

## Estrogenic and Cytotoxic Potentials of Tunisian Leachate and Textile Wastewater as Determined by *In Vitro* Bioassays

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**Abstract:** In this study, we chose to examine the estrogenic and stress induction potentials of Tunisian leachate and textile wastewater samples, given the lack of information in Tunisia concerning environmental risk assessment using bioassays. The estrogenic potential of the leachate and textile wastewater sample was determined by the modified E-Screen assay using human breast cancer MCF7 cells, methyl tetrazolium (MTT) and neutral red (NR). The cytotoxic potential of the leachate samples, on the other hand, was determined by the DNA fragmentation and the LDH assays. Leachate and textile wastewater samples show a cytotoxic effect on MCF7 cells. The cytotoxicity was clearly reduced after treatment of the textile samples, showing the efficiency of the treatment process. For DNA fragmentation, results showed that agarose gel electrophoresis did not reveal any DNA fragmentation in MCF-7 cells treated with leachate samples for 24 and 48 h; however, smearing of DNA is observed for one sample after 24 h of incubation. The LDH assay showed a dose-dependent increase in cell injury for the leachate samples in both 15 and 30 min incubation times.

**Key words:** Wastewater · Leachate Estrogenic potential · Cytotoxicity · DNA Fragmentation

### INTRODUCTION

In Tunisia, receiving ecosystems have to face solid or liquid discharges containing a complex mixture of various organic and inorganic substances. Hence, leachate wastewater and industrial wastewater are considered among the most increasing potentially harmful pollutants in the environment. Landfilling is considered as one of the main methods for the disposal of municipal and industrial solid waste. Degradation of organic fraction of landfill waste in combination with rainwater percolation produces a polluted liquid called leachate. Leachate contains larger pollutant loads than raw sewage and other industrial wastes [1]. Furthermore, if the leachate migrates into groundwater or seeps out of the landfill, significant environmental pollution can occur.

On the other hand, wastewater from textile mills, on the other hand, is strong in color and contains high amounts of organic matter, including surfactants and additives [2]. This is because the wet processes of textile

mills involve the use of a wide range of chemicals [3] and a multitude of processes, including bleaching, dyeing, printing and finishing activities [4].

In Tunisia, as in most countries, global parameters such as dissolved organic content (DOC), chemical oxygen demand (COD), biological oxygen demand (BOD) and nitrogen or phosphorus residual levels are used to evaluate wastewater quality. However, information on their possible biological effects, such as cytotoxicity and estrogenic potency, is often lacking [5].

Receiving ecosystems have to face solid or liquid discharges containing a complex mixture of various organic and inorganic substances. Thus, it is better to characterize these discharges not only by chemical means but also by the use of mammalian cells and *in vitro* bioassays, which are rapid, simple, sensitive and at the same time, cost-effective. Thus, in this study, we used various *in vitro* bioassays and molecular techniques to determine the effects of leachate and textile wastewater samples on mammalian cells.

## MATERIALS AND METHODS

**Sample Collection:** Five samples from two different municipal landfill leachates receiving urban and industrial wastes and a textile wastewater treatment plant (WWTP) in Tunisia were collected (Fig. 1). They were selected in order to study the cytotoxicity of such wastewater and their pollutant effect on receiving ecosystems. The samples include:

- Municipal landfill leachate from Henchir El Yahoudia landfill located in the southeast of Tunis City,
- Municipal landfill leachates from the relatively regulated Borj Chakir landfill [1] young leachate (fresh) sample and 1 old leachate sample] and
- Textile samples from an industrial WWTP using oxidation channels as treatment process. Samples were taken from the influent and the effluent of the WWTP.

**Sampling Site Characteristics:** The 80-hectare Henchir El Yahoudia landfill was previously described [6,7,8]. It is located at the southeast of Tunis, bordered in the southeast by Sebkhah Sejoumi, a salt lake and in the west by El Mourouj City. It was operated without any control during the 1960s and 70s; however, from 1982, the dumping was managed and the site was operated as a sanitary landfill until its closure in 1999. Most of the material that entered this landfill was municipal waste from the Greater Tunis region, as well as industrial and hospital waste.

