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SIMULTANEOUS REMOVAL OF ESTROGENS AND PATHOGENS FROM SECONDARY TREATED WASTEWATER BY SOLAR PHOTOCATALYTIC TREATMENT

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ABSTRACT

Recently, the fate of emerging compounds in environmentally relevant samples has attracted considerable attention. Solar semiconductor photocatalysis may offer an appealing methodology to treat such contaminants. At the same time the use of solar photocatalysis for water and wastewater disinfection is a topic well-documented in the literature. In this respect, the simultaneous degradation of synthetic estrogen 17α-ethynylestradiol (EE2) and *Escherichia coli* removal employing simulated solar radiation and TiO_2 as the photocatalyst was investigated. In general, the more complex the water matrix is the slower *E. coli* removal becomes, while the presence of *E. coli* in the reaction mixture did not obstruct EE2 removal.

Although EE2 removal occurred relatively fast, overall estrogenic activity was only partially removed. This implies that other species inherently present in the effluent and/or some photocatalytic transformation by-products may be proportionately more estrogenic than EE2. Overall, the use of solar radiation can constitute an advantageous treatment strategy for the simultaneous removal of micro-pollutants and pathogens from secondary treated effluent.

Keywords: disinfection; *E. coli*; solar irradiation; photocatalysis; titanium dioxide; estrogens; endocrine disruptors.

1. Introduction

Occurrence, impact, and removal of emerging contaminants from water and wastewater have been the target of many studies in the last decades (Michael *et al.*, 2013; Sumpter and Johnson, 2008). Endocrine disrupting compounds (EDCs) constitute an important class of emerging environmental contaminants, which pose an increasing threat to aquatic organisms, as well as to human health (Jjemba, 2008, Belgiorno *et al.*, 2007; Jobling *et al.*, 1998). In particular, the exposure to EDCs has been linked with altering functions of the endocrine system in male fish such as vitellogenin induction and feminized reproductive organs (Belgiorno *et al.*, 2007). Moreover, the increasing incidence of cancer and the hypothesis of a decreasing reproductive fitness of males, are thought to be attributed to EDCs (Auriol *et al.*, 2006). Natural and synthetic estrogen hormones such as 17β-estradiol, estriol and 17α-ethynylestradiol (EE2) are well-known for their estrogenic activity. These enter municipal wastewater treatment plants (WWTPs) through either excretion or disposal of unused and expired medicines in the

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sewage system (Kuster *et al.*, 2009). Currently, when EDCs enter sewage system, and afterwards conventional biological WWTP, only a small amount of these chemicals are removed via biodegradation process because many of them are non-biodegradable (Richardson, 2009). Therefore, the majority of EDCs remain soluble in the effluent and been discharged to aquatic bodies (Stasinakis *et al.*, 2008; Vajda *et al.*, 2008; Desbrow *et al.*, 1998). Thus, it is necessary to look further on the removal mechanisms, in order to improve the efficiency of the existing treatment systems and to develop new and reliable treatment strategies to remove EDCs from wastewater (Michael *et al.*, 2013; Klavarioti *et al.*, 2009; Comninellis *et al.*, 2008).

Until now, several authors have demonstrated the efficiency of various physico-chemical processes like adsorption or membrane technology for the elimination of EDCs (Gerrity *et al.*, 2011; Snyder *et al.*, 2007). The main drawback of these techniques is that the emerging contaminants transfers to another phase (adsorption), or the production of a secondary stream with a concentrated solution of EDCs, requiring an additional treatment, such as the membranes-separation technology.
On the contrary several authors reported the efficiency of advanced oxidation processes (AOPs), a group of chemical treatment procedures designed to remove organic (and sometimes inorganic) materials in water and waste water, by oxidation reactions with hydroxyl radicals (·OH). AOPs can eliminate the emerging contaminants in aqueous phase, rather than collecting or transferring pollutants into another phase (Michael *et al.*, 2013; Klavarioti *et al.*, 2009).

In the present study, solar photocatalytic process was applied for this purpose.

