



UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE TECNOLOGIA

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GC-Orbitrap-MS for investigation of micropollutants in secondary wastewater and removal evaluation by ozone during disinfection

GC-Orbitrap-MS para investigação de micropoluentes em águas residuais secundárias e avaliação de remoção por ozônio durante a desinfecção

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Dissertation presented to the School of Technology of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Technology (area: Environment).

Dissertação apresentada à Faculdade de Tecnologia da Universidade Estadual de Campinas como parte dos requisitos para a obtenção do título de Mestra em Tecnologia, na área de Ambiente.

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THIS STUDY CORRESPONDS TO THE FINAL DISSERTATION DEFENDED BY THE STUDENT, AND ORIENTED BY PROF. DR. RENATO FALCÃO DANTAS.

Limeira – SP

2020

Ficha catalográfica Universidade Estadual de Campinas Biblioteca da Faculdade de Tecnologia Felipe de Souza Bueno - CRB 8/8577

B232G		Barbacena, Raíssa Olívia, 1993- GC-Orbitrap-MS for investigation of micropollutants in secondary wastewater and removal evaluation by ozone during disinfection / Raíssa Olívia Barbacena. – Limeira, SP : [s.n.], 2020.
		Orientador: Renato Falcão Dantas. Coorientador: Joyce Cristale. Dissertação (mestrado) – Universidade Estadual de Campinas, Faculdade de Tecnologia.
		 Processo oxidativo avançado. 2. Contaminantes emergentes. 3. Cromatografia a gás. 4. Ozônio. 5. Águas residuais. I. Dantas, Renato Falcão, 1978 II. Cristale, Joyce. III. Universidade Estadual de Campinas. Faculdade de Tecnologia. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: GC-Orbitrap-MS para investigação de micropoluentes em águas residuais secundárias e avaliação da remoção por ozônio durante a desinfecção Palavras-chave em inglês: Advanced oxidation process Emerging contaminants Gas chromatography Ozone Sewage Área de concentração: Ambiente Titulação: Mestra em Tecnologia Banca examinadora: Renato Falcão Dantas [Orientador] Enelton Fagnani Fernando Fabriz Sodré Data de defesa: 24-07-2020 Programa de Pós-Graduação: Tecnologia

Identificação e informações acadômicas do(a) aluno(a) - ORCID do autor: https://orcid.org/0000-0001-8138-344 - Curriculo Lattes do autor: http://lattes.cnpq.br/5018055514970444

• FOLHA DE APROVAÇÃO •

Abaixo se apresentam os membros da comissão julgadora da sessão pública de defesa de dissertação para o Título de Mestra em Tecnologia na área de concentração de Ambiente, a que submeteu a aluna Raíssa Olívia Barbacena, em 24 de Julho de 2020 na Faculdade de Tecnologia- FT/ UNICAMP, em Limeira/SP.

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• DEDICATÓRIA •

Dedico essa dissertação à minha família, especialmente e a meus pais Heleuza e Dione e a minha irmã Giovana, que viveram comigo desde o início da minha vida acadêmica e que sofreram comigo tantas abdicações de tempo e de presença.

Dedico também ao meu marido, que sempre me apoiou em todas as decisões por mim tomadas e que é um companheiro incrível.

Agradeço aos meus orientadores, Renato e Joyce, pela paciência, apoio e ensinamentos, sem vocês nada disso teria sido possível e a estrada teria sido muito mais árdua.

Por fim, dedico esse trabalho a mim mesma, como uma prova de que as diferentes provações da vida nos sugerem outras perspectivas sobre nossos problemas, e que o esforço para superá-las, independente da dor, sempre valerá a pena.

• AGRADECIMENTOS •

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001.

ThisstudywasfinancedinpartbytheCoordenaçãodeAperfeiçoamentodePessoaldeNívelSuperior–Brasil(CAPES) – Finance Code 001.

• ABSTRACT •

GC-Orbitrap-MS was used to identify and evaluate micropollutants removal during disinfection with ozone in two secondary wastewater samples, one from a pilot-plant (PPW) and one from an urban wastewater treatment plant (UW). Two approaches were used in the identification of compounds: target analysis and suspect screening. Target analysis led to identification of 11 compounds: phthalates, organic phosphorous flame retardants (OPFRs) and 4-nonylphenol. The suspect screening method was developed from scratch for this type of sample and the data obtained from samples was comparing to the available library spectrum, leading to the identification of 14 compounds, biomolecules and pharmaceutical and personal care products (PPCPs) in both wastewaters. An individual analysis of concentration level and removal level was made for all compounds, as well as for the sum of the chemical families that the compounds belong to. As for PPW, the removal efficiency followed the order for almost all applied ozone doses: alkylphenols > PPCPs > OPFRs > phthalates > biomolecules. For UW, the order was: PPCPs> alkylphenols> phthalates> biomolecules> OPRFs. For the average micropollutant removal efficiency, PPW showed higher levels than UW for all applied ozone doses. The initial value of target micropollutants in PPW was 41 times higher than in UW, explaining the higher removal rates for PPW. In the end of the treatment, most of the target micropollutants levels were similar for both wastewaters. Disinfection evaluation using E. coli presented more efficiency for PPW than in UW, which did not show complete disinfection. Overall, the suspect screening method coupled with target analysis enabled a wide evaluation of presence of micropollutant in wastewater samples, which can potentially be applied to other studies regarding wastewater samples.

KEY WORDS: Advanced oxidation process, emerging contaminants, gas chromatography, ozone, sewage

• RESUMO •

GC-Orbitrap-MS é uma nova tecnologia que usa alta resolução para a separação de compostos em diversos tipos de amostras e foi utilizado para identificar e avaliar a remoção de micropoluentes durante a desinfecção com ozônio em duas amostras secundárias de efluentes, uma de uma planta piloto (PPW) e outra de uma estação de tratamento de águas residuais urbanas (UW). Duas abordagens foram usadas na identificação de compostos: target analysis (análise de compostos-alvo, em tradução livre) e suspect screening (varredura de compostos suspeitos, em tradução livre). Target analysis levou à identificação de 11 compostos: ftalatos, retardadores de chama organofosforados (OPFRs) e 4-nonilfenol. O método de triagem suspeito foi desenvolvido a partir do zero para este tipo de amostra e os dados obtidos a partir das amostras foram comparados com o espectro disponível na biblioteca, levando à identificação de 14 compostos, biomoléculas e produtos farmacêuticos e de cuidados pessoais (PPCPs) em ambos os efluentes. Uma análise individual do nível de concentração e do nível de remoção foi feita para todos os compostos, bem como para a soma das famílias químicas às quais os compostos pertencem. Para PPW, a eficiência de remoção seguiu a ordem de quase todas as doses de ozônio aplicadas: alquilfenóis> PPCPs> OPFRs> ftalatos> biomoléculas. Quanto a UW, a ordem foi: PPCPs> alquilfenóis> ftalatos> biomoléculas> OPRFs. Para a eficiência média de remoção de micropoluentes, PPW apresentou níveis mais altos que o UW para todas as doses de ozônio aplicadas. O valor inicial dos micropoluentes target em PPW foi 41 vezes maior que em UW, explicando as maiores taxas de remoção para PPW. No final do tratamento, a maioria dos níveis de micropoluentes target foram semelhantes para os dois efluentes. A avaliação da desinfecção com E. coli foi maior em PPW do que em UW, que não mostrou desinfecção completa. No geral, o método de suspect screening, juntamente com target analyisis, permitiu uma ampla avaliação da presença de micropoluentes nas amostras de águas residuais, método que pode ser aplicado em outros estudos sobre a presença de micropoluentes em efluentes.

PALAVRAS-CHAVE: Processo oxidativo avançado, contaminantes emergentes, cromatografia a gás, ozônio, águas residuais.

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1. Introduction

Modern society has enhanced the industrial production of goods and will continue to do so. This has increased and intensified the use of industrial products such as pharmaceutical compounds, detergent compounds, flame retardants, solvents, surfactants, plasticizers, among others. What these products have in common is that all of them, at some level, have been found in water due to their discharge, ending up on wastewater treatment plants (WWTPs) and waterbodies of any kind. These compounds receive many different names, cited here as micropollutants. Their effect on human health is only partially known, and some of these effects include carcinogenicity and disruption of the endocrine system,. These effects are only partially known because the effect of these compounds summed due to chronic exposure in human health is not yet understood. Also, possible environmental impacts are also a concern due to the presence of these compounds in water, sediment and soil (SOUSA et al., 2018). Some have been reported to be accumulative since they have been found in birds, shellfish, marine mammals and other animals (SOUSA et al., 2018), and some can cause adverse effects on algae present in the waterbodies (such as triclosan), especially at points were there are wastewater treatment plants being discharged where the concentration of these compounds is higher and then dissipate downstream (REISS et al., 2002).

When reaching the WWTPs in Brazil, wastewater is treated mostly through biological conventional systems and the majority of these micropollutants are not well removed even after the secondary treatment. Such treatment plants have become a major source of micropollutants in the environment – not to mention the amount of wastewater that has been discarded *in natura* (STARLING; LEÃO; AMORIM, 2018).

Advanced oxidation processes (AOP) are a group of technologies to treat water, urban and industrial wastewater that have in common the same feature: the use of HO• radicals. Among them, ozonation, when in basic pH is used as an AOP and it is widely applied for many types of treatments. The process of treatment with ozonation generally works via molecular ozone (highly selective) and/ or HO radicals produced from the decomposition of ozone (less selective and more reactive) (ANDREOZZI et al., 1999; HOIGNÉ, 1998). Since the 70s, the use of ozonation for wastewater disinfection has been studied due to ozone's powerful inactivation properties, even for more resistant pathogens (NASUHOGLU et al., 2018; VON GUNTEN, 2003a). In recent years, the ozonation processes has gained more attention not only because its disinfectant properties, but also because its high capacity of oxidation of micropollutants found in wastewater (NASUHOGLU et al., 2018; ZIMMERMANN et al., 2011).

In Brazil, studies regarding the presence of micropollutants in waters and wastewaters have been growing for the past decades. It is known that since most of WWTPs are based in biological processes, the hypothesis is that micropollutants are only partially or not removed in any level; therefore, they can be detected in secondary wastewater. The techniques used in this work in order to access the wastewater's micropollutants profile included a target analysis, evaluating their presence based on a pre-list of possible micropollutants and also a scanning methodology, suggesting tentative compounds that might be present in the wastewater (STARLING; LEÃO; AMORIM, 2018). Several studies have identified many emergent contaminants in different water sections In Brazil , such as polybrominated diphenyl ether congeners in aquatic sediments (ANNUNCIAÇÃO; ALMEIDA; SODRÉ, 2017), caffeine, bisphenol A, carbamazepine, nicotine, antrazine, sulfamethoxazole and others in drinking waters (SODRÉ; SAMPAIO, 2020), pharmaceuticals (such as losartan and diclofenac) and cocaine in a Brazilian coastal zone (PEREIRA et al., 2016).

In this work, ozonation was performed as a disinfection agent for two different secondary wastewaters, one from WWTPs, referred to as UW, and other from a pilotplant, referred to as PPW, both from Limeira-SP. The efficiency of disinfection of the secondary wastewater was evaluated using Total Coliforms and *E. coli* as indicators. A GC-Orbitrap-MS was used for both target analysis and suspect screening. Xcalibur Software was used to quantify the target compounds and TraceFinder 4.1 Software was used to evaluate the suspect screening compounds. The objective of this work was to determine the behavior of target and suspect micropollutants during ozonation and their removal/ transformation and decrease and to evaluate the differences between both wastewaters and how these differences affect removal and disinfection rates.

This work is divided in two chapters, which results are being published in two different scientific papers. Chapter 1 deals with the challenges of the development of the suspect screening analysis, while Chapter 2 focuses on the study of the removal of micropollutants found through both target and suspect screening analysis, comparing the removal with the disinfection of *E. coli*.

2. Objective

2.1. Main objective

The main objective of this work was to develop a suspect screening analysis for wastewater samples to be used along with target analysis and to evaluate the behavior of micropollutants present in both secondary wastewaters during the disinfection with ozone, using CG-Orbitrap-MS.

2.2. Secondary objectives

The secondary objectives of this study were to:

- Develop a reliable methodology for suspect screening of micropollutants that could be used for both wastewaters; therefore, that is reproducible,
- Evaluate the removal/ behavior of micropollutants present in secondary wastewater using different doses of ozone (before, during and after treatment), separating them into families;
- Measure the disinfection of Total Coliforms and *E. coli* of two different secondary wastewaters using different doses of ozone;
- Evaluate the relationship between micropollutants' chemical family removal and disinfection of the secondary wastewaters.

3. Review

3.1. Chromatographic analysis and mass spectrometry

Gas chromatography has been widely applied to the identification of different compounds in mixtures (BARTLE; MYERS, 2010). GC-MS combines two technologies: mass spectrometry which identifies ions and the gas chromatograph, which provided the separation of these components and then elute them individually (GRAYSON, 2016). For environmental samples, GCMS is highly used for identification of organic compounds due its many features, such as high sensitivity, accuracy and precision, high selectivity and resolution, etc. (SANTOS; GALCERAN, 2002).

With high resolution mass spectrometry (HRMS), the detection methods for organic contaminants using either HRMS coupled to liquid chromatography (LC) or to gas chromatography (GC) have evolved. It has brought new ways to detect micropollutants that don't depend on target analysis and due to its capacity to measure accurate masses to provide structural elucidation it can improve screening methods (Schymanski et al. 2015). Moreover, since HRMS techniques presents high selectivity even when screening complex matrices such as wastewaters (ZEDDA; ZWIENER, 2012), it brings new possible ways to identify possible contaminants present in the samples. LC- HRMS techniques have been employed for the analysis of environmental samples, with non-target analysis in an attempt to identify compounds in raw wastewater (ALYGIZAKIS et al., 2019), with target analysis for simultaneous investigation of over 2000 emerging contaminants in wastewater samples (GAGO-FERRERO et al., 2020), and for assessment of byproducts of ozone during wastewater treatment using non-target screening (SCHOLLÉE et al., 2018). On the other hand, GC-HRMS stands for the analysis of non-polar and semi volatile micropollutants and when coupled to electro ionization (EI) have the advantages of the disposal of databases, reproducible retention time and fragmentation pattern (ABAD et al., 2002; WANG et al., 2019a). Nevertheless, a limited number of studies have used GC coupled with quadrupole time-of-flight mass spectrometry (GC-TOF). GC-TOF was used to identify 2,4-dimethylphenol and the insecticide chlorpyrifos in water (Moschet et al. (2017) and to evaluate the occurrence and removal of micropollutants during wastewater treatment using suspect screening analysis (Wang et al. 2019). New studied using GC coupled to HRMS for environmental samples, especially wastewaters, can aim to comprise a greater number and greater variety of micropollutants that can be identified in these samples.

