UNESCO-IHE
Institute for Water Education

Universidad del Valle

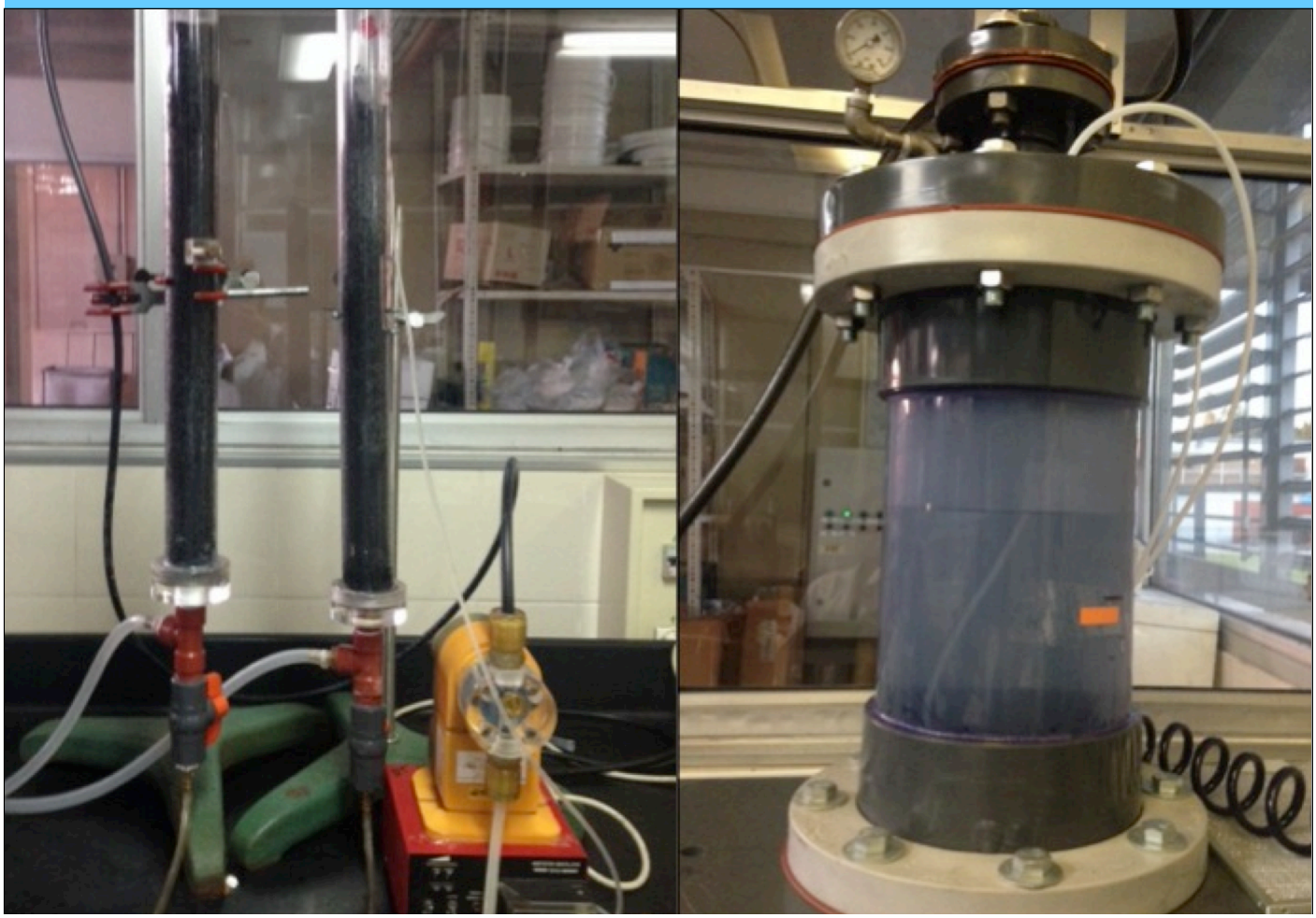
Evaluation of ozonation followed by BAC as a treatment for antimicrobial wastewater.

MSc thesis

Analía Menéndez Fagalde

MSc Thesis 46730

April 2016



Evaluation of ozonation followed by BAC as treatment for antimicrobial wastewater.

Master of Science Thesis
by
Analía Menéndez Fagalde

Mentors
Héctor García
Diana Miguez
Tineke Hooijmans
Carlos Madera

Examination committee
Diana Miguez
Tineke Hooijmans
Carlos Madera

This research is done for the partial fulfilment of requirements for the Master of Science degree at the UNESCO-IHE Institute for Water Education, Delft, the Netherlands together with Universidad del Valle, Cali, Colombia.

Delft
April 2016

Abstract

Wastewater treatment in Uruguay is in majority only primary treatment, and it does not include removal of pharmaceuticals. Toxicity is not yet regulated, and many chemical compounds are missed in the requirements of discharge and treatment. As a result, several emerging contaminants (pharmaceuticals, endocrine disruptors and others) have been accumulating in Uruguay's water basins.

In particular, this research is focused on the antibiotic establishments of Libra laboratory. Libra laboratory fabricates more than 150 products, and 4 different plants are located in Uruguay. The β-lactam antibiotics establishment (interest for this research) only works as a packing laboratory and is under inspection of DINAMA (*Dirección Nacional de Medio Ambiente*). On May 7, 2013, DINAMA took a sample from that effluent, as a part of regular inspection. Among the results, the toxicity test (lower than 11%) represented a high toxicity value, and DINAMA asked Libra to reduce the toxicity in this establishment effluent.

In the previous investigation by Silvarrey, (2015) the effluent (without any modification) of Libra antibiotics laboratory was tested for different types of ozonation treatments. The objective was to reduce the final toxicity to adequate levels (<11 %).

In order to continue with the recommendations, this investigation was focused on treatment based on ozonation and biological activated carbon. To avoid problems related to high COD concentrations, an artificial effluent was used, based on choosing the most toxic chemical component of the real effluent: the sanitizing agent. This is formed by 2 quaternary ammonium: Benzalkonium chloride and Didecyldimethylammonium chloride.

During 5 weeks of treatment, the following conclusions were achieved: toxicity effluent was reduced to “not toxic” classification, biofilm was developed effectively in the biofilter; presence of ammonium in the effluent indicates biodegradation of the quaternary ammonium; evaluation of pathogens in the effluent reach values according to the normative.

Further investigations need to be done in order to conclude about the effectiveness of the treatment in Libra effluent, especially to assess if ozonation is actually necessary.

***Key words**: ozonation, biological activated carbon, synthetic pharmaceutical water, toxicity.*

Acknowledgments

This thesis is the result of hard work only possible because of help and support of many partners, fiends, family, technicians, lecturers and mentors, who I want to say thank you.

First of all, everything was possible because the unconditional love and financial support of my mum.

Financial and academic support was also possible to ANNI (*Agencia Nacional de Investigación e Innovación, Uruguayan government*), Univalle and UNESCO-IHE.

I would also like to recognize every person in LATU for their understanding and technician support at any time, especially to Jorge Venis, Paula Mussio, Alejandro Ureta, Enrique Delgado, Lucía Boccardi and Fabiana Rey.

I am also feeling grateful to my mentors and lecturers, especially to Diana Míguez, Tineke Hooijmans, Carlos Madera, Hector García and Yness Slokar for share their experience and encouragement.

For the external supporters, which without anything in return, gave me all their knowledge and advice: Ignacio Carrera, Manuel Baruch, Alejo Silvarrey and Gregorio Iraola.

To all my friends, my brother and sister who were unconditional and made my life happier and nicer the past 2 years: Giovanna Guarinoni, Guillermo Zinola, Nicolás Cunha, Cecilia Ceiter, Pablo Kok, Ibrahim Chernor Bah, Victoria Cerecetto, Mariana Adami, Florencia Díaz, Joaquín Menéndez and Melanie Montes.
To my dad and my niece Camila Menéndez.

Finally to my boyfriend Wellington Silva, who is really proud of me and encourage every step of my life with love, trust, optimism and joy.

Table of Contents

List of Figures

List of Tables

CHAPTER 1

Introduction

- **Around the world the pharmaceutical industry recovers US$300 billion a year and the 10 largest companies control one third of this entire market. In these companies one third of the revenues are for marketing, and only half of that amount is for research (WHO, 2015).**

1.1. Background

1.1.1. Uruguay and health

Uruguay is a country located in South America with a total population of 3.4 million inhabitants (until 2013) and an area of 176.215 km². The demography is 19 inhabitant/ km² and according to the development index, Uruguay stands at 51 (PNUD, 2013).

The gross national income per capita is 18 dollars and the expenditure on health per capita is 1,715 dollars (Levcovitz, 2013), which is certainly better situation in comparison with the rest of Latin America. According to the World Health Organization (WHO), the life expectancy is 77 years (**Table** 1-1), and it has been increasing by 2 years over the period 2000-2012.

The population growth rate is 0.5% per year, which is relatively low to favour in the expansion and development of industries. Despite this fact, it is important to mention that the entire country has health care coverage. In addition, there is a strong tendency of increasing the average age of the population (13% is above 65 years old). Against this background, a high consumption of medicines is expected.

***Table** 1-1 life expectancy in years until 2012 in Uruguay (right), WHO region (Americas) and World Bank income group (WHO, 2015).*

Life expectancy (years), 2012

		Country	WHO region	World Bank income group
Life expectancy	At birth	77	76	79
	At age 60	22	22	23
Healthy life expectancy	At birth	68	67	70

The percentage of population using improved water and sanitation is extremely high, reaching almost 100% (**Figure** 1-1) (WHO, 2015).

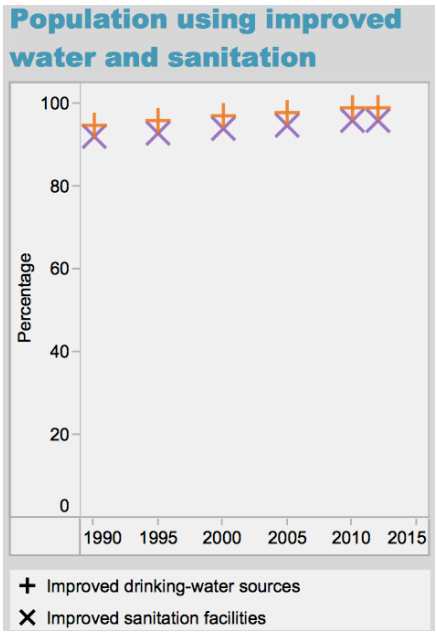


***Figure** 1-1 population using improved water and sanitation in Uruguay (WHO, 2015).*

1.1.2. Pharmaceutical industry in Uruguay

According to Miranda (2013), as at 2012, 92 companies belong to the pharmaceutical industry in Uruguay. 83 of these companies are located in the capital (Montevideo), which shows that Uruguay is a centralized country. The minister of public health controls the human use laboratories, like Libra, Roemmers, Servimedic, Urufarma, Celsius and Lazar. All of them are national or regional pharmaceutical companies, and belongs to the national association of laboratories (ALN: *Asociación de Laboratorios Nacionales*). In this research Libra is the pharmaceutical industry of interest, as is mention below.

Among the total manufacturing industries in the country, the pharmaceutical industry represents only 1%. It is possible that this modest percentage refers to the poor inversion dedicated to research. However, in the past years, the government is dedicating more capital to this field, as the "Productive Cabinet" has selected it as one of the strategic sectors to develop in the period 2010-2020 (LATINPHARMA, 2011).
In Figure 1-2 the expenses dedicated to research and development in Uruguay and in Latin America are compared.

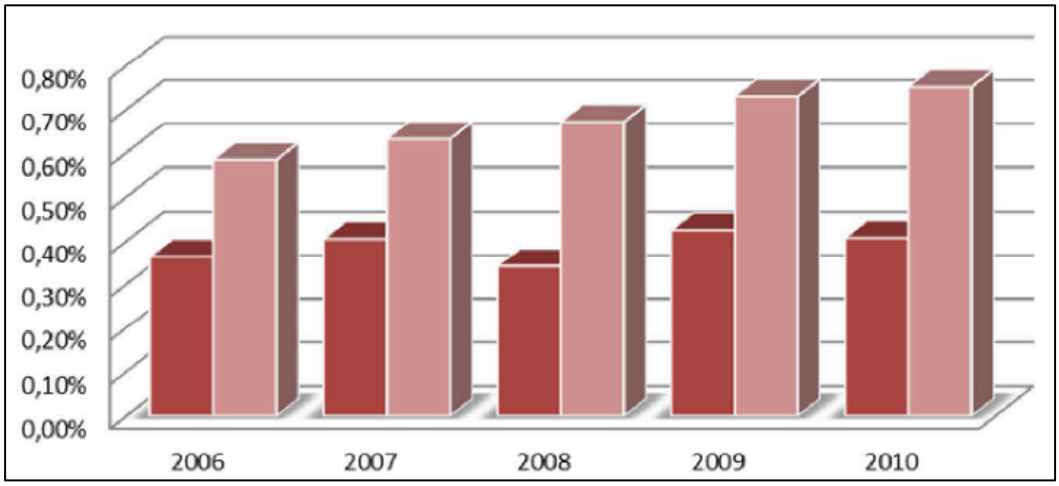


Figure 1-2 capital dedicated to research and development. In Uruguay: red and in Latin America: pink (Miranda, 2013).

A total of 475 million dollars was recovered in 2012 from the pharmaceutical industry in Uruguay (national market). In the past period 2004-2010, the expansion on the sales of around was 117.5% was seen. Despite this value, Uruguay has the lowest prices for medication across the region. The category of medicine with the highest sales is birth control pills, followed by broad-spectrum antibiotic (active ingredient: Amoxillicin) (LATINPHARMA, 2011).

1.2. Problem statement and justification

This research is focused on the antibiotic establishments of Libra, which is under inspection of DINAMA (*Dirección Nacional de Medio Ambiente*). This establishments only mixes active compounds and packaging them, they do not use water during that process. In fact, water is only used during the cleaning. Their water consumption per month is 530 m^3, and wastewater from the toilets is mixed with water from the production rooms. **Figure** 1-3 shows the percentages of water flows per activity (Silvarrey, 2015).

On May 7, 2013, DINAMA took a sample from the effluent of Libra, as part of regular inspection. The COD, BOD5, pH, settable solids and toxicity of the sample were measured for which results are shown in Table 1-2 (Silvarrey, 2015). Among the results, the toxicity test (lower than 11%) represents a high toxicity value (details of toxicity test are described below). DINAMA asked Libra to reduce toxicity in this effluent.

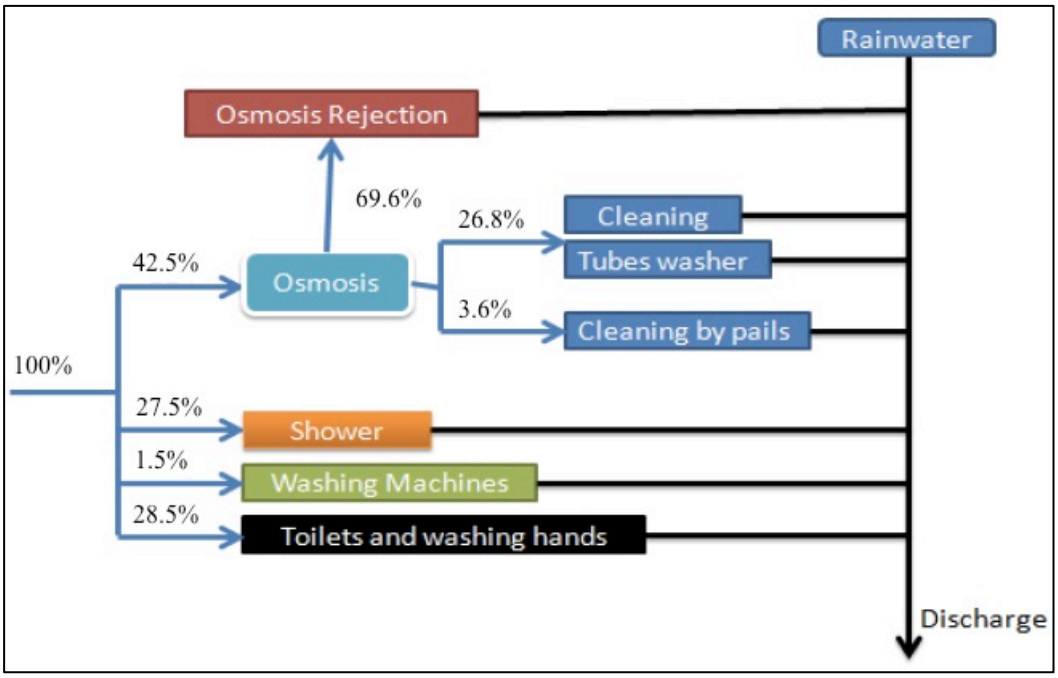


***Figure** 1-3 wastewater mass balance of Libra laboratory in percentages of flows (modified from Silvarrey, 2015).*

Table 1-2 results of DINAMA sample (Silvarrey, 2015).

Item	Value	Limit
pH	7.8	5.5-9.5
Settable solids (mg/l)	<0.5	10
BOD_5 (mgO_2/l)	210	700
COD (mgO_2/l)	440	UD
Toxicity by Microtox (%)	<11	>=100

According to the legislation number 253/979 from URSEA (*Unidad Reguladora de Servicios de Energía y Agua*), the discharge into the sewage system and natural water courses is regulated by the measurements of BOD5, pH, fats, sulphurs, cyanides, arsenic, cadmium, copper, chrome, mercury, nickel, lead, and zinc (values are showed in **Table** 1-3) (ursea, 2015).

Majority of the Wastewater treatment plants in Uruguay only primary treatment process, and removing pharmaceuticals is not part of the treatment. Toxicity is not yet regulated and several chemical compounds like pharmaceuticals are missed in the regulations of discharge and treatment. As a result of lack of regulation and control, several emerging contaminants (pharmaceuticals, endocrine disruptors and others) have been accumulating in Uruguay water basins (Silvarrey, 2015). In **Figure** 1-4 shows a summary of how pharmaceuticals have been accumulating in drinking water resources.

***Table** 1-3 wastewater discharge standards to the sewage system and to the water courses (ursea, 2015).*

Parameter	Value (sewage)	Value (water courses)
Temperature	35 ^{o}C	max. 30 ^{o}C
pH	5.5-5.9	6.0-9.0
BOD5	700 mg/l	60mg/l
Fats	200mg/l	50mg/l
Sulphurs	5mg/l	1mg/l
Cyanides	1mg/l	1mg/l
Arsenic	0.5mg/l	0.5mg/l
Cadmium	0.05mg/l	0.05mg/l
Copper	1mg/l	1mg/l
Chrome	3mg/l	1mg/l
Mercury	0.005 mg/l	0.005 mg/l
Nickel	2mg/l	2mg/l
Lead	0.3mg/l	0.3mg/l
Zinc	0.3mg/l	0.3mg/l
Amoniac	-	5mg/l
Fecal coliforms	-	5000 UFC/100ml

The consequences of the accumulation of pharmaceuticals in water are not well defined yet. This is because of the uncertainty that each compound may cause to the aquatic life (Shane, 2008). However, several studies with endocrine disruptors showed the terrible outcome in the environment and in human health. Among the most studied diseases in animals is the development of cancer. Animal experiments showed an increase in tumor formation, induced by exposure to a variety of chemicals during prenatal or neonatal life (Birnbaum, 2003).

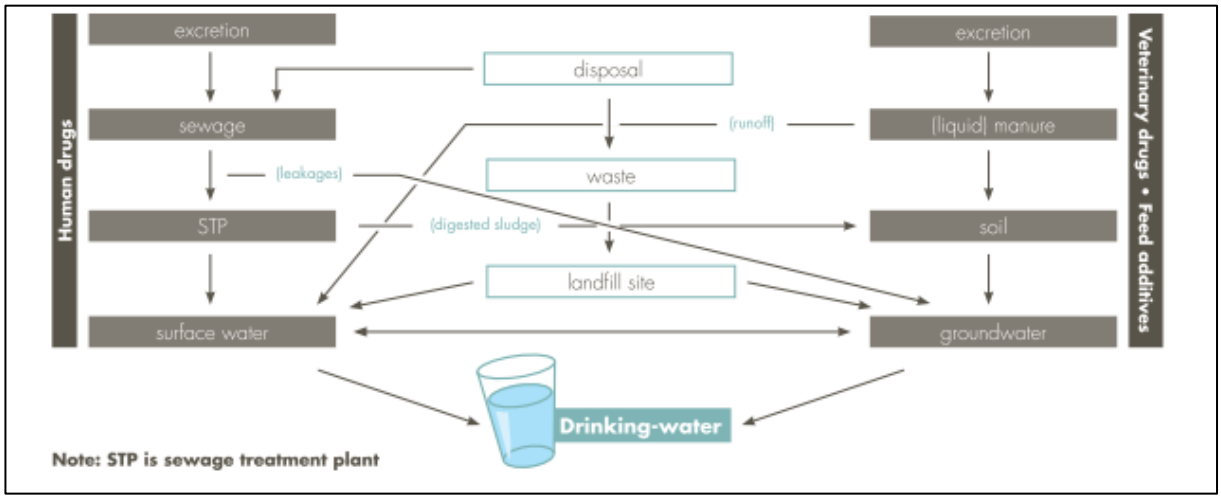


***Figure** 1-4 origin and transport of pharmaceuticals through different types of water and pharmaceutical uses. Adapted from (Ternes, 1998).*

In the previous investigation by Silvarrey, (2015) the effluent (without any modification) of Libra antibiotics laboratory was tested for different types of ozonation treatments (**Table** 1-4: details of components of the wastewater effluent). The objective was to reduce the final toxicity to adequate levels (<11 %). Results showed lot of variation between ozonation types, and in many cases, toxicity was not reduced to adequate levels. Moreover, high concentrations of COD in wastewater may affect the treatment and produce non-desirable by-products. It was concluded that it is necessary to integrate ozonation with another treatment, such as a bio filter treatment.

In order to continue with the recommendations, this investigation is focused on treatment based on ozonation and biological activated carbon. To avoid problems related to high COD concentrations, an artificial effluent will be used, based on choosing the most toxic chemical component of the real effluent.

***Table** 1-4 Libra laboratory wastewater components.*

		Libra laboratory wastewater components	
Industrial wastewater	Pharmaceuticals:	β-lactamic antibiotics	Cefazolin, Ceftriaxona, Cefepima, Meropenem, Imipenem, Ampicillin
	Santizing agent:	Quaternary ammoinums	Didecyldimethylammonium chloride, Alkyldimethylbenzylammonium chloride
Domestic wastewater	From toiletts		

1.3. Research questions

Main research question

- Is it possible to reduce the toxicity, measured as Whole Effluent Toxicity, of artificial pharmaceutical model wastewater with ozonation followed by BAC treatment?

Sub questions

- Is it possible to develop a biofilm in granular activated carbon for water with these characteristics that contribute to the toxicity reduction?
- Is there any improvement by adding a BAC system after the ozonation treatment? (Based on the previous study Silvarrey, 2015).
- Is there any difference between the toxicity reduction of artificial pharmaceutical wastewater using an activated carbon filter with or without biofilm?

Further question

- Which ones are the main bacterial strains developed in this system?

1.4. Research objectives

The main objective is to evaluate the performance and applicability of a treatment with ozonation followed by a Biological Activated Carbon filter (BAC) for an artificial pharmaceutical waste water, based on the antimicrobials produced by Libra laboratory.

The specific objectives of this research are:

- Develop a methodology to start up a process of ozonation followed by a BAC system to treat wastewater with antimicrobial chemicals.

- Evaluate the potential of this treatment by reducing the toxicity determined by Microtox or *Daphnia Magna* methods.

Further objectives of the present research are:

- Identification of the main biological strains developed in the BAC and comparison of the difference between the strains in the initial inoculum and at the end of the research period, by massive sequencing analysis.

CHAPTER 2

Literature review

2.1. Global situation: pharmaceutical and surfactants industries

The pharmaceutical industry recovers US$300 billion a year and the 10 largest companies controls one third of this entire market. In these companies one third of the revenues are for marketing, and only the half of that amount is for research. To handle this situation, the World Health Assembly adopted a principle for the rational use of drugs in 1988. However, this criteria was created for members of the International Federation of Pharmaceutical Manufacturers' Associations (PharMA), which makes it unethical due to the pharmaceuticals were making the legislation that need to be followed (WHO, 2015).

The irrational use of drugs has been generating multitude of problems related to resistance of microorganisms, pollution of the environment, waste of resources, reduction in the quality of the treatment, etc. (OPS, 2015).

Several studies have found trace concentrations of pharmaceuticals in wastewater, water sources and drinking water. In treated water this concentration was generally less than 0.05 µg/l, while for untreated water (surface water and ground water) the concentration was around 0.1 µg/l. The composition and quantity vary from place to place, depending on the characteristics of the common used drugs, but this investigation suggest the presence of pharmaceuticals in many water sources receiving wastewater effluents (WHO, 2015).

The surfactants industry is a 7140 millions pounds industry with an extensive market: from household detergents to explosives (Chemistry, 2003). This global market is projected to reach 24,037.3 KT (Kilotons), in terms of volumetric demand, and $42,120.4 million in terms of value, by 2022 (Research, 2016). Demand of surfactants has been increased in the past years, specially eco-friendly surfactants (bio-based) due to the increase of normative and regulations in Europe and North America. Despite of this, Asia-Pacific dominates the global surfactants market and is anticipated to have the highest growth rate in the next 5 years (Rohan, 2014).

Between surfactants, soaps and detergents are the largest share of the global surfactant commerce (Rohan, 2014). As is the interest of this research, quaternary ammoniums (QACs) are cationic detergents that have been accumulating in aquatic sediments near to effluent discharges due to their big amounts present in wastewater. Their concentration in wastewater effluent is 0.05 mg/l while their concentration in surface water is 0.04 mg/l (Tezel, 2009). In 4 different plants of Austria, influent of domestic wastewater treatment plants reported QACs

concentrations were from 0.001 to 0.170 mg/l, while for the effluent of industries reported concentrations were higher (1-40 mg/l). Those industries include paper processing, textile and food processing (Tezel, 2009).

