Allium Chromosome Aberration Test for Evaluation Effect of Cleaning Municipal Water with Constructed Wetland (CW) in Sveti Tomaž, Slovenia

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Abstract

We show the effectiveness of the communal waste water cleaning plant of the type "LIMNOWET® Constructed Wetlands (CW)". The tests were done with the Allium metaphase 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 genotoxic substances in the polluted water. The CW plant reduced the degree of genotoxicity 29.0% to 3.5% (the Fisher’s Exact test: p=9.2e–10<0.00001). Therefore CW is a very effective ecoremediation technique, since it removes up to 96% of waste.

Keywords: Constructed wetland (CW) LIMNOWET®; Allium metaphase test; Chromosome damage

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 atrazine [10], benzo(a)pyren [11], pharmaceutical effluents [12], 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 bedtop, 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 polluants; (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 meristeme 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.

Materials 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 3-3.5 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. 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.

Chromosome preparations

The squash technique for onion root as described [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 colchicine 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 1 h 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 400 X and 1000 X.

Macroscopic parameters

After 72 hours growth in the test solution, we measured the root length and noted related parameters as 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 chromatid (breaks), we used the treatment with colchicines. Onion *Allium cepa* L. has 16 monocentric chromosomes (2n=16), with basic number x=8 (Figure 1). The possible aberrations seen at metaphase are: (i) single break chromatide, (ii) double break chromatide, (iii) gap break and (iv) centromere break (Figure 2).

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.

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 significant results. Whichever of these levels we choose depends a great deal on the nature of the data and the problem addressed by the basic assumption. Statistical significance should not be the only determining factor when evaluating the results.

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 tables 1-3, and figures 1-5, and the physical and chemical parameters are displayed in the table 4.

Constructing wetland (CW) described here is located in village Sveti Tomaž in Slovenia (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, vineyards 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 bed 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].

General toxicity–root growth inhibition and malformation

The cleaned water (Sample II) shows longer roots and lesser general toxicity than the uncleaned waste water (Sample I), p<0.01. The latter also induces longer roots than the positive control (Sample IV). The cleaned water induces equal root length as the negative control (Sample III).

Level genotoxicity–induction of chromosome aberration in root meristems cell

The cleaned outflowing water (Sample II) is significantly less genotoxic than the inflowing polluted water (p=9.2e-13<0.00001). So the level of genotoxicity decreases from 29.5% to 3.5%. This is confirmed by NC (p=7.9e-14<0.00001). The outflowing water not statistically significantly different from PC (p=1>0.05) but is significantly different by NC (p=7.9e-14<0.00001).

Physical and chemical parameters

CW effectively reduces the concentration suspended solids for 65.52/71.43%, markedly reducing the COD (chemical need when oxygen) to 84.2/85% and BOD 5 (biochemical oxygen consumption in five days) for 94.0/95.8%.

Discussion

| Example | Number of identified metaphase cell | Average metaphase cells with chromosome damage | Average level genotoxicity (%) | Average length of root (mm) | Percentage (%) length root of negative control |
|---------|-----------------------------------|---------------------------------------------|------------------------------|-----------------------------|--------------------------|
| Wastewater; influent in CW | 200 | 58 ± 6.0 | 29.0 ± 3.0 | 17 ± 3.5 | 41.4 |
| Cleaning water effluent out CW | 200 | 7 ± 2.0 | 3.5 ± 1.0 | 42 ± 3.5 | 100.0 |
| Negative control-NC (tap water filtered with R.O.-reverse osmosis) | 200 | 6 ± 2.0 | 3.0 ± 1.0 | 41 ± 2.0 | 100.0 |
| Positive control-PC (10 ppm Methan Meth sulfonate–MMS 4016 Sigma) | 200 | 61 ± 5.0 | 31.0 ± 2.5 | 12 ± 3.0 | 29.2 |

Table 1: 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.
since there are many CWs and other waste water treatment plants in Slovenia

In our further work, we intend to expand our testing methods with the so called micronucleus test (MN) [33], on the water animals,

| Samples | NC | PC | Inflow | outflow |
|---------|----|----|--------|---------|
| NC      |    |    |        |         |
| PC      | 7.9e−14 | 0.8269 |         |         |
| inflow  | 1.7e−13 | 0.7887 |         |         |
| outflow | 0.7687 | 7.3e−11 | 9.2e−13 |

Table 2: 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.

| Samples | NC | PC | Inflow | outflow |
|---------|----|----|--------|---------|
| NC      |    |    |        |         |
| PC      | 0.01 | 0.88 |         |         |
| inflow  | 0.01 | 0.02 | 0.01   |         |
| outflow | 1   |     |        |         |

Table 3: 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.

Figure 3: The length of the roots of the test plants of Allium cepa L. treated in a sample of urban waste-municipal water.

Figure 4: The length of the roots of the test plants of Allium cepa L. treated in a sample of the cleaned water.

Figure 5: Different number chromosome damage in metaphase cells obtained from the meristeme root-type cells of onion (Allium cepa L.): one damaged chromosome (5A), four damaged chromosome (5B), eight damaged chromosomes (5C), whole chromosome garniture is damaged (5D).

Figure 6: 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 characterized as statistically significant.

Table 4: Some physical-chemical measurements of inflow waste water and outflow cleaning water CW in Sveti Tomaž. Wastewater inflow and cleaning outflow water samples taken common twice, once in July and once in October.

| Parameter       | Wastewater (inflow) | Cleaning water (outflow) | Effect CW (%) |
|-----------------|---------------------|--------------------------|---------------|
|                 | July/October        | July/October             | July/October  |
| Suspended solids (mg/l) | 35/29               | 10/10                    | 71.43/65.52   |
| COD (mg/l)      | 200/190             | 30/30                    | 85.0/84.2     |
| BOD₅ (mg/l)     | 70/50               | 3/3                      | 95.8/94.0     |

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