Estrogenic Activity in Sediments from European Mountain Lakes

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Estrogenic Activity in Sediments from European Mountain Lakes

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Superficial and bottom sediment samples from 83 European mountain lakes, ranging from Norway to the Pyrenees and East Europe, were tested for estrogenic compounds by the recombinant yeast assay. The results showed widespread potential estrogenic activity arriving at remote lakes. Tatra Mountains (Slovakia) and Scotland Highlands were the regions with the highest prevalence of lakes with high estrogenic values. Comparison of the estrogenic activity in the superficial layer of sediments with pre-industrial age sections showed that estrogenic compounds were predominantly deposited in recent times. Chemical analysis showed that highly estrogenic sediments were significantly enriched in both polycyclic aromatic hydrocarbons (PAH) and organochlorine compounds. For PAH, enrichment ratios in highly estrogenic samples versus nonestrogenic ones were inversely correlated with the vapor pressure value for each compound, indicating a significant relationship between estrogenicity and accumulation of less volatile PAH. Two PAH of predominantly diagenetic origin, retene and perylene, did not show specific enrichment in estrogenic samples. Principal component analysis revealed a strong correlation between estrogenic activity and the presence of contaminants of anthropogenic origin. These data reveal significant amounts of estrogenic compounds in remote lakes, relate them to the overall human activity, and suggest that they may affect organisms inhabiting these ecosystems.

Introduction

High mountain lakes can be used as monitoring sites for the assessment of long-range transported contamination. They are situated far away from direct human impact, and their water supplies originate from rain and snow so that they act as passive collectors of airborne anthropogenic chemical substances. Accordingly, chlorinated organic compounds (OCs), such as pesticides and polychlorobiphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAH) have been found in high mountain lakes at concentrations that were too high for their presumed conditions of pristine environments (1–3). Accumulation of OCs in remote areas is related to the so-called global distillation effect, involving the migration of these semi-volatile compounds from temperate areas to cold sites, where they become trapped (3, 4). On the other hand, PAH may also travel over long distances, currently in association with soot or other large carbonaceous particles (5), which can eventually be deposited in high mountain ecosystems.

Among environmental pollutants, endocrine-disrupting chemicals (EDCs) deserve specific attention. They are able to penetrate into exposed biota and alter their endocrine system by mimicking or counteracting natural hormones (6). Their effects range from sterility to mental deficiencies and to a variety of development defects (7, 8). They may be metabolically active at extremely low concentrations, sometimes at the nanomolar range. A wide range of chemical substances may act as endocrine disruptors, including synthetic and natural hormones, plant metabolites, pesticides and herbicides, PCBs and some of their derivatives, and many aromatic compounds (9–11). EDCs are therefore present in nearly all kinds of water bodies, including rivers (12, 13), lakes (14–16), and estuaries (17). The occurrence and concentration of non-natural EDCs is an excellent marker of human impact and a good indicator of water quality.

EDCs can be detected either by direct chemical characterization or, indirectly, by monitoring their biological effects. In water bodies with stable fauna, biomarkers such as egg protein precursors in males can be used to detect EDC exposure (18, 19). Alternatively, the organic content of water or sediments can be extracted and analyzed for EDCs using adequate bioassays (12, 13). This approach overcomes the dependence from local biota, which in some areas may be scarce, nonexistent, or simply not available for study.

The recombinant yeast assay (RYA) is one of the most convenient functional assays to evaluate the potential for endocrine disruption of a substance or an environmental sample (10, 12, 20). The version used here targets those EDCs that mimic the female steroid hormone estradiol. This subclass of EDCs is especially relevant both in terms of environmental quality and public health (21). The setup of this assay consists of an engineered yeast strain that harbors two foreign genetic elements, one acting as a sensor and the other acting as a reporter. The sensor element consists of a vertebrate receptor (in our case, a human estrogen receptor, ER) to which estrogenic EDCs bind with high to medium affinity. The reporter gene codifies a product whose concentration is easy to quantify and whose genetic expression is made dependent on binding of estrogens to the ER. This is a simplified version of the mechanism by which natural estrogens operate in vertebrates. The fundamental similarity of all eukaryotes ensures that it also works in yeast in a similar way.

