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Research Note

Dioxin-like Compounds in Lake Fish Species: Evaluation by DR-CALUX Bioassay

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ABSTRACT

Fish consumption is the principal source of intake of organochlorinated compounds in humans. Compared with other types of foods of animal origin, fish contain the highest levels of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins, and polychlorinated dibenzofurans, all of which are classified as highly toxic organochlorine compounds. Currently, lakes and fish farms in northern Italy are not regularly monitored for PCBs and dioxins in areas contaminated by industrial sources, partially because of the high costs of traditional analytical methods that limit the number of samples to be analyzed. The DR-CALUX cell bioassay is based on the uptake of the cellular aryl hydrocarbon receptor (AhR) for dioxins and dioxin-like compounds. The aim of this study was to assess the levels of dioxins and dioxin-like PCB contamination in Lake Maggiore and Lake Como, two lakes in northwestern Italy, and in nearby areas. The levels were quantified using the cell bioassay DR-CALUX and reference controls in two wild fish species, perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*), and in a farmed species, rainbow trout (*Oncorhynchus mykiss*). Tissue samples collected from the farmed rainbow trout were also submitted to immunohistochemical analysis of CYP1A expression as a marker for environmental pollutant-induced liver damage. The levels of dioxins, furans, and dioxin-like PCBs were all below the maximum levels and action limits set by European Union Regulation, suggesting no risk for human health associated with the consumption of the fish species caught or farmed in these areas.

Key words: Bioassay; Dioxins; DR-CALUX; Fish species; Screening method

Pollution of aquatic ecosystems is a growing problem worldwide. The increase in the number and amount of industrial, agricultural, and commercial chemicals discharged into the aquatic environment has had deleterious effects on aquatic organisms *(1, 20)*. The risks for wildlife and human health reside in the tendency of persistent organic pollutants in aquatic systems to bioaccumulate in the aquatic food chain *(12, 21, 30)*.

Fish consumption is the principal source of the intake of organochlorinated compounds in humans. Compared with other types of foods of animal origin, fish contain the highest levels of polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), all of which are classified as highly toxic organochlorine compounds *(12, 15)*.

Dioxins and dioxin-like compounds are a family of organic micropollutants belonging to the group of persistent organic pollutants. Highly toxic to humans, they persist in the environment and accumulate in lipid fraction of cells and tissues, thus entering the human food chain. Moreover, they are odorless, resistant to high temperatures, poorly volatile because of their high molecular weight, poorly biodegradable, and lipophilic, with a half-life lasting from several months to several years *(7)*. They are ubiquitous contaminants originating from combustion processes of organic and inorganic compounds in incinerators and smelters as well as unintentional by-products of industrial chemical production *(25, 26)*. Dioxins enter the organism through the digestive and respiratory systems and the skin, but almost 90% of human exposure comes from food intake, mainly through the ingestion of contaminated milk, meat, and fish *(7)*. According to epidemiological studies, dioxins are considered carcinogenic and are classified by the International Agency for Research on Cancer as group 1 carcinogenic substances. Their activity is under study by in vivo experiments *(7)*.

Recently, pentachlorodibenzofuran and PCBs have also been included in International Agency for Research on Cancer group 1 based on sufficient evidence of carcinogenicity in humans and experimental animals *(16)*. The use and production of PCBs have been banned in Italy since 1984; however, they still constitute a danger for the environment and human health because of their persistence and improper disposal *(33)*. Contamination by dioxins and PCBs in the aquatic ecosystems of northern Italy, especially in the area

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around Lake Maggiore, has been linked to the pollution caused by these substances *(4, 5, 8, 24)*. Contamination surveys of Italian and Swiss lakes have shown that dioxin and PCB accumulation is greatest in predatory fish species at the top of the food chain and in the oldest individuals *(2, 3, 31, 32)*.

