INVESTIGATION OF ATRAZINE AND TERBUTHYLAZINE BIODETOXIFICATION BY USING COMPLEX METHODS BASED ON DETECTION OF BIOLOGICAL EFFECTS

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1. **BACKGROUND, AIMS AND SCOPES**

Human population doubled during the last five decades, and the global agricultural production parallel with the usage of pesticides grew similarly.

However, part of the chemicals designed to protect crops and livestock do not hit the target pest, and entering the environmental compartments as pollutants their harmful effects on the non-target organisms and ecosystems could be very diverse.

Atrazine and terbuthylazine are herbicides belonging to the group of symmetric-triazines and inhibit photosynthesis by blocking the plastoquinone binding site of photosystem II. Although there is a very extensive literature about the water polluting, immunosuppressive and hormone modulating effects of atrazine, it is one of the herbicides applied in the highest amount outside of the EU. Its half-life varies between a few weeks and 1-2 years in optimal circumstances. Trapped in reductive conditions, it can persist for decades; in Hungary, atrazine is one of the most frequently detected pesticides in groundwater 10 years after its prohibition. According to the latest results, terbuthylazine is also an endocrine disrupting chemical and its adverse effects, degradation, and behavior in the soil are similar to atrazine. Terbuthylazine is currently used as an atrazine-substitute for weed control in maize in the countries of the EU. There are also toxic and endocrine disrupting chemicals among the metabolites of these herbicides.

Similarly to other pollutants, bioremediation techniques are the most eco-friendly solutions to eliminate these pollutants from the environment.

While the biodegradation of triazines has been a widely researched area in many decades with an extensive literature, investigations mainly focus on the quantitative diminution of the parent chemicals but the biological effects of the forming end-product are not examined.
Based on the discussions above, during my research the goals were the following:

I. Examination of biological degradation of atrazine and terbuthylazine by pure bacterial cultures.

II. Enhance the effectiveness of biodegradation by using bacterial consortia

III. Determination of cytotoxic effects of the active substances and their residues from biodegradation after long-term using an ecotoxicological test suitable to measure chronic cytotoxicity.

IV. Development of a combined method to detect the indirect endocrine disrupting effects of atrazine on sex steroid synthesis.

V. Monitoring the atrazine and terbuthylazine biodetoxification using the combined method and the chronic cytotoxicity test to detect endocrine disrupting and toxic effects of the residues.

VI. Selection of bacterial strains and consortia which are able to degrade the herbicides to non-harmful end-products.

To achieve my first and second aim, bacterial strains isolated from polluted environmental sites (soil, groundwater, composts) and pristine soils were chosen, which - according to our previous studies - possessed good hydrocarbon and mycotoxin degrading ability.

To enhance the efficiency of atrazine-degradation by their overlapping enzyme pools, consortia were created from strains which were able to degrade the herbicide individually with more than 50% efficiency. Terbuthylazine biodegradation experiments were carried out with the best atrazine-degrading pure strains and consortia.

To monitor the biodetoxification, the third and fourth aim had to realize first. In order to assess the sensitivity of the prolonged *Aliivibrio fischeri* assay to atrazine and terbuthylazine, acute (30 min) and chronic (25 h) *A. fischeri* assays were carried out. After determining that its sensitivity is appropriate for
monitoring the biodetoxification process, chronic *A. fischeri* assay was applied to measure the cytotoxic effects of the end-product of atrazine and terbuthylazine degradation.

To assess the potential endocrine disrupting effects of the residues, a combined method was developed, which is able to detect the disturbing impact of different chemicals on steroidogenesis, without determining specific analytical endpoints.

My final goal was to select bacterial strains and/or consortia using the complex methods to detect the adverse biological effects of residues which are able to degrade atrazine and terbuthylazine to non-harmful end-products.
2. MATERIALS AND METHODS

2.1. Degradation experiments

For atrazine-degradation, forty-three bacterial strains belonging to twenty-seven different species and fifteen genera were investigated. Twenty-one consortia were created from strains which were able to degrade atrazine individually with more than 50% efficiency. Five strains and two consortia of those with excellent atrazine-degrading capacity were used for terbuthylazine-degradation.

