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Assessing bioaccumulation of chemicals in lacustrine food webs

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Publications

This thesis is based on the following works:

Chapter III: Pascariello S., Mazzoni M., Bettinetti R., Manca M., Patelli M., Piscia R., Valsecchi S., Polesello S. 2019. Organic Contaminants in Zooplankton of Italian Subalpine Lakes: Patterns of Distribution and Seasonal Variations. Water, 11(9), 1901, doi:10.3390/w11091901

Chapter IV: Mazzoni M., Buffo A., Cappelli F., Polesello S., Valsecchi S., Volta P., Bettinetti R. 2019. Perfluoroalkyl acids in fish of Italian deep lakes: environmental and human risk assessment, Science of The Total Environment, 653, 351-358. doi.org/10.1016/j.scitotenv.2018.10.274

Chapter V: Mazzoni M., Boggio E., Manca M., Piscia R., Quadroni S., Bellasi A., Bettinetti R. 2018. Trophic transfer of persistent organic pollutants through a pelagic food web: The case of Lake Como (Northern Italy). Science of The Total Environment, 640, 98-106. doi.org/10.1016/j.scitotenv.2018.05.307

Chapter VI: Mazzoni M., Ferrario C., Bettinetti R., Piscia R., Cicala D., Volta P., Borgå K., Valsecchi S., Polesello S. "The Unbearable Lightness of" bioaccumulation in the trophic web of Lake Mergozzo (Submitted)

Chapter I

Introduction

1.1 Lakes

Lakes are lentic ecosystems where the renewal time of water is slow but continuous (Wetzel, 2001). Lakes contain 0.008% of all water present on the earth surface but this small amount of water is important for ecosystems and the society itself (Carpenter et al., 1992).

Lakes basins hold a regulatory function as temporary storage of water and subsequent gradual disposal through the emissaries. Moreover, lakes represent water reserves that can be exploited to produce electricity, for irrigation and (after appropriate treatment) for potable use (Tonolli, 1964). In spite of the great importance to humans, lakes must face several threats such as eutrophication, invasive species, shoreline alteration and destruction of wetland, overexploitation of resources, global warming and pollution (Brönmark and Hansson, 2002).

1.1.1 Lacustrine trophic web

Lacustrine ecosystems are complex systems, characterized by chemico-physical properties due to abiotic factors, to the interactions between organisms such as prey/predator or competition relationships and to the interactions between biota and their environment. For example, the spatial distribution of the organisms follows abiotic constrains like the amount of irradiance and the temperature, the

substrate characteristics and the concentration of dissolved oxygen; these with other features lead to identify specific zones with specific ecological characteristics. A simple summary of zonation system is reported in Table 1.1, taken from (Likens, 2010).

Table 1.1 Summary of zonation systems in lakes, modified from Likens (2010).

Zonation	**Spatial location**	**Temporal variability**	**Description**
Pelagic zone	Horizontal	Stable	Offshore (bottom irradiance < 1%)
Littoral zone	Horizontal	Stable	Nearshore (bottom irradiance ≥ 1%
Water column	Vertical	Stable	Water extending from lake surface to bottom
Benthic zone	Vertical	Stable	Interface of water column and lake bottom
Epilimnion	Vertical	Seasonal	Uppermost density layer
Metalimnion	Vertical	Seasonal	Middle density layer
Hypolimnion	Vertical	Seasonal	Bottom density layer
Euphotic zone	Vertical	Dynamic	Portion of lake with ≥ 1% light (photosynthesis)
Aphotic zone	Vertical	Dynamic	Portion of lake with < 1% light (no photosynthesis)

In each zonation it is possible to identify specific taxa, i.e aquatic

vegetation in littoral area or suspended phytoplankton in open waters, that build trophic dynamics and form biotic communities and trophic webs.

Food web analysis aims to elucidate and quantify the linkages of feeding relationships; traditional studies were based on gut analysis; however, this approach was subject to errors because the identification of food was not always possible. Moreover, this kind of study did not yield any long-term information, giving a picture of the situation at the moment of sampling (Vander Zanden and Rasmussen, 2001). The use of stable isotope as passive tracers may allow for a quantitative estimation of relationships between consumers and preys, the monitoring of temporal changes in diet, the perturbations in food-web with the introduction of non-native species (Post, 2003; Vander Zanden et al., 1999). More recent interesting approach have focused on detecting toxicants bioaccumulation in organisms and their transfer across the trophic chain (Mackay and Fraser, 2000).

The elements mainly used in trophic web studies are carbon and nitrogen. Heavy carbon isotope allows for the identification of the source which is at the base of food web. It is known, in fact, that isotopic C composition of consumers changes little along the trophic chain (Post, 2002). When multiple sources are available, their relative contribution in the consumer tissue may be estimated by applying a linear mixing model (Phillips et al., 2014). On the other hand, the difference between nitrogen isotopes of different organisms are useful to assess their relative trophic positions. The increase in $\delta^{15}N$ between

trophic levels is related to processes of nitrogen assimilation and excretion (Vanderklift and Ponsard, 2003). The enrichment in consumers with respect to their diet is 3-5 ‰, representing the mean of the statistical distribution of the $\delta^{15}N$ differences along the trophic chain (Minagawa and Wada, 1984); moreover, difference in nitrogen stable isotopes between consumer and diet depends on availability of environmental nitrogen (Adams and Sterner, 2000).

In lakes, the different zones are characterized by different isotopic signatures. Littoral primary producers are characterized by the least depleted $\delta^{13}C$ values, instead, pelagic phytoplankton is typically characterized by the most negative $\delta^{13}C$ (Jones et al., 1998; Grey et al., 2000; Grey and Jones, 2001). It is explained by the fact that pelagic and benthic algae have different fractionation of carbon during carbon fixation (Hecky and Hesslein, 1995). Furthermore, also the carbon source lead to a discrepancy between coastal area and open waters In fact, Grey et al. (2001) reported that the isotopic signature of allochthonous carbon, that is distributed mostly in littoral zone, is less negative ($\delta^{13}C$ ranging between -21 and -27 ‰) than that of autochthonous material ($\delta^{13}C$ around -30 ‰). These differences in isotopic signatures of producers are also reflected in those of primary consumers from different zones (Post, 2002). As already reported, $\delta^{13}C$ do not vary across different trophic levels, so, primary consumers show the same signature of their diet, maintaining different values between pelagic and littoral organisms.

Top predators and omnivore organisms like fish instead tends to show

average values because they integrate the carbon originating both from pelagic and benthic organisms (Hecky and Hesslein, 1995).

1.2 Chemicals in aquatic environment

Contaminants can be broadly defined as any synthetic or naturally occurring chemical or any microorganism that has the potential to enter the environment and cause known or suspected adverse ecological and (or) human health effects (U.S. Geological Survey, USGS, definition).

There is no single way to classify contaminants. They could be divided by their chemical structures, identifying, for example, metal ions, radioactive isotopes, inorganic compounds, organics compounds. Organic contaminants include many groups of chemicals among which polycyclic aromatic hydrocarbons, halogenated (chlorinated, fluorinated, brominated) compounds, phthalates, alkylphenols could be found.

We can distinguish between naturally occurring compounds and man-made compounds, both voluntary and as side-products, or it could be useful divide the chemicals by function (pesticide, pharmaceuticals…), regrouping together different types of chemicals that have a similar use.

Or we can divide even between “legacy compounds” and “emerging compounds (ECs)” that are, respectively, well known, regulated and monitored substances, discharged by agriculture or industry, persistent, bioaccumulative and toxic and substances that are not

(completely) regulated and monitored, are used also in consumers products, are little known and there is uncertainty on their potential negative effects. ECs are a heterogeneous group of chemicals that includes veterinary and human pharmaceuticals, Personal Care Products (PCPs), nanomaterials, additive feedings, and biocides (Picò and Barcelò, 2015).

Despite the many categories that could be identified, chemical structures give to each group specific proprieties, which have been exploited by human, but which also influence their behavior in environment, the fate and their adverse effects on organisms. They require also specific analytic techniques to be investigate.

Water covers three quarters of the earth's surface and it is not surprising that it represents the ultimate sink for most chemicals. Study of the sources, transport, fates and effects of chemicals in water and in aquatic organisms is very important for the water resources protection (Koumanova, 2006).

1.2.1 Organochlorine Compounds

Legacy persistent organic pollutants (POP) include organochlorine compounds. Among them, there are dichloro-diphenyl-trichloroethane and its metabolites (DDTs) and poly-chlorinated-biphenyls (PCBs).

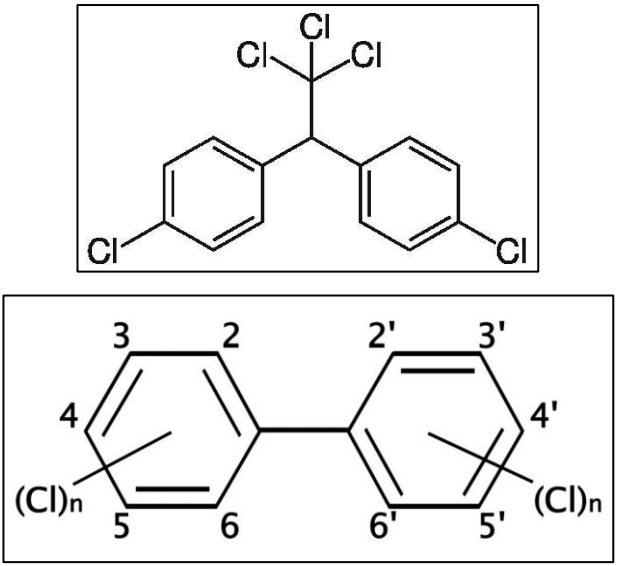

Fig. 1.1 Above: dichloro-diphenyl-trichloroethane (DDT). Under: general scheme of poly-chlorinated-biphenyls (PCBs).

DTT (Fig. 1.1) was synthesized in 1874, but its insecticidal properties were discovered around 1940s and the large-scale industrial production started after the Second War World. The low price of DDT contributed to its worldwide use (Turusov et al., 2002). In 1962 “Silent Spring” by Rachel Carson was published and studies on adverse effects of organochlorinated pesticides grow exponentially together with an environmental consciousness in the population (Carson, 2002). As result, since 1970 many countries started to ban DDT for agricultural purposes and the regulation of chemical pesticide use was strengthened. DDT has two common breakdown products: dichloro-diphenyl-dichloroethylene (DDE) formed by dehydrohalogenation

and dichloro-diphenyl-dichloroethane (DDD) formed by reductive dichlorination.

PCBs (Fig. 1.1) comprise 209 congeners from 1 to 10 chlorine atoms. They were extensively used between the 1950s and 70s for a broad range of applications because of their general chemical inertness; insulating capacity, heat stability and low burning capacity. They are used as coolants and lubricants in transformers, generators and capacitors, as well as hydraulic and heat exchange fluids (Gioia and Akindele, 2014). Therefore, PCBs are expected to be present in electronic waste (e-waste) streams (Wen et al., 2009).

DDTs and PCBs are persistent, bioaccumulative, prone to long-range transport and toxic both on wildlife and human (Jones and de Voogt, 1999; Ribeiro et al., 2017; Ruzzin, 2012; Sijm et al., 2007). They are still present in the environment, in various matrices (Mitra et al., 2019; Olatunji, 2019; Yang et al., 2019) and in biota, for their high affinity to adipose tissue (Rigét et al., 2019; Robinson et al., 2016). Many studies evidenced their biomagnification in wildlife (Corsolini and Sarà, 2017; Zhang et al., 2017)

Humans are mainly exposed through food and water; inhalation is an important exposure pathway for e-waste workers that conduct open burning activities (Helou et al., 2019)

Since 2001 they are enlisted in Stockholm Convention on POPs, an agreement to eliminate or severely restrict the use and the production of these persistent organic contaminants.

1.2.2 Poly and Perfluoroalkyl Substances

Per- and polyfluoroalkyl substances (PFASs) are synthetic chemicals produced since 1950 and used in many industrial applications and large-scale products consumption for their physical-chemical characteristics (Buck et al., 2011). They are utilised to produce fluoropolymers, in the treatments of textiles and paper, in formulations of paints for building, cosmetics and insecticide and even in firefighting foams (Buck et al., 2011). The presence of numerous carbon-fluorine bonds gives to these substances exceptional thermal and chemical stability and makes them good surfactants (Kissa, 2001) but, at the same time, allows their great mobility and persistence in the environment (Ahrens and Bundschuh, 2014).

PFASs include thousands of chemicals but the environmental studies have been focused mainly on the group of perfluoroalkyl acids (PFAAs), that have a fully fluorinated carbon chain of variable length and a hydrophilic terminal group (Buck et al., 2011). In particular, researchers have dealt with perfluoroalkyl carboxylic acids (PFCAs), that include perfluorooctanoic acid (PFOA), and perfluoroalkyl sulfonic acids (PFSAs), that include perfluorooctane sulfonic acid (PFOS) (Fig. 1.2)

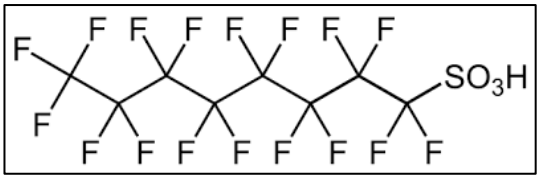

Fig. 1.2 Structure of perfluorooctane sulfonic acid (PFOS).

PFAAs are ubiquitous in the environment and, thanks to their long-term use, globally detected in various matrices, from human blood to environmental matrices, such as groundwater, surface water, sediments, soils, wastewater effluents (Ahrens and Bundschuh, 2014; Castiglioni et al., 2015; Houde et al., 2011; Lu et al., 2018; Stahl et al., 2014; Valsecchi et al., 2015). PFOS, PFOA and other long-chain PFCAs have been demonstrated to be bioaccumulative in biota and in trophic food chains (Conder et al., 2008; Houde et al., 2011). For example, PFAAs have been measured in fish in freshwater ecosystems in Europe, South Africa and Asia (Bangma et al., 2017; Berger et al., 2009; Labadie and Chevreuil, 2011; Lam et al., 2014). Data on biota monitoring collected and reviewed by Houde et al., (2011) underline that PFOS was the predominant PFASs in biota but concentrations were decreasing, at the contrary of the concentrations of long-chain PFCAs that are increasing.

The presence of high levels of PFAAs is raising concern for the end consumers, including humans and top predators, even in remote areas (Lau et al., 2007). Exposures for human population are multiple, including drinking water; dietary intake (Schwanz et al., 2016) and

consumer products (e.g. furniture, carpets, food packaging, cleaning agents) that can release PFASs in indoor dust and air (Jian et al., 2018; Karásková et al., 2016). Diet, especially fish and other seafood, has been suggested as the main route of exposure for the human population (Ahrens et al., 2016). PFOS and PFOA are absorbed in the gastrointestinal tract and then excreted in urine and faeces, they do not undergo metabolic transformations (Knutsen et al., 2018).

The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain set a tolerable daily intake (TDI) of 150 ng kg^{-1} body weight (bw) per day for PFOS and 1500 ng kg^{-1} bw per day for PFOA (European Food Safety Authority, 2008). Recently EFSA published a new risk assessment with a tolerable weekly intake (TWI) of 13 ng kg^{-1} bw per week for PFOS and 6 ng kg^{-1} bw per week for PFOA (Knutsen et al., 2018).

Laboratory studies have been carried out to evaluate the toxicity of PFAAs in many taxa, plants and animals. For algae (diatoms, green algae, cyanobacteria) a linear relationship between PFCAs toxicity and the length of carbon chains was proved (Liu et al., 2019). Even PFOS can suppress the growth of algae by increasing the permeability of the cellular and mitochondrial membrane (Liu et al., 2008). PFCAs have toxic effects on invertebrates (PFOA can significantly reduce the population density of water flee, perfluorononanoic and perfluorodecanoic acid can significantly inhibit the p-gp transport proteins in mussels) (Sanderson et al., 2003; Stevenson et al., 2006). PFOS can interfere with the activity of transport proteins in zebrafish

(Keiter et al., 2016).

In addition, PFAAs have been reported to have adverse effects on the development and immunity, carcinogenic toward the liver and may also interfere with hormone levels in mammals (Liu et al., 2019; Takacs and Abbott, 2007). High levels of PFOS can increase total cholesterol in serum in adults and decrease the antibody response at vaccination in children. For PFOA, the most critical effect is the increase of total cholesterol (Knutsen et al., 2018).

PFOS was included in the Annex B list of the Stockholm Convention on Persistent Organic Pollutants in 2009 and in 2006 and 2011 some restrictions on the marketing and use of PFOS were issued in European regulations (Directive 2006/122/UE, EU Regulation No 207/2011). On May 2019 delegates of the UN Conference of the Parties in Geneva have unanimously agreed to add the chemical, along with its salts and PFOA-related compounds, to Annex A of the Stockholm Convention (POPs). Furthermore, after risk assessment study (European Union, 2011a), the European Commission included PFOS in the priority hazardous substance list that must be monitored in all European surface waters and aquatic biota to classify the quality status of the water bodies (Directive 2000/60/EC).

1.2.3 Mercury

Mercury (Hg) is a naturally occurring element contained in many minerals (including cinnabar, the non-ferrous metals and in fossil fuels coal as an impurity) that is released by weathering of rocks,

geothermal activity, and vulcanism (Streets et al., 2019). Human activities have increased the release of mercury into the environment, raising its amount in atmosphere, soils and waters (Driscoll et al., 2013). There are two type of anthropogenic sources: the so called "unintentional" emissions, such as coal burning, mining, industrial process that use ores or other materials to produce metals cement, when mercury is emitted because it is present as an impurity in raw materials, and the emission produced after that mercury is used intentionally. Among these are enlisted: artisanal and small-scale gold mining (in which mercury is employed to extract gold), waste from consumer products (including metal recycling), the chlor-alkali industry, the production of vinyl-chloride monomer and, despite restrictions, formulation of pesticides and fungicides. The emissions from anthropogenic sources could account up to about 30% of the total amount of mercury entering the atmosphere each year (Driscoll et al., 2013; UNEP, 2013).

The chemistry of Hg is complex, and this make difficult to predict its behaviour in the natural environment (Fig. 1.3). Mercury has three oxidation states: Hg(0) (elemental mercury), Hg(I) (mercurous), or Hg(II) (mercuric). The mercurous form is not stable under typical environmental conditions and, therefore, is rarely observed. Hg(II) can complexed with various inorganic and organic ligands and also to be metabolised by microorganisms to organic forms, mainly methylmercury (MeHg) and dimethylmercury (Ullrich et al., 2001).

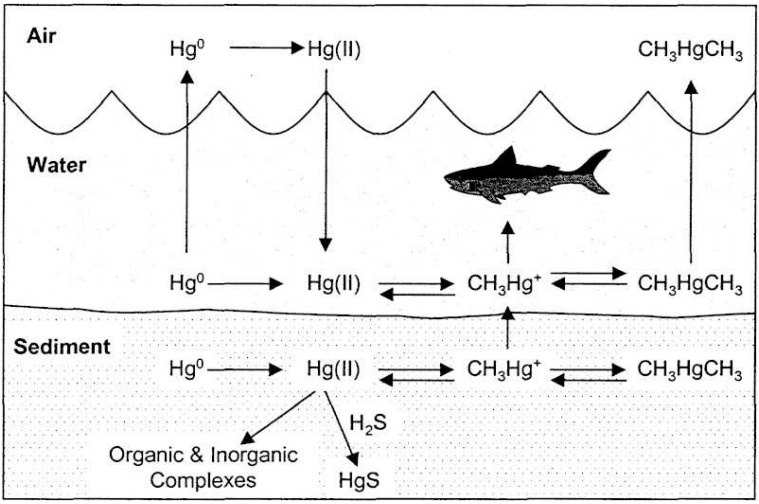

Fig.1.3 Exemplification of the cycle of Hg (CH3Hg+: methylmercury ion; CH3HgCH3: dimethylmercury; HgS: cinnabar) Adapted from EPA, 1997.

The chemical form of Hg in aquatic systems is strongly influenced by redox conditions, pH, temperature, availability of nutrients and complexing agents (Ullrich et al., 2001). Hg^0 is the most volatile of all metals (vapor pressure at 25 °C equal to 0.24 Pa) and is typically present in the atmosphere. Hg transport in the atmosphere is a major pathway for the dispersal and exchange of Hg in the global environment, even in remote areas (Driscoll et al., 2013).

The extensive use of the metal has resulted in serious contamination of freshwater, oceans, sediments and biota (Bargagli, 2016; Lin et al., 2012; Rigét et al., 2011; Wang et al., 2019) and furthermore, mercury has the capability of biomagnification in trophic webs (Lavoie et al., 2013). Humans are exposed to Hg, mainly from the ingestion of

contaminated fish and seafood; exposure occurs also though vapour of mercury, especially for workers (Rice et al., 2014).

Mercury is classified among the metals that are not essential and bioavailable (Wood, 1974). Toxicological and ecotoxicological effects are strongly dependent on the mercury species. Despite a study on marine medaka that showed that inorganic mercury may cause neurotoxicity, cytoskeletal assembly dysfunction and metabolic disorders (Wang et al., 2015), the organic methylated species are considered more toxic. Methylmercury has adverse effects on respiratory, renal, immunological, endocrine and reproductive systems (Rice et al., 2014). MeHg toxicity is associated with nervous system damage in adults, infants and children. Sadly notorious is the MeHg poisoning occurred in the 1950s and 1960s at Minamata Bay (Japan) through the ingestion of highly polluted seafood that resulted in more than 1000 deaths and many people who showed symptoms like sensory and auditory disturbances, ataxia, dysarthria, constriction of the visual field (Harada, 1995).

In fish Hg has neurotoxic effects and impacts on the development of several organs (such as abnormal eye development and cardiac malformation) (Huang et al., 2011). Hg can influence the feeding strategies, even stopping them; this may lead to a deficiency of essential nutrients for larval development (Liu et al., 2016).

In 2013, the United Nations proposed the ‘Minamata Convention on Mercury’, which aims for a more global effort in managing the risk of Hg to human health and the environment. Signed by 128 countries

(UNEP, 2016), it entered in force in 2017 (Selin et al., 2018). Furthermore, quality criteria have been set by various regulatory bodies.

1.3 Bioaccumulation

It is widely recognised that chemicals concentrations in organisms can be higher than those present in the environment. This phenomenon is referred as bioaccumulation (Mackay and Fraser, 2000) and is generally defined as the process whereby the substances in an organism reach levels that exceed those in the surrounding environment, regardless of the exposure route (Gobas et al., 2009). This fact concerns the interaction between "organisms", meaning plants, invertebrate and vertebrate animals such as fish, mammals, reptiles, and birds, and "environment", namely the medium the organisms respire (air or water), the ambient in which they stay and live (air, water, soil, or sediment) and the food that they consume. Focusing on aquatic environment, bioaccumulation is defined as the process which leads to higher chemical concentrations in an aquatic organism compared to those in water, due to uptake by all exposure routes including dietary absorption, transport across respiratory surfaces and dermal absorption (Mackay and Fraser, 2000). There are both scientific and regulatory interests in understanding and quantifying the bioaccumulation of chemicals released to the environment (Mackay et al., 2016). For example, the capacity of a substance to be accumulated can be used as indicator of environmental

contamination since it allows to better describe the fate of chemical and then to estimate the potential levels to which an organism is exposed. Indeed, bioaccumulation could be related to adverse and toxic effects, because toxic effects happen when a specific quantity of contaminant reaches target organs and the quantity of contaminant that enters in the organism is controlled by bioaccumulation phenomena. For this reason, the capability of bioaccumulation is one of the criteria to regulate, restricted or ban chemical substances according to international agreements (Gobas et al., 2009; UNEP, 1998). Bioaccumulation assessments adopt a tiered system to identify chemicals with high potential for bioaccumulation to be enlisted in regulatory frameworks and substances which are not bioaccumulative to reject at an early stage of assessment with minimal expense and effort (Mackay et al., 2016).

Researchers employ several metrics and criteria to assess bioaccumulation. The suit of the measures includes the octanol-water partition coefficient (K_{OW}), the bioconcentration factor (BCF), bioaccumulation factor (BAF), biomagnification factor (BMF), and trophic magnification factor (TMF) (Table 1.2).

Table 1.2 Definitions of bioaccumulation metrics. Modified from Gobas et al., (2009).

Metric	Definition	Formula	Unit of measure
Octanol–water partition coefficient (K_{OW})	Ratio of the chemical concentrations in 1-octanol (C_O) and water (C_W) in an octanol–water system that has reached a chemical equilibrium.	C_O / C_W	unitless
Bioconcentration factor (BCF)	Ratio of the steady state chemical concentrations in an aquatic water-respiring organism (C_B) and the water (C_W) in which the test organisms are exposed to a chemical in the water (but not in the diet).	C_B / C_W	L kg^{-1} ww

Bioaccumulation factor (BAF)	Ratio of the steady state chemical concentrations in an aquatic water-respiring organism (C_B) and the water (C_W) determined from field data in which sampled organisms are exposed to a chemical in the water and in their diet	C_B / C_W	L kg^{-1} ww
Biomagnification (BMF)	Ratio of the steady state chemical concentrations in a water respiring organism (C_B) and in the diet of the organism (C_D)	C_B /C_D	kg kg^{-1} ww or lw
Trophic magnification factor (TMF)	The average factor by which the normalized chemical concentration in biota of a food web increases per trophic level.	10^b	unitless

In fig. 1.4 a graphical representation of bioaccumulation metrics in a simple and linear trophic web is showed modified from Mackay et al., (2013). The graph proves that, even though BCF, BAF, BMF and TMF are often treated as independent measures each reflecting different exposure and uptake conditions, they are closely related from a mathematic point of view, with the high degree of interdependence (Mackay et al., 2013).

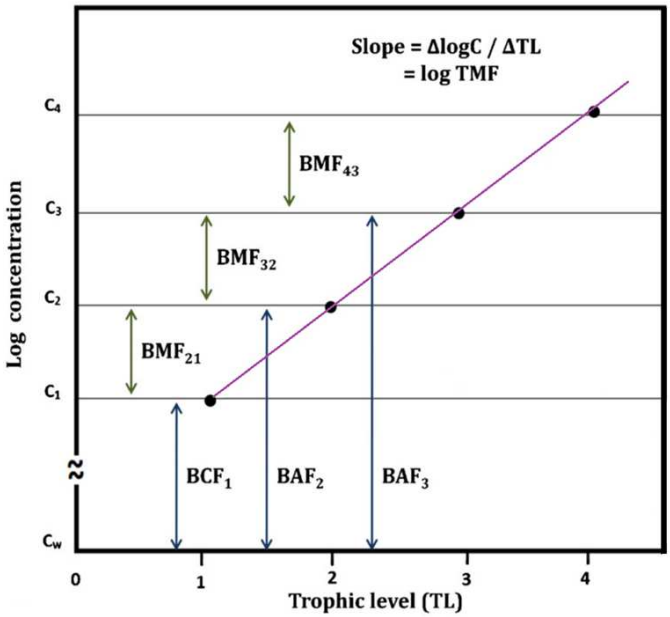

Fig. 1.4 Relationships between bioconcentration, biomagnification, bioaccumulation and trophic magnification. The round symbols are for organism in the food web. Modified from Mackay et al., (2013).

Until now, in regulatory context there is not a uniform criterion to define if a substance could be considered “bioaccumulative” or not,

and the regulation changes depending on the purpose. In Europe, for the registration of chemicals, potential bioaccumulation in aquatic organisms is evaluated based on K_{OW} of the chemicals, whose threshold value changes according to the substance considered (as example, agricultural pesticides > 3, for veterinary medicines ≥ 4, for human pharmaceuticals ≥ 4.5) (Schäfer et al., 2015). On the contrary, according to the Stockholm Convention on persistent organic chemicals (POP) a substance must be under monitoring if it presents three characteristics : first, BCF or BAF in aquatic species greater than 5000 or, in the absence of such data, log K_{OW} > 5, second, the chemical presents other reasons for concern, toxicity or ecotoxicity; third, field data in biota indicating that the potential bioaccumulation of this substance is sufficient to enlist the chemical in the Convention (Gobas et al., 2009; UNEP, 2015). The European Technical Guidance Document for Deriving Environmental Quality Standards requires that quality standards in biota (QS_{biota}) are calculated for chemicals with BCF ≥ 100 or a biomagnification factor > 1, usually determined in laboratory tests that follow OECD rules. If BCF or BMF values are not available, other indicators such as log K_{OW} ≥ 3, biota monitoring data or toxicity data of mammals and birds are required (European Union, 2011b).

1.3.1 Biomagnification metrics

Biomagnification is defined as the increase in concentration of a chemical in the tissue of consumer compared to that in prey organism

that occurs through the mechanism of dietary accumulation, in the gastrointestinal tract when food is being digested and absorbed (Conder et al., 2012). The occurrence of biomagnification through successive prey-predator relationships could results in concentrations of chemical in animals at the top of food web (e.g. birds, mammals) that greatly exceed those at the bottom, reaching levels associated with adverse effects. The metrics that explicitly account for biomagnification and describe better the scenarios presented are the biomagnification factor (BMF) and the trophic magnification factor (TMF).

TMF is a field-based metric that gives an indication about the average transfer of contaminant along the trophic web (Borgå et al., 2012; Conder et al., 2012). TMF is the antilog of the slope of the linear regression between logarithmic chemical residues in organisms and their corresponding trophic levels (TLs), calculated from stable isotope data (Fig. 1.5). The intercept of the regression may indicate inputs of the contaminant to the base of the food web (Borgå et al., 2012). For these reasons, TMF was suggested as the most conclusive tool for the assessment of contaminant bioaccumulation (Gobas et al., 2009).

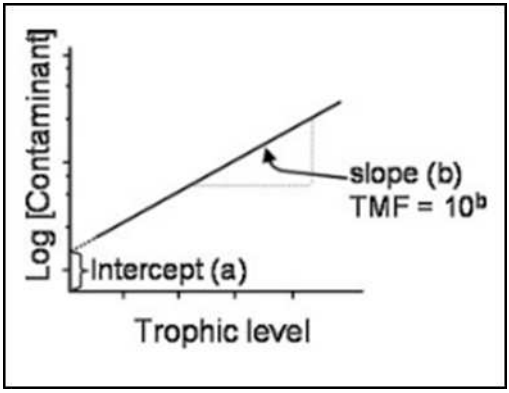

Fig. 1.5 Graphical representation of TMF, modified from Borgå et al., (2012).

The first who assessed the biomagnification of POP (dioxins and furans compounds) in the Northern Baltic Sea with an approach that considers all the food web and not only a single consumer-prey relationship were Broman and colleagues in 1992 (Broman et al., 1992). The relative trophic positions of the organisms, expressed using $\delta^{15}N$ as a proxy, were regressing against the measured contaminant concentrations to evaluate the rate of the transfer.

The method was then refined using trophic levels that are calculated by the use of a baseline to which the position of the consumer and of the enrichment factors, i.e. the increase in $\delta^{15}N$ from prey to consumer, that was called $\Delta^{15}N$ (Fisk et al., 2001), are related. Usually the baseline species are assumed to occupy a standard TL of 2.0. The equation (1) for the determination of trophic levels are:

$$TL_{consumer} = 2.0 + [(\delta^{15}N_{consumer} - \delta^{15}N_{baseline}) / \Delta^{15}N] \quad (1)$$

where TL consumer is the TL of the organism, $\delta^{15}N_{consumer}$ and $\delta^{15}N_{baseline}$ are the measured $\delta^{15}N$ data of organisms and the baseline species, and $\Delta^{15}N$ is the trophic enrichment factor that should be constant in the food web. The value of 3.4‰ for $\Delta^{15}N$ has been recommended when study a food webs without a priori knowledge of the system (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Post, 2002). But, simultaneously, the use of an average single enrichment factor introduces some uncertainties in the calculation of TMF, because the calculated TLs approximate the real ones.

There are factors that may affect the biomagnification in food webs, for example, chemical properties of contaminants, spatial and temporal variations in contaminant concentration in the ecosystem, biological aspects of biota, spatial and geographical food web characterization (Borgå et al., 2012; Franklin, 2016; Won et al., 2018). These variations increase the TMF variability, making harder the direct comparison between different ecosystems. Another important limitation for the use of TMF, also for regulatory purposes, is that the assessing of biomagnification is possible only for substances that have been quantified in environmental samples and so, commercially available for a long time.

Practical advices about how to determinate TMFs for reliable application are published by Kidd and co-authors (Kidd et al., 2019).

Contrary to TMF, BMF can be measured in laboratory and represents only a single trophic transfer. Species-specific ability to bio-transform

and eliminate chemicals can produce “false positive or negative” in BMFs values and give an erroneous indication of behaviour of the chemical in the overall food web (Hop et al., 2002). Field based BMF can be useful when data fail to meet the criteria for developing a TMF, or when the trophic food web is simple. Moreover, it can be convenient to determine whether certain combinations of individual predator–prey, with, for example, different physiological characteristics, give different indication of accumulation respect to that deduced by the TMF. However, to correctly compare TMF and BMF values, it is recommended the use of the trophic level-adjusted Biomagnification Factor (BMF_{TL}) where the concentration of the chemical is appropriately normalised and the trophic levels of consumer and diet are considered. Particularly, the ability of BMF_{TL} to accurately predict TMFs was highlighted by different studies carried out in Artic marine food web (Fisk et al., 2001; Hop et al., 2002). A significant positive correlation between BMF_{TL} and TMF was also observed by Poma et al., (2014) during the investigation of brominated flame retardants biomagnification in Lake Maggiore, a large subalpine Italian lake.

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Chapter II

Outline of the thesis

The large lakes located in the South Alpine region are among the most important freshwater resources. The central role of the lakes in history and traditions characterizing the Alpine region led to the birth of limnology and a high number of long-term studies (Salmaso et al., 2018).