Orbitrap-MS is a powerful new technology that works with a mass accuracy lower than 1 ppm and at high resolving power (up to 120,000 at *m/z* 200 FWHM) (Gómez-Ramos et al. 2019). Identification potential is provided by comparing the MS spectrum of a candidate with an electron ionization library or an in-house database. The GC Exactive Orbitrap MS combines high resolution gas chromatography and high resolution/ accurate mass (HRAM) mass spectrometry, offering a high resolution (120K, m/z 200), important to increase confidence in the identification of components, high mass accuracy (<1ppm) and high sensitivity (ppt) (DOMÍNGUEZ et al., 2020; WEIDT et al., 2016). With GC-Orbitrap-MS, as for other chromatographic techniques coupled to HRMS, there are 3 approaches to determine organic contaminants (SCHYMANSKI et al., 2015):

- Target analysis: conventional analysis based on the determination of a selected compounds or their well-established transformation products, which are characterized during method development with the corresponding standards in terms of retention time, characteristic mass fragments or MS2 transitions and their ratios.
- Suspect screening analysis: a particular case of non-target analysis that is performed when prior information regarding the presence of a structure that may be present in the sample is available (discussed in greater detail below).
- Non-target analysis: intended to determine those compounds which are not typically included in conventional target analysis nor identified by suspect screening (the remaining compounds in a sample), but can be present in samples and can be analytically determined. For that, a full identification is necessary to properly determine the presence of a given compound, including exact mass, fragmentation information, and any other parameter that need to be performed.

In this study, target analysis was used to identify a set of pre-determined compounds. Since target analysis is limited due to the number of compounds being identified, the suspect screening was performed to tentatively identify compounds that were present in the samples but were not considered in target analysis.

3.1.1. Suspect screening analysis

The identification of micropollutants can be done using a variety of techniques. Target analysis, as the name suggests, uses prior information to identify and quantify an individual substance/compound. Target analysis, alone, is not enough to capture all organic compounds that may be present in a sample such as wastewater and therefore methodologies were developed as a response to this need (CHIAIA-HERNANDEZ et al., 2014), using prior knowledge (data bases, for example) and no reference compounds (suspect screening/ semi-target analysis) and using no information whatsoever (non-target/ non-target screening) (CHIAIA-HERNANDEZ et al., 2014; MILMAN; ZHURKOVICH, 2017).

In suspect screening, specific compounds are suggested based on a database, such as libraries, that have their chemical information compared to the previously acquired data from samples (MUTER; BARTKEVICS, 2020), using the suspect compound information (exact mass derived from molecular formula) to determine its presence, investigating the peaks of a chromatogram (HUG et al., 2014a). Therefore, it depends on prior information indicates that a given compound might be present in the sample (HUG et al., 2014b). Even with the lack of standards, the information regarding the isotopic patterns and the exact mass is crucial to be able to scan for this structure in a sample, and this analysis is considered to be a prerequisite of the non-target along with the target analysis due to the possibility to identify compounds without through these methods (SCHYMANSKI et al., 2015). The suspect screening is often used as a complement for target analysis methods and the no need for reference standards increases the number of substances that can potentially be identified in samples (GAGO-FERRERO et al., 2018).

Suspect screening analysis has been widely used for identification of micropollutants, including to acquire data regarding the presence of micropollutants in wastewater (HUG et al., 2014b; SCHOLLÉE et al., 2018). By definition, the suspect screening analysis does not depend on standards and the rough systematic workflow of a suspect screening analysis works as follows (adapted from Krauss, Singer, & Hollender, 2010):

- 1. sampling, extraction and purification,
- 2. separation and high resolution mass spectrometry;
- 3. suspect screening (no reference standards);
- 4. suspect ion list;
- 5. exact mass filtering (which will depend on the resolving power and mass accuracy of the mass spectrometer that is being used;
- 6. matching isotopes: measured x theoretical isotope pattern of suspects;
- 7. matching retention time: measured x predicted;
- 8. matching measured and predicted MS/MS fragmentation of suspects.

As for confidence, a communication level has been proposed by Schymanski et al. (2014) depending on how much information is available:

- *Level 1* Confirmed structure by a reference standard: it is the ideal situation, when the compound is confirmed using a referenced standard, if available.
- *Level 2* Probable proposed structure: compounds detected through suspect screening can be marked as Level 2 confidence:
 - Level 2A: Library spectrum match: requires a compound spectrum and library spectrum match that is reliable. Additional information such as retention behavior is also desirable, such as a retention index for the matched spectrum and the proposed spectrum, available for GCMS but not sufficiently yet for LCMS.
 - *Level 2B*: Diagnostic evidence: this is the case for compounds that have no library or standard for confirmation and no other structure fit the experimental data.
- Level 3 Tentative candidates: considered to be the "gray area", where the certainty level is high due to the insufficient information to confirm a compound among different matches. Since this level is study-specific, sub

levels are not appropriate, since it would prevent from this scheme to be generically applied.

- *Level 4* Unequivocal molecular formula when a molecular formula can be attributed unequivocally using the spectral information (using, for example, fragmentation information), but there is not enough evidence to propose probable structures.
- *Level 5* Exact mass: the exact mas might be established from a sample but the lack of information leads to the impossibility to assign formulas.

The evaluation of contaminants in honeybees revealed the presence of several substances (phthalates, synthetic musks, etc) using GC-Orbitrap-MS operating in EI mode. The liability of structural characterization relied on the investigation of representative ions, including fragments and molecular ion (GÓMEZ-RAMOS et al., 2019). Suspect screening have been used to evaluate the occurrence and/or removal or micropollutants in wastewater treatments (GAGO-FERRERO et al., 2015; HERNÁNDEZ et al., 2015; WANG et al., 2019a). Few studies regarding the suspect screening of wastewater samples with the aim to evaluate the removal efficiency of unknown micropollutants using GC and HRMS are available. Using a similar flowchart from the one developed in this study for the identification of micropollutants in wastewater samples (samples > extraction > GC-TOF-MS (EI) > spectral deconvolution > library searching > data filtration > trend analysis, such as removal efficiency), Y. Wang et al., (2019b) reached Level 2 confidence for a suspect screening analysis of more than 90 compounds.

3.2. Micropollutants in water and wastewater

Micropollutants are broadly known as compounds found in the environment at concentrations rating from μ g/L to ng/L. Their occurrence in water and wastewater has sparkled a concern about their effects on ecosystems and, more specifically, their possible impact on human health (YANG; ZHOU; CICEK, 2014). These effects can vary according to the type of micropollutants and its chemical family. A compilation of some micropollutants, their major sources and human risks can be found in Table 1.

Table 1. Sources of micropollutants in the aquatic environment and potential health effects

Family	Major source *	Risk	Reference
Phthalates	Medical tubing Disposable plastic water bottles and food containing Industrial waste, domestic wastewater by leaching out of the materials Raw sewage WWTPs	Carcinogenic Endocrine disruption High levels can cause miscarriage and pregnancy complications, testicular atrophy, alteration of testosterone levels, decrease in zinc concentrations	LUO et al., 2014; ABDEL DAIEM et al., 2012; OH et al., 2006; PARK; HABEEBU; KLAASSEN, 2002.
Flame Retardants	Domestic wastewater by leaching out of the material, Sewage WWTPs	Potential carcinogenicity and accumulate in organs (i.e. TDCPP in liver and kidneys of rats), likely reduce human fertility, potentially reduce sperm mobility and density	DISHAW et al., 2011; VAN DER VEEN; DE BOER, 2012, ATSDR, 2009.
Alkylphenols	Domestic wastewater (from laundry, dishwashing, bathing, dishwashing, etc), , WWTP	Highly estrogenic. Anti-androgenic activity. Alterations in the male reproductive system	BARRIOS-ESTRADA et al., 2018; BUITRÓN; TORRES-BOJORGES; CEA- BARCIA, 2015; JAMBOR et al., 2017; LU; GAN, 2014; NING; GRAHAM; ZHANG, 2007.
РАНЅ	WWTPs Unknown sources Domestic wastewater Coal combustion	Potent carcinogen	QIAO et al., 2018; TIAN et al., 2012; WANG et al., 2018.
Pesticides	Insecticides, herbicides and fungicides, agricultural runoff, wastewater (from improper cleaning, run-off from gardens, lawns and roadways)	Alterations in the female reproductive system in mammalian, cancer, toxicity to the developing human brain	BARRIOS-ESTRADA et al., 2018; ESKENAZI et al., 2008; KIM; KABIR; ARA, 2017.

* Sources that are common for all micropollutants include industrial wastewater (from manufacturing these products) and landfill leachate (from the improper disposal of items) (LUO et al., 2014).

The concentration of micropollutants in water and in WWTPs is increasing and has become a sanitary problem. The EU water framework directive 2000/06/CE includes in its Annex X a list of 33 priority substances and groups of substances to be discontinued includes flame retardants, pesticides, phthalates, among others. In 2008, through directive 2008/105/EC, the EU establishes the limits of these compounds to be found in water. Although some of the micropollutants are regulated, limited or even banned in places such as Europe and the U.S., there is still a lack of information regarding the possible chronic exposure and the effect of such compounds in the environment.

In Brazil, not only the lack of more restricts guidelines for hazardous substances are concerning but also the lack of sewage collection, as well as the gap between collected and treated sewage. According to the Brazilian national sanitation information system (SNIS, 2018), 53.2% of the total generated wastewater was collected in 2018 and from that, 74.5% was treated. As for drinking water, the public department of national health (Ministério da Saúde) and the National Agency of Health Surveillance (ANVISA), through Annex VII of the Portaria 2914/2011 have established standards of water potability, that include a list of chemical substances (15 substances and 27 pesticides), but little have been done to prevent the continuing escalation discharge of such substances into water bodies.

In Brazil, many studies have identified these micropollutants in waterbodies and wastewater, as endocrine disruptive compounds as 4-nonylphenol (MOREIRA et al., 2009), herbicides (MACHADO et al., 2017), organochlorine compounds such as dichlorodiphenyltrichloroethane (DDT) (SOUZA BRITO et al., 2005) and PAHs (LEITE; PERALTA-ZAMORA; GRASSI, 2011). Torres et al. (2015) identified hormones in river water, with concentrations varying from 19-90 ng L⁻¹ in the Piracicaba River, in Sao Paulo. Machado et al. (2017) pointed out that the herbicide atrazine was identified in the Pardo River basin, with concentrations ranging from 0.16–0.32 μ g L⁻¹ – below the national limit of 2.0 μ g L⁻¹, but greater than the European 0.1 μ g L⁻¹. Cocaine and pharmaceutical compounds have been identified in the coastal zone (PEREIRA et al., 2016), as well as several micropollutants have been found in drinking water from the Brazilian Federal District, such as caffeine, carbamazepine, sulfamethoxazole, bisphenol A, among others (SODRÉ; SAMPAIO, 2020).

Recently, Starling, Leão & Amorin (2018) reviewed the data available about the Brazilian scenario of possible occurrence and identification of these compounds. The environmental contamination of micropollutants is enhanced by the lack of basic sanitation infrastructure, and municipal wastewater plants are unfortunately not removing these compounds satisfactorily.

3.3. Conventional wastewater treatment – removal of micropollutants

Wastewater treatment is crucial when talking about sanitary conditions. The discard of wastewaters *in natura* can lead to several problems, such as:

- Problems using waterbodies as source of potable water later on;
- Dissemination of pathogenic organisms;
- Use of these waters by human activities (recreation) and dependent biota (i. e. aquatic ecosystems) can be compromised due to the pollution.

These complications can be reduced/ extinguished by using a treatment before the discard of the wastewater and although its importance cannot be diminished, the delays on its effectiveness and consistency are still challenges for a satisfactory wastewater treatment. Generally, the so called conventional wastewater treatment is the most frequent technology worldwide. (PELCZAR et al., 1980)

The conventional treatment of wastewater is generally classified in three phases: primary, secondary and tertiary, according to the level of efficiency in terms of wellknown and broadly used parameters, such as chemical oxygen demands (COD), biochemical oxygen demand (BOD, nitrates, total suspended soils, and phosphates, among others). The conventional wastewater treatment plants (WWTPs) rely mainly on biological assets, therefore are not designed specifically for removal of other types of pollutants that are more recalcitrant or complex (CIRJA et al., 2008; GRANDCLÉMENT et al., 2017).

The conventional WWTPs are broadly used worldwide. The sewer system carries the sewage from homes, industries, etc. into the WWTPs, where the biological and natural cleansing process are enhanced and accelerated, aiming the removal of organic matter in sewage (USA EPA, 1976). The primary treatment corresponds to the removal of solids, but in some WWTPs, the primary and secondary treatments can be combined onto only one process. The secondary treatment is where the organic matter is removed from sewage. The two most common methods being used are suspended growth processes - trickling filters, rotating biological contactors, upflow anaerobic sludge blanket (UASB), active sludge, biotowers etc. - and attached growth processes, such as fixed film. (USA EPA, 2004).

Conventional treatment of micropollutants is well described in literature, as well as other biological technologies. As example, Urase & Kikuta (2005) investigated the removal of two endocrine disrupters, pharmaceuticals and three estrogens by activated sludge. Their final concentration in water environment was found to be dependent on their fate during treatment. Pharmaceuticals remained in water phase in neutral pH, neither absorbed nor removed in the activated sludge. Other studies investigated membrane bioreactors (BESHA et al., 2017; CIRJA et al., 2008) and activate sludge and biofilm (DONG et al., 2016) that can present drawbacks and not fully remove micropollutants, since the microbiota activity is dependable on many factors (temperature, pH, season, fouling, etc).

Therefore, the conventional treatments based mainly on biological processes are not yet an adequate method to remove the micropollutants. Consequently, other alternatives and technologies have been created and modified to address this issue, often being used as tertiary process in the treatment of water and wastewater for not just the removal of micropollutants but also to ensure the disinfection of water and secondary wastewater (influent from WWTPs). The advanced oxidation processes (AOPs) represent a wide range of technologies that can be applied to these purposes.

3.4. Advanced Oxidation Processes (AOPs)

The advances in chemical treatment have led to several findings regarding new technologies. The AOPs are a recognized since 1970 as suitable for pollutant degradation for water and wastewater and the commercialization of such processes have increased, evolution of the topics depending mostly on research (PARSONS et. al., 2004).

The choice of treatment depends directly on the characterization and type of wastewater. AOPs are a good alternative for a vast range of wastewaters, due to specific properties: the different AOP technologies rely on the formation of free radical species, such as hydroperoxyl radical (HO₂•), superoxide radical (O₂•-), hydroxyl radical (HO•), etc (TAI et al., 2002; WANG; XU, 2012). Among the various radicals, the hydroxyl radical is particularly important due to its high reactive and non-selective characteristic, being able to oxidize several types of pollutants. These radicals can provide completely mineralization of some compounds, and the removal/ degradation depend mainly on the concentration of HO• generated (SHARMA &FENG, 2017; SOPAJ et al., 2016). One of the sources of these radical is ozone, and it is a technology that can be applied for several aims.

3.5. Ozone

Ozone is a powerful source of HO• generation. Its existence was noticed two centuries ago, in 1785 by, the Dutch chemist Van Marum through sensorial ways (smell). But it was not until 1840 that ozone was described and named by Schönbein. Later on, in 1857, the first apparatus to produce the ozone was built, by Siemens, and the ozone formula was defined in 1867, by Soret. The first episode of water disinfection happened in 1886, by De Meritens and by the 1970s, there were more than 100 facilities using ozone for disinfection in several countries, including Russia, Canada and Germany (JOHNSON, 1975; LOURENÇÃO, 2009, VON SONNTAG & VON GUNTEN, 2012).

