Oestrogenic activity of a textile industrial wastewater treatment plant effluent evaluated by the E-screen test and MELN gene-reporter luciferase assay

UNIVERSITÀ DEGLI STUDI DI TORINO

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Response to Reviewers: Reviewer #7:

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Overall, the manuscript has been revised well taking in to account the comments on the previous submission and seems to be a much improved document. The manuscript will benefit from some minor modification before publication.

Specific comments as follows:
1. QA/QC protocol and data: Any biological tests require an elaborate QA/QC protocol and data collection, this manuscript does not adequately provide the details of the same used in the study.
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4. In the legend of Figure 1 (lines 588 - 589) it is reported: “Boxes represent the median and 25th–75th percentiles, outer lines represent the 10th -90th percentiles” .

Torino, March 26, 2012

Dear Editor,

We are sending the manuscript "*Estrogenic activity of an industrial wastewater effluent by means of the E-screen test and the MELN gene-reporter luciferase assay*" that we submit for possible publication on *The Science of the total environment.*

In the present study we were interested in the biological quantification of estrogenic activity in the effluent of a textile factory wastewater treatment plant (WWTP), in north-western Italy. Samples of the effluent were collected monthly before and after the tertiary treatment (ozonation) and all samples were tested to determine the total estrogenic activity by means of two in vitro tests: the *E-screen test* and the *MELN luciferase gene-reporter assay* by measuring the 17b-estradiol equivalent quantity (EEQ). There is a positive correlation between the two tests and the results suggest that the post-ozonation effluent had lower estrogenic activity. The study point**s** out that the environmental risk due to the input of estrogenic substances into the river via WWTP effluent is lower than the concentration at which these compounds have been reported to chronically affect the endocrine system of living organisms.

Best regards
Sincerely
Tiziana Schilirò

Ms. Ref. No.: STOTEN-D-12-00872R1

Dear Editor,

please find enclosed the new revised version of the manuscript entitled: "**Estrogenic activity of a textile industrial wastewater treatment plant effluent evaluated by the E-screen test and MELN gene-reporter luciferase assay**", hoping that it can now be accepted for publication on ***The Science of the Total Environment***.

We have changed, clarified and add what the reviewer have recommended.

The notes detailing the changes to the paper and our replies to the reviewers' comments are also enclosed. We also attach a further copy of the manuscript where changes are marked in red.

We think our manuscript falls in (7) Biomonitoring area.

Best regards
Tiziana Schilirò

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Estrogenic activity of an industrial wastewater treatment plant effluent by means of the *E-screen* test and the MELN gene-reporter luciferase assay.

Highlights

1. The two *in vitro* tests are suited for estrogenic activity assessment in textile WWTP.
2. There is a significant correlation between the results of the two *in vitro* tests.
3. The estrogenic activity of the effluent is reduced by ozonation.
4. The input of estrogenic substances into the river via textile WWTP is low.

Estrogenic activity of a textile industrial wastewater treatment plant effluent evaluated by the *E-screen* test and MELN gene-reporter luciferase assay.

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Abstract

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In the *E-screen* test, the mean EEQ values were 2.35 ± 1.68 ng/L pre-ozonation and 0.72 ± 0.58 ng/L post-ozonation; in the MELN gene-reporter luciferase assay, the mean EEQ values were 4.18 ± 3.54 ng/L pre-ozonation and 2.53 ± 2.48 ng/L post-ozonation. These results suggest that the post-ozonation IWWTP effluent had a lower estrogenic activity (simple paired t-tests, $p < 0.05$). The average reduction of estrogenic activity of IWWTP effluent after ozonation was 67 ± 26 % and 52 ± 27 % as measured by *E-screen* test and MELN gene-reporter luciferase assay, respectively. There was a positive and significant correlation between the two tests (Rho S = 0.650, $p = 0.022$). This study indicates that the environmental risk is low because estrogenic substances are deposited into the river via IWWTP at concentrations lower than those at which chronic exposure has been reported to affect the endocrine system of living organisms.

Keywords

Estrogenic activity, *E-screen*, MELN, wastewater treatment plant, textile, ozonation, citotoxicity.

1. Introduction

The potential role of endocrine-disrupting chemicals (EDCs) in the environment has been discussed extensively for several years, both in the scientific community and in the broader public (Sumpter, 2008). EDCs are defined as "exogenous substances that cause adverse health effects in an organism, or its progeny, consequent to changes in endocrine functions", and are included in the list of so-called "emerging contaminants" published by the European Union (EU Commission, 2001). EDCs are environmental contaminants that interfere with the function of the endocrine system, and in recent years, they have become one of the major subjects of environmental science research (Colborn et al., 1993; Crews et al., 2000). Recent studies on EDCs in the environment have shown that these chemicals include plastic softeners (bisphenol-A), detergents (4-nonylphenol or 4-octylphenol), heavy metals (cadmium), and natural and synthetic compounds such as 17beta-estradiol (E2) and 17alpha-ethinyl estradiol (EE2) (Ying et al., 2002; Tan et al., 2007). The scientific community has focused intensely on estrogenic EDCs, which are able to interact with human estrogen receptor alpha (hERα), mainly because the ligand-binding domain gap is larger than that required by E2 (Brzozowski et al., 1997).

