Effect-based characterization of mixtures of environmental pollutants in diverse sediments

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ABSTRACT

This study investigated whether cell-based bioassays were suitable to characterize profiles of mixture effects of hydrophobic pollutants in multiple sediments covering the remote Arctic and tropical sites to highly populated sites in Europe and Australia. The total contamination was determined after total solvent extraction and the bioavailable contamination after silicone-based passive equilibrium sampling. In addition to cytotoxicity, we observed specific responses in cell-based reporter gene bioassays: activation of metabolic enzymes (arylhydrocarbon receptor: AhR, peroxisome proliferator activated receptor gamma: PPARγ) and adaptive stress responses (oxidative stress response: AREc32). No mixture effects were found for effects on the estrogen, androgen, progesterone and glucocorticoid receptors, or they were masked by cytotoxicity. The bioanalytical equivalent concentrations (BEQ) spanned several orders of magnitude for each bioassay. The bioavailable BEQs (passive equilibrium sampling) typically were 10-100 times and up to 420 times lower than the total BEQ (solvent extraction) for the AhR and AREc32 assays, indicating that the readily desorbing fraction of the bioactive chemicals was substantially lower than the fraction bound strongly to the sediment sorptive phases. Contrarily, the bioavailable BEQ in the PPARγ assay was within a factor of five of the total BEQ. We identified several hotspots of contamination in Europe and established background contamination levels in the Arctic and Australia.

Environmental Significance Statement

Sediments are long-term reservoirs of mixtures of persistent organic pollutants. The sediments' site-specific total contamination (measured following exhaustive extraction) and bioavailable contamination (measured following silicone-based passive equilibrium sampling) of mixtures of pollutants allow prioritization of hotspots of contamination and possible remediation. Our study describes a broad characterization of mixture effects of environmental pollutants in sediment samples collected in areas from diverse sites which are supposed to vary in their contamination level. We identified three bioassays that were activated by most of the samples, showing distinct patterns across locations for the activation of metabolic enzymes and oxidative stress response, whereas the hormone receptors did not show any specific effects.

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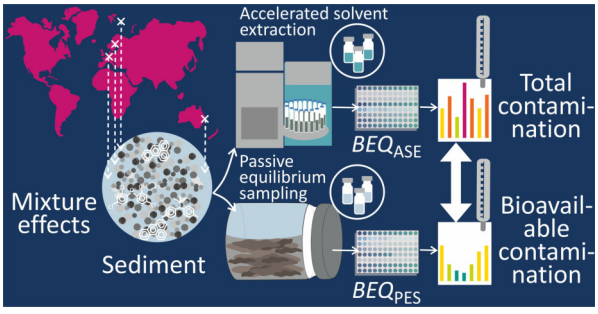
Our study distinguishes the total vs. the bioavailable contamination of mixtures of environmental pollutants in sediments from contaminated sites in Europe and more remote locations in Australia and the Arctic.

TOC art figure



INTRODUCTION

Risk assessment of sediment-bound pollutants is challenging: Firstly, organisms are hardly ever exposed to single chemicals such that complex mixtures of environmental pollutants with different modes of action and effect potencies have to be considered. Secondly, in many cases only a fraction of pollutants is freely dissolved and therefore available for partitioning and biouptake (*"bioavailable contamination"*).[1] Contrarily, the bulk of chemicals (i.e., freely dissolved plus bound chemicals) represents the *"total contamination"* that may become relevant in future scenarios ("worst case" values[2]). The bioavailable contamination can theoretically be predicted based on equilibrium partitioning theory,[3] but sediment organic carbon/water partition coefficients (K_{OC}) are highly variable.[4,5] Instead, bioavailable contamination in site-specific sediment samples determined using passive equilibrium sampling (PES)[6] can provide a more accurate assessment of exposure in contaminated,[7-9] urban[10] and moderately polluted[11, 12, 13] locations.

There is a multitude of pollutants that are both persistent and hydrophobic, such that a major fraction is being stored in sediments once emitted to the aquatic environment. The amount and characteristics of the main sorptive phase, organic carbon (OC), in combination with physicochemical properties of the pollutants, determine how strongly the pollutants are bound and which proportion is readily available for partitioning and biouptake. One part of the OC with a particularly high sorption capacity is the combustion-derived black carbon (BC) that can show by 1-3 orders of magnitude enhanced adsorption of aromatic planar hydrophobic organic compounds such as polycyclic aromatic

hydrocarbons (PAHs) or certain polychlorinated biphenyls (PCBs).[14] The authors described that sorption to BC was most relevant at low contaminant concentrations since the sorptive sites are limited.[14] Absorption into the amorphous part, OC, is thought to be reversible, whereas the adsorption onto the surface and into the pores of BC is considered to be so strong that these chemicals represent the irreversibly bound pool.

A range of studies compared the total amounts of selected (groups of) pollutants from exhaustive solvent extraction (total contamination) versus pore water concentrations from PES (bioavailable contamination). Total concentrations of PCBs, normalized to the OC content, showed larger variability than pore water concentrations in Baltic Sea sediment due to differences in sorption strength to the sediment.[11] This observation could either be due to variability in the site-specific K_{OC} values or other sorptive phases becoming more relevant. The sorptive capacities of sediments can vary considerably if different sorptive phases are involved, e.g. BC.[14, 15]

While there is a wide range of pollutants that have been detected in sediments world-wide, traditional chemical analysis cannot capture the entire mixture of pollutants, covering all compounds including those present at low concentration levels as well as their transformation products. Even if comprehensive chemical analysis was possible, no information about combined effects of the pollutants could be derived because of their unknown toxicological properties and interactions in mixtures. Contrarily, bioanalytical tools are suitable to assess combined effects of environmental mixtures of pollutants since they give integrative information about the sum of chemicals with identical mode of

action.[16] Related studies have been carried out with sediments from the Rhine Meuse estuary,[17] the River Elbe basin,[18] and Masan Bay, Korea.[19]

Li et al.[20] and Bräunig et al.[21] applied a combination of PES and total extraction on sediments from Australia followed by bioanalytical assessments of the obtained mixtures of pollutants. While the first study was of exploratory character to assess the approach of combining passive sampling of sediment with bioanalytical assessment of the mixture effects,[20] the second study extended the scope to different sorptive phases in sediment with weaker (OC) vs. stronger (BC) sorption and modeling of the partitioning of chemicals between compartments.[21]

