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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 binds 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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TABLE 1. Location and Sedimentary Estrogenic Activity of the Lakes Included in This Study[a]

lake code	lake name	latitude N	longitude E	altitude (m)	mean	max	min	lake code	lake name	latitude N	longitude E	altitude (m)	mean	max	min
							Central Norway								
CN0001	Vonavatn	61.6131	6.0054	466	bdl			CN0012	Grønevatnet	61.2011	8.0790	1184	bdl		
CN0002	Vestre Kvammavatn	60.9004	6.1556	990	54	590	23	CN0013	Kvitevatnet	61.3957	8.1781	1396	bdl		
CN0003	Slondalsvatn	60.6921	6.9489	751	8	20	4.0	CN0014	Leirvatnet	61.5478	8.2493	1401	$7.9\cdot10^4$	$1.8\cdot10^5$	$3.5\cdot10^4$
CN0004	Halsavatnet	61.2793	7.0656	820	bdl			CN0015	Øvre Heimdalsvatnet	61.4188	8.8970	1088	bdl		
CN0005	Litlosvatn	60.0738	7.1708	1172	50	680	27	CN0016	Øvre Neådalsvatn	62.7778	8.9824	728	bdl		
CN0006	Valgardsvatn	60.1028	7.3184	1324	120	750	30	CN0017	Fallbekktjørna	62.7500	9.0372	1043	bdl		
CN0007	Hornsvatnet	60.9501	7.3625	1289	24	87	17	CN0018	Høvringvatn	61.8898	9.5650	1121	bdl		
CN0008	Dargesjåen	60.0844	7.5890	1209	bdl			CN0019	Lille Innsjøen	62.5522	10.2659	938	23	91	18
CN0009	Skiftesjøen	60.3842	7.5797	1239	44	400	16	CN0020	Haltdalstjønna	62.7743	11.0670	1058	bdl		
CN0010	Holmavatnet	60.8420	7.6514	1525	$1.6\cdot10^3$	$2.9\cdot10^3$	590	CN0022	Øvlingen	62.9029	11.7848	844	bdl		
CN0011	Urdevatn	59.9734	7.7120	1329	$8.6\cdot10^3$	$4.8\cdot10^4$	$1.9\cdot10^3$	SN0023	Stavsvatn	59.6350	8.1100	1053	12	420	3.3
							Scotland								
SC0002	Coir' á Ghrunnda	57.2019	−6.2211	750	$7.7\cdot10^3$	$1.7\cdot10^4$	$3.4\cdot10^3$	SC0189	an Fhuar-thuill Mhoir	57.4524	−4.9365	770	23	830	7
SC0108		57.1429	−5.2871	720	150	360	71	SC0190	Gorm	57.6813	−4.9544	540	20	820	7
SC0153	Beag	57.3720	−5.0845	660	25	$2.1\cdot10^3$	17	SC0197	á Choire Dhairg	58.1993	−4.9764	530	$3.0\cdot10^4$	$6.7\cdot10^4$	$1.3\cdot10^4$
SC0165	á Mhadaidh	57.7126	−5.0244	570	$1.8\cdot10^4$	$4.2\cdot10^4$	$8.3\cdot10^3$	SC0399	Lochnagar	56.9591	−3.2313	790	$9.6\cdot10^3$	$2.2\cdot10^4$	$4.5\cdot10^4$
							Tatra Mountains								
TA0007	Zielony Staw Gasienicowy	49.2289	20.0010	1672	bdl			TA0011	Nizné Terianske	49.1698	20.0143	1941	37	95	19
TA0009	Dlugi Staw Gasienicowy	49.2273	20.0107	1784	$1.4\cdot10^5$	$3.1\cdot10^5$	$6.2\cdot10^4$	TA0047	Starolesnianske	49.1800	20.1678	1986	$2.6\cdot10^5$	$5.8\cdot10^5$	$1.2\cdot10^5$
							Pyrenees								
PY0080	Pondiellos sup.	42.7770	−0.2631	2745	bdl			PY0390	Llosás	42.6177	0.6548	2480	240	540	110
PY0194	Helado de Marboré	42.6966	0.0410	2592	5	12	2	PY0423	Redon	42.6421	0.7795	2235	6	16	3
PY0228	La Munia Sup.	42.7062	0.1250	2537	3	7	1	PY0569	Gelat Bergús	42.5911	0.9633	2493	bdl		
							Julian Alps								
JA0003	Zeleno	46.3514	13.7992	1983	260	580	120	JA0008	na Planini jezero	46.3111	13.8322	1430	130	320	65
JA0004	V Ledvicah	46.3403	13.7867	1830	74	170	33	JA0009	Dupeljsko	46.2892	13.7028	1340	61	140	27
JA0005	Dvojno (5.)	46.3175	13.7842	1669	65	170	33	JA0010	Krnsko	46.2858	13.6856	1383	9	20	4
JA0007	Csrno	46.2989	13.8000	1325	53	130	26	JA0014	Spodnje Kricsko	46.3997	13.8067	1880	31	770	6
							Piedmont								
PT0007	Grande	46.0028	8.0783	2269	bdl			PT1011	Crosa	46.3703	8.4826	2153	65	140	29
PT0009	Sfondato	46.0069	8.0878	2422	bdl			PT1014	Antabia	46.3865	8.4923	2189	54	120	24
PT0016	Campo	46.1294	8.1306	2293	43	83	17	PT1031	Nero	46.4495	8.5408	2387	bdl		
PT0026	Paione inferiore	46.1689	8.1908	2002	4	65	3	PT1040	Froda	46.4406	8.5594	2363	$3.5\cdot10^5$	$7.8\cdot10^5$	$1.6\cdot10^5$
PT0027	Paione superiore	46.1758	8.1908	2269	bdl			PT1051	Sascòla	46.2844	8.5708	1740	bdl		
PT0028	Paione di mezzo	46.1722	8.1919	2147	8	13	3	PT1053	Laghetto supp.	46.4767	8.5866	2128	$2.7\cdot10^5$	$6.1\cdot10^5$	$1.2\cdot10^5$
PT0029	Capezzone	45.9406	8.2100	2100	92	150	31	PT1055	Laghetto inf.	46.4770	8.5945	2074	$1.5\cdot10^3$	$3.3\cdot10^4$	260
PT0030	Variola	46.1800	8.2117	2190	28	290	11	PT1068	Mognòla	46.4311	8.6896	2003	bdl		
PT0041	Pojala	46.3294	8.3347	2305	bdl			PT1070	Tomè	46.3642	8.6907	1692	bdl		
PT0051	Matogno	46.2508	8.4014	2087	230	510	100	PT1084	Porchieirsc	46.3769	8.7451	2190	18	47	9
PT0058	Boden	46.4422	8.4531	2334	68	530	21	PT1085	Barone	46.4036	8.7529	2391	32	83	17
PT0059	Boden	46.4389	8.4533	2343	bdl			PT1087	Gardiscio	46.4288	8.7572	2580	72	560	22
PT0060	Panelatte	46.2028	8.4581	2063	$2.8\cdot10^3$	$6.2\cdot10^3$	$1.2\cdot10^3$	PT1093	Starlaresc da Sgiof	46.2757	8.7773	1875	24	61	12
							Tyrol								
TY0043	Rasasser See	46.7462	10.4555	2682	39	240	10	TY0237	Timmelsschwarzsee	46.9278	11.1627	2514	65	170	33
TY0189	Rotfelssee	47.2265	11.0080	2485	23	106	4	TY0270	Kratzbergersee	46.7046	11.2857	2119	31	80	16
TY0194	Plenderlessee Ob.	47.1988	11.0381	2344	bdl			TY0351	Schwarzsee Pojental	46.9555	12.0092	2551	bdl		
TY0195	Plenderlessee mitt.	47.2047	11.0416	2317	22	65	13	TY0362	Klammsee	46.9818	12.1280	2258	bdl		
TY0207	Milchsee	46.7257	11.0724	2540	11	21	4								

