ARTICLE 4

FOOD AND WATER ROLES IN BROWN TROUT ORGANOCHLORINE BIOACCUMULATION IN HIGH MOUNTAIN LAKES

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Food and water roles in brown trout organochlorine bioaccumulation in high mountain lakes

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Abstract

The main food components and their relative importance for brown trout were estimated by a combination of stable isotope and stomach content analyses in a high mountain lake. Organochlorine compounds (OCs) were measured in both food items and fish, and exchanges through gills and gut were estimated using a fugacity approach. For OCs with log (Kow) > 6, food showed lower concentration values than expected from an octanol-water partition and the food lipid content, indicating that the life span of those organisms (ca. 1 year) was not sufficient for reaching equilibrium. On the other hand, the degree of biomagnification in fish increased with Kow, except for largest compound analyzed (PCB # 180). For all compounds there was a net gill loss and a net gut uptake. However, according to the calculations, a pseudo stationary state was only achieved for compounds with log (Kow) < 6. As a conseuquence, an age dependency for the more hydrophobic compounds can be expected. The calculations indicate that for the most hydrophobic compounds the stationary state can hardly be achieved throughout the whole life span of the fish. Finally, our results suggest that the observed enhanced accumulation of less volatile compounds with altitude throughout Europe's mountains could be the consequence of reduced gill ventilation rates, resulting from lower water temperatures and higher oxygen concentrations with increasing elevation.

Introduction

In general atmospheric pollutants show higher concentrations at locations closer to the emission sites. However, persistent organic pollutants including some organochlorine compounds (OCs) are found in remote areas without a significant dilution effect. Natural distillation and condensation processes concurrent with atmospheric transport lead to their accumulation in ecosystems and organisms of high latitudes [1-4] or of high elevation [5]. Compounds are mobilized in areas of warm temperatures (ca. mean annual temperature > 5 ^{o}C). More volatile compounds (MVC), such as hexachlorobenzene (HCB), hexachlorocyclohexanes (HCH), and low chlorinated polychlorobiphenyls (PCBs) show high accumulation in cold areas located beyond 60^{o} N, with mean annual air temperatures below –5 ^{o}C. Less volatile compounds (LVC), such as more chlorinated PCBs (subcooled liquid vapor pressure < $10^{-2.5}$ Pa) and DDTs are selectively trapped in mountain cold areas [5], which do not reach such as low temperatures as the Arctic zone.

In the mountain areas, the accumulation of organochlorine compounds in lake sediments has been observed to be related to their specific phase exchange enthalpies [5], observed pseudoentalpies agree with summed volatilization and solubilization tabulated theoretical values. However, in fish some additional mechanism enhances temperature dependence since the observed pseudoenthalpies are two-fold higher than those expected. The origin of these higher enthalpies in fish remains open. Changes in lake or catchment trapping efficiencies cannot explain them, since they should also have an effect on the sediment values. Fish properties such as length, weight, lipid content or age do not show significative elevation gradients. Therefore, the enhanced entalpies are likely related to a temperature dependence of one or more steps in the bioaccumulation process. However, the knowledge of the food-web pathways to fish in mountain lakes is scarce [6], and no data exist on the OCs distribution in the fish food components. As a consequence the respective roles of food and water pathways for OC bioaccumulation in mountain lake fish have not been addressed without significant speculation.

Mountain lakes are relatively small in size and very oligotrophic [7], food is scarce, food chains are short, and fish show an opportunistic behavior related to the seasonal availability of food. There is not any reason to support that food-web structure changes significantly with elevation, thus it appears more likely that the observed pseudoentalpies depend on the rate of some process rather than in food-web structural differences. In this paper we present an assessment of the food pathways to brown trout in a mountain lake using stable isotopes, diet evaluation and the OCs content of the most significant fish food components. Then, we compare the food and fish OC content with that expected from simple bioconcentration from water, and discuss the roles of water and food in the bioaccumulation process, and the peculiarities for distinct OCs. Finally, we numerically analyze the gill and gut

OC fluxes based on water, food and fish fugacities and the rates of gill ventilation and food intake, which are made dependent on fish weight and water temperature following the Elliot's [8] well established model for brown trout. Based on these results, we suggest the mechanism behind the cold enhanced OC accumulation in fish of the less volatile compounds, which has been observed in remote mountain lakes.

Materials and Methods

Study site. The study was carried out in Lake Redon (42º38'N, 0º46'E) situated at 2240 m a.s.l. in Central Pyrenees (Catalonia, Spain). It has a surface area of 24 ha, a maximum depth of 73 m, a mean water residence time of about 4 yr and it is usually ice-covered from late December to June [9]. The lake is oligotrophic because most of its small watershed (155 ha) is bare rock and the rest are alpine meadows with scarcely developed soils. The productivity patterns and seasonal changes in the water column are typical for high mountain lakes [7]. The lake contains a large population of brown trout (*Salmo trutta*) [10], from which specimens up to 15 years have been collected [11]. Pollution inputs are exclusively related to atmospheric wet and dry deposition and they have been evaluated in a series of previous studies [12], as well as the OC composition of the waters [13-14], sediment and fish [5].

Sample collection and handling. Sampling was performed on two occasions during the year 2000. The aim was to cover two extremes of the fish feeding variability during the ice-free period. The availability of some food components (e.g. chironomids) decreases with water temperature as autumn advances, as it does the daily energy (food) required by fish. Therefore, June and November were selected as representative of high and low food demand and availability, respectively. Fish was collected with a series of eight individual bottom gillnets of different mesh sizes (10 – 46 mm) designed to give the best theoretical catch of brown trout over a range of 10 to 45 cm. The nets were set perpendicular to the shore at various depths and exposed in the lake for 120 min just at sunrise and sunset. All fishes were measured, dissected and determined for sex on site. Muscle fillets and stomach contents were wrapped in pre-cleaned aluminum foil and kept frozen (-20 ºC) until analysis. Brown trout analyzed for OCs (n=10) averaged (mean±SD) 265±59 mm in length, 204±118 g in weight, 0.99±0.09 in condition factor, and 7±6 years of age.

A parallel survey of the main components of the lake food chain was carried out during the same days of fish sampling. Animals were collected from distinct parts of the lake by kick sampling for littoral organisms, Ekman drags for sediment species and plankton nets for zooplankton. Samples were kept cold during transport and later in the lab were separated into distinct classes for stable-isotope and OC analysis.

Brown trout diet evaluation. Gut contents were isolated in the field and kept cold until arrival to the lab, where they were analyzed under a dissecting microscope. The food content was

determined mostly up to genus or family level, and the relative percentage on volume basis was estimated for each fish stomach. The degree of stomach fullness was categorized from 0 (empty stomach) to 5 (completely full stomach).

Dry weight and lipid content. The percentage of water content in the muscle of brown trout (74.2 ± 1.8 %, n = 8) was estimated by drying them in a vacuum sealed-dessiccator at 20 ºC to constant weight. This value was used to convert OC concentrations in fish to dry weight values for subsequent comparisons with invertebrate and algae data. The brown trout lipid content in muscle was determined gravimetrically after extraction with hexane:diclormethane (4:1, v/v). The lipid content for the other food web organisms was estimated from the measured elemental C and N by assuming that the main body constituents were lipids, proteins, ashes and chitin. The percentage of lipids was calculated by difference, after estimating proteins by multiplying the measured N by 6.25; and using literature values for ash [15-16] and chitin content [17]. Since cyanobacteria use carbohydrates as energy reserve, the lipid content for *Nostoc* from [18] was used.

Stable isotope analysis. Samples were analyzed for stable isotope ratios using a Delta C Finnigan MAT mass spectrometer coupled online with a Carlo Herba CHNS elemental analyzer, 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, and calculated as:

$$\delta^{13}C \text{ or } \delta^{15}N\ (‰) = [(R_{sample} - R_{standard})/R_{standard}] \times 10^3,$$

where R is ($^{15}N/^{14}N$) or ($^{13}C/^{12}C$). Reproducibility of $\delta^{13}C$ or $\delta^{15}N$ values were better than < 0.1‰ and 0.3‰, respectively.

Organochlorine compounds analysis. Brown trout muscle tissues were extracted and analyzed for OCs using the method described in [19]. Briefly, muscle tissue (5g) was ground with activated sodium sulfate until a fine powder was obtained. This mixture was introduced into cellulose cartridges and Soxhlet extracted with n-hexane:dichloromethane (4:1) for 18 hours. After adding TBB and PCB 209 standards, the remaining lipids were removed by sulfuric acid and extracted with n-hexane (5 times). All n-hexane fractions were combined and concentrated by vacuum rotary evaporation (20 ºC, 20 torr) to small volumes (ca. 500 µl) and further concentrated to 50 µl in isooctane under a gentle nitrogen flow.

For determining OCs in invertebrate and *Nostoc,* individuals were amalgamated in common samples until enough material was accumulated for a proper quantification. Whenever there was enough material the amalgamation was carried out at the species level; when organisms were too small or scarce in the lake, we grouped them at a higher taxonomical level amalgamating species of common feeding habits, living way, and fish predation exposure (Table 1). Samples were analyzed by a slightly modification of the method described in [20].

Briefly, all samples were dried in a vacuum sealed-desiccator at 20ºC to constant weight to determine dry weight. After the internal standards TBB and PCB 209 were added, dried tissues were Soxhlet extracted with n-hexane-dichloromethane (4:1, v/v) for 20 hours. These extracts were cleaned-up by aluminium oxide chromatographic column and eluted in two separate fractions (F1 with 16.5 ml of n-hexane:dichloromethane (19:1, v/v) and 3 ml of n-hexane:dichloromethane (1:2, v/v), and F2 with 30 ml of n-hexane:dichloromethane (1:2, v/v)). Both fractions were 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 tetrachloronaphthalene and octachloronaphthalene was added (25 μl) to the vial to correct for instrument variability. Samples were analyzed by gas chromatograph equipped with an electron capture detection (GC-ECD, Hewlett-Packard 5890 Series II) and 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 instrument operated in splitless mode and the oven temperature programme started at 90ºC (held for 1 min) to 120ºC at 10ºC/min, and then to 310ºC at 4ºC/min (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) and makeup (60 mL/min) gases, respectively. Some samples were examined by negative ion chemical ionization mass spectrometry coupled to gas chromatography (GC-MS-NICI) for structural identification. These analyses were performed in an Agilent Technologies 6890A gas chromatograph equipped with a nonpolar fused silica capillary column HP5-MS (30 m x 0.25 mm i.d. x 0.25 μm film thickness) and coupled to a MS detector 5973N. Ion source and transfer line temperatures were 150 and 280 ºC, respectively. Ammonia was chosen as ionization gas (1.6 torr) and helium was used as carrier gas (1.1 mL/min).

Chemicals. Residue analysis n-hexane, dichloromethane, iso-octane, methanol, concentrated sulfuric acid 95-97%, acetone, and analysis grade anhydrous sodium sulfate were from Merck (Darmstadt, Germany). Aluminum foil was rinsed with acetone and let dry at ambient temperature prior to use. Neutral aluminum oxide type 507C was from Fluka AG (Switzerland). Cellulose extraction cartridges (20 mm i.d. x 80 mm) were from Whatman (England). Aluminum oxide, sodium sulfate, and cartridges were previously cleaned by Soxhlet extraction with dichloromethane:methanol (2:1, v/v) for 24 h before use. Sodium sulfate and aluminum oxide were activated overnight at 400ºC and 120ºC, respectively. γ-hexachlorocyclohexane (γ-HCH) and tetrabromobenzene (TBB) were from Aldrich-Chemie (Steinheim, Germany). α-HCH and PCBs (#28, #52, #101, #118, #138, #153, #180, #209) were from Promochem (Wesel, Germany), and pp'-DDE, and pp'-DDT were from Dr. Ehrenstorfer (Augsburg, Germany).

TABLE 1. Components of the fish food-web that were analyzed for organochlorine compounds. The degree of taxonomomic resolution was conditioned by the amount of available material for analyses. Habitat, typical size analyzed, estimated life span, food type and feeding modes expected are indicated.

Operative food-web components	Group	Habitat	Size (mm)	Age (years)	Food type	Feeding mode	Comments
Salmo trutta (Brown trout)	Chordata, Fishes	Pelagic and littoral	250	7	Macroinvertebrates	Predator	
Arcynopterix compacta	Insecta, Plecoptera	Benthic	28	2-3	Macroinvertebrates	Predator	
Siphonoperla torrentium	*Insecta, Plecoptera*	Benthic	10	1-2	Macroinvertebrates	Predator	
Sialis lutaria	*Insecta, Megaloptera*	Benthic	22	2-3	Macroinvertebrates	Predator	
Polycentropodidae	Insecta, Trichoptera	Benthic	18	1-2	Macroinvertebrates	Predator	Mainly *Plectronemia*
Platambus maculatus	*Insecta, Coleoptera*	Benthic	8	2-3	Macroinvertebrates	Predator	Larvae
Chironomidae (larvae)	*Insecta, Diptera*	Benthic	6	1-2	Algae, debris	Collector-gathered	Several species but all non carnivorous
Chironomidae (pupae)	Insecta, Diptera	Pelagic	10	-	None	Non feeding stage	Mainly Diamesinae
Daphnia pulicaria	*Crustacea, Cladocera*	Planktonic	2	<0.5	Fitoplankton, bacteria	Collector-filterer	
Eurycercus lamellatus	*Crustacea, Cladocera*	Littoral	2.5	<1	Algae, debris	Collector-gathered	
Radix ovata	*Mollusca, Gastropoda*	Littoral	6	>1	Algae, debris	Scraper	
Pisidium sp.	Mollusca, Bivalvia	Littoral	3	>1	Fitoplankton	Collector-filterer	
Nostoc sp.	Cyanobacteria	Epilithic	5	-	Autotroph	Photosynthesys	Included because found in the fish stomachs

Results and discussion

Brown trout diet. The two sampled periods for fish diet evaluation corresponded to two extremes in the seasonal changes of fish food quality and availability throughout the ice-free period. In June, when larvae of aquatic insects were abundant, the average value of the stomach fullness index was 4.4, and no fish with an empty stomach was found; whereas in November, during the lake overturn and low water temperature, the average value of the index was 1.9, and 9% of the fish had a completely empty stomach. The two periods were also distinct in the characteristics of the food items found in the stomachs and in their relative contribution (Table 2). In June, chironomids, either larvae or pupae, were by large the most frequent and abundant items in the stomachs. Other organisms, such as terrestrial insects, the bivalve *Pisidium*, or the megaloptera *Sialis* were often found in the stomachs but their contribution to the food volume was low. In some stomachs, chironomid pupae were nearly the exclusive content, probably because this transient and passive stage facilitates the capture by trouts. In November, the more frequent and abundant stomach content were cladocerans, the pelagic *Daphnia* and the littoral *Eurycercus*. However, they were less dominant that chironomids in June, other items were also relevant either in abundance or in frequency (e.g. chironomids, *Radix, Pisidium*, *Sialis*). During this period, the number of unidentifiable items increased, although their contribution to food volume remained very low. It could be due to a slower food pass trough the intestine, because an overall lower activity of the fish. Unexpected food items such as colonies of the cyanobacterium *Nostoc* were also found. These two snap-shots of the trout diet at contrasting periods of the year suggested that when chironomids were abundant trouts mainly fed on them; when they become scarce, then trouts fed on alternative prey, particularly on the abundant pelagic small cladocerans, such as *Daphnia*. The large variety of other food items appeared just to complement these two main sources of food. A crude estimation of the annual average contribution to diet of the distinct food components was calculated assuming that there were about four months of high food requirement (June to September); four months of low food requirement (October to December and May) and a period of very low consumption under the ice (January to April), in which diet was considered qualitatively equivalent to the low requirement period and stomach fullness ca. 1. Other reasonable assumptions of the seasonal partition did not change significantly the relative contribution of the distinct food components.

