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Bioassays with Plants in the Monitoring of Water Quality

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<http://dx.doi.org/10.5772/50546>

1. Introduction

Currently, the concern with hydric resources has a global reach as regards the availability of this finite natural resource. The scarcity of drinking water is alarming, and many factors contribute with this scenario; the water bodies pollution occurs indiscriminately throughout the world, there is an enormous lack of control and management policies regarding the basic sanitation and proper effluents treatment. Thus, the awareness on the rational use of water, effective monitoring and treatment policies, establishing strict goals for water quality control, are more and more urgent, to ensure the survival of all forms of life.

The quality of the water springs is correlated with the human activities, since these use chemicals to reach social and economic goals and, with the absence of an ecologically correct management, human and industrial waste is dumped in these environments. Many substances with mutagenic potential can be found in food, pharmaceutical drugs, and pesticides and in the industrial and domestic effluents complexes; and some of these substances can cause harmful changes, inherited in the genetic material.

Due to the immense range of substances that compose the complex mixtures in the aquatic ecosystems, it is becoming increasingly more difficult and more expensive to make systematic and analytical analyses in these water bodies. A relatively cheap, easy and fast alternative would be the biomonitoring, because, through this method, an assessment would be made in extensive areas and, in case of positive results, chemical analyses would be made in specifically impacted locations. In this case, time and money could be spared.

The concern that the chemical agents introduced in the environment lead to possible genetic changes in the organisms was one of the main reasons that caused the development of methods to assess the genotoxicity through chemical substances.

Among the bioassays developed for detection of mutagenicity, genotoxicity, cytotoxicity and clastogenicity due to environmental pollutants, plant systems have proven to be

sensitive, cheap, and effective. Plant bioassays, which are mostly sensitive for the detection of genotoxicity, may provide a warning of environmental hazards in the water [19]. The *Allium cepa* test has been widely used for monitoring potentially cytotoxic and genotoxic effects promoted by pollutants in water, air, and soil. This test has high sensitivity and an easy and rapid execution.

2. Water pollution

Water is essential for life. The amount of fresh water on earth is limited, and its quality is under constant pressure. The quality of water sources is correlated with human activities, since they use chemicals to achieve social and economic goals, and the lack of a correct environmental management, there is discharge human and industrial waste in these environments. Preserving the quality of fresh water is important for the drinking water supply, food production and recreational water use. Water quality can be compromised by the presence of infectious agents, toxic chemicals and radiological hazards [1]. Water scarcity affects one in three people on every continent of the globe. The situation is getting worse as needs for water rise along with population growth, urbanization and increases in household and industrial uses. Almost one fifth of the world's population (about 1.2 billion people) live in areas where the water is physically scarce. Water scarcity forces people to rely on unsafe sources of drinking water. More than 10% of people worldwide consume foods irrigated by wastewater that can contain chemicals or disease-causing organisms. 1.1 billion people practised open defecation in 2010 [2].

In 2010, 783 million people still relied on unimproved drinking water sources; 2.5 billion people still lacked access to improved sanitation facilities. The MDG drinking water target, which calls for halving the proportion of the population without sustainable access to safe drinking water between 1990 and 2015, was met in 2010, five years ahead of schedule. While this a tremendous achievement, continued efforts are needed. As of 2010, 783 million people still rely on unimproved water sources (surface water from lakes, rivers, dams, or unprotected dug wells or springs) for their drinking, cooking, bathing and other domestic activities. The proportion of the world's population with access to improved drinking water sources increased from 76% to 89% globally between 1990 and 2010. While coverage is above 90% in Latin America and the Caribbean, Northern Africa and large parts of Asia, it is only 61 per cent in sub-Saharan Africa. There are also disparities between urban and rural coverage, where an estimated 96% of the urban population globally used an improved water supply source in 2010, compared to 81% of the rural population [3].

The surveillance and water quality control requires a systematic programme of surveys, which may include auditing, analysis, sanitary inspection and institutional and community aspects. It should cover the whole of the drinking water system, including sources and activities in the catchment, transmission infrastructure, treatment plants, storage reservoirs and distribution systems (whether piped or unpiped). Surveillance of drinking water quality can be defined as "the continuous and vigilant public health assessment and review of the safety and acceptability of drinking water supplies" [4-5].

One of the main sources of pollution of fresh water ecosystems refers to the discharge of industrial and urban wastes. Untreated sewage is considered an important cause to the deterioration of water quality in developing countries. Usually, this complex substance mixture the soil and the water surface of rivers and reservoirs destined for public supply. Environmental pollutants have caused a decrease in water quality, inducing harmful effects in organisms which are in direct or indirect contact with them. The effect of exposure of organisms to any potentially toxic substance depends on its concentration as well as exposure time. One of the effects frequently observed by environmental agents is the chemical alteration of the DNA affecting vital processes, like DNA duplication and transcription, gene regulation and cell division, and leading cells to pathologic processes and/or cell death. The emergence of some genetic diseases and the development of some types of cancer (e.g. leukemia, lymphomas, liver cancer, etc) are promoted by pollutants found in the water, often in drinking water.

