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Comet Assay Assessment of Wastewater Genotoxicity Using Yeast Cells

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Many natural and synthetic chemicals can damage DNA and result in either lethality or damage to cellular functions. To establish which chemicals and naturally occurring mixtures like wastewaters and industrial liquid wastes are DNA-damaging agents and to gain an understanding of how they affect cells, it is necessary to develop test systems that permit their convenient detection and evaluation.

Various test organisms are used for aquatic toxicity bioassays, ranging from prokaryotes, single cell algae, invertebrates to fish (Mitchell et al. 2002). Toxicity bioassays provide a mean of measuring the combined toxicity effects of all contaminants present and give us an integrated picture of the overall toxicity. Eukaryotic microorganisms, like protozoa, algae and yeast cells provide simple, less expensive test systems, since these organisms are easily grown in axenic laboratory cultures and provide the possibility of avoiding the use of laboratory or wild test animals.

During the last decade the single cell gel electrophoresis (SCGE) or comet assay has been widely used to detect and quantify genotoxic activities. The alkaline version of the method is a very sensitive assay for detection of single strand breaks in DNA, alkali-labile sites, and other damage that generates DNA breaks (Horváthová et al. 1998). The comet assay was originally developed for evaluating DNA damage in human blood cells (Singh et al. 1988) and it is widely used in human biomonitoring as an assay of DNA breakage, DNA damage and DNA repair following the exposure to genotoxic agents (Rojas et al. 1999; McKelvey-Martin et al. 1993). The assay is becoming a major tool for environmental biomonitoring as this technique measures the cumulative DNA damage caused by all toxic pollutants that are available to the organism (Šalagovič et al. 1996; Mitchelmore and Chipman 1998). For this purpose the SCGE assay has been applied to different eukaryotic cells and organisms.

The comet assay, developed by Singh et al. (1988), was modified and applied to *Saccharomyces cerevisiae* yeast cells in the present study. Modifications of the original protocol were made at the level of cell preparation, incubation time in

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alkaline lysis buffer and electrophoresis time. In order to overcome the problem that the yeast cell wall prevented DNA migration during electrophoresis, we let the cell walls partially disintegrate in alkaline buffer following embedding in agarose. Short time exposures of *Saccharomyces cerevisiae* cells to increasing concentrations of a well-known genotoxic compound, hydrogen peroxide, led to dose-dependent DNA damage. Hydrogen peroxide is known to produce base oxidation and single strand breaks, mediated by the highly reactive hydroxyl radicals (Horváthová et al. 1998). The protocol developed was then used to measure the genotoxic potential of influent and effluent samples from the local municipal wastewater treatment plant. Statistical analysis of the results indicated that the quantification of the genotoxic potential of the influent wastewater and the reduction of genotoxicity in effluent water was reliable.

MATERIALS AND METHODS

General reagents and chemicals used to perform the comet assay were purchased from Sigma (St. Louis, USA) and Merck (Darmstadt, Germany).

S. cerevisiae strain ZIM 1514 was used as a test organism. Cells were cultivated in rich YPD (yeast peptone D-glucose) medium (1 % (w/v) yeast extract, 2 % (w/v) bactopeptone and 2 % (w/v) glucose) (Novick and Bostein 1985) at 30 °C for 18 hr. Yeast cells were collected by centrifugation at 300 x g for 3 min, washed with water, and resuspended in S-buffer (1*M* sorbitol, 25 m*M* KH_2PO_4 pH 6.5).

To achieve a uniform background rough microscope slides were first coated with up to 400 µL of 1 % normal melting point agarose (NMP). They were left to air dry overnight. The supportive (second) agarose layer (0.6 % NMP agarose) was solidified on ice and the collected yeast cells were immobilized in the third layer. Approximately 2 x 10^4 cells were mixed with 0.7 % low melting point agarose (LMP) and spread over the slides as the third layer. After removing the cover glasses, the slides were covered with 500 µL of 0.5 % LMP agarose (the fourth layer) to prevent yeast DNA escaping during cell lysis and electrophoresis.

