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Impact of anthropic activities on eukaryotic cells in cytotoxic test

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ABSTRACT

Numerous pollution factors generate changes in water quality and in organisms that depend on this resource, as they may contain substances with mutagenic, genotoxic and cytotoxic properties that affect the genetic integrity of organisms. The Clarimundo Stream is located in the city of Cerro Largo, Rio Grande do Sul, Brazil, from which it passes through an agricultural area and crosses the city receiving discharges of untreated household waste. This work evaluated the genotoxic potential of these waters through a bioassay with *Allium cepa*, usually used in biomonitoring studies. Four water collections were conducted in each season and three points were marked for the analyses. The *Allium cepa* bulbs were germinated for 72 hours, in the proportion of three for each sample of collection points and three in distilled water for control parameters. Subsequently, the roots were submitted to the Feulgen procedure with modifications, for the preparation of the slides. The data were submitted to statistical analysis by the Tukey test with reliability of 5%. The results showed chromosomal alterations and Mitotic Index values suggesting that the water of Clarimundo Stream has the potential to cause damage to the genome of eukaryotic organisms.

Keywords: *allium cepa*, environmental toxicity, mutagenicity.

Impacto de atividades antrópicas sobre células eucarióticas em teste citotóxico

RESUMO

Inúmeros fatores de poluição geram alterações na qualidade da água e nos organismos que dela dependem, visto que podem conter substâncias com propriedades mutagênicas, genotóxicas e citotóxicas que afetam a integridade genética dos organismos. No município de Cerro Largo – RS localiza-se o Arroio Clarimundo, que no seu fluxo passa por uma área agrícola e atravessa a cidade recebendo descarga de resíduos domésticos não tratados. Este trabalho teve como objetivo, avaliar o potencial genotóxico dessas águas por meio do bioensaio com *Allium cepa* usualmente empregado em estudos de biomonitoramento. Para a realização das análises foram feitas quatro coletas de água, em cada estação do ano e em três pontos demarcados. Os bulbos de *Allium cepa* foram postos para germinar, durante 72 horas, na proporção de três para cada amostra dos pontos de coleta e três em água destilada para parâmetro de controle. Posteriormente, as raízes foram submetidas ao protocolo de Feulgen com modificações, para a confecção das lâminas. Foram observadas alterações nos valores de Índice Mitótico e Alterações Cromossômicas. Os dados obtidos foram submetidos a análise estatística pelo teste de Tukey com confiabilidade de 5%. Os resultados sugerem que a água do Arroio Clarimundo tem potencial para causar danos no genoma de organismos eucariotos.

Palavras-chave: *alium cepa*, mutagenicidade, toxicidade ambiental.

1. INTRODUCTION

Water courses are of great importance for ecosystems; they serve as a source of water and food for various vertebrate species, supply underground reservoirs that supply water for human consumption, and serve as habitats for various species of plants, vertebrates, micro -organisms and macro invertebrates (Peron et al., 2009).

Contamination of watercourses is caused by anthropogenic actions, which usually originate from agriculture, industrial and domestic waste not properly treated. All these factors of contamination generate changes in water quality, affecting organisms that depend on it, as they may contain substances with mutagenic, genotoxic and cytotoxic properties that affect the genetic integrity of living beings. Therefore, according to Fox (1995), these pollutants not only directly and indirectly affect individual organisms in toxicological terms, but also have effects on communities and ecosystems.

Since the 1970s, bioassays with higher plants have been recommended for the evaluation, monitoring and detection of genotoxic potential in the environment (Grant, 1999). Plant cells mostly have enzymes necessary for the activation and metabolization of promutagenic compounds and polycyclic aromatic hydrocarbons (PAHs), as is the case with the meristematic cells of the roots of *Allium cepa*.

Conforming to Arraes and Longhin (2012) the exclusive use of chemical and physical analyzes presents disadvantages in relation to the responses of the Bioindicators of environmental degradation, since these results show only the momentary record of the aquatic biota. Therefore, when carried out far from the polluting source and without correlation with living organisms, chemical measurements fail to detect subtle disturbances in the ecosystem (Buss et al., 2003).

The *Allium cepa* test is commonly accepted and used in laboratories around the world for the analysis of various substances and their possible mutagenic potential. This test evaluates the frequency of chromosomal aberrations and breaks indicating risks of aneuploidies and providing important information regarding the evaluation of environmental samples (Rank and Nielsen, 1993).

Many researchers have verified the mutagenic potential of water bodies through the *Allium cepa* test. Oliveira et al. (2011) analyzed the waters of the Paraíba do Sul-Tremembé-SP River, and Peron et al. (2009) analyzed the waters of the Rio Pirapó-Apucarana-Pr.

