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Suggestions for indicator based on non-target analysis

Technical Report from DCE – Danish Centre for Environment and Energy No. 321 2024

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Data sheet

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Abstract: The introduction of suspect and non-target screening (SNTS) in NOVANA monitoring is discussed and suggestions for possible indicators based on such screenings are suggested, both purely qualitative indicators on the presence of peaks and quantitative indicators based on semiquantitative concentrations of target substances, with Risk Quotients defined from comparison with PNEC or EQS value as used in the NORMAN approach in the OSPAR CONnECT and HELCOM PreEMPT projects. Some other possible indicators based on the PreEMPT and CONnECT data are suggested, and the general status for SNTS screenings and status of uncertainties using results from such are discussed. Finally, an example of how to build SNTS into a monitoring program to continuously upgrade and improve lists of substances of priority action and possible concern, is presented.

Keywords:	Non-target screening, suspect screening, hazardous substances, indicators, NOVANA monitoring
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Preface

This project is based on an interest from the Danish Ministry of Environment to use suspect and non-target screening in the Danish NOVANA monitoring program, and regional sea conventions. The report investigates the possibilities of indicators based on non-target and suspect screening of environmental samples, mainly inspired by the HELCOM PreEMPT[1] and OSPAR CONnECT[2] projects from 2020 and 2021 reported in the HELCOM HOLAS 3 and OSPAR QSR 2023.

The overall aim of this project is to suggest indicators that can be derived from non-target and suspect screening approaches, typically including hundreds or thousands of substances, and to use the PreEMPT and CONnECT datasets to exemplify the use of such indicators. Furthermore, the indicators will be divided into five possible status types in line with the HELCOM status for nutrients (high-good-moderate-poor-bad). Each class will point to what further actions could be applicable for a given substance (No further action – small concern – case for concern – priority risk assessment – risk assessment urgently needed).

The chemical analyses in the HELCOM and OPAR projects included a wide-scope target analysis against a database of >2.500 substances and suspect screening against a database of >65.000 substances. All peaks reported were assigned to a substance, according to the report, but non-assigned peaks was not reported but can be found in the underlaying data. Very little information in the PreEMPT and CONnECT is available on the certainty with which substances are identified, mostly directly against in-house databases based on retention time and mass-spectra (mainly level 3 - see chapter 1.4). Further, most substances have been associated with a semiquantitative concentration against standards of "structure related isotope labeled substances". The expected uncertainty of these concentrations for the suspect screening is rather in factors than percentages. For the wide-scope target analysis, isotopic internal standards have been added to ensure both qualification of the substance and estimation of quantification. However, this does not follow the standards used in optimized, targeted analysis for national and regional monitoring and has a much higher uncertainty.

In addition to the above-mentioned reports, this report also uses original datasets produced by the NORMAN partners Environmental Institute (Slovakia) and National and Kapodistrian University of Athens (Greece) for OSPAR (Gkotsis *et al*, 2021) and HELCOM (Alygizakis *et al,* 2023a). NORMAN[3] is a network of research laboratories, research centres and related organisations for monitoring of emerging environmental substances and has a dedicated working group for non-target screening techniques for environmental monitoring. The focus of NORMAN is to investigate new and emerging techniques for their applicability in environmental monitoring.

[1] https://helcom.fi/helcom-at-work/projects/pre-empting-pollution-by-screening-for-possible-risks-preempt/
[2] https://oap.ospar.org/en/ospar-assessments/quality-status-reports/qsr-2023/other-assessments/connect-study/
[3] https://www.norman-network.net/

In December 2023, a new set of screening data was made available to HELCOM, combining the results of the CONnECT and PreEMPT studies for Kattegat, but this updated version was not made available for use in this report. The updated versions include a re-extraction and improved evaluation of the datasets from the two reports in a unified substance database, extended with approximately 50% more potential substances in the in-house database and with updated toxicity data. This version will be reported in a future HELCOM report and used for HELCOM selection of priority substances. As this study is investigating possible indicators, this is not considered a problem, but caution should be taken using the substance list produced from the original dataset, as the risk assessments and substance list can be different from the revised version.

This report uses the following terms:

- Non-target screening (NTA): Screening for unknown substances in a sample using a defined workflow with identification criteria, but no pre-defined compound list, registering peaks, with the aim to identify substance names.
- Suspect screening (SSA): Same as above, but for a pre-defined compound list and with qualification of peaks to identified substance names, in some cases with semiquantitative determination of concentrations (e.g. within a factor of 10).
- Wide-scope analysis: An "add-on" to suspect screening with target analysis of a wide spectrum of substances (typically thousands), including isotopically marked standards of selected substances matched against a database for substances identification (in-house or other). The standards are used to qualify the run with regards to mass and retention time shifts, and semiquantitative calculation of concentration based on the isotopically marked standards improve precision for similar substances.
- Suspect and non-target screening (SNTS): All non-target screening, suspect screening and wide-scope analysis taken together with different levels of qualification and, in some cases, semiquantitative determination of concentration for the individual peaks/substances.

Suspect- and non-target analyses are based on high-resolution mass spectrometry (HRMS), usually in combination with either gas chromatography (GC) or high-performance liquid chromatography (HPLC) for non-polar and polar substances, respectively. The acquired high-resolution mass spectra can be compared with mass spectra libraries, along with supporting information such as retention times, isotope ratios etc. (González-Gaya, 2021) The NORMAN network recently published a guidance document for suspect and non-target analysis of environmental samples (Hollender *et al.,* 2023).

Sammenfatning

Dette projekt er udarbejdet efter et ønske fra miljøministeriet om at se på mulighederne for at anvende suspect og non-target screening (SNTS) i det danske NOVANA overvågningsprogram og regionale havkonventioner og fastlægge muligheder for indikatorer baseret på Suspect-, non-target- og wide scope-target screeninger. Projektet er fortrinsvis inspireret af HELCOM PreEMPT og OSPAR CONnECT projekterne fra 2020 og 2021[4].

Hovedmålet med anvendelsen af SNTS er at sikre et tidligt advarselssystem for nye kemikalier, der potentielt kan blive fremtidige hovedkontaminanter af de marine og kystnære vande for at undgå endnu et kemikalie, som først lang tid efter de er begyndt at true det marine liv (som fx TBT i bundmalinger) og menneskets sundhed (som fx PFOS i fødevarer), bliver imødegået med indgreb over for fremstilling og anvendelse i regionernes medlemslande.

To typer af indikatorer foreslås. Dels kvalitative indikatorer baseret på forekomst og relative niveauer af et stof i kromatogrammer (kapitel 3). Både semikvantitative og top-højde baserede (uden standarder) tilgange kan anvendes, og indikatorerne kan i princippet anvendes både på identificerede stoffer og signaler, hvor stoffet (endnu) ikke er identificeret, men udviser et kromatografisk signal af en betydende højde. For identificerede stoffer med semikvantificerede koncentrationer kan kvantitative indikatorer (kapitel 4) anvendes, og fastlæggelse af koncentrationer giver mulighed for at vurdere tilstedeværelsen over for relevante økotoksikologiske indikatorer og dermed give en (foreløbig) risikovurdering af de fundne koncentrationer. Økotoksikologisk relevante indikatorer kan fx være miljøkvalitetskrav (MKK/EQS) med høj konfidens, hvis de er baseret på samme matrice, som indgår i SNTS screeninger eller "Predicted No Effect Concentrations" (PNECs med lav til medium konfidens bl.a. afhængig af, hvilke matrix der er PNEC for og om det matcher SNTS screeningens matrix).

Generelt er stoffer med definerede MKK/EQS allerede en del af NOVANA, CEMP eller COMBINE overvågningen i Danmark, OSPAR eller HELCOM, og koncentrationsniveauerne fra de dedikerede analysemetoder, der bruges til overvågningen, vil give en mere pålidelig risiko vurdering for disse stoffer. Men der er få MKK/EQS-værdier til rådighed i forhold til antallet af stoffer med PNEC målværdier.

Den relevante brug af indikatorer og det generelle resultat af SNTS-screeningerne i PreEMPT og CONnECT diskuteres i denne rapport. Både fordelene ved at anvende SNTS screeninger for et stort antal stoffer og ulemper med hensyn til analysekvalitet og brugen af PNECs diskuteres, og et forslag for, hvordan SNTS-screeninger kan indgå i overvågningen, er skitseret (kapitel 5).

[4] https://www.ospar.org/work-areas/cross-cutting-issues/qsr2023 og https://helcom.fi/baltic-sea-trends/holistic-assessments/state-of-the-baltic-sea-2023/

Der er stadig mange uløste problemer med metoderne og stor usikkerhed, som gør, at det måske er for tidligt at anvende SNTS screeninger så direkte som i PreEMPT og CONnECT, men der er potentiale for at udvikle SNTS screeningerne til gode værktøjer i fremtidens NOVANA programmer og indenfor de regionale havkonventioner.

Summary

This project is based on an interest from the Danish Ministry of Environment to use suspect- and non-target screening (SNTS) in the Danish NOVANA monitoring program and investigates the possibilities for indicators based on non-target, suspect and wide-scope target screenings, mainly inspired by the HELCOM PReEMPT and OSPAR CONnECT projects from 2020 and 2021 reported in the HELCOM HOLAS 3 and OSPAR QSR 2023 marine strategy relevant reports (https://helcom.fi/Baltic-sea-trends/holistic-assessments/state-of-the-baltic-sea-2023/ and https://www.ospar.org/work-areas/cross-cutting-issues/qsr2023).

The main objective of suspect and non-target screenings is to ensure an early warning system for new chemicals and to stop usage and production before the chemicals could develop into major contaminants of the marine and coastal waters. This will hopefully avoid delayed focus on such chemicals long after they have imposed a threat to the marine life (e.g. TBT in bottom paints) or human health (e.g. PFOS in human food sources).

Two types of indicators are suggested, a qualitative set of indicators solely based on the occurrence and relative level of substances in chromatograms (chapter 3). Both semiquantitative and peak-heights approaches can be used for identified substances or chromatographic peaks, which are not identified yet, but are occurring in several samples. A quantitative set of indicators (chapter 4) are based on identified substances with at least semiquantitative concentration information. These can be used for risk assessment by comparison to relevant ecotoxicological indicators such as Environmental Quality Standards (EQS, high confidence if based on the matrix used in the SNTS screening) or Predicted No Effect Concentrations (PNECs, low to medium confidence among others depending on matrix used for SNTS screening).

In general, substances with defined EQS value will most likely be part of national or regional monitoring programs such as NOVANA, CEMP or COMBINE in Denmark, OSPAR and HELCOM, and the concentration levels from the dedicated analytical methods used here will give a more reliable risk assessment for these substances. But there is a very low number of EQS values available compared to PNECs.

The relevant use of the indicators and the general outcome of SNTS screenings are discussed, and both advantages of using SNTS screenings for coverage of a large number of substances and shortcomings regarding analytical quality and use of PNECs are discussed, and suggestions for inclusion of SNTS screening in monitoring are outlined (chapter 5).

There are still many challenges using the SNTS methods as well as an uncertainty that suggests that it may be too early to use SNTS screenings as directly as in PreEMPT and CONnECT, but there is a clear potential for developing SNTS screenings to be valuable tools in future NOVANA monitoring.

1 Sample preparation and instrumental methods for non-target analysis

This chapter describes the basic steps of extraction, clean-up and analysis of the different environmental matrices that can be used for non-target and suspect screening. In this report, the focus is on the main matrices of the NOVANA program, i.e. biota (often mussels or fish) and sediment in the marine environment. For SNTS analysis, sediments are normally sieved to the fine fraction (< 63µM) to enhance possibility of detection as most hazardous substances are concentrated in the fine fraction, but this is not the case in the NOVANA program, where whole sediments (<2 mm) are used.

The chapter also describes the methods applied in the PReEMPT and CONnECT projects as well as the scientific literature, including a recent review of sample preparation methods in NTA (Hajeb *et al* 2022) as well as the NORMAN guideline (Hollender *et al*., 2023).

1.1 Impact of extraction solvents and methods

Sample preparation is a critical step before any analysis, as it can significantly affect selectivity, sensitivity and reproducibility. While extraction and clean-up methods are highly optimized for specific substances in target analyses to ideally remove all sample components except the targets, the extraction and clean-up methods in non-target analysis should remove a minimum of sample components. As explained by Hajeb *et al.* (2022), the sample handling and processing can significantly alter the molecular composition of the samples and, consequently, the results of the analysis. In non-target analysis, the sample preparation must be as broad and non-selective as possible. Nevertheless, purification is often required to remove interfering matrix components (Hajeb *et al.,* 2022). Therefore, one of the main challenges of non-target analysis is to obtain a balance between matrix removal and preserving as many substances as possible with sufficient sensitivity, also described as the sensitivity vs. selectivity compromise (Pourchet *et al.*, 2020).

1.2 Analytical methods

High resolution mass spectrometry (HRMS) with high mass accuracy and wide mass range is the analytical technique applied for SNTS. Thus, the selectivity that is typically achieved by laboratory procedures in target analyses is introduced at the instrument stage in non-target and suspect screening. The most frequently used HRMS techniques for suspect and non-target screening studies include Quadrupole Time of Flight (QtoF) and Orbitrap mass spectrometers. These HRMS are either coupled to HPLC or GC, allowing the screening of polar and non-polar emerging contaminants, respectively.