[a] Median and range of at least three replicates. bdl, below detection limit (1 pg/g, EEQ).

TABLE 2. Estrogenicity Classes Defined by the CLUSTER Program[a]

	estrogenicity classes		
	A	B	C
sample number	26	44	13
maximal value[a]	bdl	260	3.5×10^5
minimal value	bdl	2.7	1.6×10^3
median	bdl	34.8	1.9×10^4

[a] Maximal, minimal, and median values in pg/g EEQ. bdl, below detection limits.

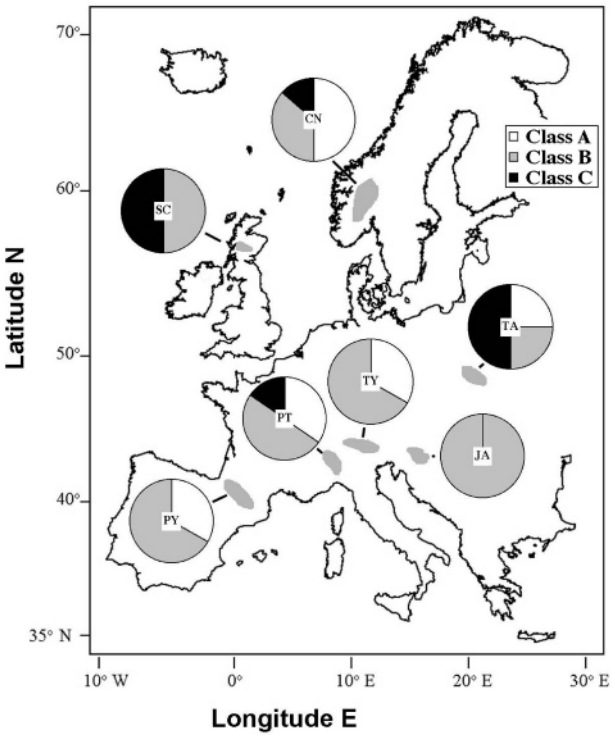


FIGURE 1. Estrogenicity in top sediments from European mountain lakes. Lake codes as in Table 1. Graphs represent frequencies of lakes belonging to three estrogenicity classes as defined by the CLUSTER: A, nonestrogenic (white); B, low estrogenicity (light gray); and C, highly estrogenic (dark gray). Approximate sampling areas are indicated in gray.