2.2. Situation in Uruguay

Several studies show how chemicals are affecting Uruguayan rivers. Uruguay river is an example of a river whose quality is negatively affected by pollution. The Uruguay River rises in southeastern Brazil and flows into the Rio de la Plata (which feeds the Atlantic Ocean), between Uruguay east and Argentina to the west. The Uruguay River together with the Parana River, the Paraguay River and other major rivers, form the Plata Basin. In the doctoral study of Míguez (2014), androgenicity and estrogenicity was found in native fishes.

Other example is the Santa Lucia river basin that is the main source of drinking water for the country. With an extension of 13,433 km^2, this basin provides water to 60% of the total population. Multiple factors are causing the deterioration of the water: the lack of an adequate system of urban sanitation, inadequate management of solid waste and dumping and untreated effluent from industries (Achkar, 2012). Between these industries are several pharmaceuticals.

Several studies also focused in Rio de la Plata basin, that is the example of Cespedes-Payret, (2009), that published a review about agro-industrial technologies and their environmental impact in Uruguay. In this review it was conclude that several changes as: erosion and flood control, pronounced droughts hydric deficit mitigation, replacement of soil nutrients with synthetic fertilisers, pest control through synthetic compounds, etc. are happening right now, and will continue happening if regulations and the government do not stop this process.

These several negative effects observed in the past years in Uruguayans rivers are the consequence of a lack of environmental laws and regulations about the discharge of wastewater effluents. As it was mentioned previously in this document, in the actual regulations only the discharge of BOD is considered, while toxic compounds are ignored in the evaluation (ursea, 2015).

2.3. Environmental risk of pharmaceuticals and surfactants in water systems

According to the World Health Organization pharmaceuticals are “synthetic or natural chemicals that can be found in prescription medicines, over-the-counter therapeutic drugs and veterinary drugs”. Pharmaceuticals can reach drinking water courses through sewage (from excreta or uncontrolled drug disposal), and they have become of emerging concern due to they are reaching drinking water sources (WHO, 2015).

Pharmaceuticals with big impact are antibiotics. Generation of antibiotic resistance in bacteria is a global issue; we are even reaching a point where bacterial infections are untreatable

(EXTER, 2015). The presence of these compounds in water reservoirs is increasing this phenomenon. According to the U.S. Geological Survey (USGS) antibiotics in the aquatic environment can delay the start of cell growth and have a negative impact in N cycle (by affecting denitrification) (USGS, 2015). Another relevant research proved how antibiotics could also affect the composition of microbial communities, which can also be the first step to generate antibiotic resistance (Haack, 2012).

Other environmental risky group is surfactants that have been converted in one of the most unwanted contaminants of water and landed surfaces. These types of chemicals are massively used, as they are part of many products like cosmetics, detergents, softeners and others (Lechuga, 2016). They are on the High Production Volume Chemicals list of USEPA (US Environmental Protection Agency) (Tezel, 2009). Because of this, surfactants easily end in water courses and their environmental risk depends on the reached concentrations in the aquatic medium (Ivanković, 2010). Due to the excessive presence of surfactants in the environment, their residues have become of special interest, in terms of ecological impact (Jardak, 2016). Environmental problems and consequences can be mentioned as a result of surfactants: they present a huge ecotoxicity as a result of surface activity and the effect produced on biological membranes (including human health damage), they caused bacteria resistance, in aquatic environment can foam and reduce re-oxygenation rate and oxygen levels, they cause variety of damages on aquatic animals, and toxic effects on aquatic plants species (because surfactants are able to break-up the chlorophyll-protein which causes difficulties in plants growth) (Tezel, 2009) (Jardak, 2016).

As it was mentioned before, Quaternary ammoniums (QACs) are cationic surfactants and represents 10% of the total surfactant demand; these molecules are present on a variety of products (**Figure** 2-1) (Schmitt, 1994). These molecules are organic micropollutants that can be considerer as emerging contaminants (Jardak, 2016). Similarly to surfactants in general, QACs are strongly adsorb on suspended solids where they can easily be transferred to aquatic sediments, where they have been accumulating (Tezel, 2009). In **Figure** 2-2 summaries of stages of accumulation and presence of QACs can be appreciated. From that figure it can be conclude that QACs have been accumulating in anoxic environments, especially on biomass, soil and sediment.

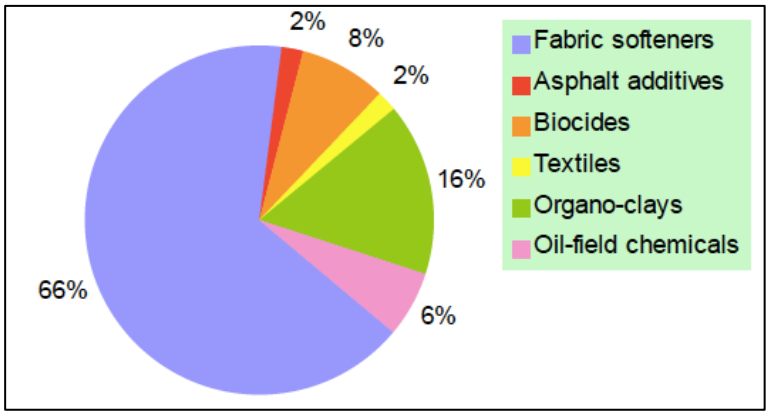


***Figure** 2-1 distribution of QACs in the market (Tezel, 2009).*

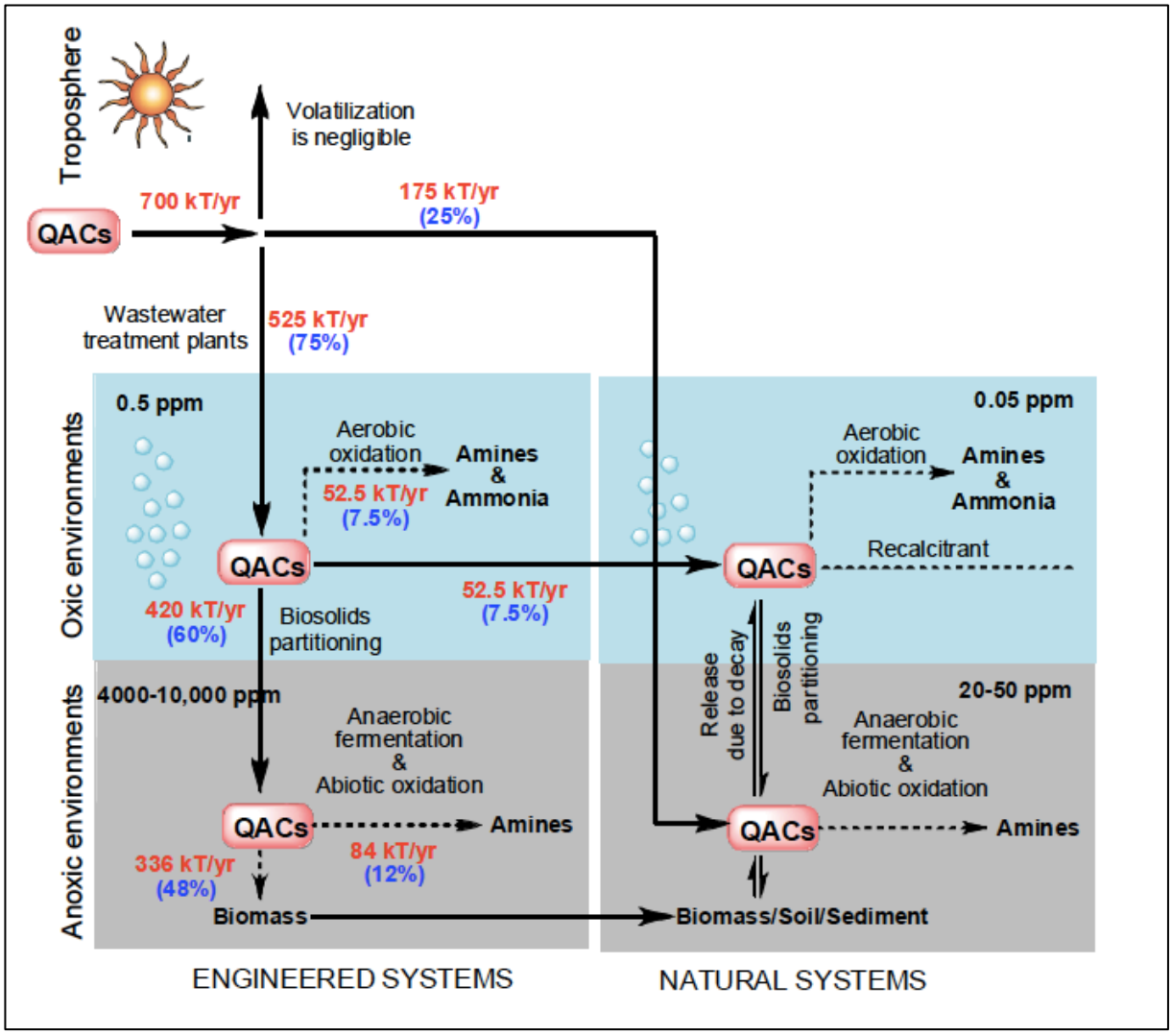


***Figure** 2-2 Summary of QACs fluxes and expected concentrations in different compartments of artificial and natural systems (Tezel, 2009).*

2.4. Wastewater treatment technologies: Ozonation

Ozone (O_3) is a very reactive molecule formed by 3 atoms of oxygen. Considering these characteristics it has to be produced *in situ.* In **Figure** 2-3 a representation of the molecule can be observed. The molecule has a resonance structure and can react as a dipole, as an electrophilic, or as a nucleophilic agent (partial negative and positive charges can be seen in **Figure** 2-3).

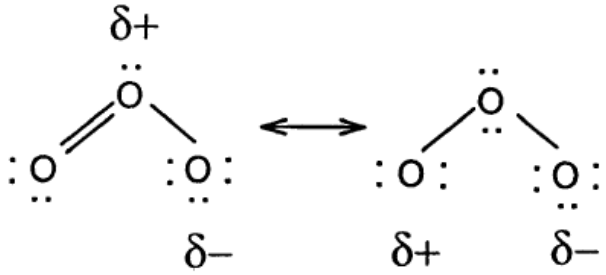


***Figure** 2-3 resonance of ozone molecule (Kasprzyk-Hordern, 2003).*

Another aspect is the instability that the molecule presents in aqueous solution, and the degradability follows a pseudo first order kinetic (**Equation** 1) (Kasprzyk-Hordern, 2003).

***Equation** 1 kinetic for the degradability of ozone in aqueous solutions (k' has constant values for given pH) (Kasprzyk-Hordern, 2003).*

$$-\left(\frac{d[O_3]}{dt}\right)_{pH} = k'[O_3]$$

Ozone decomposition is a chain reaction mechanism that produces free hydroxyl radicals ($OH^{\cdot}$). Accepted mechanisms for the decomposition of ozone in distilled water are showed in **Figure** 2-4.

Initiation Reaction

$O_3 + OH^- \xrightarrow{k_{i1}} HO_2\bullet + O_2^-\bullet$ [a]	70 $M^{-1}sec^{-1}$	(2.8)

Propagation Reactions

$HO_2\bullet \xrightarrow{k_1} O_2^-\bullet + H^+$	7.9×10^5 sec^{-1} [25]	(2.9)
$O_2^-\bullet + H^+ \xrightarrow{k_1'} HO_2\bullet$	5×10^{10} $M^{-1}sec^{-1}$ [25]	(2.10)
$O_3 + O_2^-\bullet \xrightarrow{k_2} O_3^-\bullet + O_2$	1.6×10^9 $M^{-1}sec^{-1}$	(2.2)
$O_3^-\bullet + H^+ \xrightarrow{k_3} HO_3\bullet$	5.2×10^{10} $M^{-1}sec^{-1}$	(2.11)
$HO_3\bullet \xrightarrow{k_4} O_3^-\bullet + H^+$	3.3×10^2 sec^{-1}	(2.12)
$HO_3\bullet \xrightarrow{k_5} HO\bullet + O_2$	1.1×10^5 sec^{-1}	(2.13)
$O_3 + HO\bullet \xrightarrow{k_6} HO_4\bullet$	2×10^9 $M^{-1}sec^{-1}$	(2.14)
$HO_4\bullet \xrightarrow{k_7} HO_2\bullet + O_2$	2.8×10^4 sec^{-1}	(2.15)

***Figure** 2-4 Ozone decomposition mechanisms in pure water (Beltran, 2003)*

With regards to those reactions (**Figure** 2-4), ozone effects can be classified as direct or indirect. Between ozone and any other molecule: direct reactions, while between hydroxyl radical (formed from the decomposition of the ozone or another direct reaction) and other molecules present in water: indirect reactions. Substances present in water directly affect the ozone decomposition rate. On **Figure** 2-5 ozone decomposition in buffered distilled water can be appreciated at 3 different pH. At basics pH, there is a higher and faster ozone conversion, while at acids pH this is completely the opposite. pH is also one of the main factors that influence the decomposition of ozone in water.

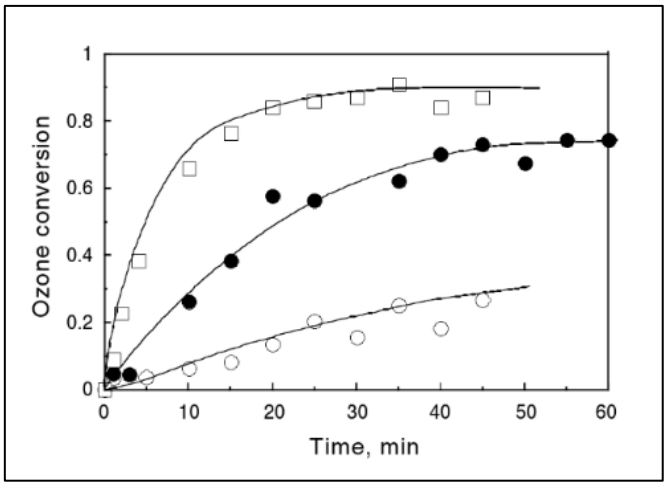


***Figure** 2-5 Decomposition of ozone in buffered distilled water at different pH. Fraction of ozone converted vs. time (minutes). Conditions 17^{0}C, buffered water with phosphates, white circle=pH 2, Black circle= pH7, square= pH 8.5 (Beltran 2003).*

Ozone can be produced by electrolysis or by UV-radiation. Some ozone applications are related to disinfection, due to the high oxidation capacity and capability of degradation of organic compounds of the molecule (Eriksson, 2005). Because of this, ozonation is a very applied technology in water treatment and is suitable for drinking water but also for wastewater (secondary effluent) (Stalter, 2010).

Despite that, some disadvantages are also part of using ozone for water treatment: low stability and solubility in water, partial oxidation of organic compounds resulting in higher toxicity at the end of the treatment (in various cases). All of them make ozonation an expensive and maybe unreliable treatment.

It was demonstrated that the utilization of ozone on pharmaceuticals has been reduced the toxicity of water with a mix of them (Andreozzi, 2004). Toxicity reduction after ozonation treatment can be affected due to the apparition of remaining by-products after water treatment, because ozone typically transforms chemical compounds but does not mineralize them entirely. For this reason, it becomes necessary to assess toxicity after the ozonation (Stalter, 2010).

Several studies focused on the removal of pharmaceuticals by ozonation. In the recent publication of Feng (2016) the antibiotic flumequine was removed by ozone oxidation, and the tentative degradation process was studied (**Figure** 2-6), showing that several low-molecular-weight carboxylic acids still remains after treatment possibly affecting the toxicity of the effluent.

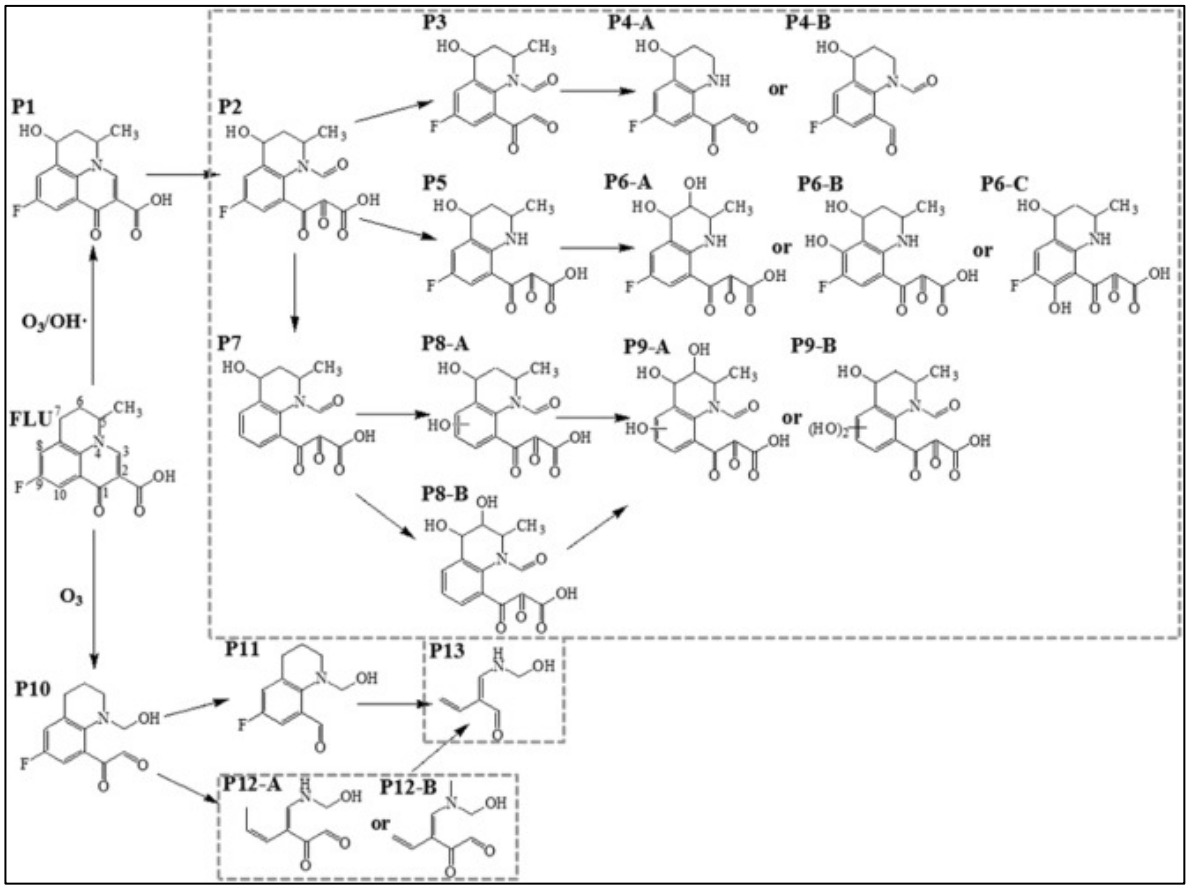


***Figure** 2-6 possible degradation pathways of antibiotic flumequine (Feng, 2016).*

Surfactants can be chemically, mechanically or biological treated (Lechuga, 2012). Oxidation is the main treatment applied to detergents and it includes ozonation, as is the case of Corless (1989). Despite they did not get relevant results, they design an ozone pathway attack of unsaturated quaternary ammoniums (**Figure** 2-7), where the double bond is essential for the ozone action (Delanghe, 1991). In the case of this research, this pathway could be similar for the double bonds of Benzalkonium chloride (section 4.1.4), and also based on the known that aromatic compounds are especially attacked by ozone (Ikehata, 2004).

Lechuga, (2012) proved that ozonation increases biodegradation of amide oxide based surfactants and reduces TOC, but mineralization caused by ozone is slow and incomplete. However, degree of mineralization after ozonation depends on the type of treatment (ozonation alone or combined with other oxidation process) and nature of surfactant (Zsilak, 2014) (Dantas, 2009).

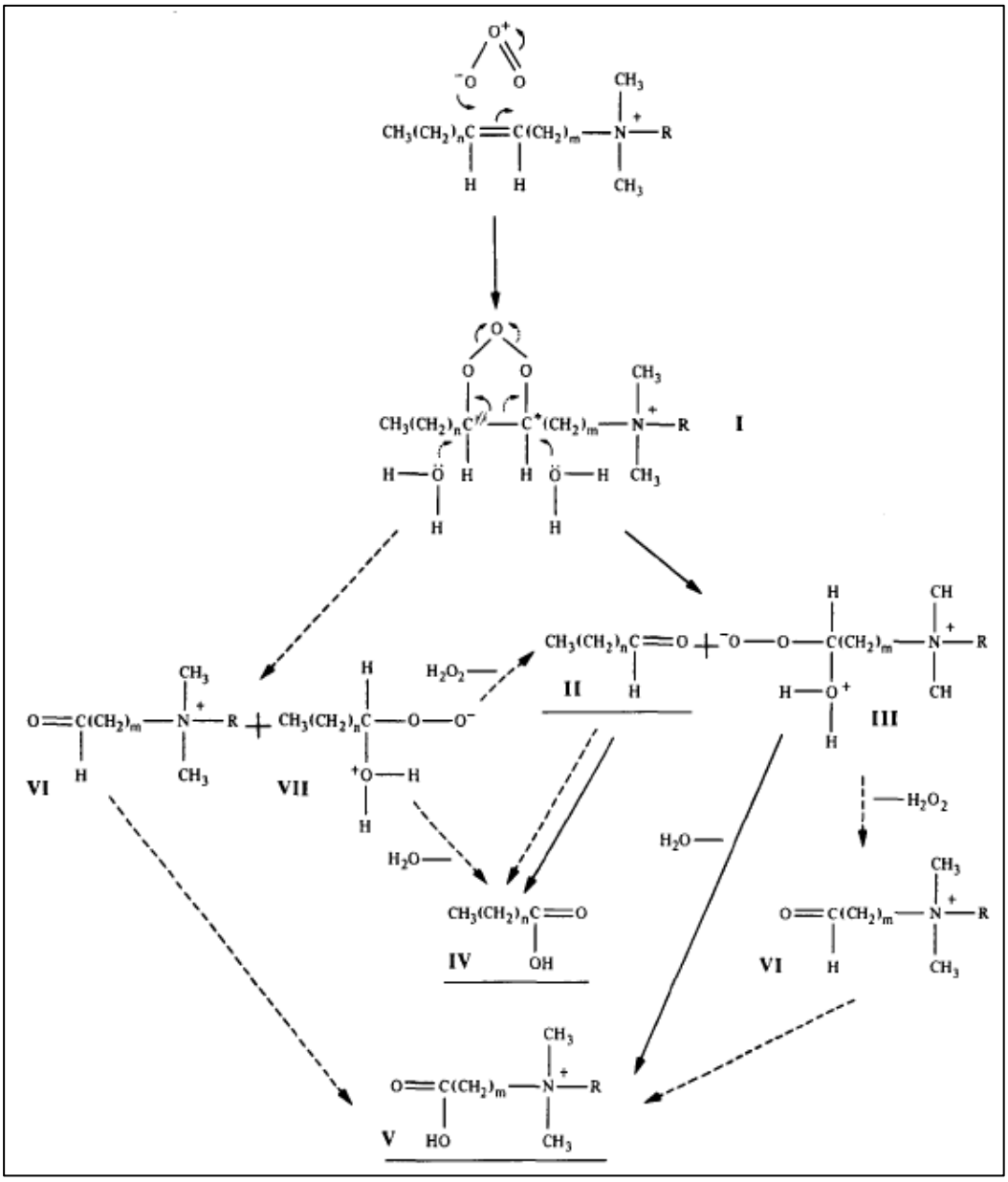


***Figure** 2-7 Proposed pathway for the reaction between ozone and the unsaturated components of the QACs. Alternative reaction pathways are represented by broken lines (Corless, 1989).*

2.5. Wastewater treatment technologies: Biological activated carbon (BAC)

A process using biological activated carbon (BAC) combines biodegradation and adsorption in order to treat water (Xiaojian, 1991). This treatment incorporates the capacity of adsorption of the activated carbon, due to its own characteristics (Bansal, 2005), and the ability of the microorganisms to degrade certain compounds (Madigan, 2015). The development and growth of the microbiota is possible because of the high degree of porosity and extended

surface area of this material (Bansal, 2005). This process was developed in 1967 on a wastewater treatment pilot plant in California, with the objective to remove organic matter (Parkhurst, 1967). However, it was in 1997 that was first used in a pilot plant for drinking water treatment (Kim, 1997). The success of this trial results in an extensively used technology.

In 2009 removal of nitromidazole antibiotics was tested with biological activated carbon treatment (Rivera-Utrilla, 2009). In this study, positive results were obtained, showing that the bioadsorption and chemical characteristics of the activated carbon are excellent to remove these types of components.
β-lactamic antibiotics were tested in batch reactors (aerobic and anaerobic) with positive results, however high retention times were needed.

There is no evidence of quaternary ammonium removal by using BAC, however, biological removal of QACs has been tested and proved, together with the identification of the main strains involved (Tezel, 2015). For this reason, it is possible to have a successful biodegradation by using BAC as treatment for quaternary ammonium compounds.

Several advantages can be mentioned when using BAC treatment; most of them are related to the properties of the activated carbon. Firstly, the adsorptive characteristic of the activated carbon increases the substrate concentration, resulting in greater time of contact with the microorganisms. In addition, the rugged surface of the activated carbon protects the microbiota environment from the water forces. Moreover, the carbon surface has a diversity of chemical functional groups, which also improve the interaction between each component (Weber, 1978). Between these are oxygen, hydrogen, combined sulphur, chlorine and nitrogen groups, which attached a variety of compounds in the surface of the activated carbon (Biniak, 1997).

With regards to the economy, regeneration of the carbon is less frequent with the biofilm attached, and a single reactor system is cheaper than individual treatment processes (Walker, 1999). Supplementary, similar filters with other supports (like sand) are not as effective as the granular activated carbon in regards to the development of the microbiota (Reungoat, 2012). Example of those supports is anthracite, sand, porous ceramsite or volcano rock (Li, 2015). Anthracite is high quality coal with similar properties to GAC, but formed by larger particles with less efficiency in chemical removals (Jeong, 2013). Porous ceramsite include mineral supports like zeolite, clay, sand, macadam, coke, anthracite coal, and plastic materials. This support is commonly used in biological aerated filters (Bao, 2014). Volcanic rock is natural and abundant material with porous structure and large surface area, which make a good adsorbent reagent in wastewater treatment (Zhang, 2014).

