Biomonitoring of chemicals in biota of two wetland protected areas exposed to different levels of environmental impact: results of the "PREVIENI" project

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Abstract The PREVIENI project (funded by the Ministry of Environment) investigated the exposure to endocrine disrupters in samples of human population and environmental biota in Italy. The environmental biomonitoring considered two Italian WWF Oasis, with the aim to compare the presence and effects of endocrine disruptors in organisms from two protected natural areas, respectively, upstream and downstream a chemical emission site. Chemical analysis of pollutants tissue levels was made on tissues from earthworm, barbell, trout, and coot, selected as bioindicator organisms. The contaminants considered were as follows: the perfluorinated compounds perfuoroctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), polychlorinated biphenyls (PCBs 58 congeners), polybrominated diphenyl ethers (PBDEs, 13 congeners), polycyclic aromatic hydrocarbons (PAHs, 16 compounds), toxic trace elements, the phthalate di-2-ethylexyl phthalate (DEHP) and its primary metabolite, bisphenol A, synthetic musk compounds (musk xylene, musk ketone, tonalide, and galaxolide), and *p*-nonylphenol. The analyses showed low concentrations of most pollutants in all species from both areas, compared to available literature; noticeable exceptions were the increases of DEHP's primary metabolite, PBDE, PAHs, Hg, and Pb in barbells, and of PCB and Cd in earthworms from the downstream area. The results showed the presence of endocrine disruptors, including those considered as "non-persistent," in bioindicators from protected areas, albeit at low levels. The results provide a contribution to the evaluation of

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reference values in biota from Mediterranean Europe and support the relevance of monitoring exposure to pollutants, in particular for freshwater environment, also in protected areas.

Keywords Perfluorinated compounds · Phthalates · Phenolic compounds · Organohalogen compounds · Polycyclic aromatic hydrocarbons · Bioindicators

Introduction

The environment represents, besides a life context, the bond among the territory, animal populations, agro-farming, and ultimately human health. Following this conceptual framework, the interdisciplinary Project PREVIENI (http://www.iss.it/prvn), funded by the Italian Ministry of Environment, integrated pilot biomonitoring studies on humans and environmental biota, through the analysis of known and emerging contaminants, identified as endocrine-disrupting chemicals (EDCs). Results of the human biomonitoring studies performed within PREVIENI revealed a widespread exposure to EDCs, such as bisphenol A (BPA), in Italian adults of both sexes, albeit with noticeable differences among geographical areas (La Rocca et al. 2014, 2015).

The monitoring study of environmental biota examined two of the Italian World Wildlife Found (WWF) Oasis, nature sanctuaries managed by WWF Italy; the aim was to assess the extent of impact of EDCs using sentinel species, through the determination of EDCs and other priority pollutants.

The two protected areas selected for the study are wetlands, located along the River Pescara, in the hilly Abruzzi region (central Italy): the Regional Natural Reserve "Pescara Springs" and the Oasis of the Wildlife Protection "Alanno Dam." The two selected areas allow the comparison between different exposures to high-impact activities, as the "Pescara Springs" and "Alanno Dam" areas are upstream and downstream, respectively, from a large chemical emission site. To assess the state of contamination, four sentinel species were chosen on the basis of the presence in the areas, versatility of use as bio-indicators as well as their representativeness of different habitats and different trophic levels: earthworm (*Lumbricus terrestris*), barbell (*Barbus tyberinus*), trout (*Salmo trutta*), and coot (*Fulica atra*).

Specimens sampled were analyzed for the levels of environmental contaminants considered by the PREVENI project: bisphenol A (BPA), para-nonylphenol (*p*-NP), perfluorinated alkylated substances (PFAS, namely, perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA)), phthalates (di-2-ethylhexyl-phthalate (DEHP) and its primary metabolite mono-2-ethylhexyl-phthalate (MEHP)), polybrominated diphenyl ethers (PBDEs, 13 selected congeners), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs, 16 prioritary compounds), synthetic musk compounds (SMCs: musk ketone, musk xylene, tonalide, and galaxolide), and toxic trace elements (arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb)).

Materials and methods

Characterization of the study areas

The sampling design is a typically "upstream-downstream" scheme. The "Pescara Springs" Reserve, the "upstream" sampling site, hosts some of the most important karst springs of the Apennines (central Italy); the area is a Site of Community Interest, part of the European Natura 2000 network, and it is located in the Aterno-Pescara river basin. The Aterno-Pescara River runs 152 km across the Apennine from west to east and touches some of the major centers in the region, including the capital. The actual length is 145 km, but it is also generally understood the short stretch of Pescara from the sources in the same reserve in Popoli (PE) until the union with Aterno. Pescara springs are a result of deep karst groundwater circulations of a wide Apennine catchment area; resurgence waters at 250 m a.s.l. form a 30 ha lake from which they flow through a 2-km channel into the Aterno River which comes from upstream. From the confluence downstream the name of the river changes in Pescara River. The Aterno River is long about 100 km and is a second-order stream. From the downstream confluence, the Pescara River, a first-order stream, is long about 50 km and ends in the Adriatic Sea. Regarding pollution sources which could affect the Aterno River, there are several towns (L'Aquila, about 72,000 inhabitants; Sulmona 25,000) and scattered small villages delivering their sewage discharge water. Data from monitoring of the civil depuration system indicate very poor treatment capacity (ARTA, http://www.artaabruzzo.it/

scarichi.php?id_page=1). L'Aquila and Sulmona hold also industrial and craft areas, which usually deliver discharges also in the civil depuration plants. After the confluence Pescara springs-Aterno river forming the Pescara River, there is a 15-km stream tract within the Bussi-Piano d'Orta reclamation area, one of the most polluted industrial areas in Italy, along with illegal industrial waste dump sites.

