Atrazine toxicity in marine algae Chlorella vulgaris, in E. coli lux and gfp biosensor tests

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Keywords: Ecotoxicology, atrazine, Chlorella vulgaris, bioluminescent biosensors, water contamination

Abstract: Atrazine (ATR) is a widely used chlorinated herbicide from the s-triazine group. Due to the widespread use of ATR, it leaks into the environment and is detected in drinking water, exceeding the WHO-acceptable concentration of atrazine in drinking water, which is 2 μg/L. The aim of our study was to determine toxicity, protein degradation and genotoxicity of ATR at concentrations of 10; 1; 0.1; 0.01 mg/L on Chlorella vulgaris and with the application of E. coli bioluminescent biosensor strains. We measured the content of chlorophyll a, b, carotenoids in Chlorella vulgaris and the inhibition of this algae culture growth. E. coli RMF443 strains with gene constructs grpE::luxCDABE, lac::luxCDABE, recA::luxCDABE and E. coli strain MM294 trc::luxCDABE were used to determine toxicity, degradation of cellular proteins and genotoxicity. On the base of the obtained results, we concluded that ATR in the tested concentrations shows a toxic effect in relation to Chlorella vulgaris. ATR is toxic and genotoxic in E. coli RMF443 strains with grpE, lac, recA promoters and causes degradation of cellular proteins. Moreover, we have detected ATR toxicity toward the GFP protein in E. coli strain MM294-GFP. Taking into account the toxicity and genotoxicity of ATR documented in our research and in the experiments of other authors, we conclude that the presence of this herbicide in surface waters and drinking water is a serious threat to living organisms.

Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, ATR) due to high efficiency, low cost, and application flexibility is one of the most widely used pesticide that is sold in more than 100 countries worldwide with annual consumption of about 70,000–90,000 tons. ATR is a chlorinated herbicide from the s-triazine group that blocks photosynthesis and is used to protect many crops, including corn, sorghum, tea, sugarcane, and various fruit crops. ATR is a carcinogen that can disrupt the endocrine system of frogs at concentrations detected in the environment (Kamaz et al. 2020, Fareed et al. 2021, Hu et al. 2021). It exerts mutagenic, genotoxic, defective cell division, erroneous lipid synthesis and hormonal imbalance in fish, amphibians, and aquatic reptiles (Sun et al. 2020). To date, it has been documented that human exposure to ATR can result in the development of cancers, such as lung, breast, pancreatic, prostate, and ovarian cancer, as well as leukemia (Chen et al. 2021). Atrazine is detected in various agri-food products, such as corn, apples and animal-based products, e.g., meat, milk and eggs. ATR neurotoxic potential in the dopaminergic system, glutamatergic neurons and astrocytes in the human neural differentiation model based on human embryonic stem cells (hESC) was demonstrated (Shan et al. 2021). Research conducted in recent years has also presented that atrazine can pass through the placental barrier and the blood-brain barrier. So far, atrazine has been detected in living organisms, including humans, in the brain, placenta, milk and breast milk, urine, blood, and hair (Shan et al. 2021).

The annual high level of ATR agricultural use leads to the wide introduction of this herbicide into the environment. ATR is very stable and resistant to biodegradation in environmental matrices (Kamaz et al. 2020, Sun et al. 2020, Zhao et al. 2023). Atrazine has a prolonged half-life in soil from 4 to
even 57 weeks, while in water it is about 100 days (Sun et al. 2020). Because of the long half-life and runoff elution, ATR is washed by rain to deeper layers of the soil and has a longer duration in the surface and underground water (Shan et al. 2021). Surface and ground waters are mainly used for drinking and irrigation purposes (Faried et al. 2021). In wastewater treatment plant effluents in the US, concentrations of ATR ranging from 49 to 870 ng/L have been detected (Kamaz et al. 2020). In several countries, such as China, the USA and Brazil the detected concentrations of atrazine in surface and ground waters ranged up to 0.992 mg/L (Sun et al. 2020). The highest recorded concentrations of atrazine in surface waters in the United States were as high as 691 μg/L (Qian et al. 2008). Whereas, in tap water, a high level of up to 3.04 μg/L of ATR and its metabolites was found in Northeastern China (Shan et al. 2021). This is a concentration exceeding the WHO-acceptable concentration of atrazine in drinking water, which is 2 μg/L (Shan et al. 2021). Atrazine dissolved in water sources is harmful to aquatic organisms and human health (Sun et al. 2020, Faried et al. 2021).

Algae are one of the main components of marine phytoplankton, which are often used as bioindicators of stability of aquatic ecosystems (Su et al. 2022). Algae are among the aquatic organisms most susceptible to atrazine pollution in water (Qian et al. 2008). *Chlorella vulgaris* is one of the most frequently used model organisms in ecotoxicological studies (Su et al. 2022). Therefore, in this study, we selected *Chlorella vulgaris* to investigate the toxic effect of atrazine. The toxicity of atrazine was determined based on its effect on growth and the content of photosynthetic pigments such as chlorophyll *a*, *b* and carotenoids. Autotrophic organisms, thanks to the process of photosynthesis, synthesize organic compounds (saccharides) from simple inorganic compounds (water and carbon dioxide) using a system of photosynthetic pigments that allow energy storage in the form of chemical bonds (Kopcewicz et al. 2020). This is an important process that allows algae to grow. The photosynthetic system consists of two systems, photosystem I (PSI) and photosystem II (PSII), which are sensitive to stress factors. PSII is often used in assessing the toxicity of the test compound on microalgae (Węgrzyn and Mazur 2020, Lu et al. 2023).

