BioTechnologia vol. 97(3) • pp. 195-201 • 2016
Journal of Biotechnology, Computational Biology and Bionanotechnology RESEARCH PAPERS
DOI: 10.5114/bta.2016.62357

PCR-RAPD based estimation of hospital wastewater genotoxicity on *Allium cepa*

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Abstract

The Chief Sanitary Inspectorate Reports in Poland showed that more than 60% of the hospital wastewater is discharged directly into the sewage system and directed further to municipal wastewater treatment plants. Moreover, 26% of the hospital wastewater is discharged directly into the receivers without any treatment processes and 20% of the hospitals do not disinfect the wastewater that requires sanitization. Such situation can occur not only in Poland. Thus, an estimation of raw wastewater genotoxic impact on environment is understandable, justified, and necessary. Genotoxicity tests performed on plants are becoming more and more popular because they are cheap and easy in the usage compared to other tests based on e.g., cell cultures. DNA isolated from the root meristem cells of onion (*Allium cepa*) exposed to the increasing concentrations of hospital wastewater were tested using PCR-RAPD. The results obtained in this study confirmed the genotoxic effect of raw hospital wastewater on genetic material, which at concentrations between 75% and 100% caused the genotoxic effect less visible than for wastewater in concentrations up to 75%.

Key words: *Allium cepa*, hospital wastewater, genotoxicity, PCR-RAPD

Introduction

The hospital wastewater is a particular sewage, dangerous for humans, animals, and plants (Ekhaise and Omavwoya, 2008). Its composition is diverse and similar to both communal and industrial wastewater, containing drugs and their metabolites, large amounts of pathogenic microorganisms, radioactive substances, and other toxic compounds. Some of the substances, such as anticancer drugs, radioactive iodine ^{131}I, or AOX (absorbable organic halides), are not removed during biological treatment processes and are directed into the water bodies, affecting the biological balance with a cumulative effect. Due to its composition, hospital wastewater requires special treatment before it enters the communal sewerage. It also contains pathogenic bacteria and therefore, additional disinfection should also be carried out. Under Polish law, disinfection is required for the wastewater from infectious hospital wards and blood donation stations (Polish Journals of Law 2001 no. 72 pos. 747). However, such disinfection may have a negative effect on the activated sludge community during the biological treatment of hospital wastewater and can also lead to the formation of disinfection by-products. Recent reports revealed that more than 60% of the hospital wastewater is discharged directly into the sewage system and directed further to municipal wastewater treatment plants. This certainly disturbs the biological wastewater treatment processes. More than 26% of the hospital wastewater is directed to the receivers without any post-treatment and becomes a potential source of environmental risk (Chief Sanitary Inspectorate Reports, 2008). Substances present in hospital wastewater may persist in the ecosystem causing short- or long-term damage to the environment, plants, and animals. Considering the amount and diversity of hospital wastewater produced, the ecotoxicological and sanitary risks should be assessed (Pauwels and Verstraete, 2006). Thus, constant monitoring of what happens to wastewater released into the environment would be advisable and it is the current research topic.

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Previous research has revealed that hospital wastewater can be potentially genotoxic due to the presence of cytostatic drugs (Bagatini et al., 2009). However, considering the wide range of parameters influencing the research results (types ofhospitals and wards, number of patients, volume and composition of wastewater produced, and different genotoxicity tests), these data cannot be compared directly and require an individual approach (Jolibois and Guerbet, 2005).

Plants, among other genetic models for genotoxic research, are known to be useful for environmental mutagens' studies (Leme and Marin-Morales, 2009). Some of the plants are used for short-term tests and are known to be reliable and sensitive. Onion (*Allium cepa*) has been widely used in chromosomal aberration research caused by chemical mutagens since 1940s. *Allium cepa* tests are cheap and the results can easily be analyzed, contrary to other short-term tests (Leme and Marin-Morales, 2009; Ferreti et al., 2007).

