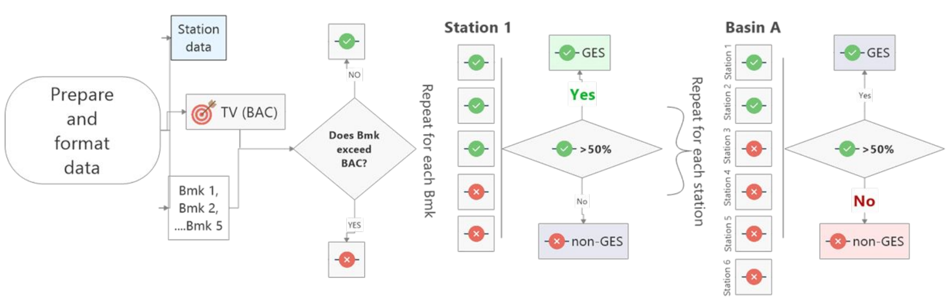
BEACON Project

Report

Deliverable 1.3

Integrated workflow for assessing biological effects of contaminants in the Baltic Sea



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Overview of iBEC

iBEC (integrated Biological Effects of Contaminants) is a systematic and reproducible workflow designed for analyzing biological effects to assess environmental stress, particularly from contaminants. It covers both suborganismal (e.g., biochemical and cellular) and organismal (e.g., reproduction and growth) responses, aiming to offer a comprehensive biological effect assessment for Descriptor 8.2 (D8.2) under the EU Marine Strategy Framework Directive (MSFD). The applicability of the workflow is demonstrated for the Bothnian Sea and the Gulf of Riga using the data compilation provided by the BEACON and H-BEC projects. By streamlining the data management process, iBEC facilitates the efficient and accurate assessment of environmental stressors on marine life, supporting both regulatory compliance and scientific research.

Key Points of iBEC

- **Scope and purpose**: iBEC is designed to collate and analyze biological effect measurements for assessing contaminant exposure in marine environments. Specifically developed for D8.2 implementation in the Baltic Sea, iBEC employs local species suitable for evaluating environmental stress from pollutants in this ecosystem characterized by high environmental variability and species-poor communities.

- **Biological effects are measured** at different levels of biological organisation. *Suborganismal* responses (biomarkers) include biochemical, cellular, and molecular markers that indicate stress at a microscopic level. *Organismal* responses (physiological health parameters) encompass metrics like reproduction, condition indices, and growth, providing a broader picture of organism health. iBEC includes biomarker responses and physiological health parameters that have been tested in the Baltic biota over the last decade and found responsive to contaminant exposure.

- **Applications**: (1) **Environmental contaminant assessment** to support D8 evaluation by targeting biological responses to pollutants (D8.2); (2) **Ecotoxicological surveys** in the field and experimental studies to understand and predict pollution impacts.

- **Data organization**: Information is systematically organized in a series of spreadsheets, which helps manage and analyze available datasets. The spreadsheets are complemented by a separate *Standardized and automated spreadsheet* provided by Ifremer (France) for calculating the data distribution for biological effect parameters in relation to the background variability at each station and visualization of the assessment outcome. Other additional files inlude data used for case studies providing the example assessments.

- **Data requirements for D8.2 assessment**: To ensure consistency and reliability, criteria for data collection and data aggregation principles across stations and years to represent subbasin status over defined time periods (e.g., 6 years for HOLAS) are defined.

Background

Assessment needs

The traditional approach of assessing chemical pollution through single-chemical concentrations is increasingly questioned. Measuring pollutant concentrations in seawater has its advantages, such as the ease of conducting targeted analyses and the ability to directly link results with contamination levels. However, there are also drawbacks, including the challenge of detecting low concentrations, the influence of random spatial and temporal variations, and the potential oversight of factors like bioavailability, mixture effects, and varying environmental conditions.

The Descriptor 8.2 allows for including biological effects in assessments of contaminant impacts to determine if harm is occurring. This requires biological effects data and an integration system to evaluate the environmental status of contaminants (ICES, 2021). OSPAR Convention (Convention for the Protection of the Marine Environment of the North-East Atlantic) and JAMP (Joint Assessment and Monitoring Programme) have recommended a set of biological effect techniques and assessment criteria (OSPAR Commission, 2012) as well as the integrated approach developed over decades (Vethaak et al., 2017). Several national and international projects have concurrently assessed the potential and benefits of biological effect monitoring and assessment in the Baltic Sea, offering robust recommendations to complement current contaminant effect assessments led by HELCOM. These projects have been instrumental in developing the iBEC for enhanced environmental monitoring and D8 assessment.