Due to the constantly increasing number of scientific reports confirming the harmful effects of ATR on living organisms the aim of our research was to estimate toxicity, protein degradation and genotoxicity of ATR at concentrations of 10; 1; 0.1; 0.01 mg/L on *Chlorella vulgaris* and with the application of *E. coli* bioluminescent biosensor strains. Both model organisms used in the research occur in their respective environmental matrices, such as surface waters and wastewaters. Determining the toxicity and genotoxicity of ATR against *Chlorella vulgaris* and *E. coli* will allow the assessment of a potential impact of ATR on environmental organisms. In the wastewater treatment plants (WWTPs), ATR may have a toxic effect on activated sludge microorganisms and disturb its proper functioning, which may lead to the reduction of the efficiency of biodegradation of pollutants in wastewaters.

ATR toxicity in *Chlorella vulgaris* was determined based on the content of chlorophyll *a*, *b*, carotenoids and the inhibition of this algae culture growth. In the present work three different recombinant bioluminescent *E. coli* strains containing the promoters *recA* (DNA damage), *grpE* (protein damage) and *lac* (constitutive expression) fused to the bacterial operon luxCDABE from *P. luminescens* were used to describe the mechanism of toxicity of ATR. Moreover, for toxicity estimation, we applied *E. coli* MM294 strain with *gfp* (green fluorescent protein) reporter gene under the control of a strong *trc* promoter (constitutive expression). For the last quarter of a century, microbial biosensors containing *gfp* and *lux* reporter genes have been used in the study of the toxicity of metals in polluted soils, polychlorinated biphenyls (PCBs) in wastewater, organic pollutants in treated wastewater, chromium, arsenic, lead, pesticides, and petroleum hydrocarbons in waters (Moraskie et al. 2021, Zhu et al. 2022). Bacterial biosensors with the *lux* gene were also used for the detection and monitoring of toxic compounds in waters, including drinking water (Woutersen et al. 2011). *E. coli* RFM443 *grpE:luxCDABE* and *E. coli* RFM443 *lac:luxCDABE* strains were successfully applied for soil toxicity assessment (Bae et al. 2020). So far, microorganisms, including *E. coli* strains containing the *recA:*luxCDABE plasmid gene construct, have been used to determine the genotoxicity of many chemical compounds. These compounds include antibiotics (Melamed et al. 2012), phenol, chromium (Cr⁶⁺), lead (Pb²⁺) and the potential effects of UV radiation exposure on bacterial cells (Song et al. 2014, Jiang et al. 2017). In environmental monitoring, *E. coli* strains *recA:*luxCDABE were used to determine the genotoxicity of crude oil in surface water samples and the genotoxicity of municipal wastewater (Zhu et al. 2022, Rojas-Villacorta et al. 2022). In the work of Zappi et al. (2021), two microbial *E. coli* biosensors containing the *gfp* and *lux* genes were used to determine the toxic effects of nitrification inhibitors (allylthiourea, phenol, and copper) in industrial and municipal wastewater. *Gfp* bacterial biosensors were involved in the cytotoxicity detection of 17β-estradiol and aromatic hydrocarbons in wastewater as well as formaldehyde and heavy metals in surface waters (Ali et al. 2021, Moraskie et al. 2021, Zhu et al. 2022, Rojas-Villacorta et al. 2022).

**Materials and methods**

**Chemicals preparation**

ATR was purchased from Sigma-Aldrich. For sample preparation ATR was dissolved in dimethyl sulfoxide (DMSO). To reduce the impact of DMSO on bacterial cells, atrazine solutions were diluted in 0.86% NaCl in a ratio of 1:9 (100 μL DMSO and 900 μL 0.86% NaCl). ATR was added to the *E. coli* cultures for a final concentration of 0.01; 0.1; 1 and 10 mg/L. According to the literature, ATR in the environmental matrices is detected even at concentration of mg/L (Sun et al. 2020, Shan et al. 2021). In our research, the range of atrazine concentrations tested included the herbicide concentration in wastewaters, surface and ground waters (0.1 and 0.01 mg/L) and higher concentrations. Such a range of tested ATR concentrations allowed for the validation of the bioluminescent biosensors used in the work for both higher and lower herbicide concentrations.

**Chlorella vulgaris used in the study**

Algae of the species *Chlorella vulgaris* BA02, obtained from the Culture Collection of Baltic Algae (CCBA) at the Institute of Oceanography, Faculty of Oceanography and Geography,
University of Gdańsk in Gdynia, were used in the study. Fig. 1 shows a photo of the *Chlorella vulgaris* BA02 algae taken under the Olympus BX61 microscope.

**E. coli strains used in the study**

For investigation of toxic and protein damage potency, we applied *E. coli* RFM443 strains with a transcriptional fusion of *grpE* and *lac* promoters with *luxCDABE* reporter gene and *E. coli* MM294-GFP strain with *trc* promoter fused to *gfp* gene. In previous work, the authors reported the usefulness of *E. coli* strains with plasmid constructs with reporter genes *luxCDABE* and *gfp* in toxicity studies (Bae et al. 2020, Rojas-Villacorta et al. 2022). For genotoxicity measurement, we used *E. coli* RFM443 *recA:luxCDABE* with *recA* promoter. *E. coli* strains with plasmid genetic constructs with the *recA* – genotoxin sensitive promoter are commonly used for genotoxicity analysis of chemicals (Melamed et al. 2012, Zhu et al. 2022, Rojas-Villacorta et al. 2022). The *E. coli* strains used in the research come from the laboratory collection of microorganisms of Prof. Shimshon Belkin, Hebrew University of Jerusalem, Israel and Prof. Imrich Barák, Slovak Academy of Sciences, Bratislava. *E. coli* strains used in this study are presented in Table 1.

