A review on ozonation and17a-ethinylestradiol

-formation and effects in the environment of the by-products

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MVEM12 EXAMENSARBETE FÖR MASTEREXAMEN 30 HP
MILJÖVETENSKAP | LUNDS UNIVERSITET

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Lund 2017

Abstract

Today, waste water treatment plants do not remove pharmaceutical residues from the water (as there are currently no regulations for wastewater treatment in regards to pharmaceuticals residues in Sweden) resulting in a high concentration of pharmaceuticals reaching the environment. The Swedish environmental protection agency have recently concluded that there is a need for advanced treatment of pharmaceuticals in wastewater. One of the most promising techniques is ozonation however, the process can generate ecotoxic by-products. This study provides an overview of the current knowledge of by-products of 17α-ethinylestradiol (EE2), generated during the ozonation process. Special emphasis is on showing if by-products are produced in the ozonation process when removing EE2 and in what concentrations. Additionally, whether the by-products have an ecotoxicological effect on the environment and if there are any measured that can be taken to reduce the production of by-products. Method used to conduct this study was a systematic search over five databases and one search engine.

This study shows that by-products of EE2 are generated during ozonation. However, the results shows that there is a huge gap in knowledge, in regards to the amount of by-products created and in what concentration also their ecotoxcity and possible risk to the environment are mostly unknown. Some by-products have estrogenic activity and have shown to have detrimental effect on fetal rats testosterone secretion. This study shows therefore possible post ozonation treatment steps that could be implemented to reduce the potential toxicity of by-products reaching the environment. The knowledges gap needs to be corrected with future studies, as ozonation is one of the most likely treatment techniques for pharmaceuticals to be implemented in the future.

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Abbreviations

AC - Activated carbon
E1 - Estrone
E2 - 17β-estradiol
EE2 - 17α-ethinylestradiol
EQS - Environmental quality standards
GAC – Granulated active carbon
NDMA - N-Nitrosodimethylamine
NOEC – No observed effect concentration
WTP - Water treatment plant

Introduction

Currently, there are no regulations for wastewater treatment in regards to pharmaceuticals residues in Sweden. Water treatment plants (WTP) do therefore not remove pharmaceutical residues from the water which results in a high concentration of pharmaceuticals reaching the environment. The main reason for the lack of regulations is that it is still a relatively new topic of discussion and there is no consensus on what levels the pharmaceuticals should be regulated or which treatment techniques should be used (Sehlén et al., 2015). The municipalities and counties of Sweden has however, developed a national drug strategy which have three goals firstly, effective and safe use of medication, secondly, accessible medicines and equal use and lastly, economically and environmentally sustainable use of medicines (Ministry of health and social affairs & Ministry of health and social affairs, 2016). Thus, there are indications that regulation of pharmaceutical residues from WTPs are very probable in the future, which drugs that are to be regulated and to what level is still to be determined (Sehlén et al., 2015). The Swedish agency for marine and water management set, during the years 2014-2017, aside 32 million SEK in towards projects aimed at advanced wastewater treatment from pharmaceuticals residues and substances seen as a threat to the environment (Swedish agency for marine and water management, 2016). Since the traditional treatment process does not include pharmaceutical residues an additional treatment step is needed. The Swedish environmental protection agency concluded, in a recently published report, that there is a need for advanced treatment of pharmaceuticals in wastewater (Sundin et al., 2017). The implementations of such techniques are extra attractive as they also have the ability to remove other unwanted substances (Sundin et al., 2017).

The removal of the active compounds and their residues in hormonal contraceptive such as the oral pills would be included in the new treatment step. The active substance in the majority of contraceptive pill is 17α-ethinylestradiol (EE2), a synthetic estrogen (Colborn et al., 1993). In 2013, EE2 was along with the anti-

inflammatory agent diclofenac and natural estrogen 17β-estradiol (E2), added to the European Commission Watchlist for priority substances (The European parliament and of the council, directive 2013/39/EU). The commission shall no later than September 2017 propose measures for member states and/or EU on how to handle pharmaceutical residues in wastewater (The European parliament and of the council, directive 2013/39/EU). EE2 is a very persistent substance and tend to bioaccumulate in different organisms (Larsson et al., 1998). EU have a proposed environmental quality standards (EQS) for EE2 at 0.035 ng/L (Tiedeken et al., 2017) and a study by Sehlén et al. (2015) suggest a no observed effect concentration (NOEC) of 0.03 ng/L. However, the majority of studies measuring endocrine disrupting substances do only report the concentration of the parent compound (Skotnicka-Pitak et al., 2008). They do not including the occurrence of metabolites and abiotic degradation product due to lack of data. Therefore, there is a possibility that the predicted environmental concentration underestimate the risk of EE2 and other endocrine disrupting substances in the environment (Skotnicka-Pitak et al., 2008).

