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PHARMACEUTICAL PSYCHOTROPICS DEGRADATION BY PHOTOCATALISYS AND ELECTROCHEMICAL OXIDATION AND NEUROTOXICITY EVALUATION

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Tese de Doutorado apresentada ao Programa de Pós-graduação em Engenharia Química, COPPE, da Universidade Federal do Rio de Janeiro, como parte dos requisitos necessários à obtenção do título de Doutor em Engenharia Química.

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DEGRADAÇÃO DE COMPOSTOS FARMACÊUTICOS POR PROCESSOS OXIDATIVOS AVANÇADOS E AVALIAÇÃO DA NEUROTOXICIDADE

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Uma grande quantidade e variedade de compostos recalcitrantes são eliminados diariamente em corpos hídricos, por esta razão é necessário utilizar processos que sejam capazes de eliminar estas substâncias. Este trabalho avaliou a degradação de alprazolam, clonazepam, diazepam, lorazepam e carbamazepina em diferentes matrizes aquosas por fotocatálise heterogênea e oxidação eletroquímica. Neste último processo também foi avaliada a neurotoxicidade de algumas soluções. Na fotocatálise heterogênea os melhores resultados foram obtidos ao se utilizar TiO_2 (0.1 g L^{-1}) em suspensão. Foi detectado que a a combinação de mais de um processo oxidativo avançado pode é necessária para que ocorra simultaneamente a mineralização e a desinfecção da solução tratada. Com relação à oxidação eletroquímica, o aumento da densidade da corrente e da concentração de eletrólito estão diretamente ligados ao aumento na degradação dos compostos. Os melhores resultados foram detectados com a utilização de BDD, aplicando-se uma densidade de corrente de 75 A m^{-2}, os compostos foram completamente degradados em menos de 5 min. Ao se utilizar efluente de uma indústria farmacêutica, foi possível remover 71 e 75 % de COT e DQO, respectivamente. A neurotoxicidade foi avaliada em diferentes soluções antes e depois da oxidação eletroquímica, a aplicação do processo oxidativo avançado foi eficiente para eliminar a neutotoxicidade do efluente proveniente da industria farmacêutica. Foi possível detectar que as soluções estudadas podem influenciar diretamente na produção de espécies reativas de oxigênio no interior das células.

Abstract of Thesis presented to COPPE/UFRJ as a partial fulfillment of the requirements for the degree of Doctor of Science (D.Sc.)

PHARMACEUTICAL PSYCHOTROPICS DEGRADATION BY PHOTOCATALISYS AND ELECTROCHEMICAL OXIDATION AND NEUROTOXICITY EVALUATION

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A large amount and variety of recalcitrant compounds are eliminated daily in water bodies, for this reason it is necessary to use processes that are capable of eliminating these substances. This work evaluated the degradation of alprazolam, clonazepam, diazepam, lorazepam and carbamazepine in different aqueous matrices by heterogeneous photocatalysis and electrochemical oxidation. In the electrochemical oxidation the neurotoxicity of some solutions was also evaluated. In the heterogeneous photocatalysis the best results were obtained when using TiO_2 (0.1 g L^{-1}) in suspension. It has been found that the combination of more than one advanced oxidative process may be required for both mineralization and disinfection of the treated solution to occur simultaneously. In electrochemical oxidation the increase in current density and electrolyte concentration are directly linked to the increase in the degradation of the compounds. The best results were detected with the use of BDD, applying a current density of 75 A m^{-2}, the compounds were completely degraded in less than 5 min. When using effluent from a pharmaceutical industry, it was possible to remove 71 and 75% of COT and COD, respectively. Neurotoxicity was evaluated in different solutions before and after the electrochemical oxidation, the application of the advanced oxidative process was efficient to eliminate the neutotoxicity of the effluent from the pharmaceutical industry. It was possible to detect that the solutions studied could directly influence the production of reactive oxygen species inside the cells.

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CHAPTER I – Introduction

I.1 Introduction

The combination of climate change and water pollution influences the quality of water used by industries, agriculture and population. A wide variety of substances are found in rivers, surface waters, lakes and drinking water (Kosma, Lambropoulou and Albanis, 2014; Rzymski, Drewek and Klimaszyk, 2017; Mandaric *et al.*, 2019; Williams *et al.*, 2019). Pharmaceuticals are some of the compounds found in the environment (Jongh *et al.*, 2012; Guang-guo *et al.*, 2013; Houtman *et al.*, 2014; Gurke *et al.*, 2015; Dotan *et al.*, 2016; Archer *et al.*, 2017; Gogoi *et al.*, 2018; Sharma *et al.*, 2019).

In large urban centers there is complaint of insomnia and depression caused by stress. The consequences of these diseases involve of sleep, concentration and productivity losses (Cheng *et al.*, 2016). As a consequence consumption of medicines for the treatment of insomnia and depression grows every year (Wickwire, Shaya and Scharf, 2016).

In general, medicines are not completely metabolized in the human body and are excreted in the urine, for this reason they are found in domestic sewage treatment plants, aquatic environments and even in water treatment plants (West and Rowland, 2012). Many substances used in daily life as pharmaceuticals, illicit drugs and agrochemicals are found in water bodies (Petrie, Barden and Kasprzyk-Hordern, 2014). In Spain, substances such as illicit drugs (13.2 – 294.0 ng L^{-1}), amphetamines (49.8 – 1020.0 ng L^{-1}) and anxiolytics (2.9 – 149.0 ng L^{-1}) have been found in surface waters (Mendoza *et al.*, 2014).

This group of compounds are classified as contaminants of emerging concern (CEC) and many of them have the potential to cause adverse effects on local biota and humans. Some contaminants are not regulated and it is common to find them in the environment and even in drinking water (Noguera-Oviedo and Aga, 2016). Among the CEC are the pharmaceutical compounds, which are often found mainly in aquatic matrices. Even at low concentrations (e.g., nanograms) long-term exposure to these compounds may cause adverse effects on animals and plants (Bautitz and Nogueira, 2010). Some medicines are resistant to biological degradation and bioaccumulative (Catalá *et al.*, 2015). Studies show the occurrence of pharmaceutical compounds in the effluent of sewage treatment plants comprising biological treatment, demonstrating that this method is not effective for the removal of some compounds (Stumpf *et al.*, 1999; Bautitz and Nogueira, 2010). Antidepressant compounds were detected at the outflow of domestic sewage treatment plants at a concentration of 285.1 ng L^{-1} at 249.5 ng L^{-1} (Baker and Kasprzyk-Hordern, 2013).

An alternative for the degradation of these recalcitrant substances are advanced oxidative processes (AOPs), which are an efficient alternative to eliminate organic substances showing difficult degradation and often detected in low concentrations (Chaplin, 2014).

Heterogeneous photocatalysis is a process characterized by the redox reactions induced by radiation on the surface of catalysts such as TiO_2, ZnO, CdS and Fe_2O_3. TiO_2 is the most used catalyst because it has characteristics such as low cost, non-toxic, water insolubility, photo stability, possibility of activation by sunlight, and chemical stability over a wide pH range (Yunus *et al.*, 2017). One theory of the mechanisms of heterogeneous photocatalysis is the change of the electron from the valence band to the conduction band, forming oxidizing and reducing sites that catalyze chemical reactions and oxidize organic compounds in CO_2 and H_2O (Costacurta *et al.*, 2010; Thu *et al.*, 2016).

Another advanced oxidative process that also mineralizes the organic compounds is the electrochemical oxidation, which uses different configurations of electrodes such as Ti/Pt, BDD, RuO_2 and IrO_2 (Haidar *et al.*, 2013; Oturan *et al.*, 2013). This process is based on the direct oxidation that occurs on the surface of the anode, mainly by the production of the hydroxyl radical (•OH), which has high oxidizing power and no selectivity (Bai *et al.*, 2017), and indirect oxidation that occurs by the production of radicals generated as a result of the type of electrolyte used in the experiment (Martínez-Huitle and Brillas, 2009). In this process some parameters such as current density, type and electrolyte concentration, anode type and pH are directly related to process efficiency (Hasa *et al.*, 2013).

The degradation of compounds and the generation of by-products is a concern in the treatment of wastewaters, since substances, previously inert, can generate toxic substances when subjected to treatment (Li, 2014). Therefore, it is important to evaluate the solutions before and after the treatment to check the level of toxicity, phytotoxicity that evaluate the germination of the seeds, the growth and quality of the plants (Ozdener and Kutbay, 2009; Schmidt and Redshaw, 2015; Pan and Chu, 2016) and the neurotoxicity in the CA3 region that evaluates the impact of wastewaters on the production of ROS (Hormuzdi *et al.*, 2004; Matias *et al.*, 2006; Trama-Freitas *et al.*, 2017) it is necessary to evaluate whether the advanced oxidative treatment decreases or increases the toxicity.

In view of the above scenario, the analysis of domestic, industrial and other wastewaters and the most appropriate type of treatment becomes extremely important from the public health and environmental standpoints. In Figure I.1 it is possible to observe the scheme of the processes performed in the thesis and the best detailing of the neurotoxicity procedure is in the appendix of this thesis.

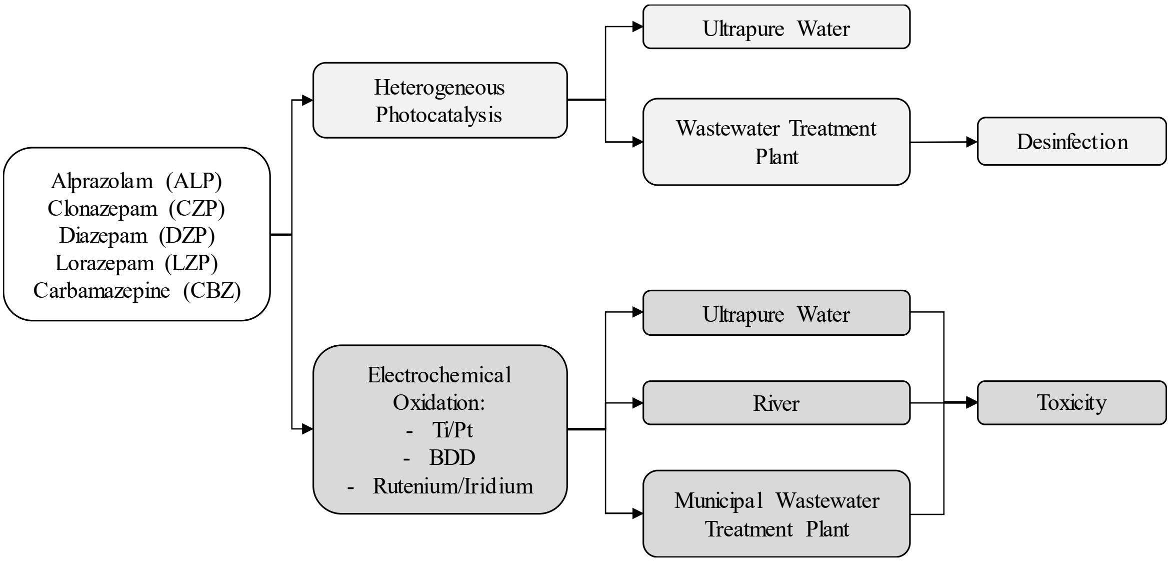

Figure I.1 - Processes performed in the thesis.

I.2 Objective

The main objectives of this thesis are (i) evaluate the degradation of the psychotropic pharmaceutical compounds Alprazolam, Clonazepam, Diazepam, Lorazepam and Carbamazepine by heterogeneous photocatalysis and electrochemical oxidation, (ii) evaluate and optimize the optimal operating conditions of the heterogeneous photocatalysis and electrochemical oxidation systems, (iii) analyze the behavior of drug degradation by using different aqueous matrices, (iv) investigation of phytotoxicity and neurotoxicity before and after electrochemical oxidation.

The idea behing this thesis is to evaluate how new technologies can treat wastewaters efficiently in such a way that thay can be disposed in the environment without compromising the well being of the local biota and to preserve the quality of water bodies.

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CHAPTER II – Fundamentals and Literature Overview

II. 1 Problems of water scarcity and pollution of water bodies

The world has faced severe climate change, causing extreme droughts in some cases and in other situations large pluviometric volumes, directly affecting the water supply and floods, mainly in large urban centers (Liu, Liu and Yang, 2016; Zhang *et al.*, 2019). Water is a basic resource for the development of basic activities such as personal hygiene, agriculture, industry and also for the maintenance of the environment (Zeng, Liu and Savenije, 2013; Liu *et al.*, 2017; Brunner, Zappa and Stähli, 2019). Another relevant factor regarding the availability of water is the quality it presents in the water bodies, from where it will be collected for later population supply (Vörösmarty *et al.*, 2010).

A solution for the maintenance of the quality of the available water would be the monitoring of the evictions of cities and industries, so that the waste streams meet the parameters required in each country (Kummu *et al.*, 2016). However, the discharge of wastewaters without any type of treatment still occurs, with the aggravation of the population increase and consequently the quantity of pollutants that reach the water bodies (Siebert *et al.*, 2015). Although wastewaters undergo conventional treatments processes such as activated sludge (Sodhi, Bansal and Jha, 2018; Zhao *et al.*, 2018) and moving bed biofilm reactor (MBBR) (Xu *et al.*, 2015; Brink, Sheridan and Harding, 2018), the municipal and industrial sewage can contain several recalcitrant substances that are not degraded biologically (Quan *et al.*, 2013; Alamelu and Jaffar Ali, 2018). Pollution of water bodies in large urban centers undoubtedly demands much more structure and logistics, since potable water needs to be captured farther (Salmivaara *et al.*, 2015).

II. 2 Contaminants of Emerging Concern - Benzodiazepines and Carbamazepine

Among the various substances that end up in the water bodies and wastewaters, there is a class of pollutants commonly regarded as contaminants of emerging concern (CECs). Among these pollutants are pharmaceuticals, personal care products, flame retardants, pesticides, hormones, disinfection products, gasoline additives, surfactants and industrial agents and artificial sweeteners (Petrović, Gonzalez and Barceló, 2003; Rodil *et al.*, 2012; Salimi *et al.*, 2017). Many of these substances are recalcitrant and cumulative in the environment and in biological tissues (Rosal *et al.*, 2010; Ebele, Abdallah and Harrad, 2017) and their harmful effects vary and some even have carcinogenic effects (Mendoza *et al.*, 2014).

Due to the recalcitrant characteristics of some compounds and the lack of adequate treatment, many of these substances are released directly into water bodies (Gavrilescu *et al.*, 2015). Figure II.1 schematizes in a simplified way how the CECs reach the environment.

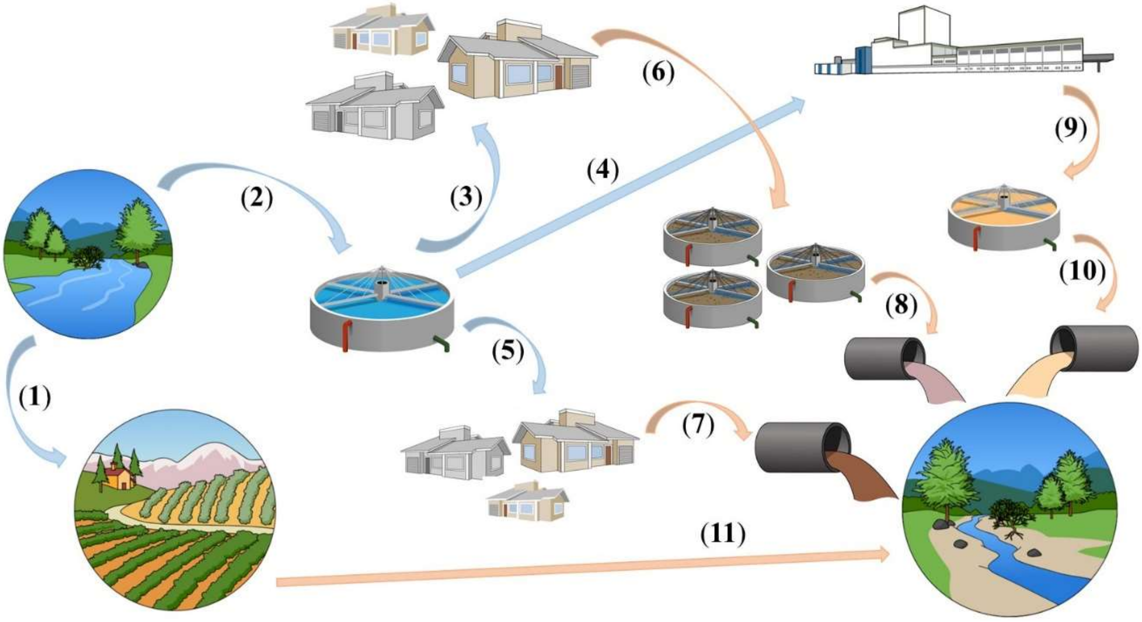

Figure II.1 – (1) – Water for agricultural use; (2) - Capture of water from the environment for treatment; (3) - Water for domestic and commercial use; (4) - Water for industrial use; (5) - Water for domestic and commercial use; (6) - Disposal of sewage in wastewater treatment plant.; (7) - Dumping of untreated sewage; (8) - Disposal of treated sewage in the environment; (9) - Disposal of industrial effluent in wastewater in treatment plant. (10) – Disposal of treated sewage in the environment and (11) Dumping of untreated agricultural effluent. (Stuart *et al.*, 2012; Klatte, Schaefer and Hempel, 2016; Mendoza *et al.*, 2016).

It can be seen in Figure II.1 that the industrial, agricultural and residential sectors are the main generators of wastewaters. This aggravates the pollution of water bodies in large urban centers and aggravates the situation when waste does not undergo any type of treatment. For this reason many substances are found and detected in rivers in concentrations that can vary from ng L^{-1} to µg L^{-1} (Sangion and Gramatica, 2016).

A group of CECs used and found in the environment are the pharmaceutical compounds, many of which are detected in relatively low concentrations, however it is not safe to say that this does not cause any deleterious effects with long exposure (Jiang, Zhou and Sharma, 2013; Sangion and Gramatica, 2016). Table II.1 shows the main drugs consumed.

Table II.1 – Main pharmaceutical compounds used (Li, 2014; Tayo *et al.*, 2018).

Categories of pharmaceuticals	**Specific drug**
Analgesics/anti-inflammatories	Acetaminophen, aspirin, codeine, methadone, morphine, oxycodone, ibuprofen, diclofenac, fenoprofen, ketoprofen, mefenamic acid, indomethacin and naproxen.
Classes of antibiotic	β-lactams, macrolides, fluoroquinolones, aminoglycosides, sulfonamide and tetracycline.
Psycho-stimulants	Caffeine
Estrogens and hormonal compounds	Estriol, estradiol, estrone and 17α-ethinylestradiol.
Antiepileptic drug	Carbamazepine

Among the drugs, a class that is also widely used today mainly in large urban centers, are benzodiazepines. These drugs act directly in the central nervous system with different effects such as anticonvulsant, hypnotic, anxiolytic and sedative.

The action of benzodiazepines occurs as follows, they increase the effect of a natural neurotransmitter called gamma-Aminobutyric acid (GABA), which is responsible for inhibiting nerve impulse transmission, enhance the GABA receptor affinity increase or make more effective the coupling of this neurotransmitter (Cornett *et al.*, 2018; Sigel and Ernst, 2018). Alprazolam, clonazepam, diazepam and lorazepam are in the group of the most used benzodiazepines. In Figure II.2 it is possible to observe the different structures of the drugs.

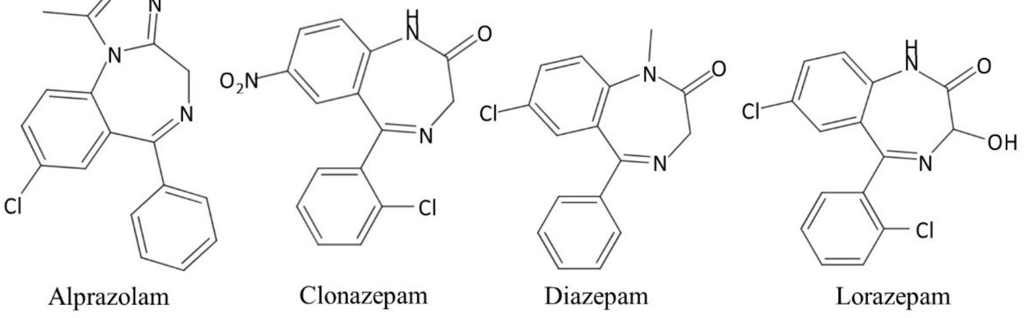

Figure II.2 – Chemical Structure of Alprazolam, Clonazepam, Diazepam and Lorazepam (Bautitz and Nogueira, 2010; Mohd *et al.*, 2013; Sruthi *et al.*, 2013).

Each benzodiazepine has a specific type of action with different time of action in the body, as exemplified in Table II.2.

Table II.2 – Indication and time of action of benzodiazepines (Lindim *et al.*, 2016).

Compound	**Application**	**Body action start time (h)**	**Percentage not metabolized by the body (%)**
Alprazolam	Anxiety disorder	1.2 – 1.7	21
Clonazepam	Epileptic disorders, anxiety disorder, mood disorders	2-3	2
Diazepam	Anxiety disorder, muscle relaxant, epileptic disorders, insomnia	0.5 - 2	-
Lorazepam	Anxiety, sedation	0.8 – 1.6	0.3

Another widely used drug is carbamazepine, an anticonvulsant that acts on the nervous system, mainly indicated for the treatment of people who suffer epileptic seizures. Its use is detected worldwide and is one of the most prescribed remedies. The time for CBZ to be absorbed varies from 4 to 8 hours, and it has a bioavailability of 75-85%. As it is not completely metabolized by the body, it is often detected in surface water from all over the planet (Serralheiro *et al.*, 2014).

Although found in low concentrations of mg L^{-1} to ng L^{-1}, it may have toxic effects on living organisms in aquatic environments and may have potential to impact humans. Carbamazepine is resistant to biodegradation and is not degraded in conventional sewage treatment plants (Wang and Wang, 2017). Figure II.3 shows the chemical structure of carbamazepine.

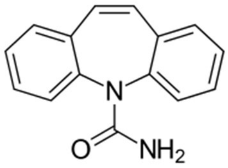

Figure II.3 – Chemical structure of carbamazepine (Wang and Wang, 2017).

However, more than eighty thousand different synthetic chemicals are released annually into the environment (Naidu *et al.*, 2016). In many cases it is hard to say what exposure to these compounds could generate in the human body and future generations (Stuart *et al.*, 2012), since there is a multitude of substances in all parts of the world. In Table II.3 it is possible to observe CECs detected in rivers, surface waters, municipal wastewater treatment plant (MWWTP) and water treatment plant (WTP). Based on this data, it is possible to infer that biological treatment processes are not fully efficient in the removal of some compounds.

Table II. 3 – Pharmaceutical compounds and their respective concentrations in rivers, MWWTP and WTP.

Compound	**Concentration**	**Samplim site**	**Country**	**Reference**
17β-Estradiol	8.60 ng L^{-1}	MWTTP influent	Czech Republic	(Vymazal et al., 2016)
	1.00 ng L^{-1}	MWTTP effluent		
Alprazolam	2.50 ng L^{-1}	River	Xanguai China	(Wu et al., 2015)
	7.60 ng L^{-1}	MWTTP influent		
	4.90 ng L^{-1}	MWTTP effluent		
Alprazolam	2.20 pg L^{-1}	River	Switzerland	(Lindim et al., 2016)
Androstenedione	0.60 ng L^{-1}	Drinking water sources	China	(Sun et al., 2015)
Atenolol	156.20 ng L^{-1}	River	Gauteng province of South Africa	(Archer et al., 2017)
Azithromycin	35.66 ng L^{-1}	Surface water	Portugal	(Pereira et al., 2017)
Bezafibrate	15.52 ng L^{-1}	Surface water	Portugal	(Pereira et al., 2017)
Bisphenol-A	239.00 ng L^{-1}	River	Gauteng province of South Africa	(Archer et al., 2017)
Caffeine	812.20 ng L^{-1}	River	Gauteng province of South Africa	(Archer et al., 2017)
Carbamazepine	1.10 µg L^{-1}	River	German	(Ternes, 1998)
Carbamazepine	1.78 µg L^{-1}	MWTTP influent	German	(Heberer and Heberer, 2002)
	1.63 µg L^{-1}	MWTTP effluent		
Carbamazepine	82.00 ng L^{-1}	River	Spain	(González Alonso et al., 2010)
Carbamazepine	3.60 µg L^{-1}	Underground water	United Kingdom	(Stuart *et al.*, 2012)
Carbamazepine	215.00 ng L^{-1}	WTP raw water	Spain	(Azzouz and Ballesteros, 2013)
	1.40 ng L^{-1}	WTP treated water		
Carbamazepine	1.90 ng L^{-1}	MWTTP influent	Portugal	(Bahlmann *et al.*, 2014)
	2.00 ng L^{-1}	MWTTP effluent		

	0.47 ng L^{-1}	MWTTP inffluent	German	
	0.52 ng L^{-1}	MWTTP effluent		
Carbamazepine	17.80 ng L^{-1}	Surface water	Portugal	(Gaffney et al., 2015)
Carbamazepine	3.50 ng L^{-1}	Drinking water sources	China	(Sun et al., 2015)
Carbamazepine	25.30 ng L^{-1}	River	Xanguai China	(Wu et al., 2015)
Carbamazepine	506.90 pg L^{-1}	River	Switzerland	(Lindim et al., 2016)
Carbamazepine	157.10 ng L^{-1}	River	Gauteng province of South Africa	(Archer et al., 2017)
Carbamazepine	10.90 ng L^{-1}	Surface water	Portugal	(Pereira et al., 2017)
Clarithromycin	21.00 ng L^{-1}	Drinking water sources	China	(Sun et al., 2015)
Cotinine	88.50 ng L^{-1}	Drinking water sources	China	(Sun et al., 2015)
Diazepam	9.50 ng L^{-1}	MWTTP inffluent	Xanguai China	(Wu et al., 2015)
	9.70 ng L^{-1}	MWTTP effluent		
Diclofenac	316.00 ng L^{-1}	WTP raw water	Spain	(Azzouz and Ballesteros, 2013)
	68.00 ng L^{-1}	WTP treated water		
Diclofenac	1660.00 ng L^{-1}	MWTTP inffluent	Murcia, Spain	(Fernández-López et al., 2016)
	430.00 ng L^{-1}	MWTTP effluent		
Diclofenac	51.24 ng L^{-1}	Surface water	Portugal	(Pereira et al., 2017)
Erythromycin	30.00 ng L^{-1}	Surface water	Portugal	(Gaffney et al., 2015)
Erythromycin	38.80 ng L^{-1}	Surface water	Portugal	(Pereira et al., 2017)
Estriol	13.00 ng L^{-1}	MWTTP inffluent	Czech Republic	(Vymazal et al., 2016)
	10.00 ng L^{-1}	MWTTP effluent		
Fluoxetine	14.00 ng L^{-1}	River	Spain	(González Alonso et al., 2010)
Fluoxetine	34.40 ng L^{-1}	River	Gauteng province of South Africa	(Archer et al., 2017)

Fluxetine	25.37 ng L^{-1}	Surface water	Portugal	(Pereira et al., 2017)
Fluxinin	145.00 ng L^{-1}	WTP raw water	Spain	(Azzouz and Ballesteros, 2013)
	0.03 ng L^{-1}	WTP treated water		
Ibuprofen	357.00 ng L^{-1}	WTP raw water	Spain	(Azzouz and Ballesteros, 2013)
	102.00 ng L^{-1}	WTP treated water		
Ibuprofen	22.00 ng L^{-1}	Surface water	Portugal	(Gaffney et al., 2015)
Ibuprofen	2800.00 ng L^{-1}	MWTTP inffluent	Murcia, Spain	(Fernández-López et al., 2016)
	720.00 ng L^{-1}	MWTTP effluent		
Ketoprofen	250.00 ng L^{-1}	WTP raw water	Spain	(Azzouz and Ballesteros, 2013)
	37.00 ng L^{-1}	WTP treated water		
Lorazepam	4.00 ng.L^{-1}	River	Xanguai China	(Wu et al., 2015)
	35.80 ng L^{-1}	MWTTP inffluent		
Naproxen	321.00 ng L^{-1}	WTP raw water	Spain	(Azzouz and Ballesteros, 2013)
	2.40 ng L^{-1}	WTP treated water		
Naproxen	5.70 ng L^{-1}	Surface water	Portugal	(Gaffney et al., 2015)
Naproxen	1180.00 ng L^{-1}	MWTTP inffluent	Murcia, Spain	(Fernández-López et al., 2016)
	190.00 ng L^{-1}	MWTTP effluent		
Naproxen	224.30 ng L^{-1}	River	Gauteng province of South Africa	(Archer et al., 2017)
Nimesulide	27.00 ng L^{-1}	Surface water	Surface water	(Gaffney et al., 2015)
Oxazepam	30.00 ng L^{-1}	River	Spain	(González Alonso et al., 2010)
Paracetamol	69.15 ng L^{-1}	Surface water	Portugal	(Pereira et al., 2017)
Paraxanthine	116.00 ng L^{-1}	Drinking water sources	China	(Sun et al., 2015)
Propylparaben	31.80 ng L^{-1}	River	Gauteng province of South Africa	(Archer et al., 2017)

Sertraline	23.30 ng L^{-1}	Surface water	Portugal	(Pereira et al., 2017)
Sulfadiazine	25.90 ng L^{-1}	Surface water	Portugal	(Gaffney et al., 2015)
Sulfamethoxazole	757.40 ng L^{-1}	River	Gauteng province of South Africa	(Archer et al., 2017)
Triclocarban	1150.00 ng L^{-1}	MWTTP inffluent	India	(Subedi and Kannan, 2015)
	49.00 ng L^{-1}	MWTTP effluent		
Triclosan	892.00 ng L^{-1}	MWTTP inffluent	India	(Subedi and Kannan, 2015)
	202.00 ng L^{-1}	MWTTP effluent		
Triclosan	1230.00 ng L^{-1}	WTP raw water	Israel and Palestine	(Dotan *et al.*, 2016)
	100.00 ng L^{-1}	WTP treated water		
Venlafaxine	0.10 ng L^{-1}	Drinking water sources	China	(Sun et al., 2015)

II. 3 Advanced Oxidative Processes

The development of cities, industries and the emergence of new products is not accompanied at the same pace in the treatment of domestic and industrial wastewaters. Most of the wastewater treatment plants have only biological treatment, which is not enough to completely degrade recalcitrant compounds. An alternative to solve this problem is the application of advanced oxidative processes (AOPs), which have the ability to partially or completely mineralize hardly degradable compounds.

The AOPs are characterized by the formation of the hydroxyl radical (•OH), whose characteristic is to be strongly and non-selective oxidative, being the second species more reactive, losing only to fluorine. In the degradation reactions the radical •OH has the capacity to mineralize the organic compounds in CO_2 and H_2O, which makes the OAPs versatile and efficient because there are different processes with which the hydroxyl radical can be produced (Bethi *et al.*, 2016; Cheng *et al.*, 2016). Table II.4 shows the different oxidation potentials of some oxidizing agents.

Table II. 4 – Main oxidizing agents and their respective reduction potentials (Turhan and Turgut, 2009).

Oxidizing agents	**Oxidation potential (eV)**
Fluorine	3.06
Hydroxyl radical	2.8
Atomic oxygen	2.42
Ozone	2.08
Hydrogen peroxide	1.78
Chlorine	1.36
Chlorine dioxide	1.27

The hydroxyl radical stands out not only for its oxidation potential, but also for its reaction kinetics with organic compounds. Compared with the kinetics of other oxidants, the hydroxyl radicals can have the kinetic constant up to a billion times faster than the constant of other oxidants.

Figure II.4 shows the classification of the advanced oxidative processes, which have a great variety. They are assentially classified in heterogeneous processes with semiconductors in the solid phase and whose reaction occurs at the interface of the two phases, and in homogeneous processes, in which the catalyst and the system constitute only one phase and may or may not be associated with UV light (Bethi *et al.*, 2016).

Some advantages of AOPs include: ability to completely mineralize pollutants and not just transfer to another phase; partially degrade some compounds and increase their biodegradability, therefore potentially serving as a suitable pre-treatment for biological processes; degrade many substances, including toxic compounds; can be applied to compounds whose concentration is relatively low (as $\mu g\ L^{-1}$); in some cases the non-generation of waste. Although they present many advantages, there are also some disadvantages such as: high cost, mainly due to the expense of energy; the generation of by-products that may be more toxic than the original compound; in some cases restrictions when there is a high concentration of pollutants (Quero-Pastor *et al.*, 2014; Moreira *et al.*, 2016).

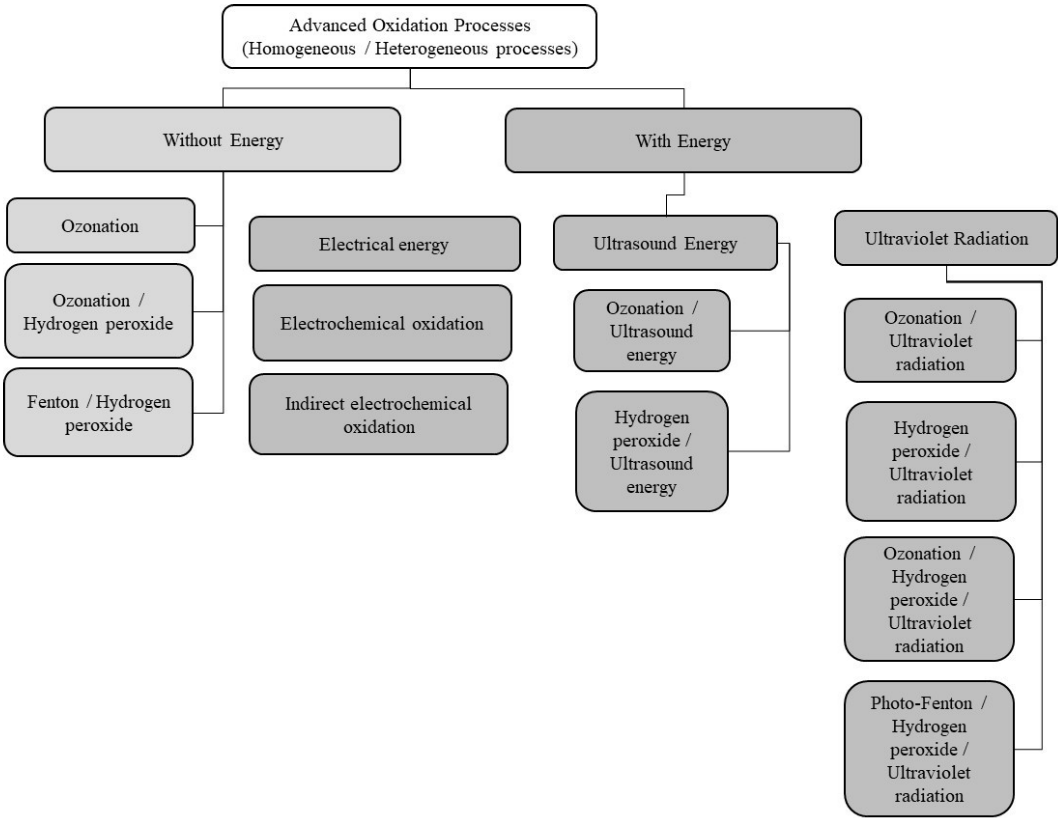

Figure II.4 – Advanced Oxidative Processes (Sharma, Ruparelia and Patel, 2011).

II. 3.1 Heterogeneous photocatalysis

Heterogeneous photocatalysis is defined as the acceleration of a photoreaction by the presence of a catalyst. They are processes that involve redox reactions induced by radiation on the surface of mineral semiconductors. The most common catalysts are: TiO_2, CdS, ZnO, WO_3, ZnS, BiO_3 and Fe_2O_3. Among these, what stands out most is TiO_2, which is non-toxic compound, has low cost, is insoluble in water, has photo stability, it is possible to activate with sunlight, has chemical stability over a wide pH range and is easy to obtain (Meribout *et al.*, 2016).

The photocatalysis process requires a sufficient amount of energy to promote the transition of the electron from the semiconductor, from the valence band (VB) to the conduction band (CB). This energy must be greater than or equal to the "bang gap" energy, which is required to excite the electron. With the transition of the electron from VB to CB, highly oxidizing and reductive gaps are formed. As VB loses electron, it is highly reductive, so this layer reacts with the •OH formed from water, which in turn reacts with the organic molecule by degrading it. In the oxidizing CB, the reaction happens with O_2, which loses an electron and reacts with water, forming H_2O_2, which also forms a hydroxyl radical and can degrade the organic molecule (Nakata and Fujishima, 2012; Ahmad *et al.*, 2016; Meribout *et al.*, 2016).

An advantage of heterogeneous photocatalysis is the electron can be excited by natural (solar) light, which is available for a long period of the day and has no additional cost to the process.

Oxidation of organic compounds can occur in several ways such as: the hydroxyl radical can attack a molecule adsorbed by TiO_2, can directly attack the compounds in solution, can diffuse on the surface and only then react with some molecule or can leave the surface of the semiconductor and migrate to the solution as a free radical (Ziolli and Jardim, 1998).

Within heterogeneous photocatalysis, some parameters can directly influence the mineralization of organic compounds:

- pH: The pH of the solutions may change the semiconductor / liquid interface, leading to catalyst adsorption and desorption modifications, in some cases the pH may also modify the chemical structure of the compounds to be degraded, such modifications may cause degradation of compounds to occur more efficiently (Tanaka and Saha, 1994; Zielinska *et al.*, 2003; Thu *et al.*, 2016).
- Temperature: The efficiency of the degradation increases with the temperature increase, this happens by the influence in the collision of the molecules and in the equilibrium of adsorption (Costacurta *et al.*, 2010).

- Initial concentration of the compounds to be degraded: The rate of degradation of the compounds increases as the concentration of the compounds increases. At a saturation concentration, if the pollutant absorbs a large amount of UV, the compounds can cover the surface of the catalyst and prevent light from being absorbed, reducing the efficiency of the catalytic process (Bhatkhande *et al.*, 2004; Chong *et al.*, 2009).
- Initial concentration of the photocatalyst: This is a factor that directly influences the efficiency of the process, the increase of the photocatalyst in the solution increases the reaction rate. However, upon reaching certain photocatalyst concentrations, the reaction rate can decrease. The high concentrations may also cause turbidity in the medium and disrupt the penetration of light (Yunus *et al.*, 2017).

Some studies were carried out with the heterogeneous photocatalysis process and prove the efficacy of this system:

The first studies of heterogenous photocatalysis using TiO_2 are dated from the 1980s with Pruden and Ollis (1983), which demonstrated the complete degradation of trichlorethylene in CO_2, inorganic species and chloride ion and Matthews (1986), which studied the degradation of several organic compounds and converted them to CO_2 at significant rates.

Prairie *et al.*, (1993) evaluated the treatment of water contaminated with metals (Au, Cd, Cr, Hg, Pt) and organic compounds such as methanol, formic acid, salicylic acid, phenol and nitrobenzene and described the kinetic and electrochemical relationships between oxidation processes of organic matter and the reduction of metals.

Bekbölet *et al.*, (1996) used heterogeneous photocatalysis in the degradation of landfill leachate, which had previously undergone biological treatment, and observed the reduction of about 70% of TOC after 5 hours of exposure.

The association of biological processes with heterogeneous photocatalysis proved to be very efficient in the degradation of wastewater with high color and COD. A study carried out by Li and Zhao (1997) led to complete removal of color and more than 90% of COD removal.

In studies conducted by Topalov, Molnár-Gábor and Csanádi (1999) it was possible to completely degrade the metalaxyl fungicide and the intermediates formed during heterogeneous photocatalysis.

Khataee, Fathinia and Joo (2013) evaluated the degradation of a mixture of drugs (metronidazole, atenolol and chlorpromazine) by photocatalysis under UV radiation, using TiO_2 immobilized on ceramic plates by the sol-gel method. A TOC removal of 90% was reached and the mineralization of the compounds proved.

Gar Alalm, Tawfik and Ookawara, (2016) used photocatalysis with TiO_2 to degrade amoxicillin, ampicillin, diclofenac and paracetamol. It took 90 min of UV radiation to remove 100, 100, 85 and 70%, respectively.