72-hours old inocula with the same density were created, and they were used individually or equally mixed with other strain in the degradation experiments. The initial concentration of the herbicides was 50 mg/L. After 7 days of incubation, aliquots were removed from the flasks and the remaining samples for their herbicide concentrations were analyzed by gas chromatography-mass spectrometry. Degradation capacity (%) of strains and consortia was calculated comparing atrazine concentrations of inoculated samples to negative controls.

2.2. Cytotoxicity of atrazine and terbuthylazine using Aliivibrio fischeri test

Cytotoxic effects of atrazine and terbuthylazine as pure chemicals were assessed by the standard (ISO 11348-1) acute (30 min) Aliivibrio fischeri (DSM 7151) test and its chronic version adapted to microtiter plates with prolonged contact time (25 h). EC$_{50}$ values of the chronic test were determined and compared to those of the acute assay. Chronic A. fischeri assay was applied to measure the cytotoxic effects of the end-product from atrazine and terbuthylazine degradation tests, after determining that its sensitivity is appropriate for monitoring the biodetoxification process.
2.3. Method development for detection of indirect endocrine disrupting effects

The aim was to develop a rapid, cost-effective combined testing method to assess the indirect effect of compounds interfering with sex-steroid synthesis and to determine the complex effects of atrazine and its end-products from bacterial degradation on estrogen and androgen hormone synthesis in vitro on the H295R human cell line. The steroidogenic assay was performed on H295R human adrenocortical carcinoma cell line as described in OCSPP Guideline 890.1550. Instead of standard analytical methods, bioluminescence bioreporter assays (Saccharomyces cerevisiae BLYES and BLYAS) were used to measure the estrogenic and androgenic effect of sex steroid hormones released by human cells in response to atrazine and its residues. During development in parallel with atrazine the known endocrine disruptors forskolin and prochloraz (as positive control chemicals) were also tested. The schematic method is presented in Figure 1.

2.4. Assessment of indirect endocrine disrupting effects of end-products of atrazine-degradation

For their endocrine disrupting effects, three non-cytotoxic atrazine-residues with excellent degradation values were tested using the successfully
developed combined method. Two of the samples aroused from bacterial consortia, and the third was the end-product of a pure culture. H295R human adrenocortical cells were exposed to filtered samples and after 48 hours of incubation, supernatants were tested for their estrogenic and androgenic effects by BLYES and BYLAS tests.

2.5. Data analysis

Statistical analyses of bioluminescence data have been performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA). Significant differences among all treatments have been determined using analysis of variance (one-way ANOVA) followed by the Tukey's and Dunnett’s post hoc tests. For curve fitting and EC$_{50}$ calculations, normalized bioluminescence values (CPS) versus the log of chemical concentrations (M) were plotted using the four-parametric logistic equation as a non-linear regression model to generate concentration-response curves. Bioluminescence intensification and inhibition values were expressed as a percentage compared to the control values.
3. RESULTS AND DISCUSSION

3.1. Degradation of atrazine and terbuthylazine by bacterial strains and consortia

Herbicide degradation capacity of individual bacterial strains and consortia was investigated by analytical method (GC-MS). Thirteen individual strains and nine consortia were able to degrade atrazine with 50-98% efficiency monitored by chemical analysis (GC-MS). Detailed results of degradation are represented in Table 1. 4 of these belong to *Rhodococcus aetherivorans*, *R. qingshengii*, *Serratia fonticola* and *Olivibacter oleidegradans* which species’ atrazine degrading ability has never been reported before.

