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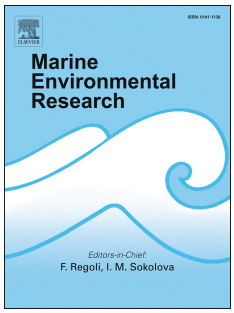

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Carbamazepine, cadmium chloride and polybrominated diphenyl ether-47, synergistically modulate the expression of antioxidants and cell cycle biomarkers, in the marine fish cell line SAF-1

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Abstract

A wide range of contaminants, industrial by-products, plastics, and pharmaceutics belonging to various categories, have been found in sea water. Although these compounds are detected at concentrations that might be considered as sub-lethal, under certain conditions they could act synergistically producing unexpected effects in term of toxicity or perturbation of biochemical markers leading to standard pathway. In this study, the *Sparus aurata* fibroblast cell line SAF-1, was exposed to increasing concentrations of carbamazepine (CBZ), polybrominated diphenyl ether 47 (BDE-47) and cadmium chloride ($CdCl_2$) until 72 h, to evaluate the cytotoxicity and the expression of genes related to antioxidant defense, cell cycle and energetic balance. In general, both vitality and gene expression were affected by the exposure to the different toxicants, in terms of antioxidant defense and cell cycle control, showing the most significant effects in cells exposed to the mixture of the three compounds, respect to the single compounds separately. The synergic effect of the compounds on the analyzed biomarkers, underlie the potential negative impact of the contaminants on health of marine organisms.

Keywords: Cadmium chloride; carbamazepine; polybrominated diphenyl-ether; oxidative stress; Sparus aurata fibroblast; biomarkers.

Introduction

The World Health Organization (WHO) has been working for years to draw public attention to the issue of environmental pollution, which can be found in air, water, and food and, in a dose-dependent manner, cause several types of metabolic diseases (Harrison and Dawson, 2016; Konduracka, 2019). In marine environments, organisms are subjected to complex mixtures of pollutants which might interact and produce abnormal effects from single compounds exposures, comprising cumulative, synergistic or antagonistic effects, depending on the nature of the toxicant and their action mechanisms (Bound and Voulvoulis, 2004). The presence of industrials sub-products (such as heavy metals, polyfluorinated compounds, polychlorinated biphenyls (Giesy et al., 2010; Trocino et., 2012; Morcillo et al., 2016), plastics sub-products (as microplastics, flame retardants) (Espinosa et al., 2017, 2019a) or pharmaceuticals metabolites (antihypertensive, anti-inflammatories) (Fernández et

al., 2010) are examples of contaminants that coexist in the aquatic system (Andreu et al., 2016). Nevertheless, most of the toxicological research on these contaminants is mainly aimed on single exposures using both *in vivo* and *in vitro* approaches, while the research evaluating the relationships and effects between pharmaceutical drugs, metals and others toxicant is limited, being only a few studies available (Almeida et al., 2018; Alsop and Wood, 2013; Cleuvers, 2003; Pires et al., 2016).

In this respect, polybrominated diphenyl ethers (PBDEs) comprises a group of compounds extensively used as flame retardant in several plastics and polymers, being characterized by its high stability (Reynier et al., 2001; Teuten et al., 2009). Because of these properties, PBDEs are able to remain in the environment for many years (Eljarrat and Barceló, 2011), and their persistence in sediments, water and marine organisms have been profoundly studied by many authors (Boer et al., 2001; Horri et al., 2018; Hu et al., 2010; Kierkegaard et al., 2004; Kim and Stapleton, 2010; Oberg et al., 2002; Sellstrom and B. Jansson, 1995; Sjödin et al., 2001; Zhu and Hites, 2006). In fact, the 2, 2′, 4, 4′-tetrabromodiphenyl ether (BDE-47) is among the most abundant found in the oceans (Bi et al., 2007; Leung et al., 2006), which has been found in a magnitude ranged from ng g^{-1} to µg g^{-1}, depending of the region (Parolini and Binelli, 2012; Vidal-Liñán et al., 2015). The effects of PBDEs on marine organisms, as well as the mechanisms implied in their toxicity has been intensively studied by several experimental procedures, such as *in vivo, ex vivo* and *in vitro* experiments using diverse marine models. In this sense, several studies have demonstrated PBDEs produces an alteration of behavior, growth, reproductive, hepatic, and renal functions as well as of the immune and the endocrine systems in fish (Berg et al., 2011; Daouk et al., 2011; Espinosa et al., 2019c, 2019b, 2019a,; Han et al., 2013, 2011; Liqin et al., 2015; Lyche et al., 2011; Péan et al., 2013).

Among the heavy metals, cadmium is one of the most relevant contaminants, being included by the USA Environmental Protection Agency' in their "Priority List of Chemicals" and classified as a human carcinogen by the International Agency for Research on Cancer (Nordberg et al., 1992). In mammals, the acute toxicity produced by cadmium entail liver, lung and testis damages (Nandi et al., 1969), as long as the chronic exposure to this heavy metal has been reported to produce obstruction of pulmonary disease, disturbance of metabolism, dysregulation of blood pressure, obstruction of kidney function, structure of bones and immune system (Järup et al., 1998; Jin et al., 1998; Nordberg et al., 1992). In marine environments, cadmium has been found at concentration ranged approximately 0.01–42 µg L^{-1} (Soares et al. 2008). In this sense, cadmium has been demonstrated both *in vivo* and *in vitro* to produce cytotoxicity, oxidative stress and severally affect to immune system from marine organisms (Guardiola et al., 2013, 2015; Lee et al., 2016; Morcillo et al., 2016).

As ~~is~~ described above, in the marine environments different toxicants do not occur isolated but they can be found in combination with other kind of compounds, including pharmaceutical drugs. Carbamazepine (CBZ) is one of the most representative antiepileptic pharmaceuticals that could be commonly found in aquatic ecosystems (Mohapatra et al., 2014). CBZ has different antiepileptic and psychotropic properties, whose mechanisms are associated with the blocking of the sodium channels in excitatory neurons (Malarvizhi et al., 2012). The elevated levels of consumption as well as the limited degradation rate upon wastewater treatment plants (WWTPs) (<10%) (Zhang et al., 2008) are the main reason for the occurrence of CBZ in the aquatic environment in concentrations which can range from 0.03 to 11.6µg L^{-1} (Bahlmann et al., 2009; Ferrari et al., 2003; Palm et al., 2002; Ternes, 1998). In marine organisms, these kind of compounds variates from 10 to 2000 ng L^{-1} (Burkina et al., 2018).

The exposure to these kind of compounds have been related to a ROS increase (Espinosa et al., 2019a; Fredriksson et al., 2014; Wu et al., 2017). A treatment with polyphenols (GAE) or β-carotene

(two kind of antioxidants which are known to prevent both oxidative stress and chemical toxicity (Espinosa et al., 2014; Kornhauser et al., 1994; Tanaka et al., 2018) before the exposure to these toxicants could help to attest that ROS production is succeeding.

