Efficacy assessment of peracetic acid in the removal of synthetic 17α-ethinyl estradiol contraceptive hormone in wastewater

Rita Maurício 1,*, Flávia Semedo 2, Rita Dias 2, João P. Noronha 3, Leonor Amaral 1, Michiel A. Daam 1, António P. Mano 2, Mário S. Diniz 4

1 CENSE, Center for Environmental and Sustainability Research, NOVA School of Science and Technology, NOVA University Lisbon, Quinta da Torre, 2829-516 Caparica, Portugal
2 Department of Environmental Sciences and Engineering, NOVA School of Science and Technology, NOVA University Lisbon, Quinta da Torre, 2829-516 Caparica, Portugal
3 REQUIMTE/FCT, NOVA School of Science and Technology, NOVA University Lisbon, Quinta da Torre, 2829-516 Caparica, Portugal
4 Biotox Lab, UCIBIO, Department of Chemistry, NOVA School of Science and Technology, NOVA University Lisbon, Quinta da Torre, 2829-516 Caparica, Portugal

A B S T R A C T

Increasing concerns have been raised on endocrine disrupting chemicals like the sex hormone 17α-ethinylestradiol (EE2), the more since traditional wastewater (WW) treatments appear to be ineffective for their removal. The efficacy of the relatively novel disinfectant peracetic acid (PAA) in EE2 removal was evaluated, as well as its potential effects on WW quality parameters. The treatments tested for EE2 removal were also evaluated in terms of toxicity, through the determination of biochemical responses (antioxidant enzymes, lipid peroxidation and vitellogenin induction) using zebrafish (Danio rerio) as a biological model. PAA contact times less than 20 min appeared insufficient regardless of the PAA dose tested, but a 100% EE2 removal was attained at a PAA concentration of 15 mg/L with a contact time of 20 min. Total suspended solids, chemical oxygen demand and pH in PAA treatments remained well within levels set in European legislation for WW discharge. EE2 induced significant increased vitellogenin (VTG) levels in both female and male fish, indicating increased estrogenic activity, especially in males suggesting an endocrine disruption effect. With the addition of PAA (15 mg/L), however, VTG levels in both sexes returned to control values. Although this PAA treatment showed increased levels of the antioxidant enzyme catalase, the lipid peroxidation levels were similar or even lower than in controls. Overall the results suggest that the use of PAA appears a promising way forward as a less toxic alternative to chlorine disinfection with high efficiency in the removal of EDC like EE2.

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* Corresponding author.
E-mail address: rmr@fct.unl.pt (R. Maurício).
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Introduction

In the past years, increasing concerns have been raised with the presence of endocrine disrupting chemicals (EDC) in environmental compartments and drinking water, since they may negatively affect the endocrine system of wildlife and humans (Adeel et al., 2017; Chen et al., 2017; Vilela et al., 2018). The sex hormone 17α-ethinylestradiol (EE2) is among the EDC with the highest estrogenic potency ($>$1), bioaccumulation potential, detection frequency and resistance to biodegradation (Aris et al., 2014; Barreiros et al., 2016; Chen et al., 2017; Maurício et al., 2018). For these reasons, EE2 was included in the European Union watch list of emerging aquatic pollutants (EC, 2015).

EE2 is used in oral contraceptives, as well as in hormone replacement therapies, suspension of breastfeeding, motor deficiencies associated with menopause, veterinary pharmacology, aquaculture and livestock (Aris et al., 2014; Liu et al., 2017). Wastewater treatment plants (WWTP) have been reported as the main point source of EE2, the more since conventional WWTP were not specifically designed, and hence have been reported to be ineffective, for the removal of EDC like EE2 (Barreiros et al., 2016; Adeel et al., 2017; Wu et al., 2017; Maurício et al., 2018; Vilela et al., 2018). For example, Cargou et al. (2004) reported EE2 removal efficiencies between 34% and 45% in WWTPs in the Paris area. In addition, traditional disinfection by chlorination may lead to the formation of toxic disinfection by-products (DBPs) through interaction with organic matter present in WW (e.g., Antonelli et al., 2013; Coyle et al., 2014; da Costa et al., 2014; Maurício et al., 2018). Regarding EE2, Pereira et al. (2011) demonstrated that EE2 metabolites resulting from the interaction with chloride may be up to ten times more toxic than parent EE2.

