Occurrence of perfluorooctanesulfonate and perfluorooctanoic acid and histopathology in eels from north Italian waters

Luisa Giari[a]*, Cristiana Guerranti[b], Guido Perra[b], Mattia Lanzoni[a], Elisa Anna Fano[a], and Giuseppe Castaldelli[a]

[a] *Department of Life Sciences and Biotechnology, University of Ferrara, St. L. Borsari 46, 44121 Ferrara, Italy*

[b] *Department of Physical, Earth and Environmental Sciences, University of Siena, St. P.A. Mattioli 4, 53100 Siena, Italy*

* Corresponding author: Tel.: +39 0532 455707; fax: +39 0532 455715

E-mail address: grilsu@unife.it (L. Giari)

ABSTRACT

A perfluorinated alkylated substances (PFAS) biomonitoring study was conducted in European eel (*Anguilla anguilla*) in Italy for the first time. Perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) concentrations were assessed in the organs of 35 wild eels from two locations, the highly impacted Po River and the Comacchio Lagoon along the north-western Adriatic coast. PFAS were extracted by ion-pairing liquid extraction procedure and measured using high performance liquid chromatography with electrospray ionization tandem mass spectrometry. There were no significant differences in mean PFAS concentrations ($p > 0.05$) between samples from the two sites. PFOS and PFOA were detectable (>0.4 ng g^{-1} wet weight, w.w.) in 73% and 31% of the total samples, respectively. PFOS concentrations ranged from <0.4 to 6.28 ng g^{-1} w.w. and PFOA from <0.4 to 92.77 ng g^{-1} w.w. The highest PFAS levels were observed in blood and the lowest in muscle. Histology showed macrophage aggregates and hepatocytic vacuolation in some liver samples. No tissue anomalies were seen in the gonads, suggesting no reproductive impairment. The PFAS contamination levels observed were comparable to, or lower than, those reported in fish in other European countries, seeming to indicate that PFAS pollution of the study area is not remarkable.

Keywords: perfluorinated alkylated substances, fish, endocrine disrupters, histology, Po River, Comacchio Lagoon

1. Introduction

The distinctive physico-chemical properties of the anthropogenic chemicals, perfluorinated alkylated substances (PFAS), have made them useful for over 50 years in a wide variety of applications: surfactants, surface protectors, food packaging, plastics (Teflon), products for personal care and domestic hygiene, fire-fighting foams, and pesticides (Prevedouros et al., 2006; Suja et al., 2009). PFAS have been recently recognized as organic pollutants of worldwide concern, being persistent, bioaccumulative, toxic, and ubiquitous in the environment and in biota, including in humans (Giesy et al., 2001; La Rocca et al., 2012). The high and increasing number of publications on this topic clearly reflects the relevance of PFAS to both the scientific world and the public, and contamination levels should be monitored (EFSA, 2008; ATSDR, 2009; Lindstrom et al., 2011).

Perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) are the predominant and most studied PFASs, often considered reference or key substances of this family of contaminants (Rumsby et al., 2009; Suja et al., 2009). PFOS is included in Annex B of the Stockholm Convention on Persistent Organic Pollutants (2011). Few national measures for control of PFOA have been adopted worldwide, although it is identified as carcinogenic (ECHA, 2012) and toxic to reproduction (Cat 1B). PFOS is the most prevalent PFAS found in both freshwater and saltwater fish species worldwide (Houde et al., 2006, 2011). A broad spectrum of adverse consequences has been reported in animals exposed to PFAS, chiefly in liver; however, these effects occur primarily at concentrations higher than those expected to be found in the environment (Suja et al., 2009). Recent research has focused on endocrine disrupting properties of both PFOA (Wei et al. 2007; White et al., 2011) and PFOS (Han and Fang, 2010; La Rocca et al., 2012), which can exert estrogen-like activity affecting fertility. Knowledge of the toxicological effects of PFOS and PFOA in fish is currently limited (Kim et al., 2010).

69 The present study focused on an edible species, the European eel, *Anguilla anguilla*
70 (L.), which is in decline in most of its geographical distribution (Dekker, 2002). Although the
71 reasons for the population collapse are unclear, pollution and the consequent bioaccumulation
72 of contaminants may play a role, as suggested by Geeraerts and Belpaire (2010). Two recent
73 works (Bettinetti et al., 2011; Quadroni et al., 2013) dealt with the contamination of Italian eel
74 populations, but PFAS were not included in the list of studied pollutants. The present
75 investigation was conducted (1) to provide initial information on the levels of PFOS and
76 PFOA in eel populations in Italy; (2) to compare PFAS concentrations in eels from the Po
77 River, receiving continuous emissions from various sources, and the Comacchio coastal
78 lagoon, a semi-enclosed environment free of contamination; and (3) to evaluate the impact of
79 these compounds on the main target organs, with particular attention to their potential
80 endocrine disruption activity. Fish gonad histology is a pivotal technique or end-point in field
81 monitoring of estrogenic contaminant effects (Sumi et al., 2007; Tanna et al., 2013).
82
83 **2. Materials and methods**
84 *2.1. Study area and sample collection*
85 Thirty-five yellow eels were caught by local fishermen between January and June 2012
86 in northern Italy: 19 from the lower stretch of the Po River near the city of Ferrara and 16
87 from the Adriatic coastal Lagoon of Comacchio. The two sampling locations were selected
88 based on their presumed differing levels of contamination. The Po River is the main Italian
89 watercourse, originating in the Alps and flowing from west to east for 653 km across the
90 entire width of northern Italy to the Adriatic Sea. This river irrigates the largest and most
91 fertile plain of the country and creates one of Europe's most important wetland systems. The
92 river water at different locations is affected by industrial activity, agriculture, and municipal
93 effluents. The Comacchio Lagoon is a 115 km^2 saltwater semi-enclosed lagoon located in the

