

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

**This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/107646> since 2016-10-04T16:05:28Z

*Published version:*

DOI:10.1016/j.scitotenv.2012.06.008

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)



# UNIVERSITÀ DEGLI STUDI DI TORINO

This Accepted Author Manuscript (AAM) is copyrighted and published by Elsevier. It is posted here by agreement between Elsevier and the University of Turin. Changes resulting from the publishing process - such as editing, corrections, structural formatting, and other quality control mechanisms - may not be reflected in this version of the text. The definitive version of the text was subsequently published in:

**[Science of the total environment, 432, August, 2012 , DOI:  
10.1016/j.scitotenv.2012.06.008]**

You may download, copy and otherwise use the AAM for non-commercial purposes provided that your license is limited by the following restrictions:

- (1) You may use this AAM for non-commercial purposes only under the terms of the CC-BY-NC-ND license.
- (2) The integrity of the work and identification of the author, copyright owner, and publisher must be preserved in any copy.
- (3) You must attribute this AAM in the following format: Creative Commons BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/deed.en>), [+ Digital Object Identifier link to the published journal article on Elsevier's ScienceDirect® platform]

Manuscript Number: STOTEN-D-12-00872R2

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

Article Type: Full Length Article

Section/Category: Biomonitoring and Surveillance

Keywords: Estrogenic activity; E-screen; MELN; wastewater treatment plant; textile; ozonation; cytotoxicity.

Corresponding Author: Dr. Tiziana Schilirò, M.D.

Corresponding Author's Institution: University of Torino

First Author: Tiziana Schilirò, M.D.

Order of Authors: Tiziana Schilirò, M.D.; Arianna Porfido; Federica Spina; Giovanna Cristina Varese; Giorgio Gilli

**Abstract:** This study quantified the biological estrogenic activity in the effluent of a textile industrial wastewater treatment plant (IWWTP) in northwestern Italy. Samples of the IWWTP effluent were collected monthly, both before and after tertiary treatment (ozonation). After solid phase extraction, all samples were subjected to two in vitro tests of total estrogenic activity, the human breast cancer cell line (MCF-7 BUS) proliferation assay, or E-screen test, and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay, to measure the 17 $\beta$ -estradiol equivalent quantity (EEQ). In the E-screen test, the mean EEQ values were 2.35  $\pm$  1.68 ng/L pre-ozonation and 0.72  $\pm$  0.58 ng/L post-ozonation; in the MELN gene-reporter luciferase assay, the mean EEQ values were 4.18  $\pm$  3.54 ng/L pre-ozonation and 2.53  $\pm$  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  $\pm$  26 % and 52  $\pm$  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.

Response to Reviewers: Reviewer #7:

This is the revised version of this manuscript incorporating the reviewers' comments. The authors also include the item wise response to comments along with the manuscript. The study aims to quantify the biological estrogenic activity in the effluent of a textile industrial wastewater treatment plant (IWWTP) in northwestern Italy. Samples of the IWWTP effluent were collected monthly, both before and after tertiary treatment (ozonation). After solid phase extraction, all samples were subjected to two in vitro tests of total estrogenic activity, the human breast cancer cell line (MCF-7 BUS) proliferation assay, or

E-screen test, and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay, to measure the 17 $\beta$ -estradiol equivalent quantity (EEQ). The 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.

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.
2. Table-1, replace "commas" used for the decimal places and the standard notation of "." to avoid confusion.
3. Table-2, for "nd", the information is not complete unless the detection limit is also provided.
4. Figure-1, the box and whisker plot needs details on what it showing (e.g., ranges showing X percentiles, the thick line in the box is the mean or the median etc.), these details can be provided as a legend in the figure or in manuscript text.

Response to Reviewer comments:

1. Thanks to reviewer comments we add and highlight the QA/QC at lines: 151-152, 194-200, 232-236, 280, 313-314.

We initially did not report about QA/QC protocol because the in vitro tests were performed by authors in other manuscripts (1. Schilirò T, et al. 2004. Toxicity and Estrogenic Activity of a Wastewater Treatment Plant in Northern Italy. *Arch Env Contam Tox* 47(4):456-462 - 2. Bicchi C, et al. 2009. Analysis of environmental endocrine disrupting chemicals using the E-screen method and stir bar sorptive extraction in wastewater treatment plant effluents. *Sci Total Env* 407:1842-1851 - 3. Schilirò T, et al. 2009. The endocrine disrupting activity of surface waters and of wastewater treatment plant effluents in relation to chlorination. *Chemosphere* 75:335-340 - 4. Schilirò T, et al. 2011. Endocrine disrupting activity in fruits and vegetables evaluated with the E-screen assay in relation to pesticide residues. *J Steroid Bioch Mol Bio* 127:139-146).

2. We replace "commas" in Table 1.

3. We provided the detection limit at lines 274 - 278 and 309 - 311.

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 17 $\beta$ -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 points 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

### **Response to Reviewer comment:**

#### **Reviewer #7:**

*This is the revised version of this manuscript incorporating the reviewers' comments. The authors also include the item wise response to comments along with the manuscript. The study aims to quantify the biological estrogenic activity in the effluent of a textile industrial wastewater treatment plant (IWWTP) in northwestern Italy. Samples of the IWWTP effluent were collected monthly, both before and after tertiary treatment (ozonation). After solid phase extraction, all samples were subjected to two in vitro tests of total estrogenic activity, the human breast cancer cell line (MCF-7 BUS) proliferation assay, or E-screen test, and the luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay, to measure the 17 $\beta$ -estradiol equivalent quantity (EEQ). The 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.*

*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.*

**Thanks to reviewer comments we add and highlight the QA/QC at lines: 151-152, 194-200, 232-236, 280, 313-314.**

**We initially did not report about QA/QC protocol because the *in vitro* tests were performed by authors in other manuscripts (1. Schilirò T, et al. 2004. Toxicity and Estrogenic Activity of a Wastewater Treatment Plant in Northern Italy. *Arch Env Contam Tox* 47(4):456-462 – 2. Bicchi C, et al. 2009. Analysis of environmental endocrine disrupting chemicals using the E-screen method and stir bar sorptive extraction in wastewater treatment plant effluents. *Sci Total Env* 407:1842–1851 - 3. Schilirò T, et al. 2009. The endocrine disrupting activity of surface waters and of wastewater treatment plant effluents in relation to chlorination. *Chemosphere* 75:335–340 - 4. Schilirò T, et al. 2011. Endocrine disrupting activity in fruits and vegetables evaluated with the E-screen assay in relation to pesticide residues. *J Steroid Bioch Mol Bioy* 127:139-146).**

2. *Table-1, replace "commas" used for the decimal places and the standard notation of "." to avoid confusion.*

**We replace “commas” in Table 1.**

3. *Table-2, for "nd", the information is not complete unless the detection limit is also provided.*

**We provided the detection limit at lines 274 – 278 and 309 – 311.**

4. *Figure-1, the box and whisker plot needs details on what it showing (e.g., ranges showing X percentiles, the thick line in the box is the mean or the median etc.), these details can be provided as a legend in the figure or in manuscript text.*

**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” .**

**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.



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

3  
4  
5 3  
6  
7 4 Tiziana Schilirò<sup>\*a</sup>, Arianna Porfido <sup>a</sup>, Federica Spina<sup>b</sup>, Giovanna Cristina Varese<sup>b</sup>, Giorgio  
8  
9 5 Gilli<sup>a</sup>

10  
11 6  
12  
13  
14 7 <sup>a</sup>Department of Public Health and Microbiology, University of Torino, Via Santena 5bis,  
15  
16  
17 8 10126 Torino, Italy.

18  
19 9 <sup>b</sup>Department of Life Sciences and Systems Biology, University of Torino, Viale Mattioli 25,  
20  
21  
22 10 10125 Torino, Italy.

23  
24 11  
25  
26 12  
27  
28  
29 13 \*Corresponding Author:

30  
31 14 Tiziana Schilirò

32  
33  
34 15 Department of Public Health and Microbiology, University of Torino.

