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

<table>
<thead>
<tr>
<th>著者別名</th>
<th>入江 光輝 □ 磯田 博子</th>
</tr>
</thead>
<tbody>
<tr>
<td>原出所</td>
<td>京都大学大学院農学研究科</td>
</tr>
<tr>
<td>種類</td>
<td>紙</td>
</tr>
<tr>
<td>ページ数</td>
<td>論文</td>
</tr>
<tr>
<td>権利</td>
<td>この論文の使用権は著者に帰属します。使用権についての質問は著者にお問い合わせください。</td>
</tr>
</tbody>
</table>

doi: 10.1016/j.ecoenv.2012.06.020
Assessment of wastewater-irrigated soil containing heavy metals and establishment of specific biomarkers

F. Ben Fredj¹, J. Han¹, ², M. Irie¹, ², N. Funamizu³, A. Ghrabi⁴, H. Isoda*¹, ²

¹Alliance for Research on North Africa (ARENA), University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8572, Japan.
²Graduate School of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8572, Japan.
³Graduate School of Engineering, Hokkaido University, Kita 13, Nishi 8, Kita-ku, Sapporo, Hokkaido 060-8628, Japan
⁴Wastewater Treatment and Recycling Laboratory, Center of Research and Technologies of Water, Technopark Borj Cedria, University of Tunis El Manar, P.O. Box 273-8020. Soliman, Tunisia.

E-mail addresses: fahmi.ben.fredj.gm@u.tsukuba.ac.jp, han.junkyu.gn@u.tsukuba.ac.jp, irie.mitsuteru.fu@u.tsukuba.ac.jp, funamizu@eng.hokudai.ac.jp, ahmed.ghrabi@certe.rnrt.tn, isoda.hiroko.ga@u.tsukuba.ac.jp.

*Corresponding author:
Tel.: + 81 2 98535775; fax: + 81 2 98535776; email: isoda.hiroko.ga@u.tsukuba.ac.jp
ABSTRACT - Irrigation with treated wastewater (TWW) is a vital alternative for arid and semi-arid lands but it poses pollution-risk to soil, vegetation and groundwater. Therefore, in the present study, in vitro bioassays were used to evaluate the adverse effects of TWW and irrigated-soil extract sample, on mammalian cells, with respect to heavy metal - Ni, Cd, Pb, Fe, Al-content. The heat shock protein (HSP) 47, E-screen, and transepithelial electrical resistance (TEER) assays served to investigate the stress response of treated-HSP47-transfected Chinese hamster ovary (CHO) cells, the estrogenic activity of the samples in MCF-7 breast cancer cells, and the barrier function (BF) of Caco-2 cells. Furthermore, proteomics analyses were performed to shed light on involved mechanisms and to establish pollution biomarkers. Results showed that the TWW elicited a stress response on HSP cells from 0.1% concentration while soil extract samples exhibited a stress at 1%. TWW induced an estrogenic activity at 10%; up-regulating cell proliferation and tumor-related proteins. Soil extract triggered the enhanced expression of HSP70 family proteins as survival mechanisms against their cytotoxicity toward MCF-7 cells. Moreover, depending on the concentration, 1% of soil extract from 20 cm depth (T20) resulted in a disruption of BF in Caco-2 cells involving cell metabolism, protein synthesis and tumor marker proteins, whereas, 5% of T20 induced the expression of BF-related proteins associated to heat shock, oxidative stress, cell proliferation and glycolytic metabolic pathway. These biological techniques were found to be extremely useful to evaluate the impact of wastewater reuse and to establish specific biomarkers that are common proteins for humans, other mammals and plants. Future studies should focus on exposure quantifications.

