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An alert from Italian rivers**

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1 **NDL-PCBs in muscle of the European catfish (*Silurus glanis*): an alert from Italian rivers**

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3

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8

9 **Abstract**

10 The non-dioxin-like polychlorinated biphenyls (NDL-PCBs) highly contribute to the PCB dietary
11 intake of total PCBs. Most of the NDL-PCBs are assumed through ingestion of contaminated fish
12 and fishery products. Therefore, it is important to quantify their presence in aquatic organisms to
13 evaluate human risks associated with fish consumption. The European catfish is a top food-chain
14 predator and is considered a reliable bio-monitoring tool reflecting the state of the environmental
15 organic pollution. From 2006 to 2009, 54 European catfish were captured in four sites covering the
16 area of the Po River (North Italy), and their muscles were analysed to determine the levels of 18
17 PCBs congeners. All samples presented detectable levels of 18 congeners and, on average, results
18 showed an important presence of NDL-PCBs. The sum of the six congeners
19 (28,52,101,138,153,180 IUPAC) was used as indicator of the total PCBs concentration. The 33% of
20 the samples analysed exceeded the maximum levels of 125 ng g⁻¹ set by European regulations in
21 fish. The values measured ranged from 19.7 to 1015.4 ng g⁻¹ (mean 135.6 ± 149.8 ng g⁻¹).

22 The concentrations of NDL-PCBs were not related to fish weight or sex, while a significant
23 variability was found among sites (p < 0.05), according to the geographical location of many
24 industrial activities in the catchment area of the Po River. PCB 153 and 138 were present in higher
25 concentrations (40% and 30% respectively). We hypothesise that this is due to their high resistance
26 to metabolic degradation.

27

28 *Keywords:* NDL-PCBs; Freshwaters; *Silurus glanis*

29

30 **1. Introduction**

31 Freshwater fish are considered reliable indicators for the presence of persistent bio accumulative
32 and toxic lipophilic compounds in river basins (Roche et al., 2000; Patrolecco et al., 2010; Pacini et
33 al., 2013). Monitoring fish tissues has a distinctive advantage in relation to monitoring inert
34 environmental compartments. Sediment bound organic contaminants rendering the latter refractory
35 to chemical and biological transformation and release, while the fraction of organic compounds
36 detected in fish tissues represents a bioavailable portion that cycles through aquatic food webs.
37 Since organic contaminants have a great affinity for the lipids in animal tissues, fish are able to
38 accumulate the contaminant concentrations not detectable in the water column.

39 Polychlorobiphenyls (PCBs) are a group of persistent organic contaminants including 209
40 compounds (congeners), exhibiting different degrees and patterns of chlorination (WHO, 1993).
41 During the 1930s and for approximately 50 years, these chemicals were commercially produced in
42 different industrialized countries as technical mixtures (e.g., Aroclors[®], Clophens[®], Fenclores[®],
43 Kanechlors[®], Pyralenes[®]) to be used mainly as dielectric fluids, organic diluents, plasticizers,
44 adhesives, and flame retardants. Although banned in the 1970s and 1980s in the United States and
45 Europe, respectively, PCBs are still present in the environmental and can be traced in animal
46 tissues. Due to their high persistence and bioaccumulation and toxic potential, PCBs can occur at
47 levels of concern. Despite the large number of theoretical congeners, only about 130 are likely to
48 occur in the technical mixtures and, among them, fewer were and are environmentally relevant.

49 According to their toxicological properties, PCBs are usually recognized to possess a dioxin-like
50 (DL-PCBs) or a non-dioxin-like (NDL-PCBs) activity. The DL-PCB group comprises 12 congeners
51 characterized by non- or mono-*ortho* chlorosubstitution. These congeners exert their toxicity
52 primarily through the binding of the aryl hydrocarbon receptor (*AhR*), similarly to

53 polychlorodibenzodioxins (PCDDs) and polychlorodibenzofurans (PCDFs) (van den Berg et al.,
54 2006). The NDL-PCB group includes the remaining congeners, analytically predominant in
55 environmental matrices and animal tissues. These congeners appear to act *via* different modes and
56 some direct effects on neuronal cells – such as the reduction of dopamine neurotransmitter levels or
57 the interference with calcium homeostasis (Brown et al., 1998, Tilson et al., 1998) – may be
58 peculiar for those chemicals.