In Italy, subalpine lakes provide fundamental ecosystem services; they are important economic resources as tourist destinations and for commercial and recreational fishing activities as well as they are intensively exploited for water supply in agriculture, industry and for drinking purposes.

The main aim of this Ph.D. study is to highlight the dynamics of redistribution of selected chemicals with different physico-chemical properties in trophic webs of four major Italian southern alpine deep lakes (Lake Maggiore, Lake Como, Lake Iseo, Lake Garda) and a smaller one (Lake Mergozzo). Specifically, the contaminants include DDTs and PCBs, PFASs and mercury.

The chemicals chosen in this study aims to represent a board spectrum of different typologies of pollutants (ranging, for examples, from lipophilic substances to more polar ones, from natural to man-made chemicals, from legacy compounds still present in the environment to emerging compounds that are not completely regulated and monitored) to give a most complete description of the behaviour of

pollutants in lacustrine environment. Furthermore, the selected chemicals are known to have a worldwide distribution and to have (or have had) punctual sources in our country; in particular, a chemical factory that produced DDT using mercury-cells was located in the basin of Lake Maggiore and had a great impact on this lake. Finally, these pollutants could give concerns for human well-being, especially as endocrine disruptors and, in case of mercury, for adverse effects on nervous and respiratory systems; therefore, it is important to evaluate their concentrations.

Based on this premise, three aspects have been fully evaluated in the present work. In particular:

(2.1) the determination of pollutants concentrations in biota in order to establish the sources of chemicals and delineate possible risks for humans and top predators;

(2.2) the description of the trophic interactions between organisms in order to elucidate chemicals behaviour in biota;

(2.3) the evaluation of bioaccumulation and biomagnification of chemicals based on the biological implications of lacustrine food webs.

2.1 Concentrations and sources of pollutants in subalpine lakes

In the large South Alpine (or sub/peri- alpine) lakes, zooplankton was widely investigated, mainly focusing on the community characterization and how eutrophication, oligotrophication and climatic fluctuations impacted on taxa composition and biomass

(Salmaso et al., 2018). Previous studies about chemical contamination were focused predominantly on polycyclic aromatic hydrocarbons (PAHs) and legacy compounds (e.g., DDTs, PCBs), especially in Lake Maggiore, where zooplankton has been seasonally analysed since 2008 to determine the variation in DDTs and PCBs levels (CIPAIS, 2009).

Fish are essential components of various ecosystems (i.e. sea, rivers, lakes, etc.) as well as important food source for humans (Squadrone et al., 2014). In perialpine lakes, fish communities were the object of studies focused on the ecology and distribution of fish species, their management, the impacts of large-scale meteorological factors and climate changes on population dynamics (Salmaso et al., 2018). In this biological matrix the presence of POPs and heavy metals and their bioaccumulation were investigated, especially in Lake Maggiore (Guzzella et al., 2018), but also in Lake Como (Bettinetti et al., 2016).

In this study, the determination of the concentrations of organic pollutants and mercury in zooplankton and fish was discussed in **Chapter III, IV, V and VI**, together with the comparison with old available data for the same basins and the identification of the sources of the contamination. In particular, **Chapter III** focused on DDTs, PCBs and PFASs levels in zooplankton of Lake Maggiore, Lake Como and Lake Iseo. In **Chapter IV,** the work addressed PFASs contamination in shad (*Alosa agone*), a representative species of the Italian subalpine lacustrine fish community, sampled in all selected lakes; in **Chapter V,** research focused specifically on organochlorine

compounds both in zooplankton and in two zooplanktivorous fish of Lake Como. Finally, in **Chapter VI** all organic contaminants (DDTs, PCBs, PFASs) and mercury were determined in 13 fish species and zooplankton samples of Lake Mergozzo.

PFASs contamination in shad was analysed in the context of fish monitoring within the European Union. Collected data were useful to assess both a possible human and predator health risk. The publication in **Chapter IV** addressed specifically this theme.

2.2 Interactions between organisms to understand chemicals behaviour

In aquatic food webs, zooplankton has an important role because it transfers energy and organic matter from basal producers (phytoplankton and bacteria) to higher trophic levels up to large predators (Frederiksen et al., 2006). In the trophic chain, zooplankton is also a source of contaminant exposure for predators (Bettinetti and Manca, 2013). It is important to study in detail the contamination of zooplanktonic organisms and how contaminant levels could vary (for example, by the size of organisms, their abundance or the taxa composition or by abiotic factors) (Kainz and Mazumder, 2005; Taylor et al., 1991). In **Chapter III**, zooplankton samples were seasonally collected for two years in order to identify the possible influence of certain biological and environmental parameters on contaminants concentrations in these organisms.

Stable isotope analysis of carbon and nitrogen signatures allows to understand trophic webs structure and prey-predator relationships and

also can reveal seasonal changes in $\delta^{13}C$ and $\delta^{15}N$ of zooplankton and fish, due to changes in food sources or in taxonomic composition of pelagic communities (Grey et al., 2001; Matthews and Mazumder, 2003; Post, 2002; Visconti and Manca, 2011). Therefore, when evaluating the biomagnification in an aquatic ecosystem, it is important to know the specific relationships among the different organisms of the trophic web.

Chapter V and **Chapter VI** focused on the description of trophic web structures by means of stable isotopes**.** Specifically, in **Chapter V** simple pelagic relationships between two zooplanktivorous fish and crustacean zooplankton in the four seasons in Lake Como are explained, while in **Chapter VI** the general fish feeding sources in a more complex trophic web in Lake Mergozzo are discussed.

2.3 Evaluation of biomagnification in lacustrine trophic web

To evaluate the potential degree of bioaccumulation of a substance, different types of bioaccumulation metrics and data can be used (Mackay et al., 2013). Trophic magnification factor and biomagnification factor (trophic level-adjusted) consider the diet as the major route of contaminant exposure and its bioaccumulation directly related to the trophic positions of the sampled organisms (Conder et al., 2012). The potential biomagnification could be affected by several factors, including chemical properties of contaminants, biological aspects of biota, spatial and geographical characterization of food web and trophic positions measurements of specimens (Borgå

et al., 2012).

The specific goal of the evaluation of the trophic transfer of chemicals was achieved in **Chapter V** where the estimations of the BMF_{TL} of DDTs and PCBs in two pelagic fish species were described, considering their real trophic relationships with the other organisms throughout the year. In **Chapter VI** this specific argument was addressed as well, with the calculation of TMFs for selected chemicals in the trophic web of Lake Mergozzo. Moreover, in this chapter the use of different sampling design and different type of calculation of organism trophic levels were discussed. Lake Mergozzo was chosen as "test" site to study trophic food web and the transfer of contaminants because, although it is a deep lake with distinct pelagic and littoral zones, it is smaller and easier to sample than the larger subalpine lakes.

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Chapter III

Organic Contaminants in Zooplankton of Italian Subalpine Lakes: Patterns of Distribution and Seasonal Variations

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Abstract

Zooplankton is a key node in many trophic webs, both for food that for persistent organic contaminants that can accumulate in biota. Zooplankton of different size was seasonally sampled for two years in three deep Italian subalpine lakes (Maggiore, Como, Iseo) with the aim of determining the concentrations of perfluoroalkyl substances (PFASs), DDTs, and PCBs, and assessing the seasonality impacts on contaminants concentrations. In general, Lake Maggiore showed the highest concentrations for each group of contaminants, with mean values of 7.6 ng g^{-1} ww for PFASs, 65.0 ng g^{-1} dw for DDTs, and 65.5 ng g^{-1} dw for PCBs. When considering the composition pattern, perfluorooctane sulfonate (PFOS) was detected in 96% of the samples and it was the predominant PFAS compound in all of the lakes. pp' DDE was the most detected congener among DDTs and their metabolites, while for PCBs, the prevalent group was hexa-CB that constituted 35.4% of the total PCBs contamination. A seasonal trend was highlighted for all contaminant groups with concentrations in colder months greater than in spring and summer; it was evident that the contaminant concentrations were more dependent from seasonality than from size, trophic levels, and taxa composition of zooplankton. Principal component analysis showed that one of the main drivers for the accumulation of most of the studied contaminants is their lipophilicity, except for perfluorooctanoic acid (PFOA) and octachlorobiphenyl.

3.1 Introduction

In aquatic food webs, zooplankton has an important role because it transfers energy and organic matter from basal producers (phytoplankton and bacteria) to higher trophic levels up to large predators (Frederiksen et al., 2006). In the trophic chain, zooplankton is also a source of contaminant exposure for predators but its role in processes of bioaccumulation/biomagnification is not well elucidated. Moreover, in ecotoxicological model, zooplankton was usually considered as a single functioning entity, despite the richness of taxon, sizes, trophic levels that compose this heterogeneous group. Only recently some zooplankton subsets have been the subject of specific studies (Travers et al., 2007), which showed that size fractions (e.g. mesozooplankton and macrozooplankton) differ in their taxonomic, elemental and biochemical composition (Harmelin-Vivien et al., 2019) and their contaminant concentrations could vary with the size, as described for MeHg by Kainz and Mazumder (2005). Size is not the only important variable, but it is also necessary to consider the abundance, biomass and composition of zooplankton community for a significant evaluation of contamination data (Carlotti et al., 2015). For example, Taylor et al. (1991) found a negative relationship between plankton biomass and DDTs and PCBs concentrations in Ontario lake, which is stronger for more hydrophobic compounds. Back et al. (2003) described the decrease of mercury levels (about 50-70%) during spring and summer, concurrently with the biomass increase; however, they could not discriminate whether the mercury

concentrations were diluted by the increased zooplankton or phytoplankton biomasses.

In the large sub-alpine (or perialpine) lakes, zooplankton was widely investigated, mainly focusing on the food-webs characterization and the impact of eutrophication, oligotrophication and climatic fluctuations (Salmaso et al., 2018). Previous studies regarding chemical contamination were focused predominantly on polycyclic aromatic hydrocarbons (PAHs) and legacy compounds (e.g., DDTs, PCBs), especially in Lake Maggiore where zooplankton has been seasonally analysed since 2008 to verify the variation in DDTs and PCBs levels (CIPAIS, 2009).

DDTs and PCBs are organochlorine compounds banned in many countries since 1970s and 1980s. These contaminants have been demonstrated to be persistent and bioaccumulative in the trophic chain for their chemical properties (Corsolini and Sarà, 2017). In fact, they are still widely detected in the water environment (Bettinetti et al., 2016), like per- and polyfluoroalkyl substances (PFASs) (Valsecchi et al., 2015). These substances are synthetic chemicals that are utilised in many industrial and consumer products (Buck et al., 2011); some of them (perfluorooctane sulfonate, PFOS, and related compounds) have been regulated in Annex B list of the Stockholm Convention on Persistent Organic Pollutants in 2009. The effects of PFOS and perfluorooctanoic acid (PFOA) on the structure of zooplanktonic community have been studied in laboratory microcosm (Jeong et al., 2016; Liang et al., 2017) and few studies were devoted to its role in

the trophic transfer of PFASs in freshwater food webs (Houde et al., 2008; Xu et al., 2014).

In the present study, zooplankton samples of different size were seasonally sampled for two years in three deep subalpine lakes (Maggiore, Como, Iseo) with the aim to i) determine the concentrations of DDTs, PCBs and PFASs in the zooplankton of these subalpine lakes; ii) assess the influence of some parameters of zooplankton community on contaminants concentrations (e.g. size fractions, biomass, feeding behaviour); iii) identify external variables (e.g. contaminant sources, seasonality, temperature) that could influence the zooplankton bioaccumulation in lakes.

3.2 Material and Methods

3.2.1 Study Area

The deep lakes Maggiore, Como, Iseo are located within the River Po basin in the pre-alpine area (Fig. 3.1) and constitute a large part of all freshwater Italian resources. Table 8.1.S1 reports the main characteristics of the lakes. They have similar morphological features since they have the same fluvio-glacial origin. They are classified as olo-oligomictic because they have long period of incomplete mixing during the spring and only occasional overturns after frosty and windy winters. Furthermore, the complete homogenization of their water has recently become rare and irregular because of climate change (Mosello et al., 2010).

These lakes have a remarkable environmental value and satisfy the drinking water need of towns (e.g. Como and Lecco) and villages in the provinces of Como, Lecco and Brescia, as well as the agricultural and industrial water requests in Northern Italy. Furthermore, they sustain significant local economic activities, such as tourism and fishery.

Lake Como, the deepest Italian lake, is characterized by an "upside-down Y" shape (Fig. 3.1); in the southern part a bathymetric ridge separates two branches: the deep western branch, with no outflow and a longer water renewal time, and the more open eastern branch with an emissary and more regular bathymetry. Lake Iseo is the fourth largest Italian lake that is fed by waters coming from the Valcamonica Valley. The shoreline area is due to undergoing sewage treatment by two treatment plants that are located at the northern and southern ends of lake (Garibaldi et al., 1999). Lake Maggiore, the second largest and deepest Italian lake, is divided between Italy (Piedmont and Lombardy Regions) and Switzerland (Canton Ticino). Most of population and the main industrial activities are in the southern part (Mosello et al., 2001). Until 1990s a chemical factory producing technical DDT and using a mercury-cell chloralkali plant discharged wastewaters into the Toce River which carried pollutants to the lake, where DDTs and Hg accumulated in sediment and biota causing an important contamination (Bettinetti et al., 2012b).

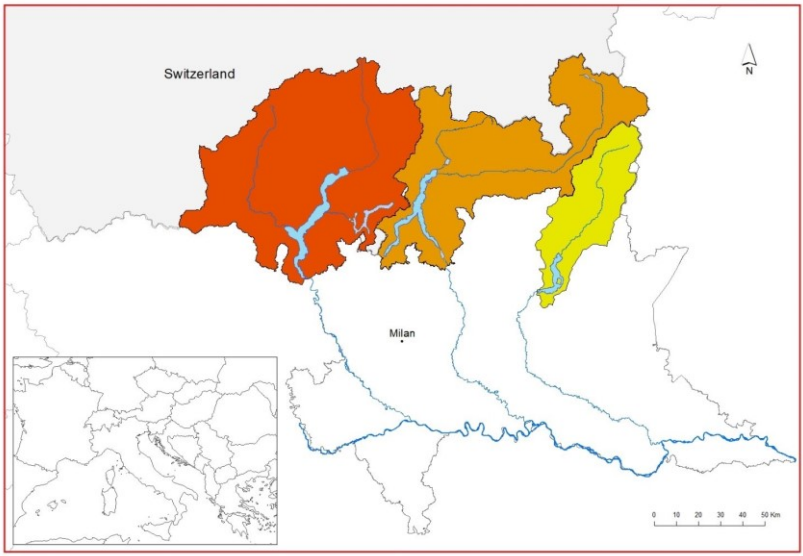

Fig. 3.1 Study area. From west to east: Lake Maggiore (red area), Lake Como (orange) and Lake Iseo (yellow) basins. The map is obtained with ArcGIS® software.

3.2.2 Sampling and Determination of Biomass

Crustacean pelagic zooplankton samples were seasonally collected for two consecutive years (2016-2018) and they were analysed together with some samples archived from previous years, as described in Table 8.1.S2. The samples were vertically caught from a boat in the middle of the lake using nylon nets with mesh of 200, 450 and 850 µm to 20 m of depth. Those sizes were chosen in order to avoid large phytoplankton colonies and rotifer taxa, while the depth was chosen considering the average transparency of lakes and where phytoplankton lives in order to collect most of the crustacean

zooplankton community. Every sampling was repeated until we got sufficient biomass for analysis.

The collected zooplankton included Copepoda (Cyclopoida and Calanoida) and Cladocera (*Daphnia longispina* group, *Eubosmina coregoni*, *Diaphanosoma brachyurum*, *Leptodora kindtii* and *Bythotrephes longimanus*).

Density and biomass were calculated using optical microscope at 40x and equations of length–weight regression for samples of Lake Como and Lake Maggiore (Manca and Comoli, 2000).

For chemical analysis two aliquots of each sample were filtered on 2 µm pore glass-fibre filters (GF/C, 4.7 cm of diameter, Whatman, Maidstone, UK) and then frozen at -20°C.

3.2.3 Chemical Analysis

3.2.3.1 PFASs

For the analysis of zooplankton, about 5 g of wet weight sample was weighed and then spiked with 100 µL of 40 µg L^{-1} stable isotope-labelled solution used as internal standard. SIL-IS was prepared from a mixture of mass-labelled MPFAC-MXA and mass-labelled M3PFPeA solutions purchased from Wellington Laboratories Inc., (Guelph, Ontario, Canada). The extraction method for PFASs is described extensively in Mazzoni et al. (2016). Briefly, a mixture of water and acetonitrile (10:90 v/v) and few µL of formic acid were added to the spiked samples. The samples were subjected to ultra-sonication extraction, centrifugation and a treatment with

$MgSO_4$/NaCl. Afterwards, the extracts were partially evaporated and filtered by HybridSPE®-Phospholipid Ultra cartridges. The final extracts were analysed by liquid chromatography coupled to mass spectrometry (UHPLC – MS/MS) after an online purification with turbulent flow chromatography (TFC).

External standard solutions with increasing concentrations were prepared by diluting PFAC-24PAR Standard Solution (Wellington Laboratories Inc., Guelph, ON, Canada) containing certified native PFASs in acetonitrile to obtain the calibration curve. The obtained solutions were acidified with 50 µL of concentrated formic acid and then spiked with 100 µL of SIL-IS. Limit of detection (LOD) and limits of quantification (LOQ) were estimated, according to the ISO Standard 6107-2:2006, as respectively, threefold and tenfold the standard deviation of an extract of biological tissue fortified at 1 µg L^{-1}, as described in (Mazzoni et al., 2016).

Details on the analyte names, abbreviations and corresponding SIL-IS are reported in Table 8.1.S3. A full list of chemicals and solvent is provided in the Supporting Information.

3.2.3.2 Organochlorine Compounds

Organochlorine compounds (OC) were analysed following the method described in Bettinetti et al. (2012b). Briefly, each sample of zooplankton was freeze-dried, about 0.5 g were put into a glass fibre thimble (19 mm I.D., 90 mm length, Whatman, Maidstone, UK) and then extracted in a modified Soxhlet equipment (ECO 6

Thermoreactor, Velp Scientifica, Usmate, Italy) for two hours with a n-hexane and acetone (1:1) mixture (pesticide analysis grade, Carlo Erba Reagents s.r.l, Cornaredo, Italy). The lipid content was gravimetrically determined, then the extract was digested with 2 mL of H_2SO_4 (98%, Carlo Erba Reagents s.r.l, Cornaredo, Italy) all night long. The supernatant was cleaned up on a Florisil® column (40×7 mm I.D.), eluted by 25 mL of 85:15 mixture n-hexane and dichloromethane (pesticide analysis grade, Carlo Erba Reagents s.r.l, Cornaredo, Italy) and finally, concentrated to 0.5 mL. The analysis was carried out by gas chromatography (GC Top 8000, Carlo Erba Instruments, Rodano, Italy) that was equipped with an on-column injection system (injected volume: 1 µL), a WCOT fused silica CP-Sil-8 CB column (50 m×0.25 mm I.D., film thickness 0.25 µm, Varian Inc., Palo Alto, CA, USA) and a ^{63}Ni electron capture detector (ECD 80, Carlo Erba Instruments, Rodano, Italy).

The external standards Custom Pesticide Mix (o2si, USA), Custom PCB Calibration Mix (o2si, USA) and Aroclor 1260 (Alltech, Nicholasville, KY, USA) were used for DDT and PCB quantitation. The solution of DDT homologues contained pp'DDT, op'DDT, pp'DDD, op'DDD, op'DDE and pp'DDE while the analysed PCBs congeners were: PCB 18, 28+31, 44, 52, 101, 118, 149, 138, 153, 170, 180, 194 and 209. LOD for zooplankton is 0,1 ng g−1 dry weight.

Routinely, standards reference materials SRM NIST-1947 "Lake Michigan Fish Tissue" and NIST-1946, "Lake Superior Fish Tissue" were analysed in triplicate in order to test good laboratory practices,

respectively for DDT homologues and PCB residues. The percentage recoveries of DDTs were between 106.2 ± 3% and 107.5 ± 4%, while those for PCBs ranged between 91.3% (±1.1%) and 102.2% (±1.6%).

3.2.4 Data Analysis

3.2.4.1 Statistical Analysis

Statistical analysis was performed by R software (R version 3.5.1). For ANOVA analysis, the significance level was set at p-value <0.05. The data were not normally distributed, so they were log-transformed before the analysis. After every analysis we checked the distribution of residual, according to R package. Principal Component Analysis (PCA) was chosen to describe the internal structure of the data explaining the variance of contaminant concentrations in the dataset. Analysis were performed using FactoMineR, and factoextra R-packages.

3.2.4.2 Spatial Analysis

Geometry of basins were obtained from geographical hydrological portal of ARPA-Lombardy and geoportal geo.admin.ch of Swiss Confederation (ARPA Lombardia; geo.admin.ch). Available spatial data about anthropic pressures were selected for each basin through ArcGIS software (ArcGIS version 10.3.1). In order to describe the study area, the degree of urbanisation (DEGURBA), that classifies local administrative units in three classes, was used. The classes are: densely populated area or cities/large urban area (class 1), intermediate area or towns and sub-urbs/small urban area (class 2) and

thinly populated area or rural area (class 3) (European Commission, 2014). For each basin the percentage of area occupied by each class was estimated. We collected also spatial data for wastewater treatment plants (WWTPs) and their dimensions (population equivalent), populations and municipalities and basin area (Istat; Ministero dell'Ambiente e della Tutela del Territorio e del Mare; Regione Lombardia; Regione Piemonte).

3.3 Results and Discussion

3.3.1 Concentrations of Organic Contaminants

Figure 3.2 shows the concentrations of each contaminant group in the different lakes. The results are expressed as sum of 12 congeners of PFASs (ng g^{-1} ww), sum of the three congeners and three respective metabolites of DDTs (ng g^{-1} dw) and sum of 14 congeners of PCBs (ng g^{-1} dw) in zooplankton samples. Detailed data regarding contaminants concentrations are reported in Table 8.1.S4-S6.

Differences between lakes were statistically significant (Anova one-way, *p-value* <0.001 for all compounds; N=51 for PFASs, N=72 for OCs). In detail, PFASs concentrations in zooplankton from Lake Iseo were lower than those in Lake Como and Maggiore (Tukey test, *p-value* <0.001) which showed no differences between them; DDTs concentrations in Lake Maggiore were significantly higher than in the other lakes (Tukey test *p-value* <0.001) and PCBs levels in zooplankton in Lake Como were lower than in samples collected in Lake Maggiore and Iseo (Tukey test *p-value* <0.001).

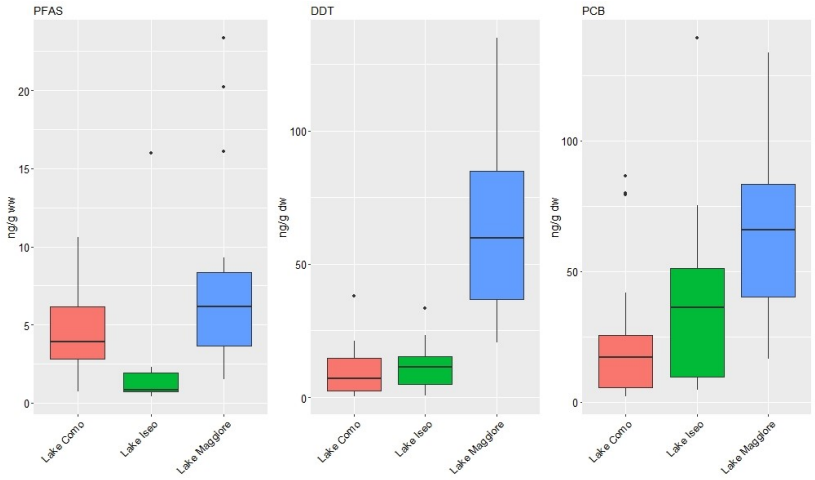

Fig. 3.2 Total concentrations of PFASs (ng g^{-1} ww) and DDTs and PCBs (ng g^{-1} dw) in zooplankton collected from lakes Como, Iseo, Maggiore.

In general, Lake Maggiore showed the highest concentrations for each group of contaminants with mean values of 7.6 ng g^{-1} ww for PFASs, 65.0 ng g^{-1} dw for DDTs and 65.5 ng g^{-1} dw for PCBs. High levels of DDT and its metabolites in this lake are due to the presence of a point source from a chemical plant located on the River Toce, an important tributary of Lake Maggiore, as already explained in the section "Study Area". The factory produced technical DDT from 1948 to 1996, but the contamination is still present because accumulated into the soils around the industrial area (Bettinetti et al., 2012b).

There are not factories that produce PFASs in this area, but lakes are subjected to effluents of both industrial and urban wastewater treatment plants (WWTPs) and to diffuse pollution from atmospheric

deposition. PFASs are not removed in standard treatments of wastewater and enter in water bodies (Loos et al., 2013). The basin of Lake Maggiore is characterized by the most extended area, the highest number of inhabitants and the highest percentage of densely populated area (2%) among the basins of the studied lakes in accord to the European report on degree of urbanization (European Commission, 2014) (Table 3.1). The same ranking of PFASs contamination has been highlighted in fish sampled in the same areas (Mazzoni et al., 2019), and in that work the source of PFASs for Lake Maggiore was hypothesised to be Lake Lugano, that belongs to Lake Maggiore basin. Lake Iseo, which collects the waters of the smallest basin with the lowest population and number of WWTPs, showed the lowest PFASs concentrations among the studied lakes (mean value: 3.2 ± 5.7 ng g^{-1} ww).

It is more difficult to address the differences in PCBs in zooplankton because the contamination is very old, and no point sources can be identified in the lake basins. In fact, the differences in concentrations cannot be directly related to the basin areas or the inhabitant number. Nonetheless, Lake Iseo has significantly higher mean concentration of total PCBs than Lake Como (40.6 ± 40.1 and 20.9 ± 21.1 ng g^{-1} dw, respectively), and this result could be linked to the great exploitation of hydroelectric power plants in Valcamonica during the economic development after the second World War, which largely used PCBs as dielectric fluid in transformers.

Regarding Italian subalpine lakes, this is the first study of PFASs contamination in zooplankton, while data of DDTs and PCBs are abundant for Lake Maggiore (Guzzella et al., 2018), but sporadic for the other lakes. The last determination of DDTs and PCBs concentrations in zooplankton in Lake Iseo, dating back to 2010, showed that current DDTs concentrations are lower while PCBs concentrations are stable (Bettinetti et al., 2012a). In Lake Como the comparison with older data showed a decrease in the concentrations of both organo-halogenated compounds (Bettinetti et al., 2016; Mazzoni et al., 2018), but there are not enough data to claim a significant decreasing trend.

Concentrations of PFASs in pelagic invertebrates in this study are higher than those reported in Baltic Sea (Gebbink et al., 2016), where the sum of PFSAs and PFCAs in zooplankton were only 0.11 ±0.02 and 0.12 ±0.01 ng g^{-1} ww respectively. On the contrary, they are comparable with the concentrations measured in the Gironde estuary (France) (Munoz et al., 2017) and in the Arctic Canadian Lakes that are not contaminated by local airport (Lescord et al., 2015).

Table 3.1 Information about the degree of urbanization (DEGURBA), wastewater treatment plants (WWTPs) and population in basins of Lake Maggiore, Como, Iseo.

Lake Basin	DEGURBA		WWTPs		Administrative Data		
	Class	Area %	Total WWTPs	Total population equivalent	Total municipalities	Population (2011)	Area (km^2)
Maggiore	1	2.01	ND	ND	207	923861	6815.6
	2	21.4					
	3	76.6					
Como	1	1.6	143	759461	191	556769	4611.6
	2	18.8					
	3	79.5					
Iseo	1	0.0	69	191709	73	191527	1842.5
	2	24.2					
	3	75.8					

3.3.2 Pattern of Contamination

When considering the composition pattern of PFASs accumulated in zooplankton (Fig. 3.3), PFOS was detected in 96% of the samples and was the predominant compound in all lakes, reaching the maximum concentration of 18.9 ng g^{-1} ww in Lake Maggiore. It represented 32% of total PFASs concentrations in zooplankton in Lake Iseo, 52% in Lake Como and 67% in Lake Maggiore. The other two perfluoroalkyl sulfonic acids detected (PFBS, PFHxS) were determined in significant concentrations only in Lake Iseo. Regarding perfluoroalkyl carboxylic acids (PFCAs), long-chain compounds (C>9) predominated in zooplankton, while short-chain compounds (with 6-7 carbon atoms) were detected only in few samples (about 10%) and at lower concentrations (maximum value: 0.9 ng g^{-1} ww). PFOA was detected in about 65% of samples, but it represented only 11.5% of the total PFASs concentration in Lake Iseo, and about 6% in the other two lakes.

Concentrations of PFTrDA (C13) and PFTeDA (C14) were lower than those of other long-chain PFCAs, probably because these compounds have higher affinity for particles and sediment is their main sink (Loi et al., 2011).

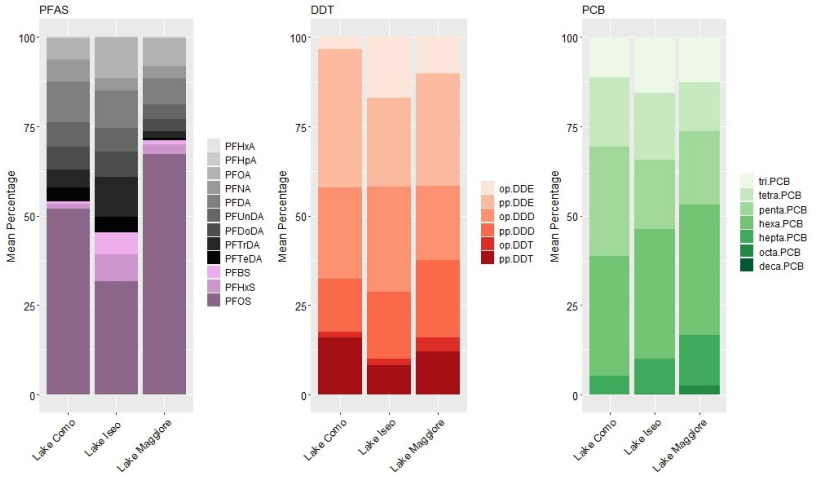

Fig. 3.3 Composition pattern of contaminants in zooplankton samples.

We analysed the whole dataset of individual congeners of PFASs by a Principal Component Analysis (PCA) (Fig. 3.4). Loading plot on the first two components, which globally explain 54% of the total variance, helps to identify common behaviours among the individual PFASs congeners (Fig. 3.4). Three different groups are gathered in the loading plot: PFOA, PFBS and PFHxSthe compose the first, which is maximum on the second component and orthogonal to the first one. PFOA shows K_{OW} similar to PFHxS (Khairy et al., 2019), and this group of compounds was higher in samples of Lake Iseo than in the other lakes, suggesting a specific contamination source for this lake. The second group is formed by PFTrDA (C13) and PFTeDA (C14) and it is orthogonal to the first component and parallel to the second one, but in the negative direction. The third group shows PFOS (C8) laying in the same direction of the other long-chain PFCAs ($8 < C <$

13); it is rather orthogonal to the other two groups and includes the most bioaccumulable and biomagnificable PFASs congeners. In fact, PFBS and PFHxS are the most soluble congeners and PFTrDA (C13) and PFTeDA (C14) are not readily bioavailable because of their molecular size (Conder et al., 2008; Martin et al., 2003). The coefficients in the second eigenvector are correlated with K_{OW} of PFASs substances (Khairy et al., 2019), except for PFOA which is absolutely uncorrelated (Fig. 8.1.S2), suggesting that lipophilicity cannot be used to model bioaccumulation of PFOA in zooplankton.

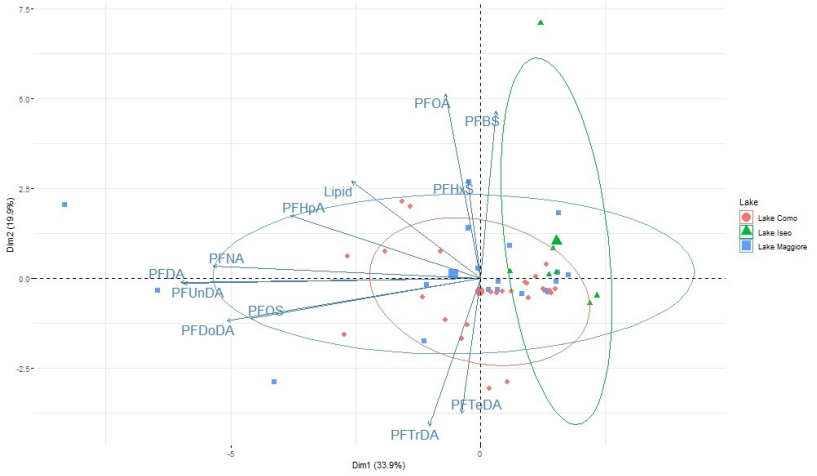

Fig. 3.4 PCA biplot characterizing individual PFAS in zooplankton samples. Studied variables are mapped with arrows, samples shape is showed in legend. Percentages in brackets refer to the proportion of variance explained by the different axes.

Looking at the bidimensional score plot, Lake Como and Lake Maggiore samples cannot be distinguished, while Lake Iseo data are better described by the second component where PFOA, PFBS and PFHxS loadings predominate.