In order to start up and develop this type of biofilter is necessary to run a process called: immobilization of the microorganisms in the carriers (IBAC). The length of this phase can be 3/4 months, and according to Zhang (2015) the removal of pharmaceuticals is only 10% in this period, but after this phase is over, the removal can be 90%.

Remarks to take into account in IBAC: microorganisms should being nonpathogenic (depending on the discharge standards and the final usage of the treated effluent), they should

be species with strong oxidation capacity and adaptation to survive poor nutritional conditions. However, to achieve these conditions, biological engineer technology is necessary to select the best community (Jin, 2013). To evaluate the presence of pathogens at the end of the treatment, molecular analysis (PCR and sequencing) for the most common pathogens can be done, and/or add another ozonation step at the end of the treatment. Immobilization of microorganisms is an important step to start up the system, due to the energy barrier (**Figure** 2-8).

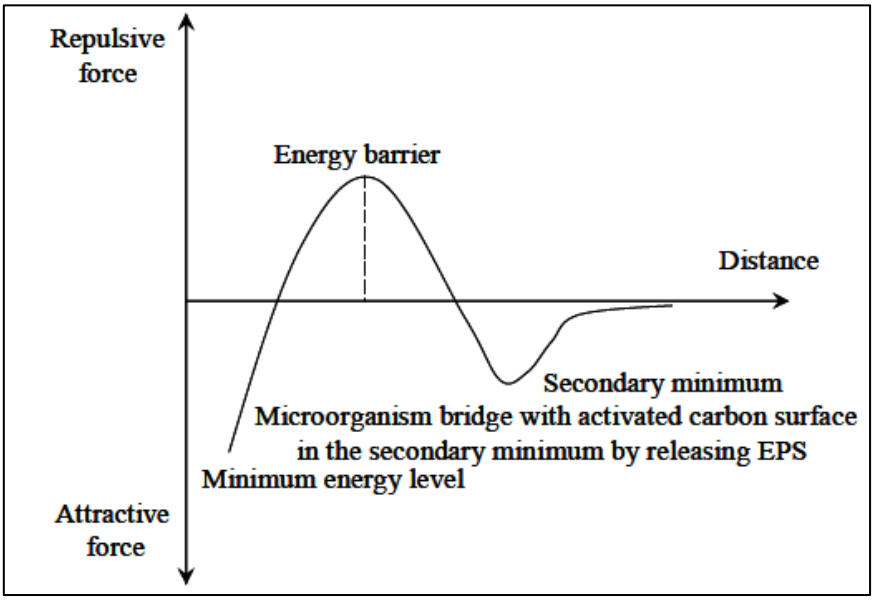


***Figure** 2-8 Total potential energy to express microbial immobilization on activated carbon (Jin, 2013).*

2.6. Wastewater treatment technologies: Ozonation-BAC treatment

In order to improve the removal efficiency and make molecules of interest in treatment more biodegradables, the BAC process in the past years was used with a pre-step of ozonation. These techniques together are commonly applied for potable water as well as domestic and industrial wastewater, perfecting the conventional treatment in order to remove organic components.

Residual ozone in water is decomposed into oxygen (described in section 2.4), allowing the development of aerobic, and anaerobic microbiota (Jin, 2013), so the presence of ozone (now degraded into O2) in the BAC filter helps to develop different types of microorganisms depending on their location in the column.

Other types of oxidation processes are also combined with a biofilter, as is the case, for example, of Jiang (2011) were Fenton oxidation was combined with biofiltration for recycling water. Fenton oxidation is the reaction involving ferrous and hydrogen peroxide for generating hydroxyl radical under acidic pH (Mofrad, 2015).

Typical flow diagrams for ozonation-BAC processes are showed in figure **Figure** 2-9.

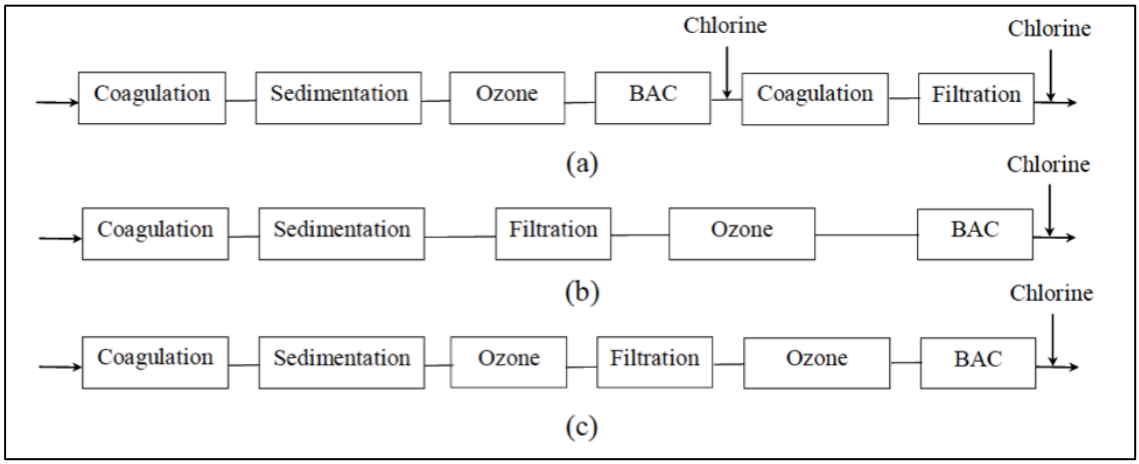


***Figure** 2-9 typical processes applied for advanced drinking water treatment (Jin, 2013).*

By combining these treatment processes, ozonation followed by BAC, some disadvantages of the ozonation are solved. The unidentified by-products produced by the oxidation can be removed with the BAC filter, and as a result, toxicity is also reduced (Reungoat, 2012).
In **Figure** 2-10 a model of action of the ozonation-BAC treatment is shown.

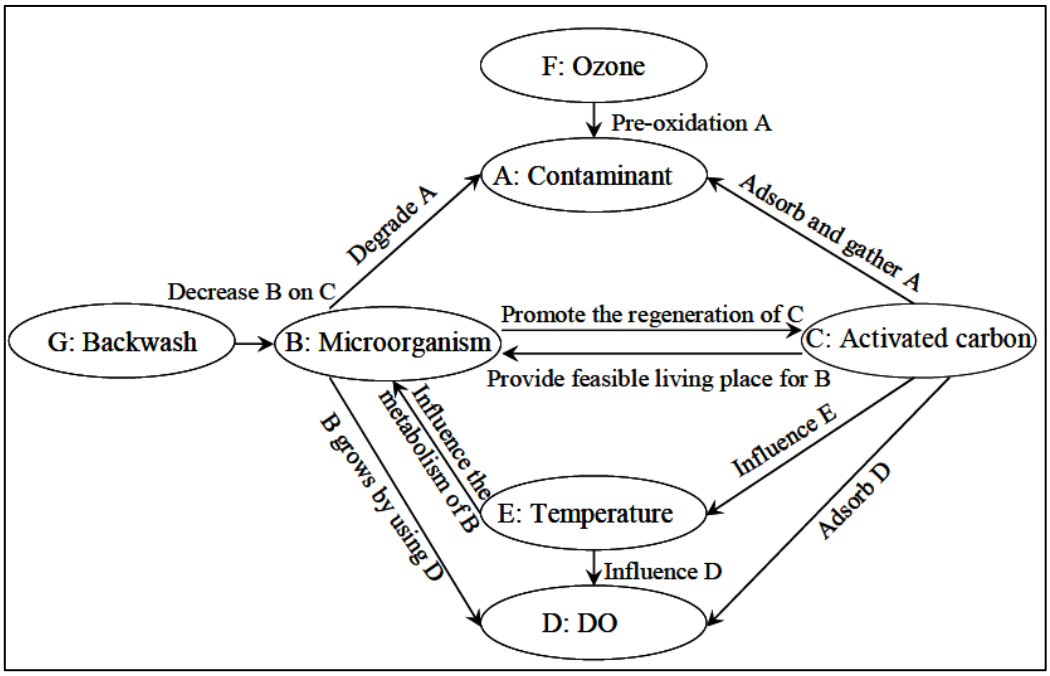


***Figure** 2-10 model of action of the ozonation-BAC treatment (Jin, 2013).*

According to Lee (2012), the removal of pharmaceuticals and diverse micropollutants, from treated wastewater, with this technique can be an environmentally friendly substitute of

reverse osmosis (RO). The objective of the research (Lee, 2012) was to look for alternatives (cheaper and better treatments) to remove pharmaceuticals and personal care products (PPCs) in order to improve treated water quality for the reuse. To prove this alternative, a pilot-scale membrane bioreactor (MBR), ozone contactor, biofilter, and RO systems were constructed in Alburqueque (New Mexico), according to the flow diagram showed in figure 15. The MBR effluent was treated in parallel by an ozone-biofilter system vs. an RO system to compare the removal efficiency of both treatments in terms of energy consumption and waste production. The ozone biofilter system consisted of three PVC columns in series for the ozonation with a downflow-upflow-downflow pattern in constant flowrate; and a 1 m length PVC column filled with anthracite as biofilter. For a successful treatment the author recommends taking special care on using sufficient ozone doses.

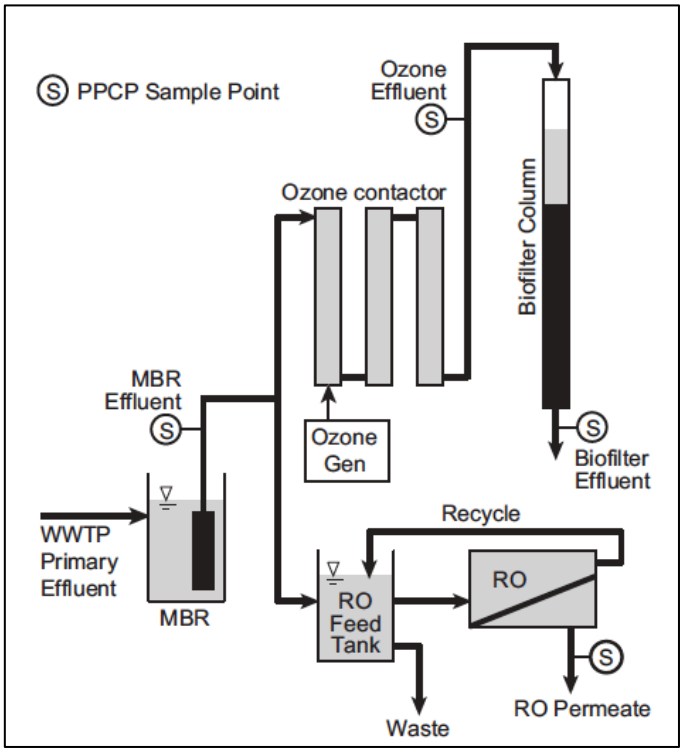


***Figure** 2-11 flow diagram of the pilot treatment system installed in Alburqueque, New Mexico (Lee, 2012).*

According to Zhu (2015), efficiency of this type of treatments depends on ozone dosage and biofilter media. In **Table** 2-1 comparison of these characteristics for different publications taking into account water reuse from secondary effluent treatment, can be appreciated. Some considerations about this table are: in general DOC removal with GAC is better than with sand removal of DOC is higher with O3+BAC than each treatment alone DOC removal will be around 10% to 30% with this techniques empty bed contact time, for the filter, is always around 15 minutes (with exceptions) different ozone dosages (in the same case study) generates different results of DOC removal (higher ozone dosage, higher removals).
Some of these characteristics are helpful for the design of the system, but the lack of sufficient design information results in the use of pilot systems these investigations.

***Table** 2-1 Summary of literature reports for secondary effluent treatment using ozone-enhanced biofiltration (Zhu, 2015).*

References	Efficiencies	EBCT *min*	Ozone Dose *mg/L*
Reaume et al. 2012	DOC removals were 43% with GAC (0.8–1.0 mm) and 17% with sand.	40	3.5
Kalkan et al. 2011	DOC removals of 45.9% and 37.8% were achieved with the steam-activated carbon (ES = 1.2 mm) and the chemically activated carbon, respectively, on day 170 of operation.	18	NA
	Similar performance was achieved at the 8.3-min EBCT ports, indicating the bioactivity was in the upper layer.	8.3	NA
Ho et al. 2011	DOC removals were <10% with biologically active sand and <20% after 200 days of operation with GAC. Biologically active sand removed little atrazine, E1, EE2, NDEA, NDMA, and NMOR.	15	NA
Hallé et al. 2015	DOC removal was <15% with the biologically active dual media (anthracite and sand).	5 and 14	NA
Levine et al. 2000	Preozonation favored the breakdown of high-molecular-weight organic matter (>1,000 D); DOC removal was 20–30% for ozonation and GAC.	15	15
Wang et al. 2008	COD, NH_3-N, and TOC were reduced from 40–52, 10–19, and 9–13 mg/L to 18–23, 0.5–1.5, and 7–8.5 mg/L, respectively (equating to removal efficiencies of 58, 89, and 25%, respectively). Media was clay-based, approximately 2–4 mm in diameter.	55-223	10
Li et al. 2006	Without ozone, BAC removed on average 14% DOC. Ozone followed by BAC removed DOC by 34% (3 mg/L O_3), 40% (6 mg/L O_3), 45% (9 mg/L O_3), and 48% (12 mg/L O_3).	15	3, 6, 9, 12
Reungoat et al. 2010	DOC removals of 10% by ozonation and 20–30% removal by GAC	18	5
Li et al. 2005	Without ozone, BAC removed on average 14% DOC after maturation. DOC removals were 34% (3 mg/L O_3) for the system (O_3 + BAC) and 12% for oxidation alone.	15	3, 6, and 9

BAC—biologically active carbon, COD—chemical oxygen demand, DOC—dissolved organic carbon, E1—estrone, EE2—17α-ethynylestradiol, EBCT—empty bed contact time, GAC—granular activated carbon, NA—not applicable, NDEA—*N*-nitrosodiethylamine, NDMA—*N*-nitrosodimethylamine, NH_3-N—ammonia nitrogen, NMOR—*N*-nitrosomorpholine, O_3—ozone, TOC—total organic carbon

To conclude, to develop this type of treatment, the following parameters must to be taken into account in order to have good results:

- It is important to give enough time to develop the biofilm
- Adsorption and biodegradation can be affected changing the filtering media used for the biofilter
- Take special care in giving the best ozone dosage (related with time, cost and type of wastewater)
- Variations in the treatment results depend on the type of wastewater to treat, the components to remove, and how many treatment steps are involved (1 reactor versus many reactors in serie, etc.)
- Depending on the objectives of the treatment, the order of the treatment train (ozonation followed by BAC versus BAC followed by ozonation)

Finally, the relatively easy way to operate the equipment and the low costs of the treatment itself (comparing with Reverse Osmosis technology), make this treatment an excellent one for water reuse and or wastewater treatment, specially for secondary effluent treatment.

The relatively easy way to operate the equipment and the low costs of the treatment itself, make this treatment an excellent one for water reuse and or wastewater treatment, especially for secondary effluent treatment.

2.7. β-lactam antibiotics

Between Libra laboratory pharmaceuticals are β-lactam antibiotics, except for the Sulbactam (β-lactamase inhibitor) (Drugs.com, 2015). These types of antibiotics were first isolated from the mold: *Penicillium chrysogenum* (Page, 2012*)*. Their action produces inhibition of growth and cell death followed by lysis trough the inhibition of bacterial cell wall biosynthesis (Tipper, 1979).
Different classes of β-lactam antibiotic correspond to different types of β-lactams (different structure).
The combination of the inhibitor Sulbactam with Ampicillin has improved their activity against penicillinase-producing staphylococci and Gram-negative bacteria (Page, 2012).

Structures of Libra antibiotics are shown in **Figure** 2-12, **Figure** 2-13, **Figure** 2-15, **Figure** 2-15.

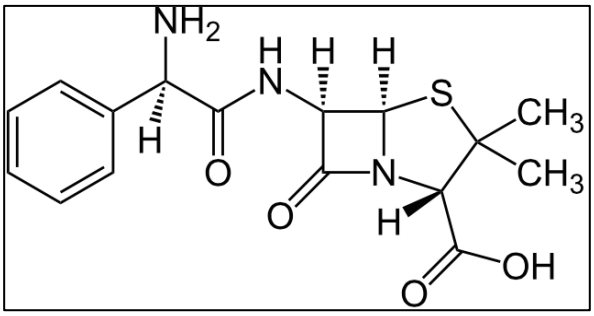


***Figure** 2-12 structure of Ampicillin (Page, 2012).*

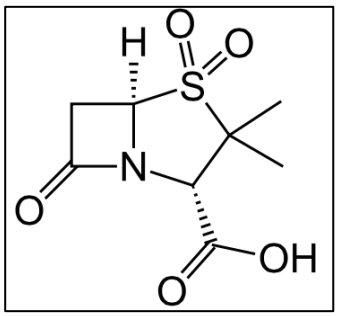


***Figure** 2-13 structure of Sulbactam (Page, 2012).*

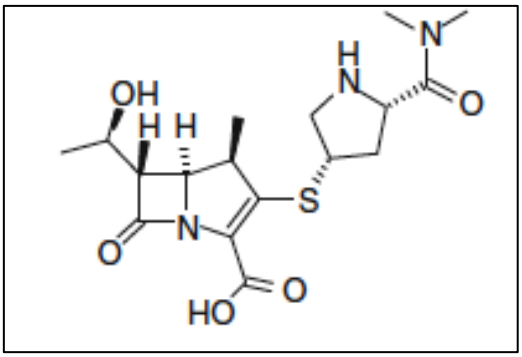


***Figure** 2-14 structure of Meropenem (Page, 2012).*

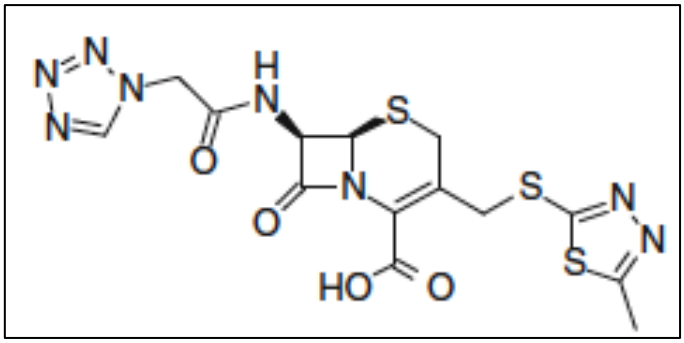


***Figure** 2-15 structure of Cefazolin (Page, 2012).*

Not much has been reported in reference to β-lactam antibiotics toxicity. Park, (2008) published toxicity values for Ampicillin and Amoxicillin in *V. fischieri* toxicity test after 15 min: IC50= 2627mg/l and IC50= 3527mg/l respectively. For 5 min of exposure these values were: IC50 Ampicillin= 1056 mg/l and IC50 Amoxicillin= 1320mg/l. Results for D. magna (EC50, 24h and 48h), M. macrocopa (EC50, 24h and 48h) and O. latipes (LC50, 48h and 96h) toxicity test were undetectable values, all reported as >1000mg/L.

2.8. Quaternary ammoniums

Actually 4 different types of surfactants can be found: anionic, non-ionic, zwitterionic and cationic surfactants (Jardak, 2016). Quaternary ammoniums are cationic surfactants molecules with either linear or branched alkyl chain with 10 to 20 carbon atoms, methyl or benzyl groups as the hydrophobic part of the molecule. The hydrophilic element of cationic surfactants is a central positive charged nitrogen atom (**Figure** 2-16) (Ikehata, 2004) (Tezel, 2009).

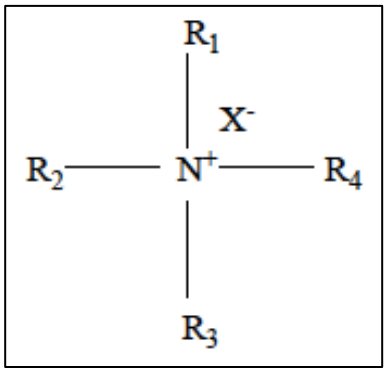


***Figure** 2-16 quaternary ammoniums general structure. R1, R2, R3 and R4 represent functional groups (alkyl chain, methyl or benzyl groups). X- represents a counter ion such as Cl-, Br-, or NO3- (Tezel, 2009).*

The aqueous solubility of QACs and their sorption to organic surfaces (biomass, sediments) depends on the length of the alkyl chain: more length, less solubility; more length, more sorption. These physical and chemical characteristics are responsible of QACs toxicity and biodegradability in the environment (Tezel, 2009).

Lavorgna, (2015) studied acute and chronic toxicity of the benzalkonium chloride. Their results showed strong toxic (EC50= 1.29 µg/l) and genotoxic potential in *Daphnia Magna* (crustacean), and they suggest further studies to evaluate the molecules as hazardous and their potential risk on human health. Furthermore, in other investigations, toxicity of 15 QACs was assess for: *Microtox* (bacterium *Vibrio fischeri*), *Spirotox*, *Protoxkit F* (two ciliated protozoa: *Spirostomum ambiguum* and *Tetrahymena thermophila*) and *Artotoxkit M* (an anostracean crustacean Artemia franciscana). Results showed toxicity for every organisms, EC50 for Microtox= 0.6 to 50µM. It was also stated that toxicity decreases with the length of the alkyl chain of QACs (Tezel, 2009).

As it was mentioned before, bacteria resistance is one of the major complains and negative effects of QACs. Bacteria have acquired resistance because of their action and usage as antimicrobial agents: sub-inhibitory concentrations coupled with microbial diversity ended with the emergence of resistant DNA mutations (Tezel, 2015). QACs in high concentration break the lipid bilayer membrane of the Gramm-negative cells, producing the release of cytoplasmatic organelles ended in the death of the microorganism (Oh, 2013) (Tezel, 2009). Their resistance, then involved, the change in their membrane molecules (Tezel, 2009).
There is evidence that suggests a link between antibiotic resistance favoured by exposure to QACs, which makes QACs research even more important (Tezel, 2015).

QACs biodegradation is produced majority under aerobic conditions (90%-99%) and half-lifes for ultimate degradation are between hours and days depending on QACs concentration, adaptation of microorganisms and presence of resistant/degrading bacteria (Tezel, 2009) (Zhang, 2011). These degrading microorganisms that utilize QACs as carbon and energy source have been identified (Zhang, 2011). They are predominantly related to bacterial community of *Pseudomonas spp.*, but some studies also found out that property on

Xanthomonas sp. and *Aeromonas hydrophila sp. K* (Tezel, 2009) (Zhang, 2011) (Oh, 2013) (Brycki, 2014) (Tezel, 2015) (Khan, 2015).
3 Biodegradation pathways were identified (**Figure** 2-17): a. hydroxylation of the alkyl chain at the C-terminal (with multiple β-oxidations resulting in 2 C liberation in each one), b. hydroxylation of C that is adjacent to central N, resulting in separation of the hydrophobic from the hydrophilic part of the molecule, **c.** hydroxylation of the methyl-C attached to the central N, followed by fission of the methyl group (Tezel, 2009) (Brycki, 2014) (Tezel, 2015).

In the case of Benzalkonium chloride molecule (one of the QACs of interest in this research), the degradation occurs through pathway b (described above), where the alkyl group is separated from the nitrogen resulting in the formation of benzyl dimethyl amine, which is then converted into ammonia (Zhang, 2011).

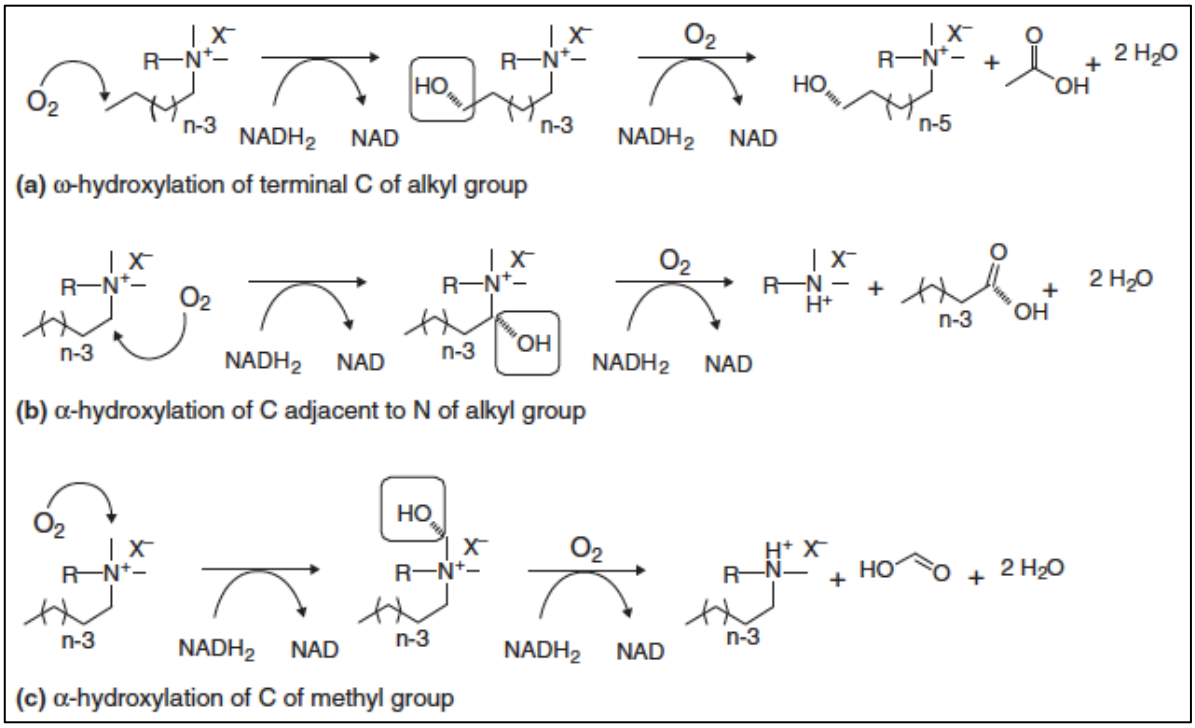


***Figure** 2-17 QACs biodegradation pathways (Tezel, 2015).*

It is important to remark that generally more than one bacterium run this biodegradation process, in fact is the adapted microorganisms community together (Tezel, 2009).
Khan, (2015) reported biodegradation of Benzalkonium chloride (laboratory batch experiment) in 100 hours at 100mg/l concentration of a single Benzalkonium chloride, while it took 150 hours at 100mg/l concentration of a mixture of Benzalkonium chlorides. The chosen bacterium for this degradation was *Pseudomonas sp.* Strain 505.