Chromatographic separation is usually still necessary to avoid co-elution and the simultaneous ionization of substances and matrix components, thus minimizing negative effects of interferences. Furthermore, retention time indices are important tools to support correct identification (Aalizadeh *et al.*, 2021; Boelrijk *et al.*, 2023). For example, isomers will have the same mass, but usually different chromatographic retention. Suspect screening aims to confirm the presence of suspected substances in a sample, typically without a reference standard, but by using preliminary information on exact mass and isotope pattern from the

molecular formula or the expected adduct(s). In non-target screening, no information is available on the substances present in the sample, and the information about the substances is derived solely from the chromatograms and mass spectra. The associated uncertainties are addressed in different confidence levels, which should be reported together with any tentative identifications (Schymanski *et al.*, 2015; Alygizakis *et al.*, 2023b).

1.3 Metals and organometals

Most of the periodic table of elementals (incl. all metals) can be measured by Inductively Coupled Plasma (ICP)-MS and will always be very specific (targeted) analysis. However, some organometallic substances are much more toxic than the inorganic metals, e.g. methylmercury, organotins (e.g. TBT) and ethyllead. These can be analysed using a GC-ICP-MS or LC-ICP-MS setup, where the metal part is measured targeted, but the organic part will only be distinguishable by the chromatographic separation, unless running through a non-destructive detector before introduction to the plasma (e.g. fluorometer, diode array detector or similar for LC). Use of isotope ratios of e.g. lead or mercury can give some insight into sources of the metals, but usually (except for lead and, to some extent, mercury) such isotope ratio differences are very small and require high-resolution ICP-MS, preferably with a multi-collector MS detector to ensure precision of isotope ratios of more than 3 significant digits.

1.4 Data analysis

Data analysis of non-target screening involves a sophisticated approach aimed at identifying and characterizing substances present in environmental samples without prior knowledge of their existence. A generic SNTS data analysis workflow always involves several steps: raw data acquisition, data pre-processing, prioritization and identification (Figure 1.1). Preprocessing raw data from analytical instruments can be time-consuming and requires careful optimization of parameters such as baseline correction, peak detection and alignment, which may vary depending on the analytical technique used. Nowadays, there are many types of commercial software (e.g., Compound Discoverer®, SciexOS®, TraceFinder®) and open-source software (e.g., MS-DIAL (Tsugawa *et al.,* 2020), Mzmine (Pluskal *et al.,* 2010), patron (Helmus *et al.,* 2021)), which are able to conduct data pre-processing of NTA and suspect screening. There are also more specific tools, for instance for the analysis of particular classes of contaminants (e.g. FluoroMatch (Koelmel *et al.,* 2022)).

After pre-processing, features are aligned and grouped across replicate injections and/or samples within an analytical sequence, yielding a so-called feature list or feature table for further investigation. Given the labor-intensive, time-consuming and computationally challenging nature of SNTS, prioritization strategies employing chemical signatures, statistical methods and effect-directed approaches play a crucial role to focus on most relevant features in the complex environmental matrices (Hollender *et al.,* 2023). In some studies, suspect screening of potential substances of interest serves as a form of prioritization of the non-target screening data.

According to Schymanski *et al.,* (2014), the substances identified by suspect and non-target screening are classified at five confidence levels: Level 1 confirmed structure with a reference standard, Level 2 probable structure with high matching with a spectral library, Level 3 tentative candidates, Level 4

unequivocal molecular formula, and Level 5 an exact mass of interest. Identification with spectral library matching is mostly used and is able to identify substances at identification confidential level 2 or 3. However, due to the lack of comprehensive libraries, the identification can vary based on the libraries used in the same workflow. Furthermore, SNTS approaches may yield false positive or false negative results due to factors such as matrix interferences, analytical artifacts or limitations in instrumental sensitivity and resolution. Distinguishing true signals from background noise or matrix components requires careful validation and verification steps.

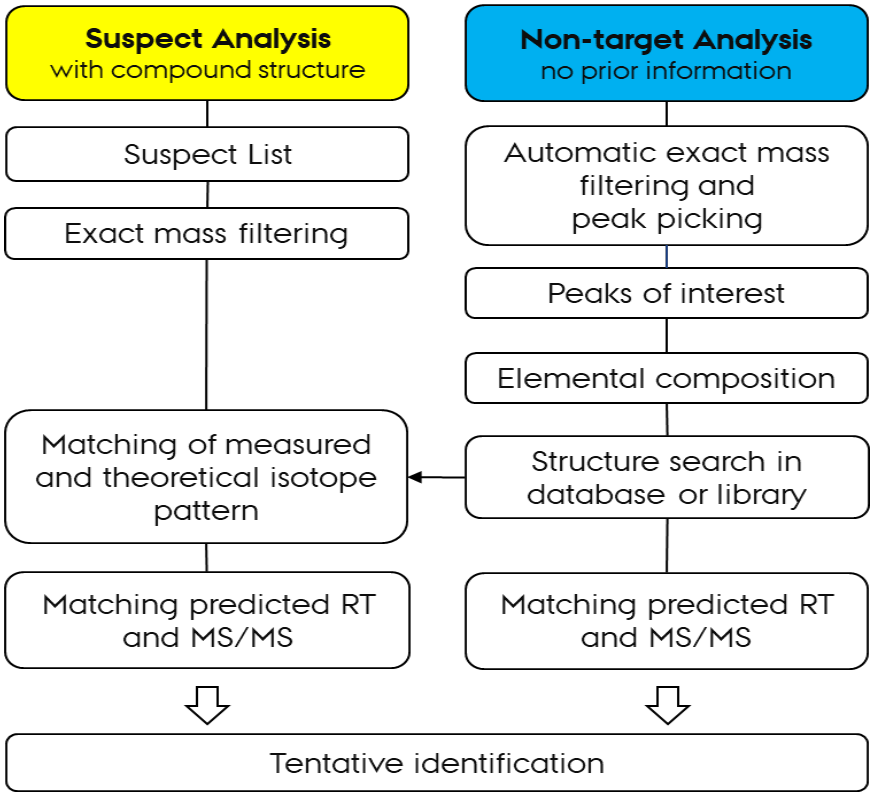

Figure 1.1. Data analysis workflow of suspect and non-target screening. RT is retention time, and MS/MS is mass breakdown in mass spectrometer for the individual peak masses

1.5 Method used in the PreEMPT and CONnECT screenings

The projects OSPAR CONnECT included biota and HELCOM PreEMPT biota and sediment samples. The biota and sediments both employed two extraction methods for LC- and GC-based analyses, respectively. The analysis was performed using Reversed phase high pressure liquid chromatography with electrospray ionization and QtoF HRMS (LC-ESI-QtoF) and gas chromatography with atmospheric pressure chemical ionization and QtoF HRMS (GC-APCI-QtoF).

Specific methods used for PreEMPT and CONnECT studies are described in appendix 9.4 as background information for the interpretation and use of their data, and the pitfalls of the methods are described in the individual sub-methods used. Note that there is no agreed overarching NTA/SNTS screening method without shortcomings (as described above), so a compromise must be weighed with regard to the individual use of the data for ensuring the data are fit for purpose.

The methods used have been described in detail in the final reports (Gkotsis *et al* 2021; McHugh *et al.,* 2022 and Alygizakis *et al* 2023b). A summary of the methods used, with emphasis on where choices have been made that could impact the detectable substances, is shown in appendix 9.4. This appendix is included for a full understanding of the origin of the data used for the indicators, but as discussed in chapter 1.1 to 1.4, there are many alternative methods. The method is just the one chosen by OSPAR and HELCOM for consistency and not a general recommendation for future use, as research is progressing fast in the field of SNTS.

2 Examples of NTA/Suspect screenings

Non-target analysis is a field in rapid development with new environmental studies being published every week. It would go beyond this study to provide a full review of studies on SNTS in sediment and biota. Recent review articles were published by e.g. González-Gaya *et al.* (2021), Minkus *et al.* (2022), Paszkiewicz *et al.* (2022), Manz *et al.* (2023) and Renner and Reuschenbach (2023). The scientific journal Chemosphere recently published a special issue on "Applications and challenges of non-target analysis with high resolution mass spectrometry for addressing chemicals of emerging concern". Since SNTS is a field of active research, we only present a few selected studies here that we consider particularly relevant for the introduction of SNTS in the NOVANA monitoring program and studies for the Danish EPA, including wastewater treatment effluents and sludge as potential sources for pollution of biota and sediments in the marine and freshwater environment.

2.1 PreEMPT and CONnECT screenings

In the PreEMPT project, 94 samples (sediment, mussels, fish muscle), and in the CONnECT project, 52 samples (mussels and fish muscle) were analysed. The screening included comparison with a 65,000 substances database based on the GC-APCI-HRMS and LC-ESI-HRMS method (Damalas, 2019; Thomaidis *et al.*, 2022), including semi-quantification. In addition, the project team performed a "wide-scope target analysis". This could be considered a suspect screening analysis, i.e. it includes a list of pre-defined substances, but with more emphasis on relevant standards, including isotopically labelled internal standards for qualification and quantification of substances. However, it did not include full specifications of a specifically developed targeted analysis with regard to neither the specificity of the substance identification nor the uncertainty of the quantification. This part of the study included comparisons to an in-house database with 2500 substances and semiquantitative approaches using LC-ESI-HRMS and GC-APCI-HRMS methods.

For the wide-scope target analysis, the semiquantitative results were further compared with existing PNEC and other risk assessment criteria for an initial risk screening for each station and the overall status in the Baltic and North Sea region (see 2.4 below).

In the Baltic Sea, the PreEMPT project found 126 substances in at least one of the matrices, and the North Sea CONnECT project identified 152 substances, out of the 2500 substances in the wide scope screening and 65.000+ in the suspect screening, indicating many substances were undetectable with the current detection limits.

Of the 126 substances found in the PreEMPT project, the top 40 substances were detected in at least 50% of the samples, 10 substances were found in more than 75% and 2 substances were found in more than 90% of the samples. All the 40 substances found in at least 50% of the samples were found in mussels and fish, whereas 35 were found in sediments. The 126 substances detected included several polyaromatic hydrocarbons (PAHs), fluorinated substances (PFAS) and chlorinated substances, which are already part of the NOVANA monitoring program. Further, many of the Danish samples were taken from the same stations as used in NOVANA (collected at the same time but

with two sub portions dissected for NOVANA and SNTS studies respectively).

2.2 Screening of wastewater treatment plants

Since NOVANA also includes wastewater monitoring, an example from this field is included as well. Many Danish WWTPs also have direct outlet to marine areas, particularly in The Sound, but also many in coastal cities. These examples are chosen for their relevance in a Danish/European context.

In a target screening survey studying 56 effluent samples from 52 European wastewater treatment plants (WWTPs), Finckh et al (2022) analysed 499 emerging chemicals. The authors aimed at quantifying the detected substances to perform a risk-based assessment for chemical mixtures from WWTPs. They used different approaches to assess the risk of the levels found for these chemicals using PNEC/EQS based risk quotients, hazard units based on species sensitivity distribution (SSD) as used for setting EQS and finally toxic units (TUs) for algae, crustaceans and fish. Only water-soluble substances were included in the list, using solid-phase extraction and LC-HRMS instrumentation similar to the suspect screening in PreEMPT and CONnECT.

The survey identified 366 of the 499 pre-defined substances. 299 of these were identified as mixture risk contributors, and 32 chemicals were classified as high concern risk contributors, mainly pesticides and biocides. WWTPs using advanced ozonation or activated carbon treatment were consistently estimated to have much lower risk levels.

Many substances (107, ~30% of the investigated substances) were found in ≥90% of the samples. About one third of the identified substances could be assigned to pharmaceuticals, pesticides and biocides (as one group) , plastic additives and surfactants (20%), food ingredients and PFAS at 13% each. The rest was "others", primarily consisting of industrial chemicals.

None of the top 30 substances found in the WWTP risk screening approaches by Finckh *et al.* (2022) were detected in biota samples from the PreEMPT and CONnECT studies, so the substances do not seem to be widely transported and biomagnified in the marine environment.

In a SNTS study of sewage sludge from five Danish WWTPs (Hansen *et al*, 2022; Nanusha *et al*, 2024), substances like methyl- and ethylparaben (100% of WWTPs), ibuprofen (80%), nicotin (60%) and perfluorononanoic acid (PFNA) (60%) were found in the sludge during the HITLIST4 project. These substances were also among those detected in the CONnECT and/or PreEMPT projects, indicating that not all is caught in the sludge, but leaks into the marine environment exist as well. The HITLIST series of SNTS studies for the Danish EPA consisted of a range of different projects adressing Danish drinking water (Frøkjær *et al.*, 2023), xenobiotics in the aquatic environment (Frøkjær, 2021) and pesticides and biocides in freshwaters (Hansen, 2021).

2.3 Use of SNTS in biota

SNTS has also been applied to analysis of biota samples, mainly for aquatic organisms. The extraction of biota matrices is particularly challenging due to the complex matrices with elevated levels of lipids, proteins and other biological molecules interfering with the detection of environmental contaminants.

Rebryk and Haglund (2021) developed a method to achieve sufficient lipid removal for NTA with GC-HRMS to investigate lipophilic contaminants. In addition, they developed a workflow designed to systematically find and identify frequently occurring and biomagnifying pollutants in a marine food web (Baltic Sea), including blue mussels, fish, and marine mammals (Rebryk and Haglund, 2021; 2022). The authors concluded that it was most efficient to focus on tissues from top predator species (e.g. harbor porpoise blubber) that are rich in contaminants in order to establish a custom library that can be used for further investigation of lower trophic levels, where concentrations are expected to be lower and, thus, more difficult to detect with a SNTS approach. The same research group also studied time trends with the SNTS approach (Rebryk *et al.*, 2022).

