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# Hormonal activity, cytotoxicity and developmental toxicity of UV filters

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ABSTRACT

Ultraviolet (UV) filters are commonly used compounds in personal care products and polymer based materials, as they can absorb solar energy in the UVA and UVB spectrum. However, they are able to bind to hormone receptors and have several and different types of hormonal activities determined by *in vitro* assays. One of the aims of this work was to measure the hormonal and cytotoxic activities of four frequently used UV filters using bioluminescence based yeast test organisms. Using *Saccharomyces cerevisiae* BLYES and BLYAS strains allowed the rapid and reliable detection of agonist and antagonist hormonal activities, whereas BLYR strain served to measure cytotoxicity. Results confirmed that all tested UV filters show multiple hormonal activities. Cytotoxicity is detected only in the case of benzophenone-3. Research data on the toxic effects of benzophenone-3, especially on aquatic organisms are scarce, so further investigations were carried out regarding its cytotoxic and teratogenic effects on bacteria and zebrafish (*Danio rerio*) embryos, respectively. Results revealed the cytotoxicity of benzophenone-3 not only to yeasts but to bacteria, as well as its ability to influence zebrafish embryo hatching and development.



## 1. Introduction

In recent years, a growing attention has been granted to the deleterious effects of ultraviolet (UV) radiation, as it may contribute to the development of skin cancer (IARC, 1992; Ichihashi et al., 2003) and deteriorate polymer based materials. Organic UV filters can absorb the energy of photons in the UVA (320–400 nm) and UVB (290–320 nm) interval, so they are widely used in personal care products (e.g. suntan lotions, body lotions, shampoos, lipsticks) and in technical materials for protecting human health and enhancing the light stability of products.

UV filters can enter surface waters through wastewater effluents and can be washed off from the skin during recreational activities. It can also be observed that there is a seasonal variation to the environmental concentration of these compounds. The highest concentrations are measured in summer in the case of benzophenone-3 (BP-3), 3-(4-Methylbenzylidene)camphor (4MBC), ethylhexyl methoxycinnamate (EHMC) and octocrylene (OC) in water samples from lakes in Switzerland (Poiger et al., 2004). UV filters have been found in different matrices, for example wastewater influents (Loraine and Pettigrove, 2006), effluents (Li et al., 2007), rivers (Cuderman and Heath, 2007), lakes (Cuderman and Heath, 2007; Rodil and Moeder, 2008), soil (Jeon et al., 2006), sludge (Gago-Ferrero et al., 2011; Rodríguez-Rodríguez et al., 2012), sediment (Kameda et al., 2011) and biota (Buser et al., 2006; Fent et al., 2010) in trace level concentration (ng/L; ng/g; μg/g). Moreover, UV filters were also detected in human milk (Hany and Nagel, 1995; Schlumpf et al., 2010) and urine (Calafat et al., 2008).

Although UV filters can provide protection against the harmful effects of UV radiation, they also have some disadvantages. UV filters can bind to hormone receptors and show agonistic and/or antagonistic activities towards human estrogen receptor α (hERα) and human androgen receptor (hAR) (Klann et al., 2005; Kunz and Fent, 2006; Ma et al., 2003; Schlumpf et al., 2001; Schmitt et al., 2008; Suzuki et al., 2005). The most comprehensive study was carried out by Kunz and Fent (2006) who examined *in vitro* multiple hormonal activities of eighteen UV filters and one metabolite with recombinant yeast systems. All compounds proved to be hormonally active; moreover, ten UV filters showed three distinct hormonal activities each, and most of them elicited antiandrogenic and antiestrogenic activities.

There are only few assays that are concerned with the effects of UV filters to aquatic organisms but there are some evidence that the presence of UV filters in surface waters and sediment can be harmful to aquatic organisms. Coronado et al. (2008) experienced significant vitellogenin synthesis in juvenile rainbow trout (*Oncorhynchus mykiss*) and male Japanese medaka (*Oryzias latipes*) at

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749 μg/L and 620 μg/L concentrations of BP-3. In addition to this, a 21-day treatment of the parent Japanese medaka with 620 μg/L BP-3 significantly decreased the percentage of hatched eggs. Kaiser et al. (2012) studied the effects of EHMC on zebrafish (*Danio rerio*) in a 48 h sediment assay. Exposing the fish to 1000 mg/kg dw EHMC resulted in serious developmental disorders. The freshwater populations of invertebrates can also be adversely affected by hormonally active UV filters. Schmitt et al. (2008) exposed *Potamopyrgus antipodarum* snails to 4MBC. The number of unshelled embryos was significantly enhanced and the mortality of snails significantly increased. The same mortality was detected in the case of *Lumbriculus variegatus* worms. Kaiser et al. (2012) found that EHMC significantly decreased the number of embryos per snail of *Potamopyrgus antipodarum* and *Melanoides tuberculata* species. Liu et al., (2015a) carried out an acute toxicity test with *Daphnia magna* testing thirteen benzophenone-type UV filters. The $EC_{50}$ value was 2.01 mg/L for BP-3.

In this work, four commonly used UV filters were selected for the purpose of analyzing their hormonal and cytotoxic activities using bioluminescent yeast bioreporters, namely BLYES (Sanseverino et al., 2005), BLYAS and BLYR (Eldridge et al., 2007). These low cost, rapid bioassays have been validated for many compounds (Sanseverino et al., 2009), but not tested for UV filters yet. As the majority of *in vitro* tests focus only on the detection of agonistic activities, our aim was to measure antagonistic activities, too. When finding cytotoxic effect, that has not been referred yet our purpose was to investigate toxicity by standard aquatic test organisms, namely the Microtox test which uses *Aliivibrio fischeri* and the fish embryo toxicity test which uses zebrafish. These test organisms are widely used and zebrafish embryos have the advantage of transparency, so main morphological changes can be followed up easily (Hill et al., 2005).