In the group of DDTs compounds, metabolites of DDT and their isomers were predominant over the parental compounds (op' DDT and pp' DDT). Technical DDT products generally contained about 75% of pp' DDT, 15% op' DDT and other compounds in very small amounts. DDT isomers are known to degrade into DDE and DDD under aerobic and anaerobic conditions. Therefore, the increase of the percentage of DDE and/or DDD and a >1 ratio DDE/DDT indicate that there are not new inputs to the environment (Kim et al., 2002). DDE represented more than 40% of the total concentrations in all lakes and its ratios with DDT were 2.4, 5.3 and 2.3 for Lake Maggiore, Lake Iseo and Lake Como respectively, suggesting that the contamination is old and no recent inputs of parental compound occurred (Fig. 3.3).

pp' DDE was the main compound detected in zooplankton and it was measured in all samples with concentrations that ranged from 0.3 ng g^{-1} dw in Lake Como to 38.3 ng g^{-1} dw in Lake Maggiore (Table 8.1.S5).

PCB 153 was the congener with the highest frequency of detection (>94%), followed by PCB 101 (91.5%), PCB 44, PCB 180 and PCB 138 (all up to 70% of total samples). In Lake Maggiore PCB 153 was the congener with the highest concentrations (11.0 ± 8 ng g^{-1} dw),

while PCB 149 prevailed in Lake Como (6.6 ± 12 ng g^{-1} dw) and PCB 52 in Lake Iseo (11.0 ±18 ng g^{-1} dw). When we grouped PCBs congeners in seven classes based on their number of chlorine atoms, concentrations raised with the increase of number of chlorine atoms until the hexachlorobiphenyl (hexa-CB) group, then tended to decrease (Fig. 3.3). Accordingly, the prevalent group was hexa-PCB which constituted 35.4% of total PCBs concentration, reaching a maximum of 60.5 ng g^{-1} dw in Lake Maggiore. The pattern of PCBs congeners probably reflected the Aroclor mixtures (Aroclor 1256 e 1260) most used in the past in Italy (Binelli and Provini, 2003).

While examining loading plot in the PCA of PCBs and DDTs compounds, gathered in isomer groups (Fig. 3.5), we can see that the coefficients of PCBs and DDTs isomer groups in the second component are significantly correlated with K_{OW} (Fig. 8.1.S2), except for octachlorobiphenyl (octa-CB). The peculiar octa-CB behaviour cannot be easily explained but it could be related to the fact that octa-PCBs were determined only in Lake Maggiore. As in the case of PFASs, the second component is related to the contaminant hydrophobicity and explains 12% of the total variance. Nevertheless, it should be noted that the slope of the correlation between coefficients in the second eigenvector and K_{OW} of PCBs and DDTs is five-times higher than that interpolated for PFASs (Figure 8.1.S2).

The score plot shows that data from different lakes cannot be distinguished, but Lake Maggiore has the highest variability while the lowest one is showed by Lake Como, as also evident in Fig. 3.2.

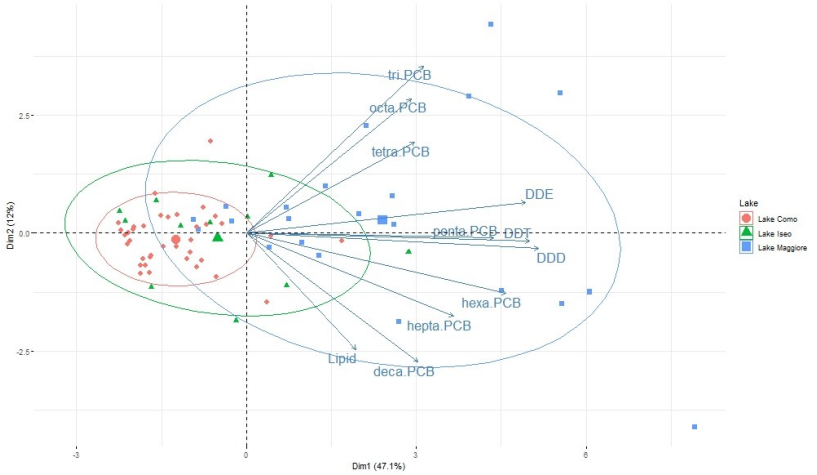

Fig. 3.5 PCA biplot characterizing isomer groups of DDTs and PCBs in zooplankton samples. Studied variables are mapped with arrows, samples shape is showed in legend. Percentages in brackets refer to the proportion of variance explained by the different axes.

3.3.3 Role of zooplankton size and seasonality on contaminant levels

Data that were collected in this study allowed for in-depth insight of the role of zooplankton ecology in the contaminant accumulation. Zooplankton has been sampled in different size fractions in order to separate species that are characterized by different trophic levels. Details on size fractions collected in the different lakes can be found in Table 8.1.S2 and Table 8.1.S7 reports data on biomass and taxa composition.

The smallest and intermediate fractions (≥200 and ≥450 μm) included all crustacean species living in the lakes but had different total biomass, because, in the former we could collect also the smallest and

youngest specimens, having a more complete picture of the zooplankton community. The greatest size fraction (≥ 850 μm) contained mainly the biggest individuals of Cladocera (generally *Daphnia* for primary consumers and predators).

We only analysed zooplankton data from Lake Como and Lake Maggiore, because, for Lake Iseo, there were enough data for the lowest size fraction (200 μm), but the total sampled biomass for the other two fractions was insufficient to carry out all the chemical analyses. PFASs data have been analysed as a whole dataset since we have shown that there are no statistically significant differences for PFASs data (Fig. 3.2), while for DDTs and PCBs the datasets have been analysed separately for each lake (Fig. 3.2).

No significant differences were observed between zooplankton size fractions for all contaminants (Fig. 8.1.S1). According to a biomagnification hypothesis, the biggest fraction, which contains more predators than filter-feeder or herbivores crustaceans, should be the preferred fraction for contaminant accumulation. On the contrary, our results showed that the biggest fraction had no statistically significant differences with the others, and in Lake Maggiore the 850 μm-fraction was clearly less contaminated than the 450 μm-one for DDTs and PCBs. Piscia et al. (2019) suggested that in the smaller fractions there were more copepods, richer in lipids than Cladocera species, and therefore, more available to bioaccumulate organic contaminants. Principal Component Analysis of taxonomic compositions and contaminant concentrations, expressed as total

concentrations of each chemical family, (Fig. 3.6) showed that chemical concentrations were orthogonal to (i.e. independent from) the taxa of planktonic organisms, but inversely correlated with the total zooplankton biomass and temperature. The score plot showed that the colder seasons (autumn and winter) positively correlated with all the contaminant concentrations in zooplankton.

This result is partially confirmed by comparing concentrations in the different seasons (Figure 3.7), which shows a similar qualitative trend for all compounds: the concentrations were higher in colder months than in spring and summer, with a characteristic U-shape from winter to autumn.

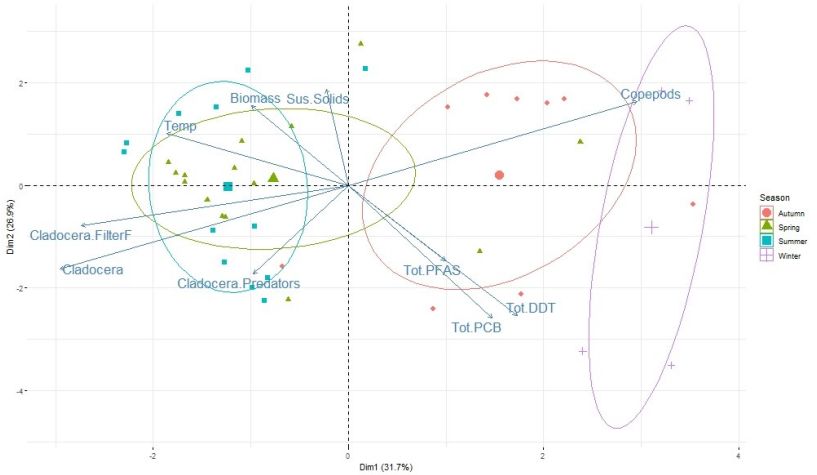

Fig. 3.6 PCA biplot characterizing contaminants and biomass of zooplankton samples. Variables are mapped with arrows; samples shape are showed in legend. Percentages in brackets refer to the proportion of variance explained by the different axes.

For PFASs these differences were not statistically significant, while winter DDTs concentrations in Lake Como were significantly higher than spring ones (*p-value* <0.05, Anova and Tukey tests), and, in Lake Maggiore there were significant differences between winter and both warmer seasons and between autumn and summer. PCBs followed the same trend as DDTs and both lakes showed significant differences between seasons: in Lake Como (*p-value* <0.01) there were significant differences between winter and both warmer seasons and between autumn and spring; in Lake Maggiore (*p-value* <0.05) there were significant differences between summer and colder seasons. No interaction between the considered variables (size and seasons) was evidenced by two-way-Anova test.

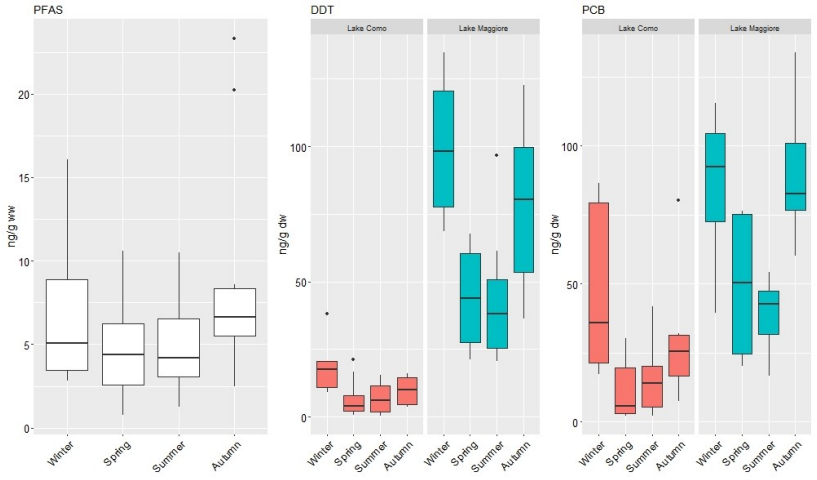

Fig. 3.7 Contaminants trend between seasons.

The characteristic U-shaped trend of DDTs and PCBs concentrations in Lake Maggiore was observed since the beginning of the monitoring activities and did not vary between years (Guzzella et al., 2018). The inverse relationship between concentrations in zooplankton and zooplankton biomass might be associated with the shift in diet of zooplanktonic specimens because of the different availability of nutrient along the year. Changes in resource availability and environmental conditions (the decrease of food availability or the increase of metabolic costs) can lead to changes in trophic interactions (Calizza et al., 2012). For example, $\delta^{15}N‰$ of all zooplanktonic species changed along the years, increasing in the cold seasons as showed in Mazzoni et al. (2018). During spring and summer, phytoplankton is easily available and filter feeders rely on this food source, while, during autumn and winter they need to eat also bacteria, protozoa or organic particles to obtain enough energy to live. Additionally, Campbell et al. (2000) observed that organisms which live in cold water from glaciers in an unproductive environment and low nutrients, often become richer in lipid and OC content, indicating that nutrient limitation at the base of the food web can affect the uptake of contaminants at higher trophic levels.

The differences of concentrations throughout the year may be also explained by “the biomass dilution effect”, proposed by Taylor et al. (1991), who observed that DDTs and PCBs concentrations varied across lakes according to an inverse relationship with their planktonic biomass. The same effect, as observed for polycyclic aromatic

hydrocarbons in plankton of the Mediterranean and Black Seas, was explained by a reduction of water concentrations by adsorption on dissolved organic matter and suspended sediments which peak during summer algal bloom (Berrojalbiz et al., 2011).

In Italian lakes, which were studied in the present work, the seasonal trend was much stronger for chlorinated compounds than for PFASs. Variations in PFASs remained quite limited as in the Gironde estuary, where PFASs varied only up to a factor of 2.5x for zooplankton and 2.3x for shrimps in different seasons (Munoz et al., 2019).

3.4 Conclusions

The biannual campaign of monitoring of persistent organic compounds (PCBs, DDTs, and PFASs) in zooplankton of the Italian subalpine lakes allowed for inferring some conclusions on the relationships among zooplankton ecology, physico-chemical characteristics of the compounds, and bioaccumulation. It was evident that the contaminant concentrations depend on seasonality more than on size, trophic levels, taxa composition, and feeding behaviour of zooplankton. This evidence might indicate that the contaminants are mainly accumulated from water, with a minor contribution from the diet. The good correlation between log K_{OW} and eigenvector coefficients in Principal Component Analysis (Figure 8.1.S2) showed that a significant driver for the accumulation of most of the studied contaminants is their lipophilicity, except for PFOA and octa-CB.

Analysis of zooplankton, as bulk, could be considered a practical alternative for monitoring purposes using a size mesh that collects more biomass (such as e.g., 200 or 450 μm), both for the description of community composition and for analytical determinations, since the determination of the studied compounds in lake water is often difficult due to their low concentrations and the need for high volume concentrations. Moreover, we suggest sampling during winter or late autumn, when the concentrations are higher, even if the collection of sufficient biomass could require more catches.

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Chapter IV

Perfluoroalkyl acids in fish of Italian deep lakes: Environmental and human risk assessment

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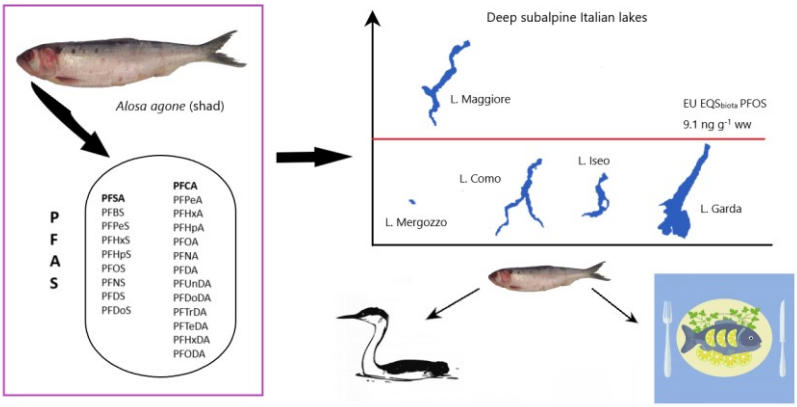

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Abstract

Determination of 20 PFASs in a fish species (*Alosa agone*) of commercial interest has been carried out in five Italian subalpine lakes to assess the risk for humans and predators for fish consumption. PFOS still presents the highest concentrations (0.9-16.6 ng g^{-1} ww) among the analysed PFASs, in spite of its normative restrictions. PFOS concentrations measured in all lakes, except in Lake Maggiore, are homogeneous with an average of 3.1 ± 1.9 ng g^{-1} ww, which could be considered the "anthropogenic background concentration" of PFOS in fish of lakes located in an industrialised and urbanised region but without point sources. In Lake Maggiore, fish concentrations always exceed the EU EQS_{biota} (9.1 ng g^{-1} ww) based on human fish consumption. Considering the effective consumption of fish in this area, an actual risk for fish consumption by humans is not evidenced, while a moderate risk of secondary poisoning for predators is highlighted. PFOA has been detected in significant concentration only in one sample in Lake Maggiore, while long chain PFCAs have been detected without significant differences among the lakes (0.3 to 2.7 ng g^{-1} ww). The present study demonstrates that biota monitoring of fish can be used as a valuable tool to classify the quality status of water bodies regarding bioaccumulative PFAAs, even if the water concentrations are close to the reachable detection limits.

4.1 Introduction

Per- and polyfluoroalkyl substances (PFASs) are synthetic chemicals applied in many industrial processes and consumer products for their exceptional thermal and chemical stability, which makes them unique (Buck et al., 2011). PFASs are ubiquitous in the environment and globally detected in human blood and all environmental matrices, such as groundwater, surface water, sediments, soils, wastewater effluents and biota (Ahrens and Bundschuh, 2014; Castiglioni et al., 2015; Houde et al., 2011; Lu et al., 2018; Stahl et al., 2014; Valsecchi et al., 2015). PFASs include thousands of chemicals but the environmental studies have been concentrated mainly on perfluoroalkyl acids (PFAAs), such as perfluorooctanesulfonic acid (PFOS), and perfluorooctanoic acid (PFOA). PFOS and PFOA have been demonstrated to be persistent in the environment and bioaccumulative in trophic food chains (Conder et al., 2008), raising concern about the risks for the end consumers, including humans and top predators, even in remote areas (Lau et al., 2007).

PFOS meets the criteria for being included as a persistent organic pollutant (POP) in the Annex B list of the Stockholm Convention on Persistent Organic Pollutants in 2009. Restrictions on the marketing and use of PFOS and its derivatives were stated in 2006 by Directive 2006/122/UE and in 2011 by EU Regulation No 207/2011. As concerns the protection and improvement of the quality of the aquatic environment, after a risk assessment study (European Union, 2011a), the European Commission included PFOS in the list of priority

hazardous substances which must be monitored in all EU water bodies. EU Water Framework Directive (WFD) (Directive 2000/60/EC) claims biota monitoring as an alternative to the measurement of bioaccumulable substances in water. According to the Technical Guidance for EQS derivation (TGD) (European Union, 2011b), two different standards for biota must be derived, i.e., one for the protection of top predators from secondary poisoning ($QS_{biota,\ sec\ pois}$), and the second for the protection of human health from consuming fisheries products ($QS_{biota,\ hh}$); finally the lower one has to be adopted as the overall environmental quality standard for biota (EQS_{biota}). In the case of PFOS, the $QS_{biota,\ hh}$ of 9.1 ng g^{-1} ww in fish, derived from EFSA tolerable daily intake (TDI) (European Food Safety Authority, 2008), has been adopted as overall EQS_{biota} (Directive 2013/39/EU), which should be compared with annual average of concentrations in fish tissues for water body classification. PFAAs have been widely found in fish not only in freshwater ecosystems in Europe (Berger et al., 2009; Labadie and Chevreuil, 2011), but also in other parts of the worlds, e.g. in South Africa (Bangma et al., 2017) and Asia (Lam et al., 2014). Data on biota monitoring were collected and reviewed by Houde et al. (2011), who concluded that PFOS concentrations were decreasing but PFOS was still the predominant PFAAs detected, in addition to increasing concentrations of long-chain perfluorocarboxylic acids (PFCAs).

Sources of PFAAs for human population exposure are multiple, including drinking water; dietary intake (Schwanz et al., 2016);

furniture, carpets and other consumer products (e.g. food packaging, cleaning agents, textiles) that can release PFASs, resulting in wide occurrence of these compounds in indoor dust and air (Jian et al., 2018; Karásková et al., 2016). Diet has been suggested as the main route of exposure to PFAAs in the human population, with fish and other seafood being considered the major contributors (Ahrens et al., 2016). The European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain set a tolerable daily intake (TDI) of 150 ng kg^{-1} body weight (bw) per day for PFOS and 1500 ng kg^{-1} bw per day for PFOA (European Food Safety Authority, 2008). Considering a person of 70 kg, the threshold dose is 105 µg per day for PFOA and 10.5 µg per day for PFOS, based on daily per capita consumption (Estimated Daily Intake, EDI) of 115 grams of fish (European Union, 2011a). Applying the following equation (1),

$$QS_{biota,hh}\,[ng\ g^{-1}] = \frac{0.1 \times TDI\,[ng\ kg^{-1}\ d^{-1}] \times 70\ [kg]}{EDI\,[g\ d^{-1}]} \qquad (1)$$

biota quality standards for human fish consumption ($QS_{biota,hh}$) for PFOS and PFOA of 9.1 and 91 ng g^{-1} ww, respectively, are obtained. Exposure scenarios and the consequent EDIs for PFAAs are based on limited available monitoring data on food which allow only for a limited exposure assessment. EFSA recommended upgrading the database, improving measurements and monitoring of PFAAs in food in order to improve the accuracy of the chronic dietary exposure risk to the European populations (European Food Safety Authority, 2012). Therefore, high-sensitivity analytical methods that increase the quantified data are required, improving the reliability of the exposure

assessments of PFAAs contamination (Chiesa et al., 2018; Valsecchi et al., 2013).

The aim of the present research is the determination of the PFAAs contamination in a fish species representative of the Italian subalpine lacustrine fish communities, in the context of fish monitoring within the European Union. Monitoring data are used to assess both a possible human health risk due to the consumption of fish and the secondary poisoning of predators.

4.2 Material and methods

4.2.1 Study area

Located in the pre-alpine area within the River Po basin, the deep lakes Maggiore, Como, Iseo and Garda form the subalpine Italian lacustrine district (Fig. 4.1) and constitute about 70% of all freshwater Italian resources. They share the same origin (in fact, they occupy valleys dug by rivers and remodelled by the action of glaciers), so they have some morphological common features: they are narrow, elongated in a north-south direction, delimited by steep shores and their current backdrop is generally flat and in crypto-depression (Mosello et al., 2010). Lake Mergozzo is a deep lake despite its small area. It is located near Lake Maggiore from which it originated about five centuries ago, due to the accumulation of sediments carried out by Toce river (Fig. 4.1). The characteristics of the lakes are reported in Table 8.2.S1.

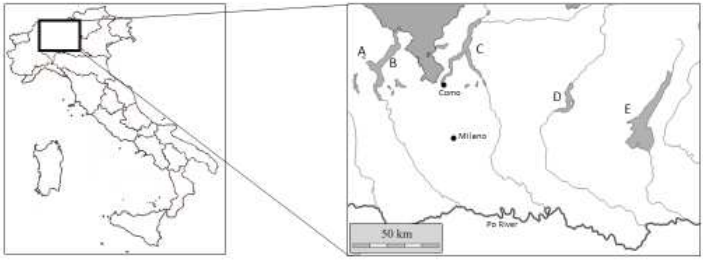

Fig 4.1 Study area. A: Lake Mergozzo; B: Lake Maggiore; C: Lake Como, D: Lake Iseo, E: Lake Garda.

The deep subalpine lakes should belong to the warm monomictic type; however, their depth is such that the complete homogenization is not frequent, showing long periods of incomplete spring mixing, interrupted by occasional and irregular complete overturns after harsh and windy winters, hence they are classified as olo-oligomictic lakes. In recent years, following climate changes, the complete homogenization of their waters seems destined to become rare and irregular (Mosello et al., 2010).

Lake Como is characterized by an "upside-down Y" shape (Fig. 4.1); in the southern part a bathymetric ridge separates two branches: the deep western branch (Como branch) and the more open eastern branch (Lecco branch). Como branch has no outflow and its deep basin is hydrologically closed determining a longer water renewal time. In contrast, the Lecco branch has an emissary, is shallower and has a more regular bathymetry (Fanetti et al., 2008). In this study we consider the two branches separately.

All lakes represent an important water source for drinking, agricultural and industrial purposes in Northern Italy and are a resource for fishery and for recreational-touristic use. To our knowledge there are no significant industrial point sources of PFASs in these sites, but lakes are impacted by effluents of wastewater treatment plants and diffuse pollution due to the atmospheric transport. Table 8.2.S1 reports some information on the catchment pressures.

4.2.2 Study species

Alosa agone (Scopoli 1786) is an endemic shad of deep subalpine Italian lakes (Kottelat and Freyhof, 2007). It is a pelagic non-migratory species. Fish begin to reproduce at 2 years, and most of them reproduce only once or twice. The spawning period ranges from June to August, along the shores near the surface at nights with bright moonlights. This species is mainly zooplanktivorous, but adult specimens of greater size feed on small fish. Usually they have a high fat content (>5% on wet weight).

This fish species was selected for monitoring because of its abundance in the study area and it is caught in all lakes both by professional and amateur fishermen. Only in the Italian part of Lake Maggiore the fishery of shad is forbidden because DDT concentrations in fillets exceed the Italian legal threshold for human consumption (100 ng g^{-1} ww) (Provincia Verbano-Cusio-Ossola, 2018). Shad represents a good portion of annual catch, for example in Lake Como it covers

approximately 18% of the total catch (i.e. 35 t, (Quadroni and Bettinetti, 2017)) and is widely consumed both fresh and preserved.
Finally, according to the EU TGD (European Union, 2011b) the optimum fish species for WFD biota monitoring and classification should be a predator of trophic level (TL) 4, and *Alosa agone* is classified of trophic level 3.8 ±0.4, according to the database FishBase (www.fishbase.org), which is used also for all the TL attribution in the following text.

4.2.3 Sample Collection and Preparation

Water was sampled by a bucket from the superficial layer, collected in polypropylene (PP) centrifuge tubes and refrigerated at 4 °C until analysis.
Shad specimens (about two years old) were caught by professional fishermen by drifting gill nets (i.e. pendent), usually nightly, in the most superficial layer (i.e. epilimnion) of the lakes. The fish were seasonally collected from 2016 to 2017, whenever possible, because of the local restrictions and the adverse climatic conditions. Fish specimens were weighed and their lengths were measured. A summary scheme of the catches and the dimension of the specimens is reported in Table 8.2.S2.
The muscle was removed from the dorsal fin to the head and separated from the skin. The muscle tissues of about six fish were pooled for following analysis according to European Union (2014).

Some selected fish samples were subsequently dissected to separate fillet from the rest of the body separately. Muscle samples (pooled sample consisting of 6 specimens) were homogenized in 50 mL polypropylene (PP) vials by Ultra-Turrax T25 (Janke & Kunnel, IKA®-Labortechnik), whereas the rest of the body was frozen at -18°C and crumbled with an ice crusher before the extraction.

The samples were dried in oven at 105°C overnight to measure the dry weight. The whole fish data were obtained as the sum of fillet and the rest of the body concentrations.

4.2.4 PFASs chemical analysis

4.2.4.1 Chemicals and Standards

A full list of chemicals and solvents is provided in the Supporting Information. Certified PFASs native compounds and isotope-labelled internal standards (ISs) were purchased from Wellington Laboratories, Inc. (Guelph, Ontario, Canada). PFAC-MXC Stock Solution containing 21 native PFCAs and PFASs was diluted in acetonitrile to prepare calibration standard solutions. Mass-labelled MPFAC-MXA and mass-labelled M3PFPeA solutions were diluted in acetonitrile (40 μg L^{-1}) for the preparation of the stable isotope labelled solution used as internal standard mixture (SIL-IS).

Details on the analyte names, abbreviations and corresponding IS are reported in Table 8.2.S3.

4.2.4.2 Analysis

Extraction and analysis of PFASs in water samples were carried out following the on-line SPE-HPLC-MS method described in Mazzoni et al. (2015).

For the analysis of fish, few grams of pooled and homogenized samples were weighed (muscle: 10 g; rest of the body: 8 g) into a 50 mL PP centrifuge tube and spiked with 100 µL of SIL-IS solution (40 µg L^{-1}) before extraction. The extraction was carried out using the method described in Mazzoni et al. (2016). Briefly, samples were extracted by sonication in an acidified water and acetonitrile 10:90 v/v solution (1.5 mL of water and acetonitrile solution per gram of fresh sample) and subsequent purification on $MgSO_4$/NaCl. To remove phospholipids, partially evaporated extracts were filtered through HybridSPE® Phospholipid Ultra cartridges, previously cleaned with 3 mL of acetonitrile and 50 µL of formic acid (1 cartridge for carcass and muscle extract and 2 cartridges for viscera extracts). PFASs in the final extract were determined by liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) coupled to a turbulent flow chromatography (TFC) for the online purification of the extracts (Mazzoni et al., 2016). A procedural blank was run every extraction batch. Full validation data are shown in Mazzoni et al. (2016).

Table 8.2.S3 lists the MS/MS transitions and collision energies applied for the different target analytes and isotope labelled standards. For all the analytes, one precursor and two product ions were monitored. Calibration curves were prepared using mixed standard

solutions in acetonitrile, which were acidified to pH 3 and spiked with SIL-IS by adding 50 µL of concentrated formic acid and 100 µL of the diluted SIL-IS solution (40 µg L^{-1}) to 0.9 mL of native standard solution before injection. Quantification was performed by using the isotopic dilution method and calibration curves were acquired before each analytical run.

Limits of detection (LOD) and limits of quantification (LOQ) in fish tissue and water were estimated, according to the ISO Standard 6107-2: 2006, as respectively, three-fold and tenfold the standard deviation of an extract of biological tissue fortified at 1 g L^{-1}. For fish, LODs and LOQs ranged from 0.03 to 0.4 and from 0.1 to 1.2 ng g^{-1}ww respectively (Table 8.2.S4). LODs of water are reported in the last row of table 8.2.S7. Additional method performance characteristics are reported in Mazzoni et al. (2015, 2016).

4.2.5. Statistical analysis

One-way ANOVA was used to detect significant differences in mean PFOS concentrations among lakes, after the log-normalization of data. Data met the assumptions (homogeneity of variances and normality of the distribution of the dependent variable) for this analysis. The significance level was set at $p<0.05$. Mann-Whitney-Wilcoxon test was performed to detect differences between PFOS and the sum of PFCAs concentrations (significance level: $p <0.05$). Statistical analysis was performed by STATISTICA 12 software.

4.3 Results and discussion

4.3.1 PFASs concentrations in lake fish and waters

The occurrence of 13 perfluoroalkyl acids (PFAAs) has been surveyed in fish caught from five subalpine Italian lakes. In Table 4.1 the concentrations of PFOA, PFOS and PFCAs ranging from 5 to 14 atoms of carbon, expressed as sums of short chain (<C8), and long chain (>C8) congeners, measured in every pooled fillet of sampled fish, are reported. Detailed data sets for each analysed compound are given in Tables 8.2.S5 and 8.2.S6. PFHxDA, PFODA and perfluoroalkyl sulfonates (PFSAs) other than PFOS were never detected. PFOS concentrations were the highest of all PFAAs in almost all samples, apart in Lake Iseo in December where the sum of long chain-PFAAs was slightly higher (0.9 and 1.3 ng g^{-1}ww respectively). However, the difference between PFOS and the total sum of PFCAs concentrations for all samples analysed is statistically significant (Mann-Whitney-Wilcoxon test, $p<0.003$).

PFOS was detected in all the samples, with concentrations ranging from 0.4 to 16.6 ng g^{-1} ww and a mean value of 5.0 ng g^{-1} ww. PFOS concentrations of pooled fish from different lakes are plotted in Fig. 2.

Lake Maggiore showed significantly higher PFOS concentrations (ANOVA, $p<0.05$), with a mean of 13.0 ± 3.2 ng g^{-1} ww, which exceeded the European EQS_{biota} (9.1 ng g^{-1}ww), plotted as a horizontal line in Figure 4.2.

Table 4.1 Concentrations of PFOA, PFOS and PFCAs (ng g^{-1} ww) of pooled fish fillets (*A. agone*), as sum of short-chain compounds (C5-C7) and long-chain compounds (C9-C14).

Lake	**Data**	**PFOA**	**Sum C_5-C_7-PFCA**	**Sum C_9-C_{14}-PFCA**	**PFOS**
Mergozzo	October 2016	< LOD	0.2	2.7	5.7
Maggiore	October 2016	1.2	0.2	0.9	16.6
Maggiore	August 2017	< LOD	< LOD	0.7	11.7
Maggiore	October 2017	< LOD	< LOD	1.3	10.6
b. Como	March 2017	< LOD	< LOD	3.1	4.2
b. Como	July 2017	0.1	0.4	1.4	6.0
b. Como	October 2017	< LOD	< LOD	0.5	4.9
b. Lecco	November 2016	< LOD	< LOD	2.1	2.8
b. Lecco	March 2017	< LOD	< LOD	0.4	1.6
b. Lecco	July 2017	0.1	0.6	0.7	4.3
Iseo	December 2016	< LOD	< LOD	1.3	0.9
Iseo	May 2017	< LOD	< LOD	0.3	1.0
Iseo	June 2017	0.3	1.3	0.5	2.5
Iseo	September 2017	< LOD	< LOD	0.3	0.4
Garda	October 2017	< LOD	0.8	0.7	1.7
Garda	December 2017	< LOD	< LOD	0.8	4.8

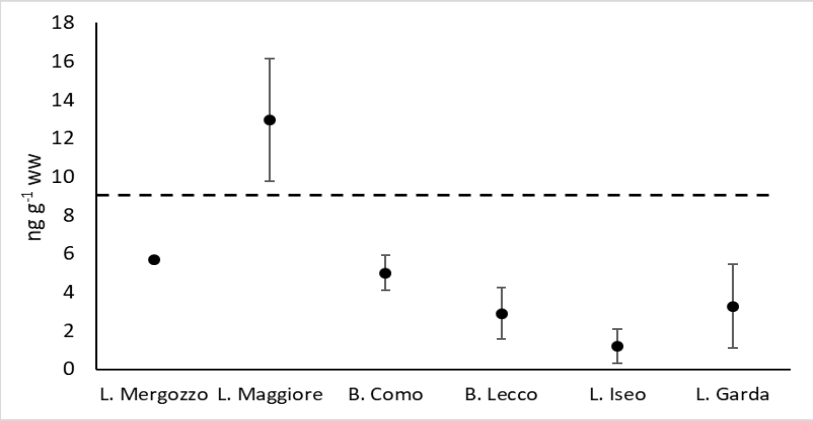

Fig. 4.2 PFOS concentrations (ng g^{-1} ww) in the different lakes. Dotted line represents EQS (9.1 ng g^{-1} ww).

Remaining lakes were not statistically different from each other, with an overall average of 3.1 ± 1.9 ng g^{-1}ww. Differences among lakes are not attributable to significant differences in fish weight and lengths (Table 8.2.S2). The weight average of full fish dataset was 119 ± 34.6 (CV=29%) and length average was 20.4 ± 2.5 (CV=12%).

