ARTICLE 1

METHOD FOR INTEGRATED ANALYSIS OF POLYCYCLIC AROMATIC HYDROCARBONS AND ORGANOCHLORINE COMPOUNDS IN FISH LIVER

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Method for integrated analysis of polycyclic aromatic hydrocarbons and organochlorine compounds in fish liver

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Abstract

An analytical method for integrated analysis of organochlorine compounds and polycyclic aromatic hydrocarbons (PAH) in large numbers of fish liver samples has been developed using one single clean-up step. Tissues are homogenized with anhydrous sodium sulphate and Soxhlet extracted with *n*-hexane–dichloromethane (4:1, v/v) for 24 h. The extracts are cleaned-up and fractionated with an alumina chromatographic column allowing the separation of the extracts in two fractions. One containing most organochlorine compounds, including hexachlorobenzene, DDTs and polychlorobiphenyls, and the other the hexachlorocyclohexane isomers and PAH. These two fractions are subsequently analysed by GC–MS. Tests of repeatability result in relative standard deviations mainly under 20%. Evaluation by the standard addition method shows good linearities and recoveries.

Keywords: Polycyclic aromatic hydrocarbons; Organochlorine compounds

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and organochlorine pesticides are ubiquitous contaminants in the environment [1,2]. These compounds have been widely studied because of their adverse effects on organisms, including humans [3–5]. Although their environmental concentrations are low they tend to accumulate in organic tissues due to their lipophilic character and persistence to degradation [6] which may eventually result in toxic concentration levels for organisms such as fish [7].

These compounds are persistent organic pollutants (POPs). Once released into the environment they may be transported over long distances and pass through many biogeochemical cycles, e.g. food web, without undergoing sensible degradation. Thus, despite the discontinued use of some of them, e.g. PCBs and most organochlorine pesticides, they are currently found in all sorts of environmental samples, including those collected in both polluted and remote sites [2]. In other cases their occurrence also reflects present production or increased delivery rates in recent decades. This is the case, for instance, of most PAHs whose concentration increased considerably in the 19th century as a consequence of the rise in fossil fuel combustion [8].

In these conditions, research is prompted to elucidate whether these compounds may have toxic

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effects on remote ecosystems, to ascertain how these potential effects are reflected in the species inhabiting these ecosystems and, most importantly, to identify the specific relationships between individual POPs and toxicity. In this respect, fish are ideal species for the study of remote aquatic systems since they are on top of the food web and therefore accumulate the highest POP concentrations.

However, studies of fish in remote sites such as high mountain lakes have to overcome major analytical difficulties such as low sample amounts and large numbers of POP to be determined. In addition to the sampling difficulties in these environments, the large number of measurements needed for the assessment of their health status limits the amount of material available for study. This is specifically the case for liver which, in turn, is the type of material from which more determinations may need to be performed, e.g. POPs, metals, histology, glycogen, enzymes and others [9]. In addition, high numbers of samples have to be analyzed in order to have representative measurements in each lake.

Thus, analytical methods allowing the analysis of large amounts of small size samples (ca. 1 g) have to be developed. Most of the presently available methods are devoted to the analysis of specific POP groups such as PCB and organochlorine pesticides (e.g. Refs. [7,10–13]) and PAHs (e.g. Refs. [14–16]). However, the stringent restrictions on sample amount require methods for the simultaneous analysis of organochlorine compounds and PAHs in fish liver. Development of these integrated methods in marine organisms has only been undertaken on a few occasions [17,18]. The present manuscript reports one of these methods based on a simple column chromatography clean-up procedure.

2. Materials and methods

2.1. Materials

Residue analysis *n*-hexane, dichloromethane, isooctane, methanol, acetone, NaOH pellets and anhydrous sodium sulphate for analysis were from Merck (Darmstadt, Germany). Aluminium foil was rinsed with acetone and let dry at ambient temperature prior to use. Neutral aluminium oxide type 507C was from Fluka AG (Switzerland). Cellulose extraction cartridges of 20 mm I.D. and 80 mm long were from Whatman (UK). The purity of the solvents was checked by gas chromatography–electron capture detection and no peaks were detected. Aluminium oxide, sodium sulphate, cartridges and NaOH pellets were cleaned by Soxhlet extraction with dichloromethane–methanol (2:1, v/v) for 24 h before use. Sodium sulphate and aluminium oxide were activated overnight at 400 °C and 120 °C, respectively.

γ-Hexachlorocyclohexane (γ-HCH) and tetrabromobenzene (TBB) were from Aldrich-Chemie (Steinheim, Germany). α-, β- and δ-HCH and PCBs (#28, #52, #101, #118, #138, #153, #180, #209) were from Promochem (Wesel, Germany), and pp′-DDE, pp′-DDT, PAHs mix9 and perdeuterated PAHs were from Dr. Ehrenstorfer (Augsburg, Germany). A standard mixture was prepared with the above mentioned HCH isomers, pp′-DDT, pp′-DDE, HCB, PCBs and PAHs (acenaphthene, acenaphthylene, naphthalene, phenanthrene, fluorene, fluoranthene, pyrene benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(ah)anthracene, indeno(1,2,3-cd)pyrene and benzo(ghi)perylene) at 50 ppb in isooctane.

Fish liver tissues were used to validate the methodology. They were cut, wrapped in pre-cleaned aluminium foil and kept frozen until extraction.

2.2. Soxhlet extraction

Liver tissue was ground with activated anhydrous sodium sulfate (7–9 g) and was introduced into a previously cleaned cellulose cartridge. TBB, PCB#209 [19] and perdeuterated anthracene, pyrene and benzo(ghi)perylene were used as internal standards. These five compounds used as internal standard mixtures were added for the study of the recoveries for the organochlorine compounds and polycyclic aromatic hydrocarbons. The cartridge was then Soxhlet extracted with *n*-hexane–dichloromethane (4:1, v/v) for 20 h. Then, the extract was vacuum evaporated until 1 ml and further concentrated to 50 μl in isooctane under a gentle nitrogen flow.

2.3. Alkaline digestion

The same internal standard mixture used for Soxhlet extraction was added to fish liver. This was

then introduced in an alkaline solution of 6 *M* NaOH for 14 h at 40 °C. A mixture of *n*-hexane–dichloromethane (4:1, v/v) was used to extract the compounds from the alkaline media. Then, the extract was vacuum evaporated until 1 ml and further concentrated to 50 μl in isooctane as described above.

