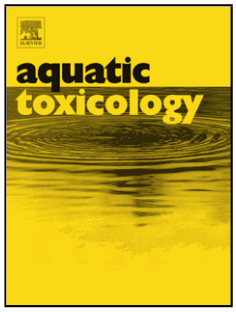

Title: Sub-lethal doses of polybrominated diphenyl ethers affect some biomarkers involved in energy balance and cell cycle, via oxidative stress in the marine fish cell line SAF-1

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Sub-lethal doses of polybrominated diphenyl ethers affect some biomarkers involved in energy balance and cell cycle, via oxidative stress in the marine fish cell line SAF-1.

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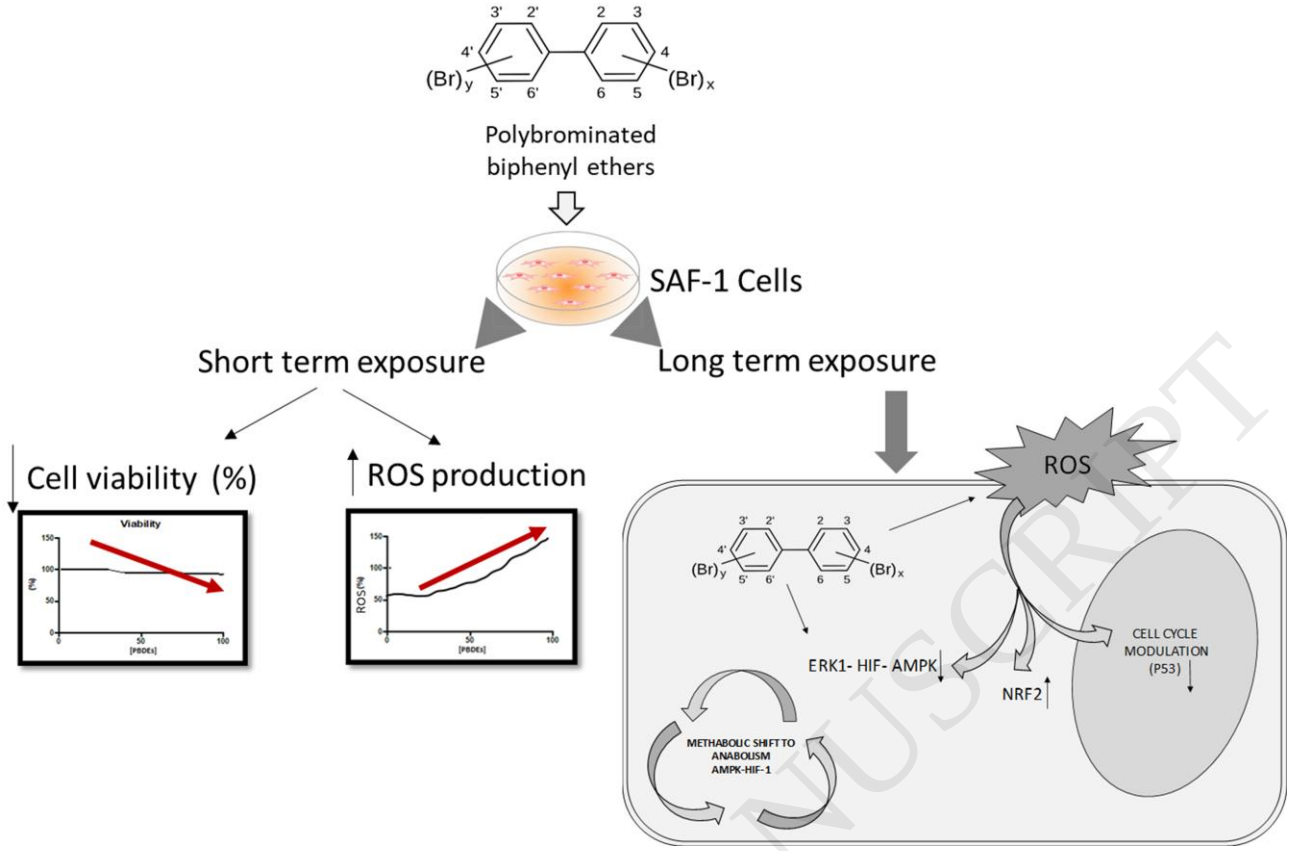
Graphical abstract



Highlights

- PBDEs are persistent pollutants that bioaccumulate in the marine environment.
- Rising concentrations of PBDEs affected SAF-1 vitality while induced ROS production.
- PBDES via oxidative stress affected the cell cycle and energetic balance pathways.
- Long term exposure to sub-lethal concentrations of PBDEs may lead cells transformation.

Abstract

Polybrominated diphenyl ethers (PBDEs) are a class of persistent contaminants which are found all over the world in the marine environment. *Sparus aurata* fibroblast cell line (SAF-1) was exposed to increasing concentrations of PBDEs 47 and 99, until 72 hours to evaluate the cytotoxicity, reactive oxygen species (ROS) production and the expression of some selected molecular markers related to cell cycle, cell signaling, energetic balance and oxidative stress (*p53, erk-1, hif-1α* and *nrf-2*), by real-time PCR. Furthermore, SAF-1 cells were exposed for 7 and 15 days to sub-lethal concentrations, in order to evaluate the response of some biomarkers by immunoblotting (p53,

ERK-1, AMPK, HIF-1α and NRF-2). After 48 and 72 hours, the cells showed a significant decrease of cell vitality as well as an increase of intracellular ROS production. Gene expression analysis showed that sub-lethal concentrations of BDE-99 and 47, after 72 hours, up-regulated cell cycle and oxidative stress biomarkers, although exposure to 100µmol L^{-1} down-regulated the selected markers related to cell cycle, cell signaling, energetic balance. After 7 and 15 days of sub-lethal doses exposure, all the analyzed markers resulted affected by the contaminants. Our results suggest that PBDEs influence the cells homeostasis first of all via oxidative stress, reducing the cell response and defense capacity and affecting its energetic levels. This situation of stress and energy imbalance could represents a condition that, modifying some of the analyzed biochemical pathways, would predispose to cellular transformation.