Currently, lakes and fish farms in northern Italy are not regularly monitored for PCBs and dioxins in areas contaminated by industrial sources; this is partially due to the high costs of traditional analytical methods that limit the number of samples to be analyzed, making monitoring outcomes poorly reliable.

The dioxin-responsive chemically activated luciferase gene expression cell bioassay (DR-CALUX, BioDetection Systems, Amsterdam, The Netherlands) is based on the uptake of the cellular aryl hydrocarbon receptor (AhR) for dioxins and dioxin-like compounds. Because of its relatively short run times and low costs, it has been widely proposed and adopted as a reference method for the screening of food contamination *(6, 29)*.

The aim of this study was to assess dioxins and dioxin-like PCB contamination levels on fish of two lakes and nearby areas in northwestern Italy. The levels were quantified using the cell bioassay DR-CALUX in two wild fish species, perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*), and in a farmed species, rainbow trout (*Oncorhynchus mykiss*) *(14)*. Tissue samples collected from the farmed rainbow trout were also submitted to immunohistochemical (IHC) analysis of CYP1A expression as a marker of environmental pollutant-induced liver damage, as described elsewhere *(23)*. The CYP1A enzyme has a role in the biotransformation of several compounds, including dioxins, furans, PCBs, and polyaromatic hydrocarbons. CYP1A isoforms transform the lipophilic xenobiotics into water-soluble metabolites, which is the first step in their excretion and detoxification. An increase in CYP1A expression and synthesis has been described by molecular analysis, in situ hybridization, immunochemical techniques, and enzymatic assays in the liver and gut of diverse fish species exposed to toxicants. Furthermore, increased CYP1A expression and synthesis were observed in rainbow trout after exposure to benzo[*a*]pyrene or pentachlorobiphenyl *(17–19)*.

MATERIALS AND METHODS

Chemicals and reagents. *n*-Hexane (purity 99%), isopropanol (purity >99%), and diethyl ether (purity >99%) were supplied by Biosolve Chimie (Diueze, France). 2,3,7-8 Tetrachlorodibenzo-*p*-dioxin (TCDD) standards and luciferin were purchased from BioDetection Systems, silica 63-200 was from Merck (Serono, Italy), sulfuric acid (purity 95 to 98%) was from Carlo Erba Reagents (Cornaredo, Italy), dimethyl sulfoxide (purity >99%) was from Acros Organics (Rodano, Italy), and Lysis Mix was from Sigma (Milan, Italy). For cell culture, Gibco minimum essential medium (MEM) α was supplied by ThermoFisher Scientific (Monza, Italy).

Formaldehyde, ethanol, xylene, and hematoxylin-eosin were purchased from Carlo Erba and paraffin was from Diapath (Martinengo, Italy). Specific reagents for IHC analysis are reported below.

Study area, fish species, and sampling. The two lakes were selected for sampling based on their proximity to polluted areas and on the results of previous surveys *(8, 9, 22)*. Pollution problems have been documented for both lakes *(4, 10)*: Lake Maggiore (middle area) and Lake Como (Lecco arm).

The wild perch and roach from the lakes selected for this study are located at the top and the bottom of the trophic chain, respectively, and can therefore be used as indicators. The farmed rainbow trout were obtained from a farm located in Marano Ticino (Novara, Italy).

To minimize individual biological variability, effects of confounders, and variability of results, the fish were selected by age, size, and sex. To detect an overall contamination prevalence of 15%, with a 95% confidence level, a minimum sample size of at least 19 individuals per site was calculated.

Random samplings were performed directly on site by professional fishermen operating in different districts of the lakes or directly at the farming sites. Samples were collected from Lake Maggiore ($n = 30$ perch and $n = 35$ roach), Lake Como ($n = 19$ perch and $n = 25$ roach), and the fish farm ($n = 25$ trout) between March and October 2015.