Nowadays, photocatalytic processes are also used as an alternative disinfection technology replacing the chlorination step that is widely used in WWTPs (Rizzo *et al.*, 2014; Malato *et al.*, 2009; Melemeni *et al.*, 2009). It is well-known that when chlorine is used as a disinfectant, it reacts with the natural organic matter present in water and wastewater to produce various undesirable chlorinated disinfection by-products (DBPs) (Hua and Reckhow, 2007). Of the wide variety of chlorinated DBPs formed, trihalomethanes and haloacetic acids are of primary concern since many of them have been found to be carcinogenic and/or mutagenic (Richardson, 2003). Therefore, ongoing research focuses on the development of alternative disinfection methods (Lazarova *et al.*, 1999). It is worth noting that usually the application of advanced oxidation processes is limited by their high cost. However, according to Vidal *et al.*, (1999), the estimated total costs for solar photocatalytic degradation of 500 μg l^{-1} of selected pesticides to maximum permitted levels (0.1 μg l^{-1}) and the four-log inactivation of E. *faecalis* bacteria, is competitive with conventional technologies and estimated to be about US$ 0.7 per m^3.

In recent years, various studies have been conducted to demonstrate photocatalytic disinfection under visible light, using mostly *E. coli* as a model microorganism, which is the well known bacterial indicator of faecal pollution (Rizzo *et al.*, 2014; Suri *et al.*, 2012; Chatzisymeon *et al.*, 2011; Dalrymple *et al.*, 2010; Gamage and Zhang, 2010; McCullagh *et al.*, 2007), however, the majority of studies focus strictly on the removal of *E. coli* without considering the presence of other emerging contaminants. Hence, this work reports the efficacy of solar TiO_2-mediated photocatalytic process to remove simultaneously EE2 and *E. coli* from secondary treated wastewaters.

2. Materials and Methods

2.1. Materials

EE2 ($C_{20}H_{24}O_2$), whose main physicochemical properties are given in Table 1, was purchased from Sigma–Aldrich (CAS number: 57-63-6) and used as received. Aeroxide P25 titania (formerly known as Degussa P25, 75:25 anatase:rutile, 50 m^2 g^{-1} BET area, 21 nm particle size, supplied by Evonik Industries) was employed as the photocatalyst. The bacterial reference strain used in this study was *E. coli* ATCC 23716 (American Type Culture Collection, Rockville, MD, USA). Colonies of the reference strain were inoculated in sterile distilled water, which was used as sample for synthetic wastewater disinfection experiments. The concentration of bacterial cells in the suspension was estimated measuring its optical density at 600 nm (Shimadzu UV1240 spectrophotometer) where, according to McFarland scale, an absorbance of 0.132 corresponds approximately to a cell density of1.5 10^8 CFU ml^{-1}. Plate counts were also performed

for accurate bacterial count. The concentration of the inocula was within the range of 10^3–10^7 CFU ml^{-1}, depending on the experiment conducted.

Table 1. Properties of 17α-ethynylestradiol

Compound	**17α-Ethynylestradiol (EE2)**
IUPAC name	17α-Ethynyl-1,3,5(10)-estratriene-3,17β-diol
CAS number	57-63-6
Molecular formula	$C_{20}H_{24}O_2$
Molar mass, g mol^{-1}	296.40
Appearance	White crystalline powder
Melting point, °C	182-183
*Solubility in water, mg l^{-1}	9.2±0.09

*Data taken from (Shareef *et al.*, 2006)

Wastewater (WW) collected from the outlet of the secondary treatment of a municipal WWTP (Chania, W. Crete, Greece). The matrix was characterized by standard methods as follows: the chemical oxygen demand (COD) and dissolved organic carbon (DOC) was 24 and 8.4 mg l^{-1}, respectively, while its pH was about 8 and the conductivity 820 μS cm^{-1}. Moreover, it contained 172 mg l^{-1} chlorides, 194 mg l^{1} bicarbonates, 54 mg l^{-1} sulfates, 37 mg l^{-1} nitrates and 37 mg l^{-1} nitrites.