EDCs are ubiquitous in the environment because of their large number of uses in residential, industrial, and agricultural applications. It has been suggested that industrial and municipal effluents and urban and agricultural runoff are the major sources of EDCs discharged into the aquatic environment (Routledge et al., 1998; Boyd et al., 2003; Liu et al., 2009). Wastewater treatment plant (WWTP) effluents likely affect reproductive processes in exposed freshwater and marine organisms (Purdom et al., 1994; Orlando and Guillette, 2007). Furthermore, it has been hypothesised that the increasing incidences of breast and testicular cancers in humans may be caused by exposure to EDCs, especially via drinking water (Carlsen et al., 1995; Safe, 2005). For these reasons, it is important to note that surface waters are often used as a source

of drinking water (Itoh et al., 2004). The presence of EDCs in the water cycle (wastewater-aquatic systems-drinking water) is considered a major environmental issue. European environmental regulation has prioritised the reduction of surface water pollution due to municipal and industrial wastewater effluents (Directive 2000/60/EC). The current Italian regulations prescribe emission limits for WWTP effluents for a wide range of chemical compounds, though not specifically for ECDs (Decree Italian Law 152/2006).

Textile industry effluents contain high concentrations of organic and inorganic compounds (Laing, 1991). Considering both the volume and the composition of the effluents, the textile industry is rated as the most polluting among all industrial sectors. Textile effluents are one of the most difficult-to-treat wastewaters due to their considerable suspended solids content and their high concentrations of dyes, salts, additives, detergents and surfactants. The presence of EDCs in textile effluents has been demonstrated (Pothitou and Voutsa, 2008; Prigione et al., 2008; Shannon et al., 2008). The physical–chemical technologies used for wastewater treatment, including advanced oxidation processes (i.e., the application of ozone, hydrogen peroxide or ultraviolet radiation), are expensive, not always effective and often do not reduce the toxicity of the effluents (Tehrani-Bagha et al., 2010; Anastasi et al., 2011).

In general, in a WWTP, natural and synthetic EDCs are subjected to a variety of treatment processes (e.g., coagulation, sedimentation and filtration) similar to those used to inactivate other compounds, but they are only partially removed. Little is known about the ultimate fate of endocrine disruptors, particularly after disinfection steps. However, recent studies on the effects of chlorination (Deborde et al., 2004; Lee et al., 2004; Schilirò et al., 2009) and ozonation (Irmak et al., 2005; Bila et al., 2007; Stalter et al., 2011) on endocrine disruptors suggested that they may be degraded via oxidation (Silva et al., 2012).

Several screening programs using a variety of chemical analyses, as well as *in vitro* and *in vivo* bioassays, have been implemented to assess the potential hazard of EDCs in aquatic

environments (Campbell et al., 2006). Considering the large number of EDCs that may be present in a complex environmental sample, target chemical analyses are not sufficient to comprehensively define its EDC content. Furthermore, the total biological effects of the whole sample cannot be determined in these analyses (Nelson et al., 2007; Bicchi et al., 2009). In contrast, several *in vitro* bioassays based on the interaction between the EDCs and estrogenic receptors can be used to determine the total estrogenic EDC activity of an environmental sample. These assays allow the evaluation of additive, synergistic and antagonistic effects (Balaguer et al., 1999; Korner et al., 1999; Leusch et al., 2010). Moreover, these evaluations can be made more comprehensive with the parallel use or combination of two or more tests (Leusch et al., 2010).

In this study, the total estrogenic activity in the effluent of an industrial wastewater treatment plant (IWWTP) in northwestern Italy was measured. Samples of the IWWTP effluent were collected from a textile factory each month from November 2009 to July 2010. After solid phase extraction, all samples were subjected to two *in vitro* tests to measure the 17beta-estradiol equivalent quantity (EEQ): the human breast cancer cell line (MCF-7 BUS) proliferation test or, *E-screen* test, and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay. The IWWTP effluent was evaluated before and after tertiary treatment (ozonation) to assess the potential impact of disinfection on estrogenic activity. The results of the two *in vitro* tests were compared.

2. Materials and Methods

2.1 Characteristics and sampling of industrial wastewater treatment plant (IWWTP) effluents.

The effluents monitored in this study were sampled from a small IWWTP associated with a textile factory in northwestern Italy. The mean treated wastewater flow is approximately

3,000 m^3/day, mainly released by cotton dyeing processes. The dyes are synthetic organic indanthrenes, derived from anthraquinones, that are particularly stable in response to light, weathering and the detergents/disinfectants used for dyeing various fibres. The plant has separate water and sludge treatment lines; the former includes primary (chemico-physical), secondary (activated sludge) and tertiary processes (ozonation). Twenty-four hour composite samples of the final IWWTP effluents were sampled after the secondary treatment (pre-O_3) and after the tertiary treatment (post-O_3) to evaluate the effects of ozonation on estrogenicity. Table 1 describes the physico-chemical characteristics of the effluent samples and the relative Italian threshold limit values (Decree Italian Law 4/2008). The ozonation treatment was carried out in columns (0.2 m in diameter and 4.5 m in height) by adding in countercurrent (effluent flow rate of 60 L/hr) 30 ppm of ozone. Samples were taken on 7 different sampling dates from November 2009 to July 2010. The samples (2 L) were stored in brown glass flasks at 4°C. Two-litre aliquots were extracted for the *E-screen* test and the MELN gene-reporter luciferase assay.