The 124-hectare Borj Chakir municipal dump located in the southwest of Tunis City was opened in 1999 and operated by the private sector since 2000. It receives 1,800 to 2,000 tons of waste per day and now contains as much as 2.4 million tons. Domestic waste from surrounding cities and municipalities comprise 85% of the waste, while 11% is from industry. The leachate production is 200 to 250 m<sup>3</sup> per day.

The textile WWTP, situated in the area of an industrial manufactory of textile in Ksar Helal City in the Eastern center of Tunisia, close to Monastir City. The WWTP utilizes oxidation channels for the biological treatment section and handles an influent flow of 1800 m<sup>3</sup> per day. The biological capacity of this plant is kg BOD<sub>5</sub>/inhabitant/day.

**Cell Culture:** The MCF-7 cells were maintained in 75 cm<sup>2</sup> flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Sigma) and 1% penicillin-streptomycin. They were incubated in an atmosphere of 5% CO<sub>2</sub> at 37 °C.

### Modified E-screen Assay

**Modified MTT Assay:** MCF-7 cells were plated onto 96-well plates at an initial concentration of 1,000 cells per well in 100 µl of experimental medium (phenol red-free RPMI +10% charcoal-treated FBS + 1 % penicillin-streptomycin solution). After allowing the cells to attach overnight, samples were added at final concentrations of 5, 10, 15 and 20% and the plates were incubated for a total of 6 days. The medium was then replaced and 10 µl of

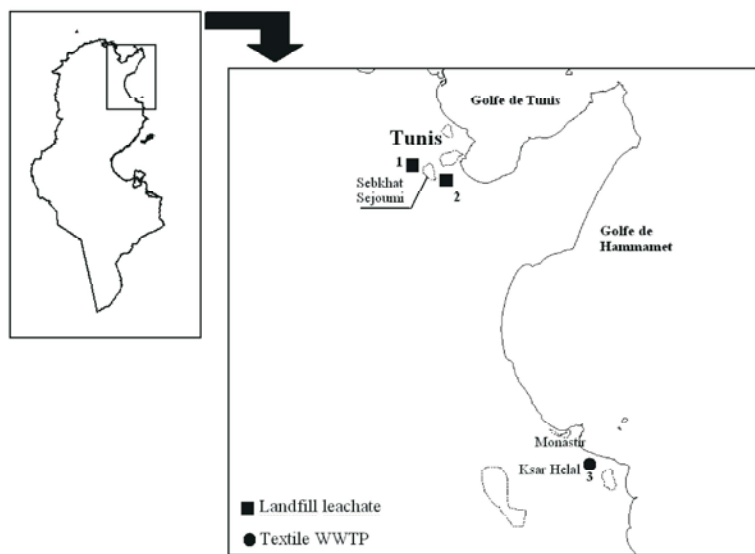


Fig. 1: Location of sampling sites in Tunis City and Ksar Helal City (Tunisia).

(1) Henchir El Yahoudia landfill, (2) Borj Chakir landfill and (3) Ksar Helal Textile WWTP.

5 mg/ml MTT was added to each well followed by overnight incubation. Then, 100 µl of 10% SDS was added to each well, the plates were incubated overnight and the absorbance was read at 570 nm using a plate reader.

**Modified NR Assay:** Similar to the MTT assay, MCF-7 cells were plated onto 96-well plates and samples were also added at the same concentrations and incubated for a total of 6 days. Then the medium was replaced with neutral red (NR) medium (1% NR solution, Sigma N2889) and the plates were incubated for 3 h. Cells were then washed and fixed with 200 µl of 0.5 % formalin-1% CaCl<sub>2</sub> and 100 µl of NR Desorb (ETOH/acetic acid) solution was added to all wells. After allowing the plates to stand at room temperature for 10-15 minutes followed by agitation on a microplate shaker for approximately 30 minutes, the absorbance at 540 nm was measured.