The alkaline version of the comet assay was performed with the major modifications of the original protocol described by Singh et al (1988). In protocol 1 agarose embedded yeast cells were exposed to increasing concentrations of hydrogen peroxide (0.01, 0.05 and 0.1 m*M* H_2O_2) for 5 min and then washed with PBS (phosphate-buffered saline) buffer. The dose response was followed by further steps of the comet assay protocol. Four layered slides were incubated in 30 m*M* NaOH, 1*M* NaCl, 0.1 % N-laurylsarcosine, 100 m*M* DMSO and 1 % Triton-X 100 for 75 min to lyse the yeast cells. The slides were rinsed three times for 20 min in 30 m*M* NaOH, 2 m*M* EDTA, pH 12.4 to unwind the nuclear DNA and then subjected to electrophoresis in the same buffer. The electrophoresis was carried out for 5 min at 25 V and 300 mA. Following electrophoresis the gels were neutralized in 400 m*M* Tris-HCl pH 7.5 for 15 min. During electrophoresis the damaged DNA traveled towards the anode and formed an image of a "comet" tail upon staining with ethidium bromide (20 µg/mL) that was detected and quanitified as described below.

To test the necessity of using a cell wall disintegrating enzyme, an additional step was introduced in the protocol. In protocol 2 aliquots of approximately 2 x 10^4 cells were mixed with 0.7 % LMP agarose containing 2 mg/mL of the enzyme preparation Lyticase (1,3-β-glucosidase, EC 3.2.1.39; Sigma, L-2524) and spread over the slides. Covered with cover glasses, slides were incubated at 30 °C for 20 min, to disintegrate the yeast cell wall. The enzyme was inactivated on an ice cold surface (4 °C) for 5 min and then covered with the fourth layer of 0.5 % LPM agarose (protocol 2). Further steps of protocol 2 (exposure to increasing concentrations of the genotoxic agent H_2O_2, cell lysis, unwinding of DNA, electrophoresis and neutralization) were the same as described in protocol 1.

To test the genotoxicity of complex water samples by protocol 1 influent and effluent wastewater samples from the largest Slovenian biological wastewater treatment plant were chosen. The plant is of a conventional type, designed for organic carbon elimination (200 000 population equivalents; daily inflow of 13 500 kg BOD – biochemical oxygen demand), treating municipal (70 %) and industrial (30 %; pharmaceutical, electroplating, oil, tanning, food, furniture, textile and color production industries) wastewater. Sampling of the wastewater was done according to the recommended standard method (SIST ISO 5667-10). Freshly taken influent and effluent wastewater samples were brought to the laboratory in 500 mL glass flasks and stored at +4 °C for 1 hr until performance of the assay. When testing the genotoxic effect of wastewater samples slides were incubated in three different dilutions of influent and undiluted effluent water for 25 min and then washed with PBS buffer. 10 % and 50 % influent waste water samples were prepared in PBS buffer. Slides representing the negative control were exposed to PBS buffer for 25 min (protocol 1 and protocol 2).

Slides were analysed with an epifluorescent microscope (Olympus BX50), using a BP 515-560 nm excitation filter and a barrier filter of LP 590 nm at 400 x magnification. Microscopic images of comets were captured by a digital camera (Hamamatsu Orca 2), connected to a computer, and comets scored by Komet 5.0 Computer Software (Kinetic Imaging Ltd. 2001). Under the alkaline conditions of the comet assay electrophoresis step, the damaged nuclear DNA migrates towards the anode forming an image of a "comet" tail upon staining with ethidium bromide. The head of the "comet" corresponds to the undamaged nuclear DNA. The migration length and fluorescence intensity of the "comet" tail are related to the degree of DNA damage. Kinetic's Komet analysis software is based upon the principle of integrated image intensity profile analysis and calculation of various comet parameters. In our study the tail length and percentage of DNA in the comet's tails and heads were determined. These were further used to calculate the Olive tail moment = (Tail mean-Head mean) X % DNA Tail/100 (arbitrary units) which represents the degree of nuclear DNA damage (Olive et al. 1990). Within one experiment, data for 100 cell nuclei per slide per treatment were pooled. Three independent experiments for the genotoxic agent H_2O_2 and wastewater samples were carried out.