Concentrations measured in this study in shad of Lake Maggiore were slightly lower than those reported by Squadrone et al. (2014), who measured an average of 20 and 22 ng g^{-1} ww in muscle of lake whitefish (TL = 3.2 ± 0.2) and European perch (TL = 4.4 ± 0.0) respectively, caught in Lake Maggiore in 2012. Fillets of perch and shad sampled in Lake Lugano, and analysed by our group in 2015, showed mean values of 23.2 and 13.5 ng g^{-1} ww respectively (Commissione internazionale per la protezione delle acque italo-svizzere, 2016), and the latter was very similar to that measured in

shad from Lake Maggiore in the present study. This evidence suggests that a possible source of PFOS for Lake Maggiore is Lake Lugano which is connected to Lake Maggiore by its outflowing stream, Tresa River. This river enters in Lake Maggiore close to the town of Luino, right where Squadrone collected their fish samples (Squadrone et al., 2014). Lake Lugano with the capital city Lugano is an important lake for both commercial and recreational fishing and receives wastewater of anthropic and industrial sources (Commissione internazionale per la protezione delle acque italo-svizzere, 2016).

Fish in the other subalpine lakes, even Lake Mergozzo, that is very close to Lake Maggiore, had lower PFOS concentrations, and their overall mean concentration (3.1 ± 1.9 ng g^{-1}ww) could be considered as the "anthropogenic background concentration" (i.e. not influenced by specific releases) of PFOS in fish of lakes located in an industrialised and urbanised region but without point sources of chemical plants. Indeed, the Lombardy region (where the lakes Como, Iseo and Garda are located) is densely populated (420 inhab. km^{-2} for the whole region).

In Lake Garda similar mean PFOS concentration was measured in eels (2.2 ± 1.7 ng g^{-1}ww) (Chiesa et al., 2018), which is a species with a similar trophic level (TL eels = 3.6 ± 0.3). A PFOS average concentration of 9.6 ± 3.8 ng g^{-1} ww has been measured in European perch in Lake Varese, a small lake located between Lake Maggiore and Lake Como (Squadrone et al., 2015). The last highest value cannot be explained only by the slightly higher TL of European perch

(4.4±0.0), but also by the different characteristics and pressures of this small and shallow lake which has been historically impacted by high load of domestic organic pollution (Agenzia Regionale per la Protezione dell'Ambiente della Lombardia, 2013).

There are few other studies on PFASs in lake ecosystems in Europe. Some of them reported data of fish caught before the restrictions on PFOS use, reaching maximum values up to 225.4 ng g^{-1}ww in Lake Griebnitzsee, in Germany (Berger et al., 2009; Schuetze et al., 2010). Other researches focused on lakes in alpine area and pristine zones (Ahrens et al., 2010; Åkerblom et al., 2017). Ahrens et al. (2010) reported PFOS concentrations of fish liver in four lakes in French Alps ranging from 3.61 to 4.24 ng g^{-1} ww. It is well known that PFOS accumulates more in liver than in the muscle tissue (Labadie and Chevreuil, 2011), so PFOS concentrations in fillets at this high altitude area might be of the same order of magnitude of those measured in pristine and remote lakes in Sweden (0.01-0.93 ng g^{-1} ww) (Åkerblom et al., 2017). These PFOS fish levels should represent the range of background European concentration attributable only to atmospheric transport, as demonstrated by the good correlation with total Hg (Ahrens et al., 2010).

Concentrations recently measured in fish in significant European rivers are more variable, ranging from 2.6 to 14 ng g^{-1} ww in Rhône River (Babut et al., 2017), 17.9-39 ng g^{-1} ww in Loire River and estuary (Couderc et al., 2015), <LOD-154 ng g^{-1} ww in Ebro River (Pignotti et al., 2017) and 0.58–61.3 ng g^{-1} ww in Vltava and Labe

rivers (Svihlikova et al., 2015). The concentration range is very wide and concentrations sometimes reach very high peaks largely exceeding the EU EQS_{biota} for PFOS.

Considering the PFCAs group, there were no significant differences among lakes for every compound (Fig. 4.3). These substances seem to follow the same pattern of distribution in all lakes, being long-chain compound concentrations generally higher than their short-chain homologues. PFPeA and PFHpA were detected only in 13% of the samples, followed by PFOA and PFHxA, with 25% and 31% respectively. Also, PFNA was found only in 5 fillet pools. Conversely, substances with 10 to 14 atoms of carbon were detected in more than 75% of the samples, with PFDoDA and PFTrDA always above the detection limits.

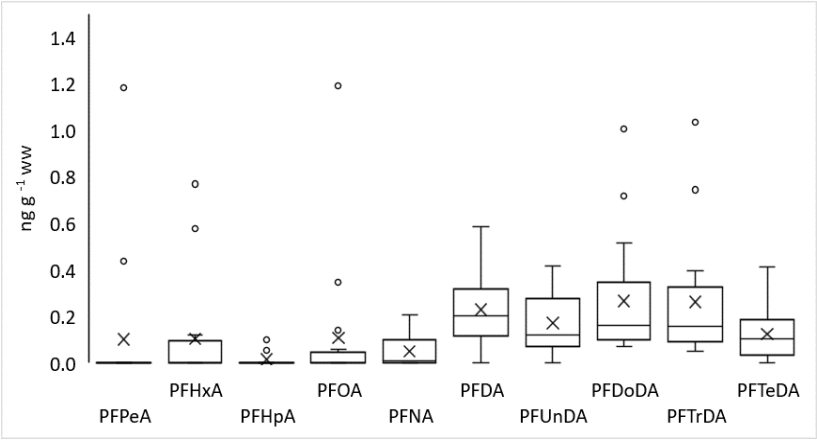

Fig.4.3 PFCAs concentrations (for compounds ranging from 5 to 14 carbon atoms).

The mean values for PFCAs ranged from <LOD to 0.27 ng g^{-1} ww (Fig. 4.3). It is possible to identify two groups of perfluoroalkyl carboxylic acids: compounds with 5 to 9 carbons and with 10 to 14. The statistical analysis confirmed that the differences between the two groups were statistically significant (t-test $p<0.05$). PFOA has been measured in a significant amount only in the sample of October 2016 in Lake Maggiore (1.2 ng g^{-1} ww), while all remaining samples were close or below the detection limit (0.1 ng g^{-1} ww).

In freshwaters, concentrations of short chain PFCAs with 5 to 7 carbon atoms were often below the detection limits in fish tissues (Babut et al., 2017; Couderc et al., 2015; Svihlikova et al., 2015) or detected only in sporadic samples (Stahl et al., 2014). PFOA was never detected above LOD in Italian lake shads (Squadrone et al., 2015, 2014), while it was detected in 77% of the samples, with an average of 0.2 ng g^{-1} ww, in Lake Garda eels (Chiesa et al., 2018).

The concentrations of long chain compounds detected in this study are usually lower than those reported in other studies conducted on European freshwaters, which reported a wide range of concentrations within the same ecosystem, from parts of nanograms to tens of nanograms per gram (Babut et al., 2017; Couderc et al., 2015; Pignotti et al., 2017).

In Como and Maggiore lakes we determined PFAAs also in water (Table 8.2.S7), but the concentrations were generally below the detection limits for most of the compounds, particularly long-chain PFCAs. The most frequently measured compounds were PFBS (<1 -

6.1 ng L^{-1}), which does not bioaccumulate in fish, and PFOS (<2.5 - 3.3 ng L^{-1}), whose measured concentrations exceeded the EU EQS_{water} (0.65 ng L^{-1}) even if they were close to the detection limit. PFOS concentrations were lower than those measured in Lake Maggiore in 2006 (Loos et al., 2007), indicating a reduction in PFOS emission because of the EU restrictions. The results of this study confirm that PFOS and the long chain PFCAs are the most bioaccumulative substances among the PFASs group, while the short chain PFCAs have a very low or negligible bioaccumulation potential (Conder et al., 2008; Valsecchi et al., 2017).

4.3.2 PFAAs risks derived from fish consumption for humans and top predators

Risk assessment requires the knowledge of the toxicity of the compounds and the exposure scenarios of the different human categories and animal or vegetal species. Toxicity data for the most frequently found PFAAs, such as PFOS and PFOA, are nowadays available (European Union, 2011a; Valsecchi et al., 2017), while data on exposure scenarios are very complex and variable, depending on local habits (Trudel et al., 2008). The most general approach is to compare monitoring data with threshold limits or quality standards derived from large scale (national or international) conventional exposure scenarios, usually considering the most sensitive human categories. As regards human food consumption, biota standards or thresholds are usually derived from PFOS and PFOA tolerable daily intakes (TDI) stated by different institutions around the world. Table

4.2 collects and compares the limits and TDIs for PFOS and PFOA in the different countries. No fish limits are available for any other PFAAs, at the best of our knowledge.

As described in the Introduction section, in the European Union PFOA and PFOS $QS_{biota,hh}$ have been derived by TDIs established by the European Food Safety Agency (EFSA) considering a person of 70 kg (European Food Safety Authority, 2008) . Nevertheless, PFOS EU quality standard for biota for the protection of human consumption of fish ($QS_{biota,\ hh}$: 9.1 ng g^{-1}) has been derived considering a European daily fish consumption of 115 g, which is much higher than the Italian fish daily intakes (38.8 g d^{-1} for general population and 71.0 g d^{-1} for high fish consumers), estimated by the Italian National Institute for Research on Food and Nutrition (INRAN) (Leclercq et al., 2009). By inserting Italian daily intakes in equation (1), we obtained $QS_{biota,hh}$ for PFOS equalling 27.6 ng g^{-1} ww and 14.8 ng g^{-1} ww for Italian general population and high fish consumers respectively. These limits are more representative for comparison with the concentrations measured in the Italian lakes.

Australia and New Zealand proposed TDIs (20 ng kg^{-1} bw per day and 160 ng kg^{-1} bw per day, per PFOS and PFOA respectively) which are ten-fold lower than EFSA ones. These discrepancies may be due to updated toxicological datasets which drastically grew in the last ten years. TDIs have been used to derive trigger points of verification (5.2 and 41 ng g^{-1} ww, for PFOS and PFOA respectively), which are the maximum concentrations of these chemicals to be tolerated in

individual foodstuffs or food groups to protect high consumers of these foods with dietary exposures exceeding the relevant TDI (Food Standards Australia New Zealand-FSANZ, 2017). Maximum concentrations were selected to ensure protection of all population groups, according to a range of consumption scenarios: in the case of PFOS and PFOA the reference population is 2-6 years old children.

If we compare fillet concentrations in the studied lakes (Table 4.1) with limits for fish human consumption (Table 4.2), we can conclude that no samples exceed any proposed limits for PFOA.

In the case of PFOS, whereas all samples from Lake Iseo are lower than any food criteria for PFOS, many samples from lakes Mergozzo, Lecco, Como and Garda are in the range of the PFOS Australian trigger point (5.2 ng g^{-1} ww), while Lake Maggiore shows concentrations even higher than the EU $QS_{biota,hh}$ 9.1 ng g^{-1}. But, applying the specific Italian daily fish intakes, only one sample from Lake Maggiore (October 2016) is slightly higher than the $QS_{biota,hh}$ (14.8 ng g^{-1} ww) calculated for a high consumer scenario. These data show that for a local risk assessment the choice of the realistic exposure scenario is crucial. In the case of Italian part of Lake Maggiore, we need also to mention that shad fishing for commercial uses is already forbidden because of the historical DDT pollution in this lake.

Ecological risk evaluation for predators in the studied lakes is even more complex, because we must know local scenarios for each predator species. A general risk assessment can be carried out by

comparing measured fish concentrations with quality standards for the protection of predators from secondary poisoning ($QS_{biota,\ sec\ pois}$). Few limits are available for this kind of protection goal: Canadian Environmental Quality Guidelines for PFOS (4.6 - 8.2 ng g^{-1} ww), EU quality standard for PFOS (33 ng g^{-1} ww) and Italian quality standard for PFOA (0.9 ng g^{-1} ww), derived following the prescriptions of the Technical Guidance for Deriving EQS (TGD) (European Union, 2011b) (Table 4.2).

All concentrations measured in shad fillets in the present work (Table 4.1) are lower than any PFOA and PFOS limits, apart from a single sample collected on October 2016 in Lake Maggiore which exceeds the $QS_{biota,sec.pois}$ for PFOA (1.2 ng g^{-1} ww).

But we have to underline that the assessment of the risk for predators in lacustrine environment should require that concentrations are measured in whole fish and not in the fillet, even if the latter is the most common procedure in most of the monitoring programs. This crucial point for monitoring programs is discussed in the following section.

4.3.3 Implication for monitoring programs

Determination of hazardous substances in aquatic biota is included in surface water monitoring programs with a two-fold aim of protecting top predators from secondary poisoning and human health from the fish consumption. Most of the monitoring programs are focused on the protection of human health from deleterious effects resulting from the

consumption of food (fish, molluscs, crustaceans, etc.) contaminated by chemicals and therefore the chemical contaminants are measured in edible parts such as the fish fillets.

The second aim, i.e. protecting top predators such as piscivorous birds or mammals from secondary poisoning by consumption of contaminated preys, would require the analysis of whole fish.

The concept that every protection goal requires its own biota monitoring strategy caused difficulties in the implementation of biota monitoring in WFD monitoring plans. CIS-WFD Guidance on Biota Monitoring (European Union, 2014) concluded, for example, that the use of whole-fish concentrations for PFOS monitoring may overestimate the risk toward human health, and conversely, the use of fillet concentrations may underestimate the risk toward top predators.

In our campaign, we were able to analyse separately both fillets and whole fish for a limited set of samples (Table 8.2.S8) and verified that the PFOS amount in fillets represented only from 5 to 28% (mean percentage 15%) of the PFOS accumulated in whole fish, because muscle is not the preferential tissue for PFOS accumulation in fish, as already demonstrated in the literature (Conder et al., 2008; Loos et al., 2017, Martin et al., 2003). The ratio between fillet and whole-body concentrations (Table 8.2.S8) ranged between 1.2 - 6.5 and was consistent with previous observations (de Vos et al., 2008; Munoz et al., 2017).

With the collected data, a log-log regression between PFOS concentrations in fillet vs whole fish has been derived (Figure 8.2.S1),

as recommended by the Guidance on Biota Monitoring (European Union, 2014). Using this empirical regression, we estimated the PFOS concentrations in whole fish in all the samples (Table 8.2.S8), in order to compare them with the EU $QS_{biota,sec.pois}$ (33 ng g^{-1} ww). While concentrations in the other lakes are significantly lower than the quality standard, most of the estimated concentrations for Lake Maggiore were close to the limit, confirming that in this lake there is a moderate risk from PFOS bioaccumulation. Comparing our analytical results with the Canadian Environmental Quality Guidelines for mammals and birds that consume aquatic biota (4.6-8.2 ng g^{-1} ww; Environment and Climate Change Canada, 2017), not only the possible contamination threat in Lake Maggiore was confirmed, but even the other deep lakes were potentially hazardous to top predators. Further analyses on aquatic birds and mammals are needed for a better definition of ecological risks.

Our results showed that, in the case of PFAAs monitoring campaign, it is strongly necessary to analyse both fillets and whole fish if monitoring aim is to evaluate risks for both human and top predators, because the simple fillet analysis is not sufficient to give information on ecological risks.

Table 4.2 PFOS and PFOA limits and Tolerable Daily Intakes (TDI) for fish in worldwide regulation according to the protection goals. TDI expressed as ng kg^{-1} bw per day.

Protection goal	State or country	Quality Standard ng g^{-1} ww fish	TDI	Reference
PFOS				
Human health via consumption of fishery products	EU		150	(European Food Safety Authority, 2008)
	EU	9.1 ($QS_{biota, hh}$)		Directive 2013/39/EU; (European Union, 2011a, 2011b)
	Australia-New Zealand	5.2 (trigger points)	20	(Food Standards Australia New Zealand-FSANZ, 2017)
	Minnesota (USA)	<40 no limits of consumption	80	(Minnesota Department of Health - MNDOH, 2008)
		40-800 restrictions		
		>800 do not eat		
	Canada		60	(Health Canada, 2016)
Predators from secondary poisoning	EU	33 ($QS_{biota, sec pois}$)		(European Union, 2011a, 2011b)
	Canada	4.6-8.2 (Federal Environmental Quality Guidelines for mammalian and avian species)		(Environment and Climate Change Canada, 2017)

Protection goal	State or country	Quality Standard ng g^{-1} ww fish	TDI	Reference
PFOA				
Human health via consumption of fishery products	EU		1500	(European Food Safety Authority, 2008)
	Italy	91 ($QS_{biota, hh}$)		(Valsecchi et al., 2017)
	Australia-New Zealand	41 (trigger points)	160	(Food Standards Australia New Zealand-FSANZ, 2017)
Predators from secondary poisoning	Italy	0.9 ($QS_{biota, sec pois}$)		(Valsecchi et al., 2017)

4.4 Conclusions

Notwithstanding the normative restrictions on PFOS, this compound still presented the highest concentrations among the analysed PFAAs and concentrations in fish caught in Lake Maggiore always exceeded the EU EQS_{biota}.. We suppose that the main contamination source is Lake Lugano, because the two lakes are connected by river Tresa, but, to confirm this hypothesis, a complete survey of PFASs losses and sources should be carried out in these lakes. However, considering the national consumption of fish, we don't evidence an actual risk for fish consumption by humans, while a moderate risk of secondary poisoning for predators is highlighted. It is important to stress that the evaluation of the ecological risk for this class of compounds is possible only when concentrations in whole fish are measured.

Finally, with the present study we demonstrate that biota monitoring on fish can be used as a valuable tool to classify the quality status of water bodies regarding bioaccumulative PFAAs, even if the water concentrations are lower than the limits of detection of available instrumentations and methods.

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Chapter V

Trophic transfer of persistent organic pollutants through a pelagic food web: The case of Lake Como (Northern Italy)

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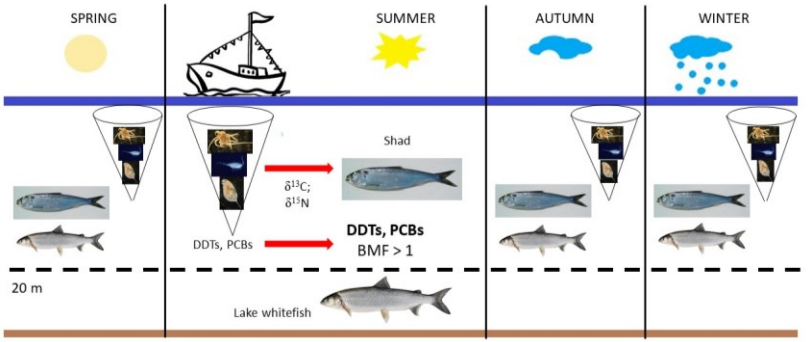

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Abstract

Despite DDTs and PCBs having been banned for about 40 years, they are still detectable in the environment. In the present research we specifically investigated the trophic transfer of these organochlorine contaminants (OCs) through a pelagic food web of a deep lake in Northern Italy (Lake Como) over time. Zooplankton and fish were sampled each season of a year and OC concentrations and the carbon and nitrogen isotopic ratios were measured. By using stable isotopes, the direct trophic relationship between pelagic zooplankton and zooplanktivorous fish was confirmed for *Alosa agone* only in summer. Based on this result, the biomagnification factor normalized on the trophic level (BMF_{TL}) for organic contaminants was calculated. BMF_{TL} values were within the range 0.9 – 1.9 for DDT isomers and 1.6 – 4.9 for some PCBs congeners (PCB 95, PCB 101, PCB 149, PCB 153, PCB 138–present both in zooplankton and in fish and representing > 60% of the PCBs contamination), confirming the biomagnification of these compounds in one of the two zooplanktivorous fish species of the lake.

5.1 Introduction

Legacy persistent organic pollutants, such as dichloro-diphenyltrichloroethane and its metabolites (DDTs) and poly-chlorinated biphenyls (PCBs), extensively used for sanitary, agricultural and/or industrial purposes from the mid-1940s and then banned/restricted, are still present in the environment for their long-time persistence. Organochlorine compounds (OC) are known to have effects on development, metabolism and reproduction of aquatic organisms (Berg et al., 2016; Gauthier et al., 2018; Jenkins et al., 2018). Sorption and distribution of these hydrophobic compounds in biota are influenced by their *n*-octanol/water partition coefficients and by the elimination rates being lower than the accumulation rates, which in turn results in their trophic accumulation along the food web (Coelhan et al., 2006; Bettinetti et al., 2016; Jurgens et al., 2016; Corsolini and Sarà, 2017; Zhang et al., 2017).

The biomagnification approach can be used to describe the potential bioaccumulation of toxicants in an organism relating it to its trophic position (Conder et al., 2012). A specific metric has to be used (as for example the biomagnification factor normalized on the trophic level - BMF_{TL}) (Conder et al., 2012), and the trophic position of each species within a food web and its temporal and spatial variations must be known too (Post, 2002). Stable isotope analysis (SIA) of carbon (C) and nitrogen (N) signatures allows the understanding of the structure of the trophic webs, prey-predator relationships, and the nature of trophic relationships among organisms. The description of the trophic

relationships and the organisms' diet reconstruction can be carried out using the difference between isotopic ratios of the consumer and its diet (i.e. the diet discrimination factors). The stable carbon isotope ratio ($\delta^{13}C‰$) reveals the contributions of different food sources, while the N isotope ratio ($\delta^{15}N‰$) indicates the trophic role since a consumer is typically enriched compared to its diet (Caut et al., 2009; Post, 2002; Visconti and Manca, 2011).

In lake ecosystems, SIA can reveal seasonal changes in $\delta^{13}C‰$ and $\delta^{15}N‰$ of zooplankton and fish, due to changes in food sources (Grey et al., 2001) or changes in taxonomic composition of pelagic communities, with different species having variable isotopic signatures (Matthews and Mazumder, 2003) that transfer this variability up to the food chain. The analysis can also underline the spatial variation in a single lake ecosystem (Syväranta et al., 2006), particularly when lakes are large and deep. Grey et al. (2001) reported that the pelagic phytoplankton has a more negative $\delta^{13}C$ value than littoral producers. Therefore, when evaluating the biomagnification in an aquatic ecosystem, it seems scientifically relevant to know the specific relationships among the different organisms of the trophic web, which can change with seasons.

The specific goals of the present study were: (i) to describe the pelagic trophic web structure of Lake Como, one of the deepest lake in Europe, located in Northern Italy; (ii) to assess the DDTs and PCBs contamination in the organisms; (iii) to estimate the BMF_{TL} of DDTs

and PCBs in two pelagic fish species considering their real trophic relationships with the other organisms throughout the year.

5.2 Materials and methods

5.2.1 Study site

Lake Como (Northern Italy, 198 m a.s.l., Fig. 5.1) is the third largest Italian lake by both volume (22.5 km^3) and surface area (145 km^2) and the deepest one with the maximum depth of 425 m at Argegno. It is characterised by an upside down "Y" shape, where three sub-basins can be identified. It is a warm monomictic lake, with a complete circulation of the water column at temperatures around 6.5°C in late winter. However, water mixing typically involves the first 150-200 m and complete turnover happens only periodically, after particularly cold and windy winters. The lake is therefore defined as olo-oligomictic (Ambrosetti et al., 1992). As concern its trophic status, Lake Como is classified as meso-eutrophic, with total phosphorus concentrations of about 25 µg L^{-1} (at the maximum depth in spring) (Salmaso et al., 2014).

Lake Como is located in a heavily industrialized area. It has been of interest for several decades because of water quality problems, in particular its south-western branch, where the major city (the city of Como) is located, no river outlet is present and the theoretical time of water renewal is 12.7 years. The city of Como represents a PCB hotspot (Bettinetti et al., 2014; 2016). Moreover, in recent years unexpected high levels of DDTs in the lake were best explained by

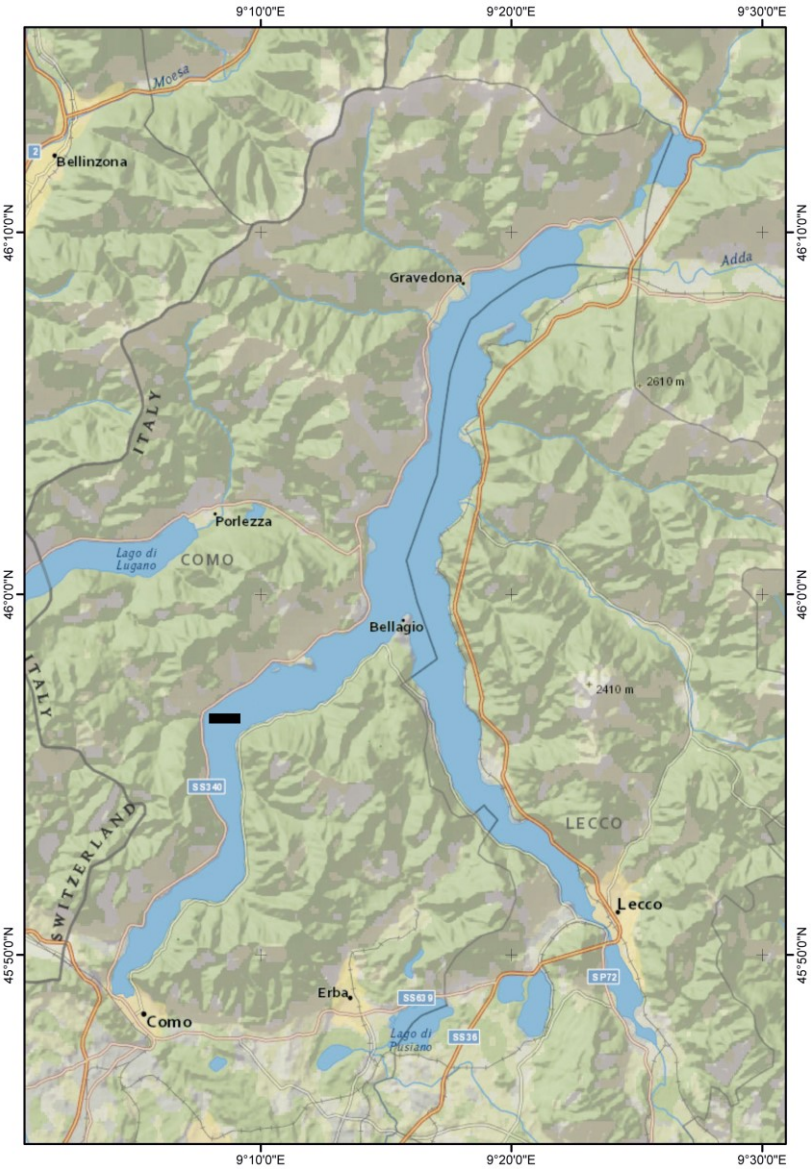

Fig.5.1 Lake Como. Black box indicates where zooplankton and fish were sampled.

glacial release (Bettinetti et al., 2008): DDT, previously used for fruit tree pest control in the valleys below glaciers, was carried up to mountain in the air and fell on glaciers trapped in snow. As climate warming has caused glaciers to retreat, the trapped contaminants were released back into the environment in melt water, flowing through streams and rivers and accumulating in lake organisms and sediments.

5.2.2 Sampling of zooplankton and fish

Zooplanktonic pelagic samples (N = 4) were collected close to Argegno (45° 56′ 36″ N, 9° 7′ 42″ E, Fig. 5.1) seasonally from spring 2013 to winter 2014, following the main changes in zooplankton biomass and density. Live crustacean zooplankton were gathered in the late morning with a 200 μm-mesh nylon net with a diameter of 58 cm by several vertical hauls (in the range 4-20, depending on the season) at 0 – 20 m depth, in order to reach around 1 g dry weight (dw) for each sample. Since the average transparency of Lake Como is 6.43 m within the year this depth range seems to be representative of the whole zooplankton community.

Each sample was divided in three parts: one part was fixed and preserved in alcohol 95% until the identification of taxa using compound microscopy at 40x and the calculation of biomass from length–weight regression equations (Manca and Comoli, 2000; McCauley, 1984); a second part was allocated for SIA; the rest of each sample was filtered on a 2 μm pore glass–fibre filters (GF/C, 4.7 cm of diameter) and then frozen at -20°C until OC analysis.

Six specimens of landlocked shad (*Alosa agone*) (Volta et al., 2011) and six specimens of lake whitefish (*Coregonus morpha hybrida*) were collected by professional fishermen near Argegno in the central part of the lake using pelagic gillnets, every season. After capture, fish were stored at 4°C and subsequently measured (Table 8.3.S1). Muscle fillets were collected from the dorsal region, between head and dorsal fin, both for the OC determination and SIA. For both the analysis the six fillets of each species were then pooled (N = 4 for both landlocked shad and lake whitefish). Fish species were selected for their dietary habits, since they are known as zooplanktivorous fish (Berg and Grimaldi, 1966; 1965).

5.2.3 C and N SIA

To determine C and N isotopic compositions of zooplankton, samples were defrosted and specimens for each taxon were isolated. Each group of zooplankton organisms and the fish tissue were oven-dried at 60°C for 48 hours and ground into fine powder. A subsample of 1 mg dry weight (dw) was weighed in aluminium capsules (5 x 9 mm) and sent to G.G Hatch Stable Isotope Laboratory (University of Ottawa, Canada). Stable isotopes were determined using a Carlo Erba 1110 Elemental Analyzer coupled with Thermo Finnigan DeltaPlus Advantage IRMS with a Conflo III interface. The analytical precision of analysis (i.e. standard deviation), based on laboratory internal standard, was usually < 0.2‰ for both elements.

Isotope ratios were calculated according to the following equation 1:

$$\delta^{13}C \text{ and } \delta^{15}N = [(R_{sample} / R_{standard}) - 1] * 1000 \quad (1)$$

where, R equalled $^{13}C/^{12}C$ for $\delta^{13}C$ and $^{15}N/^{14}N$ for $\delta^{15}N$ and standards were PeeDee Belemnite and atmospheric N_2 for C and N respectively.

5.2.4. OC chemical analysis

OC were analysed as described in Bettinetti et al. (2012a). About 0.5 g of freeze-dried zooplankton samples and freeze-dried aliquots of fish muscle fillet were put into a glass fibre thimble (19 mm internal diameter, 90 mm length, Whatman, England) and extracted in a modified Soxhlet equipment (Velp Scientifica, ECO 6 Thermoreactor, Italy) with 50 mL of a mixture (1:1) of *n*-hexane and acetone (Carlo Erba, pesticide analysis grade) for 2 hours. The determination of lipids was performed gravimetrically: the extract was dried with a gentle stream of nitrogen and several weighings were performed until balance to assess the lipid content. Lipids were then suspended in 2 mL of *n*-hexane and degraded with 2 mL of H_2SO_4 (98%, Carlo Erba) all night long. The supernatant was collected and mixed with successive *n*-hexane washings.

The extract was reduced to 1 mL by Rotavapor and cleaned up on a Florisil® column (40×7 mm length x internal diameter). The column elution was carried out by 25 mL of a mixture *n*-hexane and dichloromethane (Carlo Erba, pesticide analysis grade, 85:15). Finally, 1 mL of iso-octane (Carlo Erba, pesticide analysis grade) was

added to the obtained sample and the extract was concentrated to 0.5 mL and analysed by gas chromatography (GC Carlo Erba, Top 8000) coupled with ^{63}Ni electron capture detector (Carlo Erba ECD 80) using an on-column injection system (volume injected 1 μL) and a WCOT fused silica CP-Sil-8 CB column (50 m×0.25 mm length x internal diameter, film thickness 0.25 μm, Varian, USA). GC analysis was performed at the following temperatures: from 60 to 190°C at 20°C min^{-1}, from 190 to 250°C at 1.5°C min^{-1}, then from 250 to 270°C at 3°C min^{-1}, and, at last, the final isotherma at 270°C for 17 min, with helium as carrier gas (1 mL min^{-1}) and N as auxiliary gas (30 mL min^{-1}).

The external standards Custom Pesticide Mix (o2si, USA), Custom PCB Calibration Mix (o2si, USA) and Aroclor 1260 (Alltech, USA) were used for DDT and PCB quantitation. The solution of DDT homologues contained pp'DDT, op'DDT, pp'DDD, op'DDD and pp'DDE while the analysed PCB congeners were: PCB 18, 28+31, 44, 52, 95, 101, 118, 149, 138, 153, 156, 170, 174, 177, 180, 183, 187, 194, 195, 201, 203, 206 and 209.

The detection limit for single OC was 0.1–0.5 ng g^{-1} lipid weight (lw), depending on the compounds.

For zooplankton samples, good laboratory practices were routinely tested on standard reference materials BCR-598 for DDT homologues and BCR-349 for PCB residues (Community Bureau of Reference-BCR Brussels) analysing samples in triplicate. The percentage recovery of DDTs was between 106.2 ± 3% and 107.5 ± 4%, while

those for PCB ranged between 91.3% (±1.1%) and 102.2% (±1.6%). In fish, method performance was evaluated using SRM 1947 Lake Michigan Fish Tissue purchased from National Institute of Standard and Technology (NIST, Gaithersburg, Maryland). The recoveries were above 90% for each chemical compound. Blank samples were injected every five samples. No carryover and contamination were detected.

5.2.5 Data analysis

The use of isotopic mixing models allows the estimation of the potential diet of a given individual or species, based on the composition of different sources and assuming specific fractionation factors (Phillips and Gregg, 2001). To analyse the data the Bayesian models FRUITS (Fernandes et al., 2014), following the instruction of Phillips et al. (2014), was used.

The trophic level of a species is a function of the ^{15}N content; to estimate the seasonal trophic level (TL) of sampled zooplankton and fish the following equation 2 (Post, 2002) was used:

$$TL = \lambda + [(\delta^{15}N_{consumer} - \delta^{15}N_{pelagic\ baseline}) / 3.4 \quad (2)$$

where: λ was the TL of the pelagic baseline; $\delta^{15}N_{pelagic\ baseline}$ was the $\delta^{15}N$ values of the baseline at each season; 3.4 was the mean increase in $\delta^{15}N$ from one TL to the next in the specific trophic web. In the present study *Daphnia* spp. was chosen as a reference organism to

compare the isotopic signals of fish and other zooplanktonic taxa because its feeding behaviour seems to be non-selective and seasonally consistent (Matthews and Mazumder, 2003; Perga and Gerdeaux, 2006). *Daphnia* has been already used as pelagic baseline in other deep lakes with similar morphological features and composition of the pelagic zooplanktonic community (Leoni, 2017; Perga and Gerdeaux, 2006; Visconti and Manca, 2011).