Our downstream site is the Oasis of the Wildlife Protection Alanno Dam, which is located about 10 km downstream from the chemical site Bussi sul Tirino and about 2 km downstream from the chemical site of Piano d'Orta. The two chemical plants have hosted, during the last century, production chains of numerous toxic and harmful substances such as organic solvents at the Bussi plant and fertilizers at Piano d'Orta plant. The latter was also destroyed in the Second World War in carpet bombing. For decades, raw materials and effluents from these plants were discarded directly in the Pescara River or accumulated on the ground in the fields near the plants. The Bussi plant, which is still operating, was characterized in the last 5 years revealing very high level of pollution both in the ground and in the groundwater. Also, the Piano d'Orta plant area, which was definitively abandoned without remediation in 1962, has to be considered a heavily polluted site. In the Bussi area, monitoring from authorities revealed heavy ground and groundwater pollution from solvents, dioxins, and metals (IZSAM 2016). PFOS/PFOA, DEHP/MEHP, PBDE, BPA SMC*s*, and *p*-NP have not been sampled by authorities since these chemicals were not produced in the long-life history of the plant; however, the presence of these chemicals in urban effluents or in illicit chemical waste disposals cannot be ruled out.

The area of the Alanno Dam, being downstream along the Pescara River, is potentially exposed by contamination due to both industrial and civil effluents along the Aterno and Pescara. In 2008, the area comprising the Bussi Plant, the Piano d'Orta Plant, and the Alanno Dam was gazetted as one of the 57 Italian National Reclamation Areas.

Considering our set of toxic compound, PFOS/PFOA, DEHP/MEHP, PBDE, the SMC*s*, and *p*-NP are ubiquitary pollutants that could be present in untreated effluent along the Aterno and Pescara River, both of industrial and civil origin. PAH contamination could enter in the Aterno-Pescara river system both from effluents as from fall-out of main infrastructures such as the Pescara-Rome highway and the Tiburtina national route which for a long tract run along the Aterno-Pescara River. In this case, also, the Pescara Springs Reserve could be affected because the highway overlooks the spring lake with a bridge. PCBs and trace elements could come from industrial effluents along the Aterno-Pescara river, the latter especially from the Bussi-Piano d'Orta which area heavily polluted by Pb, As, and Hg (IZSAM 2016).

The Alanno Dam was therefore chosen assuming its potential for downstream collection of the pollutants of interest, whose release into the environment takes place largely at the industrial level (Colles et al. 2008) but also from untreated civil discharge system.

Sampling

Following the achievement of the necessary permissions, the sampling was carried out, in correspondence with the points indicated in Fig. 1. Earthworms and barbells were sampled at both sites, in spring; soil portion (20 cm dept) was collected by bucket, and then, earthworms were manually taken, while fish specimens were caught by electro fishing.

At the Pescara Springs, specimens of trout and eggs of coots which were not present in the Alanno dam area were also found and sampled, for further information and to provide possible indications for further research.

Immediately after collection, fish blood was taken from the caudal vein through a heparinised syringe. Portions of muscle and liver were dissected and stored at − 20 °C until analysis.

The analyses were carried out on five pools (of 4–5 different specimens) for sampling point, with the exception of the organic compounds in earthworms, for which two pools of organisms in total (one for each area) were analyzed and of coot's eggs, for which six pools were analyzed (Table 1).

Chemical analysis

All chemicals and reagents used were analytical grade, and glassware was carefully washed and rinsed with solvents, to avoid contamination.

Lipid concentrations of the samples have been determined gravimetrically.

PFASs

The analytical method for the determination of PFOS/PFOA and the equipment used was described by Guerranti et al. (2013a). PFOS/PFOA was extracted by