JAMP/OSPAR approach

The iBEC concept is based on the existing multistep data aggregation approach to assess contaminant and biological effects data together; the approach is advocated by the OSPAR and JAMP. Within this framework, Background Assessment Concentrations (BAC) and Environmental Assessment Criteria (EAC) are key tools used to evaluate the levels of contaminants and their potential impacts on the environment and organisms:

- **Background Assessment Concentration:** BAC values represent the baseline condition of a contaminant or biological marker in the environment to provide a benchmark against which observed data from potentially contaminated sites can be compared. They are usually determined through long-term environmental monitoring data analysis to understand the natural variability of contaminant levels and biomarkers. A statistical approach, using either the lower or the upper (depending on the effect direction) bound of the 90%-confidence interval, is employed to establish the BAC value, ensuring that it reflects the upper/lower limit of the natural variation.

- **Environmental Assessment Criteria:** EAC values are thresholds used to assess the potential risk or harm that contaminants may pose to biota. They represent biomarker levels above which there may be adverse organismal effects. EACs are typically derived from ecotoxicological studies, including laboratory toxicity tests and field observations, as well as species sensitivity distribution (SSD) models, and are used to guide regulatory actions. To account for uncertainties and variability, safety factors are applied to toxicological thresholds such as NOEC (No Observed Effect Concentration), LOEC (Lowest Observed Effect Concentration), LC50 (Lethal Concentration for 50% of the population), and EC50 (Effective Concentration for 50% of the population).

- **Risk identification:** Further investigation may be warranted if observed levels exceed BAC but are below EAC. When observed levels exceed EAC values, there is a clear indication of potential adverse effects, prompting risk management actions.

Baltic biota: selecting relevant target species and markers

Biological responses to toxic substances vary across different levels of organization, from molecular to ecosystem scales. Molecular and cellular responses are highly sensitive and specific to particular toxins, but their ecological significance remains unclear. Responses at higher levels, such as changes in population abundance or biodiversity, are ecologically relevant but cannot always attribute differences solely to pollutants. Balancing these factors is crucial for effective environmental monitoring, aiming for sensitivity as an early warning signal, ecological relevance in terms of population fitness, and practicality in terms of standardization and cost-effectiveness.

Due to its variable hydrographic conditions and strong environmental gradients, the Baltic Sea presents a unique and challenging environment for biological assessments. Selecting relevant target species and biomarkers for monitoring contaminant impacts requires careful consideration of these factors (Appendix A, Table A1). To ensure comprehensive and reliable assessments, target species and biomarkers in the Baltic Sea must account for strong environmental gradients, benthic-pelagic coupling, physiological sensitivity to stressors, and representative species distribution.

The existing monitoring of the Baltic environment guided by HELCOM is crucial because it ensures consistent data collection and comparability across different regions. This monitoring provides comprehensive datasets that reflect the overall status of the Baltic Sea ecosystem, aiding in the identification of trends and the assessment of long-term impacts from contaminants and other stressors. Therefore, selecting target species for biological effect assessment should align with existing monitoring efforts to enhance data collection and optimize the use of results.

As indicated by our inventories, pertinent target species, such as fish (perch, herring, eelpout) and invertebrates (amphipods, blue mussel, clam), have been validated for monitoring contaminant impacts in the Baltic Sea. Several biomarkers have been established and tested across these species in various research projects, demonstrating their applicability for long-term monitoring. Integrating sensitive subcellular responses with organismal indicators, such as changes in reproductive success, growth, and physiological indices, ensures early detection of environmental threats and identification of the severity of adverse effects.

iBEC description

Overall structure

iBEC is a workflow developed by the BEACON (Interreg project; 2023-2024) to organize, collate and analyze biological effect measurements for assessing contaminant exposure in the context of D8.2. The workflow is organized in *iBEC tool.xlsx* file comprised a set of spreadsheets and an additional *Standardized and Automated Spreadsheet* provided by Ifremer. **The primary output** is an evaluation of the environmental status that integrates multiple lines of evidence for the biological effects of contaminants reflecting *in-situ* exposure to the ambient contaminant mixtures and under existing environmental conditions.

Information in the spreadsheets

Each spreadsheet within the *iBEC tool.xlsx* is dedicated to specific information concerning, e.g., sampling sites, species, biomarkers, and the required BAC/EAC values as well as the assessment output. All fields in the spreadsheets are color-coded to indicate their usage (Table 1). In short, grey fields are designated for manual data entry only, while all other fields should either be populated by copying data from other spreadsheets (green fields) or calculated automatically (blue fields). Yellow and purple fields contain information that should not be changed for each assessment but, if needed, can be revised (e.g., when the approach is developed further). In addition to the spreadsheets needed for the analysis, a set of examples for the primary data structure is provided; these examples are located as the last spreadsheets in the *iBEC tool.xlsx*; see section *Examples of biological effect assessment in different subbasins* for the list of the data provided.

Table 1. Color coding for data entry in spreadsheets of the *iBEC tool.xlsx.*

Color code	Usage	Description
Green	Copy from another spreadsheet	Data are automatically copied from another spreadsheet within the file, no manual entries are allowed
Blue	Automatically calculated	Calculated using Excel functions, no manual entries are allowed
Grey	Manual data entry only	Data is to be entered manually. These are usually the primary data for each assessment round/area
Yellow	Review if needed	The standard values/variables are provided. Add if the parameters you need are missing, use the bottom rows. Do not change/remove the existing data/parameters/descriptions
Purple	Review if needed	This information is likely to overlap for all national laboratories; if the information used by your laboratory is missing, add it using the bottom rows.