Abbreviations

AREs: Antioxidant response elements

AMPK: Adenosine 5′-monophosphate-activated protein kinase

CYP: Cytochrome P450

ERK-1: Extracellular signal–regulated kinase 1

HIF-1: Hypoxia inducible factor 1

NRF-2: Nuclear factor (erythroid-derived 2)-like 2

PBDEs: Polybrominated diphenyl ethers

ROS: Reactive oxygen species

Keywords: PBDEs, oxidative stress, energetic balance, cell cycle**,** *Sparus aurata* fibroblast.

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are a group of chemical compounds that are used in synthetic polymers and plastics with the aim of diminishing the flammability of combustible materials (Reynier et al., 2001; Teuten et al., 2009). These organobromide compounds are designed to be very stable for many years, which means they are able to remain in the environment for a considerable time (Eljarrat and Barceló, 2011). Many studies on PBDEs have shown that these compounds are ubiquitous, toxic, persistent and bioaccumulated in the environment (Boer et al., 2001; Hong et al., 2010; 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). Unfortunately, they accumulate also in aquatic environments by different ways, and the 2, 2′, 4, 4′-tetrabromodiphenyl ether (BDE-47) and the 2, 2′, 4, 4′, 5-pentabromodiphenyl ether (BDE-99) are among the most abundant found in this ambience (Bi et al., 2007; Leung et al., 2006). Because of these properties, some flame retardants have been prohibited in the USA and European Union (ATSDR, 2004; Directive 76/769/EEC., 1986; Off. J. Eur. Union, 2003; World Health Organization, 2003).

In the organism, PBDEs may be metabolized to more polar compounds, such as hydroxylated, methoxylated and/or conjugated metabolites (OH-, MeO- and GS-PBDEs), via phase I and phase II metabolic enzymes, in order to be eliminated via urines or bile, as demonstrated in exposed mice and rats (Malmberg et al., 2005; Qiu et al., 2007). The enzymes of the phase I, II, and III of the xenobiotic metabolism, play a fundamental roles in biotransformation and elimination of PBDEs, although cytochrome P450 enzymes (CYPs) (as CYP1A2, CYP3A4 and CYP2B) have been suggested to display a relevant role (Stapleton et al., 2009; Szabo et al., 2009). However, the products of PBDEs metabolytes (such as hydroxylated BDE congeners (OH-BDE)) have been demonstrated to produce greater toxicity than the parent BDE congeners in laboratory bioassays (Roberts et al., 2011; Stapleton et al., 2009, 2004). Several studies have been described PBDEs acts

as endocrine disruptors, the thyroid system being their main target (Legler, 2008; Yu et al., 2015). Furthermore, some PBDEs have been shown to interfere with the reproductive function both in human (Abdelouahab et al., 2011; Main et al., 2007; McDonald, 2005) and laboratory/wild animals (Gilchrist et al., 2014; Kuriyama et al., 2004; Stoker et al., 2004). Among these organisms, teleost have been attracted much attention, as they may be exposed to waterborne contaminants during a part or all their life span (Carr and Patiño, 2011). In fact, it has been proposed that xenobiotics have contributed to the decline of some wild marine fish populations (Hamilton et al., 2016) and the productivity of some marine fish stocks, due to the nursery habitat degradation in relation to pollutant accumulation (Gilliers et al., 2003; Riou et al., 2001; Rochette et al., 2010). However, it is difficult to determine in wild orgnisms the relationship between one class of compound and its effects on organism, due to the presence of multiple stresses in natural environments, which can include also many chemicals (Baillon et al., 2016). On the contrary, the experimental approach allows to control most of the parameters, avoiding for potential crosstalks and/or interferences. The effects and the underlying mechanisms of action of PBDEs at the individual level have thus been intensively studied through experimental exposure, notably in fish. These studies have demonstrated 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; Han et al., 2013, 2011; Lyche et al., 2011; Péan et al., 2013; Yu et al., 2015). As for others contaminants or xenobiotics, the use of cell lines to analyze the toxicity of aquatic pollutants, including PBDEs, is a consistent alternative to *in vivo* trials with fish and have contributed to significant advance in our knowledge of the toxicology and its mechanisms (Morcillo et al., 2015).

Several *in vitro* studies reported different alteration at molecular level produced by PBDEs exposure, as the induction of thyroxine-like and estrogen-like activities in Wistar WU rats microsomes (Meerts et al., 2000), as well as the increase of cell proliferation, micronucleus formation (Barber et al., 2006; Llabjani et al., 2010; Ukpebor et al., 2011) and CYP1A1 activation

in human MCF-7 breast cancer cells (Mercado-Feliciano and Bigsby, 2008). The induction of proliferation by PBDEs has been described also on human cancer cells from the female reproductive system (OVCAR-3) and normal ovarian CHO cells (Chinese hamster ovary cells) mediated via phosphorylation of PKCα and ERK-1/2 proteins (Li et al., 2012). Cell cycle disruption was described in Neuro-2a cells from mouse, in which PBDEs produced an increase of p53 and p21 expression (Chen et al., 2017a). A significant reduction of vitality, ROS production and cell cycle arrest were reported in spermatocytes from mouse (Huang et al., 2016). Moreover, cytotoxic effects, alteration of cell cycle and DNA damage were showed in a cell line of hepatocytes from human (HepG2) treated with PBDEs (An et al., 2011).

Although PBDEs have been suggested to affect the cell cycle, reducing the vitality and modifying the regulation of some cell cycle factors, the mechanisms involved in this process remain still unclear even if the promotion of oxidative stress is recognized as a relevant factor. Oxidative stress has been suggested as a relevant factor in cells exposed to PBDEs. In fact, it has been described *in vitro* the decrease of vitality together with an elevation of reactive oxygen species (ROS) in HepG2 (An et al., 2011; Hu et al., 2007), and the induction of NRF-2 pathway in Neuro-2a cells (Chen et al., 2017b), modulation of others molecular markers related to cell cycle, oxidative stress and energy management (Manuguerra et al., 2019) . However, others mechanisms could be implicated on disturbance of cell homeostasis by PBDEs, as the increase of ATP, or mitochondrial signaling pathway (Chen et al., 2017b; Huang et al., 2016).