The BMF_{TL} was calculated using the equation 3 (Conder et al., 2012):

$$BMF_{TL} = (C_{predator} / C_{prey}) / (TL_{predator} - TL_{prey}) \quad (3)$$

where $C_{predator}$ and C_{prey} expressed the lipid normalized concentrations of DDTs and PCBs in the predator and in its prey, and $TL_{predator}$ and TL_{prey} were the trophic levels of the predator and its prey.

Kruskal-Wallis test was performed to detect differences in isotopic signatures between seasons for each pelagic species or group of species (significance level: $p < 0.05$). Statistical analysis were performed with STATISTICA 12 software.

5.3 Results

5.3.1 Seasonal changes in zooplankton community composition

Seasonal pattern of zooplankton biomass and density showed the highest peak in spring, reaching a value of approximately 120 mg m^{-3} dw and 13,511 ind m^{-3} respectively, followed by an autumn lower

peak of 40 mg m^{-3} dw and 6060 ind m^{-3} respectively (Fig. 8.3.S1, Table 8.3.S2).

In spring, cladocerans were clearly predominant, *Daphnia longispina* gr. represented 80% of total zooplankton biomass; while copepods (i.e. cyclopoids and calanoids) were generally dominant in summer and winter, reaching 76% and 87% of the total biomass respectively. In autumn, in terms of biomass, cladocerans and copepods showed similar values.

Among copepods, cyclopoids represented 95% of the biomass in summer and about 70% in winter. The same percentage occurred in calanoids in spring and autumn.

Between primary consumers cladocerans, *Daphnia longispina galeata* gr. outnumbered all the other species in spring and autumn. *Eubosmina coregoni* prevailed in winter (92% of cladocerans biomass) and in summer (53% of cladocerans biomass). The biomass of the predatory cladocerans, i.e. *Leptodora kindtii* and *Bythotrephes longimanus,* was always under 0.3% of the total zooplankton community biomass.

5.3.2 Isotopic signatures

Daphnia $\delta^{13}C$ signature ranged between -26.83‰ in summer and -35.39‰ in spring, with intermediate values in autumn and winter (Fig. 5.2). Calanoids spp. had the same seasonal trend (Fig. 5.2). Also *Eubosmina* and *Bythotrephes* $\delta^{13}C$ signatures reached the maximum in summer while the minimum in winter (Fig. 5.2).

The other taxa (cyclopoids, *Leptodora* and *Diaphanosoma*) seemed to follow the same seasonal pattern but, due to a low abundance of specimens, no data in winter and/or spring were available (Fig. 5.2).
The lake whitefish stable isotope ratios were more stable than the ones of landlocked shad, ranging from -27.51 to -27.85 and from -26.66 to -28.81 respectively (Fig. 5.2).
Contrary to $\delta^{13}C$, zooplankton $\delta^{15}N$ signature reached the highest values in autumn and winter (Fig. 5.2). Generally, taxa showed a trend toward an increase in $\delta^{15}N$ from spring to autumn; in winter, instead, three different situations were found: an important decrease of *Eubosmina*, constant values of *Daphnia* and *Bythotrephes* and increased values of copepods.
Daphnia and *Eubosmina* $\delta^{15}N$ values fully overlapped except for winter (10.23 and 6.24‰ respectively); *Diaphanosoma*, analysed in summer and autumn, had quite similar values (Fig. 5.2). Calanoids shared the same signature as predatory cladocerans, with $\delta^{15}N$ values ranging between 7.02 and 15.60‰ over the year. Cyclopoids were always less enriched, with a maximum value of 10.78‰ in autumn (Fig. 5.2). Fish $\delta^{15}N$ signatures were steadily around 10.50‰. In spring and summer, their $\delta^{15}N$‰ values were higher than those of zooplankton, while in autumn and winter they were intermediate between cladocerans primary and secondary consumers (Fig. 5.2).

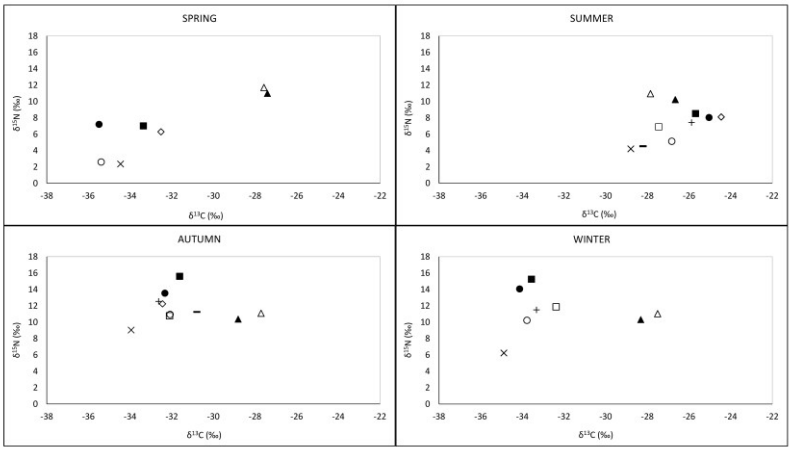

Fig. 5.2 Seasonal variations in carbon and nitrogen isotopic signatures in pelagic organisms of Lake Como. Byt: *Bythotrephes longimanus* (■)*;* Lep: *Leptodora kindtii* (◊); Cyc: cyclopoids (□); Cal; calanoids (●); Eub: *Eubosmina coregoni* (x); Dia: *Diaphanosoma brachyurum* (−), Dap: *Daphnia longispina galeata* gr. (○), landlocked shad (▲), whitefish (Δ), bulk 200 (+).

The seasonal changes of zooplanktonic isotopic signatures were significant ($\delta^{13}C$ Kruskal-Wallis test: H (3, N=28) = 21.9 p=0.001; $\delta^{15}N$ Kruskal-Wallis test: H (3, N=20) = 12.6 p=0.0056). Instead, both the fish species, *Alosa agone* and *Coregonus morpha hybrida,* did not show a significant seasonal pattern: values were similar during the year, reflecting a slower metabolic turnover in fish than in zooplankton.

Therefore, an evaluation of the diet of zooplanktivorous fish was carried out for each season (Fig. 8.3.S2). The explanatory data analysis showed that $\delta^{13}C$ and $\delta^{15}N$ of landlocked shad fell within the

range of the food source isotopic values only in summer, while in spring and in cold months the $\delta^{13}C$ fish values are intermediate between those of pelagic and littoral zone. $\delta^{13}C$ and $\delta^{15}N$ values of lake whitefish instead were always outside the polygon identified by the sources.

5.3.3 Concentrations and biomagnification of DDTs and PCBs in biota samples

On annual basis, the zooplankton average value of lipid content (expressed on dw) was 10.1%, with a minimum of 6.0% and a maximum of 14.7% in summer and spring respectively (Table 8.3.S3). Differences in seasons may be ascribed to changes in taxa composition in bulk samples. Fish presented a clear difference between the two species: the average lipid content (expressed on wet weight – ww) of landlocked shad was 12.1% while of lake whitefish was 2.1% (Table 8.3.S3). The lowest values of lipid content were observed in correspondence to the spawning period (in winter for lake whitefish and in summer for landlocked shad). In this study the fraction of lipids detected in landlocked shad was comparable with those reported in Bettinetti et al. (2016) that include fish caught from 2006 to 2009 in Lake Como. Table 5.1 and Fig. 5.3 report zooplankton and fish DDTs and PCBs contamination (spring 2013 - winter 2014) respectively.

Table 5.1 DDTs concentrations (ng g^{-1} lw.) in zooplankton and fish of Lake Como.

	Zoo	Landlocked shad	Lake whitefish	Zoo	Landlocked shad	Lake whitefish
	Spring			Summer		
pp'DDE	58.9	207.6	333.8	47.3	98.2	86.9
op'DDD	36.5	98.4	166.5	42	53.5	51.1
pp'DDD	17.6	34	48.1	9.6	19.3	21
op'DDT	8.6	26.8	53.5	8.3	13.8	13.9
pp'DDT	22.8	42.2	48.3	22.4	21.5	22.2
Total DDTs	144.4	409.1	650.2	129.5	206.5	195.1
	Autumn			Winter		
pp'DDE	70	55.3	90	72.8	10	141.6
op'DDD	51.1	47.9	108.5	75	7.4	92.2
pp'DDD	18.2	18.5	44.6	15.1	2.8	32.7
op'DDT	15.1	13.6	51.7	11.4	1.8	23.4
pp'DDT	26	54.1	53.4	35.6	2.8	35
Total DDTs	180.5	189.3	348.1	209.9	24.7	324.9

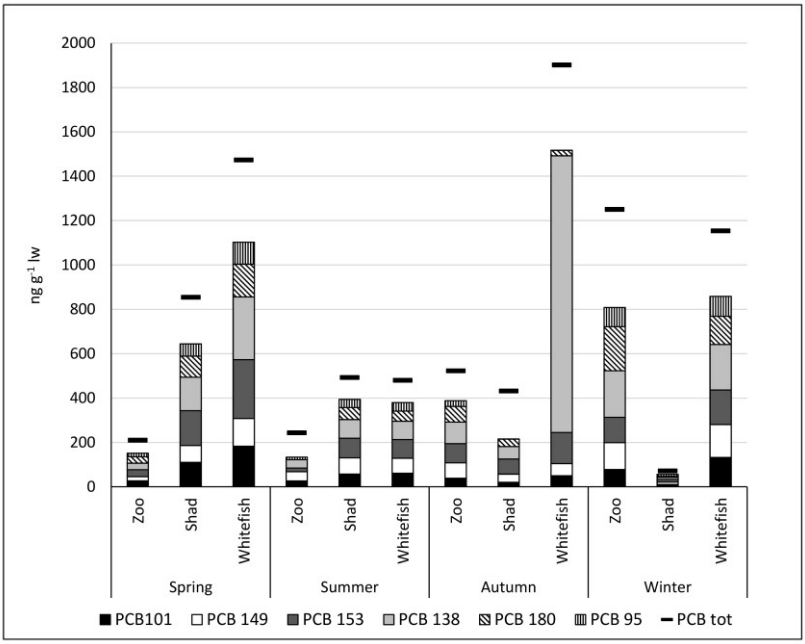

Fig. 5.3 Some PCBs congeners and total PCBs concentrations (ng g^{-1} lw) in zooplankton and fish of Lake Como.

Total DDTs concentrations in zooplankton ranged between 129.5 ng g^{-1} lw in summer and 209.9 ng g^{-1} lw in winter. pp'DDE was the main compound measured in spring, summer and autumn, representing 40.8%, 36.5% and 38.8% of total DDTs respectively, followed by op'DDD, with approximately 28% of the total contamination, while in winter op'DDD prevailed. pp'DDT was always detected, being approximately 15% of the total DDTs in each sample. The ratio between op'DDT/pp'DDT and op'DDD/pp'DDD was within the range 0.05-0.08 and 2.07-4.97, respectively.

In zooplankton, total PCBs concentrations were generally higher than DDTs; PCBs concentrations increased from spring to winter. PCB 95, 101, 149, 138, 153 and 180 represented more than 60% of the total PCB.

The highest concentration of total DDTs in fish tissue has been recorded in spring; in both species pp'DDE was the dominant compound in most samples (in whitefish collected in autumn op'DDD was predominant) followed by op'DDD, except in fish tissue collected in autumn, when pp'DDT in landlocked shad and pp'DDE in whitefish were the most detected second compound. The ratio between op'DDT/pp'DDT and op'DDD/pp'DDD was within the range 0.06-0.07 and 2.59-2.89 respectively in landlocked shad, and the ratio between op'DDT/pp'DDT and op'DDD/pp'DDD was within the range 0.07-0.14 and 2.43-3.46 respectively in lake whitefish.

Regarding PCBs, in the landlocked shad the sum of all congeners showed the same trend of DDTs contamination, reaching a maximum in spring and a minimum in winter. In lake whitefish the lowest value occurred in summer (480.1 ng g^{-1} lw), while in the other seasons, levels exceeded 1000 ng g^{-1} lw. In fish, the congeners with the highest concentrations were the same of zooplankton; their fraction reached over 75% of the total, with a prevalence of PCB 138 and 153. An exception was recorded in autumn in landlocked shad, when PCB 118 was the main congener (98.7 ng g^{-1} lw, 22% of the total PCBs).

For landlocked shad, BMF_{TL} higher than 1 were measured for all DDTs isomers except for the parental one (Table 5.2). For PCBs

congeners (both present in zooplankton and fish) the BMFTL was greater than 1, ranging between 1.6 (PCB 149) and 4.9 (PCB 153) (Table 5.2).

Table 5.2 BMF_{TL} calculated for landlocked shad

	BMF_{TL} landlocked shad
pp'DDE	1.9
op'DDD	1.2
pp'DDD	1.8
op'DDT	1.5
pp'DDT	0.9
PCB 95	2.9
PCB101	2.0
PCB 149	1.6
PCB 153	4.9
PCB 138	2.0

5.4 Discussion

5.4.1 Seasonal structure of Lake Como trophic web

Information on zooplankton in Lake Como is quite scanty and fragmentary. The species composition of the zooplanktonic community remained similar over time (Chiaudani and Premazzi, 1993; Gruppo di Lavoro Lago di Como, 2006). A significant decrease of cladocerans population of *Eubosmina coregoni* starting from the 1990s to the early 2000s, probably due the progressive improvement

of the lake trophic status in the last decades, was observed (Gruppo di Lavoro Lago di Como, 2006).

The $\delta^{13}C$ values of *Daphnia* were similar to those measured in lakes Maggiore, Geneva and Iseo (Perga and Gerdeaux, 2006; Visconti and Manca, 2011; Leoni, 2017), confirming that carbon isotopic signature seems to be influenced by the lake typology. All lakes showed less depleted values in summer, while the most negative $\delta^{13}C$ values for *Daphnia* were measured in spring in Lake Como and in winter in the other lakes. However, a significant correlation ($r = 0.7$) between water temperature in the upper 20 m and *Daphnia* carbon signature was found, as observed in Lake Maggiore and in Lake Geneva (Perga and Gerdeaux, 2006; Visconti and Manca, 2011). $\delta^{13}C$ signature increases with increasing temperature and thermal stratification (Perga and Gerdeaux, 2006; Visconti and Manca, 2011), probably reflecting changes in phytoplankton carbon isotopic signature that follow the different availability of dissolved inorganic carbon for photosynthesis along the year (Visconti and Manca, 2011; Caroni et al. 2012).

Nitrogen signature of pelagic baseline tends to increase with increasing trophy (Cattaneo et al., 2004; Grey et al., 2001; Vander Zanden et al., 1999). $\delta^{15}N$ values of *Daphnia* were higher than those measured in Lake Maggiore, which is oligotrophic, and consistent with those measured in Lake Geneva, where the mean total phosphorus concentration (35 $\mu g\ L^{-1}$) (Perga and Gerdeaux, 2006; Visconti and Manca, 2011) is similar to the Lake Como one (Salmaso et al., 2014).

The values of $\delta^{13}C$ and $\delta^{15}N$ of both fish are in step with those of Poma et al. (2014), who analysed lake whitefish and landlocked shad from Lake Maggiore.

Performing the "mixing model" to evaluate the diet of landlocked shad and lake whitefish, the littoral contribution was the main source of sustenance for both species, especially for landlocked shad during cold months (Fig. 5.4). In winter whitefish and landlocked shad usually migrate to the littoral; the first species for spawning and reproducing (Visconti et al., 2014) and the other probably because pelagic sources are not enough to support its diet (Poma et al., 2014); later both species return towards the pelagic zone when productivity increases.

In summer, only landlocked shad relied on pelagic trophic web (Fig. 8.3.S2); whitefish shows a $\delta^{13}C$ value that could overlap the one of *Eubosmina,* and probably it finds food in the pelagic area However, whitefish preferring colder water (Berg and Grimaldi, 1965), reasonably feeds deeper than 20 m, at a depth where waters in summer are colder. The isotopic signature of profundal zooplankton should be different from those of epilimnion (Matthews and Mazumder, 2006), explaining the whitefish diet.

The results of the mixing model on landlocked shad summer data showed that all species of crustacean zooplankton are included in the fish diet and cyclopoids, which is the taxon with the greater biomass (about the 70% of the total) represent about the 40% of the diet (Fig. 5.4). Therefore, it seems that zooplanktivorous fish try to be selective,

preferring secondary consumers over primary ones, likely because of their larger body size (Visconti et al., 2014) but they integrate their diet with the available preys. Volta (2010) described a similar behaviour for the landlocked shad in Lake Maggiore calculating a very positive Ivlev electivity index for *Bythotrephes* and *Leptodora* and a scarce one for cyclopoids. In summer in Lake Como, four trophic levels could be distinguished: *Daphnia*, *Diaphanosoma* and *Eubosmina* at the bottom, cyclopoids at an intermediate level, then *Bythotrephes*, *Leptodora* and calanoids and, on the top, the landlocked shad.

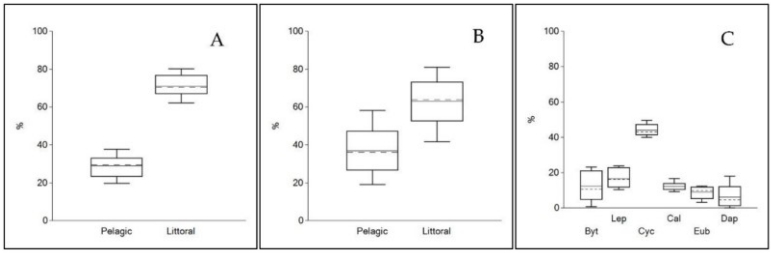

Fig. 5.4 A: diet of landlocked shad in autumn; B: diet of whitefish in autumn; C: diet of landlocked shad in summer. Byt: *Bythotrephes longimanus;* Lep: *Leptodora kindtii*; Cyc: cyclopoids; Cal; calanoids; Eub: *Eubosmina coregoni*; Dap: *Daphnia longispina* gr.

5.4.2 OCs contamination along the trophic web

Zooplankton showed a quite constant op'DDT/pp'DDT during the sampling year and the values were within the range reported by other studies discussing the analysis of pp' and op' DDT in biota (Ricking

and Schwarzbauer, 2012); instead op'DDD/pp'DDD was quite variable and slightly higher compared to the reported range ratio (Ricking and Schwarzbauer, 2012). It seems that biota in Lake Como presents high levels of op' DDD, more than expected by the composition in the original technical mixtures. The presence of an industrial plant which produces Dicofol (trade name Kelthane) not far from the lake may represent an air source of this compound but more focused analyses will follow to confirm it in the next future. In zooplankton samples, the highest values of both DDTs and PCBs were detected in winter, while the lowest ones during the cooling season. In Lake Maggiore a similar trend, repeated for some years, was recorded (CIPAIS, 2016). The biomass of the zooplankton community may explain these changes in the contaminant levels, since in winter and spring a reverse relationship between values of biomass and contaminations was observed; at the moment it can be only a hypothesis because data are few and not representative of more years. Updated and comparable studies on POP contaminations on lacustrine zooplankton are rare. Regarding Italian subalpine lakes, the last determination of DDTs and PCBs levels in Lake Iseo was in 2010. The values were comparable to Lake Como in autumn (173 and 181 ng g^{-1} lw of DDTs and 104 and 97 ng g^{-1} lw of PCBs respectively) while in summer Lake Iseo zooplankton showed higher contamination (Bettinetti et al., 2012a). Zooplankton in Lake Maggiore, analysed starting from 2010, had a DDTs contamination higher than Lake Como: in Lake Maggiore average DDTs levels fluctuated from 530.4

to 977.4 ng g^{-1} dw (Guzzella et al., 2018), while in Lake Como was around one fourth. This fact is not surprising since in Lake Maggiore a DDT contamination of industrial origin was present (Guzzella et al., 2018). As regards the distribution of DDT metabolites, pp'DDE was the main compound followed by op'DDD in both lakes (CIPAIS, 2013). On the contrary, the average contamination of PCBs was similar in both lakes (Lake Como about 49.0 ng g^{-1} dw and Lake Maggiore about 60.0 ng g^{-1} dw) (Guzzella et al., 2018).

In 2013, the annual average DDTs level in fish in Lake Como was slightly lower than in Lake Maggiore fish, (about 23 and 73 ng g^{-1} ww in landlocked shad; 7 and 31 ng g^{-1} ww in lake whitefish respectively) (Guzzella et al., 2018).

In both fish species op'DDT/pp'DDT and op'DDD/pp'DDD remained at the same level; the values of DDTs isomers were within the range reported for biota by Ricking and Schwarzbauer (2012) while op'DDD/pp'DDD was slightly higher (Ricking and Schwarzbauer, 2012), as already observed for zooplankton; as a possible explanation a plausible source may be represented by wastes from a dicofol production plant as mentioned above, since the input level of op' DDD from glacier was not reported (Bettinetti et al., 2008). Even if the contamination by Dicofol use after 1991 in Europe is negligible, the local contamination related to the production has been significant in some cases (Jurgens et al., 2016).

Moreover DDTs concentrations in Lake Como fish were lower than those measured in fish in other European water bodies where hotspot

pollution can be recognized: Ebro River (Spain) where a dicofol factory discharged its wastewaters, Lee River (England) where DDT has been manufactured for long time, Elbe River (in different countries) where there is a high industrial and urban impact (Huertas et al., 2016; Jurgens et al., 2016).

pp'DDE was confirmed to be one of the most prevalent compounds, but not always dominant, as in other studies (Bettinetti et al., 2012a; 2012b; 2016). The ratio of (pp'DDE + pp'DDD) and pp'DDT, used to evaluate the source of DDTs (Tavares et al., 1999), being higher than 1 (ranging from 1.4 to 7.9), suggests that the origin of contamination is old and no recent inputs of parental compound occurred even if a release of DDTs from melting glacier was recorded, as previously reported in the paragraph 5.2.1.

Unlike DDTs contamination, PCBs values in Lake Como were higher than those in Lake Maggiore, both for landlocked shad and lake whitefish (about 50 and 35 ng g^{-1} ww in landlocked shad; 25 and 15 ng g^{-1} ww in lake whitefish respectively) (Guzzella et al., 2018). They were generally comparable or lower than those of other studies around the world (0.81–44.2 ng g^{-1} ww in Indus River fish, Pakistan, Robinson et al., 2016); 42–260 ng g^{-1} lw in Lake Huron fish, Canada, Paterson et al., 2016); 300–3,000 ng g^{-1} lw in Lake Victoria fish, Kenya, Oluoch-Otiego et al., 2016).

Hexa-CBs were the most abundant group (50% on average) as in McGoldrick and Murphy (2016), Ondarza et al. (2014) and Sun et al. (2018), followed by hepta- and penta-CBs, with an average presence

of 20% for both groups. This specific composition was probably due both to their molecular structure that make them more stable and persistent than other congeners and to their past use in Aroclor mixture production (Bettinetti et al., 2016; Quadroni and Bettinetti, 2017).

5.4.3 OCs biomagnification

To evaluate the biomagnification of DDTs and PCBs in fish, we considered the summer concentrations measured in landlocked shad and zooplankton, since SIA underlined that only in summer fish relied on pelagic trophic web and it is directly related to zooplankton.

As previously mentioned, to calculate the BMF_{TL} the trophic level of consumer and its prey are needed. However, the use of the general trophic level of the pelagic baseline (level 2) could not describe the complexity of the relationships between zooplankton taxa that have been highlighted by SIA in the present research. The use of the trophic level of predatory cladocerans (the favourite source of pelagic fish) seems not appropriate too, because it is not representative of the biomass of the analysed sample. Consequently, the trophic level of the bulk zooplankton can represent a reasonable compromise. In fact, $\delta^{13}C$ and $\delta^{15}N$ bulk, which contained all zooplankton > 200 µm taxa, reflected the relative average percentage contribution of taxa-specific biomass. For example, in summer, when cyclopoids were predominant, bulk and cyclopoids had similar enrichment values and, therefore, similar trophic level.

The results confirmed the hypothesis that the biomagnification of these chemicals occurred in the landlocked shad. The obtained results are in good agreement with BMF values of landlocked shad in Lake Maggiore reported in Bettinetti et al. (2012b) but slighty different with those calculated by Guzzella et al. (2018). In the latter study BMF for total PCBs are lesser than 1, probably due to the young age of the considered fish.

However, there were some differences in BMF_{TL} calculated in fish collected in Lake Como in 2009, when BMF_{TL} pp'DDE and hexa-PCBs showed values of 6.6 and 3.7 respectively (Bettinetti et al. 2016), probably because the concentrations of DDTs and PCBs in fish and zooplankton were not at equilibrium, after the input of pollutants from Alpine glaciers (Bettinetti et al., 2008).

This study provides new insights in the trophic relationships between two zooplanktivorous fish species and the pelagic zooplanktonic community, suggesting new ideas on the investigation of the variations of isotopic signatures. Furthermore, these results allow a real quantification of biomagnification prey/predator, highlighting which data are suitable for this kind of assessment.

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Chapter VI

"The Unbearable Lightness of" bioaccumulation in the trophic web of Lake Mergozzo

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Abstract

Zooplankton is a key node in many trophic webs, both for food that for POP contaminants that can accumulate in biota. Zooplankton of different size was seasonally sampled for two years in three deep Italian subalpine lakes (Maggiore, Como, Iseo) with the aim to determine the concentrations of PFASs, DDTs and PCBs and assess the seasonality impacts on contaminants concentrations. In general, Lake Maggiore showed the highest concentrations for each group of contaminants with mean values of 7.6 ng g^{-1} ww for PFASs, 65.0 ng g^{-1} dw for DDTs and 65.5 ng g^{-1} dw for PCBs. Considering the composition pattern, PFOS was detected in 96% of the samples and was the predominant PFASs compound in all the lakes; for what concerns DDT and its metabolites, pp' DDE was the most detected congener; in the case of PCBs, the prevalent group was hexa-PCB that constituted 35.4% of the total PCBs contamination.

A seasonal trend was highlighted for all contaminant groups with concentrations in colder months greater than in spring and summer; it was evident that contaminant concentrations were more dependent from seasonality than from size, trophic levels and taxa composition of zooplankton.

Principal component analysis showed that one of the main drivers for the accumulation of most of the studied contaminants is their lipophilicity, except for some compounds such as PFOA.

6.1 Introduction

Bioaccumulation is the process which leads to a higher chemical concentration in an aquatic organism compared to that in water, due to uptake by all exposure routes including dietary absorption, transport across respiratory surfaces and dermal absorption (MacKay and Fraser, 2000). To evaluate the potential degree of bioaccumulation of a substance different types of bioaccumulation metrics and data could be used (Mackay et al., 2013). One of these metrics, trophic magnification factor (TMF), considers the diet as the major route of exposure to a contaminant and its bioaccumulation directly related to the trophic positions of the sampled organisms; particularly, TMF is a field-based metric that gives an indication about the average transfer of contaminant along the trophic web (Borgå et al., 2012; Conder et al., 2012). Trophic magnification factor was suggested as the most conclusive tool for the assessment of contaminant bioaccumulation but, limited to substances that have been quantified in environmental samples and so, commercially available for a long time (Borgå et al., 2012; Gobas et al., 2009). Trophic magnification factor is calculated using the slope of the linear regression between logarithmic concentrations of a given chemical in biota and trophic positions of the sampled biota. If TMF is greater than 1, the chemical shows bioaccumulation behaviour, instead, a TMF less than 1 indicates trophic dilution (Gobas et al., 2009). The potential biomagnification, and subsequent evaluation of TMFs, could be affected by many factors, including chemical properties of contaminants, biological

aspects of biota, spatial and geographical food web characterization and the measure of trophic positions (Borgå et al., 2012; Franklin, 2016; Won et al., 2018). Stable nitrogen and carbon isotope values provide reliable calculations of organism trophic levels. Ratio of nitrogen ($^{15}N/^{14}N$) is more enriched in consumers' tissues than in prey whereas the ratio of carbon ($^{13}C/^{12}C$) has very small trophic fractionation in the same trophic web and is typically used to determine original sources of dietary carbon (Jardine et al., 2006; Post, 2002).

PCBs and DDTs, that belong to the group of Persistent Organic Pollutants (POPs), have been extensively used for sanitary, agricultural and/or industrial purposes from the mid-1940s and then banned/restricted in many countries (UNEP, 2002). They are persistent, "bioaccumulative", subjected to long-range transport and toxic both on wildlife and human (Jones and de Voogt, 1999; Ribeiro et al., 2017; Ruzzin, 2012; Sijm et al., 2007) and, so, they were studied for many decades and investigated today in various matrices (Atmaca et al., 2019; Mitra et al., 2019; Olatunji, 2019; Rigét et al., 2019; Yang et al., 2019). Mercury (Hg) is a toxic trace element, widely distributed in the environment and naturally present on the Earth in low concentrations (Driscoll et al., 2013; Streets et al., 2019). However, the extensive use has resulted in serious contamination of water, sediments and biota (Azad et al., 2019; Vardè et al., 2019; Wang et al., 2019). The outbreak of Minamata disease in Japan has shown Hg magnification within the food web and its associated risks to the health

of humans and ecosystems (Driscoll et al., 2013; Lavoie et al., 2013). TMFs of organo-halogens compounds and mercury have been determined in various freshwater environment (Gantner et al., 2010; Houde et al., 2008; Kidd et al., 2001). TMF are greater than 1, thus, these bioaccumulative substances can serve as benchmarks for assessing the bioaccumulation potential of other contaminants of concern, which showed unclear pattern, such as PFASs (Franklin, 2016; Kelly et al., 2009).

Polyfluoroalkyl and perfluoroalkyl substances (PFASs) are chemical compounds ubiquitarian in every-day products and industrial formulations and so continuously release via point and nonpoint sources into the aquatic environment (Ahrens and Bundschuh, 2014). Their physico-chemical properties give them persistence, capability for long-range transport and possible adverse effects on living organisms (Ahrens and Bundschuh, 2014; Lau et al., 2007). PFASs include thousands of chemicals, the most prominent are perfluorooctanesulfonic acid (PFOS) and perfluoroalkyl carboxylic acids (PFCAs) that have been demonstrated to be bioaccumulative (Houde et al., 2011). The accumulation in the aquatic trophic web poses concern about the risks for the end consumers, included humans. For that reason, the European Commission included PFOS in the list of priority hazardous substances which must be monitored in the EU water bodies, setting an Environmental Quality Standard (EQS) of 9.1 ng g^{-1} ww for fish.

The main goal of this study is the evaluation of TMF of various

contaminants (Hg, PCBs, DDTs, PFASs) in an Italian lacustrine trophic web. The study area is a small but deep lake (Lake Mergozzo, Northern Italy) included in the protected areas of EU Natura 2000 network. The absence of direct sources of pollution and the richness in biodiversity made it as an ideal area to study biomagnification in aquatic trophic web. In particular, the aim was achieved by: 1) defining the status of the lake by the comparison between chemicals concentrations detected in fish from Lake Mergozzo and those recorded in same species samples from other European freshwater; 2) discussing the calculation of relative trophic positions of sampled organisms, 3) deriving TMFs, with emphasis on the sampling design and the use of trophic levels from literature database.

6.2 Materials and methods

6.2.1 Study Area

Lake Mergozzo is a small lake located in the north-west of Italy, in the Piedmont region (Fig. 6.1; Table 8.4.S1) which belongs to the river Ticino basin. In ancient times the lake represented the western branch of the nearby Lake Maggiore. The two lakes were divided ca. five centuries ago by the progressive accumulation of sediments carried by River Toce. L. Mergozzo, located at 193 m a.s.l., is a deep (maximum depth: 73 m) but relatively small (surface area: 1.83 km^2) lake located in Piedmont region (45°57′N, 8°27′E). L. Mergozzo is oligotrophic (TP ca. 4 μg L^{-1}). Since the eighties, this condition was preserved by the diversion of domestic sewage discharges from the lake to the

adjacent River Toce after treatment. Furthermore, the use of motorboats has long been forbidden. L. Mergozzo is classified as a warm monomictic water body and its catchment basin is mainly composed by granitic and metamorphic rocks. The littoral substrate consists mostly of sand and cobble, with a minor percentage of boulder and gravel. Submerged macrophytes are extremely scarce, while a reed bed (*Phragmites australis*) is found along the shoreline (Volta, unpublished data). L. Mergozzo is filled by underwater springs and is connected to Lake Maggiore by a canal. Usually the water flows from L. Mergozzo to L. Maggiore, but in case of big floods, L. Maggiore waters comes to L. Mergozzo. Water residence time in L. Mergozzo is about 6 years.

The fish community of L. Mergozzo is quite similar to the one of the nearby lakes, including littoral warm water-, sublittoral cold water- and open water species (Volta et al., 2016).

6.2.2 Zooplankton, benthos and fish sampling

Two zooplankton samples were collected in October 2016 in the deepest zone of the lake using a 58-cm diameter zooplankton net (450-µm mesh size) hauled vertically in the layer 0-50 m 15 times, in order to obtain a sufficient number of organisms necessary to perform the analyses (total volume filtered = ca. 390 m^3). Being zooplanktivorous fish visual predators, we used a net with a large mesh size to avoid the capture of small body size organisms (i.e. rotifers, early developmental stages of crustacean zooplankton and large

Fig. 6.1 Lake Mergozzo satellite view (from Google Earth).

phytoplankton colonies) which do not transfer pollutants actively to the upper trophic levels (Zaret, 1980). One sample of alive organisms was concentrated in ca. 1L of lake water, frozen at -20°C and subsequently used for carbon (^{13}C) and nitrogen (^{15}N) Stable Isotope Analysis (SIA). The second sample was filtered on glass fibre filters (GF/C, 4.7 cm of diameter, pore size ca. 1.2 µm) and frozen at -20°C and subsequently used for chemicals analysis.