Anaerobic degradation of QACs is not as well as described for aerobic degradation, but it was demonstrated that occurs on anaerobic reactors. However, there is no evidence of mineralization of QACs containing alkyl or benzyl groups (Tezel, 2009).

Metagenomic analysis of a long-term adapted community to quaternary ammonium biocides was published by Oh, (2013). Despite the community study in this research have been adapted for 2 months, similar phyla and generous are expected, and it is a great source of comparison (**Figure** 2-18).

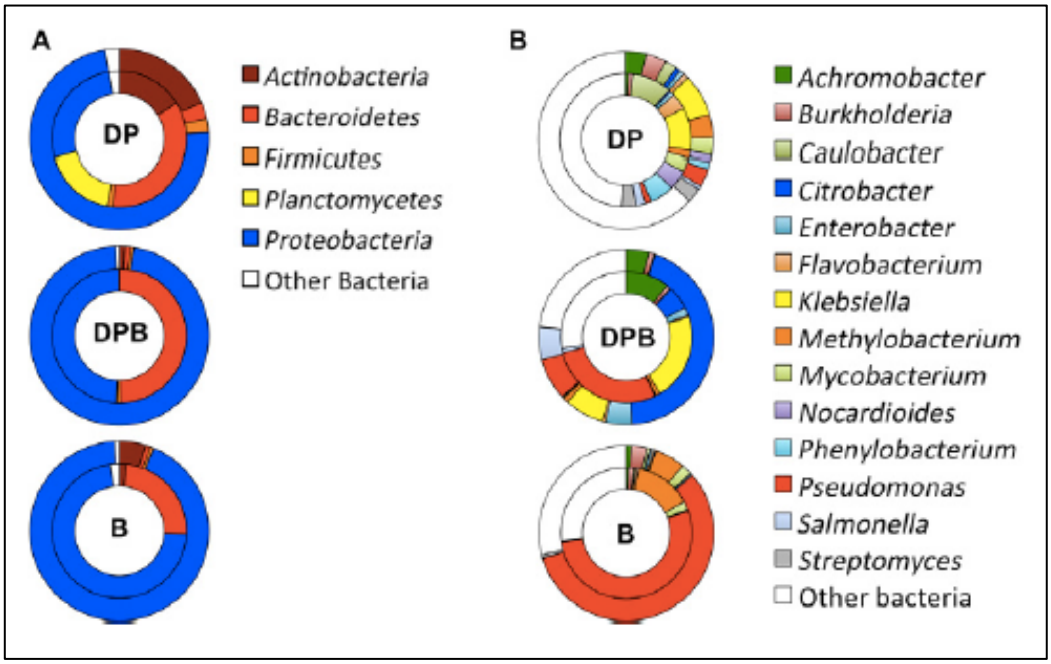


***Figure** 2-18 Phylogenetic composition of microbial communities. A: Colours represent the major phyla. B: Colours represent the major genera. DP represents the control community, B is the community with only QACs as energy and carbon source, DPB B is the community with QACs, dextrin/peptone as energy and carbon source, DP B is the community with QACs and a dextrin/peptone-fed one as energy and carbon source (Oh, 2013).*

CHAPTER 3

Research approach

3.1. Companies and agencies involved

3.1.1. Libra pharmaceutical

The pharmacist Carlos Scherschener Kohn created the Uruguayan pharmaceutical company called Libra in 1961. In 1991 was the first exportation to Paraguay, and that date was followed by an exponential growth of the market and its subsidiaries in the region (Chile and Brazil). In the past 2 years the company is making and expansion buying and rebuilding new places (LIBRA, 2015).

In the local market Libra represented 3.35% in 2010 (LATINPHARMA, 2011). In **Table** 3-1 the main laboratories and their representation in the local market between 2004 and 2010 can be appreciated.

***Table** 3-1 representation in percentage of the main pharmaceuticals Uruguayan industries in the local market in the period 2004-2010 (LATINPHARMA, 2011).*

Company	Business Chamber	2004	2005	2006	2007	2008	2009	2010
Roemmers	A.L.N.	8.14	7.87	807	8.70	8.39	8.94	10.37
Roche	C.E.F.A.	7.40	6.99	7.11	6.91	6.83	6.82	6.98
Bayer	C.E.F.A.	5.87	5.20	6.20	5.46	5.55	6.35	6.08
Celsius	A.L.N.	5.80	6.21	5.11	6.15	5.91	5.60	5.30
Urufarma	A.L.N.	3.27	3.53	3.47	3.64	4.31	4.65	4.99
Spefar	A.L.N.	3.97	4.00	4.17	3.79	3.67	3.82	4.35
Fármaco uruguayo	A.L.N.	3.06	3.58	3.83	4.10	4.06	4.12	4.29
Abbot	C.E.F.A.	3.03	3.15	2.96	2.93	3.07	3.56	3.50
Libra	A.L.N.	3.61	3.44	3.45	3.21	3.13	3.39	3.35
Lazar	A.L.N.	3.46	3.28	3.45	3.34	3.45	3.38	3.03

Libra has created its own drugs, but also they market foreign drugs (invented by other pharmaceuticals). In 2012 Libra spent 1,9 million dollars in importation, and the exportations represented 10% of the total pharmaceuticals (8,555 thousand dollars) (Miranda, 2013).

In total, Libra produces more than 150 products and 4 different plants are located in Uruguay. The β-lactam antibiotics establishment (interest for this research) only works as a packing laboratory, they do not produce the antibiotics there (Silvarrey, 2015).
With regards to the environment, their solid waste is destroyed by the Faculty of Chemistry of the public university (LIBRA, 2015).

3.1.2. DINAMA

DINAMA (*Direccion Nacional de Medio Ambiente*) is the national environmental agency and is a governmental dependency of the Minister of land use and environment (*Ministerio de Vivienda, Ordenamiento Territorial y Medio Ambiente*).

DINAMA and the municipalities are responsible of controlling wastewater discharge characteristics (domestic and industrial), according the decree 253/79. In this decree is also included a section (Article 14) that allows DINAMA to increase the demands for any discharge. This article was applied in one of the regular inspections to Libra, mentioned above.

3.1.3. LATU

LATU is the Technological laboratory of Uruguay and is a mixed public-private organization. It was created in 1965, and their mission is to "promote sustainable development of the country and its international insertion through innovation and the transfer of solutions on value on: analytical services, metrological, technological, management and conformity assessment in accordance with applicable regulations" (LATU, 2014).
Every assay for this research will be using the equipment, resources and installations of LATU, under the direction of Dr. Diana Míguez (Project Manager of LATU).

3.2. Description of the system and research phases

The system was located in LATU, inside of Biotechnology department in Montevideo, Uruguay in a 3m x 6m laboratory room with 2 exit doors.
Between the system components are 2 activated carbon filters, 3 pumps: 2 for the filters and 1 for the ozonation tank, 3 reservoir tanks: 2 made of plastic and 1 made of metal, 1 ozonation tank, 1 oxygen tank, several measurement devices, 1 ozone generator, 1 ozone trap, 1 extraction hood and several connection hoses (details and figures described and showed in chapter 4).

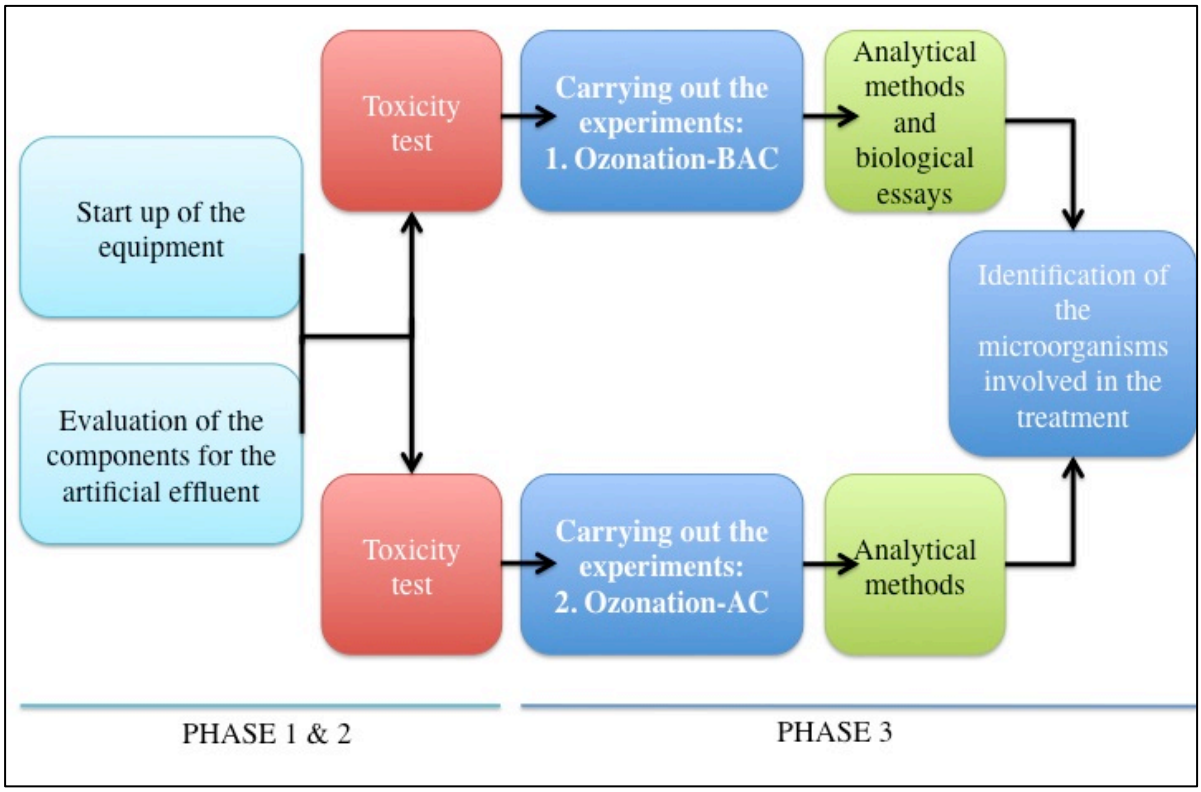


***Figure** 3-1 summary of the activities of phase 1 and 2 for this research.*

This research was divided in 3 phases: toxicity evaluation and decision of the components of the artificial effluent; ozonation and start up (IBAC) of the filters, running and evaluation of the system.

For the start up phase, the equipment was connected as is indicated in section 4.1.1. The ozonation equipment was already used for a similar purpose (Silvarrey, 2015) and the filters system were installed from the beginning. Specific artificial effluent was created containing increasing concentrations of the chosen components, to ensure the right development of the BAC filter.

With regards to the running phase, the performance and results of the system will be evaluated by toxicity tests, dissolved organic carbon (DOC) measurements, pH, temperature, presence of pathogens. The activated carbon filter (AC), without inoculum, was used as a control to compare the degradation between AC and BAC to determine the effect of the biology added to the filter.

CHAPTER 4

Materials and methodology

4.1. Equipment and experiments

4.1.1. Equipment

For this research ozonation equipment, Biological Activated Carbon (BAC) filter and Activated Carbon (AC) filter was installed for the synthetic water treatment.
The following components were used to mount it (Silvarrey, 2015):

- O_2 Tank - Pure Oxygen by Linde group
- Pressure regulator
- Ozone Generator
- 2 Gas flow meters (Rotometers) - King in SLPM calibrated with Air
- Ozone concentration meter - Mini Hicon Ozone Analyzer
- Ozone Disruptor - ODS-H series Ozone Destruct Unit
- Ozone dissolve meter - Model Q45H/64 Dissolved Ozone by Analytical Technology, Inc.
- Water pump (A3 Serie peristaltic pump with flow max capacity 120 l/h)
- pH meter
- Ambient ozone monitor - Ozone Tester Series AQ 200
- 2 Manometers

The ozone gas was bubbled from a hose with holes inside the tank, in order to have full contact between the ozone gas and water (**Figure** 4-1, **Figure** 4-2).

The 2 filters (BAC and AC) were operated as a continuous system (**Figure** 4-3). The following materials were necessary:

- Acrylic cylinders filled with granular activated carbon. Diameter: 2.360-0.50 mm (Suggested by Jin, 2013). To achieve these diameters 2 sieves were used (N^0 8=2.360mm and N^0 35=500μ)
- Connection in the bottom to the tap water for backwash (clogging of the activated carbon filters is common, so it is necessary to have a connection for backwash to solve the problem).
- 2 Water tanks as a reservoir for the ozonized synthetic water

The BAC filter sizes used in the research of Jin (2013) were: inner diameter of 70 mm and height of 1650 mm (effective height: 1050 mm). In order to use the same scale of volumes, but with a smaller filter, inner diameter of 38 mm and height of 890 mm (660 mm of effective height) were chosen. Calculations are showed below (Equation 2, Equation 3).

***Equation** 2 Volume of cylinder*

$$V = \pi \times r^2 \times h$$

$$V_1 = \pi \times 35^2 \times 1650 = 6.35l$$

***Equation** 3 relation between 2 volumes in the same scale*

$$E^3 = \frac{V_2}{V_1}$$

1 Liter filter volume is chosen for this research: V_2=1 l, and the relation between them (E):

$$E = 0.54$$

$$E \times h_1 = \boldsymbol{h_2} = 890mm$$

$$E \times r_1 = r_2 = 19mm \qquad r_2 \times 2 = d_2 = 38mm$$

Schematic diagrams of the systems with the connections of the equipment are showed in **Figure** 4-1, **Figure** 4-2. The system was assembled with the support of the LATU Maintenance Department.

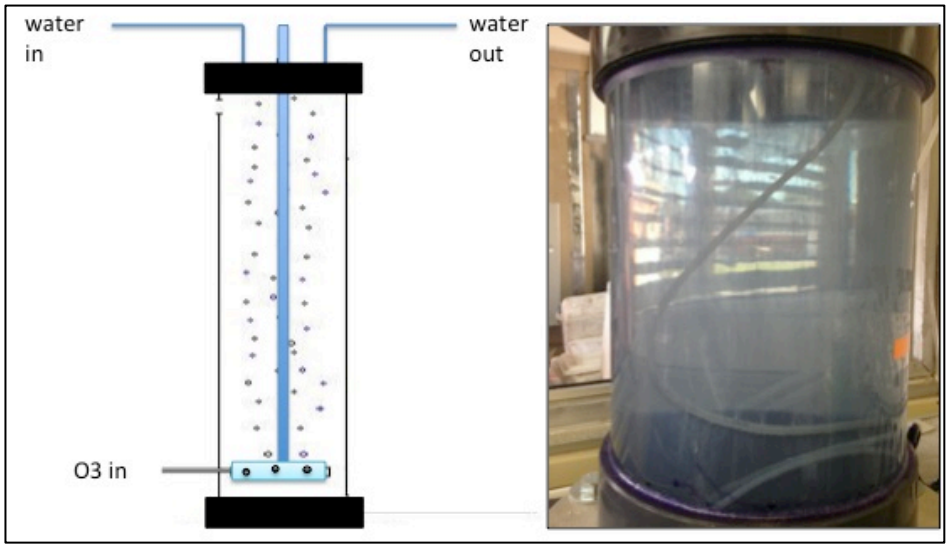


***Figure** 4-1 **left:** ozonation tank running, **right:** configuration of the ozonation tank.*

***Figure** 4-2 ozonation equipment installed at the laboratory.*

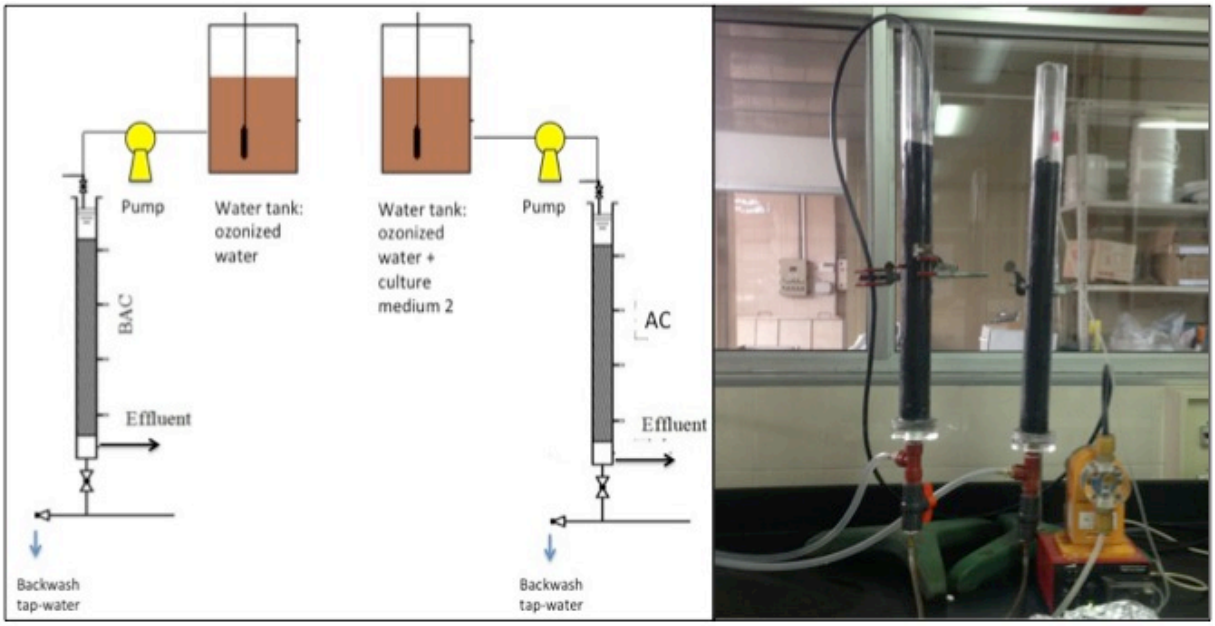


***Figure** 4-3 **left:** BAC and AC filters running in the laboratory, **right:** configuration of the Biological Activated Carbon (BAC) filter and the Activated Carbon (AC) filter equipment.*

4.1.2. Nutritive medium

For the microbiota growth, a nutritive medium (solution 1) with these characteristics was implemented (Wunder, 2010):

For 1 litter of solution 1:

- 43.8 mg/l K_2HPO_4
- 17 mg/l KH_2PO_4
- 62.4 mg/l Na_2HPO_4
- 45.0 mg/l $MgSO_4$
- 0.5 mg/l $FeCl_3$-$6H_2O$
- 5.4 mg/l NH_4Cl
- 55 mg/l $CaCl_2$
- 100 mg/L of acetate (CH_3COOH)

According to this medium, the relation between the addition of carbon and nitrogen is approximately 24C:N. Calculations are shown in Annex 1.

4.1.3. Model water (PHASE 1)

In order to synthetize representative artificial water, the data of the components of the effluent of Libra laboratory was obtained (showed below). The initial step of this research was to choose the most toxic one taking into account toxicity results and frequency of production/usage.

Possible components:

- Cefazolin: cephalosporin class, β-lactam antibiotic
- Ceftriaxona: cephalosporin class, β-lactam antibiotic
- Cefepima: cephalosporin class, β-lactam antibiotic
- Meropenem: carbapenem class, β-lactam antibiotic
- Imipenem: carbapenem class, β-lactam antibiotic
- Ampicillin: aminopenicillin family, β-lactam antibiotic + Sulbactam: β-lactamase inhibitor, usually the drugs contain a mix: Ampicillin/Sulbactam
- Sanitizing agent: Didecyldimethylammonium chloride (quaternary ammonium)
  Alkyldimethylbenzylammonium chloride (quaternary ammonium)

As it is explained and justified in results (section 5.1), all the experiments were realized with synthetic water containing the 2 sanitizing agents.

4.1.4. Start up of the system (PHASE 2)

First, it was necessary to prepare the place looking for the best solution for the aeration, due to the ozone gas toxicity for humans (Menzel, 1984). To start up the development of the microbiota on the BAC filter, it is necessary to first grow the microbiota for 24 hours (dark room at 24°C) in a nutritive medium (Wunder, 2010) and then used as a seed (culture medium 1).

Source of bacteria: the inoculum was a mixture between activated sludge from wastewater treatment plant and water from an aerobic treatment pond.

Culture medium 1*: growth of the microbiota (Wunder, 2010)*

- Inoculum
- 2-liter Erlenmeyer flask
- 1 liter of solution 1

24 hours, dark room at 24°C

This culture medium 1 was added every 2 days: 100 ml of the inoculum were added to one of the AC filters while the system was closed at the bottom (few minutes) to give enough time of attachment and contact between the inoculum and the activated carbon. Acetate was also added to the biofilter reservoir tank in first period (January and 2 weeks of February), in order to give sufficient carbon source for the development and adaptation of the biofilm to the activated carbon.

Ozonation

Ozonation was realized in a batch mode with 1-hour treatment, accordingly to what was described by Dantas (2009) previously, in order to give sufficient time to break antibiotic molecules and make them more accessible for microorganism assimilation. Ozone mass balance to calculate ozone dosage was performed in a serie of experiments: the ozonation tank (15 litters) was filled with distilled water, measuring ozone concentration (gas input, gas output and accumulated in the water) for every 5 minutes. Volumes, flows, pressures and temperatures were taken into account.

The same experiment was performed for the synthetic water (containing the sanitizing agent at 10 mg/l). With both balances (for distilled water and for the synthetic water) losses, ozone dosage and ozone reacting with the different components can be calculated. Theoretical ozone dosage can be calculated by using the following relation: 1.5 mg O_3/mg TOC (Jin, 2013). By calculating theoretical TOC of the two sanitising agents used, it was confirmed that 1 hour ozone treatment is sufficient. Calculations for the components of the sanitizing agents are showed below.

Didecyldimethylammonium chloride (DDAC) - $C_{22}H_{48}ClN$ (**Figure** 4-4)

Molecular Weight (MW)= 361.45 g/mol

$$Cmass = n^0 \times C\ atomic\ weight\ (AW)$$
$$Cmass = 22 \times 12g/mol = 264g/mol$$
$$Cmass\ portion = \frac{264g/mol}{361.45g/mol} = 0.73$$
$$[\text{DDAC}] = 10mg/l$$

$$TOC = 0.73 \times 10mg/l = 7.3mg/l$$

$$[O_3] = 1.5 \times 7.3mg/l = 10.95mg/l$$

Alkyldimethylbenzylammonium chloride (Benzalkonium chloride) (**Figure** 4-4)

n variable (**Figure** 4-4), average: n=13 - $C_{22}H_{40}ClN$

Molecular Weight (MW)= 353.46 g/mol

$$Cmass = n^0 \times C\ atomic\ weight\ (AW)$$
$$Cmass = 22 \times 12g/mol = 264g/mol$$
$$Cmass\ portion = \frac{264g/mol}{353.46g/mol} = 0.74$$
$$[\text{BAC}] = 10mg/l$$

$$TOC = 0.74 \times 10mg/l = 7.4mg/l$$
$$[O_3] = 1.5 \times 7.4mg/l = 11.2mg/l$$

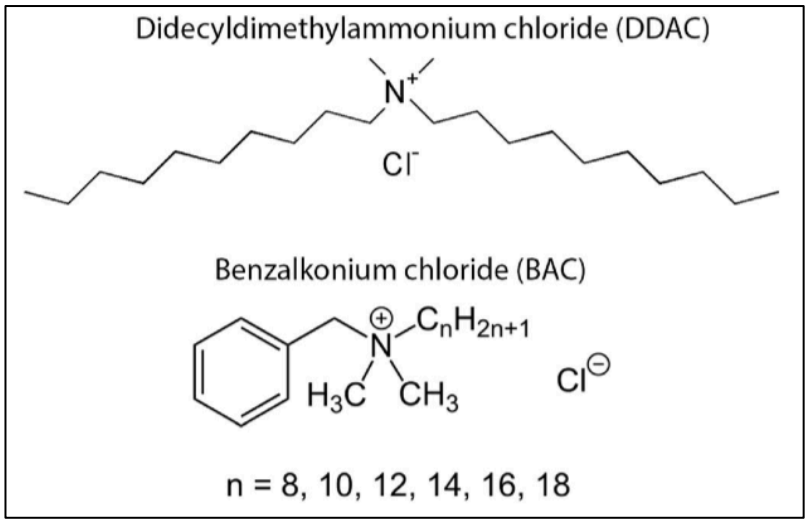


***Figure** 4-4 Didecyldimethylammonium chloride (DDAC) in the top, and Benzalkonium chloride (BAC) in the bottom, chemical structures (Díez, 2016).*

4.1.5. Running the system (PHASE 3)

BAC filter

The system was run for 5 weeks as a continuous system, were ozonized synthetic wastewater was added with increasing concentrations, as it is indicated in **Table** 4-1. Arbitrary concentrations were chosen per week, taking into account 10mg/l as the highest dilution, (explained in results, section 5.1). To get those concentrations, the following dilutions were done: 1st week with 1/8 of the most concentrated, 2nd week ¼ of the most concentrated, 3rd week ½ of the most concentrated, 4th week ¾ of the most concentrated. Hydraulic details of the system are mentioned below.

***Table** 4-1 concentration of the sanitizing agent vs time in weeks for the syntetic water.*

Synthetic water	
concentration of the sanitizing agent (mg/l)	time (weeks)
1,25	1
2,5	2
5,0	3
7,5	4
10,0	5

Activated carbon filter

The activated carbon column serves as a comparison against the results of BAC filtration. If the results are statistically equal, the development of the microbiota in the activated carbon is not sufficient or specific for this type of pharmaceuticals. This second column was operated in equal conditions among the entire period.

Calculations of hydraulic parameters

To define parameters is necessary to take into account that quality of the outflow is influenced by the filtering velocity, height of the carbon layer, the retention period and the gas-water ratio.

