

Environmental Risk Assessment of Water Resources in Arid and Semi-arid Lands Using Bioassays Systems

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Abstract: Control of effluents has traditionally been regulated using methods with unspecified responses and measuring global parameters, such as the chemical oxygen demand (COD) or Biological Oxygen Demand (BOD). Moreover, information about biological effects is unknown. Thus, the introduction of *in vitro* assays using mammalian cells to investigate the presence of environmental contaminants is more than needed. At first, physicochemical parameters of municipal wastewater samples from Tunisia were estimated. On a further step, *in vitro* bioassays systems were applied intending to find out the biological effects of these samples on mammalian cells. Results using the E-screen assay reveal that the wastewater samples induced estrogenic activity at different concentrations in 6 days incubation period. Besides, the same samples were able to generate stress response on animal cells. These findings correlate with the physicochemical parameters data and thus evaluate the efficiency of treatment system to remove such toxic compounds.

Keywords: Estrogenic activity, Risk assessment, Stress response, Wastewater

1. Introduction

Water environments have to face solid or liquid discharges containing a complex mixture of various organic and inorganic substances. Wastewater reclamation and reuse projects have been developed in arid and semiarid lands as an essential response to growing water needs. The treated water was used for recharging the ground water or irrigating some specified cultures, public green areas and sports facilities such as golf courts or football fields. Through pathways such as drinking from aquifers or the food chain, humans are confronted with the threat of residual pollutants in effluents. In arid and semiarid lands, particularly where the water resources are scarce, the detection of contaminants and the measurement of their harmful impact has become a major issue in environmental risk assessment. Therefore, in a primary step, the quality of treated water, along the wastewater treatment process, was investigated by the mean of conventional physicochemical parameters. Moreover, *in vitro* bioassays systems were used to evaluate the biological effects of wastewater samples on mammalian cells. Heat Shock Protein 47 assay for stress response and E-screen assay for estrogenic activity were carried out to assess the risk of wastewater samples from an activated sludge treatment system plant in semi-arid environment.

2. Materials and Methods

2.1. Sampling site

Water samples from the wastewater treatment system in Soliman, Tunisia, were obtained along the treatment process from the influent input, the pretreatment pond, the oxidation channel based on activated sludge treatment and the effluent pond (**Fig. 1**). Soliman STP system renewed in 2004, receives influents (12300 m³/day) from a population of 30 000 residents (www.onas.nat.tn, 2007). The primary treatment is carried out to remove the coarse fraction inclusive of oil, fatty acids and suspended solids. The biological processing is used to remove microorganisms, nitrogen and phosphorus, and lower the organic load as defined by the BOD. The biological treatment, based on the natural ability of wastewater containing bacteria to degrade organic material, is roughly divided into aerobic and anaerobic treatments including conventionally the oxidation pond and the activated sludge process.

2.2. Sampling procedures

Sterilized glass bottles served for the samples to be used for physicochemical parameters investigation,

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sterilized plastic bottles were used for the samples intended for the bioassays. For the latter samples, a primary filtration using a 0.45 μm filter was carried out on the sampling day. Samples were stored at $-20\text{ }^{\circ}\text{C}$ until transport to Japan inside an icebox to the laboratory where the same conditions were maintained. A secondary filtration using a 0.22 μm filter is required prior to use in the bioassays following pH adjustment between 3 and 6.

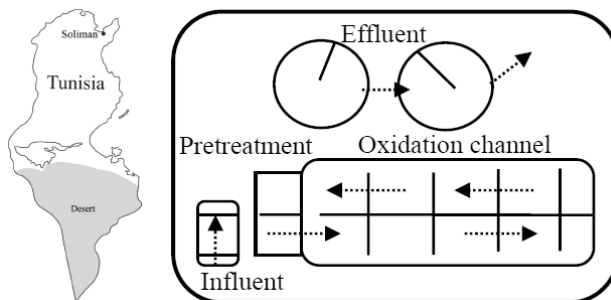


Fig. 1. Sampling points from Soliman activated sludge treatment system plant in Tunisia.

2.3. Water quality parameters

The following in situ parameters were immediately determined prior to filtration as follows: Temperature and pH using a pH meter 330i, dissolved oxygen using an oxymeter 340i, and electric conductivity using a conductimeter 330i (all from Wissenschaftlich-Technische-Werkstätten, Weilheim, Germany). Other physicochemical parameters, such as Chemical Oxygen Demand (COD), 5d-Biological Oxygen Demand (BOD₅), suspended sludge (SS) were analyzed in the laboratory according to standard methods (Rodier, 1984).