2.2 Extraction of effluent samples

The extraction of effluent samples was evaluated using a previously described method (Schilirò et al., 2009). Briefly, solid phase extraction was performed on columns with 1 g polystyrene copolymer resin ENV and a 6-mL reservoir (Varian, Inc. Agilent technologies, USA) (Kinnberg K, 2003). Two-litre aliquots of each sample were extracted. Samples were first equilibrated to room temperature; then, methanol was added, the pH was adjusted to 2.5, and NaCl was added to achieve a conductivity of 8500 µS. Samples were then drawn through the column after its activation. Elution was performed with 5 mL acetone. Each extract was evaporated to 1 mL under a stream of nitrogen. A 100 µL aliquot of dimethylsulfoxide (DMSO 99.5%) was added to the extract, and the acetone was then completely removed under the nitrogen stream. The extracts were stored in glass vials at 4°C until evaluated in the *E-*

screen test and the MELN gene-reporter luciferase assay. Two-litre Milli-Q water were extracted and analyzed as a QC laboratory sample.

2.3 Sample preparation for bioassays

Each sample extract was mixed with 9.90 mL steroid-free experimental medium, and each sample was then homogenised for approximately 2 min and filtered through a 0.22 µm Millex-GV filter (Millipore). These stock solutions containing 1% v/v DMSO were stored in sterile glass vials at 4°C. They were diluted 10- to 100,000-fold (0.05 to 500 L final volume) with steroid-free experimental medium in sterile glass vials. This ensured that the maximum solvent concentration in the culture medium did not exceed 0.1%, a concentration that was found to have no effect on cell viability.

2.4 Cell lines and culture conditions

Estrogen-sensitive human MCF-7 BUS breast cancer cells were kindly provided by Dr. A.M. Soto and Dr. C. Sonnenschein (Tufts University School of Medicine, Boston, Massachusetts, USA) and cultivated in Dulbecco's modified Eagle's medium (DMEM) with 15 mg/L phenol red, 10% foetal calf serum (FCS), 2% L-glutamine 200mM, 2% HEPES buffer 1M, 1% sodium pyruvate 100mM and 1% penicillin–streptomycin 10 mg/mL, at 37°C in an atmosphere containing 5% carbon dioxide and 95% air under saturating humidity. A stock solution of 1 mM 17-β-estradiol (E2) was prepared in ethanol, stored at -20°C and then diluted to the desired concentration in steroid–free experimental medium.

MELN cells, provided by Dr. P. Balaguer (INSERM, Montpellier, France), are MCF-7 cells stably transfected with the estrogen-responsive gene (ERE-βGlob-Luc-SVNeo) via integrated plasmid. These plasmids contain both an antibiotic resistance selection gene (SVNeo) and the estrogen-responsive elements to which the estrogen receptor-ligand complex can bind, thus inducing the transcription of the luciferase reporter gene (Berckmans et al., 2007). Therefore, the luciferase activity in these cells is proportional to the concentration of estrogenic

compounds (Hernandez-Raquet et al., 2007). MELN cells were cultured in Dulbecco's Modified Eagle Medium Nutrient Mixture F12 Ham (DMEM-F12) with phenol red, supplemented with 5% foetal calf serum (FCS), 2% 200 mM L-glutamine, 1% penicillin/streptomycin and 1 mg/ml G418 sulphate. The cells were maintained in an incubator at 37°C, a relative humidity of 95% and a CO_2 concentration of 5%. The cells were subcultured once per week, and the medium was refreshed between passages. Cells from passage number 4 to passage number 15 were used for experiments. Cells were regularly checked for mycoplasm infection to guarantee that the experiments were performed with mycoplasm-free cells and to comply with the guidelines for good cell culture practice (GCCP). Unless otherwise specified, all chemicals and materials for cell culture were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

2.5 E-screen test

The *E-screen* test was carried out as initially described by Korner et al. (1999) and modified by Schilirò et al. (2009). Briefly, subconfluent MCF-7 BUS cells were trypsinised and resuspended in the steroid-free experimental medium, which consisted of phenol-red–free DMEM supplemented with 5% stripped FCS, 2% L-glutamine 200mM, 2% HEPES buffer 1M, 1% sodium pyruvate 100mM and 1% penicillin–streptomycin 10 mg/mL. Cells were seeded into 24-well plates at a density of 30000 cells/well. After 24 hours, the medium was replaced with experimental medium containing one of five dilutions of water extracts. Each dilution was tested in six replicates per assay. Moreover, one dilution (100-fold) of each effluent sample was tested in combination with 5 nM of the antiestrogen tamoxifen (Tam) and with 0.1 nM E2. Six wells without hormones were used as negative controls, and E2, in five concentrations between 1 pM and 10 nM, was used as the positive control in each assay. Each *E-screen* assay was also performed on a QC laboratory sample (treated with the same method

of samples). The assays were completed after six days by determining the absorbance (595 nm) in each well after crystal violet staining.

The proliferative effect (PE) of a sample is the ratio between the highest cell number achieved with the sample or E2 and the cell number of the negative control:

(1) PE = (max cell number)sample/(cell number)negative control.

The estrogenic activity of a sample is evaluated by determining the relative efficacy, called the relative PE (RPE%). The RPE compares the maximum proliferation induced by a sample with that induced by E2:

(2) RPE % = [(PE-1)sample/(PE-1)E2] × 100.

Full agonistic activity, RPE ≥ 100%, can be distinguished from partial agonistic activity, in which RPE is less than 100% (Soto, 1995).

