Co-occurrence of polychlorinated biphenyls, cyanotoxins and trace elements in commercial fish species from a freshwater protected area (Pertusillo Lake, Southern Italy).

Abstract

A total of 79 fish samples covering nine species were collected in a preliminary investigation on a SCI (Site of Community Importance) water reservoir (Pertusillo Lake, Southern Italy) created for drinking purpose and located in a territory used for drilling activities. Analyses for microcystins (MYCs) and cylindrospermopsins (CYLs) presence were performed using Elisa assays, while 10 fish samples were analyzed also for trace elements by atomic adsorption spectrophotometry and for polychlorinated biphenyls (PCBs) by GC-MS operated in EI mode. The results showed the compresence of important cyanotoxins and industrial contaminants in fish. More extended studies are needed to evaluate the combined effects of these contaminants on the lake ecosystem and ichthyic fauna, in order to establish an available risk assessment for human population in the lake region.

Keywords: microcystins, cylindrospermopsins, trace elements, polychlorinated biphenyls, fish, bioaccumulation, Pertusillo Lake.

Introduction

In the past century, the development of industry and agriculture often caused the release or production of organic and inorganic pollutants in the environment, posing threats to wildlife and human health. Several studies have shown the presence of anthropic contaminants in inland waters of various continents, as found in lakes polluted by industries (1). A particular class of these contaminants, microcystins (MYCs), the commonest biotoxins of Cyanobacteria, are a family of more than 90 potent eptapeptide hepatotoxins acting as specific inhibitors of protein phosphatases

(PPs) of type 1, 2A, 3 (for MC-LA) 4 and 5, and to a lesser extent of type 2B (2). The inhibition of PP1 and PP2A results in an increased phosphorylation of proteins in liver cells, affecting several cellular processes. MYCs are responsible for liver failure and death in humans, wild animals, livestock and aquatic life. Indirect evidence supporting tumour promotion of human cancer from MYCs exposure has been reported by several studies (2). MYCs can induce oxidative DNA damage, genotoxicity, and cause oncogenes activation (3). In addition, MYCs from contaminated lakes can percolate and contaminate groundwater proportionally to the duration of toxic bloom events (4). Their association with primary carcinogens in the aquatic environment is a problematic event. Several large scale fish death outbreaks have been associated to massive occurrence of Cyanobacteria in waterbodies, MYCs concentrations between 0.34 µg/kg and 36.42 µg/kg (5) were measured in the muscle tissue of wild or farmed fish, indicating that even the consumption of contaminated fish muscle might constitute a threat for human health. Cylindrospermopsin (CYN), another common cyanotoxin, is a sulfated-guanidinium alkaloid with hepatotoxic, nephrotoxic and thymotoxic effects, *in vitro* and *in vivo* mutagenic, endocrine-disrupting and carcinogenic activity (6), showing neurotoxic activity in fish (7). Aside from microcystins, other toxic substances of major concern contaminating the environment are toxic metals, namely mercury (Hg), cadmium (Cd) and lead (Pb), and organic contaminants, including polychlorinated biphenyls (PCBs). As a consequence of their environmental persistence and potential for bioaccumulation, these chemicals are widespread throughout the ecosystem, causing toxic problems to all life forms. Fish, in particular, have the ability to accumulate these contaminants and, often, have been employed to assess environmental contamination (8). Fish is an important food source and a major part of many natural food chains; so more attention should be devoted to contaminant levels in fish especially when significant alterations in industrial development can result in large pollutant releases into the environment. Common carp is a good species for bioaccumulation monitoring, being bottom feeder fish that do not migrate extensively, reproduce rapidly and have long life spans (up to 38 yrs.) (9).

The objective of the present study was to investigate the simultaneous presence of these contaminants in ichthyic fauna from lake Pertusillo, an extended Italian reservoir part of a national park interested by intense drilling activities, often accused of causing serious water and sediment pollution in the lake.

Materials and methods

Site description

Lake Pertusillo is an artificial reservoir of the Italian region Basilicata, located at the conjunction of the three municipal lands of Grumento Nova, Montemurro and Spinoso towns (fig.1). Created between 1957 and 1962 by damming the River Agri, its surface area is 7.5 km^2 and its depth reaches 90 m. The mean renewal time is six months (10). Thick and beautiful woods surround it, covering its shores; the lake is a Site of Community Importance (SCI) for the preservation of natural habitats (European Commission Habitats Directive 92/43/EEC) and a Special Protection Zone (SPZ) (European Union Directive on the Conservation of Wild Bird Directive 79/409/EEC). As part of the National Park of Val d'Agri the lake is used for angling and rowing, and its waters are used for drinking and irrigation purposes by the Basilicata and Apulia Regions. Lake Pertusillo is about eight kilometers distant from a center of petroleum refining and in 2016, during an incident, 400 oil tons were spilled from this center in the site groundwater. From 2010 to 2015 fish deaths were reported in the lake, which cause was not found. During the spring and winter of years 2010- 2012 and 2017 occurred a huge dinoflagellate bloom, covering the lake surface.

Sample collection

Samplings from June, 2010 to March, 2013 and in May, 2016 and April, 2017 were carried out in six stations (Rifreddo, **R**; Madonna Grumentina, **MG**; Spinoso, **S**; Montemurro Bridge, **MB**; Lake Damming, **LD**; Masseria Crisci; **MC**) of the lake. Seventy nine adult fish covering 10 species and thirty water samples were analyzed. The analyzed fish species were the zoobenthivorous species *Cyprinus carpio* (carp, 30 individuals), *Carassius carassius* (crucian carp, 10 individuals) and *Cyprinus carpio specularis* (mirror carp, 2 individual), the carnivorous species *Lepomis gibbosus* (pumpkinseed, 2 individuals), *Perca fluviatilis* (perch, 9 individuals), *Scardinius erythrophtalmus* (rudd, 1 individual), *Ictalurus melas* (catfish, 1 individual), *Alburnus alborella* (bleak, 9 individuals), *Squalius cephalus* (chub, 5 individuals) and *Micropterus salmoides* (black bass, 10 individuals). Fish captured by angling were ice–stored and transported to the laboratory. Thirty surface water samples were collected in 20 samplings by filling 1 L Pyrex glass bottles 10-20 cm below the water surface from two stations (**S** shore and **R** shore).