2.4. Chromatographic fractionation and clean-up

The extracts obtained by Soxhlet extraction were fractionated by adsorption chromatography with glass columns (30 cm length and 0.8 cm I.D.) containing 5 g of aluminium oxide. Two fractions were collected: F1, 16.5 ml of *n*-hexane–dichloromethane (19:1, v/v) and 3 ml of *n*-hexane–dichloromethane (1:2, v/v); F2, 13 ml of *n*-hexane–dichloromethane (1:2, v/v). Both fractions were concentrated by vacuum rotary evaporation to 1 ml, transferred to vials, and further concentrated until 50 μl in isooctane as described above.

2.5. Recovery and standard addition test

Recoveries were calculated using 0.4-g liver fractions which were spiked with solutions containing 50 ng/ml of the standard mixture PAH and organochlorine compounds, 25 μl and 50 μl, respectively (Section 2.1).

Overall linearity of the method was calculated from concentrations of 0, 12, 25 and 62 ng/g for PAHs and 0, 0.4–3.1, 1.8–16 and 4–31 ng/g for organochlorine compounds which were prepared from aliquots of the standard mixture added to 0.4-g liver portions of the same freshwater trout. These subsamples were Soxhlet extracted and chromatography fractionated as previously described.

2.6. Instrumental analysis

Before chromatographic analysis, an internal standard of tetrachloronaphthalene and octachlororaphthalene was added (25 μl) to the vial to correct for instrument variability. Samples were analyzed by GC (Carlo Erba GC 8000) coupled to a quadrupole mass spectrometer (MS, Fisons MD800) with a 50-m HP-5MS column (0.25 mm I.D. and 0.25 μm film thickness). The machine operated in splitless mode (isooctane; hot needle technique) and electron impact mode (EI, 70 eV). The oven temperature program started at 90 °C (held for 1 min) to 120 °C at 10 °C/min, and then to 310 °C at 4 °C/min (holding time 15 min). Injector, transfer line, and ion source temperatures were 280 °C, 280 °C and 200 °C, 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. Under these conditions, repeated analysis of pp′-DDT standards at 250 °C, 270 °C and 280 °C showed no formation of pp′-DDE derivatives. Injection at 280 °C was preferred for the high molecular mass of some PCB congeners present in the samples. Helium was used as carrier gas (flow-rate 1.1 ml/min). Data acquisition was in selected ion recording mode at 40 ms of dwell time. The ion mass programs used for quantification are described elsewhere [1,20]. Quantification was performed by reference to calibration curves of the compounds included in Table 1 and subsequent correction using the internal standards for extraction and injection. The curves were generated by progressive dilution of standard mixtures and subsequent instrumental analysis. The regression lines of the curves closer to the lowest detectable concentration ranges were used to evaluate injector performance for linearity and sensitivity.

3. Results and discussion

3.1. Comparison of extraction methodologies

The method for the simultaneous analysis of organochlorine compounds and PAHs in fish liver developed in the present study is shown in Fig. 1. The recoveries of both organochlorine compounds and polycyclic aromatic hydrocarbons are better for Soxhlet extraction than alkaline digestion (Fig. 2). For Soxhlet extraction PAH and organochlorine compound recoveries were between 78.5–99% and 79–99%, respectively. In alkaline digestion these recoveries were between 67–95% and 0–81%, respectively. HCHs and pp′-DDT are destroyed as a consequence of the alkaline digestion which prevents their analysis [21,22]. These results show that Soxhlet extraction should be the method of choice.

Table 1
Results of the repeatability (relative standard deviation) and standard addition (recovery and r^2) tests for the integrated method of organochlorine compound and PAH analysis in fish liver (spiking standard mixture indicated in Section 2.5)

Compound	Relative standard deviation (%)	Recovery (%)	r^2	Limits of detection (pg/g)	Limits of quantitation (pg/g)	Compound	Relative standard deviation (%)	Recovery (%)	r^2	Limits of detection (pg/g)	Limits of quantitation (pg/g)
Acenaphthene	6.7	71	0.9988	15	16	α-HCH	6.4	110	0.9916	7.8	8
Fluorene	11	86	0.9945	17	17	γ-HCH	4.7	90	0.9896	6	6.2
Phenanthrene	15	130	0.994	8	8	HCB	3.7	68	0.9906	6.6	5.8
Anthracene	14	130	0.9789	10	10	PCB#28	5.5	110	0.9821	1.4	1.8
Fluoranthene	16	110	0.9988	11	13	PCB#52	10	79	0.9984	11	11
Pyrene	1.3	110	0.9987	12	12	PCB#101	11	83	0.9951	13	13
Benz(a)anthracene	16	75	0.9517	18	18	PCB#118	7.3	100	0.9986	21	21
Benzo(b)fluoranthene	16	79	0.9015	20	20	pp'-DDE	5.9	80	0.9988	1	1.2
Benzo(a)pyrene	16	84	0.8138	21	21	PCB#153	9.1	93	0.9996	23	25
Indeno(1,2,3-cd)pyrene	21	83	0.7803	26	26	PCB#138	8.4	88	0.9936	13	14
Dibenzo(ah)anthracene	23	80	0.7507	28	30	pp'-DDT	8.2	100	0.9968	22	22
Benzo(ghi)perylene	23	74	0.8135	25	26	PCB#180	3.0	92	0.9999	7.8	9.6

Fig. 1. Scheme of the analytical method for the integrated study of organochlorine compounds and PAH.

