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Determination of Toxicity of Pulp-Mill Effluents by Using Allium Test

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Abstract: In order to evaluate the effect of wastewater treatment process on toxicity of Kraft mill effluent, wastewater samples prior (A) and after the treatment (B) was examined by using the modified Allium test. Treatment of *A. cepa* bulbs with wastewater samples has caused inhibition to root growth when compared with the controls. Growth inhibition values for wastewater samples, A and B, were determined 55.72 % and 48.33 %, respectively. Treatment of root tip cells with wastewater samples led to cytological abnormalities including c-mitosis, anaphase with laggard chromosome, fragment and bridges and chromosome stickness in root-tip cells. Percentages of the c-mitosis were 47.7% and 53.3% for root-tip cells treated with A and B samples, respectively. Taking the total aberrations into account, percentages of laggard chromosomal fragment and bridge formations were 26.6% for those treated with sample A and 33.3% with sample B. Chromosome stickiness were determined 12.2% and 10.6% for samples A and B, respectively. Banded chromosome was observed at the frequency of 13.3% of aberrant cells caused by sample A. Banded chromosome and multipolar cell formation were rarely observed in treatments with sample B.

Key Words: allium test, toxicity , water pollution, kraft-mill, wastewater treatment plant, mitosis

Kağıt Fabrikası Atık Sularının Toksik Etkisinin Allium Testi ile Belirlenmesi

Özet: Arıtım uygulamasının kağıt fabrikası artık sularının toksititesi üzerinde etkisi arıtım öncesi (A) ve arıtım sonrası atık suları (B) ile ve Allium testi kullanılarak çalışılmıştır. Allium cepa' nın atık su örnekleri ile muamele edilmesi kontrolleri ile karşılaştırıldığında kök büyümesinde inhibisyona neden olmuştur. Büyüme inhibisyon değerleri A ve B örnekleri için sırasıyla % 55.72 ve %48.33 olarak belirlenmiştir. Kök ucu hücrelerinin atık su örnekleri ile muamele edilmesi bu hücrelerde c-mitozu, laggard kromozom, fragment ve köprüler içeren anafaz, kromozom sıkışıklığı şeklinde sitolojik anomalilere neden olmuştur. A ve B örnekleri ile muamele edilen hücrelerde c-mitoz sırasıyla %47.7 ve %53.3 olarak tespit edilmiştir. Toplam kromozom anomaliliği dikkate alındığında laggard kromozomal fragmentler ve köprü formasyonu A örneği ile muamele edilenler için % 26.6 ve B örneği ile muamele edilenler için ise %33.3 olarak tespit edilmiştir. Kromozom yapışıklığı A ve B örnekleri için sırasıyla %12.2 ve %10.6 olarak belirlenmiştir. A örneğinin neden olduğu banded kromozom anomalisi hücrelerde %13.3 oranında gözlenmiştir. B örneği uygulamalarında banded kromozom ve multipolar hücre formasyonu çok düşük sıklıkta görülmüştür.

Anahtar Kelimeler: allium testi, toksisite, su kirliliği, kağıt fabrikası, arıtım tesisi atık suları, mitoz

Introduction

Today, the pulp and the paper industries preferably use kraft process in production of cellulose pulp from woody materials. Because of its residual lignin content, kraft pulp has brownish colour. Removal of residual lignin, which is known as bleaching process, is necessary to produce white paper products from the pulp. Treatment of kraft pulp with chlorine or chlorine compounds is a very common chemical bleaching process used by most of the pulp and the paper industries world-wide. However, considerable amounts of chlorinated lignin derivatives, formed during the application of chlorine-based bleaching process, are discharged into receiving waters through the splint bleaching liqueurs. In the environment, some of these compounds exhibit toxicity or mutagenic effects. Cytotoxic and cytomutagenic effects of the bleacher effluents have been proved by several investigators using various toxicity and mutagenicity test systems (Bjorseth 1981, Priha et al. 1985). Allium test is considered as most suitable among the plant test systems for toxicity monitoring and commonly used in many laboratories (Fiskesjö 1975, Fiskesjö 1985, Hoda and Sinha 1993, Fiskesjö 1988). This test system has basically been developed to evaluate the macroscopic (e.g. growth) as well as microscopic changes (e.g. in c-mitosis, chromosome breaks, mitotic index, mitotic abnormalities, anaphase bridge and chromosome stickness) occurring in the root tip cells of *A. cepa*.

The aim of this study was to determine the toxic effects of bleacher effluents of a kraft mill before and after traetment in the wastewater traetment plant using the modified allium test procedure.

Materials and Methods

Two types of wastewater samples, prior treatment (A) and after the traetment (B) waste water samples were provided from bleached-cellulose pulp from kraft mill nearby the province Afyon, Turkey. Parameters

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associated with the chemical and physical characteristics of the wastewater samples that are presented in Table 1 have been determined according to standard methods (APHA 1985).

