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Mitosis inhibitory and clastogenic effects of waters from anthropogenically affected areas

Митоинхибиращ и кластогенен ефект на води от антропогенно повлияни зони

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ABSTRACT

The present study aims to analyse the effect of waters, anthropogenically influenced by various pollutants, on the mitotic division and chromosomal apparatus of cells by establishing their potential mitoinhibitory and clastogenic effect. The cytotoxic and mutagenic effect of contaminated water was examined by the application of the *Allium cepa* test system. Mitotic depression has been established for samples with available anthropogenic contamination. Microscopic analysis showed an increased incidence of chromosomal aberrations in the test samples compared to the control, resulting from the genotoxic effect available. Chromosomal abnormalities of the type of lagging and 'vagrant' chromosomes, chromosomal fragments, anaphase and telophase bridges, micronuclei, as well as deviations from normal cell division such as K-mitoses and asynchronous mitoses have been observed. The analysis of the spectrum of chromosomal aberrations shows some differences in the frequency of occurrence of the different types of disorders, which reflects the specificity of the genotoxic effect of the water samples from the surveyed areas.

Keywords: *Allium test*, chromosomal aberration, cytotoxicity, genotoxicity, mitotic index

РЕЗЮМЕ

Настоящото проучване има за цел да анализира влиянието на антропогенно повлияни от различни замърсители води върху митотичното делене и хромозомния апарат на клетките чрез установяване на потенциалния им митоинхибиращ и кластогенен ефект Чрез прилагане на *Allium cepa* тест-системата е проучено цитотоксичното и мутагенното действие на замърсените води. Констатирана е митотична депресия за пробите с налично антропогенно замърсяване. Микроскопският анализ показва увеличена честота на хромозомните аберации в опитните проби в сравнение с контролната, което е резултат от наличен генотоксичен ефект. Констатирани са хромозомни аномалии от типа на изоставащи и „скитащи" хромозоми, хромозомни фрагменти, анафазни и телофазни мостове, микроядра, както и отклонения от нормалното клетъчно делене като К-митози и асинхронни митози. Анализът на спектъра на хромозомните аберации показва известни различия в честотата на срещане на отделните типове нарушения, което отразява спецификата на генотоксичното действие на водните проби от проучваните зони.

Ключови думи: *Allium тест*, хромозомни аберации, цитотоксичност, генотоксичност, митотичен индекс

INTRODUCTION

Anthropogenic water pollution is generally understood to mean the adverse impact of man and his economic activity on the composition and properties of water. Factories, agricultural centres, construction sites and other sites produce or use toxic materials that often enter surface and groundwater. Although many chemicals acting alone can damage the body's normal functions, the combined action of many pollutants is of particular importance because of the possible synergistic effect. Higher plants are the preferred genetic models for detecting mutagens in the environment and are often used in monitoring studies. Among the plant species, *Allium cepa* is used to evaluate DNA damage by registering chromosomal aberrations as well as to detect disturbances in the mitotic cycle. The *Allium* test is recommended as a suitable and highly effective method for the study of almost all chemical, physical and biological factors.

Genetic changes in *Allium cepa* cells caused by industrial effluent waters impact and associated with the expression of cytotoxic and genotoxic effects have been described by Rank and Nielsen (1993, 1994), Shahaby et al. (2003), Egito et al. (2007) and Andrade et al. (2008). Using the *Allium cepa* test system, the overall toxicity and genotoxicity of different sewage water concentrations were analysed (Ukaegbu and Odeigah, 2009), as well as the cyto- andandgenotoxic effects of various pesticides (Chauhan and Gupta, 2005; Singh et al., 2007; Aydemir et al., 2008; Fisun and Rasgele, 2009).

The purpose of this study is to investigate the potential toxic, cytotoxic and genotoxic effect of waters from anthropogenically contaminated areas by analysing germination, mitotic parameters, type and frequency of chromosomal aberrations detected in the *Allium cepa* species used as an experimental test-system.

