Environ. Sci. Technol. **2007**, *41,* 5154−5160

Physiological Response to Persistent Organic Pollutants in Fish from Mountain Lakes: Analysis of Cyp1A Gene Expression in Natural Populations of *Salmo trutta*

LAIA QUIRÓS,[†] SERGIO JARQUE,[†] REINHARD LACKNER,[‡] PILAR FERNÁNDEZ,[§] JOAN O. GRIMALT,[§] AND BENJAMIN PIÑA*,[†]

Institute of Molecular Biology (IBMB-CSIC), Jordi Girona, 18, 08034-Barcelona, Catalonia, Spain, Institut fur Zoologie und Limnologie, Universität Innsbruck, Technikerstrasse 25, Innsbruck, A-6020, Austria, and Department of Environmental Chemistry (IIQAB-CSIC), Jordi Girona, 18, 08034-Barcelona, Catalonia, Spain

Cytochrome p450 1A (CYP1A) gene expression in fish liver increases upon exposure to a variety of chemical compounds, including organochlorine compounds (OCs) and polycyclic aromatic hydrocarbons (PAHs). To use this physiological response as a marker of environmental impact, we developed and validated a set of primers to quantify CYP1A expression by qRT-PCR in the brown trout, *Salmo trutta*. These primers were used to explore the natural variability of CYP1A expression in 8 isolated populations (65 samples) from European remote lakes, in a geographical distribution encompassing the Tyrolean Alps, Pyrenees, Rila, Tatras, and Norwegian and Scottish mountains. CYP1A expression values varied more than 2 orders of magnitude among samples, with strong variations within each population. CYP1A expression values were significantly elevated in Tatras and Pyrenees fish populations, whereas the lowest median values were found in populations from the Tyrolean Alps and Rila. These values correlated with the content of different environmentally relevant pollutants in the sediments of the lakes harboring each fish population, particularly with HCB and 4,4′-DDE contents. To our knowledge, this works represents a first report of a physiological response linked to persistent organic pollutants in fish from mountain lakes.

Introduction

Fish populations inhabiting high mountain lakes provide unique opportunities to evaluate the effects of airborne pollution. Studies in European remote mountain lakes show detected concentrations of long-range transported pollutants, including polycyclic aromatic hydrocarbons (PAHs) and organochlorine compounds (OCs) (*1*−*8*), both in sediments and in biota. Long-range transport of these compounds is related to their physicochemical properties as semivolatile compounds. In addition, OC concentration in remote areas is enhanced by the global distillation effect that involves their migration from temperate to cold areas, where they can become trapped. This effect occurs both at planetary scale (*9*) and at regional level, where mountain ranges serve as cold traps (*3*).

Fish, as top predators in mountain lakes, constitute excellent biomarkers for monitoring the pollution status of these ecosystems. Persistent organic compounds (POPs) accumulate in fish inhabiting mountain lakes (*3*, *8*), showing a significantly increasing trend with the fish age and the altitude of the lake for some of them (*8*). Their presence is associated with a significant estrogenic activity detected both in lake sediments and in fish fat (*10*, *11*). In sediments, estrogenic activity correlated with the concentration of several POPs (*10*). In fish muscle, estrogenic activity correlated with fish age and with the content of semivolatile OCs, such as polychlorobiphenyls, which accumulated in aged fish (*11*). The present study aims to assess the putative physiological impact of airborne anthropogenic pollution in fish populations inhabiting these remote mountain lakes.

Cytochrome P450 1A (CYP1A) is an established biomarker of exposure to different environmental pollutants in many animal species, including fish (*12*−*14*). Expression of CYP1A becomes increased upon exposition of the so-called dioxin-like compounds, which include 2,3,7,8-tetrachlorodibenzo-(*p*)dioxin (TCDD), benzo[a]pyrene (Bz[a]Py), and other polycyclic aromatic hydrocarbons (PAHs) and many coplanar polychlorobiphenyl congeners or PCBs (*15*−*17*). This effect on CYP1A expresion is usually evaluated by measuring one of its associated enzymatic activities, the ethoxyresorufin *O*-deethylase activity (EROD, *18*-*20*), although the use of mRNA-quantification methods is rapidly becoming a convenient alternative (*12*−*14*, *21*−*24*), as some of their characteristics, such as the potential use of small samples and the possibility of sample preservation in RNA stabilizing solutions, are especially relevant for in-field surveys (*25*).

The present study is aimed to develop a real-time quantitative PCR assay (qRT-PCR) to estimate CYP1 expression in natural populations of *Salmo trutta* from isolated populations in European high mountain lakes. The method was first tested in fish captured in the wild and treated with CYP1 inducers in experimental conditions. Once validated, the protocol was applied to samples from 8 natural fish populations from high mountain lakes, in order to correlate the levels of CYP1A expression with the anthropogenic pollution accumulated in the sediments of their natural habitats.

Materials and Methods

Fish and Sediment Sampling. Fish (*Salmo trutta*, brown trout) were sampled by net fishing. They were killed by cervical dislocation, weighed, measured, and dissected at the sampling site, reducing any stress or undue suffering. Condition factor, an indicator of fish health, was calculated as $K = (W/L^3) \times 100$, where W = wet weight in g, and L = fork length in cm. Liver samples (50−100 mg) were either deep-frozen in liquid nitrogen or preserved in RNA*later* (Sigma-Aldrich, St. Louis, MO) as previously described (*25*). Sediment samples were obtained by sediment coring in the deepest point of each lake in which fish were captured. The upper 1 cm of sediment was collected in situ and stored frozen as previously described (*3*). Table 1 shows the lakes used in this study and the number and characteristics of fish processed from each lake. Lake properties, including air

* Corresponding author phone: +34 93 4006157; fax: +34 93 2045904; e-mail: bpcbmc@cid.csic.es.
[†] Institute of Molecular Biology (IBMB-CSIC).
[‡] Universität Innsbruck.
[§] Department of Environmental Chemistry (IIQAB-CSIC).