Bulbs of equal size were selected from a population of a commercial variety of common onion, *Allium cepa* L. (2n=16), and series of onions were grown in each test sample. The most suitable weight of the bulbs selected was 10-12 g, with a diameter of about 2.0 cm. They were stored under dry conditions at 10 ^{0}C. Initially, the outer scales of the bulbs were removed, leaving the ring of root primordia intact. The bulbs were then placed directly in beher glasses (4 x 5.3 cm size) containing either i) only tap water (control), ii) sample A or iii) sample B. The experiment was carried out at +20 ^{0}C in a growth chamber under a 16/8 h day/night photoperiod. Water samples were refreshed at daily. Forty hours after application, ten slides were prepared for each treatment by using one root tip for each slide and approximately 1000 cells per slide were scored. The following days macroscopic observations were made and samples photographed. After 6 days of treatment, root length expressed as the mean length of the roots in a bundle was taken as a macroscopic parameter. Relative growth value was expressed as the mean % values of 10 measurements of the controls. The analysis of variance of mean root lengths was carried out and differences were determined according to Newman Keuls test.

Table 1. The results of physical and chemical analyses of wastewater samples collected from the pulp-mill, prior to (A) and after treatment (B)

Parameters	Sample A	Sample B
pH (20 ^{0}C)	9,36	7,86
Colour (pt-co)	383	550
Odour	intensive	intensive
NTU	125,95	50.5
Suspended matter (mg/L)	352,85	19,8
COD (mg l^{-1})	432	424
EC (mmho/cm)	1525	1583
CATIONS, ANIONS (mg/L)		
Sodium (Na)	227,2	236,4
Potassium (K)	24	29
Calcium (Ca)	72,77	80,05
Magnesium (Mg)	2,016	2,356
Iron (total)	0,126	0,083
Manganese (total)	< 0,2	< 0,2
Chromium (total)	< 0,4	< 0,4
Nickel (Ni)	< 0,2	< 0,2
Copper (Cu)	< 0,1	< 0,1
Zinc (Zn)	0,028	0,027
Lead (Pb)	< 0,5	< 0,5
Phosphate(PO_4)	0,66	0,2

NTU : turbitidy
COD : chemical oxygen demand
EC : electrical conductivity

Microscopical slides were prepared in accordance with the standard procedure for orcein staining of squashed material. The root tips were fixed in 1 volume acetic acid: 3 volume ethanol and then hydrolysed in 1N HCl at 50 ^{0}C for 5 min, and finally squashed in 2% orcein prepared with 45% acetic acid. Permanent slides were prepared by the use of alcohol evaporation and were mounted in Canada balsam (Gömürgen 1993). These slides were observed and photographed under a light microscope (Leitz-Wetzlar). The following microscopic parameters were calculated; i) *mitotic index* (MI), expressed as the ratio of the number of the dividing cells to the total number of cells, and ii) *characterisation of mitosis*, expressed as the number of cells at normal, metaphase, anaphase and telophase and early anaphase. Mitotic damage was determined by the observation of stickness (sticky chromosomes indicate highly toxic, usually not reversible effect - probably leading to cell death), c-mitosis (weak toxic effect may be reversible occurring after deformation of spindle structure), chromosome bridges and/or fragments (results from chromosome- and chromatid-breaks) and banded chromosomes (broken chromosomes, the chromosome segments were not dislocated laterally, suggesting a form of chromosome pattern with negatively "banded" areas along the chromosome arms).

For each concentration of treatment and control, 8000-12000 cells were analysed. X^2 test was used for statistical analysis. Differences at frequencies of cells with mitotic aberrations, compared to the controls, were evaluated statistically at p= 0.05.

Results and Discussion

The toxic effects of treated and untreated wastewater samples were determined through Allium test. The results of experiment are shown in Table 2. Some parameters such as the root length and growth, mitotic index, mitotic chromosome aberration following exposure to wastewater were compared with the control. The most important macroscopic parameter was the root length, both wastewater samples affected the root length negatively when compared to the controls. Root lengths determined as 55.72% and 48.33% of control in Samples A and B, respectively. The difference between the means of control and the samples was significant but not significant between samples A and B.

The cell cycle is a very complex process because it consists of a series of different reactions, each of which can be affected by an adverse environmental factor (Lopez Saez et al. 1966). Plant meristem can be affected by changes in temperature, water quality, irradiance and other biotic or physicochemical environmental stresses (Levitt 1972), involving changes in cell division and elongation (Yee and Rost 1982). Treating roots with samples A and B resulted in color changes in root cap cells, causing them to turn brownish. This may be regarded as an indirect effect of the wastewater. pH of sample A was 9.36 (Table 1) and this high pH might have caused biological damage to the root tip cells.

