

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

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1	Phthalate metabolites in the European eel (Anguilla anguilla) from
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19 Abstract

The levels and fate of phthalate metabolites have been poorly evaluated in fish, despite their 20 potential ecotoxicological impacts. The present study aims to characterized the levels of 21 phthalate metabolites in muscle tissue of yellow eels (Anguilla anguilla) from two coastal 22 Mediterranean lagoons, during three sampling periods. Nine phthalate metabolites were 23 detected in more than 70% of the samples. Slightly higher levels of phthalate metabolites 24 were detected in March and June compared to October, suggesting possible seasonal 25 variations in environmental release and/or phthalate metabolization process by eels. The large 26 27 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-28 29 pathologies. Body length and estimated age poorly correlated with phthalate metabolites, suggesting that eels did not accumulate phthalates during growth, contrary to persistent 30 31 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 32 33 phthalate metabolites levels, supporting the hypothesis that eels with damaged liver are less able to metabolize xenobiotics. 34

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36 Keywords

37 Phthalate monoesters, plasticizers, by-product, fish

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39 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.

45 **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.

53 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 54 55 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 56 57 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, 58 59 phthalate toxicokinetics and interspecific differences remain poorly described. Phthalate diesters are degraded by hydrolysis in the digestive lumen, which leads to a monoester. Then, 60 hydroxylation (phase I) occurs in liver and kidney through the cytochrome P-450 system 61 (Barron, 1995). At last, oxidized monoesters are conjugated (phase II, generally by 62 glucuronidation), which enhances the solubility of the compounds and facilitates their urinary 63 excretion. Monoester can also be directly conjugated (Silva et al., 2003). Erkekoglu et al. 64 (2010) reported that the monoesters might display the same toxic potencies as the parent 65 compounds, or even greater, as for mono-ethyl-hexyl phthalate (MEHP, Zhai et al., 2014). 66 Despite the potential ecotoxicological impacts, the occurrence and levels of phthalate 67 metabolites have been poorly investigated in free-living fish (Blair et al., 2009; Valton et al., 68 2014). Moreover, little is known about the extrinsic and intrinsic factors that drive their 69 70 accumulation, metabolization and excretion in wildlife.

The present study investigates the occurrence of phthalate metabolites in the European 71 72 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, 73 74 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 75 76 because of its life cycle (benthic, predatory, long-lived and semelparous fatty species, 77 Belpaire and Goemans, 2007; Geeraerts et Belpaire, 2010). To date, the levels and effects of 78 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 79 80 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 81 important public health issues. In this study, eels were collected in two Mediterranean lagoons 82 that hosts an important fishing activity (37 tons for Bages-Sigean and 10 tons for Canet-Saint 83 Nazaire in 2009, Vouvé et al. 2014) and that have to deal with increasing agricultural, 84 industrial, urban and recreational pressures. This study attempts to explain individual 85 variations of nine phthalate metabolites' levels in eels muscles (N = 117) according to 86 sampling sites and periods, as well as age, length, body condition, and histological 87 abnormalities in the liver. 88

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90 2. Materials and methods

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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''N3°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''N2°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 99 (BN), and in the south (BS), of the Bages-Sigean lagoon as well as in on station in the Canet-100 St Nazaire lagoon. The collection campaigns were conducted during three periods in 2010: 101 beginning of March (N=60), June (N=60) and October (N=60), using passive fyke-nets. 102 Among the 180 sampled eels, 117 muscle samples were analyzed for levels of phthalate 103 104 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. 105 106 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 107 anus then stored at -20°C until further analysis. Length varied among the three sites 108 (ANOVA: $F_{2,177} = 9.9$, p < 0.001), with longer eels in BN and BS (mean \pm SD: 36.1 \pm 3.3 cm) 109 compared to Canet (33.9 \pm 3.7 cm, Tukey post hoc tests: p < 0.001). 110

111 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 113 (F_{2.177} = 28.2, p < 0.001), with greater body condition in June (1.8 \pm 0.2) compared to March 114 $(1.5 \pm 0.2, \text{Tukey post hoc test: } p < 0.001)$ and October $(1.6 \pm 0.2, \text{Tukey post hoc test: } p < 0.001)$ 115 0.001). The age was estimated by analyzing the sagittal otoliths. Otoliths were removed from 116 the head of each eel and cleaned with distilled water. Small otoliths (young eels) were 117 examined in toto in camomile oil under stereomicroscope. For older eels (more than 4 years 118 old) and when the reading was not clear, the otolith was embedded in resin and ground to the 119 sagittal plane and polished until the nucleus was exposed. After using an EDTA solution, 120 otoliths were stained with toluidine blue prior to microscopic examination. The age of each 121 individual was estimated by counting the winter rings (Lecomte-Finiger 1985; ICES 2009). 122 The estimated age varied between 1 and 6 years. The eels from BS were significantly older 123 (mean \pm SD: 3.1 \pm 1.0) than those from BN (2.5 \pm 1.0) and Canet (2.2 \pm 0.9) (Kruskal Wallis 124 125 test and Bonferroni post hoc test: p < 0.001).