Keywords – Biomarkers; Wastewater reuse; Heavy metals; E-screen; Barrier function
1. Introduction

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

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

Previous studies focused on physicochemical parameters, heavy metal content, and bacterial indicators (Al-Khashman, 2009; Palese et al, 2009) to assess the potential hazard related to wastewater reuse for irrigation purposes. Hazard assessment of wastewater effluent is traditionally based on the evaluations of individual chemicals, including heavy metals, EDCs or other toxic compounds identified through chemical analyses (Baun et al., 1998). However, these
techniques could not overcome the problem of chemical mixtures present in environmental samples and are unable to characterize the hazardous effects on human health. Thus, in vitro bioassays using bacteria, sentinel marine organisms or mammalian cells, as well as in vivo animal experiments, are essential to provide information regarding the possible biological effects of the chemical mixture, such as cytotoxicity, stress (Ben Fredj et al., 2010), estrogenic activity or paracellular BF disruption (Narita et al., 2007). Nevertheless, these assays in turn do not allow understanding the mechanisms behind such biological effects. Therefore, proteome analyses of environmental samples, known as environmental proteomics, has investigated many organisms, ranging from microorganisms and plants to invertebrates and vertebrates not only to gain insights into underlying mechanisms of toxicity but also to discover specific biomarkers of several environmental pollution origins. Nonetheless, proteomics is a field of environmental research still in its infancy, due to a number of caveats, such as the limited number of organisms fully covered in the sequence databases, the high genetic variability, and the dependence on environmental factors (Nesatyy and Suter, 2007). Besides, there have been few studies using environmental proteomics that have considered mammalian or human cells to assess the hazard of environmental samples. Hence, the direct assessment of wastewater reuse on mammalian cells deserves attention.

A complementary investigation of representative environmental samples should combine chemical analyses to detect the compound levels, in vitro bioassays and proteomics. These bioassays are able to elucidate several effects such as stress response induced by stressors including heavy metals, estrogenic response due to the presence of EDCs or paracellular BF sensitive to several contaminants. This approach not only allows discovery of new protein
biomarkers of TWW-irrigated soil pollution but, more importantly, provides the basis to shed light on underlying mechanisms of toxicity.

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

2. Materials and methods

2.1. Chemicals

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

2.2. 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(+). The cells were provided by S. Yokota (Kaneka, Osaka, Japan) and
were grown as adherent monolayer in 75-cm² tissue culture flasks using F12 Medium (Invitrogen, Carlsbad, CA, U.S.A.), 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 (5000 µg/mL)-streptomycin (5000 IU/mL) solution in 75-cm² tissue culture flasks. Human intestinal Caco-2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% non essential amino acids. The cultures were maintained in a 5% CO₂ incubator at 37 °C. Cell passage was carried out at 80% confluence at one on two ratio using 0.25% trypsin (1 mM EDTA).

2.3. Sample preparation and water quality parameters

The irrigated perimeter of Zaouit Sousse (lat. = N35°46’37.2”, long. = E10°39’9.3”, and alt. = 20 m N.G.T) is located in Tunisia (Fig. 1). It is situated 7.5 km from Sousse South WWTP which uses trickling filters coupled to an activated sludge (TF/AS) system with a treatment capacity of 18700 m³ wastewater per day. The TWW is mainly reused for the irrigation of olive trees surrounded by sorghum pasture. The surface irrigation started 20 years ago. A TWW sample was collected from the irrigation pond upstream of the perimeter. A soil profile from the wastewater-irrigated area was sampled at 5 depths: 10, 20, 40, 60 and 90 cm to cover the root zone of the irrigated olive trees. For irrigated culture the roots system is not so deep; most of the roots are concentrated within 70 to 80 cm depth (Klay et al., 2010). In addition, a control soil
profile was taken from the rain-fed area at only two depths: 10 and 40 cm, due to the soil homogeneity beyond this depth (Fig. 1). Klay et al. (2010) antecedently found that the soil has a sandy-clay texture up to 20 cm depth followed by a sandy texture for deeper horizons. Soil extracts one on five (2 g of soil in 10 mL of deionized water) were prepared. The samples were filter-sterilized using a 0.45-µm membrane filter for chemical analysis. For the sample volume used for bioassays experiments, pH was adjusted using a pH meter MP220 (Mettler Toledo, Schwerzenbach, Switzerland) from alkaline (7.93 ± 0.3) to the pH range of 7.2 to 7.4 to maintain optimum conditions for cell cultures prior to 0.22-µm membrane filtration. Electrical conductivity (EC) and pH were measured using a YK-22CT conductivity meter (Sato Shouji Incorporation, Tokyo, Japan) and a UC-23 digital pH meter (Central Kagaku Corporation, Tokyo, Japan), respectively. The heavy metal levels were determined using DR/4000 Spectrophotometer (Hach, Loveland, CO, USA) (Hach, 2003) following the standard methods of analysis 8150 PAN (Detection limit (DL) = 5 µg/L), 8033 Dithizone (DL = 1.3 µg/L), 8317 fast column extraction (DL = 2 µg/L), 8147 Ferrozine (DL = 4 µg/L), and 8112 Aluminon (DL = 5 µg/L) respectively.