Fish tissue cylindrospermopsin (CYN) extraction

Cylindrospermopsin extraction from muscle tissue samples was performed according to Saker et al. (11) mod.: tissue (5 g, muscle) was homogenized in 10 ml 100% MeOH for 15 min. using a Potter Homogenizer (Polytron), then sonicated 5 min. at 30–40 °C in an ultrasonic bath (Elgasonic Swiss made, 25 kHz) at room temperature, to disrupt cells. The sample was then centrifuged for 5 min. at 5000 g and the supernatant decanted and filtered. The extraction was repeated on the pellet, and the second supernatant filtered on the same filter previously used. The filter and the funnel were washed three times with little volumes of MeOH; the two extracts and washings were collected together, then dried by rotavapor at 40 °C; the residue re-suspended in 2 mL distilled water was then stored at -30 °C until analysis.

Fish tissue microcystin (MYC) extraction

Five grams (wet weight) of muscle tissue from each fish was extracted as in (5). Briefly, the sample was homogenized in 10 ml MeOH for 15 min. using an Ultra-Turrax T8 (IKA Werke, Staufen, Germany) grinder and then sonicated for 5 min. at 30–40∘C in an ultrasonic bath (Elgasonic Swiss made, 25 kHz) to disrupt cell membranes. The sample was centrifuged for 5 min. at 5000 g and the supernatant decanted and filtered on a paper filter. The extraction was repeated on the pellet, and the second supernatant filtered on the same filter previously used. The filter and the funnel were washed three times with little volumes ofMeOH; the two collected supernatants and the washings were gathered, then reduced to a small volume (1-2 ml) by rotary evaporator (Bùchi, Switzerland) at 40∘C, and diluted to 5 ml with MeOH. One ml (for fish) of the extract (corresponding to 1 g of tissue) were then added with 1 ml of distilled water and loaded onto a HLB SPE Waters OASIS cartridge, preconditioned with 1 ml MeOH followed by 1 ml of distilled water. The column was washed with 1 ml of 5% MeOH in distilled water. Microcystins were eluted by 1 ml of MeOH. The MeOH eluate was dried by rotary evaporator at 40°C; the residue, dissolved in 2 ml distilled water, was stored at −30 °C for subsequent microcystin analysis with the EnviroGard Elisa kit.

*CYN and MYC analysis by ELISA assays*Muscle tissue extracts from 17 fish caught in 2012 in MG and S stations were analyzed using the Abraxis Cylindrospermopsin ELISA Microtiter Plate immunoassay (Abraxis Bioscience CA).ELISA assays were performed in accordance with the manufacturer's instructions using the calibration concentrations suggested. The Abraxis immunoassay declares the detection limit is 40 ppb, with percentage coefficients of variation below 10% for standard and below 15% for samples. The final reaction solution absorbances of the kit were measured at 450 nm with an Anthos 2010 microplate spectrophotometer (Anthos – Labtech, Salzburg, Austria).

123 Muscle tissue extracts from 79 fish samples were analyzed using the EnviroGard Microcystins Plate
124 Kit (Strategic Diagnostics Inc., Newark, DE, USA), a direct competitive ELISA for quantitative
125 detection of microcystins and nodularins (limit of quantification, LOQ = 0.1 ppb). This
126 immunoassay does not differentiate between microcystin-LR and two other microcystin variants
127 (MC-RR and MC-YR) but detects their presence to differing degrees. The concentrations at 50%
128 inhibition (50% B/Bo absorbance signal) for these compounds (ppb) are: microcystin-LR 0.31,
129 microcystin-RR 0.32, microcystin-YR 0.38. The final reaction solution absorbances of the kit were
130 measured at 450 nm with an Anthos 2010 microplate spectrophotometer (Anthos – Labtech,
131 Salzburg, Austria). The analytical method to determine microcystins in water and fish samples was
132 previously validated according to the decision 2002/657/CEE (12).
133
134 *Sample handling and trace elements and PCB analysis*
135
136 Ten specimens of *Cyprinus carpio* (common carp) caught from two stations (MC, LD) of Pertusillo
137 Lake in April, 2017 (figure 2,3) were analysed also for trace elements and PCBs. After sampling,
138 the specimens were stored in ice boxes with dry ice, transferred to the laboratory and immediately
139 kept in a deep freezer. Subsequently, the frozen fish samples were thawed and biometric
140 measurement were made (weight range: 868–3195 g, mean: 1296±697 g; length range: 37.0–60.0
141 cm, mean: 43.1±6.9 cm). From each specimen the muscle tissue was dissected, homogenized and
142 analyzed. The extractive analytical procedure and the instrumental conditions to determine trace
143 element concentrations have been described in detail elsewhere (13). Briefly, about 0.5 g of the
144 samples were digested to a transparent solution with a mixture of HNO_3-$HClO_4$ (8:3) for cadmium
145 (Cd), lead (Pb), chromium (Cr), copper (Cu) and zinc (Zn) determination and with a mixture of
146 H_2SO_4-HNO_3 (1:1) for mercury (Hg). The completely digested samples were allowed to cool
147 temperature and diluted with deionized water according to the method recommended by Official