southernmost part of the Po River delta and is free of direct sources of contamination. An overview of the two sites and the corresponding eel data are provided in supporting information (Table S1).

Sampled eels were transported live to the laboratory, measured for total length and wet weight, and killed by severing the spinal cord. A standard necropsy (see Ferguson, 2006) was performed, and liver, kidney, and gonad were removed along with samples of whole blood and muscle. Body size and macroscopic examination showed all specimens to be female. Liver and ovary were weighed for calculation of the hepato-somatic and gonado-somatic indices, respectively, according to the formula HSI/GSI = liver/gonad weight (g)/ body weight (g) x 100). Fragments of liver and gonad were fixed in 10% neutral buffered formalin for 24 h and subsequently stored in 70% ethanol. Portions of liver, gonad, and kidney, as well as muscle and blood samples, were flash-frozen and stored at -20 ° C for chemical analysis. Otoliths were extracted for age estimation (ICES, 2009).

2.2. Analysis of PFAS and other endocrine disrupting chemicals in tissues

Concentrations of PFOS and PFOA in whole blood, liver, kidney, gonad, and muscle were analysed to determine the accumulation and distribution patterns. PFOS and PFOA were extracted using an ion-pairing extraction procedure and measured using high performance liquid chromatography with electrospray ionization tandem mass spectrometry, following a widely used method (Perra et al., 2010; Guerranti et al., 2013a, b). About 1 g of sample was homogenised with 5 mL of Ultrapure water supplied by a Milli-Q system from Millipore (Watford, UK). One millilitres of 0.5 M tetrabutylammonium (TBA) hydrogen sulphate (Sigma Aldrich, St. Louis, MO) solution and 2 mL of sodium carbonate buffer (Sigma Aldrich, St. Louis, MO) (0.25 M, pH 10) were added to 1 mL of the homogenate samples in a polypropylene tube and thoroughly mixed for extraction. Five millilitres of methyl tert-butyl ether (MTBE) (Sigma Aldrich, St. Louis, MO) were added to the above mixture and shaken

for 20 min. The organic and aqueous layers were separated by centrifugation, and an exact volume of MTBE (4 mL) was removed from the solution. The aqueous mixture was rinsed with MTBE and separated twice; both the rinses were combined in a second polypropylene tube. The solvent was evaporated under nitrogen and replaced with 0.5 mL of HPLC grade methanol (Sigma Aldrich, St. Louis, MO). This extract was passed through a nylon filter (0.2 μm, Supelco, Bellefonte, PA) into an HPLC vial. Extraction blanks were prepared using Milli-Q water. Analytes separation was performed using a Finnigan Surveyor Plus HPLC System, consisting of a quaternary pump, vacuum degasser, and autosampler. Chromatographic separation was achieved using a Betasil C18 column (50 _ 2.1 mm i.d., 5 lm) supplied by Thermo Electron Corporation, San Jose, CA. For quantitative determination, the HPLC system was interfaced to a Finnigan LTQ linear ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA) operating in negative electrospray mode. Instrumental parameters were optimised to transmit the [M-H]- ions for the analytes. Primary and product ions monitored for PFOS and PFOA determinations were 412.8>168.8, 218.8, and 498.8>368.9, respectively. Ten microliters of each extract were injected in the LC–MS with 2 mM LC–MS grade ammonium acetate (>99%, from CNW, Dusseldorf, Germany /HPLC grade methanol (Sigma Aldrich, St. Louis, MO) as the mobile phase starting at 10% methanol. At a flow rate of 300 μL/min the gradient increased to 95% methanol at 10 min before reverting to original conditions at 15 min. Column temperature was maintained at 25°C. Standards for the five-point calibration curve were prepared by progressive dilution with methanol from a neat standard purchased from Dr Ehrenstorfer (Augsburg, Germany) and concentrations were evaluated in comparison to this unextracted standard curve and were not corrected for the recoveries or for the purity of the standards (more than 98%). Individual stock solutions of the target analytes were prepared in methanol and stored in polypropylene bottles at -20°C. LOD, determined as three times the signal-to-noise (S/N) ratio, was 0.4 ng/g

144 wet weight (w.w.). Teflon coated labware were avoided during the whole process of
145 sampling, pre-treatment and analysis to minimize contamination of the samples. Data quality
146 assurance and quality control protocols included matrix spikes, laboratory blanks, and
147 continuing calibration verification. Blanks were analysed with each set of five tissue samples
148 as a check for possible laboratory contamination and interferences; recoveries, assessed using
149 spiked matrix with a concentration of 5 ng/g of each analytes, were over 93% in blood, and
150 over 89% in tissues, for both the two analytes.