Ozone is 10 times more soluble in water than oxygen itself, and its solubility decreases when temperature increases. Its stabilization is affected by the concentration of ozone, the concentration of scavengers, pH, ultraviolet light (UV light). Due to its instability, ozone needs to be generated on site (WHITE, 1972; JOHNSON, 1975).

3.5.1. Applications and overview

In sanitation, applications of ozone are mostly disinfection and/or removal of specific contaminants in water and all application of ozone involves oxidative reactions. When aimed to both, ozonation can achieve a successful disinfection and satisfactory removal of compounds (KRITŠEVSKAJA, [s.d.]; VON GUNTEN, 2003b). Due to its instability, ozone can be decomposed to HO• in aqueous solutions. For disinfection, the ozone plays a more important role than HO• and, in oxidation processes such as removal of micropollutants, both ozone and HO• are significant (FACILE et al., 2000; VON GUNTEN, 2003b). Due to the high reactivity and oxidation power, ozonation can be applied to disinfection of water, turbidity control, and industrial waters containing phenols, pesticide degradation, among others. Some of the drawbacks of the ozone treatment are the short lifetime of ozone in water, price, and possible formation of byproducts (JOHNSON, 1975; (HOIGNÉ, 1998).

Ozone is a powerful disinfectant and has been used in the treatment of drinking water throughout the world. It can effectively destroy bacteria more rapidly than any other disinfectant (KRITŠEVSKAJA, [s.d.]; VON GUNTEN, 2003a). The disinfection of water and treated wastewaters is the process of inactivation of pathogenic microorganisms, and, differently from sterilization, it does not lead to complete destruction of all organisms that might be present (WERF, 1995; LOURENÇÃO, 2009). The mechanism for pathogen inactivation can be divided in three: (US EPA, 1999):

- The disinfectant can interfere with biosynthesis by preventing the synthesis of fundamental compounds for the cell, such as proteins and nucleic acids;
- By attacking major cells constituents (cell wall, for example), the disinfectant can destroy or impair cellular structural organization;
- The disinfectant can also interfere with energy-yielding metabolism, rendering the enzymes not functional.

3.5.2. Generation and use of ozone

In this work, ozone generation followed the process described as corona discharge. This method works mimicking the results of light on oxygen found the atmosphere. Ozone is formed using an ozone generator, and it works as follows (HOIGNÉ, 1998; COSTA, 2003; LOURENÇÃO, 2009):

- Silent electric discharge (corona discharge) in an air gap between two electrodes.
- The electrons generated by this discharge break the oxygen molecules, as shown above:

Corona discharge

$$O_2 \rightarrow 2 \bullet O$$

The now free and unstable oxygen atoms react with other oxygen molecules, forming ozone (gas) as shown:

$$\bullet O + O_2 \rightarrow O_3$$

For the past decades, the studies of ozonation of the micropollutants have been increasing. Commercial azo dyes degradation (SHU; HUANG, 1995), anti-estrogenic activity related to organic matter (TANG et al., 2014), phthalates (CHEN; SHANG; HSIEH, 2008; GAO; WEN, 2016; STAPLES et al., 1997), sulfamethoxazole (DANTAS et al., 2008) are some examples of micropollutants that have been targeted, and the applications of not only ozone but several AOPs used for this purpose have increased.

4. Material and Methods

4.1. Chemical Reagents

Nicotine, dimethyl phthalate (DMP), diethyl phthalate (DEP), hexachlorobenzene (HCB), hexachlorobutadiene (HCBD), tris (2 chloroethyl) phos- phate (TCEP),), δ hexachlorocyclohexane (δ-HCH), pesticide mix (containing 2,4'- DDD, 4,4'-DDD, 2,4'-DDE, 4,4'-DDE, 2,4'-DDT and 4,4'-DDT), hexachlorocyclohexane (γ -HCH)), tris (2-butoxyethyl) phosphate (TBOEP), tris (2-ethylhexyl) phosphate (TEHP) 4 octylphenol (OP), diisobuthyl phthalate (DiBP), dibuthyl phthalate (DBP), a hexachlorocyclohexane (a-HCH), PCB mix (containing PCB28, PCB52, PCB101, PCB118, PCB138, PCB153 and PCB180), β hexachlorocyclohexane (β-HCH), γnonylphenol (NP) were purchased from Sigma-Aldrich (Darmstadt, Germany and St. Louis, MO, USA). A PAH solution mix, containing the sixteen EPA priority PAHs, at 200 ngµL-1 was purchased from AccuStandard, Inc. (NewHaven, CT, USA). Standads used in this study presented a purity grade higher than 90%. Isotope labeled PAHs mix, containing acenaphthene d-10, chrysene d-12, naphthalene d-8, pervlene d-12, and phenanthrene d-10 was used as internal standard (IS) and it was purchased from Sigma-Aldrich (Darmstadt, Germany and St. Louis, MO, USA). DEHP, DBP, 4NP, TBOEP, TCIPP and TPHP were the initial target compounds and added to the PPW. Methanol, dichloromethane, hexane and acetone were acquired from Honeywell Riedel-de-Haën (USA). Ethyl acetate was acquired from Merck (Germany). The OASIS HLB (200 mg) solid phase extraction (SPE) cartridges were acquired from Waters (USA).

4.2. Sampling and Physicochemical Analyses

The secondary wastewaters used in this study are defined above.

- 1) Pilot Plant samples from a pilot-scale wastewater treatment plant, located in Limeira São Paulo, at the School of Technology campus (UNICAMP) were collected immediately after secondary treatment. The biological reactor is a hybrid: septic tank and anaerobic filter. This wastewater receives the sewage from all campus and also wastewater from chemical labs. The 20 L bottled samples were collected and stored at 4°C for further analysis for a maximum of 24 hours. This wastewater will be referred to as pilot plant wastewater (PPW).
- 2) Wastewater Treatment Plant This wastewater, also from Limeira São Paulo (Aguas da Serra), was collected after secondary treatment. The biological process is a hybrid: anaerobic (upflow anaerobic sludge blanket UASB) and aerobic (activated sludge), that receives sewage from about 50.000 habitants, with an average flow of 76 L s⁻¹. The 20 L bottled samples were collected and stored at 4°C for further analysis for a maximum of 24 hours. This wastewater will be referred to as urban wastewater (UW).

All physicochemical analysis: pH, temperature (°C), alkalinity, UV 254nm, true color and chemical oxygen demand (COD) (mg L^{-1}) were carried out following the procedures described in literature (APHA, 2012).

4.3. Ozone Treatment

Since no previous evaluation of the presence of the micropollutants in the secondary wastewaters was possible and to represent industrial contamination before the treatment with ozone, the raw secondary samples from PPW were spiked with the initial chosen compounds (DEHP, DBP, 4NP, TCIPP, TBOEP and TEHP) before ozonation. A standard was prepared with ethyl acetate containing the targeted compounds and added to the PPW before ozonation so the final concentration was 10 mg L⁻¹ of each compound. The samples from the WWTP were not spiked with the micropollutants, once it was believed that the compounds would already be present due to the highest complexity of the urban wastewater.

The ozonation treatment was carried out in a 2L cylindrical glass reactor (Ozonar GT 8000, Brazil), which bubbled ozone through porcelain porous diffusers (Figure 1). The ozone dose was later measured by iodometry (APHA,2015). The ozone dose was based on previous studies using the same wastewater and micropollutants that used a total dose of 11 mg L^{-1} in 50 minutes (MALVESTITI et al., 2018; MALVESTITI; DANTAS, 2018). 2L of wastewater was used in order to achieve a greater sampling volume for extraction procedures and posterior chromatography analysis. The treatments were performed in duplicate, for each wastewater.



Figure 1. Schematic representation of the bench- scale ozone reactor

In order to access the degradation behavior of the micropollutants, samplings were collected before, during and after the ozone dose for disinfection, in a continuous treatment. In this work, the treatment time is referred as the sampling times and ozone dose for each sample.

- Raw wastewater 0': before ozonation;
- Half of the disinfection dose -3.5mg O₃ L⁻¹ (17,5 minutes);
- Disinfection dose $-7.6 \text{ mg O}_3 \text{ L}^{-1}$ (35 minutes);
- Two times the disinfection dose $15.2 \text{ mg O}_3 \text{ L}^{-1}$ (70 minutes);
- Three times the disinfection dose $-22.8 \text{ mg O}_3 \text{ L}^{-1}$ (105 minutes).

4.4. Disinfection assessment

The disinfection was evaluated using Total Coliforms and *E. coli* as indicators, with Colilert® method that uses Define Subtract Technology (DST), for all samples and times. The Colilert method was done using 100mL samples and 6 dilutions from each ozone reaction time. For each dilution, the reagent was added to the 100mL sample, which was then transferred to the tray, sealed and incubated at 35°C for 24 hours. Then, the positive cavities for total coliforms (yellow) and the positive for *E. coli* (blue under ultraviolet light 366 nm) were counted to quantify the most probable number of both.

4.5. Extraction procedure

The 100 mL of samples were collected using a 100 mL volumetric pipette and stored in previously cleaned (Extran 5%, 24h) and dried amber glass bottles of 200mL. For the extraction, Oasis HLB (200 mg, WATERS) SPE cartridges were pre conditioned with 10 mL of hexane, followed by 10 mL of dichlorometane, 10 mL of methanol and 15 mL of ultrapure water, at a flowrate of 5 mL/min. Samples (100 mL) were then percolated through the cartridges at a flow rate of 10 mL/min. Then, the cartridge was dried under vacuum during 30-40 minutes. Elution was sequentially performed with cartridge rinsed 3 times with 10 mL of dichloromethane/ hexane (1:1, v/v), then 10 mL of dichloromethane/ acetone (1:1, v/v). The extracts were evaporated under nitrogen current at 35°C using water bath. (SÁNCHEZ-AVILA et al., 2011). Since the chromatography analysis was done in another country, the extracts were then reconstituted with 1000 uL of ethyl acetate and spiked with 0.1 ng L⁻¹ of internal standards: naphthalene d-8, acenaphthene d-10, phenanthrene d-10, chrysene d-12, and perylene d-12. The extraction procedure is depicted in the Figure A3 of the Appendix. The internal standards were used to quantify all 59 target compounds.

4.6. Chromatography analysis

Gas chromatography - high resolution mass spectrometry (GC-HRMS) analysis was performed using a ThermoScientific Q Exactive GC Orbitrap MS (combination of high- resolution gas chromatography and high resolution/ accurate mass (HRAM) Orbitrap mass spectrometry), with a TriPlus RSH autosampler and a TRACE 1300

series GC, with a Zebron Capillary GC column ZB- 5ms, dimensions length 30m x internal diameter 0,25 mm x film thickness 0,25 μ m. The methodology for injection is described in Table 2.

Item	Value
TriPlus RSH autosampler method	
Syringe volume	10 uL
Sample volume	2 uL
TRACE 1300 Series GC method	
Maximum temperature	350° C
Initial Temperature	70° C
Carrier flow	1 mL/ min
Ramp 1	6°C/ min until 175°C, hold time
Ramp 2	3° C/ min until 235°C, hold time
Ramp 3	7°C / min until 305°C, hold time
S/SL mode	Splitless, 1 min
Q Exactive GC	
Method duration (time)	60 min
Mass tolerance (+/-)	5 ppm
Ionization – Electron energy	70 eV
Scan range	70 to 1000 m/z

Table 2. Instrumental conditions for chromatography analysis

The identification and quantification of the 59 micropollutants was based on previous work from Velázquez-Gómez et al (2018). The list of the targeted compounds and their retention times can be found in Table 6. Standards were also injected in the same conditions and used for the calibration curve and quantification of target compounds in the concentrations of 0.001, 0.005, 0.01, 0.025, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ppm, using Quan Browser from ThermoFisher Xcalibur Software.

4.7. Identification method

In this study, target analysis and suspect screening analysis were performed in order to evaluate the presence of micropollutants in two secondary wastewaters. Each method had different approaches and characteristics. When referring to target analysis, it means that the identification was made using standards of previously selected compounds, and when referring to suspect screening analysis, the identification was made using a library and comparing the samples' peaks spectra to the library, with no previous knowledge of the presence of these compounds in the secondary wastewaters.

4.7.1. Target analysis

Target analysis was performed to evaluate the presence of micropollutants and also to help to set up the suspect screening methodology. The calibration curve was built using standard solutions in the concentrations of 0.001, 0.005, 0.01, 0.025, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 ng μ L⁻¹. Internal standard quantification of the target compounds was made using Xcalibur Software (Thermo Fischer Scientific).

4.7.2. Suspect screening method development

Software configuration changes were necessary before any sample processing or method creation. The parameters values were based following the supplier recommendations (Thermofisher, Waltham, Massachusetts, EUA) and can be found in Table 3. In the software, "unknown screening" was the equivalent for "suspect "screening", the term used in this study to refer to the method of identification of compounds with no previous knowledge of their presence, but using well known library (National Institute of Standards and Technology -NIST).

Parameter	Value
Detector type	MS
Mass tolerance	5 ppm
RT window	30 seconds
RT view width	1 minute
Ion ratio window type	Relative
Ion ratio indow (+/-%)	20
Ion coelution (min)	0.1
Detection algorithm	Avalon
Peak detection strategy (Analyte)	Highest peak
Peak detection strategy (ISTD)	Highest peak
Peal threshold type	area
Smoothing	5
Extraction window (min)	on, 3.0
Detection Algorithm	Genesis
S/N threshold	3
Enable valley detection	off
Expected width (sec)	off
Constrain peak width	off
Peak height (%)	off
Tailing factor	off
Min peak height	3 S/N
Peak S/N cutoff	200
Valley rise	2 %
Valley S/N	1.10
#background scans	5
Report noise as	peak to peak

 Table 3. TraceFinder Application configuration

The detection capability with the parameters proposed by the manufacturer was applied to process a standard. With those settings, peak detection capability was very low when testing the standard solution at 0.4 μ g/ nL because only one third of the compounds were properly detected and identified. Further, to enhance peak detection, the parameters that were selected for optimization were: "MS signal threshold", "TIC intensity threshold", "penalize molecular ion", "mass tolerance", "*m/z*", and "signal to noise threshold". The optimized conditions can be found at Table 4 and 5. Table 4 refers to the method detection parameters that were set before any processing of samples.

Parameter	Value	
MS Signal Threshold	1000-100000000	
Peak width	1-15	
RT shift	0.5 min	
RT window	30 sec	
RT limits	No RT limits	
Mass tolerance	5 ppm	
Alignment and gap filling	on	
All peaks	on	
Highest point analysis	on, exhaustive search	
Gap filling	on	
Library selection	National Institute of Standards and	
	Technology (NIST) type: mainlib,	
	replib	
	GC Orbitrap Contaminants Library	
	GC Orbitrap Other Environmental	

Table 4. Master Method - peak detection settings

Table 5 shows the settings specifically for the deconvolution of the peaks in the samples.

