

**LABORATORY
FOR PLANT CYTOGENETICS**



***ALLIUM METAPHASE GENOTOXIC TEST FOR TESTING DRINKING
WATER, ENVIRONMENTAL SAMPLES, AND CHEMICALS***

FORCED SCREEN POLARIZATION USING H2Q TECHNOLOGY

Evaluation of quality improvement of tap water treated in accordance with the
FORCED SCREEN POLARIZATION USING H2Q TECHNOLOGY
in MOLECULE – Pet Bowl

Client: FTT BG LTD – BULGARIA, Street Korenacite 63, 1619 Sofia

Researched samples: Tap Water, *Ljubljanska c 50, SI – 1236 Trzin* (sampled 5th, July 2018)
Equally – treatment using the magnetic informed element with H2Q Water Activator

Cytogenetic research was performed between 5th July and 10th July, 2018

DOMŽALE 15th AUGUST, 2018

THE TEST REPORT CONTAINING THE REASEARCH RESULTS IS SUPPORTED BY:

1. Protocol on *ALLIUM* methods
2. Results on the general toxicity and genotoxicity level of the well tap water, as treatment using
FORCED SCREEN POLARIZATION USING H2Q TECHNOLOGY
3. The treated well tap water's quality improved ($p = 0,0153 < 0,05$)
4. This report consists of 7 pages, 2 tables and 3 figures.

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TEST PROTOCOL ALLIUM METAPHASE GENOTOXICITY TEST FOR THE TESTING OF ENVIRONMENTAL SAMPLES, CHEMICALS, AND DRINKING WATER

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1. Introduction

The *ALLIUM* metaphase test is a test for determining overall cellular toxicity (cytotoxicity) and the level of genotoxicity in water, land, and air ecosystems, where potential genotoxicity agents have already been discovered. The test is short and shows the different effects (complex mix) of pollution, and any interaction between the tested plant (*Allium cepa* L.) and potential genotoxics, after which the results are statistically evaluated. The Fisher's Exact Test is used for statistical calculations of the research results. In the 2x2 frequency tables, the p-value determines the statistical characteristics or non-characteristics of two categorical patterns.

The *ALLIUM* biological test or onion test reveals any holistic impact on the growth and development of living cells or organisms, and detects the presence of harmful substances in those concentrations which are significantly equally than the limited capabilities of the analytical methods.

The first genotoxicity biomarker is the chromosome damage induced by pollution of genotoxic substances proved by biological tests. There is substantial evidence that those chemicals causing chromosome damage, known as genotoxic agents, can induce carcinogenic effects, as well as HDC features – hormone disturbers' chemicals (hormone disturber chemisation or chemicals from hormone disturbtions). By using biological (genotoxic) tests, we can ascertain those responses of the tested onion plant (*Allium cepa* L.), which result in eventual damage to its genetic material (chromosomes) regardless of the tolerance limits that can be caused by various contamination samples within an environment. In regard to the universality of the living organisms' genetic codes, the research results are transferable (applicable) to human beings.

2. Material and methods

The test is performed in compliance with: Firbas (2004, 2011), Kumar P. & Panneerselvam N. (2007); Ragunathan I. & Panneerselvam N. (2007) and Panneerselvam et al. (2012). Cytogenetic research is conducted with the aid of the OLYMPUS - BX 41 research microscope (Japan) having an automatic PM 10 SP photo system, with 400x and 1000x magnification.

2.1. *ALLIUM* metaphase test parameters

ALLIUM metaphase (M) test parameters provide: general toxicity, the level of genotoxicity, metaphase index, and a parallel control test.

GENERAL TOXICITY signifies the root lengths of the young onion testing plants (*Allium cepa* L.); it is inversely proportional to the lengths of the testing plants' roots. The longer the roots, the lower the general toxicity, and the shorter the roots, the greater the general toxicity (Figure 1).

GENOTOXICITY LEVEL signifies the damage to the chromosomes within the cells of the young onion testing plants' root-tips (*Allium cepa* L.), showing the percentage relationship between all metaphase cells and those metaphase cells with chromosome damage (Al-Sabti 1985, 1989; Firbas 2004, 2011). Inspection of up to 200 metaphase cells, at a high level of genotoxicity up to a maximum of 100 cells (Figures 2, 3).

METAPHASE INDEX: number of metaphase cells per 1000 examined cells. Index of values between 20 and 100 ‰ (per thousand points).



Figure 1. Examples of series of onions cultivated for 72 hour in different concentration MMS: 100 ppm (Figure 1a), 10 ppm (Figure 1c), 1 ppm (Figure 1e), 0.1 ppm (Figure 1f), 0.01 ppm (Figure 1d) and 0.001 ppm (Figure 1b).