Nevertheless, the evaluation of the relationship between the presence of the different compounds in the environment and the cellular homeostasis could present several difficulties, due to the presence of multiple stressors in natural systems (Baillon et al., 2016). Differently, the experimental approach, as *in vitro* assays, provides the control of most of the parameters, eluding potential interferences and/or crosstalk. An *in vitro* assay with three representative contaminants under controlled conditions could stablish a first approach for future in vivo experiments while, at the same time, validates effective biomarkers. In this study we evaluated the cytotoxic effect of different environmentally-relevant concentrations and mixture of BDE-47, CBZ and $CdCl_2$ using *Sparus aurata* fibroblast cell line (SAF-1) as *in vitro* model from marine organism to study synergetic effects. Impact of the contaminants and mixtures on cell cycle, cell signaling, cell metabolism, apoptosis and oxidative stress was evaluated through the expression of specific genes related to the mentioned pathways (*p53, erk-1, hif-1, bcl-2, nrf-2, cat and sod*).

1 Material and Methods

1.1 SAF-1 cell culture

The established cell line SAF-1 (ECACC n°00122301) was seeded in $25cm^2$ plastic tissue culture flasks (Nunc, Germany) cultured in L-15 Leibowitz medium (Sigma, UK), supplemented with 10% fetal bovine serum (FBS, Sigma, UK), 2mmol L^{-1} L-glutamine (Sigma, UK), 100i.u. mL^{-1} penicillin (Sigma, UK) and 100g L^{-1} streptomycin (Sigma, UK). Cells were grown at 25°C under humidified atmosphere (85% humidity), pH 7.2-7.4 and 296-328mOs/kg. The cells were maintained in culture for 3 weeks before the beginning of any trial.

Exponentially growing cells were detached from culture flasks by brief exposure to 0.25% of trypsin in PBS, pH 7.2-7.4, according to the standard trypsinization methods. The detached cells were collected by centrifugation (1000rpm, 5min, 25°C) and the cell vitality was determined by the trypan blue exclusion test.

1.2 Cytotoxicity assay on SAF-1 cell line

The PBDE standard was provided by SPECTRA (Rome, Italy); stock solution of BDE-47 at a concentration of 25mmol L^{-1} was prepared by dissolving the powder compounds in dimethyl-sulfoxide DMSO. Carbamazepine (CBZ) and cadmium chloride ($CdCl_2$) were obtained from Sigma-Aldrich (St. Louis, MO, USA) (C4024 and 202908, respectively); stock solutions of CBZ and $CdCl_2$ at 25nmol L^{-1} were prepared by dissolving the powder compounds in dimethyl-sulfoxide (DMSO) and phosphate buffer saline solution (PBS), respectively.

Cytotoxicity assay was performed in six replicates. When SAF-1 cell lines were approximately 80% confluent (1 week after the last trypsinization), they were detached from flasks culture with trypsin (as described before), and aliquots of 100mL containing 10,000 cells $well^{-1}$ were dispensed in 96-well tissue culture plates and incubated (24h, 25°C). This cell concentration was previously determined in order to obtain satisfactory absorbance values in the cytotoxic assay and avoided cell over-growth. After that, the culture medium was replaced by 100mL $well^{-1}$ of the BDE-47, CBZ and $CdCl_2$ to be tested at the appropriate dilution.

A first approach was done with concentrations of BDE-47 that ranged from 1nmol L^{-1} to 10µmol L^{-1} (1, 10, 100, 1000 and 10,000nmol L^{-1}). On the other hand, a second experiment with different concentrations of CBZ and $CdCl_2$ ranged from 1nmol L^{-1} to 100µmol L^{-1} (1, 10, 100, 1000, 10,000 and 100,000nmol L^{-1}), as well as the same concentrations containing 1µmol L^{-1} of BDE-47, was developed. After this, another trial with CBZ and $CdCl_2$ at concentrations ranged 10 to 100µmol L^{-1} (10, 50 and 100µmol L^{-1}) and a mixture of the three compounds BDE-47, CBZ and $CdCl_2$ (1:10:10µmol L^{-1}, 1:50:50µmol L^{-1} and 1:100:100µmol L^{-1} for BDE-47, CBZ and $CdCl_2$, respectively). Selected doses are consistent with found on the marine environment, which could variate according to region and pollution (Binelli, 2012; Burkina et al., 2018; Parolini and Binelli, 2012; Soares et al. 2008; Vidal-Liñán et al., 2015).

In all trials, cells were incubated for 24, 48 and 72h in three different plates at 25°C. Control samples received the same volume of culture medium and DMSO 0.1%, although the absence of the effects by the vehicle is well known (Abbes et al., 2013; Messina et al., 2016). After the incubation for 24, 48 and 72h at 25°C, their vitality was determined using the MTT assay.

With the aim to evaluate the oxidative stress produced by the contaminants, β-carotene and GAE (gallic acid) were used as antioxidant standards. Then, a last trial was done exposing separately the SAF-1 cells to two different concentrations of β-carotene and GAE (0.07 and 0.15µg mL^{-1}, 0.05% ethanol) for 24 hours. After this, the cells were exposed to a mixture of the three compounds BDE-47, CBZ and $CdCl_2$ (1:100:100µmol L^{-1} for BDE-47, CBZ and $CdCl_2$, respectively) for 48 hours. Appropriate vehicle controls were done.

The MTT assay is based on the reduction of the yellow soluble tetrazolium salt (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT, Sigma-Aldrich, Saint Louis, USA) into a blue, insoluble formazan product by the mitochondrial succinate dehydrogenase (Berridge and Tan, 1993; Denizot and Lang, 1986). After incubation with the PDEs, SAF-1 cells were washed with phosphate buffer saline solution (PBS) and 200mL $well^{-1}$ of MTT (1g L^{-1}) were added. After 4h of incubation, cells were washed again and the formazan crystals solubilized with 100mL $well^{-1}$ of DMSO. Plates were shacked (5 min, 100rpm) in dark conditions and the absorbance at 570 nm and 690 nm determined in a microplate reader (Multiscan-Sky Microplate Reader, Thermo-Scientific TM, USA). After the individuation of the sub-lethal concentrations, the next experiments were done in order to assess molecular markers related to the different biochemical patterns.

1.3 Gene expression assay

SAF-1 cells (500.000 cells $well^{-1}$) were incubated in 12 well plates (Nunc, Germany) for 72 hours with different concentrations of PBDEs (vehicle (control), sub-lethal doses of BDE- 47 (1µmol L^{-1}), CBZ (10µmol L^{-1}), CdCl2 (10µmol L^{-1}) and a mixture of the three compounds (1:10:10µmol L^{-1} BDE-47, CBZ and $CdCl_2$, respectively). Each concentration was tested in four different wells (500.000 cells $well^{-1}$). Then, medium was removed, cells were washed using PBS and 1mL of PUREzol (Bio-Rad, USA) was added to the flask. The PUREzol containing the RNA from cells was obtained and storaged at -80ºC to posterior analyses.

