Estrogen-like and dioxin-like organic contaminants in reclaimed wastewater: transfer to irrigated soil and groundwater
O. Mahjoub, A. Escande, D. Rosain, C. Casellas, E. Gomez and H. Fenet

ABSTRACT
In semi-arid regions, treated wastewater reuse for irrigation is a common practice since wastewater is considered as a non negligible water resource in these areas. However, treated wastewater contains traces of organic compounds which may contaminate the receiving environment i.e. soil and groundwater. Some of these organic compounds have the ability to bind to estrogen receptor (ER) or dioxin receptor (AhR, aryl hydrocarbon receptor). The fate of these compounds in effluent reused for irrigation, irrigated soils and groundwater is not well addressed yet. In the present study, estrogenic and dioxin-like activities were studied in three media: i) effluents reused for irrigation, ii) soils samples collected from the reclaimed water irrigated plot during six month irrigation, and iii) groundwater sampled before and after irrigation periods. Effluents reused for irrigation exhibited ER and AhR activities at 38.5 ± 9.9 ng estradiol-equivalent/L (ng E2-EQ/L) and 113.3 ± 27.7 ng dioxin-equivalent/L (ng TCDD-EQ/L), respectively. Soils showed ER activity (0.05 ng E2-EQ/g) only after 4 months of irrigation. AhR activities detected in all soil samples have not changed during irrigation. In groundwater, ER activities were detected in two piezometers indicating transfer of some estrogenic compounds.

Key words | dioxin-like substances, irrigation, wastewater reuse, xenoestrogens

INTRODUCTION
By 2025, about 60% of the world population is expected to suffer of water shortage (Cosgrove & Rijsberman 2000). Arid and semi-arid countries like Southern Mediterranean are facing water scarcity and quality degradation. Population growth, urbanization and industrialization are generating increasingly volumes of wastewater being discharged in the receiving environment (Kamizoulis et al. 2003). In these countries, there is a potential to reuse treated wastewater (TWW), and agricultural reuse represents the main way to preserve freshwater for water supply and more appropriate uses. Tunisia launched a national water reuse program in the early 1980’s and nowadays about 30% of treated wastewater is reused for agricultural and landscape irrigation (Qadir et al. 2009). In Tunisia, as in countries where reuse was practiced for a long time, studies dealing with the impact on soil, groundwater and crops have mainly focused on salts, nutrients, and trace metals (Bahri 1998). However, several case studies have demonstrated that organic contaminants such as polycyclic aromatic hydrocarbons (PAH), and pharmaceuticals detected in TWW were also present in TWW irrigated soil (Kinney et al. 2006). Research work dealing with occurrence of organic pollutants in TWW showed the presence of substances having the potential to disrupt the endocrine system, called endocrine disrupting compounds (EDC) (Desbrow et al. 1998). Thus, introduction of EDCs to the environment via irrigation should be considered in arid and semi-arid regions where TWW is reused. Among EDC present in TWW, natural and synthetic hormones, some alkylphenols have the ability to bind to the estrogen receptor (ER) (Balaguer et al. 1999). Some PAHs may bind to the aryl hydrocarbon receptor (AhR). ERα binding based assays have been largely used to evaluate the estrogenic activity of
wastewater effluents (Gomez et al. 2007; Dagnino et al. 2009). Likewise AhR binding assays have been frequently used to follow sediments contamination by urban runoff and industrial waste (Louiz et al. 2008), but rarely for TWW contamination assessment (Dagnino et al. 2009). These bioassays offer a useful and rapid tool to detect ER and AhR compounds in TWW and in impacted soil and waters.

The objectives of this study were to determine: i) ER and AhR activities in TWW reused for irrigation, ii) ER and AhR activities in soil during the six months irrigation period, and iii) ER activities in groundwater before and after spread of TWW on soils either by irrigation or artificial recharge.

