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1 Polycyclic aromatic hydrocarbon levels in European catfish from the upper Po

2 **River Basin**

3

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16 Abstract

Polycyclic aromatic hydrocarbons (PAHs) are a major concern in environmental studies as many of them have been labeled as probable carcinogens by the International Agency for Research on Cancer (IARC 1983). Due to their lipophilic properties and resistance to degradation, PAHs can accumulate in organic tissue. As a consequence, alarming concentrations of these compounds have been found in many aquatic species.

22 The European catfish (Silurus glanis) is a top food-chain predator that is considered to be a reliable bio-indicator of environmental pollution. From 2009 to 2011, 54 specimens of S. glanis were 23 captured from four different sites covering the area of the Po River basin (Northern Italy). Fish 24 25 muscles were analyzed in the laboratory to determine the levels of nine PAHs, namely: naphthalene, 26 acenaphthene, fluorene, phenanthrene, anthracene, pyrene, benz[a]anthracene, chrysene, and benz[a]pyrene (BaP), which were detected by High-Performance Liquid Chromatography (HPLC). 27 The total average concentration of PAHs was $26.90 \pm 49.50 \text{ ng g}^{-1}$ (min 0.60, max 275.75 ng g⁻¹). 28 Analysis showed that 9.20% of the fish muscles exceeded the maximum levels of 2 ng g^{-1} set for 29 BaP by European regulations (1881/2006 EC). Values measured for benz[a]pyrene ranged from 30 0.05 to 8.20 ng g⁻¹ (mean 1.07 \pm 1.58 ng g⁻¹). Chrysene and benz[a]anthracene, both considered 31 potential human carcinogens (PAH2), were found at levels of 4.40 ng g⁻¹ and 0.05 ng g⁻¹ (mean 32 values), respectively. The highest mean concentration was recorded for anthracene (12.92 ng g⁻¹), 33 34 which has been recently included in the list of Substances of Very High Concern (SVHC) by the 35 European Chemicals Agency (2009).

- 36
- 37 Keywords: PAHs, European catfish, Po river basin, HPLC
- 38

39 **1. Introduction**

Monitoring of PAH contaminants in food has received a growing interest in recent years. Dietary intake has been reported as the main cause of human exposure to PAHs (Scherer et al. 2000; Falcó et al. 2003). Indeed, for non–occupationally exposed subjects (and nonsmokers), 70% of PAH exposure arises from the diet. Moreover, several epidemiological studies have shown that a large proportion of human cancers are attributable, at least in part, to dietary factors (Doll et al. 1996). Consequently, one of the main reasons for concern about the exposure of humans to environmental contaminants is the evidence that a number of these contaminants are potentially carcinogenic.

Pollution by persistent chemicals significantly affect the organisms at higher trophic levels in the food chain. Marine fish can accumulate several-fold higher concentration of PAHs than the surrounding water (Johnson-Restrepo et al. 2008). Fish is a major source of protein and healthy lipids for people worldwide and long-chain omega-3 fatty acids in particular have been proven to have a variety of beneficial roles in human health and wellbeing (Ismail et al. 2005). Despite these proven benefits of a fish-based diet, the potential risk arising from exposure to toxic chemicals is an issue of grave concern (Domingo et al. 2007; Sion et al. 2008).

54 Polycyclic aromatic hydrocarbons (PAHs) mainly originate from incomplete combustion of fossil 55 fuels and organic material as well as directly from petroleum contamination. Due to their 56 hydrophobic nature, PAHs rapidly bind to suspended particles in an aquatic environment, and 57 deposit in bottom sediments (Latimer and Zheng 2003). PAHs have been demonstrated to pose 58 toxicity both in fish and birds (Payne et al. 2003; Albers 2003) by interfering with cellular 59 membrane function and the associated enzyme systems (Neff 1985). Moreover, PAH metabolites 60 can bind to proteins and DNA causing biochemical disruption and cell damage (Varanasi et al., 61 1987). In addition, carcinogenicity of PAHs can be increased by exposure to ultraviolet radiation, 62 and 11 out of the 16 PAHs listed by the US Environmental Protection Agency as priority pollutants 63 are considered to be photomutagenic (Yan and Wang 2004). Payne et al. (2003) suggested that 64 elevated PAH levels, commonly found in many aquatic environments, are a significant risk factor 65 for fish health. Moreover, Hart et al. (1998) reported an adverse histopathological and 66 immunological response in tilapia exposed to PAHs.