Among molecular tools for genotoxicity assessment, the PCR-RAPD (polymerase chain reaction-random amplified polymorphic DNA) is often used. Although RAPD analyzes do not reveal the cause or the level of damage to the genetic material, the RAPD fingerprints differences can show the DNA damage, mutation, or DNA rearrangement visible as DNA sequence alterations (De Wolf et al., 2004).

Following the disturbing information found in the reports about the toxic impact of hospital wastewater to the environment, which are directed to the receivers without any post-treatment (Chief Sanitary Inspectorate Reports, 2008), we decided to assess the genotoxicity of the raw wastewater derived from St Joseph's Hospital for Lung Diseases in Pilchowice, Poland, on the root meristem cells of *Allium cepa* using PCR-RAPD. This research includes an analysis performed on disinfected wastewater to compare their genotoxicity.

Materials and methods

Hospital wastewater characteristics

In this experiment hospital wastewater derived from St. Joseph's Hospital for Lung Diseases in Pilchowice, Poland, was used. One of the wards in this hospital is an infectious diseases ward. Wastewater from the hospital is pre-treated and disinfected in the hospital wastewater treatment plant according to the Regulation of the Ministry of Health (Polish Journals of Law, 2001 No. 7 item 747). In this experiment we analyzed the raw wastewater from the primary settler from which wastewater is directed into the hospital sewerage. The sewage was stored at 4 °C until the experiment started. Table 1 presents the physicochemical parameters of the wastewater used in the experiment.

Table 1. Physicochemical parameters of the wastewater used in the experiment

Parameter	Value	Unit
pH	7.2	–
Biological oxygen demand (BOD_5)	460	[mg O_2/l]
Chemical oxygen demand (COD)	860	[mg O_2/l]
Total organic carbon (TOC)	520	[mg C/l]
Phosphates	5.8	[mg PO_4^3/l]

Before the experiment, the wastewater was mixed and filtered using paper filters (MN-GF1 Ø150 mm, Macherey-Nagel GmbH & Co. KG) to remove suspended solids. Clarified wastewater was diluted in distilled water to obtain the following concentrations: 100%, 75%, 50%, 25%, 12.5%, and 6.25%.

Plant material preparation

The experiment was performed on *Allium cepa* germinated seeds with roots ("Barletta" onion, Gartenl and GmbH Aschersleben). The seeds were washed with tap water and detergent, then with 10% acetic acid and rinsed with distilled water. Before germination in a sprouter (Bio-natura), the seeds were soaked in warm boiled water for 4 h. During germination they were watered with warm boiled water for 7 days. After this period 10 seeds per sample with roots approx. 2-4 cm in length were used for further procedure. Eppendorf tubes were filled with wastewater at concentrations: 100%, 75%, 50%, 25%, 12.5%, and 6.25%. Ethyl methanesulfonate (EMS, Sigma, 10 mg/l) and 3% H_2O_2 were used as positive controls, while tap water constituted a negative control, as recommended (Cotelle and Férrard, 1999; Pourrut et al. 2015). Ten germinated seeds were placed in one tube. The tubes were incubated at room temperature for 96 h. After the incubation period, the meristem of the root (approx. 2 cm) was cut off and kept in 70% ethanol at 4 °C until DNA isolation.

DNA isolation, PCR-RAPD procedure and the results obtained in the analysis

For total genomic DNA isolation of the plant, a Genomic Mini AX Plant Spin (A&A Biotechnology) was used. DNA concentration was measured with Qubit (Invitrogen) and uniformed to 2.5 ng/µl for PCR reaction. The electrophoresis was performed in 1% agarose gel (Promega) in 1 × TBE buffer (100 mM Tris pH = 8.3, 90 mM boric acid, 1 mM EDTA) with ethidium bromide (10 µl/ml, Promega). The electrophoresis was performed at 100 V for 90 min. The gel was visualized under UV light and photographed.

