Genotoxicity Testing with *Allium* M Test Daring the Cleaning Process for Constructed Wetlands (CW) and Environmental Quality Samples, Slovenia

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**Abstract**

We show the effectiveness of the communal waste water cleaning plant of the type “LIMNOWET® Constructed wetlands (CW)”. Constructed wetlands (CW) fit nice into environment and contribute to a favorable appearance of degraded areas and important new characteristics biotype for plant and animals. Constructed wetlands (CW) imitate the self-cleaning ability of nature. The most important CW characteristics are: (i) new biotope for plant and animals; (ii) reduction of nitrogen, phosphorus substances, metals and other toxic compounds, and bacteria, (iii) efficient elimination of faecal and other bacteria up to 90–99%; (iv) cost-effective construction, performance and maintenance; (v) no need for energy or mechanical equipment; (vi) easy to set-up and maintain; (vii) nice fit into the environment. The tests were done with the *Allium* metaphase (*Allium* M) test and show the degree of Genotoxicity by observing the aberrations of the metaphasic chromosomes of the plant *Allium cepa* L. that are evoked by Genotoxicity substances in the polluted water. The CW plant reduced the degree of Genotoxicity 29.0% to 3.5% (the Fisher’s Exact test: \( p = 9.2 \times 10^{-13} < 0.00001 \)). Therefore CW is a very effective ecoremediation technique since it removes up to 96% of waste. The so-called “onion tests” are considered to be the standard in environmental monitoring, and are used as the bio-indicator for evaluating environmental pollution. This is deemed a very efficient scientific procedure for water quality research. *Allium* M assay is a test used for establishing Genotoxicity of earth ecosystems – water, ground, air, and other media. Frequency of chromosomal aberrations is established in root tip cells of the test plant roots – common onion (*Allium cepa* L.) where evidence of potential Genotoxicity substances is provided. The test takes into account comprehensive influence and mutual action between Genotoxicity substances and genetic material (chromatin, chromosomes). Onion (*Allium*) test give unyielding evidence of the quality of water and unlike chemical tests show the comprehensive pollution effects, not only the presence of substances sought by methods employed by analytical chemistry.

**Keywords**

Constructed Wetland (CW) LIMNOWET®-LIMNOS, SLO; *Allium* Metaphase (M) test; Chromosome Damage

1. Environmental Studies: Monitoring and Evaluation

The purpose of environmental studies is to preserve and improve the quality of the environment, to assure reasonable use of natural sources and thereby to preserve the natural balance. Work aims to introduce methods, which incorporate principles of integrated natural

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resource management, as well as to promote sustainable development and the use of co-natural environmental solutions. Studies cover the following areas: (i) sampling and field parameter measurements; (ii) assessment of the state of water and riparian zone ecosystems; (iii) sustainable water management plants for protected areas; (iv) waste water management strategies for settlements; (v) environmental damage evaluation.

2. Ecoremediation in Integrated Water Management

Ecoremediation (ERM) comprises systems and processes which function in natural and artificial ecosystems; it protects and restores the environment. It is comparatively inexpensive and highly efficient in protection of water resources, streams, rivers, lakes, groundwater and the sea. The basic characteristics of ERM, which can be utilized and improved, are its high buffer and self-protective capacities, and preservation of natural habitats and biological diversity. In the tens of thousands of years, the nature and ecosystems evolved exceptional defensive and self-protective capacity to safeguard themselves against sudden and powerful impacts and to remove their harmful consequences. Aquatic and waterside ecosystems and other wetlands have a high retention capacity and could prevent flooding as well as severe and specific physical, chemical and toxic pollution. The ecosystems neutralize toxic and efficiently reduce various pathogenic organisms. Moreover, they increase biodiversity and contribute to many so far unknown or hardly known processes maintaining the equilibrium on our planet.

