Evaluation of cytotoxicity and genotoxicity of the upstream Citarum River using *Allium cepa* assay

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Abstract. Citarum River is the longest and largest river in West Java, and its existence greatly influences the lives of surrounding communities. Many industries are built around the area. It is important to assess the quality of the water, because certain heavy metal might leak to the body of water. This research aimed to investigate the mitotic index (MI), root length, frequency and types of chromosomal aberration which determined the cytotoxicity and genotoxicity by using *Allium cepa* L. as biomarker. The Completely randomized design with seven treatments and four replications were used. Observation was done 96 hours after onion bulb soaked in water. Data was analysed using Analysis of Variance and continued with Duncan post-hoc. Results showed that root length was not affected. Water samples were affecting the MI, frequency, and types of chromosomal aberration. The highest number of chromosome aberrations was recorded on Dayeuh Kolot stations and the most common type of aberration was stickiness. The chromosome aberrations observed were; stickiness, chromosome loss, chromosome bridge, chromosome break, binucleated cells, multipolar, micronuclei, and c-mitosis. Based on the results, *Allium* assay is beneficial to evaluate the level of cytotoxicity and genotoxicity in the upstream Citarum River.

Introduction

The Citarum River faces serious problems related to pollution and a decrease in its environmental carrying capacity. The main sources of pollution are known to come from industrial and domestic activities [1]. The existence of manufacturing industries such as textiles, chemicals, paper, leather, metal/electroplating, pharmaceuticals, food and beverage products, and others can have a polluting impact on the Citarum river [2].

One of the pollutants found in the Citarum river is heavy metals [3]. Heavy metals can cause health problems and are dangerous to human health [4]. Heavy metals can induce genetic changes that can be inherited in subsequent generations and thus can affect future generations [5]. Heavy metals are elements that cannot be decomposed (persistent) and can accumulate through the food chain (bioaccumulation), with long-term detrimental effects on living things [6]. At low concentrations, heavy metals can disrupt physiological or metabolic processes and cause damage to animal organs [7-8].

The standard and sensitive *Allium* assay method has been widely used to test drinking water quality and water pollution. This method has been used for over forty years. Mainly used for testing water pollution such as rivers, rain and snow, soil, wastewater monitoring, atrazine pesticides, benzo(a)pyrene, pharmaceutical waste, hospital discharge, and radioactive waste [9]. In this research, *Allium cepa* L. was used as a bioindicator to observe the effect of water samples from the upstream of the Citarum River on cell and chromosome division. Research regarding cytotoxicity and genotoxicity testing using this type of assay has not yet been carried out for this river. This research is important to accomplish scientific information about cytotoxicity and genotoxicity and to determine the level of danger of cytotoxic and genotoxic compounds in the upstream Citarum River.

2 Research Method

This study was carried out on July 10, 2018 at 08:00 a.m until 17:00 p.m. in sunny conditions (no rain). The research method used was an experimental method using a completely randomized design (CRD) and descriptive. The preparation of *A. cep*a root tips were based on a technique performed by [10].

Cytotoxicity was assessed by recording the mitotic index (MI) at each mitotic phase obtained from counting a total of 1000 cells and measuring the length of *A. cepa* roots after 96 hours of immersion. Genotoxicity was evaluated by counting the number and type of chromosomes aberrations in 100 dividing cells with a microscope magnification of 400x [11].

Living systems are put at risk of death if the MI rate drops below 22% of the control. A reduction of less than 50% is considered to have a sublethal effect and is known as the genotoxicity limit value [12-13].

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A positive control used a material that usually induces a high level of toxicity, it is necessary to compare the test response to the tested sample. Samples whose test results were closer to negative control had a better water quality. The positive control used was Na azide (NaN_3) with a concentration of 0.3 ppm. The negative control used was distilled water [9]. The statistical analysis used was ANOVA with Duncan's post hoc test ($\alpha = 0.05$) if the ANOVA analysis was significant.

2.1 Water Sampling

The method used a survey method to determine the sampling location which was considered representative and based on a study conducted by [14]. Water samples were taken at five different places, i.e., Situ Cisanti, Majalaya, Cikeruh, Dayeuh Kolot and Batujajar. Water sampling referred to the SNI 6989.57: 2008 method (Indonesian National Standard).

