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.

Keywords
Caretta caretta, Dermochelys coriacea, Microplastics, Phthalates, Mediterranean Sea

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 (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 centimeters 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 centimeters 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 Figure 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 minutes 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 2.1x50 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 figures 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 Figure 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.

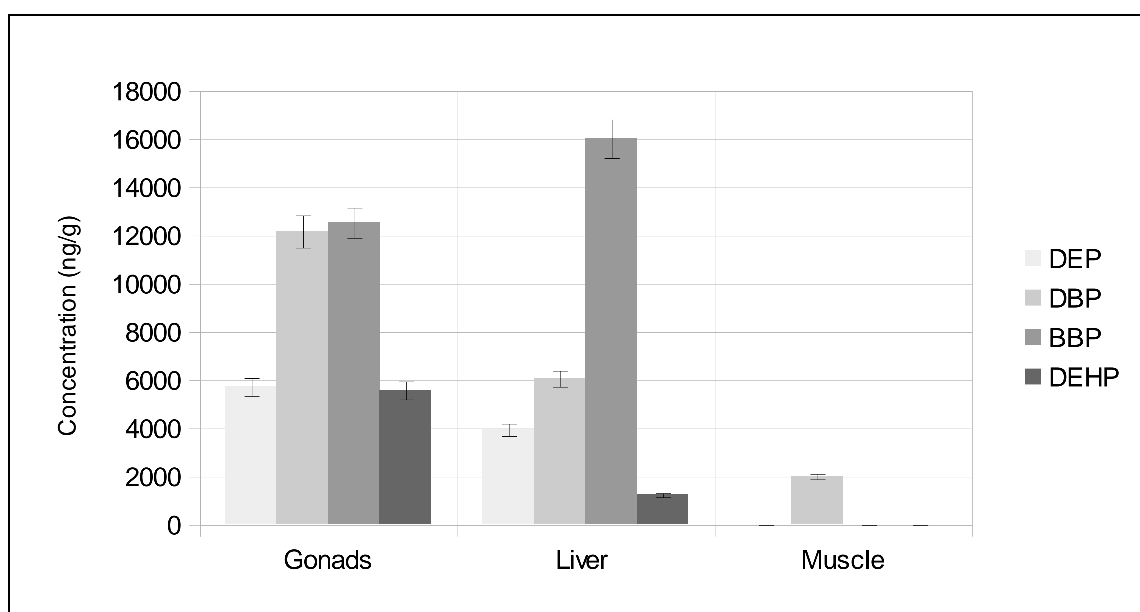

Figure 1. Phthalates concentration in tissues in one specimen of *Dermochelys coriacea.*

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 monitored phthalates. Indeed, DBP was found in muscular tissues only in samples taken from medium size specimens (56-64 cm CCL) and with a concentration between 1250-3200 ng/g. On the other hand, the analyses of seven gonads (Figure 2) and eight liver samples (Figure 3), taken from eleven specimens of *C. caretta*, showed the presence of four phthalates (DBP, BBP, DEHP, and DOTP), with a different distribution with respect to *D. coriacea* where a prevalence of BBP was found. In fact, for *C. caretta* specimens, a prevalence of DBP (2600-19000 ng/g) was found in both organs, with high quantities of BBP (700-9100 ng/g) registered only in liver tissues, while DEP was absent in all samples.

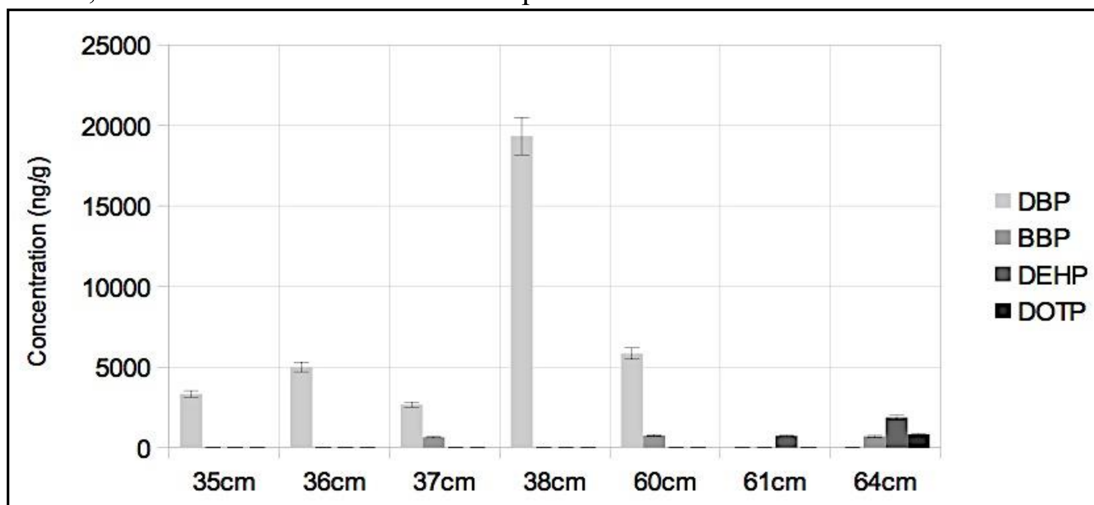

Figure 2. Phthalates concentration in gonad tissues of seven specimens of *Caretta caretta* of various sizes. Each size class corresponds to an individual.

