
Szent István University

Ph.D. Thesis

# Adverse effects and biodetoxification of water polluting UV filters and 5α-dihydrotestosterone

Adrienn Balázs

Gödöllő

2018

**Ph.D. School**


**Name:** Ph.D. School of Environmental Sciences

**Discipline:** Environmental Sciences

**Head:** Erika Csákiné Michéli, D.Sc.

Professor

SZIU, Faculty of Agricultural and Environmental Sciences

Department of Soil Science and Agricultural Chemistry

**Supervisor:** Balázs Kriszt, Ph.D.

Associate professor

SZIU, Faculty of Agricultural and Environmental Sciences

Department of Environmental Safety and Ecotoxicology

**Supervisor:** Csilla Krifaton, Ph.D.

Senior lecturer

SZIU, Faculty of Agricultural and Environmental Sciences

Department of Environmental Safety and Ecotoxicology


..... .....

Approval of Head of Ph.D. School Approval of Supervisors

## TABLE OF CONTENTS

# 1. INTRODUCTION AND AIMS

The use of natural and especially synthetic compounds is part of our everyday life but many of them can have endocrine disruptor (ED) effect. ED compounds have been frequently detected in effluents, which are introduced almost always into surface waters, of wastewater treatment plants. First of all, ED effects of thousands of compounds in our environment should be investigated by fast and cost-effective methods. Unicellular bioreporters can be suitable for this purpose. It is also necessary to clarify the adverse effects of these compounds to the ecosystem by ecotoxicological test methods.

Steroid androgens are getting more and more attention next to estrogens during analytical measurements of wastewaters and surface waters. 5α-dihydrotestosterone (DHT) is one of the natural steroid androgens that is detected in the highest concentration in environmental matrices. The elimination of steroid estrogens and androgens from the environment is still an unsolved problem. Among removal processes biodegradation based technics are also important next to physical and oxidation methods.

Components of personal care products, for example UV absorbing chemicals (UV filters), are also widespread in surface waters. Although they are applied in huge amounts we have only limited information about their toxicity and risk to aquatic species. For example they may have agonist or antagonist hormonal activity moreover most of them can be characterized by several and different types of these hormonal effects. Currently only few and sometimes controversial data can be found in the scientific literature in connection with the ED effects of UV filters. Nevertheless more and more articles report on such adverse effects to aquatic organisms like oxidative stress, growth inhibition or lethality. On that basis, it is important to find out more about the ED effect and

toxicity of UV filters by different ecotoxicological tests and to develope methods for the biodegradation of these compounds.

My aim was to test DHT among natural ED compounds, which has the highest androgenic activity, and UV filters like benzophenone-3 (BP-3), 4-methylbenzylidene camphor (4MBC), ethylhexyl methoxycinnamate (EHMC) and octocrylene (OC) among synthetic ED compounds, which are the most frequently detected UV filters in surface waters and are the most often applied in cosmetic products.

In the case of UV filters my aims were to determine:

- the estrogenic, androgenic, antiestrogenic and antiandrogenic effect and cytotoxicity of the selected UV filters by bioluminescent bioreporters.
- the toxicity of BP-3 to prokaryotic and eukaryotic aquatic testorganisms (*Aliivibrio fischeri* and *Danio rerio*).

In the case of DHT my purposes were:

- to biodetoxificate the compound by microbes.
- to find out whether biodegradation of DHT is carried out by induced or constitutive enzymes in case of successful biodegradation.

# 2. MATERIALS AND METHODS

## 2.1. Biological effects of UV filters

### 2.1.1. Measurement of hormonal activity and cytotoxicity by bioreporters

#### 2.1.1.1. Measurement of estrogenic and androgenic activity and cytotoxicity

Estrogenic and androgenic activities were tested on bioluminescent *Saccharomyces cerevisiae* BLYES (hERα) and BLYAS (hAR) strains whereas cytotoxicity was studied by *S. cerevisiae* BLYR strain. Stock solutions of selected compounds were serially diluted (1:2) in methanol in three parallels in 96-well microplates. After the optical density of the culture had been set, the suspension was placed into the appropriate wells of microplates that were thereafter incubated at 30°C for 5 hours. For curve fitting bioluminescence (cps) measured in the fifth hour versus concentration (μM) was plotted. NOEC (No-Observed-Effect Concentration) value was determined by calculating the sum of the mean and the threefold of the standard deviation of the background bioluminescence (Microsoft Excel). Furthermore $EC_{50}$ and $IC_{50}$ values were determined in the case of agonist and cytotoxicity assays, respectively (GraphPad Prism 5.03. program).