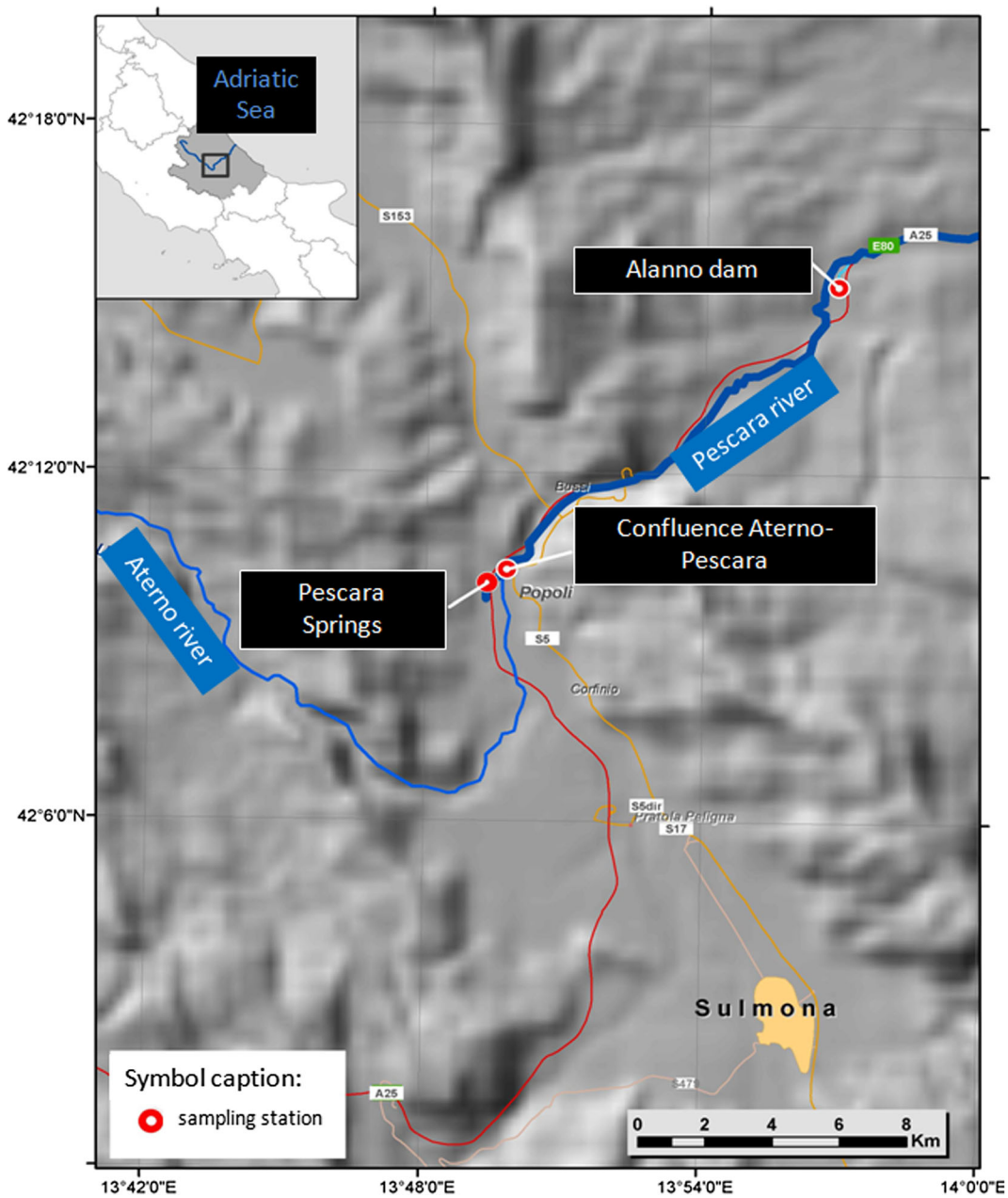

Fig. 1 Study area with sampling stations

ion-pairing and measured using high-performance liquid chromatography (HPLC) with electrospray ionization (ESI) mass spectrometry (MS). Standards for the five-point calibration curve were prepared by progressive dilution with methanol from a neat standard, and analyte concentrations were evaluated in comparison to this unextracted standard curve and not corrected for the recoveries or for the purity of the standards (> 98%). Stock solutions of the target analytes were prepared in methanol and stored at − 20 °C. Teflon-coated labware was avoided to minimize sample contamination. Blanks were analyzed with each set of six tissue samples as a check for possible laboratory contamination and interferences; recoveries, assessed using four similar samples, spiked with each analyte, were over 92%, for both the two molecules. The limit of detection (LOD) of

Table 1 Contaminants analyzed, divided according to kind of sample

Contaminant analyzed	Sample	Method's reference
PFASs	Trout and barbell's liver and muscle; coot's egg; earthworm	Guerranti et al. (2013a)
Phthalates	Trout and barbell's muscle; coot's egg; Earthworm	Guerranti et al. (2013b)
PCBs, PBDEs, PAHs trace elements (As, Cd, Hg, Pb)	Trout and barbell's liver muscle; coot's egg; earthworm	US EPA (1996) method 3545A (modified by Perra et al. 2011); EPA 6020A method (1998)) Guerranti and Focardi 2011
BPA, *p*NP, SMCs	Trout and barbell's muscle; earthworm	US EPA (1996) method 3545A (modified by Perra et al. 2011); Guerranti et al. (2014)

individual compounds was evaluated as the mean blank + 3SD, and it was 0.4 ng/g.

The repeatability and reproducibility were performed on six different injections of the same standard and were 84 and 90%, respectively, for PFOS and 82 and 85% for PFOA.

Phthalates

The determination of DEHP and MEHP followed the method described by Guerranti et al. (2013b) and employing the same equipment, with a preliminary homogenization of the samples. DEHP and MEHP were extracted by ion pairing and determined through HPLC-ESI-MS. A five-point calibration curve, prepared by progressive dilution with acetonitrile of a solution of the two analytes MEHP (> 99.5%) and DEHP (> 98.5%) for the quantitative analysis, 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 92% for both the analytes. Following the indications of Guo and Kannan (2012) and Schecter et al. (2013), three procedural blanks were analyzed with each set of four samples as a check for possible laboratory contamination. When the concentrations of DEHP in the three procedural blanks varied widely, and if the difference in concentrations among the blanks exceeded 30 ng, the data were discarded. 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, pre-treatment, and analysis to minimize contamination of the samples. The LODs (mean blank + 3SD) were 2 ng/g for MEHP and 10 ng/g for DEHP. The repeatability and reproducibility were based on six different injections of the same standard and were 82 and 83%, respectively, for DEHP and 86 and 88% for MEHP; the recovery was evaluated by the analysis of four spiked samples (similar matrices) and was always over the 86%.

PCBs and PBDEs

PCB congeners IUPAC numbers CB18, CB22, CB26, CB28, CB31, CB33, CB40, CB42, CB44, CB49, CB52, CB77, CB81, CB95, CB99, CB101, CB105, CB110, CB114, CB118, CB123, CB126, CB128, CB134, CB136, CB137, CB138, CB141, CB146, CB149, CB151, CB153, CB156, CB157, CB158 CB167, CB169, CB170, CB171, CB172, CB174, CB176, CB177, CB178, CB180, CB183, CB185, CB187, CB189, CB194, CB195, CB196, CB199, CB201, CB202, CB205, CB206, and CB207 and PBDEs IUPAC numbers BDE17, BDE28, BDE49, BDE71, BDE47, BDE66, BDE77, BDE100, BDE99, BDE85, BDE154, BDE153, BDE 209 were considered. Throughout the paper, PCBs and PBDEs are represented by their IUPAC numbers.

PCBs and PBDEs were analyzed according to Guerranti and Focardi (2011)) and using the same equipment. Samples were Soxhlet extracted and purified by a multi-layer silica gel column. PCBs were analyzed by gas chromatography using a Perkin Elmer Autosystem gas chromatograph with electron capture detector, and sample results were confirmed with a GCQ plus ion trap MS by ThermoFinnigan. The LODs of the compounds were the concentrations detected in blanks + 3SD and on the average were 0.01 ng/g for PCB congeners and 0.04 pg/g for PBDE congeners.