According to the calculations showed in section 3.2.1 total volume of the filter will be 1 l, free space volume will be 0.25 l and the carbon layer plus water space will be 0.75 l (**Figure** 4-5). Considering 50% of granular activated carbon, the water space volume will be 0.375 l.
According to the calculations (showed below), if:

$$HRT = \frac{V}{Q}$$

- HRT= 3 hours: Q= 3 l/d 2 columns: 42 l/week

Three (3) hours were chosen in order to give the microorganisms enough time for the degradation of ozonation by products. The flow rate will be 3 l/d and for 2 columns 42 l/week of ozonized wastewater effluent were needed to running the system continuously. This water was stored in specific tanks and prepared per week:

1. 5 l distilled water at 10 mg/l of the chosen component (explained in results) was ozonized
2. ozonized water was diluted (or not, in the case of the 5th week) with distilled water according to the week
3. water (influent for filters) was storage in 20 l plastic tanks, where the pumps take the water for the filters

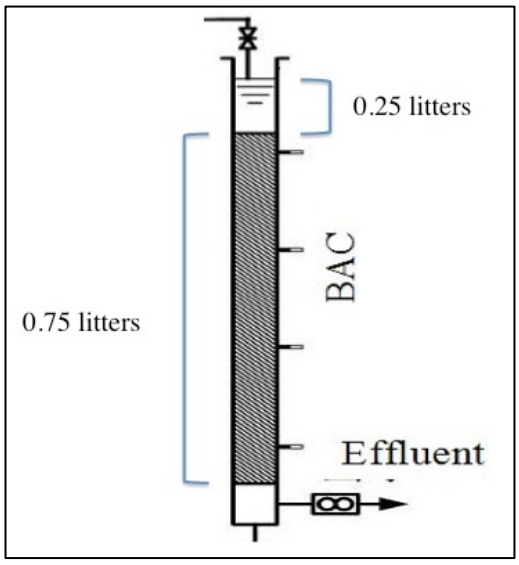


***Figure** 4-5 volumes for the activated carbon filters.*

4.1.6. Analytical methods and biological essays

Dissolved organic carbon

DOC was measured in order to follow organic matter consumption in every step of the treatment (COD measurements have interferences with O3 (Dos Santos Costa, 2006)). DOC measurements were done for samples of the synthetic water before ozonation, after ozonation, effluent from BAC and AC for the 1st and 2nd week of dilutions (section 4.1.5).

An external laboratory called Koro, which is specialized in chemical analysis, did the procedure. They are in agreement with the normative from Pharmacopeia USPC 643 TOTAL ORGANIC CARBON (USPC, 2008). The utilized equipment is called DOHRMANN model PHOENIX 8000, which follow EPA Method 415.1 (NPDES, 1974).
Because the test was too expensive, only the mentioned samples could be done.

Toxicity

The U.S. Environmental Protection Agency refers to the Whole Effluent Toxicity (WET) as the aggregate toxicity in aquatic organisms produced by wastewater pollutants (effluent). WET test measure ability of the selected organisms to survive, grow and reproduce (EPA, 2015).
The following test were selected for this research:

Microtox®: is a standardized toxicity test that employs the bioluminescent marine bacterium (*Vibrio fischeri*) as the test organism. The decreasing of the emitted light is measured at different concentrations of the wastewater. With the plot obtained, the EC50 value can be calculated. This is the value of the concentration producing 50% reduction in light (Leederconsulting, 2015).

Daphnia Magna: this organism is a species of water flea and is favorable to measure toxicity because is highly sensitive to toxic substances, multiplies very rapidly, easily acclimatizes in laboratory condition, has short generation time, cultured in a small space and can be measured in a relatively short period. This test is approved and standardized in several countries (Villegas-Navarro, 1999). In the *Daphnia Magna* reproduction test LC50 is determined by different concentrations of the wastewater effluent, and is the value of the concentration where half of the population of organisms are dead (Silvarrey, 2015).

In order to evaluate the components for the synthetic water, Microtox® and *Daphnia Magna* toxicity test for chemicals was applied (PHASE 1). (Assays based on OECD 202 for the assessment of chemicals toxicity (Chemicals, 2004)).

On the other hand, toxicity was measured every week of PHASE 2 at every concentration of the synthetic water before ozonation. After ozonation and after the filters, applying *Daphnia Magna* toxicity test for effluents (Biological Test Method: Reference Method EPS 1/RM/14 for Determining Acute Lethality of Effluents to *Daphnia magna* (Services, 2000)).

pH and temperature

Temperature and pH of the ozonized synthetic water in the tanks was measured twice in order to control and avoid unnecessary death of microorganisms and to follow the evolution of the treatment and effluent quality.

Ammonium

Ammonium was measured with Spectroquant® test number 114752 (Millipore, 2016), which is a photometric analysis with a detection range between 0.02-1.30 mg-NH_4/l. Procedure described in **Table** 4-2 was followed according to the indications of manufacturer. This method is analogous to EPA (US Environmental Protection Agency) 350.1, APHA (American Public Health Association) 4500-NH3 F, ISO (International Organization for Standardization) 7150-1, and DIN (German Institute for Standardization) 38406-5.

***Table** 4-2 Spectroquant® ammonium 114752 photometric test procedure (Millipore, 2016).*

Pretreated sample **(20 - 30 °C)**	5.0 ml	Pipette into a test tube.
Reagent NH_4-1 **(20 - 30 °C)**	0.60 ml	Add with pipette and mix.
Reagent NH_4-2	1 level blue microspoon (in the cap of the NH_4-2 bottle)	Add and shake **vigorously until the reagent is completely dissolved.**
Leave to stand for 5 min (reaction time A).		
Reagent NH_4-3	4 drops[1)]	Add and mix.
Leave to stand for 5 min (reaction time B), then fill the sample into the cell, and measure in the photometer.		

Volatile suspended solids

VSS measurements were done in order to prove and follow biofilm growth.

* VSS in the effluent, procedure:
Dry a porcelain dish with a filter, cool it down in a desiccator and determine the weight.
Filter 100 ml water sample (effluent of the columns)
Dry over night at 110 ^{0}C
Determine dry weight of filter + sample
Dry for 1 hour at 550 ^{0}C
Determine dry weight of filter + sample

* VSS in the activated carbon in both columns (BAC and AC), procedure:
Dry a porcelain dish with a filter, cool it down in a desiccator and determine the weight.
Add 4g of wet activated carbon to the porcelain.
Dry the porcelain with wet activated carbon over night at 110 ^{0}C.
Determine the weight of porcelain + dry activated carbon.
Put the porcelain and its content in a pre-heated oven (figure 5) at 550 ^{0}C for 1 hour.
Cool it down in a desiccator and determine the weight of the porcelain and its content.

Samples extracted during week 2 (17/2):
-3 Effluent BAC samples
-3 Effluent AC samples
-1 activated carbon BAC sample
-1 activated carbon AC sample
Only one sample each of activated carbon was done in order to keep the system (filter) as equal as possible without any internal intervention.
In the case of the effluent 3 samples for each filter were tested, in order to have repetitions of each sample.

Biofilm visualization

Several agar plates were incubated with the biofilm attached to BAC in order to visualize and developed the appropriate biofilm and the variety between bacterial strains.
To achieve this objective, it is necessary to elute bacteria from the particles of the activated carbon without breaking the membranes. A modification if the procedure and solutions described by (Camper, 1985) was followed:

- Remove and weight 1g of activated carbon from the filter
- Clean by gravity with distilled water (3 times)
- Elution: buffer 1 and 2 without detergent + peptone (0.1%)
- Elution buffer 1 and 2 + detergent + peptone (0.1%)
- Incubation of agar plates with TSA medium at 30^0C for 24 hours

2 dilutions of detergent were applied in order to compare the effect of the detergent. Control of the essay: washing water with 2 dilutions of detergent.

-Buffer 1: EDTA 10-3 M
-Buffer 2: TRIS 0.01 M, pH7.0
-Detergent dilution 1: Tween 20 (125ul + 225ml PBS) + 225ml distilled water
-Detergent dilution 2: Tween 20 (125ul + 225ml PBS)

Components:

- TSA medium: Tryptic Soy Agar (Pancreatic digest of casein 15 g/l, Papaic digest of soya bean 5 g/l, Sodium Chloride 5 g/L, Agar 15 g/l) (Further information in Annex 2).
- Tween 20: complex mixtures of polyoxyethylene with chemical formula: $C_{26}H_{50}O_{10}$ (PubChem, 2016).
PBS: phosphate buffered saline with NaCl, KCl, Na_2HPO_4, KH_2PO_4
- EDTA: Ethylenediaminetetraacetic acid with chemical formula: $C_{10}H_{16}N_2O_8$ (PubChem, 2016)
- TRIS: 2-Amino-2-hydroxymethyl-propane-1,3-diol with chemical formula: $C_4H_{11}NO_3$ (PubChem, 2016)

DNA extraction

Biofilm DNA extraction was performed with Powersoil DNA isolation kit (**Figure** 4-6), a kit exclusively from solid and/or soil samples, recommended by Zheng (2012) in the publication named "Evaluation of DNA extraction methods for the analysis of microbial community in biological activated carbon."

This is a specific kit for environmental, all soil types, fecal, stool and biosolid samples with patented removal technology. The high level of resulting purity of the isolated genomic DNA enables a successful sequencing (MO BIO Laboratories, 2016), as is the objective for this research.

Assessment of the presence of isolated DNA was done by Agarose electrophoresis gel: samples were run by 1 hour at 100V in a 2% Agarose gel stained with GelRed™ (intercalating nucleic acid stain). Electrophoresis is a technique where charged molecules migrate under the influence of an electric field through gel to be separated by size, charge or shape (Adler, 2015). In this case it only serve as DNA visualization and to confirm presence or absence of genome.

Samples extracted:

- Activated sludge from aerobic pond (figure 30)
- Inoculum growth in the nutritive medium incubated for 24 hrs.
- 2 samples of BAC: 1st week
- 2 samples of AC: 1st week
- Negative control only with dry activated carbon

- 2 samples of BAC: 4th week
- 2 samples of AC: 4th week

***Figure** 4-6 aerobic pond activated sludge to use it as inoculum (right) Power soil DNA isolation kit used for this research. Laboratory: MO BIO Laboratories (left)*

Molecular analysis

To identify bacterial strains in the activated carbon, molecular tests were performed. According to (Ferrando, 2015) there are many possibilities of studying a bacterial community, but massive sequencing is the most effective and accurate technique (Annex 3). It can describe precisely every genome of the community. This metagenomic analysis was done with the collaboration of Pasteur Institute of Montevideo in the bioinformatics department.

Pathogen assessment

Effluent from BAC and AC was evaluated for pathogens, especially for the presence of fecal coliforms. According to Gibert (2013) total coliforms, E. Coli, Enterococci and Clostridium perfringes must be analysed to assess quality of drinking water after biofiltration, consequently it is supposed that for wastewater, pathogens will be also present, especially fecal coliforms. According to Jin (2013), IBAC process (section 2.5) should end up with dominant microflora, which does not include pathogens. However, this process is not perfect and screening for fecal coliforms is necessary to assess the effluent quality.

The procedure was evaluated applying Most Probable Number method. According to U.S. Food and Drug Administration (FDA), this is a suitable method for diluted samples, like water, with low concentration of microorganisms (>100/g). It consists on the incubation of the sample in tubes with different dilutions. After bacteria multiply, there is an estimation of the concentration according to the dilution of the tube with growth (Blodgett, 2010).

CHAPTER 5

Results and discussions

5.1. Model water composition

5.1.1. Toxicity evaluation

The toxicity of antibiotics produced and sanitising agent used at Libra was determined.

Antibiotics toxicity test:

Antibiotics toxicity was assessed by Microtox at 10g/l and it was performed by private laboratory (Manuel Baruch). By analysing toxicity results, concentrations for EC50 and EC20 (depending on the sample) can be calculated. Values are showed in table 8. As we can appreciate in the table, solutions 4, 5 and 6 were reported for EC20, due to the high error of the result for EC50. This result means that those samples are less toxic than samples 1, 2 and 3, and EC20 is more accurate for the case. (EC20 represents 20% reduction of the light emitted by *Vibrio fischeri* (Microtox method), while EC50 represents 50% of reduction).

Frequency of production of the antibiotic in Libra was also considerer for the decision of the component for the synthetic water, in order to evaluate which antibiotic affects the effluent, in terms of frequency and toxicity.

***Table** 5-1 toxicity concentrations for EC50 and EC20.*

	EC50 (g/l)	EC20 (g/l)	FREQUENCY OF PRODUCTION	ANTIBIOTIC
SOL 1	2.078	-	frequent	Cefazolin
SOL 2	1.974	-	not frequent	Cefepima
SOL 3	2.905	-	not frequent	Imipen
SOL 4	high error	6.074	frequent	Ceftram
SOL 5	high error	1.611	not frequent	Meropenem
SOL 6	high error	2.912	frequent	Ampicillin + Sulbactam

In the previous research performed by Alejo Silvarrey (2015), toxicity values were reported as EC50. In order to compare and report the same type of values, samples with EC50 with a high error were discarded. From samples 1, 2 and 3 the most frequently used and most toxic is

sample number 1. In conclusion, this was the best antibiotic (Cefazolin) to perform the research, taking into account toxicity and frequency of production in Libra.

Sanitizing agent toxicity test:

The toxicity of the sanitizing agent was assessed by *Daphnia Magna* by laboratory from LATU. The original dilution from the factory was 10%, in Libra the agent is diluted 1:100, resulting in a dilution of 0.1% (see **Figure** 5-1).
-Dilution 1 (in the laboratory who makes the agent to sell it): 10,0000 ppm = 10,0000 mg/l = 100 g/l = 10% (**Figure** 5-1) (Annex 4).
-Dilution 2: in Libra before the agent is used is diluted 1:100. In this point concentration = 100 mg/l = 0.1% (**Figure** 5-2).

In the toxicity test results a concentration <6.25% of the sample was still highly toxic.

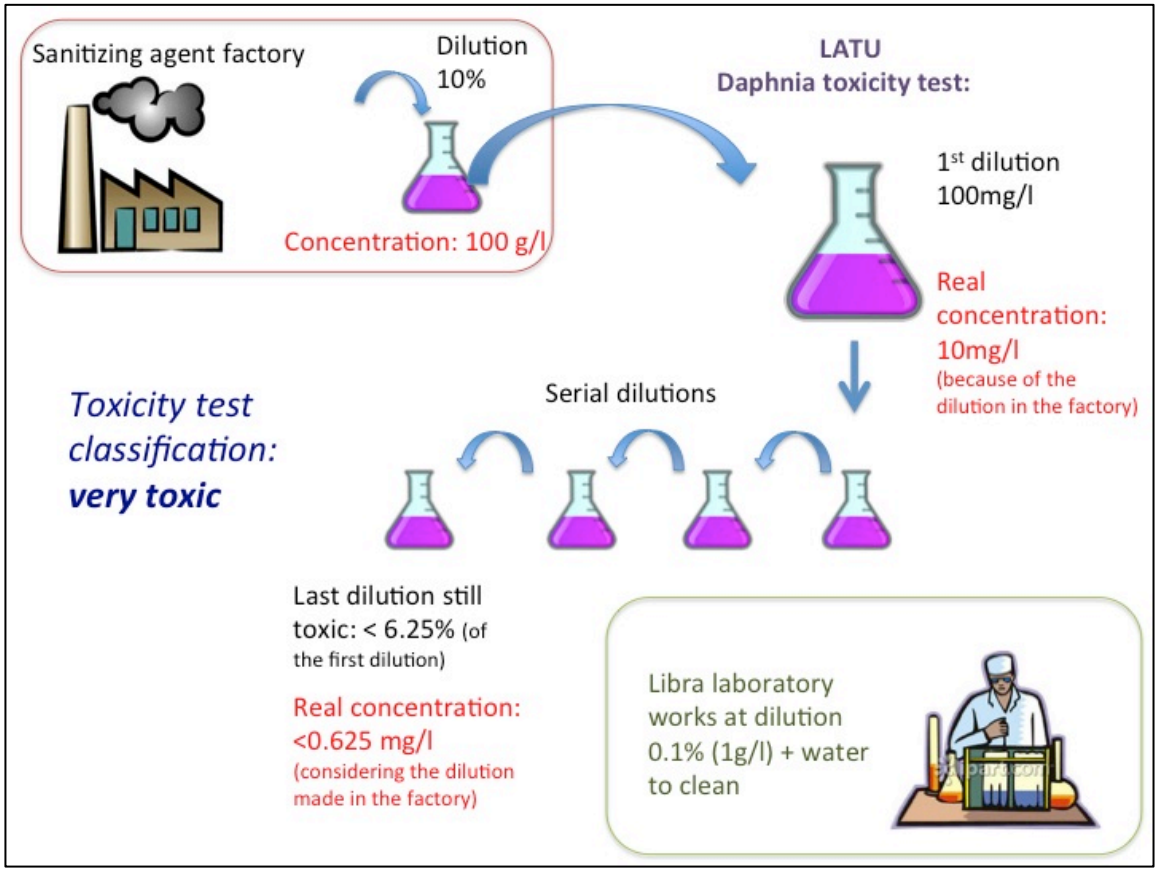


***Figure** 5-1 dilutions made for the sanitizing agent: 1. In sanitizing agent factory, 2. In Libra laboratory, 3. In Daphnia toxicity test.*

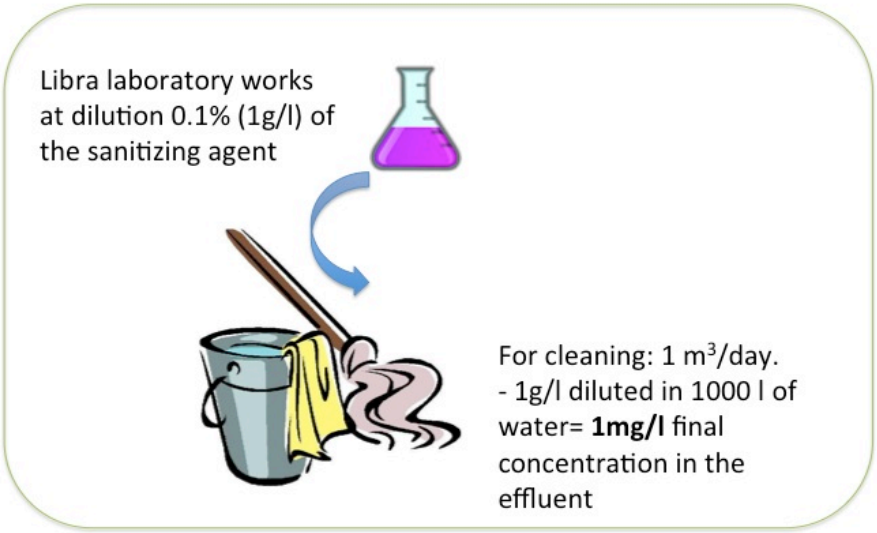


***Figure** 5-2 dilution 2: made by Libra, used for cleaning*

In the case of Microtox test, results were similar; the sample is too toxic to achieve LC50 value. After 9 dilutions in series: 0.32%, 0.64%, 1.28%, 2.56%, 5.12%, 10.24%, 20.48%, 40.95%, 81.90 %, more than 90% of bacteria were still killed (toxic effect of 95.79% in the most diluted sample: 81.90% dilution).

5.1.2. Discussion

Difficulties and disadvantages of using Cefazolin: if Cefazolin EC50 = 2.078 g/l and the ozonation tank has a capacity of 15 l, then 31.17 g of Cefazolin are necessary to ozonize 15 l of water at that concentration. If we need a minimum of 15 l per week during minimum 5 weeks running the experiment, a total of 155.85 g of Cefazolin are required for the investigation. That amount of antibiotics for a research demands a special permission form the Minister of Health of Uruguay, which was impossible to get and Libra did not want to give that amount. For this reason, it was decided to focus the research on the sanitizing agent.

Concerning toxicity results of the sanitizing agent, it was decided to choose 10 mg/l as the concentration for ozonation, taking into account the dilution used in Libra laboratory for cleaning with the sanitizing agent (1g/l) and the value of 1 m3 of water used in Libra for every cleaning day (twice a week) (Silvarrey, 2015). Trying to reproduce dilution of the sanitizing agent in the real effluent: if 1g/l is diluted in 1 m3 (1000 litters), the final concentration is 1 mg/l (**Figure** 5-2). To use the worst-case scenario, to avoid problems testing DOC (limit: 0.02 mg/l), and in order to have enough organic matter for the biofilm, 10 mg/l was chosen as the dilution for ozonation.

5.2. Water mass balance

As it was explained in section 4.1.3, water mass balance was done in order to obtain ozone dosage for comparison with the theoretical one (previously calculated), and with values reported in literature. This is important to evaluate necessary ozone dosage in order to mineralize QACs molecules. Values obtained from the experiment with distilled water are showed in **Table** 5-2 and values obtained from the experiment with distilled water + sanitizing agent 10mg/l are showed in **Table** 5-4.
Calculations taking into account document mailed by Jessica Hart (consultant from HyDOZ equipment, Annex 5) are showed below (**Table** 5-3, **Table** 5-5).

***Table** 5-2 values obtained from the ozonation of distilled water.*

Flow (SLPM)	O3 IN (GNM3)	O3 OUT (GNM3)	O3 water (mg/l)	Time (min)
0.4	14.9	44.8	0.4	5
0.4	14.9	44.8	0.2	10
0.4	14.9	44.9	0.1	15
0.4	14.9	44.8	0.5	20
0.4	14.8	44.8	0.4	25
0.4	14.8	44.9	0.7	30
0.4	14.7	44.7	0.7	35
0.4	14.7	44.8	1.1	40
0.4	14.7	44.8	1.5	45
0.4	14.7	44.9	1.3	50
0.4	14.7	44.7	1.3	55
0.4	14.7	44.7	1	60

Calculations of flow were corrected taking into account pressure, temperature and density; and with these values O_3 IN and O_3 OUT in g/day were calculated (Annex 6).
Values for O_3 in water (expressed in g/day) were calculated taking into account volume of water and time for each sample (Annex 6).

***Table** 5-3 final mass balance of distilled water.*

MASS BALANCE: IN=OUT+WATER+LOSSES			
IN (g/day)	OUT (g/day)	ACCUMULATED WATER (g/day)	LOSSES (g/day)
5758.4	4966.3	1.7	790.4
5758.4	4966.3	0.4	791.7
5758.4	4966.3	0.1	792.0
5758.4	4966,3	0.5	791.6

5758.4	4966.3	0.3	791.8
5758.4	4966.3	0.5	791.7
5758.4	4966.3	0.4	791.7
5758.4	4966.3	0.5	791.6
5758.4	4966.3	0.6	791.5
5758.4	4966.3	0.5	791.6
5758.4	4966.3	0.4	791.7
5758.4	4966.3	0.3	791.8

Distilled water + sanitizing agent mass balance:

***Table** 5-4 Data extracted for the distilled water + sanitizing agent mass balance during 1 hour ozonation.*

Flow (SLPM)	O3 IN (GNM3)	O3 OUT (GNM3)	O3 water (mg/l)	Time (min)
0.4	25.9	46	3	5
0.4	25.5	49	2.8	10
0.4	25.4	50	2.6	15
0.4	25.5	51	3.8	20
0.4	25.5	51.3	6.3	25
0.4	25.5	51.9	5.8	30
0.4	25.6	52.5	7.6	35
0.4	25.5	52.6	7.9	40
0.4	25.6	51.9	10.3	45
0.4	25.6	52.9	8.4	50
0.4	25.7	53.4	9.8	55
0.4	25.7	53.9	11.7	60

Average losses= 791.6 g/day

As in the mass balance above (only distilled water), calculations of flow were corrected taking into account pressure, temperature and density; and with these values O_3 IN and O_3 OUT in g/day were calculated (Annex 7).
Values for O_3 in water (expressed in g/day) were calculated taking into account volume of water and time for each sample (Annex 7).

***Table** 5-5 Final mass balance of distilled water + sanitizing agent 10mg/l.*

IN (g/day)	OUT (g/day)	ACCUMULATED WATER (g/day)	LOSSES + REACTION WITH WATER (g/day)	REACTION WITH WATER (g/day)	Ozone dosage= O3 IN- O3 OUT- LOSSES (g/day)
9951.3	5094.3	13.0	4844.0	4052.5	4065.4
9951.3	5426.6	6.0	4518.8	3727.2	3733.2
9951.3	5537.3	3.6	4410.4	3618.8	3622.4
9951.3	5648.1	3.9	4299.3	3507.7	3511.7
9951.3	5681.3	5.2	4264.9	3473.3	3478.5
9951.3	5747.7	3.9	4199.7	3408.1	3412.0
9951.3	5858.5	4.3	4088.5	3296.9	3301.3
9951.3	5825.3	3.9	4122.2	3330.6	3334.5
9951.3	5747.7	4.4	4199.2	3407.6	3412.0
9951.3	5858.5	3.2	4089.7	3298.1	3301.3
9951.3	5913.9	3.3	4034.2	3242.6	3245.9
9951.3	5969.2	3.6	3978.5	3186.9	3190.5

Ozone dosage average= 3467.4 g/day

5.2.1. Discussion

Some considerations were taking into account for the distilled water mass balance. Firstly, ozone has a normal decomposition in distilled water (section 2.4.), which represents ozone losses. Moreover, losses in the system, errors in measurements, etc. are also reductions in total available ozone for oxidation. All these components together were considered as LOSSES and were calculated as following:

- LOSSES is the difference between O_3 IN and (O_3 OUT+O_3 in water): LOSSES = IN - (OUT + O_3 in water).

Considerations for the distilled water + sanitizing agent mass balance: same losses (obtained for the water mass balance) were considered for the calculation of O3 reacting with the sanitizing agent. The system is supposed to have same losses for every experiment.
The difference between accumulated ozone in water, and water losses (in order to establish a correct mass balance) was considered as the ozone consumed by the substances in water: MASS BALANCE: IN – OUT = LOSSES + OZONE CONSUMED

The calculated value for ozone dosage was= 3467 gO3/day (144 gO3/h), which is an extremely high number. Values reported in literature for bubbled ozone are between 5.44 and 7.57 gO3/h (Dantas, 2009). A possible explanation for this phenomenon is the lack of

appropriate devices to measure ozone concentration devices. Every experiment for water mass balance needs to be done twice, since in the equipment, there is only one ozone concentration meter. The first time of the experiment ozone IN (GNM3) is measured, while in the second time ozone OUT (GNM3) is measured. This repetition may introduce high errors in the balance.

