Phthalate metabolites in the European eel (Anguilla anguilla) from Mediterranean coastal lagoons

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Abstract

The levels and fate of phthalate metabolites have been poorly evaluated in fish, despite their potential ecotoxicological impacts. The present study aims to characterized the levels of phthalate metabolites in muscle tissue of yellow eels (*Anguilla anguilla*) from two coastal Mediterranean lagoons, during three sampling periods. Nine phthalate metabolites were detected in more than 70% of the samples. Slightly higher levels of phthalate metabolites were detected in March and June compared to October, suggesting possible seasonal variations in environmental release and/or phthalate metabolization process by eels. The large sample size (N = 117) made it possible to explore correlations between phthalate metabolites' levels and individual parameters, such as body length, age, body condition and hepatic histo-pathologies. Body length and estimated age poorly correlated with phthalate metabolites, suggesting that eels did not accumulate phthalates during growth, contrary to persistent compounds. Eels presented different grades of hepatic fibrosis and lipidodis. A negative correlation was found between the severity of these pathologies in the liver and the sum of phthalate metabolites levels, supporting the hypothesis that eels with damaged liver are less able to metabolize xenobiotics.

Keywords

Phthalate monoesters, plasticizers, by-product, fish

Highlights

- Phthalate metabolites were characterized in 117 European eels' muscle samples.
- Phthalate metabolites' levels were higher in March/June than in October.
- Body condition, length and age did not explain variations in metabolites' levels.
- Phthalate metabolites' levels declined with increasing lesions in the liver tissue.

1. Introduction

Some synthesized chemicals are rapidly transformed through photodegradation, bacterial degradation and metabolization, which may limit the bioaccumulation and biomagnification of the parent compound (Mackintosh et al., 2004). Because of the intensive and continuous release of these degradable pollutants into the environment, some of their products of transformation may pose a significant environmental concern. Hence, a recent challenging task in ecotoxicology has focused on the evaluation of the fate and impacts of these biodegradable chemicals and their metabolites upon wildlife.

Phthalates have been extensively synthesized since the 1930s and used as plasticizers mainly for polyvinyl chloride (PVC), but also as solubilizing or stabilizing agents. Their worldwide production was estimated to reach 8.1 million tons per year (Crinnion, 2010). Phthalate are omnipresent in all environmental compartments (Mankidy et al., 2013), and induce deleterious effects, including endocrine disruption (Oehlmann et al., 2009). They are easily metabolized and excreted in vertebrates, such as fish (Barron et al., 1995). However, phthalate toxicokinetics and interspecific differences remain poorly described. Phthalate diesters are degraded by hydrolysis in the digestive lumen, which leads to a monoester. Then, hydroxylation (phase I) occurs in liver and kidney through the cytochrome P-450 system (Barron, 1995). At last, oxidized monoesters are conjugated (phase II, generally by glucuronidation), which enhances the solubility of the compounds and facilitates their urinary excretion. Monoester can also be directly conjugated (Silva et al., 2003). Erkekoglu et al. (2010) reported that the monoesters might display the same toxic potencies as the parent compounds, or even greater, as for mono-ethyl-hexyl phthalate (MEHP, Zhai et al., 2014). Despite the potential ecotoxicological impacts, the occurrence and levels of phthalate metabolites have been poorly investigated in free-living fish (Blair et al., 2009; Valton et al., 2014). Moreover, little is known about the extrinsic and intrinsic factors that drive their accumulation, metabolization and excretion in wildlife.

The present study investigates the occurrence of phthalate metabolites in the European eel (*Anguilla anguilla*), according to environmental and individual parameters. European eel has been listed in 2010 on the IUCN red list as a species under critical danger of extinction, after the catastrophic stock decline since the 1970s. Yellow eel is used as a sentinel species for monitoring the chemical status of surface waters with respect to hazardous substances because of its life cycle (benthic, predatory, long-lived and semelparous fatty species, Belpaire and Goemans, 2007; Geeraerts et Belpaire, 2010). To date, the levels and effects of PCBs, pesticides and trace metals have been extensively studied in European eels (Geeraerts

et Belpaire, 2010; Amilhat et al., 2014), while the levels of phthalate parents and metabolites have been virtually neglected. Moreover, eel represents a targeted species in the important economic activity of fisheries and the accumulation of hazardous substances in eels may raise important public health issues. In this study, eels were collected in two Mediterranean lagoons that hosts an important fishing activity (37 tons for Bages-Sigean and 10 tons for Canet-Saint Nazaire in 2009, Vouvé et al. 2014) and that have to deal with increasing agricultural, industrial, urban and recreational pressures. This study attempts to explain individual variations of nine phthalate metabolites' levels in eels muscles (N = 117) according to sampling sites and periods, as well as age, length, body condition, and histological abnormalities in the liver.

2. Materials and methods

2.1 Study area, species and sample collection

Eels were collected from two lagoons on the south-western French Mediterranean coast of the Gulf of Lions (Languedoc-Roussillon). The Canet-St Nazaire lagoon (42°39'42.28''N-3°1'27.51''E) has an area of 4.9 km² and average depth is 0.4 m (maximum: 1.0 m), with a proportionally extended catchment area of 260 km². Bages-Sigean lagoon (43°4'24.81''N-2°59'49.98''E) covers 38 km² with an average depth of 1.5 m (maximum: 3.75 m) and a catchment area of 443 km².

Yellow eels (juveniles of *A. anguilla*) were collected at two locations in the north (BN), and in the south (BS), of the Bages-Sigean lagoon as well as in on station in the Canet-St Nazaire lagoon. The collection campaigns were conducted during three periods in 2010: beginning of March (N=60), June (N=60) and October (N=60), using passive fyke-nets. Among the 180 sampled eels, 117 muscle samples were analyzed for levels of phthalate metabolites. Eels were transported to the laboratory in containers with aerated sampling site water. Eels were anesthetized by speed freezing (5 min, 4-6°C) and immediately decapitated. The total length (Lt) was recorded to the nearest millimeter and the total weight (W) to the nearest g. A portion (10-20 g) of fillet muscle was removed from between the pectoral fin and anus then stored at -20°C until further analysis. Length varied among the three sites (ANOVA: $F_{2,177} = 9.9$, $p < 0.001$), with longer eels in BN and BS (mean ± SD: 36.1 ± 3.3 cm) compared to Canet (33.9 ± 3.7 cm, Tukey post hoc tests: $p < 0.001$).