Only few papers have evaluated the effect of PBDE in marine fish cell lines (Browne et al., 2009; Yang and Chan, 2015, Espinosa et al., 2019). With the aim to bring some light into this field, we evaluated the effect of PBDEs using *Sparus aurata* fibroblast cell line (SAF-1), previously used to assess the mechanisms of action of others contaminants (Morcillo et al., 2016).

Our study focused on some aspects of the cellular response to PBDEs, such as cell cycle and

proliferation, energetic balance and oxidative stress production at short and long time of exposure, by studying some representative molecular markers of each event. Among these: the ROS production, in order to assess if our experimental system is susceptible to oxidative stress determined by PBDE; the protein p53, involved in the perception of a risk for the DNA integrity and in the management of a complex of enzymatic responses aimed at promoting cell cycle arrest for DNA repair or cell death via apoptosis; the protein ERK-1, a kinase involved on cell proliferation through a complex MAP kinase signaling pathways; the HIF-1 protein, regulated by oxygen availability and in relation to that, able to modulate the metabolic pathway, ATP production and angiogenesis; the protein AMPK, which is a sensor of AMP/ATP ratio; NRF-2, considered as an oxidative stress sensor which activates the antioxidant and detoxifying response.

2. Material and methods

2.1 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). 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 (1000 rpm, 5min, 25°C) and the cell vitality was determined by the trypan blue exclusion test.

2.2 Cytotoxicity assay

Cytotoxicity assay was performed in five replicates. When SAF-1 cell lines were approximately 80% confluent, they were detached from flasks culture with trypsin (as described before), and aliquots of 100mL containing 10000 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 avoid cell over-growth. After that, the culture medium was replaced by 100mL well^{-1} of the PBDEs to be tested at the appropriate dilution.

The PBDE standards were provided by SPECTRA (Rome, Italy); stock solution of BDE-47 and 99 at a concentration of 25mmol L^{-1} were prepared by dissolving the powder compounds in dimethyl-sulfoxide DMSO. Tested concentrations of BDE-47 and 99 ranged from 1 to 100µmol L^{-1} (1, 10, 50, 75, 100). Cells were then 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 of the vehicle is well known (Abbes et al., 2013; Messina et al., 2016). After 24, 48 and 72h at 25°C, the vitality was determined using the MTT assay.

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 produced by the mitochondrial succinate dehydrogenase (Berridge and Tan, 1993; Denizot and Lang, 1986). After incubation with the PBDEs, 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 (5min, 100rpm) in dark conditions and the absorbance at 570nm and 690nm were determined in a microplate reader (Opsys MR™ Microplate Reader, USA). After the determination of the sub-lethal concentrations for each compound, the next experiments were done in order to assess molecular markers related to the different biochemical patterns.

2.3. Evaluation of intracellular ROS

SAF-1 cells were incubated with different concentration of BDE 47 and 99, as described above. After 72h of treatment, intracellular ROS were analyzed on cell seeded in 96 microplate, by the dichlorodihydrofluorescein-diacetate (DCF-DA) method (Kang et al., 2005) with some modifications (Messina et al., 2016). DCF-DA is oxidized to dichlorodihydrofluorescein (DCF) by ROS. Each well was exposed to 10 µL of DCF-DA in HBSS (5mg L^{-1}), incubated for 5 minutes at 37ºC to allow the oxidation of the DCF-DA and successively read on a spectrofluorometer 485exc - 530em (Varian Cary Eclipse, Mulgrave, Victoria, Australia). The results have been expressed as relative fluorescence/µg of total proteins (rf µg tp^{-1}).

2.4.1 Gene expression assay in cells exposed to increased concentration of PBDE for 72 hours.

SAF-1 cells (500.000 cells/well) were incubated in 12 well plates (Nunc, Germany) for 72 hours with different concentrations of PBDEs (vehicle (control), sub-lethal doses of BDE-99 and 47 (1µmol L^{-1}) and the highest dose of BDE-99 and 47 (100µmol L^{-1}). The highest concentration was selected as it was responsible of the highest cell mortality at 72 hours (67.4±4.6 for BDE-47 and 68.9±4.5 for BDE-99 respectively). Each concentration was tested in four different wells (500.000 cells/well). 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 stored at -80ºC prior to analyses.

2.4.2 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 the 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* and *nrf-2*] gene expression was evaluated after normalization with the reference genes. Data processing and statistical analyses 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.

2.5.1 Evaluation of molecular markers by immunoblotting in cells exposed to sub-lethal concentrations of PBDE for 15 days

For the evaluation of molecular markers related to the different pathways of oxidative stress, cell cycle progression, apoptosis and energy balance managements, a long term experiment, lasting 15 days, was carried out in flask, for each compounds, at only one sub-lethal concentration (1µmol L^{-1}). SAF-1 cells (40,000cells cm^{-2}) were incubated in a 25cm^2 flask (Nunc, Germany) and exposed to BDE-99, BDE 47 at 1µmol L^{-1}, plus a Mix of the two compounds at the same concentration (each compound at 1µmol L^{-1}). The compounds were added in the culture medium each time that this was renewed. The sampling for proteins evaluation by immunoblotting was done after 7 and 15 days. For each compound and each sampling time two replicates were performed. After the treatment, the cells were recovered by tripsinization and centrifugation, as previously described, incubated 30 min on ice in lysis buffer (1:4) (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, cocktail of protease inhibitors) and sonicated. Proteins concentration was measured in total lysate, according to the method described by Lowry (Lowry et al., 1951). Experiments were carried out in duplicate.