DR-CALUX bioassay. The DR-CALUX bioassay is a validated method for screening dioxins and dioxin-like PCBs in foods according to international standards *(6)*. Nine grams of fillet collected from wild and farmed fish and the reference control were submitted to extraction of fatty substances with a mixture consisting of *n*-hexane and isopropanol (ratio of 1:5 and 1:2, respectively) in vertical agitation. The reference control is a fish previously examined by high-resolution gas chromatography–mass spectrometry, with the sum of PCDD/PCDF/dioxin-like PCB at an upper bound of 0.172 pg/g of product, to control the analytical recovery of the analyzed compounds. The percent recovery obtained in the various analytical sessions is between 80 and 105%.

To monitor the reagents used during the extraction, a control (blank) consisting of the same reagents and ultrapure water in an amount equal to the matrix weight was used. The recovered supernatant was evaporated until dry using a centrifugal humidifier (Gruppo Strola, Turin, Italy).

The fat extracted from each sample was weighed and then purified on a glass column by using a double layer of silica (63 to 200 μm) acidified with sulfuric acid at two different concentrations (33 and 20%); the eluent consisted of a mixture of *n*-hexane and diethyl ether (ratio 97:3). The cleaned extracts were completely evaporated by means of a nitrogen flow evaporator (Gruppo Strola) and then diluted in 25 μL of dimethyl sulfoxide.

Dioxins and dioxin-like compounds were determined using the H4IIE cell line consisting of rat hepatoma cells genetically modified with the construct of the luciferase reporter gene linked to the cellular AhR. The cells were cultured and grown in MEM α to confluence in 96-well plates and then incubated for 24 h at 37°C in a 5% CO_2 atmosphere with the sample extract, blank, control, and standard of analysis. The standard consisted of six different concentrations of 2,3,7,8-TCDD (0, 0.0375, 0.075, 0.125, 0.25, 0.375 nM; BioDetection Systems), diluted in dimethyl sulfoxide to create a calibration curve (range, 0 to 3 pM).

Each extract (undiluted and diluted 1:3 and 1:10) was tested in triplicate. After incubation, the cells were washed once with phosphate-buffered saline and lysed with a solution of Lysis mix; the substrate luciferin (BioDetection Systems) was added and luciferase activity was quantified using a chemiluminometer with a dual injector (Luminoskan Ascent microplate luminometer,

TABLE 1. *Median BEQ TCDD values of three fish species*

Species	Median BEQ TCDD (pg/g of fresh product)[a]
Perch	0.093
Roach	0.046
Rainbow trout	0.046

[a] Kruskal-Wallis test, $P = 0.181$.

TABLE 2. *Median BEQ TCDD of three fish species from the three sampling areas*

Site	Median BEQ TCDD (pg/g of fresh product)[a]
Lake Como	0.037
Lake Maggiore	0.130
Fish farm	0.046

[a] Kruskal-Wallis test, $P = 0.024$.

ThermoFisher Scientific). The amount of light emitted is proportional to the amount of dioxin.

Luminescence was converted to a toxic biological equivalence (BEQ CALUX) by direct comparison of the response for a given sample with a dose-response curve obtained by TCDD. The bioanalytical equivalent (BEQ) values were correlated with the toxicity equivalent (TEQ) values from high-resolution gas chromatography–mass spectrometry. The raw data, represented by relative luminescence units, were processed in Excel (Microsoft, Redmond, WA) according to the mathematical formula $y = aX + b$, where y is the response in relative luminescence units, X is the picomolar concentration of TCDD per well, a is the curve slope, and b is the intercept.

