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ENVIRONMENTAL COMPATIBILITY OF WASTEWATER TERTIARY TREATMENTS TO CONTROL ORGANIC MICROPOLLUTANTS

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Abstract

Recent advances in environmental chemistry have brought increasing focus on the presence of anthropogenic substances in the environment: even though the concentrations of these compounds are in the range of µg/L or even ng/L (therefore called "*micropollutants*"), adverse effects on human health cannot be excluded. One such group of anthropogenic substances is represented by endocrine-disrupting compounds (EDCs).

The application of conventional wastewater treatment does not provide complete elimination of all micropollutants and, consequently, residues of EDCs enter the aquatic ecosystem through WasteWater Treatment Plants (WWTPs) effluents. In order to prevent this kind of water pollution, an advanced treatment downstream the biological process should be implemented: in particular, the application of ozone proved to be a suitable technology for EDCs removal.

In this work, an integrated assessment procedure was used, based on chemical and biological analyses, in order to evaluate the performance of the tertiary chemical oxidation (with ozone). Nonylphenol (NP) and bisphenol A (BPA) were chosen as model EDCs, together with the parent compounds mono- and di-ethoxylated nonylphenol (NP1EO and NP2EO, respectively), and quantified by means of GC-MS. Water estrogenic activity was evaluated by applying the human breast cancer MCF-7 based reporter gene assay.

Experimental work was conducted at both pilot- (reactor volume = 1,500 L, flow-rate up to 6 m^3/h, treating the effluent of a municipal WWTP) and full-scale (a 140.000 p.e. WWTP equipped with a tertiary ozonation stage, treating both domestic and industrial wastewater) plants.

As pilot plant is concerned, influent trace pollutants concentrations were in the range 0.14-0.43 µg/L. Chemical oxidation was described by first order kinetics, rate constants being in the range 1.7-5.6 h^{-1}, depending on reagent dosage.

In the full-scale plant, the WWTP effluent prior to chemical oxidation recorded average concentrations of 1.21 and 0.59 µg/L, respectively for NP and BPA, and the removal efficiency of ozonation (60 min. effective contact time and 11 mgO_3/L dosage) turned out to be only around 50%: the presence of a significant industrial input (with organic biorecalcitrant molecules) reduced the abatement of target EDCs, with respect to the previous case study.

In the case of pilot plant, biological analyses confirmed ozone beneficial effect on the reduction of estrogenicity. However, unlike analytes, estrogenic activity abatement was not significantly affected by ozone dosage.

On the contrary, as a result for the full-scale plant, the bioassay outcomes clarified that ozone is only partially able to reduce the estrogenic activity (less than 20% decrease); moreover, the effective estrogenic abatement was significantly lower than the one predictable based on EDCs concentration reduction. This was explained considering that: a) a mixture of compounds is responsible for the estrogenic activity, in addition to analyzed EDCs, b) synergistic and potentiating effects between EDCs can occur and c) active by-products can originate, as oxidation intermediates, during ozonation.

Finally, the environmental compatibility of the treatment process was assessed, by means of an environmental appraisal framework. Indeed, the comparison of different scenarios (without and with ozonation treatment) highlighted the onset of a conflict (water *vs* atmospheric pollution): the increase of effluent quality, obtained via an energy-intensive treatment, is responsible for air quality decrease. This conflict can be solved only through the definition of an endpoint category: in particular, in this work the damage on human health was identified as final indicator. This approach, coupled with biological assays, made the comparison possible, displaying similar damage values for both the options, with a slight decrease for the scenario involving O_3 process. This analysis, anyway, confirmed that the optimal solution seems to be the control at the source of the pollution (green chemistry), rather than end-of-pipe, energy-intensive approaches.

Keywords: Wastewater; EDCs; NP; BPA; estrogenic activity; MCF-7 *in vitro* bioassay; mutagenicity; tertiary ozonation; environmental compatibility; damage on human health; cross-media effects; DALY.

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CHAPTER 1

EXECUTIVE SUMMARY

Recent advances in environmental chemistry have brought increasing focus on the presence of anthropogenic substances in the environment. One such group of anthropogenic substances is represented by Endocrine-Disrupting Compounds (EDCs), "exogenous agents that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development and behavior" (EPA, 1997). The EDCs best known to produce specifically estrogenic effects are the naturally occurring (17β-estradiol, estrone and estriol) and synthetic (17α-ethinyl-estradiol) steroidal estrogens, and synthetic organic compounds, mainly nonylphenol and bisphenol A: though at very low concentrations, every xenoestrogen may add incrementally to the total estrogenic effect. Recent scientific literature reports data about their trace concentration in water, sediments and aquatic organisms, as well as removal efficiencies of different wastewater treatment schemes. Despite the availability of a huge amount of data, some doubts still persist due to the difficulty in evaluating synergistic effects of trace pollutants in complex matrices.

WWTPs (WasteWater Treatment Plants) effluents are considered to be a major source for the release of these compounds in the aquatic environment: the application of conventional wastewater treatment, indeed, does not provide complete elimination of all micropollutants; subsequently, an advanced treatment downstream of biological process should be implemented. Several technologies for further micropollutants removal have been investigated: in particular, the application of ozone has proven to be a suitable technology for EDCs removal.

Anyway, although chemical analyses can successfully reveal the presence of EDCs in the aquatic environment, they are generally focused on determining only target substances in the matrices of interest. Because of the large number of EDCs that can be present in a complex environmental sample, chemical analyses are not suited for determining the actual estrogenic potential. Moreover, chemical analyses do not consider mutual and synergic interactions and the biological effects of the whole sample. On the contrary, bioassays are based on the interaction between EDCs and estrogenic

receptors and can measure the total estrogenic activity, regardless of the individual compounds identification.

In this research, a strongly multidisciplinar approach was adopted, involving both chemical and biological issues and the work of different research groups. Nonylphenol and bisphenol A were chosen as model EDCs, together with the parent compounds mono- and di-ethoxylated nonylphenol, and quantified by means of GC-MS (*Environmental Chemistry* group). Water estrogenic activity, furthermore, was evaluated by applying the human breast cancer MCF-7 based reporter gene assay (*General Pathology and Immunology* group). Finally, to complete biological assays, the *Institute of Hygiene, Epidemiology and Public Health* performed mutagenic analyses.

After the executive summary (*Chapter 1*), in *Chapter 2* an integrated assessment procedure, based on both chemical and biological analyses, was adopted to evaluate the performance in the removal of target EDCs from municipal wastewater of: i) biological treatments (conventional activated sludge, CAS, and membrane bioreactor, MBR, systems), in order to verify their efficiency and to characterize WWTPs effluents (partially conducted in the previous PhD work), and ii) chemical oxidation via ozonation (the focus of the present PhD research). The following estrogen-like substances were considered: 4-nonylphenol (NP), its parent compounds 4-nonylphenol monoethoxylate (NP1EO) and 4-nonylphenol diethoxylate (NP2EO), and bisphenol A (BPA). These substances were chosen as model EDCs since they are diffusely detected in the aquatic environment and are included in the EU priority lists on environmental quality standards in the field of water policy.

Experimental work was conducted, for biological treatments, at two full scale WWTPs located in Northern Italy equipped with either conventional settling tanks (CAS: Verona municipality) or with an ultrafiltration unit (MBR: Brescia municipality); as far as tertiary chemical oxidation concerns, the tests were performed by means of an ozone pilot plant (reactor volume = 1,500 L, flow-rate up to 6 m^3/h), treating the effluent of the Verona municipal WWTP (experimentation #1). Hormonal activity in water samples was measured by means of human breast cancer MCF-7 based reporter gene assay, using 17β-estradiol as a standard (in this initial case-study, data were processed by means of a simplified methodology).

In *Chapter 3*, the same integrated (chemical + biological) assessment procedure was adopted to evaluate the performance of a full-scale WWTP (daily treated flow-rate: $\approx$ 30,000 m^3/d) equipped with a tertiary ozonation stage (experimentation #2), treating both domestic and industrial (textile) wastewater. The primary objective of this study was to provide key baseline information concerning

the concentrations of individual estrogenic compounds (measured with chemical analysis) and the estrogenicity (measured with *in vitro* bioassay: in this case, data were deeper processed with a dose-response pattern) of untreated and treated effluent, together with the removal efficiencies of ozonation process. Furthermore, an analysis of the correlation between bio- and chemical assays was performed. Finally, wastewater genotoxicity (and the related effect of ozonation treatment) was evaluated with three different tests, in order to assess the ability to induce genetic damage in target cells of different organisms (bacterial, plant and mammalian cells), to detect point and chromosomal mutations, and DNA damage.

In conclusion, the last question that this work has wanted to answer regarded the assessment of environmental compatibility of O_3 treatment. In *Chapter 4*, indeed, a cost-benefit analysis has been undertaken to determine if the application of an advanced technology such as a tertiary ozonation stage to WWTP effluent is economically/environmentally desirable when financial costs, energy consumption, and associated atmospheric emissions are taken into consideration. In order to achieve this goal, an environmental appraisal framework can provide a means by which issues of long-term sustainability in the aquatic environment may be addressed.

The environmental compatibility of the wastewater tertiary ozonation was performed on the basis of a preliminary order-of magnitude calculation of damage on human health, expressed as an economic value. In particular, for water pollution (for which there was no established method), the rates of the negative human health impacts were evaluated in terms of global burden of disease (GBD) and measured in units of DALY (disability-adjusted life years, i.e. the sum of the life years lost due to disability and premature death), in order to compare various negative impacts on a linear scale. It can be reasonably considered an innovative procedure: far few studies, indeed, have been attempted to evaluate the attributable burden of disease caused by a certain source, despite the fact that the result could help policy makers in assessing pollution phenomena more directly in relation to human health.

Beyond the environmental compatibility of the actual full-scale ozonation plant, in order to increase the options to be compared, the same treatment was evaluated also under the hypothesis of strong O_3 dosage, in particular, equal to the higher applied to the pilot plant of experimentation #1.

Figure 1 summarizes the structure of the thesis and the contents of each chapter, as above described.
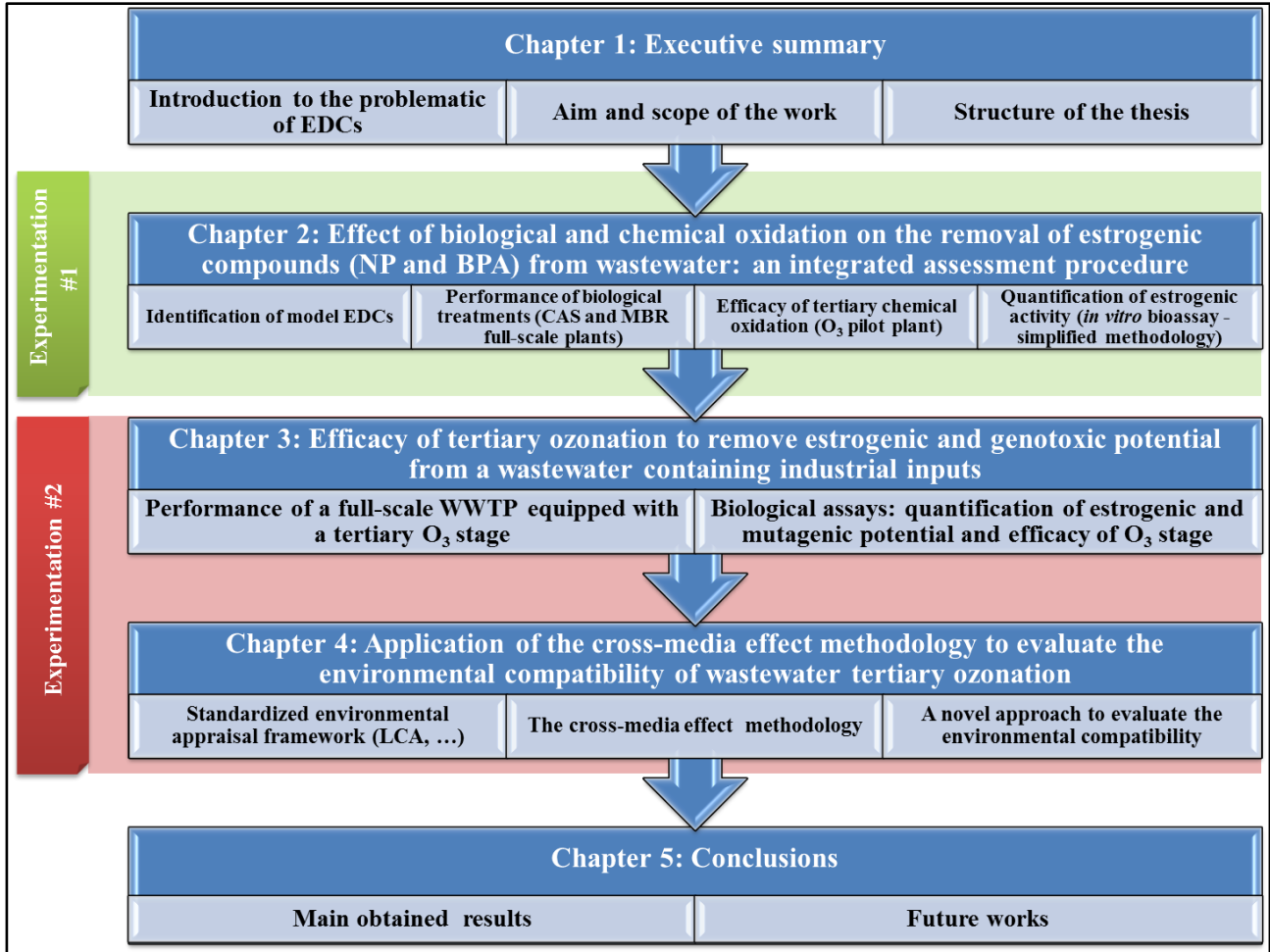
Figure 1. Structure of the thesis and summary of chapters content.

CHAPTER 2

EFFECT OF BIOLOGICAL AND CHEMICAL OXIDATION ON THE REMOVAL OF ESTROGENIC COMPOUNDS (NP AND BPA) FROM WASTEWATER: AN INTEGRATED ASSESSMENT PROCEDURE[1]

[1] This chapter is adapted from the following pubblications:

Bertanza, G., Pedrazzani, R., **Papa, M**., Mazzoleni, G., Steimberg, N., Caimi, L., Montani, C. and Dilorenzo, D. (2010): Removal of BPA and NPnEOs from secondary effluents of municipal WWTPs by means of ozonation - Ozone Science & Engineering 32(3) 204-208

Bertanza, G., Pedrazzani, R., Dal Grande, M., **Papa, M**., Zambarda, V., Montani, C., Steimberg, N., Mazzoleni, G. and Dilorenzo, D. (2011): Effect of biological and chemical oxidation on the removal of estrogenic compounds (NP and BPA) from wastewater: An integrated assessment procedure - Water Research 45(8) 2473-2484

SUMMARY

2.1. INTRODUCTION

In recent decades, concerns regarding the occurrence of Endocrine Disrupting Compounds (EDCs) in the environment have rapidly increased worldwide. Municipal sewage and Waste Water Treatment Plant (WWTP) effluents are considered to be major sources of pollution due to the documented presence of such compounds at relevant concentrations (see, *inter alia*: Auriol *et al.*, 2006; Ternes and Joss, 2006; González *et al.*, 2007; Stasinakis *et al.*, 2008, Ying *et al.*, 2009; Sanchez-Avila *et al.*, 2009).

EU Directive 2008/105/EC (amending and subsequently repealing Council Directives 82/176/EEC, 83/513/EEC, 84/156/EEC, 84/4/9/EEC, 86/280/EEC and amending Directive 2000/60/EC) sets strict quality standards for water bodies (many EDCs being included among priority substances). Therefore, in the future, efforts to adopt feasible and reliable treatment techniques for wastewater cleaning will be made. Accordingly, two important tasks should be pursued: 1) the assessment of the removal capacity of conventional biological processes and, consequently, 2) the evaluation of possible requirements for additional (tertiary) treatment.

Even though data is available in the literature on both issues, some lack in knowledge still persists: 1) the removal potential of many EDCs by conventional activated sludge plants is well-established (see for instance Farré *et al.*, 2002; Ternes and Joss, 2006; González *et al.*, 2007; Joss *et al.*, 2008; Pothitou and Voutsa, 2008; Press-Kristensen *et al.*, 2008), nevertheless, data are not easily comparable due to different treatment conditions, sampling procedures and analytical methods; 2) tertiary chemical oxidation has been successfully tested (Rosenfeldt and Linden, 2004; Auriol *et al.*, 2006; Zhang *et al.*, 2006; Esplugas *et al.*, 2007; Gultekin and Ince, 2007; Ning *et al.*, 2007; Bolong *et al.*, 2009; Racz and Goel, 2010) but technical-economic feasibility is still to be fully demonstrated (Auriol *et al.*, 2006; Gultekin and Ince, 2007, Koh *et al.*, 2008); 3) chemical analysis alone is not useful to investigate synergistic effects among mixtures of different pollutants and their degradation by-products (a well-known phenomenon in the case of endocrine disruptors: Hjelmborg *et al.*, 2006; Bjorkblom *et al.*, 2008; Mnif *et al.*, 2010). Several authors (Svenson *et al.*, 2003; Hashimoto *et al.*, 2007; Fernandez *et al.*, 2008; Mispagel *et al.*, 2009) have pointed out that water biological activity should also be monitored in order to better evaluate treatment suitability; actually, endocrine activity assays have been proposed in the last few years (Harris *et al.*, 1997; Céspedes *et al.*, 2003; Isobe *et al.*, 2003; Korner *et al.*, 2004; Tan *et al.*, 2007; Fernandez *et al.*, 2009; Jugan *et al.*, 2009; Creusot *et al.*, 2010; Sousa *et al.*, 2010).

In this chapter, an integrated assessment procedure, based on both chemical and biological analyses, was adopted to evaluate the performance of biological and chemical oxidation in the removal of target EDCs from municipal wastewater. The following estrogen-like substances were considered: 4-nonylphenol (NP), its parent compounds 4-nonylphenol monoethoxylate (NP1EO) and 4-nonylphenol diethoxylate (NP2EO), and bisphenol A (BPA). These substances were chosen as model EDCs since they are diffusely detected in the aquatic environment (Kolpin *et al.*, 2002; Belmont *et al.*, 2006; Gultekin and Ince, 2007; Loos *et al.*, 2007; Sun *et al.*, 2008; Ying *et al.*, 2009) and are included in the EU priority list (EU Directive 2008/105/EC).

Experimental work was conducted at two full scale WWTPs located in Northern Italy equipped with either conventional settling tanks (CAS, Conventional Activated Sludge: Verona municipality) or with an ultrafiltration unit (MBR, Membrane Biological Reactor: Brescia municipality). Tertiary chemical oxidation was tested by means of an ozone pilot plant located at the Verona WWTP.

The duration of the analytical campaigns was extended so as to enable the accurate calculation of mass balances of target compounds. Hormonal activity in water samples was measured by means of human breast cancer MCF-7 based reporter gene assay, using 17β-estradiol (E2) as a standard. This cell line was chosen due to its high concentration of estrogenic receptors and sensitivity (Pons *et al.*, 1990; Urban *et al.*, 2001; Soto *et al.*, 2006; Higashi *et al*, 2007).

2.2. MATERIALS AND METHODS

2.2.1 TREATMENT PLANTS

Verona WWTP. This is a CAS plant (design size 370,000 p.e.) treating mainly domestic wastewater. The process scheme includes primary settling (volume = 10,400 m^3, 3 parallel basins); pre-denitrification (volume = 7,200 m^3, 5 parallel basins); oxidation-nitrification (volume = 16,600 m^3, 5 parallel basins); secondary settling (volume = 26,100 m^3, 6 parallel basins).

The sludge treatment line consists of: dynamic thickening, anaerobic digestion and mechanical dewatering.

The following are the main operational data (typical values): influent water flow = 92,000 m^3/d (dry weather); dissolved oxygen concentration in aerated tanks = 2.0-2.2 mg/L; total suspended solids concentration in biological reactors = 4.0-4.5 gTSS/L; influent characteristics (after screens and grit-oil removal): 450 mgCOD/L, 200 $mgBOD_5$/L, 240 mgTSS/L, 50 mgTKN/L, 5 mgP_{TOT}/L;

effluent characteristics: 30 mgCOD/L, 5 mgBOD$_5$/L, 12 mgTSS/L, 6.5 mgTKN/L; 4 mgNH$_4^+$-N/L, 4 mgNO$_3^-$-N/L, <0.1 mgNO$_2^-$-N/L, 1.3 mgP$_{TOT}$/L.

Brescia WWTP. This consists of 2 CAS lines and 1 MBR line (design size 380,000 p.e.), treating domestic and industrial wastewater. The process scheme includes equalization/homogenization (volume = 24,000 m^3); pre-denitrification (volume = 11,100 m^3, 3 parallel basins); oxidation-nitrification (volume = 20,600 m^3, 3 parallel basins); secondary settling (for conventional lines, volume = 7,800 m^3, 2 parallel basins) and ultrafiltration (for MBR line). This configuration enabled the comparison of the CAS process with the MBR technique.

The sludge treatment line consists of: dynamic thickening, anaerobic digestion and mechanical dewatering.

The following are the main operational data (typical values): influent water flow = 71,500 m^3/d (dry weather); dissolved oxygen concentration in aerated tanks = 1 mg/L; total suspended solids concentration in biological reactors = 2.0 and 5.2 gTSS/L in CAS and MBR lines, respectively; influent characteristics (after screens and grit-oil removal): 310 mgCOD/L, 140 mgBOD$_5$/L, 140 mgTSS/L, 29 mgTKN/L, 5 mgP$_{TOT}$/L; effluent characteristics: 15 (CAS line) and 8 (MBR line) mgCOD/L, <5 mgBOD$_5$/L, <5 mgTSS/L, 2.1 mgTKN/L, 3.1 (CAS line) and 0.5 (MBR line) mgNH$_4^+$-N/L, 3.5 (CAS line) and 5 (MBR line) mgNO$_3^-$-N/L, <0.2 mgNO$_2^-$-N/L, 0.6 mgP$_{TOT}$/L.

Pilot scale ozonation plant. Supplied by SIAD SpA, Bergamo, Italy, this consists of a stainless steel tubular reactor (volume = 1,460 L) and is equipped with a pure oxygen supply system (capacity = 400 gO$_3$/h). The reactor can be fed with a flow-rate up to 6 m^3/h in a continuous mode of operation.

2.2.2 MONITORING CAMPAIGN AND TREATMENT TESTS

FULL SCALE CAS AND MBR WWTPS

The Verona WWTP monitoring campaign was conducted in winter (dry weather) from 5 to 20 February: sampling points were located as shown in Fig. 1 (top). It is important to note that sewage entering the primary settling tanks includes supernatants from the sludge treatment line.

The Brescia WWTP was monitored during a dry weather summer period (23 June - 11 July). Sampling points are shown in Fig. 1 (bottom); unlike the Verona plant, influent samples were not affected by supernatants from the sludge line.

For both plants, wastewater was collected daily, over 24 hours, by automatic refrigerated auto-samplers equipped with Teflon pipes and dark glass containers (pre-washed with hydrochloric acid and acetone); sludge was sampled instantaneously and submitted immediately to analysis. The following parameters were measured on collected samples: NP (mixture of 4-nonylphenol isomers), NP1EO (mixture of 4-nonylphenol monoethoxylates isomers), NP2EO (mixture of 4-nonylphenol diethoxylates isomers), BPA, COD, total suspended solids (TSS). Estrogenic activity was measured only at the Brescia WWTP, on three 24-hour samples collected during the monitoring campaign.
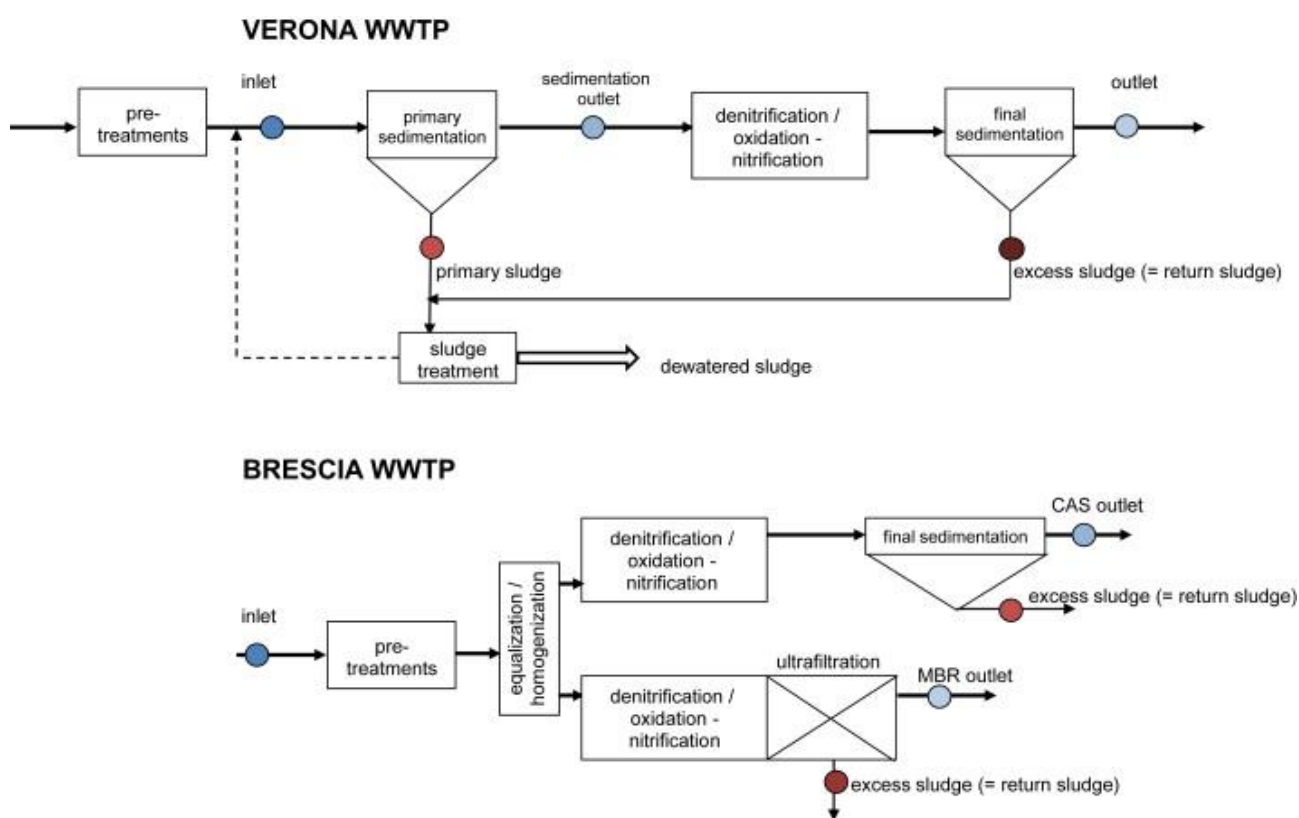
Figure 1. Sampling points for the Verona (top) and Brescia (bottom) WWTPs (bold line = wastewater; fine line = sludge; dotted line = supernatant from sludge treatment; double line = dewatered sludge).

OZONATION PLANT

Two series of tests were conducted in order to assess the effect of ozone dosage (12 and 20 mgO$_3$/L) and, for each ozone concentration, three runs were performed at increasing contact times (15, 22 and 30 minutes, respectively). During each test, at 1, 2 and 3 HRT (Hydraulic Retention

Time) time intervals, grab samples of influent and effluent wastewater were taken and immediately submitted to chemical (NP, NP1EO, NP2EO, BPA), microbiological (total coliforms and *E. coli*) and biological (estrogenic activity) analyses. Based on instrumentally detected data (ozone production and residue in offgas), the actual ozone dissolution percentage was calculated.

2.2.3 CHEMICAL ANALYSES

The method of Gatidou *et al*. (2007) was successfully adopted for the extraction of analytes from liquid phase.

The following chemicals were purchased from Sigma Aldrich (Taufkirchen, Germany): a) standard reagents: bisphenol A, NP1EO, NP2EO, 4-NP technical mixture of isomers, as proposed by ISO 18857-1 (2005); b) derivatization reagents: MSTFA and pyridine; c) internal standard: bisphenol A-d_{16}. Influent samples were filtered on glass fiber filters (Whatman GF/A ϕ=1.6 μm) in order to separate particulate matter from the liquid phase. Liquid samples were submitted to enrichment on SPE C18 (Supelco, Bellefonte, USA) and consequent elution. Filters were weighed prior to filtration; solids retained by the filter were weighed by using a thermobalance set at 60°C. Afterwards, filters were placed into 50 mL vials, and 9 mL dichloromethane-hexane 4:1, 1 mL BPA-d_{16} (500 ppb) and 100 μL HCl 6 N were added. Vials were submitted to sonication for 30 minutes at 50°C.

Derivatization was performed with 900 μL MSTFA (5% in isooctane) and pyridine (100 μL).

Instrumental analysis was conducted using a gas-chromatograph 5975B inert XL EI/CI MSD equipped with a split/splitless injector and autosampler (Agilent Technologies, Palo Alto, USA).

The RDS% (Recovery Determination Standard) varied from 7.3 to 13.7, depending on the target molecule; mean recovery percentage referring to internal standard of BPA-d_{16} was more than 80% (water samples) and about 60% (sludge samples); the lowest concentration of the calibration curve was equal to 100 ppb for each pollutant (for further details about the analytical procedure, see Pedrazzani *et al.*, in preparation).

COD and TSS were measured as prescribed by the Italian Standard Methods (APAT IRSA CNR No. 5130 and 2090, 2003), the former after $K_2Cr_2O_7$ oxidation, and the latter after 0.45 μm filtration and 105 °C drying process, respectively.

2.2.4 MICROBIOLOGICAL ANALYSES

Raw samples of ozonation plant influent and effluent wastewater were diluted in sterile NaCl 0.1% and submitted to total coliforms and *E. coli* determination, accordingly with the MPN (Most Probable Number) technique (Italian Standard Methods: APAT IRSA CNR No. 7010B and 7030B, 2003). DST Colilert® (IDEXX Laboratories, Westbrook, USA) was employed, based on specific enzymatic reactions with ONPG (o-nitrophenyl β-D-galactopyranoside) and MUG (4-methyl-umbelliferyl β-D-glucuronide). Multiplates trays were placed in an incubator at 36±1°C for 24 hours and positive results were read and interpreted as prescribed.

2.2.5 BIOLOGICAL ANALYSES

The pollutant extraction and clean-up procedure was the same as reported for the chemical analyses; extracts were resuspended in 1 mL DMSO (dimethyl-sulfoxide). Human breast cancer cell line MCF-7 stably transfected with the ERE-tK-LUC construct was maintained in DMEM (Modified Dulbecco's Medium, Euroclone, Milan), supplemented with 5% calf serum, at 37°C and 5% CO_2. 24 hours before treatment with pollutants, cells were plated at a density of $6.0·10^5$ cells/well in six-well plates containing phenol red free DMEM and 5% charcoal-stripped fetal calf serum.

Cells were treated with either reference estrogen (E2) or pollutants culture medium solutions; dishes were kept at 37°C for 24 h (Chau *et al.*, 1998; Spink *et al.*, 2003). Cells were then harvested in TEN buffer (10 mM Tris, 10 mM EDTA, 150 mM NaCl, pH 8.0) and pellets were lysed in luciferase assay buffer (25 mM Tris, 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 0.5% Triton X-100, pH 8.0). Lysate was spun for 20 s at 13,000 *g* and supernatant submitted to luciferase activity quantification, which was performed in triplicate by means of a luminometer (Centro 960, Berthold Tech., Germany) over 10 s (de Wet *et al.*, 1987), expressed as RLU (Relative Light Units) and normalized towards protein concentration. Reference estrogen E2 (dissolved in absolute ethanol) was employed for calibration curve definition, at concentrations corresponding to physiological/sub-physiological doses, i.e., from 10^{-13} to 10^{-7} M (the lower approaching LOD - Limit of Detection).