ERM is used for multipurpose management of watercourses, lakes and wetlands, which enables integrated development of particular areas and contributes to the coexistence of man and nature. Therefore, the ERM is among the most successful and sustainable methods of environment protection, from the economic and ecological point of view. Although there is still some consideration present as regards “technical indeterminateness”, slow functioning and difficult regulation of ERM systems, their utilization is spreading steadily and gaining ground among environmentally aware people. Possible utilization of ERM: (i) remedy of long-term consequences of harmful human activities in the environment; (ii) enhancement of disburdening and self-protective capabilities of habitats and running waters from non-point pollution sources; (iii) remedy of seasonal pollution impacts, e.g. tourism; (iv) tertiary or supplementary treatment of communal, stockfarming, industrial and other harmful wastewaters; (v) conditioning of water for various usus (watering, irrigation, drinking water; retention basins); (vi) protection of nature reserves; (vii) protection of ground water, water-point and other sensitive areas; (viii) protection against polluted water discharges in lakes and thesea; (ix) sustainable maintenance of amelioration ditches; (x) buffer zones (“vegetation zones”); (xi) revitalization (biological restoration) of degraded watercourses, lakes, gravel pits, clay pits, paddles; (xii) construction and restoration of ecosystems for rare and endangered plant and animal species.

3. Lakes, Reservoirs and Wetlands

Lakes and reservoirs are of great ecological and economic importance and therefore require careful and rational management. Lake management needs to consider the natural balance of water. The use of lakes is strongly dependent on the quality of water. Multipurpose use can only be assured by showing down eutrophication (control, nutrient balance), by preventing large sediment accumulation and pollution from toxic substances, and by preserving the natural balance of lake and lakeshore ecosystems.

ERM in lakes, reservoirs and wetlands include: (i) assessment of the actual state (trofic status); (ii) identification of the desired state (multipurpose use); (iii) preparation of expert recommendations for protection measures and restoration methods; (iv) development of management proposals; (v) design of monitoring programmes.

4. Waste Water Treatment with Constructed Wetlands

During the treatment process, the toxic compounds degrade, partly build in the plans and partly remain in the substrate from where they can be removed from the first basins in cyclic periods and at moderate costs. If necessary, the system can end with an open lagoon for multipurpose use of purified water (irrigation or watering of green areas, fire extinguishments, aqua cultures) or as a land scope element. The sludge from mechanical treatment is composted in the composting bed, which is basically similar to constructed wetlands (CW) beds. For 1 PE (person), by which environmental burden is expressed, 2 to 2.5 m² of surface is needed for the efficient treatment of municipal waste water (Figures 2 and 3). CW treat: (i)
sewage (individual houses, settlements, tourist centers, natural parks); (ii) technological waste water (industries, farm); (iii) landfill leachate (municipal landfill sites); (iv) non-point sources of pollution (highway run-off, agricultural run-off with toxic substances e.g. pesticides, phenol, metals); (v) tertiary waters with the potential for reuse (irrigation, fire fighting).

**Figure 2:** Cross Section of the Constructed Wetlands (CW) bed. – LIMNOWET® – LIMNOS, Company for Applied Ecology d.o.o, SLOVENIA

5. Constructed Wetlands

Constructed wetlands (CW) fit nice into environment and contribute to a favorable appearance of degraded areas and important now characteristics biotype for plant and animals. Constructed wetlands (CW) imitate the self-cleaning ability of nature. The most important CW characteristic are: (i) new biotope for plant and animals; (ii) reduction of nitrogen, phosphorus substances, metals and other toxic compounds, and bacteria, (iii) efficient elimination of faecal and other bacteria up to 90–99%; (iv) cost-effective construction, performance and maintenance; (v) no need for energy or mechanical equipment; (vi) easy to set-up and maintain; (vii) nice fit into the environment.

6. *Allium* metaphase (M) chromosome damage test for evaluation effect of cleaning municipal water with Constructed wetland (CW)

6.1 Introduction

Urban and agricultural waste can add significant amounts of contaminants to surface water and sediments. The resulting water pollution presents a serious problem for the health of the biota and humans that interact with these aquatic ecosystems [1].

The standardized and sensitive testing method “*Allium* test” is widely used for testing the quality of drinking water and environmental water pollution [2, 3]. This officially accepted method has been in use for more than forty years. It is especially useful for testing the possibility contaminated water streams like rivers [4-6], rain and snow [7], earth [8], waste water monitoring [9], pesticides like atrazin [10], benzo (a) piren [11], pharmaceutical effluents [12], and outflows from hospitals [13] including possible radioactive wastes [14]. *Allium* test shows a good correlation with other plant [15] and animal tests [16, 17]. The test procedures described here show the effective degree of combined genotoxic activity of the pollutants on the metaphase chromosomes. Complex interactions may occur in vivo because component pharmacokinetics increases the unpredictability of pharmacodynamic outcomes [18].