2.2 Preparation of Onion Bulbs

Onion bulbs (*A. cepa*) (2n=2x=16) weighing ± 3-5 g and measuring ± 3-4 cm in diameter were purchased from the Jatinangor traditional market. The root tubers of *A. cepa* were cut and then soaked in a test water sample until new roots grew. The tubers were placed by piercing the bottom of the tuber using a toothpick as a support and then placed on top of a container containing distilled water as a negative control, 0.3 ppm Na azide (NaN_3) solution as a positive control and Citarum river water samples with the bottom part tubers submerged.

2.3 Measurement of Root Length After Soaking 96 Hours

Root length measurements were taken before cutting the roots for squash. Roots were measured in length using a ruler and recorded as well as observing changes. The roots that have been measured were then made the average length of the observed root length of the observed onion tubers. The 96-hour soaking was used to make sure all the *A. cepa* bulbs had enough exposure to river water samples, negative and positive controls [15].

2.4 Preparation of the roots of *A. cepa*

The squash method was used to make *A. cepa* root slide preparations. Harvesting the tips of onion roots was carried out at 08.00-09.00 WIB because it was considered that this time was the optimum time for cleavage [16].

The root tips of *A. cepa* were prepared based on the technique performed by [10] with the first preparation procedure, the roots were cut about 1-2 mm from the root tips. The cut roots of each seed were placed in a different glass vial. After that root tips were fixed with Mc. Clintock solution of for 24 hours. Then, the root tips were hydrolysed in 1 N HCL solution for 15 minutes. After hydrolysis, the roots were rinsed with distilled water, then fixed again with Carnoy fixative solution for 20 minutes. After fixation, the root tips were given 2% aceto-carmine dye and left for 10-15 minutes. After that, the root tips were transferred to a glass slide and then dripped again with 1-2 drops of aceto-carmine. Then covered with a cover glass, and squashed using the thumb and observed under a light microscope with a magnification of 400x.

2.5 Cytotoxicity Parameters

Cytotoxicity was observed through two parameters: average root length (ARL) after 96 hours of immersion and mitotic index (MI). Calculation of root length and MI was carried out in the following way:

$$MI = \frac{\text{Prophase+Metaphase+Anaphase+Telophaase}}{\text{Total Number of Cells Observed}} \quad (1)$$

$$ARL\ (cm) = \frac{Total\ Observed\ Root\ Length}{Total\ Number\ of\ Roots\ Observed} \quad (2)$$

2.6 Calculation for Chromosomal Aberrations

Calculation of chromosomal aberrations is carried out by looking directly at a microscope using a counter. Frequency of chromosomal aberrations (FCA) were calculated for 100 dividing cells for each treatment using the following formula [11]:

$$FCA\ (\%) = \frac{\text{Total Observed Chromosomal Aberrations}}{\text{100 cells divide}} \quad (3)$$

2.7 Data Analysis

Data analysis was carried out by comparing root length parameters, MI values for cytotoxicity, type and frequency of chromosomal aberrations for genotoxicity that occurred, analysed using ANOVA ($\alpha = 0.05$) using the IBM SPSS statistics 20 program. The further test carried out was the Duncan test if F count < F table. Further tests were carried out to see the significance of the differences or effects in each treatment.

3. Results and Discussions

3.1 Heavy metal

The results of measurements of heavy metal content of Cr^{6+}, Cd, Pb and Hg in the upstream water of the Citarum river are showed in Table 3.1 We compared the results with Indonesian Government Regulations (GR) quality standard. No. 22 of 2021 about river water quality standards that can be used for drinking water. Heavy metal measurement carried out using Atomic Absorption Spectrometry (AAS).

Table 3.1 Result of Heavy Metal Analysis in The Upstream of the Citarum River

No	Station	Heavy Metal Concentration (ppm)			
		Cr^{6+}	Cd	Pb	Hg
1	Situ Cisanti	<0.021	0.002	<0.05	<0.0 0006
2	Majalaya	<0.021	nd	<0.05	<0.0 0006
3	Cikeruh	0.027	nd	0.09*	0.000 6
4	Dayeuh Kolot	<0.021	nd	<0.05	<0.0 0006
5	Batujajar	0.025	0.003	<0.05	<0.0 0006
Quality standards (ppm)		0.05	0.01	0.03	0.001

Notes:

nd : Not detected (levels below the AAS detection limit)
(*) : Metal content in river water exceeded quality standards.

