# *Carassius auratus* as a bioindicator of the health status of Lake Trasimeno and risk assessment for consumers

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## Abstract

Fish are good bio-indicators of the health status of the aquatic environment and can be used as biomarkers to assess the aquatic behavior of environmental pollutants, the exposure of aquatic organisms, and the health risk for consumers. Goldfish are a significant bioindicator in the Lake Trasimeno aquatic system (Umbria, Italy). This study aimed to characterize the health status and the chemical and biotic contamination of Lake Trasimeno to define its anthropogenic and natural pressures and the risk associated with consuming its fishery products. 114 determinations were performed on *Carassius auratus* samples from 2018 to 2020, and the occurrence of brominated flame retardants, non-dioxin-like polychlorinated biphenyls, heavy metals, and microplastics was analytically investigated. Dietary exposure assessment, risk characterization, and benefit-risk evaluation were performed for schoolchildren from 3 to 10 years old. Flame-retardants registered high levels of non-detects (99% for polybrominated diphenyl ether and 76% for hexabromocyclododecanes), while polychlorinated biphenyls were found in all samples with a maximum level of 56.3 ng/g. Traces of at least one heavy metal were found in all samples, though always below the regulatory limit. Microplastics were found with a 75% frequency of fish ingesting at least one particle. Dietary exposure and risk characterization reveal negligible contributions to the reference values of all contaminants, except for mercury, which reached up to 25% of admissible daily intake. The benefit-risk assessment highlighted that the benefits of freshwater fish intake outweigh the associated risks. The examination of goldfish as indicator fish reveals the quality of Lake Trasimeno's aquatic environment and the safety of its products.

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## Introduction

Fish represents a versatile nutrient-rich food product with varied tastes and textures and is low in saturated fat and calories; therefore, it is classified as a healthy diet food. Fish consumption offers important nutritional and health benefits, as it is one of the best sources of protein and provides polyunsaturated fatty acids, liposoluble vitamins, and essential minerals for human health (Domingo *et al*., 2016).

Given these health benefits, several campaigns have been launched to promote fish consumption and increase the number of fishery products, especially in school canteen menus, to encourage healthy dietary choices from an early age (Pieniak *et al*., 2010; Bonanomi *et al*., 2019). However, some preoccupations have emerged in relation to the presence of chemical pollutants in fish products, particularly those from lentic and anthropized ecosystems (Orban *et al*., 2007).

Like all aquatic organisms, fish are exposed to pollutants coming directly from the water and indirectly from food chains, and although persistent organic pollutant use was restricted (Stockholm Convention in 2001), their presence in water and soil is ubiquitous and tends to accumulate along aquatic food web (EPA, 2009; Christensen *et al*., 2017). Similarly, small plastic debris, *i.e*., microplastics (MPs), have been documented in all environmental matrices and various foodstuffs, including 201 edible species (Toussaint *et al*., 2019). There is currently no regulato-

ry framework regarding the presence of MPs in these products; however, it is needed to increase human food safety. When advising individuals about fish consumption, the presence of environmental contaminants in fish meat and products and the risks and benefits need to be considered, counting for the specific characteristics of consumers and considering that contaminants levels in fishery products can vary by location, age, fish species, and several other factors (EPA, 2009; Domingo *et al.*, 2016).

Due to the variety of fish species consumed in Europe, the European Food Safety Authority (EFSA) recommends that each country consider its own fish consumption pattern and carefully assess the risk of exceeding contaminants' safety levels while also evaluating the health benefits (EFSA, 2015).

Fishes, as inhabitants of aquatic ecosystems, are frequently exposed to contaminated water, particularly in those areas where contaminants are stable and have a high potential for accumulation and biological effects (Bernet *et al*., 2001). Thus, fish are good bioindicators of environmental health status because of their position in the trophic chain and their responsiveness to low stimuli of pollutants (Aliko *et al*., 2018; Burgos-Aceves *et al*., 2018). Considering the above, fish may be identified as biomarkers to describe the aquatic environment's health status, define the aquatic behavior of environmental pollutants, assess exposure of aquatic organisms, and the risk for consumers (Van der Oost *et al*., 2003). Accidentally introduced in 1988 during stocking activities with juvenile common carp (*Cyprinus carpio,* Linnaeus), Goldfish (*Carassius auratus,* Linnaeus) is nowadays the most abundant species in the lake fish community due to their high invasiveness (Carosi *et al.*, 2017). Goldfish presence is also favored by climate change, leading to a progressive decline in water levels due to decreased precipitation and evapotranspiration, increased water temperature, and reduced dissolved oxygen concentration and transparency (Ludovisi and Gaino, 2010). Many non-native fish species, such as goldfish, characterized by wider habitat preferences than native species, have taken advantage of climate-induced changes in the lake. Nowadays, for the aforementioned reasons, goldfish are a significant bioindicator in the Lake Trasimeno aquatic system (Umbria, Italy).

This study aimed to characterize the chemical and biotic contamination of goldfish and define the anthropogenic and natural pressures that burden Lake Trasimeno.

The dietary exposure of schoolchildren aged 3 to 10 years to chemical pollutants related to the consumption of fishery products from Lake Trasimeno was assessed, focusing on the benefits and risks of the consumption of the above-mentioned products as part of the school canteen menu.