Relative potency, called estradiol equivalency quantity (EEQ) is thus calculated as:

(3) EEQ = (EC50)E2/(EC50)sample.

The EC50 value for the *E-screen* test (concentration at which 50% of PE is achieved) was calculated with a probit regression (SPSS, Chicago, IL). The PE and EC50 values of each sample were calculated from the mean dose–response curves established from each experiment. The EEQ, expressed in ng/L, is defined as the total concentration of estrogenic active compounds in an environmental sample normalised to the natural estrogen 17-β-estradiol.

2.6 MELN gene-reporter luciferase assay.

MELN gene- reporter luciferase assay has been widely used for the detection of estrogenic activity in complex environmental samples (Cargouët et al., 2007; Combalbert et al., 2012; Fenet et al., 2003; Hernandez-Raquet et al., 2007; Jugan et al., 2009; Kinani et al., 2010;). The test was carried out as described by Balaguer et al. (1999).

Because phenol red and FCS exhibit estrogenic activity, *in vitro* experiments were carried out in DMEM F12 without phenol red, supplemented with 5% dextran-coated charcoal-treated foetal calf serum (DCC-FCS), 2% L-glutamine 200 mM and 1% antibiotics (penicillin/streptomycin). To adapt the cells to DCC-FCS, the growth medium was replaced with fresh test medium 3 days prior to the experiment. Cells were then harvested and seeded in 96-well plates with a flat, clear bottom (Corning) at a density of 40000 cells/well in 100 µl of DCC-FCS per well. After 24 hours, the test medium was removed, and 100 µl of each sample dilution was added to three replica wells. Cells were treated with samples for 20 h. Negative controls, without hormones, and positive controls, E2 in concentration between 1 pM and 10 nM, were included in each assay. One dilution (100-fold) of each effluent sample was tested together with 5 µM antiestrogen tamoxifen (Tam) and with 0.1 nM E2. Each *MELN gene-reporter luciferase assay* was also performed on a QC laboratory sample (treated with the same method of samples). All experiments were performed in triplicate.

2.6.1 Luciferase activity measurement in MELN cells.

We used the One-Glo Luciferase Assay System (Promega, USA) to measure luciferase activity according to manufacturer's instructions. Briefly, at the end of the incubation, 100 µl of One-Glo Reagent (containing fluoroluciferin) was added to each well and mixed for optimal consistency. After at least 3 minutes, to allow complete cell lysis, and within 30 minutes of reagent addition, luminescence was measured by a luminometer (Tecan, Infinite M200 PRO). The luciferase activity of the MELN cells relative to the positive control E2 was represented as transactivation % (TRANS %): the maximum increase in luciferase gene expression triggered by estrogenic compounds present in the samples. The induction of luciferase activity was expressed as a percentage, and the 100% value was obtained in the presence of E2 (Balaguer et al., 1999; Fenet et al., 2003). The estrogenic activity was

expressed as 17-beta estradiol equivalent estrogenic activity (EEQ) in ng/L. EEQ was thus calculated as (EC50)E2/(EC50)sample.

2.6.2 Cytotoxicity assessment in MELN cells.

The Multitox-Fluor Multiplex Cytotoxicity Assay (Promega, cat. no. 9200) is a fluorescence-based assay that use membrane integrity changes to measure cell viability or cytotoxicity in conjunction with luciferase measurements on the same test plates (Berckmans et al., 2007). This assay technology simultaneously measures two distinct protease activities, with rapid catalytic cleavage rates, as markers of cell viability or cytotoxicity.

The cytotoxicity test was applied to dilutions 1:10 and 1:100 (equal to 5 L and 0.5 L dilution volumes) of the pre- and post-ozonation effluents.

At the end of incubation, 100 µl aliquots of reagent were added to all wells, mixed and allowed to incubate for at least 30 minutes at 37°C. Fluorescence was determined at excitation/emission wavelengths of 360 nm/460 nm for cell viability and 485 nm/528 nm for cytotoxicity using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT). The mean values and standard deviations of replicate wells and the mean fold increase of cytotoxicity relative to the negative control were calculated.

2.7 Statistical analyses.

Data were analysed by means of a probit regression analysis (EC50), Spearman's test (correlation) and t-test (means comparison) using SPSS 18.0 (SPSS for Windows, Chicago, IL, USA).

3. Results and Discussion

3.1 Estrogenic activity in IWWTP effluent samples as measured by E-screen test.

The mean EC50 value of E2 for the *E-screen* was 15.24 ± 13.73 ng/L (56.44 ± 50.83 pM); EC50 values were calculated from the control curves obtained from each of the bioassays.

Maximum cell proliferation was generally induced by 0.1 nM E2. The detection limit of the *E-screen* could be defined as the concentration of a single compound or a sample inducing a cell proliferation significantly higher than the hormone-free negative control; the treatment with 17beta-estradiol showed a detection limit of 1 pM (Körner et al. 1999; Schilirò et al., 2004). The proliferative response of the samples was dose dependent; the 1:100 dilution of the samples (equal to a 0.5 L dilution volume) always stimulated the maximum proliferative response. The QC laboratory sample did not induce any significant cell proliferation. To verify that the cell proliferation induced by estrogenic activity was mediated by ER, we observed that co-incubation with Tam inhibited the proliferative response (28 ± 23 %), while co-incubation with E2 led to a greater proliferative response (114 ± 39 %). The difference was statistically significant in both cases (t-tests, $p < 0.05$).