Genotoxic agents cause primary DNA lesions (e.g. formation of DNA-adducts, oxidation of bases, base-dimerization, or cross-links), which are either repaired or otherwise lead to irreversible alterations of the DNA or cause cell death. Mutations occur on both, the gene level (gene mutations) and on the chromosomal level (structural and numerical aberrations) [6]. The pollutants can promote breaks in the genetic material are known as clastogenic and those who undertake to chromosome disjunction during division are aneugenic. Micronuclei and bridges are examples of results clastogenic, while c-mitosis and stickness, aneugenics.

3. Plant bioassays

The concern that the chemical agents introduced in the environment might lead to possible genetic changes in the organisms was one of the key reasons that promoted the development of methods to assess the genotoxicity by chemicals. An international collaborative study on the use of bioassays with plants for biomonitoring of genotoxicity of environmental pollutants was initiated and organized by global bodies such as the International Program Chemical Safety (IPCS), the United Nations Environmental Program (UNEP) and World Health Organization (WHO). Since then, the aim of the International Program on Plant Bioassays (IPPB) was to put in practice the applicability of bioassays: micronucleus test and chromosomal abnormalities and/or mitotic anomalies in the *Allium/Vicia*; micronucleus test and stem hairs mutations in *Tradescantia*. Thus, many laboratories in different cities throughout the world have been using these bioassays to assess the genotoxicity in air, water and soil. Sentinel bioassays are biological indicators of environmental contamination with the concept of detection and prevention of disease [7]. For assessing chromosome damage, the most frequently used plant species is *Vicia faba* which has large chromosomes amenable to the study of chromosome aberrations in somatic cells (1) during mitotic divisions and (2) as micronuclei in root tip cells following the mitotic divisions. *Vicia faba* was used in radiation experiments as early as 1913. *Allium cepa* and *Hordeum vulgare* are the next most commonly used species for studying chromosome aberrations although *Crepis capillaris* has been used by many Russian investigators. A few plants such as *Hordeum vulgare*, *Lycopersicon esculentum*, *Pisum sativum* and *Zea mays* can be used for the study of both

gene mutations and chromosome aberrations. The crucifer *Arabidopsis thaliana* is only used for mutation studies as the chromosomes are very small and the total genome contains only about 70000 kb in contrast to over a million kilobases in most other plants [7].

Among the higher plants used as test organisms for the detection of genotoxic substances in the environment are the following: *Tradescantia*, *Arabidopsis thaliana*, *Hordeum vulgare*, *Pisum sativum*, *Crepis capillaris*, *Vicia faba*, *Zea mays*, *Allium cepa*, among others.

Tradescantia, commonly called spiderwort, is a herbaceous plant which has almost worldwide adaptation and can grow around the year in the field of subtropical regions of the world or in green-houses everywhere. Its relative small plant size (most species are less than 50 cm in height) and six pairs of relatively large chromosomes in its somatic cells made it a favorable experimental material for cytogenetic studies [8]. Using chromosome damage as the indicator of the carcinogenic properties of environmental agents, the Trad-MCN bioassay is a quick and efficient tool for screening carcinogens in gaseous, liquid and solid forms. Test results can be obtained within 24-48 h after the exposure either on site or in the laboratory, thus, the Trad-MCN can be used in a global scale to detect carcinogens as a preventive measure of cancer [9]. The *Tradescantia* assays currently utilize a diploid hybrid clone 4430 heterozygous for flower color with blue dominant and pink recessive that is highly sensitive to chemical mutagens. *Tradescantia* has two assay systems: (1) the *Tradescantia* stamen hair assay which can be used to detect airborne, soil, and aqueous mutants and (2) the *Tradescantia* micronucleus assay (Figure 1) for the detection of chromosomal aberrations from airborne, soil, and aqueous clastogens. The *Tradescantia* stamen hair bioassay has been an important research tool in the field of genetic toxicology for several decades. This has been exploited as a somatic mutation test in the fields of radiobiology, chemical mutagenesis, and ambient air monitoring [7]. For water pollutants from runoffs of industrial sites or power plants, monitoring can be conducted either by growing plants in nearby fields or taking water samples to be tested in the laboratories [8].