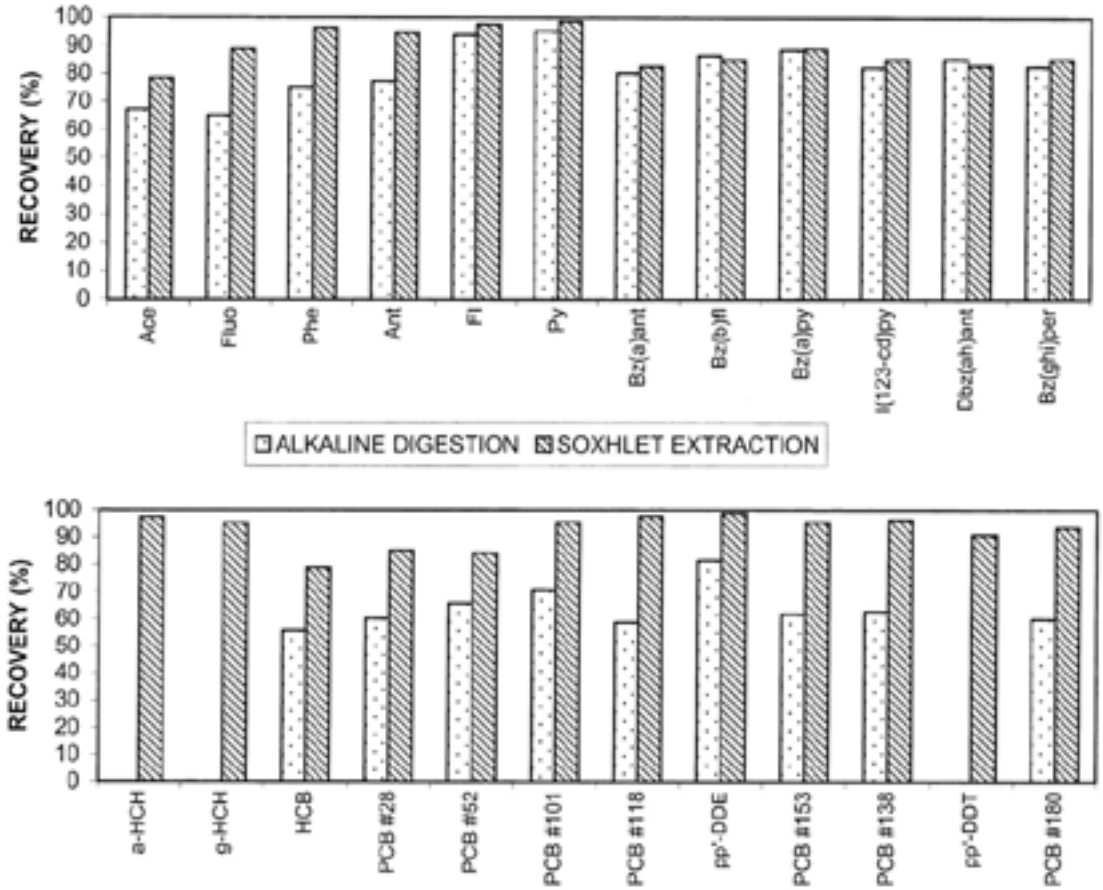

Fig. 2. Recovery values of alkaline digestion and Soxhlet extraction.

3.2. Development of chromatographic fractionation

The column chromatography method allowed the separation of the contaminants of interest in two fractions, the former containing all organochlorine compounds except HCH and the latter HCH and PAH (Fig. 3). The small dimensions of the column selected for clean up afford an easier and quicker method of preparation and sample treatment than in previous studies [23,24]. Thus, previously reported alumina/silica columns involve elution volumes of 65 ml for the recovery of PAH and additional 45 ml for the complete recovery of organochlorine compounds [14,23]. In the case of florisil columns, the complete elution of both compound groups is achieved with 45 ml [23]. In addition, the column chromatography method reported in the present study affords a better separation between the two compound groups than in these previous studies since all PAH are collected in the second fraction (together with HCH) and most organochlorine compounds, including HCB, DDTs and PCBs, in the former.

3.3. Repeatability, detection and quantification limits

The relative standard deviation (RSD) of the method was obtained from three replicates of one fish sample from the harbour of Barcelona. All compounds had levels higher than the limits of quantification. For PAHs, the RSD were around 16% or lower, except for indeno(1,2,3-cd)pyrene, dibenzo-

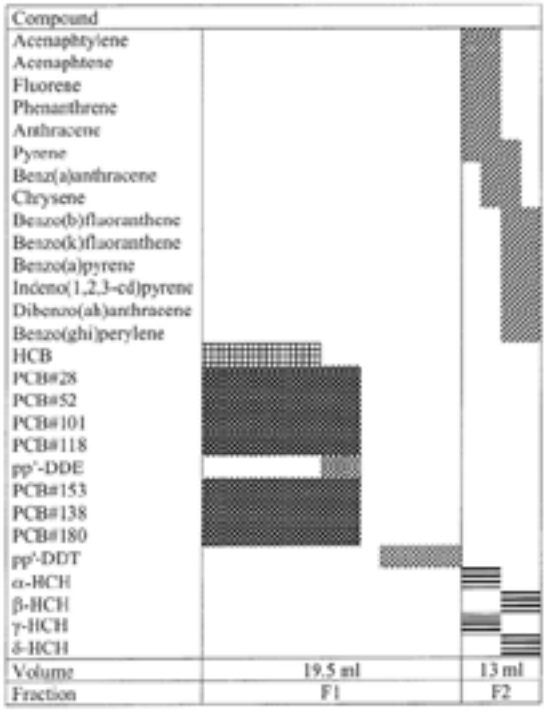

Fig. 3. Elution of PAH and organochlorine compounds in the column chromatography clean up developed in the present study.

(ah)anthracene and benzo(ghi)perylene in which it was between 20 and 24%. These values are similar to those reported in reference materials [18] and lower than those described in other studies on fish PAH [14]. All organochlorine compounds show good repeatability, with RSDs lower than 12% (Table 1). Previously reported RSD repeatabilities for organochlorine compounds were about 23% in whale tissues (including liver) [26] and between 0.1 and 22% for reference materials [18].

Detection and quantitation limits (Table 1) were calculated as described in Ref. [19]. They range between 8–30 pg/g and 1–25 pg/g for PAH and organochlorine compounds, respectively. In the case of the organochlorine compounds, these values are similar to those reported for fish muscle [19].

3.4. Standard addition method

The standard addition method has been used to evaluate the linearity (r^2) of the overall methodology, not specifically calibration. The good linearity observed, higher than 0.90 for most PAHs and higher than 0.98 for all the organochlorine compounds (Table 1), indicates that the method is adequate for liver samples containing a wide range of concentrations of the studied compounds. Some higher molecular mass PAH exhibit lower regression coefficients, 0.73–0.82. Nevertheless, these regression coefficient values also show the feasibility of the method for these compounds.

The spiked samples used in this test have also been used to calculate the recoveries of the whole method. They are all higher than 70% for PAHs and 80% for organochlorine compounds, except in the case of HCB whose recovery is 68% (Table 1). These recoveries are similar or higher to those reported elsewhere, both in the case of organochlorine compounds [17–19,25] and PAH [14,17,18]. Similar results have also been reported in the analysis of sewage sludges by supercritical fluid extraction [27]. However, in one of these studies recoveries of the order of 50% were obtained for the three-ring PAH [18].