There are six bioassays used in this technique: *Allium and Vicia* root tip chromosome break, *Tradescantia* chromosome break, *Tradescantia* micronucleus, *Tradescantia*-stamen-hair mutation and *Arabidopsis*-mutation bioassays were establish from four plant systems that are currently in use for detecting the Genotoxicity of environmental agents [15]. The *Allium* root tip chromosome aberration assay has been adopted by the International Program on Plant Bioassay (IPPB) for monitoring or testing environmental pollutants [19]. Higher plants are recognized as excellent genetic models to detect environmental mutagens and are therefore frequently used in monitoring studies [20].

Constructed wetlands (CW) imitate the self-cleaning ability of nature for the treatment of polluted waters. In general, CWs operate without machine power and need no electricity to run. This saves construction, maintenance and operation costs. The system consists of several successive beds filled with substrate (e.g. stone pebbles) and at bottom isolated with plastic foil. The flow of water through the bed substrate follows gravitational gradient. There is also added dry substrate on the biotope which reduces unpleasant odors and drives away insects. Perkins and Hunter [21] report the findings of an investigation of faecal coliform (FC) bacteria and faecal streptococci (FS) removal in four small, parallel *Typha*-dominated, surface flow reed beds. They constitute the tertiary phase of treatment at the Crow Edge sewage treatment works near Holmfirth in Yorkshire. Reduction in concentration was observed between inflow and outflow wastewater for both indicator bacteria, giving mean bed removal efficiency values of approximately 85-94%. The water is treated with the help of microorganisms, wetland plants as well as physical and chemical processes.

Onion (*Allium cepa* L.) is very suitable for genotoxic studies. Let us list some of its advantages: (i) The root growth dynamics is very sensitive to the pollutants; (ii) The mitotic phases are very clear in the onion; (iii) It has a stable chromosome number; (iv) Diversity in the chromosome morphology; (v) Stable karyotype; (vi) Clear and fast response to the genotoxic substances; (vii) Spontaneous chromosomal damages occur rarely. Therefore this test has become well established for the determination of the genotoxic substances in various environments. In this study we describe various chromosome and chromatide damages in the root meristem cells of the onion (*Allium cepa* L.), which serve as biomarkers for the different types of environmental pollution. With *Allium* test one can obtain both the effective degree of cleaning the waste water and the impact factor of the so cleaned waste water on the stream into which it flows.
6.2 Material and Methods

Sample Example

Influent and effluent water - Constructed wetlands (CW).

Onion Preparation for the Test

Small onion (Allium cepa L.) bulbs of the same size 16-18 mm, weighing about 3.0 – 3.5 g, aged maximum 6 months were denuded by removing the loose outer scales and scraped so that the root primordia were immersed into the tested liquids.

Experimental Procedure

The exposure time of small onion (Allium cepa L.) bulbs in each experiment was 72 hours at 22°C and protected against direct sunlight (Figures 1, 4 and 5). In order to eliminate the influence of daylight rhythms the plants were exposed to constant artificial light of middle intensity. In an alternative version of our Allium metaphase test, the five onion seeds were placed directly in the experimental water containers. The water sample under investigation was divided into three portions which were successively applied to the onion roots in 24 hour periods. So each 24 hours the roots obtained a fresh bath of the sample solution. After 72 hours the samples were removed from water bath. The macroscopic and microscopic tissue morphology investigation followed.

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)

Chromosome Preparations

The squash technique for onion root as described in [7, 22] was used for the chromosome investigation. Chromosome samples were taken from root meristems containing actively growing cells. The developing roots with bulbs were pretreated with 0.1 % water solution of colchicines for 3 hours at 21°C. After washing in distilled water for 20 min the terminal developing roots of 2 mm length were fixed for 1h in methanol: propionic acid mixture (3:1 or 1:1). Then they were macerated and stained in order to obtain a cellular suspension. This sample was stained with 0.5 % aceto-carmine for 4-5 min at 60°C without hydrolysis, and squashed in aceto-carmine [23]. For observation was used optical microscope Olympus - BX 41 with the photo system PM 10 SP, typical magnifications used were 400x and 1000x.