#### 2.1.1.2. Measurement of antiestrogenic and antiandrogenic activity

For the measurement of antagonist hormonal activity the method described in Section 2.1.1.1 was modified according to Sohoni and Sumpter (1998) who developed an antagonist assay procedure for the recombinant *Saccharomyces cerevisiae* strain. This assay is based on the principle that an antiestrogenic or antiandrogenic compound is able to decrease the

bioluminescence induced by the 65% effect concentration ($EC_{65}$) of agonist 17ß-estradiol (E2) or DHT, respectively.

BLYES and BLYAS tests have not yet been adapted to analyse antagonist effects so at first the sensitivity of these strains was determined to the antiestrogenic 4-hydroxytamoxifen (4HT) and antiandrogenic flutamide (FT), respectively. Serial dilutions of compounds were prepared in three parallels and E2 or DHT was added to the appropriate wells in 65% effect concentration, respectively. For data analysis NOEC and $IC_{50}$ values were calculated (GraphPad Prism 5.03. and Microsoft Excel).

### 2.1.2. Investigation of toxic effects by aquatic test organisms

#### 2.1.2.1. *Aliivibrio fischeri* bioluminescence inhibition test

The standard (MSZ EN ISO 11348) acute *Aliivibrio fischeri* bioluminescence inhibition test was carried out in triplicates and in two parallels. Stock solution was prepared in 3.3 V/V % DMSO (dimethyl sulfoxid) + 2 m/V % NaCl solution in distilled water. This diluent was also used as negative control in the test. Stock solution was serially diluted (1:1.5) and 9 concentrations were generated between 8.33 and 0.52 mg/L. Bioluminescence inhibition was determined after 30 min contact time by Microtox$^{TM}$ 500 luminometer. Inhibition concentrations ($IC_{20}$ and $IC_{50}$) were calculated according to the concentration-response curve.

#### 2.1.2.2. Zebrafish (*Danio rerio*) embryo test (OECD 236)

The acute zebrafish embryo toxicity test was carried out according to the OECD guideline 236 with the technical assistance of the Department of Aquaculture, Szent István University. Stock solution was prepared in DMSO and 6 concentrations (25 mg/L, 18 mg/L, 12 mg/L, 7 mg/L, 5 mg/L and 1 mg/L) were generated in recirculation system water. The duration of the assay was increased from 96 hours to 120 hours in order to observe developmental

disorders manifested after hatching, too. The test was carried out in quadruplicates in 24-well tissue culture plates and 40 embryos were examined for each concentration. Embryos were incubated at 27 ± 1ºC. Developmental disorders and mortality were checked 24 hours and hatching success was recorded at the 96$^{th}$ hour. Observations were made by Leica M205FA stereo microscope. Effect concentrations were calculated by GraphPad Prism 5.03. program.

## 2.2. Biodegradation of 5α-dihydrotestosterone

### 2.2.1. Biodegradation experiment by microbes

Biodegradation experiment was carried out with 8 *Rhodococcus* species (*R. erythropolis* AK35, *R. globerulus* AK36, *R. pyridinivorans* AK37, *R. gordoniae* AK38, *R. ruber* AK41, *R. aetherivorans* AK44, *R. coprophilus* N774, *R. pyridinivorans* K402) and 2 *Cupriavidus* species (*C. basilensis* BRB6A and *C. basilensis* ŐR16) that can be found in the microbe collection of the Department of Environmental Safety and Ecotoxicology, Szent István University. The experiment was set in 3 parallels in Erlenmeyer flasks containing LB medium and were incubated for 72 hours (28$^{o}$C, 170 rpm). The initial concentration of DHT was adjusted to 100 μg/L.

### 2.2.2. Biodegradation experiments by extracellular extracts

For this experiment *R. pyridinivorans* AK37 was selected as its complete genome sequence is available on which many genes responsible for steroid degradation have already been identified (Kriszt et al., 2012). The culture was centrifuged (19721.52 g, 4$^{o}$C, 20 min) after 72 hours of incubation thereafter the supernatant was filter-sterilezed and pipetted into centrifuge tubes. After the initial DHT concentration had been set, the tubes were incubated for 12 hours at 28$^{o}$C. LB medium containing DHT, autoclaved (121$^{o}$C, 20 min, 1 bar) extracellular extract and extracellular extract incubated with 1 mg/mL proteinase

K and 0.1% SDS (sodium dodecyl sulphate) for 6 hours at 37$^{o}$C served as controls in this test.

The aim of the next experiment was to determine whether the production of enzymes taking part in DHT degradation is a constitutive or an induced activity. The experiment was carried out by the technical assistance of Ákos Tóth, Ph.D. (National Agricultural Research and Innovation Centre) and Anita Risa, MSc (SZIU). For the test two types of extracellular extract of *R. pyridinivorans* AK37 were prepared: extracellular extract from culture without DHT preincubation and extracellular extract from culture preincubated with 100 µg/L DHT. Protein profiles of both types of the extracellular extract were compared after being separeted in 2 parallels by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) method (Laemmli 1970).