The following spreadsheets are included:

- **Assessment Units** {fixed data for all assessments}: HELCOM Assessment Units, Scale 2.
- **Stations** {input data}: List of stations with their geographical coordinates for each subbasin included in the assessment. Add stations as needed but check first for the existing station coding and spelling in national register and/or other databases (e.g., ICES DOME).
- **Species** {input data}: List of species used in the assessment. The list of species can be extensive, and not every species is required for each assessment. However, at least two species from each basin are necessary to ensure a valid assessment.
- **Sex** {input data}: Sex of the test animals (*male/female*) must be specified as many biomarkers have sex-specific BAC values. For juveniles in many species, however, the sex is often not feasible to determine (*unknown*). If individuals of different sex are mixed in a composite sample (*mixed*), the variability for a response variable should be calculated using relative proportion of males and females in the sample.
- **Tissues** {input data}: List of tissues used in the analysis.
- **BE Rationale** {modify if necessary}: List of Biological Effect (BE) parameters included in the assessment with corresponding units, effect direction, and rationale for their interpretation. This list can be extensive, and not every biomarker/physiological variable is required for each assessment. However, **at least two exposure and two effect markers** should be included to ensure a reliable assessment.
- **TV** {input data on Target Values}: Exposure and effect biomarkers used for each species and their target values estimated using the BAC/EAC approach (i.e., 90%-confidence interval with non-parametric bootstrapping for 1000 observations based on measurements from reference sites). For the case studies, the reference sites were defined using data on chemical contaminants provided by the national laboratories (Appendix B). **At least five observations for each BE parameter** are required for a valid assessment.
- *Standardized Automated Spreadsheet* {a separate file} for evaluating data distributions. Currently, identical to the Ifremer template. For each BE parameter/species/station, primary data are used to calculate whether the observed 90-percentile deviates from the respective BAC/EAC value.
- **Data Summary** {input data}: Summary of the data for the BE parameters and target species used for the assessment. Data are entered as mean values for each BE parameter/species/station. For MSFD assessment (a 6-year cycle), at least three years of observations are required.
- **BAC Exceedance** {input data using the output generated by *Standardized Automated Spreadsheet*}: Comparing observed vs target values for each BE parameter and scaling to 0/1 [0: exceeding BAC/EAC (=sub-GES), 1: not exceeding BAC/EAC (=in-GES)].

- **Assessment** {output data}: Assessment summary for each station and the entire basin based on the BE parameter percentage exceeding their respective BAC/EAC values.
- **Visualization** {output data}: A diagram summary for each station and the entire basin based on the assessment outcome.

Data processing: step-by-step guide

Important Notice: Please Read Before Using iBEC.

This spreadsheet contains built-in functions and equations designed to calculate values based on the data you provide. These formulas are essential for maintaining the integrity and accuracy of the calculations. To ensure proper functionality: (i) Do not delete or overwrite any cells containing formulas unless you aim for a specific change. (ii) Be extra cautious when making changes to the structure of the spreadsheet, as it may break important relationships between data and formulas. (iii) If you're unsure about any edits or find errors, please consult elena.gorokhova@su.se for assistance.

Let's go!

Step 1: For each subbasin, provide a list of stations with geographic coordinates and auxiliary information (**->Stations**).

Step 2: Provide a list of species used for BE analysis with auxiliary information (**->Species**).

Step 3: Examine the list of BE parameters to confirm that it contains all variables used in the analysis; add new variables only if necessary (**->BE Rationale**).

Step 4: For each subbasin, select species, tissue and, if needed, sex for each BE parameter analyzed (->**TV**).

Step 5: For each subbasin and BE parameter, define species-, tissue- and, if needed, sex-specific BAC/EAC values referred to as target values (**->TV**). The values provided for the case studies are based on the biomarker and contaminant data provided for the Bothnian Sea and the Gulf of Riga (Appendix B); these values are based on the 90%-confidence interval estimated for the observations at the reference sites. See supporting file *GoR_Biomarker_BACs.xlsx* for BAC calculations using Gulf of Riga data.

Step 6: Prepare your primary data for BE parameters in a separate file (see spreadsheets with the structure of the input data, e.g., *GoR_Data_PE,PF* for embryo aberration data, *GoR_Raw_Data_Ma_Biomarkers* for biochemical markers, etc.; the input data used for the case studies are placed in the iBEC file as spreadsheets with no tab color). Observe that different types of data need different structure of the input and different approaches to calculate values that will be used for the assessment. When preparing the data, check for outliers and ensure that the measurement units are correct, corresponding to the **BE Rationale.** We recommend using these spreadsheets to enter your data into the assessment tool. Some small edits might be necessary if different biomarkers are used. The 90 or 10 percentile values for each BE parameter/station will be calculated automatically and displayed in the **[Basin name] Data Summary** spreadsheet.