Parameter	Value
Accurate mass tolerance	3 ppm
TIC intensity threshold	10000
m/z sig to noise threshold	20
Use minimum RT	off
Ions to use	use all ions
Ion overlap window	99%
Smoothing factor	5
Ions to use	Use all ions
RT window	3 seconds
Library search type	normal
SI	500
Penalize molecular ions	on
Annotate fragments	on

Table 5. Peak deconvolution settings – processing parameters

The optimized conditions were applied to a set of raw and treated wastewater extracts. The detection of compounds using the TraceFinder software starts with the deconvolution of all m/z high signals (in relation to background noise). Then, the software groups these ions from the same retention time (RT). For each RT (group of
ions), the software proposes matches from a library, which are compounds that had similar spectra to that deconvoluted spectra from the samples, here referred to as *pre-candidates*. Then, the software allows the user to perform a RT alignment across all samples, and a list of each sample can then finally be exported. By manually grouping the sample files, the final list of *candidates* was manually evaluated based on the criteria established in this study (discussed with detail in the next sections) resulting in the generation of a final list of *elected* candidate compounds.

4.8. Removal efficiency determination

The evaluation of the removal efficiency (RE) using ozonation was evaluated for both compounds identified using target and suspect screening method. Suspect screening analysis does not allow the quantification of compounds, given that no calibration curve is used in this case due to the lack of standards for all detected compounds, so the peak area of each compound was used for removal calculation. In this study, the removal of target compounds previously identified in raw samples from both wastewaters were evaluated based on measured concentration (using Xcalibur software) and peak area (from TraceFinder data), in order to estimate the accuracy of the suspect screening method. In order to be able to compare them both, the removal efficiency was calculated based on Equation 1:

Where Cb and Ca refers to the concentration of the compound before and after ozonation, respectively. The higher the values of RE, the higher is the removal of a certain compound. The use of peak area to the suspect screening approach assumes that the matrix effect is similar before and after the treatment. Li et al. (2018) have proposed an adaptation of Equation 1, that is:

$RE (\%) = \frac{Ab-Aa}{Ab} \times 100 \%$ Equation 2

Where Ab and Aa are the measured areas of compounds before and after treatment. In this work, Equation 1 is used to determine the RE of target compounds and Equation 2 is used to estimate the RE of elected candidates through suspect screening analysis.

5. Chapter 1

Identification of micropollutants through suspect

screening approach using GC-Orbitrap-MS

Chromatography techniques have been used for decades for the identification of pollutants in water and wastewater, and although there is a need for methodologies that can simplify this identification and decrease the time consumed in these analysis, there is also a concern about the reliability of the methodology that is being used. Not just the identification of these micropollutants can be challenging but also the questions and hypothesis that are raised are often numerous and complex (micropollutant behavior during treatment, level of confidence of candidate compounds, confirmation parameters, etc).

Since wastewater can contain a variety of pollutants, the hypothesis of this chapter was: can suspect screening methodology be used to identify as many micropollutants as possible from secondary wastewaters? And with that hypothesis, several other questions were raised; some of them were well answered throughout the data analyses, some of them still need answers. How these candidate compounds, identified through the suspect screening, behave during the disinfection with ozone; how to analyze, in a systematic approach, all my samples at once; and how to assure that the candidate compounds are considered *elected* compounds were some of the relevant questions raised along the study.

5.1. Results

5.1.1. Target analysis

The deconvolution conditions were set up using analytical standards to identify target compounds in the calibration standards and to calculate the mass error, in parts per million (ppm), for each analytes. The theoretical molecular mass, experimental molecular mass, theoretical base ion, experimental base ion, mass errors, instrumental detection limit (IDL), method detection limit (MDL) and concentration in both PPW and UW can be found in table 6. Some compounds did not have the molecular ion identified in the standard samples and so the base ion was also used to evaluate the identification of standard compounds. Mass errors below 5 ppm were observed in the virtually all cases.

10 compounds were detected in the raw wastewater samples using the target analysis approach, including 4 phthalates (DMP, DEP, DBP, and DEHP), 5 organophosphorus flame retardants (TCIPP, TDCPP, TBOEP, TEHP, and TPHP), and 1 alkylphenol (NP). The information regarding the targeted compounds detected in the raw wastewater samples can be found in Table 6.

Base ion

41

E1

2.1

1,320

9.300

E2

 $(ng L^{-1})$

1.9

25

4.400

580

110

RT **Experimental** Theoretical IDL **MDL** Compound molecular ion molecular ion ion error error $(ng L^{-1})$ $(ng L^{-1})$ (min) base ion mass base ion mass (pg) mass (m/z) mass (ppm) (ppm) 9.44 128.0619 128.0626 -5.47 128.0619 128.0620 -0.510.60 1.50 Naphthalene Hexachlorobutadiene (HCBD) 9.88 257.8126 257.8134 -3.10224.8407 224.8407 -0.142.005.00 Nicotine 12.89 162.1150 162.1158 -4.93 84.0808 84.0803 6.08 2.005.00 Dimethyl phthalate (DMP) 14.99 194.0573 194.0579 -3.09 163.0388 163.0383 3.25 0.14 0.34 -5.52 152.0619 -0.27 1.88 Acenaphthilene 15.14 152.0618 152.0626 152.0619 0.75 154.0773 154.0783 152.0621 15.82 -6.49152.0619 -1.261.20 3.00 Acenaphthene 17.77 * 222.0892 * 149.0233 149.0240 -4.56 0.25 Diethyl phthalate (DEP) 0.10 17.81 166.0774 166.0783 165.0698 2.14 Fluorene -5.42165.0699 0.46 0.86 20.01 * 287.8604 * 180.9373 180.9370 1.71 1.20 3.00 alpha-Hexachlorocyclohexane (a-HCH) 5.00 20.04 281.8126 281.8134 -2.84283.8096 283.8096 0.06 2.00 Hexachlorobenzene (HCB) 25.09 220.1822 220.1828 -2.73 107.0491 107.0492 -0.93 0.67 1.67 39,900 Nonvlphenol (NP) β-Hexachlorocyclohexane (β-HCH) * 21.28 * 287.8604 180.9372 180.9373 -0.55 1.50 3.75 * 287.8604 * 180.9373 180.9373 0.00 3.00 gamma-hexachlorocyclohexane (γ -HCH) 21.63 1.20 * 283.9541 * 248.9845 21.65 248.9844 -0.402.00 5.00 Tris(2-carboxyethyl)phosphine (TCEP) 21.82 206.1665 206.1672 -3.40107.0491 107.0491 0.38 2.005.00 Octylphenol (OP) 178.0783 -3.37 178.0776 0.67 22.46 178.0777 178.0777 0.29 1.67 Phenanthrene Tris(2-chloroisopropyl)phosphate (TCIPP) 22.47 * 326.0010 * 124.9998 124.9998 0.00 5.00 2.00178.0777 22.76 178.0777 178.0783 -3.37 178.0776 0.29 0.67 1.67 Anthracene 23.43 * 287.8604 * 180.9371 180.9372 -0.55 3.00 1.20 delta-Hexachlorocyclohexane (δ -HCH) * * Diisobutyl phthalate (DiBP) 24.67 278.1519 149.0233 149.0233 -0.34 0.04 0.09 25.14 * 266.0398 * 255.9608 255.9607 0.21 0.04 0.09 **PCB 28** 27.25 289.92175 289.9226 -2.86 291.9186 291.9187 -0.210.22 0.56 **PCB 52** 27.69 * 278.1519 * 149.0234 149.0233 0.08 0.20 0.41 10,900 Dibutyl phthalate (DBP) Malathion 27.89 * 330.0362 * 124.9821 124.9821 0.07 6.00 15.00 * * 28.12 348.9265 257.8942 257.8943 -0.40 0.08 0.21 Chlorpyrifos 30.89 202.0774 202.0783 202.0774 202.0773 0.45 Fluoranthene -4.45 0.75 1.88 30.95 * 357.9697 * 266.9375 266.9383 -2.88 3.00 7.50 Chlorphenvinfos 32.25 315.9373 315.9382 -2.85 245.9997 245.9997 0.07 2.4'-DDE 0.00 0.03

Theoretical

Molecular

Table 6. List of targeted compounds used in this study, IDL, MDL and mass spectral information.

Experimental

Pyrene	30.86	202.0774	202.0783	-4.45	202.0777	202.0774	1.48	0.75	1.88		
PCB 101	32.52	323.8826	323.8837	-3.24	325.8796	325.8799	-0.80	0.04	0.10		
endosulfan	32.64	*	403.8172	*	240.8955	240.8906	20.25	2.00	5.00		
4,4'-DDE	34.33	315.9373	315.9382	-2.85	245.9997	245.9997	0.00	0.04	0.10		
2,4'-DDD	34.68	*	317.9539	*	235.0075	235.0075	0.09	0.06	0.15		
β-endosulfan	36.29	*	403.8172	*	236.8408	236.8408	0.11				
PCB 118	36.96	323.8827	323.8837	-3.09	325.8797	325.8800	-0.92	0.04	0.09		
2,4'-DDT	36.96	*	351.9150	*	235.0075	235.0075	-0.03	0.35	0.88		
4,4'-DDT	36.93	*	351.9150	*	235.0075	235.0075	-0.04	0.35	0.88		
4,4'-DDD	36.93	*	317.9539	*	235.0075	235.0075	-0.03	0.04	0.09		
PCB 153	37.59	357.8437	357.8447	-2.85	359.8406	359.8414	-2.22	0.04	0.09		
Tris(1,3-dichloroisopropyl)phosphate (TDCPP)	38.37	*	427.8843	*	98.9841	98.9843	-1.62	0.04	0.10	145	410
Benzyl butyl phthalate (BBzP)	39.16	*	312.1362	*	149.0234	149.0237	-2.35	0.30	0.75		
PCB 138	39.15	357.8437	357.8447	-2.79	359.8407	359.8417	-2.78	0.04	0.10		
Triphenyl phosphate (TPhP)	40.47	326.0686	326.0709	-7.05	325.0621	325.0592	8.93	0.10	0.26	15,700	200
Tris(2-butoxyethyl) phosphate (TBOEP)	41.04	*	398.2435	*	124.9998	124.9999	-0.80	3.00	7.50	307,700	460
2-ethylhexyl diphenyl phosphate (EHDPHP)	41.24	*	362.1648	*	251.0467	251.0463	1.39	3.00	7.50		
1,2-benzanthracene	41.86	228.0931	228.0940	-3.95	228.0931	228.0931	0.00	0.86	2.14		
Chrysene	42.09	228.0931	228.0940	-3.95	228.0931	228.0931	0.18	0.86	2.14		
Tris(2-ethylhexyl) phosphate (TEHP)	42.29	*	434.3527	*	98.9842	98.9841	0.20	2.00	5.00		25
PCB 180	43.08	391.8048	391.8058	-2.55	393.8017	393.8019	-0.63	0.03	0.08		
Bis(2-ethylhexyl) phthalate (DEHP)	44.08	*	390.2771	*	149.0233	149.0234	-0.87	0.55	1.36	4140	2,350
o,o,o-tricresyl phosphate (o,o,o-TCP)	46.17	368.1165	368.1178	-3.53	368.1164	368.1164	-0.11	0.08	0.21		
o,m,p-tricresyl phosphate (o,m,p-TCP)	46.67	368.1165	368.1178	-3.53	368.1163	368.1164	-0.27	0.07	0.18		
p,p,p-tricresyl phosphate (p,p,p-TCP)	47.16	368.1165	368.1178	-3.53	368.1166	368.1167	-0.16	0.11	0.27		
Benzo[b]luoranthene	47.92	252.0932	252.0940	-3.17	252.0932	252.0931	0.40	3.00	7.50		
Benzo[k]fluoranthene	48.05	252.0932	252.0940	-3.17	252.0932	252.0929	1.19	3.00	7.50		
Benzo[a]pyrene	49.15	252.0932	252.0940	-3.17	252.0932	252.0929	1.19	3.00	7.50		
Indeno(1,2,3-cd)pyrene	52.81	276.0934	276.0940	-2.17	276.0934	276.0933	0.36	6.00	15.00		
Dibenz(a,h)anthracene	52.96	278.1090	278.1096	-2.16	278.1090	278.1090	0.00	6.00	15.00		
Benzo(g,h,i)perylene	53.56	276.0934	276.0940	-2.17	276.0934	276.0933	0.36	6.00	15.00		

5.1.2. Suspect screening method development

To test the manufacturer recommended conditions, a sample of the standard solution at 0.4 ng μ L⁻¹ was used as a tryout. A method, "unknown screening method" in the software was created using the recommended conditions. At default conditions only 27 chromatographic peaks were found by the TraceFinder software out of the 59 target analytes present in the standard solution. At this point, it was concluded that the optimization of the parameters was necessary. Also, some pre-candidates (compounds proposed for each peak) were proposed by the software based on similarity against the NIST Mass Spectrum library, demonstrating the need to establish criteria for the identification of possible candidates. Because of that, a strategy for unknown screening optimization and identification of peaks was developed, resulting in the workflow presented at Figure 2, where Phase I details the creation of a pre-candidate compounds that attended the established acceptance criteria for candidates.



Figure 2. Workflow for the suspect screening methodology

5.1.3. Phase I – Creation of a pre-candidate list for a set of samples

The goal of this phase was to test if the target compounds from the standard solution could be identified, testing the detection conditions, and subsequently in the raw wastewater sample, but avoiding an excessive number of irrelevant peaks (such as peaks related to background noise or to the stationary phase of GC).

• Step 1: It was possible to understand how the changes in the software parameters were impacting the deconvolution of peaks using the standard solution (59 previously analyzed target compounds at 0.4 ng μ L-1) and the raw wastewater samples as reference. Different values from the master methods were tested and the deconvolutions of peaks were observed. Only the identification of target compound

was evaluated. Table 7 presents the most significant parameters and the modifications from the default conditions (when applicable).

Parameter	Parameter	Default	This	Comments
type		value	study	
Peak settings	MS Signal	$10^7 - 10^9$	$10^3 - 10^9$	The number of peaks using a
(master	Threshold			minimum of 10 ⁷ was
method)				considered not sufficiently
				representative of a complex
				matrix such as urban
				wastewatess, so the value was
				diminished to 10^3 . Lower
				values increased the number of
				irrelevant peaks. Maximum
				threshold remained the default
		5	4	value.
Processing	Total ion	105	104	Lower values were explored,
parameters	chromatogram			but it increased the number of
(local	intensity (TIC)			irrelevant peaks.
method)	threshold	10	•	
	m/z signal to	40	20	It was observed that higher
	noise threshold			values of S/N limited the
				detection of compounds known
				to be present in the wastewate
				concentration Lower values
				were also tested but the
				number of irrelevant peaks
				(peaks that had no library
				match or poor score) increased.
	Penalizing	-	on	Facing the relevance of the
	molecular ion			presence of molecular ion for
				confirmation of a candidate
				compound without a standard,
				it was set as on and reflected in
				the Score value given by the
				software for each pre
				candidate.

Table 7. Parameters and modifications from the recommended conditions

• **Step 2**: After steps described so far, the same conditions were applied to all samples (26 samples). This stem was the most time demanding (up to 6 hours), justifying the previous optimization of parameters.

• **Step 3**: The retention time alignment (RTA) is a tool that aligns detected peaks based on their RT and spectra similarity. This allowed exporting the data to excel files, gathering the information on mass fragments of each compound and which compound was present or absent from which samples. An excel file was manually generated for each sample, and then manually compiled into one excel sheet.

Each detected peak (individual RTs) presented a list of possible matches that were proposed by the software. They are referred to in this study as *pre-candidates*. Figure 3 shows an example of the TraceFinder window, where it can be observed a deconvoluted peak list of each sample, the pre-candidates for each peak, the extracted m/z ions for each peak, and the comparison between the fragment components and the library mass spectra.