Isotope structure of the food web. Differences in carbon and nitrogen stable isotope ratios between the distinct components of the food web provide information on trophic relationships. Commonly used trophic fractionation values are 1 ‰ for $\delta^{13}C$ and 3.4 ‰ for $\delta^{15}N$ [21-22], which are similar to mean values found in recent studies of the variation in the trophic fractionation (0.05 ± 0.63 ‰ $\delta^{13}C$, 3.49 ± 0.23 ‰ $\delta^{15}N$) [23]. Because its lower trophic fractionation, carbon is considered to indicate primary energy sources (e.g. benthic vs. pelagic

photosynthesis), and nitrogen is used for the discrimination among trophic levels. In Fig.1 we show the isotopic signatures of the organisms and primary carbon sources involved in the food web pathways to fish in Lake Redon. The main primary carbon sources for the food web - namely littoral sources (epilithon and *Nostoc*), pelagic sources (seston) and organic detritus (sediment)- showed significantly distinct isotopic signatures. The $\delta^{13}C$ depletion was larger in seston and deep sediment than in littoral algae. These may be due to the fact that pelagic primary production mainly occur in the hypolimnion of the lake, below the seasonal thermocline, due to the extreme transparency of the water column [9]; growing temperature is significantly lower than in the littoral (ca. 5-10 ^{o}C) and available CO_2 have a larger contribution from within lake respiration [24]. On the other hand, benthic algae tend to be enriched in ^{13}C, due to a boundary layer effect, which causes diffusion limitation to the cells and favors the use of bicarbonate as a carbon substrate [25]

TABLE 2. Brown trout diet in Lake Redon during two distinct periods of the ice-free season: early summer when food requirements were high; and late autumn when aquatic insect larvae were scarce and requirements were lower because of the low water temperature. Commonness in the diet is indicated by the frequency that a certain item was found in the stomachs examined (21 and 22, respectively for high and low food periods). Contribution to the diet is indicated by the percentage of food volume, obtained by weighting percentages of food volume in individual stomachs by the degree of stomach fullness. Annual average contribution to diet was calculated assuming four month of high requirements, four month of low, and four month of very low consumption (stomach fullness < 20%) during the ice covered period with a diet similar to the low food requirement period of the ice-free season. Other reasonable assumptions do not change significantly the average values.

	Frequency in stomachs (%)		Food volume (%)		
Food requirement period	High	Low	High	Low	Annual average
Chironomidae (pupae)	71.4	0	55.91	0	33.7
Daphnia pulicaria	4.8	45.5	3.1	45.8	20.1
Chironomidae (larvae)	38.1	27.3	14.0	10.5	12.6
Sialis lutaria	23.8	18.2	4.7	4.6	4.6
Terrestrial insects	33.0	5.0	5.6	2.3	4.3
Eurycercus lamellatus	0.0	32.0	0.0	14.1	5.6
Radix ovata	0.0	27.0	0.0	11.7	4.7
Polycentropodidae	5.0	0.0	4.8	0.0	2.9
Platambus maculatus	10.0	0.0	4.1	0.0	2.5
Pisidium sp.	29.0	14.0	0.5	1.7	1.0
Siphonoperla torrentium	5.0	0.0	0.6	0.0	0.3
Nostoc sp.	0.0	9.0	0.0	0.6	0.2
Other organisms	33.0	18.0	6.5	7.2	6.8
Unidentifiable material	5.0	45.0	0.2	1.5	0.7

TABLE 3. Organochlorine concentrations (ng g^{-1} dry weight) in the most significant organisms involved in the brown trout diet in Lake Redon (Pyrenees).

Taxa	Lipid (%)	α-HCH	γ-HCH	HCB	4,4'-DDE	4,4'-DDT	PCB -28	PCB -52	PCB -101	PCB -118	PCB -153	PCB -138	PCB -180
							ng g^{-1} dry weight						
Salmo trutta (muscle)	2.8	0.57	3.54	2.40	57.2	4.19	0.72	1.93	2.82	1.52	9.88	8.52	5.86
Chironomidae (pupae)	39.9	1.72	16.29	9.18	275	18.76	0.93	1.92	6.45	5.36	22.2	14.4	20.5
Daphnia pulicaria	54.4	0.03	0.16	0.20	0.41	0.11	0.31	0.46	0.11	0.03	0.08	0.08	0.03
Chironomidae (larvae)	30.4	0.17	6.05	3.08	58.5	4.98	0.90	1.94	2.33	1.02	4.35	3.32	3.18
Sialis lutaria	21.1	0.17	9.38	1.58	14.4	4.75	0.97	0.67	2.38	2.12	6.77	4.92	4.22
Eurycercus lamellatus	37.9	0.49	5.19	1.98	60.6	4.34	2.75	1.82	3.07	1.98	2.90	1.64	0.83
Radix ovata	0.6	0.02	0.19	0.23	0.20	0.00	0.47	0.41	0.18	0.08	0.11	0.10	0.10
Polycentropodidae	29.8	0.97	6.79	2.65	3.04	1.43	0.94	1.50	2.15	2.27	5.41	3.52	3.72
Platambus maculatus	9.9	0.00	14.65	2.94	21.0	4.95	1.76	9.34	8.56	3.18	1.72	1.40	2.35
Pisidium sp.	13.2	0.04	1.24	0.87	10.0	1.72	0.73	3.05	2.85	0.97	0.60	0.98	0.13
Siphonoperla torrentium	37.7	0.51	19.26	4.60	25.5	11.29	3.60	14.15	9.41	5.63	2.64	3.05	1.24
Nostoc sp.	1.7	0.03	0.22	0.18	0.10	0.00	0.55	0.25	0.10	0.03	0.01	0.03	0.03
Arcynopterix compacta	26.5	0.99	19.70	3.69	18.1	7.77	4.82	21.72	15.00	7.05	6.77	5.69	4.82

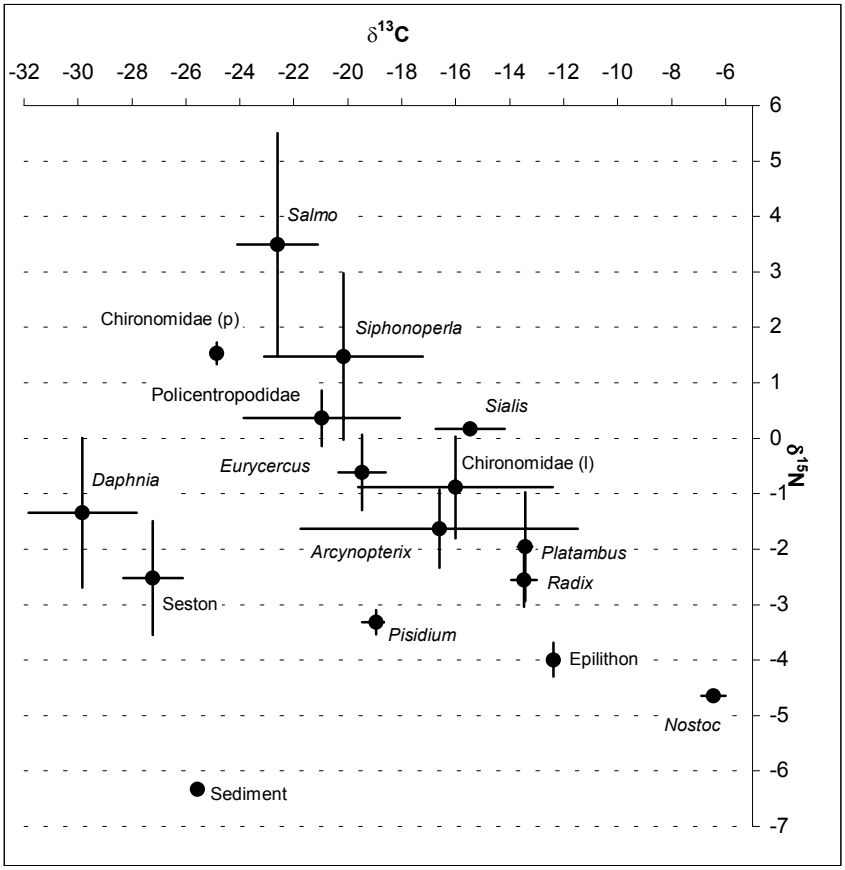

FIGURE 1.Isotopic signature of the main food-web components in Lake Redon.

Fractionation during nitrogen assimilation by algae (phytoplankton and phytobenthos) can be –4 to –5 ‰ [26]. In our data, pulled epilithon (mainly diatoms) and *Nostoc* agreed with

these values if we assumed nitrogen sources close to 0 ‰ $\delta^{15}N$ as expected from its mainly atmospheric origin. However, seston was slightly richer in the heavy isotope than epilithon, suggesting a mixture of phytoplankton and allocthonous matter in seston. Since *Daphnia* - a planktonic cladoceran mainly feeding on phytoplankton - had a $\delta^{15}N$ similar to other herbivores feeding on littoral algae, we assumed a common $\delta^{15}N$ baseline for the herbivore food web with values around –4 ‰.

As expected, brown trout appeared as the unique top predators (Fig. 1), however, the average food chain from primary producers to them was very short. Assuming an enrichment factor of 3.5 ‰ $\delta^{15}N$ per trophic level change, the average number of energy transfer steps from primary producers to trout was only of 2.2. This is not surprising for a high mountain lake, since food availability is scarce because of the oligotrophy of the system, inputs from the watershed are low, and the lake size is small [27].

The distribution of the distinct organisms throughout the $\delta^{15}N$ gradient indicated a high degree of omnivory, being the differences between successive organism in any trophic chain significantly lower than 3.5 $\delta^{15}N$. Chironomid pupae showed higher $\delta^{15}N$ values than larvae, although in both cases the species measured were herbivorous. The isotope discrimination could be due to the metamorphosis that occur from larvae to pupae, the new form is rebuilt from old tissues, which may cause an enrichment in ^{15}N in a similar way as it occurs in starving animals [28]; there is no food intake during the pupae stage. On the other hand, some consumers showed a significant contribution of detritus in their diet, particularly the bivalve *Pisidium*. The signature of some supposed predators (*Arcynopterix*, *Platambus*) did not agree with a exclusive diet of macroinvertebrates, being closer to a scavenger feeding. The narrowing of the $\delta^{13}C$ range with increasing $\delta^{15}N$ indicated a progressive mixing of the pelagic, littoral and sediment initial carbon sources.

The isotopic signatures confirmed that trout mainly predated on the herbivore level constituted by chironomids and cladocerans, with some contribution from other invertebrates. Using the estimated diet proportions (p_i) from Table 1, and the measured isotopic signatures of the distinct food items ($\delta^{13}C_i$, $\delta^{15}N_i$) we calculated

$$\delta^{13}C_{trout} = \Sigma\, p_i\, \delta^{13}C_i + 0.05 \quad \text{and} \quad \delta^{15}N_{trout} = \Sigma\, p_i\, \delta^{15}N_i + 3.5$$

the expected isotopic signature for brown trout (-22.7±1.8 ‰ $\delta^{13}C$; 3.4±0.80 ‰ $\delta^{15}N$) which turned out to be quite similar to the measured one (-22.6±1.5 ‰ $\delta^{13}C$; 3.5±2 ‰ $\delta^{15}N$), thus indicating that our assumptions about the average diet composition were acceptable, and were close to the annual average composition.

Food and fish organochlorine levels. Differences in OC concentrations among the distinct food-web components were significant, partially reflecting the large heterogeneity in lipid content (Table 2). However, when corrected for lipids, brown trout showed the highest values

for all compounds. Among the organisms more common in the trout diet, *Daphnia* showed lower OC concentration values, perhaps because of its shorter life span. When the concentrations of the individual organisms were combined to estimate the OC content in food (Fig. 2), it appeared that the concentrations were slightly lower during the low feeding period, because of the higher relative contribution of *Daphnia* to the diet. However, the differences between the two periods were not significant enough to prevent using a mean OC food concentration for comparison with fish values (Fig. 2). The mean OC values were calculated by weighting the lipid corrected concentrations in each food item by the respective estimated mean contributions to diet in Table 2.

TABLE 4. Concentrations of organochlorine compounds dissolved in water of lake Redon, episodic measurements from 1996 to 2000 (data from [x] and unpublished).

pg L^{-1}	Mean	SD	Mininum	Maximum
αHCH	483	225	267	952
γ–HCH	2671	1226	856	4846
HCB	9.9	12	0.5	35
DDE	7.4	2.3	4.1	9.7
DDT	20	5.6	12	28
PCB #28	4.3	5.3	0.4	12
PCB #52	8.8	11	1.3	33
PCB #101	6.2	5.4	1.9	17
PCB #118	3.4	2.5	1.1	8.3
PCB #153	7.5	9.3	1.4	27
PCB #138	8.8	9.5	1.4	25
PCB #180	4.5	5.4	0.4	14

At thermodynamic equilibrium it could be expected that the concentration in each organism (C_i) corrected by the lipid fraction (L_i) will be proportional to the concentration in water (C_w), being the constant of proportionality close to the octanol-water partition coefficient (K_{ow}) [29]. Therefore, the expected values (K_{ow} C_w) for each OC were a good reference to discuss the observed concentrations in food. Using OC concentrations in lake Redon water (Table 4), we estimated the mean and the range of values expected in food at thermodynamic equilibrium (Fig. 2). A significant number of OCs showed values close to those expected at equilibrium, namely HCHs, HCB, DDT, and PCB congeners #28 and #52. DDE showed concentration values much higher than expected at equilibrium (Fig. 2), which could be a consequence of the DDT conversion to DDE within the organisms [30]. Whereas DDT

concentration was always significantly higher than DDE concentration in water (Table 4), it was the opposed within all the organisms analyzed (Table 3). Excluding the above mentioned less chlorinated congeners, PCBs also deviated from equilibrium, we found lower values than expected from water concentrations, the deviation progressively increasing with the degree of chlorination (Fig. 2). A closer look to the deviation from octanol-water partition indicated a relationship with the value of log(Kow) (Fig. 3a). Equilibrium was not reached for OCs with log(Kow) values above ca. 6, indicating that the life span (< 1 yr) of the organisms more relevant in the trout diet was not sufficient long.

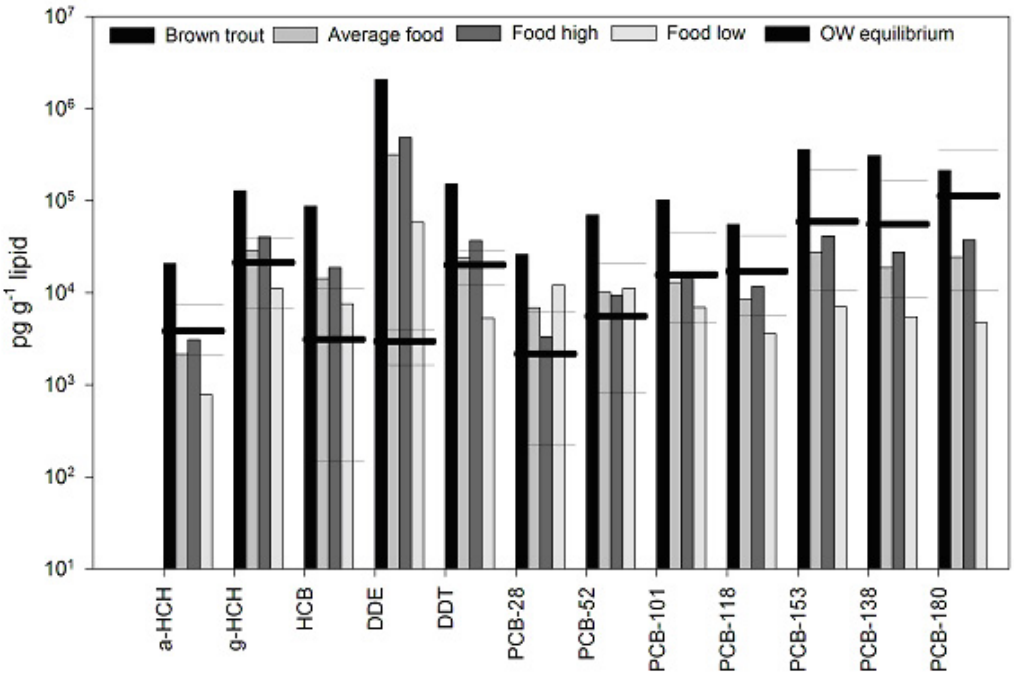

FIGURE 2. Organochlorine compounds concentrations in fish and food corrected by the respective lipid content. Annual average food and food during high and low feeding periods are distinguished. Horizontal bars indicate values expected according to the water concentration and the octanol-water partition coefficient (mean, minimum and maximum values are indicated).