When studying the quality of the water in a reservoir in Illinois, [9] collected water samples from the reservoir for two years and assessed the frequency of micronuclei through the *Tradescantia* micronucleus (Trad-MCN) test, and found an average of 12-14 MCN/100 tetrads, showing the samples mutagenicity. Drinking water from the tap was tested in parallel with lake water, and its mutagenicity tended to follow with the mutagenicity of the lake water. The Trad-MCN bioassay was used to determine the clastogenicity of wastewater samples collected from the Arena canal which contains effluent industrial in the city of Queretaro, Mexico; micronucleus frequencies of all the exposed groups at the Conalep site, a predominantly industrial area, were higher than that of the laboratory control groups throughout the 2 year period [10]. The genotoxicity of untreated and treated sewage from two municipal wastewater treatment plants in the municipality of Porto Alegre, in the southern Brazilian state of Rio Grande do Sul, was evaluated over a one year period using the *Tradescantia pallida* var. *purpurea* (Trad-MCN) bioassay. Inflorescences of *T. pallida* var. *purpurea* were exposed to sewage samples in February (summer), April (autumn), July (winter) and October (spring) 2009, and the micronuclei (MCN) frequencies were estimated in each period. The results indicated that the short-term Trad-MCN genotoxicity assay may be useful for regular monitoring of municipal wastewater treatment plants [11].

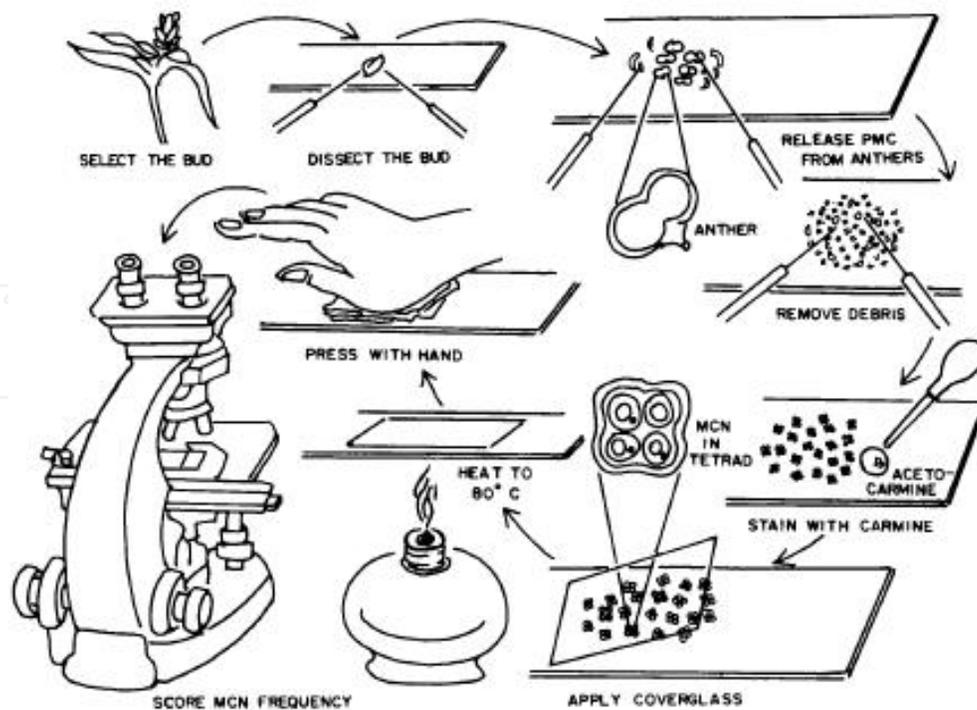


Figure 1. *Tradescantia* micronucleus assay - TRAD-MCN [8].

Vicia root tip mitotic and pollen mother cell meiotic tests are two major kinds of cytogenetic tests for environmental mutagens. Mitotic tests to determine the frequencies of chromosome or chromatid aberrations and/or sister chromatid exchange from root-tip meristematic cells can be used. Treatment of root tip meristem can be done by allowing the newly germinated roots to absorb the chemical mutagens from a water solution. Pollen mother cells can be treated by spraying the solution or pipetting the liquid over the flower buds. After an appropriate recovery time, the samples are fixed and stained, and the slides are prepared for metaphase or anaphase figures for scoring aberration frequencies. Slides for meiotic tests are prepared for metaphase I and/or Anaphase I stages for scoring chromosome aberration frequencies. Results of both cytogenetic tests should be expressed in terms of number of breaks per cell or per 100 cells. The *Vicia* root tip mitotic test is reliable, efficient, and relatively inexpensive [12]. A collaborative study involving laboratories in six countries was initiated under the sponsorship of the International Programme on Chemical Safety (IPCS) to determine the sensitivity, efficiency and reliability of the *Vicia faba* root tip meristem chromosomal aberration assay using a standardized protocol. The conclusions from this study suggest that the *Vicia faba* chromosomal aberration bioassay is an efficient and reliable short-term bioassay for the rapid screening of chemicals for clastogenicity [13]. *Vicia* root micronucleus assay was used to determine the clastogenicity of water samples from Xiaoqing River that passes through Jinan City. Positive results were obtained from eight water collecting sites. This indicates that the water in most areas of this river was polluted with industrial waste and municipal sewage. Results of this study proves that biomonitoring with *Vicia* root micronucleus test is an efficient way to assess the water quality of this river [14].