### 2.2.3. Analysis of samples from biodegradation experiments

DHT concentration of samples taken from biodegradation experiments was determined by gas chromatography-mass spectrometry (GC-MS) (Wessling Hungary Ltd.). Androgenic activity and cytotoxicity of supernatant samples were measured according to the method described in Section 2.1.1.1. Androgenic activity of the supernatant samples were expressed in bioluminescence intensification (%) whereas cytotoxicity was expressed in bioluminescence inhibition (%).

# 3. RESULTS

## 3.1. Biological effects of UV filters

### 3.1.1. Measurement of hormonal activity and cytotoxicity by bioreporters

#### 3.1.1.1. Measurement of cytotoxicity

According to the result of the BLYR test, only BP-3 was cytotoxic in the tested concentration interval. The calculated $IC_{50}$ value is 46.7 ± 2.66 μM, the 95% confidence interval (CI) is between 35.9 and 60.8 μM, the $R^2$ value is 97.5%, whereas the Hill Slope is -1.2. Due to cytotoxicity hormonal activity of BP-3 can only be determined up to the NOEC value (11.2 ± 1.20 μM) calculated in the BLYR test.

#### 3.1.1.2. Estrogenic activity of UV filters

BP-3 showed inverse U-shaped curve in the estrogen test due to cytotoxicity of the compound so only NOEC value could be determined (Table 1). 4MBC displayed partial estrogenic activity and showed sigmoidal concentration-response curve. In the assay of Kunz and Fent (2006) 4MBC displayed no estrogenic activity measured by the recombinant *S. cerevisiae* strain. However adding the cell wall digesting enzyme lyticase to the test, partial estrogenic activity could be detected (Schmitt et al., 2008) as it was also experienced in the BLYES test. EHMC and OC were not estrogenic in the BLYES test.

**Table 1: The mean and standard deviation of NOEC and $EC_{50}$ values, the 95% confidence interval (CI), the $R^2$ value and Hill Slope of benzophenone-3 and 4-methylbenzylidene camphor in the estrogen test. (n.d.= no data)**

| Compound | NOEC (μM) | $EC_{50}$ (μM) | 95% CI (μM) | $R^2$ (%) | Hill Slope |
|---|---|---|---|---|---|
| **Benzophenone-3** | 1.22 ± 0.06 | n.d. | n.d. | n.d. | n.d. |
| **4-methylbenzylidene camphor** | 13.2 ± 2.52 | 40.4 ± 1.62 | 30-54.4 | 97.8 | 2.2 |

3.1.1.3. Androgenic activity of UV filters

None of the UV filters proved to be androgenic in the BLYAS test (data not shown).

3.1.1.4. Adaptation of BLYES and BLYAS tests to measure antagonist hormonal effects

4HT and FT exhibited sigmoidal concentration-response curve in antagonist hormonal tests. The calculated NOEC and $IC_{50}$ values are summarized in Table 2. $IC_{50}$ values of 4HT and FT were 0.49 ± 0.21 μM and 4.32 ± 1.81 μM, respectively in the experiment of Kunz and Fent (2006) carried out by recombinant yeast test organism. These values are in the same order of magnitude as the values calculated from the BLYES and BLYAS tests (Table 2).

**Table 2.: The mean and standard deviation of NOEC and $IC_{50}$ values, the 95% confidence interval (CI), $R^2$ value and Hill Slope of 4-hydroxytamoxifen and flutamide in the antiestrogen and antiandrogen tests**

| Compound | NOEC (μM) | $IC_{50}$ (μM) | 95% CI (μM) | $R^2$ (%) | Hill Slope |
|---|---|---|---|---|---|
| **4-hydroxytamoxifen** | 0.14 ± 0.04 | 0.47 ± 0.08 | 0.42-0.51 | 99.3 | -1.47 |
| **Flutamide** | 2.11 ± 0.39 | 9.45 ± 1.03 | 8.41-10.47 | 98.9 | -1.74 |

According to the results it can be concluded that both BLYES and BLYAS strains are suitable for applying in antagonist tests as test organism.

3.1.1.5. Antiestrogenic activity of UV filters

BP-3 showed no antiestrogenic activity under cytotoxic concentrations. 4MBC proved to be antiestrogenic however the concentration-response curve was not full below the limit of solubility of this compound. Concentration-response curves were full in the case of EHMC and OC. NOEC and $IC_{50}$ values are summarized in Table 3. According to the results of this experiment 4MBC, EHMC and OC have no significant antiestrogenic activity in comparison to the NOEC and $IC_{50}$ values of 4HT (Table 2).