Taking into account dissolved organic carbon values (results in section 5.3.3.); the applied ozone dosage can be estimated by using the relation published by Jin, (2013): 1.5 mgO_3/mg TOC (for this case TOC and DOC are equivalents, there are no suspended solids in the solution). Calculations are showed below:

$$DOC\ bef\ O_3 - DOC\ aft\ O_3 = 16.89mg - C/l - 12.73mg - C/l$$

$$DOC\ reduced\ in = 4.16mg - C/l$$

$$4.16mg - C/l \times 1.5 = 6.24mg - O_3/l$$

If 1.5 mgO_3 is equivalent to 1 mg DOC, then at least 6.24 mg- O_3/l were approximately applied during 1 hour ozonation treatment. As it was calculated previously 11.2 mg- O_3/l were necessary for mineralize both components.

5.3. Performance of the system: Chemical results

5.3.1. pH and temperature

pH measurements were done for ozonation influent, ozonation effluent, BAC filter effluent (1st and 3rd week), AC filter effluent (1st and 3rd week):
Before ozonation, pH=5.91
After ozonation, pH=6.84
After 1 week treatment: pH BAC effluent= 6.73
pH AC effluent= 7.18

After 3 week treatment pH BAC effluent= 6.51
pH AC effluent= 6.55

Discussion

Slightly acidic values in the effluent indicate the presence of some component, which are producing that change. According to literature review, this component could be ammonium.

5.3.2. Ammonium

After pH measurements it was decided to analyse ammonium in order to explain the pH drop in BAC effluent (between week 1 and 3). Furthermore, as it was justified in the literature review, QACs biodegradation release ammonium, therefore it is another approach to verify QACs biodegradation.
Ammonium results for weeks 4/5 are showed below:

- Ozonized water (distilled water + sanitizing agent 10mg/l)= 0.01 mg-NH_4/l
- BAC effluent= 0.14 mg-NH_4/l
- AC= underrange

Underrange result means that the spectrophotometer cannot measure that value due to is higher or lower of the limits of it (0.02-1.30 mg-NH_4/l).

Discussion

Presence of ammonium in BAC effluent can be due to 4 phenomena **Figure** 5-3:

1. It may indicate that biodegradation of QACs is occurring, as is described in section 2.8 (strongest and main hypothesis).
2. Ammonium existence can also be due to ammonification. Ammonification is a step on the N-cycle where NO_2^-/NO_3^- is converted into NH_4 by some denitrifying bacteria, under anaerobic conditions. Nitrifiers convert NH_3/NH_4 into NO_2^-/NO_3^- under aerobic conditions. Depending on the high of the filter the first process or the opposite can occur (Shan, 2014), (Menéndez, 2014), (Markov, 2015), (Madigan, 2015).
3. The nutritive medium where bacteria were growth (solution 1, section 4.1.2) contains NH_4Cl, so it is possible that some ammonia has this origin.
4. Bacteria inoculum from activated sludge may also contain NH_4.

In this case of AC it is supposed that no ammonium is present because there is almost nothing biofilm developed in the control filter (AC), so there is no possibility of biodegradation and ammonium release. In conclusion, underrange results corresponds to lower values: > 0.02 mg-NH_4/l.

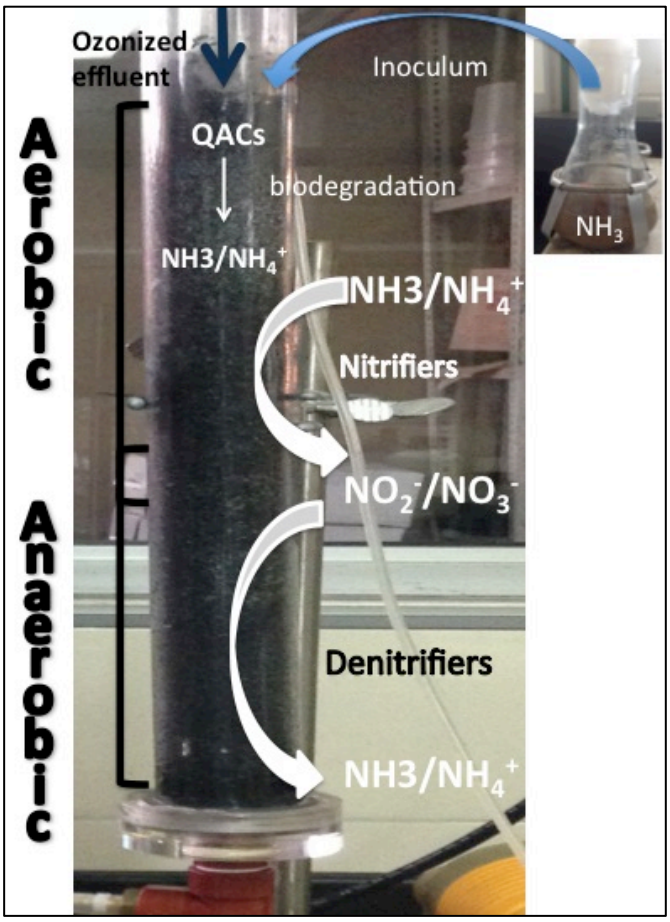


***Figure** 5-3 N possible transformations and pathways in BAC.*

5.3.3. Dissolved organic carbon

DOC was analysed before ozonation, after ozonation, effluent from BAC and AC for weeks 1 and 2:

1. before ozonation= 16.89 mg-C/l
2. after ozonation= 12.73 mg-C/l
3. effluent first week BAC= (10.98 mg-C/l) x 1.25 (dilution of the week)= 13.725 mg-C/l
4. effluent first week AC= (174.80mg-C/l) x 1.25(dilution of the week)= 218.5 mgC/l
5. effluent second week BAC= (510 mg-C/l) x 2.5(dilution of the week)= 1275mgC/l
6. effluent second week AC= 5.94 mg-C/l x 2.5(dilution of the week)= 14.85 mgC/l

Discussion

From the values above it can be conclude:

- Value of sample 1 is in agreement with theoretical value (theoretical TOC= 14.7 mg/l)
- Sample 2 shows a reduction of DOC after ozonation, which mean ozonation is working and it is oxidizing sanitizing agent molecules.

- From the 6 analyzed samples, values of samples 4 and 5 are distant from the rest and they are not from the same carbon filter. These extreme values can be due to biofilm presence in the effluent. Despite the sample is diluted and centrifuged, some parts of bacteria organic matter can be disturbing the measurement.

In **Figure** 5-4, **Figure** 5-5 O3 attack pathway for this research is proposed, based on literature (Bailey, 1982). It is anticipated than only Benzalkonium chloride double bonds are oxidized by O3 under these experimental conditions (section 2.4). Oxidation of this molecule can be done through 2 steps: by basic ozonolysis and/or by phenol formation. In the first case, benzyl group is disrupted and 4 molecules are formed in the end of the reaction. In the second case, benzyl group is oxidized into a phenol. Both reactions can occur at the same time. These reactions, especially the first one, make the molecule more biodegradable, which is the propose before biofiltration.

After phenol formation occurs, further phenol oxidation is possible (**Figure** 5-6), ending in CO_2 releases. These CO2 formations can explain the difference between DOC results of samples before ozonation and after ozonation (before ozonation 16.89 mg-C/l - after ozonation 12.73 mg-C/l = 4.16 mg-C/l). It is suggested that 4.16 mg-C/l corresponds to CO_2 formations.

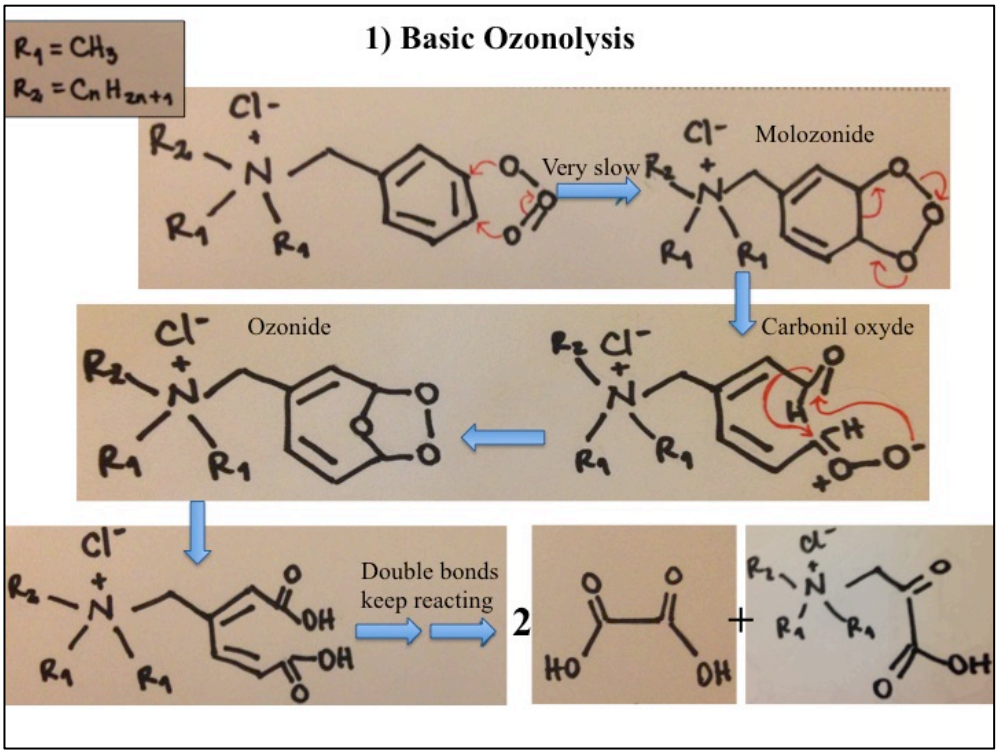


***Figure** 5-4 possible O3 attack pathway to Benzalkonium chloride: basic ozonolysis.*

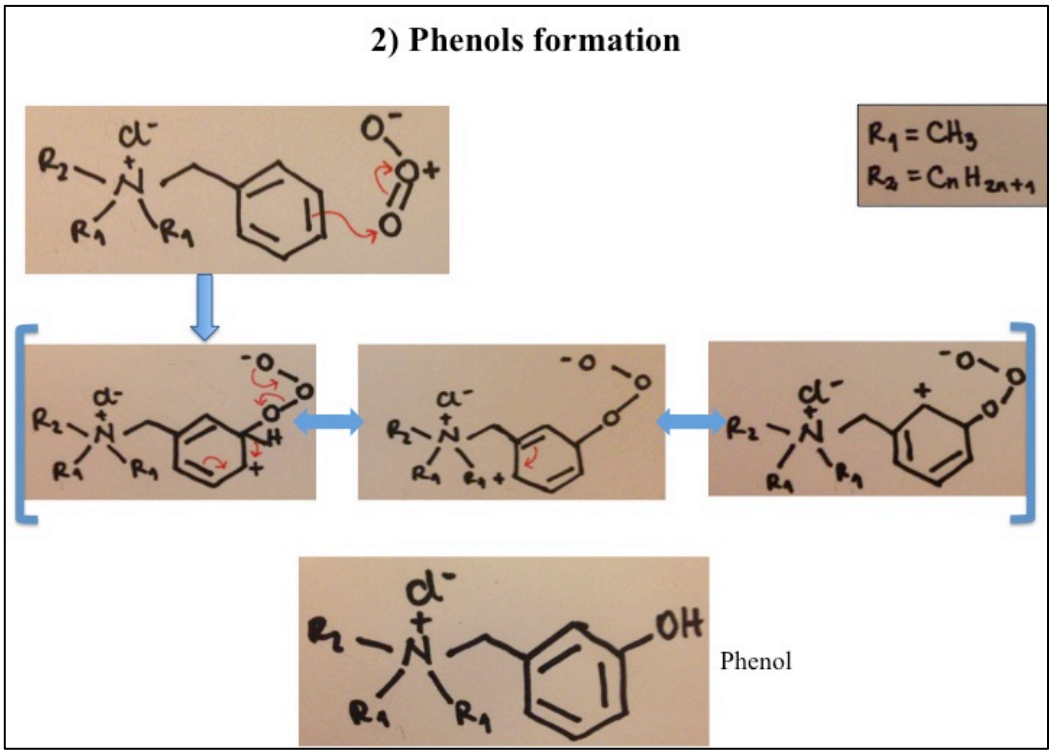


***Figure** 5-5 possible O3 attack pathway to Benzalkonium chloride: phenols formation.*

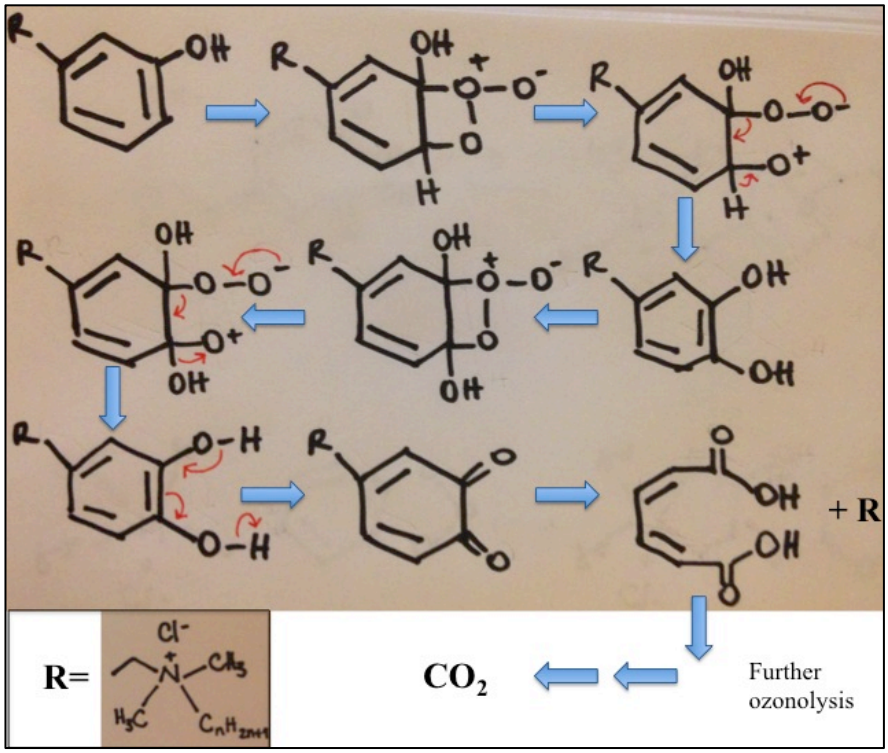


***Figure** 5-6 phenol ozonolysis, final product: CO_2.*

5.3.4. Toxicity

Total Daphnia samples:

1. Synthetic water after ozonation = 56.7% (moderate toxicity)
2. First week:
   effluent from BAC= 50.1% (moderate toxicity)
   effluent from AC = 53.1% (moderate toxicity)
3. Second week:
   effluent from BAC= not toxic
   effluent from AC = 36.9% (toxic)
4. Third week:
   effluent from BAC= not toxic
   effluent from AC = 17.9% (very toxic)
5. Fourth/fifth week:
   effluent from BAC= not toxic
   effluent from AC = 56,6 (moderate toxicity)

6. Fourth/fifth week filtered (0.45 μm filter):
   effluent from BAC= not toxic
   effluent from AC = 56,6 (moderate toxicity)

Discussion

Results of toxicity for the first week showed that the system was still on adaptation (IBAC), but for the 2nd and 3rd week the BAC system was successful removing toxicity. Toxic values from AC are inconsistent, so it was decided to filter all samples from 4th/5th week (0.45 μm filter) in order to evaluate any interference from activated carbon particles. However, results filtered and unfiltered are the same, so it was not the case. Anyhow, BAC effluent toxicity was reduced until “not toxic” classification, which was the one of the objectives. AC effluent achieve moderate toxicity, which demonstrates that biofilm is effectively part of the treatment and only activated carbon particles are not enough.

5.4. Performance of the system: Biofilm development

5.4.1. Volatile suspended solids analysis

***Table** 5-6 data extracted from VSS analysis.*

	porcelain dish+ filter (g)	Volume (ml)	wet activated carbon (g)	Dry porcelain (g)	2nd dry porcelain (g)	TSS (g)	VSS (g)	VSS (mg/l)
AC effluent	61,8221	100		61,8219	61,8193	-0,0002		
AC effluent	63,1208	100		63,1207	63,1189	-0,0001		
AC effluent	61,7846	100		61,7843	61,7831	-0,0003		
BAC effluent	61,5957	100		61,5962	61,5938	0,0005	0,0024	24
BAC effluent	64,2099	100		64,2100	64,2066	1E-04	0,0034	34
BAC effluent	63,0106	100		63,0113	63,0078	0,0007	0,0035	35
BAC carbon	62,6199		4,0200	65,1408	64,9126	-1,4991		
AC carbon	63,2828		4,0125	65,5528	65,2900	-1,7425		

- TSS was calculated as: dry porcelain (over night 110^0C) – initial weight of porcelain dish + filter
- VSS was calculated as: 2^{nd} dry porcelain (550 ^{0}C for 1 hour) – dry porcelain (over night 110^0C)

***Figure** 5-7 pre-heated oven at 550 ^{0}C*

Discussion

Values in red (showed in **Table** 5-6) are incorrect: TSS content cannot be negative and VSS content cannot be higher than the TSS content, as TSS= IS + VSS.
From the obtained values, it can be conclude that the effluent is relatively clean in terms of solids due to the high error of the measures and small differences of weight. Moreover, it appears that is better to find another method for the assessment of attached biofilm in the activated carbon particles.
More sensitive methods are required to visualize and follow the growth of the biofilm in this research.

5.4.2. Agar plate analysis

In **Figure** 5-8 agar plates for Tween dilutions 1 and 2 are showed: 3 activated carbon samples (1g each) were taken from the biofilter in order to have triplicates of the essay. Dilution 2 is 2 times concentrated than dilution 1.
Figure 5-9 represents the control of the essay of **Figure** 5-8, where washing waters of dilutions 1 and 2 are showed.

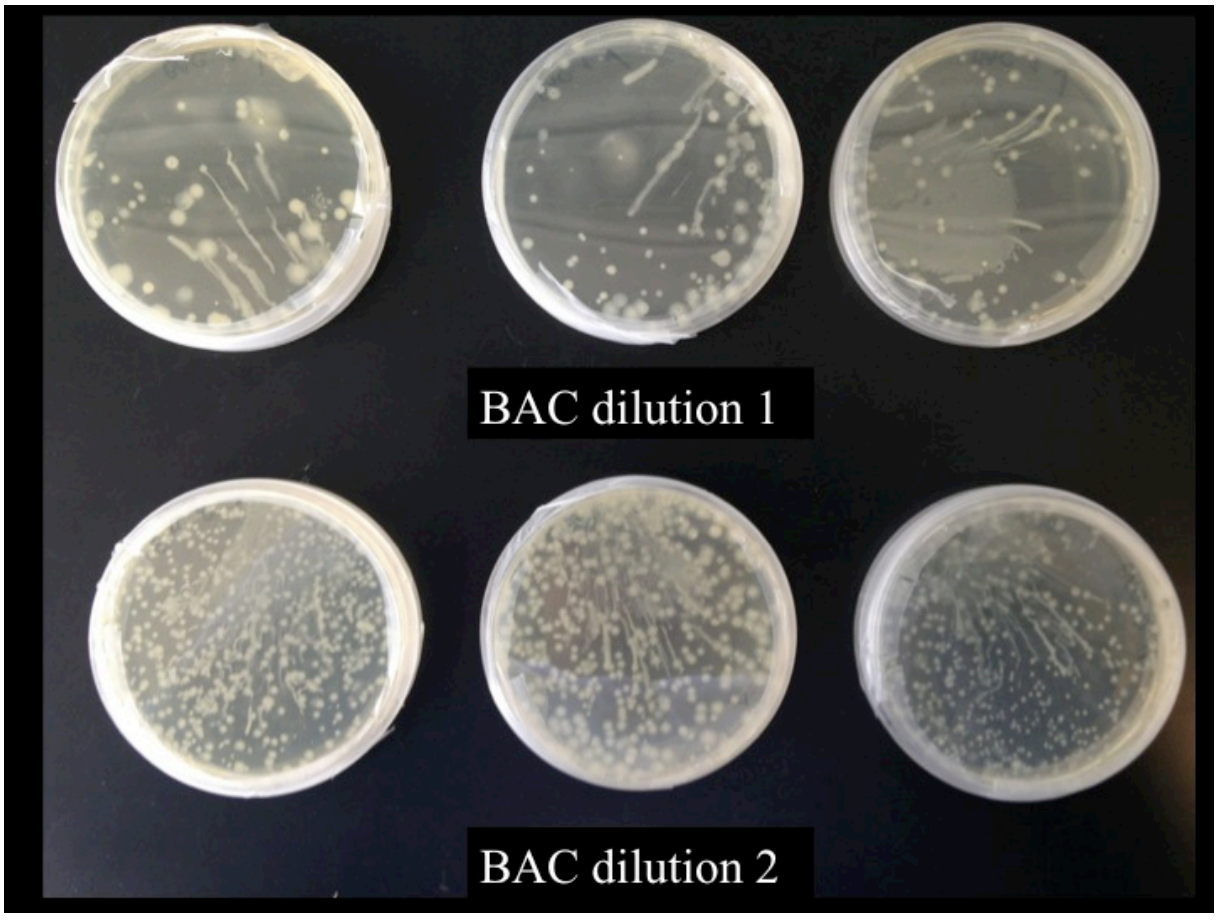


***Figure** 5-8 biofilm elution for BAC with Tween (dilution 1 in the top and dilution 2 in the bottom).*

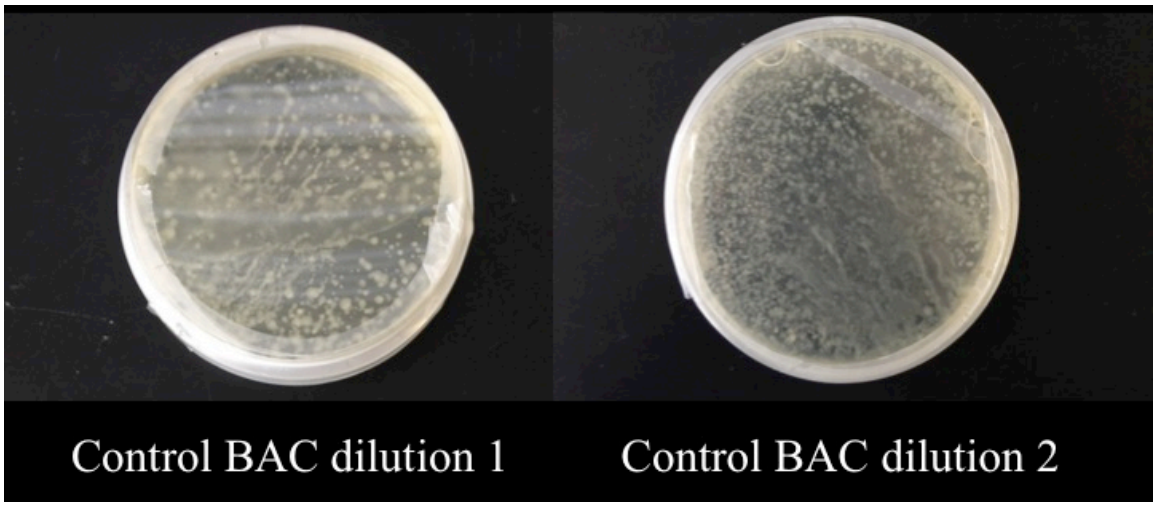


***Figure** 5-9 control of bacteria in solution for Tween with dilutions 1 and 2 (BAC).*

Because activated carbon filter (AC) was installed exposed to open air, it was possible that bacteria from the environment developed some kind of biofilm. In order to evaluate this, the same agar plate assay was done for 2 samples (1g each) of activated carbon from AC. Results are showed in **Figure** 5-10.
The same control with washing solution was done for these samples and is showed in **Figure** 5-11.

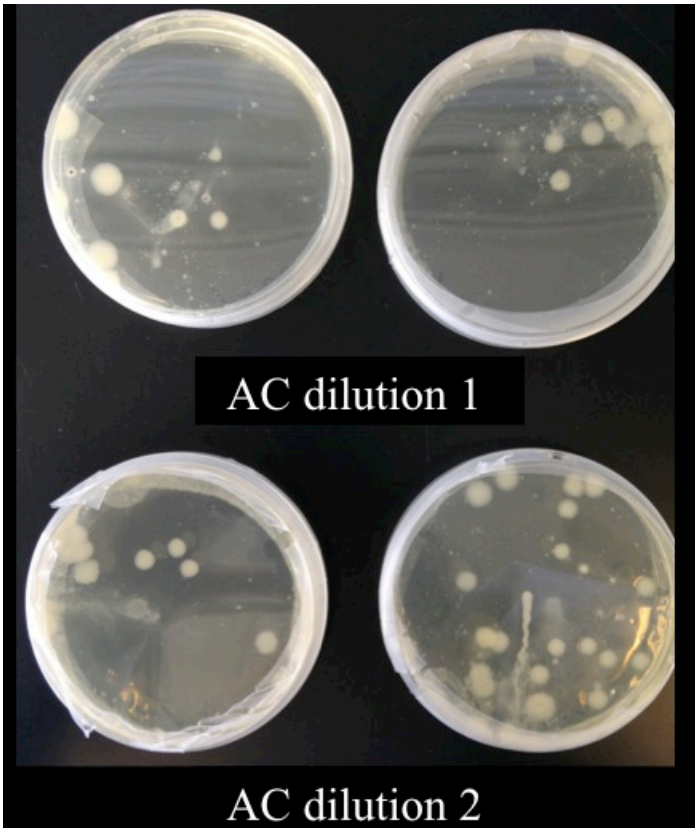


***Figure** 5-10 biofilm elution for AC with Tween (dilution 1 in the top and dilution 2 in the bottom).*

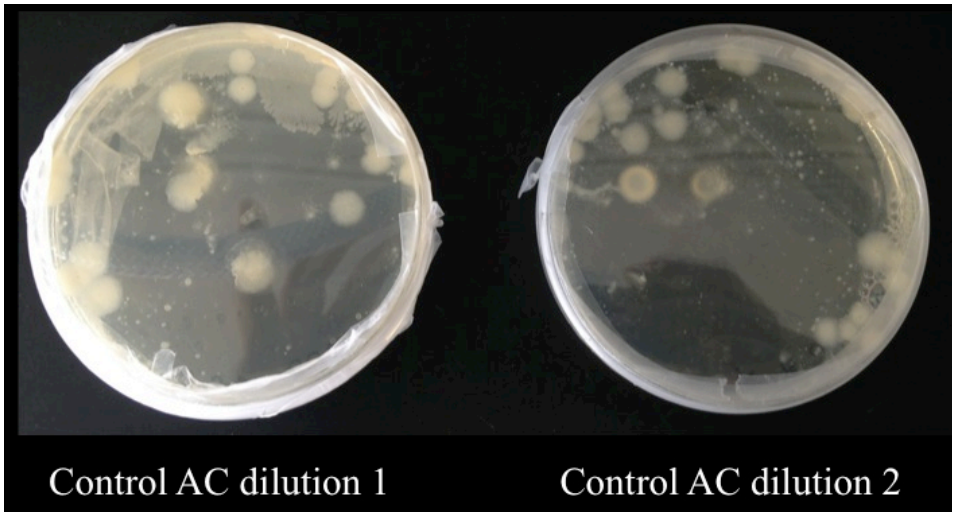


***Figure** 5-11 Control of bacteria in solution for Tween with dilutions 1 and 2 for AC.*

Discussion

From images above, it can be appreciated that more bacteria are attached to activated carbon particles of BAC (**Figure** 5-10) than activated carbon particles of AC (**Figure** 5-8), because more bacteria colonies were developed in BAC agar plates.
Different dilutions of detergent were done in order to probe this is the cause of biofilm elution, the aim is to elute bacteria attached to activated carbon particles and to separate them from bacteria in solution. Effectively, higher concentration of detergent improves elution of bacteria from the activated carbon particles.
Moreover, presence of bacteria in solution is also higher for BAC (**Figure** 5-9) than for AC (**Figure** 5-11).
The control agar plates propose was to probe that detergent does not affect concentration of bacteria in solution; it only works as an elution liquid. This fact was demonstrated in **Figure** 5-9 and **Figure** 5-11 were there is no substantial difference between dilutions 1 and 2 in each case.