Although benz[a]pyrene is considered a reliable marker for the occurrence and toxicity of total
PAHs in food (Wenzl et al. 2006), this relationship is still not fully convincing. Therefore, the
European Food Safety Agency (EFSA) has recently suggested that other PAHs, including
benz[a]anthracene and chrysene, should also be taken into account when considering the occurrence

71 and associated carcinogenicity of contaminated foods (EFSA 2008a). Following a recommendation 72 to further investigate PAH levels in certain foods (2005/108/EC), 18 Member States submitted 73 almost 10,000 results for PAH levels in different food commodities. Evaluation of these data performed by the EFSA in June 2007, and updated in June 2008, demonstrated that benz[a]pyrene 74 75 is present in about 50% of all samples. Furthermore, other carcinogenic and genotoxic PAHs were 76 detected in about 30% of samples that were negative for benz[a] pyrene. Among the individual 77 PAHs, chrysene was most commonly found in samples negative for benz[a] pyrene, and the highest level detected was 242 ng g^{-1} . 78

Moreover, it has been observed that the correlation between the sum of benz[a] pyrene and chrysene (PAH2) and the sum of the PAHs (PAH8) used by Culp et al. (1998) to assess carcinogenicity risk (benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene, dibenz[a,h]anthracene and indeno[1,2,3-cd]pyrene: benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene, dibenz[a,h]anthracene and indeno[1,2,3cd]pyrene) is 0.92.

Considering other PAHs that can pose an environmental threat, anthracene has been recently included in the list of Substances of Very High Concern (SVHC) edited by the European Chemicals Agency (2009). Indeed, it is considered to be persistent, bio-accumulative and toxic for both freshwater and marine ecosystems. In particular, when released in freshwater environment, anthracene is rapidly adsorbed by the sediment and suspended particulate without being hydrolyzed. Moreover, Hall et al. (1989) reported that anthracene bio-concentrates in species which lack microsomal oxidase, an enzyme that allows rapid metabolization of poly-aromatic hydrocarbons.

92 Results about PAH contamination of fish species allow consumers to make decisions about food 93 preferences in order to reduce risks from contaminant exposure and increase health benefits. 94 However, there is limited information on PAH levels in fish muscle in the literature. In particular, to 95 date no studies have been performed on Silurus glanis, an invasive species accidentally introduced 96 about 30 years ago in the Po river and now widespread in Northern and Central Italy. This species 97 was also introduced in several European Countries (i.e. Spain and France), where it is now 98 successfully acclimatized. Being a top food-chain predator, Silurus glanis can be considered a 99 reliable species for bio-monitoring studies, as it accurately reflects the state of the environmental 100 organic and chemical pollution.

101 The aim of this study was to evaluate PAH contamination in European catfish muscles from Rivers 102 within the area of the upper Po river basin (Northern Italy). In particular, compliance with the 103 maximum levels established for BaP by the European Commission Regulation (1881/2006) in fish 104 muscle was verified, and the distribution of other PAHs, known as being the most appropriate

- indicators for the presence of carcinogenic and genotoxic PAHs in foodstuffs, with a particularattention to PAH2 (EFSA 2008b), was discussed.
- 107 Results of the present study may provide reliable information about the environmental impact of
 108 PAHs in North-West Italian Rivers, which is essential for the monitoring and management of the
 109 study area.
- 110

111 **2. Materials and methods**

112 2.1 Study species

113 The European catfish (Silurus glanis), also known as Wels catfish, is one of the biggest European freshwater fish. This species is native to Eastern Europe and Western Asia and is abundant in the 114 Danube and Volga basins. The European catfish inhabits the lower reaches of large rivers and 115 116 muddy lakes, and usually feeds on fish that are smaller than would be expected for its size and 117 mouth gape (Adàmek et al. 1999; Wysujack and Mehner 2005). Silurus glanis is a night predator, 118 foraging both on the river bottom and in the water column. Fry and juveniles are benthic, feeding on 119 a wide variety of invertebrates and fish, while adults prey on fish and other aquatic vertebrates. Sexual maturity is reached at 2-3 years of age, and this species can survive for over 30 years in 120 121 natural conditions.