Contaminants were determined by comparing the results with those of external standards of known concentration and composition and at least 99% purity: Aroclor 1260 (Supelco Inc., US EPA-certified) and PBDE mix standard solution containing the 13 analytes of interest (Wellington Laboratories Inc.). The internal standard used was PCB14 ^{13}C in isooctane (Cambridge Isotope Laboratories). The data quality assurance and quality control protocols also included daily calibration verification.

PAHs, SMCs, and p-NP

Samples were extracted by accelerated solvent extractor (ASE), according to US EPA (1996) method 3545A (modified by Perra et al. 2011). The extract was divided in two and then evaporated under a stream of nitrogen; the two rates were brought to final volume (1 ml) with acetonitrile and with hexane, respectively, for the analysis of PAHs and SMCs/*p*NP.

The US EPA has classified 16 of the PAHs as priority pollutants, based on toxicity, potential for human exposure, frequency of occurrence, and amount of existing information (ATSDR 2005). The procedure for the quantification of PAHs was the same described by Perra et al. (2011) and made with the same equipment and conditions. The LODs for the PAHs selected for analysis were: 0.25 ng/g *w*/*w* for naphthalene and acenaphthylene, 0.5 ng/g for acenaphthene, 0.05 ng/g for fluorene and pyrene, 0.04 ng/g for benzo[*g,h,i*]perylene, 0.03 ng/g for benzo[*a*]anthracene, chrysene, benzo[*a*]pyrene and indeno[*1,2,3-cd*]pyrene, 0.02 ng/g for phenanthrene and fluoranthene, 0.01 ng/g for anthracene, benzo[*b*]fluoranthene and benzo[*k*]fluoranthene, and 0.1 ng/g for dibenzo[*a,h*]anthracene.

For SMCs and *p*NP analysis, the second rate of the extract was purified on a chromatographic column packed with 5 g of Florisil PR (Fluka, Italy) activated at 130 °C for 16 h. The column was conditioned with 10 ml of hexane and the sample eluted with a mixture of diethyl ether/hexane (100 ml, 1:10), then evaporated under a stream of nitrogen, and brought to final volume (50 μl) with nonane. For the quantitative analysis, a GC/MS ion trap Polaris coupled to a gas chromatograph GC TraceTM 2000 (provided with AS3000 autosampler) (ThermoFinnigan) was used. The capillary column used was RTX-5MS (30 m × 0.25 mm in inner diameter, 0.25 μm) provided by Restek. Two microliters of sample was injected in splitless mode, at 250 °C. The temperature program applied was as follows: oven at 90 °C for 2 min, then at 180 °C with an increase of 20 °C/min up to 225 °C (for 10 min) with an increase of 4 °C/min, up to a maximum of 300 °C (for 5′) with an increase of 30 °C/min. The energy of the filament was set to 70 eV. The mass spectrometer has functioned with EI+ source (200 °C), with a transfer line temperature of 300 °C. SMCs and pNP were quantified using a standard mix containing four SMCs (xylene, ketone, tonalide, and galaxolide) and *p*NP at four different concentrations as external standard. LOD was 1 ng/g for each compound. A blank, prepared following the same procedure used for real samples, was included in every batch of 5. The repeatability and reproducibility were performed on six different injections of the same standard and were 85 and 88%, respectively; the recovery was evaluated by the analysis of four similar spiked samples and was always over 89%.

BPA

For BPA analyses, the homogenized samples were extracted using by ion pairing for three times; for the analytical determination, a HPLC-ESI-MS system was used. To measure the concentration of total BPA (free BPA and BPA conjugates), samples were spiked with 2000 IU of β-glucuronidase; then, each sample was vortexed, incubated at 37 °C for 12 h, and subsequently analyzed. The analytical method and the equipment used were the same as described by Guerranti et al. (2014). A standard solution of BPA at four different concentrations was used as a calibration curve for analysis. The LOD (mean blank + 3SD) was 0.5 ng/g. A blank prepared with the same procedure was included every four samples, and the results were not correct, being the blanks free from contamination. The repeatability and reproducibility were performed on six different injections of the same standard and were 88 and 90%, respectively; the recovery was evaluated by the analysis of three spiked samples (similar matrices) and was always over the 90%.

Toxic trace elements

For the analysis of As, Pb, Cd, and Hg, samples have been lyophilized, homogenized, and analyzed according to the US-EPA 6020A method (1998)). About 120 mg for each sample has been mineralized with 2.5 ml HNO3 and 1 ml H2O2, in Teflon containers, under high

pressure, at 120 °C, for 8 h. During each cycle of mineralization, in one of Teflon container, only nitric acid has been placed, to assess the cleanliness of containers. To evaluate the accuracy of analytical method, in another container, 120 mg of a reference standard material has been mineralized. Determination of element concentrations has been carried out using a calibration curve, obtained with the method of "internal addition." Analytical determination of Pb and Cd has been carried out by an atomic absorption spectrophotometer (Perkin-Elmer THGA 4100 ZL), with atomization in graphite furnace and Zeeman background correction. Hg has been assessed by Flow Injection Mercury System (FIMS 400, Perkin-Elmer), and As by atomic absorption spectrophotometer (ZETASS 4100 ZL, Perkin-Elmer), after generation of its hydride form (AsH_3).

Statistical analysis

Comparisons between sampling sites were evaluated by the Mann-Whitney non-parametric test, and $p = 0.05$ was taken as significance cut-off, using Statistica 7.1.

