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RESEARCH ARTICLE

Risk assessment of heavy metal toxicity of soil irrigated with treated wastewater using heat shock proteins stress responses: case of El Hajeb, Sfax, Tunisia

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Abstract Heavy metal contamination of soil resulting from treated wastewater irrigation can cause serious concerns resulting from consuming contaminated crops. Therefore, it is crucial to assess hazard related to wastewater reuse. In the present investigation, we suggest the use of biomarker approach as a new tool for risk assessment of wastewater reuse in irrigation as an improvement to the conventional detection of physicochemical accumulation in irrigated sites. A field study was conducted at two major sites irrigated with treated wastewater and comparisons were made with a control site. Different soil depths were considered to investigate the extent of heavy metal leaching, the estrogenic activity, and the biomarker response. Results have shown that a longer irrigation period (20 years) caused a slight decrease in soil metal levels when compared to the soil irrigated for 12 years. The highest levels of Cr, Co, Ni, Pb, and Zn were detected at 20 and 40 cm horizons in plots irrigated with wastewater for 12 years. The latter finding could be attributed to chemical leaching to deeper plots for longer irrigation period. Furthermore, the treated wastewater sample showed a high estrogenic activity while none of the soil samples could induce any estrogenic activity. Regarding the stress response, it was observed that the highest stress shown by the HSP47 promoter transfected cells was induced by a longer irrigation period. Finally, the treated wastewater and the irrigated soils exhibited an overexpression of HSP60 in comparison with reference soil following 1 h exposure. In conclusion, in vitro techniques can be efficiently used to assess potential hazard related to wastewater reuse.

Keywords Risk assessment · Wastewater reuse · Stress response · Biomarkers · Heavy metals · Estrogenic activity

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Introduction

Wastewater reuse is becoming essential in arid and semiarid regions where the water shortage represents a serious problem. It is used to irrigate fruit trees, vineyards, fodder, cotton, cereals, golf courses, and public gardens (Bahri and Brissaud 1996). Moreover, the increased urbanization discharging larger amounts of wastewater encouraged the reuse of the nonconventional water resource. Even though the reuse for irrigation of crops and landscapes or groundwater recharge provides socioeconomic benefits, it might contaminate the plant–soil–water matrix and deteriorate groundwater quality. Therefore, it is crucial to assess hazard related to wastewater reuse.

Guidelines for treated wastewater (TWW) reuse usually include the levels of treatment needed for the different types of reuse (Blumenthal et al. 2000). Nonetheless, the rely on TWW physicochemical parameters, coliforms, or nematodes levels as pollution indicators is no longer sufficient in case of

irrigation (Angelakis et al. 1999). Guidelines for soil monitoring should be equally included or setup in countries facing water shortage such as Tunisia (Bahri and Brissaud 1996).

To assess the impact of wastewater reuse on groundwater quality, Katz et al. (2009) used a combination of geochemical and microbiological indicators including pharmaceutical and other organic wastewater compounds, stable isotopes, major ions, nutrients, pesticides, volatile organic chemicals, trace elements, and fecal indicator bacteria and human enter viruses were considered (Katz and Griffin 2008).

The introduction of in vitro techniques is quite new to investigate the potential hazard related to wastewater reuse. In this context, we previously considered in vitro bioassays specific for stress response, estrogenic activity, and intracellular tight junction to assess the transfer of contamination from TWW to irrigated soil in Zaouit Sousse, Tunisia (Ben Fredj et al. 2012). Such techniques revealed to be very efficient to assess the effect of a mixture compound and to simulate the field conditions rather than testing one single compound separately. Besides, the introduction of biomarker approach is even pioneering for the assessment of pollution hazard related to TWW irrigation. We recently investigated several biomarkers specific for wastewater reuse pollution (Ben Fredj et al. 2012). Most of the identified biomarkers were common for plants, mammalians, and humans with high relevance to several organisms. Some biomarkers were related to several molecular and biological functions such as protein folding and stress response, heat shock, oxidative stress, cell proliferation, cell metabolism, protein synthesis, and glycolytic metabolic pathway. Other biomarkers were associated to cancer pathogenesis, apoptosis, tumors, or diseases like neuropathy. Among all, the heat shock proteins (HSP) family biomarkers are known to be ubiquitous in all organisms without considering cell specificity.

For the present study, we suggest considering real-time polymerase chain reaction (RT-PCR) as a new tool for hazard assessment of wastewater reuse. The PCR is a method which produces multiple copies of a target DNA. Briefly, the PCR method uses a thermostable polymerase enzyme (e.g., the Taq enzyme) to create multiple copies of target DNA. Detection of target DNA in the human genome is achieved through the use of short sections of synthetic, single-stranded DNA known as oligonucleotide primers. These primers can be designed to be specific for different species. The PCR works by using a cycling of different temperatures. The most common method employs three distinct temperatures. Double-stranded DNA is separated into individual strands using a high temperature, commonly over 90 °C. A lower temperature is then used to anneal the primers to the target section of DNA. At an intermediate temperature between the previous two temperatures, the polymerase enzyme produces a mirror copy of the target DNA. This cycle of temperatures is then repeated several times. Over 40 cycles, due to the exponential nature of the PCR method, more than 10^9 copies of the target DNA can be theoretically produced (Toze 1999). Previous studies from Rose et al. (2006) used RT-PCR to identify microbial strains in wastewater or fresh water as a sign of environmental contamination.