For biota as for other matrices, there are more LC- than GC-based SNTS studies published in the literature investigating polar substances. Most studies focus on water systems directly impacted by emission from industry or wastewater municipal plants. Fu *et al.* (2022) conducted a study on a freshwater food web to screen potential polar contaminants and evaluate their trophic transfer behavior. Plankton, blue mussels, and fish samples were included in the study. SNTS allowed the identification of 27 new substances, including plasticizers, flame retardants, other industrial chemicals and pharmaceutical metabolites.

Our own work includes SNTS on ringed seals *(Phoca hispida)* and pilot whales *(Globicephala melas)* collected in Greenland (Zhu *et al.*, 2024). Blubber and liver samples were extracted for GC- and LC-based analyses, respectively, using a sequence of solvents of different polarity. The lipid-rich blubber was extracted with four solvents or solvent mixtures, of which the first one (hexane) contained the lipids. This fraction, but not the others, was treated with acid to remove lipids and subsequently mixed with the other fractions. Using the GC approach, 43 and 34 substances were identified in pilot whale and ringed seal blubber, respectively, whereas six and nine substances were identified with LC-HRMS in pilot whale and ringed seal liver, respectively. Besides well-known persistent organic pollutants, including PFAS, the study indicated the presence of current-use pesticides and flame retardants, plasticizers, PAHs, pharmaceuticals and personal care products.

A recent interlaboratory comparison for fish samples (bream, *Abramis brama*) organized under the auspices of NORMAN revealed serious challenges with comparability and accuracy of the SNTS results (Dürig *et al.*, 2023). Some substances were spiked to the matrix (and some of these disclosed to the participants), of which 9-69% and 20-60% were correctly identified using LC- and GC-based methods, respectively. For GC-analyses, no laboratory identified all the five disclosed substances. Further, identification of other substances showed very little agreement between the participants. The main source of variation was evaluated to be in the data analysis, as samples prepared with the same method still showed substantial variability.

2.4 Risk assessment approach

Risk assessment possibilities in NTA approaches are currently limited because of the lack of concentration data. Most NTA approaches aim at identifying a compound. While developments exist towards semi-quantification (Malm *et al.*, 2021), these estimated concentrations are highly uncertain. At the same time, effect threshold values, such as PNECs, typically carry some uncertainty as well. This combination of uncertainty must be considered when discussing a risk assessment based on semiquantitative NTA results.

These precautions are also needed when handling SNTS results, as all concentration data are semiquantitative in nature and can be off by a factor from the "real" value. Future developments in both analytical techniques and calibration standards used can alter detection limits and the precision of the measurements significantly.

PreEMPT and CONnECT NORMAN Risk approach

The NORMAN risk approach is mainly based on the results from the wide scope analysis using the semiquantitative results for concentrations in biota and sediment. Following is a description of how the risk values used in PreEMPT and CONnECT were calculated, but discussions on the use of PNECs are ongoing between NORMAN and OSPAR/HELCOM, and the use below is considered very preliminary with ample room for improvements.

Risk assessment of the detected target and suspected substances was based on comparing the estimated concentrations of detected substances against their Predicted No-Effect Concentrations (PNECs), which represent their ecotoxicological threshold values (Alygizakis *et al* 2023b; Gkotsis *et al* 2021). All PNEC values used in this project were extracted from the NORMAN Ecotoxicology database (https://www.norman-network.com/nds/ecotox). For risk assessment purposes, the lowest PNEC was selected in the order of (a) EQS values; (b) experimental PNEC values from reference laboratories; (c) in silico predicted PNECs. PNECs applied in the work to date are an area that likely needs further development in the future, as the setting and application of threshold values are complex and form a key step in this evaluation. The priority (or risk ranking) was evaluated based on three 'elements': (i) Frequency of Appearance (FoA); (ii) Frequency of PNEC Exceedance (FoE) and (iii) Extent of PNEC Exceedance (EoE).

The frequency of appearance (FoA) expresses in how many sites (samples) the compound was detectable, given as a percentage of the total number of samples analysed.

The frequency of exceedance (FoE) considers the frequency of monitoring sites with observations of a compound above a certain effect threshold. It is calculated using the following equation:

FoE = (No. of samples with concentration > PNEC) / No. of samples

The final element is the extent of exceedance (EoE), which ranks substances with regard to the maximum risk observed compared to the effect threshold. It is calculated using the following equation:

EoE = maximum measured concentration / PNEC,

followed by the subsequent categorical step:

Table 2.1. Assignment of category score based on Extend of Exceedance

EoE value found	Assigned category score
EoE <1	0
≥1 EoE ≤10	0.1
>10 EoE ≤100	0.2
>100 EoE ≤ 1000	0.5
>1000	1.0

Each parameter has a maximum value of 1 and a summed risk of maximum 3. In the CONnECT program, fish and mollusks were treated separately for each substance, so the summed risk score could be up to 4.

Using the NORMAN risk assessment approach on the HELCOM dataset, 46 substances were found with a total risk assessment factor above 1, and 19 were found between a risk assessment factor of 0.5 and 1. Many of these substances are known and include dioxins, PAHs, PFAS and DDT that are already covered by the HELCOM monitoring programs. This leaves 73% of the samples with a risk assessment factor > 0.5 that at present are not monitored in the HELCOM program, mainly pharmaceuticals (51%), industrial chemicals (8%) and stimulants and personal care products (each at 6%).

2.5 Conclusions

There are many different methods within NTA and SNTS screening, each with its own benefits and problems, no "golden standard" is currently accepted. The variation from targeted (fairly optimized) analytical screening methods to the wide scope screening and suspect screening is going from high certainty on the identification of the substances and close to standard methods in analytical quality control (e.g. Finchk *et al.*, 2022) to the suspect screenings of PreEMPT and CONnECT with lower certainty for the identification and quantification.

Methods have been used with success to identify hitherto unknown substances in both wastewater, biota and sediment, highlighting both existing substance classes in monitoring programs (e.g. PAH and PFAS), but also many new classes not monitored routinely (e.g. plasticizers, pharmaceutical metabolites and parabens). Unfortunately, intercalibration between methods indicated that the identification of substances varied, with only between 9% and 64% of spiked substances identified correctly by the laboratories. This must be taken into account when evaluating results and substances against PNECs or as frequency of appearance.

In OSPAR and HELCOM, the NORMAN approach was chosen due to the whole package, where the semiquantitative results in combination with the PNEC database made it possible to calculate both FoA, FoE and EoE, mostly automated and with a "digital freezing" platform for the chromatograms making it possible to go back and repeat analysis, as done for the PreEMPT dataset. This is also the basis for the indicators presented in the next chapters, but other methods could yield other results, both in identification of substances and particularly the quantification step. For use in risk assessment procedures, quantification and relevant effect thresholds are needed, with the final aim to determine which substances need monitoring and are a risk to the (marine) environment.

3 Qualitative indicators based on occurrence

Both OSPAR and HELCOM have the long-term goal of stopping inputs of hazardous substances to the marine environment, subsequently keeping concentrations below thresholds to ensure that chemicals are not causing harm to marine life.

The occurrence of substances in the screening (based on peak occurrences in the NTA) can be used to indicate the extent of presence for each substance detected. For peaks that are detected as ubiquitous (>90% of samples), identification should be confirmed with an analytical standard and quantitative target analyses should be initiated. It will also be relevant to investigate whether the possible substances are present in other samples, for example in chromatograms stored in a digitally frozen database or in samples from environmental specimen banks.

Gros et al (2017) developed a prioritization system for micropollutants in wastewater and came up with a scoring system based on chemical properties of substances including removal efficiency in WWTP, half-life in all matrices, bioconcentration factors (BCFs) and the hazards quotient (HQs) (measured environmental concentrations/PNEC), combined with screening of maximum concentration in effluents and frequency of detection in effluents. However, this system is also based on quantitative data, i.e. it requires an analytical standard for quantification, or a semiquantitative approach based on a chemically similar compound. A score of 1 was assigned in the case of: low removal efficiency (<75%), BCF>10.000, and an HQ>1 together with detection in all samples at maximum concentrations above 1 µg/L. BCF > 1.000 and HQ > 0.1 together with 75% detection and maximum concentrations of 0.5 µg/l results in a score 2, and for more efficient removal (>75%) together with lower BCF, HQ and max. concentration of 0.1 µg/L and 50% detection a score 3. 25% detection score 4 and no detection score 5.

Using this scale, a simplified indicator based on just occurrence (not considering the BCF, HQs) can be suggested (table 3.1) either for identified substances (to a defined certainty) or for a specific recurring chromatographic peak observed in the samples. Methods using concentrations, BCF and HQs are described in the next chapter. It is important to note that false positive identification of substances can occur, so any substances found to have a large HQ should be checked carefully (e.g. appropriate blanks for checking contamination, by further investigating breakdown mass spectra or making a standard addition/external calibration of the substance using the same method) before committing resources to developing an EQS.

Table 3.1. Indicator 1 based on the occurrence of substances in the NTA samples (found in x% samples either as defined substance or a recurring chromatographic peak without substance identification). For all substances of concern, the compound identity should be thoroughly confirmed before continuing further investigations.

FoA	Indicator Status
None detected	No evidence for further action on substance
<25%	Little to small cause for concern of given substance
25-50%	Case for concern of given substance
50-75%	Substance should be given priority for risk assessment
75-100%	Substance should be risk assessed as soon as possible

If the main contributor of overall occurrence is in sediment, recommended action towards confirmation of compound identity and quantification should be focused on the same matrix. Equally, if quantitative data are available in sediment, a potential risk assessment should focus on sediment dwelling organisms.

If peak heights or concentrations are known, these can be included in a hotspot-detection indicator, which will be particularly informative for sediment results (table 3.2). This could be used to locate sources of specific substances and identify areas where sensitive species might need special protection. If substances are already in the European Chemicals Agency database (ECHA) (echa.europe.eu), estimates of production and use are available to support decisions on the urgency of the action.

Table 3.2. Indicator 2 – test for hotspot potential and spreading of hotspot chemicals in the general environment (intensity is either peak height for unknown or uncalibrated substances or expected concentrations for suspect screening or wide scope screenings).

Relative intensity of substance in highest sample	FoA	Indicator status
<10	<10%	No evidence for further action
>10	<25%	No to little cause for concern for hotspots given substance
>100	<50%	Case for concern of hotspots of given substance
>100	<75%	Substance should be given priority for hotspot investigations
>100/ >1000	<90%/ <75%	Substance should be risk hotspot investigated immediately

4 Quantitative indicators based on concentrations and risk

To use quantitative indicators in the absence of target analysis, SNTS approaches with semiquantitative approaches are needed. To improve confidence in estimated concentrations and identifications of substances, both internal standards and a calibration solution should be analysed together with the samples. Both need to be of suitable similarity to the identified substances to allow the concentration estimation. Thus, the substances are not calibrated directly, but semi quantified using standard curves for other substances. If the relevant analytical standard is available, an SNTS approach can increase the identification level as well as increase the precision of the substance quantification (e.g. wide scope screening).

For risk assessments, a threshold value is needed where effects are expected. For very few substances, EU Environmental Quality Standards (EQS), regional (OSPAR/HELCOM) or national criteria are available for biota and sediment, and further, in most cases only water based PNECs are available, for example from the NORMAN Ecotoxicology Database (https://www.norman-network.com/nds/ecotox/lowestPnecsIndex.php). The conversion from a water based PNEC to biota or sediment criteria is challenging and adds to the uncertainty of the PNEC derivation (as well as the semi-quantification of SNTS results). Even for EU EQS dossiers, the recalculations between water and biota are often based on a large range of conversion factors, leading to uncertainties of a factor of 10 or more. There is a clear need for more data, with possibilities of using Quantitative Structure–Activity Relationship models (QSAR models) to fill data gaps.

In cases where the preliminary risk assessment is above one, or in such cases where the PNEC value is lower than the respective method's LOD, the analysis should be followed up by a conventional target analysis. These are more sensitive and precise methods, which should be applied to draft reliable conclusions on the associated risk, by producing a more reliable HQ before committing resources on developing an EQS.

4.1 Approach in PreEMPT and CONnECT

A risk quotient was calculated in the PReEMPT project based on semiquantitative data and the PNEC values from the NORMAN database. This approach was described as follows in the PreEMPT final report (Alygizakis, 2023a): In cases where PNECs were not available, no risk assessment could be carried out. For extracting the Frequency of Appearance (FoA) for every contaminant, concentration levels above the LOD were considered (including also substances that were detected below the limit of quantification (LOQ)), while only concentrations above the LOQ were considered for the risk assessment.

The outcome of the results is shown in appendix 9.2. Note that for many substances (marked with a * in the annex), the PNEC or EQS was lower than the LOD for the method, and only values above LOQ were counted as being a risk. The risk score developed by NORMAN is taken as:

$$\text{Risk} = \text{FoA} + \text{FoE} + \text{EoE (PreEMPT)}$$

For the CONnECT project, FoA was determined for both mollusks and fish individually, but the Risk was only determined as the sum of exceedances:

$$\text{Risk} = \text{FoE} + \text{EoE (CONnECT)}$$

Hence, the upper limit for Risk is 2 for each of biota and sediment samples (4 if found in both, see chapter 2.4), but the highest FoA should be added to bring this in line with PreEMPT risk quotions, in this case 0.08 (Sotalol in mollusks) to 1 (Methylparaben in fish). Only five substances were found with a risk score above 1, and adding the FoA to the risk brought only four more substances up to a risk score of above one.

The PNECs were converted to biota using very simple factors, so the risk results can only be viewed as indicative results, unless actual biota EQS's are available for the substances used. However, for these substances environmental monitoring based on target analysis is usually already in place. Furthermore, given the high uncertainties of the semiquantitative approaches, the results will remain indicative.