3.5. Case studies

Examples of the applicability of the method described in the present study are shown in Table 2. Fish livers from a remote high altitude lake in the Pyrenees (Redó Lake) and a polluted site (Barcelona harbor) have been analyzed. The concentrations of organochlorine compounds found in the fish liver from Redó Lake are similar to those found in fish muscle from the same lake [28,29]. RSD of the organochlorine compound concentrations in liver and muscle [30] of fish from this lake are similar. Method repeatability (RSD in Table 1) is significantly lower than the observed within lake and harbor variabilities of the fish analyzed individually, PAH RSD 44–230% and 35–140%, respectively, and organochlorine compound RSD 30–100% and 27–76%, respectively.

In the case of PAH, exposure experiments with benzo(a)pyrene have shown that concentrations in liver are about 100 times higher than in muscle [30,31]. The method is therefore useful for the analysis of organochlorine compounds and PAH

Table 2
Representative examples of the concentrations of organochlorine compounds and PAH in fish liver (ng/g wet weight) from remote high altitude lakes (Redó; $n = 11$) and polluted sites (Barcelona harbour; $n = 3$)

Compound	Redó lake		Barcelona harbour		Compound	Redó lake		Barcelona harbour	
	Mean	Standard deviation	Mean	Standard deviation		Mean	Standard deviation	Mean	Standard deviation
Acenaphthylene	0.55	0.46	2.3	2.2	Indeno(1,2,3-cd)pyrene	0.19	0.18	4.9	3.2
Acenaphthene	0.39	0.30	15	21	Dibenzo(ah)anthracene	0.12	0.19	5.5	3.8
Fluorene	1.5	0.66	9.0	8.7	Benzo(ghi)perylene	0.087	0.14	2.6	2.9
Phenanthrene	8.8	5.9	19	17	HCB	0.5	0.15	3.4	2.6
Anthracene	0.50	0.37	2.0	1.5	PCB#28	0.38	0.16	13	3.5
Fluoranthene	1.8	1.1	6.9	3.4	PCB#52	1.2	0.93	31	14
Pyrene	1.6	0.83	6.5	3.0	PCB#101	2.0	1.1	62	33
Benz(a)anthracene	0.17	0.085	1.1	0.38	PCB#118	1.3	0.74	43	12
Chrysene	0.49	0.36	1.1	0.96	pp'-DDE	11	9.8	52	36
Benzo(b)fluoranthene	0.52	1.4	2.5	1.9	PCB#153	3.4	3.0	120	48
Benzo(k)fluoranthene	0.40	0.64	1.5	1.8	PCB#138	3.4	2.4	110	43
Benzo(a)pyrene	0.16	0.18	3.5	3.2	PCB#180	1.8	1.8	110	46

from the same samples avoiding combination of several clean up steps [17,18].

4. Conclusions

The method selected for study (Fig. 1) is based on one single clean up step and useful for the analysis of PAHs, organochlorine pesticides and PCBs in large numbers of fish liver samples. Soxhlet extraction of liver tissues ground with anhydrous sodium sulphate provides better recoveries than alkaline digestion avoiding the degradation of pp'-DDT and HCH. The column chromatography fractionation methods allows the separation of all PCBs and DDTs from PAH. Repeatability tests show RSDs under 23%, with average values of 7% and 15% for organochlorine compounds and PAH. Good linearity and recoveries are obtained after evaluation of the method by the standard addition test.

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ARTICLE 2

AGE DEPENDENCE OF THE ACCUMULATION OF ORGANOCHLORINE POLLUTANTS IN BROWN TROUT (*Salmo trutta*) FROM A REMOTE HIGH MOUNTAIN LAKE (REDÓ, PYRENEES)

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Age dependence of the accumulation of organochlorine pollutants in brown trout (*Salmo trutta*) from a remote high mountain lake (Redó, Pyrenees)

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Abstract.

The study of polychlorobiphenyls (PCBs), hexachlorocyclohexanes (HCHs), hexachlorobenzene (HCB) and DDTs in fish muscle of brown trout (*Salmo trutta*) from a high mountain lake located in the Pyrenees (Catalonia, Spain) that is taken as model example of these lacustrine environments has shown that age dependence is the main factor of variability among specimens in this population submitted to atmospheric inputs of these organochlorine compounds (OC). Increases of 2-20 times between fish of 1 and 15 years old are found. The observed trends cannot be explained in terms of fish size, conditioning factor or muscle lipid content.

Higher molecular weight compounds (higher lipophilicity) are better correlated with age than low molecular weight compounds. A transformation from 4,4'-DDT to 4,4'-DDE after fish ingestion is observed resulting into amplified age dependent signals, namely among male specimens. In contrast, PCB congener #180 show lower age dependence than the general OC group which could be due to its high hydrophobicity (log(Kow) > 7). In any case, the selective accumulation of hydrophobic compounds is already observed among the younger fish (1 year old). Due to this effect, the relative OC composition does not reflect the main OC pollutants in the lake waters.

Introduction

Organochlorine compounds (OC), such as polychlorobiphenyls (PCBs), hexachlorocyclohexanes (HCHs), hexachlorobenzene (HCB) and DDTs, are ubiquitous contaminants in the planet. Once released into the environment they may be transported over long distances (Wania and Mackay 1996) and be incorporated to many biogeochemical cycles, e.g. food web, without undergoing important degradation. Despite the discontinued use of many of them, their presence in cold and remote sites, such as high mountain lakes, has been documented (Grimalt et al. 2001; Vilanova et al. 2001) where they are trapped by condensation due to low temperatures (Grimalt et al. 2001). Moreover, organochlorine compounds tend to accumulate in organic tissues due to their lipophilicity and persistence to degradation (de Voogt and Brinkman 1989) The combination of these two effects may eventually result in concentration levels that are toxic for the organisms living in these sites. The accumulation of OC in fish tissues may result from water intake (bioconcentration) and/or from prey injestion (biomagnification).

Bioconcentration and biomagnification ultimately depend on the octanol-water partition coefficient (Kow) of each compound (Chiou, 1985; Mackay, 1982; Sijm, 1992; Hawker and Connell, 1998; Burreau et al., 1997; Fisk et al., 1998) but as observed from field data the relationship between these processes and Kow is not straighforward (Swackhamer and Hites 1988; Thomann and Connolly 1984). Moreover, fish biology may also be relevant for OC accumulation, e.g. species, sex, age, reproductive stage, trophic status (Rosseland et al., 1999; Rognerud et al., 2002). Therefore, OC accumulation in fish depends on a large number of biological factors which complicates the environmental significance of the observed concentrations.