Lin *et al.*, (2017) tested the degradation of drugs by heterogeneous photocatalysis and after 180 min of UV radiation it was possible to reach 54, 81, and 92% removal of carbamazepine, ibuprofen and sulfamethoxazole, respectively.

Simsek (2017), using photocatalysis after 4 hours of UVA radiation and TiO_2 catalyst, obtained degradation of dichlorophenol (75%), bisphenol A (97%), ibuprofen (89%) and flurbiprofen (93%).

II. 3.2 Electrochemical Oxidation Process

The first stage of electrochemical oxidation is the breaking of the water molecule on the surface of the anode (Equation II.1) to form hydroxyl radicals, where X designates the anode (Dominguez *et al.*, 2018b).

$$X + H_2O \rightarrow X(\bullet OH) + H^+ + e^- \qquad \text{(II.1)}$$

This step occurs in active (Pt, IrO_2 and RuO_2) and non-active electrodes (PbO_2, SnO_2 and BDD) (Zhu *et al.*, 2008; Martínez-Huitle and Brillas, 2009). However, the following steps depend on the nature of the electrode material. The active anodes have a higher oxidation state on the surface and interact more with •OH, leading to the formation of superoxide (XO), Equation II.2 (Moreira *et al.*, 2017).

$$X(\bullet OH) \rightarrow XO + H^+ + e^- \qquad \text{(II.2)}$$

Different interactions can occur in the active anode such as the chemisorbed active oxygen which results in the partial oxidation of the organic compounds on the anode (Equation II.3), whereas in the not active anode the interaction can form physiosorbed active oxygen, which can result in the complete mineralization of the compounds (Equation II.4) (Panizza and Cerisola, 2009; Oturan *et al.*, 2013).

$$XO + R \rightarrow X + RO \qquad \text{(II.3)}$$

$$X(\bullet OH) + R \rightarrow X + CO_2 + H_2O + H^+ + e^- \qquad \text{(II.4)}$$

Another relevant fact regarding electrochemical oxidation is the composition of the anode, which has different oxidation potential (V) depending on the constituent material, as can be observed in Table II.5.

Table II. 5 – Oxidation potencial and overpotencial of O_2 evolution of some electrodes (Comninellis *et al.*, 2008).

Electrode material	**Oxidation Potencial (V)**	**Overpotencial of O_2 Evolution (V)**
RuO_2-TiO_2	1.4-1.7	0.18
IrO_2-Ta_2O_5	1.5-1.8	0.25
Ti/Pt	1.7-1.9	0.30
Ti/PbO_2	1.8-2.0	0.5
Ti/SnO_2-SbO_5	1.9-2.2	0.7
p-Si/BDD	2.2-2.6	1.3

Degradation of the compounds can occur in two ways, by direct oxidation, when the compounds are degraded on the surface of the anode or by indirect oxidation, which occurs when the compounds are degraded by the radicals produced in the solution. Both mechanisms, are exemplified in Figure II.5. It is important to note that direct and indirect oxidation can co-exist.

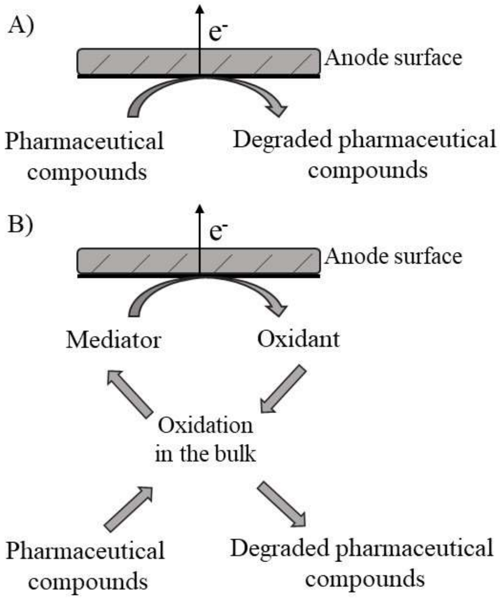

Figure II.5 – Direct (a) and indirect (b) electrochemical oxidation (Anglada, Urtiaga and Ortiz, 2009; Wang and Xu, 2012).

For the reactions to occur, conductivity in the reaction medium is necessary. For this reason, electrolytes are used, the most usual are NaCl and Na_2SO_4. In addition to providing the conductivity required for the process to occur, the electrolytes generate radicals that provide the indirect EO of the organic compounds present in the reaction medium. The most common reactions for NaCl are represented in Equations II.5, II.6 and II.7 (Baddouh *et al.*, 2018). Some compounds formed in the indirect EO from sulphate, carbonate and phosphate are presented in Equations II.8, II.9 and II.10, respectively (Bai *et al.*, 2017; Garcia-Segura, Ocon and Chong, 2018).

$$2Cl^- \rightarrow Cl_2 + 2e^- \qquad \text{(II.5)}$$

$$Cl_2 + H_2O \leftrightarrow HOCl + H^+ + Cl^- \qquad \text{(II.6)}$$

$$HOCl \leftrightarrow OCl^- + H^+ \qquad \text{(II.7)}$$

$$2SO_4^{2-} \rightarrow S_2O_8^{2-} + 2e^- \qquad \text{(II.8)}$$

$$2CO_3^{2-} \rightarrow C_2O_6^{2-} + 2e^- \qquad \text{(II.9)}$$

$$2PO_4^{3-} \rightarrow P_2O_8^{4-} + 2e^- \qquad \text{(II.10)}$$

Some advantages of electrochemical oxidation are: the operation of the system is simple, the equipment is easy to operate and with a low retention time; great versatility in relation to the variation of compounds that can be treated – pulp and paper industry (El-ashtoukhy, Amin and Abdelwahab, 2009), textile industry (Vlyssides *et al.*, 2000), food industry (Piya-areetham, Shenchunthichai and Hunsom, 2006), urban wastewater (Dialynas, Mantzavinos and Diamadopoulos, 2008; Lazarova and Spendlingwimmer, 2008), landfill leachate (Deng and Englehardt, 2007), agro-industry (Gotsi *et al.*, 2005; Cañizares *et al.*, 2006; Giannis, Kalaitzakis and Diamadopoulos, 2007; Deligiorgis *et al.*, 2008; Mavros *et al.*, 2008); and the volume of operation can vary from a few to millions of liters; the operation requires low temperatures and pressure, especially when compared with other processes; complex process automation is not required, since the electrical variables used are easily suitable for each case. However, there are some disadvantages associated with electrochemical oxidation: relatively high energy consumption, making the process more expensive; in some cases the addition of electrolytes is necessary and the electrode can be obstructed when there is deposition of materials on the surface of the electrode.

The electrochemical oxidation process is very diverse in relation to applicability, as displayed in Table II.6, showing some studies carried out with this advanced oxidative process.

Table II. 6 – Electrochemical oxidation process applied to the treatment of different wastewater and degradation of several pollutants.

Wastewater/Polluent	**Characteristics**	**Anode**	**Operating conditions**	**Main conclusions**	**Reference**
Tannery wastewater	COD = 2080 mg dm^{-3}	$Ti/TiRuO_2$	J = 600 A m^{-2} pH = 10	COD_{rem} = 90 %	(Panizza and Cerisola, 2004)
Textile wastewater	COD = 3635 mg L^{-1}	Iron electrode	J= 10 mA cm^{-2} pH = 6.9	COD_{rem} = 78 %	(Kobya, Demirbas and Akyol, 2009)
Herbicide atrazine	30 mg L^{-1}	BDD	J= 100 mA cm^{-2} pH = 3	TOC_{rem} = 90 - 92 %	(Borrás *et al.*, 2010)
Pharmaceutical effluent	COD = 12000 mg dm^{-3}	BDD	J= 50 mA cm^{-2} pH = 8.5	COD_{rem} = 85 %	(Domínguez *et al.*, 2012)
Pharmaceutical wastewater	COD = 20650 - 23200 mg L^{-1}	Carbon electrode	J = 40 - 120 A m^{-2} pH = 7.2	$Color_{rem}$ = 31 - 34 % COD_{rem} = 32 - 40 %	(Deshpande, Ramakant and Satyanarayan, 2012)
Real textile wastewater	COD = 650 mg L^{-1}	BDD	J= 40 mA cm^{-2}	$Color_{rem}$ = 100 % COD_{rem} = 100 %	(Martínez-huitle *et al.*, 2012)
Petroleum effluent	COD = 1588 mg L^{-1}	Ti/Pt BDD	J= 60 mA cm^{-2}	COD_{rem} = 80 % COD_{rem} = 98 %	(Rocha *et al.*, 2012)
Nitrobenzene	256 mg L^{-1}	BDD	J= 60 mA cm^{-2} pH = 3	TOC_{rem} = 92 %	(Rabaaoui *et al.*, 2013)
Real textile effluent	COD = 1000 mg L^{-1}	BDD	J= 60 mA cm^{-2}	$Color_{rem}$ = 100 % COD_{rem} = 100 %	(Solano *et al.*, 2013)
Sulfamerazine	50 mg L^{-1}	BDD	J= 15 mA cm^{-2} pH = 6.4	$Sulfamerazine_{rem}$ = 92 %	(Fabianska *et al.*, 2014)
Real textile wastewater	COD = 124 mg L^{-1}	Ti/Pt	J= 177 mA cm^{-2}	$Color_{rem}$ = 100 %	(Sala and Gutiérrez-bouzán, 2014)

Carmoisine (Acid Red 14)	209.3 mg L^{-1}	BDD	J= 100 mA cm^{-2}	Color$_{rem}$ = 70 - 82 %	(Thiam *et al.*, 2015)
Cinnamic acid	100 mg L^{-1}	PbO$_2$	J= 20 mA cm^{-2} pH = 4	COD$_{rem}$ = 35 % Cinnamic acid$_{rem}$ = 80 %	(Dai *et al.*, 2016)
M-dinitrobenzene	125 mg L^{-1}	BDD	J= 10 mA cm^{-2} pH = 7	M-dinitrobenzene$_{rem}$ = 82.7 %	(Bai *et al.*, 2017)
Wastewater containing pharmaceuticals	COD = 81 mg L^{-1}	BDD	J= 4.34 mA cm^{-2}	COD$_{rem}$ = 100 %	(Lan *et al.*, 2017)
Ciprofloxacin	76 µg L^{-1}		pH = 8	Ciprofloxacin$_{rem}$ = 100 %	
Phenol	50 mg L^{-1}	PbO$_2$	J= 100 mA cm^{-2} pH = 3	TOC$_{rem}$ = 98 % Phenol$_{rem}$ = 100 %	(Wang *et al.*, 2017)
Dye Blue BR	50 mg L^{-1}	BDD	pH = 2.8-3.0	Color$_{rem}$ = 53 - 99 %	(Alcocer *et al.*, 2018)
Violet SBL	50 mg L^{-1}		J= 2.0 - 18 mA cm^{-2}	COD$_{rem}$ = 78 - 92 %	
Rhodamine B	50 mg L^{-1}	SnO$_2$	J= 20 - 40 mA cm^{-2}	Color$_{rem}$ = 70 - 95 %	(Baddouh *et al.*, 2018)
Bisphenol A	TOC = 100 mg L^{-1}	BDD	J= 33.3 mA cm^{-2} pH = 3	TOC$_{rem}$ = 50.8 %	(Burgos-Castillo *et al.*, 2018)
Winery wastewater	COD = 3490 mg L^{-1}	BDD	J= 20 - 60 mA cm^{-2}	COD$_{rem}$ = 50 - 70 %	(Candia-Onfray *et al.*, 2018)
2,4-dichlorophenol	250 mg L^{-1}	BDD	J= 30 mA cm^{-2}	TOC$_{rem}$ = 64 -75 %	(Chen *et al.*, 2018)
Procion red MX-5B	100 mg dm^{-3}	BDD	J= 10 mA cm^{-2}	TOC$_{rem}$ = 98 %	(Cotillas *et al.*, 2018)
Lindane	10 mg L^{-1}	BDD	J= 8.33 mA cm^{-2} pH = 6.5	TOC$_{rem}$ = 70 %	(Dominguez *et al.*, 2018a)
Paper mill wastewater	COD = 300 mg L^{-1}	BDD	J= 5 mA cm^{-2} pH = 6.8	COD$_{rem}$ = 74 %	(Klidi *et al.*, 2018)
Iopromide	50 mg L^{-1}	BDD	J= 4.76 mA cm^{-2}	Iopromide$_{rem}$ = 32 %	(Loos *et al.*, 2018)
Sulfamethoxazole	50 mg L^{-1}			Sulfamethoxazole$_{rem}$ = 61 %	

17-α-ethinylestradiol	50 mg L^{-1}			17-α-ethinylestradiol$_{rem}$ = 50 %	
Diclofenac	50 mg L^{-1}			Diclofenac$_{rem}$ = 70 %	
Real wastewater	TOC = 210 mg L^{-1}	BDD	J= 5.3 - 53 mA cm^{-2} pH = 6.2	TOC$_{rem}$ = 45 - 80 %	(Ma *et al.*, 2018)
Acid orange 7	100 mg L^{-1}	PbO_2	J= 30 mA cm^{-2} pH = 7	TOC$_{rem}$ = 90 % Acid orange 7$_{rem}$ = 95 %	(Qiao *et al.*, 2018)
Thiamethoxan	30 mg L^{-1}	PbO_2/CeO_2	J= 30 mA cm^{-2} pH = 6	COD$_{rem}$ = 63 % Thiamethoxan$_{rem}$ = 71 %	(Yao *et al.*, 2018)
Amaranth dye	0.015 mM	PbO_2	J= 25 mA cm^{-2}	Color$_{rem}$ = 75 %	(Elaissaoui *et al.*, 2019)
Water-oil efflunet	DOC = 2034 mg L^{-1}	BDD	J= 60 mA cm^{-2}	COD$_{rem}$ = 95 %	(Escalona-Durán *et al.*, 2019)
Crytal violet dye	25 mg L^{-1}	BDD	J= 38 mA cm^{-2}	Crytal violet dye$_{rem}$ = 100 %	(Klidi *et al.*, 2019)
Acetaminophen Diclofenac Sulfamethoxazole	15 µM 15 µM 15 µM	Graphite	J= 4 mA cm^{-2}	Acetaminophen$_{rem}$ = 100 % Diclofenac$_{rem}$ = 100 % Sulfamethoxazole$_{rem}$ = 100 %	(Liu, Hu and Lo, 2019)
Norfloxacin	0.031 M	BDD	J= 20 mA cm^{-2} pH = 4	COD$_{rem}$ = 100 % Norfloxacin$_{rem}$ = 100 %	(Silva *et al.*, 2019)
Acredine orange	30 mg L^{-1}	PbO_2/ZrO_2	J= 50 mA cm^{-2} pH = 5	Acredine orange$_{rem}$ = 100%	(Yao, Ren, *et al.*, 2019)
Acetamiprid	10 - 80 mg L^{-1}	PbO_2	J= 50 - 250 mA cm^{-2} pH = 3 - 11	COD$_{rem}$ = 30 - 74 % Acetamiprid$_{rem}$ = 40 - 98.96 %	(Yao, Teng, *et al.*, 2019)

II. 4 Neuronal Studies

The study of neuronal toxicity was avalable in Wistar rats brain (Figura II.6 - A and B). More precisely in the hippocampus, which has important functions in the role of memory formation, attention control, emotions and olfactory function (Andersen *et al.*, 2007). The hippocampus has a complex "C" shape that can be observed in blue in Figure II.6 - C and with the main subdivisions CA1, CA2 and CA3 (Figure II.6 - D). Zone CA3 is represented by mossy fibers, which are composed of the axons of the granular cells of the dentate fascia.

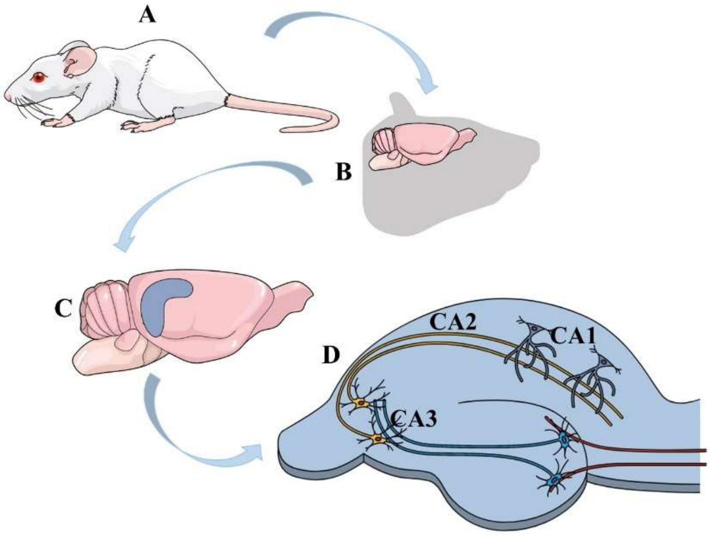

Figure II.6 – (A) Wistar rat; (B) Rat head and brain; (C) Rat brain and hippocampus (blue); (D) Transversal slice of hippocampus rat.

The main characteristic of the central nervous system is the ability of communication and interaction between cells. This property that is inherent to the neurons is due to two mechanisms of fundamental signalization, the axonic conduction and the synaptic transmission (Deshmukh and Knierim, 2012). Only one neuron can form up to two hundred thousand synapses, these connections between cells are called pre and post synaptic signals (Andersen *et al.*, 2007).

The synapses allow to send signals to other neurons or to non-neuronal cells in an extremely fast time. The synapses are divided into two types (Hormuzdi *et al.*, 2004; Pereda, 2014; Nagy, Pereda and Rash, 2018):

- Chemical synapses, which are characterized by the transmission of neurotransmitters from a neuron (presynaptic region), to an adjacent receptor neuron (postsynaptic region). The presynaptic region is characterized by the presence of exocytosis vesicles where the neurotransmitter molecules are found and the postsynaptic region where the receptors are located in the membranes of the cells. These two regions are separated by a spacing called the synaptic cleft.

- Electrical synapses are intercellular channels that allow the passage of ions or small molecules from one cell to another. The electrical signal is connected by a structure called gap junction, in this case there is no pre- or post-synaptic region, since the signal flow can vary. Both synapses can best be seen in Figure II.7.

The synapses performed are classified according to the types of neurotransmitters that are released: excitatory and inhibitory synapses. In the excitatory synapses there is an increase in the permeability of the membrane in relation to sodium and potassium, causing a depolarization of the neuron that can lead to a nerve impulse. In contrast, in the inhibitory synapses there is an increase in membrane permeability to chlorine, thus generating a hyperpolarization, not triggering a nerve impulse (Matias *et al.*, 2006; Matias, Saggau and Quinta-ferreira, 2010). The most abundant excitatory neurotransmitter in the hippocampus is glutamate, while the inhibitory is GABA (γ-aminobutyric acid).

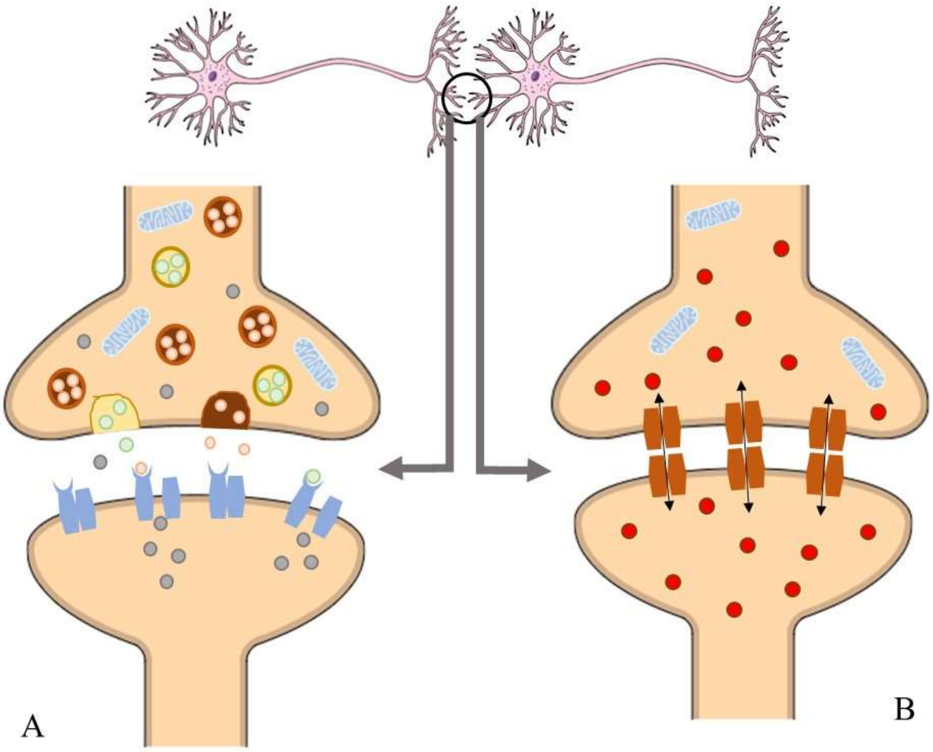

Figure II.7 – (A): Chemical synapse and (B): Electrical synapse (Hormuzdi *et al.*, 2004; Pereda, 2014; Nagy, Pereda and Rash, 2018).

For the normal functioning of the neuronal system four ions are necessary: potassium (K^+), sodium (Na^+), calcium (Ca^{2+}) and chlorine (Cl^-), these ions are in different concentrations inside and outside the cells, creating a potential difference, which is essential for the neuron. When some chemical, mechanical or electrical stimulation occurs, changes in the cellular membrane permeability of the neurons can occur, which can lead to the entrance and the exit of ions, provoking the depolarization of the membrane (Purves *et al.*, 2004).

The formation of ROS happens mainly in the mitochondria, extremely important organelles since they are the main source of energy of the cells. Present in eukaryotic cells, these organelles are responsible for storing energy in the form of adenosine triphosphate (ATP), this energy is used in reactions that occur inside cells (Stewart *et al.*, 2005). However, ROS can also be produced in other organelles such as ribosomes, endoplasmic reticulum, plasma membrane, peroxisomes, among others. The formation of ROS is accompanied by other reactions, such as the auto-oxidation of the cells own components, which can lead to the inactivation of biomolecules and some inactivation inside the cell (Wijk *et al.*, 2008).

The production of reactive oxygen species is crucial for the survival and proper functioning of the organism. At normal concentrations, they play an important protection role. However, excess production of these species may lead to the development of some neurodegenerative diseases. When ROS production occurs in large quantities, it is combated by antioxidants, species that have the ability to react with ROS and leave the system in equilibrium. If there is a not normal increase in ROS production and if the body cannot detoxify, it remains in a state of oxidative stress. For this reason, the balance between these species is crucial for the proper functioning of the organism (Freinbichler *et al.*, 2011).

The main compounds involved in ROS are superoxide anion (O_2^-), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and oxygen (O_2) (Schieber and Chandel, 2014; Zorov, Juhaszova and Sollott, 2014). Reactive oxygen species are apparently involved in the regulation of various channels such as calcium and potassium (Massaad and Klann, 2011). Ca^{2+} promotes the synthesis of adenosine triphosphate (ATP) that stimulates Krebs cycle enzymes and oxidative phosphorylation in the mitochondria, which leads to an increase in the metabolic rate and consequently ROS levels (Brookes *et al.*, 2004). When the membrane potential is high and ATP synthesis does not occur, Ca^{2+} uptake leads to a decrease in ROS production (Adam-Vizi and Starkov, 2010).

The detection of ROS in biological organisms depends on some type of indicator such as H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate). Its molecular structure is shown in Figure II.8. This compound reacts with the reactive oxygen species and becomes fluorescent which allows the capture of the generated signals. The greater the ROS production the greater the signal capture (Kalyanaraman *et al.*, 2012).

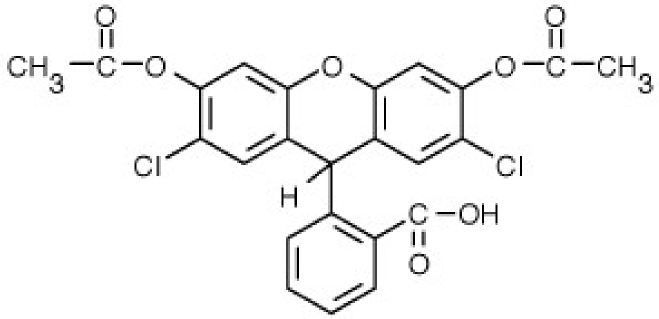

Figure II.8 – H_2DCFDA molecular structure.

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CHAPTER III – Photocatalysis Process

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Removal of pharmaceutically active compounds from synthetic and real aqueous mixtures and simultaneous disinfection by supported-TiO_2/UV-A, H_2O_2/UV-A and TiO_2/H_2O_2/UV-A processes

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ABSTRACT

Pharmaceutically active compounds are carried into aquatic bodies along with domestic sewage, industrial and agricultural wastewater discharges. Psychotropic drugs, which can be toxic to the biota, have been detected in natural waters in different parts of the world. Conventional water treatments such as activated sludge, do not properly remove these recalcitrant substances, so the development of processes able to eliminate these compounds becomes very important. Advanced oxidation processes are considered clean technologies, capable of achieving high rates of organic compounds degradation, and can be an efficient alternative to conventional treatments. In this study the degradation of alprazolam, clonazepam, diazepam, lorazepam and carbamazepine was evaluated through TiO_2/UV-A, H_2O_2/UV-A and TiO_2/H_2O_2/UV-A, using sunlight and artificial irradiation. While using TiO_2 in suspension, best results were found at $[TiO_2] = 0.1\ g \cdot L^{-1}$. H_2O_2/UV-A displayed better results under acidic conditions, achieving from 60 to 80% of removal. When WWTP was used, degradation decreased around 50 % for both processes, TiO_2/UV-A and H_2O_2/UV-A, indicating a strong matrix effect. The combination of both processes was shown to be an adequate approach, since removal increased up to 90 %.

H_2O_2/UV-A was used for disinfecting the aqueous matrices, while mineralization was obtained by TiO_2-photocatalysis.

Keywords: benzodiazepines; carbamazepine; supported-TiO_2; H_2O_2; wastewater; disinfection

III.1 Introduction

Pharmaceutical compounds have been detected in the aquatic environment worlwide, mostly in concentrations ranging from $\mu g \cdot L^{-1}$ to $ng \cdot L^{-1}$. Several sources of these contaminants exist, such as domestic sewage and industrial and agricultural wastewaters (Luo *et al.,* 2014; James *et al.,* 2016). Psychotropic drugs, a class of pharmaceutical compounds commonly used in psychiatric treatments, due to their muscular relaxant, sedative and antiepileptic activities (Sruthi *et al.,* 2013; Karpinska *et al.,* 2015; Shah *et al.,* 2015) have been detected in natural waters. Population growth, easier access to medicine and pharmaceutical industry growth contribute to an increasing intake of psychotropics worldwide (Song and Guo 2013; Meng et al. 2013; Miranda *et al.,* 2017). Jelic *et al.,* (2011) detected diazepam (DZP), lorazepam (LZP) and carbamazepine (CZP) in a wastewater treatment plant at concentrations up to 1000 $ng \cdot L^{-1}$ in the influent and up to 800 $ng \cdot L^{-1}$ in the affluent wastewater in Catalonia (Spain). Another study, carried out by Yuan *et al.,* (2013) in a wastewater treatment plant from a psychiatric hospital in Beijing (China), detected alprazolam (ALP) and LZP, respectively, at 30 and 294 $ng \cdot L^{-1}$ in the influent, and at 29 and 205 $ng \cdot L^{-1}$ after treatment. Alonso *et al.,* (2010) observed 1.1 $\mu g \cdot L^{-1}$ of CBZ in fresh waters in Madrid, while Baker and Kasprzyk-Hordern (2013) found DZP up to 8 $ng \cdot L^{-1}$ in influent and effluent streams of a domestic sewage treatment plant in England. The continuous release of these substances into the environment, contaminating surface water and groundwater, can affect the aquatic biota, even at low concentrations (Sangion and Gramatica 2016). Brodin *et al.,* (2017) tested oxazepam effects on the fish *Rutilus rutilus*, and observed that the fish became agitated when exposed.

Since these drugs cannot be completely degraded by conventional routes, such as activated sludge, there is a need for the development of processes able to eliminate these substances. Advanced oxidation processes (AOPs) can be an efficient alternative, since they are considered a clean technology capable of high rates of organic compound degradation (Luo *et al.,* 2014). In AOPs the main reactive species formed is the hydroxyl radical (•OH), which non-selectively attacks organic pollutants, possibly achieving complete mineralization (CO_2, H_2O and inorganic ions) (Ribeiro *et al.,* 2015). Some AOPs combine UV radiation with hydrogen peroxide (H_2O_2/UV) and/or titanium dioxide (TiO_2/UV) (Ferrando-Climent *et al.,* 2017). The TiO_2 catalyst is the most common semiconductor employed in heterogeneous photocatalysis, due to its non-toxicity, low cost, activation by sunlight, chemical stability in a broad pH range and the possibility of solid immobilization surfaces (Meribout *et al.,* 2016). A sufficient amount of energy is required to promote an electron from the valence band (VB) to the conduction band (CB), forming highly oxidative and reductive gaps (Equation III.1). The positive hole (h^+) formed in the VB can react with water, hydroxyl ions or organic compounds (Equations III.2, III.3 and III.4). The promoted electron in the CB can react with O_2, forming superoxide radical ions (Equation III.5), or with hydrogen peroxide, forming •OH (Equation III.6). The produced superoxide radical can react with hydrogen peroxide, again producing •OH (Equation III.7) (Bethi *et al.,* 2016; Garza-Campos *et al.,* 2016).

$$TiO_2 + hv \rightarrow e^- + h^+ \tag{III.1}$$

$$TiO_2(h^+_{VB}) + H_2O \rightarrow TiO_2 + \bullet\,\mathrm{OH} + H^+ \tag{III.2}$$

$$TiO_2(h^+_{VB}) + OH^- \rightarrow TiO_2 + \bullet\,\mathrm{OH} \tag{III.3}$$

$$TiO_2(h^+_{VB}) + RX \rightarrow TiO_2 + RX^{\bullet +} \tag{III.4}$$

$$TiO_2(e^-_{CB}) + O_2 \rightarrow TiO_2 + O_2^{\bullet -} \tag{III.5}$$

$$TiO_2(e^-_{CB}) + H_2O_2 \rightarrow TiO_2 + \bullet\,\mathrm{OH} + OH^- \tag{III.6}$$

$$H_2O_2 + O_2^{\bullet -} \rightarrow \bullet\,\mathrm{OH} + OH^- + O_2 \tag{III.7}$$

Another process that can be applied in the degradation of organic substances is H_2O_2/UV, where H_2O_2 dissociation leads to the formation of •OH (Equation III.8). However, excess H_2O_2 may act as a hydroxyl scavenger, generating hydroperoxide radicals (Equation III.9) (Boutiti *et al.,* 2017).

$$H_2O_2 + hv(254nm) \rightarrow 2^{\bullet}OH \tag{III.8}$$

$$H_2O_2 + \bullet\, OH \rightarrow HO_2^{\bullet} + H_2O \tag{III.9}$$

Several researchers have studied AOP applications in the degradation of pharmaceutically active compounds such as ALP, LZP, DZP and CZP (Calisto *et al.,* 2011; Martínez *et al.,* 2011; Haroune *et al.,* 2014; Mohapatra *et al.,* 2014; Carabin *et al.,* 2015; Tomi *et al.,* 2015; Finčur *et al.,* 2016), individually. However, there is still a shortage of studies on the degradation of psychotropic pharmaceutical compounds under conditions closer to reality, such as environmentally relevant concentrations and in a complex multiresidue aquatic matrix. There is also a lack of research on the coupling of these processes, as well as simultaneous evaluation of degradation and disinfection.

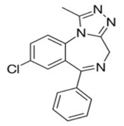
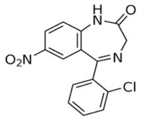
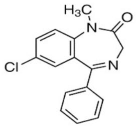
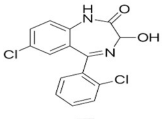
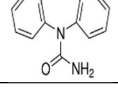
In this context, this study aimed to assess the degradation of the benzodiazepines ALP, CZP, DZP, LZP and the anti-epileptic drug CBZ, pharmaceutically active compounds (PhACs) commonly found in aquatic environment matrices. Degradation was evaluated through TiO_2/UV-A, H_2O_2/UV-A and TiO_2/H_2O_2/UV-A using natural sunlight and artificial irradiation. Different operational parameters that could affect PhAC degradation in supported-TiO_2 photodegradation and in different aquatic matrices were evaluated. Furthermore, the main photoreactive species involved was retrieved, as well as a simultaneous disinfection and degradation scenario.

III.2 Material and Methods

III.2.1 Chemicals and reagents

ALP, CZP, DZP, LZP and CBZ (Table III.1) were obtained from commercial sources and used as received.

Table III.1 - Physical and chemical PhACs characteristics

			Physical and chemical properties	
Name	**CAS number**	**Chemical structure**	**pK_a**	**$\log K_{ow}$**
Alprazolam	28981-97-7		2.4	2.12
Clonazepam	1622-61-3		1.5	2.41
Diazepam	439-14-5		3.4	2.82
Lorazepam	846-49-1		1.3	2.39
Carbamazepine	298-46-4		13.9	2.45

Powdered P25 TiO_2 (surface area = 50 $m^2.g^{-1}$; particle size = 20 nm) was supplied by Evonik. Hydrogen peroxide (30%) and acetonitrile were acquired from Merck and Tedia, respectively. Titanium (IV) butoxide (≥ 97%, Sigma–Aldrich), nitric acid (Merck, Germany), polyethylene glycol PEG600 (Sigma-Aldrich) and isopropanol (≥99.5%, Sigma–Aldrich) were used to immobilize the catalyst. Potassium iodide (MW=166 $g.mol^{-1}$) and Tiron (4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt) were supplied by Sigma-Aldrich. All standard solutions were prepared using ultrapure water (Milli-Q®, conductivity <0.1 $mS{\cdot}cm^{-1}$).

III.2.2 Wastewater Treatment Plant (WWTP) Effluent

An effluent sample (WWTP) was collected at the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil), from the secondary wastewater treatment plant Szachna Elias Cynamon, where institutional sewage is treated using activated sludge (extended aeration with denitrification) (medium flow rate = 512 m^3/day; sludge retention time = 18–30 days; hydraulic retention time = 16–24 h). The physical-chemical characterization was performed following APHA (1998) procedures, as displayed in Table III.2.

Table III.2 - Physical and chemical WWTP characteristics

Analyzed Parameter	
COD (mg L^{-1})	37.2
Inorganic carbon (mg L^{-1})	3.72
Total organic carbon (mg L^{-1})	37.14
Total carbon (mg L^{-1})	40.87
Total nitrogen (mg L^{-1})	17.18
Conductivity (µS cm^{-3})	506.0
pH	7.55
True color (uC)	58.0
Apparent color (uC)	262.0
Turbidity (NTU)	11.13
Dissolved oxygen (mg.L^{-1})	6.1
Alkalinity (mg L^{-1})	133.0
Total solids (mg L^{-1})	281.0
Total volatile solids (mg L^{-1})	121.0
Total fixed solids (mg L^{-1})	160.0
Cl^- (mg.L^{-1})	54.3
NO_3^- (mg.L^{-1})	1.8
F^- (mg.L^{-1})	0.6
PO_4^{-3} (mg.L^{-1})	26.4
SO_4^{-2} (mg.L^{-1})	28.4

III.2.3 Preparation of TiO_2 immobilized on glass beads

Glass spheres (φ = 5 mm) were prepared by washing as follows: (i) tap water, (ii) ultrapure water, (iii) sulfuric acid 0.27 N (T = 25°C; t = 30 min). In order to coat the glass spheres with TiO_2, a thin film was prepared using the sol–gel technique (Jiménez *et al.,* 2015). First, 1.8 L of distilled water were acidified with 13 mL of nitric acid under continuous magnetic stirring. Then, titanium (IV) butoxide (TTIB) was added to the solution and the suspension was stirred for 24 h until complete peptization. Once the sol was formed, a 20 mg L^{-1} solution of PEG600 was prepared in isopropanol and added to the TTIB sol under continuous magnetic stirring. The pH was adjusted to 2.4–2.5, and an adequate amount of P25 TiO_2 was incorporated, to obtain a final concentration of 10 $g{\cdot}L^{-1}$, by constant shaking. Finally, the glass spheres were dip-coated with the TTIB sol, dried at 110°C for 90 min and calcined at 400°C for 5 h (Saggioro *et al.,* 2014).

III.2.4 Irradiation experiments

III.2.4.1 Irradiation apparatus

The experiments were carried out in a batch magnetically stirred reactor (500 mL), irradiated by a polychromatic high-pressure 125 W mercury vapour lamp ($UV_{365\ nm}$ = 2.38 W m^{-2}) (Saggioro et al., 2015). Natural sunlight irradiated (UV_{365} nm = 2.4 $W.m^{-2}$) experiments were performed (using the same reactor) in Rio de Janeiro, Brazil (S 22° 52′ 37.3″; W 43° 15′ 0.9″) during the summer time (March, 2017, 11 a.m. to 2 p.m.), under clear sky conditions. The radiant flux at 365 nm in both cases was measured with a radiometer (Cole-Parmer Instrument Co; model 9811-50). The samples were collected at different reaction times, and, since the efficiency of the processes depends on the intensity of the UV-A radiation, PhACs degradation was expressed as a function of the accumulated UV-A energy in the reactor ($Q_{UV,n,}$ kJ L^{-1}) by applying Equation III.10 (Malato *et al.,* 2000):

$$Q_{UV(365nm),n} = Q_{UV(365\ nm),n-1} + \Delta t_n . UV_{G\ (365\ nm),n} . \frac{A_i}{V_T}\ ;\ \Delta t_n = t_n - t_{n-1} \qquad \text{(III.10)}$$

where t_n is the time corresponding to n water sample, V_T is the total reactor volume (0.5 L), A_i is the illuminated surface area (0.015 m^2) and $U_{VG,n}$ is the average UV-A (365 nm) measured during the period Δt_n.

III.2.4.2 Photodegradation set-up

PhACs (100 µg.L^{-1}) were spiked in ultrapure water and the WWTP effluent. The TiO_2/UV-A process was performed at different catalyst concentrations (0.1, 0.05 and 0.005 g L^{-1}) and under different pH conditions (3, 7 and 10). In addition, the TiO_2/UV-A process was performed using synthesized TiO_2 (TTIB) supported on glass beads (50 g) and TiO_2 in suspension (0.1 and 0.05 g L^{-1}). Furthermore, in order to determine the main species involved in the TiO_2 degradation, scavengers were added to the solution (Table III.3).

Table III.3 - Strategy followed to determine the main reactive species involved (Jiménez *et al.,* 2014).

Scavenger added	Reactive species	Evaluation strategy
2-propanol (0.14 mol L^{-1})	$^{\bullet}OH$	If degradation occurs, other species are involved (e^-, $O_2^{\bullet -}$/$HO_2^{\bullet}$, H_2O_2, h^+)
2-propanol (0.14 mol L^{-1}) and Tiron (3x10^{-5} mol L^{-1})	$^{\bullet}OH$ and $O_2^{\bullet -}$	If degradation occurs, other species are involved (e^-, H_2O_2, h^+)
2-propanol (0.14 mol L^{-1}) and KI (1.2x10^{-3} mol L^{-1})	$^{\bullet}OH$ and h^+	If degradation occurs, other species are involved (e^-, $O_2^{\bullet -}$/$HO_2^{\bullet}$, H_2O_2)

Before the irradiation experiments, all samples were kept in the dark for 30 min. H_2O_2/UV-A processes were performed using different H_2O_2 concentrations (250, 200, 50, 25 and 10 mg L^{-1}) and pH (3, 7 and 10). The TiO_2/H_2O_2/UV-A process was performed in the WWTP effluent with a TiO_2 concentration of 0.1 g L^{-1} and different concentrations H_2O_2 (100, 50 and 25 mg L^{-1}). The disinfection evaluation of the WWTP was carried out during the different studied processes. *Escherichia coli* and total coliform bacteria (TCB) were determined before, during, and after WWTP phototreatments. The experimental design is reported in Table III.4.