In order to compare the data generated using PES directly with those from total extraction, the data need to be transformed to a $\mu g/kg_{OC}$ basis. Li et al.[20] reported that regression lines of K_{OC} and the partition coefficient between silicone and water ($K_{silicone/w}$) were roughly parallel for pollutants with a broad range of hydrophobicity (log octanol/water partition coefficient, K_{OW}, between 2 and 8).[20] Hence, a largely constant partition coefficient between OC and silicone ($K_{OC/silicone}$) was derived for a large number of chemicals[20, 22], and $K_{OC/silicone}$ was determined to be 2.0. Hence, it can be used to transform data from a silicone basis to an OC basis for comparison with ASE data that are also given on a $\mu g/kg_{OC}$ basis. Following the assumption of a relatively constant, K_{OW}-independent $K_{OC/silicone}$, the original mixture composition from the sample is expected to be transferred into the silicone during equilibration without substantial changes, and then quantitatively transferred into the solvent used for silicone extraction. Using ASE

133 assures exhaustive extraction of the organic pollutants present in a sediment
134 sample and hence quantitative transfer into the solvent.[22]

135 Vethaak et al.[23] also combined PES and total extraction with chemical analysis
136 and selected bioassays on sediments from the North Sea, Baltic Sea,
137 Mediterranean Sea and Icelandic waters. Differences were observed between the
138 total contamination (from accelerated solvent extraction, ASE) and the
139 bioavailable contamination (from PES), but without clear trends. For the
140 arylhydrocarbon receptor (AhR) assay, more than two thirds of the effects
141 remained unexplained, and the attempt to link chemical and bioanalytical results
142 was largely unsuccessful for the other assays due to the complexity of the matrix
143 and associated contaminants.

144 In the present study, we aim to identify patterns of contamination on an
145 extended geographical scale covering sediments with widely varying sources and
146 degrees of contamination, and spanning a battery of relevant cell-based reporter
147 gene bioassays to characterize the effects of pollutants present in sediments. Our
148 goal was to assess the usefulness of PES vs. exhaustive extraction in combination
149 with effect-based tools for improved hazard and risk assessment, both in remote
150 and urban locations. The sampling locations were selected to provide a broad
151 perspective about the pollution load and corresponding effects, including
152 locations dominated by different point sources (e.g., a steelwork site) or diffuse
153 sources (e.g., different streams flowing into a large river). The sites covered
154 presumably pristine versus highly populated sites from freshwater, estuarine
155 and marine locations. The sediment samples were extracted using ASE and PES,

and the total vs. bioavailable contamination were characterized in cell-based bioassays (*Figure 1*).

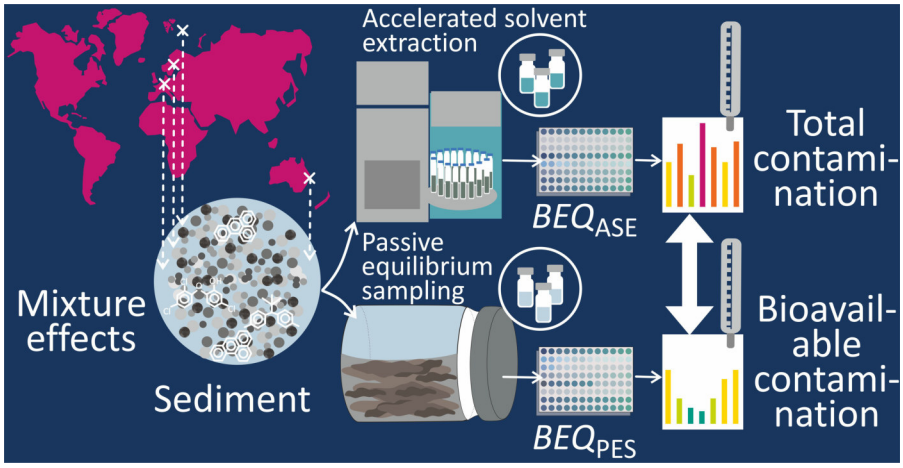


Figure 1: Summary of sampling and analytical steps. Sediment samples were collected in four major regions, processed by accelerated solvent extraction (ASE) and passive equilibrium sampling (PES) and submitted to a battery of cell-based bioassays to determine and compare the bioanalytical equivalent concentrations (BEQ) caused by the total contamination vs. the bioavailable contamination.

Methods

Sediment samples. Sediments were collected in Sweden, in Germany in a French-German river catchment, in four rivers/coastal areas in Queensland (Australia) and in the European Arctic (coastal Svalbard and offshore deep sea). Surface sediments were collected during various sampling campaigns carried out between 2013 and 2016. The samples were stored cold or frozen, and the Australian samples were freeze-dried prior to shipment to the UFZ laboratories. The sampling locations are shown in *Figure 2*, and the details of the sites and sample characteristics (including their fraction of OC) are given in *Table S1* in the Supporting Information (SI). Before processing the samples, stones and other large items such as leaves or branches were removed.

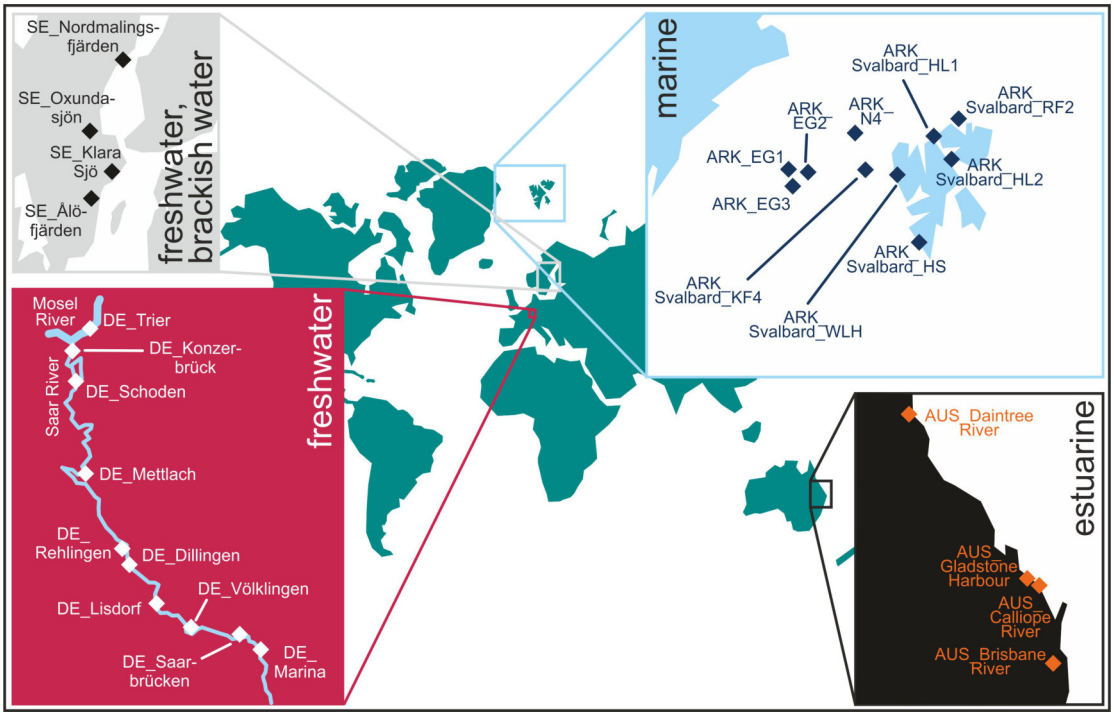


Figure 2: Map of the sampling locations in the European Arctic (n = 9, Svalbard vs. offshore deep sea), Sweden (n = 4), Germany (n = 10) and Australia (n = 4).