High mountain lakes offer unique environments for the assessment of some of these biological factors since they contain controlled populations of fish that have been exposed exclusively to known OC inputs. These ecosystems offer “natural experiments” of exposure of low pollution inputs in real environments. In the present paper, Lake Redo (Pyrenees, Catalonia, Spain) has been selected for study (42º38’N, 0º46’E). This lake (7.7 Hm^3) is situated at 2240 m above sea level, is oligotrophic, has a surface area of 24 ha, a maximum depth of 73 m, and a water residence time of 4 yr (Ventura et al., 2000). The ice-free period is from May to December (Catalan, 1992). Its watershed is small (155 ha) and scarcely vegetated. Pollution inputs are exclusively related to atmospheric deposition (wet and dry) and there is only one outflow. Having in mind previous studies (Grimalt et al., 2002), this lake can be taken as model example of these lacustrine water bodies in high mountain systems.

The lake contains a large population of brown trout (*Salmo trutta*) in which specimens between 1 and 15 years have been collected. These fish are on top of the trophic food web but do not contain piscivorous specimens. The inputs of PCBs, HCB, HCHs, DDTs entering into

this lake have been determined in a previous study (Carrera et al., 2002) as well as the OC composition of the waters (Vilanova et al., 2001a and b). The study of specimens (n = 29) from the same lake avoids geographical differences in OC input and provides a good case for the evaluation of the age and sex dependence of the accumulation of these compounds in fish.

Materials and methods

Sample Collection and Handling

Fish sampling followed standard test fishing procedures with multifilament gillnets. All fish were measured, dissected and sexed on site. Muscle fillets were wrapped in a pre-cleaned aluminium foil and kept frozen until analysis. Otholits and scales were kept for aging which was performed at NIVA.

Chemicals

Residue analysis n-hexane, dichloromethane, iso-octane, methanol, concentrated sulphuric acid 95-97%, acetone, and anhydrous sodium sulphate for analysis were from Merck (Darmstadt, Germany). Aluminium foil was rinsed with acetone and dried at ambient temperature prior to use. Cellulose extraction cartridges of 20 mm i.d. and 80 mm long were from Whatman (England). The purity of the solvents was checked by gas chromatography coupled to electron capture detection (GC-ECD). Sodium sulphate and cartridges were pre-cleaned by Soxhlet extraction with dichloromethane: methanol (2:1, v/v) for 24 h before use. Sodium sulphate was activated overnight at 400ºC.

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 4,4'-DDE, and 4,4'-DDT were from Dr. Ehrenstorfer (Augsburg, Germany).

Organochlorine compound analysis

Muscle tissues were extracted and analysed for OC using the method described in Berdie and Grimalt (1998). Briefly, muscle tissue (5 g) was ground with activated sodium sulphate 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. Lipid content was determined gravimetrically using 20% of the extract. TBB and PCB 209 standards were added to the rest of the extract which was subsequently cleaned up with sulphuric acid (5 times). All n-hexane solutions were combined and concentrated by vacuum rotary evaporation (20 ºC, 20 torr) to small volumes (ca. 500 µl), further concentrated to nearly dryness under a gentle nitrogen flow and redissolved in 50 µl of iso-octane.

Before chromatographic analysis, an internal standard of tetrachloronaphthalene (TCN) and octachloronaphthalene (OCN) was added to correct for instrument variability. Samples were analyzed by GC-ECD (Hewlett-Packard 5890 Series II) with a 50 m x 0.25 mm i.d. DB-5 capillary column (J&W Scientific, Folsom, CA) coated with 5% phenyl 95% methylpolysiloxane (film thickness 0.25 μm). The instrument was operated in splitless mode and the oven temperature program 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 confirmation of the analysed compounds. A GC system from Agilent Technologies 6890A coupled to an MS detector 5973N was used. The system was equipped with a HP-5MS (30 m x 0.25 mm i.d. x 0.25μm film thickness) and run under the same oven temperature program as described above. Helium was used as carrier gas (1 ml/min) and ammonia was chosen as ionization gas (1.6 10^{-4} Pa). Transfer line and quadrupole temperatures were 280ºC and 150ºC, respectively. The selected ion program is reported elsewhere (Chaler et al. 1998).

Quality Assurance

A detailed evaluation of the method used in the present study is reported elsewhere (Berdie and Grimalt 1998). Procedural blanks were analyzed for every set of six samples. The recovery of the surrogate standards (TBB and PCB #209) was calculated for each sample. Identification and quantification of all studied compounds were performed by injection of external standards at different concentrations. The relative responses to TCN and OCN were used in order to correct for instrumental variabilities and this value was also corrected by the recovery of the surrogate standards.

Results and Discussion

Fish characteristics

The length of the collected specimens was 265 ± 58 mm (mean ± standard deviation), weight was 200 ± 99 g and conditioning factor was 0.98 ± 0.10. A continuous increase between weight and length is observed when comparing specimens between 20-200 g (Figure 1). Then, the rate of length increase flattens but still an increase is observed. Similar length-weight

distributions have been observed in other fish such as perch (LeCren, 1951; Olsson et al., 2000). The conditioning factors of the collected specimens are generally low and not correlated to weight (Figure 1).

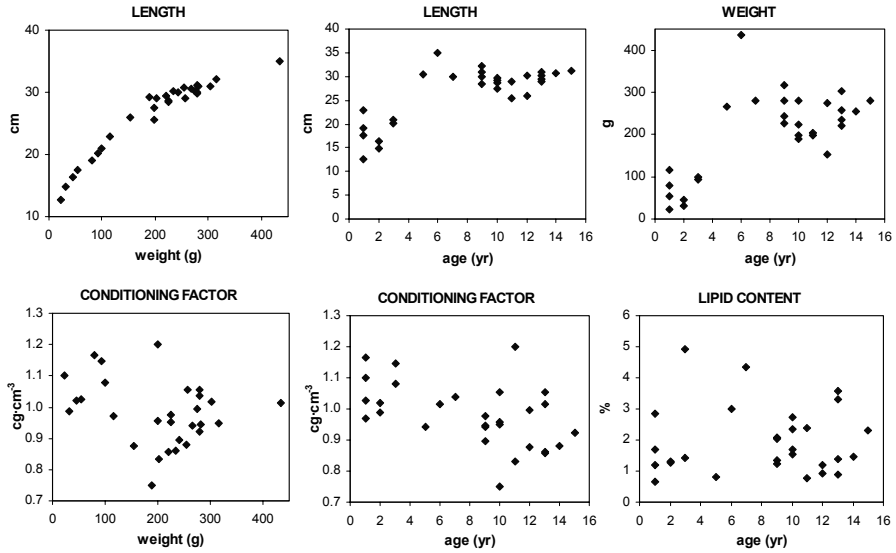

FIGURE 1. Fish characteristics of the fish from Lake Redo included in the study.