MATERIALS AND METHODS

Object of research

For the purpose of the study, three wetlands have been selected, located along the Maritsa River. Two of them are rice fields in the lands belonging to the village of Tsalapitsa and city of Plovdiv, and the third is a protected area near the village of Zlato pole. The 'Plovdiv rice fields' wetland is located to the west of Plovdiv's North Industrial Zone, in the immediate vicinity of Ring Road 805. It is located in a densely populated area and is subject to intense anthropogenic pressure from intense human activities. 'Tsalapitsa rice fields' is a complex of water areas used for rice production, surrounded by low dikes and channels, and moist meadows located right next to them. The intensification of rice production is associated with significant use of pesticides, fertilizers and other chemicals, which causes a change in water quality. The waters used to grow rice in this area come from the Topolnitsa Dam and are often contaminated with heavy metals (Angelova, 2008; Ivanova et al., 2003; 2005; 2008). 'Tsalapitsa ricefields' has been designated as a protected area for the purpose of protecting birds and their habitats under Directive 2009/147/EU. Despite this status, a strong anthropogenic influence is observed there due to the inexpedient use of agrochemicals (Zhelev et al., 2017).

'Zlato pole' is the largest wetland of natural character in the Maritsa River (MOEW, 2007). It includes several water basins of different surface area and depth, reed and rush areas, islands and pastures. 'Zlato pole' was declared a protected area in 2001 by Order No.RD-476/11.07.2001 of the MOEW.

Water samples were taken from each of the zones described to study their overall toxic, cytotoxic and mutagenic effects. Distilled water was used as a control sample to assess the impact of water in the wetlands studied. The physicochemical parameters of the water samples from the rice fields were analysed in an accredited testing laboratory. Data from the physicochemical monitoring of the waters of the 'Zlato pole' protected zone have been provided by the Basin Directorate – Plovdiv (Table 1).

Cytogenetic methods

Allium cepa test-system (according to Fiskesjö (1985), with modifications of Ivanova et al., 2005; Staykova et al., 2010) was used for screening the mutagenic activity

Table 1. Physicochemical parameters of the water samples

Type of test	Unit of magnitude	Standards	Test results rice fields	Test results Zlato pole
pH	-	BSS EN ISO (2012)	7.27±0.03	8.0
Conductivity	µS/cm	BSS EN (2000)	448±11	334
BOD_5	mg/l	BSS EN (2004a)	4.1±0.5	1.9
COD	mg/l	ISO (2002)	9±1	4.9
Ammonium nitrogen	mg/l	BSS ISO (2002a)	0.61±0.06	<0.08
Orthophosphates (PO_4)	mg/l	BSS EN ISO (2005)	<0.02	0.44
Total phosphorus	mg/l	BSS EN ISO (2005)	0.073±0.007	0.46
Total nitrogen	mg/l	BSS EN (2004b)	1.8±0.4	1.9
Calcium carbonate hardness	$mgCaCO_3/l$	BSS ISO (2002b)	215±6	-
Calcium	mg/l	BSS ISO (2002c)	63±2	-
Magnesium	mg/l	BSS ISO (2002b)	14±1	-
Nitrogen nitrite	mg/l	BSS EN ISO (2009)	<0.03	0.036
Nitrogen nitrate	mg/l	BSS EN ISO (2009)	0.43±0.03	1.75
Chlorides	mg/l	BSS EN ISO (2009)	6.5±0.2	-
Sulphates	mg/l	BSS EN ISO (2009)	47±1	-
Aluminum	µg/ml	BSS EN ISO (2016)	18.1±0.9	-

of the analysed water samples. It has been adopted by the International Program on Plant Bioassays (IPPB) for monitoring or testing pollutants in the environment (Ma, 1999). For the purposes of this study, annual seeds of *Allium cepa* were used.

Total toxicity was calculated as the percentage of seeds germinated from the total number of seeds planted in the control and test samples.

Temporary squash preparations from the root apical meristem of *Allium cepa* were prepared from the fixed samples. From each test and control sample, a minimum of 2000 cells from at least 5 microscopic preparations from different root tips were analysed. The number of dividing cells in the different phases of mitosis and the total number of cells were registered when conducting the microscopic analysis. Mitotic index and phase indices, which are indicators of cell proliferation, were used to analyse the cytotoxic action of the tested water samples.

In the present study, the genotoxic effect of the tested water samples was analysed by determining the frequency of the observed chromosomal abnormalities and deviations from normal cell division of *Allium cepa*. Micronucleus test and anaphase analysis were used to register the genotoxic effect.

Statistical analysis

The data were analyzed through the SPSS Statistics software package, version 21.0. Descriptive statistics was used to characterize the frequency of the groups compared and crosstabs. Data were presented as a mean ± standard deviation (SD), number of cells and a percentage. Differences between the groups were analyzed by the Pearson Chi-Square test. Statistical significance was defined as $P<0.05$. In addition, to compare the mean values, ANOVA and post-hoc tests (LSD) analysis was used.