## Materials and Methods

### Sampling

From 2018 to 2020, 114 determinations have been performed on *C. auratus* samples (average length 25.34±2.04 cm, estimated age 3-4 years old) caught in Lake Trasimeno. Fish was sampled according to Regulation 2017/644 (European Commission, 2017) within the framework of the official monitoring programs and subsequently transferred to the Experimental Zooprophylactic Institute of Umbria and Marche Togo Rosati for chemical analysis. Lake Trasimeno is one of the largest Italian lakes (128 km$^2$), albeit quite shallow (max depth of 6 m). It hosts 19 fish species dominated by the Cyprinidae family, particularly *C. auratus* (Branciari *et al*., 2020). Goldfish is an allochthonous species introduced to the lake around 1988, and since then they have grown exponentially.

### Chemical analysis

#### *Brominated flame retardants*

Twenty-six determinations were performed for 15 congeners of polybrominated diphenyl ethers [(PBDEs) 28, 49, 47, 66, 77, 85, 99, 100, 138, 153, 154, 183, 197, 206, 209] and hexabromocyclododecanes (HBCDs) flame retardants (3 isomers: α-, β-, γ-HBCD). Brominated flame retardants analysis was performed in isotopic dilution, as already described by Tavoloni *et al*. (2020). 20 g of sample were weighed in a polypropylene centrifuge tube, spiked at 1 ng/g with PBDEs and HBCDs labeled internal standards and submitted to QuEChERS extraction adding 5 mL of ultrapure water, 15 mL of EtOAc, 3 g of NaCl and 6 g of anhydrous $MgSO_4$. After shaking (10 minutes) and centrifuging (4000 rpm, 10 minutes), 10 mL of the upper organic layer was transferred into a clean glass tube and reduced in volume at 35°C using the Genevac EZ-2 concentrator (SP Scientific, Ipswich, Suffolk, UK). The residue was purified on $H_2SO_4$ Extrelut NT-3/SPE Si 1 g/6 mL tandem columns assembly and gel permeation chromatography [(GPC) Gilson GPC system equipped with ASPEC XL auto sampler, 307 HPLC pump and UV-vis detector (Gilson, Middleton, WI, USA)]. The GPC collected eluent was evenly divided into two fractions and reduced to dryness. Before analysis, each residue was re-suspended with the respective PBDEs (fraction 1) or HBCDs (fraction 2) syringe standard. PBDE analysis was performed in GC-QqQ-MS/MS (7890A GC-7000B MS; Agilent Technologies, Palo Alto, CA, USA) using large volume injection (PTV inlet), and chromatographic separation was achieved on an RTX1614 column (15 m  250 m 0.10 m; Restek) using helium as carrier gas. HBCDs were analyzed by an ACQUITY I-Class Ultra Performance Liquid Chromatography system (Waters, Milford, MA, USA), and the separation was achieved on a Kinetex XB-C18 column (2.6 m 100 Å, 100 2.10 mm; Phenomenex, Torrance, CA, USA). Background contamination at any stage of the analytical process was carefully monitored and subtracted from the results when not negligible (>20%). To monitor method performances, two procedural blanks, a blank sample and the same blank sample spiked at 100 pg/g for PBDEs and 50 pg/g for HBCDs, were processed in each analytical batch. External quality assurance was guaranteed by regular participation in inter-calibration exercises organized by the European Union reference laboratory for halogenated POPs in feed and food (https://food.ec.europa.eu/horizontal-topics/european-union-reference-laboratories_en). Limits of quantification (LOQs) for all the analytes were equal to 10 pg/g except for PBDE 206 and 209, which was 100 pg/g.

#### *Non-dioxin-like-polychlorinated biphenyls*

Thirty-one independent determinations were performed for non-dioxin-like-polychlorinated biphenyls (NDL-PCB) considering the six indicator congeners [(PCB) -28, -52, -101, -153, -138, -180] (European Commission, 2006).

PCB analyses were conducted in isotopic dilution: a sample amount yielding roughly 1 g of fat was weighted, spiked with 2.5 ng of the six 13C12-labelled indicator PCB, and freeze-dried (5 hours). The sample powder obtained was mixed with diatomaceous earth and extracted with ASE 200 (Dionex Corporation, Sunnyvale, CA), using a mixture of hexane/acetone (1:1 v/v). The extracts, after solvent evaporation, were purified on $H_2SO_4$ acidic Extrelut NT-3/SPE Si 1 g/6 mL tandem columns assembly, and the

