ES S27

Assessment of Estrogenic Activity in Tunisian Water and Wastewater by E-Screen Assay

Atef Limam†, Terence P.N. Talorete[1,†], Mourad Ben Sik Ali, Mitsuko Kawano[1], Amel Ben Rejeb Jenhani[2], Yukuo Abe[1], Ahmed Ghrabi and Hiroko Isoda[1,*]

Centre of Research and Technologies of Water, BP. 273, 8020 Soliman, Tunisia
[1]Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8572, Japan
[2]Laboratoire Aquaculture et Milieu, Institut National Agronomique de Tunisie, 43, Avenue Charles Nicolle, 1082 Tunis-Mahrajène, Tunisia

(Received June 28, 2007, accepted October 23, 2007)

*E-mail: isoda@sakura.cc.tsukuba.ac.jp

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Wastewater and surface water samples from three wastewater treatment plants (WWTPs) and three rivers in Tunisia were assayed for estrogenic activity using the E-screen assay and enzyme-linked immunosorbent assay (ELISA). Results showed that all the Tunisian raw wastewater samples as well as the Roriche river water sample induced a strong proliferative response in human MCF-7 breast cancer cells. Tunisian raw wastewater had an average 17β-estradiol content of 2,705.4 pg/ml, whereas that of the Roriche river was 36.7 pg/ml, which is sufficient for inducing endocrine-mediated responses in aquatic organisms. Results further showed that the Mornag WWTP, which uses the activated-sludge treatment system, has a higher estrogen removal efficiency than the stabilization ponds of the Gammart and pilot WWTPs. This study, which is the first of such studies in Tunisia, and probably the first in the North African region, underscores the need to detect and monitor the estrogenic activity of water and wastewater, given the scarcity of water in Tunisia and the detrimental impact of endocrine-disrupting compounds on the physiology of both animals and humans.

1. Introduction

The detection of estrogenic compounds in water and wastewater has become increasingly important to the water industry and regulatory authorities, particularly in semiarid countries such as Tunisia, where water is scarce. To date, no such study has been conducted in Tunisia and information on the presence of these compounds in Tunisian water and wastewater is nil.

The endocrine disruption phenomenon is a relatively new area of concern, first brought to light during the 1980s when deformities in fish were observed in certain stretches of UK rivers.(1) Endocrine-disrupting compounds (EDCs) are known to interfere with endocrine function(2) and have been linked to changes in sex ratio, embryonic damage, and reduced fecundity in various vertebrate species.(3–7)

Aquatic organisms are particularly vulnerable to the effects of EDCs as aquatic systems are a repository of chemicals derived from human activity. Effluent from sewage treatment plants (STPs) may be a significant source of EDCs for aquatic systems. The contamination of sewage effluent with EDCs can be caused by natural human and ani-

†Both authors contributed equally to this study.

mal hormones such as estradiol, testosterone and estrone, and from birth control pills containing 17α-ethynylestradiol.[8,9]

In recent years, wastewater treatment has increasingly become important for the sustainability of human society, particularly in arid and semiarid environments. Because of its harsh climate conditions and water scarcity, Tunisia is facing drought problems that are becoming increasingly frequent. Indeed, reclaimed water has recently been playing a more significant role in agriculture and in ensuring the sustainability of water resources.[10]

Wastewater treatment plants (WWTPs) receive a large spectrum of compounds from domestic and industrial wastes, which are not totally eliminated during the treatment processes.[11,12] At the outlets of WWTPs, a complex mixture of molecules including not only partially eliminated wastewater molecules but also metabolites formed during treatment processes are finally discharged into rivers.[13] Thus, industrial and domestic wastewater effluents have been identified as sources of active estrogenic compounds that contaminate the aquatic environment. Several studies have shown a correlation between reproductive abnormalities in fish and exposure to WWTP effluents even several kilometers downstream from outfalls.[7,9,14,15]

In this study, we examined the estrogenic activity of water and wastewater samples from three WWTPs and three rivers in Tunisia using the E-screen assay.[16] We also attempted to detect the presence of known estrogens and xenoestrogens by high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA). Moreover, we show the capacity of Tunisian WWTPs to decrease the estrogenic activity of the wastewater from the influent to the effluent. Our findings, which are the first of such findings in Tunisia, and probably the first in the entire North African region, demonstrate the need for the detection and monitoring of estrogenic activity in wastewater, sewage effluents and rivers.