The current study focused on evaluating the potential hazard related to wastewater reuse for irrigation in semiarid areas in Tunisia. The main objective was to investigate the stress and the estrogenic activity of the reused (TWW) and irrigated soils with possible regard to their heavy metals content, physicochemical properties, and the duration of the irrigation period. Furthermore, gene expression assays (RT-PCR) were performed aiming to explore whether heat shock biomarkers are specific for wastewater reuse pollution in semiarid land. Heat shock protein 47, E-screen in vitro bioassays were carried out on the latter samples and heat shock biomarker expression was tested via RT-PCR.

Materials and methods

Chemicals

The list of reagents that were used to prepare the culture medium and the required solutions are available in the supporting information (Text S1).

Cells and culture conditions

Chinese hamster ovary (CHO) cells stably transfected with (+) or without (−) a HSP47 promoter were used for this experiment. Heat shock protein 47 promoter transfected cells will be abbreviated into HSP(+); HSP47 promoter transfected cells. The cells were provided by S. Yokota (Kaneka, Osaka, Japan) and were grown as adherent monolayer in 75-cm^2 tissue culture flasks using F12 Medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10 % fetal bovine serum (FBS), 200 μg/mL of Geneticin (G418), and 0.1 g/L kanamycin solution. Estrogen receptor-positive human breast cancer MCF-7 cells were obtained from H. Shinmoto (National Food Research Institute, Ministry of Agriculture, Fishery, and Forestry, Tsukuba, Japan) and routinely maintained in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % FBS and 1 % penicillin (5,000 μg/mL)–streptomycin (5,000 IU/mL) solution in 75-cm^2 tissue culture flasks. Human intestinal Caco-2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % FBS, 1 % penicillin–streptomycin, and 1 % nonessential amino acids. The cultures were maintained in a 5 % CO_2 incubator at 37 °C. Cell passage was carried out at 80 % confluence at one on two ratio using 0.25 % trypsin (1 mM EDTA).

Sample preparation and physicochemical parameters

The irrigated perimeter is part of El Hajeb Town, Sfax Prefecture which is located in southeast Tunisia (Long.=E10°38′

06.8″, Lat.=N34°40′49.5″) and being irrigated with a TWW from Sfax South Wastewater Treatment Plant (WWTP) since 1989.

Originally, a wetland system used to treat a volume of 24,000 m^3 per day until 2007. Later on, the treatment system was improved to activated sludge system with successive nitrification/denitrification processes in order to scale up the treatment capacity to 49,500 m^3 per day by (Belaid 2010). The TWW is mainly reused for the irrigation of olive trees surrounded by pasture plants. A TWW sample was collected from the outlet of the effluent pond of Sfax South WWTP. Three soil sampling sites were identified: the first one has been irrigated for almost 12 years. The second for more than 20 years and the third one rainfed, referred as the control site. From each location, four soil samples at different depths of 10, 20, 40, and 60 cm were collected to cover the rhizosphere area. To get a homogenous representative sample, each depth sample corresponds to a mixture of three sampling corners forming a triangle in the center of the field and having edges of 2 m length. In total, 12 soil samples were obtained in addition to the TWW sample and their physicochemical characteristics and heavy metal contents were determined. For this purpose, the soil samples were firstly spread on plastic trays in fume cupboard, air-dried for 10 days at room temperature. They were crushed with an agate mortar and sieved through a 125-μm mesh screen. The prepared samples were finally stored in labeled polypropylene containers at room temperature. For bioassays experiments, soil extracts one on five (2 g of soil in 10 mL of ultra pure water or 70 % EtOH) previously dried for 2 h at 40 °C and sieved through a 2-mm mesh screen were prepared. The soil extracts were sonicated, shaked overnight, and then centrifuged at 1,000 rpm for 30 min. The TWW and soil extract samples were filter-sterilized using a 0.45-μm membrane and pH was adjusted using a pH meter MP220 (Mettler Toledo, Schwerzenbach, Switzerland) from alkaline (7.73±0.14) to the pH value of 6.5 to maintain optimum conditions for cells culture prior to 0.22-μm membrane filtration.

Soil pH and conductivity were measured in a soil–water suspension (1:5 *w*/*v* extraction ratios) according to the method described in FAOUN (1984). Five grams of each soil was mechanically shaken with 25 mL of ultra pure water in a beaker during 30 min. The mixtures were left to rest for 1 h and the pH of the overlying solution was measured using a precalibrated WTW330/SET-2 pH meter (Weilheim, Germany). The electric conductivity (EC) was measured, with a LF 330/SET conductivity meter (Weilheim, Germany) using the same suspension, which was kept overnight to allow the bulk of the soil to settle. Dissolved organic matter in soil was extracted by adding 20 mL of 0.01 M KNO_3 solution (ultra pure grade) to 10 g of soil in 30-mL polyethylene centrifuge tubes (Stephan et al. 2008). The supernatants (soil solutions) were separated by shaking the 30-mL centrifuge tubes overnight and then centrifuging at 3,000 rpm for 10 min to be finally injected in a total organic analyzer (aj-Analyzer multi N/C 2100 S, Jena, Germany) for organic matter evaluation.