Peracetic acid (PAA) has emerged in recent years as one of the most promising alternatives for chlorine disinfection since PAA (1) has been shown to be cost-effective and can be easily installed in existing wastewater treatment facilities; (2) does not generate carcinogenic or mutagenic DBPs; (3) is less persistent as compared to chlorine; and (4) has a large spectrum of microbial activity with low dependency on pH and WW suspended solid size (Azzellino et al., 2011; Antonelli et al., 2013; Coyle et al., 2014; Block et al., 2015; Collivignarelli et al., 2017; Eramo et al., 2017; McFadden et al., 2017; Henao et al., 2018; Sun et al., 2018). The main disadvantages of the use of PAA as WW disinfectant are the higher costs as compared to chlorine disinfection and the increase in organic matter content in the effluent due to the formation of acetic acid during PAA degradation, which may potentially result in a microbial regrowth (Kitis, 2004; Collivignarelli et al., 2017; Luukkonen and Pehkonen, 2017). Most studies evaluating the efficacy of PAA have focussed on the removal of microorganisms like pathogenic bacteria and viruses, indicating that its potential to remove EDC like EE2 remains poorly known (Bonetta et al., 2017; Rizzo et al., 2019). There is no evidence, however, of any endocrine disruption potential of PAA itself in human health and ecotoxicological studies (Henao et al., 2018) and therefore there is a lack of knowledge concerning these issues.

The disinfecting action of PAA is due to the release of active oxygen or the production of reactive hydroxyl radicals that attack the bacterial cell causing the destruction of the cell wall and membrane as well as certain enzymes and DNA (Karpova et al., 2013; Luukkonen et al., 2015; Collivignarelli et al., 2017). Thus, this dictates that PAA may also potentially provoke oxidative stress to beneficial organisms in waterbodies receiving PAA-treated WW (Chhetri et al., 2014).

The main aim of the present study was to evaluate the efficacy of EE2 removal using PAA. This was achieved by determining the reduction in EE2 concentrations in jar tests and by measuring the estrogenic activity (vitellogenin - Vtg), antioxidant enzyme activities ([γ-glutathione-S-transferase - GST, and catalase - CAT] and oxidative stress (lipid peroxidation - LPO) following zebrafish (Danio rerio) exposure to different concentrations of PAA. Effects of PAA on water quality (pH, chemical oxygen demand – COD, total suspended solids - TSS) were also assessed to evaluate whether PAA-treated WW remained within the limits set in the EU.

1. Materials and methods

1.1. Wastewater

The wastewater used in the jar tests (see Section 1.2) was obtained from the wastewater treatment plant (WWTP) in Quinta do Conde, located near Lisbon (Portugal). This WWTP has a treatment capacity of 19,300 m$^3$/day and receives urban wastewater from about 94,000 equivalent inhabitants. The final effluent is discharged in the Coína stream, which leads to the Tagus river (Leite, 2014). This WWTP has a tertiary treatment level, including three treatment phases: liquid, solid and gaseous, and a water reuse system. The liquid phase includes a secondary treatment by oxidation ditches followed by secondary decantation and disinfection by ultraviolet (UV) radiation. The wastewater used in the jar tests was collected between the secondary treatment and the disinfection and kept in a refrigerator at 3°C until testing. The physical-chemical characteristics determined in the wastewater were: pH = 7.9; total suspended solids (TSS) $<$ 10 mg/L; chemical oxygen demand (COD) = 67 mg O$_2$/L TSS and COD were determined using the methods described in APHA (1998), whereas pH was measure by means of a WTW inoLab pH/Ion 735 m.

1.2. Jar tests

The wastewater collected as described above was filtered through a 1.2 μm micro fiber glass filters (Filter-Lab; ø 47 mm) and 0.4 μm paper filter (Macherey-Nagel; ø 45 mm). This filtration process was conducted by means of a vacuum pump (KNF Neuberger; type N035AN.18; Motor IP20), with an operating pressure of 4 bar. The filtrate was transferred to a 1 L Erlenmeyer flask and used to perform the jar tests.