2. Materials and Methods

2.1 *Sample collection*

Sixteen samples from five different sites and WWTPs in Tunisia (Fig. 1) were collected on November 24 and 25, 2005. They include six urban wastewater samples from the different stabilization ponds of the Gammart WWTP plant, namely, three urban wastewater samples from different stabilization ponds of a pilot WWTP and three urban wastewater samples from different points of an activated-sludge WWTP in Mornag, and four surface water samples from three different rivers, namely, the Ariana River (upstream and downstream of the Lot 4 Channel), the Medjerda River (pumping station of Medjerda-CapBon Channel) and the Roriche River (at the connection between the Roriche River and the Charguia highway stream) (Table 1). They were selected to represent a wide range of receiving ecosystems and wastewater treatment types, namely, stabilization ponds and activated-sludge treatment systems. Upon collection in the morning from the sampling points of the different sites using inactinic glass bottles that were initially washed and rinsed with acetone and MilliQ water, the samples were placed in a cool box and brought to the laboratory within approximately 1 h. They were immediately filter-sterilized using a 0.45-µm filter and stored at –80°C until use.

2.2 *Treatment plant characteristics*

The 81-hectare Gammart WWTP handles an influent flow of 25,000 m^3 per day with a retention time of three months and serves up to about 180,000 inhabitants. The approximately 400-m^2 pilot WWTP, on the other hand, handles 30 m^3 per day with a retention time of 17 days. The Mornag plant handles an influent flow of 3,200 m^3 per day and serves up to 35,000 inhabitants. The biological capacities of the Gammart, pilot and Mornag plants are 5,000, 700 and 1,700 kg biological

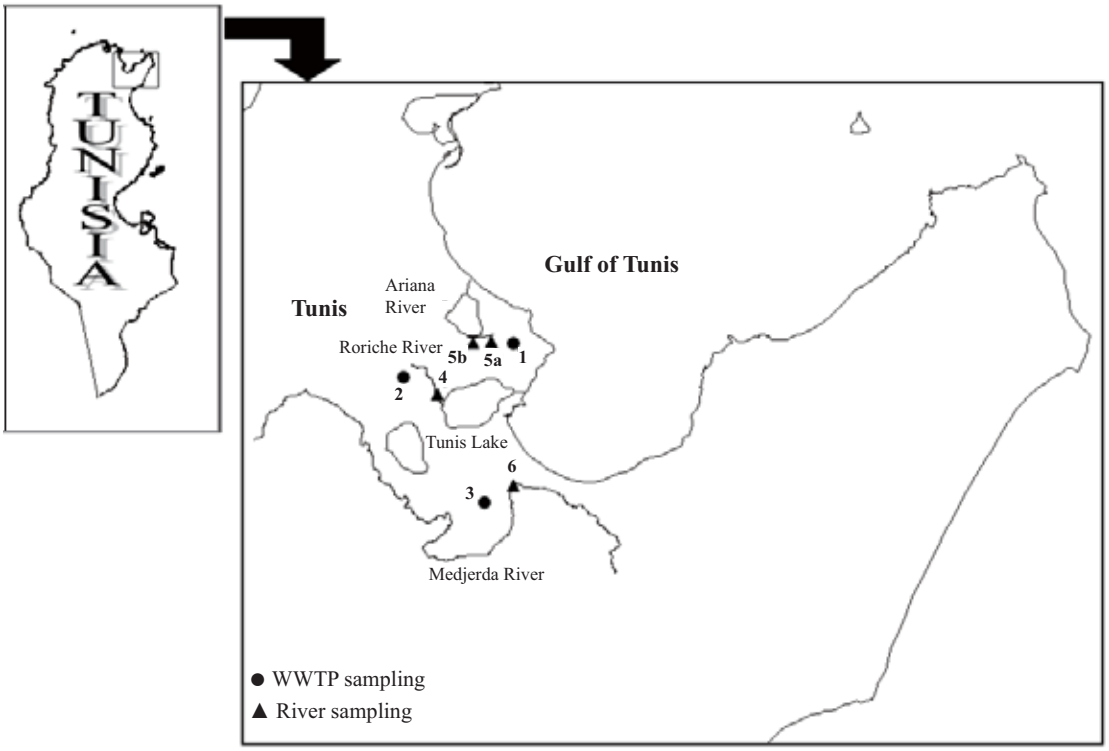

Fig. 1. Locations of sampling sites in the Tunis area (Tunisia). (1) Gammart WWTP, (2) pilot WWTP, (3) Mornag WWTP, (4) Roriche River, (5) Ariana River (5a is upstream and 5b is downstream), (6) Medjerda River.