Measured concentrations of EE2 varies between 0.00-34.00 ng/L in water and <0.10-133.64 ng/g dry weight in sediments around the world (Aris et al., 2014). It is suggested that 2-25% of the total length of Europe's rivers could exceed the proposed EQS for EE2 (Johnson et al. 2013). The most exposed countries are Germany, the Netherlands, Poland and Romania, with almost a third of their total river length exciding the EQS. Finland, Norway and Sweden along with Estonia, Latvia and Lithuania has the best water quality in regards to EE2 concentrations (Johnson et al., 2013).

There are correlations between EE2 and widespread sexual disruption in wild populations of vertebrate as well as reproductive and developmental effects (Jobling et al., 1998). Endocrine disruption seems to be particularly widespread in freshwater fish populations. Besides the reproductive impairment in fish disturbed foraging performance have been observed as a result of EE2 exposure (Hallgren et al., 2014). Despite the very low concentrations normally found in the environment, EE2 have negative effects on fish populations and consequences for freshwater communities both on structure and function (Jobling et al., 1998; Larsson et al., 1999; Hallgren et al. 2014).

One of the most alarming effects documented is the feminisation of male fish, resulting in an increasing amount of intersex fish (Jobling et al., 1998; Larsson et al.,

1999; Kidd et al., 2007). EE2 have severe effects on the sustainability of fish populations, shown in an in lake study performed over seven years (Kidd et al., 2007). It further showed that chronic exposure to EE2 almost resulted in the extinction of the fathead minnow (*Pimephales promelas)* population in the lake, due to feminisation of male fish and affecting the female egg cells. Fish populations with shorter life span may therefore be at risk from instability when exposed to EE2 over a long period (Kidd et al., 2007).

Ozonation

There are currently two WTP in Sweden that uses ozonation as an additional step in the treatment process to remove pharmaceutical residues; Tekniska verken in Linköping and Knivsta WTP (Sehlén et al., 2015: Roslagsvatten, 2017). Ozonation is one of the most common techniques used when removing pharmaceuticals residues from water, it has shown that it has high removal efficiency (Sehlén et al., 2015; Ternes et al., 2003; Baresel et al., 2017). Ozonation is an oxidation processes that uses ozone as the oxidizing agent (Sehlén et al., 2015). Ozone is a strong oxidant that also can decompose into hydroxyl radicals, which are even stronger oxidizing agents (Sehlén et al., 2015). Therefore ozonation incorporates the so-called indirect oxidation afforded by hydroxyl radicals (Mantzavinos D. & Psillakis E., 2004). Tekniska verken shows an average removal efficiency of 90 % when comparing the influent and effluent (Sehlén et al., 2015). The use of ozonation however, does result in the creation of ecotoxic by-products from the parent substance (Hübner et al., 2015; Margot et al., 2013; Stalter et al., 2010). Known effects of by-products from ozonation of pharmaceutical residues, such as bromate, nitrosamines and formaldehyde (Hübner et al., 2015; Margot et al., 2013; Stalter et al., 2010), results in higher mortality and genotoxicity in fish, maggots and clams (Margot et al., 2013). Bromate is today the only ozonation product that is regulated (The council of the European Union, directive 98/83/EG).

Switzerland have several WTP using ozonation because of a new water protection law, implemented 2016, that required instalment of technological measurement for removing micropollutants at larger plants and at heavily-used bodies of water (The federal council of Switzerland, 2016). It is predicted that

around 100 WTP will be upgraded the coming 20 years. Besides ozonation is also activated carbon (AC) considered to method used in the future (The federal council of Switzerland, 2016).

A pilot study, performed in the Czech republic, shows that using ozonation with the purpose of removing endocrine disrupting substances (such as E1, E2, estriol and EE2) from wastewater is an effective method (Pešoutová et al., 2014). The concentrations of the estrogens in the influent were 1.65 – 3.59 µg/L. All estrogen had a removal efficiency of 99 % when using an ozone dose of 1.8 mg/L and removal efficiency between 99.7-99.9 % at an ozone dose of 4.4 mg/L (Pešoutová et al., 2014). It is well documented that EE2 and E2 are the two strongest endocrine disrupting substances found in the environment however, there are lack of knowledge regarding their degradation products (Skotnicka-Pitak et al., 2008).