4.2 Outcome of NORMANs approach in PreEMPT and CONnECT

The main results of PreEMPT and CONnECT are shown in appendices 9.1 to 9.3 for the whole HELCOM/OSPAR dataset. In this paragraph, the results for the Danish samples are discussed.

The substances and indicators based on the Danish results are shown in table 4.1 for biota and 4.2 for sediments. The biota results were previously reported in the NOVANA report "Marine Områder 2020" chapter 11 (Hansen & Høgslund, 2021)

The biota samples (from CONnECT) have been classified according to PNEC values when possible (table 4.1). For the wide scope screening 67 substances were identified, no classification has been performed (substances in green). For the suspect screening resulting in 76 substances, the colour indicates the concentrations found in relation to the PNEC value from NORMAN: with a graduate colour scale from good (green) to bad (red), where yellow and red indicate samples with a risk factor of >1. For fish, the reference stations OSPAR (O) and HELCOM (H) were the ones with most substances found (8 respectively 9). For molluscs, fewer substances were found at the reference stations. But still, two samples from the OSPAR reference station scored the highest factors above PNEC, indicating that the reference stations were probably not the least polluted stations, despite being chosen as stations the farthest from known sources.

The sediment samples (table 4.2) have been arranged according to their maximum concentrations. For 28 substances, one of the five Danish sediment samples contained the highest concentrations overall of the 30 screened sediment samples in PreEMPT (table 4.2). Further 14 substances were found in the 75th percentile range of all samples, so 42 of the 81 substances found in the Danish sediment samples were in the top quartile of the concentration range for all Baltic Sea samples. Most of the substances were in the ECHA database (39). Of these, 23 was substances classified as industrial chemicals, followed by eight pharmaceuticals and eight agricultural substances (pesticides etc.), and two substances related to personal care products.

Table 4.1. Substances found in at least four of 13 Danish biota samples (F= fish, M=molluscs; CONnECT study). Results are given as < (not detected), P for below quantification but detected og K for quantified. The colour scale graduates risk factors from dark green (0,1<RF<1), light green (1<RF≤5), yellow (RF>5) up to dark red (highest RF 550). O indicate Ospar, H Helcom.

Sample type		**F (O)**	**F (H)**	**F**	**F**	**F**	**F**	**M (O)**	**M (H)**	**M**	**M**	**M**	**M**	**M**
Sample No.		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Reference station for		O	H					O	H					
Substance	Usage													
Methylparaben	Personal care	K	K	K	K	K	K	K	K	K	K	K	K	K
Pyrene	Industrial	<	K	<	<	K	K	K	K	K	K	K	K	K
Antipyrine- 4-Acetamido	Pharma	P	P	K	K	K	K	K	P	K	K	K	<	<
N,N-Dimethyldodecylamine	Industrial	K	P	K	K	P	P	<	<	K	K	<	K	<
Chrysene	Industrial	<	<	<	<	<	<	<	K	K	K	<	K	K
Lidocaine-N-oxide	Pharma	<	<	<	<	<	<	K	K	<	K	<	K	<
Acetyl tributyl citrate	Pharma	K	<	<	<	<	<	<	K	K	K	K	K	K
Alminoprofen	Farming	K	<	<	<	<	<	<	K	K	K	K	K	K
Nicotine met 3-166	Farming	<	K	<	<	K	<	<	K	K	K	K	K	<
2-Methylpyridine	Surface	<	<	<	<	<	<	K	<	K	K	K	K	K
CAPRYLOYL SALICYLIC ACID	Metabolite	<	<	<	K	<	<	<	K	K	<	K	K	K
Nicotine met 8-194	Pharma	<	<	<	<	<	<	K	K	<	K	K	K	K
2-[1-(4-hydroxyphenyl)-1-methy-lethyl]-Phenol	Pharma	<	<	<	<	<	<	K	K	K	K	K	K	<
Butyrophenone	Industrial	<	<	<	<	<	<	K	K	<	K	K	K	K
Hexanoic acid, 2-ethyl-, hexadecyl ester	Industrial	K	<	<	K	<	<	<	K	K	K	K	<	<
N,N-Bis(2-hydroxyethyl)-4-pyridine-carboxamide	phthalat	<	<	<	K	<	<	K	K	<	K	K	K	<
9-?-D-ribofuranosyl-9H-purine	Industrial	<	<	<	<	<	<	<	K	<	K	K	K	K
Hexadecyl hydrogen phthalate	phthalat	K	<	<	<	<	<	<	K	<	K	K	<	K
Indole-3-acetic acid	Industrial	<	<	<	<	<	<	<	K	K	K	<	K	K
Phenallymal	Landbrug	<	<	<	K	<	<	K	K	<	<	<	K	K
2-Quinolinecarboxylic acid, 4-hy-droxy-	Industrial	<	<	<	<	<	<	<	K	K	<	<	K	K
Nicotine met 12-326	Pharma	<	<	<	<	<	<	<	<	K	<	K	K	K
Valerophenone	Industrial	K	<	<	K	<	<	<	<	K	K	<	<	<
Amines, C15-alkyldimethyl, N-oxides	Pharma	<	<	<	K	<	<	<	<	K	K	<	K	<
Pentaethylene glycol monododecyl ether	Industrial	<	K	<	<	K	K	K	<	<	<	<	<	<
N-(2-Hydroxyethyl)octa-Decanamide	Pharma	<	K	<	<	K	K	K	<	<	<	<	<	<
PPG n8	Surface	<	K	<	<	K	K	K	<	<	<	<	<	<
Phenoxyethyl caprylate	Industrial	<	K	<	<	<	<	<	K	<	K	K	<	<

Table 4.2. Substances and usage type found in the five Danish sediment samples (PreEMPT study). Results are given as < (not detected), P for below quantification, but detected, and K for quantified. There are no PNEC values recalculated to sediments, so no classification has been done for the sediment samples.

Sample no.		**1**	**2**	**3**	**4**	**5**
Substance	Usage					
3,5-Di-tert-butyl-4-hydroxybenzaldehyde	industrial	K	K	K	K	K
2-Naphthylamine	Industrial	K	K	K	K	K
Methacrylamide	industrial	K	K	K	K	K
(3S-trans)-hexahydro-3-isobutylpyrrolo[1,2-a]pyrazine-1,4-dione	industrial	<	<	K	<	K
Octadecanamide	industrial	K	K	K	<	K
N-Methyl-2-pyrrolidone	industrial	K	K	K	K	K
Benzoic acid, 4-methoxy-	industrial	K	K	<	K	K
4,4-Dimethyl oxazolidine	Pharma	K	K	K	K	K
Telbivudine	Pharma	<	<	<	<	K
Threonate	Pharma	K	K	K	K	K
stearic acid, monoester with glycerol	industriel	<	K	K	<	K
4-Morpholinecarboxaldehyde	industrial	K	K	K	K	K
Octinoxate	Ultraviolet	K	K	<	K	K
2-[2-(Dimethylamino)ethoxy]ethanol	industrial	K	K	K	K	K
Tris(2-ethylhexyl) phosphate	industrial	<	<	K	<	K
5'-Methylthioadenosine	Pharma	K	K	K	K	K
N-(2,4-Dimethylphenyl)formamide	Pesticide	K	K	K	K	K
N-dodecyl-4-methoxybenzamide	Industrial	K	K	K	<	K
TP1/Isophorone	Industrial	<	<	K	K	K
N,N-Bis(2-hydroxyethyl)dodecanamide	Industrial	<	<	K	<	K
Monoethyl phthalate	phthalate	K	K	K	K	K
1-Ethenylazepan-2-one	Industrial	K	<	K	K	<
Hexaprofen	Pharma	K	K	K	K	K
2,3-Dihydroxypropyl pentadecanoate	Industrial	K	<	<	<	K
Ethyl 3-(N-butylacetamido)propionate	Insectiside	<	<	<	2,1	<
1,3-Benzenedimethanamine	Industrial	K	K	K	K	<
N,N-Diethylaniline	Industrial	K	<	K	<	K
Perfluorononanoic acid	Industrial	<	<	<	<	K

5 Other approaches

5.1 Rarity score

Krauss et al. (2019) suggested a rarity score as a way to implement a rapid screening approach for surface waters without the need for PNECs or even knowledge about the substance, only using the intensity (figure 5.1). If available, concentrations can be used instead of the peak height (intensity) of the signal. Non-detects are substituted with minimum intensity threshold or the detection limit for targeted analysis. The lowest rarity score is 1 if the median = maximum intensity.

The rarity score used in the example of Krauss *et al.* (2019) included 80% of the samples that had an RS_x between 10-100. Only about 1% of the samples had a rarity score above 1000 and, in many cases, this was attributed to only one sample. However, for other substances found with high intensities, up to around 10 of the 31 samples could have $RS_x > 1000$.

Figure 5.1. Rarity score

$$RS_x = \frac{maximum\ intensity\ across\ all\ sites(x)}{median\ intensity\ across\ all\ sites(x)} \frac{Total\ number\ of\ samples}{Number\ of\ detected\ samples}$$

The use of RS_x is mainly to find hot spots, and the outcome is very dependent on the number of samples analysed, but no minimum number of samples was given. Based on the discussion of Krauss *et al.* (2019), the 31 samples used as an example seemed to be a reasonable number, making approximately 25 samples in the lower limit. As an indicator value for each substance x, the RS_x could be used in parallel with EoE setting up a classification system of ranges of RS_x of e.g. 10, 100, 1000 and 5000, as summarized in table 2.1 (chapter 2.4). Where EoE is based on actual concentration estimates, RS_x only needs an intensity, so it can be used on peaks that have not been identified as specific substances and, hence, more versatile than EoE for true non-target screenings.

For the PreEMPT dataset, the highest RS_x score was 43.8, with a median of 3.5 for the 126 detected substances. However, the use of biota and sediment as monitoring matrices will probably reduce the span of results compared to surface waters. For the PreEMPT dataset only one substance above 40 (1-Propanone, 1-(4-dodecylphenyl)-2-hydroxy-2-methyl-, ECHA 10-100 tons production) and two above 30 (Telbivudine, pharmaceutical and 2H-1-Benzopyran-2-one, 7-amino-4-methyl-, an ECHA registered textile dye of 1-10 tons production) and further five additional substances above 20 were identified.

The rarity score method is possibly more suitable for pinpointing hotspots directly from effluents compared with more complex bio-uptake or sedimentation processes. The classification system should be modified to accommodate an approximately 100-fold lower range of outcomes.

5.2 Extension of the risk-based approach

The basic idea of a risk quotient is described as:

RQ= concentration/assessment criteria

The NORMAN derived risk factors were based on the PNECs from the NORMAN database. Other sources of assessment criteria could include EQS values as the top tier RQ assessment criteria. Single study assessment criteria might exist for specific substances. If these should be used, we suggest defining a confidence level of 1 if the same species and substance(s) are investigated, for same biological and substance class a confidence level 0.5, and finally, PNECs can be based on mixtures of known substance groups, products without specified substances or for another biological class, these are set to confidence level between 0.1 and 0.2.

The final RQ could be classified with both a maximum risk quotient RQ_{max} for a given substance (hot spot identification) and a median risk quotient RQ_{med} for all substances evaluated. The confidence level of the RQ should be based on the median confidence level for the individual substances in RQ_{med} and the median confidence levels for the top 5 to 10 highest values in the case of RQ_{max}.

5.3 Spatial analysis

If enough samples have been taken, it would also be possible to make a division into the individual spatial areas, e.g. assessment units in HELCOM or the North Sea, Kattegat and Baltic for the Danish Sea areas. The areas should be at a size so there are at least 5-6 samples per spatial area. The indicator could be mapped in a pie diagram of the substances categorized according to their use (reduced to <10 different use cases for better overview) and the Frequency of Appearance or other parameters used as sizes of the chart together with the number of total substances detected in the area. Figure 5.2 shows the location of the samples in PreEMPT.

