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Chasing phthalates in tissues of marine turtles from the Mediterranean sea

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ABSTRACT

Tissues from thirteen specimens of marine turtles, one *Dermochelys coriacea* and twelve *Caretta caretta*, found dead along the Sicilian coasts in 2016 were analyzed for the presence of phthalates. Four phthalates (DEP, DBP, BBP, and DEHP) were found at different significant concentrations in liver and gonads, while only DBP was found in muscle tissues and at a fourfold lower concentration than other phthalates in *Dermochelys coriacea*. No traces of DEP were detected in *C. caretta* tissues where DOTP was also revealed. The presence of phthalates in fat tissue in specimens of *C. caretta* showed a major prevalence of the most lipophilic phthalates DEHP and DOTP. The total concentration of all analyzed phthalates, showed high values in all tissues. Results suggested that for monitoring purposes from live specimens sample collection should be addressed to fat tissue with accurate manipulations.

1. Introduction

The impact of macroplastics, i.e. plastics of larger size than five millimeters, on the marine environment has been thoroughly studied in the last three decades, mostly focusing on their physical effect on the environment (Ryan et al., 2009; Gregory, 2009). More recently, due to the increased presence of microplastics deriving from the disaggregation or partial decomposition of macroplastics and whose size can range from five millimeters down to nanometers, global concern is raising about the impact of such “invisible plastics” on marine organisms (Andrady, 2011; Cole et al., 2011).

Direct ingestion is generally the most common way for plastics to penetrate in a living organism: this is the case, for example, of plastic bags that could be mistaken for jellyfishes by sea turtles (Caracappa et al., 2017) or for squids by sperm whales (Stamper et al., 2006; Mrosovsky et al., 2009; Schuyler et al., 2014; Poli et al., 2015). Moreover, the accidental ingestion of plastics is also a cause of death for fishes (Sazima et al., 2002) and marine birds (Ryan et al., 2009).

While plastics have been considered as biochemically inert materials due to their size (Avio et al., 2016), microplastics can release chemical substances with molecular weight smaller than a thousand Dalton able to penetrate cell membranes (Roy et al., 2011; Teuten et al., 2009). These substances, are, for example, phthalates and bisphenol A that have been detected in marine water and organisms (Rudel et al., 2003), thus increasing awareness about negative effects on wildlife and humans. (Meeker et al., 2009; Oehlmann et al., 2009; Thompson et al., 2009).

Phthalates are phthalic acid esters, colorless substances usually soluble in hydrophobic matrices and scarcely soluble in water. They are widely used in plastics industry as plasticizers, principally for the production of PVC, but they can also be found in a variety of products such as glues and adhesives, mural paintings (Barreca et al., 2014) as well as in electronics, toys, packaging and personal care products (Chan and Shuang, 2012). Not being covalently bound to, but simply mixed with the plastic polymer, phthalate plasticizers can be released in the environment especially when plastics products are degraded to debris and microplastics. Despite phthalates exposure is a threat for the health of mammals and other classes of animals, human ingestion of phthalates is low. Nevertheless, the US Environmental Protection Agency (US EPA) has listed phthalates among endocrine disruptors and inhibitors of male fertility (Sparling, 2016).

According to Fossi's studies, the presence of phthalates in the common fin whale (*Balaenoptera physalus*) in the Mediterranean Sea suggests that phthalates can be used as tracers for microplastic intake and that this approach can also be used in other marine organisms (Fossi et al., 2012). Moreover, the Marine Strategy Framework Directive (MSFD) remarks the importance of monitoring the presence of plastics and microplastics in the sea as indicator to improve the knowledge of the qualitative descriptor n.10 (Marine Litter). This descriptor is used to evaluate whether the Good Environmental Status

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(GES) is being achieved (Galgani et al., 2013). Therefore, sea turtles and marine mammals can be considered as appropriate sentinel species to study how marine litter can interact with marine animals (Fossi and Panti, 2017). The three most common sea turtles species presents in the Mediterranean Sea are listed by IUCN as vulnerable or endangered species also due to the negative impact of accidental ingestion of plastics (bags, fragments, fishing lines, etc.) (Seminoff and Shanker, 2008; Dobbs, 2001). Considering that risks of plastic ingestion are higher in marine environment, several studies have been conducted in this field also regarding sea turtles (Nelms et al., 2015; Schuyler et al., 2016; Tomás et al., 2002; Deudero and Alomar, 2015). However, these studies concerned only the quali-quantitative determination of plastics in living organisms (Meeker et al., 2009; Oehlmann et al., 2009; Talsness et al., 2009; Wagner and Oehlmann, 2009) while, to the best of our knowledge, there are no reports about the presence of phthalates in marine turtles tissues.

This study reports for the first time the determination of phthalate levels in different organs and tissues of sea turtles from the Mediterranean Sea found stranded along the coasts of Sicily and highlights the potential for in vivo monitoring of phthalates level as an indicator of both animal and marine environment conditions.

In particular, this study focused on the monitoring of six phthalates that are most commonly used as additives: dimethyl phthalate (DMP), diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), butyl benzyl phthalate (BBP), bis(2-ethylhexyl) phthalate (DEHP), and di-*n*-octyl phthalate (DOTP).

2. Materials and methods

2.1. Sample collection

All tools and glassware used for sample collection have been preliminary washed with acetonitrile (from the same batch used for phthalates extraction) to avoid sample contamination from external phthalate source during every stage of the analytical procedure, including sampling and sample preparation (extraction, cleanup, and concentration).