Chloride, nitrate, and sulfate ions were quantified in a soil–water solution using an ionic chromatography (HIC-6A Shimadzu type, Kyoto, Japan) equipped with a conductivity detector and a Shim-Pack Column. The separation was achieved using an isocratic elution at a flow rate of 1 mL/min. A mobile phase of 1 mmol/L of tris-hiroxymethyl-aminomethane and 1 mmol/L of phthalic acid were used to quantify Cl^-, NO_3^-, and SO_4^{2-}. Exchangeable cations, K^+, Na^+, Mg^{2+}, and Ca^{2+} were extracted with 1 M ammonium acetate at 1:20 dilution according to the method NF X 31-108 described in AFNOR (2004) and then analyzed using atomic absorption spectrophotometer (Thermo Scientific iCE 3000 Series, Waltham, MA, USA).

To assess the soil pseudo-total metal concentrations, 1 g of soil was digested with a mixture of concentrated nitric acid and hydrochloric acid applied as Aqua Regia reagent in a screw-capped Teflon beaker as described by Pereira et al. (2008). The mixture was gently heated on a hotplate at 100 °C until almost dryness. Afterwards, 10 mL of HNO_3 (4 N) was added to the Teflon vessels and the solution was filtered through Whatman filter paper into a 25-mL volumetric flask to remove all coarser particles. The filtrate was adjusted to 25 mL with ultra pure water and analyzed by atomic absorber similarly to the exchangeable cations.

The composite index method (Nemerow index) according to Liang et al. (2011) was adopted to evaluate the soil quality of the study area. The composite index method used the single pollution index (Pi)=Ci/Cref (where Ci represents the mean concentration of each heavy metal for different depths and *C*ref indicates the evaluation criteria value), which is an environmental indicator of pollution. The Nemerow composite index method considers the single evaluation factors and highlights the most contaminating element as expressed by Nemerow index $(P_s) = \sqrt{\left(P_{av}{}^2 + P_{max}{}^2\right)/2)}$ (where P_{av} is the average of single pollution indexes of all metals and P_{max} is the maximum value of the single pollution index of all metals). The quality of soil environment is classified into five grades from the Nemerow index (P_s<0.7, safety domain; 0.7≤P_s<1.0, precaution domain; 1.0≤P_s<2.0, slightly polluted domain; 2.0≤P_s<3.0, moderately polluted domain; and 3.0≤P_s, seriously polluted domain (Ogunkunle and Fatoba 2013).

HSP47 assay

The cell line used for HSP47 assay in the present study was developed in a previous study (Katz et al. 2009). In brief, the promoter–reporter construct, which carried the 5′-upstream promoter sequences of murine HSP47 gene ligated to

upstream of β-galactosidase coding sequences, was transfected to CHO cells and a stable transgenic cell line was established.

Concisely, the HSP47 promoter transfected cells were plated in 96-well plates and were allowed to attach for 48 h before adding samples diluted in medium for 3 h. Heat shock protein 47 expressed is accompanied by the enzymatic release of β-galactosidase. The assay was performed following the protocol detailed in Ben Fredj et al. (2010) by measuring the β-galactosidase activity (fluorescence at 365 nm exCitation/450 nm emission) in response to sample-induced stress, as described previously with some modifications (Isoda et al. 2003).

Modified E-screen assay

The estrogenic activity of the samples was investigated using the modified E-screen assay. Human breast cancer MCF-7 cells containing estrogenic receptors were plated onto 96-well plates at 1.0×10^4 cells/mL of phenol-red-free RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % charcoal-treated FBS. The cells were then allowed to attach for 24 h. The TWW and soil extract samples were added to the cells at the same concentrations tested for the HSP47 assay. The cells were incubated for 6 days, after which, cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay as described in Ben Fredj et al. (2010).

Total RNA isolation, cDNA synthesis, and real-time PCR

DNA-free total RNA of cultured cells was isolated with Isogen kit (Molecular Research Center, Japan) according to manufacturer's instructions. Briefly, human intestinal Caco-2 cells were seeded at 2.0×10^5 cells/mL and treated with wastewater and soil extract samples for indicated times. Isolated RNA was ethanol precipitated, quantified, and quality assessed by Nanodrop ND-1000 spectrophotometer. About 1 μg of total RNA was submitted to reverse transcription with Superscript III (Invitrogen) using oligo (dT) primers according to manufacturer's protocol and cDNA was synthesized using thermal cycler (Applied Biosciences). Taqman master mix and gene taqman probes (AHSA1, HSPA1, HSPA5, HSPA8 and HSPD1) (Applied Biosystems) were used for RT-PCR and taqman probe (Applied Biosystems) was used as an internal control. cDNA amplification reactions were run on Applied Biosystems 7500/7500 fast RT-PCR system.

Statistical analyses

Two to three independent experiments were carried out for each test. For statistical analysis, all data were tested for normality (Kolmogorov and Smirnov) and homogeneity of variances (Levene test) and were found to satisfy the assumption for analysis of variance (ANOVA). Statistical significance ($p<0.05$) was evaluated by one-way ANOVA, and if significant, group means were compared using Bonferroni's post hoc test and homogeneous subsets of significance were determined by Duncan's post hoc test.

Results and discussion

Physicochemical parameters

Preliminary studies showed that the investigated soils (three sites) were sandy-alkaline type. Tables 1 and 2 provided the general characteristics of the TWW and the examined soils. Comparison between the tree sites showed a similar pH at all the depths (Table 1). Whereas, the pH of the TWW was relatively alkaline (pH = 8.28) due to a high concentration of bicarbonates (719.42 mg/L). Unexpectedly, the irrigated sites did not exhibit higher pH values in comparison with TWW. The latter can be explained by the buffer capacity of the soil which is probably related to its physicochemical characteristics.