Three jar tests were conducted to evaluate the removal of 50 μg EE2/L (Sigma-Aldrich; reference 48767-1G; purity $\geq$ 98%) by PAA (Merck KGaA; concentration 38%–40%). In the first jar test, PAA concentrations of 1, 5, 10 and 15 mg/L with a contact time of 10 min were evaluated. These EE2 and PAA
concentrations were derived by diluting stock solutions prepared in methanol and distilled water, respectively. In the second and third jar tests, the same PAA concentrations were tested, but with a contact time of 15 and 20 min respectively. Each treatment consisted of three replicates, each consisting of a glass jar containing 1 L treatment solution, which were positioned in a Velp Scientifica FC 6S jar tester with stirring at 200 r/min throughout the duration of the test. After the required contact time had elapsed, the PAA reaction was stopped through the addition of 100 mg sodium thiosulfate/L (after Gehr et al., 2003).

To determine the EE2 removal efficiency of the different PAA treatments, EE2 concentrations were measured after the treatments. This was done through stir bar sorptive extraction (SBSE) followed by high performance liquid chromatography with diode-array detection (HPLC-DAD), as detailed in Mauricio et al. (2018). Under these analytical conditions, the limits of detection (LOD) and quantification (LOQ) for EE2 were 1.6 μg/L and 4.1 μg/L, respectively. In addition, to evaluate the influence of the different treatments on the physical-chemical properties of the wastewater, COD and pH were determined as described in Section 1.1 at the beginning and at the end of the test.

### 1.3. Bioassays

Zebrafish (D. rerio), wild type, were obtained from a commercial supplier (Aquataplante, Portugal) and acclimatized to laboratory conditions for two weeks prior to testing. Animals were housed in a closed-circuit system consisting of a 100 L volume aquarium filled with filtered dechlorinated tap water with pH: 7.2 ± 0.1; temperature: (24 ± 1)°C; photoperiod: 12 hr:12 hr (light: dark) and continuous aeration (>6 mg O₂/L).

At the beginning of the tests, 35 adult fish of both sexes (weight: 0.3 ± 0.1 g; length: 2.4 ± 0.2 cm; mean ± SD) were randomly distributed over five polystyrene test tanks containing 10 L water volume to assess the different treatments on the physical-chemical properties of the wastewater, COD and pH were determined as described in Section 1.1 at the beginning and at the end of the test.

### 1.4. Biochemical responses

Glutathione-S-transferase (GST) activity was determined at 340 nm, following a procedure first described by Habig et al. (1974) and adapted for 96-well microplates (Diniz et al., 2013). The lipid-peroxidation was assessed by adapting the TBARS method, which is based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) resulting in a compound absorbing at 532 nm (Ohkawa et al., 1979). The catalase (CAT) activity was determined spectrophotometrically at 540 nm, as described in Johansson and Borg (1988) and adapted for 96 well-microplates. The determination of VTG was carried out by the ELISA (enzyme-linked immunosorbent assay) method adapted from Denslow et al. (1999), and following the same procedure as described in Diniz et al. (2010). The total amount of proteins in samples was carried out by the Bradford method (1976).

All the spectrophotometric measures were carried out using a microplate reader (Bio-Rad, Benchmark, USA). Enzyme activities and VTG concentrations were expressed according to samples total protein.

### 1.5. Data analysis

Results were analyzed using the non-parametric test Mann-Whitney U since statistics assumptions were not fulfilled. Statistical analyses were performed with the Statistica software (Statistica version 8.0; Statsoft Inc., Tulsa, OK, USA, 2007) at a significance level of 5%.

### 2. Results and discussion

#### 2.1. EE2 removal efficacy of PAA

The EE2 removal efficiency obtained for the different PAA concentrations and contact times are visualised in Fig. 1. As can be deducted from Fig. 1, the lowest contact time (10 min) did not show a dose-response relationship between PAA concentration and EE2 removal. At the intermediate contact time (15 min), a removal efficiency of approximately 50% could be attained at a PAA concentration of 5 mg/L. Increasing the PAA concentration at this contact time, however, did not

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**Fig. 1** – EE2 removal efficiency as a function of PAA concentration and contact time.
result in higher removal efficiencies (Fig. 1). Only at a contact time of 20 min, a clear dose-response could be denoted, with a removal efficiency of 100% at 15 mg PAA/L (Fig. 1).

