

GENOTOXICITY OF WASTEWATER SAMPLES FROM THE TEXTILE INDUSTRY DETECTED BY BROAD BEAN (*VICIA FAB*A) MICRONUCLEUS TEST ASSAY

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Abstract. The textile wastewater induces a significant number of environmental problems, and while its toxicity has been extensively described in literature, its genotoxic and cytotoxic potential is not well known. The presence of non-biodegradable and highly toxic compounds in textile effluents make textile wastewater a possible source of ecological concern. Therefore, new data in subject matter may be very useful for their risk assessment. In the study the cytotoxicity and genotoxicity of textile wastewater derived from leather dyeing in reference to meristematic *Vicia faba* (*V. faba*) root cells has been studied. The study results show that textile wastewater decreased the mitotic index (MI) as well as significantly enhanced the micronucleus frequencies (MCN) and number of chromosomal aberrations (CA) in root cells. The MCN frequencies for wastewater samples were in the range of tested concentration 6.5 – 43.5 times higher than the negative control (NC). Also the correlation between the decreasing MI ratio and increasing wastewater concentration were detected. For the highest wastewater concentration the MI value was over 9 times lower than in the NC. The results indicate that textile wastewater has a genotoxic effect on plant cells and exposure to untreated wastewater can pose a potential cytotoxic and genotoxic risk.

Keywords: cytotoxicity, dye, chromosome aberrations, micronuclei, mitotic index

Introduction

Environmental impact of textile and dyeing industry is directly associated with significant amount of water consumption and significant quantities and types of chemicals used along all of the textile processing steps. Textile wastewater is a heterogeneous, poorly characterized complex mixture of several contaminants which contains a large amounts of dyes, pigments, biocides, metals, salts, surfactants, solvents, detergents and other non-biodegradable organic matters (Liang et al., 2018; Castro et al., 2019). Most of these substances poses a serious threat to exposed ecosystems. Toxicity and biodegradability of individual textile wastewater components has been the subject of many studies. Many of dyes and pigments widely used in the textile industry have been found to be toxic and genotoxic. The toxicity of dyes as individual components of textile wastewater has been widely described by authors who applies a battery of ecotoxicological tests contest organisms belonging to different trophic level and with a wide range of sensitivities (e.g. *Daphnia magna*, *Artemia salina*, *Raphidoceli subcapitata*, *Aliivibrio fischeri*, *Lemna minor*, *Cucumis sativum*, *Lycopersicon esculentum*, *Eisenia fetida*, etc.) (de Souza et al., 2007; Liang et al., 2018; Oliveira et al., 2018; Castro et al., 2019). Additionally, the publication describing the cytotoxicity, genotoxicity and mutagenicity of dyes detected using the techniques like mouse lymphoma assay, bone marrow micronucleus test and comet assay can also be found. The above-mentioned topics were the subject of work Fernandes et al. (2019) and Jha et al. (2016).

However, in relation to textile wastewater, it should be noticed that wastewater are a complex mixture in which the dyes are only a part. Thus, the assessment of the toxicity of a single component is not sufficient to predict the interactions and joint toxicity of pollutant present in mixture even at low concentration and does not reflect the real environmental threat. While information about the potential toxicity of textile wastewater as a complex mixture can be found in trade literature (Castro et al., 2019), the knowledge about the genotoxicity and cytotoxicity of textile wastewater is still insufficient. Therefore, in order to enrich knowledge about the environmental threat posed by untreated or insufficiently treated textile wastewater the aim of the present study was to evaluate the genotoxic potential of raw textile wastewater by the use of *V. faba* micronucleus test assay. *V. faba* micronucleus test is a low cost, easy to handle short term assay which has been extensively used for environmental monitoring and genotoxicity assessment of various types of chemicals and wastewater (Duan et al., 1999; Iqbal, 2016). *V. faba* micronucleus test does not only provide information about cytotoxic and genotoxic potential of analyzed samples but also its mechanism of action on genetic material.