This study provides an overview on the current knowledge of by-products generated during the ozonation process of EE2. Special emphasis will be put on showing if by-products are produced in the ozonation process when removing EE2 and in what concentrations. Additionally, a second aim is to conclude whether the by-products have an ecotoxicological effect on the environment and if there are any measured that can be taken to reduce the amount of produced by-products reaching the environment.

Method

A systematic search performed to collect data.

Data collection

A survey of the scientific literature using the databases Web of science, Lubsearch, Scopus, PubMed, Embase and the search engine Google scholar. The search was performed with no restrictions on publication year and used the search phrase; ("EE2" OR "17α-ethinylestradiol") AND ("by-product*" OR "byproduct*" OR "ozonation product*" OR “transformation product*”) AND "ozonation". The one other criteria was the need for the article to be peer-reviewed.

Restriction to the analysis was made to only sample of articles reporting (a) ozonation of ethinylestradiol resulting in by-products, (b) ozonation of ethinylestradiol resulting in no by-products and (c) possible measures to reduce the generated by-products reaching the environment.

The sampling was made in three steps to evaluate the relevance of the articles in regards to the three criteria (a-c), first step was selection based on the relevance of the title, second steps selection was based on the abstract and the third step was a read through of the article. If the relevance of the article not able to be made in one step was it included to the following step. The articles in other languages was excluded from the sampling.

The search on Lubsearch resulted in 29 articles between the years 2004-2016, of them was eight duplicates. The first sampling step resulted in 20 articles, the second 16 and the third identified 14 articles to be used in the analysis.

The search on Web of Science resulted in 16 articles between the years 2004-2016, zero duplicates. The first sampling step resulted in 15 articles, the second 14

and the third identified 11 to be used in the analysis. Two articles was not able to be accessed.

The search on Google scholar resulted in 643 articles between the years 2003-2017. Of the articles found was nine duplicates, four not accessible and 18 in another languish. The search also resulted in 10 "quotes" from articles these was disregarded. The first sampling step resulted in 171 articles, the second 87 and the third 51 articles identified to be used in the analysis.

The search in Scopus resulted in the 11 articles publicized between 2004-2016, zero duplicates. All of the articles was concluded to be used in the analysis.

The search in PubMed resulted in 2 articles between 2004-2013, zero duplicates. Both articles was assessed to be relevant and used in the analysis.

The search in Embase resulted in the 11 articles publicized between 2004-2016, zero duplicates. All of the articles was concluded to be used in the analysis.

In total 51 articles were identified to be used in the analysis across the six searches, due to the same article being found in multiple searches, resulting in duplicates.

Result

The systematic search resulted in 14 studies that presented results either generating by-product or no by-products. There were 11 studies that showed by-products of EE2 created through the ozonation process, of those were 11 identified and four unidentified (Table 1). Four of the studies showed no by-product production in the ozonation process (Table 1).

There was a difference in the amount of by-products that were identified in the different studies. The amount ranged between 1-11, by-products and in some cases the amount were undisclosed in the studies (Table 3). An average of 2.3 by-products were found in the studies analysed.

Table 1. The studies that generated by-product or no by-products of EE2.

BY-PRODUCTS	NUMMBER OF STUDIES	STUDY
Identified	9	Beltrán et al., 2009; Chon et al., 2015; Huber et al., 2004; Guedes Maniero et al., 2008; Larcher et al., 2012; Larcher & Yargeau., 2013; Lee et al., 2008; Nakrst et al., 2011; Zhang et al., 2006
Unidentified	2	Lee et al., 2008; Alum et al., 2004
Not found	4	Hashimoto et al., 2006; Lassonde et al., 2015; Liu et al., 2005; Amaral et al., 2017

By-products identified and methods used

The studies utilized different methods to find and identify the by-products.

The majority of the studies used GS/MS analysis, GC/MS analysis or LC/MS analysis or a combination (Guedes Maniero et al., 2008; Huber et al., 2004; Larcher et al., 2012; Larcher & Yargeau, 2013; Nakrst et al., 2011). GS/MS analysis identified two by-products, both with a phenolic ring (Table 2) (Guedes Maniero et al., 2008). The ozonation was performed at different pH (3, 7, 11), at pH 3 none of by-products were identified, at pH 7 was one identified and at pH 11 was both discovered (Table 2). However, the by-products were isomers and were unable to be confirmed with a standard (Guedes Maniero et al., 2008). Eleven by-products determined (Table 2) using LC-MS/MS and GC/MS and with model compounds 5,6,7,8-tetrahydro-2-naphthol and 1-ethinyl-1-cyclohexanol, which were used to represent the reactive phenolic moiety and the ethinyl group of EE2 (Huber et al., 2004). These are the two reactive moieties will be attacked by the ozone but have a significant difference in its reactivity, the phenolic moiety is highly reactive compared to the ethinyl group (Huber et al., 2004). LC-MS analysis identified two by-products, both muconic acid derivatives with open phenolic ring structures (Table 2) (Larcher et al., 2012). Using the same analytical method was another two by-products (Table 2), both muconic acid derivatives with open phenolic ring structures (Larcher & Yargeau, 2013). Utilized GC-MS analysis was one by-product with 93.0% similarities to dibutylftalate determined (Table 3) (Nakrst et al., 2011).