Data were statistically analyzed with the SAS/STAT package (SAS 2000). Bauer et al. (1998) suggested that the distribution of the Olive tail moment (OTM) follows a chi-

square (χ^2) distribution, which is a special case of the gamma distribution. Boxplots (Figure 1 and 2) revealed the same conclusion for our data. Therefore a generalized model with a gamma distribution and an identity link function was used to model the data in the present study. We modeled the treatment effect (peroxide concentration and percentage of wastewater) by classes (B_j) in model 1, as well as by covariate (x_{ijk}) - regression in model 2. The effect of protocol/experiment (A_i) and interaction (AB_{ij}) between treatment and protocol/experiment effect in model 1 (Equation 1) and nested regression (b_i) within protocol/experiment in model 2 (Equation 2) were also included. The symbols y_{ijk}, μ, e_{ijk} in the models represent values for OTM, overall mean, corresponding unexplained deviations and errors from expected values, respectively. The last class of wastewater (the effluent) was excluded from the regression analysis, due to its distinct characteristics. Linear regression adequately described the increasing trend of tail moments with increasing concentration, while quadratic regression was not significant (p>0.25). Box plots revealed some measurements (outliers) which had substantial influence on the the results, and were therefore excluded from the analyses (crossed dots in box plots - Figure 1 and 2).

Model 1: $$y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk} \quad (1)$$

Model 2: $$y_{ijk} = \mu + A_i + b\left(x_{ijk} - \bar{x}\right) + b_i\left(x_{ijk} - \bar{x}\right) + e_{ijk} \quad (2)$$

Physical and chemical parameters (pH, chemical oxygen demand (SIST ISO 6060), biochemical oxygen demand (SIST EN 1899/2), total organic carbon (SIST ISO 8245), Kjeldahl nitrogen (SIST EN 25663), ammonium nitrogen (SIST EN 25664), nitrite nitrogen (SIST EN 26777), nitrate nitrogen (SIST ISO 7890/1), total phosphorus, copper, zinc, cadmium, chromium, nickel, lead, iron (SIST ISO 5815), total cyanide (SIST ISO 6703) and phenol (SIST ISO 6439)) of influent and effluent samples from the local wastewater treatment plant were determined according to the recommended ISO standards.

RESULTS AND DISCUSSION

The aim of the study was to design the most appropriate *in vitro* test protocol to assure that the assay would be sensitive enough to detect and quantify the genotoxic effects of chemically known and unknown substances and their mixtures. Due to the cytological traits of yeast cells, some technical problems were expected. Yeasts have complex cell walls and to achieve their successful disintegration to allow the migration of DNA in an electric field, two modified protocols of the comet assay were tested: without enzyme application (protocol 1) and with the application of the Lyticase (protocol 2).

In general OTM values, representing the degree of DNA damage, increased with increasing hydrogen peroxide concentration and showed a broader dispersion at the highest hydrogen peroxide concentration both in protocol 1 and protocol 2 (Figure 1), as well as for wastewater dilutions in both experiments. Dose-response effects were

recorded for both protocols with hydrogen peroxide (Figure 1) and for both experiments with wastewater (Figure 2).

The analysis of variance showed, as expected, the significant effect ($p \leq 0.0118$, by model 1 and 2) of enzyme application when testing the genotoxic effect of hydrogen peroxide. OTM values were higher and more dispersed when the lysing enzyme was applied (Figure 1 – b). The effect of treatment (hydrogen peroxide concentration) was also significant ($p=0.0001$) in the case of both models. Least square mean values (LSM) for all tested concentrations of hydrogen peroxide were statistically different ($p<0.05$). The interaction of the protocol and the treatment was significant in the case of hydrogen peroxide ($p=0.0002$), which is in agreement with the significantly different slopes ($p=0.0001$) of linear regression within the protocol . The use of enzyme preparation Lyticase (protocol 2) caused an approximately one fold (0.0019 vs 0.0037) increase in OTM, as modelled by linear regression.