According to some authors, PAA disinfection efficacy is more dependent on its dosage than on contact time (e.g., Azzellino et al., 2011; Luukkonen et al., 2014). Contrarily, other authors indicated that PAA disinfection depends more on contact time than the PAA concentration used (e.g., Dell’Erba et al., 2007; Chhetri et al., 2014). According to Coyle et al. (2014), contact time and the applied disinfectant are both significant factors in achieving a satisfactory disinfection level. Microbial inactivation models also typically rely on both PAA contact time and concentration, besides model parameters (e.g., Santoro et al., 2007; Antonelli et al., 2013). In the present study, it may be concluded that a sufficient contact time is a prerequisite, after which the PAA dose is also crucial for an efficient EE2 removal (Fig. 1). To evaluate this further, the EE2 removal efficiency as a function of the PAA concentration multiplied with PAA contact time was assessed both including (Fig. 2a) and excluding (Fig. 2b) the data for the 10 min contact time. Whereas no correlation could be demonstrated when including all data ($r = 0.42; DF = 10; p > 0.05; \text{Fig. 2a}$), a positive correlation was obtained when excluding the 10 min contact time data ($r = 0.91; DF = 6; p < 0.01; \text{Fig. 2b}$).

To the best of our knowledge, Block et al. (2015) is so far the only other study that evaluated the EE2 removal efficiency of PAA. These authors evaluated 1, 5, 10 mg PAA/L at contact times of 10 and 20 min to disinfect 9 $\mu$g EE2/L. Efficacies above 79% were obtained for all treatments, although not always with a clear dose-response relationship. The lower removal efficiency in the present study at the lower contact times and PAA concentrations as compared to the study by Block et al. (2015) may have several reasons. Firstly, different PAA sources were used in the two studies. Different PAA formulations contain different components and equilibrium compositions, which makes that results in the literature would be different from one case to another (Luukkonen and Pehkonen, 2017). Secondly, Block et al. (2005) conducted their tests with distilled water, whereas filtered wastewater was used in the present study. Since PAA efficacy is known to be greater in neutral to acidic solutions with lower initial COD (Luukkonen et al., 2014; Eramo et al., 2017), this is also likely to at least partly explain these different PAA efficiencies. In line with this, removal efficiencies in preliminary tests that were conducted in the present study showed EE2 removal efficiencies of 100% even at the lowest PAA concentration (1 mg/L) and contact time (10 min) (data not shown). Thirdly, the initial EE2 test concentration in the present study (50 $\mu$g/L) was higher than that in Block et al. (2015; 9 $\mu$g/L). EE2 concentrations measured in surface waters are typically in the ng/L range (Aris et al., 2014; Adeel et al., 2017; Vilela et al., 2018), although they may reach the lower $\mu$g/L range in waterbodies downstream of WWTP (Pereira et al., 2011). There is thus a need to continue monitoring EE2 in WWTP effluents and receiving waterbodies and to evaluate the EE2 removal efficiency of PAA at the environmental-realistic concentrations obtained from such studies.

### 2.2. Effects of PAA on wastewater quality

The degradation products of PAA are acetic acid, hydrogen peroxide, and water (Chhetri et al., 2014). As a result of the acetic acid formation, chemical oxygen demand (COD) and total organic carbon (TOC) may increase following peracid dosing (Collivignarelli et al., 2017; Luukkonen and Pehkonen, 2017). Typical reported (theoretical or measured) COD increases range between 2 and 4 mg/L per 1 mg/L of PAA dosing (Kitis, 2004; Cavallini et al., 2013; Luukkonen and Pehkonen, 2017). Based on these values, the expected COD increase in the highest PAA concentration tested in the present study (15 mg/L) would have been between 30 and 60 mg/L. The actually measured COD increases, however, were 9.4 mg/L (distilled water) and 18 mg/L (WW). It is known that the actual increase in COD depends on the method used and the chemical PAA composition tested (Luukkonen et al., 2014; Luukkonen and Pehkonen, 2017). The open reflux boiling method used in the present study, for example, is known to potentially lead to the volatilization of organics (Baldry et al., 1995). In any case, both the measured (60 mg/L) and theoretical (max. 101 mg/L) are below the trigger value of 125 mg/L for WW discharge in waterbodies (EC, 1991).