Materials & Methods

Wastewater sampling

Textile wastewater samples were obtained from textile industry located in GZM Metropolis (Silesia, Poland). Within the study an average daily wastewater sample were used. The samples were collected directly from the dyeing process line in July 2019. The daily average sample was a mixture of hourly samples taken during the 24-hour production cycle. During the daily production cycle, a sample of wastewater was taken every hour and placed in a collecting vessel. Two containers of 5 L capacity were taken for the test. Directly after collection, a part of wastewater was sent for physicochemical analysis the rest was portioned and storage in refrigerator until use. Before each genotoxicity test the wastewater samples were thawed and filtered.

Physico-chemical analysis

Within the study an average daily wastewater sample were used. The collected textile wastewater sample was analyzed for physico-chemical parameters like: pH, temperature, biochemical oxygen demand (BOD₅), chemical oxygen demand (COD), electrical conductivity (EC), total phosphorus (P_{tot}), total nitrogen (N_{tot}), total organic carbon (TOC) and suspended solids. Within the analyses the concentration of sulfate and chloride ions in wastewater sample was also determined. *Table 1* showed some special phasico-chemical parameters of the analyzed wastewater samples.

Test concentration and control

The essence of the micronucleus test is the assessment of damage of genetic material occurring during the cell division. The genetic damages are observed in plant cells in the division phase, therefore it is important to use the samples that do not affect the total inhibition of the mitosis process. In order to determine an appropriate range of textile wastewater concentrations for *V. faba* micronucleus test assay an initially the root growth inhibition test for meristematic part of broad bean was carried out. The root growth inhibition test was performed for 8 wastewater concentration in a range 0.78%-

100.00% (OECD, 1984). Based on root growth inhibition test result the EC₅₀ value for meristematic part of *V. faba* was estimated (Table 2). According to the guidelines the target *V. faba* micronucleus test was carried out for wastewater concentrations in the range below the EC₅₀. Therefore, the *V. faba* micronucleus test was carried out for 5 test concentrations. The highest concentration corresponded to estimated EC₅₀ value (15.40%) and amounted 12.50%. Subsequent wastewater concentrations were prepared by the dilution method, maintaining a dilution factor of 1: 2. Simultaneously, according to OECD (2014) recommendations in the field of genetic toxicology studies, the test was carried out using two types of test control: negative control (NC) and positive control (PC). As a NC an aerated tap water was used. The NC was used as a medium ensuring optimal culture growth conditions. A PC was used to observe damage induced by compounds with proven cancerogenic and mutagenic activity. In the micronucleus test a 1.12 g/L solution of maleic hydrazide (Sigma Aldrich, C₄H₄N₂O₂) was used as a PC. This compound is considered a potential carcinogen whose ability to cause micronuclei and chromosomal aberrations in plant cells has contributed to the widespread use of this compound in genotoxicity assessment (Hajjouji et al., 2007; Marcato-Romain et al., 2009; Sta et al., 2012).

Table 1. Characteristics of untreated textile wastewater

Parameter	Unit	Value	±SD
pH	-	6.6	0.2
Electrical conductivity (EC)	Ms/cm	7 500	380
Temperature	°C	14.5	-
COD	mg/L	17 500	2 600
BOD ₅	mg/L	2 750	
TOC	mg/L	3 100	460
N _{tot}	mg/L	380	57
P _{tot}	mg/L	27.5	2.8
Suspended Solid	mg/L	2 000	200
Chloride	mg/L	635	64
Sulphates	mg/L	2 070	210

Table 2. Impact of raw textile wastewater on growth inhibition of *V.faba* root

Sample	Control		Textile wastewater concentration [%]							
	NC	PC	100	50	25	12.5	6.25	3.13	1.56	0.78
Mean root length [cm]	4.68	1.10	0.68	0.91	1.94	2.37	2.88	3.54	4.57	5.38
± SD	±1.52	±0.21	±0.37	±0.31	±0.22	±0.66	±0.88	±2.47	±2.00	±3.75
Growth inhibition [%]	-	76.5	85.3	80.5	58.5	49.3	38.3	24.3	2.32	-15.1
EC ₅₀ [%]	-	-	15.45							

Procedure for seeds treatment

In the test untreated broad bean (*V. faba*) seeds variety of White Windsor were used. The seeds were obtained from a local farmers' market. 72 hours before the proper micronucleus test, healthy, equal size *V. faba* seeds were pre-sterilized using a 2% H₂O₂ solution. After sterilization the seeds were soaked in tap water for 24 h and placed on moist surface to germinate for 2÷3 days at 20÷22°C. Incubations were carried out until the roots reached the required length of 1.5-2.5 cm. The required length should be

understood as the length of the root obtained after seed germination, which allows to immerse the root in the tested medium without soaking the seed which protects the tested material against rotting. For each of the test concentrations and controls five seeds of *V. faba* were used. One seed means one measurement series.