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2.2 Chemicals and reagents

Solvents for cleaning and extraction purposes of ultrapure quality and free from phthalate 128 residues were supplied by Merck-Chimie (Fontenay-sous-Bois, France). Oasis HLB cartridges 129 (6 mL/200 mg) were provided by Waters (Guyancourt, France). Mobile phase for high 130 performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) was 131 prepared with acetonitrile (liquid chromatography - mass spectrometry quality solvent; 132 Merck), formic acid (Carlo-Erba for analysis) and ultrapure water from a Milli-Q system. 133 Seven phthalate monoesters: mono-methyl phthalate (MMP), mono-ethyl phthalate (MEP), 134 mono-iso-butyl phthalate (MiBP), mono-nbutyl phthalate (MnBP), mono-benzyl phthalate 135 (MBzP), mono-n-octyl phthalate (MnOP), MEHP and two oxidized by-products, mono (2-136 ethyl-5-oxohexyl) phthalate (MEOHP) and mono (2-ethyl-5-hydroxyhexyl) phthalate 137 (MEHHP) were purchased from Cambridge Isotope Laboratories, Inc. (UK). A mixture of 138 four surrogates (from Cambridge Isotope Laboratories, purity > 95%), 4-139 methylumbelliferone- ${}^{13}C_{12}$ (4-MU- ${}^{13}C_{12}$), MnBP- ${}^{13}C_{12}$, MEHP- ${}^{13}C_{12}$ and MiNP- ${}^{13}C_{12}$ each 140 one at 25 ng. μ L⁻¹ in acetonitrile (ACN) was prepared and used as 10 μ L per sample. β -141 Glucuronidase from Escherichia coli K12, 200 units.mL⁻¹ was provided by Roche Biomedical 142 (France). Basic buffer (pH of 11) was prepared with NH₄OH/ACN/H₂O (0.5:50:50, v/v/v). 143 Acid buffer (pH of 2.8) was prepared with NaH₂PO₄ (1.93 g for 100 mL) in H₃PO₄ (85 144 %)/H₂O (1:99, v/v). 145

147 2.3 Phthalate monoesters extraction and purification

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

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

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

After deconjugation and centrifugation (4 500 RPM, 10 min), the supernatants were 167 collected into a 15 mL glass tube and 3 mL of ammonium acetate (1 M) were added to the 168 deconjugated sample and homogenized with an Ultra-Turrax, centrifuged and the supernatants 169 collected (step repeated with 2 mL of ammonium acetate (1 M)). The combined supernatants 170 were extracted twice on SPE (solid phase extraction) cartridges (Oasis HLB 6 mL/200 mg) 171 for purification. Prior to the first elution, the cartridge were conditioned with 3 mL of ACN 172 and 6 mL of basic buffer. After loading, the samples were cleaned-up (twice) with 2 mL of 173 174 ammonium acetate (1 M) and eluted with 6 mL of basic buffer, and collected in a 22 mL glass tube with 10 mL of acid buffer. The acidified sample was then purified through a second 175 conditioned SPE cartridge (1 mL of ACN then 1 mL of H₂O and 2 mL of acid buffer) and 176 washed (twice) with 2 mL of acid buffer then 2 mL of H₂O. The analytes were eluted with 10 177 178 mL of ACN then, 10 mL of ethyl acetate. The purified eluate was evaporated under nitrogen stream in a thermostatic bath (65° C), until dry. The residue was resuspended in 0.5 mL of 179 ACN, filtered under centrifugation (6 000 RPM, 5 min) and transferred in vial. This procedure 180

was repeated three times. Finally, the filtrated samples were combined and concentrated under nitrogen stream (65° C) at 500 μ L 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 %.

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2.4 LC-MS/MS analysis

Phthalate monoesters were quantified by liquid chromatography (1200 series - Agilent 187 Technologies, Massy, France) - tandem mass spectrometry (6410-triple quadrupole MS, in 188 negative ion mode for the electrospray source - Agilent Technologies (LC- MS/MS), 189 equipped with a silice upti-prep strategy column (100 A, C18-2, 2x100 mm, 2.2 µm -190 Interchim) heated at 40°C. The source was in ESI negative mode (N2: 350°C; gas flow: 660 191 L/h; capillary: 4000 V) and the injected sample volumes were 10 µL. The LC gradient flow, 192 the MRM parameters for LC-MS/MS and limits of quantification (LOQs) are indicated in the 193 Supplementary Material (Table SM.1, 2, 3 and 5). 194

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2.5 Method validation

197 The analytical method performance was determined by evaluating the linearity, recovery 198 rates, instrument detection limits (IDLs), limits of detection (LODs) and limits of 199 quantification (LOQs). Performance in terms of linearity and dynamic range was checked by 200 injecting reference standard solutions in duplicate. The range tested was 0.3-10 ng on column 201 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).