The condition of each eel was estimated using Fulton's condition factor $K = 100.W(g)/Lt(cm)^3$ (Ricker 1975). Body condition did not differ among the three sampling

sites ($F_{2,177}$ = 2.25, p = 0.11). Body condition significantly differed among sampling periods ($F_{2,177}$ = 28.2, p < 0.001), with greater body condition in June (1.8 ± 0.2) compared to March (1.5 ± 0.2, Tukey post hoc test: p < 0.001) and October (1.6 ± 0.2, Tukey post hoc test: p < 0.001). The age was estimated by analyzing the sagittal otoliths. Otoliths were removed from the head of each eel and cleaned with distilled water. Small otoliths (young eels) were examined in toto in camomile oil under stereomicroscope. For older eels (more than 4 years old) and when the reading was not clear, the otolith was embedded in resin and ground to the sagittal plane and polished until the nucleus was exposed. After using an EDTA solution, otoliths were stained with toluidine blue prior to microscopic examination. The age of each individual was estimated by counting the winter rings (Lecomte-Finiger 1985; ICES 2009). The estimated age varied between 1 and 6 years. The eels from BS were significantly older (mean ± SD: 3.1 ± 1.0) than those from BN (2.5 ± 1.0) and Canet (2.2 ± 0.9) (Kruskal Wallis test and Bonferroni post hoc test: p < 0.001).

2.2 Chemicals and reagents

Solvents for cleaning and extraction purposes of ultrapure quality and free from phthalate residues were supplied by Merck-Chimie (Fontenay-sous-Bois, France). Oasis HLB cartridges (6 mL/200 mg) were provided by Waters (Guyancourt, France). Mobile phase for high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) was prepared with acetonitrile (liquid chromatography – mass spectrometry quality solvent; Merck), formic acid (Carlo-Erba for analysis) and ultrapure water from a Milli-Q system. Seven phthalate monoesters: mono-methyl phthalate (MMP), mono-ethyl phthalate (MEP), mono-iso-butyl phthalate (MiBP), mono-nbutyl phthalate (MnBP), mono-benzyl phthalate (MBzP), mono-n-octyl phthalate (MnOP), MEHP and two oxidized by-products, mono (2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono (2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) were purchased from Cambridge Isotope Laboratories, Inc. (UK). A mixture of four surrogates (from Cambridge Isotope Laboratories, purity > 95%), 4-methylumbelliferone-$^{13}C_{12}$ (4-MU-$^{13}C_{12}$), MnBP-$^{13}C_{12}$, MEHP-$^{13}C_{12}$ and MiNP-$^{13}C_{12}$, each one at 25 ng.μL^{-1} in acetonitrile (ACN) was prepared and used as 10 µL per sample. β-Glucuronidase from Escherichia coli K12, 200 units.mL^{-1} was provided by Roche Biomedical (France). Basic buffer (pH of 11) was prepared with NH_4OH/ACN/H_2O (0.5:50:50, v/v/v). Acid buffer (pH of 2.8) was prepared with NaH_2PO_4 (1.93 g for 100 mL) in H_3PO_4 (85 %)/H_2O (1:99, v/v).

147 *2.3 Phthalate monoesters extraction and purification*

148 In order to avoid sample contamination by phthalates the equipment used (flask, pipet, tubes)
149 was glassware or polycarbonate tubes for storage. After washing twice with acetone and with
150 n-hexane, the glassware (except for volumetric material) was baked at 400° C in a furnace, for
151 4 h and stored with glass stoppers in aluminum foils. For micro-volumes, automated
152 analytical glass syringes (Thermo Fisher Scientific, UK) were used. Moreover laboratory
153 blanks were performed every 20 samples, following the same treatment steps than the
154 samples.

155 Prior to analysis, tissues were freeze-dried for 48 h and then grounded to obtain a fine
156 powder. Surrogates were added to the muscle samples, prior to their extraction.
157 Concentrations of seven monoesters (MMP, MEP, MiBP, MnBP, MBzP, MnOP and MEHP)
158 and two phase I metabolites of DEHP (MEOHP and MEHHP) were determined in eel
159 muscles (N = 117).

160 First, 500 mg of freeze-dried muscle tissue were spiked with surrogates in a 25 mL
161 polycarbonate tube and crushed with an Ultra-Turrax in 4 mL of ammonium acetate (1 M).
162 Then, a deconjugation step was used (90 min, 37° C) with β-glucuronidase from E. coli K 12
163 (25 µL) free from any detectable esterase activity upon phthalate diesters (Blount et al. 2000).
164 The glucuronide conjugate of 4-MU, the 4-methylumbelliferyl-β-D-glucuronide, was used
165 (140 mg by incubation) for controlling the deconjugation reaction. Thus, for each parent
166 phthalate, total metabolites were quantified including deconjugated compounds.

167 After deconjugation and centrifugation (4 500 RPM, 10 min), the supernatants were
168 collected into a 15 mL glass tube and 3 mL of ammonium acetate (1 M) were added to the
169 deconjugated sample and homogenized with an Ultra-Turrax, centrifuged and the supernatants
170 collected (step repeated with 2 mL of ammonium acetate (1 M)). The combined supernatants
171 were extracted twice on SPE (solid phase extraction) cartridges (Oasis HLB 6 mL/200 mg)
172 for purification. Prior to the first elution, the cartridge were conditioned with 3 mL of ACN
173 and 6 mL of basic buffer. After loading, the samples were cleaned-up (twice) with 2 mL of
174 ammonium acetate (1 M) and eluted with 6 mL of basic buffer, and collected in a 22 mL glass
175 tube with 10 mL of acid buffer. The acidified sample was then purified through a second
176 conditioned SPE cartridge (1 mL of ACN then 1 mL of H_2O and 2 mL of acid buffer) and
177 washed (twice) with 2 mL of acid buffer then 2 mL of H_2O. The analytes were eluted with 10
178 mL of ACN then, 10 mL of ethyl acetate. The purified eluate was evaporated under nitrogen
179 stream in a thermostatic bath (65° C), until dry. The residue was resuspended in 0.5 mL of
180 ACN, filtered under centrifugation (6 000 RPM, 5 min) and transferred in vial. This procedure

was repeated three times. Finally, the filtrated samples were combined and concentrated under nitrogen stream (65° C) at 500 µL before analysis. Concentrations (ng.g^{-1} dry weight, *dw*) are given for individual phthalate metabolites as well as the sum of all nine metabolites (MPA). Water content in eel muscles was 40.2 ± 6.1 %.