**Measurement methods**

The pigment contents of chlorophyll *a*, *b* and carotenoids were measured spectrophotometrically using a PerkinElmer Lambda Bio+ spectrophotometer on the base of the method described by Xiong et al. (2016). For this purpose, a sample of 2 mL of the algae culture was collected and centrifuged at 4500 rpm for 10 minutes. The supernatant was removed, and 2 mL of 90% methanol was added to the centrifuged mass of algae. Then the tubes were secured with a stopper, and the contents of the sample were mixed and placed in a thermoblock at 60°C for 10 minutes. After this time, the sample was centrifuged again for 10 minutes at 4500 rpm, and the obtained supernatant was subjected to spectrophotometric analysis at wavelengths of 470, 652, and 665 nm. The content of pigments was calculated according to formulas 1–3 (Xiong et al. 2016):

**ATR toxicity determination in Chlorella vulgaris**

Research stands and the culture medium

ATCC (American Type Culture Collection) Medium 616 – BG 11 medium (Blue Green Algae) was selected as the culture medium. Medium in one liter of distilled water contains 1.5 g NaNO₃, 0.04 g K₂HPO₄, 0.075 g MgSO₄ · 7H₂O, 0.036 g CaCl₂ · 2H₂O, 6.0 mg citric acid, 6.0 mg ferric ammonium citrate, 1.0 mg EDTA, 0.02 g Na₂CO₃, 1.0 mL Trace Metal Mix A5 (which consists of: 2.86 g H₃BO₃, 1.81 g MnCl₂ · 4H₂O, 0.222 g ZnSO₄ · 7H₂O, 0.039 g Na₂MoO₄ · 2H₂O, 0.079 g CuSO₄ · 5H₂O, 49.4 mg Co(NO₃)₂ · 6H₂O per 1000 mL distilled Water) (source: https://www.atcc.org/, access date January 20, 2023). Algae culture was carried out in bioreactors, in which the volume of the culture medium was 100 mL. The algae count in the substrate was adjusted to the same optical density of OD₆₈₀ ≈ 0.375 which corresponds to the initial cell concentration of 2.5 · 10⁶ cell/mL. Atrazine was then added to the medium at the appropriate concentration. The control sample contained the test algae culture suspended in DMSO (dimethyl sulfoxide) solvent in the appropriate proportions in which the atrazine solutions had been prepared, but no atrazine. The medium was aerated with atmospheric air which passed through a Midisart 2000, pore size 0.45 μm from Sartorius. The bioreactors were shaken on a PSU-10i shaker from Biosan. During the experiments, algae were illuminated by LED (light-emitting diode) panels with warm white light with an intensity of 250 μmol·m⁻²·s⁻¹ for 10 hours, followed by a period of 14 hours without lighting. The algae were incubated at room temperature (25°C).

**Table 1. E. coli biosensor strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene promoter acting as sensing element</th>
<th>Reporter gene</th>
<th>Type of stress sensed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> RFM443</td>
<td><em>grpE</em></td>
<td><em>Lux</em></td>
<td>Protein damage and general toxicity</td>
<td>Bae et al., 2020</td>
</tr>
<tr>
<td><em>E. coli</em> RFM443</td>
<td><em>LacZ</em></td>
<td><em>Lux</em></td>
<td>General toxicity</td>
<td>Bae et al., 2020</td>
</tr>
<tr>
<td><em>E. coli</em> RFM443</td>
<td><em>RecA</em></td>
<td><em>Lux</em></td>
<td>Genotoxicity</td>
<td>Melamed et al., 2012, Matejczyk et al., 2020a, 2020b, 2020c</td>
</tr>
<tr>
<td><em>E. coli</em> MM294</td>
<td><em>trc</em></td>
<td><em>Gfp</em></td>
<td>General toxicity</td>
<td>Baumstark-Khan et al., 1999, 2007, Rabbow et al., 2002, Matejczyk et al., 2022</td>
</tr>
</tbody>
</table>
decreases. The level of luminescence inhibition is proportional to luminescence constitutively. In this strain when the cells are measured spectrophotometrically at 600 nm (OD600). For each sample the bacterial concentration was determined by the OD value at 600 nm (OD600). The luminescence values were normalized to the bacterial concentration measured spectrophotometrically at time zero, after 2 and 24 h of incubation. The luminescence intensity was calculated according to the formula: 

\[ L = \frac{IL A}{OD600} \]

where: \( L \) – luminescence, \( IL A \) – luminescence intensity read from the apparatus, \( OD600 \) – optical density of the sample. GrpE and lac promoter responses in tested samples are presented as the percentage (%) of promoters’ induction or inhibition, compared to the control. The experiment was carried out in three independent series.