analytes were eluted with 13 mL of n-hexane. The purified extract was re-dissolved in 0.25 mL of a 13C12-PCB-155 iso-octane solution at 10 ng/mL (syringe standard) and submitted to GC–MS/MS analysis (Agilent 7890A GC coupled to Agilent 7000 QqQ; Agilent Technologies, Palo Alto, CA, USA). The injection was carried out in pulsed splitless mode, and the chromatographic separation was achieved in temperature-programmed mode (120 °C, ramp to 200 °C at 20 °C/min, ramp to 270 °C at 3 °C/min, ramp to 300 °C at 15 °C/min, hold 4.67 min, total run time 34 min) on an SGE-HT8 PCB capillary column (60 mm _ 0.25 mm _ 0.25 _m; SGE analytical science, Ringwood Victoria, Australia), using helium at 1 mL/min as the carrier gas. The transfer line was held at 280 °C, the source at 230 °C, and the quadrupoles at 150°C. The method was submitted to multi-level validation in intra-laboratory reproducibility conditions, following the prescription of Regulation 2017/644 (European Commission, 2017). The procedure enabled the measurement of 0.10 ng/g fresh weight for each of the six PCB congeners (LOQ). To monitor method performances, a procedural blank, a blank sample, and the same blank sample spiked at a level near the LOQ were processed in each analytical batch; moreover, internal standard recoveries were acceptable and included between 60 and 120%, as requested by Regulation 2017/644 (European Commission, 2017).

### *Heavy metals*

Heavy metals were analyzed in 1 g of sample after microwave digestion with 6 mL $HNO_3$ (67-69%, v/v), 2 mL of $H_2O_2$ (30%, v/v), and 100 LHF (40%, v/v) (Milestone-Ethos1-HPR1000). The appropriately diluted solutions were analyzed by inductively coupled plasma mass spectrometry (Elan DRCII Perkin Elmer, Waltham, USA) in standard mode using specific mass-to-charge ratios (m/z) for each element [206+207+208 lead (Pb), 111 cadmium (Cd), 202 mercury (Hg), 60 nickel (Ni), 52 chromium (Cr), 75 arsenic (As)]. 103Rh was used as the internal standard, and quantification was matrix-matched. The analytical methods were fully validated in intra-laboratory reproducibility conditions, following Regulation 333 (European Commission, 2007). The LOQs (mg/kg) of the method were: Pb=0.010; Cd=0.005; Hg=0.010, Ni, Cr, As=0.040. Batch-to-batch precision and accuracy were evaluated by analyzing a certified reference material (Dorm4, NIST Canada).

## Extraction and characterization of microplastics

To assess MP ingestion, the gastrointestinal tracts of 12 goldfish specimens were processed according to the method validated by Avio *et al*. (2015, 2020) and already applied to extract MPs from tissues of a range of marine species (Bour *et al*., 2018; Bessa *et al*., 2019; Avio *et al.*, 2020). A recovery yield higher than 90%, for particles smaller than 100µm, and 95%, for greater ones, was demonstrated, with no alteration in particle characteristics such as shape, size, and color. Samples were prepared for MP density separation by drying and careful triturating using a mortar and pestle and combining them with a saturated solution of NaCl salt (1.2 gr $cm^3$). After the mixed solution had settled, the supernatants were collected and vacuum-filtered; the dried filters were then inspected under a stereomicroscope [GZ 808 Optech with Optech IS 4K-8 digital camera (Optech, Vaughan, Canada)]. To isolate the potential MPs, manual sorting was performed through tweezers. Particles holding their shape or stretching and resistance to easy breakage when poked with tweezers, bright and unnaturally colored particles, fragments with sharp geometrical shapes, and sheet-like and thread-like particles were all collected (Primpke *et al*., 2020). Collected items were transferred onto a clean cellulose acetate membrane [Ø 47 mm, 0.45µm pore size (Sartorius Stedim Biotech, Gottinga, Germany)], located on a microscope slide for the polymer characterization, and classified based on their shape according to definitions of fibers, fragments, films, and pellet (Avio *et al*., 2020).

Items were additionally measured using an image analysis software (Optika Vision Lite 2.1 Image View): the length was recorded for fibers, whereas other types of items (*e.g.*, fragments, films, and pellets) were measured based on the largest dimension following the most widespread criterion (Avio *et al*., 2020). Fibers longer than 5 mm were not considered microfibers and were excluded from the results. Chemical identification was performed through µFTIR spectroscopy, using a Spotlight 200i FT-IR microscope system (Perkin Elmer, Waltham, USA) equipped with Spectrum Two and driven by Spectrum 10 software. Measurements were taken in the MidIR region (wavenumber range: 4,000-600 cm-1) by attenuated total reflection (µATR-FTIR), with the resolution set at 4 cm-1 and the optical aperture's dimension of 100×100µm. Infrared spectra were acquired after 32 scans per sample, and several backgrounds were performed throughout the working session. For their interpretation, comparison was carried out with commercial spectra libraries (Perkin Elmer, Waltham, USA) that were implemented with personal-created ones, resulting from the characterization of microplastics extracted during previous studies, and with that compiled within the framework of the JPIOCEANS project BASEMAN: polymers matching with reference spectra for a hit quality index ≥0.7 were validated, after a careful examination of peak characteristics (Avio *et al*., 2020).