Table 1
Description of Tunisian water and wastewater samples.

Type	Sampling site	Sample	Sampling point
Urban wastewater	Gammart WWTP (stabilization ponds)	G1	Influent
		G2	Decantation pond (input)
		G3	Primary pond (output)
		G4	Secondary pond (output)
		G5	Tertiary pond (output)
		G6	Effluent
	Pilot WWTP (stabilization ponds)	P1	Raw wastewater
		P2	Facultative pond (output)
		P3	Maturation pond (output)
	Mornag WWTP (activated sludge)	M1	Influent
		M2	Activated-sludge pond
		M3	Effluent
Surface water	Roriche River	R	Roriche River
	Medjerda River	MC	Medjerda-CapBon
	Ariana River	A1	Upstream
		A2	Downstream

oxygen demand (BOD_5)/inhabitant/day, respectively. In Tunisia, the bulk of wastewater is of domestic origin (76%), whereas 19 and 5% are from industrial and tourism sources, respectively. The three WWTPs studied receive urban wastewater of mainly domestic origin but also partly include wastewater from industrial and tourist sites.

2.3 *Water quality parameters*

While collecting the samples from the different sites, the following *in situ* parameters were immediately determined: temperature and pH using a pH meter 330i type WTW, dissolved oxygen concentration using an oxymeter 340i type WTW, and electric conductivity and salinity using a conductimeter 330i type WTW. Other physicochemical parameters, such as total organic carbon (TOC) concentration, chemical oxygen demand (COD), BOD_5, suspended sludge (SS) concentration, and total nitrogen and orthophosphorus ($P(PO_4)$) concentrations were analyzed in the laboratory according to standard methods.[3–7]

2.4 *Modified E-screen assay*

Estrogen receptor-positive human breast cancer MCF-7 cells were routinely maintained in 75-cm^2 tissue culture flasks containing Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal calf serum (Sigma) and 1% penicillin (5,000 IU/ml)-streptomycin (5,000 µg/ml) solution (ICN Biomedicals Inc.). Cells were incubated at 37°C in a 95% air-5% CO_2 incubator and passage was carried out at 70–80% confluence at a 1:2 ratio using 0.25% trypsin-1 mM ethylenediaminetetraacetic acid (EDTA). For the modified E-screen assay, the cells were cultured in phenol-red-free Roswell Park Memorial Institute (RPMI) medium supplemented with 10% charcoal-treated fetal bovine serum (FBS). They were then plated onto 96-well plates at 1,000 cells per well in 100 µl of medium and allowed to attach for 24 h. The 16 filter-sterilized samples (urban wastewater and surface water) at final concentrations of 5, 10, 15 and 20%, 17β-estradiol (E_2) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS(–)) (29 nM final concentration), and PBS(–) (5, 10, 15 and 20% final concentrations) were then added to the cells. E_2 and PBS(–) were used as positive and negative controls, respectively. The cells were incubated for six days, after which, cell number was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as follows: 10 µl of 5 mg/ml MTT (Dojindo, Japan) 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, USA). For comparison, additional experiments were carried out. Cell number was assessed by the neutral red (NR) dye uptake assay, as follows: after six days of incubation, the medium was replaced with NR medium (1% NR solution, Sigma N2889) and the plates were incubated for 3 h. The cells were then washed and fixed with 200 µl of 0.5% formalin-1% $CaCl_2$, and 100 µl of NR Desorb (EtOH/acetic acid) solution was added to all the wells. After allowing the plates to stand at room temperature for 10–15 min followed by agitation on a microplate shaker for approximately 30 min, the absorbance at 540 nm was determined. For both assays, the results are presented as a percentage of the negative control (*e.g.*, the results in wells with 5% sample were compared with those with 5% PBS(–)). The positive control was used to confirm the responsiveness of the cells to estrogenic compounds and to confirm the validity of the assay.