2.2. Photocatalytic experiments

Simulated solar radiation experiments took place in a batch-type, laboratory-scale photoreactor. The volume of the reactant mixture was 300 ml. Radiation was provided by a Newport 67005 - 150 W solar simulator system. Chemical actinometry based on 2-nitrobenzaldehyde photoreaction (Galbavy *et al.*, 2010) was performed to measure the photon flux emitted from the solar simulator system which was found equal to 5.8×10^{-7} einstein l^{-1} s^{-1}). In a typical photocatalytic run, the effluent was loaded in the reaction vessel and the solution was slurried with the proper amount of TiO_2. The resulting TiO_2 suspension was magnetically stirred for 30 min in the dark to ensure complete equilibration of adsorption/desorption of EE2 and *E. coli* onto the catalyst surface. At specific time intervals, samples of about 2-3 ml were drawn from the reaction vessel and were centrifuged at 13200 rpm to remove catalyst particles and then analyzed for residual estrogens concentration. *E. coli* survival was also estimated for the same samples before undergoing any centrifugation process.

2.3. Analytical and molecular biology techniques

High performance liquid chromatography (Alliance 2690, Waters) was employed to monitor the concentration of EE2. Separation was achieved on a Luna C-18(2) column (5 μm, 250 mm×4.6 mm) and a security guard column (4 mm×3 mm), both purchased from Phenomenex. The mobile phase consisting of 35 : 65 ultrapure water: acetonitrile eluted isocratically at 1 ml min^{-1} and 30 °C, while the injection volume was 100 μl. Detection was achieved through a fluorescence detector (Waters 474 Scanning Fluorescence detector), in which the excitation wavelength was 280 nm and the emission wavelength was 305 nm. Under these conditions, the retention time was 5.1 min, the limit of detection (LOD) was 0.63 μg l^{-1} and the limit of quantitation (LOQ) was 2.11 μg l^{-1} (Frontistis *et al.*, 2012a).

The detection and enumeration of *E. coli* in the reaction solution were performed using the serial dilution streak plate agar technique. Serial dilutions of the reaction solution were performed in sterile 0.8% (w/v%) NaCl aqueous solution and 200 μl of each dilution (including neat sample) were streaked onto HiCrome Coliform Agar plates (HiMedia Laboratories), which is a selective *E. coli* culture medium. The plates were incubated at 37 °C for 20–24 h before viable counts were determined. The minimum detectable number of bacteria in these experiments was 1 CFU ml^{-1} (a reflection of the fact that 1000 μL of neat sample (5 × 200 μl) were cultured out from each neat sample) (Venieri *et al.*, 2014).

Genomic DNA was extracted performing chemical lysis and phenol/chloroform/isoamyl alcohol (25:24:1) extraction using lysozyme and proteinase K, according to our previous work (Venieri *et al.*, 2013). The

quantity and purity of all DNA samples were determined measuring their absorbance at 260 nm and estimating the ratio of absorbance values at 260 nm and 280 nm, respectively.

The SYBR green qPCR method was chosen for the detection and quantification of gadAB gene of residual *E. coli* cells after each photocatalytic experiment using the StepOne Plus System (Applied Biosystems Inc., Foster City, CA, USA). The gadAB gene encodes glutamate decarboxylase and has both sensitivity and specificity to *E. coli*. The primers for the target gene were as follows: forward primer 5′-GCG TTG CGT AAA TAT GGT TGC CGA-3′ (gadrt-1) and reverse primer 5′-CGT CAC AGG CTT CAA TCA TGC GTT-3′ (gadrt-2) (Chen *et al.*, 2006). The product size for this primer set is 305 bp. Triplicate PCR reactions were carried out with Quantimix Easy SYG Kit (Biotools) to a final volume of 20 μL. The mixed qPCR solution contained 2XPCR master mix, 0.5 mM of each primer, 30 nM of reference dye (Rox) all diluted to the final volume of the reaction mixture with DNase/RNase free water. PCR reactions were carried out at a temperature profile of 10 min initial denaturation at 95 °C, followed by 40 cycles each of denaturation at 95 °C for 0.5 min, annealing at 57 °C for 1 min, and extension at 72 °C for 0.5 min. Deionized water and DNase-treated *E. coli* served as negative controls. Melting curve analyses were conducted from 55 to 95 °C (1 °C per cycle of 10 s) with simultaneous measurements of the SYBR Green signal intensities.