Moreover, as shown in Table 1, EC increased overall with depth for the tree sites probably because of initial soil characteristics and properties. Indeed, Cl^-, SO_4^{2-}, and NO_3^- concentrations in shallow soils were higher than the upper layers for the three sites (Table 2). Moreover, high correlation was observed between EC and the latter ions (R^2=0.99, 0.70, and 0.69; respectively). Nonetheless, irrigated sites showed lower conductivity compared to the reference site (EC-Ref>EC-T12>EC-T20). This is probably due to salt leaching and infiltration to deeper layers following long-term irrigation. These findings corroborate positively with the results of Corwin and Lesch (2005), who reported that a similar phenomenon was linked to the increase of sand fraction in depth leading to a higher drainage. Concerning the exchangeable elements (K^+, Mg^{2+}, Ca^{2+}, Na^+) in Table 2, the negative correlation with EC could be hardly explained. We would speculate that there is a competition between the organic matter and the iron–manganese oxides in the soils matrix for retaining these elements.

Heavy metals usually present in soil naturally or due to anthropologic activities. Among them, Al, Fe, and Mn are major constituents important for mineral structure (Baize and Sterckeman 2001; Horckmans et al. 2005). The irrigated and control soils exhibited almost the same concentration of Cu, Mn, Ni, and Fe suggesting that the TWW does not contribute to the enrichment of soil during both irrigation periods. However, a slight accumulation was noticed for Cr and Zn, Pb especially for the site irrigated for 12 years. This can be explained by the fact that the metal load conveyed by the TWW was either leached to deeper depths or taken by plants as presented by Wang et al. (2007).

Table 1 Physicochemical parameters in wastewater and soil extracts

Sample	pH			EC (mS/cm)			COD (mg/L)		
Wastewater									
TWW	8.28			7.27			–		
WWTP[a]	7.1–8.7			4–7.7			123-700		
TN[b]	6.5–8.5			7			90		
Soil 125 μm fraction							DTC (mg/kg)		
	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20
10 cm	7.8	7.7	7.8	0.46	0.53	0.34	69.8	48.9	78.1
20 cm	7.6	7.7	7.7	0.47	0.53	0.34	69.5	54.6	99.9
40 cm	7.8	7.7	7.9	1.02	0.53	0.44	65.4	66.9	69.5
60 cm	7.8	7.7	7.7	1.06	0.67	0.42	71.6	73.0	68.2

EC electrical conductivity, *DTC* dissolved total carbon

[a] Sfax South values from 1989 to 2011, ONAS 2011

[b] Tunisian standards for wastewater reuse NT 106-003, 1989

Moreover, concentrations of selected heavy metals in the 12 and 20 years irrigated soil profiles (Table 3) indicated a difference related to irrigation period. Indeed, the highest levels of Cr, Pb, and Zn were found at 20 and 40 cm horizons in plots irrigated with wastewaters for 12 years. A similar trend was reported by Xua et al. (2010) following 8 years irrigation with TWW while no accumulation was observed after 20 years irrigation. Furthermore, Smith et al. (1996) results indicated that there was no change in the concentration of heavy metals in soils irrigated with TWW for 4 years. These findings support our above-mentioned hypothesis of chemical leaching to deeper depths for longer irrigation period.

In the present study, longer irrigation period (20 years) caused a slight decrease in soil metal levels when compared with the soil irrigated for 12 years. These results were unexpected considering the continuous long-term input of metals even with a very low concentration and the adsorption of trace metals on soil particles. The latter could be attributed to plant uptake and/or leaching under sandy soil conditions as explained above and demonstrated by Lin et al. (2008). Furthermore, the soil investigated in the present work was classified as sandy soil which would facilitate metal leaching to deeper soil layers. In addition, the low soil organic matter (DTC) content (Table 1) of the examined soils can lead to low adsorption affinity and retention capacity of the sandy soils toward metals. Actually, soil DTC is regarded as the major component able to fix Cu, Ni, Zn, and Cr in soils (Lin et al. 2008). Regardless of the metal load brought by the (TWW), the rapid decomposition of the added organic matter due to the high year-round temperatures in Tunisia contributes to the reduction of the DTC concentration throughout the three sites.

Based on previous studies by Martin et al. (2006) and Al-Kashman and Shawabkeh (2006), correlation coefficients can provide suggestive information regarding heavy metal sources and pathways. It was shown that Mn, Cu, Pb, Zn, Cr, and Ni were significantly positively correlated with each others. Especially, Mn was highly correlated with Cu, Zn, Cr, and Pb corresponding to R^2 coefficient values of 0.67, 0.8, 0.73, and 0.68, respectively. This correlation indicated that these metals had potentially similar origins. Besides, it was reported that hydroxide manganese usually has a high affinity for metallic ions such as Ni, Cu, and Pb (Mason et al. 1999).

Rattan et al. (2005) studied the effect of untreated WW irrigation period on the soil accumulation of exchangeable metals. They found out that metal concentration levels increased with irrigation period for Zn, Cu, Fe, Pb, and Ni. However, the same authors demonstrated that Mn concentration decreased throughout the irrigation years due to the leaching of originally present amount.

For Nemerow composite pollution method, we considered the following metals Ni, Cr, Zn, Pb, and Cu since no concentrations of references (Cref) were established for Fe and Mn. The adopted evaluation criteria was the recommended values for agricultural soil by the Canadian Council of Ministers for the Environment (2007), as Tunisia has no stipulated guideline for heavy metals in soil. The calculated values of the Nemerow index (P_s) were, respectively, 0.075, 0.077, and 0.08 for the reference, T12, and T20 samples showing a slight trend of pollution risk but still within the safety domain grade (P_s<0.7).