**Table 3.: The mean and standard deviation of NOEC and $IC_{50}$ values, the 95% confidence interval (CI), $R^2$ value and Hill Slope of ethylhexyl methoxycinnamate and octocrylene in the antiestrogen test**

| Compound | NOEC (μM) | $IC_{50}$ (μM) | 95% CI (μM) | $R^2$ (%) | Hill Slope |
|---|---|---|---|---|---|
| **Ethylhexyl methoxycinnamate** | 709 ± 33.3 | 4700 ± 406 | 3166-7184 | 97.5 | -1.01 |
| **Octocrylene** | 4030 ± 2950 | 15300 ± 4780 | 5210-66880 | 97.5 | -1.19 |

3.1.1.6. Antiandrogenic activity of UV filters

All of the 4 UV filters showed antiandrogenic activity in the BLYAS test moreover the concentration-response curve was non-monotonic in the case of OC (Figure 1).

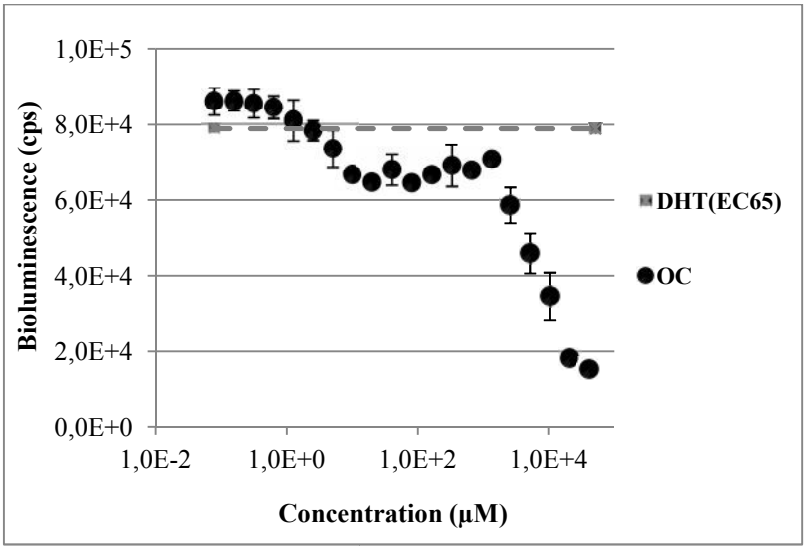


**Figure 1.: Antiandrogenic activity of octocrylene (OC) measured by *Saccharomyces cerevisiae* BLYAS strain and the mean bioluminescence induced by 65% effect concentration ($EC_{65}$) of 5α-dihydrotestosterone (DHT)**

The NOEC value of BP-3 (Table 4) was in the same order of magnitude as the NOEC value of the positive control FT (Table 2). 4MBC can also be characterized by significant antiandrogenic activity according to the BLYAS test (Table 4). Although EHMC and OC showed antiandrogenic activity (Table 4), their $IC_{50}$ values were 3 order of magnitude higher than the $IC_{50}$ value of FT.

**Table 4.: The mean and standard deviation of NOEC and $IC_{50}$ values, the 95% confidence interval (CI), $R^2$ value and Hill Slope of the 4 UV filters in the antiandrogen test (n.d.= no data)**

| Compound | NOEC (µM) | $IC_{50}$ (µM) | 95% CI (µM) | $R^2$ (%) | Hill Slope |
|---|---|---|---|---|---|
| **Benzophenone-3** | 1.82 ± 0.18 | n.d. | n.d. | n.d. | n.d. |
| **4-methylbenzylidene camphor** | 4.27 ± 2.24 | 30.4 ± 14.8 | 21.25-38.92 | 99.2 | -0.89 |
| **Ethylhexyl methoxycinnamate** | 1080 ± 531 | 6830 ± 2450 | 4811-10088 | 99.2 | -1.026 |
| **Octocrylene** | 0.44 ± 0.2 | 7340 ± 2410 | 5598-9506 | 99.0 | -1.831 |

#### 3.1.1.7. **Novel scientific results (by the results of Section 3.1.1)**

**Thesis 1: Cytotoxic effect have been detected in the case of benzophenone-3 by *Saccharomyces cerevisiae* BLYR strain. This compound can reduce the bioluminescence of this test organism by 50% in 46.7 ± 2.66 µM concentration.**

**Thesis 2: *Saccharomyces cerevisiae* BLYES strain is able to detect the partial estrogenic activity of 4-methylbenzylidene camphor without the enzyme lyticase in contrast to the recombinant *Saccharomyces cerevisiae* strain (Genetics Department, Glaxo Wellcome plc, United Kingdom).**

**Thesis 3: *Saccharomyces cerevisiae* BLYES and BLYAS strains are suitable for measuring antiestrogenic and antiandrogenic activity. These strains showed antiandrogenic activity in the case of benzophenone-3 and both antiestrogenic and antiandrogenic activities in the case of 4-methylbenzylidene camphor, ethylhexyl methoxycinnamate and octocrylene. Furthermore antiandrogenic activity of octocrylene measured by the BLYAS strain can be described by non-monotonic concentration-response curve.**

### 3.1.2. Investigation of toxic effects by aquatic test organisms

In next subsections results of aquatic ecotoxicological tests carried out by BP-3 are presented.