35  
36 16 Via Santena, 5bis - 10126 Torino – ITALY

37  
38  
39 17 tel. + 39 011 670 5820

40  
41 18 Fax + 39 011 670 5874

42  
43  
44 19 e-mail: [tiziana.schiliro@unito.it](mailto:tiziana.schiliro@unito.it)

45  
46 20

47  
48 21

49  
50  
51 22

52  
53 23

54  
55  
56 24

57  
58 25

59  
60

61

62

63

64

65

## 26 **Abstract**

1  
2 27 This study quantified the biological estrogenic activity in the effluent of a textile industrial  
3  
4 28 wastewater treatment plant (IWWTP) in northwestern Italy. Samples of the IWWTP effluent  
5  
6  
7 29 were collected monthly, both before and after tertiary treatment (ozonation). After solid phase  
8  
9  
10 30 extraction, all samples were subjected to two *in vitro* tests of total estrogenic activity, the  
11  
12 31 human breast cancer cell line (MCF-7 BUS) proliferation assay, or *E-screen* test, and the  
13  
14 32 luciferase-transfected human breast cancer cell line (MELN) gene-reporter assay, to measure  
15  
16  
17 33 the 17 $\beta$ -estradiol equivalent quantity (EEQ).

18  
19 34 In the *E-screen* test, the mean EEQ values were  $2.35 \pm 1.68$  ng/L pre-ozonation and  $0.72 \pm$   
20  
21  
22 35  $0.58$  ng/L post-ozonation; in the MELN gene-reporter luciferase assay, the mean EEQ values  
23  
24  
25 36 were  $4.18 \pm 3.54$  ng/L pre-ozonation and  $2.53 \pm 2.48$  ng/L post-ozonation. These results  
26  
27 37 suggest that the post-ozonation IWWTP effluent had a lower estrogenic activity (simple  
28  
29 38 paired t-tests,  $p < 0.05$ ). The average reduction of estrogenic activity of IWWTP effluent  
30  
31  
32 39 after ozonation was  $67 \pm 26$  % and  $52 \pm 27$  % as measured by *E-screen* test and MELN gene-  
33  
34  
35 40 reporter luciferase assay, respectively. There was a positive and significant correlation  
36  
37 41 between the two tests (Rho S = 0.650,  $p = 0.022$ ). This study indicates that the environmental  
38  
39 42 risk is low because estrogenic substances are deposited into the river via IWWTP at  
40  
41  
42 43 concentrations lower than those at which chronic exposure has been reported to affect the  
43  
44 44 endocrine system of living organisms.

## 46 **Keywords**

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

## 51           **1. Introduction**

1  
2 52   The potential role of endocrine-disrupting chemicals (EDCs) in the environment has been  
3  
4 53   discussed extensively for several years, both in the scientific community and in the broader  
5  
6  
7 54   public (Sumpter, 2008). EDCs are defined as “exogenous substances that cause adverse health  
8  
9 55   effects in an organism, or its progeny, consequent to changes in endocrine functions”, and are  
10  
11 56   included in the list of so-called “emerging contaminants” published by the European Union  
12  
13 57   (EU Commission, 2001). EDCs are environmental contaminants that interfere with the  
14  
15 58   function of the endocrine system, and in recent years, they have become one of the major  
16  
17 59   subjects of environmental science research (Colborn et al., 1993; Crews et al., 2000). Recent  
18  
19 60   studies on EDCs in the environment have shown that these chemicals include plastic softeners  
20  
21 61   (bisphenol-A), detergents (4-nonylphenol or 4-octylphenol), heavy metals (cadmium), and  
22  
23 62   natural and synthetic compounds such as 17beta-estradiol (E2) and 17alpha-ethinyl estradiol  
24  
25 63   (EE2) (Ying et al., 2002; Tan et al., 2007). The scientific community has focused intensely on  
26  
27 64   estrogenic EDCs, which are able to interact with human estrogen receptor alpha (hER $\alpha$ ),  
28  
29 65   mainly because the ligand-binding domain gap is larger than that required by E2 (Brzozowski  
30  
31 66   et al., 1997).

32  
33  
34 67   EDCs are ubiquitous in the environment because of their large number of uses in residential,  
35  
36 68   industrial, and agricultural applications. It has been suggested that industrial and municipal  
37  
38 69   effluents and urban and agricultural runoff are the major sources of EDCs discharged into the  
39  
40 70   aquatic environment (Routledge et al., 1998; Boyd et al., 2003; Liu et al., 2009). Wastewater  
41  
42 71   treatment plant (WWTP) effluents likely affect reproductive processes in exposed freshwater  
43  
44 72   and marine organisms (Purdom et al., 1994; Orlando and Guillette, 2007). Furthermore, it has  
45  
46 73   been hypothesised that the increasing incidences of breast and testicular cancers in humans  
47  
48 74   may be caused by exposure to EDCs, especially via drinking water (Carlsen et al., 1995; Safe,  
49  
50 75   2005). For these reasons, it is important to note that surface waters are often used as a source  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

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

82 Textile industry effluents contain high concentrations of organic and inorganic compounds  
83 (Laing, 1991). Considering both the volume and the composition of the effluents, the textile  
84 industry is rated as the most polluting among all industrial sectors. Textile effluents are one of  
85 the most difficult-to-treat wastewaters due to their considerable suspended solids content and  
86 their high concentrations of dyes, salts, additives, detergents and surfactants. The presence of  
87 EDCs in textile effluents has been demonstrated (Pothitou and Voutsas, 2008; Prigione et al.,  
88 2008; Shannon et al., 2008). The physical-chemical technologies used for wastewater  
89 treatment, including advanced oxidation processes (i.e., the application of ozone, hydrogen  
90 peroxide or ultraviolet radiation), are expensive, not always effective and often do not reduce  
91 the toxicity of the effluents (Tehrani-Bagha et al., 2010; Anastasi et al., 2011).

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

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

101 environments (Campbell et al., 2006). Considering the large number of EDCs that may be  
102 present in a complex environmental sample, target chemical analyses are not sufficient to  
103 comprehensively define its EDC content. Furthermore, the total biological effects of the  
104 whole sample cannot be determined in these analyses (Nelson et al., 2007; Bicchi et al.,  
105 2009). In contrast, several *in vitro* bioassays based on the interaction between the EDCs and  
106 estrogenic receptors can be used to determine the total estrogenic EDC activity of an  
107 environmental sample. These assays allow the evaluation of additive, synergistic and  
108 antagonistic effects (Balaguer et al., 1999; Korner et al., 1999; Leusch et al., 2010).  
109 Moreover, these evaluations can be made more comprehensive with the parallel use or  
110 combination of two or more tests (Leusch et al., 2010).

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

## 121 2. Materials and Methods

### 122 2.1 Characteristics and sampling of industrial wastewater treatment plant (IWWTP) 123 effluents.

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

126 3,000 m<sup>3</sup>/day, mainly released by cotton dyeing processes. The dyes are synthetic organic  
127 indanthrenes, derived from anthraquinones, that are particularly stable in response to light,  
128 weathering and the detergents/disinfectants used for dyeing various fibres. The plant has  
129 separate water and sludge treatment lines; the former includes primary (chemico-physical),  
130 secondary (activated sludge) and tertiary processes (ozonation). Twenty-four hour composite  
131 samples of the final IWWTP effluents were sampled after the secondary treatment (pre-O<sub>3</sub>)  
132 and after the tertiary treatment (post-O<sub>3</sub>) to evaluate the effects of ozonation on estrogenicity.  
133 Table 1 describes the physico-chemical characteristics of the effluent samples and the relative  
134 Italian threshold limit values (Decree Italian Law 4/2008). The ozonation treatment was  
135 carried out in columns (0.2 m in diameter and 4.5 m in height) by adding in countercurrent  
136 (effluent flow rate of 60 L/hr) 30 ppm of ozone. Samples were taken on 7 different sampling  
137 dates from November 2009 to July 2010. The samples (2 L) were stored in brown glass flasks  
138 at 4°C. Two-litre aliquots were extracted for the *E-screen* test and the MELN gene-reporter  
139 luciferase assay.

## 2.2 Extraction of effluent samples

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

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

### 153 *2.3 Sample preparation for bioassays*

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

### 161 *2.4 Cell lines and culture conditions*

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

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

176 compounds (Hernandez-Raquet et al., 2007). MELN cells were cultured in Dulbecco's  
177 Modified Eagle Medium Nutrient Mixture F12 Ham (DMEM-F12) with phenol red,  
178 supplemented with 5% foetal calf serum (FCS), 2% 200 mM L-glutamine, 1%  
179 penicillin/streptomycin and 1 mg/ml G418 sulphate. The cells were maintained in an  
180 incubator at 37°C, a relative humidity of 95% and a CO<sub>2</sub> concentration of 5%. The cells were  
181 subcultured once per week, and the medium was refreshed between passages. Cells from  
182 passage number 4 to passage number 15 were used for experiments. Cells were regularly  
183 checked for mycoplasma infection to guarantee that the experiments were performed with  
184 mycoplasma-free cells and to comply with the guidelines for good cell culture practice  
185 (GCCP). Unless otherwise specified, all chemicals and materials for cell culture were  
186 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



200 of samples). The assays were completed after six days by determining the absorbance (595  
 1  
 2 201 nm) in each well after crystal violet staining.