**ATR toxicity analysis with gfp gene as reporter**

ATR toxicity was also determined using the *E. coli* strain MM294 with the gfp reporter gene. This strain contains the plasmid gene construct pHK233-2 in which the gfp gene is under the control of the strong trc promoter. According to this method, the determination of the toxic effect was based on the quantification of the fluorescence intensity of the GFP protein in the *E. coli* MM294-GFP culture containing the plasmid gene construct with the gfp gene, after its 2- and 24-hour incubation with ATR. If the bacterial cells are exposed to a toxic chemical, there is a decrease in fluorescence intensity, which is the result of the toxic effect on the GFP protein. The *E. coli* MM294-GFP culture was incubated in Luria-Bertani (LB) broth with ampicillin at a concentration of 100 μg/mL at 37°C for 24 hours. After that, the bacterial culture was diluted in LB broth with ampicillin (100 μg/mL) and incubated at 37°C with shaking (150 rpm) to the early log phase (OD≈0.2). Black 96-well plates for fluorescence measurement were used for the study. To each plate well, 100 μl of the appropriate atrazine solutions was added to 100 μL of cultures of *E. coli* MM294-GFP. Samples were incubated at 37°C for 2 and 24 hours. A control sample, an *E. coli* MM294 culture without chemical compounds, was carried out in parallel and under the same conditions. The fluorescence intensity of the GFP protein in *E. coli* MM294 cultures incubated with ATR and in a control culture was measured at an excitation wavelength of 405 nm and an emission wavelength of 505 nm using a GloMax®. To standardize the fluorescence of the *E. coli* MM294-GFP culture response, the optical density (OD) value of bacterial cultures was determined spectrophotometrically at a wavelength of 600 nm. Fluorescence values for each sample were calculated according to the formula: 

\[ F = \frac{FA}{OD_{600}} \]

where: \( F \) – fluorescence, \( FA \) – fluorescence intensity read from the apparatus, \( OD_{600} \) – optical density of the sample. Fluorescence inhibition (FI) values after 2 and 24 hours of incubation of *E. coli* MM294 with ATR were calculated according to the formula:

\[ FI(%) = \frac{F_{CS} - F_{TS}}{F_{TS}} \]

where: \( F_{CS} \) – fluorescence of the tested sample (%), \( F_{TS} \) – fluorescence of the control sample (%). The experiment was conducted in three independent series.

**ATR genotoxicity determination**

Genotoxicity was determined using *E. coli* strain RF443 with a plasmid gene construct containing the recA promoter fused to the luxCDABE reporter gene (Melamed et al. 2012). The recA promoter is a DNA damage-inducible promoter of the bacterial SOS response regulon. It is involved in several different DNA repair mechanisms, including the elimination of daughter-strand DNA gaps, double helix breaks, as well as a replication error tolerance mechanism called SOS mutagenesis. The transcriptional fusion of the recA promoter with the luxCDABE reporter gene has the features of an effective genotoxicity biosensor (Melamed et al. 2012, Zappi et al. 2021, Zhu et al. 2022, Rojas-Villacorta et al. 2022).
Biosensors with the luxCDABE reporter gene rely on the production of luminescence by the action of the luciferase enzyme (Melamed et al. 2012). The genotoxic effect was determined based on the quantification of the luminescence signal intensity in the E. coli RFM443 culture containing the plasmid gene construct with the luxCDABE gene, after its 2- and 24-hour incubation with ATR, compared to a control culture not treated with a chemical. The luminescent signal is proportional to the level of recA promoter induction and genotoxic potency of ATR. E. coli RFM443 recA::luxCDABE culture was incubated for 24 h at 37°C with ampicillin at a concentration of 100 μg/mL. Next, the culture was diluted in fresh LB broth with ampicillin at a concentration of 100 μg/mL and incubated at 37°C with shaking (150 rpm) to the early logarithmic growth phase (OD<sub>600</sub>=0.2). In a 96-well white plate, to 100 μl of E. coli recA::luxCDABE culture we added 100 μL of the appropriate concentration of ATR. Samples were incubated for 2 and 24 hours at 37°C. The luminescence intensity measurement was performed using a GloMax® plate reader after 2 and 24 hours of incubation of the bacterial culture with the test chemical. To standardize the luminescent culture response of E. coli recA::luxCDABE for all samples, the optical density (OD) value was determined spectrophotometrically at a wavelength of 600 nm. The control sample, the culture of E. coli RFM443 recA::luxCDABE, did not contain any chemical compounds. The luminescence intensity for each sample was calculated according to the formula: L = IL<sub>y</sub>/OD<sub>recA</sub>, where: L – luminescence, IL<sub>y</sub> – luminescence intensity read from the apparatus, OD<sub>recA</sub> – optical density of the sample. RecA promoter induction values after 2 and 24 hours of E. coli recA::luxCDABE culture incubation with test compounds are presented as the percentage of promoter induction, compared to the control. The experiment was carried out in three independent replications.

**Potential limitations or challenges faced during the experimental procedures**

Organisation for Economic Co-operation and Development (OECD) recommends that the initial concentration of algae in toxicity tests be from 2·10<sup>3</sup> to 5·10<sup>5</sup> cell/mL and the intensity of lighting should be in the range of 60–120 μmol·m<sup>-2</sup>·s<sup>-1</sup> (OECD 2011). However, it is estimated that higher initial concentrations of cells may provide more information about the mechanisms of action of toxic substances on microalgae (Sun et al. 2020). The use of other than recommended light intensities in algae cultures contributes to different effects of the tested compounds (Camuel et al. 2017). For algae of the Chlorella vulgaris species, the optimal growth conditions are in the range of light intensity from 232 to 465 μmol·m<sup>-2</sup>·s<sup>-1</sup> (Dębowski 2018). The research using microbial biosensors was carried out in such a way as to avoid the influence of the limitations of the method used on the results obtained. Firstly, microbial biosensor strains were cultured in LB broth and algae in BG 11 medium, which contained the necessary nutrients to ensure the right growth rate of algae and bacteria and to sustain the bioluminescent and fluorescent signal in the bacterial cells. Secondly, readings of the results obtained were conducted after 2 and 4 days of algal culture and after 2 and 24 hours of bacterial culture. In order to prevent the inhibitory effect of metabolic products accumulated in the medium and to avoid loss of nutrients, cultures were not carried out for more than it was planned in the experiments (Melamed et al. 2012).