2.5.2 Immunoblotting

Equivalent amounts of proteins (20µg) were loaded on pre-cast gel for SDS–polyacrylamide electrophoresis (SDS-PAGE), (Bio-Rad, Hercules, CA, USA) and blotted using a Trans Blot Turbo Transfer System (Bio-Rad, Hercules, CA, USA). The correct amount of protein loading was confirmed by red Ponceau staining. Filters were used for protein detection by primary antibodies (AbI) specifics for p53 (Ab monoclonal from mouse), for total Extracellular signal-regulated kinase 1 (ERK-1) (Ab monoclonal from rabbit), phospho-AMP-activated protein kinase alpha (AMPK α) (Ab polyclonal from rabbit), hypoxia-inducible factor 1-alpha (HIF-1α) (Ab monoclonal from

mouse) and Nuclear factor (erythroid-derived 2)-like 2 (NRF-2) (Ab monoclonal from mouse) (Sigma-Aldrich, Dorset, UK; Santa Cruz, CA, USA). In relation to the origin of the AbI, the appropriate secondary antibodies were used (anti mouse or anti-rabbit, anti-goat secondary antibody conjugated with horseradish peroxidase (GAR/M-HRP Bio-Rad, Hercules, CA, USA). The signals originated by immunoreaction were detected using enhanced chemo-luminescent (ECL) reagents (Bio-Rad). Images were obtained, photographed and digitalized with Chemi Doc XRS (Bio-Rad, Hercules, CA, USA), and further analyzed with Image Lab software (Bio-Rad, Hercules, CA, USA). The results were expressed as fold increase of each treatment in relation to the respective control, representing the mean value of three separate experiments.

2.6 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).

3. Results

3.1 Cytotoxicity assay

The effects of BDE 47 and 99 on vitality and cytotoxicity of SAF-1 cells were evaluated by MTTs. Results from the cytotoxicity test showed that incubation of SAF-1 cells with BDE 47 and 99 significantly affected their vitality ($P<0.05$), indeed showing high levels of cytotoxicity at the higher concentration tested (50 and 100µmol L^{-1} at 48-72 hours of BDE-47 and BDE-99) (Figure 1a and b, respectively).

3.2 Evaluation of intracellular ROS

In this study BDE 47 and 99 were also tested for their effects on intracellular ROS production in SAF-1 cells. While lower concentrations of compounds did not determine a significant increase in ROS, the exposure to 100µmol L^{-1} of BDE 47 and BDE 99, after 72 hours of incubation, significantly increased the intracellular ROS production in SAF-1, with respect to control samples ($P<0.05$) (Figure 1c and d, respectively).

3.3 Evaluation of molecular markers by gene expression and immunoblotting

The expression of some genes related to cell cycle (p53), cell proliferation (*erk-1*), energetic balance (*hif-1*) and oxidative stress (*nrf-2*) were evaluated in cells exposed for 72 hours to two doses of BDE-99 and 47 (1µmol L^{-1} and 100µmol L^{-1} (Figure 2). *p53* expression was significantly up-regulated by the exposure to sub-lethal concentrations (1µmol L^{-1}) of BDE-99 and 47 while was down-regulated after the exposure to 100 µmol L^{-1} of BDE-47 after 72 hours ($P<0.05$). By contrast, the highest dose of BDE-99 did not affect the expression of *p53* (Figure 2). e*rk-1* expression was significantly down-regulated by the exposure to 100µmol L^{-1} of both PBDEs ($P<0.05$) at 72 hours. The expression of *hif-1* was significantly decreased by the exposure to the highest dose of both compounds at 72 hours ($P<0.05$) (Figure 2). Finally, *nrf-2* expression was significantly up-regulated

by the exposure to sub-lethal concentrations ($P<0.05$), although highest concentrations of both compounds did not affect the oxidative stress marker (Figure 2).

Most of the proteins previously analyzed by gene expression, were studied by immunoblotting, in SAF-1 cell treated for 15 days with one sub-lethal dose of each compound (1µmol L^{-1}), plus a mix of the two at the same concentration; the results are showed in Figure 3.

Regarding the p53, it was observed a significant reduction of the protein levels in SAF-1 cells treated with the mix and with BDE-99 at 7 days, *vs* the control ($P<0.05$) (Figure 3).

The marker of cell proliferation, ERK-1, resulted decreased in almost all PBDEs treatments, after 7 and 15 days, respect to the control (Figure 3) ($P<0.05$).

The selected markers related to the energetic balance (AMPK and HIF-1α) were affected by the PBDEs treatment, respect to the control (Figure 3). The levels of the AMPK protein significantly decreased in cells treated with BDE 47 and Mix ($P<0.05$) and the decrease was more pronounced for HIF-1α in cells treated with all compounds ($P<0.05$).

Finally, the marker related to oxidative stress, the NRF-2, resulted significantly increased in all exposed cells, respect to the control ($P<0.05$ (Figure 3).

4. Discussion

4.1 Effects of PBDEs on cytotoxicity and ROS production

The objective of this study was to use the SAF-1 cell line as *in vitro* model to investigate the molecular mechanisms involved on cell cycle, energetic metabolism and oxidative stress produced by BDE-47 and 99, which are the most abundant PBDEs in the environment and wildlife (Bi et al., 2007; Leung et al., 2006; Yang et al., 2016). The cytotoxic effects of PBDEs exposure at short time and ROS production, along with the gene expression of some markers were evaluated on the SAF-1 cell line. Furthermore, the effect of PBDEs exposure at middle/long time at sub-lethal doses on the protein profile involved on cell cycle, cell metabolism and oxidative stress were studied.

Our findings showed that PBDEs exposure affected the cell vitality in a time-dose dependent manner (Figure 1a-c). In our study, BDE-47 and 99 significantly decreased the cell vitality of SAF-1 above a threshold concentration (50μmol L^{-1}) after 48 and 72 hours of exposure. These observations agrees with other works developed *in vitro*, which reported that BDE-47 significantly affected to HepG2 cell proliferation (An et al., 2011; L. Wang et al., 2012) or works that showed that BDE-99 produced a significant inhibition of Neuro-2 cells (Chen et al., 2017a; Slotkin et al., 2017).