Results and discussion

Results are reported on Table 3 and in the graphics of Fig. 2. As overall picture, the analyses showed that in the sampled specimens of both sites, the concentrations of the contaminants of interest were comparable or lower than those reported in similar organisms by the available literature; nevertheless, for some contaminants, concentrations in the high-pressure area were clearly higher, as discussed in the paragraphs below. The lipid content of the samples in the matrices involved the analysis of lipophilic contaminants and showed remarkably consistent results in the context of samples of the same type (Table 2).

PFASs

PFASs were always below the LOD. These results can be considered as indicator of minimum or, indeed, null environmental contamination, considering the results of the papers present in scientific literature, which attest the accumulation of the contaminants considered, by organisms, when they are present in the environment.

Up to now, most studies on PFOS and PFOA focus on marine species, while fewer studies consider the presence of these molecules in freshwater or terrestrial species, even if high levels of PFOS, PFOA, and other PFASs have been found in rural soils (Renner 2009). Several studies found the presence of detectable levels of PFAS in freshwater fish of different areas; in particular, PFOS was recently found in barbell from the Don River (barbell muscle, 76 ng/g *w/w*, Rose et al. 2015). In Italy, Giari et al. (2015)) found the following values (as ng/g) in eels from the Po River: PFOS < 0.4–2.47 and PFOA < 0.4–24.71 in muscle and PFOS < 0.4–4.29 and PFOA < 0.4–84.63 in liver, pointing out that the sampling area (northern Italy) is exposed to PFOA emission sources. Overall, fish species appears as good collectors of PFAS, especially PFOS, due to the PFAS ability to bioaccumulate; therefore, our findings actually indicate that in both sampling areas, there are no significant sources of PFAS detected.

As for fish, comparisons with the literature data regarding worms and coot lead to hypothesis minimum levels of contamination or absence by PFOS/A. Concerning the coot's eggs, no direct comparisons with literature data is possible, being the first time that PFAS have been analyzed in this matrix. Nevertheless, literature reports numerous evidences of the presence of PFASs in bird's eggs (e.g., Herzke et al. 2009). To the authors knowledge, no data are available in scientific literature about the levels of PFASs in earthworm or comparable organisms sampled from the environment, but PFAS bioaccumulation was demonstrated in experiments made on earthworm exposed to contaminated biosolid-amended soils (Navarro et al. 2016).

Phthalates

The concentrations of phthalates considered (DEHP and MEHP) in coot eggs, earthworm, and muscle of trout are all < LOD. The concentrations of DEHP are also < LOD in all samples of muscle of barbell analyzed; MEHP, instead, a biomarker of exposure to DEHP, was observed at higher concentrations (almost three times higher) at the Alanno dam compared to the low-pressure area, in accordance with the initial hypothesis (Table 3) (Mann-Whitney, $p = 0.047$). MEHP is the primary metabolite of DEHP and the active EDC in mammalian organisms; thus, the detection of higher MEHP concentrations in barbells from Alanno likely indicates a past and/or indirect exposure to DEHP, at