Macroscopic Parameters

After 72 hours growth in the test solution we measured the root length and noted related parameters as

Figure 4: The Length of the Roots of the Test Plants of Allium cepa L. Treated in a Sample of Urban Waste-Municipal Water

Figure 5: The Length of the Roots of the Test Plants of Allium Cepa L. Treated in a Sample of the Cleaned Water
the shape of the roots, number, color and turgescence [2, 3]. Toxicity and Genotoxicity measurements were performed with 10 ppm concentration of the test MMS chemicals as positive control (PC).

Microscopical Parameters
To study the damage of chromosome and chromatide (breaks) we used the treatment with colchicines. Onion Allium cepa L. has 16 monocentric chromosomes (2n = 16) with basic number x = 8 (Figure 6). The possible aberrations seen at metaphase are: (i) single break chromatide, (ii) double break chromatide, (iii) gap break and (iv) centromere break (Figure 7). (Figure 6&7)

**Figure 6:** Diploid Metaphase Chromosome from the Root Cells of the Onion (Allium cepa L.) Containing 2n of 16 (2n =16)

**Figure 7:** Damaged Chromosomes: 4a – Break in Centromere, 4b – Double Break Chromatide, 4c – Single Break Chromatide, 4d – Gap Chromatide

Level Genotoxicity
This is the percentage between all the metaphase cells and the cells with their chromosomes damaged. The total number studied was 200 metaphase cells.

Parallel Control Test
Integral parts of Allium test are the so called negative and positive control. Negative control shows the degree of toxicity in unexposed onions and serves as control of the test efficiency. Positive control is used with known material which normally induces a high degree of toxicity and is necessary for controlling the test response. In other words - nearer the results of tested samples to negative control, the better the quality of water. On the other hand - farther the values from the results of negative control and nearer to the positive control – this points to poorer water quality.

Control Inhibition Test
Toxicity and Genotoxicity assay is performed with 6 concentrations of the test MMS chemicals as control. Distribution Genotoxicity (induction of chromosome damage in root cell) and general toxicity (root growth inhibition and malformation) in Allium cepa L. exposed MMS (Methyl methanesulfonate) 100 ppm to 0,001 ppm (1 ppb) caused in Table 1 and Figure 1a-f. Results for 10 mg/l MMS, varied from 18,4 % to 28,6 % aberrant cell [3]. (Table 1)

Physical and Chemical Parameters
The physical and chemical properties of the effluent sample were determined in accordance with standard analytical methods [24]. Suspended substances in the sample were determined by the standard (SIST ISO 11923), COD with standard (SIST ISO 6060) and BOD with the standard (SIST EN 1899-1).

Statistic Calculation
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 [25]. These pairs (investigated samples) are either different (statistically significant) or the same (statistically insignificant), and tell us what the risk is. The most common values are 0.05, followed by 0.01, 0.001 and 0.0001. With regard to these limits we can also speak about 5%, 1%, 0.1% and 0.01 % levels of
6.3 Results

We used the *Allium* metaphase test (*Allium* M test) which indicates partially damaged chromosomes or chromatids. The results of the *Allium* M test are shown in Table 2-4, and Figures 4-8 and the physical and chemical parameters are displayed in the Table 5.

Constructed wetland (CW) described here is located in village Sveti Tomaž in Slovenske gorice (46°29′ N; 16°4′ E); Ev. No.13/56-08/P (Limnos, Slovenia). CW follows the system Limnowet® [26]. Its GPS coordinates are YX: 583011 149381. The village Sveti Tomaž has 254 inhabitants. It is surrounded by fields, wine yards and forests [27].

CW was built in the year 2000. It is intended for cleaning of household waste water and effluent of urban-municipal activities. Load CW is 250 PE (population units) with an area of 700 m². The system consists of two stage beveled settler, filtration bed, treatment beds and polishing bed. The slope of the shaft is 1%, the substrate is composed of different grades of sand. Filtration bed is planted with bent-grass (*Carex*) and rush (*Juncus*), treatment bed the shaft with reds (*Phragmites*), and rush (*Juncus*) polishing bed [26]. If necessary, the system can end with an open lagoon for multipurpose use for purified water (irrigation or watering of green areas, aqua culture) or as a landscape element. The sludge from mechanical treatment is composted in the composting bed, which is basically similar to CW beds [26].

6.4 Discussion

Our testing has shown the high efficiency of the CW plant in Sveti Tomaž. CW reproduce the self-cleaning ability of nature. They coexist nicely with environment, improve the appearance of degraded areas and provide new biotopes for plants and animals. Even most of the pharmaceutical substances (e.g. antibiotics and other drugs) get decomposed there.