#### 3.1.2.1. *Aliivibrio fischeri* bioluminescence inhibition test

BP-3 inhibited the bioluminescence of *A. fischeri* by 20% and 50% in 1.16 ± 0.51 mg/L and 5.55 ± 0.97 mg/L concentrations, respectively.

### 3.1.2.2. Zebrafish (*Danio rerio*) embryo test (OECD 236)

BP-3 increased the number of dead zebrafish embryos concentration-dependently. $LC_{50}$ values decreased time-dependently. Furthermore BP-3 caused tail deformity, inhibited the inflation of swim bladder and the hatching of embryos concentration-dependently. $LC_{50}$, $EC_{50}$ and $IC_{50}$ values are summarized in Table 5.

**Table 5.: The mean and standard deviation (SD) of 50% lethal ($LC_{50}$), inhibition ($IC_{50}$) and effect ($EC_{50}$) concentrations calculated from the zebrafish (*Danio rerio*) embryo test**

| **Effect** | **Lethality** | | | **Tail deformity** | **Inhibition of hatching** | **Inhibition of swim bladder inflation** |
|---|---|---|---|---|---|---|
| **Time (hpf)** | 72 | 96 | 120 | 72 | 96 | 120 |
| **$LC_{50}$/$IC_{50}$/$EC_{50}$ (mg/L)** | **17.6** | **16** | **12.14** | **10.16** | **11.94** | **7.28** |
| **SD** | 0.36 | 0.74 | 0.98 | 1.51 | 0.27 | 0.52 |

Above the previously mentioned developmental disorders BP-3 also caused jaw deformity to several zebrafish embryos. The number of embryos with jaw deformity has not increased with concentration but this deformity could be observed in many concentration groups (1 mg/L, 7 mg/L, 12 mg/L, 18 mg/L).

### 3.1.2.3. **Novel scientific results (by the results of Section 3.1.2)**

**Thesis 4: By the results of the 120 hours long zebrafish (*Danio rerio*) embryo test it can be concluded that benzophenone-3 (BP-3) is lethal to the embryos ($LC_{50,120hpf}$= 12.14 ± 0.98 mg/L), furthermore it can cause developmental disorders like tail deformity, lack of swim bladder inflation and jaw deformity. Moreover BP-3 can reduce the hatching success of zebrafish embryos.**

## 3.2. Biodegradation of 5α-dihydrotestosterone

### 3.2.1. Biodegradation experiment by microbes

According to the analytical measurement of samples (Table 6) taken from DHT biodegradation experiment the selected strains have excellent DHT biodegradational ability as DHT concentration has been reduced under the limit of quantification in both pellet and supernatant samples.

Cytotoxicity could not be experienced during the 72 hours long experiment according to the BLYR test as supernatant samples did not cause bioluminescence inhibition (Table 6).

**Table 6: 5α-dihydrotestosterone concentration of samples taken at the 72nd hour from the biodegradation experiment and analysed by gas chromatography-mass spectrometry (GC-MS). Androgenic activity and cytotoxicity of samples measured by *Saccharomyces cerevisiae* BLYAS and BLYR strains. Values in the table are means with standard deviations. (n.d.= no data)**

| Microbe strain | GC-MS (µg/L) | | BLYAS test | BLYR test |
|---|---|---|---|---|
| | | | Bioluminescence (%) | |
| | Supernatant | Pellet | Supernatant | |
| **Control** | **117 (± 14.8)** | **n.d.** | **550.3 (± 29.2)** | **10.5 (± 8.6)** |
| *Escherichia coli* TOP10 | 70 (± 9.3) | 10.4 | 484.6 (± 36.2) | 6.5 (± 5.4) |
| *Rhodococcus gordoniae* AK38 | < 1 | < 0.66 | 24.5 (± 9.0) | -9.2 (± 8.0) |
| *Rhodococcus coprophilus* N774 | < 1 | < 0.66 | 30.7 (± 15.9) | -14.1 (± 4.5) |
| *Cupriavidus basilensis* BRB6A | < 1 | < 0.66 | 31.6 (± 7.5) | -9.2 (± 3.5) |
| *Rhodococcus globerulus* AK36 | < 1 | < 0.66 | 41.2 (± 10.6) | -11.5 (± 7.2) |
| *Rhodococcus erythropolis* AK35 | < 1 | < 0.66 | 41.7 (± 2.0) | -10.7 (± 4.2) |
| *Rhodococcus pyridinivorans* AK37 | < 1 | < 0.66 | 42.2 (± 24.3) | -8.2 (± 7.6) |
| *Rhodococcus aetherivorans* AK44 | < 1 | < 0.66 | 46.7 (± 13.7) | -9.6 (± 6.0) |
| *Rhodococcus pyridinivorans* K402 | < 1 | < 0.66 | 51.5 (± 7.9) | -7.8 (± 4.8) |
| *Rhodococcus ruber* AK41 | < 1 | < 0.66 | 63.7 (± 24.8) | -18.6 (± 4.3) |
| *Cupriavidus basilensis* ŐR16 | < 1 | < 0.66 | 198.3 (± 69.2) | -8.1 (± 0.8) |