The addition of an acidic substance like PAA to WW could hypothetically lead to a decrease in pH, although previous studies have indicated that this is not significant (Cavallini et al., 2013; Luukkonen and Pehkonen, 2017). Luukkonen et al. (2014), for example, determined that the decrease in pH can be calculated by multiplying the PAA dose (in mg/L) with $p < 0.05$; Fig. 2b).

![Fig. 2 — Correlation between EE2 removal efficiency and PAA concentration multiplied with PAA contact time (i.e., PAA concentration × time), by including (a) or excluding (b) the 10 min contact time data.](image-url)
with 0.033. In line with this, the pH values in the PAA-treated WW indeed remained the same or only slightly dropped from 7.9 (value prior to PAA treatment) to 7.9 (0 mg PAA/L), 7.9 (1 mg PAA/L), 7.8 (5 mg PAA/L), 7.6 (10 mg PAA/L), 7.5 (15 mg PAA/L). Subsequently, the pH values remained within the limits (pH: 6.0–9.0) set in EC (1991). Similarly, TSS (<10 mg/L) also adhered to this Directive (TSS < 10 mg/L; EC 1991).

2.3. Biochemical responses

No significant fish mortality (<10%) was observed in any of the tested treatments. Given the 4d-LC50 of 1.7 mg EE2/L for adult zebrafish (Versonnen et al., 2003), the 50 µg EE2/L used in the present study was indeed not expected to result in fish mortality. For PAA, however, Henao et al. (2018) reported 4d-LC50 of 0.35 and 1 mg/L for adult zebrafish. In line with this, studies performed by da Costa et al. (2014) showed 50% zebrafish mortality in wastewater treated with 5 mg PAA/L. Thus, it could be expected that the concentrations tested in the present work had an effect on the survival of the animals. The absence of such an effect may be due to the fast degradation of PAA. A follow-up study conducted in our jar test system demonstrated that 15 mg PAA/L reduced to about half after 10 min (7.6–7.9 mg PAA/L) and to about one-third after 15 min (4.8 mg/L). In addition, the degradation products of PAA have been demonstrated to have negligible toxicity to aquatic life (Chhetri et al., 2014).

The results obtained for GST, CAT, LPO (MDA content) and VTG are shown in Fig. 3a, 3b, 3c and 3d, respectively. Despite the absence of effects on fish survival, PAA caused a significant increase in GST and CAT (Fig. 3a and 3b), which may be explained as an antioxidant defense mechanism against oxidative stress after exposure to PAA, which is known to form hydrogen peroxide during PAA degradation (Chen et al., 2017; Chupani et al., 2016). The LPO (MDA content) results show a decrease of MDA levels in comparison to controls (Fig. 3c). Therefore, this can suggest that the increase in CAT activity, which is considered reversible (Chupani et al., 2014), seems to be capable to combat oxidative stress in organisms’ cells. In addition, as compared to PAA, chlorine compounds (sodium hypochlorite, chlorine dioxide) are known to have greater potential to induce toxicity on organisms (Elia et al., 2006).

However, future (pilot) experiments evaluating the use of PAA in WWTP should consider an optimized residence time to avoid or minimize (sublethal) toxic effects on aquatic organisms in WW receiving waters. Similar biochemical responses were also observed for the EE2 and EE2 + PAA treatments, besides a slight but significant increase in GST activities was detected in the latter treatment (Fig. 3a). EE2 is indeed also known to exert its toxic effects by the creation of ROS and hence to induce antioxidative immune responses (Aris et al., 2014; Chen et al., 2017; Liang et al., 2018). Phase II metabolism (GST-activity) was also demonstrated by Maranho et al. (2014) to play a less significant role in the detoxification of EE2. Other studies, using different species, however, did not indicate effects of EE2 and PAA on CAT activity, but encountered increased levels of GST (Elia et al., 2006; Wang et al., 2013; Chupani et al., 2014). This shows the complexity and still unraveled mechanistic toxic pathways of both EE2 and PAA, which may be different in different species and sexes of the same species, and even change in time following exposure (Greco et al., 2007; Kaptaner et al., 2009).