High-performance liquid chromatography (HPLC) was another method utilized to detect by-products (Beltrán et al., 2009). At times, HPLC was used in combination with other methods, HPLC/UV system or a combination of HPLC and GC/MS analysis (Lee et al., 2008; Zhang et al., 2006). Using only the HPLC method, the amount of by-products was not determined however, it identified the majority as carboxylic acids, such as oxalic acid (Table 3) (Betrán et al., 2009). The HPLC/UV system showed that EE2 degraded to products with lower molecular weight and with greater polarity. It further indicated that the by-products were semiquinoes, which in turn oxidized to organic acid with smaller molecular weight (Table 2; Table 3) (Zhang et al., 2006). Using the combined HPLC/UV system six different initial by-products was determined, as well as five more as a result of EE2 reaction with ozone and three as a result of the reaction between EE2 and hydroxyl radicals (Table 2) (Lee et al. 2008) Some of the by-product have higher reactivity than the parent compounds and will further transform into other products, however, these were not identified (Lee et al., 2008).

Bromate was identified as the sole by-product of EE2 in two separate studies (Table 3) (Chon et al., 2015; Meunier et al., 2006). They were determining it using

> "a novel method based on size exclusion chromatography followed by a post-column reaction was developed and calibrated against an existing electrochemical method"

(Chon et al., 2015) and using ion chromatography (Dionex DX-500) with post-column reaction and detection with conductivity and UV (Meunier et al., 2006).

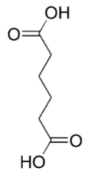
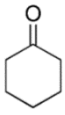
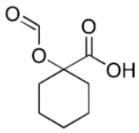
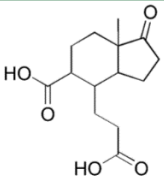
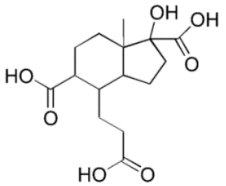
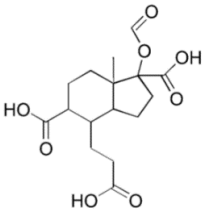
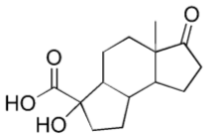
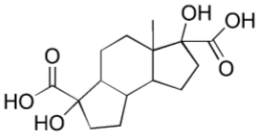
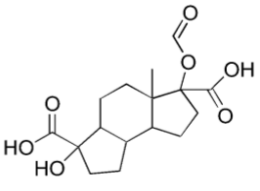
E1 was determined as a degradation product using the Enzyme-Linked Immunosorbent Assay (ELISA) method (Table 3) (Manickum & John., 2013).

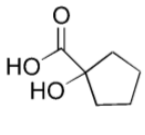
By-products were not measured directly, they were observed in the study by Alum et al. (2004) however, the amount of unknown by-products were not analysed.

Four studies reported no EE2 ozonation by-products (Table 1). Using LC/MS analysis observed one study no by-products with estrogenic activity and in another no by-products at all (Hashimoto et al., 2006; Liu et al., 2005) Using the HPLC–MS analysis was no by-products identified (Lassonde et al., 2015). ESI-MS also used and detected no by-products (Amaral et al., 2017).

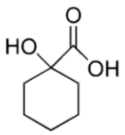
None of the studies where by-products were found, performed measurements of their concentration (Alum et al., 2004; Beltrán et al., 2009; Chon et al., 2015; Huber et al., 2004; Guedes Maniero et al., 2008; Larcher et al., 2012; Larcher & Yargeau., 2013; Lee et al., 2008; Nakrst et al., 2011; Zhang et al., 2006). The by-products discovered were not identified and no further tests were made with the exception of few estrogenicity tests (Guedes Maniero et al., 2008; Lee et al., 2008).

