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Abstract

PAH concentrations, $\delta 13C$ and $\delta 15N$ isotopes signatures were analysed in different specimens of a high mountain lake food web (Estany Redon, Pyrenees) from primary producers to top predators. $\delta 15N$ varied between –4.6 and 3.6 °/°° with *Salmo trutta* at the top of the food chain. $\delta 13C$ ranged from –29.8 to –6.5 °/°°, covering species from benthic to pelagic habitats. Mean PAH concentrations ranged from 18 to 903 ng·g dw^{-1}, found in *Daphnia pulicaria* and *Siphonoperla torrentium*, respectively. Phenanthrene dominated in most of the species, except in *Polycentropodidae* and *Chironomidae* pupae where pyrene and fluoranthene were present at a higher proportion, respectively. Generally, significant correlations were found between PAH composition in the organism and water phases. No correlation was observed with the sediments. Organism concentration divided by dissolved phase water concentration gave higher values than k_{ow} for PAH with $K_{ow} < 10^6$ for most of the species. Most of the species accumulated lower concentrations of most PAH than Salmo trutta, except *Radix ovata* and *Platambus maculatus*. PAH concentration of an estimated annual diet of the fish presents similar values as found in fish for the majority of PAHs. Exceptions of this trend are found for indeno(1,2,3-cd)pyrene and benzo(ghi)perylene that can be explained by the steric difficulties of these compounds to pass tissue membranes in fish.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous compounds, generally formed as the result of incomplete combustion of organic material (1), whose input into the environment has increased extensively in the 20th century (2). They are directly released to the atmosphere, both in the form of gas and associated to particles, where they are transported over long distances and become global contaminants (3) (4). In this respect, recent studies on sediments, water and air have demonstrated that these compounds are relevant pollutants in high mountain lakes (5) (6) (2) (7) (8).

PAHs have been widely studied because of the carcinogenic and mutagenic properties of some of them and their metabolites (9) (10) (11) (12, 13). For this reason, some PAH are included in lists of priority pollutants from the US and EU. Toxico-hepatic lesions in fish have been related to PAH exposure (14).

To our knowledge less has been published on PAHs in food webs, because they are rapidly metabolised by vertebrates and, thus, they are often not detected in higher organisms (15) (16). While fish possess mixed-function oxygenase systems and rapidly metabolise PAH compounds (15) (17) (18) (19) (20), invertebrates are poorly developed on these enzymes systems and consequently present a slow rate of metabolism (21). However, PAHs are frequently found in benthic and pelagic organisms, even in areas that are not considered to be highly polluted (22) (23). Because benthic and pelagic invertebrates are important components of aquatic food webs (24) (25), accumulated PAHs can be directly transferred to higher trophic levels, such as fish, through their diet. Low PAH concentrations in higher trophic levels do not necessarily imply that these organisms are not at risk, because if an organism is exposed to PAHs but subsequently metabolises the compounds at a rapid rate, the body burden will be low but the potential hazard high. The factors determining PAH accumulation in trophic chains are still to be elucidated.

High altitude mountain lakes offer unique environments for the assessment of atmospherically transported pollution inputs into biota. The mountain lake selected for study avoids receiving water flows, e.g. rivers, streams, from other aquatic systems. The organisms living in this system are only exposed to atmospherically transported pollution levels during all their life. Thus, this type of lake constitute "natural experiments" of long-term exposure to low doses of pollutants, as it is the current case for most ecosystems. The study of PAH body burdens at the different steps of the lake food web provides the opportunity of relating intake and accumulation of these compounds in different species to well-monitored ecosystem pollution inputs .

MATERIALS AND METHODS

Study area. The study site was the remote mountain lake Estany Redon (42º38'N, 0º46'E), in the central Pyrenees (Catalonia, Spain). This lake is located at 2240 m above sea level and above the regional tree line, far from local pollution sources. This system is oligotrophic, has a surface area of 24 ha, a maximum depth of 73 m, a volume of 7.7 Hm^3, and a water residence time of 4 yr (26). The ice-free period is from May to December (27). Pollution inputs are exclusively dominated by atmospheric deposition (wet and dry) and there is only one outflow from the lake.

Sample Collection and Handling. Brown trouts (*Salmo trutta*) were collected for both PAH and stable isotope analysis. Fish sampling followed standard test fishing procedures with multifilament gillnets. All fish were measured and dissected and their sex was determined on site. Sex could not be identified in two specimens. Liver and individually identifiable stomach content organisms were separately wrapped in a pre-cleaned aluminium foil and kept frozen (-20ºC) until analysis.

Invertebrate samples were collected for PAH and stable isotope analysis. Different habitats were sampled to collect organisms from different trophic levels and with different isotope signature. Zooplankton was obtained by trolling a mesh net through the water. *Daphnia pulicaria* was the main specie found in zooplankton samples. Species living in the sediment were collected with a dredger and by "kicking technique" to up-well the sediment and trap them in nets. Insect larvae and pupae, molluscs and crustacean were the main communities collected. Littoral species were sampled by whipping off the rock surfaces along the shoreline. By this method, algae, insect larvae, mollusc gasteropodes were collected. All invertebrate samples were stored in glass vials kept frozen until analysis.

Chemicals. Residue analysis n-hexane, dichloromethane, iso-octane, methanol, acetone, and analysis grade anhydrous sodium sulphate were from Merck (Darmstadt, Germany). Aluminium foil was rinsed with acetone and let dry at ambient temperature prior to use. Neutral aluminium oxide type 507C was from Fluka AG (Buchs, Switzerland). Cellulose extraction cartridges (20 mm i.d. x 80 mm long) were from Whatman (England). PAHs mix9 and perdeuterated PAHs were purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Aluminium oxide, sodium sulphate and the cellulose cartridges were pre-cleaned by Soxhlet extraction with dichloromethane:methanol (2:1, v/v) for 24 h before use. Sodium sulphate and aluminium oxide were activated overnight at 400ºC and 120ºC, respectively.

PAH analysis. Liver was extracted and analysed for PAHs as described elsewhere (28). Briefly, liver was ground with activated sodium sulphate spiked with perdeuterated anthracene, pyrene

and benzo[g,h,i]perylene, and introduced in a pre-clean cellulose cartridges. The tissue was Soxhlet extracted (n-hexane:dichloromethane, 4:1, v/v) for 20 hours and purified through an aluminium oxide chromatographic column. Elution with hexane:dichloromethane (1:2; v/v; 30 ml) contained all the studied PAHs. Further on, extracts were concentrated to 2 ml by vacuum rotary evaporation (20 ºC, 20 Torr), then to near dryness under a gentle nitrogen flow and redissolved to 50μl with iso-octane.

Mean per cent H_2O composition in tissue was calculated for brown trouts (74.2±1.8 %, n=8) by drying them in a vacuum sealed-dissecator at 20ºC to constant weight. This value was used to convert wet weight based PAH concentrations in fish to dry weight values for subsequent comparisons with invertebrate and algae data.

PAH in invertebrate, algae and classified stomach samples were analysed by a slightly modification of the method of Vives and Grimalt (2002) (28). Briefly, all samples were dried in a vacuum sealed-dissecator at 20ºC to constant weight to determine dry weight. Tissues were Soxhlet extracted with n-hexane-dichloromethane (4:1, v/v) for 20 hours, after the above mentioned perdeuterated PAHs were added. These extracts were cleaned-up by adsorption on 5g aluminium oxide column and eluted with 30 ml of n-hexane:dichloromethane (1:2, v/v)). This extract contained all the PAH compounds of interest and was concentrated by vacuum rotary evaporation to 1 ml and further concentrated until 50 μl isooctane as described above.

Before chromatographic analysis, an internal standard of perdeuterated perylene was added to all the sample vials to correct for instrument variability. Samples were analysed by gas chromatograph coupled to mass spectrometer (GC-MS, Trace, Thermo, Bremen, Germany). This instrument was equipped with a 50 m x 0.25 mm i.d. HP-5MS capillary column coated with 5% phenyl 95% methylpolysiloxane (film thickness 0.25 μm). Samples were injected in splitless mode. The oven temperature program started at 90ºC (held for 1 min) to 120ºC at 10ºC·min^{-1}, and then to 310ºC at 4ºC·min^{-1} (holding time 15 min). Injector, transfer line and ion source temperatures were 280ºC, 280ºC and 200ºC, respectively. Stringent precautions were kept for maintenance of the injector under clean conditions avoiding adsorptions that could deviate the system from linearity and increase the limits of detection and quantification. Helium at a flow of 1.1 mL·min^{-1} was used as carrier gas. Data acquisition was in electron impact (70 eV) and selected ion monitoring (40 ms dwell time). The ion mass program is reported elsewhere (6) and (29).

The PAHs analysed were fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene+triphenylene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenz[a,h]anthracene, indeno[c,d-1,2,3]pyrene and benzo[g,h,i]perylene. All of them are included in the EPA list and some of them are described as mutagenic, carcinogenic and teratogenic by IARC, WHO (30). Although the analysed PAHs may represent a minor

fraction of the total amount of PAHs in the environment, they may be indicative for the total PAH concentrations.

Procedural blanks were analysed for every set of six samples and the recovery of the surrogate standards was calculated for each sample. Identification and quantification of all studied compounds were performed by an external standard method. Relative response to perylene-d_{10} was used in order to correct for instrumental variabilities and this value was also corrected by the recovery of the surrogate standards.

Stable Isotope Analysis. Samples were analysed for stable isotopes ratios in a Delta C Finningan MAT mass spectrometer coupled online with a Carlo Herba CHNS elemental analyser, via a Finnigan conflo 2 interface. Results are reported using atmospheric nitrogen and Peedee Belemnite (PDB) carbonate as reference for nitrogen and carbon isotopes, respectively. Nitrogen isotope signatures were used for the discrimination among trophic levels of the food web. Carbon isotope signatures of the food web indicated the relative importance of benthic and pelagic autochthonous carbon as energy sources.

Lipid Content Determination. The lipid content for the food web organisms was estimated from measured elemental carbon and nitrogen by assuming that the main body constituents were lipids, proteins, ashes and chitin. The percentage of lipids was calculated by difference, after estimating proteins multiplying N by 6.25; and using literature values for ash and chitin content. Since cyanobacteria use carbohydrates as energy reserve, the lipid content for *Nostoc* reported in the literature was used. Lipid content for each specie is reported in Table 1.

Table 1. Number of samples, lipid content and concentrations of individual PAH in each species analysed (units ng/g dry weight).Compound abbreviations: Fluorene (Fl), Phenanthrene (Phe), Anthracene (A), Fluoranthene (Fla), Pyrene (Py), Benz(a)anthracene (BaA), Chrysene+tripherylene (Chr), Benzo(b)fluoranthene+Benzo(k)fluoranthene (BFlas), Benzo(a)pyrene (BaPy), Indeno(cd-1,2,3)pyrene (IndPy), Benzo(g,h,i)Perylene (BPer) and Dibenz(a,h)anthracene (DibahA).

Species	n	Lipid content (%)	Fl	Phe	A	Fla	Py	BaA	Chr	BFlas	BaPy	IndPy	BPer	DibahA
Nostoc sp.	1	1,7	3,6	31	1,8	3,5	2,9	0,08	0,36	0,52	0,23	0,15	0,18	0,15
Radix ovata	1	0,6	6,7	46	3,4	5,6	4,4	0,13	0,54	1,1	0,31	0,55	0,63	<LOD*
Chironomidae larva	2	30,4	19 ± 6,7	110 ± 21	11 ± 3,2	39 ± 38	24 ± 26	4,3 ± 15	19 ±41	25 ±11	7,9 ± 7,8	9,6 ± 16	9,8 ± 9,1	13 ± 10
Daphnia pulicaria	1	54,4	1,4	10	0,74	2,2	1,1	0,22	0,54	0,72	0,31	0,21	0,28	<LOD
Chironomidae pupa	4	39,9	10 ± 10	12 ± 7,5	3,0 ± 2,9	64 ± 97	3,9 ± 1,4	1,2 ± 3,2	4,8 ± 9,6	3,5 ± 3,1	2,2 ± 4,0	2,6 ± 4,8	2,5 ± 3,9	0,24 ± 0,14
Sialis lutaria	1	21,1	41	144	96	87	3,3	2,5	7,9	2,9	2,0	3,7	7,4	<LOD
Polycentropodidae	1	29,8	2,9	11	5,3	105	280	3,5	5,3	2,0	1,2	1,9	<LOD	<LOD
Arcynopterix compacta	1	26,5	92	268	26	117	7,1	4,2	35	14	11	20	14	<LOD
Platambus maculatus	1	9,9	93	312	31	136	80	14	43	23	12	25	18	<LOD
Pisidum sp.	1	13,2	14	45	4,9	22	13	2,2	12	9,0	1,9	4,6	3,8	<LOD
Siphonoperla torrentium	1	37,7	117	341	34	162	97	13	74	<LOD	<LOD	37	28	<LOD
Eurycercus lamellatus	1	37,9	23	92	9,3	65	45	18	55	42	24	18	14	11
Salmo trutta	15	2,8	9,8 ± 3,4	47 ± 16	3,1 ± 1,9	8,7 ± 5,2	7,5 ± 3,7	0,60 ± 0,60	2,4 ± 1,6	1,5 ± 0,65	0,55 ± 0,60	0,15 ± 0,10	0,20 ± 0,20	0,20 ± 0,15

* Below Limit of Detection (0,1-0,14 ng/g dw)

RESULTS AND DISCUSSION

Fifteen liver samples of brown trout (*Salmo trutta*) collected in Estany Redó, Pyrenees, were analyzed. The length of the collected specimens was 286 ± 26 mm (mean ± standard deviation) and weight was 230 ± 58 g. Average conditioning factors of male and female specimens (n = 6 and n = 7, respectively) were 0.94 ± 0.08 and 0.99 ± 0.10 g·cm^{-3}, respectively, involving no significant difference (ANOVA, $p < 0.05$). Average ages of the males and female groups were 9 ± 4 and 12 ± 3 year old, involving again no significant difference (ANOVA, $p < 0.05$). The two groups of male and female specimens examined do not reflect significant population size or age differences. Averages of conditioning factor and age of the trouts analysed were 0.97 ± 0.09 g·cm^{-3} and 11 ± 4 year old.

