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

(Article begins on next page)

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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 17b-estradiol equivalent quantity (EEQ). 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.

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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 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

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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.

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

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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 17β-estradiol equivalent quantity (EEQ).

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

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

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 ECDs (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 Voutsa, 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.

120

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^3/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$_3$)

132 and after the tertiary treatment (post-O$_3$) 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.

140   *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 µm

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-β-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-βGlob-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_2 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 mycoplasm infection to guarantee that the experiments were performed with

184 mycoplasm-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).

187   *2.5 E-screen test*

188 The *E-screen* test was carried out as initially described by Korner et al. (1999) and modified

189 by Schilirò et al. (2009). Briefly, subconfluent MCF-7 BUS cells were trypsinised and

190 resuspended in the steroid-free experimental medium, which consisted of phenol-red–free

191 DMEM supplemented with 5% stripped FCS, 2% L-glutamine 200mM, 2% HEPES buffer

192 1M, 1% sodium pyruvate 100mM and 1% penicillin–streptomycin 10 mg/mL. Cells were

193 seeded into 24-well plates at a density of 30000 cells/well. After 24 hours, the medium was

194 replaced with experimental medium containing one of five dilutions of water extracts. Each

195 dilution was tested in six replicates per assay. Moreover, one dilution (100-fold) of each

196 effluent sample was tested in combination with 5 nM of the antiestrogen tamoxifen (Tam) and

197 with 0.1 nM E2. Six wells without hormones were used as negative controls, and E2, in five

198 concentrations between 1 pM and 10 nM, was used as the positive control in each assay. Each

199 *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

201   nm) in each well after crystal violet staining.

202   The proliferative effect (PE) of a sample is the ratio between the highest cell number achieved

203   with the sample or E2 and the cell number of the negative control:

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

205   The estrogenic activity of a sample is evaluated by determining the relative efficacy, called

206   the relative PE (RPE%). The RPE compares the maximum proliferation induced by a sample

207   with that induced by E2:

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

209   Full agonistic activity, RPE ≥ 100%, can be distinguished from partial agonistic activity, in

210   which RPE is less than 100% (Soto, 1995).

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

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

213   The EC50 value for the *E-screen* test (concentration at which 50% of PE is achieved) was

214   calculated with a probit regression (SPSS, Chicago, IL). The PE and EC50 values of each

215   sample were calculated from the mean dose–response curves established from each

216   experiment. The EEQ, expressed in ng/L, is defined as the total concentration of estrogenic

217   active compounds in an environmental sample normalised to the natural estrogen 17-β-

218   estradiol.

219   *2.6 MELN gene-reporter luciferase assay.*

220   MELN gene- reporter luciferase assay has been widely used for the detection of estrogenic

221   activity in complex environmental samples (Cargouët et al., 2007; Combalbert et al., 2012;

222   Fenet et al., 2003; Hernandez-Raquet et al., 2007; Jugan et al., 2009; Kinani et al., 2010;).

223   The test was carried out as described by Balaguer et al. (1999).

224 Because phenol red and FCS exhibit estrogenic activity, *in vitro* experiments were carried out

225 in DMEM F12 without phenol red, supplemented with 5% dextran-coated charcoal-treated

226 foetal calf serum (DCC-FCS), 2% L-glutamine 200 mM and 1% antibiotics

227 (penicillin/streptomycin). To adapt the cells to DCC-FCS, the growth medium was replaced

228 with fresh test medium 3 days prior to the experiment. Cells were then harvested and seeded

229 in 96-well plates with a flat, clear bottom (Corning) at a density of 40000 cells/well in 100 μl

230 of DCC-FCS per well. After 24 hours, the test medium was removed, and 100 μl of each

231 sample dilution was added to three replica wells. Cells were treated with samples for 20 h.

232 Negative controls, without hormones, and positive controls, E2 in concentration between 1

233 pM and 10 nM, were included in each assay. One dilution (100-fold) of each effluent sample

234 was tested together with 5 μM antiestrogen tamoxifen (Tam) and with 0.1 nM E2. Each

235 *MELN gene-reporter luciferase assay* was also performed on a QC laboratory sample (treated

236 with the same method of samples). All experiments were performed in triplicate.