Figure 3. An example of the RT alignment window across all samples (upper right) in TraceFinder Software. It is the tentative identification of the substance caffeine. Parts that are highlighted 1) red: the list of deconvoluted and merged peaks of all samples, organized by RT; 2) blue: list of precandidate matches for each peak. Here, 55 pre-candidates were proposed for the same peak, with different Score rates. Caffeine has the higher Score; 3) yellow: list of the extracted m/z ions for each peak (here, "all ions" option was on); 4) green: comparison between component (upper portion) versus library spectra (bottom portion).

5.1.4. Phase II – Sorting elected candidate compounds

Several criteria were adopted in order to sort the candidates and to extract reliable information regarding each compound and to exclude any doubtful or irrelevant compounds. In order to maintain a pattern, for each peak, the first pre candidate (first library hit; Figure 2, red box) was considered the most accurate match (highest Score) and was referred in this study as *candidate compound*. The other pre-candidate options were discarded. A total of 1,006 candidates were manually verified in order to exclude unfeasible compounds.

Step 4: Pre-candidates are ranked by the software according to a Score that is calculated based on:

- *High Resolution Filtering (HRF) Score* the percentage of the explainable measured fragment from the proposed chemical formula in the library (in what percentage the fragments can be explained by the proposed chemical formula);
- *Forwarded search index* (*SI*) the search index score (0-999) for each compound detected returned by the NIST library search;
- *Molecular Ion* presence/ absence of the molecular ion.

As aforementioned, the first pre-candidate option for each peak was selected as the best match, as it was also the pre-candidate with the highest score.

• **Step 5:** The candidates were classified using the set of filters, created to exclude irrelevant candidates (evaluating the presence in sample and absence in blanks) and then, from the 256 candidates remaining, another set of filters was applied and they were manually checked before concluding that 18 candidates could be considered *elected candidates*. The description of filters can be found in Table 8.

Filter Type	Filter description	Comments
nt	Peaks present in the blanks and not in samples	Discarded (no relevance).
rrelevar tes	Peaks randomly present	Discarded (no comparison could be performed among the samples)
lusion of i candida	Peaks only present in one sample	Discarded (no comparison could be performed among samples, even if only present in raw samples).
Exc	Irrelevant peaks with library matches	Such as column bleed-related compounds.
S	Compounds that had only one RT	Some candidate compounds were identified twice (two or more RT), so they were discarded.
remaining candidate	Presence of the molecular ion	The confirmation of a candidate compound as an elected compound was assured by the presence of molecular ion. Also, the candidates with the absence of molecular ion had lower Score, justifying the next criterion (Score above 80).
firmation of	TraceFinder Score	A minimum Score of 80 was established (GÓMEZ-RAMOS et al., 2019) and peaks below this limit were discarded.
Con	Matching spectra: comparing the candidate compound mass spectrum to the NIST library.	At least the three highest fragments in intensity were evaluated and compared, as well as the spectra as a whole.

 Table 8. Set of filters for the suspect screening candidate compounds' confirmation

5.1.5. Target analysis versus Suspect Screening

5.1.5.1. Standard solution

It was observed that from 59 standard compounds, 44 peaks were deconvoluted and identified (CAS number checked), meaning that their names and retention times corresponded to the target analysis identification, when the optimized suspect screening conditions were applied to a standard mix sample (0.4 ng L⁻¹). The other 15 compounds not properly identified by CAS number were: EHDPhP, TEHP, o,o,o-TCP, γ -HCH, DiBP, endosulfan, 4,4'-DDT, PCB 28, PCB 52, PCB 101, PCB 118, PCB 153, PCB 138, PCB 180, and dibenz[*a*,*h*]anthracene. These compounds were considered not identified. Although TraceFincer could deconvolve the PCB peaks and all had their respective molecular ion present (except for PCB 28), they were not properly identified by their CAS number, possibly due to their similar spectra. Except for dibenz[*a*,*h*]anthracene, the other 7 non identified compounds were all missing the molecular ion from library.

Among the 44 identified standards, 26 compounds presented the molecular ion (natphalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, 1,2-benzanthracene, chrysene, benzo[b]fluoranthene, benzo[a]pyrene, indeno(1,2,3-cd)pyrene, benzo[g,h,i]perylene, HCBD, nicotine, DMP, HCB, 4NP, OP, 2,4'-DDE, 4,4'-DDE, TPhP, o,m,p-TCP, p,p,p-TCP), 16 standards did not present the molecular ion in either the library or the standard sample spectra (α -HCH, β -HCH, δ -HCH, TCEP, TCIPP, TDCPP, β -endosulfan, malathion, chlorpyrifos, chlorphenvinfos, 2,4'-DDT, 4,4'-DDD, BBzP, DBP, DEP, and DEHP) and lastly 2 had their molecular ion missing from the standard sample but it was present in the library (2,4'-DDD and TBOEP).

Since the standard solution mix was prepared using high purity solvents and standards, these results show the good performance of TraceFinder software to deconvolve and identify compounds in low complexity samples. Although presence of molecular ion was not mandatory for identification of compounds in the standard solution (due to the previous known since they were target compounds), 59% of identified compounds presented the molecular ion.

5.1.5.2. Wastewater samples

The presence of the molecular ion was the key criteria to establish the list of elected candidates. The elected candidate list for the Wastewater samples did not included DEP, DBP, DEHP, TCIPP, TDCPP, TBOEP and TEHP due to the lack of molecular ion, as described above. In other words, these compounds were included in the pre-candidate and candidate lists, but excluded at Step 5 of the workflow (Figure 2). It means that their presence would not have been considered without the target analysis and previous knowledge about them, since they did not attend all the criteria presented in Table 8.

A total of 21 compounds were included in the elected candidate list using the developed suspect screening workflow, from which 3 compounds were also detected by target analysis (DMP, TPHP and 4-NP). Other 18 elected candidates were detected only based on the developed suspect screening methodology (no analytical standard was used). The results extracted from TraceFinder and compiled into an excel sheet can be found in Table 9. All compounds had a Score (further explained) above 90.

Compound name	Chemical Formula	CAS Number	RT	Score	Fragments ^a	$\mathbf{M}^{+\mathbf{b}}$	Present M ⁺	Theoretical Reference m/z ^c	Reference present m/z ^d
1,8-Naphthyridine, 2,4,7- trimethyl-	$C_{11}H_2N_2$	14757- 44-9	17.536	97.6	9/13	172.10016	172.099426	120.0205	120.020584
5-Acetyl-2-methylpyridine	C ₈ H ₉ NO	42972- 46-3	17.702	96.5	14/26	135.06847	135.067947	120.044418	120.044472
Benzophenone	C ₁₃ H ₁₀ O	119-61-9	18.660	95.9	13/16	182.0732	182.072586	105.03344	105.033508
Cotinine	$C_{10}H_{12}N_2O$	486-56-6	20.207	95.4	20/21	176.09506	176.094376	98.060051	98.060074
Caffeine	$C_8H_{10}N_4O_2$	58-08-2	24.112	94.5	13/18	194.0805	194.079697	194.079834	194.079697
Tonalid	C ₁₈ H ₂₆ O	21145- 77-7	24.329	86.8	21/22	258.19848	258.197937	243.174362	243.174393
Antipyrine	$C_{11}H_{12}N_2O$	60-80-0	25.165	96.8	58/64	188.09506	188.094208	188.094299	188.094208
Acridine 9 carbaldehyde	C ₁₄ H ₉ NO	885-23-4	30.874	95.6	35/27	207.06847	207.067764	179.0727	179.072784
Cyclic octaatomic sulfur	S ₈	10544- 50-0	30.551	92.6	10/8	255.7768	255.776001	159.859726	159.859787
Triclosan	$C_{12}H_7Cl_3O_2$	3380-34- 5	32.151	93.3	26/22	287.95131	287.950684	218.012955	218.012924
Carbamazepine	$C_{15}H_{12}N_2O$	298-46-4	39.144	97	17/17	236.09506	236.09436	193.088623	193.088623
Pentoxifylline	$C_{13}H_{18}N_4O_3$	6 493-05- 6	42.445	95.8	20/21	278.13804	278.137451	221.103256	221.103333
Cholest-2-ene, (5a)-	C ₂₇ H ₄₆	570-73-0	48.935	90.1	66/64	370.36018	370.359375	215.179398	215.179413
Γ-Tocopherol	$C_{28}H_{48}O_2$	7616-22- 0	51.486	97.9	18/18	416.36564	416.364838	416.364838	416.364838
Cholestan-3-ol, $(3\beta,5\beta)$ -	C ₂₇ H ₄₈ O	360-68-9	51.861	94.1	93/93	388.37074	388.369904	215.17952	215.17952
Cholest-7-en-3-ol, (3)-	C ₂₇ H ₄₆ O	6036-58- 4	52.180	93.9	121/121	386.35508	386.354248	316.312469	316.312286

Table 9. Data extracted from the suspect screening methodology regarding the elected compounds

Cholesterol	$C_{27}H_{46}O$	57-88-5	52.252	95	112/112	386.35508	386.35434	301.288971	301.288971
Vitamin E	$C_{29}H_{50}O_2$	59-02-9	52.306	97.7	6/6	430.3813	430.380341	430.38028	430.380341

^a: Number of fragments used to propose a candidate compound/total number of fragments for that peak. For most compounds, the software did not use the totality of the deconvoluted fragments for each

^b:Calculated exact mass.

^c: Theoretical referenced m/z from Tracefinder for each candidate compound. ^d: Presence of the theoretical referenced m/z in the identification of each candidate compound.

5.1.6. Removal Efficiency and Error

A comparison of removal rates calculated using Equations 1 (based on measured concentrations) and Equation 2 (based on peak areas) can be found in Figure 4, for the target compounds identified in both PPW and UW through target and suspect screening. Higher initial levels in PPW led to higher initial peak areas and reflected on the removal efficiency rates. For PPW, almost all compounds showed higher suspect screening RE levels were higher than target analysis RE values, for all ozone doses. Among OPFRs in PPW, TCIPP, TBOEP and TPHP showed similar RE levels for target analysis and suspect screening. Higher RE variation was observed for TDCPP (up to 9% of difference), but RE values were higher than 80% for all ozone doses. 4NP RE levels were equal for both methods, reaching 100% removal rate for the first doses applied (3.5 mg $O_3 L^{-1}$). Among phthalates, DBP presented the faster removal rate for both methods, followed by DMP and DEP. DMP showed the lowest initial concentration for phthalates in this wastewater (2 ng L^{-1}), and may not have been detected through suspect screening, showing a false complete removal. DEHP showed recalcitrant behavior when submitted to ozone for both wastewaters and methods.

Overall, for UW, the RE was lower than for PPW, possibly due to the lower initial concentration of compounds. To illustrate this difference, the values for the target analysis removal at the first ozone dose applied ($3.5 \text{ mg O}_3 \text{ L}^{-1}$) ranged from 3 to 14%, while for PPW, these values ranged from 52 and 100%. In UW, DEP was the compound that presented the faster RE for both methods, reaching 95 and 100% of removal rate for suspect screening and target analysis, respectively, for 7.6 mg O₃ L⁻¹. By the end of the treatment, among phthalates, DEHP was the most resistant compounds, similarly to PPW, not surpassing 40 and 42% RE values for both target analysis and suspect

screening, respectively. For OPFRs, suspect screening RE levels were higher for TEHP and TCIPP for all ozone doses. By the end of the reaction time, TDCPP presented higher RE for both detection methods. Overall, none of the OPFRs surpassed 50% RE for the UW wastewater.



Figure 4. Removal efficiencies comparison for the suspect screening and target detection of target compounds for PPW (upper) and UW (bottom).

The errors obtained for RE calculated using Equation 2 in relation to RE calculated using Equation 1 were considered in order to compare both removal rates and are presented in Figure 5. Higher errors were observed for UW than for PPW. Higher initial levels in PPW led to higher initial peak areas and reflected on the removal efficiency rates. Also, it is important to stress that compounds at lower concentration

due to the treatment with ozone were sometimes only identified in samples from the treated wastewater by target analysis, resulting in a false complete removal rate by the suspect screening RE approach (Equation 2), contributing to higher errors (>50%).



Figure 5. Calculated error between suspect screening and target identification of target compounds for PPW (up) and UW (bottom) wastewaters.

5.2. Discussion

New technologies such as GC-Orbitrap-MS providing high resolution mass spectral data, coupled with powerful tools as TraceFinder Software and Mass Spectral Libraryes can improve the identification of pollutant in complex matrices such as wastewater samples. The high resolution technologies can be an alternative to tentatively identify several compounds at once through suspect screening, along with target analysis. Nevertheless, due to the presence of contaminants that can be at trace concentrations, the suspect screening needs to be optimized in order to detect these compounds. Also, due to lack of standards, manual verification is also necessary in order to establish a list of elected candidate compounds that meet the criteria for confirmation. A detailed discussion about the aspects involved in the suspect screening method development is presented below.

5.2.1. Peak detection and deconvolution adjustment

In this study some of the detection parameters were optimized in order to identify a larger number of chromatographic peaks. This was considered necessary since the default conditions did not detect some compounds that were known to be present in the standard and samples (target compounds) since they were previously identified using target analysis. After the parameters adjustment, 84 peaks were detected in the standard sample containing the 59 target compounds at 0.4 ng L^{-1} , including unknown peaks that were not chemically identified. The optimized suspect screening conditions were accepted once the target compounds were convolved by the suspect screening method in the standard sample. This combination of target and suspect screening analysis was important due to the complexity of a wastewater extract compared to a standard solution, and also to try to identify compounds that were at trace concentrations, establishing a proper peak detection setting (Table 4) and peak deconvolution (Table 5). The number of peaks increased with the changes in the master method and peak detection, as well as the number of irrelevant or unidentified peaks; but was necessary in order to properly identify relevant peaks with lower areas. It is important to point out that the necessity of higher or lower screening sensitivity (number of convolved peaks in a sample) will impact on the changes necessary in the peak deconvolution parameters.

5.2.2. Sample set processing and RT Alignment

In this study, the RT alignment was crucial in order to compare the presence/absence of a given compound throughout the treatment with ozone and to understand the removal behavior. Since the basic point of RT alignment is to relate peaks that have same RT, to account for batch deviations in terms of RT, RT shift

(dislocation) was set at 0.5 seconds, as predetermined by the manufacturer (Table 4). RT alignment is a feature that, depending on the number of samples, can take many hours of processing, justifying the previous optimization of parameters previously to sample processing. RT alignment is a useful tool to perform suspect screening analysis in environmental samples and also to compare the results obtained from different wastewater treatment options, including ozone, including ozonation transformation products of micropollutants in wastewater (SCHOLLÉE et al., 2018) and to evaluate different treatment options from an urban WWTP (NÜRENBERG et al., 2015).