Increases in length (from 12 to 35 cm) are observed when comparing specimens between 1 and 6 years. In this age interval weight also increases from 20 to 450 g (Figure 1). However, at higher age (between 6 and 15 years) both the length and weight remain constant. No relationship between conditioning factor and age is observed. Muscle lipid content is generally low (0.5-5%; Figure 1). Again, no relationship between lipid content in muscle and age is found.

Average conditioning factors of male and female specimens (n = 18 and n = 11, respectively) were 0.96 ± 0.11 and 0.99 ± 0.10, respectively, involving no significant difference ($p < 0.05$). Average ages of the males and female groups were 7.9 ± 4.3 and 8.5 ± 5.2 involving again no significant difference ($p < 0.05$). Correlation of length vs age gives rise to similar functions in male (length = 5.2·ln(age) + 18, $r^2 > 0.66$) and female (length = 5.5·ln(age) + 15, $r^2 > 0.80$). Therefore, the two groups of male and female specimens examined do not reflect significant population size or age differences.

POP levels in muscle tissue

The mean concentrations of HCHs (α-HCH and γ-HCH), DDTs (4,4'-DDT and 4,4'-DDE), HCB, and PCBs (congeners #28+31, #52, #101, #118, #153, #138 and #180) in muscle from brown trouts are 1.6 ± 0.9, 19 ±13, 0.60 ±0.4 and 8.2 ±4.8 ng g^{-1} ww, respectively (Table

1). These results are comparable to the concentrations found for the same species in other European high mountain lakes and in fish from low altitude freshwater systems (Swackhamer and Hites 1988; Andersson et al 1988; Leiker et al. 1991; Grimalt et al. 2001). The relative standard deviation of most compounds, 40-85%, is smaller than that reported in previous studies.

Significant differences between water and fish composition are observed (Table 1). Thus, HCH largely predominate in the waters but these compounds range among those in lower concentration in fish muscle. This contrast is already observed in the young fish. Thus, even in the group of one year old specimens there is not HCH predominance (Table 1).

Among DDTs, the major OC group, 4,4'-DDT is found in higher abundance than 4,4'-DDE in the water but this last one is more abundant in fish. Again, the contrast is already observed among the one year old specimens examined (Table 1).

The more chlorinated PCB congeners are more abundant than less chlorinated congeners in fish but the predominant compounds are #52 and #101 in the waters (Table 1). The relative abundance of the more chlorinated congeners is higher among older (13-15 years old) than younger (1 year old) fish. However, even among younger fish PCB#138 and 153 predominate over #52 and #101. Thus, incorporation of OC into fish muscle proceeds through a selective process that accumulates the more hydrophobic compounds. This process already occurs at the ages of early development.

TABLE 1. Averages and standard deviations of the concentrations of the major OC compounds in fish from Redo.

Compound	All individuals Mean±standard deviation ($ng \cdot g^{-1}$ ww)	One year old fish Mean±standard deviation ($ng \cdot g^{-1}$ ww)	13-15 year old fish Mean±standard deviation ($ng \cdot g^{-1}$ ww)	All male Mean±standard deviation ($ng \cdot g^{-1}$ ww)	All female Mean±standard deviation ($ng \cdot g^{-1}$ ww)	Water (disolved + particulated) ($pg \cdot l^{-1}$) (Vilanova et al., 2001a)	Log(Kow) Hawker and Connell (1988); Ballschmitter and Wittlinger (1991)
α-HCH	0.26±0.10	0.13±0.04	0.25±0.23	0.26±0.13	0.27±0.19	410	3.9
γ-HCH	1.4±0.80	0.60±0.18	1.4±1.2	1.3±0.60	1.5±0.98	2500	3.9
HCB	0.60±0.40	0.26±0.05	0.60±0.38	0.53±0.32	0.70±0.41	8.4	5.5
4,4'-DDE	18±13	1.7±0.68	30±11	19±13	16±13	8.9	5.6
4,4'-DDT	1.2±0.60	0.41±0.25	1.6±0.54	1.2±0.52	1.2±0.71	20	6
PCB 28	0.14±0.20	0.06±0.11	0.14±0.04	0.15±0.19	0.13±0.076	8.6	5.7
PCB 52	0.30±0.30	0.38±0.32	0.22±0.08	0.36±0.42	0.20±0.13	14	5.8
PCB 101	0.94±0.50	0.38±0.27	1.1±0.46	1.0±0.55	0.83±0.43	14	6.4
PCB 118	0.74±0.60	0.15±0.10	0.82±0.30	0.89±0.71	0.50±0.31	5.5	6.7
PCB 153	2.4±1.6	0.48±0.16	4.3±1.7	2.5±1.8	2.2±1.5	8.9	6.9
PCB 138	2.2±1.4	0.41±0.16	3.9±1.1	2.3±1.3	2.1±1.5	10	6.8
PCB 180	1.5±1.1	0.35±0.06	2.9±0.85	1.5±1.0	1.5±1.1	3.6	7.4

Age dependence

As shown in Figure 2, the logarithms of the concentrations of most OC exhibit a significant linear dependence from age. This dependence is significant at the $p < 0.01$ level for 4,4'-DDE, 4,4'-DDT and all PCBs except congener #28 (Table 2). In the case of HCB the statistical significance is at the $p < 0.05$ level (Table 2). Only the HCH and PCB congener #28 do not exhibit this trend. HCH are less lipophilic than the other compounds, log(Kow) = 3.9, and they are not accumulated irrespectively of being the predominant OC in the lake waters (Table 1). PCB congener #28 is also less lipophilic than the other PCB congeners considered in the study. The log(Kow) of this congener (5.7; Table 1) is similar to that of PCB congener #52 (5.8; Table 1) which exhibits an age dependence. However, these two congeners also have different vapour pressure constants, $10^{-1.5}$ and $10^{-1.8}$, respectively, which are consistent with their different degree of chlorine substitution (3 and 4 chlorine atoms, respectively). These two vapour pressures involve that congener #28 will be less retained in the lake waters, and ultimately in fish, than the heavier molecular weight PCB.