Passive Equilibrium Sampling. For PES, the freeze-dried Australian samples were reconstituted using deionized water to yield a slurry suitable for the silicone-based extraction. Other samples were kept as received, or small aliquots of deionized water were added if necessary to obtain suitable consistency. The sorptive capacity of water for the hydrophobic pollutants causing the effects is much smaller than that of the sediment as demonstrated by Bräunig et al.[21] using sediment/water distribution coefficients ($D_{\text{sediment/w}}$) in the range of 100 to 1,000,000. Therefore, aliquots of water can be added, including freeze-drying and reconstitution of the sediment, without changing the sediment slurry's capacity substantially. Eleven blanks were generated using bi-distilled water, and one solvent blank was prepared.

The chemicals in the pore water of the sediment samples were equilibrated with thin coatings of silicone (20 µm, corresponding to 147 ± 15.7 mg of silicone) on

191 the inner vertical walls of 120 mL glass jars by horizontal rolling for 3
192 weeks.[10, 11, 24, 25] For each jar, 90-120 g of sediment were used, and approx. 0.1 %
193 of sodium azide (Merck) was added to preclude microbial degradation during
194 equilibration. For blanks, we used bi-distilled water with sodium azide. The
195 equilibration time was extended from two weeks, which had been shown to be
196 sufficient for the indicator PCBs,[11, 26] to three weeks in order to ensure reaching
197 an equilibrium between the samples and the silicone if even more hydrophobic
198 contaminants were present. Negligible depletion was demonstrated for the
199 pentachlorinated PCB 118 by plotting the mass of PCB 118 sampled in the
200 silicone versus the mass of silicone in jars with different coating thicknesses (5
201 µm, 10 µm and 20 µm). Proportionality was observed, confirming that
202 equilibrium was achieved and showing the absence of sample depletion.[24]

203 Subsequently, the sediment was removed, and the jars were cleaned thoroughly
204 with a few mL of deionized water and lint-free tissues. Then, the chemicals in the
205 silicone were extracted with two aliquots of 2 mL ethyl acetate (Merck), by
206 horizontal rolling for 30 min each, and the extracts were combined. In order to
207 generate enough extract for broad bioanalytical screening, three glass jars were
208 equilibrated with three subsamples of sediment for each location. The extracts
209 were combined, evaporated to dryness and reconstituted in 1 mL of methanol
210 (Merck) for subsequent dosing in the bioassays.

211 **Total solvent extraction**. For ASE of the pollutants present in the sediment,
212 aliquots of the samples from the Arctic, Germany and Sweden were freeze-dried
213 and subsequently ground with a mortar and pestle. Approximately 5 g of the
214 dried sediment samples were mixed with 1 g of hydromatrix (high purity, inert

diatomaceous earth sorbent, Biotage), filled into ASE cells, and the cells were closed. For each sample, 2-3 replicates were processed. Thirteen ASE cells without sediment (with hydromatrix only) were processed as blanks. The total amount of chemicals present in the sediment was extracted with a mixture of ethyl acetate and acetone (1:1, v:v, Merck), in two cycles at 100 °C and 150 psi in a method optimized for wide-scope multitarget screening as described by Massei et al.[27] The extracts were blown down to dryness and reconstituted in 1 mL of methanol for testing. Aliquots of the methanol extracts were transferred into cell-based reporter gene bioassays.[22] The methanol was completely evaporated before the assay medium was added for transfer to the cells.

Cell-based reporter gene bioassays. To avoid changing the obtained mixture composition, the extracts were not submitted to any clean-up step before dosing in the bioassays. This measure to conserve the mixture as much as possible is supported by several studies that have shown that the potencies of sediment extracts to elicit effects were reduced after treatment with sulfuric acid.[17, 23, 28, 29]

The extracts were dosed into seven cell-based reporter gene bioassays (*Table S2*, SI) indicative of metabolism of xenobiotic compounds, specific receptor-mediated effects and adaptive stress response. Cell viability was assessed in parallel in all the assays as a quality assurance/quality control measure[30] to ensure that cytotoxicity did not interfere with the observed effect. Cell viability was quantified as the confluence of the cells in each bioassay well. The cutoff above which the data were no longer considered valid was set at the cell viability decreasing to less than 90 %, i.e., the concentration at which 10 % of cytotoxicity occurred (inhibitory concentration, IC_{10}, *Figure 3*). At concentrations just above

the IC_{10} value, the cells can non-specifically show activity as a result of general stress that even triggers specific cell stress pathways, a phenomenon referred to as 'cytotoxicity burst'.[31] At even higher concentrations, reporter gene effects decreased due to the reduced viable cell number (*Figure 3*).

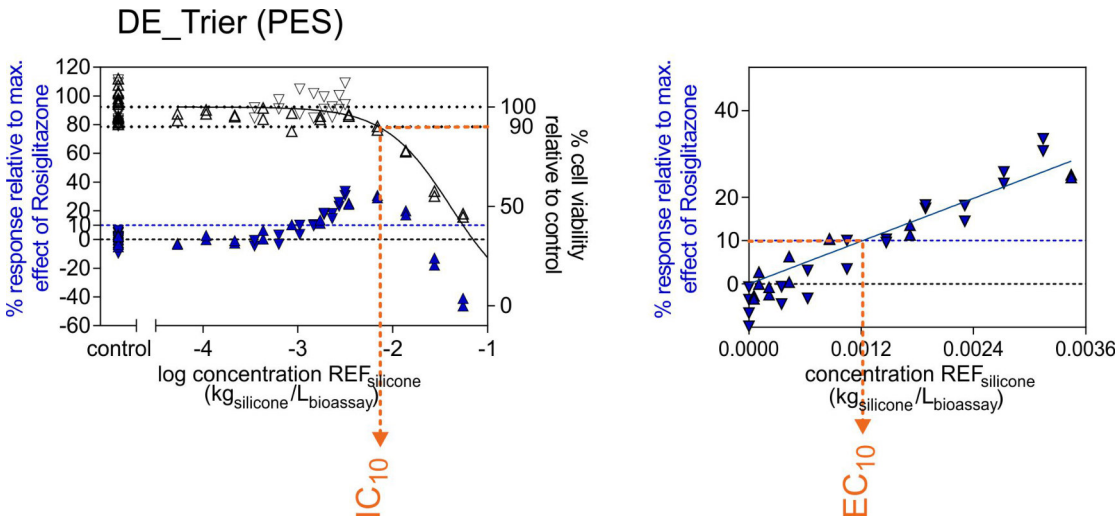


Figure 3: Concentration-effect curves for sample DE_Trier processed with PES dosed into the PPARγ assay. Independently repeated experiments are represented by different symbols. Specific effects (filled triangles, left axis) and cell viability (open triangles, right axis) are given. Left: full dosing range with the derivation of the IC_{10} cutoff; right: linear range, from which the effect concentration eliciting 10 % of the maximum effect of the reference compound (EC_{10}) is derived. $REF_{silicone}$ = relative enrichment factor, the equivalent mass of silicone dosed per volume of bioassay.