For both E-screen assays, the results are presented as a percentage of the nontreated control.

**DNA Fragmentation Assay:** MCF7 cells were plated onto Petri plates at 10<sup>6</sup> cells per plate in 10 ml of medium and incubated overnight. Leachate samples in addition to distilled water dH<sub>2</sub>O (control) were added at 10% final concentration and the cells incubated for 24 or 48h. DNA extraction was carried out using a genomic DNA purification kit (Promega, USA). DNA purity was determined by measuring absorbance ratios ( $A_{260}/A_{280}$ ) by a spectrophotometer (Beckman, USA). A 2% agarose gel was prepared using electrophoresis-grade agarose (Nippon Gene, Japan) and 1x Tris-acetate EDTA (TAE) buffer containing 0.5 µg/mL ethidium bromide (Wako). DNA samples (containing 1 µg of DNA) as well as molecular weight marker solution (Marker 1 ( $\lambda$ /Hind III digest), Wako) were mixed thoroughly with loading buffer (Wako) and loaded onto the gel in 1x TAE buffer solution. Electrophoresis was carried out for 25 min at 100 V. The gel was then directly photographed under UV illumination.

**Lactate Dehydrogenase (LDH) Assay:** The LDH-Cytotoxicity Test kit from Wako (Osaka, Japan) was used for this assay. The amount of lactate dehydrogenase released from cells following cell membrane disruption is determined with this kit. Sterile 96-well plates were inoculated with 5000 MCF-7 cells per well in 100 µL of medium and then incubated overnight in a 5% CO<sub>2</sub> incubator at 37 °C. The medium was carefully removed and cells washed twice with PBS (-). After dispensing 50 µl of PBS (-) into each well, leachate samples diluted in PBS (-) were added at 5, 10, 15 and 20% final

concentrations. PBS (-) and 0.2% Tween 20 dissolved in PBS were used as negative and positive controls, respectively. After a treatment time of 15 and 30 min at room temperature (RT), 50 µl of supernatant was transferred to a nonsterile plate to which 50 µl of coloring solution [Nitrotetrazolium blue (3.7 mg/vial), diaphorase and NADH dissolved in buffer solution (50 g/l DL-Lithium lactate)] was added into each well to induce the production of purple-blue diformazan. The plate was allowed to stand for 30 min at RT. The addition of 100 µl of reaction terminator (0.5 N HCl) into each well stops the reaction. Finally, the absorbance of the reaction mixture is measured at 570 nm in a microplate reader (Powerscan HT, Biotek Instruments, USA). Cell injury rate was quantified using the following equation:

$$\text{Cell injury rate} = [(S - N) \times 100] / (P - N),$$

where S, N and P are the absorbances of the sample, negative control and positive control, respectively.

## RESULTS

**E-Screen Assay:** Analyses of the three leachate samples show a clear cytotoxic effect which is, broadly speaking, dose-dependent. Moreover, results clearly show that textile wastewater entering the plant is cytotoxic; however, this cytotoxicity is considerably decreased after the treatment process.

Figure 2 shows that all leachate samples are cytotoxic towards MCF-7 cells, except for the leachate samples at 5% concentration in Fig. 2B, which show that they have estrogenic activity. In this E-Screen assay, MTT is used to quantify live and actively metabolizing cells that can reduce the yellow MTT to the purple formazan product through the action of mitochondrial and cytoplasmic enzymes. On the other hand, NR dye uptake does not require metabolic activity but simply measures the number of live cells that have an intact plasma membrane and can thus absorb the neutral red dye. It is therefore hypothesized that the difference between Figs. 2A and 2B in the case of the leachate samples at 5% concentration can be attributed to the inhibitory effect of the samples on cell metabolism. NR data, therefore, provides a better description of cell number in this case.