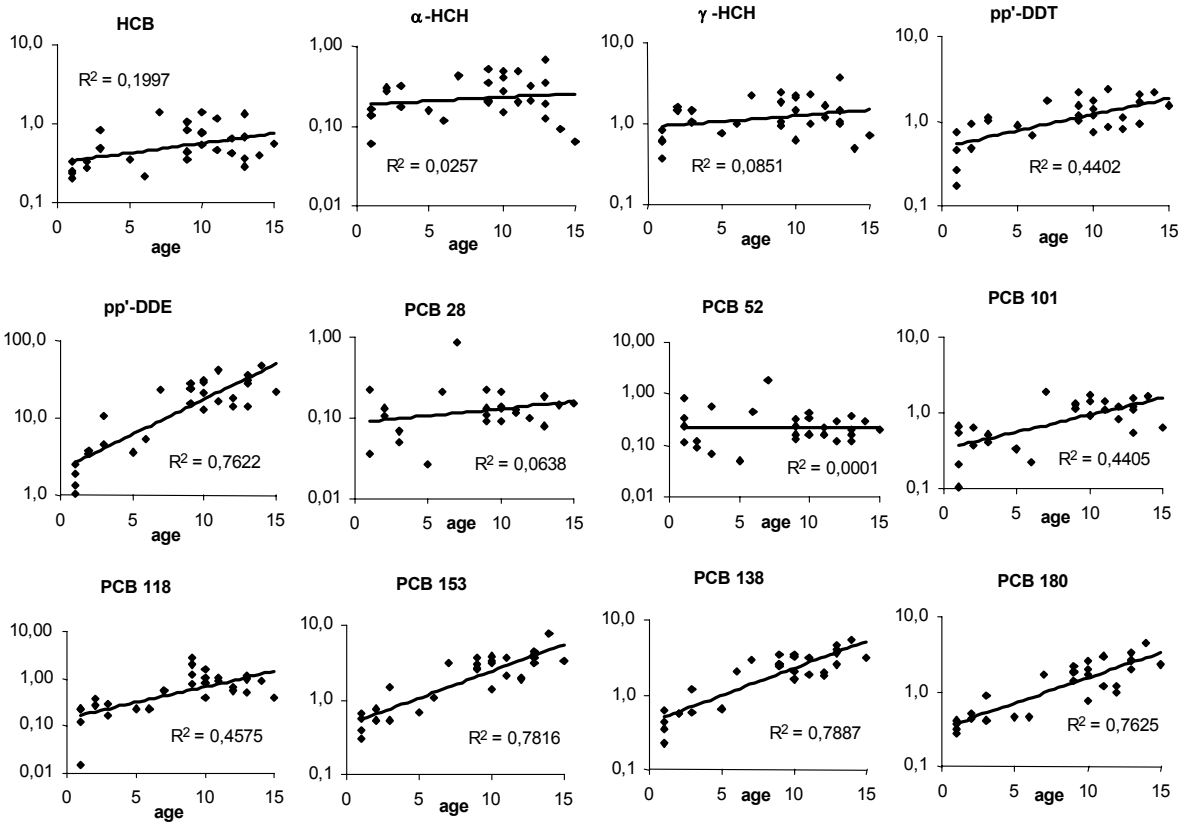

FIGURE 2. Dependence of the concentrations of the organochlorine compounds (ng·g^{-1} ww) from age (years).

In all cases the dependence involves an increase of OC in the older specimens, representing increases of 2-20 times between specimens 15 and 1 year old (Table 2). Furthermore, the correlations show a continuous trend with age. Thus, five year old fish exhibit higher values than one year old fish and similar increases are observed when comparing between ten and five year old specimens (Figure 2). These constant increments cannot be explained in terms of fish size since length and conditioning factor are not continuously correlated to age (Figure 1). Likewise, the continuous distribution with age is consistent with the lack of piscivorous fish in the lake since abrupt increases of about 3-4 times would be expected when comparing specimens of different trophic levels (MacDonald et al., 2002; Madenjian et al., 2002; Rognerud et al., 2002).

PCB congeners show higher increments at higher degree of chlorination (from 4 to seven chlorine groups). However, the compound that exhibits highest age-increase is 4,4'-DDE (19 times) which only has four chlorine groups. The different behaviour of this compound suggests that its accumulation in fish follow other metabolic processes than PCB. In this respect, 4,4'-DDT does not exhibit a similar or higher age-dependent accumulation than 4,4'-DDE despite containing five chlorine atoms. Maybe part of the 4,4'-DDT incorporated by fish is transformed into 4,4'-DDE and accumulated in the form of this more chemically stable molecule. This transformation may also explain the difference between water and fish muscle composition (Table 1).

TABLE 2. Correlation values of the age dependence of the concentrations of the main organochlorine compounds in fish (ng·g^{-1} ww) from Lake Redo (lg(conc) vs age).

	All specimens				Female				Male			
Compound	R^2	slope	Inter	O/Y ratio[a]	R^2	slope	inter	O/Y ratio[a]	R^2	slope	inter	O/Y ratio[a]
α-HCH	0.026	-[b]	-	-	0.0093	-	-	-	0.048	-	-	-
γ-HCH	0.085	-	-	-	0.196	-	-	-	0.024	-	-	-
HCB	0.200*	0.024	2.5	2.2	0.612**	0.040	2.4	3.7	0.029	-	-	-
4,4'-DDE	0.762**	0.091	3.3	19	0.837**	0.087	3.3	16	0.766**	0.096	3.4	22
4,4'-DDT	0.440**	0.038	2.7	3.4	0.736**	0.056	2.5	6.0	0.234**	0.024	2.8	2.2
PCB 28	0.064	-	-	-	0.039	-	-	-	0.081	-	-	-
PCB 52	0.0001	-	-	-	0.289**	0.026	2. 1	2.3	0.062	-	-	-
PCB 101	0.440**	0.045	2.5	4.3	0.359**	0.030	2.6	2.6	0.547**	0.060	2.4	6.8
PCB 118	0.457**	0.066	2.2	8.5	0.583**	0.043	2.2	4.0	0.531**	0.089	2.1	18
PCB 153	0.782**	0.072	2.7	10	0.856**	0.068	2.6	9.1	0.77**	0.076	2.7	12
PCB 138	0.789**	0.073	2.6	10	0.925**	0.075	2.5	11	0.731**	0.072	2.7	10
PCB 180	0.762**	0.068	2.5	8.8	0.781**	0.066	2.5	8.4	0.772**	0.070	2.5	9.5

[a]Ratio between the concentration at 15 and 1 years. [b]When the correlation was not significant values are not given.* significant at $p < 0.05$. ** significant at $p < 0.01$.

Influence of lipid content

Lipid content in fish muscle is not correlated to age (Figure 1). The log-transformed lipid normalized concentrations show the same significant age-dependent correlations (Table 3) as those observed when referring to wet weight content (Table 2). The observed age dependence cannot therefore be attributed to increasing lipid accumulation with age.