## Dietary exposure assessment

With the intent of promoting local healthy products and Km 0 food consumption, goldfish patties (GP) have been recently introduced in the canteen menu of schools around Lake Trasimeno to increase fish consumption from an early age. Specifically, in the targeted school canteens, GP are served for lunch once a week in a portion of 3 patties of 40 g each (portion size 120g). GP are produced by a local facility (*Cooperativa dei Pescatori del Trasimeno, San Feliciano, Perugia, Italy)* with the following ingredients: goldfish pulp, potatoes, breadcrumbs, and sea salt. The targeted population was represented by children attending schools in the Lake Trasimeno area and consuming lunch at the school canteen: subjects aged from 3 to 5 years attending kindergarten (KS) and from 6 to 10 attending primary school (PS), weighing on average 16 and 28.6 kg, respectively (Branciari *et al*., 2020). The estimated dietary intake (EDI) of the chemical compounds was calculated using a deterministic approach combining the normalized daily consumption of GP (g/die) considering the portion size and the consumption frequency with the mean concentration of contaminants in the product. The EDI of the considered chemical compounds was derived from the selected population consumption data combined with the contaminant occurrence data in the selected food item, according to the formula reported in the literature (Branciari *et al*., 2020) and adjusted as follows in Equation 1:

$$EDI_i = \sum_{k-1}^{n} \left( \frac{I_{ik}\, C_k}{BW_i} \right) \qquad \text{[Eq.1]}$$

where $EDI_i$ is the total dietary exposure to chemical compounds of subject $i$ (mg/kg bw/day); $I_{ik}$ is the intake of food item $k$ by subject $i$ (g/d), $C_k$ is the contaminant's concentration in food item $k$ (mg/kg), $BW_i$ is the mean body weight of subject $i$ (kg), and $n$ is

the total number of food items consumed by subject *i*. When dealing with dietary exposure assessment of chemical substances, distinguishing between non-detect values and true zeroes is crucial. It was reported that for certain groups of substances, such as persistent organic pollutants, true zero concentrations are unlikely to exist due to their ubiquitous and persisting presence in the environment (EFSA, 2010). The same concept applies to persistent inorganics, such as heavy metals (EFSA, 2011b). Given the above, to perform a realistic estimation of consumers' exposure to the targeted chemical pollutants, the left censored data was handled with the substitution method, applying the middle bound approach (MB) (EFSA, 2010). Therefore, a value equal to half LOQ (1/2 LOQ) was attributed to the undetected values (<LOQ)

## Risk characterization

Aiming to quantitatively estimate the severity of potential adverse health effects in the targeted population, the risk characterization was performed by comparing the exposure assessment results with the available health-based guidance value (Roila *et al*., 2021).

### *Acceptable daily intake approach*

The comparison of the derived EDI with the acceptable daily intake (ADI), "the amount of a chemical in food or drinking water that can be ingested daily over a lifetime without appreciable health risk to the consumer, expressed on a body weight basis" (Benford, 2013), is used for PCB and heavy metal risk characterization. For these molecules, the EDI result was compared to the health-based guidance values and expressed as a percentage contribution to the ADI (% ADI). The specific reference values were the following: Pb=0.004 mg/kg bw/d (FAO/WHO, 2001), Hg=0.571 ug/kg bw/d (EFSA, 2018), Cd = 0.35 ug/kg bw/d (EFSA, 2011b).

### *Margin of exposure approach*

The margin of exposure (MOE) is adopted for those compounds for which there may not be a threshold dose (Benford *et al*., 2013; EFSA, 2015).

Despite not giving a precise quantification of the risk, MOE indicates the level of health concern about a substance's presence in food, and it is a valuable tool for risk characterization and guiding risk managers in prioritizing risk management decisions (Benford *et al*., 2013). The risk characterization of flame retardants (PBDEs and HBCDs) was performed using the MOE by comparing the MB EDI for the targeted molecules with the chronic human intake associated with the body burden at the benchmark dose lower confidence limit for a benchmark response of 10% (BMDL10) for neurodevelopmental effects in mice, identified as the critical endpoint for some PBDEs (EFSA, 2011a). In a recent reevaluation of the risk assessment of HBCDs in food, the EFSA panel of experts concluded that, due to some limitations in the assessment, the endpoint for changes in spontaneous behavior in mice [lowest observed adverse effect level (LOAEL) of 0.9 mg/kg b.w.] was not suitable for the establishment of a reference point; therefore, a BMDL10 was not defined, and the LOAEL was used to define the chronic human dietary intake (EFSA, 2006). The chronic human dietary intake (Dr,h), which reflects the steady state body burden at the calculated BMDL10 or the LOAEL, considering the fraction of the absorbed daily intake and the constant body rate of the elimination of the compounds, was used for the calculation of the MOE values according to the Equation 2 (EFSA, 2005):

$$MOE = \frac{D_{r,h}}{EDI} \quad \text{[Eq.2]}$$

where *MOE* is the margin of exposure, *Dr,h* is the chronic human dietary intake (g/kg b.w.), and *EDI* is the estimated dietary intake (g/kg b.w.).

EFSA states that relevant toxicity data are available only for PBDE -47, -99, -153 and -209; therefore, in the present study, the risk assessment was only performed for these four PBDE congeners (EFSA, 2021). Body burdens at the BMDL10 of 0.172, 0.0042 and 0.0096 g/kg b.w./day for PBDE -47, -99 and -153, respectively, were considered. In contrast with the other PBDE, for PBDE -209, the BMDL10 of 1700 g/kg b.w./day expressed as an external dose can be compared with the estimated human dietary exposure (EFSA, 2011b). Concerning HBCDs (considered as the sum of α-, β- and ɣ- HBCD), Dr,h of 2.35 g/kg b.w./day was used for risk characterization (EFSA 2021). The MOE, calculated as described, was compared to the EFSA reference values and resulted above 24 for HBCDs and above 2.5 for PBDEs -47, -99, -153 and -209, indicating a low health concern, with the risk decreasing as the MOE increases (EFSA 2011b, 2021).

