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Rifiuti marini plastici ed i loro additivi: valutazione *in vitro* della citotossicità e genotossicità degli ftalati su linee cellulari di spigola e tursiope

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Plastic marine debris and their additives: *in vitro* assessment of phthalates cytotoxicity and genotoxicity on European sea bass and bottlenose dolphin cell lines

s.s.d. BIO/07

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Riassunto

L'inquinamento marino causato dai rifiuti antropogenici è diventato un serio problema ambientale a livello mondiale, con molteplici conseguenze ecologiche. Circa 6,4 milioni di tonnellate di rifiuti entrano ogni anno negli oceani, rendendo così non trascurabili i loro effetti sull'ambiente marino. I rifiuti marini sono ampiamente distribuiti nell'ambiente marino ed i detriti plastici rappresentano la maggior parte dei rifiuti in mare (60 - 90%), diventando una fonte importante di inquinanti. Tra questi, il di (2-ethylhexyl) ftalato (DEHP) è l'additivo delle plastiche più utilizzato, risultando essere un contaminante potenzialmente onnipresente in mare. Il DEHP e uno dei suoi principali metaboliti, il mono (2-etilesil) ftalato (MEHP), sono stati ampiamente studiati negli ultimi decenni, dimostrando la loro influenza sui processi biochimici sia nell'uomo che nella fauna selvatica. Tuttavia, gli studi sui loro effetti tossicologici sugli organismi marini sono ancora scarsi, nonostante tali studi possano rivestire un ruolo fondamentale per sollecitare l'avvio dei provvedimenti necessari per fronteggiare questa emergenza ambientale. In questo progetto di dottorato, abbiamo studiato gli effetti citotossici, genotossici e mutageni del DEHP e dell'MEHP, applicando specifici test *in vitro,* in due linee cellulari di specie costiere di elevato valore ecologico ed economico, utilizzando una linea cellulate embrionale di spigola (DLEC) ed una di cute di tursiope (TT); quest'ultima è stata valutata a confronto con la linea cellulare standardizzata di ovaio di criceto cinese (CHO).

I risultati del DEHP sulle DLEC hanno mostrato una significativa diminuzione della vitalità cellulare a partire da 0,01 mM dopo 24 ore di trattamento, insieme ad un significativo aumento di apoptosi e necrosi, a cambiamenti morfologici delle cellule ed a un aumentato distacco cellulare. Il trattamento con MEHP ha mostrato un leggero effetto negativo sulla vitalità delle DLEC ma nessun effetto sull'induzione di apoptosi e necrosi a tutte le concentrazioni testate. La maggiore tossicità del DEHP potrebbe essere attribuita ad un effetto sinergico sia del DEHP che dei suoi metaboliti, che vengono prodotti dal metabolismo cellulare a partire da quest'ultimo. Inoltre, il trattamento con DEHP ha portato ad un moderato aumento delle lesioni al DNA a partire da 0,02 mM, mentre l'MEHP non ha incrementato frammentazione del DNA a nessuna delle concentrazioni testate. Tuttavia, sia il DEHP che l'MEHP hanno causato un aumento dose-dipendente della frequenza dei micronuclei, considerati marcatori di danno permanente al DNA, producendo la metà dei micronuclei indotti dall'MEHP, rispetto al DEHP, alla stessa concentrazione (10 μM). Questi risultati suggeriscono un possibile effetto aneugenico di questi composti sulla linea cellulare DLEC, probabilmente associato principalmente all'azione dell'MEHP. I nostri risultati dimostrano che l'esposizione *in vitro* al DEHP ha effetti citotossici e genotossici dose-dipendenti nella linea cellulare DLEC, mentre l'MEHP risulta più pericoloso del suo precursore, poiché induce un'instabilità genomica nella linea cellulare DLEC senza innescare la morte cellulare.

I risultati sulle linee di mammifero mostrano una riduzione dose-dipendente nella vitalità delle cellule di TT dopo 24 ore di trattamento a partire dalle dosi basse/intermedie (0.02 e 0.1 mM di DEHP) e un leggero e non significativo aumento delle cellule necrotiche, fatta eccezione che per la dose di 5 mM, mentre nelle CHO è stata osservata una significativa diminuzione della vitalità cellulare, insieme ad un significativo aumento delle cellule necrotiche, a tutte le dosi di DEHP. Sebbene il trattamento con DEHP di entrambe le linee non abbia incrementato la frammentazione del DNA a nessuna delle concentrazioni testate, è stato osservato un aumento dose-dipendente della frequenza dei micronuclei, accompagnato da una progressiva diminuzione della proliferazione cellulare. Questi dati supportano ulteriormente l'ipotesi che il DEHP potrebbe avere un effetto aneugenico, probabilmente dovuto alla produzione dei suoi metaboliti, in particolare dell'MEHP.
Inoltre, il confronto di questi ultimi risultati con quelli ottenuti nello studio sulle cellule embrionali di spigola europea, suggerisce un ordine di tossicità del DEHP come segue: DLEC > CHO > TT.
In conclusione, i risultati dell'intero lavoro sottolineano l'importanza di approfondire lo studio degli effetti citotossici e genotossici degli ftalati sui sistemi *in vitro*, soprattutto per le specie protette, e di intraprendere ulteriori indagini sui loro effetti nei sistemi cellulari *in vivo* e/o *ex vivo* di organismi marini.

Abstract

Marine pollution from anthropogenic litter has become a serious worldwide environmental concern, resulting in multiple ecological consequences. Around 6.4 million tonnes of litter enter the oceans each year, thus rendering not negligible litter impacts on the marine environment. Marine litter is extensively distributed in the marine environment, and plastic debris accounts for the majority of litter items in the sea (60 – 90 %), becoming a major source of pollutants. Among them, Di(2-ethylhexyl)-phthalate (DEHP) is the most abundantly used plastic additive, resulting to be a potential ubiquitous contaminant to the marine environment. DEHP and one of its primary metabolites, mono(2-ethylhexyl) phthalate (MEHP), have been extensively studied in the last decades, demonstrating their influence in biochemical processes both in humans and wildlife. However, studies on its toxicological effects on marine organisms are still scarce, despite these studies could play a key role to solicit the development of necessary measures to face this environmental emergency. In this PhD project, we studied the cytotoxic, genotoxic, and mutagenic effects of DEHP and MEHP, by applying specific *in vitro* tests, in two coastal species of ecological and economic value: the European sea bass embryonic cell line (DLEC) and bottlenose dolphin skin cell line (TT); the latter was evaluated in comparison to the standardized Chinese Hamster Ovary cell line (CHO).

DEHP results on DLEC showed a significant decrease in cell viability starting at 0.01 mM after 24 h together with a significant increase in apoptosis and necrosis, morphological changes and cell detachment. MEHP displayed a slight effect on DLEC viability but no effect in the induction of apoptosis and necrosis at all tested concentrations. The higher toxicity of DEHP could be attributed to a synergic effect of both DEHP and its metabolites, which are produced by the cellular metabolism of the DEHP. Furthermore, DEHP caused a moderate increase in DNA strand breaks from 0.02 mM, whereas MEHP did not enhance DNA fragmentation at the tested concentrations. Yet, both DEHP and MEHP caused a dose-dependent increase of micronucleus frequency, which is considered a marker of permanent DNA damage, displaying half of the micronuclei induced by MEHP, with respect to DEHP, at the same concentration (10 µM). These outcomes suggested a possible aneugenic effect of these compounds on DLEC cell line, probably mainly ascribed to MEHP. Our results demonstrate that *in vitro* exposure to DEHP had a dose-dependent cytotoxic and genotoxic effects in DLEC cell line, whereas MEHP resulted more dangerous than its precursor because it induces genomic instability in the DLEC cell line without triggering cell death.

Results on the mammalian cell lines show a dose-dependent reduction in the viability of TT after 24 hours of treatment, starting from low/intermediate doses (0.02 and 0.1 mM of DEHP), and a slight but not significant increase in necrotic cells, except for the 5 mM dose. Instead in CHO a significant decrease in cell viability was observed, along with a significant increase in necrotic cells, at all tested doses of DEHP. Although DEHP treatment of both lines did not increase DNA fragmentation at any of the concentrations tested, a dose-dependent increase in the frequency of micronucleus was observed, accompanied by a progressive decrease in cell proliferation. These data further support the hypothesis that DEHP could have an aneugenic effect, probably due to the production of its metabolites, in particular MEHP.

Furthermore, comparing the current results with those obtained in the study on the European sea bass embryonic cell line, the degree of DEHP toxicity on the tree cell lines resulted in this decreasing order: DLEC > CHO > TT.

In conclusion, the results of the whole work underline the importance of deepening the study of the cytotoxic and genotoxic effects of phthalates on *in vitro* systems, especially for protected species, and to undertake further investigations on their effects in *in vivo* and/or *ex vivo* cellular systems of marine organisms.

1. Introduction

Marine pollution from anthropogenic litter has become a serious worldwide environmental concern, resulting in multiple ecological consequences (Rochman et al., 2013). Marine litter is defined as "any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment" (UNEP, 2009). Litter enters the marine environment both from sea-based sources, like shipping and fishery, and from land-based sources, like rivers run-off and coastal anthropogenic activities (UNEP, 2009; Rech et al., 2014). It has been estimated that 6.4 million tonnes of litter enters the oceans each year (UNEP, 2009; Pham et al., 2014), thus rendering not negligible litter impacts on the marine environment (Pham et al., 2014). Furthermore, litter can be accidentally ingested by a wide variety of marine organisms. Accidental ingestion, together with entanglement, is considered one of the primary forms of direct damage to wildlife caused by marine litter (UNEP, 2009; Galgani et al., 2013; Bergmann et al., 2015).

Many studies have shown that plastic debris accounts for the majority of litter items in the sea (60 – 90 %) and around 80 - 95% of litter stranded on beaches and shorelines (Galgani et al., 2015), with an estimation of a continue increment in the global annual production of plastic (PlasticsEurope, 2017). These debris, after entering the marine environment, degrade to tiny particles due to mechanical, chemical and biological processes (Lambert and Wagner, 2016). When these synthetic polymer reach dimensions smaller than 5 mm, commonly called microplastics, they become particularly dangerous and persistent (Collignon et al., 2012; Galgani et al., 2013). Several studies have revealed that microplastics are often accidentally ingested by marine organisms, causing them harm because of their physical and chemical properties (Deudero and Alomar, 2015) and consequently propagating over higher trophic levels of the marine food webs (Farrell and Nelson, 2013; Setälä et al., 2014). Furthermore, plastic particles effectively collect on their surface organic pollutants from surrounding water, thus becoming even more toxic (Andrady, 2011).

Plastic is composed by a wide range of hydrocarbon polymers, often in combination with additives (e.g. bisphenol A and phthalates), which can leak and leach from the polymers into the marine environment (Teuten et al., 2009). Phthalates, which are phthalic acid esters, are widely used in many plastic products to increase their plasticity and, due to their susceptibility to leach into the environment, have the potential to enter the food chain and be transported through the trophic levels (Andrady, 2011; Bergmann et al., 2015). Among phthalates, di-(2-ethylhexyl) phthalate (DEHP) is the most extensively used plasticizer, resulting to be a ubiquitous contaminant to the environment to which marine organisms, and potentially humans, are exposed through multiple routes (Caldwell, 2012; Rowdhwal and Chen, 2018). DEHP and one of its primary metabolites, mono(2-ethylhexyl) phthalate (MEHP), have been extensively studied in the last decades, demonstrating their influence

on multiple biochemical processes, both *in vitro* and *in vivo*, in humans and animals (Caldwell, 2012; Mankidy et al., 2013; Maradonna et al., 2013; Ye et al., 2014; Rowdhwal and Chen, 2018). Despite the many studies dealing with the presence and the harmful effects of DEHP and MEHP, there are very few information about their effects on marine organisms, especially on marine fishes and marine mammals (Fossi et al., 2012; Ye et al., 2014).

In order to coordinate marine environment protection, the European Commission has developed the Marine Strategy Framework Directive 2008/56/EC (MSFD), with the objective to achieve or maintain a Good Environmental Status (GES) for the marine environment by 2020. In Annex I of MSFD, among the 11 descriptors, the Descriptor 8, identified as "Concentrations of contaminants are at levels not giving rise to pollution effects", is based upon monitoring programmes covering the concentrations of chemical contaminants and also biological measurements relating to the effects of pollutants on marine organisms (Law et al., 2010). To achieve Descriptor 8 objectives, the biological effects of xenobiotics should be evaluated taking into account environmental target levels, expressed as concentrations of contaminants or levels of biological response, for which significant harm to the organisms concerned has been assessed. The final aim of Descriptor 8 is to prevent pollution effects occurring at the organism, population, community and ecosystem level (Law et al., 2010). In this framework, *in vitro* studies on marine organisms offer pivotal information for the generic toxicity and genotoxicity of xenobiotics, evaluating risks to wild organisms and to humans as consumers of marine resources (Benedetti et al., 2015).

This PhD project is aimed to provide the first data about the effects of phthalates, such as DEHP and its metabolite MEHP, on two coastal marine organisms using *in vitro* cell lines: one of European sea bass (*Dicentrarchus labrax*) embryonic stem cells, DLEC (Buonocore et al., 2005), and one of bottlenose dolphin (*Tursiops truncatus*) skin cells, TT; the latter was compared with the standardized Chinese Hamster Ovary cell line (CHO). Since most marine debris comes from land-based sources (Carlson et al., 2017), inshore species are particularly exposed to the risks associated with plastic pollution. Moreover, the potential effects of plastic pollution should be primarily evaluated on predator species, which are highly exposed to the ingestion of plastic and bioaccumulation of its additives (Rochman et al., 2013). Both European sea bass and bottlenose dolphin feed primarily on fishes and are frequently found in coastal areas. These two species are considered of ecological importance and are good indicators of the status of marine waters. Given their importance and their potential exposure and vulnerability to anthropogenic pollutants, inasmuch mainly coastal species, cell lines of these two organisms have been selected to investigate the effects of phthalates at cellular, DNA and chromosomal level and to determine the impact of these pollutants on coastal species of high ecological and economic value and, through them, on marine/coastal ecosystems.

In particular, this work focuses on three main topics: i) DEHP toxic effects on DLEC cell line (Ch.1); ii) MEHP toxic effects on DLEC cell line (Ch. 2); iii) compared toxicity of DEHP on TT and CHO cell lines (Ch. 3).

Chapter 1 is represented by a paper (Molino et al., 2019) about *in vitro* test to evaluate the potential impact of DEHP on the European sea bass embryonic cell line. In this paper were performed specific test to assess cell viability, cell death, and DNA damage on DLEC after exposure to increasing concentrations of the phthalate. The results showed a significant decrease in cell viability starting from lower doses of DEHP after 24 h of exposure, coupled with a significant increase in cell apoptosis and necrosis accompanied by morphological changes and cell detachment. Genotoxicity tests also detected a moderate increase in DNA strand breaks and a higher increase in the frequency of MN accompanied by a significant and progressive inhibition of cell proliferation, suggesting possible aneugenic effects of this phthalate on DLEC cell line. These results show the importance of improving research on the effects of DEHP on marine fishes, since it is constantly introduced into the marine environment through marine litter, becoming a potential threat for marine ichtyofauna.

In Chapter 2 the toxicity of MEHP, one of the primary and most toxic metabolites of DEHP, has been assessed. Considering that, as a primary metabolite, MEHP should display a higher toxicity than DEHP, the objective of this study was to evaluate the potential adverse effects of MEHP, using specific *in vitro* tests to evaluate cytotoxicity and genotoxicity on the sea bass cell line after exposure to increasing concentrations of MEHP. The results showed a slight dose dependent decrease in cell viability, resulting in a statistically significant difference at the intermediate and higher doses of MEHP. No significant increase of cell death was observed. Genotoxicity tests detected no increase in DNA strand breaks but a higher increase in the frequency of MN accompanied by a slight inhibition of cell proliferation. These results suggest that MEHP treatment on DLEC cell line could lead to chromosomal loss events instead of chromosomal brakes. Comparing DEHP and MEHP effects, it is conceivable that the latter is the most responsible for the aneugenic effect on the DLEC cell line, underlining the greatest danger of this metabolite on the European sea bass and, consequently, on other marine organisms.

In Chapter 3, data of the toxicity of DEHP on bottlenose dolphin cell line in comparison with CHO cell line, are presented with the purpose to evaluate the potential adverse effects of this persistent pollutant. Specific test to assess cell viability, cell death, and DNA and chromosomal damage on TT and CHO cell lines to increasing concentrations of the phthalate, were performed. The results showed, in both cell lines, a significant decrease in cell viability starting from lower doses of DEHP after 24 h of exposure, coupled, only for CHO cells, with a significant increase in cell necrosis. Even though the Comet assay did not detect an increase in DNA fragmentation at any of the concentrations tested,

a significant increase in the frequency of MN accompanied by a significant and progressive inhibition of cell proliferation was observed in both TT and CHO cell lines, supporting the hypothesis that DEHP could have an aneugenic effect.

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2. Chapter 1: *In vitro* effects of Di(2-ethylhexyl)-phthalate

2.1 Molino C., Filippi S., Stoppiello G.A., Meschini R., Angeletti D. (2019)

In vitro evaluation of cytotoxic and genotoxic effects of Di(2-ethylhexyl)-phthalate (DEHP) on European sea bass (*Dicentrarchus labrax*) embryonic cell line

Toxicology *in Vitro* 56: 118–125

Abstract

Marine litter is extensively distributed in the marine environment, and plastic debris, of which litter is mostly composed, can be a major source of pollutants. Among them, Di(2-ethylhexyl)-phthalate (DEHP) is the most abundantly used plastic additive, and it has been reported to affect biochemical processes both in humans and wildlife; however, studies on its toxicological effects on marine organisms are still scarce. In this survey, we studied the cytotoxic, genotoxic, and mutagenic effects of DEHP in European sea bass embryonic cell line (DLEC) by applying specific *in vitro* tests. Results showed a significant decrease in cell viability starting at 0.01 mM of DEHP after 24 h together with a significant increase in apoptosis and necrosis, morphological changes and cell detachment. Consistently, we detected a moderate increase in DNA strand breaks from 0.02 mM, and a dose-dependent increase in of micronucleus frequency from 0.01 mM, accompanied by a significant inhibition of cell proliferation, which suggested a possible aneugenic effect of this phthalate. Our results demonstrate that *in vitro* exposure to DEHP had a dose-dependent cytotoxic and genotoxic effects in DLEC cell line, encouraging further investigation into its effects in *in vivo* and/or *ex vivo* cell systems of marine organisms.