2.4. HSP 47 assay

The cell line used for HSP47 assay in the present study was developed in a previous study (Isoda et al., 2003). 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 HSP (+) cells were plated in 96-well plates and were allowed to attach for 48 h before adding samples diluted in medium for 3 h. The TWW sample was diluted to 0.01, 0.1, 1, 5, 10, and 20% of total volume of the medium (100 µL), whereas the soil extracts were used at
0.001, 0.01, 0.1, 1, 5, and 10% concentration. For higher concentrations than 1%, the results in
wells with 5% sample were compared with those with 5% PBS(-) and so on, to distinguish
between the sample effect and the decrease of medium volume. Heat shock protein 47 expression
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).

2.5. 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 1000 cells per well in 100 μL 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 (MTT) assay
as described in (Ben Fredj et al., 2010).

2.6. TEER Assay

For TEER measurement, the Caco-2 cells were grown at a density of 5 × 10^5 cells/mL in 8-
well-10-electrodes (8W10E+) culture ware (Applied Biophysics, NY, USA) coated with Type
I collagen derived from bovine placenta and washed twice with PBS(-). The real time TEER measurements were performed using the electrical cell-substrate impedance sensing (ECIS) equipment (Applied Biophysics, NY, USA). The TEER increase is correlated with the closure of tight junction (TJ) which functions as a barrier, whereas, a TEER decrease denotes a disruption of BF following the exposure to toxicants. The cells were cultured to establish monolayer integrity. After 48h culture period, the cell monolayer was washed with medium, the samples were added for 4h and their effect is expressed as the TEER relative to that at zero time. Following the results of E-screen assay, TWW and T20 samples were selected. A preliminary MTT proliferation assay showed that both samples were not cytotoxic toward Caco-2 cells up to 20% or 10% concentrations, respectively (Supplementary data Fig. S1). Hence, the TWW sample was added at 10 and 20% concentration, whereas, the soil extract sample T20 was added at 1 and 5% concentration.

2.7. Proteomics Analyses

Proteomics analyses were carried out for both MCF-7 and Caco-2 cells. Human breast cancer 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 and then allowed to attach for 24 h and 48 h, respectively. For sample treatment, concentrations derived from the E-screen and TEER assay results were selected. The cells were incubated with medium (control cells) or with samples for 24 h (MCF-7) and 3 h (Caco-2), after which the total proteins were extracted and quantified following the protocol detailed in Talorete et al. (2008).