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 oxyde 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, Arochlor 1260, and EPA 16 PAH standards were from Dr. Ehrenstorfer (Ausburg, Germany).

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

TABLE 3. Estrogenicity of Top and Bottom Sediment Samples in Different European Mountain Lakes[a]

lake	top (0−0.5 cm)	bottom (10−15 cm)
CN0006	120	bdl
CN0009	44	bdl
CN0011	$8.7 \cdot 10^3$	bdl
CN0015	bdl	bdl
CN0016	bdl	13
CN0017	bdl	bdl
PT0007	bdl	3.1
PT0009	bdl	bdl
PT0016	43	bdl
PT0026	4.3	10
PT0027	bdl	bdl
PT1040	$3.5 \cdot 10^5$	13
PT1053	$2.7 \cdot 10^5$	720
PT1055	$1.6 \cdot 10^3$	bdl
PT1070	bdl	17
PY0423	9.7	1.8
TY0043	39	bdl
TY0170	87	bdl
TY0189	23	bdl
TY0194	bdl	bdl
TY0195	22	bdl
TY0207	11	bdl
TY0237	65	bdl
TY0270	31	19
TY0362	bdl	bdl

[a] Lake codes as in Table 1. bdl, below detection limit (1 pg/g EEQ, approximately).

Plasmid pVITB2x contains two copies of the pseudopalindromic estrogen responsive element ERE2 from *X. laevis* vitellogenine B1 gene (5′-AGTCACTGTGACC-3′) inserted into the unique *Kpn*I site of pSFLΔ-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, Arochlor 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:

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

where R and R_{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_{10}, 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

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

	total		estrogenicity class						statistical tests					
			A		B		C		variance homogeneity[a]	ANOVA[b]		Kruskal-Willis[c]		
	mean	SD[e]	mean	SD	mean	SD	mean	SD		F-test	σ	χ^2	σ (assintotic)	signif.[d]
TOC (mg/g)	64	48	56	44	66	51	72	48	+	0.57	0.57			
PAHs (ng/g)														
Flu	5.8	6.2	3.8	4.4	6.1	6.5	8.7	7.2	+	2.9	0.06			
Anthr	71	8.2	5.7	8.1	5.9	5	13	13	−			12	0.003	**
Phen	67	90	42	52	58	54	150	180	−			15	5.0×10^{-4}	***
MethylPhe[f]	35	46	17	16	34	42	72	72	−			16	3.0×10^{-4}	***
Fla	170	280	100	160	140	170	410	550	−			12	0.0023	
Ace	6.8	8.6	4.2	4.3	5.5	5.3	16	16	−			13	1.5×10^{-3}	**
Pyr	110	180	71	98	100	120	240	350	-			9	0.011	
DimethylPhe	37	76	23	46	40	93	56	52	+	0.9	0.41			
B[ghi]Fla	51	77	24	30	44	59	130	130	−			15	6.5×10^{-4}	***
Cyclop[cd]pyr	10	15	6.6	5.7	9.4	10	24	31	−			5.4	0.066	
B[a]A	43	65	27	38	34	39	100	130	−			11	0.004	**
Chrys+Triphe	170	280	89	120	140	140	460	560	−			14	9.3×10^{-4}	***
Ret	9.8	36	7.8	19	13	47	2.4	1.9	+	0.5	0.61			
BFlas	400	600	210	240	330	370	1000	1100	−			16	4.2×10^{-4}	***
B[e]Pyr	150	210	76	86	120	140	360	400	−			15	6.9×10^{-4}	***
B[a]Pyr	64	110	36	38	50	48	160	230	−			9.3	0.0096	*
Per	140	280	190	310	140	300	70	55	+			0.75	0.48	
I[7,1,2,3-cdef]Ch	60	77	32	34	55	69	130	120	−			16	4.3×10^{-4}	***
I[1,2,3-cd]Pyr	170	290	79	67	150	200	440	560	−			16	3.1×10^{-4}	***
B[ghi]Per	130	220	56	44	110	150	320	440	−			12	0.0021	**
DB[a,h]Ant	34	49	19	15	33	52	69	67	−			12	0.003	**
Cor	54	130	17	14	39	47	180	300	−			13	0.0018	**
PAHs-Ret-Per[g]	1800	2700	970	990	1500	1500	4300	5200	−			13	1.2×10^{-3}	***
sum PAHs	1900	2600	1100	1100	1600	1600	4400	5200	−			13	0.0018	**
HCB OCs (pg/g)	550	1300	380	410	320	250	1700	3000	−			5	0.079	
pp'DDE	3800	8000	2200	2100	3000	4400	9700	17000	−			5	0.081	
PCB 28+31	350	570	320	410	230	230	780	1200	−			4.7	0.095	
PCB 52	340	710	170	100	230	280	1000	1600	−			9	0.011	*
PCB 90+101	610	800	450	360	490	380	1400	1700	−			6.4	0.04	*
PCB 118	480	750	260	200	380	320	1200	1600	−			12	0.0026	**
PCB 138	970	1600	610	320	770	540	2400	3700	−			8.5	0.014	*
PCB 153	730	740	560	370	630	470	1400	1400	−			5.9	0.053	
PCB 180	790	1200	490	350	690	720	1700	2600	−			4.6	0.098	
sum PCBs	4300	5900	2900	1600	3400	2400	9900	1300	−			8.1	0.017	*

[a] Levene's test, 95% confidence level. [b] 2 degrees of freedom (df), only parameters with homologous variances. [c] 2 df, parameters with heterologous variances. [d] Confidence levels: *, >95%; **, >99%; ***, >99.9%. [e] SD, standard deviation. [f] PAH abbreviations as in Figure 3. [g] Sum of all PAH except Ret and Per.