237 *2.6.1 Luciferase activity measurement in MELN cells.*

238 We used the One-Glo Luciferase Assay System (Promega, USA) to measure luciferase

239 activity according to manufacturer's instructions. Briefly, at the end of the incubation, 100 μl

240 of One-Glo Reagent (containing fluoroluciferin) was added to each well and mixed for

241 optimal consistency. After at least 3 minutes, to allow complete cell lysis, and within 30

242 minutes of reagent addition, luminescence was measured by a luminometer (Tecan, Infinite

243 M200 PRO). The luciferase activity of the MELN cells relative to the positive control E2 was

244 represented as transactivation % (TRANS %): the maximum increase in luciferase gene

245 expression triggered by estrogenic compounds present in the samples. The induction of

246 luciferase activity was expressed as a percentage, and the 100% value was obtained in the

247 presence of E2 (Balaguer et al., 1999; Fenet et al., 2003). The estrogenic activity was

248 expressed as 17-beta estradiol equivalent estrogenic activity (EEQ) in ng/L. EEQ was thus

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-

252 based assay that use membrane integrity changes to measure cell viability or cytotoxicity in

253 conjunction with luciferase measurements on the same test plates (Berckmans et al., 2007).

254 This assay technology simultaneously measures two distinct protease activities, with rapid

255 catalytic cleavage rates, as markers of cell viability or cytotoxicity.

256 The cytotoxicity test was applied to dilutions 1:10 and 1:100 (equal to 5 L and 0.5 L dilution

257 volumes) of the pre- and post-ozonation effluents.

258 At the end of incubation, 100 μl aliquots of reagent were added to all wells, mixed and

259 allowed to incubate for at least 30 minutes at 37°C. Fluorescence was determined at

260 excitation/emission wavelengths of 360 nm/460 nm for cell viability and 485 nm/528 nm for

261 cytotoxicity using a Packard EL340 microplate reader (Bio-Tek Instruments, Winooski, VT).

262 The mean values and standard deviations of replicate wells and the mean fold increase of

263 cytotoxicity relative to the negative control were calculated.

264 *2.7 Statistical analyses.*

265 Data were analysed by means of a probit regression analysis (EC50), Spearman's test

266 (correlation) and t-test (means comparison) using SPSS 18.0 (SPSS for Windows, Chicago,

267 IL, USA).

268

3. Results and Discussion

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

271 The mean EC50 value of E2 for the *E-screen* was 15.24 ± 13.73 ng/L (56.44 ± 50.83 pM);

272 EC50 values were calculated from the control curves obtained from each of the bioassays.

273 Maximum cell proliferation was generally induced by 0.1 nM E2. The detection limit of the

274 *E-screen* could be defined as the concentration of a single compound or a sample inducing a

275 cell proliferation significantly higher than the hormone-free negative control; the treatment

276 with 17beta-estradiol showed a detection limit of 1 pM (Körner et al. 1999; Schilirò et al.,

277 2004). The proliferative response of the samples was dose dependent; the 1:100 dilution of

278 the samples (equal to a 0.5 L dilution volume) always stimulated the maximum proliferative

279 response. The QC laboratory sample did not induce any significant cell proliferation. To

280 verify that the cell proliferation induced by estrogenic activity was mediated by ER, we

281 observed that co-incubation with Tam inhibited the proliferative response (28 ± 23 %), while

282 co-incubation with E2 led to a greater proliferative response (114 ± 39 %). The difference was

283 statistically significant in both cases (t-tests, $p < 0.05$).

284 Estrogenic activity, expressed as EEQ, was detected in all but the sixth sample and ranged

285 from 0.10 to 4.73 ng/L (Table 2). The proliferative effect of the different effluent samples on

286 MCF-7 BUS cells relative to the positive control E2 is represented as RPE % in Table 2. The

287 RPE of the effluent both before and after ozonation generally showed partial agonist activity

288 (RPE < 100%) ranging between 15 to 90 %. The EEQs of the effluent extracts, determined in

289 the *E-screen* test, are reported in Figure 1.

290 The highest mean EEQ was found in the second pre-ozonation sample (4.73 ± 2.42 ng/L), and

291 the lowest mean EEQ was found in the last post-ozonation sample (0.10 ± 0.13 ng/L). The

292 mean EEQ values were 2.35 ± 1.68 ng/L pre-ozonation and 0.72 ± 0.58 ng/L post-ozonation.

293 The average reduction in the estrogenic activity of IWWTP effluent from pre- to post-

294 ozonation was 67 ± 26 %. Despite the low number of analysed samples, the difference

295 between EEQ values before and after ozonation was statistically significant (simple paired t-

296 test, $p = 0.038$), suggesting that the ozonation process significantly reduced the estrogenic

297 activity of the IWWTP effluent.