2.2.6 ECONOMIC EVALUATION

In order to assess economic feasibility of tested process, an evaluation of operating costs was carried out. The following data were assumed: electric energy consumption for ozone production: 10 kWh/kgO$_3$; pure oxygen cost: 0.07 €/kg; efficiency of ozone production: 7% (wO$_3$/wO$_2$). Required treatment conditions were calculated based on kinetic coefficients obtained from experimental data, considering an initial concentration of 1 µg/L for studied trace pollutants and different removal percentages.

2.3. RESULTS AND DISCUSSION

2.3.1 VERONA WWTP: CAS PROCESS

The mass balance of target compounds was calculated based on measured concentrations and recorded flow-rates of different streams (wastewater and sludge). It should be highlighted that the daily flow-rate was quite stable during the entire period (average value: 82.500 m^3/d ± 5%), thus yielding reliable calculations, despite an expected slight variability of influent concentrations (similar patterns were observed for EDCs and conventional pollutants COD and TSS: Fig. 2).

Average weighted concentrations of pollutants in different plant sections as well as solid-liquid phase partition percentages are detailed in Table 1; the complete mass balance is shown in Fig. 3.

As far as influent wastewater is concerned, the results confirm the data from the literature, even though NP1EO and NP2EO concentrations are close to the lowest values found by several authors (Di Corcia *et al.*, 1994; Solé *et al.*, 2000; Körber *et al.*, 2000; Fuerhacker *et al.*, 2001; Farré *et al.*, 2002; Planas *et al.*, 2002; Fauser *et al.*, 2003; Laganà *et al.*, 2004; Vethaak *et al.*, 2005; Mart'ianov *et al.*, 2005; Fountoulakis *et al.*, 2005; Lee *et al.*, 2005; Jiang *et al.*, 2005; Clara *et al.*, 2005[a]; Shen *et al.*, 2005; Komori *et al.*, 2006; Cantero *et al.*, 2006; Vogelsang *et al.*, 2006; Nakada *et al.*, 2006; Belmont *et al.*, 2006; Levine *et al.*, 2006; González *et al.*, 2007; Clara *et al.*, 2007; Loyo-Rosales *et al.*, 2007; Stasinakis *et al.*, 2008).

Average concentrations (Table 1) indicate that primary sedimentation exerted negligible removal of trace pollutants, notwithstanding an appreciable abatement of TSS (50%: data not shown) and the relevant percentage of pollutants associated with particulate matter. As a confirmation, mass

balance revealed that only 5-6% (Fig. 3) of the influent amount of these contaminants was in primary sludge, detected concentrations being in the range 3-7 mg/kgTSS. This is in agreement with published data (González *et al.*, 2004; Levine *et al.*, 2006), even though removal percentages up to 20% - 30% are reported as well (in particular for NPnEO, Ahel *et al*., 1994). However, an exhaustive comparison with the literature is not possible because primary settling performance is likely to be influenced by hydraulic retention time and sewage temperature, and these data are often missing.

Taking into account final effluent, it can be observed that biological process was able to reduce the concentrations of target organics to a significant extent. These results are in accordance with the data from the literature (Koh *et al.*, 2005; Auriol *et al.*, 2006; Huntsman *et al.*, 2006; Levine *et al.*, 2006; Nakada *et al.*, 2006; Vogelsang *et al.*, 2006; Clara *et al.*, 2007; Loos *et al.*, 2007; Loyo-Rosales *et al.*, 2007; Stasinakis *et al.*, 2008). It must be noted that the residual amount of NP, NP1EO and NP2EO in the effluent is the result of both removal (by means of biodegradation/sorption) and generation (as metabolites of parent compounds) processes. Therefore, while in the case of BPA we focus on primary degradation, for NP, NP1EO and NP2EO we refer to an apparent degradation. Trace pollutants were also detected in excess sludge at concentrations ranging from 0.26 mg/kgTSS (BPA) to 4.08 mg/kgTSS (NP1EO); however, mass balance showed that the amount found in excess sludge accounted for less than 0.5% of the mass entering the biological system. As already noted for primary sludge, these pollutants were not removed with solid phase (sludge). Based on the comparison between TSS (data not shown) and trace pollutant concentrations in final effluent, a clear correlation could not be evidenced, as already stated in the literature (see, among others, Jiang *et al*., 2005), who observed that tertiary filtration does not improve the removal of EDCs.
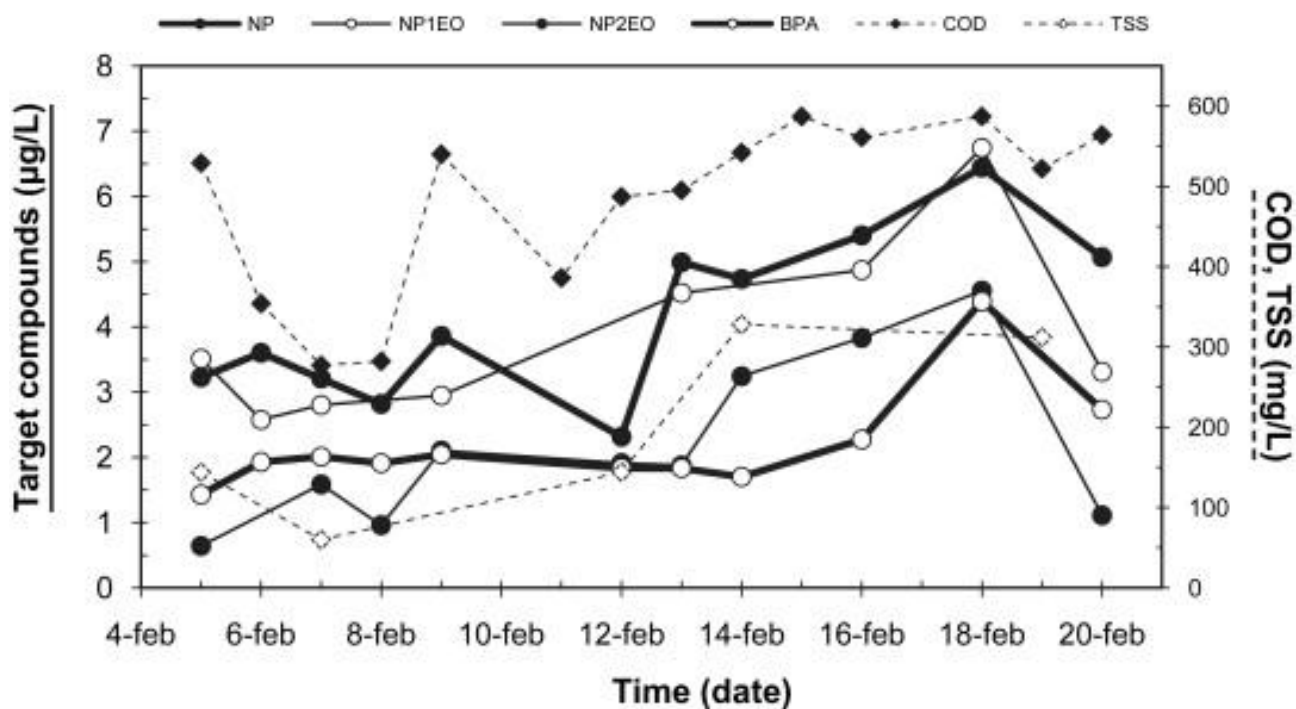
Figure 2. Verona WWTP: daily average concentration of pollutants in influent wastewater.

	Influent		Primary settling tank effluent		Final effluent
	Total (μg/L)	Particulate (%)	Total (μg/L)	Particulate (%)	Total (μg/L)
NP	4.15	47	3.65	41	0.85
NP1EO	3.90	49	3.96	33	0.52
NP2EO	2.18	38	2.15	39	0.70
BPA	2.19	41	2.43	30	0.31

Table 1. Verona WWTP: average concentrations of target EDCs and percentage attached to 1.6 μm particulate fraction.

Figure 3. Verona WWTP: mass balance of trace pollutants. "*Degraded*" mass obtained by subtracting the sum of each effluent mass flow (final effluent, primary and excess sludge) from influent load.

2.3.2 BRESCIA WWTP: CAS AND MBR PROCESSES

CHEMICAL ANALYSES

Weighted mean concentrations of trace pollutants are reported in Table 2 while mass balance is shown in Fig. 4 for CAS and MBR processes, respectively. Also in this case, no appreciable scattering (± 5%) with respect to average value was evidenced for sewage flow-rate during the monitoring campaign.

Considering influent wastewater characteristics, while NP and BPA were detected in similar concentrations as in the Verona WWTP, NP1EO and NP2EO values were higher. This may be due to several factors:

- the origin of influent wastewater (Brescia is located in a heavily industrialized area);

- influent wastewater temperature (higher during the Brescia monitoring campaign), which influences NPnEO degradation pathways, hence metabolite generation by biodegradation processes;
- sewer pipeline features (length, hydraulic retention time, etc.).

Both CAS and MBR lines yielded a noticeable reduction of trace pollutants and, like in Verona the plant, amounts detected in excess sludge were very low: from 1.1% to 5.3% of total influent mass (concentrations ranging from 0.38 mg/kgTSS for NP to 1.51 mg/kgTSS for NP1EO).

	Influent		Final effluent (CAS)	Final effluent (MBR)
	Total (µg/L)	Particulate (%)	Total (µg/L)	Total (µg/L)
NP	4.70	64	0.74	0.79
NP1EO	7.89	51	0.29	0.30
NP2EO	5.01	45	0.64	0.96
BPA	1.94	63	0.47	0.50

Table 2. Brescia WWTP: average concentrations of target EDCs and percentage attached to 1.6 µm particulate fraction.

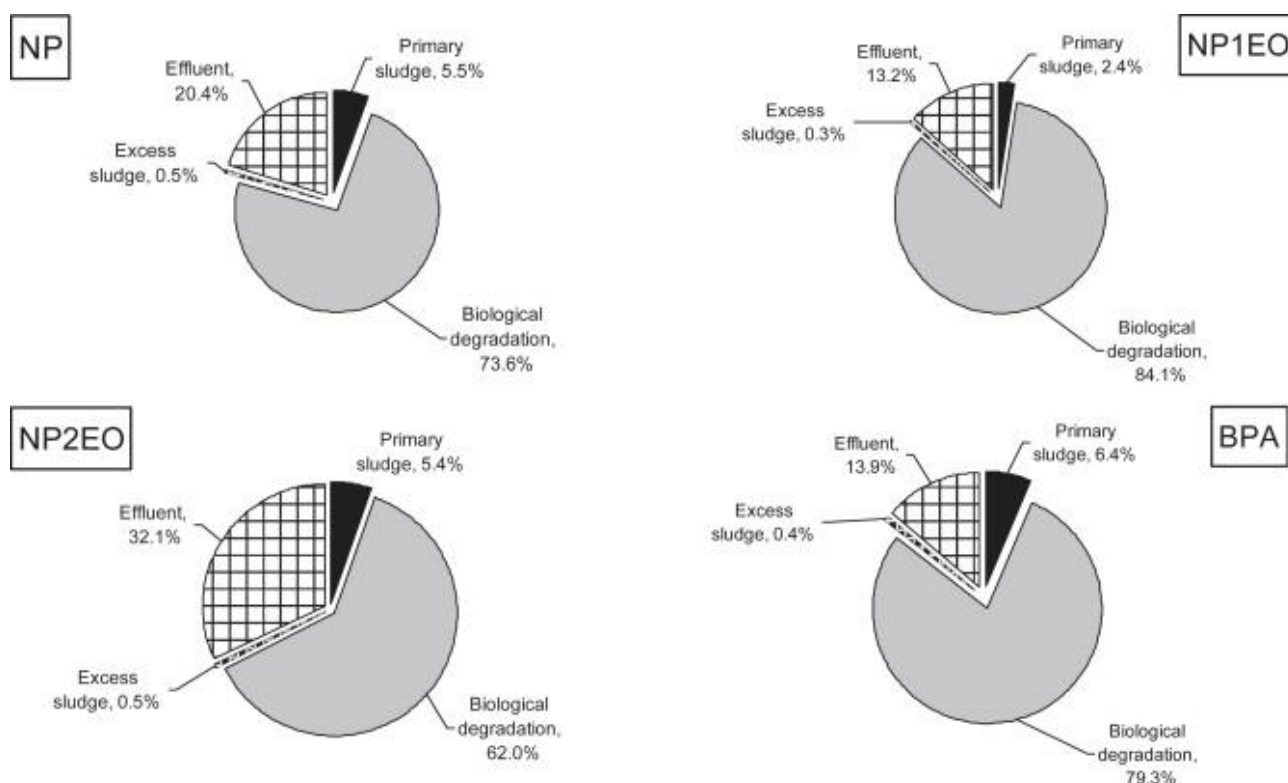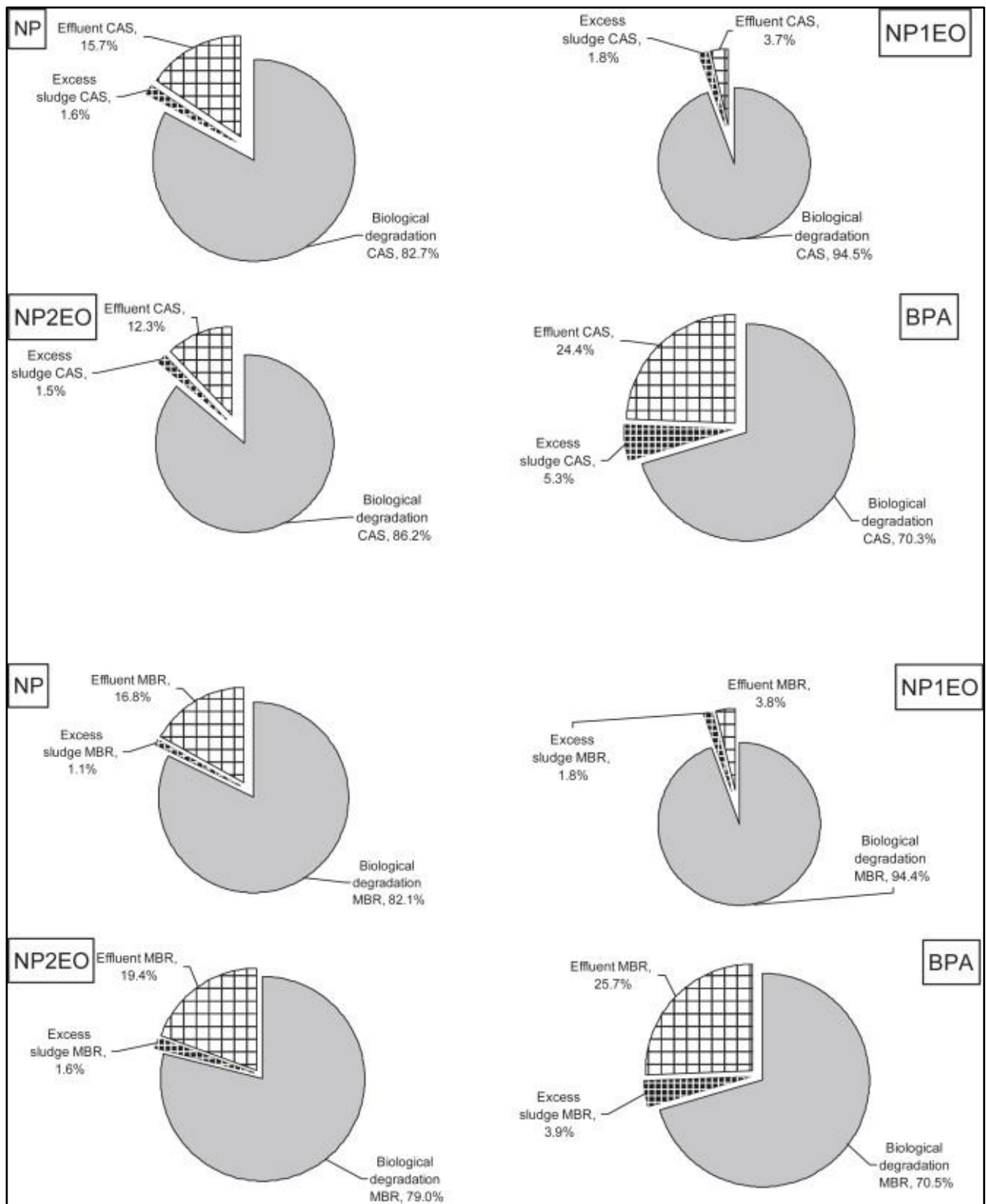Figure 4. Brescia WWTP: mass balance of trace pollutants: CAS (top) and MBR (bottom) line, respectively. "*Degraded*" mass obtained by subtracting the sum of each effluent mass flow (final effluent and excess sludge) from influent load.

BIOLOGICAL ANALYSES

Water samples (influent and both CAS and MBR effluents), taken on three different days of consecutive weeks (W1, W2 and W3) during the monitoring period, were submitted to biological assays, which were repeated twice (experiment #1 and #2). Prior to each experiment, cell responsivity to E2 was checked and a calibration curve was plotted (an example is presented in Fig. 5).

Fig. 6 shows the results of the biological analyses. It is clear that estrogenic activity was significantly reduced by both treatments, and, in five of six cases, with greater efficiency by the MBR system. This is a relevant outcome which emphasizes the importance of biological analyses: actually, while EDC (NP + BPA) concentrations were similar in outlet samples taken from both lines (Fig. 6), estrogenic activity exerted by CAS effluent was almost always higher.

Figure 5. Biological assay: calibration curve with the reference estrogen E2.

Figure 6. Brescia WWTP: comparison between estrogenic activity (measured in two different experiments and in three different days W1, W2, W3) and EDC concentration (NP + BPA). Error bars represent maximum and minimum values measured in 3 replicates in the case of biological data, while they show variation percentage in the case of chemical analyses.

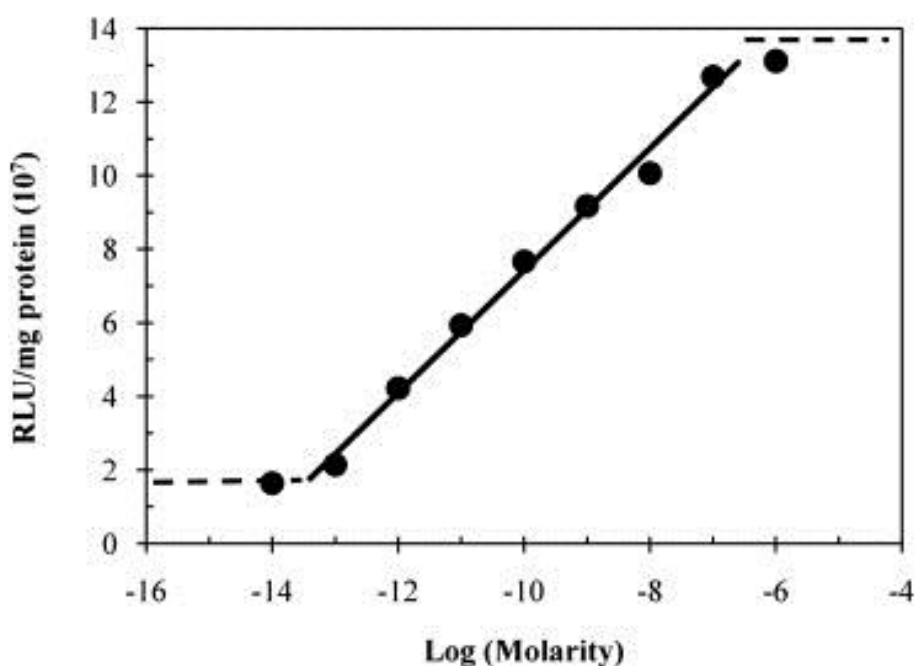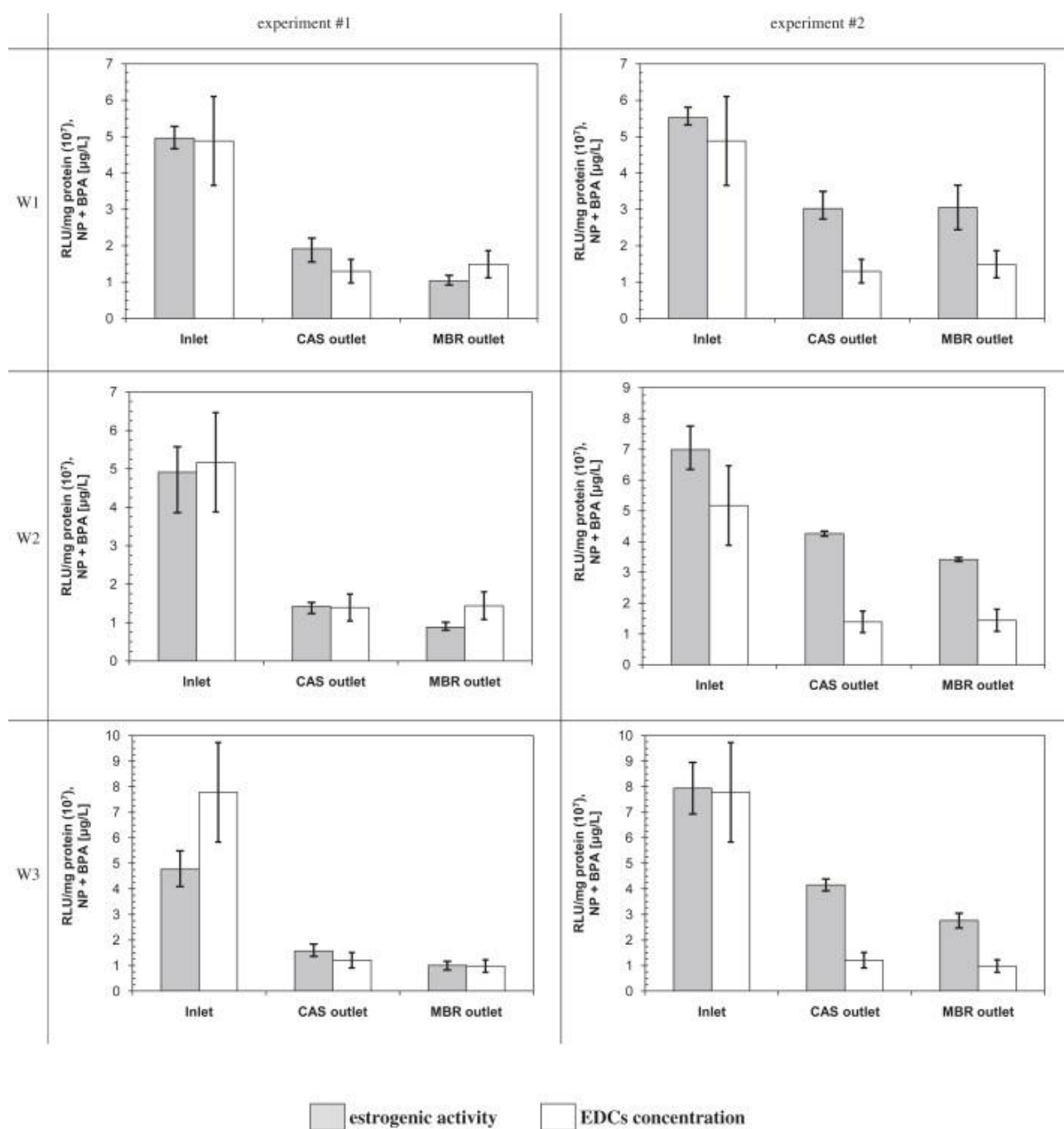## 2.3.3 OVERALL COMPARISON BETWEEN CAS AND MBR PROCESSES PERFORMANCE

Removal efficiency and residual effluent concentration of target compounds for all studied plants and processes are compared in Fig. 7.

The experimental results show that, while the Brescia CAS and MBR lines, where different sludge ages were kept (9 d for CAS and 15 d for MBR, respectively), yielded similar performances, the Verona CAS plant, having the same sludge age as the Brescia MBR line, yielded on the contrary to slightly lower removal efficiencies (apart from BPA). This phenomenon might be due to different sewage temperature (16°C and 23°C for the Verona and Brescia WWTPs, respectively).

Actually, it is well known, that sludge age and temperature are crucial parameters: Clara *et al*. (2005[b]) argue that the minimum required sludge age is 10 d at 10°C, and further increases do not lead to noticeable improvements. Moreover, several authors (e.g. Auriol *et al*., 2006; Koh *et al*., 2008; Koh *et al*., 2009) conclude that EDC removal occurs only in plants equipped with nitrification stages (as in the Brescia and Verona WWTPs). In addition, Clara *et al*. (2004) report that possible MBR efficiency improvements might be ascribed to an increase in sludge age, rather than to filtration.

Nevertheless, biological measurements carried out in this work showed that estrogenic activity was reduced to a greater extent by a MBR process with respect to CAS treatment, even if analytes were removed at a comparable level. While the reason is still under investigation; it might be attributed to metabolic pathways exhibited by different microbial consortia growing in MBR plants (Cicek *et al*., 1999; Clouzot *et al*., 2010).
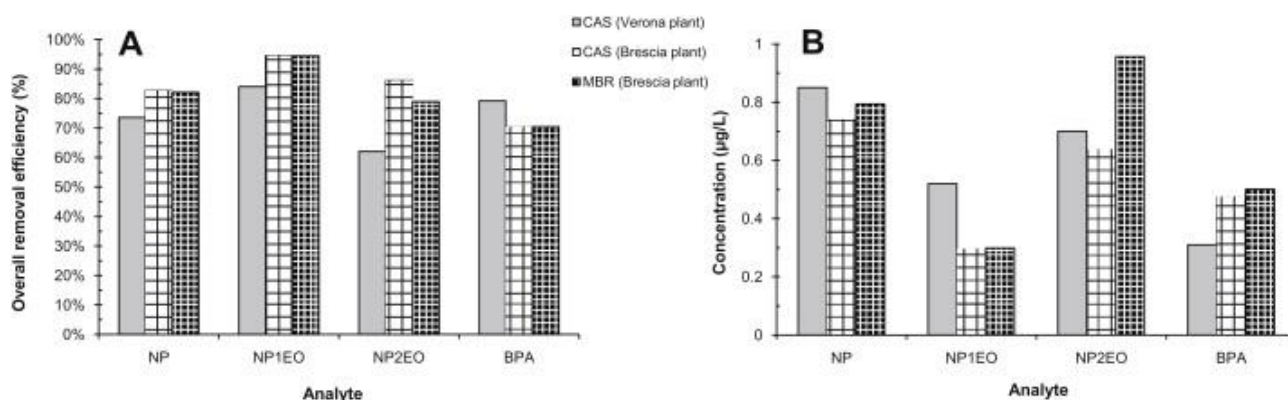
Figure 7. Comparison among studied processes: treatment efficiency (A) and effluent residual concentrations (B).

2.3.4 TERTIARY OZONATION

CHEMICAL AND MICROBIOLOGICAL ANALYSES

Actual ozone dosages (calculated based on dissolution efficiency) were 8 and 11 mg/L, respectively, during the two series of tests.

Disinfection performance was very high: total coliforms and *E. coli* were abated from 3.2 log efficiency (8 mg/L actual ozone dosage, 15 min contact time) up to 4.2 (11 mg/L actual ozone dosage, 30 min contact time), notwithstanding the initial concentration ($2.4 \cdot 10^5$ - $1.0 \cdot 10^6$ MPN/100 mL total coliforms, $4.3 \cdot 10^4$ - $1.9 \cdot 10^5$ MPN/100 mL *E. coli*).

Influent trace pollutants concentrations were in the range 0.14-0.30 µg/L and 0.20-0.43 µg/L for NP and BPA, respectively, while both NP1EO and NP2EO were below 0.20 µg/L. Time profiles of NP and BPA normalized concentration are shown in Fig. 8; NP1EO and NP2EO are omitted since they were below detection limits. Assuming first order kinetics (and under the hypothesis of plug-flow reactor), it was possible to estimate reaction rate constants, which resulted, for both pollutants, in the range 0.028-0.093 min^{-1} depending on ozone dosage.
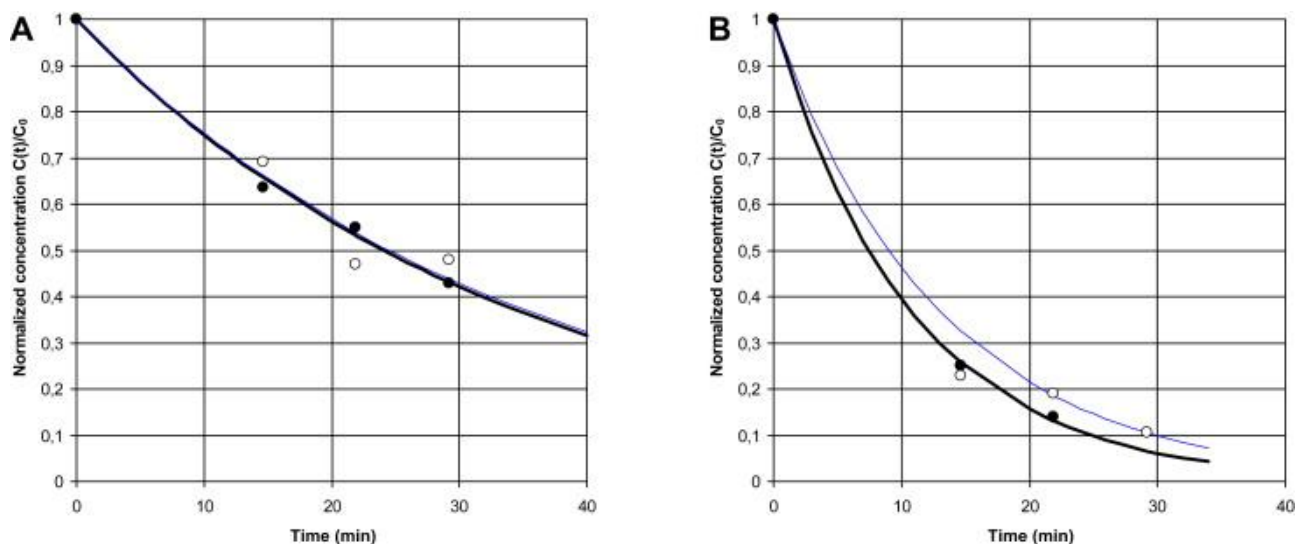
Figure 8. Ozonation: target pollutants normalized concentration *vs*. reaction time. White marks and fine line: BPA; black marks and bold line: NP. Actual ozone dosages: (A) = 8 mg/L; (B): = 11 mg/L. C_0 = influent concentration.

BIOLOGICAL ANALYSES

The influence of O_3 dosage on estrogenic activity abatement is shown in Fig. 9 (average values). Error bars indicate results obtained during different experiments (i.e., reaction time conditions). Chemical oxidation was able to reduce estrogenicity of wastewater remarkably. Nevertheless, while a higher O_3 dosage led to an appreciable improvement of EDC (NP + BPA) removal, only a slight additional reduction of hormonal activity was achieved. This may be due to the persistence of endocrine disruptors (e.g., including natural hormones) or the formation of active by-products, as recently found by other authors (Huber *et al.*, 2004; Bila *et al.*, 2007).
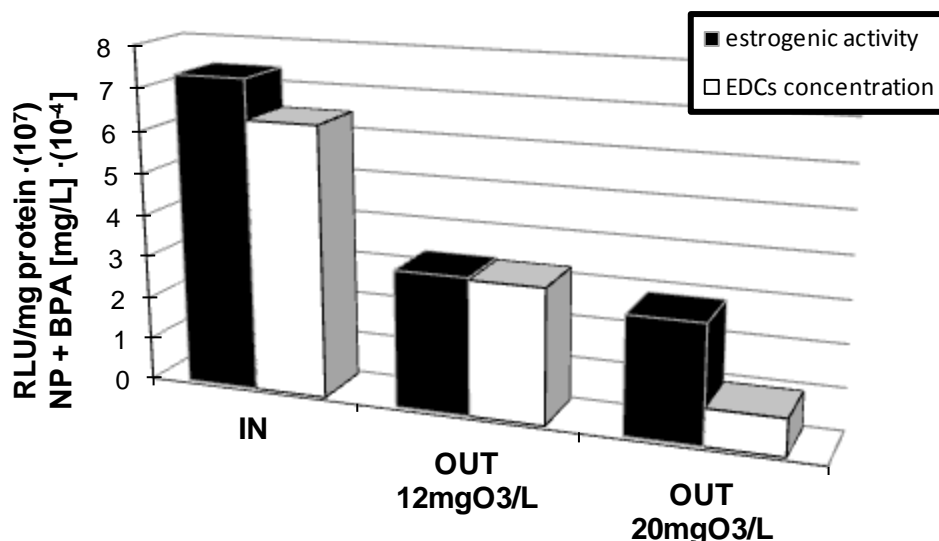
Figure 9. Ozonation: comparison between the estrogenic activity and EDC concentration (NP + BPA) as a function of O_3 dosage. Error bars indicate results obtained during different experiments (i.e., reaction time conditions).