The Clarimundo Stream, chosen for this study, is located in the municipality of Cerro Largo - RS in Brazil, which at its source receives discharge of industrial waste. It then passes through an agricultural area crossing the city from one end to another, receiving directly the household waste of a large part of the population, since the small town does not have a waste treatment center. The region where this stream is located is predominantly of family agriculture that has as a common factor the use of many fertilizers and agrochemicals, which highlights the importance of environmental biomonitoring.

In this way, the present study proposed a qualitative analysis of the genotoxic potential of the water of the Clarimundo Stream, using the Allium cepa test to analyze the chromosomal alterations, the mitotic index. Finally, a statistical test was applied to analyze the data collected.

2. MATERIAL AND METHODS

The Clarimundo Stream is located in the small town of Cerro Largo, in the state of Rio Grande do Sul, Brazil (Figure 1), latitude 28°08'49" south, longitude 54°44'17" west and 211 meters above sea level. The stream suffers high pressure of anthropic factors, since at its head (P1) there are large agricultural areas with declivity towards the river, then in the mediations of the urban zone. There are also the presence of the food sector industries (P2), which discharge their previously treated waste in the river, but there is no information that these treatments are carried out properly. Moreover, in the urban perimeter (P3), the stream crosses the city receiving directly untreated domestic effluent, since the small town does not have a treatment center for sewage.

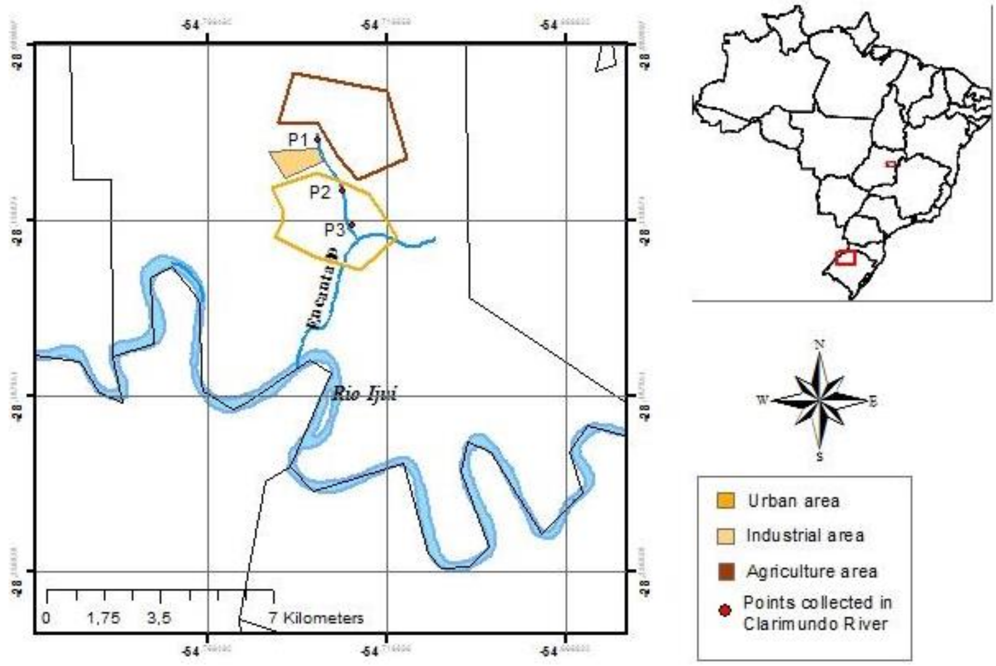

Figure 1. Location of the Clarimundo Stream with the three sample collection points.

The samples were collected in the four seasons of the year (summer, autumn, winter and spring) in three points of the Clarimundo Stream, determined as: (P1) source, before the arable areas; (P2) downstream from the city, just past the agricultural and industrial areas; (P3) upstream from the city, after crossing the urban area. The stream is characterized as having shallow water not exceeding 1.50 m in depth. The samples were collected with 2-liter PET bottles at a depth of 0.5 m. The water samples were taken to the Genetic Laboratory of the Federal University of the South Frontier - Campus Cerro Largo - RS (UFFS) and stored in refrigeration until the tests were conducted.

In this study, the *Allium cepa* test was used as an experimental model, which is able to indicate the presence of genotoxic agents in liquid through cellular and nuclear alterations. The *Allium cepa* bulbs were exposed to the water samples collected from each point and the control in distilled water for 72 hours, and then the roots were removed with the hood to a size of approximately 2 cm in length. The entire test series were performed in triplicate, which means that occurred three repetitions of each series in all the seasons.