298   The *in vitro E-screen* test can be used to screen a large number of compounds and integrate

299   the effects of chemicals that may not be measured in an analytical screen (Vega-Lopez, 2007).

300   It has been studied extensively, and no false positives or false negatives have yet been found;

301   it is also one of the most sensitive of all published assays (Soto et al., 2006; Vanparys et al.,

302   2010). The *E-screen* has been shown to be appropriate for the determination of estrogenic

303   activity in environmental extracts from IWWTP effluent samples.

304   *3.2 Estrogenic activity in IWWTP effluent samples by MELN gene-reporter luciferase*

305   *assay*

306   The EC50 values of E2 in the MELN gene-reporter luciferase assay were calculated from

307   control curves obtained from each bioassay, and the mean EC50 value was 1.76 ± 0.87 ng/L

308   (6.52 ± 3.22 pM). Maximum luciferase activity was generally induced by 0.1 nM E2. The

309   stable transfectants MELN used in this study exhibited good responsiveness following

310   treatment to 17beta-estradiol with a detection limit of 1 pM (Balaguer et al., 1999). The

311   luciferase activity of the samples was dose dependent, and the 1:100 dilution (equal to a 0.5 L

312   dilution volume) always stimulated the maximum luciferase activity. The QC laboratory

313   sample did not induce any significant luciferase activity. To confirm that the luciferase

314   activity was mediated by the ER, we observed that co-incubation with Tam led to an

315   inhibition of the proliferative response (51 ± 29 %), while co-incubation with E2 led to a

316   greater proliferative response (113 ± 34 %). The difference was statistically significant in both

317   cases (t-tests, $p < 0.05$). Figure 2 shows the MELN luciferase activity of E2 and of the post-

318   ozonation effluents expressed in dose-response curves and compared with the negative

319   control.

320   Estrogenic activity was detected in all but the sixth sample and ranged from 0.14 to 8.50 EEQ

321   (ng/L) (Table 2). The luciferase activity of the different effluent samples in the MELN cells

322   relative to the positive control E2 is represented as TRANS % in Table 2. The TRANS % of

323 the effluent before and after ozonation ranged between 27 to 62 %. The EEQs of the effluent

324 extracts, determined in the MELN gene-reporter luciferase assay, are reported in Figure 1.

325 The highest mean EEQ was found in the first pre-ozonation sample (8.50 $\pm$ 4.36 ng/L), and

326 the lowest mean EEQ was found in the second post-ozonation sample (0.14 $\pm$ 0.05 ng/L). A

327 lower mean value was found for the effluents post-ozonation: the mean EEQ values were 4.18

328 $\pm$ 3.54 ng/L pre-ozonation and 2.53 $\pm$ 2.48 ng/L post-ozonation; this difference was

329 statistically significant (simple paired t-test, p = 0.048). The average reduction of estrogenic

330 activity of IWWTP effluent after ozonation was 52 $\pm$ 27 %.

331 The *in vitro* MELN gene-reporter luciferase assay can rapidly screen a large number of

332 chemicals, and it is transferable, robust and reproducible, enabling the ranking of chemical

333 compounds from strong to weak affinity for the estrogen receptor (Witters et al, 2010). This

334 assay has been shown to be appropriate for the determination of estrogenicity in

335 environmental extracts from IWWTP effluent samples.

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

337 The 1:10 dilution of the effluent pre-and post-ozonation was shown to decrease cell

338 proliferation compared to negative controls, in terms of PE, in both assays. The sample, which

339 is less diluted, might be able to mask the real estrogenic activity of the compounds present in

340 it. To determine whether the effluent might have a toxic effect on the cells, 1:10 and 1:100

341 dilutions (equal to 5 L and 0.5 L dilution volume) were tested for cytotoxicity (Table 3).

342 The 1:10 mean fold increase of cytotoxicity relative to the negative control was 50.3 $\pm$ 30.0

343 for pre-ozonation effluents and 6.3 $\pm$ 6.0 for post-ozonation effluents (t-tests, p < 0.01 and p <

344 0.05, respectively). The mean fold increase of cytotoxicity produced by the 1:100 dilution

345 compared to the negative control was 1.2 $\pm$ 0.8 for the pre-ozonation effluent and 1.0 $\pm$ 1.1 for

346 the post-ozonation effluent (t-tests, p > 0.05). The sample without estrogenic activity (n°6) did

347 not exhibit a significantly higher cytotoxicity; therefore, the absence of estrogenicity can not

348 be ascribed to the toxicity of the sample.