## Benefit-risk assessment

Beneficial and adverse effects may simultaneously occur in a specific food item within the same range of dietary intake. To weigh the benefits and risks associated with food consumption, they should be evaluated and expressed comparably following the benefit-risk assessment (BRA) paradigm (Tijhuis *et al*., 2012).

In the present study, the benefit assessment of fish consumption refers mainly to the ingestion of omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), identified as active factors in chronic disease prevention and health promotion. Risk factors were attributed to the ingestion of chemical compounds, which have been proven toxic to humans.

Aiming to perform a quantitative estimation of the health benefits of Lake Trasimeno fish consumption, the EPA and DHA contents of these fishes were defined according to Branciari *et al*. (2020). The exposure assessment of the target population to such nutrients was performed as mentioned above, multiplying EPA and DHA levels in the portion by its weight for toxicologically relevant contaminants. Subsequently, benefit characterization was determined as the contribution of the nutrient level in the portion with respect to the suggested recommended dietary intake (RDI) of 250 mg/die for EPA and DHA (EFSA, 2012).

The benefit-risk quotient (BRQ) was applied to compare benefits and risks deriving from the simultaneous ingestion of omega-3 fatty acids and contaminants through freshwater fish consumption, as reported in the literature (Geng *et al*., 2015; Barchiesi *et al*., 2020) (Equation 3):

$$BRQ = \frac{Q_{FA}}{Q_T} \quad \text{[Eq.3]}$$

where *QFA* is defined as follows (Equation 4):

$$Q_{FA} = \frac{R_{FA}}{C_{FA}} \quad \text{[Eq.4]}$$

where $R_{FA}$ (mg/day) is the RDI of EPA+DHA. In this study, the RDI of 250 mg/d for a healthy adult (EFSA, 2012) was applied.

$C_{FA}$ (mg/g) represents the concentration of EPA+DHA in fish muscles. The maximum allowable fish consumption related to toxic effects (QT) can be defined according to Equation 5:

$$Q_T = \frac{RfD * bw}{c} \quad \text{[Eq.5]}$$

where *RfD* (mg/kg b.w./day) is the reference dose of the chemical considered; *bw* is the standard bodyweight set as mentioned above; and *c* (mg/g) is the concentration of each toxic molecule in the targeted fish muscle. The values of *RfD* considered for the definition of QT were 100 ng/kg b.w/day for PBDE-47 and -99, 200 ng/kg b.w./day for PBDE-153, 7000 ng/kg b.w./day for PBDE-209 and 200 ng/kg b.w./day for HBCDs (USEPA-IRIS 2008a, 2008b, 2008c, 2008d; Besis *et al*., 2017). BRQ values <1 suggest that achieving the recommended intake of EPA+DHA associated with the intake of flame retardants through fish consumption poses no evident risk to human health (Geng *et al*., 2015; Barchiesi *et al*., 2020)

### Health status of fish

Seventy-three goldfish were harvested, with sampling occurring every 15 days to evaluate the health status of this fish population. One sampling was carried out during a mortality outbreak in late summer/autumn 2019. According to the laboratory standard operating procedures, each fish underwent necropsy, parasitological, bacteriological, histological, and virological examinations. Specifically, the necropsy examination was performed by documenting external abnormalities, including lesions on the body surface and fins, eyes and gills, and external and gill parasites. Subsequently, the abdominal cavity was opened using scissors, cutting from the anal area to the operculum; then the flap of muscle was removed to expose internal organs. Internal abnormalities, including general or focal discolorations of organs, presence of raised areas, cysts, parasites, and size abnormalities were registered. Skin, gill, and intestinal scrapings were performed to search for possible ectoparasites and endoparasites by microscopic observation. Organ samples were tested for the presence of fish pathogenic bacteria by means of the culture-dependent approach through the inoculation of organ homogenates on culture media [blood agar, McConkey agar, and thiosulfate-citrate-bile-sucrose – (TCBS) agar], incubation at 22°C for 24-48 hours, and subsequent identification by biochemical tests and/or by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The search for viral agents was performed by end-point polymerase chain reaction, for the following diseases: carp spring viremia, koi herpesvirus, carp edema virus, and cyprinids herpes virus type 2.

Furthermore, muscle samples were taken for testing for the two main lake environment zoonotic parasites: *Eustrongylides* spp. by macroscopic observation and *Opisthorchis* spp. by artificial digestion and following search of metacercariae under a stereomicroscope.