In comparison to the control, the mitotic index was reduced. Variations in the mitotic index were not significant. Chromosomal aberrations were more frequent in samples A and than the control cells. Differences between control and the samples were significant ($P<0.05$). On the other hand, differences between the mean frequencies of total aberrations in samples A and B were not significant (Table 2).

The treatment of the root-tip cells with wastewater samples for 40 h caused cytological abnormalities. In both wastewater samples, the most frequent aberrations were c-mitosis, anaphase bridges and fragments and sticky chromosomes from metaphase and anaphase groups (Fig 1.).

The reason for such an effect could be the fact that toxic substance in the liquid medium may disturb the division, causing relatively high number of aberrations (Vidacovic et al. 1993).

The analyses of the root-tip cells treated for 40 h have showed many c-mitosis, 43 c-mitosis out of 90 aberrations (47.7%) and 40 c-mitosis out of 75 aberrations (53.3%) in samples A and B, respectively. Anaphase with laggard chromosome fragment and bridges observed was 26.6% in sample A and 33.3% in sample B. The waste water samples also caused stickiness at 12.2% and 10.6% frequencies in sample A and B, respectively. 13.3% of total aberrant cells in sample A contained banded chromosomes. Banded chromosomes and multipolar cells were rarely found in sample B (Table 2). Similarly, it was reported that chromosome breaks are highly related to the mutagenic events in the cell (De Serres 1978).

The observed most frequent aberrations were laggard chromosomes and disturbed anaphase (Fig 1). Such effects could be caused by binding the toxic substances to microtubules that formed mitotic spindle (Bond 1986). Binding some substances to kinetochore (Brinkley et al. 1985) could also cause this effect. After these processes it was possible to observe aberrations in root-tip cells, like abnormal movements and distribution of chromosomes, whose ultimate effect could be aneuploidy (Liang and Brinkley 1985).

Fiskesjö (1985) reported that the macroscopic parameter seems to be the most sensitive. This result is actually more respected since growth restriction as a parameter is the total damage effects. Plant cells usually possess important enzymes necessary for the activation of certain mutagenes; this has been shown to be the case for *Allium* root tip cells. *Allium* root cells posses the important MFO-system (the mixed function oxydase). This ability to activate promutagens is shared by the *Allium* material and many other plant materials (Vig 1978). It was shown that both wastewater samples contained heavy metals (Table 1). In our samples, nickel and copper concentrations were <0.2 mg/L and < 0.1 mg/L, respectively and such concentrations may cause chromosomal aberrations. Copper is shown to be far more toxic than other metals such as manganese and sodium. It has been already demonstrated that a solution of specific concentration of heavy metals causes chromosomal and mitotic aberrations in root-tip cells (Fiskesjö 1988). For example, certain concentrations of copper, cadmium, nickel and manganese caused c-mitosis and chromosome stickness (Bond 1986). Fiskesjö (1988) found that copper causes aberrations in 0.0635 mg/L and Vidacovic et al. (1993) also reported similar effects at 0.50, and even lower than 0,0635 mg/L concentrations. The same worker found that nickel remarkably increased the frequency of c-mitosis and laggard chromosomes at concentrations higher than 0.5869 mg/L. Also in tested material, the similar aberrations were observed at lower concentrations (<0.05 mg/L nickel). Experiments have shown that the roots usually tolerate rather high concentrations of ions such as Cl^-, SO_2^-, NO_3^-, Na^+, K^+ occurring under normal conditions (Fiskesjö 1985). In our study, increases in chromosomal aberrations in both tests samples may be a result of high Cl concentration. This supports the earlier study of Vidakovic (1993).

As a result, it could be concluded that the significance of the differences between treated and control cells shows that mitotic index and chromosome aberrations is a reliable criteria for the measurement of toxicity. The differences between effluent of pulp-mill

Table 2. Morphological and cytological effects of waste water samples

Treatment	Root length	Mitotic index	N. of total cells	Mitosis	Microscopical effects (%)								
					Normal metaphase	Normal anaphase	Telophase	Early anaphase	Sticky chromo somes	c-mitosis	Bridges, fragments	Banded chromo somes	multipolar
Control	100	4,84	9331	450	60,2	22,9	11,6	5,3	0	0	0	0	0
Sample A	55,72	2,77	1268 3	352	31,3	21	14,5	7,7	3,1	12,2	6,8	3,4	0
Sample B	48,33	3,28	8735	282	26,6	31,9	4,2	10,6	2,8	14,2	8,9	0,4	0.4