Estrogenic activity, expressed as EEQ, was detected in all but the sixth sample and ranged from 0.10 to 4.73 ng/L (Table 2). The proliferative effect of the different effluent samples on MCF-7 BUS cells relative to the positive control E2 is represented as RPE % in Table 2. The RPE of the effluent both before and after ozonation generally showed partial agonist activity (RPE < 100%) ranging between 15 to 90 %. The EEQs of the effluent extracts, determined in the *E-screen* test, are reported in Figure 1.

The highest mean EEQ was found in the second pre-ozonation sample (4.73 ± 2.42 ng/L), and the lowest mean EEQ was found in the last post-ozonation sample (0.10 ± 0.13 ng/L). The mean EEQ values were 2.35 ± 1.68 ng/L pre-ozonation and 0.72 ± 0.58 ng/L post-ozonation. The average reduction in the estrogenic activity of IWWTP effluent from pre- to post-ozonation was 67 ± 26 %. Despite the low number of analysed samples, the difference between EEQ values before and after ozonation was statistically significant (simple paired t-test, $p = 0.038$), suggesting that the ozonation process significantly reduced the estrogenic activity of the IWWTP effluent.

The *in vitro E-screen* test can be used to screen a large number of compounds and integrate the effects of chemicals that may not be measured in an analytical screen (Vega-Lopez, 2007). It has been studied extensively, and no false positives or false negatives have yet been found; it is also one of the most sensitive of all published assays (Soto et al., 2006; Vanparys et al., 2010). The *E-screen* has been shown to be appropriate for the determination of estrogenic activity in environmental extracts from IWWTP effluent samples.

3.2 Estrogenic activity in IWWTP effluent samples by MELN gene-reporter luciferase assay

The EC50 values of E2 in the MELN gene-reporter luciferase assay were calculated from control curves obtained from each bioassay, and the mean EC50 value was 1.76 ± 0.87 ng/L (6.52 ± 3.22 pM). Maximum luciferase activity was generally induced by 0.1 nM E2. The stable transfectants MELN used in this study exhibited good responsiveness following treatment to 17beta-estradiol with a detection limit of 1 pM (Balaguer et al., 1999). The luciferase activity of the samples was dose dependent, and the 1:100 dilution (equal to a 0.5 L dilution volume) always stimulated the maximum luciferase activity. The QC laboratory sample did not induce any significant luciferase activity. To confirm that the luciferase activity was mediated by the ER, we observed that co-incubation with Tam led to an inhibition of the proliferative response (51 ± 29 %), while co-incubation with E2 led to a greater proliferative response (113 ± 34 %). The difference was statistically significant in both cases (t-tests, $p < 0.05$). Figure 2 shows the MELN luciferase activity of E2 and of the post-ozonation effluents expressed in dose-response curves and compared with the negative control.

Estrogenic activity was detected in all but the sixth sample and ranged from 0.14 to 8.50 EEQ (ng/L) (Table 2). The luciferase activity of the different effluent samples in the MELN cells relative to the positive control E2 is represented as TRANS % in Table 2. The TRANS % of

the effluent before and after ozonation ranged between 27 to 62 %. The EEQs of the effluent extracts, determined in the MELN gene-reporter luciferase assay, are reported in Figure 1.

The highest mean EEQ was found in the first pre-ozonation sample (8.50 ± 4.36 ng/L), and the lowest mean EEQ was found in the second post-ozonation sample (0.14 ± 0.05 ng/L). A lower mean value was found for the effluents post-ozonation: the mean EEQ values were 4.18 ± 3.54 ng/L pre-ozonation and 2.53 ± 2.48 ng/L post-ozonation; this difference was statistically significant (simple paired t-test, $p = 0.048$). The average reduction of estrogenic activity of IWWTP effluent after ozonation was 52 ± 27 %.

The *in vitro* MELN gene-reporter luciferase assay can rapidly screen a large number of chemicals, and it is transferable, robust and reproducible, enabling the ranking of chemical compounds from strong to weak affinity for the estrogen receptor (Witters et al, 2010). This assay has been shown to be appropriate for the determination of estrogenicity in environmental extracts from IWWTP effluent samples.

3.3 Cytotoxicity of IWWTP effluent samples in MELN cells.

The 1:10 dilution of the effluent pre-and post-ozonation was shown to decrease cell proliferation compared to negative controls, in terms of PE, in both assays. The sample, which is less diluted, might be able to mask the real estrogenic activity of the compounds present in it. To determine whether the effluent might have a toxic effect on the cells, 1:10 and 1:100 dilutions (equal to 5 L and 0.5 L dilution volume) were tested for cytotoxicity (Table 3).

The 1:10 mean fold increase of cytotoxicity relative to the negative control was 50.3 ± 30.0 for pre-ozonation effluents and 6.3 ± 6.0 for post-ozonation effluents (t-tests, $p < 0.01$ and $p < 0.05$, respectively). The mean fold increase of cytotoxicity produced by the 1:100 dilution compared to the negative control was 1.2 ± 0.8 for the pre-ozonation effluent and 1.0 ± 1.1 for the post-ozonation effluent (t-tests, $p > 0.05$). The sample without estrogenic activity (n°6) did

not exhibit a significantly higher cytotoxicity; therefore, the absence of estrogenicity can not be ascribed to the toxicity of the sample.