## Results

### Brominated flame retardants

A total of 26 specimens of goldfish were tested for 15 PBDE congeners (PBDE -28, -47,-49, -66, -77, -85, -99, -100, -138, -153, -154, -183, -197, -206, -209) and for 3 HBCD isomers (α-, β-, ɣ-HBCD). PBDE congeners were below LOQ (10 pg/g; 100 pg/g for PBDE -206 and -209) for all the samples tested, except for one specimen where PBDE -47 (70 pg/g), -49 (11 pg/g), and -154 (15 pg/g) were measured. Similar results were already reported for goldfish from the same basin in a previous study (Roila *et al*., 2021). Similarly, HBCD isomers were below LOQ (10 pg/g) in 20 of the 26 samples; only in 6, α-HBCD was quantified, ranging from 12 to 22 pg/g and in 2 samples, the γ isomer (17 and 39 pg/g) was also measured. It is interesting to notice that in these 2 cases, the γ isomer concentrations are higher than the α isomer's, which is different from what is usually reported for freshwater fishes (Fliedner *et al*., 2018). In HBCD technical mixtures, the γ- isomer is the major component; however, as reported by some authors in animal tissues and food of animal origin, α-HBCD is usually found to be predominant, followed by ɣ-HBCD and β- (Barghi *et al*., 2016; Fernandes *et al*., 2016).

### Non-dioxin-like-polychlorinated biphenyls

Thirty-one independent goldfish samples were analyzed for NDL-PCB. Contrary to what was previously reported for flame retardants, all 6 analyzed congeners were above the LOQ in all samples. The 6 congeners sums ranged from 0.62 to 5.0 ng/g, except for 3 specimens for which 9.28, 10.1 and 56.3 ng/g were measured. The sample contamination pattern is similar irrespective of the level of contamination, being the congener 153>138>180>101>52>28. This result is in line with the most frequent PCB concentrations in freshwater for wild brown trouts (*Salmo trutta trutta* L.) from rivers in the Marche region, Central Italy (Piersanti *et al*., 2012). Furthermore, the congeners 153, 138, 180 contribute to 77% of the total PCB contamination.

### Heavy metals

Analysis for heavy metals was conducted on 31 samples of goldfish from Lake Trasimeno. In particular, Pb, Cd, Hg, and As were investigated. Traces of at least one heavy metal were found in every sample, albeit at low levels and always below the regulatory limit [Hg=0.5 mg/kg; Pb=0.30 mg/kg; Cd=0.050 mg/kg, in Regulation 1881 (European Commission, 2006)]. Pb ranged from <0.010 to 0.103 mg/kg, Cd from <0.005 to 0.013 mg/kg, and Hg from 0.023 to 0.183 mg/kg.

### Microplastics

MPs were determined in 12 goldfish samples, and 9 tested positive for ingestion of plastic material, with a 75% frequency of fish ingesting at least one particle. 12 particles were extracted (average 1.33±0.71) per organism. 83.3% of the extracted plastics were fibers, while the remaining 16.6% were fragments. Regarding the polymer composition, 83.3% of the particles were polyesters (PEST), 8.3% polyethylene (PE), and 8.3% polyamide. These preliminary results seem to show that freshwater fish exhibit a higher frequency of MP ingestion-positive specimens than reported in the literature for marine organisms (50% *vs* 30%, respectively) (Shim *et al*., 2015) (Figure 1).

### Dietary exposure

To assess the potential risks associated with consuming fish products from the Trasimeno Lake, the mean EDIs were calculated, considering the GP consumption of the targeted population and the above-reported MB occurrence data. The EDIs for PCBs were 6.54 and 3.66 ng/kg bw/die for KS and PS, respectively. For flame retardants, the values of EDI were 456.9 and 255.6 pg/kg bw/die for PBDEs and 28.9 and 16.18 pg/kg bw/die for HBDEs, for KS and PS, respectively. The EDIs associated with heavy metals are below 0.001 mg/kg bw/die for all the metals tested.

In terms of risk characterization, the contribution to ADIs was calculated for heavy metals based on EDIs results, and the values were 0.56 and 0.31% for Pb, 25.1 and 14.1 % for Hg, and 1.63 and 0.91% for Cd, for KS and PS respectively. As reported in the literature, the highest contribution to ADI is registered for Hg, while the consumption of GP has a low contribution to the reference value for the other two metals tested. The risk characterization of toxicologically relevant PBDE congeners and HBCDs was performed employing the MOE approach by comparing the MB dietary intake for the different molecules with the estimated population exposure associated with the body burden at the BMDL10, in line with EFSA scientific opinions (EFSA 2011, 2021). The calculated MOEs are largely above the EFSA reference values (data not shown).

## Benefit-risk assessment

Recently, monitoring programs have been implemented by several countries to assess the presence of chemical pollutants in foods and to define human health risks resulting from dietary exposure to these contaminants. In this study, the BRQ was applied to evaluate the simultaneous effects on human health of EPA and DHA ingestion and contaminants present in GP and the values registered were always <1 (*data not shown*).

## Health status of fish

Overall, the specimens examined showed an anatomopathological spectrum in the normal range, except for a few cases of modest gill anemia, sometimes associated with hypermucosity, and rare cutaneous hemorrhages mainly distributed on the pinniferous insertions (lesions presumably attributable to death by asphyxia). The specimens that were part of the mortality episode showed a generalized hyperemic status both externally and viscerally, with cases of mono- and bilateral exophthalmos and anal protrusion (Figure 2). The scraping results were in the normal range. The bacteriological examination results were all negative, except for *Aeromonas sobria* isolated from the specimens subject to seasonal mortality. All virology tests were negative, in line with the virological health trend of the fish fauna of Lake Trasimeno, also concerning other species sampled according to the current monitoring plans issued by Umbria. All muscle samples examined were negative for both *Opisthorchis* spp. and *Eustrongylides* spp. parasites.