In the present study, RYA has been used to detect estrogenic compounds in organic extracts of sediments from 83 European mountain lakes, spanning from the Scandinavian mountains to the Pyrenees and East Europe (Table 1). These lakes were selected to be above the local tree line, far from direct anthropogenic influence, and only receiving rain and snow as water inputs. The levels of estrogenic activity between the top and bottom sections of sediment columns have been compared in order to evaluate the difference between present times and pre-industrial ages. Finally, the estrogenicity values in sediments have been compared with their composition in semi-volatile organic pollutants, namely, OCs and PAH for assessment of possible anthropogenic chemical origins of the observed activities.
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* Median and range of at least three replicates. bdl, below detection limit (1 pg/g, EEQ).
FIGURE 1. Estrogenicity in top sediments from European mountain lakes. Lake codes as in Table 1. Graphs represent frequencies of lakes belonging to three estrogenicity classes as defined by the CLUSTER: A, nonestrogenic (white); B, low estrogenicity (light gray); and C, highly estrogenic (dark gray). Approximate sampling areas are indicated in gray.

TABLE 2. Estrogenicity Classes Defined by the CLUSTER Program

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<td>median</td>
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<td>34.8</td>
<td>$1.9 \times 10^4$</td>
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*Maximal, minimal, and median values in pg/g EEQ. bdl, below detection limits.

Material and Methods

**Sampling.** Sediment cores were obtained using a gravity corer (Glew, 7.5 cm diameter, 30 cm length) in the deepest areas of the lakes selected for study. Two sediment sections were obtained in the field, the top layer (0–0.5 cm) and a bottom sample (10–12 cm). They were wrapped in pre-rinsed aluminum foil and stored at −20 °C until analysis.

**Materials.** Residue analysis n-hexane, dichloromethane, isooctane, methanol, and KOH were from Merck (Darmstadt, Germany). Neutral aluminum oxide type 507C was from Fluka AG (Buchs, Switzerland). Aluminum oxide was cleaned by Soxhlet extraction with dichloromethane:methanol (2:1, v/v) during 24 h and activated overnight at 120 °C before use. KOH pellets were cleaned by sonication with hexane. PCB congeners, HCB, 4,4'-DDT, Aroclor 1260, and EPA 16 PAH standard mixtures were from Dr. Ehrenstorfer (Augsburg, Germany).

**Yeast Strains and Plasmids.** Yeast strain BY4741 (MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0) was obtained from EURO-SCARF (Frankfurt, Germany). Expression plasmid pH5HE0 contains the human estrogen hormone receptor HE0 cloned into the constitutive yeast expression vector pAAH5 (12).

Plasmid pVITB2x contains two copies of the pseudopalindromic estrogen responsive element ERE2 from *X. laevis* vitellogenine B1 gene (5'-AGTCAGCTGACG-3') inserted into the unique *KpnI* site of pSFLA-178K (12). Yeast manipulations, including transformation by the LiAc method, were performed as described in ref 22.

**Tests with Standards.** Specific endocrine activities of individual OCs, Aroclor 1260 and EPA 16 PAH standard mixture diluted in methanol were analyzed by serial dilutions as indicated above. The resulting dose–response curves were assumed to follow the classical equation for ligand–receptor equilibrium:

$$\frac{R}{R_{\text{max}}} = \frac{1}{1 + \frac{K_d}{[H]}}$$

where $R$ and $R_{\text{max}}$ represent the value of the response in β-galactosidase units at specific ([H]) and saturating ligand concentrations, respectively. $K_d$ is the dissociation constant of the ligand–hormone complex, its apparent value coinciding with $EC_{50}$. These different parameters were calculated by nonlinear regression methods, using data from three independent experiments, each one representing seven dilution points.

**Sediment Extraction.** Wet samples were extracted by sonication with methanol (20 mL; 20 min) to separate the interstitial water. Subsequent extractions were performed with dichloromethane:methanol (2:1; 3 × 20 mL; 20 min). The combined extracts to be analyzed chemically were spiked with a mixture of surrogate standards, PCB 30, PCB 209, anthracene-$d_{10}$, pyrene-$d_{12}$, benz[a]anthracene-$d_{12}$, and benzo[ghi]perylene-$d_{12}$. Then, they were vacuum evaporated to 10 mL and hydrolyzed overnight with 20 mL of 6% KOH in methanol. The neutral fraction was recovered with n-hexane (3 × 10 mL), concentrated by vacuum rotatory evaporation to approximately 500 μL, and to 100 μL under a gentle stream of nitrogen. Samples for chemical analysis were further fractionated by column chromatography with aluminum.
oxide as described elsewhere (1, 3). Two fractions were collected. The first by elution with 5 mL of n-hexane: dichloromethane (95:5), mostly containing OCs, including PCBs, hexachlorobenzene (HCB), and DDTs, and the second with 10 mL of n-hexane:dichloromethane (1:2), mostly containing PAH, from fluorene (Flu) to coronene (Cor). In the first fraction sulfur was removed with activated copper. Both fractions were vacuum and nitrogen concentrated to almost dryness; 50 μL of internal standard mixture containing tetrachloronaphthalene and perylene-d12 were added prior to instrumental analysis. Extracted sediment samples were lyophilized for dry weight determination. Samples for RYA analysis were treated similarly, except that no surrogate standards were added and total extract was dissolved in 200 μL of methanol. For total organic carbon (TOC) analysis, sediment samples were treated with HCl to remove inorganic carbon, neutralized, and dried at 60 °C. TOC determination was performed by flash combustion at 1025 °C followed by thermal conductivity detection in a CHNS elemental analyzer EA1108. Detection limit was 1 mg/g.

