

# Assessment of wastewater-irrigated soil containing heavy metals and establishment of specific biomarkers

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1 **Assessment of wastewater-irrigated soil containing heavy metals and establishment of**  
2 **specific biomarkers**

3

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20

21 **ABSTRACT** - Irrigation with treated wastewater (TWW) is a vital alternative for arid and semi-  
22 arid lands but it poses pollution-risk to soil, vegetation and groundwater. Therefore, in the  
23 present study, *in vitro* bioassays were used to evaluate the adverse effects of TWW and irrigated-  
24 soil extract sample, on mammalian cells, with respect to heavy metal - Ni, Cd, Pb, Fe, Al-  
25 content. The heat shock protein (HSP) 47, E-screen, and transepithelial electrical resistance  
26 (TEER) assays served to investigate the stress response of treated-HSP47-transfected Chinese  
27 hamster ovary (CHO) cells, the estrogenic activity of the samples in MCF-7 breast cancer cells,  
28 and the barrier function (BF) of Caco-2 cells. Furthermore, proteomics analyses were performed  
29 to shed light on involved mechanisms and to establish pollution biomarkers. Results showed that  
30 the TWW elicited a stress response on HSP cells from 0.1% concentration while soil extract  
31 samples exhibited a stress at 1%. TWW induced an estrogenic activity at 10%; up-regulating cell  
32 proliferation and tumor-related proteins. Soil extract triggered the enhanced expression of HSP70  
33 family proteins as survival mechanisms against their cytotoxicity toward MCF-7 cells. Moreover,  
34 depending on the concentration, 1% of soil extract from 20 cm depth (T20) resulted in a  
35 disruption of BF in Caco-2 cells involving cell metabolism, protein synthesis and tumor marker  
36 proteins, whereas, 5% of T20 induced the expression of BF-related proteins associated to heat  
37 shock, oxidative stress, cell proliferation and glycolytic metabolic pathway. These biological  
38 techniques were found to be extremely useful to evaluate the impact of wastewater reuse and to  
39 establish specific biomarkers that are common proteins for humans, other mammals and plants.  
40 Future studies should focus on exposure quantifications.

41

42 **Keywords** – Biomarkers; Wastewater reuse; Heavy metals; E-screen; Barrier function

43

## 44 **1. Introduction**

45

46 Wastewater reuse for irrigation and groundwater recharge is considered a sustainable option  
47 to satisfy a sharply increasing water demand in arid and semi-arid regions. Despite of the  
48 potential socio-economic benefits derived from wastewater reuse, irrigation with wastewater  
49 treatment plant (WWTP) effluents poses a number of potential environmental and health risks.  
50 Effluents may contains considerable amounts of pathogenic microorganisms, heavy metals,  
51 harmful organic chemicals such as endocrine disrupting compounds (EDCs) that are able to  
52 reduce soil quality, accumulate in plants or contaminate the groundwater (Stagnitti, 1999). These  
53 harmful compounds represent a potential risk to the environment.

54 Guidelines for the reuse of TWW have focused on defining appropriate levels of treatment  
55 needed for different types of reuse (Blumenthal et al., 2000). Several pollution indicators such as  
56 the physicochemical parameters (chemical oxygen demand (COD), biological oxygen demand  
57 (BOD), suspended solids (SS), chlorine, phosphates, and nitrates, the levels of pesticides, heavy  
58 metals, fecal coliforms or intestinal nematodes are widely used (Angelakis et al., 1999). Treated  
59 wastewaters are used to irrigate fruit trees, vineyards, fodder, cotton, cereals, golf courses, and  
60 public gardens (Bahri and Brissaud, 1996). Nevertheless, in many countries, including Tunisia,  
61 guidelines for soil monitoring have not yet been set up.

62 Previous studies focused on physicochemical parameters, heavy metal content, and bacterial  
63 indicators (Al-Khashman, 2009; Palese et al, 2009) to assess the potential hazard related to  
64 wastewater reuse for irrigation purposes. Hazard assessment of wastewater effluent is  
65 traditionally based on the evaluations of individual chemicals, including heavy metals, EDCs or  
66 other toxic compounds identified through chemical analyses (Baun et al., 1998). However, these

67 techniques could not overcome the problem of chemical mixtures present in environmental  
68 samples and are unable to characterize the hazardous effects on human health. Thus, *in vitro*  
69 bioassays using bacteria, sentinel marine organisms or mammalian cells, as well as *in vivo*  
70 animal experiments, are essential to provide information regarding the possible biological effects  
71 of the chemical mixture, such as cytotoxicity, stress (Ben Fredj et al., 2010), estrogenic activity  
72 or paracellular BF disruption (Narita et al., 2007). Nevertheless, these assays in turn do not allow  
73 understanding the mechanisms behind such biological effects. Therefore, proteome analyses of  
74 environmental samples, known as environmental proteomics, has investigated many organisms,  
75 ranging from microorganisms and plants to invertebrates and vertebrates not only to gain insights  
76 into underlying mechanisms of toxicity but also to discover specific biomarkers of several  
77 environmental pollution origins. Nonetheless, proteomics is a field of environmental research  
78 still in its infancy, due to a number of caveats, such as the limited number of organisms fully  
79 covered in the sequence databases, the high genetic variability, and the dependence on  
80 environmental factors (Nesatyy and Suter, 2007). Besides, there have been few studies using  
81 environmental proteomics that have considered mammalian or human cells to assess the hazard  
82 of environmental samples. Hence, the direct assessment of wastewater reuse on mammalian cells  
83 deserves attention.

84 A complementary investigation of representative environmental samples should combine  
85 chemical analyses to detect the compound levels, *in vitro* bioassays and proteomics. These  
86 bioassays are able to elucidate several effects such as stress response induced by stressors  
87 including heavy metals, estrogenic response due to the presence of EDCs or paracellular BF  
88 sensitive to several contaminants. This approach not only allows discovery of new protein

89 biomarkers of TWW-irrigated soil pollution but, more importantly, provides the basis to shed  
90 light on underlying mechanisms of toxicity.

91 The present study focused on evaluating the potential hazard related to wastewater reuse for  
92 irrigation in semi-arid areas in Tunisia. The objective of the study was to investigate the stress,  
93 the estrogenic activity, and the effect on paracellular BF of the reused TWW and the irrigated  
94 soil with possible regard to their heavy metal content. Furthermore, proteomics analyses were  
95 performed aiming to understand the mechanisms involved in such effects and to establish  
96 specific biomarkers of wastewater reuse pollution. Heat shock protein 47, E-screen,  
97 transepithelial electrical resistance (TEER) *in vitro* bioassays were carried out on the latter  
98 samples and specific biomarkers were identified by the proteomics analyses.

99

## 100 **2. Materials and methods**

101

### 102 *2.1. Chemicals*

103

104 The list of reagents that were used to prepare the culture medium and the required solutions  
105 are available in the supplementary data (Text S1).