151 In addition to PFOS and PFOA, the concentrations of a phthalate di-2-ethylhexyl
152 phthalate (DEHP) and of its primary metabolite, mono-2- ethylhexyl phthalate (MEHP), were
153 measured in blood, and the alkylphenols 4-para nonylphenol (*p*-NP), 4-nonylphenol
154 monoethoxylate (NP1EO), 4-nonylphenol diethoxylate (NP2EO), and 4-nonylphenol
155 triethoxylate (NP3EO) as well as some synthetic musks (SMCs) were measured in muscle,
156 since these endocrine disrupting compounds can affect reproduction and thus the histological
157 endpoint of gonads under investigation in this study.

158 The methods used to quantify phthalates were detailed in Fossi et al. (2012) and
159 Guerranti et al. (2013c), with improvement of QA/QC described by Guo and Kannan (2012)
160 and Schecter et al. (2013). Each sample was thawed and weighed, and acetone (HPLC grade,
161 Sigma Aldrich, St. Louis, MO), was added. The resulting mixture was sonicated, stirred and
162 centrifuged. Then, the supernatant was placed in a further 15 ml tube and precipitant was
163 again added to 1 ml of acetone, sonicated, agitated and centrifuged for a further separation.
164 The supernatant phase was then recovered and rebuilt with what resulted from the first
165 extraction. The supernatants were then mixed and evaporated in a centrifugal evaporator. The
166 extract was re-suspended with 0.5 ml of acetonitrile (HPLC grade, Sigma Aldrich, St. Louis,
167 MO) and passed through a nylon filter (0.2 μm, Supelco, Bellefonte, PA). Subsequently, the
168 sample was placed in an auto-sampler vial and injected into an LC-ESI-MS system. The

instrumental analysis was performed with a Finnigan LTQ Thermo LC/MSn with an ESI interface. A total of 5 μL of the extracted sample was injected via the auto-sampler into the HPLC system. A HPLC column ODS-2 HYPERSIL, 150 X 2.1 mm, particle size 5 μ (Thermo Electron Corporation, San Jose, CA) was used. The mobile phases consisted of 100% acetonitrile (HPLC grade, Sigma Aldrich, St. Louis, MO) (A) and 0.05% aqueous acetic acid (≥99.5%, supplied by Sigma Aldrich, St. Louis, MO) (B). Elution was performed using an isocratic mode (A/B: 15/85, v/v) at 0.25 ml/min. ESI-MS was operated in the negative or positive ion mode depending on the analytes (MEHP was detected in the negative mode, whereas DEHP was detected in the positive mode). Column temperature was maintained at 25°C. The heated capillary and voltage were maintained at 500°C and ±4.0 kV, respectively. Standards of MEHP (≥99.5%) and DEHP (≥98.5%) were purchased from Dr Ehrenstorfer (Augsburg, Germany); for the quantitative analysis, a five-point calibration curve, prepared by the progressive dilution with acetonitrile (HPLC grade, Sigma Aldrich, St. Louis, MO) of a solution of the two analytes of interest, was used, evaluating the concentrations in comparison to this unextracted standard curve and not correcting for the recoveries or for the purity of the standards. Recoveries, assessed using matrix spiked with a concentration of 20 ng/g for DEHP and 10 ng/g of MEHP, were over 93% in blood, and over 90% in tissues for both the two analytes. Following the indications of Guo and Kannan (2012) and Schecter et al. (2013), three procedural blanks were analysed with each set of five samples as a check for possible laboratory contamination and interference. When the concentrations of DEHP in the three procedural blanks varied widely, and if the difference in concentrations among the blanks exceeded 30 ng, then all the data were discarded and samples were reanalysed. Mean blank values were subtracted from sample values for each batch. The data quality assurance and quality control protocols also included daily calibration verification. Plastic labware was avoided during the whole process of sampling, pretreatment

and analysis to minimize contamination of the samples. The limits of detection (LODs) for the compounds analysed were the values of the compound in the blanks +3 SD. The LODs were 2 and 10 ng/g, respectively, for MEHP and DEHP.