59 Several evidences suggest that even low DL- and NDL-PCB doses can cause subtle effects when
60 exposure is prolonged over time, and particularly, if it occurs during the prenatal and postnatal
61 development in mice (Haave et al., 2011). For these reasons, a more specific concern has been
62 raised as to the effects on children's neurological development (Walkowiak et al., 2001;
63 Vreugdenhil et al., 2004). Moreover, possible relations with specific neurobehavioral changes in
64 human adults, such as the attention-deficit/hyperactivity disorders, have been reported (Schoeters &
65 Birnbaum, 2004).

66 The consumption of contaminated fish is one of the most relevant pathways for transfer PCBs from
67 the environment to humans (US EPA, 2007). A recent report of the European Food Safety
68 Authority (EFSA) showed that particularly high levels of non dioxin-like PCBs (NDL-PCBs) can
69 be found in fish and fishery products (EFSA, 2010). Furthermore, a relevant number of national and
70 international regulatory bodies have established fish consumption guidelines with a particular
71 respect for those fish who are known to accumulate a variety of chemicals. The European Union has
72 also provided recommendations of alternative diets in order to avoid consumption of contaminated
73 products. Moreover, the regulation 1259/2011/EU (enforced since January 1th 2012) has set *de novo*
74 a maximum tolerable levels (MLs) for the sum of the six “indicators” NDL-PCBs 28, 52, 101, 138,
75 153 and 180 (Σ_6 NDL-PCBs) in fish flesh.

76 Fish can be considered a valid bio indicator for the level of pollution in freshwater environment.
77 European catfish (*Silurus glanis*) is a top food-chain predator in the freshwater ecosystem, and can
78 reflect the environmental contamination. This species is nowadays popular among European anglers

79 and, for this reason, it has been introduced in many European countries, including France, the
80 Netherlands, Spain, and the UK (Elvira, 2001). In Italy this species has received an increasing
81 interest also for commercial purposes, as it is the case for the Eastern European market, where its
82 flesh is greatly appreciated. In a previous study, Squadrone et al. (2012) estimated the
83 concentrations of mercury, cadmium, lead, arsenic and chromium in several organs of this predator
84 within the area of the Po river basin (Northern Italy). They found levels of mercury exceeding the
85 Maximum Levels (MLs).

86 The aim of this study is to evaluate, in the same area, the levels of NDL-PCBs in *Silurus glanis*, in
87 order to evaluate the reliability of this fish species as a bio-indicator of organic and chemical
88 pollution.

89 In particular, the compliance with the maximum levels established by the European Commission
90 Regulation (1259/2011) were verified, and the distribution of the six indicators congeners, their
91 variations with sampling sites, gender, age and size were discussed.

92

93 **2. Materials and methods**

94 *2.1 Study species*

95 The European catfish (*Silurus glanis*), also known as wels catfish, is one of the largest European
96 freshwater fish. This species is native in Eastern Europe and Western Asia and is abundant in the
97 Danube and Volga basins. The European catfish inhabits the lower reaches of large rivers and
98 muddy lakes, tends to prey on fish smaller than could be expected for its size and mouth gape
99 (Adámek et al., 1999; Wysujack & Mehner, 2005). *Silurus glanis* is a bottom dwelling nocturnal
100 predator, feeding in the whole water column. Fry and juveniles are benthic, feeding on a wide
101 variety of invertebrates and fish, while adults prey on fish and other aquatic vertebrates. The sexual
102 maturity is reached at 2-3 years, and this catfish species can live for over thirty years.

103 Only the flesh of young specimens is valued as food, and is palatable when the catfish weighs less
104 than 15 kg (33 lb). Larger than this size, the fish is highly fatty and not recommended for

105 consumption.