Table III.4 - Summarized conditions of the photodegradation experiments.

	TiO_2 P25 ($g.L^{-1}$)	**H_2O_2 ($g.L^{-1}$)**	**Water matrix**	**Compounds (100 $\mu g.L^{-1}$)**	**pH**	**Synthesized TTIB supported on glass beads (g)**	**Synthesized TTIB suspensions ($g.L^{-1}$)**
TiO_2/UV	0.1	-	Ultrapure	All	7	-	-
	0.05	-	Ultrapure	All	7	-	-
	0.005	-	Ultrapure	All	7	-	-
	0.05	-	Ultrapure	All	3	-	-
	0.05	-	Ultrapure	All	10	-	-
	0.01	-	Ultrapure	Alprazolam	7	-	-
	0.01	-	Ultrapure	Clonazepam	7	-	-
	0.01	-	Ultrapure	Diazepam	7	-	-
	0.01	-	Ultrapure	Lorazepam	7	-	-
	0.01	-	Ultrapure	Carbamazepine	7	-	-
	0.1	-	WWTP	All	7.55	-	-
	-	-	Ultrapure	All	3	50	-
	-	-	Ultrapure	All	7	50	-
	-	-	Ultrapure	All	10	50	-
	-	-	Ultrapure	All	7	-	0.1
	-	-	Ultrapure	All	3	-	0.05
	-	-	Ultrapure	All	7	-	0.05
	-	-	Ultrapure	All	10	-	0.05
UV/H_2O_2	-	250	Ultrapure	All	7	-	-
	-	200	Ultrapure	All	7	-	-
	-	50	Ultrapure	All	7	-	-
	-	25	Ultrapure	All	7	-	-
	-	10	Ultrapure	All	7	-	-
	-	200	Ultrapure	All	3	-	-
	-	200	Ultrapure	All	10	-	-
	-	100	WWTP	All	7.55	-	-
TiO_2/H_2O_2/UV	0.1	100	WWTP	All	7.55	-	-
	0.1	50	WWTP	All	7.55	-	-
	0.1	25	WWTP	All	7.55	-	-
TiO_2/UV Solar light	0.1	-	Ultrapure	All	7	-	-
TiO_2/UV (2-propanol)	0.1	-	Ultrapure	All	7	-	-
TiO_2/UV (2-propanol and Tiron)	0.1	-	Ultrapure	All	7	-	-
TiO_2/UV (2-propanol and KI)	0.1	-	Ultrapure	All	7	-	-

III.2.4.3 Analytical determinations

High-performance liquid chromatography (HPLC-UV/DAD - Series, 1200 Agilent Technologies) was used to determinate ALP, CZP, DZP, LZP and CBZ concentrations, at 224, 310, 229, 232 and 286 nm, respectively. A C8 analytical column (Poroshell 120 PFP 3 x 150 mm, 4 µm) was used, maintained at 25ºC. A linear gradient from 40:60 acetonitrile:water (t=0) to 82:18 acetonitrile:water (t = 7 min) was applied, at a flow rate of 0.7 mL min^{-1}. The limits of quantification (LOQ) for ALP, CZP, DZP, LZP and CBZ were 8.8, 24.81, 9.21, 6.39 and 6.38 µg L^{-1}, respectively, and the limits of detection (LOD) for each compound was 2.64, 7.44, 2.76, 1.91 and 1.91 µg L^{-1}. Before the HPLC analysis, the samples were filtered (0.2 µm) with nylon filter supplied by Analitica.

Total coliform bacteria (TCB) and *E. coli* were quantified by the Colilert method as described by the USEPA (1978). TOC determinations were carried out in filtered samples (Nylon 0.2-µm filters) with a Shimadzu-TOC-V SCN analyzer. Total suspend solid (TSS) were determined by the gravimetric method. Turbidity was measured by the nephelometric method using a portable turbidimeter HACH (2100P/1991-1998). A colorimetric method with closed reflux was employed for the chemical oxygen demand (COD) determinations. pH was measured with a digital pHmeter (Marte MB-10). Anions were analyzed by ion chromatography (Metrohm Personal IC Model 2.790.010 pre column Metrosep A Supp 4/5 Guard 4 and Metrosep A Supp 5 150/4.0 column). Hydrogen peroxide was analyzed by the spectrophotometric method using ammonium metavanadate (Jiménez *et al.,* 2011) .

III.3. Results and discussion

III.3.1. PhACs photocatalysis by TiO_2 P25

III.3.1.1. Effect of TiO_2 concentrations

No adsorption or photolysis was observed (Fig. III.1A and III.1B), in agreement with previous findings (Carlson *et al.,* 2015; Rozas *et al.,* 2016; Alharbi *et al.,* 2017).

PhACs removal increased with increasing TiO_2 concentrations (Fig. III.2A), as has been reported out previously for LZP and CBZ, individually (Sousa *et al.,* 2013; Georgaki, Vasilaki and Katsarakis, 2014; Tokumura *et al.,* 2016). A removal close to 85% was observed when applying 100 mg L^{-1} of TiO_2 after 10 min and Q_{uv} = 0.04 kJ L^{-1}. This phenomenon was expected, since increasing TiO_2 concentrations lead to increased availability of active sites, improving compound degradation (Garza-Campos *et al.,* 2016). However, this effect is limited, since the concentration of the photocatalyst can be high enough to increase turbidity, decreasing light transmission and, consequently, TiO_2 activation (Georgaki, Vasilaki and Katsarakis, 2014), as observed by Martínez *et al.,* (2011) for CBZ using TiO_2 at 500 mg L^{-1}.

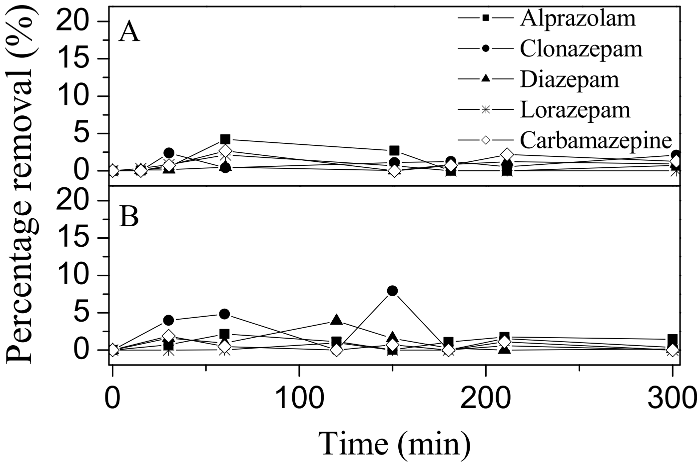


Figure III.1 - Effect of PhACs adsorption and photolysis: (A) $[TiO_2]$ = 100 mg L^{-1}; (B) artificial irradiation. T = 25 ºC; pH 7.

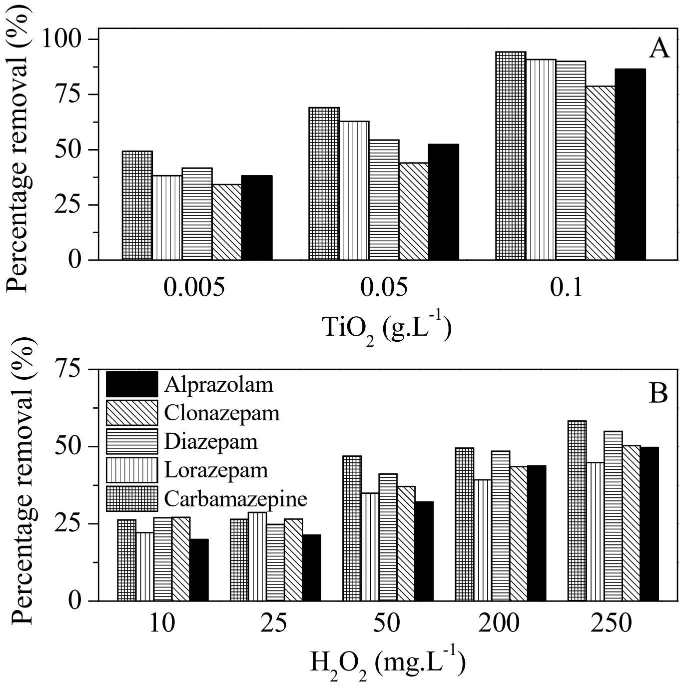


Figure III.2 - PhACs photodegradation in ultrapure water. (A) Effect of TiO_2 concentrations (Q_{uv} =0.04 kJ L^{-1}) and (B) H_2O_2 concentrations (Q_{uv} = 0.6 kJ L^{-1}). T = 25 ºC; pH=7.

The lowest degradation rate was achieved by CZP. On the other hand, CBZ displayed the highest degradation rate for all evaluated TiO_2 concentrations. Degradation depends not only on the catalyst, but also on the nature and chemical structure of the target compound (Gaya and Abdullah 2008). CBZ has the highest pKa (13.9) among the tested compounds (pKa = 1.3 - 3.4), which could be the reason for its higher degradation when compared to the other evaluated compounds (Molinari *et al.,* 2006; Haroune *et al.,* 2014). In addition, CBZ does not possess chlorinated groups (Table 1). Thus, oxidation reactions occur by hydrogen abstraction and electrophilic addition, whereas benzodiazepine compounds possess one or two chlorinated groups, and electron transfer is the primary oxidation reaction (Nakata and Fujishima 2012; Pelaez *et al.,* 2012).

III.3.1.2 Effect of pH

pH plays an important role in the photocatalytic process, affecting the interaction between the photocatalyst and the target compounds. The point of zero charge (pzc) of the applied TiO_2 (P25) is 6.5, which means that at pH < 6.5 the TiO_2 surface is positively charged, and at pH > 6.5 is negatively charged, as illustrated by Equations III.11 and III.12 (Chong *et al.,* 2010).

$$pH < pzc \rightarrow TiOH + H^+ \leftrightarrow TiOH_2^+ \qquad \text{(III.11)}$$

$$pH > pcz \rightarrow TiOH + OH^- \leftrightarrow TiO^- + H_2O \qquad \text{(III.12)}$$

Experimental runs were carried out at different pH values (3, 5 and 10) (Fig. III.3A) using 0.5 mg·L^{-1} of TiO_2.

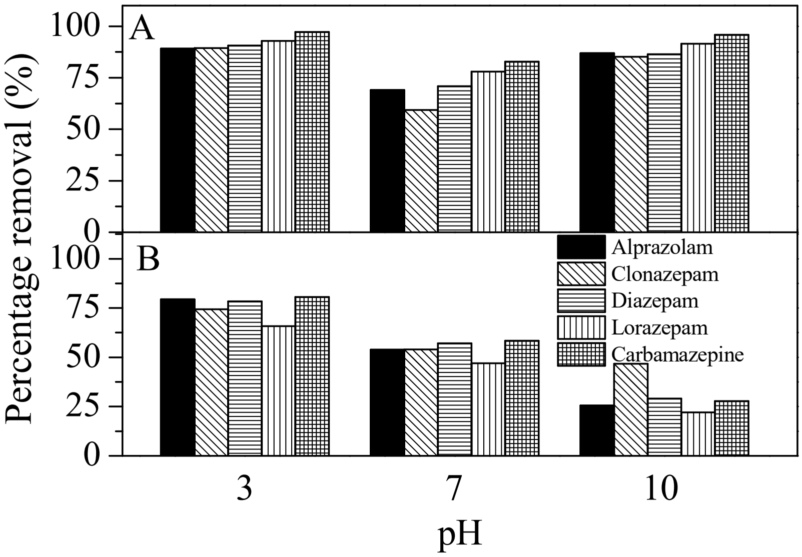


Figure III.3 - Effect of pH (3, 7 e 10) during PhACs photodegradation. (A) TiO_2-photocatalysis ([TiO_2] = 50 mg L^{-1} and Q_{uv} = 0.07 kJ L^{-1}); (B) H_2O_2/UV ([H_2O_2] = 200 mg L^{-1} and Q_{uv} = 0.7 kJ L^{-1}). T= 25 ºC.

At pH 3 and 10, degradation was approximately 90%, while, at pH 7, degradation was much lower, and mainly CZP was removed (60%). This behavior can be explained not only by the TiO_2 pzc, but also by the pKa of the studied compounds. At pH 7, TiO_2

is practically neutral, since it is close to the isoelectric point of the compound, which decreased the interaction between TiO_2 and the PhACs, affecting degradation, which is lower than at pH 3 or 10 (Das and Basu *et al.,* 2015).

III.3.1.3 Effect of UV radiation source

Fig. III.4 displays the higher degradation observed when sunlight radiation is used instead of the UV-A lamp. CBZ, ALP, and LZP were degraded in 6 min (0.03 kJ L^{-1}), whereas CZP and DZP were removed after 12 min (0.06 kJ L^{-1}) under sunlight radiation (Fig. III.4A). On the other hand, when artificial irradiation was applied, the removal of studied compounds was almost the same in 15 min (0.07 kJ L^{-1}). Sousa et al. (2013) also observed more efficient LZP degradation by sunlight radiation.

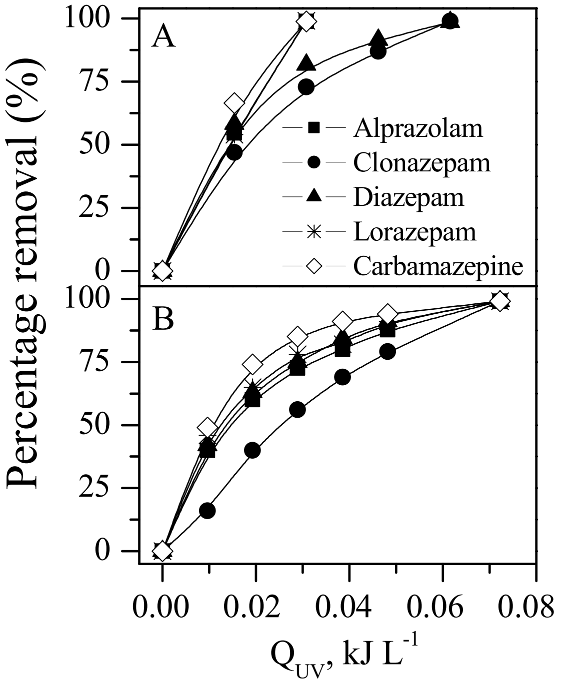


Figure III.4 - Effect of UV-A radiation source on PhACs TiO_2 photocatalysis: (A) sunlight radiation; (B) artificial irradiation. [TiO_2] = 100 mg L^{-1}; T = 25 ºC; pH 7.

III.3.2. PhACs photocatalysis by the TiO_2-supported process

TiO_2 was synthesized and immobilized onto glass spheres, and degradation was followed at different pH (3, 7, and 10). Previous studies (Miranda-García *et al.,* 2010, 2011; Jiménez *et al.,* 2015) have demonstrated that TiO_2 supported onto glass spheres displays durability, repeatability, and photoactivity. Those studies employed the same synthesized method and characterized by X-ray diffraction (XRD), thermogravimetric analysis (TGA), surface area determination (BET), and scanning electronic microscopy (SEM) analyses. Herein, degradation was performed using the synthesized TiO_2 both in suspension (Fig. III.5A) and immobilized (Fig. III.5B). The highest percentage removal (< 85 %) was achieved at pH 3 using the TiO_2 in suspension, while the highest degradation for immobilized TiO_2 was at pH 7 (60 – 90%). The efficiency of the synthesized TiO_2 was lower than the commercial P25. This is probably due to lower superficial area, larger particle size of synthesized TiO_2 (BET area 53 $m^2\ g^{-1}$, unpublished data), when compared to commercial TiO_2 (BET area 50 $m^2\ g^{-1}$), which can explain its low performance, affecting the production of hydroxyl radicals (Lee, Kim and Kim, 2004; Léonard *et al.*, 2016).

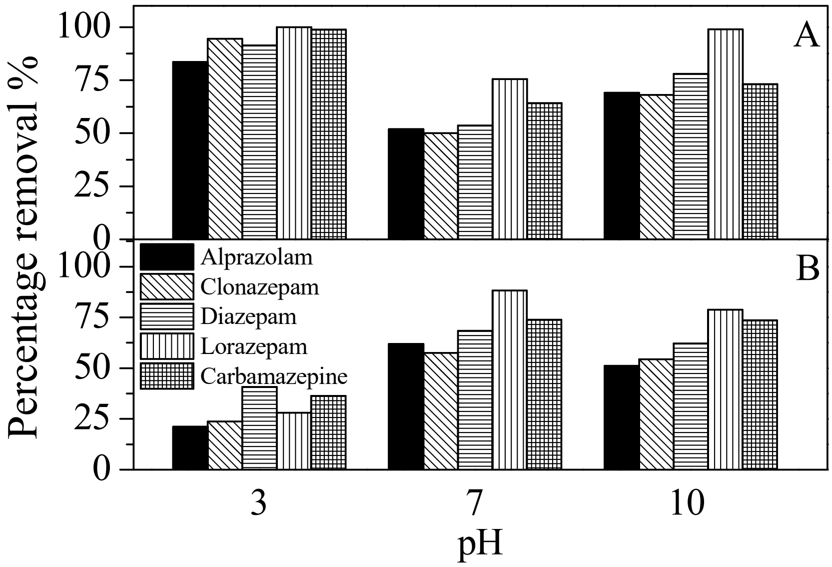


Figure III.5 - Effect of pH on PhACs TiO_2 photocatalysis using synthesized TiO_2: (A) in suspension (100 $mg \cdot L^{-1}$) and (B) immobilized onto glass spheres. t = 120 min; $Q_{uv} = 0.6$ $kJ\ L^{-1}$; T = 25 ºC.

For the immobilized TiO_2 system to be as efficient as the TiO_2 in suspension, a longer exposure time is required. A decrease in 60-70% of performance was observed when using immobilized TiO_2 in comparison to suspended TiO_2 (Kabra, Chaudhary and Sawhney, 2004). Immobilized TiO_2 requires more accumulated energy to archive satisfactory degradation. However, it is possible to use sunlight as an irradiation source (topic 3.1.3) and, in large-scale, immobilized TiO_2 eliminates the catalyst removal step, reducing treatment costs (Saggioro *et al.,* 2014).

III.3.3 Influence of photogenerated species

Different quenchers were used with TiO2 P25 suspensions, namely 2-propanol ($^{\bullet}OH$ scavenger), Tiron ($O_2^{\bullet-}$ scavenger) and potassium iodate (h^+ scavenger), in order to identify the roles of the different radicals produced during photodegradation, as displayed in Fig. III.6 (Zhao *et al.,* 2013; Jiménez *et al.,* 2015). Low or no degradation of AZP, DZP and CBZ in the presence of the $^{\bullet}OH$ scavenger (2-propanol) was observed, while CZP and LZP were degraded in about 60 and 40%, respectively. When $O_2^{\bullet-}$ is also suppressed, along with $^{\bullet}OH$, all PhACs were affected, displaying degradation lower than 10%, apart from CZP, which presented degradation close to 40%. In the last case, h^+ along with $^{\bullet}OH$, was quenched, and, in this case AZP, DZP and CBZ were the most affected, probably due to $^{\bullet}OH$ suppression, observed when only 2-propanol was added. LZP degradation (50 %) was lower than when only 2-propanol was used, indicating a small h^+ contribution. CZP displayed a more pronounced decrease in degradation (25%) when h^+ was quenched, along with $^{\bullet}OH$. As observed in Fig. III.6, the main reactive species that degrades AZP, DZP and CBZ is $^{\bullet}OH$, while $^{\bullet}OH$ and $O_2^{\bullet-}$ degraded LZP. CZP displayed inhibition towards all scavengers ($^{\bullet}OH$, $O_2^{\bullet-}$, h^+) but $^{\bullet}OH$ and h^+ demonstrated greater contribution.

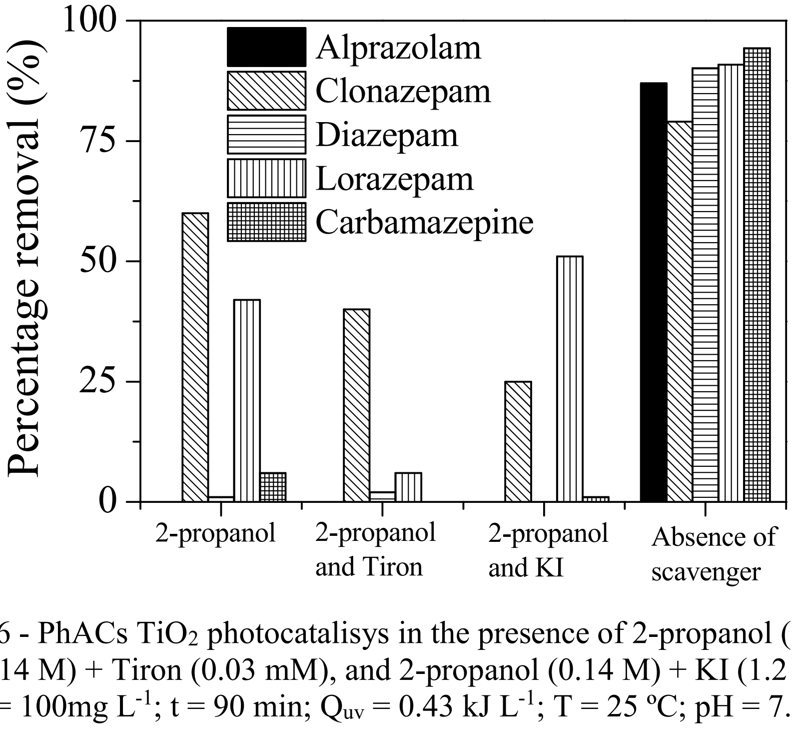


Figure III.6 - PhACs TiO_2 photocatalisys in the presence of 2-propanol (0.14 M), 2-propanol (0.14 M) + Tiron (0.03 mM), and 2-propanol (0.14 M) + KI (1.2 mM). $[TiO_2]$ = 100mg L^{-1}; t = 90 min; Q_{uv} = 0.43 kJ L^{-1}; T = 25 ºC; pH = 7.0

III.3.4 PhACs photodegradation by H_2O_2/UV

III.3.4.1 Effect of H_2O_2 concentrations

H_2O_2 concentrations affects the degradation of all PhACs (Fig. III.2B), with the same pattern observed for all concentrations of the oxidant. CBZ displayed the highest degradation. However, a limitation for concentrations higher than 50 mg·L^{-1} was detecetd, since the degradation does not follow the increases in H_2O_2 concentrations. Despite increasing the oxidant concentration from 50 mg·L^{-1} to 250 mg L^{-1}, degradation increased in only 10%. This limitation is due to the fact that, at high H_2O_2 concentrations, there is also the consumption of $^\bullet OH$ (E_0 = + 2.80V) by the oxidant (Equation 9), due to the formation of the hydroperoxide radical (e.g. $HO^\bullet_2$; E_0 = + 1.42V), decreasing the process efficiency (Alaton and Balcioglu 2001; Schrank *et al.,* 2005; Chong *et al.,* 2010).

III.3.4.2 Effect of pH

The degradation of PhACs was followed at pH 3, 7, and 10 using 200 mg L^{-1} of H_2O_2 (Fig. III.3B). A faster degradation at acidic pH was observed. In this condition (pH = 3), AZP, CZP, DZP, and CBZ displayed around 80% removal, while LZP was removed up to 65%. At pH 7, AZP, CZP, DZP, and CBZ were removed close to 60 %, while, LZP was degraded by 45 %. On the other hand, at alkaline conditions (pH = 10), CBZ achieved the highest removal (43%), whereas AZP, CZP, DZP, and LZP degradation ranged between 20 and 30%. The lower degradation at basic pH is in accordance to Yang *et al.,* (2017) and Lachheb *et al.,* (2017), explained by H_2O_2 decomposition (Equation III.13) in basic media, 7-fold higher than in acid media and about 3-fold times higher than in neutral media.

$$2H_2O_2 \rightarrow 2H_2O + O_2 \qquad \text{(III.13)}$$

III.3.5 Photodegradation in WWTP

The photodegradation of the WWTP was performed using TiO_2/UV-A, H_2O_2/UV-A and TiO_2/H_2O_2/UV-A (Fig. III.7). When TiO_2/UV-A was used (Fig. III.7A), CBZ displayed the highest percentage removal (30%) and AZP, the lowest (15%). CZP showed the highest degradation (30%), while the other removals were around 20% when

H_2O_2/UV-A was used (Fig. III.7B). WWTP causes a matrix effect on the degradation of the studied PhACs, which decreases the efficiency of the processes. The matrix effect is caused by scavengers present in the WWTP, such as dissolved organic matter (DOC), either natural or anthropogenic, carbonates, and anions. These compounds react with the formed radicals, preventing the reaction with the target PhACs (Bautitz and Nogueira 2010; He *et al.,* 2016). Furthermore, anions can compete for the absorption site on the catalyst, or still, modify the superficial charge, interfering with the degradation efficiency (Jallouli et al. 2018). Moreover, natural waters are enriched with DOC, such as humic acids, and nitrates, which absorb UV light, preventing the activation of TiO_2 and H_2O_2 for •OH generation (Chiron *et al.,* 2006; Rosario-Ortiz *et al.,* 2010).

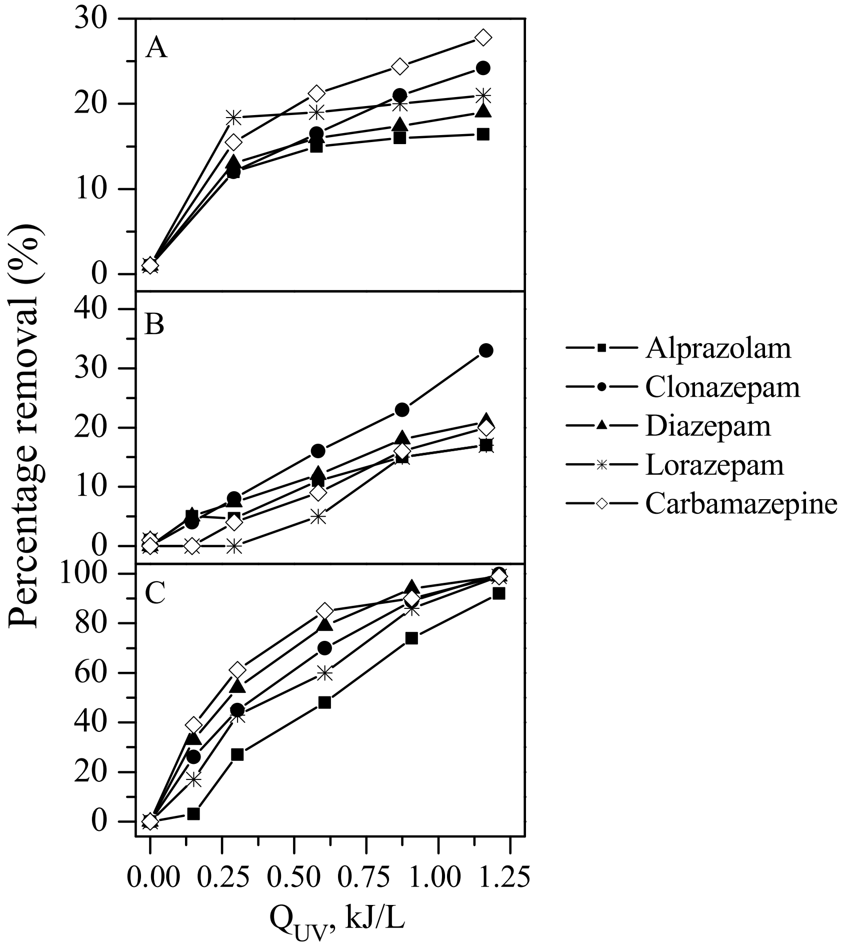


Figure III.7 - PhACs degradation in WWTP. (A) TiO_2/UV; (B) H_2O_2/UV; (C) TiO_2/H_2O_2/UV. [TiO_2] = 100 mg L^{-1}; [H_2O_2] = 100 mg L^{-1}; t = 240 min; pH = 7.55; T = 25 ºC.

Conversely, the combined process, TiO_2/H_2O_2/UV-A, was able to achieve complete degradation of the studied compounds, apart from AZP, whose degradation was close to 90% (Fig. III.7C). The combination of TiO_2/UV-A and H_2O_2/UV-A enhanced the degradation due to the reaction between H_2O_2 on the TiO_2 surface. H_2O_2 is absorbed and acts as an electron acceptor, which prevents the electron-hole recombination, promoting superoxide formation and increasing $^\bullet OH$ via H_2O_2 photodecomposition (Equation III.8), increasing degradation efficiency (Auguliaro *et al.,* 1990; Li, Chen and Zhao, 2001).

III.3.6. Disinfection assessment

The disinfection (fecal coliforms and *E. coli*) in WWTP during the photodegradation of the evaluated PhACs using TiO_2/UV-A e H_2O_2/UV-A was assessed. Disinfection occurs due to the reaction between the radicals formed during the photoprocesses and the plasmatic membrane of bacteria, which is fragmented, killing the cells. The results are displayed in Table III.5, and it is possible to observe that the TiO_2/UV-A process was not able to perform the disinfection, while H_2O_2/UV-A achieved satisfactory results. Considering that many studies report the TiO_2/UV-A process as an efficient disinfection method (Gelover *et al.,* 2006; Paleologou *et al.,* 2007), the exposure time might be one of the reasons for the fact that TiO_2 was unable to deactivate microorganisms in this study. In accordance to the obtained results (Fig. III.2 A and III.8), the TiO_2/UV-A process displayed a higher efficiency in promoting PhACs degradation in comparison to H_2O_2/UV-A. On the other hand, H_2O_2/UV-A demonstrated greater efficiency for disinfection. Thus, the combination of TiO_2/H_2O_2/UV-A processes could be an alternative for simultaneous degradation and disinfection.

Table III.5 - Fecal coliforms and *E. coli* disinfection during PhACs photodegradation in WWTP by TiO_2/UV and H_2O_2/UV. $[TiO_2] = 100$ mg L^{-1}; $[H_2O_2] = 200$ mg L^{-1}, pH 7.55, temperature 25 ºC.

Process	**Microorganisms**	**Accumulated energy (W.m^{-2})**					
		0	**0.3**	**0.6**	**0.9**	**1.2**	**1.5**
TiO_2/UV	**Fecal coliforms**	< 2419.6	< 2419.6	< 2419.6	< 2419.6	< 2419.6	< 2419.6
	E. coli	< 2419.6	< 2419.6	< 2419.6	< 2419.6	< 2419.6	< 2419.6
UV/H_2O_2	**Fecal coliforms**	< 2419.6	< 2419.6	< 2419.6	< 124.6	< 1	< 1
	E. coli	< 2419.6	< 2419.6	< 2419.6	< 30.1	< 1	< 1

III.3.7 Mineralization evaluation

Mineralization occurs when the degradation is complete, which means the organic compounds were degraded to CO_2 and H_2O. It is common to observe only partial mineralization, meaning there are still organic molecules in the solution, by-products from the degradation of the parent compounds. Despite the observed degradation of all the studied compounds when H_2O_2/UV-A was applied, mineralization did not occur (Fig. III.8), in both ultrapure water and WWTP. On the other hand, the TiO_2/UV-A process displayed 80% and 12% of mineralization in ultrapure water and WWTP, respectively (Fig. III.8). A lower mineralization in WWTP is expected, due to its complex matrix.

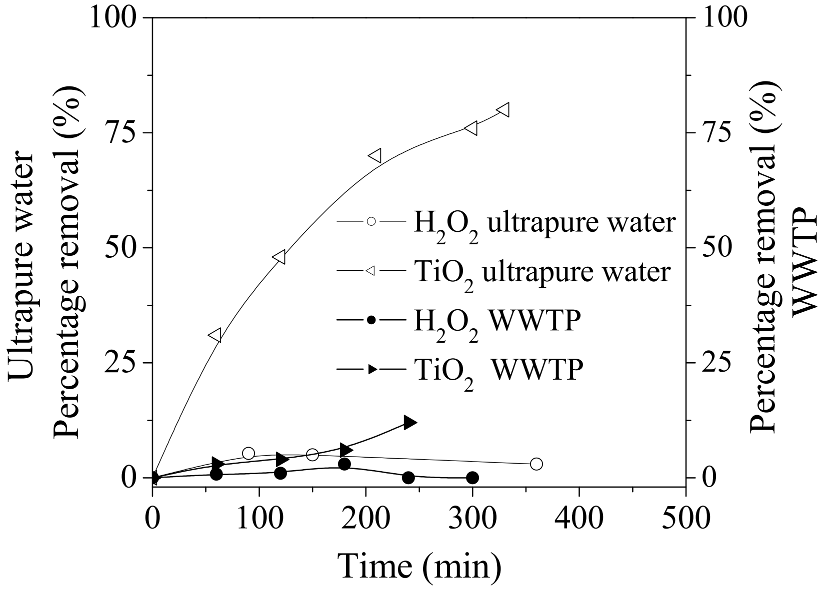


Figure III.8 - TOC rate during PhACs photodegradation in ultrapure water and WWTP by TiO_2/UV and H_2O_2/UV. $[TiO_2] = 100$ mg L^{-1}; $[H_2O_2] = 200$ mg L^{-1} pH = 7.55, T = 25 ºC.

III.4 Conclusions

Increasing TiO_2 concentrations led to higher PhACs degradation, although higher amounts of H_2O_2 in the solution did not promote significant variation in the degradation rate, indicating that excess H_2O_2 affects the process. The TiO_2/UV-A process is more efficient under acidic and alkaline conditions, while degradation is lower under neutral pH, due to the proximity to TiO_2 pHpzc. For the H_2O_2/UV-A process, acidic pH enhanced the degradation efficiency. Photolysis was not observed, while photodegradation under solar light was more efficient than photodegradation by artificial light. The photodegradation process using synthesized TiO_2 was more efficient under acidic pH, while results obtained with immobilized TiO_2 were similar in both neutral and alkaline conditions. The degradation of the evaluated compounds in WWTP was much less efficient, since other compounds present in the wastewater compete for the radicals formed in the process. Disinfection occurred in the H_2O_2/UV-A process, whereas mineralization ensued in the TiO_2/UV-A process. For simultaneous disinfection and mineralization to occur during treatment, the TiO_2/H_2O_2/UV-A process could be employed. However, this process must be further investigated.

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CHAPTER IV – Electrochemical Oxidation (Ti/Pt) - 1

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Neurotoxicity and Phytotoxicity of Residual Pharmaceutical Compounds from Electrochemical Oxidation Using a Platinum-Coated Titanium Anode

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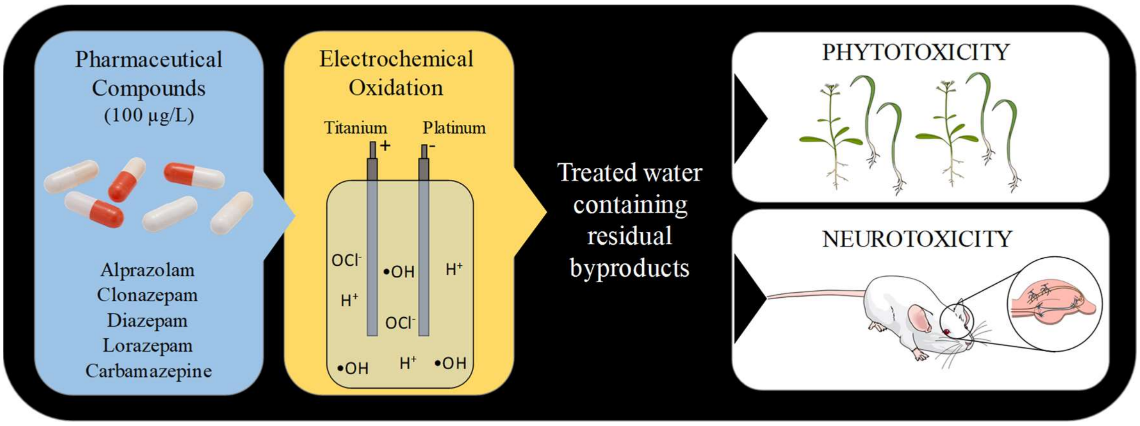
ABSTRACT

Contaminants of emerging concern (CECs) have been released daily into surface water and their recalcitrant properties often require a tertiary treatment. Advanced oxidation processes (AOPs) are often used as an alternative way to eliminate these compounds from water, but literature barely addresses phyto- and neuro-toxicological effects of AOPs residual byproducts. Here we investigated (i) the performance of electrochemical oxidation (EO) to degrade a mixture of five CECs (alprazolam, clonazepam, diazepam, lorazepam and carbamazepine) with initial concentration of 100 $\mu g\ L^{-1}$ and (ii) the neurotoxicity and phytotoxicity of the residual EO byproducts. Different current densities (13-75 $A\ m^{-2}$) and pH values (3-10) were tested and the best EO condition achieved using synthetic wastewater was also investigated with real aqueous matrices (surface water and municipal wastewater). The neurotoxicity of the EO treated solution was assessed investigating the reactive oxygen species (ROS) production in hippocampal slices of the Wistar rat brain. Seed germination of *Lepidium sativum* method was used to verify the phytoxocicity of treated solution containing the residual pharmaceutical compounds. As a result, the drugs were successfully degraded by EO process and concentrations below the limit of detection were reached after 5 min of reaction, at current density of 75 A m^{-2}. Neutral pH demonstrated a better performance in terms of drugs removal if compared to acid and alkaline conditions. In the tests using municipal wastewater, the degradation of the compounds reached 40% after 120 min of reaction. No significant changes were observed in the morphology of the anode material, even after several experiments. Neurotoxicity studies showed that for both real matrices, the treated solutions provoked a decrease in the reactive oxygen species (ROS) signals. However, when the artificial cerebrospinal fluid (ACSF) medium was reapplied, the signal recovered and increased to a value above baseline, indicating that the cells recovered part of their normal activity but remained in a different condition. Phytotoxicity assays revealed effects of residual benzodiazepines and carbamazepine on seed germination when real water was used and the phytotoxicity was not reduced by electrochemical oxidation.

Keywords: Advanced oxidation processes; benzodiazepines; carbamazepine; neuronal activity; ROS indicator; *Lepidium sativum.*

Graphical Abstract



IV.1 Introduction

The indiscriminate use of synthetic substances has brought a series of negative effects to the aquatic ecosystems worldwide. The contaminants of emerging concern (CECs), a category of non-regulated pollutants that includes pharmaceuticals and personal care products (PPCPs), endocrine-disrupting compounds (EDCs), pesticides, disinfection by-products and other substances at very low levels, have potential to cause adverse effects on the environment and on animal and human health. CECs are constantly being released into the environment in large quantities and many of them are persistent, lipophilic, bioaccumulative and have biological effects. Some CECs are partially metabolized by the human body and, as a consequence, the metabolites enter into the environment in concentration levels ranging from mg L^{-1} to ng L^{-1} (Puckowski *et al.*, 2016), sufficient to be a risk to the biota.

Among the CECs, a group of particular concern is formed by the psychoactive drugs. The consumption of anxiolytics and anticonvulsants has increased considerably in the last years and several ecological effects have been reported, such as anxiolytic-like effects in animals as rodents and fish (Bourin *et al.*, 2007; Brodin *et al.*, 2014) and the increased migration behaviour in salmon (Brodin *et al.*, 2017). The constant detection of these pollutants in wastewater treatment plant effluents (Kot-Wasik, Jakimska and Śliwka-Kaszynska, 2016; Archer *et al.*, 2017; Yang *et al.*, 2017) suggests the inefficacy of conventional treatment processes in removing CECs (Kummu *et al.*, 2016). Some substances are not degraded in conventional processes due to their recalcitrant characteristics and complex structures (López-serna, Petrovi and Barceló, 2012; Paíga *et al.*, 2016). The recalcitrant nature of benzodiazepines suggests that advanced treatments processes are necessary to degrade these compounds (Kosjek *et al.*, 2012). Several anxiolytic and anticonvulsant drugs have been detected not only in rivers and surface waters (for example, carbamazepine was detected in rivers of India (Ramaswamy *et al.*, 2011), diazepam/carbamazepine in rivers of Kenya (K'oreje *et al.*, 2016), and alprazolam/diazepam in rivers of Europe (Fick *et al.*, 2017)), but also in municipal wastewater treatment plants (MWWTPs) at concentration levels ranging from ng L^{-1} to µg L^{-1} (Papageorgiou, Kosma and Lambropoulou, 2016; Afonso-Olivares, Sosa-Ferrera and Santana-Rodríguez, 2017).