Step 7 (optional): Use *Standardized Automated Spreadsheet* (provided as a supporting file) and insert the BAC/EAC values for each BE parameter/station as well as raw data to calculate the 90 or 10 percentile values and to compare whether the observed data differ from the corresponding BAC/EAC values. This assessment in this file is station-based; therefore, if several stations are used to assess a subbasin, each should be evaluated separately.

Step 8: The station-based assessment for each biomarker/target organism combination is conducted in the **BAC exceedance** spreadsheet using 0/1 coding (0: BAC is exceeded, 1: BAC is not exceeded) to provide an overview of each measured BE parameter. The comparisons are done using Excel built-in functions to compare the observed values with their respective target values (**->TV**).

Step 9: Complete the integration of the station-based assessments and aggregation at the subbasin level (-> **[Basin] Assessment**).

Step 10: Visualize the assessment outcome. An example of assessment visualization is provided in the [Basin] Assessment spreadsheet.

Examples of biological effect assessment in different subbasins

See Appendix B for detailed descriptions of the study areas, sampling methods, and variables utilized in the two case studies: the Bothnian Sea (all data from 2018) and the Gulf of Riga (spanning 2012–2022). These case study data represent a compilation of available observations and are not designed for year-specific assessments. The varying time spans, species, and biomarkers illustrate how biological effects (BE) data can be processed and applied for assessment purposes.

The following spreadsheets with input data and basic statistics are provided in the iBEC file for each case study.

Bothnian Sea

- *BoS Raw Data_PE, PF*: data for embryo aberrations in amphipods the amphipod *Monoporeia affinis*;
- *BoS Raw Data_Ma_Biomarkers*: data for biochemical markers in the amphipod *Monoporeia affinis*;
- *BoS Raw Data_FishBiomarkers*: data for physiological condition parameters and biochemical markers in perch.

Gulf of Riga

- *GoR Data_PE, PF*: data for embryo aberrations in amphipods;

- *GoR Raw Data_Ma_Biomarkers*: data for biochemical markers in the amphipod *Monoporeia affinis*;
- *GoR Raw Data_Mb_Biomarkers*: data for biochemical markers in the clam *Macoma balthica*;
- *GoR Raw Data_Poro_Biomarkers*: data for biochemical markers in the amphipod *Pontogammarus robustoides.*

Supporting files

In the current version, two supporting files are provided:

- *GoR_Biomarker_BACs.xlsx* contains data on biomarkers measured by Estonian and Latvian laboratories in their respective reference stations.
    - The data distributions for each species/biomarker combination are compared between the countries using a t-test. If the distributions are not significantly different, a common BAC value is calculated. This was the case for all data provided.
    - All BAC calculations are executed using built-in functions in Excel and transferred to **TV** spreadsheet.
    - If significant differences in data distributions are observed between countries, follow-up actions should be recommended. These could include intercalibration activities or similar initiatives to identify and address the source of the discrepancies. Once the reasons for the differences are understood, steps should be taken to harmonize analytical methods and instrumentation. Additionally, statistical approaches, such as normalizing data to laboratory long-term mean values, can be considered to enhance the compatibility of data for joint assessments in shared basins.

- *Standardized Automated Spreadsheet.xlsx* contains calculations for the biological effect assessment prepared by T. Burgeot and A. Mauffret (Ifremer, France) for the integrated assessment published in the OSPAR QSR 2023 (Burgeot et al., 2023). The species and biomarker selection is based on the monitoring and screening studies in the OSPAR area. Available at: https://oap.ospar.org/en/ospar-assessments/quality-status-reports/qsr-2023/other-assessments/integrated-biological-effects

Appendix A. Confounding factors in the Baltic Sea

The Baltic Sea presents a unique and challenging environment for biological assessments due to its variable hydrographic conditions and strong environmental gradients. A recent example of delineating non-chemical and chemical drivers of the biological responses is a cross-basin analysis of reproductive aberrations in amphipods to identify contaminant effects under varying temperatures, oxygen and salinity (Kolesova et al., 2024). Moreover, regular activities need to be established to ensure consistency in effect data from national and international monitoring programmes in the HELCOM area.

Table A1. *Features of the Baltic Sea that require special considerations when selecting target species and biomarkers for biological effect assessment.*

Feature	Explanation
Variable hydrographic conditions	Variations in temperature, salinity, and oxygen levels influence organisms' physiological responses. Target species and markers must be resilient to these changes to provide consistent and reliable biomarker data.
Strong environmental gradients	North-south gradients in temperature and salinity drive species distribution and their responses to stressors. Selected biomarkers need to be sensitive enough to detect effects across these gradients while remaining applicable to multiple regions within the sea. Also, reference conditions should be selected to reflect responses in the relevant range of environmental factors.
Species distribution	Only a few species are universally distributed across the different subbasins of the Baltic Sea, limiting the choice of target species that must be representative of the broader ecosystem while being present in sufficient numbers in various regions to allow for meaningful comparisons and assessments.
Physiological optima	Due to challenging environmental conditions, many species in this system live at the edge of their physiological optimum. This makes them particularly sensitive to additional stressors, such as pollutants. At the same time, selecting species that are already under environmental stress can provide early warnings of contaminant effects.
Benthic-Pelagic coupling	Due to the relatively shallow depth, interactions between the water column and the seabed are strong. As a result of this benthic-pelagic coupling, contaminants can quickly move between sediments and the water. Therefore, targets should include both pelagic and benthic organisms to capture the full extent of contaminant exposure and effects.