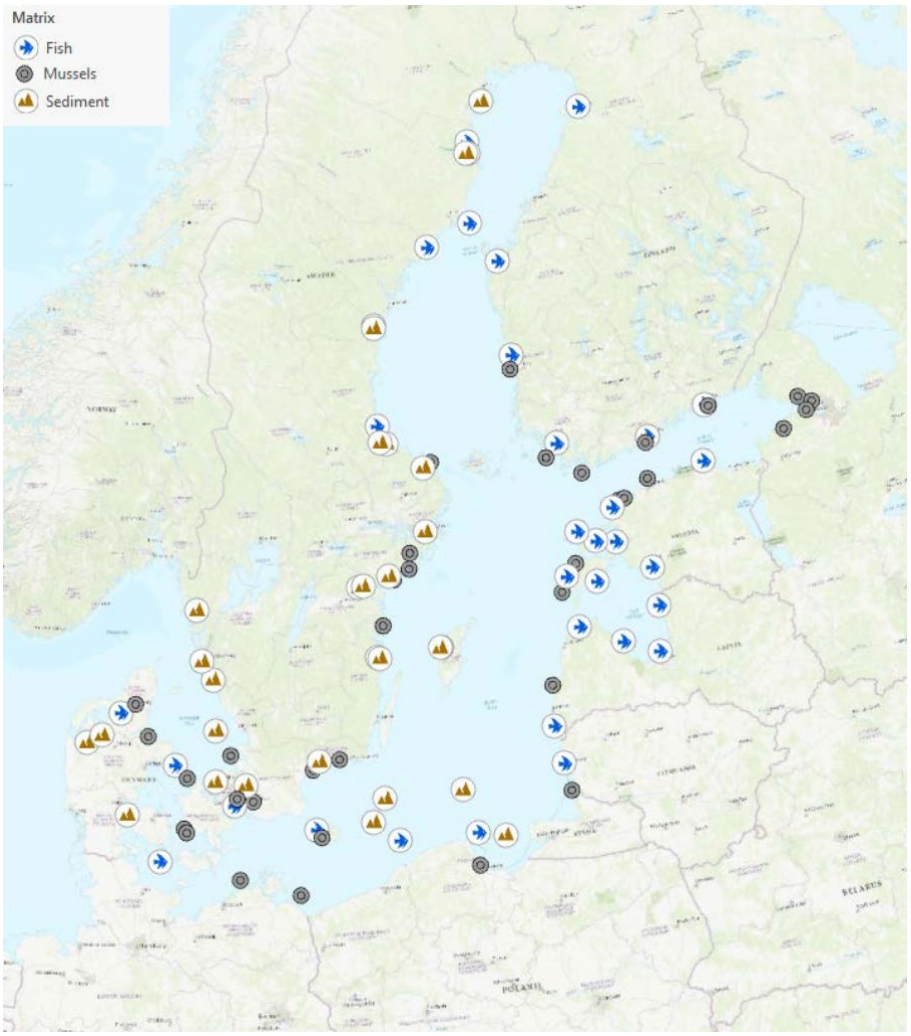

Figure 5.2. Stations from HELCOM PreEMPT for the Baltic Sea

An example of pie charts, based on the data from PreEMPT, is given in figure 5.3 of each area of OSPAR (North Sea plus Kattegat) and HELCOM (Belt Sea + western Baltic Sea out to Bornholm). The areas have respectively 5 mussels and fish stations and 8 mussel and fish stations. For both areas, 76 substances were detected in the wide scope/suspect screening in at least one sample. For each substance, the concentration was divided by the median value for the substance for all analysed samples (in this case corresponding to the 31 samples from the CONnECT study). For each substance group, pie charts were constructed based on median and maximum values using all seven substance groups that were identified. For each substance group, the number of substances included is given next to the name as #x. Finally, for each pie chart the number of substances above detection limit is indicated to the right of the pie chart (see figure 5.3).

Of the 76 substances found in the Danish samples for OSPAR (Kattegat + North Sea) respectively HELCOM (The Belt Sea + Western Baltic Sea out to Bornholm), the distribution between detections is shown for each area (HELCOM = 8 stations; OSPAR = 5 stations in all) in figure 5.3. Note that seven types of chemicals were found in both OSPAR and HELCOM, but in different proportions (more agricultural substances for OSPAR, versus more surfactants for HELCOM). For the median detected, it is noticed that pharmaceuticals and industrial substances were found in OSPAR samples, whereas phthalates and substances related to smoking were found in HELCOM, in both cases together with agricultural substances and personal care products as well as UV substances.

Similar pie charts and total findings could be made for the "EoE" indicators for substances with PNECs to indicate which substance groups are most related to the individual assessment units, and eventually per station to map out the hot spots.

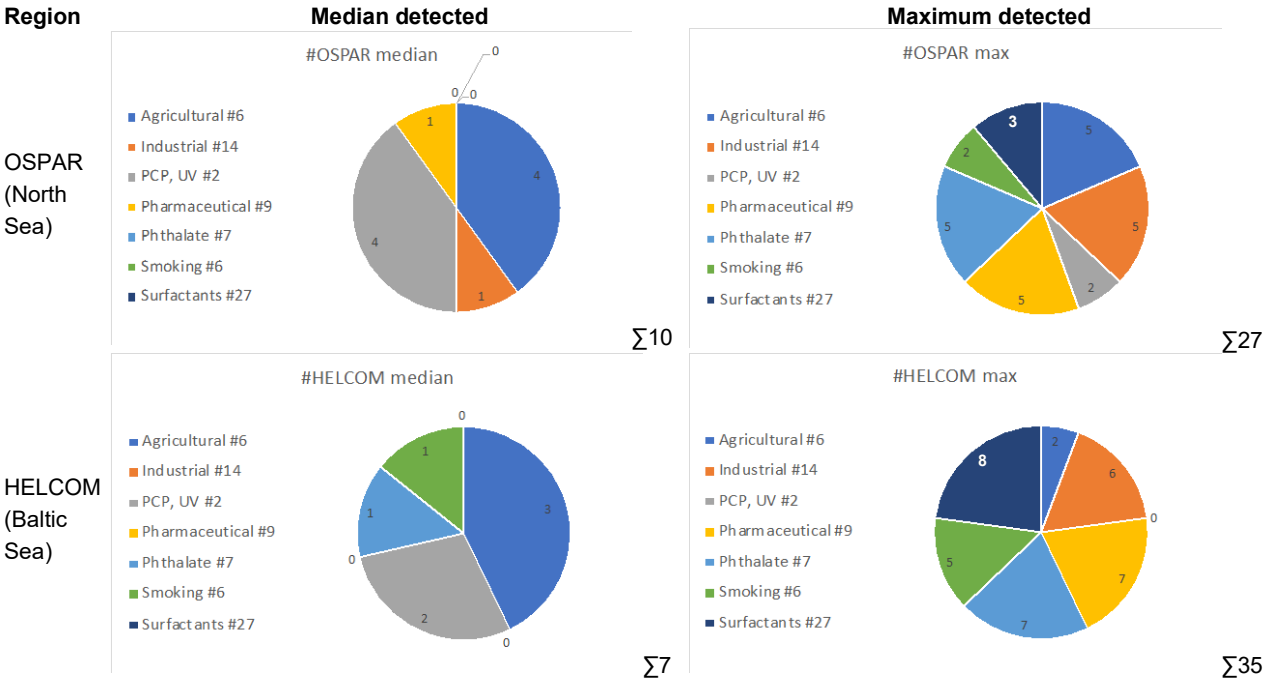

Figure 5.3. Number of stations with median and maximum concentrations above detection limit based on substance type (with number of substances for each type indicated behind the #). The total number of substances above detection limit is given in the lower right corner for each region and median/maximum detected figure.

6 Monitoring matrix and frequency

The CONnECT and PreEMPT screenings focused on biota and sediments, the background being that findings in biota indicate potential bioaccumulative substances, and findings in sediment indicate long term pollution and substances prone to adsorption to particles.

The screening of water, be it drinking water, freshwater or marine waters, requires some sort of preconcentration and will typically be very restricted in time (typically spot samples), i.e. not representable of time-averaged levels, unless passive sampler are used. But wastewaters are often sampled as time- or volume averaged samples over longer time periods and could be a good measure of the water-soluble substances added to the environment. Measurements in the WWTP sludge could indicate adsorptive substances, which could reach the freshwater or marine environment if the sludge is used on fields close to watersheds or beaches.

SNTS screenings could be even more useful, if agreement within OSPAR and HELCOM could be made to enhance the number of stations and coverage in the North Sea and the Baltic Sea to detect substances, which in time could enter Danish waters from neighbouring waters. Work is underway in OSPAR and HELCOM to repeat and build SNTS screenings into their respective regional sea monitoring commitments.

6.1 Biota samples

SNTS screening of biota samples sampled in the NOVANA program can be used to indicate potential bioaccumulative substances in both coastal (potentially polluted areas) and open waters (potentially pristine areas). The selection of stations could be based on the distance to known sources (e.g. river outlets, harbours or WWTP outlets) to investigate in what range the substances are actually found as well as an indication of local pollution. The open water samples can be used to indicate substances that could be ubiquitous in the marine environment, with a potential for long-range transportation out in the more open areas. The biota samples represent a fairly recent occurrence in the range of weeks to months and elucidate also a potential for bioaccumulation in the organs/biota investigated.

Pretreatment such as freeze-drying will lose some volatile chemicals but will also act as a pre-concentration step for those that are less volatile. In general, keeping samples frozen is a better option, unless samples are to be sent long distances where thawing can occur during transportation.

The indicators for occurrence can be used to point out chromatographic peaks of potential interest, even if the substance is currently unidentified. Further, use of chromatographic information (retention time) combined with the mass-spectra of fragments might be used for a preliminary identification, followed up by further chromatographic and breakdown-spectra to assist in a final positive identification of the substance. For identified substances in the suspect or wide scope screenings, the semiquantitative result can be compared with EQS or PNECs to produce a Risk Quotient, which if >1 can be indication of some urgency in assessing the possible sources as well as the general occurrence and need for further development of PNEC and EQS values for the

species/target organ combination. The uncertainty of the Risk Quotients still must also be taken into account when prioritizing substances for further analysis.

6.2 Sediment samples

SNTS screening of sediment samples collected in the NOVANA program will have a similar application to that of biota samples, but is more directed at more long-term (probably years) occurrence and substances with low degradability in potentially anoxic sediments. Pretreatment to ensure comparability between samples and better detection limits include sieving to <63µm fraction. Sieving can be performed either as wet sieving and freezing, or freeze-drying and dry sieving, with potential loss of some substances (volatiles) during freeze drying. Freeze drying is only recommended if samples are to be sent over long distances, e.g. biota. As for all sediment monitoring, the main areas where pollution will be expected to occur have high sedimentation rates and small particle sizes, where the higher (and potentially charged) surface area to volume is favourable for substances with high K_{ow} (potentially bioaccumulative). Normalization to organic carbon content for organic substances is common, as this is where organic substances usually concentrate in.

The indicators for occurrence can, again, be used to appoint substances of potential interest, even if the substance is currently unidentified, and use of chromatographic information (retention time) combined with the mass-spectra of fragments can be used for a preliminary identification. This can be followed by further chromatographic and mass-spectra information in a final positive identification of the substance. For identified substances, semiquantitative results can be compared with sediment QS or PNECs to produce a Risk Quotient. For sediment, however, the long-term stability/lack of degradation is of the same concern as the concentration levels for "forever chemicals" that can be released again, if sediment is being moved during dredging or during storm events.

As sediments are generally easy to sample, sediments are most relevant for SNTS sampling for long range transportation substances, but also as a tool for assessing hitherto unfound connections with e.g. oil and gas drilling in the North Sea.

6.3 Water samples

In the Danish marine NOVANA program, water samples are not currently inclued. The chance of finding substances in marine water is also very low due to the high dilution factor in seawater. Water sampling is therefore most relevant in freshwater systems or wastewater outlets to increase the likelihood of detection. Using WWTP outlets also has the advantage that the source is well described, but WWTP can contain unexpected substances, which, if possible, can be backtracked to original sources.

Monitoring programs usually rely on grab samples to detect polar contaminants. However, the concentrations are usually too low to be detected directly by SNTS. Hence, pretreatment of water samples usually includes a preconcentration step(s) on different columns with specific chemistries that include/exclude the range of substances possible to detect. Tadicét *et al.* (2022) found that passive sampling using e.g. Polar Organic Chemical Integrative Sampler (POCIS) showed a clear advantage in comparison to grab sampling,

reflected in the higher number of identified substances and higher level of confidence in samples collected with passive sampling. It should be noted that most POCIS only work for water soluble polar substances, with different uptake rates depending on the substances and environmental conditions.

The use of PNECs and EQS for water samples are better developed and, in most cases, mainly developed for use in (fresh) water systems. Water sampling from WWTPs that are close to where streams enter the sea can be used to verify inputs of point sources to the marine from the freshwater systems and assist in tracing, where specific substances enter the marine environment. To overcome the spot-sampling strategy, passive or active samplers can be used to take average samples over days or weeks, but this requires at least two visits to sites.

6.4 SNTS analysis sampling strategy for NOVANA

In general, SNTS analysis is a useful tool for expanding the knowledge of potential substances that are not routinely monitored and should be carried out one to two times per 6 years cycle of the Water Framework Directive WFD and/or the Marine Strategy Framework Directive (MSFD) monitoring. Sampling in the WFD area could focus on expected problematic areas (areas with large cities, industry outlets, extensive farming etc.), whereas the MSFD area could be used for assessing chemicals with long range transportation traits. A tiered approach (figure 6.1) would be the most efficient: the first-year targets biota in areas with major sources together with a wider general sampling to establish which (if any) substances are of concern. To have enough data for a realistic assessment, 33-50 samples should be considered. By using the geographical knowledge gained from biota screening, sediments samples should be taken from the areas with indication for raised levels for long-term pollution estimates. This could be combined with, or followed by, sampling from streams or WWTP that could potentially contribute to the biota and sediment stations. This could be done in a three-year cycle, potentially with sediments being sampled together with biota, but not analysed before outcome of biota samples have shown which areas are problematic.

Alternatively, all samples could be collected in the beginning of a new cycle, and a follow up can be made during the cycle with either more SNTS screenings in areas with similar sources or more specific screenings of substances of special concern. In the latter case, target analysis could be a possibility, also ensuring better detection limits and validated methods, and further follow up with development of EQS if concentration levels indicate preliminary risk quotients > 1 after the first round.