For first-dimension electrophoresis, 24 cm-immobilized pH gradient (IPG, pH 3-10) dry strips (Amersham Biosciences, Uppsala, Sweden) were used. Using the Ettan IPGphor II
(Amersham Biosciences, Uppsala, Sweden) apparatus, the proteins were then separated according to their isoelectric point under conditions provided in the 2D-protocol. For second-dimension, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were separated according to their molecular weight using Ettan DALTsix electrophoresis system (Amersham Biosciences, Uppsala, Sweden) (Talorete et al., 2008). The coomassie brilliant blue (CBB)-stained gels were scanned using Image-Scanner (Amersham Biosciences, Uppsala, Sweden) and the spots were analyzed using the ImageMaster 2D-Elite software (Amersham Biosciences, Uppsala, Sweden). For mass spectrometry, protein spots of interest were excised from the CBB-stained gel, washed, and digested in-gel with trypsin (sequencing grade; Promega, Tokyo, Japan). All liquid chromatography tandem mass spectrometry (LC/MS/MS) mass spectra were acquired on a 3200 QTAR Pulsar (Applied Biosystems, Foster, CA, USA) mass spectrometer. The obtained peptide sequence tags were used to identify proteins by searching databases using the Mascot search engine (Matrix Science; http://www.matrixscience.com).

2.8. Statistical analyses

Three to four 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.
3. Results

3.1. Water quality parameters and heavy metal levels

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

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

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

The stress response of HSP(+) cells exposed to the TWW sample was estimated using the HSP47 assay (Fig. 2). Interestingly, the results showed that the TWW samples exhibited a dose dependent stress with a peak registered at 1% 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. When the stress response of HSP(+) cells was tested, 3 h-treatment with irrigated soil extract samples at 1% concentration induced the highest effect, similar to the TWW effect except
for T90 sample. Interestingly, no significant stress was observed for the control soil extract samples at both depths C10 and C40. Moreover, the highest stress was induced by T20 and T90 samples.

3.3. Estrogenic activity of the TWW and soil extract samples

The modified E-screen assay was carried out for the latter 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. 3, the effluent sample at 10% concentration was able to induce an estrogenic activity of 2.2-times of the control cell activity. The latter concentration caused an estrogenic activity almost equal to the positive control (E2) and was consequently chosen for further proteomics analyses.

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

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

Following the results given by the modified E-screen assay, the TWW sample at 10%, the sample T20, and the control soil C40 at 1% concentration were selected for investigating the
protein expression relative to the estrogenic activity and the cytotoxic effect. Two-dimensional-SDS-PAGE was performed for MCF-7 cells treated with the latter samples. Following the spot analyses using image master software, eight spots of interest were retained. The selection was based on the differential protein expression of TWW and T20-treated cells in comparison with the control cells and C40-treated cells. Two main groups arise depending on the treatment with the TWW sample or the soil extract T20 including common proteins for both target effects.

3.4.1. Estrogenicity-associated proteins

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

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

3.4.2. Cytotoxicity-related proteins


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

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

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

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

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

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

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

3.6.2. Barrier function associated proteins

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

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

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

Previous results revealed the presence of several heavy metals in the TWW and the soil extracts (Klay et al., 2010). For this reason, we targeted five heavy metals Ni, Cd, Pb, Al, and Fe, highly relevant to human exposure, through detecting their residual levels in the samples (Table 1). Previously, Angelakis et al. (1999) sustained the idea that heavy metals predominantly accumulate in sludge and not in the liquid wastewater. However, the Cd level in the TWW exceeded local guidelines. The migration of metals to the soil will especially depend on the concentration of organic carbon and the soil texture. Klay et al. (2010) antecedently found that
the irrigated soil has a sandy-clay texture up to 20 cm depth followed by a sandy texture for
deeper horizons. The results shown in Table 1 demonstrated high heavy metal content at 20 and
90 cm depth. The sandy nature will allow the infiltration of pollutants, while the clay fraction
might be responsible for adsorption of pollutants. In Tunisia, no guidelines for monitoring the
soil quality have been established yet.

We hypothesized that the samples including a heavy metal mixture, with a high level of Cd,
could induce stress on mammalian cells. Thus, the HSP47 assay was carried out aiming to verify
this assumption. 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 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).