106

### 107 *2.2. Cells and culture conditions*

108

109 Chinese hamster ovary (CHO) cells stably transfected with (+) or without (-) a HSP47  
110 promoter were used for this experiment. Heat shock protein 47-promoter-transfected cells will be  
111 abbreviated into HSP(+). The cells were provided by S. Yokota (Kaneka, Osaka, Japan) and

112 were grown as adherent monolayer in 75-cm<sup>2</sup> tissue culture flasks using F12 Medium (Invitrogen,  
113 Carlsbad, CA, U.S.A.), supplemented with 10% fetal bovine serum (FBS), 200 µg/mL of  
114 Geneticin (G418), and 0.1 g/L kanamycin solution. Estrogen receptor-positive human breast  
115 cancer MCF-7 cells were obtained from H. Shinmoto (National Food Research Institute,  
116 Ministry of Agriculture, Fishery, and Forestry, Tsukuba, Japan) and routinely maintained in  
117 RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 1% penicillin  
118 (5000 µg/mL)-streptomycin (5000 IU/mL) solution in 75-cm<sup>2</sup> tissue culture flasks. Human  
119 intestinal Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)  
120 supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% non essential amino acids.  
121 The cultures were maintained in a 5% CO<sub>2</sub> incubator at 37 °C. Cell passage was carried out at  
122 80% confluence at one on two ratio using 0.25% trypsin (1 mM EDTA).

123

### 124 *2.3. Sample preparation and water quality parameters*

125

126 The irrigated perimeter of Zaouit Sousse (lat. = N35°46'37.2", long. = E10°39'9.3", and alt.  
127 = 20 m N.G.T) is located in Tunisia (Fig. 1). It is situated 7.5 km from Sousse South WWTP  
128 which uses trickling filters coupled to an activated sludge (TF/AS) system with a treatment  
129 capacity of 18700 m<sup>3</sup> wastewater per day. The TWW is mainly reused for the irrigation of olive  
130 trees surrounded by sorghum pasture. The surface irrigation started 20 years ago. A TWW  
131 sample was collected from the irrigation pond upstream of the perimeter. A soil profile from the  
132 wastewater-irrigated area was sampled at 5 depths: 10, 20, 40, 60 and 90 cm to cover the root  
133 zone of the irrigated olive trees. For irrigated culture the roots system is not so deep; most of the  
134 roots are concentrated within 70 to 80 cm depth (Klay et al., 2010). In addition, a control soil

135 profile was taken from the rain-fed area at only two depths: 10 and 40 cm, due to the soil  
136 homogeneity beyond this depth (Fig. 1). Klay et al. (2010) antecedently found that the soil has a  
137 sandy-clay texture up to 20 cm depth followed by a sandy texture for deeper horizons. Soil  
138 extracts one on five (2 g of soil in 10 mL of deionized water) were prepared. The samples were  
139 filter-sterilized using a 0.45- $\mu$ m membrane filter for chemical analysis. For the sample volume  
140 used for bioassays experiments, pH was adjusted using a pH meter MP220 (Mettler Toledo,  
141 Schwerzenbach, Switzerland) from alkaline ( $7.93 \pm 0.3$ ) to the pH range of 7.2 to 7.4 to maintain  
142 optimum conditions for cell cultures prior to 0.22- $\mu$ m membrane filtration. Electrical  
143 conductivity (EC) and pH were measured using a YK-22CT conductivity meter (Sato Shouji  
144 Incorporation, Tokyo, Japan) and a UC-23 digital pH meter (Central Kagaku Corporation, Tokyo,  
145 Japan), respectively. The heavy metal levels were determined using DR/4000 Spectrophotometer  
146 (Hach, Loveland, CO, USA) (Hach, 2003) following the standard methods of analysis 8150 PAN  
147 (Detection limit (DL) = 5  $\mu$ g/L), 8033 Dithizone (DL = 1.3  $\mu$ g/L), 8317 fast column extraction  
148 (DL = 2  $\mu$ g/L), 8147 Ferro zine (DL = 4  $\mu$ g/L), and 8112 Aluminon (DL = 5  $\mu$ g/L) respectively.

149

#### 150 2.4. HSP 47 assay

151 The cell line used for HSP47 assay in the present study was developed in a previous study  
152 (Isoda et al., 2003). In brief, the promoter-reporter construct, which carried the 5'-upstream  
153 promoter sequences of murine HSP47 gene ligated to upstream of  $\beta$ -galactosidase coding  
154 sequences, was transfected to CHO cells and a stable transgenic cell line was established.

155 Concisely, the HSP (+) cells were plated in 96-well plates and were allowed to attach for 48  
156 h before adding samples diluted in medium for 3 h. The TWW sample was diluted to 0.01, 0.1, 1,  
157 5, 10, and 20% of total volume of the medium (100  $\mu$ L), whereas the soil extracts were used at



158 0.001, 0.01, 0.1, 1, 5, and 10% concentration. For higher concentrations than 1%, the results in  
159 wells with 5% sample were compared with those with 5% PBS(-) and so on, to distinguish  
160 between the sample effect and the decrease of medium volume. Heat shock protein 47 expression  
161 is accompanied by the enzymatic release of  $\beta$ -galactosidase. The assay was performed  
162 following the protocol detailed in (Ben Fredj et al., 2010) by measuring the  $\beta$ -galactosidase  
163 activity (fluorescence at 365-nm excitation/ 450-nm emission) in response to sample-induced  
164 stress, as described previously with some modifications (Isoda et al., 2003).

165

### 166 *2.5. Modified E-screen Assay*

167

168 The estrogenic activity of the samples was investigated using the modified E-screen assay.  
169 Human breast cancer MCF-7 cells containing estrogenic receptors were plated onto 96-well  
170 plates at 1000 cells per well in 100  $\mu$ L of phenol-red-free RPMI medium (Invitrogen, Carlsbad,  
171 CA, USA) supplemented with 10% charcoal-treated FBS. The cells were then allowed to attach  
172 for 24 h. The TWW and soil extract samples were added to the cells at the same concentrations  
173 tested for the HSP47 assay. The cells were incubated for 6 days, after which, cell proliferation  
174 was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay  
175 as described in (Ben Fredj et al., 2010).

176

### 177 *2.6. TEER Assay*

178

179 For TEER measurement, the Caco-2 cells were grown at a density of  $5 \times 10^5$  cells/mL in 8-  
180 well-10-electrodes (8W10E<sup>+</sup>) culture ware (Applied Biophysics, NY, USA) coated with Type

181 I collagen derived from bovine placenta and washed twice with PBS(-). The real time TEER  
182 measurements were performed using the electrical cell-substrate impedance sensing (ECIS)  
183 equipment (Applied Biophysics, NY, USA). The TEER increase is correlated with the closure of  
184 tight junction (TJ) which functions as a barrier, whereas, a TEER decrease denotes a disruption  
185 of BF following the exposure to toxicants. The cells were cultured to establish monolayer  
186 integrity. After 48h culture period, the cell monolayer was washed with medium, the samples  
187 were added for 4h and their effect is expressed as the TEER relative to that at zero time.  
188 Following the results of E-screen assay, TWW and T20 samples were selected. A preliminary  
189 MTT proliferation assay showed that both samples were not cytotoxic toward Caco-2 cells up to  
190 20% or 10% concentrations, respectively (Supplementary data Fig. S1). Hence, the TWW  
191 sample was added at 10 and 20% concentration, whereas, the soil extract sample T20 was added  
192 at 1 and 5% concentration.

193

## 194 *2.7. Proteomics Analyses*

195

196 Proteomics analyses were carried out for both MCF-7 and Caco-2 cells. Human breast cancer  
197 MCF-7 cells and Caco-2 cells were plated at  $2 \times 10^5$  cells/ mL and  $1 \times 10^6$  cells/ mL of medium  
198 and then allowed to attach for 24 h and 48 h, respectively. For sample treatment, concentrations  
199 derived from the E-screen and TEER assay results were selected. The cells were incubated with  
200 medium (control cells) or with samples for 24 h (MCF-7) and 3 h (Caco-2), after which the total  
201 proteins were extracted and quantified following the protocol detailed in Talorete et al. (2008).