Estrogenic activity of the TWW and soil extract samples

The modified E-screen assay was carried out for the TWW and the soil extract samples to detect the presence of estrogenic compounds and estrogen-like EDCs in the TWW samples as well as their potential accumulation in the soil. As shown in Fig. 1, the effluent sample between 1 and 10 % concentrations was able to induce an estrogenic activity

Table 2 Exchangeable cations and water soluble ions in wastewater and soil extracts

Sample	Na^+			K^+			Mg^{2+}			Ca^{2+}			Cl^-			NO_3^-			SO_4^{2-}		
Wastewater (mg/L)																					
TWW	1,218.70			56.28			150.27			97.29			nd			nd			nd		
WWTP[a]	780–2,100			17–105			129–209			103–521			903–2,580			0.35–50			508–1,950		
TN[b]	–			0			0						2,000			–			–		
Soil 125 μm fraction (mg/kg)																					
	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20
10 cm	126.5	102.9	253.9	457.5	345.4	304.3	164.8	224.3	254.4	3,553.5	3,394.4	3,749.4	231.9	293.6	56.3	90.5	258.3	48.6	265.4	232.2	126.5
20 cm	130.3	99.0	283.6	440.9	398.0	398.9	141.4	239.7	255.8	3,141.2	3,614.6	3,675.3	314.0	359.6	78.1	163.9	90.0	59.4	222.1	289.2	131.5
40 cm	107.5	358.5	192.2	363.5	328.1	293.0	147.4	247.1	171.6	3,603.9	3,865.5	3,367.3	847.5	405.6	191.8	312.8	87.4	192.9	457.9	302.5	203.8
60 cm	102.7	250.5	297.8	376.6	421.2	471.8	248.1	295.3	236.1	3,743.6	3,645.5	3,437.2	935.2	482.6	117.6	240.6	70.4	46.8	728.7	399.1	643.7

[a] Sfax South values from 1989 to 2011, ONAS 2011

[b] Tunisian standards for wastewater reuse NT 106-003

varying from 1.3 to 1.8 times of the control cell activity. The highest concentration caused an estrogenic activity almost equal to the positive control (E2). Within this range, 5 % concentration was chosen for the heat shock protein genes biomarker approach. These results confirmed our previous investigation on TWW from Sousse South WWTP, Tunisia, where the same concentrations induced an estrogenic activity varying from 1.4 to 2.2 times of the control cell activity (Ben Fredj et al. 2012). Sfax south WWTP processes a mixture of domestic (47 %) and industrial (21 %) influents containing a large load of EDCs including natural and synthetic estrogenic compounds or xenoestrogens from female hormones, contraceptive pills containing 17α-ethynylestradiol, or detergent industry in Sfax area.

Using the modified E-screen assay, we determined whether the 70 % ethanol soil extract samples can induce a significant estrogenic activity of MCF-7 cells (Fig. 1). The soil extraction was carried out in 70 % ethanol and not in water to target the estrogenic compounds from the soil (Zhang et al. 2006). To prevent from the cytoxic effect of ethanol at high concentration, the highest treatment concentration did not exceed 0.1 %. As shown in Fig. 1, while the TWW sample showed the high estrogenic activities from 1 to 10 % concentrations, none of the soil samples could induce any estrogenic activity. Previous results by Ying and Kookana (2005) showed the sorption and degradation of estrogen-like EDCs in sandy to clay soil associated with wastewater reuse. The biodegradation might be carried out by bacteria present in animal manure. This was confirmed by the absence of estrogenic activity for all the soil extract samples. Overall, the samples were more cytotoxic in depth (40–60 cm) rather than on the surface soil (0–20 cm). Among the different profiles and concentrations, S40 samples at 0.001 and 0.01 % concentrations were the most cytotoxic. Moreover, the possible presence of cytostatic or cytotoxic compounds accumulated in the soil may explain the significant decrease in cell number for soil horizons (S40 and S60) versus that of the nontreated control. Results of Tables 2 and 3 showed the highest levels of heavy metals (Cr, Zn, Pb, Fe, Cu, and Mn) and nitrates at the same depths. Comparison of irrigation period showed that the longer irrigation slightly increased the accumulation of cytotoxic compounds in T20-S40 rather than T12-S40 sample probably due to the lack of oxygen.

Stress response effect of the TWW and soil extract samples on HSP47 promoter transfected cells

Even though the TWW samples showed tiny concentrations of heavy metal levels, we hypothesized that the mixture effect could induce stress on mammalian cells. The stress response system, in particular the HSP inducing system, functions in all mammalian tissues and cells. Therefore, in bioassay systems utilizing this stress response, it is not necessary to take into