By the result of the BLYAS test (Table 6) initial DHT concentration in control samples induced the background bioluminescence of the test organism by 550%. Only 4-12% of effect induced by control samples could be detected in the case of 9 out of 10 strains (24.5-63.7% bioluminescence intensification). Although androgenic activity of supernatant samples of *C. basilensis* ŐR16 strain decreased in comparison to control samples, biodetoxification potential was insufficient as almost 200% bioluminescence intensification could be measured (Table 6). Consequently the biodegradation of DHT resulted in metabolites with high androgenic activity. Even by increasing the incubation time to 7 days, this strain could still not fully biodetoxificate the compound, as significant androgenic activity (237%) could be detected at the 7$^{th}$ day in comparison to control samples (447%) (data not shown).

### 3.2.2. Biodegradation experiments by extracellular extracts

According to the result of GC-MS analysis DHT concentration in control samples containing medium and DHT was the same as in autoclaved and 1 mg/mL proteinase K + 0.1% SDS treated extracellular extract amended media (Table 7). In spite of this DHT concentration of medium amended with non-treated extracellular extract of *R. pyridinivorans* AK37 decreased below the limit of quantification by the 12$^{th}$ hour (Table 7).

**Table 7: The mean and standard deviation of 5α-dihydrotestosterone (DHT) concentration of active and inactive extracellular extracts of *Rhodococcus pyridinivorans* AK37 taken after 12 hours long incubation and determined by gas chromatography-mass spectrometry (GC-MS)**

| Samples | DHT concentration (µg/L) |
|---|---|
| **DHT control** | 65 ± 6.54 |
| **Heat treated (autoclaved) extracellular extract** | 61 ± 8.26 |
| **Proteinase K + 0.1% SDS treated extracellular extract** | 61 ± 4.35 |
| **Non-treated (active) extracellular extract** | < 1 |

Androgenic activity ceased by the 9$^{th}$ hour according to the result of BLYAS test (data not shown).

The aim of the next experiment carried out with extracellular extracts was to determine whether the producing of DHT degrading enzymes are constitutive or induced. By the result of this experiment protein profile based on SDS-PAGE method of DHT preincubated extracellular extract of *R. pyridinivorans* AK37 was the same as the protein profile of extracellular extract without DHT preincubation (data not shown). According to the SDS-PAGE method it is assumable that DHT degrading enzymes are produced constitutively.

### 3.2.3. Novel scientific results (by the results of Section 3.2)

**Thesis 5: In the 5*α*-dihydrotestosterone (DHT) 72 hours long biodegradation experiment bacterial strains from which 8 belong to the *Rhodococcus* genus and 1 belongs to the *Cupriavidus* genus were found to have excellent biodegradation and biodetoxification potential. Out of these strains extracellular extract of *R. pyridinivorans* AK37 reduced the concentration of DHT below the limit of quantification during 12 hours. Androgenic activity ceased by the 9$^{th}$ hour.**

# 4. CONCLUSIONS

During the measurement of the agonist and antagonist ED activity of the 4 UV filters *S. cerevisiae* BLYES and BLYAS strains were not more sensitive than other *in vitro* test organisms. The only exeption was the case of the investigation of estrogenic activity of 4MBC where BLYES strain proved to be more sensitive than recombinant *S. cerevisiae* strain. BLYES strain can detect the partial estrogenic activity of 4MBC without the enzyme lyticase so the cell wall of the BLYES strain is more permeable than the cell wall of the recombinant *S. cerevisiae* strain.

OC showed non-monotonic concentration-response curve in the antiandrogenic test. It is assumable that OC can bind to the secunder binding site of the receptor in the lower concentration interval whilst it competes for the primery binding site with DHT in the higher concentration interval.