In contrast to food, fish showed concentrations significantly higher than those expected at equilibrium for all OC compounds, indicating a biomagnification process (Fig. 2). The ratio between the fish and food concentrations, both corrected by lipid content, ranged from 3.8 for PCB #28 to 16.5 for PCB #138. Figure 3b shows how the OC biomagnification factor and the deviation from octanol-water equilibrium in food were related. This is a spurios relationship that appears because, for the two ratios, the differences among OCs bases on their different water-lipid partition capacity: i) in the case of the food items, the higher the Kow, the longer it takes to build up the internal equilibrium concentration, since the supplying rate (gill ventilation, food consumption) is the same for all OCs; ii) in the case of biomagnification, the higher the

hydrophobicity of an OC compound, the lower its loss through the gills. The exception to that relationship was PCB #180 (Fig. 3b), it presented a significant lower biomagnification than expected from its large deviation from octanol-water equilibrium in food. The observed anomaly could be due to a the lower membrane permeation expected for large molecules [31], which seems to be species dependent [29]. The trout gut uptake efficiency for PCB#180 seems to be significantly lower than those of other congeners.

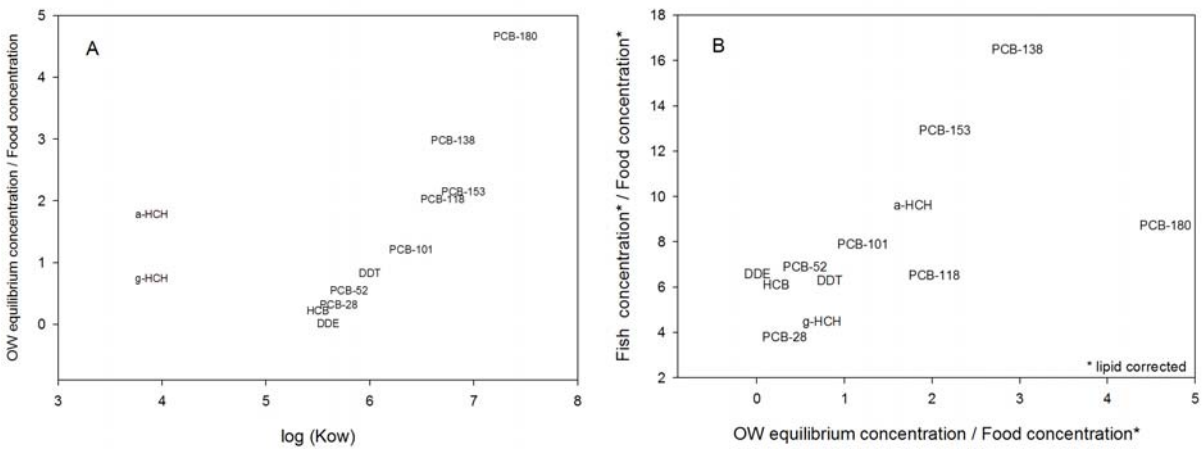

FIGURE 3. Degree of departure from octanol-water equilibrium of the organochlorine compounds in the average food composition against the octanol-water partition coefficient (A) and the biomagnification ratio between fish and its average food (B).

Food and water roles in trout OC bioaccumulation. The OC concentration in fish is the result of the balance between exchanges at the gills during fish respiration, uptake from diet, elimination by fecal egestion and metabolism and dilution by fish growth. Metabolic elimination can be considered of little relevance compare to the other processes for OCs [32]. Even in the case of DDT, at first it was not quite apparent the conversion to DEE in our fish data (Fig. 2); in contrast to what we saw in the invertebrates of the food web. Growth may be significant depending on the time scales of the gill and gut fluxes. In our calculations we initially assumed that growth was irrelevant.

The results showed above, it appeared that for all OCs gill exchange resulted in net loss, because fish fugacity was larger than water fugacity, and that there was a net uptake from the gut, because all OCs showed biomagnification (Fig. 2). However, there was no reason to believe that all, if any, OC exchanges were part of a whole fish pseudo steady state [29]. In principle, we could expect that the steady state was more likely for those OCs that presented a similar or higher fugacity in food than in water (HCB, DDTs, PCB # 28, PCB # 52). To investigate the fact, we calculated the gill and gut fluxes for a “typical” fish of the average size measured (204

g) and submitted to the seasonality of feeding at four month periods of 8, 4 and 2 ^{o}C in an environment of ca. 9 mg O_2 L^{-1}, which is representative of the lake [9].

For the calculations of the gill and gut fluxes, we assumed that all parts of the fish body had the same fugacity. We considered the gill as a well-mixed compartment into and out of which water flows with oxygen and OCs being transferred to the fish by diffusion [29]. With this appraoch, there is no reason for assuming that conductivities (Dw) for uptake and loss in the gills are different, therefore, the two were equally considered dependent on the gill ventilation rate (Gw). The net flux in the gill exchange (Fg) was determined by the difference between water (f_W) and fish (f_F) fugacities.

$$Fg = Dw (f_W - f_F)$$

The exchanges in the gastrointestinal tract are more complex, because between food uptake and egestion there is a fraction of matter that is removed, and digestion and hydrolysis of lipids occur. Therefore, the intestine flux (Fi) required the separate consideration of uptake (D_A) and loss (D_E) conductivities.

$$Fi = D_A f_A – D_E f_F$$

where f_A is food fugacity. It is common to model D_A as depending on the food consumption rate (G_A) and a gut absorption efficiency (E_A) [29, 32-33]. However, D_E is addressed in a number of different ways, in our case we selected to consider it as proportional to G_A (1-ß), where ß was the fraction of ingested diet absorbed by the organism. Developing the two equations according to the definitions in Table 5, we obtained the following expressions for the two fluxes:

$$Fg = Gw (Cw - C_F / (L_F K_{ow}))$$

$$Fi = G_A (C_A E_A – C_F (1-ß))$$

Apart from water and food concentrations, the relative flux differences among OCs arise from the values of the K_{ow} and E_A coefficients. The latter being particularly relevant for differentiating the PCB#180 behavior. The absolute flux values are determined by Gw and G_A. The two rates depending on the fish daily energy requirements [34], which under optimal conditions depends on the body weight and temperature in non-linear way. In our calculations, we used the model of Elliot for brown trout [8], from which we derived the food consumption rate to achieve the daily energy requirements in the three feeding periods distinguished. The ventilation rate, in addition to the oxygen consumption determined by the energy requirement, was made dependent on the oxygen in the water and the efficiency of uptake [33] (Table 5).

The calculations indicated that a number of OCs were closed to a pseudo steady state, namely HCB, DDE, PCB #28 and PCB #52 (Fig. 4). These were most of the compounds for which we thought it was more likely to be in a steady state, since they were also in equilibrium between water and food. Therefore, we can consider that the assumptions for the calculations of exchanges in the gills and the intestine were acceptable, at least in relative terms between them. As mentioned above, HCHs and DDT were also candidates to be close to a steady state. In the

case of DDT, the gut uptake appeared to be higher than gill net loss, which may reflect a certain metabolic transformation within the fish. For HCHs the gill loss was much higher than gut uptake, which is unlikely unless the water fugacity assumed did not correspond to the actually experienced by the fish. This can certainly be the case, as we use long time averaging for water concentrations and the fish renewal time of those more volatile compounds are a matter of days. In fact, for the compounds in an apparent steady state we could calculate an average residence time in fish. For HCHs, it was of the order of some days to a few weeks, for HCB and DDE was about one year, and for DDT, PCB # 28 and # 52 of 2 or 3 years. For the rest of compounds the steady-state was not achieved but the actual turnover indicate characteristic times around a decade for PCB # 101, and two or three decades for PCBs # 110 to # 153. In the case of PCB # 180, a fish could hardly achieve a steady state at present exposures unless it lived for centuries.

TABLE 5. Definition of symbols and summary of the parameters used in the OC fish flux calculations.

Parameter	Units	Definition
f_W, f_A, f_F	Pa	water, food and fish fugacities, $f_i = C_i Z_i^{-1}$
C_i	Pg L^{-1}	Concentration
Z_i	Pg L^{-1} Pa^{-1}	Fugacity capacity, $Z_i = L_i K_{ow} H^{-1}$
K_{ow}		Octanol-water partition coefficient
H	Pa L pg^{-1}	Henry's law constant
Dw	Pg d^{-1} Pa^{-1}	Conductivity at gills, Dw = Gw Zw
Gw	L d^{-1}	gill ventilation rate, $Gw = K k_{eo} O_{2w}^{-1} E_{ox}$
K	Cal d^{-1}	fish daily energy requirement, K= f(T,W) [8]
T	^oC	Temperature
W	G	fish weight
k_{eo}	mg cal^{-1}	Energy to oxygen consumption coefficient, 0.047
O_{2w}	mg L	water oxygen concentration
E_{ox}		Efficiency of oxygen uptake, 0.45
D_A	Pg d^{-1} Pa^{-1}	gut uptake conductivity, $D_A = E_A G_A Z_A$
G_A	L h^{-1}	food consumption rate, $G_A = K k_{ef}$
k_{ef}	L cal^{-1}	Energy to food volume consumption coefficient, 10^{-6}
E_A		gut uptake effiency, 0.75 (except for PCB # 180, 0.45)
D_E	Pg d^{-1} Pa^{-1}	gut loss conductivity, $D_E = G_A (1-ß) Z_F$
ß		Fraction of ingested diet absorbed by the fish

On the origin of increased bioaccumulation with altitude. The analysis of HCB, HCHs, PCBs and DDTs in muscle of fish from high mountain lakes have shown that a proportion of their variance depends on lake altitude [5, 35] and, in a less extent, on fish age [35] for those compounds with log-transformed vapor pressure (Vp) lower than $10^{-2.5}$ Pa. The two effects were found independent, since no correlation between fish age and lake altitude was observed. Our results indicate that the age effect for the less volatile compounds might be related to the slow achievement of the steady state, thus older fish are closer to it, although even fish of 10 years

would be far from it for some of the less volatile compounds. Since food consumption and growth are both related to the daily energy requirements, it is not expected that growth dilution could be significant.

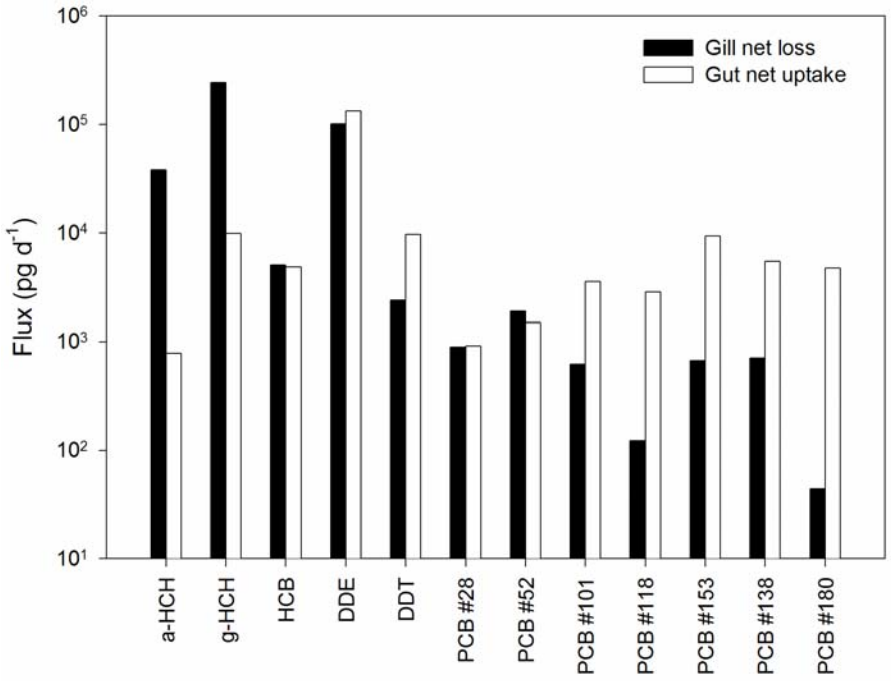

FIGURE 4. Comparison of the calculated organochlorine net gill loss and net gut uptake for a fish of 204 g in lake Redon according to the water, food and fish concentrations measured.

The bioaccumulation increase with altitude of the less volatile compounds were partially explained by condensation effects such as those described for the latitudinal trends that support the global distillation theory [36] . The additional temperature-dependent amplification which has been observed could be related to the fact that the mean water temperature that fish experience decreases with altitude, and hence it does the daily energy requirements of the fish. As a consequence, the food consumption and the gill ventilation rate decrease as well. The key aspect being that gill ventilation probably decreases more than food consumption rates, since to the effect of a lower oxygen demand in the fish, it adds the higher oxygen concentration. In those alpine environments of very low productivity oxygen tends to be at saturation in water, thus the lower the temperature, the higher the oxygen concentration.

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Literature Cited

1.- S.L. Simonich and R.A. Hites. Global distribution of persistent organochlorine compounds. *Science* **269,** 1851-1854 (1995).

2.- F. Wania, and D. Mackay. Global fractionation and cold condensation of low volatility organochlorine compounds in polar regions. *Ambio* **22,** 10-18 (1993).

3.- J.M. Blais, D.W. Schindler, D.C.G. Muir, L.E. Kimpe, D.B. Donald and B. Rosenberg. Accumulation of Persistent Organochlorine Compounds in Mountains of Western Canada. *Nature***, 395,** 585-588 (1998).

4.- P. Fellin, L. Barrie, D. Doughtery, D. Toom, D. Muir, N. Grift, L. Lockhart and B. Billeck. *Environ. Toxicol. Chem.* **15**, 253-261 (1994).

5.- J.O. Grimalt, P. Fernandez, L. Berdié, R.M. Vilanova, J. Catalan, R. Psenner, R. Hofer, P.G. Appleby, B.O. Rosseland, L. Lien, J.C. Massabuau and R.W. Battarbee. Selective trapping of organochlorine compounds in mountain lakes of temperate areas. *Environ. Sci. Technol.* **35**, 2690-2697 (2001).

6.- Buscar referència food-web i peixos a l'alta muntanya (Marc)

7.- Catalan, J., M. Ventura, A. Brancelj, I. Granados, H. Thies, U. Nickus, A. Korhola, A. F. Lotter, A. Barbieri, E. Stuchlík, L. Lien, P. Bitušík, T. Buchaca, L. Camarero, G.H. Goudsmit, J. Kopáček, G. Lemcke, D.M. Livingstone, B. Müller, M. Rautio, M. Šiško, S. Sorvari, F. Šporka, O. Strunecký and M. Toro. 2002. Seasonal ecosystem variability in remote mountain lakes. Implications for detecting climatic signals in sediment records. *J. Paleolimnol.* 28: 25-46.

8.- Elliott, J. M., and Hurley, M. A. 1998. A new functional model for estimating the maximum amount of invertebrate food consumed per day by brown trout, Salmo trutta. *Freshwater Biol.* **39**: 339-349.

9.- Ventura, M., Camarero, L., Buchaca, T., Bartumeus, F., Livingstone, M.D., and Catalan, J. 2000. The main features of seasonal variability in the external forcing and dynamics of a deep mountain lake (Redó, Pyrenees). *J. Limnol.* **59**: 97-108.

10.- Buscar referència treball densitat (Marc)

11.- Vives, I., J.O. Grimal, M. Ventura, J. Catalan, and B. Rosseland. 2003. Age dependence of the accumulation of organochlorine pollutants in brown trout (Salmo trutta) from a remote high mountain lake (Redó, Pyrenees). (submitted)

12.- Carrera, G., Fernández, P., Grimalt, J.O., Ventura, M., Camarero, Ll., Catalán, J., Nickus, U., Thies, H., and Psenner, R. 2002. Atmospheric deposition of organochlorine compounds to remote high mountain lakes of Europe. *Environ. Sci. Technol.* **36**: 2581-2588.