For alkylphenols and SMCs, the procedure adopted was that described by Guerranti et al. (2014). Homogenized samples (approximately 5 g) were extracted by accelerated solvent extractor (ASE 200, Dionex Corporation, Sunnyvale, CA), according to US-EPA (1996) method 3545A. The extract was purified on a chromatographic column packed with 5 g of Florisil PR 60/100 mesh (Supelco, Bellefonte, PA) activated at 130 °C for 16 h. The column was conditioned with 10 ml hexane (Sigma Aldrich, St. Louis, MO) and the sample eluted with a mixture of diethyl ether (Sigma Aldrich, St. Louis, MO)/hexane (100 ml, 1:10), then evaporated under a stream of nitrogen and brought to final volume (50 µL) with nonane (Sigma Aldrich, St. Louis, MO). For the quantitative analysis, a GC/MS ion trap Polaris coupled to a gas chromatograph GC TraceTM 2000 (provided with AS3000 autosampler) (ThermoFinnigan, San Jose, CA) was used. The capillary column used was RTX-5MS (30 m × 0.25 mm, 0.25 µm) (Restek Corporation, Bellefonte, PA). Two µL of sample was injected in splitless mode at 250 °C. The energy of the filament was set to 70eV. The mass spectrometer has functioned with EI+ source (200 °C), with a transfer line temperature of 300 °C. SMCs and *p*-NP were quantified using a standard mix containing 4 SMCs (xylene, ketone, tonalide, and galaxolide) and *p*-NP (Dr Ehrenstorfer, Augsburg, Germany) at 4 concentrations as external standard. Plastic labware and soap use were avoided during the whole process of sampling, pre-treatment and analysis to minimize contamination of the samples. Data quality assurance and quality control protocols included matrix spikes, laboratory blanks, and continuing calibration verification. Blanks were analysed with each set of five tissue samples as a check for possible laboratory contamination and interferences; recoveries, assessed using spiked matrix with a concentration of 10 ng/g of each analytes,

were over 90% in blood, and over 85% in tissues, for SMCs and *p*NP, and over 83% in blood and tissues, for the ethoxylates. The LODs calculated as the mean blank +3SD were 1 ng g^{-1} for *p*NP and SMCs, 5 ng g^{-1} for NP1EO, NP2EO, NP3EO.

2.3. Histology

The fixed samples were processed by routine techniques for paraffin embedding to produce 5 µm sections for histopathological assessment. Sections stained with haematoxylin and eosin were examined by light microscopy using a Nikon Microscope ECLIPSE 80i and computerised image analysis software (Nis Elements AR 3.0). All slides were evaluated for pathology without knowledge of the sampling location or the level of PFAS contamination. Liver was scored based on severity of hepatocyte vacuolation. Based on US-EPA (2006) and Wolf et al. (2008), the lesion scores were 0 (not remarkable) = no vacuoles; 1 (minimal) = few scattered cells with vacuolation, less than 20% of the tissue in the section is involved; 2 (mild) = scattered clusters of vacuolated hepatocytes, 30-50% of the tissue involved; 3 (moderate) = most areas with vacuolated hepatocytes, 60-80% of the tissue involved); 4 (severe) = majority of the hepatocytes filled with large clear vacuoles, greater than 80% of the tissue involved. The abundance of macrophage aggregates (MAs) in liver was evaluated at 400 x magnification in ten fields of 70 000 μm^2 from one section for each fish; the numbers of MAs counted in the ten fields were used for a single mean value.

2.4. Statistics

The statistical package Statistica v 7.1 (StatSoft Inc., Tulsa, OK) was used to analyze all data, with significance set at the 0.05 level. The mean, standard deviation, and range were determined for PFOS and PFOA in each tissue/organ of all specimens and separately in fish from the two sampling locations. To allow inclusion of all samples in the statistical analyses,

measured concentrations below the detection limit (0.4 ng g^{-1} wet weight) were assigned a value of one-half the LOD.

Prior to analysis, the Shapiro–Wilk's and Levene's Tests were used to assess normality and the homogeneity of variance of the data. One-way ANOVA for PFOS and Mann–Whitney U test for PFOA were used to compare concentrations of the Po River and Comacchio Lagoon samples. To assess potential difference in histopathological score among eel groups with different PFAS levels, one-way ANOVA was again applied. Correlations between the measured PFAS and vacuolation score, MA abundance, and fish size were evaluated using the Pearson coefficient.

3. Results and Discussion

3.1. Occurrence and distribution of PFOS and PFOA in eels

While pollutants such as PCBs, heavy metals, and pesticides are relatively well studied, the impact on the eel of more recently developed chemicals, such as PFAS and phthalates, remains poorly understood (Geeraerts and Belpaire, 2010).

The concentrations of DEHP, MEHP, pNP, NP1EO, NP2EO, NP3EO, musk xylene (MX), musk ketone (MK), galaxolide (HHCB), and tonalide (AHTN), were below or near the LOD in the vast majority of the samples (Table S2). Prior to this study no information was available about contamination by phthalates and synthetic musks in fish from Po River and Comacchio Lagoon. Higher levels of MX, MK, HHCB, and AHTN were found in eel muscles from some waters in Germany (Fromme et al., 1999; Gatermann et al., 2002). With regard to alkylphenols, bile samples of fish from the middle section of Po River showed moderately low levels of estrogenic chemicals comprising 4-nonylphenol (Viganò et al., 2006).