5.4.3. DNA extraction

Agarose gel visualization of DNA extractions performed with Power Soil DNA extraction kit are showed in **Figure** 5-12 Figure 5-13. As it was already mentioned in section 4.1.6, in this case, electrophoresis serves to visualize and evaluate quality of DNA. It was important to assess DNA presence before applying molecular analysis (massive sequencing) in order to save money and time.

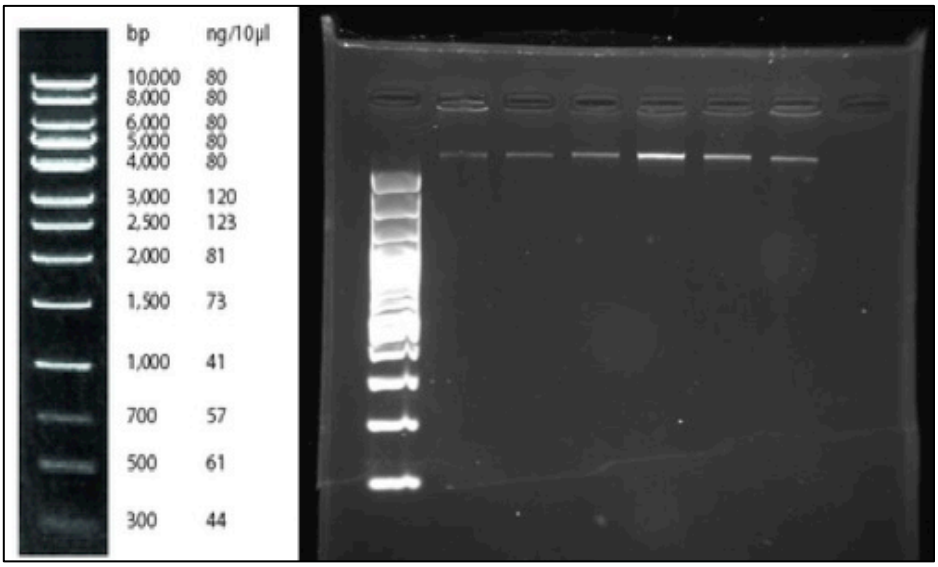


***Figure** 5-12 1 Kb DNA Ladder marker (It works as a comparative marker for DNA sizes (right)). Agarose gel electrophoresis for the extracted DNA. In order from left to right: 1- 1 Kb ladder DNA marker (figure 10), 2- activated sludge from anaerobic pond, 3- inoculum growth in acetate and minerals, 4- / 5- activated carbon particles from BAC (1st week), 6-/7- activated carbon particles from AC (1st week), 8- dry activated carbon particles (negative control) (left).*

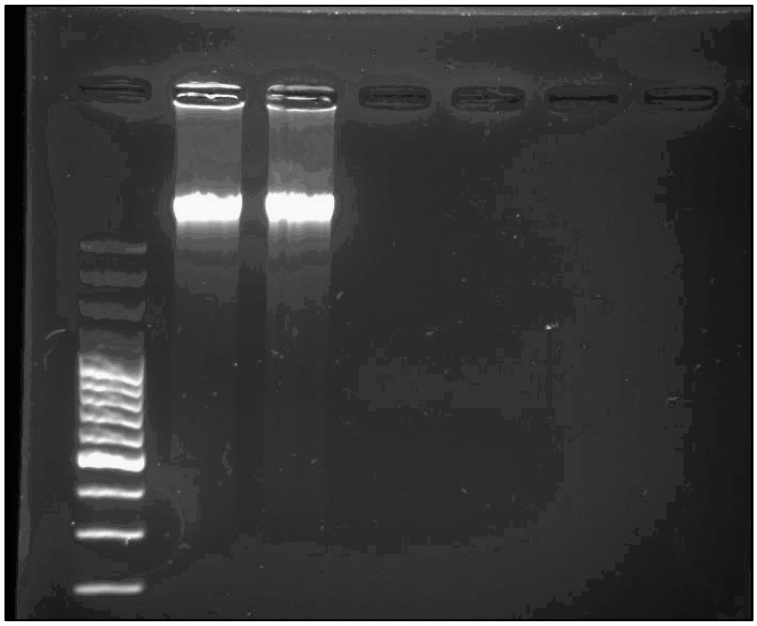


Figure 5-13 Agarose gel electrophoresis for the extracted DNA. In order from left to right: 1- 1 Kb ladder DNA marker, 2-/3- activated carbon particles from BAC (4th week), 4-/5- activated carbon particles from AC (4th week).

Discussion

From figure **Figure** 5-12, DNA extracted can be appreciated for samples 2 to7 as one single white line above the 1 Kb ladder. Every bacterial DNA has a length of around 10^6 base pairs

(bp) (Batut, et al., 2014). If electrophoresis gel samples are compared with 1 Kb (10^4 bp) ladder, genome bacteria samples must be above that value (considering that the small DNA fractions run faster in electrophoresis gel).
From **Figure** 5-12 it can be conclude:
-DNA extraction with an excellent quality (genome DNA is not broken, if it was broken several lines were appreciated for every sample)
-Presence of DNA in both filters: BAC and AC. AC presence of bacteria can be justified with the development of bacteria attached from the air.

To have more information, DNA extraction was repeated in the 4th week for BAC and AC samples, with the difference that carbon particles were washed with distilled water several times. This was done in order to remove bacteria in solution and to keep only attached bacteria (forming biofilm). 2 samples for each filter were analysed. From Figure 5-13 it can be conclude:
- DNA extraction with an excellent quality (same as before)
- Presence of biofilm only in BAC samples, which was expected because samples were washed. There is no appreciable DNA in AC samples.

Nothing can be concluded about the type of strains for every sample. That is investigated with massive sequencing.

5.4.4. Molecular analysis

Analysis is in progress. It is expected to have similar results to those showed in **Figure** 2-18.

5.4.5. Effluent pathogen evaluation

Result for fecal coliforms is the same for BAC and AC effluent= >18 MPN/100ml.

Discussion

According to the regulation, there is no limit of fecal coliforms discharge through the sewage system, so this effluent is allowed to be released into the sewage system of Uruguay (in terms of fecal coliforms).

CHAPTER 6

6.1. General discussion and conclusions

In a previous research by Silvarrey, (2015), Libra effluent was treated in order to reduce toxicity. By that time, it was unknown which effluent component contributes more to the high toxicity levels. In this opportunity, by analyzing toxicity results, it was expected major contribution from antibiotics. Results demonstrated that the sanitizing agent was the most toxic chemical component of the real effluent. However toxic effects of mixture of compounds were not analyzed. It is highly demonstrated that complex joint effect, as antagonism or synergisms, are produced by a combination of compounds, so almost nothing can be concluded about the cause of elevated toxicity in the real effluent (Liu, 2015) (Wang, 2016). Due to this reason, only one chemical compound was chosen for the research.

As it was shown above, one of the sanitizing agent molecules is not oxidized by ozone because it does not possess unsaturated bonds. Harder oxidizing treatments may be necessary to attack and make more biodegradable didecyldimethylammonium chloride molecules.

On the other hand, in terms of biofilm development, IBAC process was effectively done, despite it is unknown if longest times of development were necessary to obtain better results.
It was a clear difference between presence and development of biofilm in BAC than in AC. Biofilm existence in the control filter (AC) can be due to environmental bacteria present in the air.

Because BAC effluent has no toxicity, and some ammonium was found, it can be strongly suggested that specific QACs biodegradation is occurring, which was one of the main objectives of the research.

In conclusion, in terms of effluent discharge limits, toxicity and pathogens are in agreement with the normative, so it can allow effluent discharge in the swage system. With regard to organic carbon limits, not much can be concluded with the obtained DOC results. Further analysis must be done considering that the normative only explicit about BOD_5.

In conclusion, research objectives of this thesis were achieved: an ozonation treatment followed by biofiltration was successfully developed, and it was demonstrated it effectiveness in terms of toxicity reduction.

6.2. Recommendations

Further analyses are necessary to prove toxicity diminution with this treatment in the real effluent. As it was already mentioned, toxicity of mixture of compounds has a variety of effects, so the result of the treatment could be extremely different.

In this particular case, ozonation may not be the best choice as a pre-treatment for biofiltration, due to there is no much variation in DOC values, and one of the components of the sanitizing agent (didecyldimethylammonium chloride) is not oxidized by ozone.
Knowing the exact molecules formed after ozonation and biodegradation could help a lot how to improve the treatment and make it more specific, to ensure that objective complex and more expensive analysis must be done. It can also help to evaluate ozonation effectively.

It is also recommended to take more time for the IBAC process, in order to have a more specific biofilm attached to the activated carbon particles. The recommended time for pharmaceuticals is about 3 months; bacteria would differentiate better and acquire resistance in that time, which improve efficiency of the treatment (Zhang, 2015).

References

114752 Test Amonio (2016) http://www.merckmillipore.com/INTL/es/product/Ammonium-Test,MDA_CHEM-114752#anchor_TI

Acerca de LATU (2014) http://www.latu.org.uy/index.php/acerca-de-latu2015

Achkar M, Dominguez A, Pesce F (2012) Cuenca del Río Santa Lucía - Uruguay. Aportes para la discusión ciudadana. In: Uruguay RadlT (ed).

Adler R (2015) Electrophoresis Salem Press.

Akhtar J, Amin NS (2015) Ozonation of Cephalexin Antibiotic Using Granular Activated Carbon in a Circulating Reactor. J Chem Soc Pak 37: 653-663

Andreozzi R, Campanella L, Fraysse B, Garric J, Gonnella A, Lo Giudice R, Marotta R, Pinto G, Pollio A (2004) Effects of advanced oxidation processes(AOPs) on the toxicity of a mixture of pharmaceuticals. Water Sci Technol 50: 23-28

Andreozzi R, Canterino M, Marotta R, Paxeus N (2005) Antibiotic removal from wastewaters: The ozonation of amoxicillin. J Hazard Mater 122: 243-250 DOI http://dx.doi.org/10.1016/j.jhazmat.2005.03.004

Bailey PS (1982) CHAPTER III - Ozonation of Aromatic Compounds: Benzene and Substituted BenzenesOzonation in Organic Chemistry:18-42.

BAM Appendix 2: Most Probable Number from Serial Dilutions (2010) http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm 2016

Bansal RC, Goyal M (2005) Activated Carbon Adsorption. In: Press C (ed).

Bao T, Chen T, Liu H, Chen D, Qing C, Frost RL (2014) Preparation of magnetic porous ceramsite and its application in biological aerated filters. Journal of Water Process Engineering 4: 185-195 DOI http://dx.doi.org/10.1016/j.jwpe.2014.10.004

Batut B, Knibbe C, Marais G, Daubin V (2014) Reductive genome evolution at both ends of the bacterial population size spectrum. Nature Reviews Microbiology 12: 841-850 DOI 10.1038/nrmicro3331

Beltran FJ (2003) Ozone reaction kinetics for water and wastewater systems CRC Press

Biniak S, Szymański G, Siedlewski J, Świtkowski A (1997) The characterization of activated carbons with oxygen and nitrogen surface groups. Carbon 35: 1799-1810 DOI http://dx.doi.org/10.1016/S0008-6223(97)00096-1

Birnbaum LS, Fenton SE (2003) Cancer and developmental exposure to endocrine disruptors. Environ Health Perspect 111: 389-394

Brycki B, Waligórska M, Szulc A (2014) The biodegradation of monomeric and dimeric alkylammonium surfactants. J Hazard Mater 280: 797-815 DOI http://dx.doi.org/10.1016/j.jhazmat.2014.08.021

Camper AK, LeChevallier MW, Broadaway SC, McFETERS GA (1985) Growth and persistence of pathogens on granular activated carbon filters. Appl Environ Microbiol 50: 1378-1382

Cespedes-Payret C, Piñeiro G, Achkar M, Gutiérrez O, Panario D (2009) The irruption of new agro-industrial technologies in Uruguay and their environmental impacts on soil, water supply and biodiversity: a review. International Journal of Environment and Health 3: 175-197

Chu W, Gao N, Yin D, Deng Y, Templeton MR (2012) Ozone–biological activated carbon integrated treatment for removal of precursors of halogenated nitrogenous disinfection by-products. Chemosphere 86: 1087-1091 DOI http://dx.doi.org/10.1016/j.chemosphere.2011.11.070

Corless C, Reynolds G, Graham N, Perry R, Gibson TM, Haley J (1989) Aqueous ozonation of a quaternary ammonium surfactant. Water Res 23: 1367-1371 DOI http://dx.doi.org/10.1016/0043-1354(89)90075-4

Dantas RF, Darcissac M, Lesueur C, Contreras S, Sans C, Fuerhacker M, Esplugas S (2009) Assessment of cationic surfactants mineralization by ozonation and photo-Fenton process. Water Environ Res 81: 201-205

Delanghe B, Mekras C, Graham N (1991) Aqueous ozonation of surfactants: a review. Ozone: science & engineering 13: 639-673

Díez C, Feinberg M, Spörri AS, Cognard E, Ortelli D, Edder P, Rudaz S (2016) Evaluation of quantification methods to compensate for matrix effects in the analysis of benzalkonium chloride and didecyldimethylammonium chloride in fruits and vegetables by LC-ESI-MS/MS. Food Analytical Methods 9: 485-499

Dos Santos Costa H, Daniel LA (2006) Experimental Study Using Ozone: applicability of DPD Method and interference in analytic methods for hydrogen peroxide. REVISTA DE CIÊNCIA & TECNOLOGIA 12

Drugs.com (2015) Drugs.com Know more. Be sure.

EPA (2015) Whole Effluent Toxicity

Eriksson M (2005) Ozone chemistry in aqueous solution - Ozone decomposition and stabilisation. Licenciate, Royal Institute of Technology

Feng M, Yan L, Zhang X, Sun P, Yang S, Wang L, Wang Z (2016) Fast removal of the antibiotic flumequine from aqueous solution by ozonation: Influencing factors, reaction pathways, and toxicity evaluation. Sci Total Environ 541: 167-175 DOI 10.1016/j.scitotenv.2015.09.048

Ferrando L (2015) Análisis de comunidades microbianas. Introducción a la Microbiología Ambiental y Agrícola.

Gerrity D, Gamage S, Holady JC, Mawhinney DB, Quiñones O, Trenholm RA, Snyder SA (2011) Pilot-scale evaluation of ozone and biological activated carbon for trace organic contaminant mitigation and disinfection. Water Res 45: 2155-2165 DOI http://dx.doi.org/10.1016/j.watres.2010.12.031

Gibert O, Lefèvre B, Fernández M, Bernat X, Paraira M, Calderer M, Martínez-Lladó X (2013) Characterising biofilm development on granular activated carbon used for drinking water production. Water Res 47: 1101-1110 DOI http://dx.doi.org/10.1016/j.watres.2012.11.026

Giudice R, Marotta R, Pinto G, Pollio A (2004) Effects of advanced oxidation processes (AOPs) on the toxicity of a mixture of pharmaceuticals. Water Sci Technol 50: 23-28

Haack SK, Metge DW, Fogarty LR, Meyer MT, Barber LB, Harvey RW, LeBlanc DR, Kolpin DW (2012) Effects on groundwater microbial communities of an engineered 30-day in situ exposure to the antibiotic sulfamethoxazole. Environ Sci Technol 46: 7478-7486

Ikehata K, El-Din MG (2004) Degradation of recalcitrant surfactants in wastewater by ozonation and advanced oxidation processes: a review. Ozone: science & engineering 26: 327-343

Information sheet: Pharmaceuticals in drinking-water

http://www.who.int/water_sanitation_health/emerging/info_sheet_pharmaceuticals/en/ . Cited September 2015
Ivanković T, Hrenović J (2010) Surfactants in the environment. Arhiv za higijenu rada i toksikologiju 61: 95-109
Jardak K, Drogui P, Daghrir R (2016) Surfactants in aquatic and terrestrial environment: occurrence, behavior, and treatment processes. Environmental Science and Pollution Research 23: 3195-3216 DOI 10.1007/s11356-015-5803-x
Jeong S, Bae H, Naidu G, Jeong D, Lee S, Vigneswaran S (2013) Bacterial community structure in a biofilter used as a pretreatment for seawater desalination. Ecol Eng 60: 370-381
Jiang W-x, Zhang W, Li B-j, Duan J, Lv Y, Liu W-d, Ying W-c (2011) Combined fenton oxidation and biological activated carbon process for recycling of coking plant effluent. J Hazard Mater 189: 308-314 DOI http://dx.doi.org/10.1016/j.jhazmat.2011.02.037
Jin P, Jin X, Wang X, Feng Y, C X (2013) Biological Activated Carbon Treatment Process for Advanced Water and Wastewater Treatment DOI 10.5772/52021
Kasprzyk-Hordern B, Ziółek M, Nawrocki J (2003) Catalytic ozonation and methods of enhancing molecular ozone reactions in water treatment. Applied Catalysis B: Environmental 46: 639-669 DOI http://dx.doi.org/10.1016/S0926-3373(03)00326-6
Khan AH, Topp E, Scott A, Sumarah M, Macfie SM, Ray MB (2015) Biodegradation of benzalkonium chlorides singly and in mixtures by a Pseudomonas sp. isolated from returned activated sludge. J Hazard Mater 299: 595-602 DOI http://dx.doi.org/10.1016/j.jhazmat.2015.07.073
Kim WH, Nishijima W, Shoto E, Okada M (1997) Pilot plant study on ozonation and biological activated carbon process for drinking water treatment. Water Sci Technol 35: 21-28 DOI http://dx.doi.org/10.1016/S0273-1223(97)00147-9
LATINPHARMA (2011) Pharmaceutical Industry in Uruguay. In: exportaciones UXPdiy (ed).
Lavorgna M, Russo C, D'Abrosca B, Parrella A, Isidori M (2015) Toxicity and genotoxicity of the quaternary ammonium compound benzalkonium chloride (BAC) using Daphnia magna and Ceriodaphnia dubia as model systems. Environ Pollut 210: 34-39 DOI 10.1016/j.envpol.2015.11.042
Lechuga M, Fernández-Serrano M, Jurado E, Fernández-Arteaga A, Burgos A, Ríos F (2012) Influence of ozonation processes on the microbial degradation of surfactants. Procedia Engineering 42: 1038-1044
Lechuga M, Fernández-Serrano M, Jurado E, Núñez-Olea J, Ríos F (2016) Acute toxicity of anionic and non-ionic surfactants to aquatic organisms. Ecotoxicol Environ Saf 125: 1-8 DOI http://dx.doi.org/10.1016/j.ecoenv.2015.11.027
Lee CO, Howe KJ, Thomson BM (2012) Ozone and biofiltration as an alternative to reverse osmosis for removing PPCPs and micropollutants from treated wastewater. Water Res 46: 1005-1014 DOI 10.1016/j.watres.2011.11.069
Levcovitz E (2013) Uruguay World Health Organization
LIBRA WEB SITE (2015) http://www.laboratoriolibra.com
Liu Y, Vijver MG, Qiu H, Baas J, Peijnenburg WJGM (2015) Statistically significant deviations from additivity: What do they mean in assessing toxicity of mixtures? Ecotoxicol Environ Saf 122: 37-44 DOI http://dx.doi.org/10.1016/j.ecoenv.2015.07.012

Madigan MT (2015) Brock biology of microorganisms 14 edn Benjamin Cummings
Markov SA (2015) Nitrogen Cycle Salem Press.
Menéndez A, Bejarano E, Daza J, Vásquez C (2014) Descripción de las principales rutas metabólicas microbianas en canales para el tratamiento de aguas residuales domésticas. Universidad del Valle.
Menzel DB (1984) Ozone: an overview of its toxicity in man and animals. J Toxicol Environ Health 13: 183-204
Microtox toxicity Testing (2015) http://www.leederconsulting.com/toxicology_services_microtox.html
Míguez D (2014) Un aporte al desarrollo sostenible. Evaluación integrada de riesgos de disruptores endocrinos en el Río Uruguay. Uruguay Ciencia LATU 17
Miranda AR, González C (2013) Estudio cadena de valor de la Industria Farmacéutica en Uruguay (Salud Humana). In: FLACSO/Argentina Cap (ed).
Mofrad MR, Akbari H, Nezhad ME, Atharizade M, Miranzadeh MB (2015) Evaluation of Efficacy of Advanced Oxidation Processes Fenton, Fenton-like and Photo-Fenton for Removal of Phenol from Aqueous Solutions. J Chem Soc Pak 37: 266-271
Ocaña C (2005) Banco de especímenes ambientales y biomonitorización de disruptores endócrinos. . Paper presented at the VII Conferencia sobre disruptores endócrinos, Universidad da Coruña, Galicia, España.2005
OECD (2001) Guidance Document on the Use of the Harmonised System for the Classification of Chemicals which are Hazardous for the Aquatic Environment OECD Publishing
Oh S, Tandukar M, Pavlostathis SG, Chain PS, Konstantinidis KT (2013) Microbial community adaptation to quaternary ammonium biocides as revealed by metagenomics. Environ Microbiol 15: 2850-2864 DOI 10.1111/1462-2920.12154
OPEN CHEMISTRY DATABASE (2016) https://pubchem.ncbi.nlm.nih.gov/compound/Tween_20#section=Top2016
OPS (2015) Problems of Irrational Drug Use Organización Mundial de la Salud.
Paca J, Halecky M, Fitch M (2009) Steady-state performance of an activated carbon biofilter degrading styrene: effects of residence time and inlet concentration. J Air Waste Manage Assoc 59: 45-51
Page MP (2012) Beta-Lactam Antibiotics. In: Dougherty TJ, Pucci MJ (eds) Antibiotic Discovery and Development:79-117.
Park S, Choi K (2008) Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. Ecotoxicology
Parkhurst JD, Dryden FD, McDermott GN, English J (1967) Pomona Activated Carbon Pilot Plant. Journal (Water Pollution Control Federation) 39: R70-R81 DOI 10.2307/25035846
Pharmaceutical Industry (2015) http://www.who.int/trade/glossary/story073/en/. Cited September 2015
Porte C, Janer G, Lavado R, Martin-Skilton R, Thibaut R (2005) Disruptores endócrinos en el medio acuático: efectos sobre vertebrados e invertebrados. . Paper presented at the VII Conferencia sobre disruptores endócrinos, Universidad da Coruña, Galicia, España.2005
PowerSoil® DNA Isolation Kit (2016) https://mobio.com/powersoil-dna-isolation-kit.html
PowerSoil® DNA Isolation Kit (2016) https://mobio.com/powersoil-dna-isolation-kit.html
Research GV (2016) Global surfactants market to be worth $46.20 bn by 2022: Grand View

Research. Focus on Surfactants 2016: 3 DOI http://dx.doi.org/10.1016/j.fos.2016.01.011
Reungoat J, Escher BI, Macova M, Argaud FX, Gernjak W, Keller J (2012) Ozonation and biological activated carbon filtration of wastewater treatment plant effluents. Water Res 46: 863-872 DOI http://dx.doi.org/10.1016/j.watres.2011.11.064
Rivera-Utrilla J, Prados-Joya G, Sánchez-Polo M, Ferro-García M, Bautista-Toledo I (2009) Removal of nitroimidazole antibiotics from aqueous solution by adsorption/bioadsorption on activated carbon. J Hazard Mater 170: 298-305
Schmitt TM (2001) Analysis of surfactants, 2nd edn CRC Press
Services MoPWaG (2000) Biological Test Method: Reference Method for Determining Acute Lethality of Effluents to Daphnia magna. In: Canada E (ed) Environmental Technology Centre, National Library of Canada Cataloguing in Publication Data.
Shan M, Zhang Y, Kou L (2014) Nitrogen balance and transformation in the nitrification process of coking wastewater and the influence on nitrification kinetics. Water Sci Technol 69
Shane C-C, Lue-Hing C (2008) Pharmaceuticals in the Water Environment.
Silvarrey A (2015) Evaluation of ozonation and Advance Oxidation Process as methods to alleviate the toxicity of pharmaceutical industries wastewater. MSc, UNESCO-IHE
Simpson DR (2008) Biofilm processes in biologically active carbon water purification. Water Res 42: 2839-2848 DOI 10.1016/j.watres.2008.02.025
Soto, Sonnenscein (2010) Environmental causes of cancer: endocrine disruptors as carcinogens. Nature Reviews Endocrinology 6
Stalter D, Magdeburg A, Weil M, Knacker T, Oehlmann J (2010) Toxication or detoxication? In vivo toxicity assessment of ozonation as advanced wastewater treatment with the rainbow trout. Water Res 44: 439-448
Surfactants Market worth $42,120.4 Million by 2020 (2014) http://www.marketsandmarkets.com/PressReleases/surfactants.asp2016
Surfactants: the ubiquitous amphiphiles (2003) http://www.rsc.org/chemistryworld/Issues/2003/July/amphiphiles.asp2016
Targeted genomics to reveal antibiotic resistance genes disseminated by transduction (2015) http://www.ecehh.org/research-projects/targeted-genomics-to-reveal-antibiotic-resistance-genes-disseminated-by-transduction/2015
Tena-Sempere (2005) Nuevos biomarcadores y mecanismos moleculares de disrupción endócrina por compuestos estrogénicos: análisis en la rata como especie modelo. Paper presented at the VII Conferencia sobre disruptores endócrinos, Universidad da Coruña, Galicia, España.2005
Ternes TA (1998) Occurrence of drugs in German sewage treatment plants and rivers. Water Res 32: 3245-3260
Test No. 202: Daphnia sp. Acute Immobilisation Test (2004) http://www.oecd-ilibrary.org/environment/test-no-202-daphnia-sp-acute-immobilisation-
Tezel U (2009) Fate and effect of quaternary ammonium compounds in biological systems ProQuest
Tezel U, Pavlostathis SG (2015) Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology. Curr Opin Biotechnol 33: 296-304 DOI http://dx.doi.org/10.1016/j.copbio.2015.03.018
Tipper D (1979) Mode of action of β-lactam antibiotics. Review of Infectious Diseases 1: 39-53 ursea (2015) Texto compilado de normativas de ursea.