- In recent years, the European catfish has received an increasing interest for consumption in Northern Italy. Particularly, only the muscle of young specimens (weigh less than 15 kg or 33 lb) is considered palatable. Larger specimens are highly fatty and not recommended for consumption.
- 125

126 2.2 Field sampling

- 127 Fifty-four specimens of *Silurus glanis* were collected in the following four sites from 2009 to 2011:
- 128 1. Po River (Lat. 45.138098, Long. 8.558135)
- 129 2. Tanaro River (Lat. 44.919446, Long. 8.6099719)
- 130 3. Bormida River (Lat. 44.906940, Long. 8.646197)
- 131 4. Parma River (Lat. 44.832150, Long. 10.314585)

132 Sampling sites belong to the hydrographical basin of the Po (the largest river in Italy) and were

- 133 selected according to accessibility and fish abundance.
- 134 Fish were captured using an electro fisher IG 600. Specimen sizes were 60 to 120 cm (age from < 1
- to 12 years) with weights ranging from 1.5 to 10.5 Kg.
- 136 Captured fish were immediately euthanized and kept refrigerated while transported to the
- 137 laboratory. Fish were sexed and dissected to obtain muscle samples, which were immediately
- 138 frozen and stored at -20 °C.

Twenty-four samples were collected from the Po River (Alessandria district), ten from the Tanaro
River (Alessandria district), nine from the Bormida River (Alessandria district), and eleven from the
Parma River (Parma district).

142

143 2.3 Analytical Methods

144 5 g of freeze-dried sample was extracted for 15 minutes with *n*-hexane/acetone 1:1 (33 mL). The extraction procedure was performed using the Accelerated Solvent Extraction System (Dionex 145 146 ASE® 200 Solvent Extractor - Dionex Corporation). This system increases the efficiency of the extraction process using conventional liquid solvents at elevated temperatures (100 °C) and 147 148 pressures (102 atm). The solvent was gently removed by evaporation in a rotary evaporator, 149 followed by addition of 50 mL of Ethanol 95% and 6 ml of Potassium Hydroxide 50% to the dried 150 residue. Saponification was performed for 2 hours on a hot plate, connecting the flask with a watercooled reflux condenser (Teledyne Isco, Lincoln NE, USA). When samples were returned to the 20 151 152 °C ambient temperature, a liquid-liquid extraction with *n*-hexane and distilled water was performed 153 in a separatory funnel. PAHs were collected in the non-polar phase. After this procedure, the solvent was evaporated and the residue was loaded on top of a silica cartridge (Supelco Analytical 154 S.p.A., Milano). Elutions were carried out with 5 mL of a mixture of *n*-hexane and diethyl ether 9:1 155 (v/v). Finally, dried extracts were suspended in isooctane for instrumental analysis, which was 156 157 performed using an HPLC (1100 series, Agilent Technologies, Santa Clara, CAL, USA) equipped 158 with auto sampler, quaternary pump and fluorescence detector. For the separation an Envirosep 159 reversed phase for PAHs column 125 x 4.60 mm, 5 µm (Phenomenex Inc., CA, USA) was used. 160 Acetonitrile and distilled water were used as eluents, at a flow rate of 1 mL min⁻¹. The gradient elution program was: 50% acetonitrile initially until 5 min, then increasing linearly to 100% 161 162 acetonitrile in 20 min, holding in 100% for 15 min and finally back to initial conditions in 10 min. Column temperatures were maintained at 25 ± 0.1 °C. The fluorescence detector was programmed 163 164 to optimize sensitivity for all peaks while minimizing interference. Fluorescence intensity was 165 measured at the following excitation/emission wavelengths pairs: 275/325 nm for Na, Ac, F, Phe, 166 250/375 nm for Ant, Pyr, 285/475 for BaA, Chr and 270/380 for BaP.