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 almost to dryness; 50 μL of internal standard mixture containing tetrachloronaphthalene and perylene-d_{12} 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 gas chromatography 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 NH_3 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 pH5HE0 and pVITB2x 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

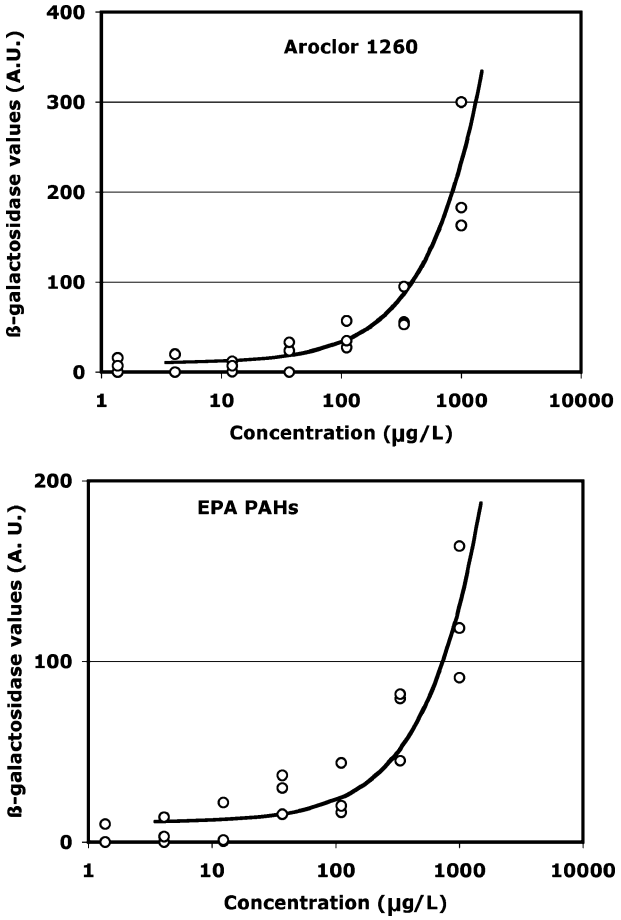


FIGURE 2. 2. Dose−response curves for Arochlor 1260 and EPA PAH standard mixture. Dots represent individual RYA measurements; lines indicate theoretical dose−response equations.

μL aliquots for analysis in 300 μL glass tubes baked at 400 °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.

Statistics. All calculations were performed using the SPSS v.10.0.6 package (SPSS Inc., Chicago, IL). Normality of distributions was checked by the Kolmogorov−Smirnov test. Principal component analysis (PCA) was performed using the Varimax method for orthogonal rotation of the resulting eigen vectors. Unless otherwise noted, significance levels were set at $p < 0.05$.

Results and Discussion

Estrogenic Activity of Sediment Samples across Europe. The estrogenicity values of all 83 top core sediment samples analyzed are shown in Table 1. Twenty-six samples showed no estrogenicity in the RYA at any dilution, indicating that their content in estrogenic compounds does not reach the limit of detection of the RYA test, which is estimated in 1 pg/g EEQ, approximately. On the contrary, a small subset of 13 samples show estrogenicity levels above the nanogram per gram EEQ, with maximal values as high as 350 ng/g EEQ. This wide range of estrogenic activities can be used to divide the samples into three estrogenicity classes with the CLUSTER program (Table 2). Further division into four or more categories result in one or more classes with low number of samples (5 or less). The geographical distribution of sediment samples falling into the three estrogenic classes is shown in Figure 1. Highly estrogenic samples (class C) occur in four out of the seven lake regions, with Scotland (SC) and the Tatra Mountains (TA) being those with the highest proportion of highly estrogenic lakes (50%, Figure 1). Previous studies have shown that the lake sediments in the Tatra Mountains stand out among the European high altitude lakes for their PAH concentrations, which are about 10−20 times higher than the average values of the other mountain ranges (*1*). Conversely, only nonestrogenic or low-estrogenicity samples (classes A and B, respectively) are found in Pyrenees (PY), Tyrol (TY), and Julian Alps (JA, Figure 1).