2.4 LC-MS/MS analysis

Phthalate monoesters were quantified by liquid chromatography (1200 series - Agilent Technologies, Massy, France) – tandem mass spectrometry (6410-triple quadrupole MS, in negative ion mode for the electrospray source - Agilent Technologies (LC- MS/MS), equipped with a silice upti-prep strategy column (100 A, C18-2, 2x100 mm, 2.2 µm – Interchim) heated at 40°C. The source was in ESI negative mode (N2: 350°C; gas flow: 660 L/h; capillary: 4000 V) and the injected sample volumes were 10 µL. The LC gradient flow, the MRM parameters for LC-MS/MS and limits of quantification (LOQs) are indicated in the Supplementary Material (Table SM.1, 2, 3 and 5).

2.5 Method validation

The analytical method performance was determined by evaluating the linearity, recovery rates, instrument detection limits (IDLs), limits of detection (LODs) and limits of quantification (LOQs). Performance in terms of linearity and dynamic range was checked by injecting reference standard solutions in duplicate. The range tested was 0.3-10 ng on column for phthalate metabolites and 0.002-2 ng for 4-MU.

Recovery experiments were performed on eel muscle samples (N = 3) spiked at high concentration levels, i.e. at least 10 times higher than the mean concentrations of each endogenous phthalate metabolite in eel muscle samples (Table SM.4, Supplementary Material).

IDLs were determined using the less concentrated surrogate as a signal/noise ratio of 3 peak to peak.

Eight procedural blanks that underwent all the sample extraction and purification, were analyzed every 20 samples. All samples were blank-corrected by subtracting the mean phthalate metabolite concentration in the batch blanks from each sample, prior to recovery correction. The LOD was calculated as the mean value plus three standard deviations of the analyte in blank samples, and LOQ was calculated as the mean value plus ten standard deviations of the analyte in blank samples.

2.6 Histological approach

Small pieces of liver from 62 eels were fixed with Bouin Hollande's solution, dehydrated with graded series of ethanol, placed in butanol-1 for at least 24 h and then embedded in paraffin wax. Liver sections (6 µm) were stained with nuclear fast red (NR) and picro-indigo-carmin (PIC) (Gabe 1968). Microphotographies were taken with a light microscope (Laborlux S – Leitz wetzlar Germany) equipped with a digital camera (Imagingsource) and analysis were realized with the Archimed software (Version 5.6.0.). Three different liver areas (25 mm^2) randomly selected on different sections were analyzed for each eel. Scoring notes for 5 hepatic parameters: lysis-necrosis, lipidosis, fibrosis, melano-macrophage (MMC) aggregates and nuclear alterations, were established and an individual global score was calculated as the mean value of the different scores (Jaffal et al., 2015). The normal histological state of the eel liver (score 0) corresponding to healthy uncontaminated yellow eels was established according to studies realized 30 to 20 years before, when it was possible to collect eels that were not exposed to phthalates (Barni et al., 1985; Affandi and Biagianti, 1987; Lemaire-Gony and Lemaire, 1992). The higher scores of histopathological alterations were established taking into account the grade of alterations reported in literature for eel but also for different fish species (Affandi and Biagianti, 1987; Braunbeck et al., 1990; Costa et al., 2011; Jaffal et al., 2011). The global histopathological score (on 10 grade) was calculated with the formula:

$$\text{Global score} = [\text{score}_{\text{lysis-necrosis}} + \text{score}_{\text{MMC}} + \text{score}_{\text{fibrosis}} + \text{score}_{\text{nucleus}} + \text{score}_{\text{lipid}}]/ 5.$$

Lysis-necrosis score was established as follow: 0 = less than 10 %, 2.5 = 10 to 25 %; 5 = 25 to 50 %; 7.5 = 50 to 75 %, 10 = more than 75 % of death hepatocytes in the parenchyma. To establish the nuclear alterations score, the quantity of altered nucleus among 40 randomly observed was converted in a score on 10 points. The lipid score was established on the size and the amount of the lipid droplets observed in hepatocytes: 0 = no lipid droplet; 2.5 = few little lipid droplets; 5 = lipid droplets present in 25 to 50% of the hepatocyte; 7.5 = more than 50% of the hepatocytes presented large lipid droplets and have a double sized; 10 = all hepatocytes were filled with huge lipid droplets and have at least a double size. The fibrosis score was determined by observation of the collagen deposits localization in liver : 0= only big blood vessel (portal section) had collagen deposits, 2.5 = some blood vessel and bile duct presented collagen deposit, 5= the majority of the blood vessel and bile duct presented collagen deposit, 7.5= abnormal large collagen deposits were observable around sinusoids, veins, small bile ducts and some hepatocytes, 10= collagen deposit present throughout all the parenchyma, particularly between hepatocytes. Melano macrophage aggregate (MMA) were

counted and the score was the quantity of MMA per area with a maximum of 10 (score10 = 10 or more MMC observed on each of the three areas).

2.7 Statistical analyses

All statistical tests were performed using R software version 3.2.2 (R Development Core Team 2015). Data were log-transformed to make the data fit the normal distribution. Significant differences among groups (i.e. sampling periods or sampling sites) were evaluated by ANOVA (or Kruskall-Wallis tests for non-parametric data), followed by post hoc Tukey test (or Bonferroni tests for non-parametric data) if ANOVA indicated a significant difference between groups. Correlations between phthalate metabolites and individual parameters (age, body condition, body length, score of histological abnormalities in liver) were expressed by Pearson (or Spearman for non-parametric data) correlation coefficient.

