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Disinfection By-products of Emerging Concern in Drinking Water: Monitoring and Hazard Assessment

Raquel Maria Santos Chaves

Orientador (es):

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências e Tecnologias da Saúde, especialidade em Saúde Ambiental

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Thesis outline

This thesis is composed by six chapters. In Chapter I it is presented a general introduction of the background information related with the aim of this thesis, including the current knowledge about water treatment plant (WTP), mass spectrometry-based methodologies applied for the detection of Disinfection By-products (DBPs) in drinking water, and characterization of risk assessment process.

The Chapters II to V represent four different studies made during this thesis project. In chapter II, the characterization of a water supply system based on chemical and microbial parameters is described. Chapter III presents the validation and implementation results of mass spectrometry-based methods for the measurement of the selected DBPs in drinking water. In Chapter IV, a review considering hazard, risk characterization and mode of action of DBPs was made, with a strong focus on identifying and suggesting new research priorities. Chapter V describes a toxicological assessment of target DBPs using an *in vivo* animal model. All these chapters are composed by the full reproductions of the scientific publications. All the studies were performed in collaboration with co-authors. I declare that I have contributed to the development of the ideas in this thesis, to produce and to analyze the data presented and to the writing of all chapters.

This thesis is composed by the following published or submitted manuscripts:

Chaves, R.S., Salvador, D., Nogueira, P., Santos, M.M., Aprisco, P., Neto, C., Cardoso, V.V., Rodrigues, J.E., Carneiro, R.N., 2021. Assessment of Water Quality parameters and their seasonal behavior in a Portuguese Water Supply System: a 6-year monitoring study. *Environmental Management* https://doi.org/10.1007/s00267-021-01572-w (published) - CHAPTER II

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À minha avó,
97 anos de inspiração.

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Resumo

A água é essencial para a vida, promovendo a regulação dos ecossistemas, a manutenção da biodiversidade, a saúde e o bem-estar humanos. A monitorização da qualidade da água é uma ferramenta essencial para a gestão dos recursos hídricos e para a consequente segurança da água potável. O consumo de água potável é essencial para a promoção de saúde. O acesso a água segura é um direito humano básico para a vida e uma componente integrante das políticas em proteção de saúde.

A qualidade e a quantidade dos recursos de água disponíveis para consumo humano estão sob crescente pressão, devido a diversos fatores, como o crescimento demográfico, a variabilidade climática ou a mudança do clima, os níveis crescentes de urbanização e de densificação populacional (alterando o ambiente paisagístico e social) e as diversas atividades antropogénicas. A indústria, a agricultura e o estilo de vida, em constante alteração, desempenham um papel fundamental neste âmbito, desde logo, pela proliferação de xenobióticos com impacte ambiental. Ainda que dependendo do seu percurso, a maioria desses compostos têm como destino final a água, quer através das redes de esgoto, quer através de infiltrações no solo, resultando, na sua maioria, na contaminação dos diversos reservatórios de água, como rios, albufeiras ou aquíferos. O grau de contaminação pode variar sazonal e localmente, dependendo não só das diversas fontes de contaminantes, mas também das características geológicas intrínsecas dos locais. Devido ao aumento dos níveis de poluição ambiental em alguns sistemas, é fundamental avaliar, periodicamente, a qualidade da água, aplicando um conjunto integrado de ferramentas, como a avaliação de risco ambiental e humano, aferindo os impactos nos recursos naturais e na consequente segurança da água potável. Considerando a importância da qualidade da água para consumo humano na promoção da saúde, o tratamento da água representa uma das maiores conquistas do século. Com o tratamento da água, e em particular com o processo de desinfeção, asseguramos a inativação de organismos patogénicos ao longo do sistema de distribuição e eliminamos matéria inorgânica, permitindo ao consumidor o acesso a água segura e de qualidade. No entanto, durante o processo de tratamento da água, por reação do conteúdo a tratar (tal como matéria orgânica natural, fármacos, pesticidas, entre outros), com os agentes químicos e /ou desinfetantes, formam-se diversas espécies de subprodutos de desinfeção (DBPs), com efeitos ainda pouco conhecidos. Dada a sua ocorrência generalizada, alta dispersão e diversidade de vias de exposição, os impactos para a saúde humana e a (eco) toxicidade associada à exposição a DBPs são de particular interesse devido à potencial

carcinogenicidade associada e vários outros efeitos não carcinogénicos, como a disrupção endócrina.

O trabalho desenvolvido no âmbito desta tese teve como principal objetivo melhorar os planos de segurança da água de consumo e produzir novos conhecimentos no sentido de uma avaliação de risco mais abrangente, considerando a ocorrência e toxicidade de DBPs alvo.

No capítulo I é feita uma revisão geral da temática, abordando aspetos como o tratamento da água, a consequente formação de DBPs, as principais metodologias analíticas, para monitorização destes compostos, em água de consumo, e a toxicidade reportada em associação a estes compostos.

No capítulo II, com o objetivo de melhor caracterizar o sistema de abastecimento de água da EPAL- Empresa Portuguesa das Águas Livres, SA, foram avaliados parâmetros de qualidade da água, correlacionando diversos parâmetros microbiológicos e químicos, alguns regulamentados pelo regime jurídico português / UE e outros ainda não regulamentados, mas com relevância ambiental e para a saúde humana, durante um período de 6 anos (2014-2019). Para o efeito, duas Estações de Tratamento de Água (ETA) convencionais, utilizando tecnologias de tratamento de água amplamente implementadas em diferentes regiões do globo, foram avaliadas utilizando uma abordagem integrada a fim de melhorar a compreensão dos processos, avaliar a eficiência do tratamento e a qualidade global da água. Os resultados deste primeiro estudo contribuíram para o estabelecimento de uma estrutura de análise de dados, para avaliar a qualidade do sistema de abastecimento, identificando relações de ocorrência relevantes e fornecendo informações essenciais, que poderão ser utilizadas pelos gestores e decisores na construção de planos de segurança da água mais eficazes e eficientes.

No capítulo III, com o objetivo de caracterizar o sistema de abastecimento da EPAL considerando a presença de DBPs não regulamentados (UR-DBPs), foram desenvolvidas e validadas novas metodologias analíticas, contrariando a escassa informação analítica documentada sobre os compostos em estudo. Para tal, foi utilizada cromatografia gasosa acoplada a espectrometria de massa (GC-MS), para a deteção e quantificação de 15 UR-DBPs alvo, em matrizes de água de consumo. Os compostos alvo incluíram DBPs de 4 classes quimicamente diferentes, nomeadamente nitrosaminas, haloacetonas, aldeídos e álcoois. Considerando as características físico-químicas dos compostos, foram selecionados dois métodos de preparação da amostra distintos. A extração em fase sólida (SPE) foi aplicada para a análise do grupo de nitrosaminas, e a microextração em fase sólida (SPME), para os restantes grupos de DBPs. Após validação das metodologias analíticas, estas foram

aplicados a amostras de água de consumo, provenientes de diferentes pontos do sistema de abastecimento da EPAL- Empresa das Águas Livres, SA, incluindo duas ETA convencionais, com capacidades de produção e localização geográfica distintas. As amostras foram recolhidas periodicamente durante um período de 4 meses. Dos 15 UR- DBPs analisados, 8 deles foram detetados nas amostras de água de consumo, sendo os DBPs mais representativos pertencentes à classe dos aldeídos. Este estudo representa o primeiro contributo na deteção destes DBPs alvo, em matrizes de água de consumo, considerando um dos maiores sistemas de abastecimento público de Portugal.

No capítulo IV, foi feita uma revisão da literatura recente considerando os potenciais efeitos de DBPs presentes na água para consumo humano, particularmente focada em compostos não regulamentados e no seu modo de ação, considerando os dados disponíveis com resultados adversos para a saúde. As principais lacunas de conhecimento neste campo foram identificadas e as prioridades de investigação futuras também foram discutidas.

No capítulo V, a fim de contribuir para a melhoria da avaliação de risco, sete dos UR-DBPs mais prevalentes, considerando a informação disponível na literatura e as deteções analítica, foram selecionados para serem incluídos numa abordagem toxicológica. Neste estudo, a toxicidade dos UR-DBPs alvo, quimicamente diferentes, foi avaliada usando o modelo *in vivo* peixe-zebra (*Danio rerio*). O desenvolvimento embrionário foi avaliado, considerando três parâmetros distintos, a considerar, % de alterações morfológicas, % de mortalidade e % de alterações no comportamento. Neste estudo, observaram-se efeitos toxicológicos no desenvolvimento de *D. rerio*, em níveis ambientalmente relevantes, de 7 UR-DBPs. Com base nos resultados toxicológicos foram calculados valores toxicológicos de LOEC, NOEC, EC10 e EC20. Com base nos efeitos demostrados, o estudo realça a especial atenção que deve ser dada aos UR-DBPs, destacando o 2-EH (EC10 = 0,04 mg / L) e NDMA (EC10 = 0,06 mg / L), uma vez que apresentaram os menores valores de ECs. Os resultados estão em consonância com a crescente alerta das entidades reguladoras face à presença de uma grande diversidade e quantidade de DBPs de cariz emergente na água com potencial toxicológico e para o quais a investigação continuada é fundamental.

Com o objetivo de contribuir para uma melhor compreensão do modo de ação (MoA) destes compostos, extraiu-se o mRNA de vários grupos de embriões de *D. rerio* expostos aos dois compostos com maior toxicidade (NDMA e 2-EH), e efetuou-se o seu transcriptoma. Foi realizada uma análise transcriptómica exploratória de forma a identificar os genes e as vias metabólicas potencialmente alterados, dando os primeiros contributos para melhor entender

os mecanismos de ação e a funções biológicas potencialmente alteradas por estes UR-DBPs alvo.

Até à data de conclusão deste trabalho, não se encontravam publicadas informações sobre percentagens de ocorrência e dados toxicológicos referentes a alguns dos DBPs alvo analisados. No contexto nacional, este estudo representa a primeira contribuição na caracterização e avaliação destes UR-DBPs alvo em água de consumo, considerando um dos maiores sistemas de abastecimento público portugueses. Paralelamente, foi realizada, pela primeira vez, uma análise integrada das principais ETA da EPAL, contribuindo para planos de segurança da água mais eficazes e eficientes.

Palavras-chave: água de consumo; compostos de interesse emergente; subprodutos de desinfeção; avaliação toxicológica; qualidade da água.

Abstract

The access to safe drinking-water is essential to assure health. It is a basic human right and a component of effective policy for health protection. Due to the increase levels of environmental pollution in some systems, it is crucial to assess water quality applying an integrative approach, combining environmental and human risk assessments, attending to surface sources pressure and the consequent drinking water security.

Water treatment plants (WTPs) represent an important instrument to promote the quality of drinking water. Disinfection of water system is an essential strategy to protect human health from pathogens and prevent their regrowth during water distribution, but the reaction of disinfectant agents with organic matter can lead to the formation of disinfection by-products (DBPs). Given their widespread occurrence, potential human health impacts and (eco)toxicity associated with exposure to DBPs are of particular interest due to their potential carcinogenicity and vary non-carcinogenic effects, such as endocrine disruption. The work developed in the frame of this thesis had the ultimate aim to improve water safety plans and produce new knowledge towards a more comprehensive risk assessment, considering the occurrence and toxicity of target DBPs.

In chapter I, a general description of the subject is made, considering aspects such as water treatment, the DBPs formation, main analytical methodologies to measure these compounds in drinking water, and their reported toxicity.

In chapter II, with the aim of better characterizing the EPAL- Empresa Portuguesa das Águas Livres, S.A water supply system, we assessed the water quality parameters, correlating several microbiological and chemical parameters, some regulated under Portuguese /UE legal framework and others still not regulated but with environmental and human health relevance, during a 6-year period (2014-2019). For that purpose, two conventional WTPs with a technology largely implemented in many different regions, were assessed by an integrative approach as a proxy to improve processes understanding, treatment efficiency and global water quality. The outcomes of this first study contributed to the establishment of a framework to evaluate the quality of the water supply system, identifying relevant occurrence relationships, and providing meaningful information that decision makers can use for more effective water safety plans.

In chapter III, we aim to characterize EPAL water supply system attending to some target UR-DBPs and given the limited validated analytical methods to detect and quantify UR-DBPs, a new multi-residue gas chromatography coupled with mass spectrometry

methodologies for the detection and quantification of 15 UR-DBPs in drinking water matrices was validated. The target compounds included DBPs from 4 chemically different classes, namely nitrosamines, haloketones, aldehydes and alcohols. The selected sample preparation methods included solid phase extraction (SPE), applied for nitrosamines group, and solid phase micro extraction (SPME), for the remaining DBPs. The developed analytical methods were applied to drinking water samples from different points of EPAL water supply system, including two conventional WTPs, geographically different, with distinct production capacities, periodically collected during a 4-month period. From the 15 UR-DBPs analyzed, 8 of them were detected in the analyzed drinking water samples, being the most representative DBPs belonging to aldehydes class. To the best of our knowledge, this study represents the first insights on the measurement of some of the target DBPs in drinking water matrices, considering one of the biggest Portuguese public distribution systems.

In chapter IV, we review the recent literature on the effects of DBPs present in water for human consumption, mainly focusing in unregulated compounds and the putative underlying mode of action (MoA), linking the available data with adverse health outcomes. The main knowledge gaps in this field were identified, and future research priorities discussed.

In chapter V, in order to improve hazard and risk assessment, 7 of the most prevalent UR-DBPs were selected to be included in a toxicological approach. In this study, the developmental toxicity of the chemically-different UR-DBPs was evaluated using zebrafish (*Danio rerio*) embryo bioassay as a proxy animal model, and considering morphological abnormalities, % mortality and behavior endpoints. We demonstrate here toxicological effects on *D. rerio* development, at environmentally relevant levels, of 7 UR-DBPs. To gain additional insights into the biological functions and potential pathways disrupted by the exposure to 2 of the most toxic DBPs studied, 2-EH and NDMA, a comprehensive transcriptome assembly was also produced, and an exploratory analysis of the transcriptomic profiles were made.

Before this work, no information was available regarding % of occurrence and toxicological data for some of the target DBPs analyzed. At the national context, it represents the first insights regarding the presence and characterization of target UR-DBPs, considering one of the largest Portuguese public water supply systems. In addition, an integrative analysis of the main WTPs of EPAL was made, contributing to more effective water safety plans.

Keywords: drinking water; emerging compounds; DBPs; toxicological assessment; water quality.

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List of abbreviations

1,1,1-TCA: 1,1,1-trichloroacetone

1,1-DCA: 1,1-dichloroacetone

1,3- DP: 1,3-dichloro-2-propanol

1,3-DCA: 1,3- dichloroacetone

2-EH: 2-ethyl-1-hexanal

2-HN: 2-ethyl-1-hexanol

3-CDMP: 3-chloro-2,2-dimethyl-1-propanol

ACPN: acetophenone

AdP: Águas de Portugal

APOs: adverse outcome pathways

DBPs: Disinfection By-products

DCAN: dichloroacetonitrile

EC10: effective concentration with 10% effect

EC20: effective concentration with 20% effect

ECs: effective concentrations

ECD: electron capture detector

EDCs: endocrine disrupting chemicals

EPAL: Empresa Portuguesa das Águas Livres, S.A.

EPs: Emerging Pollutants

ERA: Environmental Risk Assessment

ERSAR: Entidade Reguladora de Serviços de Águas e Resíduos

EU: European Union

FID: flame ionization detector

GC: gas chromatography

HAAs: Haloacetic acids

HKs: Haloketones

IDL: instrumental detection limit

LC: liquid chromatography

LOD: limit of detection

LOEC: lowest observed effect concentration

LOQ: limit of quantification

MDL: method detection limit

MoA: mode of action

MQL: method quantification limit

mRNA: messenger ribonucleic acid

MS: mass spectrometer

NDMA: N-nitrosodimethylamine

NDPhA: N- nitrosodiphenylamine

NMOR: N- nitrosomorpholine

NOEC: no observed effect concentration

NPIP: N- nitrosopiperidine

NPYR: N-nitrosopyrrolidine

OM: organic matter

PAHs: polyaromatic hydrocarbons

PAM: polyacrylamide

RNA: ribonucleic acid

RSD: relative standard deviation

THMs: trihalomethanes

TNF: tumor necrosis factor

UR-DBPs: Unregulated Disinfection By-products

UV: ultraviolet

WL: watch list

WTP: water treatment plant

WWTP: wastewater treatment plant

CHAPTER I. General Introduction

1. Water quality and human health

Several models have been proposed to describe the social and ecological determinants of health: the ways in which elements of the social, economic, and physical environments interact with individual biological factors and behaviors and shape health status. In terms of physical environment determinants, that influence directly the public health condition, the water quality and sanitation have been highlighted over the years (Evans and Stoddar, 1990). In fact, water and the production technologies involved are actually listed in the environmental, economic and sustainable priorities.

Water is essential for life, promoting ecosystems regulation, biodiversity maintenance and human health and well- being. Water quality monitoring is a fundamental tool in the management of freshwater resources and for the consequent drinking water security.

The quality and available quantity of water resources are under increased pressure due to the demographic growth and population activities, climate change and higher rates of urbanization, changing the social and environmental landscape (EEA, 2019). The anthropological pressure plays an important role in this topic, attending to the impact of activities such as lifestyle, industry, veterinary and agriculture, increasing the load of xenobiotics in the environment. Most of these xenobiotics can reach the environment through sewer or soil infiltration, but in the end, they will play an important role in water contamination, arriving to water reservoirs, such as rivers or aquifers. The contamination type could be dependent of local climate conditions, geographical and geological characteristics and type of water source. All these variables could change the water source quality, seasonally and locally (Srebotnjak et al., 2012). The water sources can include different origins such as ground and surface waters, being the later the most abundant resource (WHO, 2017).

Contaminated water may contain pathogens such as bacteria, viruses or protozoa, or chemicals such as heavy metals, polyaromatic hydrocarbons (PAHs), toxins, pesticides and herbicides, pharmaceuticals, endocrine disrupting chemicals (EDCs), microplastics and even disinfection by-products (DBPs) (COM, 2000; WHO, 2017).

The exposure to contaminated water could be through direct water ingestion, or recreation use through adsorption or inhalation during activities such as swimming. This exposure may cause several diseases, including diarrhea, cholera, dysentery, typhoid, among others

(WHO 2017; Teixeira et al. 2020). Chronic exposure can cause several adverse effects like cancer and vary non-cancer effects such as endocrine disruption, birth defects, neuronal problems, among others (Ding et al., 2017; Holmes et al., 2017; Richardson et al., 2015).

Due to the diversity and vast degrees of contamination during urban water cycle, it is crucial to improve risk assessment to support more effective regulatory limits and human health and ensure that water for human consumption present high levels of security and quality. In accordance, water treatment plant (WTP) is the key tool to guarantee safe, clean and affordable drinking water (WHO, 2019; Teixeira et al. 2020). Towards obtaining a more sustainable urban water cycle, it is essential to achieve an efficient removal or inactivation of potential contaminants that result from the different human activities. Wastewater treatment plants (WWTPs) play a crucial role in chemical contaminant and pathogen removal in the treated effluent, ensuring a safe environmental water reuse.