Stratification of the Observed Estrogenic Activity. Comparison of the estrogenicity 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 (2.7×10^5 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 (pp′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

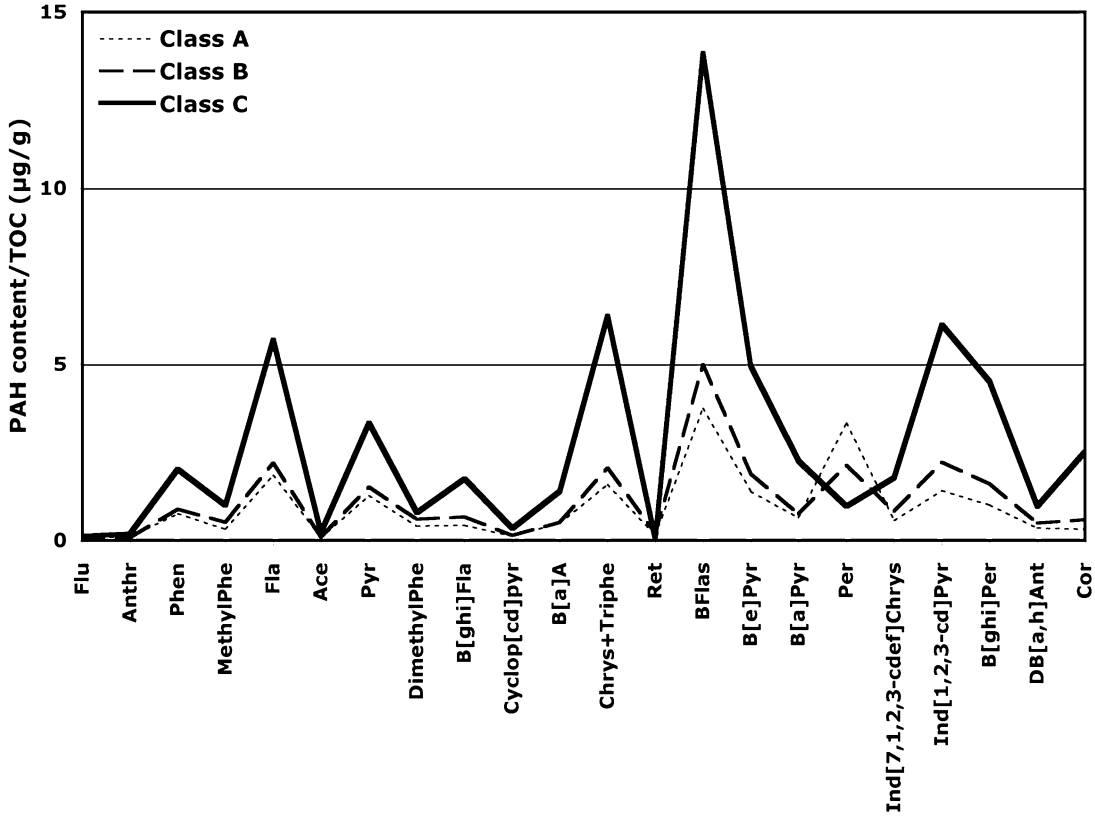


FIGURE 3. Averaged PAH content profiles for the three estrogenicity classes: A, thin dotted line; B, thick discontinuous line; and C, thick continuous line. Values are expressed as μg of each compound/g of TOC. PAH abbreviations: Flu, fluorene; Anthr, anthracene; Phe, phenanthrene; Methylphe, methylphenanthrene; Fla, fluoranthene; Ace, acephenanthylene; Pyr, pyrene; Dimethylphe, dimethylphenanthrene; Ret, retene; B(ghi)fla, benzo[*ghi*]fluoranthene; B(a)A, benz[*a*]anthracene; Chr+Triph, chrysene + triphenylene; Bflas, sum of different benzofluoranthenes; B(e)pyr, benzo[*e*]pyrene; B(a)pyr, benzo[*a*]pyrene; Per, perylene; Ind[1,2,3-cd]pyr, indeno[1,2,3-*cd*]pyrene; Ind[7,1,2,3-cdef]chrys, indeno[7,1,2,3-*cdef*]chrysene; B(ghi)per, benzo[*ghi*]perylene; DB(ah)a, dibenz[*ah*]anthracene; Cor, coronene.

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 EC_{50} 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*).