*S. cerevisiae* BLYES and BLYAS strains could detect the estrogenic, antiestrogenic and antiandrogenic activity of the analysed UV filters above presently environmentally relevant concentrations. Consequently these test organisms are not sensitive enough to detect the agonist and antagonist ED activity of the selected UV filters at presently environmentally relevant concentrations. However they can be suitable for monitoring microbial biodetoxification of these compounds in case of lab-scale experiments. There are only a few experiments reported the endocrine activity of the 4 UV filters in which the maximum concentrations that are detected in the environment were tested. Based on the results of this Ph.D. thesis and data from the scientific literature I suggest to investigate the ED effect of these 4 UV filters under discussion at environmentally relevant concentrations by analysing the expression of genes related to the endocrine system of aquatic test organisms.

However humans are mostly exposed to UV filters by the application of cosmetics, and not by the environment. UV filters absorbed through the skin can reach such concentrations in the human body at which ED activity or toxicity could be detected by BLYES/BLYAS/BLYR strains or aquatic test organisms. So it would be important to supervise the permissible concentrations of UV filters in cosmetics.

According to the results of aquatic tests BP-3 had effect on testorganisms in concentrations several order of magnitude higher than the maximum environmental concentration of this compound. However it would be necessary to carry out chronic tests next to acute tests as $LC_{50}$ values followed a decreasing tendency in the course of time in the zebrafish embryo test. So it is possible that BP-3 has similar effect in environmental concentrations in the long term as it has in the acute test.

BP-3 caused different developmental disorders to zebrafish embryos like tail deformity, lack of swim bladder inflation and jaw deformity so BP-3 is teratogen to zebrafish embryos. In the future it would be important to find out the mode of action of this compound in the case of these malformations.

Tail deformity of zebrafish embryos could be caused by apoptosis in the tail and impairment of muscle fibres beyond the disorder of blood circulatory system. Investigation of apoptosis can be carried out by acridine orange staining and fluorescence microscope whereas the impairment of muscle fibres can be studied by microscopic tissue analysis.

On the one hand the cause of the lack of swim bladder inflation could be the deformation of the tail that could prevent zebrafish embryos from swimming up to the water surface. On the other hand it would be practical to find out whether BP-3 can inhibit the biosynthesis of surfactant responsible for the inflation of swim bladder in the gas gland cells of zebrafish. It would also be

useful to determine whether there were difference in the volumes of inflated swim bladders between treated and control groups.

Teraoka et al. (2002) found out that 2,3,7,8-TCDD affected the lower jaw of zebrafish embryos via an aryl hydrocarbon receptor (AhR)-dependent mechanism so I suggest to investigate whether BP-3 is able to bind to the AhR. For this purpose bioluminescence *Saccharomyces cerevisiae* strain harbouring *ahr* gene on its genom could be a suitable test organism. As Fong et al. (2016) experienced jaw deformity of zebrafish embryos also in the case of BP-2, so it would be useful to study whether the other BP-type UV filters can cause jaw deformity and are able to bind to the AhR.

BP-3 also reduced the hatching success of zebrafish embryos. The reason for this could be the deformity of tail and on the other hand it would be important to determine whether BP-3 can influence the transcription of ZHE1 encoding gene responsible for hatching.

Taking into account the results of cytotoxicity and embryo toxicity tests in the case of BP-3 and data from the scientific literature it would be necessary to carry out microbial biodetoxification of this compound.

In practice, for biodegradational purposes it is favourable to examine what kind of pollutants could be biodetoxificated sufficiently by excellent DHT degrading strains and what kind of microbes can be co-cultured and whether separately or in consortium have these microorganisms higher biodetoxification potential. It is also important to analyse the biofilm forming ability of these strains.

Results in the case of *Cupriavidus basilensis* ŐR16 revealed the importance of biotests next to analytical measurements as significant androgenic activity can be experienced along with excellent DHT biodegradation.

As *C. basilensis* ŐR16 could not cease androgenic activity in DHT biodegradation experiment even during 7 days, so it is assumable it does not contain all of the enzymes necessary for steroid degradation.

Results of DHT biodegradation test also draw attention to the fact that significant difference may be established between the biodetoxification potential of strains belonging to the same species. *C. basilensis* BRB6A had excellent whereas *C. basilensis* ŐR16 insufficient biodetoxification potential. For revealing the cause of this difference, it would be useful to monitor DHT biodegradation by analytical methods and identify the formed metabolites. Herewith side/end-products of the DHT degradation could be determined. Furthermore genes responsible for steroid degradation can be identified by genome analysis.

Extracellular extract of *R. pyridinivorans* AK37 could excellently degrade DHT in 12 hours. A following research project could be the identification of DHT degrading extracellular enzymes of AK37 strain and the determination of the degradation pathway.

According to SDS-PAGE protein profiles the production of DHT degrading extracellular enzymes is assumably constitutive. However to confirm this result my future aim is to examine the enzyme production also by 2D PAGE method that enables much more efficient protein separation.