13.- Vilanova, R., Fernández, P. Martínez, C., and Grimalt, J.O. 2001a. Organochlorine pollutants in remote mountain lake waters. *J. Environ Qual.* **30**: 1286-1295.

14.-Vilanova, R.M., Fernandez, P., and Grimalt, J.O. 2001b. Polychlorinated biphenyl partitioning in the waters of a remote mountain lake. *Sci. Total Environ.* **279,** 51.

15.- Salonen, K., and J. Sarvala. 1978. Estimation of the inorganic fraction of total carbon in aquatic invertebrates. *Verh.Internat.Verein.Limnol.* **20**:1221-1225.

16.- Stelzer, R. S., and G. A. Lamberti. 2002. Ecological stoichiometry in running waters: periphyton chemical composition and snail growth. *Ecology* **83**:1039-1051.

17.- Cauchie, H.-M. 2002. Chitin production by arthropods in the hydrosphere. *Hydrobiologia* **470**:63-96.

18.- Ahlgren, G., I.-B. Gustafsson, and M. Boberg. 1992. Fatty acid content and chemical composition of freshwater microalgae. *J. Phycol.* **28**:37-50.

19.- Berdié, L.; Grimalt, J. Assessment of the sample handling procedures of a man power minimized method for the analysis of organochlorine compounds in large numers of fish sampling. *Journal of Chromatography A* **1998**, *823*, 373-380.

20.- Vives, I.; Grimalt, J. Method for integrated analysis of polycyclic aromatic hydrocarbons and organochlorine compounds in fish liver. *Journal of Chromatography B* **2002**, *768*, 247-254.

21. Minagawa, M. and Wada, E. 1984. Stepwise enrichment of 15N along food chains: Further evidence and the relation between $\delta^{15}N$ and animal age. Geochim. Cosmochim. Acta 50: 2143-2146.

22. Michener, R.H.; and Schell, D.M. 1994. Stable isotope ratios as tracers in marine aquatic food webs, p. 138-157. In K. Lajtha and R. Michener (eds.) Stable isotopes in ecology and environmental science. Blackwell Scientific.

23. Vander Zanden, M.J. and J. B. Rasmussen. 2001. Variation in $\delta^{15}N$ and $\delta^{13}C$ trophic fractionation: Implications for aquatic food web studies. *Limnol. Oceangr.* **46** (8): 2061-2066.

24. Rau, G. H. 1980. Carbon-13/carbon-12 variation in sub-alpine lake aquatic insects: food source implications. Can. J. Fish. Aquat. Scien. 37:742-746.

25. Hecky, R. E., and R. H. Hesslein. 1995. Contributions of benthic algae to lake food webs s revealed by stable isotope analysis. *Can. J. Fish. Aquat. Scien.* **52**: 1195-1201.

26. Fogel, M. L.; Cifuentes, L.A. 1993. Isotope fractionation during primary production, p. 73-100. In M.H. Engel, and S.A. Macko (eds.). Organic geochemistry. Plenum.

27. Post, Pace, Hairston, Jr. 2000. Ecosystem size determines food-chain length in lakes. Nature 405:1047-1049.

28. Hobson, K.A., Alisauskas, R.T., and Clark, R.G. 1993. Stable-nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: Implications for isotopic analysis of diet. Condor 95: 388-394.

29. Clark, K.E., Gobas, F.A.P.C., Mackay, D. 1990. Model of organic chemical uptake and clearance by fish from food and water. Environ. Sci. Technol. 24: 1203-1213.

30. Heberer, T. And Dünnbier, U. 1999. DDT metabolite bis(chlorophenyl)acetic acid: the neglected environmental contaminant. *Environ. Sci. Technol*. **33**, 2346-2351.

31. Mackay, D. 1982. Correlation of bioconcentration factors. Environ. Sci. Technol. 16: 274-278.

32. Campens, J. and Mackay, D. 1997. Fugacity-based model of PCB bioaccumulation in complex aquatic food webs. Environ. Sci. Technol. 31:557-583.

33. Morrison, H.A., Gobas, F.A.P., Lazar, R., Whittle, D.M. and Haffner, G. D. 1997. Development and verification of a benthic pelagic food web bioaccumulation model for PCB congeners in Western Lake Erie. Environ. Sci. Technol. 31: 3267-3273.

34. Elliott, J. M. 1994. Quantitative ecology and the brown trout. Oxford University Press. Oxford. UK. 286 pp.

35. Vives, I.; Grimalt, J. O.; Catalan, J.; Rosseland, B. O. and Battarbee, R. W. (submitted). Influence of altitude and age in the accumulation of organochlorine compounds in fish from high mountain lakes. Environ. Sci. Tech.

36. Wania, F.; Mackay, D. Sci. Total Environ. 1995, 160/161, 211-232.

ARTICLE 5

LEVELS OF ENDOCRINE DISRUPTION ACTIVITY ASSOCIATED TO ORGANOCHLORINE COMPOUNDS IN FISH EXTRACTS FROM EUROPEAN MOUNTAIN LAKES

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ENDOCRINE DISRUPTION ACTIVITY IN ORGANOCHLORINE COMPOUNDS EXTRACTED FROM FISH INHIBITING EUROPEAN MOUNTAIN LAKES

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ABSTRACT

We analyzed the presence of endocrine disruptors in fish from 10 European mountain lakes, spanning from Norway to Bulgaria and Spain. Organic components from total of 55 fish muscle samples were extracted using a protocol that eliminates natural hormones and analyzed for estrogenic activity with a designed version of the Recombinant Yeast Assay. We combined these data with a GC-MS analysis of the organic contaminants present in muscle and liver from the same animals. Both highly- and low-estrogenic samples were found in most lakes studied; the lakes showing the highest proportion of highly estrogenic samples were Velke Hinçovo in the Tatra mountains (Slovakia) and Redo in the Pyrenees (Spain). Endocrine disruption activity was strongly correlated with fish age, as well as with the concentration, in muscle and/or in liver, of several organochlorine compounds, like pp'-DDE, HCHs, and high-number PCBs. The data provided evidence for bioaccumulation of endocrine disruptors, and for a significant contribution of non-accumulative organochlorine compounds, such as γ-HCH. Considering both contributions, we could interpret more than 80% of observed variability between different lakes and among fish from individual lakes. We consider that our data may help on the future characterization an control of endocrine disruptors in remote areas.

INTRODUCTION

Endocrine disrupting chemicals (EDCs) are pervasive chemical pollutants able to penetrate into exposed biota and alter their endocrine system, by mimicking or counteracting natural hormones (*1*). These alterations may lead to multiple deleterious effects, from sterility to mental deficiencies and to a variety of development defects (*2*), (*3*). EDCs may have physiological effects at extremely low concentrations, some times under the nM range. In addition, many of them bioaccumulate along trophic chains, which increases their potential toxicity (*4*).

Among EDCs, those that mimic the female steroid hormone estradiol are specially relevant, both in terms of environment and of public health (*5*). These compounds bind to the estrogen receptor (ER), the key factor that elicits estrogenic response in vertebrates (*5*). There are several structural trends that could mark a given molecule as a putative ligand for the ER (*6*); however, the definitive characterization of a compound as an endocrine disruptor can only be attained by functional assays, either *in vivo* or *in vitro* (*7*), (*8*).

The recombinant yeast assay (RYA) is one of the most convenient functional assays to evaluate the potential for endocrine disruption of a substance, or, as in the present case, an environmental sample (*9*), (*10*), (*11*). It consists of an engineered yeast strain that harbors two foreign genetic elements: a vertebrate receptor (in our case, a human estrogen receptor, ER) and a reporter gene whose expression is made dependent on the presence of estrogens and whose final product concentration is easy to quantify. This is a simplified version of the mechanism by which natural estrogens operate in vertebrates; the fundamental similarity of all eukaryotes ensures that it also works in yeast in a similar way.

Mountain lakes rank among the most fragile ecosystems on Earth. The EMERGE project aims to the study a large number of European remote lakes in order to assess their actual status, to evaluate their signs of degradation or recovery, and to forecast their future. In this context, mountain lakes were defined as those water bodies lying above the local tree-line, devoid of hydric inputs except for rain and snowfall, and suffering a minimal human impact. Despite to their remote location, mountain lakes show important levels of organic contamination, essentially due to polycyclic aromatic hydrocarbons (PAHs) and organochlorine compounds (OCs) (*12*), (*13*), (*14*). The presence of OCs in remote areas is potentiated by their ability to migrate from temperate to cold areas, where they can become trapped. This occurs both at planetary scale (*15*) and at regional level, where mountain ranges serve as cold traps (*14*). As some of these compounds are suspected or *bona fide* EDCs (*16*), (*11*), (*4*), there is a need for direct measurement of estrogenic activity in the field, in order to known whether higher organisms inhibiting remote sites are truly exposed to estrogenic effects and whether those

compounds reported to have estrogenic activity may really act as EDCs at the concentrations found in these environments.

Fish from remote lakes constitute excellent biomarkers for monitoring the pollution status of the lakes. They suffer from several stresses, including low temperatures, low ionic content of the water, and chemical contamination (*17*), (*18*), (*19*). In the present work, we aimed to detect the presence of EDCs in muscle of fish from European remote lakes (mountain lakes and Arctic lakes) by using a version of the RYA specially developed for testing highly hydrophobic compounds. The experimental setup was designed to avoid interference from natural estrogens by eliminating them by chemical treatment. Our goal is to check whether semi-volatile compounds deposited on the lakes by rain or snow may contribute to the low reproductive efficiency of remote lake fish populations (*18*) by acting as endocrine disruptors.

MATERIAL AND METHODS

Samples

Fish muscle and sediment samples were collected as described in http://www.mountain-lakes.org/methods/index.html. Table 1 lists the names, codes and *Fish collection and determination of physical parameters.*

Materials

Potassium hydroxide, *n*-hexane, dichloromethane, methanol, concentrated sulphuric acid 95-97% and anhydrous sodium sulphate were from Merck (Darmstadt, Germany). Cellulose cartridges (20 mm x 80 mm, Whatman Ltd, UK) and sodium sulphate were Soxhlet-extracted before use, and sodium sulphate subsequently activated overnight by heating at 400ºC.

Yeast strains and plasmids. Yeast strain BY4741 (MAT**a** *ura3Δ0 leu2Δ0 his3Δ1 met15Δ0*) was obtained from EUROSCARF, Frankfurt, Germany. Expression plasmid pH5HE0 contains the human estrogen hormone receptor HE0 cloned into the constitutive yeast expression vector pAAH5 (*9*). Plasmid pVITB2x contains two copies of the pseudo-palindromic estrogen responsive element ERE2 from *X. laevis* vitellogenine B1 gene (5'-AGTCACTGTGACC-3') inserted into the unique *Kpn*I site of pSFLΔ-178K (*9*). Yeast manipulations, including transformation by the LiAc method, were performed as described in (*20*).

Fish muscle extraction and clean-up.

Protocols for fish muscle dissection and sample treatment and extraction are described in (*21*) (see also http://www.mountain-lakes.org/methods/index.html). In brief, each sample was divided in two halves, one for chemical analysis and the other for RYA assay. In both cases,

between 1 and 3 g of muscle tissue were mixed with activated sodium sulphate and grounded to a fine powder. The mixture was introduced into cellulose cartridges and Soxhlet-extracted with 100 ml of *n*-hexane-dichloromethane (4:1) for 18 h. For chemical analysis, surrogate standards (tetrabromobenzene –TBB- and PCB 209) were added to the extracts. Then, they were concentrated under vacuum to 2 ml and 2 ml of sulphuric acid were added. After vigorous stirring in a Vortex-mixer (2 min) the mixture was centrifuged to remove any foam in the interface, and the sulphuric acid layer was discarded. The clean-up step was repeated 3 times to obtain a colorless *n*-hexane layer, which were concentrated by vacuum rotatory evaporation to approximately 500 µL. A final concentration step to near dryness was attained by evaporation under a gentle stream of nitrogen. Samples for chemical analysis were redissolved to 50 µL with an internal standard mixture of tetrachloronaphthalene (TCN) and octachloronaphthalene (OCN) in isooctane for instrumental analysis, while 1 mL of methanol was added to the samples for RYA assay, which were concentrated again to 300 µL. Methanol-dissolved extracts can be used directly for RYA, as it is relatively non-toxic for yeast cells.

Instrumental analysis.

OCs analysis were carried out by gas chromatography with electron capture detection (Hewlett-Packard Model HP-5890). Detailed description of chromatographic conditions are described in (*14*). In addition, compound identification was confirmed by gas chromatography coupled to mass spectrometry, operating in the negative ion chemical ionization mode (GC-MS-NICI), using NH_3 as reagent gas as described elsewhere (*22*). Quantitative levels were determined by the internal standard method, the response factors being referred to the internal standard mixture. OCs reported levels were corrected by blank and surrogate recovery (*21*), (*13*), (*12*).

Estrogenic activity test

RYA assays using fish extracts were performed as described (*9*), except for the use of 300 µL glass tubes (baked at 400°C overnight) instead of polypropylene titration plates. Serial dilutions from 1:10 to 1:1250 (in 5-fold steps) were performed for each sample; some samples required dilutions up to 1:26250 to dilute out their estrogenic potential. The RYA does not provide a direct measurement of the molar (or mass) concentration of endocrine disruptors, but of their estrogenic activity. For simplification, results were calculated as "estradiol equivalents," defined as the amount of estradiol that should be present to account for all the observed response in a given sample. This was calculated from the lowest dilution in which the ß-galactosidase activity was indistinguishable from that of the control (only vehicle). Based on a series of experiments with muscle extracts from non-contaminated trout from a local fishery (24 assays in total), we established a threshold equivalent to 40% of the ß-galactosidase value for the negative controls in each set of experiments. Values for each fish were calculated from at least two independent

serial dilutions (4.3 replicates per fish on average). When necessary, the lowest active dilution was calculated as the geometric mean of the results from the different replicates. The estrogenic activity in this lowest active dilution was considered equivalent to the detection limit for estradiol (2 ppt in these conditions, data not shown, (*9*)). This value was then corrected for the wet weight of fish muscle corresponding to the initial inoculum (in g) to give the final equivalency in ppt of estradiol (ppt E2 eq.).

RESULTS

1.- Estrogenic activity in fish from Europe remote lakes.

The recombinant yeast assay using muscle extracts from 55 fish from 10 Europe mountain lakes showed very broad ranges of estrogenic activity values, being RD and VH the lakes showing the highest levels of estrogenic activity and OK, BL and OH the ones giving the lowest values (Table 1, geographical coordinates of each lake in http://www.mountain-lakes.org/districts/index.php). Estrogenicity values followed a extremely skewed distribution, with most samples (34/55) showing very low estrogenic activity (less than 10 ppt E2 eq) and a reduced number of samples (9/55) showing extremely high levels, over 1000 ppt E2 eq. This skewed distribution was also observed within fish populations from six out of the ten lakes studied (Table 1, see also Figure 1). This type of distribution prompted us to use median and ranges, instead of average and standard deviation, to describe the aggregated values for samples from a given lake (Table 1).

To study the distribution of estrogenicity activity in different lakes, we divided the complete set of data in quartiles. We slightly modified the distribution by including in the first (lowest estrogenicity value) quartile all 16 samples giving estrogenicity values below detection level, i.e., showing no estrogenic activity at any dilution in the RYA. The rest of quartiles included data from 13 samples each. Figure 1 shows absolute frequencies of samples falling in each quartile for each studied lake. As indicated by median and range values in Table 1, VH and RD gave the highest proportion of fishes with high estrogenicity levels (fourth quartile), whereas BL, OK and OH gave the lowest. It is also evident the co-existence of high and low-estrogenicity samples (first and fourth quartiles) in many lakes.