202 For first-dimension electrophoresis, 24 cm-immobilized pH gradient (IPG, pH 3-10) dry  
203 strips (Amersham Biosciences, Uppsala, Sweden) were used. Using the Ettan IPGphor II

204 (Amersham Biosciences, Uppsala, Sweden) apparatus, the proteins were then separated  
205 according to their isoelectric point under conditions provided in the 2D-protocol. For second-  
206 dimension, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the  
207 proteins were separated according to their molecular weight using Ettan DALTsix  
208 electrophoresis system (Amersham Biosciences, Uppsala, Sweden) (Talorete et al., 2008). The  
209 coomassie brilliant blue (CBB)-stained gels were scanned using Image-Scanner (Amersham  
210 Biosciences, Uppsala, Sweden) and the spots were analyzed using the ImageMaster 2D-Elite  
211 software (Amersham Biosciences, Uppsala, Sweden). For mass spectrometry, protein spots of  
212 interest were excised from the CBB-stained gel, washed, and digested in-gel with trypsin  
213 (sequencing grade; Promega, Tokyo, Japan). All liquid chromatography tandem mass  
214 spectrometry (LC/MS/MS) mass spectra were acquired on a 3200 QTAR Pulsar (Applied  
215 Biosystems, Foster, CA, USA) mass spectrometer. The obtained peptide sequence tags were used  
216 to identify proteins by searching databases using the Mascot search engine (Matrix Science;  
217 <http://www.matrixscience.com>).

218

## 219 2.8. Statistical analyses

220

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

## 227 **3. Results**

228

### 229 *3.1. Water quality parameters and heavy metal levels*

230

231 The physicochemical parameters as well as the heavy metal content in the TWW and soil  
232 extract samples are presented in Table 1. For most of the heavy metals, the levels in the TWW  
233 sample were within the local guidelines. However, the Cd content in the TWW was broadly  
234 much higher than the Tunisian standard of 10 $\mu$ g/L (over 17-times).

235 Overall, the heavy metal levels in the irrigated soil were higher than the control soil (C10 and  
236 C40) due to the prolonged irrigation (20 years). For the irrigated soil, Ni, Al, and Cd profiles  
237 showed a similar trend with high levels in the top soil at 20 cm and in depth beyond 90 cm.  
238 Nevertheless, only the Cd level was revealed to be higher than EU and US guidelines for both  
239 irrigated and control soils.

240

### 241 *3.2. Stress response effect of the TWW and soil extract samples on HSP(+) cells*

242

243 The stress response of HSP(+) cells exposed to the TWW sample was estimated using the  
244 HSP47 assay (Fig. 2). Interestingly, the results showed that the TWW samples exhibited a dose  
245 dependent stress with a peak registered at 1% concentration.

246 To investigate whether the irrigation with TWW conveys any harmful effect on soil, the  
247 HSP47 assay was carried out using the irrigated or rain-fed soil extract samples taken at several  
248 depths. When the stress response of HSP(+) cells was tested, 3 h-treatment with irrigated soil  
249 extract samples at 1% concentration induced the highest effect, similar to the TWW effect except

250 for T90 sample. Interestingly, no significant stress was observed for the control soil extract  
251 samples at both depths C10 and C40. Moreover, the highest stress was induced by T20 and T90  
252 samples.

253

### 254 *3.3. Estrogenic activity of the TWW and soil extract samples*

255

256 The modified E-screen assay was carried out for the latter samples to detect the presence of  
257 estrogenic compounds and estrogen-like-EDCs in the TWW samples as well as their potential  
258 accumulation in the soil. As shown in Fig. 3, the effluent sample at 10% concentration was able  
259 to induce an estrogenic activity of 2.2-times of the control cell activity. The latter concentration  
260 caused an estrogenic activity almost equal to the positive control (E2) and was consequently  
261 chosen for further proteomics analyses.

262 Using MCF-7 cells, we determined by the modified E-screen assay whether the soil extract  
263 samples can induce a significant estrogenic activity (Fig. 3). Overall, the samples were more  
264 cytotoxic on the surface soil (0-20 cm) rather than in depth (90 cm). Among the different profiles  
265 and concentrations T20 at 1% concentration, representative of the cytotoxic effect, was selected  
266 for further investigation by the proteomics analyses in addition to the control soil C40 at the  
267 same concentration.

268

### 269 *3.4. Proteomics analyses for MCF-7 cells treated with TWW and soil extract samples*

270

271 Following the results given by the modified E-screen assay, the TWW sample at 10%, the  
272 sample T20, and the control soil C40 at 1% concentration were selected for investigating the

273 protein expression relative to the estrogenic activity and the cytotoxic effect. Two-dimensional-  
274 SDS-PAGE was performed for MCF-7 cells treated with the latter samples. Following the spot  
275 analyses using image master software, eight spots of interest were retained. The selection was  
276 based on the differential protein expression of TWW and T20-treated cells in comparison with  
277 the control cells and C40-treated cells. Two main groups arise depending on the treatment with  
278 the TWW sample or the soil extract T20 including common proteins for both target effects.

279

#### 280 3.4.1. Estrogenicity-associated proteins

281

282 Proteomics analyses (Fig. 4A) showed that estrogenicity-associated proteins (spots M1 to  
283 M3) were over-expressed, by 2 to 4-folds in comparison with control cells, following the TWW  
284 sample exposure at 10%. Nevertheless, the soil extract T20 was able to reduce the protein  
285 expression by more than 2-fold for spots M1 and M2. Spots M1, M2 and M3 were identified as  
286 activator of HSP90 ATPase homolog, proliferating cell nuclear antigen (PCNA), and  
287 nucleophosmin (NPM); respectively.

288 In contrast to spots M1, M2, and M3, spot M4 identified as transitional endoplasmic  
289 reticulum ATPase (TEPR) was significantly inhibited by the addition of TWW up to 50%. This  
290 effect was less visible but still significant (25%) for the cells treated with T20 due to the soil  
291 interaction with certain compounds present in the TWW.

292

#### 293 3.4.2. Cytotoxicity-related proteins

294

295 Proteomics analyses (Fig. 4A) revealed that MCF-7 cells incubated for 24 h with the irrigated  
296 soil extract T20 at 1% final concentration showed down-regulation of cytotoxicity-related  
297 proteins (spots M5 to M8). These spots were identified as HSP70 1A/1B, 78 glucose-regulated  
298 protein, HSP cognate 71, and T-complex protein 1 subunit  $\epsilon$ ; respectively.

299

300 *3.5. Transepithelial electrical resistance (TEER) of Caco-2 cells treated with TWW and soil*  
301 *extract samples*

302

303 In the presence of soil extract T20 at 5% concentration, 3h of incubation is required to  
304 significantly increase ( $p < 0.05$ ) the TEER in comparison with control cells (**Fig. 5**). Thus, 3h  
305 appears to be the threshold incubation time that is sufficient to trigger the BF. On the other hand,  
306 the TWW sample at 20% concentration was not able to induce a significant closure of tight  
307 junction (TJ) (i.e., no barrier function). Interestingly, the same samples at lower concentrations  
308 (i.e., T20 at 1% and TWW at 10%) induced TEER reduction following 3h incubation with the  
309 cells. However, only T20 at 1% concentration disrupted meaningfully ( $p < 0.05$ ) the monolayer  
310 integrity.