In line with the water quality agenda, the European Union (EU) Water Framework Directive (2000/60/EC), published in 2000, established provisions for a list of Priority Substances and environmental monitoring of surface water bodies. Following, in 2013, EU directive 2013/39/EU established a list of the priority substances to be monitored, including pesticides and herbicides, endocrine disrupting chemicals (EDCs), polyaromatic hydrocarbons (PAHs), among others. A watch list (WL) mechanism was also proposed (2015) as a guideline of substances, such as pharmaceuticals and hormones, for which data should be obtained to support future prioritization decisions (Carvalho et al., 2015). In 2020, the EU published the third Watch List, which repealed the 2st WL(2018) (Loos et al., 2018). This review, identifies more than 20 compounds or group of compounds taking in consideration the current state of knowledge on the reported occurrence and concentrations of the respective chemicals (Cortes et al., 2020). This watch list mechanism was introduced also in the European Directive on water quality for human consumption (2020), addressing the presence of emerging compounds in the supply chain and the growing concern about the effects on human health through the water use (EU, 2020).

Since 2010, the United Nations General Assembly recognized the access to safe and affordable water and sanitation as a human right, defining also that everyone has the right to get sufficient, continuous and physically accessible water, in acceptable characteristics for the cultural context (WHO, 2019). However, according to the World Health

Organization (WHO) in 2017 only 71% of the global population had access to safe and contamination-free drinking water. About 785 million people have no access to potable water service and at least 2 billion used a source of contaminated water (WHO, 2019). Thus, the correct management of these water bodies and the monitoring of the higher diversity of contaminants is of extreme importance (Behmel et al 2016; Dunca, 2018; WHO, 2019).

More recently, the sixth objective defined on the Global Sustainable Development Goals of the United Nations for 2030, also reinforces the need of ensure availability and management of water and sanitation for all.

2. Risk assessment

Risk assessment is an important tool to estimate the nature and the probability of adverse effects at an organism level, biological system or population, through exposure to certain environmental stressors or xenobiotic, taking in consideration the intrinsic characteristics of the agent and the organism (Haas and Eisenberg, 2001). Environmental Risk Assessment (ERA) is necessary to access and characterize the interactions between contaminants, humans and ecological resources. ERA is an important and comprehensive tool based on human population description, combined with environmental characterization, contaminants and exposure evaluation. Thus, the main stages of ERA include hazard identification, problem formulation, analysis and risk characterization. It is also important to define uncertainties, to manage correctly the risk, and the effective communication of the information. Toxicological properties are defined as hazard endpoints by which we can measure or assess the adverse effects of substances or chemicals on human health (Klaassen and Watkins, 2015). At this point, it is important to distinguish hazard and risk.

Risk is broadly defined as a potential harmful consequence of an action, behavior or condition. It involves the evaluation of the hazard's characteristics and the exposure conditions.

A Hazard represents a chemical, physical or biological substance that has the potential to produce harm to health. If the substance is present in the environmental, it comes in contact with individuals or populations through the exposure vias, such as ingestion, absorption or inhalation. The hazardous properties of an environmental agent are defined

in accordance with their nature and severity of its harmful consequences. Risk is usual measured according to the equation: Risk= probability of exposure x severity of consequences (EPA, 2019).

In the risk management process, three activities should be collectively comprise in order to effectively support the decision: risk estimation, risk evaluation and risk control.

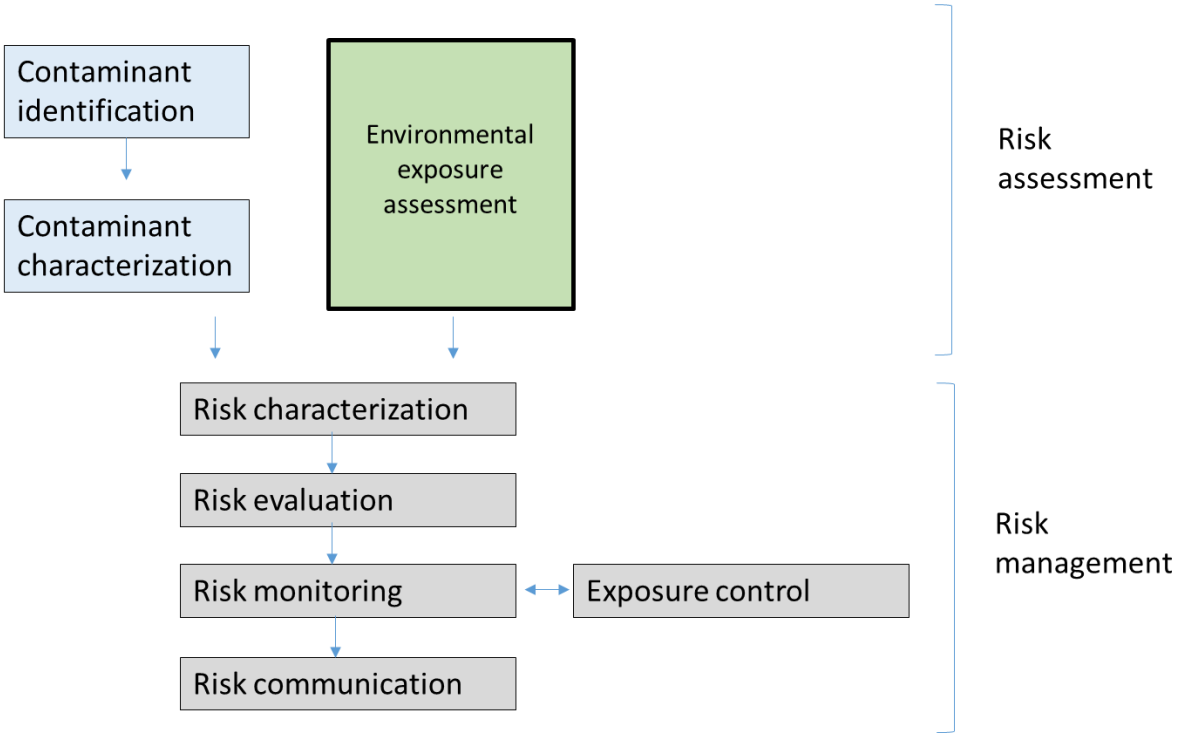

Figure 1- Risk process framework. Adopted from EPA, 2019

Human Health Risk Assessment address questions, such as: What is the chance that people will experience health problems when exposed to different levels of environmental stressors?; what types of health problems may be caused by environmental stressors such as chemicals and radiation?; Is there a level below which some chemicals don't pose a human health risk?; What environmental stressors are people exposed to and at what levels and for how long?; Are some people more likely to be susceptible to environmental stressors because of factors such as age, genetics, pre-existing health conditions, ethnic practices, gender, etc.?; Are some people more likely to be exposed to environmental stressors because of factors such as work place, where they play, what they like to eat, etc.?. The answers to these types of questions involve a large effort of scientific researchers and risk assessors in order to help decision makers, (EPA, 2019).

The concepts of precaution and prevention are the most important aspects related with public health protection and risk definition. The precautionary principle has become a guiding principle in modern thinking in environment and health. This principle addresses uncertain risks and seeks to shift the ways in which science informs policy and regulatory makers supporting a strategic response of precaution in contrast with a strategy of reaction. Taking together different approaches such as health risk assessment and environmental risk assessment, precaution provides a useful means of guiding public health decisions under conditions of uncertainty and also addressing essential issues such as social equity and dignity (WHO, 2003). Actually, substantial evidence supports the conclusion that environmental health risks result from complex interactions among genetics, nutrition, behavior, environmental and socioeconomic factors. In this way, precautionary principle should be used to encourage research, innovation, and contributing to solve complex cross-disciplinary problems.

3. Water treatment plant

Disinfection of water supplies is among the most successful public health measures ever implemented to control pathogens and protect the populations from infectious waterborne diseases. Contamination by sewage or animal feces is one of the greatest dangers associated with drinking water, by the presence of causative organisms of many communicable diseases. Due to the technical advances, millions of people worldwide receive quality drinking water every day from their public water systems (WHO, 2004).
While disinfectants (chlorine, chlorine dioxide, chloramines and ozone) are effective for killing harmful microorganisms in drinking water, their highly reactive oxidizing nature causes interaction with organic and/or inorganic substances naturally present in most source waters (rivers, lakes, and many groundwater sources) (WHO, 2011).
Globally, it is essential that water treatment plants be designed based on a full investigation of site conditions, including chemical and microbiological analysis of the water to be treated, and on a risk assessment process with laboratory (or pilot scale tests) to determine the effectiveness of the process and the chemical dosing requirements. These requirements may vary according to seasonal events, such as rainfall, catchment activity, water flow or others. Although monitoring may indicate that the water is bacteriologically safe for most sampling campaigns, it is essential the continuous assessment of

bacteriological challenges, due to the potential environmental variations (Zhou et al., 2014). In practice, this means that many supplies will require a disinfection stage unless the supply can be shown based on risk assessment and frequent surveillance to be likely to be consistently pathogen free (EPA, 2011).

At the water treatment plant level, it is important to choose equipment, suppliers and consultants carefully. For water suppliers, it is crucial to ensure the ongoing management and maintenance requirements of all the equipment and treatment process levels, taking care of the total variations of raw water (Bartrand-Krajewski et al., 2000; Mian et al., 2018). For some contaminants, potentially several techniques could be appropriate but cheaper and simpler alternatives may be just as effective in particular cases. It is likely that a combination of processes will be required to deal with the majority of raw waters, for example filtration followed by UV are commonly used to remove particles and inactivate microorganisms (EPA, 2011).

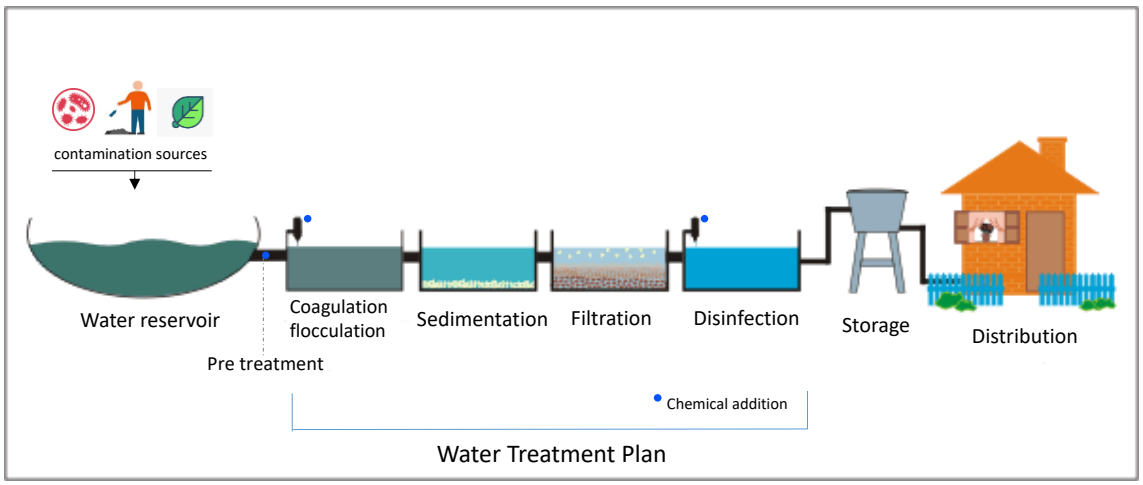

Figure 2- Representation of the different steps involved in a common/conventional water treatment plan. (EPA, 2011)

Substances in water which affect its appearance, odor or taste may lead to consumer's rejection. Microorganisms can be associated with particles and turbidity in water, physical contamination may also represent a health risk as it makes disinfection more difficult. Most treatment systems are designed to remove microbiological contamination and those physical constituents, such as suspended solids (turbidity) that affect aesthetic acceptability, preventing at the same time the effective disinfection. A final disinfection stage is always included at the end of the treatment process to inactivate any remaining

pathogens. When a persistent disinfectant, such as chlorine, is applied this also provides a residual disinfection that will act as a preservative to prevent biological regrowth during storage and/or distribution systems (EPA, 2011; Li et al., 2019).

Treatment processes are based on the physical removal of contaminants through filtration, settling, by some form of chemical addition, or biological removal of microorganisms. Usually, treatment consists of a number of stages, with initial pre-treatment by settling or pre-filtration, pre-oxidation, filtration followed by chlorination. This is called the multiple barrier principle approach (EPA, 2011). This approach is an important concept as it provides the basis for effective treatment of water and allows each individual process stage to treat water to a suitable quality for subsequent downstream process stages (e.g., filtration can prepare water to ensure it is suitable for UV disinfection). The multiple barrier principle applies throughout the supply from catchment all the way to the consumer's tap, reinforcing the importance of all process steps in the effectiveness of treatment. Proper selection and protection of water sources are of prime importance in the provision of safe drinking water. It is always better to protect water from contamination than to treat it after it has been contaminated. Effective source protection, careful choice of water intake, well designed processes and maintained infrastructures are all important variables for the multiple barrier approach (EPA, 2011).

Coagulation and flocculation are used to remove color, turbidity, algae and other microorganisms from surface waters. The addition of a chemical coagulant to the water causes the formation of a precipitate, or floc, which entraps these impurities. The floc is separated from the treated water by sedimentation and/or filtration, although flotation processes may be used in place of sedimentation (WHO, 2004). Some of the most commonly used coagulants are aluminum sulphate and ferric sulphate, although other cationic polymers such as polyacrylamide (PAM) and polyDADMAC have also been used. The coagulants dosed rate is determined by raw water quality in the mixing tank or flocculator. Usually, the coagulant is rapidly and highly dispersed on dosing by adding it at a point of high turbulence. The water is allowed to flocculate and then passes into the sedimentation tank to allow aggregation of the flocs, which settle out to form sludge. This sludge will need to be periodically removed. The major advantages of coagulation are the time required to reduce the suspended solids and the effective removal of fine particles that are otherwise very difficult to remove. Thus, coagulation can be effective in the removal of many protozoa, bacteria and viruses. The principal disadvantages of using coagulants during the water treatment is the potential formation of chemical by-products,

the accurate dosing and frequent monitoring and the cost, especially in small supplies (Postigo and Richardson, 2014). The efficiency of the coagulation process depends on the raw water properties, the coagulant type and operational factors including mixing conditions, temperature, coagulant dose rate and pH value (Chen et al., 2017; Z. Li et al., 2017; Yang et al., 2018).

Sedimentation may be also used to reduce turbidity and solids in suspension. Sedimentation tanks are designed to reduce the velocity of water flow in order to place the suspended solids under gravity. Without the aid of coagulation step, sedimentation will only remove large or heavy particles. Sedimentation tanks require regular cleaning, to maintain the best performance. The process of organic matter removal can be also made using filtration of waters with screens, gravel filters, slow sand, rapid gravity filters or cartridge filters (WHO, 2011). Depending on the water source origin, the inorganic content may represent different problematics and approaches to control the water treatment plan parameters. In groundwater, iron is usually present as dissolved ferrous compounds, for example. To remove iron in this form, it is necessary to oxidize ferrous iron, usually by aeration, to the insoluble ferric hydroxide and to remove the precipitated material in a subsequent filtration stage. In surface waters, iron and manganese are usually present in their oxidized forms and are associated with the suspended solids, which can be removed by filtration. Where coagulation is used for the removal of color and turbidity, iron removal may be achieved simultaneously. Iron and manganese may be combined with organic matter in very stable forms. The usual treatment in this case is coagulation followed by oxidation with chlorine and filtration. Nitrate removal is usually achieved by ion-exchange. Water is passed through a column of synthetic resin beads that remove anions including nitrate and exchange them for equivalent amounts of chloride. Nitrate-selective resins preferentially remove nitrate and also add less chloride to the treated water because of the lower sulphate removal (WHO, 2000a; Yang et al., 2018).

3.1. Disinfection

Surface waters including feeding springs and shallow wells may contain between a few tens of *Escherichia coli* per 100ml in a source derived from a protected upland catchment to many thousands of *E. coli* per 100ml in a source derived from a lowland river containing treated sewage effluents (WHO, 2000b). Groundwater is generally less microbiologically active, although contamination may occur through geological features

like swallow holes, fissures or through poor construction and protection of borehole headworks. In general, microbiological contamination in drinking water may include pathogens such as bacteria, viruses or protozoa increasingly released from the different human activities and animal sewage. Actually, the regular water quality monitoring plans includes the assessment of microbiological indicators such as total coliform bacteria, fecal coliforms, their subgroup *E. coli*, and intestinal enterococci. The occurrence and seasonal behavior profiling of these parameters are crucial to establish more effective water quality plans. Due to the pertinence of this topic, a comprehensive characterization of water quality parameters along the water supply system and the identification of potential correlations between them were described in Chapter II, using the EPAL Water Supply System as study model. Considering the microbiological parameters, the vast majority of surface water samples studied presented high occurrence percentage (above 98%) of microbial contamination, being Coliforms bacteria the most representative group.

Considering recent results of WISE Water Framework Directive database (2019), which presented data from the 1st and 2nd River Basin Management Plans reported by EU Members States, the area of Tagus River was identified as the most polluted area in Portugal. The awareness increases since Tagus River is one of the major Portuguese surface water sources, being an important sub-system for drinking water production to supply Lisbon region. In Portugal, around 68% of the water used in 2018 was from surface water source. There are 295 surface water sources and 3601 treatment sites, of which 269 are conventional WTPs and 3332 are treatment facilities that only perform disinfection and/or aggressiveness correction operations (ERSAR, 2019). Treated drinking water reaches about 96% of the national consumers and, regarding the compliance of water quality standards established within the Portuguese and UE, the drinking water was classified as of excellent quality with 98.6 % of compliant results.

Consistently, the results presented in Chapter II, showed a significant decrease in occurrence and counts of microorganisms during the water treatment step, confirming the effectiveness of the process.

Considering the contamination scenarios and the increasing environmental pressure it is essential to ensure the efficient water disinfection, leading to the inactivation of pathogens in water for human consumption, without neglecting the need to better understand their differential contribution for DBPs formation.

Several disinfection methods are used in water treatment. Disinfection with chlorine is the most widely used method for large water supplies but its application is less common in small supplies (EPA, 2011; Postigo and Richardson, 2014; Simard et al., 2018; Villanueva and Cordier, 2015).

Different microorganisms have different susceptibilities to disinfectants, and disinfectants vary in their potency. The disinfection efficiency is affected especially by disinfectant concentration and contact time, and also by original properties of raw water and consequent affinity to treatment, pH and temperature. The exposure to disinfectant called Ct (in mg/L. min) is commonly the product of disinfectant concentration C (in mg/L, measured at the end of the contact period) and time (t in minutes) and is commonly expressed by following: $Ct = C \times t$.

Values of Ct can be useful to compare the efficiency of disinfectants. Lower Ct values means that stronger and more effective the disinfectant is to neutralize the pathogens and clean the water. The Ct value can also be used to rank the relative susceptibility of different microorganisms; higher Ct values are necessary to kill the more resistant microorganism.