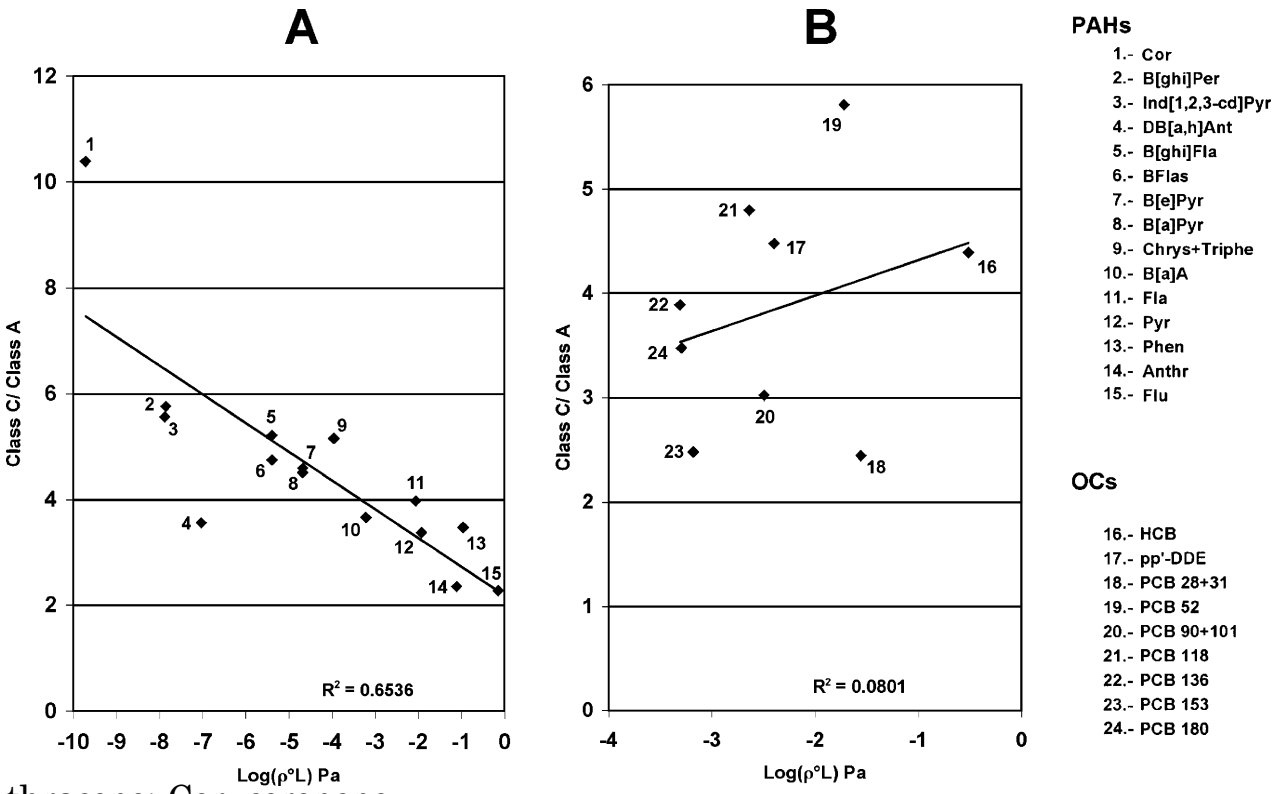
The distribution of individual PAH in the three estrogenicity classes, normalized by TOC, is shown in Figure 3. These profiles are similar to those commonly observed in the European high mountain lakes (*1*) and reflect an origin from pyrolytic sources. Retene and perylene stand out as the only compounds 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 estrogenicity 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 EC_{50} (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



thracene; Cor, coronene.

FIGURE 4. Linear correlation between the logarithm of vapor pressure (p_L°, in Pa) and enrichment factors for PAHs (A) and OC (B) Enrichment factors are defined from the ratios between average concentrations in the high estrogenicity C class and nonestrogenic A class groups. The values for Ret (0.30) and Per (0.38) are not included. Values for p_L° were obtained from refs *32* and *33*.

TABLE 5. Correlation Coefficients Between Estrogenicity and Different Compounds

parameters	correlation coefficient	σ (unilateral)
TOC	0.059	0.300
sum PCBs	0.483	3.7×10^{-6}
HCB	0.498	1.8×10^{-6}
p,p'-DDE	0.475	5.7×10^{-6}
PAHS except retene and perylene	0.448	1.9×10^{-5}
retene	-0.053	0.320
perylene	-0.059	0.310

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 (49% 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