Tucker T, Marra M, Friedman JM (2009) Massively parallel sequencing: the next big thing in genetic medicine. The American Journal of Human Genetics 85: 142-154

Uruguay en breve (2013) http://www.uy.undp.org/content/uruguay/es/home/countryinfo/2015

USGS (2015) Antibiotics in Groundwater Affect Natural Bacteria, Environmental Health-Toxic Substances.

Vicenta Paparella C, Pavesi AB, Feldman RN, Bouvet BR (2011) El efecto de los agroquímicos en la espermatogénesis. Revista Habanera de Ciencias Médicas 10: 0-0

Villegas-Navarro A, González MR, Lopez ER, Aguilar RD, Marcal WS (1999) Evaluation of Daphnia magna as an indicator of toxicity and treatment efficacy of textile wastewaters. Environ Int 25: 619-624

Walker GM (1999) Biological activated carbon treatment of industrial wastewater in stirred tank reactors. Chemical Engineering Journal 75

Wang T, Wang D, Lin Z, An Q, Yin C, Huang Q (2016) Prediction of mixture toxicity from the hormesis of a single chemical: A case study of combinations of antibiotics and quorum-sensing inhibitors with gram-negative bacteria. Chemosphere 150: 159-167 DOI http://dx.doi.org/10.1016/j.chemosphere.2016.02.018

Weber WJ (1978) Biological Growth on Activated Carbon: An Investigation by Scanning Electron Microscopy. American Chemical Society 12

WHO (2015) Country statistics and global health estimates by WHO an UN partners Global Health Observatory.

Wunder DB (2010) Fate and Impact of Antibiotics in Slow-rate Biofiltration Processes. UNIVERSITY OF MINNESOTA

Xiaojian (1991) Simple combination of biodegradation and carbon adsorption-The mechanism of the biological activated carbon process. Wat Res 25

Zhang C, Tezel U, Li K, Liu D, Ren R, Du J, Pavlostathis SG (2011) Evaluation and modeling of benzalkonium chloride inhibition and biodegradation in activated sludge. Water Res 45: 1238-1246 DOI http://dx.doi.org/10.1016/j.watres.2010.09.037

Zhang C, Zhong Z, Feng YJ, Sun L, Qi L (2014) Potential for Phosphorus Removal in Wastewater Using Volcanic Rock as AdsorbentAdvanced Materials Research Trans Tech Publ, pp. 202-206.

Zhang Y, Zhu H, Szewzyk U, Geissen SU (2015) Removal of pharmaceuticals in aerated biofilters with manganese feeding. Water Res 72: 218-226 DOI http://dx.doi.org/10.1016/j.watres.2015.01.009

Zhou P, Su C, Li B, Qian Y (2006) Treatment of high-strength pharmaceutical wastewater and removal of antibiotics in anaerobic and aerobic biological treatment processes. J Environ Eng 132: 129-136

ZHU IX, Wang J, Wieland A (2015) Ozone-Enhanced Biologically Active Filtration for Wastewater Reuse. JOURNAL AWWA 107: 12

Zsilak Z, Fonagy O, Szabo-Bardos E, Horvath O, Horvath K, Hajos P (2014) Degradation of industrial surfactants by photocatalysis combined with ozonation. Environ Sci Pollut Res Int 21: 11126-11134 DOI 10.1007/s11356-014-2527-2

ANNEX 1

Relation between carbon and nitrogen

Nutritive medium (Wunder, 2010)

- Sodium acetate (CH3COONa), Molecular Weight (MW): 82 g/mol (2C)
- m= 100 mg in 1 liter
- Ammonium chloride (NH4Cl), Molecular Weight (MW): 51 g/mol (1N)
- m= 5.4 mg in 1 liter

$$mol\ (n) = \frac{m}{MW}$$

- CH3COONa $n = \frac{0.1g}{82\ g/mol} = 0.0012\ mol$

1 mol CH3COONa is equivalent 2 mol C, in 0.0012 mol CH3COONa there are 0.0024 mol C.

- NH4Cl $n = \frac{0.0054g}{51\ g/mol} = 0.000106\ mol$

1 mol NH4Cl is equivalent 1 mol N, in 0.000106 mol NH4Cl there are 0.000106 mol N.

Relation C/N

$$\frac{C}{N} = \frac{0.0024\ mol\ C}{0.000106\ mol\ N} = 24\ C/N$$

Relation 24 C : 1 N

ANNEX 2

Tryptic Soy Agar (TSA)

For microbiological control only

PRINCIPLE
The Tryptic Soy Agar (T.S.A.) is a multi-purpose medium that contains two peptones to grow a variety of germs either fastidious or non-fastidious.
This medium is used in pharmaceutical industry for the enumeration of total aerobic microbial count (TAMC) in non-sterile products and for the preparation of reference strains with the aim of growth promotion tests of culture media.
The medium can also be used as a base for a blood agar : if so, add 5% of sterile defibrinated horse or sheep blood to agar base that has been previously melted and cooled down to 42°C ± 1°C.
The Tryptic Soy Agar can also be a base for the preparation of the "chocolate agar".
Our control of the manufacturing process enables us to provide you with triple wrapped ionised ready to use products whose shelf-life, from manufacturing day, extends to 9 months for Petri plates.

THEORICAL FORMULA
In grams per litre of purified water.
Pancreatic digest of casein 15,00
Papaic digest of soya bean 5,00
Sodium Chloride 5,00
Agar 15,00
Final pH: 7.3 ± 0.2 at 25°C

PREPARATION
Suspend 40 grams of powder into 1 litre of purified water.
While bringing to the boil stir to achieve complete dissolution.
Autoclave for 15 minutes at 121°C.
Place flasks or tubes of medium in a boiling water bath until the medium is completely liquefied. Transfer the contents in a water bath at 47°C ± 1°C. Pour the agar in sterile Petri plates. Let them set on a plane surface plane before storage at refrigerated temperature.

PROCEDURE
Dry the plates before inoculation.
When adding 0.5ml of a 1% tellurite potassium solution, the Tryptic Soy Agar can be used for the growth and the selective isolation of Corynebacterium diphteriae, Candida albicans, L. isteria and Streptococcus.

PACKAGING
Dehydrated medium (Store between 1 and 30°C) AEB152852 : 500 g Flask Made by AES CHEMUNEX – Combourg – France

152852_en : 02/05/13 - O
Formula can be adjusted to meet performances criteria.
Radiosterilized T.S.A. is enriched with growth factors to guarantee an optimal fertility.

Harmonized method of Pharmacopoeias protocol :
Prepare the sample according to § 2.6.12. and according to the nature of the product to be tested, carry out a membrane filtration or a direct inoculation, by pour-plate method or by surface method, of the primary solution and its decimal dilutions onto T.S.A. plates and incubate the plates at 30-35°C for 3 to 5 days.

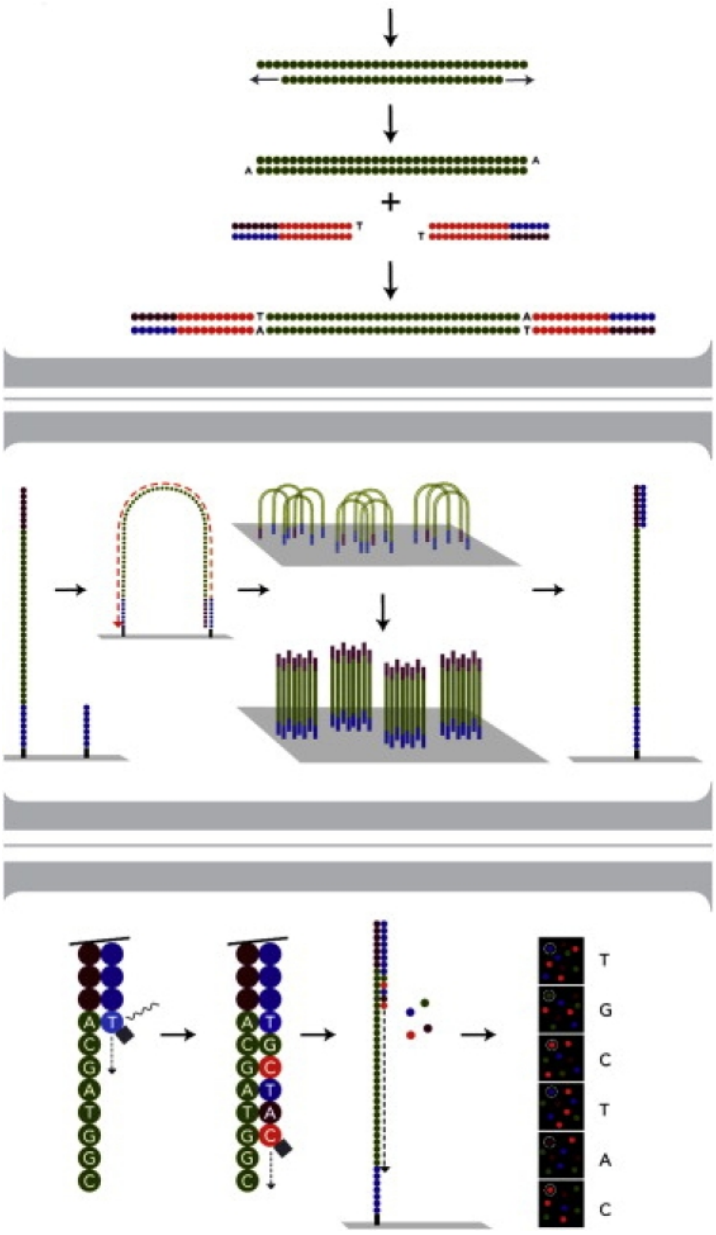
BIBLIOGRAPHY
1. Abbot J.D.and Graham J.M. 1961. Mon Bull. Min Hlth. Pub. Hlth. Lab. Serv. 20 : 51-58.
2. Mitchell T.G. 1964. J. Appl. Bact. 27(1) :45-52.
3. Barnes E.M. and Shrimpton D.H. 1958. J.Appl. Bact. 2(2) :313- 329.
4. Facklam R.R. and Carey R.B. 1985. Manual of Clinical Microbiology4th Ed.-ASM–154-175
5. European Pharmacopeia. Harmonized method. Edition 7th.

URL: http://www.lab-diagnostic.cl/uploads/fichatecnica/c4aea4fef8982f957239eed9c98d6ac0b7ae811a.pdf

ANNEX 3

Massive sequencing

Extracted from the review: Massively Parallel Sequencing: The Next Big Thing in Genetic Medicine (Tucker, 2009).



"Sequencing libraries are generated by fragmenting genomic DNA, denaturation, and adaptor ligation. Fragments are added to the flow cell chamber coated with oligonucleotides

complementary to the adaptors. Hybridization forms a “bridge,” and amplification is primed from the 3′ end and continues until it reaches the 5′ end. After several rounds of amplification, discrete clusters of fragments, all with the same sequence, are formed. The clusters are denatured, and sequencing primers, polymerase, and fluorescently labeled nucleotides, each with their 3′OH chemically inactivated, are added. After each base is incorporated, the surface is imaged, the 3′OH-inactivating residue and label are removed, and the process repeated. Reprinted with permission from Illumina, Inc.”

ANNEX 4

Sanitizing agent

BS101
BACTERICIDA CONCENTRADO

El ***Bactericida Concentrado BS101*** es un detergente multiuso concentrado desarrollado para la limpieza y desinfección en una sola tarea, de ambientes y distintas superficies.

BS101 está compuesto por un detergente y un bactericida de última generación que contiene agentes limpiadores que actúan sobre toda superficie que admita ser lavada con agua.

El bactericida utilizado es un tipo de amonio cuaternario, que es considerado un biocida de alto espectro, activo por tanto frente a un gran número de microorganismo entre los que se incluyen virus, algas, hongos, bacterias.

De alta efectividad en destruir bacterias Gramm positivas y Gramm negativas impidiendo que se formen nuevamente colonias bacterianas.

Por lo tanto el ***BS101*** es un poderoso agente antimicrobiano cuya actividad no se ve disminuida por la presencia de materia orgánica manteniendo su estabilidad en condiciones extremas de pH y temperatura y siendo activo aún en presencia de aguas duras.

Por sus características este ***BACTERICIDA CONCENTRADO (BS101)*** no produce corrosión sobre ninguna superficie en la que es aplicado y por sus condiciones de seguridad tampoco produce riesgos ocupacionales en el personal que lo maneja.

El ***BS101*** puede ser utlizado como *Detergente desinfectante de uso general, Desinfectante de instalaciones y equipamientos, Desinfectante bactericida y fungicida específico* en plantas de procesamiento de alimentos, fábricas de productos lácteos y afines, tambos, industrias cerveceras, plantas envasadoras de bebidas sin alcohol.

DOSIFICACIÓN
De acuerdo al microorganismo a combatir se diluye entre 10 ml y 40 ml de ***BS101*** por litro de agua.

No es tóxico, ni abrasivo, no es corrosivo, ni cáustico, ni inflamable.
Es 100% Biodegradable y se descompone con facilidad en tanques sépticos y sistemas de desagües.
No contribuye a la polución de suministros de agua y no contiene fosfatos.

FICHA TÉCNICA
BS101 (Bactericida Concentrado)
Detergente orgánico completamente soluble en agua
PROPIEDADES
Apariencia:líquido viscoso de color verde
Peso Específico: 0.98 - 1.02
Punto de Ebullición: 100° C
Solubilidad en agua: Total en cualquier proporción.
Punto de inflamación: No inflamable.
Toxicidad: Nula.
Estable al frío
PH: 6-7

COMPONENTES
Activo: no iónico
Cloruro de Didecil Dimetil Amonio
Cloruro de Aquil Amido Propil Dimetil benzil Amonio
Colorante
Agua destilada

ANNEX 5

Ozone Calculation Reference Data

The intent of this document is to provide some basic guidance on calculations and units of measure common in ozone systems.

Reference Conditions:

There are 2 sets of reference conditions commonly used for ozone measurements:
Standard Temperature and Pressure (STP)
Normal Temperature and Pressure (NTP)
These are both defined as 1 Atmosphere, with STP at 0°C and NTP at 20°C.
It is important to understand these reference conditions because flow and concentration measurements are typically 'corrected' to one or the other. The 'correction' is calculated based on a fixed mass of gas.

Flow translation:
Flows referenced to STP (SLPM, SCFH) may have to be converted to NTP. Flows referenced to NTP (most rotometer readings) may have to be converted to STP. The Ideal gas law is used to make the conversion:
If $PV_1 = nRT_1$ describes the fixed mass of gas at one condition, and $PV_2 = nRT_2$ describes the same mass of gas at the other condition then:
P: pressure is the same (1 ATM)
N: Number of molecules is the same
R: Gas Constant is, well, constant
T: defined by the reference conditions (absolute temp)

So:
$V_1 / T_1 = V_2 / T_2$
Or:
$V_2 = V_1 * (T_2 / T_1)$
For the case of a sample at NTP, the volume would decrease by a factor of (273.15)/(20+273.15),
Or 0.93
NLPM * 0.93 = SLPM
$Mg/NM_3 * 0.93 = Mg/SM_3$
The same approach may be used to convert to one of the reference conditions from any other condition (from room temperature, for example)

Flow Measurements:

Rotometers:
Flows in ozone systems are typically measured with 'rotometers' – the 'raw' measurements can rarely be used without significant correction. Sources of 'error' include:
/
Outlet Temp / Pressure other than NTP (typical of most manufacturers)
Gas composition (most off-the-shelf rotometers are set up for air)
When rotometers are used at conditions other than those for which they are designed, the indicated values must be 'adjusted'. The following corrections are typical:
[Note that absolute Temps/Pressures must be used in all calcs]
Outlet Pressure not equal to calibrated outlet pressure:
Actual flow = Indicated flow * sqrt(P_{ACT} / P_{CAL})

Outlet Temp not equal to calibrated Temp:
Actual flow = Indicated flow * sqrt(T_{CAL} / T_{ACT})
Gas not the same density as Cal gas:
Actual flow = Indicated flow * sqrt(1 / SG)
Where SG = (Density of Actual gas / Density of Cal gas)
Sqrt(1/SG) = 0.95 for actual = Oxygen and Cal = Air
It is (obviously) important to understand the calibration data for the rotometer that one is trying to use. Rotometer direct readings are worthless (for precise calculation) without these corrections.

Mass Flow Meters:
There are a variety of mass flow meters available. They typically 'correct' their outputs to STP. They may be calibrated for any of a number of gases – where a gas other than the calibration gas is used, the manufacturer typically provides a linear correction factor.

Errata:
Calculations for ozone production are typically based on a measured flowrate and a measured concentration. Because ozone is incompatible with so many things, the flow measurement is usually of the feed gas and not of the ozone/oxygen exhaust gas. If the inlet volumetric flow is assumed to be the same as the outlet volumetric flow, a small error is introduced to the calculation; that error grows as ozone concentration increases.
The act of turning O2 into O3 reduces the number of molecules. The 'n' in the ideal gas law is not the same at the inlet and the outlet. The 'error' may be corrected as follows:
(Assume oxygen feed gas)

$FLOW_{OUT} = FLOW_{in} * (100/((1.5 * OZ\%) + (100 - OZ\%))$
Where OZ% is the volumetric ozone percentage
For an ozone concentration of 10% (Volume), the correction is 0.95
For an ozone concentration of 5% (Volume), the correction is 0.975
For an ozone concentration of 1% (Volume), the correction is 0.995

Flow Calculations:

Reference Data:
O_3 density at STP: 2.144 g/L
O_2 density at STP: 1.429 g/L
O_3 density at NTP: 1.998 g/L
O_2 density at NTP: 1.332 g/L
Typically, flow and concentration are measured. The following text is intended to demonstrate how everything of any use can be derived from those quantities.
Mass fraction (A.K.A. percent by weight):
All that is required for this calculation is a concentration. The concentration must be in explicit units. It really doesn't matter what the units are, so long as the temperature and pressure are specified. Analyzers that operate at ozone generator concentrations typically use a mass/volume format – g/NM_3 is common.
Mass fraction may be calculated from g/NM_3 as follows:
Mass Fraction = $(g/NM_3) / [(g/NM_3) + (1000-(g/NM_3/1.998))*1.332]$
100 g/NM_3 = 7.32% (mass)
It must be noted that this factor is useful ONLY for the case where the concentration is NTP – it must be modified for all other cases.
Volume Fraction (A.K.A. percent volume):

As in the case of Mass Fraction, only a concentration is required (this calculation is actually a subset of the mass fraction calculation).
Volume fraction may be computed from g/NM^3 as follows:
Volume Fraction = (g/NM^3 / 1.998) / 1000
100 g/NM^3 = 5.01% (volume)
Note:
There is apparently a commonly held belief that a constant 'conversion factor' between % mass and % volume exists. This is simply incorrect. The value is in the range of 3(mass) :2(vol) for low concentrations of ozone, but that ratio decreases (approaches 1:1) with increasing ozone concentration.

Mass Flow Rate:
In ozone applications, Mass flows are often expressed in units of 'grams/hour' or 'pounds per day'. The difference between these units is a simple conversion factor. The confusion in these calculations is usually in getting flow and concentration data in consistent units. Specifically, both flow and concentration must be in 'normal' or 'standard' units (any reference condition may be used, but these are the most common).

Given a flow in SLPM and a concentration in g/NM^3 , proceed as follows:
Compute Volume Fraction as described above
Compute ozone volumetric flowrate:
Ozone volume flowrate = SLPM * Volume Fraction [SLPM]
Compute Ozone mass flowrate;
Ozone mass flowrate = volume flow * ozone density (@ STP) [L/min*g/L =g/min]
The ozone mass flowrate will be in units of g/min. Some useful conversion factors:
60 minutes / hour
1440 minutes / day
1000g / Kg
453.6 g / pound

Example:
Given a 10 SLPM flow of 100 g/NM^3 gas, calculate mass flow in LB/Day
The Volume Fraction is .0501 (5.01%)
The ozone volume flowrate = 10 SLPM * .0501 = .501 SLPM ozone
The ozone mass flowrate = .501 SLPM * 2.144 g/L = 1.074 g/min
Converted to LB/Day:
1.074 g/ min * 1 LB/453.6g * 1440 min / day = 3.41 LB/Day

Notes:
If flowrates are given in at normal (rather than 'standard') conditions, they may be 'corrected' by using the Temperature correction described in the 'Reference Conditions' section, above.
Before ANY raw flow measurement is used in the mass flow calculation, it must be evaluated to determine what corrections are required.

ANNEX 6

Distilled water mass balance

Calculations to correct flow taking into account pressure, temperature and density:

Real values IN			
Preassure (atm)	2,3	correction factor	1,516575089
Temperature (K)	306	correction factor	0,944540556
Density (g/l)	2,144	correction factor	4,399403922
		Actual flow corrected	2,520799809
Real values OUT			
Preassure (atm)	1	correction factor	1
Temperature (K)	307,5	correction factor	0,94
Density (g/l)	2,144	correction factor	1,90
		Actual flow corrected	0,72

Calculations of FLUX for O3 IN:

average	14,78333333

mass fraction (%)	1,105769031	%
volume fraction (%)	0,739906573	%
Ozone volume flowrate	1,865156348	SLPM ozone
Ozone mass flowrate (g/min)	3,998895211	g/min
Ozone mass flowrate (g/day)	5758,409104	g/day

Calculations of FLUX for O3 OUT:

average	44,8	
mass fraction (%)	3,326074045	%
volume fraction (%)	2,242242242	%
Ozone volume flowrate	1,608583438	SLPM ozone
Ozone mass flowrate (g/min)	3,448802891	g/min
Ozone mass flowrate (g/day)	4966,276163	g/day

Calculations of FLUX very 5 minutes for O3 accumulated in distilled water:

O3 water (mg/l)	time (min)	Volume water (l)	mg/min	g/day
0,4	5	15	1,2	1,728
0,2	10	14,8	0,296	0,42624
0,1	15	14,6	0,097333333	0,14016
0,5	20	14,4	0,36	0,5184

0,4	25	14,2	0,2272	0,327168
0,7	30	14	0,326666667	0,4704
0,7	35	13,8	0,276	0,39744
1,1	40	13,6	0,374	0,53856
1,5	45	13,4	0,446666667	0,6432
1,3	50	13,2	0,3432	0,494208
1,3	55	13	0,307272727	0,442472727
1	60	12,8	0,213333333	0,3072

ANNEX 7

Distilled water + sanitizing agent mass balance

Calculations to correct flow taking into account pressure, temperature and density:

Real values IN			
Preassure (atm)	2,3	correction factor	1,516575089
Temperature	306,2	correction factor	0,944232034
Density	2,144	correction factor	4,396530372
		Actual flow corrected	2,518330454

Real values OUT			
Preassure (atm)	1	correction factor	1
Temperature	307,7	correction factor	0,941927714
Density	2,144	correction factor	1,902216445
		Actual flow corrected	0,716700155

Calculations of FLUX for O3 IN:

average	25,57272727	
mass fraction (%)	1,907666155	%
volume fraction (%)	1,27991628	%
Ozone volume flowrate	3,223252146	SLPM ozone
Ozone mass flowrate (g/min)	6,910652601	g/min
Ozone mass flowrate (g/day)	**9951,339746**	**g/day**

Calculations of FLUX for O3 OUT:

Example of calculation of flux for flow OUT= 46 GNM3, the rest values are showed in the table below.

46 GNM3		
mass fraction (%)	3,41415141	%
volume fraction (%)	2,302302302	%
		SLPM
Ozone volume flowrate	1,650060417	ozone
Ozone mass flowrate (g/min)	3,537729535	g/min
Ozone mass flowrate (g/day)	**5094,33053**	**g/day**

O3 OUT (GNM3)	g/day
46	5094,33
49	5426,57
50	5537,32
51	5648,06
51,3	5681,29
51,9	5747,73
52,5	5858,48
52,6	5825,26
51,9	5747,73
52,9	5858,48
53,4	5913,85
53,9	5969,23

Calculations of FLUX very 5 minutes for O3 accumulated in the liquid:

O3 water (mg/l)	time (min)	Volume liquid (l)	mg/min	g/day
3	5	15	9	**12,96**
2,8	10	14,8	4,144	**5,96736**
2,6	15	14,6	2,530666667	**3,64416**
3,8	20	14,4	2,736	**3,93984**
6,3	25	14,2	3,5784	**5,152896**
5,8	30	14	2,706666667	**3,8976**
7,6	35	13,8	2,996571429	**4,315062857**
7,9	40	13,6	2,686	**3,86784**
10,3	45	13,4	3,067111111	**4,41664**
8,4	50	13,2	2,2176	**3,193344**
9,8	55	13	2,316363636	**3,335563636**
11,7	60	12,8	2,496	**3,59424**