Micronucleus test procedure

The *V. faba* micronucleus test was conducted based on the modified procedure described by Minissi and Lombi (1997). *V. faba* micronucleus test assay is a modification of the micronucleus test procedure recommended by OECD (2014) guidelines. After reaching by the *V. faba* roots a required length the seeds were peeled from the skin covering the endosperm. The seeds were handled carefully in a way that prevents damage the visible germ or seed shell. Prepared seeds were placed on styrofoam platforms covering the glass cuvette filled with adequate concentrations of textile wastewater and respectively controls solutions. The Styrofoam platform has been constructed in a way ensuring permanent contact of plant roots with the tested medium (form of hydroponic farming). After 44 h of incubation, *V. faba* roots were cut at 1.5-2 cm length and transferred to freshly prepared Carnoy solution (99% ethanol and glacial acetic acid in a 3: 1 ratio). Prepared samples were stored in 4±7°C in dark for 24 h. After 24 h, the roots were rinsed with distilled water. Within the test the possibility of indirect microscopic analysis was used. For this purpose, the roots were fixed in a 70% ethyl alcohol solution (PPH Stanlab sp. J.). Samples for analysis were stored in a refrigerator (4°C). In order to prepare microscopic preparations, the roots were washed in dH₂O and subjected to hydrolysis in 1N hydrochloric acid solution (POCH S. A.), at a temperature of 55±60°C for a time of 5-7 minutes. After hydrolysis the roots were washed in dH₂O and placed on microscope slides. 1.0±1.5 mm meristem was cut, stained with a 2% orcein solution (2% orcein in 45% glacial acetic acid) and left for 2±5min (dye penetration time). A cover slip was applied. Microscopic observation was carried out at 1000x magnification (10 × 100) using a Motic light microscope. Pictures of the cells were taken with the MOTICam 100 camera and developed in the Motic Images program. For each of the test concentrations and controls, the test was performed in 5 replicates. In each replication, microscopic observations were made for 1000 cells (1 series = 5000 cells analyzed / concentration). Based on the results of microscopic analysis, the following parameters were assessed: the value of the mitotic index (MI), the frequency of chromosome aberrations in cells during mitotic division (CA), as well as the incidence of micronucleus aberrations in the observed cells (MCN).

Scoring the mitotic indexd (MI)

Five slides for each wastewater concentration and control were prepared. Nearly 5000 cells (1000 cells per slide) were subjected to microscopic analysis to determine the mitotic index (MI). The MI value was calculated as the quotient of a total number of dividing cells to total number of cells examined.

Scoring the micronucleus (MCN) and chromosomal aberrations (CA) frequency

About 1000 meristematic cells were scored for each slide. The MCN frequency was calculated from the number of cells with scored MCN aberrations divided by the total cells scored for slide and expressed in terms of MCN/1000 cells. Adequately, the CA

were analyzed by microscope observing about 400 cells at the stage of mitosis per slide. During the analyses a different types of the aberration cells were observed and recorded.

Statistical analysis

Statistical analysis was performed using MS EXcel software. The obtained results are expressed as an average value and standard errors.

Results & Discussion

MI is considered as an indicator that allows to estimate the frequency of cellular division. The results of the analysis (Table 3) show that textile wastewater induced mitotic delay and decrease MI frequency in meristematic root cell of *V. faba*. The MI index was significantly different for all analyzed samples, however in all test series a reliable and reproducible results were obtained. Compared to NC, MI frequencies decreased with increasing wastewater concentration. Textile wastewater affected the inhibition of the cell division from 13.67% to 54.85%. The 54.85% reduction of MI versus NC were detected in the *V. faba* root tips after treatment with the highest wastewater concentration (12.50%). Similar results were obtained by Giorgetti, which evaluates the genotoxicity of raw effluents from textile industry. Estimated MI value was heavily reduced from 19.58% in the NC to 3.82% for raw effluent. For undiluted textile wastewater the mitosis activity inhibition was 80.49% (Giorgetti et al., 2011).