Cyanobacterium (*Nostoc sp*.), molluscs (*Radix ovata* and *Pisidum sp*.), insects (*Chironomidae, Sialis lutaria, Polycentropodidae, Arcynopterix compacta, Platambus maculatus* and *Siphonoperla torrentium*) and pelagic and littoral cladocerans (*Daphnia pulicaria* and *Eurycercus lamellatus*) were the rest of species collected to characterise the food web of this lake and its PAH impact. Samples were analysed pooling individuals from the same species to obtain a minimum wet weight of 0.5 g. When enough material was available, replicates were analysed.

Isotope signatures. Food web interrelationships of the biota from Estany Redó are shown in Figure 1, representing the nitrogen (δ^{15}N) and carbon (δ^{13}C) isotopes signatures. These biota represented several trophic levels, ranging in mean δ^{15}N between –4.6 and 3.6 °/°° in *Nostoc sp*. and *Salmo trutta*, respectively. Assuming an enrichment factor of 3.5 °/°° δ^{15}N per trophic level change, a short food chain from primary producers to top predators is observed (factor of 2.2 of total energy transference). This is common in small sized oligotrophic high mountain lakes where food is not abundant and depends on seasonal productivity patterns. Fish (*Salmo trutta*), located at the higher position of the food web, do not contain piscivorous specimens. Although slightly periodical differences in fish dietary habits (Catalan et al 2003 in press), stable isotopes signature for *Salmo trutta* did not show any significance seasonal differences.

The range in δ^{13}C signature of the food web was -29.8 and –6.5 °/°° in *Daphnia pulicaria* and *Nostoc sp*., small pelagic cladocerans and benthic primary producers, respectively. Organic detritus (sediment) showed significantly distinct isotope signatures. Organisms living in the littoral of the lake present signatures of δ^{13}C between –15 and –6 °/°°. This group is mainly composed by algae (*Nostoc sp* and *Epilithon*), insect larvae (*Platambus maculatus*) and gasteropode mollusc (*Radix ovata*). Pelagic organisms show δ^{13}C signatures between –30 and –25 °/°°. *Daphnia pulicaria* and *Seston* are the more characteristic organisms living in the water

column. Organisms with a benthic habitat or sediment interaction give signatures between –24 and –15 $°/°°$ for $\delta^{13}C$. *Eurycercus lamellatus*, *Pisidum sp.* and Chironomidae larvae are benthic organisms that eat micro-organisms from sediment or sediment particles. Although three different habitats can be distinguished, the $\delta^{13}C$ signatures show a continous gradient in the trophic web.
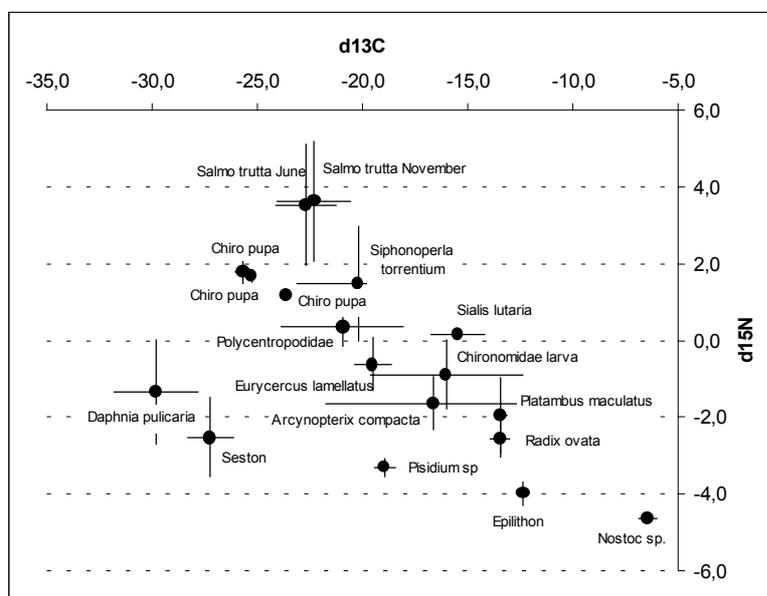

Figure 1. Carbon and nitrogen isotopes signatures (°/°°) of the food web of Estany Redon. Mean (dots) and standard deviations (lines) are represented.

Food web concentrations of PAH. Mean concentrations of individual parent PAH are presented in Table 1. Minimum and maximum ΣPAH concentrations were 18.1 and 902.83 ng g^{-1} dw, found in *Daphnia pulicaria* and *Siphonoperla torrentium* respectively. Involving a difference of nearly two orders of magnitude. Considering that sum low molecular weight PAHs (LMW) includes from Fl to Py and sum high molecular weight PAHs (HMW) from BaA to DibahA, mean LMW PAHs ranged in the same order as mentioned above and the minimum and maximal values were found in the same species cited earlier. However, HMW PAHs values ranged between 1.67 and 182.44 ng g^{-1} dw in *Nostoc sp.* and *Eurycercus lamellatus*, respectively. The difference in HMW PAHs involve only one order of magnitude.

Relative distribution of PAHs. LMW PAHs are more abundant than HMW PAHs as is shown in Table 2. In the majority of the species, the PAH distributions are dominated by phenanthrene (ranging between 22-70 % of the total analysed PAHs), followed by fluorene, fluoranthene and pyrene. *Polycentropodidae* and C*hironomidae* pupae present a PAH profile dominated by pyrene (67 %) and fluoranthene (75 %), respectively. Anthracene is present in smaller proportions (between 1-5 % of the total analysed PAH), with the exception of *Sialis lutaria*,

where its contribution reaches around 24 %. Higher abundance of chrysene and tripherylene (8-13 %) is found in *Eurycercus lamellatus*, *Pisidum sp.* and plecoptera (*Siphonoperla torrentium*). The other PAH involve individually less than 6% in all the species.

Table 2. PAH pattern in each species analysed. Relative distribution expressed as percentage of individual PAH concentration from the sumation of the total analysed PAH concentrations. Compound abbreviations as reported in Table 1. Sum LMW includes from Fl to Py and Sum HMW from BaA to DibahA.

Species	Fl	Phe	A	Fla	Py	BaA	Chr	BFlas	BaPy	IndPy	Bper	DibahA	Sum LMW	Sum HMW
Nostoc sp.	8	70	4	8	6	0,2	1	1	1	0,3	0,4	0,3	96	4
Radix ovata	10	66	5	8	6	0,2	1	2	0,4	1	1	0	95	5
Chironomidae larva	6	35	3	12	8	1	6	8	2	3	3	4	68	26
Daphnia pulicaria	8	57	4	12	6	1	3	4	2	1	2	0	87	13
Chironomidae pupa	12	14	4	75	5	1	6	4	3	3	3	0,3	77	20
Sialis lutaria	10	36	24	22	1	1	2	1	0,5	1	2	0	93	7
Polycentropodidae	1	3	1	25	67	1	1	0	0,3	0,4	0,02	0	97	3
Arcynopterix compacta	15	44	4	19	1	1	6	2	2	3	2	0	84	16
Platambus maculatus	12	40	4	17	10	2	5	3	1	3	2	0	83	17
Pisidum sp.	11	34	4	16	10	2	9	7	1	3	3	0	75	25
Siphonoperla torrentium	13	38	4	18	11	1	8	0	0	4	3	0	83	17
Eurycercus lamellatus	5	22	2	16	11	4	13	10	6	4	3	3	56	44
Salmo trutta	12	55	4	10	9	1	3	2	1	0,2	0,2	0,2	95	4
Sediment	1	8	1	15	9	4	15	27	3	10	7	1	33	67
Dissolved water	9	41	2	23	8	1	9	3	1	1	1	1	83	17
Particulate water	3	31	1	13	20	1	5	9	2	8	5	1	68	31

The average distribution is remarkably similar among *Salmo trutta* and *Daphnia pulicaria*. The clear predominance of phenanthrene in fish is consistent with the PAH profiles found in fish liver from other freshwater (31) and marine systems (32) (28). *Daphnia pulicaria* is a pelagic specie living in the water column. Previous studies in Estany Redon have shown that phenanthrene is also the dominant PAH in high mountain lake waters, both in the dissolved and the particulate fractions (Table 2; (8)). However, water exhibits a higher proportion of the heavy molecular weight compounds, namely among the suspended particles. Chrysene, benzofluoranthenes, indeno[1,2,3-*cd*]pyrene and benzo[ghi]perylene are commonly found in higher proportion. These mentioned PAH are individually present in less than 3 % in fish and *Daphnia*.

All the organisms studied present more than 65 % of abundance of LMW PAHs, except for *Eurycercus lamellatus* that presents an equal abundance of LMW and HMW PAHs (56 % and 44 %, respectively) (Table 2). PAH profile of Estany Redon sediments are also dominated by fluoranthene, chrysene, benzofluoranthenes, indeno[1,2,3-*cd*]pyrene and benzo[ghi]perylene (Table 1). The relative abundance of HMW PAHs in sediments is around 67 %, in compare with of 33 % of LMW PAHs. This similarity in PAH relative distributions of *Eurycercus lamellatus* and sediments may indicate an direct interaction of this specie with the sediment compartment in compare with other organisms.

PAH exposure pathways. Correlations of relative PAH distribution in the abiotic compartments (sediment and water phases) versus relative PAH distribution in each organism help better to elucidate whether correlations exist between PAH concentrations in the organism and its surrounding environment (Table 3). Significant correlations with slopes similar to one (between 0.7 and 1.5) are found for most of the organisms in relation with the water phases (ANOVA, $0.05 < p < 0.0001$). Correlation coefficients are higher with the dissolved phase ($0.65 < r^2 < 0.97$) than with the particulate phase ($0.35 < r^2 < 0.83$). *Eurycercus lamellatus* presents lower slopes (0.46 and 0.55 for dissolved and particulate phases, respectively), although these correlations are still significant. *Polycentropodidae* and *Chironomidae* pupae are the only organisms which do not significantly correlate with any of the water phases. In the case of *Chironomidae*, this may be explained due to the fact that there is no food intake and a metamorphological reorganisation is going on during the pupae stage .

Table 3. Linear correlations between relative PAH distributions in the organisms and the different environmental compartments (sediment, dissolved water phase and particulate water phase).

	Sediment		dissolved water		particulate water	
	slope	r^2 and significance	slope	r^2 and significance	slope	r^2 and significance
Nostoc sp.	-0,0596	0,0005	1,4600	0,8131*****	1,8116	0,7083****
Radix ovata	-0,0501	0,0004	1,3820	0,8150*****	1,7135	0,7088****
Chironomidae larva	0,2116	0,0306	0,7161	0,9218*****	0,8707	0,7710*****
Daphnia pulicaria	0,0823	0,0015	1,2166	0,8751*****	1,4692	0,7221****
Chironomidae pupa	0,8235	0,0892	0,8967	0,2778*	0,6937	0,0941
Sialis lutaria	-0,1432	0,0076	0,8241	0,6592****	0,8045	0,3554**
Polycentropodidae	0,2400	0,0083	0,2518	0,0240	1,0263	0,2256*
Arcynopterix compacta	0,1612	0,0092	1,0114	0,9481*****	1,1190	0,6566***
Platambus maculatus	0,1604	0,0117	0,8986	0,9676*****	1,1099	0,8352*****
Pisidum sp.	0,3091	0,0609	0,7619	0,9720*****	0,9323	0,8235*****
Siphonoperla torrentium	0,1265	0,0076	0,8787	0,9662*****	1,0848	0,8332*****
Eurycercus lamellatus	0,3789	0,2154*	0,4614	0,8392*****	0,5521	0,6798****
Salmo trutta	0,0258	0,0002	1,1924	0,8675*****	1,4902	0,7665*****
Sediment	n.a.[a]	n.a.	0,1119	0,0329	0,1439	0,0308
Dissolved water	0,2941	0,0329	n.a.	n.a.	1,1510	0,7495*****
Particulate water	0,2139	0,0308	0,6512	0,7495*****	n.a.	n.a.

[a]Not applicable. *$p<0,1$. **$p<0,05$. ***$p<0,005$. ****$p<0,001$. *****$p<0,0001$.

In the case of sediments, all the organisms differ significantly in their PAH composition in compare with what is found in the sediments (Table 3). This can be observed in the slopes that are not close to one, suggesting that PAH concentrations in the organisms hold no relation with the PAH composition in the sediments. Moreover, these correlations are in all cases not significant and present often very small r^2 values ($0.0002 < r^2 < 0.089$). A slightly better correlation coefficient ($r^2 = 0.2$) is only found for *Eurycercus lamellatus*, which indicates a certain relationship with the PAH in sediments as it was previously observed, although its relation with the sediment is again not significant. Therefore, this value of r^2 is lower than what

is observed in water correlations and indicate a large error in the sediment comparison. These results show once more that PAH composition in the organisms is more related to PAHs in the water column than PAHs in the sediments.