Samples of muscle, liver, gonads, and fat tissues were collected during necroscopic analyses performed at the Istituto Zooprofilattico Sperimentale della Sicilia “A. Mirri”: Centro di Referenza Nazionale sul Benessere, Monitoraggio e Diagnostica delle Malattie delle Tartarughe Marine (National Reference Center on Well-being, Monitoring and Diagnosis of Marine Tortoise Diseases).

A total of thirteen turtles found dead stranded in 2016 along the Sicilian coasts were analyzed: a 134 cm of curve carapace length (CCL) of *Dermochelys coriacea* (see for more details Caracappa et al., 2017) and twelve *Caretta caretta* with size ranging between 25 and 64 cm of CCL.

Dead turtles have been dissected in order to extract organs and tissues to be analyzed. However, we could not collect the same organs from all the turtles due to their state of conservation. In fact, the different state of decomposition of the internal organs of the turtles did not allow us to sample all four tissues from all thirteen animals and consequently to do the analysis of phthalates for the entire tissue-by-animal matrix. In particular, we sampled and analyzed seven gonads samples, eight liver samples and three fat samples all coming from different turtles from which only a few samples could be obtained, because of the different state of preservation/conservation of each tissues. Additionally, the uptake of muscle tissues was affected by animal size and we were able to collect only seven samples from *D. coriacea* and six *C. caretta* of 38–64 cm CCL size range. Each turtle has been labelled by its size expressed in cm. An apex index was added to the labelling to differentiate animals of the same size (e.g. 37 cm and 37 cm in Fig. 3).

2.2. Samples preparation, extraction, and phthalate analysis

The procedure for phthalate extraction was adapted from the literature (Chan and Shuang, 2012; Wenzl, 2009).

Typically, a 500 mg tissue sample was homogenized and apportioned into two samples (200 mg each) before extraction. The extraction of phthalates was performed by adding 10 mL of acetonitrile (for LC-MS grade) to each homogenized sample (200 mg) in a glass vial and by sonication of the resulting mixture for 20 min at room temperature. The extraction mixture was then centrifuged for 15 min at 3500 rpm. A portion of the extract supernatant (5 mL) was added to a volumetric flask and diluted to 10 mL before analyses, which were run in triplicates. A standard mixture of six commercial phthalates in hexane (EPA Phthalate Esters Mix), containing dimethyl phthalate (DMP), diethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), butyl benzyl phthalate (BBP), bis(2-ethylhexyl) phthalate (DEHP), and di-n-octyl phthalate (DOTP), was used as reference for calibration in the range 10–0.0001 ppm each. Analyses were performed on an HPLC-ESI-QTOF Agilent 6540 operating in the positive ion monitoring mode and injecting 10 μL of sample in a Zorbax Extend C-18 2.1 × 50 mm 1.8 μ column using a mixture of water and acetonitrile as eluents with a fixed flow of 0.7 mL/min with the following gradient: from Water/ACN 80/20 (vol/vol) to ACN (100%) in 10 min, and maintaining elution with 100% ACN for further 4 min before returning to initial conditions. Phthalate esters were monitored as protonated (M + H, at m/z = MW + 1) and sodiated molecular adducts (M + Na, at m/z = MW + 23) under single ion monitoring conditions. Under the used conditions, higher formation of protonated adducts was recorded and values for quantitation are referred to [M + H] species. The following retention times (min) were recorded: DMP 2.15; DEP: 4.05; DBP: 6.90; BBP: 6.75; DEHP: 10.40; DOTP: 10.60. The whole analytical procedure (sampling and analysis) was validated by simulation of a real sampling situation. The linearity was measured in the concentration range from 0.1 ppb to 10 ppm. The RSDs on three replicates are below 10%. LOD and LOQ were quantified by IUPAC method and range from 0.1 ppb to 1.0 ppb.

In order to avoid any cross contamination from subsequent samples, besides the washing segment present in each run, a pure ACN analysis was performed in between two sample analyses. Quantitative determination of phthalate presence in samples was determined by subtracting chromatogram values referred to pure ACN from those referred to sample extracts. Values were then reported as nanograms of phthalates per gram of tissue sample and data are illustrated in Figs. 1–4 only for phthalates detected in the samples. Recovery efficiencies were checked by analyzing uncontaminated samples (see below) spiked with a known quantity of phthalate standards. Uncontaminated samples of gonads, liver, muscle, and fat, were obtained by preliminary extraction for three times with acetonitrile. For all determined analytes, average recoveries ranged from 70% to 108%. The relative standard deviations on the phthalates measurements of recovery were less than 12%. Relative differences for triplicate samples were less than 15%.

3. Results and discussion

In *Dermochelys coriacea*, concentration of phthalate expressed in nanograms of phthalate per gram (ng/g) of sampled tissue (gonads, liver, and muscle) is shown in Fig. 1, where phthalates absent in all the samples, i.e. DOTP and DMP, have been omitted. Four phthalates (DEP, DBP, BBP, and DEHP) were found at different concentrations in liver and gonads, whereas in muscle tissues only DBP was found at much lower values than the others.

The lower amount of phthalates found in *D. coriacea* muscle, induced us to investigate the level of phthalates distribution in muscle tissues available from other turtles to verify if muscle could be an appropriate tissue to assess the animal exposure to phthalates sources. The analysis of muscle tissues from six specimens of *Caretta caretta* with a size range of 38–64 cm CCL evidenced the presence of only one of the