Benthos was sampled with a hand net along the shore. All the items collected were brought to the laboratory where they were individually frozen at -20°C.

Fish were sampled by means of benthic and mesopelagic gill nets and point abundance sampling electric fishing along the shore in the first two weeks of November 2016 (see Volta et al., 2018, for sampling design and details). All fish captured were identified to species, measured (total length to nearest 0.1 cm, LT) and weighed (total weight to nearest 0.1 g, WT). When a lot of fish of different sizes for each species were sampled, specimens were divided in three groups as follows: small sized fish= length <30% of max theoretical total length; medium sized fish= length comprised among 30% and 60% of the max theoretical total length; large sized fish= length >60% of the max theoretical total length. Fish were brought to the laboratory and a portion of dorsal muscle was taken and frozen at -20°C.

6.2.3 Stable Isotopes Analysis (SIA)

SIA was performed on pooled samples of the two size fractions ≥450µm and on fish samples (dorsal muscle tissues). Samples were oven-dried for at least 24 h at 60 °C, homogenized and transferred into tin capsules (size = 5 x 9 mm). The isotopic composition of organic carbon and nitrogen was determined by Ján Veizer from the analyses of CO2 and N2 by the G. G. Hatch Stable Isotope Laboratory at the (Ottawa University of Ottawa, Ontario, Canada) following method already described in previous studies (Visconti et al., 2018; Visconti and Manca, 2011), using a CE 1110 Elemental Analyser (Vario EL III manufactured by Elementar, Germany) and a DeltaPlus Advantage isotope ratio mass spectrometer (Delta XP Plus Advantage manufactured by Thermo, Bremen, Germany) coupled to a ConFlo III interface (Conflo II manufactured by Thermo, Bremen, Germany). The standard deviation of the analyses (SD) based on laboratory internal standards (C-55) was < 0.2h for both ^{13}C and ^{15}N. Isotope ratios were expressed as the parts per thousand (‰) difference from a standard reference of PeeDee Belemnite for carbon and atmospheric N2 for nitrogen according to the equation:

$$(\delta^{13}C),(\delta^{15}N)=[(R_{sample}/R_{standard})-1]\times 1000 \quad (1)$$

where R is the isotopic ratio: $^{13}C/^{12}C$ and $^{15}N/^{14}N$.

6.2.4 Analysis of contaminants

A total of 31 compounds, including Hg, 14 PCBs and 6 DDTs congeners and 10 PFASs were analysed in zooplankton and fish samples. Quantity of benthic organisms was not enough to perform chemical analysis.

The extraction method for DDTs and PCBs compounds is described extensively in Bettinetti et al. (2012) and in Supplementary Materials. Summarising, about 0.5 g of freeze-dried samples were extracted with Soxhlet equipment with n-hexane and acetone. Lipids were determined after repeated weighing of dried extracts. Then, the prepared samples were degraded with H_2SO_4 and cleaned up on a Florisil® columns. OCs compounds in the final extracts were analysed by gas chromatography coupled with ^{63}Ni electron capture detector using an on-column injection system.

Complete extraction and analysis method for perfluoroalkyl acids in biota samples were reported in Supplementary Materials as well as in Mazzoni et al. (2016). Briefly, few grams of fresh pooled and homogenized samples were extracted by sonication with acetonitrile and then purified on $MgSO_4$/NaCl. To remove phospholipids, extracts were filtered through special cartridges. PFASs in the final extract were determined by liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) coupled to a turbulent flow chromatography (TFC) for a better purification of the samples.

Mercury was analysed as total mercury. Approximately 30 mg of freeze-dried samples were weighted, put in quartz boats and analysed

by atomic absorption spectrometry with Direct Mercury Analyzer (DMA-80, Milestone). The quantification was carried out with an external calibration curve. Analysis of blanks and certified reference material (DORM-4 fish protein; DOLT-5 dogfish liver, National Research Council Canada) were performed together with the analysis of samples. Recoveries of the certified reference materials were within 10% of the official reported concentrations. Every type of sample was analysed at least in duplicate to ensure precision of measurements. The detection limit of the instrument was 0.05 ng (Hitchcock et al., 2019).

6.2.5 Determination of Trophic Levels and Trophic Magnification Factors

The two-end-member-mixing model allows for the differentiation between two sources (Post, 2002). In the present study, littoral and pelagic zone in Lake Mergozzo were identified and used according to the following equation to estimate the trophic levels (TL) of zooplankton and fish (Post, 2002):

$$TL = \lambda + \{\delta^{15}N_{consumer} - [\alpha*\delta^{15}N_{base1} + (1 - \alpha)*\delta^{15}N_{base2}]\} / 3.4 \quad (2)$$

where λ was the standard trophic level of baseline organism. In the present study, λ was equal to 2 because primary consumers of zooplankton primary consumers and *Corbicula* were chosen as reference organisms for, respectively, pelagic and littoral signal.
δ^{15}Nbase1 and δ^{15}Nbase2 were the mean δ^{15}N values of littoral and

pelagic baseline; 3.4 was the average increase in $\delta^{15}N$ from a trophic level to the next one in aquatic trophic web.

α was the coefficient represent the proportion of nitrogen in the consumer derived from each food source and could be estimated using carbon stable isotopes following the equation (Post, 2002):

$$\alpha = (\delta^{13}C_{consumer} - \delta^{13}C_{base2}) / (\delta^{13}C_{base1} - \delta^{13}C_{base2}) \quad (3)$$

When α was near 0 the contribution of pelagic source was predominant; at the contrary, α near 1 indicated a strong influence of littoral source.

The lipid content of an organism influenced the $\delta^{13}C$ values, and tissues rich in lipid were more depleted in $\delta^{13}C$ that those rich in protein and carbohydrates (McConnaughey and McRoy, 1979). Moreover, the heterogeneity of lipid content among samples could influence the carbon values. In aquatic organisms a strong relationship between lipid content and the carbon-to-nitrogen ratio (C:N) was found (Post et al., 2007). To obtain the corrected $\delta^{13}C$ values, we chose to perform a mathematical normalization proposed by Post et al. (2007), that used C:N ratio as parameter of conversion:

$$\delta^{13}C_{normalised} = \delta^{13}C_{untreated} - 3.32 + 0.99 * C:N \quad (4)$$

We normalized all data, as suggested by Post et al. (2007) when many samples showed C:N values above the threshold of 3.5.

A linear regression between log-transformed contaminants concentrations (expressed as lipid-normalized concentrations for DDTs and PCBs, as dry weight concentrations for Hg and as wet weight concentrations for PFASs) and the trophic positions of organisms calculated by Eq. 6.2 was conducted.

Trophic Magnification Factors were determined as the antilog with base 10 of the slope b of the linear regression ($TMF = 10^b$) (Borgå et al., 2012).

The R statistical software (R version 3.5.1, R Core Team, 2018) were used to conduct data analysis. Statistical significance was set at $p < 0.05$.

6.3 Results and discussion

6.3.1 Chemicals concentrations in biota of Lake Mergozzo

The occurrence of selected PFASs and OCs and of Hg has been surveyed in specimens collected from Lake Mergozzo in 2016. Detailed data regarding chemicals concentrations are reported in Tables 8.4.S2-S5.

An overall picture of the analysed substances underlines that no sample was completely free of contaminants. In particular, results show that PFASs concentrations, expressed as a sum of congeners, ranged from 0.36 to 60.18 μg kg^{-1} ww. Among these groups of compounds, PFOS and C10-C12 PFCAs displayed the highest detection frequency (DF) (95-100%) while PFNA was only sporadically found (DF = 30%). On the contrary, C6-C8 PFCAs and

PFHxS were always below the detection limit. When considering the composition pattern, PFOS was the most detected PFASs in the majority (84%) of collected fish while PFUnDA was the predominant ones in zooplankton.

On the other hand, OCs were normalised with lipid content. DDTs concentrations, expressed as a sum of two congeners and four respective metabolites of DDT, ranged from 15.91 to 13153.07 μg kg^{-1} lw while PCBs levels, reported as a sum of 14 congeners, were between 17.73 and 14777.73 μg kg^{-1} lw. In terms of detection frequency, pp' DDD and the DDE isomers have been found in the majority of samples (DF = 80-95%), contrary to parental compounds (DDT isomers DF = 40-45%). Moreover, pp' DDD was also the predominant DDTs in all collected specimens except for zooplankton and pike perch. Regarding PCBs, the most frequently detected congeners were PCB 153 and PCB 180 (DF=100%) whose concentrations ranged from 0.24 to 3345.47 μg kg^{-1} lw and from 1.10 to 1768.30 μg kg^{-1} lw, respectively. Lastly, Hg levels were between 20 and 501.06 μg kg^{-1} ww.

To the best of our knowledge, a very few studies has been carried out to investigate the presence of the selected pollutants in Lake Mergozzo. Based on the Directive 2000/60/EC, this lake has been considered in high ecological and good chemicals status (ARPA Piemonte, 2017). The optimal quality conditions of this waterbody are surely facilitated and preserved by the urban sewers diversion, which was realized in the early 80s, and by the prohibition of navigation with

motorboats (ARPA Piemonte, 2017): these practices aid in avoiding high nutrient intake and pollution of urban and industrial origin

Other than by water analysis, environmental assessment is supported by biota monitoring which has become a valuable tool for the chemical status assessment, especially for substances which is prone to accumulate in organisms (Fliedner et al., 2018). An example of this is represented by two of the compounds investigated in the present study. Indeed, the European Water Framework Directive (WFD) requires the assessment of compliance with environmental quality standards (EQSs) in biota for PFOS and Hg. Regarding this last compound, Lake Mergozzo was generally considered unpolluted by heavy metals, so much to be used as clean reference during microbial community test carried out to evaluate the long-term effects caused by exposure to these chemicals (Di Cesare et al., 2016). However, Hg concentrations exceeded the EQS_{biota} limits in all fish collected during this study. In fact, the recorded Hg levels ranged from two to twenty-five-fold the EQS_{biota} referred to fish, that is equal to 20 μg kg^{-1} ww (EC 2013, D. Lgs. 172/2015). Differently, PFOS concentration was over the limit (9.1 μg kg^{-1} ww, EC, 2000; EC, 2013) only in the 32% of samples. Moreover, the Italian legislation on priority substances also defines the EQS_{biota} for DDTs, setting limit at 100 μg kg^{-1} ww (D. Lgs. 172/2015). Based on this, DDTs concentrations never exceed the EQS_{biota} with only one exception represented by pikeperch.

As already explained above, samples collected in Lake Mergozzo belonged to different species. Among them, shad, whitefish, roach and

perch are the most frequently fish studied to investigate European lakes contamination by means biota monitoring. For this reason, these species were chosen to compared chemicals levels detected in the present study with concentrations reported in literature, as Figure 6.2 shows. Particular attention was given to records from Lake Maggiore and Lake Lugano, which belong to the same basin of Lake Mergozzo. Completed data about comparison were reported in Table 8.4.S6. More in details, the obtained results highlighted that PFASs concentrations detected in shad of Lake Mergozzo were very similar to levels found in samples of the same species caught in other European lakes. As expected, PFOS concentrations detected in the present study were lower than Lake Maggiore and Lake Lugano levels. In fact, Mazzoni and co-workers (2019) already suggested that, while Lake Lugano is a possible source of PFOS for Lake Maggiore, Lake Mergozzo could be not influenced by specific releases of this compound. This conclusion seems to be supported also by OCs and Hg results. Indeed, Figure 6.2 showed once more that levels found in shad from Lake Mergozzo were placed between the lowest values recorded in samples of the European lakes. In this case, the higher levels of DDTs and Hg in Lake Maggiore were explained by the presence of a chemical factory producing technical DDT and using a mercury-cell chloralkali plant discharged wastewaters into the River Toce, an important tributary of this lake (Pascariello et al., 2019). Likewise, the average values measured in whitefish samples from Lake Mergozzo did not differ from European levels, confirming the

hypothesis that probably there is not a point source of the investigated contaminants in this lake. However, this is not completely true for roach and perch samples. In particular, the OCs concentrations in roach from Lake Mergozzo were higher than levels recorded in specimens of the same species collected in Lake Maggiore and in Lake Lugano (Figure 6.2). In addition, the concentrations of selected contaminants detected in perch caught during this study were not lower than the levels recorded in fish of this specie collected in lakes belong to the same basin.

Based on what has been said so far, it is difficult to define ecological status of a waterbody through biota monitoring. Indeed, present results highlighted that the final judgment is strongly depend on the considered species and especially on organisms feeding behaviour. For this reason, it is necessary to make a more detailed analysis trying to verify if there is a relationship between chemicals bioaccumulation in biota and samples trophic level.

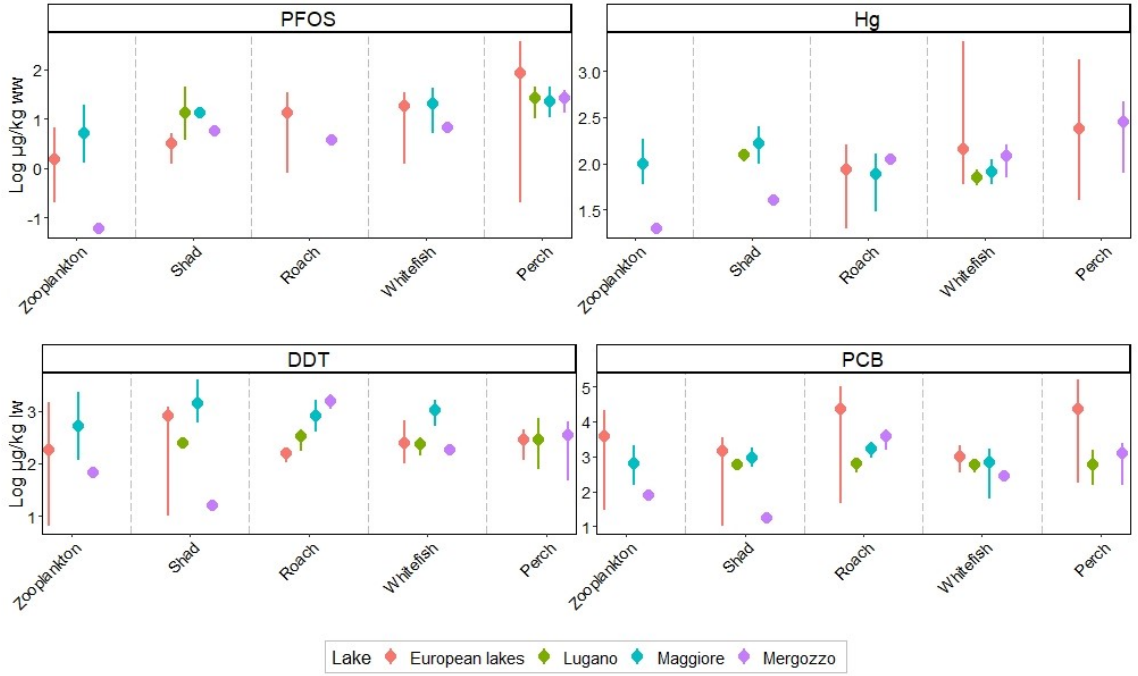

Fig. 6.2. Data comparison among results of this study (in violet) and chemicals concentrations recorded in European lakes (in red). Levels detected in lakes belonging to the same basin of Lake Mergozzo (Lake Lugano in green and Lake Maggiore in blue) were individually represented. Filled dots and bars represent average value and range, respectively. Detailed data are reported in Tables 8.4.S6.

6.3.2 Trophic Magnification Factors

Stable isotope techniques produce a continuous measurements of trophic positions (as opposed to discrete trophic levels) of all organisms, considering magnitude of energy and mass fluxes through different food web pathways and complex interactions such as omnivory (Post et al., 2000). To measure correctly the trophic positions, an appropriate choice of baseline is needed. This choice is one of the most difficult issues to dealt with using stable isotopes for food web characterisation (Jardine et al., 2006). In lakes, the two major sources of available energy are littoral and pelagic productions and $\delta^{13}C$, that reflects the different fractionation carried out by primary producers during the uptake of dissolved inorganic carbon (DIC), is particularly useful to distinguish the two zonations (Post et al., 2000). The chosen baselines had to show consistent differences between their $\delta^{13}C$, typical of littoral and pelagic food webs. As reported by previous studies (Post, 2002; Vander Zanden and Rasmussen, 2001), long-lived primary consumers, such as molluscs, are recommended to standardize the baseline of food webs. In this study, the bivalve *Corbicula fluminea* was sampled for littoral signal, but a different type of organism was necessary for pelagic signal. Zooplankton primary consumers, i.e *Daphnia* sp., *Eubosmina longispina* and *Diaphanosoma brachyurum*, were chosen as the reference organism. *Daphnia* has been already used as pelagic baseline in other deep lakes with similar composition of the zooplanktonic community (Leoni,

2017; Perga and Gerdeaux, 2006; Visconti and Manca, 2011).

Each consumer was allocated to one or the other of these food chains through the α coefficient: when α was 1, the organism was totally benthic, instead, when α = 0 the specimen was totally pelagic. If α values were comprised between 0 and 1, the organism fed on both pelagic and benthic sources. The α values calculated in this study are reported in Table 6.1.

Table 6.1 α values and trophic levels of analysed samples

Species common name	**TL**	**α**
Zooplankton	2.51	0.02
Ruffe	5.01	0.27
Shad	4.10	0.30
Burbot	5.53	-0.03
Chub	4.46	0.34
European whitefish	5.04	0.09
European whitefish	4.84	0.19
Roach	4.40	0.35
Roach	4.77	0.37
Pikeperch	5.11	0.36
Pike	5.28	0.39
European perch	5.22	0.34
European perch	4.68	0.46
Pumpkinseed	4.70	0.45
Largemouth bass	5.21	0.45
Largemouth bass	5.32	0.39
Largemouth bass	5.14	0.40
Char	5.70	0.02
Rudd	4.55	0.31
Rudd	4.67	0.37

Some of the α values were very similar to zero and all were less than 0.5. This implied that the pelagic source was predominant in the diet of zooplankton community (that include copepods and cladoceran predators) as expected, but it was important also for all fish species analysed. These results confirmed the visual observation and the previous knowledge of the morphological characteristics of the Lake Mergozzo. Indeed, this lake presents a short and steep littoral area, that does not allow the growth of many aquatic plants and the settlement of big populations of mollusc and long-live primary consumers, limiting the littoral resources. Based on this, all samples were analysed together as part of a unique food web.

Considering the enrichment factor used to calculate the trophic positions, the mean trophic fractionation of 3.4‰ suggested by Post (2002) for aquatic food webs was selected. The use of unique value across many trophic levels is a simplification, which could lead to under or overestimate the calculated TMF, but is also the only solution propose in the European Union Directive 2013/39/EU and related paper (Kidd et al., 2019).

Calculated trophic levels (TLs) are reported in Table 6.1. They varied from 2.51 to 5.70 and the difference between the highest and the lowest trophic level is 3.2, higher than the minimum spam suggested by Kidd et al., (2019).

TMFs have been derived for those substances which have more than 90% of data >LOQ. Censored data were substituted by ½ LOQ. TMFs were derived also for total DDTs and the sum of isomeric PCBs. Data

are reported in Table 6.2.

Regression slope of Hg was significant (p-value < 0.001) and the derived TMF was greater than 1 as expected, with values of 2.3 and 2.4, using concentration expressed on dry weight and on wet weight respectively, which were consistent with the worldwide data analysis of Lavoie et al. (2013) who calculated a mean TMF value for freshwater sites of 4.3 ± 4.8. Normalization of Hg concentrations to dry weight provides more information because dry weight is probably more correlated with tissue protein content, but, the wet weight–based TMF and the dry weight–normalized TMF were not statistically different as reported in other studies (Kidd et al., 2019; Wyn et al., 2009).

TMF of PCBs and DDTs were in the upper range of literature data (PCB-153: 6 ± 6.74; DDT: 1.64 ± 0.39) collected and reviewed by Walters et al. (2016). It was observed that oligotrophic lakes showed higher TMF values for organochlorine substances (Houde et al., 2008) and their biomagnification in food webs of deep lakes was greater when trophic nets were more dependent on pelagic carbon sources (Borgå et al., 2012). Both these two factors might concur to higher TMF for PCBs and DDTs in Lake Mergozzo.

Concentrations data were sufficient to derive TMF for PFOS and long chain PFCAs with 10 to 12 carbon atoms. TMF was significant for PFOS but not significant for long chain PFCAs. PFOS value (TMF = 3.00) was in the range of those measured in different trophic webs around the world (Franklin, 2016), but most of these TMF were

Table 6.2. Derived TMFs of selected chemicals (in bold significant p values < 0.005).

Chemical	**slope**	**TMF**	**[95%]**	**R^2**	**p value**
Hg (mg/kg dw)	0.36	2.27	[1.45; 3.55]	0.42	**0.001**
Hg (mg/kg ww)	0.38	2.38	[1.57; 3.61]	0.49	**0.000**
PCB 153 (µg/kg lw)	1.00	10.01	[3.55; 28.27]	0.52	**0.000**
Penta_CB (µg/kg lw)	0.99	9.66	[1.05; 88.76]	0.16	**0.045**
Hexa_CB (µg/kg lw)	1.10	12.49	[4.14; 37.67]	0.54	**0.000**
Hepta-CB (µg/kg lw)	0.69	4.92	[1.68; 14.36]	0.32	**0.006**
pp'DDD (µg/kg lw)	1.15	14.09	[4.78; 41.56]	0.57	**0.000**
pp'DDT tot (µg/kg lw)	0.47	2.92	[1.05; 8.13]	0.17	**0.042**
PFOS (µg/kg ww)	0.48	3.00	[1.17; 7.71]	0.21	**0.025**
PFDA (µg/kg ww)	0.32	2.07	[0.87; 4.92]	0.10	0.094
PFUnDA (µg/kg ww)	0.08	1.21	[0.53; 2.72]	-0.04	0.631
PFDoDA (µg/kg ww)	0.13	1.34	[0.53; 3.43]	-0.03	0.515

derived in trophic webs which included top predators such as birds and mammals

6.3.3 Discussion about the use of literature TLs and on sampling design

We tested the possibility to use fish trophic levels taken from on-line available database (www.fishbase.org) to derive TMFs instead of TLs derived by experimental SIA. TLs in Fishbase database were derived from diet composition data mainly based on observations of the stomach content of fish as they occur in the wild on a global scale. This approach is suggested by Italian Guideline on Biota Monitoring issued by Italian Environmental Agency (ISPRA) as an alternative when SIA data are not available (ISPRA, 2016). TMFs values obtained by this approach were reported in Table 8.4.S7.

To evaluate if the two approaches were comparable, we confronted TLs between each other. Calculated trophic levels were systematically higher than those reported in the online database, with marked differences in fish that occupied low trophic levels as shown in figure 6.3. Paired t-test between experimental and literature TLs confirmed that the differences are statistically significant ($p<0.001$).

However, the determination of TMFs did not depend by the reported trophic levels values, but by the relationships between them that should remain constant. Lines that interpolated the two datasets, obtained with linear regressions, had two slopes statistically different, consequently, the two lines were not parallel to each other and the two approaches were not comparable.

Therefore, TMFs values obtained by using literature TLs, even if not different by TMFs obtained by stable isotope approach, were not useful in this trophic web. Then, the use of SIA approach is highly recommended.

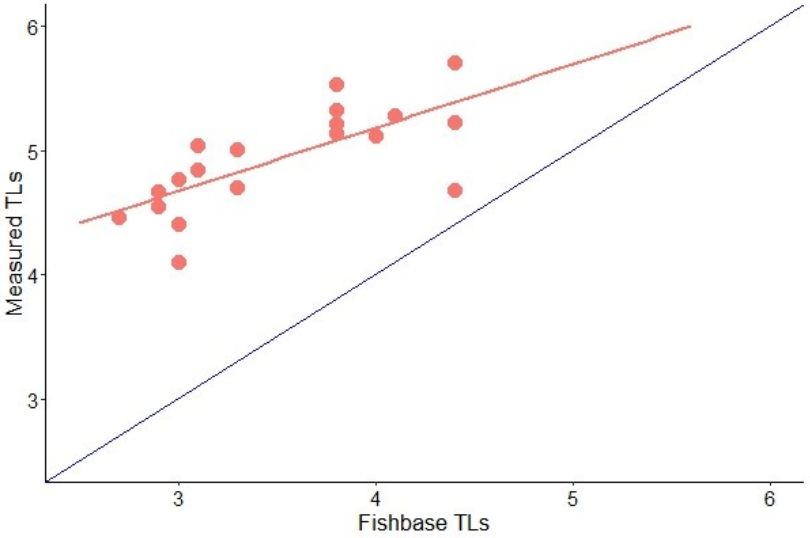

Fig. 6.3. Comparison between measured TLs and Fishbase TLs. In red: calculated data and their regression line. In blue: 1:1 regression line.

Observing experimental design of the present study, the main gap is the disequilibrium between fish and invertebrate data. Then, the effect of the elimination of zooplankton on TMF regressions was tested. Results are reported in Table 6.3. After the zooplankton elimination, the dataset was composed by 19 samples from 13 different fish species, with TL spanning from 4.10 to 5.70. According to literature reviews, it is important to "benchmark" the results for the study design

against a substance universally recognized to be prone to biomagnification, for the evaluation of the biomagnification potential for other chemicals (Borgå et al., 2012 and Franklin, 2016). For that reason, in our study we included Hg as a positive control. In particular, our results demonstrated that its TMFs were still significant even if they were derived for fish-only food web and the values (2.85-2.99) were still consistent with the available literature data (Lavoie et al., 2013).

In the case of most of PCBs and DDTs, the reduction of TL range led to an expected increase of the p-values of regression slopes with a loss in statistical significance. But the most interesting variation was found for PFOS, whose TMF became not significant and <1, suggesting that biomagnification is not an effective process for PFASs in fish in this environment. Similar results were highlighted by Kelly et al. (2009), who obtained a narrower TL range for pure piscivorous part of the food web with a TMF of 0.47 for PFOS and from 0.6 to 1.1 for C10-C12 PFCAs. For what concerns freshwater lentic environments, negative relationships were found between PFASs and $\delta^{15}N$ in some lakes, suggesting that no biomagnification of PFASs occurred through these food webs and that was the carbon source and not the trophic position in the food web which affected PFASs concentrations in biota in the studied environments (Lescord et al., 2015).

TMFs for any PFASs, including PFOS, were not significant in Baiyangdian lake, China (Zhou et al., 2012), while Fang et al. (2014) measured TMF from 2.25 to 2.59 for long chain PFCAs and 3.74 for

PFOS, analysing only fish species in lake Taihu, China. It is interesting to note that in an estuarine environment demersal food web, TMFs were in the range 0.18-1.5, with a TMF of 0.94 for L-PFOS, while derived TMF for PFOS in benthic food webs was 2.5 (Munoz et al., 2017). The re-analysis of the same data with advanced statistical techniques showed that none of the investigate PFASs could be considered biomagnificable in the whole Gironde estuarine food web (Ballutaud et al., 2019).

The lack of significance for PFOS biomagnification in our only-fish food web can be explained by the high pelagic character of this food web, due to the absence of degrading littoral areas. Furthermore, the fact that for fish the contribution of bioconcentration from water is at least as significant as the food assumption can also be hypothesised. Thus, fish concentrations are more regulated by the concentrations of specific binding proteins (Ng and Hungerbühler, 2013) or membrane phospholipids (Armitage et al., 2013) in the bioconcentration process than by the trophic position of different fish species.

Another confounding factor leading to the overestimation of TMFs at some sites might be the occurrence of unidentified precursors and their enhanced biotransformation in fish compared to invertebrates (Simmonet-Laprade et al., 2019), but no data on PFASs precursors are available for Lake Mergozzo.

Table 6.3. Derived TMFs for fish-only food web (in bold significant p values < 0.005).

Chemical	slope	TMF	[95%]	R^2	p value
Hg (mg/kg dw)	0.46	2.85	[1.33; 6.13]	0.29	**0.01**
Hg (mg/kg ww)	0.48	2.99	[1.47; 6.06]	0.35	**0.00**
PCB 153 (µg/kg lw)	0.60	4.02	[0.73; 22.24]	0.10	0.10
Penta_CB (µg/kg lw)	1.36	23.03	[0.51; 1049.81]	0.10	0.10
Hexa_CB (µg/kg lw)	0.69	4.88	[0.79; 30.26]	0.12	0.08
Hepta-CB (µg/kg lw)	0.87	7.45	[1.17; 47.24]	0.19	**0.03**
pp'DDD (µg/kg lw)	0.60	4.02	[0.73; 22.12]	0.10	0.10
pp'DDT tot (µg/kg lw)	0.62	4.16	[0.71; 24.41]	0.10	0.11
PFOS (µg/kg ww)	-0.09	0.80	[0.2; 3.30]	-0.05	0.75
PFDA (µg/kg ww)	-0.07	0.86	[0.21; 3.47]	-0.06	0.82
PFUnDA (µg/kg ww)	-0.06	0.87	[0.21; 3.51]	-0.06	0.83
PFDoDA (µg/kg ww)	0.05	1.13	[0.22; 5.74]	-0.06	0.88

6.4 Conclusions

Despite Lake Mergozzo does not receive direct inputs of contaminants in its water, it does not represent a pristine site so much to be used as clean reference. Indeed, how demonstrated by the obtained results, the levels of Hg in all fish species were above the allowed EQS_{biota} and some species showed high concentrations of the other investigated organic substances. The bioaccumulation along trophic web was well characterised in this food web for OCs compounds and Hg, instead results for PFASs were inconsistent when reference organisms changed. This should lead to new studies for a better understanding of PFASs accumulation in fish.

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Chapter VII

General conclusions

This Ph.D. study has been focused on current challenges in environmental sciences and ecotoxicology with the purpose to improve the knowledge of the mechanisms of organic pollutants accumulation in lacustrine organisms, with a specific focus on regional lacustrine environments in order to provide new data for local and European risk assessments and implementation of EU legislation. In particular, the present work has evaluated the presence of legacy and emerging contaminants in some Italian subalpine lakes with the aim of better understanding the environmental fate of these chemicals, their sources and their possible adverse impacts on humans and top predators. On the other hand, the attention has been focused on the relationships between organisms, first in the pelagic zooplankton community, then between zooplankton and fish and finally among more general lacustrine trophic web. Especially, the efforts were devoted to understand the potential links between biological populations and contaminants behaviour (seasonal trends, bioaccumulation…).

In conclusion, biota monitoring, both on zooplankton and fish, can be used as a valuable tool to classify the quality status of water bodies when the water concentrations are lower than limits of detection of available instrumentations and methods. Analysis of zooplankton as bulk could be considered a practical alternative both for the

description of community composition and for analytical determinations.

Notwithstanding the normative restrictions, DDTs, PCBs, PFOS and Hg still reached quantifiable concentrations in biota of the studied lakes and moreover, PFOS concentrations in fish caught in Lake Maggiore exceeded the EU EQS_{biota}. However, there were not evidences of a risk for fish consumption by humans, while a moderate risk of secondary poisoning for predators is highlighted. Regarding the evaluation of the ecological risk of PFASs, these data established that concentrations must be measured in the whole fish, since the use of fillet would underestimate the exposure of predators who feed also on the interior organs of prey that are the most contaminated part of the body.

For many of these chemicals it was not possible to identify specific point sources of contamination, such as industrials plants, except for DDTs pollution in Lake Maggiore. In this case, pollution comes from the presence of the old chloro-alkali plant in Pieve Vergonte (VCO) on river Toce, a tributary of the Lake Maggiore. Regarding PFASs, this research underlined a correlation between the levels of the pollutants with the dimension of the basins area, the number of inhabitants and the presence of industrial area, confirming that wastewater treatment plants were significant input source points for these contaminants.

This study provided also new insight on the seasonally trend in contaminants concentration in zooplankton, that seemed to be

inversely related to the total biomass and not to the size of specimens or zooplankton taxa composition.

The analysis of trophic webs with the use of carbon and nitrogen stable isotopes revealed that feeding relationships are not fixed and stable over time but they are probably ruled by the availability of resources. For instance, the zooplanktivorous fish in Lake Como changed their diet during cold season when the pelagic resources and the zooplanktonic biomass were scarce, preferring to rely on littoral preys. Furthermore, stable isotopes values of different fish species showed how the classical division in pelagic/benthic organism approximate the real picture that is more complex. In fact, the area of feeding could be influence by the morphological characteristic of lakes or by other abiotic factors. The analysis of diet also showed a situation far from the subdivision in primary consumers, secondary consumers and predators because many fish resulted as omnivore, with different feeding strategies also within the same taxon.

A detailed description of the trophic relationships between zooplanktivorous fish species and pelagic zooplanktonic community allow a real quantification of contaminant biomagnification when the organisms are actually in direct feeding interaction. Biomagnification of OCs compounds was well characterised considering both a single interaction consumer/prey and an average transfer, calculated by the means of trophic magnification factor. Higher values of TMF observed in Lake Mergozzo than BMF_{TL} calculated in Lake Como could be explained by the fact that bioaccumulation of organochlorine

substances was higher in oligotrophic lakes than in the more eutrophic lakes such as Lake Como.

Hg showed a significant increase of concentration related to the trophic position of organisms as widely documented in literature, confirming that both the approach and sampling methods for the calculation of bioaccumulation were correct. On the contrary, results of biomagnification for PFOS were inconsistent when the reference food web was not composed by both invertebrate and fish, but only by fish species. This imply that further studies should be carried out for a better understanding of PFOS accumulation in fish.