1.3.1 Quantitative real-time PCR

Total cellular RNA was isolated from the samples in PUREzol using Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, USA), and the concentration was assessed spectrophotometrically at 260 nm. The absorbance ratios A260/A280 and A260/A230 were evaluated as indicators of RNA purity. Then, 1µg of RNA were reverse-transcribed for each sample, in a volume of 20µL, by the 5X

iScript Reaction Mix Kit (Bio-Rad, USA) according to manufacturer's instructions. The amplification was performed in a total volume of 20µL, which contained: 0.4µmol L^{-1} of each primer, cDNA diluted 1:10 of the final reaction volume, 1X IQ SYBR Green Supermix (Bio-Rad, USA) and nuclease-free water. Conditions for real-time PCRs were optimized in a gradient cycler (C1000 Touch Thermal Cycler, Bio-Rad, USA) using the following run protocol: an initial activation step at 95°C for 3min, followed by 39 cycles of 95°C for 10s and 60°C for 30s, with a single fluorescence measurement. Melting curve program was achieved at 65-95°C with heating rate of 0.5°C/cycle and a continuous fluorescence measurement. All reactions were performed in triplicate. For each PCR, we checked linear range of a standard curve of serial dilutions. The relative quantification of [*p53*, *erk-1*, *hif-1*, *bcl-2*, *nrf-2*, *cat* and *sod*] gene expression was evaluated after normalization with the reference genes. Data processing and statistical analysis were performed using CFX Manager Software (Bio-Rad, USA). The primers used are shown in Table 1. The relative expression of all genes was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using *Sparus aurata* β-actin and 18S as the endogenous reference.

Table 1. Gilthead seabream primer sequences used for real-time PCR

Gene	Accession number	F/R Primer sequence (5'–3')
p53		F- CCTCATCCTCATCATCGCCT
		R- AGCTCGTTGAATTTGCAGGG
erk-1		F- GCTCTATGGCAAGGCTGAC
		R- TGCCTGGAAACGAGCTGTT
hif-1		F- CTCAGCCACAGTGTGTTGTC
		R- TACATCAACCTCGGGCAACT
bcl-2		F- TCAGGAGTGATGTCGAGCTG
		R- CAGCCAGGTGCTGACATAGA
nrf2		F- GTTCAGTCGGTGCTTTGACA
		R- CTCTGATGTGCGTCTCTCCA
cat		F- TTCCCGTCCTTCATTCACTC
		R- CTCCAGAAGTCCCACACCAT
sod		F- CCATGGTAAGAATCATGGCGG
		R- CGTGGATCACCATGGTTCTG
β-actin		F- GTCATGGATTCCGGTGATGG
		R- TGGTGAAGGAGTAGCCACGC
18S	AM490061	F- CTTCAACGCTCAGGTCATCAT
		R- AGTTGGCACCGTTTATGGTC

1.4 Statistical analysis

Statistical differences among the groups were assessed by one-way ANOVA analyses, followed by the Bonferroni or Games Howell test, depending on the homogeneity of the variables. The normality of the variables was confirmed by the Shapiro–Wilk test and homogeneity of variance by the Levene test. The significance level was 95% in all cases ($P < 0.05$). All the data were analyzed by the computer application SPSS for Windows® (version 20.0, SPSS Inc., Chicago, USA).

2 Results

The effects of BDE-47, CBZ and $CdCl_2$ on the vitality of SAF-1 cells was evaluated by MTT assay. No differences were denoted in SAF-1 cells exposed to rising concentrations of BDE-47 (from 1nmol L^{-1} to 10µmol L^{-1}) until 72 hours (figure 1). However, the exposure to CBZ and $CdCl_2$ significantly affected the vitality of SAF-1 cells.

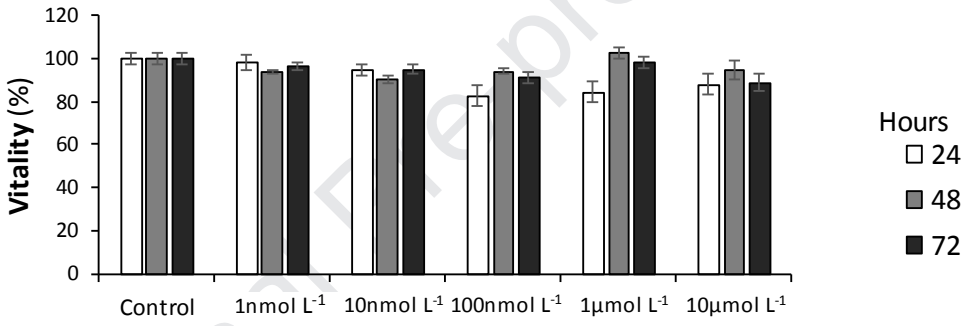

Figure 1. Cytotoxicity of SAF-1 cells exposed to different concentrations of BDE 47 (1nmol L^{-1}-100µmol L^{-1}) for 24, 48 and 72 h. Bars represent the mean ± SEM (n=6). Non statistical differences were found between treatments.

SAF-1 cells were exposed to rising concentrations of CBZ (from 1nmol L^{-1} to 10µmol L^{-1}) mixed each one with 1µmol L^{-1} of BDE-47 until 72 hours (figure 2). At 24 hours, the vitality of cells was significantly reduced with the higher concentrations of CBZ (1 and 10µmol L^{-1} of CBZ) (10.0 and 13.9% of vitality reduction, respectively), as well as by higher concentration of the mixture of CBZ+BDE47 (10µmol L^{-1} CBZ + 1µmol L^{-1} of BDE-47) (15.8% of vitality reduction) ($p<0.05$). At 48 hours, the vitality of cells was significantly affected by the exposure to 10µmol L^{-1} of CBZ (14.6% of vitality reduction) as well as the exposure to 10µmol L^{-1} CBZ + 1µmol L^{-1} of BDE-47 (7.9% of vitality reduction) ($p<0.05$). At 72 hours, the vitality of cells was significantly decreased by the exposure to 10 µmol L^{-1} of CBZ, (21.7% of vitality reduction), although the vitality of cells was significantly increased by the exposure to the mix of 100nmol L^{-1} CBZ + 1µmol L^{-1} of BDE-47 and 1µmol L^{-1} CBZ + 1µmol L^{-1} of BDE-47 (115.1% and 117.7%, respectively) ($p<0.05$).

Concomitantly, SAF-1 cells were exposed to rising concentrations of $CdCl_2$ (from 1nmol L^{-1} to 10µmol L^{-1}) mixed each one with 1µmol L^{-1} of BDE-47 until 72 hours (figure 3). At 24 hours, the vitality of cells was significantly decreased by the exposure to the mix of 10µmol L^{-1} $CdCl_2$ + 1µmol L^{-1} of BDE-47 (16.8% of vitality reduction) ($p<0,05$). At 48 hours, the vitality of cells was

significantly decreased by the exposure to 10µmol L^{-1} of $CdCl_2$ (42.9% of vitality reduction) as well as the exposure to the mix of 1µmol L^{-1} $CdCl_2$ + 1µmol L^{-1} of BDE-47 and 10µmol L^{-1} $CdCl_2$ + 1µmol L^{-1} of BDE-47 (20.0% and 35.2%, respectively) ($p<0.05$). Finally, at 72 hours, the cells vitality showed to be significantly decreased by the exposure to 10µmol L^{-1} of $CdCl_2$ (61.0% of vitality reduction), as well as the exposure to the mix of 1µmol L^{-1} $CdCl_2$ + 1µmol L^{-1} of BDE-47 and 10µmol L^{-1} $CdCl_2$ + 1µmol L^{-1} of BDE-47 (31.0% and 59.5% of vitality reduction, respectively) ($p<0.05$).