Advanced Oxidation Processes (AOPs) are a set of technologies based on the *in situ* reactive oxygen species (ROS) generation. ROS are atoms or molecules that contain at least one unpaired electron such as hydroxyl radical (•OH), superoxide radical ($O_2^{\bullet-}$) and hydroperoxyl radical ($HO_2^{\bullet}$). These species have the capacity to degrade complex substances due to their high oxidation potential (Wang and Xu, 2012). The AOPs lead to the formation of •OH, a non-selective and highly reactive radical, which can oxidize refractory micropollutants (R) according to Eq. IV.1. After this reaction, the organic compounds become unstable and can react with the molecular O_2 generating the peroxyl radical (Eq. IV.2) or may be capable of direct electron transfer (Eq. IV.3),which can produce oxidized intermediates or even the complete mineralization of the compounds in CO_2, H_2O and inorganic acids (Kanakaraju, Glass and Oelgem, 2018; Tayo *et al.*, 2018).

$$HO^{\bullet} + R \rightarrow M^{\bullet} + H_2O \qquad \text{(IV.1)}$$

$$R^{\bullet} + O_2 \rightarrow RO_2^{\bullet} \qquad \text{(IV.2)}$$

$$HO^{\bullet} + RX \rightarrow RX^{\bullet +} + HO^{-} \qquad \text{(IV.3)}$$

Electrochemical oxidation (EO) is a treatment within AOPs where the organic degradation may occur through (i) direct oxidation, where the compounds are oxidized directly on the anode surface by direct charge transfer and (ii) indirect oxidation, where the compounds are attacked in the bulk by the radicals produced in the anode surface (Ganzenko *et al.*, 2014). Usually, •OH, ozone (O_3) and hydrogen peroxide (H_2O_2) are the main oxidizing agents formed in the indirect oxidation and, depending on the type of electrolyte, other reactive species can contribute to the degradation of the organic compounds. For example, when using NaCl as electrolyte, the production of active chlorine species is strongly pH dependent (Thiam et al 2015). In acidic environment, the main species produced in the anode is the Cl_2 (Eq. IV.4), while at high pH values the HClO (Eq. IV.5) and ClO^- (Eq. IV.6) are the predominant species (Deborde and Gunten, 2008; Anglada, Urtiaga and Ortiz, 2009; Baddouh *et al.*, 2018). These species may form organochlorine compounds from the reaction with organic compounds and by the process of electrochemical oxidation, organochlorine compounds may often be persistent, bioaccumulative and more toxic than the starting compounds (Jayaraj, Megha and Sreedev, 2016). Electrochemical oxidation process is a polishing treatment widely used in several WWTPs, therefore the study of EO by-products toxicity and their effects on animal and human health is essential.

$$Cl^{-} \rightarrow \frac{1}{2}Cl_2 + e^{-} \qquad \text{(IV.4)}$$

$$Cl_2 + H_2O \overset{K_{Cl2}}{\longleftrightarrow} HClO + H^{+} + Cl^{-} \qquad \text{(IV.5)}$$

$$HClO \overset{K_{HClO}}{\longleftrightarrow} OCl^{-} + H^{+} \qquad \text{(IV.6)}$$

Converging lines of research suggest that the hippocampus has a role in many psychological disturbances, like major depressive disorder (MDD). The production of ROS inside the cells is necessary for all animals to survive, however when exposed to adverse situations a change in ROS production may occur. This phenomenon known as 'oxidative stress' may affect ROS production inside the cells, more precisely in the mitochondria, and lead to irreversible damages (Lenaz, 1998; Shokolenko *et al.*, 2009; Bratic and Trifunovic, 2010). Therefore, an alternative way to investigate the influence of anxiolytics and anticonvulsants in ROS production is through neurotoxicity tests, where rat hippocampal slices are placed in contact with these drugs (Sena and Chandel, 2013; Murphy *et al.*, 2015; Brand, 2016).

Another approach to investigate the effects of CECs is through phytotoxicity tests. Considering the negative effects that anxiolytics and anticonvulsants may bring to the environment, a rapid way to identify temporary or long-term effects of these pharmaceutical compounds in plants is through the seed germination test. The phytotoxicity is determined by color variation, root deformation and changes in seed germination in relation to a control group not exposed to the target compounds. This test is relatively quick, easy and inexpensive (Walter, Martı and Cala, 2006; Nalcaci, Ozgen and Ovez, 2010; Pinho *et al.*, 2017).

Electrochemical oxidation has been extensively reported for the degradation of CECs using synthetic effluents (Vieira *et al.*, 2013; Singh *et al.*, 2016; Jager *et al.*, 2018; Loos *et al.*, 2018; Siedlecka *et al.*, 2018) and real wastewater (Woisetschläger *et al.*, 2013; Lan *et al.*, 2017; Sánchez-sánchez *et al.*, 2018). Literature data on the use of EO for a specific contaminant removal in water is extensive, however studies using a mixture of drugs and real wastewater are scarce. As far as we know, there are no reports on the neurotoxicity and phytotoxicity effects of EO by-products using real matrices.

In this study, the electrochemical oxidation process was applied to remove four benzodiazepines (anti-anxiety drugs) and carbamazepine (antiepileptic drug) in three different water matrices: ultrapure water, surface water and municipal wastewater. The effect of different process variables such as pH (3-10) and current density (13-75 A m^{-2}), was assessed with ultrapure water and the best conditions were analysed with the real matrices. The toxicity of EO treated water was evaluated through phytotoxicity and neurotoxicity tests. For this, ROS production in hippocampal slices of the Wistar rat brain and seed germination of *Lepidium sativum* were assessed.

IV.2 Material and Methods

IV.2.1 Chemicals and reagents

Alprazolam (ALP, MW: 308.76 g mol^{-1}), clonazepam (CZP, MW: 315.71 g mol^{-1}), diazepam (DZP, MW: 284.74 g mol^{-1}), lorazepam (LZP, MW: 321.16 g mol^{-1}) and carbamazepine (CBZ, MW: 236.27 g mol^{-1}) were obtained from a commercial source and used as received. Acetonitrile (CH_3CN, purity, ≥99.9%), potassium chloride (KCl, purity, ≥99.0%), sodium dihydrogen phosphate (NaH_2PO_4, purity. ≥99.0%), magnesium dichloride ($MgCl_2$, purity, ≥98.0%), calcium chloride ($CaCl_2$, purity, ≥99.0%), sodium chloride (NaCl, purity, ≥99.5%), sodium bicarbonate ($NaHCO_3$, purity, 99.7%) and D-glucose ($C_6H_{12}O_6$, purity, ≥99.5%), all analytical grade, were obtained from Sigma-Aldrich. The ROS indicator H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate), was purchased from Life Technologies. Sodium hydroxide (NaOH), and sulfuric acid (H_2SO_4), used for pH adjustment, were obtained from Labkem and Honeywell/Fluka, respectively. For phytotoxicity, filter papers from Deltalab with 78 g m^2 were used. The ultrapure water was obtained using a Direct-Pure Interlab water system.

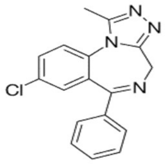
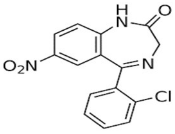
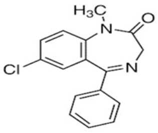
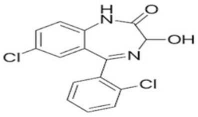
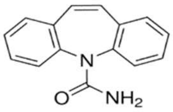
IV.2.2 Analytical techniques

The pharmaceutical compounds were quantified by high performance liquid chromatography (HPLC, UFLC, Shimadzu) equipped with a diode-array detector (DAD). The C18 analytical column (AQ C18 Silia Chrom, 5 μm, 100 Å, 4.6 x 250 mm) was kept at 25° C. The solution was delivered with a flow rate of 1.0 mL min^{-1} in linear gradient mode, using the mixture acetonitrile:ultrapure water ranging from 45:55 v/v ($t = 0$ min) to 70:30 v/v ($t = 15$ min) and an injection volume of 100 μL. Physicochemical characteristics including the limits of quantification (LOQ) and the limits of detection (LOD) of the pharmaceutically active compounds ALP, CZP, DZP, LZP and CBZ are presented in Table IV.1. Before HPLC analysis, all samples were filtered with cellulose acetate filter (0.45 μm) supplied by Sartorius and the chromatographic analyses were performed immediately after the experiments. To better evaluate the performance of the Ti/Pt anode in EO, the kinetic profiles were studied based on the variation of the individual concentrations of ALP, CZP, DZP, LZP and CBZ, and the results followed the pseudo-first-order kinetics according to Eq. IV.7.

$$ln\frac{C}{C_0} = -kt \tag{IV.7}$$

Where C_0 is the initial concentration (μg L^{-1}), C is the concentration of ALP, CZP, DZP, LZP and CBZ (μg L^{-1}) at time intervals, k is the reaction rate constant (min^{-1}) and t is time (min). The Ti/Pt anode morphological properties were assessed using a Vega3 SBH-Tescan scanning electron microscopy (SEM) at 500x and 5000x magnification, before and after the reactions. In electrochemical oxidation experiments the pH of the synthetic solution was adjusted to values between 4 and 10 using NaOH (2 M) or H_2SO_4 (1 M), as needed. The temperature of the perfusing media was controlled by a thermostatic bath (Variomag – Electronicrührer).

Table IV.1 - Characteristics of the pharmaceutical compounds (Bosio *et al.*, 2018).

Common name	LOQ (µg L^{-1})	LOD (µg L^{-1})	pKa	Chemical structure
Alprazolam	35.3	10.6	2.4	
Clonazepam	15.1	4.5	1.5	
Diazepam	4.0	1.2	3.4	
Lorazepam	1.0	1.3	1.3	
Carbamazepine	3.82	1.1	13.9	

IV.2.3 Aqueous matrices

The synthetic matrix was prepared with ultrapure water (UP) spiked with the pharmaceutically active compounds (PhACs) ALP, CZP, DZP, LZP and CBZ, at the concentration of 100 µg L^{-1} each. In order to maintain a high conductivity, NaCl or Na_2SO_4 were used in experiments as electrolytes. The surface water (SW) was collected from Mondego River, located in the city of Coimbra, Portugal. The municipal wastewater (MWW) was collected after the secondary process of the municipal wastewater treatment plant (MWWTP), located in Coimbra, Portugal. After collection, SW and MWW were stored under refrigeration (4° C). The characterization of the different aqueous matrices is presented in Table IV.2. The real matrices were spiked with all PhACs (100 µg L^{-1} each drug) in all experiments.

Table IV.2 - Characterization of the aqueous matrices. The synthetic effluent was prepared with ultrapure water and spiked with 100 µg L^{-1} of each compound (UP_{100}).

	Aqueous Matrix		
Parameters	**Synthetic effluent**	**SW (surface water)**	**MWW (municipal wastewater)**
Alprazolam (µg L^{-1})	99.70 ± 1.82	<LOD	<LOD
Clonazepam (µg L^{-1})	100.03 ± 1.34	<LOD	<LOD
Diazepam (µg L^{-1})	98.31 ± 2.52	<LOD	<LOD
Lorazepam (µg L^{-1})	97.84 ± 1.18	<LOD	<LOD
Carbamazepine (µg L^{-1})	101.09 ± 1.01	<LOD	<LOD
pH	3, 7 and 10	7.1	7.43
Conductivity (µS cm^{-1})	120-230	110	748

* LOD = Limit of Detection

IV.2.4 Electrochemical oxidation

Electrochemical oxidation experiments were carried-out in a lab-scale batch-stirred reactor (total volume of 1 L) with 500 mL of UP, SW or MWW at 25° C and atmospheric pressure. A titanium electrode cell coated with 50 g Pt m^{-2} (Ti/Pt) was used as anode and stainless steel (SS) as cathode with an effective area of 20 cm^2 (8.0 cm x 2.5 cm). The electrodes were placed in parallel with a gap distance of 1 cm and connected to a DC power supply HY3010 Kaise (current (I) = 1.2 - 2.5 A).

IV.2.5 Neurotoxicity studies

All neurotoxicity assays were performed using samples collected after 1 h of electrochemical oxidation process with a current density of 25 A m^{-2}. Three samples were studied: (i) the synthetic effluent spiked with 100 µg L^{-1} of PhACs and 1.5 g NaCl L^{-1} used as electrolyte (UP_{100}), (ii) the effluent from MWWTP in the absence of PhACs (MWW_0) and (iii) the effluent from MWWTP spiked with 100 µg L^{-1} of PhACs (MWW_{100}). After EO, all solutions were preserved in refrigerator and at the time of the neurotoxicity experiments they were spiked with the compounds of artificial cerebrospinal fluid (ACSF). The pH was measured to ensure that it was around 7.4. A schematic configuration of the operational setups is displayed in Figure IV.1.

The neurotoxicity evaluation was performed using the brain of Wistar rats (10-16 weeks old; 14-18 days of gestational) provided by the Center of Neuroscience and Cell Biology (CNC) of the University of Coimbra. The rats were anesthetized and had a quick cervical displacement followed by decapitation and only the number of animals needed to produce reliable scientific data were used. These procedures follow the European Communities Council Directives (2010/63/UE) with the aim of minimizing the suffering of animals. The brain was promptly extracted and stored in a refrigerated ACSF solution with the following composition (in mM): KCl 3.5; NaH_2PO_4 1.25; $MgCl_2$ 2.0; $CaCl_2$ 2.0; NaCl 124.0; $NaHCO_3$ 24.0 and D-glucose 10.0, pH 7.4. A few minutes after the brain extraction, the hippocampus was manually removed and sliced by transversal cuts with blades spaced 350 µm. Then, the slices were transferred to the ACSF oxygenated solution (95% O_2 and 5% CO_2) at room temperature.

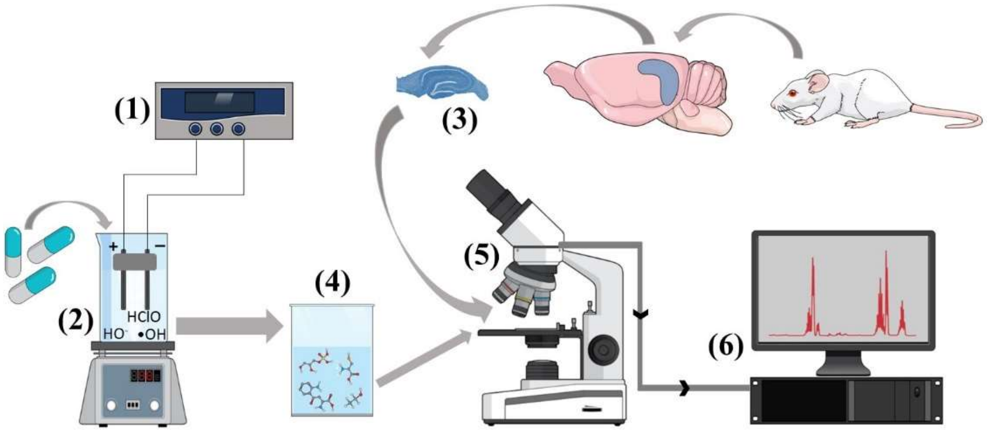


Figure IV.1 – Schematic configuration of the operational setups. (1) – Power supply; (2) – Electrochemical oxidation process; (3) – Rat hippocampal slice; (4) – Treated effluent; (5) - Fluorescence microscope and (6) – Data acquisition system.

Afterwards, the slices were incubated for 1 h in an oxygenated ACSF solution containing the fluorescent ROS indicator (H_2DCFDA, 20 µM). After the period of incubation, the hippocampal slices were placed again in the oxygenated ACSF solution and used within the next 10 h. For an experiment one hippocampal slice was transferred to the experimental chamber coupled to the fluorescence microscope (Zeiss Axioskop) in which oxygenated ACSF solution, or an EO treated medium, was perfused (1.5-2.0 mL min^{-1}, at the controlled temperature of 30-32 ºC). The microscope has a tungsten/halogen lamp (12 V, 100 W) and narrow band (10 nm) excitation and high-pass emission filters of 480 and 500 nm, respectively. The light was focused on the slice using a water immersion lens (40x, N.A. 0.75). The optical signals were obtained with a photodiode (1 mm^2, Hammamatsu), passing the response through an I/V converter (1 GΩ feedback resistance) and a 16 bits analog/digital converter (National Instruments). The signals were processed at 0.017 Hz and displayed using the Signal Express^{TM} software (Labview, National Instruments). The responses were acquired at the mossy fiber synapses from hippocampal CA3 region. Each point in an interval of one minute represents the average of 100 measurements. All experiments were run in triplicate.

IV.2.6 Phytotoxicity assays

The influence of PhACs in the germination of *Lepidium sativum* seeds was assessed under different conditions, before and after the electrochemical oxidation process, according to Table IV.3. A control experiment containing only ultrapure water (UP$_0$, no pharmaceutical compounds) was also performed and all experiments were made in duplicate. The seeds were wetted with 5 mL of the intended solution and kept at 25 ºC for 48 h in the absence of light. Each petri dish contained 10 seeds placed on filter paper. After this period, the seeds were analysed and the relative seed germination (SG), relative root growth (RG), and germination index (GI) were calculated according to Eqs. 8, 9 and 10, respectively. The degree of phytotoxicity was classified into different indices: no inhibition for GI > 80%, mild inhibition for 80% > GI >60%, strong inhibition for 60% > GI > 40% , and severe inhibition for GI < 40% (Pinho *et al.*, 2017).

Table IV.3 - Phytotoxicity experiments.

Experiment	**PhACs [μg L^{-1}]**	**After electrochemical oxidation process?**	**Time of EO reaction (min)**	**NaCl [g L^{-1}]**	**Current density (A m^{-2})**
UP$_0$ (Control)	0	No	-	-	-
UP$_{100,\ \text{no EO}}$	100	No	-	1.5	-
UP$_{100,\ \text{EO, 60min}}$	100	Yes	60	1.5	25
MWW$_{100,\ \text{no EO}}$	100	No	-	-	-
MWW$_{100,\ \text{EO 60 min}}$	100	Yes	60	-	25
MWW$_{100,\ \text{EO 120 min}}$	100	Yes	120	-	25

$$SG(\%) = \frac{Number\ of\ seeds\ germinated\ in\ tested\ solution}{Number\ of\ seeds\ germinated\ in\ control\ solution}\ x\ 100 \qquad (8)$$

$$RG(\%) = \frac{Mean\ root\ length\ in\ tested\ solution}{Mean\ root\ leng\quad in\ control\ solution}\ x\ 100 \qquad (9)$$

$$GI(\%) = \frac{SGxRG}{100} \tag{10}$$

IV.3 Results and Discussion

IV.3.1 Electrochemical oxidation experiments

IV.3.1.1 Effect of current density

The current density may affect the production of some radicals such as ROS and chlorine species. Higher amounts of these oxidizing agents allow the reactions to occur more rapidly (Ellouze *et al.*, 2016). In order to investigate the effect of this parameter on the degradation of ALP, CZP, DZP, LZP and CBZ drugs during the electrochemical oxidation, experiments were carried out using current density values of 13, 25 and 75 A m^{-2} applied to a synthetic solution containing 100 µg L^{-1} of each compound (UP$_{100}$), at 25 ºC (Fig. IV.2 A-C).

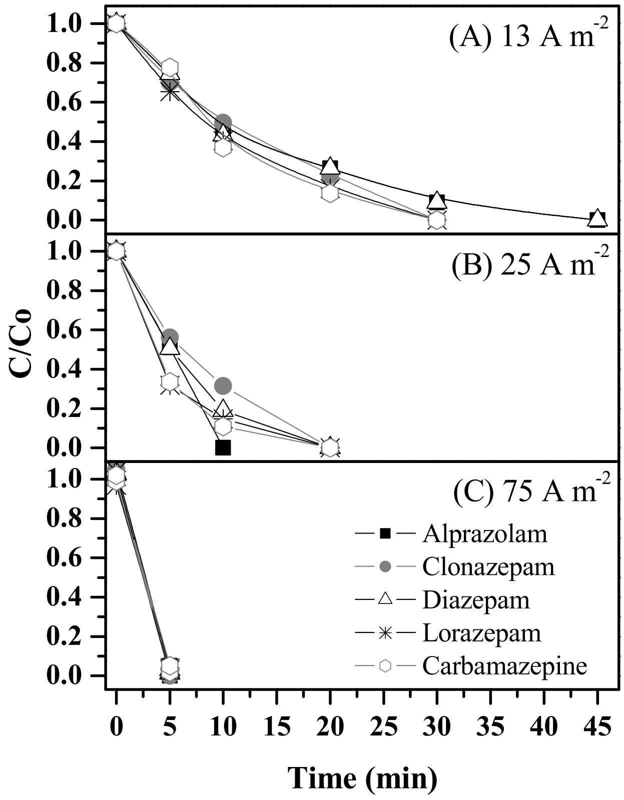


Figure IV.2 – Effect of current density on PhACs degradation using Ti/Pt anode. (A) 13 A m^{-2}, (B) 25 A m^{-2}, and (C) 75 A m^{-2}. (1.5 g NaCl L^{-1}, 100 µg PhACs L^{-1}, pH = 7, T = 25 ºC).

As observed in Figure IV.2, increasing the current density from 13 to 75 A m^{-2} all drugs were completely removed (< LOQ, limits of quantification) in less than 5 min.

Under 13 A m^{-2}, at least 30 min were needed to completely remove CZP, LZP and CBZ drugs. The faster degradation of these compounds compared with ALP and DZP is probably associated with the difference between their p*K*a values. Carbamazepine has the highest p*K*a (13.9), while CZP and LZP have the lower p*K*a (1.5 and 1.3, respectively), thus being the interactions between the structures and the radicals produced more intense, leading to degrade the compounds more quickly. The pseudo-first-order kinetic constants are presented in Table IV.4 and according to the experiments 1 – 2, higher kinetic constants were observed for the compounds CBZ and LZP.

Table IV.4 – Initial conditions of the EO experiments and pseudo-first-order kinetic constants.

Exp.	**Initial Conditions**						**Pseudo-first-order kinetic constants**	
	PhACs	**[µg L^{-1}]**	**Current density (A m^{-2})**	**Water**	**pH**	**NaCl [g L^{-1}]**	**k (min^{-1})**	**R^2**
1	ALP	100	25	UP	7	1.5	0.17 ± 0.06	0.91
	CZP	100					0.12 ± 0.02	0.98
	DZP	100					0.15 ± 0.02	0.98
	LZP	100					0.21 ± 0.01	0.99
	CBZ	100					0.22 ± 0.01	0.99
2	ALP	100	13	UP	7	1.5	0.07 ± 0.01	0.98
	CZP	100					0.08 ± 0.01	0.98
	DZP	100					0.06 ± 0.01	0.98
	LZP	100					0.09 ± 0.01	0.99
	CBZ	100					0.09 ± 0.01	0.96
3	ALP	100	25	UP	3	1.5	0.21 ± 0.07	0.91
	CZP	100					0.10 ± 0.02	0.93
	DZP	100					0.14 ± 0.02	0.98
	LZP	100					0.18 ± 0.01	0.99
	CBZ	100					0.25 ± 0.06	0.96
4	ALP	100	25	UP	10	1.5	0.14 ± 0.03	0.96
	CZP	100					0.09 ± 0.02	0.91
	DZP	100					0.13 ± 0.02	0.98
	LZP	100					0.16 ± 0.01	0.99
	CBZ	100					0.14 ± 0.01	0.99
5	ALP	100	25	SW	7.1	-	0.027 ± 0.002	0.98
	CZP	100					0.031 ± 0.003	0.97
	DZP	100					0.030 ± 0.003	0.97
	LZP	100					0.040 ± 0.003	0.98
	CBZ	100					0.034 ± 0.002	0.98
6	ALP	100	25	MWW	7.4	-	0.003 ± 0.0001	0.99
	CZP	100					0.004 ± 0.0002	0.98
	DZP	100					0.002 ± 0.0002	0.94
	LZP	100					0.003 ± 0.0001	0.99
	CBZ	100					0.004 ± 0.0002	0.98

UP = Ultrapure water; SW = Surface water; MWW = Municipal wastewater

Applying the current density of 25 A m^{-2}, the highest kinetic constants were also those of CZP, LZP and CBZ, being 0.12, 0.21 and 0.22 min^{-1}, respectively. However, variations in the degradation pathways of the compounds may also occur depending on the nature and chemical structure of the compounds (Gaya and Abdullah, 2008) and also the reaction conditions (Witte *et al.*, 2011), Therefore, increasing the current density led to an increase in the production of •OH on the anode surface and, consequently, increased the oxidation of pharmaceutical compounds in the diffusion layer and the occurrence of other reactions simultaneously (Zou *et al.*, 2017). These results are in accordance with literature data (Costa et al. 2009; Clematis et al. 2017; Flores et al. 2017; Salazar et al. 2017). Based on the data acquired in these experiments, the current density of 25 A m^{-2} was chosen for the next experiments carried out in this study.

IV.3.1.2 Effect of pH

Taking into consideration that benzodiazepines contain a chlorine in their structure and that the degradation of these PhACs may lead to the formation of reactive chlorine species (Garcia-Segura, Ocon and Chong, 2018), the influence of the initial pH value (3, 7 and 10) was assessed. The results of the experiments, performed using 1.5 g NaCl L^{-1}, a current density of 25 A m^{-2} and 5 min of reaction, are shown in Fig. IV.3 and the calculated pseudo-first-order kinetic rates are presented in Table IV.4.

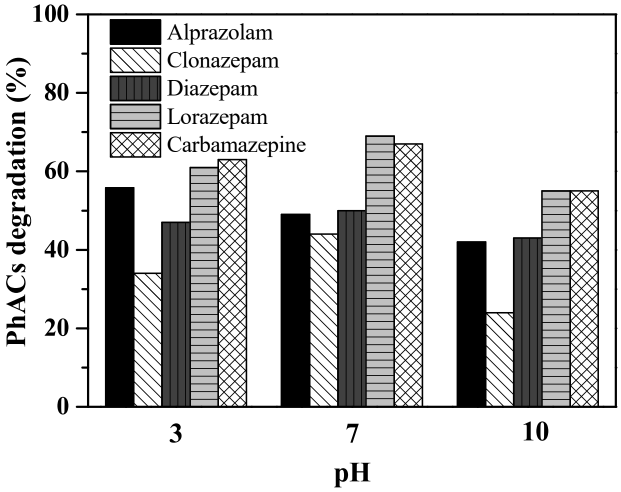


Figure IV.3 – PhACs degradation under different initial pH values (1.5 g NaCl L^{-1}, 100 μg PhACs L^{-1}, t = 5 min, current density = 25 A m^{-2}, T = 25 ºC).

According to Fig. IV.3, in acidic and neutral medium the degradation of the compounds performed better than in alkaline solution. Depending on the pH, the main reactive chlorine species present in the solution are chlorine (Cl_2) (pH < 3), hypochlorous acid (HClO) (3 < pH < 8) and hypochlorite ion (ClO^-) (pH > 8). This can be explained, since the chloride (Cl^-) mediated oxidations are stronger for $Cl_{2(aq)}$ ($E^0(Cl_{2(aq)}/Cl^-) = 1.36$ V) and HClO ($E^0(HClO/Cl^-) = 1.49$ V) than for ClO^- ($E^0(ClO^-/Cl^-) = 0.89$ V) (Rajkumar et al. 2005; Deborde & Gunten 2008; Fajardo et al. 2017a). However, a fact that may explain why alkaline degradation has been equated with other media is the fact that in alkaline medium evaporation of free chlorine is minimized (Fajardo et al. 2017b).

The lowest degradation was observed for pH 10 and for CZP (23 %, $k = 0.09 \pm 0.02$ min^{-1}). However LZP and CBZ showed the better performance at all pH values, reaching kinetic rates 1.55-fold and 1.77-fold higher compared to CZP at pH 10 and degradation of 69 % and 67%, respectively. This variation may be related to the difference of p*K*a of the benzodiazepines and the strong molecular interactions with the different radicals produced during the electrochemical oxidation (Eqs. IV.11-14) (Bosio et al. 2018; Poyatos et al. 2010). The •OH radical has the highest oxidative power and is not selective, so it is difficult to identify a degradation pattern of the compounds, since the degradation reactions occur very rapidly and simultaneously (Deng and Zhao, 2015).

$$H_2O \rightarrow H^+ + (\bullet OH)_{ads} + e^- \qquad \text{(IV.11)}$$

$$2(\bullet OH)_{ads} \rightarrow O_2 + 2H^+ + 2e^- \qquad \text{(IV.12)}$$

$$(\bullet \mathrm{OH})_{ads} \rightarrow (O)_{ads} + H^+ + e^- \qquad \text{(IV.13)}$$

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2 \qquad \text{(IV.14)}$$

The pH 7 was the best pH value to degrade the compounds and for this reason this pH was maintained in the next experiments.

IV.3.1.3 Effect of the aqueous matrix

In order to investigate the efficacy of EO in the removal of the target compounds using different water matrices, benzodiazepines and carbamazepine degradation profiles were evaluated with surface water (SW) (Fig. IV.4A) and municipal wastewater (MWW) (Fig. IV.4B) at pH 7.1 and 7.4, respectively, and applying a current density of 25 A m^{-2}. The results were compared with UP_{100} (1.5 g NaCl L^{-1}, 100 µg PhACs L^{-1}, 25 A m^{-2} and 25 ºC). Due to the high conductivity of the real matrices (SW and MWW), the addition of electrolyte was dispensable in these waters.

According to Fig. IV.2B, with the synthetic effluent all pharmaceutical compounds reached values below the LOD in 20 min of reaction. Using SW, the efficiency of the EO process decreased, although the compounds were completely degraded in 90 min (Fig. IV.4A). For MWW the maximum degradation was around 40% after 120 min for CLZ, LRZ and CBZ. Similar results were reported by Bosio et al. 2018, which detected a decrease of the degradation in MWWTP effluent, possibly by the presence of a great variety of organic, inorganic and biological pollutants in the real matrices. These compounds may be competing with the radicals produced by direct and indirect pathways in the electrochemical oxidation (Parsa and Abbasi, 2007; Moreira *et al.*, 2017; Garcia-Segura, Ocon and Chong, 2018).

Experiments 1, 5 and 6 (Table IV.4) show the kinetic constants of the reactions using UP, SW and MWW, respectively. In UP, the reaction rates of ALP, CZP, DZP, LZP and CBZ were 6, 4, 5, 5 and 6 times higher than in SW, respectively. Comparing UP with MWW, the degradation of ALP, CZP, DZP, LZP and CBZ was about 50, 30, 75, 70 and 55-times higher, respectively, compared to MWW. Due to the incomplete degradation of the compounds in the MWW, these effluents were further selected for the toxicity tests for comparison with the degradation of the compounds in the synthetic effluent.

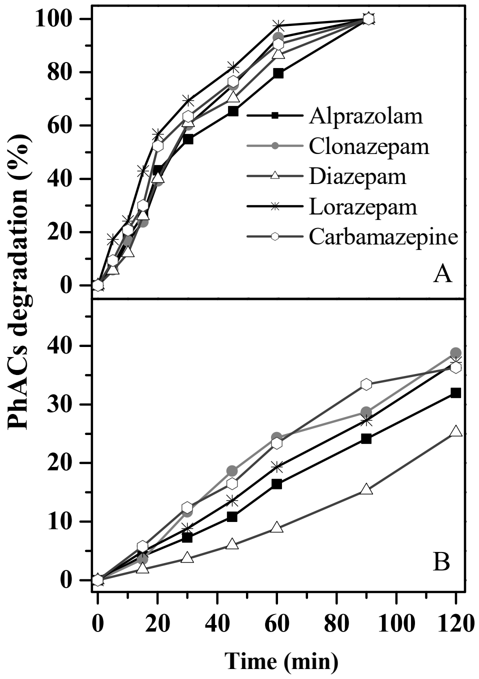
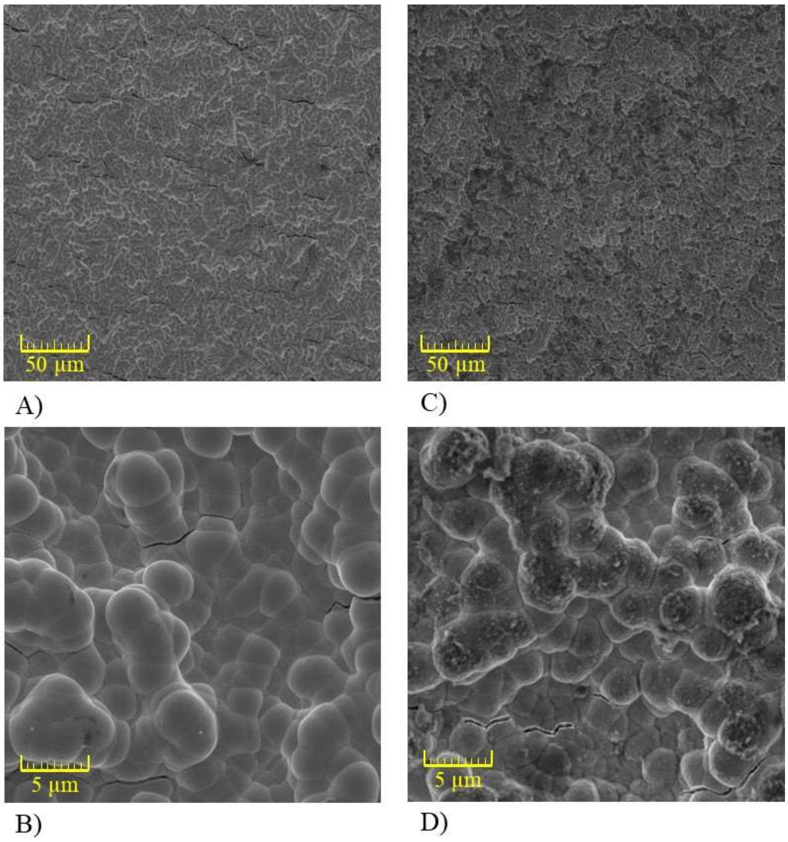


Figure IV.4 – PhACs degradation under different water matrices using 100 μg PhACs L^{-1}, $T = 25$ ºC, and current density of 25 A m^{-2}: (A) SW at pH = 7.1, and (B) MWW at pH = 7.4.

V.3.1.4 Ti/Pt anode surface analysis

A scanning electron microscopy (SEM, Fig. IV.5) was performed to evaluate the morphology of the Ti/Pt anode before and after the electrochemical oxidation treatment, in order to observe if corrosion of the anode occurred. The analysis was performed after all the experiments of this study. According to Figure IV.5 (A-D), it is possible to observe the morphology of the anode prior to and after use, with magnifications of 500x (Figures IV.5 A-B) and 5000x (Figures IV.5 C-D),



A) C)

B) D)

Figure IV.5 – SEM images of Ti/Pt anode material before and after electrochemical oxidation process. Images were taken before reaction at 500x (A) and 5000x (B), and after reaction at 500x (C) and 5000x (D).

In Figure IV.5A the film has a homogeneous appearance, however in Figure IV.5C, it is possible to observe that irregular granules are present and some cracks, these characteristics are generated at the moment of the thermal decomposition of the compounds on the anode surface (Isarain-Chávez et al. 2017). The characteristics of the surface of the anode reveal that the surface area increases, favoring catalytic sites and thus generation of radicals (•OH) (Yang *et al.*, 2018). After repeated uses, a slow wear of the morphological structure of the anode can be detected. However, it is possible to say that the anode can be reused in a variety of reactions, without a large decay in the efficiency of the electrochemical process. This is very attractive in terms of maintenance and to minimize process costs.

IV.3.2 Neurotoxicity tests

The effects of the degradation of the mixture of ALP, CZP, DZP, LZP and CBZ by the electrochemical oxidation process were evaluated in neuronal ROS signals from brain slices applying different treated solutions. Figure IV.6A represents the data obtained with the UP_{100}, Figure IV.6B shows the results caused by MWW_0 and Figure IV.6C indicates the action of MWW_{100}.

Experiments carried out in previous studies indicated that the synthetic effluent before EO with PhACs (100 μg L^{-1}) and without electrolyte showed a decreased in amplitude of 0.10 ± 0.01 S.E.M, n = 3. However, it can be observed in Figure IV.6A that in the presence of the UP_{100}, applied during the period indicated by the bar, the ROS signal decreases reaching a steady level with an amplitude of 0.13 ± 0.02 S.E.M, n = 3. Upon washout the signal recovers during the next 20 min and stabilizes at 0.11 ± 0.02 S.E.M, n = 3, above baseline. This proves that the electrochemical oxidation with the parameters used was not enough to eliminate the neurotoxicity of the solution. A similar behaviour was reported by Fajardo et al (2017a; 2017b).

In Figure IV.6B it can be seen that MWW_0 caused no effect in ROS signal. In this case the ROS signal remained stable throughout the period of application of the effluent increasing slightly following its removal, reaching a steady level of 0.01 ± 0.01, n = 3. Thus, in the presence of the treated municipal wastewater containing no pharmaceutical compounds there was no clear positive or negative effect on ROS production but a small enhancement was observed afterwards. Figure IV.6C shows that the ROS signal decreased during the first 10 min of application of MWW_{100} and remained constant, with an amplitude of 0.08 ± 0.03, n = 3, in the last 20 min of perfusion. When the effluent solution was replaced by ACSF, the ROS signal increased reaching a maximum in relation to baseline of 0.11 ± 0.03, in the period 65-70 min, n = 3. Thus the signal did not return to baseline during the recording period, suggesting that the effect is not reversible.

In this study the ROS signals are considered to be directly associated with synaptic activity and to have mitochondrial origin (Chen et al 2003; Kasischke et al 2004). If something interferes with the normal cellular functioning it can cause oxidative stress, with a direct manifestation in the production of ROS. One possible explanation for the decrease of the signals is that the PhACs affect membrane voltage dependent and receptor channels altering the movement of potassium, calcium and zinc ions through them (Lima, Trejo and Urbina, 1995) leading to the decrease in ROS production.

The solutions containing PhACs (UP_{100} and MWW_{100}) caused a depression in the ROS signals. However, when the ACSF medium was reapplied the signal recovered and increased to a value above baseline, indicating that the cells recovered part of their normal activity but remained in a different state.

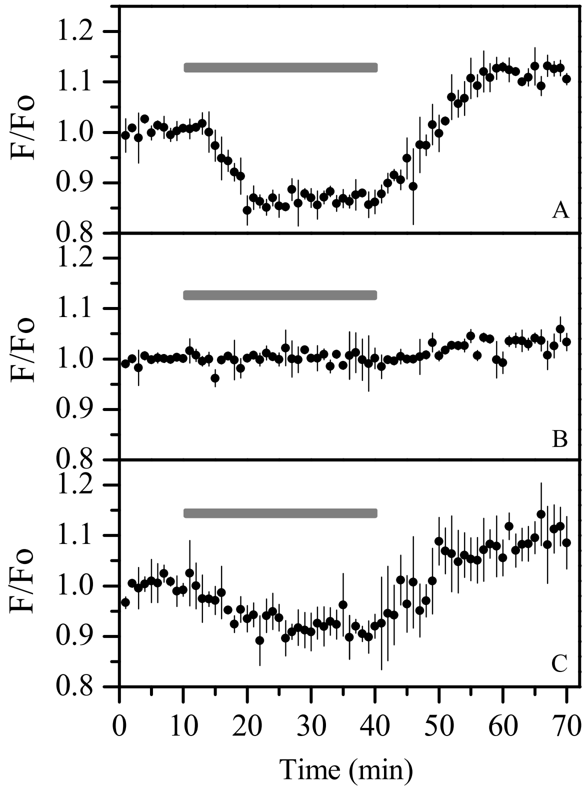


Figure IV.6 – Effect of the PhACs, degraded by EO, on neuronal ROS signals. The data were acquired using the fluorescent ROS indicator H_2DCFDA (20 μM) and are normalised by the average of the first 10 responses and represented as mean ± S.E.M (n = 3). The treated mixture of PhACs was perfused in hippocampus slice during the period indicated by the grey bar. (A) UP_{100}, (B) MWW_0, and (C) MWW_{100}.