349 *3.4 Comparison of estrogenic activity in the E-screen test and in the MELN gene-*

350 *reporter luciferase assay*

351 A comparison of the results obtained in these two assays highlights the non-significance of

352 differences in average EEQs (t-test, p > 0.05). The correlation between the EEQs obtained

353 with the MELN gene-reporter luciferase assay and with the *E-screen* was positive and

354 significant (Rho S = 0.650 and p = 0.022). The mean EC50 values relative to E2 were 15.24 ±

355 13.73 ng/L for the *E-screen* and 1.76 ± 0.87 ng/L for the MELN gene-reporter luciferase

356 assay; these values were significantly different (t-test, p = 0.015). The MELN gene-reporter

357 luciferase assay generally showed higher EEQ mean values.

358 These differences could be partially explained by the end-points of the two tests: the *E-screen*

359 is based on a binding mechanism that causes proliferation as a cellular response, which could

360 be affected by other external factors, while the MELN assay is receptor specific. Another

361 important difference between the two tests is in the assay duration; stimulation with the test

362 compounds lasts for 16-20 hours in the MELN assay but 120 hours for the *E-screen* (Soto et

363 al., 2006; Witters et al, 2010).

364

4. Conclusions

366 In general, the *E-screen* test and the MELN gene-reporter luciferase assay are well suited for

367 the determination of estrogenic activity in environmental matrices; in this specific study, they

368 were used to evaluate estrogenic activity in wastewaters from a textile factory, before and

369 after treatment with ozone. The two *in vitro* tests are particularly suitable for environmental

370 monitoring because of their sensitivity, speed, reading and low cost. The estrogenicity values

371 in the final effluent (post O_3) in the present work were similar to results reported in the

372 literature for other IWWTP effluents (Korner et al., 1999; Onda et al., 2002; Vethaak et al.,

373 2005; Tan et al., 2007; Salste et al., 2007). Our study suggests that the input of estrogenic

374 substances into the river via IWWTP is low (EEQ range: 0.72 – 2.53 ng/L) and that the

375 environmental risk could be even lower due to the dilution effect connected with discharge

376 and the self-depuration capacity of the receiving river. In a previous study, this reduction was

377 approximately one order of magnitude for EEQs (Schilirò et al., 2009), resulting in

378 concentrations lower than those at which these compounds have been reported to chronically

379 affect the endocrine systems of living organisms (approximately 1-10 ng/L; Lopez de Alda &

380 Barcelo, 2001). The analysed effluent shows lower EEQs values relative to a municipal

381 effluent (Fenet et al., 2003; Pothitou and Voutsa, 2008; Pereira et al., 2011). This difference

382 in the estrogenic activity of urban and industrial wastewater is certainly attributable to the

383 presence of different types of substances; municipal wastewater contains more natural EDCs,

384 such as E2 and EE2, that have strong estrogenic power even at very low concentrations, while

385 industrial wastewater typically contains more synthetic EDCs that are weakly estrogenic

386 relative to natural EDCs (Soto et al., 1995; Pereira et al., 2011).

387 Ozonation is considered an economically feasible option for the advanced treatment of

388 WWTP effluents (Joss et al., 2008). It is a very effective method for the degradation of

389 persistent organic dyes from colored textile wastewater (Tehrani-Bagha et al., 2010). In

390 general, ozonation and other oxidation processes seem to be the best alternatives for treating

391 WWTP effluents containing estrogens (Pereira et al., 2011). Some authors have noted that the

392 decrease in EDCs may be due to the oxidation effects of ozonation (Lee, 2004; Larcher et al.,

393 2012). To our knowledge, few reports in the literature describe estrogenic activity in textile

394 IWWTP. This work shows that this type of effluent does have estrogenic activity and that

395 ozonation decreases its overall estrogenic effects. However, ozonation is known to lead to the

396 formation of transformation products that have largely not been identified to date, and there

397 are concerns about their potential impact on the environment and human health (Benner and

398 Ternes, 2009; Radjenovic et al., 2009; Dodd et al., 2010; Stalter et al., 2010, 2011; Anastasi et

399 al., 2011; Reungoat et al., 2012). This highlights the need to evaluate the presence of

400 disinfection by-products after water treatment has been performed.