PCR-RAPD was performed with primers OPA04 (5′ AATCGGGCTG 3′), OPA19 (5′ CAAACGTCGG 3′), OPA20 (5′ GTTGCGATCC 3′), and OPB11 (5′ GTAGAC CCGT 3′) (Williams et al., 1990). To optimize the PCR reaction Taguchi method was used (Cobb and Clarkson, 1994). The PCR optimization procedure was as previously described (Szulc et al., 2012). The reaction mixture, volume of 30 µl, consisted of 15.5 µl MiliQ water, 6 µl 5 × Green GoTaq Flexi Buffer (Promega), 2.4 µl $MgCl_2$ (25 mM, Promega), 1.3 µl dNTPs Mix (1 mM, Promega), 0.5 µl of the primer, and 0.3 µl GoTaq DNA Polymerase (5 u/µl, Promega). Genetic material was added in a volume of 4 µl (2.5 ng/µl). PCR amplification was performed in C-1000 Thermocycler (BioRad) as previously described (Szulc et al., 2012). PCR products were visualized in 1% agarose gel as described earlier. A 1 kb DNA Ladder (Promega) was used for DNA size estimation. The fingerprints were analyzed with Quantity One 1D Software (BioRad) and based on it diagrams of genetic similarities and dendrograms presenting genetic distance were constructed. The dendrograms were constructed with neighbor-joining algorithm using Dice coefficient.

Results and discussion

The disturbing information found in the Polish Chief Sanitary Inspectorate Reports stating that more than 26% of hospital wastewater is directed to the clean water supplies without any treatment, made it obvious that the genotoxicity assessment of hospital wastewater is an important part of environmental monitoring. In this experiment we used PCR-RAPD for genotoxicity research performed on raw hospital wastewater.

The DNA isolated from root meristems of *A. cepa* during the experiment differed in concentration. The concentration of DNA isolated from *Allium cepa* root meristems (equal in weight), incubated in increasing concentrations of hospital wastewater was measured with Qubit (Invitrogen). The results of DNA concentration measurements for hospital wastewater concentration from 6.25% to 100% were: 4.9; 6.11; 11; 8.59; 8.56; and 31.8 ng/µl, respectively. For positive controls, EMS (10 mg/l) and H_2O_2 (3%), and negative control,water, the DNA concentration was 2.53, 5.4, and 38.9 ng/µl, respectively (Fig. 1).

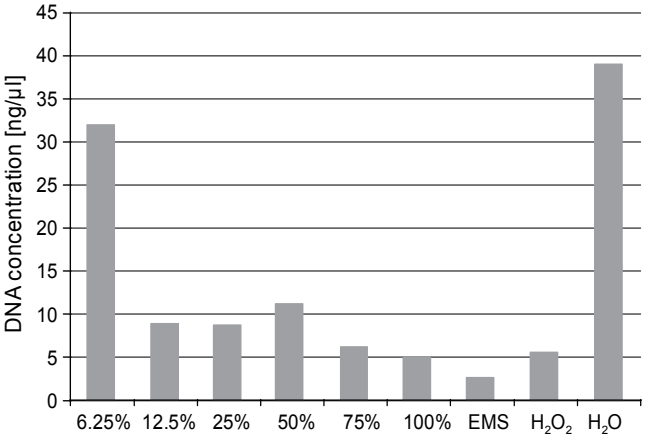

Fig. 1. The concentration of DNA samples treated with increasing concentrations of hospital wastewater; EMS and H_2O_2 – positive controls, H_2O – negative control

The highest DNA concentration was in the negative control and a sample incubated in not diluted hospital wastewater, the lowest was determined for the positive control in EMS. Such a high concentration of DNA isolated from not diluted wastewater sample is baffling, while for other wastewater concentrations the DNA level is almost 6 times lower. It was previously reported that for *A. cepa*, DNA extraction has a relatively low efficiency due to the presence of secondary metabolites in its cells (Abu-Romman, 2011). The onion cells possess, among others elements, essential oils containing sulfur compounds, flavonoids, phenol acids, saponins, and steroles. Selenium, zinc, iron, magnesium, phosphorus, and calcium salts are also present inside their cells (Ekholm et al., 2007). Flavonoids, phenols, and sterols can be released from vacuoles interfering in DNA isolation (Abu-Romman, 2011).