Bioaccumulation of aqueous PAHs. To evaluate the bioaccumulation phenomena from dissolved water phase an empirical approach was used. The individual PAH ratios were deduced from concentration in each organism (ng·kg lw^{-1}) and previously reported field concentration of freely dissolved PAH concentration in water of this lake (ng·L^{-1}) (8) to obtain real bioaccumulation factors (oc/wc) (Figure 2). For the majority of species, the oc/wc was higher than the theoretical value for PAHs with $K_{ow}<10^6$, indicating that PAH accumulation is higher than the predicted theoretical octanol-water equilibrium. Exception to this trend is *Daphnia pulicaria*, which have not reached the equilibrium for most of the PAHs. This fact may be due to the younger age of these organisms (only two moths all) in the time of sampling. *Radix ovata, Pisidum sp., Arcynopterix compacta, Platambus maculatus* and *Siphonoperla torrentium* presented as well oc/wc values higher than the K_{ow} for the more hydrophobic compounds, except for dibenzo(ah)anthracene. *Nostoc sp , Eurycercus lamellatus, Chironomidae* larvae and pupae, *Sialis, Polycentropodidae* and *Salmo trutta* show concentrations smaller than what is predicted for the most hydrophobic compounds. These results confirm that positive relationship between experimental and theoretical bioaccumulation values tend to break down when dealing with very hydrophobic ($K_{ow}>10^6$) compounds which may be less bioavailable, due to their size, even though partitioning would favour their bioaccumulation (21).

PAH ingestion in brown trout. *Salmo trutta* is located at the top of the food web of Estany Redó. Ratios of lipid normalised individual PAH concentration in each organism divided by the lipid normalised concentration in trout (oc/fc) were calculated (Figure 3). Most of the species accumulated significantly lower concentrations of most PAH than Salmo trutta, except *Radix ovata* and *Platambus maculatus*. For the rest of species, their concentration only exceeded trout values in some cases for the HMW PAH. *Daphnia pulicaria* is the only species that its concentration does not exceed in any case the amounts found in trout.

The annual diet composition for the brown trout in lake Redó has been described in other studies (Catalan et al 2003, in press). The average PAH concentrations of the diet components was used to calculate the PAH intake by food. Figure 3 shows a very good agreement between the PAH levels of most of the individual compounds in liver of trout (ng g^{-1} lw) and estimated food level (ng g^{-1} lw), which is expressed in a high correlation between both relative PAH compositions (r2 = 0.9727, ANOVA, p<0.0001). The LMW PAH show quite similar concentrations in fish in compare with food, while the HMW PAH (indeno(1,2,3-

cd)pyrene and benzo(ghi)perylene) present higher levels in the food. These observations may indicate that the food intake plays an important role in the PAH intake in these fish and that there is only little magnification, if any.
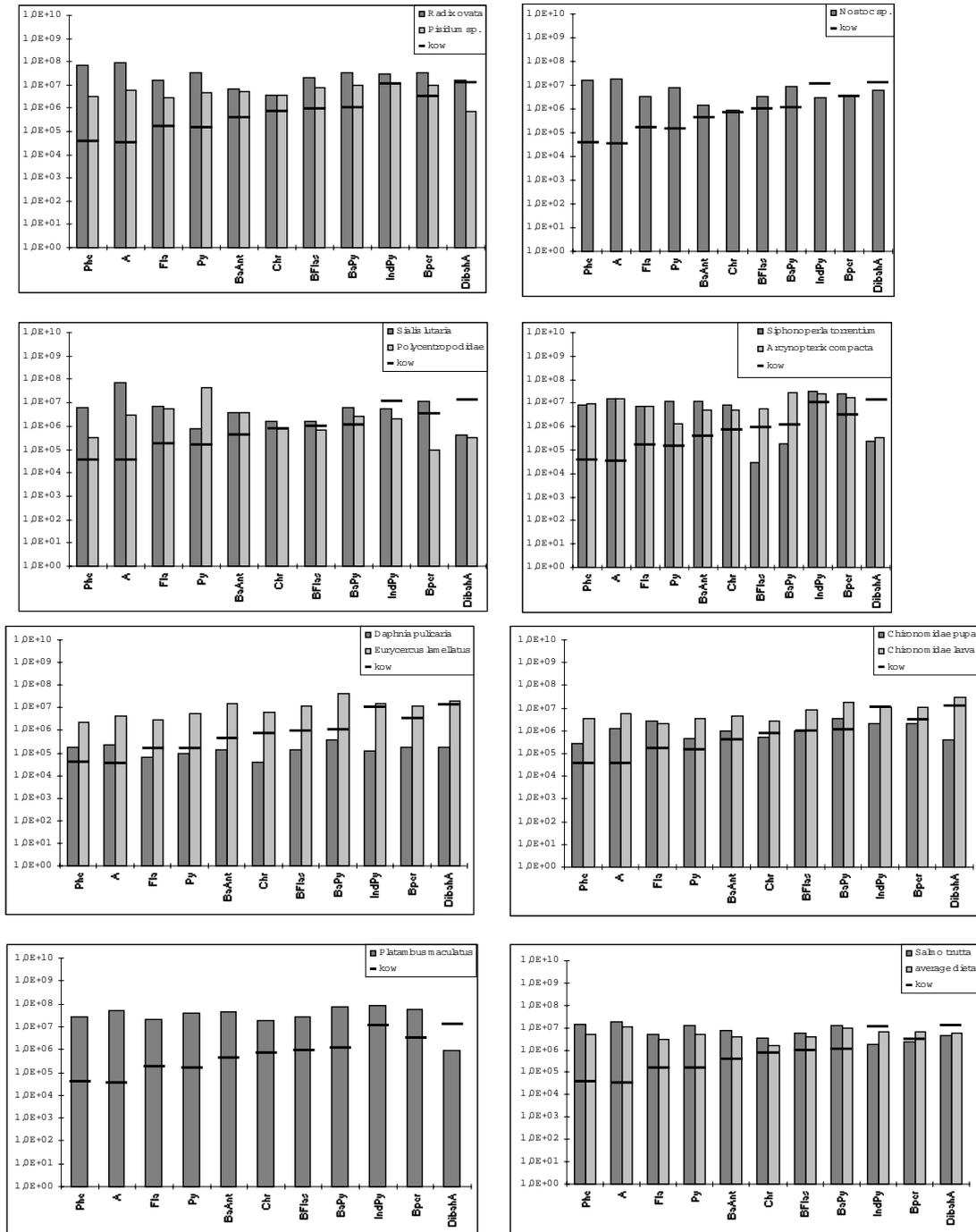

Figure 2. Bioaccumulation factors (concentration in the organism expressed in lipid weight divided with dissolved water phase concentration) for the different PAH compounds. Compound names as described in Table 1. Kow values are represented as reference (34-35).

The concentrations of PAH in fish were analysed in liver tissue, so the levels and composition may represent a degraded fraction of the total PAH uptake by fish (food uptake +

respiration + dermal uptake), since they can be metabolically transformed as described for other fish species ((15), (33)). However, both PAH composition and levels are very similar between trout and average diet, so it can be considered that there is not too much metabolic transformation taking place. The higher concentrations of HMW PAH in food than in liver may indicate a transformation effect. But because of their large molecular size this compounds may pass tissue membranes very slowly, and thus may be excreted before they can be assimilated.
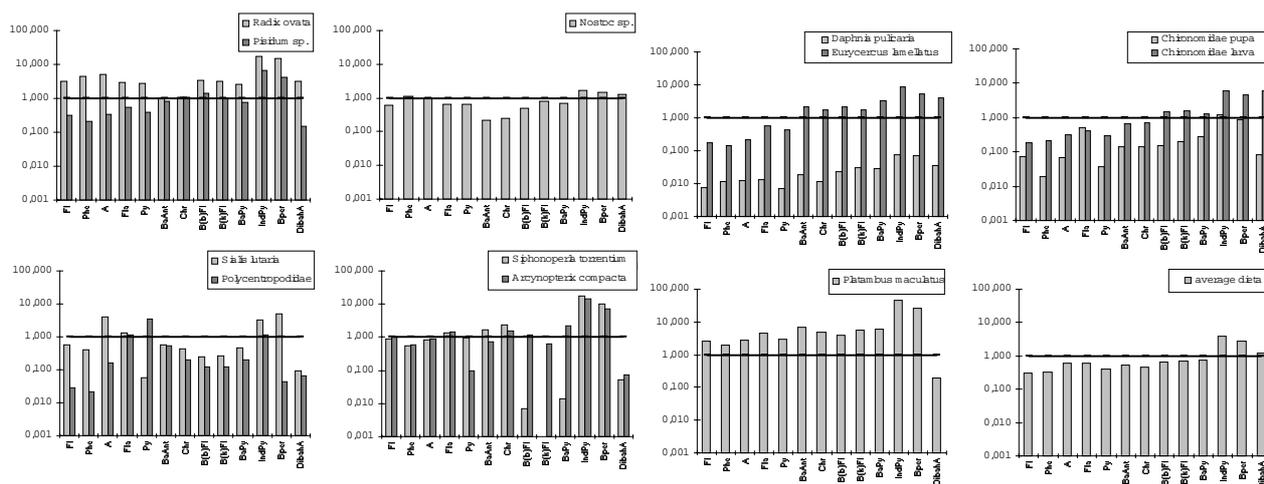

Figure 3.Organism concentration relative to fish concentration for all the individual PAH compounds. All the concentrations were expressed as lipid weight.

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ARTICLE 8

INFLUENCE OF DEPOSITED PAHS ON THE HEALTH STATUS OF FISH IN EUROPEAN MOUNTAIN LAKES

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Sotmès per a publicació

Influence of deposited PAHs on the health status of fish in European mountain lakes

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Abstract

Brown trouts (*Salmo trutta*) from four European high mountain lakes (Norway, the Pyrenees, the Alps and the Tatras) were analyzed for metal and organic pollution, histopathological abnormalities and biochemical parameters. Fish from all lakes appeared well nourished with conditioning factors close to unity. Their mean age ranged from 4 to 14 years old in Gossenköllesee and Vel'ké Hincovo, respectively. Lead and cadmium concentrations were higher in fish from Vel'ké Hincovo in comparison with the other studied lakes. All fish presented similar and lower mercury levels in muscle. While organochlorine compounds concentrations are consistent with the pattern observed in the sediments and water of the lakes, polycyclic aromatic hydrocarbons (PAHs) in liver do not correlate with what is found in the sediments. All fish presented similar PAH concentration in liver. Higher numbers of melanomacrophages and increased amounts of lipid degradation and degeneration in liver were found in trouts from Vel'ké Hincovo and Øvre Neådalsvatn. Signs of oxidative stress, such as elevated concentrations of glutathione disulphide (GSSG) and G6PDH, were also detected in specimens of these two lakes. This metabolic activation probably due to higher detoxifying activity was corroborated with previous findings of elevated number of PAH metabolites in bile of fish from these two locations.

Introduction

Anthropogenic pollutants are nowadays world-wide spread and several of them are detected in remote and pristine areas where they were never produced nor used ((1), (2), (3), (4)). Organochlorine compounds exhibit a latitudinal or altitudinal fractionation from lower to higher latitudes and even a concentration gradient from lower to higher colder altitudes ((5), (4)). These findings are consistent with the global distillation theory for persistent organic pollutants (POPs) ((6)).

Although a global distillation effect is not observed for polycyclic aromatic hydrocarbons (PAHs), they are transported over large distances usually related to adsorbtion onto soot particles ((7), (8)). Thus, PAHs are partly protected from photolysis by the sun by these particles. Nevertheless, a consistent fraction of the atmospheric PAHs are present in the gas phase ((9)). After atmospheric deposition, PAHs can enter high mountain lake systems, where they can be incorporated in the lake's food web. Once taken up by an organism they may be dissolved in the lipid rich tissues where they can be metabolized by the fish cells into metabolites ((10), (11)). In this sense, fish tissue concentrations of parent PAHs are not always an adequate measure for xenobiotic impact on organisms. Their availabilaty, i.e. the PAH fraction that reaches the target tissue and provokes a toxicological effect, might be more important than the concentration accumulated in the whole body ((11)). Furthermore, for most toxic PAHs it is necessary to enter a metabolic pathway or to influence a reaction to exert a toxic effect ((12), (13). (14)). In most cases, toxicological effects of xenobiotic chemicals on organisms is described by the interactions of different chemical compounds ((15)). All these factors have to be considered when examining the possible effects of organic xenobiotic chemicals on aquatic organisms.

High altitude mountain lake offer natural experiments for the assessment of the anthropogenic impact into fish that is long-term exposed to known atmospherically transported pollution inputs ((16), (17), (18), (19)). The lakes selected for the study are located above the local treeline, far from any local pollution sites and their hydrology depends exclusively on atmospheric deposition. Trout is the organism located at the top of the food chain and considerable concentrations of POPs and metals have been detected in its tissues ((20), (4)). In this paper evidence is given that the presence of anthropogenic contaminants causes enzymatic and pathological alterations to the fish populations of these remote mountain lakes.

Material and methods

Sample collection. This study is based on data from several European high mountain lakes, located in the region of southern Norway (Øvre Neådalsvatn), the Austrian Alps (Gossenköllesee), the Pyrenees (Redó) and the Tatra mountains (Vel'ké Hinçovo) (Figure 1).