311

312 *3.6. Proteomics analyses for Caco-2 cells treated with TWW and soil extract samples*

313

314 As shown in Figure 4B, soil extract T20 treatment at 1% concentration triggered mainly up-  
315 regulation (1.5 to 4.5-folds) of proteins associated to the disruption of Caco-2 cell BF. On the  
316 other hand, 5% treatment with T20 seemed to affect BF-related proteins (from 0.25 to 3.5-folds)

317

### 318 3.6.1. Disruption of barrier function-associated proteins

319

320 When Caco-2 cells were treated with soil extract T20 at 1%, proteomics analyses revealed the  
321 up-regulation of eight proteins (spots C1 to C8) in comparison with the control soil C40 and the  
322 control cells. Among them, four proteins (spots C1 to C4) showed an over-expression higher  
323 than 2.5-folds. The latter spots correspond to NPM, mitochondrial HSP60, 26S protease  
324 regulatory subunit 6B and heterogeneous nuclear ribonucleoprotein (hnRNP)-C1/C2,  
325 respectively (Figure 4B).

326 Contrary to spots C1 to C8, spots C9 to C11; identified as eukaryotic initiation factor,  
327 hnRNP-F, and triosephosphate isomerase (TPI); were significantly inhibited by the addition of  
328 T20 at 1% concentration.

329

### 330 3.6.2. Barrier function associated proteins

331

332 Fig. 4B revealed that Caco-2 cells incubated for 3 h with the irrigated soil extract T20 at 5%  
333 final concentration showed differential protein expressions of four BF related-proteins (spots  
334 C12 to C15). Two proteins serpin-E3 and kelch-like protein 21(KEAP) were up-regulated (1.5 to  
335 3.5-folds), whereas polyadenylate-binding protein-interacting protein 1 (PAIP) and enolase- $\alpha$   
336 were down-regulated (less than 0.5-folds).

337



#### 338 **4. Discussion**

339

340 In the arid and semi-arid region, countries such as Tunisia are facing increasingly more  
341 serious water shortage problems. Population growth and accelerated urbanization has lead to  
342 both an increase in water consumption and pollution of water resources. Policy makers have then  
343 been compelled to develop additional water resources (Bahri, 2002). Consequently, TWW reuse  
344 has been made as an integral part of overall environmental pollution control and water  
345 management strategy. Thus, it is essential to characterize TWW composition, to establish  
346 impacts of TWW application on the water-soil-plant system, and to evaluate the long-term  
347 changes in soil properties connected to irrigation practices. In fact, the TWW and potentially the  
348 irrigated-soil contain a complex mixture of organic compounds and chemicals including several  
349 heavy metals likely to threaten human health through the food chain.

350 In the present study, we examined the effect of TWW and soil samples from Tunisia on  
351 animal and human cells specific for stress response, estrogenic activity and paracellular BF.  
352 Furthermore, we checked the protein expression change following sample treatment aiming to  
353 establish specific biomarkers of water pollution.

354 Previous results revealed the presence of several heavy metals in the TWW and the soil  
355 extracts (Klay et al., 2010). For this reason, we targeted five heavy metals Ni, Cd, Pb, Al, and Fe,  
356 highly relevant to human exposure, through detecting their residual levels in the samples (Table  
357 1). Previously, Angelakis et al. (1999) sustained the idea that heavy metals predominantly  
358 accumulate in sludge and not in the liquid wastewater. However, the Cd level in the TWW  
359 exceeded local guidelines. The migration of metals to the soil will especially depend on the  
360 concentration of organic carbon and the soil texture. Klay et al. (2010) antecedently found that

361 the irrigated soil has a sandy-clay texture up to 20 cm depth followed by a sandy texture for  
362 deeper horizons. The results shown in Table 1 demonstrated high heavy metal content at 20 and  
363 90 cm depth. The sandy nature will allow the infiltration of pollutants, while the clay fraction  
364 might be responsible for adsorption of pollutants. In Tunisia, no guidelines for monitoring the  
365 soil quality have been established yet.

366 We hypothesized that the samples including a heavy metal mixture, with a high level of Cd,  
367 could induce stress on mammalian cells. Thus, the HSP47 assay was carried out aiming to verify  
368 this assumption. The stress response system, in particular the HSP inducing system, functions in  
369 all mammalian tissues and cells. Therefore, in bioassay systems utilizing this stress response, it is  
370 not necessary to take into consideration the basic problems regarding cell specificity (Isoda et al.,  
371 2003). It has already been revealed that the production of stress proteins is induced as a result of  
372 the reaction of cells with a stressor such as heat, a chemical substance or a heavy metal. We  
373 previously developed this highly sensitive system, HSP47 assay, for detecting trace amounts of  
374 environmental pollutants including heavy metals and natural toxins (Isoda et al., 2003).

375 As presented by Figure 2, the stress induced by the TWW samples might be related to the Cd  
376 content (Table 1) combined to a mixture of heavy metals responsible of a significant stress in  
377 accordance with our recent works where we demonstrated that Cd combined with Ni and Pb was  
378 responsible for inducing high stress response (Ben Fredj et al., 2010). Moreover, the highest  
379 stress shown by HSP(+) cells was induced by T20 and T90 in accordance with the heavy metal  
380 profile showing the highest content for the same samples. While the stress response at 20 cm  
381 depth is a sign of a potential hazard to the rhizosphere (up to 70-80 cm), the stress inducing  
382 effect of T90 sample might endanger the groundwater by infiltration through the sandy texture  
383 (Klay et al., 2010).

384 Wastewater Effluent may be a significant source of EDCs caused by natural human and  
385 animal hormones such as estradiol, testosterone and estrone, and from birth control pills  
386 containing 17 $\alpha$ -ethynylestradiol (Ying and Kookana, 2005). Although we did not analyze for  
387 such compounds, Garcia Morales et al. (1994) showed that Cd has an estrogen mimetic effect  
388 that induced the growth of MCF-7 cells, mediated by the estrogen receptor (ER). Since the  
389 TWW and soil extract samples exhibited a high level of Cd, we aimed to examine their potential  
390 effect on MCF-7 cells and inquire about the presence of estrogens or other estrogen-like-EDCs  
391 that are able to stimulate their estrogenic activity.

392 As shown in Figure 3, while the TWW sample showed the highest estrogenic activity at 10%  
393 concentration none of the soil sample could induce any estrogenic activity. Ying and Kookana  
394 (2005) suggested that several EDCs including alkylphenols and estrogens, which may be present  
395 in reclaimed wastewater, would not persist, either by sorption or degradation, in sandy to clay  
396 soil associated with wastewater reuse. This was confirmed by the absence of estrogenic activity  
397 for all the soil extract samples. Moreover, the possible presence of cytostatic or cytotoxic  
398 compounds accumulated in the soil may explain the significant decrease in cell number for most  
399 of soil horizons (T20 to T60) versus that of the non-treated control.

400 For proteomics purposes, TWW at 10% concentration was obviously selected while T20 at  
401 1% concentration was preferably considered among the soil horizons. Indeed, the T10 sample  
402 showed more significant cytotoxic effect than T20. However T10 sample was not considered  
403 because such effect is probably due to a number of factors, such as grazing of livestock, presence  
404 of wild animals and surface water runoff from adjacent agricultural areas (Palese et al., 2009).  
405 On the other hand, the level of statistical significance for T20 at 1% concentration (Fig. 3) was  
406 exhibited by most of the profiles (T10 to T60). Hence, it was selected to reflect the soil extract-

407 induced cytotoxicity and to check the impact of wastewater reuse at protein level. In addition, the  
408 control soil C40 at 1% concentration was also considered for further proteomics analyses to  
409 distinguish between the control soil component effect and the wastewater-conveyed component  
410 effect.