PCR-RAPD was performed to present the genotoxic effect of the hospital wastewater used in the experiment. Four primers, characteristic for *A. cepa*, were used: OPA04, OPA19, OPA20, and OPB11. Figure 2A-D pre-

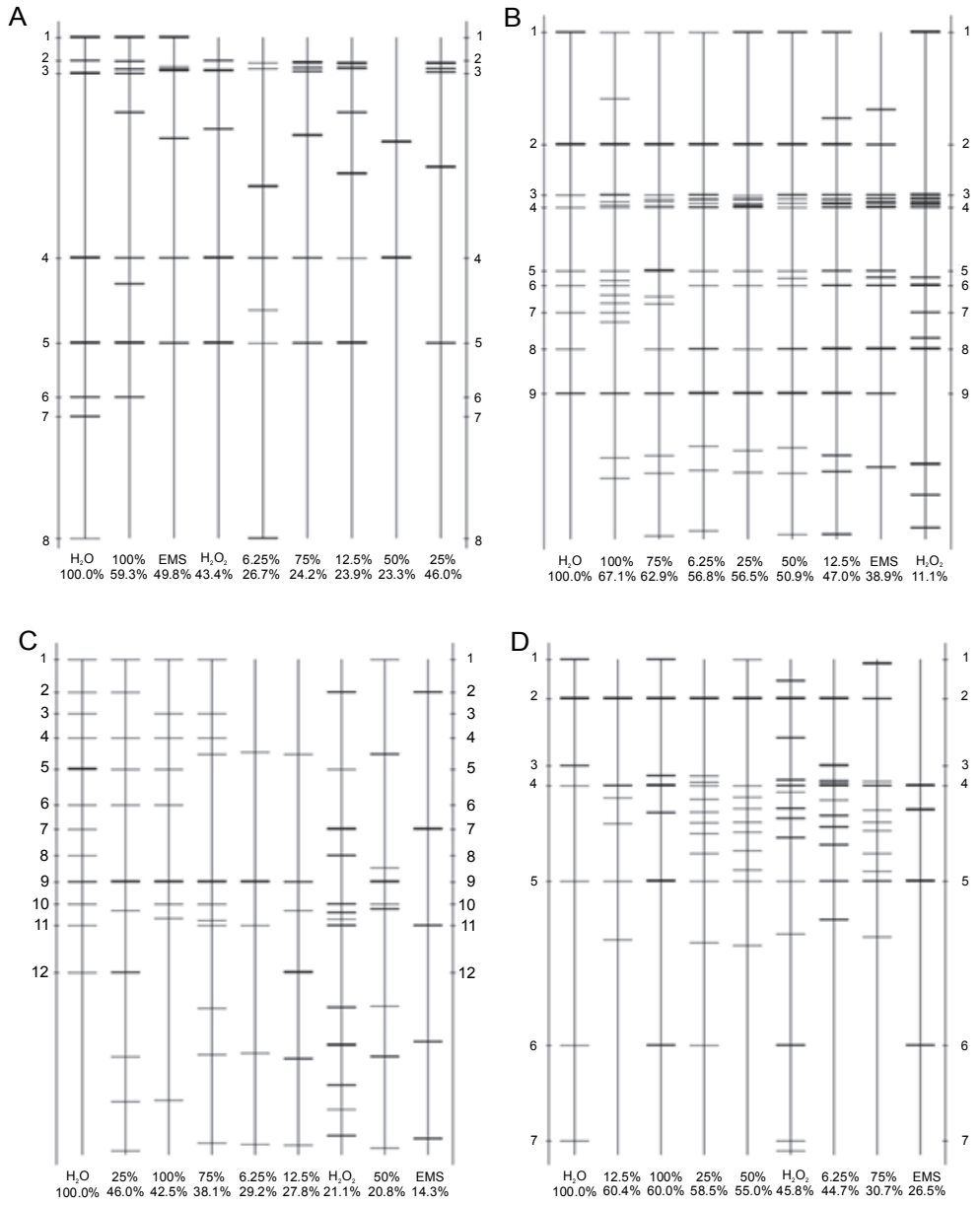

Fig. 2. Similarity diagram for DNA fingerprints generated with primers: A) OPA04; B) OPA19; C) OPA20 and D) OPB11 for *A.cepa* root meristem cells incubated in increasing concentrations of hospital wastewater ranged between 6.25% and 100%; EMS and H_2O_2 – positive controls, H_2O – negative control; the fingerprints are compared in descending order of the similarity to the negative control fingerprint, in which DNA bands are marked with numbers

sents the diagrams of similarities, Figure 3A-D presents genetic distance dendrograms. The similarity profiles for RAPD fingerprints calculated with Quantity One 1D Analysis Software (BioRad) were analyzed statistically. The results of the analysis are presented in Figure 4.

RAPD profiles obtained in this study were repeatable. These results stand in contradiction to Qari (2010), who obtained repeatable results only for OPA04 and OPA20 primers. However, Qari (2010) did not perform PCR optimization which could be the reason for non-repeatable results for amplification with OPA19 and OPB11 primers (Cob and Clarkson, 1994; Atienzar and Jha, 2006). For this experiment we used the Taguchi method for PCR optimization (Szulc et al., 2012). The results of the dendrograms (Fig. 3) generated based on all optimized PCR-RAPD performed were also repeatable, which underlined the need of such procedure before initiation of the PCR-based experiment. The RAPD profile analysis obtained with primers OPA04, OPA19, OPA20, and OPB11 suggests that all concentrations