2.5 *HPLC of estrogenic compounds*

The reversed-phase HPLC of estrogenic compounds was performed to confirm the presence of estrogenic compounds in the samples showing estrogenic activities and to identify their nature. Ten different samples were selected for analysis

by HPLC. As standards, eight natural and synthetic estrogens were used, namely, estrone, 17β-estradiol, 17α-ethynylestradiol, estriol, bisphenol A, dibutylphtalate, 4-*tert*-octylphenol and 4-nonylphenol (technical isomer mixture). The standards were purchased from Sigma–Aldrich and WAKO (Japan). HPLC-grade acetonitrile, methanol and acetic acid were purchased from WAKO (Japan). Analysis was performed using a Gilson HPLC system equipped with a double plunger pump, a diode array UV detector and an autosampler. The separation of compounds was achieved using a BetaMax Neutral RP-C18 column (150 × 4.6 mm, 5 μm particle size). Mobile-phase solvents were water-acidified with 2% acetic acid (A) and acetonitrile (B) at an initial ratio (A:B) of 69:31. Separation and analysis were carried out at room temperature. The detection of EDCs was accomplished at 280 nm. The injection volume was 100 μl. The limit of detection (LOD), which is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value, was 100 ng/ml.

2.6 *ELISA for 17β-estradiol detection*

The YK170:17β-Estradiol EIA kit from Yanaihara Institute Inc. (Japan) was used for the quantitative determination of 17β-estradiol in the samples (detection range: 16.5–4,000 pg/ml) following the manufacturer's instructions. Similarly to the HPLC analyses, 10 samples were also selected for ELISA. By using a standard curve, the estradiol concentration in each sample was determined by comparing the logit value of each sample with the corresponding concentration calculated from this curve (Ln (x)) and expressed in pg/ml.

2.7 *Statistical analyses*

Student's t-test was carried out using Microsoft Excel and SPSS. Differences in means were considered significant at $p<0.05$.

3. Results

The E-screen assay results clearly show that all the Tunisian wastewater samples had estrogenic activity (Fig. 2). In the case of the Gammart WWTP, which uses stabilization ponds, the raw wastewater (G1), the input of the decantation pond (G2) and the output of the primary pond (G3) showed high estrogenic activities compared with those in the case of the negative control (more than 200 and 150% in the MTT and NR results, respectively). This estrogenic activity, however, is significantly reduced as the water passes through the secondary (G4) and tertiary (G5) ponds, with the resulting effluent (G6) having a relative estrogenic activity of less than 150%. Although this value is still high, it does show the relative efficiency of the treatment system in decreasing the estrogenic activity of the wastewater.

However, in the case of the pilot WWTP, although the TOC concentration, COD and BOD_5 reductions are considerable (Table 2), these were not reflected in the decrease in the estrogenic activity of the wastewater. The estrogenic activities of the raw wastewater (P1), as well as the outputs of the facultative (P2) and maturation (P3) ponds, remained almost unchanged at more than 150% compared with those of the negative control.

The Mornag WWTP, on the other hand, which uses the activated-sludge treatment system, decreased the estrogenic activity in the wastewater to almost the same level as the negative control (100%). Although the influent (M1) showed an estrogenic activity of more than 140%, the output of the activated-sludge pond (M2) as well as the effluent (M3) showed estrogenic activities of less than 110%. This is also reflected in the relative efficiency of the plant in reducing TOC concentration, COD and BOD_5 (Table 2).