Fish samples studied here belong to three species, *Salvelinus alpinus* (RF), *Salvelinus fontinalis* (OK) and *Salmo trutta* (rest of lakes, Table 1). Although none of *Salvelinus* samples felt into the fourth quartile, the distribution in quartiles of *Salvelinus* samples were not significantly different from that of the whole set of data (χ^2 test, p=0.30). In addition, samples from the two lakes from Rila (OK and BL) gave very similar values in contents of the main organic

contaminants analyzed, despite the divergence on the fish species (not shown). This suggests that species diversity was irrelevant in terms of the content of estrogenic contaminants in fish samples.

Table 1. Fish samples analyzed in this work.

					Estrogenic activity (ppt E2 eq.)	
District	**Lake**	**Lake code**	**Fish species**	**N# specimens**	**Median**	**Range**
Central Norway	Øvre Neådalsvatn	ON	*S. trutta*	5	4.1	1600 - bdl
	Nedre Neådalsvatn	NN	*S. trutta*	4	4.2	186 - bdl
	Ø. Heimdalsvatnet	OH	*S. trutta*	4	1.4	7 - bdl
	Fallbekktjørna	FK	*S. trutta*	6	7.4	325 - 3
Scotland	Lochnagar	LN	*S. trutta*	4	13.0	2800 - bdl
Pyrenees	Redon	RD	*S. trutta*	11	140.7	36000 - bdl
Central Alps	Rotfelssee	RF	*S. alpinus*	5	3.0	64 - 2
Tatra Mountains	Velké Hinçovo	VH	*S. trutta*	6	315.7	37000 - 15
Rila	Bliznaka	BL	*S. trutta*	5	2.1	3 - bdl
	Okoto	OK	*S. fontinalis*	5	bdl	8 - bdl

bdl: Below detection limits

We observed a strong correlation between fish age and estrogenicity of muscle extracts. The age average of animals included in the fourth quartile were more than twice the age average of animals in the first or second quartiles, a statistically significant difference ($p=6x10^{-4}$, Table 2) Other physical parameters, such as length, weight, did not show any significant correlation with estrogenicity among the examined samples (Table 2). Although there was an increased proportion of males in the fourth quartile (Table 2), the observed differences were not statistically significant (χ^2 test, $p=0.45$).

2.- Estrogenicity as a function of chemical contamination by organochlorine compounds.

Parallel chemical and RYA analysis of samples allowed us to correlate estrogenicity with concentrations of different organic pollutants. At this point, it should be noted that the extraction procedure and clean-up that we used limited the number of compounds to analyze to several OCs families: polychlorobiphenyls (PCBs), hexachlorobenzene (HCB), DDT derivatives, and hexachlorocyclohexanes (HCHs). Most non-derivatized organic compounds (including natural sex hormones) were destroyed during the sulphuric digestion of the samples

(see M&M); therefore, they were not only not detectable, but also they could not influence the RYA results.

We analyzed OCs in muscle and liver from the same fish samples. There was a strong correlation between high estrogenic activity and the concentration of total PCBs in both liver and muscle (Table 2). There were also significant correlations between estrogenicity and the content in α-HCH and pp'-DDE in liver (but not in muscle), and the content in γ-HCH in muscle, but not in liver (Table 2). When the different PCBs congeneres were analyzed separately, we observed a selective enrichment in high number PCB congeners in the third and fourth quartiles relative to the first quartile (Figure 2).

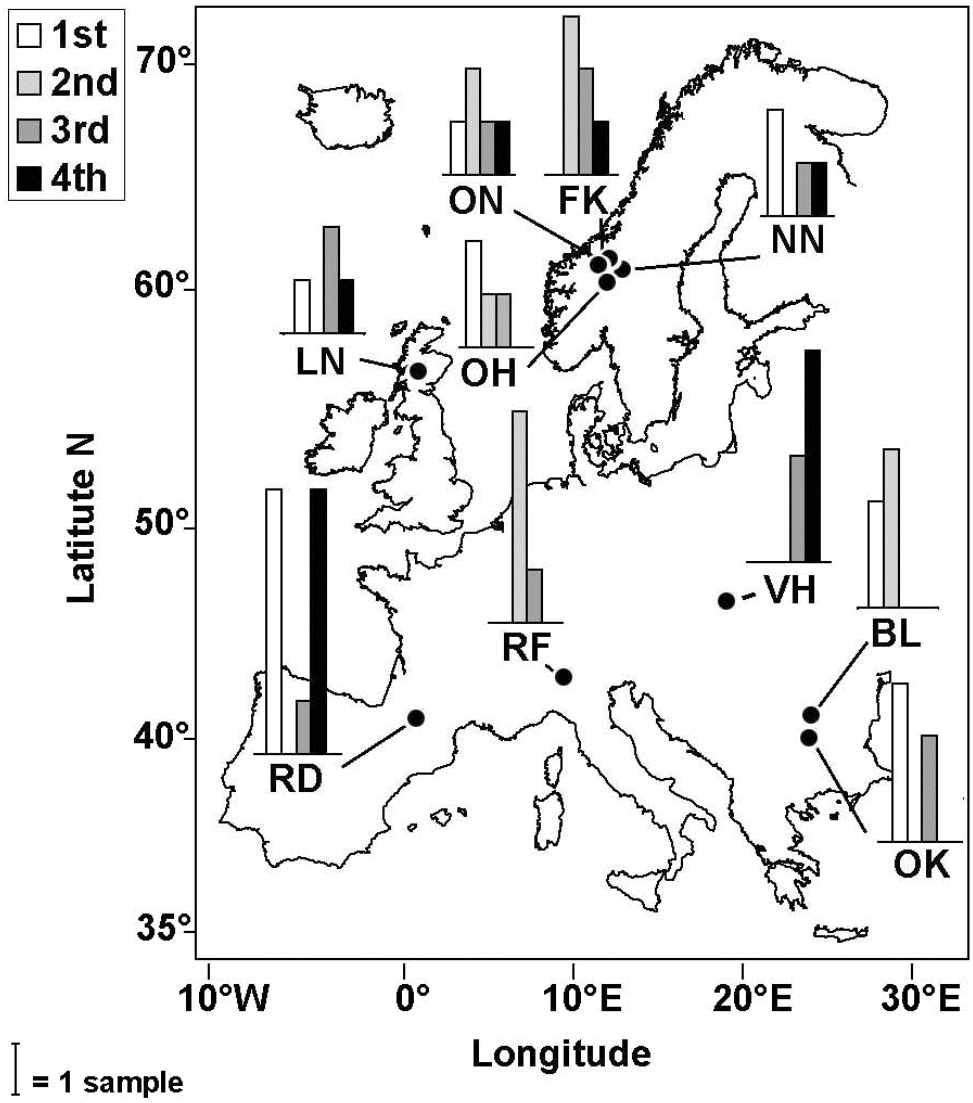

Figure 1.- Map of estrogenicity in fish from European mountain lakes. Lakes codes are indicated in Table 1. Graphs represent absolute frequencies of fish samples belonging to the first (empty), second (light gray), third (dark grey) and fourth (solid) quartiles, according to their increasing estrogenicity values. The line at the bottom left corner indicates the bar size corresponding to one sample.

Table 2. Physical and chemical data from fish samples distributed in quartiles.

	Quartile			
	1st	**2nd**	**3rd**	**4th**
Length [cm]	26.69 ± 6.96	26.05 ± 4.10	28.67 ± 6.95	28.45 ± 6.53
Weight [g]	259.33 ± 214.00	185.4 ± 101.0	274.62 ± 198.1	246.8 ± 180.6
Sex ratio	0.78	0.63	1.60	2.25
Age [years]	4.25 ± 3.38	5.23 ± 1.88	8.38 ± 5.38 *	10.50 ± 5.14 ***
Content in organochlorine compounds [ppb]				
Muscle				
α-HCH	0.12 ± 0.12	0.15 ± 0.13	0.08 ± 0.08	0.15 ± 0.13
γ-HCH	0.34 ± 0.28	0.17 ± 0.12 *	0.37 ± 0.37	0.74 ± 0.68 *
HCB	0.48 ± 0.59	0.30 ± 0.26	0.26 ± 0.18	0.58 ± 0.51
Sum PCB	4.19 ± 4.04	4.13 ± 3.76	5.17 ± 4.99	11.19 ± 7.68 **
DDT	1.31 ± 1.83	0.52 ± 0.59	0.87 ± 0.97	1.47 ± 1.30
DDE	19.66 ± 41.75	13.78 ± 42.72	10.08 ± 14.63	21.68 ± 18.46
Liver				
α-HCH	0.08 ± 0.06	0.20 ± 0.21	0.21 ± 0.32	1.58 ± 1.49 **
γ-HCH	0.68 ± 1.09	0.29 ± 0.40	0.60 ± 1.13	2.42 ± 2.51
HCB	0.71 ± 0.44	1.45 ± 1.64	1.14 ± 1.27	0.73 ± 0.38
Sum PCB	5.86 ± 4.40	13.45 ± 6.26 **	16.38 ± 13.16 *	21.31 ± 14.13 **
pp'-DDT	1.00 ± 1.29	1.74 ± 0.94	1.53 ± 1.28	1.84 ± 0.73
pp'-DDE	7.43 ± 9.23	6.86 ± 4.27	19.07 ± 16.36 *	48.42 ± 36.62 ***

Average ± Standard deviation for samples belonging to each quartile.

Significancy of the differences relative to the first quartile (t-test): *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

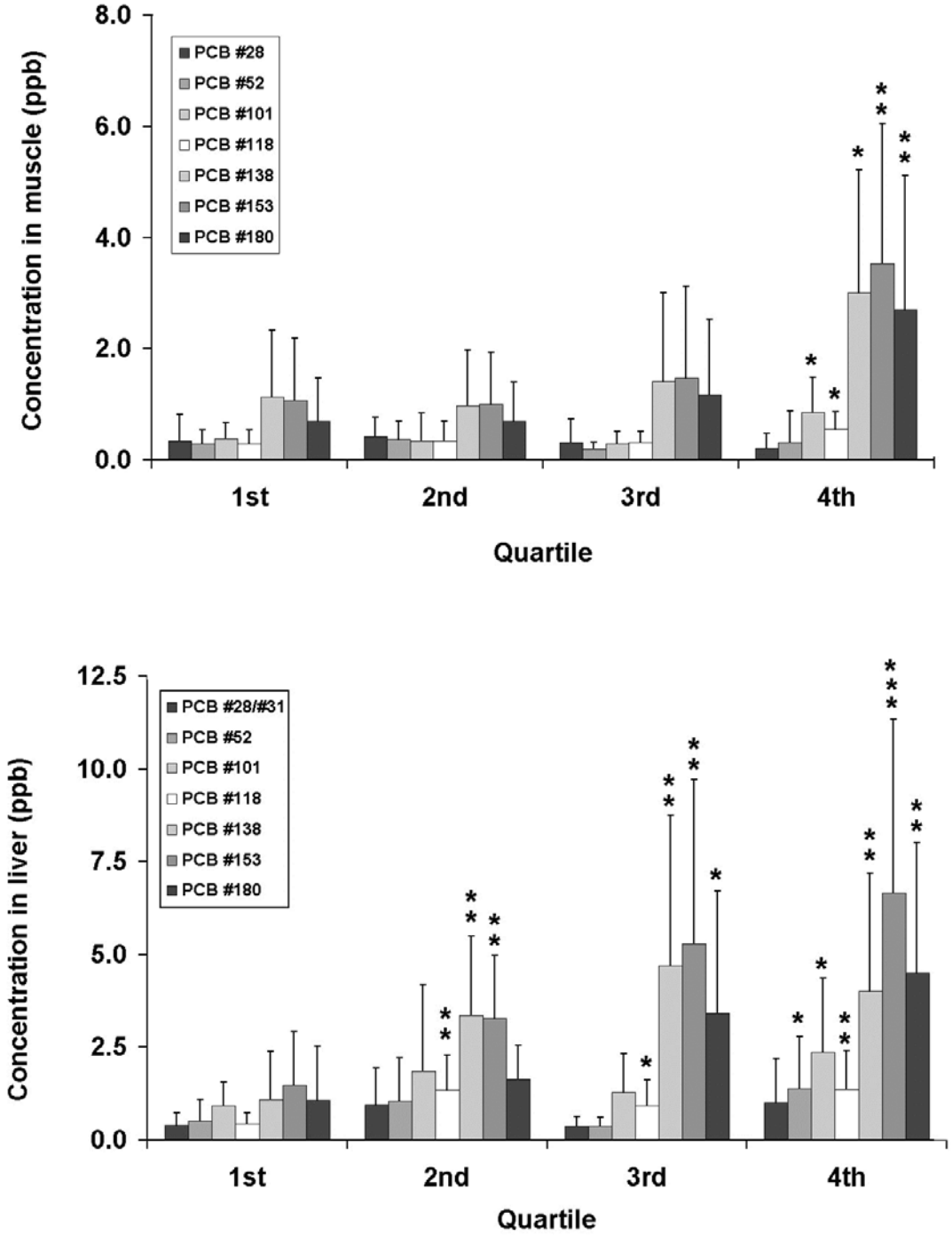

Figure 2.- PCB-congener composition profiles in muscle (top) and liver (bottom) of fish samples separated according to their estrogenicity in quartiles. Bars represent averages of all samples belonging to each quartile, lines indicate standard deviations. Stars indicate significant differences relative to the corresponding values for the first quartile (non-estrogenic samples) at 95% (*), 99% (**) and 99.9% (***) of confidence, calculated by the t-test.

The correlation between high number PCB concentration and estrogenicity is consistent with the correlation between age and estrogenicity, for high number PCBs accumulate in mountain lake fish, due to their high K_{ow} values. However, we wanted to explore the contribution of non-accumulative OCs to the observed distribution of estrogenicity among fish samples. Figure 3A shows a plot of age *versus* γ-HCH concentration in muscle for all individual samples analyzed. By marking differently samples according to their estrogenicity, we can define two sectors separated by a straight lane (Figure 3, top). Sector A (low age/low γ-HCH content) included most samples with low estrogenicity values (first and second quartiles), whereas sector B included all samples from the fourth quartile and most of samples from the third one (Figure 3, bottom). Note that none of the two parameters by itself can define such a neat division between high and low-estrogenicity samples; in fact, no single of the parameters studied by us could (unpublished observations). When individual samples were labeled according to their geographical origin in the same plot (Figure 4), their distribution reflected the geographical variability in estrogenicity of the samples. For example, samples from the Rila district (OK and BL, gray triangles), with low or no estrogenic activity, grouped in the A sector; all high-estrogenic samples from VH (Tatra district, solid squares) grouped in the B sector (Figure 4). Samples from Norway (ON, NN, OH and FK, empty circles) and Pyrenees (RD, solid diamonds), which showed broad distributions in estrogenicity (Figure 1), were distributed among the two sectors accordingly (Figure 4).

DISCUSSION

Functional bioassays for hydrophobic estrogenic compounds encounters several difficulties. It requires a sophisticated organic extraction, similar to the one used for their chemical analysis. Many of the targeted compounds are relatively insoluble in water, implicating the use of a semi-polar vehicles compatible with both the extraction procedure and the viability of the bioassay. Finally, hydrophobic compounds show a very strong tendency to adsorb to any organic material, including pipette tips, Eppendorf tubes, cell culture dishes, and microtitration plates. We solved these difficulties by developing a RYA version free from plastic material at any step, thus avoiding both adsorption of compounds to plastic surfaces and the release of estrogenic substances from plasticware into water solutions (*9*). In addition, we scaled the assay down to only 100µL of culture, to maximize the sensitivity of the assay and to reduce the amount of sample required --a critical aspect in samples from remote areas. This system allowed us to detect estrogenic activity in compounds that were difficult to analyze in culture due to their hydrophobicity, such as unmodified PCBs and other halogenated organic compounds (in

preparation). Estrogenicity values in fish muscle from mountain lakes is probably the result of a complex mixture of pollutants. The age of the animals appeared to be the most determinant single factor to explain estrogenicity values, suggesting that one or several bioaccumulative compounds may act as EDCs. This is consistent with the correlation between estrogenicity and high number PCBs, which bioaccumulate in mountain fish. It is worth considering that the correlation between high order PCBs concentration and estrogenicity was not only observed for muscle extracts, which were very similar to the ones used for the RYA, but also for liver extracts from the same fishes. These extracts were obtained in a completely different way and treated in a totally independent form. The fact that the correlation between liver extract concentrations and estrogenicity was indeed better than the correlation with muscle extracts value may reflect the better correlation of high order PCBs concentration with age in liver (not shown).