METHODS

Site description and sampling

The Oued Souhil irrigated area (276 ha) is situated in Nabeul region, North-East of Tunisia. Our experimental site was a citrus cultivated irrigated plot located in this area (Figure 1). The TWW used for irrigation was a secondary treated wastewater provided by an activated sludge sewage treatment plant (STP). The TWW further stored in a basin of 4,000 m$^3$ and reused during the irrigation period (from June to September-October) is called irrigation water (IW). IW was sampled in July and September 2008 at the valve supplying the irrigated plot. The latter was irrigated every 10 to 15 days, hence consuming about 4,000 m$^3$ from June to October. Before each irrigation event, soil samples (0–10 cm and 10–20 cm horizons) were collected randomly with a stainless steel scoop according to the following dates: June 16$^{th}$, July 1$^{st}$, July 23$^{rd}$, August 26$^{th}$, September 15$^{th}$, and October 7$^{th}$.

This area is the first site where research experiments on the impact of treated wastewater reuse were carried out in the 80’s with a battery of piezometers installed to control ground-water quality. Groundwater samples were collected in three of these piezometers (P1, P2, and P3) and two wells (WA, WB). Groundwater sampling was performed in March and September 2008. Before samples collection, all glassware, equipment and containers were thoroughly washed and rinsed with distilled water and organic solvent.

Water and soil samples treatment

Reagent and solvent. All solvents, methylene chloride, acetone, n-hexane, ethyl acetate, methanol, water were HPLC quality or pesticide-grade and were purchased from Carlo Erba Reactifs (Val de Reuil, France). Materials for cell culture were obtained from Invitrogen (Cergy-Pontoise, France). Luciferin was purchased from Promega (Charbonnières-les-Bains, France). 17β-estradiol (E$_2$, CAS #50-28-2) was purchased from Sigma Aldrich (Saint-Quentin Fallavier, France), and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, dioxin, CAS #1746-01-6) from Promochem (Wesel, Germany). All effectors were dissolved in dimethyl sulfoxide at 10 mM and successive dilutions were performed in culture medium.

Waters. The following parameters: pH, electrical conductivity (EC), total suspended solids (TSS), biochemical oxygen demand (BOD$_5$), and chemical oxygen demand (COD) were measured in water samples collected in polyethylene bottles. For bioassays, IW and groundwater (1000 ml) collected in amber glass bottles were filtrated within 24 h using a 0.70 μm retention fibre glass filter (Fioroni, Ingré, France) pre-washed with distilled water and methanol. All samples were preserved refrigerated at 4°C until extraction within 24 to 48 hr. Waters were extracted by LC-18 reversed-phase cartridge (500 mg, 8 mL) provided by Alltech (Carquefou, France) conditioned with 5 mL ethyl acetate, 5 mL methanol and 5 mL of 2% methanol/HPLC-quality water. Sample was applied at a rate of 5 mL/min. After solid-phase extraction, cartridges were rinsed with 5 mL of HPLC-quality water. Elution was performed with 10 ml methanol, 10 ml ethyl acetate/methanol (1:1, v/v), 10 ml ethyl acetate/hexane (1:1, v/v) and 10 ml hexane. Extracts were then filtrated through anhydrous sodium sulfate (Na$_2$SO$_4$) on a glass microfiber filter and rotary evaporated to dryness at 37°C. Residues were taken up with 500 μl of methanol.

Soil. Composite soil samples collected at 0–10 and 10–20 cm horizon depths were dried, ground, sieved at 2 mm and 0.2 mm (for organic carbon analysis) and preserved wrapped in aluminium foils refrigerated at −15°C. Electrical conductivity and pH were determined on saturated paste extract. Organic carbon (OC) content was determined according to Anne's method consisting in oxidising the
organic carbon by sulphuric acid and potassium dichromate, and titrating the latter in excess by Mohr's salt (NF X 31–109) (AFNOR 1993). Then organic matter (OM) content is obtained by multiplying the OC values by 1.72 (Nelson & Sommers 1996). For bioassays, soil samples from the 0–10 cm horizon were extracted following a microwave-assisted extraction (MAE) procedure, using a Multiwave 3000 (Anton Paar). 5 g of soil were weighed out and 20 mL of hexane-acetone mixture (1:1, v/v) was added. Extraction was performed at 115°C temperature, 5 bars pressure and 70% power during 15 min. After elapsed time, the device was allowed to cool. Supernatant was separated and liners were rinsed three times with 5 mL hexane:acetone (1:1) fractions which were added to the extract. The latter was dried with 10 g Na₂SO₄ then evaporated to dryness at 37°C, and residues were taken up with 250 μL of methanol.