**Statistical analysis**

The individual variables adopted for statistical analysis were characterised by a normal distribution according to the Shapiro-Wilk test and heterogeneity of variance according to the Bartlett test. The Tukey test for equal samples was used to compare the statistical significance of the individual measurement results. The level of statistical significance was taken as α= 0.05. Statistical calculations were carried out using Statistica 13.1 software running on a Windows 11 platform. The statistical analysis examined the difference in the values of the parameter’s chlorophyll a, b, carotenoids content and algae growth tested in relation to the control sample and between days 2 and 4 of the research work. The second stage of statistical analysis considered the effects of the tested doses of atrazine on the induction or inhibition of the grpE promoter in E. coli strain RFM443 grpE::luxCDABE, the lac promoter in E. coli strain RFM443 lac::luxCDABE, induction of the recA promoter in E. coli strain RFM443 recA::luxCDABE and inhibition of GFP protein fluorescence in E. coli strain MM294-GFP compared to the control sample after exposure times of 2 and 24 hours.

**Results**

**ATR toxicity determination in Chlorella vulgaris**

**Inhibition of chlorophyll a content**

Toxicity on Chlorella vulgaris algae was determined based on a decrease in the content of chlorophyll a, b, carotenoids, and inhibition of the growth of algae cultures. After two days of incubation of the algae culture with ATR and after 4 days, inhibition of chlorophyll a content was observed (Fig. 2). Our studies have shown that the content of chlorophyll a in algae cells decreases with increasing concentration of atrazine in the culture medium. After two days of exposure of algae to this herbicide, the highest level of inhibition of chlorophyll a content was observed at the highest tested concentration of ATR 10 mg/L and it was over 57% in comparison to the control sample. A subsequent 48-hour ATR treatment of algae enhanced the toxic effect of the herbicide on the content of chlorophyll a. The highest level of chlorophyll a inhibition up to over 70% was observed at the highest concentrations of ATR 10 and 1 mg/L tested.

**Inhibition of chlorophyll b**

ATR also had a similar toxic effect on the content of chlorophyll b in Chlorella vulgaris. The highest level of inhibition of chlorophyll b content was detected after a 4-day incubation of the algae culture with the tested herbicide concentrations of 10; 1; 0.1 and 0.01 mg/L (Fig. 3). For the tested concentrations, the inhibition of chlorophyll b ranged from over 9 to over 79%, with the highest values for the highest ATR concentrations tested.

**Inhibition of carotenoids**

As in the case of chlorophylls a and b, the toxic effect of ATR on the content of carotenoids was observed. Both 2- and 4-day exposure of algae to the tested herbicide concentration led
to a significant, even 80% inhibition of the content of these pigments in *Chlorella* cells (Fig. 4). In relation to carotenoids, higher inhibition values of their content were observed after a 4-day incubation of algae compared to a 2-day incubation.

**Inhibition of Chlorella vulgaris growth**

In addition to the toxic effect of ATR on the content of chlorophyll *a* and *b* pigments and carotenoids, this herbicide also inhibited the growth of *Chlorella vulgaris* cultures at all tested concentrations of 10; 1; 0.1 and 0.01 mg/L, both after 2 and 4 days of exposure (Fig. 5). The highest values of inhibition of the growth of algae cultures, up to more than 60%, at the highest herbicide concentrations of 10 and 1 mg/L were obtained after 4 days of incubation.

**Toxicity and protein damage estimation with bioluminescent E. coli strains**

The toxicity and protein damage potential of ATR was estimated with the use of *E. coli* RFM443 *grpE:luxCDABE* strain. The obtained results indicated that ATR in all tested concentrations of 10, 1, 0.1 and 0.01 mg/L influenced the *grpE* promoter after 2 and 24 hours of incubation (Fig. 6). A stronger effect of

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**Fig. 2.** Effect of studied doses of atrazine on chlorophyll *a* content in *Chlorella vulgaris*, presented as a percent (%) of the inhibition. *- statistically significant difference at α= 0.05 between control sample, + - statistically significant difference at α= 0.05 between sample after 2 days. Error bars indicate the standard error of the mean of three independent replicates.

**Fig. 3.** Effect of studied doses of atrazine on chlorophyll *b* content in *Chlorella vulgaris*, presented as a percent (%) of the inhibition *- statistically significant difference at α= 0.05 between control sample, + - statistically significant difference at α= 0.05 between sample after 2 days. Error bars indicate the standard error of the mean of three independent replicates.
toxicity and protein degradation was observed after 24 hours of incubation of bacteria culture compared to 2 hours of *E. coli* RFM443 *grpE:luxCDABE* exposure to ATR. After 24 hours of bacteria culture incubation with ATR at the concentration of 10 mg/L, almost 50% inhibition of the activity of the *grpE* promoter compared to the control was obtained. This was probably due to the global toxic effect of ATR on bacteria, which inhibited the expression of the *luxCDABE* gene from the *grpE* promoter. For the remaining concentrations tested, induction of the *grpE* promoter was observed with the highest level of response of the *grpE:luxCDABE* gene construct in *E. coli* of over 60% at concentrations of 1 and 0.01 mg/L. Based on the obtained results, it was found that the time of exposure of the bacterial culture to ATR was the main factor affecting the sensitivity of the *grpE:luxCDABE* gene construct in *E. coli*.