Table 1. – Atrazine degrading capacity of bacterial strains and consortia. Data are expressed as a percentage of residual atrazine concentrations after 7 days of incubation compared to those of the control.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Atrazine biodegradation (% compared to the control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PT1/1</td>
<td><em>S. fonticola</em></td>
<td>51.66±1.00</td>
</tr>
<tr>
<td>NI1</td>
<td><em>R. erythropolis</em></td>
<td>52.98±1.51</td>
</tr>
<tr>
<td>OR16</td>
<td><em>C. basilensis</em></td>
<td>53.13±3.66</td>
</tr>
<tr>
<td>AK44</td>
<td><em>R. aetherivorans</em></td>
<td>59.07±3.58</td>
</tr>
<tr>
<td>CHB15P</td>
<td><em>R. pyridinivorans</em></td>
<td>65.65±18.39</td>
</tr>
<tr>
<td>TBF2/20.2</td>
<td><em>O. oleidegradans</em></td>
<td>67.29±3.99</td>
</tr>
<tr>
<td>GD2A</td>
<td><em>R. erythropolis</em></td>
<td>77.21±13.70</td>
</tr>
<tr>
<td>CW25</td>
<td><em>R. rhodochrous</em></td>
<td>78.41±11.59</td>
</tr>
<tr>
<td>BA4.9</td>
<td><em>R. qingshengii</em></td>
<td>85.0±1.10</td>
</tr>
<tr>
<td>K408</td>
<td><em>R. pyridinivorans</em></td>
<td>87.05±5.85</td>
</tr>
<tr>
<td>PT2/14B</td>
<td><em>R. qingshengii</em></td>
<td>93.12±1.08</td>
</tr>
<tr>
<td>K402</td>
<td><em>R. pyridinivorans</em></td>
<td>97.55±1.29</td>
</tr>
<tr>
<td>K404</td>
<td><em>R. pyridinivorans</em></td>
<td>98.12±1.22</td>
</tr>
</tbody>
</table>

Among the pure strains and consortia with excellent atrazine-degrading capacity, only the *Rhodococcus pyridinivorans* K404 strain was able to degrade terbuthylazine with moderate, 46% efficiency.
NOVEL SCIENTIFIC RESULT (by the results of chapter 3.1.):
(Thesis 1) Thirteen of the examined forty-one strains and nine consortia of twenty-one were able to degrade more than 50% of atrazine in pure cultures after 7 days. Among these, 5 strains belong to the *Rhodococcus aetherivorans* (1), *R. qingshengii* (2), *Olivibacter oleidegradans* (1), and *Serratia fonticola* (1) species, which atrazine degrading ability was proved firstly based on my research. Only the *Rhodococcus pyridinivorans* K404 strain of the 5 strains and 2 consortia with excellent (>75%) atrazine-degrading capacity was able to degrade terbutylazine with 46% efficiency, although the two chemicals differ structurally only by one methyl-group.

Our results with regard to atrazine were published in an international publication (Háhn et al., 2017).

3.2 *Aliivibrio fischeri* cytotoxicity assays

*Cytotoxicity of pure active substances*

The chronic *A. fischeri* bioluminescence assay was found to be the most sensitive to atrazine and terbutylazine at 10 and 15h contact times when even at the lowest concentrations of the herbicides resulted in significant decrease in the bioluminescence compared to the solvent control. Results of non-linear regression analysis to determine the EC\(_{50}\) values for the given contact times are described in Table 2. The EC\(_{50}\) values were 2.7 μg/mL and 8.3 μg/mL after 10 and 15 h of contact time, respectively. *A. fischeri* with prolonged contact time showed notably higher sensitivity to atrazine compared to that of the acute (Table 2.) test based on my results: the EC\(_{50}\) values were significantly lower at 3.5, 10 and 15 h than that of the acute standard test (ISO 11348-1) at 30 min, where inhibition in bioluminescence was not found even at the highest tested concentrations (125 and 100 mg/L, respectively). According to the results, the sensitivity of *A. fischeri* assay with elongated exposure time is suitable to assess
the cytotoxic effects of bacterial end-products originating from atrazine and terbuthylazine degradation experiments.