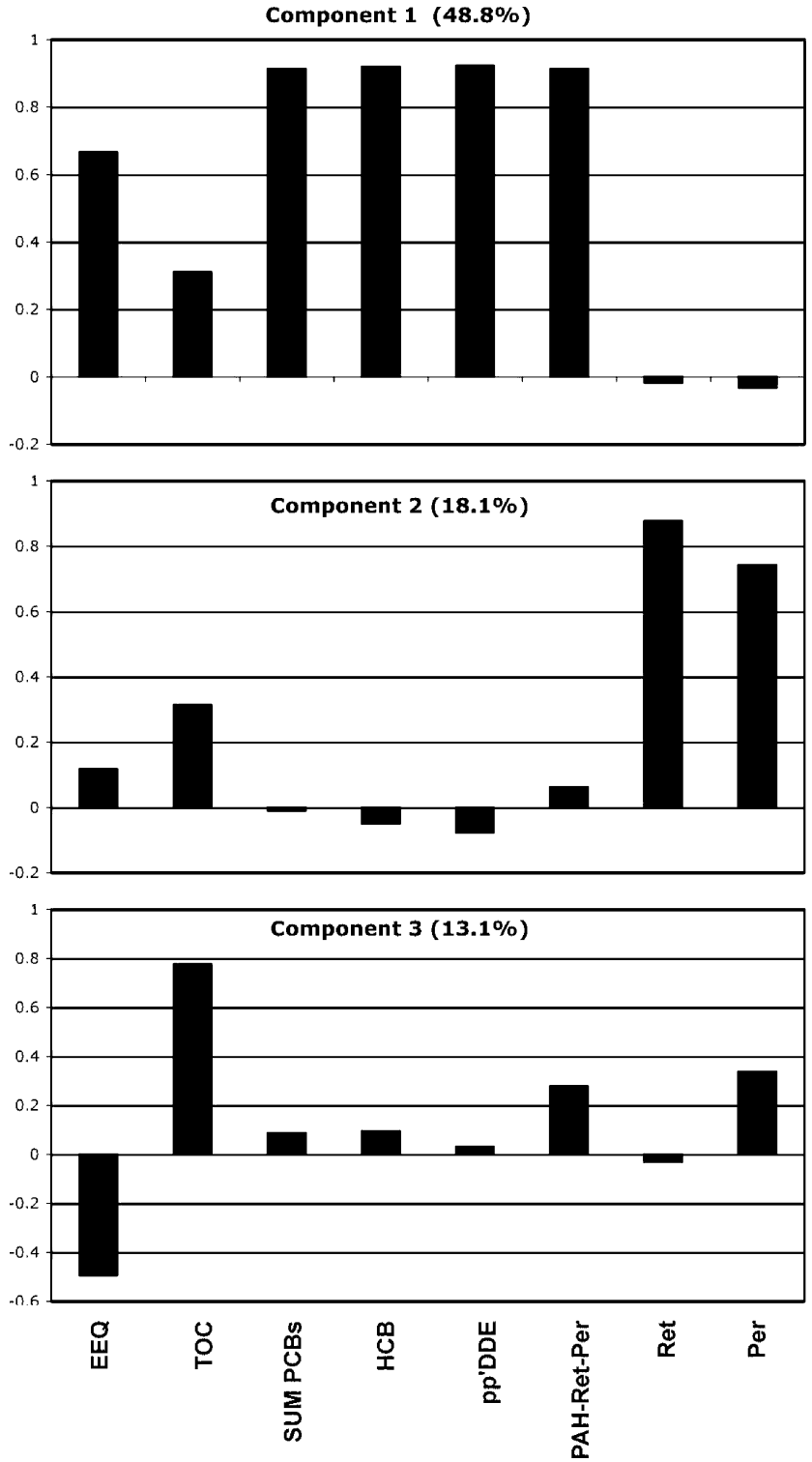


FIGURE 5. PCA loading plot. The amount of total variability explained by each component is indicated in parentheses. PAH-Ret-Per represents the sum of all PAH except retene and perylene.

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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Literature Cited