The yeast estrogen screening (YES) test was employed to evaluate overall estrogenicity according to the procedures described elsewhere (Frontistis *et al.*, 2011a).

3. Results and discussion

3.1. Effect of catalyst concentration

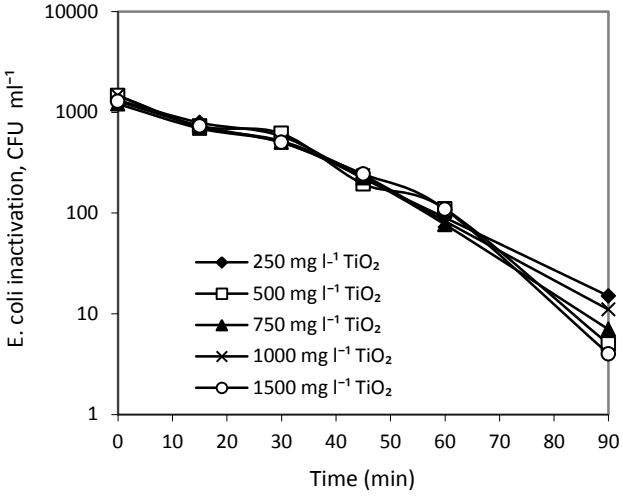
The effect of catalyst loading in the range 250-1500 mg l^{-1} on the photocatalytic disinfection in the presence of 100 μg l^{-1} EE2 was investigated and the results are shown in Figure 1.

As can be seen, increasing catalyst loading up to 750 mg l^{-1}, photocatalytic efficiency increased in terms of both EE2 and *E. coli* removal. The increase in the efficiency with increasing catalyst loading indicates a heterogeneous catalytic regime, since the fraction of incident light absorbed by the catalyst progressively increases in suspensions containing higher amounts of titanium dioxide (Herrmann, 2010). Increasing catalyst loading apparently results in an increased number of catalyst active sites that are available for photocatalytic reactions and this occurs up to a point where all catalyst particles become fully illuminated (Poulios and Kyriacou, 2002).

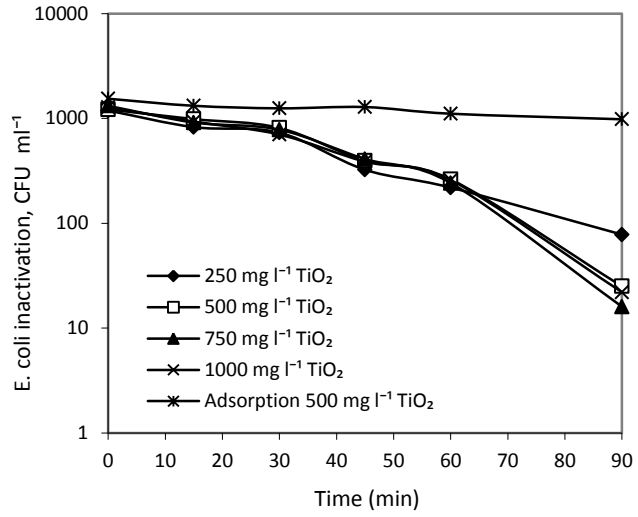
3.2. Effect of wastewater matrix

The organic and inorganic species typically found in environmentally relevant matrices may interfere with the oxidizing agents (e.g. photogenerated holes, as well as hydroxyl radicals) and affect the rate of disinfection. This is evident in Figure 2 showing that the matrix has an adverse effect on conversion and/or reaction rate; for instance, after 90 min of treatment at 500 mg l^{-1} titania disinfection efficiency for real wastewater was 3 times less than the respective value for the synthetic effluent (initial bacterial concentration 1×10^3 CFU ml^{-1}). This behaviour has been observed several times from our group in the degradation of organic compounds by heterogeneous photocatalysis (Frontistis *et al.*, 2012a, Frontistis *et al.*, 2012b) and photocatalytic disinfection (Chatzisymeon *et al.*, 2011).