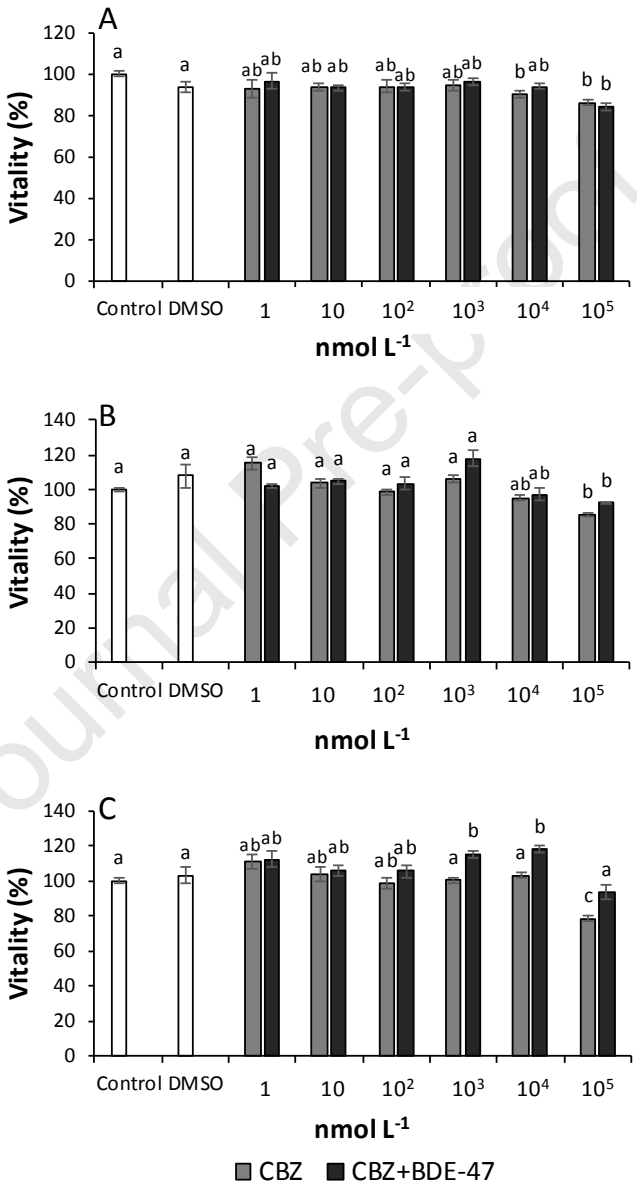

Figure 2. Cytotoxicity of SAF-1 cells exposed to different concentrations of carbamazepine (CBZ) (1nmol L^{-1} - 100µmol L^{-1}) as well as a mixture of CBZ (1nmol L^{-1}-100µmol L^{-1}) + BDE-47 1µmol L^{-1} for 24h (A), 48h (B) and 72h (C). Bars represent the mean ± SEM (n=6). Statistically significant differences (ANOVA; $P\leq0.05$) were denoted using different letters.

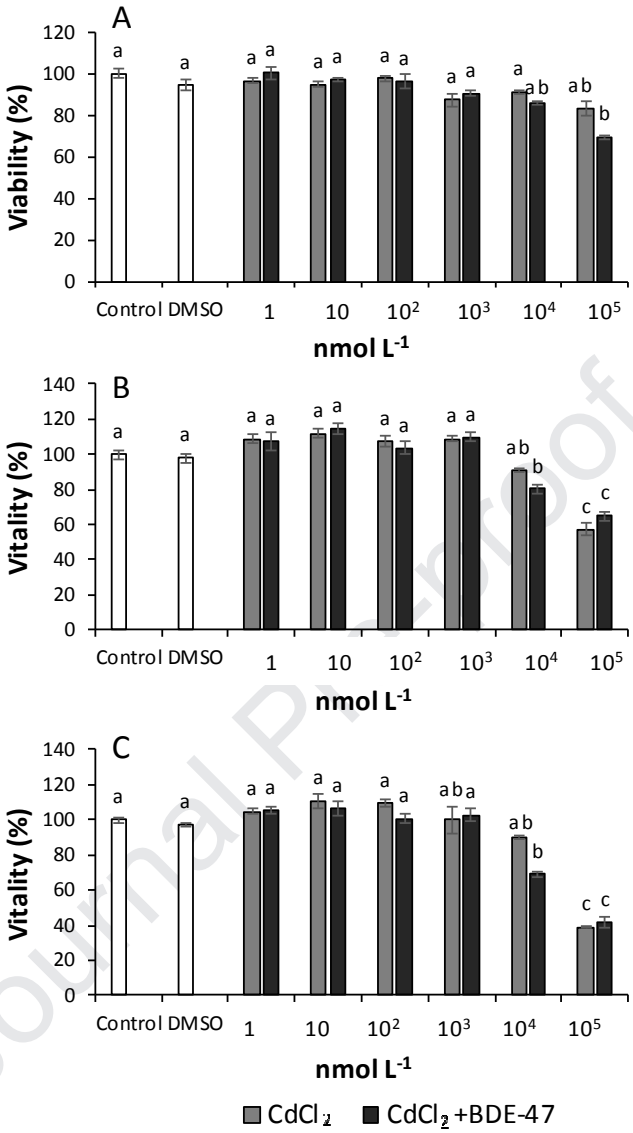

Figure 3. Cytotoxicity of SAF-1 cells exposed to different concentrations of $CdCl_2$ (1nmol L^{-1} - 100µmol L^{-1}) as well as a mixture of CdCl2 (1nmol L^{-1}-100µmol L^{-1}) + BDE-47 1µmol L^{-1} for 24h (A), 48h (B) and 72h (C). Bars represent the mean ± SEM (n=6). Statistically significant differences (ANOVA; P≤0.05) were denoted using different letters.