2.3.5 ECONOMIC EVALUATION

Based on estimated rate constants, operating costs were calculated considering an initial concentration of 1 µg/L of NP and different removal percentages. Results are summarised in Figure 10. Treatment conditions required for an appreciable removal of trace pollutants are more severe with respect to those which lead to an efficient disinfection: in fact, while above 4 log *E. coli* abatement was achieved with 12 mg/L ozone addition and 30 min contact time, 70% reduction of

NP and BPA requires 42 minutes reaction time under the same ozone dosage conditions; reaction time can be reduced down to 15 minutes, if ozone dosage is increased to 20 mg/L. Estimated operating costs depend only on ozone dosage and, for considered treatment conditions, range between 2 and 4 Euro cents/m^3.
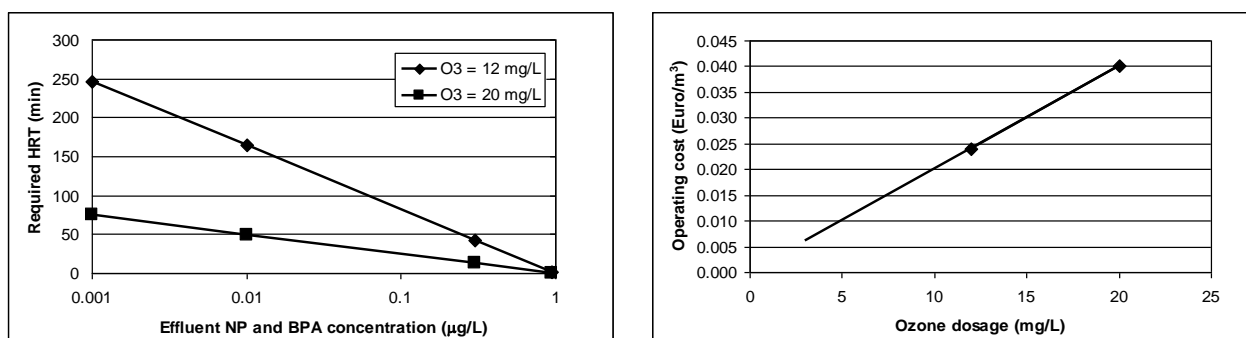
Figure 10. Ozonation. Required contact time and operating cost for achieving different effluent NP and BPA concentrations with different O_3 dosages.

2.4. CONCLUSIONS

In this work, the fate of selected trace pollutants (NP, NP1EO, NP2EO and BPA) in two full scale WWTPs was investigated.

Monitoring campaigns showed that the contribution of primary settling in the removal of studied pollutants was negligible, their content in primary sludge being quite low (<10 mg/kgTSS). Biodegraded fractions ranged from 62.0% (NP2EO, Verona plant) to 94.5% (NP1EO, Brescia plant); final effluent concentrations were always < 1 µg/L and excess sludge concentrations ≤ 5 mg/kgTSS for all analytes. Although the WWTPs considered have different process schemes (CAS and MBR, respectively) similar performances were observed. In fact this finding was expected based on the literature, since the most influential process parameters (sludge age and temperature) were always within the optimal range for EDC biodegradation.

On the contrary, biological assays showed that MBR was more efficient in estrogenicity reduction: this is a very important finding of this research, which would not have been highlighted if only chemical analysis had been performed.

As far as tertiary ozonation is concerned, chemical oxidation of trace pollutants was described by first order kinetics, rate constants being dependent on reagent dosage: for instance, a 90% removal

of BPA and NP could be achieved either after 80 min at 8 mgO_3/L, or 27 min at 11 mgO_3/L. From the economic point of view, starting from 1 µg/L NP (or BPA) concentration in the effluent of a WWTP, a further reduction of 70% (which means an effluent concentration of 0.3 µg/L, environmental quality standard for surface waters proposed by the EU Directive 2008/105/EC) can be achieved with ozonation at operating costs around 2-4 Euro cents/m^3.

Biological analyses confirmed the beneficial effect of ozonation on the reduction of estrogenicity of CAS effluent. However, unlike analytes, estrogenic activity abatement was not significantly affected by ozone dosage.

In summary, CAS treatment enabled a satisfactory reduction of EDCs and estrogenicity, thanks to adequate process conditions; a further decrease of biological activity was achieved by means of MBR and ozonation, but the latter, at the same time, yielded an additional reduction in pollutants.

Finally, the efficacy of an integrated (chemical + biological) approach in evaluating performances of wastewater treatment processes was demonstrated: bioassays account for synergistic effects of dozens of pollutants, the simultaneous determination of which might be actually unfeasible.

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CHAPTER 3.

EFFICACY OF TERTIARY OZONATION TO REMOVE ESTROGENIC AND GENOTOXIC POTENTIAL FROM A WASTEWATER CONTAINING INDUSTRIAL INPUTS

SUMMARY

3.1. INTRODUCTION

Recent advances in environmental chemistry have brought increasing focus on the presence of anthropogenic substances in the environment: even though the concentrations of these compounds are in the range of µg/L or even ng/L (therefore called "*micropollutants*"), adverse effects on human health cannot be excluded. Government and the public awareness of the impacts that these cocktails of chemicals have on aquatic resources have recently become a significant driver for reducing levels of micropollutants in the environment (EU Water Framework Directive 2008/105/EC; Rowsell *et al.*, 2010), but the lack of standardized monitoring techniques does not always allow for comparability of results between countries, nor are the results always totally reliable (Lepom *et al.*, 2009). In many cases, countries simply cannot afford to carry out expensive analytical methods.

One such group of anthropogenic substances is represented by endocrine-disrupting chemicals (EDCs). EDCs are defined as "exogenous agents that interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body, which are responsible for the maintenance of homeostasis, reproduction, development and behavior" (EPA, 1997); in other words, they can alter the normal function of the endocrine system, which is responsible for growth and development in vertebrates.

The EDCs best known to produce specifically estrogenic effects are the naturally occurring steroidal estrogens, i.e. hormones such as 17β-estradiol (E2), estrone (E1) and estriol (E3) from urinary excretion; synthetic compounds used in medicine, as contraceptives and in some hormonal therapies, i.e. 17α-ethinyl-estradiol (EE2); and synthetic organic compounds that have been shown to interact with estrogen receptors, including the alkylphenols (poly-ethoxylates) AP(EOs), mainly nonylphenol (NP) and octylphenol (OP), which are more estrogenically potent than their short chain ethoxylated precursors, and other phenolic compounds, mainly bisphenol A (BPA) (Hu and Chen, 2006; Frassinetti *et al,*. 2011). Table 1A summarizes the main estrogenic compounds and their relative strength, having taken a value equal to 100 for the more potent estrogen, estradiol (deeper details are reported in *Materials and Methods* section).

In particular, they may interfere with regulation mechanisms controlled by estrogens by competing for the binding sites of the estrogen receptors: Routledge *et al.* (1998) documented that steroidal hormones can induce feminization in fish and other aquatic organisms at concentrations of 1 ng/L of Estradiol EQuivalent (EEQ) or less; on the contrary, Witters *et al.* (2001) reported that significant reproduction effects in male and female fish might appear at levels above 10 ng/L-EEQ;

however, though at very low concentrations, every xenoestrogen, even weak one, may add incrementally to the total estrogenic effect (Rajapakse *et al.*, 2002).

Furthermore, among many environmental contaminants, EDCs residues in the environment are of emerging concern also because of their intrinsic genotoxic activity, which may cause adverse effects, particularly at chronic exposure: EDCs are capable of interfering with regulatory systems of different types of cells and of causing DNA damage, and chronic exposure to low doses of these chemicals may increase the risk for cancer development (Frassinetti *et al.*, 2011).

In addition to these documented adverse effects on human health, the presence of estrogenic EDCs in the environment is becoming an increasingly serious problem, because they are widely prevalent in aquatic environments and present in higher concentrations than other EDCs: indeed, APEOs products are reported to comprise approximately 6% of the total surfactant production in the world (Ning *et al.*, 2007) and, of all the APEOs production, 80–85% is sold as nonylphenol ethoxylates (NPnEOs, where *n* is the number of ethoxylic units in the molecule), non-ionic surfactants widely used in several industrial applications, such as textile and leather processing, paper industry, formulation of pesticides, paints and washing cleaners (Johnson *et al.*, 2005); NPnEOs are easily bio-converted into short chain NPnEOs (*n* = 1-3) and NP, but these compounds appear to be recalcitrant to further microbial attack. BPA, on the contrary, is widely used as a monomer for epoxy resins and polycarbons (Snyder *et al.*, 2003).

The application of conventional wastewater treatment does not provide complete elimination of all micropollutants and, subsequently, residues of EDCs enter the aquatic ecosystem through wastewater: natural waters (both surface and groundwater) represent the most affected environmental media and the most significant exposure pathways (Ning *et al.*, 2007), because of the presence of EDCs in WasteWater Treatment Plants (WWTPs) effluents. This poses new challenges for wastewater purification (Schaar *et al.*, 2010). Indeed, the continuous disposal of WWTPs discharges results in a risk for aquatic systems and consequently for human health. In order to prevent this kind of water pollution, an advanced treatment downstream of biological process effluent should be implemented.

Several technologies for further micropollutants removal, such as ozonation (Huber *et al.*, 2005), advanced oxidation (Huber *et al.*, 2003), activated carbon (Westerhoff *et al.*, 2005) and filtration (Poseidon, 2004), have been investigated. In particular, the application of ozone in laboratory, pilot and full-scale experiments proved to be a suitable technology for EDCs removal (Leusch *et al.*, 2005; Ning *et al.*, 2007; Schaar *et al.*, 2010). From the chemical point of view, ozone reacts with organic pollutants through direct and indirect pathways, with molecular ozone involved in the former and hydroxyl radicals involved in the latter. Under acidic conditions, or in the presence of

radical scavengers which inhibit the chain reaction that causes the decomposition of molecular ozone, direct ozone reactions predominate. Under neutral pH or basic conditions, or in the presence of solutes that promote the radical chain reaction and hydroxyl radical formation, indirect reactions predominate because hydroxyl radical reactions are non-selective and extremely rapid (Ning *et al.*, 2007).

In general, however, it is difficult to quantify the overall impact of ozonation only on a chemical basis (i.e., by measuring the concentrations/loads of individual compounds): for example, NPnEOs present a mixture of nonylphenol ethoxylates of different chain lengths, which can be metabolized into various intermediates during the ozonation process; in any case, full mineralization of the EDCs during ozonation is difficult to achieve. Consequently, oxidation products will develop and be present in the treated waters, but very little is known about these oxidation intermediates and their biological activity (Ning *et al.*, 2007).

An alternative approach consists in the direct measure of the change in estrogenic activity, as a result of the ozonation process, through *ad hoc* bioassays: although chemical analyses can successfully reveal the presence of EDCs in the aquatic environment, they are generally focused on determining only target substances in the matrices of interest. Because of the large number of EDCs that can be present in a complex environmental sample, target chemical analyses are not always sufficient to determine all EDCs it may contain. Moreover, chemical analyses do not consider mutual and synergic interactions and the biological effects of the whole sample. On the contrary, bioassays are based on the interaction between EDCs and estrogenic receptors and can measure the total estrogenic activity of a sample (Bicchi *et al.*, 2009), regardless of the individual compounds.

Another strength of estrogenic bioassays lies in the consideration that the removal of a target xenobiotic compound does not necessarily means that the biodegradation process is detoxifying, as toxic intermediates or dead-end products may be produced below the threshold detection level or may not be detected by the analytical system employed (Frassinetti *et al.*, 2011): when biological response being measured is highly sensitive, *in-vitro* bioassays can also account for compounds which can exert a biological effect at concentration less than analytical detection limits (Hu and Chen, 2006).

In addition, another biological effect can be assessed: some wastewater pollutants, indeed, display mutagenic activity in different organisms, inducing point mutations, chromosome breakage and rearrangement, and DNA damage. Studies on surface water receiving effluents from wastewater treatment plants have shown that genotoxins can accumulate in the environment and have adverse effects on water biocenosis and, consequently, on human health.

Wastewater treatments, particularly ozonation, have generally been shown to reduce or eliminate genotoxicity. In some cases, however, wastewater treatments have been shown to enhance genotoxicity (Isidori *et al.*, 2007; Petala *et al.*, 2008; Misik *et al.*, 2011).

As well as for estrogenicity assessment it is extremely difficult to quantify the genotoxic risk associated with such chemical pollutants, because they usually occur at concentrations too low to allow analytical determination. In addition, using only physico-chemical analysis, it is impossible to predict the mutagenic properties of complex water samples, especially if synergistic, antagonistic or potentiating effects between the components occur (Zegura *et al.*, 2009). Alternative methodologies to characterize the genotoxicity of complex water samples are biological tests, which produce a global response to the complex mixture of chemicals, regardless of prior knowledge of the mixture composition or its chemical properties: short-term mutagenicity tests, which are rapid and predictive of carcinogenic activity, allow an evaluation of the presence of mutagenic compounds in complex environmental mixtures, such as wastewater. The evaluation of effluents using mutagenicity assays may provide useful data for risk assessment and also on the effectiveness of wastewater treatment processes.

In conclusion, simple and effective bioassays aimed at truly establishing the detoxification efficiency of a treatment process are looked forward, and a framework for bioanalytical quantification of organic micropollutants should be applied, with bioassays for specific modes of toxic action that are indicative for groups of chemicals of particular relevance for human (and environmental) health, primarily including aspects of estrogenicity and genotoxicity (Macova *et al.*, 2010).

In this work, an integrated assessment procedure, based on both chemical and biological analyses, was adopted to evaluate the performance of a tertiary chemical oxidation (with ozone) in order to remove target EDCs (NP, NP1EO, NP2EO and BPA) and biological (estrogenic and mutagenic) activity from wastewater.

Experimental work was conducted at a full-scale WWTP (daily treated flow-rate: $\approx$ 30,000 m^3/d) equipped with a tertiary ozonation stage, treating both domestic and industrial (textile) wastewater.

The primary objective of this study was to provide key baseline information concerning the concentrations of individual estrogenic compounds (measured with chemical analysis) and the estrogenicity (measured with *in vitro* bioassay) of untreated and treated effluent, together with the removal efficiencies of ozonation process. Furthermore, an analysis of the correlation between bio- and chemical assays was performed.

As estrogenic activity is concerned, human breast cancer MCF-7 based reporter gene assay (E-SCREEN) was chosen as *in vitro* bioassay due to its high concentration of estrogenic receptors and

sensitivity (Pons *et al.*, 1990; Urban *et al.*, 2001; Soto *et al.*, 2006; Higashi *et al*, 2007). Table 1B, furthermore, provides a matrix summarizing the performance of the most common estrogenic bioassays and their identified strengths and weaknesses: it is highlighted that each bioassay has its advantages and limitations, and may be suitable for testing estrogenic activity in environmental samples as long as their restrictions and technical requirements are clearly understood by the researcher.

Compound name	Abbr.	Source	Estrogenic strength (%)
Estrone	E1	Natural hormones	3
Estradiol	E2	Natural hormones	100
Estriol	E3	Natural hormones	15
Ethynil-estradiol	EE2	Contraception/hormonal therapies	90
Octylphenol	OP	Degradation of OPnEO	0.015
Octylphenol monoethoxylates	OP1EO	Surfactants	0.000065
Octylphenol diethoxylates	OP2EO	Surfactants	0.000081
Nonylphenol	NP	Degradation of NPnEO	0.004
Nonylphenol monoethoxylates	NP1EO	Surfactants	0.000013
Nonylphenol diethoxylates	NP2EO	Surfactants	0.000014
Bisphenol A	BPA	Resin production	0.003

Table 1A. Main estrogenic compounds, source and relative potency.

assay	YES	ER-CALUX	MELN	KBluc	E-SCREEN
performance					
environmental samples EEq	+	+++	+	++	++
likeness to other assays	++	+++	−	+++	+++
method quantification limit (MQL)	−	+++	+++	+++	+++
predicted vs measured comparison	+	+++	+	++	++
strengths and weaknesses					
analysis of model compounds	+++	+++	++	+++	+++
analysis of environmental samples	−	+++	+	++	+++
ease of use	++	+	+	+	+
simple training	++	−	−	−	−
low maintenance and consumables cost	+++	−	+	+	+
free access to cell line for nonprofit use	+++	−	+++	++	+++
sensitivity	−	+++	++	++	++
robustness	−	++	++	++	++
reproducibility	++	+++	+	++	++
maturity (widespread use)	+++	++	+	+	+++
high-throughput screening	+++	+++	+++	+++	+++
quick results	++	++	++	++	−

Notes: "-", below average; "+", fair; "++", good; "+++", excellent;

Table 1B. Summary of performance, strength and weaknesses of the most common estrogenic bioassays (adapted from Leusch *et al.*, 2010).

Finally, wastewater genotoxicity (and the related effect of ozonation treatment) was evaluated with three different tests, in order to assess the ability to induce genetic damage in target cells of different organisms (bacterial, plant and mammalian cells), to detect point and chromosomal mutations, and DNA damage.

3.2. MATERIALS AND METHODS

3.2.1 THE ANALYZED WWTP

Fino Mornasco (Como, Italy) WWTP is a plant (design size 140,000 p.e.) treating domestic and industrial (textile and printing) wastewaters; specifically, the industrial discharge contributes up to 50% (as COD load) to the total influent sewage. Textile wastewaters are typically characterized by strong color and presence of recalcitrant compounds, such as dyes, surfactants and sizing agents (Chiavola, 2009).

Moreover, it has to be strongly underlined that the plant receives wastewaters from a combined sewer system, and run-off and infiltration waters contributed in a predominant extent to the total influent flow during the experimentation: Figure 1 and Table 2 display the data recorded during the monitoring period.
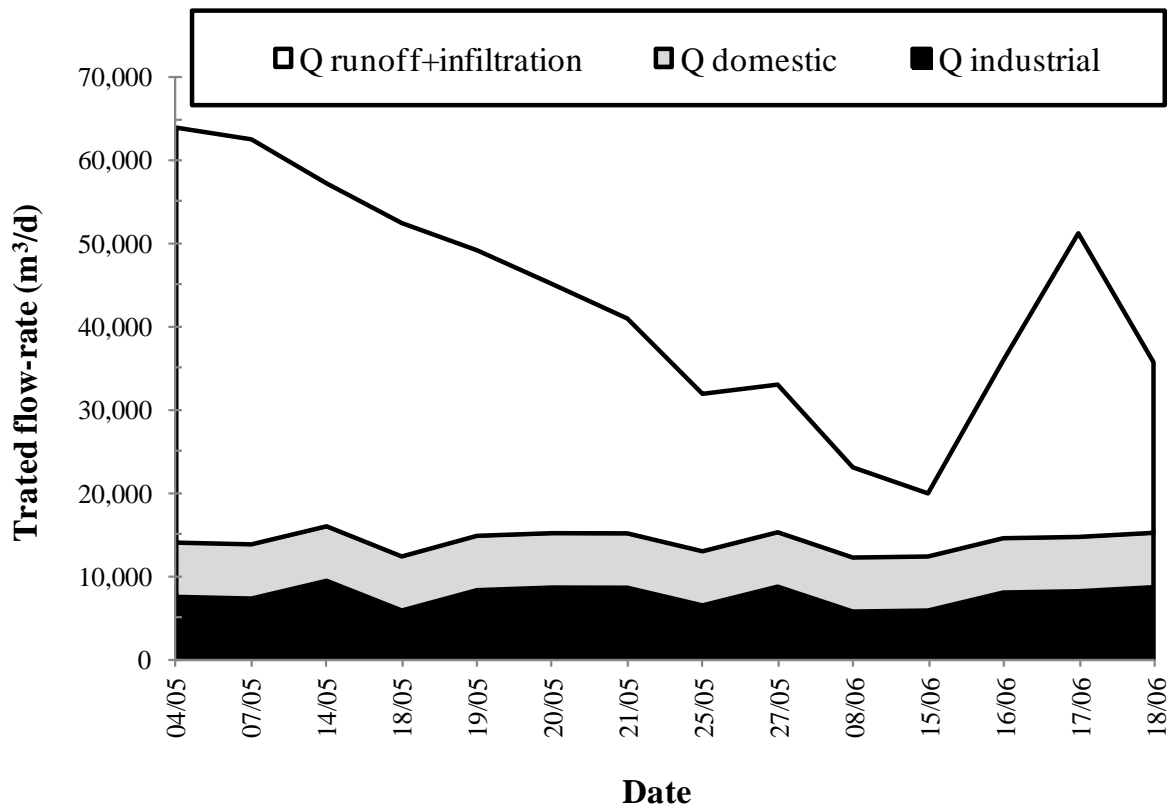
Figure 1. Treated flow-rate during the monitoring campaign, split up for source.

Q treated [m³/d]	SOURCE [%]		
	Run-off + infiltration	Industrial	Domestic
43,000 (± 13,500)	63.4 (± 12.1)	19.1 (± 5.6)	17.5 (± 6.7)

Table 2. Treated flow-rate during the monitoring campaign, split up for source (mean ± standard deviation).

The process scheme of the plant (Figure 2) includes both conventional and tertiary treatment: the former consists of pre-denitrification (volume = 3,600 m^3), oxidation-nitrification (volume = 10,500 m^3) and secondary settling, the latter of a coagulation-flocculaton-sedimentation stage (for suspended solids reduction) and a chemical oxidation with ozone - the object of the experimentation (volume = 2,000 m^3, O_3 dosage= 11mg/L, contact time (on a Q_{design}=1,300 m^3/h) = 90 min), for the abatement of residual color and surfactants; the effective contact time of ozonation basin decreased during the experimentation down to around 60 min (average value), because of the higher treated flow-rate.

The sludge treatment line consists of: dynamic thickening and mechanical dewatering; the ozonation of a fraction ($\approx$ 20%) of the recycle sludge flow, moreover, allows a remarkable improvement of sludge settleability.

The following are the main operational data (typical values referred to the yearly average). Influent characteristics (after screens and grit-oil removal): 300 mgCOD/L, 150 mgBOD$_5$/L, 110 mgTSS/L, 35 mgN$_{TOT}$/L, 3,5 mgP$_{TOT}$/L, 10 mg/L surfactants (90% non ionic); effluent characteristics (after ozonation): 50 mgCOD/L, 10 mgBOD$_5$/L, 10 mgTSS/L, 16 mgN$_{TOT}$/L, 1.2 mgNH$_4^+$-N/L, 12 mgNO$_3^-$-N/L, <0.1 mgNO$_2^-$-N/L, 0.8 mgP$_{TOT}$/L, 0.6 mg/L surfactants.
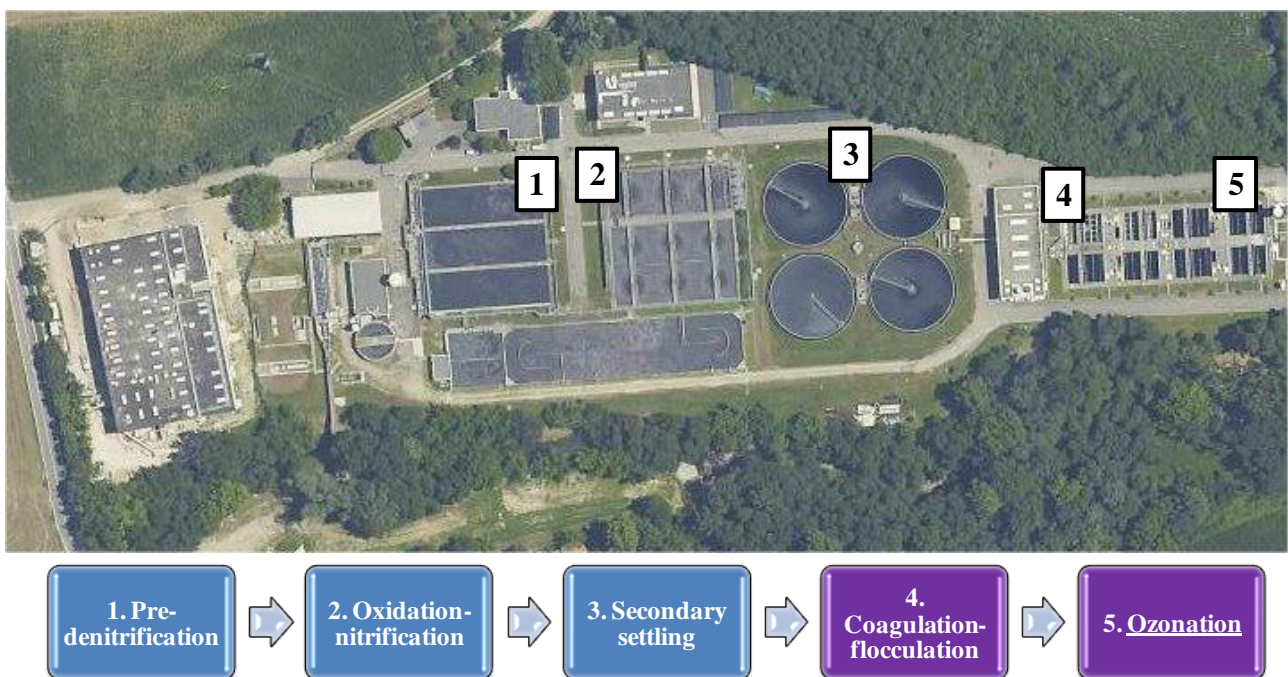
Figure 2. Aerial view and treatment units of the analyzed WWTP.

3.2.2 THE MONITORING CAMPAIGN

The monitoring campaign was conducted in 14 days of six consecutive weeks during spring-time (dry and wet weather, alternatively), from 4 May to 18 June, on the inlet and outlet flow of the tertiary ozonation stage.

Samples were collected daily, over 24 hours (a rate of 75 mL every 30 min), by automatic refrigerated auto-samplers equipped with Teflon pipes and dark glass containers, except for mutagenic analyses (grab samples).

The measured EDCs are listed in Table 3, whose chemical structure is, moreover, displayed in Figure 3.

Substance	Abbr.	CAS No.	EQS [μg/L]	List in Directive 2008/105/EC
Bisphenol A	BPA	80-05-7	1.6^{aa}	Substances subject to review for possible identification as priority substances or priority hazardous substances
Nonylphenol	NP	25154-52-3	0.3^{aa} / 2.0^{mac}	Priority substances, identified as priority hazardous substance
Nonylphenol monoethoxylate	NP1EO	104-35-8		
Nonylphenol diethoxylate	NP2EO	20427-84-3		

Table 3. Analyzed compounds, CAS number and Environmental Quality Standards (EQS), as annual average (aa) and maximum allowable concentration (mac) for inland surface waters (EU Water Framework Directive 2008/105/EC for NP; other National EQS - Austrian BGBl. II 96, 2006 - for BPA).
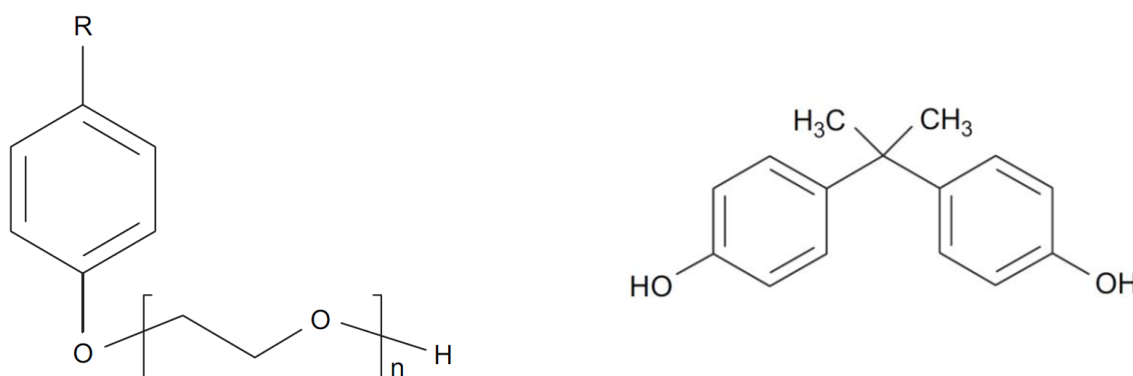

Figure 3. Structure of NP[nEOs] (left), where R is the alkyl-chain (i.e. nonyl-, C_9H_{19}), and BPA (right).

Furthermore, estrogenic and mutagenic activities were measured on several samples collected during the monitoring campaign.

Table 4 summarizes the monitoring campaign scheme and the analyses carried out during the experimentation.

	date	weather conditions	chemical analysis	biological analyses	
				estrogenic activity	*mutagenic activity*
week 1	04 May	heavy rain	X		
	07 May	heavy rain	X		
week 2	14 May	heavy rain	X		
week 3	18 May	dry	X		
	19 May	dry	X		
	20 May	dry	X	X	
	21 May	dry	X	X	
week 4	25 May	dry	X		
	27 May	dry	X	X	
week 5	08 June	dry	X	X	
week 6	15 June	rain	X		
	16 June	rain	X		
	17 June	dry	X		
	18 June	dry	X	X	X

Table 4. Summary of the chemical and biological analyses carried out during the experimentation.

3.2.3 CHEMICAL ANALYSES AND DATA PROCESSING

The method of Gatidou *et al*. (2007) was successfully adopted for the extraction of analytes from liquid phase.

The following chemicals were purchased from Sigma Aldrich (Taufkirchen, Germany): a) standard reagents: bisphenol A, NP1EO, NP2EO, 4-NP technical mixture of isomers, as proposed by ISO 18857-1 (2005); b) derivatization reagents: MSTFA and pyridine; c) internal standard: bisphenol A-d_{16}. Influent samples were filtered on glass fiber filters (Whatman GF/A ϕ=1.6 μm). Liquid samples were submitted to enrichment on SPE C18 (Supelco, Bellefonte, USA) and consequent elution.

Derivatization was performed with 900 μL MSTFA (5% in isooctane) and pyridine (100 μL).

Instrumental analysis was conducted using a gas-chromatograph 5975B inert XL EI/CI MSD equipped with a split/splitless injector and autosampler (Agilent Technologies, Palo Alto, USA).

The RDS% (Recovery Determination Standard) varied from 7.3 to 13.7, depending on the target molecule; mean recovery percentage referring to internal standard of BPA-d_{16} was more than 80%;

the lowest concentration of the calibration curve was equal to 50 ppb for each pollutant (for further details about the analytical procedure, see Pedrazzani *et al.*, in preparation).

Data were, then, elaborated by means of the SPSS 15.0 statistics program (SPSS for Windows, Chicago, IL) and a linear regression analysis (ANOVA: one-way ANalysis Of VAriance) was performed; differences were considered to be significant for $p<0.05$.