Zea mays (maize), a member of the Poaceae, is the third most important crop plant in the world. Maize is the oldest plant to have a fully established gene map with the basic genome consisting of 10 chromosomes, is an excellent plant for testing for mutations. The maize bioassay is a particularly favorable experimental assay for the study of chromosome aberrations that may be scored in both mitotic and meiotic cells and pollen. The sister chromatid exchanges have been induced in root tips of maize. Plants of maize have also been used to detect urban air particulate matter, mutagenicity from lake water, municipal water, bottom sediment of a water reservoir, municipal sewage sludge, waste water and soil contaminated with coal fly ash [15].

In this context, toxicity and genotoxicity biological tests are mandatory for the evaluation of reactions of living organisms to environmental pollution, as well as for the identification of potential synergetic effects of several pollutants [16].

One of the principal objectives of using chromosomes as a monitoring system is to determine whether or not a particular chemical is a clastogen – that is, capable of breaking chromosomes. If chemical is a clastogen, then this would permit exchanges with subsequent cytological or genetic damage. At the same time it has been recognized that turbagens (chemicals which cause mitotic disturbance) while not necessarily affecting DNA directly, may result in chromosome segregation errors, and therefore, should not be considered genetically insignificant [17]. Pollutants with mutagenic and cytotoxic potentials produce effects such as DNA fragmentation, induction of chromosome aberrations, inhibition of cellular division, and arrest of the cellular cycle, that can be cytologically detected [18]. Plant roots are generally useful tools in biological tests, because they are the first structures to be exposed to chemical variations in the water and soil [19]. Many types of assays for evaluation of cytotoxicity and genotoxicity employing microorganisms and mammalian cells have been used for monitoring complex environmental samples such as river water. Plant assays and the *Allium cepa* test, particularly, have some advantages over microbial and mammalian cell tests because they are highly sensitive to many environmental pollutants, including heavy metals, and are also useful for monitoring the potential synergistic effects of mixtures of pollutants, including hydrophylic and lipophylic chemicals. Furthermore, test plants can be directly exposed to complex mixtures or environmental samples either in the laboratory or in situ. Because of the large size of their chromosomes, some higher plants are suitable for cytological analysis and the responses so obtained are highly correlated with those seen in other biological systems, thus making plant tests also good candidates for evaluating the genotoxicity of environmental samples.

A. cepa has been indicated as an efficient bioindicator in genotoxicity testing, due to cellular proliferation kinetics, the rapid growth of its roots, large numbers of cells in division, high tolerance to different cultivation conditions, year-round availability, easy management and reduced number of large chromosomes ($2n = 16$) [20-21]. The *Allium cepa* test has been used since the 1930s [22] and subsequently standardized by [20] in other studies (1985). Since then, many studies adopt this methodology and the results confirm its efficiency in detecting the effects caused by toxic substances found in the environment. A literature review by [23] shows that 148 chemicals had the clastogenic potential when tested through the *Allium* test. Among

these substances, 76% had positive behavior for the induction of chromosome aberrations. The author then suggested the introduction of the *Allium* test to the tests routinely used for the detection of chromosomal damage induced by chemicals substances. Considering only the analysis of aberrations in anaphase and telophase cells, the *A. cepa* test is the simplest and considerably one of the most reliable among the investigation methods.

3.1. *Allium cepa* test 'in focus'

Among the several classes of contaminants detected by the sensitivity of the *Allium* test are: heavy metals, domestic and industrial sewage, landfill extracts and samples of water from rivers and lakes, whose solutions encompass a complex mixture of substances of different compositions, thus attesting the sensitivity and efficacy of this bioassay. Some heavy metals, such as iron, nickel and chrome, are among the contaminants commonly found in the waters of rivers and lakes, act as toxic agents, and can influence the mitotic rates, induce chromosomal aberrations and the formation of micronuclei in tests conducted with *Allium cepa*. Certain metals in aqueous solution can cross the cellular membrane or enter through phagocytosis or pinocytosis and can cause damages to the DNA molecule structure [25].

The first studies conducted in *Allium cepa* roots were performed by [23] when investigating the toxic action of colchicine. Since then, numerous works have used this protocol to estimate the environmental danger in several situations.