MELN and HAhLP bioassays. The stably transfected luciferase reporter cell lines were obtained as already described by (Balaguer et al. 1999; Pillon et al. 2005). Briefly, MELN cell line used for ER bioassays was obtained by transfecting ERα positive breast cancer MCF-7 cell line with the estrogen responsive element cloned upstream of the luciferase reporter gene construct ERE-βGlob-Luc-SVNeo. For AhR, HAhLP cell lines were obtained by transfecting HeLa cells with the CYP1A1-Luc and pSG5-puro plasmids. Cells were seeded in 96-well tissue culture plates (Greiner, France). Compounds and extracts were incubated with cells for 8 or 24 hr for HAhLP cell lines and 24 hr for MELN at various concentrations in quadruplicate. At the end of incubation, effectors containing medium was removed and replaced by Dulbecco’s Modified Eagle Medium F-12 phenol red free media containing 0.3 mM luciferin. Results are expressed as a percentage of maximum luciferase activity. The maximum value, taken as 100%, was obtained in presence of 10 nM E₂ for MELN and 10 nM TCDD for HAhLP. The basal activity (in the absence of ligands) is 15% of the maximal activity for MELN and 25% for HAhLP. For data analysis, GraphPad Software (version 5.0 Inc., San Diego, CA, USA) was used to determine dose-response curves and EC₅₀ (concentration corresponding to 50% of cell transactivation) for reference compounds (E2 and TCDD) and samples. Results were expressed as nanogram equivalent per liter, for water (ng E₂-EQ/L and ng TCDD-EQ/L) and per gram, for soil (ng E₂-EQ/g and ng TCDD-EQ/g).

RESULTS AND DISCUSSION

In the area of Oued Souhil, the IW has a neutral to slightly alkaline pH (7.5). The EC was 2.6 mS/cm (Table 1). The IW showed higher TSS (49 mg/L) and COD (137 mg O₂/L) than values recommended by the irrigation standards NT 106.03 (30 mg/L and 90 mg O₂/L, respectively for TSS and COD). These values could result from an overload of the wastewater treatment plant during summer due to tourism activity. However, BOD₅ with 27 mg O₂/L fulfilled the limit values recommended by NT 106-03 (1989).

Because of the high levels of TSS and COD in IW, ER and AhR activities were expected. Mean values were 31 ng estradiol-equivalent/L (ng E₂-EQ/L) in July and 46 ng E₂-EQ/L in September. Mean AhR activities were 134.5 ng TCDD-EQ/L in July and 92 ng TCDD-EQ/L in September (Table 1).

Table 1 | Physico-chemical characteristics, ER, and AhR activities of the irrigation water

<table>
<thead>
<tr>
<th>Parameter</th>
<th>m</th>
<th>sd</th>
<th>n</th>
<th>Irrigation standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHs</td>
<td>7.5</td>
<td>0.1</td>
<td>5</td>
<td>6.5–8.5</td>
</tr>
<tr>
<td>EC (mS/cm)</td>
<td>2.6</td>
<td>0.1</td>
<td>5</td>
<td>7.0</td>
</tr>
<tr>
<td>TSS (mg/L)</td>
<td>49</td>
<td>34</td>
<td>4</td>
<td>30</td>
</tr>
<tr>
<td>COD (mg O₂/L)</td>
<td>137</td>
<td>63</td>
<td>5</td>
<td>90</td>
</tr>
<tr>
<td>BOD₅ (mg O₂/L)</td>
<td>27</td>
<td>14</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>TOC (mg C/L)</td>
<td>61</td>
<td>36</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Activity</td>
<td>July</td>
<td>September</td>
<td>Mahjoub et al. (2009)</td>
<td></td>
</tr>
<tr>
<td>ER activity (ng E₂-EQ/L)</td>
<td>25–37</td>
<td>45–47</td>
<td>21 (77.6 pmol/L)</td>
<td></td>
</tr>
<tr>
<td>AhR activity (ng TCDD-EQ/L)</td>
<td>127–142</td>
<td>78–106</td>
<td>103 (319.5 pmol/L)</td>
<td></td>
</tr>
</tbody>
</table>