167

168 2.4 Statistical analysis

169 Data were tested for normality by using the Kolmogorov-Smirnov test. Since the assumptions for 170 parametric analyses were not met, a Kruskal–Wallis analysis of variance by ranks followed by 171 Mann–Whitney U tests for pairwise comparisons were performed to assess differences in the 172 concentration of BaP in the muscles of fish from different rivers. Differences were considered to be 173 significant with a p value < 0.05.

- 174 Moreover, correlations between PAH2 and BaP as well as BaP and fish size/gender were examined
- 175 for each site using linear regression models.
- 176

177 **3. Results and Discussion**

178 Most of the PAHs detectable in the aquatic environment are localized to rivers, estuaries and coastal

179 waters (Hellou et al. 1994). Ascertaining contamination levels in rivers is instrumental to evaluate

the risks posed by human activity to the freshwater environment and consequently to human health.
In the present study, the levels of nine PAHs (naphthalene, acenaphthene, fluorene, phenanthrene,

182 anthracene, pyrene, benz[a]anthracene, chrysene, benz[a]pyrene) were measured in 54 samples of

183 *Silurus glanis* muscle from Northern Italy.

- 184 The values measured for BaP in fish muscle ranged from 0.05 to 8.2 ng g^{-1} (mean 1.07 ± 1.58 ng g^{-1}
- ¹). Five (9.2%) of the 54 analyzed samples exceeded the maximum levels (ML) of 2 ng g^{-1} set for BaP by European regulations in fish muscle (1881/2006 EC). In particular, ML were exceeded in
- bar by European regulations in fish muscle (1001/2000 EC). In particular, will were exceeded in
- three samples from the Tanaro River with values of 3.3, 4.1, 2.8 ng g⁻¹ and in two samples from the Po River (7.8 and 8.2 ng g⁻¹) (Fig. 1). Mean values registered for BaP (Table 1) in the sampling sites were 1.07 (Po River), 2.15 (Tanaro River), 0.52 (Bormida River) and 0.57 ng g⁻¹ (Parma River), respectively. A significantly different concentration of BaP was found in fish muscles from the four sampling rivers (Kruskal–Wallis, p < 0.005, df 3). However, multiple comparison Mann-Whitney U tests showed that differences only exist between the Po and Tanaro (U = 35, p < 0.001),
- 193 the Po and Bormida (U = 67, p < 0.05), and the Tanaro and Parma (U = 16, p < 0.005) Rivers.

Results regarding the relationship between gender and total PAH concentrations (data not shown) suggested that accumulation levels are similar for males and females in all sites. However, literature concerning this subject is very limited. Moreover, we did not observe any relationship between PAH concentration in fish muscle and size. Although some authors reported this correlation in fatty fish (e.g. chub mackerel and sardine), suggesting that PAH bioaccumulation mostly occurs in lipidrich tissues (Meador et al. 1995), this topic still remains controversial, as fish are known to be capable of rapidly metabolizing PAHs (Perugini et al. 2007; Akpambang et al. 2009).

201 The EFSA CONTAM Panel recently pointed out that benz[a] pyrene alone is not a suitable indicator 202 for the total PAHs in food. Indeed, benz[a]pyrene was detected in only 50% of the 10,000 samples 203 analyzed in the EFSA report (2008). Moreover, in approximately 30% of all the samples considered, other carcinogenic and genotoxic PAHs were detected (most frequently chrysene), 204 205 despite the absence of benz[a]pyrene. Consequently, the CONTAM Panel concluded that the 206 toxicity and carcinogenicity risk characterization should be based upon the PAHs for which oral 207 carcinogenicity data are available. Therefore, PAH2 (the sum of chrysene and benz[a]anthracene), PAH4 (the sum of benz[a]pyrene, chrysene, benz[a]anthracene and benz[b]fluoranthene) and PAH8 208

209 (the sum of benz[*a*]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene,chrysene, 210 dibenz[*a*,*h*]anthracene and indeno[*1,2,3-cd*]pyrene: 211 benz[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[ghi]perylene, chrysene, 212 dibenz[a,h]anthracene and indeno[1,2,3-cd]pyrene) can be considered suitable indicators for total 213 PAH contamination in food. Accordingly, in the present study, a linear regression model showed a strong relationship (R^2 = 214

215 0.98; p < 0.001) between PAH2 and BaP levels in the muscles of fish from the Tanaro River (Fig.
216 2).