Histology and immunohistochemistry for CYP1A expression in rainbow trout specimens. Liver and intestine specimens were collected from the farmed rainbow trout ($n = 25$) and fixed in 4% buffered formaldehyde. Serial histological sections (5 μm) were submitted to IHC analysis for CYP1A detection. Briefly, tissues were exposed to enzymatic treatment with 0.5% trypsin and 0.5% $CaCl_2$ (Sigma) for 15 min at 37°C. After preincubation with normal goat serum (Sigma), the sections were incubated for 2 h with a rabbit polyclonal antibody specific for rainbow trout CYP1A (CP-226, Biosense Laboratories, Bergen, Norway) diluted 1:200 and then incubated for 30 min with a biotin-conjugated antibody anti-rabbit immunoglobulin (Dako, Glostrup, Denmark) developed in goat, diluted 1:1,000. Reaction was revealed by the avidin-biotin peroxidase complex (Vectastain, Vector Laboratories, Burlingame, CA) by using 3,3-diaminobenzidine (Sigma) as substrate and observed under fluorescence microscopy (DMRB microscope, Leica Microsystems, Wetzlar, Germany) to determine the presence and location of positive cells in the tissue. Significant results were documented with a digital camera.

To assess method validity, the IHC analysis also included positive controls, i.e., liver samples collected from the rainbow trout with degenerative liver damage ascribable to intoxication (available at the Sezione di Scienze Animali e Veterinarie, Udine University), and in technical negative controls in which the primary antibody was omitted.

Statistical analysis. A nonparametric Kruskal-Wallis test was used to identify statistically significant differences in the concentration values between groups (sampling sites and species). Based on the 75th percentile of the distribution (0.18 pg/g), the samples were classified as highly contaminated or not.

Logistic regression models were applied to detect a possible association between fish species or sampling area and highly contaminated status: the risk of being highly contaminated is expressed as the odds ratio with its associated 95% confidence limit. All analyses were performed using Stata 13 (StataCorp, College Station, TX).

RESULTS AND DISCUSSION

The contamination levels of dioxins, furans, and dioxin-like PCBs in the fish species were lower (Tables 1 and 2) than the maximum level and action limit set by European Union Regulation 1259/2011 (3.5 pg/g [wet weight] for sum of dioxins [WHO-PCDD/F-TEQ] and 6.5 pg/g [wet weight] for sum of dioxins and dioxin-like PCBs [WHO-PCDD/F-PCB-TEQ]) *(13),* suggesting that the pollution levels of Lake Maggiore and Lake Como are not particularly high. These data are consistent with published data about pollution levels *(10),* which showed a gradual decrease in PCB concentrations since 2006. According to a more recent Commissione per la Protezione delle Acque Italo-Svizzere report *(11),* the dioxin concentrations found in the fish from Lake Maggiore have declined. Also, the final report on the monitoring plan of fish in the lakes and rivers in Lombardy showed that the PCB levels in perch are within the limits set by European Union regulations, confirming the hypothesis that PCB contamination has decreased over the past years *(27)*. The median BEQ CALUX TCDD value was higher in Lake Maggiore than that measured in Lake Como and in the fish farm (0.13 versus 0.037 versus 0.046, respectively) (Table 2). The dioxin-like contamination level was higher in the perch and roach from Lake Maggiore than in those sampled from Lake Como. The BEQ CALUX TCDD value was higher in the perch than in the roach from Lake Maggiore (1.8 versus 1.1 pg/g), higher in the perch than in the roach from Lake Como (0.55 versus 0.1 pg/g), and slightly higher than the 1.1 pg/g found for the rainbow trout collected from the fish farm (Fig. 1). The differences could be related to the different degrees of water contamination of the two lakes because of the larger number of contamination sources identified for Lake Maggiore compared with Lake Como.