As presented by Figure 2, the stress induced by the TWW samples might be related to the Cd
content (Table 1) combined to a mixture of heavy metals responsible of a significant stress in
accordance with our recent works where we demonstrated that Cd combined with Ni and Pb was
responsible for inducing high stress response (Ben Fredj et al., 2010). Moreover, the highest
stress shown by HSP(+) cells was induced by T20 and T90 in accordance with the heavy metal
profile showing the highest content for the same samples. While the stress response at 20 cm
depth is a sign of a potential hazard to the rhizosphere (up to 70-80 cm), the stress inducing
effect of T90 sample might endanger the groundwater by infiltration through the sandy texture
(Klay et al., 2010).
Wastewater Effluent may be a significant source of EDCs caused by natural human and animal hormones such as estradiol, testosterone and estrone, and from birth control pills containing 17α-ethynylestradiol (Ying and Kookana, 2005). Although we did not analyze for such compounds, Garcia Morales et al. (1994) showed that Cd has an estrogen mimetic effect that induced the growth of MCF-7 cells, mediated by the estrogen receptor (ER). Since the TWW and soil extract samples exhibited a high level of Cd, we aimed to examine their potential effect on MCF-7 cells and inquire about the presence of estrogens or other estrogen-like-EDCs that are able to stimulate their estrogenic activity.

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

For proteomics purposes, TWW at 10% concentration was obviously selected while T20 at 1% concentration was preferably considered among the soil horizons. Indeed, the T10 sample showed more significant cytotoxic effect than T20. However T10 sample was not considered because such effect is probably due to a number of factors, such as grazing of livestock, presence of wild animals and surface water runoff from adjacent agricultural areas (Palese et al., 2009). On the other hand, the level of statistical significance for T20 at 1% concentration (Fig. 3) was exhibited by most of the profiles (T10 to T60). Hence, it was selected to reflect the soil extract-
induced cytotoxicity and to check the impact of wastewater reuse at protein level. In addition, the control soil C40 at 1% concentration was also considered for further proteomics analyses to distinguish between the control soil component effect and the wastewater-conveyed component effect.

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.

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

Proliferating cell nuclear antigen (PCNA) is a nuclear protein that plays a key role in cell proliferation, DNA repair, and cell cycle control associated with hepatocellular carcinoma. Moreover, Lottering et al. (1996) showed that E2 metabolites, suspected in the TWW sample
(Narita et al., 2007), elevated PCNA levels during S-phase of the MCF-7 cell cycle through interaction with ERα. Genes encoding PCNA and/or its products have been identified in a wide variety of diverse organisms such as animals, yeast and higher plants including Arabidopsis, bean, carrot, soybean and tobacco (Strzalka and Ziemienowicz, 2011).

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

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

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 against the stress-inducing compounds such as the heavy metals detected in the samples (Table 1). 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 over-expressed following the addition of both the TWW and the soil extract probably due to the Cd abundantly present within the heavy metal mixture in the samples. Nevertheless, the former members of HSP70 family were not affected by TWW sample and were down-regulated only by T20-treatment in accordance with the cytotoxic effect induced by the soil extract on MCF-7 cells. 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 down-regulation of spot M1 (AHSA1) (Ruden et al., 2005).

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

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

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

We previously demonstrated that activated sludge effluent induced disruption of BF due to lipopolysaccharide (LPS) produced with microbial decay (Narita et al., 2007). We can also assume that the disruption of BF is linked to the Cd present in the samples as elicited by Rossi et al. (1996). It could occur by way of altered calcium homeostasis following Cd uptake and flux across the cell monolayer (Rossi et al., 1996). The soil extract T20 at 5% and 1% concentrations...
were therefore selected for the proteomics analyses to gain insight into the mechanisms behind the induction of BF and the disruption of monolayer integrity, respectively.

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

Mitochondrial HSP 60 (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). Moreover, substances that disrupt BF cause various stresses on the cells (Konsula and Baril, 2005). Thus, the disruption of BF is consistent with the over-expression of HSP60.