Table 2. The determined by-products of EE2 from different studies, where the structure was included. *No name included in the study

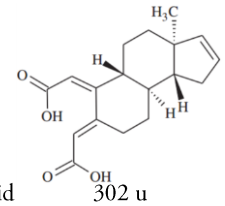
STRUCTURE OF BY-PRODUCTS				
				
Adipic acid (Huber et al., 2004)	Cyclohexanone (Huber et al., 2004)	Product 12 (Huber et al., 2004)	Product 14 (Huber et al., 2004)	Product 15 (Huber et al., 2004)
				
Product 16 (Huber et al., 2004)	Product 17 (Huber et al., 2004)	Product 18 (Huber et al., 2004)	Product 19 (Huber et al., 2004)	



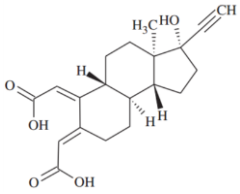
1-hydroxy-cyclopentanecarboxylic acid
(Huber et al., 2004)



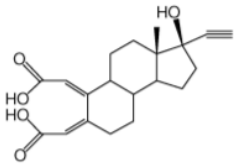
1-hydroxycyclohexanecarboxylic acid
(Huber et al., 2004)



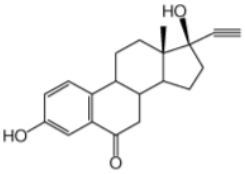
302 u
(Larcher et al., 2012)



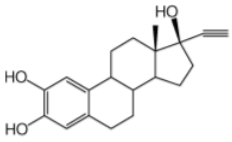
344 u
(Larcher et al., 2012)



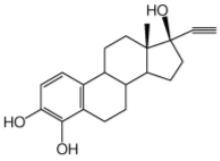
Muconic EE2
(Lee et al., 2008)



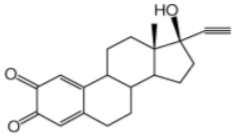
6-oxo EE2
(Lee et al., 2008)



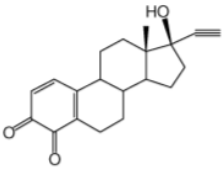
2-OH EE2
(Lee et al., 2008)



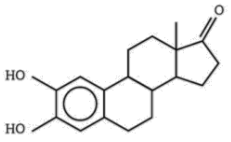
4-OH EE2
(Lee et al., 2008)



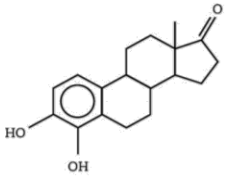
2,3-quinone EE2
(Lee et al., 2008)



2,4-quinone EE2
(Lee et al., 2008)



estra-1,3,5(10)-trien-17-one,
2,3-bis[(trimethyl)oxy
(Guedes Maniero et al., 2008)



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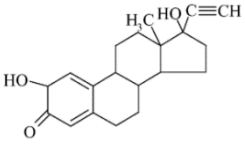
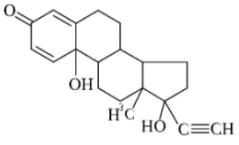
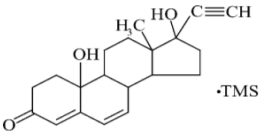
3,4-bis[(trimethyl)oxy
(Guedes Maniero et al., 2008)



(Zhang et al., 2006)*



(Zhang et al., 2006)*



(Zhang et al., 2006)*

Table 3. The determined by-products of EE2 from different studies, where the structure was not included.

STUDY	IDENTIFIED BY-PRODUCT(S) (WITHOUT STRUCTURE)
Beltran et al., 2009	Mostly carboxylic acids, such as oxalic acid.
Chon et al., 2015	Bromate
Larcher & Yargeau, 2013	O3 by-product A O3 by-product B
Manickum & John., 2013	E1
Meunier et al., 2006	Bromate
Nakrst et al., 2011	Dibutyl ftalate
Zhang et al., 2006	Bis(trimethylsiyl) oxalate Bis(trimethylsiyl) malonate Bis(trimethylsiyl) succinate Bis(trimethylsiyl) glutarate Bis(trimethylsiyl) adipate

Enhancing the treatment to ensure complete removal

Post-ozonation step

To remove by-products, the addition of a post ozonation treatment step would limit the possibility of by-products reaching the environment. Enhancing the biodegradability of generated by-products and an addition of a biofilter after ozonation would be one way of removing them from the water (von Gunten, 2003). The technique with biofiltration is especially efficient when the by-products are more easily degradable (von Gunten, 2003). The concern regarding the use of biofiltration is that there is a risk of compounds both being created and reformed during the process (Merel & Zwiener, 2016).

Sand filtration could be an effective alternative additional treatment step to lower toxicity (Stalter et al., 2010a; Stalter et al., 2010b; Hollender et al. 2009). Sand filters remove a wide range of different by-products generated through ozonation. It

have not specifically been tested on by-products of EE2 however, tests on common ozonation by-products N-Nitrosodimethylamine (NDMA) and bromate have been performed. NDMA shows a significant reduction to an agreeable level for drinking water and bromate shows no reduction (Stalter et al., 2010a; Hollender et al. 2009). Design and operation of sand filtration, as a new treatment steps, can be constructed on laboratory experiments and without expensive pilotig to be implemented on full scale (Hollender et al. 2009).