148 Italian Agencies (14). The content of elements was determined by atomic absorption
149 spectrophotometry (Shimadzu AA 7000). Zn was analysed by flame, Cd, Pb, Cr, and Cu by using a
150 graphite furnace (high density tube) (GFA-7000), Hg was measured by using a hydride vapour
151 generator (HVG-1) after reduction by $NaBH_4$. Concerning PCBs, the concentrations of indicator
152 PCBs (28, 52, 101, 138, 153 and 180) were determined using analytical procedures previously
153 described and validated (15). Briefly, about 40 g of powder were mixed with Na_2SO_4 and spiked
154 with PCB 143 used as internal standard. The mixture was extracted with hexane: acetone (9:1) and
155 the extracts were concentrated in order to determine the fat content by gravimetry. Next the extract
156 was dissolved in hexane and cleaned by passing through 8 g of acid silica (H_2SO_4, 44% w. w.),
157 using 50 mL of a mixture of hexane/dichloromethane (1/1, v/v) for elution of the analytes. The
158 eluate was evaporated to dryness and redissolved in 100 mL of iso-octane. For the analysis of
159 PCBs, a Thermo Trace GC connected with a Thermo PolarisQ MS operated in electron impact
160 ionization (EI) mode was equipped with a 30 m, i.d. 0.25 mm and 0.25 µm Rtx 200 capillary
161 column (Thermo, Austin, Texas, USA). The MS was used in the SIM mode with two ions
162 monitored for each PCBs homologue group in specific windows. One ml of the cleaned extract was
163 injected in splitless mode (injector temperature 90 °C then to 300 °C with 70 °C/min), splitless time
164 1.50 min, pulse pressure time 1.50 min, pressure pulse 25 psi. Helium was used as carrier gas at
165 constant flow (1.0 ml/min). The temperature of the Rtx 200 column was held at 90 °C for 1.50 min,
166 then increased to 180 °C at a rate of 15 °C/min, further increased to 280 °C at a rate of 5 °C/min,
167 further increased to 300 °C at a rate of 40 °C/min, held for 7 min.
168 *Quality assurance*
169
170 Reference tissue (Tort-2 Lobster Hepatopancreas, National Research Council of Canada, Ottawa,
171 Ontario, Canada) was treated and analysed in the same way as the samples. Results (Hg: 0.28 ±
172 0.03; Cd: 26.2 ± 2.4; Pb: 0.32 ± 0.18; Cr: 0.73 ± 0.16; Cu: 101 ± 13; Zn: 188 ± 12 µg g^{-1} dry

weight) were in good agreement with the certified values (Hg: 0.27 ± 0.06; Cd: 26.7 ± 0.60; Pb: 0.35 ± 0.13; Cr: 0.77 ± 0.15; Cu: 106 ± 10; Zn: 180 ± 6 μg g^{-1} dry weight) and the standard deviations were low, proving good repeatability of the methods. The results for standard reference material displayed recoveries of the elements ranging from 91 to 104% (n = 3).The limit of detection (LOD) (Hg: 5; Cd: 0.12; Pb: 10; Cr: 5; Cu: 26; Zn: 24 ng g^{-1} wet weight) was defined as the concentration corresponding to three times the standard deviation of blanks, and the standards of quantification (LOQs) were the following: Hg: 13; Cd: 0.30; Pb: 38; Cr: 16; Cu: 81; Zn: 87 ng g^{-1} wet weight. Two blank samples were analysed together with each sample batch. Metal concentrations in blanks were below the detection limits in all the analyses. Blanks and calibration standard solutions were similarly analysed as the digested sample solution, and calibration curves were constructed. Analyses were duplicated to check the reproducibility of the results. Relative standard deviations among replicates were always less than 10%. Recovery tests were performed for the investigated metals in selected samples by spiking analysed samples with aliquots of the metal standards and then carrying out digestion. The recovery percentages ranged from 96 to 99%. Metal concentrations are presented as μg g^{-1} wet weight basis. For PCBs quality control was performed through the analysis of procedural blanks, a duplicate sample and a standard reference material [CRM349 for PCBs (cod liver oils) (BCR, Brussels)] within each batch of samples. The recovery percentage of the standard reference material was within the range of 86 and 105%. For the samples and standard reference materials, the relative standard deviations (RSD) were <10% for all the detected compounds. The limit of detection (LOD) for PCBs ranged from 0.02 to 0.50 ng g^{-1} on a lipid weight basis, while the limit of quantification (LOQ) varied from 0.20 to 1.30 ng g^{-1} on a lipid weight basis. Appropriate standard solution was added to the samples and recovery values were between 82 and 104%. The trace element and PCB concentrations in the samples were expressed as μg g^{-1} and ng g^{-1} wet weight, respectively.

198 *Statistical analysis*

199

200 Kruskal-Wallis test was conducted to verify the difference in the levels of trace metal and PCB
201 accumulation, while simple linear regression coefficient was used to examine the correlations
202 between PCBs and specimen length. To investigate size influence on PCB accumulation, the length
203 of fish was chosen, because less subject to fluctuation than body weight (16). The level of
204 significance was set at $p < 0.05$.

205

206 *Microscopic observations*

207

208 The water samples were stored in ice chests and transported to the laboratory. For microscopic
209 observations water subsamples were analyzed by an inverted microscope (Leitz Labovert FS)
210 according to Utermőhl (17) and Lund et al. (18), using 25 ml sedimentation chambers for
211 phytoplankton identification and cell density estimation.