1 Introduction

The emerging issue of marine litter in the marine environment has recently received increasing attention. It has been estimated that around 60 – 90 % of litter is composed of plastic debris (Derraik, 2002; Arcangeli et al., 2018; Campana et al., 2018). Microplastics, which are fragments below 5 mm in diameter or length resulting from the degradation of plastic introduced into the environment, are a persistent form of marine pollution, since they may require centuries to be degraded (Collignon et al., 2012). Recent studies have estimated that this debris has reached maximum levels of 892,000 particles/km^2 in the Mediterranean Sea (Collignon et al., 2012; Fossi et al., 2012). Microplastics accumulate on the sea surface, particularly in the habitat of neuston (Ryan et al., 2009), and one of the main problems is their accidental ingestion by marine organisms. When ingested, these particles can affect organisms due to both their physical and chemical properties (concerning the plastic polymer itself and the additives present) and the persistent organic pollutants (POPs) collected on their hydrophobic surface (Teuten et al. 2009; Andrady, 2011; Andersson, 2014).

Plastic is composed by a wide range of different hydrocarbon polymers, often in combination with additives (e.g. bisphenol A and phthalates). Plastic polymers are considered biologically inert, but their additives can leak and leach from the polymers depending on their molecular size, temperature, and pH of the water and other chemical properties of the medium and of the compound (Teuten et al. 2009; Andrady, 2011; Andersson, 2014). Among these additives, phthalates, which are phthalic acid esters, are primarily used to increase the plasticity of industrial polymers and are used in many consumer products (Mankidy et al., 2013). Phthalates are not covalently bound to the polymer making them susceptible to be easily leached from the matrix. Once released into the environment, these compounds have the potential to be transported for long distances and eventually enter the food chain (Mankidy et al., 2013).

Recent studies have highlighted the influence of phthalates on multiple biochemical processes, both in humans and wildlife, including effects on reproduction (Huang et al., 2012; Maradonna et al., 2013; Ye et al., 2014), sperm damage (Huang et al., 2012), early onset puberty in females (Wolff et al., 2010; Mankidy et al., 2013), infertility (Tranfo et al., 2012; Mankidy et al., 2013) and adverse pregnancy outcomes (Whyatt et al., 2009; Mankidy et al., 2013), neurological development (Miodovnik et al., 2011; Mankidy et al., 2013), and onset of allergies (Bornehag et al., 2004; Mankidy et al., 2013).

Marine organisms are particularly suited and effectively used to evaluate the effect of pollutants in environmental monitoring and toxicological studies, since pollutants tend to accumulate in their tissues and cells and provide an integrated assessment of the bioavailability and of the biological effects of contaminants on biota. Such studies can allow to analyse the cellular responses and

toxicological effects of xenobiotics and to evaluate risks to wild organisms and to humans as consumers of marine resources (Benedetti et al., 2015). In particular, cell lines of marine organisms are widely used as instruments for *in vitro* assessment of environmental stressors and to study important aspects, such as epidemiology, molecular carcinogenesis, toxicology, and functional genomics (Buonocore et al., 2005). Nevertheless, studies assessing the mechanical, physical, and toxicological impacts of plastic pollution (Fossi et al., 2012) and the effects of phthalates on marine organisms are still scarce, despite the increasing concern represented by marine litter.

Since most marine debris comes from land-based sources (Carlson et al., 2017), inshore species are particularly exposed to the risks associated with plastic pollution. Moreover, the potential effects of plastic pollution should be primarily evaluated on predator species, which are highly exposed to the ingestion of plastic and bioaccumulation of its additives (Rochman et al., 2013). The European sea bass (*Dicentrarchus labrax*) is an eurytherm and euryhaline marine teleost that inhabits temperate regions and is frequently found in coastal areas, as well as in estuaries and lagoons. Juveniles feed primarily on invertebrates, while adults feed on fishes. This species is of high commercial and recreational value (sport/recreational fishing) and plays an important role in marine trophic networks. *D. labrax* has been widely used in toxicological studies of anthropogenic pollutants due to its ecological importance, easy maintenance in laboratory and high responsiveness of its biotransformation system (Alimeda et al., 2010). To date, the continuous embryonic cell line (DLEC) (Buonocore et al., 2006), derived from early embryos and formed by adherent and fibroblast-like cells, has been shown to be useful for immunological (Casani et al., 2009) and toxicological studies (Rocco et al., 2013).

In this paper, we studied the possible cytotoxic, genotoxic and mutagenic effects of DEHP, the most abundantly used plastic additive, on DLEC cell line to evaluate its potential impact on this coastal species. To this aim, we exposed the DLEC cultures to increasing concentrations of the phthalate. MTT and the Trypan Blue assays were applied to evaluate the cytotoxicity of DEHP through possible decreases in cell viability after treatments. The alkaline version of the Comet assay was employed to measure the frequency of SSBs and alkali-labile sites induced by DEHP immediately after treatments. Comet assay is a rapid and sensitive procedure to quantify DNA lesions in mammalian cells (Fairbairn et al., 1997; Berni et al., 2008; Meschini et al., 2015a), as well as the DNA damage induced by pollution in sentinel organisms (Mosesso et al., 2012; Angeletti et al., 2013) and by endogenous reactive oxygen species (Berni et al., 2008). To assess DEHP mutagenicity, we applied the Cytokinesis-block micronucleus (CBMN) assay. This test measures the final effect of an exposure to contaminants (Filippi et al., 2018), and it has a high correlation with cancer risk (Souza et al., 2016). Besides genotoxic effects, the CBMN test provides better precision, because the data

obtained are not confounded by altered cell division kinetics (Fenech, 2000) and has the potential to detect beneficial effects of agents against the genotoxic action of known carcinogens, both *in vivo* (Berni et al., 2012; Grossi et al., 2012; Pepe et al., 2013) and *in vitro* (Meschini et al., 2015b).

2 Materials and Methods

2.1 Chemicals

Leibovitz (L-15) without L-Glutamine, phosphate buffer saline (PBS) w/o Ca and Mg, and L-Glutamine were purchased from Lonza, Italy. Penicillin/Streptomycin, Trypsin-EDTA in Phosphate Buffered Saline (PBS) w/o Calcium, Magnesium, and Phenol Red were purchased from EuroClone, Italy. Bis (2-ethylhexyl) phthalate (DEHP), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Cytochalasin B (1200 µg/mL), Trypan Blue solution (0.4%), dimethyl sulfoxide (DMSO), and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich, Italy. Foetal bovine serum (FBS) was purchased from Invitrogen, Italy.

DEHP stock solutions used during this study were 1, 2, 5, 10, 20, 50, 200, and 500 mM freshly prepared in DMSO, before treatments. For the latter, its concentration for treatments never exceeded 1%.

2.2 Cell culture and DEHP treatments

For this study, a continuous adherent cell line derived from European sea bass (*Dicentrarchus labrax* L.) embryos (DLEC) (Buonocore et al., 2005), was used. DLEC cells were grown in Leibovitz (L-15) medium supplemented with 10% FBS, 1% L-Glutamine, and 1% Penicillin/Streptomycin. Cell cultures were maintained at 20–22 °C without CO_2.

For cell viability assays, DLEC cell line was treated with 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 2, and 5 mM of DEHP for 24 h. Solvent and positive control cells were treated with 1% DMSO and 100 µM H_2O_2, respectively. Doses of DEHP used to assess DNA damage were chosen based on the results of cell viability assays.

2.3 Cell viability assay

2.3.1 MTT assay

Cell viability was assessed by MTT. DLEC were seeded onto 96-well microplates at density of 10,000 cells/well and incubated for 24 h at 21 °C, to allow cell adherence. Growth medium was then replaced by fresh medium containing DEHP at concentrations previously described and incubated for 24 h. At the end of exposure, MTT was added to each well (0.5 mg/mL), and cells were incubated for an additional 3 h at 21◦C. After incubation, supernatant was replaced with 100 µL of lysis solution

(10% SDS, 0.6% acetic acid in DMSO) to dissolve the formazan crystals and produce a purple solution. Optical density measurements were obtained using a scanning spectrophotometer DTX 880 Multimode Detector (Beckman Coulter). Readings were made using a 630 nm (background) and a 570 nm filters. Viability was determined in three independent experiments conducted in triplicate for each experimental condition. Cell viability, presented as the Relative O.D. at 570, was calculated using the formula: = Absorbance of treated cells/Absorbance of control cells.

2.3.2 Trypan Blue exclusion assay

Cell viability was also assessed by Trypan blue. DLEC cell line were seeded onto 35 mm petri dishes at a density of 10,000 cells/dish and incubated for 24 h at 21 °C, to allow them to attach. DLEC were then treated with DEHP and incubated for 24 h. At the end of the exposure, cells were harvested, and 20 µL of cell suspension was mixed with 20 µL of Trypan Blue solution (1/1; w/w) for 5 min to allow cell staining. Cells were then seeded on a slide and counted under an optical microscope. Viability was determined in three independent experiments. Percentage of cell viability was calculated as the mean of the cell survival in the three experiments.

2.4 Giemsa staining proliferation assay

DLEC cells were plated into 35 mm petri dishes with a final density of 4×10^5 cells/dish. DLEC were treated with DEHP and incubated for 24 h. Then, cells were fixed with cold methanol for 10 min and stained with 5% of Giemsa solution in distilled water for 15 min, washed in PBS, and air-dried. Cell morphology was observed and photographed under an inverted light microscope at a magnitude of 100 ×.

2.5 Detection of DEHP-induced cell death by fluorescence staining

To evaluate DEHP-induced cell death, DLEC were seeded onto 60 mm petri dishes at a density of 400,000 cells/dish, incubated for 24 h at 21 °C to allow them to attach, and then treated with DEHP for 24 h. At the end of the exposure, DLEC were harvested and, to identify apoptotic and necrotic cells from viable cells, a combination of Fluorescein Di-Acetate (FDA, 0.75 mg/mL), Propidium Iodide (PI, 0.25 mg/mL), and Hoechst (HO, 0.1 mg/mL) dyes were used (Proietti De Santis et al., 2001). FDA and HO are vital dyes that stain, respectively, cytoplasm and the nucleus of viable cells. PI staining identifies necrotic and late stage of apoptotic cells. Cells in early phase (viable-HO stained) and late phase (dead-PI stained) of apoptosis displayed the characteristic pattern of chromatin fragmentation. Approximately 500 randomly chosen unfixed cells for each experimental point were microscopically analysed for cell death, and the results of two independent experiments showing good reproducibility and comparable outcomes were considered.

2.6 Single Cell Gel Electrophoresis (SCGE) analysis

The standard alkaline (pH > 13) SCGE, or Comet assay, was performed under visible fluorescent light (Tice et al., 2000). DLEC cell line was treated with 0.02, 0.05, 0.2, 0.5, 2, and 5 mM of DEHP for 24 h. After treatments, cells were collected and processed for the assay. In short, 20 µL of the cell suspension were mixed with 80 µL of 0.75% low melting-point agarose in PBS at 37 °C and immediately pipetted onto a frosted glass microscope slide pre-coated with a layer of 1% normal melting-point agarose similarly prepared in PBS. One slide for each experimental point was then incubated in lysis solution (2.5 M NaCl, 10 mM Tris-HCl, 100 mM EDTA, pH 10, with 1% Triton X-100 and 10% DMSO freshly added) for 1 day at 4 °C. After lysis, slides were placed on a horizontal electrophoresis unit containing fresh electrophoresis buffer (1 m MEDTA, 300 mM NaOH, pH 13) and incubated for 15 min to allow unwinding of DNA. Electrophoresis was then conducted for 20 min at 25 V and 300 mA at 4 °C. Subsequently, slides were gently washed three times in neutralization solution (0.4 M Tris-HCl, pH 7.5) for 5 min and examined after staining with 50 µL ethidium bromide (20 µg/mL). Stained nucleoids were analysed at 400 × magnification with an automatic image analyser (Comet Assay III, Perceptive Instruments, UK) connected to a fluorescence microscope (Axioskop 2, Zeiss). To evaluate the amount of DNA damage, computer-generated tail moment (TM) values were used. For each experimental point, a total of 100 randomly selected cells were scored, and the mean of the results of three independent experiments showing good reproducibility and comparable outcomes was considered.

2.7 Cytokinesis-block micronucleus (CBMN) assay

The CBMN assay was carried out with the standard technique proposed by Fenech (1993) with minor modifications. DLEC cell line was treated with 0.01, 0.02, 0.1, and 0.2 mM of DEHP for 24 h. Then, cells were washed with PBS and fresh medium containing 2 µg/mL of Cytochalasin B to arrest cell cytokinesis was added for 48 h. For harvesting, cells were washed twice with PBS, trypsinized with trypsin-EDTA (0.25%), and centrifuged for 10 min at 143 x g. The pellet was then re-suspended with cold hypotonic solution (0.75 mM potassium chloride). Cell suspension was centrifuged again for 10 min at 143 ×*g*, fixed first with freshly mixed methanol: acetic acid: NaCl 0.9% (4:1:5) and, after centrifugation (143 ×*g* for 10 min), fixed with freshly mixed methanol: acetic acid (4:1) and spread on a microscope slide. Air dried preparations were made and coded. The slides were stained with Giemsa (5%) for 10 min. A total of 1000 binucleated cells with intact cytoplasm were scored for the presence of micronuclei (MN) for each experimental point. MN is a biomarker of chromosome breakage or loss. For the analysis of cell cycle progression, 1000 cells per sample were scored for the

presence of one, two, or more than two nuclei, and the Cytokinesis Block Proliferation Index (CBPI) was calculated as follows: CBPI = [1 N + (2 × 2 N) + (3× > 2 N)]/TC where 1 N is the number of cells with one nucleus, 2 N with two nuclei, >2 N with more than two nuclei and TC is the total number of cells examined. Based on this parameter, the percentage of cytostasis was calculated with the formula: = 100–100[CBPIt−1/CBPIc−1] where t and c are treated and control samples, respectively (Lorge et al., 2008). The mean of the results of two independent experiments showing good reproducibility and comparable outcomes is shown.

2.8 Statistical analysis

Statistical analysis for MTT, Trypan blue, and apoptotic/necrotic cell death assays (treatments *vs* solvent) was performed by use of $\chi 2$-test. The analysis of the significance of the Comet test, in terms of differences in mean tail moment between DEHP treatments and the solvent sample was performed by applying the Student's *t*-test for paired samples. The statistical significance in the yield of micronuclei per cell and the cytostatic effect between the solvent and treated samples was evaluated by the Student's *t*-test and the Chi-squared test, respectively. The level for statistical significance was set at $p \leq .05$.

3. Results

3.1 Cell viability

3.1.1 MTT assay

Fig. 1A shows DLEC cell line viability at 24 h of DEHP treatment measured by MTT assay. The solvent shows no effect on DLEC cell survival, whereas H_2O_2 (100 µM) was highly toxic with a decrease in cell survival of ~ 65%. A dose dependent reduction in DLEC viability after treatment with different DEHP doses was observed, displaying a range of viability between 71% at the lowest dose (0.01 mM) to 20% at the highest one (5 mM). Decreasing survival rates of all DEHP treatments show a statistically significant difference with respect to the solvent ($p < .001$).

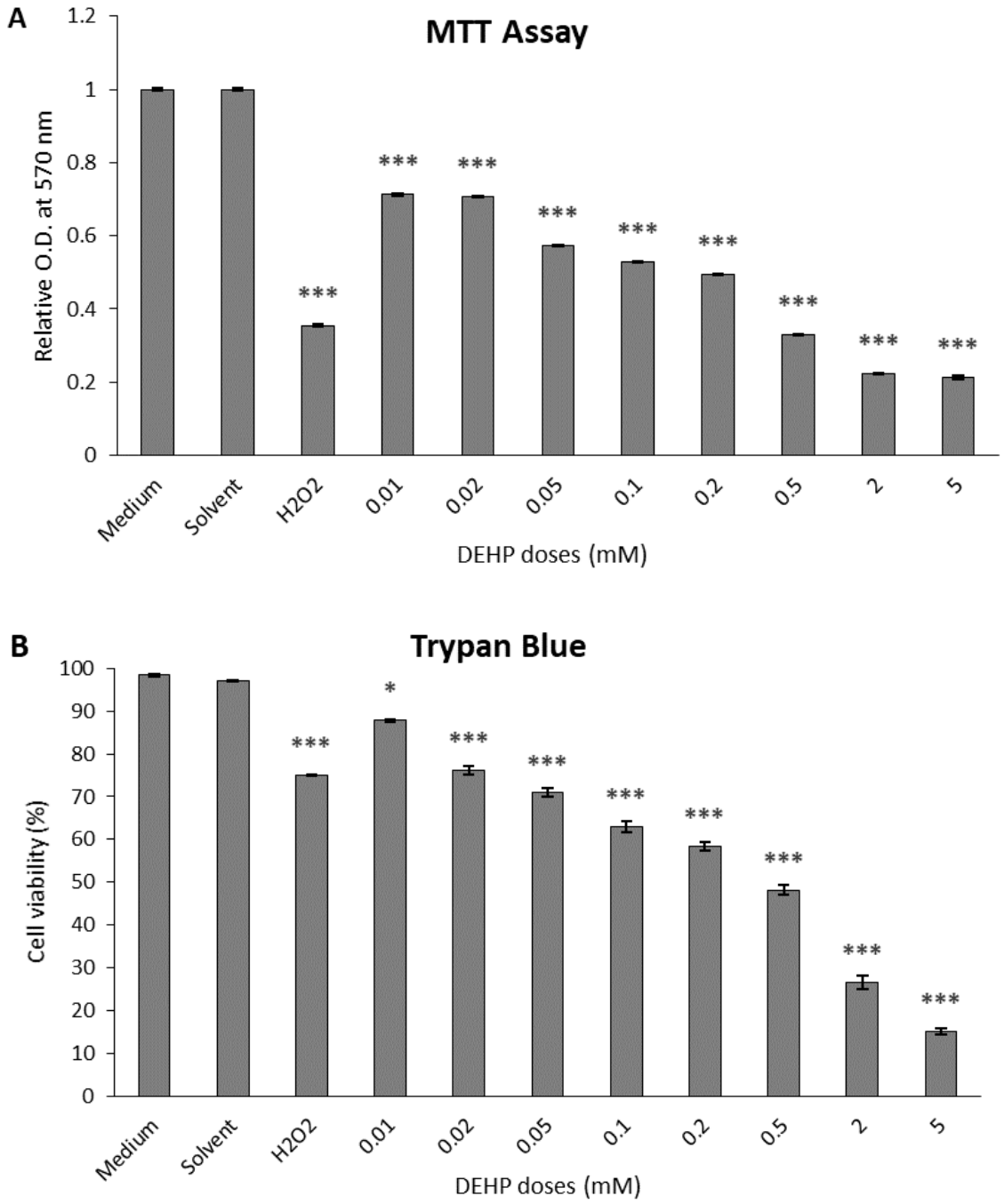

Fig. 1: Cell viability in DEHP-exposed DLEC cell line determined by MTT and Trypan Blue assay. **A)** MTT assay. Results are displayed as a mean of the optical density at 570 nm at each treatment level normalized to the solvent (DMSO). The data are expressed as mean ± SE of three experiments. *** $p \leq .001$. **B)** Trypan Blue assay. Cell viability is expressed as the percent of viable cells out of the total cells at each treatment level. The data are expressed as mean ± SE of three experiments. * $p \leq .05$; *** $p \leq .001$.