AC-filtration in combination with ozonation, is another possible post treatment (Beltrán et al., 2009). Having an AC-filter as a post treatment step would result in lower ozone consumption compared to singular ozonation (Beltrán et al., 2009). Using AC as the singular treatment technique leads to a considerable removal of pharmaceuticals however, the compound are not totally removed and can ultimately result in the saturation of the filter and loos of its capacity. The combination of ozonation and AC present lower ecotoxicity level compared to the singular use of ozonation or AC (Beltrán et al., 2009). Nevertheless, the effect on by-products of EE2 have not been directly tested in this combination and is therefore unknown (Beltrán et al., 2009).

Altering conditions

The effectiveness of ozonation is to some extent dependent on the conditions it is performed under. Both ozone concentration and pH have an effect on the removal efficiency, higher pH and ozone dose result in higher removal rate (Zhang et al., 2006). A change in pH, altered the outcome of the amount of EE2 by-products that are created during ozonation (Guedes Maniero et al., 2008; Larcher et al., 2012). pH affects the mineralization of the by-products formed during ozonation (Beltrán et al., 2009). Ozonation at pH 3, 7 and 11, resulted in the creation of zero, one and two by-products respectively (Guedes Maniero et al., 2008). The removal rate of EE2 is greater at higher pH values however, there is simultaneously a potentially larger generating of by-products (Guedes Maniero et al., 2008; Zhang et al., 2006). There are indications that the creation of by-products are ozone dose dependant (Larcher et al., 2012). High ozone dose ensure higher bromate concentrations in the effluent (Margot et al., 2013). However, at a lower ozone dose would the ethinly group not react with the ozone (Huber et al., 2004). The phenolic moiety will, at least partly, be transformed at lower ozone exposure in the same way as higher ozone exposure (Huber et al., 2004). Temperature does not seem to have an effect on the removal efficiency of EE2 when performing the ozonation step (Manickum & John, 2014). Another factor is the concentration of EE2, when using the same ozone dose the amount of by-products being generated differed (Larcher et al., 2012). Lower concentration of EE2, limits the ability to detect potential by-products (Larcher et al., 2012).

Ecotoxcicity

The systematic search did not find any material on the toxicity of by-products formed during ozonation of EE2.

What is known is that complete removal of estrogens is rapidly achieved by ozonation (Nakrst et al., 2011). The time of ozonation is however, prolonged because of the formation of by-products. Residual estrogenic activity after ozonation is probably caused by the generated by-products. Extended contact time is expected to result in complete degradation of the estrogens (Nakrst et al., 2011; Alum et al., 2004).

The disruption of the phenolic moiety is expected to lower estrogenic activity but studies show that it is, in some instances, still intact after ozonation (Guedes Maniero et al., 2008; Lee et al., 2008). The by-products would therefore have an unchanged estrogenicity. However, when performing a Yeast estrogen screen assay to investigate the change in estrogenic activity, it shows lowered estrogenicity than the parent compound (Guedes Maniero et al., 2008; Lee et al., 2008). Some by-products of EE2 have shown to have higher estrogenicity level than the endocrine disrupter BPA after ozonation (Alum et al., 2004). By-products of EE2 generated through ozonation have demonstrated to have negative effects on the testosterone secretion by fetal rats (Larcher et al., 2012). However the probability that by-products would have a higher risk than the parent compound is considered very rare (Escher et al., 2011). A read across between by-products and parent compound would reduce the uncertainty in predictions of ecotoxicity (Esher et al., 2011).

Discussion

There is a lack of studies when it comes to by-products of EE2 in connection to ozonation. This study indicates that there have been a decrease of new studies published the last couple of years. None of the studies examined, that generated by-products, measured the concentration of by-products, and by extension its effect. This is an oversight as it is an important factor to take in to consideration when electing treatment technique used in WTP. The articles analyses do solely show whether by-products were generated or not, and in those cases they were generated were the by-products either identified or not. Due to the limited amount of studies available as well as lack of quantitative data a meta-analysis was not possible to perform. This is a huge gap in knowledge, something that should be corrected with future studies.