212

213 **Results and discussion**

214

215 *Trace element and PCB concentrations*

216

217 The trace element concentrations detected in the study showed Zn values ranging from 1.15 to 4.32
218 μg g^{-1} wet weight (2.31 μg g^{-1} wet weight), while Cu showed much lower concentrations, ranging
219 from 0.15 to 0.61 μg g^{-1} wet weight (0.36 μg g^{-1} wet weight) ($p < 0.001$) (fig.2). The considerable
220 difference in levels between these two metals is not unique to the species here studied, being part of
221 a general picture suggesting muscle tissue not to be considered a specific physiological site for Cu
222 (19). Cr levels were very low too, ranging from 0.02 to 0.05 μg g^{-1} wet weight (0.03 μg g^{-1} wet

223 weight) ($p < 0.001$). Among non-essential metals the highest concentrations were recorded for Hg
224 with values between 0.27 and 0.53 μg g^{-1} wet weight (0.40 μg g^{-1} wet weight), followed by Pb
225 showing levels from 0.05 to 0.28 μg g^{-1} wet weight (0.14 μg g^{-1} wet weight), while Cd registered
226 the lowest values between 0.03 and 0.05 μg g^{-1} wet weight (0.04 μg g^{-1} wet weight) ($p < 0.001$). A
227 comparison with data in the literature shows a wide concentration heterogeneity for all metals
228 studied. However, our Hg levels are very similar to those found by Stong *et al.* (20) in common
229 carp from Lake Chapala in Mexico, but very higher than those reported by Vicarova *et al.*(21) in the
230 same species from three reservoirs in the Czech Republic. For Cd and Pb, the levels in this study are
231 in line with values reported by Yancheva *et al.* (22) in muscle tissue of common carp from
232 Topolnitsa reservoir in Bulgaria. For essential metals, our Cr values are in good agreement with
233 results found in the muscle tissues of common carp from the uncontaminated fishponds in the
234 Czech Republic (23) and Kabul River in Pakistan (24). In contrast, our Zn values are lower than
235 those reported by Yousafzai *et al.* (24) and by Čelechovská *et al.* (23) in muscle tissue of common
236 carp from the Keban Dam Lake in Turkey and the fishponds in the Czech Republic, respectively.
237 Regarding Cu concentrations, samples analysed in this study showed levels of the same order of
238 magnitude of those reported for common carp from the Czech Republic (23) To safeguard public
239 health, concentration standards in fish for some heavy metals have been established by the
240 European Commission. In particular, Hg, Pb and Cd limit values at 0.50, 0.30 and 0.05 μg g^{-1} wet
241 weight respectively, have been fixed (25). In this context, no analysed fish sample showed
242 concentrations exceeding the European Directive proposed limits for Pb and Cd while for Hg,
243 slightly exceeding levels were registered in two samples (0.51 and 0.53 μg g^{-1} wet weight).There
244 are no European guidelines for fish consumption established as regards Cu, Zn and Cr, but the UK
245 Food Standards Committee's Report fixed Zn and Cu limits at 50 and 20 μg g^{-1} wet weight
246 respectively, while the Western Australian Food and Drug Regulation List (26) fixed Cr limits at

247 5.5 μg g^{-1} wet weight. Our detected results were always lower than these human consumption
248 limits.
249 The subset of six PCB congeners here tested were selected by the International Council for the
250 Exploration of the Sea (ICES) as contamination indicators, due to their easy quantification
251 compared to the other non-dioxin-like PCBs, however representing all relevant degrees of
252 chlorination. The data analysis showed that PCBs 153 and 138 were the most frequently detected
253 congeners (detection in 100% of samples), while PCBs 101 and 180 were detected with 50% and
254 70% frequency, respectively, and PCBs 28 and 52 were below the detection limits in all samples
255 examined. The total concentrations of indicator PCBs were 95.8-202.5 ng g^{-1} lipid weight, with a
256 mean value of 148.6 ng g^{-1} lipid weight. PCBs 153 and 138 with mean values of 62.6 ng g^{-1} lipid
257 weight and 55.4 ng g^{-1} lipid weight were the highest in concentration, followed by PCB 180
258 showing a mean concentration of 18.7 ng g^{-1} lipid weight and PCB 101 exibithing the lower mean
259 value equal to 11.9 ng g^{-1} lipid weight. The PCB bioconcentration in aquatic organisms correlates
260 with the degree of chlorination, the stereochemistry and lipophilicity (27). Generally, congeners
261 with a high chlorination grade are more difficult to metabolise and eliminate than less chlorinated
262 congeners. Our data well fit this general picture, being low chlorinated congeners PCBs 28 and 52
263 below the detection limit, PCB 101 contributing for 8%, while hexa- and heptachlorinated
264 biphenyls 138, 153 and 180 together constituted a consistent percentage of the total PCB burden
265 representing 92%. Generally, the largest and potentially oldest fish exhibit higher PCB levels than
266 younger organisms. Despite of this, no correlation between fish length and total PCB concentrations
267 was observed ($R = 0.42$; $P > 0.05$) in the present study, probably as consequence of scarce PCB
268 contamination in the Pertusillo basin. These PCBs have been recommended by the EU as indicators
269 of PCB contamination because generally they represent approximately half of the total ndl-like
270 PCBs existing in food. In fact, the European Food Safety Authority (EFSA) Scientific Panel
271 regarding Contaminants in the Food Chain (CONTAM Panel) recommends the sum of these six

PCBs as an appropriate marker for risk assessment of ndl-PCBs. Regulation No. 1259/2011 of the European Union (EU) (28) has set *de novo* maximum tolerable levels for the sum of the six indicators non-dioxin-like PCBs in muscle meat of freshwater fish that, apart from some exceptions, is of 125 ng g^{-1} wet weight. Our results presented on a lipid weight basis have, hence, been converted to wet weight basis to conform to legal standard. According to this, the sum of six "indicator" congener concentrations was below the conventional permissible consumption limit in all samples examined (1.27 ng g^{-1} wet weight).

Microcystin and cylindrospermopsin concentration

Superficial fortnightly water samples taken from March to April 2012 and from October 2012 to March 2013 were analyzed for phytoplankton presence. In these winter samples only 16 species were detected; the lack of summer samples, due to difficulties in carrying out regular water samplings, did not allow a complete evaluation of phytoplanktonic composition. In a few summer samples analyzed by the Basilicata Agency for Environmental Protection (ARPAB) in 2014, 9 other species were detected (29). The poor presence of phytoplanktonic species detected in this study may also be due to the need for column samplings and more systematic monitoring. However, even in the past the lake showed the presence of a limited number of species (29 species detected, 30). No cyanotoxins were detected in the analyzed water samples.