Most reports on the occurrence of PFAS have relied on the analysis of fish liver, whole body homogenates, or fillets (Ye et al 2008a). This investigation examined blood, liver,

muscle, gonad, and kidney. Tables 1 and 2 report the PFOS and PFOA concentrations detected in the eel samples in this study. No significant differences were found in PFOS (Anova, $p > 0.05$) or PFOA (Mann–Whitney U, $p > 0.05$) levels in any organ between fish from the Po River, which is highly impacted from anthropogenic activities, and those from Comacchio Lagoon, although the latter lacks in any obvious sources of these contaminants. This is in accordance with reports of occurrence of PFAS in species inhabiting not only areas in proximity to pollution sources, but also remote locations (Houde et al., 2006, 2011; Fatihah et al., 2009).

PFOS was measured in 73% of the samples examined at mean concentrations ranging from 1 to 3 ng g^{-1} w.w, depending on the tissue. PFOS was above the LOD in all kidney and blood samples, reaching a maximum concentration of 6.28 ng g^{-1} (Table 1). The PFOS levels found were lower than those reported in *A. anguilla* tissue in other European countries (Schrap et al., 2004; Hoff et al., 2005; Kwadijk et al., 2010; Schuetze et al., 2010; Holzer et al., 2011) (Table 1). Hoff et al. (2005) reported PFOS concentrations from 17 to 9031 ng g^{-1} in liver of eels from canals and ponds in Belgium. PFOS residues ranged from 27 to 120 ng g^{-1} and from 37 to 83 ng g^{-1} in muscle of eels from the Rhine River in the Netherlands (Kwadijk et al., 2010) and Lake Mohne in Germany, respectively (Holzer et al., 2011). The mean PFOS concentration measured in *Micropterus dolomieui* and *Cyprinus carpio* muscle collected from rivers in the USA, which is considered to represent a background level in relatively uncontaminated sites, was approximately nine-fold that observed in eel muscle in the present study (0.9 ng g^{-1} w.w) (Kannan et al., 2005; Ye et al., 2008a).

Thirty-one percent of the tested samples contained PFOA above the LOD. The mean PFOA concentration varied from 2 to 14 ng g^{-1} w.w according to the organ. Peak level (92.77 ng g^{-1} w.w) was reached in a single gonad (Table 2). PFOA has often been reported low or under the LOD in fish organs (Bossi et al., 2005; Ye et al., 2008a; Quinete et al., 2009;

294 Fernandez-Sanjuan et al., 2010). Reports of quantification of PFOA in eel tissues are rare and
295 refer only to muscle, and have indicated maximum concentrations of 0.3 (Schuetze et al.,
296 2010), 2.3 ng g^{-1} w.w (Holzer et al., 2011), and 2.6 (Schrap et al., 2004). The range of PFOA
297 concentrations, from values below the LOD to 200 x LOD, found in the present study is
298 comparable to results obtained for edible benthonic fish in the Mediterranean Sea (Nania et
299 al., 2009). Mean PFOA levels were reported as 9 and 14 μg kg^{-1} in liver and muscle,
300 respectively, with a few unusually high concentrations (110 and 172 μg kg^{-1}) (Nania et al.,
301 2009).

302 Although in the examined samples the highest mean concentrations of both PFAS were
303 observed in blood and the lowest in muscle, the tissue distribution of PFOS and PFOA
304 differed slightly. PFOS accumulation followed the order blood > kidney ≥ liver > gonad >
305 muscle, with a blood/muscle ratio of 3.5, while PFOA concentrations showed the order blood
306 ≥ kidney > gonad > liver > muscle, with blood/muscle ratio of 6.6. Quinete et al. (2009) found
307 PFOS concentrations in fish from Brazil to be generally higher in liver than in muscle, while
308 hepatic PFOA concentrations were similar to, or lower than, in muscle. *Lates niloticus* and
309 *Oreochromis niloticus* from Lake Victoria, Kenya contained PFOS and PFOA in liver at
310 concentrations several orders of magnitude above that in muscle (Orata et al., 2008). A recent
311 tissue distribution analysis of perfluorinated compounds in farmed freshwater fish from China
312 indicated blood, followed by liver, brain, and finally muscle as sites of accumulation (Shi et
313 al., 2012). PFOS and PFOA are preferentially retained in plasma and in highly vascularised
314 sites, such as kidney and liver, probably due to PFAS affinity to haematic proteins (Han et al.
315 2003; Jones et al., 2003).