10.1021/es070455p CCC: $37.00
Published on Web 06/15/2007

```
S.trutta            T GTGAGGACAG GAAACTAGAT GAGAATGCCA ACATCCAGGT TTCTGATGAG AAGATT
S.salar             T GTGAGGACAG GAAACTAGAT GAGAATGCCA ACATCCAGGT TTCTGATGAG AAGATT
S.fontinalis        T GTGAGGACAG GAAACTAGAT GAGAACGCCA ACATCCAGGT TTCTGATGAG AAGATT
O.mykiss( CYP1A2)   T GTGAGGACAG GAAACTAGAT GAGAACGCCA ACATCCAGGT TTCTGATGAG AAGATT
O.mykiss( CYP1A1)   T GTGAGGACAG GAAACTAGAT GAGAACGCCA ACATCCAGGT TTCTGATGAG AAGATT
S.gairdner          T GTGAGGACAG GAAACTAGAT GAGAACGCCA ACATCCAGGT TTCTGATGAG AAGATT
S.namaycush         T GTGAGGACAG GAAACTAGAT GAGAACGCCA ACATCCAGGT GTCTGATGAG AAGATT
O.mykisS( CYP1A3)   T GTGAGGACAG GAAACTAGAT GAGAACGCCA ACGTCCAGGT GTCTGATGAG AAGATT
```

FIGURE 1. Real-time PCR amplicon regions of *Salmo trutta* for CYP1A were aligned with corresponding sequences of representative salmonids. Additional Genbank accesion numbers used were: AF361643 (CYP1A1 *Salmo salar*); AF539414 (*Salvelinus fontinalis* cytochrome P450 1A mRNA); U62797 (*Oncorhynchus mykiss* cytochrome P450 1A2); U62796 (*Oncorhynchus mykiss* cytochrome P450 1A1); M21310 (*Salmo gairdneri* cytochrome P450IA1 mRNA); AF539415 (*Salvelinus namaycush* cytochrome P450 1A mRNA); AF059711 (*Oncorhynchus mykiss* cytochrome P450 1A3 (CYP1A3) mRNA). Highlighted nucleotide residues correspond to base differences between *Salmo trutta* and other salmonid sequences.

TABLE 1. Origin, Characteristics, and CYP1A1 Expression Values of Fish Samples Studied in This Work

		lake information				fish characteristics				CYP450 1A1 expression[a]	
region[b]	lake (lake code)	latitude (N)	longitude (E)	altitude (m)	temp. (°C)[c]	N[d]	age (yr)[e]	sex[f]	condition factor (cg cm^{-3})[g]	median	range
A	Øvre Neådalsvatn (ON)	62.77778	8.98237	728	3.25	8	5.8	1.60	1.09	865	(8134−176)
A	Fallbekktjørna (FK)	62.74996	9.03719	1043	1.36	10	11.0	1.67	0.89	807	(2809−61)
A	Øvre Heimdalsvatnet (OH)	61.41887	8.89696	1088	3.75	4	n.d.	n.d.	n.d.	819	(1591−279)
B	Lochnagar (LN)	56.95914	−3.23128	790	3.70	6	n.d.	n.d.	n.d.	1041	(3272−382)
C	Velké Hinçovo (VH)	49.17970	20.06060	1946	0.50	10	5.9	1.20	1.05	1482	(3506−1014)
D	Grossenköllessee (RF)	47.22528	11.01390	2413	−0.33	10	5.4	1.57	1.06	549	(1647−113)
E	Redó (RD)	42.64208	0.77951	2235	3.18	8	9.9	1.29	1.00	1650	(2549−412)
F	Bliznaka (BL)	42.20122	23.31497	2243	1.09	9	3.0	1.75	1.02	603	(1972−135)

[a] Expressed as mRNA copies per 1000 copies of β Actin mRNA. [b] Regions: A, Central Norway; B, Scotland (UK); C, Tatra Mountains (Slovakia); D, Tyrolean Alps (Switzwerand); E, Pyrenees (Spain); F, Rila (Bulgaria). [c] Annual average air temperatures, from Vives et al. (*8*). [d] Number of fish analyzed for each lake. [e] Average age of fish analyzed in each lake. n.d., no data available. [f] Male = 1; female = 2. [g] Average condition factor for analyzed fish.

temperature values, and particulars of fish and sediment sampling methodologies, have been published elsewhere (*3*).

Sediment Extraction and Analysis. Residue analysis *n*-hexane, dichloromethane, iso-octane, methanol, and KOH were from Merck (Darmstadt, Germany). Neutral aluminum oxide type 507C was from Fluka AG (Buchs, Switzerland). Aluminum oxide was cleaned by Soxhlet extraction with dichloromethane/methanol (2:1, v/v) during 24 h, and activated overnight at 120 °C before use. KOH pellets were cleaned by sonication with hexane. PCB standards were purchased from Dr. Ehrenstorfer (Ausburg, Germany).

Top superficial core sections (1 cm, 0.1−1 g) were extracted by sonication with methanol (1 × 20 mL; 20 min) to separate the interstitial water. Subsequent extractions were performed with dichloromethane/methanol (2:1; 3 × 20 mL; 20 min). For chemical analysis, a mixture of surrogate standards (PCB 30, PCB 209, d_{10}-anthracene, d_{10}-pyrene, d_{12}-benz[*a*]anthracene, and d_{12}-benzo[*ghi*]perylene) was added to the extracts. Then, they were vacuum evaporated to 10 mL and hydrolyzed overnight with 20 mL of 6% KOH in methanol. The neutral fraction was recovered with *n*-hexane (3 × 10 mL), concentrated by vacuum rotatory evaporation to approximately 500 μL, and to 100 μL under a gentle stream of nitrogen. Samples for chemical analysis were further fractionated by column chromatography with aluminum oxide as described elsewhere (*1*, *3*). Two fractions were collected: 5 mL of *n*-hexane/dichloromethane (95:5) (OCs, including PCBs, HCB, and DDTs), and 10 mL of *n*-hexane/dichloromethane (1:2) (PAH, 30 individual compounds from fluorene to coronene). After sulfur removal with activated copper, fractions were rotary vacuum and nitrogen concentrated to almost dryness. A 50 μL aliquot of internal standard mixture containing tetrachloronaphthalene and perylene-d_{12}, was added prior to instrumental analysis. Samples for RYA analysis were treated similarly, but without addition of surrogate standards and dilution of the total extract in 200 μL of methanol. For total organic carbon (TOC) analysis, sediment samples were treated with HCl to remove inorganic carbon, washed with Milli-Q water, and dried at 60 °C. TOC determination was performed by flash combustion at 1025 °C followed by thermal conductivity detection in a CHNS Elemental Analyzer EA1108. The limit of detection was 1 mg/g.