# Discussion

Despite the ubiquitous distribution of PBDEs and HBCDs, the results of their occurrence in goldfish from Lake Trasimeno were



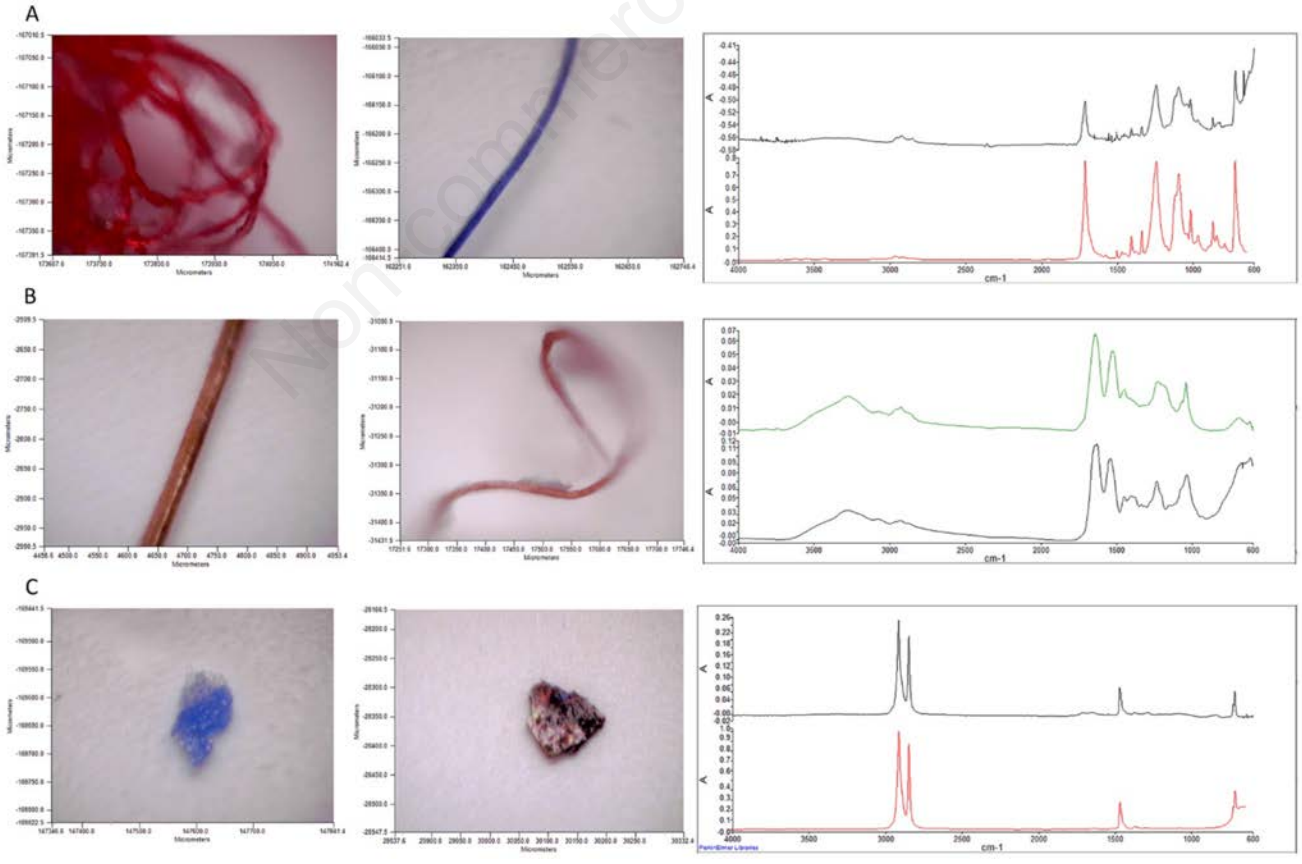


**Figure 1. A-C**) Images of microplastic fibers and fragments extracted from Lake Trasimeno goldfish (*Carassius auratus*).

characterized by a high proportion of non-detects, confirming the low level of contamination in this lake. PBDE and HBCD concentrations found in the present study are similar to or lower than those observed in previous studies on freshwater species from different countries, although the comparison of contamination levels could be difficult due to the different numbers and types of congeners and isomers analyzed and due to the different species considered. Due to goldfish's low market value, studies assessing this specie's contamination pattern are scarce. However, studies refer to a wide range of contamination in cyprinids, most probably as a result of the different aquatic environments considered. For common carp harvested in Chinese freshwater basins, PBDEs have been found in concentrations of 19.78 ng/kg l.w. in 2009, 16.48 ng/kg l.w. in 2010, and 5.54 ng/kg l.w in 2011 (Su *et al*., 2014). In the same study, the levels of HBCDs were 51.9 ng/kg l.w. in 2009, and 23.7 to 169.6 ng/kg l.w. in 2011. In crucian carp from south China, the registered PBDEs were 1430 ng/kg l.w. (Zhang *et al*., 2011). Lower levels of PBDE contamination were reported in the same year in a study (Covaci *et al*., 2006) on gibel carp samples from the Danube delta, Romania (2.73 ng/kg l.w.), and in a study (Zhang *et al*., 2011) on mud carp from south China (10.1 ng/kg l.w.). The PCBs analyzed represent the sum of six congeners (PCBs 28, 52, 101, 153, 138, 180) considered suitable indicators of total PCB contamination, due to their predominance in biotic and non-biotic environments and listed as priority food contaminants monitored by EFSA. The results on PCB goldfish contamination confirm the presence of these chemicals in the ecosystem of Lake Trasimeno, even if the registered values are always largely below the threshold set by Regulation 1881 (European Commission, 2006) (75 ng/g wet weight).