IV.3.3 Phytotoxicity studies

In order to investigate the effects of the pharmaceutical compounds on the environment, the *Lepidium sativum* germination test was performed. Although this test is mostly used to evaluate the phytotoxicity of contaminated soils (Walter, Martı and Cala, 2006; Roca-Pérez *et al.*, 2009; Oleszczuk *et al.*, 2012), its use in aqueous solution may be valuable, since the water may be used for irrigation of plantations. Table IV.5 presents the SG, RG and GI values for the studied solutions. According to Table IV.5, a small inhibition was observed for $UP_{100,\ no\ EO}$ and $UP_{100,\ EO,\ 60min}$ (GI values of 88% and 95%, respectively). A large inhibition occurred for the solution MWW_{100} (GI = 64%) and a strong inhibition was displayed for the solutions $MWW_{100,\ EO\ 60\ min}$ and $MWW_{100,\ EO\ 120\ min}$ (GI 40% and 48%, respectively). These results suggest that more toxic compounds may have been formed after the EO process, which directly affected the seed growth and root size. As can be seen by comparing $UP_{100,\ no\ EO}$ and $UP_{100,\ EO,\ 60min}$, the germination index increased, this was also detected by (Gomes *et al.*, 2017) when treating solutions of parabens by photolytic ozone. However, for the $MWW_{100,\ EO\ 60\ min}$ and $MWW_{100,\ EO\ 120\ min,}$ treatment by electrochemical oxidation the germination index decreased. This shows that in some cases treatment by AOP can make the effluent more toxic than the original one.

Table IV.5 – Phytotoxicity of the PhACs on *Lepidium sativum* germination.

Sample	Solution	SG (%)	RG (%)	GI (%)
UP_0	Water control	100	100	100
$UP_{100,\ no\ EO}$	Synthetic effluent-1	100	88	88
$UP_{100,\ EO\ 60min}$	Synthetic effluent-2	100	95	95
$MWW_{100,\ no\ EO}$	MWW	95	67	64
$MWW_{100,\ EO\ 60\ min}$	MWW	95	42	40
$MWW_{100,\ EO\ 120\ min}$	MWW	100	48	48

IV.4 Conclusions

Electrochemical oxidation using a Ti/Pt anode was successfully applied to degrade a mixture of four benzodiazepines and carbamazepine in three different water matrices: ultrapure water, surface water and municipal wastewater. In the synthetic effluent, a better performance was achieved at neutral pH conditions when applying a current density of 25 A m^2, likely due to the formation of reactive chlorines species. In surface water and municipal wastewater, the efficiency of the process was considerably reduced probably due to the complex composition of the aqueous matrices, whose components may compete with PhACs and react with the radicals produced in the electrochemical oxidation. The anode has a fairly stable morphology and can be reused in subsequent experimental procedures.

This study was successful in establishing new reliable and effective toxicity methods to investigate the influence of low levels of pharmaceutical compounds in neuronal ROS formation and in germination seeds. The neurotoxicity assays successfully demonstrated the effect of residual concentrations of pharmaceutical compounds after EO in the synaptic function of rat hippocampal slices. The samples containing PhACs (UP_{100} and MWW_{100}) decreased ROS production in the studied neuronal system and, although the cells recovered part of their normal activity, they remained in a different state. The phytotoxicity experiments confirmed the influence of the studied CECs in seed germination. The GI was strongly affected by samples containing municipal wastewater ($MWW_{100,\ EO\ 60\ min}$ and $MWW_{100,\ EO\ 120\ min}$), most likely due to the presence of complex substances in this matrix.

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CHAPTER V – Electrochemical Oxidation (Ti/Pt) - 2

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Pharmaceutical compounds electrotreatment by Pt anodes and effect on synaptic function

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ABSTRACT

Thousands of compounds are used and disposed of every day and many of them are not degraded in conventional treatment plants. It is necessary an alternative to eliminate these compounds. This can be done through electrooxidation technology, which was applied in this work to a mixture of pharmaceutical compounds including alprazolam (ALP), clonazepam (CLP), diazepam (DZP), lorazepam (LZP) and carbamazepine (CBZ) at 100 $\mu g\ L^{-1}$. The mixture was studied with different types of electrolytes and the neurotoxic effect of the treatment was evaluated. The best result was obtained with NaCl (0.5 $g\ L^{-1}$), leading to complete degradation of CLP, LZP and CBZ.

Keywords: electrooxidation; benzodiazepines; hippocampal; neurotoxicity

V.1 Introduction

A few decades ago it was possible to find many compounds in rivers and oceans, mainly plastics, discarded by humans. These visible compounds directly affect aquatic animals, in many cases leading to their death. However, there are thousands of invisible compounds that can affect the entire food chain, being released into waterbodies every day. These contaminants, ranging from beauty and cleaning products to certain medicines, are giving rise to an emerging concern. Many of these compounds, found under concentrations of ng L^{-1} to μg L^{-1} (James *et al.*, 2016), can have a cumulative action in the environment.

A major problem of some of these substances is that they are recalcitrant and bioaccumulative. Therefore, they are able to remain in the environment for a long time, affecting both animals and plants (Wang and Wang, 2017). This way, the water quality of many rivers has been affected by the discharge of both untreated and treated industrial and domestic wastewaters (Mohapatra *et al.*, 2014).

Among the different contaminants of emerging concern, benzodiazepines that are used for treatment of the central nervous system as sedative, muscle relaxant and anxiolytic, play an important role. Some studies report the existence of these compounds in rivers from many parts of the world (Wu *et al.*, 2015; Fick *et al.*, 2017; Mutiyar, Gupta and Mittal, 2018). Another compound that proves to be very influential is carbamazepine, an antiepileptic drug used in the treatment of seizure disorders. Its biodegradation cannot be performed in conventional sewage treatment plants. As a consequence many studies show that it is present in rivers and at the end of a biological treatment (González Alonso *et al.*, 2010; Ramaswamy *et al.*, 2011; Luo *et al.*, 2014).

Since the mentioned compounds are not degraded by conventional methods it is necessary to employ processes that provide for the degradation of such recalcitrant compounds. As an alternative, advanced oxidative processes , which are characterized mainly by the degradation through highly reactive radicals with the capacity to degrade more complex molecules, are used (Garza-Campos *et al.*, 2016). Electrooxidation is one of the advanced oxidation processes that can be used to degrade compounds like benzodiazepines and carbamazepines. In this process, in which the anode may be Pt, BDD (boron-doped diamond), IrO_2 or RuO_2, the hydroxyl radicals, generated from water discharge at the surface of the anode, oxidize the organic compounds (Garza-Campos *et al.*, 2014; Fajardo *et al.*, 2016; Klidi *et al.*, 2018).

The main objective of this work was to analyze the effect of Ti/Pt anode material in the oxidation of a synthetic mixture of five pharmaceutical compounds, consisting of ALP, CZP, DZP, LZP and CBZ, that are usually present in water treatment plants

(WTPs). Besides that, the neurotoxicity of a treated mixture was assessed in rat brain slices, using a fluorescent reactive oxygen species (ROS) indicator.

V.2 Material and Methods

V.2.1 Chemicals and setup

ALP, CZP, DZP, LZP and CBZ drugs were acquired from commercial sources and used as obtained. They were spiked at the concentration of 100 µg L^{-1}. HPLC-grade acetonitrile was purchased from Sigma-Aldrich. For the brain slices experiments extracellular artificial cerebrospinal fluid (ACSF) was prepared with the following composition (in mM): 124 NaCl, 3.5 KCl, 24 $NaHCO_3$, 1.25 NaH_2PO_4, 2.0 $MgCl_2$, 2.0 $CaCl_2$ and 10 D-Glucose, pH 7.4. All chemicals were obtained from Sigma-Aldrich and used as received (analytical grade) without any further purification. The ROS indicator H_2DCFDA was purchased from Life Technologies. Ultrapure water was obtained by using an Interlab Direct-Pure purification system. The reactor, with a volume of 500 mL and magnetic stirring, contained electrodes composed of Ti/Pt (anode) and stainless steel (cathode).

The concentrations of benzodiazepines and of carbamazepine were determined by HPLC (High performance liquid chromatography) from Shimadzu, with a diode-array detector, flow rate of 1 mL min^{-1} and column C18 at 25 ºC. The initial gradient of the mobile phase was 70:30 of acetonitrile:water.

A fluorescence microscope (Zeiss Axioskop) equipped with a water immersion objective (40x, N.A. 0.75, 1.6 mm working distance) was used for the detection of the optical signals. Light from a tungsten/halogen lamp (12 V, 100 W) was selected using an excitation wavelength of 480 nm (10 nm bandwidth) being emission collected for wavelengths above 500 nm. The fluorescence signals were acquired through a photodiode system (Hammamatsu K2G 1336, 1.0 mm^2) using a 16 bit analog to digital converter (National Instruments) and the data were processed using the Signal Express® software (National Instruments). The optical signals represent the ratio of the fluorescence intensity (F) over the baseline fluorescence (F_0, s the average of the first 10 points), after correction for the autofluorescence determined from non-incubated slices.

V.2.2 Toxicity tests procedure

Toxicity assays were conducted in hippocampal slices (400 µm thick) from pregnant female Wistar rats (8-16 weeks old, 14-18 days of gestation), at the mossy fiber synaptic system. The slices were incubated in the oxygenated ACSF solution containing 20 µM of H_2DCFDA (ROS indicator), for 1h, at room temperature. After that period, the slices were transferred to the control oxygenated ACSF medium until they were used. For this purpose, the incubated slices were transferred to the experimental chamber inserted in the microscope setup, being continuously perfused with the desired medium, at 32º C and a flow rate of 1.5 mL min^{-1}. Following the initial application of ACSF, the treated solution, containing the oxidized pharmaceutical compounds, was perfused for 30 min. Then, the ACSF medium was perfused again, also for 30 min, to investigate the ability of the synaptic activity to return to the initial condition. Each plotted point is an average of 100 points collected with a frequency of 1.6 Hz. All experiments were run in triplicate.

V.3 Results and Discussion

V.3.1 Electrotreatment studies

V.3.1.1 Compounds degradation

The degradation (C/Co) and the percentage of degradation of ALP, CLP, DZP, LZP and CBZ, both mixed and applied individually, are shown in Figure V.1 (A and B), after 15 min. In the mixture (A) all compounds were degraded between about 70 and 85 %, being the behavior of the degradations similar in both types of experiments. The degradation was higher when the compounds were used individually (B) than when they were mixed. In the first case, LZP and CBZ were completely removed , while ALP, CLP and DZP were partially degraded (Figure V.1, right side). The higher degradation observed for the individual solutions may be explained by the fact that there is less organic matter in the media. Thus, all radicals produced by the electrotreatment were used to degrade a single compound, while in the mixture several compounds had to be degraded by the same amount of radicals. These different levels of degradation are in agreement with previous results (Bautitz and Nogueira, 2010; He *et al.*, 2016).

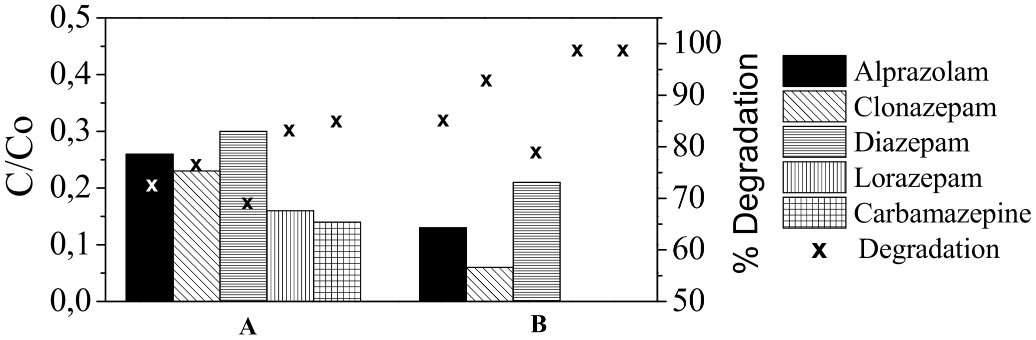

Figure V.1 – Degradation and percentage of degradation of ALP, CZP, DZP, LZP and CBZ in ultrapure water. Bar graph for comparison of the degradation (C/Co) and of the percentage of degradation (x) of the mixed (A) and individually applied (B) compounds. T = 25° C, pH = 7, t = 15 min, I = 0.05 A, NaCl = 1.5 g L^{-1}.

V.3.1.2 Effect of the electrolyte

Figure V.2 shows the degradation (C/Co) and the percentage of degradation of ALP, CZP, DZP, LZP and CBZ with different electrolyte types (NaCl and Na_2SO_4). The results indicate that NaCl was better at degrading the compounds. With NaCl, the CZP, LZP and CBZ pharmaceutical agents were completely degraded after 30 minutes, while with Na_2SO_4 the degradation of all compounds was between approximately 12 and 35 %. This difference in the amount of degradation may be explained by the formation of different oxidizing species (Fajardo *et al.*, 2017).

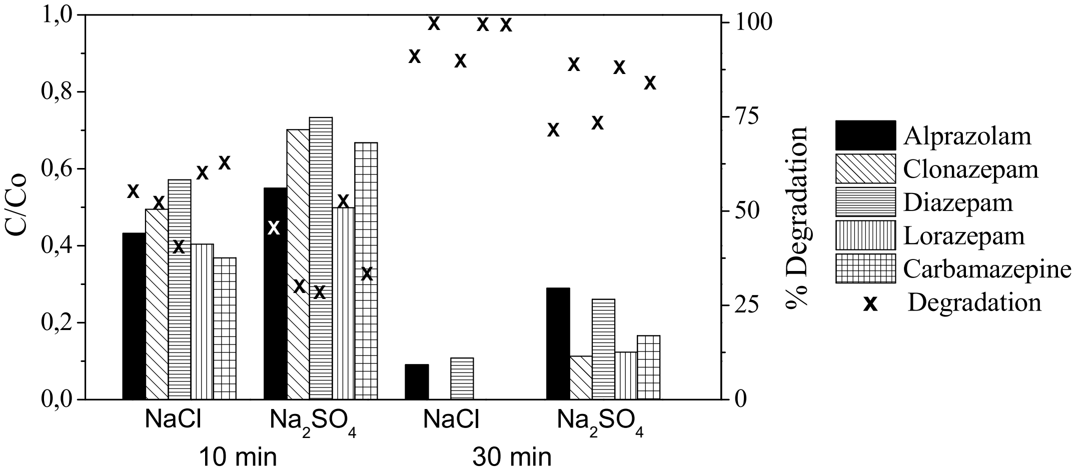

Figure V.2 – Degradation and percentage of degradation of ALP, CZP, DZP, LZP and CBZ in ultrapure water. Bar graph representing the degradation (C/Co) and the percentage of degradation (x) with the electrolytes NaCl (0.5 g L^{-1}) and Na_2SO_4 (0.5 g L^{-1}), for different electrooxidation reaction times, 10 min and 30 min. T = 25° C, pH = 7 and I = 0.05 A.

V.3.2 Neurotoxicity studies

The effect of the mixture of the pharmaceutical compounds used (100 µg L^{-1}, each) treated by the electrooxidation process, in neuronal metabolism, was evaluated in brain slices. For this purpose, fluorescence ROS signals were detected at the hippocampal mossy fiber synapses from slices incubated with the ROS indicator H_2DCFDA, (Figure V.3). In the normal medium (ACSF) the fluorescence values of the ROS signal remained stable. When the treated solution was perfused, the ROS signal decreased significantly. Upon returning to the control (ACSF) fluorescence values increased towards the baseline, reaching afterwards a potentiated level. These results suggest that, in the presence of the treated water, there is less ROS production.

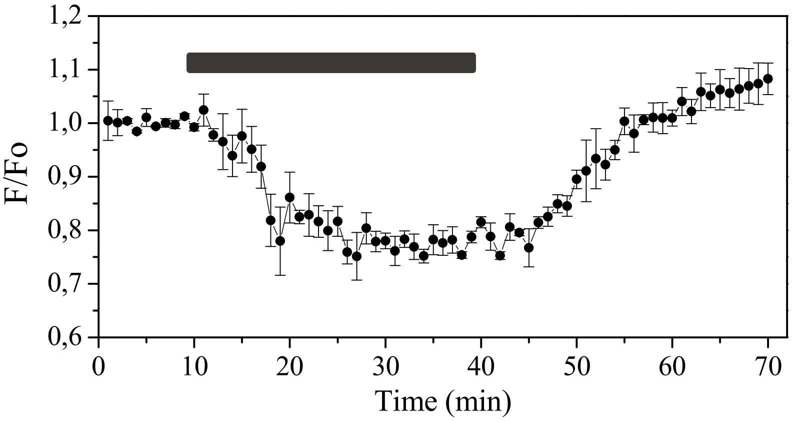

Figure V.3 - Electrotreated water depresses the fluorescence ROS signals from brain slices. The data points represent the mean ± standard deviation of normalized signals (F, fluorescence intensity; Fo, baseline fluorescence). The bar indicates the period of the treated water perfusion.

V.4 Conclusions

The individual pharmaceutical compounds (alprazolam, clonazepam, diazepam, lorazepam and carbamazepine) were better degraded by the electrotreatment (Ti/Pt) than when mixed, probably due to the fact that, in the first case, there is less organic matter to be eliminated. The use of different electrolytes (NaCl, Na_2SO_4) lead to different amounts of drug degradation, possibly because the nature of the eletrolytes generates different oxidative species. The treated water mixture caused a depression of the brain slices ROS signal in agreement with the idea that, in this medium, there was less physiological ROS formation.

Acknowledgments

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CHAPTER VI – Electrochemical Oxidation (BDD)

Article in preparation.

Electrochemical oxidation of a mixture of pharmaceuticals using BDD anode and phytotoxicological and neurotoxicological study of the treated solutions.

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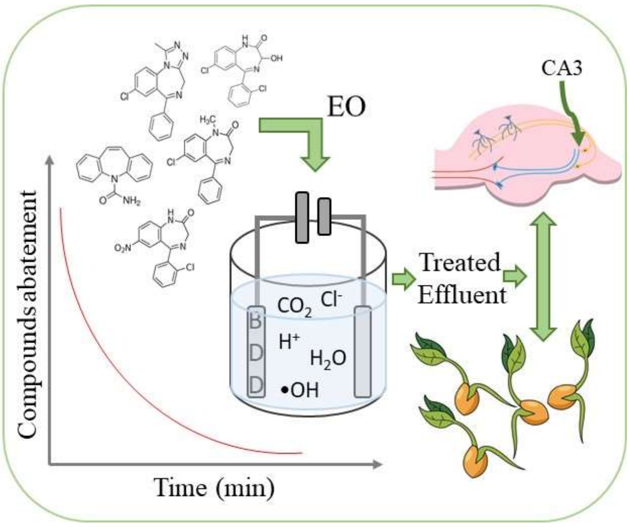
ABSTRACT

The inappropriate disposal of industrial, agricultural and domestic effluents causes daily contamination of the aquatic environment. This problem is aggravated when these substances are recalcitrant and/or bioaccumulate in plants and animals. An alternative to mitigate this problem are advanced oxidative processes, which encompass electrochemical oxidation (EO). This process was used in this study to evaluate the degradation of a mixture containing alprazolam, clonazepam, diazepam, lorazepam and carbamazepine. Some parameters were varied such as current density, pH, variation in concentration and type of electrolyte (NaCl and Na_2SO4) and different aqueous matrices. When was applied the current density of 75 A m^{-2}, the drugs were completely degraded in less than 5 min in synthetic effluent. The acid solution was the most efficient in the degradation of the compounds, however with the minimal variation when compared to the neutral and alkaline media. After 120 min of EO the drugs degradation varied between 33 and 52 % in municipal wastewater treatment plant. Tests were carried out to evaluate the effluent toxicity before and after the electrochemical oxidation treatment. The EO in municipal wastewater was not efficient to eliminate or decrease phytotoxicity. The best neurotoxicity result showed that municipal wastewater after treatment, upon contact with hippocampal slices, did not alter the production of reactive oxygen species, indicating that EO was efficient in eliminating toxicity.

Keywords: Advanced oxidative process; Electrolyte; CA3; *Lepidium sativum*.

Graphical Abstract



VI.1 Introduction

The technological and scientific advance in several areas such as pharmaceutical, agricultural and automotive products, has generated a great amount of new products (Lapworth *et al.*, 2012; Lamastra, Balderacchi and Trevisan, 2016). These substances are called compounds of emerging concern (CECs) and their effects on biota and the environment in many cases are still unknown. Some of these substances have recalcitrant properties, that is, they are not degraded in conventional biological treatments (Blair *et al.*, 2013; Souza *et al.*, 2016; Al-farsi *et al.*, 2018). An aggravation of this situation occurs in underdeveloped countries that do not even have sanitary sewage treatment and wastewater is disposed of directly in surface waters (Kay *et al.*, 2017). These compounds are found in the environment at concentrations ranging from ng L^{-1} to µg L^{-1} (Archer *et al.*, 2017; Souza *et al.*, 2017). Although they are relatively low concentrations, it is difficult to describe the consequences of exposure over time (Sui *et al.*, 2015).

The class of CECs is very broad, within this class there are the drugs, among them the benzodiazepines used in the treatment of insomnia and depression (Howard *et al.*, 2014; Hata *et al.*, 2018) and carbamazepine in the treatment of seizures (Bahlmann *et al.*, 2014). They have recalcitrant characteristics and are found at the beginning and end of sewage treatment plants and in surface water (Kanakaraju, Glass and Oelgem, 2018). For this reason it is necessary to apply some method that is capable of degrading these molecules, such as advanced oxidative processes (Ikehata *et al.*, 2007). These processes are essentially characterized by degradation through radicals with high oxidative potential (Rivera-utrilla *et al.*, 2013). Electrochemical oxidation is one of these processes, it is possible to use some types of electrodes, in which one of the most efficient is the boron doped diamond (BDD) (Burgos-Castillo *et al.*, 2018; Candia-Onfray *et al.*, 2018). One of the radical formations in the boron doped diamond (BDD) anode is the hydroxyl radical (Equation 1) which provides rapid degradation of the compounds, in some cases the results could be the mineralization of the drugs (Equation 2) (Siedlecka *et al.*, 2018).

$$BDD + H_2O \rightarrow BDD(\bullet OH) + H^+ + e^- \qquad (1)$$

$$BDD(\bullet OH) + M \rightarrow BDD + CO_2 + H_2O + H^+ + e^- \qquad (2)$$

In addition to using advanced oxidative processes in the degradation of recalcitrant compounds, it becomes very interesting to investigate the action of these compounds on the environment and effects on living beings. When treated, untreated compounds and by-products reach the water bodies, they come in direct contact with the biota present on the site, it is possible that some animals and plants have the ability to bioaccumulate these substances or that this contact generates a toxic effect by this there is a difficulty in telling what a long exposure can generate. A very interesting analysis is to evaluate how the counts of the treated and untreated effluents can influence the production of reactive oxygen species (ROS) in rat hippocampus. ROS is produced naturally and is essential for the organism to maintain its normal functions (Dröge, 2002; Wijk *et al.*, 2008). If something changes this production and the organism is not able to return to the normal state, it is in oxidative stress, which in most cases increases ROS production being harmful and could lead to the collapse of the organism (Freinbichler *et al.*, 2011).

Another way to evaluate the toxicity is with phytotoxicity, which can be used to evaluate the germination of the seeds of some species, among them *Lepidium sativum* and to detect in what way treated and untreated effluents can directly influence the development of the plant. This is a quick, simple and inexpensive way to identify the adverse effects of compounds. Phytotoxicity can be evaluated by plant color, root deformation, root length and seed germination rate (Grosu *et al.*, 2012; Carter *et al.*, 2018).

For this reason, the electrochemical oxidation by BDD anode was used in this study to degrade benzodiazepines and carbamazepine, identifying the degradation behavior in different parameters such as current density, pH, concentration and electrolyte types. In order to evaluate the toxicity of the solutions, the germination of seeds was analyzed, as well as the variation of ROS of mossy fibers on hippocampus of Wistar rats. And to evaluate the life of the anode, the surface of the anode was analyzed before and after the experiments.

VI.2 - Material and Methods

VI.2.1 – Chemical and reagents

In this study were used 5 different drugs: Alprazolam (ALP), clonazepam (CZP), diazepam (DZP), lorazepam (LZP) and carbamazepine (CBZ), physical and chemical characteristics of these pharmaceutically active compounds (PhACs) are possible to find in previous study Bosio et al. 2018.

The reagents acetonitrile (CH_3CN, purity, ≥ 99.9%), sodium chlorine ($NaCl$, purity, ≥ 99.5%), magnesium dichloride ($MgCl_2$, purity, ≥ 98.0%), calcium chloride ($CaCl_2$, purity, ≥ 99.0%), potassium chloride (KCl, purity, ≥ 99.0%), sodium bicarbonate ($NaHCO_3$, purity, ≥ 99.7%), D-glucose ($C_6H_{12}O_6$, purity, ≥ 99.5%) and sodium dihydrogen phosphate (NaH_2PO_4, purity, ≥ 99.0%) were obtained from Sigma-Aldrich. Sodium hydroxide ($NaOH$) and sulfuric acid (H_2SO_4) were obtained from Labkem and Honeywell/Fluka, respectively. The ROS indicator H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) used in neurotoxicity experiments was obtained from Life technologies. Filter paper (78 g m^{-2}) used in phytotoxicity was obtained from Deltalab. The ultrapure water was obtained from Direct-Pure Interlab system.

VI.2.2 – Electrochemical oxidation process

The type of the reactor used was batch-stirred in a laboratory scale at 25 ºC and at atmospheric pressure. In this study were used 3 different kind of water: Ultrapure water (UPW); Water from river Mondego (WRM) (Coimbra-Portugal); Water from municipal wastewater treatment plant (MWWTP) (Coimbra-Portugal). In all experiments were used

0.5 L of water and the main characteristics of the effluents is possible to see in Table VI.1. In the WRM and the MWWTP were not detected PhACs at the limit of the equipment, for this reason in all experiments were spiked PhACs at concentration of 100 µg L^{-1}.

Table VI.1 – Main characteristics of UPW, WRM and MWWTP.

Main characteristcs	**UPW**	**WRM**	**MWWTP**
Alprazolam (µg L^{-1})	98.89 ± 2.41	<LOD	<LOD
Clonazepam (µg L^{-1})	99.05 ± 1.52	<LOD	<LOD
Diazepam (µg L^{-1})	98.99 ± 1.78	<LOD	<LOD
Lorazepam (µg L^{-1})	100.74 ± 1.18	<LOD	<LOD
Carbamazepine (µg L^{-1})	102.51 ± 0.88	<LOD	<LOD
pH	3-10	7.25	7.38
Conductivity (µS cm^{-1})	120-230	117	731

LOD = Limit of Detection

The anode used was boron doped diamond (BDD) and the cathode was stainless steel, with the effective area of 20 cm^2 (8.0x2.5x0.1 cm). They were arranged in parallel with a gap distance of 1 cm and they were connected to a DC power supply HY3010 Kaise (I = 1.2-2.5 A). The pH of the synthetic solution was measured by Hanna pH meter and it was modified as needed in the beginning of the experiments between 3 and 10 using H_2SO_4 at 1 M to acidify the solution and NaOH at 2 M to make the alkaline solution. The operating system can be seen in Figure VI.1.

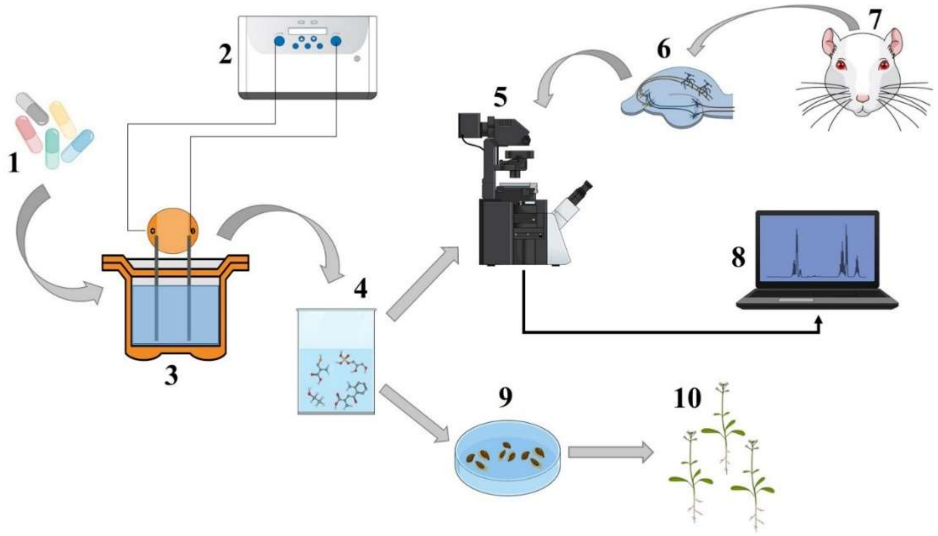


Figure VI.1 – Operational set up: (1) - Pharmaceutically active compounds; (2) – Power supply; (3) – Electrochemical oxidation reactor; (4) – Treated effluent; (5) – Neurotoxicity tests; (6) – Hippocampal slice; (7) – Wistar rat; (8) – Data analyzed; (9) – Phytotoxicity tests and (10) – Germination of plants.

VI.2.3 – Analytical Techniques

In the solutions, the concentration of alprazolam, clonazepam, diazepam, lorazepam and carbamazepine were determined by high performance liquid chromatography (HPLC) from Shimadzu with a diode-array detector. The column used was C18 analytical (AQ C18 Silia Chrom, 5 µm, 100 Å, 4.6 x 250 mm) kept at 25 ºC. The mobile phase consists of the linear gradient 45:55 acetonitrile:water at 0 min to 70:30 acetonitrile:water at 15 min, the injection volume and the flow rate were 100 µL and 1.0 mL.min^{-1}, respectively. The compounds were determined at different wave-length (ʎ), with different limits of quantification (LOQ) and detection (LOD): Alprazolam (224 nm, 35.3 µg L^{-1} and 10.6 µg L^{-1}); Clonazepam (310 nm, 15.1 µg L^{-1} and 4.5 µg L^{-1}); Diazepam (229 nm, 4.0 µg L^{-1} and 1.2 µg L^{-1}); Lorazepam (232 nm, 1.0 µg L^{-1} and 1.3 µg L^{-1}) and Carbamazepine (286 nm, 3.82 µg L^{-1} and 1.1 µg L^{-1}). Before the HPLC analysis all the samples were filtered by Sartorius cellulose acetate filter (0.45 µm) and the chromatographic analyses were performed at the same time of the experiments.

VI.2.4 – Morphological characterization of the BDD anode

For the analysis of the morphological characteristics of the BDD anode before and after used, was used the scanning electron microscopy (SEM) at 500 x magnification with a Vega 3 SBH-Tescan equipment.

VI.2.5 – Detection of ROS signals from rat hippocampal slices

Three different solutions were used for hippocampal experiments, they were represented in Table VI.2. For all experiments were used hippocampal slices from pregnant Wistar rats (Gestational 14-18 days). After anesthetized the rats had the cervical displacement and the decapitation as fast as possible. All experiments with animals were performed according to European Communities Council Directives (2010/63/UE) in order to minimize the suffering of animals and only the necessary amount of animals were used to produce reliable scientific data.

Table VI.2 – Different solution used in hippocampal experiments.

Sample	**Matriz**	**Compounds (μg L^{-1})**	**Time (min)**	**NaCl (g L^{-1})**	**Current density (A m^{-2})**
SyE - 0	Ultrapure water	PhACs (100)	0	-	0
SyE - 1	Ultrapure water	PhACs (100)	30	1.5	25
SWPh - 0	River	PhACs (100)	60	-	25
MWWTP - 0	MWWTP	PhACs (100)	60	-	25
MWWTP - 2	MWWTP	PhACs (100)	120	-	25

The brain was extracted and it was transferred for a cold artificial cerebrospinal fluid (ACSF), this solution had the composition of (in mM): D-Glucose 10.0; NaCl 124.0; NaH_2PO_4 1.25; KCl 3.5; $MgCl_2$ 2.0; $CaCl_2$ 2.0; $NaHCO_3$ 24.0. The hippocampus was extracted and it was sliced by transversal cuts with blades spaced 400 µm. The slices were transferred to ACSF oxygenated (95% O_2 and 5 % CO_2) at room temperature. The slices were incubated for 1 h in a oxygenated ACSF solution with 20 µM of the fluorescent ROS (H_2DCFDA) indicator. Sequentially the slices were transferred as soon as possible for an oxygenated ACSF solution and for the beginning of the experiments they were transferred for a chamber with ACSF perfused (1.5 – 2.0 mL min^{-1}) and kept at controlled temperature (30 – 32 ºC).

The data collection was obtained from a fluorescence microscope Zeiss Axioskop equipment. It has a tungsten/halogen lamp with 12 V and 100 W and the excitation filter of 480 nm and the emission filter of 500 nm. The lens used was a water immersion lens (40 x, N.A. 0.75) and a photodiode (1 mm^2) from Hammamatsu. The data for synapses study were obtained from hippocampus slice at the mossy fiber CA3 region. To process the data from photodiode was used a I/V converter (1 GΩ feedback Instruments) processed at 1.67 Hz and using the Signal Express^{TM} software. The measurements were an average of 100 points for the one-minute interval.

VI.2.6 – Phytotoxicity on Lepidium sativum germination

Different solutions were used to analyzed the effect of compounds on the *Lepidium sativum* germination. It is possible to observe the solutions on Table VI.3. All the experiments were made in duplicate and simultaneous with seeds growing in control solution and conditions. To analyzed the evolution of the seeds, they were kept for 48 h in the absence of light and at 25 ºC, were used petri dish contained 5 mL of the solution and 10 seeds on filter paper. To calculate and evaluate the percentages of relative seed germination (RSG), relative root growth (RRG) and germination index (GI) were used Equation 3, 4 and 5, respectively. Phytotoxicity has different classification according to the germination index, such as: no inhibition if GI is greater than 80 %; mild inhibition if GI is between 80 and 60 %: strong inhibition if GI is between 60 and 40 % and severe inhibition if GI is less than 40 % (Pinho *et al.*, 2017).

Table VI.3 - Solutions tested on phytotoxicity.

Sample	**Matriz**	**Compounds (µg L^{-1})**	**EO**	**pH**	**Time (min)**	**NaCl (g L^{-1})**	**I (A m^{-2})**
Control	Ultrapure water	-	No	7	-	-	-
SyE - 1	Ultrapure water	PhACs (100)	Yes	7	30	1.5	25
SyE - 2	Ultrapure water	PhACs (100)	Yes	3	30	1.5	25
SyE - 3	Ultrapure water	PhACs (100)	Yes	10	30	1.5	25
SWPh - 1	River	PhACs (100)	No	7.25	-	-	-
SWPh - 2	River	PhACs (100)	Yes	7.25	90	-	25
MWWTP - 1	MWWTP	PhACs (100)	Yes	7.38	90	-	25

$$RSG(\%) = \frac{Number\ of\ seeds\ germinated\ in\ tested\ solution}{Number\ of\ seeds\ germinated\ in\ control\ solution} x100 \qquad (3)$$

$$RRG(\%) = \frac{Mean\ root\ leng\quad in\ tested\ solution}{Mean\ root\ length\ in\ control\ solution} x100 \qquad (4)$$

$$GI(\%) = \frac{RSGxRRG}{100} \qquad (5)$$

VI.3. Results and Discussion

VI.3.1 Effect of current density on PhACs degradation

In this topic, the effect of the current density was discussed. This is one of the most important parameter in the degradation of the compounds (Sirés and Brillas, 2012), because with the increase of the current density occurs the increase of oxidative radicals as •OH, Cl^- and ClO_3^- that are responsible to degrade some recalcitrant compounds (Fabianska *et al.*, 2015).

For this reason, the effect of current density in the degradation of ALP, CZP, DZP, LZP and CBZ was used in three different values 13, 25 and 75 A m^{-2} and they were applied in a synthetic solution (Figure VI.2 A-E; Table VI.1 – Experiments 1, 2 and 3). For this experiments the completely (≤ LQO) degradation of CZP, DZP, LZP and CBZ occur in 5, 10 and 30 min for 75, 25 and 13 A m^{-2}, respectively. And the completely degradation of ALP occur at 5, 20 and 30 min for 75, 25 and 13 A m^{-2}, respectively. The degradation increase with the increase of the current density, because the reaction between the drugs and the oxidative radical on BDD non-active anode is characterized to a complete mineralization of the organic compounds (Baddouh *et al.*, 2018).

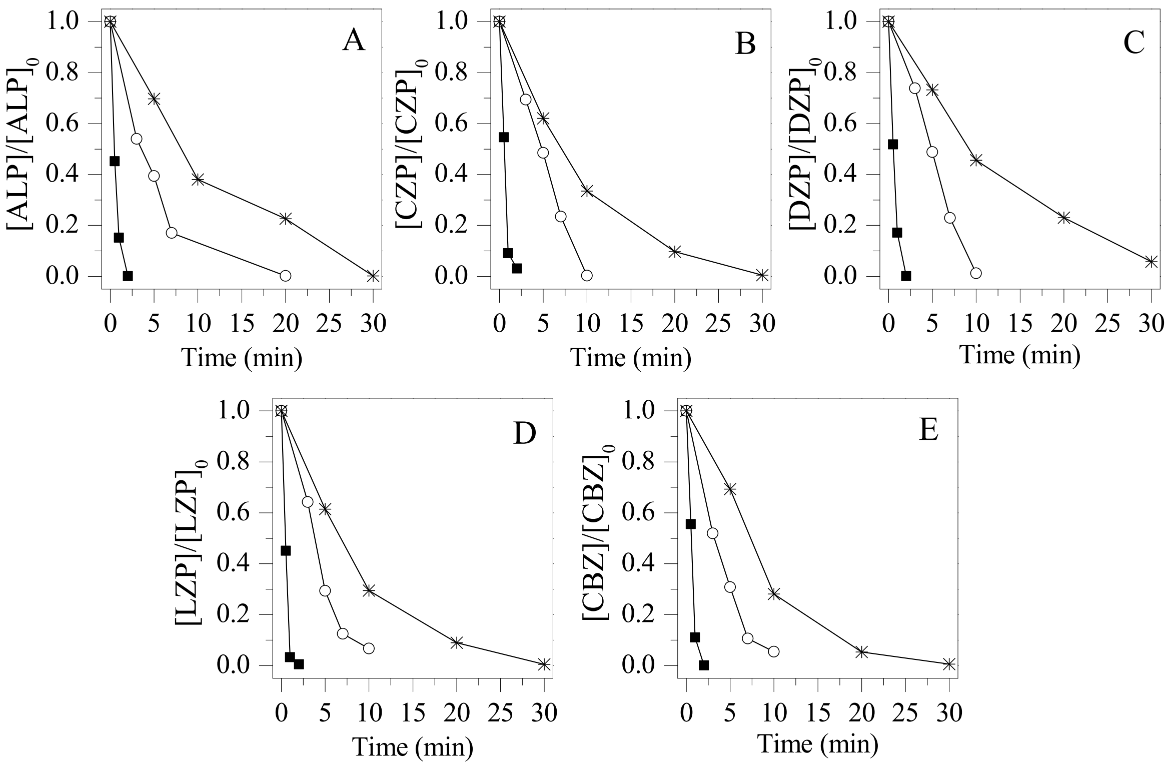


Figure VI.2 – The effect of current density (■ - 75 A m^{-2}, ○ - 25 A m^{-2} and ✳ 13 - A m^{-2}) on the ALP, CZP, DZP, LZP and CBZ degradation using BDD anode. (1.5 g NaCl L^{-1}, 100 µg PhACs L^{-1}, pH = 7, T = 25 ºC).

The pseudo first order kinetic constants were calculated for all compounds during the experiments (Table VI.4), the highest kinetic constants of ALP, CZP, DZP, LZP and CBZ were at the highest current density. As the current density doubled from 13 to 25 A m^{-2}, the kinetic constants increased about 2-fold.