In fish 86% of total tissue samples were positive for MYC presence, at concentration values ranging from a minimum of 0.19 ng/g to a maximum of 2.01 ng/g b.w. (fig. 4-6). *Micropterus salmoides*, *Carassius carassius* and *Cyprinus carpio* were the species with highest concentration capacity and averages.

ELISA analyses showed the presence of CYN in 64% of samples, with maximum concentrations at 0.78 ng/g in muscle (fig. 7). *Cyprinus carpio* and *Perca fluviatilis* were the species with highest

297 concentration capacity and averages. ARPAB phytoplankton analyses in summer 2014 showed the
298 presence of *Aphanizomenon* sp., which could take account for CYN presence (31).
299 In May, 2016 fifteen fish samples from four stations (2 carps from MG, 2 carps from MB, 5 chubs
300 and 6 perchs from MC) were analyzed for MYC presence (fig. 5), and the highest mean content
301 (0.72 ng/g) was found in perchs. In the following year (April, 2017) nine samples (5 carps from MC
302 and 4 carps from LD) showed a mean content (0.91 and 0.93 ng/g, respectively, fig.6) higher than
303 that of 2016 carps (0.29 and 0.28 ng/g, respectively). The toxicity of microcystins in fish depends
304 on the balance between accumulation and metabolism (32); the observed species-specific
305 sensitivities have been interpreted as the result of anatomical, physiological and behavioral
306 differences among the various fish orders (33; 34): such as, for example, the detoxification
307 capacities *via* the glutathione-S-transferase pathway (35).
308 CYN accumulation in ichthyic fauna was previously investigated in crayfish (*Cherax*
309 *quadricarinatus*), rainbow fish (*Melanotaenia eachamensis*) (36) freshwater mussels (*Anodonta*
310 *cygnea*) (11), salmonids (*Salmo trutta*) (31) and finfish (37).
311 The acute Tolerable Daily Intake (TDI) guideline for MC-LR, proposed by WHO in 1998 for an
312 adult of 60 kg b.w. (0.04 µg/kg body weight/day, 38) was revised by USEPA in 2006, with new
313 proposed guidelines developed for acute and chronic risk (0.006 and 0.003 microcystin µg/kg
314 b.w./day, respectively; 39), but no guidelines for cancerogenicity were proposed, due to the
315 insufficient adequacy of the available studies. In the same 2006 the International Agency for
316 Research on Cancer classified microcystin-LR as possibly carcinogenic to humans (group 2B, 40).
317 Case-control studies in southwest China recently confirmed the link between MYC serum levels
318 and occurrence of hepatocellular carcinoma in humans (41).
319 For an adult human weighing 60 kg and ingesting 300 g serving of fish muscle, the microcystin
320 level in 14.5 % of muscle samples analyzed from 2010 to 2012 was 1.6 -fold the recommended TDI
321 acute value of EPA, and 36.3% of muscle samples were even 3.3 -fold the recommended chronic

322 value. According to Italian law, cyanotoxin presence is not allowed in edible fish.

323 Contaminant classes like polycyclic aromatic hydrocarbons, trace elements, PCBs and microcystins
324 are known to produce synergistic effects on organisms: in fish heavy metals may cause enhanced
325 toxic effects if combined (42), *in vitro* and *in vivo* studies on cyanobacterial extracts, PCB 153 and
326 fluoranthene (43) provide evidence on synergistic effects of tumor promotion.

327 In Italy, microcystin contaminations in ichthyic fauna were detected in several lakes (5); (44).
328 MYCs demonstrated to be a recurrent component among the lake Pertusillo main contaminants,
329 being detected in fish tissue all along the duration of the study. No MYC producing cyanobacteria
330 were found in our phytoplankton analyses but several benthic species are MYC producers, too, and
331 an extended monitoring for phytobenthic toxic species in the sediments of the lake would be
332 needed, to investigate the reason why a higher presence of these toxins was detected in the cyprinid
333 species.

334 Zn levels detected by ARPAB lake water monitoring in 2014 (between 5 and 83 µg/l; 29) are
335 known to increase the growth and intracellular MYC production in *Microcystis aeruginosa* cultures
336 (45). A recent meta-analysis has also shown that persistent organic pollutants, among which PCBs,
337 are able to stimulate cyanobacterial growth (46).

338 A more extended monitoring is needed to define the presence of these different contaminants in
339 ichthyic fauna, their role in the recurrent fish deaths in the lake, and the exposure risk of people of
340 the lake region by consuming contaminated lake fish. As Pertusillo Lake is part of a SCI zone, the
341 PCBs content in lake fish could endanger also the fish-eating birds, through biomagnification. Lake
342 Pertusillo is mesotrophic-eutrophic (29) and several episodes of algal blooms occurred in the lake
343 during the last seven years. Organisms are usually exposed not only to isolated environmental
344 pollutants, but to chemical mixtures which individual components may be present at concentrations
345 lower than their safety threshold levels.

346 Although the concentrations of metals and PCBs detected in the analysed fish samples are not high,

the presence of these different compounds in association with microcystins suggests the possibility of future cyanobacterial toxic blooms in this environment through their growth stimulation, pointing out the need for restoration programs to improve the trophic conditions of the reservoir. Moreover, given the presence of the industrial activities of oil drilling in the area, further studies are needed to investigate the potential contamination of oil compounds in Pertusillo ichthyic fauna.

Conclusions

The ichthyic fauna of Pertusillo appears to be interested by concentration of multiple contaminants including MYCs, CYN, heavy metals and PCBs. The MYC levels in 14.5 % of fish muscle samples exceeded 1.6 -fold the recommended TDI acute value of EPA, and 36.3% of muscle samples were even 3.3 -fold the recommended chronic value.

The MYC production by cyanobacteria may be synergistically influenced and enhanced in the aquatic environment of the lake by some trace element concentrations, as Zn levels detected.