Kruskal-Wallis comparisons indicated that only the sampling site variable was significant ($P = 0.024$); there were no statistical differences in the PCDD/F and PCB values between fish species. Fish species was marginally significant ($P = 0.0493$) only when the two lakes (Maggiore and Como) were considered as covariates in the Kruskal-Wallis test. When the two variables (species and lake) were entered in the logistic regression model, a statistically significant association was found ($P = 0.016$) with lake, but not with species. Logistic regression showed a 3.7 times higher risk for Lake Maggiore compared with Lake Como ($P = 0.016$, 95% confidence interval 1.3 to 10.8). Although the differences between species were not statistically significant, higher contaminant levels were found in perch. Because it is a predator fish located at the top of the food

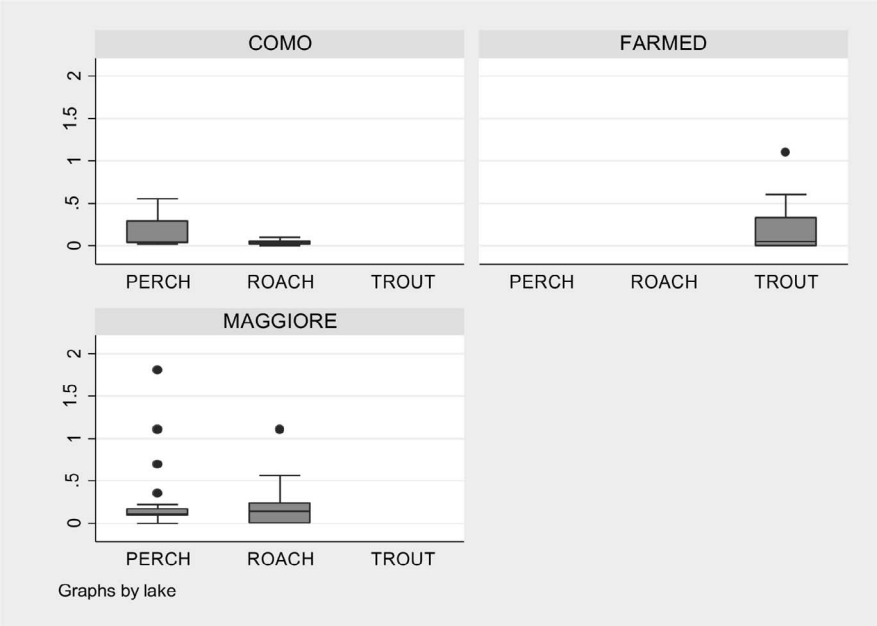

FIGURE 1. *Distribution of BEQ TCDD (picograms per gram of fresh product) values by species and sampling site.*

chain, perch is expected to accumulate higher levels of dioxins and PCBs than roach, which occupies a lower level in the food chain, and also farmed rainbow trout. Histological evaluation showed no visible pathological alterations. IHC labeling of CYP1A tested negative in the intestine samples (data not shown) and in the liver samples (hepatocytes and stromal structures including vessels and ducts) from the farmed rainbow trout (Fig. 2). The absence of IHC positivity in these tissues of rainbow trout suggests no liver P450 enzymatic system induction to detoxify environmental xenobiotics, as expected based on the DR-CALUX bioassay results. Nonetheless, in the positive control liver samples showing evident degenerative changes due to toxicant exposure, numerous CYP1A-positive cells were observed to involve the cytoplasm of macrophage-like cells and fibroblasts in both degenerated and normal areas of the liver (Fig. 3). Moreover, bile duct epithelium and vessel endothelium occasionally resulted positive. Our results are shared by a previous study *(28)* that support the application of IHC-based labeling of CYP1A as a suitable procedure to assess and quantify activation of detoxifying mechanisms in fish liver parenchyma.

In conclusion, the results of the present monitoring survey of Lake Maggiore and Lake Como reveal no serious pollution problems and indicate that the dioxin and dioxin-like PCB contamination levels in the inhabitant fish are below the current regulatory limits. These results suggest that there is no high risk for human health associated with the consumption of these fish species caught in these lakes or farmed.