[26] when assessing samples of domestic sewage from the residual water from a municipal water treatment station, observed a dramatic reduction in the mitotic index, so that it was impossible to count chromosome aberration; but the dilution is a factor that can influence these parameters. Similar results were also observed by [27] and [28] when estimating the genotoxicity in pharmaceutical/hospital effluents samples and [29] when assessing water samples from a lake environmentally affected with heavy metals. Other works also observed the environmental impact of the presence of heavy metals in rivers, lakes and soil aqueous extracts [30-34]. In addition to the domestic sewage [26,35-37], industrial effluents are also historically impacting: [38] submitted seeds of *A. cepa* to the effluent from a textile industry and observed a mutagenic effect, the type of cell damage may be transmitted to subsequent generations, possibly affecting the organism as a whole, as well as the local biota exposed to the effluent discharge. If the damage results in cell death, the development of the organism may be affected, which could also lead to its death. Chromosome aberration assay was carried out in *A. cepa* meristematic cells exposed to the Guaecá river waters, located in the city of São Sebastião, SP, Brazil, which had its waters impacted by an oil pipeline leak. Analyses of the aberration types showed clastogenic and aneugenic effects for the roots exposed to the polluted waters from Guaecá river, besides the induction of cell death. Probably all the observed effects were induced by the petroleum hydrocarbons derived from the oil leakage [39]. In the work of [40], the general toxicity (root growth inhibition and malformation) and genotoxicity (induction of chromosome aberrations in root cells) of an oil field wastewater in the Nigeria have been investigated by the *Allium* test. The wastewater is mitodepressive and increased significantly the frequency of chromosome aberrations in root cells (sticky chromosomes, c-mitosis, spindle multipolarity, bridges and fragments). At

lower concentrations c-mitosis was the most common aberration. The suitability of the *Allium* test in genotoxicity screening is highlighted and the impact and significance of positive results on the environment and human health should be discussed.

The uses of medicinal plants have always been part of human culture. The World Health Organization estimates that up to 80% of the world's population relies on traditional medicinal system for some aspect of primary health care. However, there are few reports on the toxicological properties of most medicinal plants especially, their mutagenicity and carcinogenicity. In this study, nine medicinal plants had their mutagenic potential assessed; the extracts inhibited the root growth, and promoted a mitodepressive effect and induction of chromosomal aberrations in *A. cepa* [41]. In other studies, plant extracts are assessed for the toxicity of their potential medicines: [42] detected a cytotoxic and genotoxic action when assessing extracts of *Inula viscosa*. [43] assessed the toxic effects of five medicinal plants, all the tested extracts were observed to have mitodepressive effects on cell division and induced mitotic spindle disturbance in *Allium cepa*.

3.2. Evaluated parameters in *Allium cepa* test

Among the plants commonly used as indicators for studies of potential toxicity of river water, *Allium cepa* L. constitutes a convenient system for the analysis of anatomical (root growth, deformity, twist) and microscopic parameters (chromosome abnormalities, altered mitotic index (MI), and micronucleus (MN) formation) [44]. Root growth and mitotic index are parameters of the cytotoxicity; micronucleus and chromosome abnormalities parameters of genotoxicity. The mitotic index is a parameter that allows to estimate the frequency of cells in cell division, reflects cell proliferation and is regarded as an important parameter when determining the rate of plant root growth. Therefore, a low mitotic index (mitodepressive effect) characterize a few dividing cells [45-46] and is more frequently reported in the literature. This parameter predicts the root growth behavior, which is conducted by the frequency of cellular division in the root tissue. This index has been clearly correlated to the root length in the *A. cepa* test, as for instance decreasing in value with increasing concentrations of toxic metals like chromium and cadmium [46].

Chromosomal aberration types detected may depend on DNA lesions and the cell cycle phase that is considered [46]. The mitotic anomalies often detected by the *Allium* test are: chromosome stickiness, multipolar anaphase, anaphasic bridge, c-mitosis or c-metaphase, and micronuclei (Figure 2).

The induction of micronuclei in root meristems of *A. cepa* or in any cell of any other organism is the manifestation of chromosome damage and disturbance of the mitotic process. The micronucleus is formed by the development of a new membrane around the chromatin matter that failed to move to either pole during the anaphase of mitosis. Such chromatin matter arises either from anomalous disjunction of chromosomes due to spindle abnormalities or the breakage of chromosomes resulting in formation of acentric fragments, dicentric chromosomes and chromatin bridges. Thus, the induction of micronuclei may suggest that the environmental pollutant is either a spindle inhibitor or a clastogen. Induction of micronucleus formation, the outcome of chromosome breaks/fragments or

spindle poisoning which induces an anomalous disjunction of chromosome at anaphase, has usually been considered a genotoxic indicator. On the other hand, chemicals substances can induce micronuclei promoting changes in the achromatic fuse (aneugenic effect). Micronuclei arise when the lost genetic material is involved by a nuclear envelope, independent of nuclear envelope of the principal nucleus [48]. When the interphase cell is exposed to environmental pollutants, damage is produced either in whole chromosomes - in G1, or damage in individual chromatids - S and G2 phases. Anomalies typically found in S phase are gaps and chromatid breaks [49]. Moreover, during mitosis, all types of AC may be found. Chromosome breaks, bridges and lagging chromosomes may result from sticks and are considered to induce aneuploid and polyploid cells. The stick form can be related to the depolymerization of DNA, the dissolution of nuclear proteins or with an increase in chromosome condensation.