Even if the single trace element values and PCB values detected in fish were below the Italian limits, the simultaneous presence of these multiple comtaminants could involve sinergistic effects in the ichthyic faunaand in the lake environment, still unknown.

Waiting for new tools to assess the overall impact of these pollutants on aquatic life and human health, the managing policy remains the exploration and implementation of cost-effective and appropriate remediation, coupled with the search for environmentally more benign products and processes, which should aim to minimize introduction of critical pollutants into the aquatic environment.

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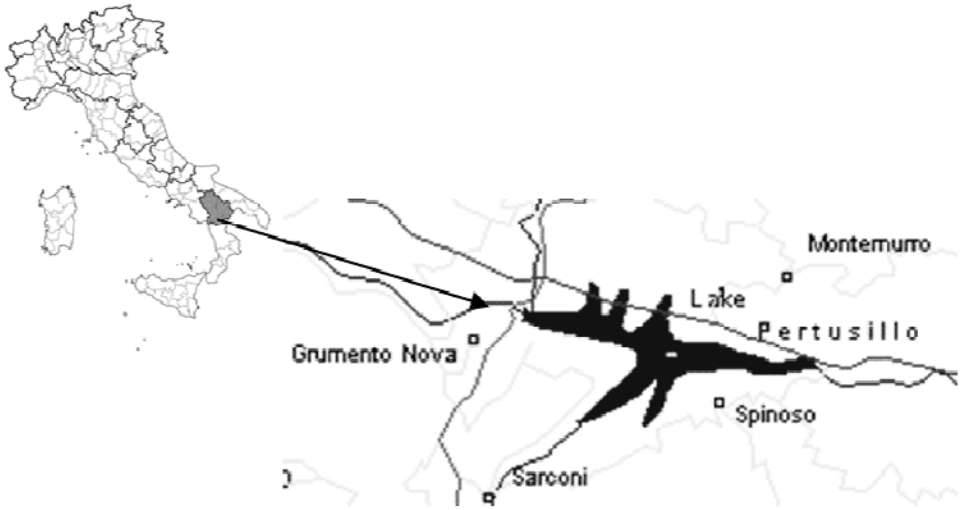
548 **Legend**

549 Figure 1. Study site and station coordinates.



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Sampling station	N	E
Masseria Crisci **MC**	40.28977	15.95180
Rifreddo **R**	40.28710	15.9527
Spinoso **S**	40.28044	15.96638
Madonna Grumentina **MG**	40.29172	15.92957
Montemurro Bridge **MB**	40.28238	15.9825
Lake Damming **LD**	40.27522	15.99157

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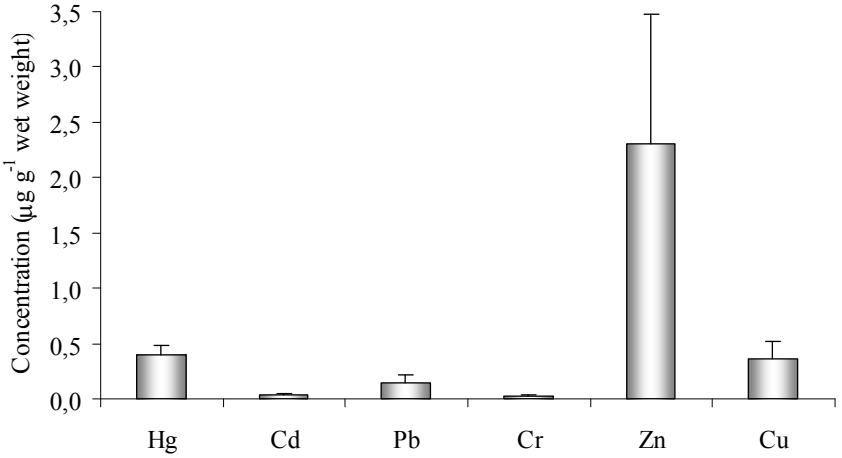
556 Figure 2. Trace element concentrations in common carp.



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558 Figure 3. Concentrations of six PCB indicator congeners in common carp.

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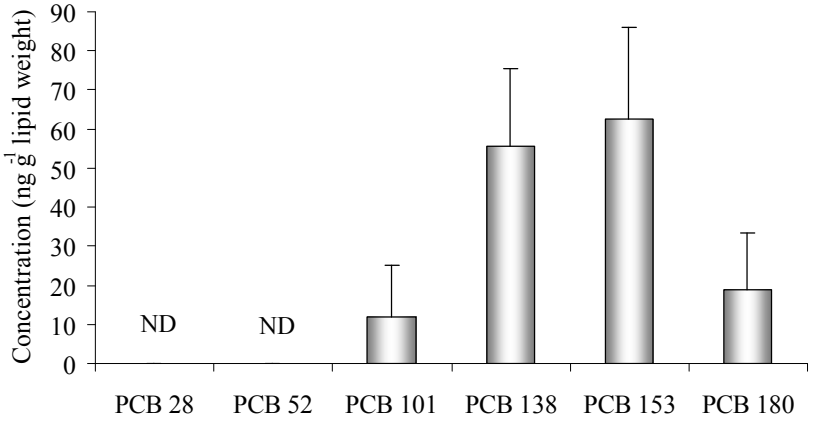

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563

564 Table 1. Phytoplanktonic species identified in the superficial samplings of 2012.

Phytoplanktonic species	
Cyanobacteria	*Coelosphaerium kutzingianum* Nageli
Diatomeae	*Asterionella formosa* Hassall
	Cyclotella kutzingiana Thwaites
	Cymbella sp. C. Agardh
	Fragilaria crotonensis Kitton
	Gyrosigma attenuatum (Kutzing) Rabenhorst
	Melosira italica (Ehrenberg) Kutzing
	Melosira varians C. Agardh
	Navicula ssp. Bory de Saint-Vincent
	Nitzschia acicularis (Kutzing) W. Smith
	Rhizosolenia sp. Ehrenberg
	Stephanodiscus astraea (Ehrenberg) Grunow
Chlorophyceae	*Oocystis lacustris* Chodat
Conjugatophyceae	*Closterium kùtzingii* Brébisson
	Closterium pronum Brébisson
Dinophyceae	*Ceratium hirundinella* (O.F.Müller) Dujardin