The root meristems were collected and conditioned for a period of 24 hours in microtubes, Ependorf® type, containing Carnoy (3: etanol/1: acetic acid) fixative. Subsequently, the roots were washed in distilled water and stored in 70% alcohol under refrigeration until the slides were made.

Five slides (corresponding to five roots of each bulb) were made per sampling point in each test series. The protocol used for the production of the slides was the Feulgen with modifications, where the roots were subjected to acid hydrolysis with HCL 1N. Then, they were immersed in Schiff's reagent (Feulgen protocol), and washed in sulfur water, and finally discarding the smooth region of the roots, only the root cap was stained with acetic Orcein 2% and closed with the cover slip.

The study observed 1000 cells per slide in Optical Microscopy Olympus® CX31 counting mitotic divisions and chromosomal damage. The data were submitted to ANOVA statistical analysis, applying the Tukey test, using the Assistat software, in order to verify the relationship between factors, sample collection points and season of the year and if there were a significant difference between the control and points.

3. RESULTS AND DISCUSSION

According to Table 1, the pH of the samples collected at the collection points show few changes in the four seasons of the year.

Table 1. Ph value on three points collected in respective seasons of the year.

	P1	P2	P3
Winter	6.25	6.50	6.70
Summer	6.42	6.76	6.90
Autumn	6.25	6.50	6.70
Spring	6.07	6.23	6.49

Bremm and Mayer (2012), in studies at the same points evaluated here, show that Point 3 did not meet the standards recommended by the legislation in relation of the organic matter (2.95 mg / L); Dissolved oxygen (5.87 mg O2 / L); BOD (5.49 mg O2 / L); COD (19.66 mg O2 / L); (88000 NMP / 100mL) and total coliforms (160000 NMP / 100mL). These are indicative values of an impacted environment, which corroborates our results regarding the damages found in the cells.

The Mitotic Index (MI) (Table 2) was calculated according to the Equation 1:

$$MI = number\ of\ cells\ in\ division\ /\ total\ number\ of\ cells\ counted\ x\ 100 \qquad (1)$$

It can be observed that the MI of the treatments was lower than the obtained in the control. These results were repeated in the data collected in all other seasons.

Table 2. Values of the Mitotic Indexes with the respective seasons and sample collection points.

	Mitotic Index % (IM)			
	Spring	Summer	Autumn	Winter
P1	4.5	6.7	5.1	5.2
P2	5.5	5.5	5.3	5.4
P3	2.1	5.6	4.1	5.5
C	9.0	9.0	9.0	9.0

The result of the statistical test indicated that the factors, sample collection points (Table 2) and seasons (Table 3 and Table 4) did not interact significantly. Sampling points 1, 2 and 3 had a significant difference at the 0.01% probability level in relation to the control. Among the seasons, there was a significant difference at the 0.05% level, only between spring and winter, since summer and autumn did not differ significantly. We can infer that the statistical difference observed between the seasons is due to the adverse weather, where the respective seasons had low levels of rainfall precipitation, increasing the concentration of pollutants in the river.

Table 3. Averages of Factor 1 (sample collection point), followed by distinct letters in the column differ statistically from each other by the Tukey test at 0.01% probability level.

P1	49.50000	b
P2	47.25000	b
P3	42.16667	b
C	91.00000	a

Table 4. Averages of the Factor 2 (seasons of the year) followed by letters in the column differ statistically from each other by the Tukey test at 0.05% probability level.

Sp	50.66667	b
Su	60.50000	ab
A	56.08333	ab
W	62.66667	a

In the analysis of the slide cells were identified the presence of chromosomal damage when compared with normal cells in mitosis. The most frequent damages identified were stickiness, anaphase bridges, micronuclei, anaphase delay, malformed nuclei and c-mitoses (Figure 2 and Figure 3).

Data observed in the spring, summer, autumn and winter seasons, respectively, showed that the number of cells in cell division of the roots submitted to treatments were below the control samples. These data suggest that the water of the stream has components which interfere in the mitotic processes of the meristematic cells of the roots of Allium cepa. The records from Oliveira et al. (2011), who analyzed the waters of the Paraíba do Sul River, and also presented results similar found in this study.

Whether we compare the M.I. (Table 1) with the four seasonal seasons, we can observe that in the spring the number of cells in mitotic division was smaller than the other seasons. It is important to emphasize that at the period of collection there was still a dairy industry activity in the town localized near to P1. This suggests that products present in the collected water interfered in the cell cycle. Our evidences corroborate the data that also found in the work of Barberio et al. (2009).