3.4 Comparison of estrogenic activity in the E-screen test and in the MELN gene-reporter luciferase assay

A comparison of the results obtained in these two assays highlights the non-significance of differences in average EEQs (t-test, $p > 0.05$). The correlation between the EEQs obtained with the MELN gene-reporter luciferase assay and with the *E-screen* was positive and significant (Rho S = 0.650 and p = 0.022). The mean EC50 values relative to E2 were 15.24 ± 13.73 ng/L for the *E-screen* and 1.76 ± 0.87 ng/L for the MELN gene-reporter luciferase assay; these values were significantly different (t-test, p = 0.015). The MELN gene-reporter luciferase assay generally showed higher EEQ mean values.

These differences could be partially explained by the end-points of the two tests: the *E-screen* is based on a binding mechanism that causes proliferation as a cellular response, which could be affected by other external factors, while the MELN assay is receptor specific. Another important difference between the two tests is in the assay duration; stimulation with the test compounds lasts for 16-20 hours in the MELN assay but 120 hours for the *E-screen* (Soto et al., 2006; Witters et al, 2010).

4. Conclusions

In general, the *E-screen* test and the MELN gene-reporter luciferase assay are well suited for the determination of estrogenic activity in environmental matrices; in this specific study, they were used to evaluate estrogenic activity in wastewaters from a textile factory, before and after treatment with ozone. The two *in vitro* tests are particularly suitable for environmental monitoring because of their sensitivity, speed, reading and low cost. The estrogenicity values in the final effluent (post O_3) in the present work were similar to results reported in the

literature for other IWWTP effluents (Korner et al., 1999; Onda et al., 2002; Vethaak et al., 2005; Tan et al., 2007; Salste et al., 2007). Our study suggests that the input of estrogenic substances into the river via IWWTP is low (EEQ range: 0.72 – 2.53 ng/L) and that the environmental risk could be even lower due to the dilution effect connected with discharge and the self-depuration capacity of the receiving river. In a previous study, this reduction was approximately one order of magnitude for EEQs (Schilirò et al., 2009), resulting in concentrations lower than those at which these compounds have been reported to chronically affect the endocrine systems of living organisms (approximately 1-10 ng/L; Lopez de Alda & Barcelo, 2001). The analysed effluent shows lower EEQs values relative to a municipal effluent (Fenet et al., 2003; Pothitou and Voutsa, 2008; Pereira et al., 2011). This difference in the estrogenic activity of urban and industrial wastewater is certainly attributable to the presence of different types of substances; municipal wastewater contains more natural EDCs, such as E2 and EE2, that have strong estrogenic power even at very low concentrations, while industrial wastewater typically contains more synthetic EDCs that are weakly estrogenic relative to natural EDCs (Soto et al., 1995; Pereira et al., 2011).

Ozonation is considered an economically feasible option for the advanced treatment of WWTP effluents (Joss et al., 2008). It is a very effective method for the degradation of persistent organic dyes from colored textile wastewater (Tehrani-Bagha et al., 2010). In general, ozonation and other oxidation processes seem to be the best alternatives for treating WWTP effluents containing estrogens (Pereira et al., 2011). Some authors have noted that the decrease in EDCs may be due to the oxidation effects of ozonation (Lee, 2004; Larcher et al., 2012). To our knowledge, few reports in the literature describe estrogenic activity in textile IWWTP. This work shows that this type of effluent does have estrogenic activity and that ozonation decreases its overall estrogenic effects. However, ozonation is known to lead to the formation of transformation products that have largely not been identified to date, and there

are concerns about their potential impact on the environment and human health (Benner and Ternes, 2009; Radjenovic et al., 2009; Dodd et al., 2010; Stalter et al., 2010, 2011; Anastasi et al., 2011; Reungoat et al., 2012). This highlights the need to evaluate the presence of disinfection by-products after water treatment has been performed.

The identification and removal of EDC pollutants from IWWTP effluents may solve many of the apparent endocrine disruption problems observed in aquatic environments, in addition to providing a cleaner source of drinking water. Further investigations should be carried out to identify the level of ozone appropriate to minimise both the estrogenic activity and the toxicity of IWWTP textile effluents.

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Figure captions

Figure 1. Estradiol equivalency quantity (EEQ ng/L) of the IWWTP effluent extracts after the secondary treatment (Pre ozonation) and after the tertiary treatment (Post ozonation) by means of *E-Screen* test and MELN gene-reporter luciferase assay. Boxes represent the median and 25th – 75th percentiles, outer lines represent the 10th -90th percentiles.

Figure 2. MELN luciferase activity of 17-beta-estradiol (E2) and of post ozonation effluents (examples: n° 5 dotted bars and n° 7 striped bars) expressed with dose-response curves and compared with the negative control.

Table 1. Physico-chemical parameters of the effluents after secondary treatment (Pre-O_3) and after tertiary treatment (Post-O_3) and Italian threshold limit values (nd: not detected).