There was no significant difference between two independent repetitions of the experiment with the same wastewater samples ($p=0.1710$). However, the effect of treatment (wastewater concentration) was significant ($p=0.0001$) in both repetitions by model 1 and 2. All differences between the control and the tested concentrations of wastewater were significant ($p<0.05$). Although there was no significant difference between 10 % and 50 % and 50 % and 100 % concentration of wastewater, where 100 % concentration represents the undilluted influent of the wastewater treatment plant, the dose-response is indicated by significant regression of OTM on percentage of wastewater ($b_1=0.0022$, $b_2=0.0012$). No difference ($p=0.1021$) in the slopes of OTM increase between independent repetitions of the experiment with wastewater samples excluding effluent was found. The interaction between experiment and treatment was also not significant ($p=0.5540$). Both results imply the reproducibility of the designed test protocol.

Based on the results shown in Figures 1 and 2 it could be assumed that the use of enzyme preparation Lyticase in protocol 2 was not crucial, although there was higher degree of DNA damage found, which occurred at lower concentrations of hydrogen peroxide in comparison with protocol 1 (Figure 1). This could be explained by the higher disintegration rate of yeast cell walls caused by the enzyme. In order to develop a sensitive and low cost *in vitro* biotest, protocol 1 without enzyme was applied further, where the incubation time of slides in cell lysis buffer was prolonged and the electrophoresis time reduced. The protocol 1 was less expensive and adequate enough for genotoxicity assessment.

The results confirm that the modified comet assay on yeast cells is reproducible and sensitive enough for rapid genotoxicity detection and quantification of wastewater samples and enables the reduction of genotoxicity in the municipal wastewater treatment process to be estimated (Figure 2).

Table 1. Physical and chemical parameters of influent and effluent wastewater samples from the local wastewater treatment plant.

Parameter	Influent	Effluent
pH	7.59	7.54
Total suspended solids	359 mg/L	28.9 mg/L
Chemical oxygen demand	1093 mg O_2/L	89.5 mg O_2/L
Biochemical oxygen demand (BOD_5)	140 mg O_2/L	20 mg O_2/L
Total organic carbon	118 mg C/L	32.7 mg C/L
Kjeldahl nitrogen	70.1 mg N/L	28.8 mg N/L
Ammonium nitrogen	29.4 mg N/L	22.7 mg N/L
Nitrite nitrogen	0.036 mg N/L	0.644 mg N/L
Nitrate nitrogen	0.781 mg N/L	6.24 mg N/L
Total phosphorus	9.33 mg P/L	3.47 mg P/L
Copper	0.0516 mg Cu/L	0.0145 mg Cu/L
Zinc	0.479 mg Zn/L	0.138 mg Zn/L
Cadmium	<0.002 mg Cd/L	<0.002 mg Cd/L
Chromium	0.041 mg Cr/L	0.023 mg Cr/L
Nickel	0.084 mg Ni/L	0.030 mg Ni/L
Lead	<0.02 mg Pb/L	<0.02 mg Pb/L
Iron	1.076 mg Fe/L	0.366 mg Fe/l
Phenol	0.152 mg C_6H_5O/L	0.003 mg C_6H_5O/L
Total cyanide	0.045 mg CN/L	0.008 mg CN/L

a, b, c, d Different letters characterize statistically significant differences ($p<0.05$); SEE – standard error of estimate.

In comparison with the only comet assay test protocol for yeast cells found in the literature (Miloshev et al. 2001), major modifications were introduced in our procedure, especially at the level of yeast cells exposure to genotoxic agents or wastewater samples. Miloshev et al. (2001) applied the comet assay on yeast as an *in vivo* assay. They added 1, 5 or 10 μM solutions of hydrogen peroxide for 1 hr to the yeast culture in the middle of the logarithmic growth phase. The present study describes the exposure of yeast cells embedded in the third layer of 0.7% LMP agarose to increasing concentrations of hydrogen peroxide (for 5 min) and to different dilutions of influent and effluent wastewaters (for 25 min). A type of test strip was designed which would provide a fast and reliable response to the presence of genotoxic agents in environmental samples.