Geographical coordinates, physical parameters and water chemistry characteristics of each studied lake are given in Tables 1 and 2. All lakes present a similar water chemistry with very low mineralized waters and ultra oligotrophic conditions. Brown trout (Salmo trutta) is the dominant fish specie present in all studied lakes (Table 1). Fish were caught by bottom multi-mesh sized gillnets and dissected immediately after capture. Fish biological characteristics are summarized in Table 1.
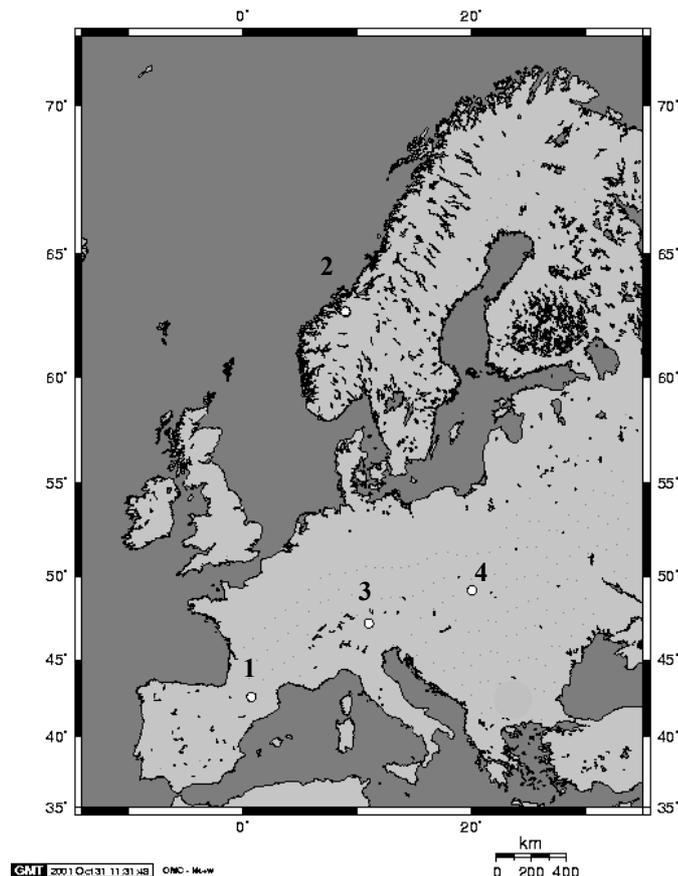
Figure 1. Map of sampling sites. 1. Redó (Pyrenees), 2. Øvre Neådalsvatn (Norway), 3. Gossenköllessee (Alps) and Vel'ké Hincovo (Tatra).

Portions of liver, gill and kidney were fixed in 5% phoshate-buffered formaline in the field for further histology analyses . Another fraction of liver was kept in liquid nitrogen or dry ice for biochemical analysis. Half of the remaining liver as well as a portion of the dorsal trunk muscle was wrapped in aluminium foil for analysis of organic pollutants. The remaining fractions of liver, dorsal trunk muscle and kidney were stored in polyethylene bags for metal analysis. All samples for organic pollutants and metal analyses were frozen at the sampling site (-20ºC) until analyses.

Pathological Analysis. The fixed tissues were gradually dehydrated in ethanol and embedded in methyl metacrylate. Microtome sections (3μm) were stained with May-Grünwald/Giemsa and

evaluated by microscopy. The method of Weibel (1979) (21) was applied for quantitative evaluation of pathological changes.

Enzyme Analysis. Liver was homogenized in 10 volumes of 10% (w/v) metaphosphoric acid. A subsample of the homogenate was used for the determination of glycogen. The remaining homogenate was centrifuged (10 min, 13.000 r.p.m.) and the supernatant used for the determination of glutathione (GSH) and glutathione disulphide (GSSG). Glycogen was digested in a mixture containing 50 µl homogenate, 150 µl 2M Na-acetate buffer, pH 6.2, 0.3 mg amyloglucosidase. The amount of glucose liberated by amyloglucosidase was determined by HPLC ((22)). GSH and GSSG were separated on a Merck LiChrospher RP select B column, 0.4x25 cm using 0.1% trifluoroacetic acid as eluent at 1 ml min^{-1}. Detection was carried out by post column reaction using 2 solutions added sequentially at 0.5 ml min^{-1}: The first one was 0.55 M NaOH with 5 % diethanolamine. This mixture was added to the eluent and heated in a reaction coil to 100°C to hydrolyze all compounds. The second solution was 200 mg L^{-1} o-phtaldialdehyde (dissolved in 4 ml dimethylformamide) and fluorescence determination was recorded at 340/420 nm after passing another reaction coil at room temperature for mixing eluent and fluorogenic reagent.

Organic pollutants analysis. Muscle tissues were extracted and analysed for OCs using the method described elsewhere ((23)). Briefly, muscle tissue (5 g) was ground with activated sodium sulphate (Merck, Darmstad, Germany) until a fine powder was obtained. This mixture was introduced into cellulose cartridges (Whatman, England) and Soxhlet-extracted with n-hexane: dichloromethane (4:1) for 18 h (both solvents of Merck, Darmstad, Germany). Lipid content was determined gravimetrically using 20% of the extract. TBB (Aldrich-Chemie, Steinheim, Germany) and PCB 209 (Promochem, Wesel, Germany) standards were added to the rest of the extract which was subsequently cleaned up with sulphuric acid (Merck, Darmstad, Germany) (5 times). All n-hexane solutions were combined and concentrated by vacuum rotary evaporation (20 ºC, 20 Torr) to small volumes (ca. 500 µl), further concentrated to near dryness under a gentle nitrogen flow and redissolved in 50 µl of iso-octane (Merck, Darmstad, Germany).

Liver samples were analysed as described elsewhere ((24)). Briefly, liver was also ground with activated anhydrous sodium sulphate and spiked with TBB, PCB#209 and perdeuterated anthracene, pyrene and benzo[ghi]perylene (Dr. Ehrenstorfer, Augsburg, Germany). Then, it was Soxhlet-extracted (n-hexane:dichloromethane, 4:1, v:v) for 20 h and cleaned up by elution through an aluminium oxide (Fluka AG, Switzerland) chromatographic column. Two fractions were collected. The first involved elution with 16.5 ml of n-hexane:dichloromethane (19:1, v:v) and 3 ml of n-hexane:dichloromethane (1:2, v:v) and provided HCB, PCBs and DDTs. The second was obtained by elution with 13 ml of n-

hexane:dichloromethane (1:2, v:v) to obtain the HCH and PAHs. The two fractions were concentrated to 50 μl in isooctane as described above.

Before chromatographic analysis of both tissues, an internal standard of tetrachloronaphthalene (TCN), octachloronaphthalene (OCN) and deuterated perylene (all from Dr. Ehrenstorfer, Augsburg, Germany) was added to correct for instrument variability. Samples for organochlorine compounds were analyzed by GC-ECD (Hewlett-Packard 5890 Series II) with a 50 m x 0.25 mm i.d. DB-5 capillary column (J&W Scientific, Folsom, CA) coated with 5% phenyl 95% methylpolysiloxane (film thickness 0.25 μm). The GC operated in splitless mode and the oven temperature program started at 90ºC (held for 1 min) to 120ºC at 10ºC·min^{-1}, and then to 310ºC at 4ºC·min^{-1} (holding time 15 min). Injector and detector temperatures were 270ºC and 310ºC, respectively. Stringent precautions were observed for maintenance of the injector under clean conditions avoiding adsorptions that could deviate the system from linearity and increase the limits of detection and quantification. Helium and nitrogen were used as carrier (0.33 mL·min^{-1}) and make-up (60 mL·min^{-1}) gases, respectively.

Samples for PAH determination were analysed by gas chromatography coupled to mass spectrometry (GC-MS, Trace, Thermo, Bremen, Germany). This instrument was equipped with a 50 m x 0.25 mm i.d. HP-5MS capillary column coated with 5% phenyl 95% methylpolysiloxane (film thickness 0.25 μm). Samples were injected in splitless mode. The oven temperature program started at 90ºC (holding time 1 min) and increased to 120ºC at 10ºC min^{-1}, and then to 310ºC at 4ºC min^{-1} (holding time 15 min). Injector, transfer line and ion source temperatures were 280ºC, 280ºC and 200ºC, respectively. Stringent precautions were observed for maintenance of the injector under clean conditions avoiding adsorptions that could deviate the system from linearity or increase the limits of detection and quantification. Helium at a flow of 1.1 mL min^{-1} was used as carrier gas. Data acquisition was in electron impact (70eV) and selected ion monitoring modes (40 ms dwell time). The ion mass program is reported elsewhere ((25) (26)).

Metal analysis. Samples for metal analysis were digested in 20 mL 7N nitric acid at 120ºC. Hg was analysed as total Hg by cold vapour AAS (Perkin Elmer FIMS 400) equipped with a hydride generator. The detection limit was 0.005 μg g^{-1} wet weight. The analysis were run in series of 30 samples, of which one was a standard reference and three were blanks. The standard reference was a fish homogenate (DORM 2, dogfish filet) from the National Research Council of Canada (certified value 4.64   g g^{-1}). Concentrations of Pb and Zn were determined by inductive coupled plasma atomic emission spectrometry (ICP-AES) and Cd and Cu were analysed by inductive coupled plasma-mass spectrometry (ICP-MS).

Table 1. Geographical and physical characteristics of studied lakes

Lake	Mountain range (Country)	Latitude	Longitude	Altitude (m)	Lake area (ha)	Catchment area (km²)	Max. depth (m)	n[a]	Fish species	Length (cm)[b]	Weight (g)[b]	CF (g cm⁻³)[b]	Age (yr)[b]
Redó	Pyrenees (Spain)	42°38' N	0°46' E	2235	24	1.5	73	32	Brown trout	26	190	0.97	9
Øvre Neådalsvatn	Caledonian (Norway)	62°46' N	8°59' E	728	50	16	18	24	Brown trout	24	184	1.17	5
Gossenköllesee	Alps (Austria)	47°00' N	11°00' E	2413	1.6	0.30	9.9	26	Brown trout	20	93	1.01	4
Velké Hincovo	Tatra (Slovakia)	49°10' N	20°03' E	1946	18	1.4	53	6	Brown trout	24	134	0.95	14

[a] Number of analyzed fish. [b] Mean of all individuals analyzed in each lake.

Table 2. Water chemical parametres of studied lakes

Lake	pH	Conduc. (mS cm⁻¹)	Alkalinity (µequ L⁻¹)	Ca²⁺ (mg L⁻¹)	Mg²⁺ (mg L⁻¹)	Na⁺ (mg L⁻¹)	K⁺ (mg L⁻¹)	SO₄⁺ (mg L⁻¹)	Cl⁻ (mg L⁻¹)
Redó	6.32	1.2	41	1.5	0.17	0.2	0.06	1.48	0.18
Øvre Neådalsvatn	6.16	0.9	26	0.38	0.09	0.76	0.12	0.65	1.04
Gossenköllesee	6.96	2.1	91	1.8	0.40	0.69	0.14	0.26	0.13
Velké Hincovo	6.85	2.3	106	2.94	0.18	0.34	0.07	2.29	0.16

Table 3. Concentration of selected metals in trout tissues. Each value represents mean ± standard deviation and concentrations are given in µg g wet weight.

		Redo	Ø. Neådalsvatn.	Gossenköllesee	Velké Hincovo
Pb	Liver	0.135 ± 0.068	0.056 ± 0.038	0.376 ± 0.218	-
	Kidney	1.789 ± 1.352	0.200 ± 0.121	5.686 ± 6.008	13.14
Cd	Liver	1.846 ± 1.301	0.110 ± 0.028	0.359 ± 0.165	-
	Kidney	8.065 ± 4.172	0.366 ± 0.100	1.634 ± 0.353	23.7
Zn	Liver	30.50 ± 4.00	28.06 ± 5.69	22.85 ± 4.30	-
	Kidney	35.70 ± 9.09	46.74 ± 11.09	24.78 ± 6.64	-
Cu	Liver	200.65 ± 226.28	74.20 ± 39.82	56.62 ± 29.47	-
	Kidney	2.62 ± 0.72	2.03 ± 0.50	3.89 ± 2.08	-
Hg	Muscle	0.050 ± 0.026	0.013 ± 0.008	0.018 ± 0.005	

- Not determined.

Table 4. *Polycyclic aromatic hydrocarbons and organochlorine compounds in liver and muscle. Each value represents mean and standard deviation. All concentrations are expressed in ng g^{-1} wet weight.*

	Tissue	Redo	Ø. Neådalsvatn.	Gossenköllesee	Velké Hincovo
Σ PAHs[a]	Liver	17.90 ± 4.43	37.74 ± 16.62	16.51 ± 8.42	33.02 ± 10.5
HCB	Muscle	0.60 ± 0.36	0.58 ± 0.21	0.33 ± 0.09	0.30 ± 0.11
	Liver	0.67 ± 0.37	0.64 ± 0.40	0.47 ± 0.36	0.69 ± 0.02
Σ HCHs[b]	Muscle	1.6 ± 0.90	0.28 ± 0.12	027 ± 0.05	0.91 ± 0.44
	Liver	2.85 ± 2.5	0.95 ± 1.5	0.61 ± 0.42	3.2 ± 1.6
p,p' DDE	Muscle	18 ± 13	0.52 ± 0.32	2.2 ± 0.85	33 ± 12
	Liver	18 ± 25	0.69 ± 0.61	3.6 ± 3.4	61 ± 11
p,p' DDT	Muscle	1.2 ± 0.59	0.22 ± 0.18	0.43 ± 0.16	2.6 ± 0.97
	Liver	0.7 ± 1.2	0.09 ± 0.15	1.3 ± 0.51	2.8 ± 0.83
Σ PCB[c]	Muscle	8.2 ± 4.8	1.5 ± 0.57	7.8 ± 2.5	17 ± 3.5
	Liver	12 ± 11	5.6 ± 3.0	0.47 ± 0.36	22 ± 4.1

[a] Sum of fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene, indeno(cd-1,2,3)pyrene and benzo(g,h,i)perylene. [b] Sum of α and γ isomers. [c] Sum of PCB congeners 28, 52, 101, 118, 138, 153, 180.

Table 5. Histopathological alterations foun in gills, liver and kidney and biochemical analysis of livers. Each value represents mean and standard deviation.