Additionally, intracellular ROS production was increased in a dose-response manner after PBDEs incubation (Fig. 1d-f). Others *in vitro* studies reported a similar relationship between PBDEs and ROS production. For example, ROS production, as well a decrease of vitality, were evidenced in HepG2 cells treated with PBDEs (An et al., 2011; Hu et al., 2007). Another study showed a significant induction of NRF-2 and its antioxidant pathway on Neuro-2a cells after BDE-47 exposure (Chen et al., 2017b). In addition to *in vitro* studies, *in vivo* experiment on mouse treated with BDE-209 showed increased lipid peroxidation on germ cell as well as decreased the levels of CAT and SOD (Sarkar and Singh, 2017). In all, we showed that PBDEs exposure determined

cytotoxicity and a significant increase of oxidative stress on SAF-1 cells. Similar results on toxicity and ROS production were obtained in our recent work on the same cell line exposed to BDE-209 (Espinosa et al., 2019) and in human fibroblast cell line exposed to BDE-47, 99 and 209 (Manuguerra et al., 2019).

4.2 Effects of PBDEs on molecular markers related to cell cycle, energetic balance, oxidative stress and proliferation

Under a stress situation, the protein p53, designed as "the genome guardian", contributes to regulate the cell cycle through the control of the cell cycle arrest (by activation of cyclins and retinoblastoma protein which entail cell cycle interruption) or addressing cell death (Yee and Vousden, 2005; Zhang et al., 2015). Our results showed that *p53* gene resulted up-regulated after short time exposure to sub-lethal concentrations of BDE-47 and 99 and down-regulated in cell exposed to the highest concentrations (figure 2); the protein seems to be still active in cells exposed to the sub-lethal doses for longer time, except than in cells exposed to the mix of PBDEs, in which its levels resulted dramatically reduced ($P < 0.05$) (figure 3). Other studies reported an increase of the p53 levels after PBDEs exposure, such as in human fibroblast (Manuguerra et al., 2019), in Neuro-2a cells, in mouse spermatocytes and HepG2 cells (Chen et al., 2017a; Huang et al., 2016; An et al., 2011), as well as in the studies done on zebrafish embryos (Lyche et al., 2016) and in human SH-SY5Y cells (Zhang et al., 2013). In all these studies both time and doses of exposure were different and this aspect render complicate the comparison with our results, also because of the complexity of these pathways. Nevertheless, the decrease of the p53 expression observed in our experiment could indicate the possibility that the cell lost one of its most important protective factor, blocking the DNA reparation. A similar results was observed in SAF-1 cells exposed to BDE-209 (Espinosa et al, 2019). This situation might increase the susceptibility to mutagenesis and cell cycle transformation, that, in fact, have been reported for PBDEs (Espinosa et al 2019; Chen et al., 2017a; Li et al., 2012).

ERK-1 is a kinase mainly implicated in cell activation, modulating cell proliferation (McCubrey et al., 2007a, 2007b; Turpaev, 2006). In our study, both the gene expression as well as the levels of ERK-1 protein, resulted depresses after PBDE exposure (figure 2 and 3). These results are consistent with our recent results obtained in the same cell line exposed to BDE-209 (Espinosa et al., 2019), but in contrast with others studies that described an increase of ERK1/2 levels 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), or in human HeLa cells (Li et al., 2012), although different doses and time of exposition were used. It was reported that other contaminants, such as 1,2-dichloroethane, can induce toxicity and apoptosis via ERK1 downregulation in HepG2 human cells (Pang et al., 2018). As ERK1/2 activation plays a protective role against oxidative stress and others cell insults, the inhibition of ERK1/2 pathway could result in a loss of protection from cells (Koinzer et al., 2014), suggesting that the inhibition of ERK1 observed in SAF-1, after PBDEs exposure, may exacerbate the negative effects of the contaminant. However, further research is needed to clarify this issue.

AMPK is an enzymatic complex activated by the increase of AMP/ATP ratio, being considered as a sensor of the cell energy levels (Shen et al., 2010). In our long term experiment, the levels of the AMPK protein resulted significantly decreased in cells treated with BDE-47 and Mix (figure 3), which is consistent with our recent results obtained in the same cell line exposed to PBDE-209 (Espinosa et al., 2019), and in human fibroblasts exposed to BDE-47, BDE-99 and BDE-209 (Manuguerra et al., 2019). However, our results contrast with those reported by others authors who showed that BDE-47 and BDE-99 exposure decreased ATP levels on insolated mitochondria from rat liver (Pazin et al., 2015), determining an AMPK increase (Shen et al., 2010). In fact, low levels of AMPK could be related to metabolic changes (anaerobic conditions) that has been described on cancer genesis (Sciacovelli et al., 2014) and different types of cancer (Li et al., 2015). As AMPK activation may regulate energy levels, enforcing metabolic checkpoints and inhibiting cell growth,

this protein is often considered as a tumor suppressor (Li et al., 2015). In this situation, the low levels of AMPK observed in our experiment could represent an early warning marker, that could lead to cancer promotion. However, the mechanisms need to be further evaluated.

The hypoxia inducible factor 1 (HIF-1) is considered the master transcriptional regulator of cellular responses to hypoxia due to its sensibility to the oxygen availability inside the cell (Kitajima et al., 2017; Qi et al., 2014). In relation to this condition, HIF-1 regulates the molecular processes that maintain the oxygen homeostasis, adapting the related metabolic pathways (Romney et al., 2011; Shao et al., 2010; Zhang et al., 2009). In view of these properties, HIF-1 activates the transcription of genes involved in cancer genesis, including angiogenesis, anaerobic glycolysis, cell survival and invasion (Lin et al, 2014).