Exposure assays using mixtures of EE2 with other chemicals, however, may influence biochemical responses in fish and leading to potentially complex antagonistic and
synergistic detoxification mechanisms (Solé et al., 2000). For example, whereas MeOH showed a slight increase in GST activity, no effects on GST activities were found for both the MeOH + EE2 and MeOH + PAA treatments (Fig. 3a), which may suggest different response effects (e.g., antagonistic, synergistic) of EE2 and PAA when assayed mixed with MeOH or when testing MeOH alone (control MeOH).

Although the GST activity in the MeOH treatment showed some variability, the difference with the controls was not statistically significant (Fig. 3a). A slightly higher methanol concentration was used in the toxicity tests (0.05%) than the maximum concentration (0.1 mL/L) indicated in the OECD protocol for acute fish testing (0.1 mL/L). However, no effects were anticipated since Liu et al. (2014) also did not encounter any difference in enzymatic activities (including for GST activity), Vtg levels and DNA damage between goldfish exposed to solvent (0.05%) and water controls. The metabolism pathway of methanol is known to depend upon glutathione (GSH) and glutathione-S-transferase (GST) (Pankow and Jagielski, 1993; Parthasarathy et al., 2006; Borah et al., 2016). Borah et al. (2016) discussed that, unlike methanol, this pathway does not play a role for ethanol. Subsequently, lower methanol concentrations should be used in future studies, or ethanol could be evaluated as an alternative solvent.

Several studies have previously showed increased VTG in both female and male fish after exposure to EE2, suggesting an effect on the fish endocrine system which can also result in a threat to their breeding success and hence survival of fish populations (e.g., Adeel et al., 2017; Aris et al., 2014; Liu et al., 2014; Solé et al., 2000; Kaptaner et al., 2009). In line with this, VTG levels in females exposed to EE2 were almost five to six times higher than those found in the controls (Fig. 3d). In addition, VTG levels in EE2-exposed males were over twice as high as in untreated females, whereas VTG was not detected in males receiving any of the other tested treatments (Fig. 3d). However, VTG was detected in female fish exposed to EE2 + PAA at levels significantly higher than controls, whereas VTG levels for females exposed to H2O + MeOH and H2O + PAA were comparable with those in the control (H2O), as shown in Fig. 3d. We can hypothesize that PAA reacting with EE2 can generate some metabolites that affected female’s endocrine system by slightly elevating the VTG levels in comparison to controls. Several studies have indicated increased levels of VTG in EE2-exposed zebrafish at concentrations as low as 5 ng/L (e.g., Van der Belt et al., 2003; Xu et al., 2014), supporting the high EE2 removal efficiency of PAA discussed in Section 2.1.

3. Conclusions

This study demonstrated that PAA has a high EE2 removal efficiency, both in terms of EE2 concentrations as by decreasing the potential toxicity effects (e.g., oxidative stress, endocrine disruption) that EE2 may exert. Together with the lack of substantial effects on wastewater quality, this may indicate the great potential of PAA as a disinfectant of EDC like EE2 in WWTP. Future studies should include evaluations at environmental-realistic EE2 concentrations, which may imply that lower PAA concentrations or contact time may be required. By testing different effluents with different physical-chemica l compositions, the applicability of PAA over the large range of existing WW can be determined. In addition, chronic toxicity studies should be conducted to ensure that organisms in WW-receiving waterbodies do not suffer from chronic exposure to WW discharges. Pilot plants installed at WWTP using PAA as disinfectant and monitoring of field communities in receiving water bodies (including biochemical parameters) could aid to evaluate the practical application of PAA as a substitute disinfectant for chlorine products. PAA treatment is easily incorporable in a WWTP, specially when compared to other alternatives for EDC removal such as membrane systems.

Conflict of interests

There are no conflict of interests.

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