Table 3 Heavy metal levels in wastewater and soil extracts

Sample	Ni			Cr			Zn			Pb			Fe			Cu			Mn		
Wastewater (mg/L)																					
TWW	0.013			0.005			0.05			0.051			0.093			0.012			0.11		
WWTP[a]	0.02–0.13			0.007–1.1			0.01–0.27			0.001–0.37			<0.013–1.69			<0.01–0.06			0.04–0.17		
TN[b]	1.00			0.10			5.00			1.00			5.00			0.50			0.50		
Soil 125 μm fraction (mg/kg)																					
	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20	Ref	T12	T20
10 cm	4.0	3.2	5.0	2.6	4.0	5.0	11.5	14.2	13.7	2.1	3.6	3.6	4,903.2	4,050.4	4,786.1	4.0	4.5	7.4	39.1	51.2	60.7
20 cm	3.4	3.7	3.3	2.0	4.2	3.8	11.2	15.6	11.9	4.4	3.8	2.9	5,728.1	4,885.4	3,876.2	7.7	5.1	6.2	60.9	57.6	51.6
40 cm	3.6	4.3	4.3	2.2	6.0	3.1	10.3	16.8	11.4	2.5	3.9	2.7	4,735.7	6,058.4	5,362.6	5.7	6.2	6.3	41.3	67.1	58.4
60 cm	3.6	4.4	3.9	2.4	3.2	2.5	9.9	12.5	9.8	2.6	2.5	2.0	4,470.9	5,202.4	4,679.8	4.9	5.1	4.9	33.5	49.5	39.7
EU[c]	0.03–0.075			0.1–0.15			0.15–0.3			0.05–0.3			–			0.05–0.14			–		
US[d]	0.21			1.5			1.4			–			–			0.75			–		

[a] Sfax South values from 1989 to 2011, ONAS 2011

[b] Tunisian standards for wastewater reuse NT 106-003

[c] European Union Framework, Directives 86/278/EEC, 2000

[d] United States, Directives NRC, 2002

consideration the basic problems regarding cell specificity (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. We previously developed this highly sensitive system, HSP47 assay, for detecting trace amounts of environmental pollutants including heavy metals and natural toxins (Isoda et al. 2003). Thus, the HSP47 assay was carried out to estimate the stress response of HSP47 promoter transfected cells exposed to the TWW sample (Fig. 2). Interestingly, the results showed that the TWW samples exhibited a slight stress regardless of the treatment concentration.

To investigate whether the irrigation with TWW conveys any harmful effect on soil, the HSP47 assay was carried out using the irrigated or rain-fed soil extract samples taken at several depths and for two irrigation periods 12 and 20 years. Contrary to the soil ethanol extracts used for the E-screen assay, water extraction was performed for the samples used in HSP47 assay to target water soluble ions and heavy metals. When the stress response of HSP47-promoter transfected cells was tested, 3-h treatment with T20-S20 and T20-S40 samples at 0.1 and 5 % concentrations, respectively, induced the highest effect, even higher than the TWW effect. Interestingly, no significant stress was observed for the control soil extract samples at all depths showing the relevance of this bioassay. Considering the longer irrigation period, T20 samples induced a higher stress that could be only explained by the lack of oxygen, the oxidation state, and the form (complexes or free) of some heavy metals.

As presented by Fig. 2, the highest stress shown by HSP47 promoter transfected cells was induced by T20-S20 and T20-S40 in accordance with the heavy metal profile showing the highest content of Zn and Mn for the same samples. The stress response at 20 and 40 cm depth is a sign of a potential hazard to the rhizosphere. However, previous results by Belaid (2010) demonstrated the infiltration of heavy metals and nitrates to the groundwater by infiltration through the sandy texture following long-term irrigation with TWW.

Heat shock protein biomarkers

To evaluate the quality of water in terms of the presence of potentially toxic substances and the accumulation of pollutants in soil, it is important to perform preliminary toxicological tests and to check cellular impacts of wastewater irrigation at protein level. Chemical analyses provide information about the fate of single compounds. Comparison of detected levels of heavy metals or other chemicals with the established guidelines can give information whether the TWW could be used for irrigation purposes. However, no guidelines are established on soils to estimate the accumulation of toxic compounds. In vitro bioassays investigate the potential effects of the whole sample composition on mammalian or human

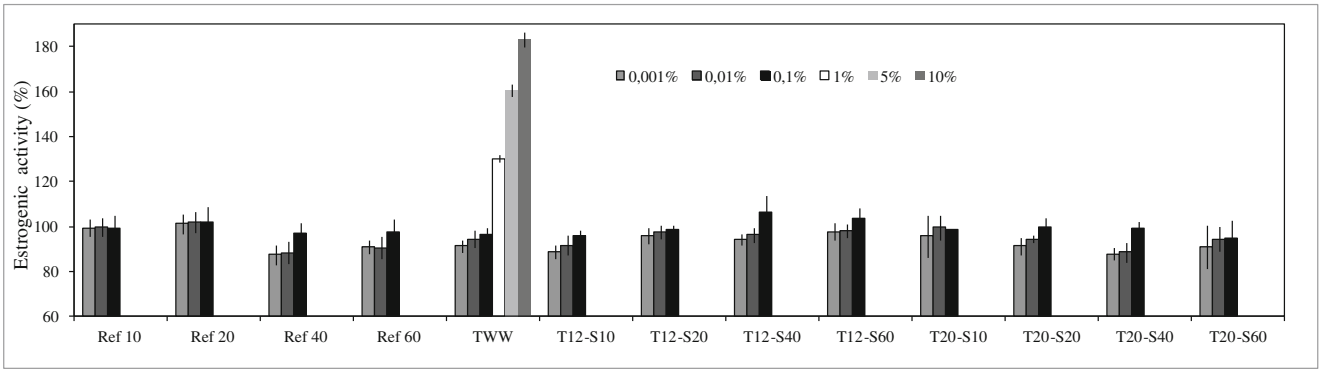

Fig. 1 Estrogenic activity of wastewater and soil samples

cells and other organisms involved in the wastewater reuse process. To gain insight into the molecular mechanisms and harmful effects induced by the environmental pollution, there is need for relevant and quick response to judge not only for the quality of the TWW but also for the safe feasibility and implementation of the wastewater reuse.