Specifically, the assays in this study targeted a) cytotoxicity, b) activation of metabolic enzymes, via binding to the AhR and the peroxisome proliferator-activated receptor gamma (PPARγ), c) specific, receptor-mediated effects covering the estrogen (ERα), androgen (AR), glucocorticoid (GR) and progesterone (PR) receptors and d) adaptive stress response, i.e., the reaction to oxidative stress (AREc32). Each assay had a specific reference compound, i.e., a chemical with high potency for the respective endpoint (*Table S2*), which was used to determine maximum effects that the effects of the environmental mixtures could be related to.

Regarding the activation of AhR-targeting dioxin-like chemicals, the method was initially described by Brennan et al.,[32] adapted by Neale et al.[33] and Nivala et al.[30] The method of Neale et al.[33] was used for activation of PPARγ by so-called "obesogens" such as phthalates and nonylphenol. Adaptive stress response (AREc32), which usually occurs due to the presence of less hydrophobic chemicals, was tested as outlined by Escher et al.[34, 35] The specific, receptor-mediated effects (ERα, AR, GR and PR GeneBLAzer) were assessed according to König et al.[36]

Data evaluation. In a first assessment, the unknown, highly concentrated sample was dosed at a high level and serially diluted to cover a broad range of concentrations. The concentrations of the sediment extracts are given in units of relative enrichment factors (REFs) that show the equivalent mass of silicone ($REF_{silicone}$ in $kg_{silicone}/L_{bioassay}$) or sediment on a dry-weight (dw) basis ($REF_{sediment}$ in $kg_{sediment,dw}/L_{bioassay}$) dosed per volume of bioassay.

Figure 3 illustrates the concentration-effect curves. The goal was to induce cytotoxicity at the highest concentration levels to define the IC_{10} cutoff, because this threshold represents the upper boundary above which assessment of specific effects is not reasonable. From the resulting concentration-effect curve, and based on the IC_{10} cutoff, at least one additional dosing was performed, usually for linear dilution focusing on the concentration range to derive the EC_{10} value. The purpose of the linear repeat was to confirm the initial results and allow for derivation of a robust effect concentration.

Environmental mixtures of chemicals seldom show full concentration-effect curves up to 100 % effect relative to the reference compound. This is partly

because of low levels of the pollutants, but also due to masking by cytotoxicity by these complex samples. In many cases it makes the derivation of effect concentrations eliciting 50 % of the maximum effect (EC_{50}) highly uncertain or impossible. Therefore, we derived EC_{10} values instead, using the linear part of the concentration-effect curves up to 40 % effect (*Figures 3 and DS1 to DS7* in the Data Supplement, DS) as suggested in *refs*.[33, 37, 38] The AREc32 assay does not show a maximum, and hence the induction ratio (IR) of 1.5, i.e., 50 % over the control (cells with medium only), was used to derive an $EC_{IR1.5}$ instead.[34]

Since small EC values represent strong effects, which may appear counter-intuitive, we derived toxic units (TUs, TU_{PES} in units of $L_{bioassay}/kg_{silicone}$ or TU_{ASE} in units of $L_{bioassay}/kg_{sediment,dw}$) as the reciprocal values of the EC data (Eqs. 1 and 2):

For AhR, PPARγ, ERα: (1)

For AREc32: (2)

The blanks were dosed into the cell-based bioassays along with the samples derived from the sediments. We quantified the blank response in each assay as TU and weighted the blanks by summing up the TUs for all the blanks for each set of samples (PES vs. ASE) and dividing them by the number of blanks (n = 11 or n = 13, respectively) according to Eq. 3:

(3)

In those cases where the TU of this weighted blank corresponded to less than 50 % of the TU of a sample, it was subtracted from the sample (Eq. 4) to generate blank-corrected TUs:

306 (4)

307 If the TU of the weighted blank was larger than 50 % of the TU of the sample, this
308 sample was excluded from further data analysis.

309 The combined effects characterized using bioanalytical tools have been
310 described using BEQs,[16, 34] which are derived from the product of the effect
311 concentrations of a potent reference chemical in a bioassay and the blank-
312 corrected TU of a sample (Eq. 5):

313 (5)

314 where EC is the effect concentration eliciting a certain effect level of the
315 maximum effect as determined by using the reference chemical.

316 We dosed either the total contamination from exhaustive solvent extraction or
317 the bioavailable contamination in silicone at equilibrium with the sediment
318 sample from silicone-based PES into the bioassays to characterize the BEQs for
319 the total BEQ (BEQ_{ASE} in $\mu g_{ref}/kg_{sediment,dw}$) and the bioavailable BEQ (BEQ_{PES} in
320 $\mu g_{ref}/kg_{silicone}$).

321 To derive OC-normalized BEQs that enable for direct comparison of the data sets
322 obtained with PES and ASE, the BEQ_{ASE} [$L_{bioassay}/kg_{sediment,dw}$] were divided by the
323 fraction of OC (*Table S2*) to yield $BEQ_{ASE,OC}$ (Eq. 6):

324 (6).

325 BEQ_{PES} [$L_{bioassay}/kg_{silicone}$] were multiplied by the OC/silicone partition coefficient of
326 2.0[20] to give $BEQ_{PES,OC}$ (Eq. 7):

(7).

In this study, we used a $K_{OC/silicone}$ value of 2.0 to convert silicone-based concentrations to concentrations in OC.[20] Since the sediment samples originated from very diverse sampling locations with different patterns and levels of contamination, a ranking was performed: The BEQ data were sorted to give ascending BEQs, and then the % rank of each data point was calculated as the rank divided by the number of samples. The probit rank was then calculated using the NORMINV function around a mean of 5 with a standard deviation of 1 in MS Excel, returning the inverse of the cumulative standard normal distribution for each data point.