Waste waters are typically very mitodepressive. That is clearly shown in the root growth inhibition of the experimental plant *Allium cepa* L [2, 3]. Mutagen parameters are represented by the damages of chromosomes like single or multiple chromatide breaks, gap damages, circular centric or acentric chromatides. Further abnormalities that appear are centric or acentric fragments, dicentric chromosomes and circular chromosomes [7, 22, 27].
Table 2: The Average Length of the Roots and Cytological Effects of Investigated Samples and Both Controls of the Test Plants of *Allium cepa* L. Cytological Effects– Investigation of Genotoxicity Level and Average Root Length of Test Plant *Allium cepa* L. – Investigation of General Toxicity. From Each of 5 Bulbs in a Series of 5, One Root Tip is Taken for Each of 5 Slides. From Each Slide, 200 Metaphase Cells are Scored. The Degree of General Toxicity of the Analysed Samples (5 bulbs per sample) was Assessment from the Mean Root Lengths Expressed as a Percentage of the Mean Root Length of the Negative Control.

<table>
<thead>
<tr>
<th>Example</th>
<th>Number of Identify Metaphase Cell</th>
<th>Average Metaphase Cells with Chromosome Damage</th>
<th>Average Level Genotoxicity (%)</th>
<th>Average Length of Root (mm)</th>
<th>Percentage (%) Length Root of Negative Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wastewater, influent in CW</td>
<td>200</td>
<td>±6.0 58</td>
<td>±3.0 *29.0</td>
<td>±3.5 17</td>
<td>41.4</td>
</tr>
<tr>
<td>Cleaning water effluent out CW</td>
<td>200</td>
<td>±2.0 7</td>
<td>±1.0 *3.5</td>
<td>±3.5 42</td>
<td>100.2</td>
</tr>
<tr>
<td>Negative control - NC (tap water filtered with R.O. - reverse osmosis)</td>
<td>200</td>
<td>6 ±2.0</td>
<td>±1.0 3.0</td>
<td>±2.0 41</td>
<td>100</td>
</tr>
<tr>
<td>Positive control - PC (10 ppm Methan Methilsulphonate – MMS 4016 Sigma)</td>
<td>200</td>
<td>±5.0 61</td>
<td>±2.5 31.0</td>
<td>±3.0 12</td>
<td>29.2</td>
</tr>
</tbody>
</table>

Table 3: Comparison of Frequency of Aberrant Cells among Controls (NC – Negative Controls, PC – Positive Controls) and Effluents Samples (two by two tables - Fisher’s Exact Test which gives the p value property between pairs of data); *p > 0.05 Samples is not Characterized as Being Statistically Significant; *p < 0.0001 Samples is Characterized as Statistically Significant

<table>
<thead>
<tr>
<th>Samples</th>
<th>NC</th>
<th>PC</th>
<th>Inflow</th>
<th>Outflow</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>7.9e-14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflow</td>
<td>1.7e-11</td>
<td>0.8269</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outflow</td>
<td>0.7887</td>
<td>7.3e-11</td>
<td>9.2e-11</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Comparison of Root Length among Controls (NC – Negative Controls, PC – Positive Controls) and Effluents Samples (two by two tables - Fisher’s Exact Test which gives the p value property between pairs of data); *p > 0.05 Samples is not Characterized as Being Statistically Significant; *p < 0.05 Samples is Characterized as Statistically Significant

<table>
<thead>
<tr>
<th>Samples</th>
<th>NC</th>
<th>PC</th>
<th>Inflow</th>
<th>Outflow</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflow</td>
<td>0.01</td>
<td>0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outflow</td>
<td>1</td>
<td>0.02</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>

Table 5: Some Physical-Chemical Measurements of Inflow Waste Water and Outflow Cleaning Water CW in Sveti Tomaž. Waste Water Inflow and Cleaning Outflow Water Samples Taken Common Twice, Once in July and Once in October