3.2.4 BIOLOGICAL ANALYSES

ESTROGENIC ACTIVITY

BIOASSAY

The pollutant extraction and clean-up procedure was the same as reported for the chemical analyses; extracts were resuspended in 1 mL DMSO (dimethyl-sulfoxide). Human breast cancer cell line MCF-7 stably transfected with the ERE-tK-LUC construct (kindly supplied by Mikko Unkila**,** Hormos Medical Corporation, Turku, Finland) was maintained in DMEM (Modified Dulbecco's Medium, Lonza, Milan), supplemented with 10% fetal bovine serum, at 37°C and 5% CO_2. 24 hours before treatment with pollutants, cells were plated at a density of $2,5 \cdot 10^5$ cells/cm^2 in twenty four-well plates containing phenol red free DMEM and 5% charcoal-stripped serum.

Cells were treated with either reference estrogen (E2 - Estradiol) or pollutants culture medium solutions; dishes were kept at 37°C for 24 h (Chau *et al.*, 1998; Spink *et al.*, 2003). As controls, cells were treated with DMSO alone (E2 control) or ethanol alone (for pollutants treated cells). Cells were then harvested and lysed in Passive lysis buffer (Promega, Italy). Lysate was spun for 15 s at 12,000 *g* and supernatant submitted to luciferase activity quantification (Luciferase Assay System, Promega, Italy), which was performed in triplicate by means of a luminometer (GloMAx, Promega, Italy) over 10 s (de Wet *et al.*, 1987), expressed as RLU (Relative Light Units) and normalized towards protein content (Bradford assay, Biorad, Italy). Reference estrogen E2 (dissolved in absolute ethanol) was employed for calibration curve definition, at concentrations corresponding to physiological/sub-physiological doses, i.e., from 10^{-15} to 10^{-8} M (the lower approaching LOD - Limit of Detection).

The standard curve for estradiol was fitted (sigmoïdal function) using Graphpad Prism 5.0 software (GraphPad Software, Inc., USA), which allowed to calculate parameters like EC_{50} (and 95% confidence limits), maximum and minimum values, useful to define the detection limits.

BIOASSAY *VS* CHEMISTRY

The estrogenicity measured through the bioassay was compared to the one predicted from chemical analysis, calculated as Pred = $\Sigma(EEF \times conc)$, where *EEF* represents the relative estrogenic potency (i.e., the Estrogenic Equivalency Factor) and *conc* is the concentration as determined by analytical chemistry. The average of the relative potencies published in the literature, related to the bioassay applied in this research (Furlong *et al.*, 2010; Leusch *et al.*, 2010) was used (Table 5): it clearly shows that, among the analyzed compounds, NP and BPA exert the most estrogenic strength.

Compound	EEF	Literature reference
NP	4.0·E-05	Leusch *et al.*, 2010
NP1EO	1.3·E-07	Furlong *et al.*, 2010
NP2EO	1.4·E-07	Furlong *et al.*, 2010
BPA	3.0·E-05	Leusch *et al.*, 2010

Table 5. Estrogenic Equivalency Factor (EEF) of analyzed compounds, relative to model compound 17β-estradiol (E2).

MUTAGENIC ACTIVITY

MUTAGENICITY TESTS

Three different mutagenicity tests were carried out on the wastewater in order to assess their behaviour in terms of ability to induce genetic damage in target cells of different organisms (bacteria, plant cells and human leukocytes).

SAMPLING

Wastewater samples (40 liters per sample) collected before and after ozonation treatment were adsorbed on trifunctional silica C18 cartridges and tested using Ames test to evidence point mutations in bacteria, and Comet test to evidence primary DNA damage in human leukocytes. Unconcentrated wastewater (2 liters) was assayed using a plant mutagenicity test to evidence chromosomal mutations in root cells of *Allium cepa*.

WASTEWATER CONCENTRATION

Wastewater samples (40 L) were passed on filter paper (Whatman 5) to eliminate the suspended solids, acidified with hydrochloric acid at pH 3.5, and passed on trifunctional silica C18 cartridges (Sep-Pak Plus tC18 Environmental Cartridges, Waters Chromatography) according to US EPA 525.2 method (EPA1994). The cartridges had previously been washed with 5 ml of ethyl acetate, 5 ml of acetone, 5 ml of methanol and 10 ml of distilled water. Two liters of wastewater samples were adsorbed on each cartridge (flow 10 ml/min), which were then eluted with 5 ml of ethyl acetate, 5 ml of acetone and 5 ml of methanol. The eluates were reduced to a small volume by means of a rotating vacuum evaporator, mixed and dried under nitrogen flow. The dry residue was dissolved in dimethylsulfoxide (DMSO) and stored in the dark at -20°C (Monarca *et al.*, 2002).

Distilled water (40 L) were adsorbed on tC18 cartridges (blank cartridge) to exclude effects related to concentration method.

AMES TEST

Wastewater concentrates were tested in duplicate at increasing doses (corresponding to 0.05, 0.1, 0.5, 1, e 2 L of water per plate) with *Salmonella typhimurium* strains TA98 and TA100, with and without exogenous metabolic activation by Aroclor-induced rat liver S9. The experimental procedure was the standard preincubation method (Ames et al., 1975; APHA, 1998; Maron and Ames, 1983). Salmonella TA98 strain detects frame-shift mutagens and TA100 strain responds to base-pair substitution.

100 ml of overnight bacterial culture in nutrient broth together with the water extracts dissolved in DMSO with and without 500 ml of S9 Mix were added to 2 ml of top agar with 0.5 mM histidine and biotine. The content obtained was mixed and poured onto minimal medium plates. Revertant colonies were hand counted after 72 h of incubation at 37 °C. Blank cartridge was tested too. All experiments were conducted in duplicate.

Positive and negative controls were included in each assay. For positive control 2-nitrofluorene for TA98 without S9 (10 μg/plate), sodium azide for TA100 without S9 (10 μg/plate), and 2-aminofluorene for both strains with S9 mix (20 μg/plate) were used, respectively. DMSO was used as negative control.

The data obtained were the average of duplicate plates and were expressed as mutagenicity ratio, dividing the revertants/plate by the spontaneous mutation rate. Results were considered positive if two consecutive dose levels or the highest non-toxic dose level produced a response at least twice

that of the solvent control, and at least two of these consecutive doses showed a dose-response relationship (APHA, 1998).

COMET ASSAY

The single cell gel electrophoresis (SCGE) assay, or comet assay, detects the primary DNA damage (Singh *et al*., 1988). Human leukocytes were treated at increasing doses (corresponding to 0.05, 0.1, 0.5, and 1 L equivalent) with organic extracts of wastewater at 37°C for 1 hour. Negative (distilled water) and positive (ethyl methanesulfonate, 2 mM) controls were performed. After treatment, the assay was performed only on samples with viability >70%, as recommended in the International Workshop on Genotoxicity Test Procedures (Tice *et al.*, 2000). Blank cartridge was tested too.

After slide preparation and cell lysis, DNA was subjected to 20-minute unwinding and 20-minute electrophoresis (pH>13, 0.78 V/cm and 300 mA). The slides stained with 75 µl of ethidium bromide (10 µg/ml) e were examined under a fluorescence microscope (Olympus CX 41RF) equipped with a BP 515-560 nm excitation filter and an LP 590 nm barrier filter. Fifty randomly-selected cells per slide (two slides per sample) were analyzed. The extent of DNA migration was evaluated by both "visual score" (based on visual classification of DNA damage) and the comet parameter "tail intensity" (percentage of DNA migrated in the tail) detected by an automatic imaging system (Komet 5, Kinetic Imaging Ltd). Significance of the effect of each dose against the negative control was determined using Dunnett's test; the dose-effect relationship for each detergent was evaluated by linear regression analysis.

ALLIUM CEPA TEST

Unconcentrated wastewater was assayed with *Allium cepa* test to detect micronuclei formation in root cells (Ma *et al.*, 1995).

In a preliminary toxicity assay, 12 equal-sized young bulbs of onion were exposed for 96 hours in the dark to different dilution of wastewater (undiluted, 1:2, 1:10, 1:100 e 1:1000). Root length mean was used to calculate the EC_{50} value and to identify the dilutions to undergo the *Allium cepa* genotoxicity assay, the highest of which corresponded to the EC_{50} value found (Fiskesjo, 1985; Fiskesjo, 1993). Other macroscopic parameters (turgescence, consistency, colour change, root tip shape) were used as toxicity indexes.

The *Allium cepa* micronucleus test was performed using six equal-sized young bulbs per sample (Ma *et al.*, 1995). After 48-hour pre-germination in mineral water, the bulbs were exposed to

undiluted, 1:2 and 1:10 dilution for 24 hours. They were then replaced in mineral water for 44 hours of recovery time, fixed in acetic acid and ethanol (1:3) for 24 hours and lastly stored in 70% ethanol. Feulgen staining was carried out on roots. Distilled water was used as negative control and maleic hydrazide (1 mg/L, 6-hour exposure) was used as positive control.

Five roots of each sample were considered for microscopic analysis (400X magnification): 10000 cells (2000 cells/slide) were scored for mitotic index (as a measure of cellular division and therefore of sample toxicity) and 20000 cells (4000 cells/slide) were scored for micronucleus frequency. The results were reported as number of micronuclei per 100 cells and the data were analyzed using the χ^2 and Dunnett's tests. All *Allium cepa* experiments were performed in duplicate.

3.3 RESULTS AND DISCUSSION

3.3.1 CHEMICAL ANALYSES

EDCS CONCENTRATION AND REMOVAL EFFICIENCIES

Figures 4 and 5 report the concentration of, respectively, NP and BPA, NP1EO and NP2EO, in the inlet and outlet flow of the ozonation basin.

It has to be underlined that several values were not taken into account, because of two possible reasons:

- the measure was under the limit of detection;
- the internal standard recover (BPA-d_{16}) was excessively low (under 50%), as a consequence of the matrix complexity and the presence of interfering compounds (due to the industrial component of wastewater).
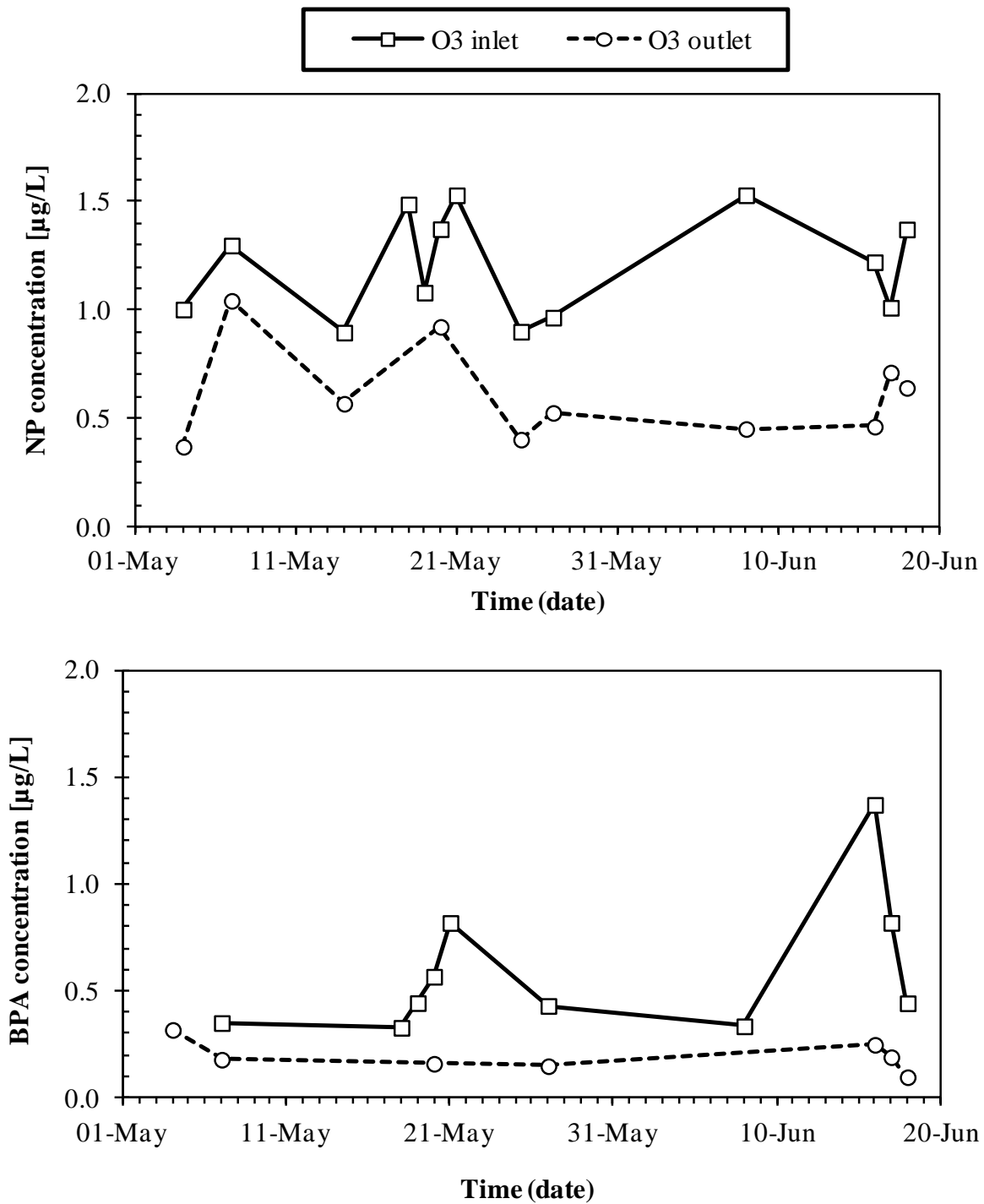
Figure 4. Chemical analysis: daily average concentration of NP and BPA (influent - solid line- and effluent -dashed line - of ozonation basin).
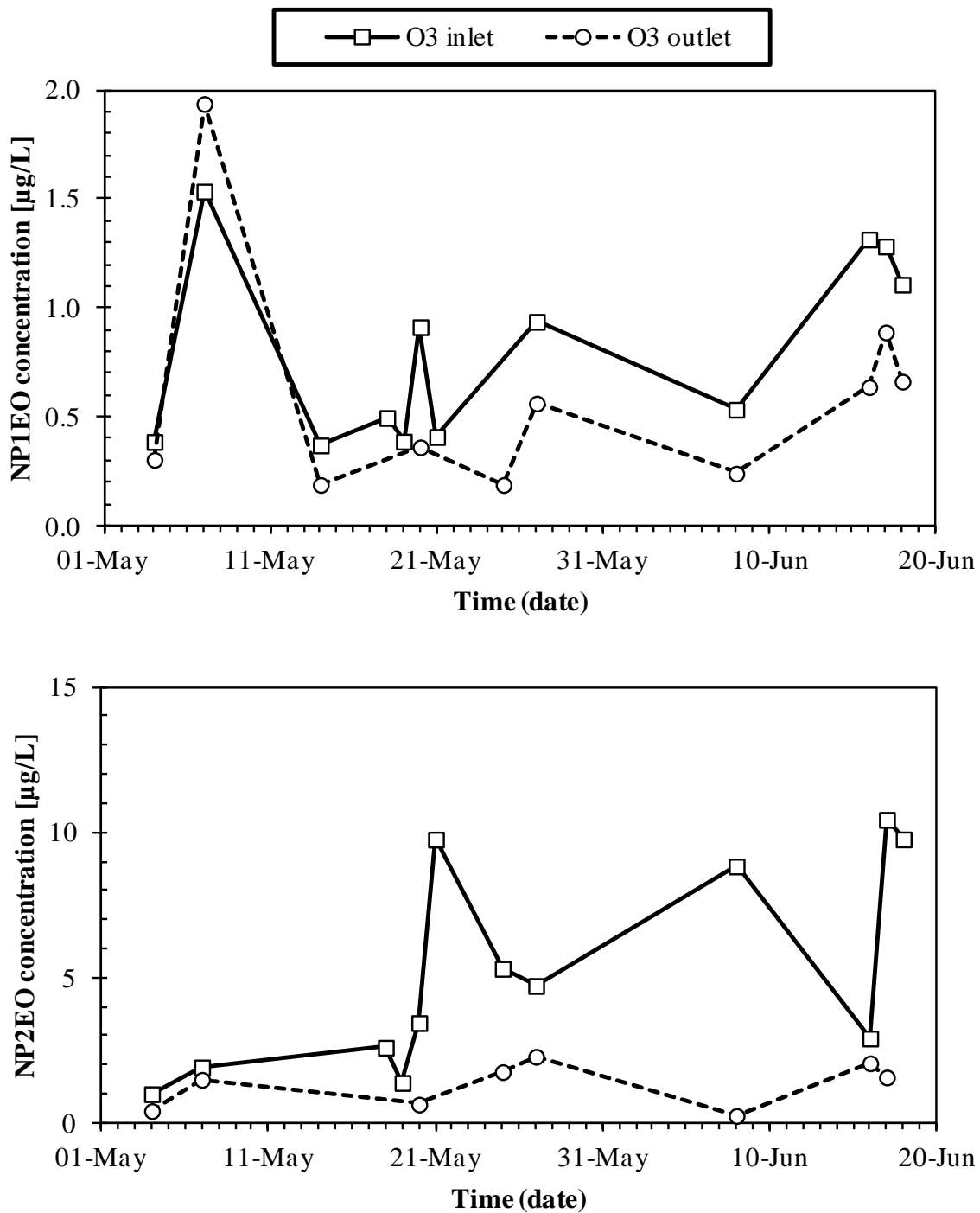
Figure 5. Chemical analysis: daily average concentration of NP1EO and NP2EO (influent - solid line- and effluent -dashed line - of ozonation basin).

Analytical results were, then, summarized as average concentrations and removal efficiencies, with the indication of the standard deviations (Table 6); the abatements were determined on the basis of the calculated loads, i.e. grams per day (data not shown), Since NP, NPnEOs and NPnECs (nonylphenol carboxylates) undergo and are the results of transformation processes, a mass balance

for NP, NPnEOs and NPnECs would have to be considered; owing to the lack of analytical data, this was not possible in the present study.

	O3 inlet [µg/L]	O3 outlet [µg/L]	Removal efficiency [%]
NP	1.21 (± 0.24)	0.61 (± 0.22)	47.5% (± 16.7%)
BPA	0.59 (± 0.33)	0.19 (± 0.07)	58.4% (± 12.0%)
NP1EO	0.81 (± 0.43)	0.60 (± 0.53)	21.1% (± 26.2%)
NP2EO	5.18 (± 3.57)	1.32 (± 0.77)	73.1% (± 26.5%)

Table 6. Chemical analysis: average concentrations and removal efficiencies, together with the standard deviations, for detected pollutants.

It has to be noted that EDCs removal efficiencies are significantly lower than:

- the abatements of experimentation #1 (the pilot plant installed at the Verona urban WWTP), where under similar operating conditions (O_3 dosage = 12 mg/L; contact time = 60 min) more than 80% reduction should be expected (based on reaction rate constants for NP and BPA obtained from experimental results);

- those reported in the most recent literature: Ning *et al.* (2007) affirmed that 1 mg/L ozone could remove 60% of 1 µgBPA/L and 90% of 2.5 µgNP/L (laboratory scale). Schaar *et al.* (2010) achieved 50% and 60% reduction for NP and BPA, respectively, with an ozonation pilot plant (O_3 dosage ≈ 6 mg/L; HRT ≈ 20min), treating the effluent of an urban WWTP.

In the *Estrogenic activity* section this finding will be deeper analyzed and explained.

STATISTICAL ANALYSIS

Data obtained from the chemical analyses were, then, elaborated using the SPSS 15.0 statistics program. Data are, firstly, presented as quantile box plots with 95% confidence interval (Figure 6 - top). The top, the bottom and the line in the middle of the box represent the 75th, the 25th and the 50th (median) percentile, respectively. The whiskers (the lines that extend out the top and bottom of the box) represent the highest and lowest values that are not outliers or extreme values. Outliers (values that are between 1.5 and 3 times the interquartile range) and extreme values (values that are more than 3 times the interquartile range) are represented by circles beyond the whiskers. In this

case, the box plots indicate the presence of only two outliers / extreme values. The statistical difference between the inlet and outlet samples is demonstrated by the observation of the error bars (displaying the 95% confidence interval of the means), shown in Figure 6 (bottom) only for NP and BPA: since there is no overlap between the confidence interval of inlet and outlet samples, the means are significantly different. Moreover, as a further confirmation, the ANOVA analysis, which was performed by taking the EDC concentration as dependent variable and the sampling site (O_3 inlet against outlet) as independent variable, indicated a significant difference ($p<0.05$).
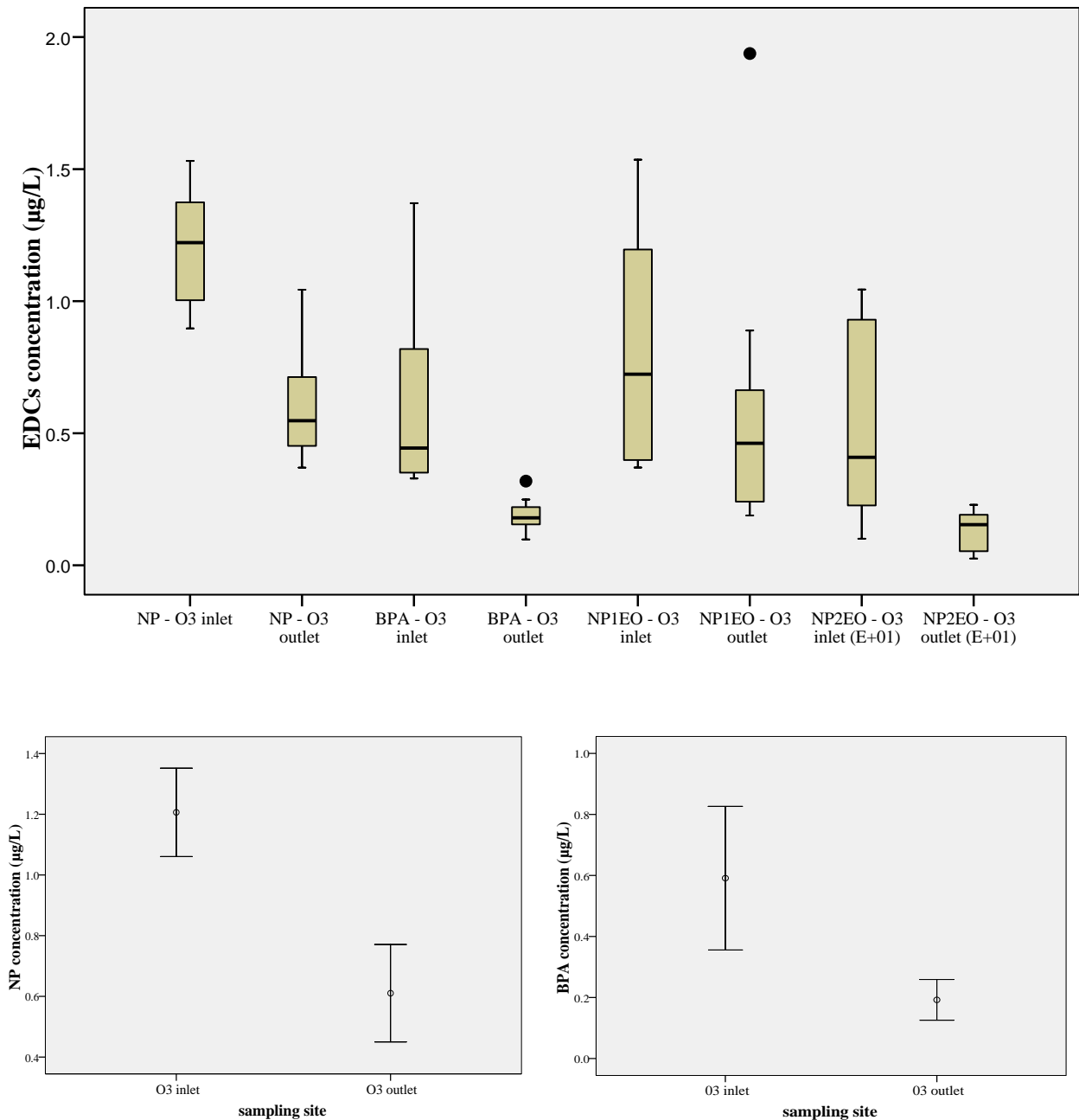

Figure 6. Statistical analysis: box plots (top) and error bars (bottom) of pollutants concentration in inlet and outlet samples.

3.3.2 BIOLOGICAL ANALYSES

ESTROGENIC ACTIVITY

BIOASSAY

Water samples (O_3 influent and effluent), taken on five different days during the monitoring period (see Table 4), were submitted to estrogenic biological assay: prior to the experiment, cell responsivity to the reference compound (estradiol, E2) was checked and a calibration curve was plotted (Figure 7). First of all, the consistency of the cell responsiveness can be highlighted: starting from the "spontaneous" activity supplied by the negative controls, the estrogenicity increases from a concentration of 100 fM-E2, up to 100 pM-E2; beyond this value, because of involved toxicity, an "apparent" decrease of activity is recorded. In particular, the calibration curve (detailed in Figure 7) shows a typical dose-response pattern between the E2 concentration (dose) and the estrogenic activity (response), whose main parameters are summarized in Table 7; as a further confirmation of the goodness of the bioassay, EC_{50} value perfectly agrees with literature data (listed in Leusch *et al.*, 2010).

Figure 8 displays the estrogenic activity, in terms of Relative Light Units (RLU) emitted per mg of protein, for tested samples: in particular, only the results of three samples are reported, because for the other two cases the data were distorted by the onset of toxicity (then confirmed by microscope observation).

Estrogenic activities in water samples were, then, expressed as estradiol equivalent concentration (ng/L-EEQ), transforming light values into concentrations, based on the calibration curve (as displayed in Figure 7), and shown together with the removal efficiencies (Figure 9). Measured EEQ values are reported as mean (of three replicates, except for #1 and #3 outlet samples), and, being aware of the modest number of statistical samples, the standard error, i.e. standard deviation·(sample number)$^{-1/2}$, was chosen to evaluate data variability.

Primarily, it can be stated that the bioassay results (tenths of ng/L-EEQ) are comparable to those reported for WWTP effluents in previous studies (among others: Körner *et al.*, 2001; Witters *et al.*, 2001; Hu and Chen, 2006; Bicchi *et al.*, 2009; Leusch *et al.*, 2010); in particular, they are closed to the lowest values indicated by these authors, thus showing that the estrogenicity of the wastewater can be considered weak. This can be explained considering that, as previously reported in Figure 1 and Table 2 and better clarified in Figure 10 for three sampling days, more than a half of the influent flow is represented by run-off and infiltration waters, and, on the contrary, domestic and industrial inputs, representing the major responsible for the estrogenic activity (Witters *et al.*, 2001),

account only for 40%. This is also confirmed by COD concentration entering the WWTP, in the range 250-300 mg/L (Figure 10), showing the dilution effect of run-off and infiltration waters.

As estrogenicity reduction concerns, Figure 9 clearly displays that ozone is only partially able to reduce the estrogenic activity: the overall removal efficiency is, indeed, equal to about 18%. This value was determined on the basis of the calculated loads, i.e. grams EEQ per day (data not shown): in this way, the highest value recorded for sample #3 was softened.

Finally, it has to be noted that the estrogenic removal efficiency is significantly lower than the abatement obtained in experimentation #1 (the pilot plant installed at the Verona urban WWTP); the latter recorded more than 50% reduction (on the basis of RLU/mg protein values), with only 30 min contact time (instead of 60 min as in the present case) and almost the same O_3 dosage. The same finding was detected for EDCs abatement (see *Chemical analyses* section). The reason of these incomparable performance lies in the different wastewater source: almost totally domestic in the case of experimentation #1 and, on the contrary, with a considerable industrial component (almost equal to the domestic one - Figure 10) in the case of experimentation #2 (the Fino Mornasco WWTP); this wastewater is, in effect, characterized by a huge amount of biorecalcitrant and/or toxic compounds, competitors with EDCs for O_3 consumption.
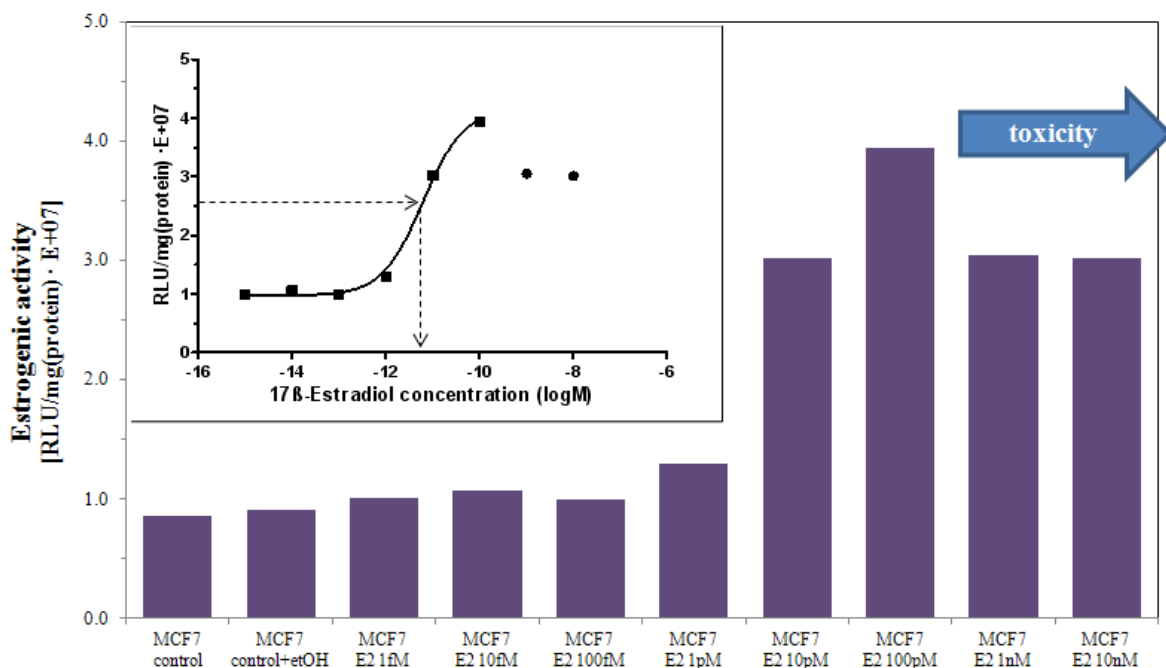
Figure 7. Estrogenic biological assay: cell responsivity, with detail of the calibration curve with the reference estrogen E2.

EC$_{50}$	Detection limits	
[95% confidence interval]	Minimum value	Maximum value
Log(Molarity) - E2	*[ng/L - E2]*	
-11.21 [-11.46, -10.97]	0.027	27.2

Table 7. Dose-response curve (R^2=0.996): EC$_{50}$ values and detection limits.

Figure 8. Biological assay: estrogenic activity obtained on three tested samples.

Figure 9. Biological assay: estrogenic activity obtained on three tested samples (as estradiol equivalent concentration), together with the removal efficiencies.

Figure 10. Composition of the influent flow, split up for source, and COD concentration entering the WWTP.