OCs analysis was carried out by gas chromatography with electron capture detection (Hewlett-Packard Model HP-5890). Chromatographic conditions are described elsewhere (*3*). 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 (*26*). PAHs were quantified by gas chromatography coupled to mass spectrometry (GC−MS) operating in electron impact and selective ion monitoring modes. A 30 m × 0.25 mm i.d. HP-5MS capillary column (film thickness of 0.25 μm) was used. Additional details of the chromatographic and spectrometric conditions are provided elsewhere (*1*). Quantification was performed by the internal standard method, with the response factors being referred to the internal standard mixture. Reported values were corrected by blank levels and surrogate recoveries (*1*, *2*, *27*).

Induction Experiments. Wild brown trout were captured in brooks from the Tyrolean Alps and transported to conditioned aquaria in Innsbruck University, and placed for several weeks in 100 L flow-through tanks at 19 °C under natural conditions of photoperiode. Ten brown trout juveniles (mean body weight 11.4 ± 2.6) were treated either with a single dose intraperitoneal injection of Naphthoflavone (25 mg/kg body weight) dissolved in corn oil (Sigma-Aldrich) or vehicle (corn oil) as controls, and kept for 4 days. They were sacrificed by neck dislocation and liver samples were stored in RNAlater.

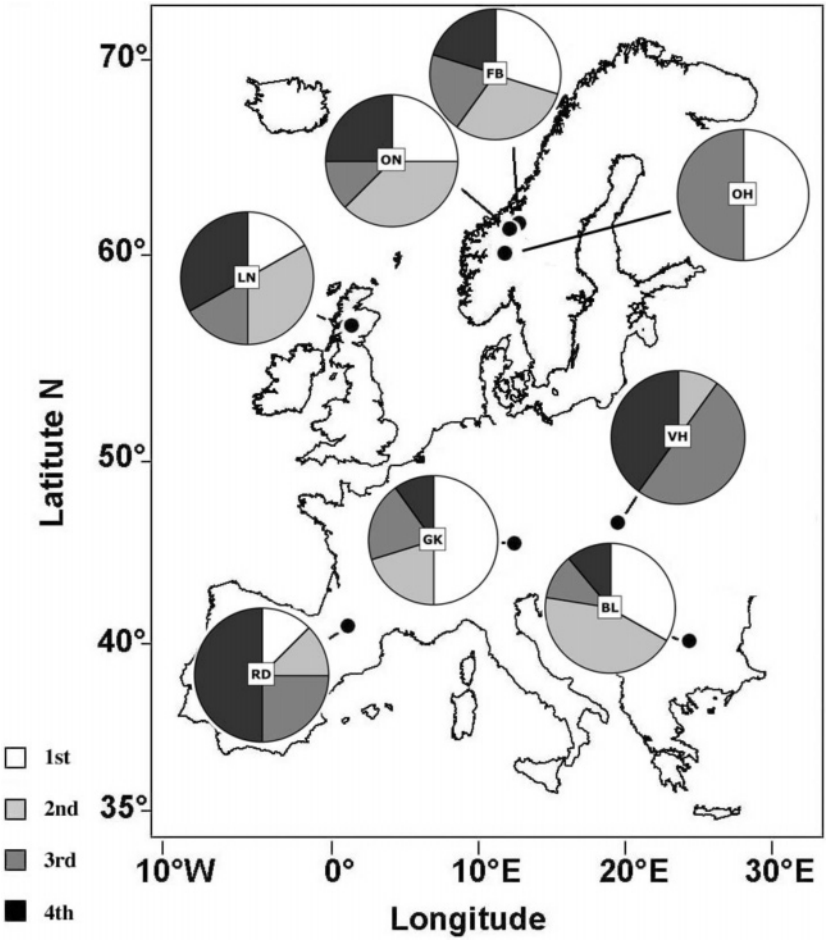

FIGURE 2. Distribution of CYP1A levels in fish samples from European lakes. Graphs represent proportion of samples in each lake falling into each quartile of the total value distribution. Empty sectors (first quartile) show the proportions of low expression samples, whereas solid sectors (fourth quartile) correspond to high expression samples. Lake codes are indicated in Table 1.

TABLE 2. CYP1A1 Expression in Brown Trout Injected with BNF

	average[a]	range	*N*
control	9720	19095−170	5
+BNF	69581	195843−21225	5
p-value[b]	0.003		

[a] CYP1A1 mRNA copies per 1000 copies of β Actin mRNA. [b] Calculated from REST too.

CYP1A mRNA Analysis by qRT-PCR. Liver samples, either frozen in liquid nitrogen or preserved in RNAlater, were stored at −80 °C. RNA isolation was performed as previously described (*25*). Samples were homogenized in TRIzol Reagent (Gibco, Paisley, UK) using Eppendorf-fitting, RNase free pestles (Iberlabo, Madrid, Spain). RNA was extracted in TRIzol as specified by the supplier. Total RNA concentration was estimated by spectrophotometric absorption at 260 nm in a Nanodrop Spectrophotometer ND-1000 (NanoDrop Technologies; Delaware) and treated with DNAse I (F. Hoffmann-La Roche Ltd, Basel, Switzerland) to remove contaminating genomic DNA. DNaseI-digested RNA (10 μg) was copied to cDNA by reverse transcriptase (Omniscript, Qiagen, Valencia, CA) and stored at −20 °C. Aliquots corresponding to 40 ng of the original RNA preparation were used to quantify specific transcripts in an Abi Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) by the SYBR GREEN method (Applied Biosystems). Cytochrome P4501A and β-Actin primers were designed from existing *Salmonidae* sequences (GenBank accession numbers AF539414 from *Salvelinus fontinalis* cytochrome P450 1A mRNA and AY262761 *Salvelinus alpinus* β-actin gene) using Primer Express 2.0 software (Applied Biosystems). The sequences of primers used in this work were: β-Actin Forward: 5′ CTGTCTTCCCCTCCATCGTC 3′; β-Actin Reverse: 5′ TCTTGCTCTGAGCCTCGTCTC 3′; CYP1A Forward: 5′ CACTGACTCCCTCATTGACCAC 3′; CYP1A Reverse: 5′ ACAGATCATTGACAATGCCCAC 3′.