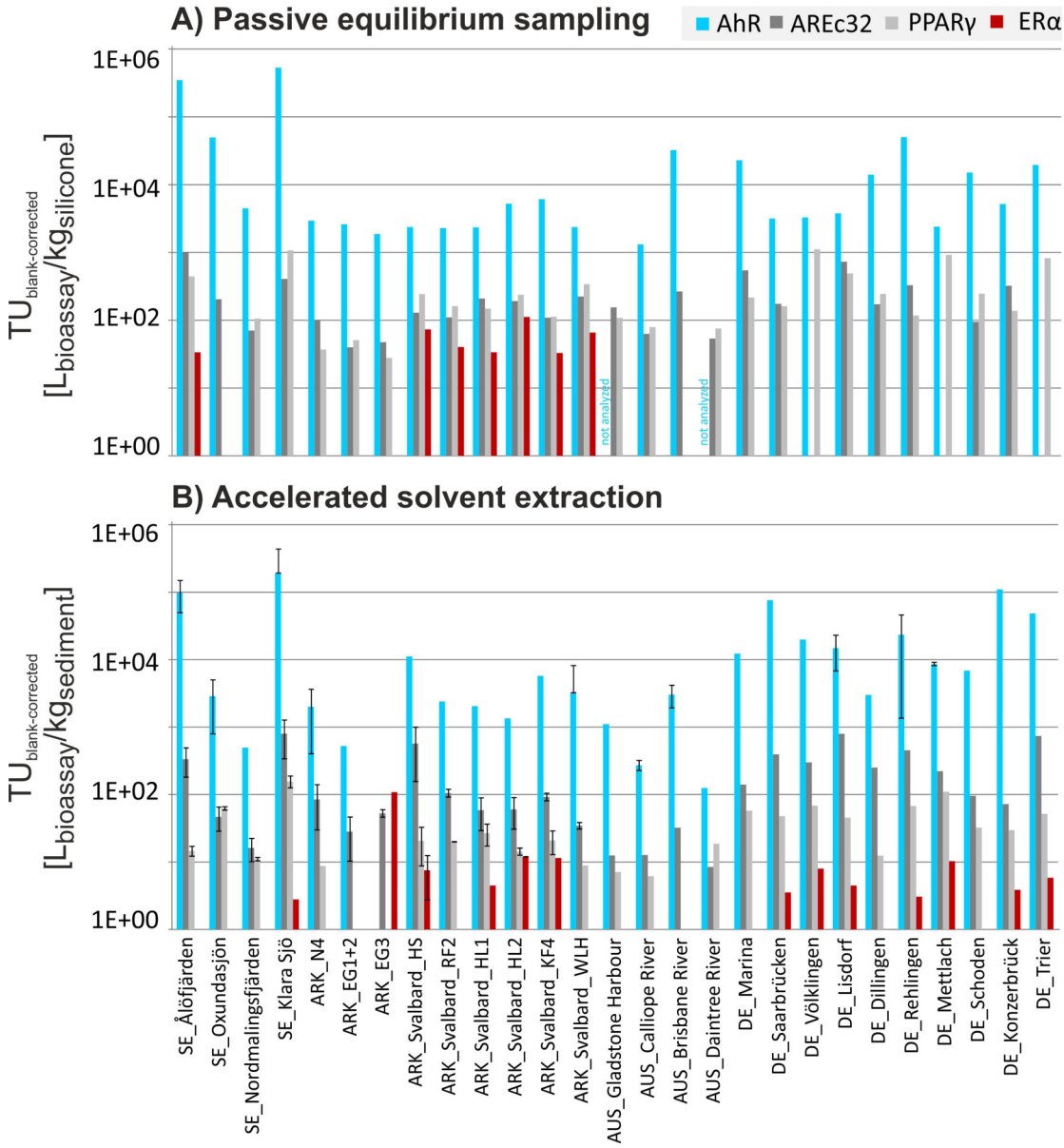
RESULTS AND DISCUSSION

Bioanalytical screening. The full concentration-effect curves and the linear part of the curves used for data evaluation of all seven bioassays and all samples including all procedural blanks are given in *Figures DS1 to DS7* (in the Data Supplement, DS). Cytotoxicity masked the effects occasionally as discussed in detail below. No cytotoxicity was observed for the blanks, giving evidence that the sodium azide used during equilibration of the sediments and blanks with the silicone coating of the glass jars was completely removed before solvent extraction of the chemicals from the silicone.

Figure 4 shows the effects expressed as TUs of the sediment samples processed using PES (A) and ASE (B) in the active bioassays obtained using Eqs. 1 and 2. The TUs and their related standard errors are additionally listed in *Tables S3 (PES) and S4 (ASE) in the SI*.

A few sediment extracts were low in response, with TUs close to the TU of the weighted blank. As described above, these data points were excluded from further data analysis when the weighted blank corresponded to more than 50 % of the TU of the sample. In total, four data points were excluded based on the blank evaluation procedure: one ASE extract in AhR, as well as one PES extract and two ASE extracts in ERα.

For PPARγ, blanks were not an issue as no blank response was observed for the PES and ASE data sets. For the PES samples in AhR, the TU of the weighted blank corresponded to less than 1 % of the TUs of the samples, whereas for the ASE data, the weighted blank corresponded to <1 % (*n* = 21), 1-10 % (*n* = 15), 10-30 % (*n* = 5) and >50 % (*n* = 1, sample ARK_EG3 (3)). In the case of AREc32, no blank response was recorded for the PES data set, whereas the TU of the weighted blank corresponded to <1 % (*n* = 10), 1-10 % (*n* = 25), and 10-30 % (*n* = 5) of the TUs of the ASE data set. Regarding the ERα assay, the TU of the weighted PES blank corresponded to <10 % (*n* = 1, sample ARK_Svalbard_HL2), 10-30 % (*n* = 6) and >50 % (*n* = 1, sample DE_Rehlingen) of the sample response, and to <10 % (*n* = 1, sample ARK_EG3), 10-30 % (*n* = 7), 30-50 % (*n* = 7) and >50 % (*n* = 2, samples ARK_N4 and ARK_Svalbard_RF2) for the ASE data. As a consequence of the relatively low response of the samples compared to the weighted blank, the ERα data have to be interpreted with caution.



370

371 ***Figure 4: Blank-corrected toxic units (TUs) in the pooled PES extracts (n = 1, panel A) and the***
372 ***average of the ASE extracts (n = 2 or 3, panel B), with standard deviation (n = 3) or absolute***
373 ***deviation (n = 2). In those cases where no error bar is displayed, only one data point is***
374 ***available. For the blanks, $TU_{blank,weighted}$ was 11 (AhR-PES), n.d. (AREc32 and PPARγ PES), 12***
375 ***(ERα PES), 46 (AhR ASE), 2.4 (AREc32 ASE), n.d. (PPARγ ASE) and 2.4 (ERα ASE). Note: if no***
376 ***bars are shown, no activity was recorded.***

377 Three of the seven bioassays were active for most of the PES and ASE extracts of
378 the sampled sediments: AhR, AREc32 and PPARγ (*Figures DS1-DS3*, DS), with
379 each cell line showing a distinct pattern throughout the sampling locations. Of
380 the hormone receptors that were investigated, only ERα was activated by some
381 sample extracts (*Figures 4 and DS4*, DS), whereas AR, GR and PR were not

activated when dosed with the sediment extracts, or the effects were masked by cytotoxicity (*Figures DS5-DS7*, DS).