### Instrumental Analysis
OCs analysis was carried out by flash combustion at 1025 °C followed by thermal conductivity detection in a CHNS (DIFCO, Basel, Switzerland) gas chromatograph with electron capture detection (Hewlett-Packard model HP-5890). Detailed description of chromatographic conditions is given elsewhere (3). Compound identification was additionally confirmed by gas chromatography coupled to mass spectrometry, operating in the negative ion chemical ionization mode (GC–MS–NICI), using NH3 as reagent gas (23). PAH were quantified by gas chromatography coupled to mass spectrometry (GC–MS) operating in electron impact and selective ion monitoring modes. A 30 m × 0.25 mm i.d. HP-5MS capillary column (film thickness of 0.25 μm) was used. Additional information on the chromatographic and mass spectrometric conditions is provided elsewhere (1).

Quantification was performed by the internal standard method, the response factors being referred to the internal standard mixture. Compounds lacking reference standard were quantified using the response factor of the standard exhibiting the closest retention time. Reported values were corrected by blank levels and surrogate recoveries (1, 2, 24).

### Estrogenic Activity Test
RYA assays using hydrolyzed sediment extracts in methanol were performed as described elsewhere (12) after some modifications. Briefly, yeast strain BY4741 was transformed with plasmids pHisHE and pVT2B2x and grown overnight in glass culture tubes. Growth was first performed in nonselective medium (YPD, 5 g/L yeast extract, 10 g/L peptone, 20 g/L glucose–PRONADISA, Madrid, Spain) and then in minimal medium (SD, 6.7 g/L yeast nitrogen base without amino acids–DIFCO, Basel, Switzerland—together with 20 g/L glucose, supplemented with 0.1 g/L of prototrophic markers as required). The final culture was adjusted to an optical density (O.D.) of 0.5 and split into 45

### Table 4: Mean Concentrations of Different Analytes in Sediment Samples from European Mountain Lakes, both Aggregated and Distributed in Estrogenicity Classes

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<tr>
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<tr>
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* Levene’s test, 95% confidence level. ** 2 degrees of freedom (df), only parameters with homologous variances. 3 df, parameters with heterologous variances. 4 Confidence levels: *, > 95%; **, > 99%; ***, > 99.9%. 5 SD, standard deviation. 6 PAH abbreviations as in Figure 3. 7 Sum of all PAH except Ret and Per.
Incubation at 30 °C overnight for each sample. Some samples required dilutions up to 1:1250 (in 5-fold steps) were performed to achieve the chromogenic substrate activity. Serial dilutions of 5 µL aliquots for analysis in 300 µL glass tubes baked at 40 °C overnight. Serial dilutions of 5 µL of extract in 45 µL of culture from 1:10 to 1:1250 (in 5-fold steps) were performed for each sample. Some samples required dilutions up to 1:26250 due to their high estrogenic potential. After overnight incubation at 30 °C, the estrogen-dependent expression of the β-galactosidase gene was measured by hydrolysis of the chromogenic substrate o-nitrophenyl-β-D-galactopyranoside (ONPG, from Sigma-Aldrich, St. Louis, MO). RYA provides measurements of estrogenic activity and not of molar (or mass) concentrations of endocrine disruptors. For simplification, results were calculated as estradiol equivalents (EEQ) defined as the amount of estradiol that should be present to account for all the observed response in a given sample. This was calculated from the lowest dilution in which the β-galactosidase activity was indistinguishable from that of the control. From a series of experiments using mock extracts (10 assays in total), we established a threshold equivalent to 140% of the β-galactosidase value for the negative controls in each set of experiments. Values for each sample were calculated from at least three independent serial dilutions (3.8 replicates per sample on average). The estrogenic activity in this lowest active dilution was considered to be equivalent to the detection limit for estradiol (10 pM in these conditions; 12). This value was then corrected for the dry weight corresponding to the initial inoculum (in g) to give the final equivalency in pg/g of EEQ.