Moreover, future researches should fill the gap of knowledge regarding contamination in those lakes, such as Lake Garda and Lake Iseo, that are mentioned in this study but not deeply investigated due to time schedules. Based on the sampling design and the results obtained in Lake Mergozzo, future investigations should be carried out to evaluate trophic transfer of contaminants in the other larger and deep subalpine lakes, linking results to the trophic status of the different environments. These studies will provide reliable data for planning sustainable use and management of freshwater resources which remain an important aspect of environmental sciences.

Chapter VIII

Supplementary material

8.1 Organic Contaminants in Zooplankton of Italian Subalpine Lakes: Patterns of Distribution and Seasonal Variations

Chemicals and solvents

All reagents were analytical reagent grade. LC–MS grade Chromasolv acetonitrile and concentrated formic acid were purchased from Sigma-Aldrich. Water (<18 MΩcm resistivity) was produced by a Millipore Direct-QUV water purification system (Millipore, Bedford, MA, USA).

HybridSPE® Phospholipid Ultra cartridges (30 mg, 1 mL SPETubes) were obtained by Sigma Aldrich (St. Louis, Missouri, USA).

Table 8.1.S1. Main morphology characteristics of deep subalpine lakes.

	Maggiore	**Como**	**Iseo**
Altitude - m a.s.l.	193	198	186
Area - km^2	213	146	61
Maximum depth - m	370	425	251
Mean depth – m	178	154	123
Volume - km^3	37	22	8
Main inflowing rivers	Ticino, Toce	Adda	Oglio
Outflowing river	Ticino	Adda	Oglio
Mean outflow discharge - $m^3\ s^{-1}$	291.3	158	58.7
Theoretical renewal time – years	4.1	12.7	4.5/7.2
O_2 hypolimnetic - mg L^{-1}	8	8	0.2
Water temperature (min-max) - °C	7.1-17	7.6-20.3	10.7-18.3
Total phosphorus (min-max) - µg L^{-1}	2.5-23	8.25-22.8	8.9-25.7
Transparency (min-max) - Secchi disk m	4.5-10	4.9-8.1	3.5-6.2
Total suspended solids (min-max) - mg L^{-1}	0.5-3.5	2.5-3.3	2.5-2.8
Total nitrogen (min-max) - mg L^{-1}	0.9-1.3	1.07-1.12	0.7-0.9
Dissolved oxygen (min-max) - mg L^{-1}	8.5-10	9.9-11.9	8.5-11

Table 8.1.S2. Monitoring Plan.

Lake	Number of samples	Monitoring periods	Number of samples per size			Number of samples per season			
			≥200	≥450	≥850	Winter	Spring	Summer	Autumn
Maggiore	23	08/2015-11/2015 11/2016 - 08/2018		12	11	4	6	8	5
Como	38	06/2013 11/2016 - 11/2018	17	13	8	15	11	7	5
Iseo	12	11/2016 - 11/2018	9	3		3	4	2	3

Table 8.1.S3. List of PFASs compounds targeted in the present study, corresponding internal standards (ISs) and LC/MS/MS parameters for all target analytes and internal standards.

Target analytes	**Abbreviation**	**Precursor ion (*m*/*z*)**	**Product ions (*m*/*z*)**	**Collision energy**	**Corresponding ISs**
Perfluoropentanoic acid	PFPeA	262.9	69.0	39	^{13}C3-PFPeA
			218.9	11	
Perfluorohexanoic acid	PFHxA	312.9	119.1	22	^{13}C2-PFHxA
			268.9	11	
Perfluoroheptanoic acid	PFHpA	362.9	169.0	18	^{13}C4-PFOA
			318.9	12	
Perfluorooctanoic acid	PFOA	412.9	169.0	19	^{13}C4-PFOA
			368.9	13	
Perfluorononanoic acid	PFNA	462.9	218.9	18	^{13}C5-PFNA
			418.9	13	
Perfluorodecanoic acid	PFDA	512.9	268.9	18	^{13}C2-PFDA
			468.9	13	
Perfluoroundecanoic acid	PFUnDA	562.9	268.8	20	^{13}C2-PFUnDA
			518.8	14	
Perfluorododecanoic acid	PFDoDA	612.9	318.8	20	^{13}C2-PFDoDA
			568.9	14	

Target analytes	Abbreviation	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	Collision energy	Corresponding ISs
Perfluorotridecanoic acid	PFTrDA	662.9	619.0	15	^{13}C2-PFDoDA
			369.0		
Perfluorotetradecanoic acid	PFTeDA	712.9	669.0	15	^{13}C2-PFDoDA
			419.0		
Pefluorohexadecanoic acid	PFHxDA	812.9	769.0	15	^{13}C2-PFDoDA
Perfluorooctodecanoic acid	PFODA	912.9	869.0	16	^{13}C2-PFDoDA
Perfluorobutane sulphonate	PFBS	298.9	80.2	44	^{13}C2-PFHxA
			99.1	32	
Perfluoropentane sulphonate	PFPeS	348.9	99.0	35	^{13}C4-PFOS
			79.9	40	
Perfluorohexane sulphonate	PFHxS	398.9	80.1	38	^{18}O2-PFHxS
			99.0	34	
Perfluoroheptane sulphonate	PFHpS	449.9	99.0	35	^{13}C4-PFOS
			79.3	40	
Perfluorooctane sulphonate*	PFOS *	498.9	80.3	45	^{13}C4-PFOS
			99.1	45	
Pefluorononane sulphonate	PFNS	548.9	99.0	40	^{13}C4-PFOS
			80.0	45	

Target analytes	**Abbreviation**	**Precursor ion (*m*/*z*)**	**Product ions (*m*/*z*)**	**Collision energy**	**Corresponding ISs**
Perfluorodecane sulphonate	PFDS	598.9	99.0	42	^{13}C4-PFOS
			79.9	46	
Perfluorododecane sulphonate	PFDoS	699.0	99.1	50	^{13}C4-PFOS
			80.0	46	
Perfluoro-n-[^{13}C3] pentanoic acid	$^{13}C_3$-PFPeA	267.0	222	11	n/a
Perfluoro-n-[^{13}C2] hexanoic acid	$^{13}C_2$-PFHxA	314.9	269.9	11	n/a
Perfluoro-n-[^{13}C4] octanoic acid	$^{13}C_4$-PFOA	416.9	371.9	13	n/a
Perfluoro-n-[^{13}C5] nonanoic acid	$^{13}C_5$-PFNA	467.9	422.9	13	n/a
Perfluoro-n-[^{13}C2] decanoic acid	$^{13}C_2$-PFDA	514.9	469.9	13	n/a
Perfluoro-n-[^{13}C2] undecanoic acid	$^{13}C_2$-PFUnDA	564.9	519.8	14	n/a
Perfluoro-n-[^{13}C2] dodecanoic acid	$^{13}C_2$-PFDoDA	614.9	569.9	14	n/a
Perfluoro-n-hexane [^{18}O2] sulphonate	$^{18}O_2$-PFHxS	402.9	103.0	34	n/a
Perfluoro-n-octane [^{13}C4] sulphonate	$^{13}C_4$-PFOS	502.9	99.1	45	n/a

Table 8.1.S4. PFASs concentrations in zooplankton samples (ng g^{-1} ww).

	PFHxA	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFUnDA**	**PFDoDA**	**PFTrDA**	**PFTeDA**	**PFBS**	**PFHxS**	**PFOS**
Lake Maggiore (n=19)												
% positive samples	0	15.8	68.4	94.7	94.7	94.7	89.5	52.6	52.6	15.8	47.4	100.0
min	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.3
median	<LOD	<LOD	0.1	0.2	0.4	0.2	0.1	<LOD	<LOD	<LOD	<LOD	3.9
max	<LOD	0.9	4.0	0.9	1.8	1.8	2.0	0.8	0.4	1.0	1.9	18.9
mean	<LOD	<LOD	0.6	0.3	0.6	0.4	0.3	0.1	0.0	<LOD	0.2	5.1
Lake Iseo (n=7)												
% positive samples	0	14.3	57.1	71.4	71.4	71.4	71.4	57.1	57.1	28.6	28.6	85.7
min	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
median	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.07	0.03	<LOD	<LOD	<LOD	0.4
max	<LOD	0.1	9.4	0.1	0.3	0.3	0.3	0.2	0.2	4.3	0.3	1.8
mean	<LOD	<LOD	1.4	<LOD	0.1	0.08	0.09	0.06	0.05	0.7	<LOD	0.6

	PFHxA	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFUnDA**	**PFDoDA**	**PFTrDA**	**PFTeDA**	**PFBS**	**PFHxS**	**PFOS**
Lake Como (n=25)												
% positive samples	3.8	26.9	69.2	92.3	96.2	96.2	96.2	76.9	80.8	19.2	30.8	96.2
min	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.0	<LOD	<LOD	<LOD	<LOD	<LOD
median	<LOD	<LOD	<LOD	0.2	0.4	0.3	0.2	0.1	<LOD	<LOD	<LOD	2.5
max	<LOD	0.1	5.0	0.9	1.4	0.8	1.0	1.0	1.0	0.9	1.3	6.7
mean	<LOD	<LOD	0.4	0.3	0.5	0.3	0.3	0.2	0.1	<LOD	0.1	2.4
LOD	0.06	0.1	0.1	0.07	0.07	0.04	0.03	0.07	0.05	0.3	0.1	0.2

Table 8.1.S5. DDTs concentrations in zooplankton samples (ng g^{-1} dw). LODs are 0.1 ng g^{-1} dw.

	op'DDE	**pp'DDE**	**op'DDD**	**pp'DDD**	**op'DDT**	**pp'DDT**
Lago Maggiore (n=22)						
% positive samples	100	100	100	100	100	100
min	1.1	4.5	2.5	4.1	0.4	1.6
median	4.0	21.4	11.6	9.7	2.0	6.3
max	20.2	38.3	36.0	37.2	11.2	21.3
mean	6.2	19.5	13.7	14.6	2.7	8.2
Lago Iseo (n=11)						
% positive samples	36.4	100	81.8	72.7	36.4	63.6
min	<LOD	0.5	<LOD	<LOD	<LOD	<LOD
median	<LOD	1.6	1.3	2.1	<LOD	0.2
max	20.0	2.7	15.8	7.6	2.3	3.3
mean	3.2	1.6	3.9	2.4	0.3	0.8

	op'DDE	**pp'DDE**	**op'DDD**	**pp'DDD**	**op'DDT**	**pp'DDT**
Lago Como (n=38)						
% positive samples	7.9	100	81.6	68.4	26.3	71.1
min	<LOD	0.3	<LOD	<LOD	<LOD	<LOD
median	<LOD	2.0	1.4	0.9	<LOD	0.9
max	7.4	14.0	23.9	14.3	1.5	12.4
mean	0.3	2.8	2.5	1.7	0.2	1.4

Table 8.1.S6. PCBs concentrations in zooplankton samples (ng g^{-1} dw). LODs are 0.1 ng g^{-1} dw.

	PCB 18	**PCB 28+31**	**PCB 52**	**PCB 44**	**PCB 101**	**PCB 149**	**PCB 118**	**PCB 153**	**PCB 138**	**PCB 180**	**PCB 170**	**PCB 194**	**PCB 209**
Lago Maggiore (n=22)													
% positive samples	100	100	100	81.8	100	100	100	100	100	100	100	86.4	13.6
min	0.9	0.2	0.8	<LOD	2.8	0.6	0.3	1.5	1.3	0.7	0.3	<LOD	<LOD
median	4.3	1.4	3.3	1.7	7.9	3.8	2.9	9.0	7.6	6.1	2.3	0.7	<LOD
max	9.1	15.9	11.7	16.3	25.6	14.1	33.1	28.2	22.1	17.8	8.0	9.8	0.6
mean	4.4	3.2	4.3	3.8	9.1	4.2	5.0	11.0	9.5	6.5	2.8	1.6	<LOD

	PCB 18	**PCB 28+31**	**PCB 52**	**PCB 44**	**PCB 101**	**PCB 149**	**PCB 118**	**PCB 153**	**PCB 138**	**PCB 180**	**PCB 170**	**PCB 194**	**PCB 209**
Lago Iseo (n=11)													
% positive samples	63.6	27.3	54.5	63.6	81.8	54.5	54.5	90.9	72.7	81.8	36.4	9.1	0
min	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
median	0.7	<LOD	0.2	1.9	3.2	0.6	0.3	2.3	3.6	0.8	<LOD	<LOD	<LOD
max	12.4	6.9	46.3	6.3	17.1	29.5	7.5	37.2	18.9	25.1	11.6	2.0	<LOD
mean	2.5	0.9	6.0	2.3	5.0	3.7	1.3	7.3	5.1	5.0	1.4	0.2	<LOD
Lago Como (n=38)													
% positive samples	34.2	47.4	50.0	76.3	89.5	34.2	42.1	92.1	57.9	57.9	23.7	5.3	0
min	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
median	<LOD	<LOD	<LOD	1.2	2.8	<LOD	<LOD	3.6	0.6	0.2	<LOD	<LOD	<LOD
max	20.1	4.6	8.8	7.2	20.7	41.6	10.8	24.2	14.8	14.8	7.2	0.1	<LOD
mean	1.8	1.0	0.6	1.8	4.3	2.2	0.6	4.3	2.3	1.6	0.3	<LOD	<LOD

Table 8.1.S7. Taxa composition of zooplankton community in Lake Como and Maggiore.

Lake	Size	Date	Season	Filter feeders (Cladocera) mg m^{-3}	Predators (Cladocera) mg m^{-3}	Cladocera mg m^{-3}	Copepods mg m^{-3}	Total biomass mg m^{-3}
Como	200	07/06/2013	Spring	96.6	0.1	96.7	19.9	116.6
Como	450	07/06/2013	Spring	34.5	0.1	34.6	4.3	38.9
Como	850	07/06/2013	Spring	14.3	0.3	14.6	0.0	14.6
Como	200	05/12/2016	Autumn	3.2	0.0	3.2	14.1	17.3
Como	450	05/12/2016	Autumn	0.3	0.0	0.3	1.1	1.4
Como	200	07/12/2016	Autumn	4.4	0.0	4.4	15.7	20.1
Como	450	07/12/2016	Autumn	1.6	0.0	1.6	4.9	6.5
Como	200	15/02/2017	Winter	1.0	0.0	1.0	20.3	21.3
Como	450	15/02/2017	Winter	1.1	0.0	1.1	3.3	4.4
Como	450	16/02/2017	Winter	0.4	0.0	0.4	1.5	2.0
Como	200	23/05/2017	Spring	38.0	3.8	41.8	33.6	75.4
Como	450	23/05/2017	Spring	30.6	2.9	33.5	4.0	37.4
Como	850	23/05/2017	Spring	22.0	3.1	25.1	0.0	25.1

Lake	Size	Date	Season	Filter feeders (Cladocera) mg m^{-3}	Predators (Cladocera) mg m^{-3}	Cladocera mg m^{-3}	Copepods mg m^{-3}	Total biomass mg m^{-3}
Como	200	07/06/2017	Spring	13.0	1.3	14.3	0.5	14.8
Como	450	07/06/2017	Spring	15.0	0.9	15.9	0.1	16.1
Como	850	07/06/2017	Spring	10.6	5.1	15.6	0.0	15.6
Como	200	28/08/2017	Summer	40.3	2.1	42.4	24.1	66.5
Como	450	28/08/2017	Summer	13.0	1.5	14.5	0.3	14.8
Como	850	28/08/2017	Summer	0.2	0.8	1.0	0.0	1.0
Como	200	29/08/2017	Summer	12.3	1.0	13.2	17.1	30.4
Como	450	29/08/2017	Summer	3.6	1.3	4.9	0.1	5.0
Como	850	29/08/2017	Summer	0.1	1.4	1.4	0.0	1.4
Como	200	15/11/2017	Autumn	6.7	0.8	7.5	11.4	18.9
Como	200	16/11/2017	Autumn	3.3	0.3	3.6	10.0	13.6
Como	200	07/02/2018	Winter	0.4	0.0	0.4	8.0	8.5
Como	200	08/03/2018	Winter	0.5	0.0	0.5	5.6	6.0
Como	200	07/05/2018	Spring	45.7	0.6	46.3	58.0	104.3
Como	450	07/05/2018	Spring	37.4	0.2	37.6	0.2	37.8
Como	850	07/05/2018	Spring	20.1	0.6	20.7	0.0	20.7

Lake	Size	Date	Season	Filter feeders (Cladocera) mg m^{-3}	Predators (Cladocera) mg m^{-3}	Cladocera mg m^{-3}	Copepods mg m^{-3}	Total biomass mg m^{-3}
Como	200	05/06/2018	Spring	21.8	2.0	23.8	9.4	33.2
Como	450	05/06/2018	Spring	22.3	1.8	24.0	0.0	24.0
Como	850	05/06/2018	Spring	33.7	4.1	37.8	0.0	37.8
Como	200	30/07/2018	Summer	64.4	1.4	65.7	20.9	86.7
Como	450	30/07/2018	Summer	19.5	1.7	21.2	0.0	21.2
Como	850	30/07/2018	Summer	1.7	3.5	5.3	0.0	5.3
Como	200	06/08/2018	Summer	61.3	0.6	61.9	12.1	74.0
Como	450	06/08/2018	Summer	17.6	1.1	18.6	0.0	18.6
Como	200	26/11/2018	Autumn	3.5	0.1	3.6	8.1	11.7
Maggiore	450	18/08/2015	Summer	12.1	1.2	13.3	0.5	13.8
Maggiore	850	18/08/2015	Summer	2.2	3.0	5.1	0.0	5.1
Maggiore	450	19/11/2015	Autumn	3.6	0.1	3.7	14.8	18.5
Maggiore	850	19/11/2015	Autumn	0.4	0.7	1.2	0.0	1.2
Maggiore	450	16/11/2016	Autumn	3.3	0.5	3.9	0.8	4.7
Maggiore	850	16/11/2016	Autumn	ND	ND	ND	ND	3.9
Maggiore	450	18/01/2017	Winter	2.0	0.0	2.0	1.7	3.8

Lake	Size	Date	Season	Filter feeders (Cladocera) mg m^{-3}	Predators (Cladocera) mg m^{-3}	Cladocera mg m^{-3}	Copepods mg m^{-3}	Total biomass mg m^{-3}
Maggiore	850	18/01/2017	Winter	0.4	0.3	0.7	0.0	0.7
Maggiore	450	17/05/2017	Spring	5.8	1.9	7.7	5.5	13.2
Maggiore	850	17/05/2017	Spring	18.5	4.5	23.0	0.0	23.0
Maggiore	450	08/08/2017	Summer	24.6	2.3	26.9	3.6	30.5
Maggiore	850	08/08/2017	Summer	1.1	1.7	2.8	0.0	2.8
Maggiore	450	20/11/2017	Autumn	11.1	1.5	12.7	6.3	19.0
Maggiore	850	20/11/2017	Autumn	0.4	0.7	1.2	0.0	1.2
Maggiore	450	24/01/2018	Winter	2.7	0.4	3.1	1.6	4.7
Maggiore	850	24/01/2018	Winter	0.5	0.1	0.6	0.0	0.6
Maggiore	450	09/05/2018	Spring	8.4	0.1	8.5	30.8	39.3
Maggiore	850	09/05/2018	Spring	2.1	0.1	2.2	0.0	2.2
Maggiore	450	07/08/2018	Summer	12.1	1.2	13.3	0.5	13.8
Maggiore	850	07/08/2018	Summer	1.4	1.6	3.0	0.0	3.0
Maggiore	450	20/11/2018	Autumn	3.6	0.1	3.7	14.8	18.5

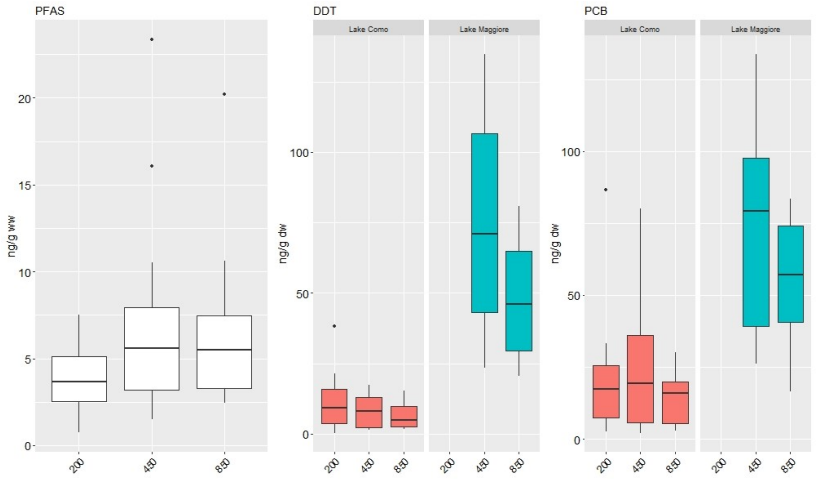

Figure 8.1.S1. Contaminant trend in different zooplankton size.

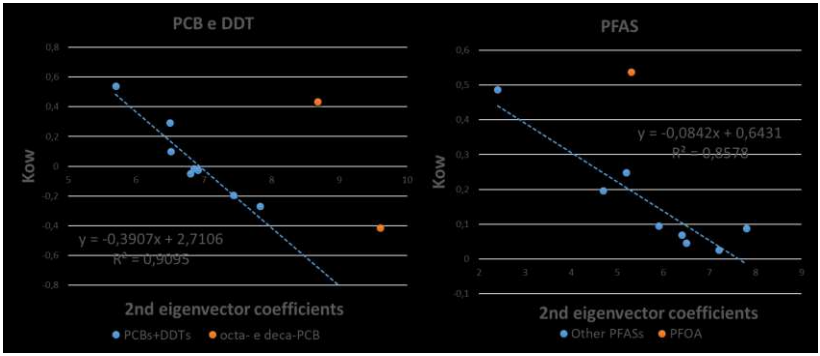

Figure 8.1.S2. Correlation between coefficients of 2nd eigenvector in PCA and K_{OW}.

8.2 Perfluoroalkyl acids in fish of Italian deep lakes: Environmental and human risk assessment

Details on chemicals and solvents

All reagents were analytical reagent grade. LC–MS grade Chromasolv acetonitrile and concentrated formic acid were purchased from Sigma-Aldrich. Water (<18 MΩcm resistivity) was produced by a Millipore Direct-QUV water purification system (Millipore, Bedford, MA, USA). HybridSPE®Phospholipid Ultra cartridges (30 mg, 1 mL SPETubes) were obtained by Sigma Aldrich (St. Louis, Missouri, USA).

Table 8.2.S1. Main morphology characteristics of deep subalpine lakes.

	Maggiore	**Como**	**Iseo**	**Garda**	**Mergozzo**
Altitude (m a.s.l.)	193	198	186	65	195
Area (km^2)	213	146	61	368	1.81
Maximum depth (m)	370	425	251	350	74
Mean depth (m)	178	154	123	133	45
Volume (km^3)	37	22	8	49	0.82
Catchment area (km^2)	6599	4508	1842	2290	10
Main inflow	Ticino, Toce	Adda	Oglio	Sarca	Rio Rescina
Outflow	Ticino	Adda	Oglio	Mincio	Mergozzo stream
Mean outflow discharge ($m^3\ s^{-1}$)	291.3	158	58.7	58.4	
Theoretical renewal time (years)	4.1	12.7	4.5/7.2	26.6	6
Total P (mg P L^{-1})	10	25	70	18	<10
O_2 hypolimnetic (mg L^{-1})	8	8	0.2	8	3
Inhabitants of the catchment area	670000	About 500000	168377	370000	In the catchment area of L. Maggiore
WWTPs which discharge directly in lakes	30	42	7	4	
Population equivalent	133007	402860	63648	17836	

Table 8.2.S2. Scheme of the catches and dimension of the specimens.

Lake	Date	Weight (g)[a]	Length (cm)[a,b]	Lipid fraction	Protein fraction	n° organisms
L. Como, branch Como	March 2017	149.9 ± 9.4	23.3 ± 0.9	0.10	68.23	6
L. Como, branch Como	July 2017	154.5 ± 33.6	23.3 ± 1.7	0.01	83.24	6
L. Como, branch Como	October 2017	184.0 ± 27.7	25.1 ± 1.7	0.07	90.51	6
L. Como, branch Lecco	November 2016	n.d.	17.5 ± 0.5	0.12	61.67	6
L. Como, branch Lecco	March 2017	81.5 ± 9.5	18.0 ± 0.7	0.11	46.65	6
L. Como, branch Lecco	July 2017	95.1 ± 16.0	19.2 ± 0.9	0.06	44.87	6
L. Garda	October 2017	100.6 ± 10.1	19.8 ± 1.3	0.13	90.16	6
L. Garda	December 2017	104.7 ± 6.3	20.8 ± 0.5	n.d.	n.d.	6
L. Iseo	December 2016	92.3 ± 11.3	18.0 ± 0.5	n.d.	n.d.	6
L. Iseo	May 2017	114.7 ± 15.0	19.7 ± 1.4	n.d.	n.d.	6
L. Iseo	June 2017	92.4 ± 11.2	18.6 ± 0.6	0.06	85.83	6
L. Iseo	September 2017	119.7 ± 14.9	20.2 ± 1.5	0.08	91.67	6
L. Maggiore	October 2016	n.d.	n.d.	n.d.	n.d.	
L. Maggiore	August 2017	n.d.	n.d.	n.d.	n.d.	
L. Maggiore	October 2017	139.0 ± 28.8	21.7 ± 1.6	n.d.	n.d.	6
L. Mergozzo	October 2016	n.d.	n.d.	n.d.	n.d.	

a= values are mean ± st. dev.; b=standard length; n.d. not determined. Lipid and protein fraction are in mg g^{-1} ww

Table 8.2.S3. List of PFASs compounds targeted in the present study, corresponding internal standards (ISs) and LC/MS/MS parameters for all target analytes and internal standards.

Target analytes	**Abbreviation**	**Precursor ion (*m*/*z*)**	**Product ions (*m*/*z*)**	**Collision energy**	**Corresponding ISs**
Perfluoropentanoic acid	PFPeA	262.9	69.0	39	^{13}C3-PFPeA
			218.9	11	
Perfluorohexanoic acid	PFHxA	312.9	119.1	22	^{13}C2-PFHxA
			268.9	11	
Perfluoroheptanoic acid	PFHpA	362.9	169.0	18	^{13}C4-PFOA
			318.9	12	
Perfluorooctanoic acid	PFOA	412.9	169.0	19	^{13}C4-PFOA
			368.9	13	
Perfluorononanoic acid	PFNA	462.9	218.9	18	^{13}C5-PFNA
			418.9	13	
Perfluorodecanoic acid	PFDA	512.9	268.9	18	^{13}C2-PFDA
			468.9	13	
Perfluoroundecanoic acid	PFUnDA	562.9	268.8	20	^{13}C2-PFUnDA
			518.8	14	
Perfluorododecanoic acid	PFDoDA	612.9	318.8	20	^{13}C2-PFDoDA
			568.9	14	

Target analytes	Abbreviation	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	Collision energy	Corresponding ISs
Perfluorotridecanoic acid	PFTrDA	662.9	619.0	15	^{13}C2-PFDoDA
			369.0		
Perfluorotetradecanoic acid	PFTeDA	712.9	669.0	15	^{13}C2-PFDoDA
			419.0		
Pefluorohexadecanoic acid	PFHxDA	812.9	769.0	15	^{13}C2-PFDoDA
Perfluorooctodecanoic acid	PFODA	912.9	869.0	16	^{13}C2-PFDoDA
Perfluorobutane sulphonate	PFBS	298.9	80.2	44	^{13}C2-PFHxA
			99.1	32	
Perfluoropentane sulphonate	PFPeS	348.9	99.0	35	^{13}C4-PFOS
			79.9	40	
Perfluorohexane sulphonate	PFHxS	398.9	80.1	38	^{18}O2-PFHxS
			99.0	34	
Perfluoroheptane sulphonate	PFHpS	449.9	99.0	35	^{13}C4-PFOS
			79.3	40	
Perfluorooctane sulphonate*	PFOS *	498.9	80.3	45	^{13}C4-PFOS
			99.1	45	
Pefluorononane sulphonate	PFNS	548.9	99.0	40	^{13}C4-PFOS
			80.0	45	

Target analytes	Abbreviation	Precursor ion (*m*/*z*)	Product ions (*m*/*z*)	Collision energy	Corresponding ISs
Perfluorodecane sulphonate	PFDS	598.9	99.0	42	^{13}C4-PFOS
			79.9	46	
Perfluorododecane sulphonate	PFDoS	699.0	99.1	50	^{13}C4-PFOS
			80.0	46	
Perfluoro-n-[^{13}C3] pentanoic acid	$13C_3$-PFPeA	267.0	222	11	n/a
Perfluoro-n-[^{13}C2] hexanoic acid	$^{13}C_2$-PFHxA	314.9	269.9	11	n/a
Perfluoro-n-[^{13}C4] octanoic acid	$^{13}C_4$-PFOA	416.9	371.9	13	n/a
Perfluoro-n-[^{13}C5] nonanoic acid	$^{13}C_5$-PFNA	467.9	422.9	13	n/a
Perfluoro-n-[^{13}C2] decanoic acid	$^{13}C_2$-PFDA	514.9	469.9	13	n/a
Perfluoro-n-[^{13}C2] undecanoic acid	$^{13}C_2$-PFUnDA	564.9	519.8	14	n/a
Perfluoro-n-[^{13}C2] dodecanoic acid	$^{13}C_2$-PFDoDA	614.9	569.9	14	n/a
Perfluoro-n-hexane [^{18}O2] sulphonate	$^{18}O_2$-PFHxS	402.9	103.0	34	n/a
Perfluoro-n-octane [^{13}C4] sulphonate	$^{13}C_4$-PFOS	502.9	99.1	45	n/a

Table 8.2.S4. Limits of detection (LODs) and limits of quantification (LOQs) of PFASs target analytes for fish tissues.

Analytes	**LOD (ng g^{-1} ww)**	**LOQ (ng g^{-1} ww)**
PFPeA	0.3	0.9
PFHxA	0.06	0.2
PFHpA	0.1	0.4
PFOA	0.1	0.4
PFNA	0.07	0.2
PFDA	0.07	0.2
PFUnDA	0.04	0.1
PFDoDA	0.03	0.1
PFTrDA	0.07	0.2
PFTeDA	0.05	0.2
PFHxDA	0.04	0.1
PFODA	0.07	0.2
PFBS	0.3	0.8
PFPeS	0.4	1.2
PFHxS	0.1	0.2
PFHpS	0.1	0.3
PFOS	0.2	0.5
PFNS	0.2	0.6
PFDS	0.2	0.6
PFDoS	0.3	0.9

Table 8.2.S5. PFCAs concentrations in all fish fillets analysed (ng g^{-1} ww).

Lake	Data	PFPeA	PFHxA	PFHpA	PFOA	PFNA	PFDA	PFUnDA	PFDoDA	PFTrDA	PFTeDA	PFHxDA	PFODA
Mergozzo	October 2016	< 0.3	0.1	0.1	< 0.1	0.2	0.6	0.4	0.5	0.7	0.3	< 0.04	< 0.07
Maggiore	October 2016	< 0.3	0.1	0.1	1.2	< 0.07	0.4	0.2	0.2	0.1	< 0.05	< 0.04	< 0.07
Maggiore	August 2017	< 0.3	<0.06	< 0.1	< 0.1	0.2	0.2	0.1	0.1	0.1	< 0.05	< 0.04	< 0.07
Maggiore	October 2017	< 0.3	<0.06	< 0.1	< 0.1	< 0.07	0.3	0.2	0.3	0.3	0.2	< 0.04	< 0.07
b. Como	March 2017	< 0.3	<0.06	< 0.1	< 0.1	< 0.07	0.2	0.3	1.0	1.0	0.4	< 0.04	< 0.07
b. Como	July 2017	0.4	<0.06	< 0.1	0.1	0.1	0.5	0.2	0.2	0.3	0.2	< 0.04	< 0.07
b. Como	October 2017	< 0.3	<0.06	< 0.1	< 0.1	< 0.07	0.1	0.1	0.1	0.2	0.1	< 0.04	< 0.07
b. Lecco	November 2016	< 0.3	<0.06	< 0.1	< 0.1	< 0.07	0.3	0.4	0.7	0.4	0.2	< 0.04	< 0.07
b. Lecco	March 2017	< 0.3	<0.06	< 0.1	< 0.1	< 0.07	0.1	< 0.04	0.2	0.1	< 0.05	< 0.04	< 0.07
b. Lecco	July 2017	< 0.3	0.6	< 0.1	0.1	< 0.07	0.2	0.1	0.1	0.2	0.1	< 0.04	< 0.07
Iseo	December 2016	< 0.3	<0.06	< 0.1	< 0.1	< 0.07	0.2	0.3	0.4	0.2	0.2	< 0.04	< 0.07
Iseo	May 2017	< 0.3	<0.06	< 0.1	< 0.1	< 0.07	< 0.07	< 0.04	0.1	0.1	0.05	< 0.04	< 0.07
Iseo	June 2017	1.2	0.1	< 0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.1	< 0.04	< 0.07
Iseo	September 2017	< 0.3	<0.06	< 0.1	< 0.1	< 0.07	0.1	0.1	0.1	0.1	< 0.05	< 0.04	< 0.07
Garda	October 2017	< 0.3	0.8	< 0.1	< 0.1	0.1	0.2	0.1	0.1	0.1	0.1	< 0.04	< 0.07
Garda	December 2017	< 0.3	<0.06	< 0.1	< 0.1	< 0.07	0.2	0.1	0.2	0.2	0.1	< 0.04	< 0.07

Table 8.2.S6. PFSAs concentrations in all fish fillets analysed (ng g^{-1} ww)

Lake	Data	PFBS	PFPeS	PFHxS	PFHpS	PFOS	PFNS	PFDS	PFDoS
Mergozzo	October 2016	< 0.3	< 0.4	< 0.1	< 0.1	5.71	< 0.2	< 0.2	< 0.3
Maggiore	October 2016	< 0.3	< 0.4	< 0.1	< 0.1	16.60	< 0.2	< 0.2	< 0.3
Maggiore	August 2017	< 0.3	< 0.4	< 0.1	< 0.1	11.70	< 0.2	< 0.2	< 0.3
Maggiore	October 2017	< 0.3	< 0.4	< 0.1	< 0.1	10.61	< 0.2	< 0.2	< 0.3
b. Como	March 2017	< 0.3	< 0.4	< 0.1	< 0.1	4.17	< 0.2	< 0.2	< 0.3
b. Como	July 2017	< 0.3	< 0.4	< 0.1	< 0.1	5.97	< 0.2	< 0.2	< 0.3
b. Como	October 2017	< 0.3	< 0.4	< 0.1	< 0.1	4.86	< 0.2	< 0.2	< 0.3
b. Lecco	November 2016	< 0.3	< 0.4	< 0.1	< 0.1	2.81	< 0.2	< 0.2	< 0.3
b. Lecco	March 2017	< 0.3	< 0.4	< 0.1	< 0.1	1.62	< 0.2	< 0.2	< 0.3
b. Lecco	July 2017	< 0.3	< 0.4	< 0.1	< 0.1	4.29	< 0.2	< 0.2	< 0.3
Iseo	December 2016	< 0.3	< 0.4	< 0.1	< 0.1	0.89	< 0.2	< 0.2	< 0.3
Iseo	May 2017	< 0.3	< 0.4	< 0.1	< 0.1	0.97	< 0.2	< 0.2	< 0.3
Iseo	June 2017	< 0.3	< 0.4	< 0.1	< 0.1	2.52	< 0.2	< 0.2	< 0.3
Iseo	September 2017	< 0.3	< 0.4	< 0.1	< 0.1	0.42	< 0.2	< 0.2	< 0.3
Garda	October 2017	< 0.3	< 0.4	< 0.1	< 0.1	1.74	< 0.2	< 0.2	< 0.3
Garda	December 2017	< 0.3	< 0.4	< 0.1	< 0.1	4.82	< 0.2	< 0.2	< 0.3

Table 8.2.S7. Concentrations of 11 PFASs in water samples (ng L^{-1}). Method LODs are reported in the last row.