3  
 4 202 The proliferative effect (PE) of a sample is the ratio between the highest cell number achieved  
 5  
 6  
 7 203 with the sample or E2 and the cell number of the negative control:

8  
 9  
 10 204  $(1) PE = (\text{max cell number})_{\text{sample}} / (\text{cell number})_{\text{negative control}}$ .

11  
 12 205 The estrogenic activity of a sample is evaluated by determining the relative efficacy, called  
 13  
 14 206 the relative PE (RPE%). The RPE compares the maximum proliferation induced by a sample  
 15  
 16  
 17 207 with that induced by E2:

18  
 19 208  $(2) RPE \% = [(PE-1)_{\text{sample}} / (PE-1)_{\text{E2}}] \times 100$ .

20  
 21  
 22 209 Full agonistic activity,  $RPE \geq 100\%$ , can be distinguished from partial agonistic activity, in  
 23  
 24 210 which RPE is less than 100% (Soto, 1995).

25  
 26  
 27 211 Relative potency, called estradiol equivalency quantity (EEQ) is thus calculated as:

28  
 29 212  $(3) EEQ = (EC50)_{\text{E2}} / (EC50)_{\text{sample}}$ .

30  
 31 213 The EC50 value for the *E-screen* test (concentration at which 50% of PE is achieved) was  
 32  
 33  
 34 214 calculated with a probit regression (SPSS, Chicago, IL). The PE and EC50 values of each  
 35  
 36 215 sample were calculated from the mean dose–response curves established from each  
 37  
 38  
 39 216 experiment. The EEQ, expressed in ng/L, is defined as the total concentration of estrogenic  
 40  
 41 217 active compounds in an environmental sample normalised to the natural estrogen 17- $\beta$ -  
 42  
 43  
 44 218 estradiol.

#### 45 46 219 *2.6 MELN gene-reporter luciferase assay.*

47  
 48  
 49 220 MELN gene- reporter luciferase assay has been widely used for the detection of estrogenic  
 50  
 51 221 activity in complex environmental samples (Cargouët et al., 2007; Combalbert et al., 2012;  
 52  
 53  
 54 222 Fenet et al., 2003; Hernandez-Raquet et al., 2007; Jugan et al., 2009; Kinani et al., 2010;).  
 55  
 56 223 The test was carried out as described by Balaguer et al. (1999).  
 57  
 58  
 59  
 60  
 61  
 62  
 63  
 64  
 65

224 Because phenol red and FCS exhibit estrogenic activity, *in vitro* experiments were carried out  
1  
2 225 in DMEM F12 without phenol red, supplemented with 5% dextran-coated charcoal-treated  
3  
4 226 foetal calf serum (DCC-FCS), 2% L-glutamine 200 mM and 1% antibiotics  
5  
6  
7 227 (penicillin/streptomycin). To adapt the cells to DCC-FCS, the growth medium was replaced  
8  
9 228 with fresh test medium 3 days prior to the experiment. Cells were then harvested and seeded  
10  
11 229 in 96-well plates with a flat, clear bottom (Corning) at a density of 40000 cells/well in 100  $\mu$ l  
12  
13  
14 230 of DCC-FCS per well. After 24 hours, the test medium was removed, and 100  $\mu$ l of each  
15  
16  
17 231 sample dilution was added to three replica wells. Cells were treated with samples for 20 h.  
18  
19 232 Negative controls, without hormones, and positive controls, E2 in concentration between 1  
20  
21  
22 233 pM and 10 nM, were included in each assay. One dilution (100-fold) of each effluent sample  
23  
24 234 was tested together with 5  $\mu$ M antiestrogen tamoxifen (Tam) and with 0.1 nM E2. Each  
25  
26 235 *MELN gene-reporter luciferase assay* was also performed on a QC laboratory sample (treated  
27  
28  
29 236 with the same method of samples). All experiments were performed in triplicate.

### 31 237 *2.6.1 Luciferase activity measurement in MELN cells.*

32  
33  
34 238 We used the One-Glo Luciferase Assay System (Promega, USA) to measure luciferase  
35  
36 239 activity according to manufacturer's instructions. Briefly, at the end of the incubation, 100  $\mu$ l  
37  
38  
39 240 of One-Glo Reagent (containing fluoroluciferin) was added to each well and mixed for  
40  
41 241 optimal consistency. After at least 3 minutes, to allow complete cell lysis, and within 30  
42  
43  
44 242 minutes of reagent addition, luminescence was measured by a luminometer (Tecan, Infinite  
45  
46 243 M200 PRO). The luciferase activity of the MELN cells relative to the positive control E2 was  
47  
48  
49 244 represented as transactivation % (TRANS %): the maximum increase in luciferase gene  
50  
51 245 expression triggered by estrogenic compounds present in the samples. The induction of  
52  
53  
54 246 luciferase activity was expressed as a percentage, and the 100% value was obtained in the  
55  
56 247 presence of E2 (Balaguer et al., 1999; Fenet et al., 2003). The estrogenic activity was  
57  
58  
59  
60  
61  
62  
63  
64  
65

248 expressed as 17-beta estradiol equivalent estrogenic activity (EEQ) in ng/L. EEQ was thus  
1  
2 249 calculated as  $(EC50)E2/(EC50)_{sample}$ .

### 250 *2.6.2 Cytotoxicity assessment in MELN cells.*

251 The Multitox-Fluor Multiplex Cytotoxicity Assay (Promega, cat. no. 9200) is a fluorescence-  
8  
9 252 based assay that use membrane integrity changes to measure cell viability or cytotoxicity in  
10  
11 253 conjunction with luciferase measurements on the same test plates (Berckmans et al., 2007).  
12  
13 254 This assay technology simultaneously measures two distinct protease activities, with rapid  
14  
15 255 catalytic cleavage rates, as markers of cell viability or cytotoxicity.  
16  
17

18  
19 256 The cytotoxicity test was applied to dilutions 1:10 and 1:100 (equal to 5 L and 0.5 L dilution  
20  
21 257 volumes) of the pre- and post-ozonation effluents.  
22  
23

24 258 At the end of incubation, 100  $\mu$ l aliquots of reagent were added to all wells, mixed and  
25  
26 259 allowed to incubate for at least 30 minutes at 37°C. Fluorescence was determined at  
27  
28 260 excitation/emission wavelengths of 360 nm/460 nm for cell viability and 485 nm/528 nm for  
29  
30 261 cytotoxicity using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT).  
31  
32

33  
34 262 The mean values and standard deviations of replicate wells and the mean fold increase of  
35  
36 263 cytotoxicity relative to the negative control were calculated.  
37  
38

### 39 264 *2.7 Statistical analyses.*

40  
41 265 Data were analysed by means of a probit regression analysis (EC50), Spearman's test  
42  
43 266 (correlation) and t-test (means comparison) using SPSS 18.0 (SPSS for Windows, Chicago,  
44  
45 267 IL, USA).  
46  
47

48 268

## 50 51 269 **3. Results and Discussion**

### 52 53 270 *3.1 Estrogenic activity in IWWTP effluent samples as measured by E-screen test.*

54  
55  
56 271 The mean EC50 value of E2 for the *E-screen* was  $15.24 \pm 13.73$  ng/L ( $56.44 \pm 50.83$  pM);  
57  
58 272 EC50 values were calculated from the control curves obtained from each of the bioassays.  
59  
60  
61  
62  
63  
64  
65

273 Maximum cell proliferation was generally induced by 0.1 nM E2. The detection limit of the  
1  
2 274 *E-screen* could be defined as the concentration of a single compound or a sample inducing a  
3  
4  
5 275 cell proliferation significantly higher than the hormone-free negative control; the treatment  
6  
7 276 with 17beta-estradiol showed a detection limit of 1 pM (Körner et al. 1999; Schilirò et al.,  
8  
9  
10 277 2004). The proliferative response of the samples was dose dependent; the 1:100 dilution of  
11  
12 278 the samples (equal to a 0.5 L dilution volume) always stimulated the maximum proliferative  
13  
14 279 response. The QC laboratory sample did not induce any significant cell proliferation. To  
15  
16  
17 280 verify that the cell proliferation induced by estrogenic activity was mediated by ER, we  
18  
19 281 observed that co-incubation with Tam inhibited the proliferative response ( $28 \pm 23 \%$ ), while  
20  
21  
22 282 co-incubation with E2 led to a greater proliferative response ( $114 \pm 39 \%$ ). The difference was  
23  
24 283 statistically significant in both cases (t-tests,  $p < 0.05$ ).