BIOASSAY *VS* CHEMISTRY

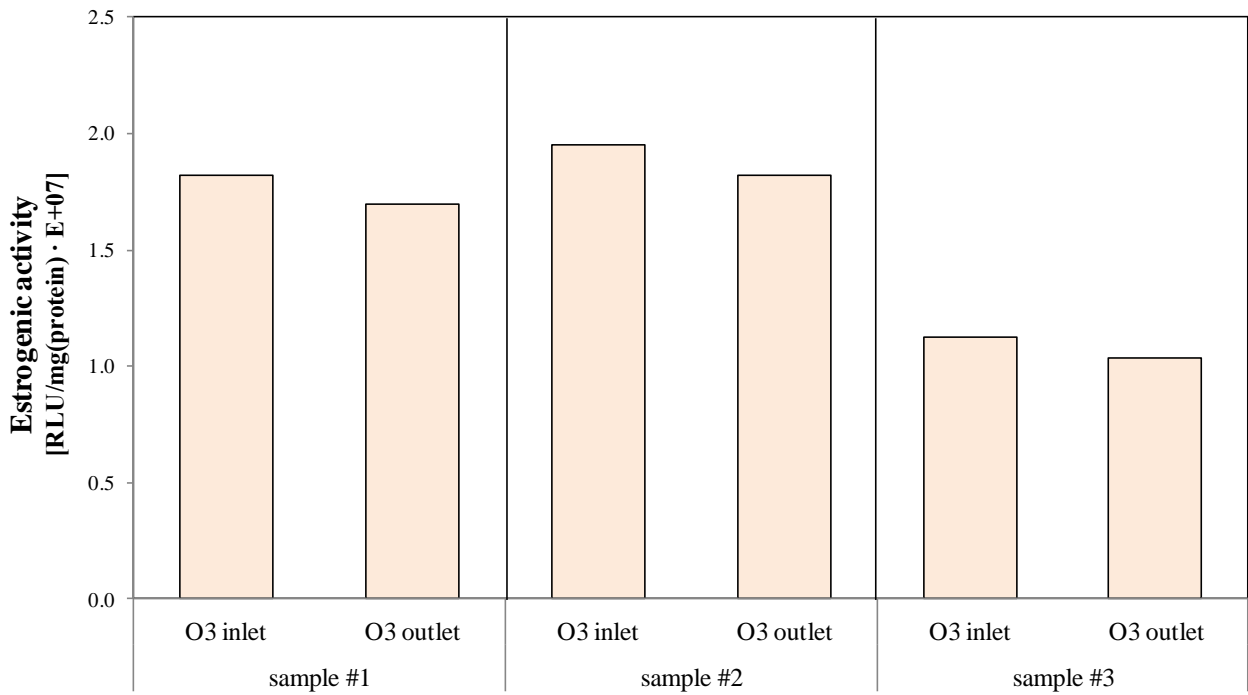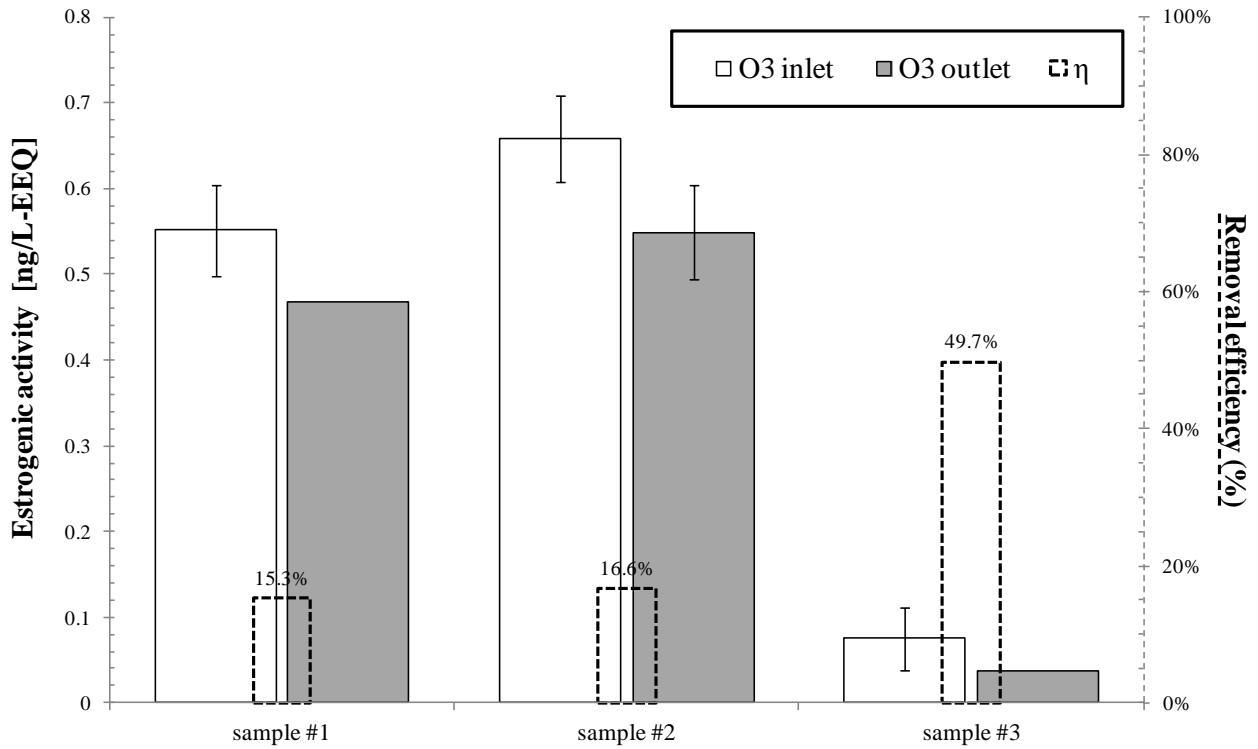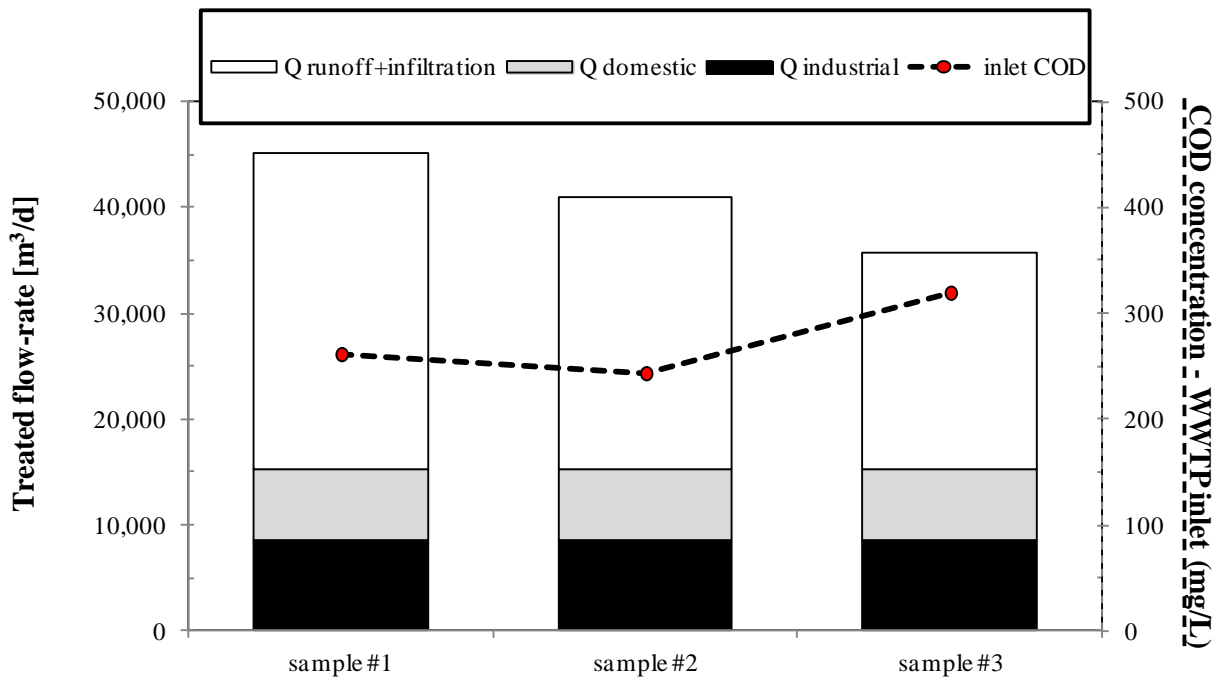As proposed in the *Materials and Methods* section, multiplying the detected concentration of each EDC by the relative estrogenic potency (Estradiol Equivalency Factor), it is possible to calculate a predicted estrogenicity for each sample: as expected considering the EEF values (see Table 5), NP (≈ 80%) and BPA (≈ 20%) are responsible for almost the whole predicted estrogenicity of analyzed samples, as Figure 11 clearly demonstrates.

The comparison of measured and predicted estrogenic activity (Figure 12) highlights, first of all, that the effective estrogenic abatement turned out to be significantly lower than the one predicted by EDCs concentration: in particular, it resulted less than a half, except for sample #3 that, anyway, recorded extremely low values of EEQ (under 0.1 ng/L, i.e. close to the detection limit). Furthermore, a discrepancy between predicted and measured estrogenicity was found: by the direct comparison of the obtained values (Figure 13), it can be inferred that, for samples #1 and #2 (both O_3 inlet and outlet), the measured estrogenicity was around 10 times higher than the predicted one; on the contrary, sample #3 exhibited a perfect matching between predicted and measured activity. This could indicate that the entire estrogenicity can be ascribed to the detected compounds, even if, as previously stated, sample #3 presented extremely low values of EEQ.

These findings can be directly explained considering the following reasons:

1) a mixture of compounds is responsible for the estrogenic activity (see, *inter alia*: Auriol *et al.*, 2006; Bicchi *et al.*, 2009; Furlong *et al.*, 2010; Leusch *et al.*, 2010): beside the analyzed EDCs, other alkyphenols, in particular octylphenol (OP), having an Estrogenic Equivalency Factor even higher of NP, i.e. around 1.5·E-04 (Leusch *et al.*, 2010), and steroidal hormones, which occur at 1,000 or less times lower concentration than alkyphenols but which are characterized by EEFs 1,000 or more times greater (Leusch *et al.*, 2010: around 2.8·E-02 for estrone; 1 for estradiol, the reference compound; around 0.9 for ethynil-estradiol; around 0.15 for estriol), strongly contribute to the overall estrogenicity;

2) the occurring of synergistic and potentiating effects between the components;

3) the formation of active by-products, as found by other authors (Huber *et al.*, 2004; Bila *et al.*, 2007): in some cases, oxidation intermediates of EDCs may be more harmful than parent compounds.

This suggests that the chemicals measured in the analytical assay cannot fully explain the estrogenicity of the sample, and that other undetected chemicals contribute to it, and/or that different EDCs present in the sample have a synergistic estrogenic effect on the MCF-7 cells (Bicchi *et al.*, 2009); on the contrary, non-additive mixture interactions, poor bioassay performance

due to matrix interference, or the presence of potent anti-estrogens could be the reasons of an opposite situation, i.e. measured lower than predicted (Leusch *et al.*, 2010).
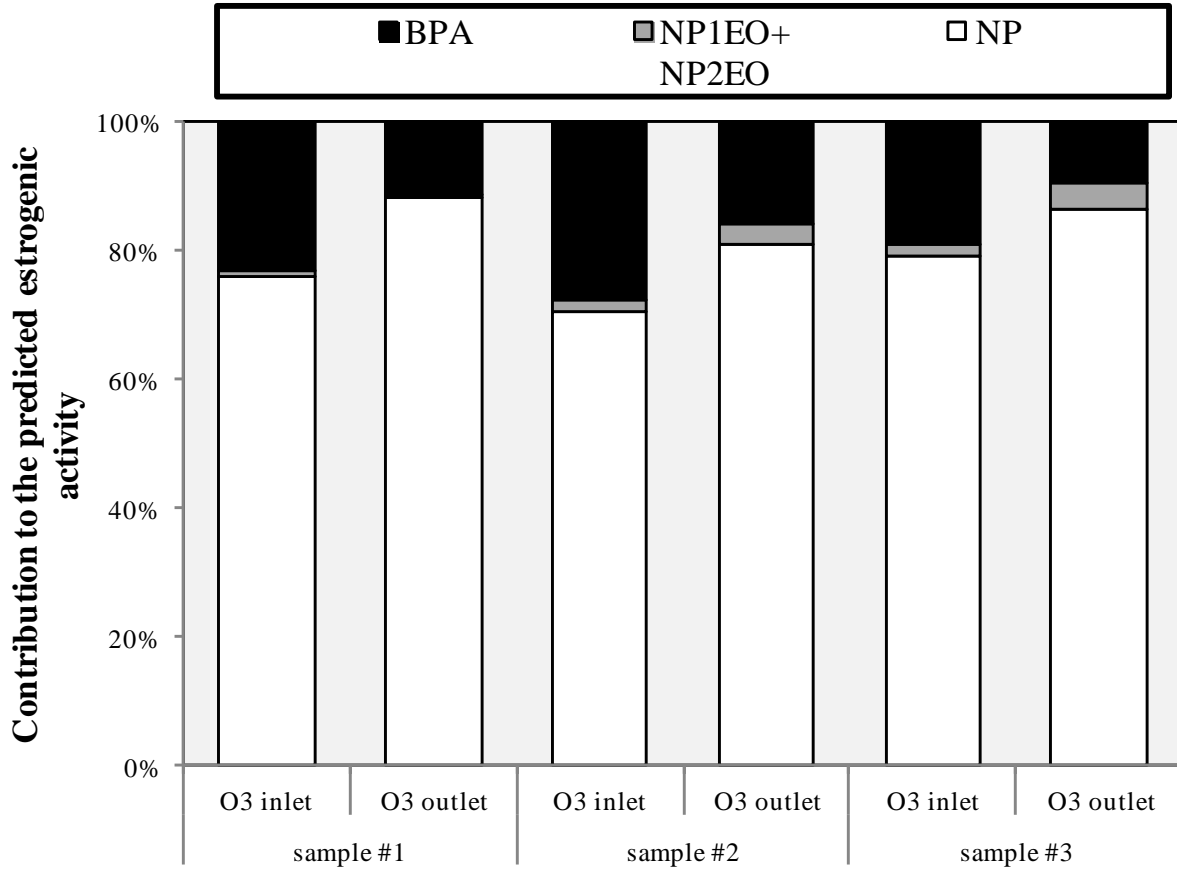
Figure 11. Contribution of each detected compound to the total predicted estrogenic activity.
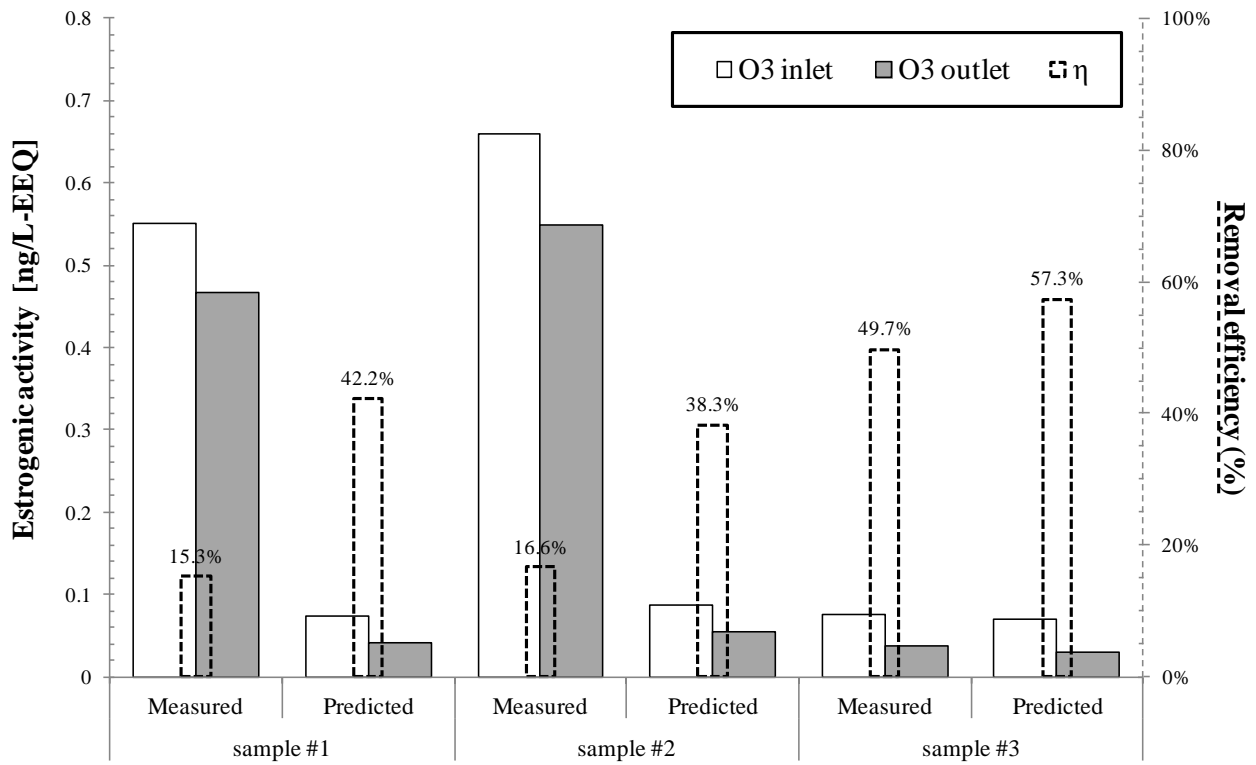
Figure 12. Results of measured (through bioassay) and predicted (with chemical analysis) estrogenic activity (as estradiol equivalent concentration), together with the removal efficiencies.
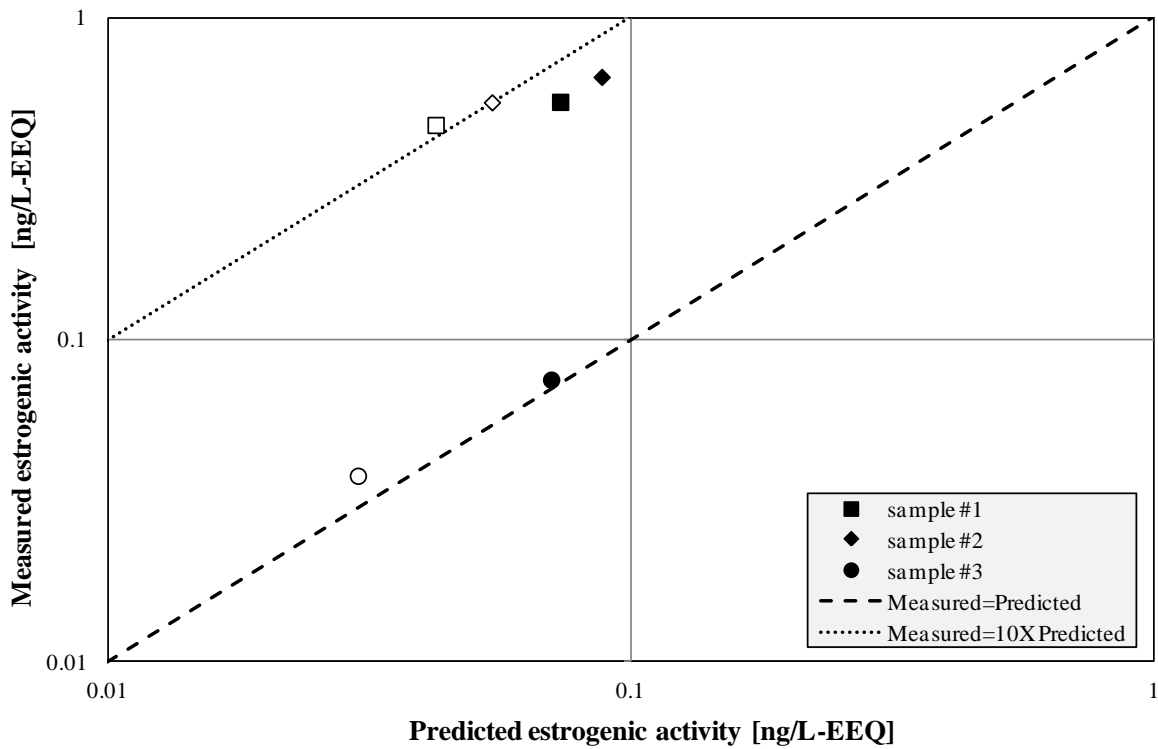
Figure 13. Comparison of the estrogenic activity (as EEQ concentration) measured *in vitro* against predicted from chemical analysis (black marker = O$_3$ inlet; white marker = O$_3$ outlet).

MUTAGENIC ACTIVITY

AMES TEST

The Ames test results are set out in Table 8 and are expressed as mutagenicity ratio (MR). All wastewater extracts assayed using the Ames test showed very high mutagenicity, particularly on TA98 strain, with and without metabolic activation (±S9), indicating the presence of direct and indirect mutagens. The detoxifying property of the enzymatic mixture (S9) determined a slight reduction in toxicity, highlighting the mutagenic effect.

The sample collected before ozonation showed very high toxicity at the highest tested dose (1 and 2 L equivalent/plate). The outlet samples showed generally lower toxicity but higher mutagenicity ratios. Both samples showed an evident dose-response relationship.

It was found that the most sensitive strain was TA98, which is sensitive to frameshift mutagens. According to the twofold rule (MR>2) for positive results, the data showed very high mutagenicity: the mutagenic effect starts from lowest tested doses, in both inelt and outlet samples (0.05 L equivalent). TA100 strain also displayed a mutagenic effect, but much lower than TA98 strain and particularly for the outlet sample. Again the addition of S9 detoxified the samples.

SAMPLES (L eq / plate)	TA98		TA100	
	- S9	+ S9	- S9	+ S9
	MR	MR	MR	MR
O_3 INLET				
0.05	5.5	3.8	1.6	1.0
0.1	10.5	6.9	1.6	1.1
0.5	23.6	14.1	2.3	1.8
1	Tox	14.2	2.0	1.4
2	Tox	Tox	Tox	Tox
O_3 OUTLET				
0.05	5.1	4.3	-	-
0.1	9.0	7.0	-	-
0.5	30.8	17.9	1.8	1.7
1	34.0	28.7	2.4	2.1
2	Tox	40.6	4.4	2.8
BLANK CARTRIDGE				
0.05	1.1	1.2	1.5	-
0.1	1.3	1.3	1.4	-
0.5	1.4	1.1	1.1	1.0

SAMPLES (L eq / plate)	TA98		TA100	
	- S9	+ S9	- S9	+ S9
	MR	MR	MR	MR
1	1.3	1.1	1.0	0.9
2	1.1	0.8	1.0	0.9
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Negative control	18.5 ± 4.0	32.0 ± 8.6	87.5 ± 14.8	113.7 ± 7.9
Positive control	>1000	>1000	>1000	>1000

Table 8. Results of the Ames test.

COMET ASSAY

The comet assay on human leukocytes carried out on organic extracts of wastewater showed strong toxicity at the highest tested doses: 1 and 2 L equivalent caused a very high loss of cell viability (Figure 14). The results of the comet assay, expressed as visual score and tail length, are given in Table 9. Both samples induced a dose-dependent response and showed mutagenicity from the lowest tested doses (0.05 L equivalent for inlet sample and 0.1 L equivalent for outlet sample). Wastewater after ozone treatment showed slightly less DNA damage than wastewater before ozonation.
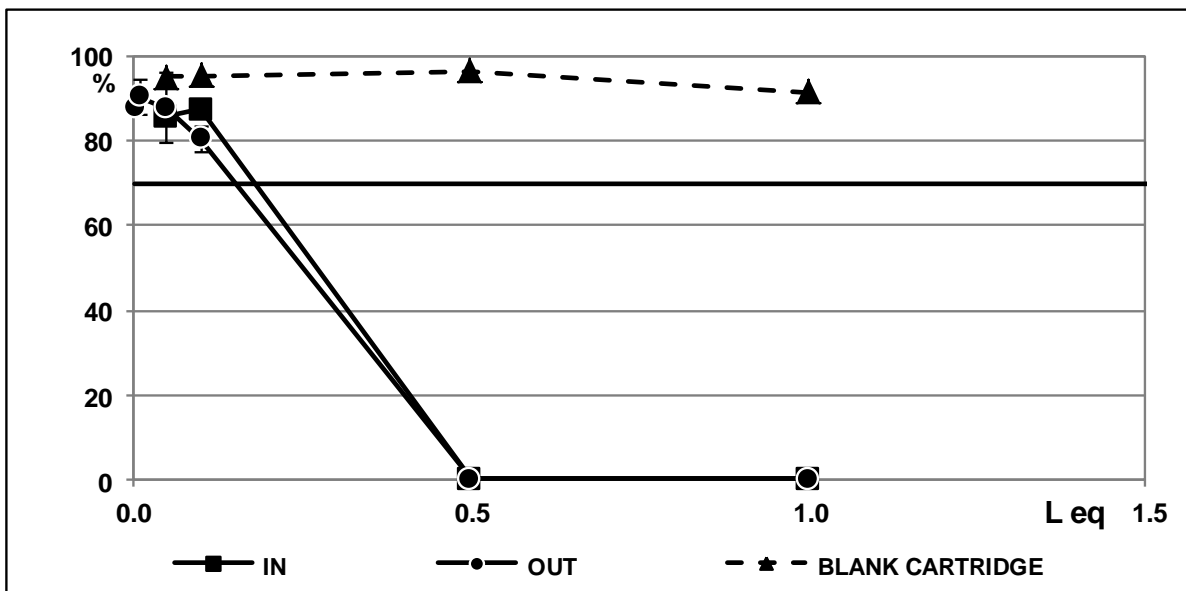

Figure 14. Cell viability, after treatment with wastewater.

SAMPLES (L eq)	VISUAL SCORE	TAIL INTENSITY (%)
O₃ INLET		
0.05	274.00 ± 11.31**	20.36 ± 2.95**
0.10	309.00 ± 18.38**	27.69 ± 0.78**
0.50	Tox	Tox
1.00	Tox	Tox
O₃ OUTLET		
0.05	173.00 ± 50.64	6.81 ± 7.51
0.10	236.00 ± 55.57*	15.27 ± 6.81*
0.50	Tox	Tox
1.00	Tox	Tox
BLANK CARTRIDGE		
0.05	117.00 ± 7.07	0.35 ± 0.02
0.10	127.00 ± 12.73	0.37 ± 0.03
0.50	121.00 ± 12.73	0.32 ± 0.02
1.00	136.00 ± 2.83	0.54 ± 0.03
Negative control	119.00 ± 5.97	0.31 ± 0.04
Positive control	366.67 ± 5.43	45.52 ± 5.02

*statistical significance vs negative control ($p < 0.05$) in accordance with Dunnett's test
**statistical significance vs negative control ($p < 0.01$) in accordance with Dunnett's test

Table 9. Results of the comet assay.

ALLIUM CEPA TEST

The *Allium cepa* test performed on unconcentrated wastewater displayed neither toxicity nor genotoxicity. No reduction in root length or morphological abnormalities was observed in *Allium* roots. Unexpectedly, the exposure of *Allium cepa* to wastewater seems to have positive effects on root growth. The presence of substances with estrogenic activity should promote the proliferation of the root meristem cells. High mitotic index values found in the lowest dilutions also confirm this. Mitotic indexes and genotoxicity results are set out in Table 10. No increase in micronucleus frequency was highlighted for all samples.

SAMPLE DILUTIONS	IM (%)	MCN (%)
O₃ INTLET		
Undiluted sample	11.8	0.7 ± 0.5
1:2 dilution	11.2	1.1 ± 1.0
1:10 dilution	10.8	0.6 ± 0.6
1:100 dilution	9.2	1.0 ± 1.2
O₃ OUTLET		
Undiluted sample	8.7	0.9 ± 0.6
1:2 dilution	11.1	1.9 ± 2.0
1:10 dilution	11.8	1.7 ± 1.9
1:100 dilution	8.6	0.8 ± 0.8
Negative control	11.0	0.8 ± 0.6
Positive control	13.3	5.5 ± 3.0

Table 10. Results of the *Allium cepa* test.

INTEGRATED DISCUSSION

The mutagenicity tests performed on wastewater showed apparently contradictory results, but very interesting ones.

No genotoxic effect was seen in *Allium cepa* cells, whereas the Ames and Comet tests, on bacteria and human cells, respectively, gave positive results. This fact could depend on the different sensitivity of the indicator organisms: *Allium cepa* may be not sensitive to compounds present in these samples. Although several authors have shown that *Allium cepa* MN test is a sensitive assay for evaluating the genotoxicity of industrial and domestic effluents, particularly in the presence of heavy metals and petroleum hydrocarbons (Morais Leme *et al.*, 2009; Radic *et al.*, 2010), other authors (Rank and Nielsen, 1998) found that this organism had low sensitivity in detecting the genotoxicity of pollutants present in sludge from municipal treatment plants influenced by industrial wastewater. In this study, the industrial contribution to total influent sewage is high and is accounted for by highly-toxic products from the textile and printing industries, but these do not include heavy metals. In addition, the use of unconcentrated wastewater determines plant cells exposure to lower amounts of pollutants compared to bacteria and human cells exposed to organic extracts obtained by adsorbing on C18 cartridges (SPE), with a 20,000X concentration factor.

The concordance observed in the Ames and the comet tests suggests the presence in wastewater of genotoxic pollutants capable of causing DNA damage in different organisms, inducing point mutations, particularly frameshift mutations, and DNA damage. It was not possible to determine whether the wastewater contained few substances with strong mutagenic activity or many compounds in traces with adding activity, or perhaps both. However, the DNA damage observed in human leukocytes indicates a severe effect, showing the potential human health risks associated with these environmental mixtures. Wastewater samples before and after ozonation showed strong toxicity and mutagenicity, with only minor differences between the samples, which suggests that the toxic and/or mutagenic compounds, already present in influent, can enter the environment via effluent, and cause a human health risk.

The samples were very rich in toxic and/or genotoxic contaminants, mainly deriving from industrial wastewater, and these genotoxic compounds were present both before and after ozone treatment. In the Ames test the outlet sample showed a slight reduction in toxicity in favour of mutagenicity. The substances responsible for mutagenicity observed in wastewater after ozonation treatment are present in wastewater before ozonation, but the concurrent presence of toxic compounds concealed the mutagenic effect, leading to a decrease in cell survival.

TA98 strain showed the most sensitivity in the Ames test, and this strain allows the detection of frameshift mutations. S9 addition causes slight detoxification of the sample, showing the presence of directly and indirectly acting mutagens. The prevalence in wastewater of direct and indirect mutagens causing frameshift mutations has been confirmed in the literature (Isidori *et al.*, 2007; Macova *et al.*, 2010; Misik *et al.*, 2011). A decrease in mutagenicity after ozone treatment in bacterial, plant and mammalian cells is widely reported in the literature (Takanashi *et al.*, 2002; Misik *et al.*, 2010), but the effectiveness of this treatment can be influenced by the chemical composition of wastewater (Misik *et al.*, 2010). In our study the presence of a large amount of precursors and persistent mutagenic chemicals in influent may have determined ozone's reduced effectiveness at decreasing mutagenicity in effluent, but the possible formation of by-products due to the reaction of contaminants with ozone may also have contributed to the observed genotoxicity.

3.4 CONCLUSIONS

In this work, an integrated assessment procedure, based on chemical and biological (estrogenic and mutagenic) analyses, was adopted to evaluate the performance of a tertiary full-scale ozonation plant installed in a WWTP treating domestic and industrial (textile) discharges, in order to remove target EDCs (NP, NP1EO, NP2EO and BPA) and biological activity from wastewater.

Primarily, chemical analyses, carried out on several samples (n=14), showed that analyzed substances occurred in the range of μg/L: in particular, focusing on two main compounds, the WWTP effluent prior to chemical oxidation recorded average concentrations of 1.21 and 0.59 μg/L, respectively for NP and BPA, and the removal efficiency of ozonation basin turned out to be around 50%. Statistical data processing revealed the presence of only two outlier values and, thanks to the ANOVA analysis, the statistically significant difference between O_3 inlet and outlet samples was ensured.

As far as estrogenic analyses are concerned, the bioassay displayed concentrations of estradiol equivalent in the order of ng/L and clarified that ozone is only partially able to reduce the estrogenic activity (less than 20% decrease). In particular, both EDCs and estrogenicity abatement were significantly lower than the ones obtained in experimentation #1 (the pilot plant installed at the Verona urban WWTP); the reason of these incomparable performance lies in the different wastewater source: almost totally domestic in the case of experimentation #1 and, on the contrary, with a considerable industrial component for experimentation #2.

Furthermore, the comparison between measured (through *in vitro* bioassay) and predicted (by way of analytical chemistry) estrogenicity highlighted a discrepancy between the obtained values (measured around one order of magnitude higher than the predicted) and an effective estrogenic abatement significantly lower than the one predicted by EDCs concentration. This can be explained considering that:

- a mixture of compounds is responsible for the estrogenic activity, not only analyzed EDCs;
- synergistic and potentiating effects between the components can occur;
- active by-products can originate, as oxidation intermediates.