The results obtained in the summer sample (Table 1) show higher M.I. in relation to the other stations. The variation found in the seasonality is a factor that can significantly influence the frequency of genetic damage and M.I., besides promoting physiological changes in exposed organisms. This variation may be due to changes caused by the level of pollution at a certain spot (Ruiz et al., 1992; Hayashi et al., 1998).

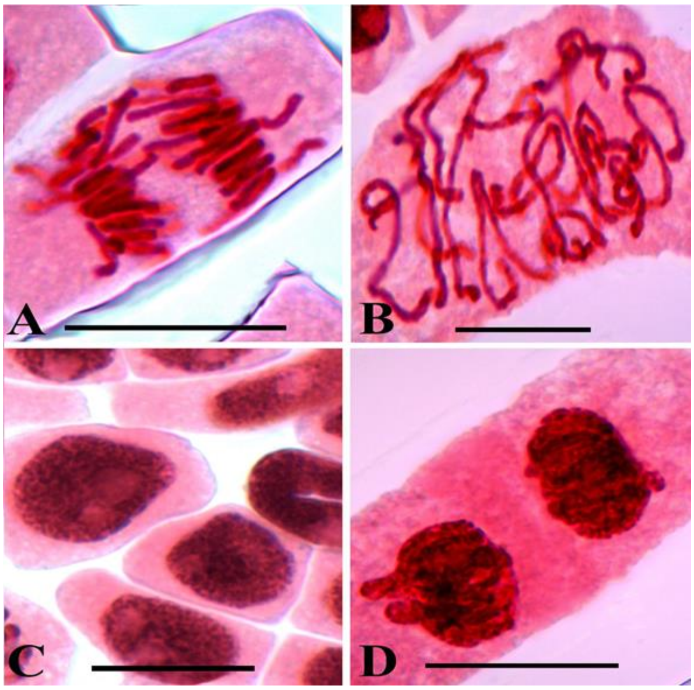

Figure 2. Chromosomal alterations found in the cells of roots submitted to the water from the Clarimundo Stream; A) anaphase bridges; B) c-mitosis; C) malformed nuclei; D) micronuclei. M.O 100X- scale bar 50µm.

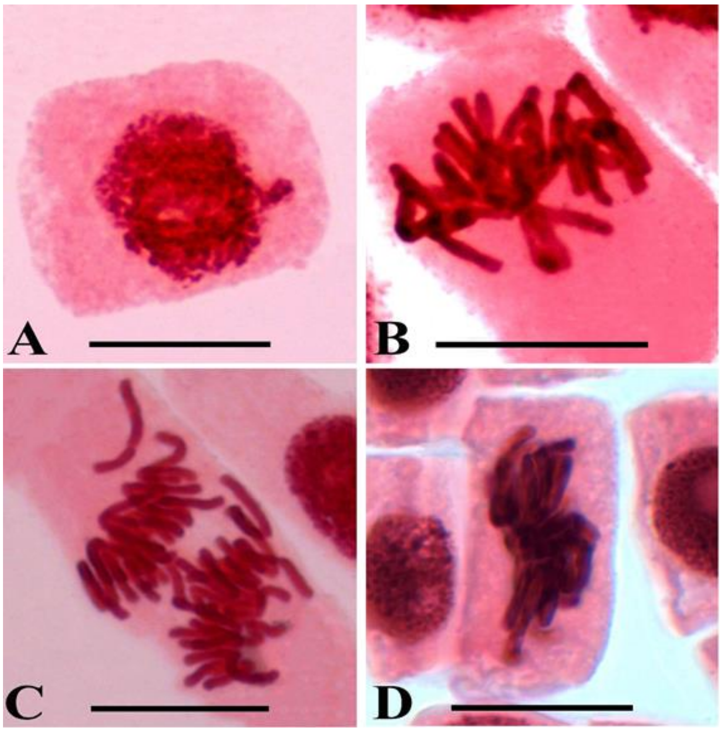

Figure 3. Chromosomal alterations found in the root cells submitted to the water from the Clarimundo Stream: A) micronucleus; B) irregular metaphase; C) loss of chromosomes; D) stickiness. M.O 100X- scale bar 50µm.

Agreeing with Cézar et al. (1997), one can determine the time and concentrations at which the chemical agent or pollutant is potentially harmful in living organisms. If the concentration of the product is low or the time of contact is insufficient the effect may not be adverse, and high concentrations may have detrimental effects at extremely short exposure times (Carniato et al., 2007) whereas small concentrations generally produce chronic, sublethal and even lethal effects during long periods of exposure.

