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Sensitivity of in Vitro Bioassays towards Several Water Origins in Tunisian Arid and Semi-arid Area

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Abstract: Arid and semi-arid lands are confronted to water resources scarcity. Thus, it is essential to prevent their contamination. Hazardous compounds present in the effluents or solid waste as single compounds or mixture may settle in receptor sites and drastically affect the human health through drinking from aquifers or the food chain. Control of effluents has traditionally been regulated using physicochemical parameters. 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 necessary. The aim of this study is to investigate the biological effects on mammalian cells of potential environmental contaminants present in river, wastewater, leachate, channel, and dam samples obtained from semi-arid area in Tunisia. This new approach for risk assessment in water environments used a combination of physicochemical parameters with in vitro bioassays relative to stress response and endocrine disrupters. Among all, the leachate sample (2520 mg/L), the river sample R1 (1792 mg/L) on the far downstream of the watershed network and the wastewater sample W5 (1056 mg/L) from Grombalia STP showed the highest levels of organic content. Tarhouna and El Bey rivers have very high estrogenic activity because they receive wastewater effluents from Beni Khalled and Grombalia STPs. As expected the highest stress response levels were observed for the industrial wastewater W3 and the leachate L samples. These specific bioassays systems, sensitive to wastewater and water components responsible for stress response or estrogenic activity are very useful in evaluating the associated hazardous risk.

Key Words: Endocrine disruptors, Leachate, 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 semi-arid lands as an essential response to growing water needs. The treated water was mainly discharged into natural bodies and eventually reused for recharging the groundwater 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 semi-arid 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, the aim of this study is to investigate the biological effects on mammalian cells of potential environmental contaminants including endocrine disrupters and heavy metals present in river, wastewater, leachate, channel, and dam samples obtained from arid and semi-arid area in Tunisia. Hence, *in vitro* bioassays systems to evaluate the biological effects on mammalian cells of water samples obtained from rivers, wastewater effluents, leachate, channels, and a dam. Heat Shock Protein 47 assay for stress response and E-screen assay for estrogenic activity were carried out to investigate the sensitivity of these *in vitro* bioassays toward several water origins.

2. Materials and Methods

2.1. Sampling sites

Water samples from several water origins in Soliman and Grombalia (lat. between N36:31:55.38 and N36:43:21.96, long. between E10:28:21.54 and E10:32:06.06) semi-arid area in Tunisia were obtained on March 24th and 25th, 2008 (**Fig. 1**). The samples includes mainly six river samples (R1 to R6) from El Bey river watershed and six wastewater effluents (W1 to W6) from Grombalia and Beni Khaled sewage treatment plants (STPs) as well as from industrial installations.

Besides, two samples from a drainage channel (D1 and

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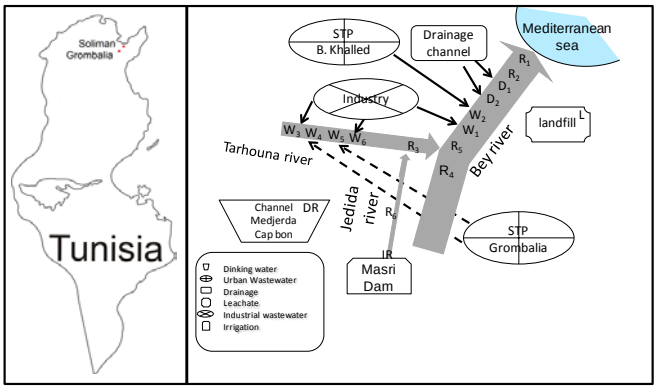

Fig. 1. Sampling sites in Soliman and Grombalia area

D2), a leachate sample from Soliman urban landfill (L) in addition to a sample (IR) from El Masri dam for irrigation purpose were collected. Moreover, a sample (DR) taken from Medjerda-Cap bon channel situated on the upstream of a drinking water treatment plant was considered. A primary filtration using a 0.45 µm filter was carried out on the sampling day. Samples were stored at -20°C until transport inside an icebox to the laboratory where the same conditions were maintained. A secondary filtration using a 0.22 µm filter was performed prior to use in the bioassays following pH adjustment between 5 and 6 in order to maintain optimum conditions for cells culture.

2.2. Water quality parameters

The following in situ parameters were immediately determined 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), and suspended sludge (SS) were analyzed in the laboratory according to standard methods (APHA, 1989).

2.3. Modified E-screen assay

The estrogenic activity of the samples was investigated using E-screen assay. 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). Estrogen receptor-positive 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 water samples at 0.01, 0.1, 1, 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. 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 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, Japan) was added to each well followed by incubation for 6 h. Sodium dodecyl sulfate (SDS, 10%) was then added at 100 µL per well, followed by incubation for another 18 h. The absorbance was then determined at 570 nm using a multidetection microplate reader (Powerscan® HT, Biotek Instruments, USA).