This can be explained taking into account that (i) the oxidizing agents are competitively consumed in reactions involving the natural organic matter present in treated WW but not in pure water and constitutes most of the matrix's total organic content; (ii) hydroxyl radicals may be scavenged by bicarbonates, chlorides and sulfates present in WW to form the respective radicals, whose oxidation potential is lower than that of hydroxyl radicals (Frontistis *et al.*, 2012a)

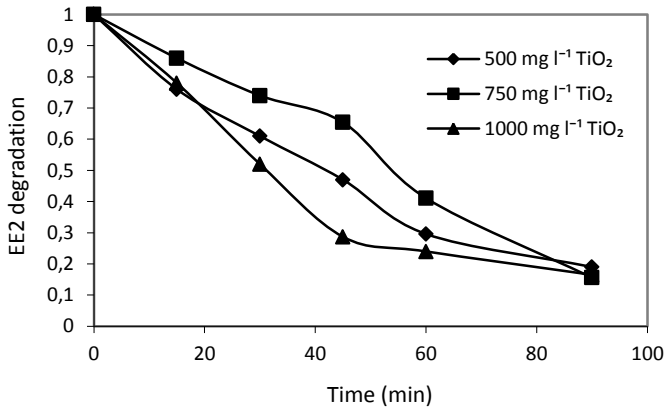


(a)



(b)

wastewater



(c)

Figure 1. Effect of catalyst loading on photocatalytic performance.
(a) *E. coli* in synthetic wastewater; (b) *E. coli* in actual wastewater; (c) EE2 in actual wastewater

3.3. EE2 & E. coli interaction

Experiments were carried out to investigate the possible interactions of EE2 with *E. coli*. Figure 2 clearly demonstrates that the influence of the presence of *E. coli* is almost negligible to EE2 removal. Particularly, EE2 removal in synthetic wastewater came up to 84.5% and 89.9%, after 90 min of treatment, under simulated solar irradiation, in the presence or absence of *E. coli*, respectively.

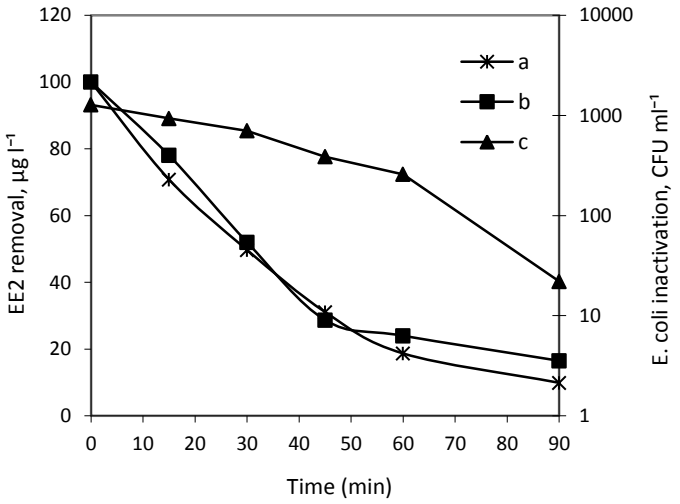


Figure 2. Photocatalytic efficiency under solar radiation with 1000 mg l^{-1} titania. (a) EE2 removal from a synthetic wastewater matrix inoculated with 100 μg l^{-1} EE2, (b) EE2 removal from a synthetic wastewater matrix inoculated with 100 μg l^{-1} EE2 and 10^3 CFU ml^{-1} *E. coli*, and (c) *E. coli* inactivation from a synthetic wastewater matrix inoculated with 100 μg l^{-1} EE2 and 10^3 CFU ml^{-1} *E. coli*.