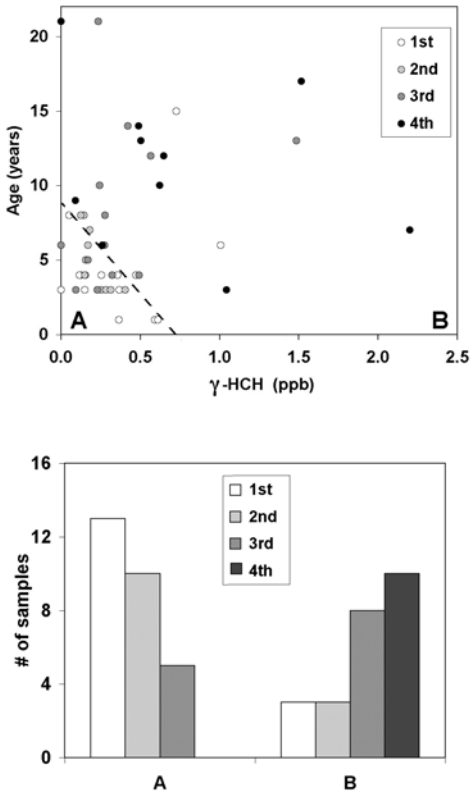

Figure 3. Distribution of high- and low-estrogenicity samples according age and γ-HCH content in muscle. The plot in the top shows individual samples; they are coded by their estrogenic activity as in Figure 1. The discontinuous line separates highly estrogenic (sector B) and low-estrogenic samples (sector A). The bottom graph shows absolute frequencies of samples in each sector, distributed in quartiles, as in Figure 1.

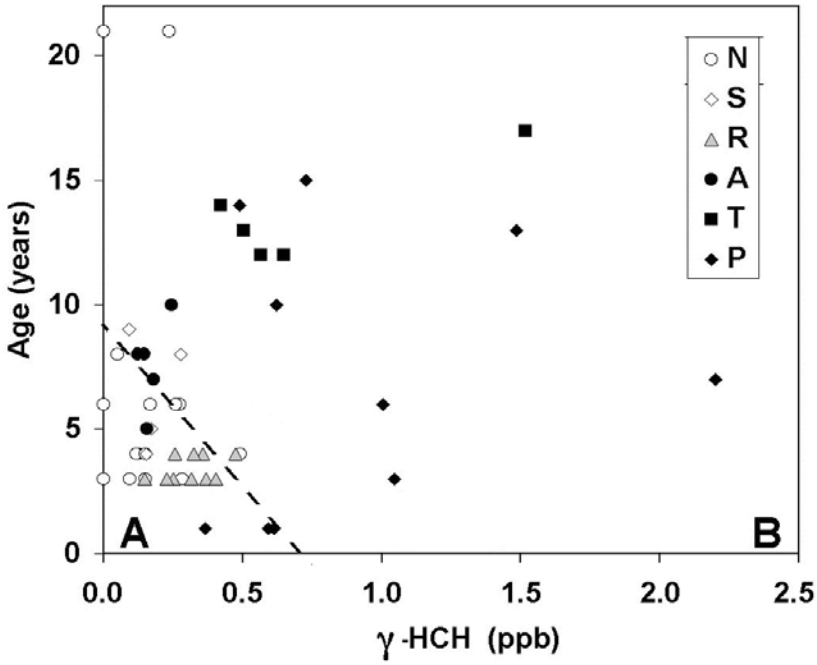

Figure 4. Geographical distribution of estrogenicity. This plot is identical to that of Figure 3, except for that geographical distribution instead of quartile distribution is indicated; low- (A) and high-estrogenicity (B) sectors are also indicated. Lakes are grouped in lake districts: N: Norway (ON, NN, OH, FK); S: Scotland (LN); R: Rila (OK, BL); A: Alps (RF); T: Tatras (VH); P: Pyrenees (RD).

Despite the good correlation of estrogenicity in muscle samples and concentration of bioaccumulative OCs, it were not sufficient to explain the enormous variation of estrogenic activity among samples from a same lake and between lakes from different districts. A better correlation was observed when we added a component representing non-accumulative OCs, such as γ-HCH. When considering both components in Figure 3, we could explain the estrogenicity values for more than 80% of samples. This result argues for a complex mixture of estrogenic compounds in the fish extracts, whose composition may vary among lake districts. This double correlation explains, for example, the absence of highly-estrogenic samples in lakes from Rila district (OK and BL) or from RF, as the corresponding samples clustered in or close to the low-estrogenicity sector. It also explains the enormous dispersion in estrogenicity values in fishes from Norway or from RD, whose samples were dispersed in a considerable area in the same plot.

Measuring estrogenicity in natural samples has the added problem of analyzing a complex mixture of compounds, from them only a small portion is characterized and an even smaller part is quantified. At the present, we do not know what compound(s) are responsible for the estrogenic activity we observed in our samples. It is doubtful that the compounds analyzed so

far (PCBs, γ̃HCH, HCB, etc) could account for the observed estrogenicity, which in some cases reached very high apparent concentrations, at ppb level of estradiol equivalents or even higher. Our results suggest that PCBs and other organochlorine compounds have estrogenic potentials between three and four orders of magnitude lower than estradiol in the RYA (in preparation); therefore the amounts of these compounds in the samples (tens to hundreds of ppb at maximum) appear too low to explain the observed responses. To account for the estrogenicity detected by the RYA in our samples we needed concentrations 10 to 50 times higher than the ones actually calculated by chemical assays. At this point, it is worth considering that there are 209 different PCB congeners, many of them capable to trigger estrogenic response in our assay (in preparation, (*4*)). In addition, it is possible that the combination of several compounds may have a synergistic effect, which could give a stronger response than the predicted addition of the single components (*23*). We have some indications that such cooperative effects occur in our samples (unpublished observations). Finally, we cannot exclude the presence of uncharacterized, highly estrogenic compound(s) in the extracts, such as polybrominated compounds, know to be also present in European mountain lakes, but whose effects are still poorly understood.

Semi-volatile organic compounds, many of them putative or confirmed EDCs, proved to be ubiquitous in the Biosphere, accumulating preferentially in cold regions, including apparently pristine areas such as mountain lakes (*15*), (*14*), (*24*). Our findings indicate that they accumulated in fish from mountain lakes at levels high enough to influence their hormonal behavior. These data only stresses the need for an accurate control of volatile and semi-volatile organic compounds that may ultimately reach remote regions anywhere in the planet. This control would require extremely sensitive chemical analyses as well as equally precise functional bioassays.

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REFERENCES

1. Kavlock, R. J., Daston, G. P., DeRosa, C., Fenner-Crisp, P., Gray, L. E., Kaattari, S., Lucier, G., Luster, M., Mac, M. J., Maczka, C., Miller, R., Moore, J., Rolland, R., Scott, G., Sheehan, D. M., Sinks, T., Tilson, H. A. *Environ Health Perspect.* **1996**, *104 Suppl 4*, 715-740.

2. Degen, G. H., Bolt, H. M. *Int Arch Occup Environ Health.* **2000**, *73*, 433-441.

3. Barlow, S., Kavlock, R. J., Moore, J. A., Schantz, S. L., Sheehan, D. M., Shuey, D. L., Lary, J. M. *Teratology.* **1999**, *60*, 365-375.

4. Bonefeld-Jorgensen, E., Andersen, H., Rasmussen, T., Vinggaard, A. *Toxicology.* **2001**, *158*, 141-153.

5. Colborn, T., vom Saal, F. S., Soto, A. M. *Environ Health Perspect.* **1993**, *101*, 378-384.

6. Fang, H., Tong, W., Shi, L. M., Blair, R., Perkins, R., Branham, W., Hass, B. S., Xie, Q., Dial, S. L., Moland, C. L., Sheehan, D. M. *Chem Res Toxicol.* **2001**, *14*, 280-294.

7. Mueller, S. O. *J Chromatogr B Analyt Technol Biomed Life Sci.* **2002**, *777*, 155-165.

8. Gaido, K. W., Leonard, L. S., Lovell, S., Gould, J. C., Babai, D., Portier, C. J., McDonnell, D. P. *Toxicol Appl Pharmacol.* **1997**, *143*, 205-212.

9. García-Reyero, N., Grau, E., Castillo, M., López de Alda, M., Barceló, D., Piña, B. *Environm. Toxicol. Chem.* **2001**, *20*, 1152-1158.

10. Rehmann, K., Schramm, K.-W., Krettrup, A. A. *Chemosphere.* **1999**, *38*, 3303-3312.

11. Coldham, N. G., Dave, M., Sivapathasundaram, S., McDonnell, D. P., Connor, C., Sauer, M. J. *Environ Health Perspect.* **1997**, *105*, 734-742.

12. Fernández, P., Vilanova, R., Grimalt, J. *Environ Sci Technol.* **1999**, *33*, 3716-3722.

13. Fernández, P., Vilanova, R., Martínez, C., Appleby, P., Grimalt, J. *Environ. Sci. Technol.* **2000**, *34*, 1906-1913.

14. Grimalt, J. O., Fernandez, P., Berdie, L., Vilanova, R. M., Catalan, J., Psenner, R., Hofer, R., Appleby, P. G., Rosseland, B. O., Lien, L., Massabuau, J. C., Battarbee, R. W. *Environ Sci Technol.* **2001**, *35*, 2690-2697.

15. Simonich, S., Hites, R. *Science.* **1995**, *269*, 1851-1854.

16. Petit, F., LeGoff, P., Cravedi, J. P., Valotaire, Y., Pakdel, F. *J Mol Endocrinol.* **1997**, *19*, 321-335.

17. Datta, S., Ohyama, K., Dunlap, D., Matsumura, F. *Ecotoxicol Environ Saf.* **1999**, *42*, 94-101.

18. Rosseland, B., Massabuau, J., Grimalt, J., Hofer, R., Lackner, R., Rognerud, S., Lien, L. *Zoology.* **1999**, *102*, 90-100.

19. Rose, N., Backus, S., Karlsson, H., Muir, D. *Environ Sci Technol.* **2001**, *35*, 1312-1319.

20. Sherman, F. *Methods Enzymol.* **1992**, *194*, 3-21.

21. Berdié, L., Grimalt, J. O. *J Chromatogr A.* **1998**, *823*, 373-380.

22. Chaler, R., Vilanova, R., Santiago-Silva, M., Fernandez, P., Grimalt, J. O. *J Chromatogr A.* **1998**, *823*, 73-79.

23. Silva, E., Rajapakse, N., Kortenkamp, A. *Environ Sci Technol.* **2002**, *36*, 1751-1756.

24. Colborn, T., Dumanoski, D., Myers, J. *Our stolen future*; Plume: New York, 1997 .

ARTICLE 6

POLYCYCLIC AROMATIC HYDROCARBONS IN FISH FROM REMOTE AND HIGH MOUNTAIN LAKES IN EUROPE AND GREENLAND

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Polycyclic aromatic hydrocarbons in fish from remote and high mountain lakes in Europe and Greenland

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) were analysed in liver of fifty-seven individual trout distributed among seven high mountain lakes in Europe and one remote lake in Greenland. In all cases, very similar distributions were observed in which phenanthrene largely predominated and fluoranthene and pyrene were the second major compounds. These distributions were similar to those observed in the dissolved fraction of the waters studied in three of these lakes. The range of concentrations of PAH in fish liver show only a five-fold variation, which is considerably smaller than the range more than two orders of magnitude of sedimentary PAH concentrations of these lakes. No correlation between PAH content in sediments and fish liver has been found both at the level of total and individual compounds. However, lake site is the main statistically significant factor of variability between PAH concentrations in fish liver. Changes in fish species explain significant differences in liver content of some PAHs. Within lake, condition factor and liver concentration are inversely correlated. Female fish display lower average concentrations than male in all lakes but the differences are not statistically significant. No correspondence between fish age and PAH content has been observed.

Keywords: Polycyclic aromatic hydrocarbons; Remote lake; Mountain lake; Fish liver PAH; Greenland; Lake sediments

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are toxic compounds, generally formed as the result of incomplete combustion of organic material (Howsan and Jones, 1998), whose input into the environment has increased extensively in the 20th century (Fernández et al., 2000). They are directly released to the atmosphere, both in the form of gas and associated with particles, where they are transported over long distances becoming ubiquitous global contaminants (Pacyna and Oehme, 1988; Wania and Mackay, 1996). In this respect, recent studies on sediments, water and air have demonstrated that these compounds are relevant pollutants in high mountain lakes (Fernández et al., 1996, 1999, 2000, 2002; Vilanova et al., 2001).

PAHs have been widely studied because of the carcinogenic and mutagenic properties of some of

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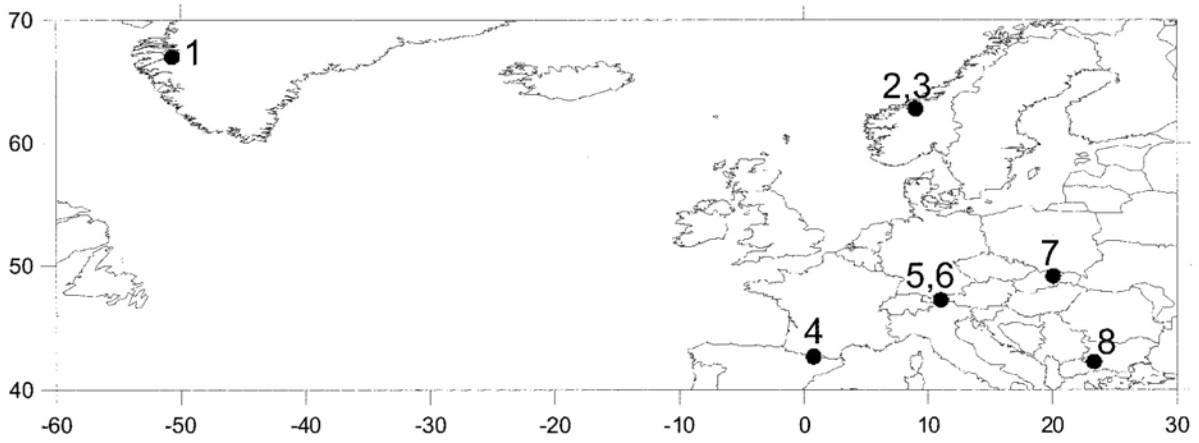

Fig. 1. Map showing the location of the lakes included in the study. 1, Ferguson; 2, Fallbekktjorna; 3, Ovre Neadalsvatn; 4, Redon; 5, Gossenköllesee; 6, Rotfelssee; 7, Vel'ké Hincovo; 8, Okoto.

them (Lehr and Jerina, 1997; Varanasi et al., 1987; Black et al., 1988; Aas et al., 2000, 2001). For this reason, some PAHs are included in the lists of priority pollutants from the US and EU. Toxico-hepatic lesions in fish have been related to PAH exposure (Myers et al., 1998).

The factors determining PAH accumulation in fish tissues are still to be elucidated. Exposure experiments with benzo[*a*]pyrene have shown that the concentrations of this compound in liver are approximately 100 times higher than that in muscle (Varanasi and Stein, 1991; Varanasi et al., 1987). PAH in fish can be metabolized rapidly to intermediates that either bind to liver DNA or form conjugates for ultimate transfer to bile (Varanasi, 1989; Collier and Varanasi, 1991; D'Adamo et al., 1997; Livingstone, 1998; Broman et al., 1990). In some studies, the occurrence of PAH in fish organs has been related to recent episodes of pollution exposure (Pointet and Milliet, 2000).