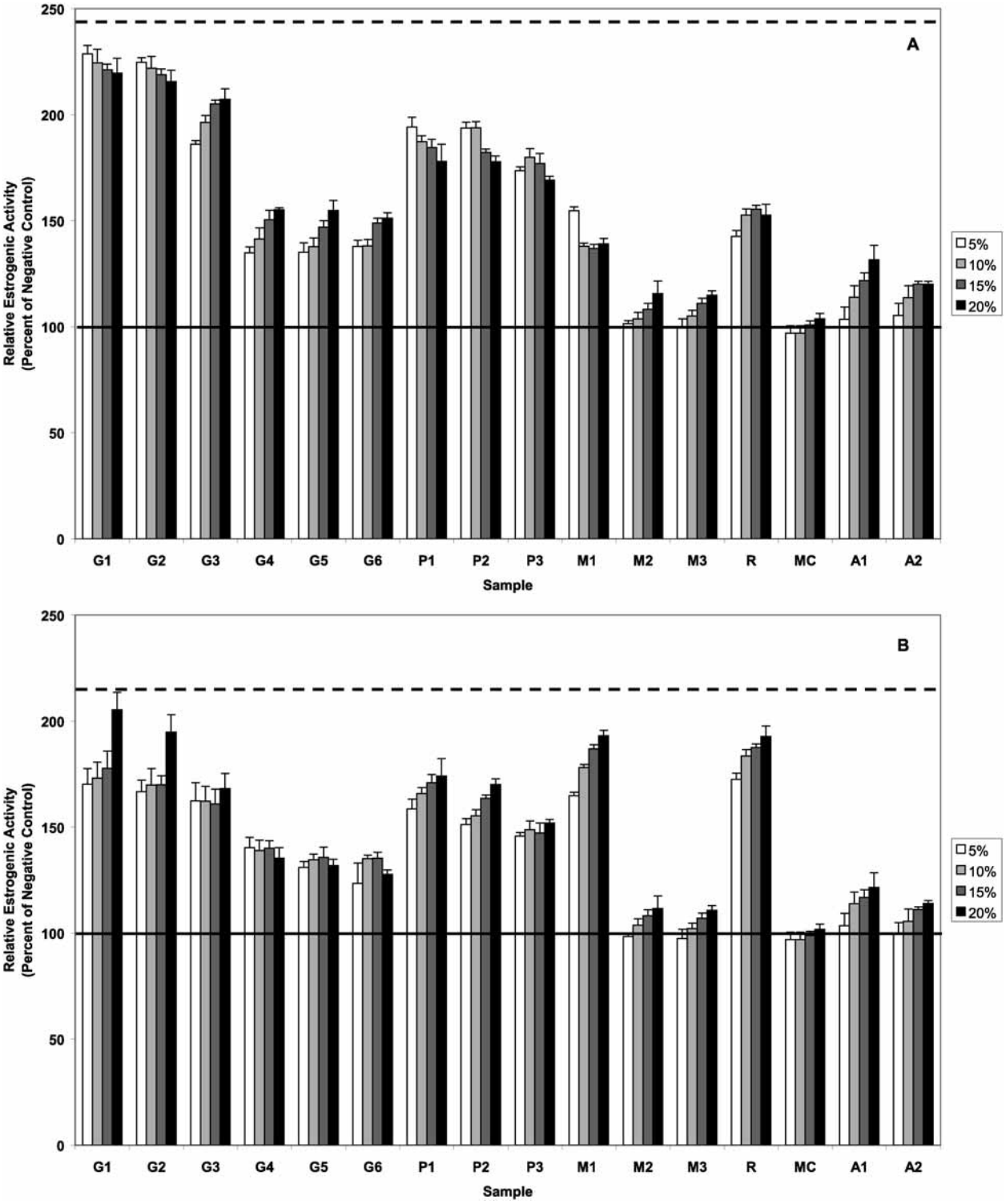

Fig. 2. Relative estrogenic activities of Tunisian water and wastewater samples at 5, 10, 15 and 20% final concentrations as determined by modified E-screen assay after six days of incubation. Cell number was assessed by MTT (A) and NR (B) assays. Samples are shown in Table 1. Results, which represent the average of two to six independent experiments, are expressed as percentages of the negative control (100%, solid line). PBS(–) (5, 10, 15 and 20% final concentrations) and 17β-estradiol (2 nM) were used as negative and positive controls, respectively. The dashed lines represent the relative estrogenic activities of the positive control, which are 244 and 215% for the MTT and NR graphs, respectively. Results are statistically significant versus the negative control ($p<0.05$, Student's t-test) for all samples except for the following: M2, M3, A1 and A2 at 5% concentration; MC at any concentration.

Table 2
In situ physicochemical analyses results.

Sample	pH	EC (ms/cm)	O_2 (mg/l)	S (g/l)	T (°C)	TOC (mg/l)	COD (mg/l)	BOD_5 (mg/l)	N (mg/l)	$P(PO_4)$ (mg/l)	SS (g/l)
G1	7.36	6.38	3.90	0.83	17.6	201	530.4	420	7	1.67	0.46
G2	7.82	7.95	0.14	4.90	16.9	45	491.2	302	8	1.51	0.27
G3	7.73	6.03	1.93	3.60	17.7	120	257.6	100	7	0.87	0.21
G4	7.82	5.95	3.06	3.60	17.7	86	239.3	100	7	0.04	0.34
G5	7.97	5.75	2.39	3.40	17.7	40	129.6	55	7	0.02	0.19
G6	7.82	5.63	2.49	3.90	17.7	65	98.4	14	1.4	0.08	0.11
P1	6.82	1.92	1.09	0.90	17.3	102	864.0	245	51.4	1.33	0.57
P2	7.43	1.80	1.98	0.80	17.4	301	848.0	240	63	0.02	0.33
P3	7.24	1.80	2.39	0.80	17.4	35	116.8	45	7	0.02	0.24
M1	7.19	2.26	0.16	1.20	20.8	102	408.0	380	5.8	0.16	0.52
M2	7.35	1.67	0.20	0.80	18.6	41	140.8	240	2.8	1.37	2.37
M3	7.46	1.76	1.33	0.80	18.6	11	119.2	25	4.2	0.07	0.01
R	7.78	2.12	2.12	1.10	18.7	21.95	139.2	15	2.8	0.01	0.03
MC	7.37	1.37	2.86	0.60	19.4	0.20	0.8	0.1	14	0.01	0.04
A1	8.00	5.64	2.25	3.40	17.2	32	121.6	90	3.5	0.87	0.10
A2	7.96	5.65	2.00	3.40	16.6	12	113.6	45	1.4	0.17	0.10