25  
26  
27 284 Estrogenic activity, expressed as EEQ, was detected in all but the sixth sample and ranged  
28  
29 285 from 0.10 to 4.73 ng/L (Table 2). The proliferative effect of the different effluent samples on  
30  
31  
32 286 MCF-7 BUS cells relative to the positive control E2 is represented as RPE % in Table 2. The  
33  
34 287 RPE of the effluent both before and after ozonation generally showed partial agonist activity  
35  
36 288 (RPE < 100%) ranging between 15 to 90 %. The EEQs of the effluent extracts, determined in  
37  
38  
39 289 the *E-screen* test, are reported in Figure 1.

40  
41  
42 290 The highest mean EEQ was found in the second pre-ozonation sample ( $4.73 \pm 2.42$  ng/L), and  
43  
44 291 the lowest mean EEQ was found in the last post-ozonation sample ( $0.10 \pm 0.13$  ng/L). The  
45  
46  
47 292 mean EEQ values were  $2.35 \pm 1.68$  ng/L pre-ozonation and  $0.72 \pm 0.58$  ng/L post-ozonation.

48  
49 293 The average reduction in the estrogenic activity of IWWTP effluent from pre- to post-  
50  
51  
52 294 ozonation was  $67 \pm 26 \%$ . Despite the low number of analysed samples, the difference  
53  
54 295 between EEQ values before and after ozonation was statistically significant (simple paired t-  
55  
56  
57 296 test,  $p = 0.038$ ), suggesting that the ozonation process significantly reduced the estrogenic  
58  
59 297 activity of the IWWTP effluent.  
60  
61  
62  
63  
64  
65

298 The *in vitro E-screen* test can be used to screen a large number of compounds and integrate  
1  
2 299 the effects of chemicals that may not be measured in an analytical screen (Vega-Lopez, 2007).  
3  
4 300 It has been studied extensively, and no false positives or false negatives have yet been found;  
5  
6  
7 301 it is also one of the most sensitive of all published assays (Soto et al., 2006; Vanparys et al.,  
8  
9 302 2010). The *E-screen* has been shown to be appropriate for the determination of estrogenic  
10  
11 303 activity in environmental extracts from IWWTP effluent samples.

### 14 304 *3.2 Estrogenic activity in IWWTP effluent samples by MELN gene-reporter luciferase* 15 16 305 *assay*

17 306 The EC50 values of E2 in the MELN gene-reporter luciferase assay were calculated from  
18  
19 307 control curves obtained from each bioassay, and the mean EC50 value was  $1.76 \pm 0.87$  ng/L  
20  
21  
22 308 ( $6.52 \pm 3.22$  pM). Maximum luciferase activity was generally induced by 0.1 nM E2. The  
23  
24 309 stable transfectants MELN used in this study exhibited good responsiveness following  
25  
26  
27 310 treatment to 17beta-estradiol with a detection limit of 1 pM (Balaguer et al., 1999). The  
28  
29 311 luciferase activity of the samples was dose dependent, and the 1:100 dilution (equal to a 0.5 L  
30  
31  
32 312 dilution volume) always stimulated the maximum luciferase activity. The QC laboratory  
33  
34 313 sample did not induce any significant luciferase activity. To confirm that the luciferase  
35  
36 314 activity was mediated by the ER, we observed that co-incubation with Tam led to an  
37  
38  
39 315 inhibition of the proliferative response ( $51 \pm 29$  %), while co-incubation with E2 led to a  
40  
41  
42 316 greater proliferative response ( $113 \pm 34$  %). The difference was statistically significant in both  
43  
44  
45 317 cases (t-tests,  $p < 0.05$ ). Figure 2 shows the MELN luciferase activity of E2 and of the post-  
46  
47 318 ozonation effluents expressed in dose-response curves and compared with the negative  
48  
49  
50 319 control.

51  
52 320 Estrogenic activity was detected in all but the sixth sample and ranged from 0.14 to 8.50 EEQ  
53  
54  
55 321 (ng/L) (Table 2). The luciferase activity of the different effluent samples in the MELN cells  
56  
57  
58 322 relative to the positive control E2 is represented as TRANS % in Table 2. The TRANS % of  
59  
60  
61  
62  
63  
64  
65

323 the effluent before and after ozonation ranged between 27 to 62 %. The EEQs of the effluent  
1  
2 324 extracts, determined in the MELN gene-reporter luciferase assay, are reported in Figure 1.

3  
4  
5 325 The highest mean EEQ was found in the first pre-ozonation sample ( $8.50 \pm 4.36$  ng/L), and  
6  
7 326 the lowest mean EEQ was found in the second post-ozonation sample ( $0.14 \pm 0.05$  ng/L). A  
8  
9  
10 327 lower mean value was found for the effluents post-ozonation: the mean EEQ values were  $4.18$   
11  
12 328  $\pm 3.54$  ng/L pre-ozonation and  $2.53 \pm 2.48$  ng/L post-ozonation; this difference was  
13  
14  
15 329 statistically significant (simple paired t-test,  $p = 0.048$ ). The average reduction of estrogenic  
16  
17 330 activity of IWWTP effluent after ozonation was  $52 \pm 27$  %.

18  
19  
20 331 The *in vitro* MELN gene-reporter luciferase assay can rapidly screen a large number of  
21  
22 332 chemicals, and it is transferable, robust and reproducible, enabling the ranking of chemical  
23  
24  
25 333 compounds from strong to weak affinity for the estrogen receptor (Witters et al, 2010). This  
26  
27 334 assay has been shown to be appropriate for the determination of estrogenicity in  
28  
29  
30 335 environmental extracts from IWWTP effluent samples.

### 31 32 336 *3.3 Cytotoxicity of IWWTP effluent samples in MELN cells.*

33  
34 337 The 1:10 dilution of the effluent pre-and post-ozonation was shown to decrease cell  
35  
36  
37 338 proliferation compared to negative controls, in terms of PE, in both assays. The sample, which  
38  
39  
40 339 is less diluted, might be able to mask the real estrogenic activity of the compounds present in  
41  
42 340 it. To determine whether the effluent might have a toxic effect on the cells, 1:10 and 1:100  
43  
44 341 dilutions (equal to 5 L and 0.5 L dilution volume) were tested for cytotoxicity (Table 3).

45  
46  
47 342 The 1:10 mean fold increase of cytotoxicity relative to the negative control was  $50.3 \pm 30.0$   
48  
49  
50 343 for pre-ozonation effluents and  $6.3 \pm 6.0$  for post-ozonation effluents (t-tests,  $p < 0.01$  and  $p <$   
51  
52 344  $0.05$ , respectively). The mean fold increase of cytotoxicity produced by the 1:100 dilution  
53  
54 345 compared to the negative control was  $1.2 \pm 0.8$  for the pre-ozonation effluent and  $1.0 \pm 1.1$  for  
55  
56  
57 346 the post-ozonation effluent (t-tests,  $p > 0.05$ ). The sample without estrogenic activity ( $n^{\circ}6$ ) did  
58  
59  
60  
61  
62  
63  
64  
65

347 not exhibit a significantly higher cytotoxicity; therefore, the absence of estrogenicity can not  
1  
2 348 be ascribed to the toxicity of the sample.  
3

### 4 349 *3.4 Comparison of estrogenic activity in the E-screen test and in the MELN gene-* 5 6 7 350 *reporter luciferase assay* 8

9 351 A comparison of the results obtained in these two assays highlights the non-significance of  
10  
11 352 differences in average EEQs (t-test,  $p > 0.05$ ). The correlation between the EEQs obtained  
12  
13 353 with the MELN gene-reporter luciferase assay and with the *E-screen* was positive and  
14  
15 354 significant (Rho S = 0.650 and  $p = 0.022$ ). The mean EC50 values relative to E2 were  $15.24 \pm$   
16  
17 355  $13.73$  ng/L for the *E-screen* and  $1.76 \pm 0.87$  ng/L for the MELN gene-reporter luciferase  
18  
19 356 assay; these values were significantly different (t-test,  $p = 0.015$ ). The MELN gene-reporter  
20  
21 357 luciferase assay generally showed higher EEQ mean values.  
22  
23  
24