3. Results

3.1. Method performance

The column separation obtained for the LC-MS/MS method were consistent with accurate quantification. The Multiple Reaction Monitoring mode (MRM) separates masses in two stages, making the instrument significantly more selective and sensitive than a single quadrupole system. As a result, compounds can be easily identified and quantified in the presence of complex matrices using MRM. Quantification of phthalate metabolites was carried out by calculating the response factor for each compound relative to the corresponding surrogate and concentrations were obtained using a linear regression analysis of the peak area versus the concentration ratio.

The results for linearity were satisfactory with an R^2 ranging from 0.997 to 0.999 (Table SM.3, Supplementary Material). Recoveries for the overall extraction procedure displayed satisfactory results (74% - 102%) for MiBP, MEHHP, MnBP, MEOHP, average score for MEHP and MBzP (46% - 61%) and poor results (12% - 32 %) for MMP, MEP, MnOP (Table SM.4, Supplementary Material). Low recovery rates for these phthalate metabolites could be due to the long lasting protocol, and especially the deconjugation step. IDLs ranged from 0.3 to 6 pg (Table SM.2, Supplementary Material). The nine phthalate metabolites were detected in the 8 blanks (Table SM.5, Supplementary Material) and were consistent with compound determinations. The LODs and LOQs were compatible with fish

concentrations and allowed quantification of all compounds (Table SM.5, Supplementary Material).

3.2. Phthalate metabolites in eel muscles

All the phthalate metabolites were detected in more than 70% of the muscle samples analyzed. Large inter-individual variations were detected (Table 1, Fig. 1). The most abundant phthalate metabolites were MEHP, followed by MiBP and MnBP, whereas the less abundant was MBzP (Table 1, Fig. 1). Positive correlations were detected between MiBP and MnBP (DBP metabolites), between MEHP, MEHHP and MEOHP (DEHP metabolites), and between MEOHP and MnOP (Table 2). A negative correlation was detected between MEHHP and MnBP (Table 2).

3.3. Sampling sites and periods

The levels of each phthalate metabolite and of the sum of all nine metabolites in eel muscle did not significantly differ among the sites Canet, South and North of Bages-Sigean (Table 3). Concerning the sampling periods, levels of MEP, MEHHP and MEOHP were higher in March and June than in October (Table 3, Fig. 2), whereas the other phthalate metabolites did not show significant differences among the three periods (Table 3).

3.4. Age, length, body condition

Muscular levels of phthalate metabolites in eels were not correlated to age, body length and body condition (Table 4), except a negative correlation between MnBP levels and age, between MnBP levels and length and a positive correlation between MEOHP levels and length.

3.5. Histological abnormalities

Histological abnormalities were detected in the liver of eels collected at the three stations. The main liver alterations were hepatocytic lysis or necrosis, nuclear abnormalities, lipid accumulation, fibrosis and presence of melano-macrophage aggregates. The score of histopathological perturbations in eels revealed clear liver pathologies in most of the individuals. The higher grade of hepatic alterations were related to important accumulation of lipid forming large lipid droplets in enlarged hepatocyte and paralleled with nuclear alteration, fibrosis and necrosis and lysis which characterize the lipoid degeneration (also called fatty

liver) (Fig.3). Muscular levels of MPA, MEP, MiBP and MnOP significantly decreased with increasing number of hepatic abnormalities (Table 4, Fig. 4).

4. Discussion

4.1. Muscular levels of phthalate metabolites

The detection of phthalate metabolites in eel muscle suggested that eels were directly exposed to phthalate metabolites from the water or the sediment, since phthalate parents can be transformed by microorganisms, or that eels were exposed to phthalate parents and were able to incorporate and metabolize them. MEHP, MiBP and MnBP were the major phthalate metabolites. Results obtained in this study and using a new method are consistent with previous studies that reported similar muscle contents of phthalate metabolites in fish collected in an urbanized marine inlet, in Vancouver: the juvenile Shiner Perch *Cymatogaster aggregata* (geometric mean ranged from 0.54 $ng.g^{-1}$ *ww*. for MBzP to 82 $ng.g^{-1}$ *ww*. for MnBP, McConnell, 2007) and the white spotted greenling *Hexogrammos stelleri* (0.24-1.1 $ng.g^{-1}$ *ww* for MEHP, 6.63-60.9 $ng.g^{-1}$ *ww* for MnBP, Blair et al., 2009), as well as in marine fish from the Yangtze River Delta sea area (China, 1.2 to 20.4 $ng.g^{-1}$ *ww,* Gu et al., 2014). It should be noted that much higher levels of phthalates metabolites were found in liver samples (mean ranged from 7.1 ± 2.6 (MEOHP) to 1607 ± 2706 (MiBP) $ng.g^{-1}$ *dw*) of roaches fished in the Seine river, France (Valton et al., 2014), which is coherent with the metabolization of phthalates in liver.

4.2. Seasonal and spatial variations

Lagoons are known to be characterized by great spatial and seasonal variabilities in environmental conditions (e.g. temperature, Viaroli et al., 2007). In the present study no significant differences of phthalate metabolite levels were observed among the three sites. This lack of spatial difference could be due to the geographical proximity of Canet and Bages Sigean lagoons and the absence of direct discharge of urban wastewater into these lagoons. The levels of PCBs and PBDEs were also relatively low in the sediment and eels muscles from these two lagoons (Labadie et al., 2010; Vouvé et al., 2014). Analyses of phthalate concentrations in water and sediments should be necessary to confirm this lack of spatial variation.