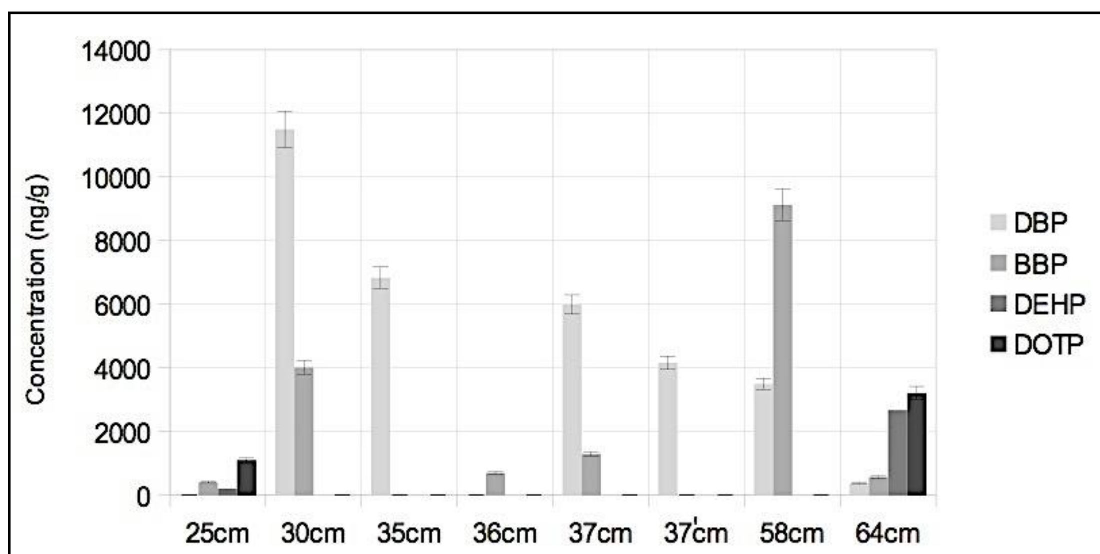

Figure 3. Phthalates concentration in liver tissues of eight specimens of *Caretta caretta* of various sizes (CCL). Each size class corresponds to an individual.

Unfortunately, due to preservation issues (see above), only three fat tissues were available from *C. caretta* specimens. In these samples (Figure 4), a major prevalence of the most lipophilic phthalates DEHP and DOTP was registered at higher level than DBP, which was the most frequent phthalate in the other organs.

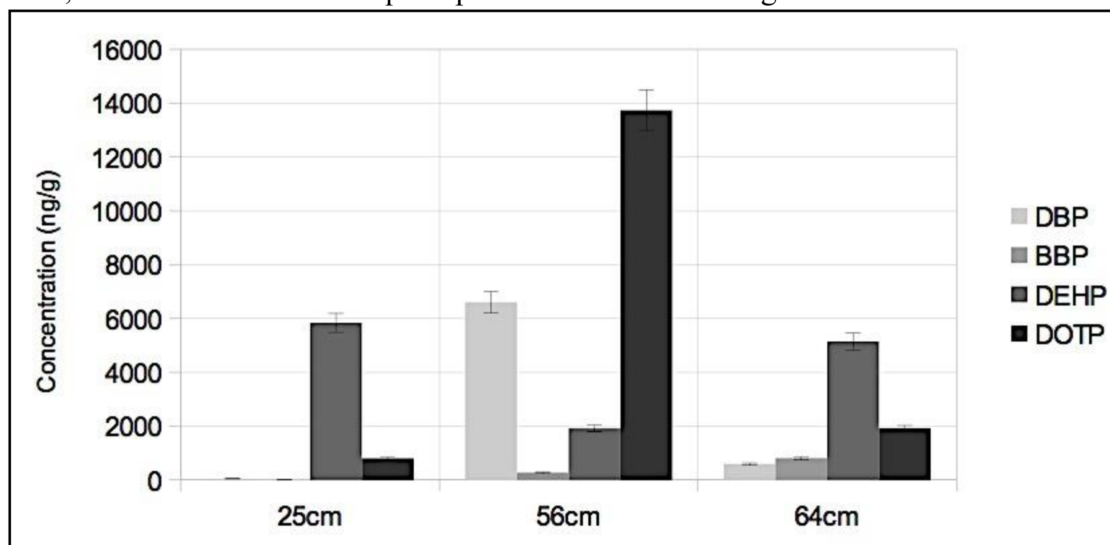

Figure 4. Phthalates concentration in fat tissues of three specimens of *Caretta caretta* of various sizes (CCL). Each size class corresponds to an individual.