Table 2. - EC$_{50}$ values for atrazine measured in chronic 	extit{Aliviibrio fischeri} assay with prolonged exposure time compared to EC$_{50}$ values of 30 min acute tests.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Contact time</th>
<th>Chronic AVF EC$_{50}$ (mg/L) (95% confidence interval)</th>
<th>r$^2$</th>
<th>Acute AVF EC$_{50}$ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine</td>
<td>10 h</td>
<td>2.67 (2.19-3.25)</td>
<td>0.8899</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15 h</td>
<td>8.28 (6.60-10.37)</td>
<td>0.8477</td>
<td></td>
</tr>
<tr>
<td>Terbuthylazine</td>
<td>10 h</td>
<td>4.97 (4.04-6.11)</td>
<td>0.9203</td>
<td>30 perc &gt;125</td>
</tr>
<tr>
<td></td>
<td>15 h</td>
<td>9.62 (6.57-14.07)</td>
<td>0.6796</td>
<td>30 perc &gt;100</td>
</tr>
</tbody>
</table>

NOVEL SCIENTIFIC RESULT (by the results of chapters 3.1. and 3.2.)

(Thesis 2) Chronic 	extit{Aliviibrio fischeri} test with prolonged contact time (25 h) is the most sensitive to atrazine and terbuthylazine at the 10 and 15 h of contact times, where the EC$_{50}$ values were 2.7 and 8.3 mg/L for atrazine and 5 and 9,6 mg/L for terbuthylazine. In comparison, cytotoxic effects were not found even at the highest applied concentrations (125 and 100 mg/L, respectively) in the acute tests. Based on the similarity in their chronic cytotoxic effects, and the fact that the most effective atrazine-degraders were not able to break it down, terbuthylazine currently used as an atrazine-substitute poses an increased environmental risk.

Our results with regard to atrazine were published in an international publication (Háhn et al., 2017).

**Chronic cytotoxicity of end-products derived from atrazine biodegradation**

Results of the chronic cytotoxicity assay with 	extit{A. fischeri} showed a remarkable variety. In total there were only three strains and four consortia which were able to degrade atrazine to non-toxic metabolites and the results did not show a linear correlation between atrazine degrading capacity and
cytotoxicity of end-products. End-products of the four consortia with non-cytotoxic end-products also showed enhanced ability to degrade atrazine, moreover, bioluminescence inhibition in the case of these consortia decreased or completely ceased compared to the individual strains.

The cytotoxic effects of the residue of K404, the only strain which was able to degrade 46% of terbuthylazine, did not cease.

**NOVEL SCIENTIFIC RESULT (by the results of chapter 3.2.)**

*(Thesis 3)* Chronic *Aliivibrio fischeri* test with prolonged contact time (25 h) is suitable to monitor the biodetoxification of atrazine and terbuthylazine. From among the residues of individual strains with high atrazine-degrading capacity only 3 strains’ (*2 Rhodococcus pyridinivorans* and 1 *Olivibacter oleidegradans*) end-products did not prove to be cytotoxic. In the case of 4 consortia the degradation rate exceeded that of the creating individual strains; therefore the bacterial cultures together are capable of more efficient degradation and detoxification. Our results with regard to atrazine were published in an international publication *(Háhn et al., 2017)*.

**3.3. Method development for detection of the indirect endocrine disrupting effects of atrazine**

Atrazine induced both estrogen and androgen production in H295R cells concentration-dependently after 48 h exposure and the elevated estrogenic and androgenic effect was detected by strains of *S. cerevisiae* BLYAS and BLYES after 5-hour contact time (Fig. 2). Lowest observed effect concentrations of atrazine were 1 and 3 μM resulting in significant increase in estrogenic and androgenic effect in the supernatants measured by BLYES and BLYAS, respectively.
Elevated hormone activity of estrogens and androgens produced by H295R cells resulted in 50% increase in maximal bioluminescence (EC$_{50}$) after exposure of 16.78 µM (BLYES) and 34.38 µM (BLYAS) atrazine, respectively.

Fig. 2 - Concentration-response curves show the concentration-dependent elevation of estrogenic (A) and androgenic (B) effect in the supernatants of H295R adrenocortical carcinoma cells after 48 h exposure to atrazine. One-way ANOVA followed by the Tukey’s post hoc test was used. Data are presented as mean ± SD from three independent experiments. (**, ++) Significantly different from control (p<0.01), (***, ++++) significantly different from control (p<0.001).

**NOVEL SCIENTIFIC RESULTS (based on the results of chapter 3.3):**

(Thesis 4) To detect the indirect effects of atrazine on sex steroid synthesis, a new, combined method was successfully developed by linking the steroidogenesis assay using H295R human adrenocortical carcinoma cell line and the yeast-based BLYES/BLYAS bioreporters for measuring estrogenic and androgenic effects.