Table VI.4 – Main conditions of the electrochemical oxidation experiments and pseudo first kinetic constants

			Initial Conditions					Pseudo first order kinetic constants	
Exp.	**PhACs**	**[μg L^{-1}]**	**Current density (A m^{-2})**	**Water**	**pH**	**NaCl [g L^{-1}]**	**Na_2SO_4 [g L^{-1}]**	**k (min^{-1})**	**R^2**
1	ALP	100	75	UPW	7	1.5	-	1.71 ± 0.15	0.99
	CZP	100						1.64 ± 0.36	0.94
	DZP	100						1.56 ± 0.19	0.98
	LZP	100						1.99 ± 0.60	0.92
	CBZ	100						1.61 ± 0.35	0.95
2	ALP	100	25	UPW	7	1.5	-	0.21 ± 0.03	0.97
	CZP	100						0.16 ± 0.02	0.93
	DZP	100						0.17 ± 0.03	0.94
	LZP	100						0.22 ± 0.04	0.91
	CBZ	100						0.25 ± 0.02	0.97
3	ALP	100	13	UPW	7	1.5	-	0.08 ± 0.01	0.97
	CZP	100						0.10 ± 0.01	0.99
	DZP	100						0.07 ± 0.01	0.99
	LZP	100						0.11 ± 0.01	0.99
	CBZ	100						0.13 ± 0.01	0.99
4	ALP	100	25	UPW	3	1.5	-	0.29 ± 0.06	0.93
	CZP	100						0.21 ± 0.04	0.93
	DZP	100						0.27 ± 0.03	0.98
	LZP	100						0.29 ± 0.05	0.96
	CBZ	100						0.44 ± 0.14	0.93
5	ALP	100	25	UPW	10	1.5	-	0.18 ± 0.04	0.90
	CZP	100						0.14 ± 0.02	0.97
	DZP	100						0.16 ± 0.01	0.99
	LZP	100						0.20 ± 0.02	0.98
	CBZ	100						0.26 ± 0.02	0.99
6	ALP	100	25	WRM	7.25	-	-	0.030 ± 0.001	0.99
	CZP	100						0.029 ± 0.002	0.99
	DZP	100						0.028 ± 0.002	0.97
	LZP	100						0.045 ± 0.002	0.99
	CBZ	100						0.041 ± 0.002	0.99
7	ALP	100	25	MWWTP	7.38	-	-	0.005 ± 0.0003	0.98
	CZP	100						0.005 ± 0.0004	0.97
	DZP	100						0.003 ± 0.0002	0.98
	LZP	100						0.004 ± 0.0001	0.99
	CBZ	100						0.006 ± 0.0006	0.95
8	ALP	100	25	UPW	7	0.5	-	0.02 ± 0.001	0.99
	CZP	100						0.05 ± 0.005	0.97
	DZP	100						0.03 ± 0.002	0.98
	LZP	100						0.04 ± 0.001	0.99
	CBZ	100						0.03 ± 0.003	0.98
9	ALP	100	25	UPW	7	-	1.5	0.019 ± 0.0004	0.99
	CZP	100						0.05 ± 0.007	0.93
	DZP	100						0.01 ± 0.0008	0.97
	LZP	100						0.02 ± 0.0007	0.99
	CBZ	100						0.02 ± 0.0008	0.99

Although the kinetic constants of the compounds vary with the increase of the parameter, if the kinetics are analyzed with a fixed current density, it is possible to observe that ALP, CZP, DZP, LZP and CBZ have similar kinetic constants. This can be explained by the molecular structures of the compounds, which are organic molecules of small size and pKa of the substances, which among the benzodiazepines, has a small variation between 1.3-3.4. This corroborates that the variations in the degradations are not so different. CBZ in turn presented the highest kinetic constants at current densities of 13 and 25 A m^{-2}, a fact that may be directly related to the high pKa (13.9) value. The increase in current density and the complete degradation of compounds is in accordance with the results of several authors (Haidar *et al.*, 2013; Cheng *et al.*, 2016; Loos *et al.*, 2018) and in other cases the increase in the current density resulted in a partial or complete mineralization of the organic compounds (García-Montoya *et al.*, 2015; Garcia-segura *et al.*, 2015; Candia-Onfray *et al.*, 2018; Vasilie *et al.*, 2018).

Among the current densities used, it was selected for the next experiments the value of 25 A m^{-2}, since the reaction time is relatively low, however it is possible to perform the collection of several points.

VI.3.2 Effect of initial pH on PhACs degradation

The pH is an important parameter to evaluated the degradation of the organic compounds, however there is a contradiction in the studies of which medium is the most efficient in the degradation of organic compounds, this can be justified by the differences in the molecular structures of the compounds used and the electrode material (Rabaaoui *et al.*, 2017). Some studies indicate that the degradation of the compounds is favored in acidic media, because in this medium the maximum formation of •OH occurs (Czupryniak, Stepnowski and Ossowski, 2012; Chen, Xia and Dai, 2015; Lin *et al.*, 2016), while other studies indicate that the maximum degradation occurs in alkaline media (Pacheco *et al.*, 2011; Wang *et al.*, 2015; Berenguer *et al.*, 2016). Based on this variation in degradation, the experiments were performed at three different pH in a synthetic effluent that is possible to observe in Figure VI.3 (A-E: ■ - 3, ○ - 7 and ✳ - 10) and the pseudo first order kinetic in Table VI.2 (Experiments 2, 4 and 5).

The degradation of ALP, CZP, DZP, LZP and CBZ was very similar in the acid, neutral and alkaline media, some studies report a similar degradation of organic compounds in different pH solution (Czupryniak, Stepnowski and Ossowski, 2012). In all cases, 10 min was required for the complete elimination of the compounds, except for carbamazepine which in acidic solution was degraded in 5 min with a pseudo first order kinetic constant of 0.44 ± 0.14, that is approximately 1.7 fold greater than the constants of the other compounds in the same solution. This more efficient degradation of carbamazepine can be explained by the fact that this compound has a high pKa, which in an acidic solution favors the molecular interactions and reactions between CBZ and the radicals produced by electrochemical oxidation (Molinari *et al.*, 2006; Zhang *et al.*, 2014).

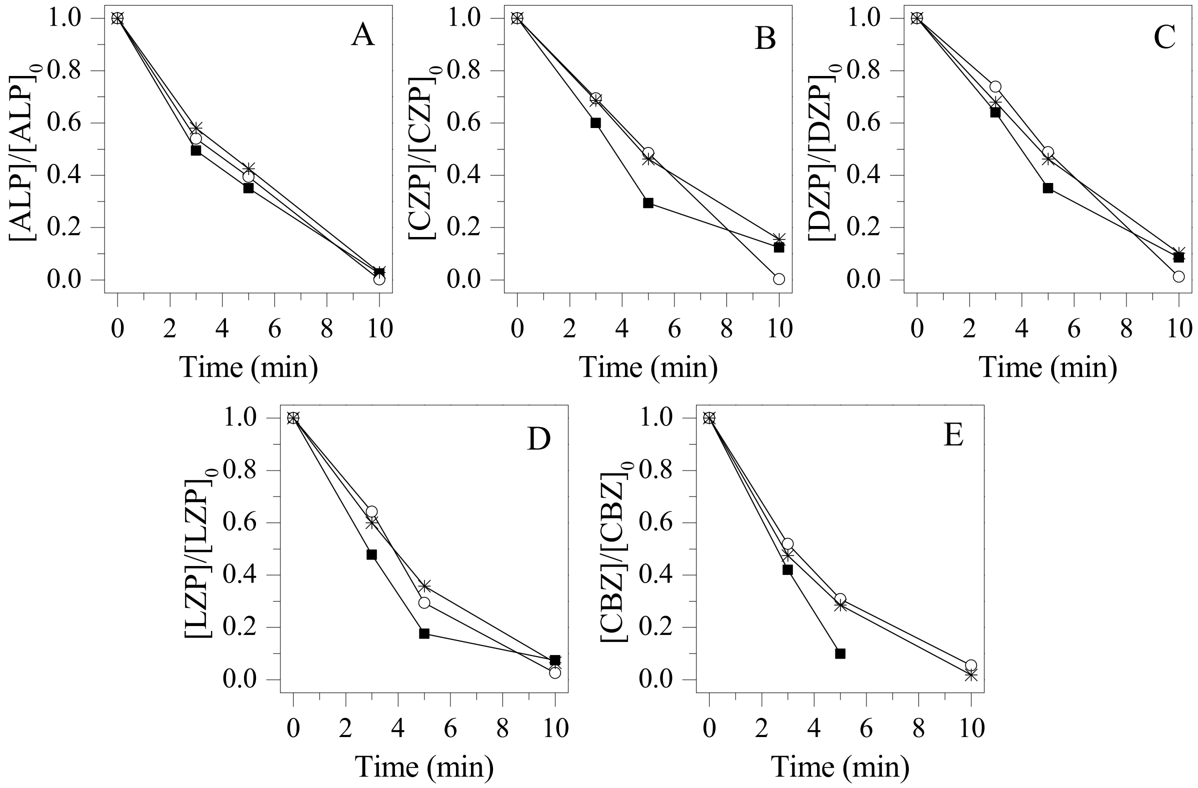


Figure VI.3 – The effect of pH (■ - 3, ○ - 7 and ✳ - 10) on the ALP, CZP, DZP, LZP and CBZ degradation using BDD anode. (1.5 g NaCl L^{-1}, 100 µg PhACs L^{-1}, $J = 25$ A m^{-2} and T = 25 ºC).

VI.3.3 Effect of aqueous matrix on PhACS degradation

The degradation of the compounds was evaluated in different aqueous matrix: (■) synthetic effluent (SyE); (○) surface water (Mondego river) spiked with PhACs (SWPh); (✳) municipal waste water treatment plant spiked with PhACs (MWWTP). In Figure

VI.4 A, B, C, D and E it is possible to observe the effect of the aqueous matrix in ALP, CZP, DZP, LZP and CBZ degradation, respectively.

The complete degradation in SyE was given in 10 min for all compounds analyzed, CBZ had the highest first order constant 0.25 min^{-1} (Table VI.4 - Experiment 2), probably due to the fact that carbamazepine has the highest pKa (13.9) and is the molecule, among those studied, with the greatest chemical structural difference, such as the absence of Cl in its composition. In SWPh to improve the completely degradation of all PhACs was necessary 90 min and the pseudo first order was smaller than in SyE around 6 fold. While in MWWTP after 120 min of electrochemical oxidation it was not possible to completely degrade the compounds and their respective degradation kinetics of the compounds are in Table VI.4 – Experiment 7. The decreases in degradations and kinetic constants in these media are caused by the presence of other organic and/or recalcitrant compounds that compete directly with the radicals produced by EO (Deng and Zhao, 2015), the decrease in the efficiency of the degradations in real aqueous media were also reported by (Song *et al.*, 2017; Bosio *et al.*, 2018; Dominguez *et al.*, 2018).

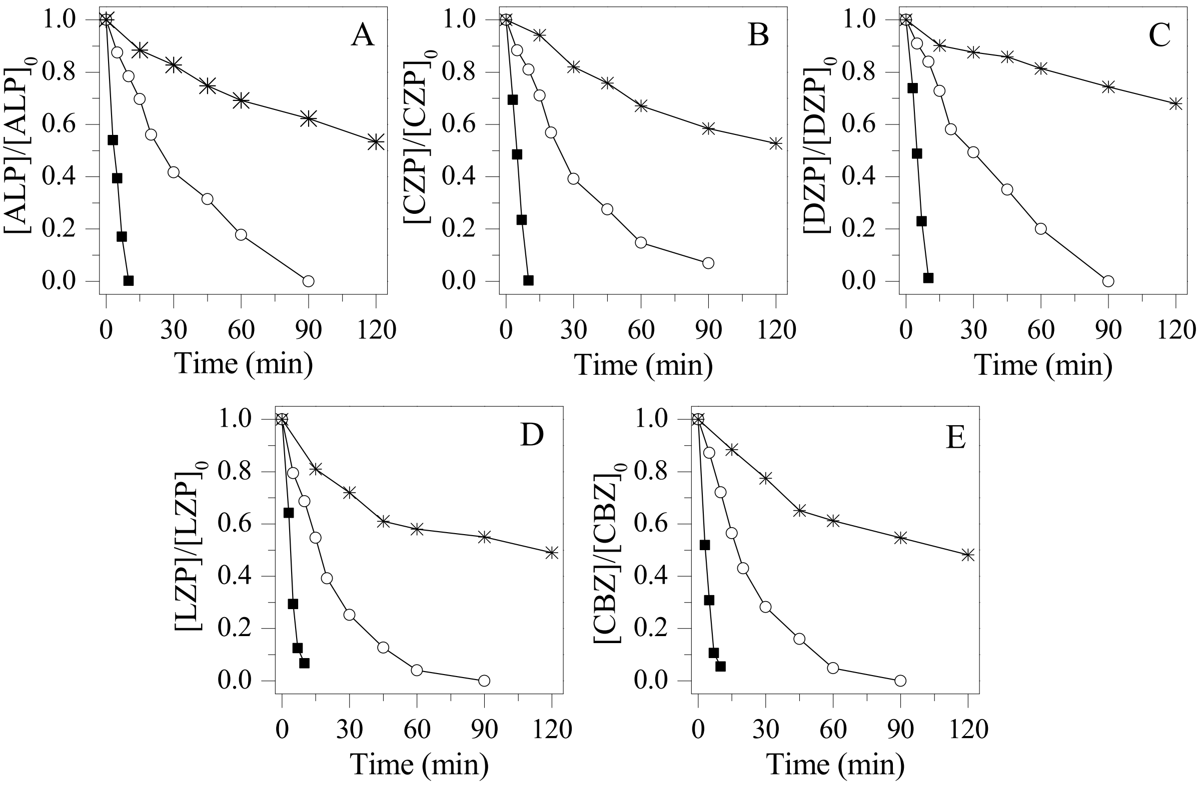


Figure VI.4 - The effect of aqueous matrix (■ - SyE, ○ - SWPh and ✳ - MWWTP) on the ALP, CZP, DZP, LZP and CBZ degradation using BDD anode. (100 µg PhACs L^{-1}, J = 25 A m^{-2}, T = 25 ºC and 1.5 g NaCl L^{-1}, the electrolyte was used only in synthetic solution).

VI.3.4 Effect of salt

The degradation of the compounds was analysed with variation of electrolytes: Na_2SO_4 (✳ - 1.5 g L^{-1}) and different concentration of NaCl (○ - 0.5 g L^{-1} and ■ - 1.5 g L^{-1}). The addition of electrolytes increases the conductivity of the solution, which generally leads to a greater degradation of the organic compounds (Chen, 2004; Martínez-Huitle and Ferro, 2006; Särkkä, Bhatnagar and Sillanpää, 2015; Migliorini *et al.*, 2017; Garcia-Segura, Ocon and Chong, 2018). Figure VI.5 - A, B, C, D and E is possible to see the different degradation of the ALP, CZP, DZP, LZP and CBZ, respectively.

Two different types of electrolytes were used in synthetic medium, NaCl generate radicals as HClO (Equation 3.1), ClO^- (Equation 3.2), ClO_2^- (Equation 3.3), ClO_3^- (Equation 3.4) and Na_2SO_4 generate radicals as SO_4^{2-} (Equation 3.5) that can aid in the degradation of the PhACs (Salazar *et al.*, 2016). These radicals that are responsible for the indirect degradation, when associated with the radicals of the direct degradation, increase the degradation of the compounds. However, it is possible to observe that the degradation with 1.5 g L^{-1} of NaCl was greater than the degradation with 1.5 g L^{-1} of Na_2SO_4.

$$HClO + Organic\ Compounds \rightarrow CO_2 + H_2O + HCl \qquad (3.1)$$

$$ClO^- + H_2O \rightarrow ClO_2^- + 2H^+ + 2e^- \qquad (3.2)$$

$$ClO_2^- + H_2O \rightarrow ClO_3^- + 2H^+ + 2e^- \qquad (3.3)$$

$$ClO_3^- + H_2O \rightarrow ClO_4^- + 2H^+ + 2e^- \qquad (3.4)$$

$$2SO_4^{2-} \rightarrow S_2O_8^{2-} + 2e^- \qquad (3.5)$$

Comparing the NaCl electrolyte concentration of 1.5 g.L^{-1} and 0.5 g L^{-1} it is possible to observe that the higher concentration of NaCl generated a decrease in the time of degradation of the compounds, being necessary only 10 min so that the complete elimination (≤ LQO) of them. Some studies also detected the increase in efficiency, with the increase in NaCl concentration, this is due to the fact that a higher concentration of electrolyte leads to an increase in conductivity and consequently the generation of more radicals (Maljaei, Arami and Mohammad, 2009; Morsi, Al-Sarawy and Shehab El-dein, 2011; Indu, Gupta and Sahoo, 2014). The increase in the reaction rate for the degradation of 0.5 g L^{-1} NaCl relative to 1.5 g L^{-1} NaCl was 10.5, 3.2, 5.6, 5.5 and 8.33 fold for ALP, CZP, DZP, LZP and CBZ, respectively. While the increase in reaction rate from 1.5 g L^-

^{1} NaCl to 1.5 Na_2SO_4 was 11.0, 3.2, 1.7, 11.0 and 12.5 for ALP, CZP, DZP, LZP and CBZ, respectively.

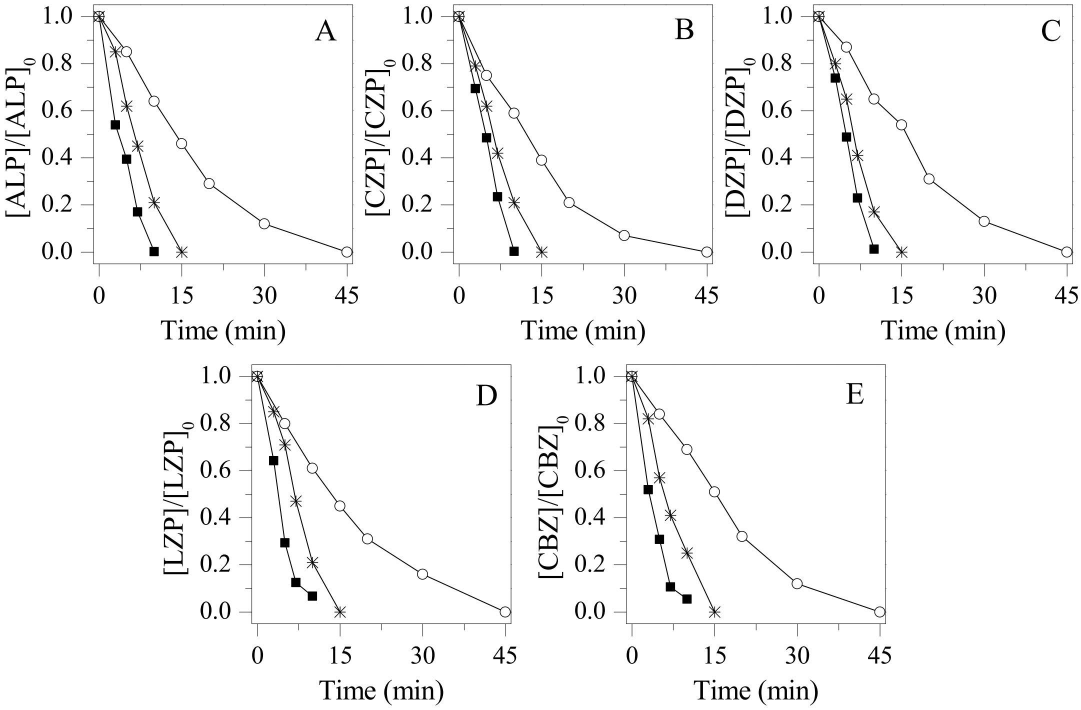


Figure VI.5 - The effect of electrolyte concentration (NaCl = ○ - 0.5 g L^{-1}; ■ - 1.5 g L^{-1} and Na_2SO_4 = ✳ - 1.5 g L^{-1}) on the ALP, CZP, DZP, LZP and CBZ degradation using BDD anode. (J = 25 A m^{-2}, 100 μg PhACs L^{-1}, pH = 7, T = 25 ºC).

VI.3.5 Effect on the anode surface before and after the electrochemical oxidation

The morphological analysis of the anode is necessary to evaluate the level of wear and if it is possible to reuse it, the reuse and effectiveness of the reactions are directly related to the cost of the process implemented. There are some situations that may interfere with electrode surface wear, such as chemical and / or physical absorption of some substances from electrochemical oxidation, corrosion of the electrode and its deactivation. However the electrode composed of BDD is characterized by low adsorption and resistance to the electrode deactivation process (Migliorini et al. 2016). Figure VI.6 A and B presents the morphological characterization of the electrode before and after use, respectively (the analysis after use was made only after all experiments were performed). The electrode analysis before use shows a linear deposition at the electrode and a high surface area, indicating high efficiency in the degradation of the compounds (Alves *et al.*, 2012; Vasconcelos *et al.*, 2016). It is possible to observe that after the reactions, the electrode continues with the initial morphology, without the presence of cracks indicating that the material is resistant and allows the reuse of the anode (Souza et al 2014; Lu et al 2018).

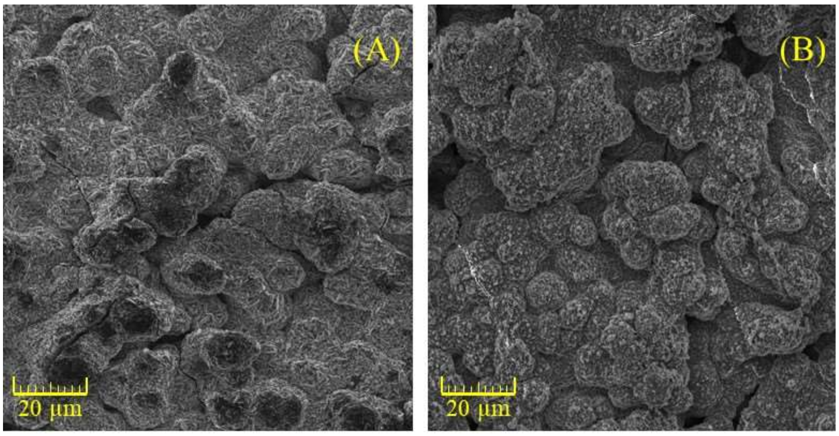


Figure VI. 6 - SEM photographs of the BDD anode material before used at 1000x (A) and after used at 1000x (B) on electrochemical oxidation process.

VI.3.6 Effect of different solutions on the neuronal studies

In order to evaluate the production of ROS in hippocampal slices from different effluents, mixtures of ALP, CZP, DZP, LZP and CBZ were used in different aqueous matrices with and without electrochemical oxidation treatment. Figure VI.7 A indicates the action of synthetic effluent before EO (SyE – 0), Figure VI.7 B represents the results obtained applying the synthetic solution after the EO (SyE – 1), Figure VI.7 C shows the results caused by surface water after EO treatment (SWPh - 0), Figure VI.7 D (MWWTP - 0) and VI.7 E (MWWTP - 2) indicate the behavior of the signals with municipal wastewater after EO treatment.

In Figure VI.7 A it can observed that the SyE – 0 was applied during the period indicated by the grey bar, the ROS signals decreased with an amplitude of 0.07 ± 0.01 S.E.M, n = 3, in relation to the baseline. With the return of the ACSF solution, the signals recovered and between 60 and 70 min stabilized with an amplitude of 0.05 ± 0.02 S.E.M, n = 3, above the baseline. In Figure VI.7 B the synthetic effluent after EO was applied in the time represented by the grey bar, it is possible to observe that a variation occurred in the amplitude of the signals 0.19 ± 0.01 S.E.M, n = 3, below the baseline. However, in relation to the untreated effluent (Figure VI.7 A), the decrease was almost 4 times higher, indicating that EO caused alterations of the compounds present in the effluent.

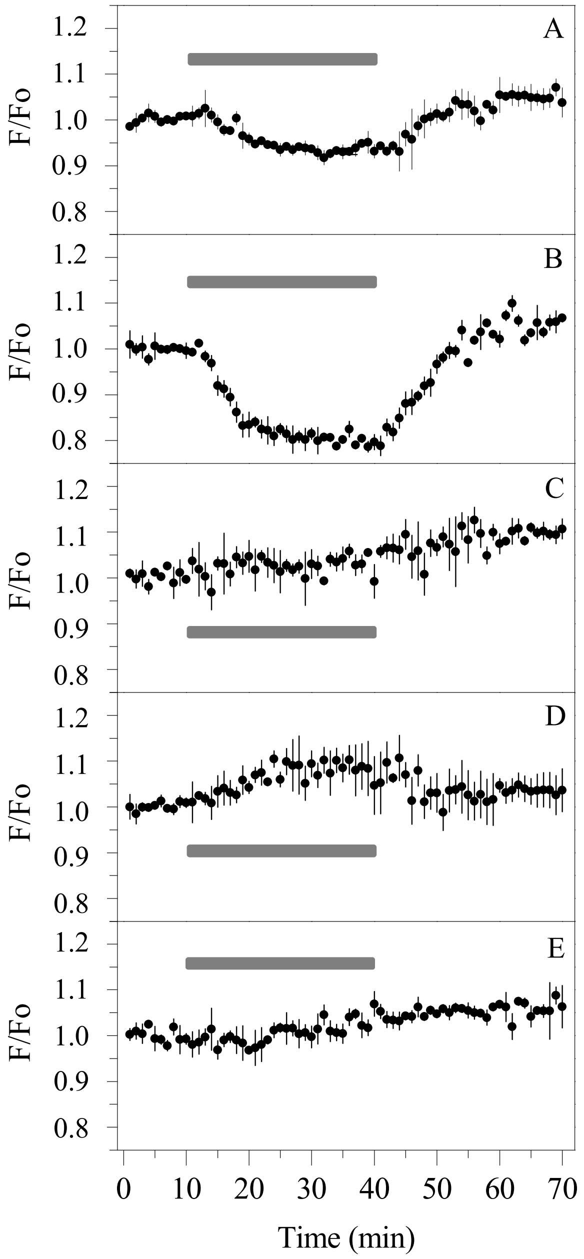


Figure VI.7 – Effects on ROS signals of benzodiazepines and carbamazepine compounds degraded by electrochemical oxidation and the mixture was applied during 30 min represented by the grey bar. In order to collect the data was used the fluorescent ROS indicator H_2DCFDA (20 µM). A (SyE - 0), B (SyE - 1), C (SWPh - 0), D (MWWTP - 0) and E (MWWTP - 2).

These new substances have altered the production of ROS inside the cells which may have caused oxidative stress (Blacker and Duchen, 2016). A situation in which the cell begins to produce ROS in quantities different from those necessary for its normal functioning, which can lead to morphological variations and even lead to the death of it (Arriba *et al.*, 1999; Sasaki *et al.*, 2018). This variation in amplitude of ROS production after EO was also reported by Bosio, Souza, et al. 2018.

In Figure VI.7 C the neurotoxicity was evaluated using surface water after EO in the interval which is represented by the grey bar. From 10 to 40 min it is possible to observe that the amplitude of the signals increases in relation to the baseline of 0.03 ± 0.02 S.E.M, n = 3. After the ACSF solution returned at 40 min, the signals continued to increase with maximum amplitude values between 60 and 70 min of 0.09 ± 0.01 S.E.M, n = 3. ROS production did not return to the baseline during the analysed period, noting that the SWPh - 0 solution caused irreversible effects on the slices. One of the possibilities for the increase of the signals was the byproducts generated during the EO.

In Figures VI.7 D and E were applied solutions from municipal wastewater after 60 and 120 min of EO, respectively. It is possible to observe that the solution with the shortest treatment time promotes a variation in the amplitude of 0.08 ± 0.03 S.E.M, n = 3. The use of this solution resulted in an increase in the production of ROS, caused by substances present in the effluent that can enter the cells or change their functioning as the channels through which they pass essential substances for the maintenance of them (Ivanov and Zilberter, 2011). The MWWTP – 2 had an amplitude of 0.01 ± 0.01, S.E.M, n = 3, the solution after 120 min of EO treatment did not cause significant variation in ROS production. This shows that advanced oxidative processes such as electrochemical oxidation are effective in eliminating the toxicity of solutions. A study carried out by Gomes, Bednarczyk, et al. 2017 with advanced oxidative process in the treatment of a mixture of parabens, obtained the same result eliminating the toxicity of the solution.

VI.3.7 Effect of different solutions on Lepidium sativum germination

The germination index (GI) of *L. sativum* in contact with different solutions is show in Table IV.5. The GI of the synthetic water untreated solution was 88 ± 3 (realized in previously study / data not shown). The samples of synthetic water SyE – 1, SyE – 2, SyE – 3 had higher GI compared to the untreated synthetic solution and all these samples have GI considered without inhibition. Although all the solutions are in the same classification, it is possible to observe that after the electrochemical oxidation, the GI of the treated samples increased. The germination index of SWPh – 1 before the electrochemical oxidation had a GI of 76 % that fits mild inhibition, whereas the same solution when passing through the advanced oxidative process had a GI of 107 % that is no inhibition. The advanced oxidative process may increase GI, since mineralization of compounds that may be more toxic prior to treatment may occur (Gomes, Leal, *et al.*, 2017; Monteiro *et al.*, 2018). However, even after the electrochemical treatment MWWTP – 1 continued with the same phytotoxicity as the solution had before treatment (data not shown), that is, the treatment time and the current density were not sufficient to decrease the phytotoxicity of the solution.

Table VI.5 – Phytotoxicity on *L. sativum* germination.

Sample	**Matriz**	**SG (%)**	**RG (%)**	**GI (%)**
Control	Ultrapure water	100 ± 1	100 ± 2	100 ± 2
SyE - 1	Ultrapure water	100 ± 2	90 ± 2	90 ± 4
SyE - 2	Ultrapure water	100 ± 3	98 ± 2	98 ± 5
SyE - 3	Ultrapure water	95 ± 3	105 ± 1	100 ± 3
SWPh - 1	River	90 ± 2	85 ± 2	76 ± 4
SWPh - 2	River	100 ± 3	107 ± 3	107 ± 6
MWWTP - 1	MWWTP	90 ± 4	72 ± 1	64 ± 4

VI.4 Conclusion

Electrochemical oxidation using BDD anode applied to treat the mixture of benzodiazepines and carbamazepine, the process was efficient in the degradation of the compounds with the variations of different parameters. When synthetic effluent was used the best degradation occurred when using the highest current density of 75 A m^{-2}. The acid medium had a small advantage in the elimination of the compounds, with little variation in relation to the neutral and alkaline medium. In relation to the use of electrolytes, the most efficient degradation occurred with the highest concentration of NaCl, this occurs due to the formation of reactive chroline species helped to increase the efficiency of the system. In surface water and municipal wastewater the efficiency has been reduced, due to the fact that in this type of water there are many substances that compete with benzodiazepines and with carbamazepine, this occurs because the radicals produced during EO are non-selective and react with all the substances present in the medium. The BDD anode after the experiments maintained the initial morphology and characteristics, proving to be stable and reusable.

On neurotoxicity, it was possible to detect that the drugs used in the study caused alterations in the synaptic functions, since the treated synthetic effluent caused changes in ROS production, advanced oxidative treatment was not effective to completely eliminate the toxicity of this solution. However, the MWWTP sample after treatment did not cause changes in neurotoxicity, indicating that EO was efficient to decrease the toxicity of this solution. In relation to phytotoxicity studies, the use of real effluents such as surface water and MWWTP were very influential. EO in the surface water was efficient to eliminate the phytotoxicity of the solution, however the same parameters applied in the MWWTP did not decrease the phytotoxicity, most probably due to the complexity of the substances present in this type of effluent.

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CHAPTER VII – Electrochemical Oxidation (Ruthenium and Iridium)

Article in preparation.

Removal of Emerging Contaminants from Aqueous Matrices Using Electrochemical Oxidation Process: Effects on Rat Hippocampus Neuronal Activity

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ABSTRACT

The present study is about the use of electrochemical oxidation (EO) process to remove contaminants of emerging concern as a strategy to reach an optimal condition to study the effects of treated water on rat hippocampus neuronal activity (CA3 area). The oxidation ability of the EO process was evaluated to degrade a mixture of carbamazepine and four benzodiazepines (alprazolam, clonazepam, diazepam, lorazepam) in pure water at different pH values (3 – 10), current density (30 – 100 A m^{-2}), and electrolyte concentrations (NaCl; 0.5 – 1.0 g L^{-1}). Ruthenium and iridium were used as anode materials and all pharmaceutical compounds had an initial concentration of 100 µg L^{-1} in the mixture. Experiments were carried out also in some conditions in river water and urban wastewater. No significant variations were observed in the degradation of compounds in relation to the pH. The optimal condition was achieved applying a current density of 100 A m^{-2}, pH 7 and 0.1 g NaCl L^{-1}, with a complete degradation of the drugs (below the limit of detection) in less than 5 min. Higher removal efficiencies were achieved using ultrapure water, probably due to the complex composition of the real matrices, which leads to greater competition for radicals produced in the electrochemical oxidation process. When using the MWWTP, residual pharmaceuticals were detected even after 120 min of reaction. In the neurotoxicity studies the solutions used caused different variations in the ROS production, the use of the synthetic solution after EO caused a decrease in ROS signals and was partially reversible, whereas the use of MWWTP after EO treatment led to an increase in ROS production and which proved to be irreversible.

Keywords: Advanced Oxidation Technologies; Benzodiazepines; Carbamazepine;
ROS indicator; Neuronal toxicity.

II.1 Introduction

It is quite common in metropolitan areas to find a higher incidence of insomnia, depression and stress related diseases, due to the dynamics of big cities and the lack of a pleasant work environment. These factors often lead to sleep loss, lack of will to interact with others, loss of concentration and a reduction in productivity (Cheng *et al.*, 2016). As a result, the consumption of medicines and health-care costs for treating insomnia and depression grows annually (Wickwire, Shaya and Scharf, 2016).

Many substances, including pharmaceuticals related to insomnia and depression, illicit drugs and personal hygiene products, are making their way into both natural and man-made bodies of water, both through direct disposal into rivers and through the incomplete degradation of some of these compounds by conventional wastewater treatment plants (WWTP). Therefore, these substances are easily found in aquatic environments such as rivers and lakes, and even in water treatment plants (WTP) (West and Rowland, 2012; Petrie, Barden and Kasprzyk-Hordern, 2014). In Spain, illicit drugs (13.2 – 294.0 ng L^{-1}), amphetamines (49.8 – 1,020 ng L^{-1}) and anxiolytics (2.9 – 149.0 ng L^{-1}) were found in superficial water, showing that even in developed countries, where urban wastewater treatment is more effective, it is still possible to find these and other related substances in sources of water (Mendoza *et al.*, 2014).

Some of the substances currently encountered in the environment are encompassed in a class of compounds known as Contaminants of Emerging Concern

(CECs), this is because these contaminants are not removed by conventional treatments in WWTPs and they are commonly considered as Persistent Organic Pollutants (POPs). Many of these have the potential to cause adverse effects both to local biota and to human beings, even in low concentrations (Noguera-Oviedo and Aga, 2016). Benzodiazepines (BZDs) and carbamazepine (CBZ) are part of CECs. CBZ is widely used in the treatment of insomnia, anxiety and seizures, and many studies report the presence of BZDs in bodies of water. Alprazolam and CBZ were detected in Swedish rivers in concentrations of 2.2 pg L^{-1} and 506.9 ng L^{-1}, respectively (Lindim *et al.*, 2016), while in Spanish rivers, alprazolam, diazepam and lorazepam were found in concentrations of 8.1, 25.8, and 86 ng L^{-1}, respectively (Mastroianni *et al.*, 2016). Carbamazepine was also observed in Indian rivers (128 ng L^{-1}) (Ramaswamy *et al.*, 2011).

When detected in drinking water, the concentration of pharmaceuticals may be much too low to cause toxic or therapeutic effects. However, the presence of drugs in drinking water poses a risk, since long-term exposure might lead to pharmaceutical effects, and some of the compounds may amplify the effect of others, thus becoming toxic to human health (Peng, Hall and Gautam, 2016).

The majority of municipal wastewater treatment plants (MWWTPs) are equipped only for biological treatment, which is insufficient to fully degrade compounds such as BZDs. One possible solution for this problem is the application of advanced oxidation processes (AOPs), which can partially or fully mineralize recalcitrant compounds. In AOPs, the powerful and non-selective hydroxyl radical (•OH) is produced, capable of degrading these substances, yielding CO_2, H_2O and inorganic ions (Bethi *et al.*, 2016; Cheng *et al.*, 2016).

Among the AOPs, electrochemical oxidation is a method easy to operate and control. In this process, compounds can be degraded with the production of •OH formed on the anode surface (AS) through the decomposition of water (Eq. VII.1). The addition of the electrolyte, for example sodium chloride (NaCl), leads to the production of active species of chlorine (Cl_2) due to the oxidation of the chloride (Cl^-) on the surface of the anode (Eq. VII.2). However other reactions can happen in the solution bulk, leading to the formation of other radicals (Eq. VII.3 and VII.4) (Panizza and Cerisola, 2009; Moreira *et al.*, 2017).

$$AS + H_2O \rightarrow AS(\bullet\, OH) + e^- + H^+ \qquad \text{(VII.1)}$$

$$2Cl^- \rightarrow Cl_2 + 2e^- \qquad \text{(VII.2)}$$

$$Cl_2 + H_2O \leftrightarrow HClO + Cl^- + H^+ \qquad \text{(VII.3)}$$

$$HClO \leftrightarrow ClO^- + H^+ \qquad \text{(VII.4)}$$

Different anode materials can be used in the electrochemical oxidation process, such as dimensionally stable anodes (DSAs), among them the anodes formed by ruthenium (Ru), iridium (Ir) and titanium (Ti), where ruthenium and iridium are catalytic agents and titanium provides mechanical stability for good adhesion of the blend. Boron-doped diamond (BDD) is a well-known anode material with the highest generation of radicals, however due to its high cost, other electrodes are being investigated with the aim of making the process more accessible, especially in regard to the degradation of large volumes of effluent.

Literature reports a large number of studies applying the electrochemical oxidation technology to treat industrial wastewater and effluents containing a specific compound (Un *et al.*, 2008; Linares-Hernández *et al.*, 2010; Barrios *et al.*, 2015; Mena *et al.*, 2018; Ochoa-Chavez *et al.*, 2018). However, only a few studies have examined the degradation of benzodiazepines and carbamazepine by EO process (García-Gómez *et al.*, 2014, 2016; García-Espinoza *et al.*, 2016). It is difficult to define the damage that some pharmaceutical compounds in aquatic environments, even at low concentrations, can cause to humans and to the local biota (Lagesson *et al.*, 2016).

The disposition and fate of many compounds is very much under-studied and therefore uncertain. Some studies have explored the possible effects that the presence of some compounds may cause in living beings. An example is the evaluation of neuronal activity and the production of reactive oxygen species (ROS) in hippocampus of some species. These studies can be carried out in small mammals such as rats, whose purpose is to evaluate direct contact and to measure the beneficial or harmful effects that these compounds can generate (Malkov *et al.*, 2014). Some of these substances may impair or alter the normal state of the cells, the variation in the production of ROS occurring and causing oxidative stress, if this state is not reversed or if the substances are very toxic, can lead to death of the cells leading to a collapse of the organism (Wijk *et al.*, 2008; Freinbichler *et al.*, 2011).

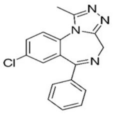
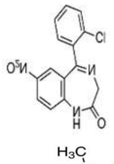
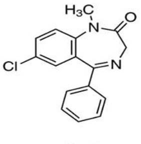
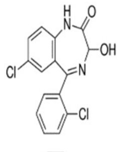
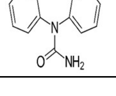
In this work, we investigated the use of EO process to remove a mixture of BDZs and CBZ, using ruthenium and iridium as anode materials. The effect of varying different parameters such as current density (100 – 30 A m^{-2}), pH (3 – 10), and electrolyte concentration (0.5 – 1.0 g L^{-1}) was analyzed. The best degradation conditions were applied in different aqueous matrices and the impact on reactive oxygen species (ROS) generation in hippocampal slices of rats was measured.