411 To evaluate the quality of water in terms of the presence of potentially toxic substances and  
412 the accumulation of pollutants in soil, it is important to perform preliminary toxicological tests  
413 and to check cellular impacts of wastewater irrigation at protein level.

414 According to Figure 4A, three proteins corresponding to spots M1, M2, and M3 are associated  
415 with estrogenicity. Activator of HSP90 ATPase homolog 1 (AHSA1) is a co-chaperone stress-  
416 regulated protein that stimulates HSP90 ATPase activity (Stark et al., 2010). Originally, ER in  
417 MCF-7 is associated with a complex of proteins including HSP90. Binding of estrogens  
418 including E2 to ER causes dissociation of the receptor from the HSP complex and allows ER to  
419 translocate to the nucleus and consequently interact with DNA, resulting in promotion of gene  
420 transcription. Interestingly, dissociated HSP90 can dimerise and translocate to the nucleus where  
421 it can activate transcription as well (Ruden et al., 2005). The increased expression of HSP90  
422 proteins exhibited in the present study may reflect a cellular response to attenuate the  
423 transcriptional activity of ER following exposure to a high concentration of estrogenic  
424 compounds. Besides, other members of AHSA1 family are soil bacterial proteins *Bacillus*  
425 *subtilis* Yndb and CalC from *Micromonospora echinosporato* (Stark et al., 2010). Alteration in  
426 such proteins may affect the soil degradation process of organic matter present in the TWW.

427 Proliferating cell nuclear antigen (PCNA) is a nuclear protein that plays a key role in cell  
428 proliferation, DNA repair, and cell cycle control associated with hepatocellular carcinoma.  
429 Moreover, Lottering et al. (1996) showed that E2 metabolites, suspected in the TWW sample

430 (Narita et al., 2007), elevated PCNA levels during S-phase of the MCF-7 cell cycle through  
431 interaction with ER $\alpha$ . Genes encoding PCNA and/or its products have been identified in a wide  
432 variety of diverse organisms such as animals, yeast and higher plants including *Arabidopsis*,  
433 bean, carrot, soybean and tobacco (Strzalka and Ziemienowicz, 2011).

434 Nucleophosmin (NPM) is an estrogen-regulated nucleolar protein that has been implicated in  
435 cancer pathogenesis. Patterson et al. (1995) showed that NPM exhibited a decrease in protein  
436 expression by apoptotic inductions; this could explain the cytotoxic effect observed for the T20-  
437 treated cells.

438 To the best of our knowledge, this is the first application of proteomics analyses to such soil  
439 extract samples. Mass spectrometry was able to identify the target proteins, to shed light on the  
440 mechanisms underlying the observed cytotoxic effect, and to establish specific biomarkers of  
441 wastewater reuse pollution. Four proteins corresponding to spots M5 to M8 are associated to  
442 cytotoxicity (Fig. 4A).

443 Heat shock protein 70 1A/1B, 78 glucose-regulated, and HSP cognate 71 proteins are  
444 members of HSP70 family and contribute in cellular defense mechanism against the stress-  
445 inducing compounds such as the heavy metals detected in the samples (Table 1). The HSP70  
446 family is the most highly conserved of the many heat shock protein families across a wide range  
447 of species from bacteria to plants and animals (Beere and Green, 2001). As shown in Fig. 2,  
448 HSP47 was over-expressed following the addition of both the TWW and the soil extract  
449 probably due to the Cd abundantly present within the heavy metal mixture in the samples.  
450 Nevertheless, the former members of HSP70 family were not affected by TWW sample and were  
451 down-regulated only by T20-treatment in accordance with the cytotoxic effect induced by the  
452 soil extract on MCF-7 cells. We assume that the interaction between pollutants, originally

453 present in the TWW, and soil components induced such toxic effect. For specific tasks such as  
454 assistance in refolding of partially denatured proteins the HSP70 cycle is coupled to the action of  
455 HSP90, in accordance with the down-regulation of spot M1 (AHSA1) (Ruden et al., 2005).

456 In general, there was a strong agreement between the modified E-screen results and the  
457 protein expression. In fact, the proliferative estrogenic activity following the treatment of MCF-7  
458 cells with TWW sample at 10% concentration was illustrated by the up-regulation of  
459 estrogenicity-related proteins, whereas, the cytotoxic effect induced by T20 at 1% concentration  
460 resulted in the down-regulation of cytotoxicity-regulated proteins.

461 Unsafe TWW irrigation might contaminate the groundwater or the irrigated fruit by residual  
462 xenobiotic substances. Thus, we explored their effect on barrier function. Indeed, Caco-2 cell  
463 layer has a BF which depends on the intracellular TJ. Hence, the TJ permeability was evaluated  
464 by measuring the TEER (Narita et al., 2007).

465 The TEER, considered as a good early marker of BF disruption, is useful for analyzing  
466 various cytotoxic substances (Narita et al., 2007). Regardless of the absence of cytotoxicity  
467 (Supplementary data-Fig.S1), we carried the TEER assay because the loss of monolayer integrity  
468 caused by toxicants occurs much earlier than the death or dysfunction of monolayer cells (Narita  
469 et al., 2007). Figure 5 has shown that high concentrations of TWW or T20 samples induced a  
470 TEER increase whereas low concentrations triggered a decrease in TEER.

471 We previously demonstrated that activated sludge effluent induced disruption of BF due to  
472 lipopolysaccharide (LPS) produced with microbial decay (Narita et al., 2007). We can also  
473 assume that the disruption of BF is linked to the Cd present in the samples as elicited by Rossi et  
474 al. (1996). It could occur by way of altered calcium homeostasis following Cd uptake and flux  
475 across the cell monolayer (Rossi et al., 1996). The soil extract T20 at 5% and 1% concentrations

476 were therefore selected for the proteomics analyses to gain insight into the mechanisms behind  
477 the induction of BF and the disruption of monolayer integrity, respectively.

478 As exhibited by Fig. 4B, eight proteins corresponding to spots C1 to C8, associated to  
479 disruption of BF, were up-regulated. Interestingly, NPM and TEPR (spots C1 and C6) were  
480 identified as well in MCF-7 cells (Fig. 4A). Conversely, the over-expression trend shown in  
481 Caco-2 cells contrasted with the absence of protein expression in MCF-7 cells (spots M3 and  
482 M4) following the treatment with T20 at the same concentration.

483 Mitochondrial HSP 60 (spot C2) is a chaperonin that assists in protein folding under both  
484 normal and stressful conditions and is ubiquitously produced in mammalian cellular  
485 mitochondria. The HSP60 protein is induced in response to heat stress and is a member of an  
486 immunologically conserved family represented in yeast (*Escherichia coli*) and in mitochondria  
487 of protozoan, plants and animals (Reading et al., 1989). Moreover, substances that disrupt BF  
488 cause various stresses on the cells (Konsula and Baril, 2005). Thus, the disruption of BF is  
489 consistent with the over-expression of HSP60.