Appendix B. Case studies: the Bothnian Sea and the Gulf of Riga

Ecological and hydrological profiles of the study areas

Bothnian Sea

The Bothnian Sea, covering approximately 66,000 km^2, is a central sub-basin in the Baltic Sea located between Sweden and Finland. With a mean depth of around 60 m and maximum depths reaching 293 m, it holds an estimated water volume of 4,345 km^3 and exhibits a water residence time of approximately 6 years. The Bothnian Sea connects to the northern Gulf of Bothnia and the southern Åland Sea, allowing limited exchange with the lower-salinity surface waters of the Baltic Proper through these shallower straits.

The region receives a significant annual freshwater inflow, mainly from the rivers along the Swedish and Finnish coasts, contributing around 60 km^3 per year, equivalent to about 1.4% of the Bothnian Sea's water volume. This continuous freshwater influx shapes the vertical and horizontal salinity gradients, with surface salinity averaging around 4–5 PSU and decreasing toward the north. Salinity levels are lower near river mouths and higher closer to the Åland Sea. Seasonal thermal stratification is a characteristic feature, generally forming in spring and lasting until early autumn, while winter and autumn bring well-mixed conditions throughout the water column. Exceptions to this mixing pattern are found near river mouths, where salinity and temperature gradients can be observed year-round.

Temperatures in the Bothnian Sea follow a seasonal trend, with surface temperatures rising in summer while near-bottom temperatures remain relatively low throughout the year, typically between 2–5°C. Dissolved oxygen concentrations also vary by season, with levels dropping to 4–6 mg/L in bottom areas during summer but rarely reaching anoxic conditions, although occasional hypoxic zones can develop.

The seabed of the Bothnian Sea consists of diverse substrates. In depths between 10–40 m, sandy and soft sediments support a variety of benthic species, with a richness of up to 15 species, including amphipods, polychaetes and bivalves. Silt and clay sediments dominate in deeper areas, typically beyond 40 m, sustaining a smaller but resilient benthic community. These sediment habitats play a crucial role in the sea's ecological function, supporting nutrient cycling and providing feeding grounds for fish and invertebrates.

Gulf of Riga

The Gulf of Riga, covering approximately 17,900 km^2, is a shallow bay in the northeastern Baltic Sea. It has an average depth of 23 m and a maximum depth of 62 m, holding a water volume of roughly 424 km^3. The gulf exhibits a water residence time of approximately 2 to 4 years. Water exchange with the Baltic Sea occurs primarily through the Irbe Strait in the northwest and the Suur Strait in the north. Both of these straits are relatively shallow, which restricts the water exchange largely to low-salinity surface waters from the Baltic Proper.

The Gulf receives a substantial amount of freshwater runoff, around 32 km^3 annually, contributing about 7.9% of the total water volume in the gulf. This freshwater influx and limited water exchange establish a strong salinity gradient. Salinity levels decrease from approximately 6.0 PSU in the Irbe Strait to as low as 2.0 PSU near the Daugava and Lielupe river mouths. Seasonal stratification is a defining feature, with the gulf being mostly well-mixed to the bottom during winter and autumn but becoming thermally stratified from April to mid-October. However, areas near straits and river mouths may experience varying levels of salinity stratification throughout the year.

Temperatures in the Gulf of Riga vary seasonally. Surface waters warm significantly in the summer, while near-bottom temperatures remain low year-round, generally between 4–6°C. Dissolved oxygen concentrations range from 2 to 6 mg/L, with around 30% of near-bottom areas experiencing hypoxic conditions during warmer months; however, the gulf does not typically reach anoxic conditions.

The gulf's benthic habitats are diverse, supporting well-developed communities. At 20–30 m depths, soft sediment and sandy substrates prevail, interspersed with boulders, which provide ideal conditions for benthic organisms, with species richness reaching up to 13. Silty and muddy sediments are common in deeper areas, supporting fewer species, including amphipods and polychaetes. These diverse benthic communities contribute to the gulf's ecological productivity, providing food sources for fish and contributing to nutrient cycling within the ecosystem.

Biological and chemical analyses overview

Data contributions

In each basin, samples of sediment, benthic invertebrates (amphipods and mussels), and fish were collected to assess contaminant levels and biological responses (Table B1). Sampling programmes and data contributions in the Gulf of Riga come from Latvia and Estonia, with Latvia primarily focusing on biomarkers (amphipods and bivalves) and contaminants in sediment and bivalves, particularly organic pollutants and metals, while Estonian data largely emphasise biomarkers for reproductive health and oxidative stress in amphipods and fish with contaminant data being only measured in sediments.