(1) Fernández, P.; Vilanova, R.; Grimalt, J. Sediment fluxes of polycyclic aromatic hydrocarbons in european high altitude mountain lakes. *Environ. Sci. Technol.* **1999**, *33*, 3716−3722.
(2) Fernández, P.; Vilanova, R.; Martínez, C.; Appleby, P.; Grimalt, J. The historical record of atmospheric pyrolytic pollution over Europe registered in the sedimentary PAH from remote mountain lakes. *Environ. Sci. Technol.* **2000**, *34*, 1906−1913.
(3) Grimalt, J. O.; Fernández, P.; Berdie, L.; Vilanova, R. M.; Catalan, J.; Psenner, R.; Hofer, R.; Appleby, P. G.; Rosseland, B. O.; Lien, L.; Massabuau, J. C.; Battarbee, R. W. Selective trapping of organochlorine compounds in mountain lakes of temperate areas. *Environ. Sci. Technol.* **2001**, *35*, 2690−2697.
(4) Simonich, S.; Hites, R. Global distribution of persistent organochlorine compounds. *Science* **1995**, *269*, 1851−1854.
(5) Fernandez, P.; Grimalt, J. O.; Vilanova, M. R. Atmospheric gas-particle partitioning of polycyclic aromatic hydrocarbons in high mountain regions of Europe. *Environ. Sci. Technol.* **2002**, *36*, 1162−1168.
(6) Kavlock, R. J.; Daston, G. P.; DeRosa, C.; Fenner-Crisp, P.; Gray, L. E.; Kaattari, S.; Lucier, G.; Luster, M.; Mac, M. J.; Maczka, C.; Miller, R.; Moore, J.; Rolland, R.; Scott, G.; Sheehan, D. M.; Sinks, T.; Tilson, H. A. Research needs for the risk assessment of health and environmental effects of endocrine disruptors: a report of the U.S. EPA-sponsored workshop. *Environ. Health Perspect.* **1996**, *104* (Suppl. 4), 715−740.
(7) Degen, G. H.; Bolt, H. M. Endocrine disruptors: update on xenoestrogens. *Int. Arch. Occup. Environ. Health* **2000**, *73*, 433−441.
(8) Barlow, S.; Kavlock, R. J.; Moore, J. A.; Schantz, S. L.; Sheehan, D. M.; Shuey, D. L.; Lary, J. M. Teratology Society Public Affairs Committee position paper: developmental toxicity of endocrine disruptors to humans. *Teratology* **1999**, *60*, 365−375.
(9) Bonefeld-Jorgensen, E.; Andersen, H.; Rasmussen, T.; Vinggaard, A. Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. *Toxicology* **2001**, *158*, 141−153.
(10) Coldham, N. G.; Dave, M.; Sivapathasundaram, S.; McDonnell, D. P.; Connor, C.; Sauer, M. J. Evaluation of a recombinant yeast cell estrogen screening assay. *Environ. Health Perspect.* **1997**, *105*, 734−742.
(11) Petit, F.; LeGoff, P.; Cravedi, J. P.; Valotaire, Y.; Pakdel, F. Two complementary bioassays for screening the estrogenic potency of xenobiotics: recombinant yeast for trout estrogen receptor and trout hepatocyte cultures. *J. Mol. Endocrinol.* **1997**, *19*, 321−335.
(12) Garcia-Reyero, N.; Grau, E.; Castillo, M.; López de Alda, M.; Barceló, D.; Piña, B. Monitoring of endocrine disruptors in surface waters by the yeast recombinant assay. *Environ. Toxicol. Chem.* **2001**, *20*, 1152−1158.
(13) Céspedes, R.; Petrovic, M.; Raldúa, D.; Saura, U.; Piña, B.; Lacorte, S.; Viana, P.; Barceló, D. Integrated procedure for determination of endocrine-disrupting activity in surface waters and sediments by use of the biological technique recombinant yeast assay and chemical analysis by LC-ESI-MS. *Anal. Bioanal. Chem.* **2004**, *378*, 697−708.
(14) Colborn, T. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *J. Toxicol. Environ. Health* **1991**, *33*, 395−453.
(15) Vilanova, R. M.; Fernández, P.; Martínez, C.; Grimalt, J. O. Polycyclic aromatic hydrocarbons in remote mountain lake waters. *Water Res.* **2001**, *35*, 3916−3926.
(16) Vilanova, R.; Fernández, P.; Martínez, C.; Grimalt, J. O. Organochlorine pollutants in remote mountain lake waters. *J. Environ. Qual.* **2001**, *30*, 1286−1295.
(17) Thomas, K.; Hurst, M.; Matthiessen, P.; Waldock, M.Characterization of estrogenic compounds in water samples collected from United Kingdom estuaries. *Environ. Toxicol. Chem.* **2001**, *20*, 2165−2170.
(18) Arukwe, A.; Goksøyr, A. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. *Comp. Hepatol.* **2003**, *2*, 4−24.
(19) Garcia-Reyero, N.; Raldúa, D.; Quirós, L.; Llaveria, G.; Cerdà, J.; Barceló, D.; Grimalt, J.; Piña, B. Use of vitellogenin mRNA as a biomarker for endocrine disruption in feral and cultured fish. *Anal. Bioanal. Chem.* **2004**, *378*, 670−675.
(20) Rehmann, K.; Schramm, K.-W.; Krettrup, A. A. Applicability of a yeast oestrogen screen for the detection of oestrogen-like activities in environmental samples. *Chemosphere* **1999**, *38*, 3303−3312.
(21) Colborn, T.; vom Saal, F. S.; Soto, A. M. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Perspect.* **1993**, *101*, 378−384.
(22) Sherman, F. Getting started with yeast. *Methods Enzymol.* **1992**, *194*, 3-21.
(23) Chaler, R.; Vilanova, R.; Santiago-Silva, M.; Fernández, P.; Grimalt, J. O. Enhanced sensitivity in the analysis of trace organochlorine compounds by negative-ion mass spectrometry with ammonia as reagent gas. *J. Chromatogr. A* **1998**, *823*, 73−79.
(24) Berdié, L.; Grimalt, J. O. Assessment of the sample handling procedures in a labor-saving method for the analysis of organochlorine compounds in a large number of fish samples. *J. Chromatogr. A* **1998**, *823*, 373−380.
(25) Pankow, J. F. Review and comparative analysis of the theories on partitioning between the gas and aerosol particulate phases in the atmosphere. *Atmos. Environ.* **1987**, *21*, 2275−2283.
(26) Pankow, J. F.; Bidleman, T. F. Interdependence of the slopes and intercepts from log−log correlations of measured gas-particle paritioning and vapor pressure. I. theory and analysis of available data. *Atmos. Environ.* **1992**, *26A*, 1071−1080.
(27) van Drooge, B. L.; Grimalt, J. O.; Torres Garcia, C. J.; Cuevas, E. Semivolatile organochlorine compounds in the free troposphere of the northeastern Atlantic. *Environ. Sci. Technol.* **2002**, *36*, 1155−1161.
(28) Bidleman, T. F. Atmospheric processes. *Environ. Sci. Technol.* **1988**, *22*, 361−367.
(29) Duinker, J. C.; Bouchertail, F. On the distribution of atmospheric polychlorinated biphenyl congeners between vapor phase, aerosols and rain. *Environ. Sci. Technol.* **1989**, *23*, 57−62.
(30) Sanusi, A.; Millet, M.; Mirabel, P.; Wortham, W. Gas-particle partitioning of pesticides in atmospheric samples. *Atmos. Environ.* **1999**, *33*, 4941−4951.
(31) Vives, I.; Grimalt, J. O.; Catalan, J.; Rosseland, B. O.; Battarbee, R. W. Influence of altitude and age in the accumulation of organochlorine compounds in fish from high mountain lakes. *Environ. Sci. Technol.* **2004**, *38*, 690−698.
(32) Mackay, D.; Shiu, W. Y.; Ma, K. C. *Illustrated Handbook of Physical−Chemical Properties and Environmental Fate for Organic Chemicals*; Lewis Publishers: New York, 1992; Vols. I and II.
(33) Agency for Toxic Substances and Disease Registry (ATSDR). *Toxicological Profile for Polycyclic Aromatic Hydrocarbons (PAHs)*. Public Health Service, U.S. Department of Health and Human Services: Atlanta, 1995.

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