High-altitude mountain lakes offer unique environments for the assessment of atmospherically transported pollution inputs into biota. The series of lakes selected for study avoids systems receiving water flows, e.g. rivers, streams, from other aquatic systems. In addition, one lake from Greenland has been included as an example of a very remote site in comparison to the European locations (Fig. 1). The hydrology of the systems under study is dominated by atmospheric inputs. The organisms living in these systems are exposed to atmospherically transported pollution levels during all their life. Thus, these lakes constitute 'natural experiments' of long-term exposure to low doses of pollutants, as is the current case for most ecosystems. The study of PAH in trout living in these systems provides the opportunity of relating intake and accumulation of these compounds in fish to pollution inputs and fish biology in controlled populations under well-monitored pollution lake intakes. On the other hand, this is the first time, to the best of our knowledge, that PAHs have been studied in fish from high mountain lakes.

2. Materials and methods

2.1. Sample collection

Fish sampling followed standard test fishing procedures with multifilament gillnets. All fish were measured, dissected and sex determined on site. Livers were wrapped in pre-cleaned aluminium foil and kept frozen (−20 °C) until analysis. Otoliths and scales of all individuals were kept for age determination in the laboratory (NIVA).

Table 1
Characteristics of the lakes selected for study and average properties of the fish sampled and analysed in each lake

Lake	Mountain range (Country)	Latitude	Longitude	Altitude (m)	Total PAH in sediments (ng/g dw)	Number of individuals analysed	Fish species	Length (cm)	Weight (g)	Conditioning factor (cg/cm^3)	Sex		Age (yr)	Lipids (%)
											Males (n)	Females (n)		
Redon	Pyrenees (Spain)	42°38′N	0°46′E	2235	560	15	Brown trout	29	230	0.97	6	7	11	4.6
Fallbekkfjorna	Caledonian (Norway)	62°45′N	9°02′E	1043	1700	3	Brown trout	31	280	0.94		3	6	6.3
Øvre Neådalsvatn	Caledonian (Norway)	62°46′N	8°59′E	728	540	12	Brown trout	27	240	1.17	8	4	5	5.9
Gossenköllesee	Alps (Austria)	47°13′N	11°00′E	2413	590	11	Brown trout	23	140	1.01	6	5	5	2.1
Rotfelssee	Alps (Austria)	47°13′N	11°00′E	2485	440	5	Arctic charr	22	86	0.76	2	3	8	3.6
Okoto	Rila (Bulgaria)	42°12′N	23°18′E	2440	900	5	Brook trout	34	600	1.54	3	2	4	3.5
Vel'ké Hincovo	Tatra (Slovakia)	49°10′N	20°03′E	1946	7500	6	Brown trout	24	130	0.95	6		14	3.4
Fergusson	Greenland (Denmark)	66°58′N	50°39′W	60	12	4	Arctic charr	37	475	0.85	2	1	10	4.3

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mo trutta) was the species found in most of them. Okoto contained brook trout (*Salvelinus fontinalis*) and Fergusson and Rotfelssee contained arctic charr (*Salvelinus alpinus*). Biological data (length, weight, condition factor, sex and age) are summarized in Table 1.

3.1. PAH composition

The PAHs selected for analysis were fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene + triphenylene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenz[*ah*]anthracene, indeno[1,2,3-*cd*]pyrene and benzo[*ghi*]perylene. All of them are included in the EPA list of priority pollutants and some of them are described as mutagenic, carcinogenic and teratogenic (IARC, 1983).

In all cases, the PAH distributions are dominated by phenanthrene ($52 \pm 10\%$ of total analysed PAH), followed by fluorene, fluoranthene and pyrene ($\sim 10\%$ each; Fig. 2). The other PAHs involve individually less than 5%. Methylated PAH compounds were below the limit of detection. This average distribution is remarkably uniform among all studied lakes (relative standard deviations between 20 and 39%). The predominance of phenanthrene is consistent with the PAH composition found in fish liver from other freshwater (Pointet and Milliet, 2000) and marine systems (Baumard et al., 1998).

Previous studies in lakes Gossenkölle, Redó and Øvre Neådalsvatn have shown that phenanthrene is also the dominant PAH in high mountain lake waters, both in the dissolved and the particulate fractions (Fig. 2; Vilanova et al., 2001). However, waters may exhibit a higher proportion of the heavy molecular weight compounds, namely among the suspended particles. Chrysene + triphenylene, benzofluoranthenes, benzo[*e*]pyrene, indeno[1,2,3-*cd*]pyrene and benzo[*ghi*]perylene are commonly found in higher proportion.

Sedimentary PAHs from high mountain lakes may also be dominated by phenanthrene but this is not the common case (Fernández et al., 1999). Currently, benzofluoranthenes, chrysene + triphenylene, indeno[1,2,3-*cd*]pyrene and benzo[*ghi*]perylene are the major PAHs. The high

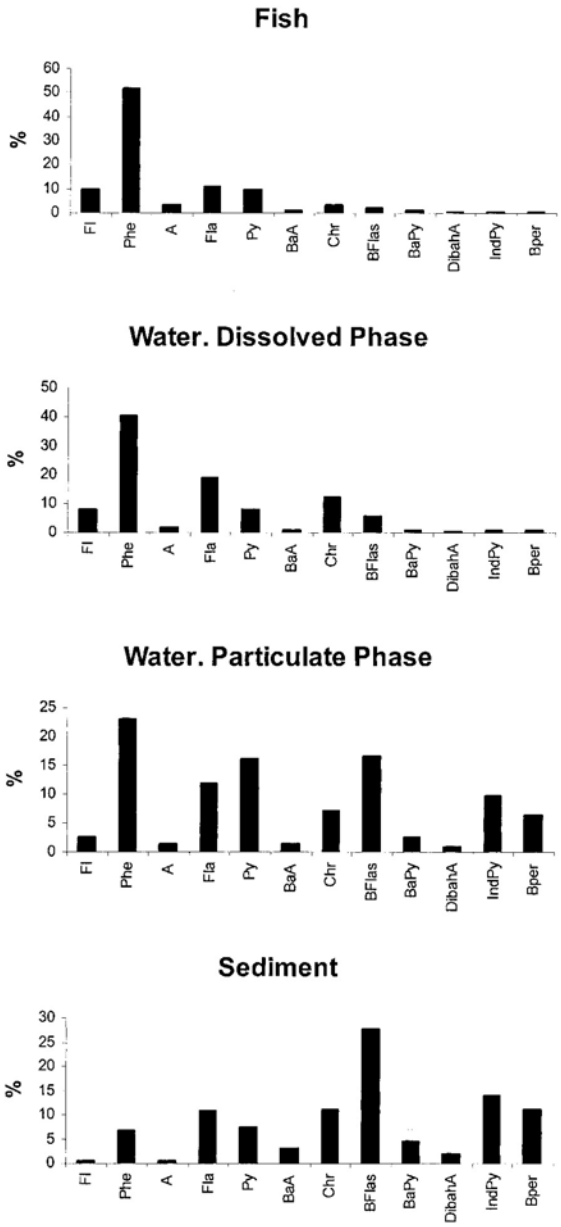

Fig. 2. Average PAH distributions in fish, water (dissolved and particulate phases) and sediments of remote mountain lakes. *Abbreviations:* Fluorene (Fl), Phenanthrene (Phe), Anthracene (A), Fluoranthene (Fla), Pyrene (Py), Benz[*a*]anthracene (BaA), Chrysene + Triphenylene (Chr), Benzo[*b*]fluoranthene + Benzo[*k*]fluoranthene (BFlas), Benzo[*a*]pyrene (BaPy), Dibenz[*ah*]anthracene (DibahA), Indeno[1,2,3-*cd*]pyrene (IndPy) and Benzo[*ghi*]perylene (BPer).

parallelism between the PAH distributions in the water-dissolved phase and fish liver is remarkable, suggesting that this environmental compartment is the most immediate PAH source for fish inhabiting high-altitude lakes.

3.2. PAH concentrations

The lowest and highest ∑PAH concentrations in fish liver (2.1 and 65.4 ng/g ww) were found in specimens from the Norwegian Region, lakes Fallbekktjørna and Øvre Neådalsvatn, respectively (Table 2). Mean PAH concentrations vary between lakes Vel'ké Hincovo and Rotfelssee (33–44 ng/g) and lakes Fallbekktjørna and Gossenköllesee (9–11 ng/g) involving a difference of 4–5 times. Normalization of the measured concentrations to lipid content gives concentration intervals that are somewhat larger, in this case ranging between 140 and 1200 ng/g lipid, i.e. nearly one order of magnitude.

Comparison of present data with other studies is limited by methodological differences, quantified compounds and concentration reporting (e.g. normalized to dry weight or to lipid weight). However, PAH concentrations in fish liver from the Mediterranean sea (2.0–13 ng/g in *Serranus scriba* and 1.4–4.7 ng/g in *Mullus Barbatus*, Baumard et al., 1998; transformed to wet weight basis as explained by Valette-Silver et al., 1999) and the Barcelona harbour (57 ± 19 ng/g in *Mugil auratus* and *Sarpa salpa*, Vives and Grimalt, 2002) exhibit values that are in the same order of magnitude as those found in the high mountain lakes. In contrast, those reported in La Camargue (96–210, 140–400 and 62–230 ng/g for *Carassius carassius*, *Anguilla anguilla* and *Anarhichas lupus*, respectively; Pointet and Milliet, 2000) exhibit higher values.

3.3. Factors of variability

Total PAH concentration is represented vs. age and conditioning factor in Fig. 3. Two types of plots have been performed, one for the results of all individual specimens and the other for Redo lake, the site in which the largest number of individuals have been analysed. As evidenced in both types of plots, no relationship between PAH concentration in fish liver and age is observed.

In contrast, the conditioning factor may be related in some way to liver PAH accumulation. An inverse trend between lower factor values and higher PAH accumulation is observed in Redo lake (Fig. 3) although the degree of significance is not very strong ($r^2 = 0.2375$; $P < 0.1$). Furthermore, Rotfelssee, the lake exhibiting the highest liver average PAH values, is the one where fish exhibit the lowest average conditioning factor (Table 1). However, representation of all individual specimens from all lakes shows no trend (Fig. 3).

Fish from most lakes considered for study are brown trout (Redo, Fallbekktjørna, Øvre Neådalsvatn, Gossenköllesee, Vel'ké Hincovo; Table 1). The *Salvelinus* genus is represented in Rotfelssee and Fergusson (arctic charr) and in Okoto (brook trout). ANOVA for comparison of liver PAH accumulation between brown trout and the *Salvelinus* group shows significant species-related associations for some compounds such as phenanthrene, chrysene, benzo[*ghi*]perylene ($P < 0.05$ in all cases) and dibenz[*ah*]anthracene ($P < 0.01$). The concentrations of the other PAHs do not exhibit significant differences (Table 3). Since phenanthrene is the dominant PAH in all fish examined, it has to be concluded that the species effect is relevant for PAH accumulation.

This observation is, in fact, consistent with the above-mentioned correspondence between conditioning factor and PAH accumulation since arctic charr is the species showing the lowest conditioning factor (Table 1). As mentioned above, fish from Rotfelssee exhibit the highest PAH concentrations whereas the PAH inputs into this lake, as measured from the amount of sedimentary PAH, range among the smaller of the whole series (440 ng/g; Table 1). Likewise, arctic charr from Fergusson, with the second smallest conditioning factor value, exhibit a rather high PAH liver content (15 ng/g; Table 2) whereas the PAH inputs recorded in this lake are very small (12 ng/g; Table 1). As indicated above, fish from the same species like brown trout in Redo lake exhibit a dependence between PAH in liver and conditioning factor (Fig. 3). The observed differences between *Salvelinus* spp. and *S. trutta* may reflect, in part,

Table 2
Lake averages and minimum and maximum concentration (between brackets) of the PAH concentrations in fish liver (ng/g ww)

	Redon	Fallbekktjorna	Øvre Neådalsvatn	Gossenköllesee	Roftelssee	Okoto	Vel'ké Hincovo	Fergusson
Fluorene	2.0 (1.3–3.6)	1.1 (0.61–1.8)	2.7 (1.0–5.6)	1.6 (0.86–4.3)	4.5 (3.2–6.5)	1.6 (0.53–2.6)	2.1 (0.85–4.0)	0.69 (0.47–0.90)
Phenanthrene	9.3 (4.5–19)	3.1 (0.40–9.3)	12 (0.97–43)	4.9 (1.8–21)	24 (17–30)	15 (11–21)	16 (8.7–28)	8.0 (5.6–12)
Anthracene	0.62 (0.24–1.6)	0.14 (0.01–0.51)	0.88 (0.26–2.4)	0.48 (0.21–1.3)	2.3 (1.4–3.7)	0.60 (0.22–0.89)	0.95 (0.64–1.6)	0.44 (0.35–0.53)
Fluoranthene	1.7 (0.77–4.4)	1.1 (0.13–3.4)	3.0 (0.55–6.9)	1.6 (0.86–2.9)	3.5 (2.6–5.3)	1.4 (0.99–2.1)	4.3 (3.0–7.1)	1.6 (1.1–2.1)
Pyrene	1.5 (0.70–3.1)	1.5 (0.40–3.1)	2.7 (0.76–5.4)	1.3 (0.56–2.3)	2.8 (1.9–4.2)	1.0 (0.71–1.5)	3.5 (2.5–5.7)	1.2 (0.88–1.5)
Benz[*a*]anthracene	0.12 (0.02–0.48)	0.11 (0.04–0.29)	0.16 (0.02–0.56)	0.08 (0.02–0.53)	0.16 (0.02–0.56)	0.13 (0.03–0.25)	0.67 (0.34–1.1)	0.24 (0.15–0.48)
Chrysene	0.48 (0.18–1.3)	0.14 (0.02–0.76)	0.37 (0.17–0.96)	0.18 (0.04–0.58)	2.1 (1.1–4.3)	0.52 (0.26–0.82)	3.0 (1.8–5.0)	0.64 (0.35–1.1)
Benzo[*b*]fluoranthene	0.21 (0.09–1.4)	0.12 (0.07–0.17)	0.17(0.02–0.29)	0.11 (0.02–0.59)	1.6 (0.68–4.3)	0.09 (0.06–0.14)	0.97 (0.56–1.8)	0.26 (0.18–0.45)
Benzo[*k*]fluoranthene	0.09 (0.02–0.58)	0.11 (0.06–0.21)	0.04 (0.02–0.14)	0.05 (0.02–0.27)	1.2 (0.53–3.5)	0.02 (0.007–0.07)	0.45 (0.22–0.83)	0.16 (0.11–0.22)
Benzo[*a*]pyrene	0.11 (0.02–0.71)	0.07 (0.03–0.13)	0.15 (0.05–0.46)	0.13 (0.04–0.56)	1.6 (0.73–4.0)	0.03 (0.02–0.04)	0.24 (0.09–0.44)	0.27 (0.18–0.40)
Dibenz[*ah*]anthracene	0.04 (0.03–0.45)	0.04 (0.03–0.06)	0.04 (0.03–0.44)	0.04 (0.03–0.14)	0.15 (0.03–2.8)	0.03 (0.03–0.03)	0.03 (0.03–0.07)	0.28 (0.13–0.43)
Indeno[*cd*-1,2,3]pyrene	0.03 (0.01–0.40)	0.05 (0.03–0.07)	0.13 (0.01–0.64)	0.05 (0.03–0.31)	0.06 (0.03–1.4)	0.03 (0.03–0.03)	0.03 (0.03–0.03)	0.27 (0.14–0.41)
Benzo[*ghi*]perylene	0.04 (0.03–0.47)	0.06 (0.02–0.16)	0.08 (0.03–0.44)	0.04 (0.02–0.24)	0.05 (0.02–1.1)	0.10 (0.07–0.19)	0.09 (0.02–0.16)	0.22 (0.11–0.32)
Sum PAHs	17 (7.8–29)	8.8 (2.1–20)	23 (3.9–65)	11 (5.6–31)	44 (32–65)	21 (15–27)	33 (22–53)	15 (11–19)

The number of individuals analysed in each lake are given in Table 1.