## 2. Materials and methods

### 2.1. Chemicals

17ß-estradiol (E2) (purity ≥98%), 5α-dihydrotestosterone (DHT) (≥99%), 4-hydroxytamoxifen (4HT) (50:50 E:Z isomers), OC (certified reference material) and EHMC (≥98%) were purchased from Sigma-Aldrich Co. LLC. Flutamide (FT), BP-3 (≥98%) and 4MBC (≥99%) were purchased from VWR International LLC. FT and 4HT were used as positive controls. Dimethyl sulfoxide (DMSO) was bought from Fischer Scientific (analytical reagent grade) and used for preparing stock solutions that were applied in the Microtox test and in the acute embryo toxicity test. Methanol (purity 99.9%) was bought from Sigma-Aldrich Co. LLC and used for preparing stock solutions that were applied in BLYAS/BLYES/BLYR experiments.

### 2.2. The measurement of hormonal activities

#### 2.2.1. Bioluminescent yeast systems

The BLYES and BLYAS strains served to detect estrogenic and androgenic activity, respectively. The constructions of these strains are detailed in Eldridge et al. (2007) and Sanseverino et al. (2005). Briefly, the BLYES and BLYAS strains contain the hERα and hAR gene in their chromosome, respectively; while two plasmids with estrogen and androgen response elements (EREs and AREs) and genes (*frp*, *luxCDABE*) are responsible for the production of bioluminescence. When an estrogenic or androgenic compound enters the cell and bind to the hormone receptor, receptor-molecule complexes form dimers and bind to the EREs or AREs to induce the transcription of genes (Eldridge et al., 2007; Sanseverino et al., 2005).

#### 2.2.2. The measurement of estrogenic and androgenic activities using yeast bioreporters

Modified yeast minimal medium (YMM leu-, ura-) is applied for growing *Saccharomyces cerevisiae* strains (Routledge and Sumpter, 1996) at 30 °C and 200 rpm to an $OD_{600}$ of 1.00. The stock solution of UV filters were serially diluted in methanol and 20 μl aliquots were transferred to sterile, flat bottom, black, 96-well microplates (Greiner Bio-one Gmbh, Germany), in 3 parallels. After methanol evaporated, 200 μl of yeast cells were added to the appropriate wells. The serial dilution of E2 and DHT served as positive controls. Negative controls (YMM and yeast cells) and solvent controls (YMM, yeast cells and methanol) were also applied. The bioluminescence was measured after 5 h of incubation at 30 °C by VictorX Multilabel Plate Reader (Perkin Elmer Inc.).

#### 2.2.3. The antiestrogen and antiandrogen assay procedure

To measure antiestrogen and antiandrogen effect, the BLYES and BLYAS assay procedures were modified, as suggested by Sohoni and Sumpter (1998). The serial dilutions of UV filters were supplemented with E2 or DHT in concentration corresponds with $EC_{65}$. The known antiestrogen and antiandrogen 4HT and FT served as positive controls. Additionally, solvent controls (YMM, yeast cells, methanol) and negative controls as E2/DHT ($EC_{65}$) were also included.

### 2.3. The measurement of toxic effects

#### 2.3.1. Saccharomyces cerevisiae BLYR strain

Cytotoxicity was followed up by the constitutive control BLYR strain. This strain also contains the genes responsible for bioluminescence but in spite of the BLYES and BLYAS strains, their transcription is continuous, so cytotoxic effects can lead to the decrease of bioluminescence (Eldridge et al., 2007). The same assay procedure was applied for the BLYR test as described in Section 2.2.2.

#### 2.3.2. Aliivibrio fischeri (MicroTox™)

In order to prove unexpected cytotoxicity measured by BLYR test, further investigations were carried out using *Aliivibrio fischeri* (DSM-7151, NRLLB-11177) bacteria according to ISO 11348-3:2007. *A. fischeri* was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). Stock solution of the tested compound was made in DMSO and was diluted in distilled water containing 2% NaCl. The final concentration of DMSO was only 1.665% v/v which is not toxic to *A. fischeri* bacteria. The NaCl and DMSO containing distilled water also served as solvent control. The test was repeated three times in two parallels. Relative bioluminescence was detected by Microtox™ 500 luminometer after 30 min incubation and bioluminescence inhibition was determined.

#### 2.3.3. Modified fish embryo acute toxicity test (OECD 236.)

Fish were bred and maintained at the Institute of Aquaculture and Environmental Safety, Szent Istvan University in a recirculation system (ZebTEC, Tecniplast Inc.) under standard laboratory conditions (recirculation system water: 25°C, 525 ± 50 μS conductivity, with 14 h light–10 h dark cycle). Fish were fed twice a day with complete fish food (zebrafish Small Gran food, Dietex International Limited, Special Diets Services G.B.) supplemented by freshly hatched live *Artemia nauplii* twice a week. The Animal Protocol was approved by the Hungarian Animal Welfare Law (XIV-I-001/2303-4/2012).

The test method was based on the OECD guideline 236 (OECD, 2013) but to be able to detect developmental disorders, the test procedure had to be modified. The applied number of zebrafish embryos was 40 in each concentration in four replicates.