However, the presence of estrogens has been associated with an increase in the risk of mutagenicity and genotoxicity. According to Roy and Liehr, (1999) various types of estrogen-induced DNA damage include: (a) direct covalent binding of estrogen quinone metabolites to DNA; (b) enhancement of endogenous DNA adducts by chronic estrogen exposure of rodents; (c) free radical generation by metabolic redox cycling between quinone and hydroquinone forms of estrogens and free radical damage to DNA. These types of DNA damage (i.e., 8-hydroxydeoxyguanine, lipid–DNA adducts and DNA strand breaks) generated by estrogen treatment or reactive metabolites of estrogens, are all potentially significant, because these lesions are capable of producing changes in the genome.

In an attempt to evaluate the consistency and integrity of microbial DNA in our wastewater samples, qPCR was performed for the detection and quantification of *E. coli* gadAB gene, isolated from residual viable cells after each photocatalytic experiment. The presence of EE2 and its photodegradation byproducts may lead to *E. coli* genomic extensive DNA damage, which subsequently may inhibit any generation of PCR products. In our case SYBR green qPCR proved to be effective in the detection and quantification of bacterial DNA during photocatalytic experiments, as there was successful generation of gadAB gene product of 305bp. In the present study, the high size/length of PCR product and the post-amplification melting curve analysis ensured the specificity and reliability of the method. Melting curve analysis is an assessment of the dissociation-characteristics of double-stranded DNA during heating. As the temperature is raised, the double strand begins to dissociate leading to a rise in the absorbance intensity, hyperchromicity. The temperature at which 50% of DNA is denatured is known as the melting point. The information gathered can be used to infer the presence and identity of possible polymorphisms with further analysis.

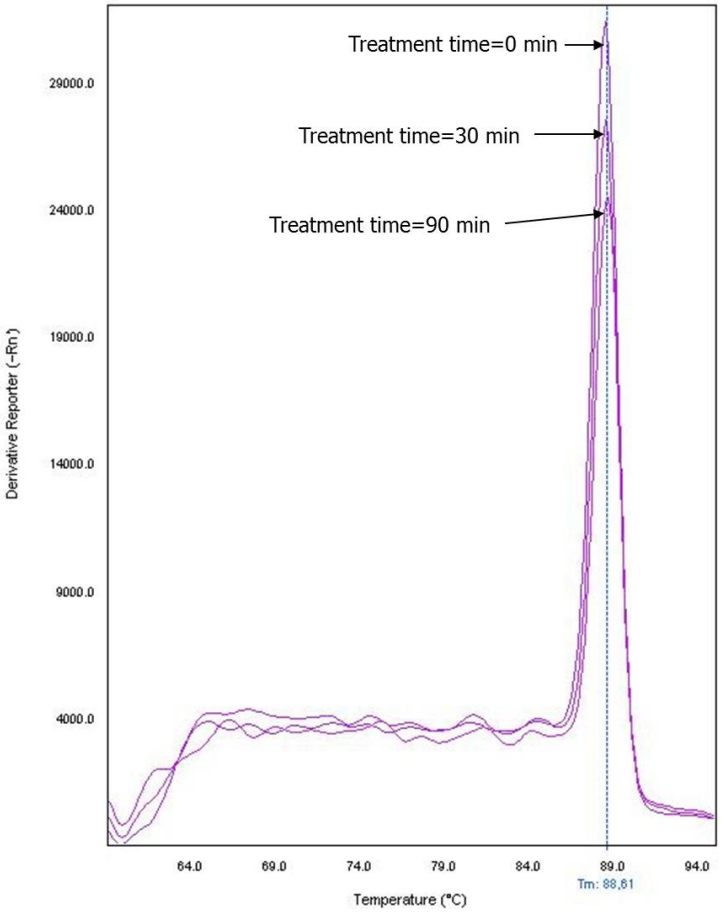


Figure 3. Melting curves in real-time for synthetic wastewater samples during their 90 min photocatalytic treatment, in the presence of 100 μg l^{-1} EE2