ALLIUM metaphase test (Firbas 2004, 2011):

1. **First day:** taking samples in the field, for use during the *ALLIUM* test research
2. **Second day:** Placement of the testing plants on specific patterns
3. **Third day:** after 24 hours, changing of water samples
4. **The fourth and fifth days:** testing plants remain in the aqueous medium for another 48 hours.
5. **Sixth day:** research of general toxicity and the genotoxicity level
6. **Seventh day:** statistical calculation (Fisher's Exact Test).

Table 1. Comparing the genotoxicity level results of different drinking water qualities (Firbas, 2011)

The genotoxicity level (expressed in percentage points – is the relationship between all metaphase cells and cells with chromosome defects, n = 200)	Threat level (risk assessment)	Samples of drinking water and some chemicals
2	Natural mutagenicity of testing organisms	Quality drinking water
3 - 5	Zero to low	5.0 mg NO ₃ /l
9 - 13	Medium	0.01 µg/l biocides, 25 mg/l nitrates
15 - 18	High	0.1 µg/l biocides, 40 mg/l nitrates
20 - 23	Critical	(>0.1 µg/l biocides, 50 mg/l nitrates)

Five bulbs of *Allium cepa* L. (size 16-18 mm, weight 3-4 g, aged max. 6 months) are used for each sample, and both controls. The testing plants of young onions are stored at a temperature of 10-14°C, and

humidity of 50%. In the water samples, as well as during both controls, the young onion bulbs of the testing plants are grown over a period of 72 hours at a temperature of 20-21°C. Exposure of the testing plants to an aqueous solution of colchicine, blocks the cell's division during the metaphase stage, where chromosomes are most visible (metaphase cells). The *ALLIUM* Metaphase (M) test provides results for general toxicity and the genotoxicity level. After coating in carmine acetic acid (3-4 min., 60°C), microscopic preparations are made - crushing (maceration). Any chromosomal damages within the chromosome sets can now be observed. After treatment with liquid CO₂ (-70°C), the crushing are placed into Euparal.

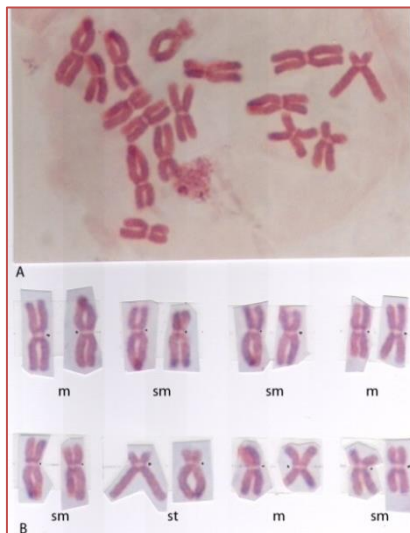


Figure 2. Diploid metaphase chromosome (Figure 2a) and its karyotype from the root cells of the onion (*Allium cepa* L.) containing 2n of 16 (2n =16) with 6 m, 8 sm and 2 st chromosomes (Figure 2b).

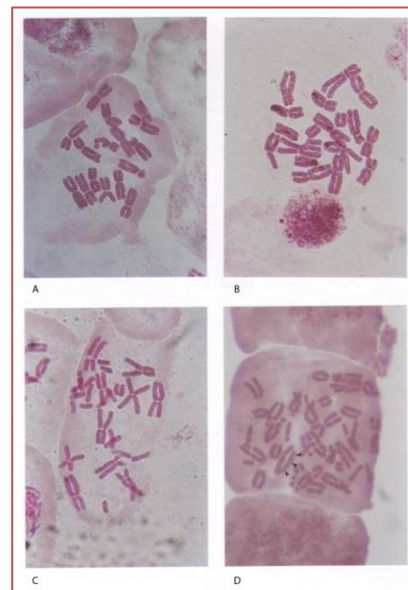


Figure 3. Different number chromosome damage in metaphase cells obtained from the meristeme root-type cells of onion (*Allium cepa* L.): one damaged chromosome (Figure 3a), four damaged chromosome (Figure 3b), eight damaged chromosomes (Figure 3c), whole chromosome garniture is damaged (Figure 3d).

Karyotypization of young onion (*Allium cepa* L.) chromosomes Before analyzing chromosome damages, the general characteristics of chromosome morphology should be mentioned, as they allow us to clarify the mechanisms of different chromosome damage origins. The common onion (*Allium cepa* L.) has 16 chromosomes (Figure 2). A chromosome is formed by two parallel strands or chromatids of equal length, each representing the longitudinal half of the chromosome. The chromosome has a primary constriction (centromere), dividing the chromosome into long and short arms. Chromosome arms may be of the same length. The ratio between the lengths of the arms gives the form of chromosomes and is the basis for chromosomal karyotypization (Levan et al. 1964, Firbas 2004).

Injuries within a chromosome set affect one or two chromosomes within that set, possibly 3 to 7 or 8, and in rare cases up to 12 (Figure 3A, 3B, 3C). However, all chromosomes within a chromosome set could be damaged (Figure 3D). The higher the level of genotoxicity, the more damaged chromosomes in the set. There can also be several different injuries to a single chromosome within the set.