The toxic effect of ATR was also estimated with the use of *E. coli* RFM443 *lac:luxCDABE* biosensor strain. The decrease in luminescence in *E. coli* RFM443 *lac:luxCDABE* culture is proportional to the toxic potency of tested ATR concentrations of 10, 1, 0.1 and 0.01 mg/L. In all tested concentrations of ATR, its strongest toxic effect was detected after 24 hours of incubation.

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**Fig. 4.** Effect of studied doses of atrazine on carotenoids content in *Chlorella vulgaris*, presented as a percent (%) of the inhibition. *- statistically significant difference at α= 0.05 between control sample, + - statistically significant difference at α= 0.05 between sample after 2 days. Error bars indicate the standard error of the mean of three independent replicates.

**Fig. 5.** Effect of studied doses of atrazine on the growth of *Chlorella vulgaris*, presented as a percent (%) of the inhibition. *- statistically significant difference at α= 0.05 between control sample, + - statistically significant difference at α= 0.05 between sample after 2 days. Error bars indicate the standard error of the mean of three independent replicates.
incubation of the E. coli RFM443 lac:luxCDABE culture in comparison to 2 hours of incubation (Fig. 7). Compared to the control sample, the strongest toxic effect of ATR was detected at the highest tested concentrations of 10 and 1 mg/L and was over 60% and about 20%, respectively.

**ATR toxicity analysis with gfp gene as reporter**

The use of the E. coli strain MM294-GFP allows for the assessment of ATR toxicity based on its effect on the GFP protein. Our results indicated that ATR at all tested concentrations had a toxic effect in E. coli MM294-GFP culture after 2 and 24 hours of incubation (Fig. 8). After 2 h of ATR treatment on a culture of E. coli MM294-GFP, the fluorescence inhibition of GFP protein ranged from over 2% to over 14% with the highest value of 14.525% at the highest tested ATR concentration of 10 mg/L. Extending the incubation time of the bacterial culture to 24 h, resulted in the toxic effect intensification of the herbicide in all tested ATR concentrations. This intensification was manifested by an increase in the GFP protein inhibition value in the E. coli MM294-GFP culture to over 15 and 30% at the highest tested ATR concentrations of 10 mg/L and 1 mg/L, respectively.

![Fig. 6](image-url)

**Fig. 6.** Effect of studied doses of atrazine on grpE promoter response in E. coli RFM443 grpE:luxCDABE (toxicity and protein damage), presented as a percent (%) of the induction or inhibition. *- statistically significant difference at α= 0.05 between control sample, + - statistically significant difference at α= 0.05 between sample after 2 days. Error bars indicate the standard error of the mean of three independent replicates.

![Fig. 7](image-url)

**Fig. 7.** Effect of studied doses of atrazine on lac promoter response in E. coli RFM443 lac:luxCDABE (toxicity), presented as a percent (%) of the induction or inhibition. *- statistically significant difference at α= 0.05 between control sample, + - statistically significant difference at α= 0.05 between sample after 2 days. Error bars indicate the standard error of the mean of three independent replicates.
**ATR genotoxicity determination**

The genotoxic potential of ATR was estimated with the use of *Escherichia coli* RFM443 recA:luxCDABE biosensor strain by analysis of the induction of recA promoter. A stronger genotoxic effect of ATR on bacterial cultures was detected after 24 hours of their exposure to the herbicide. ATR concentrations of 0.1 and 1 mg/L caused the strongest induction of the recA promoter, over 200% and almost 170%, respectively, compared to the control (Fig. 9). For the remaining concentrations of ATR, recA promoter induction levels ranged from greater than 75% to greater than 82%.

**Statistical analysis**

In the statistical analysis of the obtained results we applied Tukey’s NIR test, which indicated statistical significance between chlorophyll a, chlorophyll b, carotenoids inhibition and optical density values in the control samples and between 2 and 4 days of exposure to the atrazine doses considered. Most parameters showed statistically significant differences, the exception to this observation being chlorophyll b values at atrazine concentrations of 0.01 mg/L. In the case of induction or inhibition of the grpE, lac and recA promoters in *E. coli* RFM443, especially after 24 hours of exposure to ATR and...
in most of the tested concentrations, statistically significant differences in the values of the tested parameters were detected compared to the control and 2-hour incubation. No statistically significant differences were observed for the effect of atrazine doses of 0.01 mg/L after 2 hours of exposure to the grpE promoter in E. coli strain RFM443 grpE:luxCDABE, which may have been due to the similar value to that observed in the control sample. A similar relationship was shown for the effect of atrazine doses of 0.01; 0.1 and 1.0 mg/L after an exposure time of 2 hours during lac promoter induction studies in E. coli strain RFM443 lac:luxCDABE. In addition, no statistically significant differences were observed for atrazine doses of 0.1, 1.0 and 10 mg/L between exposure times of 2 and 24 hours for induction of GFP protein fluorescence in E. coli strain MM294-GFP.