3.1.2 Trypan Blue exclusion Assay

Results of Trypan Blue assay are illustrated in Fig. 1B. The solvent shows no effect on DLEC cell survival, whereas H2O2 (100 µM) was highly toxic with a decrease in cells survival of ~ 25%. A dose dependent reduction in DLEC viability after treatment with different DEHP doses was observed, ranging between 88% and 15% at the lowest and the highest dose, respectively. Survival decrease rates of all DEHP treatments show a statistically significant difference with respect to the solvent ($p < .05$; $p < .001$).

3.2 Giemsa staining proliferation

Proliferation of DLEC was assessed by Giemsa staining (Fig. 2). Data shows that 24 h of DEHP treatment strongly inhibited cell proliferation and affects cell morphology, especially at higher doses, with respect to control cells. DLEC cells appeared to have lost their fibroblast-like morphology and were more jagged at the edges.

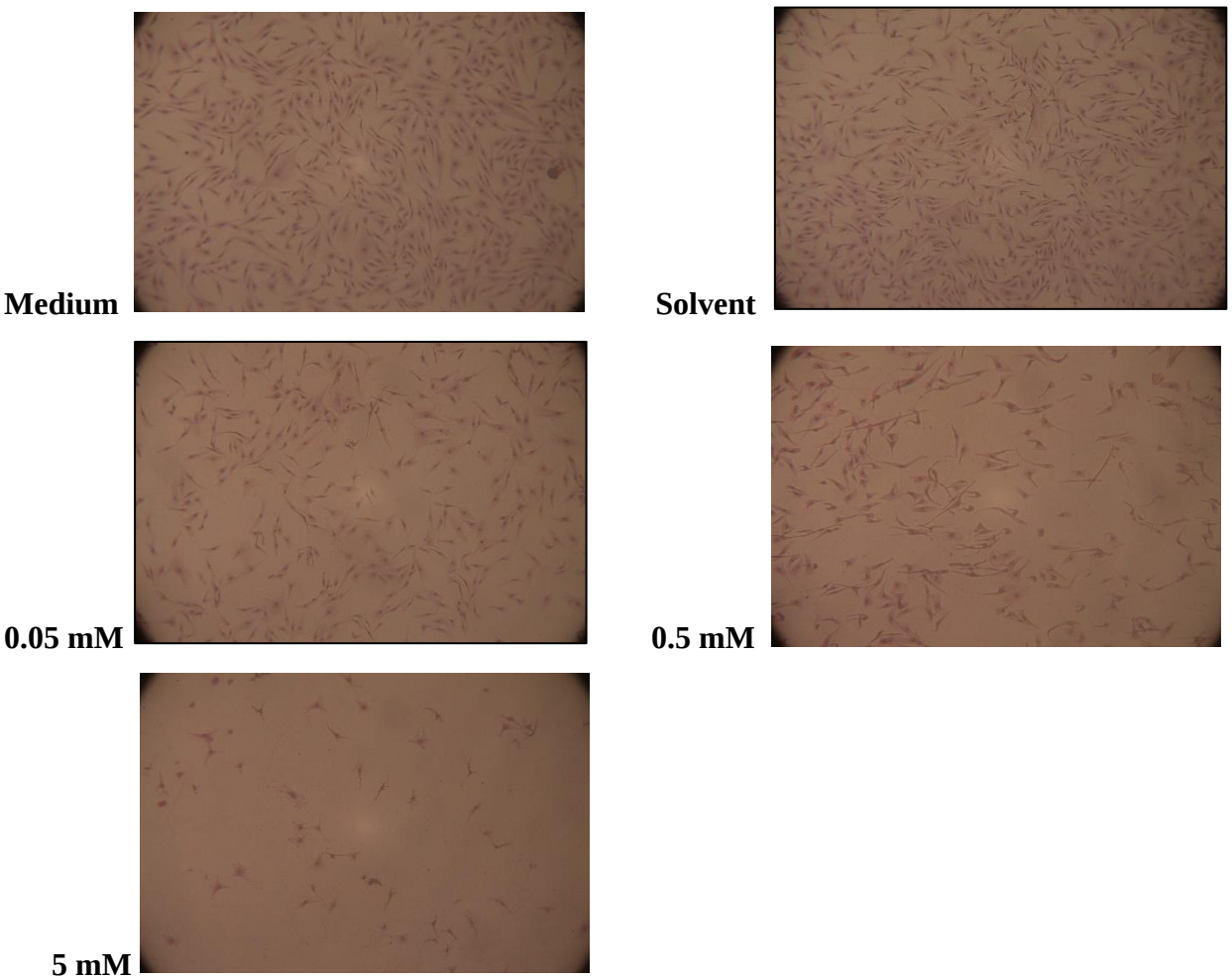

Fig. 2: Giemsa staining proliferation assay. After the 24 h treatment with DEHP, DLEC were stained with 5% Giemsa to assess cell abundance and morphology. Cells from medium, solvent, 0.05, 0.5 and 5 mM of DEHP are shown.

3.3 Cell death analysis

The apoptotic response of DLEC cells treated with different doses of DEHP is shown in Fig. 3. In the medium and solvent samples, no significant increase of necrotic and apoptotic cells was observed. H_2O_2 treatment (100 µM) resulted in a moderate induction of necrotic cells, otherwise no increase in apoptotic cells was observed. Both necrosis and apoptosis induction were statistically significant in comparison with the solvent ($p < .01$; $p < .001$) with a higher induction of necrosis respect to apoptosis starting from a DEHP dose of 0.2 mM.

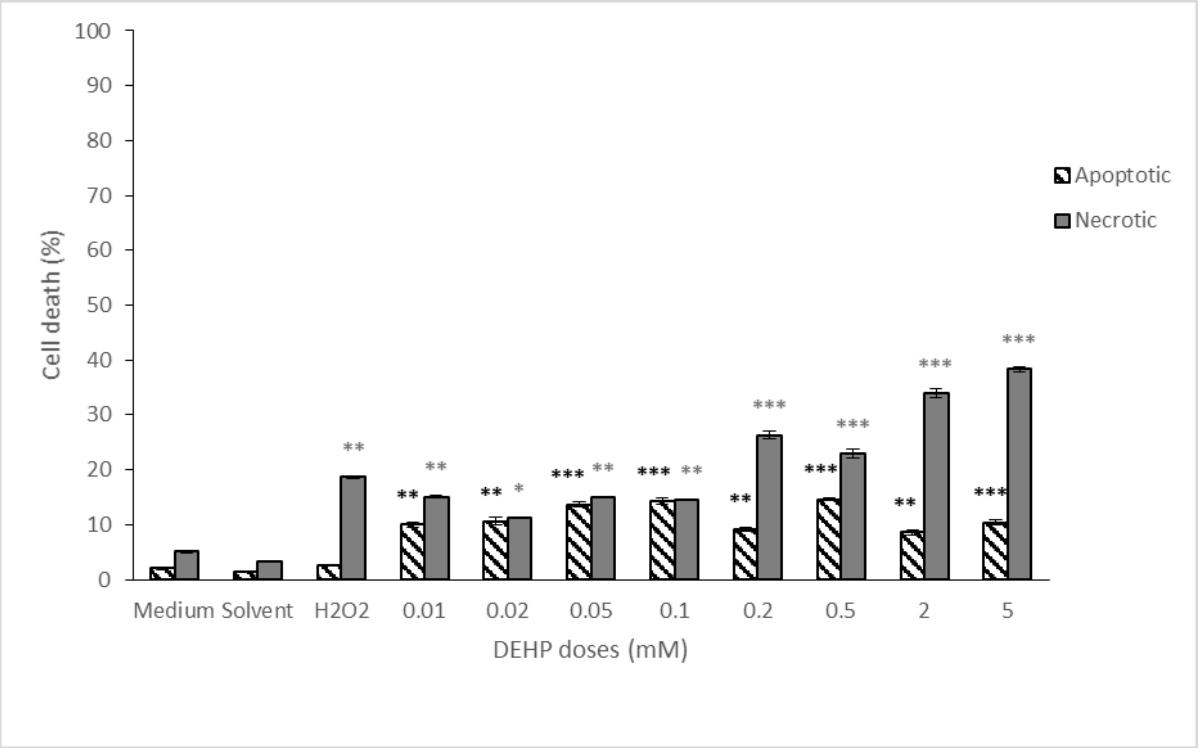

Fig. 3: Cell Death assay. Percentage of apoptosis and necrosis in DLEC cells treated with DEHP for 24 h. Data are presented as means ± SE of two independent experiments for each treatment. * $p \leq .05$; ** $p \leq .01$; *** $p \leq .001$.

3.4 Comet assay

Results of induction in DNA primary damage obtained in DLEC cells treated with DEHP are shown in Fig. 4. Control DLEC cells show a mean tail moment (TM) of 0.77. Treatment with solvent did not increase the mean TM value with respect to the medium. H_2O_2 (100 µM) significantly increased the frequency of DNA damage as detected by a mean TM of 4.51 ($p < .001$). Treatment with DEHP shows a dose-dependent increase of mean TM statistically significant in comparison with the solvent ($p < .05$; $p < .01$; $p < .001$) for all treatments, except that at the lowest DEHP dose (0.02 mM).

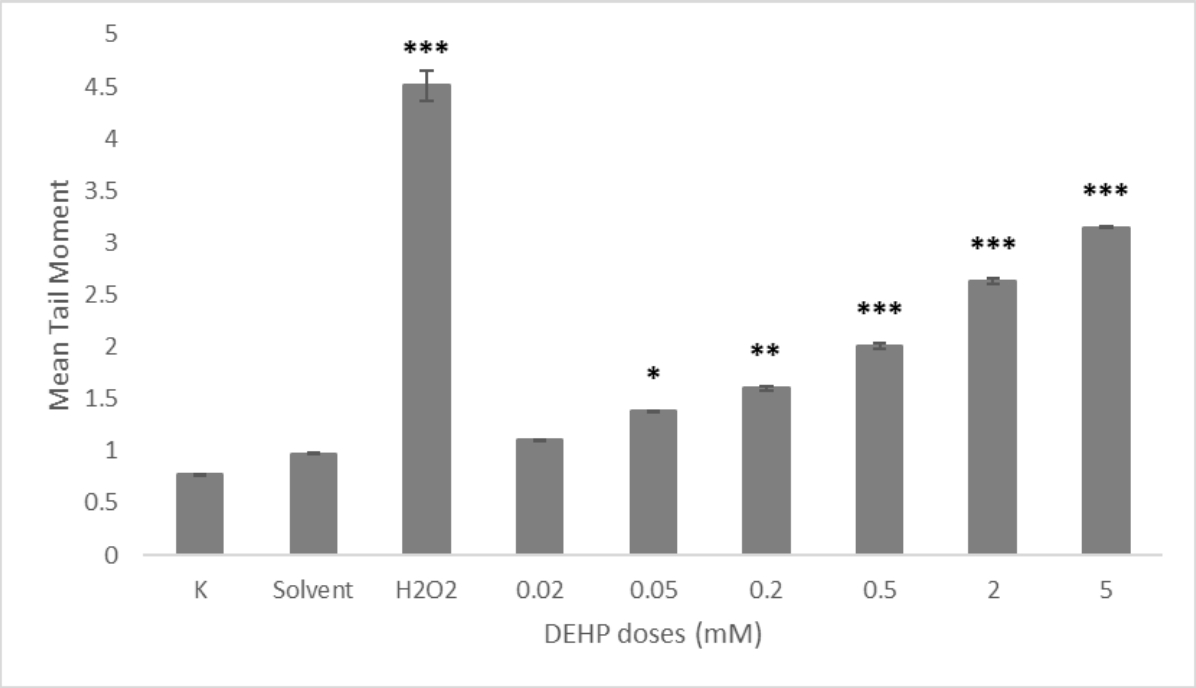

Fig. 4: Mean Tail Moment in DLEC cell line after 24 h exposure to DEHP. Data are presented as means ± SE of three independent experiments for each treatment. * $p \leq .05$; ** $p \leq .01$; *** $p \leq .001$.

3.5 CBMN assay

Results from CBMN assay are presented in Table 1. No statistically significant difference either in the frequency of MN or in CBPI values in samples treated with the solvent, in comparison to the control, was observed. Treatment with H_2O_2 (100 µM) caused a statistically significant increase in the frequency of MN ($p < .001$), a significant decrease in the CBPI value ($p < .001$), and an increase in the percentage of cytostasis when compared to the medium.

With regard to the DEHP treatments, data show a dose-dependent increase in the frequency of MN in comparison to the solvent. Student's *t*-test analysis shows a statistically significant increase in the yield of micronuclei per cell in all DEHP treatments ($p < .001$) compared to the solvent. Moreover, an increase in the percentage of cytostasis in samples treated with DEHP with respect to the solvent was found. In particular, lower doses (0.01 and 0.02 mM) display a MN mean frequency between 75.5 to 189/1000 binucleated cells and a percentage of cytostasis between 13 and 9.9, respectively. Higher doses (0.1 and 0.2 mM) exhibit a greater percentage of cytostasis between 67.3 and 73.4, and a MN frequency of 367/1000 binucleated cells at 0.1 mM DEHP. At 0.2 mM DEHP, it was not possible to evaluate the MN frequency due to the high cytotoxic effect of the phthalate at this concentration. CBPI values show a dose-dependent decrease at all DEHP doses with respect to the

solvent. The cytostatic effect of all treatments was statistically significant ($p < .001$), except at the lowest DEHP dose ($p < .01$).

Table 1. Induction of micronuclei (MN), Cytokinesis Block Proliferation Index (CBPI) and percentage (%) of Cytostasis in DLEC cell lines treated for 24 h with DEHP. Data are presented as means ± SE of two independent experiments for each treatment.

Treatment	Harvesting time after cyto-B	MN/1000 BN±S.E.	ts	CBPI±S.E.	χ_2	% Cytostasis±S.E.
Medium	48 h	4.3 ± 0.04		1.57 ± 0.0001		0 ± 0.00
Solvent	48 h	4.7 ± 0.15	NS	1.55 ± 0.0002	NS	3.7 ± 0.03
H_2O_2	48 h	30 ± 0.00	§§§	1.45 ± 0.00	§§§	28.9 ± 0.00
0.01 mM	48 h	75.5 ± 0.92	***	1.48 ± 0.0001	***	13.0 ± 0.01
0.02 mM	48 h	189 ± 0.49	***	1.49 ± 0.0003	**	9.9 ± 0.03
0.1 mM	48 h	367 ± 0.00	***	1.18 ± 0.00	***	67.3 ± 0.00
0.2 mM	48 h	/	/	1.15 ± 0.00	***	73.4 ± 0.00

Significance of Student's *t*-test (ts) and Chi-squared test (χ_2): NS: not significant.

** $p \leq .01$ treated vs solvent.

*** $p \leq .001$ treated vs solvent.

§§§ $p \leq .001$ H_2O_2 vs medium.

4. Discussion

Phthalates and their derivatives are a common group of widely used chemicals in manufacturing; however, to the best of our knowledge, the potential for *in vitro* toxicity to cells of marine organisms after DEHP exposure has not yet been evaluated, whereas a number of adverse effects resulting from exposure to this agent have been reported *in vivo* and *in vitro* in humans and other organisms. In the current study, the effects of *in vitro* exposure to DEHP on the European sea bass embryonic cell line, DLEC, including cytotoxicity, cell death, and genotoxicity were investigated, and the obtained results indicate a clear effect in all the tested cellular parameters.