The Quality standards based on GR. No. 22 of 2021 concerning river water whose designation as raw material for drinking water. The limit of detection is: AAS Cr^{6+} = 0.05 ppm; Pb = 0.03 ppm; Hg = 0.001 ppm; Cd = 0.01 ppm.

Pb metal content that exceeds quality standards found at Cikeruh station has exceeded quality standards. While Pb metal content at other stations was detected at low levels. The levels of Cd metal in several stations were not detected and some were still below the quality standard. Cr^{6+} and Hg metals were detected at all stations at levels below the quality standard.

Heavy metals are carcinogenic compounds which are indirectly genotoxic agents, especially at low levels [17]. Carcinogenic metal compounds are often also co-mutagenic. This compound increases the mutagen levels of other genotoxic agents. Carcinogenic metal compounds at low concentrations can be inhibitors of the repair of DNA damage caused either by xenobiotics or by other endogenous factors. Continuous inhibition of DNA repair and damage can cause genome instability, which can disrupt the mechanisms of accelerated cell proliferation and/or disrupt apoptosis [18].

3.2 Cytotoxicity Assessment

The results of the upstream Citarum River samples and control on onion root length growth can be seen in table 3.2.

Table 3.2. Average Root Length (ARL)

No	Station	ARL±SE
1	Negative control	1.66±0.16
2	Positive control	2.35±0.24
3	Situ Cisanti	2.61±0.12
4	Majalaya	2.34±0.29
5	Cikeruh	2.77±0.53
6	Dayeuh Kolot	1.67±0.49
7	Batujajar	2.38±0.50

The results of the ANOVA analysis with a confidence level of 95% showed that providing various water samples from the upstream Citarum River did not have a real effect on the root growth of *A. cepa* because the calculated F value was smaller than the F table. Based on the results obtained, the average root length at the Situ Cisanti, Majalaya, Cikeruh and Batujajar stations was close to the average root length of the positive control.
The average root length of the positive control was around 2.35 cm. The average length of these roots was relatively the same as the Situ Cisanti, Majalaya, Cikeruh and Batujajar stations. This average root length is thought to be the impact of toxic agents that cause abnormal root length growth. The Na azide compound is a compound that is highly toxic and capable of inducing genetic damage. The Na azide compound is mutagenic in bacteria, higher plants and human cells. This compound has been used as a positive control for *Allium* assay [19]. The highest average length of onion roots was at Cikeruh station. Based on the results of heavy metal analysis, the Cikeruh station has Pb metal levels that exceed the threshold of 0.09 ppm from the quality standard which should be around 0.05 ppm. Pb metal can disrupt the normal cell cycle which causes a decrease in the number of dividing cells [20]. Based on the results obtained, the root length at Cikeruh station is the longest compared to other stations.

[11] conducted research on cytotoxicity in the Pitimbu River, Brazil using the *Allium* assay method. The results of the research stated that the water samples that had the highest impact on cell damage were found in water samples near industrial areas. This is proven by the ability to induce a decrease in the average root length. The average root length obtained was 2.3cm compared to the negative control of 4.4 cm.

Based on the results of the Duncan Multiple Distance Test, it can be seen that the Majalaya, Cikeruh and Batujajar water samples had the same effect as the positive control on the MI parameters. Situ Cisanti and Dayeuh Kolot water samples had the same effect as the negative control, although it was not significant. The Situ Cisanti water sample had a MI value of 5.95% and the Dayeuh Kolot water sample was 6.78%. The MI value of the two samples was close to the negative control mitotic index value of 8.15%.