Advances in environmental risk assessment methodologies have triggered the research to establish early warning signals, or biomarkers, reflecting the adverse biological responses towards environmental toxins (Bucheli and Fent 1995). Biomarkers are measurements in cells or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host response. In an environmental context, biomarkers offer promise as sensitive indicators demonstrating that toxicants have entered organisms, have been distributed between tissues, and are eliciting a toxic effect at critical targets (Van der Oost et al. 2003).

Following the results of E-screen assay and HSP47 assay, a TWW sample at 5 % in addition to soil extract samples at 40 cm at 1 % was selected for the biomarkers investigation using the gene expression assay (RT-PCR). For soil extracts, a representative of each soil (reference, T12, and T20) was considered. We previously established several biomarkers relative to the wastewater reuse-induced pollution (Ben Fredj et al. 2012). The latter biomarkers are involved in different cellular functions ranging from cell proliferation, cell metabolism, protein synthesis, heat shock, oxidative stress, cell proliferation, and glycolytic metabolic pathway or associated to tumors or diseases. Among them, heat shock biomarkers related to environmental stress were selected. The HSP family biomarkers contribute in cellular defense mechanism against the stress-inducing compounds such as the heavy metals detected in the samples (Table 3). Moreover, the stress response exhibited by the HSP47 assay motivated the selection of five biomarkers namely mitochondrial HSP60, activator of HSP90 ATPase homolog 1 (AHSA1), and three members of the HSP70 family (heat shock 70 1A/1B protein (HSP71), 78 glucose-regulated protein (GRP78), and heat shock cognate 71 protein (HSP7C)).

Mitochondrial HSP60 (spot C2) is a chaperonin that assists in protein folding under both normal and stressful conditions and is ubiquitously produced in mammalian cellular mitochondria. The HSP60 protein is induced in response to heat stress and is a member of an immunologically conserved family represented in yeast (*Escherichia coli*) and in mitochondria of protozoan, plants, and animals (Reading et al. 1989). As shown in Fig. 3a, following 1-h exposure, TWW and irrigated soils exhibited an overexpression of HSP60 in comparison with the reference soil and the nontreated cells. Interestingly, T20 sample showed a higher expression than

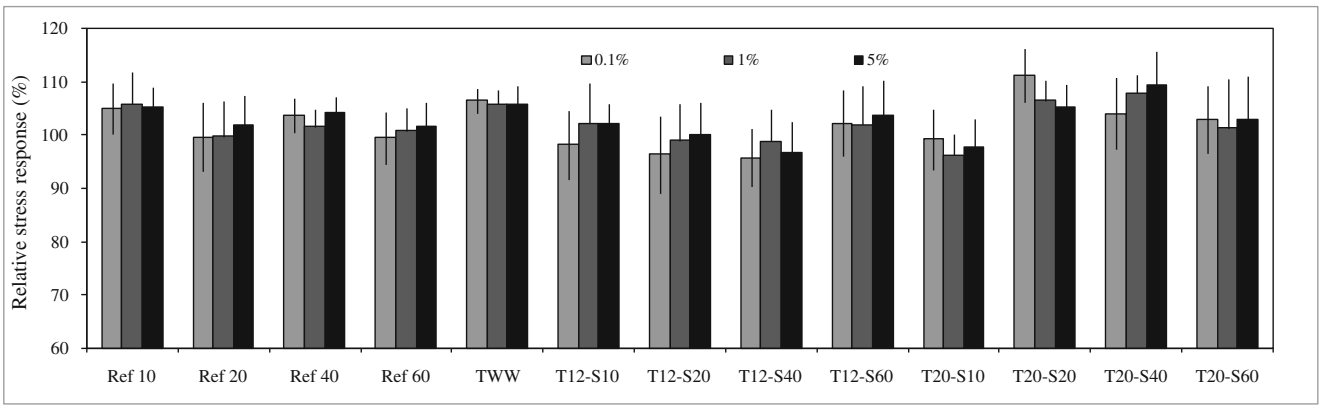

Fig. 2 Stress responses of wastewater and soil samples on HSP47 promoter transfected cells