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Waste Water (Inflow) July / October</th>
<th>Cleaning Water (Outflow) July / October</th>
<th>Effect CW (%) July / October</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Suspended solids (mg/l)</td>
<td>29 / 35</td>
<td>10-Oct</td>
<td>65.52 / 71.43</td>
</tr>
<tr>
<td>(COD (mg/l)</td>
<td>190 / 200</td>
<td>30 / 30</td>
<td>84.2 / 85.0</td>
</tr>
<tr>
<td>(BOD$_4$ (mg/l)</td>
<td>50 / 70</td>
<td>3-Mar</td>
<td>94.0 / 95.8</td>
</tr>
</tbody>
</table>
The cell is called aberrant if at least one chromosome gets damaged [31]. The onion *Allium cepa* has 16 (2n=16) chromosomes - several of them can get damaged. There is also possible to observe several types of damage on a single chromosome (Figure 8A). Typically one to two chromosomes become damaged, sometimes 3 to 7 or 8, in extreme cases even 12 or all (Figures 8B and 8C). This corresponds to the degree of pollution of the tested water. In addition multiple damages on a single chromosome point to serious Genotoxicity [31]. Effluent wastewater has a complex mixture of inorganic and organic substances and is highly mutagenic. Typically at least 4-10 chromosomes (sometimes even all) get damaged in such a water (Figure 8D). The higher the level of Genotoxicity, the more damaged chromosomes in the set [32]. The cleaned water samples from the CW show a much lesser degree of Genotoxicity – typically one chromosome, rarely two are damaged.

The discharge of wastewater into natural water courses, ponds and wetlands has been an ancient practice. Urbanization led to the development of more engineered solutions for domestic sewage as well as industrial effluents. This technical development temporarily lessened the economical importance of the earlier approaches that were closer to nature [33]. However, in the last years there has increased interest in simpler, more natural methods for wastewater treatment including Macrophyte Treatment System like CWs [33]. CWs imitate the self-cleaning ability of nature. They reduce the concentrations of nitrogen, phosphorous and other toxic substances. Their removal efficiency up to 96%; they are cheap to build and maintain and fit nice into the local environment.

In the period between 1960-1970 in Slovenia there was a common practice of melioration and regulation of small streams. So many of them were biologically destroyed. In addition there was paid very little attention to the waste water cleaning. However, from 1990 on the situation has improved very much. The projects resulting in ecoremediations and revitalizations were undertaken. This was accompanied by the testing also with the *Allium* method, which helps to monitor the general water quality. Until 2011 there was made more than 1230 *Allium* tests on 390 localities [31], such trends increasing since there are many CWs and other waste water treatment plants in Slovenia. The research was performed to year 2013 with more 410 geographical sample locations and more than 1550 *Allium* tests performed [34]. Locations were grouped into six reference group: drinking water, surface flowing water, surface standing water, soil on the surface, precipitation water, waste waters originating from household or industry which were divided into 19 sub-reference group.
Figure 9: Study Map of the Group Location of the Allium M test to Realize in Slovenia. The Research was Performed to Year 2017 with more 450 Geographical Sample Locations and more than 1670 Allium M test Performed

Table 6: Reference and Sub-Reference Groups, Which have been Investigated in Environmental Samples with Allium M test. The Research was Performed to Year 2017 with more 450 Geographical Sample Locations and more than 1670 Allium M test Performed