We were able to detect and quantify the genotoxic potential of a known genotoxic compound and of a mixture of such compounds in waste water samples by applying the developed test protocol (protocol 1).

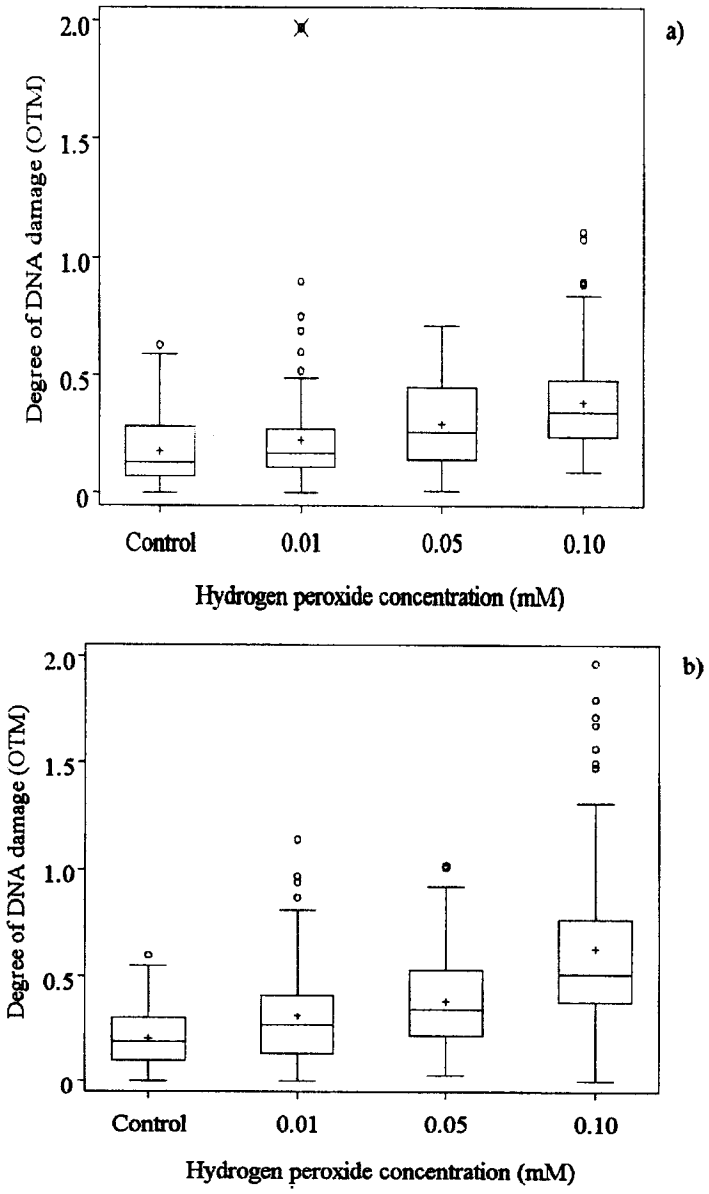

Figure 1. Degree of yeast DNA damage (represented as OTM) caused by different concentrations of H_2O_2 (a – protocol 1, b – protocol 2 with Lyticase). Results from 100 comets are shown with box-and-whisker plots. Measured values at the concentrations tested are shown as boxes that include 50 % of the data. The top and bottom of the boxes mark the 25th and 75th percentiles, the inner line marks the median value, while the plus symbol marks the mean value. 25 % of the data above the 75th percentile and 25 % of the data below the 25th percentile are marked as "whiskers" limited by the maximum or minimum values. Outliers are displayed as points.