Discussion

Chlorophylls a and b, along with carotenoids, belong to the main pigments of Chlorella vulgaris, the content of which is often determined in toxicity tests. Chlorophylls are the main photosynthetic pigments. They are very important because they start the photosynthesis process by capturing photons and directing them via resonant transfer to the transmembrane structures in the thylakoid, i.e. photosystem II (PSII) (Barsanti and Gualtieri, 2014, Malcata, 2019). Carotenoids act as additional pigments for capturing and transferring energy, protecting against excessive irradiation. They also function as antioxidants and detoxifiers, protecting cells from damage caused by free radicals, including reactive oxygen species (Barsanti and Gualtieri, 2014, Ozturk et al. 2022, Sivathanu and Palaniswamy, 2012).

In our tests of ATR toxicity, we obtained the content inhibition of chlorophyll a, b and carotenoids in Chlorella after 2 and 4 days of exposure to ATR at concentrations of 10; 1; 0.1; 0.01 mg/L for these algae. The highest values of inhibition of the content of pigments up to over 80% were obtained after 4 days of algae incubation and at the highest tested concentrations of ATR 10 and 1 mg/L.

Majewska et al. (2021) described the phytocidal mechanism of atrazine. ATR can bind to the plastoquinone b (Qb) binding site on the D1 protein of photosystem II (PSII), thus blocking the transfer of excitation energy from PSII to PSI. A decrease in the content of chlorophylls leads to a significant decrease in the efficiency of photosynthesis. Our experiments also showed a toxic effect of atrazine on the growth of Chlorella cultures after both the 2 and 4 days of algae exposure to ATR. Our results of Chlorella vulgaris growth inhibition are in agreement with the study of Sun et al. (2020), who reported that atrazine concentrations of 0.004 to 1 mg/L caused a 50% decrease in Chlorella vulgaris growth (Sun et al. 2020). In turn, Camuel et al. (2017) found over 50% inhibition of the growth of Chlorella vulgaris cultures at ATR concentrations ranging from 0.02 to 4.6 μM. In the toxicity tests with the use of Chlamydomonas reinhardtii, the IC50 values were about 78 μg/L (Majewska et al. 2021).

Disturbances in the efficiency of the antioxidant enzyme system and the oxidation product generation system in living cells lead to oxidative stress. Oxidative stress causes damage to DNA, proteins, lipids, and cellular structures. Under conditions of oxidative stress, there are changes in the activity of the main antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione-S-transferase (GST), and glutathione reductase (GR), which can reflect the oxidative damage in the cell. Changes in the activity of antioxidant enzymes SOD, POD, CAT, GST and GR are considered to be markers of oxidative stress caused by environmental pollution, including herbicides (Yang et al. 2021a: Mofeed et al. 2013).

Previous work has documented that one of the main mechanisms of atrazine toxicity in algae is oxidative stress. Mofeed et al. (2013) in their work examined the effects of different ATR concentrations of 25, 50 and 100 μg/L on growth and oxidative stress on microalgae Scenedesmus obliquus. To determine the toxic effect of ATR on the microalgae the antioxidative enzyme activities were used as biomarkers. The authors in their work discovered that ATR significantly induced antioxidative enzyme activities of glutathione-S-transferase (GST), catalase activities (CAT) and glutathione reductase (GR) at different concentrations (Mofeed et al. 2013).

E. coli microbial biosensor strains with lux and gfp reporter genes are used for toxicity, genotoxicity, and bioavailability bioassays, which respond to a variation of xenobiotics and herbicides (Ali et al. 2021). In our studies, we also detected toxic activity of ATR with the use of E. coli strains RFM 443 with the gene constructs grpE:luxCDABE and lac:luxCDABE. A 24-hour exposure of the bacterial culture to ATR resulted in an intensification of the toxic effect of the herbicide, compared to 2-hours. In the obtained results, in most of the tested ATR concentrations, the induction of the grpE promoter was observed, even up to more than 60%, which proves the toxicity of this herbicide and its potency of degradation of cellular proteins. Similar results were obtained using the lac:luxCDABE gene construct in E. coli RFM443, where 24-hour incubation with ATR caused over 26% inhibition of luminescence in bacterial cells. Also, using the E. coli strain MM294-GFP, the strongest inhibition of GFP protein fluorescence, up to over 30%, was obtained after 24-hour incubation with ATR at the highest concentration of 10 mg/L.

The widespread use of herbicides induces oxidative stress in microbial populations. We know the most about the effect of ATR on soil microorganisms. Yang et al. (2021) documented that ATR significantly reduced the diversity of bacterial populations in cultivated soil layers of chernozem. Our studies have shown that ATR at concentrations of 10; 1; 0.1 and 0.01 mg/L affects the level of expression of the luxCDABE gene and causes a toxic effect and protein degradation in E. coli RFM443 cells. Zhang et al. (2012) in their work documented the inhibitory effect of ATR on cultures of E. coli K-12 and Bacillus subtilis B19. They also showed that ATR induced superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and total antioxidant capacity (T-AOC). Exposure to atrazine led to oxidative stress responses in the shrimp Palaemonetes argentinus, in red swamp crayfish Procambarus clarkii and in copepods (Yang et al. 2021, Silverya et al. 2022). It is very possible that oxidative stress was the mechanism of ATR-induced toxicity and protein degradation in the E. coli RFM443 (luxCDABE) and E. coli MM294 (gfp) strains we studied.
In our studies, for the determination of ATR genotoxic potency we used *E. coli* RFM443 strain with plasmid transcriptional fusion of *recA* promoter with *luxCDABE* reporter gene. In our experiment with the application of *E. coli* RFM443 *recA::luxCDABE* strain the significant genotoxic activity of ATR was noticed. A very clear increase in ATR genotoxicity, even up to over 200% compared to the control, was detected after 24 hours of incubation of the bacterial culture with the herbicide. According to literature data ATR can accumulate in the tissue, destroy DNA, and cause genotoxicity which may lead to mutations, immunosuppression, as well as damage to reproduction, growth, and development (Roustan et al. 2014, Yang et al. 2021). So far, in studies with the use of fish as model organisms' teratogenicity, genotoxicity, and oxidative stress have been reported, together with several histopathological effects. In crustaceans exposed to atrazine, teratogenic effects were observed, which could be caused by damage to the genetic material of embryos (Silverya et al. 2022). Akhtar et al. (2021) in their work and with the use of Comet assay proved, the genotoxic activity of ATR at the concentration of 24 ppm to snow trout (*Schizothorax plagiostomus*). Genotoxic effects of ATR have already been observed in the fish *Puntius lineatus*. The main mechanism of genotoxicity of ATR is that this herbicide can bind directly to DNA through intercalation mechanisms and the formation of adducts between the herbicide and the adenine and guanine bases (Santos et al. 2014). This mechanism may also be the cause of the genotoxic effect of the ATR tested in this study in the *E. coli* strain RFM443 *recA::luxCDABE*.