Parameters	**Pre O_3**	**Post O_3**	**Italian threshold limits values***
pH	8.3 – 9.4	8.4 – 9.2	5.5 < x < 9.5
Colour	Often detectable after 1:20 dilution	nd, after 1:20 dilution	nd, after 1:20 dilution
Suspended solids (mg/L)	18 – 58.8	17 - 27	< 80
COD (mg/L)	53.3 - 81.0	33.7 - 89.3	< 160
BOD (mg/L)	9 - 20	8 - 14	< 40
Cl^- (mg/L)	235.6 - 925	795 - 1102	< 1200
Sulphites (mg/L)	0.44 - 0.72	< 0.2	< 1
Sulfates (mg/L)	269 - 948	479 - 561	< 1000
NH_4 (mg/L)	0.05 - 1.025	< 0.1	< 15
Nitrites (mg/L)	0.1 - 0.2	< 0.02	< 0.6
Nitrates (mg/L)	1.04 - 1.4	7.1 - 11.9	< 20
Total tensioactives (mg/L)	1.4 - 1.5	< 0.2	< 2

*Decree Italian Law 4/2008, reference values for discharge to surface waters.

Table 2. Mean estrogenic activity of the IWWTP effluent extracts after secondary treatment (Pre-O_3) and after tertiary treatment (Post-O_3) in the *E-screen* test and MELN gene-reporter luciferase assay (nd: not detected).

N° sampling	***E-screen test* EEQ (ng/L)**	***E-screen test* RPE%**	***MELN assay* EEQ (ng/L)**	***MELN assay* TRANS %**
Pre O_3				
1	1.66 ± 0.85	37	8.50 ± 4.36	29
2	4.73 ± 2.42	39	1.25 ± 0.37	42
3	1.78 ± 2.37	65	7.38 ± 0.87	27
4	4.17 ± 1.38	74	6.12 ± 3.34	58
5	0.79 ± 0.30	6	1.04 ± 0.39	46
6	nd	nd	nd	nd
7	0.99 ± 0.27	45	0.78 ± 0.28	37
Post O_3				
1	1.34 ± 0.69	32	3.92 ± 2.01	56
2	0.73 ± 0.38	21	0.14 ± 0.05	32
3	0.35 ± 0.47	52	5.33 ± 1.63	44
4	1.50 ± 0.20	90	5.03 ± 1.65	62
5	0.28 ± 0.10	26	0.38 ± 0.14	39
6	nd	nd	nd	nd
7	0.10 ± 0.13	15	0.41 ± 0.20	42

Table 3. MELN cytotoxicity and cell viability of the IWWTP effluent extracts (1:10 and 1:100 dilutions) after secondary treatment (Pre-O_3) and after tertiary treatment (Post-O_3).

N° sampling	***Cytotoxicity*** **fold increase**		***Cell viability*** **fold increase**	
	1:10	1:100	1:10	1:100
Pre O_3				
1	36.2	1.0	0.6	1.6
2	89.4	1.8	0.3	1.3
3	35.3	1.0	0.5	1.5
4	26.7	1.0	0.7	1.4
5	75.4	1.7	0.5	1.5
6	71.6	0.9	0.4	1.5
7	17.3	1.2	0.6	1.3
Post O_3				
1	1.5	0.5	1.3	1.8
2	18.5	0.5	0.7	1.6
3	4.6	0.5	1.0	1.8
4	1.6	0.6	1.4	1.8
5	7.4	0.7	1.0	1.8
6	8.1	0.6	0.9	1.5
7	2.2	3.4	1.3	1.1

Figure 1-2

Figure 1.