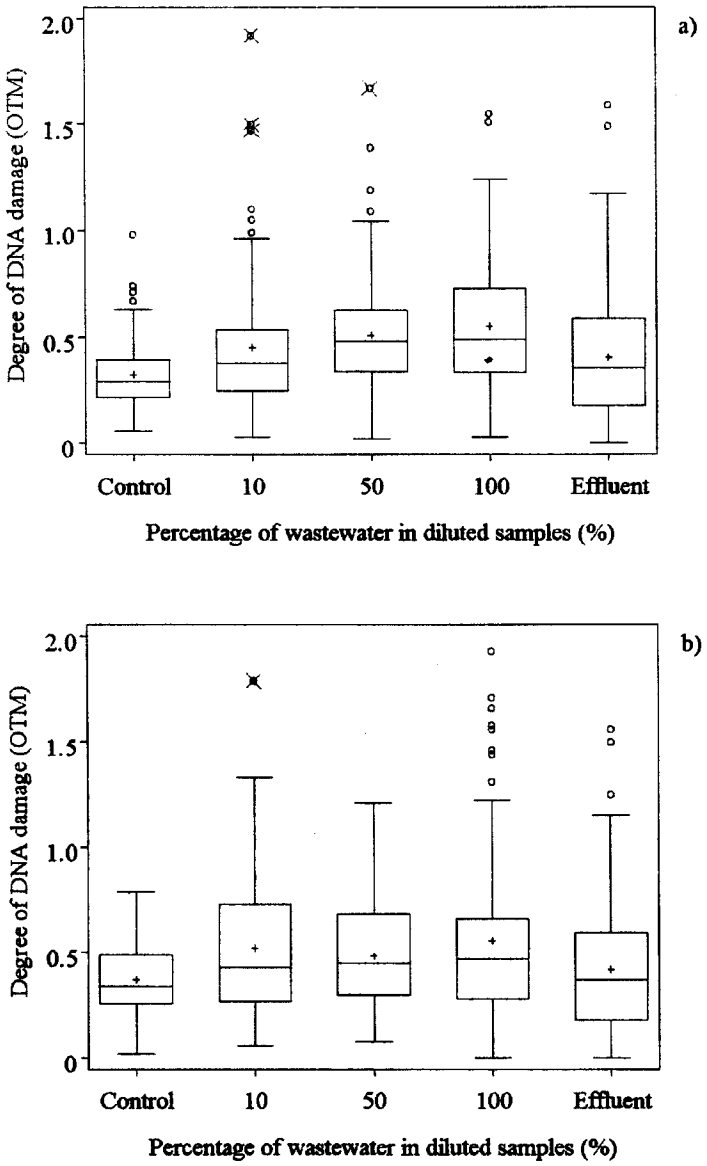

Figure 2. Degree of yeast DNA damage (represented as OTM) caused by different dilutions of influent and effluent wastewater samples from the municipal wastewater treatment plant (a – 1st experiment, b – 2nd experiment). Results from 100 comets are shown and explained with box-and-whisker plots as in Figure 1.

Our results show that the detection of DNA strand breaks by means of comet assay on yeast cells could provide an important aspect of genotoxicity monitoring in complex liquid samples, e.g. municipal and industrial wastes, surface water, potable water and soil leachates. The short time exposure to genotoxic agents provides clear evidence that yeast cells are affected by genotoxic substances, causing primary DNA damage. The detected DNA damage caused by wastewater samples from the municipal wastewater treatment plant was an integral effect of all present direct and indirect genotoxic substances causing DNA strand breaks, among them phenol, cyanide and heavy metals certainly played an important role (Table 1). The differences in susceptibility of yeast cells to genotoxic load certainly depend on biochemical and morphological variations in the cell population.

One of the first successful and reproducible experiments reporting the use of eukaryotic microorganisms for environmental genotoxicity monitoring by comet assay was performed on unicellular green algae *Chlamydomonas reinhardtii* (Erbes et al. 1997). Unicellular algae are able to modify genotoxic substances and their biological effects and they are supposed to play a key role in the activation and detoxification of pollutants in aquatic system. Like algae, yeast may be grown easily and inexpensively in axenic laboratory cultures and as such they both represent ideal biomonitors for genotoxicity. As eukaryotic microorganisms possess almost the same enzymatic "machinery" as animals, plants and humans, they could be relatively successfully used instead of laboratory and field animals for genotoxicity detection in wastewater, soil and food samples.

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