Results and Discussion

Fig. 2. Dose–response curves for Arochlor 1260 and EPA PAH standard mixture. Dots represent individual RYA measurements; lines indicate theoretical dose–response equations.

Stratification of the Observed Estrogenic Activity. Comparison of the estrogenic values in the top and bottom sediment sections of a subset of 25 lakes (Table 3) show a much higher proportion of estrogenic samples in top sections as compared to bottom sections. The difference of estrogenicity between top and bottom values is statistically significant (Wilcoxon test for non-normal distributions, \( p = 1.5 \times 10^{-3} \)). As determined in previous studies (2), the deep core sections correspond to pre-industrial periods. The observed increases of estrogenic activity in the top cores must therefore be related with the overall human impact on these remote ecosystems as consequence of industrial and human development in the 20th century. The single bottom lake sediment section exhibiting relatively high estrogenic activity (PT1055, 725 pg/g EEQ, Table 3) was topped by a surface sediment section of very high estrogenicity (2.7 \( \times 10^4 \) pg/g EEQ). Thus, it is not unlikely that, in this case, the deep sediment sample may have received influences from the estrogenic pollutants accumulated in the top section due to vertical post-depositional transport or burrowing.

Chemical Contamination in Estrogenic and Nonestrogenic Sediment Samples. The concentration of several pollutant types in top core samples with high, low and nonestrogenic activity (Table 3), namely, PCBs, hexachlorobenzene (HCB), p,p'-dichlorodiphenyldichloroethylene (p,p'DDE), and PAH, are shown in Table 4. Most analytes show a higher concentration in the high estrogenicity class than in the nonestrogenic or low-estrogenicity classes. For example, samples in the high-estrogenicity class contain, on average, four times more PAH than the nonestrogenic samples (Table 4). To assess whether these differences were statistically significant, tests for homogeneous and inhomogeneous variances (Levene’s test) were used. The parametric ANOVA test, which requires variance homogeneity, showed no significant differences between estrogenicity classes for TOC and some PAHs, including the two most volatile compounds, fluorene (Flu) and anthracene (Anthr) as well as retene (Ret) and perylene (Per). However, most piparameters showed no variance homogeneity among estrogenicity classes and required the nonparametric
Kruskal–Willis test for assessing significant differences. According to this last test, many PAHs were significantly enriched in the high estrogenicity class as well as some OCs, particularly PCB 118, PCB 138, and PCB 90 + PCB 101 (Table 4). The aggregated values of all PAH and all PCB (sum PAH and sum PCBs in Table 4) also show a significant enrichment in estrogenic samples.

**PAH and Estrogenicity.** The dose–response curve for the EPA 16 PAH standard mixture is shown in Figure 2. Precise determination of EC50 requires reaching saturating concentrations of the ligand (13) but this was not possible, probably due to the inherent toxicity of the compounds. The limit of detection (LOD) for the PAH mixture, defined as the concentration at which the β-galactosidase response reached 140% of the basal level, was calculated instead. The observed LOD for this mixture of hydrocarbons, 320 μg/L (Figure 2, top), was similar to those calculated for other weak ER ligands, such as etoxylated nonylphenol (LOD = 750 μg/L) or bisphenol A (LOD = 490 μg/L; 13).

The distribution of individual PAH in the three estrogenicity classes, normalized by TOC, is shown in Figure 3. These profiles are similar to those commonly observed in the European high mountain lakes (1) and reflect an origin from pyrolytic sources. Retene and perylene stand out as the only compounds not exhibiting a significant increase when comparing samples with low to high estrogenic activities (classes A and C). High perylene concentrations in high mountain lakes are related to diagenetic processes, although this hydrocarbon may also originate from combustion processes (1). In the present case, this discrepant behavior in relation to the other PAH points to a predominantly diagenetic origin. This observation, combined with the results from PCA (see below), gives further grounds to an association of the measured estrogenic values to anthropogenic inputs.

Enrichment ratios between average PAH concentrations in high estrogenicity and nonestrogenic samples show values varying between 2.2 (Flu) and 10 (Cor). These ratios display a significant linear correlation ($r^2 = 0.65$) with the log-transformed subcooled vapor pressure of PAH (Figure 4). Thus, estrogenic samples are progressively enriched in the less volatile PAH.