316 No significant correlations were found between hepatic PFOA concentration and total
317 length ($r = 0.20$, $p = 0.248$) or weight ($r = 0.13$, $p = 0.47$) of eels, or for PFOS with total
318 length ($r = -0.12$, $p = 0.483$) or weight ($r = -0.06$, $p = 0.753$). The present results are

consistent with those of other studies of PFAS in freshwater fish and might suggest that bioaccumulation is not linked to fish size (Hoff et al., 2005; Quinete et al., 2008, Ye et al., 2008a). Contamination in fish can be influenced by habitat and trophic level. Fish living on, or closely associated with, the bottom can absorb PFAS from both water and sediments, possibly explaining the higher levels of PFOS and PFOA detected in benthonic species compared to pelagic (Nania et al., 2009). Several studies provided evidence for biomagnification of PFAS in aquatic food webs (Bossi et al., 2005), indicating elevated concentrations of these compounds in piscivorous fishes (Martin et al., 2004; Kannan et al., 2005). Given that *A. anguilla* is a benthic species at a high trophic level, it may be an indicator of higher PFAS accumulation compared to other fish species.

Field biomonitoring studies showed that PFOS is the main PFAS detected in the biota, in terms of both prevalence and concentration (Giesy et al. 2001; EFSA, 2008). Houde et al. (2006) reported that PFOA and other PFAS were generally measured at concentrations one or two orders of magnitude lower than those of PFOS in fish. In the eel population examined, PFOS was more prevalent than PFOA but, in the individual specimens in which both PFAS were detected, PFOA was at higher levels than PFOS. Monitoring of PFAS levels in European rivers by Loos et al. (2009) identified the Po as a major PFOA source in Europe. In 2006, the PFOA concentration near the Po mouth ranged from 60 to 174 ng L^{-1}, while PFOS levels were approximately 10 ng L^{-1} (Loos et al., 2008). More recent biomonitoring of sites in the Po basin reported the mean PFOA concentration near Ferrara to be 20 ng L^{-1} and the mean PFOS level below 10 ng L^{-1} (Valsecchi, 2013). The data on PFAS in Po water and the present results on PFAS levels in eel tissue samples, suggest that the most significant source of these contaminants in the Po River is enriched in PFOA rather than to PFOS. The PFOA level in the Po has decreased since 2006, and the mean concentrations detected in eel samples in 2012 do not indicate remarkable pollution by PFAS, and are comparable to other areas of Europe.

344

345 *3.2. Histology*

346 Microscopic examination of the ovaries showed all specimens to be fully differentiated,
347 with chains of numerous large oocytes (Fig. S1a) in the previtellogenic stage or at the
348 beginning of the vitellogenic stage (Fig. S1b). No histopathological changes were found in the
349 ovaries of any eel (Fig. S1c, d). Although PFAS are considered EDs, there is limited
350 information on the histological effects of PFOS and PFOA in fish gonads. In *Gobiocypris*
351 *rarus* exposed for 28 days to PFOA (3, 10, and 30 mg L^{-1}) ovaries underwent degeneration
352 and occurrence of testes/ova were observed (Wei et al., 2007). Fathead minnows exposed to
353 0.3 and 1 mg L^{-1} PFOS for 21 days showed histopathological alterations in female gonads,
354 with elevated incidence of atretic follicles and more and/or larger MAs compared to controls
355 (Ankley et al., 2005). Contrary to the above reported data on fish experimentally exposed to
356 PFAS, the gonads of the eels examined in this study appeared normal (Fig. 1c, d), suggesting
357 a lack of reproductive impairment. This discrepancy in histological results between the
358 laboratory and field studies may depend primarily on the different concentrations and
359 conditions of exposure. Based on the species/endpoint evaluated in the present study, current
360 levels of PFOS and PFOA in wild eels would not appear to represent potential risk to
361 fecundity of females at this sexual stage.

362 The liver is the primary target organ of both acute and chronic exposure to PFAS (Cui
363 et al., 2009). Hoff et al. (2005) found high PFOS contamination in freshwater fish and showed
364 a correlation between the hepatic PFOS concentration and serum alanine aminotransferase
365 activity, a marker of hepatic damage. The present study showed liver with normal structure,
366 composed of hepatocytes arranged in typical architecture (Fig. S2a). The predominant
367 anomaly was vacuolation of hepatocytes (Fig. S2b, c), which was evaluated semi-
368 quantitatively. The majority of eels with PFOA hepatic level below or near the LOD had a