2.4. E-screen assay

The estrogenic activity of the samples was investigated using E-screen assay. Human breast cancer MCF-7 cells were initially cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS). They were then plated onto 96-well plates at 1×10^3 cells/well and allowed to attach for 24 h. The medium was removed and the wastewater samples at 5, 10 and 20% (V/V) final concentrations, in addition to 17- β -estradiol (E_2) (29 nM final concentration) as positive control, were diluted in phenol-free RPMI medium supplemented with 10% charcoal-treated FBS and then added to the cells. A separate experiment was carried out to adjust the effects of various concentrations (Phosphate Buffer Solution PBS (-) at 5%, 10% and 20%) on the survival and proliferation of MCF-7 cells. The cells were incubated for 6 days and the medium changed, after which 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Dojindo) was added to each well followed by incubation for 24 h. Sodium dodecyl sulfate (SDS, 10%) was then added at 100 μL per well, followed by incubation for another 24 h. The absorbance was then determined at 570 nm using a multidetection microplate reader (Powerscan® HT, Biotek Instruments).

2.5. HSP 47 assay

The HSP 47 assay based on the enzymatic activity of β -galactosidase (Isoda *et al.*, 2003), driven by the hsp47 gene promoter, aims to determine the stress response of HSP 47-transformed cells due to samples addition. HSP 47-transformed cells were trypsinized and plated onto 96-well plates at initial concentrations of 1×10^4 cells per well in 100 μL of F12 Medium (Gibco®, Invitrogen) supplemented with 10% FBS, 200 $\mu\text{g}/\text{mL}$ of G418 (Gibco BRL) and 0.1 $\mu\text{g}/\text{mL}$ kanamycin solution (Sigma). The cells were allowed to attach for 48 h before removing medium and adding 100 μL of samples diluted with medium followed by incubation for 3 h in a 5% CO_2 incubator at $37\text{ }^{\circ}\text{C}$. The wastewater samples were added at 5 concentrations 0.01, 0.1, 1, 5, 10 and 20% (V/V). For the highest concentrations, additional controls (5, 10 and 20% PBS(-), data not shown) were considered as references in order to consider the sample effect exclusively. The medium was then carefully removed and the cells washed twice with PBS(-). Fifty microliters of lysis buffer (Promega) was then added and the plates incubated for 30 min at room temperature (RT). Twenty microliters of cell lysate was transferred to a new plate, to which 100 μL of substrate solution (10 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 100 mM NaCl, 1% BSA, 0.005% NaN_3 , 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1% 4-methylumbelliferyl- β -galactose (MUG), pH 7.0) was added in order to trigger the conversion of MUG into β -galactose and methylumbelliferyl. After allowing the reaction to occur in the dark for 30 min at RT, 60 μL of reaction stop buffer (1 M glycine-NaOH, pH 10.3) was added and the fluorescence at 365 nm excitation/ 450 nm emission was then determined using a multidetection microplate reader.

2.6 Statistical analyses

Statistical analyses such as Student's t-test were carried out. Differences in means were considered significant at $p < 0.05$. All experiments were conducted at least three times.

3. Results and Discussion

3.1 Physicochemical parameters

Table 1 clearly shows that the wastewater samples had a higher electric conductivity (EC) ranging from 5.26 to 8.72 ms/cm as a sign of presence of salts and impurities. The sample obtained from the oxidation pond exhibited the highest concentration of organic matter, as represented by the chemical oxygen demand (COD), reaching 2 to 3 fold the levels of influent and effluent samples, respectively. On the other hand, the ratio of BOD₅ to COD for both the influent and the effluent attained 80%. This is explicit a relatively high biodegradability for the treatment system. Nevertheless, the removal rate of organic content did not exceed 35%. Thus, the residual levels of COD and BOD were broadly above the Tunisian guidelines (Table 1). Overall, the suspended sludge (SS) for all the samples was lower than the guidelines.

3.2 E-screen assay

The E-screen assay is widely used to determine the estrogenicity of environmental compounds through the ability of a substance to stimulate the growth of an estrogen-dependent cell line, commonly MCF-7. This human breast cancer cell line possesses estrogen receptors (ERs) and responds in culture to the presence of estrogens or xenoestrogens with a proliferative response (Jones *et al.*, 1998). Indeed, Estrogens play important roles in growth, development, reproduction and maintenance of a diverse range of mammalian tissues. The physiological effects of estrogens are mediated by the intracellular ERs, which regulate transcription of target genes through binding to specific DNA target sequences. As illustrated in **Figure 2**, the coregulators PELP1 (Proline Glutamic Acid-Rich Nuclear Protein) and Steroid Receptor Coactivator (SRC) act as coactivators of ER (Mishra *et al.*, 2003) to promote cell proliferation. This leads to the subsequent activation of Extracellular Signal Regulated Kinases (ERK)1/2.