**OC and Estrogenicity.** As for the case of EPA 16 PAH mixture, the toxicity of Arochlor 1260 did not allow reaching saturating concentrations in dose–response curves for calculation of EC50 (Figure 2, bottom). The LOD for this PCB mixture was calculated at 170 μg/L, which is similar to the above-described value for the EPA 16 PAH mixture or other weak ER ligands (13).

Although many OCs show high average concentrations in highly estrogenic samples and low concentrations in nonestrogenic or low-estrogenicity samples, the differences are only significant for a reduced number of PCB (Table 3). The enrichment ratios between no and highly estrogenic samples ranged between 2.4 (PCB 28) and 5.8 (PCB 52). These values are lower than those observed for individual PAH. In this case no correlation between enrichment ratios and vapor pressures was found (Figure 4).

Comparison of the measured LOD for Arochlor 1260 and EPA 16 PAH standard mixture (Figure 2) to the observed pollutant concentration in the European high mountain lake sediments suggests that only the most contaminated lakes show POP levels high enough to explain the observed values. Giving the dilution factor inherent to the extraction and RYA procedures (approximately 1 g of sediment results in 200 μL...
of organic extract, which is tested at 1/10 dilution at most), the limit of detection of these compounds is around 500 ng/g of sediment. This value essentially agrees with the actual concentrations of PAHs, whose combined values exceed 10000 ng/g in several lakes and whose average concentrations in the class C samples is around 4000 ng/L (Table 4). In contrast, the maximum concentrations for individual PCB congeners do not exceed 15 ng/g in any case. Extrapolation of these values to PCB mixtures currently encountered in high-altitude lake sediments gives rise to total PCB concentrations around 300–400 ng/g at the most, which is still clearly below the values obtained for PAH. These data suggest that PAH may be more relevant than PCBs to explain the estrogenicity values observed in the lake sediments. In any case, the detected analyte concentrations seem not high enough to explain the estrogenicity data. The occurrence of unidentified pollutants having estrogenic activities similar to or even higher than the standard mixtures tested in this study cannot be discarded.

**Linear Correlations and Principal Component Analysis.**

Examination of the linear correlations between estrogenicity values and OC and PAH concentrations gives further ground to the significance of compound origin. Thus, PCB, HCB, pp’DDE, and total PAH except retene and perylene (the compounds related to anthropogenic activity) exhibit significant correlations to estrogenicity, with $p$ values between $10^{-5}$ and $10^{-6}$ (Table 5). Conversely, the compounds related to natural sources (e.g., TOC, retene, and perylene) did not show significant correlations with estrogenicity.

PCA analysis shows consistent results with those described above. The first component (45% of total variance) has a strong contribution from human-related contaminants, such as PCBs, pp’DDE, HCB, and PAHs (excluding retene and perylene) (Figure 5), and explains most of the variability in estrogenicity values (EEQ in Figure 5). The second component (18% of total variance) shows a strong contribution of retene and perylene, and the third component (13% of total variance) is strongly related to TOC. Estrogenicity shows a very low contribution to the second component and a negative correlation with TOC in the third component.

**Estrogenicity and Modes of Pollutant Transport.**

The present results can also be related to the transport mechanism of these pollutants to high mountain lakes. OC are predominantly found in the gas phase ($27–30$). Their incorporation into high mountain lakes is mediated by cold trapping from the gas phase, which, as observed in studies encompassing fish ($3,31$) and sedimentary inventories ($3$), occurs more effectively among the low vapor pressure compounds. In contrast, PAH are long-range transported through the atmosphere, mostly in association with particles. Their distribution between gas and particle phase in the atmosphere is compound dependent, with increasing association to particulate matter at decreasing vapor pressure ($25,26$).

The significant linear correlation between vapor pressures of the individual hydrocarbons and PAH enrichment in high versus no estrogenic samples diverges from the lack of vapor...
pressure dependence in the enrichment of the cold condensation trapped OC (Figure 4). This disparity probably reflects that the largest portion of the observed estrogenicity corresponds to pollutants air transported to the mountain lake sediments in association to particles. In addition, the large abundance of highly estrogenic lakes in the Tatra Mountains suggests that the observed RYA activity could be mostly related to pollutant compounds (including PAH) transported to high mountain lakes by similar particle-associated mechanisms such as those of PAH.

In any case, the agreement between high concentrations of organic pollutants such as OC and PAH and high estrogenic levels is consistent with the overall enhancement of atmospheric contamination over Europe after the industrial and urban development.

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FIGURE 5. PCA loading plot. The amount of total variability explained by each component is indicated in parentheses. PAH-Ret-Per represents the sum of all PAH except retene and perylene.
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Literature Cited