25 358 These differences could be partially explained by the end-points of the two tests: the *E-screen*  
26  
27 359 is based on a binding mechanism that causes proliferation as a cellular response, which could  
28  
29 360 be affected by other external factors, while the MELN assay is receptor specific. Another  
30  
31 361 important difference between the two tests is in the assay duration; stimulation with the test  
32  
33 362 compounds lasts for 16-20 hours in the MELN assay but 120 hours for the *E-screen* (Soto et  
34  
35 363 al., 2006; Witters et al, 2010).  
36  
37  
38  
39  
40

41 364

## 44 365 **4. Conclusions**

45 366 In general, the *E-screen* test and the MELN gene-reporter luciferase assay are well suited for  
46  
47 367 the determination of estrogenic activity in environmental matrices; in this specific study, they  
48  
49 368 were used to evaluate estrogenic activity in wastewaters from a textile factory, before and  
50  
51 369 after treatment with ozone. The two *in vitro* tests are particularly suitable for environmental  
52  
53 370 monitoring because of their sensitivity, speed, reading and low cost. The estrogenicity values  
54  
55 371 in the final effluent (post O<sub>3</sub>) in the present work were similar to results reported in the  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

372 literature for other IWWTP effluents (Korner et al., 1999; Onda et al., 2002; Vethaak et al.,  
1  
2 373 2005; Tan et al., 2007; Salste et al., 2007). Our study suggests that the input of estrogenic  
3  
4 374 substances into the river via IWWTP is low (EEQ range: 0.72 – 2.53 ng/L) and that the  
5  
6  
7 375 environmental risk could be even lower due to the dilution effect connected with discharge  
8  
9  
10 376 and the self-depuration capacity of the receiving river. In a previous study, this reduction was  
11  
12 377 approximately one order of magnitude for EEQs (Schilirò et al., 2009), resulting in  
13  
14 378 concentrations lower than those at which these compounds have been reported to chronically  
15  
16  
17 379 affect the endocrine systems of living organisms (approximately 1-10 ng/L; Lopez de Alda &  
18  
19 380 Barcelo, 2001). The analysed effluent shows lower EEQs values relative to a municipal  
20  
21  
22 381 effluent (Fenet et al., 2003; Pothitou and Voutsas, 2008; Pereira et al., 2011). This difference  
23  
24 382 in the estrogenic activity of urban and industrial wastewater is certainly attributable to the  
25  
26 383 presence of different types of substances; municipal wastewater contains more natural EDCs,  
27  
28  
29 384 such as E2 and EE2, that have strong estrogenic power even at very low concentrations, while  
30  
31  
32 385 industrial wastewater typically contains more synthetic EDCs that are weakly estrogenic  
33  
34 386 relative to natural EDCs (Soto et al., 1995; Pereira et al., 2011).

36 387 Ozonation is considered an economically feasible option for the advanced treatment of  
37  
38  
39 388 WWTP effluents (Joss et al., 2008). It is a very effective method for the degradation of  
40  
41 389 persistent organic dyes from colored textile wastewater (Tehrani-Bagha et al., 2010). In  
42  
43  
44 390 general, ozonation and other oxidation processes seem to be the best alternatives for treating  
45  
46 391 WWTP effluents containing estrogens (Pereira et al., 2011). Some authors have noted that the  
47  
48  
49 392 decrease in EDCs may be due to the oxidation effects of ozonation (Lee, 2004; Larcher et al.,  
50  
51 393 2012). To our knowledge, few reports in the literature describe estrogenic activity in textile  
52  
53 394 IWWTP. This work shows that this type of effluent does have estrogenic activity and that  
54  
55  
56 395 ozonation decreases its overall estrogenic effects. However, ozonation is known to lead to the  
57  
58 396 formation of transformation products that have largely not been identified to date, and there  
59  
60  
61  
62  
63  
64  
65



397 are concerns about their potential impact on the environment and human health (Benner and  
1  
2 398 Ternes, 2009; Radjenovic et al., 2009; Dodd et al., 2010; Stalter et al., 2010, 2011; Anastasi et  
3  
4 399 al., 2011; Reungoat et al., 2012). This highlights the need to evaluate the presence of  
5  
6  
7 400 disinfection by-products after water treatment has been performed.

8  
9 401 The identification and removal of EDC pollutants from IWWTP effluents may solve many of  
10  
11 402 the apparent endocrine disruption problems observed in aquatic environments, in addition to  
12  
13 403 providing a cleaner source of drinking water. Further investigations should be carried out to  
14  
15 404 identify the level of ozone appropriate to minimise both the estrogenic activity and the  
16  
17 405 toxicity of IWWTP textile effluents.  
18  
19  
20

21 406

## 22 407 **5. Acknowledgments**

23  
24  
25  
26 408 This study was financed by a Research Projects of National Interest grant (PRIN 2008). We  
27  
28 409 wish to give special thanks to Felli Color Spa of Martinengo (BG) for providing the samples,  
29  
30 410 the chemical analysis and the information about the ozonation plant. The authors also thank  
31  
32 411 Drs. E. Aldieri, E. Gazzano and C. Costamagna of the Department of Genetics, Biology and  
33  
34 412 Chemistry of the University of Torino for the use of their fluorescence microplate reader and  
35  
36 413 Dr. Ivana Cirello for her helping.  
37  
38  
39  
40

41 414

## 42 415 **6. References**

43  
44  
45 416 Anastasi A, Parato B, Spina F, Tigini V, Prigione V, Varese GC. Decolourisation and  
46  
47 417 detoxification in the fungal treatment of textile wastewaters from dyeing processes. N  
48  
49 418 Biotechnol 2011;29(1):38-45.  
50

51  
52  
53  
54 419 Balaguer P, François F, Comunale F, Fenet H, Boussioux AM, Pons M, Nicolas JC, Casellas  
55  
56 420 C. Reporter cell lines to study the estrogenic effects of xenoestrogens. Sci Total Environ  
57  
58 421 1999; 233:47-56.  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4  
5  
6 422 Benner J, Ternes TA. Ozonation of metoprolol: elucidation of oxidation pathways and major  
7  
8 423 oxidation products. *Environ Sci Technol* 2009;43(14):5472-5480.  
9  
10  
11 424 Berckmans P, Leppens H, Vangenechten C, Witters H. Screening of endocrine disrupting  
12  
13 425 chemicals with MELN cells, an ER-transactivation assay combined with cytotoxicity  
14  
15 426 assessment. *Toxicol In Vitro* 2007;21(7):1262-1267.  
16  
17  
18 427 Bicchi C, Schilirò T, Pignata C, Fea E, Cordero C, Canale F, Gilli G. Analysis of  
19  
20 428 environmental endocrine disrupting chemicals using the *E-screen* method and stir bar sorptive  
21  
22 429 extraction in wastewater treatment plant effluents. *Sci Total Env* 2009;407:1842-1851.  
23  
24  
25 430 Bila D, Montalva AF, Azevedo D, Dezotti M. Estrogenic activity removal of 17 $\beta$ -estradiol by  
26  
27 431 ozonation and identification of by-products. *Chemosphere* 2007;69(5): 736-746.  
28  
29 432 Boyd GR, Reemtsma H, Grimm DA, Mitra S. Pharmaceuticals and personal care products  
30  
31 433 (PPCPs) in surface and treated waters of Louisiana, USA and Ontario, Canada. *Sci Total*  
32  
33 434 *Environ* 2003;311:135–149.  
34  
35  
36  
37 435 Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O. Molecular basis of  
38  
39 436 agonism and antagonism in the estrogen receptor. *Nature* 1997;16(389):753–758.  
40  
41  
42  
43 437 Campbell CG, Borglin SE, Green FB, Grayson A, Wozel E, Stringfellow WT. Biologically  
44  
45 438 directed environmental monitoring, fate, and transport of estrogenic endocrine disrupting  
46  
47 439 compounds in water: A review. *Chemosphere* 2006;65(8):1265-1280.  
48  
49  
50  
51  
52 440 Cargouët M, Perdiz D, Lévi Y. Evaluation of the estrogenic potential of river and treated  
53  
54 441 waters in the Paris area (France) using in vivo and in vitro assays. *Ecotox Environ Safe*  
55  
56 442 2007;67:149-156.  
57  
58  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- 443 Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Declining semen quality and  
444 increasing incidence of testicular cancer, Is there a common cause? Environ Health Persp  
445 1995;103(suppl 7):137–139.
- 446 Colborn T, vom Saal FS, Soto AM. Developmental effects of endocrine-disrupting chemicals  
447 in wildlife and humans. Environ Health Persp 1993;101:378–384.
- 448 Combalbert S, Bellet V, Dabert P, Bernet N, Balaguer P, Hernandez-Raquet G. Fate of steroid  
449 hormones and endocrine activities in swine manure disposal and treatment facilities. Water  
450 Res 2012;46:895-906.
- 451 Crews D, Willingham E, Skipper JK. Endocrine disruptors, present issues, future directions.  
452 Quart Rev Biol 2000;75:243–260.
- 453 Deborde M, Rabouan S, Gallard H, Legube B. Aqueous Chlorination Kinetics of Some  
454 Endocrine Disruptors. Env Sci Technol 2004;38:5577-5583.
- 455 Decree Italian Law 152/2006, Decreto Legislativo 3 aprile 2006, n. 152 “Testo Unico  
456 Ambientale - Norme in materia ambientale”.
- 457 Decree Italian Law 4/2008, Decreto Legislativo 16 gennaio 2008, n. 4, “Ulteriori disposizioni  
458 correttive ed integrative” del Decreto Legislativo 3 aprile 2006, n. 152 “Testo Unico  
459 Ambientale - Norme in materia ambientale”.
- 460 Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000  
461 establishing a framework for Community action in the field of water policy.
- 462 Dodd MC, Rentsch D, Singer HP, Kohler HPE, von Gunten U. Transformation of beta-  
463 Lactam antibacterial agents during aqueous ozonation: reaction pathways and quantitative