The results suggest that although there were variation between the different stations in the various treatments. There is a constant difference in the presence of chromosomal alterations when compared to the control, which reports that during the whole evaluation time the altered the cell cycle and genetic events.

The study does not aim to identify which substances in the water could have caused the differences in the cell cycle of the roots exposed to the treatments. However, according to Cazenave et al. (2009), anthropogenic activities are key factors in increasing levels of aquatic contaminants, and it is possible to detect a variety of persistent chemical compounds that have mutagenic and clastogenic properties.

It can be inferred that, because it is an agricultural region, with intense use of pesticides, the presence of these components in the water is quite probable.

The impact of each pesticide on soil biota varies according to the characteristics of the product, the physical characteristics of the environment and the type of microflora associated. The half-life of pesticides in the soil and the risk of contamination of the water table also depend on the interaction of these different factors and the pathways of degradation of the pesticide (Grisolia, 2005).

In relation to water, although agriculture is only one of the numerous non-point sources of pollution, it is generally pointed out as the largest contributor to all categories of pollutants (Ongley, 1996; Bakore et al., 2004; Potter et al., 2007).

Once in the water, depending on the physicochemical characteristics, the residue of the pesticide can bind either to suspended particulate matter or in the sediment of the bottom or still be absorbed by the organisms. When absorbed, they may be detoxified or accumulated and, in the latter case, enter the food chain, reaching man and eukaryotic organisms (Nimmo 1985, Zhou et al., 2006).

The effects derived from the action of pesticides can be diverse, such as direct reaction with nuclear DNA, incorporation into DNA during cell replication, interference in mitotic or meiotic cell division, resulting in incorrect cell divisions (Fernandes et al., 2007). Therefore, it is possible that Clarimundo Stream is being used as a place for depositing pesticide residues, and the results obtained in this study express the action of these components in the meristematic cells of the *Allium cepa* bulbs. However, in order to ensure that these components are responsible for the cytotoxic and clastogenic effects, an accurate study of the chemical quality of the water is required.

Genotoxicity can be assessed by chromosomal changes, resulting in anomalies in chromosomes, such as stickiness, micronuclei, chromosomal bridges and nucleus deformations (Fiskesjö, 1985). The first mechanism of action of genotoxic agents is to promote DNA lesions (Oliveira et al., 2011) and, according to Majer et al. (2005), these alterations can provoke an attempt to repair that is not always successful, causing the changes to become irreversible, which can lead to cell death.

In the genotoxic analysis, the chromosomal changes observed were anaphase bridges, stickiness, nucleus deformations, loss of chromosomes, micronuclei and multipolarity (Figure 2 and 3).

The action of genotoxic agents promotes lesions in the DNA (oxidation and dimerization, adducts of DNA among others). Damages as Micronucleus (MN), anaphase bridges, found in this study, are indicative of the presence of clastogenic substances in the samples tested. Other findings in this study were stickiness (Figure 3D), which is determined by faults in the disjunction process indicating the presence of aneugenic substances, which interfere in the formation of the achromatic spindle. The events of c-mitoses (Figure 2B) originate when there is inactivation of the spindle so that the chromosomes are dispersed in the cell. Many chemicals such as pesticides can promote these events.

According to Leme and Marin-Morales (2009), the chromosomal abnormalities of meristematic cells of *Allium cepa*, are efficient parameters for the investigation of genotoxicity. It is worth noting that studies with this biological model show that toxicity is not always correlated with genotoxicity, because changes related to root growth and M.I are indicative parameters of cytotoxicity. On the other hand, changes such as chromosomal abnormalities (stickiness, micronuclei, chromosomal bridges, among others) indicate genotoxicity (Friskesjö, 1985).

As the stream runs through an agricultural area and an urban area, it can be inferred that the most likely contaminants are of agricultural origin and household waste.

Generally, genotoxicity is related to environmental pollution events, and since all living beings interact with the environment they will be influenced externally, causing changes in the genetic material of their organisms. In this way, we can suggest that organisms, dwellers or users of this stream, are exposed to genotoxic damage (Minissi and Lombi, 1997; Pascalicchio, 2002; Matsumoto et al., 2006; Klauck et al., 2013).

4. CONCLUSION

Considering the results obtained through the analyzes of the water of the water body, we can conclude that, in the different seasons, the waters of the three sample collection points of the stream presented genotoxic and cytotoxic potential for eukaryotic cells. These events suggest solid evidence of the presence of aneugenic and clastogenic toxic substances that interfere in the processes of cell division and in the genetic material of the organisms present in this ecosystem.

Due to the importance of conserving natural resources, we can propose that, for this stream, it is necessary to establish biomonitoring programs, in order to assist and contribute to management and protection actions.

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