The relative amounts of cDNA present in the samples were calculated from the number of cycles required for each pair of primers to reach fluorescence above the threshold level in the qRT-PCR reaction (CT values), according to the following equation (*28*):

$$\frac{\mathrm{mRNA_{TG}}}{\mathrm{mRNA_{Act}}} = \frac{E_{\mathrm{Act}}{}^{(\mathrm{CT_{Act}})}}{E_{\mathrm{TG}}{}^{(\mathrm{CT_{TG}})}}$$

in which E_{Act} and E_{TG} correspond to real-time efficiency for β-actin and the target gene, respectively. Efficiency values for CYP1A and β-Actin amplicons were initially calculated as close to 100%; therefore E_{Act} and E_{TG} values were set at 2 for all further calculations. The suitability of β-Actin as reference gene for these samples was tested following Pfaffl et al. (*29*). Results are given in copies of CYP1A mRNA per 1000 copies of β-actin mRNA. A typical experiment calculated CTs as averages of three replicates.

Amplified PCR products were purified using GFX PCR Purification Kit (Amersham Biosciences, Backinghamshire, UK) and inserted into the pTZ57R/T plasmid (InsT/Aclone PCR Product Cloning Kit, Fermentas, Burlington, Canada).

TABLE 3. Chemical Analysis of Sediments from Sampled Lakes[a]

	Øvre Neådalsvatn	Fallbekktjørna	Øvre Heimdalsvatnet	Lochnagar	Velké Hinçovo	Gossen koellesee	Redon	Bliznaka
TOC (%)	6	8	6	4	8	9	4	5
MethylPhe (ng/g)	13	49	12	73	nq	21	15	nq
DimethylPhe (ng/g)	16	55	11	54	138	12	13	9
Flu (ng/g)	5	7	2	9	26	3	3	5
Phen (ng/g)	34	96	27	131	441	46	45	35
Anthr (ng/g)	3	16	3	19	32	4	4	3
Fla (ng/g)	60	315	61	290	1293	71	86	96
Ace (ng/g)	3	17	5	28	6	2	3	5
Pyr (ng/g)	56	232	42	205	824	49	50	66
Ret (ng/g)	9	6	1	3	14	1	2	2
B[ghi]Fla (ng/g)	12	56	17	177	340	11	18	21
Cyclop[cd]pyr (ng/g)	6	3	5	nq	69	2	2	5
B[a]A (ng/g)	22	87	17	99	265	20	20	17
Chrys+Triphe (ng/g)	60	182	51	470	1025	79	82	75
BFlas (ng/g)	143	427	176	981	1887	144	157	164
B[e]Pyr (ng/g)	58	160	78	370	689	43	50	55
B[a]Pyr (ng/g)	40	96	34	91	314	30	15	29
Per (ng/g)	118	194	1088	84	60	6	4	74
Ind[7,1,2,3−cdef]Chrys (ng/g)	20	52	42	212	207	15	28	24
Ind[1,2,3−cd]Pyr (ng/g)	69	140	105	433	862	98	56	79
B[ghi]Per (ng/g)	49	105	101	256	524	46	39	57
DB[a,h]Ant (ng/g)	12	29	18	68	78	8	8	11
Cor (ng/g)	2	22	19	133	172	17	16	28
S−PAH (ng/g)	33	42	12	69	231	19	20	15
SUM PAH (ng/g)	858	2255	1974	4058	9128	694	688	850
HCB (pg/g)	243	541	259	759	1589	199	1020	363
4,4′DDE (pg/g)	655	1726	1004	3694	33122	2253	9824	2626
PCB 28 + 31 (pg/g)	195	952	211	101	487	262	176	20
PCB 52 (pg/g)	48	408	38	349	2781	721	352	288
PCB 90 + 101(pg/g)	437	1298	236	469	4698	1384	512	199
PCB 118 (pg/g)	121	192	127	505	1302	679	544	285
PCB 153 (pg/g)	330	696	318	750	2911	3816	407	208
PCB 138 (pg/g)	256	516	416	1077	1286	3839	346	127
PCB 180 (pg/g)	110	365	202	674	3167	1989	367	119
Sum PCBs (pg/g)	1497	4427	1548	3925	16631	12690	2703	1245

[a] PAH abbreviations: Flu, fluorene; Anthr, anthracene; Phen, phenanthrene; MethylPhe, methylphenanthrene; Fla, fluoranthene; Ace, acephenanthylene; Pyr, pyrene; DimethylPhe, Dimethylphenanthrene; Ret, retene; B(ghi)Fla, benzo[ghi]fluoranthene; B(a)A, benz[a]anthracene; Chr+Triph, chrysene+triphenylene; B(ghi)Fla, benzo[ghi]fluoranthene; BFlas, sum of benzofluoranthenes; B(e)Pyr, benzo[e]pyrene; B(a)Pyr, benzo[a]pyrene; Per, perylene; Ind[1,2,3-cd]Pyr, indene[1,2,3-cd]pyrene; Ind[7,1,2,3-cdef]Chrys, indene[[7,1,2,3-cdef]chrysene; B(ghi)Per, benzo[ghi]perylene; DB(ah)A, dibenz[ah]anthracene; Cor, coronene. nq, not quantified.