In this study, the cytotoxicity was assessed using the MTT and Trypan Blue assays. A clear dose response was observed with a significantly decreased cell viability already at the lowest dose DEHP of 0.01 mM in both assays. Cell viability was lower for the MTT with respect to the Trypan blue. This might be explained by a major capacity of DLEC cells to produce formazan crystals from the metabolism of MTT solution and/or by a better sensitivity of the first assay (Wilking et al. 2014). Even though viability assays display a different sensitivity, both assays agree upon showing a cytotoxic effect of DEHP with dose/effect relationships on DLEC cell line suggesting the incapacity of DLEC cells to overcome xenobiotic damage, which affected their replication and survival. Giemsa staining proliferation assay also showed the onset of cell death accompanied by morphological changes and cell detachment, confirming the significant inhibition of cell proliferation due to DEHP treatment in the DLEC cell line. These results are in agreement with previous studies on rainbow trout and different mammalian cell lines (Erkekoğlu et al., 2010; Bath et al., 2013; Peropadre et al., 2013; Martins et al., 2015; Rajamanikyam et al., 2017), where 24 h of exposure to DEHP resulted in toxic effects and significantly induced cytotoxicity starting at 0.01 mM. Nevertheless, our results indicate that an effect at lower doses cannot be excluded since we detected a significant decrease of cell viability and a significant induction of MN even at the lowest dose. In a more recent study on GC-2spd mouse spermatocytes cells (Zhu et al., 2017) in addition to decrease in cell viability as a function of exposure time, DEHP caused a significant and dose-dependent increase in apoptosis starting from a dose of 0.1 mM. Rosado-Berrios et al. (2011) reported that DEHP and its metabolite, the mono(2-ethylhexyl) phthalate (MEHP), caused an alteration in the mitochondrial membrane potential, a hallmark of apoptosis, in Human TK-6 lymphoblast cells. Cell death by apoptosis caused by DEHP exposure has been widely demonstrated (Bhattacharya et al., 2005; Caldwell, 2012; Sun et al., 2015); however, this phthalate is also known to cause cell death by necrosis (Caldwell, 2012; Wang et al., 2013) due to the activation of a transduction pathway involving two different peroxisome proliferator-activated receptor (PPARs) isotypes, PPARalpha and PPARbeta (Martinasso et al., 2006). The current study showed that DEHP could induce, in DLEC cells, an increase in apoptosis

and necrosis at 0.01 mM and higher concentrations of the phthalate. The results of the analysis of cell death obtained in the current are thus in agreement with the data in the literature revealing an induction of both apoptosis and necrosis already at 0.01 mM also in the embryonic cell lines derived from the European sea bass.

Genotoxicity was assessed using the Comet and CBMN assays. Results clearly showed the genotoxic and mutagenic potential of DEHP in DLEC embryonic cells. The overall amount of DNA strand breaks detected by means of the Comet assay was not very high, even if it significantly increased at increasing concentrations of the phthalate. If an agent shows no or a low effect in one test, this is not enough to consider it as safe, and the result has to be confirmed with other tests that contemplate all the possible mechanisms through which a xenobiotic can cause genotoxic or mutagenic damage. Indeed, from the data of the CBMN assay, DEHP was found to be highly cytotoxic starting from the dose of 0.01 mM, with a subsequent influence on cell proliferation and a dose-dependent increase in the frequency of MN. This result evaluated under the light of a moderate increase in DNA strand breaks suggests a possible aneugenic effect of this phthalate in the DLEC cell line. Accordingly, treatment with H_2O_2, a well-known clastogenic agent, caused a higher amount of DNA strand breaks in the Comet assay but a lower increase in the frequency of MN, when compared with all doses of DEHP. This outcome further reinforces the hypothesis that this compound could have an aneugenic effect in the DLEC cells. Genotoxicity of DEHP on human and rodent cell lines has been widely demonstrated, indicating several mechanisms involved, such as the oxidative stress induction (Erkekoğlu et al., 2010; Erkekoğlu et al., 2011; Yuan et al., 2017), the inhibition of DNA replication (Li et al., 2014), lysosomal or mitochondrial damage (Li et al., 2014; Wang et al., 2015), mutation in genes, formation of DNA adducts (IARC, 2006; Caldwell, 2012) and DNA strand breaks (Okai and Okai, 2000; IARC, 2006; Caldwell, 2012) and changes in DNA methylation patterns (IARC, 2006; Caldwell, 2012). The induction of oxidative stress due to the presence of reactive oxygen species (ROS) is one of the important mechanisms underlying the toxicity of DEHP and its metabolite, MEHP (Erkekoğlu et al., 2011; Li et al., 2014). Previous studies also indicate that an increase of ROS and the resulting oxidative stress can cause aneuploidy in cultured cells (Fang and Zhang, 2011; Caldwell, 2012; Wang et al., 2013; Yuan et al., 2017). These findings suggest that the mechanisms involved in the induction of the possible DEHP aneugenic effect and, subsequently, of MN formation in DLEC cell line might be ascribed to an increase of ROS and oxidative stress.

In conclusion, the overall results of our study demonstrated the cytotoxicity and genotoxicity of DEHP in the DLEC embryonic cell line with the inhibition of cell proliferation and the induction of micronuclei as the main effects, reflecting a possible aneugenic effect of this compound. Since DNA and chromosomal damage often correlate with mutagenicity (Bruce and Heddle, 1979; Jenssen and

Ramel, 1980), a mutagenic effect of DEHP in the DLEC cell line cannot be excluded. To the best of our knowledge, there are still too scarce data to understand whether DEHP is metabolized by marine organisms, in which case, its effects (i.e. that of its metabolites) could be greater in wildlife. Consequently, to efficiently assess the effects of pollutants on marine organisms, cytotoxicity and genotoxicity assays *in vivo* and/or *ex vivo* should be included. Nevertheless, with *in vitro* assays it is possible to successfully predict systemic toxicological effects *in vivo* (Groothuis et al., 2015). With this respect, it is worth noting that, although the DEHP doses we applied in our acute tests were higher than those usually measured in the environment (Ye et al 2014; Zhang et al 2018), Junaid et al (2018) recently reported a maximum concentration of 13,050 µg/L (about 334 µM) of phthalates in river waters which is in the middle of our range of treatment. Therefore, our study shows that DEHP, which is constantly introduced into the marine environment through marine litter, is a potential threat for European sea bass and for marine ichtyofauna in general.

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3. Chapter 2: I*n vitro* effects of Mono(2-ethylhexyl)-phthalate on European sea bass (*Dicentrarchus labrax*) embryonic cell line

3.1 Molino C., Filippi S., Caccia A., Meschini R., Angeletti D.

Effects of phthalates on marine organisms: *in vitro* evaluation of Mono(2-ethylhexyl)-phthalate (MEHP) on European seabass (*Dicentrarchus labrax*) embryonic cell line

Abstract

Di(2-ethylhexyl)-phthalate is the most abundant plastic additive is considered a globally ubiquitous pollutant in marine environments, and its primary metabolite, Mono(2-ethylexyl) phthalate (MEHP), may be responsible for many of the effects of DEHP. In this study, we evaluated the cytotoxicity and genotoxicity of MEHP on European sea bass embryonic cell line (DLEC). After DLEC exposure to MEHP, effects on cell viability, cell death, and genotoxicity were investigated. The results showed a slight dose dependent decrease in cell viability that was statistically significant at the intermediate and higher doses of MEHP. No increase in necrotic cells was observed, except at the highest dose of MEHP. Genotoxicity tests detected no increase in DNA strand breaks, but resulted in a higher increase in the frequency of micronuclei, both in binucleated and mononucleated cells, accompanied by a slight inhibition of cell proliferation. These results suggest that MEHP exposition could lead to chromosomal loss events in DLEC, thus indicating MEHP as possibly being responsible for the insurgence of aneugenicity in this cell line, and underlining its great danger for the European sea bass and, potentially, for other marine organisms. To our knowledge, this is the first evidence of a possible aneugenic effect of this compound.

1. Introduction

Phthalates, which are esters of the phthalic acid, are man-made additives primarily used to increase the plasticity and flexibility of industrial polymers and are used in many consumer products (Mankidy et al., 2013; Meruvu et al., 2016). Although they confer advantageous properties to plastics, their influence on multiple biochemical processes has been widely reported (Huang et al., 2012; Mankidy et al., 2013; Maradonna et al., 2013; Ye et al., 2014a; Wang et al., 2015). Di-(2-ethylhexyl) phthalate (DEHP) is the most common phthalate (Caldwell, 2012; Chang et al., 2017; Rowdhwal and Chen, 2018) used in plastics. Due to the growing presence of plastic marine litter in the sea, DEHP is globally ubiquitous in marine environments (Caldwell, 2012; Ye et al., 2014a, 2014b; Rowdhwal and Chen, 2018). In fact, it has been reported that the concentration of phthalates in the marine surface water is up to 0–300 µg/L, whereas in the marine sediments and in the marine organisms it is up to 3 µg/g and 4.07 ng/g, respectively (Liu et al., 2009; Ye et al., 2014a). Once ingested, DEHP is rapidly metabolized into different metabolites. Mono(2-ethylexyl) phthalate (MEHP), which is the primary hydrolysed metabolite, is one of the most studied since it is responsible for many of the effects of DEHP (Albro, 1986; Caldwell, 2012; Wang et al., 2012; Tetz et al., 2013). Considering the significant presence of DEHP in the marine environment, it is expected that its metabolites are also ubiquitous (Ye et al., 2014a). Indeed, recent studies have reported that the average concentration of MEHP in superficial zooplankton/microplastic samples of the Pelagos Sanctuary ranged from 29.17 ng/g to 93.37 ng/g (Fossi et al., 2012), whereas its concentration in samples collected from the Sea of Cortez (La Paz Bay) ranged from 13.08 ng/g to 13.69 ng/g (Fossi et al., 2016). The ubiquitous presence of DEHP and MEHP in marine environments has aroused great concern for aquatic organisms (Ye et al., 2014a).

MEHP, as with many pollutant metabolites, might be more toxic than its parent compound (Huber et al., 1996; Ye et al., 2014a). As its precursor, MEHP can adversely affect developmental and reproductive functions of organisms (Wang et al., 2012; Zhao et al., 2012; Hart et al., 2014; Ye et al., 2014a; Meruvu et al., 2016). Among its major effects, MEHP is known to cause the impairment of reproductive success (Li et al., 2018), the induction of cell apoptosis (Yokoyama et al., 2003; Meruvu et al., 2016) and have genotoxic (Kleinsasser et al., 2004), mutagenic, and carcinogenic potential (Chang et al., 2017) on human and rodent cell lines. However, to our knowledge, few data are available on the effect of MEHP on aquatic organisms, especially on marine fishes (Ye et al., 2014a).

In our previous study (Molino et al., 2019), we demonstrated that DEHP has a toxic effect on the European sea bass embryonic cell line (DELC), resulting in a significant decrease in cell viability, a moderate increase in DNA strand break induction, and a dose-dependent increase in the frequency of MN accompanied by a significant and progressive inhibition of cell proliferation. Considering that

different studies have shown the higher toxicity of MEHP, the objective of this study was to evaluate the potential adverse effects of increasing concentrations of MEHP on DLEC cell line, using specific *in vitro* tests to evaluate MEHP cytotoxicity, genotoxicity, and mutagenicity. Cell viability and its possible decrease after treatment was assessed using MTT and the Trypan Blue Exclusion (TBE) assays, whereas the possible genotoxic and mutagenic effects of MEHP were analysed using the alkaline version of the Comet assay and the Cytokinesis-Block MicroNucleus (CBMN) assay, respectively. Based on the findings of our previous study on the effects of DEHP on DLEC cell line (Molino et al., 2019) and literature (Ye et al., 2014a), in the current study lower concentrations of MEHP, in comparison to DEHP doses, were tested.

2. Materials and Methods

2.1 Chemicals

Leibovitz (L-15) without L-Glutamine, Phosphate Buffer Saline (PBS) without Calcium and Magnesium, and L-Glutamine were purchased from Lonza, Italy. Penicillin/Streptomycin, Trypsin-EDTA in PBS without Calcium, Magnesium, and Phenol Red were purchased from EuroClone, Italy. Mono(2-ethylexyl) phthalate (MEHP), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Cytochalasin B (1200 µg/mL), Trypan Blue solution (0.4%), Dimethyl sulfoxide (DMSO), and Sodium Dodecyl Sulfate (SDS) were purchased from Sigma-Aldrich, Italy. Foetal Bovine Serum (FBS) was purchased from Invitrogen, Italy.

MEHP stock solutions used during this study were 0.05, 0.1, 0.25, 0.5, 1, 5, 7.5, and 10 mM freshly prepared in DMSO, before treatments. For the latter, its concentration for treatments never exceeded 1%.

2.2 Cell culture and MEHP treatments

A continuous adherent cell line derived from European sea bass (*Dicentrarchus labrax* L.) embryos (DLEC) (Buonocore et al., 2005), was used. DLEC cells were grown in Leibovitz (L-15) medium supplemented with 10% FBS, 1% L-Glutamine, and 1% Penicillin/Streptomycin. Cell cultures were maintained at 20-22°C without CO_2.

For cell viability assays, solvent and positive control cells were treated with 1% DMSO and 100 µM H_2O_2, respectively. Doses of MEHP used to assess primary DNA and chromosomal damage were chosen based on the results of cell viability assays.

2.3 Cell viability assay

2.3.1 MTT assay

DLEC were seeded into 96-well microplates at a density of 10,000 cells/well and incubated for 24 h at 21◦C to allow cell adherence. Growth medium was then replaced by fresh medium containing MEHP at concentrations of 1, 2.5, 5, 10, 50, 75, and 100 μM and incubated for 24 h. At the end of the MEHP exposure, the MTT assay was performed as described in Grossi et al. (2014) and in Molino et al. (2019). Viability was determined in two independent experiments conducted in triplicate for each experimental condition. Cell viability, presented as the Relative O.D. at 570 was calculated using the formula: = Absorbance of treated cells/Absorbance of control cells.

2.3.2 Trypan Blue Exclusion (TBE) assay

DLEC cells were seeded in 35 mm petri dishes at a density of 10,000 cells/dish and incubated for 24 h at 21°C to allow them to attach. DLEC were then treated with 1, 5, 10, 50, 75, and 100 μM of MEHP and incubated for 24 h. At the end of the exposure, cells were harvested and 20 μL of cell suspension was mixed with 20 μL of Trypan Blue solution (1/1, w/w) for 5 minutes to allow cell staining. Cells were then seeded on a slide and counted under an optical microscope. Viability was determined in two independent experiments. The percentage of cell viability was calculated as the mean of the cell survival in the two experiments.

2.4 Detection of MEHP-induced cell death by fluorescence staining

DLEC were seeded in 60 mm petri dishes at a density of 400,000 cells/dish, incubated for 24 h at 21°C to allow them to attach, and then treated with 1, 5, 10, 50, and 100 μM of MEHP for 24 h. At the end of the exposure, DLEC were harvested and, to distinguish apoptotic and necrotic cells from viable cells, a combination of Fluorescein Di-Acetate (FDA, 0.75 mg/mL), Propidium Iodide (PI, 0.25 mg/mL), and Hoechst (HO, 0.1 mg/mL) dyes were used (Proietti De Santis et al., 2001). FDA and HO are vital dyes that stain, respectively, cytoplasm and the nucleus of viable cells. PI staining identifies necrotic and the late stage of apoptotic cells. Cells in early phase (viable-HO stained) and late phase (dead-PI stained) of apoptosis displayed the characteristic pattern of chromatin fragmentation. Approximately 500 randomly chosen unfixed cells for each experimental point were microscopically analysed for cell death, and the results of two independent experiments showing good reproducibility and comparable outcomes were considered.

2.5 Single Cell Gel Electrophoresis (SCGE) analysis

DLEC cell line was treated with 1, 5, 10, 50, 75, and 100 μM of MEHP for 24 h and the standard alkaline (pH > 13) SCGE, or Comet assay, was performed according to previous works (Meschini et

al., 2015a; Egidi et al., 2018). Shortly, 20 µL of the cell suspension (1×10^4 cells) were mixed with 0.75% low melting-point agarose (80 µL) and put onto a microscope slide pre-coated with 1% normal melting-point agarose. Slides were dipped in a lysis solution for 1 day at 4 °C, subjected to electrophoresis for 20 minutes at 25 V and 300 mA at 4 °C preceded by a 15-minute incubation in electrophoresis buffer to allow DNA unwinding, neutralized, and stained with ethidium bromide (20 µg/mL, 50 µL). Nucleoids were examined at 400× magnification with a fluorescence microscope (Axioskop 2, Zeiss) associated with a Comet Assay III program. To evaluate DNA damage, computer-generated tail moment (tm) values were used. For each experimental point, a total of 100 randomly selected cells were scored, and the mean of the results of two independent experiments showing good reproducibility and comparable outcomes were considered.

2.6 Cytokinesis-Block MicroNucleus (CBMN) assay

The CBMN assay was carried out with the standard technique proposed by Fenech (1993) with minor modifications. DLEC cell line was treated with 0.5, 1, 2.5, 5, and 10 µM of MEHP for 24 h. Moreover, a dose of DEHP (10 µM) was selected in order to compare the micronuclei induced by the two phthalates. Then, cells were washed with PBS and fresh medium containing 2 µg/mL of Cytochalasin B was added for 48 h to arrest cell cytokinesis. Harvesting and fixing were carried out as previously described (Meschini et al., 2015b; Molino et al., 2019). The slides were stained with Giemsa (5 %) for 10 min. Scoring of micronuclei (MN) was performed in both 1000 mononucleated and 1000 binucleated cells with intact cytoplasm for each experimental point. For the analysis of cell cycle progression, 1000 cells per sample were scored for the presence of one, two, or more than two nuclei, and the Cytokinesis Block Proliferation Index (CBPI) was calculated as follows: CBPI = [1N + (2×2N) + (3×>2N)]/TC where 1N is the number of cells with one nucleus, 2N with two nuclei, >2N with more than two nuclei, and TC is the total number of cells examined. Then, the percentage of cytostasis was calculated with the formula: = 100–100[CBPIt−1/CBPIc−1] where t and c are treated and control samples, respectively (Lorge et al., 2008). The mean of the results of three independent experiments showing good reproducibility and comparable outcomes is shown.

2.7 Statistical analysis

The statistical analysis for MTT, TBE, and apoptotic/necrotic cell death assays (treatments vs solvent) was performed by use of $\chi 2$-test. The analysis of the significance of the Comet test, in terms of differences in mean tail moment between MEHP treatments and the solvent sample was performed by applying the Student's t-test for paired samples. The statistical significance in the yield of micronuclei per cell and the cytostatic effect between the solvent and treated samples was evaluated

by the Student's t-test and the Chi-squared test, respectively. The level for statistical significance was set at $p < 0.05$.