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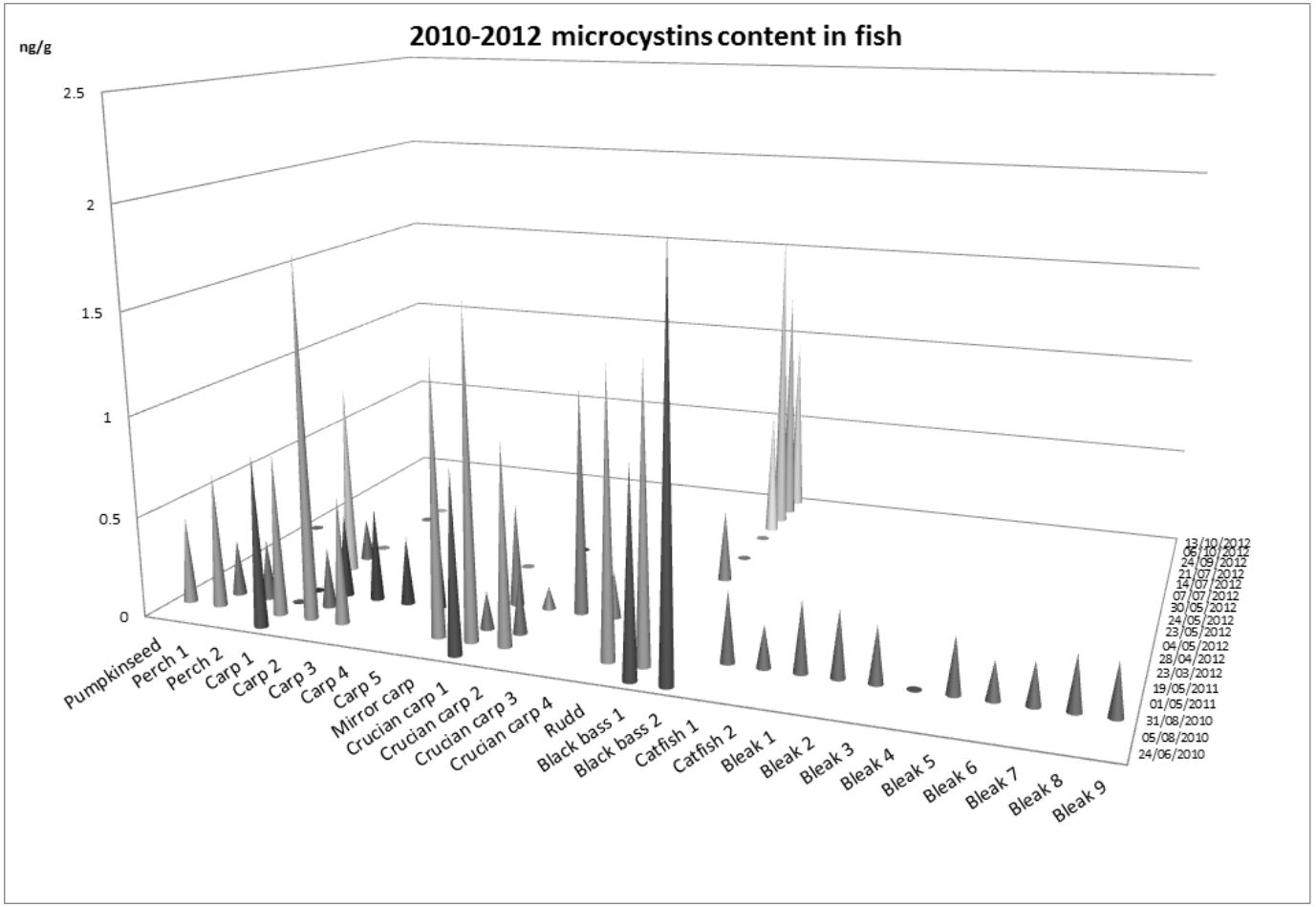
566

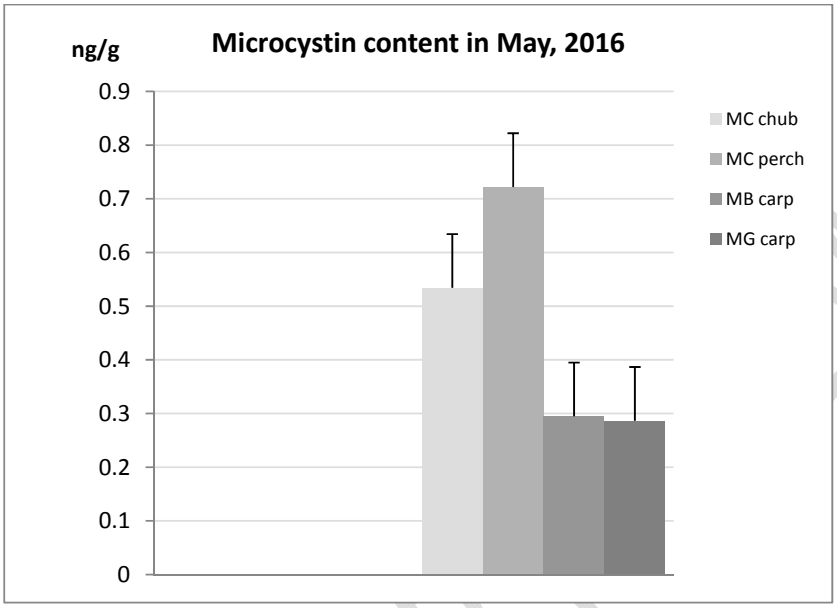
567 Figure 4. Microcystin concentration in fish muscle tissue (all fish samples) during three years (2010-2012).