106 *2.2 Field sampling*

107 Fifty-four specimens (28 males and 26 females) of European catfish were collected from late spring
108 to early fall 2009-2011 in the following 4 sites:

- 109 ◦ 1. Po River (Lat. 45.138098, Long. 8.558135)
- 110 ◦ 2. Tanaro River (Lat. 44.919446, Long. 8.6099719)
- 111 ◦ 3. Bormida River (Lat. 44.906940, Long. 8.646197)
- 112 ◦ 4. Parma River (Lat. 44.832150, Long. 10.314585)

113 All the sites belong to the hydrographical basin of the Po River - the largest river in Italy - and were
114 selected according to accessibility and fish abundance. 24 animals (12 males and 12 females) were
115 collected from Po River, Alessandria district, 10 (6 males and 4 females) from Tanaro River,
116 Alessandria district, 9 (6 males and 3 females) from Bromida River, Alessandria district, and 11 (4
117 males and 7 females) from Parma River, Parma district.

118 Fish were captured using an electro-fishing boat, providing up to 100 Hz, in agreement with the
119 animal welfare legislation prescription. Specimens were preserved on ice and transported to the
120 laboratory. Animals were dissected to obtain muscle samples, which were immediately frozen and
121 stored at $-20\text{ }^{\circ}\text{C}$. Fish age was estimated by growth bands in vertebrae. The overall sample
122 consisted of specimens ranging from a length of 60 to 120 cm and a weight between 1.5 and 10.5
123 Kg (males: $86.80\pm 22.12\text{ cm}$, $5.10\pm 4.07\text{ Kg}$; females: $83.55\pm 17.05\text{ cm}$, $4.59\pm 2.24\text{ Kg}$; mean \pm SD).

124 *2.3 Analytical methods*

125 The quantification of NDL-PCBs was performed by adapting the method of Perugini (2004). The
126 quantified congeners were the six indicators 28, 52, 101, 138, 153 and 180, and their cumulative
127 analytical concentration has been reported as Σ_6 PCBs. Other 18 NDL PCB congeners (95, 105,
128 110, 118, 146, 149, 151, 155, 170, 177, 183, and 187) were detected and their cumulative analytical
129 concentration has been reported as Σ_{18} PCBs.

130 All the samples were freeze-dried, powdered and transferred into Accelerated Solvent Extraction
131 (ASE) cells (102,1 atm and 100 °C). The extraction solvent was a mixture of n-hexane/acetone 1:1
132 (v/v). The extract was filtered and evaporated to dryness, permitting the gravimetric determination
133 of the fat content. Before the dissolution of fat in hexane for sample cleaned up , PCB 155 and PCB
134 198 were added as internal standards. The purification step was performed using silica columns.
135 The fat was removed on a Extrelut-NT3 column loaded with sulphuric acid. The final sample
136 extract was evaporated under a nitrogen stream to dryness and reconstituted by addition of 100 µL
137 of isooctane. The GC/MS detection was performed on a Thermo Focus gas chromatographer,
138 equipped with a DB-5MS column (30 m x 0.25 mm, 0.25 µm film thickness), and coupled to a DSQ
139 single quadruple mass spectrometer. The GC injector and transfer line temperatures were
140 respectively 250°C and 270 °C. The oven temperature program was: 100°C for 1 min, ramp
141 20°C/min up to 190°C (isotherm for 2 min), ramp 3°C/min up to 250°C and ramp 50°C/min up to
142 300°C (isotherm for 20 min). All analyses were performed in duplicate. To check the purity of the
143 reagents and contamination, “blanks” was analysed for each calibration run, using the same
144 procedure. Moreover, the reference material for organo-chlorine compounds CARP-2 (Ground
145 whole carp, NRC Canada) was utilized for quality control, together with control and spiked samples
146 in each round of analysis.

147 In line with European regulatory instructions (EU 1259/2011) the cumulative concentrations (Σ_6 ,
148 Σ_{18}) were expressed as "upper bound" (UB) concentrations, on the assumption that all the values of
149 the different congeners below the LOQ are equal to the LOQ. To establish the compliance of
150 samples with the ML, the expanded measurement uncertainty was subtracted to the analytical result
151 when the UB was above the ML.

152 The Limit of Quantitation (LOQ) for the analyzed PCBs was 6 ng g⁻¹. Cause the validation is
153 required for the analytical methods used in food official control, this method was validated
154 according to 2004/882/EC Regulation and ISO 17025 criteria.