490 Two members of hnRNP family, C1/C2 and A2/B1 (spots C4 and C7), were highly up-  
491 regulated. Fahling et al. (2006) showed that hnRNP-A2/B1 is relevant for a large variety of  
492 changes in cell metabolism. This confirms the involvement of these hnRNPs in the disruption of  
493 BF.

494 Tubulin- $\alpha$ -1B (TBA1B) and Tubulin- $\beta$  chain (TBB5) (spots C5 and C8) are cytoskeletal  
495 microtubules that participate in multiple cellular functions and have become a target of cancer  
496 chemotherapy (Hooven and Baird, 2008).

497 Moreover, three proteins corresponding to spots C9 to C11, associated to disruption of BF,  
498 were down-regulated (Fig. 4B). Eukaryotic initiation factor (Spot C9) down-regulation is

499 involved in dysfunction of protein synthesis (Perez-Perez et al., 2009). Heterogeneous nuclear  
500 ribonucleoprotein-F (Spot C10) is strongly associated with several colon tumors (Balasubramani  
501 et al., 2006). Triosephosphate isomerase (TPI) (Spot C11) is a central and conserved glycolytic  
502 enzyme that plays an important role in several metabolic pathways. Reduced TPI activity can  
503 increase specific oxidant resistances of model organisms (Ralsler et al., 2008). Our results  
504 confirmed their findings to demonstrate the potential hazard from the wastewater reuse.

505 Barrier function related-proteins were both up-regulated (spots C12 and C13) and down-  
506 regulated (spots C14 and C15). Serpin-E3 is a member of serpin family mainly active as protease  
507 inhibitor. In addition, some members of this family are involved in physiologic processes such as  
508 tumor suppression, neurotrophism, and heat shock (Packard et al., 1995).

509 Enolase- $\alpha$  is a transcriptional regulator. Stierum et al. (2003) have shown that the shift  
510 in enolase- $\alpha$  expression patterns during Caco-2 differentiation reflects glycolytic changes  
511 associated with differentiation.

512 Most of the identified proteins could be grouped into several molecular and biological  
513 functions, such as protein folding and stress response (HSP60, HSP70, and HSP90 families),  
514 heat shock (serpin-E3), oxidative stress (26S protease regulatory subunit 6B, KEAP), cell  
515 proliferation (PCNA and PAIP), cell metabolism (TEPR, hnRNP family), protein synthesis  
516 (eukaryotic initiation factor 1), and glycolytic metabolic pathway (TPI and enolase- $\alpha$ ). Others  
517 might be associated to cancer pathogenesis, apoptosis, tumors (NPM, tubulins and hnRNP-F), or  
518 diseases like neuropathy (T-complex protein 1 subunit  $\epsilon$ ). Consequently, the disturbance of the  
519 latter mechanisms or the induction of such diseases endangers humans and soil organisms. Thus,  
520 in addition to compliance with established standards, wastewater reuse must be implemented



521 with high precautions to prevent the risk related to such harmful diseases for humans and  
522 damaging effects for the environment.

523 If we consider effect-based criteria for selecting the biomarkers, the best candidates as  
524 estrogenicity biomarkers are activator of HSP90 ATPase homolog, PCNA and NPM, while HSP  
525 cognate 71 and T-complex protein 1 subunit  $\epsilon$  are potentials cytotoxicity biomarkers of pollution  
526 from wastewater reuse. Additionally, NPM, HSP60, 26S protease regulatory subunit 6B and  
527 hnRNP-C1/C2 are seriously postulating as biomarkers of paracellular BF disruption, whereas,  
528 serpin-E3 and enolase- $\alpha$  might be good biomarkers for BF induction.

529 To assess the potential hazard of wastewater irrigation, the best biomarkers should be  
530 common to plants, animals and humans. While plants are directly exposed to wastewater  
531 irrigation, mammals and humans are equally likely to be affected through grazing or through  
532 the food chain. Thus we propose these common proteins as biomarkers for assessing potential  
533 hazard of wastewater reuse: Activator of HSP90 ATPase homolog 1, PCNA, HSP 70 1A/1B, 78  
534 Glucose regulated protein, HSP60 mitochondria and Tubulin- $\beta$  chain (<http://biogps.org>).

535

## 536 **5. Conclusions**

537

538 In conclusion, we have shown that TWW and T20 samples, containing heavy metals  
539 including Cd, induced stress responses from cells, as shown by the enhanced expressions of HSP  
540 families (47, 60, 70 and 90). The highest stress shown by HSP(+) cells was induced by T20 and  
541 T90 in accordance with the heavy metal profile showing the highest content for the same  
542 samples. Moreover, TWW caused estrogenic activity exhibited by enhanced expressions of cell  
543 proliferation and tumor-related proteins (PCNA and NPM), whereas, T20 was able to trigger the

544 enhanced expressions of HSP70 family proteins. These expressions can be considered as survival  
545 mechanisms against T20-induced cytotoxicity. Furthermore, depending on the concentration,  
546 while 1% of T20 resulted in a disruption of BF involving cell metabolism (TEPR, hnRNP  
547 family), protein synthesis (eukaryotic initiation factor 1), and tumor markers, 5% of T20 induced  
548 the expression of BF-related proteins associated to heat shock (serpin-E3), oxidative stress  
549 (KEAP), cell proliferation (PAIP), and glycolytic metabolic pathway (enolase- $\alpha$ ). Common  
550 proteins for humans, other mammals and plants are to be considered as the best biomarkers for  
551 assessing the potential hazard of wastewater reuse.

552 Previous pilot scale field researches showed the feasibility of water reuse provided that some  
553 precautions are taken (Bahri, 2003). However, the legal and institutional framework should be  
554 strengthened by establishing guidelines for the TWW-irrigated soil. In addition to the current  
555 monitoring of chemical elements, bacteria, and parasitic content of TWW reused in agriculture,  
556 risk assessment studies on water-soil-plant-animal-human exposure pathways are needed (Bahri,  
557 2003). Out of the scope of this investigation, additional studies devoted to exposure  
558 quantification of soil microorganisms, irrigated plants and concerned farmers and consumers  
559 would be with great interest to make the present contribution more complete.

560

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562

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567

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- 663

664 **Figure captions**

665

666 **Fig. 1.** Sampling site.

667

668 **Fig. 2.** Stress response of heat shock protein-positive Chinese hamster ovary cells incubated for 3  
669 h with treated wastewater (TWW) and soil extract samples. The results, which represent the  
670 average of three independent experiments  $\pm SD$ , are presented as a percentage of control (100%  
671 i.e., medium only). Statistically significant results ( $p < 0.05$ ) were determined by one-way analysis  
672 of variance followed by Duncan's post hoc test. Means without a common letter within the same  
673 graph differ significantly.

674

675 **Fig. 3.** Relative estrogenic activities of treated wastewater (TWW) and soil extract samples after  
676 6 days of incubation. Medium and  $17\beta$ -estradiol (E2) (29 nM) were used as negative (solid line)  
677 and positive control (dashed line, 234%), respectively. The results, which represent the average  
678 of three independent experiments  $\pm SD$ , are presented as a percentage of control (100% i.e.,  
679 medium only). Statistically significant results ( $p < 0.05$ ) were determined by one-way analysis of  
680 variance followed by Duncan's post hoc test. Means without a common letter within the same  
681 graph differ significantly.