After the selection of appropriate doses of contaminants, a last trial with a mixture of the three compounds was done. SAF-1 cells were exposed to rising concentrations of BDE-47 (10µmol L-1) + CBZ (10, 50 and 100µmol L^{-1}), BDE-47 (10µmol L^{-1}) + $CdCl_2$ (10, 50 and 100µmol L^{-1}), and three mixtures of the three compounds: BDE-47 (10µmol L^{-1}) + CBZ (10, 50 and 100µmol L^{-1}) and CdCl2 (10, 50 and 100µmol L^{-1}), until 72 hours (figure 4). At 24 hours, the vitality of SAF-1 cells was significantly decreased with the higher dose of CBZ + BDE-47 (9.6% of vitality reduction) as well as the higher dose of $CdCl_2$ + BDE-47 (17.5% of vitality reduction), while the cells exposed to the mixture of contaminants showed its vitality significantly decreased under the exposure to the higher dose (10µmol L^{-1} of BDE-47, 100µmol L^{-1} of CBZ and 100µmol L^{-1} of $CdCL_2$) (16.7 % of vitality reduction) ($p<0.05$). At 48 hours, the vitality of cells was significantly reduced after the exposure to

the higher dose of CBZ + BDE-47 (18.8% of vitality reduction), as well as the cells exposed to BDE-47 (10µmol L^{-1}) + $CdCl_2$ (50 and 100µmol L^{-1}) (53.0 and 71.6% of vitality reduction), while the cells treated with the BDE-47 (10µmol L^{-1}) + CBZ and $CdCL_2$ (50 and 100µmol L^{-1}) showed to be its vitality significantly reduced (60.2 and 70.7% of vitality decrease, respectively). Finally, at 72 hours, a significant reduction was detected in the cells exposed to the BDE-47 (10µmol L^{-1}) + CBZ (50 and 100µmol L^{-1}) (32.3 and 26% of vitality reduction) ($p<0.05$). Besides, the vitality of SAF-1 cells exposed to BDE-47 (10µmol L^{-1}) + $CdCl_2$ (50 and 100µmol L^{-1}) was reduced (46.1 and 63.7% of vitality reduction, respectively) ($p<0.05$). The cells exposed to the mixture of the three contaminants showed its vitality significantly decreased with all the doses (BDE-47 (10µmol L^{-1}) + CBZ and $CdCL_2$ (10, 50 and 100µmol L^{-1}) (20.9, 73.1 and 9.1% of vitality reduction) ($p<0.05$).

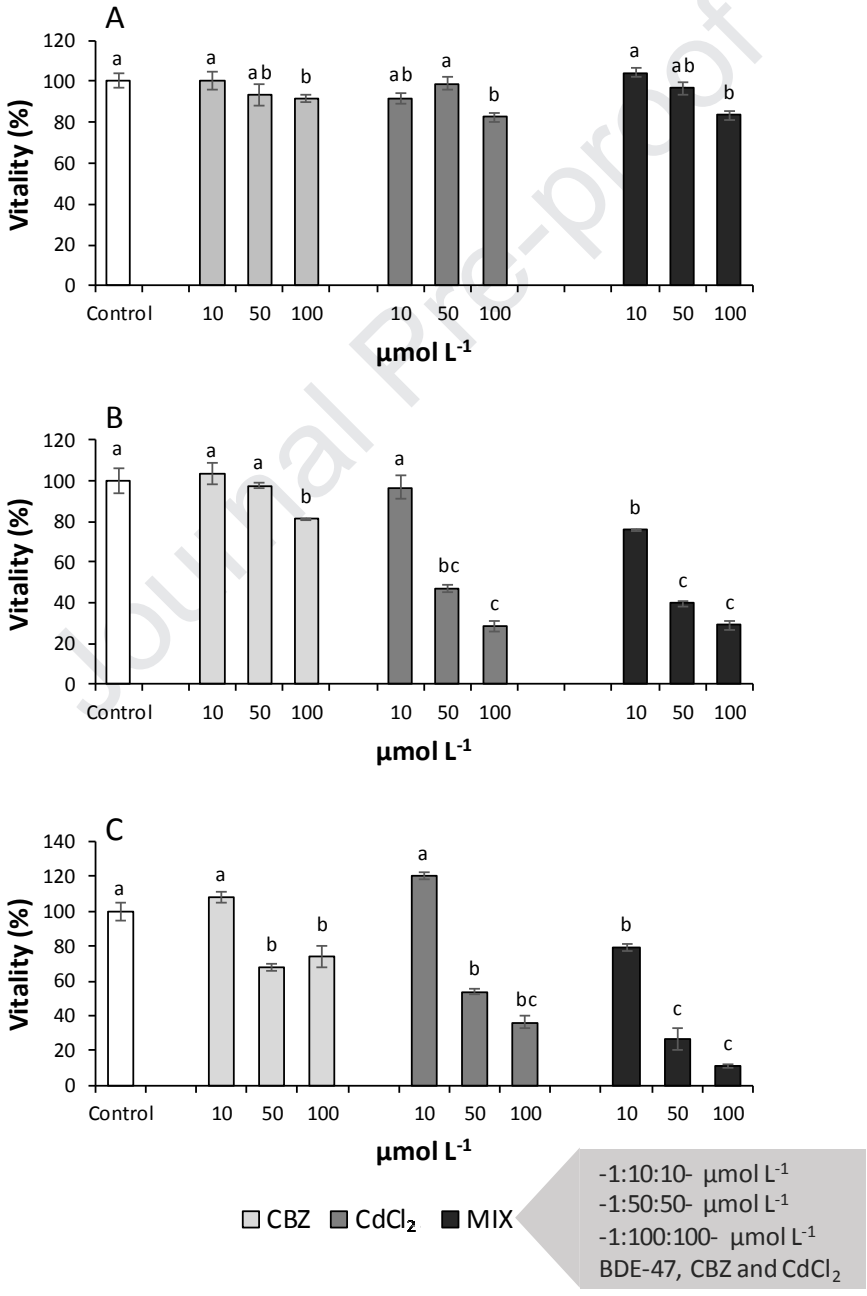

Figure 4. Cytotoxicity of SAF-1 cells exposed to different concentrations of a mixture of carbamazepine (CBZ) (10, 50 and 100µmol L^{-1}) + BDE-47 1µmol L^{-1}, a mixture of $CdCl_2$ (10, 50 and 100µmol L^{-1}) + BDE-47 1µmol L^{-1}, or a mixture of contaminants (1:10:10µmol L^{-1}; 1:50:50µmol L^{-1}; and 1:100:100µmol L^{-1} of BDE-47, CBZ and $CdCl_2$, respectively) for 24h (A), 48h (B) and 72h (C). Bars represent the mean ± SEM (n=6). Statistically significant differences (ANOVA; P≤0.05) were denoted using different letters.

In addition, SAF-1 cells were treated with β-carotene and GAE for 24 hours and then were exposed to the mixture of the three compounds BDE-47, CBZ and $CdCl_2$ (1:100:100µmol L^{-1} for BDE-47, CBZ and $CdCl_2$, respectively) for 48 hours (figure 5). The cells exposed to GAE and β-carotene at 0.07 µg mL^{-1} did not show any difference with respect to the control group, although the cells exposed to the higher concentration of β-carotene (0.15µg mL^{-1}) significantly decreased cells vitality ($p<0.05$). In addition, the mixture of contaminants affected cell vitality (40%; $p<0.05$) at 72 h and the previous exposure to β-carotene and GAE (during 24 h) protected SAF-1 cells from decrease in their vitality at 72h ($p<0.05$).