RESULTS

Total toxicity of water samples

The data on the total toxicity found by the *Allium cepa* germination test are presented in Table 2.

Cytotoxic effect of water samples

Table 3 presents the data on the mitotic and phase indices in the growing root tip of *Allium cepa* in the control and tested samples.

Genotoxic effect of water samples

Genotoxicity is an indicator of the potential of clastogenic agents to cause damage to genetic material. Various deviations from normal division have been reported in all phases of the mitotic cycle, ranging from chromosomal fragmentation to disorganization of the spindle apparatus.

Table 4 presents the frequency of types of chromosomal aberrations and deviations from normal cell division compared to the total number of cells analysed and the number of dividing cells, as well as the calculated total frequency of observed abnormalities in the test and control samples.

The tested water samples induce a wide range of chromosomal aberrations, varying in frequency and appearance depending on the level of contamination. The following types of disorders were registered: chromosomal fragments, lagging and 'vagrant' chromosomes, micronuclei, anaphase and telophase bridges, K-mitoses and asynchronous mitoses, which demonstrate the genotoxic potential of the tested water samples (Figures 1-9).

Table 2. Effect of water samples on germination of *Allium cepa* seeds

	Germination of seeds											
	Number of seeds	Number of seeds germinated								Total number of seeds germinated	Seeds not sprouted	Germinated seeds (%)
		1 day	2 day	3 day	4 day	5 day	6 day	7 day	8 day			
Control	50	3	12	14	6	1	1	1	1	39	11	78
Plovdiv rice fields	50	0	7	15	8	1	3	3	2	39	11	78
Tsalapitsa rice fields	50	1	9	16	8	4	1	1	1	41	9	82
Zlato pole	50	3	11	14	6	1	1	0	0	36	14	72

Table 3. Presents the data on the mitotic and phase indices in the growing root tip of *Allium cepa* in the control and tested samples

Samples	Mitotic index IM	Prophase index IPph	Metaphase index IMph	Anaphase index IAph	Telophase index ITph
Control	51.49	84.88	6.13	5.51	3.47
Plovdiv rice fields	49.94	87.18	4.75	4.46	3.61
Tsalapitsa rice fields	45.97	90.29	4.01	3.85	1.85
Zlato pole	52.19	89.65	4.21	3.50	2.64

Statistical significance of differences between the control sample and the tested samples: P<0.0001 (ANOVA)

Table 4. Frequency of occurrence of different types of chromosomal aberrations and deviations from normal mitosis, analyzed by the *Allium cepa* test method

Samples	Cells with micronuclei	Aschronous mitosis	K-mitoses	Cells with chromsomal fragments	Cells with chromsomal bridges	Cells with lagging chromsomes	Total number of cells with aberrations
Control	0.35	0.00	0.04	0.07	0.14	0.28	0.88
	0.68	0.00	0.07	0.14	0.27	0.54	1.70
Plovdiv rice fields	0.28	0.14	0.57	0.14	0.11	0.42	1.66
	0.28	0.28	1.13	0.28	0.21	0.85	3.33
Tsalapitsa rice fields	0.63	0.07	0.30	0.15	0.26	0.30	1.70
	0.63	0.16	0.64	0.32	0.56	0.64	3.69
Zlato pole	0.26	0.15	0.04	0.04	0.26	0.30	1.04
	0.50	0.29	0.07	0.07	0.50	0.57	2.00

For each sample, data in the first row is calculated as % of the total number of cells (N) and data in the second row is calculated as % of the number of dividing cells (N'). Statistical significance of differences between between: the control and the Plovdiv sample – $P<0.001$; the control and the Tsalapitsa sample – $P<0.01$; the Zlato pole and the Plovdiv samples – $P<0.01$ (Post Hoc Test)

Figure 1. Lagging chromosomes (sample from Plovdiv rice fields)

Figure 2. 'Vagrant' chromosomes (Control sample)

Figure 3. 'Vagrant' chromosomes (sample from Zlato pole)