However, it can be determined that the ozonation of EE2 results in the creation of by-products. Not much is known about the possible toxicity of these products yet, some of have estrogenic activity. As many as 11 by-products were observed in one study (Huber et al., 2004). Since some by-product have higher reactivity than the parent compounds, they will continue to transform into other products (Lee et al., 2008). So it can be assumed that some of the identified by-products are only initial products that will transform into something else, an aspect that should be taken into consideration when analysing the ozonation of EE2. There is a need to do a continues study over a time to analyse the "final" products.

There were no by-product that was continuously found across the studies, besides bromate. Bromate was found in two studies but its connection to EE2 is unclear, since it is often formed as result of ozonation regardless of the pharmaceuticals in the wastewater (Chon et al., 2015; Meunier et al., 2006). The fact that no by-products were found across the studies can be a result of different identifying methods, the ozone dose and the EE2 concentration in the study. Even in those cases where the identification method used was the same, the same by-products was not determined. That could indicate that the conditions of the experiments were performed at differed enough to result in differing amount of by-products. Publication bias is also an aspect to be considered especially in those cases in which no by-products were determined. The authors would perhaps choose to not include

such data as it is not considered exciting results. However, publication bias should perhaps not be considered a huge factor in this case, as the lack of data more likely is a result of few studies.

The difference in amount of by-products found in the analysed studies could be caused by the use of different identifications method. The most commonly used are GS/MS analysis, GC/MS analysis or LC/MS analysis or a combination. While LC-MS analysis can provide very informative data for the identification of by-products, other spectroscopic techniques is perhaps needed to obtain unambiguous structural information on unknown by-products (Skotnicka-pitak et al. 2008). A combination of LC-MS, 1 H-NMR and 13C-NMR analysis is perhaps a suitable option (Skotnicka-pitak et al. 2008). Utilizing alternative chromatographic separation methods such as HILIC and IC could be vital for the analysis and detection of by-products (Prasse et al., 2015). Another possibility is X-ray crystallographic analysis, it is rarely used due to difficulty in creating crystals, but could result in the absolute identification of by-products and may prove invaluable in screening process (Skotnicka-pitak et al. 2008).

Being aware of the effect of products generated in different treatment techniques are important, as there are no guarantee that the by-product created is less dangerous to the environment than the parent compound. In the case of ozonation this is stressed as it is the technique most likely to be implemented due to success in Switzerland and The Swedish environmental protection agency are recommending it when a WTP is considering adding a pharmaceutical treatment step, most likely following Switzerland implementing the technique in all WTP in Sweden in the future (Sundin et al., 2017). The disappearance of the parent compound should not be considered as the sole indicator of successful treatment (Larcher et al., 2012). The by-products generated and their characteristics compared relative to the parent compound must also be considered (Larcher et al., 2012). Majority of by-products from pharmaceuticals have only miniscule modifications on the molecular structure in comparison to their parent compounds (Escher et al., 2011). Though by-products have been observed were they had significantly altered structure compared to the parent compound (Huber et al., 2004). There are a lack of ecotoxicological data to support an evaluation of the by-products environmental risk, the same goes for the potential negative effect. There is the possibility to use a read across but the method is not yet well developed enough and have to be implemented with care, even incremental structural changes can in some cases change properties radically (Larcher et al., 2012). The lack of knowledge of the toxicity of by-products both in regards to the by-product estrogenic activity as well as other properties is huge. Further identifications, kinetic studies, chemical analysis and biological assessment of the by-products are need to model the fate of these compounds in the environment and

assess the health impact (Deborde et al., 2005; De Witte et al., 2011). The gaps hinder an in-depth and larger understanding of the possible problems originating from by-products in the environment.

There are by-products that are generated in the ozonation treatment, not directly connected to EE2, that have been shown to been toxic such as NDMA, bromate or formaldehyde (Margot et al, 2013). The presence of by-products, from pharmaceuticals, in aquatic environments are not negligible and do contribute to both human health and environmental risk of organic micropollutants (Escher et al., 2011). There are an overwhelming number of transformation pathways and products that makes a comprehensive risk assessment for all by-products, including EE2, impossible to be performed (Escher et al., 2011). Risk assessments need in the future to include by-products in all relevant regulatory assessment schemes to ensure an adequate estimation of a compounds environmental risk (Escher et al., 2011).

Countries as Switzerland have chosen to implement ozonation on a large scale at their WTP although there is lack of information in regards to the potential environmental risk. The technique was more likely chosen with a focus on the removal rate of other pharmaceuticals and their effects on the environment. EE2 and other endocrine disturbing substances are a large threat to the environment (Jobling et al., 1998; Jobling & Tyler, 2003; Kidd et al., 2007; Larsson et al., 1999). The introduction of EE2 to European Commission Watchlist for priority substances indicates an increasing awareness of its harmful effect on the environment (European Parliament, 2013). The problem, it would seem, does not disappear after the parent compounds removal with the help of ozonation. These are assumptions made with limited data but it would seem that there is still estrogenic activity after ozonation as a result of the generated by-products. The precautionary principle should be enacted in this instant and there should be further research before continued large scale implementation, nationally and internationally.