2.4. HSP 47 assay

The HSP 47 assay based on the enzymatic activity of β-galactosidase (Isoda *et al.*, 2003) aims to determine the stress response of HSP 47-transformed cells due to samples addition. Chinese hamster ovary (CHO) cells stably transfected with (+) or without (-) a HSP 47 promoter were used for this experiment. HSP 47-transformed cells were plated onto 96-well plates at initial concentrations of 1×10^4 cells per well in 100 µL of F12 Medium (Gibco®, Invitrogen, Tokyo, Japan) supplemented with 10% FBS, 200 µg/mL of G418 (Gibco BRL) and 0.1 µg/mL kanamycin solution (Sigma, USA). 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°C. The water samples were added at the same concentrations as the E-screen assay. 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 NaH_2PO_4•$2H_2O$, 100 mM NaCl, 1% BSA, 0.005% NaN_3, 1 mM $MgCl_2$•$6H_2O$, 1% 4-methylumbelliferyl-β galacticose (MUG), pH 7.0) was added in order to trigger the conversion of MUG into 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.

2.5. Statistical analyses

Statistical analyses such as Student's t-test were carried out. Differences in means were considered significant at $p<0.05$ (* or X) or highly significant at $p<0.01$ (** or XX). All

Table 1. Main physicochemical parameters of water samples.

Origin	Sample	pH	EC (mS/cm)	T (°C)	D O	COD (mg/L)	BOD	SS
River	R1	7.44	3.10	16.9	0.45	1792	1433	0.050
	R2	7.89	3.26	15.9	7.60	278	60	0.066
	R3	7.43	2.20	18.4	0.97	580	220	0.008
	R4	7.62	2.72	16.2	7.53	232	40	0.072
	R5	7.64	3.12	16.3	7.43	492	120	0.048
	R6	7.41	1.71	14.5	7.83	120	16	0.060
Wastewater	W1	8.10	4.17	17.3	2.12	354	70	0.020
	W2	7.60	2.05	17.5	7.47	492	160	0.094
	W3	7.89	14.70	21.7	0.82	926	310	0.145
	W4	7.29	2.62	17.7	0.36	646	320	0.132
	W5	7.46	2.18	17.5	2.54	1056	300	0.120
	W6	7.20	3.83	22.3	0.53	808	280	0.100
Drainage	D1	7.99	6.64	13.0	8.08	142	80	0.118
	D2	8.22	7.17	16.1	9.88	29	16	0.040
Irrigation	IR	8.13	1.56	14.4	8.75	29	16	0.050
Upstream Driniking water plant	DR	7.94	1.78	15	7.46	120	18	0.070
Leachate	L	7.62	11.32	17.7	0.05	2520	350	0.010
Guidelines (NT 106.002, 1989)		6.5-8.5	7.00	-	-	90	30	30.000

experiments were conducted at least three times.

3. Results and Discussion

3.1. Physicochemical parameters of the water samples

The physicochemical parameters of the water samples are presented in **Table 1.** For most of the samples, the EC was below the Tunisian standards (i.e., 7 mS/cm) indicating the presence of low amounts of salts and impurities. However, drainage D2, industrial wastewater W3 and leachate samples showed EC levels exceeding the guidelines by 2-fold. Even though, expected for the drainage sample, this is might be explained by the textile wastes for the industrial sample and the mixture of discharge for the leachate sample.

Apart from the drainage sample D2 and the sample IR taken from Masri dam, all the samples exhibited high concentrations of organic matter, as represented by the COD content. Among all, the leachate sample (2520 mg/L), the river sample R1 (1792 mg/L) on the far downstream of the watershed network and the wastewater samples W5 (1056 mg/L) from Grombalia STP showed the highest levels widely exceeding the Tunisian guidelines. Interestingly, the COD concentration of R1 sample reached 60-fold of IR sample located on the far upstream. On the other hand, the same trend was observed for the BOD concentrations that did not feet the guidelines for most of the samples. Moreover, the ratio of BOD to COD for R1 sample was equal to 0.8, while other samples showed a relatively low biodegradability below 0.6. The average SS content of the wastewater samples was twice the average of river sample level and 10-fold of the leachate sample level. However, none of them exceeded the guidelines.

Table 2. Estrogenic activity of water samples.

Origin	Sample	Concentration					
		0.01%	0.1%	1%	5%	10%	20%
River	R1	-	**	**	**	**	**
	R2	-	-	-	**	**	**
	R3	ND	ND	ND	**	**	**
	R4	-	**	**	**	**	*
	R5	ND	ND	ND	**	**	**
	R6	-	-	-	X	X	X
Wastewater	W1	-	-	**	*	*	*
	W2	ND	ND	ND	**	**	**
	W3	X	X	X	X	XX	XX
	W4	**	**	**	**	**	**
	W5	ND	ND	ND	-	*	**
	W6	*	*	*	**	-	X
Drainage	D1	-	-	-	-	-	-
	D2	-	-	-	-	-	-
Irrigation	IR	-	-	*	-	-	-
Upstream Driniking water plant	DR	-	-	*	-	X	X
Leachate	L	-	-	*	*	**	**