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

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

Moreover, three proteins corresponding to spots C9 to C11, associated to disruption of BF, were down-regulated (Fig. 4B). Eukaryotic initiation factor (Spot C9) down-regulation is
involved in dysfunction of protein synthesis (Perez-Perez et al., 2009). Heterogeneous nuclear ribonucleoprotein-F (Spot C10) is strongly associated with several colon tumors (Balasubramani et al., 2006). Triosephosphate isomerase (TPI) (Spot C11) is a central and conserved glycolytic enzyme that plays an important role in several metabolic pathways. Reduced TPI activity can increase specific oxidant resistances of model organisms (Ralser et al., 2008). Our results confirmed their findings to demonstrate the potential hazard from the wastewater reuse.

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

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

Most of the identified proteins could be grouped into several molecular and biological functions, such as protein folding and stress response (HSP60, HSP70, and HSP90 families), heat shock (serpin-E3), oxidative stress (26S protease regulatory subunit 6B, KEAP), cell proliferation (PCNA and PAIP), cell metabolism (TEPR, hnRNP family), protein synthesis (eukaryotic initiation factor 1), and glycolytic metabolic pathway (TPI and enolase-α). Others might be associated to cancer pathogenesis, apoptosis, tumors (NPM, tubulins and hnRNP-F), or diseases like neuropathy (T-complex protein 1 subunit ε). Consequently, the disturbance of the latter mechanisms or the induction of such diseases endangers humans and soil organisms. Thus, in addition to compliance with established standards, wastewater reuse must be implemented
with high precautions to prevent the risk related to such harmful diseases for humans and
damaging effects for the environment.

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

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

5. Conclusions

In conclusion, we have shown that TWW and T20 samples, containing heavy metals
including Cd, induced stress responses from cells, as shown by the enhanced expressions of HSP
families (47, 60, 70 and 90). The highest stress shown by HSP(+) cells was induced by T20 and
T90 in accordance with the heavy metal profile showing the highest content for the same
samples. Moreover, TWW caused estrogenic activity exhibited by enhanced expressions of cell
proliferation and tumor-related proteins (PCNA and NPM), whereas, T20 was able to trigger the
enhanced expressions of HSP70 family proteins. These expressions can be considered as survival mechanisms against T20-induced cytotoxicity. Furthermore, depending on the concentration, while 1% of T20 resulted in a disruption of BF involving cell metabolism (TEPR, hnRNP family), protein synthesis (eukaryotic initiation factor 1), and tumor markers, 5% of T20 induced the expression of BF-related proteins associated to heat shock (serpin-E3), oxidative stress (KEAP), cell proliferation (PAIP), and glycolytic metabolic pathway (enolase-α). Common proteins for humans, other mammals and plants are to be considered as the best biomarkers for assessing the potential hazard of wastewater reuse.

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

Acknowledgements

This research was supported in part by funds from the SATREPS project for Valorization of Bio-resources in Semi-arid and Arid Land for Regional Development and the Grant-in-Aid for Scientific Research (Grant No. 21226013). The authors want to thank Pr. Abdelkrim Charef for assisting in the sampling and Dr. Parida Yamada for the useful comments.
References


Stress response of heavy metal mixture present in wastewater and leachate on HSP47-transfected cells. Environ. Toxicol. Chem. 29 (7), 1637-1647.


associated with proliferation and differentiation of the colorectal cancer cell line Caco-2.

Biochimica et Biophysica Acta 1650, 73-91.


Figure captions

**Fig. 1.** Sampling site.

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

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

**Fig. 4.** Two-dimensional (2D) protein profile of human breast cancer MCF-7 cells (A) and epithelial intestinal Caco-2 cells (B). Eight spots of interest (M1 to M8 for MCF-7 cells) (A) and Fifteen spots of interest (C1 to C15 for Caco-2 cells) (B) from excised CBB-stained gels and identified by mass spectrometry are shown with their names and National Center for
Biotechnology information (NCBI) id. Comparison of spot relative expression (fold of control) among 2D protein profiles of MCF-7 cells incubated for 24 h with treated wastewater (TWW) at 10% concentration, irrigated soil extract at 20 cm depth (T20) and control soil at 40 cm (C40) at 1% concentration (A). Comparison of spot expression among 2D protein profiles of Caco-2 cells incubated for 3 h with or without T20 and C40 at 1% and 5% concentrations (B).