A temporal variation was detected, especially for MEP, MEOHP and MEHHP, whose levels were higher in March/June than in October 2010. This could be due to a seasonal change in exposure to phthalate, in abiotic factors (e.g. temperature, precipitation, salinity, oxygen rate) and thus in the hepatic enzymatic activities involved in detoxification processes in fish (Förlin et al.,1995). Moreover, March/June corresponds to the intense recreational activity, and potentially to an increasing release of plasticizers into the lagoons. Additional data are needed, however, to confirm this seasonal trend

4.3. Age, length, body condition

The large sample size of eel muscles (N = 117) made it possible to explore correlations between phthalate metabolites' levels and individual parameters. Age was not correlated to phthalate metabolites' levels, except for MnBP, whose levels decreased with age. Similarly body length was not correlated to muscular levels of phthalate metabolites, except for MnBP whose levels decreased with length and for MEOHP whose levels increase with length. These results suggest that eels did not accumulate phthalates during growth, contrary to persistent compounds. For instance, levels of polychlorobiphenyls (PCBs) and polybrominated diphenyl-ethers (PBDEs) increased with length and age from glass eels to silver eels (Tapie et al., 2011). Similarly, PCBs, hexachlorocyclohexanes (HCHs), hexachlorobenzene (HCB), dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyldichloroethylene (DDE) accumulated with age in the muscle of brown trout (*Salmo trutta*, Vives et al., 2005). It should also be noted that range of age and length were limited in our data set, which may have restricted patterns of accumulation or dilution with growth. At last, eels could have been exposed to phthalate intermittently throughout their lives.

No correlation were found between muscular levels of phthalate metabolites and body condition, suggesting that metabolization efficiency is not linked to the individual body condition.

4.4 *Histological abnormalities*

An original and innovative objective of this study was to explore whether individuals with hepatic lesions had lower metabolites' levels, because of a less efficiency of their liver to metabolize phthalate parents. The prevalent histopathological characteristics identified in eel from Mediterranean lagoons were hepatocellular necrosis and parenchymal lysis, cellular hyperplasia associated to lipid vacuolization and fibrosis who are interpreted as nonspecific responses to stress as they have been described in fish exposed to a wide spectrum of

pollutants (Hinton et al., 1992). The lipoid degeneration (fatty liver) is one of the major changes observed here. In yellow eel, this change is symptomatic of a metabolic dysfunction since their liver does not usually store lipids. Lipid droplets may sequester fat-soluble pollutants and may reduce the metabolic capacity of that tissue. Moderate lipidosis is commonly regarded as an unspecific alteration with multiple potential causes and lipoid degeneration indicates a severe alteration of fish health (Costa et al., 2011). Lipoid degeneration is linked to an high rate of hepatic alteration and fatty liver probably take place with impairment of their function (Vandemiale et al., 2001).

Muscular levels of the sum of phthalate metabolites were negatively correlated with the score of histological abnormalities in the liver. This negative correlation supports the hypothesis that individuals with numerous hepatic lesions are less efficient to metabolize xenobiotics. Such hepatic alteration of the metabolic function would imply an accumulation of phthalate parents.

Conclusion

Nine phthalate metabolites were characterized in muscle tissue of 117 European eels. No difference was found between the two lagoons, and a weak temporal variation was detected with higher contamination in March/June than in October. Body condition, length and age did not explain individual variations in muscular levels of phthalate metabolites suggesting that eels did not accumulate phthalates during growth, contrary to persistent compounds.. Individuals with numerous histological abnormalities in liver bore lower levels of phthalates metabolites, suggesting that they were less efficient to metabolize xenobiotics. Further ecotoxicological research should be conducted to improve understanding of the links between fish physiology and ecology and the metabolization of phthalates.

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Table 1. Mean ± standard deviation (SD) of phthalate metabolites in eel muscles (ng.g^{-1} *dw*, N = 117). % represents the percentage for each metabolites with respect to the sum of phthalate metabolites (MPA).

	MPA	MMP	MEP	MiBP	MnBP	MBzP	MnOP	MEHP	MEHHP	MEOHP
Mean	913	5.7	33	206	174	2.0	82	282	94	34
SD	885	6.0	108	280	333	4.1	162	403	206	94
%		0.6%	3.6%	23%	19%	0.2%	9%	31%	10%	3.7%

Table 2. Pearson or Spearman correlation matrix between phthalate metabolites levels in eel muscles (N = 117, * p < 0.05, ** p < 0.01, *** p < 0.001).

	MEP	MiBP	MnBP	MBzP	MEHP	MEHHP	MEOHP	MnOP
MMP	0.1	0.03	- 0.15	0.05	0.2 **	0.2 **	0.1	0.11
MEP		0.3 **	0.1	0.15	0.1	0.2	0.2	0.08
MiBP			0.7 ***	0.4 ***	- 0.02	0.1	0.15	0.01
MnBP				0.2 **	- 0.02	- 0.2 *	0.2	0.2*
MBzP					0.2 *	0.2	0.2 *	0.02
MEHP						0.5 ***	0.4 ***	0.2 *
MEHHP							0.5 ***	- 0.009
MEOHP								0.5 ***

Table 3. Spatial and temporal differences in the levels of phthalate metabolites in eel muscles, using ANOVA or Kruskal Wallis (* p < 0.05, ** p < 0.01, *** p < 0.001). When the difference was statistically significant within sites or periods, a Tuckey or Bonferroni post hoc test was realised.

	MPA	MMP	MEP	MiBP	MnBP	MBzP	MEHP	MEHHP	MEOHP	MnOP
Periods	$F_{2, 114}$ = 3.3 *	X^2 = 4.4 **	X^2 = 18.1 ***	$F_{2, 114}$ = 1.4	X^2 = 2.2	X^2 = 2.7	X^2 = 5.1	X^2 = 7.6 *	$F_{2, 114}$ = 8.7 ***	X^2 = 1.6
Post-hoc			Oct<Jun ** Oct<Mar ***					Oct<Jun *	Mar<Jun * Oct<Jun ***	
Sites	$F_{2, 114}$ = 0.3	X^2 = 0.9	X^2 = 1.3	$F_{2, 114}$ = 1.8	X^2 = 5.6	X^2 = 2	X^2 = 0.6	X^2 = 0.08	$F_{2, 114}$ = 0.3	X^2 = 1

Table 4: Pearson or Spearman correlation matrix between the levels of phthalate metabolites in eel muscles and individual parameters (age, length, body condition and the score of histological abnormalities in the liver (histo), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

	MPA	MMP	MEP	MiBP	MnBP	MBzP	MEHP	MEHHP	MEOHP	MnOP
Age	- 0.02	- 0.1	- 0.08	- 0.05	- 0.2 *	- 0.07	0.08	0.15	0.2	0.05
L	- 0.07	0.04	- 0.03	- 0.02	- 0.25 **	- 0.2	0.01	0.1	0.2 *	- 0.02
K	- 0.08	- 0.09	- 0.1	- 0.09	- 0.1	- 0.1	- 0.03	0.05	0.1	0.07
Histo	- 0.4 **	- 0.1	- 0.3 **	- 0.3 *	- 0.2	- 0.2	- 0.07	- 0.05	- 0.01	- 0.3 *

539 Figure legend

540

541 Figure 1: Distribution of phthalate metabolites content (log-transformed) in muscle ($ng.g^{-1}$
542 *dw*) in 117 eels specimens.