Among the surface water samples, the Roriche River sample showed a very high estrogenic activity (more than 150 and 190% in the MTT and NR results, respectively). On the other hand, the Medjerda River sample showed no estrogenic activity. Water from the Ariana River showed a maximum estrogenic activity of 131% in the MTT results, which showed slight estrogenic activity at high concentrations.

Given these findings, we attempted to determine the estrogenic compounds in the water samples by HPLC. However, our detection limit was rather high at 100 ng/ml, and thus, none of these compounds were detected by HPLC.

We then used ELISA, which has a detection range of 16.5–4,000 pg/ml, to determine the presence of 17β-estradiol in some of the water samples. Results showed that 17β-estradiol was present in the water samples at concentrations ranging from 19.3 to 3,515.9 pg/ml (Table 3). Expectedly, untreated wastewater (G1, G2 and M1) had an average 17β-estradiol concentration of 2,705.4 pg/ml. On the other hand, water treated by activated sludge (M2 and M3) had a 17β-estradiol concentration of as low as 19.3 pg/ml.

4. Discussion

The E-screen assay is widely used to determine the estrogenicity of natural and environmental compounds through their ability to stimulate the growth of an estrogen-dependent cell line, most commonly MCF-7. This assay has also been used to determine the estrogenicity of sewage effluent and surface water.[18] MCF-7 is a human breast cancer cell line that expresses estrogen receptors and shows a proliferative response in culture in the presence of estrogens.[16] The endpoint of the E-screen assay is the increase in cell number, which was assessed by both MTT and NR assays in this study.

The MTT assay 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, the NR dye uptake assay does not require metabolic activity but simply measures the number of live cells that

Table 3
17β-Estradiol concentrations (pg/ml) in some Tunisian water and wastewater samples as determined by ELISA.

G1	G2	G3	P1	P2	P3	M1	M2	M3	R
2261.4	3515.9	55.6	128	40.2	79.1	2338.8	19.3	25.1	36.7

have an intact plasma membrane and can thus absorb the NR dye. Because both assays have the same end point (cell number), a comparison of their results further strengthens the validity of the experiment, and ensures that, regardless of the assay mechanism, the increase in cell number in response to estrogen activity can be reproduced. In this study, the trends in the MTT and NR assay results are generally similar.

For each experiment, we included a positive control (cells treated with 29 nM 17β-estradiol) to confirm that our cell line indeed proliferates in response to estrogen, given that not all MCF-7 cell stocks show the same proliferative response.[19] Results were calculated relative to that of the negative control (cells treated with the same PBS(–) concentration) and expressed as relative estrogenic activity. The proliferative effects of some of the water samples, particularly the G1, G2 and G3 samples, were almost as large as that of 17β-estradiol.

The presence of numerous EDCs in natural waters and sediments has been attributed to the incomplete removal of these substances during wastewater treatment.[20] This is further confirmed by the results of this study, which showed that two WWTPs (Gammart and pilot WWTPs) that utilize the stabilization pond treatment system were not capable of completely removing the estrogenic activity of the effluent. The difference in their estrogen removal efficiencies can be attributed to their hydraulic retention times (HRTs), which are three months for the Gammart WWTP and 17 days for the pilot WWTP. It is known that the longer the HRT, the higher the estrogen removal efficiency.[21] Moreover, for the Gammart WWTP, it appears that the relative estrogen removal efficiency could also be related to the plant's capacity to reduce the TOC concentration, COD and BOD_5 (Table 2). As for the pilot WWTP, the low relative estrogen removal efficiency is probably attributable to the short HRT, aside from the fact that it is not being run continuously.