This suggests that the chemicals measured in the analytical assay cannot fully explain the estrogenicity of the samples.

As mutagenic activity regards, similar conclusions can be drawn. Indeed, the tests revealed the presence and/or formation of significant amounts of genotoxic compounds in wastewater, both before and after ozone treatment. Therefore, tertiary oxidation did not improve the mutagenic

characteristics of the wastewater under study, despite its effectiveness in reducing the analyzed trace pollutants. The scarce ability of ozone to reduce mutagenicity can be influenced by the chemical composition of wastewater, due to the high load of industrial discharges; moreover, many classes of substances can contribute to mutagenicity, not only those identified. Taking into account that chemical analysis does not reflect biological status, the mutagenicity of this complex matrix needs to be studied in view of minimizing human and environmental exposure to noxious pollutants.

In summary, in this research, even if the chemical analysis proved to be essential to identify and monitor EDCs, the bioassays were found to be appropriate to determine estrogenic and genotoxic activity in wastewater samples, having taken also into account synergistic interactions and the effects of chemicals undetected in the analytical screening: these tests showed that ozonation treatment stage was capable to reduce biological activity of the WWTP effluent only at a low extent. Next steps of our work will involve both chemical (in order to complete the spectrum of endocrine compounds to be measured, in particular with octylphenol and steroidal hormones, having a strong estrogenic strength) and biological issues, with the inclusion of additional endpoint, such as eco-toxicological assays with bacteria (*Vibrio fischeri*), algae (*Selenastrum capricornutum*) and crustacea (*Daphnia magna*).

Finally, this work demonstrated the importance of the application of an integrated bio-analytical procedure, being however aware that a battery of bioassays will never be comprehensive, because of the myriads of receptors and regulatory pathways in an organism (Macova *et al.*, 2010); another strength of *in vitro* bioassays is represented by their role of initial screening tool: a significant response in an *in vitro* bioassay could be used as a trigger for further investigation using *in vivo* test models, while a negative response would suggest the scope of these financially and ethically expensive investigations can be reduced (Leusch *et al.*, 2010). The standardization of operating protocols, including data analysis methodology and the automation of the assays (i.e., to a degree that they can be used routinely by laboratories) may definitely help the application of these tests to WWTPs effluents. Consequently, the control of these pollution phenomena may help to solve some of the emerging problems related to water environments, as well as having a positive feedback on drinking water quality: at this stage, anyway, the only answers that can be drawn regard how much biological activity is removed by the analyzed process, but the ecological and health effects cannot be yet deduced: further analyses must be performed in order to evaluate a global balance on these implications.

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CHAPTER 4

APPLICATION OF THE CROSS-MEDIA EFFECT METHODOLOGY TO EVALUATE THE ENVIRONMENTAL COMPATIBILITY OF WASTEWATER TERTIARY OZONATION

SUMMARY

4.1 INTRODUCTION

If, on the one hand, chemicals are part of our daily lives, on the other hand, they may cause diseases. Which fraction of the current disease burden do chemicals however cause? This is an important question for decision-makers in order to prioritize efforts to protect us from the harmful effects of chemicals (Prüss-Ustün *et al.*, 2011). The information on the collective role of chemicals as contributors to global disease may assist policy makers in setting priorities in view of health protection. In particular, the research on the impact of chemical pollution during the last decades has focused almost exclusively on conventional priority pollutants. Today, these compounds are less relevant for most first world countries, since emissions have been substantially reduced through the adoption of appropriate legal measures and the elimination of many of the dominant pollution sources (Jones *et al.*, 2007). The focus has consequently switched to compounds present in lower concentrations and that have only recently been thought of as pollutants: in particular, endocrine disrupting compounds (EDCs) are widely regarded as a major environmental issue, largely due to concerns over risks to human and ecological health.

Secondary biological treatment of wastewater significantly reduces the concentrations of many of these substances, but does not provide complete elimination of all micropollutants (as observed, among others, by Ning *et al.*, 2007, and in *Chapter 2* on analyzed activated sludge systems); subsequently, residues of EDCs (predominantly natural/synthetic estrogens, alkylphenols and bisphenol A), entering the aquatic ecosystem through wastewater, represent a proven risk to the environment. This has led to the growing interest of water and wastewater utilities in evaluating cost-effective methods of reducing the EDCs levels (Ning *et al.*, 2007). Notwithstanding, far few attempts has been made previous this work to calculate the economic benefits of removing endocrine disrupting compounds from the wastewater stream. This clearly depends on the inherent difficulty in assigning economic value to environmental factors (Jones *et al.*, 2007).

In order to prevent this kind of water pollution, an advanced treatment (e.g., a tertiary ozonation stage) downstream the biological process can be implemented. However, while such advanced techniques will undoubtedly reduce the discharges of micropollutants, they will also inevitably result in large financial costs, as well as environmentally undesirable increases in energy consumption and consequent atmospheric emissions, considering that at present power demand would be met mainly from non-renewable sources (Jones *et al.*, 2007). An ozone plant, for example, would therefore indirectly contribute a large amount of CO_2 to the atmosphere, with associated

ramifications for global warming and climate change; these issues can significantly increase the economic and environmental cost of the plant.

The question, then, becomes on how much should be spent for removing/decreasing amounts of pollutants from wastewater, while simultaneously elevating the amount of atmospheric pollutants through increasingly energy-intensive treatment processes, which are likely to better remove pollutants from the wastewater, but at a large environmental and financial cost, while existing (biological) technologies are cheaper but removes slightly fewer compounds (Jones *et al.*, 2007).

A paradigm of wastewater treatment can plainly explain the situation (Figure 1): namely, an increase in effluent quality can only be environmentally beneficial. In fact, when subjected to a wider analysis, the benefits of improved effluent quality are balanced by the negative effects on air quality, when energy consumption and related pollutants emissions deriving from the advanced treatment technologies are taken into account (Jones *et al.*, 2007). Basically, there is a conflict of interest between two conflicting goals, but current environmental policy has not fully recognized the cross-media effects of improving water quality (Zakkour *et al.*, 2002).

A previous study (Ross *et al.*, 2004) on UK WWTPs (WasteWater Treatment Plants), for example, concluded that the addition of end-of-pipe solutions was required as the main control measure for priority substances, based on the quality standards being proposed, and suggested that the whole life cost could be in excess of a few billion euros. With the potential costs so large and current discussions on additional indirect environmental impacts (energy usage and resulting atmospheric emissions) of advanced treatments, such investment to improve effluent quality could be seen as inefficient or not cost beneficial.

In this study, on the contrary, an integrated analysis has been undertaken to determine if the application of an advanced technology (i.e., a tertiary ozonation stage) to WWTP effluent is economically/environmentally desirable when financial costs, energy consumption, and associated atmospheric emissions are taken into consideration. In order to achieve this goal, an environmental appraisal framework could provide a means by which issues of long-term sustainability in the aquatic environment may be addressed. In terms of the paradox outlined above, this would mean that water quality improvement schemes would have to be assessed for their effects on the wider environment (Zakkour *et al.*, 2002). This approach needs the collection of consistent data about the environmental impacts of competing options, through use of *ad hoc* tools.
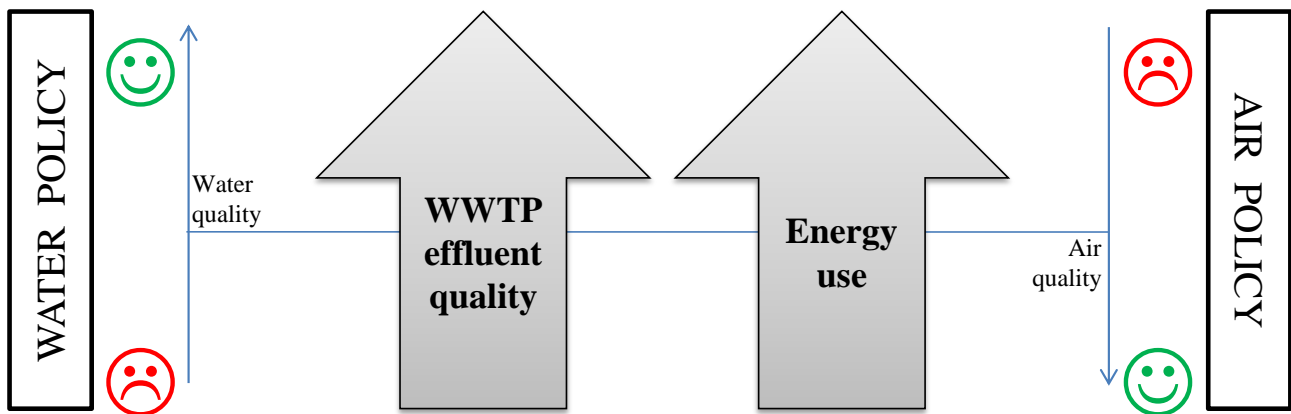
Figure 1. Diagrammatic representation of the current paradox in wastewater treatment, underscoring the conflicting interests between water and air policy (adapted from Zakkour *et al.*, 2002).

For this purpose, an useful help can be found in the Integrated Pollution Prevention and Control (IPPC) European Directive (96/61/EC), born for permitting of industrial installations in the EU Member States. The aim of this Directive is to develop an integrated approach, so as to achieve a high level of environmental protection taking into account the emissions in air, water and soil as a whole. Under the framework of the IPPC Directive, a Reference Document called "Economics and Cross-Media Effects" (EC 2006) has been developed: its first step should be the consideration of the environmental effects of the options, namely "Cross-Media Effects". Secondly, costing methodology is mentioned in order to determine the cost for each option considered. After the establishments of cross-media effects and costs of the options, their comparison is needed in order to determine which of the alternatives can be selected (Dogan *et al.*, 2010). For example, when assessing an end-of-pipe abatement plant that is powered by electricity (as O_3 plant in the current research), the environmental impact of the additional electricity used has to be traded-off, against whichever pollutant is being abated. If the abatement plant has a significant electricity demand and the abated pollutant is relatively benign, abating the pollutant may provide less *overall protection to the environment as a whole* (EC 2006).

Nevertheless, the critical point exactly lies in the determination and quantification of *overall protection to the environment as a whole*, which involves the measurement of the damage caused by different kind of pollution, and its translation in an economic value to get the comparison possible. But this point represents the weakness of Cross-Media Effects methodology: indeed, it suggests how this assessment can be performed only for air pollution (i.e., by the implementation of external costs), but no such response is given for aquatic contamination and, consequently, the requested

comparison cannot be carried out. Similarly, other traditional tools, such as the most known Life Cycle Assessment (LCA), are unlikely to prove suitable, due to the difficulty in assigning economic value to environmental factors (Zakkour *et al.*, 2002) and in accounting for all the negative effects on human health. For example, diarrhea do not include all the possible diseases due to the Water, Sanitation, and Hygiene (WaSH) category, but the burden from other diseases is not currently quantified (Prüss-Ustün *et al.*, 2011): exposure-response relationships between chemicals and their health outcomes usually are lacking and, then, it is not possible to add the different modes of action by which chemicals exert their toxic effects, such as through endocrine systems. Furthermore, if two chemicals interact (toxicokinetically and/or toxicodynamically), then these interactions are described as antagonistic or synergistic (reducing or increasing expected additive effects, respectively): such chemical interactions are not addressed in current appraisal techniques (Pennington *et al.*, 2004) and can be accounted for only through the application of biological assays (as illustrated in *Chapter 3*).

Usually, impact assessment methods of existing appraisal framework quantify the environmental impacts through the individuation of category indicator, located at any point in the cause-effect chain (Jolliet *et al.*, 2003); in particular, two main schools of methods have developed: i) classical impact assessment methods (e.g. Guinée *et al.*, 2002), which restrict quantitative modeling to relatively early stages in the cause-effect chain to limit uncertainties and group inventory results in so-called midpoint categories, according to environmental themes such as common mechanisms (e.g. climate change) or commonly accepted grouping (e.g. ecotoxicity): the term "midpoint" expresses the fact that this point is located somewhere on an intermediate position between the emissions inventory results and the damage (or endpoint) on the impact pathway; ii) damage oriented methods (e.g. Goedkoop and Spriensma, 2000), which try to model the cause-effect chain up to the endpoint, but with high uncertainties.

Starting from the fundamentals of damage oriented (also known as severity-based) methods, it is the goal of this work to present an advanced attempt at evaluating the environmental compatibility of a wastewater tertiary ozonation, based on a preliminary order-of magnitude calculation of damage on human health, expressed as an economic value. In particular, for water pollution (for which, as said, there was no established method), the rates of the negative human health impacts will be evaluated in terms of Global Burden of Disease (GBD) and measured, to compare various negative impacts on a linear scale, in units of DALY (Disability-Adjusted Life Years, i.e. the sum of the life years lost due to disability and premature death), as developed by the World Health Organization (WHO) and the World Bank (Murray and Lopez, 1996). It can be reasonably considered an innovative procedure: far few studies, indeed, have been attempted to evaluate the attributable burden of

disease caused by a certain source (Kim *et al.*, 2011), despite the fact that the result could help policy makers in assessing pollution phenomena more directly in relation to human health.

As stated above, in this work the environmental compatibility of the tertiary ozonation stage, previously described in *Chapter 3* (where the results of chemical and biological analyses, used as input data for this assessment, are also reported) was evaluated. Furthermore, in order to increase the options to be compared, the same treatment was evaluated also under the hypothesis of strong O_3 dosage, in particular, equal to the higher applied to the pilot plant of experimentation #1 (the Verona WWTP, see *Chapter 2*).

4.2 MATERIALS AND METHODS

4.2.1 THE CROSS-MEDIA EFFECT METHODOLOGY

In order to assess the environmental effects of the alternatives, the cross-media effects methodology was applied, following the guidelines illustrated in the Reference Document (EC 2006) and briefly summarized in Figure 2.

The term 'Cross-Media effects' is used to describe a conflict: choosing between alternative options might require a choice to be made between releasing different pollutants to different media. The purpose of this methodology, which takes origin from the Life Cycle Assessment (ISO 14040), is to provide guidance on how to choose which option is best for the environment in these more complex cases, going to determine the least environmentally damaging scenario.
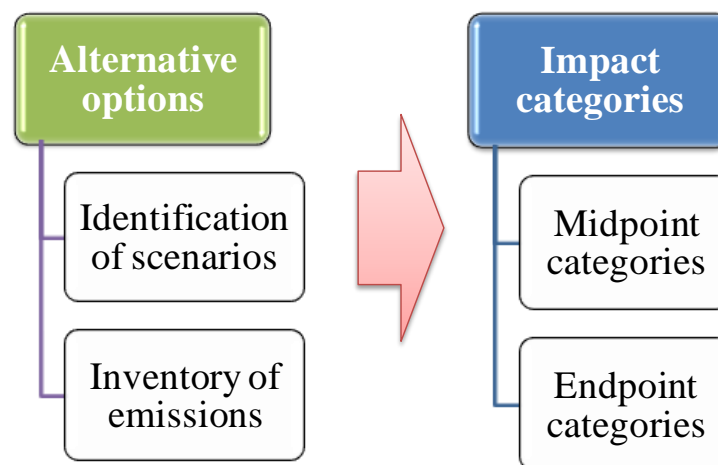

Figure 2. Flow chart for the application of the cross-media effects methodology.

4.2.2 IDENTIFICATION OF SCENARIOS

The initial step is to scope and identify the alternative options that are available and that could be implemented. The boundaries of the systems to be assessed need to be set at this stage.

In this work, as previously stated, the environmental compatibility of the tertiary ozonation (downstream a conventional activated sludge process) was evaluated, so that the alternative scenarios are: the base-case, lack of the treatment (option 0, involving the by-pass of the already existing O_3 plant), *vs* the presence of the ozonation stage operating at mild (option 1) rather than strong (option 2) dosage, as reported in Table 1.

The "impact producers" (i.e., a way to better define the boundaries of the system) were:

- for option 0, the discharge of 43,000 m^3/d of biologically treated effluent;
- for options 1 and 2, the discharge of the effluent subjected to tertiary ozonation and all the items related to the chemical oxidation stage: the on-site ozone production and dissolution, the production of pure oxygen and its transportation to the plant; on the contrary, the plant construction (and the relative impacts) were not accounted for, because, as stated above, it was already existing (and used to improve sludge settleability).

For all these points, the primary data referred to the case study (shown synthetically in Table 1) were adopted for the calculation and the sizing of the "impact producers", deriving from the following information: the distance between the oxygen production plant and the WWTP ($\approx$ 80 km), the vehicle capacity ($\approx$ 23 t), O_3/O_2 efficiency ($\approx$ 7%) and specific energy consumption for on-site ozone production and dissolution ($\approx$ 36 MJ/kgO_3).

Option	Tertiary O_3 stage	Energy for O_2 production [MJ/kgO_2]	Transportation [km/y]	Energy for O_3 generation and use [MJ/m$^3_{wastewater}$]
0	NO	0	0	0
1	YES, mild dosage (11 mgO_3/L)	$\approx$ 1.1	$\approx$ 7,500	$\approx$ 0.38
2	YES, strong dosage (20 mgO_3/L)	$\approx$ 1.1	$\approx$ 13,500	$\approx$ 0.69

Table 1. Summary of primary data for energy consumption and transportation used for the different scenarios.

4.2.3 INVENTORY OF EMISSIONS

The significant environmental releases and the resources consumed by each of the alternative techniques under consideration needed to be listed and quantified: this list should cover the emitted pollutants and the energy used in the process.

In this work, the quantification of emissions in water sector (i.e., the analyzed EDCs, NP and BPA) was performed on the basis of measured (options 0 and 1) and estimated (option 2) concentrations.

On the contrary, atmospheric releases were calculated by means of secondary data, i.e. by consulting database for both energy production and transportation. In particular, the former was a so-called "secondary energy source", i.e. generated outside the WWTP, and then supplied in the form of electricity: the environmental impact from this kind of energy will depend on the power plant technology and the fuel source that is used to generate it. The European electricity mix is a simplified approach for deriving emission factors to account for the environmental effects of the electricity used: multiplication factors have been derived for CO_2, SO_2, NO_x and PM_{10} emissions (Table 2) for Italian energy production (DM 35/2009), where oil and natural gas represent the main sources.

Pollutant	Emission factor [g/kWh$_e$]
CO_2	600
SO_2	0.8
NO_x	0.5
PM_{10}	0.03

Table 2. Emission factors for electric energy production in Italy (DM 35/2009).

The same approach was adopted for transportation and corresponding emission factors are displayed in Table 3. These elements were calculated with COPERT III (COmputer Programme to calculate Emissions from Road Transport), a free software program which is developed as a European tool for the calculation of emissions from the road transport sector.

Pollutant	Emission factor [g/km]
CO_2	750
NO_x	7

Pollutant	Emission factor [g/km]
CO	1.7
NMVOC	0.8
SO$_2$	0.5
PM$_{10}$	0.5
N$_2$O	0.03

Table 3. Average emission factors (Copert III) for Italian vehicle fleet (category: heavy duty vehicles, diesel, 16 - 32 t).

4.2.4 IMPACT ON MIDPOINT CATEGORIES

To assess the environmental effects for each alternative, the methodology set out below (EC, 2006) allows the different pollutants identified in the inventory to be collocated into some environmental themes (midpoint categories), originating from the environmental effects that the pollutants are most likely to cause. The themes taken into account in this work (according to EC, 2006) were six: aquatic toxicity, human toxicity (in fact, it refers to the toxicity due to air emissions, whereby "atmospheric toxicity" would be a more correct definition, opposite to the aquatic one), global warming, acidification, photochemical ozone creation potential and PM creation potential. One further environmental theme (abiotic depletion) could be considered: this would have given a measure of the consumed resources and would have allowed consideration of the potential depletion of the earth's resources. Although abiotic depletion remains an important issue, there were significant concerns about the reliability of the factors that had been derived to describe it: as a result, it was decided not to retain abiotic depletion in this methodology (EC, 2006).

These themes were carefully selected to give comprehensive coverage of the most relevant environmental effects whilst still ensuring that the assessment remains practical (EC, 2006). Although the coverage is comprehensive, it has not been possible to define a methodology that covers every possible impact; therefore, the user should be aware that there are environmental effects not accounted for at this stage (e.g., non-toxic aquatic effects).

To calculate the cross-media effects on midpoint categories, two different approaches were used: 1) when assessing global warming effects, acidification, photochemical ozone creation potential and PM creation potential, individual pollutants can be converted into an equivalent reference substance using multiplication factors. For example, a wide range of greenhouse gases can be expressed in

carbon dioxide equivalents to describe their Global Warming Potential (GWP). Expressing individual pollutants in terms of a reference substance allows them to be directly compared and also allows a range of pollutants to be summed together, in order to assess the significance of the total effect of the release.

On the contrary, for both human and aquatic toxicity, the mass of an individual pollutant can be divided by its toxicity threshold, to give a volume of air or water that would be needed to dilute the emission to safe levels when it is released. The methodology (EC, 2006) asserts, then, that the volume of air/water is summed to derive a total theoretical volume that is polluted to its threshold.

Table 4 summarizes the analyzed environmental themes and the approach adopted for the calculation of impacts.

Environmental themes	Approach for calculation
Global warming **Acidification** **Photochemical ozone creation potential** **PM creation potential**	Reference substance: $\Sigma_i \, M_i \cdot f_i$
Human toxicity **Aquatic toxicity**	Toxicity: $\Sigma_i \, M_i \, / \, t_i$
	where: M_i = mass (kg/y) of ith pollutant f_i = equivalency factor (-) of ith pollutant t_i = toxicity threshold (mg/L) of ith pollutant

Table 4. Analyzed environmental themes (midpoint categories) and approaches adopted for effects calculation.

The multiplication factors and toxicity thresholds used in both of the above approaches are derived from established methods that have been developed within recognized international forums: for example, the Intergovernmental Panel on Climate Change (IPCC 2001) provides the generally accepted values for GWP; for NP and BPA, the toxicity thresholds correspond to the PNEC (Predicted No-Effect Concentration) values, as reported in the EU Risk Assessment Reports (ECB 2002 and 2003), equal to 0.33 and 1.6 µg/L, respectively.

The effects on environmental themes were, then, normalized against a common reference value, defined as the Total European Load. This can be used as a mechanism for assessing the significance of the different environmental effects from the alternative options (similarly to the contribution analysis step in LCA).

The greatest difficulty within this procedure is establishing the reference point. Some work has been carried out to establish the Total European Loads and those that have been derived for the themes used in this research are listed in Table 5.

Environmental theme	Unit	Total European Load	Literature reference
Energy consumption	MJ/y	7.6 E+13	Jolliet *et al.*, 2003
Aquatic toxicity	m^3/y	2.2 E+13	Wegener Sleeswijk *et al.*, 2008
Human toxicity	m^3/y	8.4 E+15	Wegener Sleeswijk *et al.*, 2008
Global warming	$kgCO_2eq$/y	5.2 E+12	Wegener Sleeswijk *et al.*, 2008
Acidification	$kgSO_2eq$/y	2.4 E+10	Wegener Sleeswijk *et al.*, 2008
PM creation potential	$kgPM_{10}eq$/y	8.1 E+09	Wegener Sleeswijk *et al.*, 2008
Photochemical O_3 creation potential	kgNMVOCeq/y	2.8 E+10	Wegener Sleeswijk *et al.*, 2008

Table 5. Summary of Total European Loads (referred to EU_{25} and 100 years time horizon) for analyzed environmental themes.

4.2.5 COSTING EVALUATION

After the assessment of the environmental performances, the alternatives were evaluated in terms of economic considerations: investment (I ≈ 3,000,000 €) and operating (O&M ≈ 350,000 and 550,000 €/y, respectively for options 1 and 2, including power, maintenance, labor, transportation and pure oxygen) costs of each alternative were calculated on the basis of primary data, considering 25 years service lifetime and 5% discount rate.

Primarily, a cost-effectiveness analysis (a more simplified approach than a traditional cost-benefit analysis, as environmental benefits are quantified but not monetarised), useful to find out which scenario offers the most value (environmental benefits) for money (costs), was performed.

Then, once monetarised the benefits (see next paragraph), the monetary costs were evaluated together with the environmental ones.

4.2.6 IMPACT ON ENDPOINT CATEGORIES (INNOVATIVE APPROACH)

As soon as the environmental effects (and the monetary costs) had been established, the costs and benefits of the alternatives must be compared. In this work, an innovative approach was followed. First of all, the impact on endpoint categories was accounted for, allocating the midpoint categories to one (or more) damage categories, the latter representing quality changes: the damage indicator is, indeed, the quantified representation of this change; in practice, a damage indicator result is always a simplified model of a very complex reality, giving only a coarse approximation to the quality status of the item (Jolliet *et al.*, 2003).

In particular, the damage on human health was chosen as final indicator.

The application of this methodology allows to solve the cross-media conflicts: the assessment of alternative scenarios, having impact on different media (water and air in the present research), can be finally performed, on the basis a "lowest common denominator", represented by the damage indicator. To be able to make this analysis, there has to be a mechanism for attributing an economic value to the pollution that would be avoided: the following section reports the approaches used in this work.

AIR EMISSIONS

Various methodologies have been developed to derive economic values for the effects of atmospheric pollution. All of them, however, are characterized by a direct approach, i.e. the straight attribution of a cost to each pollutant emission: each mass of chemical emitted into the environment is multiplied by an associated "characterization factor" to provide an estimation of the associated impacts. The characterization factors are a measure of the potential impacts attributable to a unit mass of chemical (Crettaz *et al.*, 2002).

The European Commission derived external costs for some air pollutants. As part of the development of cost benefit analysis in the Clean Air For Europe programme (CAFE, 2005), a special report was prepared to provide a simple ready-reckoner for the estimation of the external

costs of air pollution, derived only for a few air pollutants ($PM_{2.5}$, NO_X and SO_2 , which can cause and worsen various public health problems such as cardiovascular disease, respiratory disease, low-birth weight, and lung cancer (Kim *et al.*, 2011), and (NM)VOC) and not for other environmental media.

The modeling carried out to derive these figures suggests that the generated results quantify a large fraction of total damages for most of the pollutants considered, although some effects which are undeniably important are omitted. The effect of omission of impacts has to be seen in the context of the full range of uncertainties in the assessment, including model assumptions and statistical uncertainties. It is important to underline that the external costs in the CAFE relate only to human health, while ecosystem externalities could not be monetized due to lack of data. For particulate emissions, $PM_{2.5}$ was calculated on the basis of size distribution and $PM_{2.5}/PM_{10}$ ratio, around 0.5 according to Monn (2001).

Another source of data is represented by the work of De Schryver *et al.* (2009), which calculated the damage characterization factor for CO_2 (equal to $2.6 \cdot E-07$ $DALY/kg_{emitted}$) and N_2O ($8.3 \cdot E-05$ $DALY/kg_{emitted}$). These values correspond to 0.006 and 2 $€/kg_{emitted}$, respectively (for detail on DALY, see *Water emissions* section). In particular, CO_2 (representing the main emission, as showed in the inventory) external costs, restricted in this work into two orders of magnitude variability, were calculated (De Schryver *et al.*, 2009) on the basis of three coherent scenarios, based on cultural theory perspectives (individualist, hierarchical and egalitarian, respectively with an increasing damage factor): indeed, the individualist coincides with the view that mankind has a high adaptive capacity through technological and economic development and that a short time perspective is justified. The egalitarian coincides with the view that nature is strictly accountable, that a long time perspective is justified, and a worst case scenario is needed (the precautionary principle). The hierarchical perspective coincides with the view that impacts can be avoided with proper management, and that the choice on what to include in the model is based on the level of (scientific) consensus.

Finally, the work of Kim *et al.* (2011) was applied to calculate external costs for CO emissions.

Table 6 lists the external costs for each air pollutant.

Air pollutant	External cost (€/kg$_{emitted}$)			Source
	Average	**Min**	**Max**	
PM$_{2.5}$	≈ 60	34	97	CAFE 2005
SO$_2$	≈ 11	6.1	18	CAFE 2005
NO$_X$	≈ 10	5.7	16	CAFE 2005
NMVOC	≈ 2.2	1.1	3.5	CAFE 2005
N$_2$O	≈ 2	0.1	23	De Schryver *et al.* (2009)
CO	≈ 1.5	0.4	8	Kim *et al.* (2011)
CO$_2$	$\approx 6.0 \cdot E\text{-}03$	$6 \cdot E\text{-}04$	$6 \cdot E\text{-}02$	De Schryver *et al.* (2009)

Table 6. Average, minimum and maximum external cost for analyzed air pollutants.

WATER EMISSIONS

The calculation of damage factor for water emissions was performed, on the contrary, on the basis of an indirect approach (summarized in Table 7), because of the following reasons:

- the lack of studies reporting the direct external costs for water pollutants, i.e., NP and BPA; Pennington *et al.* (2002) assert the sufficient epidemiological data do not exist to address effects (especially non-carcinogenic) for the vast majority of compounds: therefore methods to use bioassay data to account for the consequences, as well as the likelihood of an effect, are required;

- furthermore, for water emissions, the integrated evaluation of biological (estrogenic and mutagenic) and microbiological (disinfection) assays is fundamental to reach meaningful results (see *Chapter 3*), regardless of the individual compounds.

An estimation of the EBD, generally, consists of three steps: estimating the attributable fraction (through the knowledge of exposure distribution in a population and the relative risk), measuring the burden of disease associated with the risk factor at the population level, and multiplying the attributable fraction and the burden of disease (Kim *et al.*, 2011; Prüss-Ustün *et al.*, 2011;).

In our research, the first step of the indirect methodology was characterized by the individuation of the diseases related to WWTP effluent discharge: in particular, starting from the Global Burden of Diseases (Murray and Lopez, 1996), the main highlighted diseases were *Endocrine Disorders*, *Malignant neoplasms*, *Diarrhoeal diseases* and *Perinatal conditions* (only prematurity and low birth weight). These items were selected considering the work of Prüss *et al.* (2002), reporting that

infectious diarrhea is the largest contributor to the disease burden from water, sanitation, and hygiene; on the contrary, other infectious and parasitic diseases (such as intestinal nematode infections) were not accounted for, as because they have no impact in Italy.

Then (step 2), the weight of each disease was evaluated with a specific indicator, the DALY: the Disability Adjusted Life Year (see, among others, Murray and Lopez, 1997) is a health gap measure that extends the concept of potential years of life lost due to premature death to include equivalent years of 'healthy' life lost by virtue of being in states of poor health or disability. The DALY combines in one measure the time lived with disability and the time lost due to premature mortality. One DALY can be thought of as one lost year of 'healthy' life and the burden of disease as a measurement of the gap between current health status and an ideal situation where everyone lives into old age free of disease and disability. DALYs for a disease or health condition are calculated as the sum of the years of life lost due to premature mortality (YLL) in the population and the years lost due to disability (YLD) for incident cases of the health condition. The years of life lost (YLL) basically correspond to the number of deaths multiplied by the standard life expectancy at the age at which death occurs. To estimate YLD for a particular cause in a particular time period, the number of incident cases in that period is multiplied by the average duration of the disease and a weight factor that reflects the severity of the disease on a scale from 0 (perfect health) to 1 (dead). Furthermore, as proposed by Shuval (2003), the money value of the economic loss of one productive year of life (or one DALY) is about equal to the national gross domestic product (GDP) per capita per year, around 25,000 €/cap/y for Italy in 2004 (World Bank): in this way, it is possible to attribute an economic quantification (€/y) to the damage on human health.

Consequently, in the second step the national weight of each considered disease (due to all possible causes, not only WWTPs discharges) was quantified for Italy, through World Health Organization documents (WHO 2008) reporting the estimates of mortality and burden of disease for WHO Member States (including Italy) for the year 2004.