1 464 bioassay of biologically-active oxidation products. Environ Sci Technol 2010; 44(15):5940-  
2 465 5948.  
3

4  
5  
6 466 European Commission Report, Stockholm Convention. Identification of priority hazardous  
7  
8 467 substances. Brussels, Jan. 16, Adonis n° 901019, 2001.  
9

10  
11  
12 468 Fenet H, Gomez E, Pillon A, Rosain D, Nicolas JC, Casellas C, Balaguer P. Estrogenic  
13  
14 469 activity in water and sediments of a french river: contribution of alkylphenols. Environ  
15  
16  
17 470 Contam Toxicol 2003;44:1-6.  
18

19  
20  
21 471 Hernandez-Raquet G, Soef A, Delgenès N, Balaguer P. Removal of the endocrine disrupter  
22  
23 472 nonylphenol and its estrogenic activity in sludge treatment processes. Water Res  
24  
25 473 2007;41:2643-2651.  
26

27  
28  
29 474 Irmak S, Erbatur O, Akgerman A. Degradation of 17 $\beta$ -estradiol and bisphenol A in aqueous  
30  
31 475 medium by using ozone and ozone/UV techniques. J Hazard Mater 2005;126:54–62.  
32

33  
34  
35 476 Itoh S, Yoshimura Y, Okada T. Detection of estrogenic effect formation potential in  
36  
37  
38 477 chlorinated drinking water. In: 2nd IWA Leading Edge Conference on Water and Wastewater  
39  
40 478 Treatment Technologies. 2004;60-62.IWA, Prague, Czech Republic,  
41

42  
43  
44 479 Joss A, Siegrist H, Ternes TA. Are we about to upgrade wastewater treatment for removing  
45  
46 480 organic micropollutants? Water Sci Technol2008;57 (2):251-255.  
47

48  
49  
50 481 Jugan ML, Oziol L, Bimbot M, Huteau V, Tamisier-Karolak S, Blondeau JP, Lévi Y. In vitro  
51  
52 482 assessment of thyroid and estrogenic endocrine disruptors in wastewater treatment plants,  
53  
54  
55 483 rivers and drinking water supplies in the greater Paris area (France). Sci Total Environ  
56  
57 484 2009;407:3579-3587.  
58

59  
60  
61  
62  
63  
64  
65

- 485 Kinani S, Bouchonnet S, Creusot N, Bourcier S, Balaguer P, Porcher JM, Aït-Aïssa S.  
1  
2 486 Bioanalytical characterisation of multiple endocrine- and dioxin-like activities in sediments  
3  
4 487 from reference and impacted small rivers. *Environ Pollut* 2010;158:74-83.  
5  
6  
7  
8 488 Kinnberg K. Evaluation of in vitro assays for determination of estrogenic activity in the  
9  
10 489 environment. Danish Environmental Protection Agency, Environmental project No. 899,  
11  
12 Working Report No. 43, 2003. København, Denmark  
13 490  
14  
15  
16 491 Korner W, Hanf V, Schuller W, Kempster C, Mzger J, Hagenmaier H. Development of a  
17  
18 492 sensitive E-screen assay for quantitative analysis of estrogenic activity in municipal sewage  
19  
20  
21 493 plant effluents. *Sci Total Environ* 1999;225:33-48.  
22  
23  
24  
25 494 Laing IG. The impact of effluent regulations on the dyeing industry, *Rev Prog Coloration*  
26  
27 495 1991;21:56-71.  
28  
29  
30  
31 496 Larcher S, Delbès G, Robaire B, Yargeau V. Degradation of 17 $\alpha$ -ethinylestradiol by  
32  
33 497 ozonation--identification of the by-products and assessment of their estrogenicity and toxicity.  
34  
35 498 *Environ Int* 2012;39(1):66-72.  
36  
37  
38  
39  
40 499 Lee BC, Kamata M, Akatsuka Y, Takeda M, Ohno K, Kamei T, Magara Y. Effects of  
41  
42 500 chlorine on the decrease of estrogenic chemicals. *Water Res* 2004;38:733-739.  
43  
44  
45  
46 501 Leusch FD, de Jager C, Levi Y, Lim R, Puijker L, Sacher F, Tremblay LA, Wilson VS,  
47  
48 502 Chapman HF. Comparison of five in vitro bioassays to measure estrogenic activity in  
49  
50 503 environmental waters. *Environ Sci Technol* 2010;44(10):3853-60.  
51  
52  
53  
54 504 Liu Z, Kanjo Y, Mizutani S. Removal mechanisms for endocrine disrupting compounds  
55  
56 505 (EDCs) in wastewater treatment d physical means, biodegradation, and chemical advanced  
57  
58 506 oxidation: a review. *Sci Total Env* 2009;407:731-748.  
59  
60  
61  
62  
63  
64  
65

- 1  
2  
3  
4  
5  
6 507 Lopez de Alda MJ, Barcelò D. Review of analytical methods for the determination of  
7  
8  
9 508 estrogens and progestens in waste waters. *Fresenius J Anal Chem* 2001;371:437–447.  
10  
11  
12  
13  
14 509 Nelson J, Bishay F, van Roodselaar A, Ikonomou M, Law FCP. The use of in vitro bioassays  
15  
16  
17 510 to quantify endocrine disrupting chemicals in municipal wastewater treatment plant effluents.  
18  
19  
20  
21 511 *Sci Total Environ* 2007;374:80–90.  
22  
23  
24  
25  
26 512 Onda K, Yang SY, Miya A, Tanaka T. Evaluation of estrogen-like activity on sewage  
27  
28  
29 513 treatment processes using recombinant yeast. *Water Sci Technol* 2002; 46:367-373.  
30  
31  
32  
33  
34  
35 514 Orlando EF, Guillette LJ Jr. Sexual dimorphic responses in wildlife exposed to endocrine  
36  
37  
38 515 disrupting chemicals. *Environ Res* 2007;104(1):163-173.  
39  
40  
41  
42 516 Pereira RO, Postigo C, de Alda ML, Daniel LA, Barceló D. Removal of estrogens through  
43  
44  
45 517 water disinfection processes and formation of by-products. *Chemosphere* 2011;82(6):789-  
46  
47  
48 518 799.  
49  
50  
51  
52  
53 519 Pothitou P, Voutsas D. Endocrine disrupting compounds in municipal and industrial  
54  
55  
56 520 wastewater treatment plants in Northern Greece. *Chemosphere* 2008;3(11):1716-1723.  
57  
58  
59  
60  
61 521 Prigione V, Tigini V, Pezzella C, Anastasi A, Sannia G, Varese GC. Decolourization and  
62  
63  
64 522 detoxification of textile effluents by fungal biosorption. *Water Res* 2008;42:2911–2920.  
65  
66  
67  
68  
69 523 Purdom CE, Hardiman PA, Bye VJ, Eno NC, Tyler CR, Supter JP. Estrogenic effects of  
70  
71  
72 524 effluent from sewage treatment works. *J Chem Ecol* 1994;8:275–285.  
73  
74  
75  
76  
77 525 Radjenovic J, Godehardt M, Petrovic M, Hein A, Farre M, Jekel M, Barcelo D. Evidencing  
78  
79  
80 526 generation of persistent ozonation products of antibiotics roxithromycin and trimethoprim.  
81  
82  
83 527 *Environ Sci Technol* 2009;43(17):6808-6815.  
84  
85