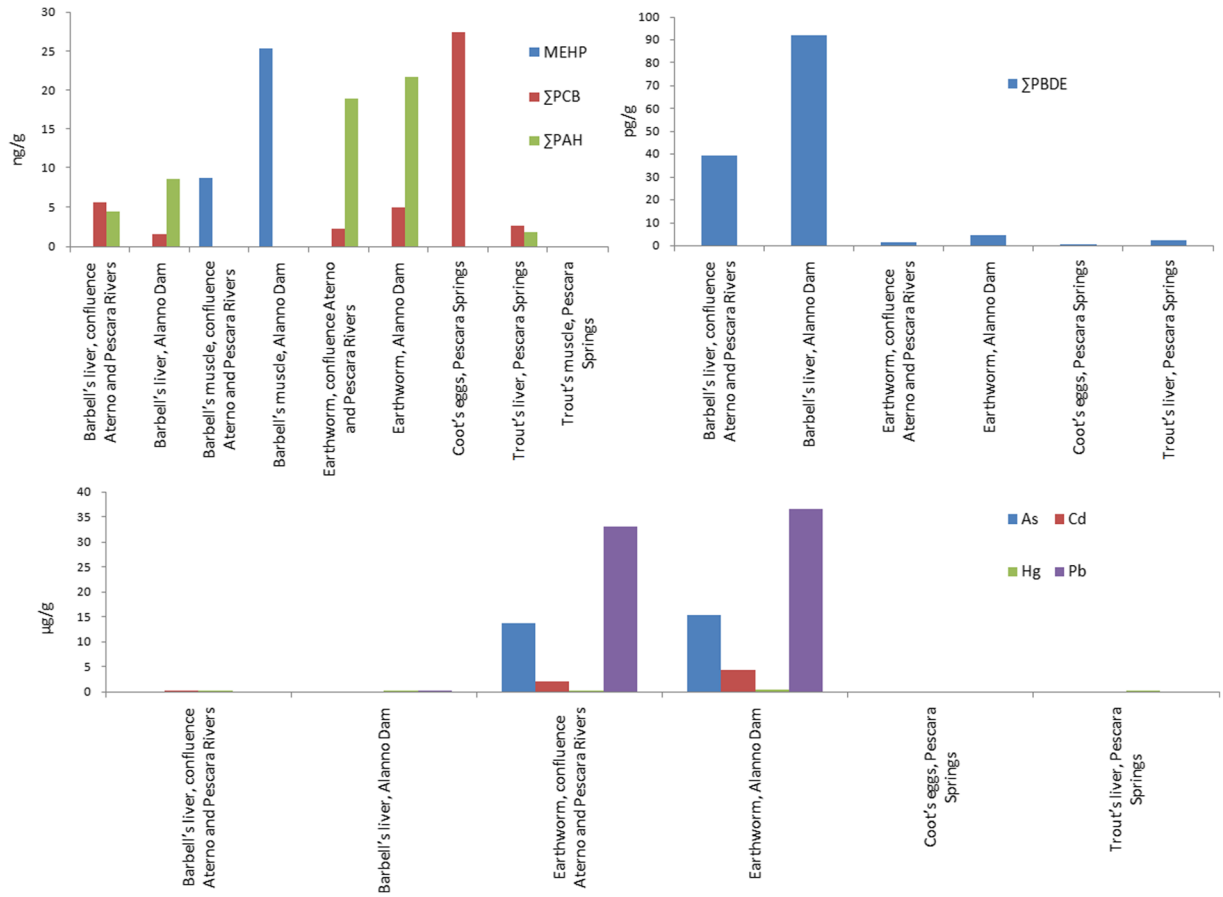

Fig. 2 Synthesis of the results

higher levels than in the low-pressure area. Since scientific literature reports very few results of studies on animal organisms which consider the levels of accumulation of MEHP (e.g., Fossi et al. 2014; Giari et al. 2015; Hu et al. 2016; Guerranti et al. 2016), it is not possible to make direct comparisons. DEHP concentrations in freshwater organisms can range widely and reach levels of 1800–3200 ng/g *w/w*, demonstrating that a recent exposure or the presence in the environment of the primary molecule can be detected the tissues of organisms exposed (Mayer et al. 1972; Peijnenburg and Struijs 2006; Cheng et al. 2013).

Other studies, on marine organisms, report the absence of the compound of origin in tissue vertebrates and support the hypothesis that the form of accumulation of DEHP is the primary metabolite MEHP (Fossi et al. 2014). MEHP levels in marine fish were very variable in different species; overall, they were of the same magnitude order as those found in our study (Guerranti et al. 2016; Hu et al. 2016), but were higher in shark's muscle (Fossi et al. 2014), hinting to a possible biomagnification in predators.

In blood samples from eels of Po River (Italy), Giari et al. (2015) reported detectable DEHP levels together with MEHP levels comparable to those found in our study (MEHP < 2–3–28).

While earthworms can hydrolyze di-(2-ethylhexyl) phthalate (DEHP) to mono-2-ethylhexyl phthalate (MEHP) and phthalic acid (PA) (Albro et al. 1993), DEHP/MEHP were undetectable in the *L. terrestris*

Table 2 Sample lipid content

Barbell's liver	11.8–12.5%
Trout's liver	10.2–10.3%
Barbell's muscle	1.1–1.9%
Trout's muscle	6.5–7.0%
Earthworm	1.1–1.2%
Coot's eggs	14.5–15.1%

Table 3 Mean ± standard deviation levels of contaminants in organisms from the study areas

Organism/matrix and sampling site	Contaminant concentration							
	MEHP (ng/g *w/w*)	∑PCB (ng/g)	∑PBDE (pg/g *w/w*)	∑PAH (ng/g *w/w*)	Trace elements (μg/g *w/w*)			
					As	Cd	Hg	Pb
Barbell's liver, confluence Aterno and Pescara Rivers	–	5.56 ± 4.79	39.42 ± 40.76	4.35 ± 3.43	< LOD	0.11 ± 0.01	0.12 ± 0.01	0.04 ± 0.01
Barbell's liver, Alanno Dam	–	1.53 ± 1.22	91.59 ± 38.27	8.58 ± 6.20	< LOD	< LOD	0.28 ± 0.02	0.27 ± 0.01
Barbell's muscle, confluence Aterno and Pescara Rivers	8.68 ± 2.93	–	–	–	–	–	–	–
Barbell's muscle, Alanno Dam	25.27 ± 15.40	–	–	–	–	–	–	–
Earthworm, confluence Aterno and Pescara Rivers	< LOD	2.23	1.27	18.97	13.60	2.13	0.25	32.97
Earthworm, Alanno Dam	< LOD	4.90	4.65	21.63	15.36	4.29	0.40	36.45
Coot's eggs, Pescara Springs	< LOD	27.36 ± 11.02	0.49 ± 0.08	< LOD	< LOD	< LOD	0.05 ± 0.01	< LOD
Trout's liver, Pescara Springs	–	2.59 ± 0.92	2.11 ± 0.93	1.80 ± 1.75	< LOD	0.01	0.10 ± 0.01	< LOD
Trout's muscle, Pescara Springs	< LOD	–	–	–	–	–	–	–

PFASs, DEHP, SMCs, BPA, and 9 PAHs (AceP, A, Phe, Py, BbF, BkF, BaP, DBA, and IP) were always below the respective LODs and are not reported in the table. pNP, with the exception of one pool of earthworms from the area of the Pescara river source, found in levels equal to the LOD (1 ng/g) was always below this treshold. The sampling points "Pescara Springs" and "confluence Aterno and Pescara rivers" are in the Regional Natural Reserve "Pescara Springs," while "Alanno Dam" is in the homonymous Oasis of the Wildlife Protection. Where the sign "–" is reported, the sample was not analyzed for insufficient quantity of material

samples analyzed; this result, together with the MEHP concentrations detected only in barbell muscle, can support the hypothesis that a contamination by phthalates is not ongoing at least in the soil of the study area of the Alanno Dam but it cannot be excluded in the water basin.

To the best authors knowledge, no data are available in scientific literature about the levels of phthalates in bird's matrices, making those presented here the first available indication.

PCBs

In barbells, the ΣPCB shows lower values in pooled samples from the Alanno Dam (1.53 ± 1.22 ng/g) compared to samples from the Pescara Springs (5.56 ± 4.97 ng/g) (Table 3). This result, albeit not statistically significant, is in contrast with the initial assumption which provided a greater contamination for the organisms collected downstream. On the contrary, PCBs in earthworms are in higher concentrations at the Alanno Dam compared to the control areas, in accordance with the initial hypothesis (ratio over 2; Table 3).