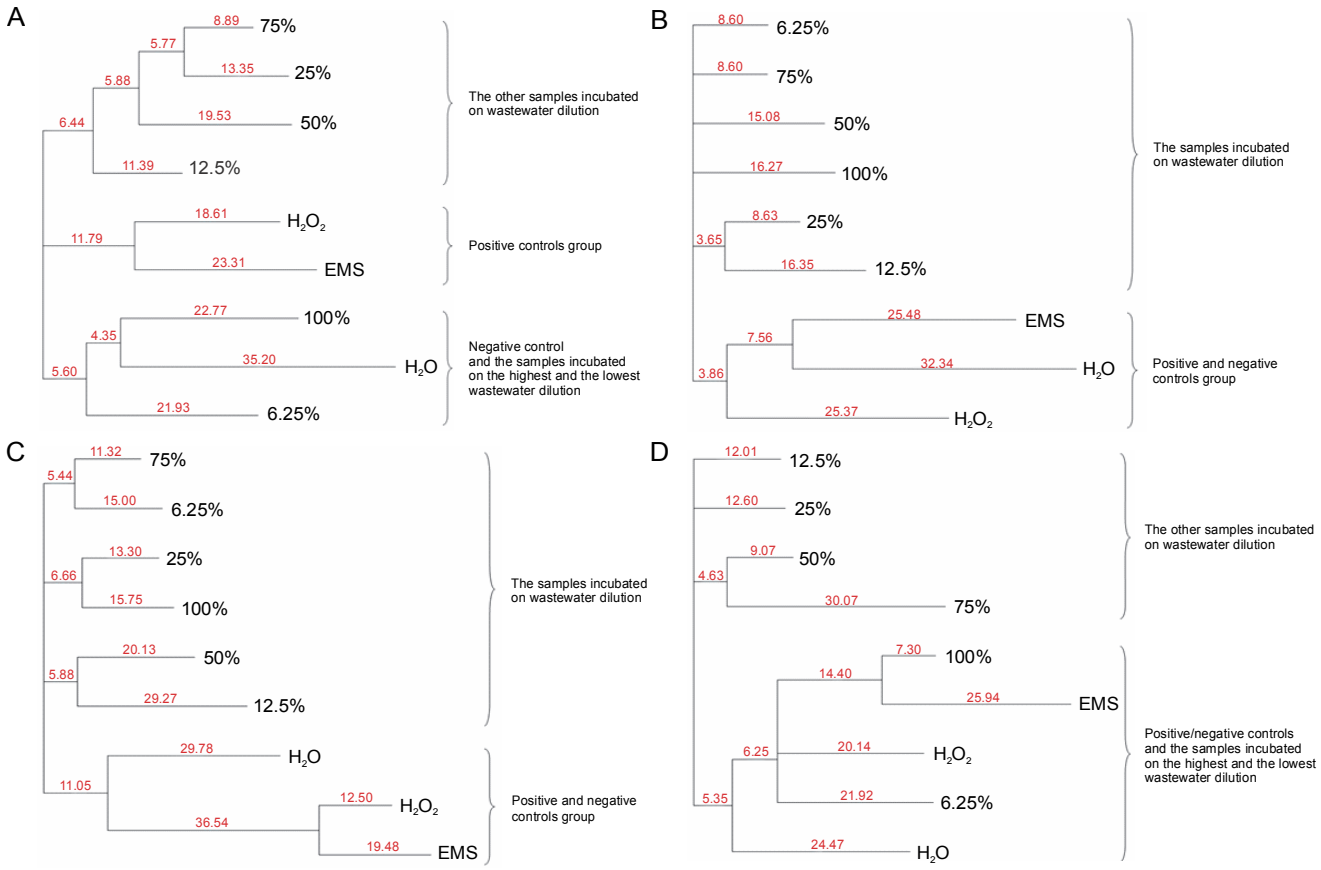

Fig. 3. Dendrograms constructed with neighbor-joining algorithm for RAPD profiles with primers: A) OPA04; B) OPA19; C) OPA20 and D) OPB11 for *A. cepa* root meristem cells; samples incubated inincreasing concentrations of hospital wastewater, ranged between 6.25% and 100%; EMS and H_2O_2 – positive controls, H_2O – negative control

of hospital wastewater used in the experiment cause changes in the DNA of the root meristem cells of *Allium cepa*.

The degree of similarity of profiles varies in relatively wide limits and is not correlated with wastewater concentration (Fig. 2). Similar results were obtained by Akinboro et al. (2011). Such a situation might occur because the DNA changes caused by hospital wastewater appear randomly. Moreover, RAPD can reveal changes causing appearance or disappearance of primer complementary sites in DNA, thus not all alterations in the material can be revealed. The usage of several types of random primers in RAPD increases the chance of the detection of other changes (Atienzar and Jha, 2006). The changes in DNA profiles in RAPD are based not only on the presence or absence of DNA bands but also on their band density change in comparison to positive and negative controls. As it was mentioned earlier, PCR-RAPD can reveal the DNA change but answering the question as to what causes such changes is not possible using this method (Atienzar et al., 2002). Therefore, we were able to confirm only the genotoxic effect of hospital wastewater with no explanation of its mechanism.