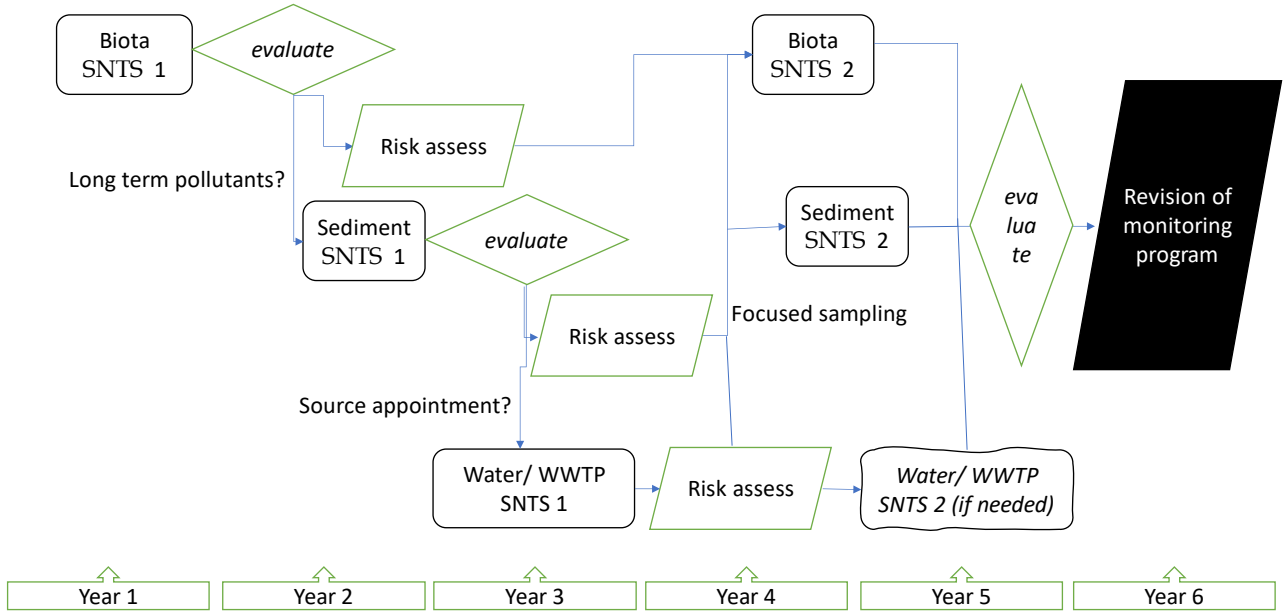

Figure 6.1. Suggestion for NTA analysis cycle for WFD and MSD for optimization of substance selection before next cycle

In the risk assessment phase, a preliminary risk assessment based on the NORMAN methodology could be used. However, consideration of the validity of the PNEC and transformation of PNECs should be part of the assessment. If outcome of the SNTS screening indicates a more general problem, establishment of an EQS for biota or sediment should be initiated.

The development of NTA protocols will make more suspect screening substances available over time, as more and more non-target substances are analysed in depth and become suspect substances. In the HITLIST4 screening of wastewater sludge, several high peaks were found in the original run, and high-level identification of these substances became a high priority in the data processing. For the PreEMPT and CONnECT screening tests, a rerun on the "digitally frozen" datasets was prepared in December 2023 and extended the suspect screening and number of peaks integrated by a factor of almost two, potentially catching many more substances than in the original run.

7 Conclusions

The use of suspect and non-target screening is a promising extension of the existing NOVANA program, which will give the opportunity to document the presence of new substances, for which there would otherwise not be any data. Based on the substance's presence-indicators, it can be decided for which substances there is a need for more investigations and targeted analysis, or if SNTS screenings should be extended to freshwater systems and WWTPs to find potential sources. For sediments, the data can establish whether substances will concentrate in sediments over time and become future problems.

The development of future monitoring can be partly based on SNTS screenings. Preliminary and tentative risk assessments could be performed if semiquantitative data are available in combination with effect thresholds, such as the PNEC database of the NORMAN network. However, this approach includes so many uncertainties (i.e. semiquantification, modelled PNECs, conversions from water to biota or sediment, etc.) that it can be questioned whether this approach is meaningful with the current state of knowledge. More research will be needed to reduce uncertainties in these approaches, including false positives due to erroneous identifications and negatives due to too high detection limits or inadequate prepation methods.

SNTS screening is generally not suited for checking compliance with EQS, as it is not a quantitative approach. Furthermore, both the limits of detection and the analytical uncertainty are typically higher than in the target analysis performed under current environmental monitoring programs, such as NOVANA or COMBINE/CEMP. Thus, SNTS screening is not typically used for determination of absolute concentrations. Regarding time trend studies, Rebryk *et al.* (2022) showed that normalized chromatographic response can be used in NTA retrospective studies with results in agreement with the time-trend direction of targeted analysis for top-predators. However, the question remains whether the detection limits are sufficient for retrospective studies of blue mussels, the main coastal species in the North Sea and Baltic Sea, or fish species used in more open waters. The main focus of time trends is to follow effects of measures taken against use or production of substances, or improvements in the WWTPs with an extra chemical cleaning step. But the compliance monitoring part comparing concentrations to environmental, or background assessment criteria is not possible without determination of absolute concentrations. This is used to calculate a "distance to target", i.e. how many years until a substance complies with the target concentrations. Furthermore, the SNTS screening "presence indicator" could be used to indicate general improvements/reductions in the number of substances that reach the freshwater or marine environment and potentially reduce the chemical status or ecological status in rivers, lakes and the ocean. This was also demonstrated by Rebryk *et al.* (2022).

The periodic use of SNTS screenings in biota, sediments and, if deemed necessary, in wastewater effluents/river outlets to pinpoint sources, would be a strong tool to combat pollutions that are not included in the ordinary robust monitoring programs for known contaminants in NOVANA, COMBINE or CEMP. The indicators can further be used to prioritize substances for development of EQS targets and risk assessment and raise alerts for hitherto unknown pollutants before they reach critical environmental levels.

Coordinated screenings within OSPAR/HELCOM will provide stronger indicators for the total marine environment. Around 40-50 samples of each matrix are estimated to give a fairly robust assessment of upcoming substances, but it will take at least a year from sampling and delivery to laboratory until reporting, as was the case for PreEMPT and CONnECT.

7.1 Konklusioner (in Danish)

Brugen af suspect og non-target screening analyser er en fornuftig udvidelse af det eksisterende overvågningsprogram, som giver mulighed for at dokumentere tilstedeværelsen af mange stoffer, der ellers ikke ville være data på. Ud fra "tilstedeværelses"-indikatorerne kan de stoffer, der potentielt skal undersøges nærmere med målrettede analyser, udvælges, eller der kan gennemføres SNTS screening af ferskvande og renseanlægsudløb for at finde potentielle kilder. Screening af sedimenter kan vise, om stofferne bliver opkoncentreret over tid i sediment og dermed kan blive fremtidige problemstoffer.

Udviklingen af fremtidens overvågningsprogrammer kan delvis baseres på SNTS screeningerne. Preliminære risikovurderinger kan gennemføres, hvis semikvantitative data er tilgængelige, sammen med effektgrænser som fx NORMAN gruppens PNEC-værdier. Der er dog mange usikkerheder ved både SNTS screeningernes kvantificering og NORMANs PNEC-værdier og deres omregning til biota-kvalitetskrav. Der kan derfor sættes spørgsmålstegn ved, om det er en meningsfuld måde at risikovurdere stoffer med den nuværende viden. Mere forskning er nødvendig for at reducere usikkerhederne ved disse fremgangsmåder, især med hensyn til falske positive på grund af fejlidentifikation af stoffer og falske negative ved forhøjede detektionsgrænser eller uoptimerede prøveforberedelsesmetoder.

SNTS-screening er ikke egnet til at kontrollere, om EQS er overholdt, fordi det ikke er kvantitative metoder. Selvom der beregnes semikvantitave koncentrationer er analyseusikkerhederne typisk er meget større end for målrettede analyser som dem, der anvendes i det eksisterende NOVANA program, og detektionsgrænserne vil i mange tilfælde ikke være tilstrækkelige til at være 10x under EQS-niveauer. Med hensyn til tidstrend-studier viste Rebryk *et al.* (2022), at normaliseret kromatografisk respons kan anvendes i NTA retrospektive studier med god overensstemmelse med tidstrends retningen for målrettede analyser af toprovdyr. For toprovdyr er detektionsgrænser i de fleste tilfælde tilstrækkelige på grund af opkoncentrering i fødekæden, men enkelte stoffer, der kun opkoncentreres lidt igennem fødekæden kan stadig blive overset. For SNTS-målinger i både blåmuslinger, den mest udbredte art i Nordsøen og den danske del af Østersøen, og fiskearter, der anvendes i de mere åbne vande, kan detektionsgrænserne være over PNEC og MKK-kravene og dermed give falske negative. Hovedfokus for tidstrend-analyserne er at følge effekten af lovgivningen mod brug eller produktion af enkeltstoffer eller stofgrupper, eller forbedringer af renseanlægs effektivitet med ekstra kemikalierensningstrin. Men kontrollen af overholdelse af MKK eller baggrundsvurderingskriterier er ikke mulig uden kvantitative koncentrationsmålinger. Sådanne målinger anvendes til at beregne et "afstand til målsætning", dvs. hvor mange år går der, før et stof er under MKK kravet. SNTS screeningernes påvisningsindikator kan anvendes som indikator for en general forbedring/reduktion i antallet af stoffer, som når det ferske eller marine miljø, og potentielt reducere den kemiske eller økologiske status i floder, søer og havet. Dette blev også demonstreret i Rebryk *et al.* (2022).

Periodisk anvendelse af SNTS-screeninger i biota, sediment og, hvis det bliver vurderet nødvendigt, også i spildevandsudløb/vandløbsmundinger for at identificere mulige kilder, vil være et stærkt redskab mod forurening med stoffer, der ikke indgår i det ordinære danske NOVANA overvågningsprogram, eller HELCOMs COMBINE og OSPARs CEMP overvågning. Indikatorerne vil give mulighed for at prioritere, hvilke stoffer, der skal udvikles MKK (EQS) kravværdier for samt fange ukendte stoffer, før de når til et koncentrationsniveau, hvor de har indflydelse på kemisk eller økologisk status under Vandrammedirektivet eller Havstrategidirektivet. Koordineret screening inden for OSPAR/HELCOM vil give meget stærkere indikatorer for det totale marine miljø, og fordele omkostningen ved de 40-50 prøver for hver matrix der skal til, for at give en rimeligt robust vurdering af potentielt nye stoffer.

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9 Appendices

9.1 PreEMPT occurrence tabel of identified substances

Relative occurrence of identified substances in PreEMPT in biota and sediments.

Detected substance	Chemical class	Mussel	Fish	Sedi-ment	Average 'occur-rence'	'Score'
4,4-Dimethyl oxazolidine	Antimicrobial, biocide, preservative	100%	97%	97%	92	****
N-Methyl-2-pyrrolidone	Industrial chemical, plasticizer	97%	94%	97%	90	****
Threonate	Pharmaceutical	100%	100%	83%	89	****
TP2/Pentanedioic acid, bis[2-[2-(2-butoxy-ethoxy)ethoxy]ethyl] ester	Surfactant, ECHA database, 100-1000 tonnage	90%	94%	97%	88	****
5'-Methylthioadenosine	Pharmaceutical, antimalarial	77%	97%	100%	86	****
2-Propen-1-yl 2-(cyclohexyloxy)acetate	Fragrance, ECHA database, 100-1000 tonnage	100%	97%	70%	84	****
Musk	ECHA database, 10-100 tonnage	94%	85%	90%	84	****
2-allyloxymethyl-2-ethylpropanediol	ECHA database, 100-1000 tonnage, resign manufacturing, plastic production	97%	76%	70%	76	****
TP1/Dimethyl succinate	ECHA database, 1000-10000 tonnage	94%	94%	53%	76	***
3,5-Di-tert-butyl-4-hydroxybenzaldehyde	ECHA database, 1-10 tonnage	94%	73%	77%	76	****
2-Naphthylamine	Industrial chemical, dye	84%	64%	87%	73	***
N-dodecyl-4-methoxybenzamide	Surfactant, ECHA, confidential tonnage	100%	100%	17%	69	**
1,3-Benzenedimethanamine	ECHA database, 10000-100000 tonnage	97%	97%	23%	69	**
1-Butanol, 3-methoxy-3-methyl-, acetate	ECHA database, 10-100 tonnage	100%	100%	7%	66	**
Misoprostol	Anti-ulcer drug	100%	100%	0%	64	**
Octylphenol diethoxylates (OP2EO)	Surfactant	100%	94%	7%	64	**
Telbivudine	pharmaceutical, antiviral drug	100%	82%	20%	64	**
TP3/Trimethylolpropane trimethacrylate	ECHA database, 1000-10000 tonnage	71%	45%	90%	64	**
3a,4,5,6,7,7a-Hexahydro-4,7-methano-1H-inden-5-yl propionate	Fragrance, ECHA database, 1000-10000 tonnage	100%	6%	100%	63	**
Dodecanedioic acid	Surfactant, ECHA, 10000-100000 tonnage	100%	91%	3%	62	**
TP1/tert-Butyl phenyl glycidyl ether	paint industry, ECHA database, 100-1000 tonnage	97%	94%	3%	62	**
Butyl acrylate	Precursor of polybutylacrylate, ECHA database, 100000-1000000 tonnage	94%	100%	0%	62	**
TP1/1,4-Bis[(ethenyloxy)methyl]cyclohexane	ECHA database, >10 tonnage	97%	88%	7%	61	**