lesion score from 0 to 2 (mean score ± SD, 0.92 ± 0.86), while, in liver with detectable PFOA residue (1-85 ng g^{-1} w.w), the hepatocyte vacuolation score ranged from 2 to 4 (mean score ± SD, 3.00 ± 0.82) (Table S3). The mean vacuolation score between the two groups was significantly different (Anova, $p < 0.05$). A significant positive correlation was found between vacuolation score and hepatic PFOA concentration ($r = 0.60$, $p = 0.000$). Hepatocytic vacuolation was reported in liver of rats after sub-chronic exposure to PFOA or PFOS (Cui et al., 2009). In mice treated with PFOA, the accumulation of cytoplasmic vacuoles in hepatocytes was correlated with a dose-dependent increase in serum and liver concentrations of PFOA (Wolf et al., 2008). With regard to fish, histological alterations, mainly vacuolation of hepatocytes, were observed in zebrafish fry chronically exposed to 250 µg L^{-1} PFOS (Du et al., 2008). Vacuolation is a common cellular response that may or may not be reversible, induced by a variety of stressful stimuli (Stevens et al., 2002; Bjerregaard et al., 2006; Giari et al., 2007). Generally it is the consequence of swelling and coalescence of intracytoplasmatic membrane-bound organelles and depends on the alteration of membrane permeability (Stevens et al., 2002). Recent research demonstrated altered membrane permeability in zebrafish exposed to PFOA (Hagenaars et al., 2013). Since vacuolation is an aspecific response, it is difficult to establish whether PFOA was the source of vacuolated hepatocytes observed in the present study. The presence of MAs of varying abundance and dimensions was observed in liver (Fig. 2b). These aggregates of pigmented cells occurred predominantly in individuals with higher levels of hepatic PFOA. A significant positive correlation was found between the abundance of MAs in liver and hepatic PFOA concentration ($r = 0.55$, $p = 0.001$). Based on an extensive literature, MAs are more numerous in fish collected from polluted sites or experimentally treated with contaminants, and their density has been evaluated as a biomarker of exposure (Giari et al., 2007; Greenfield et al., 2008; Dabrowska et al., 2012). Since their proliferation is linked to multiple physiological and pathological

conditions (Agius and Roberts, 2003), MA occurrence is an nonspecific biomarker that should be considered with caution. In general, field studies are too complex to allow definite conclusions about the causality of the observed effects, and laboratory experiments carried out under controlled conditions are necessary to establish the correlation between histological damage and a specific pollutant or class of pollutants. This is the case of the present work which is part of a broad research program investigating natural and experimental exposures of fish to PFAS.

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Table 1. PFOS concentrations (ng g^{-1} wet weight) detected in eels samples.

Location	Tissue/Organ	N[a]	N > LOD[b]	Min	Max	Median[c]	Mean[c] ± SD
Comacchio Lagoon	blood	13	13	1.30	4.73	3.19	3.14 ± 1.03
	kidney	9	9	1.03	2.75	1.94	1.93 ± 0.54
	liver	16	13	<0.4	3.72	1.81	1.73 ± 1.01
	gonad	16	12	<0.4	3.15	1.07	1.28 ± 0.98
	muscle	16	13	<0.4	2.47	1.07	1.10 ± 0.60
Po River	blood	13	13	1.47	6.28	3.18	3.10 ± 1.40
	kidney	7	7	0.99	2.75	1.97	1.82 ± 0.58
	liver	19	13	<0.4	4.29	1.53	1.76 ± 1.32
	gonad	18	8	<0.4	2.76	0.30	0.82 ± 0.76
	muscle	19	10	<0.4	1.39	0.77	0.72 ± 0.51
Total[d]	blood	26	26	1.30	6.28	3.19	3.12 ± 1.20
	kidney	16	16	0.99	2.75	1.96	1.88 ± 0.54
	liver	35	26	<0.4	4.29	1.70	1.75 ± 1.17
	gonad	34	20	<0.4	3.15	1.05	1.04 ± 0.89
	muscle	35	23	<0.4	2.47	1.01	0.89 ± 0.58

[a] number of fish samples analyzed
[b] number of fish samples with concentration above LOD (LOD = 0.4 ng g^{-1})
[c] for samples under LOD, half of LOD was assigned for the calculation of median, mean and standard deviation (SD)
[d] all eels from Comacchio Lagoon and Po River together

Table 2. PFOA concentrations (ng g^{-1} wet weight) detected in eels samples.

Location	Tissue/Organ	N[a]	N > LOD[b]	Min	Max	Median[c]	Mean[c] ± SD
Comacchio Lagoon	blood	13	7	<0.4	68.16	0.59	12.62 ± 21.39
	kidney	9	4	<0.4	49.37	0.20	15.01 ± 19.02
	liver	16	4	<0.4	27.33	0.20	5.08 ± 9.24
	gonad	16	4	<0.4	92.77	0.20	7.51 ± 22.99
	muscle	16	4	<0.4	24.71	0.20	3.55 ± 7.47
Po River	blood	13	7	<0.4	89.19	0.52	15.17 ± 26.81
	kidney	7	1	<0.4	69.33	0.20	10.08 ± 26.13
	liver	19	6	<0.4	84.63	0.20	9.12 ± 20.99
	gonad	18	6	<0.4	78.25	0.20	10.32 ± 23.10
	muscle	19	2	<0.4	12.76	0.20	0.90 ± 2.88
Total[d]	blood	26	14	<0.4	89.19	0.54	13.90 ± 23.79
	kidney	16	5	<0.4	69.33	0.20	12.85 ± 21.73
	liver	35	10	<0.4	84.63	0.20	7.27 ± 16.58
	gonad	34	10	<0.4	92.77	0.20	8.99 ± 22.74
	muscle	35	6	<0.4	24.71	0.20	2.11 ± 5.55

[a] number of fish samples analyzed
[b] number of fish samples with concentration above LOD (LOD = ng g^{-1})
[c] concentrations under LOD were given as half of LOD for the calculation of median, mean and standard deviation (SD)
[d] all eels from Comacchio Lagoon and Po River together

Supporting information

Table S1. Features of the two sample locations and corresponding eels data.