From structural point of view, HIF is a heterodimer composed by α and β subunits, that are differently active in relation to the oxygen availability: in normoxia, HIF 1-α is degraded by proteasome and its activity is inhibited. In hypoxia, HIF 1-α levels increase, binds to the β subunit, also called ARNT (aryl hydrocarbon receptor nuclear translocator), the heterodimer translocates to the nucleus, binds to the hypoxia-responsive elements (HRE), regulating the expression of target genes for angiogenesis, glucose transporters and glycolytic enzymes (Wang et al., 2012; Lin et al, 2014)

In our experiment, *hif-1* expression and protein levels were down-regulated by exposure to the highest dose of BDE-47 (100µmol L^{-1}) after 72 hours and after 15 days of exposure, which is according to our recent work on the same cell line exposed to BDE- 209 (Espinosa et al, 2019). Nevertheless, these results contrast with the data reported in others papers that showed HIF-1 is increased under oxidative stress situation (Jung et al., 2008; Li et al., 2016; Wang et al., 2012; Manuguerra et al., 2019). As far as we know, no data are available regarding the effect of PBDEs 47 and 99 on *hif-1* expression in marine organisms. In this sense, it has been reported HIF-1 activity

could be influenced by AhR (aryl hydrocarbon receptor) (Regoli and Giuliani, 2014). AhR could interact with xenobiotics, which produces its activation by separation from HSP90 (Kolluri et al., 1999; Ma and Whitlock, 1996). Then, the subunit AhR-ligand is transported into the nucleus where it interacts with ARNT (HIF-1β) and binds to specific DNA regions. Thus, AhR could activate the expression of detoxification enzymes, which includes phase I enzymes (Rowlands and Gustafsson, 1997; Whitlock Jr. et al., 1996). For this reason, AhR agonists can downregulate the HIF-1 pathway due to the competition for ARNT (Chan et al., 1999; Matikainen et al., 2001; Nie et al., 2001). This could be a plausible explanation of our results, even if this aspect must be investigated in future researches.

Interestingly, it has been reported that AMPK and HIF-1 could regulate the levels of ROS in opposite directions, suggesting that these factors are able to modulate the levels and/or activity one to another (Hwang et al., 2014). Then, HIF-1 may be downregulated by AMPK via phosphorylation, while AMPK could be downregulated by HIF-1 in a situation of oxidative stress (Hwang et al., 2014). In our experiment, both AMPK and HIF-1 showed to be significantly decreased after 7 and 15 days of PBDEs exposure. So, although these factor could be inhibited by different pathways (AhR antagonisms, etc.), it can be hypothesized that both AMPK and HIF-1 could be downregulated each other after prolonged upregulation. Anyways, as both AMPK and HIF-1 are normally activated during oxidative stress situation (Han et al., 2010; Wang et al., 2012), the decrease of these factor after long time exposure could lead to an exacerbation of the negative effects produced by PBDEs, mainly via oxidative stress. For this reason, the measurement of these biomarkers might support valuable information about the negative effects of contaminants in influence both metabolism and energetic pathways, that are preliminary conditions for cell transformation.

Multiple signaling pathways have been implicated in protecting cell from ROS overproduction and oxidative stress (Huang et al., 2015; Regoli et al., 2011). In this sense, NRF-2 is a factor which

plays a protective role against oxidative stress by interacting with AREs (antioxidant response elements) and regulates the expression of a wide range of antioxidant and phase II detoxification genes (Huang et al., 2015). In addition, NRF-2 is a transcription factor well conserved in marine species (Giuliani and Regoli, 2014). Our results showed both *nrf-2* expression and protein levels were increased by the exposure to sub-lethal doses of PBDEs for 72 hours and 7-15 days, respectively. These observations are consistent with the research described in SAF-1 cells (Espinosa et al., 2019), in human fibroblast cell line (Manuguerra et al., 2019), on mice (Shan et al., 2017), on Neuro-2a cells and human extravillous trophoblast cell line (Chen et al., 2017b; Park and Loch-Caruso, 2014), that reported the up-regulation of NRF-2 via oxidative stress induced by PBDEs exposure. By contrast, the exposure to the highest dose (100µmol L^{-1}) of these compounds for 72 hours did not affect the *nrf-2* expression. The expression of the genes analyzed on cells exposed to 100µmol L^{-1} seems to indicate a weak or diminished response to the contaminant. A line of evidence has suggested that the NRF2 pathway has various novel functions, particularly in cell proliferation and differentiation (Murakami and Motohashi, 2015). In this sense, intracellular ROS produced by PBDEs might affect different cellular processes, passing from metabolic and energetic change (Dong et al., 2017; Martinez-Outschoorn et al., 2017; Mullen and DeBerardinis, 2012; Sullivan et al., 2016), that are process preliminary to cell transformation (Valko et al., 2007).

In conclusion, our results demonstrated that rising concentrations of PBDEs exposure effected the cell vitality, mainly via oxidative stress. However, long term exposure to sub-lethal doses of PBDEs were able to affect different cell pathways related to cell cycle, cell metabolism, energetic balance and oxidative stress. Collectively, these findings suggested the cell response to low doses of PBDEs could be attenuated after long term exposure, avoiding the apoptosis mechanisms. Instead of apoptosis, the cell survival could entail the increase of cell damage, mainly via oxidative stress and, successively, cell transformation (Figure 4). Further studies are needed to ascertain the potential impact of different PBDEs on fish biology as well as the molecular mechanisms involved.

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Legend to figures

Figure 1. Cytotoxicity of SAF-1 cells exposed to different concentrations of (A) BDE 47 (1-100µmol L^{-1}) and (B) 99 (1-100µmol L^{-1}) for 24, 48 and 72 h. Reactive oxygen species (ROS) production, expressed as relative fluorescence · µg total $proteins^{-1}$ in SAF-1 cells exposed to different concentrations of (C) BDE 47 (1-100µmol L^{-1}) and (D) BDE-99 (1-100µmol L^{-1}) for 72 h. Bars represent the mean ± SEM (n=6). Statistically significant differences (ANOVA; P≤0.05) were denoted using different letters.