Table 3.3. Mitotic Index

Treatment	P	M	A	T	I	MI
Negative Control	29.5 ± 3.28	17.5 ± 1.44	10.5 ± 2.60	24.00 ± 4.02	918.5 ± 9.95	8,15 ± 1,00 (A)
Positive Control	16.75 ± 2.87	8.75 ± 2.46	6.25 ± 1.93	10.75 ± 2.29	957.5 ± 8.59	4,25 ± 0,86 (B)
Situ Cisanti	27.25 ± 3.09	16.25 ± 3.17	6.75 ± 1.11	9.25 ± 1.60	940.5 ± 5.24	5,95 ± 0,52 (AB)
Majalaya	21.25 ± 4.55	7.5 ± 2.33	5.00 ± 1.29	8.75 ± 3.15	957.5 ± 10.70	4,25 ± 1,07 (B)
Cikeruh	19.25 ± 4.99	13.25 ± 1.25	6.75 ± 0.63	10.25 ± 2.56	950.00 ± 7.38	5,00 ± 0,74 (B)
Dayeuh Kolot	23.5 ± 4.66	19.25 ± 5.12	9.75 ± 0.75	15.25 ± 3.90	932.25 ± 9.38	6,78 ± 0,94 (AB)
Batujajar	24.5 ± 5.48	9.25 ± 2.02	6.50 ± 1.32	10.50 ± 1.26	949.25 ± 5.65	5,08 ± 0,56 (B)

Notes: P = Prophase; M = Metaphase; A = Anaphase; T= Telophase; I = Interphase; MI= Mitotic Index.

The decrease in the mitotic index in onion root meristem tissue can be used to determine the level of cytotoxic pollution in the environment as an evaluation of the level of pollution in water. This parameter is also sensitive enough to be used for monitoring pollution levels in lightly polluted water [21-22].

Based on the results, the MI value in the Situ Cisanti water sample compared to the negative control MI value was 63%, the Majalaya water sample was 52%, the Cikeruh water sample was 61%, the Dayeh Kolot water sample was 83% and the Batujajar water sample was 62%. This value showed that of all the samples tested, none was sublethal because the value was still above 50%. This shows that the Citarum River water sample did not cause death.

The decrease in mitotic index values could be caused by increasing levels of heavy metals [23, 20]. However, only Pb at Cikeruh station heavy metals were detected through which exceeded the quality standards of GR No. 22 of 2021. Pb metal can disrupt the normal cell cycle which causes a decrease in the number of dividing cells. This is caused by inhibition of DNA synthesis or a block in the G2 phase which prevents cells from entering the mitotic phase. This in line with the results obtained because many interphase phases were found. The mitotic phase is stuck in the resting phase due to interference from the heavy metal Pb [20].

3.3 Genotoxicity Assessment

Genotoxicity was observed the frequency and type of chromosomal aberrations. The results of observations of chromosomal aberrations as suggested in table 3.4.

Table 3.4. Frequency and Types of Chromosomal Aberrations

	Treatment						
Type of Abberation	NC	PC	SC	M	C	DK	B
C-Mitosis	4	5	5	6	10	9	19
Loss	9	51	9	22	52	70	49
Binucleated	0	0	0	0	0	5	3
Stickiness	11	50	16	18	38	45	59
Bridge	4	33	4	12	22	15	11
Breaks	1	14	0	12	9	12	7
Others	1	3	0	2	2	5	2
Total Normal Cells	366	244	370	328	267	239	250
Total Cell Aberration	34	156	30	72	133	161	150
Frequency (%)	8.5 (A)	39.0 (B)	7.5 (A)	18.0 (AB)	33.3 (B)	40.3 (B)	37.5 (B)

Notes : NC=Negative Control ; PC = Positive Control ; SC= Situ Cisanti ; M = Majalaya ; C = Cikeruh ; DK = Dayeuh Kolot ; B = Batujajar ; ToA = Types of Aberrations.

Based on the results of the frequency of chromosomal aberrations of Citarum River Upstream water samples, it was found that the highest frequency of aberrations found was chromosome loss of 202 cells from all stations observed. Then, stickiness was 176 cells, chromosome bridge was 64 cells, C-mitosis was 49 cells, chromosome breaks were 40 cells, binucleate cells were 8 cells, and others were 11 cells.

Chromosome aberrations such as chromosome breaks and chromosome bridges are an indication of the activity of clastogenic agents. Chromosome loss, chromosome delays, stickiness, multipolarity and c-mitosis are indications of an aneugenic effect [24, 20].

Aneugenic agents are agents capables of inducing aneuploidy. Clastogenic agents can cause or encourage disruption and breakage of chromosomes, and can cause deletions, duplications and translocations of chromosomes. The activity of this clastogenic agent can be carcinogenic, leading to the formation of cancer cells [25].