	Comacchio Lagoon	Po River (Berra)
Latitude	44°39'37.12"N	44°59'10.87"N
Longitude	12°11'7.45"E	11°58'4.04"E
Salinity	22-39	0.1-0.3
Winter water temperature °C	0.5-11.5	4.8-6.2
Summer water temperature °C	23.8-30.6	26.5-28.4
Sample size	16	19
LT in cm; mean ± SD (range)	64.66 ± 5.90 (53-75)	58.32 ± 7.29 (49-75)
Body wet weight in g; mean ± SD (range)	571.19 ± 173.30 (320-885)	317.00 ± 161.31 (161-745)
Age in years; mean ± SD (range)	5.44 ± 1.03 (4-7)	6.31 ±1.14 (4-8)
CF; mean ± SD (range)	0.21 ± 0.03 (0.16-0.25)	0.15 ± 0.03 (0.10-0.20)
HSI; mean ± SD (range)	1.90 ± 0.46 (1.38-2.85)	1.04 ± 0.22 (0.51-1.46)
GSI; mean ± SD (range)	0.84 ± 0.19 (0.58-1.19)	0.75 ± 0.27 (0.29-1.19)

CF (condition factor) = body weight (g)/ $TL(cm)^3$ x 100
HSI (hepato-somatic index) = liver weight (g)/ body weight (g) x 100
GSI (gonado-somatic index) = gonad weight (g)/ body weight (g) x 100

Table S2. Contamination levels by phtalates (DEHP, MEHP) measured in blood and by alkylphenols (pNP, NP1EO, NP2EO, NP3EO) and synthetic musks (MX, MK, HHCB, AHTN) measured in muscle of 35 examined eels.

	DEHP	MEHP	pNP	NP1EO	NP2EO	NP3EO	MX	MK	HHCB	AHTN
LOD (ng ml^{-1} ww)	10	2	1	5	5	5	1	1	1	1
Values < LOD (%)	60	25	63	100	100	100	73	100	73	89
Range values > LOD	10.23-11.73	2.07-3.28	1.29-3.53	-	-	-	1.20-2.96	-	1.09-2.76	2.01-2.64

DEHP = di-2-ethylhexyl phthalate; MEHP = mono-2- ethylhexyl phthalate; pNP = 4-para nonylphenol; NP1EO = 4-nonylphenol monoethoxylates; NP2EO = 4-nonylphenol diethoxylates; NP3EO = 4-nonylphenol triethoxylates; MX = musk xylene; MK = musk ketone; HHCB = Galaxolide; AHTN = Tonalide

Table S3. Vacuolation severity scores of hepatocytes in the examined eels according to the PFOA accumulation in liver

Score	No. eels with liver PFOA ≤ LOD	No. eels with liver PFOA > LOD
0	9	0
1	10	0
2	5	3
3	1	4
4	0	3

Figure legends Supplementary figures

Fig. S1. Histological sections of European eel ovaries. (a) Fully differentiated gonad: Ovarian lamellae (arrows) regularly arranged and rich in oocytes. H&E, bar = 200 μm. (b) High magnification of oocytes at early vitellogenic stage. Nucleoli (arrowheads) at the periphery of the nucleus (N) and a few small lipid vesicles (arrows) in the cytoplasm can be seen. H&E, bar = 20 μm. (c) Micrograph of uncontaminated gonad (PFOS and PFOA < LOD): Ovarian tissue appeared normal, with previtellogenic or early vitellogenic oocytes (arrows) amid fat (F). H&E, bar = 50 μm. (d) Ovary with the highest level of PFOA residue (93 ng g^{-1}) showing normal oocytes (arrows) in previtellogenic or early vitellogenic stage. F = fat. H&E, bar = 50 μm.

Fig. S2. Histological sections of European eel liver. (a) Normal parenchyma and hepatocytes without vacuolation in an eel from Po River with hepatic PFAS concentration under the LOD. H&E, bar = 50 μm (b) Macrophage aggregates (arrowheads) and vacuolated hepatocytes in liver with high PFOA concentration (85 ng g^{-1}). H&E, bar = 50 μm. (c) Isolated hepatocytes displaying high cytoplasmic vacuolation (arrows) and one MA (arrowhead). H&E, bar = 20 μm.