3. Results

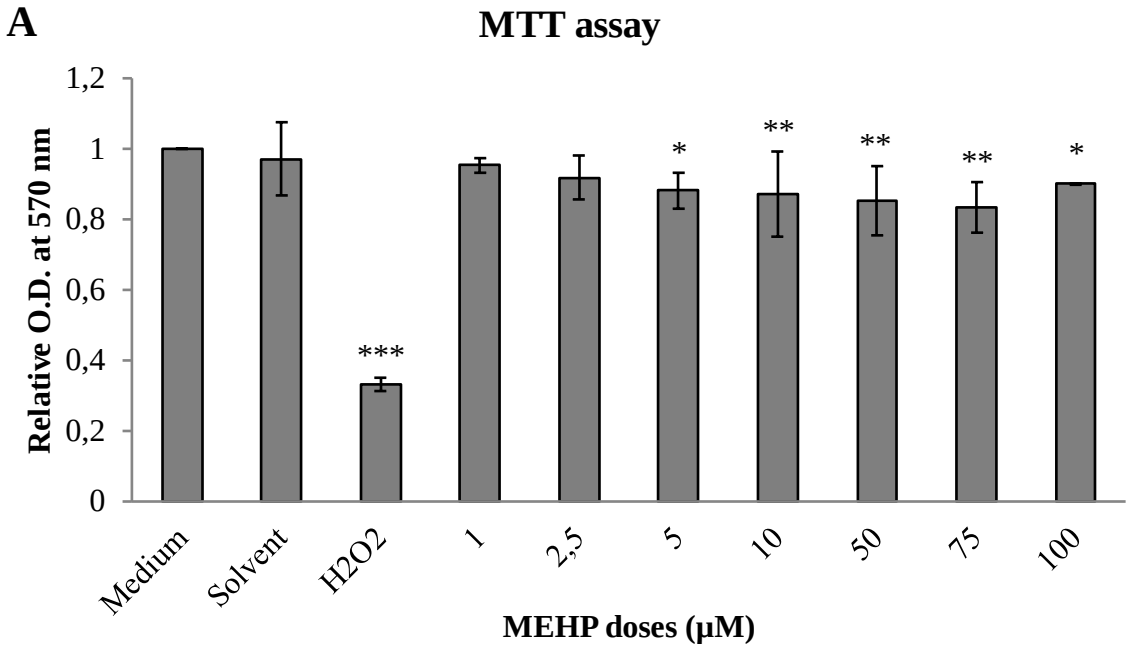
3.1 Cell viability

3.1.1 MTT assay

Figure 1A shows DLEC cell line viability at 24 h of MEHP treatment measured by MTT assay. The solvent shows no effect on DLEC cell survival, whereas H_2O_2 (100 µM) was highly toxic with a decrease in cell survival of 66.9%. A slight dose dependent reduction in DLEC viability after treatment with different MEHP doses was observed. Decreasing survival rates of MEHP treatments shows a statistically significant difference with respect to the solvent ($p < 0.05$; $p < 0.01$) starting from 5 µM of MEHP.

3.1.2 Trypan Blue Exclusion Assay

Results of TBE assay are illustrated in Figure 1B. The solvent shows no effect on DLEC cell survival, H_2O_2 (100 µM) was highly toxic with a decrease in cell survival of 28.8%. A slight dose dependent reduction in DLEC viability after treatment with different MEHP doses was observed, ranging between 91.9% (1 µM) and 85.7% (100 µM). Survival decrease rates of MEHP treatments show no statistically significant difference with respect to the solvent, except at the higher doses of 75 µM and 100 µM ($p < 0.05$).



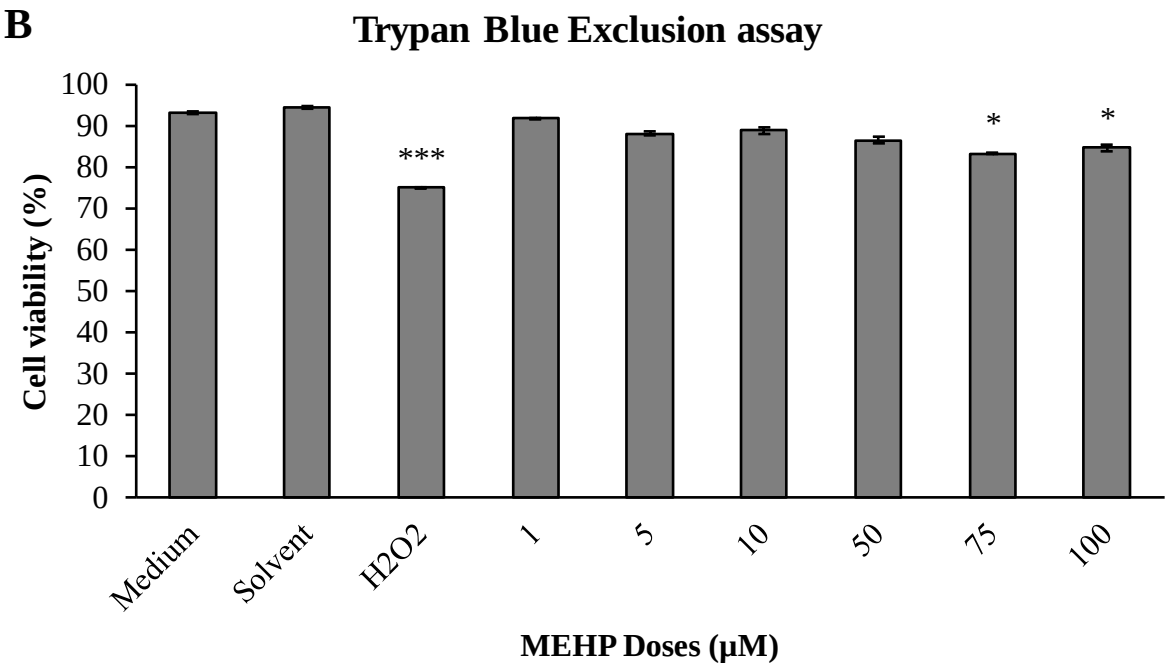


Fig. 1: Cell viability in MEHP-exposed DLEC cell line determined by MTT and TBE assays. **A)** MTT assay. Results are displayed as a mean of the optical density at 570 nm at each MEHP treatment level normalized to the solvent. The data are expressed as mean ± SD of two experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **B)** TBE assay. Cell viability is expressed as the percent of viable cells out of the total cells at each treatment level. The data are expressed as mean ± SE of two experiments. * $p < 0.05$; *** $p < 0.001$.

3.2 Cell death analysis

The apoptotic response of DLEC cells treated with different doses of MEHP is shown in Figure 2. In the medium and solvent samples, no significant increase of necrotic and apoptotic cells was observed. H_2O_2 treatment (100 µM) resulted in a moderate induction of necrotic cells and no increase in apoptotic cells; the increase of necrotic cells was statistically significant with respect to the medium ($p < 0.01$). Necrosis and apoptosis induction in MEHP treatments were not statistically significant in comparison with the solvent, except for necrosis induction at the highest dose ($p < 0.05$).

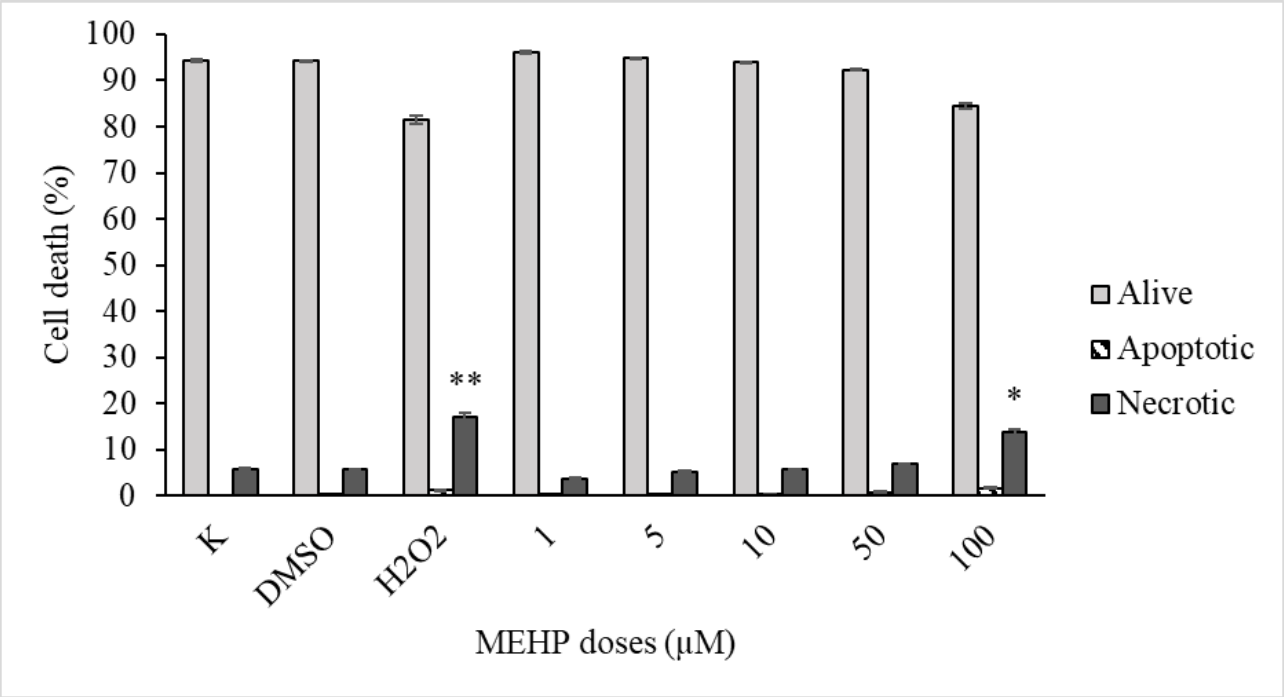


Fig. 2: Cell Death assay. Percentage of apoptosis and necrosis in DLEC cells treated with MEHP for 24 h. Data are presented as means ± SE of two independent experiments for each treatment. * $p < 0.05$; ** $p < 0.01$.

3.3 Comet assay

Results of induction in primary DNA damage obtained in DLEC cells treated with MEHP are shown in Figure 3. Control DLEC cells show a mean tail moment (TM) of 0.90. Treatment with solvent slightly increases the mean TM value to 1.17 with respect to the medium. H_2O_2 (100 µM) significantly increased the frequency of DNA damage as detected by a mean TM of 5.54 ($p < 0.001$). Treatment with MEHP showed no increase of mean TM at all doses.

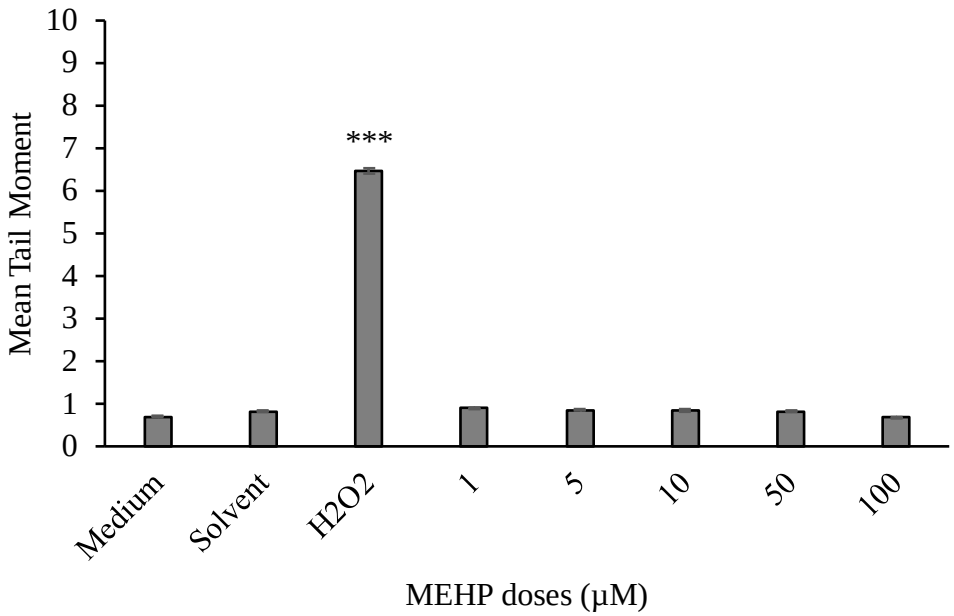


Fig. 3: DNA damage in DLEC cell line after 24 h exposure to MEHP. Data are presented as means ± SE of two independent experiments for each treatment. *** $p < 0.001$.

3.4 CBMN assay

Results from CBMN assay are presented in Table 1. No statistically significant difference either in the frequency of MN or in CBPI values in samples treated with the solvent, in comparison to the control, was observed. Treatment with H_2O_2 (100 µM) caused a statistically significant increase in the frequency of MN ($p < 0.001$), a significant decrease in the CBPI value ($p < 0.001$), and an increase in the percentage of cytostasis when compared to the medium.

With regard to MEHP treatments, data show a dose-dependent increase in the frequency of MN in comparison to the solvent. Student's t-test analysis shows a statistically significant increase in the yield of micronuclei per cell in all MEHP treatments ($p < 0.001$) compared to the solvent. The percentage of cytostasis was statistically significant at all MEHP doses ($p < 0.001$) with respect to the solvent. CBPI values show a slight dose-dependent decrease at all MEHP doses with respect to the solvent.

Table 2 shows the induction of MN in mononucleate cells. No statistically significant difference in the frequency of MN in samples treated with the solvent, in comparison to the control, was observed. Treatment with H_2O_2 (100 µM) caused a slight but statistically significant ($p < 0.05$) increase in the frequency of MN when compared to the medium.

With regard to the comparison of micronuclei induced by the two phthalates, it is worth noting that MEHP treatments show a statistically significant dose-dependent ($p < 0.01$; $p < 0.001$) increase in the frequency of MN in mononucleated cells, while in the treatment with DEHP (10 µM), no

statistically significant difference in the frequency of MN, when compared to the solvent, was observed.

Table 1. Induction of micronuclei (MN), Cytokinesis Block Proliferation Index (CBPI) and % of Cytostasis in DLEC cell line treated for 24 h with MEHP. Data are presented as means ± SE of two independent experiments for each treatment.

Treatment	Harvesting time after cyto-B	MN/1000 BN±S.E.	ts	CBPI±S.E.	χ_2	% Cytostasis±S.E.
Medium	48 h	12.0 ± 0.05		1.36 ± 0.0003		0 ± 0.00
Solvent	48 h	13.2 ± 0.08	NS	1.36 ± 0.0006	NS	0 ± 0.01
H_2O_2	48 h	35.8 ± 0.05	§§§	1.26 ± 0.0001	§§§	26.3 ± 0.07
0.5 µM	48 h	20.7 ± 0.08	**	1.32 ± 0.0002	***	12.6 ± 0.11
1 µM	48 h	25.3 ± 0.03	***	1.31 ± 0.0001	***	13.6 ± 0.16
2.5 µM	48 h	26.5 ± 0.09	***	1.29 ± 0.0003	***	21.1 ± 0.06
5 µM	48 h	32.8 ± 0.20	***	1.28 ± 0.0002	***	22.3 ± 0.12
10 µM	48 h	42.7 ± 0.26	***	1.29 ± 0.0005	***	19.4 ± 0.02

Significance of t-student test (ts) and Chi-squared test (χ_2): NS: not significant; ** $p < 0.01$ treated vs solvent; *** $p < 0.001$ treated vs solvent; §§§ $p < 0.001$ H_2O_2 vs medium

Table 2. Induction of micronuclei (MN) in mononucleate cells in DLEC cell line treated for 24 h with MEHP and one dose of DEHP. Data are presented as means ± SE of two independent experiments for each treatment.

Treatment	Harvesting time after cyto-B	MN/1000 Mono±S.E.	ts
Medium	48 h	10.3 ± 0.06	
Solvent	48 h	11.3 ± 0.09	NS
H_2O_2	48 h	15.2 ± 0.01	§
MEHP 0.5 µM	48 h	22.0 ± 0.09	**
MEHP 1 µM	48 h	28.3 ± 0.05	***
MEHP 2.5 µM	48 h	33.8 ± 0.10	***
MEHP 5 µM	48 h	40.3 ± 0.05	***
MEHP 10 µM	48 h	50.7 ± 0.11	***
DEHP 10 µM	48 h	12.9 ± 0.24	NS

Significance of t-student test (ts): NS: not significant; ** $p < 0.01$ treated vs solvent; *** $p < 0.001$ treated vs solvent; § $p < 0.05$ H_2O_2 vs medium

4. Discussion

Mono(2-ethylexyl) phthalate (MEHP) is the primary DEHP metabolite and is also one of the most studied since it is responsible for many of the effects of DEHP (Albro, 1986; Caldwell, 2012; Wang et al., 2012; Tetz et al., 2013). The presence of phthalates in the marine environment has aroused great concern for aquatic organisms, due to the growing threat posed by the plastic marine litter. However, to the best of our knowledge, the potential for MEHP to cause *in vitro* toxicity to cells of marine fishes has been scarcely investigated, whereas a number of adverse effects resulting from exposure to this compound have been reported *in vivo* and *in vitro* in humans and other organisms. In the current study, the effects of *in vitro* exposure to MEHP on the European seabass embryonic cell line, DLEC, including cytotoxicity, cell death, and genotoxicity were investigated, and the obtained results indicate a clear genotoxic effect of this compound.