Sweden contributed data for the Bothnian Sea, with sampling focused on sediment, amphipods, and perch, coordinated under Sweden’s coastal monitoring program and related initiatives. Here, the Swedish Environmental Protection Agency leads contaminant analyses, particularly for metals, PAHs, PCBs, and PFAS, supported by regional research institutes.

Bothnian Sea sampling

In the Bothnian Sea, sediment, amphipods (*Monoporeia affinis*), and fish (perch, *Perca fluviatilis*) were the primary samples collected for biological and chemical analyses as a part of the Effect Screening Study (ESS; 2017-2020), commissioned by the Swedish Environmental Protection Agency, aimed to assess biological impacts of contaminants in polluted coastal areas, including the Bothnian Sea. ESS focused on validating biomarkers in fish and benthic invertebrates to enhance contaminant monitoring programs under frameworks like the Marine Strategy Framework Directive (MSFD) and proceeded in two stages. Stage 1 (2017-2018) assessed health and biomarker responses in fish and invertebrates, revealing contamination-related effects, and in Stage 2 (2019-2020), these biological responses were linked to contaminant levels in sediments and biota, focusing on contaminants like PAHs, dioxins, PCBs, PFAS, organotins, and metals.

Sediment samples and amphipods were collected using a benthic sled, targeting the upper 2–3 cm, with macrofauna removed through sieving. Sediment samples were then homogenized and stored at -20°C. Chemical analyses focused on various contaminants, including heavy metals (e.g., As, Cd, Cr, Hg), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and per- and polyfluoroalkyl substances (PFAS). Accredited laboratories such as ALS Scandinavia and the Swedish Environmental Research Institute conducted these analyses.

Amphipods collected from the Bothnian Sea sites, particularly near Sundsvall and Norrsundet, were used for embryo aberration (gravid females) and biomarker analyses. For biomarkers, the de-brooded females were immediately frozen at -80°C to preserve sample integrity. Embryo aberrations and biomarkers, including AChE activity, RNA/DNA ratio, and oxidative status indicators (such as TBARS and ORAC), were measured to correlate biological responses with sediment contaminant levels (for detailed methods and results, refer to the section on Biomarker overview).

Perch collected from Skellefteå, Holmöarna, and other coastal sites were analyzed for physiological indicators, such as body and liver weights, and biomarkers, including EROD, GST, and AChE. Chemical analyses included all target groups; PAHs were not analysed as fish can efficiently metabolise these compounds.

Gulf of Riga sampling

In the Gulf of Riga, both Latvian and Estonian sites were sampled, targeting fish (herring, perch and round goby), bivalves (*Macoma balthica*), and amphipods (*Monoporeia affinis*), with gravid amphipod females used for reproductive studies. Fish samples were used mostly for metal analysis, with a minor portion of perch samples being analyzed for PAHs and PBDE/HBCDs.

Sampling of the benthic invertebrates occurred across various depths (5–43 m) using a Van Veen grab and sieving to remove non-target material. Biomarker analysis in these samples included enzyme activity related to oxidative stress and reproductive parameters, such as fecundity, embryo development stages, and rates of embryo aberrations. These biomarkers helped assess the impact of environmental contaminants on reproductive health.

Bivalves collected in the Latvian part of the Gulf of Riga were also analyzed for contaminant levels. Individuals larger than 1 cm were retained for contaminant analysis, which included organic contaminants and metals. Samples were held in aerated seawater for 24 hours to cleanse the intestinal tract before soft tissue was extracted and stored at -20°C for analysis.

Biomarker overview

The biomarkers used across sampling sites in the Bothnian Sea and the Gulf of Riga encompassed a range of physiological, biochemical, and reproductive indicators designed to assess the health impacts of contaminants on aquatic organisms. These biomarkers can be grouped into three main categories:

- **Reproductive and developmental disorders**: Reproductive biomarkers focused on assessing impacts on reproductive success and development, particularly in gravid female amphipods. Fecundity (number of eggs per female), embryo development stages, and rates of embryo aberrations were analyzed to detect reproductive impairments potentially linked to contaminant exposure. Elevated embryo malformation rates often correspond with exposure to toxic substances, including trace metals and organic contaminants like PAHs, suggesting potential long-term impacts on population viability; this is used in the *Reproductive disorders in amphipods* (ReproIND), a supplementary indicator for D8/MSFD (https://indicators.helcom.fi/indicator/reproductive-disorders/).
- **Oxidative status and antioxidant defense**: Biomarkers like TBARS (Thiobarbituric Acid Reactive Substances) and ORAC (Oxygen Radical Absorbance Capacity) measured oxidative stress levels, providing insight into the cellular damage and antioxidant defenses in organisms such as amphipods and bivalves. Additionally, enzymes involved in detoxification, such as catalase and glutathione peroxidase, were used to evaluate how organisms respond to prooxidative impacts.
- **Enzymatic activity**: Biomarkers, such as EROD (Ethoxyresorufin-O-deethylase; in fish) and GST (Glutathione S-Transferase; in fish, bivalves and amphipods), provided insights into specific physiological responses to contaminants. EROD activity indicates exposure to PAHs and dioxins, while GST detoxifies xenobiotic compounds.
- **Neurotoxicity**: AChE (Acetylcholinesterase) activity, associated with nervous system health, can be inhibited by neurotoxic substances like organophosphates, providing insights into responses related to neurobehavioral impacts.
- **Metabolic activity indicators:** The RNA/DNA ratio was a key biomarker of metabolic activity and growth potential in amphipods. Higher ratios indicate active growth and metabolic processes, which may signal increased energy expenditure in response to contaminants, while lower ratios could reflect suppressed metabolic function under environmental stress. This ratio provides an understanding of the broader metabolic impacts of contaminants on organismal health.

Contaminant overview

The chemicals analyzed in the Bothnian Sea and Gulf of Riga highlight the ecological significance of various contaminant classes that persist, bioaccumulate, and exert toxic effects across multiple trophic levels. Metals and POPs, including PCBs, OCPs, and PAHs, pose chronic threats due to their persistence and ability to induce oxidative stress, hormonal disruptions, and reproductive harm. Emerging contaminants like PFAS and pharmaceuticals add a new layer of risk, reflecting the evolving complexity of pollution impacts in biota. Below is a synthesis of the key chemical classes and their ecological significance:

- **Metals:** Trace metals such as mercury (Hg), lead (Pb), cadmium (Cd), arsenic (As), chromium (Cr), and zinc (Zn) were analyzed across sites. Metals are often persistent in the environment and can bioaccumulate in aquatic organisms, leading to toxic effects even at low concentrations. For instance, in its methylated form, Hg is neurotoxic and can disrupt reproductive health in fish and invertebrates. Lead and cadmium are also highly toxic, affecting neurological and reproductive systems.
- **Polychlorinated Biphenyls (PCBs) and Organochlorine Pesticides (OCPs):** PCBs and OCPs (e.g., DDT and its metabolites) are persistent organic pollutants with high bioaccumulation potential. Despite being banned or restricted, they remain prevalent due to their resistance to degradation. PCBs are toxic to fish and invertebrates, impacting growth, reproduction, and endocrine systems. Organochlorine pesticides like DDT are known for their ecotoxicity, particularly in affecting reproductive and developmental health in wildlife. These compounds tend to accumulate in higher trophic levels, leading to long-term ecological consequences, including hormonal disruptions and reproductive/developmental impairments.
- **Polybrominated Diphenyl Ethers (PBDEs) and Dioxins/Furans:** PBDEs (flame retardants) and dioxins/furans are another classes of POPs detected at these sites. PBDEs are associated with neurological, immune, and endocrine effects in marine organisms, while dioxins and furans are highly toxic compounds formed as industrial byproducts. Known to induce cancer and reproductive toxicity, dioxins and furans can persist in sediment, impacting benthic organisms and bioaccumulating in food webs.
- **Per- and Polyfluoroalkyl Substances (PFAS):** PFAS, including PFOS (perfluorooctanesulfonic acid), are synthetic compounds used in various industrial applications for their water-repellent properties. PFAS are highly persistent and mobile in aquatic environments, with strong bioaccumulation potential. They are linked to immune, reproductive, and developmental toxicity in marine life, impacting endocrine functions and survival rates, especially in long-lived species.
- **Polycyclic Aromatic Hydrocarbons (PAHs):** PAHs are a group of organic pollutants commonly released from incomplete combustion processes, such as oil spills and urban runoff. PAHs are known carcinogens and mutagens, with significant toxicity for aquatic organisms. In benthic invertebrates and fish, PAHs induce oxidative stress, impair growth, and lead to developmental and reproductive abnormalities. Given their tendency to accumulate in sediments, PAHs can pose long-term risks to organisms that inhabit or interact with sediment environments.
- **Organotins:** These compounds, including tributyltin (TBT), are used in antifouling paints and are highly toxic to aquatic organisms, particularly affecting invertebrates. TBT disrupts endocrine functions and is notorious for causing imposex in mollusks, a condition where females develop male characteristics, severely impacting reproductive success.

Contaminant analysis in the Gulf of Riga and Bothnian Sea encompassed a comprehensive range of chemical classes, although there were substantial differences in the data availability between the subbasins (Table 2). Both regions analyzed heavy metals such as mercury, cadmium, and lead, although additional metals like vanadium were included in the Bothnian Sea. With varying frequency, PCBs and OCPs were assessed across both basins, with industrial areas in the Bothnian Sea showing notably higher levels. Both areas also examined PBDEs, HBCDs, dioxins, and furans. Measurements of organotin compounds, particularly

TBT, are available in both regions, with elevated levels near polluted sites in the Bothnian Sea.