VII.2 Material and Methods

VII.2.1 Chemicals and reagents

All pharmaceutical active compounds (PhACs): Alprazolam (ALP), clonazepam (CZP), diazepam (DZP), lorazepam (LZP) and carbamazepine (CBZ) were obtained from a commercial supplier and used as received. Table VII.1 displays some of the characteristics of the pharmaceuticals used in this study. All drugs were used in a concentration of 100 µg L^{-1}. CH_3CN (Acetonitrile, purity, ≥ 99.9%), NaCl (Sodium chlorine, purity, ≥ 99.5%), KCl (Potassium chloride, purity, ≥ 99.0%), $NaHCO_3$ (Sodium bicarbonate, purity, ≥ 99.7%), NaH_2PO_4 (sodium dihydrogen phosphate, purity, ≥ 99.0%), $MgCl_2$ (magnesium dichloride, purity, ≥ 98.0%), $CaCl_2$ (calcium chloride, purity, ≥ 99.0%), $C_6H_{12}O_6$ (D-Glucose, purity, ≥ 99.5%) were purchased from Sigma-Aldrich and used without further purification. NaOH (Sodium hydroxide) and H_2SO_4 (Sulfuric acid) were acquired from Labkem and Honeywell/Fluka, respectively. The ROS indicator H_2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) was obtained from Life Technologies. Ultrapure water obtained from Interlab Direct-Pure purification system was used for all solutions.

Table VII. 1 - Chemical characteristics of alprazolam, clonazepam, diazepam, lorazepam and carbamazepine.

Common name	Molecular formula	Molecular weight ($g.mol^{-1}$)	Chemical structure
Alprazolam	$C_{17}H_{13}ClN_4$	308.8	
Clonazepam	$C_{15}H_{10}ClN_3O_3$	315.7	
Diazepam	$C_{16}H_{13}ClN_2O$	284.7	
Lorazepam	$C_{15}H_{10}Cl_2N_2O_2$	321.2	
Carbamazepine	$C_{15}H_{12}N_2O$	236.3	

VII.2.2 Aqueous Matrices

In the experiments three different types of water were used: Synthetic water (SW) with ultrapure water and PhACs; The river water matrix (RWM) was collected from the Mondego River, located in the city of Coimbra (Portugal), and the municipal wastewater treatment plant (MWWTP) obtained from the same city. Table VII.2 displays how each aqueous matrix was used throughout the experiments. At the time of the experiments, PhACs were added in all matrices at concentrations of 100 µg L^{-1}. The NaCl was chosen as electrolyte because it is one of the compounds used in neurotoxicity experiments. And it was necessary to use it only in the SW to promote the conductivity of the solution, however in the other aqueous matrices no type of electrolyte was added, since RWM and MWWTP already had sufficient conductivity for EO.

Table VII. 2 - Characteristics of the aqueous matrices.

Characteristics	**SW**	**RWM**	**MWWTP**
Alprazolam (µg L^{-1})	101.54 ± 1.12	<LOD*	<LOD
Clonazepam (µg L^{-1})	100.85 ± 1.75	<LOD	<LOD
Diazepam (µg L^{-1})	99.86 ± 2.64	<LOD	<LOD
Lorazepam (µg L^{-1})	98.16 ± 2.84	<LOD	<LOD
Carbamazepine (µg L^{-1})	99.13 ± 1.51	<LOD	<LOD
pH	3, 7 and 10	7.1	7.43
Conductivity (µS cm^{-1})	120-230	115	751

* Limits of detection

VII.2.3 Electrochemical oxidation experiments

The reactor electrodes were composed of Iridium and Ruthenium with 200 cm^2 (10 x 20 cm) of the effective area with 1 cm distance between the plates and they were obtained from a commercial source. Electrochemical oxidation experiments were carried out in a 1 L reactor with magnetic stirring. The electrodes, in parallel, were submerged in the solution and connected to a power supply (Kaise model HY3010) (current (I) = 1.2 – 2.5 A). The conductivity of the solutions was measured using Consort C863 equipment. The pH of the synthetic effluent was measured with a Hanna pH meter and varied from 3 to 10 by adding NaOH (2.0 M) and H_2SO4 (1.0 M) when needed. During the experiment, the pH of the solution was not adjusted.

VII.2.4 Analytical Determinations

The concentration of pharmaceuticals in the mixture was determined by high performance liquid chromatography (HPLC) using Shimadzu equipment with a diode-array detector. A gradient mobile phase was used and was initially composed of acetonitrile and water (45:55) at a flow rate of 1 mL min^{-1}, with the acetonitrile ratio rising to 70% in 15 min. A C18 Shimadzu column with a temperature of 25 °C was used as the stationary phase. Prior to the HPLC analysis, the samples were filtered through cellulose acetate filters (0.45 μm) obtained from Sartorius. The chromatographic analyzes were performed whilst the experiments were in progress. Limits of detection (LOD) and limits of quantification (LOQ) for alprazolam (10.6/35.3 μg L^{-1}), clonazepam (4.5/15.1 μg L^{-1}), diazepam (1.2/4.0 μg L^{-1}), lorazepam (1.3/1.0 μg L^{-1}) and carbamazepine (1.1/3.82 μg L^{-1}) were determined.

VII.2.5 Neuronal Studies

The hippocampal slices (400 μm) were obtained from pregnant Wistar rats (Gestational day 14-18) provided by Center for Neuroscience and Cell Biology (University of Coimbra, Portugal). The animals were killed by cervical dislocation and decapitation, with prompt brain extraction and placement into cold (5-8 °C) artificial cerebrospinal fluid (ACSF), followed by hippocampus extraction and slicing. The slices were then immersed in an oxygenated ACSF solution (95% O_2 e 5% CO_2). The ACSF solution was prepared with the following composition (in mM): 124 NaCl, 3.5 KCl, 24 $NaHCO_3$, 1.25 NaH_2PO_4, 2.0 $MgCl_2$, 2.0 $CaCl_2$ and 10 D-Glucose, pH 7.4. The slices were kept for one hour in an oxygenated ACSF solution to which 20 μM of ROS indicator (2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA)) was added. Afterwards, the slices were re-transferred to a pure oxygenated ACSF solution and then used in the experiments. At the beginning of each experiment, the slices were placed in a chamber in which oxygenated ACSF (1.5 - 2.0 mL min^{-1}) was perfused and maintained at a temperature between 30 - 32 °C. The slices were perfused with the ACSF solution during the first 10 minutes, then with the treated synthetic effluent for 30 minutes and finally again with the ACSF medium for another 30 min. Table VII.3 shows the parameters of the neurotoxicity experiments that were performed in triplicate. A Zeiss Axioskop microscope with trans-fluorescence setup was used for the optical measurements. The synapses studied were of the hippocampal CA3 region. The equipment had an excitation and emission filter of 480 nm and 500 nm respectively and contained a tungsten/halogen lamp (12V, 100W). The light emitted in the experiments that passed through the hippocampus was collected via a water immersion lens (40x, N.A. 0.75) by a photodiode (1 mm^2, Hammamatsu). Data were analyzed using Signal Express^{TM} software. The mean value of each group of 100 points for each one-minute interval was calculated.

Table VII.3 - Neurotoxicity experiments.

Experiments	**PhACs [µg L^{-1}]**	**Current density (A m^{-2})**	**Time (min) of EO reaction**	**NaCl [g L^{-1}]**
$\text{CZP}_{500,\ \text{no EO}}$	500 (Clonazepam)	No EO	-	-
$\text{SW}_{100,\ \text{EO},\ 15\ \text{min}}$	100	50	15	1
$\text{RWM}_{100,\ \text{EO},\ 90\ \text{min}}$	100	50	90	-
$\text{MWWTP}_{100,\ \text{EO},\ 120\ \text{min}}$	100	50	120	-

All experiments were performed following European Communities Council Directives in order to minimize animal suffering and to use only the number of animals needed to generate reliable scientific data. The operating system of electrochemical oxidation and neurotoxicity tests is further detailed in Figure VII.1.

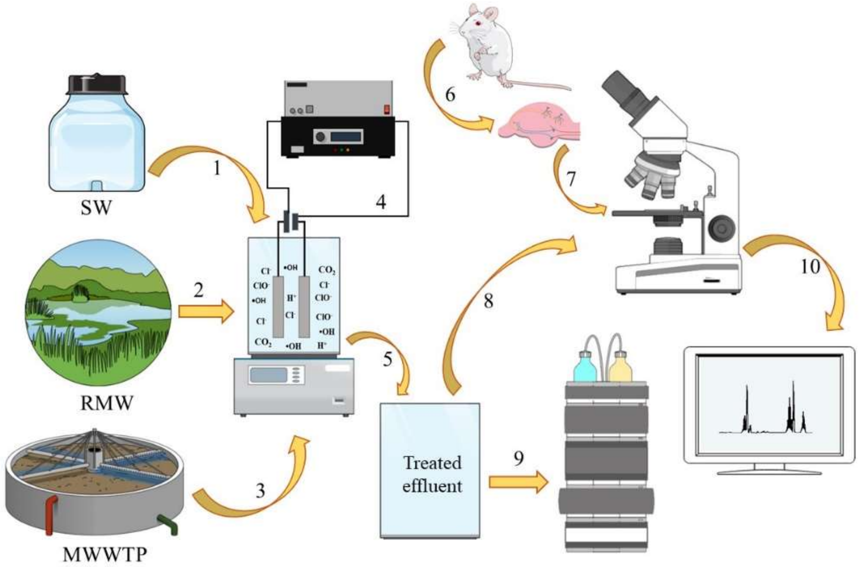


Figure VII.1 – The operating system: 1 – Synthetic effluent (ultrapure water and PhACs), 2 – Water from the Mondego river spiked with PhACs, 3 – Water from MWWTP spiked with PhACs , 4 – Electrochemical oxidation (Reactor and power supply), 5 – Treated effluent, 6 – Wistar rat, 7 – Hippocampal slice, 8 – Treated effluent used in neurotoxicity studies, 9 – Samples to be analyzed on HPLC and 10 - Acquisition of ROS production data.

VII.3 Results and Discussion

VII.3.1 Effect of current density

One of the most important parameters in electrochemical oxidation is current density. This parameter directly influences the generation of the radicals which degrade the compounds present in any medium, This happens because with the increase in current density, a greater generation of radicals occurs (Sanchez-Sanchez). Figure VII.2 shows the degradation of alprazolam, clonazepam, diazepam, lorazepam and carbamazepine under the current densities of 30 A m^{-2} and 50 A m^{-2} (Figure VII.2 A and B, respectively). These experiments were conducted in a synthetic medium, with 100 µg L^{-1} of all PhACs, pH 7 and 0.1 g L^{-1} of NaCl, as a function of the applied load over time. The degradation of the compounds under the same conditions and current density of 100 A m^{-2} (data not shown) was also evaluated and it was possible to observe that all compounds were completely degraded in less than 5 min. Figure VII.2 A shows that applying 50 A m^{-2} of current density led to a complete degradation over a 15 min interval. Lorazepam was fully degraded after 5 min, while alprazolam and clonazepam took 15 min to completely degrade. In comparison, Figure VII.2 B shows that under 30 A m^{-2}, lorazepam and diazepam were the first compounds to be degraded, taking 15 min, while the total removal of alprazolam, carbamazepine and clonazepam required 20 min, 20 min, and 30 min, respectively. Similar behavior, where the removal of compounds was influenced by current density variations, was observed by Garcia-segura et al. (2015) and Fajardo et al. (2017).

Results obtained by the authors suggest that the degradation of the compounds is directly influenced by current density, as higher degradation was noticed under higher current density. The degradation reactions occur both on the surface of the electrode and on the indirect reactions by some radicals as •OH and OCl^-, these oxidants have little time and do not have selectivity in the attack of the organic compounds (Anglada, Urtiaga and Ortiz, 2009; Rocha *et al.*, 2014). Although these drugs have similar structure chains, the difference in degradation time is directly linked to the molecular interactions between the radicals formed in the electrochemical oxidation. Many studies have shown that the use of electrochemical oxidation led to complete mineralization of organic compounds in concentrations higher than those used in this study (García-Montoya *et al.*, 2015; Garcia-segura *et al.*, 2015; Candia-Onfray *et al.*, 2018; Vasilie *et al.*, 2018). For the best evaluation of the results in the experiments of pH variation and electrolyte concentration, the current density of 30 A m^{-2} was used.

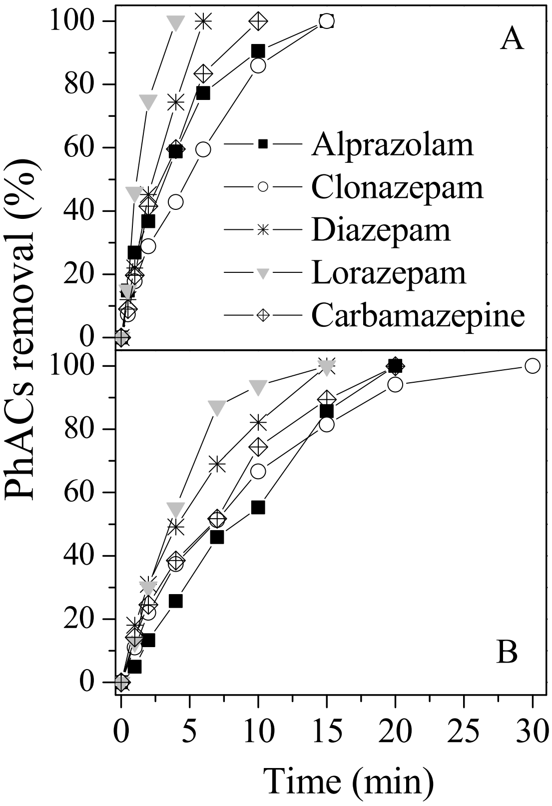


Figure VII.2 - Degradation of BZDs and CBZ under the current densities of (A) 50 A m^{-2} and (B) 30 A m^{-2}. (pH = 7, T = 20 ºC and 0.5 g NaCl L^{-1}).

VII.3.2 Effect of initial pH

The pH of the solution can have a direct effect on the degradation of compounds, since Cl_2 predominance occurs in acidic pH. In the range of 3 to 8, the hypochlorous acid (HOCl) is predominantly formed, and in alkaline pH there is a higher generation of OCl^- (Fajardo, Seca, Martins, Corceiro, Freitas, *et al.*, 2017a; Kong *et al.*, 2018). The variation in the degradation of PhACs was evaluated at three different values of pH as presented in Figure VII.3.

In the three pH conditions which were studied (pH 3, 7 and 10), the degradation behavior of the compounds was similar. At pH 3 (Figure VII.3 A) the degradation of alprazolam, diazepam and carbamazepine occurred within a maximum time of 30 min, with 93 % of clonazepam being removed in 30 min and the complete degradation of lorazepam being observed in 20 min, this may be due to the fact that under acidic conditions, a higher generation of hydrogen peroxide occurs, which is produced by the electrolysis of water and it is very effective for the removal of aromatics compounds (Cotillas *et al.*, 2018).

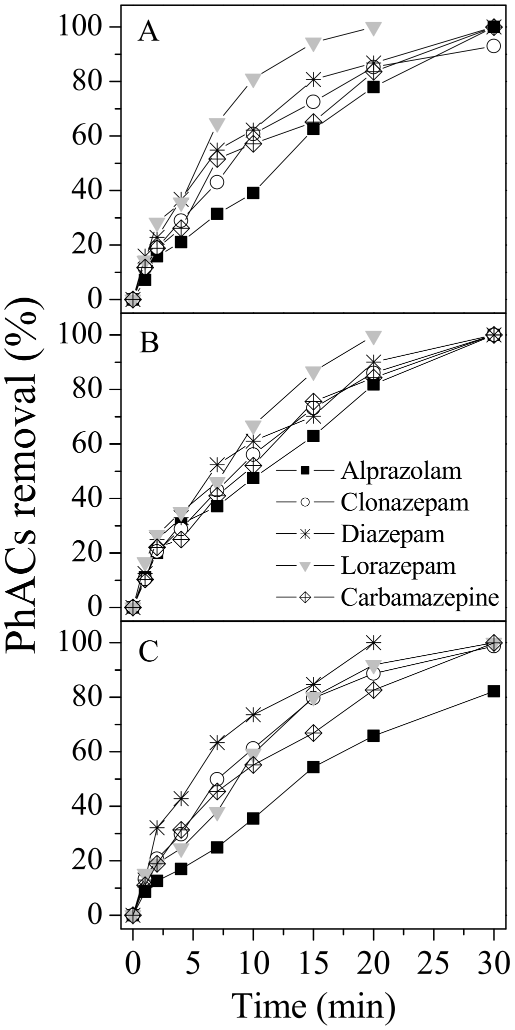


Figure VII.3 - Degradation of BZDs and CBZ at (A) pH 3, (B) pH 7 and (C) pH 10 over time. (T = 20 ºC, 0.5 g NaCl L^{-1}, current density of 30 A m^{-2}).

As shown in Figure VII.3 B, complete degradation of the compounds at pH 7 required 30 min, with the exception of lorazepam, which was fully degraded in 20 min. Regarding pH 10 (Figure VII.3 C), diazepam was completely degraded in 20 min, clonazepam, lorazepam and carbamazepine in 30 minutes while only 82% of alprazolam degradation was observed in 30 min. It is worth mentioning that compound removal occurs not only through reactions with chlorinated groups, but also through reactions to other radicals produced, such as the hydroxyl radical (HO•), which is not selective and possesses high oxidative power (Martínez-Huitle and Brillas, 2009). This may be one explanation for why no significant variation was observed in the degradation of compounds in relation to the pH.

VII.3.3 Effect of the concentration of the supporting electrolyte

In order to evaluate the removal of the compounds within mediums containing different concentrations of electrolyte, experiments with 1.0 g L^{-1} and 0.5 g L^{-1} of NaCl were performed. Samples were collected at 4 and 10 min of reaction. Figure VII.4 shows the percentage removed of alprazolam, clonazepam, diazepam, lorazepam and carbamazepine at different times according to the NaCl concentrations used. Among the concentrations and times analyzed, the best result was observed using a concentration of 1.0 g L^{-1} NaCl and a reaction time of 10 min. At this point, alprazolam and lorazepam obtained the best results, both with a removal rate of 93%, while clonazepam and carbamazepine were the least degraded with removal rates of 66% and 74%, respectively. The experiment using 0.5 g L^{-1} NaCl and a reaction time of 4 min resulted in the least efficient result obtained, likely due to the low production of radicals responsible for the degradation of the compounds. The degradation profile of the drugs was similar, however, with the removal efficiencies for alprazolam, clonazepam, diazepam, lorazepam and carbamazepine being 35%, 28%, 32%, 35% and 24%, respectively.

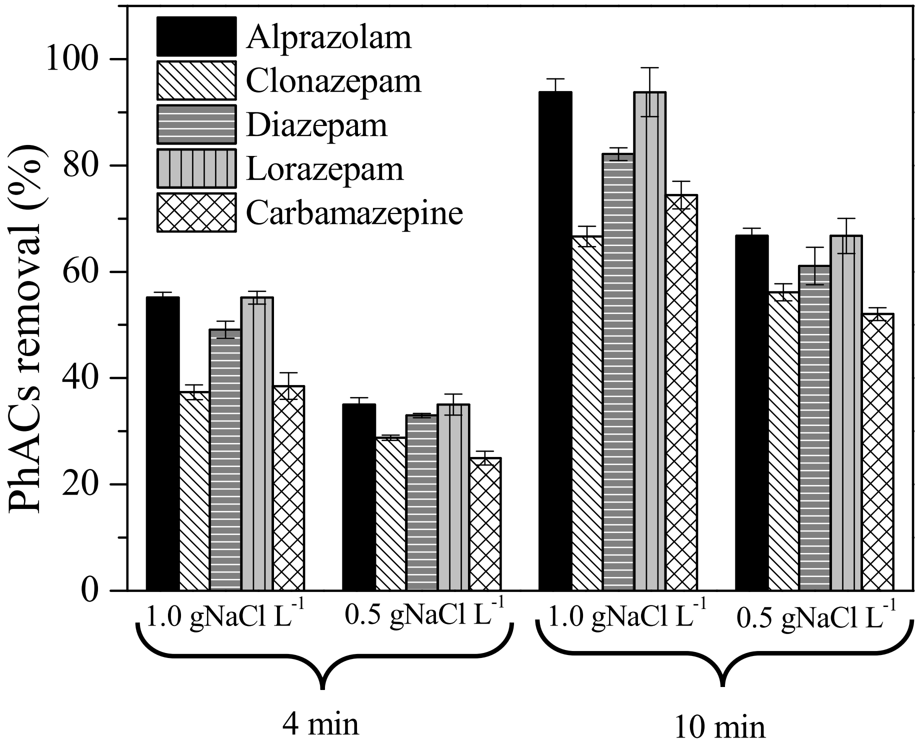


Figure VII.4 - Degradation of BZDs and CBZ at different times of reaction (4 and 10 min) and initial NaCl concentrations (0.5 and 1.0 g L^{-1}). (pH 7, T = 20 ºC and 30 A m^{-2}).

Regarding the amount of electrolyte present in the solution, it was possible to detect that with a higher concentration of NaCl the removal of the compounds occurred more efficiently. This difference in removal rates can be explained because with a lower concentration of NaCl, lower concentrations of Cl^- ions are obtained in solution that promote the generation of oxidants. In addition, the higher the NaCl concentration, the higher the conductivity of the solution and consequently the greater generation of oxidizing radicals (Fajardo, Seca, Martins, Corceiro, Vieira, *et al.*, 2017).

VII.3.4 Effect of different water matrices

To examine the effect of the drugs in different aqueous matrices, three water sources were used: Ultrapure water, river water and wastewater from a sanitary sewage treatment plant. The different degradation profiles may be associated with the compounds nature, their chemical structures, interactions with other compounds present in the medium and the nature of the aqueous matrix (Gaya and Abdullah, 2008). In Figure VII.5 A it is possible to observe the degradation of the compounds in Ultrapure water. Lorazepam and CBZ drugs were degraded after 5 and 10 min, respectively, while alprazolam and clonazepam needed 15 min for a complete degradation (below the limit of detection). In Figure VII.5 B, the degradation of the compounds in river water is displayed, with diazepam, lorazepam and carbamazepine requiring 90 min for full degradation, and alprazolam and clonazepam needing 120 min. Finally, Figure VII.5 C shows the results obtained in wastewater, where none of the pharmaceutical compounds were fully degraded after 120 min, and the percentages of removal attained for clonazepam, alprazolam, lorazepam, carbamazepine and diazepam were 29%, 32%, 36%, 42% and 43%, respectively.

As expected, the best results were obtained in ultrapure water, since only the compounds of interest were present in the test solution and all the radicals produced in the electrochemical oxidation were free to react with BDZs and CBZ. In river water and urban wastewater, dozens or even thousands of other compounds may be present, which could contribute to the increase of the chemical oxygen demand, and could in turn compete with the radicals produced in the electrochemical oxidation, thus decreasing the efficiency in the degradation of the compounds of interest (Rosario-ortiz, Wert and Snyder, 2010). Similar degradation efficiency decrease in different aqueous matrices was also observed in a study by Sousa et al. (2013) and Bosio et al. (2018).

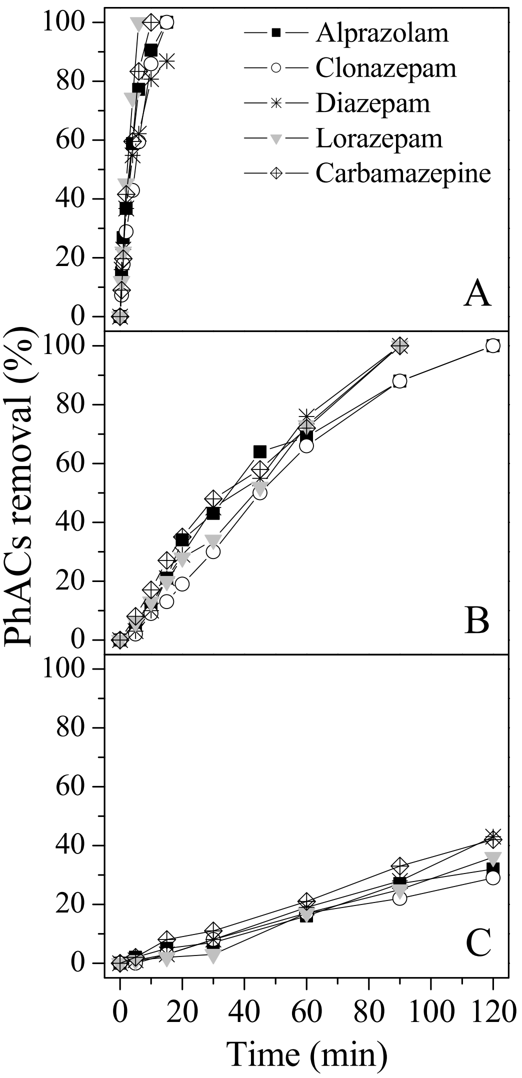


Figure VII.5 - Degradation of BZDs and CBZ in (A) Ultrapure water (1.0 g NaCl L^{-1}), (B) river water and (C) MWWTP. (T = 20 ºC, and current density of 50 A m^{-2}).

VII.3.5 Impact of the residual compounds in neuronal ROS activity

To evaluate the effect of an electrochemical oxidation treated effluent in neuronal signals, rat hippocampal slices were incubated with the ROS fluorescent indicator H_2DCFDA. The experiment was carried out with untreated synthetic effluent (100 µg PhACs L^{-1}) (data not shown), as the slices came into contact with the solution being studied, a decrease in the signals from the baseline occurred 7.4 ± 1.3 % S.E.M, n=3, in the period 25-40 min and upon washout A recovery of the signals occurred in the time of 60-70 min of 5 ± 2 % S.E.M, n=3, above the baseline. For comparison, a solution of clonazepam (500 µg L^{-1}) in ultrapure water without EO was tested for the effect of the isolated benzodiazepine. In Figure VII.6 A the effects of this solution on ROS production are demonstrated, on contact with the CZP solution (500 µg L^{-1}) the signs decreased 8.91 ± 2.08 % S.E.M, n=3 in the interval of 25-40 min. In the return of the ACSF solution the signals increased and were 4.57 ± 1.38 % S.E.M, n=3 above the baseline in the period of 60-70 min.

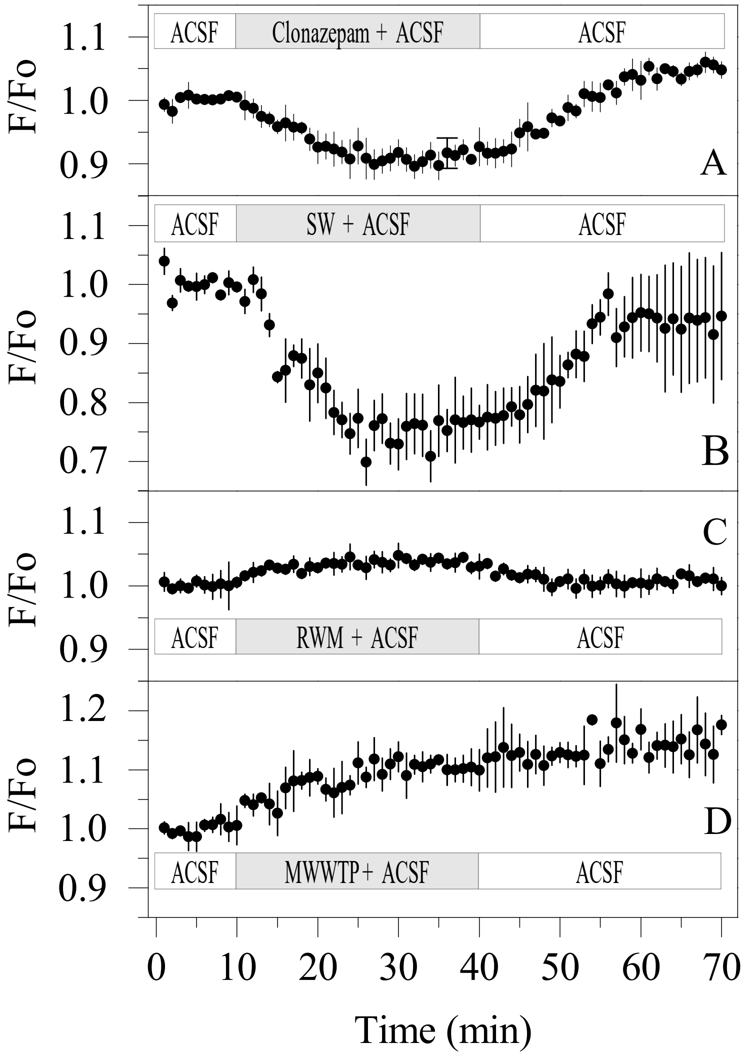


Figure VII.6 - ROS signals from brain slices evoked by: A = ultrapure water, pH 7.4 and 500 µg Clonazepam L^{-1}); B = ultrapure water, pH 7.4, 50 A m^{-2}, 15 min and 1 g NaCl L^{-1}; C = RWM, pH 7.4, 50 A m^{-2} and 90 min; D = MWWTP, pH 7.4, 50 A m^{-2} and 120 min. The data were acquired in triplicate using the fluorescent ROS indicator H_2DCFDA and were normalised by the average of the first 10 responses. The ROS changes measured in the presence of the studied mixture, correspond to the period indicated by the grey.

In Figure VII.6 B it is observed that the ROS signal is stable during the baseline, formed by the first ten points in ACSF. In the presence of the synthetic effluent after EO, the signals decrease reaching a steady level, being the amplitude of the depression 21.9 ± 4.9 % S.E.M, n=3, in the period 35-40 min. Upon washout there is a partial recovery of the signal to a level 5.9 ± 10.5 % S.E.M, n=3, at 65-70 min, below that of the baseline. It is considered that the decrease of the measured ROS signal reflects synaptic metabolic changes of mitochondrial origin, due to flavoproteins, since the release of neurotransmitters depends on the production of energy by this organelle (Kasischke *et al.*, 2004; Zorov, Juhaszova and Sollott, 2014). In the absence of the effluent during the last 30 min, the ROS signal increases indicating that it is largely reversible, thus suggesting that the cells recovered almost completely normal function.

In Figure VII.6 C it is possible to observe the variations of ROS with the use of RWM, from 10 min there was an increase in the signals that reached 3.71 ± 1.09% S.E.M, n = 3 above the baseline in the period of 25-40 min with the return of the ACSF solution the ROS production returns to the baseline practically without variations, this shows that in this case the solution caused growth in the ROS production, however the effects of the solution were not permanent and there was total recovery of the signals.

Figure VII.6 D, between 10 and 40 min, shows neurotoxicity data with MWWTP after EO. During this interval there is an increase in ROS signals and between 25-40 min this increase is 10.46 ± 2.45 % S.E.M, n=3, a possible explanation for this change is that the solution used in this experiment contained several compounds and after EO process, different reactions may have led to the production of toxic substances that directly affected the hippocampal slices and caused an increase in ROS production (Sánchez-Carretero *et al.*, 2011). From 40 min the ACSF solution returns to the experiment, however the signals continue to increase reaching a variation of 14.53 ± 3.80 % S.E.M, n=3 between 60-70 min. One reason that the signals do not return to the baseline, it may be that the toxic substances have affected the calcium channels, these channels are responsible for the passage of Ca^{2+} into the cells and the organelles and the calcium is directly linked to ROS production (Görlach *et al.*, 2015).

VII.4 Conclusion

The removal of BDZs and CBZ emerging pollutants was successfully achieved with the electrochemical oxidation technology using different aqueous matrices. The increase in current density directly influenced the degradation of the compounds, since a greater amount of radicals was generated and, consequently, a greater efficiency in the removal of the drugs was reached. Different pH values were studied, and no relevant influence was observed, which suggests that the pH of the medium does not interfere in the degradation of the pharmaceutical compounds. An improvement in the degradation of the compounds was observed with the increase of the electrolyte concentration (NaCl), likely due to the high production of radicals responsible for the oxidation reactions. Lower degradation efficiencies were achieved using river water and urban wastewater, probably because other compounds present in these water sources compete for the radicals produced in the electrochemical oxidation system. In neuronal studies the effluents treated by EO, demonstrated effects on ROS production on rat hippocampal in the CA3 region. Experiments CZP_{500} and SW_{100} had an effect on decreased ROS production and partial recovery of the signals. The $MWWTP_{100}$ solution caused an increase in the signals and the slices were affected in an irreversible way.

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CHAPTER VIII – Electrochemical Oxidation (Pharmaceutical Wastewater)

Article in preparation.

Degradation of real wastewater by electrochemical oxidation with different anodes and neurotoxicity studies.

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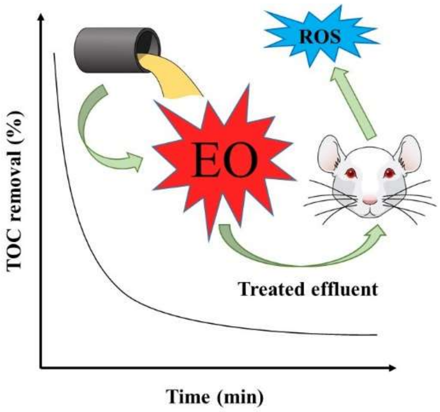
ABSTRACT

The pharmaceutical industry produces a wide variety of remedies and consequently a complex effluent to be discarded. Conventional effluent treatments are not capable of degrading many compounds of this industrial activity. However, the emergence of new technologies has led to the degradation of recalcitrant and toxic compounds. Electrochemical oxidation (EO) is an alternative, this process is characterized by the production of strong oxidative agents such as the hydroxyl radical (•OH) and the possibility of using various combinations of electrodes. In this study the degradation of the pharmaceutical wastewater by EO was evaluated with different electrodes (BDD and Ti/Pt) under different conditions and the neurotoxicity of the samples before and after the EO was analyzed. The removals of TOC and COD were 71.03 and 75.12%, respectively, demonstrating more efficiency when using BDD anode. Neurotoxicity tests showed that the effluent before EO caused a decrease in ROS formation and this formation was normalized with the application of the ACSF solution. The solutions after EO with BDD and Ti / Pt indicated that the treatment was efficient to reduce the neurotoxicity of the samples.

Keywords: Pharmaceutical wastewater; BDD; Ti/Pt, CA3; ROS

Graphical Abstract



VIII.1 Introduction

The growth of the population aggravated by climate change increases the concern with the availability of water and the disposal of domestic, industrial and agro-industrial effluents (Ebele, Abdallah and Harrad, 2017; Saaristo *et al.*, 2019). For this reason it is necessary to develop and implement technologies that are capable of treating effluents with different characteristics (Sirés *et al.*, 2014). The parameters for effluent disposal must comply with the legislation of each country.

The industrial effluents have an enormous variability of parameters such as the amount of organic matter, inorganic compounds, pH, temperature, color, turbidity, conductivity and toxicity, so different types of treatment can be performed (Zazou *et al.*, 2019). In some cases only the biological treatment is enough to eliminate the organic compounds, however in some cases it is necessary to use treatments that can degrade recalcitrant compounds, in these cases the advanced oxidative processes are applied (Zou *et al.*, 2017; Tang *et al.*, 2019).

Electrochemical oxidation (EO) is one of these processes and is characterized by the in situ production of hydroxyl radicals (•OH) with high potential redox E^{o} (•OH/H_2O) = 2.80 V/SHE that is not selective and degrades the majority of organic compounds and recalcitrant, in some cases this degradation can lead to complete mineralization of the compounds in CO_2 and H_2O (Moreira *et al.*, 2017). Some of the advantages of electrochemical oxidation are operating conditions that can be maintained under ambient temperature and pressure, reactors that require less physical space, easy combination with other types of effluent treatment and easy automation of the process (Grafias *et al.*, 2010; Moreira *et al.*, 2015; Rivera *et al.*, 2015). Several studies were carried out with electrochemical oxidation treatment in synthetic effluents (Costa *et al.*, 2009; Fabianska *et al.*, 2015; García-Montoya *et al.*, 2015; Ochoa-Chavez *et al.*, 2018; Siedlecka *et al.*, 2018; Urtiaga, Soriano and Carrillo-abad, 2018), however in real effluent this number is lower (Zhu *et al.*, 2011; Zou *et al.*, 2017; Loos *et al.*, 2018).

In addition to the degradation of the effluents it is necessary to evaluate how the solutions can affect the environment and the living beings. One of the alternatives is the toxicity study as neurotoxicity in rats hippocampus, this study is focused on the CA3 region of the hippocampus and evaluates how the effluents can alter the production of reactive oxygen species (ROS). The production of ROS is essential for the functioning of cells, however the contact with some substances can alter the normal production of ROS leading to oxidative stress (Cai *et al.*, 2007; Ahmad *et al.*, 2018). If the state of the cells is not normalized can cause cell death and later death of the organism (Malinska *et al.*, 2010; Keng *et al.*, 2017). Some studies evaluating the effect of effluents on neurotoxicity were performed (Fajardo, Seca, Martins, Corceiro, Freitas, *et al.*, 2017; Gomes *et al.*, 2017; Bosio *et al.*, 2018), however there are many doubts to be clarified around this subject.

Based on these perspectives, this study was carried out with industrial effluent from a pharmaceutical company, applying the electrochemical oxidation with variation of current density (75 – 1000 A m^{-2}), type of anode (BDD and Ti/Pt), concentration of the raw effluent (100 – 30 %) and effluent neurotoxicity before and after electrochemical oxidation.

VIII.2 Material and Methods

VIII.2.1 Chemicals and reagents

The chemicals were purchased from Sigma-Aldrich: calcium chloride ($CaCl_2$, purity, ≥99.0%), D-glucose ($C_6H_{12}O_6$, purity, ≥99.5%), magnesium dichloride ($MgCl_2$, purity, ≥98.0%), potassium chloride (KCl, purity, ≥99.0%), sodium bicarbonate ($NaHCO_3$, purity, 99.7%), sodium chloride (NaCl, purity, ≥99.5 %) and sodium dihydrogen phosphate (NaH_2PO_4, purity. ≥99.0%) all analytical grade. The ROS indicator 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was obtained from Life Technologies. The ultrapure water was obtained using a Direct-Pure Interlab water system.

VIII.2.2 Analytical analysis

The temperature in neurotoxicity studies was controlled by a thermostatic bath (Variomag - Electronicrührer) and pH by HANNA. The chemical oxygen demand (COD) was determined according to the standard method (Greenberg et al 1985). The total organic carbon (TOC) and total nitrogen (TN) were measured using a TOC analyzer (TOC-V CPN model, Shimadzu, Japan) coupled to an autosampler (model V-ASI, Shimadzu, Japan).

VIII.2.3 Industrial effluent

The raw wastewater was obtained from the final effluent from a pharmaceutical industry located near Lisbon, after collection the wastewater was maintained at 4 °C. The composition of the wastewater is presented Table VIII.1.

Table VIII.1 – Composition of pharmaceutical wastewater.

Parameter	**Average value**	**Unit of measurement**
TOC	3085.00 ± 78.52	mg L^{-1}
COD	9770.00 ± 69.41	mg L^{-1}
TN	205.00 ± 21.05	mg L^{-1}
Cl^-	1801.00 ± 57.47	mg L^{-1}
SO_4^{2-}	354.00 ± 24.04	mg L^{-1}
pH	7.76 ± 0.41	
T	20.20 ± 2.58	°C
Condutivity	4.95 ± 0.45	mS cm^{-1}

VIII.2.4 Electrochemical oxidation

The experiments for EO were carried-out in a lab-scale batch-stirred reactor with 500 mL at atmospheric pressure. Two types of anodes were used with the effective area of 20 cm^2 (8.0 x 2.5 cm): titanium electrode cell coated with 50 g Pt/m^2 (Ti/Pt) and boron doped diamond (BDD) and the cathode was stainless steel (SS). The electrodes in parallel with a distance of 1 cm were connected to a power supply (DC HY3010 Kaise – Current I = 1.2 – 2.5 A). In Table VIII.2 is possible to observe the parameters used in the experiments of electrochemical oxidation.

Table VIII.2 – Experiments and parameters used in electrochemical oxidation.