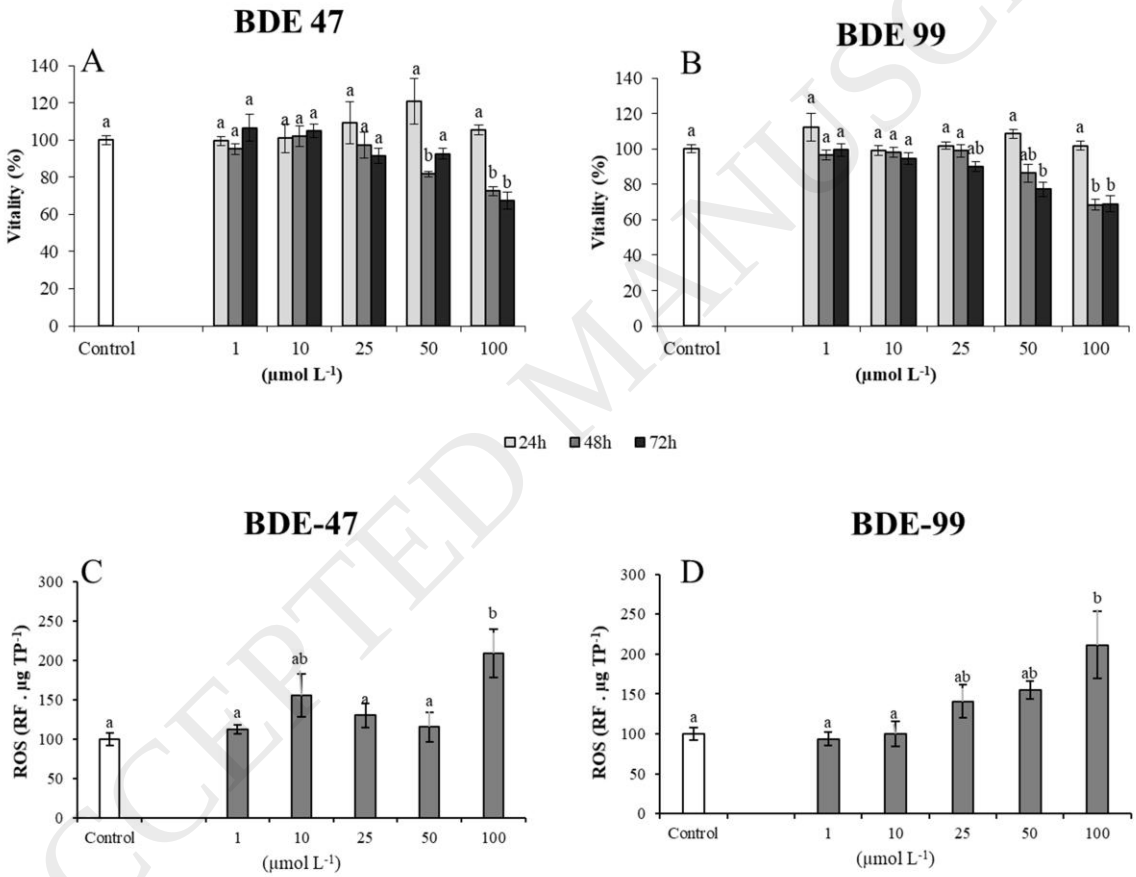

Figure 2. Relative gene expression of some genes related to cell cycle (*p53*), proliferation (*erk-1*), energetic balance (*hif-1*) and stress (*nrf-2*) from SAF-1 cells exposed to vehicle (Control), 1µmol L^{-1} or 100 µmol L^{-1} doses of BDE 99 and 47 for 72 hours. Bars represent the mean ± SEM (n=4). Statistically significant differences (ANOVA; P≤0.05) were denoted using different letters.

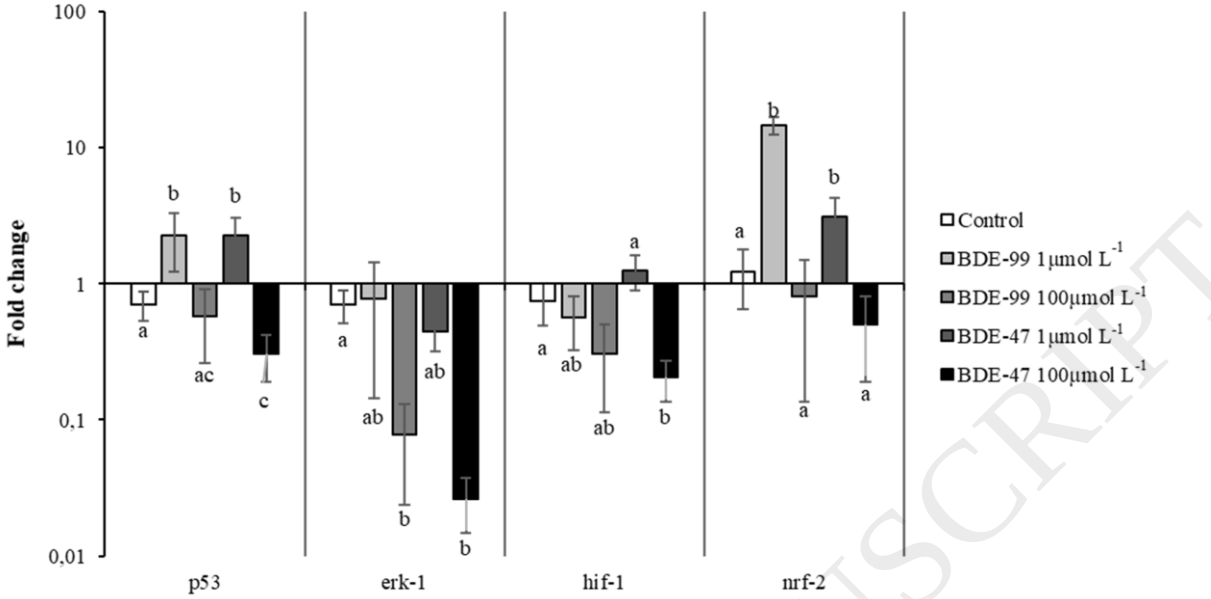

Figure 3. Immunoblotting of some proteins related to cell cycle (p53), proliferation (ERK-1), energetic balance (HIF-1α, AMPK) and stress (NRF-2) from SAF-1 cells exposed to vehicle (Control) or 1µmol L^{-1} of BDE 99 and 47 or a Mix of the three compounds for 7 or 15 days. Immunoblotting of actine protein is used as a control.