(Thesis 5) Based on the result of the combined method, atrazine induces the synthesis of both estrogenic and androgenic hormones in H295R cells (EC$_{50}$ values were 16.78 µM and 34.38 µM for estrogenic and androgenic effects, respectively). The method is suitable for detecting indirect endocrine disrupting effects of compounds without the determination of predetermined chemical/immunoanalytical endpoints, providing the opportunity to explore unknown biological effects.
Our results were published in an international publication (Háhn et al., 2016).

3.4. Assessment of the indirect endocrine disrupting effects of residues originated from atrazine-degradation experiments

In a total of 3 strains and 4 consortia were able to degrade atrazine with high efficiency forming non-cytotoxic end-product. 3 of these residues were investigated using the developed combined method for their indirect endocrine disrupting effects. These end-products originated from strain K404 (atrazine-degradation: 98%), consortia containing strains AK44+CHB15p+TBF2/20.2 (78%) and AK44+CHB15p (93%). Based on the results (Fig. 3.), it can be concluded, that the end-product of the mixture of *R. aetherivorans* AK44 + *R. pyridinivorans* CHB15p was the only one, which did not cause any difference in the synthesis of neither the estrogenic nor the androgenic hormones in the H295R cells compared to the control.

![Graph](image-url)

**Fig. 3 – Estrogenic and androgenic effects in BLYES and BLYAS tests by sex hormones produced by H295R cells exposed to non-cytotoxic atrazine residues.** (+++) significantly different from control (H295R+medium) (p<0.002); (+++,***) significantly different from control (H295R+medium) (p<0.0002). Red outline – elevated hormone effect compared to the control. Green outline - no elevated hormone effect compared to the control. BLANK ATR – abiotic control in the degradation experiment (medium + atrazine); 1 – K404; 2 – AK44+CHB15p+TBF2/20.2; 3 - AK44+CHB15p
NOVEL SCIENTIFIC RESULT (based on the results of chapters 3.1., 3.2. and 3.4.):

(Thesis 6) Based on the results of the applied biological assays, the mixture of strains *Rhodococcus pyridinivorans* CHB15p and AK44 belonging to the newly described atrazine degrading *Rhodococcus aetherivorans* species was able to convert atrazine to degradation end-products that are non-cytotoxic and have no disrupting effects on sex hormone synthesis. Therefore, application of complex methods to detect biological effects of residues arising from xenobiotics, such as pesticide biodegradation is essential.
4. CONCLUSIONS AND SUGGESTIONS

Based on the chemical analytical results testing biodegradation by bacterial strains and consortia, atrazine degradation can be widely observed among different genera; however, the biodegradation efficiency of strains belonging the same species but isolated from different environments could differ drastically.

The members of *Rhodococcus* genus displayed the best results in the herbicide degradation experiments. Moreover, members of other genera were also able to utilize atrazine: PT1/1 and TBF2/20.2 strains belonging to the *Serratia fonticola* and *Olivibacter oleidegradans* species were also able to degrade the compound with good efficiency.

According to the analytical results, I verified the hypothesis in several cases that consortia indeed can achieve better efficiency in atrazine degradation than the forming strains individually.

However, the strains and consortia with great atrazine degrading ability were not able to degrade terbuthylazine (except for the 46% degradation of K404), therefore the great structural similarity between the two compounds does not certainly mean that the same microbe can utilize both herbicides with the same effectivity.

While testing atrazine and terbuthylazine as pure chemicals in the chronic *Aliivibrio fischeri* test used for measuring the cytotoxicity, the chronic assay showed a significantly greater sensitivity than the standard acute test. In the case of the latter according to my experiments, the two compounds do not cause bioluminescence inhibition even at the highest applied concentrations at a contact time of 30 minutes.