- 1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65
- 528 Reungoat J, Escher BI, Macova M, Argaud FX, Gernjak W, Keller J. Ozonation and  
529 biological activated carbon filtration of wastewater treatment plant effluents. *Water Res* 2012;  
530 46(3):863-72.
- 531 Routledge EJ, Sheahan D, Desbrow C, Brighty GC, Waldock M, Sumpter JP. Identification of  
532 estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environ*  
533 *Toxicol Chem* 1998;32:1559–1565.
- 534 Safe S. Clinical correlates of environmental endocrine disruptors.’ *Trends Endocrin Met*  
535 2005;16(4):139-144.
- 536 Salste L, Leskinen P, Virta M, Kronberg L. Determination of estrogens and estrogenic  
537 activity in wastewater effluent by chemical analysis and the bioluminescent yeast assay. *Sci*  
538 *Total Environ* 2007;378:343–351.
- 539 Schilirò T, Pignata C, Fea E, Gilli G. Toxicity and estrogenic activity of a wastewater  
540 treatment plant in Northern Italy. *Arch Environ Contam Toxicol* 2004; 47: 456–462.
- 541 Schilirò T, Pignata C, Rovere R, Fea E, Gilli G. The endocrine disrupting activity of surface  
542 waters and of wastewater treatment plant effluents in relation to chlorination. *Chemosphere*  
543 2009;75(3):335-340.
- 544 Shannon MA, Bohn PW, Elimelech M, Georgiadis JG, Mariñas BJ, Mayes AM. Science and  
545 technology for water purification in the coming decades. *Nature* 2008;452:301–310.
- 546 Silva CP, Otero M, Esteves V. Processes for the elimination of estrogenic steroid hormones  
547 from water: A review. *Environ Poll* 2012;165:38-58.

1 548 Soto AM, Maffini MV, Schaeberle CM, Sonnenschein C. Strengths and weaknesses of in  
2 549 vitro assays for estrogenic and androgenic activity. *Best Pract Res Clin Endocrinol Metab*  
3  
4 550 2006; 20(1):15-33.

5  
6  
7  
8 551 Stalter D, Magdeburg A, Oehlmann J. Comparative toxicity assessment of ozone and  
9  
10 552 activated carbon treated sewage effluents using an in vivo test battery. *Water Research*  
11  
12 553 2010;44(8):2610-2620.

13  
14  
15  
16 554 Stalter D, Magdeburg A, Wagner M, Oehlmann J. Ozonation and activated carbon treatment  
17  
18 555 of sewage effluents: removal of endocrine activity and cytotoxicity. *Water Research* 2011;45  
19  
20 556 (3):1015-1024.

21  
22  
23  
24  
25 557 Sumpter JP. The ecotoxicology of hormonally active micropollutants. *Water Science and*  
26  
27 558 *Technology* 2008;57:125-130.

28  
29  
30  
31 559 Tan BLL, Hawker DW, Müller JF, Leusch FDL, Tremblay LA, Chapman HF.  
32  
33 560 Comprehensive study of endocrine disrupting compounds using grab and passive sampling at  
34  
35 561 selected wastewater treatment plants in South East Queensland, Australia. *Environ Int*  
36  
37 562 2007;33:654–669.

38  
39  
40  
41  
42 563 Tehrani-Bagha AR, Mahmoodi NM, Menger FM. Degradation of a persistent organic dye  
43  
44 564 from colored textile wastewater by ozonation. *Desalination* 2010; 260, 34–38.

45  
46  
47  
48 565 Vanparys C, Depiereux S, Nadzialek S, Robbens J, Blust R, Kestemont P, De Coen W.  
49  
50 566 Performance of the flow cytometric E-screen assay in screening estrogenicity of pure  
51  
52 567 compounds and environmental samples. *Sci Total Environ.* 2010;408(20):4451-60.

53  
54  
55  
56  
57 568 Vega-López A, Ramón-Gallegos E, Galar-Martínez M, Jiménez-Orozco FA, García-Latorre  
58  
59 569 E, Domínguez-López ML. Estrogenic, anti-estrogenic and cytotoxic effects elicited by water  
60  
61  
62  
63  
64  
65



570 from the type localities of the endangered fish *Girardinichthys viviparous*. *Comp Biochem*  
1  
2 571 *Phys C* 2007;143:394–403.  
3

4  
5  
6 572 Vethaak AD, Lahr J, Schrap SM, Belfroid AC, Rijs GB, Gerritsen A, de Boer J, Bulder AS,  
7  
8 573 Grinwis GC, Kuipe RV, Legler J, Murk TA, Peijnenburg W, Verhaar HJ, de Voogt P. An  
9  
10 574 integrated assessment of estrogenic contamination and biological effects in the aquatic  
11  
12 575 environment of The Netherlands. *Chemosphere* 2005;59(4):511-24.  
13  
14

15  
16  
17 576 Witters H, Freyberger A, Smits K, Vangenechten C, Lofink W, Weimer M, Bremer S, Ahr  
18  
19 577 PH, Berckmans P. The assessment of estrogenic or anti-estrogenic activity of chemicals by  
20  
21 578 the human stably transfected estrogen sensitive MELN cell line: results of test performance  
22  
23 579 and transferability. *Reprod Toxicol* 2010;30(1):60-72.  
24  
25

26  
27  
28 580 Ying G, Williams B, Kookana R. Environmental fate of alkylphenols and alkylphenol  
29  
30 581 ethoxylates—A review. *Environ Int* 2002;28:215–226.  
31  
32

33  
34 582

35  
36 583 **Figure captions**  
37

38  
39 584

40  
41 585 **Figure 1.** Estradiol equivalency quantity (EEQ ng/L) of the IWWTP effluent extracts after the  
42  
43 586 secondary treatment (Pre ozonation) and after the tertiary treatment (Post ozonation) by  
44  
45 587 means of *E-Screen* test and MELN gene-reporter luciferase assay. Boxes represent the median  
46  
47 588 and 25th – 75th percentiles, outer lines represent the 10th -90th percentiles.  
48  
49

50  
51 589

52  
53 590 **Figure 2.** MELN luciferase activity of 17-beta-estradiol (E2) and of post ozonation effluents  
54  
55 591 (examples: n° 5 dotted bars and n° 7 striped bars) expressed with dose-response curves and  
56  
57 592 compared with the negative control.  
58  
59  
60  
61  
62  
63  
64  
65

- 1 **Table 1.** Physico-chemical parameters of the effluents after secondary treatment (Pre-O<sub>3</sub>) and  
 2 after tertiary treatment (Post-O<sub>3</sub>) and Italian threshold limit values (nd: not detected).

| Parameters                 | Pre O <sub>3</sub>                   | Post O <sub>3</sub>     | 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 <sup>-</sup> (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 <sub>4</sub> (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                              |

3 \*Decree Italian Law 4/2008, reference values for discharge to surface waters.  
 4  
 5

- 6 **Table 2.** Mean estrogenic activity of the IWWTP effluent extracts after secondary treatment  
 7 (Pre-O<sub>3</sub>) and after tertiary treatment (Post-O<sub>3</sub>) in the *E-screen* test and MELN gene-reporter  
 8 luciferase assay (nd: not detected).