All phthalates found in this study are used as additives in a wide variety of consumer products, such as plastics, detergents, cosmetics, and fragrances (Hubinger, 2010). However, several studies report different phthalate profiles composition for many commercial products. For instance, DEHP is mostly used as plasticizer in PVC (Strauss, 2004), while DBP is used primarily as plasticizer to add flexibility to plastics (Manikkam et al., 2013).
Since in samples from *C. caretta*, DBP was the most abundant phthalate found, it is reasonable to suppose that phthalate detected in *C. caretta* came from plastic materials either by direct ingestion of plastics or by exposure to phthalates released in the environment during plastics degradation.
The monitoring of exposure to DBP is crucial for animal health since experiments in rats (Swan, 2008) and wrinkled frogs (Ohtani et al., 2000) have confirmed the toxicological action of DBP as environmental endocrine disruptors, even at low exposures. Indeed, in a qualitative study, phthalates and other chemical pollutants were found in the lipid contents of egg yolk olive ridley (*Lepidochelys olivacea*) and leatherback (*Dermochelys coriacea*), highlighting the transfer from the mother to the offspring through vitellogenesis (Cerón et al., 2000). Therefore, considering that *C. caretta* reaches sexual maturity at about 20-25 years, one might expect that the concentration of phthalates in the tissues could increase in time, thus interfering with the reproductive processes including embryonic development. . In fact, phthalates are liposoluble molecules that in animals tend to accumulate in fatty tissues with great difficulty in eliminating them quickly. Indeed, phthalates level found in fat tissues from *C. caretta* were comparable with levels found in fat tissue of the Mediterranean

fin whale (Fossi et al., 2012) suggesting that also sea turtles are particularly exposed to plastic ingestion, despite the different form of foraging of the two species.
However, at a first sight, our data did not show any correlation between turtle size and phthalate concentrations, demonstrating that size, probably, is not a good parameter to evaluate this relationship. This could be ascribed to the uncertainty regarding the specific marine area where the animal might have spent most of its lifetime as well as to the lack of further information on the animal life (i.e. ingestion and permanence in the gastrointestinal tract of micro and macroplastics).
These aspects have been considered also in view of recent studies conducted in the Mediterranean Sea on the presence of plastics and microplastics and their potential impact on the marine organisms (de Lucia et al., 2014; Fossi et al., 2014, 2017; Lazar and Gracan 2011; Guerranti et al., 2017; Campani et al., 2013).
As for the amount of total phthalates, the concentrations found in this work in *D. coriacea* gonads (36 ppm) and in *C. caretta* fat (22 ppm) were higher than values recently reported for other marine animals such as the *Rutilus rutilus* fish (Valton et al., 2014), as well as terrestrial species such as broiler chicks (Jarosova et al., 2009).

Conclusions

Ingestion of plastics and microplastics by marine organisms may have physical and toxicological noxious effects creating a serious threat to marine species. Therefore, there is a need to develop methods to evaluate the plastic exposure in free-ranging marine wildlife particularly exposed to microplastic ingestion. Reported results showed how the monitoring of phthalates concentration in sea turtles tissues could be chosen as benchmark for exposure to plastics in the marine environment. However, relying only on the fortuitous recovery and collection of dead animals may limit the correlation between analyzed data and other environmental parameters. On the other hand, *in vivo* collection of gonads and liver samples is not feasible for monitoring purposes and muscles tissues have not shown suitable levels of phthalates to suggest their use as typical tissue to be monitored. Conversely, sample collection should be addressed to fat tissue where phthalates are usually more easily found. Thus, by using a mini invasive technique it will possible to withdraw a small amount (500 mg) of fat tissue from the axillary or inguinal area without stressing the animal.
In conclusion, despite the lack of a complete tissue-by-animal data matrix, this work represents the first quantitative study determining the distribution of phthalates in four different tissues in turtles. Consequently, a comparison between our data with literature can be reasonably made only based on type of analyzed tissues or total phthalate concentration.
Our work revealed that in *C. caretta* the most frequent phthalate was DBP followed by DEHP, while a significant amount of BBP was found in *D. coriacea*. Additionally, found phthalates were distributed differently in the four type of analyzed tissues with low concentrations of DBP found in muscles and a prevalence of the most lipophilic phthalates, DEHP and DOTP, in fat tissues. Phthalates levels found in fat tissues of *C. caretta* are comparable with those found in fat tissue of larger marine mammals. The high values of total phthalates found in this work remark the need for adopting a common plastic waste management policy among all Mediterranean countries.
As a final comment, the different phthalates distribution among the tissues opens the way to the interpretation of the impact that metabolic pathways of these substances may have in marine organisms. Therefore, a continuous monitoring of sea turtles as *living probes* for environmental assessment would be a promising approach from both points of view of animal health and marine condition evaluation.

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