m: mean; sd: standard deviation; n: observations; Irrigation Standard NT 106.03 (1989) (Institut National de la Normalisation et de la Propriété Industrielle 1989)
ER and AhR activities are in accordance with values recorded in TWW used for irrigation of a plot located in the same area (Mahjoub et al. 2009). However, in other studies carried out previously, ER activities were lower in TWW (Korner et al. 2000; Cargouët et al. 2004; Pawlowska et al. 2004). Overall, ER compounds elimination rate through activated sludge treatment may exceed 80% (Svenson et al. 2003). AhR activities found in the present study are higher than those found by Dagnino et al. (2009) in activated sludge secondary effluents where 15 ng TCDD-EQ/L were detected. In literature, few data on AhR activities are available for comparison, since these activities have been rarely measured in TWW. In the present study, as mentioned before, the hydraulic and biological capacities of the STP may be exceeded during summer which is probably associated to a lower removal efficiency of estrogenic and dioxin-like compounds.

In Oued Souhil area, soils are poor sandy to sandy-silty. Before irrigation, soil samples showed pH of 7.5, EC between 0.9 and 1.1 mS/cm and OM between 2.0 and 2.5% on both 0–10 and 10–20 cm horizons. OM content is relatively high due to organic amendment in early spring, vegetation residues and grazing animals.

TWW used for irrigation has been reported to change soil characteristics such as pH and OM content (Rusan et al. 2007; Jahantigh 2008; Walker & Lin 2008). In our case, during irrigation pH does not seem to be affected by IW (Figure 2). However, some previous studies have reported increase (Smith et al. 1996) or decrease of pH (Kizilöglu et al. 2008) because of ammonia nitrification. Soil EC increased in both horizons because of salts and nutrients transport by the IW, as observed in previous works (Vazquez Montiel et al. 1996; Mohammad & Mazahreh 2003; Kizilöglu et al. 2008). After a first decrease, soil OM content increased probably due to irrigation with IW that carries suspended solids exceeding the standards values, and to weeds growth. Reuse of TWW for irrigation commonly contributes to the organic load of soil (Walker & Lin 2008). Two months after the end of the irrigation, a slight decrease in OM content was observed.

AhR activities were detected in all samples collected before, during and after irrigation (Figure 3). AhR activation by dioxin and dioxin-like compounds was reported to be stable from 8 hr to 24 hr incubation, whereas the activity of PAH-like compounds, such as Benzo(a)pyrene decreases with longer incubation time (24 hr activation < 8 hr activation) (Louiz et al. 2008). This is due to degradation processes in the cells occurring rapidly for PAH-like compounds but more gradually for dioxin-like compounds. Therefore, in our study AhR activities were assessed after 8 hr and 24 hr incubation.

All soil samples showed the same pattern: AhR activities detected after 24 hr incubation time were lower than those detected after 8 hr (Figure 3). Thus, the AhR activities detected in our samples were partly due to PAH-like compounds. PAH are frequently found in TWW (Blanchard et al. 2003) and could contribute to the AhR activities in soil, however as observed, the IW did not markedly increase AhR activity in soil.