The cytotoxicity of MEHP was assessed using the MTT and TBE assays. A slight but significant decrease in cell viability was observed at MEHP doses between 5 and 100 µM and 75 and 100 µM respectively in the MTT and TBE assay. Although there was some variability in cell survival results, probably due to a different sensitivity of the two tests (Wilking et al., 2014), both assays agree upon showing a minor cytotoxic effect of MEHP on DLEC cell line with respect to DEHP. These results are also confirmed by a weak and not significant induction of apoptosis and necrosis at all tested concentrations of this metabolite. In literature, large differences in sensitivity to MEHP and in its resulting cytotoxicity have been noted in both human and rodent cell lines (Caldwell, 2012). Some *in vitro* studies have reported various indicators of cytotoxicity starting from low concentrations of MEHP. For example, Erkekoğlu and colleagues (2010a, 2010b) reported a cytotoxic effect of MEHP, after 24 h of exposure, in MA-10 (mouse Leyding tumour) and LNCaP (human prostatic cancer) cell lines starting from 3 µM treatment. On the other hand, other studies detected scarce cytotoxic effects of MEHP on rodent or human cell lines, if not at higher doses. In GCs (rat ovarian granulosa cells) and HepG2 (human liver) cell lines, a significant decrease in cell viability at 50 µM MEHP has been reported (Yang et al., 2012; Li et al., 2018), whereas HRT-8/SVneo (human placenta) cell line were more resistant with a decrease in cell viability at a MEHP dose of 180 µM. The decreased viability of both HepG2 and HRT-8/SVneo was associated with an increase in apoptotic cells starting from 100 µM MEHP (Yang et al., 2012; Meruvu et al., 2016). Great variability in the toxicity range of MEHP can also occur within the same cell line. Other studies on the MA-10 cell line, indeed, reported significantly different results than Erkekoğlu and colleagues (2010a, 2010b), by finding a cytotoxic response starting at 300 µM (Fan et al., 2010) or at even higher doses, such as 1 to 3 mM of MEHP (Dees et al., 2001; Piché et al., 2012), coupled with occasional encounters of apoptotic cell bodies in all MEHP tested treatments (Dees et al., 2001). The reasons for these discrepancies might lie in

several factors: differences in the experimental designs, cell culturing conditions, cell density/number, cell source, purity of the MEHP, employment of secondary compounds (Erkekoğlu et al., 2010a), as well as the different responses of the diverse cell lines to this metabolite.

In the present study, genotoxicity was assessed through both the Comet and the Cytokinesis-Block MicroNucleus assays, and the results of the latter clearly indicate a genotoxic effect of MEHP in DLEC embryonic cells. Indeed, induction of DNA strand breaks revealed by the Comet assay was not high, with a slight significant increase just at the highest concentrations of the phthalate (100 µM), while the treatment with H_2O_2, a clastogenic agent, induced a significant higher yield of DNA strand breaks. This outcome suggests that MEHP does not have a clastogenic effect in the DLEC cell line. Conversely, from the data of the CBMN assay, MEHP was found to display a slight cytotoxicity and a minor influence on cell proliferation, but a high and significant dose-dependent increase in the frequency of MN, while H_2O_2 induced a lower increase in the frequency of MN, with respect to all MEHP treatments. Besides, treatment with MEHP induced a dose-dependent enhancement of MN not only in the binucleated but also in the mononucleated cells, strongly suggesting an anuegenic action of this compound in the DLEC cell line. The genotoxic effects of DEHP and MEHP have been investigated in a number of different tissues and with various genotoxicity assays (Caldwell, 2012; Štrac et al., 2013). MEHP genotoxic potential has been investigated in several studies by means of the Comet assay. For instance, Erkekoğlu and colleagues (2010a, 2010b) reported high levels of DNA damage associated with an increase in both Tail Moment and Tail Intensity by several folds at very low doses of MEHP (3 µM). Other authors detected an enhancement of DNA migration only at higher doses of MEHP (Kleinsasser et al., 2004; Chang et al., 2017) and also a relationship between urinary concentrations of phthalate metabolites, including MEHP, and sperm DNA damage in humans (Hauser et al., 2007). However, to our current knowledge, no studies have been conducted on the induction of micronuclei by MEHP. The micronucleus assay is a methodology that allows to obtain a measure of both chromosome breaks and whole chromosome loss (Fenech, 1993). With the CBMN assay, it is possible to detect between 60% and 90% of acentric fragments and, in combination with kinetochore/centromere detection or other genotoxicity assay (e.g. Comet assay), it is an optimal procedure for measuring whole chromosome loss events (Fenech, 1993; Araldi et al., 2015). Moreover, scoring MN in mononucleated cells could be a further end-point able to distinguish agents with clastogenic action from aneugenic ones (Elhajouji et al., 1998). Indeed, Elhajouji and co-workers (1995) first and later Kirkland (2010) demonstrated that increasing MN in mononucleated cells is a clear and sensitive index for detection of aneugenic compounds. Therefore, given the results of the Comet and CBMN assays, it can be hypothesized that the dose-dependent increase in the frequency of the MN caused by MEHP treatments on DLEC cell line could represent chromosomal loss events

rather than chromosomal breaks. Thus, to the best of our knowledge, this is the first experimental evidence of an aneugenic effect of MEHP.

5. Conclusion

The overall results of this study show a significant difference between the cytotoxic and genotoxic effects of DEHP and MEHP on the European sea bass embryonic cell line. In our previous study (Molino et al., 2019) we found that the cytotoxicity of DEHP was much higher compared to that caused by its metabolite. Even the induction of apoptosis and necrosis were significantly higher after treatment with the precursor compound when compared to MEHP. Since pollutant metabolites are usually more toxic than their precursors, an explanation of the higher DEHP cytotoxicity could be probably a synergic effect of both DEHP itself and its metabolites, including barely MEHP (Ye et al., 2014a). On the other hand, the induction of MN was very high for both phthalates, displaying half of the micronuclei induced by MEHP, with respect to those induced by DEHP at the same concentration (10 µM). This outcome, coupled with the slight or absent induction of DNA strand breaks by DEHP and MEHP, respectively, suggests that the possible aneugenic effect on DLEC cell line could be mostly ascribed to MEHP. Under this hypothesis, MEHP should probably be considered even more harmful than its precursor, because it induces genomic instability in the DLEC cell line at lower doses and without triggering cell death. In conclusion, our study shows that MEHP as the primary DEHP metabolite, which is ubiquitous to the marine environment, is an even more dangerous threat for the European sea bass and probably for other marine organisms.

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4. Chapter 3: *In vitro* effects of DEHP on Bottlenose dolphin (*Tursiops truncatus*) and Chinese Hamster Ovary (CHO) cell lines

4.1 Cetaceans and effects of marine litter

Cetaceans are long living species, that occupy different trophic levels in the food chain. Most cetaceans are wide ranging, showing seasonal movements and changes in habitat use, which can be very variable even at individual level. Due to their position at the top of the food chain, the low rate of their metabolism and the presence of a layer of blubber, which accumulates lipophilic pollutants (Carvan III et al., 1994), cetaceans bioaccumulate high quantities of marine contaminants. Furthermore, contaminants, besides having a cytotoxic and genotoxic potential on these organisms (Marsili and Focardi, 1977), may act as immunosuppressant, as they lead to a decrease in leukocytes proliferation, potentially increasing cetacean's disease susceptibility (Beineke et al., 2010). For these reasons, cetaceans are considered good indicators of the status of marine waters and are protected under an extensive legislative framework (CITES, 1973; Convention, 1979; Convention on Biological Diversity, 1992; Habitat Directive, 1992; ACCOBAMS, 2001; Marine Strategy Framework Directive, 2008).

Considering cetacean's high rates of accumulation of xenobiotics, in the last 25 years the toxic effects of these persistent pollutants have raised great concerns. As previously mentioned, the blubber layer plays a fundamental role in the accumulation and the toxic effects of contaminants. Indeed, lipophilic pollutants tend to accumulate in this layer, rendering their subsequent excretions difficult (Wagemann and Miur, 1984). Bioaccumulation of pollutants it's also strictly correlated with the age and reproductive status of an individual. The levels of contamination in males of many species result to increase with their age and, in general, to be greater than those of sexually mature females. This difference can be explained since sexually mature females transfer appreciable amounts of persistent contaminants to the offspring during gestation and lactation (Wagemann and Miur, 1984). These characteristics are unique to cetaceans and can have a strong impact on their state of conservation and well-being.

In more recent years, many studies have associated the death of many cetaceans to the ingestion of marine litter, mainly plastic debris (Derraik, 2002). The massive presence of plastics debris in the marine environment causes negative effects of physical nature due to ingestion and entanglement. Furthermore, plastic debris became a vector of pollutants that bind to their surfaces or that are already present in their composition (e.g. additives). Although plastic litter has become a serious worldwide environmental concern, plastic debris accumulates in semi-enclosed basins to a greater degree than in the open oceans. It has been estimated that in the Mediterranean Sea plastic debris has reached

maximum levels of 892,000 particles/km^2 (Collignon et al., 2012; Fossi et al., 2012) rendering this form of persistent pollution very hazardous for both wildlife and the marine ecosystem (Collignon et al., 2012). Indeed, previous studies have highlighted that bioaccumulation of contaminants (e.g. organochlorine) tend to be greater in marine mammals from enclosed basins, particularly in cetacean odontocetes (Aguilar et al., 2002). Plastic litter is principally carrier of contaminants such as polycyclic aromatic hydrocarbons and phthalates, which are its principal constituents. In recent years, phthalates heave raised many concerns due to their ubiquity into the marine environment and, furthermore, due to their influence on multiple biochemical processes, both in humans and wildlife (Caldwell, 2012; Ye et al., 2014; Rowdhwal and Chen, 2018). For these reasons, in recent years phthalates have been used in many studies as tracers to the intake of microplastic by marine mammals. Fossi and collaborators (2012) have detected high concentrations of MEHP, ranging from 1.00 ng/g to 99.93 ng/g, in the blubber of five stranded fin wales. The results suggested that the fin whales could have suffered from chronic exposure to these persistent contaminants as a result of microplastic ingestion. This study represented one of the first evidence of the potential impact of phthalates in a baleen whale in the Mediterranean Sea.

Despite many studies evaluate the presence and the harmful effects of phthalates, to date, probably due to the difficulty to obtain samples from wild marine mammals, the physiological and toxicological effects of plastic ingestion and the resulting transfer of persistent pollutants are still poorly investigated and understood (Fossi et al., 2012).

4.2 Bottlenose dolphin and *Tursiops truncatus* cell line

Bottlenose dolphin (*Tursiops truncatus*), of the Delphinidae family, is a cosmopolitan odontocete cetacean that inhabits tropical and temperate regions and inhabits both coastal and pelagic areas (Yu et al., 2005). This species feeds manly on fishes and search for prey primarily using echolocation. Natural and anthropic factors may pose a threat to the well-being of marine mammalian communities, especially those inhabiting coastal waters, due to exposure to runoff of agricultural and industrial chemicals, wastewater, marine litter and possible ingestion of contaminated prey. Increase in number and frequency of bottlenose mortality has led to increased concern about the potential role of environmental pollutants in the disappearance of the marine mammal community (Carvan III et al., 1994). These events are worrisome not only for local populations of marine mammals, but also because they can be indicators of the state of coastal ecosystems. Since bioaccumulation of contaminants, such as phthalates, tend to be greater in cetacean odontocetes (Aguilar et al., 2002) and since the concentration of contaminants is influenced by the trophic level at which the species feed

(Borrell, 1993), it stand to reason that the bottlenose dolphin, as an inshore and predator species, could be particularly exposed to the risks associated with plastic pollution.

In vitro culture of cells of marine mammals has been shown to be difficult, due to the nature of cetacean cells and specimen limitations as they are protected species and there are rare opportunities for sampling tissue samples, suitable for cell culture, from living individuals. Only a few of successes have been reported in cellular cultures of marine mammals. Some cell lines derived from kidney, lung, and skin tissues of cetaceans have been developed and reported to be suitable for genomic analysis and *in vitro* toxicological studies (Carvan III et al., 1994; Pine et al., 2004; Yu et al., 2005; Garrick et al., 2006; Marsili et al., 2012; Jin et al., 2013) to evaluate the effects of contaminants on this species. In this study, a *T. truncatus* cell line has been selected to investigate the effects of phthalates, in particular of DEHP at cellular level and to determine the impact of these pollutants on this coastal species of high ecological value. Moreover, since the *T. truncatus* cell line has not been used for cytotoxic and genotoxic studies, results were compared and discussed with DEHP effects detected on the standardized Chinese Hamster Ovary cell line (CHO).

4.3 Materials and Methods

4.3.1 Cell cultures and DEHP treatments

The *T. truncatus* cell line (TT) is a cell line (provided by the Bank for Mediterranean Marine Mammals, University of Padua, Italy) derived from the skin of a 40 years old dolphinarium individual, died of natural causes. This cell line is characterized by two different populations of cells: a population with a compact, round cell body with short extroflexions probably related to cells of epithelioid origin (endothelial-like cells); the second presents a more irregular, often fusiform form with an elongated body, typical of fibroblasts (fibroblastic-like cells). These adherent cells are left in culture for about 3-4 days in order to reach a good density (90-100 %) for the sub-culture steps. Cells are grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% L-Glutamine, and 1% Penicillin/Streptomycin and maintained in an incubator at 37 °C with 5 % of CO_2.

Chinese hamster ovary (CHO) is an epithelial line derived from the ovary of Chinese hamster. CHO are cultivated in Ham’s F10 medium, supplemented with 10% FBS, 1% L-Glutamine, and 1% Penicillin/Streptomycin, and grown in an incubator at 37°C with 5 % of CO_2. This cell line has been chosen since routinely used in mutagenicity testing as indicated in the guidelines of EEC Council 79/831.

For cell viability assays, both TT and CHO cell lines were treated with 0.01, 0.02, 0.05, 0.2, 0.5, 2, and 5 mM of DEHP for 24 h. Solvent is represented by 1% DMSO and positive control cells were

treated with 100 µM H_2O_2 for 1 h in medium without FBS. Doses of DEHP used to assess DNA and chromosomal damage were chosen based on the results of cell viability assays.

4.3.2 Cell viability assay

4.3.2.1 MTT assay

Cell viability was assessed by MTT, as described in Molino et al. (2019). TT cells were seeded onto 96-well microplates at a density of 5 x 10^3 cells/well, whereas CHO where seeded at a density of 1 x 10^3 cells/well. Both cell lines were incubated at 37 °C during the experiment. Viability was determined in two independent experiments conducted in triplicate for each experimental condition for both cell lines. Cell viability, presented as the Relative O.D. at 570, was calculated using the formula: = Absorbance of treated cells/Absorbance of control cells.

4.3.2.2 Trypan Blue exclusion assay

Trypan Blue exclusion assay was performed as described in Molino et al. (2019). TT and CHO cells were seeded in 35 mm petri dishes at a density of 1.5 x 10^5 and 1 x 10^5, respectively. Viability was determined in two independent experiments. The percentage of cell viability was calculated as the mean of the cell survival in the two experiments.

4.3.3 Detection of DEHP-induced cell death by fluorescence staining

TT and CHO cells were seeded in 35 mm petri dishes at the same density of the TBE assay. The DEHP-induced cell death by fluorescence staining was performed as described in Molino et al. (2019). The results of two independent experiments showing good reproducibility and comparable outcomes were considered.

4.3.4 Single Cell Gel Electrophoresis (SCGE) analysis

Standard alkaline (pH > 13) SCGE was performed according to Molino et al. (2019). Cells were seeded in 35 mm petri dishes at the same density of the TBE assay. Both TT and CHO cells were treated with 0.02, 0.05, 0.2, 0.5, 2, and 5 mM of DEHP. To evaluate DNA damage, computer-generated tail moment (TM) values were used and the mean of the results of two independent experiments showing good reproducibility and comparable outcomes were considered.

4.3.5 Cytokinesis-Block MicroNucleus (CBMN) assay

The CBMN assay was carried out according to Molino et al. (2019). TT and CHO cell lines were seeded in 60 mm petri dishes at a density of 2.5 x 10^5 and 3 x 10^5, respectively. After 24 h, cells were

treated with 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 mM of DEHP. Then, cells were washed with PBS and fresh medium containing 6 µg/mL of Cytochalasin B was added for 24 h to arrest cell cytokinesis. Only for medium, H_2O_2 and 0.01 mM of DEHP scoring of micronuclei (MN) was performed in both 1000 mononucleated and 1000 binucleated cells. The mean of the results of two independent experiments showing good reproducibility and comparable outcomes is shown.

4.3.6 Statistical analysis

The statistical analysis for MTT, TBE, and apoptotic/necrotic cell death assays (treatments vs solvent) was performed by use of χ2-test. The analysis of the significance of the Comet test, in terms of differences in mean tail moment between DEHP treatments and the solvent sample was performed by applying the Student's t-test for paired samples. The statistical significance in the yield of micronuclei per cell and the cytostatic effect between the solvent and treated samples was evaluated by the Student's t-test and the Chi-squared test, respectively. The level for statistical significance was set at $p < 0.05$.

4.4. Results

4.4.1 Cell viability

4.4.1.1 MTT assay

Figure 4.1 shows TT and CHO cell lines viability at 24 h of DEHP treatment. In both experiments, the solvent shows no significant effect on the cell lines survival, whereas H_2O_2 (100 µM) was highly toxic with a decrease in cell survival of ~ 70%. A dose dependent reduction in TT cells viability (Fig. 4.1 A) after treatment with different DEHP doses was observed, displaying a statistically significant difference with respect to the solvent starting from 0.02 mM of DEHP ($p < 0.01$). CHO cell line (Fig. 4.1 B) shows a dose dependent and statistically significant decrease with respect to the solvent at all doses of DEHP.