As shown in Figure 3, the point of infection (melting temperature of the amplicon) occurs at 88.6 °C and remains stable during photocatalytic treatment, showing that there are no considerable alterations to the bacterial DNA or PCR byproducts. Also, it is shown that DNA quantity is substantially decreased during the 90 min photocatalytic treatment and this is consistent with the decrease of bacteria survival, which was quantified by the serial dilution culture technique (Figure 2). In addition, it can be seen that no contaminating products are present in this PCR reaction, since contaminated DNA or primer dimers would show up as an additional peak separate from the desired melt curve amplicon peak. Consequently, it is interesting to note that there are no extensive DNA alterations, at least within the studied gene during the 90 min photocatalytic treatment of *E. coli* in the presence of EE2 in synthetic wastewater.

3.4. Removal of estrogen activity

Figure 4 shows changes in effluent's overall estrogen activity (as assessed by the YES assay) during 100 μg l^{-1} EE2 degradation at 750 mg l^{-1} titania in wastewater. As seen, estrogenicity decreases by only 6% after 30 min of reaction and this coincides with 30% EE2 removal. Estrogen activity slightly increases and then drops again leading to a final (i.e. after 90 min) reduction of 14%; these findings imply that certain EE2 and/or effluent organic matter transformation byproducts are proportionately more estrogenic than the parent compounds. Similar behaviour has been reported in recent studies from our group dealing with EE2 and estrogenicity removal by ultrasound irradiation and electrochemical oxidation (Frontistis *et al.*, 2011b, Frontistis and Mantzavinos, 2012).

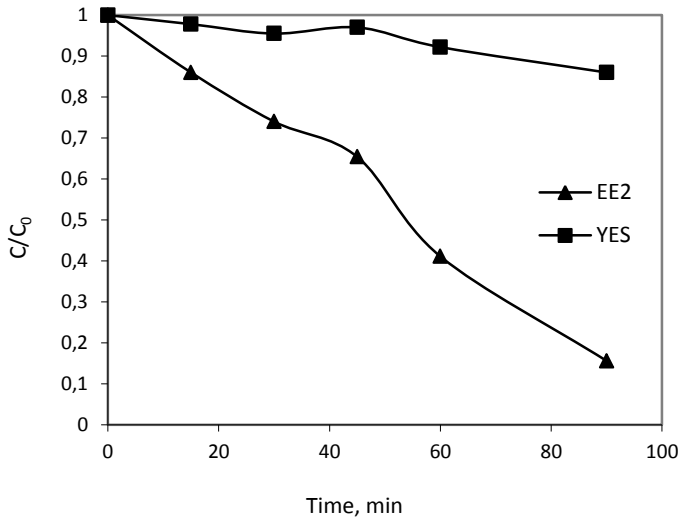


Figure 4. EE2 and estrogenicity removal during photocatalytic treatment of actual wastewater with 750 mg l^{-1} titania

These results show the need for further research on the correlation of the change in estrogenicity and EE2 in real aqueous matrices in order to assess the influence of the transformation byproducts of natural organic matter on estrogen activity.

At the same time, the effluent's dissolved organic carbon remains practical unchanged, thus indicating insignificant mineralization to carbon dioxide.

4. Conclusions

In this study, TiO_2-mediated solar photocatalytic treatment was successfully applied for the simultaneous removal of estrogens and pathogens from secondary treated effluent. Hence, the main conclusions drawn from this study are summarized as follows:

1) The more complex the water matrix is the slower the disinfection rate becomes; this is due to the non-target species inherently present in the matrix behaving as scavengers of the photogenerated oxidants. Nevertheless, the presence of *E. coli* in the reaction mixture did not obstruct EE2 removal.
2) The generation and quantification of *E. coli* gadAB gene during the photocatalytic treatment in the presence of EE2 in synthetic wastewater was successful through qPCR. Melting curve analysis showed no significant alterations in the specific gene segment or the formation of any byproducts.
3) Although EE2 removal occurred relatively fast, overall estrogenic activity was only partially removed. This implies that other species inherently present in the effluent and/or some photocatalytic transformation byproducts may be proportionately more estrogenic than EE2.

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