5.2.3. Tentative identification and removal of candidate compounds

As mentioned before, Trace Finder software is able to process sample raw data and generate a pre-candidate list for each chromatographic peak. This tentative identification is done by the comparison of the deconvoluted peaks data with the NIST nominal mass spectra library (>200.000 compounds). For each peak, the software ranks the pre-candidate, scoring them from the highest score match to the lowest, using a set of parameters to create the Score as aforementioned. Peaks with similar/equal chemical formulas and similar exact masses had similar Score rates, sometimes differing tenths; the first pre-candidate was selected for all peaks to set a pattern . These parameters (Score, retrieval score (SI) and a high resolution filtration (HRF) score were relevant to understand how the software proposes the pre-candidates. To obtain data with compounds that can be distinguished even with similar mass to ratios (m/z), HRF values were obtained using the high resolution and high mass accuracy obtained from Orbitrap-MS, similar to other studies using the GC-Orbitrap-MS to identify micropollutants in complex samples, such as fly ashes (YANG et al., 2019)

In this study, the presence of molecular ion was one of the criteria established for considered a candidate as elected candidate due to its relevance to confirm its chemical elemental composition, as well as the spectra similarity to the library reference (SCHYMANSKI et al., 2015). Despite its relevance, the absence of the molecular ion discarded a major portion of the deconvoluted peaks (approximately 85% of the candidate after applying the empirical filters (Table 4). The absence or the low intensity of the molecular ion is often owed to the condition of the electron ionization (EI) at 70 eV and also to the common temperature, at 230 °C (LAU et al., 2019). EI at 70 eV is the most common condition, used in 90% of the published work between 2014 and 2017 (TRANCHIDA et al., 2018). Lower electron energy (12 eV) and lower temperature (200°C) is associated with highest molecular ion intensity, and lower electron energy can increase mass accuracy (LAU et al., 2019). In this study, the standardized EI at 70 eV and the ion source temperature of 260° C were used. As mentioned before, some target compounds (standard solution mix) did not present the molecular ion in its mass spectrum, and some of them were also absent from the library. Without a standard, these compounds would not have been identified. Thus, a lower EI value could possibly increase the number of identified compounds through increasing the number of candidate compounds with the molecular ion. Aside from the molecular ion, the base ion was also relevant when comparing the candidate compound to its match in the library, as showing in Table 3. The base ions were able to be identified for all targeted compounds and were the most abundant for the majority of the compounds.

The software was set to use "all ions" in order to propose pre-candidates for each peak. When a fragment had no chemical explanation for their presence in the identification of a candidate compound, the software rejected the fragment and showed which ones were used to propose the candidate compounds. The software also indicates whether a fragment was used or not, and ranks these used fragments according to their intensity. From the 18 elected candidates, 6 included all fragments, and the others used at least half of the fragments deconvoluted for each RT, as shown in Table 9.

For the purpose of having the same criteria for the selection of all pre-candidate compounds, the first library hit and consequently the best Score value proposed by the software was kept for all compounds, meaning that no selection of second or third candidate compound on the list was made, even when the scores were close. 1,8-Naphthyridine, 2,4,7-trimethyl-, cotinine, cyclic octaatomic sulfur, pentoxifylline, tonalid and triclosan were the compounds that were the best fit and higher score rates and that the next compound on the candidate list had a score below 85 (53, 84.2, 40, 75.7, 51.3 and 61.7, respectively), indicating that the first pre-candidate had a low false positive probability in relation to the compounds included in the NIST library. Other pre-candidate compounds such as caffeine (score 94.5) and benzophenone (score 95.9) had their upcoming pre-candidates on the list with small difference in the score values, 93.9 and 95.7, respectively.

A confidence level system from 1 (highest confidence) to 5 (lowest confidence) have been proposed by Schymanski et al. (2014) for the identification of compounds via HRMS to improve communication of confidence of the detected compounds. The elected compounds in this study reached a level 2 confidence (exact mass, molecular formula, tentative candidate and probable structure using a library spectrum match). The highest confidence level 1 was not achievable due to the lack of standards for confirmation. A similar study using UHPLC- Orbitrap-MS/MS for the evaluation of RE of several polar compounds in wastewater also reached a level 2 confidence for 288 compounds and level 1 for 31 compounds confirmed with standards, and also used a comparison between target analysis and non-target analysis to identify the differences in the removal rates obtained for both methods (LI et al., 2018).

As for the suspect screening method, the developed workflow is in accordance to previous schemes used in other studies. Gómez-Ramos et al. (2019) used GC-TOF-MS and GC-Orbitrap-MS to exploit the environmental contaminants in honeybees, also using TraceFinder 4.1 to convolve peaks, using the molecular formula of the representative ions (fragments and molecular ion) to improve confidence for the characterization of the tentative compounds (PAHs, phthalates and synthetic musks), similarly to the data presented in this study (Text S1 and Table 4), in which the fragments information was considered part of the criteria to establish the list of elected candidates. Another study used LC-QTOF-MS to evaluate emerging micropollutants of a Mediterranean river basin, using the compounds that were not identified through suspect screening as candidates for the non-target screening approach (CCANCCAPA-CARTAGENA et al., 2019), which can be a start point for a further non-target screening of wastewater samples with the aim to understand the performance of advanced treatments, especially to identify unknown ozonation transformation products (OTP) for which individual substance transformation information is still scarce, since suspect screening can give information about known OTPs but non-target analysis is needed for a broader picture (SCHOLLÉE et al., 2018). In this work, no OTP was identified through suspect screening, since the library did not include these substances.

An individual analysis of each elected candidate compares the results with information on the compounds from literature, mostly from low resolution libraries, since high resolution information regarding all elected candidates is still scarce. The low mass error for each fragment also increases confidence for the confirmation of their elemental composition proposed by the software. The elected candidates were considered to fit the Level 2 confidence for suspect screening analysis: exact mass, library match and tentative candidate led to a probable structure for all compounds (SCHYMANSKI et al., 2014). Table 10 brings information regarding the fragmentation and proposed chemical formula for elected candidates. The mass error for each fragment was given by TraceFinder, comparing the theoretical m/z calculated by the software with the obtained m/z value. The top fragments (F1, F2, F3), were ranked according to their intensity (M⁺). The most abundant high resolution mass fragments of candidate compounds were also compared to a low resolution mass spectral library (NIST), using the three most abundant fragments of each candidate compound.

Table 10. Top fragments from candidate compounds extracted from TraceFincer compared with data from Pubchem (NIST spectra for GC-MS analysis) and theoretical data from TraceFinder (NIST mainlib library) obtained from the raw secondary wastewater samples.

Candidate Compound and top peaks ^a	Score	$\mathbf{M}^{+\mathbf{b}}$	Chemical formula/ Theoretical <i>m/z^c</i>	Mass error (ppm)	NIST m/z ^d
1,8-Naphthyridine, 2,4,7- trimethyl-	97.56	172.0994	C ₁₁ H ₁₂ N ₂ / 172.0995	0.4	
F3		173.1029	C ₁₁ H ₁₁ N ₂ /173.1028	0.38	173
F1		172.0994	$C_{10}^{13}CH_{12}N_2/172.0994$	0	172
F2		171.0916	$C_{11}H_{12}N_2/171.0916$	0	171
5-Acetyl-2-methylpyridine	96.47	135.0679	C ₈ H ₉ NO/ 135.0678	0.6	
F1		120.0444	C ₇ H ₆ NO/ 120.0443	0.68	120
F2		135.0679	C ₈ H ₉ NO/ 135.0678	0.61	135
F3		92.04947	C ₆ H ₆ N/ 92.0494	0.02	92
Benzophenone	95.99	182.0725	C ₁₃ H ₁₀ O/ 182.0726	0.16	
F1		105.0335	C ₇ H ₅ O/ 105.0334	0.16	55*
F2		182.0725	CH ₁₀ O/ 182.0726	0.16	77
F3		77.03858	C ₆ H ₅ / 77.0385	0.07	182
Cotinine	95.41	176.0943	C ₁₀ H ₁₂ N ₂ O/ 176.0944	0.21	
F1		98.06007	C ₅ H ₈ NO/ 98.0600	0.34	*
F2		147.0680	C ₉ H ₉ NO/ 147.0678	0.97	*
F3		176.0943	C ₁₀ H ₁₂ N ₂ O / 176.0944	0.21	*
Caffeine	94.53	194.0797	C ₈ H ₁₀ N ₄ O ₂ / 194.0798	0.66	
F1		194.0797	C ₈ H ₁₀ N ₄ O ₂ / 194.0798	0.66	194
F2		193.0719	C ₈ H ₉ N ₄ O ₂ / 193.0720	0.37	109
F3		109.0397	C ₅ H ₇ N ₃ / 109.0634	0.79	55*
Tonalid	86.8	258.1979	C ₁₈ H ₂₆ O/ 258.1978	0.47	
F1		243.1743	C ₁₇ H ₂₃ O/ 243.1743	0.21	243
F2		187.1117	C ₁ H ₁₅ O/ 187.1117	0.16	43*
F3		159.1168	C ₁₂ H ₁₅ / 159.1168	0.35	258
Antipyrine	96.78	188.0942	C ₁₁ H ₁₂ N ₂ O/ 188.0944	1.09	
F1		188.0942	C ₁₁ H ₁₂ N ₂ O/ 188.0944	1.09	188
F2		96.04435	C ₅ H ₆ NO/ 96.0443	0.42	96
F3		77.03858	C ₆ H ₅ / 77.0385	0.08	77
Acridine 9 carbaldehyde	95.66	207.0677	C ₁₄ H ₉ NO/ 207.0678	0.48	

F1		179.0727	C ₁₃ H ₉ N/179.0729	0.93	207
F2		207.0677	C ₁₄ H ₉ NO/ 207.0678	0.48	179
F3		178.065	C ₁₃ H ₈ N/178.0651	0.52	178
Cyclic octaatomic sulfur	92.56	255.776	S ₈ / 255.7760	0.06	
F1		159.8597	S ₅ /159.8598	0.11	64*
F2		255.776	S ₈ /255.7760	0.06	256
F3		127.8877	S ₄ /127.8877	0.13	160
Triclosan	93.27	289.9476	C ₁₂ H ₇ Cl ₃ O ₂ / 289.9476		
F1		218.0129	C ₁₂ ClH ₇ O ₂ / 218.0129	0.07	290
F2		287.9506	C ₁₂ Cl ₃ H ₇ O ₂ / 287.9506	0.25	288
F3		289.9477	C ₁₂ Cl ₂ ³⁷ Cl ₁ H ₇ O ₂ / 289.9476	0.21	218
Carbamazepine	97.06	236.0943	C ₁₅ H ₁₂ N ₂ O/ 236.0944	0.22	
F1		193.0886	C ₁₄ H ₁₁ N/ 193.0886	0.12	193
F2		192.0806	C ₁₄ H ₁₀ N/ 192.0807	0.61	192
F3		236.0943	C ₁₅ H ₁₂ N ₂ O/ 236.0944	0.22	236
Pentoxifylline	95.83	278.1374	$C_{13}H_{18}N_4O_3/278.1373$	0.4	
F1		221.1033	$C_{10}H_{13}N_4O_2/221.1033$	0.14	221
F2		193.0720	C ₈ H ₉ N ₄ O ₂ / 193.072	0.19	193
F3		180.0641	$C_7H_8N_4O_2/$ 180.0641	0.16	180
Cholest-2-ene, (5a)-	90.13	370.3594	C ₂₇ H ₄₆ /370.3593	0.07	
F1		215.1794	C ₁₆ H ₂₃ /215.1794	0.06	384
F2		355.3359	C ₂₆ H ₄₃ /355.3359	0.05	316
F3		316.3124	C ₂₃ H ₄₀ /316.3124	0.04	105
Γ-Tocopherol	97.94	416.3648	C ₂₈ H ₄₈ O ₂ / 416.3648	0.1	
F1		416.3648	416.3648	0.1	151
F2		151.0754	$C_9H_{11}O_2/151.0753$	0.36	416
F3		417.3684	$C_{27}^{13}C_1H_{48}O_2/417.3682$	0.41	417
Cholestan-3-ol, $(3\beta,5\beta)$ -	94.09	388.3699	C ₂₇ H ₄₈ O/388.3699	0.16	
F1		215.1795	C ₁₆ H ₂₃ / 215.17943	0.43	215
F2		373.3462	C ₂₆ H ₄₅ O/ 373.3464	0.56	370
F3		355.3359	C ₂₆ H ₄₃ / 355.3359	0.11	355
Cholest-7-en-3-ol, (3β)-	93.93	386.3542	C ₂₇ H ₄₆ O/ 386.3543	0.18	
F1		316.3122	C ₂₃ H ₄₀ / 316.3124	0.52	385
F2		231.1743	C ₁₆ H ₂₃ O/ 231.1743	0.09	255
F3		161.1324	$C_{12}H_{17}/$ 161.1324	0.01	

Cholesterol / F3	95.04	386.3543	C ₂₇ H ₄₆ O/ 386.3543	0.06	
F1		301.2889	C ₂₂ H ₃₇ / 301.2889	0.02	*
F2		353.3202	C ₂₆ H ₄₁ / 353.3202	0.16	*
Vitamin E	97.78	430.3803	C ₂₉ H ₅₀ O ₂ / 430.3805	0.44	
F1		430.3803	C ₂₉ H ₅₀ O ₂ / 430.3805	0.44	165.1
F2		165.091	C ₁₀ H ₁₃ O ₂ / 165.0910	0.02	137.1
F3		431.3836	$C_{28}^{13}C1H_{50}O_2/431.3838$	0.58	166.1

^a:Candidate compounds and top fragments (F1, F2, F3): fragments with highest intensity for each candidate compound. Some compounds, such as Triclosan, had the three peaks matching the expected peaks from the NIST library, but not in the same order of intensity.

^b:m/z for each proposed fragment, found in samples after RT alignment.

^c: Data proposed by TraceFinder. When not specified differently, isotopes are: ${}^{12}C$, ${}^{35}Cl$, ${}^{32}S$.

^d:GC/MS spectral top fragments (top fragment, 2nd highest and 3rd highest) consulted in Pubchem.

*: not found/ below detected m/z.

An individual analysis of each elected candidate compares the results with information on the compounds from literature, mostly from low resolution libraries, since high resolution information regarding all elected candidates is still scarce. All data presented below are based on GC-MS techniques of identification of compounds, found in literature. The low mass error for each fragment also increases confidence for the confirmation of their elemental composition proposed by the software.

- Benzophenone: is a pharmaceutical commonly used as sunscreen ingredient and often present in WWTPs due to its resistance to conventional treatments (DE LUCA et al., 2017). Low resolution fragments of identification of this elected candidate ranked according to their abundance were reported to be 105, 77 and 182 (ALTUNTAS; HITAY; OZCELIK, 2016). Main high resolution fragments (HRF) for this compound were found by the software as: 105.0335, 182.0725, and 77.03858.
- Cotinine: cotinine is the major metabolite of nicotine and is eliminated by body fluids, such as urine (LEITE; CHATKIN, 2010), consequently being present in wastewaters. Major ion peaks for cotinine in low resolution were reported to be *m/z* 98 and 176 (CULEA; NICOARA, 2006; DURAL, 2017). Main HRF values were *m/z* 98.06007 and 176.0943.