Detected substance	Chemical class	Mussel	Fish	Sedi-ment	Average 'occur-rence'	'Score'
Methylhexahydrophthalic anhydride	ECHA database, 1000-10000 ton-nage	87%	36%	70%	60	**
4-tert-Butylbenzoic acid	ECHA database, 100-1000 tonnage	87%	27%	80%	60	**
Methacrylamide	Industrial chemical, polymer produc-tion	94%	6%	93%	59	**
1-Propanone, 1-(4-dodecylphenyl)-2-hy-droxy-2-methyl-	ECHA database, 10-100 tonnage	90%	91%	3%	59	**
Ethyl 3-(N-butylacetamido)propionate	Insect repellent, ECHA database	94%	82%	7%	58	**
4-Morpholinecarboxaldehyde	ECHA database, 1000-10000 ton-nage	48%	42%	97%	58	*
Piperonal	Pesticide, ECHA database, 100-1000 tonnage	90%	9%	80%	55	**
hexa-2,4-dienoic acid	Antibacterial drug, fungicide, ECHA database, 1000-10000 tonnage	77%	30%	70%	55	**
Iloprost	Pharmaceutical	97%	73%	0%	54	**
Sodium levulinate	ECHA database, 100-1000 tonnage	45%	61%	67%	54	
1-Eicosanol, phosphate, compd. with 2,2'-iminobis[ethanol]	Surfactant	74%	91%	0%	53	**
Tolazoline	alpha-adrenergic antagonist, antihy-pertensive agent, vasodilator agent	90%	73%	0%	52	**
TP1/Isophorone	ECHA database, >100 tonnage	87%	55%	23%	52	*
TP2/1-Propanamine, 3,3'-[1,4-buta-nediylbis(oxy)]bis-	ECHA database, 10-100 tonnage	100%	58%	3%	51	*
Benzoic acid, 4-(1,1-dimethylethyl)-, 1-meth-ylethyl ester	Metabolite of benzoic acid	74%	6%	83%	50	**
Jasmonic acid	Pesticide, plant growth regulator	68%	27%	67%	50	
Dacarbazine	Pharmaceutical	58%	6%	100%	50	*
3-Pyridinol	Agricultural chemical, pesticide	90%	39%	27%	49	*
Octanedioic acid	ECHA database, 10000-100000 ton-nage, plastics manufacture	97%	48%	7%	48	*
2H-1-Benzopyran-2-one, 7-amino-4-methyl-	Dye, paiting, textile, ECHA database, 1-10 tonnage	90%	6%	60%	48	*
3-Methylbenzoic acid	ECHA database, 100-1000 tonnage	97%	15%	40%	47	*
1,3-Dioxolane, 2,4-dimethyl-2-(5,6,7,8-tetra-hydro-5,5,8,8-tetramethyl-2-naphthalenyl)-	ECHA database, fragrance	94%	45%	10%	47	*
Perfluorononanoic acid	Industrial chemical, perfluorinated substance	32%	100%	13%	47	*
Nonanedioic acid	Industrial chemical, pharmaceutical, antineoplastic agent	90%	55%	0%	46	*
N-Vinyl-2-pyrrolidone	Precursor of PVC, ECHA database, > 10000 tonnage	52%	58%	37%	46	
2-[2-(Dimethylamino)ethoxy]ethanol	ECHA database, 1000-10000 ton-nage	16%	36%	97%	46	*
N-(2,4-Dimethylphenyl)formamide	Agricultural chemical, pesticide, in-secticide, acaricide	71%	12%	63%	45	*

Detected substance	Chemical class	Mussel	Fish	Sedi-ment	Average 'occur-rence'	'Score'
PEMA (2-Phenyl-2-ethylmalonamid)	Pharmaceutical, metabolite of pri-midone	45%	91%	0%	44	*
2-(2-(2-(4-nonylphenoxy)ethoxy)ethoxy)ethanol	ECHA database	35%	100%	0%	44	*
Miristalkonium	Surfactant	61%	12%	67%	43	
TP1/2,4,6-Trimethylbenzaldehyde	Additives for resins, agrochemical and pharmaceutical intermediate, fla-vor, fragrance	97%	18%	20%	42	*
Naphthalene-1-sulfonic acid	Surfactant, ECHA database, 10000-100000 tonnage	58%	55%	20%	42	
Cetylpyridinium	Surfactant	52%	9%	77%	42	*
N-hexyl-N-(3-phenylpropyl)hexan-1-amine	Cationic surfactant	52%	9%	77%	42	*
Camphor	Antiinfective agent, antipruritic drug, antiseptic drug	84%	24%	20%	40	*
Glycerol monomyristate	ECHA database, cosmetics, >1 ton-nage	39%	70%	17%	40	*
N,N-Dimethylformamide	ECHA database, >10000 tonnage	39%	39%	50%	40	
C14 Alkyl amine oxide	ECHA database, 10000-100000 ton-nage	81%	36%	0%	37	*
(3S-trans)-hexahydro-3-isobutylpyrrolo[1,2-a]pyrazine-1,4-dione	Industrial chemical	48%	61%	7%	37	
isobutyric acid, monoester with 2,2,4-trime-thylpentane-1,3-diol	ECHA database, 10000-100000 ton-nage	81%	30%	0%	35	*
Diethylmethylbenzenediamine	ECHA database, 1000-10000 ton-nage	74%	36%	0%	35	*
Decanedioic acid	Industrial chemical, plasticizer	71%	21%	20%	35	*
Tricyclodecanedimethanol	ECHA database, 1000-10000 ton-nage	68%	0%	47%	35	
TP1/Hydroxycitronellal dimethyl acetal	Perfume, fragrance	39%	15%	60%	35	
2,3-Dihydroxypropyl pentadecanoate	Industrial chemical, ECHA database	23%	70%	13%	34	*
Erucamide	Industrial chemical, plasticizer	35%	21%	50%	33	
TP3/tert-Butyl phenyl glycidyl ether	Paint, ECHA database, 100-1000	23%	0%	87%	33	*
Hexaprofen	Anti-inflammatory drug	23%	0%	87%	33	*
Glycine, N-(1-oxooctyl)-	ECHA database, 100-1000 tonnage	81%	12%	10%	32	*
Octinoxate	Ultraviolet filter, ECHA database, 1000-10000 tonnage	39%	3%	63%	32	
tetradecane-7-sulfonic acid	Surfactant	23%	27%	53%	32	
1,3-Diphenylguanidine	Rubber accelerator, ECHA data-base, 10000-100000 tonnage	6%	18%	80%	32	*
1-Ethenylazepan-2-one	ECHA database, 100-1000 tonnage	35%	12%	53%	31	
9,10-Phenanthrenedione	Phenanthrene, dyes, preservative, pesticide in farming	6%	52%	40%	31	
Tris(2-ethylhexyl) phosphate	Industrial chemical, phosphate plasti-cizer	52%	18%	27%	30	
Isobutyl hydrogen phthalate	Industrial chemical, plasticizer	61%	24%	7%	29	

Detected substance	Chemical class	Mussel	Fish	Sedi-ment	Average 'occur-rence'	'Score'
Bis(2-ethylhexyl) decanedioate	Plasticizer, ECHA database, 1-10 tonnage	48%	15%	30%	29	
Bis(2-chloro-1-methylethyl) 2-chloropropyl phosphate	Flame retardant	35%	42%	13%	29	
N,N-Bis(2-hydroxyethyl)dodecanamide	Industrial chemical, surfactant	39%	30%	20%	28	
CGA 353042	Pesticide, TP	0%	0%	90%	27	*
Sodium hydroxy- methane sulfonate	ECHA database, 10-100 tonnage	0%	0%	90%	27	*
Amines, C10-16-alkyldimethyl, N-oxides	ECHA database	58%	3%	23%	26	
Cyclohexylamine	ECHA database, 1000-10000 ton-nage	42%	3%	40%	26	
Bethanidine	Antihypertensive agent	32%	45%	3%	26	
N-Butyl-1-butanamine	ECHA database, 1000-10000 ton-nage	23%	58%	0%	26	
N,N-Diethylaniline	ECHA database, 100-1000 tonnage	65%	3%	13%	25	
N,N-Dimethyldecylamine oxide	Industrial chemical, surfactant, ECHA database, 100-1000 tonnage	52%	27%	0%	25	
Tetradecylamine	Industrial chemical	23%	39%	17%	25	
Succinic acid, sodium adduct	ECHA database, 10000-100000 ton-nage	61%	9%	7%	24	
stearic acid, monoester with glycerol	ECHA database, 10000-100000 ton-nage	26%	39%	10%	24	
N,N-Diethylethanolamine	ECHA database, 1000-10000 ton-nage	35%	36%	0%	23	
(Z)-1,1,1,4,4,4-Hexafluoro-2-butene	PFAS, ECHA database, 100-1000 tonnage	13%	55%	0%	22	
Penicillic acid	Mycotoxin, major degradation prod-uct of penicillin	3%	9%	60%	22	
Acetyl tributyl citrate	ECHA database, 10000-100000 ton-nage	55%	3%	10%	21	
Octadecanamide	ECHA database, 1000-10000 ton-nage	3%	15%	50%	21	
Monoethyl phthalate	phthalate	3%	9%	57%	21	
Vernakalant	Pharmaceutical	65%	0%	0%	20	
RP 12913 (TP of Carbetamide)	TP of the herbicide Carbetamide	65%	0%	0%	20	
2-Propanol, 1,1'-[[3-(dimethylamino)pro-pyl]imino]bis-	ECHA database, 1000-10000 ton-nage	6%	52%	3%	20	
TP1/1-(2-Aminoethyl)piperazine	TP of 1-(2-Aminoethyl)piperazine, asphalt additive	26%	30%	3%	19	
TP1/2-Propenoic acid, 2-(2-hydroxyeth-oxy)ethyl ester	ECHA database, 100000-1000000 tonnage	10%	48%	0%	19	
Ornithine	Anticholesteremic drug	58%	0%	0%	18	
1-(2-Aminoethyl)piperazine	ECHA database, 1000-10000 ton-nage, asphalt additive	52%	6%	0%	18	
GLYCERYL LINOLENATE	Cosmetic ingredient, ECHA data-base, 1-10 tonnage	39%	15%	3%	18	
Benzoic acid, 4-methoxy-	ECHA database, 100-1000 tonnage	32%	3%	23%	18	

Detected substance	Chemical class	Mussel	Fish	Sedi-ment	Average 'occur-rence'	'Score'
2-Butenedioic acid (2Z)-, monobutyl ester	ECHA database, 10-100 tonnage	55%	0%	0%	17	
Bis-(2-ethylhexyl) phthalate	Industrial chemical, phthalate	39%	6%	10%	17	
2,2,6,6-tetramethyl-4-oxopiperidinooxy	ECHA database, 100-1000 tonnage	0%	0%	57%	17	
Methyldopa	Antihypertensive agent	48%	3%	0%	16	
Bis(2-ethylhexyl) phosphate	Phosphate, ECHA database, 100-1000 tonnage	39%	6%	3%	15	
1-(2-Hydroxyethyl)-2,2,6,6-tetramethyl-4-pi-peridinol	ECHA database, 1000-10000 ton-nage	6%	12%	20%	12	
1H-Indole-3-methanamine, N,N-dimethyl-	ECHA database, 1-10 tonnage	3%	33%	0%	12	
Pentaethylene glycol	Industrial chemical, surfactant	3%	6%	30%	12	
TP1/Phosphoric acid, trihexyl ester	Phosphate, ECHA database	13%	18%	3%	11	
Diethylene glycol monoisobutyl ether	Surfactant, ECHA database, 10000-100000 tonnage	16%	15%	0%	10	
Stearic acid, compound with 2,2',2''-nitrilotri-ethanol (1:1)	Surfactant	3%	0%	30%	10	
Benzenesulfonamide, N,4-dimethyl-N-ni-troso-	TP of Benzenesulfonamide (ECHA, 1000-10000 tonnage)	23%	0%	7%	9	
3,6,9,12-tetraoxatricosan-1-ol	Surfactants, ECHA database, 100000-1000000 tonnage	3%	21%	0%	8	
bisoprolol TP M1	Pharmaceutical	19%	3%	0%	7	
reaction mass of isomers of: C7-9-alkyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propio-nate	ECHA database, >10000 tonnage	0%	18%	3%	7	
Hymexazol	Antibacterial drug, fungicide	16%	3%	0%	6	
Tris(4-methylphenyl) phosphate	ECHA database, 1000-10000 ton-nage	0%	0%	20%	6	
N,N-Diethyldodecanamide	ECHA database	0%	0%	10%	3	

9.2 PreEMPT and CONnECT RISK assessment with quotient >1

Wide-scope screening project results for CONnECT and PreEMPT, the substances with risk quotients at or above 1 are mainly PAHs, PFOS, some pharmaceuticals and breakdown products (Pharm&TPs) and personal care products (PCP&TPs). RISK quotient reported by NORMAN screening shown for HELCOM PreEMPT (in **S**ediment, **F**ish, **M**ussels, red indicate Risk≥1 found in HELCOM area) and OSPAR CONnECT (in **F**ish, **M**ussels and other shellfish). – indicate substance not found in the specific matrix, 0 that substance was detected but not quantified (Risk could not be evaluated), * indicate that the PNEC is below the LOD of the substance (i.e. RISK>1 if detected, but RISK<1 cannot be documented).