DNA sequencing was performed using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems) at the DNA Sequencing Facility in IBMB. Amplified sequences were compared to previously reported sequences of homologous genes from different *Cyprinidae* (GenBank accession numbers in the Figure 1 legend) using ClustalW from Bioedit Sequence Alignment Editor (BioEdit v7.0.5, Ibis Therapeutics, Carlsbad, CA).

Statistics. All statistical calculations were performed using the SPSS v. 13.0.1 package (SPSS Inc., Chicago, IL). Normality of the total distribution of CYP1A expression values was assessed by the Kolmogorov−Smirnov test. Unless otherwise noted, significance levels were set at $p < 0.05$. Results from qRT-PCR were analyzed (confidence limits and p values) by the REST tool (*30*).

Results

Identification and Quantitation of *S. trutta* CYP1A Transcript. Total RNA isolated from different liver *S. trutta* samples was retrotranscribed and used as template in a standard polymerase chain reaction (PCR) using the CYP1A-specific oligonucleotide pair. The reaction produced a single DNA fragment (amplicon) of 102 bp, the expected size for *S. trutta* CYP1A gene. The sequence of this amplicon was 100% identical to the published *Salmo salar* CYP1A sequence and very similar to that of other *Salmonidae* CYP1A gene sequences (Figure 1). The same pair of oligonucleotides, combined with the β-Actin specific oligonucleotide pair, was then used to quantify the relative expression of CYP1A in control and BNF-treated fish by qRT-PCR. As shown in Table 2, BNF treatment increased the levels of CYP1A mRNA in liver of treated fish by a factor of 7 relative to untreated samples, using β-Actin gene as a reference ($p = 0.003$, REST tool). This increase, typical of CYP1A expression in liver (*14*), confirmed our identification of the 102 bp amplicon as belonging to the *S. trutta CYP1A* gene.

CYP1A Expression Levels in Natural *S. trutta* Populations. Gene expression of CYP1A was calculated for 65 fish samples using β-Actin as reference gene (Table 1). CYP1A expression levels varied some 200-fold among *S. trutta* specimens from different high mountain lakes (Table 1). A substantial fraction of this variability occurred within each lake population; when median values from each fish population were considered, the difference between the highest (Redon) and the lowest (Grossenköllessee) median values was about 3-fold. Figure 2 shows the distribution of CYP1A values in the different fish populations, distributed by quartiles. No significant differences in CYP1A expression were found between males and females from the same lake (Student's T test, $p > 0.05$). Samples with high levels of CYP1A expression (third and fourth quartiles) were predominant in two lakes, Redon (75%) and Velké Hinçovo (88%), whereas most samples from Bliznaka and Grossenköllessee showed low expression levels (78% and 70% of samples below median,

TABLE 4. Correlation between CYP1A1 Expression Values (log Values) and Chemical Content in Lake Sediments

	CYP1A1 expression (log values)	
	R	*p*-value[a]
air temperature	0.144	0.251
age	−0.158	0.331
length	−0.313	0.056
weight	−0.294	0.066
condition factor	0.298	0.070
TOC	−0.159	0.204
MethylPhe	−0.021	0.887
DimethylPhe	0.235	0.060
Flu	0.262	0.035*
Phen	0.268	0.031*
Anthr	0.209	0.094
Fla	0.252	0.043*
Ace	−0.009	0.946
Pyr	0.245	0.050*
Ret	0.239	0.055
B[ghi]Fla	0.286	0.021**
Cyclop[cd]pyr	0.300	0.021**
B[a]A	0.246	0.048*
Chrys+Triphe	0.284	0.022**
BFlas	0.278	0.025**
B[e]Pyr	0.278	0.025*
B[a]Pyr	0.238	0.056
Per	−0.102	0.420
Ind[7,1,2,3−cdef]Chrys	0.280	0.024**
Ind[1,2,3−cd]Pyr	0.281	0.023**
B[ghi]Per	0.277	0.025*
DB[a,h]Ant	0.252	0.043*
Cor	0.286	0.021**
S−PAH	0.284	0.022**
SUM PAH	0.266	0.032*
HCB	0.348	0.004***
4,4′DDE	0.316	0.010**
PCB 28 + 31	−0.115	0.363
PCB 52	0.234	0.061
PCB 90 + 101	0.200	0.110
PCB 118	0.258	0.038*
PCB 153	0.000	0.997
PCB 138	−0.114	0.366
PCB 180	0.159	0.207
Sum PCBs	0.106	0.400

[a] *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$.

respectively, Figure 2). The distributions of CYP1A expression values in these four Southern- and Central-European fish populations were significantly different ($p = 0.004$, ANOVA). In contrast, the four fish populations from Northern European Lakes (Øvre Neådalsvatn, Fallbekktjørna, Øvre Heimdalsvatnet, and Lochnagar) showed statistically indistinguishable distributions of CYP1A expression values.

Correlation between CYP1A Expression Levels and Pollutant Concentration in Sediments. CYP1A expression levels in fish populations were correlated with the concentration of airborne pollutants in the sediments from the lakes they inhabit, including TOC and the concentration of 23 PAH and 9 OC species, as well as the total PCBs, PAHs, and sulfur-containing PAHs, S-PAHs (Table 3). Twenty of these compounds showed significant correlation with CYP1A expression levels (log-transformed values, Table 4), 10 of them at $p < 0.025$. The highest correlation was observed with HCB content ($p = 0.004$), followed by 4,4′-DDE ($p = 0.010$). Several PAHs showed significant correlation with log CYP1A expression values, some of them strongly linked to anthropogenic pollution, such as Cor ($p = 0.021$) and sulfur-containing PAHs (S-PAH, $p = 0.022$). These data indicate a link between airborne pollution and CYP1A expression levels in fish from high mountain lakes. No significant correlation was found between log CYP1A expression values and average air temperatures of the sample lakes, nor with the age, length, weight, or condition factor of the analyzed fish (Table 4)

Correlations between CYP1A expression levels in fish liver and the concentrations of HCB and 4,4′-DDE in sediments are plotted in Figure 3. The plots show a good correlation for the median values (empty dots in Figure 3) and for the central portion of values (second and third quartiles, vertical bars) from each fish population. However, extreme values in each population seemed not to follow this general trend, especially in the case of maximal values (horizontal dashes). Therefore, the data indicate large variations in CYP1A expression within individuals from a single fish population, and a good correlation between the central values of these distributions and the presence of exogenous AhR inducers in the environment. The dispersion of CYP1A values likely results from both the genetic variability occurring in natural populations and changes in expression levels through the lifespan of a single individual according to its physiological state.