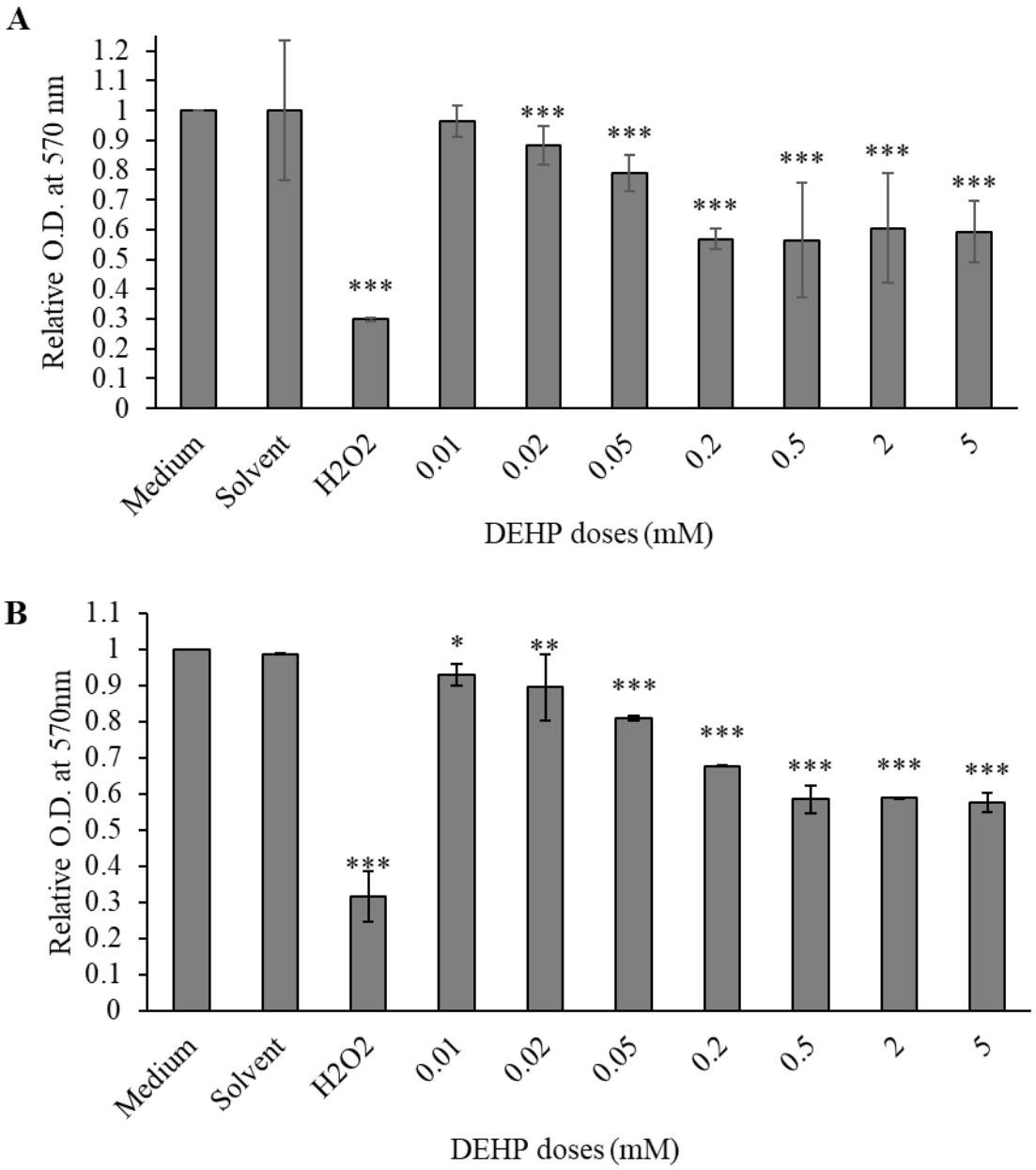


Fig. 4.1: Cell viability in (**A**) TT and (**B**) CHO cell lines determined by MTT. Results are displayed as a mean of the optical density at 570 nm at each DEHP treatment level normalized to the Solvent. The data are expressed as mean ± SD of two experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.4.1.2 TBE assay

Results of TBE assay are illustrated in Figure 4.2. The solvent shows no significant effect on cell survival of both cell lines. H_2O_2 (100 µM) was highly toxic with a decrease in cell survival of 24.5% for TT cells and of 26.8% for CHO cells. A dose dependent reduction in TT viability (Fig. 4.2A) after treatment with different DEHP doses was observed, ranging between 89% (0.01 mM) and 63.1% (5 mM). Survival decrease rates of DEHP treatments show statistically significant difference with

respect to the solvent starting from 0.2 mM ($p < 0.05$). CHO cell line (Fig. 4.2B) shows a higher sensitivity to DEHP, displaying a dose dependent and statistically significant decrease with respect to the solvent at all doses of DEHP ($p < 0.001$).

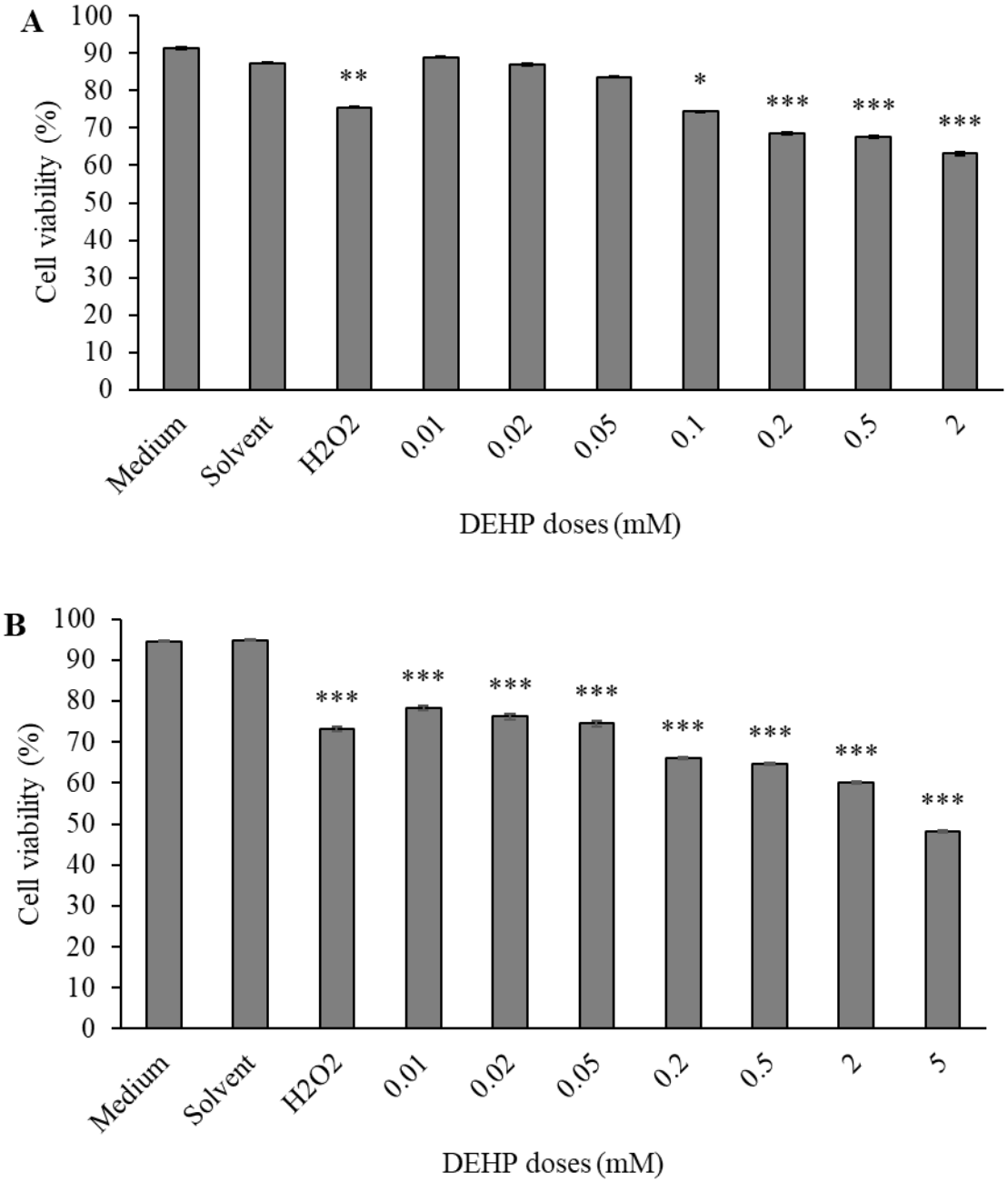
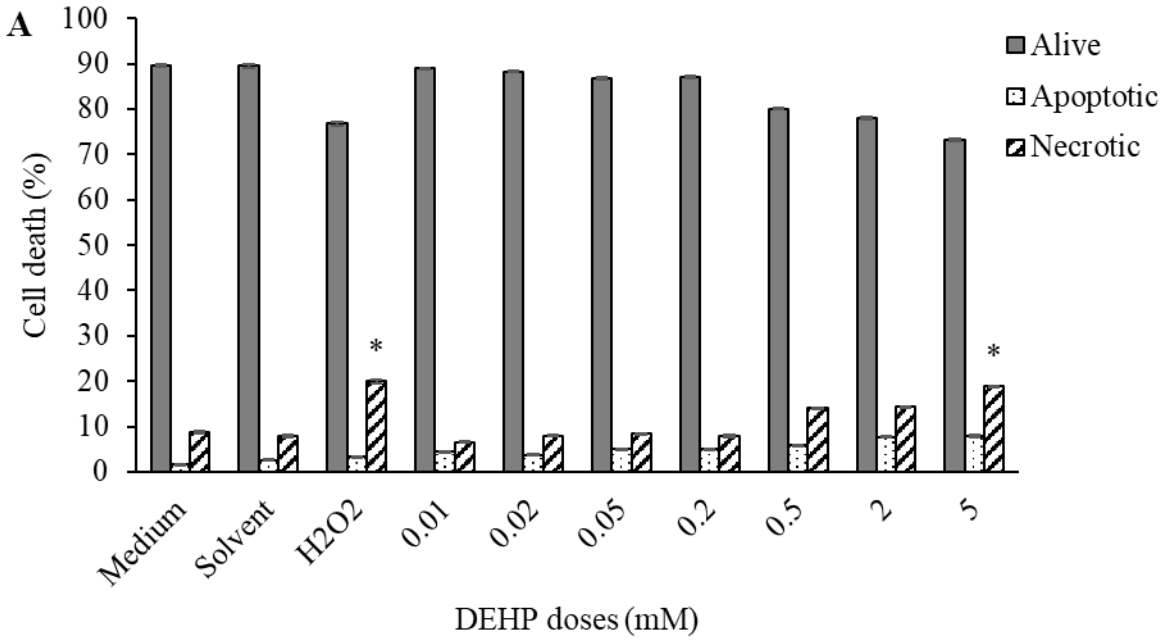


Fig. 4.2: Cell viability in (**A**) TT and (**B**) CHO cell lines determined by Trypan Blue Exclusion assay. Cell viability is expressed as the percent of viable cells out of the total cells at each treatment level. The data are expressed as mean ± SE of two experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.4.2 Cell death analysis

The apoptotic response of TT and CHO cells treated with different doses of DEHP is shown in Figure 4.3A and 4.3B, respectively. For TT cell line, a slight increase in necrotic cells in the medium and solvent samples was observed, of 8.7% and 7.9%, respectively; no significant increase in apoptotic cells was observed. In the medium and solvent samples of CHO, no significant increase of necrotic and apoptotic cells was observed. H_2O_2 treatment (100 µM) resulted in a moderate induction of necrotic cells and no increase in apoptotic cells in both cell lines; the increase of necrotic cells was statistically significant with respect to the medium ($p < 0.05$). Necrosis and apoptosis induction in DEHP treatments in TT cell line were not statistically significant in comparison with the solvent, except for necrosis induction at the highest dose ($p < 0.05$). For CHO, necrosis induction was statistically significant in comparison with the solvent ($p < 0.001$) with a clear dose-dependent increase. Apoptosis induction was very slight and not statistically significant.



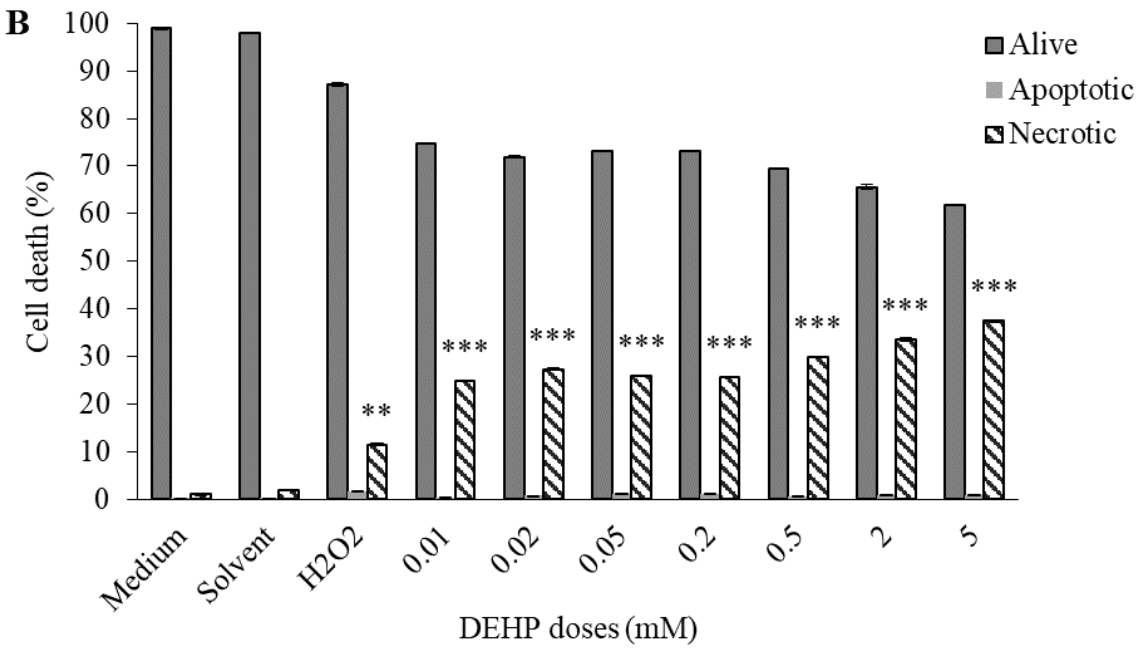


Fig. 4.3: Cell Death assay. Percentage of apoptosis and necrosis in TT (**A**) and CHO (**B**) cell lines treated with DEHP for 24 h. Data are presented as means ± SE of two independent experiments for each treatment. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4.4.3 Comet assay

Results of induction in DNA primary damage obtained in TT and CHO cells treated with DEHP are shown in Fig. 4.4A and 4.4B, respectively. Control TT and CHO cells show a mean tail moment (TM) of 1.70 and 0.91, respectively. In both cell lines, treatment with solvent did not increase significantly the mean TM value with respect to the medium. H_2O_2 (100 µM) significantly increased the frequency of DNA damage as detected by a mean TM of 5.05 for TT ($p < 0.001$) and 3.59 for CHO ($p < 0.001$). Even thought there was a slight variability for both cell lines, treatment with DEHP shows no dose-dependent nor statistically significant increase of mean TM for all treatments in comparison with the solvent.

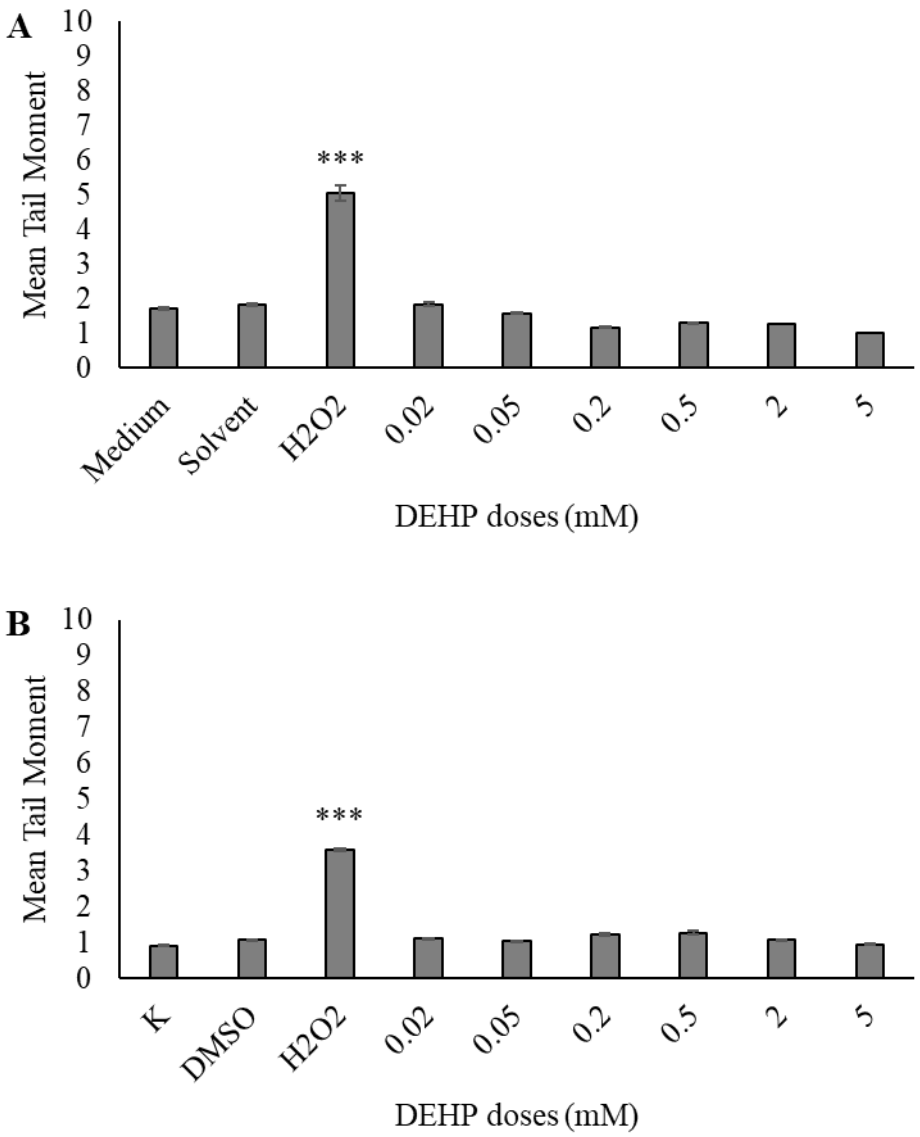


Fig. 4.4: Mean Tail Moment in TT (**A**) and CHO (**B**) cell lines after 24 h exposure to DEHP. Data are presented as means ± SE of three independent experiments for each treatment. *** $p \leq 0.001$.