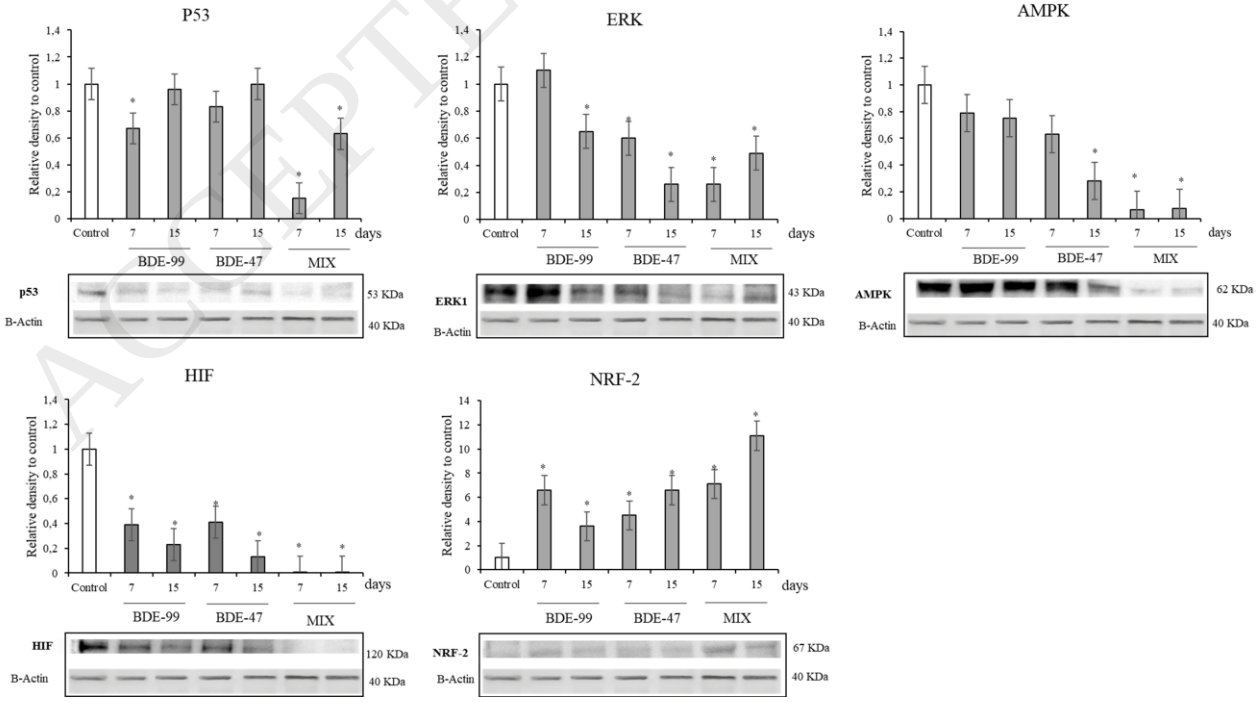

Figure 4. Diagram showing the effects of sub-lethal doses of PBDEs exposure on the cell signaling, cell cycle and cell metabolism via oxidative stress.

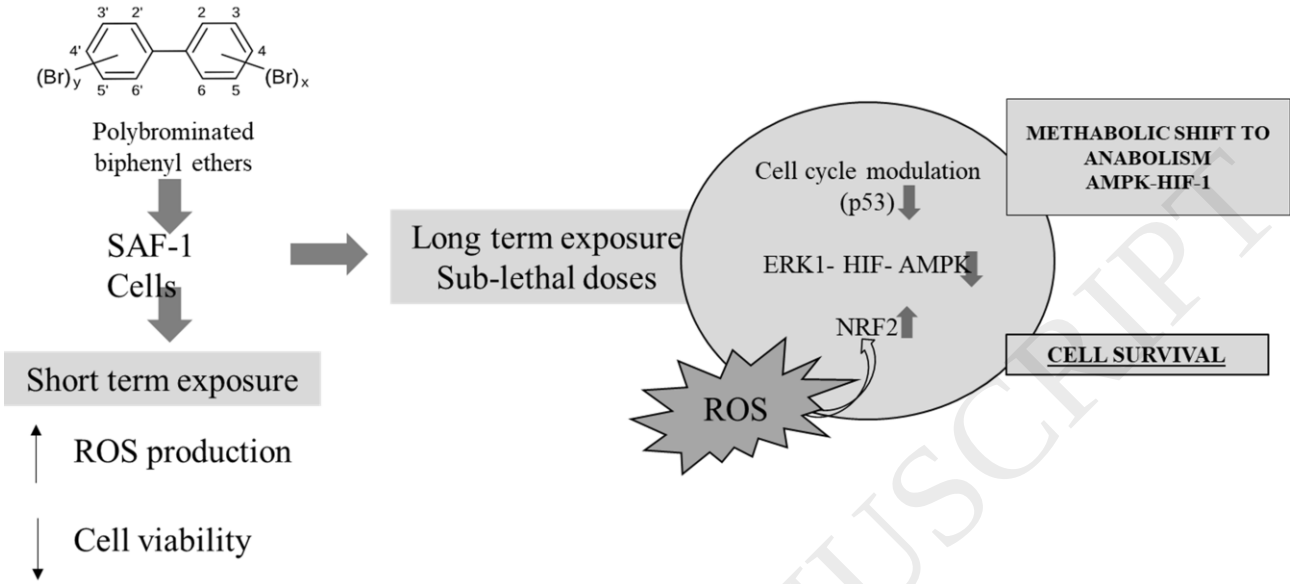

Table 1. Gilthead seabream primer sequences used for real-time PCR.

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
nrf2		F- GTTCAGTCGGTGCTTTGACA
		R- CTCTGATGTGCGTCTCTCCA
ef1a	AF184170	F- CTTCAACGCTCAGGTCATCAT
		R- GCACAGCGAAACGACCAAGGGGA
18S	AM490061	F- CTTCAACGCTCAGGTCATCAT
		R- AGTTGGCACCGTTTATGGTC