This pattern may indicate two different phenomena. Earthworms may be directly exposed to PCB through the organic fraction of the soil, to which PCB bind. Conversely, the unexpected finding in barbells may indicate that current PCB levels reflect past environmental emissions and their transfer through the food web, consistent with highly persistent and bioaccumulative characteristics of these pollutants, phased out since more than 20 years. Indeed, the levels found in our study are much lower than those found in coastal or freshwater fish from Eastern Europe (Olsson et al. 1999; Falandysz et al. 2002; Covaci et al. 2006).

Among the samples considered, coot eggs showed the greatest concentrations of PCBs; this is due, probably to the higher trophic level of coot compared to the other organisms considered in this study, to a possible biomagnification process and to the high lipid content of eggs; lipophilic contaminants, such as PCB, are transferred from the body fat of the animal to egg being formed (Fiedler 2003). The average concentrations of PCBs found in the coot eggs from the Pescara Springs are comparable to those found in eggs of the same species from the Danube delta (Romania) (approximately 20 ng/g) (Aurigi et al. 2000) and from the Büyük Menderes River (Turkey) (25 ± 21 ng/g *l*/*w*) (Kocagöz et al. 2014). Our findings suggest a PCB contamination, even low, in the area, and support eggs of wildlife birds as a suitable indicator of PCB pollution.

Overall, PCB-138 is the congener prevalent in the individual pools analyzed. This congener is particularly resistant to metabolic degradation, allowing high levels of accumulation in tissues. Furthermore, PCB 138, together with 153, 180, 187, and 170, can be considered the most widespread congeners in commercial mixtures and in the environment owing to their persistence (Safe 1990). Conversely, the undetectable presence in our samples of congeners with low chlorine content, such as PCB-31 for example, can be related to such factors as the lower presence in commercial mixtures, the lower environmental persistence, and a greater efficiency of the organisms to metabolize PCB congeners with low chlorination.

PBDE

In both the barbell and the earthworm, the ΣPBDE shows higher values (almost triple for barbell, almost four times higher for earthworms) in the pools from the Alanno Dam area than the pools from the Pescara Springs (Table 3), albeit the difference does not achieve statistical significance by Mann-Whitney test. As expected, PBDE concentrations in barbell are one magnitude order higher than those in earthworm, due to the higher trophic level and to a possible biomagnification phenomenon. PBDE are persistent and lipophilic contaminants like PCB; however, contrary to PCB, their widespread production and use as flame retardants have been drastically restricted only in the last decade. Therefore, the PBDE exposure of biota is due to emissions much more recent as compared to PCB.

In both sampled area, PBDE were present in liver but not in muscle, consistent with the toxicokinetic features of these pollutants. The PBDE levels found in fish liver in our study are somewhat higher than those found in fish from Danube delta (< 0.1–14.03 ng/g *l*/*w*, Covaci et al. 2006) but are comparable and even lower than concentrations reported for fish muscle from other areas (Eljarrat et al. 2007; Schmid et al. 2007) including barbell muscle from Don river (105.4 ng/g *w*/*w*, Rose et al. 2015). Even taking into account the marked interspecies differences among fishes in regard to the bioaccumulation of lipophilic pollutants, our results do not point a high level of contamination of fish biota by PBDE even in the Alanno Dam area.

Among the samples considered, and contrary to PCBs, coot eggs showed the lowest concentrations of PBDE; in fact, PBDE levels were lower by two orders of magnitude compared to PCB in coot eggs from the same area (Pescara Springs). To the best of Authors' knowledge, just one comparison datum is available for *F. atra* eggs: PBDE levels of 7 ± 3 ng/g *l*/*w* have been found by Kocagöz et al. (2014) in samples from the Büyük Menderes River (Turkey). This value is significantly higher than that found in the present study.

Overall, in our study, the predominant congener was BDE47 followed by BDE-99. A study on the presence of PBDE in animal food products (Schecter et al. 2004), made in USA, confirms that the PBDE-47 and PBDE-99 are among the dominant congeners in fish. Low-brominated congeners, such as the PBDE-47 and PBDE-99, are considered relatively soluble, therefore more mobile in water compared to congeners with a degree of bromination (Watanabe and Sakai 2003); this, together with the fact that these congeners were also those mainly present in the most used commercial mixtures, may explain their prevalence in the samples analyzed.

PAHs

Both study areas showed low levels of PAH contamination, with many PAHs exhibiting concentrations < LOD (Table 3). Earthworms showed the highest concentrations of the 16 PAHs, but no significant differences were observed among the two areas (Table 3, Figs. 2 and 3), even if the ∑16PAH were 18.97 and 21.63 ng/g in Pescara Springs and Alanno dam samples, respectively. In earthworms naphthalene, phenanthrene, fluoranthene, and chrysene were accumulated to a measurable extent. Comparable, albeit slightly lower levels (8.4 ng/g total PAH) were found in earthworms in soil from a Manufactured Gas Plant site (Parrish et al. 2006). However, higher levels were measured in livers of barbell from Alanno Dam as compared to Pescara Springs (∑16PAH: 8.58 ± 6.20 vs. 4.35 ± 3.43 ng/g). It is not clear why barbell liver, but not earthworms, presents a higher PAH contamination difference between high pressure and low pressure area; it can be speculated that the freshwater fish lifecycle might be more exposed to the, predominantly airborne, pollution by PAHs.

PAH levels < LOD were observed in trout liver tissue from Pescara Springs except for benzo(g,h,i)perylene, which was 1.80 ng/g. In all samples of eggs of coot PAH concentrations below the LOD. To our knowledge, this is the first study in which PAH levels have been evaluated in coot eggs. Although the results gave no indication that PAHs may concentrate in waterfowl eggs, at least in a low pressure area, the paucity of publications on the levels of organic pollutants in water fowl eggs is surprising, in Italy and elsewhere.