All the vitality results are resumed in different tables (supplementary table 1-5).

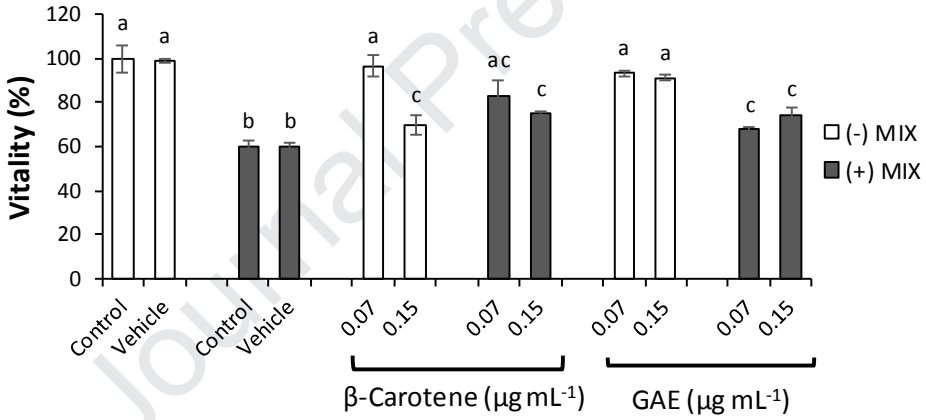

Figure 5. Cytotoxicity of SAF-1 cells exposed to 0.07 and 0.15µg mL^{-1} of β-carotene and GAE for 24 hours. After that, the cells were exposed to a mixture of contaminants (1:100:100µmol L^{-1} of BDE-47, CBZ and $CdCl_2$, respectively) (black bars) or without mixture treatment (phosphate buffered saline) (white bars). Bars represent the mean ± SEM (n=6). Statistically significant differences (ANOVA; P≤0.05) were denoted using different letters.

The analysis of some relevant genes related to the cell cycle (*p53*), cell signaling (*erk-1*), modulation of metabolism to restrictions (*hif-1*), apoptosis (*bcl-2*) and oxidative stress (*nrf-2, cat* and *sod*) were evaluated in SAF-1 cells exposed for 72 hours to selected concentrations of BDE-47 (10µmol L^{-1}), CBZ (10µmol L^{-1}), CdCl2 (10µmol L^{-1}) and a mix of three (10µmol L^{-1} of BDE-47 + 10µmol L^{-1} of CBZ + 10µmol L^{-1} of $CdCl_2$) (figure 6). The expression of *p53* was significantly up-regulated in cells exposed to 1µmol L^{-1} of BDE-47 and 10µmol L^{-1} of $CdCl_2$ with respect to the control group ($p<0.05$), although the expression of *p53* showed to be significantly up-regulated with respect to all the groups in cell exposed to the mixture of compounds ($p<0.05$). At the same time, the expression of *erk-1*, *hif-1, bcl-2, nrf-2, cat* and *sod* showed to be up-regulated in cells exposed to the mixture of compounds

(1:10:10 µmol L^{-1} of BDE-47, CBZ and $CdCl_2$, respectively) compared with its respective control group ($p<0.05$).

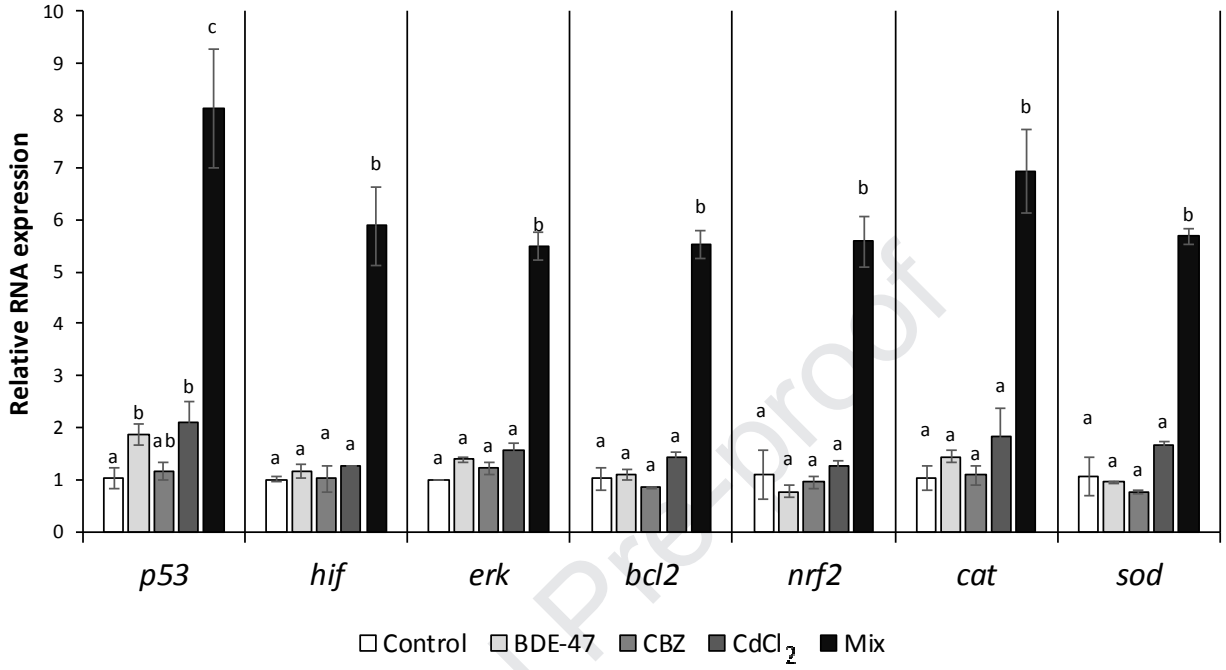

Figure 6. Relative gene expression of some genes related to cell cycle (*p53*), proliferation (*erk1*), energetic balance (*hif1*), apoptosis (*bcl2*) and oxidative stress (*nrf2*, *sod* and *cat*) from SAF-1 cells exposed to vehicle (Control), 1µmol L^{-1} of BDE 47, 100µmol L^{-1} of carbamazepine (CBZ), 100µmol L^{-1} of $CdCl_2$ or a mixture of contaminants (1:100:100µmol L^{-1} of BDE-47, CBZ and $CdCl_2$, respectively) for 72 hours. Bars represent the mean ± SEM (n=4). Statistically significant differences (ANOVA; $P\leq0.05$) were denoted using different letters.

3 Discussion

The objective of this study was to use the SAF-1 cell line as in vitro model to investigate the effects on vitality as well as the impact on the expression of stress-responsive genes related to cell cycle, energetic metabolism and oxidative stress produced by different mixtures of contaminants such as BDE-47, which is one of the most abundant PBDEs in the environment and wildlife (Bi et al., 2007; Leung et al., 2006; Yang et al., 2016), $CdCl_2$, which is one of the most relevant contaminants among the heavy metals (Nordberg et al., 1992), and CBZ, which is one of the pharmaceuticals compounds that could be found in marine environment (Mohapatra et al., 2014). The cytotoxic effects of BDE-47, $CdCl_2$ and CBZ exposure at short time, along with the gene expression of some markers were evaluated on the SAF-1 cell line.