The endpoint of the E-screen assay used in this study is the number of cells as determined by the absorbance of the purple formazan product, which is produced by the metabolic conversion of MTT (Mosmann, 1983). The proliferation levels of the human breast cancer MCF-7 cells treated with Soliman wastewater samples were significant in comparison with the nontreated control (T-test, $p < 0.05$). Overall, the wastewater samples contain estrogenic or xenoestrogenic compounds, hormones or chemicals known to act like estrogen. In fact, Steroid estrogens have been detected in influents and effluents of STPs in different countries such as Italy, Brazil, Netherlands and Japan (Cui *et al.*, 2006; references cited in there). The removal rate of these estrogenic compounds in such municipal STPs were approximately above 80%. Drewes *et al.* (2005) reported a significant correlation between the BOD₅ loading and the total estrogenic activity exhibited in the E-screen in seven full-scale water reclamation facilities. Moreover, it is difficult for these chemicals to be degraded by microorganisms because of complexity and anti-biodegradation of estrogen containing wastewater (Cui *et al.*, 2006). Indeed, in a recent investigation, Narita *et al.* (2007) found out that the by-product of activated sludge process induced higher activity than the influent due to the production of some metabolites during biological process. On the other hand, the estrogenic activity shown by the samples correlate with the BOD₅ loading and with previous investigations by Limam *et al.* (2007) and consequently, might put under question the efficiency of the treatment system.

Table 1. Main physicochemical parameters of wastewater.

Sample	pH	EC (ms/cm)	T (°C)	DO (mg/L)	COD (mg/L)	BOD ₅ (mg/L)	SS (µg/L)
Influent	7.96	8.72	18.7	1.31	900	720	224
Pretreatment	8.09	5.26	18.6	0.93	994	795	38
Oxidation pond	7.75	6.20	17.2	1.02	1996	1596	86
Effluent	7.58	8.32	16.7	0.76	602	482	60
Guidelines (NT 106.002, 1989)	7-8	-	-	-	< 90	< 30	< 3000

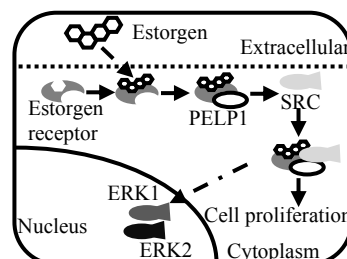


Fig. 2. Estrogen Pathway Overview.

3.3 HSP 47 assay

Heat shock proteins (HSPs) are a distinctive class of molecules that protects cells against a wide range of injuries (Georgopoulos and Welch, 1993). They assist in the recovery of stress either by repairing damaged proteins or by removing them to restore protein homeostasis (Hartl, 1996). Heat shock protein 47 (HSP47) is a collagen-specific molecular chaperone that is required for molecular maturation of various types of collagens. HSP47 activity can be determined using HSP47-transformed Chinese Hamster Ovary (CHO) cells by incubating the latter with the test compound and measuring the enzymatic activity of β -galactosidase upon stress induction. In the presence of β -galactosidase, methylumbelliferyl- β -galactose (MUG) is converted to β -galactose and methylumbelliferyl. Since it is difficult to measure HS protein activity, the gene protein β -galactosidase activity is thus measured spectrophotometrically to detect chemicals or toxins which may disrupt or disturb homeostasis in organisms (Isoda *et al.*, 2003).

It has already been revealed that the production of stress proteins is induced as a result of the reaction of cells with a stressor such as heat, a chemical substance or a heavy metal (**Fig. 3**). Isoda *et al.* (2003) developed this highly sensitive system, HSP 47 assay, for detecting trace amounts of environmental pollutants and natural toxins. Following 3 h treatment time, the combination of the stress response and the cytotoxicity effect of Soliman STP samples on HSP cells showed that they induced a significant increase in β -galactosidase activity in comparison with the control. This result can be correlated with the estrogenic activity and the physicochemical parameters of the same samples as shown by the E-screen assay.

4. Conclusion

These specific bioassays systems allowed the detection of wastewater samples components responsible for stress response or estrogenic activity in order to evaluate the efficiency of the treatment system. These findings will help establish, in a future purpose, some specific environmental biomarkers for wastewater pollutants

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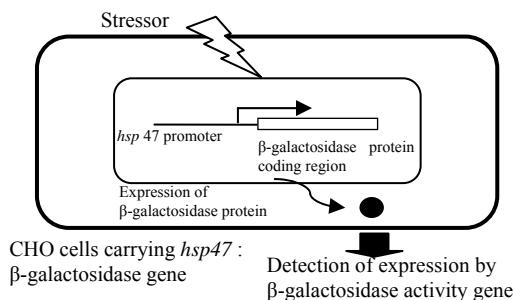


Fig. 3. HSP 47 assay overview.