Ultraviolet (UV) irradiation is the preferred method for disinfection of small supplies with small distribution networks or retention time. However, chlorination may be more suitable for larger schemes in which it is necessary to maintain a residual disinfectant during storage and distribution. UV disinfection efficiency is particularly affected by water quality and flow rate. The water to be disinfected must be of good quality and particularly low in color and turbidity. Disinfection will only be effective provided that a sufficient dose of UV is applied and is dependent of microorganism susceptibility and pre-filtration is almost required prior to UV treatment (EPA, 2011). Color and turbidity will both affect radiation intensity in the reactor and turbidity may protect microorganisms from the radiation, decreasing the effectiveness of disinfection. The principal disadvantage is the absence of any residual effect during storage and distribution system, promoting the potential regrowth of pathogens. Advantages includes short contact time and the low amount of disinfection byproducts with significant effect to health.

Chlorine, whether in the form of pure chlorine gas, sodium hypochlorite or calcium hypochlorite, dissolves in water to form hypochlorous acid (HOCl) and hypochlorite ion

(OCl^-). For example, chlorine gas dissolves rapidly in water, initially forming hypochlorous and hydrochloric acids:

$$Cl_2 + H_2O \Leftrightarrow HOCl + HCl$$

Hypochlorous acid is a weak acid which undergoes partial dissociation to produce a hydrogen ion (H^+) and a hypochlorite ion (OCl^-):

$$HOCl \Leftrightarrow H^+ + OCl^-$$

The total concentration of chlorine, hypochlorous acid and hypochlorite ions is referred to as the free available chlorine. If ammonia is present in raw water, the hypochlorous acid can react to produce chloramines (WHO, 2000b, 2000c). The total concentration of the chloramines and any organic nitrogen chlorine containing compounds is referred to as the combined available chlorine. Combined available chlorine is a less powerful disinfectant than free available chlorine but gives a more persistent residual. The formation of combined chlorine is due to a sequence of reactions whereby hydrogen in ammonia is progressively replaced by chlorine as follows:

$$(1)\ NH_3 \Rightarrow (2)\ NH_2Cl \Rightarrow (3)\ NHCl_2 \Rightarrow (4)\ NHCl_3$$

(1) Ammonia (2) Mono-dichloramine (3) Dichloramine (4) Chloramine trichloride

If a large chlorine dose is applied (relative to ammonia), as is practiced in breakpoint chlorination, then nitrogen is formed.

$$2NH_2Cl + Cl_2 \Rightarrow N_2 + 4HCl$$

The effectiveness of chlorine for disinfection depends on the form of chlorine, its concentration and the contact time. Hypochlorous acid is a more powerful disinfectant than the hypochlorite ion and chlorination is usually practiced at values of pH favorable to its formation. The World Health Organization recommends that for the effective disinfection of drinking water “the pH should preferably be less than 8.0 and the contact

time greater than 30 minutes, resulting in a free chlorine residual of 0.2 to 0.5mg / L"(WHO, 2011, 2000b, 2000d).

Chlorination processes need to be carefully controlled in order to minimize the formation of taste and odor forming compounds. There may also be a need to control the formation of disinfection by-products including THMs and a vast amount of emerging ones (Mian et al., 2018; Richardson, 2011).

3.2. Chlorine sources

Chlorination can be achieved by using liquefied chlorine gas, sodium hypochlorite solution or calcium hypochlorite granules. Chlorine gas is very reactive and highly toxic and must be carefully stored and handled (WHO, 2011). It is used for treatment of large public supplies but the inherent danger of using chlorine gas has resulted in an increased use of sodium hypochlorite or the electrolysis of brine (electro-chlorination) as alternative sources of chlorine.

Several regimes of chlorination can be used, including marginal (simple) chlorination, breakpoint chlorination, superchlorination/dechlorination and chloramination (Aragone et al., 2012; Weinberg et al., 2006). On small supplies, it is probable that only marginal chlorination would be used in most cases. Marginal chlorination involves the dosing of chlorine to produce a suitable residual free available chlorine concentration. Sufficient chlorine is added to exceed the demand for chloramine production and to ensure a free available chlorine residual (Li et al., 2019). The chlorine dose must be carefully controlled to avoid forming dichloramine and nitrogen trichloride which can cause taste and odor problems. Breakpoint chlorination requires a dose of around 10 mg/l chlorine dosed per mg/l ammonia removed. The actual dose depends on water quality and has to be determined for each water. The resultant free available chlorine residual should remain in the range 0.2 to 0.6mg/L (DL152, nation law).

Chlorine residual control is the most common method of control the way in which chlorine is dosed continuously into the water. If the quality of the water and hence the chlorine demand varies appreciably, it is necessary to use a control system to maintain a constant chlorine residual. The signal from the chlorine analyzer system is used to adjust the chlorine dose thus maintaining the required residual chlorine concentration. Key challenges have included the maintenance of stable concentrations of disinfectant

residuals and the control of disinfection by-products that may form as a consequence of residual decay processes also during distribution (Li et al., 2019).

3.3. Chemical control during treatment

3.3.1. pH

The pH value of water may need to be adjusted during treatment and before distribution for several reasons, including:

- to improve the effectiveness and efficiency of disinfection
- to control corrosion in the distribution system and consumers' installations
- to ensure the final water quality standards
- for removal of water hardness, inorganic elements such as iron and manganese and some metals.
- to facilitate the removal of turbidity by chemical coagulation

Many raw surface waters are slightly acidic and coagulation processes further increase acidity. In these cases, an increased pH can be achieved removing the excess carbon dioxide by aeration, dosing with sodium hydroxide, calcium hydroxide or sodium carbonate or passing the water through a bed of alkaline medium.

Where necessary, reduction of pH can be achieved by dosing with a suitable acid such as sulphuric acid, hydrochloric acid, sodium hydrogen sulphate or carbon dioxide (WHO, 2004).

3.3.2. Corrosion

High chloride concentrations and chloride to bicarbonate ratios are associated with increased corrosion of certain metals. Corrosion consists in the dissolution of the materials constituting the treatment and supply systems, tanks, pipes, valves, and pumps. It may lead to structural failure, leaks, loss of capacity, minimizing the chemical and microbiological water quality. The internal corrosion of pipes and fittings can have a direct impact on the concentration of some water constituents, including lead, copper and nickel. Corrosion control is therefore an important aspect of the management of a water

supply system. The corrosion control involves many parameters, including the concentrations of calcium, bicarbonate, carbonate, and dissolved oxygen, as well as pH. The detailed requirements differ depending on water quality and for each distribution system material. (WHO, 2004).

3.3.3. Tap water taste and odor

Taste and odor can be removed by several methods, including aeration, ozonation and adsorption on activated carbon. The method used will depend on the source of the taste and odor. Adsorption on activated carbon is generally the most effective method for the removal of earthy or moldy taste and odor. However, activated carbon is an ideal medium for the accumulation and growth of microorganisms. Activated carbon removes chlorine from the water but bacterial growth can occur even on filters treating chlorinated water. Thus, there is concern that direct consumption of water from activated carbon devices may cause health problems due to bacteria released into the water. Bacteria could be inhaled when dispersed by aerosols, for example during washing, and this could also be harmful.

4. DBPs formation, toxicity and risk characterization

It was in the early 1970s that scientists first became aware of DBPs. In 1974, Rook and Bellar (Bellar, 1974; Rook, 1974) reported the identification of the first DBPs in chlorinated drinking water; chloroform and other three trihalomethanes (THMs), bromoform, bromodichloromethane and dibromochloromethane (Hrudey, 2009; Richardson, 2011; Richardson and Postigo, 2011). In 1976, the U.S. Environmental Protection Agency (U.S. EPA) published the results of a national survey that showed that chloroform and the other THMs were very common in chlorinated drinking water. In the same year, the U.S. National Cancer Institute published a report that chloroform was carcinogenic in laboratory animals. Also, the first reports appeared in the late 1970s showing that organic extracts of drinking water were mutagenic in the Salmonella mutagenicity assay (Richardson et al., 2007). Based on these observations, an important public health issue was recognized, since DBPs may cause developmental, reproductive and carcinogenic effects (Florina et al., 2020).

Disinfection By-products (DBPs) are formed during water treatment, through the reaction of chemical agents with organic matter (OM), inorganic constituents and a vast diversity of anthropogenic contaminants, presents in raw water. These occur particularly in the disinfection and coagulation steps, with the addition of oxidant agents, such as chlorine, chlorine dioxide, chloramine, and ozone, with the purpose of inactivate microbial organisms and coagulate the inorganic content. The characteristics of OM, temperature, pH, disinfectant/coagulant type and applied dose, and disinfectant residual stability in drinking water distribution system were found to be among the most important factors to understand DBPs formation (Chaukura et al., 2020; Li et al., 2019). Most DBPs could be defined as emerging pollutants (EPs).

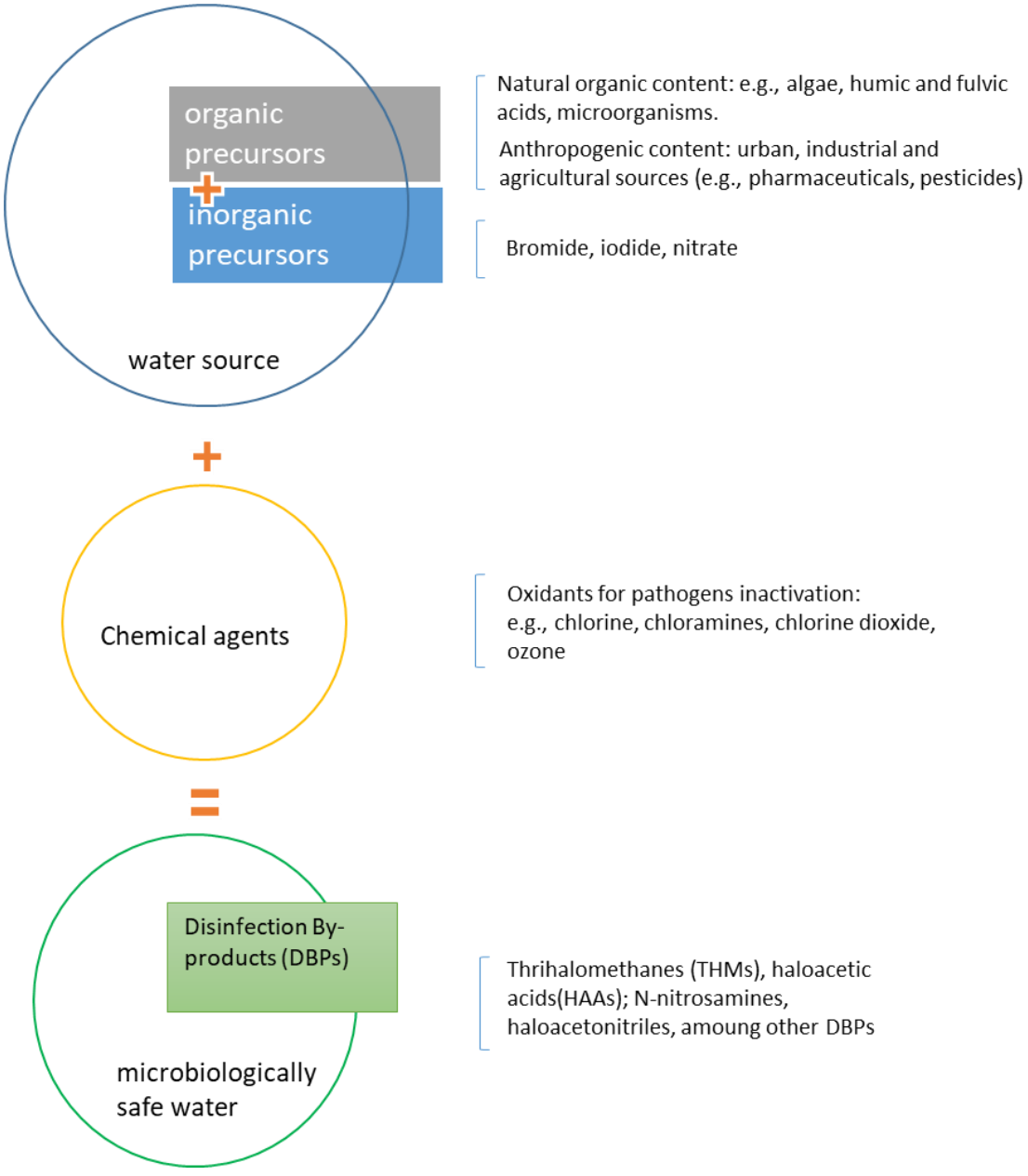

Figure 3- schematic summary of DBPs formation, during water treatment plant

Several reactions for the DBPs formation have been broadly described (Ding et al., 2017; Richardson, 2011; Richardson et al., 2015; Yang et al., 2018). Depending on the type of disinfectant used, dose, pH, temperature, and contact time applied, different types of DBPs are formed. Example of DBPs formation is presented in figure 4, highlighting individual species of both regulated and nonregulated DBPs formed by the reaction of some water contaminants and the disinfectant agents. Chloroform (CF) is one of the most reported THM and their formation mechanism has been broadly described, attending to the many potential water contaminant precursors. N-nitrosodimethylamine (NDMA) (nitrosamines group) and dichloroacetonitrile (DCAN) (haloacetonitriles) are unregulated DBPs with emerging interest.

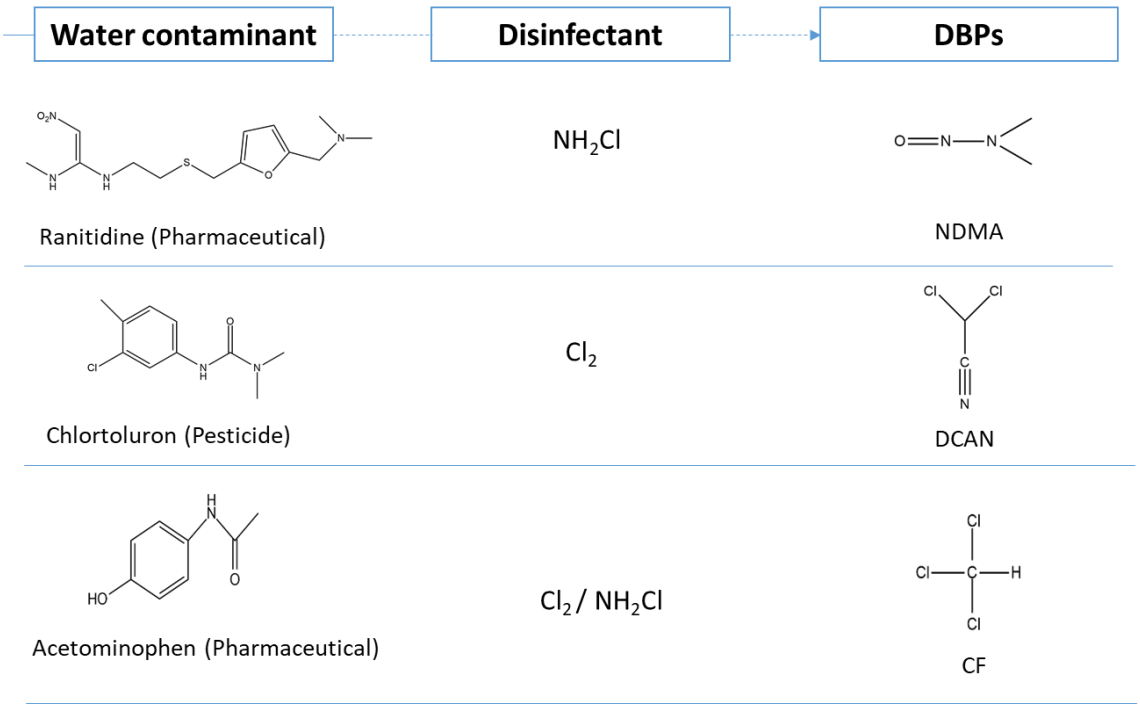

Figure 4-Examples of different regulated and unregulated DBPs formation (CF, DCAN and NDMA) by the reaction of different water contaminant, Acetaminophen, Ranitidine (pharmaceuticals) and Chlortoluron (pesticide/herbicide) with the disinfectants, chloramine and chlorine. Acetaminophen is a broadly used analgesic and antipyretic medication. Ranitidine belongs to a group of drugs called histamine-2 blockers It is commonly used in treatment of ulcer disease or gastroesophageal reflux disease. Chlortoluron is a widely used herbicide to control broadleaf and annual grass weeds. Adapted from (Richardson et al., 2015).

Nitrosamines were considered a DBPs class in 2002 (Choi and Valentine, 2002; Mitch and Sedlak, 2002) and have been of significant interest ever since because several, including N-nitrosodimethylamine (NDMA), are known carcinogens (Richardson et al., 2007). Nitrosamines are a group of DBPs with potential interest in the context of this project, mostly considering the toxicological potential and the non-regulated status. Considering the diversity of the disinfectants applied during water treatment, diverse water technologies used and the quantity and quality of the precursors, many DBPs formation mechanisms can occur. NDMA is generally found at highest levels in chloraminated drinking water, although ozonation and chlorination can also form these and other nitrosamines. In this last case, nitrite and other nitrogen precursors such as natural ammonia in the water source, nitrogen-containing coagulants or ion-exchange resins used in the water treatment process can promote a NDMA production. Amino acids and hydrophilic/low molecular weight dissolved organic nitrogen, shampoos, laundry detergents, dish washing liquids, and fabric softeners, personal care products, as amine-

based pharmaceuticals, can also serve as nitrosamine precursors (Krasner et al., 2013; Richardson et al., 2015).