Looking at the silicone-based extracts, the activation of the AhR, known to be triggered by dioxin-like chemicals, was by far the most sensitive endpoint, and TUs could be derived for the vast majority of the samples. The other three assays showed responses only at higher enrichment. The AREc32 and PPARγ assays also showed effects for most of the samples, but their TUs were 5.2-1,300 (AREc32, on average 130) or 2.6-790 (PPARγ, on average 100) times lower than for AhR. Furthermore, a selection of PES extracts triggered a response in ERα, with TUs 32-10,000 (on average 1,500) times lower than for AhR (Figure 4).

The TUs for the ASE extracts showed a corresponding picture: Again, the AhR was the most responsive assay, while the other assays required substantially higher enrichment factors to observe effects. In this case, the TUs were even lower in comparison the AhR assay with 12-1,500 (AREc32, on average 130), 6.7-6,800 (PPARγ, on average 750) and 110-68,000 (ERα, on average 12,000) times for the AREc32, in comparison with the AhR assay.

Focusing on AhR, we observed some variability in which site elicited the highest response for samples extracted with PES (bioavailable contamination) and ASE (total contamination), respectively. As an example, in the River Saar, the ASE sample from station DE_Konzerbrück showed the highest effect (a factor 4.7 higher than at station DE_Rehlingen), whereas the PES data from DE_Rehlingen gave evidence of 9.7 times higher exposure than at DE_Konzerbrück, indicating differences in the sorptive capacities of these sediments. For other sampling regions, it was the same site that dominated both the ASE and the PES response,

406 but the relative importance may differ. These effect-based data strongly support
407 the importance of considering the PES-derived bioavailable contamination from
408 sediment in hazard and risk assessments of contaminated sediments since the
409 total contamination might lead to prioritization of less important locations for
410 remediation actions. Another pollutant pool that could be worth considering is
411 the accessible fraction of chemicals. It represents the fraction that can become
412 available, e.g. if the bioavailable pool is removed or if the environmental
413 conditions change substantially. The accessible chemicals can be studied
414 following extraction with mild sorbents[20, 21] or depletive extraction with
415 polymers such as silicone (e.g., the "multi-ratio" approach[39]).

416 **Specificity of the bioanalytical results**. The cytotoxicity assessment led to a
417 cutoff of the valid bioanalytical results once the cell viability sank below 90 %,
418 and all data with REFs above the IC_{10} value were not considered (see *Figure 3*
419 and the dotted vertical lines in *Figures DS1-DS7*, DS). In general, cytotoxicity did
420 not differ substantially between the various bioassays, as supported by *Figure 5*,
421 which shows a plot of the specific effects (EC_{10} or $EC_{IR1.5}$) vs. cytotoxicity (IC_{10}) for
422 PES (A) and ASE (B). Here, the IC_{10} data fell into a narrow range across bioassays
423 (grey area), whereas the specific effects showed substantially larger variability.
424 Cytotoxicity of complex environmental mixtures is expected to be rather non-
425 specific and hence the similarity of IC_{10} across cell lines was expected. We
426 suggest that the distance the data have from the 1:1 line can be used as a
427 measure of the importance of the specific effect ("specificity ratio"), because the
428 more distant the EC_{10} data is from the 1:1 line, the more specific is the effect (Eq.
429 8):

(8).

Figure 5. Specific effects (EC_{10} or $EC_{IR1.5}$ values) plotted vs. cytotoxicity (IC_{10}), with the 1:1 perfect fit line and a factor 10 deviation (blue area) also given. The further the data are from the 1:1 line, the more specific the observed effects are ("specificity ratio"). The grey shadings demonstrate the similarity of the IC_{10} data across bioassays.

The plots demonstrate that the effects observed in the AhR bioassay have the highest specificity, i.e., the largest distance from the 1:1 perfect fit line. Most other data were also more than a factor 10 away, except for one data point for AREc32, a few data points for PPARγ and all the ERα data. The limited data set that we obtained using the ERα cell line is non-specific as all the data fell within a factor 10 of the 1:1 line (blue area, *Figure 5*) and could hence be an artefact of the cytotoxicity burst.[31] This concern is supported by the fact that known agonists for ERα are highly specific and usually do not sorb strongly to sediment. Hence, we exclude the ERα data set from the discussions in the following sections.

Risk versus hazard assessment. By comparison of the effects caused by the bioavailable contamination (PES) and the total contamination (ASE), we can derive important site-specific information on the different sediments. BEQ_{PES} gives an indication of the potency of the mixture of chemicals that are at present available for partitioning and biouptake. Contrarily, BEQ_{ASE} can be considered as a measure of the potency of the total contamination that might in the future become available if substantial changes occurred in the ecosystem.

To allow for direct comparison of the data sets, the data were translated to an OC basis as described above (Eqs. 6 and 7). The relationship between $BEQ_{ASE,OC}$ and $BEQ_{PES,OC}$ is shown in *Figure 6*. In this context, $BEQ_{ASE,OC}$ should be equal to (if all

chemicals are readily available) or larger than $BEQ_{PES,OC}$ (if part of the chemicals are irreversibly bound to sediment components such as BC). The scatter around the 1:1 line, in particular below and just above the 1:1 line, represents the measurement/modeling uncertainty. A version of Figure 6 including standard errors is given as *Figure S1 (SI)*.

Figure 6. Bioanalytical equivalent concentrations (BEQ) from ASE vs. silicone-based PES, normalized to OC. The 1:1 line indicates that the complete contaminant mixture captured by ASE was also captured by PES, whereas the broken lines mark differences of 1-3 orders of magnitude in both directions.

In this data set, many data points (n = 47 of 71, i.e., 66 %) scatter around the 1:1 line and can be found in the dark grey area, within a factor of 10, which means that in many samples the chemicals are mostly available for partitioning and biouptake. For example, those sample extracts that activate the PPARγ assay scatter around the 1:1 line, indicating that most of the chemicals that are active in these assays are present in the sediment interstitial pore water and hence readily available for partitioning to the silicone, which is consistent with the discussion above.

The fact that no data are found below the 1:10 line indicates that the uncertainty of this approach, including the conversion to the $\mu g/kg_{OC}$ basis, is less than a factor of 10. For other data that are between the 10:1 and the 100:1 lines, only a minor fraction (1-10 %) is currently available, whereas the larger fraction is bound to the sorptive phases present in the sediment; this is the case for many sample extracts in the AREc32 and AhR assays (in total 22 of 71, 31 %). For two samples in the AhR assay (2.8 %), less than 1 % is available (data points above the 100:1 line) since the effects in the ASE-derived samples are 210

(DE_Konzerbrück) or 420 (DE_Saarbrücken) times higher than in the corresponding samples processed using PES. The response in the AhR assay is to a large degree caused by very hydrophobic chemicals such as PAHs, PCBs and dioxins, hence the observed differences are plausible because these chemicals are likely to bind strongly to BC as outlined above. Regarding the chemicals that activate the AREc32 assay, the current data set indicates that even here, the bioavailability of active chemicals might be strongly reduced due to strong binding to other sorptive phases such as BC, which has been demonstrated previously.[21]

To enable a comparison with literature data, we transformed the PES-derived data set from Vethaak et al.[23] to a $\mu g/kg_{OC}$ basis according to Eq. 7. The data set reflecting the total contamination (from ASE) was 11-65 (on average 24) times higher than the bioavailable contamination (from PES). These factors show that in that study,[23] roughly 1-10 % of the active chemicals were present in their bioavailable form, which is similar to the observations we made with our data set.