568



569

570 Figure 5. Microcystin concentration in fish samples from three lake stations (MG, MB, MC) in
571 May, 2016.



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575 Figure 6. Microcystin concentration in fish samples from two lake stations (MC, LD) in April,
576 2017.

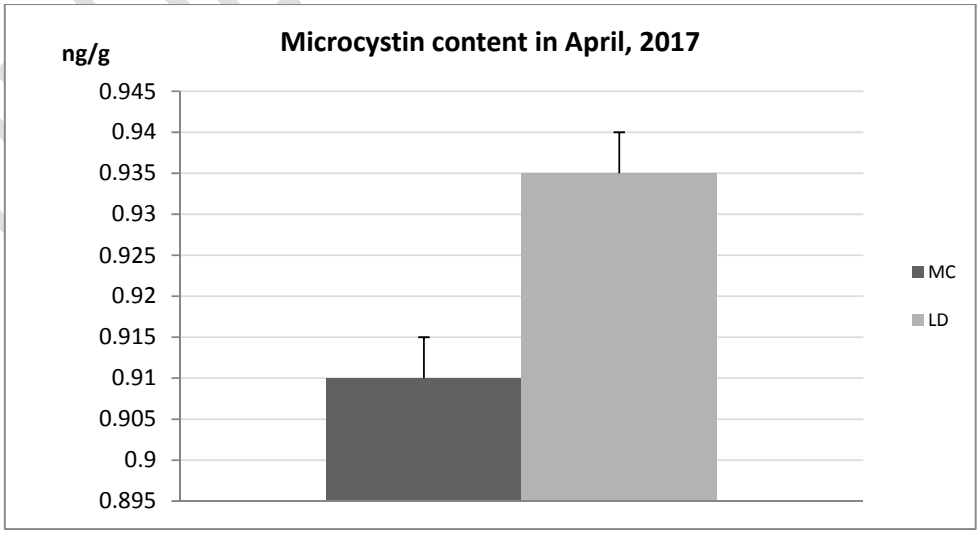

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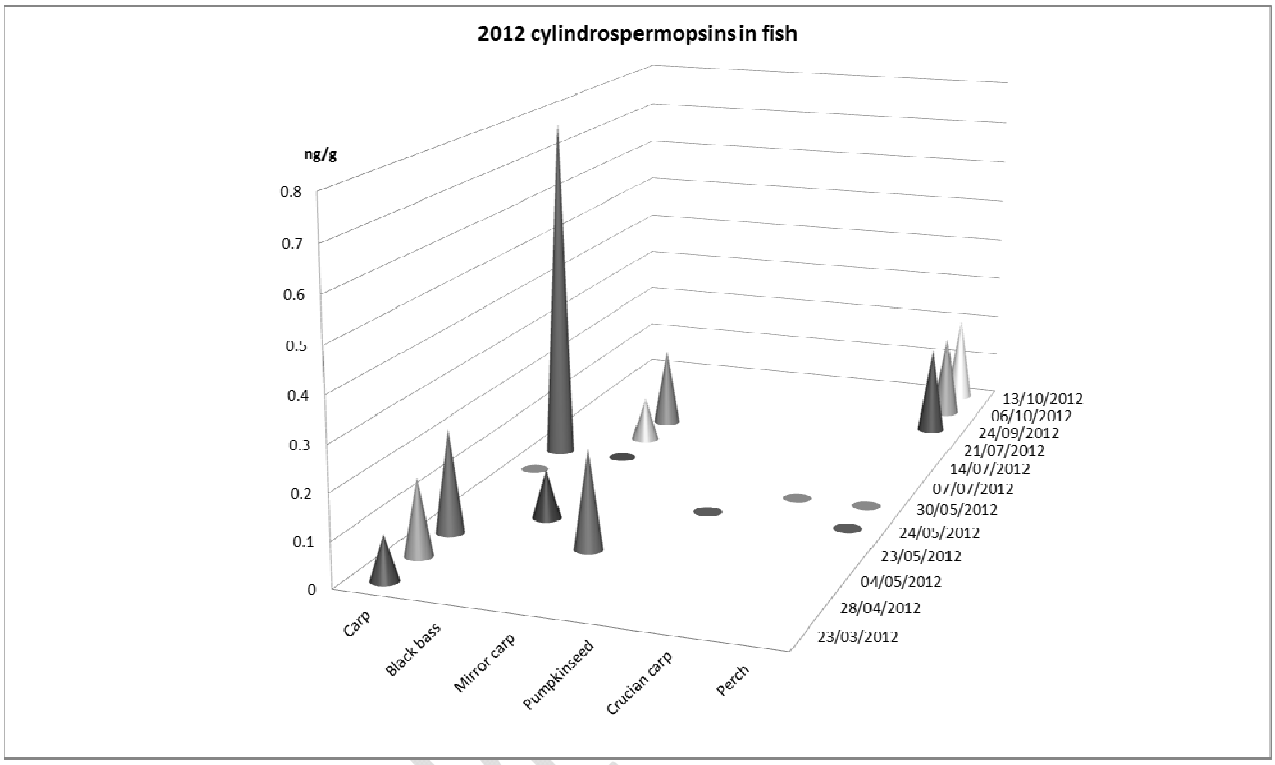
Figure 7. Cylindrospermopsin concentration in fish muscle tissue during 2012.



592 **Figure legend**

593

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595

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597

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599

600 Figure 4. Microcystin concentration in fish muscle tissue (all fish samples) during three years
601 (2010-2012).

602

603 Figure 5. Microcystin concentration in fish samples from three lake stations (MG, MB, MC) in
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606 Figure 6. Microcystin concentration in fish samples from two lake stations (MC, LD) in April,
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611 **Table legend**

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614