- Caffeine: caffeine is widely consumed and is considered a human-related chemical. The presence of caffeine in surface waters can be an indicator of water pollution by domestic wastewaters (BUERGE et al., 2003; PETEFFI et al., 2019). NIST top peaks for caffeine mass spectrum are *m*/*z* 194, 109 and 55, as shown in Table 4. The *m*/*z* values for identification of caffeine have been reported to be 194 (molecular ion) and ions m/z 165, 138, 109 and 82 (VERENITCH; LOWE; MAZUMDER, 2006). Main HRF for caffeine were found to be 194.0797, 193.0719 and 109.0397. The intensity of peaks differed, with the *m*/*z* 109 appearing as third and not second most abundant. Even so, comparing the chemical formulas proposed by the software and their theoretical masses with the obtained fragments with low mass errors (<0.8 ppm) were considered sufficient to confirm their elemental compositions.</p>
- Tonalid: tonalid is a musk used in a variety of personal care products such as deodorants, soaps and detergents, known to be resistant to WWTPs treatments and to be bio accumulative due to their lipophilic nature (CLARA et al., 2011; KANNAN et al., 2005; REINER; BERSET; KANNAN, 2007). Ions for tonalid identification *m*/*z* 243, 187, 258, 201 (MOTTALEB et al., 2008) were in accordance to the ones observed in this study, main HRF being: 243.1743, 187.1117, 258.1979. The *m*/*z* 201 was also identified, (201.1273), with a mass error of 0.20 ppm and proposed chemical formula C₁₄H₁₇O, appearing as the seventh most abundant ion. The proposed elemental compositions for fragments, their exact masses and their low mass error were considered accurate to consider this candidate compound a confirmed candidate.
- Antipyrine: this anti-inflammatory medication has been found in surface waters and wastewater treatment plants (LUO et al., 2014). The peaks (m/z) 188, 159, 130, 105, 96 and 77 were reported to the identification of antipyrine (CAI et al., 2013). Main HRF found in this study were 188.0942, 96.0443, and 77.03858. These three ions also match the NIST GC-MS m/z spectrum for antipyrine. The other fragments from literature, 159, 130 and 105 were also present among the peaks identified for this candidate compound, with the following m/z: 159.0916 (mass error: 0.38, chemical formula: C₁₀H₁₁N₂, seventh highest option), 130.0652 (mass error: 0.7 ppm, chemical formula C₉H₈N, fourteenth highest option), and 105.0336 (mass error: 1.32 ppm, chemical formula C₇H₅O, fortieth highest option).

- Acridine 9 carbaldehyde: this compound has been identified as a transformation product of carbamazepine degradation by advanced oxidation processes and it is one of its intermediates (KOSJEK et al., 2009). Kinect constant for OH attack to acridine have been reported (5.8 x 10⁹ lmol⁻¹s⁻¹), so degradation of acridine can happen concomitantly with the formation of carbamazepine during degradation (Vogna et al. 2004; Milano et al, 1995). NIST *m*/*z* library reported the ions 207, 179 and 178 for this compound. From literature, *m*/*z* were reported to be, in order of abundance: 207, 179, 151, 125, 100, 87, and 75 (VOGNA et al., 2004). As shown in table 5, the top HRF identified for this compound in this study, in order of abundance, are 179.0727, 207.0677, 178.0650. Other fragments *m*/*z* (with associate mass error in parenthesis) were: 151.0542 (0.17 ppm), 75.0229 (0.2 ppm), and 125.0386 (0.16 ppm).
- Cyclic octaatomic sulfur: the molecular ion was present (*m/z* 207.06776), the *m/z* 160 appears as 159.8597 from the high resolution library (mass error 0.10 ppm) and appears as the highest peak in abundance; and the second expected highest (64) was not identified.
- Triclosan: is present in personal care products, acting as a antimicrobial agent and household products, such as shampoos, soaps, detergents and toothpaste that are disposed down the drains, end up in domestic wastewaters and increasing the input of triclosan in waterbodies through wastewater (BUTLER et al., 2012; ORHON et al., 2017; REISS et al., 2002). Ions used for confirmation and identification of triclosan in environmental samples are 218, 288, and 290 (BUTLER et al., 2012; RAJENDRAN et al., 2011). The top peaks *m/z* 218.0129, 287.9506, and 289.9477 were identified, all with mass error lower than 0.3 ppm.
- Carbamazepine: carbamazepine is a drug used as antiepileptic and reported to be present in WWTPs and other waterbodies (KOSJEK et al., 2009; VOGNA et al., 2004). Mass ions for carbamazepine identification have been reported to be 193, 149, 165, 236, 280 and 180 (DURÁN-ALVAREZ et al., 2009; RAJENDRAN et al., 2011; TERNES; HIRSCH; MUELLER, 1998). Top ions found for this elected candidate in are *m/z* 193.0886, 192.0806 and 236.0943. The ions 280 and 180 were not present in the library spectra or the samples.
- **Pentoxifylline:** Pentoxifylline is a pharmaceutical, used as vasodilator that reduces blood viscosity and increases red blood cell flexibility and has been

found in wastewaters and in waters due to its resistance to conventional wastewater treatments (KAMIŃSKA et al., 2018; YU-CHEN LIN; HAN-FANG HSUEH; ANDY HONG, 2015). Monitoring ions have been reported in previous studies from literature to be (m/z): 278, 221, 193, 180, and 109 (MAURER, 2008) and m/z 193 and 221 (TERNES; HIRSCH; MUELLER, 1998). NIST top fragments are m/z 221, 193 and 180. Top HRF found in samples in this study are m/z 221.1033, 193.0720 and 180.0641. Other monitoring fragments that also matched with the literature included the molecular ion, m/z 278.1374 and m/z 109.0635 (theoretical HRF m/z 109.0634, mass error 0.9 ppm, chemical formula C₅H₇N₃).

- Cholest-2-ene, (5α) NIST low resolution fragments are m/z 384, 316 and 105.
 Top HRF for this compound were found to be m/z 215.1794, 355.3359, and 316.3124. The ion 105 also appears as sixth highest, m/z 105.0699 (theoretical m/z 105.0698, mass error 0.6 ppm, chemical formula C₈H₉).
- Cholestan-3-ol, (3β,5β)-: also known as coprostan-3-ol or coprostanol is a sterol (combination of alcohol and steroid) originate in the intestinal microbiota by the reduction of cholesterol, and is also marker of human activity (CAIN et al., 2004; GRIMALT et al., 1990). Along with cholesterol, it is used as a biomarker of human fecal matter in the environment (MOLINER-MARTINEZ et al., 2010; REICHWALDT et al., 2017). The concentration ratio of coprostanol and cholesterol indicates faecal matter in waters, and a ration greater than 0.2 is considered contaminated water (MOLINER-MARTINEZ et al., 2010). Monitoring ions were reported to be *m/z* 215,355 and 373 (CATHUM; SABIK, 2001). In this study, the first top HRF were *m/z* 215.1794, 373.34628 and 355.3359. The molecular ion appeared as seventeenth higher fragment (388.3699).
- Cholest-7-en-3-ol, (3β)- is a sterol precursor of the cholesterol sinthesis known as lathosterol (LUZÓN-TORO; ZAFRA-GÓMEZ; BALLESTEROS, 2007), and reported to be a compound in tobacco (Rodgman & Perfetti, 2016). NIST library top ions are *m*/*z* 385, 255 and 55. HRMS ions were, according to the abundance, 316.3132, 231.1743 and 161.1324.

5.2.4. Removal comparison of target compounds

The removal of elected candidates was assessed with Equation 2, using the deconvolved peak areas for each compound. This method can be applied when no quantification of compound is made. Although this method has less accuracy than target analysis due to the lack of a standard, it can be used to evaluate a wide range of chemicals, that can provide more information regarding which compounds are present in the samples, especially when evaluating the oxidant efficiency of different technologies to remove micropollutants, as well as to indicate which compound or family is more resistant to degradation.

As to the absence of some compounds after treatment, two main relevant points are worth mentioning: i) some compounds could have actually been removed in its totality, ii) some compounds were not identified after different ozone doses due to the minimum area threshold setting, even though the minimal threshold was set lower than the proposed one. The area threshold parameters could have led to peaks with smaller areas to be undetected. For compounds which are degraded during ozonation, there would be a need of a library containing the possible byproducts and metabolites of such compounds, that could help clarify patterns during the treatment and to better understand the removal pathways.

Removal efficiency have been used to compare two detection methodologies to track wastewater treatment efficiency, such as non-target and target methods to evaluate the efficiency of an advanced oxidation pilot scale reactor to remove organic micropollutants, achieving good correlation values for both methods (PARRY; YOUNG, 2016) and to evaluate the removal of micropollutants in a wastewater treatment plant, where good agreement was also found between the detection of compounds through non-target and target screening methods (LI et al., 2018). In this study, the correlation is expressed using the removal rates from both suspect screening and target analysis (Figure 4). In PPW, for almost all the ozone doses and compounds, suspect screening removal efficiency was higher or equal to target analysis removal, similarly to other studies regarding this type of comparison, in which target analysis presented lower removal rates (28% of contaminants removal rates) than non-target analysis for organic contaminants (Z. Li et al., 2018) In UW, the calculated error

reflected the difference between both detection methods, indicating that the matrix effect could have played a role in the removal and detection of some compounds and the RE value could have been underestimated (LI et al., 2018), such as for DBP and DEP. For other compounds, such as TCIPP and TDCPP, the RE presented higher rates with the suspect screening than for target analysis. Regarding the calculated errors in PPW, the errors increased as the ozone dose increased, except DBP and TBOEP. TBOEP was the most abundant compound, with the concentration reaching 307700 ng L⁻¹. Its absence in the suspect screening identification throughout the treatment can be due to the possible high difference between the initial area (raw sample) and the following areas (treated sample), since the software uses highest point analysis to convolve peak areas, which uses the highest response peak to perform searches on and these identification results are passed through remaining samples in order to save processing time. Even with the changes in the software configuration to tentatively identify peaks with lower areas and some were identified (DMP, which presented low concentration level and were identified through suspect screening is an example), it is yet not clear how this feature affects this type of case, when a compound is abundantly identified and has its area decreased in the following samples.

6. Chapter 2

Target and suspect screening analysis for a wide evaluation of micropollutant removal in wastewater during disinfection with ozone

In this chapter, the removal of micropollutants from secondary wastewater was evaluated. The removal was established for both target compounds (using concentration levels and Xcalibur Software) and for suspect screening elected compounds (using peak areas from TraceFinder 4.1 Software), through equations 1 and 2. Also, the removal was individually assessed for each micropollutant, as well as for the chemical family, comparing it to the disinfection of the wastewaterss by measuring *E. coli* levels.

6.1. Results and Discussion

6.1.1. Physicochemical evaluation of wastewater samples

The physicochemical evaluation presented in Table 11 of both PPW and UW wastewaters before and after treatment gives information concerning the initial conditions and the changes along the treatment. PPW showed initial higher pH, alkalinity, COD and color compared to UW. Different removal rates were observed according to the wastewater. In UW, about 30% of COD removal was observed while in PPW, despite the higher initial COD and alkalinity, the COD removal reached near to 70% after 22.8 mg $O_3 L^{-1}$. Higher removal in PPW for color was also observed. These results can be attributed to the different composition and characteristics of PPW and UW samples since the type of wastewater matrix can interfere on the performance of ozonation by affecting O_3 decomposition and its reaction in water (MALVESTITI; DANTAS, 2018; VON GUNTEN, 2003a).

	PW wastewater							
Ozone dose (mgL ⁻¹)	рН	Total alkalinity (mg CaCO ₃ L ⁻¹)	$\frac{\text{COD}}{(\text{mg } \text{L}^{-1} \text{ O}_2)}$	Color (CU)				
0	8.2 ± 0.3	434 ± 11	147.3 ± 2.8	455±1.41				
7.6	8.5 ± 0.3	374 ± 9	130 ± 15.7	29±0.7				
22.8	8.5 ± 0.4	314 ± 3	66 ± 6	20±2.8				
	UW wastewater							
Ozone dose (mgL ⁻¹)	рН	Total alkalinity $(mg CaCO_3 L^{-1})$	$\begin{array}{c} \text{COD} \\ (\text{mg } \text{L}^{-1} \text{ O}_2) \end{array}$	Color (CU)				
0	6.5 ± 0.2	56 ± 2	71± 8.5	189±2				
7.6	6.6 ± 0.1	64 ± 4	57 ± 1	138±0.1				
22.8	6.3 ± 0.5	48 ± 6	54 ± 2.6	82±0.7				

Table 11. Physico-chemical characterization of PPW and UW before and after ozonation.

6.1.2. Micropollutants removal by chemical family

Using target and suspect screening analysis by GC-OrbitrapMS, a total of 24 compounds were detected in the wastewaters. Target compounds included 6 OPFRs (TBOEP, TCPP, TCEP, TDCPP, TEHP, and TPhP), 1 alkylphenol (NP), 4 phthalates (DBP, DEHP, DMP, DEP). The suspect screening allowed the identification of 7 pharmaceuticals and personal products (benzophenone, cotinine, caffeine, tonalid, triclosan, carbamazepine, pentoxifylline) and 7 biomolecules (androstane-3, 17-dione, (5β) -, cholest-2-ene, (5α) -, Γ -tocopherol, cholestan-3-ol, $(3\beta, 5\beta)$ -, cholest-7-en-3-ol, (3β) -, cholesterol and vitamin E.

Figure 6 presents the concentration of each chemical family for target compounds (sum of all detected compounds separated by chemical family) in PPW and UW wastewater before, during and after treatment with ozone. The initial concentration of each compound was highly relevant when analyzing removal performance since micropollutants in lower concentration tend to be removed slower than ones in higher concentration, and that difference can reflect on the percentage of removal. Without target analysis and quantification, the concentration of micropollutants is not accessed. Besides, the type of wastewater can influence in the scavenging and formation of radicals, also influencing in the removal of micropollutants. Although in PPW the removal was almost 90% after 3.5 mg $O_3 L^{-1}$, in UW the removal was around 40% with the same dose. These two different trends indicate that the efficiency of ozonation for pollutant load), and thus a comprehensive analysis is needed if the intention of the treatment is the removal of micropollutants.

In UW, the concentration ranged between 2 and 580 ng L^{-1} and PPW, it was between 2 and 307700 ng L^{-1} , whereas. This difference in concentration led to a higher removal rate from PPW than from UW. At the first applied ozone dose (3.5 mg L^{-1}) more than 80% of the sum concentration of all detected micropollutants was removed from PPW. However, after that ozone dose, the degradation rate decreased and their concentration remained similar until the end of the reaction time, showing that the concentration of micropollutants is also relevant when comparing removal rates between two wastewaters. Phthalates and OPFR remained at similar concentration even at very high ozone doses indicating that these contaminants are more recalcitrant than others. Contrarily, 4NP (alkylphenols) were highly removed during ozonation in both wastewaters.



GW OPFRs GW Phthalates GW Alkylphenols GW All



■ UW OPFRs ■ UW Phthalates ■ UW Alkylphenols ■ UW All

Figure 6. PPW (up) and UW (bottom) chemical family's concentration along the treatment with ozone.

The ozonation performance for each chemical family was also assessed considering that among the same chemical family; the compounds can have different reactivity with ozone.

6.1.2.1. **OPFRs**

The detected OPFR were TPHP, TBOEP, TDCPP, TCPP, with concentrations ranging from 145 to 307700 ng L^{-1} in PPW and from 20 to 580 ng L^{-1} in UW. The levels in PPW were 160 times higher than in UW. The result showed that ozone
treatment was not sufficient for their total removal. Similar to other chemical families, the removal rates varied significantly depending on the wastewater.