Discussion

Fish *Salmonidae* populations have long been used to monitor the ecological status of mountain lakes. These lake fish accumulate unexpectedly high concentrations (pg/g to ng/g in fish tissue) of long-range transported anthropogenic pollutants, such as OCs, pyrolythic PAHs, and, more recently, polybromodiphenylethers (PBDEs) (*1*, *5*, *8*, *31*). The data shown here link their occurrence to physiological impacts.

High proportions of samples with high CYP1A expression levels were found in lakes Velké Hinçovo, Redon, and Lochnagar. These three lakes are those exhibiting the highest concentrations of HCB and 4,4′-DDE (Velké Hinçovo, Redon) and PAH (Velké Hinçovo, Lochnagar) among those included in the present study (Table 3). Velké Hinçovo and Redon fish populations have been shown to accumulate significantly higher levels of OC in both fish liver and muscle when compared with the rest of the surveyed lakes (*8*). These two fish populations also show high proportion of fish with significant estrogenic activity related to human activities (*11*). Although there is no direct link between CYP1A expression and estrogenic compounds, many OCs interact with at least one of the relevant receptors (aryl hydrocarbon receptor and estrogen receptor, respectively) or with both (*32*). These combined data strongly indicate an elevated anthropogenic impact in these fish populations likely having physiological consequences.

There is little information on specific pathologies in mountain lake fish, although focal liver inflammation and accumulations of melano-macrophages have been observed in brown trout from Lochnagar and Velké Hinçovo (*33*). As ectopic CYP1A expression has been related to immunity suppression in Japanese medaka (*34*), these pathologies may be related to the elevated CYP1A levels found in these fish populations. It is not clear whether the presence of fish specimens with high levels of CYP1A expression even in the less polluted lakes (Fallbekktjørna, Øvre Heimdalsvatnet, or Bliznaka) is due to individual differences, indicates other pathologies (e.g., infections), or simply reflects physiological changes during the normal life cycle. Many physiological parameters may influence both pollutant contents and CYP1A expression, such as sex, age, or condition factor. In this work, we found no convincing correlation between CYP1A expression and any of these parameters, but we cannot exclude that they may contribute to the intra-lake variability of the data. This aspect could only be addressed by a more extensive study, involving a much larger set of fish samples, but probably limited to a reduced number of lakes in selected locations. Conversely, the strategy followed in the present study was designed to compare fish populations exposed to very different levels of chemical pollution. To this purpose, we covered a rather extended geographic range, at the price

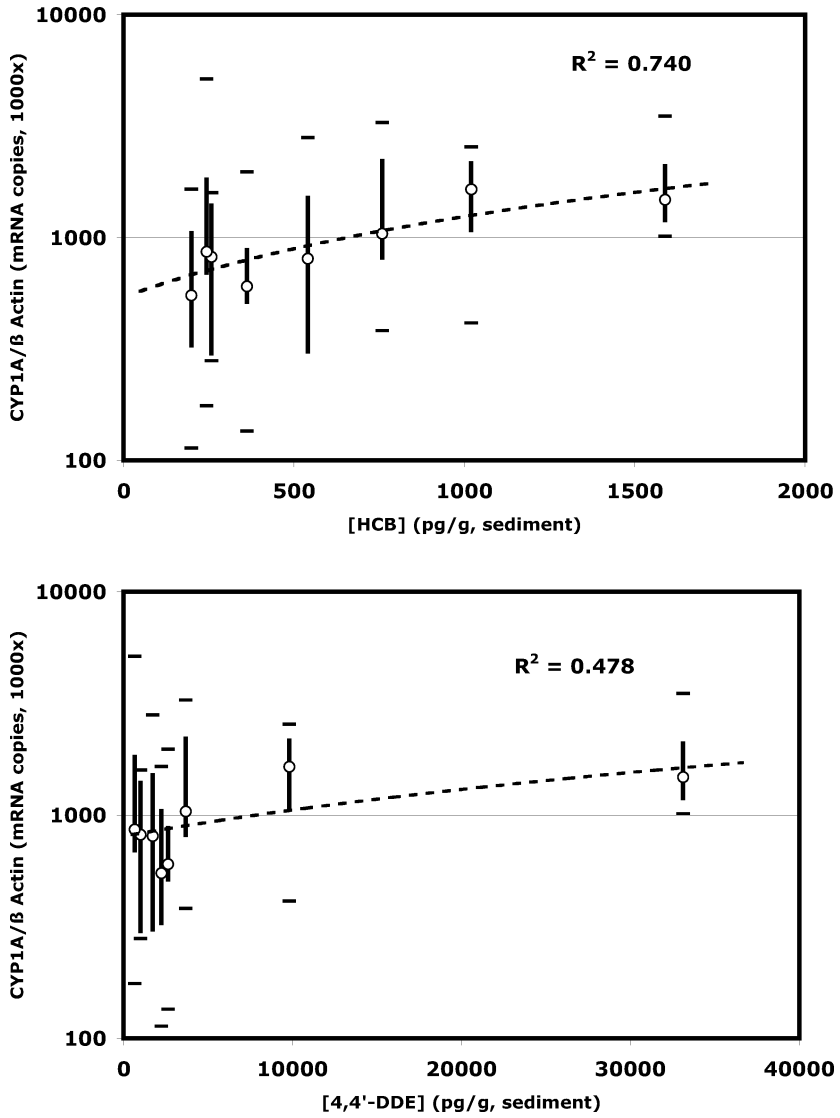

FIGURE 3. Semilogarithmic plots between CYP1A expression levels in fish liver and HCB (top) and 4,4′-DDE (bottom) concentrations in sediments of the corresponding lakes. Empty dots represent medians of CYP1A expression values for each fish population, vertical bars indicate the range of central values (second and third quartiles) for each distribution. Extreme values (maximum and minimum) are indicated by dashes. Discontinuous lines correspond to linear regressions between CYP1A expression median values for each fish population and compound concentration for each lake; R^2 values from these regressions are indicated.