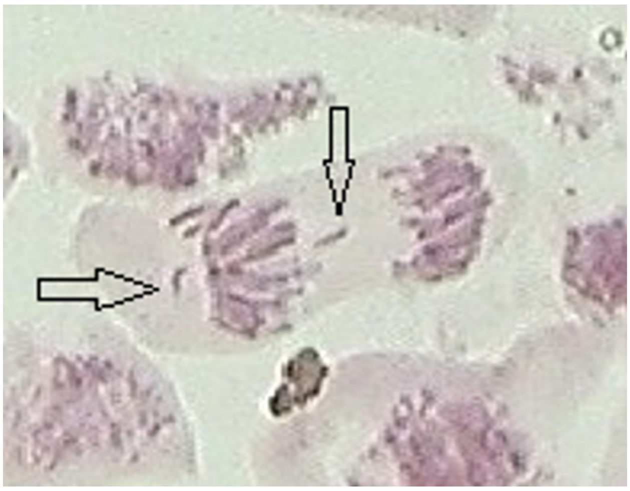

Figure 4. Chromosomal fragments (sample from Tsalapitsa rice fields)

Figure 5. Telophase bridge (sample from Tsalapitsa rice fields)

Figure 6. Anaphase bridge (sample from Tsalapitsa rice fields)

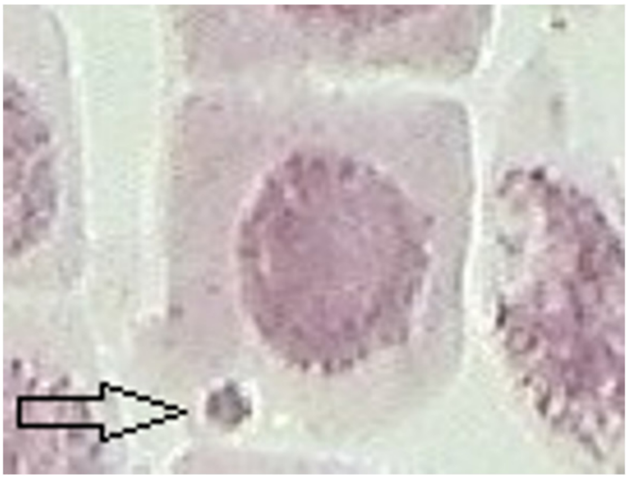

Figure 7. Micronucleus (sample from Tsalapitsa rice fields)

Figure 8. K-mitosis (sample from Plovdiv rice fields)

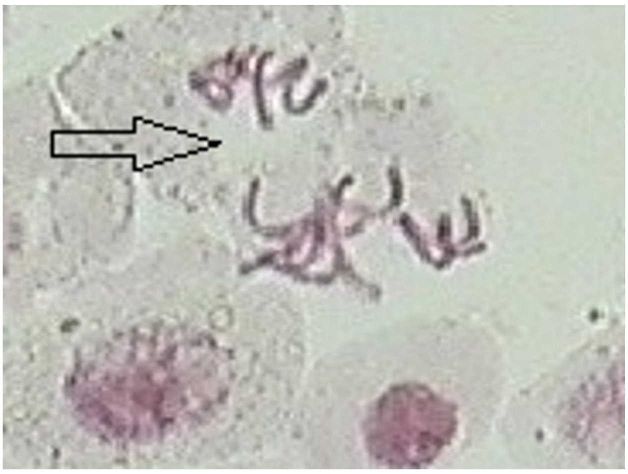

Figure 9. Multipolar anaphase (sample from Plovdiv rice fields)

DISCUSSION

In contemporary conditions, water bodies are exposed to the general influence of anthropogenic factors of different nature. In the water basins in nature there is a complex of dissolved, suspended and colloidal substances, the composition of which varies over time and according to the place. Organisms that are in contact with water contaminated with mutagenic substances are not exposed to individual substances but to the whole complex, which is why the mutagenic effect can be enhanced by synergistic action or attenuated by neutralizing interaction. For this reason, the traditional approach by which pollutants are evaluated separately for mutagenicity does not take into account the actual mutagenic effect of contaminated water (Turusov et al., 1998). In the present study, data from physicochemical analysis show elevated levels of ammonium nitrogen, aluminum and BOD_5 in the water samples collected from the rice fields, as well as of the total phosphorus and orthophosphates in the 'Zlato pole' sample (Table 1).

Total toxic effect of anthropogenically contaminated waters

The degree of total toxicity was established by the inhibitory effect on seed germination. The germination of seeds from the control sample and those treated with water from Zlato pole started fastest. Data show that water samples from areas with high anthropogenic loading (Plovdiv and Tsalapitsa rice fields) show an initial retention effect in relation to seed germination but do not have a general toxic effect, as indicated by the high germination rate reported (Table 2).