Table 3. Value of mitotic index (MI) in root tip cells of *V. faba* treated with textile wastewater in various concentrations

Wastewater concentration	Mitotix index per 1000 <i>Vicia faba</i> cells in 5 root tips [%]							
	Series	1	2	3	4	5	Mean	±SE
12.5%		2.13	1.45	0.74	2.56	2.09	1.79	±0.71
6.25%		7.05	8.14	8.62	6.99	6.2	7.40	±0.97
3.125%		8.3	7.12	8.34	9.76	8.98	8.45	±0.99
1.56%		10.15	10.78	11.14	15.12	11.23	11.68	±1.97
0.78%		13.56	15.24	14.98	10.77	16.2	14.15	±2.11
NC		14.23	16.53	18.34	15.23	17.65	16.39	±1.69
PC		1.89	1.76	0.96	1.45	1.15	1.44	±0.39

The determined values of MI confirms the results obtained in growth inhibition test. Root growth reflects the toxicity in the elongation plant zone, as root growth inhibition is caused by the occurrence of chromosol aberration and a reduction in the root length of *V. faba* exposed to textile wastewater (Bhat et al., 2017). Both test results (growth inhibition, micronucleus test) confirm that textile wastewater in concentration above 12.50% are highly toxic and affect significant growth inhibition, which exceeds the 80% for wastewater concentration above 50%. Maximum inhibition of cell division noted in the micronucleus test directly coincides to a EC₅₀ (15.45%) estimated in growth inhibition test for meristematic part of *V. faba* (Table 2). A comparable impact level of textile wastewater on plant cells has been shown by Samuel (2010). Estimated EC₅₀ value of textile industry effluent for rhizosphere part of *Allim cepa* amounted 16% after 96 h of exposition.

The genotoxic effects expressed as the number of micronuclei (MCN) induced in *V. faba* root cells are given in *Table 4*. The reference point in the text is the PC that induced a significantly high number of micronuclei in root tips of *V. faba* (24.42‰). The number of micronuclei induced by textile wastewater was lower according to PC (twice lower) and simultaneously much higher in reference to NC (43 times higher). Conducted studies proves that textile wastewater, even at low concentrations, causes damage to the genetic material of tested plants. The number of micronuclei identified in root cells exposed to wastewater sample at the lowest concentration reach 1.58‰ (wastewater concentration 0.78%) and increased with increasing wastewater concentration up to value 10.44‰ (wastewater concentration 12.50%). Examples of micronucleus aberrations observed in *V. faba* meristematic cells are presented in *Figure 1*.

Table 4. MCN frequencies in root tip cells of *V. faba* treated with textile wastewater in individual concentrations

Wastewater concentration	MCN per 1000 <i>Vicia faba</i> cells in 5 root tips [‰]					Mean	
	1	2	3	4	5	Mean	±SE
Series							
12.5%	11.89	10.98	9.77	11.02	8.56	10.44	±1.29
6.25%	6.54	9.28	7.13	7.25	6.14	7.27	±1.21
3.125%	4.56	10.56	5.12	4.16	6.55	6.19	±2.61
1.56%	2.94	3.15	3.89	2.06	3.85	3.18	±0.75
0.78%	1.03	0.99	1.45	2.43	2.01	1.58	±0.63
NC	0.78	0.00	0.00	0.12	0.32	0.24	±0.33
PC	24.32	21.34	19.87	26.75	29.84	24.42	±4.03

The study result shows a clastogenic effect, evidenced by the induction of CA at used concentration. During the micronucleus test the major CA were noted as fragments, laggards, vagrants and chromosomal bridges (*Figure 2*). The lowest CA frequency was observed to be 0.39% in control sample (NC). In turn the highest frequency of CA (18.78%) was found to be in 12.50% concentration of textile wastewater. A PC, whose genotoxicity has been confirmed, induced chromosomal aberrations at the level 3.52%. An increasing with wastewater concentration percentage of CA showed positive correlation with sample concentration (*Table 5*).