	Redo	Ø. Neådalsvatn.	Gossenköllesee	Velké Hincovo
Gill fil. gob. [a]	3.0 ± 4.0	2.1 ± 2.2	26.9 ± 16.7	-
Gill lam. gob. [b]	1.4 ± 2.0	9.4 ± 8.8	2.6 ± 2.7	-
Kidney macrophages [c]	44.3 ± 23.8	17.8 ± 6.9	16.6 ± 6.8	-
Liver macrophages [d]	6.6 ± 8.8	10.4 ± 11	1.71 ± 1.70	54.3 ± 54.2
Liver lipid degeneration. [e]	0.481 ± 1.132	4.008 ± 3.421	0.224 ± 0.386	-
Liver inflammation [f]	1.17 ± 0.39	1.57 ± 1.00	1.13 ± 0.45	2.4 ± 0.67
Glycogen ($\mu M\ g^{-1}$)	16.2 ± 16.2	5.0 ± 4.9	55.8 ± 30.1	-
GSH ($\mu M\ g^{-1}$)	1.3 ± 0.6	1.5 ± 0.4	1.22 ± 0.39	0.56 ± 0.4
GSSG ($nM\ g^{-1}$)	24.1	48.6	14.2	69
Vitamina C ($\mu g\ g^{-1}$)	88.92	174.41	173.60	129
Riboflavin ($nM\ g^{-1}$)	1.75	0.80	0.41	12.8
G-6-PDH ($U\ g^{-1}$)	90.8 ± 41.8	127.9 ± 43.2	97.0 ± 68.9	-

[a]Number of goblet- (mucus-) cells on filament / 100 lamellae. [b]Number of goblet cells on lamellae / 100 lamellae. [c]Kidney melanomacrophages, % of area in section. [d]Liver melanomacrophages mm^{-2}. [e]Lipid depeneration (storage) in liver, % of area in section. [f]1 Normal liver, 2 Moderate liver inflammations 3 Severe liver inflammations.

Results and Discussion

Fish population characteristics. Fish appeared well nourished as indicated by the condition factor (Table 1). This factor should be close to one for ideally shaped trout, a criterion which is met by the fish from all lakes. Average ages of the fish collected range between 4 (Gossenköllesee) and 14 years (Vel'ké Hinçovo). Fish from this last lake presented significant older ages than the rest of the studied lakes and higher proportion of male specimens than females (80 % of the total individuals of this lake). Fish caught from lakes in Norway, the Alps and the Pyrenees were in similar number of both sexes. No correlation is found between altitude or latitude and age (Figure 2). Conditioning factor shows no trend with altitude, but correlates good with latitude (Figure 2). Lakes located more north contain fish with higher conditioning factors, although, as it has been pointed out before, the conditioning factors are not significantly different between lakes.

Metals and organic pollutants levels. Concentrations in fish tissue of metals and organic pollutants are presented in Tables 3 and 4, respectively. Most of the metals that were analyzed in liver and kidney show higher values in the latter. Copper was more abundant in liver. Lower levels of lead and cadmium were found in Øvre Neådalsvatn, while Gossenköllesee presented the smallest concentrations for zinc and copper. Higher concentrations of cadmium and copper were observed in Redó when Vel'ké Hincovo measurements were not available. Fish from Tatra mountains presented clearly higher values of lead and cadmium (13 and 24 μg g^{-1} ww, respectively). Mercury is only analyzed in muscle tissue to evaluate its bioaccumulation and it is specially useful for detecting pscivorous fish in a population ([20]). All fish presented similar mercury levels in muscle (around 0.1 μg g^{-1} ww), indicating the absence of carnivorous fish in these lakes. Metal concentrations do not show any significant correlation with age or conditioning factor of fish, and neither with altitude or latitude of the lake (ANOVA test).

Hexachlorobenzene (HCB), hexachlorocyclohexanes (HCHs), DDTs, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) were analyzed in liver and muscle tissue. Organochlorine compounds present are consistent with the pattern observed in the sediments of the same lakes ([4]). In general, slightly higher concentrations are found in liver than in muscle. HCB presented similar levels in all lakes (0.30-0.69 ng g^{-1} ww). Concentrations of HCHs were higher in lakes Redó and Vel'ké Hincovo (between 0.91-3.2 ng g^{-1} ww), probably due to the actual use of γ-HCH in these regions. These results are is in accordance with previous studies of water and air samples of these locations ([17]). Concentrations of HCHs in lakes Øvre Neådalsvatn and Gossenköllesee do not exceed (0.95 ng g^{-1} ww) for both tissues. pp'-DDE, pp'-DDT and PCBs show a similar pattern (significant correlation ANOVA, $p < 0.05$) with higher concentrations in fish from Tatra mountains, followed

by lake Redó and, at 1-2 orders of magnitude lower, by Øvre Neådalsvatn and Gossenköllesee. PCBs and DDTs present a good correlation with age (ANOVA, $p<0.05$), a feature that is not found with HCHs and HCB.

A completely different picture is seen for PAHs. The concentration of PAHs in trouts is similar in all studied lakes (Table 4), while sediment PAH concentrations of Tatra mountains (7500 ng g^{-1} dw) are 2 orders of magnitude higher than the rest of the European lakes. Sediment levels of Redó, Gossenköllessee and Øvre Neådalsvatn do not differ significantly with each other (560, 590 and 540 ng g^{-1} ww, respectively). Furthermore, observed PAH concentrations in liver do not correlate with age or conditioning factor of the fish, and neither with altitude or latitude of the lake (ANOVA test).

Histopathological analysis. Highest numbers of melanomacrophages in liver, i.e. macrophages having accumulated melanin granules, are most abundant in Ve'lké Hincovo and Øvre Neådalsvatn followed by one order of magnitude lower Gossenköllessee and Redó (Table 5). Salmonid liver usually do not store high amounts of lipids in the liver. Thus increased lipid storage in this organ can be interpretated as a pathological sign. Highest amount of lipid degradation and inflamation in liver was again found in fish from Norway and Tatra mountains (Table 5). According to the higher age of the Redo fish (Table 1) melanomacrophages are more abundant in the kidney of these fish (Table 5) in compare with the younger fish from other lakes.
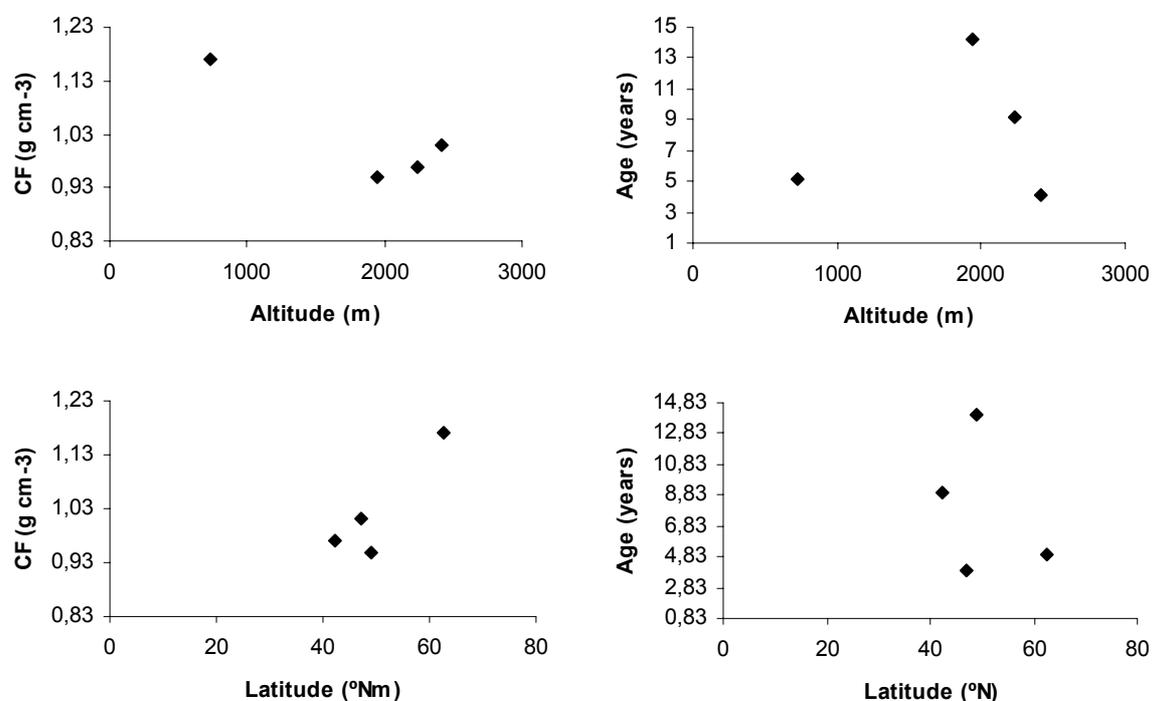

Figure 2. Altitude and latitude correlations with average conditioning factor and mean age of each lake

Liver glycogen, a major energy store of salmonids is highest in Gossenköllesee followed by Redo and Øvre Neådalsvatn (Table 5). Glutathione (GSH) the most abundant thiol compound in most tissues is a powerful antioxidant and shows highest concentrations again in Øvre Neådalsvatn (Table 5). Glutathion disulphide (GSSG) is formed when GSH is oxidized. Therefore GSSG is an indicator for oxidative stress in tissues and higher values of this parameter indicate a high activity of the detoxification enzyme system ((13)). GSSG concentration is between 4 and 7 times higher in Øvre Neådalsvatn and Vel'ké Hincovo than the Alps and Pyrenees lakes (Table 5). Another parameter which is usually high in fish suffering oxidative stress is glucose 6-phosphate dehydrogenase (G-6-PDH). This enzyme is highest in fish liver from Øvre Neådalsvatn followed by Gossenköllessee and Redo (Table 5).

State of fish health and pollution impact. Looking at condition factors as a measure for fish health (Table 1) fish from all locations appear well nourished and healthy. In contrast to this parameter liver glycogen (Table 5) is lowest in Øvre Neådalsvatn indicating that despite the general good appearance of the fish additional energy is required for more processes, possibly detoxification and repair. This is corroborated by histological findings. Llipid degeneration and macrophage densities in the livers of Vel'ké Hincovo and Øvre Neådalsvatn are higher than in the other lakes. The number of macrophages in the liver is a measure for tissue turnover in this organ where they remove dead cells and debris. Their number increases with age or when pollutants damage liver tissue ((27), (28), (29)). Elevated numbers of macrophages in liver from Vel'ké Hincovo fish could be attributed in part to the older age of this population. However, Øvre Neådalsvatn fish are younger. This histological damage is further fortified by biochemical data. GSSG concentrations in liver from fish of these lakes indicate oxidative stress and the high G-6-PDH activity suggests increased detoxification activity. Both biochemical and histological analyses give evidence that the fish from Øvre Neådalsvatn and Vel'ké Hincovo suffer health impact.

The relative homogeneous distribution of POPs among fish tissue for the different lakes may indicate a similar POP input and availability of these compounds to fish. In the case of PCBs and DDTs this maybe true, since between lake sediments and fish tissue a parallelism is observed between their concentrations ((4)). For HCHs, highest fish tissue levels are coincidence with high lake water concentrations ((17)). However, there is a large discrepancy between PAH levels in fish and other lake compartments, such as sediments and atmospheric deposition. While PAH concentrations in fish are relatively homogeneous among lakes, the atmospheric and sediment concentrations in Øvre Neådalsvatn and Vel'ké Hincovo show levels that are magnitudes higher than the other lake sites ((3)). This discrepancy may indicate that PAH in Øvre Neådalsvatn and Vel'ké Hincovo are metabolized in a higher rate by fish after uptake.

Upon all the pollutants analyzed in the fish samples, concentration of PAHs in liver shows a significant correlation with lipid degradation and inflammation (ANOVA, $p < 0.01$), although no significant relation is found with parameters describing a high detoxifying activity like GSSG and G6PDH. However, PAH concentration may only be a measure of tissue burden and do not describe the turnover rate of these compounds. PAHs are easily metabolized by the phase I enzymes to more hydrophilic products products ([30]) and conjugated by phase II enzymes of the mixed function oxygenase system (MFO). Both products may further on bind to DNA and other structures within the cells leading to unspecific lesions, mutation and cancer. The occurrence of hydroxilated PAHs (OH-PAHs) in fish bile is evidence that PAHs are metabolized by the fish ([31], [32], [33], [34]). The rate of PAH metabolism in turn is a measure for the possible toxic action ([35], [13]). Escartín and Porte (1999) [36] reported OH-PAH concentrations in bile of fish of these mountain lakes. Fish from East European mountain lake presented the highest OH-PAH concentrations in bile (990 ng mL^{-1}) followed by fish from Øvre Neådalsvatn (454 ng mL^{-1}). Bile levels in Gossenköllessee and Redó were not over 200 ng mL^{-1}. These PAH metabolite concentrations correlate significantly with liver macrophages and GSSG levels, histopathological effect and biochemical response respectively (ANOVA, $p < 0.05$). The differences to PCB metabolism must be sought in the different availability to the activating system and consequently a slower rate of biotranformation of these compounds. PCBs and most other low volatile organochlorines are too lipophilic and remain dissolved in the adipose tissues without excerting any toxicity when living conditions for the organism remain adequate.