<table>
<thead>
<tr>
<th>REFERENCE GROUP</th>
<th>SUB-REFERENCE GROUP</th>
<th>LOCALITY AND ENVIRONMENTAL SAMPLES (LEVEL GENOTOXICITY - %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drinking Water</td>
<td>Tap Water</td>
<td>City: Domžale (3.0), Jesenice (5.80), Slov. Bistrica (9.52), Ormož (7.5), Murska Sobota (8.0), Celje (14.0), Trzin (9.0), Muretinci (18.5 in 2011 and 14.0 in 2015) Ptuj (19.50) of water pump Skorba-Ptuj (Agrochemicalization and Golf course (54 ha))</td>
</tr>
<tr>
<td></td>
<td>Water Pump</td>
<td>D2-Mirna in Mirenksa dolina (2.0), Vrbanski plato (3.0), VO-Vodice (5.35), in natural protect, Podroteja-Idrija (18.56) mine mercury A3-Apače (21.21); agrochemization on Apaško polje</td>
</tr>
<tr>
<td></td>
<td>To Scoop Water</td>
<td>To scoop water: Repce, Mrzli studenec, Tresilo (Kobarid); Peričnik-Vrata valley (5.0); Dolge Poljane (Ajdovščina)</td>
</tr>
<tr>
<td></td>
<td>Well Water</td>
<td>Vodnjak Savarin (3.0) Dragomelj, Moškanjci (3.0) Vodnjak Mali (7.5) Podreče; Vodnjak Kozel (13.88) Žiberci (Apaško polje), Vodnjak Resnik (4.0) Vransko</td>
</tr>
<tr>
<td>REFERENCE GROUP</td>
<td>SUB-REFERENCE GROUP</td>
<td>LEVEL GENOTOXICITY (%)</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Surface Flowing Water</td>
<td>Spring</td>
<td>3.0 – 10.5</td>
</tr>
<tr>
<td></td>
<td>Rivers</td>
<td>4.0 – 24.0, 5.5 – 44.0</td>
</tr>
<tr>
<td></td>
<td>Brock</td>
<td>4.0 / 33.33</td>
</tr>
<tr>
<td></td>
<td>Canals</td>
<td>9.0 / 31.0 / 48.5</td>
</tr>
<tr>
<td>Standing Water</td>
<td>Natural Lakes</td>
<td>3.5 – 7.5</td>
</tr>
<tr>
<td></td>
<td>Artificial Lakes</td>
<td>15.0 – 26.29 – 34.37</td>
</tr>
<tr>
<td></td>
<td>Level Ground</td>
<td>10.78 – 24.57</td>
</tr>
<tr>
<td></td>
<td>Grassland</td>
<td>9.09 – 14.51 – 24.0</td>
</tr>
<tr>
<td></td>
<td>Hobsfeld</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>Rain</td>
<td>12.20 / 10.0</td>
</tr>
<tr>
<td></td>
<td>Hail</td>
<td>21.0 / 22.5</td>
</tr>
<tr>
<td>Industrial Waste Waters</td>
<td>To Stain Out to Deposit</td>
<td>43.35 – 76.66</td>
</tr>
<tr>
<td></td>
<td>Wastewater (Sewage)</td>
<td>19.0 – 27.0 – 55.0</td>
</tr>
<tr>
<td></td>
<td>Final Effluent (Outflow) Cleaning Ww</td>
<td>6.5 – 14.5 – 23.5, 3.5</td>
</tr>
</tbody>
</table>
In our further work we intend to expand our testing methods with the so called fish micronucleus test (MN) [35] on the water animals especially when testing larger water volumes like lakes and rivers [36]. There are more than 200 lakes in Slovenia and more than 1300 water habitats [37]. They are sensitive ecosystems where one needs to monitor the quality of water in order to keep the Slovene waters clean and healthy.

Introduction of Genotoxicity research in the environment protection policies is of great importance since it enables us to understand the impacts and consequences of genotoxic substances present in water. The goal of our research is to give an immediate and important contribution to preserving the health of the most precious life source – water. Due to our lack of knowledge and carelessness we have already polluted some water sources, therefore it is our obligation to correct our mistakes. We should be aware that as regards Genotoxicity there are no safe “maximum permissible concentrations” (MPC) which would ensure a good and reliable quality of water.

Physico-chemical analysis alone does not provide any reliable answer to the question of how healthy the water is. We should be well aware that as regards Genotoxicity there are no safe “maximum permissible concentrations” (MPC) which would ensure a good and reliable quality of water. Complementary research, however, in association with biological and chemical researches is reveals an integrated impact on the growth and development of living cells or organisms, and detects the presence of harmful substances within the limits and capabilities of analytical methods. 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.

Onion (Allium cepa L.) is very suitable for genotoxic studies because: (i) The root growth dynamics is very sensitive to the pollutants; (ii) The mitotic phases are very clear in the onion; A stable chromosome number; (iii) Diversity in the chromosome morphology; (iv) Stable karyotype; (v) Clear and fast response to the genotoxic substances; (vi) Spontaneous chromosomal damages occur rarely. Therefore this test has become well established for the determination of the genotoxic substances in various environments. In this study we describe various chromosome and chromatid damages in the root meristeme cells of the onion (Allium cepa L.), which represent the biomarkers for the different types of environmental pollution. Plant cytogenetic, using Allium metaphase test, identifies the same chromosome damages as they are identified in human cytogenetic such as: chromosome and chromatid damages, aneuploidy, euploidy and translocation [32]. Mentioned chromosome aberrations cause clinical defects on human body [38, 39].

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. The cause or source of the pollution can be determined using the above Allium M 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.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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