Additionally, the studies proved the usefulness of *V. faba* micronucleus test for assessing the genotoxic potential of such complex mixtures as textile wastewater. Similar conclusions based on research were formulated by Bhat (2017). Author indicate in his work that the *V. faba* (broad bean) bioassay is used extensively to evaluate toxic substances in the environment and is an excellent bioassay for toxicological observations. As an advantage of the method author indicates the high sensitivity and economic profitability of the test. Moreover, author marks the that broad bean cells are quite large which makes the chromosomes more visible and finally makes the chromosomal-aberration evaluation easier (Bhat et al., 2017). The study result confirm that plant test model based on the analysis of induced changes in meristematic cells of *V. faba* is effective and appropriate assay for detection of genotoxicity and cytotoxicity of environmental samples.

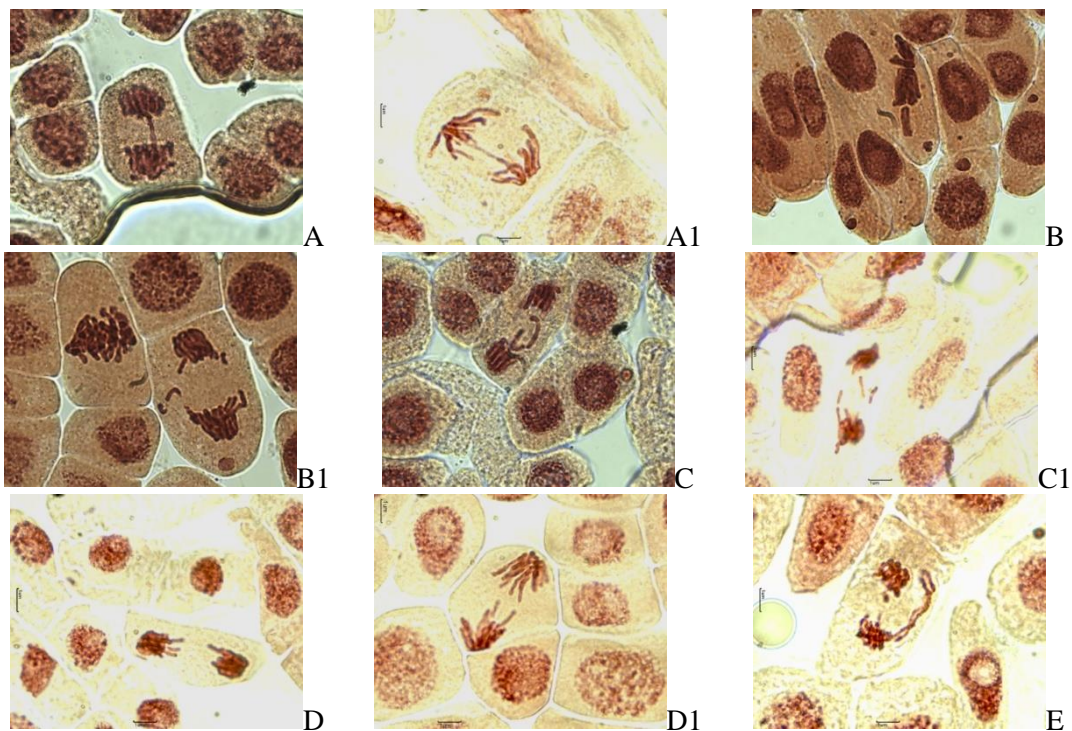


Figure 1. Examples of micronucleus and chromosomal aberrations observed in *Vicia faba* root cells after exposure to textile wastewater: ^{A,A1}chromosome bridge; ^{B,B1}fragments of chromosome; ^{C,C1}larger chromosome; ^{D,D1}vagrant chromosome; ^Emulti aberration