Acknowledgements

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ARTICLE 9

POLYBROMINATED DIPHENYL ETHER (PBDE) FLAME RETARDANTS IN FISH FROM LAKES IN EUROPEAN HIGH MOUNTAIN AND GREENLAND

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Polybromodiphenyl Ether (PBDE) Flame Retardants in Fish from Lakes in European High Mountains and Greenland

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Abstract

Individual polybromodiphenyl ethers (PBDE) were investigated in liver and muscle tissue of trout from 11 high mountain lakes in Europe and one in Greenland. Trouts in these lakes, brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*) and arctic charr (*Salvelinus alpinus*), are important sentinel species because they are located in the top of the food chain and pollution can only reach these ecosystems by atmospheric transport. The major PBDE congeners were BDE-47 and BDE-99, followed by BDE-100, BDE-153, BDE-154 and BDE-28. These compounds were found in all the samples examined. Their average concentrations, 110-1300 and 69-730 pg g^{-1} ww in liver and muscle, respectively, (2400-40000 and 2900-41000 pg· g lipid weight^{-1} –pg·glw^{-1}-, respectively) were in the lower range when compared with those of fish from other less remote locations. The highest levels of PBDEs in liver and muscle are found in Lochnagar, Scotland, 11000 and 1200 pg g^{-1} ww, respectively (366000 and 177000 pg·glw^{-1}, respectively). Male specimens exhibited higher PBDE concentrations in liver than female. The concentrations of most PBDE in liver were correlated with fish age ($p < 0.01$) and, inversely, with condition factor ($p < 0.01$). Muscle PBDE concentrations did not correlate with age and only some congeners showed significant positive correlations with condition factor ($p < 0.05$). Main differences between species were found in the accumulation of the more abundant PBDE, brook trout showing the highest concentrations in muscle and the lowest in liver. No correlation between the occurrence of these compounds in high mountain fish and altitude, latitude or temperature was observed. This fact and the lack of correlation between muscle concentrations and age suggest that the fluxes of PBDE arriving to high mountain lakes are still not constant. In view of the present use of these compounds they are probably increasing.

Introduction

Polybromodiphenyl ethers (PBDE) are used as flame retardants for plastics, printed circuit boards, textile and polyurethane foam in furniture and cars. PBDEs are very hydrophobic (log Kow values 5.7-8.3) (1) and resistant to degradation. Their subcooled vapor pressures have been observed to be lower than those of polychlorobiphenyls (PCBs) and decrease at increasing bromine substitution (2). Tetra- and penta-substituted PBDEs have been shown to be persistent, lipophilic and environmentally ubiquitous (3-4). In this respect, congeners BDE#47 and BDE#99 have shown higher bioaccumulation factors than PCB despite the larger molecular size of the bromine substituents (5). Biotransformation of PBDEs is also though to be relatively slow, leading to their accumulation in biota (6). Biomagnification of PBDEs through trophic levels has recently been observed (7-9).

PBDE, unlike PCB, are still being used as additives for consumer products. In 1999, European consumption of penta-BDE, octa-BDE and deca-BDE was estimated to be 210, 450 and 7500 metric tones, respectively, and in north and south America 8290, 1375 and 24300 metric tones, respectively (10). The widespread occurrence of these compounds in the environment deserves growing concern because of their stability, lipophilicity and potential toxicity. In this respect, the use of penta BDE has recently been banned in the European Union.

Few data on PBDE occurrence are available for remote regions (8, 11, 12). However, their chemical properties suggest that they should also be distributed in high mountain regions such as other persistent organic pollutants (13-15). In this respect, high mountain lakes are ideal ecosystems for assessment of the impact of these compounds in these remote regions. These lakes are those situated far from local pollution emission sources, above the regional tree line and with hydrology dominated by atmospheric processes. Contaminants only arrive to them by aerial transport (13-16). Fish as top chain predators in these environments are ideal sentinel organisms for examining the importance of these compounds in these aquatic systems. For this purpose, liver and muscle tissue of trouts inhabiting these lakes have been examined. To the knowledge of the authors, this is the first time that concentrations of PBDE are investigated in mountain lake ecosystems. In addition, one lake from Greenland is also included in the study to examine the potential impact of these compounds in very remote regions.

Materials and Methods

Sampling sites and fish collection. Eleven high mountain lakes (728-2485 m above sea level) in Europe and one in Greenland were selected for study (Figure 1 and Table 1). These lakes are distributed among the main European mountain ranges (the Tatra, the Alps, the Pyrenees, the Rila and the mountains from Norway and Scotland) and Greenland. Geographical, physical and biological information of the studied lakes is summarized in Table 1. Fish sampling was performed during the summer months of 2000 and 2001 and followed standard

test fishing procedures with multifilament gillnets. All fish were measured, dissected and their sex determined in the field. Muscle fillets and liver tissues were wrapped in a pre-cleaned aluminium foil and stored at –20ºC until analysis. Otoliths and scales were kept for age determination at the Norwegian Institute of Water Research. Age was primarily estimated by counting the number of annual ridges formed during winter (17).
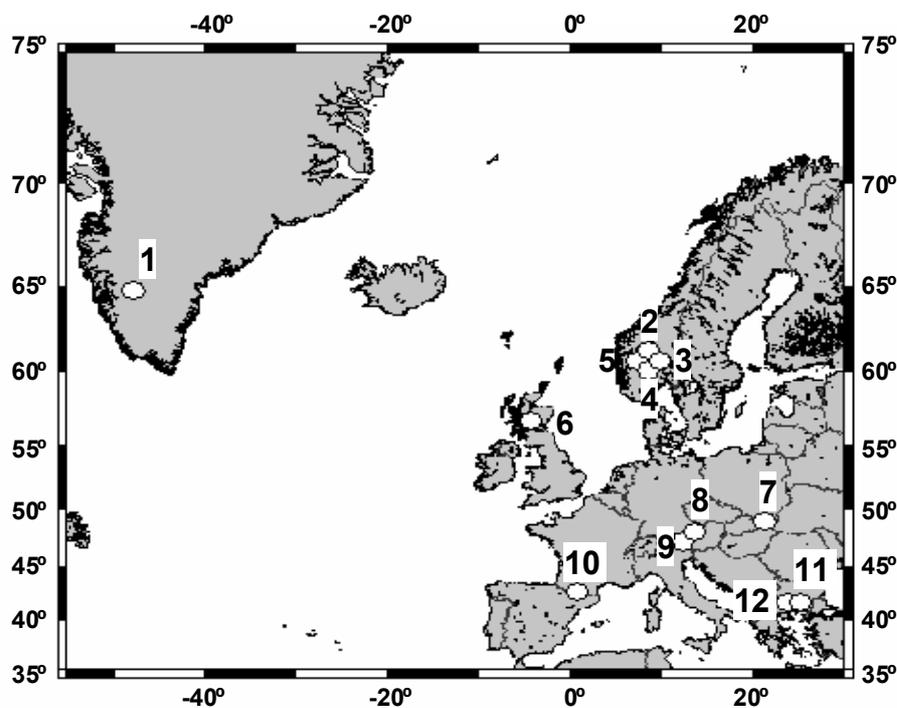
Figure 1. Location of the Greenland and European high mountain lakes selected for this study. Lake numbers as in Table 1.

Sample handling. PBDE in muscle were analyzed following methods proposed elsewhere (18) after some modifications for the joint analysis of organobromine and organochlorine compounds (OC). Briefly, muscle tissue (5 g) was ground with activated sodium sulphate (Merck, Darmstadt, Germany) until a fine powder was obtained. This mixture was introduced into cellulose cartridges (Whatman, England) and Soxhlet extracted with n-hexane:dichloromethane (4:1) (Merck, Darmstadt, Germany) for 18 hours. Lipid content was determined gravimetrically using 20% of the extract. The remaining lipids, after adding PCB#209 standard (Promochem, Wesel Germany), were removed by oxidation with concentrated sulfuric acid (Merck, Darmstadt, Germany) and PBDE extracted with n-hexane in successive repeated steps (5 times). All n-hexane solutions were combined and concentrated by vacuum rotary evaporation (20 ºC, 20 Torr) to small volumes (ca. 500 µl). Then, they were further concentrated to nearly dryness under a gentle nitrogen flow and redissolved in 50 µl of isooctane (Merck, Darmstadt, Germany).

Table1. Description of the high mountain lakes and fish included in the study.

#	Lake Name	Latitude (N)	Longitude (E)	Altitude[a] (m)	Temperature[b] (°C)	Number of fish analyzed	Species	Age[c] (yr)	Conditioning Factor[d] (cg.cm⁻³)	Sex[e]	Lipid in muscle[f] (%)	Lipid in liver[f] (%)
1	Fergusson	66,96667	-50,65000	60	-[g]	4	arctic charr	7,7	0,8[h]	1,3	1,90	4,29
2	Øvre Neådalsvatn	62,77778	8,98237	728	3,25	5	brown trout	4,6	1,08	1,4	2,90	5,91
3	Fallbekktjørna	62,74996	9,03719	1043	1,36	5	brown trout	11	0,89	1,7	0,74	6,27
4	Nedre Neådalsvatn	62,41243	7,98756	566	4,22	2	brown trout	4,3	1,08	2,0	2,25	-
5	Øvre Heimdalsvatnet	61,41877	8,89696	1088	1,09	3[i]	brown trout	3,7	0,85	1,5	0,94	-
6	Lochnagar	56,95914	-3,23128	790	3,7	4	brown trout	6,5	0,82	1,0	0,65	-
7	Velké Hincovo	49,17970	20,06060	1946	0,5	5	brown trout	14	0,95	1,0	1,17	3,41
8	Gossenkoellesee	47,22528	11,01390	2413	-0,33	5[j]	brown trout	5,4	1,08	1,6	-	2,15
9	Rotfelssee	47,22647	11,00796	2485	-0,74	5	arctic charr	8,2	0,78	1,5	1,16	3,59
10	Redon	42,64208	0,77951	2235	3,18	7	brown trout	8,6	0,98	1,5	3,12	4,59
11	Okoto	42,19964	23,30584	2440	-0,09	5	brook trout	3,8	1,54	1,6	4,14	3,53
12	Bliznaka	42,20122	23,31497	2243	1,09	5	brown trout	3,0	1,01	1,8	2,50	-

[a]Meters above sea level. [b]Annual average air temperature. Meteorological data were supplied by the Department of Geology and Geophysics from the University of Edinburgh, UK. Lake site air temperatures were calculated from WMO data and corrected with daily altitudinal lapse rates. [c]Average age of the fish analyzed in each lake. [d]Average CF of the fish analyzed in each lake. [e]Male= 1, Female= 2. Average value of the fish analyzed in each lake. [f]Average value of the fish analyzed in each lake. [g]Not determined. [h]Conditioning Factor calculated as weight*100/length³. [i]Only muscle tissue was analyzed. [j]Only liver was analyzed.

Liver tissues were analysed following a slightly modification of the methodology published elsewhere (19) for the joint analysis of PBDE, OC and polycyclic aromatic hydrocarbons. The resulting method was similar to the one proposed specifically for PBDE (17). Briefly, liver samples (0.5 g) were ground with activated sodium sulphate and spiked with PCB#209 as surrogate standard. This mixture was Soxhlet extracted with n-hexane:dichloromethane (4:1) for 18 hours. A clean-up step was performed with an open aluminum oxide (Fluka Type 507C, Fluka AG, Switzerland) column (5 g) eluted with 15 ml of n-hexane:dichloromethane (19:1, v:v) and 3 of ml n-hexane:dichloromethane (1:2, v:v). The extract was concentrated by vacuum rotary evaporation (20ºC, 20 torr) to small volumes (ca. 500 µl) and further concentrated to nearly dryness under a gently nitrogen flow and redissolved in 50 µl of iso-octane.

Lipid content was determined using 20% of the Soxhlet extract which was dried to constant weight under a gentle nitrogen flow. Then, lipid content was measured gravimetrically and reported as percent to wet weight. Occasionally, when the lipid measurement of an individual sample was not available, the mean lipid content of all fish from the same lake was taken as reference. Estimates of lipid in liver from fish of lakes Øvre Neådalsvatn, Lochnagar and Bliznaka were calculated by interpolation in regression equations between lipid muscle and lipid content (e.g., lipid content in liver = 0.89 x lipid content in muscle + 2.52, $r^2 = 0.70$) calculated from lakes with the same trout species.

Instrumental Conditions and PBDE quantification. Samples were analyzed by negative ion chemical ionization mass spectrometry coupled to gas chromatography (GC-MS-NICI). A GC system from Agilent Technologies 6890A (USA) coupled to an MS detector 5973N was used. The instrumental conditions and quantification methodology for PBDE is described elsewhere (20). The system was equipped with a HP-5MS (30 m x 0.25 mm i.d. x 0.25 µm film thickness) and the oven temperature program was from 110ºC (held for 1 min.) to 180ºC (held for 1 min.) at 8ºC·min^{-1}, then from 180ºC to 240ºC (held for 5 min.) at 2ºC·min^{-1}, and then to 300ºC (held for 6 min.) at 2ºC·min^{-1}. Helium was used as carrier gas at 10 psi and ammonia was chosen as ionization gas (1.6 10^{-4} Pa). Transfer line and quadrupole temperatures were 280ºC and 150ºC, respectively. PBDE analysis was performed at m/z values of 79/81 [Br]$^-$, 161 [HBr$_2$]$^-$, 327, 405, 483, 563 and 643, corresponding to [M]$^-$ or [M-HBr$_2$]$^-$. Quantification was performed at m/z value of 79, which is the base peak of all PBDEs monitored. Retention time shifts could not be higher than 1 sec The mass ion acquisition program is described in detail elsewhere (20).