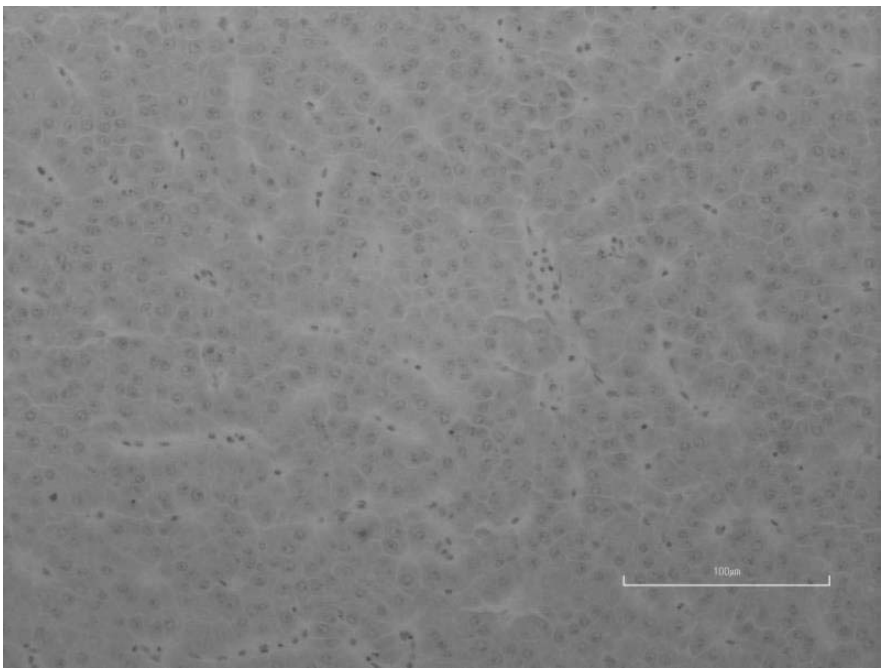

FIGURE 2. *Histological section of liver tissue sample from farmed rainbow trout showing no CYP1A response on IHC analysis.*

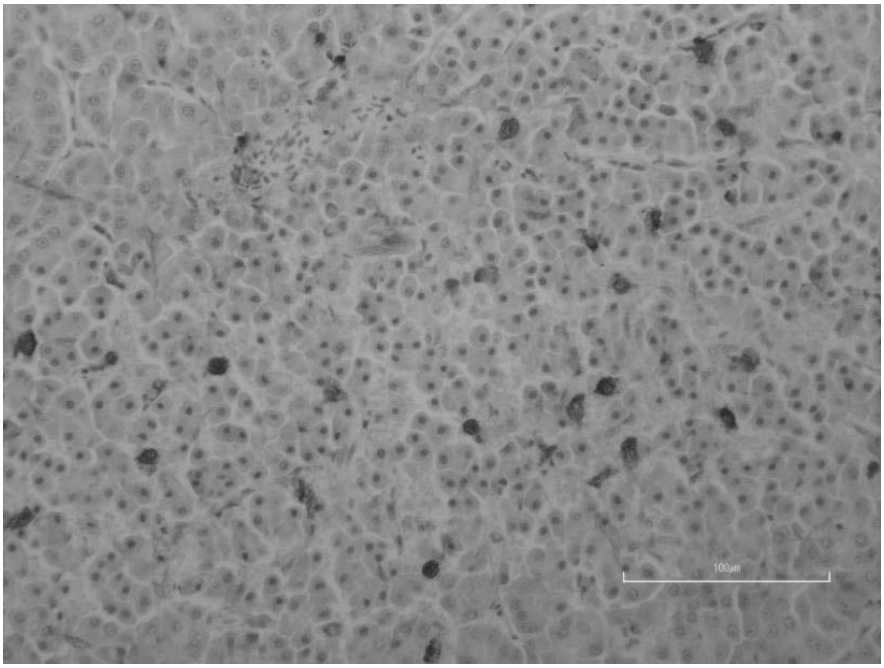

FIGURE 3. *Histological section of liver positive control showing IHC detection of CYP1A.*

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