**DNA Fragmentation Assay:** As for DNA fragmentation, MCF-7 cells incubated with the leachate samples for 24 and 48 h did not undergo apoptosis or programmed cell death as revealed by the absence of DNA fragmentation (Fig. 3).

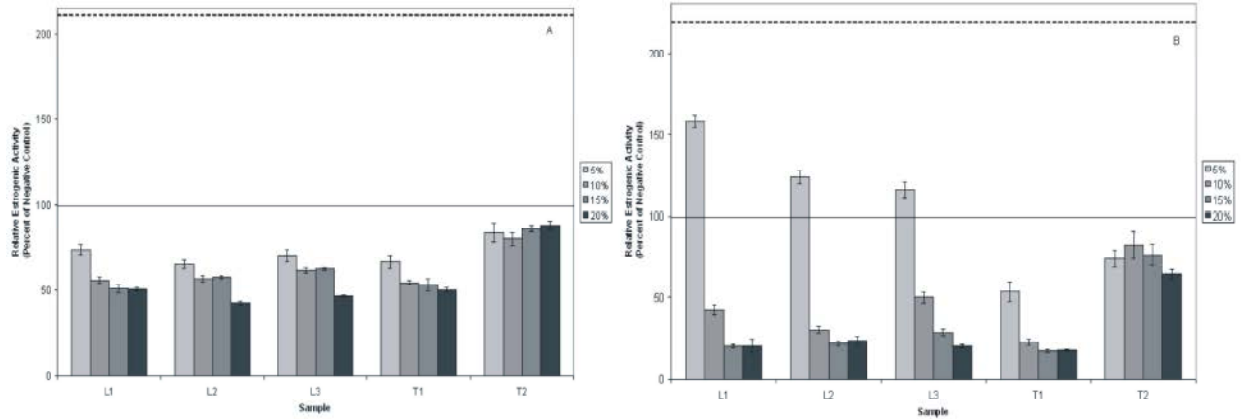


Fig. 2: Relative estrogenic activity of Tunisian leachate and textile samples at 5, 10, 15 and 20% final concentrations as determined by the modified E-screen assay after 6 days incubation. Cell number was assessed by the MTT (A) and neutral red assays (B).

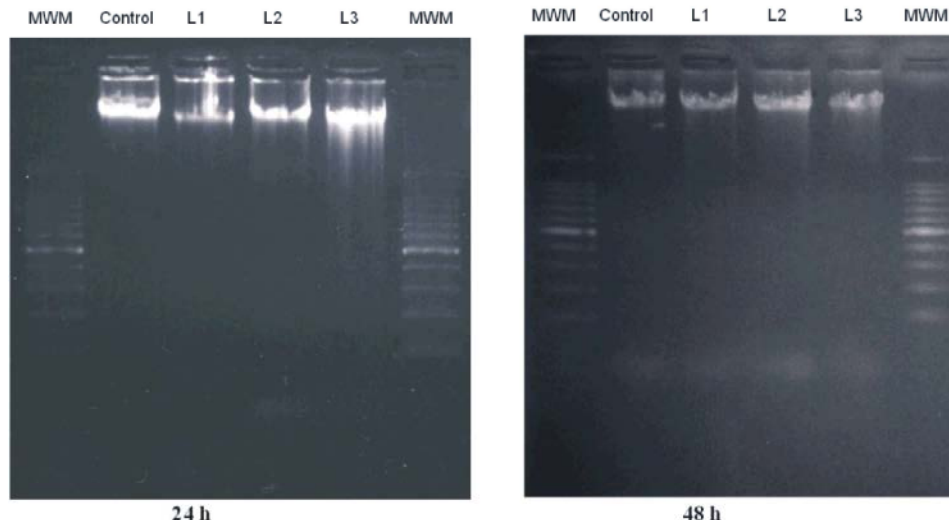


Fig. 3: Ethidium bromide-stained gel showing DNA from MCF-7 cells treated for 24 and 48 h with PBS (-) (control) or L1, L2 and L3 samples at 10% final concentration.