Quality Assurance. Procedural blanks were analyzed for every set of six samples. Average values ranged between 4.2-27 and 12-25 pg g ww^{-1} for liver and muscle, respectively (Table 3). These levels accounted for 3-10% of the individual PBDE concentrations and in a few cases up to 40%. The recoveries of the surrogate standard, PCB#209, were calculated for

each sample being 89±18 % (average ± standard deviation) for muscle and 86±18 % for liver. Relative recoveries of individual PBDE to surrogate standard recovery were between 77-100%. Each congener was corrected with their relative recovery for calculation of the concentrations in each sample. Identification and quantification of all studied compounds were performed by injection of external standards at different concentrations using the PBDE Analytical Standard Solution EO-5099 (Cambridge Isotope Laboratories, Inc., MA. USA) that contains 39 individual congeners (# 1, #2, #3, #7, #8, #10, #11, #12, #13, #15, #17, #25, #28, #30, #32, #33, #35, #37, #47, #49, #66, #71, #75, #77, #85, #99, #100, #116, #118, #119, #126, #138, #153, #154, #155, #166, #181, #183, and #190) and 11 ^{13}C-labeled PBDE compounds (# 3, #15, #28, #47, #77, #99, #100, #118, #126, #153, and #183) in the same concentration as their native congeners. Therefore, quantification was performed by the external standard method with PBDE relative recovery correction. Since some congeners of the standard mixture are in the native and the isotope labeled form, the area of the GC-MS-NICI base peak records was divided by two for compilation of the external calibration lines. Limits of detection (LOD) were calculated from real samples as the mean of noise signal plus 3 times the standard deviation (n = 5). They were in the order of 9.2-13 pg·g^{-1} ww in muscle and 3.4-18 pg·g^{-1} ww in liver (Table 3).

Results

  Fish population characteristics. Fifty-five trout specimens were analyzed (Figure 1 and Table 1). Brown trout (*Salmo trutta*) was the species found in most lakes (Table 1). Okoto contained brook trout (*Salvelinus fontinalis*) and Fergusson and Rotfelssee contained arctic charr (*Salvelinus alpinus*).

  The ages of the individual fish specimens range between 1 and 21 years, in Redon and Fallbekktjørna, respectively. Average ages of fish collected in each lake range between 3 (Bliznaka) and 14 years (Veľké Hincovo) (Table 1). A slightly higher number of male (n = 33) than female (n = 29) fish were analyzed although the overall figure is close to 50% each (Table 1). The mean condition factors of the fish species were 0.97, 1.54, and 0.79 cg·m^{-3} for brown trout, brook trout and arctic charr, respectively. No significant differences in condition factor were found between species (ANOVA p > 0.01). Thus, no further interspecific difference was considered in relation to this variable. An inverse significant linear correlation (p < 0.01; n = 62; $r^2 = 0.108$) between condition factors and ages of the individual specimens was observed. Older specimens presented lower condition factors. Condition factor was not significantly different between both sexes (ANOVA p > 0.1) being 0.94 and 1.04 cg·cm^{-3} for male and female, respectively.

  Age did not show any significant correlation with lipid content but the condition factor correlated significantly with lipids in muscle (p < 0.0001; n = 62; $r^2 = 0.242$).

No correlation was found between lake location or altitude and age, condition factor, sex or lipid content in muscle.

PBDE distributions. BDE#47 is the most abundant congener in the majority of the samples (Figure 2) as observed in many other environmental studies (9, 21, 22). In the majority of the lakes, this congener represented more than 30% of total PBDEs in muscle and more than 40% in liver. BDE#99, #100, #153, #154 and #28 were the other major congeners in all samples (Figure 2). Nearly all samples exhibited rather uniform PBDE distributions. This uniformity is rather significant since it includes lakes from Greenland, the Alps, the Tatra, the Rila, the Pyrenees and Norway.
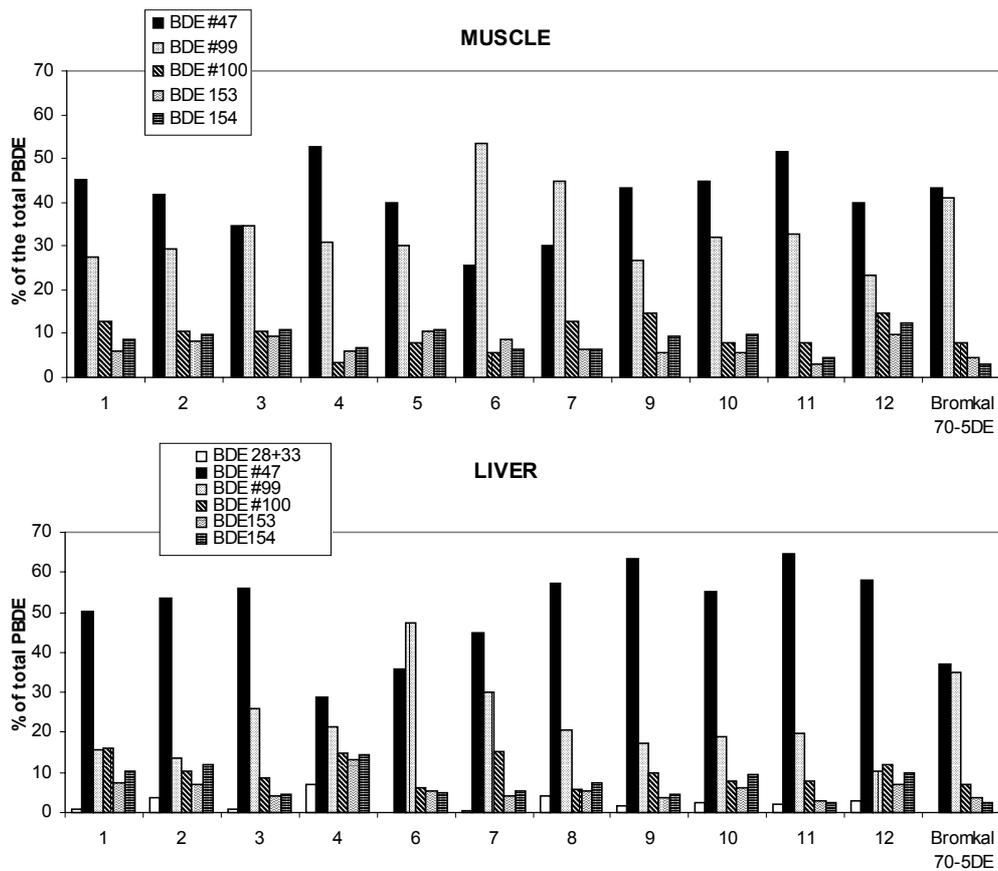
Figure 2. Relative distribution of PBDE in muscle and liver expressed as percentage of total PBDE. Lake numbers as in Table 1. The composition of Bromkal 70-5DE is taken from (39).

In contrast, significant differences were found in muscle and liver tissues of fish from Lochnagar, showing a double fold BDE#99 concentration than BDE#47 for muscle and slightly higher concentrations of BDE#99 than BDE#47 in liver of each individual specimen (Figure 2). In Veľké Hincovo fish, BDE#99 was in higher concentration than BDE#47 in muscle but BDE#47 predominated in liver following the general trend. The ratio between these two congeners, BDE #47 and #99, in Bromkal 70-5DE is around 1.1 (Figure 2). As it will be shown later, fish from Lochnagar are also those exhibiting highest PBDE concentrations. Thus, the

distinct pattern in this lake points to a direct contamination by PBDE from a nearby source whereas the other lakes exhibit the regular PBDE distribution found in other environmental studies (9, 21, 22). The close parallelism between PDBE distributions in specific sites and commercial mixtures has also been interpreted to reflect episodes of point source contamination in other studies (23).

The higher proportion of BDE#47 in liver than in muscle is also a major feature of these distributions. Having in mind the chemical structures of BDE#47 and BDE#99, 2,2',4,4'-tetrabromodiphenyl and 2,2',4,4',5-pentabromodiphenyl eters, respectively, it could be hypothesized that the increase in the former could originate, at least in part, by debromination of the later at position 5 (or *meta*) as consequence of the action of some liver enzymatically-mediated detoxification mechanism. Consistently, higher proportions of BDE#100 (2,2',4,4',6-pentabromodiphenyl eter) than BDE#154 (2,2',4,4',5,6'-hexabromodiphenyl eter) are generally found in liver than in muscle that is again in agreement with this debromination mechanism at *meta* positions in the phenyl rings. However, higher proportions of BDE#47 vs. BDE#99 have also been found in other environmental samples (9, 21, 22) including organisms of lower metabolic activity (9). Thus, the occurrence of less brominated congeners in livers could also reflect a better parallelism between environmental PBDE distributions in liver than in muscle, as generally observed with the polychlorinated compounds (26).
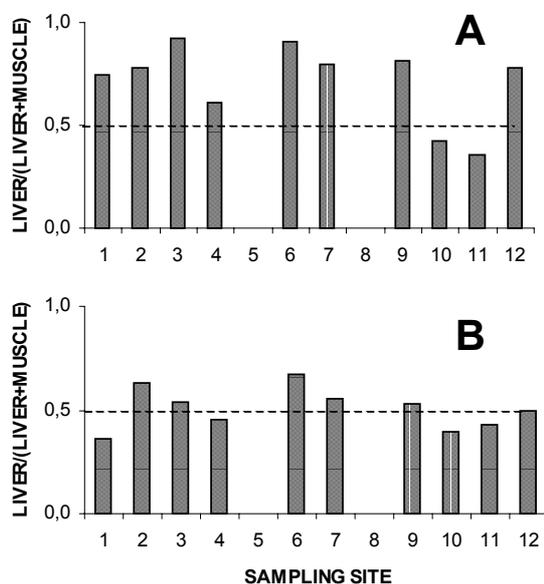

Figure 3. Lake-averaged relative content of PBDE in fish liver and muscle. The ratio is calculated by division of the lake-averaged concentrations in liver by the summation of the lake-averaged concentrations in liver and muscle. Concentrations are referred to wet weight (A) and to lipid weight (B). Lake numbers as in Table 1.

In most lakes, the PBDE concentrations were higher in liver than in muscle (Figure 3). Only fish from Okoto and Redon showed the opposite situation. In previous studies on marine fish slightly higher concentrations in liver than muscle have been observed in cod and whiting and the other way around in herring (9). However, when the results of the high altitude lakes are normalized to tissue lipid weight nearly the same concentrations are observed in the two types of tissues (Figure 3).

Comparison of the log-transformed muscle and liver concentrations in the specimens where measurements in both tissues were available (n = 19) did not show significant correlations (Table 2) except in the case of BDE#99 ($r^2 = 0.304$; p<0.05). However, significant positive correlations between both tissues for all congeners are observed when concentrations are normalized to lipid content ($r^2 = 0.43$-0.64; p <0.001-0.0001; Table 2). Therefore, normalization to lipid content reveals a situation of uniform distribution of all congeners between the two tissues.

Lake-averaged PBDE concentrations. PBDE congeners #47, #99, #100, #153 and #154 were found in all samples, representing a relative proportion of more than 75% of the total 39 PBDEs analyzed (Figure 2; Table 3). BDE#28 was also found in all liver samples examined but it could not be quantified in muscle due to a coeluting compound. This congener may be a metabolite of BDE with higher degree of bromination (25). Less than 20% of the samples occasionally presented concentrations of other congeners similar to the detection limit levels and therefore were not considered. The mono-, di- and heptabromo congeners were not detected in any of the samples. PBDE of higher degree of bromination, e.g. BDE209, were not found.

Lake-averaged total PBDE concentrations were calculated by summation of the lake-averaged concentrations of the congeners reported in Table 3 after individual analysis of all specimens available from each lake. The values obtained from all lakes except Lochnagar range between 69-730 pg·g ww^{-1} (2900-41000 pg·gram of lipid weight^{-1} (pg·glw^{-1}) and 110-1300 pg·g ww^{-1} (2400-40000 pg·glw^{-1}) in muscle and liver, respectively, being in the lower range of the concentration values previously reported in biota from other environments (Table 4). In turn, these concentrations are about 2-3 orders of magnitude lower than those of organochlorine compounds such as PCB or DDE found in fish from high mountain lakes (13).

Table 2. Table 2. Correlation coefficients (r²), enrichment ratios, and ANOVA significance of the log-transformed concentrations of PBDE normalized to wet weight and lipid content in liver and muscle when compared to fish age, sex, species and conditioning factor.

COMPOUND	LIVER vs MUSCLE[a]	LIVER[b]						MUSCLE[b]				
		conditioning factor[c]		age[c]		species[c]	sex[c]	conditioning factor[d]		age[d]	species[d]	sex[d]
	r²	r²	ratio[e] 2.08/0.71	r²	ratio[e] 21/1 year old	sign[f]	sign[f]	r²	ratio[e] 2.08/0.71	r²	sign[f]	sign[f]
wet weight												
BDE #28+33	n.a.	0,002	_g	0,068	-	*	-	n.a.[h]	n.a.	n.a.	n.a.	n.a.
BDE #47	0,041	0,07	-	0,152**	4	-	-	0,215*	13	0,01	***	-
BDE #99	0,304*	0,06	-	0,265****	12	-	-	0,185*	11	0,005	**	-
BDE #100	0,066	0,139**	8	0,174**	5	-	-	0,018	-	0,021	*	-
BDE#153	0,162	0,178**	7	0,043	-	-	-	0,021	-	0,008	-	-
BDE#154	0,06	0,179**	9	0,061	-	*	-	0,02	-	0,016	-	-
lipid weight												
BDE #28+33	n.a.	0,01		0,063		-	-	n.a.[h]		n.a.	n.a.	n.a.
BDE #47	0,45***	0,028		0,127**		-	-	0,045		0,009	*	-
BDE #99	0,646****	0,032		0,243****		-	-	0,058		0,058	-	-
BDE #100	0,43***	0,089*		0,168**		-	-	0,226**		0,089	*	-
BDE#153	0,45***	0,107*		0,04		-	-	0,263**		0,058	-	-
BDE#154	0,48***	0,113*		0,058		-	-	0,289**		0,089	-	-

[a]Concentrations of the individual specimens (n = 19). [b]All lakes, except Lochnagar (#6). [c]Concentrations of the individual specimens (n = 46). [d]Concentrations of the individual specimens (n = 28). [e]Maximum increases for the largest conditioning factor, age and lipid intervals represented by the fish included in Table 1 as predicted by the corresponding curve fitting equations. [f]Significance of the ANOVA test. [g]Only values for the significant correlations are given. [h]n.a. = not applicable. *p<0.05. **p<0.01. ***p<0.001. ****p<0.0001.