The third step was based on the reduction of the overall DALYs values only to the ones directly related to water pollution (from Global to Environmental Burden of Diseases, EBD (Kay *et al.*, 2000)): in particular, World Health Organization documents (Prüss-Üstün and Corvalán, 2006) reports an analysis, for each disease, of estimates of the environmental attributable fraction and indicative values for specific environmental risk factor (in particular, the WaSH category - *Water, Sanitation and Hygiene*).

Then, a further decrease was introduced (step 4): first of all, only the studied WWTP and its basin (corresponding to around 0.2% of total Italian capacity) were taken into account for the attribution of DALYs. Furthermore, a model based on the national situation of wastewaters

collection/treatment systems (ISTAT 2006) was applied (Figure 3A), so that different treatment classes (uncollected, untreated and only partially treated, in addition to the secondary treatment discharges) were evaluated and counted for damage attribution; in particular, the effect on effluent quality for each class was assessed on the basis of the results obtained on CAS systems in *Chapter 2* (regarding estrogenic and microbiological analyses), and in the works of Monarca *et al.* (2000) and Zegura *et al.* (2009) (for mutagenicity). These data allowed the quantification of the microbiological and biological activity reduction exerted by a secondary treatment and, considering lower efficiencies, by the other treatment category, as displayed in Figure 3B.

Finally (step 5), the result corresponding to the secondary treatment class was associated to the option 0 (negligible presence of tertiary treatments). On the contrary, the damage related to options 1 and 2 was decreased on the basis of the ozonation removal efficiencies obtained from the biological (estrogenic and mutagenic activity) and microbiological (disinfection) assays (see *Chapter 3*). In particular, the following assumptions were considered:

- for endocrine disorders (option 1), the result of the estrogenic *in vitro* assay was directly applied;
- the value related to endocrine disorders (option 2) was estimated on the basis of the results of the experimentation #1;
- for malignant neoplasms (option 1), the results of Ames tests were applied, considering a carcinogenicity/mutagenicity ratio equal to 80%, as suggested by Benigni and Bossa (2011);
- the value referred to malignant neoplasms (option 2) was assumed from the work of Takanashi *et al.* (2002) and Cao *et al.* (2009);
- for diarrhoeal diseases, the result of microbiological analyses on disinfection performance (*E.coli* determination) was chosen as indicator;
- for perinatal conditions, the mean of estrogenic and mutagenic assays was evaluated.

The indirect methodology adopted to define the damage on human health for water emissions is briefly summarized in Table 7 and Figure 3.

Step 1: Individuation of diseases	Step 2: Global Burden of Diseases [DALYs/y]	Step 3: WaSH attributable fraction [average (min - max)]	Step 5: Damage reduction (opt. 1; opt. 2)
Endocrine Disorders	≈ 100,000	6% (1-20)^	20%; 28%
Malignant neoplasms	≈ 1,200,000	0.5% (0.1-1)	13.5%; 18%
Diarrhoeal diseases	≈ 13,000	45% (23-90)	≈ 100% (both opt.)
Perinatal conditions	≈ 23,000	0.15% (0.06-0.3)	17%; 23%

Table 7. Summary of steps involved in the determination of the damage on human health for water emissions.

^ the mean values of similar diseases were used, because "population health impacts associated with environmental exposures to endocrine-disrupting substances were not deemed to be perfectly quantifiable at present" (WHO 2006)
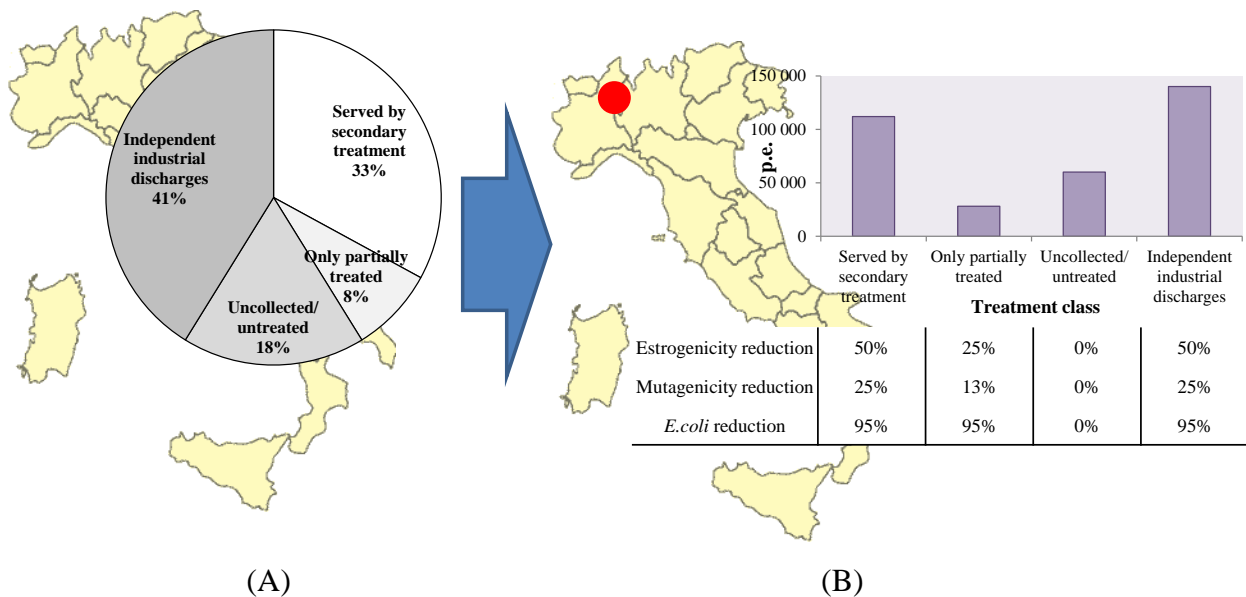

Figure 3. Representation of step 4: Italian state-of-art for wastewaters collection/treatment systems (A) and treatment classes composition and removal efficiencies (B).

Finally, a comparison of the monetary costs of the measure against the social cost of the damage to the human health that is avoided by implementing the measure was performed.

4.2.7 SENSITIVITY ANALYSIS

In the final stage, there is a need to evaluate the variability of input data and carry out a sensitivity analysis, based on the accuracy of the factors that have been used, in order to discern which factor was more determinative (Kim *et al.*, 2011) and to increase the objectivity in assessing alternatives (EC 2006): this analysis allow to know the impact of using an alternative input value on output model results.

Indeed, the interpretation of damage results will largely depend on the level of confidence/uncertainty in various input factors, in particular the external costs (for air emissions) and the WaSH attributable disease fraction (for water emissions), both characterized by relatively high variability (and quantified by means of minimum and maximum values).

Primarily, one-way sensitivity analysis was performed (only one parameter changed at one time), by varying each factor to the highest and lowest possible damage values.

Finally, a multi-way sensitivity analysis was executed, by varying all the parameters to their best and worst case. In particular, four scenarios were tested:

a) minimization of damage (lowest damage values for each factor);
b) maximization of damage (highest damage values for each factor);
c) water-dominant damage (lowest damage values for air, highest for water emissions);
d) atmospheric-dominant damage (lowest damage values for water, highest for air emissions).

4.3 RESULTS AND DISCUSSION

4.3.1 INVENTORY OF EMISSIONS

Figure 4 shows the inventory of emissions for the analyzed options. A cross-media conflict between water and atmospheric emissions appears clear: the more the WWTP effluent quality increases (corresponding to a decrease in discharged micropollutants, by means of O_3 treatment), the more the atmospheric pollution raise-up (as a consequence of air emissions for energy consumption and transportation).
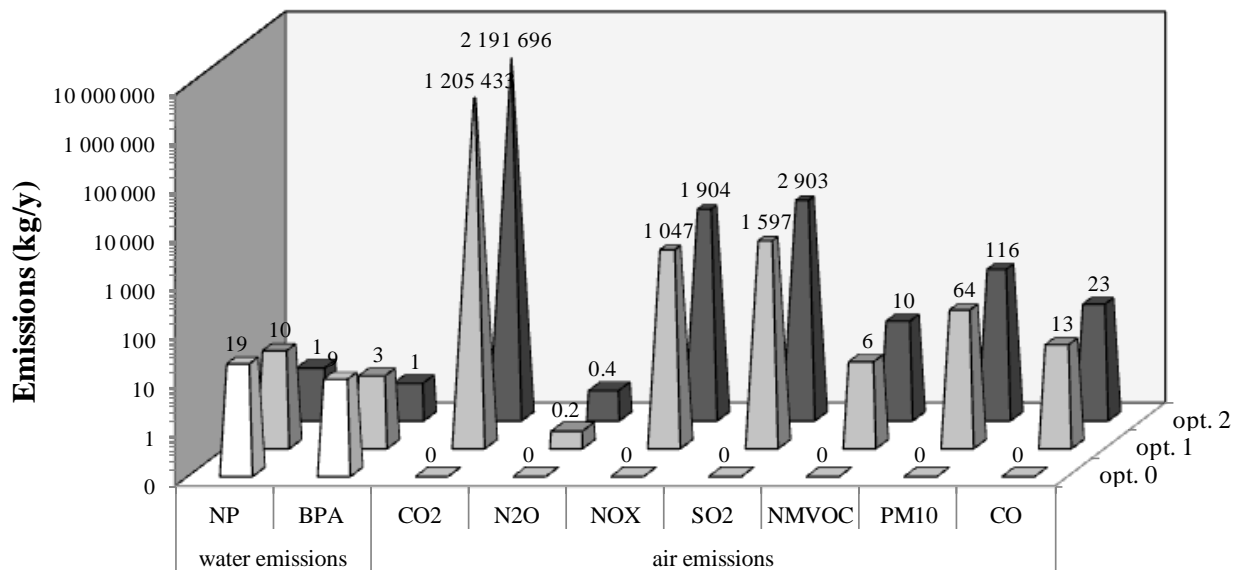
Figure 4. Inventory of emissions for three analyzed scenarios.

4.3.2 IMPACT ON MIDPOINT CATEGORIES

The quantification of impact on midpoint categories was performed, according to the methodology described in the *Materials and Methods* section, considering which emissions will affect each environmental theme, as summarized in Table 8. The results of impacts are, then, reported in Figure 5, where also the category "energy consumption" was added, in order to immediately visualize the energy-intensive options.

Environmental theme	Pollutants
Aquatic toxicity	NP, BPA
Human toxicity	SO_2, NO_X, CO
Global warming	CO_2, N_2O
Acidification	SO_2, NO_X
PM creation potential	PM_{10}
Photochemical O_3 creation potential	NMVOC, NO_X, SO_2, CO

Table 8. List of emissions affecting each environmental theme of midpoint categories.
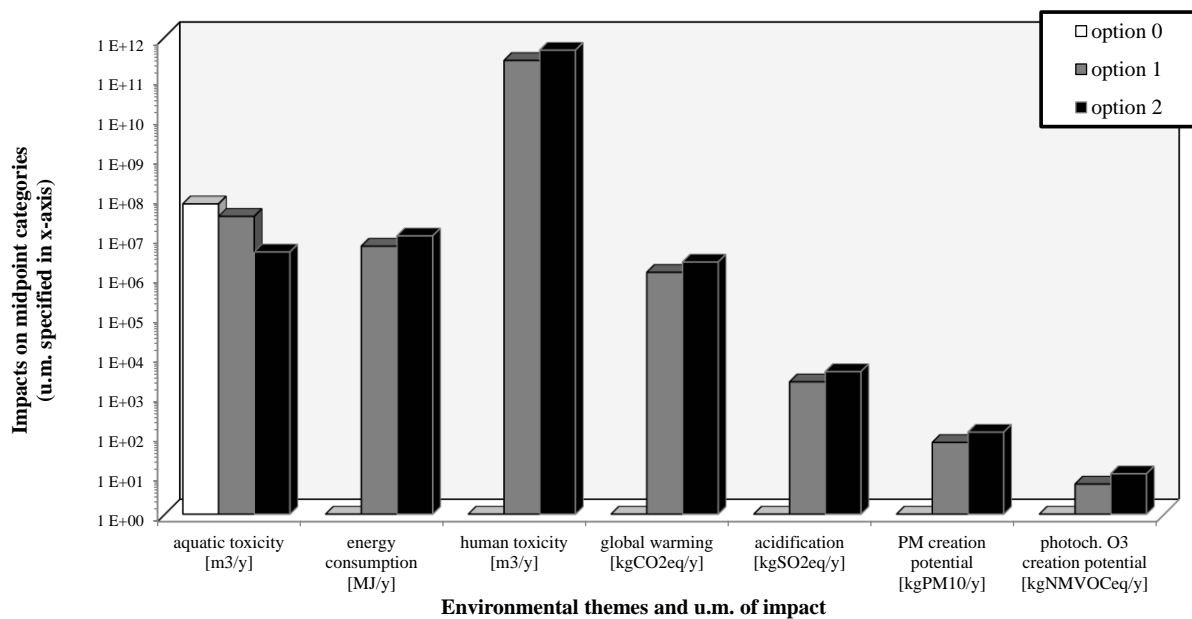
Figure 5. Quantification of midpoint categories impact.

The analysis carried out on midpoint categories showed that option 0 would seem to be the preferred choice for most of the environmental themes (impact), but the worst performer for aquatic toxicity, as clearly displayed in Table 9 that reports a synthetic comparison of different options.

Environmental theme	Option 0	Option 1	Option 2
Aquatic toxicity	☹	☺	☺
Human toxicity	☺	☺	☹
Global warming	☺	☺	☹
Acidification	☺	☺	☹
PM creation potential	☺	☺	☹
Photochemical O₃ creation potential	☺	☺	☹

Table 1. Synthetic comparison of environmental effects on midpoint categories of three scenarios (☺ preferred option, ☺ mid performance, ☹ worst performance).

Furthermore, a comparison against the Total European Load was performed, as showed in Figure 6: aquatic toxicity is the theme with the greatest impact on the European total, so option 0 would appear to have the most evident environmental effects, on the basis of this analysis.
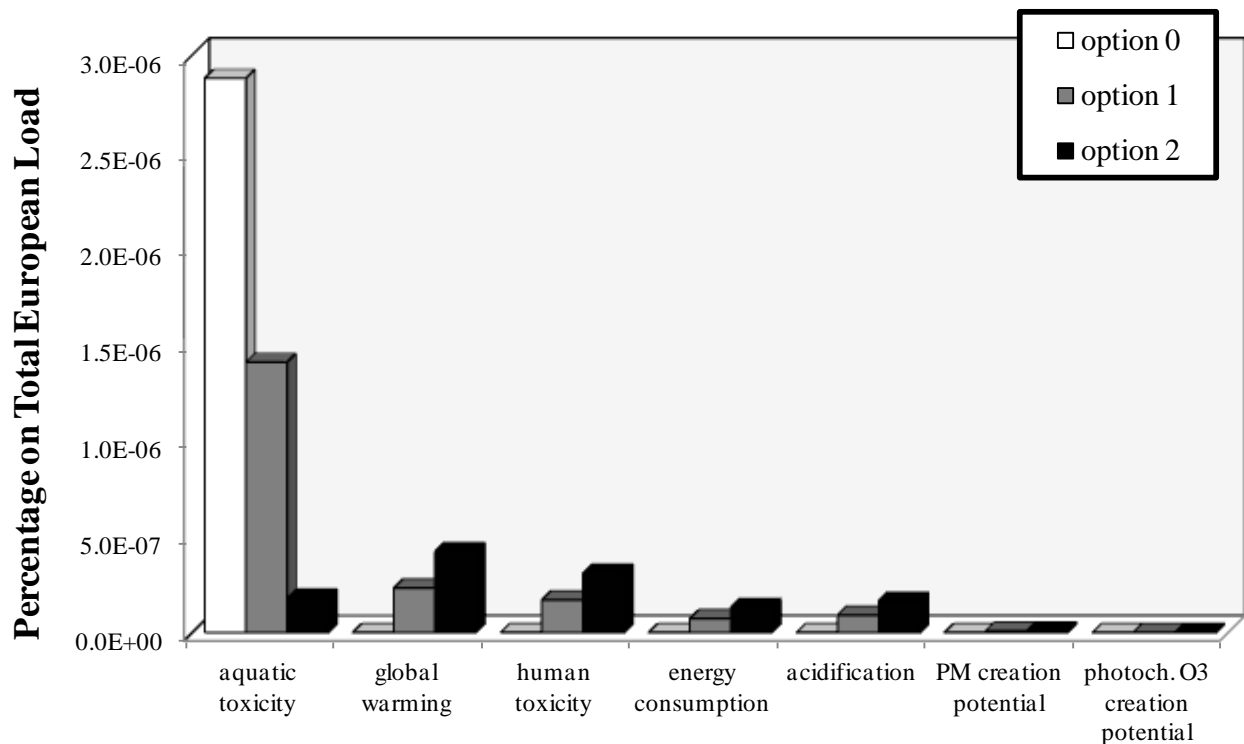
Figure 6. Impact on midpoint categories, as percentage on Total European Load.

In conclusion, no obvious decision on the environmental compatibility of O_3 treatment can be drawn in this phase, due to the persistence of the cross-media conflicts also in the midpoint categories.

4.3.3 COSTING EVALUATION

Capital and operating costs of the treatment are presented in Figure 7 (left): option 0 is taken as the base case and costs are presented as additional to that. On the contrary, Figure 7 (right) displays the cost-effectiveness analysis: option 2 appears the more cost effective for micropollutants (NP and BPA) removal.
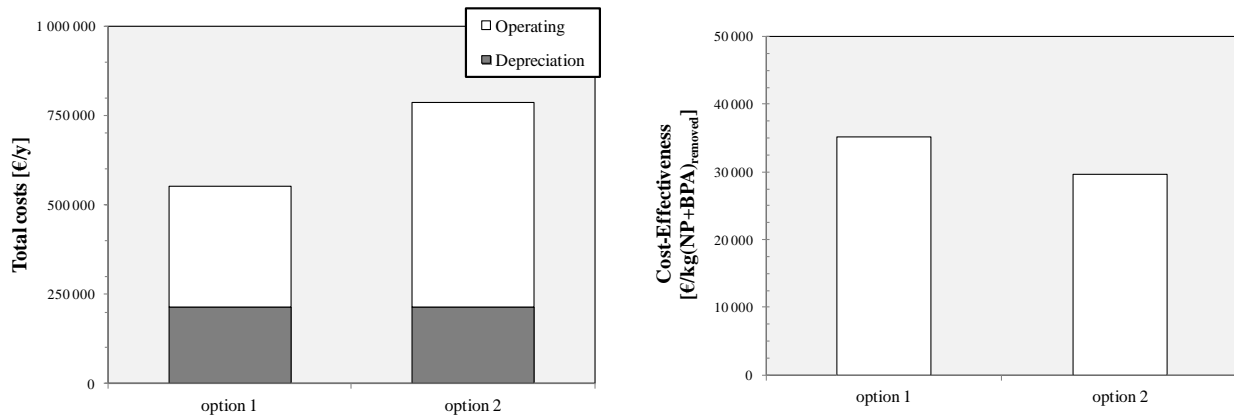
Figure 7. Total additional costs, with respect to option 0 (no treatment), for both (mild and strong) dosages (left), and cost-effectiveness analysis (right).

4.3.4 IMPACT ON ENDPOINT CATEGORIES

The solution of the cross-media conflict can be allowed only by the analysis of an endpoint category, as described in the *Materials and Methods* section. Figure 8 clearly shows that the damage on human health is quite similar for all the analyzed scenarios (considering the unavoidable approximations of methodology), but option 1 seems to be the better case: the damage *vs* O_3 dosage curve highlights a minimum corresponding to mild dosages (around 7-8 mgO_3/L), suggesting that highest benefits can be obtained with neither too low nor excessive dosages (Figure 8 - detail).

Moreover, as expected, the damage composition is different (see also Figure 12 at the end of the section): for option 0, indeed, all the effects can be attributed to water emission and, in particular, the main diseases are represented by endocrine disorders ($\approx 45\%$), malignant neoplasm ($\approx 40\%$) and diarrhoeal disease ($\approx 15\%$). Option 1 and 2 are characterized, on the contrary, by both water and air pollution, the former consisting only of endocrine disorders ($\approx 50\%$) and malignant neoplasm ($\approx 50\%$), the latter due to SO_2 ($\approx 45\%$), NO_X ($\approx 30\%$), CO_2 ($\approx 20\%$), and PM_{10} ($\approx 5\%$) emissions.
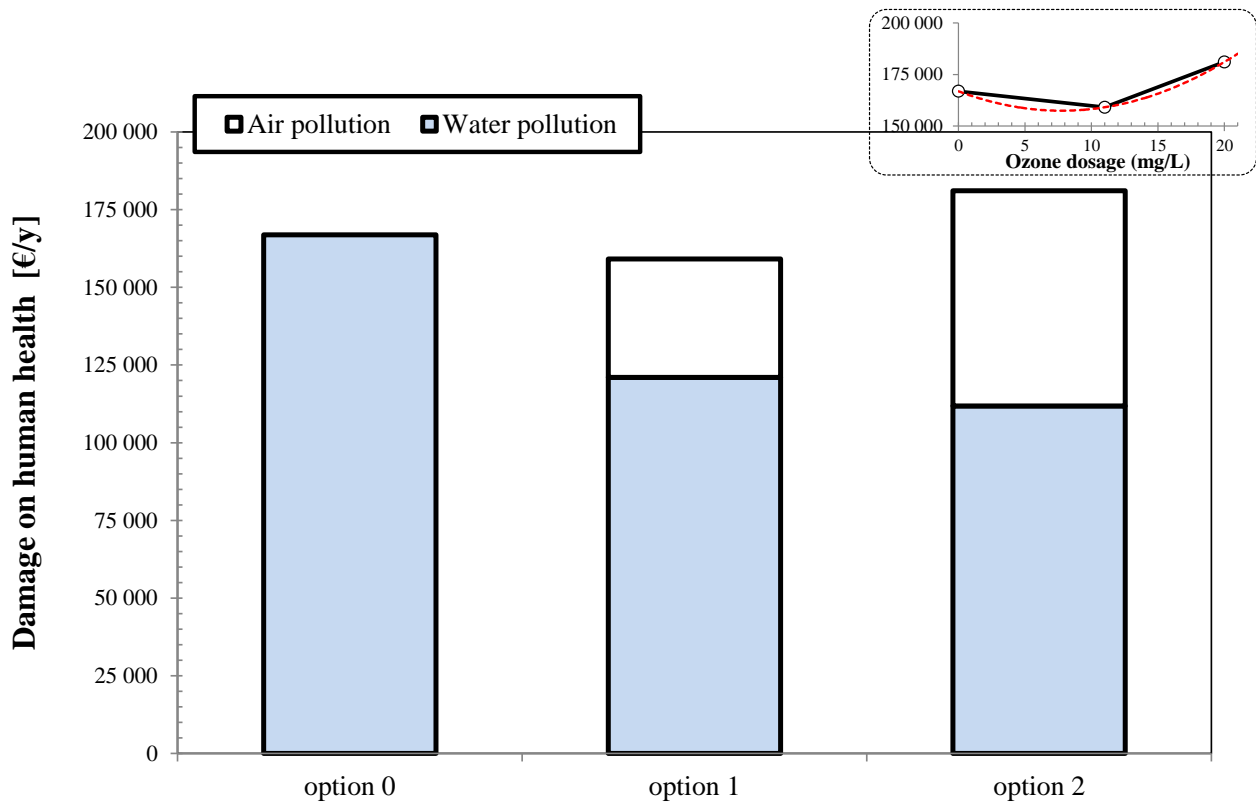
Figure 8. Impact on endpoint category (as damage on human health) for three analyzed scenarios and damage-ozone dosage pattern (detail in the upper right corner).

A similar comparison can be performed also considering the monetary costs of treatment: as expected and displayed in Figure 9, options 1 and 2 are characterized by total costs (monetary and social) much higher than option 0.

Moreover, it can be highlighted that, in order to reduce the social cost of a pollution phenomenon (related to the water sector), the monetary costs that must be employed represent a huge amount, even more than the same initial damage: nevertheless, this great economic effort does not yield an appreciable damage reduction. In this assessment, however, the monetary cost has been compared with the social one, although the latter represents an inestimable value, perhaps, being linked to human life and its quality.
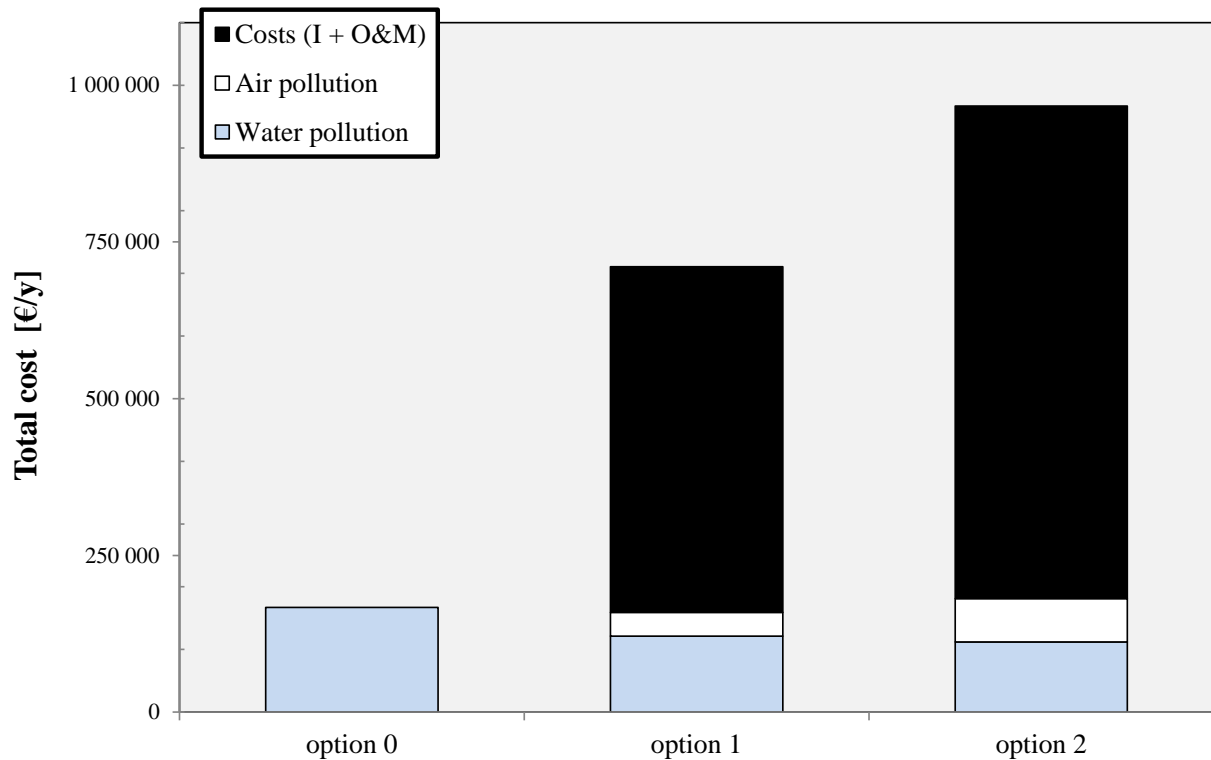
Figure 9. Total costs (monetary *plus* social) for the analyzed scenarios.

4.3.5 SENSITIVITY ANALYSIS

The results of one-way sensitivity analysis are graphically reported in the form of *tornado diagrams* (Figure 10), which records the percentage impact on the model's outcome (damage on human health) for each parameter change, respectively for option 0 (Figure 5A), option 1 (Figure 5B) and option 2 (Figure 5C). The test shows that the key drivers of the model variability can be identified in two main parameters: endocrine disorders (for water) and CO_2 emissions (for atmospheric pollution). Indeed, they are able to generate heavy variation (more than 50%, took up as threshold level), when assuming the highest possible damage value (i.e., 0.06 €/$kg_{emitted}$ for CO_2 emission, and 20% WaSH attributable fraction for endocrine diseases). This, anyway, is an expected finding, considering the high variability associated with both CO_2 external costs (related to the damage of global warming, whose quantification is characterized by a high level of complexity and hypothesis to be adopted, as briefly reported in the *Materials and Methods* section) and WaSH attributable fraction for endocrine diseases (as above stated, "population health impacts associated with

environmental exposures to endocrine-disrupting substances were not deemed to be perfectly quantifiable at present" (WHO 2006)).
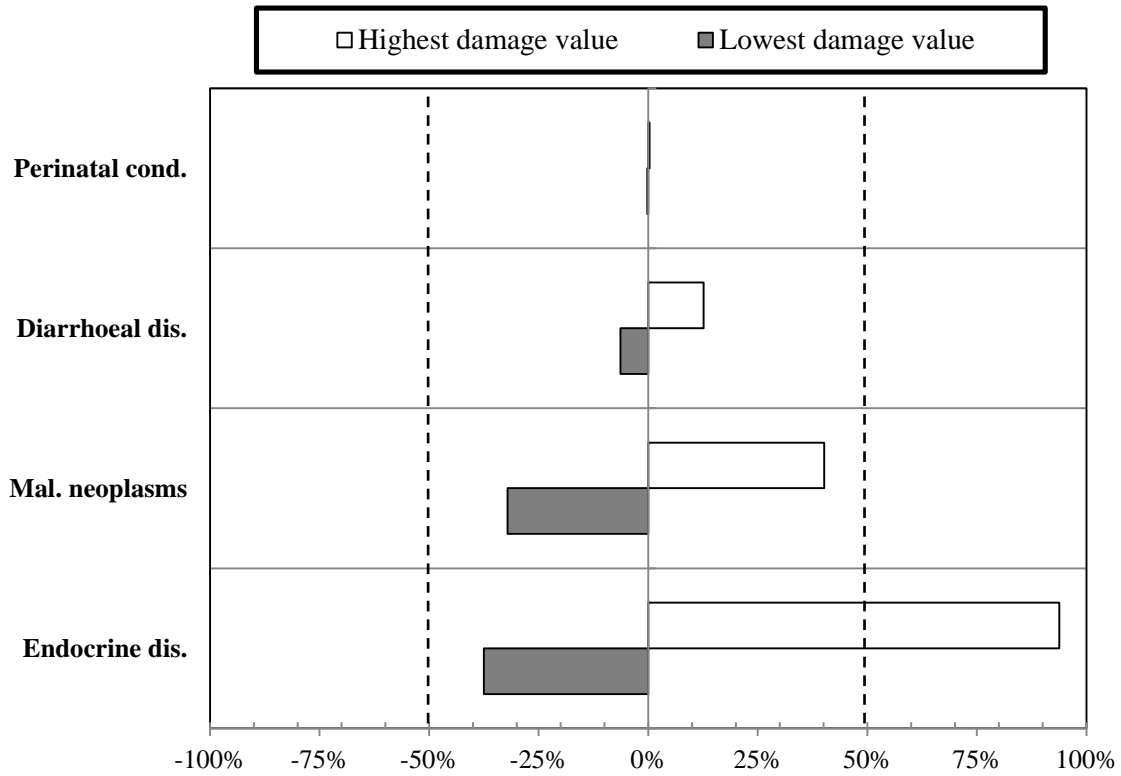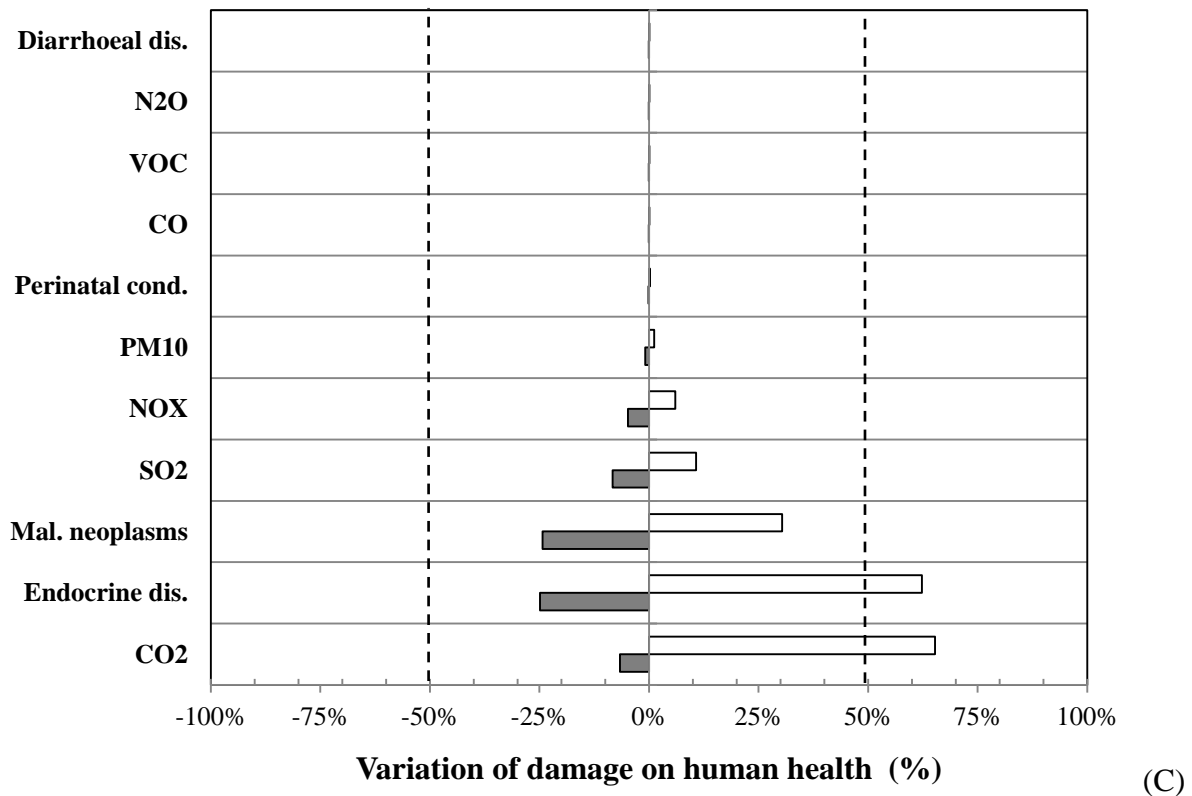