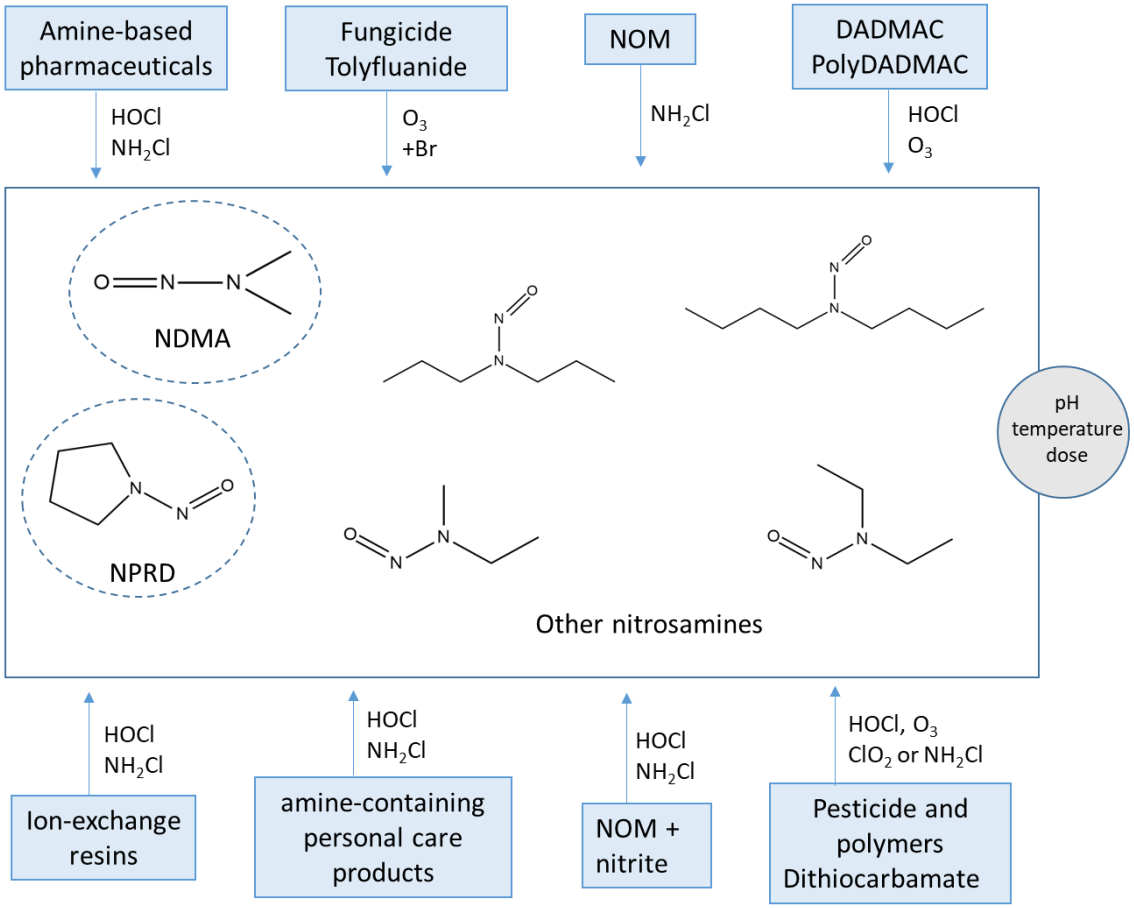

Figure 5- Nitrosamine's formation mechanisms, attending to the different variables during water treatment. Adapted from (Richardson et al., 2015)

Given DBPs widespread occurrence, there are strong evidences of potential human health impacts that include carcinogenic and vary non-carcinogenic effects, such as endocrine disruption. Understanding the public health implications of this emerging issue is crucial for societies and decision-makers, supporting more effective water safety plans.

Humans are mostly exposed to water DBPs through direct ingestion of water, via inhalation and dermal absorption while showering, bathing and swimming in treated pools, since DBPs are (semi-) volatile and skin permeable.

Toxicological studies, using cell cultures and animals, suggested potential genotoxicity, cytotoxicity, carcinogenicity, teratogenicity and endocrine disruption associated with the chronic exposure to several DBPs, depending of the origin of water, disinfection

procedure and disinfectant nature (table 1) (Cortés and Marcos, 2018; Dong et al., 2017; Hanigan et al., 2017; Richardson et al., 2007). Epidemiological studies involving exposure to DBPs reported an association with cancer and non-cancer outcomes, i.e., bladder, lung and colon cancer, birth defects, asthma and skin rashes. These outcomes were associated with different contact routes such as respiratory, gastrointestinal tract and dermal contact. Most biological samples used in the former studies included blood, urine and exhaled bread (Allen et al., 2017; Cantor et al., 2010; Rahman et al., 2010; Tardiff et al., 2006; Villanueva and Cordier, 2015; Wright et al., 2017). Table 1 summarizes toxicological effects listed and disinfectant sources of some of our target DBPs classes.

Table 1- Toxicity, disinfectant sources, and potential health effects of target DBPs classes. Retrieved from (Florina et al., 2020)

DBPs classes	Recognized as EP´s	Disinfectant precursor				Toxicity
		Cl	ClO_2	NH_2Cl	O_3	
Total Trihalomethanes (TTHMs or THM4) Bromodichloromethane Bromoform Dibromochloromethane Chloroform	yes	x	x	x		Possible carcinogenic for liver, kidney, intestine; central nervous and reproductive system diseases; also causing bladder, colon, rectal, or pancreatic cancer.
Unregulated THMs Dibromomethane Bromochloromethane Tetrachloromethane Dibromodichloromethane	yes	x		x		Possible carcinogenic for liver, kidney, intestine; central nervous and reproductive systems diseases.
Iodo-THMs	yes					Possible genotoxic and cytotoxic effects, causing bladder cancer and endocrine diseases
Haloacetic acids (HAAs or HAA5) Dichloroacetic acid Trichloroacetic acid Monochloroacetic acid Monobromoacetic acid Dibromoacetic acid	yes	x		x		Possible carcinogenic for liver, kidney, lung, causing leukemia; effects on reproductive system, skin and eyes irritation.
Unregulated HAAs Bromochloroacetic acid Bromodichloroacetic acid Dibromochloroacetic acid Tribromoacetic acid	yes	x		x		Possible carcinogenic for liver, lungs, kidney and reproductive system diseases; leukemia.
Nitrosamines (NDMA) N-Nitrosodimethylamine N-Nitrosopyrrolidine N-Nitrosomorpholine N-Nitrosopiperidine N-Nitrosodiphenylamine N-nitrosomethylethylamine N-nitrosodiethylamine N-nitroso-di-n-butylamine	yes	x		x		Possible carcinogenic for liver, stomach, esophagus, bladder, lung, breast and brain.
Aldehydes Chloroacetaldehyde Dichloroacetaldehyde Bromochloroacetaldehyde Tribromoacetaldehyde	yes	x	x		x	Possible carcinogenic for stomach and lung; reproductive system toxicity; causes depression and DNA damage.
Haloketones (HKs) Hexachloropropanone 1,1-Dibromopropanone 1,1,3-Trichloropropanone 1,1,1,3-Tetrachloropropanone 1,1,3,3- Tetrachloropropanone 1,1,1,3,3-Pentachloropropanone	yes	x		x		Possible carcinogenic or mutagenic effects.

The vast majority of the identified DBPs remains with unknown biological activities, which supports the need to understand how exposure to these compounds may impact the

human health. Recent studies in emerging DBPs, including, haloacetaldehydes, nitrosamines, halo benzoquinones, among others, suggest they can display higher genotoxicity and cytotoxicity potential than those that are currently regulated, such as THMs (Florina et al., 2020). Expanding the knowledge on the mode of action (MoA) of unregulated and poorly studied DBPs will improve risk assessment; increasing knowledge of new potential impacted signaling pathways and identifying specific biomarkers to support a robust human health risk assessment. Ideally, adverse outcome pathways (APO) should be identified and characterized for target DBPs. This topic is discussed in more detail in chapter IV.

5. Methods for DBPs identification

Considering the chronic effects of long-term exposure to DBPs and the related public health issues, increasing research to advance understanding of DBPs nature, formation, concentrations and health hazards have been required in the recent years. In accordance, analytical methodologies for monitoring drinking water have been in the first line of scientific research, serving a large number of related purposes, including occurrence studies in community water systems, determination of the DBPs in the different water system approaches and identification of emerging organic compounds (Ding et al., 2020; Hanigan et al., 2017a; Plewa et al., 2017; Richardson et al., 2007). These methodologies also support toxicological studies and the consequent drinking water regulations (Hrudey, 2009; Yang et al., 2018).

Considering the analytical methodologies, the choice of separation method is primarily based on analyte properties; with gas chromatography (GC) most suitable for volatile and semi-volatile analytes, and liquid chromatography (LC) most suitable for highly polar, non-volatile and thermally unstable analytes. Several detectors have been used in tandem with GC, being the most common the flame ionization detector (FID), the electron capture detector (ECD) and the mass spectrometer (MS). In fact, due to its versatility, robustness and sensitivity, MS is the most used detector to measure compounds at trace concentrations present in complex environmental matrices. In the context of this work, we selected the GC-MS separation method, attending to the volatile and semi-volatile characteristics of the target compounds. Therefore, GC-MS method is briefly described.

GC is based on the physical separation method in which the components in a complex mixture are selectively distributed between the mobile phase (an inert carrier gas) and a stationary phase (coating of column packaging or of the inner column wall). The GC instrumentation consists, essentially, of a sample introduction device (injector), a column housed in the temperature-programmable oven, an interface, the mass spectrometer and a data collecting system (McMaster, 2008). The chromatographic process consists in repeated sorption/desorption steps, occurring during the movement of the analytes along the stationary phase caused by the carrier gas (Karasek and Clement, 1986).

The most widely applied GC injector is the split/splitless injector, which can work in three different types of injection: split, splitless and on-column. In split injection, only a small fraction of the sample will be sent to the column, minimizing the possible overloading of the column, being applied when the analytes are present in high concentrations. In splitless injection, the split vent is closed for a period of time and remains so while the sample is vaporized in the liner and transferred to the column, concentrating the analytes. Splitless injection has the benefit of higher sensitivity. This type of injection is particularly applied in samples with trace amounts of compounds. Finally, in on-column injection the sample is directly introduced into the column, being useful for analyzing components with high boiling point and/or for analytes that could degrade at high temperatures (Grob, 2007).

In general, GC-MS allowed researchers to separate and identify the individual contaminants/ analytes in complex mixtures. GC-MS involves the introduction of a small amount of sample extract into a heated injection port, where the chemical mixture is vaporized, and introduced onto a chromatographic column. The individual compounds are separated on this column and swept by a carrier gas into the mass spectrometer, where they are ionized and analyzed. The most common carrier gases are hydrogen, helium or, to a lesser extent, nitrogen. The principle of MS is the production of gas-phase ions that are subsequently separated according to their mass-to-charge (m/z) ratio. The resulting mass spectrum is a plot of the (relative) abundance of the generated ions as a function of the m/z ratio.

Large mass spectral libraries (NIST and Wiley databases, which contain >200,000 spectra) enable rapid identification. Selected ion monitoring (SIM) mode are used with GC/MS to maximize the sensitivity and provide low detection limits.

Gas chromatography-mass spectrometry (GC-MS) has been playing a crucial role in the discovery of disinfection by-products (DBPs) in drinking water, due to their major volatile and semi-volatile properties. The first known DBP identified, the chloroform (CF), was characterized using GC-MS (Bellar et al., 1974; Postigo et al., 2018). Since then, GC-MS became the key tool used for measuring of volatile DBPs, such as THMs, haloacetonitriles (HANs) and HKs (Richardson, 2010). For the detection of more polar DBPs with ionizable functional groups, such as HAAs, derivatization is necessary prior to separation with GC being the most commonly used derivatizing agents diazomethane and acidic methanol (Andersson et al., 2018; Postigo et al., 2020; Xie, 2001). Since drinking water is a complex matrix, a sample preparation step is usually required.

6. Conventional methods for sample preparation

The concentrations of DBPs in drinking water samples are usually in the range of micrograms, nanogram or picogram per milliliter. Additionally, most analytical instruments cannot handle direct injection of water samples. Therefore, a sample preparation step is necessary prior to analysis, which serves a number of purposes including elimination or reduction of disturbing components of the sample matrix (clean-up), trace concentration of analytes (enrichment), medium exchange (gas phase, solvent or sorbent) for introduction to analytical instruments and, if needed, analytes conversion to more suitable form, for separation and detection (derivatization). Compared with other sample matrices such as biological and wastewater samples, sample preparation of drinking water with relatively clean matrix and low amounts of DBPs is usually less complex and often restricted to extraction and preconcentration of analytes from aqueous medium. However, challenges still arise from hydrophilicity, high polarity, non-volatility and instability of some analytes, which renders their efficient extraction and detection difficult to achieve. Obviously, the more soluble the analytes in water, the greater is the difficulty to extract them.

The conventional sample preparation techniques for analysis of micropollutants in water, such as DBPs, are solid phase extraction (SPE) and Solid Phase Microextraction (SPME). Both techniques were used during this project development.

In SPE procedure, an aqueous sample is passed over a sorbent packed cartridge or disk, which has initially been conditioned by one or more solvents. The analytes are thus adsorbed and retained on the sorbent. After washing and drying steps of the sorbent bed with ultra-pure water, solvents and vacuum or nitrogen, the analytes are recovered by eluting with a small volume of appropriate organic solvent through the cartridge or disk. The extract is then filtered and evaporated to the desired volume before chromatographic separation. SPE tends to be less selective, and matrix components can be extracted as well. Thus, the choice of elution solvent can be critical, particularly if the analytes are highly volatile (Fritz, 2000). The solvent should be sufficiently volatile and nonpolar, and it should be able to elute the target analytes from the SPE sorbent in a small volume. Compared to the other conventional techniques, SPE offers higher sensitivity due to its exhaustive extraction nature, decreased use of organic solvents, shorter analysis times, automation capability and suitability for field analysis. Also, with a wide range of stationary phases now available, including hydrophilic and dual-type stationary phases, SPE can be tailored to extract a broader range of polar and non-polar analytes which is an advantage for profiling type of analysis, i.e., when all or most of the sample components are of interest. SPE can increase the concentration factor for target analytes by more than 3 orders of magnitude, achieving low parts per trillion (ng/L) detection limits.

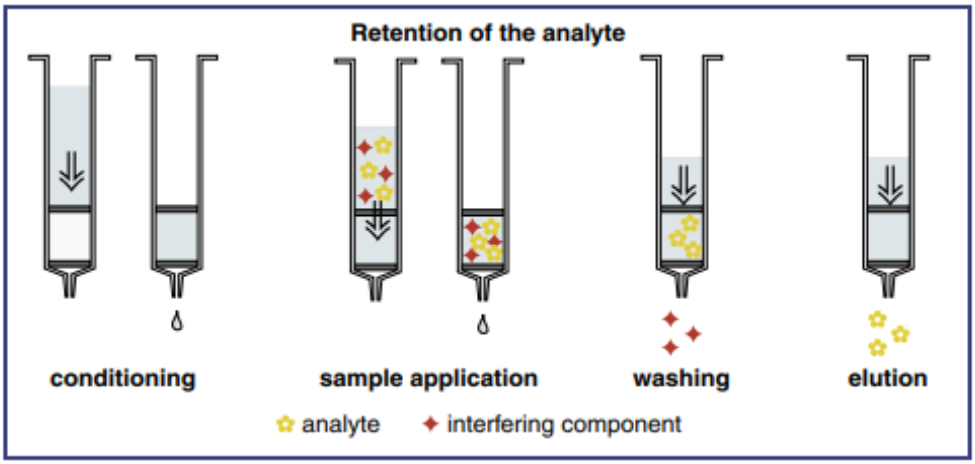

Figure 6-Scheme of the steps involved in Solid Phase Extraction. Adapted (Fritz, 2000)

SPME has addressed the need for a simple, rapid, sample preparation method as it integrates sampling, isolation, enrichment and sample introduction into a single solvent-free step. Moreover, the trend of implementing green analytical chemistry favors the use of "solvent-free" sample preparation methods. SPME complies with the solvent-free requirement and affords the promise for rapid, cost-effective sample handling along with good sensitivity and automation capability for drinking water analysis.

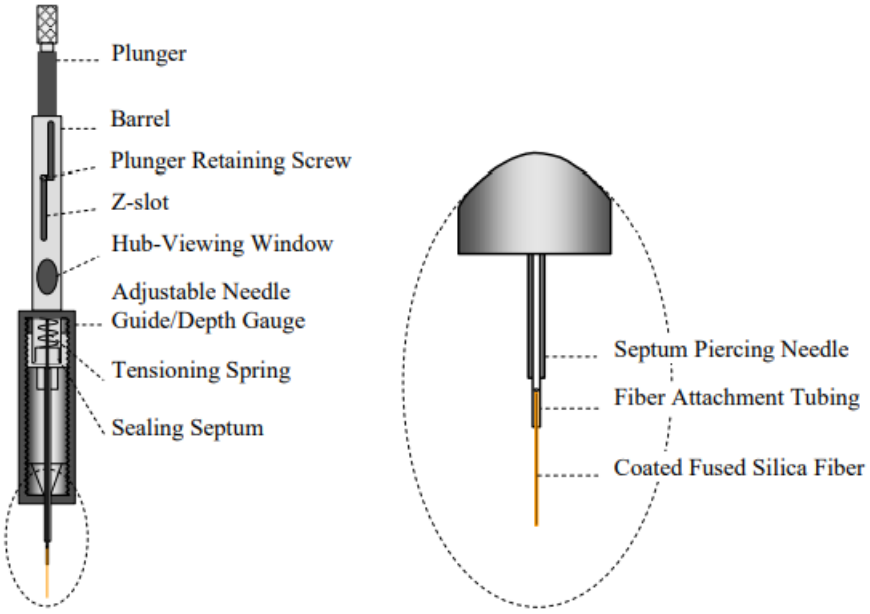

Figure 7- Design of the commercial SPME device made by Supelco (Sigma-Aldrich)

The most commonly used form of SPME is fiber geometry, in which a thin layer of a polymeric material, neat or blended with solid particles, is coated and immobilized on a bare fiber support (e.g., fused silica or metal) for extraction purpose. To protect the fiber, an assembly has been designed that contains a septum piercing needle and inner needle tubing to which the coated fiber is attached. The outer needle is sealed with a septum to keep it from leaking during extraction and desorption. Figure 7 illustrates the design of a manual fiber assembly and its holder made by Supelco. The manual fiber assembly contains a spring that helps to retract the fiber after exposure for extraction or desorption. The inner needle has a color-coded screw-type hub at the end, which indicates the type of coating and facilitates attachment of the assembly to the holder. Different types of coatings such as DVB/CAR/PDMS and PDMS/DVB were tested in the present work to identify the best extraction approaches.

SPME procedure includes two main steps that are extraction of analytes by the coating from sample and desorption of the extracted analytes from the coating (figure 8). The extraction process is carried out either by direct immersion of the fiber into the sample or by its exposure to the headspace above the sample. The headspace mode is usually applicable to volatile and semi-volatile species, while the direct immersion can be applied regardless of the analyte volatility. Headspace mode is advantageous considering the lifetime of SPME fiber and modification of the matrix, such as pH adjustment and salt addition without damaging the fiber (Sarrión et al., 2000).

Immediately after exposing the fiber coating to the sample, partitioning of analyte begins rapidly between the two phases. The amount of analyte extracted depends on the exposure time at initial stages of extraction. If a sufficient time is allowed for the equilibrium to be established, further increases in extraction time do not affect the amount of analyte extracted and microextraction is considered complete. When two phases are involved, the equilibrium sampling can be described by the equation according to the law of mass conservation:

$$C_0 V_s = C_f^{\infty} V_f + C_s^{\infty} V_s$$

where C_0 is the initial concentration of the analyte in the sample; C_f^{∞} and C_s^{∞} are the equilibrium concentrations of the analyte in the fiber coating and in the sample, respectively; V_s and V_f are the volumes of the sample and the fiber coating, respectively.