543

544 Figure 2. Seasonal distribution of phthalate metabolites content (log-transformed) in 117
545 muscle samples ($ng.g^{-1}$ *dw*).

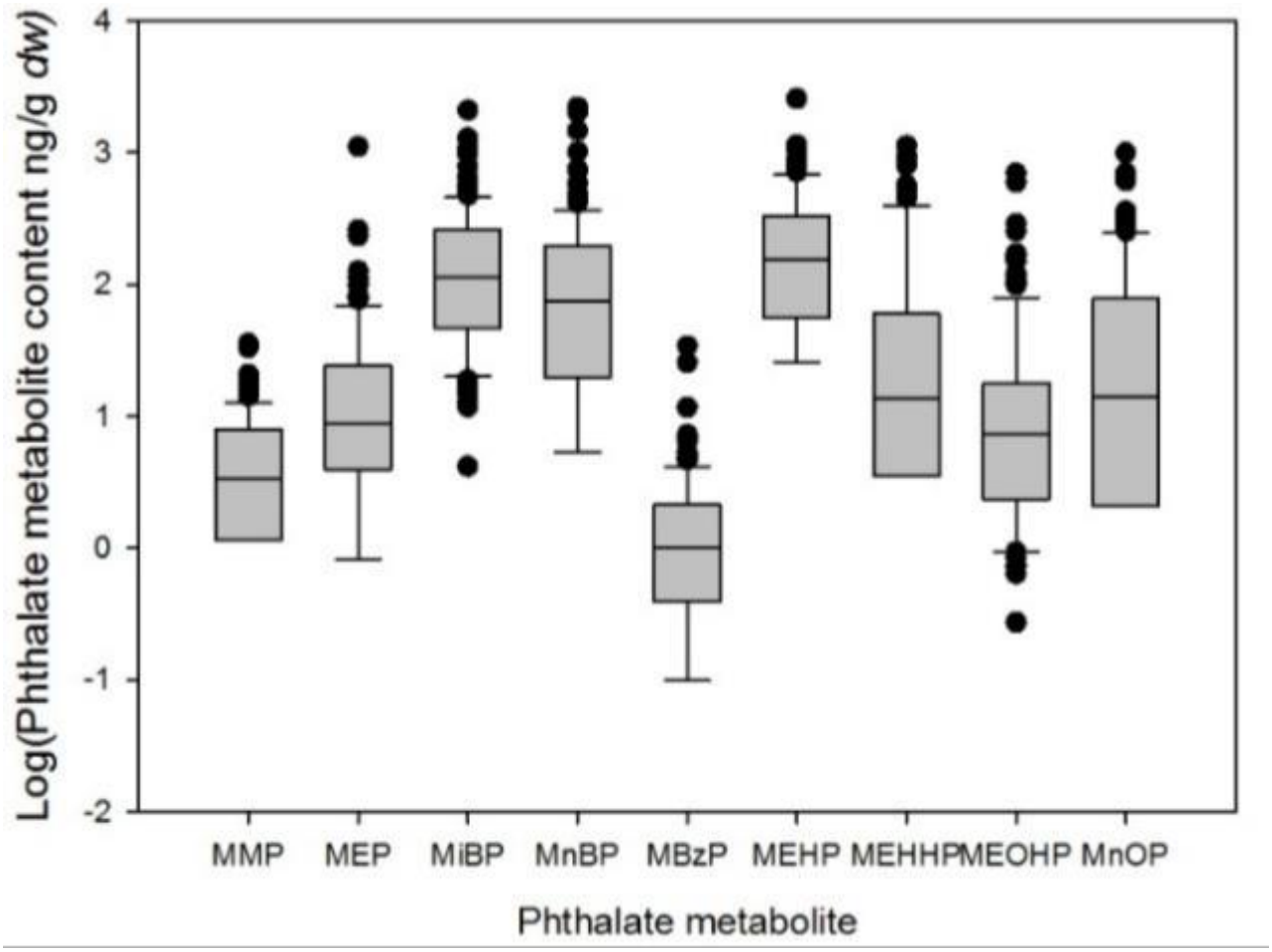
546

547 Figure 3. Micrographies of eel liver showing contrasted grade of hepatic alterations : [A] low
548 perturbations in liver of an eel from BN in March (score 1/10); [B] moderate grade of
549 alterations in the liver of an eel from BN in June with a beginning of lipidosis (score 3/10);
550 [C] steatosis in liver of eel from BS in October (score 5/10) and [D] high grade of alterations
551 in liver of an eel from C in October undergoing lipoid degeneration with lipidosis, fibrosis
552 and frequent necrosis and lysis (score 7/10). Bar = 20 µm, AN : altered nucleus, FV: fibrotic
553 vessel, MMA: melanomacrophage aggregate, nN : normal nucleus, N: necrosis, Ly: lysis, li:
554 lipidic droplet, V: vessel.

555

556 Figure 4. Correlations between the sum of all the phthalate metabolites (MPA) content in
557 muscle (log-transformed, $ng.g^{-1}$ *dw*) and the individuals' histo-pathological score.

558 Figure 1



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564 Figure 2.