In general, the PAH profile of the samples analyzed indicates a predominance of low molecular weight over high molecular weight PAHs, suggesting the airborne origin of the contamination and, contrary to what was seen for other contaminants, not supporting the influence of the location of sampling points with respect to the high impact site.

p-NP

p-NP was < LOD (1 ng/g *w*/*w*) in all the samples analyzed, with the exception of one pool of earthworms from the area of the Pescara river source, found in levels equal to the LOD.

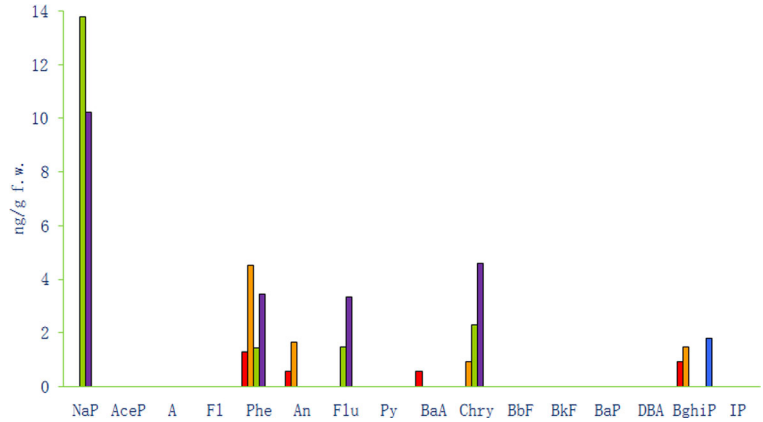

Fig. 3 PAHs fingerprint. Red = Barbell's liver, confluence Aterno and Pescara Rivers; orange = Barbell's liver, Alanno Dam; green = earthworm, confluence Aterno and Pescara Rivers; purple = earthworm, Alanno Dam; blue = coot's eggs, Pescara Springs

Scientific literature reports data on *p*NP in the wild freshwater fishes ranging 1.01 to 277 μg/kg *w/w* (Shao et al. 2005; Vethaak et al. 2005; Wei et al. 2011). Since the *p*-NP in the terrestrial environment is related to sewage sludge or its presence in fertilizer, it can be concluded that in both areas, there are no significant sources of exposure

SMCs

SMCs were below the LOD in all analyzed samples. Although no data on SMC levels in the species analyzed have been found in literature, the possibility to accumulate this contaminants has been reported for freshwater fish, with levels very variable and up to 42 ng/g for galaxolide (Schmid et al. 2007). As for *p*-NP, our data did not indicate any significant source of exposure to SMCs in the study areas.

BPA

As for SMCs, BPA levels were below the LOD in all analyzed samples.

Literature reports that wild freshwater fishes contain higher concentrations of BPA than the farmed or marine fishes (Lee et al. 2015); freshwater fish muscles have contain 0.18–2.60 ng/g *w/w* of BPA (Shao et al. 2005; Vethaak et al. 2005; Wei et al. 2011). It has been suggested that the use of BPA-containing products in household activities factories is the main discharge sources of this compound (Lee et al. 2013). Our results suggest that in both study areas, there are no significant sources of BPA exposure.

Toxic trace elements

There are several indications of a potential difference between the two areas: the two-fold increase of Hg levels in the barbell's liver and earthworms from Alanno dam as compared to Pescara Springs, the two-fold increase of Cd levels in earthworms from Alanno dam as compared to Pescara Springs (4.29 vs. 2.13 ng/g) and the seven-fold increase of Pb levels in the barbell's liver from Alanno dam as compared to Pescara Springs. This trend is confirmed by a monitoring study with the same "upstream-downstream" rationale for dioxins and trace elements along the Aterno-Pescara River (IZSAM 2016). Levels of Hg and Pb in muscle tissue of *B. tyberinus* and other fish species sampled at Bussi plant and along the Aterno River and downstream along the Pescara River resulted overall comparable to the results of the present study. In the cited monitoring study, micronuclei were also assessed in fish erythrocytes: trout and barbell sampled downstream showed an increase of micronuclei. Fishes transferred in non-contaminated water for 30 days showed a time-related decrease of micronuclei, suggesting a relationship with chronic contamination of the Bussi waters.

Noticeably, Hg is known to bioaccumulate in fish, whereas Cd is a recognized soil pollutant, coming also from fertilizers. Thus, freshwater fish and earthworms may be suitable indicators to monitor the environmental trends of Hg and Cd, in these areas as well as other potentially exposed sites.

However, a comparison with literature data relating to the same tissues and species, or species with similar trophic niches, indicates that the observed concentrations do not show signs of exposure of ecotoxicological relevance and the overall picture of the results indicates generally medium-low contamination conditions (e.g., Ernst et al. 2008).

Conclusions

From the biomonitoring carried out on the tissues of various organisms, generally low levels of contamination emerged for both study areas. This aspect found both in samples from the Pescara Springs, control zone, and from the Alanno Dam, area characterized by strong human impact, leads to hypothesize low levels of environmental contamination, at least for the contaminant object of study, giving, in this sense, an indication of conservation of the natural environment. Nevertheless, bioindicators from Alanno Dam, downstream of the industrial site, give indication of the widespread and increased presence of some EDCs and other toxic contaminants (MEHP, PBDEs, PCB, PAHs, Hg, Pb, Cd) in particular in the freshwater environment.

The levels of endocrine disruptors and related biomarkers measured in wild species represent values associated to a state of background exposure, offering a novel contribution to the evaluation of reference values.

The study results deserve to be implemented to obtain an indication on the diffusion and movement of contaminants in the study area; furthermore, in-depth studies would help to clarify the possible contribution of

PAHs from sources other than those in the high impact site, such as airborne contamination.

The indications provided by this study may be used as a basis for furthering the toxicological implications of contamination levels found, in order to assess possible risks for ecosystems and food webs.

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