Partitioning of the analyte between the sample and the fiber coating is governed by the partition coefficient, K_{fs}, also called the distribution constant as given by:

$$K_{fs} = \frac{C_f^{\infty}}{C_s^{\infty}}$$

The number of moles of the analyte extracted by the coating at equilibrium, $n^{\infty} = C_f^{\infty} V_f$, can be expressed by:

$$n^{\infty} = \frac{K_{fs} V_f \quad V_s}{K_{fs} V_f + V_s} C_0$$

In accordance with the latter equation, the amount of analyte extracted by the coating at equilibrium is linearly proportional to the initial concentration of analyte in the sample, and that the amount is independent of extraction time. Moreover, when the fiber capacity ($K_{fs} V_f$) is insignificant relative to V_s, the amount of analyte extracted at equilibrium is no longer dependent on the sample volume. The figure 8 shows the summary of SPME procedure

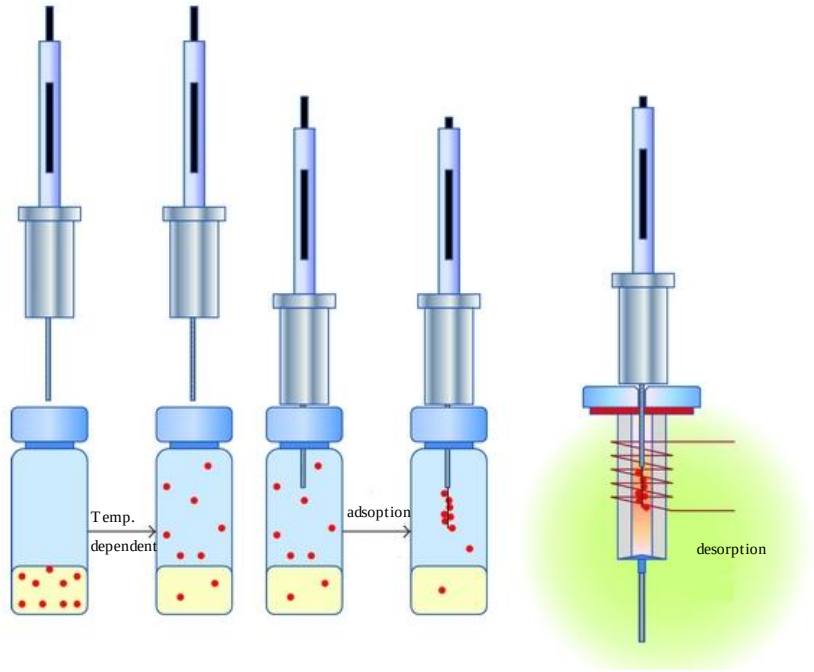

Figure 8- SPME procedure schematics. Adapted from Thermo fisher scientific.

7. Validation of analytical methods

The current legislation concerning quality of water for human consumption refers the need to make periodical analysis considering the regulated target compounds, preferentially carried out by accredited laboratories.

Accreditation is the recognition of technical competence for the laboratory to carry out a specific set of Tests / Calibrations, in full compliance with the EN ISO / IEC 17025: 2018 (General requirements for the competence to carry out research tests and calibrations) (ISO, 2018). This standard refers to management and technical requirements that a

laboratory must implement, with a view to accreditation. In the technical requirements are included the test methods and validation procedures.

Analytical methods should be validated to ensure reliability, consistency, and accuracy of analytical data. Method validation can be described as the process used to confirm that the developed analytical procedure is suitable for its intended use. Typical validation includes the evaluation of performance characteristics, such as:

- Specificity/selectivity
- Precision (Repeatability; Intermediate Precision)
- Linearity and working range
- Analytical threshold: limits of detection and quantitation
- Accuracy
- Uncertainty measurement

Selectivity of an analytical method is its ability to measure accurately an analyte in a sample matrix without any interferences. Precision is a measure of the degree of closeness of the values obtained by the analytical method when analyzing, repetitively, the same sample, under specific and controlled conditions. It is expressed as the relative standard deviation (RSD) of the replicate measurements. This parameter can be evaluated at three different levels: repeatability, intermediate precision and reproducibility (Causon, 1997; RELACRE, 2000).

Linearity demonstrates the ability of the method to provide a response (analyte signals) directly proportional to the concentration of an analyte in the sample within a working range (Shabir et al., 2007). Linearity is usually evaluated by the coefficient of determination (r^2, values ≥ 0.990) and coefficient of variation of the method (CVm values $\leq 5\%$) Several statistical tests, such as residual analysis ($\pm$ 15%), Mandel test ($PG \leq F$ (1; N-3; 95%), RIKILT test ($\pm$ 10%) and normalized areas test ($\pm$ 15%), are also applied to assess the linearity in the selected concentration range, in accordance with ISO 8466-1: 1990 (ISO, 1990). The working range of an analytical method is the interval between the upper and lower levels that have been demonstrated to be determined with precision, accuracy and linearity.

Analytical thresholds assess the method performance, being expressed as limit of detection (LOD) and limit of quantification (LOQ). LOD is defined as the lowest amount

of analyte reliably detected in a mixture above the reference noise (commonly three times the noise level), but not necessarily quantified. LOD may be divided into two components, the method detection limit (MDL) and instrumental detection limit (IDL). MDL should be applied when extraction step followed by analytical measurement is performed for the analysis of specific analytes within a matrix. LOQ refers to the lowest analyte concentration that can be quantified with an appropriate level of precision and accuracy and above the reference noise. Similarly to LOD, LOQ can also be defined into two components, method quantification limit (MQL) and instrumental quantification limit (IQL). LOD and LOQ can be determined based on three different approaches i) the calibration curve (LOD = 3 × relative standard deviation of calibration curve ($S_{Y/X}$) / slope; LOQ = 10 × $S_{Y/X}$ / slope); ii) repeatability or intermediate precision conditions (n=10, LOD=3 x standard deviation (SD); LOQ=10 x SD); and iii) signal-to-noise (n=10, LOD=3 x signal-to-noise (S/N); LOQ=10 x S/N) (Shabir et al., 2007).

The accuracy of an analytical measurement describes how close the result is to its true value, including the effects of both precision and trueness (expressed in the form of bias, assessed by recovery tests)(Rao, 2018; RELACRE, 2000). Accredited laboratories use approved methods to analyze drinking water compliance samples from public water systems.

Uncertainty can be defined as a parameter associated with a result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand (EURACHEM/ CITAC, 2012). Uncertainty of measurement does not imply doubt about the validity of a measurement, on the contrary, knowledge in uncertainty contributes to increase the confidence in the validity of the measurement. Uncertainty may arise from several possible sources including incomplete definition of measurand, sampling, matrix effects and interferences, environmental conditions, uncertainties of masses and volumetric equipment, approximations and assumptions incorporated in the measurement method and procedure, and random variations (EURACHEM/ CITAC, 2012).

In accordance, the ISO/IEC 17025, standard by which EPAL is accredited, specifies the general requirements for competence of testing and calibration laboratories, referring the need of uncertainty determination. The calculations are performed taking into account the requirements of Eurachem/ CITAC (see supplementary information).

8. EPAL- Empresa Portuguesa das Águas Livres, S.A.

EPAL’s water supply system includes the production, storage, adduction/ transport and the distribution systems. Currently, the company is responsible for provide the supply to consumers in a total area of 7,095 km², representing a population around 2.9 million inhabitants. EPAL’s drinking water distribution system provides water for Lisbon region, representing over than 1,447 km of distribution network, in 2019. It further includes 14 reservoirs, 10 pumping stations and over 86 thousand branch lines connected with urbanization.

EPAL's Laboratories and Water Quality Control Department has been accredited since 1999 according to standard NP EN ISO/IEC 17025 - 'General requirements for the competence to carry out tests and/or calibrations' for the activities, such as

- Collection, preservation, and transport of water samples for human consumption;
- Analysis of 110 water quality parameters (corresponding to 198 compounds) where some parameters/compounds have been accredited for more than one testing method;
-Testing organic materials in contact with water for human consumption (with one of the two national laboratories holds this certification).

EPAL's testing laboratories is a Portuguese and European reference in water quality control. This is consistent with the national institutions that resort its analytical services, such as Directorate-General of Health, "Entidade Reguladora de Serviços de Águas e Resíduos" (ERSAR), and municipalities by means of City Halls and their Municipal Services, as well as companies belonging to Grupo AdP, among others.

9. Aims of the work

The ultimate purpose of this work was to improve water safety plans with new knowledge about DBPs formation and their potential toxicity, contributing to improve risk assessment and (a) to implement and validate analytical methods to detect and quantify emerging DBPs, such as nitrosamines and haloketones, in drinking water, attending to different water treatment plants (WTPs) and (b) to improve toxicological data of unregulated DBPs with human health relevance, using embryo bioassays with the vertebrate animal model zebrafish, *Danio rerio*.

Secondary goals of the project included (a) to estimate the occurrence of emerging DBPs, namely nitrosamines and haloketones in drinking water (b) to compare concentrations of DBPs in waters from different surface water sources, different WTPs; characterization of EPAL's water supply system attending to both regulated and non-regulated DBPs (c) to estimate seasonal variation along the year, attending to different geographic area and WTPs technologies involved (d) to evaluate effects of DBPs in zebrafish embryonic development and (e) to evaluate the mode of action (MoA) of selected DBPs on zebrafish embryos, using molecular tools, i.e., RNA sequencing (RNA-seq).

These objectives were implemented in four chapter. In Chapter II, entitled "Assessment of water quality parameters of Lisbon water supply system, a 6-year monitoring study", we characterized EPAL's water supply system, correlating microbiological and chemical water quality parameters. Seasonal behavior of the target parameters was assessed, attending to different surface waters, different WTPs technologies and three geographically different points of Lisbon distribution network, during a 6-year period. In chapter III, entitled "Development of multi-residue gas chromatography coupled with mass spectrometry methodologies for the measurement of 15 chemically-different Disinfection By-products (DBPs) of emerging concern in drinking water from two different Portuguese water treatment plants." we validated new methods for detection and quantification of UR-DBPs attending to four different classes of compound, aldehydes, alcohols, haloketones and nitrosamines. The % of occurrence of the target DBPs were assessed, attending to two different WTPs. In chapter IV, entitled "Hazard and mode of action of disinfection by-products (DBPs) in water for human consumption: evidences and research priorities", we reviewed the literature regarding DBPs formation and forcing agents, legal context, potential effects, exposure assessment and risk characterization, in water for human consumption. This review was particularly focused in UR-DBPs and their putative underlying MoA, linking the available data with adverse health outcomes. The main knowledge gaps in this field are also identified, and future research priorities discussed. In chapter V, entitled "Toxicological Assessment of seven unregulated drinking water Disinfection By-products (DBPs) using the zebrafish embryo bioassay" we used the Zebrafish (*Danio rerio*) embryo bioassay (OECD TG 236), to evaluate the toxicity of seven UR-DBPs, including aldehydes, alcohols, haloketones and nitrosamines. Multiple endpoints, including mortality, morphological abnormalities and locomotor behavior were assessed, at specified developmental stages (24, 48, 72 and 96 hpf). The

lowest observed effect concentration (LOEC) and effective concentration 20 (EC20) and 10 (EC10) values were determined, and the findings discussed considering the current risk assessment framework for this group of compounds. Based on this toxicological assessment, and to gain additional insights regarding the potential MoA and signaling pathways disrupted, two of the 7 target UR-DBPs were selected for a preliminary exploratory transcriptomic analysis. The first results of this analysis are presented in supplementary information.

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CHAPTER II. Assessment of Water Quality parameters and their seasonal behaviour in a Portuguese Water Supply System: a 6-year monitoring study

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Abstract

Water quality monitoring is a fundamental tool in the management of freshwater resources. The purpose of monitoring is to provide meaningful quality data for local action planning and catchment-wide decision making. The assessment of water quality is crucial to guarantee the efficient operation of the Water Treatment Plants (WTPs), promoting the health conditions and contributing for a more sustainable urban water cycle. In accordance, the objective of this study was to evaluate key target chemical and microbiological water quality parameters, some of them already monitored within the Portuguese/EU legal framework and some which are still not regulated, but with environmental and human heath relevance.

A local monitoring database model, using a 6-year period set (from 2014 to 2019) of water quality data, regarding water samples collected on representative sampling locations covering the freshwater abstraction sites, conventional WTPs and distribution network was assessed. This work provides new knowledge regarding the occurrence and seasonal behaviour for both microbiological and chemical water quality parameters, essential to understand/manage the water supply system. In addition, correlation between the target variables were also assessed. Particularly, strong correlations were identified between TOC and THMs formation at distribution network ($r = 0.69$; $p \leq 0.001$); nitrates were the water quality parameter that revealed the best correlation between surface water source and treated water ($r = 0.81$; $p \leq 0.001$), suggesting that treatment yield/performance is dependent on surface water load. The local and continuous monitoring of water systems are crucial to implement new approaches to guarantee the best quality of drinking water throughout the supply system.

1. Introduction

The quality and available quantity of water resources are under increasing pressure due to demographic growth and population activities, climate change and higher rates of urbanization, changing the social and environmental landscape. The water sources can include different origins such as ground and surface waters, the latter being the most abundant resource. In fact, at least 144 million people worldwide depend directly on surface waters resources (UNWATER, 2020; WHO, 2017).The anthropological pressure plays an important role in this topic. The impact of activities such as lifestyle, industry,

veterinary and agriculture activities, increase the proliferation of contaminants in the environment and consequent water contamination (Bartrand-Krajewski et al., 2000; Behmel et al., 2016; Geissen et al., 2015; UNWATER, 2020). Contaminated water may contain pathogens such as bacteria, viruses or protozoa, or chemical compounds such as trihalomethanes, arsenic, nitrosamines, aluminum, and others of emerging concern (Chaves et al., 2020; Mian et al., 2018; Nawrocki and Andrzejewski, 2011; Salvador et al., 2020; WHO, 2017). The exposure to these contaminants, through water ingestion or recreational use, may cause several diseases, including diarrhea, cholera, dysentery, typhoid, among others (WHO, 2017). The outcomes of chronic exposure can also include cancer and several non-cancer effects, such as neuronal problems, birth defects, asthma and skin rashes (Cortés and Marcos, 2018; Villanueva and Cordier, 2015; Wright et al., 2017).

In this context, water treatment plants (WTPs) represent a crucial tool to guarantee safe and clean drinking water, promoting health and sanitation. Towards obtaining a more sustainable urban water cycle, it is essential to achieve an efficient removal or inactivation of potential contaminants released from different human activities. Wastewater treatment plants (WWTPs) play also a crucial role in chemical contaminant and pathogen removal in the treated effluent, ensuring a safe environmental water reuse. Thus, the supply of safe water in both quantity and quality for human consumption and the treatment of urban wastewaters are two of the biggest challenges of the twenty-first century (Bartrand-Krajewski et al., 2000).

The disinfection processes used to produce drinking water are particularly important in the inactivation of pathogens, contributing towards a safe delivery of drinking water to the consumers. In order to minimize the microbial risk during distribution, secondary disinfectants such as chlorine, have been widely applied in drinking water supply systems. Some of the current challenges related with disinfection include the maintenance of stable concentrations of the residual disinfectants throughout the supply system, the control of the factors that influence the residual disinfectant's stability and/or decay reactions, and the consequent formation of disinfection by-products (DBPs)(Li et al., 2019). This is a complex process due to the different factors that influence the nature and activity degree of natural and anthropogenic organic content in the water. Some of the factors that affect DBPs formation include temperature, water age, piping material, corrosion products, nutrients, flow conditions and residual disinfectant type and dosage. The disinfectant type

and dose applied were found to be among the most important factors to understand the knowledge gaps and research needs in DBPs formation (Li et al., 2019).

In 2010, the United Nations General Assembly recognized the access to safe and affordable water and sanitation as a human right, defining also that everyone has the right to get *sufficient, continuous* and *physically accessible* water, in *acceptable* characteristics for the cultural context. However, according to the World Health Organization (WHO) in 2017 only 71% of the global population had access to safe and contamination-free drinking water (WHO, 2017). Around 785 million people have no access to potable water service and at least 2 billion used a contaminated water source. In 2025, it is estimated that half of the human population will live in water stress areas, which will be a huge challenge for the water supply systems (Morton et al., 2017; UNWATER, 2020) . Thus, the correct management of these water bodies and the monitoring of a wide diversity of contaminants is extremely important (Behmel et al., 2016; Geissen et al., 2015; Srebotnjak et al., 2012).

The European Union (EU) Water Framework Directive (2000/60/EC), published in 2000, established provisions for a list of Priority Substances and environmental monitoring of water bodies. In 2013, EU Directive 2013/39/EU established a list of the priority substances to be monitored, including pesticides and herbicides, endocrine disrupting chemicals (EDCs), polyaromatic hydrocarbons (PAHs), among others. A watch list mechanism was also proposed as a guideline of substances, such as pharmaceuticals and hormones, for which data should be obtained to support future prioritization decisions (Carvalho et al., 2015). More recently, the sixth objective defined on the Global Sustainable Development Goals of the United Nations for 2030, also reinforced the need of ensure availability and management of water and sanitation for all (UNWATER, 2020).

In Portugal, 68.18% of the water used in 2018 was from surface water source. There are 295 surface water sources and 3601 treatment sites, of which 269 are conventional WTPs and 3332 are treatment facilities that only perform disinfection and/or aggressiveness correction operations (ERSAR, 2019). Treated drinking water reaches about 96% of the national consumers and, regarding the compliance of water quality standards established within the Portuguese and EU, the drinking water was classified as of excellent quality with 98.63% of compliant results. This annual water quality assessment is done by ERSAR, (Water and Waste Services Regulation Authority of Portugal), taking into

account the results of more than 567 000 analytical assays, which are legally carried out each year by the Portuguese water companies, within their Water Quality Control Programs (WQCP), which are annually submitted and approved by ERSAR. These WQCP comply with the current Portuguese legislation on water quality for human consumption (DL 152/2017, 2017), which defines the guidelines, the sampling frequency and water quality parameters to monitor, in order to support an effective protection of the national water resources and the public health (ERSAR, 2019).
Understanding the public health implication of water quality and monitoring is crucial for improving human health risk assessment, monitoring environmental quality, and implementing effective water safety plans.

Thus, the objective of this study was to assess the occurrence and seasonal behaviour of some target chemical and microbiological water quality parameters, considering the Portuguese and EU legal context, from EPAL - Empresa Portuguesa das Águas Livres, SA monitoring database, between 2014 and 2019. Different sampling points of EPAL's water supply system were evaluated, from water areas abstraction to the inlets of the distribution network sampling points in the city of Lisbon. For this study, we selected EPAL's main water sources used to produce drinking water (2 surface water sources), the corresponding conventional WTPs and three inlets/deliveries points of the transport system to the distribution network of the city of Lisbon. Currently, the company is responsible for the water supply of 2.9 million consumers in a total area of 7,095 km^2.
The selection of this water supply system was related with their huge water production capacity, number of supplied consumers and, at the same time, the potential environmental pressure and contamination on the surface water sources (Figure s1, supplementary information).
This study can be transposed, to some extent, to other drinking water supply systems, as the technologies employed in the selected WTPs are also used worldwide.