Natural and synthetic estrogens that are not removed by wastewater treatment eventually end up in the aquatic environment. The Roriche River is a typical example of a contaminated river. Figure 2 shows that water from this river has very high estrogenic activity; this is primarily because it receives wastewater effluent from the Charguia WWTP. The latter processes not only domestic wastewater from two highly populated areas but also wastewater from hospitals, research institutes and various industries of the Charguia industrial zone. It is safe to assume that, similarly to the Gammart and pilot WWTPs, the Charguia WWTP cannot also completely remove estrogenic compounds from wastewater. A 17β-estradiol concentration of 36.7 pg/ml (=ng/L) in water of the Roriche River (Table 3) represents a significant estrogenic input. This concentration is sufficient to induce endocrine-mediated responses in aquatic organisms.[22] The slight estrogenic activity of water of the Ariana River can also be attributed to the fact that it receives effluent from the Gammart WWTP.

This study has likewise confirmed that the activated-sludge treatment system is more effective than systems using stabilization ponds for decreasing estrogenic activity. This finding is similar to that of Leusch *et al.*[23] who showed that the levels of estrogenic activity in treated municipal sewage from 15 activated-sludge WWTPs in Australia and New Zealand were lower than those reported by researchers in the United Kingdom, whose WWTPs use a different technology. According to Khanal *et al.*,[21] natural estrogen compounds are mainly removed from the aqueous phase by adsorption onto associated solid phases, such as sludge in wastewater treatment.

The ELISA results shown in Table 3 confirmed the presence of 17β-estradiol in the water samples and provided a clear reason for the increase in the relative estrogenic activity of the treated cells as shown in Fig. 2. However, a direct correlation between the results shown in Table 3 and Fig. 2 could not be statistically established because of the very wide range (19.3 to 3,515.9 pg/ml) of 17β-estradiol concentrations in the water samples, whereas the differences in estrogenic activity shown in Fig. 2 are not as wide. This is because only very low concentrations of 17β-estradiol are required to induce a proliferative response in MCF-7 cells, but whose growth rate is limited by enzyme kinetics and the availability of space and nutrients.

The significance of the ELISA results is strengthened by one study that showed that the natural estrogens estrone and 17β-estradiol make up more than 98% of the total estrogen equivalent concentration in STP effluent, whereas the contribution of phenolic compounds is less than 2%.[24] This denotes that 17β-estradiol is a highly predominant estrogen in WWTP effluent. Moreover, endogenous steroidal estrogens such as 17β-estradiol are 10,000- to 100,000-fold more potent than exogenous endocrine disruptors or synthetic chemicals such as organochlorine aromatic compounds.[25]

In this study, we showed for the first time the estrogenic activities of Tunisian surface water as well as raw and treated wastewaters. We also showed and compared the capabilities of three WWTPs for decreasing estrogenic activity. Given the detrimental impact of endocrine-disrupting compounds on the physiology of both animals and humans, their detection and monitoring in water and wastewater are increasingly required.

References

1 Gomez, R.L., Scrimshaw, M.D. and Lester, J.N. (2003): Determination of endocrine disrupters in sewage treatment and receiving waters. *Trends Anal. Chem.* **22**: 697–707.

2 Colborn, T. and Clement, C. (1992): *Induced Alteration in Sexual Development: the Wildlife Human Connection.* Princeton Scientific Publishing Company, Princeton, N. J., p. 403.

3 Birnbaum, L.S. (1995): Development effects of dioxins. *Environ. Health Perspect.* **103**: 89–94.

4 Fry, D.M. and Toone, C.K. (1981): DDT-induced feminization of gull embryos. *Science* **213**: 922–924.

5 Guillette Jr., L.J., Gross, T.S., Masson, G.R., Matter, J.M., Percival, H.P. and Woodward, A.R. (1994): Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ. Health Perspect.* **102**: 680–688.

6 Sumpter, J.P. (1995): Feminized responses in fish to environmental estrogens. *Toxicol. Lett.* **82**/**83**: 737–742.

7 Jobling, S., Nolan, M., Tyler, C., Brighty, G. and Sumpter, J. (1998): Widespread sexual disruption in wildfish. *Environ. Sci. Technol.* **32**: 2498–2506.