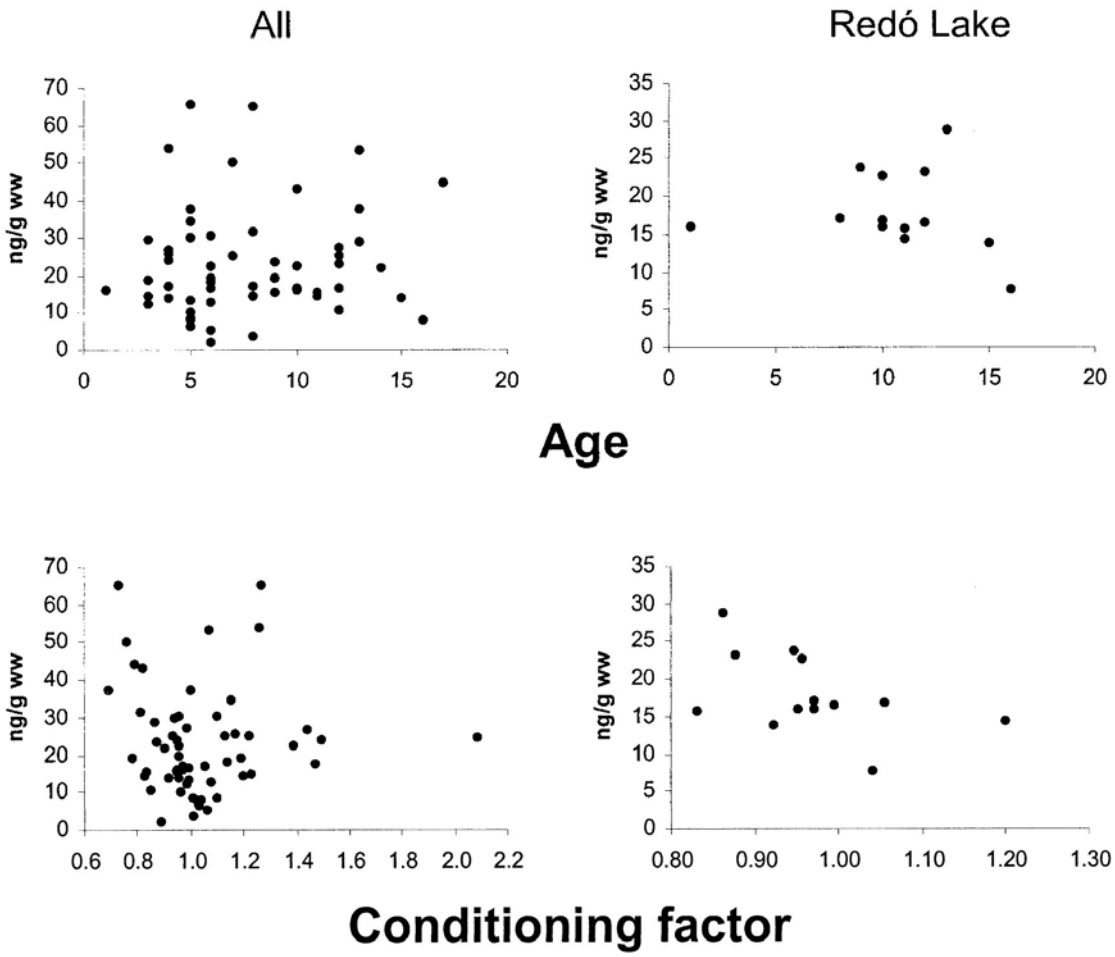

Fig. 3. Concentration of ΣPAH in liver (ng/g ww) plotted against age (years) and condition factor. Graphs on the left side include all analysed individuals; whereas only specimens from lake Redon (Pyrenees) are represented on the right. Condition factor is calculated as 100×weight/length^3. Weight and length in g and cm, respectively.

conditioning factor differences besides species-related metabolic effects.

In any case, the most significant difference for PAH concentration in fish liver is lake site. ANOVA between lakes shows that the differences are significant for all compounds ($P<0.0005$ in most cases; Table 3). Inter-lake differences are therefore one of the main factors determining PAH accumulation. These results prompt us to examine whether this difference could be related to PAH input.

3.4. Fish–sediment relationship

The PAH contamination load arriving to these high mountain lakes can be monitored from the concentrations in the sediments (Fernández et al., 1999). In this respect, previous studies have identified that the lakes in the Tatra mountains stand out among the European high-altitude lakes, currently two orders of magnitude more than the others, which reflects regional pollution sources (Fernández et al., 1999, 2000).

Table 3
Results of the ANOVA analysing differences between fish species and lake

	Species		Lakes	
	F	*P*	*F*	*P*
Fluorene	0.26	–[a]	7.4	<0.0005
Phenanthrene	4.8	<0.05	4.7	<0.0005
Anthracene	2.3	–	5.8	<0.0005
Fluoranthene	0.15	–	3.6	<0.001
Pyrene	1.7	–	5.1	<0.0005
Benz[*a*]anthracene	0.21	–	3.6	<0.001
Chrysene	5.7	<0.05	14	<0.0005
Benzo[*b*]fluoranthene	2.5	–	11	<0.0005
Benzo[*k*]fluoranthene	3.3	–	15	<0.0005
Benzo[*a*]pyrene	2.3	–	13	<0.0005
Dibenz[*ah*]anthracene	12	<0.05	4.4	<0.001
Indeno[*cd*-1,2,3]pyrene	0.86	–	4.6	<0.0005
Benzo[*ghi*]perylene	4.5	<0.05	2.3	<0.05
Sum PAHs	3.2	–	5.9	<0.0005

[a] Not statistically significant.

The PAH concentrations in the sediments of the lakes of the present study are given in Table 1. As expected, the sediment of the lake from the Tatra mountains, Vel'ké Hincovo, exhibits the highest values, 7500 ng/g of total PAH. This concentration is much higher than those of the other lakes. Thus, Fallbekktjørna and Okoto exhibit values equal to or higher than 900 ng/g and the others from continental Europe have concentrations in the range of 440–590 ng/g. Fergusson in Greenland has the lowest PAH content, 12 ng/g, which is consistent with the remoteness of the area from human influence.

Total PAH concentrations in fish liver range between 9 and 44 ng/g, reflecting a much narrower span compared with that of the sediments (~5 and 600 times between highest and lowest values, respectively). Correlation of the concentrations in fish liver and sediments show no significant correspondence both for total PAH and the individual compounds. This is illustrated in Fig. 4 where plots for phenanthrene (a volatile PAH), benzofluoranthenes (non-volatile PAH) and total PAH are shown. In these plots, PAH fish liver concentrations were expressed by reference to wet weight; normalization to lipid content not changing the trend. The sedimentary PAH data of Fig. 4 were normalized to total organic content but correlations with sedimentary values referring to sediment dry weight do not change the results. No correspondence between sediment and fish concentrations is therefore observed.

As mentioned above, other aspects such as species and conditioning factor are also relevant for PAH accumulation. Thus, arctic charr from lake Rotfelssee exhibit the highest average values in liver (Table 2) but its sedimentary PAH concentrations are the lowest among the mountain lakes studied (Table 1). However, representations of the phenanthrene, benzofluoranthenes and total PAH plots of Fig. 4 for only the lakes containing brown trout again show no significant correspondence.

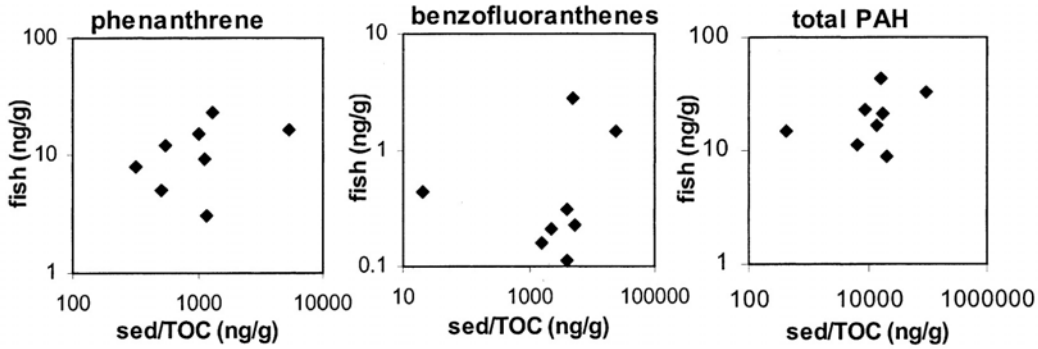

Fig. 4. Examples illustrating the lack of correspondence between PAH in fish liver (lake average values in ng/g ww.) and sediments (normalized to total organic carbon, ng/g) from the lakes where fish were collected.

This lack of correlation indicates that PAHs accumulated in fish liver are influenced by many processes as to reflect the pollution load arriving at these high mountain lakes. As indicated above, conditioning factor and species have some influence. Another aspect to be considered is sex. In this respect, brown trout collected in Fallbekktjørna, all female, exhibit the lowest values of the whole series whereas those from Vel'ké Hincovo, all male, show the highest. Examination of the male–female differences in fish liver PAH concentrations from lake Redo also shows lower values in female than male (16 and 20 ng/g, respectively) but the differences are not statistically significant. Likewise, in Øvre Neådalsvatn female fish exhibit lower total PAH concentrations than male (20 and 32 ng/g, respectively). Nearly the same concentrations in both groups are observed in Gossenköllesee but still females show lower values than males (13 and 14 ng/g, respectively). However, in none of these lakes are the mean differences statistically significant. Besides species, conditioning factor and maybe sex, other aspects specific of the fish populations in each lake must be relevant for the overall PAH accumulation in liver.

4. Conclusions

PAHs in liver from fish inhabiting high mountain lakes and one remote lake in Greenland exhibit uniform distributions irrespective of location. These distributions are largely predominated by phenanthrene and, in much lower concentrations, fluoranthene and pyrene. Higher molecular weight compounds are in very low abundance. Methylated PAHs if detected are only found at trace levels. These distributions are rather similar to the PAH composition of the dissolved phase of high mountain lake waters, suggesting that this water compartment is the most immediate PAH source for fish in these environments.

Total PAH content in fish liver and sediments range between 5 and 600 times, respectively. This contrast, and the lack of correlation between sedimentary and fish liver PAH, indicate that the concentrations of these hydrocarbons in liver are not in direct correspondence with PAH inputs. However, the most statistically significant factor of differentiation between fish liver concentrations is lake site, pointing to some properties of these oligotrophic systems as the most relevant for the accumulation of PAH in fish. Differences in fish species, namely between *S. trutta* and *Salvelinus* spp., have been found to be significant for some compounds. In addition, comparison of fish from the same lake shows significant correlations between condition factor and PAH concentrations. Female fish display lower concentrations than males in all lakes but the differences are not statistically significant. No significant relationship between fish age and PAH liver content has been found.

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References

Aas E, Baussant T, Balk L, Liewenborg B, Andersen O. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. Aquat Toxicol 2000;51:241–258.

Aas E, Beyer J, Jonsson G, Reichert W, Andersen O. Evidence of uptake, biotransformation and DNA binding of polyaromatic hydrocarbons in Atlantic cod and corkwing wrasse caught in the vicinity of an aluminium works. Mar Environ Res 2001;52:213–229.

Baumard P, Budzinski H, Garrigues P, Sorbe J, Burgeot T, Bellocq J. Concentrations of PAHs (polycyclic aromatic hydrocarbons) in various marine organisms in relation to those in sediments and to trophic level. Mar Pollut Bull 1998;36:951–960.

Black JJ, MacCubbin AE, Johnson CJ. Carcinogenicity of benzo(*a*)pyrene in the rainbow trout from embryo microinjection. Aquat Toxicol 1988;13:297–308.

Broman D, Näf C, Lundenbergh I, Zebühr Y. An in situ study on the distribution, biotransformation and flux of polycyclic aromatic hydrocarbons (PAHs) in an aquatic food chain (Seston—*Mytilus edulis* L.—*Somateria mollissima* L.) from the Baltic: an ecotoxicological perspective. Environ Toxicol Chem 1990;9:429–442.

Carrera G, Fernandez P, Vilanova R, Grimalt J. Analysis of trace polycyclic aromatic hydrocarbons and organochlorine compounds in atmospheric residues by solid-phase disk extraction. J Chromatogr A 1998;823:189–196.

Collier T, Varanasi U. Hepatic activities of xenobiotic metabolizing enzymes and biliary levels of xenobiotics in English sole (*Parophrys vetulus*) exposed to environmental contaminants. Arch Environ Contam Toxicol 1991;20:462–473.

D'Adamo R, Pelosi S, Trotta P, Sansone G. Bioaccumulation and biomagnification of polycyclic aromatic hydrocarbons in aquatic organisms. Mar Chem 1997;56:45–49.

Fernández P, Grimalt J, Vilanova R. Atmospheric gas-particle partitioning of polycyclic aromatic hydrocarbons in high mountain regions of Europe. Environ Sci Technol 2002;36:1162–1168.

Fernández P, Vilanova R, Grimalt J. PAH distributions in sediments from high mountain lakes. Pol Arom Comp 1996;9:121–128.

Fernández P, Vilanova R, Grimalt J. Sediment fluxes of polycyclic aromatic hydrocarbons in European high altitude mountain lakes. Environ Sci Technol 1999;33:3716–3722.

Fernández P, Vilanova R, Martínez C, Appleby P, Grimalt J. The historical record of atmospheric pyrolitic pollution over Europe registered in the sedimentary PAH from remote mountain lakes. Environ Sci Technol 2000;34:1906–1913.

Howsan M, Jones K. Sources of PAHs in the environment. In: Neilson AH, editor. Handbook of Environmental Chemistry vol. 3, Part 1, PAHs and Related Compounds, 1998.

IARC. Monographs on the evaluation of the carcinogenic risk of chemicals to humans: polynuclear aromatic hydrocarbons, vol. 32. Lyon (France): WHO; 1983.

Lehr RE, Jerina DM. Metabolic activations of polycyclic hydrocarbons: structure activity relationship. Arch Toxicol 1997;39:1–6.

Livingstone DR. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. Comp Biochem Physiol 1998;120:43–49.

Mosello R, Lami A, Marchetto A, Rogora M, Wathne B, Lien L, Catalan J, Camarero L, Ventura M, Psenner R, Koinig K, Thies H, Sommaruga-Wögrath S, Nickus U, Tait D, Thaler B, Barbieri A, Harriman R. Trends in the water chemistry of high altitude lakes in Europe. Water Air Soil Pollut 2002;2:75–89.

Myers M, Johnson L, Olson O, Stehr C, Horness B, Collier T, McCain B. Toxicopathic hepatic lesions as biomarkers of chemical contaminant exposure and effects in marine bottomfish species from the Northeast and Pacific Coasts, USA. Mar Pollut Bull 1998;37:92–113.

Pacyna J, Oehme M. Long-range transport of some organic compounds to the Norwegian Arctic. Atmos Environ 1988;22:243–257.

Pointet K, Milliet A. PAHs analysis of fish whole gall bladders and livers from the Natural Reserve of Camargue by GC/MS. Chemosphere 2000;40:293–299.

Valette-Silver N, Hameedi MJ, Efurd DW, Robertson A. Status of the contamination in sediments and biota from the Western Beauford sea (Alaska). Mar Pollut Bull 1999;38:702–722.

Varanasi U. Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. Boca Raton, FL: CRC Press, Inc., 1989.

Varanasi U, Stein J. Disposition of xenobiotic chemicals and metabolites in marine organisms. Environ Health Perspect 1991;90:93–100.

Varanasi U, Stein J, Nishimoto M, Reichert W, Collier T. Chemical carcinogenesis in feral fish: uptake, activation, and detoxification of organic xenobiotics. Environ Health Perspect 1987;71:155–170.

Vilanova R, Fernández P, Martínez C, Grimalt J. Polycyclic aromatic hydrocarbons in remote mountain lake waters. Water Res 2001;35:3916–3926.

Vives I, Grimalt J. Method for integrated analysis of polycyclic aromatic hydrocarbons and organochlorine compounds in fish liver. J Chromatogr B 2002;768:247–254.

Wania F, Mackay D. Tracking the distribution of persistent organic pollutants. Environ Sci Technol 1996;30:390A–396A.