2. Material and methods

2.1 Sampling sites

EPAL's water supply system includes the production, storage, adduction/transport and the distribution network systems. EPAL's drinking water distribution network system provides water for Lisbon region, representing over than 1,447 km of distribution network. The distribution system's management is supported by a series of infrastructure and bodies, namely 14 reservoirs, 10 pumping stations, 6 chlorination posts and about 86,000 branches connecting the system to buildings. EPAL currently manages and operates a water supply system that is made up of two sub-systems: Castelo do Bode reservoir (supply 1; S1), built across the River Zêzere, opened in 1987 and extended in 2007, connected with WTP Asseiceira with a water production capacity of around 625,000 m^3 daily; and the Tagus River (supply 2; S2), opened in 1940 and extended in 1963 and 1976, connected with WTP Vale da Pedra with a water production capacity of 240,000 m^3 daily (Figure 1).

Of the two above-mentioned sub-systems, the largest and most relevant is Castelo do Bode, as it accounts for about 75% of the company's production capacity. The water treatment process in Asseiceira WTP (Santarém District, Portugal) has the following steps: pre-oxidation with chlorine, remineralization, correction of aggressiveness, coagulation, filtration with sand filters, pH correction and disinfection with chlorine. This WTP has two independent treatment lines with the capacity to produce 500,000 m^3/day and 125,000 m^3/day (EPAL, 2020a).

The water treatment process in Vale da Pedra WTP (Santarém District, Portugal) has the following steps: pre-oxidation with chlorine, pH adjustment, activated carbon adsorption, coagulation/flocculation, sedimentation, filtration with sand filters, pH correction and disinfection with chlorine. In the last year of this study the pre oxidation system was optimized replacing chlorination by ozonation. This WTP (treatment 2; T2) is composed of two independent treatment lines, each with the capacity to produce 240,000 m^3/day (EPAL, 2020a)

In this work, water samples from the two main surface water sources, Castelo de Bode Dam e Tagus River were analyzed (Figure1). These sampling points were selected based on their relevance considering water production capacity of the company, since they are the two major sub-systems for drinking water production, being located in geographically different sites. Drinking water samples were collected at sampling points located at the outlet of the WTP and at the end of the delivery points (D) of the three main transport subsystems to Lisbon's distribution network.

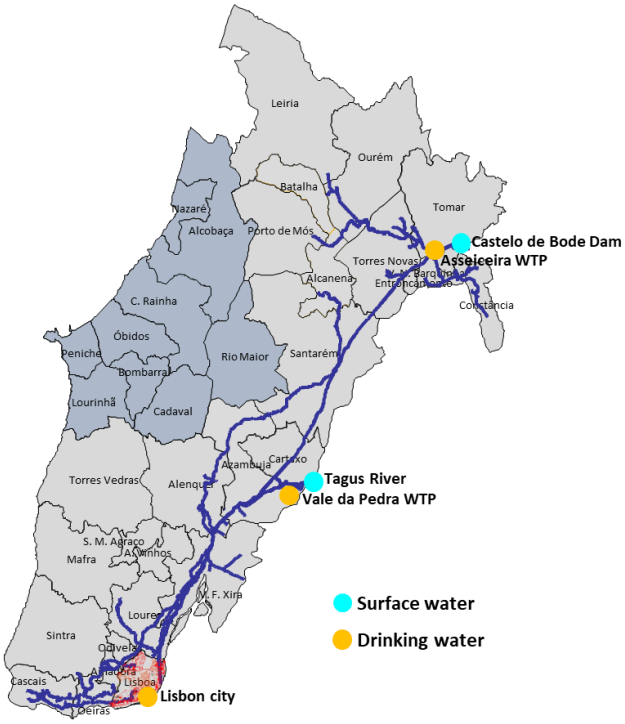

Figure 1. Global representation of sampling sites, between 2014 and 2019. Adapted from EPAL, 2020.

2.2 Sampling

The sampling frequency and the water quality parameters analyzed are described in Table 1 and were established in accordance with WHO guidelines, national legislation and EPAL's Water Quality Control Program (WQCP). The fieldwork was carried out from January 2014 to June 2019. Sampling frequency varied between daily and biannual, depending on the sampling location, the water matrix, and the water quality parameter. All samples were collected in adequate containers and stored at 5 ± 3 °C until processing and analysis. According with some procedures, and in order to inactivate the residual chlorine in the samples, sodium thiosulfate was added to water samples. Microbiological and chemical parameters were analyzed according to internal standard procedures based on international standards; the sampling volume were procedures depending.

Table 1. Target water quality parameters and their sampling frequency. Adopted from EPAL's WQCP (EPAL, 2020b).

Sampling point	Parameter	Frequency
Castelo de Bode reservoir (S1)	Ammonia	Monthly
	Coliform bacteria	Monthly
	Fecal coliforms	Monthly
	E. coli	Monthly
	Intestinal enterococci	Monthly
	Nitrates	Monthly
	Nitrites	Monthly
	Nitrogen Kjeldahl	Monthly
	Temperature	Monthly
	TOC	Monthly
	Trihalomethanes	Monthly
Tagus River (S2)	Ammonia	Daily
	Coliform bacteria	Fortnightly
	Fecal coliforms	Monthly
	E. coli	Fortnightly
	Intestinal enterococci	Fortnightly
	Nitrates	3 per week
	Nitrites	Fortnightly
	Nitrogen Kjeldahl	Monthly
	Temperature	Fortnightly
	TOC	Fortnightly
	Trihalomethanes	Monthly
Asseiceira WTP (T1)	Ammonia	Monthly
	Coliform bacteria	Monthly
	Fecal coliforms	Monthly
	E. coli	Monthly
	Intestinal enterococci	Monthly
	Nitrates	Monthly
	Nitrites	Monthly
	Nitrogen Kjeldahl	Monthly
	Temperature	Monthly
	TOC	Monthly
	Free chlorine	Monthly
	Combined chlorine	Monthly
	Total chlorine	Monthly
	Haloacetic acids	Monthly
	Bromates	Biannual
	Trihalomethanes	Monthly
Vale da Pedra WTP (S2)	Ammonia	Monthly
	Coliform bacteria	Monthly
	Fecal coliform	Monthly
	E. coli	Monthly
	Intestinal enterococci	Monthly
	Nitrates	Monthly

	Nitrites	Monthly
	Nitrogen Kjeldahl	Monthly
	Temperature	Monthly
	TOC	Monthly
	Free chlorine	Monthly
	Combined chlorine	Monthly
	Total chlorine	Monthly
	Haloacetic acids	Monthly
	Bromates	Monthly
	Trihalomethanes	Monthly
All the delivery points to the distribution network (D)	Ammonia	Weekly
	Coliform bacteria	5 per week
	E. coli	5 per week
	Intestinal enterococci	5 per week
	Nitrates	Weekly
	Nitrites	Weekly
	Temperature	5 per week
	TOC	Monthly
	Free chlorine	5 per week
	Combined chlorine	5 per week
	Total chlorine	5 per week
	Haloacetic acids	Monthly
	Bromates	Biannual
	Trihalomethanes	Weekly

2.3 Determination of physical-chemical parameters

The temperature was measured at the sampling sites with a Model E 905 000 thermometer (Amarell-Electronic).

Free chlorine and total chlorine were measured in drinking water samples by molecular absorption photometry (colorimetric method) with N, N-diethyl-p-phenylenediamine (Sigma-Aldrich) using portable photometers (Palintest) (Standard Methods for the Examination of Water and Wastewater, 2017). Total organic carbon (TOC) was measured using UV persulfate oxidation or catalytic combustion method (EN1484), depending of the water matrix analyzed.

Kjeldahl and ammoniacal nitrogen, nitrates and nitrites were measured using segmented flow analysis with photometric detection ("ISO 10304-1:2007," 2007).

Haloacetic acids (HAAs) were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Total HAAs were measured based on the sum of monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), trichloroacetic acid

(TCAA), bromochloroacetic acid (BCAA), monobromoacetic acid (MBAA), bromodichloroacetic acid (BdCAA), and dibromoacetic acid (DBAA).
Trihalomethanes (THMs) were analyzed by gas chromatography with electron capture detection using headspace as sample treatment. Total THMs were measured based on the sum of 4 individual species: chloroform (ClF), bromodichlorometane (BDCM); dibromochlorometane (DBCM), and bromoform (BrF).

2.4 Determination of microbiological parameters

In surface water samples, the concentration of coliform bacteria and, *Escherichia coli* (*E. coli*) were performed using Colilert^{R} with Quanti-Tray^{R} (IDDEX Laboratories), at 36 °C, following the ISO 9308-2:2012 ("ISO 9308-2:2012," n.d.) . Fecal coliforms were performed using the Colilert test with Quanti-Tray (IDDEX Laboratories), at 44.5 °C, following the manufacturer's instructions.
In drinking water samples, the concentration of coliforms bacteria and *E. coli* was determined by the membrane filtration method, according with ISO 9308-1:2014 ("ISO 9308-1:2014," 2014). The concentration of fecal coliforms was determined by the filter membrane method, according with ISO 19458:2006 ("ISO 19458:2006," n.d.) .
The concentration of intestinal enterococci in surface and drinking water was determined by the membrane filtration method, according to ISO-7899-2:2000 ("ISO 7899-2:2000," n.d.) .

2.5 Statistical analysis

The statistical analyses were conducted using Microsoft Excel 2017 (Microsoft Inc., Redmond, WS, USA) and R software, version 4.0.0, combined with Rstudio version 1.3.1093, an integrated development environment for R.
Boxplots were built by using an R script, package "ggplot2" (Wickham, 2016). Since environmental data are in general non-stationary, non-parametric statistical methods were used for data analysis. In accordance, non-parametric Spearman correlations between the target parameter were determined by using an R script, package "Performance Analytics" (Peterson et al., 2020). The obtained correlation charts display the distribution of each

variable; the bivariate scatter plots (bottom of the diagonal); and the correlation values and significance level stars (top of diagonal).

3. Results and discussion

3.1 Occurrence assessment of microbiological and physical-chemical parameters

The summary of the monitoring results for the target compounds is shown in Table 2.
Regarding natural surface waters, fecal contamination bacteria (FIB), such as fecal coliforms, *E. coli*, and the intestinal enterococci, presented higher concentration in water samples from Tagus River (S2) catchment than in water samples from Castelo de Bode Dam reservoir (S1) (Table 2). In S2, coliform bacteria presented 100% of occurrence (% occ.), with median values of 3255 MPN/100mL and maximum (max.) value of 24196 MPN/100mL; fecal coliforms (99.5% occ.) presented median values of 199 MPN/100mL, with a max. value of 1 6867 MPN/100mL. *E. coli* (99.4 % occ.) and intestinal enterococci (99.4% occ.) have been found with median values (max. value) of 103 (12033) MPN/100 mL and 28(630) cfu/100 mL, respectively. In contrast, in catchment S1, the same parameters presented lower occurrence % when compared with S2 (Table 2), namely for fecal coliforms (52.1%), *E. coli* (50.6%) and intestinal enterococci (40.0%). The high levels of fecal contamination in S2 may be related with the strong industrial, livestock and agricultural activities in the involving area of Tagus River. Since these human activities are some of the most important causes for the current contamination of surface water sources, proper regulatory restrictions and decision-maker awareness could play an important role to minimize the impact of this kind of environmental contamination.
Considering the Portuguese legal context, there is no mandatory maximum admissible value for the presence of microorganisms in surface water eligible to produce drinking water. There is only a recommended maximum value defined in the Portuguese Decreto-Lei n. º 236/98, for coliforms bacteria, fecal coliforms, and intestinal enterococci. In the six years of study, the presence of these pathogens in S1 were always below the maximum recommended value, whereas in S2 the counts for coliform bacteria and fecal coliforms presented 31.8 % and 1.1 % count values above the maximum recommended, respectively.

Regarding the chemical content, the concentration of nitrates and TOC presented higher values in S2, with a median value of 3.20 mg/L NO_3 (maximum value of 5.80 mg/L NO_3) and 3.91 mg/L C (maximum value of 6.92 mg/L C). In consistency with microorganism's results, the presence of these compounds is mostly related with agriculture and industrial activities (Seçkin et al., 2018). Considering Kjeldahl nitrogen and ammonia (Table 2) parameters, no quantified results were observed in the water supply system. For this reason, we decided to only include ammonia in the Table 2.

Regarding the measurement of microbiological parameters at the WTPs (T1 and T2), samples, T1 presented an overall occurrence for coliforms bacteria of 0.80 % (1 positive/124 measures) whereas T2 shown an occurrence of 3.60 % (4/111) (Table 2). For the remaining microbiological contamination, no occurrences were verified.
At the delivery points (D), more than 97.2 % of the samples did not show the presence of microorganisms (Table 2). Positive measurements were only observed for coliform bacteria (2.80 %; 112/3957) and *E. coli* (0.20 %; 6/3958). The occasional occurrence of bacteria in the water distribution network can be related to several causes, namely with the water matrix, development and/or resuspension of biofilms, cleaning of filters during the treatment process, some failure in the chlorination process, or occasional problems in the piping (Liu et al., 2002; WHO, 2004). Whenever anomalous concentrations of these microorganisms were detected in the water distribution network, the situation was promptly solved by the operations department of EPAL and the consumer safety and compliance with current legislation was ensured.

Regarding the occurrence of organic compounds in drinking water samples, the variation of total HAAs in the treated water and distribution network (T1, T2 and D) was assessed based on the 7 individual compounds. Results showed that the total HAAs content was mainly dependent on the prevalence of two individual compounds: DCAA (80.2 %, 68.4 % and 58.5 % occ for T1, T2 and D, respectively) and TCAA (10.3 %, 59.8 % and 21.6 % occ for T1, T2 and D, respectively) (Table 2).
When comparing the two different WTPs, the total HAAs occurrence % was slightly lower in T2 (70.4 % occ, with median concentration of 52 µg/L) when compared with T1 (81.0 % occ, with 12 µg/L median value), although the median concentration of total HAAs in T2 was around 4-fold higher than in T1. However, regarding the individual HAAs, the occurrences of TCAA was higher in T2 (59.8 %) when compared with T1

(10.3 %). These differences can be related with different chemical affinity according to physical-chemical parameters, such as temperature, pH adjustments or different oxidizing agents used in the two WTPs. Considering Vale da Pedra WTP (T2), the pre-oxidation step was changed from chlorine to ozone followed by disinfection with chlorine in 2017 (in contrast with Asseiceira WTP (T1), where the pre-oxidation and final disinfection steps are carried out with chlorine) and these could explain the decrease of DBPs formation, after 2017. This is consistent with literature, suggesting that ozone, when used as primary treatment, followed by chlorine could decrease the amount of DBPs such as HAAs (Richardson and Postigo, 2011).

HAAs were non-regulated DBPs in the European Union before 2020, thus information on their occurrence in drinking water is critical to understand the potential differences between the water treatment processes of the two WTPs studied. The knowledge of HAAs formation associated with the type of water treatment process is useful in the optimization of the water treatment process, in order to decrease HAA levels in drinking water. More recently, a new Directive 2020/2184 (EU) of the European Parliament and of the Council on the quality of water intended for human consumption has defined a parametric value of 60 µg/L, for the sum of 5HAAs (MCAA, DCAA, TCAA, MBAA and DBAA) (EU, 2020). Regarding this new recommended value, only one sampling date presented a total HAAs concentration above the parametric value (89 µg/L). Total HAAs presented a 60.8% occurrence, at the delivery points (D). The frequent occurrence of this group of DBPs in water samples suggest the importance of monitoring these compounds of emerging interest, providing background data that can be used to support their recent inclusion in the legal context.

Considering THMs, the occurrence percentage in both WTPs was 100%, with median value of 20 µg/L and 42 µg/L for T1 and T2, respectively. The parametric value established by European Directive 2020/2184 for THMs total is 100 µg/L. In both cases, the percentage of compliance with this water quality standard was 100 % (Table 2). At the delivery points (D), THMs were detected in 99.2% of the water samples. However, the concentration never exceeded the parametric value.

Many and diverse groups of unregulated and emerging DBPs are formed during these common chlorinated WTP (Chaves et al., 2020, 2019). Therefore, it is extremely important to improve the knowledge in unregulated DBPs, along with the continuing monitoring of the already regulated ones, in order to guarantee water safety.

Table 2. Summary of the monitoring results obtained for the target parameters in the surface water sources (S1, S2), post-treatment sampling points (T1, T2) and delivery points to the distribution network (D). LOQ refers to the limit of quantification.