of increasing potential sources of variability among the selected fish populations. Despite all these possible sources of variation, this study clearly differentiates between low- and high-impacted fish populations, with the most characteristic indicator for highly impacted populations being the virtual absence of individuals with low CYP1A levels, which predominate in non-impacted populations. These results are to be compared with previous reports in which low or nil differences on CYP1A expression were found between fish populations inhabiting polluted and reference areas (*35*, *36*), which was attributed to adaptation of fish to chronic pollution. At this point, it is important to consider the fundamental differences between acute and chronic exposure to chemical stress. For example, injection of AhR agonists or immersion of caged specimens in polluted areas results in a strong induction of CYP1A and of their associated enzymatic activities (*12*−*14*, *21*−*24*, this work). Our results indicate that the qRT-PCR method reported in this study is able to analyze the response of high mountain lake fish to chemical impacts. They also indicate that an accurate evaluation of the correlation between this physiological response and the actual chemical burden requires a population-oriented approach, rather than an individual-oriented one, in order to separate pollution effects from the natural variability on CYP1A expression (and, presumably, on other physiological parameters) present in wild fish populations.

Acknowledgments

Authors L.Q. and S.J. contributed equally to this work. G. Carrera, B. van Drooge, and B. Martrat are thanked for the analysis of some sediment samples described in this work. We are also grateful to the groups of J. Catalán (CEAB-CSIC, Spain), E. Stuchlik (Charles University Prague, Czeck Republic), R. Psenner (Innsbruck University, Austria), D. Wright (NIVA, Norway), and N. Rose (University College London, UK), for sampling. This work has been supported by the EU Projects EUROLIMPACS (GOCE-CT-2003-505540) and EMERGE (EVK1-CT-1999-00032) and the Spanish Ministry of Science and Technology (BIO2005-00840). Part of this work has been carried out in the framework of an Austrian−Spanish cooperation (HU2020043-04AT0001). We also acknowledge the support of the Centre de Referència en Biotecnologia of

Generalitat de Catalunya. Technical assistance by M. Casado, R. Chaler, D. Fanjul, and R. Mas is acknowledged.

Literature Cited

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

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

(3) Grimalt, J. O.; Fernández, P.; Berdie, L.; Vilanova, R. M.; Catalán, J.; Psenner, R.; Hofer, R.; Appleby, P. G.; Rosseland, B. O.; Lien, L.; Massabuau, J. C.; Battarbee, R. W. Selective trapping of organochlorine compounds in mountain lakes of temperate areas. *Environ. Sci. Technol.* **2001**, *35*, 2690−2697.

(4) Carrera, G.; Fernández, P.; Grimalt, J. O.; Ventura, M.; Camarero, L.; Catalán, J.; Nickus, U.; Thies, H.; Psenner, R. Atmospheric deposition of organochlorine compounds to remote high mountain lakes of Europe. *Environ. Sci. Technol.* **2002**, *36*, 2581−2588.

(5) van Drooge, B. L.; Grimalt, J. O.; Camarero, L.; Catalán, J.; Stuchlik, E.; Torres Garcia, C. J. Atmospheric semivolatile organochlorine compounds in European high-mountain areas (central Pyrenees and high Tatras). *Environ. Sci. Technol.* **2004**, *38*, 3525−32.

(6) Grimalt, J. O.; van Drooge, B. L.; Ribes, A.; Fernández, P.; Appleby, P. Polycyclic aromatic hydrocarbon composition in soils and sediments of high altitude lakes. *Environ. Pollut.* **2004**, *131*, 13−24.

(7) Grimalt, J. O.; van Drooge, B. L.; Ribes, A.; Vilanova, R. M.; Fernández, P.; Appleby, P. Persistent organochlorine compounds in soils and sediments of European high altitude mountain lakes. *Chemosphere* **2004**, *54*, 1549−1561.

(8) Vives, I.; Grimalt, J.; Catalán, J.; Rosseland, B.; Battarbee, R. Influence of altitude and age in the accumulation of organochlorine compounds in fish from high mountain lakes. *Environ. Sci. Technol.* **2004**, *8*, 690−698.

(9) Simonich, S.; Hites, R. Global distribution of persistent organochlorine compounds. *Science* **1995**, *269*, 1851−1854.

(10) Garcia-Reyero, N.; Piña, B.; Grimalt, J. O.; Fernández, P.; Fonts, R.; Polvillo, O.; Martrat, B. Estrogenic activity in sediments from European mountain lakes. *Environ. Sci. Technol.* **2005**, *39*, 1427−1435.

(11) Garcia-Reyero, N.; Vives, I.; Fernández, P.; Grimalt, J.; Piña, B. Endocrine disruption activity associated to organochlorine compounds in fish extracts from European Mountain Lakes. *Environ. Pollut.* **2007**, *145*, 745−752.

(12) Arukwe, A. Complementary DNA cloning, sequence analysis and differential organ expression of beta-naphthoflavone-inducible cytochrome P4501A in Atlantic salmon (*Salmo salar*). *Comp. Biochem. Physiol., Part C: Toxicol., Pharmacol., Endocrinol.* **2002**, *133*, 613−624.

(13) McClain, J.; Oris, J.; Burton, G., Jr.; Lattier, D. Laboratory and field validation of multiple molecular biomarkers of contaminant exposure in rainbow trout (*Oncorhynchus mykiss*). *Environ. Toxicol. Chem.* **2003**, *22*, 361−370.

(14) Rees, C.; Li, W. Development and application of a real-time quantitative PCR assay for determining CYP1A transcripts in three genera of salmonids. *Aquat. Toxicol.* **2004**, *66*, 357−368.

(15) Hankinson, O. The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.* **1995**, *35*, 307−340.