Substance	Group	Risk PreEMPT (S/F/M)	Risk CON-nECT (F/M)	Matrix Risk≥1
Lopinavir	Pharms & TPs	-/-/-	61.1/3515	M, F
Reproterol	Pharms & TPs	-/-/-	0/3059	M
Pilocarpine*	Pharms & TPs	-/0.71/1.31	0/2100	F
Darunavir	Pharms & TPs	-/-/-	59.8/1686	M, F
Methylparaben	PCPs	1.5/1.44/2.5	12.3/1001	M, F, S
Atrazine-desisopropyl	Pharm & TPs	-/-/-	0/465	M
alol	Pharms & TPs	-/-/-	0/165	M
Harman	Stimulants	-/-/-	6.7/150	M, F
Octocrylene	PCP&PTs	-/-/-	3.2/91.9	M, F
Dapiprazole	Pharms & TPs	-/-/-	0/83.2	M
Procainamide	Pharms & TPs	-/-/-	4/46.9	M, F
Nicotine	Stimulants	-/-/-	0.4/22.1	M
Ketoprofen	Pharms & TPs	-/-/-	15.9/0	F, M
N,N-Dimethyltetradecylamine	Ind. Chems	-/-/-	0/14.5	M
Diethofencarb	Pharm & TPs	-/-/-	0/11.3	M
N,N-Dimethyldodecylamine	Ind. Chems	-/-/-	0.1/10.9	M
Lidocaine-N-oxide	Pharms & TPs	-/-/-	0/9.9	M
Didecyldimethylammonium (DADMAC (C10:C10))	Ind. Chems	-/-/-	0.1/7.9	M
otine-Nor	Stimulants TPs	-/-/-	0.1/6.7	M
Molindone	Pharms & TPs	-/-/-	0/4.9	M
Butylparaben	PCP&PTs	-/0.65/-	0/4.9	M
Albuterol / Salbutamol	Pharms & TPs	-/-/-	0/4.4	M
Maprotiline	Pharms & TPs	-/-/-	0/3.6	M
Guaifenesin	Pharms & TPs	-/-/-	0.2/3.5	M
Ritalinic acid	Pharms & TPs	-/-/-	0/3.1	M
Antipyrine- 4-Acetamido	Pharms & TPs	-/-/-	0.2/2.9	M
Mexiletine	Pharms & TPs	-/-/-	0/2.8	M
Anthracene*	PAHs	2.6/0.19/0.13	0/0.6	S
Budesonide	Pharms & TPs	-/-/-	0/2.6	M
Benzo(a)pyrene*	PAHs	2.3/-/-	-/-	S
PFOS*	PFAS	2.2/1.74/0.39	0/0	S, F
Bisoprolol	Pharms & TPs	-/-/-	0/2.1	M
Phenazone	Pharms & TPs	-/-/-	0.2/2.1	M
p,p'-DDE*	Pharm & TPs	0.43/2.03/1.19	-/-	F, M
Terbumeton*	Pharm & TPs	1.7/-/-	-/-	S
Aspartame	Sweeteners	-/-/-	1.6/0	F
Pyrene	PAHs	0.93/0.91/1.58	0/0.1	M
Prometon	Pharm & TPs	1.4/-/-	-/-	S
Chrysene	PAHs	1.17/-/-	0/8.8	S

Substance	Group	Risk PreEMPT (S/F/M)	Risk CONnECT (F/M)	Matrix Risk≥1
Fluorene	PAHs	1.17/0.64/0.26	0/0	S
Chlordimeform	Pharm & TPs	-/-/-	0/1.2	M
Methiocarb-sulfone	Pharm & TPs	-/-/-	0/1.2	M
Acenaphthylene	PAHs	1.07/-/-	-/-	S
Simazine	Pharm & TPs	1.07/-/-	-/-	S
Benz(a)anthracene	PAHs	1/-/-	-/0.3	S
Fluoranthene	PAHs	1/0.03/0.23	0/0.4	S

9.3 PreEMPT and CONnECT RISK assessment with quotient ≥0.5 to <1

Substances in the range of Risk quotiont $0.5 \geq$ RISK < 1 in HELCOM PreEMPT (in **S**ediment, **F**ish, **M**ussels) and OSPAR CONnECT (in **F**ish, **M**ussels and other shellfish). – indicate substance not found in the specific matrix. Matrix with Risk <0.5 in all matrices have not been reported in this table.

Substance	Group	Matrix Risk ≥ 0.5	Risk PreEMPT	Risk CONnECT
N,N-Dimethyldecylamine	Ind. Chems	F, M	0.91	-/-
Acamprosat	Pharms & TPs	M	0.9	0.9
Perfluorononanoic acid (PFNA)	PFAS	F, S	0.88/0.5	-/-
3,3-Pentamethylene-4-butyrolactam	Pharms & TPs	M, F	0.84/0.71	-/-
Ethylparaben	& TPs	M	0.8	0.8
Fludioxonil	Pharm & TPs	S	0.8	-/-
Caffeine*	Stimulants	M	0.75	-/-
N-Methyldodecylamine	Ind. Chems	S	0.73	-/-
Phenanthrene	PAHs	M, S	0.71/0.7	0.03
Acenaphthene	PAHs	S	0.7	-/-
Galaxolide	PCPs	S	0.67	-/-
Naproxen*	Pharms & TPs	M	0.66	-/-
Triethylcitrate	Ind Chems	F	0.6	0.6
2-Trifluoromethyl-benzenesulfonamide	Pharm & TPs	S	0.6	-/-
Tributylamine	Ind Chems	M	0.58	0.04
2,2,4,5,5'-Pentochlorobiphenyl (PCB 101)*	PCBs	F	0.56	-/0.1
2,2',3,4,4',5'-Hexachlorobiphenyl (PCB 138)*	PCBs	F	0.5	-/0.4
2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153)*	PCBs	F	0.5	-/0.03
Perfluorooctanoic acid (PFOA)	PFAS	S	0.5	-/0.03

9.4 Method descriptions for the PreEMPT screening

The method- descriptions below are given as information to assess the validity of the results used in the above indicator assessments and are a shortened version of the final report to HELCOM (Alygizakis, 2023). This is the actual method used, and not a recommendation for performing SNTS studies. Methods always have a bias of which components they retain, and possibly lose, during processing, some of these have been highlighted below for each substance group.

Extraction of LC-amenable contaminants from sediment samples

The extraction protocol was based on the study by Nikolopoulou *et al.* (2022) and used for PreEMPT and CONnECT samples. The intake was based on freeze-dried and sieved sediments, which made the samples stable and easy to transport, but also gave a risk of loss of volatile substances. Some samples were freeze-dried before being sent to Athens, others were freeze-dried in Athens after transportation in frozen form. The freeze-drying itself can also give rise to some oil-product contaminants, but probably below the detection limits of the methods used.

Extraction of sediment sample was done three times with a methanol/water/formic acid/EDTA mixture by Vortex stirring followed by centrifugation. Extracts were evaporated to dryness and reconstituted with Methanol/water, filtered and transferred to a glass vial for LC-ESI-QToF MS analysis.

The evaporation to dryness can lead to loss of volatiles, and the filtration can give rise to pollutants from the filters and equipment used, if blanks are not checked thoroughly.

Extraction of GC-amenable contaminants from sediment samples

Extraction of sediment sample was done two times with a dichloromethane/hexane mixture by Vortex stirring followed by centrifugation. Extracts were evaporated to 1 ml and cleaned up on activated silica aluminium oxide and activated anhydrous Na_2SO. Then re-eluted with dichloromethane/hexane and hexane/acetone. Isooctane was added as a keeper and the extract was evaporated to near dryness. The sample was reconstituted to hexane, filtered and transferred to a glass vial for GC-APCI-QToF MS analysis.

As for LC-amenable extraction, the filtration can result in pollution of the extract, but the introduction of keeper and evaporation to only near dryness reduces loss of volatiles during the evaporative concentration of the sample.

Extraction of LC-amenable contaminants from biota samples

Accelerated solvent extraction (ASE), was used for the extraction of freeze-dried biota samples, followed by a clean-up step using mixed mode SPE cartridges that were developed by the authors of the study (Gkotsis *et al.*, 2022). As for sediments, the use of freeze-dried materials risk loss of volatile substances and the introduction of oil pollution from the freeze-drying process.

Each sample was mixed Na_2SO_4 (1+4g) and spiked with isotopically labelled internal standards, representing different chemical classes to allow for suspect screening and semiquantitative analysis. Samples were ASE extracted with methanol/acetonitrile and filtered if not transparent. Extracts were pre-concentrated on a rotary evaporator and cleaned up for lipids by extraction with n-hexane using Vortex stirring followed by centrifugation and discarding the hexane layer. This was followed by a second step to remove other matrix components using a SPE-cartridge with a layered 'mixed bed' cartridge, consisting of Oasis HLB (200 mg) and a mixture of Strata-X-AW (weak anion exchanger), Strata-X-CW (weak cation exchanger) and Isolute ENV+. The cartridge was conditioned with methanol and milli-Q water before loading the extracts. The cartridge was air-dried, and then extracted with basic ethylacetate/methanol/ammonia hydroxide followed by acidic ethylacetate/methanol/formic acid. The combined extract was evaporated to dryness and reconstituted using a mixture of methanol and milli-Q water, and the final extract filtered

through a RC filter into the glass vial used for LC-ECI-QToF MS analysis. As for sediments, loss of volatiles and pollution from filtration should be checked.

The use of ASE extraction and freeze drying of biota can give loss of volatile substances, and other substances that are bound hard to the ASE column material. ASE extraction only works on dried biota samples, so some sort of drying or chemical removal of free water is needed for ASE extractions, usually with the risk of contaminating the samples or loss of volatile substances. The benefits of ASE is a relative simple sample handling and use of less organic solvents than traditional multiple-extractions or soxleth-extraction methods.

Extraction of GC-amenable contaminants from biota samples

ASE extraction was used for the extraction of freeze-dried biota samples, followed by a clean-up step using mixed mode SPE cartridges that were developed by the authors of the study (Badry *et al.,* 2022), similar to the LC-amenable method.

1g of sample was mixed with 4 g of Na_2SO_4. A mix of isotopically labelled internal standards was spiked into each sample, representing different chemical classes to allow for suspect screening and semiquantitative analysis. Samples were ASE extracted with hexane/dichloromethane and filtered if not transparent. Extracts was pre-concentrated on a rotary evaporator using isooctane as keeper. The extract was cleaned up using a SPE Strata® FL-PR Florisil cartridge and extracted with dichloromethane: hexane followed by hexane. The combined extract was evaporated in a rotary evaporator to 10 ml and then by nitrogen stream to 250 µl hexane, keeping the temperature below 30°C. The final extract was filtered into the glass vial used for GC-APCI-QToF MS analysis.

As for sediments, pollution from the filtration should be checked.

Reversed-Phase Liquid Chromatography High Resolution Mass Spectrometry

The sediment and biota samples prepared for LC analysis were analysed with an Ultra High Performance Liquid Chromatography (UHPLC) apparatus with an HPG-3400 pump (Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific), using an Acclaim TM RSLC 120 C18 column and the Maxis Impact Hybrid Quadropole Time of Flight Mass Analyser (QTOF-MS) from Bruker Datonics.

A gradient elution programme of the reversed-phase liquid chromatographic system was used with a mixture of water/methanol/ammonium formate gradient to methanol, both acidified with formic acid, for the positive ionization ESI mode and water/methanol to methanol, both with ammonium acetate buffer, for the negative ionization ESI mode.

Samples was run in full scan mode MS spectra and MS/MS spectra, followed by a full scan MS with extract of the 5 most abundant ions MS/MS spectra. m/z scan range was 50-1000 Da at scan rates of 2 Hz.

To assist in the semiquantitave suspect screening, an external calibration of the QToF-MS was performed just before analysis with 10 mM of sodium formate

in a mixture of water/isopropanol (50/50, v/v). The theoretical exact masses of calibration ions with formulas Na(NaCOOH)1-14 in the range of 50−1000 Da were used. Also, internal calibration was performed by calibrant injection at the beginning of each chromatogram (1st segment, 0.1−0.25 min).

The use of external calibration is less precise than internal standardization, but given that only semiquantitative analysis is performed, this is a simpler way of getting results from many substances. The in-house database was used for automatic identification of substances, and no information is available on which isotopic labelled substances was used, except that they were representative for different classes of the in-house LC target list. A number of internal standards was mixed into each sample before extraction, and every 10 injection was the external standard.

Gas Chromatography High Resolution Mass Spectrometry

The sediment and biota sample prepared for GC analysis were analysed with a GC-APCI-QTOF system consisted of a Bruker 450 GC, a CP-8400 Auto Sampler and the same QTOF as used for liquid chomotography.

Splitless injection mode with the splitless purge valve activated 1 min after injection was used, with injection volume of 1 µL. A 30 m Restek Rxi-5Sil MS column (0.25 mm i.d. x 0.25 µm film thickness) was used with helium as carrier gas at the constant flow of 1.5 mL min^{-1}. GC oven was started at 55°C with a 15°C/minute ramp to 180 °C then to 280°C at lower climb rate and hold until final increase to 300°C in 2 minutes, followed by hold. Inlet, transfer line and source were kept at temperatures of 250°C to 290°C.

As for LC, external calibration of the QTOF was made prior to each analysis using perfluorotributylamine (FC43). The details of the APCI interface are given in the paper, and the MS scans was performed like the LC method above, except at 8 Hz scan rat

The use of external calibration is less precise than internal standardization, but given that only semiquantitative analysis is performed, this is a simpler way of getting many results. The in-house database was used for automatic identification of substances, and no information is available on which isotopic labelled substances was used, except that they were representative for different classes of the in-house GC target list. A number of internal standards was mixed into each sample before extraction, and every 10 injection was the external standard.

SUSPECT AND NON-TARGET SCREENING IN ENVIRONMENTAL MONITORING

Suggestions for indicator based on non-target analysis

The introduction of suspect and non-target screening (SNTS) in NOVANA monitoring is discussed and suggestions for possible indicators based on such screenings are suggested, both purely qualitative indicators on the presence of peaks and quantitative indicators based on semiquantitative concentrations of target substances, with Risk Quotients defined from comparison with PNEC or EQS value as used in the NORMAN approach in the OSPAR CONnECT and HELCOM PreEMPT projects. Some other possible indicators based on the PreEMPT and CONnECT data are suggested, and the general status for SNTS screenings and status of uncertainties using results from such are discussed. Finally, an example of how to build SNTS into a monitoring program to continuously upgrade and improve lists of substances of priority action and possible concern, is presented.

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