In line with the literature, heavy metals were detected in all samples investigated; this is probably due to their inability to degrade under normal environmental conditions and consequently deposit in sediments or bioaccumulate (Yang *et al*., 2014). Their presence, especially in aquatic environments, is unavoidable. Therefore, fish can accumulate a considerable amount of heavy metals that can harm consumers' health, highlighting the importance of a thorough dietary exposure evaluation (Yi and Zhang, 2012). The risk characterization performed in this study confirms that the heavy metal exposure through GP does not exceed admissible daily intakes, albeit only in the case of Hg, where it reaches 25%, thus indicating the need for continuous monitoring.

Freshwater ecosystems located in low anthropizated areas are vulnerable to MP contamination and are usually more contaminated than marine ecosystems. The use of goldfish as a bioindicator showed that not even the waters of Lake Trasimeno are free from MPs. Overall, the registered level of abundance is slightly higher than the one found in other Italian lakes, such as Lake Bracciano (Cera *et al*., 2022), albeit the study considered different fish species (mean abundance 0.16±0.67 for *Atherina boyeri* and 0.05±0.40 for *Coregonus lavaretus*). Concerning *C. auratus*, a Chinese study registered a higher MPs abundance, from 0 to 18 items/fish, and reported that the relatively higher MP abundance might be attributed to the high level of pollution in the surrounding environment. MPs are a complex, persistent contaminant class consisting of an array of sizes, polymers, colors, and morphologies and may act as vectors for pathogens, additive chemicals such as pigments, plasticizers, or flame retardants, and other concerning contaminants that could be adsorbed from the surrounding environment (Cera *et al*., 2022).

In the present study, MPs extracted from fish are predominantly linear-shaped PEST particles, in line with the results reported in the literature for other lacustrine basins (Cera *et al*., 2022). It has been reported that in marine species, fragment-shaped PE particles are usually found, and this difference is presumably due to the fact that, while in inland waters the presence of plastic particles comes mainly from domestic discharges, in the sea they are mostly formed as a result of fragmentation of macroscopic material (Avio



**Figure 2.** Goldfish (*Carassius auratus*) necropsy, external (skin hemorrhages, monolateral oesophtalmous), and internal (generalized hyperemic status) lesions.

*et al*., 2020). The occurrence of MPs in the gastrointestinal tract of fish does not provide direct evidence for human exposure as this organ is usually removed before consumption, but the ingestion and permanence in the gut can cause a series of biological alterations indirectly affecting meat quality and nutritional value of fish (Ašmonaite *et al*., 2018).

The purpose of BRA, built on the risk assessment framework, is to study the combined potential adverse and beneficial health impact associated with the consumption of foods. In this study, we considered BRA by combining information on actual exposure with a characterization of adverse and beneficial effects (dose-response functions or established thresholds).

The outcome of BRA implies that healthy children eating enough GP to achieve the RDI for EPA+DHA would not be exposed to an increased health risk due to the simultaneous exposure to the toxic compounds analyzed. As already reported by other authors in different environments, these results confirm that the benefits of freshwater fish intake should outweigh the associated risks (Roila *et al*., 2021). As mentioned above, the bacteriological analysis was able to isolate *Aeromonas sobria* from the specimens subject to seasonal mortality. This germ is normally present in the aquatic environment, and several researchers have suggested that infections caused by *Aeromonas* spp. can result from a compromised immune system of animals under stressful conditions closely associated with water temperature change, hypoxia, eutrophication, organic pollution and rough weather conditions. Temperature influences the expression of virulence genes in pathogenic bacteria such as *Aeromonas*. Furthermore, an increase in water temperature in Lake Trasimeno increases metabolism and exerts stress on fish. This stress causes an increase in corticosteroid production, which makes fish more susceptible to infection. Increasing stress on potential hosts due to climate change makes this pathogen perfectly suited to profit and makes it plausible that it will even more troublesome in the future (Majtán *et al.,* 2012). The zoonotic potential of these microorganisms is still debated (Majtán *et al.,* 2012; Abdi *et al*., 2014); however, a thorough monitoring plan (necropsy and microbiological analysis) combined with proper cooking procedures would minimize the potential risk for consumers.

## Conclusions

Examining indicator fish, like goldfish, reveals the caliber of the aquatic environment of Lake Trasimeno, where these fish reside. Furthermore, the obtained results on consumers' exposure indicate that even in the globalization era, it is possible to locate areas with low pollution pressure from which fish can be safely harvested for human consumption.

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