Mitoinhibitory effect of anthropogenically polluted waters

The mitotic index can be used to quantify changes in the rate of cell division resulting from changes in the environment. According to Fernandes et al. (2007) the level of cytotoxicity of an agent can be demonstrated by increasing or decreasing the mitotic index. Water samples from anthropogenically contaminated areas showed an inhibitory effect on the frequency of cell proliferation, as demonstrated by lower mitotic index values compared to control sample (Table 3). The lowest degree of cell division in the sample of Tsalapitsa rice fields was reported, which provoked the most significant cytostatic effect among the tested water samples. Weaker inhibition of cell proliferation was observed in the sample of Plovdiv rice fields. The reduction of the mitotic index in plant test systems, depending on the degree of contamination of water bodies, has been established also by other authors (Shahaby et al., 2003; Egito et al., 2007; Singh et al., 2007; Ivanova et al., 2008; Fisun and Rasgele, 2009; Mesi and Kopliku, 2014; Mishra et al., 2015).

Phase mitotic indices allow the determination of the relative duration of the various stages of cell division. Different distribution of cells in the mitosis phases was found for the different experimental versions. In the meristem cells of *Allium cepa* germinated in water samples from the anthropogenically affected areas, a certain delay of the mitotic cycle was observed compared to the cells from the control sample. This retention of cell division was observed at the prophase stage (Table 3). The results obtained for the phase indices in this study are similar to the data of the study of Liman et al. (2010, 2011), which found an increase in the prophase index and a decrease in metaphase, anaphase and telophase indices according to the concentration of the biocides analysed by them.

Clastogenic effect of anthropogenically polluted waters

The chromosomal aberration reporting test in meristem cells has been validated and widely used in analysing the genotoxic effect of various contaminants. In this study, the analysis of the microscopic preparations clearly shows the presence of clastogenic effect with respect to the water samples examined.

In the experimental versions, the incidence of meristem cells with various disorders, including structural aberrations of chromosomes or abnormal mitoses, significantly exceeds that in the control sample. The highest genotoxic effect was reported in the water sample from Tsalapitsa rice fields, where the highest overall aberration rate was recorded - 1.70% (Table 4). A high percentage of chromosomal abnormalities were also detected in the test sample from Plovdiv rice fields, whose value exceeds significantly the percentage of detected violations in the control and the sample from the protected wetland area of Zlato pole.

The analysis of the wide range of aberrations in the experimental versions shows that the main disorders observed are lagging and 'vagrant' chromosomes (Figures 1-3), cells with K-mitosis and micronuclei. All analysed samples also showed the presence of anaphase and telophase bridges, which is an indicator of DNA damages. In some cells, lagging chromosomal fragments were observed along with the bridges (Figure 4). Fragmentation of chromosomes is an indicator of the destruction of their structure associated with the lysis of DNA molecules and serves as an indicator of genomic instability (Leme and Marin-Morales, 2009). Samples from Tsalapitsa and Zlato pole rice fields provoke the highest number of aberrations of the type of chromosomal bridges (Figures 5-6). A high percentage of cells with micronuclei were detected in all samples tested. The highest number of micronuclei in cells of *Allium cepa* was found in the sample of Tsalapitsa rice fields (Figure 7). The higher frequency of mutations in the sample from Plovdiv rice fields compared to the control sample was largely due to the higher percentage of cells with K-mitosis (Table 4). K-mitosis results from the destruction of the filaments of the spindle apparatus, which results in characteristic configurations of chaotically dispersed chromosomes in the cells (Figure 8). Damages during the formation of the achromatic apparatus can lead to severe abnormalities associated with uneven distribution of the genetic material (Figure 9). Control sample cells have been found to have abnormalities that result from an auto-mutagenic effect and have a lower frequency. A slightly higher percentage of cells with chromosomal abnormalities than the control sample were reported in the Zlato pole sample, which showed aberrations such as anaphase and telophase bridges, micronuclei, and 'vagrant' chromosomes.

CONCLUSIONS

Water samples from the anthropogenically influenced areas tested in this study demonstrate mitosis inhibitory activity on cells of the root apical meristem of *Allium cepa*, with a maximum level of mitotic depression in relation to Tsalapitsa rice field waters. The analysed water samples have a clastogenic effect and induce chromosomal aberrations with a high frequency and wide spectrum of expression, as well as deviations from normal cell division. Specificity was found in the genotoxic action of the tested water samples, expressed by the frequency of occurrence of reported types of disorders.

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