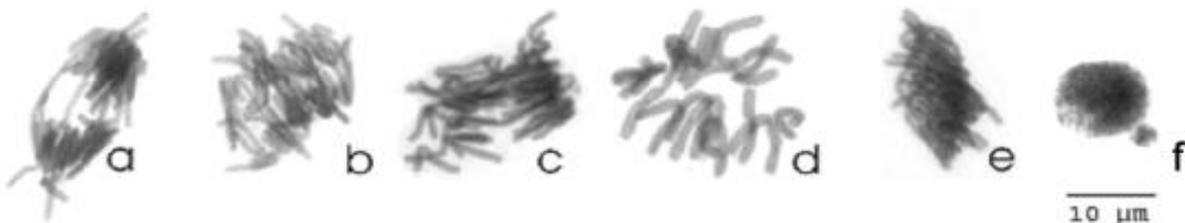


Figure 2. Mitotic anomalies and micronucleus observed in root meristematic cells of *Allium cepa* exposed to water samples of the Paraíba do Sul river - Brazil: a. chromosome bridges; b. not identified; c. multipolar anaphase; d. c-mitose; e. stickiness; f. micronuclei [47].

The use of *Allium cepa* as a test system was introduced by [23], when the effects of colchicine were investigated. This author defined it as an inactivation of segments achromatic spindle fibers by condensed chromosomes randomly dispersed throughout the cytoplasm cell. The formation of c-mitotic inhibitors relates to fibers of the spindle. Calmodulin, involved in regulating the concentration of calcium in the cell was specifically localized in the zone achromatic of mitotic cells, suggesting its involvement in the movement of chromosomes by controlling the polymerization and depolymerization of microtubules [50]. The storage irregular calcium in the cell inhibits the polymerization of microtubules, causing the formation of c-mitosis [51]. The c-mitosis may be defined, as well as the permanence of the equatorial plate anaphase chromosomes rather than move to their respective poles [21]. C-mitosis results from inactivation of the mitotic spindle followed by a random scattering of the condensed chromosomes [46].

The chromosome stickiness arises from improper folding of the chromosome fiber into single chromatids and chromosomes. As a result there is an intermingling of the fibers, and the chromosomes become attached to each other by means of subchromatid bridges. Chromosome stickiness has generally been inflicted by highly toxic agents, usually of an irreversible type, and probably leads cells to death [18,46]. Chromosome breaks - Any descriptive classification must include a category for the break or discontinuity, a simple severance of the chromosome or chromatid to give an acentric fragment, and which is not clearly associated with any exchange process [52]. Usually, the mitotic anomalie is the most frequently observed in studies conducted with *A. cepa*.

The chromosome bridges can involve one or more chromosomes. Bridges associated with stickiness promote a usually irreversible toxic effects.

The chromosome aberration terminology used in cytogenetic experiments generally comprehends chromosome changes designated as numerical (euploidy and aneuploidy) and structural (deletions, inversions, duplications, and translocations) aberrations. However, in experiments involving the *A. cepa* test, other parameters have also been considered to be important tools to inform on chromosome abnormalities as induced by cytotoxic or genotoxic agents. In this respect, we consider the term mitotic anomalies more appropriate for chromosomal changes observed in the *Allium* test [46].

3.3. The *Allium cepa* protocol

In the *Allium cepa* test protocol (based in Invitox Protocol n. 08 by [53]) for complex mixtures (as is the case of rivers and lakes waters), after having their ring of the root primordia carefully cleaned, the onion bulbs are exposed to clean water (it may be good quality tap water, Milli-Q water, distilled water, Hoagland solution, or mineral water, etc) for 48 hours in order to allow for the rooting of the bulb. After this period, bulbs were exposed to treatment for 24 hours: solution or water which needs to be tested, a negative control (no effect on the cell samples; the same solution for rooting is usually used) and a positive control (a drug used to promote the formation of abnormalities). A 20 L bucket was used for collecting the samples. The water was collected at bridges over the River passing alongside the mentioned cities (Figure 3). A mixture was composed from water sampled from the margins and the middle of the River, and transferred to 40 L containers.

All root tips are fixed in absolute ethanol-glacial acetic acid 3:1 (volume/volume) for 5 minutes and subjected to the Feulgen reaction en bloc. The acid hydrolysis pertinent to the Feulgen reaction is carried out in 4 M HCl at 24 °C for 75 minutes. Each stained root was squashed between slide and coverslip, and the squashes frozen in liquid nitrogen for the coverslip removal, and air dried. The preparations were then counterstained with Fast Green at pH 2.7 [54], rinsed in distilled water, air dried, cleared in xylene, and mounted in Canada balsam (Figure 3).