Table 3. Lake-averaged concentrations (± standard deviation), blank values (± standard deviation) and limit of detection of PBDE congeners and total PBDE in liver and muscle of trout from Greenland and European high mountain lakes (in pg·g⁻¹ ww except as noted).

#	Lake	LIVER									MUSCLE							
		n	BDE-28	BDE-47	BDE-99	BDE-100	BDE-153	BDE-154	Sum PBDEs	Sum PBDEs[a] (pg g⁻¹ lw)	n	BDE-47	BDE-99	BDE-100	BDE-153	BDE-154	Sum PBDEs	Sum PBDEs (pg g⁻¹ lw)
1	Fergusson	4	6.9[b]±11[c]	450±310	140±110	140±170	68±79	91±100	900	23000	4	140±63	86±38	40±19	19±7	27±11	310	41000
2	Øvre Neådalsvatn	5	18±16	260±300	67±73	50±77	35±20	59±60	490	8100	3	59±24	41±15	15±7.0	<LOD[d]	14±9.0	140	4800
3	Fallbekktjørna	5	10±5.0	620±170	290±120	97±76	46±42	49±45	1100	19000	2	33±17	34±2	<LOD	<LOD	<LOD	97	16000
4	Nedre Neådalsvatn	2	7.8±2.0	31±21	23±2.0	16±6.0	14±3.0	16±4	109	2400	2	36±18	21±9.0	<LOD	<LOD	<LOD	69	2900
5	Øvre Heimdalsvatnet										3	68±18	52±20	14±5.0	18±5.0	19±11	170	29000
6	Lochnagar	2	22±1.0	4100±2600	5400±4400	700±630	620±510	570±370	11000	370000	4	290±240	610±510	65±52	98±87	73±62	1100	180000
7	Velké Hincovo	5	6.2±2.0	610±420	410±350	205±250	54±52	71±68	1400	40000	1	100	153	43	21	22	340	32000
8	Gossenkoellesee	5	25±22	350±240	130±180	35±39	33±42	44±63	615	29000								
9	Rotfelssee	4	8.4±4.0	370±160	100±47	57±25	21±5.0	25±7.0	580	16000	3	57±12	35±8.0	19±3.0	<LOD	<LOD	130	14000
10	Redon	7	11±8.0	240±120	83±45	35±19	26±20	42±32	440	9000	2	270±320	190±200	47±34	34±26	58±44	600	14000
11	Okoto	5	7.6±8.0	260±89	81±27	31±8.0	12±5.0	11±3.0	400	13000	4	380±270	240±170	58±58	21±13	34±33	730	17000
12	Bliznaka	5	10±6.0	220±140	39±30	45±31	25±18	36±32	370	7800	4	43±13	25±9	16±4.0	<LOD	13±9.0	110	8000
	Blank values	10	4.2±2.4	21±5.8	27±16	17±4.5	<LOD	<LOD			6	25±9.8	15±5.8	<LOD	<LOD	<LOD		
	Limit of Detection	5	3,4	18	8,5	15	8,7	7,6			5	13	9,2	12	13	13		

[a]Sum of BDE congeners presented in the table. [b]Mean. [c]Standard deviation. [d]Below Limit of Detection.

Table 4. Comparison of the PBDE levels in fish from Greenland and European mountain lakes and previous studies (units in ng·g⁻¹ wet weight)

Location	Matrix	PBDE#47	Sum PBDEs	PBDE congeners considered	References
Remote lake Fergusson, Greenland	arctic charr liver	0,45	0,90	#28, #47, #99, #100, #153, #154	In this study
	arctic charr fillet	0,14	0,31	#28, #47, #99, #100, #153, #154	
High mountain lakes, Europe	trout liver	0,031-0,62	0,11-1.3	#28, #47, #99, #100, #153, #154	
	trout fillet	0,033-0,38	0,070-0,73	#28, #47, #99, #100, #153, #154	
Lochnagar, high mountain lake, Scotland	brown trout liver	4,1	11	#28, #47, #99, #100, #153, #154	
	brown trout fillet	0,3	1,1	#28, #47, #99, #100, #153, #154	
Viskan River, Sweden	pike fillet	0,22-2.5	0,27-3.3	#47, #99, #100	(11)
Lake Michigan, US	salmon fillet	52	80	#47, #66, #99, #100, #153, #154	(30)
Hadley Lake, US	carp fillet	6,5	13	#47, #99, #100, #153, #154, #190, #290	(31)
Lake Superior, US	smelt fillet	5,7	9,1	#47, #99, #100, #153, #154, #190, #290	
Lake Ontario, US	smelt fillet	10	18	#47, #99, #100, #153, #154, #190, #290	
Coast of Southern Greenland	shorthorn sculpin fillet	3,5	3,7	#47, #99, #100, #153	(8)
	uvak fillet	7,5	9,5	#47, #99, #100, #153	
	spotted wolffish fillet	1,2	1,2	#47, #99, #100, #153	
	starry ray fillet	1,1	1,4	#47, #99, #100, #153	
North Sea	herring liver	1,5	3,0	#28, #47, #99, #100, #153, #154	(9)
	herring fillet	4,5	7,6	#28, #47, #99, #100, #153, #154	
	cod liver	42	63	#28, #47, #99, #100, #153, #154	
	cod fillet	0,2	0,4	#28, #47, #99, #100, #153, #154	
	whiting liver	41	65	#28, #47, #99, #100, #153, #154	
	whiting fillet	0,2	0,3	#28, #47, #99, #100, #153, #154	
Umea River, Sweden	salmon fillet	167*	298*	#47, #99, #153	(6)
	herring fillet	27*	36*	#47, #99, #153	
Virginia rivers, US	freshwater fish species	20-100	1140	#47, #99, #100, #153, #154	(32)
Detroit River, US	carp fillet	3,0	5,4	#47, #99, #100, #153, #154, #181, #183, #190	(33)
	large mouth bass fillet	2,8	5,2	#47, #99, #100, #153, #154, #181, #183, #190	
Des Plaines River, US	carp fillet	1,9	12	#47, #99, #100, #153, #154, #181, #183, #190	
Washington State rivers, US	rainbow trout	0,68	1,4	#47, #99, #100, #153, #154	(34)
	mountain white fish fillet	520	1250	#47, #99, #100, #153, #154	
	carp fillet	21	22	#47, #99, #100, #153, #154	
Coast of British Columbia, Canada	English sole fillet	0,88-10.4	1.74-22.1	#15, #17, #28/33, #47, #49, #66, #75, #99, #100, #119, #153, #154, #155	(35)
The Netherlands	flounder fillet	0,07-3.2	0,09-4.2	#47, #85, #99, #138, #153	(36)
	bream fillet	0,04-30	0,05-32	#47, #85, #99, #138, #153	
Schwarzee ob Soelden, Austrian Alps	arctic charr fillet	0,6	1,1	#47, #99, #100	(37)
Great Lakes, US	lake trout fillet	33	49	#47, #66, #99, #100, #153	(38)

The highest lake-averaged concentrations of PBDEs among all lakes studied were found in muscle and liver of fish from Lochnagar (1200 pg g^{-1} ww -177000 pg·glw^{-1}- and 11000 pg g^{-1} ww -366000 pg·glw^{-1}- of total PBDEs in muscle and liver, respectively) representing about one order of magnitude higher values than in the other high mountain sites. Other persistent organic pollutants, such as PCBs, DDTs, hexachorocyclohexanes, and hexachlorobenzene, in fish from this lake do not show higher concentrations than in other high mountain lakes (13). The organohalogen pollution episode of Lochnagar is therefore specifically related to PBDEs. A study with invertebrates from the North Sea has evidenced high levels of PBDEs in the organisms collected near the English coast when compared with the concentrations along the coastline of continental Europe (9, 26). These coincident results between independent studies could be indicative of higher industrial PBDE emission sources in UK than in other European countries.

Discussion

PDDE dependence from fish condition factor. The log-transformed liver PBDE concentrations of the individual fish specimens exhibit significant inverse correlations with condition factor for BDE#100, BDE#153 and BDE#154 ($p < 0.01$; Table 2). These and the forthcoming correlations were calculated without inclusion of the Lochnagar data due to its specificity in the context of the lakes examined. Fish with lower condition factor exhibited higher liver concentrations involving decreases of 7-9 times between specimens with 0.71 and 2.08 g·cm^{-3} (Table 2). These differences were more significant for the more brominated BDEs showing higher correlation coefficients and higher significance level. The same correlations were observed for the lipid normalized concentrations but with lower statistical significance ($p < 0.05$).

Muscle concentrations of congeners PBDE#47 and #99 correlated positively with condition factor ($p < 0.05$) (Table 2) when expressed as concentrations relative to wet weight. These two correlations go against the general trend of higher concentrations at lower condition factor. In fact, they do not reflect a general trend among fish included in the study but the contribution of two fish from Okoto Lake that have very large condition factor. No correlation is observed if the concentrations of these two fish are removed. In contrast, when the concentrations were normalized to lipid content significant inverse correlations for congeners BDE#100, BDE #153 and BDE #154 ($p < 0.01$) were found in agreement with the trends observed in liver and the general correlation of the lipid normalized concentrations of both tissues.

PBDE dependence from fish age. Significant positive correlations between age and log-transformed liver concentrations were observed for BDE#47, BDE#99 and BDE#100 (Table

2). Normalization to lipid content showed the same correlations with the same degree of statistical significance. These dependences are expected in view of the significant inverse correlations of the concentrations of these congeners and condition factor. As mentioned above, age and condition factors are inversely correlated. Concentration increases of 4-12 times were found between 1 and 21 year old individuals. BDE#99 was the congener exhibiting the highest age increase.

In contrast, no significant correlation was found between PBDE concentrations in muscle and age irrespectively of referring PBDE levels to wet weight or lipid content. In principle, this difference could be attributed to lower PBDE concentrations in this tissue than in liver which results in a shorter range of values for age correlation. However, higher liver than muscle concentrations are only observed when PBDE amounts are referred to wet weight and not to lipid content (Figure 3). Having in mind that concentrations in muscle tissue generally represent longer periods of contamination than in liver (26), the difference between both tissues could also reflect that the amounts of PBDE arriving to high mountain fish are still not constant. In agreement with these observations, the air concentrations of these compounds are not yet globally homogeneous (21) and increasing trends of biota burdens have been reported (27-28).

PBDE differences between fish species and sex. PBDE concentration differences between species were significant for BDE#28 and #154 in liver and BDE#47, #99 and #100 in muscle (ANOVA, Table 2). Mean concentrations of BDE#28 in liver were 13 and 7.6 pg·g^{-1} ww for *Salmo trutta* and *Salvelinus alpinus*, respectively. Mean BDE#154 concentrations in liver were 11, 48 and 58 pg·g^{-1} ww for *Salvelinus fontinalis, Salmo trutta* and *Salvelinus alpinus*, respectively. Muscle BDE#47 showed values of 78, 105 and 379 pg·g^{-1} ww in *Salmo trutta*, *Salvelinus alpinus* and *Salvelinus fontinalis*, respectively. Muscle BDE#99 concentrations were 241 pg·g^{-1} ww in *Salvelinus fontinalis* and 60 pg·g^{-1} ww in *Salmo trutta* and *Salvelinus alpinus*. Mean BDE#100 values in muscle were 18 and 58 pg·g^{-1} ww in *Salmo trutta* and *Salvelinus fontinalis*, respectively. *Salvelinus fontinalis* shows therefore the highest concentrations in muscle and the lowest in liver.

No significant sex differences were found for the PBDE liver and muscle concentrations either as referred to wet weight or lipid content (ANOVA $p < 0.05$; table 2). However, males exhibited higher liver concentrations of BDE#47, #99, #100, #153 and #154 (411, 181, 104, 43 and 59 pg·g^{-1} ww, respectively) than females (299, 103, 41, 25, and 33 pg·g^{-1} ww, respectively). This contrast is consistent with the observations of other persistent organic pollutants since some of these compounds are excreted by female specimens during spawning (29). However, the difference is much smaller when referring to muscle concentrations, exhibiting BDE#47, #99, #100, #153 and #154 values in male of 130, 92, 26, 21 and 16 pg·g^{-1} ww, respectively, and 120, 78, 27, 22 and 14 pg·g^{-1} ww, respectively, in female.

Global PBDE concentration patterns. Examination of uniform distribution trends between the occurrence of PBDEs in mountain lakes and altitude, latitude or temperature shows no statistically significant correlation with fish muscle or liver concentration (either referred to wet weight or lipid basis). These calculations have been performed after exclusion of the Lochnagar data since this lake represents a specific case. The lack of correlation contrasts with previous observations on the distributions of organochlorine compounds in fish from these high mountain lakes. In this case, statistically significant correlations were found for concentrations in muscle of the compounds with volatilities lower than 10^{-5} Pa and altitude or temperature ($p < 0.01$) (13).

The relevance of the global distillation effect for the planetary distribution of PCBs and other organochlorine compounds was recognized about 50 years after their use and about 30 years after their banning in most of countries. PBDEs are still introduced into the environment more recently. Thus, their environmental distribution has likely not reached steady-state conditions. In addition, these compounds are less volatile than PCBs and other organochlorine compounds which maybe may delay further the achievement of a widespread distribution reflecting steady-state conditions. Nevertheless, the information already available on PCB studies could be taken as an early warning of their likely environmental fate and action could be addressed towards anticipation of their possible deleterious effects in organisms from remote sites.

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