Root length: Differences between control and the samples were significant but between samples A and B not significant at p= 0.05.
Mitotic index: Differences between control and the samples were not significant at p= 0.05.
Aberrations: Differences between control and the samples were significant but between samples A and B not significant at p= 0.05

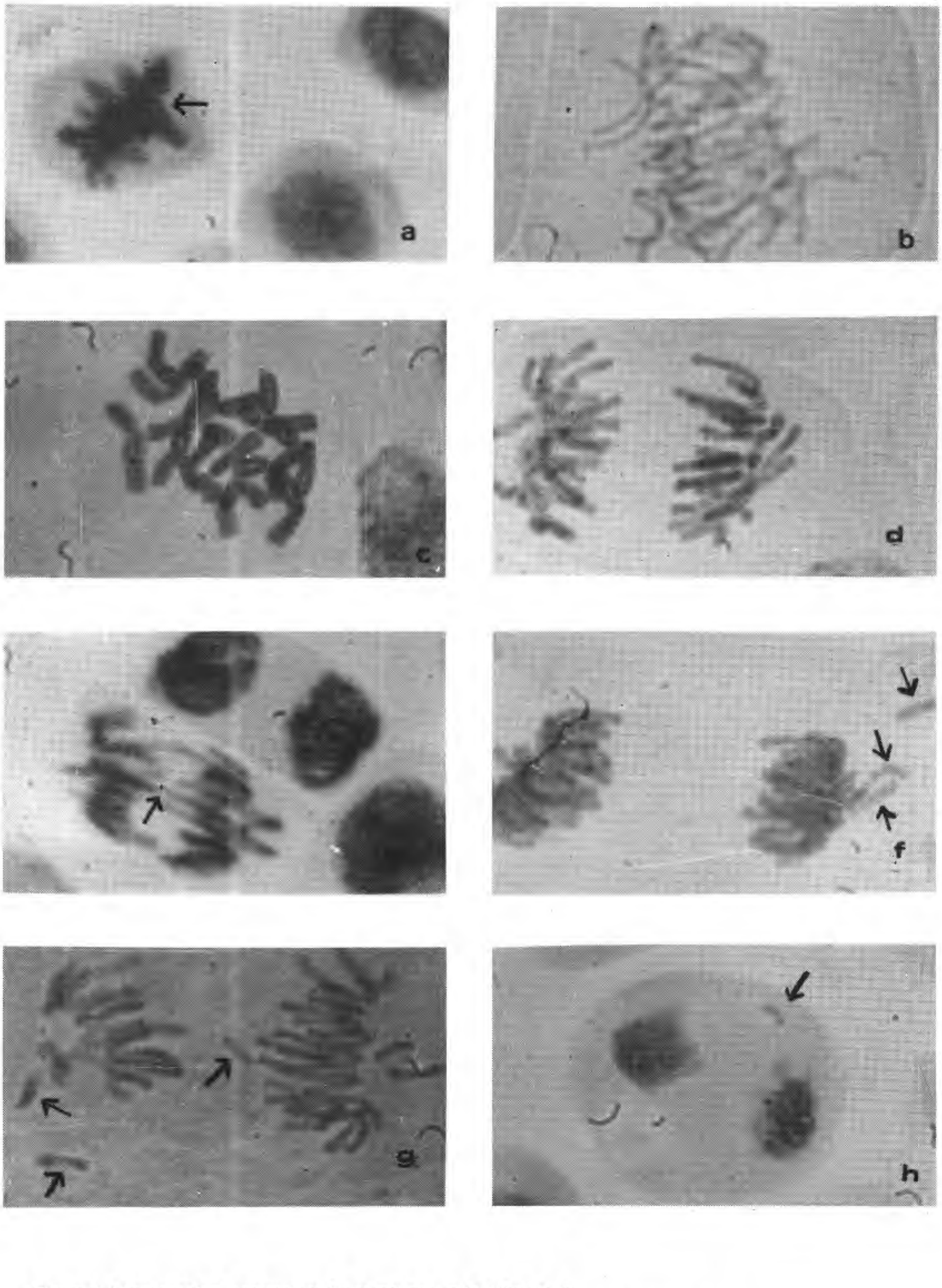

Figure 1. Microscopical parameters for standard use in Allium test,
(a) sticky metaphase, (b) early anaphase, (c) c-mitosis, (d) normal anaphase,
(e) bridges, (f) vagrant chromosomes (g) vagrant chrosomes and fragments,
(h) fragment in telophase

and effluent of wastewater treatment were at minimum. In addition, the *Allium* test which provide the rapid screening of wastewater of pulp mill toxicity combines two test targets; toxicity and mutagenicity. Toxicity is easily measured by observation of growth inhibition, and since mutagenicity is correlated to the rate of chromosome breaks, the risk of mutagenic events may be evaluated by the frequency of chromosome breaks induced by various treatments.

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