(*) Significant estrogenicity (-) Non significant effect

(X) cytotoxic effect (ND) Non determined

3.2. Estrogenic activity of the water samples

The modified E-screen results clearly show that the river, wastewater and leachate samples had a high estrogenic activity (**Table 2**) while samples R6 and W3 induced a cytotoxic effect. Among the river samples, R3 sample was able to generate a very high estrogenicity (more than 180% of the control cells at 10% sample concentration) whereas other river samples reached a maximum estrogenic activity of 140 or 150%. In the case of wastewater samples, the highest estrogenicity was shown by W4 sample from Gromabalia STP (170% of the negative control). On the other hand, other wastewater effluents induced estrogenic activities between 120 and 137%. Besides, the leachate sample taken from Soliman landfill showed an estrogenicity of about 150% at 20% sample concentration.

The presence of numerous endocrine-disruptor compounds (EDCs) such as estrogens in natural waters and sediments has been attributed to the incomplete removal of these substances during wastewater treatment (Farré *et al.*, 2005). This was further confirmed by the results of this study, which showed that the activated sludge treatment system, implemented in Grombalia and Beni Khalled STPs, was not capable of completely removing the estrogenic compounds from the effluent. Natural and synthetic estrogens that are not removed by wastewater treatment eventually end up in the aquatic environment. Tarhouna and El Bey rivers are typical examples of contaminated rivers. These rivers have very high estrogenic activity because they receive wastewater effluents from Beni Khalled and Grombalia STPs as well a

Table 3. Stress response of water samples.

Origin	Sample	Concentration					
		0.01%	0.1%	1%	5%	10%	20%
River	R1	*	*	*	**	**	**
	R2	-	*	**	**	**	**
	R3	**	**	**	**	*	-
	R4	*	**	**	*	*	-
	R5	-	-	*	*	**	*
	R6	*	**	**	*	*	-
Wastewater	W1	-	*	**	**	*	*
	W2	-	*	**	X	X	X
	W3	-	-	**	**	**	**
	W4	-	-	**	**	**	-
	W5	-	-	*	-	-	-
	W6	*	**	**	**	*	-
Drainage	D1	-	-	-	-	*	-
	D2	*	-	X	X	X	XX
Irrigation	IR	*	**	**	-	-	-
Upstream Driniking water plant	DR	*	**	**	**	**	*
Leachate	L	**	**	**	**	**	**

(*) Significant stress response (-) Non significant effect

(X) cytotoxic effect (ND) Non determined

myriad of industrial wastewater effluents. The latter processes not only domestic wastewater from highly populated areas but also wastewater from hospitals and various industries from the industrial zone in Grombalia and Soliman. According to Khanal *et al.* (2006), natural estrogen compounds are mainly removed from the aqueous phase by adsorption onto associated solid phases, such as sludge in wastewater treatment. However, these findings showed that the levels of estrogenic activity in the effluents form Beni Khalled and Grombalia are quite high.

3.3. Stress response effect of the water samples

The stress response of HSP(+) cells exposed to the water samples was estimated using the HSP47 assay (**Table 3**). Overall, the results showed that the water samples exhibited a significant stress response with a peak registered at 1% concentration or above. As expected the highest stress response levels were observed for the industrial wastewater W3 and the leachate L samples (142% and 139% of the control cells level). Interestingly, the river samples R1, R2, R3 and R6 induced a significant stress response of about 135%. Surprisingly, the sample taken from Masri dam showed a similar stress response (i.e. 134% of the control cells). Out of 17 water samples, 10 samples showed a correlation with the modified E-screen results. Indeed R1 and R2 samples were able to induce a concentration dependent stress response while a peak was observed for R4, R5, W1, W4, W6, D1, IR and DR samples between 1 and 10% concentration depending on the sample. Moreover, the wastewater samples W2 and the drainage sample D2 resulted cytotoxic for the HSP cells at Concentrations above 1%. The high stress response induced by the river samples might be attributed to the discharge of wastewater effluents from Beni Khalled and Grombalia STPs in addition to the industrial wastewater effluents (Fig. 1).

4. Conclusion

The introduction of in vitro bioassays systems for water environment risk assessment is relatively new in semi-arid land and particularly in Tunisia. The physicochemical parameters results have shown high levels of BOD or COD in the leachate and wastewater samples. Moreover, an increase in the organic contamination was observed from the upstream of El Bey river watershed (IR, Masri dam) until the downstream point (R1). The E-screen assay results elucidated the presence of high amounts of estrogenic compounds in the wastewater samples and consequently in their receptor sites, the rivers. Some alarming values of estrogenic activities in samples R3 or W4 could not be predicted by the physicochemical parameters. The HSP 47 assay results showed a correlation with the E-screen results. However, this stress response system has shown higher sensitivity toward trace compounds present in Masri dam (IR) or the channel Medjerda-Cap bon (DR). It was thus concluded that this strategy developed is sufficiently sensitive to be applied alone or coupled with physicochemical parameters in the water preservation strategy.

Aknowledgement

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