217 Considering PAH2 values in all investigated sites, we can conclude that the major contribution was 218 due to chrysene. In fact, the highest value detected for BaA was 0.3 ng g^{-1} in a sample from the Po 219 River.

220 Chrysene contamination was negligible (mean value 0.05 ng g^{-1}) in the Po River, scarce in Bormida 221 and Parma Rivers (mean values respectively 0.62 and 0.68 ng g⁻¹) while the Tanaro River revealed

to be significantly more contaminated with the highest value of 119.1 ng g^{-1} and a mean value of 223 22.63 ng g^{-1} (Fig. 3).

The highest mean concentration recorded for anthracene was 28.0 ng g⁻¹, in the Tanaro River (min 224 225 0.05, max 119.1 ng g⁻¹) (Fig. 3). Considering the mean values of PAHs detected (Table 1), the Tanaro River was revealed to be the most polluted of the four rivers examined, with a decreasing 226 227 concentration of the following PAHs: anthracene > chrysene > acenaphthene > phenanthrene >naphthalene > benz[a]pyrene > pyrene > fluorene > benz[a]anthracene. In Figure 4, the mean 228 229 concentrations of Σ PAHs in the four locations are shown. The Tanaro River had the highest mean value of Σ PAH (80.92 ng g⁻¹), with a maximum value of 275.75 ng g⁻¹. The Bormida, Parma and Po 230 rivers showed mean Σ PAH values of 24.56, 16.02 and 10.26 ng g⁻¹, respectively. 231

232 All PAHs are soluble in water and quickly become absorbed in organic and inorganic particulate 233 matter, depositing in bottom sediments. Most PAHs remain relatively near to the point source and 234 their concentrations decrease approximately logarithmically with the distance from the source. The 235 basin of the Po river crosses the major industrial and populated area of Northern Italy. Therefore, it 236 has a legacy of pollution which is reflected in the water quality of local rivers including the Tanaro, 237 Parma and Bormida. In particular, the Tanaro was heavily affected by the presence of PAH 238 contaminants. The Tanaro River is the most significant right-side tributary to the Po. The river 239 flows close to an highway from the town of Alba to Alessandria. It is well-known that a large 240 amount of PAHs are from incomplete combustion of fuels, especially in heavy-duty and diesel 241 engines (Barakat, 2002). We hypothesize that vehicles could be a source of persistent, toxic 242 pollutants that flows into the Tanaro and that seriously affect the water quality and river ecology.

243 Finally, the Council treatment plants cannot completely remove the entire load of pollutants and, as

- a consequence, this river receives some quantities of incompletely treated wastewater and sewage
- from the urban areas.
- 246

247 4. Conclusions and recommendations

248 The European catfish is one of the world's largest (and the largest European) fish species (Stone 249 2007). Moreover, it is considered to be a suitable bio-indicator of environmental pollution. In this 250 study, benz[a]pyrene (the most hazardous compound for human health) was detected in all the 251 specimens collected, and the levels measured exceeded the limits set by the European Union in five 252 out of 54 fish. As a consequence, we underline the importance of an adequate and more accurate 253 treatment of industrial effluents prior to their drainage in the main water courses. Moreover, a 254 continuous monitoring system of PAH residues in sentinel fish is necessary to prevent consumption 255 of contaminated food. Finally, fishing areas must be maintained at a suitable distance from sources 256 of industrial effluents.

257

258 **5. Acknowledgements**

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263 **6. References**

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- 345

346 Figure captions

- 347
- 348 Figure 1

349	Benz[a]pyrene distribution in <i>Silurus glanis</i> samples
350	
351	Figure 2
352	Regression model: correlation between benz[a]pyrene and the sum of benzo[a]pyrene and chrysene
353	(PAH2)
354	
355	Figure 3
356	Chrysene and Anthracene levels in the four sampling sites
357	
358	Figure 4
359	ΣPAH in Tanaro, Po, Bormida and Parma Rivers