In comparing contaminant data availability across the Gulf of Riga and the Bothnian Sea (Table 2), three matrices—sediment, fish, and mussels (*Macoma*)—revealed notable differences in data coverage. Sediment samples in both basins included Metals, Organotins, PAHs, and PFAS, while PBDE/HBCDs, OCPs and PCBs were only analyzed in the Bothnian Sea. For fish, only Metals and PBDE/HBCDs were consistently analyzed in both basins, with the Bothnian Sea offering broader coverage for other contaminants. *Macoma* samples were analysed only in the Gulf of Riga, mainly for Metals and PCBs, with limited data for other contaminants but not OCPs and PFAS. Overall, the Bothnian Sea demonstrated a more extensive dataset across sediment and fish for both traditional and emerging contaminants, while the Gulf of Riga emphasized Metals and PAHs in fish and mussels.

Table B1. *Overview of sampling methods and analytical approaches for biological and sediment samples in the Gulf of Riga (A) and Bothnian Sea (B). Sampling locations, collection methods, sample processing, and types of biological and chemical analyses are provided. SMNH: Swedish Museum of Natural History.*

Test organism	Sampling location	Collection method	Sampling depth	Sample processing	Analysis type	Collection time
A. Gulf of Riga						
Monoporeia affinis	Latvian part	Van Veen grab (0.1 m^2)	Latvian part: 20-40 m;	Sieved through 0.5 mm; dissected for biomarkers, frozen at -80°C	Biomarkers	Year-round
Macoma balthica				Sieved through 1 mm; foot and digestive gland frozen at -80°C		
Gravid females *Monoporeia affinis*	Estonian and Latvian parts		Estonian part: 5–40 m Latvian part: 20-40 m;	Sieved through 0.5 mm mesh; transported to lab	Embryo analysis (fecundity, development stage, and aberrations)	Winter-early spring
Gravid females *Pontogammarus robustoides*	Latvian part	Hand net	Shallow waters (< 1 m)			June
Macoma balthica (>1 cm)		Van Veen grab (0.1 m^2)	Estonian part: 5–40 m Latvian part: 20-40 m;	Held in aerated seawater for 24 h to clear intestines; stored at -20°C until analysis	Metals and organic contaminants (e.g., PAHs, PCBs)	Year-round
Sediment	Latvian and Estonian parts			Macrobenthos removed by sieving, sediment homogenized, frozen at -20°C and stored until analysis		
Fish (herring, perch, round goby)	Latvian part	Trawling (herring), Gill nets (others)	Offshore (herring), Coastal (others)	Muscle and liver samples dissected; stored at -20°C		

Table B1, Cont.

Test organism	Sampling location	Collection Method	Sampling depth	Sample processing	Analysis type	Collection time
A. Bothnian Sea						
Fish (*Perca fluviatilis*)	Skellefteå, Holmöarna, Sundsvall, Norrsundet	Gill nets; SMNH guidelines for fish sampling and analysis	Water column	Muscle and liver samples dissected, epidermis and subcutaneous fatty tissue removed; stored at -20°C	Physiological indicators (body weight, gonad weight, liver weight), biomarkers (EROD, GST, AChE)	September
					Metals (Hg, Cd, Pb), PCBs, PFAS, HCBs, PFOS	
Gravid females *Monoporeia affinis*	Several sampling sites near Sundsvall and Norrsundet	Benthic sled, upper 2–3 cm layer of sediment	18-95 m	Within a week after collection, aminals kept in ambient water and temperature	Embryo analysis (fecundity, development stage, and aberrations)	January
				Collected in concert with embryo analysis, frozen immediately and stored at -80°C	Oxidative status (TBARS, ORAC/TBARS, RNA/DNA), neurotoxicity (AChE) and metabolic indices (RNA/DNA)	
Sediments				Macrobenthos removed by sieving, sediment homogenized, frozen at -20°C	Metals (As, Cd, Co, Cr, Cu, Hg, Ni, Pb, V, Zn), organic tin compounds, TBT, PAHs, PFAS, dioxins, PCBs, OCPs, PBDEs, HBCD	

Table B2. *Comparison of contaminant data availability across matrices in the Gulf of Riga and Bothnian Sea. Color coding facilitates data visualization.*

Contaminant class	Gulf of Riga	Bothnian Sea
Sediment		
Metals	Yes	Yes
Organotins	Yes	Yes
PAHs	Yes	Yes
PBDE/HBCDs	No	Yes
OCPs	No	Yes
PFAS	Yes	Yes
PCBs	No	Yes
Fish		
Metals	Yes	Yes
Organotins	No	Yes
PAHs	Yes	No
PBDE/HBCDs	Yes	Yes
OCPs	No	Yes
PFAS	No	Yes
PCBs	No	Yes
Mussels		
Metals	Yes	No
Organotins	Yes	No
PAHs	Yes	No
PBDE/HBCDs	Yes	No
OCPs	No	No
PFAS	No	No
PCBs	Yes	No

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