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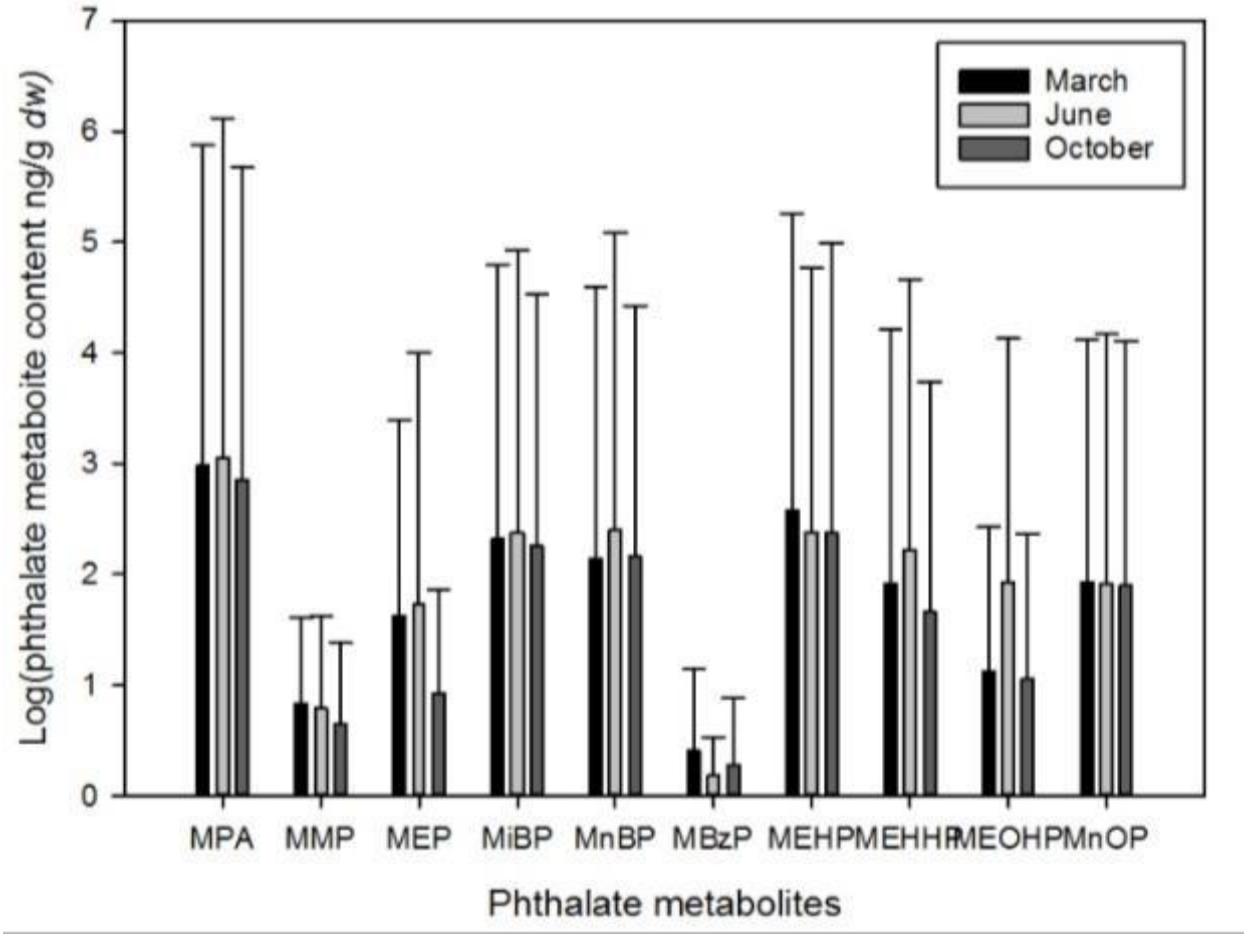

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567 Figure 3.
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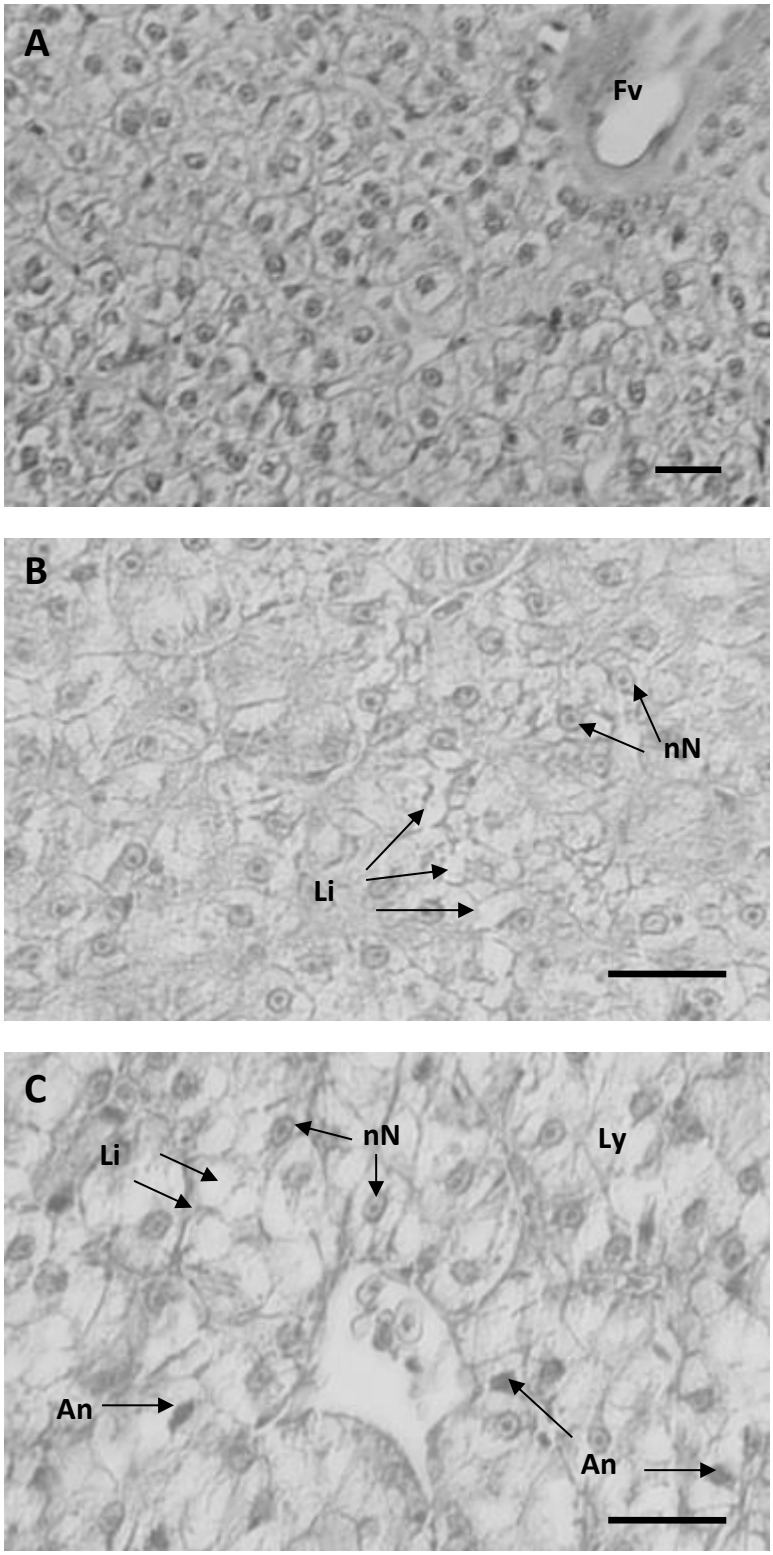