682

683 **Fig. 4.** Two-dimensional (2D) protein profile of human breast cancer MCF-7 cells (**A**) and  
684 epithelial intestinal Caco-2 cells (**B**). Eight spots of interest (M1 to M8 for MCF-7 cells) (**A**) and  
685 Fifteen spots of interest (C1 to C15 for Caco-2 cells) (**B**) from excised CBB-stained gels and  
686 identified by mass spectrometry are shown with their names and National Center for



687 Biotechnology information (NCBI) id. Comparison of spot relative expression (fold of control)  
688 among 2D protein profiles of MCF-7 cells incubated for 24 h with treated wastewater (TWW) at  
689 10% concentration, irrigated soil extract at 20 cm depth (T20) and control soil at 40 cm (C40) at  
690 1% concentration (A). Comparison of spot expression among 2D protein profiles of Caco-2 cells  
691 incubated for 3 h with or without T20 and C40 at 1% and 5% concentrations (B).

692

693 **Fig. 5.** Changes in transepithelial electrical resistance (TEER) in Caco-2 cells monolayers  
694 incubated for 4 h with or without treated-wastewater-irrigated soil extract at 20 cm depth (T20)  
695 and control soil at 40 cm (C40) at 1% and 5% concentrations. The results, which represent the  
696 average of three independent experiments  $\pm SD$ , are presented as TEER of incubated cells relative  
697 to that at zero time. Statistically significant results ( $p < 0.05$ ) were determined by one-way  
698 analysis of variance followed by Duncan's post hoc test. Means without a common letter within  
699 the same graph differ significantly.

**Table 1.** Physicochemical parameters and heavy metal levels.

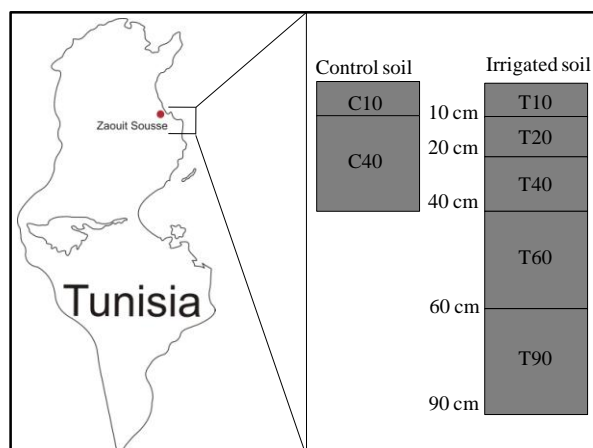
Sample	pH	EC (mS/cm)	Ni	Cd	Pb	Al	Fe
TWW	8.18	1.990	25	172.50	ND	140	105
T.S <sup>a</sup>	6.50-8.50	7.000	200	10.00	1000	5000	5000
C10	7.67	0.056	10	73.08	ND	150	50
C40	7.78	0.100	5	42.50	ND	250	65
T10	7.82	0.393	50	92.50	ND	160	85
T20	7.73	0.174	285	85.00	ND	1310	130
T40	7.95	0.271	95	65.72	ND	830	50
T60	8.17	0.274	95	65.00	ND	530	80
T90	8.17	0.203	150	205.00	ND	830	30
EU.S <sup>b</sup>	NS	NS	6-15	0.20-0.60	10-60	NS	NS
US.S <sup>c</sup>	NS	NS	6-42	4	NS	NS	NS

<sup>a</sup> Tunisian standards for wastewater reuse, NT 106-003, 1989 (Angelakis, 1999).

<sup>b</sup> European Union Framework, Directives 86/278/EEC, 2000 (Klay et al., 2010).

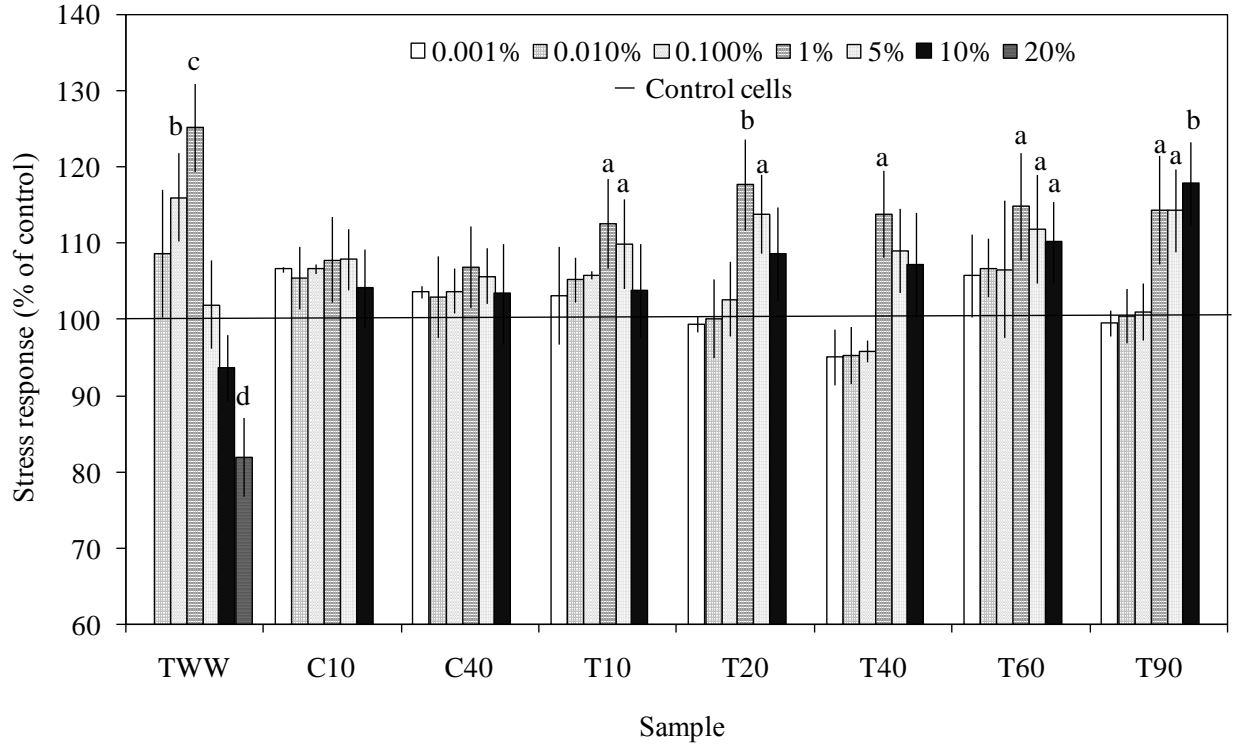
<sup>c</sup> United States, Directives NRC, 2002 (Klay et al., 2010); ND: Not detected; NS: No standards.