In PPW, at the end of the treatment, the removals were 99, 88, 83 and 72%, for TPHP, TCPP, TBOEP and TDCPP, respectively (Figure 6). In UW, they showed significant resistance to ozonation, especially the chlorinated OPFRs (Figure 4B), which is in agreement with literature (Cristale et al., 2016; Wang et al., 2018). After the maximum dose of ozone (22.8 mg $O_3 L^{-1}$), only 49% of removal was achieved for the 6 OPFR detected in this wastewater, showing that these contaminants are highly resistant to ozonation. During the treatment, as shown in Figure 7, a difference in the decrease of total OPFRs load was observed. Their removal in PPW more significant than in UW due to the higher initial concentration, which led to higher removal rates. As an example, TBOEP initial concentration in PPW was 670 times higher than in UW, and TBOEP reached 640 and 360 ng L^{-1} for PPW and UW at 7.6 mg O₃ L^{-1} , respectively. and 97 and 78 ng L^{-1} for PPW and UW, respectively, at 22.8 mg O₃ L^{-1} . In UW, TCPP showed low removal rate, with the concentration remaining constant after all applied ozone doses and in PPW, TCPP had 20% of the initial load after 7.6 mg $O_3 L^{-1}$ (1800) ng L^{-1}) and more than 80% of removal rate was observed at the end of treatment but yet the final concentration remained at 1100 ng L⁻¹. TPHP showed high removal rates for PPW, but the levels for this compound after 22.8 mg $O_3 L^{-1}$ were of 200 and 250 ng L^{-1} for PPW and UW, respectively, indicating again that the concentration levels are also important when comparing the efficiency of micropollutants removal.



*Initial TBOEP concentration was 100 times higher the showed value for PPW.

Figure 7. Removal of OPFRs from PPW and UW (top) during ozonation and OPFRs concentration PPW and UW (bottom) during ozonation.

6.1.2.2. Phthalates

In terms of phthalate concentration in both wastewaters, the initial PPW was 6.5 times higher than in UW, with levels between 2 and 10900 ng L^{-1} (Figure 3). An ozone dose of 3.5 mg O₃ L^{-1} reduced the PPW phthalate concentration to similar levels found in UW. After that dose, the phthalate level was slowly reduced, depending of the compound and type of wastewater.

The individual removal rates of all detected phthalates during ozonation of PPW and UW are indicated in Figure 8 along with their concentration. In PPW, DBP was the most abundant phthalate, showing 99% of removal with 3.5 mg $O_3 L^{-1}$, going from 10900 ng L^{-1} to 60 ng L^{-1} and being completely removed after 7.6 mg $O_3 L^{-1}$. At 7.6 mg $O_3 L^{-1}$ the removal rates for DEHP, DEP and DMP ranged from 50 to 69%. The behavior of removal efficiency for both DMP and DEP was similar; but DMP initial

concentration was much lower (2 ng L⁻¹) and DEP showed higher initial level (1320 ng L⁻¹) and was not completely removed after all ozone doses, with a final concentration of 75 ng L⁻¹. DEHP had 50% of removal after 7.6 mg L⁻¹ and remained stable until the end of the treatment (22.8 mg O₃ L⁻¹) with levels not exceeding 2000 ng L⁻¹, being the most recalcitrant compound among the detected phthalates. Except for DEHP, the other phthalates namely (DBP, DEP and DMP) were removed with 22.8 mg O₃ L⁻¹.

Phthalates removal in UW had a similar behavior; at 7.6 mg $O_3 L^{-1}$ DEP and DBP were significantly removed, having 99 and 64% of degradation, respectively. DEHP (2350 ng L^{-1}) and DMP (2 ng L^{-1}) showed lower levels of removal during treatment. Low micropollutant concentration can influence the detection and treatment processes (NAS et al., 2017), which may explain the presence of DMP after treatment. As in PPW, DEPH was the most recalcitrant phthalate since it was detected at 51% of the initial concentration at 22.3 mg $O_3 L^{-1}$.



Figure 8. Removal of phthalates from PPW and UW (top) and phthalate concentration in PPW and UW (bottom) during ozonation.

6.1.2.3. Alkylphenol

Fast oxidation of NP by ozone was previously indicated in the US EPA's Drinking Water Treatability Database (US EPA, n.d.). and same behavior was observed in this study. PPW presented higher levels of NP than in UW, as shown in Figure 9, their concentration decreases abruptly after the application of the first ozone dose (3.5 mg $O_3 L^{-1}$), showing a very fast removal. Regarding UW, despite the fact that similar levels of NP were detected in both wastewaters, a higher ozone dose was needed for their complete elimination in UW. In the case of NP, ozonation was highly effective as NP rapidly reacted with ozone and HO• due to their higher reactivity when compared to the other studied compounds (NING; GRAHAM; ZHANG, 2007b, 2007a).



Figure 9. Removal of alkylphenols from PPW and UW (top) during ozonation and alkylphenols concentration in PPW and UW (bottom) during ozonation.

6.1.2.4. Pharmaceuticals and personal care products (PPCPs)

Using the suspect screening approach, the removal of several pharmaceuticals in the wastewaters during ozonation was evaluated. It is important to highlight that only the compounds with moderate polarity were extracted and detected due to the extraction method and instrumental technique used in this work, other more-polar pharmaceuticals usually detected in WWTP samples might be undetected due this methodological limitation. Similar to the other compounds, PPCPs removal rates after application of different ozone doses differed for each compound and dependent on the wastewater matrix (Figure 10). PPW ozonation promoted the complete removal of caffeine, triclosan and pentoxifylline, while cotinine and benzophenone were the most recalcitrant. In UW, the identified compounds were benzophenone, tonalid, carbamazepine and triclosan, which presented also a fast removal, with exception of benzophenone.

PPCPs removal by ozonation has been widely discussed in the last decades. Carbamazepine, a very recalcitrant compound, has shown high susceptibility to be degraded by ozone (up to 77% of removal with 0.2 g O_3 /g TOC) (WILT et al., 2018). Caffeine was completely removed with the same dose of ozone. It has been reported that benzophenone had good degradability by ozonation, with a removal of 95% after 40-50 minutes under 85.7 µmol L_{gas}^{-1} of ozone (GAGO-FERRERO et al., 2013), and with about 5 mg L⁻¹ of ozone, 98% of triclosan is removed in 10 min (ORHON et al., 2017). In this study, about 80% of the initial benzophenone was removed in PPW and about 37% in UW using 7.6 mg L⁻¹ of ozone.



Figure 10. Removal of pharmaceuticals and personal care substances in PPW (left) and UW (right) by ozonation.

6.1.2.5. Biomolecules

The biomolecules cholest-2-ene (5α) -, androstane-3,17-dione (5β) -, cholestan-3ol, $(3\beta,5\beta)$, vitamin E, cholesterol, and Y tocopherol were identified. The removal rates for PPW and UW are presented in Figure 11.



Figure 11. Removal of biomolecules in PPW (left) and UW (right) by ozonation.

As presented, the removal rates for biomolecules were different for each compound. Cholestan-3-ol, $(3\beta,5\beta)$ -, also known as coprostan-3-ol or coprostanol is a sterol (combination of alcohol and steroid) originated in the microbial reduction of cholesterol, and is also used as a marker of human activity (CAIN et al., 2004). Along with cholesterol, it is used as a biomarker of human fecal matter in the environment (MOLINER-MARTINEZ et al., 2010). In UW, cholesterol showed a 75% removal rate after 7.6 mg L⁻¹ and in PPW, the removal of cholesterol reached 99% and cholestan-3-ol, $(3\beta,5\beta)$ - was totally removed ozone for the same applied dose of ozone.

Vitamin E and Γ -Tocopherol were detected in PPW and presented 90 and 100% removal rates after 7.6 mg L⁻¹ of ozone, respectively. For UW, vitamin E removal level for the same dose was similar, reaching 90% of removal. Cholest-7-en-3-ol, (3 β)-, detected in both wastewaters, is a sterol also known as lathosterol, is a cholesterol-like molecule (ChEBI, s.n.), precursor of the cholesterol sinthesis (LUZÓN-TORO;

ZAFRA-GÓMEZ; BALLESTEROS, 2007), and reported to be a compound in tobacco (Rodgman & Perfetti, 2016). This compound did not show any removal with ozonation, being the most recalcitran molecule found in this study.

Androstane-3,17-dione (5 β)-, also known as etiocholanedione, is a natural metabolite of dehydroepiandrosterone produced from cholesterol. It was identified in PPW after the beginning of the treatment and showed initial resistance, being removed with 22.8 mgL⁻¹ of ozone. This compound can also be a result of transformation of β -sistosterol (major plant sterol present in sugarcane) and other sterols by aerobic and anaerobic microorganisms (Chandra and Kumar, 2017; Taylor et al., 1981); this derivate can potentially cause disturbances in the endocrine system and can cause morphological defects such as hermaphroditism. Masculinization of fish and reduction in fish population has also been associated with this compound (Jenkins et al., 2003).

6.1.3. Wastewater disinfection vs. micropollutants removal

Ozonation is an well-known method for disinfection of water and municipal wastewaters and a possible alternative for micropollutants removal, with many uses in WWTP around the world such as Switzerland (BOURGIN et al., 2018), China (LI et al., 2015) Japan, United States and Canada (LOEB et al., 2012). To understand the degree in which micropollutants can be removed along the disinfection by ozone is relevant to understand the potential of this alternative to improve wastewater quality when discarded. Figure 11 presents the percentage of removal for the micropollutants families in relation to the disinfection results for each used ozone dose.

In PPW, the disinfection was achieved with 3.5 mgL⁻¹ of ozone. The pilot plant is used to treat wastewater from a university campus, where no industrial-related activities are performed; therefore, it is less complex wastewater than UW that corresponds to wastewater of 50.000 people. For UW the complete disinfection was not achieved since higher ozone doses were necessary to reach 2 logs of inactivation even though the initial levels of total coliforms and *E. coli* were lower.



Figure 12. Disinfection rates applied and removal (%) of chemical families in PPW (top) and UW (bottom) during ozonation.

For PPW (Figure 12, upper), a removal of micropollutants higher than 62% was reached with 3.5 mg $O_3 L^{-1}$, and after 7.6 mg $O_3 L^{-1}$, more than 80% of micropollutants and *E. coli* (<1 MPN per 100 mL) removals were reached. It is important to point out that even with an ozone dose of 7.6 mg $O_3 L^{-1}$, necessary for disinfection and for a high micropollutants removal, there is still the presence of diverse organic molecules in the wastewaters and also, as aforementioned, some compounds showed resistance to ozonation. At 7.6 mg $O_3 L^{-1}$ in PPW, OPFR and PPCPs had more than 80% of removal while others chemical such as phthalates and biomolecules achieved around 65% of removal. Even if after PPW disinfection with ozonation the organic pollutants were highly removed, there still the potential risks in this treated wastewater due to the remaining compounds and their concentration level. As for disinfection in UW (Figure

12, bottom), the highest level of disinfection was achieved with 22.8 mg $O_3 L^{-1}$. Since most of the compounds showed around 60% of removal, the presence of great part of these compounds was observed at the end of the reaction time. Similar study using the same micropollutants as the representation of industrial contamination (NP, DBP and DEHP) indicated that the micropollutants can act as a scavenger and reduce the inactivation of *E. coli* and total coliforms (MALVESTITI; DANTAS, 2018). In UW, *E. coli* inactivation might have been influenced by the scavenger of ozone, and 2 log of bacteria removal was achieved after 22.8 mg $O_3 L^{-1}$, while a micropollutant removal greater than 50% could only be achieved after 70 min of ozonation (15.2 mg $O_3 L^{-1}$). Thus, these results show that the ozone dose used for disinfection was able to lower the level of micropollutants identified in the samples but not the most recalcitrant compounds such as DEHP, which were present after the end of treatment.

7. Conclusion

GC-Orbitrap-MS was used for suspect screening analysis of a wide range of micropollutants in two secondary samples. The identification of unknown compounds reached a level 2 confidence, since no standards were available in this study to propose confirmation but library spectrum match was achieved. This approach of screening for micropollutants in wastewater is promissing since it saves time and gives a broader data regarding which micropollutants there are in the samples, not depending on a list of the most common compounds used in target analysis since their presence or absence is study-specific and cand change from a location to another. The combination of target and suspect analysis is a major shortcut to identify micropollutants in wastewater wastewaters, saving time, resources and speeding up the process of compound identification. Aditionally, the removal efficiency of these compounds by ozone could also be established, by comparing the initial and final deconvolved areas, even without quantification. Nevertheless, byproducts of the ozonation treatment were not assessed.

As for the TraceFinder 4.1 software, major aspects of the method development were the need of optimization of identification and deconvolution parameters to fully characterize contaminants in water - these parameters also seem to be study-specific, and when optimized, TraceFinder 4.1 provides a wide range of candidate compounds and together with retention time alignment and library spectrum match, proved to be a powerful software to evaluate the presence of suspect compounds across many samples. Adjusting the parameters for a better peak deconvolution outcome can be time demanding, and it dependents on the complexicity of a sample. Also, even with a powerful software such as TraceFinder 4.1, the outcome has to be manually and visually confirmed, mainly the spectra comparison with library search and presence of molecular ion, before a peak can be identified as a suspect compound. Understanding configuration parameters settings and their impact on the deconvolution of peaks is still challenging and there is a need of understanding the interaction between the parameters, peaks and candidate compounds so that peaks are properly identified. Deconvolution parameter set up is crucial for enhanced detectability of contaminants in complex matrices so that the concentration of contaminants can be elucidated.

As for removal efficiency, a total of 14 elected and 11 target compounds had their removal by ozone evaluated during the disinfection of 2 secondary wastewaters with ozone. As for UW, the log level of *E. coli* decreased but was not completely inactivated, although the initial level of *E. coli* was lower and micropollutants were removed in 44%. In this wastewater, the greatest removal was 70%, at 22.8 mg $O_3 L^{-1}$, indicating that micropollutants remained even after ozonation. The ozone dose of 7.6 mg L^{-1} led to almost completely removal of *E. coli* in PPW (<1 MPN) and 79% for micropollutants. Phthalates and OPFRs were the chemical families that showed higher resistance to ozonation and 4NP (alkylphenol) was completely removed in PPW and 96% removed in UW by the end of the treatment. As for the elected compounds, PPCPs showed higher removal rates than biomolecules, for all doses and for both wastewaters. In PPW, PPCPs and biomolecules showed a 95 and 71%, respectively, of removal by the end of the reaction time and for UW, the removal rates were 86 and 58%, respectively.

The evaluation of micropollutants removal during ozonation of WWTP samples showed that the efficiency levels need to consider the initial concentration of micropollutants in each wastewater and wastewater complexity. Some chemical families have demonstrated to be more resistant to ozonation than others, and also behave differently depending on the initial concentration, wastewater matrix and complexity (particles, carbonate, nitrate, etc) that need to be evaluated in further studies to better understand how the wastewater matrix can influence the micropollutant removal and disinfection.

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9. Appendix



Figure A1. Ozone generator and reactor (beginning of the treatment)



Figure A2. Ozone generator and reactor (after treatment)



Figure A3. Example of one of the extration procedures performed