Our findings showed that exposure to BDE-47 did not affect the vitality of SAF-1 cells at concentrations ranging from 1nmol L^{-1} to 10µmol L^{-1}, which agree with our previous research (Espinosa et al., 2019a, 2019b; Manuguerra et al., 2019). Regarding the CBZ and $CdCl_2$ exposure, these compounds decreased the vitality of SAF-1 cells in a time-dose dependent way, which is

consistent with other works that showed the cytotoxic effects in vitro of CBZ (Fredriksson et al., 2014; Grewal et al., 2017) as well as $CdCl_2$ (Skipper et al., 2016; Song and Koh, 2012). In comparison with other works in vitro, our results could contrast with the reported by other authors that did not find any toxicity in mouse BV-2 microglia cells exposed to CBZ, although both concentrations and time of exposure were different (1 to 20 µmol L^{-1} of CBZ and the BV-2 cells were exposed to CBZ for 15 min) (Wang et al., 2014). Of course, it could be hypothesized that marine cells could be more sensitive than murine or mammals' cells. For example, difference of sensibility to the same contaminant (BDE-47) could be observed between human fibroblast (HS-68 cell line) and sea bream fibroblast (SAF-1 cell line) exposed to BDE-47 (Espinosa et al., 2019a; Manuguerra et al., 2019). A trial with mammals and marine cell lines using the same doses and time of exposure could elucidate some light on this issue.

In general, the exposure to the mix of $CdCl_2$ + 1µmol L^{-1} of BDE47 produced higher levels of toxicity in SAF-1 cells than each compound by itself, which agree with others works that reported the effects of the exposure to the mixture of these compounds (Chen et al., 2018; Feng et al., 2018; L. Wang et al., 2018). In addition, the exposure to the mix of CBZ + 1µmol L^{-1} of BDE47 produced a decrease of the vitality at 24 and 48 hours with the higher doses. Curiously, the exposure to the mixture of 1 and 10µmol L^{-1} CBZ + 1µmol L^{-1} of BDE47 increased the vitality of SAF-1 cells with respect to the control at 72 hours, while the cells exposed to 100µmol L^{-1} CBZ + 1µmol L^{-1} of BDE47 did not show any difference with respect to the control. These results suggested that specific concentrations of the mixture of CBZ and BDE-47 could promote proliferative patters and increase the survivor of SAF-1 cells. In general, both CBZ (Fredriksson et al., 2014; Grewal et al., 2017) and BDE-47 (Espinosa et al., 2019a; Jin et al., 2010) have been reported to reduce the cells vitality in vitro. However, these compounds have been shown to promote proliferative patters under some conditions, reducing the cells mortality by the induction of survivor signals in the cells, as it has been reported in liver from mice (Kawaguchi et al., 2013), where CBZ promoted hepatocyte proliferation via mTOR signaling pathway; or the work that reported in OVCAR and MCF-7 cell lines exposed to BDE-47 metabolites (Karpeta and Gregoraszczuk, 2017), where the reduction in the apoptosis was produced by decrease of *caspase 6*, *caspase 9* and *bcl-xl* expression. The mixture of both compounds (CBZ and BDE-47) at the appropriate concentrations could entail the induction of these proliferative pathways, promoting the increase of vitality observed in our results, although at higher doses (100µmol L^{-1}) these effects are not showed, maybe for the toxicity at higher doses. More research is needed to shed light in this issue.

The exposure to a mixture of CBZ, $CdCl_2$ and BDE47 at rising concentrations for 24, 48 and 72 hours showed the vitality of cells has decreased in a higher degree, compared to the decrease observed in cells exposed to the respective mixture of CBZ + 1µmol L^{-1} of BDE47 as well as $CdCl_2$ +1µmol L^{-1} of BDE47. ~~The exposure to these kind of compounds have been related to a ROS increase (Espinosa et al., 2019a; Fredriksson et al., 2014; Wu et al., 2017). With the aim to evaluate the mechanisms involved in the increase of cell mortality, a treatment with two doses of polyphenols (GAE) or β-carotene (two kind of antioxidants which are known to prevent both oxidative stress and chemical toxicity (Espinosa et al., 2014; Kornhauser et al., 1994; Tanaka et al., 2018) was done before the exposure to the higher dose of the mix (1µmol L^{-1} of BDE-47, 100µmol L^{-1} of CBZ and $CdCl_2$).~~ Although our results showed the higher dose of β-carotene resulted toxic for SAF-1 cells and significantly decreased its vitality (as has been described previously for high levels of β-carotene and others antioxidants (Heywood et al., 1985; Pisoschi and Pop, 2015), both β-carotene and GAE were able to partially avoid the negative effect of the mixture exposure. For this reason, our results suggested the toxicity caused by the mixture of contaminants may be produced via oxidative stress,

as it has been demonstrated by the contaminants alone (Espinosa et al., 2019a; Fredriksson et al., 2014; Wu et al., 2017).

On the other hand, the expression of different genes related to cell cycle (*p53*), cell signaling (erk1), stress (*hif1*), apoptosis (*bcl2*) and oxidative stress (*nrf2, sod* and *cat*) was analyzed in SAF-1 cells exposed to 1µmol L^{-1} of BDE-47, 1µmol L^{-1} of BDE-47+ 10µmol L^{-1} CBZ, 1µmol L^{-1} of BDE-47+ 10µmol L^{-1} $CdCl_2$ and the mixture of 1µmol L^{-1} of BDE-47, 10µmol L^{-1} of CBZ and $CdCl_2$ for 72 hours.

The role of *p53* is well known for protection of cell genome, contributing in cell cycle regulation by the control of cell cycle arrest (via cyclins and retinoblastoma) or inducing cell death under stress situation (Yee and Vousden, 2005; Zhang et al., 2015). In our experiment, the *p53* expression was up-regulated on cells exposed to 1µmol L^{-1} of BDE-47, 10µmol L^{-1} of $CdCl_2$ and the mixture of 3 contaminants, that suggested the DNA damage was produced by these treatments. These results agree with other works that reported the induction of *p53* under exposure to cadmium (Lee et al., 2016) and BDE-47 (Manuguerra et al., 2019; You et al., 2018), and with our previous research on SAF-1 cells that showed the *p53* expression after the exposure to 1 µmol L^{-1} of BDE-47for 72 hours (Espinosa et al., 2019a).