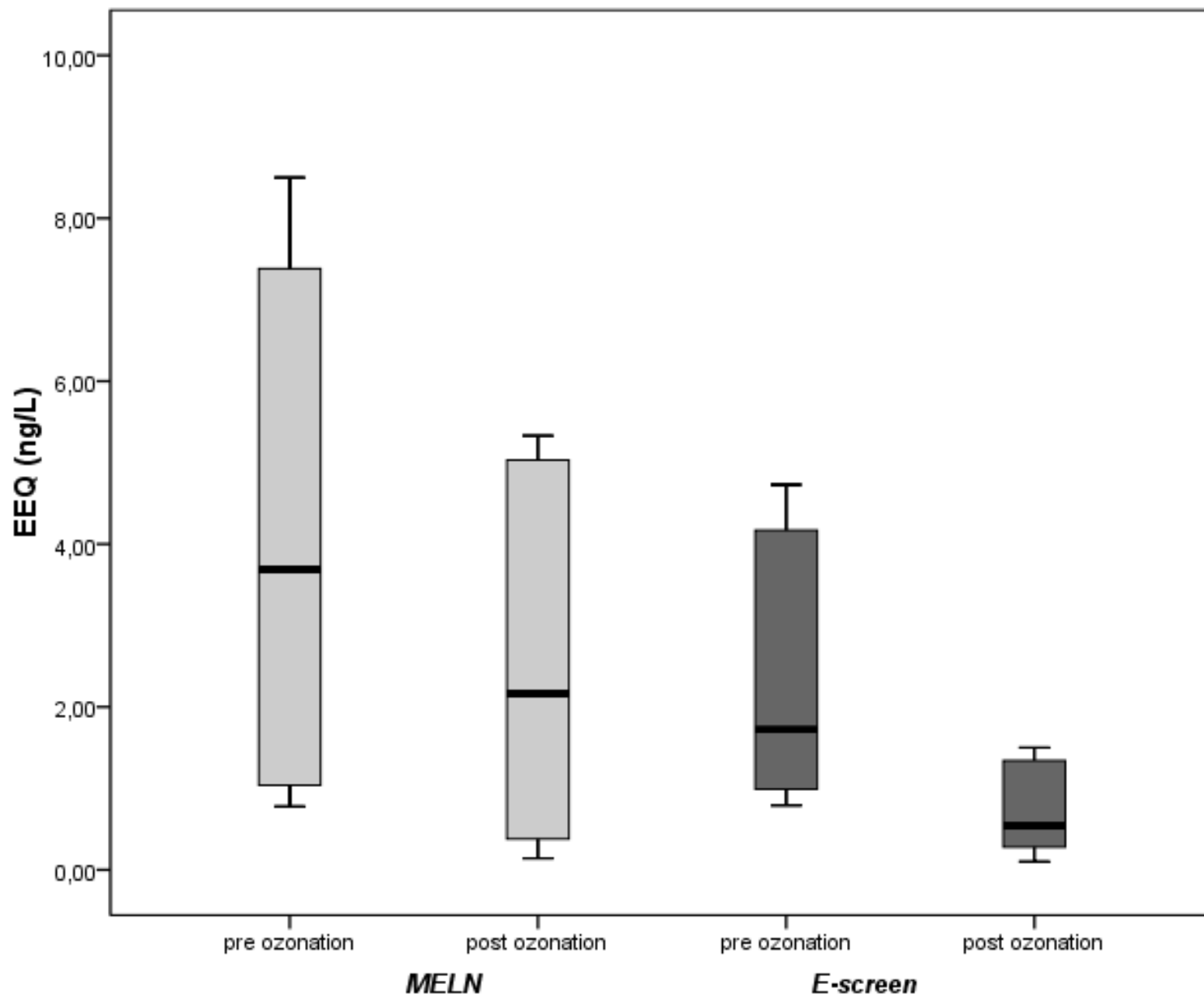
| N° sampling               | <i>E-screen</i> test<br>EEQ (ng/L) | <i>E-screen</i> test<br>RPE% | <i>MELN</i> assay<br>EEQ (ng/L) | <i>MELN</i> assay<br>TRANS % |
|---------------------------|------------------------------------|------------------------------|---------------------------------|------------------------------|
| <b>Pre O<sub>3</sub></b>  |                                    |                              |                                 |                              |
| 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                           |
| <b>Post O<sub>3</sub></b> |                                    |                              |                                 |                              |
| 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                           |

10 **Table 3.** MELN cytotoxicity and cell viability of the IWWTP effluent extracts (1:10 and  
 11 1:100 dilutions) after secondary treatment (Pre-O<sub>3</sub>) and after tertiary treatment (Post-O<sub>3</sub>).

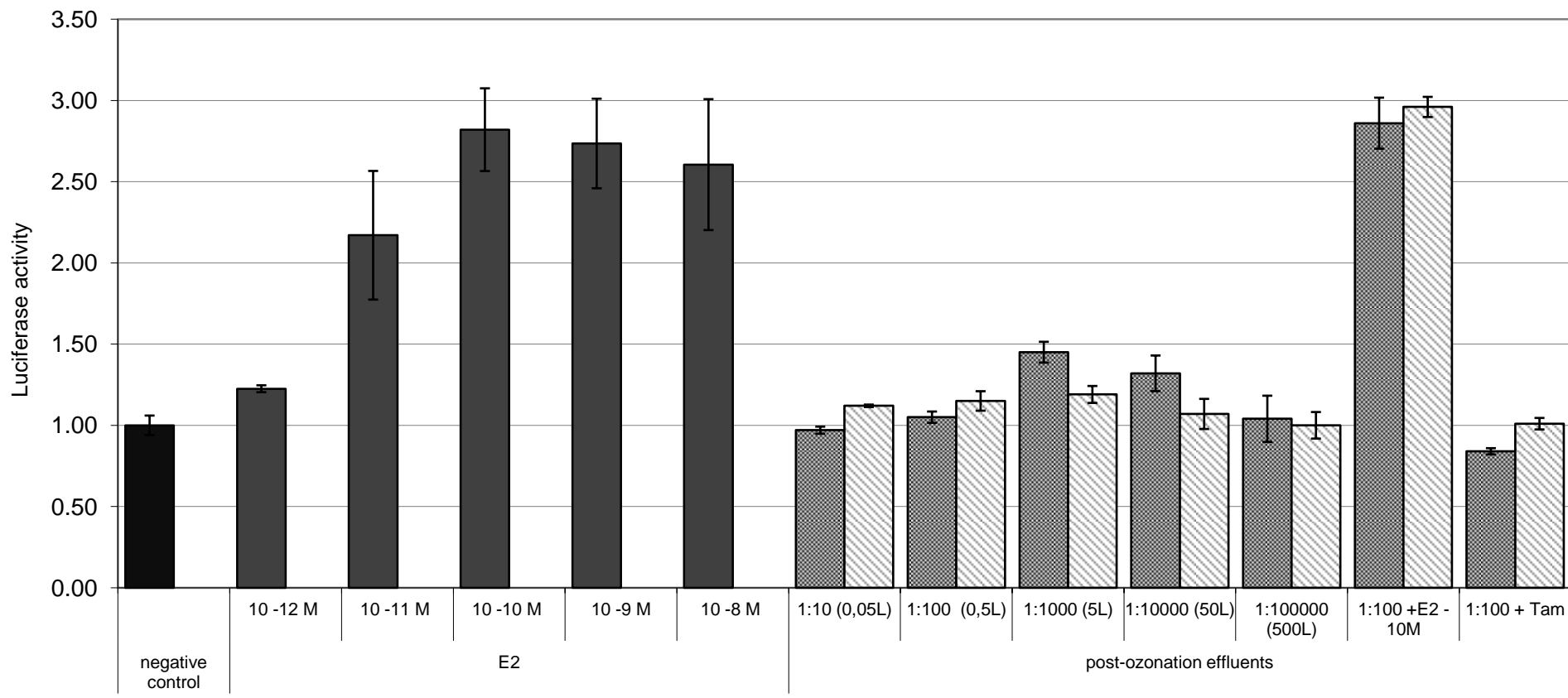
| N° sampling               | <i>Cytotoxicity</i><br>fold increase |       | <i>Cell viability</i><br>fold increase |       |
|---------------------------|--------------------------------------|-------|--|-------|
|                           | 1:10                                 | 1:100 | 1:10                                   | 1:100 |
| <b>Pre O<sub>3</sub></b>  |                                      |       |  |       |
| 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   |
| <b>Post O<sub>3</sub></b> |                                      |       |  |       |
| 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   |

12

Figure 1.2.  
[Click here to download Figure: Figure 1.2..pptx](#)



**Figure 2.**



Manuscript tracks-changes

[Click here to download Supplementary Material: paperFelli\\_12\\_stoten2\\_markedREV.doc](#)