The sensitivity of the chronic test is important in more aspects. On the one hand, it can provide a help in measuring the ecological risk of atrazine and terbuthylazine more accurately as most of the ecotoxicological tests focus on the acute effects, but an investigation with a long contact time may be able to reveal
the long-term and delayed harmful effects of compounds present at low concentrations in environmental compartments. On the other hand, it has been also proved that chronic test carried out on a microtiter plate is a simple, cost-effective and time-efficient method for the simultaneously examining the cytotoxic effect of a large number of residues from the degradation of atrazine and terbutylazine. Based on the results, it can be concluded that according to the chemical analysis, the great decrease in the concentration of the parent compound is not always directly proportional to the decrease or cessation of toxicity. In many cases, highly toxic residues were forming even with a more than 90% biodegradation. Moreover, it is also can be observed that the biodetoxification ability of strains belonging to the same species can be different. The application of strains in consortia also provides advantages in this case as well: in the cases of 4 consortia, not only the extent of degradation but also the detoxification effectiveness have also increased compared to the creating individual strains, presumably due to their complementary enzyme systems.

The results of the combined method developed to assess biodetoxification of atrazine with other aspects, i.e. the indirect endocrine disrupting effects, showed that, aside of the expected increase in estrogenic activity, atrazine could also induce the synthesis of androgenic hormones in H295R cells. The molecular genetic background of this phenomenon should be further investigated, and the expression of genes encoding the steroidogenic enzymes of H295R cells would be expedient.

The currently used methods focus on specific and predetermined endpoints (e.g. testosterone and 17β-estradiol). The developed combined test allows for rapid and simple detection of the complex, indirect effect of chemicals, even combination of chemicals, certain metabolites on sex steroid synthesis without the need for costly chemical and/or immunoanalytical evaluations and related preparation and extraction procedures. Additionally, the
method is also applicable in cases when the effect of the test substance(s) on steroid synthesis, hence the analytical endpoints are not possible to pre-specify accurately. By using the complex effect evaluation the false negative results due to inadequate selection and use of measurement endpoints can be avoided and the method can be a useful tool to determine the most suitable further tests and assays more efficiently.

These properties of the combined method meet many of the recommendation proposed by EDSTAC (1998) considering the scope of the Tier 1 screening and testing strategy, such as that the assays should: i. be inexpensive, quick, easy to perform; ii. capture multiple endpoints. Moreover, it fulfills the need that the test should be predictive across species; hence most of the genes and enzymes involved in adrenal steroidogenesis are present and serve the identical biochemical role in several different species and classes.

Using the combined method, 3 non-cytotoxic atrazine degradation residues were examined for their endocrine disrupting effects. Based on the results, only one consortium consisting two *Rhodococcus* species was found to cease both the cytotoxicity and the inductive effects on sex steroid synthesis.

Aside of the analytical measurement of degradation, the results highlight the importance of the complex assessment of the residues’ biological effects in several aspects, which has been successful regarding the atrazine and can be used in the future not only for atrazine but also for other xenobiotics.

Hereinafter, further attention should be paid to the search for microbes capable of biodetoxification of terbuthylazine.

Also, potential endocrine disrupting effects of terbuthylazine should be assessed using the combined method, as beyond the fact that its dealkylated degradation products are partially the same than that of atrazine, which has been shown to be aromatase inducers, the parent compound may have unknown effects.
In Europe and in countries where atrazine is prohibited, terbutylazine is used as an atrazine-substitute. Considering this and the chronic toxicity of terbutylazine and the fact that it is almost completely resistant to the best atrazine degrading bacterial strains, we have to face a real environmental risk due to the large-scale use of terbutylazine.

Continuing the research, attention should be paid to the investigation not only of the pure active substances but also the formulations and plant protection products. Forming agents may pose an ecological risk in themselves, and they may drastically change the behavior of the active substances.

It is also necessary to know the effects of different oxygen concentrations and temperature conditions on the biodegradation and biodetoxification of pesticides in different environmental elements. For this investigation, it may be appropriate to carry out microcosm experiments to model and analyze the physical, chemical and biological interactions between pollutants and environmental elements. For the proper execution of such microcosm experiments, the experimental results of my doctoral research can serve as a good starting point.
5. PUBLICATIONS

Scientific paper:


Proceeding:


**Total impact factor (IF): 8.427**