However, both r^2 values and age increases are slightly lower when they are calculated after lipid normalization than over wet weight. Thus, introduction of lipid content in the calculations does not reveal a more defined age trend but increases in the scatter of the data. This result is expected in view of the random distribution of lipid content with age (Figure 1) and indicates that OC are stored in fish muscle in all sorts of organic tissues, not only fat.

TABLE 3. Correlation values of the age dependence of the lipid-normalized concentrations of the main organochlorine compounds in fish (ng·g^{-1} lipid) fromLake Redo (lg(conc) vs age).

Compound	R^2	Slope	Intercept	old/young ratio[a]
α-HCH	0.007	-[b]	-	-
γ-HCH	0.040	-	-	-
HCB	0.179**	0.021	4.3	2.0
4,4'-DDE	0.681**	0.087	5.1	17
4,4'-DDT	0.341**	0.035	4.5	3.1
PCB 28	0.035	-	-	-
PCB 52	0.002	-	-	-
PCB 101	0.273**	0.042	4.3	3.9
PCB 118	0.341**	0.063	4.0	7.7
PCB 153	0.698**	0.069	4.5	9.2
PCB 138	0.751**	0.069	4.4	9.4
PCB 180	0.615**	0.064	4.3	7.9

Sex differences

Female show age dependence in the accumulation of all PCB except congener #28 (Table 2). 4,4'-DDT, 4,4'-DDE and HCB are also correlated. Male exhibit similar trends but the less lipophilic compounds of this series, that is PCB congeners #28 and #52, and HCB are not correlated (Table 2). Since there is no difference between the amount of muscle lipids in females (1.93 ± 1.0%) and males (1.97 ± 1.2%), the observed differences in OC accumulation cannot be attributed to distinct lipid content.

The OC showing an age dependent accumulation pattern in both sexes generally exhibit higher old/young ratios in male than female (Table 2). However, the curve fitted straight lines from which these ratios were calculated are not statistically significant. Only in the case of 4,4'-DDT the male-female lines are significantly different at $p < 0.1$. Previous observations on fish related to size have also shown higher accumulations in male than female (Olsson et al. 2000)

and probably reflects a slightly higher OC detoxification capacity of female, e.g. during spawning.

The two DDT compounds exhibit a distinct behaviour. Thus, whereas the old/your ratio for 4,4'-DDE is higher in male than in female (22 and 16, respectively) the ratio for 4,4'-DDT is lower in male than female (2.2 and 6.0, respectively; Table 2). The higher detoxification capacity of female than male suggested above could explain in part these results since female would incorporate OC rich in 4,4'-DDT from the lake waters, e.g. after spawning. However, the large difference in old/young ratio of the two compounds also suggest that male may have higher capacity of transformation of 4,4'-DDT into 4,4'-DDE than female.

Kow dependence.

A consistent relationship between log(Kow) and the slopes (S_{CA}) of the curve fitted straight lines is observed for the compounds showing age dependent accumulation in fish (Figure 3) with the exception of 4,4'-DDE. This trend is independent of OC water concentrations and reflects higher bioconcentration and/or biomagnification at higher Kow.

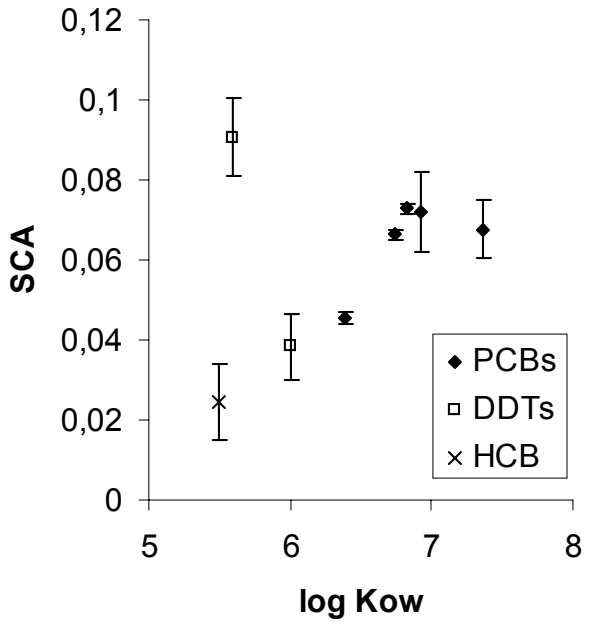

FIGURE 3. Correlation between Figure 2 slopes (S_{CA}) and logarithms of the octanol-water partition constants.

PCB congener #180 shows a lower S_{CA} than expected from its Kow according to the general trend (Figure 3). This lower value could be due to difficulties in the transfer of this highly hydrophobic compound (log(Kow) > 7) into fish tissues, e.g. reduced membrane passage in the gills (Gobas et al. 1986) and/or in the gastrointestinal tract (Opperhuizen et al. 1985;

Gobas et al. 1993)), and therefore a high degree of elimination through feces (Gobas et al., 1989) or difficulties for fish absorption due to low water solubility (Chessells et al., 1992). In both cases, the ultimate effect would involve lower bioconcentration than expected from the Kow constant.

In contrast, 4,4'-DDE deviates from the general trend involving a higher S_{CA} than expected from its Kow according to the general trend (Figure 3). This higher increase may indicate that 4,4'-DDE is more difficult to excrete or metabolize than PCB or HCB (Olsson et al., 2000). However, it may also reflect the above mentioned transformation of 4,4'-DDT into 4,4'-DDE as consequence of fish metabolism.

In conclusion, age is the main factor explaining the variance of OC levels in muscle from a brown trout population in a high mountain lake (Redó, Pyrenees) showing increases of 2-20 times between fish of 1 and 15 years old. This dependence is observed for the compounds with log(Kow) > 5. In fact, compounds with a low degree of chlorination, e.g. 3 chlorine atoms, do not show these age increases. The increase for 4,4'-DDE is higher than expected when considering the overall dependence of the correlation slopes from log(Kow). The observed values likely reflect transformation from 4,4'-DDT to 4,4'-DDE after fish ingestion. In contrast, the lower slope values found for PCB congener #180 may reflect steric restrictions for fish intake. Due to this effect, the relative abundances of OC accumulated in muscle tissue exhibit major differences from those observed in the lake waters (dissolved + particulate fractions). The observed changes in concentration are not related to fish size, conditioning factor or muscle lipid content. Higher age-dependent OC accumulation is generally observed in male than female but the differences are not statistically significant.

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