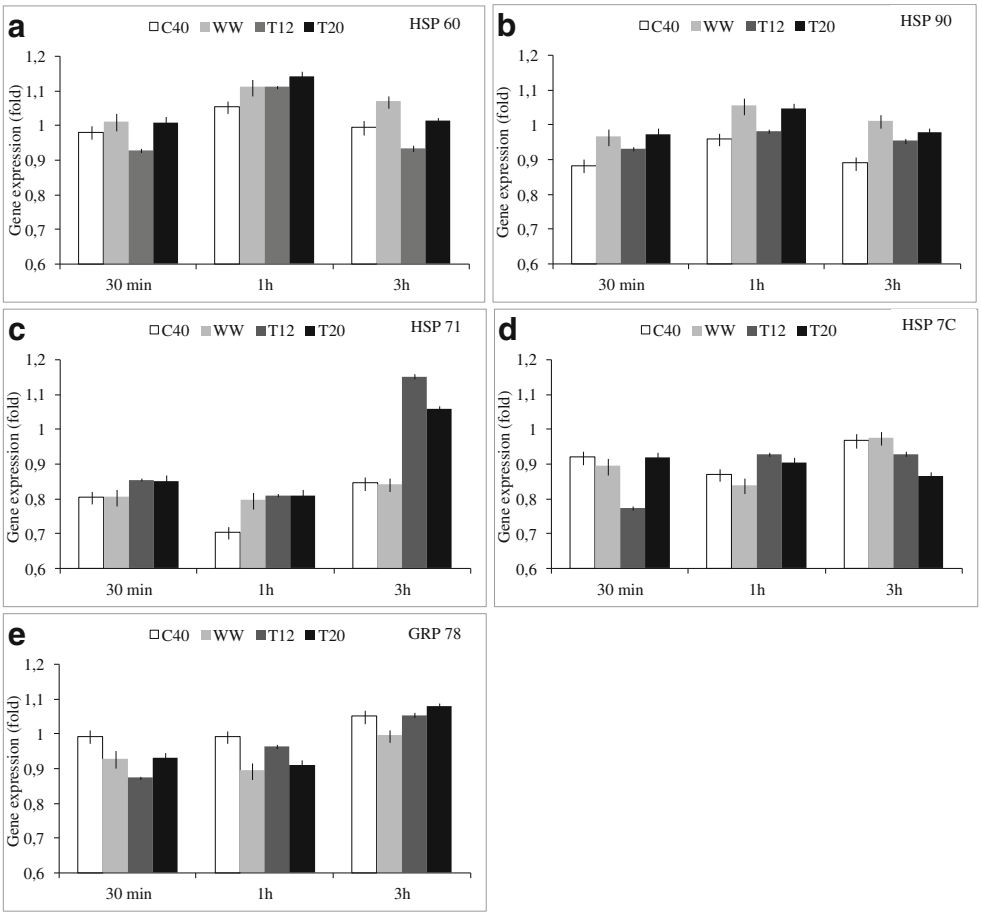

Fig. 3 Stress-related gene expression

T12 and TWW in total accordance with HSP47 assay results (Fig. 2). These results could be explained by the accumulation effect due to an extended irrigation period (20 versus 12 years). Moreover, no time-dependent manner was observed due to the specificity of chaperone gene that is expressed in early stage of incubation to trigger the expression of protein. Besides, we previously demonstrated that the overexpression of HSP60 is associated to the disruption of intracellular barrier function following the treatment of Caco-2 cells with TWW- and TWW-irrigated soil samples.

Activator of HSP90 AHSA1 is a co-chaperone stress-regulated protein that stimulates HSP90 ATPase activity (Stark et al. 2010). According to the results in Fig. 3b, TWW as well as T20 exhibited an increased expression of HSP90 gene following 1-h treatment. These results correlate with the estrogenic activity shown by the same samples (Fig. 1). Besides, other members of AHSA1 family are soil bacterial proteins *Bacillus subtilis* Yndb and CalC from *Micromonospora echinosporato* (Stark et al. 2010). Alteration in such proteins may affect the soil degradation process of organic matter present in the TWW.

Heat shock protein 70 1A/1B, 78 glucose-regulated, and HSP cognate 71 proteins are members of HSP70 family and contribute in cellular defense mechanism. The HSP70 family is the most highly conserved of the many heat shock protein families across a wide range of species from bacteria to plants and animals (Beere and Green 2001). As shown in Fig. 2, HSP47 was overexpressed following the addition of both the TWW and the soil extract probably due to the heavy metal mixture in the samples. For HSP71, 3-h incubation of Caco-2 cells with the samples showed an overexpression of the latter gene due to T12 and T20 treatment while reference soil and TWW showed a downregulation (Fig. 3c). The results of HSP7C expression assay elicited a downregulation of HSP7C following 3 h treatment with T12 and T20 (Fig. 3d). Conversely, the reference soil and the TWW were ineffective following 3 h incubation. Finally, when the Caco-2 cells were treated for 3 h, the TWW-irrigated soils for 12 and 10 years (T12 and T20) induced the upregulation of GRP78 gene in comparison with reference soil as shown by Fig. 3e. Furthermore, T20 resulted more effective than T12 due to accumulated load of stress-inducing compounds on the TWW-irrigated soil. We assume that the interaction between pollutants, originally present in the TWW, and soil components induced such toxic effect. For specific tasks such as assistance in refolding of partially denatured proteins, the HSP70 cycle is coupled to the action of HSP90, in accordance with the downregulation of AHSA1 (Ruden et al. 2005).

Conclusions

The present study investigated on the quality of a treated wastewater in terms of potentially toxic substances and their eventual accumulation in irrigated soils following wastewater reuse. It was shown that physicochemical properties of the treated wastewater fit Tunisian standards for wastewater reuse. However, heavy metal concentrations in soil showed a slight accumulation following 12 years irrigation while a prolonged irrigation period (20 years) caused a slight decrease of heavy metal levels in soil resulting probably from the leaching to deeper layers or an uptake by plants.

Treated wastewater samples showed high estrogenic activities while none of the soil samples could induce any estrogenic activity. The absence of estrogenic compounds in soil was explained by the sorption and degradation of estrogen-like EDCs in sandy to clay soil associated with treated wastewater. Moreover, the present results showed that longer irrigation period (20 years) increased the accumulation of cytotoxic compounds in the 40 cm fraction when compared to 12 years irrigated soil samples collected from the same depth. The highest stress was shown using HSP47 promoter transfected cells and induced an increased expression of HSP90 gene after 1 h treatment using the 20 years irrigated soils.

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