(16) Nebert, D. W.; Puga, A.; Vasiliou, V. Role of the Ah receptor and the dioxin-inducible toxicity, cancer, and signal transduction. *Ann. N. Y. Acad. Sci.* **1993**, *685*, 624−640.

(17) Sutter, T. R.; Tang, Y. M.; Hayes, C. L.; Wo, Y. Y.; Jabs, E. W.; Li, X.; Yin, H.; Cody, C. W.; Greenlee, W. F. Complete cDNA sequence of a human dioxin-inducible mRNA identifies a new gene subfamily of cytochrome P450 that maps to chromosome 2. *J. Biol. Chem.* **1994**, *269*, 13092−13099.

(18) Solé, M.; Raldúa, D.; Barceló, D.; Porte, C. Long-term exposure effects in vitellogenin, sex hormones, and biotransformation enzymes in female carp in relation to a sewage treatment works. *Ecotoxicol. Environ. Saf.* **2003**, *56*, 373−380.

(19) Lavado, R.; Urena, R.; Martin-Skilton, R.; Torreblanca, A.; Del Ramo, J.; Raldúa, D.; Porte, C. The combined use of chemical and biochemical markers to assess water quality along the Ebro River. *Environ. Pollut.* **2006**, *139*, 330−339.

(20) Machala, M.; Dusek, L.; Hilscherova, K.; Kubinova, R.; Jurajda, P.; Neca, J.; Ulrich, R.; Gelnar, M.; Studnickova, Z.; Holoubek, I. Determination and multivariate statistical analysis of biochemical responses to environmental contaminants in feral freshwater fish *Leuciscus cephalus*, L. *Environ. Toxicol. Chem.* **2001**, *20*, 1141−1148.

(21) Dixon, T. J.; Taggart, J. B.; George, S. G. Application of real time PCR determination to assess interanimal variabilities in CYP1A induction in the European flounder (*Platichthys flesus*). *Mar. Environ. Res.* **2002**, *54*, 267−270.

(22) Tom, M.; Shmul, M.; Shefer, E.; Chen, N.; Slor, H.; Rinkevich, B.; Herut, B. Quantitative evaluation of hepatic cytochrome P4501A transcript, protein and catalytic activity in the striped sea bream (*Lithognathus mormyrus*). *Environ. Toxicol. Chem.* **2003**, *22*, 2088−2092.

(23) Tom, M.; Chen, N.; Segev, M.; Herut, B.; Rinkevich, B. Quantifying fish metallothionein transcript by real time PCR for its utilization as an environmental biomarker. *Mar. Pollut. Bull.* **2004**, *48*, 705−710.

(24) Tom, M.; Auslander, M. Transcript and protein environmental biomarkers in fish - a review. *Chemosphere* **2005**, *59*, 155−162.

(25) Garcia-Reyero, N.; Raldúa, D.; Quirós, L.; Llaveria, G.; Cerdà, J.; Barceló, D.; Grimalt, J.; Piña, B. Use of vitellogenin mRNA as a biomarker for endocrine disruption in feral and cultured fish. *Anal. Bioanal. Chem.* **2004**, *378*, 670−675.

(26) Chaler, R.; Vilanova, R.; Santiago-Silva, M.; Fernández, P.; Grimalt, J. O. Enhanced sensitivity in the analysis of trace organochlorine compounds by negative-ion mass spectrometry with ammonia as reagent gas. *J. Chromatogr., A* **1998**, *823*, 73−79.

(27) Berdié, L.; Grimalt, J. O. Assessment of the sample handling procedures in a labor-saving method for the analysis of organochlorine compounds in a large number of fish samples. *J. Chromatogr., A* **1998**, *823*, 373−380.

(28) Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res.* **2001**, *29*, e45.

(29) Pfaffl, M. W.; Tichopad, A.; Prgomet, C.; Neuvians, T. P. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper−Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* **2004**, *26*, 509−515.

(30) Pfaffl, M. W.; Horgan, G. W.; Dempfle, L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucl. Acids Res.* **2002**, *30*, e36.

(31) Vives, I.; Grimalt, J. O.; Lacorte, S.; Guillamón, M.; Barceló, D. Polybromodiphenyl ether flame retardants in fish from lakes in European high mountains and Greenland. *Environ. Sci. Technol.* **2004**, *38*, 2338−2344.

(32) Noguerol, T.; Boronat, S.; Casado, M.; Raldúa, D.; Barceló, D.; Piña, B. Evaluation of vertebrate receptor interaction with persistent pollutants and antifouling pesticides by recombinant yeast assays. *Anal. Bioanal. Chem.* **2006**, *285*, 1012−1019.

(33) Rosseland, B. O.; Rognerud, S.; Collen, P.; Grimalt, J.; Vives, I.; Massabuau, J.-C.; Hofer, R.; Lackner, R.; Fjellheim, A.; Harriman, R.; Piña, B. Brown trout in Lochnagar. Population and contamination by metals and organic micropollutants. In *Lochnagar: The Natural History of a Mountain Lake*; Rose, N., Ed.; Springer: Dordrecht, The Nederlands, 2007; pp 253−285.

(34) Carlson, E. A.; Li, Y.; Zelikoff, J. T. Benzo a pyrene-induced immunotoxicity in Japanese medaka (*Oryzias latipes*): relationship between lymphoid CYP1A activity and humoral immune suppression. *Toxicol. Appl. Pharmacol.* **2004**, *201*, 40−52.

(35) Brammell, B. F.; Price, D. J.; Birge, W. J.; Elskus, A. A. Apparent lack of CYP1A response to high PCB body burdens in fish from a chronically contaminated PCB site. *Mar. Environ. Res.* **2004**, *58*, 251−255.

(36) Greytak, S. R.; Champlin, D.; Callard, G. V. Isolation and characterization of two cytochrome P450 aromatase forms in killifish (*Fundulus heteroclitus*): differential expression in fish from polluted and unpolluted environments. *Aquat. Toxicol.* **2005**, *71*, 371−389.

Received for review February 22, 2007. Revised manuscript received May 18, 2007. Accepted May 18, 2007.

ES070455P