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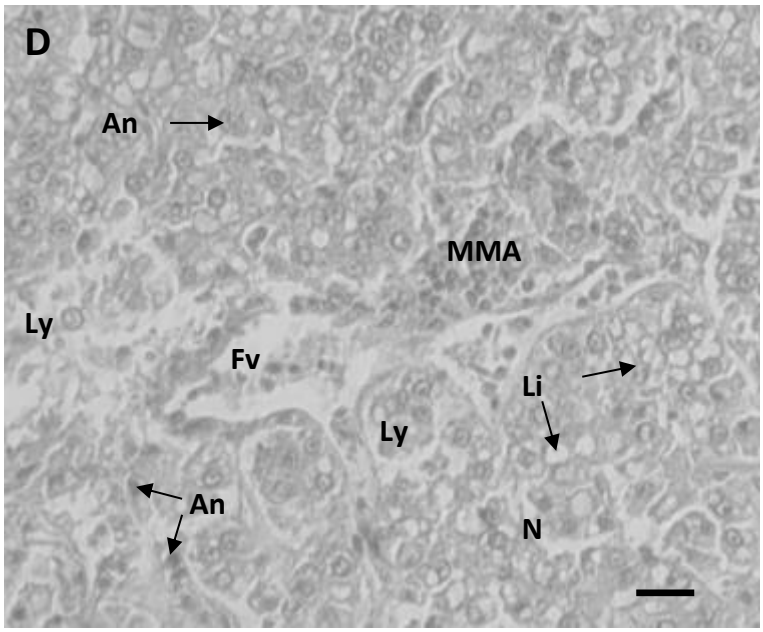

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578 Figure 4.

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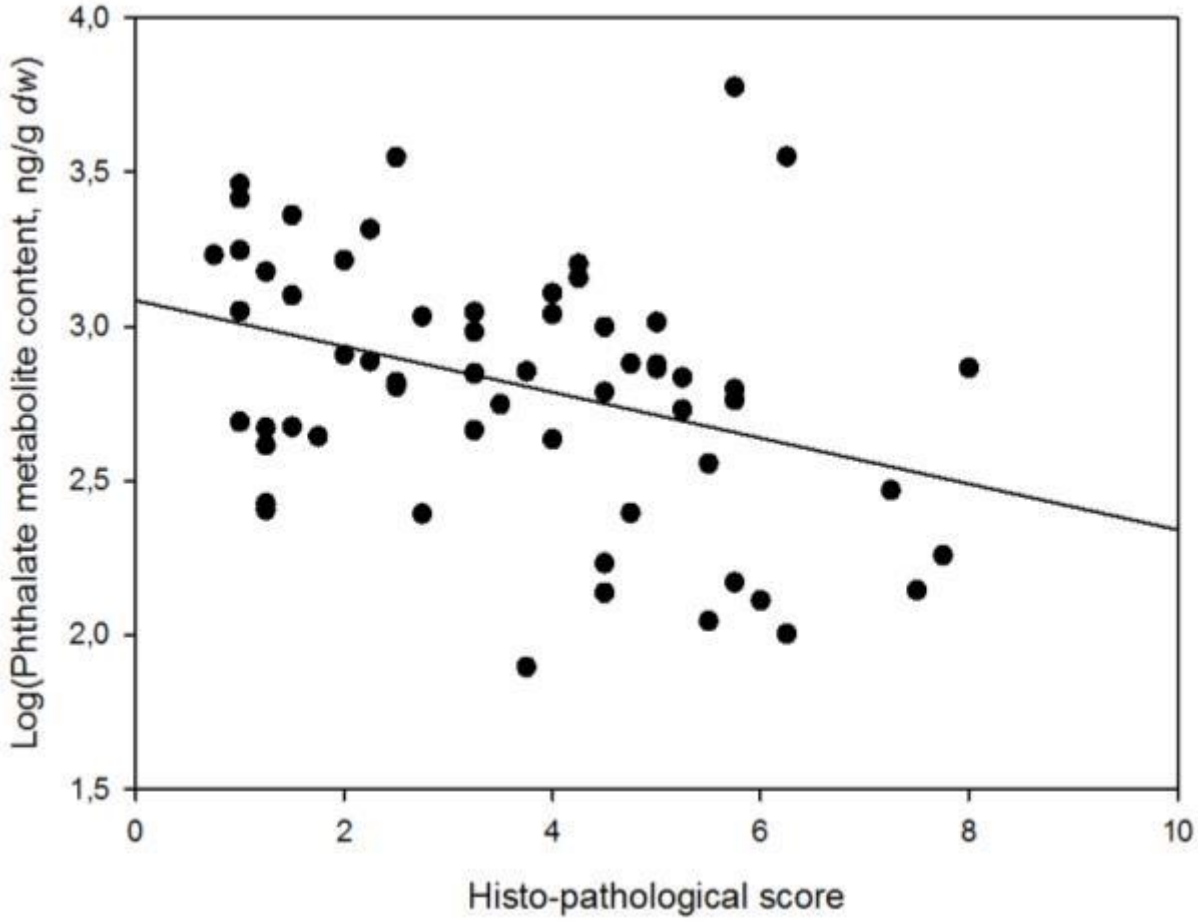

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