Geographical trends and hot spots. Since the sediment samples used in this study were collected in very diverse regions, covering a broad range of pollution types and degrees, the obtained data allow us to derive geographical trends as illustrated in *Figure 7*. The figure shows one panel for each (active) assay (A-C) with the data ranked using probit units as described above.

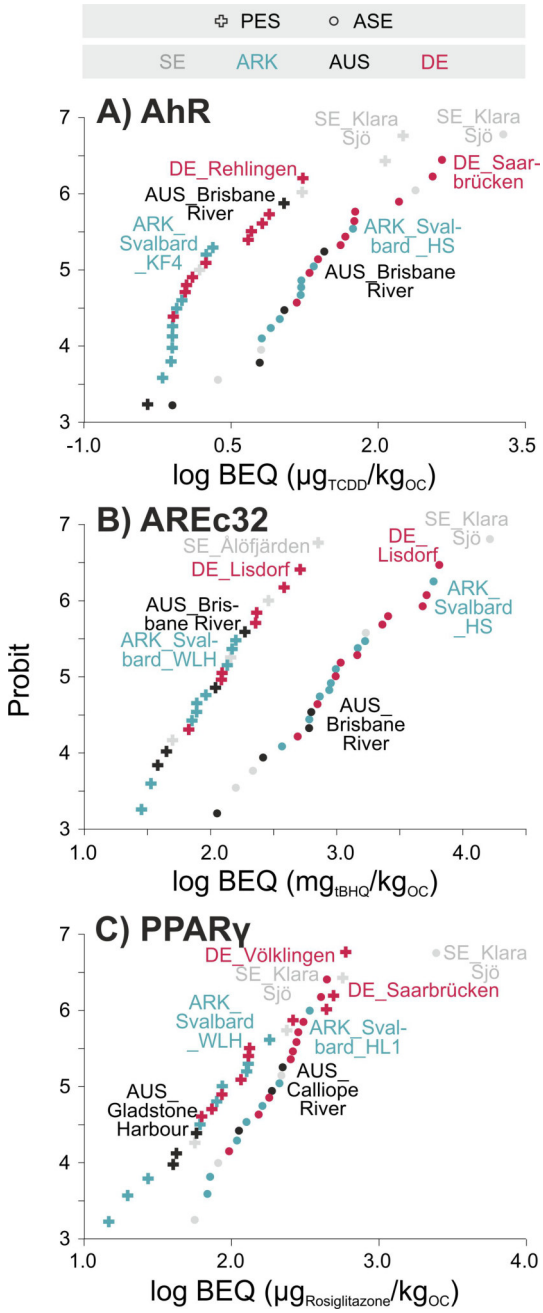


Figure 7. Probit-ranked bioanalytical equivalent concentrations (BEQs) on an OC basis for the AhR (A), AREc32 (B) and PPARγ (C) assays derived from silicone-based PES (crosses) or ASE (dots). The source regions are color-coded in grey (Sweden), blue (Arctic), black (Australia) and red (Germany).

The highest ranked sites for each sampling region in some cases overlap for the silicone-based PES and the total concentrations from ASE (such as SE_Klara Sjö in AhR), whereas in other assays, different sites are dominant (e.g., SE_Ålöfjärden (PES) vs. SE_Klara Sjö (ASE) in AREc32).

511 Overall, the samples from the Arctic were included in our set of samples to
512 represent background areas. In general, the responses of the extracts in the AhR,
513 AREc32 and PPARγ assays were in the mid to low range, whereas they showed
514 substantial responses for selected samples in other assays (such as the ASE
515 sample of ARK_Svalbard_HS in AREc32). Together with the samples from the
516 Arctic, those from Australia showed less explicit effects, with the exception of the
517 sample from an urban estuary, the Port of Brisbane (AUS_Brisbane River). Given
518 the medium to low responsiveness of the samples from the Arctic and Australia,
519 these results indicate that even in remote areas, environmental mixtures of
520 chemicals can elicit effects as has also been observed by Vethaak et al.[23] Indeed,
521 analyses of passive sampling devices deployed for a year close to the Arctic deep
522 sea sites included in this study indicated the prevalence of polybrominated
523 diphenyl ethers (PBDEs), PCBs and organochlorine pesticides in deep waters.[13]
524 In addition, sediment samples taken near the Arctic offshore sites contained high
525 levels of microplastic, which can function as vectors of numerous pollutants and
526 could have transferred sorbed chemicals to the sediments.[40] For a more detailed
527 comparison with literature data, see below.

528 One general observation is that the sampling location SE_Klara Sjö was highly
529 responsive. This sample was collected at a location contaminated with PAHs
530 from a former gas works and creosote production. In addition, there is pollution
531 from road runoff and storm water drainage. Dredging activities two decades ago
532 have not succeeded in fully remediating the site. The ASE extracts from SE_Klara
533 Sjö elicited strong effects in the AhR, AREc32 and PPARγ assays, followed by
534 SE_Ålöfjärden and several locations along the German part of the River Saar. This

535 river is known for its contamination with persistent organic pollutants such as
536 PBDEs, dioxins and dioxin-like PCBs, particularly downstream of the industrial
537 region around Völklingen and Saarbrücken.[41, 42] The PES data of SE_Klara Sjö
538 showed the highest response in AhR, too, while the PPARγ response was
539 outcompeted by sample DE_Völklingen, and the AREc32 response was ranked as
540 number four in this data set.

541 The data from silicone-based PES were clearly separated from the ASE data for
542 the AhR and AREc32 assays (*Figures 7 A and B*). Hence, the bioavailable
543 contamination of the compounds that were active in these assays differed
544 substantially from the total contamination, meaning that a substantial fraction of
545 the chemicals eliciting effects in AhR and AREc32 were bound to sorptive sites in
546 the sediments. Contrarily, we did not observe large differences between the PES
547 and the ASE data sets for PPARγ, in particular for the higher ranked samples. In
548 general, most of the sample sets already covered a relatively large range of
549 contamination.