401 The identification and removal of EDC pollutants from IWWTP effluents may solve many of

402 the apparent endocrine disruption problems observed in aquatic environments, in addition to

403 providing a cleaner source of drinking water. Further investigations should be carried out to

404 identify the level of ozone appropriate to minimise both the estrogenic activity and the

405 toxicity of IWWTP textile effluents.

406

5. Acknowledgments

414

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582

583 **Figure captions**

584

585 **Figure 1**. Estradiol equivalency quantity (EEQ ng/L) of the IWWTP effluent extracts after the

586 secondary treatment (Pre ozonation) and after the tertiary treatment (Post ozonation) by

587 means of *E-Screen* test and MELN gene-reporter luciferase assay. Boxes represent the median

588 and 25th – 75th percentiles, outer lines represent the 10th -90th percentiles.

589

590 **Figure 2**. MELN luciferase activity of 17-beta-estradiol (E2) and of post ozonation effluents

591 (examples: n° 5 dotted bars and n° 7 striped bars) expressed with dose-response curves and

592 compared with the negative control.

1   **Table 1.** Physico-chemical parameters of the effluents after secondary treatment (Pre-O_3) and

2   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

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_3) and after tertiary treatment (Post-O_3) in the *E-screen* test and MELN gene-reporter

8   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

9

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_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 O₃				
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 O₃				
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