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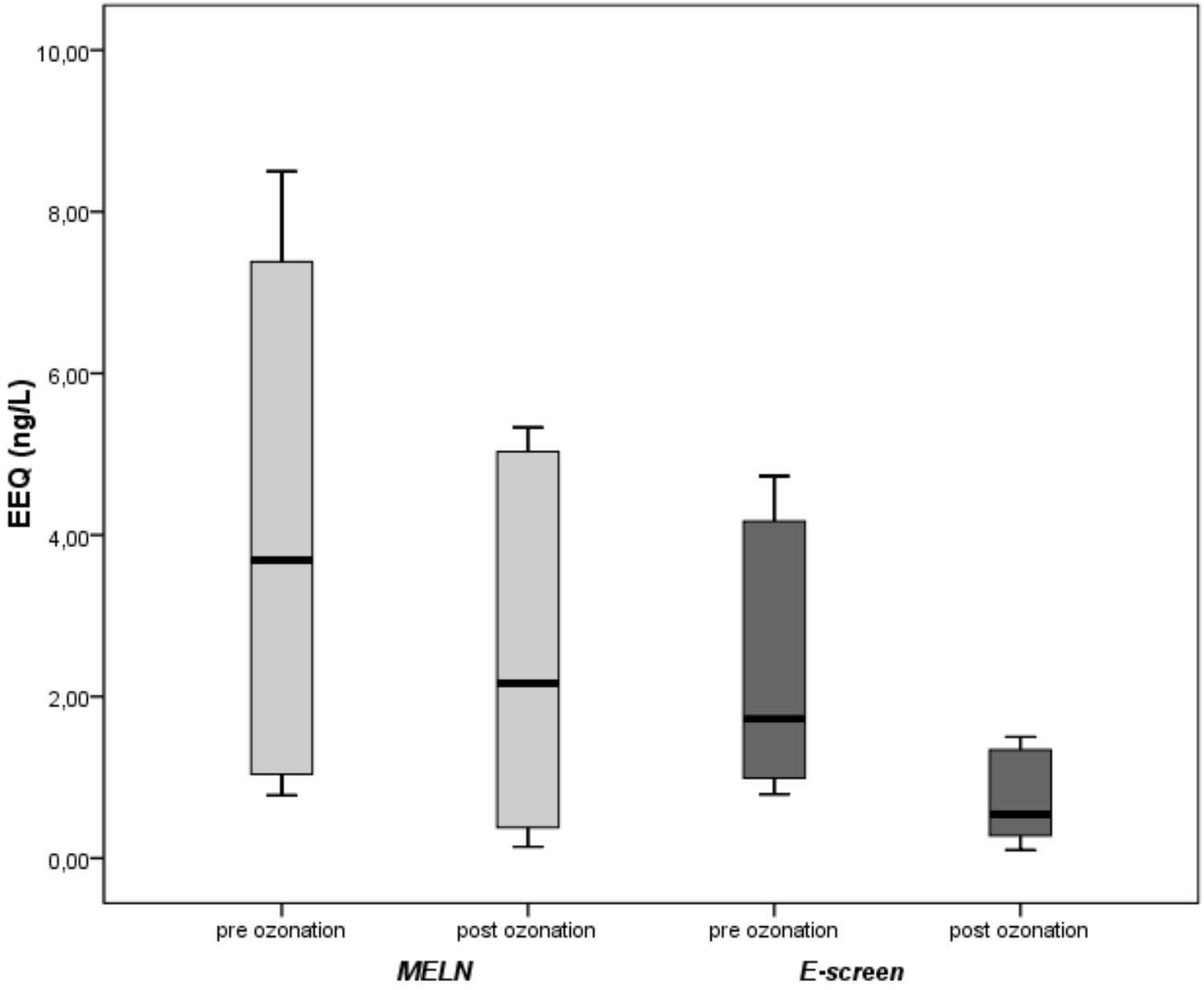
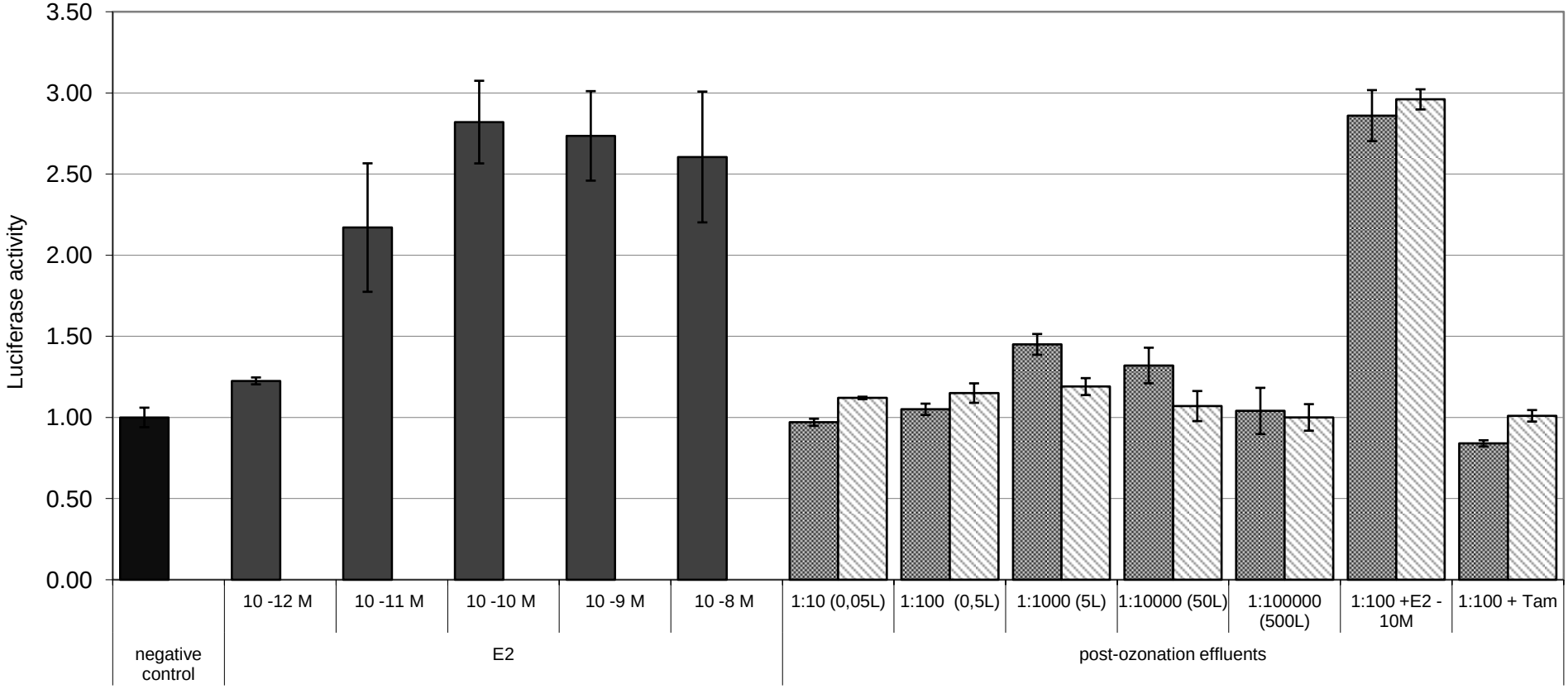


Figure 2.


Luciferase activity
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
negative control
10 -12 M
10 -11 M
10 -10 M
10 -9 M
10 -8 M
E2
1:10 (0,05L)
1:100 (0,5L)
1:1000 (5L)
1:10000 (50L)
1:100000 (500L)
1:100 +E2 - 10M
1:100 + Tam
post-ozonation effluents

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