Sampling point		Parameter	n (samples)	% positives (nr occurrence/ nr samples)	Average	Median	Max	LOQ	Legal limit*	% above legal limit
Surface Water	S1	Coliform bacteria (MPN/100 mL)	125	98.4	466	131	3445	0	5000 **	0.0
		Fecal coliforms (MPN/100 mL)	125	52.1	8.4	1.0	430	0	2000 **	0.0
		E. coli (MPN/100 mL)	125	50.6	14.5	1.0	667	0		
		Intestinal enterococci (cfu/100 mL)	125	40.0	2.3	0.0	23.0	0	1000 **	0
		Nitrates (mg/L NO3)	177	75.1	1.2	1.4	3.4	1	50	0
		Nitrites (mg/L NO2)	177	2.3	<LOQ	<LOQ	0.023	0.01	0.1	0
		TOC (mg/L C)	174	100.0	1.81	1.78	2.62	0		
		Ammonia (mg/L NH_4)	177	0.0	<LOQ	<LOQ	0.00	0.05	0.5	0.0
		BDCM (µg/L)	90	1.1	<LOQ	<LOQ	5.0	1.0		
		BrF (µg/L)	90	0	<LOQ	<LOQ	---	1.0		
		ClF (µg/L)	90	1.1	<LOQ	<LOQ	10.0	2.0		
		DBCM (µg/L)	90	1.1	<LOQ	<LOQ	1.6	1.0		
		THMs Total (µg/L)	88	1.1	<LOQ	<LOQ	16.0	2.0		
	S2	Coliform bacteria (MPN/100 mL)	179	100.0	4618	3255	24196	0	5000 **	31.8
		Fecal coliforms (MPN/100 mL)	183	99.5	356	119	6867	0	2000 **	1.1
		E. coli (MPN/100 mL)	180	99.4	402	103	12033	0		
		Intestinal enterococci (cfu/100 mL)	180	99.4	63.3	28	630	0	1000 **	0
		Nitrates (mg/L NO3)	787	98.1	3.2	3.2	5.8	1	50	0
		Nitrites (mg/L NO2)	182	35.7	0.022	0.020	0.092	0.01	0.1	0
		TOC (mg/L C)	179	100.0	3.91	3.91	6.92	0		
		Ammoniacal nitrogen (mg/L NH_4)	1534	31.6	<LOQ	<LOQ	0.7	0.05	0.5	0.07
		BDCM (µg/L)	69	0.0	<LOQ	<LOQ	---	1.0		

		BrF (µg/L)	69	0.0	<LOQ	<LOQ	---	1.0		
		ClF (µg/L)	69	0.0	<LOQ	<LOQ	---	2.0		
		DBCM (µg/L)	69	0.0	<LOQ	<LOQ	---	1.0		
		THMs Total (µg/L)	69	0.0	<LOQ	<LOQ	---	2.0		
Treated water	T1	Coliform bacteria (cfu/100 mL)	124	0.8	0	0	8	0	0	0.8
		Fecal coliforms (cfu/100 mL)	126	0.0	0	0	0	0	0	0
		E. coli (cfu/100 mL)	114	0.0	0	0	0	0	0	0
		Intestinal enterococci (cfu/100 mL)	126	0.0	0	0	0	0	0	0
		Combined chlorine (mg/L Cl2)	126	92.9	0.04	0.03	0.16	0		
		Residual chlorine (mg/L Cl2)	126	100.0	0.88	0.90	1.11	0.6	>0.6	100
		Total chlorine (mg/L Cl2)	126	100.0	0.92	0.94	1.20	0		
		Nitrates (mg/L NO3)	126	100.0	2.1	1.99	4.9	1	50	0
		Nitrites (mg/L NO2)	126	0.0	<LOQ	<LOQ	---	0.01	0.5	0
		TOC (mg/L C)	120	100.0	1.05	1.03	1.46	0		
		Ammonia (mg/L NH_4)	126	0.0	<LOQ	<LOQ	---	0.05		
		BCAA (µg/L)	114	0.0	<LOQ	<LOQ	---	8.0		
		BdCAA (µg/L)	34	0.0	<LOQ	<LOQ	---	25.0		
		DBAA (µg/L)	114	0.0	<LOQ	<LOQ	---	5.0		
		DCAA (µg/L)	116	80.2	11.1	12.3	46.0	5.0		
		MBAA (µg/L)	116	0.0	<LOQ	<LOQ	---	5.0		
		MCAA (µg/L)	114	0.0	<LOQ	<LOQ	---	8.0		
		TCAA (µg/L)	116	10.3	4.6	<LOQ	22.0	15.0		
		HAAs Total (µg/L)	116	81.0	13.1	12.0	46.0	5.0		
		BDCM (µg/L)	124	99.2	6.5	6.0	12.0	1.0		
		BrF (µg/L)	124	0.0	<LOQ	<LOQ	---	1.0		
		ClF (µg/L)	124	100.0	12.0	12.0	33.0	2.0		
		DBCM (µg/L)	124	100.0	2.0	2.0	3.7	1.0		

		THMs Total (µg/L)	124	100.0	20.4	20.0	48.0	2.0	100	0
	T2	Coliform bacteria (cfu/100 mL)	111	3.6	0	0	7	0	0	3.6
		Fecal coliforms (cfu/100 mL)	99	0.0	0	0	0	0	0	0
		E. coli (cfu/100 mL)	111	0.0	0	0	0	0	0	0
		Intestinal enterococci (cfu/100 mL)	111	0.0	0	0	0	0	0	0
		Combined chlorine (mg/L Cl2)	117	99.1	0.2	0.14	0.54	0		
		Residual chlorine (mg/L Cl2)	117	100.0	1.0	0.99	1.61	0.6	>0.6	97.4
		Total chlorine (mg/L Cl2)	117	100.0	1.2	1.15	1.96	0		
		Nitrates (mg/L NO3)	107	100.0	3.5	3.4	22.3	1	50	0
		Nitrites (mg/L NO2)	111	0.0	<LOQ	<LOQ	---	0.01	0.5	0
		TOC (mg/L C)	108	100.0	2.69	2.69	4.35	0		
		Ammonia (mg/L NH_4)	111	0.0	<LOQ	<LOQ	---	0.1		
		BCAA (µg/L)	92	0.0	<LOQ	<LOQ	---	8.0		
		BdCAA (µg/L)	31	0.0	<LOQ	<LOQ	---	25.0		
		DBAA (µg/L)	96	0.0	<LOQ	<LOQ	---	5.0		
		DCAA (µg/L)	98	68.4	16.8	17.0	47.0	5.0		
		MBAA (µg/L)	97	0.0	<LOQ	<LOQ	---	5.0		
		MCAA (µg/L)	93	0.0	<LOQ	<LOQ	---	8.0		
		TCAA (µg/L)	97	59.8	32.5	35.0	118	15.0		
		HAAs Total (µg/L)	98	70.4	47.8	52.0	154	5.0		
		BDCM (µg/L)	109	100.0	9.2	9.0	18.0	1.0		
		BrF (µg/L)	109	1.8	<LOQ	<LOQ	1.0	1.0		
		ClF (µg/L)	108	99.1	26.3	32.5	67.0	2.0		
		DBCM (µg/L)	109	97.2	2.6	2.3	7.1	1.0		
		THMs Total (µg/L)	109	100.0	37.9	42.0	85.0	2.0	100	0
Delivery points	D	Coliform bacteria (cfu/100 mL)	3957	2.8	0	0	100	0	0	2.8
		Fecal coliforms (cfu/100 mL)	---	---	---	---	---	0	0	0

		E. coli (cfu/100 mL)	3957	0.2	0	0	21	0	0	0.2
		Intestinal enterococci (cfu/100 mL)	4007	0.2	0	0	8	0	0	0
		Combined chlorine (mg/L Cl2)	4027	96.2	0.04	0.04	0.04	0		
		Residual chlorine (mg/L Cl2)	4030	100.0	0.65	0.64	0.64	0.6	>0.2	100.0
		Total chlorine (mg/L Cl2)	4030	100.0	0.69	0.68	0.68	0		
		Nitrates (mg/L NO3)	839	99.5	2.1	2.04	14.6	1	50	0
		Nitrites (mg/L NO2)	841	0.0	<LOQ	<LOQ	---	0.01	0.5	0
		TOC (mg/L C)	194	96.4	1.03	1.09	1.82	0		
		Ammonia (mg/L NH_4)	841	0.0	<LOQ	<LOQ	---	0.05		
		BCAA (µg/L)	176	0.0	<LOQ	<LOQ	---	8.0		
		BdCAA (µg/L)	176	0.0	<LOQ	<LOQ	---	25.0		
		DBAA (µg/L)	176	0.0	<LOQ	<LOQ	---	5.0		
		DCAA (µg/L)	176	58.5	6.8	6	37	5.0		
		MBAA (µg/L)	176	0.0	<LOQ	<LOQ	---	5.0		
		MCAA (µg/L)	176	0.0	<LOQ	<LOQ	---	8.0		
		TCAA (µg/L)	176	21.6	7.2	3	60	15.0		
		HAAs Total (µg/L)	176	60.8	11.6	6	89	5.0		
		BDCM (µg/L)	746	99.1	8.4	9.0	15.0	1.0		
		BrF (µg/L)	745	31.5	1.1	0.2	12.0	1.0		
		ClF (µg/L)	746	98.1	17.6	19.0	45.0	2.0		
		DBCM (µg/L)	746	98.8	3.5	3.3	20.0	1.0		
		THMs Total (µg/L)	746	99.2	30.5	31.0	60.0	2.0	80	0

*applied when the legal limit is different the internal limit used to measure the % of occurrence; **recommended maximum value for natural waters (Decreto- Lei n. º 236/98); MPN: most probable number; cfu: colony forming unit.

3.2 – Seasonal profiling of target parameters (2014 – 2019)

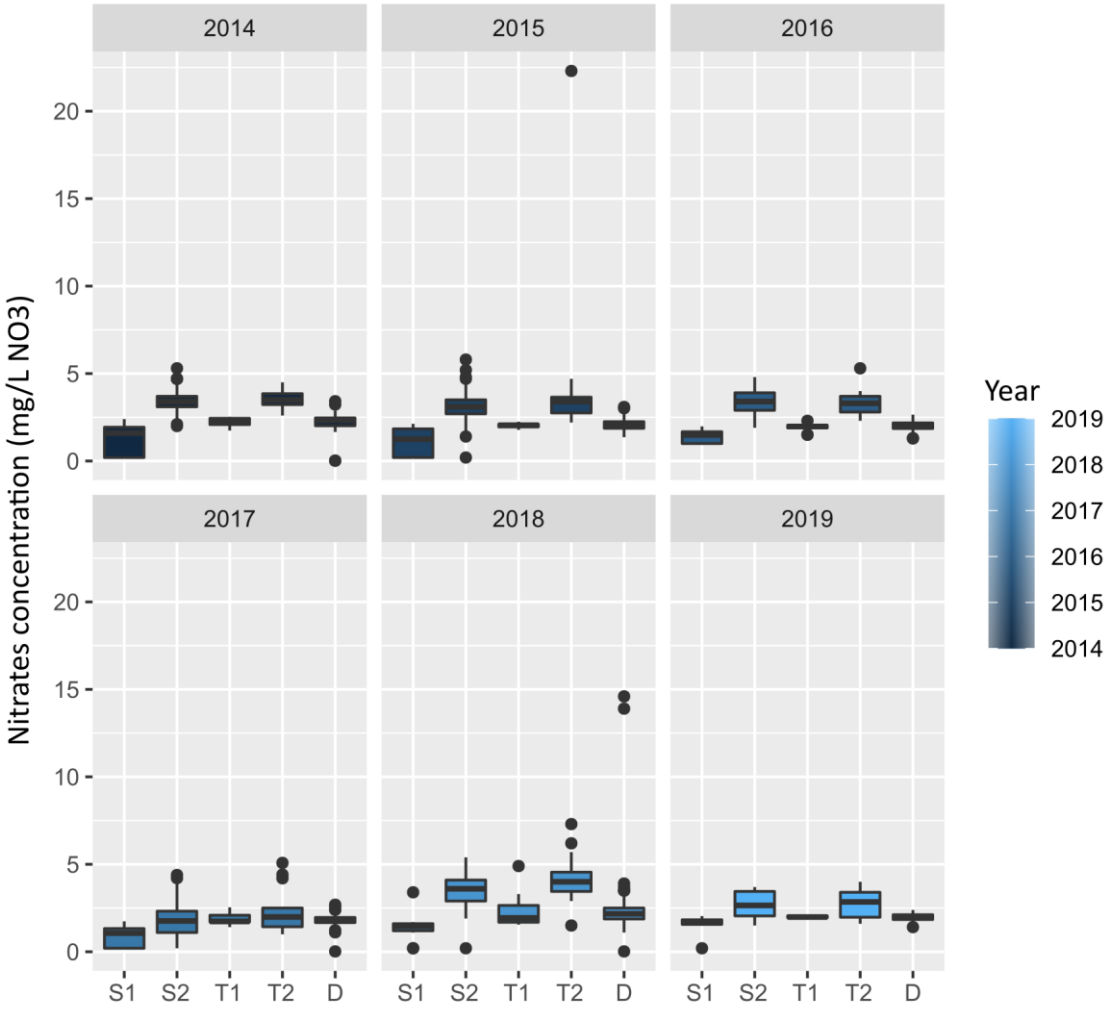

Figure 2. *Nitrates seasonal profile throughout the water supply system, considering the two studied surface water sources (S1 and S2); both treatment plants (T1 and T2) and all delivery points to the distribution network (D).*

Nitrates content was measured at all the three components of the water supply system. A consistent profile between surface water source and treated water at the outlet of the WTP was identified, meaning that the concentrations were similar before and after water treatment in most of the sampling campaigns carried out. Comparing nitrate results in the two surface water sources, S2 presented higher concentrations than S1, which justifies the corresponding relation of T2 > T1 (Figure 2). In 2018, both S2 and T2 presented slightly higher concentration levels, when compared with the other studied years. These 2018 results could be related with several specific environmental factors, such as climate (with a prolonged drought period) and industrial contamination, that lead to higher levels of biomass in the

Tagus river. Regarding the delivery points to the distribution network (D) results, their seasonal profile presents a median concentration similar with the T1 values, however with a higher dispersion of results. This wider range of measurements could be related with the fact that the water at D represents a mixture of waters from both WTPs (with a higher contribution of S1) and a small contribution of groundwater sources. In addition, environmental contamination factors of the surface water sources related with each WTP should also be considered.

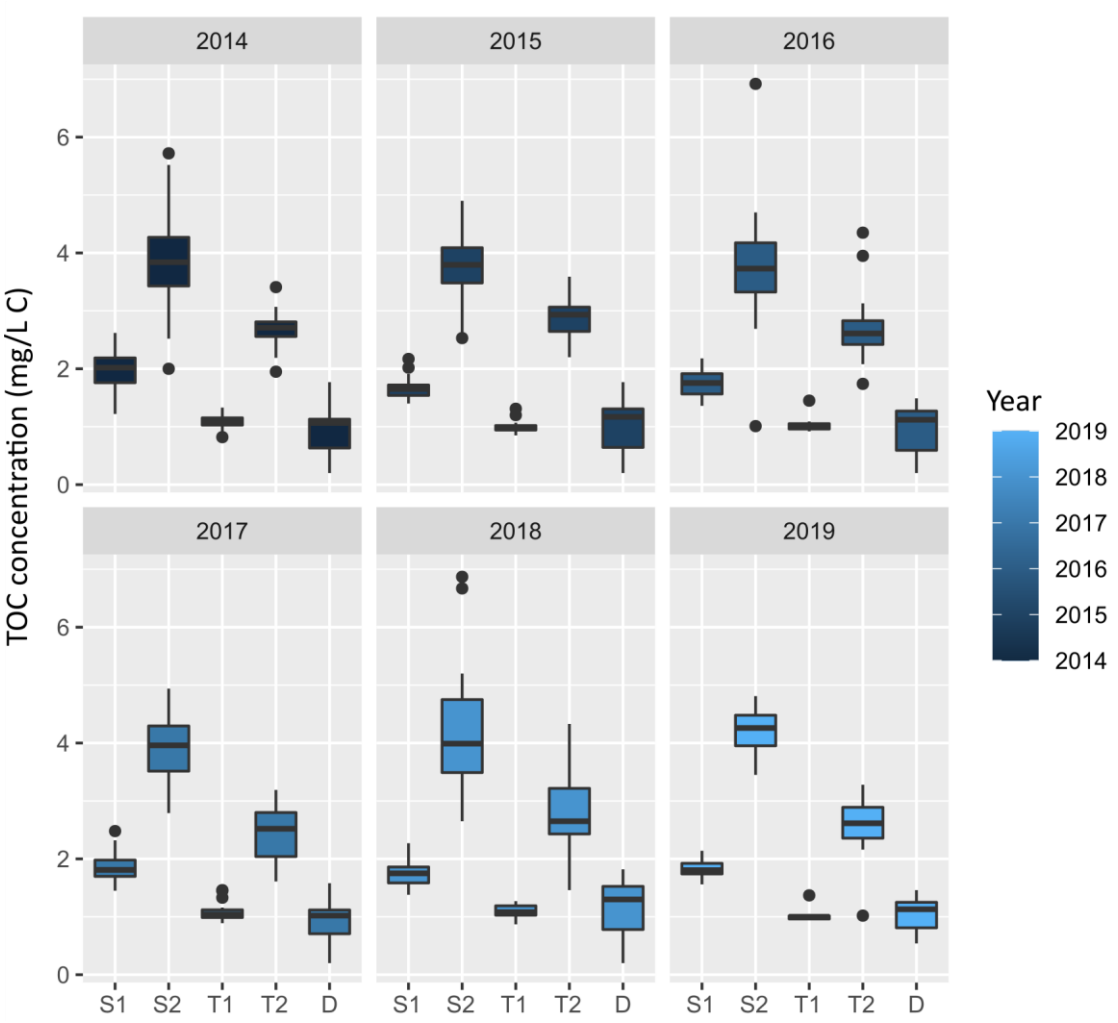

Figure 3. *TOC seasonal profile throughout the water supply system, considering both surface water sources (S1 and S2); both WTPs (T1 and T2) and all the delivery points to the distribution network (D).*

As observed with nitrates, TOC presented higher concentration levels in S2 when compared with S1 (Figure 3), with the same profile being observed between the corresponding WTPs

(T2 > T1). This is consistent with the higher levels of environmental contamination expected in S2. The decrease of TOC content after treatment is notable, which means that the treatment technologies are removing organic matter efficiently. Comparing the two WTPs and the corresponding surface waters, it is observed that even the treated water T2 (Vale da Pedra WTP) presented higher TOC levels that surface water source S1 (Castelo de Bode reservoir) and the corresponding treated water T1 (Asseiceira WTP). These results support the current EPAL's decision of selecting surface water from Castelo de Bode as the main contributor for the global distribution network. In accordance, the delivery sampling points (D) presented low concentration values, similar to T1 levels, despite the influence of the other water sources.

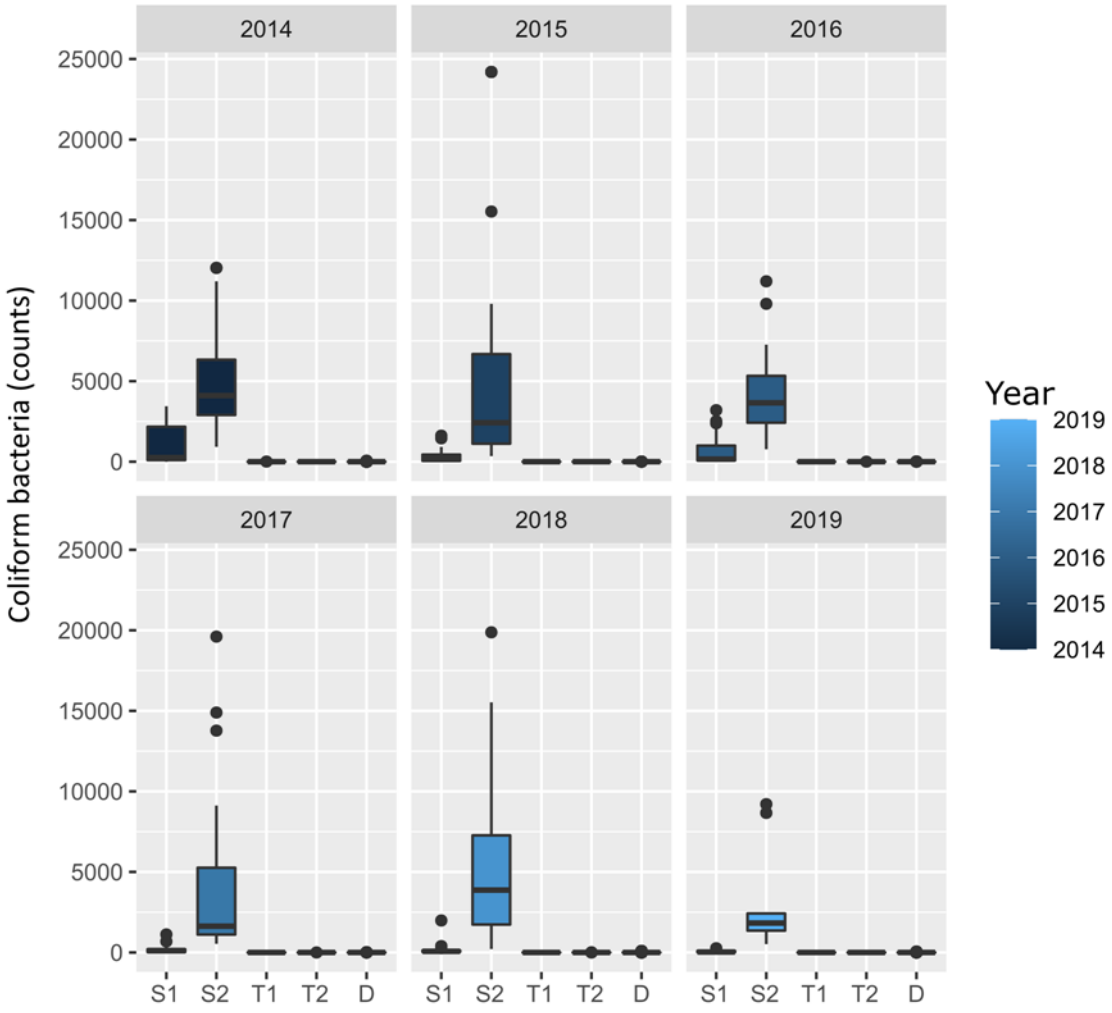

Figure 4. *Coliform bacteria seasonal profile throughout the water supply system, considering both surface water sources (S1 and S2); both treatment plants (T1 and T2) and all the delivery points to the distribution network (D).*

In terms of microorganisms (Figure 4), S2 presented higher levels of contamination by coliforms bacteria than S1. This is in line with the trend already observed in the inorganic parameters, possibly due to the higher pollution levels in the S2 catchment area. Results showed that the water treatment processes were effective, significantly reducing the presence of coliforms in treated water, with seasonal occurrence varying between 0.0% and 8.7 % for T2 and 0.0 % and 4.2 % for T1. Comparing the results of the outlets of the WTPs and D, it is observed that in specific dates, the regrowth of coliforms occurred, with maximum counts of 8 cfu/100 mL in T1 (1/124, total occurrence 0.8 %), 7 cfu/100 mL in T2 (4/111, total occurrence of 3.6 %) and 100 cfu/100 mL in D (112/3957, total occurrence of 2.8 %).