8 Desbrow, C., Routledge, E.J., Brighty, G.C. Sumpter, J.P. and Waldock, M. (1998): Identification of estrogenic chemicals in STW effluent. I. Chemical fractionation and *in vitro* biological screening. *Environ. Sci. Technol.* **32**: 1549–1558.

9 Purdom, C.E., Hardiman, P.A., Bye, V.J. Eno, N.C., Tyler, C.R. and Sumpter, J.P. (1994): Estrogenic effects of effluents from sewage treatment works. *Chem. Ecol.* **8**: 275–285.

10 Al Atiri, R. (2007): Integration of wastewater reuse in the overall water resources management, Tunisian experience. In: *MEDA WATER Int. Conf. Sustainable Water Management.* 21–24 March 2007, Tunis, Tunisia, pp. 287–292.

11 Ternes, T.A. (1998): Occurence of drugs in German sewage treatment plants and rivers. *Water Res.* **32**: 3245–3260.

12 Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.D. and Servos, M. (1999): Behavior and occurrence of estrogens in municipal sewage treatment plants. I. Investigations in Germany, Canada and Brazil. *Sci. Total Environ*. **225**: 81–90.

13 Cargouët, M., Perdiz, D., Mouatassim-Souali, D., Tamisier-Karolak, S. and Levi, Y. (2004): Assessment of river contamination by estrogenic compounds in Paris area (France). *Sci. Total Environ.* **324**: 55–66.

14 Harries, J.E., Sheahan, D.A., Jobling, S., Matthiessen, P., Neall, P., Routledge, E.J., Rycroft, R., Sumpter, J.P. and Taylor, T.A. (1996): Survey of estrogenic activity in UK inland waters. *Environ. Toxicol. Chem.* **15**: 1993–2002.

15 Harries, J., Janbakhsh, A., Jobling, S., Matthiessen, P., Sumpter, J. and Tyler, C. (1999): Estrogenicity potency of effluent from two sewage treatment works in the United Kingdom. *Environ. Toxicol. Chem.* **18**: 932–937.

16 Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N. and Serrano, F. O. (1995): The E-screen assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ. Health Perspect.* **103**: 113–122.

17 Rodier, J. (1984): *Analysis of Water: Natural Water, Wastewater and Marine Water*. Dunod, BORDAS, 7th ed., p. 1365.

18 Korner, W., Spengler, P., Bolz, U., Schuller, W., Hanf, V. and Metzger, J.W. (2001): Substances with estrogenic activity in effluents of sewage treatment plants in southwestern Germany. 2. Biological analysis. *Environ. Toxicol. Chem.* **20**: 2142–2151.

19 Villalobos, M., Olea, N., Brotons, J.A., Olea-Serra, M.F., Almodovar, J.M.R. and Pedraza, V. (1995): The E-Screen assay: a comparison of different MCF7 cell stocks. *Environ. Health Perspect.* **103**: 844–850.

20 Farre, M., Brix, R. and Barcelo, D. (2005): Screening water for pollutants using biological techniques under European Union funding during the last 10 years. *Trends Anal. Chem*. **24**: 532–545.

21 Khanal, S.K., Xie, B., Thompson, M.L., Sung, S., Ong, S.-K. and Van Leeuwen, J.H. (2006): Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. *Environ. Sci. Technol.* **40**: 6537–6546.

22 Tabata, A., Kashiwada, S., Ohnishi, Y., Ishikawa, H., Miyamoto, N., Itoh, M. and Magara, Y. (2001): Estrogenic influences of estradiol-17β, p-nonylphenol and bisphenol-A on Japanese medaka (*Oryzias latipes*) at detected environmental concentrations. *Water Sci. Technol.* **43**: 109–116.

23 Leusch, F.D.L., Chapman, H.F., van den Heuvel, M.R., Tan, B.L.L., Gooneratne, S. R. and Tremblay, L.A. (2006): Bioassay-derived androgenic and estrogenic activity in municipal sewage in Australia and New Zealand. *Ecotoxicol. Environ. Saf.* **65**: 403–411.

24 Nakada, N., Nyunoya, H., Nakamura, M., Hara, A., Iguchi, T. and Takada, H. (2004): Identification of estrogenic compounds in wastewater effluent. *Environ. Toxicol. Chem.* **23**: 2807–2815.

25 Hanselman, T.A., Graetz, D.A. and Wilkie, A.C. (2003): Manure-borne estrogens as potential environmental contaminants: A review. *Environ. Sci. Technol.* **37**: 5471–5478.