(A)
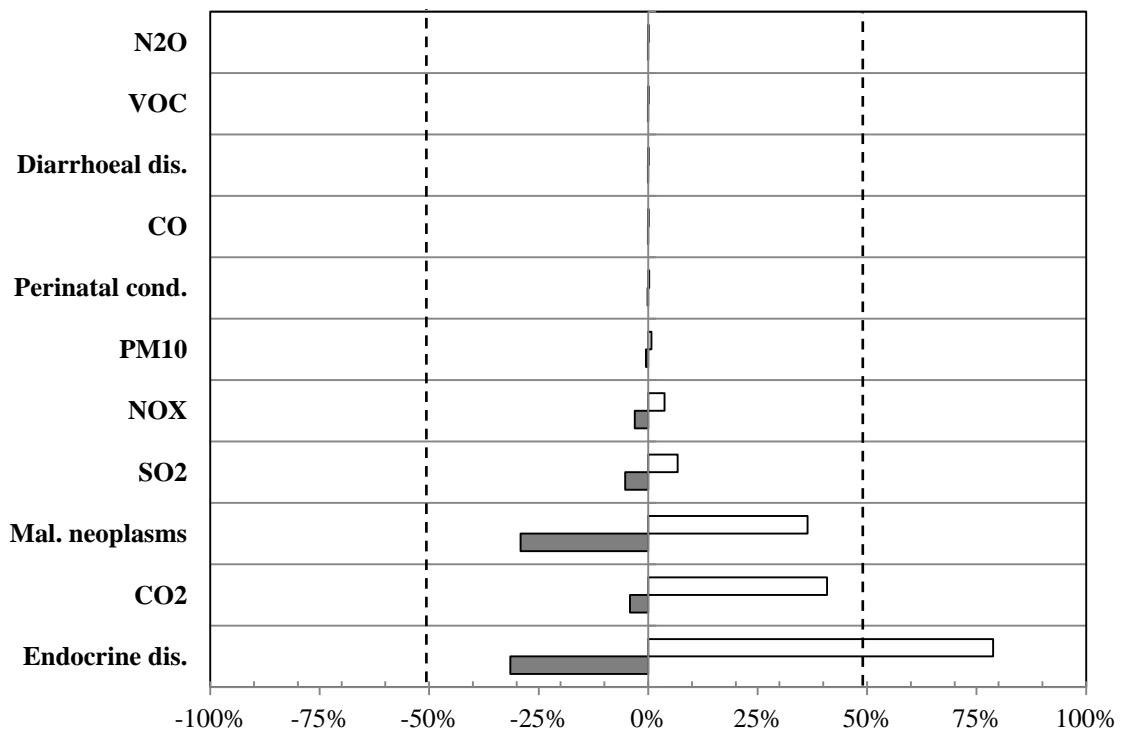
(B)

Figure 10. *Tornado diagrams* representing the change (%) in damage on human health, as result of one-way sensitivity analysis, for option 0 (A), 1 (B) and 2 (C), respectively.

On the contrary, Figure 11 displays the outcome of multi-way sensitivity analysis, respectively for tested scenarios (A: minimization of damage; B: maximization of damage; C: water-dominant damage; D: atmospheric-dominant damage). According to the previous analysis on average values, quite similar damages for each option are recorded in A and B scenarios, even if the better case would seem to be option 0; furthermore, the high variability of damage factors generates one order magnitude gap between the minimum (A) and the maximum (B) scenario. The crossing scenarios (C and D) shows, as expected, a damage prevailing in option 0 and option 2, respectively.

In synthesis, then, Figure 11E reassumes the total average damage on human health for three analyzed options, together with the maximum and minimum values (represented by error bars), as derived by sensitivity analysis. The final result is characterized by a high level of uncertainty, thus directly deriving from the variability of assumed external costs (mainly for CO_2 emissions and for WaSH attributable fraction of endocrine diseases). It has to be strongly underlined that extreme (i.e., minimum and maximum) values have been applied for this evaluation, because no information on statistical distribution of parameters (that would help to contain the spread of results and to increase the significance of methodology) was known.
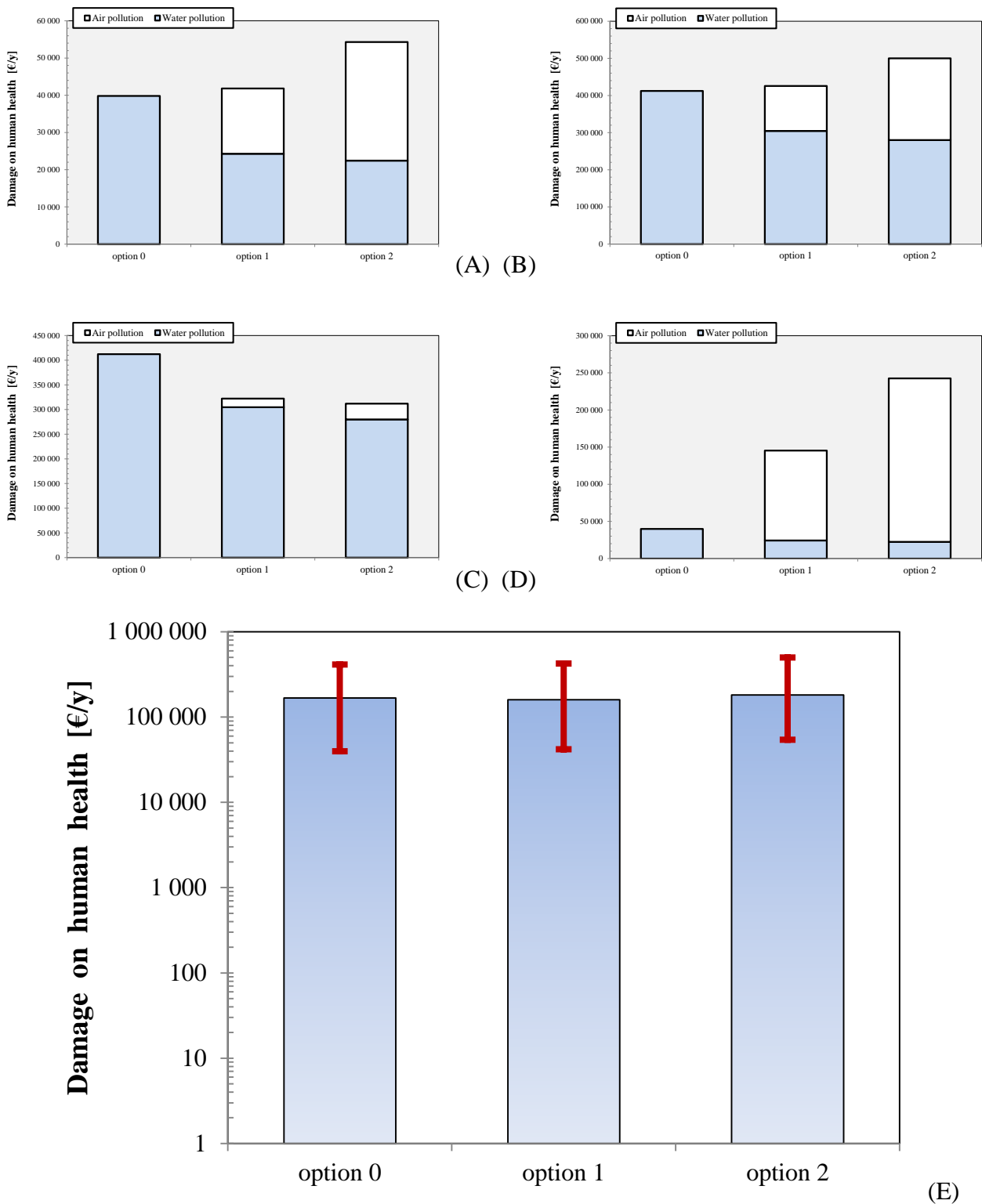
Figure 11. Multi-way sensitivity analysis outcomes, for tested scenarios (A: minimization of damage; B: maximization of damage; C: water-dominant damage; D: atmospheric-dominant damage; E: synthetic representation of lowest, average and highest scenario).

Figure 11E shows, in conclusion, very similar damage values, with no statistically significant differences between the options. Probably, the actual impacts could be carried out with a more focused site-specific analysis, by means of peculiar data of the case study, but then losing in generalization of results.

Finally, Figure 12 summarizes the damage composition (%) for each scenario (lowest, average and highest effects), in order to better highlight the weight of each factor.

In particular, for water pollution, the distinction of three options is also accounted for: anyway, for all the scenarios, the main diseases are represented by endocrine disorders and malignant neoplasm while diarrhoeal disease damage is completely eliminated with ozonation treatment (both option 1 and 2). Furthermore, endocrine diseases become strongly predominant in the highest damage scenario, as a consequence of their high variability.

Instead, air pollution maintains the same damage distribution for both option 1 and 2 (thus represented with a pie chart), but the key parameters differ, depending on the scenario: mainly SO_2 and NO_X for the lowest and the average and, on the contrary, CO_2 for the highest one, as expected considering the high spread recorded in one-way sensitivity analysis.
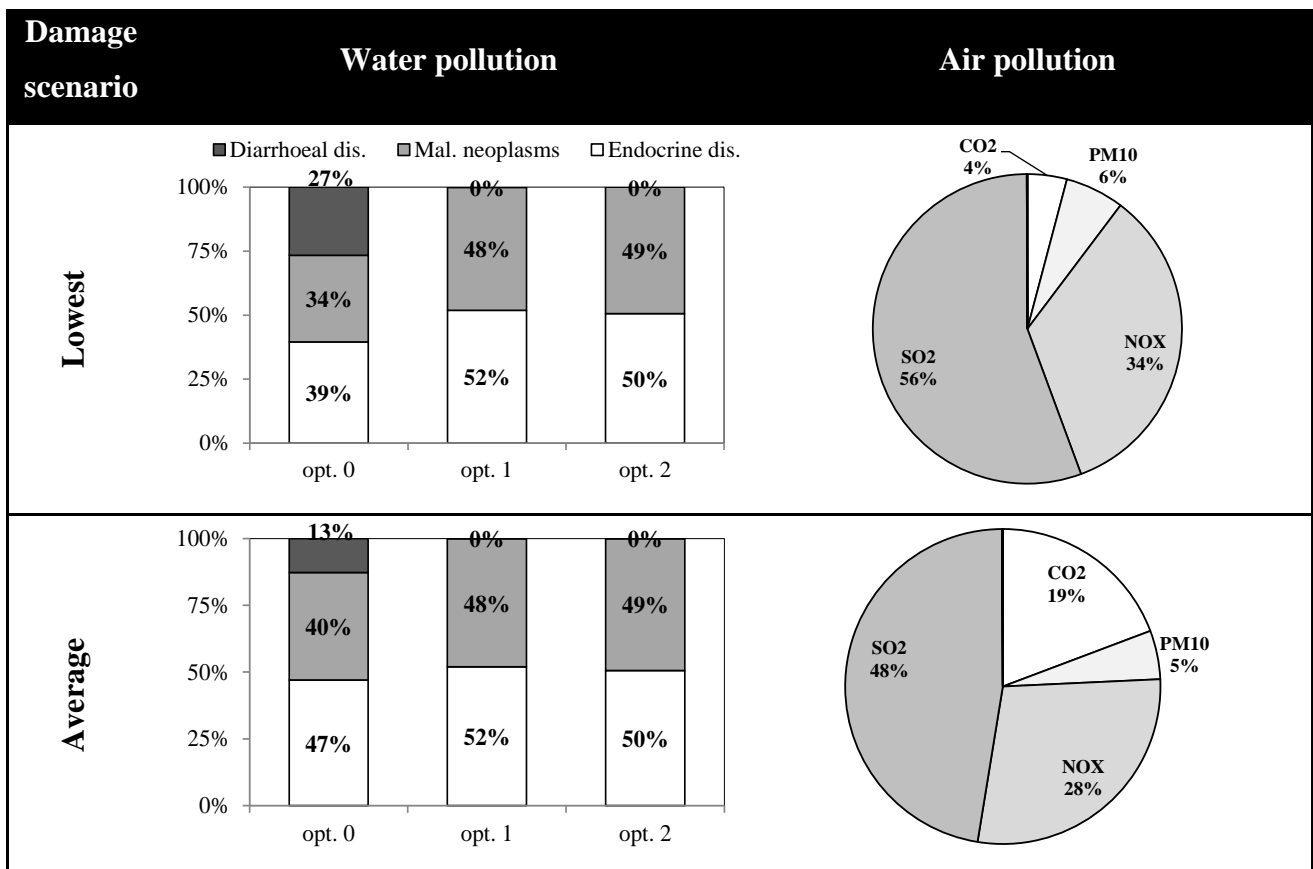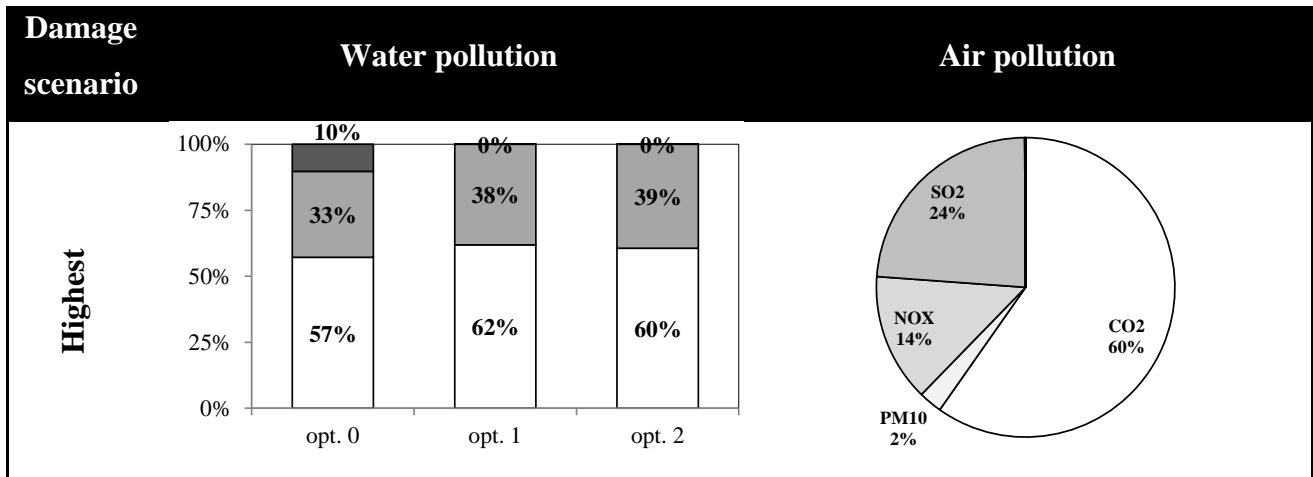
Figure 12. Summary of damage composition (%) imputable to water rather than air pollution, for each scenario (lowest, average and highest effects).

4.4 CONCLUSIONS

In this study, an advanced attempt at evaluating the environmental compatibility of a wastewater tertiary ozonation to control organic micropollutants was performed, based on a preliminary order-of magnitude calculation of damage on human health, expressed as an economic value. In order to achieve this goal, the effects on both water and air sectors were evaluated. For water pollution, an innovative environmental appraisal framework was applied: indeed, other traditional tools, such as LCA, are unlikely to prove suitable, due to the difficulty in assigning economic value to environmental factors and in accounting for all the negative effects on human health, which can be detected only through appropriate bioassays. For atmospheric emissions, on the contrary, the results of previous studies on the damage generated by specific atmospheric pollutants were used.

In particular, the assessment was conducted considering the lack (option 0) *vs* the presence (option 1, 11 mgO_3/L) of a tertiary ozonation stage. Furthermore, in order to increase the options to be compared, the same treatment was evaluated also under the hypothesis of strong O_3 dosage (option 2, 20 mgO_3/L).

Primarily, following traditional assessment tools, the inventory of emissions was established, showing a cross-media conflict between water and air matrix: the more the WWTP effluent quality increased, the more the atmospheric contamination raised-up. The analysis carried out on midpoint categories (expressed as environmental themes such as common mechanisms, e.g. climate change, or commonly accepted grouping, e.g. ecotoxicity), then, showed that option 0 would seem to be the preferred choice for most of the environmental themes (zero impact), but the worst performer for

aquatic toxicity. Anyway, no obvious decision on the environmental compatibility of O_3 treatment could be drawn in this phase, due to the persistence of the cross-media conflicts also at this level.

The solution of the conflict can be allowed only by the analysis of an endpoint category, the damage on human health: it's just at this stage that the innovative appraisal framework took place. In particular, this study propose, for water pollution, a health impact assessment through the measure of the environmental (WaSH category) burden of disease, attributable to a specific emission source (WWTP discharge) taking into consideration the health status of citizens (measured by means of DALY values). Therefore, the impacts caused by both water (due to WWTP effluent) and atmospheric (due to O_3 plant) pollution on human health were calculated and, as result, quite similar effects (expressed as economic values) were recorded for all the analyzed scenarios, considering the unavoidable approximations of methodology. Anyway, option 1 (O_3 treatment with mild dosage) seemed to be the better case: in particular, the lowest effects were put down with dosages around 7-8 mgO_3/L, suggesting that highest benefits could be obtained with neither too low nor excessive doses. On this side, however, it has to be reminded that several impacts (i.e., the construction of the ozonation plant, assumed already existing, and the collection and transport of fuels to the thermoelectric power plants) were not accounted for: their assessment would have played against option 1 (and 2, as well).

Moreover, as expected, the damage composition was different: for option 0, indeed, all the effects could be attributed to water emission and, in particular, the main diseases were represented by endocrine disorders ($\approx$ 45%), malignant neoplasms ($\approx$ 40%) and diarrhoeal diseases ($\approx$ 15%). Option 1 and 2 were characterized, on the contrary, by both water and air pollution, the former consisting only of endocrine disorders ($\approx$ 50%) and malignant neoplasm ($\approx$ 50%), while diarrhoeal diseases were completely eliminated through chemical oxidation; the latter, instead, due to SO_2 ($\approx$ 45%), NO_X ($\approx$ 30%), CO_2 ($\approx$ 20%), and PM_{10} ($\approx$ 5%) emissions.

A similar comparison was performed also considering the monetary costs of treatment: this analysis showed that, in order to reduce the social cost of a pollution phenomenon (related to the water sector), the economic costs that must be employed represent a huge amount, even more than the same initial damage: nevertheless, this great economic effort does not yield an appreciable damage reduction. In this assessment, however, the monetary cost has been compared with the social one, although the latter represents an inestimable value, perhaps, being linked to human life and its quality.

Finally, a sensitivity analysis was carried out in order to take into account the inherent uncertainty of various input factors, mainly the external costs (for air emissions) and the WaSH attributable disease fraction (for water emissions), which were both characterized by a relatively strong

variability, quantified by means of minimum and maximum values. One-way sensitivity analysis, from one hand, showed that endocrine disorders and CO_2 emissions were the main drivers of model variability, able to generate heavy variation (even more than 50%) when assuming the highest possible damage value. This, anyway, was an expected outcome, considering the high unevenness associated with both CO_2 external costs (related, indeed, to the damage of global warming, whose quantification is characterized by high complexity) and the WaSH attributable fraction of endocrine disorders (not yet fully recognized). Multi-way sensitivity analysis, on the other hand, displayed a final damage result characterized by a prominent degree of uncertainty, with no appreciable differences between the various options: in any case, it has to be strongly underlined that extreme (i.e., minimum and maximum) values were applied for the evaluation, because any information on statistical distribution of parameters (that would help to contain the spread of results) was known.

In summary, the assessment of the environmental compatibility of a wastewater tertiary ozonation highlighted the onset of a conflict (water *vs* atmospheric pollution), that was solved only through the definition of a common endpoint category and, consequently, the determination of damage on human health for both the matrices. This approach, coupled with biological (estrogenic and mutagenic) and microbiological (disinfection) assays, made the comparison possible. The results of the assessment performed in the present work, then, displayed similar damage effects for all analyzed scenarios, nevertheless the complexity of its structure, whose weaknesses have been pointed out in the text. Probably, a more focused, site-specific analysis could be carried out with peculiar data of the case study, but then losing in generalization of results.

All these findings, therefore, suggested that the optimal solutions to control organic micropollutants and, thus, improving WWTPs effluents quality can be prioritized as a function of the secondary impacts generated on atmosphere:

- first of all, the control at the source of the pollution (i.e., green chemistry), representing a zero-impact answer;
- then, the reconfiguration of existing biological treatment processes: for instance, it has previously been shown that WWTPs utilizing both nitrification and denitrification treatment steps and/or high sludge ages (15 days or more) exhibit better removal rates for endocrine disrupting compounds than those using high food-to-microorganism (F/M) ratio (as reported in *Chapter 2* and, among other authors, by Jones *et al.*, 2007). This solution would minimally deteriorate air quality;
- finally, only at the last place of a proper planning, an additional energy-intensive treatment (e.g. a tertiary ozonation stage), responsible for an atmospheric pollution almost comparable

(in terms of damage on human health, as displayed in this work) to that one avoided to water compartment.

On this side, however, thanks to greater take-up of energy efficient processes and renewable energy schemes (Zakkour *et al.*, 2002), the protection of the aquatic environment by means of end-of-pipe solutions may be delivered at an environmental cost lower than is presently achieved. Next step of the research will involve the evaluation of the effectiveness of tertiary treatments with less environmental effects (i.e., less energy-intensive).

Finally, it has to be highlighted that the methodology cannot make the final decision on environmental compatibility, but it is just a tool that allows the decision maker to set out the main issues and to fairly consider the options: notwithstanding the extensive uncertainties affecting external costs analysis, these benchmarks can still be a useful guide when discussing whether the specific advantages generated by implementation of a new treatment represents a benefit for the wider environment.

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CHAPTER 5

CONCLUSIONS

The primary objective of this study was to provide key baseline information concerning the concentrations of individual EDCs (measured using gas chromatography with mass spectrometry method) and the estrogenic activity (measured through an *in vitro* bioassay) of i) biological (CAS and MBR systems) treated sewage and ii) chemically oxidized (with ozonation) secondary effluents. Another objective of this study included the evaluation of bioassays to analyze wastewater samples, and the correlation between bio- and chemical assays. A third scope that was pursued involved the assessment of environmental compatibility of tertiary ozonation.

In particular, in this work two tertiary ozonation plants were investigated: a pilot- (reactor volume = 1,500 L, flow-rate up to 6 m^3/h, treating the effluent of a municipal WWTP) and a full-scale (140.000 p.e. WWTP equipped with a tertiary ozonation stage, treating both domestic and industrial wastewater) plants, namely experimentation #1 and #2 respectively.

As far as the pilot plant is concerned (experimentation #1), chemical oxidation of trace pollutants was described by first order kinetics, rate constants being dependent on reagent dosage: for instance, a 90% removal of BPA and NP could be achieved either after 80 min at 8 mgO$_3$/L, or 27 min at 11 mgO$_3$/L. From the economic point of view, starting from 1 µg/L NP (or BPA) concentration in the effluent of a WWTP, a further reduction of 70% (which means an effluent concentration of 0.3 µg/L, equal to the environmental quality standard for surface waters, as proposed by the EU Directive 2008/105/EC on water policy) can be achieved with ozonation at operating costs around 2-4 Euro cents/m^3.

Biological analyses, on the other hand, confirmed the beneficial effect of ozonation on the reduction of estrogenicity. However, unlike analytes, estrogenic activity abatement was not significantly affected by ozone dosage.

This study, although at an early stage (e.g., bioassay data were processed by means of a simplified methodology), demonstrated the efficacy of an integrated (chemical + biological) approach in evaluating performances of wastewater treatment processes: bioassays account for synergistic

effects of dozens of pollutants, the simultaneous determination of which might be actually unfeasible.

As far as the full-scale plant is regarded (experimentation #2), chemical analyses showed that analyzed substances occurred in the range of μg/L in WWTP effluent, and the removal efficiency of ozonation turned out to be around 50%.

The bioassay results, then, processed by means of a dose-response pattern, displayed concentrations of estradiol equivalent in the order of ng/L and clarified that ozone is only partially able to reduce the estrogenic activity (less than 20% decrease). In particular, both EDCs and estrogenicity abatement were significantly lower than the ones obtained in experimentation #1 (the pilot plant); the reason of these incomparable performances lies in the different wastewater source: almost totally domestic in the case of experimentation #1 and, on the contrary, with a considerable industrial component for experimentation #2. Furthermore, the comparison between measured (through *in vitro* bioassay) and predicted (by way of analytical chemistry) estrogenicity highlighted a discrepancy between the obtained values (i.e., measured around one order of magnitude higher than the predicted) and an effective estrogenic abatement significantly lower than the one predicted by EDCs concentration.

These findings can be explained considering that:

- a mixture of compounds is responsible for the estrogenic activity, not only analyzed EDCs;
- synergistic and potentiating effects between the components can occur;
- active by-products can originate, as oxidation intermediates;

and suggesting that the chemicals measured in the analytical assay cannot fully explain the estrogenicity of the samples.

To complete the spectrum of biological assays, mutagenic activity was also measured, and similar conclusions could be drawn. Indeed, the tests revealed the presence and/or formation of significant amounts of genotoxic compounds in wastewater, both before and after ozone treatment: tertiary oxidation did not improve the mutagenic characteristics of the wastewater under study, despite its effectiveness in reducing the analyzed trace pollutants. The scarce ability of ozone to reduce mutagenicity can be influenced by the chemical composition of wastewater, due to the high load of industrial discharges; furthermore, as previously stated for estrogenic activity, many classes of substances can contribute to mutagenicity, not only those identified.

Therefore, taking into account that chemical analysis does not reflect biological status, the biological activity of this complex matrix needs to be studied in view of minimizing human and environmental exposure to noxious pollutants.

Moreover, as treatments themselves are as a source of (secondary) pollution, consequently the assessment of environmental compatibility of the full-scale tertiary ozonation was performed. In particular, this study proposed for water pollution an innovative environmental appraisal framework: indeed, other traditional tools, such as LCA, are unlikely to prove suitable, due to the difficulty in assigning economic value to environmental factors and in accounting for all the negative effects on human health, which can be detected only through appropriate bioassays. The health impact was measured through the environmental burden of disease, attributable to a specific emission source (WWTP discharge) taking into consideration the health status of citizens (measured by means of DALY values): the main accounted diseases were endocrine disorders, malignant neoplasms and diarrhoeal diseases. For atmospheric emissions, on the contrary, the results of previous studies on the damage generated by specific atmospheric pollutants (SO_2, NO_X, CO_2, and PM_{10}) were used.

The results of the impacts caused by both water (due to WWTP effluent) and atmospheric (due to O_3 plant) pollution on human health showed quite similar effects (expressed as an economic value) for all the analyzed scenarios (the lack (option 0) *vs* the presence (option 1, 11 mgO_3/L, and option 2, 20 mgO_3/L) of the treatment), considering the unavoidable approximations of methodology; anyway, option 1 (O_3 treatment with mild dosage) seemed to be the better case: in particular, the lowest effects were put down with dosages around 7-8 mgO_3/L, suggesting that highest benefits could be obtained with neither too low nor excessive doses. On this side, however, it has to be reminded that several impacts (i.e., the construction of the ozonation plant, assumed already existing, and the collection and transport of fuels to the thermoelectric power plants) were not accounted for: their assessment would have played against option 1 (and 2, as well).

Finally, considering the inherent uncertainty of various input factors, a sensitivity analysis was carried out: the final damage result was characterized by a high degree of variability, with no appreciable differences between the various options: in any case, it has to be strongly underlined that extreme (i.e., minimum and maximum) values were applied for the evaluation, because any information on statistical distribution of parameters (that would help to contain the spread of results) was not known.

Probably, a more focused, site-specific analysis could be carried out with peculiar data of the case study, but then losing in generalization of results.

In summary, this research highlighted, first of all, the role of bioassays, found to be appropriate to determine estrogenic and genotoxic activity in wastewater samples, having taken also into account synergistic interactions; without neglecting the chemical analysis, essential to identify and monitor

EDCs concentrations: as stated above, the integrated (chemical + biological) approach represents a powerful tool in evaluating performances of wastewater treatment processes. The standardization of operating protocols, including data analysis methodology and the automation of the assays (i.e., to a degree that they can be used routinely by laboratories) may definitely help the application of these tests to WWTPs effluents.

On this side, next steps of our work will involve both chemical (in order to complete the spectrum of endocrine compounds to be measured, in particular with steroidal hormones, having a strong estrogenic strength) and biological issues, with the inclusion of additional assays such as eco-toxicity tests with bacteria (e.g. *Vibrio fischeri*), algae (e.g. *Selenastrum capricornutum*) and crustacea (e.g. *Daphnia magna*).

Then, this work underlined the fundamental importance of the proper definition of an endpoint category (the damage on human health, expressed as an economic value) to solve the conflict (water *vs* atmospheric pollution) born in the assessment of the environmental compatibility of tertiary ozonation. To get this calculation possible for water pollution, an innovative appraisal framework was applied, coupled with biological (estrogenic and mutagenic) and microbiological (disinfection) assays. The results of the assessment, although displaying similar damage effects for all analyzed scenarios, are useful to prioritize the possible solutions to control organic micropollutants and, thus, improving WWTPs effluents: i) first of all, the control at the source of the pollution (i.e., green chemistry), representing a zero-impact answer; ii) then, the upgrading of existing biological processes (e.g. with nitrification and denitrification treatment steps); iii) finally, only at the last place of a proper planning, an additional energy-intensive treatment (e.g. a tertiary ozonation stage), responsible for an atmospheric pollution almost comparable (in terms of damage on human health, as displayed in this work) to that one avoided to water compartment.

On this side, however, thanks to greater take-up of energy efficient processes and renewable energy schemes, the protection of the aquatic environment by means of end-of-pipe solutions may be delivered at an environmental cost lower than is presently achieved. For example, next step of the research will involve the evaluation of the effectiveness of tertiary treatments with less environmental effects (i.e., less energy-intensive).