1 Fig. 1.



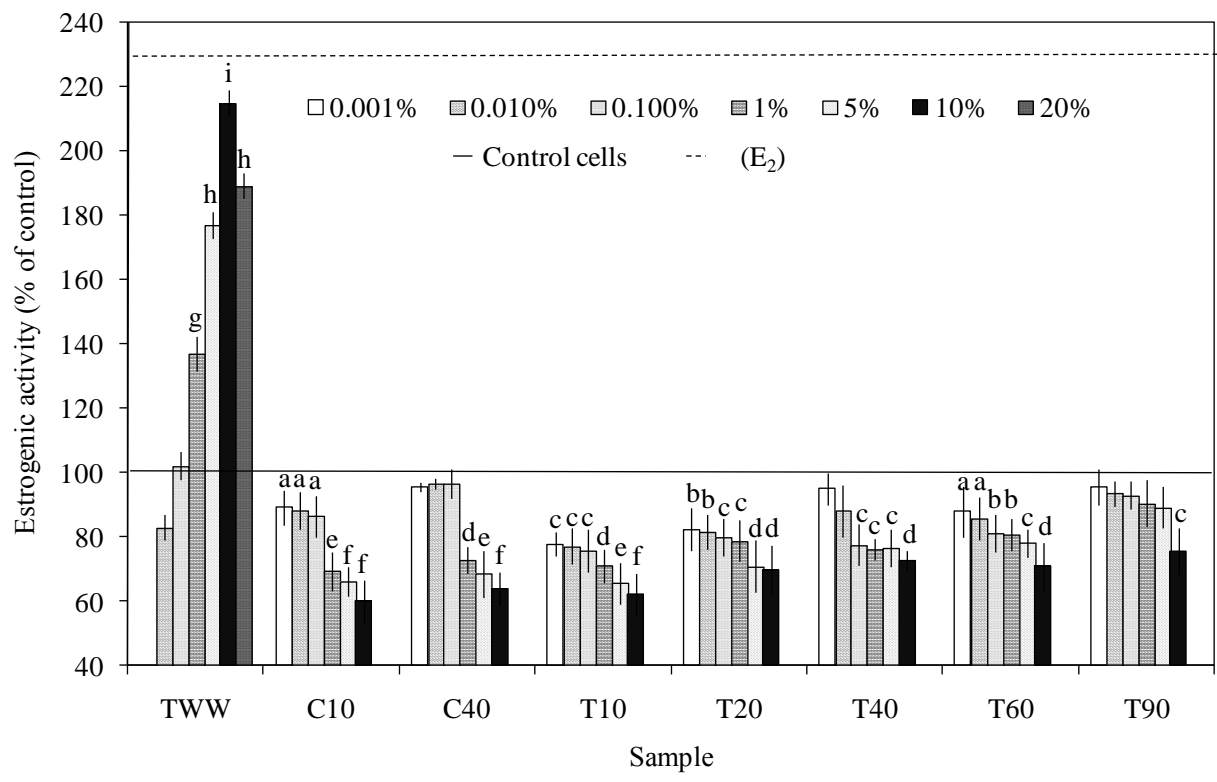
2

1 **Fig. 2.**



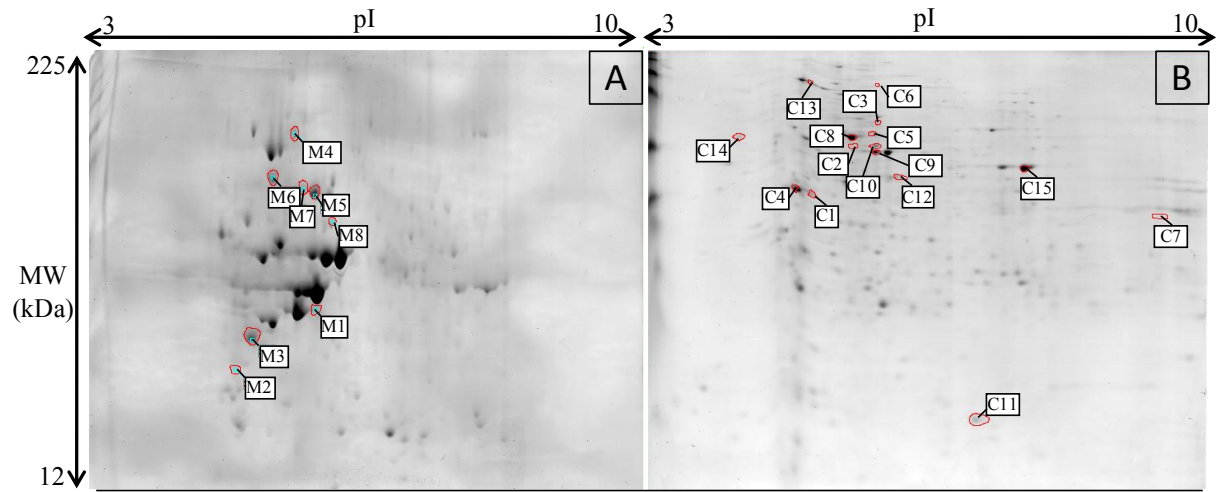
2

1 **Fig. 3.**



2

1 Fig. 4.



Spot	M1	M2	M3	M4	M5	M6	M7	M8	
Protein	Activator of HSP90 ATPase homolog 1	Proliferating cell nuclear antigen	Nucleophosmin	Transitional endoplasmic reticulum ATPase	HSP 70 1A/1B	78 Glucose regulated protein	HSP cognate 71	T complex protein 1 subunit ε	
NCBIid	O95433	P12004	P06748	P55072	P08107	P11021	P11142	P48643	
Relative expression (fold of control)	TWW-10%	4.073 ± 0.002	2.148 ± 0.019	2.109 ± 0.006	0.544 ± 0.007	0.971 ± 0.023	1.258 ± 0.015	1.223 ± 0.011	0.956 ± 0.009
	C40-1%	1.084 ± 0.012	0.837 ± 0.027	0.853 ± 0.009	1.053 ± 0.002	0.877 ± 0.086	1.116 ± 0.022	0.722 ± 0.007	1.080 ± 0.018
	T20-1%	0.472 ± 0.021	0.298 ± 0.016	0.781 ± 0.001	0.755 ± 0.004	0.669 ± 0.062	0.585 ± 0.013	0.459 ± 0.004	0.393 ± 0.024
Spot	C1	C2	C3	C4	C5	C6	C7	C8	
Protein	Nucleophosmin	HSP 60, mitochondrial	26S protease regulatory subunit 6B	Heterogeneous nuclear ribonucleoproteins C1/C2	Tubulin-α-1B	Transitional endoplasmic reticulum ATPase	Heterogeneous nuclear ribonucleoproteins A2/B1	Tubulin-β chain	
NCBIid	P06748	P10809	P43686	P07910	P68363	P55072	P22626	P07437	
Relative expression (fold of control)	C40-1%	1.473 ± 0.001	1.499 ± 0.005	0.934 ± 0.012	0.583 ± 0.007	1.262 ± 0.004	0.463 ± 0.0017	1.027 ± 0.027	0.982 ± 0.006
	T20-1%	4.747 ± 0.003	2.602 ± 0.003	2.504 ± 0.002	2.419 ± 0.004	2.108 ± 0.007	1.685 ± 0.004	1.586 ± 0.001	1.427 ± 0.012
Spot	C9	C10	C11	Spot	C12	C13	C14	C15	
Protein	Eukaryotic initiation factor	Heterogeneous nuclear ribonucleoprotein F	Triosephosphate isomerase	Protein	Serpin E3	Kelch-like protein 21	Polyadenylate-binding protein-interacting protein 1	Enolase-α	
NCBIid	P60842	P52597	P60174	NCBIid	A8MV23	Q9UJP4	Q9H074	P06733	
Relative expression (fold of control)	C40-1%	0.835 ± 0.011	1.104 ± 0.009	0.866 ± 0.008	C40-5%	0.849 ± 0.007	0.86731 ± 0.014	0.919 ± 0.011	1.038 ± 0.008
	T20-1%	0.645 ± 0.001	0.412 ± 0.003	0.219 ± 0.004	T20-5%	3.298 ± 0.002	1.56704 ± 0.005	0.443 ± 0.007	0.305 ± 0.002

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