996 onwards, except for $C_{24}H_{41}NO_4$, PD-12804, and 6,9,12,15-octadecatetraenoic acid. Data gaps (i.e., below detection limit) for all detected features occurred mainly before 1990 for the tentatively identified compounds, indicating that fewer environmental stressors were released to the environment some decades ago. From 1991 to 2011, a rapid increase was observed for 6,9,12,15-octadecatentraenoic acid (an increase of 2100%), PD12804 (+2600%) and $C_{24}H_{41}NO_4$ (+1500%) (Fig. 2). The European Chemical Agency (ECHA) has pre-registered 6,9,12,15-octadecatentraenoic acid, as it is produced by at least one company in volumes of up to 10 tons and is suspected to be bioaccumulative (BCF > 2 000 L kg^{-1}), and therefore could be investigated for influences on the aquatic environment (ECHA, 2020). Chloamide is a tetracyclic diterphenoid used as an antiviral and cytotoxic agent for cancer treatment which is not registered at the Swedish Medical Product Agency. Tolterodine is approved for medical use in Sweden since 1998 and used as treatment for incontinence (National Board of Health and Welfare, 2021). According to ECHA, this drug is in the hazardous class 3 and 2 of acute and reproductive toxicity, respectively (ECHA, 2021a). The cosmetic and surfactant Octoxynol-2 is on the NORMAN Suspect List Exchange for its high presence in the environment (NORMAN Suspect List Exchange, 2021). ECHA classified Octoxynol-2 as hazardous class 4 acute toxicity, 1B skin corrosion, 2 reproductive toxicity, 1 aquatic acute toxicity and 1 aquatic chronic toxicity (ECHA, 2021b). White-tailed sea eagles are migrating birds and may accumulate contaminants from different sources and countries making it possible that usage pattern of the compounds in Sweden do not reflect the results obtained in our study. The drop in intensity for most tentatively identified compounds after 2011 indicates reduced emissions of these chemical stressors to the environment.

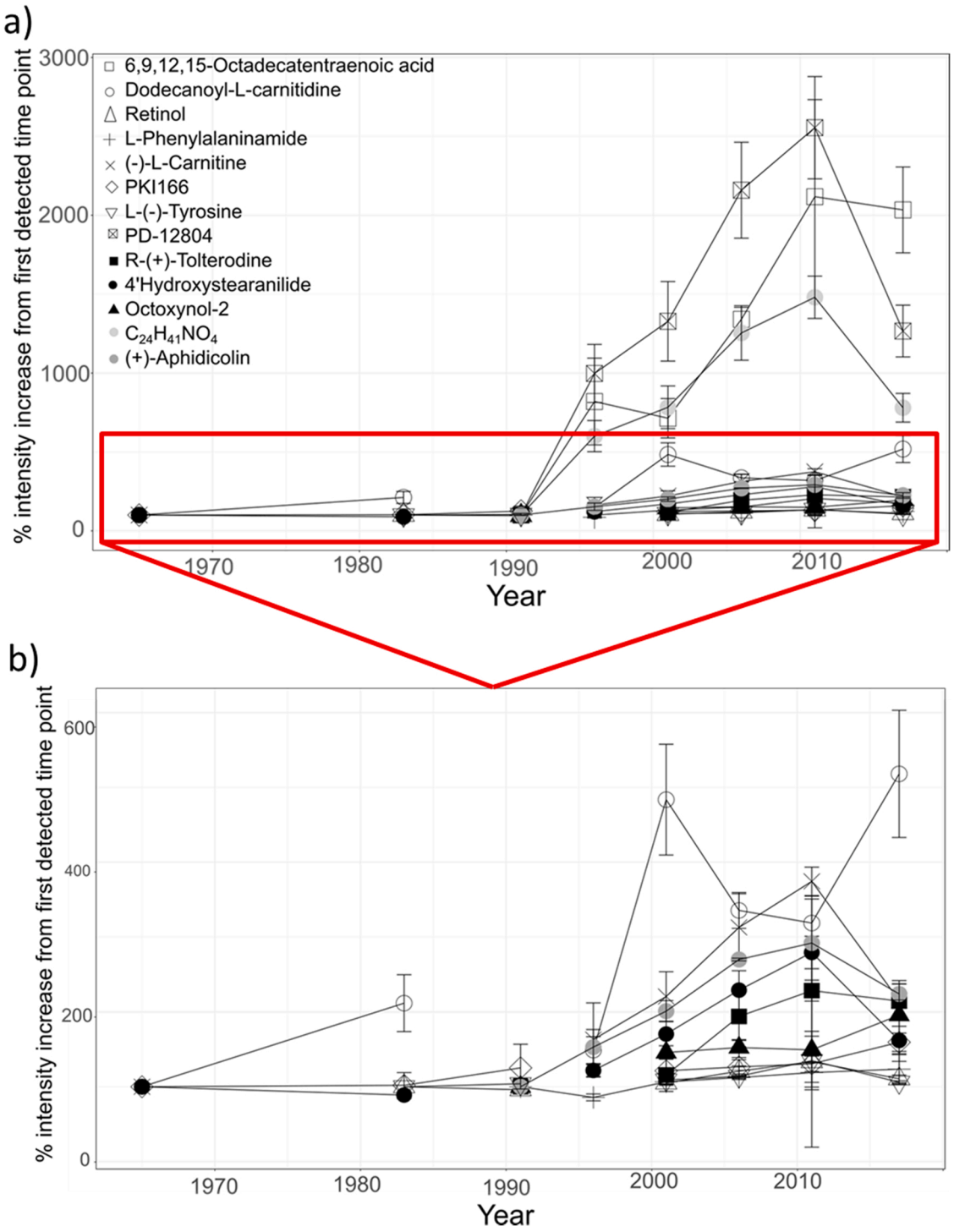


Fig. 2. a) Time trends in tentatively identified compounds displaying statistically significant (Mann-Kendall $p < 0.05$ and Spearman rank $p > 0.8$) increasing trends from 1965 to 2017 ($n = 3$) b) Magnified view on the lower intensity time trends.

4. Conclusion

Ultimately, non-target analysis of biological matrices is of high relevance to pinpoint unknown, potentially harmful compounds with an increasing trend, which can indicate persistency and bioaccumulation potential of these compounds. Univariate statistics proved to be useful for prioritisation of increasing intensity trends in LC-HRMS data. The strategies developed here can be used as a complement to traditional target screening monitoring. The use of archived biological tissue provides more possibilities of successful prioritisation of CECs in biota using non-target screening.

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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. **Frank Menger:** Conceptualization, Validation, Formal analysis, Writing – review & editing. **Oksana Golovko:** Conceptualization, 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 that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.127331.

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