**Conclusions**

In the presented studies, the toxicity of a known herbicide – atrazine was determined on *Chlorella vulgaris* and *E. coli* microbial biosensor strains. In the assessment of ATR toxicity, we defined the inhibition of the content of chlorophyll *a*, *b*, carotenoids in *Chlorella vulgaris* and the inhibition of the growth of this alga culture. *E. coli* RFM443 strains with gene constructs grpe:*luxCDABE*, lac:*luxCDABE*, recA:*luxCDABE* and *E. coli* strain MM294 *trec::luxCDABE* were used to determine toxicity, degradation of cellular proteins and genotoxicity. Based on the obtained results, the following conclusions were made.

1. Atrazine in the tested concentrations shows a toxic effect in relation to *Chlorella vulgaris*.
2. ATR is toxic in *E. coli* RFM443 strains with grpe:*luxCDABE*, lac:*luxCDABE* gene constructs and causes degradation of cellular proteins.
3. ATR is toxic to the GFP protein in *E. coli* strain MM294-GFP.
4. ATR is genotoxic in *E. coli* strain RFM443 *recA::luxCDABE*.

Atrazine in surface and drinking water is detected in small concentrations (μg/L), but long-term exposure of organisms to low concentrations of pesticides contained in water intended for consumption may adversely affect sensitive populations. Caution should be exercised, because scientific studies have shown a correlation between human exposure to ATR and cancer incidence.

**Conflict of interest**

No conflict of interest has been declared.

**Funding**

Research carried out as part of statutory project WZ/WB-IIŚ/2/2023.

**Acknowledgements**

Authors are very grateful to Prof. Shimshon Belkin, Dr Tal Elad and Dr Sharon Yagur-Kroll, The Hebrew University of Jerusalem, Jerusalem, Israel for providing bioluminescent biosensor strains and as well as for scientific cooperation. Authors’ gratitude also go to Prof. Imrich Barak, Slovak Academy of Science, Bratislava for providing *E. coli* MM294-GFP fluorescent biosensor strain.

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Journal of Environmental Research and Public Health, 17, pp. 2567–2578, DOI: 10.3390/ijerph1702567


Atrazine toxicity in marine algae *Chlorella vulgaris*, in *E. coli lux* and *gfp* biosensor tests


**Toksyczność atrazyny w glonach *Chlorella vulgaris* oraz w biosensorowych testach *E. coli* z genami *gfp* i *lux***

**Streszczenie:** Atrazyna (ATR) to szeroko stosowany na całym świecie chlorowany herbicyd z grupy s-triazyn. Ze względu na powszechne stosowanie, ATR przedostaje się do środowiska i jest wykrywana w wodzie pitnej, przekraczając dopuszczalne przez WHO stężenie, które wynosi 2 μg/L. W przedstawionych badaniach określono toksyczność ATR w stężeniach 10; 1; 0.1; 0.01 mg/L na glonach *Chlorella vulgaris* oraz z zastosowaniem *E. coli* mikrobiologicznych biosensorów z genami reporterowymi *gfp* i *lux*. Toksyczność oszacowano na podstawie zawartości chlorofilu a, b, karotenoidów w *Chlorella vulgaris* oraz zahamowania wzrostu tej kultury alg. Szczepy *E. coli* RFM443 z konstruktami genowymi *grpE:*luxCDABE, *lac:*luxCDABE, *recA:*luxCDABE i szczep *E. coli* MM294 trc:*luxCDABE wykorzystano do określenia toksyczności, degradacji białek komórkowych i genotoksyczności. W przeprowadzonych badaniach wykryto, że ATR w analizowanych stężeniach wykazuje działanie toksyczne w stosunku do *Chlorella vulgaris*. W przypadku ATR stwierdzono właściwości toksyczne i genotoksyczne oraz potencjał degradacji białek w szczepach *E. coli* RFM443 z promotorami *grpE*, *lac*, *recA*. Ponadto wykryto toksyczność ATR w stosunku do bialka GFP w szczepie *E. coli* MM294-GFP. Biorąc pod uwagę udokumentowaną w badaniach własnych oraz w doświadczeniach innych naukowców toksyczność i genotoksyczność ATR, obecność tego herbicydu w wodach powierzchniowych i wodzie pitnej stanowi poważne zagrożenie dla organizmów żywych.