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Figure 1-2.
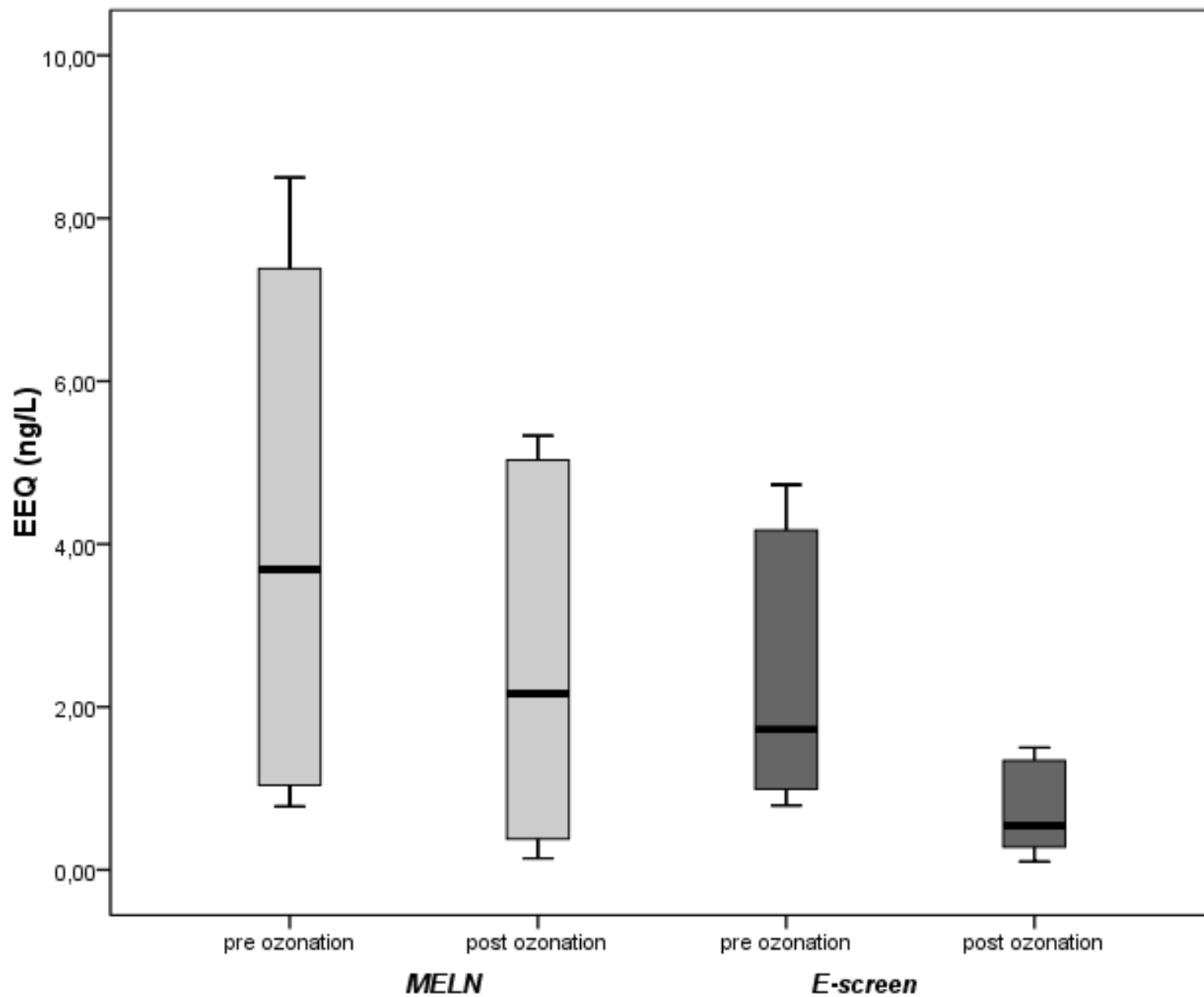
Figure 2.
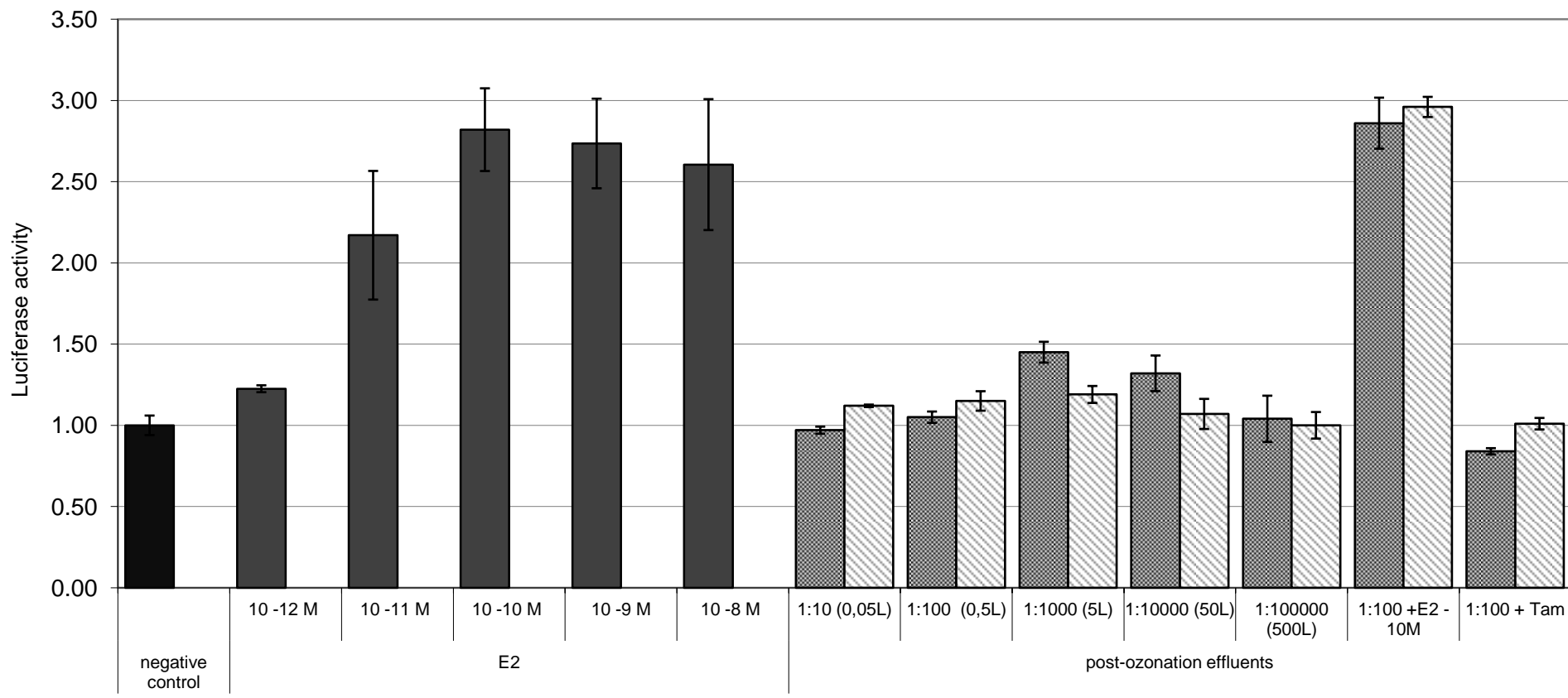
Manuscript tracks-changes