In relationship with this, ERK-1 is a kinase mainly implicated in cell activation, modulating cell proliferation (McCubrey et al., 2007a, 2007b; Turpaev, 2006). The incubation with 1µmol L^{-1} of BDE-47 for 72 hours failed to affect the expression of *erk1*, which is consistent with our previous research in SAF-1 cells (Espinosa et al., 2019a). Besides, our results showed the exposure to the mixture of three contaminants was able to up-regulate the *erk1* expression. Although the concentrations, time of exposure and cell lines were different, the increase of ERK1/2 levels has been previously described after PBDEs exposure in human fibroblast cells (Manuguerra et al., 2019), in human OVCAR-3 cells (Karpeta et al., 2016), in cerebellar granule neurons from Long–Evans rat (Fan et al., 2010), as well in human HeLa cells (Li et al., 2012); besides, an increase in ERK1 levels has been described after CBZ exposure in 3T3 (Turpin et al., 2013), in human embryonic cells (Pomati et al., 2006); as well as an increase of ERK1 after the exposure to $CdCl_2$ was described on human placental JEG-3 trophoblast cells (Paniagua et al., 2019) and OVCAR3 and SKOV3 cell lines (Ataei et al., 2019). So, our data demonstrated the exposure to the mixture of these three compounds is able to induce the activation of ERK1. In according to this, ERK1/2 signal pathway has been suggested to take part in 'hormesis' phenomenon by phosphorylation of ERK1/2 and activation of dependent genes (Ataei et al., 2019). It was described by Stebbing (2002) that the exposure of lower doses of metals, cells showed an overcompensation response to with the aim to protect and adapt against the damage produced by the toxicant, although at higher concentrations they produce serval toxic effects that induce cell death. The activation of ERK1, which entails proliferation, should be the explanation for our vitality results in cells exposed to CBZ+BDE47 at low concentrations. However, more research is needed.

The hypoxia inducible factor 1 (HIF-1) is known as a transcription factor that regulates the cellular response to hypoxia because of its sensibility to the oxygen concentrations inside the cell (Kitajima et al., 2017; Qi et al., 2014). For this reason, HIF-1 controls the molecular processes related to the oxygen homeostasis, regulating the related metabolic pathways (Romney et al., 2011; Shao et al., 2010; Zhang et al., 2009). HIF-1 activates the expression of gene implicated in cancer genesis, as angiogenesis, anaerobic glycolysis, cell survival and invasion (Qi et al., 2014). Our results are consistent with our previous research on SAF-1 that showed the exposure to 1µmol L^{-1} of BDE-47 for 72 hours failed to affect the *hif1* expression (Espinosa et al., 2019a). In addition, our results

showed the expression of *hif-1* was up-regulated on SAF-1 cells exposed to the mixture of contaminants, which could suggest the mixture of these contaminants could promote patterns related to cells transformation. Other authors reported the increase of HIF-1 on human fibroblast exposed to BDE-47 (Manuguerra et al., 2019), or in human bronchial epithelial cells exposed to $CdCl_2$ (Jing et al., 2012), although the times of exposure and the kind of cells were different with respect to our experiment.

Regarding to the *bcl2* expression, our results contrast with other works that reported the expression of *bcl2* significantly decreased on human embryonic stem cells after exposure to BDE-209 (Du et al., 2016), on rats exposed to $CdCl_2$ (Refaie et al., 2018), on neonatal murine engineered cardiac tissue exposed to $CdCl_2$ (Yu et al., 2018), although the CBZ showed to increase the *bcl2* levels in brain cells from rats with acid-induced temporal lobe epilepsy (Jia et al., 2018). The mixture of compounds could entail the cells survivor, as indicated the levels *bcl2* together with the levels of expression of *p53* and *erk1*.

Under oxidative stress and ROS overproduction, several signaling pathways are involved in cell protection. (Huang et al., 2015; Regoli et al., 2011). In this respect, NRF-2 is a transcription factor which plays a role against oxidative stress by the interactions with the AREs (antioxidant response elements) and regulations of the expression of several antioxidants and phase II detoxification genes (Hayes et al., 2015; Huang et al., 2015), being a well-recognized and well-conserved factor in marine organisms (Giuliani and Regoli, 2014). NRF-2 has the ability to promote the expression of antioxidant genes as superoxide dismutase or catalase, focused in the ROS detoxification (Hayes et al., 2010). Our results showed the expression of *nrf2*, sod and cat resulted upregulated in the cells exposed to the mixture of toxicants, with respect to the control group, that suggested the exposure to this kind of compounds entails oxidative stress. These results are consistent with other works that reported these compounds are able to induce oxidative stress separately, as our previous work on SAF-1 cells exposed to BDE-47 that showed the nrf2 expression significantly up-regulated (Espinosa et al., 2019a), or the work developed on mice that showed the NRF-2 pathway activated by CBZ exposure (Lu et al., 2008), or the works that reported cadmium exposure activated the NRF-2 response *in vitro* (He et al., 2019; Qu et al., 2019; Y. Wang et al., 2018). In addition, the fact that the decrease of vitality produced by the higher doses of the mixture of contaminants was partially avoided by the incubation with β-carotene and GAE, suggest the oxidative stress as one of the main mechanisms by the mixture of these compounds affect the cells.

In this respect, the oxidative stress produced by the mixture could affect diverse cellular processes, from metabolic to energetics (Dong et al., 2017; Martinez-Outschoorn et al., 2017; Mullen and DeBerardinis, 2012; Sullivan et al., 2016), as indicated the upregulation of *hif1* expression. These kind of changes are preliminary to cell transformation (Valko et al., 2007).

As is well known, the 2D cell culture models fail to reconstitute the *in vivo* cellular microenvironment (Huh et al., 2011). Although the *in vitro* approach is a limitation of our study, our observations suggested that marine organisms exposed to a similar mixture of these agents may probably evidence liver damage as well as to show unbalanced its antioxidant enzymes, as has been described for the same toxicant separately for cadmium (De Conto Cinier et al., 1998; Eleawa et al., 2014), PBDEs (Espinosa et al., 2019c) and CBZ (Almeida et al., 2015), being all these negative effects mostly aggravated by the synergic effect of the toxicants mixture.

Overall, our results demonstrated that rising concentrations CBZ and $CdCl_2$ along BDE-47 exposure affected the cell vitality, being suggested the oxidative stress as one of the main mechanism as

indicated our trial using GAE and β-carotene. In addition, the mixture of the three compounds affected in higher degree the vitality of cells, promoting the expression of different genes related to pathways involved into cell cycle, stress, cell survival and oxidative stress. The exposure to diverse mixture of contaminants severally exacerbates the negative effects that the same agents produces separately, mainly via oxidative stress, magnifying its toxicity and promoting cell mechanisms that could entail cell transformation. Further research is needed to clarify the potential impact of the mixture of pollutants on fish biology. Future *in vivo* trials on marine models exposed to mixtures of theses contaminants might be developed for the evaluation of their effects on immunity, stress and oxidative markers.

4 Declaration of interest

Declarations of interest: 'none'

5 Author Contributions

Concetta M. Messina designed the experiment; Cristobal Espinosa Ruiz and Simona Manuguerra performed molecular analyses. Eleonora Curcuraci provided and tested the antioxidant power. Cristobal Espinosa drafted the manuscript; Concetta M. Messina and Andrea Santulli finalized the manuscript, supervisioned the study and funded the research.

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- The mix modulates cell cycle biomarkers
- The mix modulates metabolism-related biomarkers
- The mix exerted a negative synergic effect