This indicates that the chromosomal aberrations observed in the upstream Citarum River water were the impact of an aneugenic agent. Because many chromosome losses, chromosome delays, stickiness, and c-mitosis were found during observations. However, the aneugenic agent found in the upstream Citarum River were not yet been identified with certainty which compound acts as an aneugenic agent. Herbicide might be a source of aneugenic. Research by [25], regarding the genotoxic effects of the herbicides glyphosate and 2,4-dichlorophenoxyacetic acid was done using the *Allium* assay method. The results of this study showes that all the compounds tested were able to induce chromosomal aberrations in *A. cepa* onion root cells.

3.4 Type of Chromosomal Aberrations

Based on the observations, water samples from the Citarum River are known to be able to induce aberrations in the *A. cepa* onion root chromosome. The types of chromosomal aberrations observed in the study can be seen in Figure 3.1.

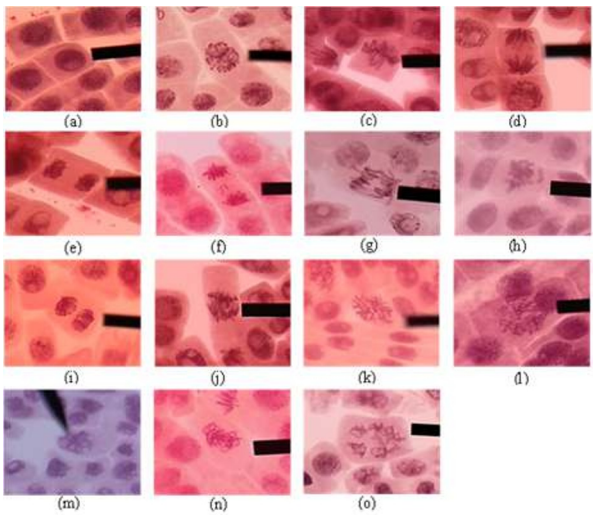

Fig. 3.1 Normal and aberration chromosomes *Allium cepa* L.

Notes: (a) Normal Interphase, (b) Normal Prophase, (c) Normal Metaphase, (d) Normal Anaphase, (e) Normal Telophase, (f) Metaphase *Chromosome break* (g) Anaphase *Chromosome bridge*, (h) Metaphase *Chromosome loss*, (i) Binukleat cell, (j) Anaphase *Sticky*, (k) C-mitosis, (l) Premature chromosome condensation (m) nuclear disintegration (n) Sticky metaphase (o) *Irregular* Prophase. Microscope magnification 400x.

Several plant species have been used as bioassays to evaluate environmental pollution. Some of them are *A. cepa, V. faba, Z. mays, Tradescantia, N. tabacum, C. capillaris* and *H. vulgare*. Among these species, *A. cepa* is considered as a species that has advantages for assessing chromosome damage and disturbances in the mitotic cycle. The *Allium* assay shows a high level of sensitivity in detecting chemicals in the environment [24].

There are advantages of doing *Allium* assay i.e. due to *Allium* large chromosomes, it is easily to observed using a light microscope; in addition to its lengthy history of use as a cytotoxicological test, this assay may disclose an effect even at relatively low levels of contact between the tested material and the genetic material [26-27]. For higher plants, the *Allium* test is a very sensitive, dependable, quick, affordable, and straightforward test that is frequently used to find pesticides, genotoxins, and mutagens in the environment [28]. In order to do this assay, researchers who do the chromosome analysis must train themselves well to recognize different types of aberration.

This study has shown that *Allium* assay is a promising method to assess the quality of Citarum Rivers, along with other assays. Genetics method has been used in many other places to study of cytotoxicity and genotoxicity in river as well as other water ecosystems.

4 Conclusion

Based on the results it can be concluded that cytotoxicity in upstream Citarum River was not in sublethal category. However, the genotoxicity is high based on chromosomes aberration parameters. The present study provides compelling scientific evidence in favour of the potential inclusion of the *A. cepa* bioassay test system as an easy-to-use and cost-effective supplementary tool in effluent management regulations, particularly in Indonesia. This system would allow for the quick screening of complex industrial effluents discharged into public water supplies, while taking ecological and public health into consideration.

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