155 *2.3 Statistical analysis*

156 Data were tested for normality by using Kolmogorov-Smirnov test. Since the assumptions for
157 parametric analyses were not met, a Kruskal–Wallis analysis of variance by ranks, followed by
158 Mann–Whitney U tests for pairwise comparisons, was performed to assess differences in the Σ_6
159 PCBs among fishes from different rivers. Significant differences were considered to occur if $p <$
160 0.05. Moreover, correlations between Σ_6 PCBs and Σ_{18} PCBs across samples, and between fish's
161 weight and Σ_6 PCBs were examined using linear regression models.

162

163 **3. Results and discussion**

164 The six PCB congeners 28,52,101,138,153 and 180 were chosen as indicators not for their toxicity,
165 but because they are easily quantified compared to the other NDL-PCBs, and they represent all
166 relevant degrees of chlorination. Indeed, the EFSA (European Food Safety Authority) Scientific
167 Panel concerning Contaminants in the Food Chain (CONTAM Panel) decided to use the sum of
168 these six PCBs as the basis for the evaluation, because these congeners are appropriate indicators
169 for different PCB patterns in various sample matrices and are most suitable for a risk assessment of
170 NDL-PCBs. The CONTAM Panel underlines in its Scientific Opinion related to the presence of
171 non-dioxin-like PCBs in feed and food that the sum of the six indicator PCBs represents about 50 %
172 of the total NDL-PCB in food (EFSA, 2005). According to this, in our findings, a linear regression
173 model showed an highly significant relationship ($R^2 = 0.98$; $p < 0.001$) between Σ_6 PCBs and Σ_{18}
174 PCBs (Figure 1). For this reason, we can confirm that in our study the Σ_6 PCBs well represents the
175 environmental pollution of the study area. Our results show that 33.3% of the analysed fish samples
176 had a NDL-PCBs content (Σ_6 PCBs) that exceeded the maximum levels of 125 ng g⁻¹ fresh weight
177 (fw) set by UE 1259/2011 (Figure 2). In particular, 50% of the specimens collected from Po River,
178 20% of the specimens collected from Tanaro River, 11% of the specimens collected from Bormida
179 River and 27% of the specimens collected from Parma River were not compliant with EU ML
180 (Figure 3). Σ_6 PCBs in total ranged from 19.7 ng g⁻¹ to 1015.4 ng g⁻¹, with a mean concentration of
181 135.6 ng g⁻¹. Considering each location, the Po River registered the highest presence of NDL-PCB,

182 with Σ_6 PCBs ranging from 19.7 ng g⁻¹ to 1015.4 ng g⁻¹, and a mean concentration of 187.6 ng g⁻¹.

183 In the other three sampling sites concentrations were similar: in the Tanaro River, the levels of Σ_6

184 PCBs ranging from 25.3 ng g⁻¹ to 266.5 ng g⁻¹, mean concentration of 94.2 ng g⁻¹, in the Bormida

185 River, Σ_6 PCBs ranging from 36.6 ng g⁻¹ to 195.6 ng g⁻¹, mean concentration of 95.6 ng g⁻¹; in

186 Parma River Σ_6 PCBs ranging from 26.1 ng g⁻¹ to 240.1 ng g⁻¹, mean concentration of 92.4 ng g⁻¹.

187 The statistical analysis confirmed significantly different mean concentrations in Σ_6 PCBs among the

188 fishes from the four sampling sites (Kruskal–Wallis, $p < 0.05$, $df = 3$). Multiple comparisons

189 performed with the Mann-Whitney U tests showed that differences exist only between the Po and

190 the other three rivers (Table 1).

191 Considering the contribution of single NDL-PCB congeners to the sum of the six indicators, NDL-

192 PCBs 153 and 138 were analytically predominant (40% and 30% respectively) followed by the

193 other congeners 101 (9.7%), 180 (9.3%), 52 (6.9%) and 28 (3.5%) as shown in Table 2. Our

194 results are in line with findings reported in other studies which demonstrated that PCB-153 has an

195 average contribution of roughly one third to the sum of the six indicator PCBs (EFSA, 2005; BFR,

196 2006; Jursa et al., 2006). Indeed, the mean contribution of PCB-153 and PCB-138 across food

197 groups ranged from 23% to 44% and from 19% to 32%, respectively (EFSA, 2010). Together their

198 contribution was at least 50% in each food group. PCB-180 and PCB-101 contributed between 10%