Although the *Allium cepa* test has been widely used to identify potentially cytotoxic and genotoxic pollutants in aquatic environments, variable non-standardized choices have been made regarding the number of plant bulbs and roots analyzed. We propose numbers for bulbs and roots per bulb when comparing the frequencies of micronuclei and mitotic anomalies with this test. When comparing quantitative biological data, one of the most frequent issues is the selection of an adequate number of samples. This decision is influenced by several factors, including the purpose of the study, the size of the population, the risk of using a sample that differs markedly from the population, and the sample error allowed. Determining the sample size is therefore of great importance; exceeding the necessary sample size may prove detrimental in terms of time and human and financial resources. However, too small of a sample size may lead to inconsistencies. As sample sizes increase, their variability tends to decrease, leading to a better hypothesis testing, a higher statistical power, and smaller confidence intervals. Nevertheless, there has not been a standardization of the ideal sample size of bulbs and their roots to be analyzed with the *A. cepa* test. The sampling of ten bulbs and five roots per bulb was adequate for comparative

studies to evaluate the potential damage inflicted by pollutants in aquatic environments. Furthermore, even one bulb and one root per bulb was sufficient in discerning this damage, thereby shortening the time required to attain a statistically confident comparative evaluation. However, to allow for the use of statistical programs based on the evaluation of average values, and to avoid criticism based on genetic variability, we propose that three bulbs and three roots per bulb be considered as standard sample sizes for the *A. cepa* test [46].



Figure 3. *Allium cepa* bioassay: a 20 L bucket was used for collecting the samples and transferred to 40 L containers (up); bulbs were exposed to treatment for 24 hours; growth root and root stained with Schiff (down).

4. Biomonitoring in the Vale do Paraíba region, São Paulo state, Brazil

The São Paulo state is the most developed area of Brazil, with a high level of industrialization, expanding urbanization and high demographic growth rates. Consequently, there is a trend towards the worsening of some situations, as regards the deficiency in several aspects, such as infrastructure (difficulties to accommodate a population that grows every year), basic sanitation, lack of hydric resources before the demand and damage to their quality, mainly resulting from the direct dumping of urban and industrial sewage, untreated or inadequately treated, in rivers, lakes and reservoirs, among others. The main source of water pollution in the state of São Paulo is the dumping of domestic and industrial effluents, as well as the diffuse load of urban and agricultural.

The Paraíba do Sul river basin is located in the country's key economic area, which highlights for the diversity of its industrial park, especially the aeronautical and automotive industries, paper and cellulose, chemical, mechanical, electrical and electronic, and

extrativist industries, in addition to technological research centers. In agriculture, the predominance is of cultivations destined for animal production (cattle), extensive areas with the cultivation of eucalyptus, as well as the presence of the cultivations of rice, beans and corn. The basin draining area is about 55 500 km², encompassing the states of São Paulo, Minas Gerais and Rio de Janeiro [47].

The Paraíba do Sul river (Figure 4) is one of the components of the Paraíba do Sul basin, of importance to the southeast region of Brazil, as it serves for urban supply, irrigation, generation of energy, and assimilation of urban, industrial and agricultural discharges in the mentioned region. For this reason, since 2005, samples of water of this important river, collected in cities along its course, have been periodically evaluated. Anatomomorphologic parameters (root growth), mitotic indices (MI), cell division phase indices (PI), frequency of micronuclei (MN) and chromosome anomalies (CA) are investigated.



Figure 4. Indication of the cities (Tremembé and Aparecida) ‘triangles’ at which the water was sampled from the Paraíba do Sul river [19].

The Environmental Sanitation and Technology Company (CETESB) performs the monitoring of the water bodies in the state of São Paulo, and has been trying to contribute with the pollution control and water quality recovery actions in the rivers and reservoirs in the state of São Paulo developed by the city, state and federal governments. In this monitoring, CETESB use 50 water quality variables (physical, chemical, hydrobiological, microbiological and ecotoxicology) [17]. In the last 20 years, 1007 surface water samples were analyzed through the Ames test at CETESB, and 137 (14%) of those showed mutagenic

activity. The reason for this contamination was, usually, the discharge of industrial effluents [55-56]. Other ecotoxicological tests, such as those conducted with *Ceriodaphnia dubia*, have shown, according to the CETESB 2007 Report, chronic toxicity in 21% of samples collected in the Paraíba do Sul River.

Considering the data involving root length growth and especially MI values, a cytotoxic potential is suggested for the water of the river Paraíba do Sul at Tremembé and Aparecida, in 2005. On the other hand, since in this year the MN frequency was not affected by the river water treatments, genotoxicity is not assumed for the river water sampled at the mentioned sites. By considering the frequencies of MN, CA and PI, a cytotoxic and genotoxic effect is supposed for *A. cepa* treated with the water collected from the river Paraíba do Sul at sites of Tremembé and Aparecida, in 2007. The *Allium* test has proved to be a sensitive tool for detecting cytotoxicity and genotoxicity effects promoted by the Paraíba do Sul water in the mentioned sites. Present findings reinforce the sensitivity of the *Allium cepa* test, especially concerning the MI evaluation, for monitoring river waters, thus serving as a tool for the early warning of the presence of cytotoxins in the hydric environment. We consider that this test could even be recommended for the prescreening of cytotoxicity in wastewaters [17,19]. In this study, MI and the MN frequency were determined by examination of 2,000 cells per slide, using 3-5 root tips from each bulb. The number of bulbs used depended on the availability of roots produced. The slides were examined from right to left, up end down, and the first 2,000 cells were scored for MI and MN frequency. Data on root length, MI, and the MN frequency were compared using ANOVA and the Tukey test. Variance heterogeneity was tested. Data on root length regarding the Tremembé site and the negative control in the year of 2005 were compared by a Welch test (t-test for different variances). The analyses were done using Statistica and Statsdirect software [19].