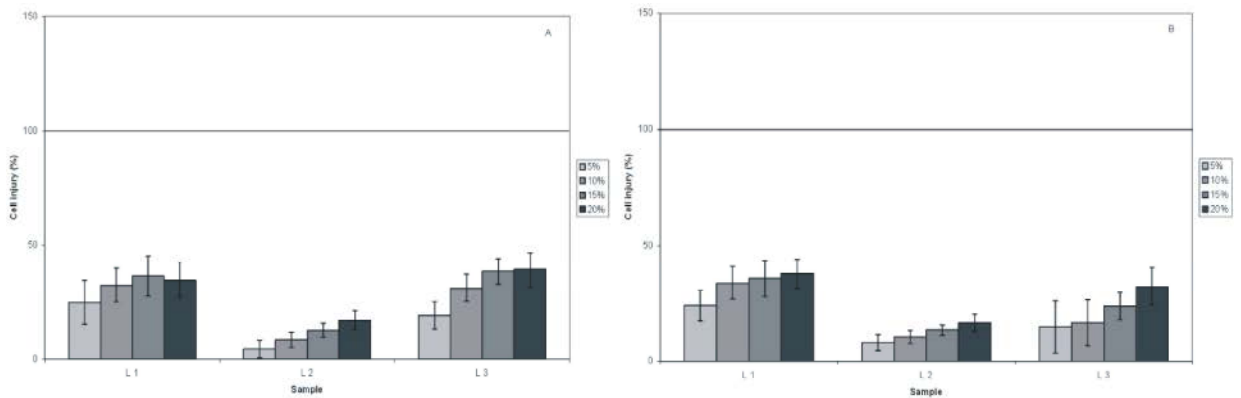


Fig. 4: Lactate dehydrogenase activity (expressed as cell injury) of MCF-7 cells after 15min (A) and 30 min (B) incubation with leachate samples (5, 10, 15 and 20% final concentrations).

However, it was revealed from the lactate dehydrogenase (LDH) assay that cells incubated with the leachate samples for 15 or 30 min underwent cellular necrosis in a dose-dependent manner (Fig. 4). Nonetheless, while samples L1 and L3 induced the most damage, cell injury rates did not exceed 40%. The positive (0.2% Tween 20 in PBS(-)) and negative (PBS(-) at 5, 10, 15 or 20% final concentration) controls induced respectively 100% (solid line) and 0% cell injury.

### DISCUSSION

In this study, we examined the effect of water samples from two municipal landfills and a textile WWTP on mammalian cells using *in vitro* bioassays. Concerning the textile samples, results clearly showed the importance of wastewater treatment by industrial plants. Wastewater entering textile plant is cytotoxic; however, this cytotoxicity is considerably decreased after the treatment process. Textile influent sample characterized by a high residual COD, which is an indicator of the dissolved organic matter content, may include some synthetic organic compounds. In this context, Dantas *et al.* [2] reported that effluents from textile industries are generally high in organic content and have strong colour, as well as high amounts of surfactants and additives.

On the other hand, the results of the E-screen assay showed that leachate samples generally do not contain estrogenic compounds; on the contrary, they were cytotoxic towards MCF-7 cells after 6 days of incubation. An exception, however, was shown by the leachate samples at 5% concentration, as shown in Fig. 2B. Moreover, it is widely known that leachate from landfills, particularly the older types, is one of the main sources of groundwater pollution. Human health problems such as developmental abnormalities, low birth weights and cancer have been linked to exposure to water pollution from old-style landfills [9]. Concerns are compounded when the fills content is unknown because of weak dumping restrictions

The mechanism by which the leachate samples induced cytotoxicity on the cells was then determined by the DNA fragmentation and LDH assays. Results in Figs. 3 and 4 show that the cytotoxicity was not caused by apoptosis but by necrosis during the early stages of incubation. Cell injury however did not exceed 40%; thus, cells that can survive the initial insult have the potential to proliferate, albeit at a slower pace, as proven by the results in Fig. 2.

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