206 IDLs were determined using the less concentrated surrogate as a signal/noise ratio of 3207 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.

215 *2.6 Histological approach*

Small pieces of liver from 62 eels were fixed with Bouin Hollande's solution, dehydrated with 216 graded series of ethanol, placed in butanol-1 for at least 24 h and then embedded in paraffin 217 wax. Liver sections (6 µm) were stained with nuclear fast red (NR) and picro-indigo-carmin 218 (PIC) (Gabe 1968). Microphotographies were taken with a light microscope (Laborlux S – 219 Leitz wetzlar Germany) equipped with a digital camera (Imagingsource) and analysis were 220 realized with the Archimed software (Version 5.6.0.). Three different liver areas (25 mm²) 221 randomly selected on different sections were analyzed for each eel. Scoring notes for 5 222 223 hepatic parameters: lysis-necrosis, lipidosis, fibrosis, melano-macrophage (MMC) aggregates 224 and nuclear alterations, were established and an individual global score was calculated as the 225 mean value of the different scores (Jaffal et al., 2015). The normal histological state of the eel 226 liver (score 0) corresponding to healthy uncontaminated yellow eels was established 227 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-228 229 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 230 231 fish species (Affandi and Biagianti, 1987; Braunbeck et al., 1990; Costa et al., 2011; Jaffal et 232 al., 2011). The global histopathological score (on 10 grade) was calculated with the formula:

233 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 to234 235 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 236 237 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 = few238 little lipid droplets; 5 = lipid droplets present in 25 to 50% of the hepatocyte; 7.5 = more than 239 50% of the hepatocytes presented large lipid droplets and have a double sized; 10 = all240 hepatocytes were filled with huge lipid droplets and have at least a double size. The fibrosis 241 score was determined by observation of the collagen deposits localization in liver : 0= only 242 big blood vessel (portal section) had collagen deposits, 2.5 = some blood vessel and bile duct 243 presented collagen deposit, 5= the majority of the blood vessel and bile duct presented 244 collagen deposit, 7.5= abnormal large collagen deposits were observable around sinusoids, 245 veins, small bile ducts and some hepatocytes, 10= collagen deposit present throughout all the 246 parenchyma, particularly between hepatocytes. Melano macrophage aggregate (MMA) were 247

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).

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251 *2.7 Statistical analyses*

All statistical tests were performed using R software version 3.2.2 (R Development Core 252 Team 2015). Data were log-transformed to make the data fit the normal distribution. 253 Significant differences among groups (i.e. sampling periods or sampling sites) were evaluated 254 by ANOVA (or Kruskall-Wallis tests for non-parametric data), followed by post hoc Tukey 255 test (or Bonferroni tests for non-parametric data) if ANOVA indicated a significant difference 256 between groups. Correlations between phthalate metabolites and individual parameters (age, 257 body condition, body length, score of histological abnormalities in liver) were expressed by 258 Pearson (or Spearman for non-parametric data) correlation coefficient. 259

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261 **3. Results**

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3.1. Method performance

264 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 265 stages, making the instrument significantly more selective and sensitive than a single 266 quadrupole system. As a result, compounds can be easily identified and quantified in the 267 presence of complex matrices using MRM. Quantification of phthalate metabolites was 268 carried out by calculating the response factor for each compound relative to the corresponding 269 surrogate and concentrations were obtained using a linear regression analysis of the peak area 270 271 versus the concentration ratio.

The results for linearity were satisfactory with an R² ranging from 0.997 to 0.999 272 (Table SM.3, Supplementary Material). Recoveries for the overall extraction procedure 273 displayed satisfactory results (74% - 102%) for MiBP, MEHHP, MnBP, MEOHP, average 274 score for MEHP and MBzP (46% - 61%) and poor results (12% - 32%) for MMP, MEP, 275 276 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. 277 IDLs ranged from 0.3 to 6 pg (Table SM.2, Supplementary Material). The nine phthalate 278 metabolites were detected in the 8 blanks (Table SM.5, Supplementary Material) and were 279 consistent with compound determinations. The LODs and LOQs were compatible with fish 280

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

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284 *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).

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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).

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300 *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.

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306 *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).