Experiments	Aqueous Matrix	Current density (A m^{-2})	EO time (min)	Type of anode
1-WW$_{\text{100BDD75}}$	100 % pharmaceutical wastewater	75	180	BDD
2-WW$_{\text{70BDD250}}$	70 % pharmaceutical wastewater 30 % ultrapure water	250	180	BDD
3-WW$_{\text{50BDD250}}$	50 % pharmaceutical wastewater 50 % ultrapure water	250	180	BDD
4-WW$_{\text{50BDD500}}$	50 % pharmaceutical wastewater 50 % ultrapure water	500	180	BDD
5-WW$_{\text{50BDD1000}}$	50 % pharmaceutical wastewater 50 % ultrapure water	1000	180	BDD
6-WW$_{\text{30BDD250}}$	30 % pharmaceutical wastewater 70 % ultrapure water	250	180	BDD
7-WW$_{\text{100TiPt75}}$	100 % pharmaceutical wastewater	75	180	Ti/Pt
8-WW$_{\text{70TiPt250}}$	70 % pharmaceutical wastewater 30 % ultrapure water	250	180	Ti/Pt
9-WW$_{\text{50TiPt250}}$	50 % pharmaceutical wastewater 50 % ultrapure water	250	180	Ti/Pt
10-WW$_{\text{50TiPt500}}$	50 % pharmaceutical wastewater 50 % ultrapure water	500	180	Ti/Pt
11-WW$_{\text{50TiPt1000}}$	50 % pharmaceutical wastewater 50 % ultrapure water	1000	180	Ti/Pt
12-WW$_{\text{30TiPt1000}}$	30 % pharmaceutical wastewater 70 % ultrapure water	1000	180	Ti/Pt

VIII.2.5 Neurotoxicity studies

Neurotoxicity studies were performed on hippocampus of Wistar rats (10-16 weeks old and 14-18 days of gestational). Wistar females were anesthetized and had cervical dislocation. The brain was rapidly extracted and stored in oxygenated artificial cerebrospinal fluid (ACSF) with the following composition (in mM): NaH_2PO_4 1.25; $MgCl_2$ 2.0; KCl 3.5; $NaHCO_3$ 24.0; $CaCl_2$ 2.0; NaCl 124.0 and D-glucose 10.0, pH 7.4. After manual removal of the hippocampus, it was rapidly sliced by transversal cuts (350 μm), then the slices were transferred to ACSF oxygenated solution (95 % O_2 and 5 % CO_2) solution at room temperature. Only the necessary number of Wistar rats was used with minimization of the suffering of the animals following the European Communities Council Directives (2010/63/UE). After a few minutes, the slices were incubated for one hour in ACSF oxygenated solution (95 % O_2 and 5 % CO_2) containing ROS indicator (H_2DCFDA, 20 μM). After incubation the slices were transferred to ACSF solution and after a few minutes to the experimental chamber with ACSF perfused (1.5 – 2.0 mL min^{-1}) and controlled temperature (30 - 32 °C). The characteristics of the operating system were previously described by (Fajardo, Seca, Martins, Corceiro, Freitas, *et al.*, 2017). The experimental steps are shown in detail in Figure VIII.1.

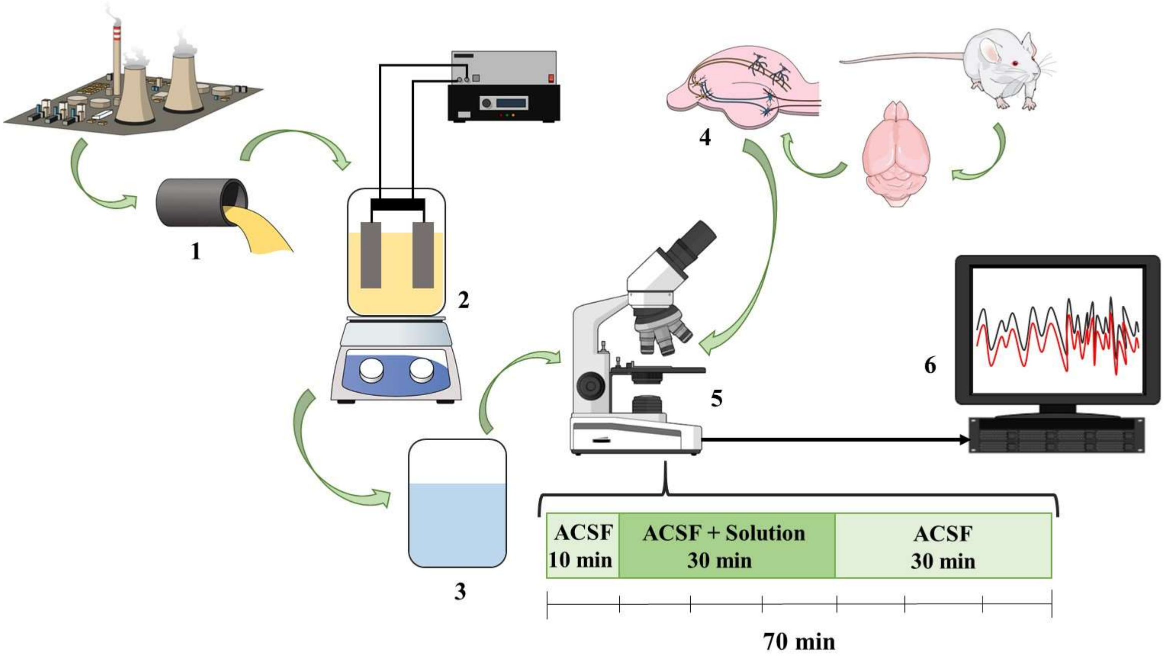


Figure VIII.1 – Operating system of the experiments: 1 - Pharmaceutical wastewater; 2 – Electrochemical oxidation process; 3 – Treated effluent; 4 – Hippocampal slice; 5 – Microscope and 6 – Data acquisition system.

Table VIII.3 shows the samples used in the neurotoxicity experiments. All samples used were refrigerated and spiked with the ACSF compounds at the time of the neurotoxicity experiments.

Table VIII.3 – Different solutions used in neurotoxicity studies.

Sample	Aqueous Matrix	Current density (A m^{-2})	EO time (min)	Type of anode
WW$_{50noEO}$	50 % pharmaceutical wastewater 50 % ultrapure water	No electrochemical oxidation		
5-WW$_{50BDD1000}$	50 % pharmaceutical wastewater 50 % ultrapure water	1000	180	BDD
11-WW$_{50TiPt1000}$	50 % pharmaceutical wastewater 50 % ultrapure water	1000	180	Ti/Pt
WW$_{100cycle}$	100 % pharmaceutical wastewater	No electrochemical oxidation		

VIII.2.6 Energy consumption

To calculated the energy consumption (EC) was used Equation 1, this represent the volume of treated effluent in terms of kWh m^{-3}.

$$EC(kWh\,m^{-3}) = \frac{EIt}{V} \tag{1}$$

Where E represents the average potential difference of the cell (V), I represents the current intensity (A), t represents the electrochemical oxidation time (h) and V represents the volume of the solution (L).

VIII.3 Results and Discussion

VIII.3.1 Electrochemical oxidation experiments

VIII.3.1.1 TOC removal

In some experiments it was necessary to dilute the pharmaceutical wastewater, this occurred to reduce the concentration of the compounds present in the raw effluent, many studies reveal the need for a pre-treatment before the electrochemical oxidation (Jara and Fino, 2010; Tejedor-Sanz, Ortiz and Esteve-Núñez, 2017). One of the pre-treatments may be electrocoagulation which is widely used in conjunction with electrochemical oxidation (García-garcía, Martínez-miranda and Martínez-cienfuegos, 2015; Gilpavas *et al.*, 2017; Flores *et al.*, 2018; Costa *et al.*, 2019), electrocoagulation has the ability to remove large amounts of insoluble particles and COD (Sharma and Simsek, 2019), while electrochemical oxidation efficiently removes recalcitrant compounds (Zazou *et al.*, 2019).

Figure VIII.2 shows the results of TOC removal with different parameters (Table VIII.2) and different anodes: BDD (Figure VIII.2 A) and Ti / Pt. (Figure VIII.2 B). When using non-diluted pharmaceutical wastewater and applied current density of 75 A m^{-2} the removal of TOC was minimal, 3.01 and 2.51 for $WW_{100BDD75}$ and $WW_{100Ti/Pt75}$, respectively. In the experiments with BDD anode: $WW_{70BDD250}$, $WW_{50BDD250}$ and $WW_{100BDD500}$ the TOC removal in 180 min was 8.48, 9.91 and 16.93%, respectively. The best results obtained with BDD were $WW_{50BDD1000}$ and $WW_{30BDD250}$ whose TOC removal was 71.03 and 55.45% respectively.

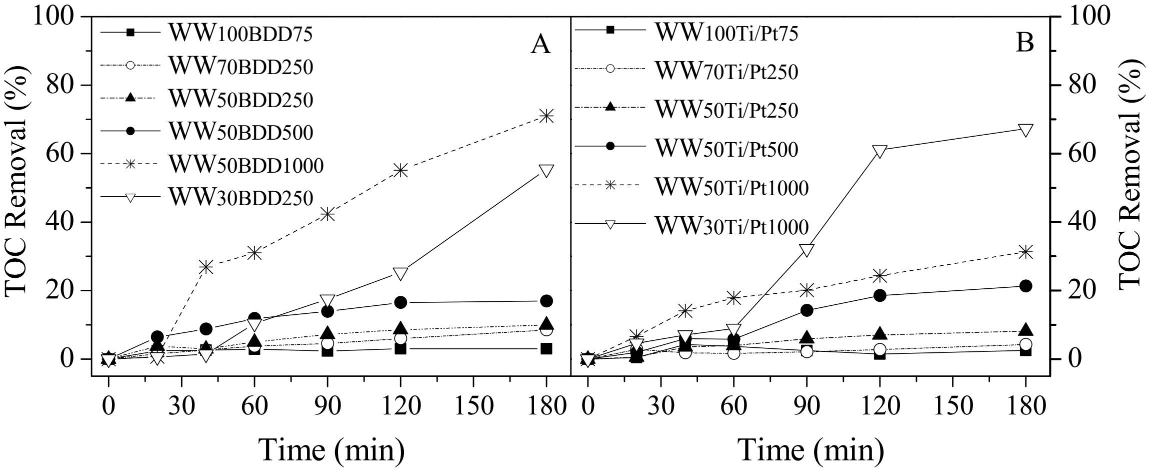


Figure VIII.2 – TOC removal during the electrochemical oxidation: (A) BDD and (B) Ti/Pt.

With the use of the Ti/Pt anode only the experiment $WW_{30Ti/Pt1000}$ was efficient in the removal of TOC, 67.27%. It has been shown that the increase in current density is directly related to the increase in TOC removal, since the increase of this parameter promotes a higher production of radicals that degrade several compounds present in the effluent (Anglada, Urtiaga and Ortiz, 2009; Chen, Jhou and Huang, 2014; Alcocer *et al.*, 2018). When comparing the BDD and Ti / Pt anodes, it is possible to notice that the removal of TOC is greater with the use of BDD, this electrode is more powerful in the production of radicals and has great ability to mineralize the intermediates formed (Ridruejo *et al.*, 2017). The BDD was established as the best anode to produce X(•OH)

(Equation 2), which is related to large overvoltage for O_2 evolution and the small adsorption of •OH on the surface of the anode (Flox *et al.*, 2006; Özcan *et al.*, 2008; Santos *et al.*, 2010; dos Santos *et al.*, 2015).

$$X + H_2O \rightarrow X(\bullet OH) + H^+ + e^- \qquad (2)$$

VIII.3.1.2 COD removal

Figure VIII.3 shows the EO results in COD removal at the BDD anode (Figure VIII.3 A) and Ti/Pt anode (Figure VIII.3 B). Some experiments showed small percentages of COD removal in 180 min: WW$_{100BDD75}$ (6.21 %), WW$_{70BDD250}$ (8.51 %), WW$_{50BDD250}$ (11.64 %), WW$_{50BDD500}$ (22.34 %), WW$_{100Ti/Pt75}$ (5.94 %), WW$_{70Ti/Pt250}$ (7.44 %), WW$_{50Ti/Pt250}$ (10.0 %) and WW$_{50Ti/Pt500}$ (22.15 %). This small COD removal can be related to the high concentration of substances present in the sample, low current density and the production of weak oxidizing agents, represented by equations 3, 4, 5 and 6 (Marselli *et al.*, 2003; Panizza and Cerisola, 2009; Sirés and Brillas, 2012).

$$X(\bullet OH) \rightarrow X + \frac{1}{2}O_{2(g)} + H^+ + e^- \qquad (3)$$

$$X(\bullet OH) \rightarrow X + \frac{1}{2}H_2O_2 \qquad (4)$$

$$H_2O_2 + X(\bullet OH) \rightarrow X(HO_2 \bullet) + H_2O \qquad (5)$$

$$3H_2O \rightarrow O_{3(g)} + 6H^+ + 6e^- \qquad (6)$$

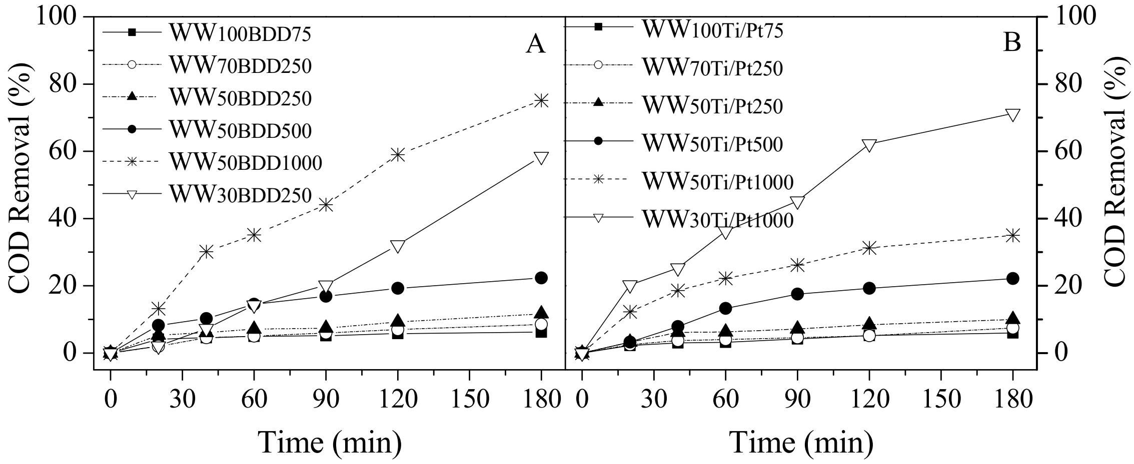


Figure VIII.3 – Abatement of COD during the electrochemical oxidation: (A) BDD and (B) Ti/Pt.

Samples WW$_{50BDD1000}$ and WW$_{30Ti/Pt1000}$ had the highest COD removal efficiencies of 75.12 and 71.23%, respectively, removal is directly linked to the increase in current density which produces more radicals capable of degrading recalcitrant substances, the increase of this parameter and the increase in the efficiency of DOC removal was also reported by Florenza et al. (2014). For sample $WW_{30Ti/Pt1000}$ the relationship between DOC removal and raw effluent dilution was also reported by Fajardo et al. (2017)b, this is because by promoting the dilution of pharmaceutical wastewater the amount of recalcitrant substances present in the sample decreases.

VIII.3.1.3 TN removal

Total nitrogen removal is shown in Figure VIII.4, samples $WW_{100BDD75}$, $WW_{100Ti/Pt75}$ and $WW_{70Ti/Pt250}$ removed 7.79, 3.15, 2.01%, repectively, total nitrogen removal was not efficient in these experiments. Experiments $WW_{70BDD250}$, $WW_{50Ti/Pt250}$ and $WW_{50Ti/Pt250}$ showed a more efficient TN removal of 28.02, 36.30 and 21.02%, respectively, however this removal was not very high. $WW_{50BDD500}$, $WW_{50BDD1000}$, $WW_{30BDD250}$, $WW_{50Ti/Pt500}$, $WW_{50Ti/Pt1000}$ and $WW_{30Ti/Pt1000}$ were the samples with the best TN removal results of 77.7, 80.09, 73.25, 61.23, 78.60 and 81.15%, respectively.

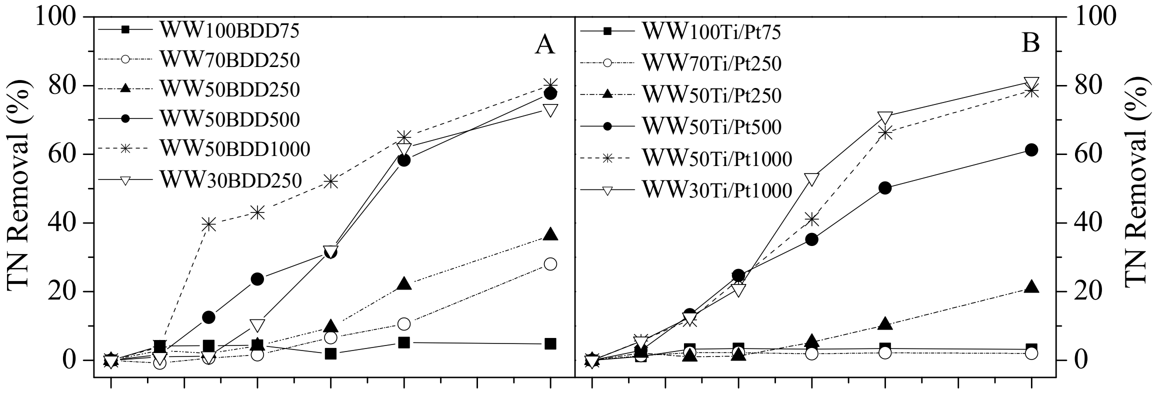


Figure VIII.4 – Removal of total nitrogen in solution during electrochemical oxidation. (A) – BDD anode and (B) Ti/Pt anode.

The increase in current density and greater removal of nitrogen occurs due to the fact that it increases the production of radicals responsible for the degradation of the compounds (Hu, Gong and Feng, 2009; Díaz *et al.*, 2010; Ghimire *et al.*, 2019). The large amount of total nitrogen removed suggests the conversion of nitrogen into volatile compounds, probably N_2 and N_xO_y species (Cavalcanti *et al.*, 2013).

VIII.3.2 Energy consumption

In Figure VIII.5 is possible to observe the energy consumption over COD and TN removal, for EO to be applied in a real scale it is very important to evaluate this parameter. The increase in energy consumed led to an improvement in COD and TN degradation efficiency. This is related to the increase of oxidative radicals produced and the high current density caused by the ions present in the pharmaceutical wastewater (Aquino *et al.*, 2014; Coledam *et al.*, 2014).

The BBD anode and Ti/Pt anode were effective to remove COD and TN, however the energy consumption was high to be implemented in a real context, so some pre-treatment can be implemented to reduce energy consumption (Elaoud *et al.*, 2011).

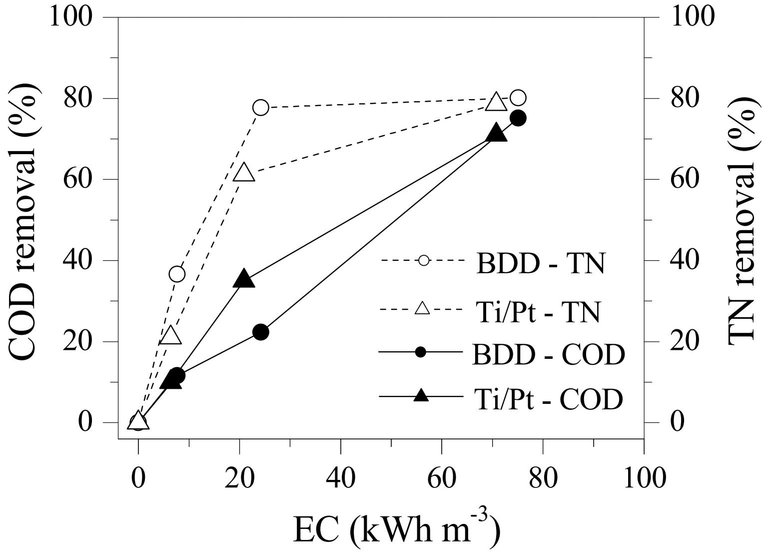


Figure VIII.5 – Analysis of energy consumption (EC) over COD and TN removal.

VIII.3.3 Effect of the pharmaceutical wastewater in neuronal ROS signals

In Figure VIII.6 A are shown results of the sample WW_{50noEO} applied in the CA3 region to evaluate ROS production, there was a decrease in the ROS signals, with the highest values in relation to the baseline of 0.23 ± 0.04 S.E.M, n = 3, between 25-40 min. Signal reduction may be related to alteration of calcium channels, which are directly linked to ROS production (Görlach et al., 2015). At 40 min the ACSF solution was perfused again in the slice and the signals were recovered with values of 0.03 ± 0.02 S.E.M, n = 3 in the period of 60 and 70 min. The recovery of the signals suggests that the cells can recover their activities and that the oxidative stress to which they were submitted was converted, same result was obtained by Fajardo et al. (2017)b and Bosio et al. (2018).

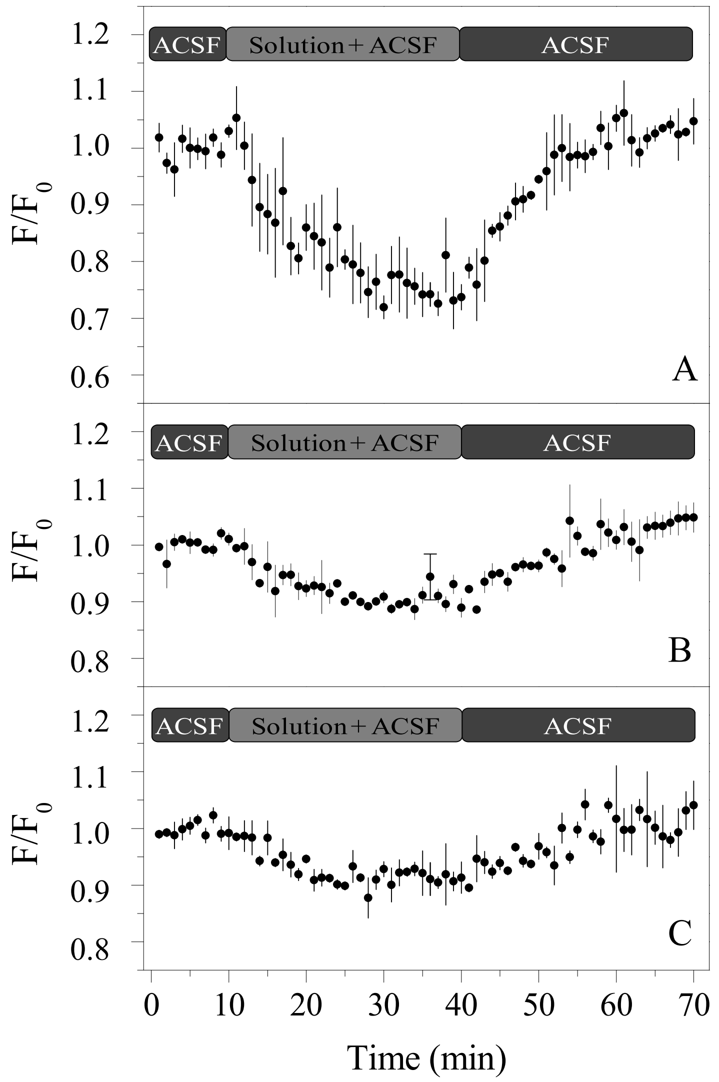


Figure VIII.6 – Effects on ROS signals of pharmaceutical wastewater before (WW_{50noEO}) and after ($5\text{-}WW_{50BDD1000}$ and $11\text{-}WW_{50TiPt1000}$) EO treatment. The fluorescente ROS indicator H_2DCFDA (20 µM) was used for data acquisition.

In Figures VIII. 6 B and C it is possible to observe the results obtained in the production of ROS with solutions after the electrochemical oxidation with BDD anode ($5\text{-}WW_{50BDD1000}$) and Ti/Pt anode ($11\text{-}WW_{50TiPt1000}$). In both cases there was a decrease in ROS production with values of 0.09 ± 0.01 and 0.08 ± 0.02 S.E.M, n = 3 (25-40 min) relative to baseline for BDD and Ti/Pt, respectively. It is possible to detect that the EO was efficient to reduce the toxicity of the medium, the results obtained showed an improvement of 60 and 65 % for BDD and Ti/Pt, respectively in relation to the untreated effluent. Electrochemical oxidation had the ability to degrade and mineralize some of the substances responsible for increased toxicity (Thiam *et al.*, 2015). The decrease in toxicity after advanced oxidative process was also reported by Gomes et al. (2017).

To evaluate the long-term effect of effluent on ROS production, the experiment was carried out (Figure VIII.7) with application of the pharmaceutical wastewater in cycles ($WW_{100cycle}$).

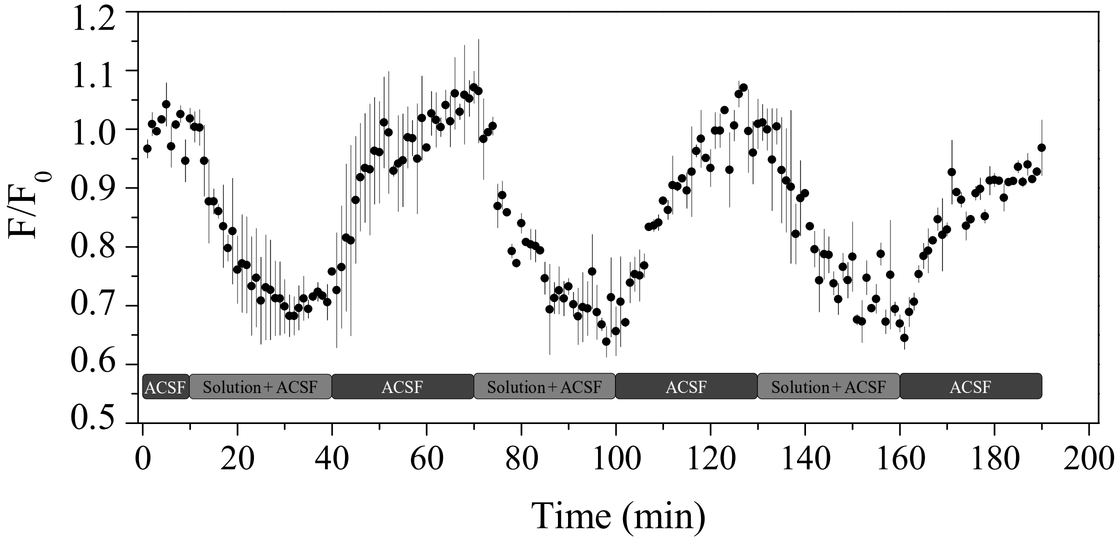


Figure VIII.7 - Pharmaceutical wastewater solution applied in cycles to evaluate ROS production. To collect the data the fluorescente ROS indicator H_2DCFDA (20 µM) was used.

From the moment the solution with real effluent comes into contact with the hippocampal slices (10 min), the ROS signals decrease 0.29 ± 0.04 S.E.M, n = 3 in the period of 25-40 min and with the return of the ACSF solution the signals become recovered with values of 0.01 ± 0.04 S.E.M, n = 3 above the baseline in the period of 55 - 70 min. Then the solution with pharmaceutical wastewater was applied again between 70 and 100 min, in this period the signs decreased to values of 0.29 ± 0.04 S.E.M, n = 3 between 85 and 100 min. With the return of the ASCF solution a recovery of the signals of 0.02 ± 0.03 S.E.M, n = 3 below the baseline occurred between 115 - 130 min. And in the third and last stage of the experiments, the perfusion of pharmaceutical wastewater was between 130 and 160 min, in this interval it was possible to detect the decrease in ROS production with mean values of 0.27 ± 0.02 S.E.M, n = 3 between 145 and 160 min and with the return of the ACSF solution was detected the recovery of the signals with values of 0.09 ± 0.01 S.E.M, n = 3 below the baseline in the period of 175 and 190 min. The effect of the application of pharmaceutical wastewater on the production of ROS increased with the number of cycles and the recovery power of the slices decreased. This may be related to the long-term contact of some substances, which can be harmful to cells and lead to a decrease in efficiency in the recovery of vital activities of the organism.

VIII.4 Conclusion

Two types of anodes (BDD and Ti/Pt) were used for the degradation of the pharmaceutical effluent by electrochemical oxidation, both electrodes were efficient in the removal of TOC, COD and TN. Some parameters were evaluated and it was possible to observe that the current density had a strong influence on the removal of TOC, COD and TN. The best results for TOC removal (71.03%) were with 50% dilution of pharmaceutical wastewater, BDD anode and 1000 A m^{-2}. The BDD anode was more efficient compared to Ti/Pt. In studies of neurotoxicity, the pharmaceutical wastewater caused a depression of the signals, these were recovered to initial values upon washout. With the treatment of the effluent by electrochemical oxidation with BDD and Ti/Pt anode, the effects caused by the variation of the ROS signals were lower, EO was effective in partially eliminating toxicity. Evaluation of ROS production with the application of several cycles of untreated effluent demonstrated that the long-term effect may alter the functionality of the cells and the recovery response decreases over time.

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CHAPTER IX – Conclusion

The evaluated the advanced oxidative processes for the degradation of the benzodiazepines (alprazolam, diazepam, clonazepam and lorazepam) and carbamazepine, as well as the degradation of these compounds in different aqueous matrices and the electrochemical oxidation used in the raw effluent from a pharmaceutical. In addition to the degradation of the different aqueous matrices, toxicity studies were performed: phytotoxicity evaluated seed germination and root growth when exposed to different solutions before and after electrochemical oxidation; and neurotoxicity detected the influence of different solutions on the production of reactive oxygen species in the CA3 region of hippocampus of Wistar rats. The degradation of the compounds and the comparison with the effects of toxicity are fundamental to evaluate the impact of pollutants on the environment.

In Chapter III the degradation of benzodiazepines and carbamazepine in the process of heterogeneous photocatalysis and hydrogen peroxide/UV was discussed. It was possible to evaluate that the concentration of TiO_2 and H_2O_2 influence in the efficiency of the process and that the excess of these reagents can be harmful in these advanced oxidative processes. The degradation of the compounds into a real aqueous matrix requires more energy expenditure, as there are more compounds to be degraded and increase the competition for the oxidants produced. The combination of two processes may be necessary for the mineralization of the compounds and the simultaneous disinfection of the solution.

In Chapters IV and V was studied the degradation of the pharmaceutical compounds alprazolam, diazepam, clonazepam, lorazepam and carbamazepine by electrochemical oxidation with Ti/Pt anode. It was possible to detect the efficiency of the degradation of the compounds in different aqueous matrices, however, when applying the water from the domestic sewage treatment plant, the efficiency of the process decreased, due to the presence of a greater amount of other substances in the solution. It was possible to conclude that the anode can be used several times, since its morphology is not decharacterized with the use and the nature of the electrolyte can affect the degradation of the compounds, since the degradation by indirect electrochemical oxidation is directly affected by the production of different species. Solutions after electrochemical oxidation continued to affect the decrease of signal generation in ROS production and the seed germination index was also affected.

Electrochemical oxidation with BDD anode of benzodiazepines and carbamazepine was evaluated in Chapter VI. With the increase in current density the process became more efficient, due to the formation of more radicals such as •OH. The formation of reactive chlorine species helps in the degradation of the compounds and with the increase of NaCl concentration, the system was more efficient. The EO with BDD was more efficient when compared to Ti/Pt. However, effluent from sewage treatment plant treated and applied to neurotoxicity and phytotoxicity showed no improvement in the elimination of toxicities.

In Chapter VII benzodiazepines and carbamazepine were removed in different aqueous matrices by EO. The pH variation did not significantly alter the degradation of the compounds and the increase in NaCl concentration was responsible for an increase in the degradation of the pharmaceutical compounds. The use of aqueous matrices with higher concentration of organic matter decreased the efficiency of the electrochemical oxidation. The use of ultrapure water and superficial water in the neurotoxicity, altered the signs of ROS, however the partial recovery of the signs occurred, this fact was not observed when using water from a domestic sewage treatment plant.

Finally, in chapter VIII the degradation of a pharmaceutical effluent by electrochemical oxidation with different anodes (BDD and Ti/Pt) was evaluated. Both electrodes removed TOC, COD and TN, however the BDD was more efficient. For the removal of a higher percentage of COD, TOC and TN it was necessary to increase the current density which led to an increase in energy expenditure. In the solutions used in neurotoxicity tests after EO, it was possible to observe that the changes caused in the ROS production by these solutions decreased compared to the solutions before EO. However, when the untreated effluent was applied in different cycles, it was possible to observe that the increase in the number of cycles leads to a decrease in the recovery of the ROS signals.

In electrochemical oxidation the degradation of benzodiazepines and carbamazepine was more efficient when using BDD anode, one of the reasons is that this electrode has the highest oxidation potential among the studied anodes. In the electrochemical oxidation independent of the anode used, the pH variation of the solution showed no significant variation in the degradation results of the studied compounds. However, when the current density was varied in all experiments, the degradation of the compounds was higher in the higher applied current density.

The degradation in the electrochemical oxidation of the compounds was more efficient than the heterogeneous photocatalysis, requiring an energy expenditure in the EO and the heterogeneous photocatalysis of 0.065 and 0.31 kWh m^{-3}, respectively, for the same percentage of degraded compound. However, when the EO was compared with the heterogeneous photocatalysis with H_2O_2, the energetic demands were 0.065 and 0.033 kWh m^{-3}, respectively, which makes the heterogeneous photocatalysis process with H_2O_2 more efficient. This demonstrates that combining more than one process may favor the elimination of wastewater compounds.

The use of a raw aqueous matrix in the heterogeneous photocatalysis and in the electrochemical oxidation, directly influenced the decrease of the efficiency of both processes. This is due to the fact that real matrices have a large load of organic compounds and other substances. The high concentration of compounds requires a longer process time or an increase in energy demand. For this reason complex wastewater should be studied in advanced oxidative processes.

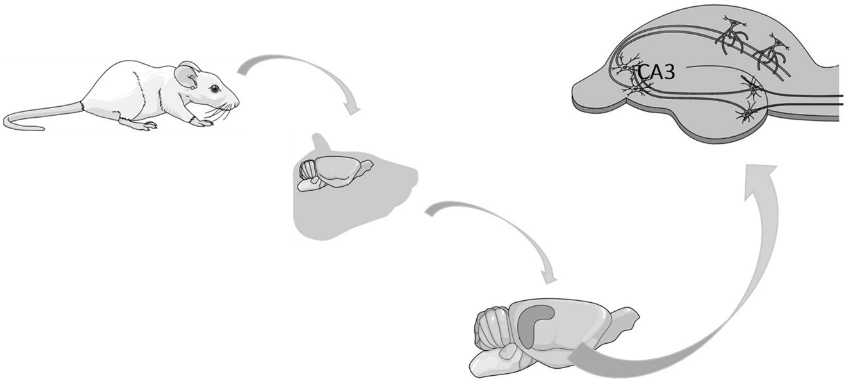
The results of the neurotoxicity tests clearly demonstrated the difficulty of completely eliminating the toxicity of the solutions. It was observed in all experiments that some kind of variation in ROS production occurred. The decrease in toxicity was observed mainly in the effluent of the pharmaceutical industry after electrochemical oxidation with BDD and Ti/Pt anode.

Innovative use of rat brain in toxicity studies becomes quite interesting, these animals have a rapid maturation of the organism and biological and functionalities characteristics similar to those of humans. For this reason the experiments performed on these animals indirectly indicate what effects may be caused in humans.

APPENDIX

Anexo: metodologia dos testes de neurotoxicidade

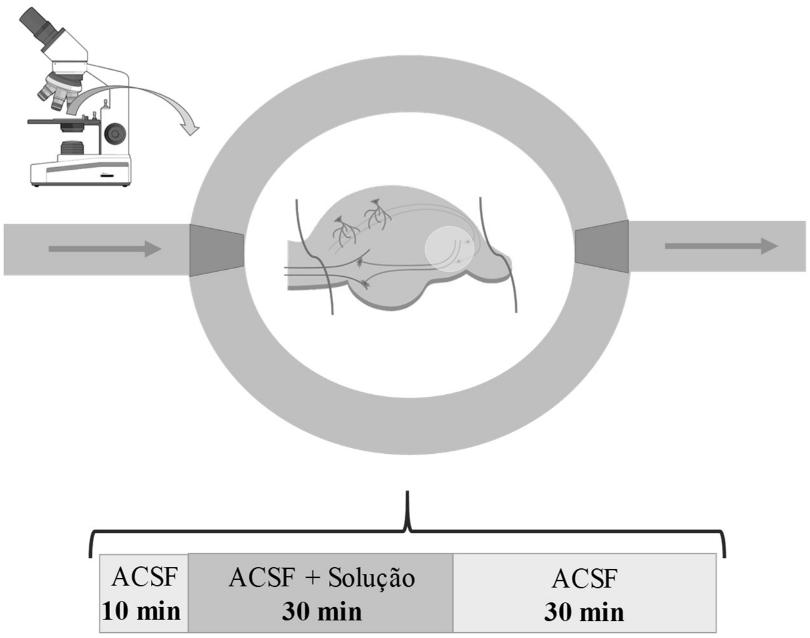
Etapa 1: Abatimento do rato Wistar. A rata com período gestacional entre 14-18 dias era anesteziada e em seguida ocorria o deslocamento cervical, instantaneamente ocorria a decapitação e remoção do cérebro que era depositado na solução de ACSF resfriada (5º C). Em seguida o cérebro era cortado para a extração do hipocampo de ambos os lados. O hipocampo era fatiado com lâminas de 350 μm de espaçamento e as fatias eram instataneamente imersas em solução de ACSF oxigenada com 95 % de O_2 e 5 % de CO_2. A solução de ACSF era preparada no dia dos experimentos com a seguinte composição e concentração: KCl (3.5 mM), NaH_2PO_4 (1.25 mM), $MgCl_2$ (2.0 mM), $CaCl_2$ (2.0 mM), NaCl (124.0 mM), $NaHCO_3$ (24.0 mM) e D-glucose (10.0 mM), pH 7.4.



Etapa 2: Solução de indicador de ROS. Depois das fatias serem extraídas e acondicionados em solução de ACSF, ocorria a adição de 20 μM do indicador fluorescente de ROS (H_2DCFDA) na solução de ACSF oxigenada, na qual as fatias permaneciam por 1 h para o que indicador pudesse permear para dentro das célular. O H_2DCFDA entra nas células e enzimas naturais presentes na mesma, reagem com o H_2DCFDA formando um compost impermeável que se liga ao ROS e se torna fluorescente. Após esse período as fatias eram novamente transferidas para uma solução de ACSF oxigenada.

Etapa 3: Utilização das fatias para os experimentos. As fatias podiam ser utilizadas por um período de aproximadamente 10 horas. O início do experimento ocorria com a colocação da fatia de hipocampo em um camara experimental, acoplada a um microscópio (Zeiss Axioskop). Nesta camara era perfundida a solução de ACSF oxigenada, com temperatura controlada (30 – 32 ºC) e vazão entre 1.5 e 2.0 mL min^{-1}. Inicialmente a fatia permanecia com a solução de ACSF por 30 min, entretanto são utilizados apenas os 10 minutos finais, depois desse período iniciava-se a perfusão da solução de estudo (com

ACSF) por mais 30 min e no final novamente ACSF por mais 30 min, em seguida a fatia era descartada. Os experimentos eram realizados em triplicata com diferentes ratas.



Etapa 4: Especificações do microscópio. O microscópio Zeiss Axioskop continha uma lâmpada de tungstênio/halogênio (12 V, 100 W), com lente de imersão na solução líquida (40x). Os sinais eram obtidos por sinais de fotodiodo e convertidos posteriormente utilizando um conversor analógico/digital (National Instruments). Os sinais eram processados utilizando o software Signal Express^{TM} (Labview, National Instruments). Os sinais eram obtidos da região CA3 do hipocampo, o foto nesta região foi realizado com o auxílio de micromanipuladores. Cada ponto foi representado por uma media de 100 pontos a cada minuto.