A post-ozonation step would be preferable to guarantee that no by-products, or at agreeable concentrations, are released into the environment. Based on the knowledge gap on the toxicity of by-products could it be argued that ozonation should not be applied without appropriate barrier for by-products (Stalter et al., 2010b; Snyder et al., 2013). Studies show that a biologically active filter could be of great importance if the aim is to reduce the negative effects of by-products (Stalter et al., 2010; Hollender et al. 2009). Using ozonation to ensure the complete removal of EE2 and subsequently having a biological treatment step to degrade the by-products, would be an effective way to ensure a good wastewater treatment (Larcher & Yargeau, 2013). The alternatives taken up in this study are all viable measures to lessen the ecotoxicological effect. There are no concrete answers whether sand filtration as extra treatment step will have a positive impact on the overall survival of

fish downstream from WTP (Margot et al., 2013; Stalter et al., 2010b). Studies are showing different result in regards to effectiveness of sand filtration, one where there is no observed effect on fish population (Margot et al., 2013) and another that shows a positive impact on the survival rate (Stalter et al., 2010b).

Unfortunately, there have not been any studies performed to determine the effect sand filtration, or any post-ozonation treatment, have an effect on by-products of EE2 as a result of ozonation. However, sand filtration have been proven to reduce the concentration of NDMA post ozonation. It does not affect bromate but it do not degrade in biological filtration processes (von Gunten, 2003).

AC is a plausible treatment step to implement after ozonation. Ozonation in combination with granulated active carbon (GAC) would have both an oxidative and biological degradation and an adsorption of pollutants and by-products, resulting in a close to complete removal of all pharmaceuticals (Sundin et al., 2017; Baresel et al., 2017). The use this technique would make it possible to have lower ozone dose when performing ozonation (Beltrán et al., 2009). Which could mean that only phenolic moiety would be transformed not the ethinyl group and may result in less created by-products (Huber et al., 2004). Although there are no data on the direct effect AC have by-products of EE2 after ozonation, it has been showed that the by-products generated during ozonation are removed by GAC. The GAC-filters do overtime get saturation however, this is not a problem as they are regenerate and therefore reusable (Baresel et al., 2017). The by-products are actually destroyed in the regeneration process, the removal of by-products, and the environmental risk, are completely removed first after the regeneration process (Belrán et al., 2009; Sundin et al., 2017; Baresel et al., 2017). This, in comparison to other biofilters that can generate by-products on their own (Merel & Zwiener, 2016). The combination, ozonation and GAC, is already implemented in Knivsta WTP and Hammarby Sjöstadsverk (Baresel et al., 2017; Baresel et al., 2015).

A possibility to perform a large scale study on a WTP would generate results that could answer many of the unanswered questions in regards to by-products and ozonation. Suggestion for the future is to perform more studies, both laboratory studies and if possible large scale studies on WTP, on by-products of EE2 during ozonation and specifically on the concentrations of these by-products. Furthermore, if the by-products have an effect on the environment then the question is what kind of effect and how large is the impact. There need to be actions taken to ensure that the by-products, if it assessed as an environmental threat, are neutralised before reaching the environment.

Conclusions

This study show that there are a huge knowledge gap in regards to by-product of EE2 generated during the ozonation process.

There are a lack of studies that analyse the formation of by-products, and those that do mostly conclude whether there are or not. However, there are no conclusions to be made on the potential ecotoxicity of these substances, since the studies performed have not measured concentration of the determined by-products. There are too many uncertainties if by-products are not included when a risk assessment of ozonation of EE2, or other pharmaceuticals are performed. As seen they have some estrogenic activity but there is an obvious lack of knowledge about the by-products ecotoxicity that needs to be corrected with further studies.

There are post ozonation step that could be implemented to limit the potential toxicity reaching the environment, different biological treatments, sand filtration and AC-filter are alternatives.

More studies are need in general in regards to EE2, and other pharmaceutical, by-products generated during ozonation before the technique is implemented on a larger scale. A working pharmaceutical treatment is vital to ensure the sanitation of water and the continued protection of the environment and human health. The knowledges gap needs therefore to be corrected with future studies, as ozonation is one of the more likely treatment techniques for pharmaceuticals to be implemented in the future.

Thanks

I like to thank my family for their support during this study and I like to thank my supervisor Lina Nikoleris for all the help.

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