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317 **4. Discussion**

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4.1. Muscular levels of phthalate metabolites

The detection of phthalate metabolites in eel muscle suggested that eels were directly exposed 320 to phthalate metabolites from the water or the sediment, since phthalate parents can be 321 transformed by microorganisms, or that eels were exposed to phthalate parents and were able 322 to incorporate and metabolize them. MEHP, MiBP and MnBP were the major phthalate 323 metabolites. Results obtained in this study and using a new method are consistent with 324 previous studies that reported similar muscle contents of phthalate metabolites in fish 325 collected in an urbanized marine inlet, in Vancouver: the juvenile Shiner Perch Cymatogaster 326 aggregata (geometric mean ranged from 0.54 ng.g⁻¹ ww. for MBzP to 82 ng.g⁻¹ ww. for 327 MnBP, McConnell, 2007) and the white spotted greenling Hexogrammos stelleri (0.24-1.1 328 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 329 fish from the Yangtze River Delta sea area (China, 1.2 to 20.4 ng.g⁻¹ ww, Gu et al., 2014). It 330 should be noted that much higher levels of phthalates metabolites were found in liver samples 331 (mean ranged from 7.1 \pm 2.6 (MEOHP) to 1607 \pm 2706 (MiBP) ng.g⁻¹ dw) of roaches fished 332 in the Seine river, France (Valton et al., 2014), which is coherent with the metabolization of 333 phthalates in liver. 334

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4.2. Seasonal and spatial variations

Lagoons are known to be characterized by great spatial and seasonal variabilities in 338 environmental conditions (e.g. temperature, Viaroli et al., 2007). In the present study no 339 significant differences of phthalate metabolite levels were observed among the three sites. 340 341 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. 342 The levels of PCBs and PBDEs were also relatively low in the sediment and eels muscles 343 from these two lagoons (Labadie et al., 2010; Vouvé et al., 2014). Analyses of phthalate 344 concentrations in water and sediments should be necessary to confirm this lack of spatial 345 variation. 346

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

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355 *4.3. Age, length, body condition*

The large sample size of eel muscles (N = 117) made it possible to explore correlations 356 357 between phthalate metabolites' levels and individual parameters. Age was not correlated to phthalate metabolites' levels, except for MnBP, whose levels decreased with age. Similarly 358 359 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 360 361 results suggest that eels did not accumulate phthalates during growth, contrary to persistent compounds. For instance, levels of polychlorobiphenyls (PCBs) and polybrominated 362 363 diphenyl-ethers (PBDEs) increased with length and age from glass eels to silver eels (Tapie et al., 2011). Similarly, PCBs, hexachlorocyclohexanes (HCHs), hexachlorobenzene (HCB), 364 dichlorodiphenyltrichloroethane (DDT) and dichlorodiphenyldichloroethylene (DDE) 365 accumulated with age in the muscle of brown trout (Salmo trutta, Vives et al., 2005). It should 366 also be noted that range of age and length were limited in our data set, which may have 367 restricted patterns of accumulation or dilution with growth. At last, eels could have been 368 exposed to phthalate intermittently throughout their lives. 369

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.

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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 381 changes observed here. In yellow eel, this change is symptomatic of a metabolic dysfunction 382 since their liver does not usually store lipids. Lipid droplets may sequester fat-soluble 383 pollutants and may reduce the metabolic capacity of that tissue. Moderate lipidosis is 384 commonly regarded as an unspecific alteration with multiple potential causes and lipoid 385 degeneration indicates a severe alteration of fish health (Costa et al., 2011). Lipoid 386 degeneration is linked to an high rate of hepatic alteration and fatty liver probably take place 387 with impairment of their function (Vandemiale et al., 2001). 388

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.

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395 Conclusion

Nine phthalate metabolites were characterized in muscle tissue of 117 European eels. No 396 397 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 398 not explain individual variations in muscular levels of phthalate metabolites suggesting that 399 eels did not accumulate phthalates during growth, contrary to persistent compounds.. 400 Individuals with numerous histological abnormalities in liver bore lower levels of phthalates 401 metabolites, suggesting that they were less efficient to metabolize xenobiotics. Further 402 ecotoxicological research should be conducted to improve understanding of the links between 403 fish physiology and ecology and the metabolization of phthalates. 404

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- 409

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- 515

Table 1. Mean \pm 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).

519

	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%

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521

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).

524

	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 ***

525

526

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.

531

	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 ***</mar </jun 					Oct <jun *</jun 	Mar <jun * Oct<jun ***</jun </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).

536

	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 *

537

539 Figure legend

540

Figure 1: Distribution of phthalate metabolites content (log-transformed) in muscle (ng.g⁻¹ dw) in 117 eels specimens.

543

Figure 2. Seasonal distribution of phthalate metabolites content (log-transformed) in 117 muscle samples (ng.g⁻¹ dw).

546

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

555

556 Figure 4. Correlations between the sum of all the phthalate metabolites (MPA) content in

muscle (log-transformed, $ng.g^{-1} dw$) and the individuals' histo-pathological score.









567 Figure 3.