The lowest genetic similarity in RAPD profiles was for OPA04 primer, while other primers presented profiles that were far more similar (Fig. 4). This result allows us to state that among the primers used in the experiment, OPA04 is the most efficient in DNA changes detection in the studied material for hospital wastewater. The exception is the non-diluted wastewater sample (100%) in which for all used primers, the profiles generated in the analysis were similar in approx. 60% compared to the negative control. This may support the hypothesis that after passing the critical level of the DNA damage (induced by hospital wastewater in concentration ranged between 75% and 100%), the efficient cellular repairing processes are being turned on. It is worth mentioning that genetic similarity for DNA pro-

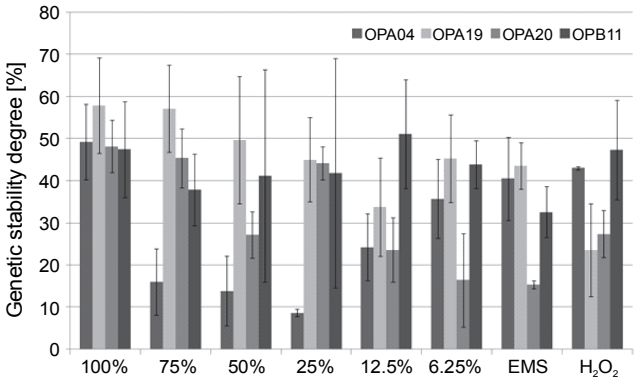

Fig. 4. The comparison of PCR-RAPD genetic stability degree obtained for the samples treated with hospital wastewater in concentrations of 6.25-100% and positive controls (EMS and H_2O_2) with the negative control (H_2O) obtained for primers: OPA04, OPA19, OPA20, and OPB11

files obtained from samples from hospital wastewater at the concentration of 6.25% and EMS positive control is similar for all primers used. It allows us to assume that even in such a low concentration wastewater may generate changes at the level of the positive control of EMS (10 mg/l). On the other hand, it should be underlined that the genetic profiles for RAPD are far different for both positive controls: EMS and H_2O_2, which might be caused by different genotoxic mechanism of these molecules and various change induction pathway (Konat, 2003; Gocke et al., 2009).

The dendrogram generated for OPA04 primer from its RAPD profiles (Fig. 3A) consists of three clusters. As can be seen in Figure 3A, positive controls create one cluster of the dendrogram, the other created by samples incubated at the highest and the lowest wastewater concentrations together with the negative control, the last cluster group created by the samples incubated in 25%, 50%, and 75% wastewater concentrations. Similar results were obtained for OPB11 primer (Fig. 3D). The wastewater concentrations 75%, 50%, 25%, and 12.5% caused mutations creating a separate cluster. The dendrograms for primers OPA19 (Fig. 3B) and OPA20 (Fig. 2C) present clearly the clusters for the samples incubated in hospital wastewater and for the positive/negative controls, which suggests a different genotoxic mechanism. Additionally, a longer genetic distance between these profiles suggests a higher level of hospital wastewater genotoxicity than for positive controls with proven mutagenic effect. The dendrograms presented in this study confirm the randomness of genotoxic mechanisms of hospital wastewater because the genetic distances are not correlated with the wastewater concentration used in the experiment.

Conclusions

Based on the research performed in this study it could be stated that:

- Raw wastewater obtained from St. Joseph's Hospital for Lung Diseases in Pilchowice, Poland, presents genotoxic effect on the DNA from root meristem cells of *Allium cepa* with no clear correlation between wastewater concentration and mutagenic effect.
- The hospital wastewater at concentrations between 75% and 100% caused genotoxic effect that was less visible in PCR-RAPD fingerprints than for wastewater in concentrations up to 75%. These results suggest that hospital wastewater at lower concentrations could affect the DNA much stronger than its high concentrations. It could be suspected that the damage of DNA in samples influenced with hospital wastewater in concentrations higher that 75% was so strong that it could turn on the DNA repairing mechanisms. This statement requires further research.
- PCR-RAPD after optimization is a useful tool for genotoxicity estimations in *Allium cepa* meristem root cells.
- OPA04, OPA19, OPA20, and OPB11 primers enable to obtain repeatable PCR-RAPD profiles in *A. cepa* DNA, when PCR optimization is performed. Genetic profiles analyzes reveal similar results.

Acknowledgement

This research was supported by the Ministry of Science and Higher Education, grant no: N N523 561038.

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