Still in the year 2007, the genotoxic and cytotoxic potential in water samples from the basin of Tapanhon river, Pindamonhangaba, São Paulo, using the *Allium* test was evaluated. Water samples of the Tapanhon river and its affluents (the Primeira Água and Segunda Água Streams, the Galega Brook) as well as a sample from the Borba Spring have been collected for negative control. Six bulbs of *Allium cepa* have been submitted to each of the samples for 24 hours. As such, 6000 cells have been collected for evaluation of the mitotic index (MI) and micronuclei (MNC) as well as 300 cells in the metaphase and anaphase phases, to evaluate chromosome aberrations (CA). No MNC has been found in the samples tested. The MI values have shown to be elevated in all samples tested when compared to the control with statistical relevance, however, only for the samples from the Primeira Água Stream and the Galega Brook ($p < 0,05$). Chromosome aberrations have been observed in all samples ($p < 0,05$). The averages of the parameters assessed were compared through ANOVA (0.05) of a factor, and the heteroscedasticity premise was assessed through the Levene test. The averages obtained were compared to one another through the Tukey test. The analyses were done using Statistica and Statsdirect software [25].

In 2008 a new study was carried out to characterize the mutagenic potential of the pollutants in the water of the Paraíba do Sul river, in Tremembé city, São Paulo State, Brazil, analyzing

chromosomal changes in the meristematic cells of *Allium cepa*, in the summer (April) and winter (August). The bulbs were exposed for 72 h to the treatments: water from river, Hoagland solution (negative control) and 15 µg/L from MMS – methyl methanesulfonate (positive control). In each treatment, three bulbs were exposed and for each bulb, five slides were prepared. For mitotic index (MI) and micronucleus (MN) frequency rate, a total of 2,000 cells per root/slide were analyzed and 100 cells for the chromosome aberrations (CA). In April, the pollutants induced a high mutagenic potential in the meristematic cells of *Allium cepa*, the frequency rate of MN, stickiness and CA from non identified type were greater than the negative control. In August, the only significant change found was the chromosome bridges. There was no significant change for MI. These results bioindicator, therefore, it is important to keep biomonitoring and adopting effluents control measures.

Macroscopic Parameters (root growth) were evaluated in the roots of 5 to 10 bulbs of *Allium cepa* submitted to samples of water of the Paraíba do Sul river collected in the city of Tremembé in August 2009 and 2010. Tap water was used as negative control. After 48 hours the roots of each bulb were measured in 72 hours (1st day) e 168 hours (5th day). There was no difference in the growth of roots in 2009, however, there was a difference in 2010, as well as between the years of 2009 and 2010 for the samples of the river. Several factors may have influenced this toxicity. August 2010 was the driest month in the state of São Paulo, with only 11 mm of rainfall registered. The low rainfall index may have promoted less dispersion of substances along the river. In the same period, the ecotoxicological result carried out by the CETESB using *Ceriodaphnia dubia* was chronic, what shows the presence of toxic substances present in water which inhibited the reproduction of this organism. The same toxicity was observed in our study for the decrease of the index of cellular division in the meristematic root of *A. cepa*.

Positive results, detected by the analysis of some parameters in bioassays with superior plants, indicate the presence of genotoxic and/or citotoxic substances in the environment, demonstrating a direct or indirect potential risk for living beings in contact with it.

Water is an essential resource to sustain life. As governments and community organizations make it a priority to deliver adequate supplies of quality water to people, individuals can help by learning how to conserve and protect the resource in their daily lives. In conclusion, for being an efficient and low cost, and easy to be executed tool, the *Allium* test is recommended for use in order to magnify the system of biomonitoring carried out by the inspection agencies.

5. Conclusion

The physical-chemical monitoring process is expensive, once it demands time, equipment, solutions and specialized labor. The biomonitoring techniques do not replace the assessments of the different parameters assessed through the conventional methods, but have great usefulness for a first diagnosis of the monitored and non-monitored areas, in order to effectively contribute in the pollution control process. The bioassays that use plants are effective, fast, easy to apply to any area, have low cost, do not need sophisticated

equipment and can be performed in partnership with teaching institutions, by providing specialized labor in the environmental area. In this sense, it is suggested to the official pollution control bodies that they implement the use of bioassays with plants as a screening before the expensive physical-chemical analyses, in order to spare time and money; and that partnerships with private initiative and teaching institutions can build on the relationship of the world with the quality of life throughout the terrestrial biome as regards the pollution control and environmental preservation.

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