199 and 29%, and 4% and 19%, respectively. PCB-52 and PCB-28 contributed both between 1% and

200 17% to the sum of the indicator NDL-PCBs (EFSA, 2010). In fish species from Danube River in

201 Serbia (Janković et al., 2010) congeners 138 and 153 were similarly the most abundant, and the

202 same results were obtained by Pacini et al. (2013) in different fish species from southern Italy. In

203 this latter study, the commonest congeners were 153, 138, and 101, in decreasing priority, and the

204 concentrations they found for the Σ_6 PCBs ranged from 1.30 to 195 ng g⁻¹ fw. Similarly, Brazova et

205 al. (2012) found that PCB 153 was present in higher concentrations than other congeners in muscle

206 of top predators as the European catfish, with an average of 29% of Σ_6 PCB, while the 138 congener

207 accounted for approximately 24 % of Σ_6 PCBs.

208 Our analytical NDL-PCB patterns were coherent also with those observed in different fish specie
209 from the middle and lower stretches of the Po river (Viganò et al., 2000), from the Orbetello lagoon
210 (Mariottini et al., 2006), and from several Campania rivers (Pacini et al., 2013) where the presence
211 of PCBs was associated with residues of commercial technical mixtures.

212 Congeners 138 and 153 are characterized as less hydrophobic and not so tightly bound to sediment
213 than higher chlorinated octa-, nona-, and deca-PCBs, reason why they are more readily available to
214 water organisms (McFarland & Clarke, 1989). Moreover, these congeners with chlorine atoms in
215 positions 2, 4, and 5 in one (PCB 138) or both rings (PCB 153) could have a greater resistance to
216 metabolism and elimination from fish organism than the lower congeners such as 28, 52, and 101
217 (Jacob & Boer, 1994; Nie et al., 2006). The high proportion of 138 and 153 PCB compounds found
218 in our samples of fish muscle could explain their low rates of biotransformation and inability to be
219 metabolized (Brazova et al., 2012).

220 In the previous study performed in the same area, in order to detect the presence of toxic metals in
221 muscle of *Silurus glanis*, we found that length, weight and age were significantly
222 related to Hg content. This suggested an increasing bio-accumulation with the increasing
223 size of this fish species (Squadrone et al., 2012). In this investigation no significant
224 relationship was found between Σ_6 PCBs concentration and fish weight both considering sex
225 together ($R^2 = 0.02$; $p > 0.05$) and separately (Male: $R^2 = 0.08$, $p > 0.05$; Female: $R^2 = 0.01$, $p >$
226 0.05). We can assess that several factors including the metabolic activity of individual organs, fish
227 species, age, size, feeding habits, or the complex PCB transport in an organism may control PCB
228 accumulation (Ashley et al., 2000; Brázová et al., 2012).

229

230 **4. Conclusions**

231 Toxicological data indicate that NDL-PCBs alter a number of physiological processes important
232 during the development of the species, in particular in the nervous and endocrine systems
233 (Vreugdenhil et al., 2004). The European Union has undertaken short and long term actions, aimed

234 to reduce environmental contamination and human exposure, that have recently been extended to
235 incorporate NDL-PCBs. Analysis of NDL-PCBs in fish muscle of a top predator as *Silurus glanis* in
236 northern Italy water bodies reflects the severe pollution by organic compounds in this area.
237 Moreover, it represents a way to assess the presence of these substances in rivers and to improve the
238 understanding of the environmental and human risks. Fish and fishery products are the major
239 contributors of the dietary exposure and evaluating the levels of these contaminants is necessary to
240 protect consumers from NDL-PCBs intake. The Σ_6 PCBs well represented total NDL-PCBs levels,
241 and the contribution of both PCB-153 and PCB-138 was about 70% of the overall sum of the six
242 congeners, higher than previous reported in literature. The concentration of NDL-PCBs in a top
243 food-chain predator well reflects the level of the aquatic environment pollution. Despite the fact that
244 commercial production of PCBs has been previously banned or severely restricted, fish continue to
245 accumulate these chemicals from sediments polluted during the previous decades. It is of great
246 importance to consider this persisting contamination and additional investigations in the interested
247 water bodies should be performed.

248

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252

253 **6. References**

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