The following chromosomal aberrations were observed: the most frequent are chromosome breaks in the primary constriction (centromere region); in a chromosomal set a maximum of two chromosomes is damaged. In positive control samples single-strand and double-strand chromatide breaks were also observed as well as circular chromosomes.

3. Research results and discussion

Localities of the tap water samples: *Ljubljanska c 50, SI – 1236 Trzin* (sampled 5th, July 2018)

Equally – treatment with the INFORMATION TECHNOLOGY - POZNIK

- I. Tap Water: *Ljubljanska c 50, SI – 1236 Trzin*
- II. Equally - Treated with the FORCED SCREEN POLARIZATION USING H2Q TECHNOLOGY
- III. Negative control (tap water filtered through R. O. – Reverse Osmosis)
- IV. Positive control (1 mg/l or 1 ppm methane-methyl-sulphamide – MMS 4016, SIGMA)

3.1. Genel toxicity and genotoxicity level

Results of general toxicity and genotoxicity level are given in Table 2.

As regards general toxicity well water samples (Samples I, II, III and IV) vary minimally.

As regards average root length all samples of well tap water have statistically longer roots than the roots of positive control samples (Sample VI).

The sample of treated well tap water (Sample II) exhibits the equally level of general toxicity (longest root length).

Table 2. Cytological effects of the examined samples (Genotoxicity level research) and the average length of tested *Allium cepa* L plant roots (General toxicity research)

Sample	Metaphase index (n/1000 - ‰)	Number of metaphase cells	Number of metaphase cells with chromosome aberrations	Genotoxicity level (%)	Average root length (mm)
I	97	200	20	10,0*	35,5
II	103	200	7	3,5*	39,0
III*				2,0-2,5	40-44
IV*				19,5-23,5	23,0

*-K (III); +K (IV). Citat: Firbas & Amon. 2014. *Caryologia*. 67(1):25-35.

4. Fisherjev eksaktni test (Fisher's Exact Test).

Sample of well tap water (Samples I) and sample of treated well tap water (Samples II) are statistically different because the treatment reduces the genotoxicity level of tap water.

All the tap water samples are statistically different from positive control (Sample VI), where the results of genotoxicity level (Samples I, II, III) are lower than in positive control (Sample VI).

4. Conclusion

The results show that the level of genotoxicity in the drinking tap water, after treatment by **FORCED SCREEN POLARIZATION USING H2Q TECHNOLOGY in subject PET BOWL** is reduced and the quality improved by using original technology. This means that the quality of the well water changes from a medium-quality risk assessment (10.0 percentage points) to a zero to medium risk assessment (3.5 percentage points) (Compare Tables 1 and 2). The difference in the root growth and development of young onion testing plants (*Allium cepa* L.) as a cytogenetic study into root-tip cells meristemic testing plants, shows that the **FORCED SCREEN POLARIZATION USING H2Q TECHNOLOGY in subject PET BOWL**, improves the quality of well water, consequently, affects our well-being and health.

1. Following the results of general toxicity and genotoxicity level between positive and negative control treated tap water (orig. **FORCED SCREEN POLARIZATION USING H2Q TECHNOLOGY in subject PET BOWL**) has a lower genotoxicity level than ordinary tap water.

2. Treatment in accordance with original technology (**FORCED SCREEN POLARIZATION USING H2Q TECHNOLOGY in subject PET BOWL**) reduces the level of general toxicity as well as the level of genotoxicity ($p = 0,0153 < 0,05$ – Fisher's Exact Test). The quality of tap water is improved.

5. The aims of the research and its statistical characteristic

The aim of this research was undoubtedly to establish whether or not there is pollution and, in particular, what the risk is for living organisms, including human beings. The cause or source of the pollution can be determined using the above *ALLIUM* test. Only when we know the origin can measures be taken, and thus contribute to maintaining a healthy environment, as much as possible. The inclusion of genotoxicity researches into environmental protection is of great importance, since it allows understanding of the impacts and consequences of genotoxic substances on living organisms. It is time for us to act responsibly, thus ensuring a healthy environment which also includes high quality drinking water.

Statistically-established significant differences among the investigated samples are confirmed by the statistical calculation of paired data analysis using the two-way Fisher's exact test, which gives the p value property between pairs of data. These pairs (investigated samples) are either different (statistically significant) or the same (statistically insignificant), and tell us what the risk is.

Which risks are insignificant enough? The most common values are 0.05, followed by 0.01, and 0.001.

With regard to these limits we can also speak about 5%, 1%, and 0.1% levels of significant results.

Statistical significance should not be the only determining factor when evaluating the results.

6. Sources and Literature

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Use of *Allium* M test for purpose of establishing quality of waters; since 1998

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