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

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


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

*c United States, Directives NRC, 2002 (Klay et al., 2010); ND: Not detected; NS: No standards.
Fig. 1.
Figure 2
Fig. 3

Figure 3
### Fig. 4.

<table>
<thead>
<tr>
<th>Spot</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
<th>M6</th>
<th>M7</th>
<th>M8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Activator of HSP90 ATPase homolog 1</td>
<td>P12004</td>
<td>P06748</td>
<td>P55072</td>
<td>P08107</td>
<td>P11021</td>
<td>P11142</td>
<td>P48643</td>
</tr>
<tr>
<td>NCBIid</td>
<td>O95433</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative expression (fold of control)</td>
<td>TWW-10%</td>
<td>4.073 ± 0.002</td>
<td>2.148 ± 0.019</td>
<td>2.109 ± 0.006</td>
<td>0.544 ± 0.007</td>
<td>0.971 ± 0.023</td>
<td>1.258 ± 0.015</td>
<td>1.223 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>C40-1%</td>
<td>1.084 ± 0.012</td>
<td>0.837 ± 0.027</td>
<td>0.853 ± 0.009</td>
<td>1.053 ± 0.002</td>
<td>0.877 ± 0.086</td>
<td>1.116 ± 0.022</td>
<td>0.722 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>T20-1%</td>
<td>0.472 ± 0.021</td>
<td>0.298 ± 0.016</td>
<td>0.781 ± 0.001</td>
<td>0.755 ± 0.004</td>
<td>0.669 ± 0.062</td>
<td>0.585 ± 0.013</td>
<td>0.459 ± 0.004</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Spot</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>C7</th>
<th>C8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Nucleophosmin</td>
<td>P66748</td>
<td>P10809</td>
<td>P43686</td>
<td>P07910</td>
<td>P68363</td>
<td>P55072</td>
<td>P22626</td>
</tr>
<tr>
<td>NCBIid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative expression (fold of control)</td>
<td>C40-1%</td>
<td>1.473 ± 0.001</td>
<td>1.499 ± 0.005</td>
<td>0.934 ± 0.012</td>
<td>0.583 ± 0.007</td>
<td>1.262 ± 0.004</td>
<td>0.463 ± 0.007</td>
<td>1.027 ± 0.027</td>
</tr>
<tr>
<td></td>
<td>T20-1%</td>
<td>4.747 ± 0.003</td>
<td>2.602 ± 0.003</td>
<td>2.504 ± 0.002</td>
<td>2.419 ± 0.004</td>
<td>2.108 ± 0.007</td>
<td>1.685 ± 0.004</td>
<td>1.586 ± 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Spot</th>
<th>C9</th>
<th>C10</th>
<th>C11</th>
<th>C12</th>
<th>C13</th>
<th>C14</th>
<th>C15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>Eukaryotic initiation factor ribonucleoprotei n F</td>
<td>P66842</td>
<td>P52597</td>
<td>P60174</td>
<td>NCBIid</td>
<td>A8MV23</td>
<td>Q9UJP4</td>
</tr>
<tr>
<td>NCBIid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative expression (fold of control)</td>
<td>C40-1%</td>
<td>0.835 ± 0.011</td>
<td>1.104 ± 0.009</td>
<td>0.866 ± 0.008</td>
<td>0.849 ± 0.007</td>
<td>0.86731 ± 0.004</td>
<td>0.919 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>T20-1%</td>
<td>0.645 ± 0.001</td>
<td>0.412 ± 0.003</td>
<td>0.219 ± 0.004</td>
<td>3.298 ± 0.002</td>
<td>1.56704 ± 0.005</td>
<td>0.443 ± 0.007</td>
</tr>
</tbody>
</table>