550 Looking at the AREc32 data (*Figure 7B*), the observed effects are most explicit for
551 the ASE sample SE_Klara Sjö, whereas SE_Ålöfjärden dominates the effects of the
552 PES samples. The sampling location SE_Ålöfjärden is a contaminated Baltic Sea
553 bay in the direct vicinity of an active steelworks site, located approx. 100 km
554 south of Stockholm. The sample from the River Saar that showed the most
555 explicit effect in the AREc32 assay was DE_Lisdorf. As in the AhR assay, the
556 response of the Australian samples in the AREc32 assay occurred at medium to
557 high REFs, with AUS_Brisbane River eliciting the most explicit activation. The
558 samples from the Arctic showed medium to low response for the PES samples,

559 but high to medium response for those generated using ASE, with sediments
560 collected close to Svalbard showing the largest effects, indicating the island
561 population as a source of pollutants.

562 The effects in the PPARγ assay (*Figure 7C*) were dominated by samples collected
563 at locations in Germany (PES: DE_Völklingen) and Sweden (ASE: SE_Klara Sjö).
564 Medium to low response was observed for the samples from the Arctic, again
565 showing higher response when taken close to Svalbard. Low (PES) or medium
566 (ASE) effects were recorded in the Australian samples. In the latter case,
567 proximity to the Port of Brisbane was not relevant in the PPARγ assay, since
568 other locations triggered the most explicit response (PES: AUS_Gladstone
569 Harbour, ASE: AUS_Calliope River).

570 While the analysis of similarities (ANOSIM, multivariate ANOVA) routine
571 revealed no significant overall regional differences between the stations based
572 on PES data (Global R = 0.084, p = 0.175), it showed significant differences when
573 applied to the ASE-derived BEQs (Global R = 0.227, p = 0.01) as illustrated in
574 *Figure S2* (SI). Despite the differences between the PES and ASE results, the
575 routine RELATE indicates that these data sets are correlated (ρ = 0.316, p =
576 0.013). The samples from Germany differed significantly from those from
577 Sweden (R = 0.65, p = 0.003) and Australia (R = 0.45, p = 0.01). BEQs derived
578 from PPARγ contributed most to the dissimilarity between Swedish and German
579 samples (36 %), and BEQs derived from AREc32 were most relevant for the
580 dissimilarity between Australian and German samples (47 %). In addition, the
581 sediments collected in Sweden differed from those taken near Svalbard (R = 0.30,

582 p = 0.04). In this case, the BEQ derived from PPARγ contributed most to the
583 dissimilarity (44 %).

584 Our results agreed fairly well with data by Bräunig et al.[21] for the identical
585 samples: the PES data agreed within an average factor of 19 (AhR) and 4.3
586 (AREc32), providing evidence that the freeze-drying of the Australian samples
587 did not change the freely dissolved concentrations, whereas the total extraction
588 data sets differed by an average factor of 220 (AhR) and 5.7 (AREc32). The
589 different combinations of solvents used in these two studies (acetone:hexane[21]
590 vs. acetone:ethyl acetate, this study), may be part of the reason for the observed
591 differences.

592 To compare our data to the data set published by Vethaak et al.[23], we
593 transformed the literature data to a μg/kg_{OC} basis. For the AhR response of the
594 PES data, our data is similar to the published data set,[23] but includes more
595 variability, covering both more (Sweden) and less contaminated samples
596 (Arctic). The AhR results of the samples in the present study processed using PES
597 for the samples from the Arctic and Australia were on average a factor of 1.6
598 lower or 3.5 higher than the data from the background station in Iceland, and the
599 published ASE data were an average factor of 1.8 (Arctic) or 3.0 (Australia)
600 higher than our data, respectively. The data sets generated using ASE were very
601 similar across studies and differed by less than one order of magnitude. Vethaak
602 et al.[23] also reported estrogenicity data, but given that no specificity ratios were
603 calculated, it might be that these data were a result of the cytotoxicity burst as
604 observed in our study.

An additional comparison can be made with Li et al. [29] (AhR data from Lake Tai Basin, China), showing good agreement for the maximum response from Australia and the Arctic (within a factor 6.3), whereas our most contaminated samples from Sweden and Germany showed an up to 62 times higher response.

CONCLUSIONS.

The present study provides further evidence of the usefulness of (1.) passive sampling data giving important information about the bioavailable contamination as opposed to the total contamination that is often of limited relevance for exposure and risk assessments; and (2.) bioanalytical tools that give integrative information of the sum of chemicals with the same mode of action, serving as a complementary tool to chemical analysis. By combining different extraction methods, the bioavailable contamination from PES can be compared to the total contamination as extracted using ASE. Bioanalytical tools are useful in the evaluation of sediments as they have good sensitivity, and thus facilitate assessment of sediments both from contaminated and background areas. Depending on the bioassay, the response of the total contamination was up to 420 times higher than the bioavailable contamination (DE_Saarbrücken in AhR), and on average 41 (AhR), 16 (AREc32) and 2.2 (PPARγ) times higher for ASE than for PES. The reduced availability of a substantial fraction of the chemicals relevant for the different assays may be due to strong binding to sorptive phases such as BC, which is expected to be more explicit for certain hydrophobic pollutants that show aromaticity and planarity.[14] These observations underline the importance of monitoring the bioavailable

628 contamination using PES for accurate risk assessment of the real exposure
629 situation.

630 As recently pointed out by Brack et al.,[43] assessing the current status and
631 pollution potential of sediments is extremely important to judge the
632 environmental status of river basins according to the European Water
633 Framework Directive (WFD). In many freshwater and coastal areas, the sediment
634 may strongly influence the degree of contamination of the water phase. The
635 chemical status determined under the WFD is driven by comparison of
636 environmental concentrations of single priority chemicals (in total 42) to risk-
637 based environmental quality standards, thus excluding both potential effects of
638 the mixture, and contributions of the multitude of chemicals that are not on the
639 priority list. Including effect-based assessments in combination with passive
640 sampling techniques as demonstrated in this study would allow for a more
641 holistic and environmentally relevant approach.

642 The presented work covers the screening of a wide range of endpoints in cell-
643 based reporter gene bioassays after dosing of sediment extracts collected across
644 a range of pristine, remote vs. polluted, urban areas covering different types of
645 pollution sources and degrees. A next step could be to combine bioanalytical data
646 with results from chemical analytical profiling with the aim of identifying those
647 chemicals that explain a major part of the observed effect, as has been done, e.g.,
648 for water samples[31, 44, 33, 45] and to quantify the contribution of the unidentified
649 mixture to the total effect. Another option is to apply effect-directed analyses in
650 cases where single chemicals are expected to be responsible for the mixture
651 effects[46, 47, 48], which is the case, e.g., at sites of known contamination.

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ADDITIONAL MATERIAL

The *Supporting Information* provides additional details about the sampling sites and procedures, the bioassays and gives raw data compilations, whereas the *Supplementary Material* shows the concentration-effect curves for all samples in the applied battery of cell-based bioassays.

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