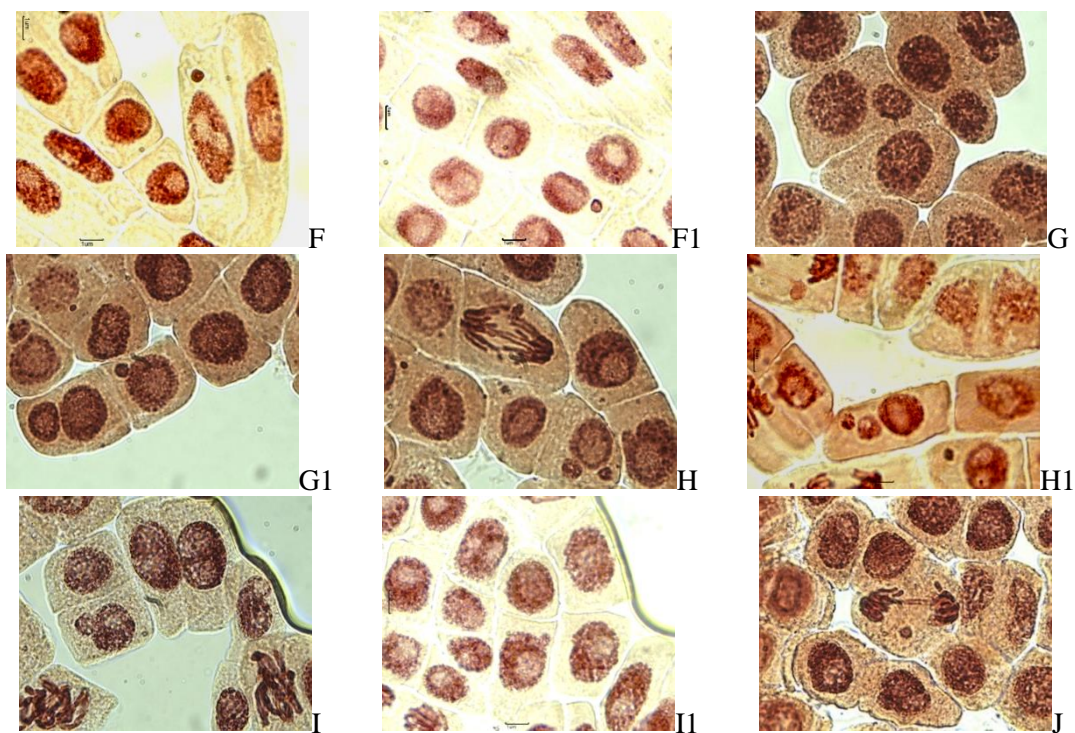


Figure 2. Examples of micronucleus aberration observed in *V.faba* root cells after exposure to textile wastewater: ^{F,F1}micronuclei; ^{G,G1}meganuclei; ^{H,H1}multinuclei; ^{I,I1}bud chromosome; ^Jmicronuclei in anaphase

Table 5. CA frequencies in root tip cells of *V. faba* treated with textile wastewater in individual concentrations

Wastewater concentration	CA per 400 <i>Vicia faba</i> cells in 5 root tips [%]							
	Series	1	2	3	4	5	Mean	±SE
12.5%		18.67	17.34	21.14	18.32	18.45	18.78	±1.41
6.25%		11.23	10.98	9.87	11.34	12.14	11.11	0.82
3.125%		7.85	7.64	8.14	6.14	7.33	7.42	±0.76
1.56%		3.87	4.1	2.88	3.95	4.13	3.79	±0.52
0.78%		2.65	1.89	2.54	2.32	1.99	2.28	±0.33
NC		0.65	0.45	0.00	0.87	0.00	0.39	±0.39
PC		3.45	4.21	2.98	3.21	3.75	3.52	±0.48

Conclusion

The results of the study showed toxic effect posed by untreated textile wastewater on the meristematic cell of *V. faba*. The test results demonstrated a positive correlation between the wastewater concentration and toxic effect expressed in the indicators form as MI, MCN frequencies and CA. Conducted research proves that textile wastewater have a genotoxic potential. The results imply that the wastewater could result in environmental contamination even at low concentration and exposure to wastewater might posed potential risk conducted cytogenetic damages induced in exposed organisms cells. Additionally, the study results imply that *V. faba* micronucleus assay as a sensitive and repeatable method can successfully use for testing cytotoxic and genotoxic risk for both, individual (chemical) substances, which have been already proved, as well as complex mixtures, such as textile wastewater which often forming a difficult to identify. Moreover, described method is cheaper than other techniques that may be used in genotoxicological studies (e.g. FISG, GISH or RT-PCR) and, what is particularly important, does not require sterile working conditions.

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