# 5. REFERENCES


Fong, H. C. H., Ho, J. C. H., Cheung, A. H. Y., Lai, K. P., Tse, W. K. F. (2016): Developmental toxicity of the common UV filter, benzophenone-2, in zebrafish embryos. *Chemosphere* 164: 413-420.

Kriszt B., Táncsics A., Cserháti M., Tóth Á., Nagy I., Horváth B., Nagy I., Tamura, T., Kukolya J., Szoboszlay S. (2012): De Novo Genome Project for the Aromatic Degrader *Rhodococcus pyridinivorans* Strain AK37. *Journal of Bacteriology* 194(5): 1247.

Kunz, P. Y. & Fent, K. (2006): Multiple hormonal activities of UV filters and comparison of *in vivo* and *in vitro* estrogenic activity of ethyl-4-aminobenzoate in fish. *Aquatic Toxicology* 79: 305–324.

Laemmli, U. K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259): 680-685.

Schmitt, C., Oetken, M., Dittberner, O., Wagner, M., Oehlmann, J. (2008): Endocrine modulation and toxic effects of two commonly used UV screens on the aquatic invertebrates *Potamopyrgus antipodarum* and *Lumbriculus variegatus*. *Environmental Pollution* 152(2): 322–329.

Sohoni, P. & Sumpter, J. P. (1998): Several environmental oestrogens are also anti-androgens. *Journal of Endocrinology* 158: 327–339.

Teraoka, H., Dong, W., Ogawa, S., Tsukiyama, S., Okuhara, Y., Niiyama, M., Ueno, N., Peterson, R. E., Hiraga, T. (2002): 2,3,7,8-tetrachlorodibenzo-*p*-dioxin toxicity in the zebrafish embryo: Altered regional blood flow and impaired lower jaw development. *Toxicological Sciences* 65(2): 192-199.

# 6. PUBLICATIONS

**Scientific papers:**

**A. Balázs**, C. Krifaton, A. Risa, M. Cserháti, J. Kukolya, S. Szoboszlay, J. Háhn, M. Eldridge, J. Wang, B. Kriszt (2014): Biodegradation of 5α-dihydrotestosterone to non-androgenic products. *International Biodeterioration & Biodegradation* 93: pp. 162-167. **(IF: 3.562)**

**A. Balázs**, C. Krifaton, I. Orosz, S. Szoboszlay, R. Kovács, Z. Csenki, B. Urbányi, B. Kriszt (2016): Hormonal activity, cytotoxicity and developmental toxicity of UV filters. *Ecotoxicology and Environmental Safety* 131: pp. 45-53. (**IF: 3.974**)

**A. Balázs**, I. Orosz, C. Krifaton (2015): Hormonmoduláns hatású szerves UV-szűrő vegyületek. *Biokontroll* (accepted: 20.02.2015)

**Conference proceedings:**

**A. Balázs**, B. Kriszt, I. Orosz, S. Szoboszlay, R. Kovács, Z. Csenki, B. Urbányi, C. Krifaton (2015): A benzofenon-3 káros hatásai és biodetoxifikációs lehetőségei. V. Ökotoxikológiai Konferencia, Budapest, 2015.11.20. p. 7-8. Darvas B. főszerk. V. Ökotoxikológiai Konferencia előadás és poszter kötete. Magyar Ökotoxikológiai Társaság, 2015. ISBN 978-963-89452-5-9

**A. Balázs**, B. Kriszt, I. Orosz, C. Krifaton (2015): Adaptation of bioluminescent yeast strains for measuring the multiple hormonal and the cytotoxic activities of four UV filters. SETAC Europe 25th Annual Meeting, Barcelona, 2015.05.03-07. (http://meetings.setac.org/frontend.php/presentation/listForPublic)

**A. Balázs**, B. Kriszt, I. Orosz, C. Krifaton (2014): Hormonal activities of four UV filters measured by bioluminescent yeast bioreporters - A Magyar Mikrobiológiai Társaság 2014. évi Nagygyűlése és EU FP7 PROMISE Regional Meeting: Absztraktfüzet. pp. 4-5.

**A. Balázs**, C. Krifaton, A. Risa, M. Cserháti, Z. Padla, B. Kriszt (2013): Biodegradation of dihydrotestosterone as a micropollutant with androgen-disrupting potency. In: K. Márialigeti (szerk.) Acta Microbiologica et Immunologica Hungarica: Abstracts of the 4th Central European Forum for Microbiology. Keszthely, Magyarország, 2013.10.16-2013.10.18. Budapest: Akadémia Kiadó, pp. 112-113. (ISSN: 1217-8950)

**Total impact factor (IF): 7.536**