Lake	**Date**	**PFPeA**	**PFHxA**	**PFHpA**	**PFOA**	**PFNA**	**PFDA**	**PFUnDA**	**PFDoDA**	**PFBS**	**PFHxS**	**PFOS**
L. Maggiore	February 2017	<LOD	<LOD	1.0	<LOD	<LOD	<LOD	<LOD	<LOD	1.7	<LOD	3.3
L. Como, b. Lecco	February 2017	<LOD	1.3	<LOD	2.7	0.7	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
L. Como, b. Como	February 2017	<LOD	<LOD	0.7	<LOD	<LOD	<LOD	<LOD	<LOD	1.2	<LOD	2.6
L. Como, b. Lecco	February 2017	<LOD	<LOD	<LOD	<LOD	0.6	<LOD	<LOD	<LOD	1.8	<LOD	<LOD
L. Como, b. Como	May 2017	<LOD	1.1	<LOD	0.6	<LOD	0.7	<LOD	<LOD	4.1	<LOD	2.7
L. Como, b. Como	August 2017	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	6.1	<LOD	<LOD
L. Como, b. Lecco	August 2017	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.5	<LOD	<LOD
LOD		2	0.2	0.5	0.5	0.5	0.5	0.5	1	1	5	2.5

Table 8.2.S8. Measured PFOS concentrations in fish fillets and whole fish and estimated PFOS concentrations in whole fish (ng g^{-1}ww).

Lake	Data	Fillet (measured)	Whole fish (measured)	Whole fish (estimated)*
L. Mergozzo	October 2016	5.7		13.0
L. Maggiore	October 2016	16.6		25.5
L. Maggiore	August 2017	11.7		20.4
L. Maggiore	October 2017	10.6		19.2
L. Como, b. Como	March 2017	4.2		10.6
L. Como, b. Como	July 2017	6.0	7.2	13.3
L. Como, b. Como	October 2017	4.9	11.7	11.7
L. Como, b. Lecco	November 2016	2.8		8.3
L. Como, b. Lecco	March 2017	1.6		5.8
L. Como, b. Lecco	July 2017	4.3	11.8	10.8
L. Iseo	December 2016	0.9		4.0
L. Iseo	May 2017	1.0		4.2
L. Iseo	June 2017	2.5	4.0	7.7
L. Iseo	September 2017	0.4	2.6	2.5
L. Garda	October 2017	1.7	5.4	6.1
L. Garda	December 2017	4.8		11.7

*concentrations estimated by the equation reported in figure 8.2.S1.

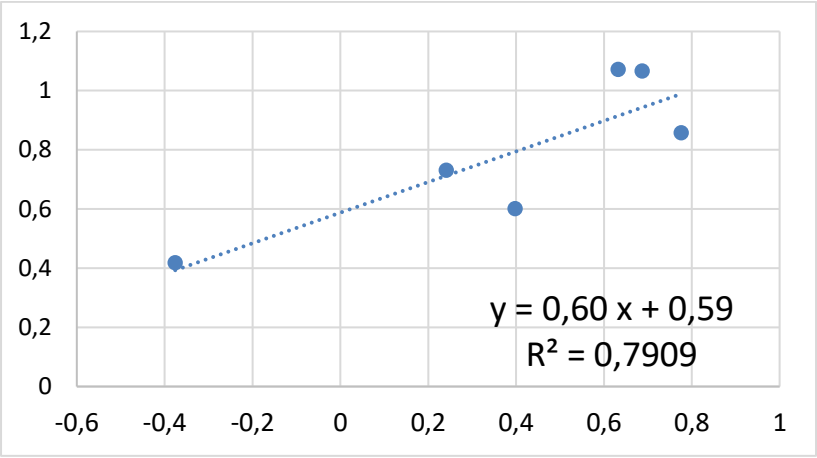

Figure 8.2.S1. Log-log regression between PFOS concentrations in fillets and in whole fish.

8.3 Trophic transfer of persistent organic pollutants through a pelagic food web: The case of Lake Como (Northern Italy)

Table 8.3.S1. Fish length (expressed in cm).

Shad				Lake whitefish			
Spring	**Summer**	**Autumn**	**Winter**	**Spring**	**Summer**	**Autumn**	**Winter**
27.0	27.0	19.0	20.0	28.5	29.0	27.0	28.5
26.5	21.0	20.0	21.0	28.0	29.0	28.0	27.5
30.0	21.5	20.0	21.0	27.5	28.5	28.0	26.0
29.0	20.0	22.0	21.5	27.0	27.0	28.5	26.5
26.0	21.0	19.0	21.0	26.0	28.0	28.0	29.0
31.5	19.5	21.5	21.5	27.0	29.0	29.0	

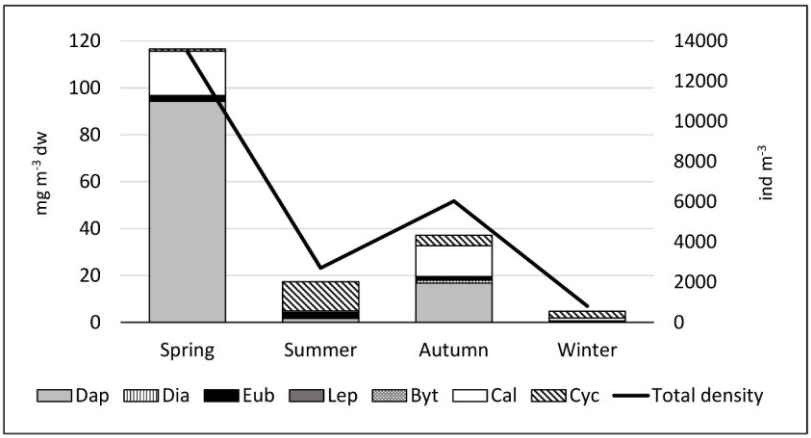

Fig. 8.3.S1. Seasonal changes in zooplankton biomass and total density. Dap: *Daphnia longispina* gr.; Dia: *Diaphanosoma brachyurum*; Eub: *Eubosmina coregoni*; Lep: *Leptodora kindtii*; Byt: *Bythotrephes longimanus;* Cal; calanoids; Cyc: cyclopoids.

Table 8.3.S2. Seasonal changes in zooplankton community density, expressed as ind m^{-3}

	Spring	**Summer**	**Autumn**	**Winter**
Bythotrephes longimanus	7.6	1.9	1.5	0.0
Leptodora kindtii	2.5	11.4	0.0	0.0
Cyclopoids	126.2	2010.8	651.3	419.9
Calanoids	4483.6	170.4	3108.2	299.6
Eubosmina coregoni	348.4	323.8	218.1	86.7
Diaphanosoma brachyurum	0.0	75.7	554.4	0.8
Daphnia longispina gr.	8500.1	113.6	1507.2	4.2
Total	13511.4	2764.4	6060.4	811.2

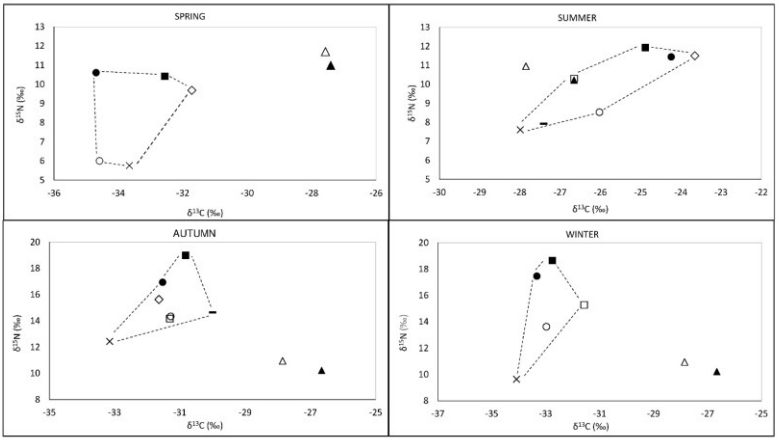

Fig. 8.3.S2. Plot of $\delta^{13}C$ and $\delta^{15}N$ values of zooplankton and fish in the four seasons. Byt: *Bythotrephes longimanus* (■)*;* Lep: *Leptodora kindtii* (◊); Cyc: cyclopoids (□); Cal; calanoids (●); Eub: *Eubosmina coregoni* (x); Dia: *Diaphanosoma brachyurum* (−), Dap: *Daphnia longispina galeata* gr. (○), landlocked shad (▲), whitefish (Δ), bulk 200 (+).

Table 8.3.S3. Lipid content (%) on dry weight (dw) for zooplankton and on wet weight (ww) for fish.

	Spring	**Summer**	**Autumn**	**Winter**
200 µm	14.7	6.0	13.0	6.7
Landlocked shad	9.8	8.3	15.5	14.6
Lake whitefish	1.3	3.1	2.7	1.3

8.4"The Unbearable Lightness of" bioaccumulation in the trophic web of Lake Mergozzo

Table 8.4.S1. Lake Mergozzo morphometric characteristics.

Lake altitude (m a.s.l)	194
Volume (m^3)	$83*10^6$
Area (km^2)	1.83
Maximum depth (m)	73
Catchment basin area (km^2)	10.4

Extraction method for DDTs and PCBs

About 0.5 g of sample (freeze-dried zooplankton or an aliquot of freeze-dried muscle fish) were added to 50 mL of mixture *n*-hexane and acetone (1:1, Carlo Erba, pesticide analysis grade) into a glass fibre thimble (19 mm I.D., 90-mm length, Whatman, England) and extracted in a modified Soxhlet equipment (Velp Scientifica, ECO 6 Thermoreactor, Italy) for 2 hours. After the complete evaporation of solvent, the lipid weight was measured. Lipids were then suspended in 2 mL of *n*-hexane and digested with 2 mL of H_2SO_4 (98%, Carlo Erba) all night long. The day after, several washings of the extract were performed with *n*-hexane and the resulting supernatants were added together. Collected supernatants was reduced with Rotavapor and was cleaned up on a Florisil® column (40×7 mm I.D.) that was eluted with 25 mL of a mixture *n*-hexane and dichloromethane (Carlo Erba, pesticide analysis grade, 85:15). Then, 1 mL of iso-octane

(Carlo Erba, pesticide analysis grade) was added to the final extract, that was subsequently concentrated to 0.5 mL.

Analysis was performed by gas chromatography (GC Carlo Erba, Top 8000) coupled with ^{63}Ni electron capture detector (Carlo Erba ECD 80) using an on-column injection system (volume injected 1 μL) and a WCOT fused silica CP-Sil-8 CB column (50 m×0.25 mm length x internal diameter, film thickness 0.25 μm, Varian, USA). The thermal ramp followed was: from 60 to 190°C at 20°C min^{-1}, from 190 to 250°C at 1.5°C min^{-1}, then from 250 to 270°C at 3°C min^{-1}, and, at last, the final isotherma at 270°C for 17 min, with helium as carrier gas (1 mL min^{-1}) and N as auxiliary gas (30 mL min^{-1}).

DDT and PCB quantification were performed by building calibration curves with Custom Pesticide Mix (o2si, USA), Custom PCB Calibration Mix (o2si, USA) and Aroclor 1260 (Alltech, USA) standards. The solution of DDT compounds contained pp'DDT, op'DDT, pp'DDD, op'DDD, op'DDE and pp'DDE while the analysed PCBs congeners were: PCB 18, 28+31, 44, 52, 101, 118, 149, 138, 153, 170, 180, 194 and 209. LOD is 0,1 ng g^{-1}.

Routinely, standards reference materials SRM NIST-1947 "Lake Michigan Fish Tissue" and NIST-1946, "Lake Superior Fish Tissue" were analysed in triplicate in order to test good laboratory practices, respectively for DDT homologues and PCB residues. The percentage recoveries of DDTs were between 106.2 ± 3% and 107.5 ± 4%, while those for PCBs ranged between 91.3% (±1.1%) and 102.2% (±1.6%).

Extraction method for PFASs

Few grams of homogenized sample (10 g of fish fillet and 0.5 g of freeze-dried zooplankton) were added into a 50 mL PP centrifuge tube together with 1.5 mL of water and acetonitrile solution (10:90 v/v) per gram of fresh sample and 200 µL of formic acid. This mix was spiked with 100 µL of 40 µg L^{-1} stable isotope-labelled solution used as internal standard (SIL-IS) and vigorously shaken. The tube with sample were subjected to ultra-sonication extraction for 15 min and then centrifuged for 10 min at 11,000 rpm at 10°C. The extraction was repeated twice. The mixed supernatants were transferred in a new 50 mL PP tube where 0.6 g $MgSO_4$ and 0.2 g NaCl per gram of fresh sample were added. Afterwards, the extract volume was reduced to 1 mL under a gentle nitrogen stream and filtered through Hybrid SPE® Phospholipid Ultra cartridge (previously washed with acetonitrile) to remove phospholipids. Finally, the extract was acidified with 50 L of formic acid and transferred into the autosampler vial. A procedural blank was run every extraction batch.

The analysis was performed by liquid chromatography coupled to mass spectrometry (UHPLC-MS/MS) after an online purification with turbulent flow chromatography (TFC). PFASs analysed include: PFHxA, PFHpA; PFOA; PFNA; PFDA; PFUnDA, PFDoDA, PFHxS, PFOS. Explanation of acronyms are in Mazzoni et al. (2016). For all the analytes, one precursor and two product ions were monitored.

Calibration curves were prepared using mixed standard solutions in acetonitrile spiked with 50 µL of formic acid and 100 µL of the diluted

SIL-IS solution (40 µg L^{-1}) before injection. Quantification was performed by using the isotopic dilution method and calibration curves were acquired before each analytical run. Limits of detection (LOD) and limits of quantification (LOQ) ranged from 0.03 to 0.4 and from 0.1 to 1.2 ng g^{-1}ww respectively.

Lists the MS/MS transitions, collision energies applied for the different target analytes and isotope labelled standards, additional method performance characteristics and full validation data are reported in Mazzoni et al. (2016).

Table 8.4.S2. Hg concentrations (µg kg^{-1} ww) in samples.

ID_sample	Scientific name	Common name	Hg
LMerg450nov16	Zooplankton	Zooplankton	20.00
MERG 1	*Alosa agone*	Agone	35.89
MERG 3	*Rutilus rutilus*	Roach	111.01
MERG 6	*Squalius cephalus*	Chub	54.85
MERG 7	*Micropterus salmoides*	Largemouth bass	143.19
MERG 9	*Lepomis gibbosus*	Pumpkinseed	72.95
MERG 10	*Coregonus lavaretus*	Whitefish	160.26
MERG 11	*Coregonus lavaretus*	Whitefish	70.94
MERG 12	*Scardinius erythrophthalmus*	Rudd	68.73
MERG 15	*Scardinius erythrophthalmus*	Rudd	103.39
MERG 16	*Salvelinus alpinus*	Char	87.60
MERG 17	*Sander lucioperca*	Pikeperch	501.06
MERG 18	*Esox lucius*	Pike	187.77
MERG19	*Lota lota*	Burbot	259.81
MERG 20	*Perca fluviatilis*	Perch	472.28
MERG 21	*Micropterus salmoides*	Largemouth bass	221.95
MERG 22	*Micropterus salmoides*	Largemouth bass	212.63
MERG 24	*Rutilus*	Roach	107.42
MERG 25	*Perca fluviatilis*	Perch	84.36
MERG 26	*Gymnocephalus cernuus*	Ruffe	50.11
% positive samples			**100**

Table 8.4.S3. DDTs concentrations (µg kg^{-1} lw) in samples. Common name and scientific name are reported in Table 8.4.S2.

ID_sample	op' DDE	pp' DDE	op' DDD	pp' DDD	op' DDT	pp' DDT	∑DDT
LMerg450nov16	<LOD	26.88	21.63	<LOD	<LOD	17.80	**66.31**
MERG 1	1.95	1.54	0.32	11.00	0.39	0.71	**15.91**
MERG 3	119.14	130.63	<LOD	815.18	4.88	<LOD	**1069.83**
MERG 6	6.81	<LOD	<LOD	109.85	<LOD	<LOD	**116.67**
MERG 7	25.61	23.67	<LOD	98.21	<LOD	<LOD	**147.49**
MERG 9	33.05	20.76	<LOD	528.59	3.93	<LOD	**586.32**
MERG 10	1.19	13.81	<LOD	194.95	1.42	<LOD	**211.37**
MERG 11	5.26	14.57	<LOD	130.35	<LOD	<LOD	**150.18**
MERG 12	158.00	<LOD	<LOD	382.26	<LOD	167.75	**708.02**
MERG 15	71.74	47.78	<LOD	626.80	44.26	<LOD	**790.58**
MERG 16	445.80	417.37	52.58	1429.67	79.20	<LOD	**2424.62**
MERG 17	7516.00	553.66	195.69	3804.38	229.31	854.03	**13153.07**
MERG 18	51.25	25.23	<LOD	1784.02	<LOD	175.99	**2036.48**
MERG19	<LOD	10.35	<LOD	359.72	<LOD	<LOD	**370.07**
MERG 20	46.19	12.26	<LOD	470.52	<LOD	100.47	**629.43**
MERG 21	<LOD	<LOD	<LOD	41.72	<LOD	<LOD	**41.72**
MERG 22	293.03	63.16	<LOD	1120.06	<LOD	417.74	**1894.00**
MERG 24	433.12	162.62	18.16	1348.12	19.21	60.14	**2041.37**
MERG 25	<LOD	<LOD	<LOD	47.41	<LOD	<LOD	**47.41**
MERG 26	98.88	12.06	7.12	231.65	<LOD	113.15	**462.86**
% positive samples	**80**	**80**	**30**	**95**	**40**	**45**	**100**

Table 8.4.S4. PCBs concentrations (μg kg^{-1} lw) in samples. Common name and scientific name are reported in Table 8.4.S2.

IDsample	PCB 18	PCB 28+31	PCB 44	PCB 101	PCB 149	PCB 118	PCB 153	PCB 138	PCB 180	PCB 170	PCB 194	PCB 209	Tot PCB
LMerg450 nov16	35.15	<LOD	35.38	1.91	<LOD	<LOD	0.24	<LOD	4.76	<LOD	<LOD	<LOD	**77.45**
MERG 1	<LOD	0.12	2.10	2.47	2.12	1.14	5.30	3.08	1.10	<LOD	<LOD	<LOD	**17.43**
MERG 3	81.47	<LOD	<LOD	52.03	82.95	253.14	551.39	470.02	83.79	<LOD	21.31	<LOD	**1596.10**
MERG 6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	68.54	<LOD	27.73	60.86	11.17	<LOD	**168.29**
MERG 7	<LOD	7.77	<LOD	<LOD	41.72	4.20	176.82	<LOD	132.23	54.83	15.78	<LOD	**433.35**
MERG 9	186.20	74.32	<LOD	688.08	28.92	25.43	135.48	<LOD	25.22	67.81	8.01	<LOD	**1239.46**
MERG 10	<LOD	<LOD	<LOD	13.30	18.66	27.39	127.66	150.09	37.39	<LOD	<LOD	<LOD	**374.49**
MERG 11	<LOD	<LOD	<LOD	88.61	3.16	4.33	61.42	<LOD	12.31	17.07	<LOD	<LOD	**186.90**
MERG 12	167.42	48.36	21.61	1271.5	75.84	204.74	458.26	399.34	110.74	1.12	<LOD	<LOD	**2758.88**
MERG 15	83.87	<LOD	187.60	420.78	81.35	95.76	484.34	7.99	131.25	88.87	<LOD	40.75	**1622.56**
MERG 16	48.50	<LOD	86.03	681.80	328.06	395.93	1547.5	856.49	357.65	233.87	40.11	<LOD	**4575.92**
MERG 17	487.24	53.91	1099.5	3408.9	898.76	821.60	3345.5	1699.7	1768.3	642.48	551.8	<LOD	**14777.73**
MERG 18	209.81	16.02	282.49	2335.2	64.41	111.87	602.90	325.36	323.37	148.87	13.75	<LOD	**4434.00**
MERG19	<LOD	<LOD	<LOD	59.34	2.73	55.07	176.50	162.79	91.21	<LOD	7.81	<LOD	**555.44**
MERG 20	<LOD	<LOD	55.39	196.33	71.75	1314.9	253.21	229.47	123.35	121.69	15.17	<LOD	**2381.31**
MERG 21	<LOD	<LOD	<LOD	105.08	<LOD	8.44	40.21	16.53	19.85	<LOD	<LOD	<LOD	**190.11**
MERG 22	285.16	<LOD	<LOD	12016	194.93	231.05	98.89	396.92	637.35	180.27	54.22	<LOD	**14094.63**

IDsample	PCB 18	PCB 28+31	PCB 44	PCB 101	PCB 149	PCB 118	PCB 153	PCB 138	PCB 180	PCB 170	PCB 194	PCB 209	Tot PCB
MERG 24	20.44	15.92	78.81	919.28	737.31	308.37	1316.7	1320.7	687.89	223.24	68.09	72.74	**5769.44**
MERG 25	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	76.04	<LOD	38.72	40.79	<LOD	<LOD	**155.55**
MERG 26	95.04	9.76	135.07	440.32	120.31	92.27	350.98	172.56	161.15	100.14	76.60	<LOD	**1754.21**
% positive samples	**55**	**40**	**50**	**85**	**80**	**85**	**100**	**70**	**100**	**70**	**60**	**10**	**100**

Table 8.4.S5. PFASs concentrations (μg kg^{-1} ww) in samples. Common name and scientific name are reported in Table 8.4.S2.

ID_sample	PFHxA	PFHpA	PFHxS	PFOA	PFOS	PFNA	PFDA	PFUnDA	PFDoDA
LMerg450nov16	<LOD	<LOD	<LOD	<LOD	0.06	0.05	0.05	0.62	0.34
MERG 1	<LOD	<LOD	<LOD	<LOD	5.71	0.21	0.58	0.41	0.52
MERG 3	<LOD	<LOD	<LOD	<LOD	3.35	<LOD	0.78	1.45	1.28
MERG 6	<LOD	<LOD	<LOD	<LOD	2.42	<LOD	0.46	1.20	1.06
MERG 7	<LOD	<LOD	<LOD	<LOD	6.21	<LOD	1.35	2.75	2.02
MERG 9	<LOD	<LOD	<LOD	<LOD	3.71	<LOD	0.64	1.96	2.31
MERG 10	<LOD	<LOD	<LOD	<LOD	5.37	0.06	1.10	0.95	1.01
MERG 11	<LOD	<LOD	<LOD	<LOD	8.09	0.18	1.79	1.19	0.47
MERG 12	<LOD	<LOD	<LOD	<LOD	0.87	<LOD	0.41	2.49	<LOD
MERG 15	<LOD	<LOD	<LOD	<LOD	13.67	<LOD	2.10	2.19	2.64
MERG 16	<LOD	<LOD	<LOD	<LOD	1.74	<LOD	0.30	0.53	0.61
MERG 17	<LOD	<LOD	<LOD	<LOD	13.27	<LOD	2.30	4.23	1.71
MERG 18	<LOD	<LOD	<LOD	<LOD	2.88	<LOD	1.16	3.27	3.34
MERG19	<LOD	<LOD	<LOD	<LOD	0.27	<LOD	<LOD	0.05	0.04
MERG 20	<LOD	<LOD	<LOD	<LOD	38.40	0.12	7.92	8.93	4.81
MERG 21	<LOD	<LOD	<LOD	<LOD	13.75	<LOD	2.40	3.43	1.02
MERG 22	<LOD	<LOD	<LOD	<LOD	13.72	<LOD	2.24	3.66	2.65
MERG 24	<LOD	<LOD	<LOD	<LOD	3.82	<LOD	0.54	0.78	0.18
MERG 25	<LOD	<LOD	<LOD	<LOD	12.92	<LOD	2.53	2.68	1.44
MERG 26	<LOD	<LOD	<LOD	<LOD	3.61	0.26	0.54	1.17	0.75
% positive samples	**0**	**0**	**0**	**0**	**100**	**30**	**95**	**100**	**95**

Table 8.4.S6. Data comparison among results of this study and fish concentrations in European lakes. Concentrations are expressed in µg kg^{-1} lw for OCs, in µg kg^{-1} ww for PFASs and for Hg. For each specie mean, minimum and maximum (in brackets) values are reported.

Name	Lake (Country)	Sampling year	∑ C6 - C7 PFAC	PFOA	PFOS	∑ C9- C12 PFAC	PCB 153	∑PCB	∑DDT	Hg	References
Zooplankton	Mergozzo (Italy)	2016	<0.16	<0.10	0.06	1.01	0.24	77.45	66.31	20	present study
	Maggiore (Italy)	2013-2018	<0.16 (<0.16-0.90)	0.60 (<0.10 -4.00)	5.10 (1.30-18.90)	1.60 (<0.21-6.50)	156.65 (16.00-364.58)	606.18 (156.25-2115.88)	506.19 (114.58-2272.50)	100 (60-180)	a; b; c
	European lakes	2009-2018	<0.16	0.9 (<0.10 -9.40)	1.50 (<0.20-6.70)	0.84 (<0.21-4.10)	68.34 (4.99-362.37)	3885.02 (30.23-20240.00)	182.10 (6.37-1458.05)		a; d; e; f
Shad *Alosa agone*	Mergozzo (Italy)	2016	0.20	<0.10	5.71	1.70	5.30	17.43	15.91	35.89	present study; e
	Maggiore (Italy)	2009-2017	0.18 (<0.16-0.20)	0.47 (<0.10 -1.2)	12.97 (10.61-16.60)	0.54 (0.15-0.87)		930.00 (500.00-1800.00)	1417.00 (600.00-3900.00)	165 (100-250)	b; c; d; e
	Lugano (Swiss-Italy)	2009-2015			13.51 (3.70-45.10)	2.4 (0.70-8.80)		561.88 (475.25-673.27)	247.52 (188.12-306.93)	127 (105-130)	g; h
	European lakes	2006-2016	0.31 (<0.16-0.54)	0.11 (<0.10 -0.15)	3.10 (1.20-5.03)	0.64 (0.43-0.93)	81.43 (11.79-157.27)	1370.94 (10.00-3500.00)	786.33 (10.00-1168.00)		e; i; j; k

Name	Lake (Country)	Sampling year	∑ C6 - C7 PFAC	PFOA	PFOS	∑ C9- C12 PFAC	PCB 153	∑PCB	∑DDT	Hg	References
Whitefish *Coregonus lavaretus*	Mergozzo (Italy)	2016	<0.16	<0.10	6.73 (5.37-8.09)	3.38 (3.12-3.63)	94.54 (61.42-127.66)	280.70 (186.90-374.49)	180.77 (150.18-211.37)	115.60 (70-160)	present study
	Maggiore (Italy)	2004-2017		<0.10	19.85 (5.00-42.30)			669.78 (62.12-1700.00)	1018.83 (500-1586.86)	82 (60-110)	b; c; d; l; m;
	Lugano (Swiss-Italy)	2009						578.03 (350.88-798.83)	230.54 (140.35-315.79)	71 (57-85)	g; n
	European lakes	2001-2016		<0.10	18.43 (1.21-34.00)	0.54 (0.41-2.36)	330.63 (84.13-800.00)	956.66 (338.00-2000.00)	241.29 (97.60-650.20)	146 (60-2070)	k; o; p; q; r; s; t; u
Roach *Rutilus rutilus*	Mergozzo (Italy)	2016	<0.16	<0.10	3.59 (3.35-3.82)	2.51 (1.49-3.52)	934.04 (551.39-1316.68)	3682.77 (1596.10-5769.44)	1555.6 (1069.8-2041.4)	109.22 (111-107)	present study
	Maggiore (Italy)	2009-2017						1700 (900-2500)	800.00 (400-1600)	75 (30-130)	b; c; d
	Lugano (Swiss-Italy)	2009						617.98 (337.08-842.70)	323.03 (168.54-449.44)		g
	European lakes	2006-2011	<0.05	<1.00	13.08 (0.81-34.80)		18.60 (11.82-25.38)	22100.49 (44.55-99410.00)	152.12 (105.00-199.23)	85 (20-160)	p; v; w; x; y, z

Name	Lake (Country)	Sampling year	∑ C6 - C7 PFAC	PFOA	PFOS	∑ C9- C12 PFAC	PCB 153	∑PCB	∑DDT	Hg	References
Perch *Perca fluviatilis*	Mergozzo (Italy)	2016	<0.16	<0.1	25.66 (12.92-38.40)	14.21 (6.65-21.77)	164.62 (76.04-253.21)	1268.43 (155.55-2381.31)	338.42 (47.41-629.43)	278.32 (80-470)	present study
	Maggiore (Italy)	2012		<0.5	22.25 (11.00-45.80)						m
	Lugano (Swiss-Italy)	2009-2015			26.25 (10.00-45.00)	9.30 (3.70-16.80)		575.00 (150.00-1600.00)	287.50 (75.00-700.00)		h; g
	European lakes	2001-2017	<0.05	0.50 (0.11-1.00)	82.56 (0.20-370)	1.35 (0.95-2.68)	60.00	22554.27 (176.67-155940.0)	288.09 (111.00-438.00)	238 (40-1330)	f; o; p; q; r; t; w; x; y; aa; ab; ac

a) Pascariello et al., 2019; b) CIPAIS (Lake Maggiore), 2014; c) CIPAIS (Lake Maggiore), 2017; d) Bettinetti et al., 2012; e) Mazzoni et al., 2019; f) Figueiredo et al., 2014; g) CIPAIS (Lake Lugano), 2009; h) CIPAIS (Lake Lugano), 2015; i) Bettinetti et al., 2016; j) Quadroni et al., 2017; k) Mazzoni et al., 2018; l) Blank et al., 2013; m) Squadrone et al., 2014; n) Guzzella et al., 2018; o) Berger et al., 2009; p) Holzer et al., 2011; q) Villa et al., 2011; r) Moreno et al., 2015; s) Keva et al., 2017; t) Łuczyńska et al., 2016; u) Masset et al., 2019; v) Mazej et al., 2010; w) Petkovšek et al., 2012; x) Komov et al., 2014; y) Filipovic et al., 2015; z) Łuczyńska et al., 2016; aa) Ahrens et al., 2015; ab) Georgieva et al., 2015; ac) Łuczyńska et al., 2018.

Table 8.4.S7. Derived TMFs with TL from Fishbase (in bold significant p values < 0.005)

Chemical	**slope**	**TMF**	**[95%]**	**R^2**	**p value**
Hg (mg/kg dw)	0.38	2.41	[1.54; 3.76]	0.46	**0.004**
Hg (mg/kg ww)	0.37	2.37	[1.52; 3.70]	0.45	**0.001**
PCB 153 (µg/ kg lw)	0.70	5.01	[1.26; 19.86]	0.21	**0.02**
Penta_CB (µg/ kg lw)	0.61	4.05	[0.34; 47.92]	0.02	0.25
Hexa_CB (µg/ kg lw)	0.76	5.82	[1.31; 25.67]	0.22	**0.02**
Hepta-CB (µg/ kg lw)	0.60	4.00	[1.22; 13.13]	0.21	**0.03**
pp'DDD (µg/ kg lw)	0.79	6.15	[1.36; 27.68]	0.22	**0.02**
pp'DDT tot (µg/ kg lw)	0.34	2.17	[0.7; 6.69]	0.05	0.17
PFOS (µg/ kg ww)	0.50	3.13	[1.18; 8.28]	0.21	**0.02**
PFDA (µg/ kg ww)	0.40	2.48	[1.05; 5.85]	0.17	**0.04**
PFUnDA (µg/ kg ww)	0.22	1.65	[0.74; 3.69]	0.04	0.21
PFDoDA (µg/ kg ww)	0.30	2.01	[0.8; 5.01]	0.08	0.13

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