4.3.4 CBMN assay

Results from CBMN assay in TT cell line are presented in Table 4.1 for TT and 4.2 for CHO. For both cell lines, no statistically significant difference either in the frequency of MN or in CBPI values in samples treated with the solvent, in comparison to the control, was observed. Treatment with H_2O_2 (100 µM) caused a statistically significant increase in the frequency of MN ($p < 0.001$), a significant decrease in the CBPI value ($p < 0.001$), and an increase in the percentage of cytostasis when compared to the medium.

For TT cell line, data show a plateau trending increase in the frequency of MN in comparison to the solvent. Student's t-test analysis shows a statistically significant increase in the yield of

micronuclei per cell in all DEHP treatments ($p < 0.001$) compared to the solvent. CBPI values show a dose-dependent decrease and the percentage of cytostasis was statistically significant ($p < 0.001$) with respect to the solvent, starting from the lowest dose. CBPI and induction of MN could not be analysed at 0.2 and 0.5 mM of DEHP due to the lack of binucleated cells.

For CHO cell line, data show a dose-dependent increase in the frequency of MN in comparison with the solvent. A statistically significant (Student's t-test) increase in the yield of micronuclei per cell ($p < 0.001$) was detected, while MN frequencies could not be analysed at 0.2 and 0.5 mM of DEHP due to the paucity of binucleated cells. CBPI values show a dose-dependent decrease at all DEHP doses and the percentage of cytostasis was statistically significant at all DEHP doses ($p < 0.01$; $p < 0.001$) with respect to the solvent.

Table 4.3 shows the induction of MN in mononucleate cells. For both samples treated with H_2O_2 (100 µM) and DEHP (0.01 mM) no difference was observed in the frequency of MN in when compared to the medium.

Table 4.1. Induction of micronuclei (MN), Cytokinesis Block Proliferation Index (CBPI) and % of Cytostasis in TT cell line treated for 24 h with DEHP. Data are presented as means ± SE of two independent experiments for each treatment.

Treatment	Harvesting time after cyto-B	MN/1000 BN±S.E.	ts	CBPI±S.E.	χ_2	% Cytostasis±S.E.
Medium	24 h	21.5 ± 0.03		1.65 ± 0.0006		0 ± 0.00
Solvent	24 h	19.5 ± 0.08	NS	1.64 ± 0.0007	NS	2.4 ± 0.04
H_2O_2	24 h	75.2 ± 0.72	§§§	1.31 ± 0.002	§§§	53.0 ± 0.40
0.01 mM	24 h	35.8 ± 0.18	***	1.54 ± 0.001	***	16.3 ± 0.13
0.02 mM	24 h	49 ± 0.28	***	1.52 ± 0.002	***	16.6 ± 0.19
0.05 mM	24 h	45.8 ± 0.09	***	1.49 ± 0.001	***	22.4 ± 0.10
0.1 mM	24 h	51.3 ± 0.04	***	1.31 ± 0.002	***	50.2 ± 0.36
0.2 mM	24 h	/	/	/	/	/
0.5 mM	24 h	/	/	/	/	/

Significance of t-student test (ts) and Chi-squared test (χ_2): NS: not significant; *** $p \leq 0.001$ treated vs solvent; §§§ $p \leq 0.001$ H_2O_2 vs medium

Table 4.2. Induction of micronuclei (MN), Cytokinesis Block Proliferation Index (CBPI) and % of Cytostasis in CHO cell line treated for 24 h with DEHP. Data are presented as means ± SE of two independent experiments for each treatment.

Treatment	Harvesting time after cyto-B	MN/1000 BN±S.E.	ts	CBPI±S.E.	χ_2	% Cytostasis±S.E.
Medium	24 h	17 ± 0.08		1.69 ± 0.002		0 ± 0.00
Solvent	24 h	18.3 ± 0.05	NS	1.67 ± 0.003	NS	3.4 ± 0.1
H_2O_2	24 h	84.6 ± 0.04	§§§	1.28 ± 0.0006	§§§	59.9 ± 0.06
0.01 mM	24 h	39.9 ± 0.12	***	1.67 ± 0.002	**	3.6 ± 0.03
0.02 mM	24 h	44.7 ± 0.15	***	1.62 ± 0.003	***	8.1 ± 0.02
0.05 mM	24 h	51.9 ± 0.06	***	1.57 ± 0.003	***	16.2 ± 0.04
0.1 mM	24 h	76.5 ± 0.18	***	1.44 ± 0.0002	***	33.2 ± 0.35
0.2 mM	24 h	/	/	1.16 ± 0.0006	***	75.7 ± 0.19
0.5 mM	24 h	/	/	1.09 ± 0.0003	***	85.4 ± 0.10

Significance of t-student test (ts) and Chi-squared test (χ_2): NS: not significant; ** $p \leq 0.01$ treated vs solvent; *** $p \leq 0.001$ treated vs solvent; §§§ $p \leq 0.001$ H_2O_2 vs medium

Table 4.3. Induction of micronuclei (MN) in mononucleate cells in TT and CHO cell lines treated for 24 h with H_2O_2 and one dose of DEHP. Data are presented as means ± SE of two independent experiments for each treatment.

		TT		CHO	
Treatment	Harvesting time after cyto-B	MN/1000 Mono±S.E.	ts	MN/1000 Mono±S.E.	ts
Medium	24 h	15 ± 0.07		15 ± 0.02	
H_2O_2	24 h	17 ± 0.07	NS	14 ± 0.00	NS
0.01 mM	24 h	17 ± 0.02	NS	17 ± 0.02	NS

Significance of t-student test (ts): NS: not significant

4.5 Discussion

Marine mammals, in general, and dolphins, in particular, have an extremely high rate of accumulation of contaminants, therefore the toxic effects of marine pollutants have raised concerns over the last 25 years. In particular, the growing threat posed by the plastic marine litter and the ubiquitous presence of its additives, particularly phthalates, in the marine environment, has recently received increasing attention. Due to the strong legislation aimed at protecting marine mammals (CITES, 1973; Convention, 1979; Convention on Biological Diversity, 1992; Habitat Directive, 1992; ACCOBAMS, 2001; Marine Strategy Framework Directive, 2008) and for obvious ethical reasons, *in vivo* studies on these species are essentially impossible to conduct. For this reason, *in vitro* cell lines afford the opportunity to study the cellular responses and toxicological effects of xenobiotics and to evaluate risks to wild organisms. In the current study, the effects of *in vitro* exposure to DEHP on a *T. truncatus* skin cell line, TT, and on the CHO cell line, were evaluated. The latter, being routinely used in mutagenicity testing as indicated in the guidelines of EEC Council 79/831, has been chosen for comparison. The results indicate a clear cytotoxic and genotoxic effect of this phthalate, on both cell lines.

Cytotoxicity results show a clear dose response with a decrease in cell viability. The TBE assays showed a slight lower sensitivity of TT cells: cell viability significantly decreased starting from 0.1 mM, whereas in CHO all treatment with DEHP resulted statistically significant. Furthermore, in the MTT, TT viability decrease tended to a plateau starting from the intermediate doses (from 0.2 mM), whereas in CHO viability decreased, although very slightly, at the higher doses as well (from 0.5 to 5 mM). These results are also confirmed by a weak induction of apoptosis and necrosis in TT cell line starting from 0.5 mM of DEHP and significant only at the higher dose of 5 mM, whereas in CHO cells, DEHP induced a significant increase of necrotic cells at all tested concentrations. It is interesting to notice that, in the two cell lines, treatment with H_2O_2 caused approximately the same number of necrotic cells and the same decrease in cell viability in both MTT and TBE assays. Hydrogen peroxide is a well known reactive oxygen species (ROS) and it can have immediate effects when diffusing through cells and tissues (Sies, 2017). Therefore, our results could be explained by a direct effect of H_2O_2 on TT and CHO cells, whereas DEHP requires metabolic activations to exhibit its toxic effect (Caldwell, 2012). The different response in cell viability and cell death of TT and CHO to DEHP exposure could be explained by a slightly higher resistance of TT to the phthalate. Indeed, it has been reported a greater resistance of marine mammal cells to the cytotoxicity of persistent pollutants, probably due to adaptations and development of more efficient detoxification mechanisms (Betti and Nigro, 1996; Chen et al., 2009; Chen et al., 2012; Gui et al., 2014). Even so, the results are in agreement with previous studies on other mammalian cell lines, which report that 24 h of exposure

to DEHP could significantly decrease cell viability (Erkekoğlu et al., 2010; Bhat et al., 2013; Peropadre et al., 2013) and increase cell death by necrosis (Martinasso et al., 2006; Caldwell, 2012). Furthermore, comparing the current results with those obtained in our study on the European sea bass embryonic cell line (Molino et al., 2019), the degree of toxicity to DEHP of the tree cell lines resulted in this decreasing order: DLEC > CHO > TT.

As far as genotoxicity results are concerned, a clear genotoxic potential of DEHP in TT and CHO cells is revealed. In both cell lines, the Comet assay showed no significant induction of DNA strand breaks caused by DEHP, while the treatment with H_2O_2 induced a significant higher yield of DNA strand breaks. Instead, results of the Cytokinesis-Block MicroNucleus assays showed a high cytotoxicity of DEHP starting from the dose of 0.01 mM, with a subsequent influence on cell proliferation and an increase in the frequency of MN. TT cell line displayed a gradual increase in the induction of MN, tending to a plateau, and a higher cytostatic effect when compared with CHO, suggested by the failure to analyse the CBPI at 0.2 and 0.5 mM. Indeed, an increase of cytostasis does not allow to detect the possible cytogenetic damage, leading to a plateau in the induction of MN in TT cells, whereas CHO cell line exhibits a clear dose-dependent increase in the frequency of MN. When comparing these data with DLEC results (Molino et al., 2019), it is evident a greater sensitivity in terms of chromosomal damage and apoptotic induction of DLEC with respect to both TT and CHO cell lines. This can be explained by a hypersensitivity of embryonic cell lines to DNA damaging agents leading to the onset of apoptosis after a considerable genotoxic insult (Heyer et al., 2000; Maynard et al., 2008; Momcilovic et al., 2010; Fan et al., 2011). Lastly, the analysis of the frequency of MN in TT and CHO mononucleated cells confirms what has been observed in the DLEC cell line (Ch. 2), further supporting the hypothesis that DEHP treatments could lead to chromosomal loss events, suggesting a genotoxic effect of DEHP stemming from its metabolism, in particular to MEHP.

In conclusion, the results of this study show a significant cytotoxic and genotoxic effects of DEHP on both TT and CHO cell line, occurring primarily as inhibition of cell proliferation and induction of micronuclei. Moreover, the current study further supports the hypothesis of a possible aneugenic effect of this compound. With this respect, our study additionally underlines that DEHP, a ubiquitous pollutant to the marine environment, is a potential threat for the bottlenose dolphin and possibly to all marine mammals, which are constantly exposed to plastic marine litter. Considering the legislative and ethical limitations concerning these species, our results confirm the necessity of *in vitro* studies to evaluate the potential threat of plastic additives and marine litter, in general, on marine mammal species.

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5. Conclusions

In environmental monitoring and in toxicology studies, marine organisms are effectively used to evaluate the effect of pollutants, since pollutants accumulate in their tissues, providing an integrated assessment of the bioavailability and of the biological effects of contaminants on biota. In this contest, cell lines of marine organisms are very effective instruments to the assessment of environmental stressors *in vitro*, and as such widely used.

In the framework of ever-increasing pollution due to the dispersion of plastic debris in the marine environment, coastal marine organisms result to be more exposed and vulnerable to anthropogenic pollutants derived from this persistent litter. Therefore, the assessment of the presence and toxicological effects of these contaminants allows to obtain a first evaluation of the threats to which those organisms are exposed and of their "state of health". In the current study, an investigation of the potential effects of one of the marine environment ubiquitous pollutant, di-(2-ethylhexyl) phthalate (DEHP), and its metabolite, mono-(2-ethylhexyl) phthalate, on two coastal species was carried out. *In vitro* tests were selected to assess the effects of the two phthalates at cellular, DNA and chromosomal level and to determine their impact on European sea bass and bottlenose dolphin, which are considered of high ecological and economic value. The results of this study provide a first indication of the threats of plastic additives on marine organisms, and, through them, on marine/coastal ecosystems.

The study on the toxic effects of DEHP on the European sea bass embryonic cell line (Ch. 1) highlighted the cytotoxic and genotoxic potential of this compound on this species. All the tests conducted showed that 24 h treatment of DEHP could significantly affect cell survivals, induce the apoptotic process and increase cell death due to necrosis. Furthermore, the moderate increase in DNA fragmentation and the higher increase in the frequency of micronucleus, accompanied by a significant and progressive inhibition of cell proliferation, suggested a possible aneugenic effect of DEHP on DLEC cell line. On the other hand, in the second study (Ch. 2) a significant difference between the cytotoxic and genotoxic effects of DEHP and MEHP on the European sea bass cell line was detected. MEHP resulted to have a slight effect on DLEC viability, whereas no effect was detected in the induction of apoptosis and necrosis at all tested concentrations of this metabolite. Perhaps, the high toxicity of DEHP on cell viability and cell death could be caused by a synergic effect of both DEHP itself and its metabolites, including MEHP. Yet, MEHP displayed a high dose-dependent increase in the frequency of micronucleus in both binucleated and mononucleated cells which, coupled with an absent induction of DNA strand breaks, suggests that MEHP could be one of the major compounds involved in the possible aneugenic effect detected on DLEC cell line.

These results underline, firstly, the importance of improving research on the effects of DEHP on marine fishes, as a potential threat for marine ichtyofauna since it is constantly introduced into the marine environment through marine litter. Furthermore, the comparison between DEHP and MEHP effects on DLEC cell line, highlight the greatest danger of this metabolite, since its effects could lead to chromosomal loss events, inducing genomic instability in the DLEC cell line without triggering the removal of the over damaged cells through cell death.

Marine mammals, which are at the top of the food webs, have an extremely high accumulation rate of contaminants with respect to other marine organisms; for this reason, over the past 25 years the toxic effects of persistent pollutants on cetaceans have raised many concerns. Considering the ecological relevance of cetaceans and the extensive legislative framework under which they are protected, it results difficult to assess the biological effects of contaminants on these species. Cetacean's *in vitro* cell cultures are a sensitive and non-destructive instrument, suitable for genomic analysis and toxicological studies, to evaluate the effects of contaminants on these organisms. As a coastal and predator species, the bottlenose dolphin is considered particularly exposed to the risks associated with plastic pollution, so much so that its increasing mortality has led to great concern about the potential role of environmental pollutants in affecting the conservation of the marine mammal communities. The study on the toxic effects of DEHP on the bottlenose dolphin skin cell line in comparison with those on the standardized Chinese Hamster Ovary cell line (Ch. 3) further highlighted the cytotoxic and genotoxic potential of this compound. All the tests conducted showed that 24 h treatment of DEHP could significantly affect cell survivals in both cell lines, and could increase cell death due to necrosis only in CHO. Overall cytotoxicity data suggest a slightly higher resistance of TT cell line to the phthalate. Furthermore, the absent increase in DNA fragmentations and the higher increase in the frequency of micronucleus, accompanied by a significant and progressive inhibition of cell proliferation, further support the hypothesis of an aneugenic effect of DEHP. This hypothesis is further supported by the analysis of the induction of micronucleus in mononucleated cells, where no induction was detected in both TT and CHO cells.

Finally, comparing the current results with those obtained in our study on the European sea bass embryonic cell line (Molino et al., 2019), the degree of toxicity to DEHP of the tree cell lines resulted in this decreasing order: DLEC > CHO > TT.

In conclusion, results of this whole study underline the importance of contributing to the enrichment of the data concerning the cytotoxic and genotoxic effect of phthalates on *in vitro* systems. In particular, given the economic importance of the European sea bass and the ecological importance of the bottlenose dolphin, this research indicates how much these marine organisms are endangered from the ubiquitous presence of phthalates into the marine environment, potentially impairing the

health of all marine fauna and of humans as final consumers of marine resources. Since abundant concentrations of plastic litter in the marine and coastal environments is common, a better understanding of the toxicity of the persistent pollutants associated with plastic is an important prerequisite to evaluating the ecological risk in the marine environment and to encourage the establishment of regulations concerning the reduction of their discharge into marine waters.

List of Papers/Contributions

- **Molino C**., Filippi S., Stoppiello G.A., Meschini R., Angeletti D., 2019. *In vitro* evaluation of cytotoxic and genotoxic effects of Di(2-ethylhexyl)-phthalate (DEHP) on European sea bass (*Dicentrarchus labrax*) embryonic cell line. Toxicology *in Vitro* 56, 118–125. https://doi.org/10.1016/j.tiv.2019.01.017.

- **Molino C**., Angeletti D., Oldham V.E., Goodbody-Gringley G., Buck K.N., 2019. Effect of marine antifouling paint particles waste on survival of natural Bermuda copepod communities. Marine Pollution Bulletin 149, 110492. https://doi.org/10.1016/j.marpolbul.2019.110492.

- **Molino C**., Filippi S., Caccia A., Meschini R., Angeletti D., Effects of phthalates on marine organisms: *in vitro* evaluation of Mono(2-ethylhexyl)-phthalate (MEHP) on European seabass (*Dicentrarchus labrax*) embryonic cell line. Aquatic Toxicology (Submitted and Under Review).

Conferences participations

- **Molino** C., Angeletti D., "Cytotoxic and genotoxic effects of priority substances on sea bass and bottlenose dolphin cell lines". **Oral presentation**. 2nd meeting "Ecotoxicology and Health", 25- 26 March 2019, Istituto Superiore di Sanità, Rome, Italy.

- **Molino** C., Filippi S., Giovani G., Peruffo A., Centelleghe C., Marchini S., Angeletti D., Meschini R., "*In vitro* evaluation of cytotoxic and genotoxic effects of Di(2-ethylhexyl)-phthalate (DEHP) on bottlenose dolphin (*Tursiops truncatus*) skin cell line". **Poster**. WMMC'19, World Marine Mammal Conference, 9-12 December 2019, Barcellona, Spain.