On soil samples collected at 0–10 cm, ER activities were detected only at the end of the irrigation season (October, 7th) on two samples at the quantification limit (0.05 ng E2-EQ/g - data not shown). ER ligands present in IW might have been transferred to soil during irrigation. The absence of activity in soil collected before irrigation could be due to degradation of estrogen-like compounds as the soil has not received IW since 6 months at least. During irrigation, ER ligands may biodegrade in the meantime of the 10 to 15 days that separate two irrigation events (sampling was performed before irrigation, i.e. each 10–15 days). In fact, hormones half-lives were reported to vary between 2 to 6 days in water and sediments (Williams et al. 1999). Other substances as alkylphenols may contribute to estrogenic activities. Transfer of compounds to deeper soil layers or groundwater is likely to occur because of the surface irrigation technique known to introduce high water volumes, and the high permeability of light sandy soils. However, (Ternes et al. 2007) suggested that steroid estrogens are removed when TWW used for irrigation passes through the soil.

AhR activity in soil.
The Oued Souhil aquifers are shallow (about 10 m depth) and have received TWW since the 80's with irregular water spread. Water sampling showed that groundwater has a neutral to slightly alkaline pH (7.0–7.3) with a relatively high EC showing salt contamination (Table 2). ER activities were detected in the three piezometers samples collected in March with values between 0.1 and 0.9 ng E2-EQ/L; the values observed in September were similar. However, no estrogenic activity was detected on samples collected from wells WA and WB. This could be due to continuous water drawing for several purposes like irrigation of plots in the research experimental unit. Knowing that the aquifer is 10 to 12 m deep and that the piezometer P3 (16 m depth) is situated on the groundwater flow axis with a filter zone between 0.5 and 15.5 m, the P3 (windowed piezometers) can catch all the water column in the vadose zone (P.N.U.D/O.P.E. 1987). The piezometers P1 and P2 (punctual piezometers) with filter zones between 5 and 13 m, and 9 and 11 m, respectively, (P.N.U.D/O.P.E. 1987) are located downstream P3. Given this situation, the P3 may have been contaminated by IW spread irregularly on the infiltration basins during the sampling period, while for P1 and P2, estrogenic substances should be fewer or more diluted resulting in lower ER activity. In groundwater, detection of ER activities indicates transfer of dissolved xenoestrogens through irrigation/artificial aquifer recharge. For further studies, identification of these ER organic contaminants is recommended. At this scale, transfer of ER and AhR organic contaminants to soil and groundwater and their fate should be determined for each practice (irrigation and aquifer recharge). Contamination of the agricultural environment and associated risks need to be investigated through temporal and spatial studies and in different conditions (irrigation, aquifer use, soils, and plants).

**CONCLUSIONS**

TWW reused for irrigation contains ER and AhR organic contaminants in the aqueous phase. For a more comprehensive study of the occurrence and distribution of these contaminants in the irrigation water, ER and AhR activities need to be determined in the particulate phase as well. Irrigation of the sandy soil with TWW resulted in a hardly detectable ER activity while AhR activity did not clearly increase. Hence, degradation/transfer of compounds should be assessed in depth. In groundwater, detection of ER activities indicates transfer of dissolved xenoestrogens through irrigation/artificial aquifer recharge. For further studies, identification of these ER organic contaminants is recommended. At this scale, transfer of ER and AhR organic contaminants to soil and groundwater and their fate should be determined for each practice (irrigation and aquifer recharge). Contamination of the agricultural environment and associated risks need to be investigated through temporal and spatial studies and in different conditions (irrigation, aquifer use, soils, and plants).

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**REFERENCES**

AFNOR 1993 NF X31-109, Qualité des sols- Méthodes Chimiques- Détermination du carbone organique par oxydation sulfochromique.


Kiziloglu, F. M., Turan, M., Sahin, U., Kuslu, Y. & Dursun, A. 2008 Effects of untreated and treated wastewater irrigation on some chemical properties of cauliflower (Brassica oleracea L. var. botrytis) and red cabbage (Brassica oleracea L. var. rubra) grown on calcareous soil in Turkey. Agricultural Water Management 95(6), 716–724.