The results of years 2017 and 2019 showed a significant decrease in coliforms counts in samples collected at the surface water sources (S1 and S2). In fact, for S1, the median values in 2017 and 2019 were 115 and 28, respectively, compared with medians values above 160 obtained in the previous years (2014-2016). For S2, the same trend was observed with median values of 1630 and 1826 for years 2017 and 2019, respectively, in comparison with medians above 3650 for previous years.

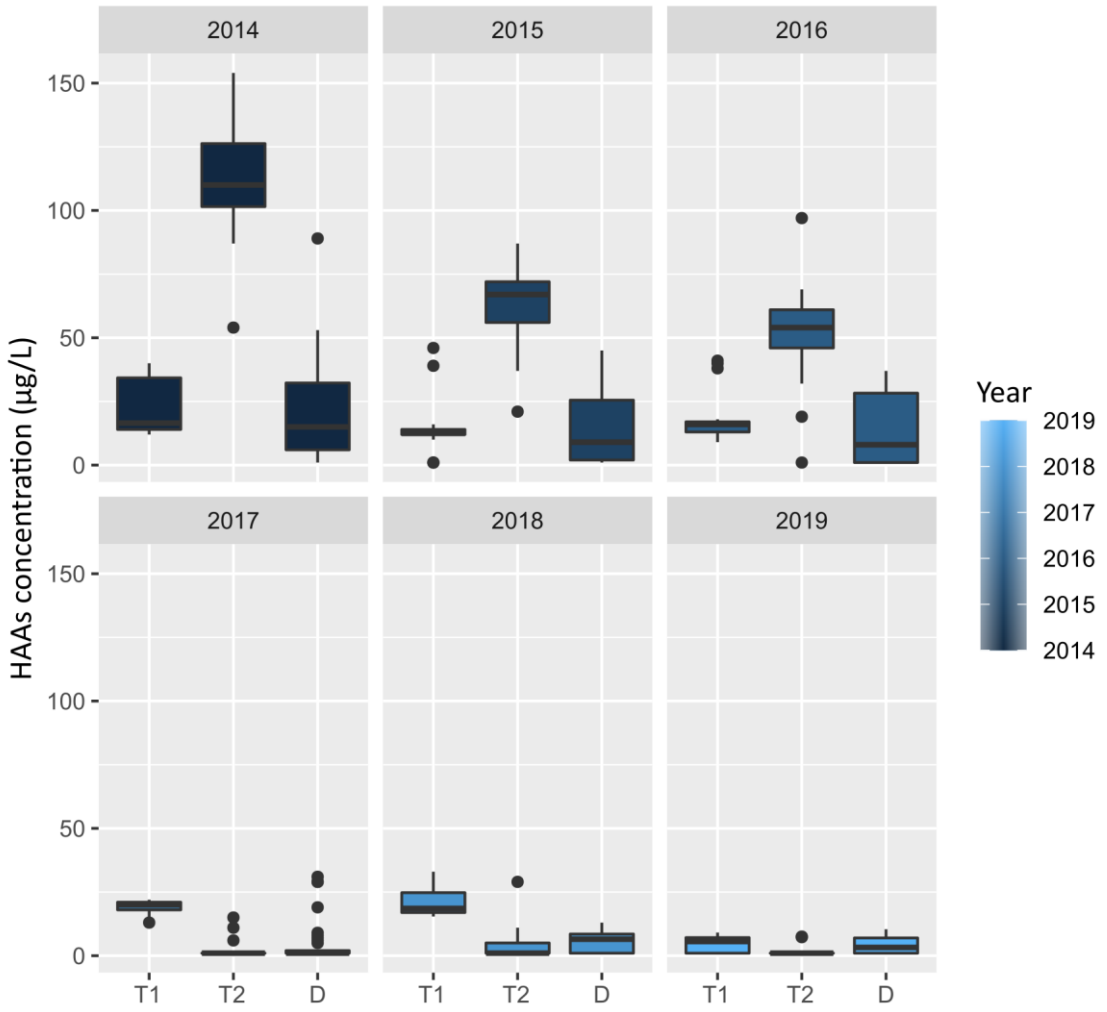

Figure 5. Seasonal HAAs behaviour, considering both WTPs (T1 and T2) and all the delivery points to the distribution network (D).

In terms of DBPs, the results indicated a similar behaviour of HAAs concentration along the water distribution network system, with a trend of lower HAAs levels in the delivery sampling points in comparison with the outlet of the WTPs.

Considering the WTPs, higher levels of HAAs were detected in T2 in comparison with T1, 3 to 7-fold (Figure 5), in the period 2014-2016. After 2017, the HAAs levels trend have changed, with lower levels of HAAs identified in T2. This reduction at T2 is related with the reformulation/optimization of the treatment processes carried out in this WTP, mainly the use of ozone instead of chlorine in the pre- oxidation step. This is consistent with other reported studies, that identified the decrease of HAAs concentration when ozone is used as a primary oxidant (Gunten, 2003; Karnik et al., 2005; Papageorgiou et al., 2014; Richardson et al., 2015). T1 levels have maintained a similar profile along the years.

In fact, in the period 2017-2019, the HAAs range of concentrations became lower and narrower throughout the distribution network system, particularly in T2. In this period, HAAs presented median values lower than 6 µg/L for T1; lower than LOQ for T2; and lower than 6.5 µg/L for D, significantly lower than previous years occurrence, with values above 13 µg/L for T1, above 54 µg/L for T2 and above 8 µg/L for D. This is consistent with other reported studies, which identified the decrease of HAAs concentration when ozone is used as a primary oxidant.

The decrease of HAAs levels in T2 lead to a narrow amplitude of values in D.

Although chlorination is still the most widely applied disinfection method to inactivate pathogenic microorganisms, ozone has been recognized as an effective alternative, reducing the volume of some chlorine-based DBPs formed, such as THMs and HAAs (Karnik et al., 2005; Park et al., 2016; Richardson et al., 2015). However, ozonation can result in a higher production of other DBPs, such as aldehydes, ketones and bromate (Aragone et al., 2012; Richardson et al., 2015). Bromate is particularly problematic because it is not biodegraded in biological filters which usually follow an ozonation step and it is particularly toxic (Gunten, 2003). Due to that, in this study we assessed the bromate content in treated water and delivery points. However, all the measurements were lower than the LOQ.

HAAs are an unregulated group of DBPs, so it is very important to evaluate the behaviour of this type of compounds to understand the effects of different water technologies in HAAs formation, because sometimes the concentration levels of regulated organic compounds in drinking water can be minimized but some other emerging and unregulated organic compounds might be formed. In consistency with literature, emerging and unregulated DBPs presented higher toxic effects when compared with some already regulated ones (Chaves et al., 2019; Mian et al., 2018). Understanding the target parameters behaviour is crucial to improve the safety of a water supply system and the development or improvement of water technologies.

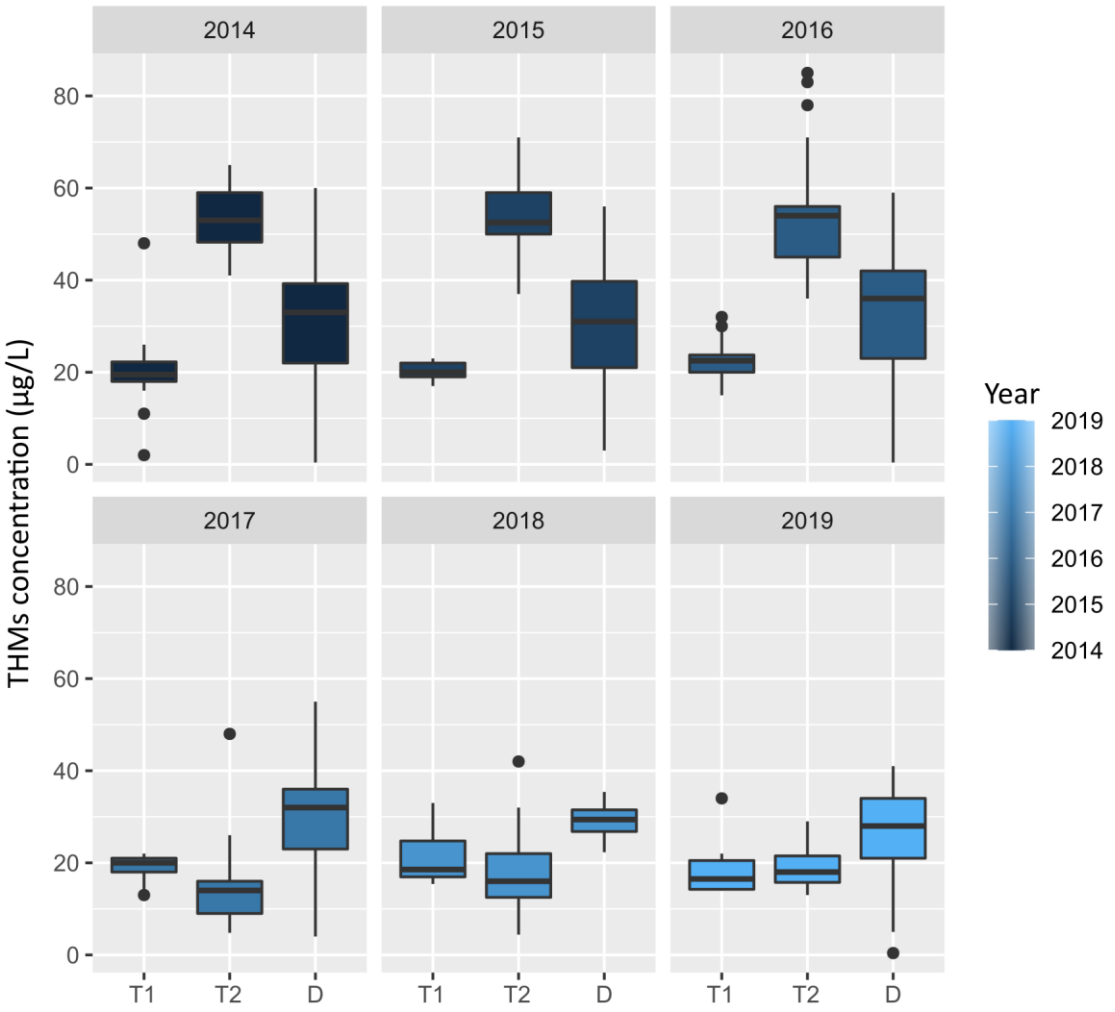

Figure 6. *Seasonal THMs behaviour, considering both WTPs (T1 and T2) and all the delivery points to the distribution network (D).*

Regarding THMs, in the period of 2014-2016, T2 presented a higher content when compared with T1, approximately 2-fold higher. After 2017, concentration levels of THMs in T2 decreased significantly, while T1 did not show seasonal variations. This behaviour was similar to the observed in HAAs, proving that the reformulation/optimization in the treatment scheme used in T2, leading to the overall decrease of THMs content.

Globally, THMs in D samples showed a similar profile throughout the years, with annual median values varying between 36 µg/L (2016) and 27 µg/L (2019). THMs levels in deliveries (D) presented a higher median concentration when compared with the outlet of WTPs, considering the bigger contribution of T1 to the distribution network. This increasing behaviour could be related with the nitrification levels, reducing the disinfectant stability, stimulating the residual disinfectant decay and THMs production during the water distribution network system (Li et al., 2019; Zeng and Mitch, 2016).

Temperature, pH, nutrients, water age and disinfectant residual have been found to have high impacts on nitrification occurrence since they were reported to influence both chemical and microbiological changes. In fact, water quality in piped drinking water distribution systems depends on complex interactions between the microbial community (abundance, diversity and growth rates of microorganisms), and chemical and physical conditions (Kennedy et al., 2021). Nitrification in distribution systems, were reported to depend on pipe materials and nutrient concentrations, and their extension along the water supply system could be related with higher levels of nitrifiers activity and population activities (Cruz et al., 2020; Li et al., 2019; Lipponen et al., 2002).

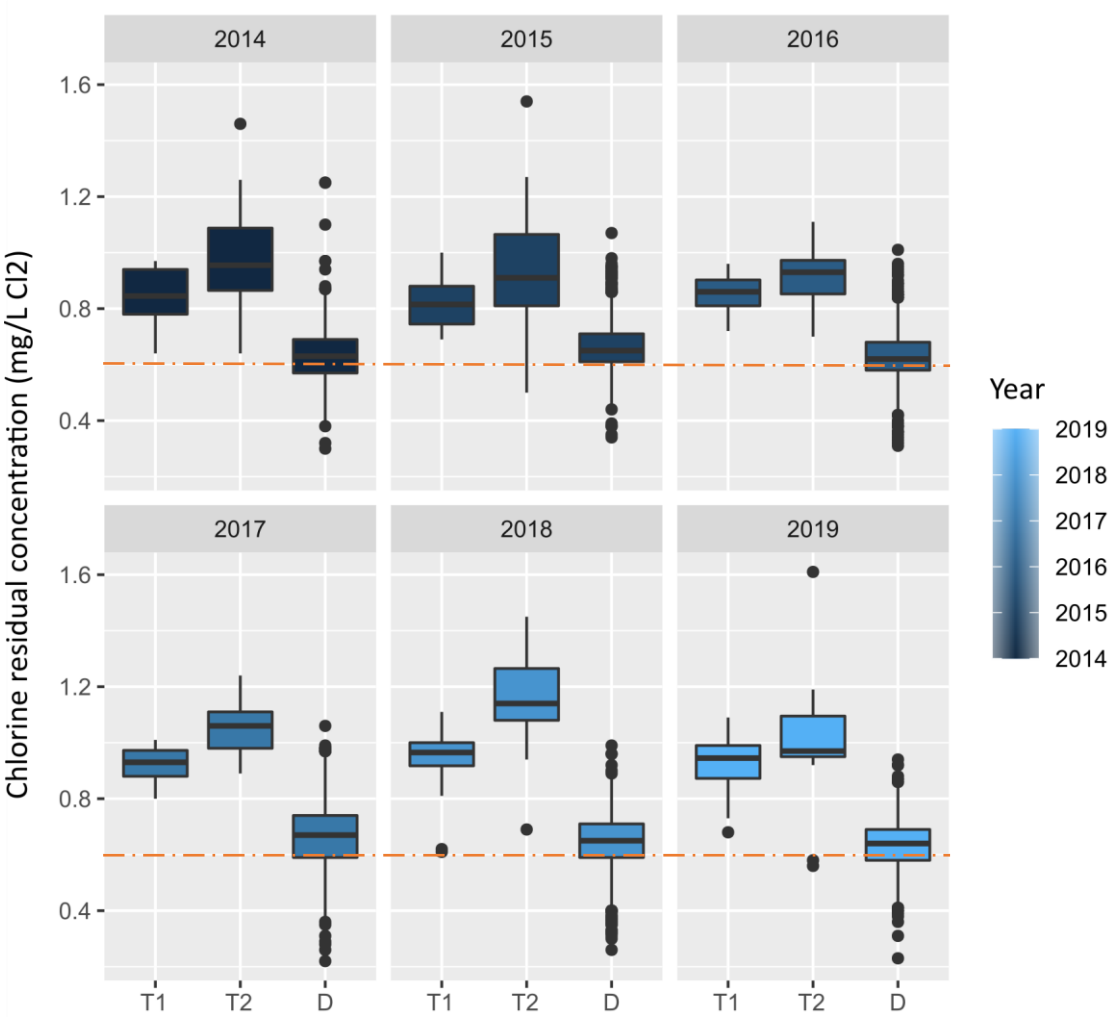

Figure 7. *Seasonal residual chlorine behavior, considering both WTPs (T1 and T2) and all the delivery points to the distribution network (D). Red dashed line: 0.6 mg/L Cl_2, minimum recommended value of residual chlorine after treatment.*

Chlorine results (Figure 7) showed a consistent seasonal variation, with slightly higher levels measured in T2 when compared with T1, and these could be related with the higher amounts

of organic matter present in the surface water source S2. It is important to maintain appropriate chlorine concentration throughout the distribution network, considering factors such as quality of surface water, extension of distribution network, operational conditions (i.e. pH and temperature), properties and age of pipe materials, among others (Kim et al., 2014; Li et al., 2019). In accordance with the Portuguese legal context, the levels of residual chlorine should be higher than 0.6 mg/L Cl_2, after treatment (DL 152/2017, 2017). This requirement was totally fulfilled in T1 samples (100 %) whereas in T2 there were 3 dates with values lower than the recommended value, but higher than 0.5 mg/L CL_2, representing 97 % of compliance.

3.3. Correlations between target parameters

Correlation approaches were applied in order to assess the possible quantitative relations between the target quality parameters measured (Figures 8 and 9). The correlation plots expressed the distribution of each variable (diagonal); the bivariate scatter plots between each pair of parameters; and the correlation values and significance levels expressed in stars (*). Correlation plots between i) parameters that characterized each surface water source and the corresponding water treatment plant, namely S2 and T2 (see Figure 8) and between S1 and T1 (see supplementary information, Figure S2); and ii) deliveries parameters (Figure 9) were built.

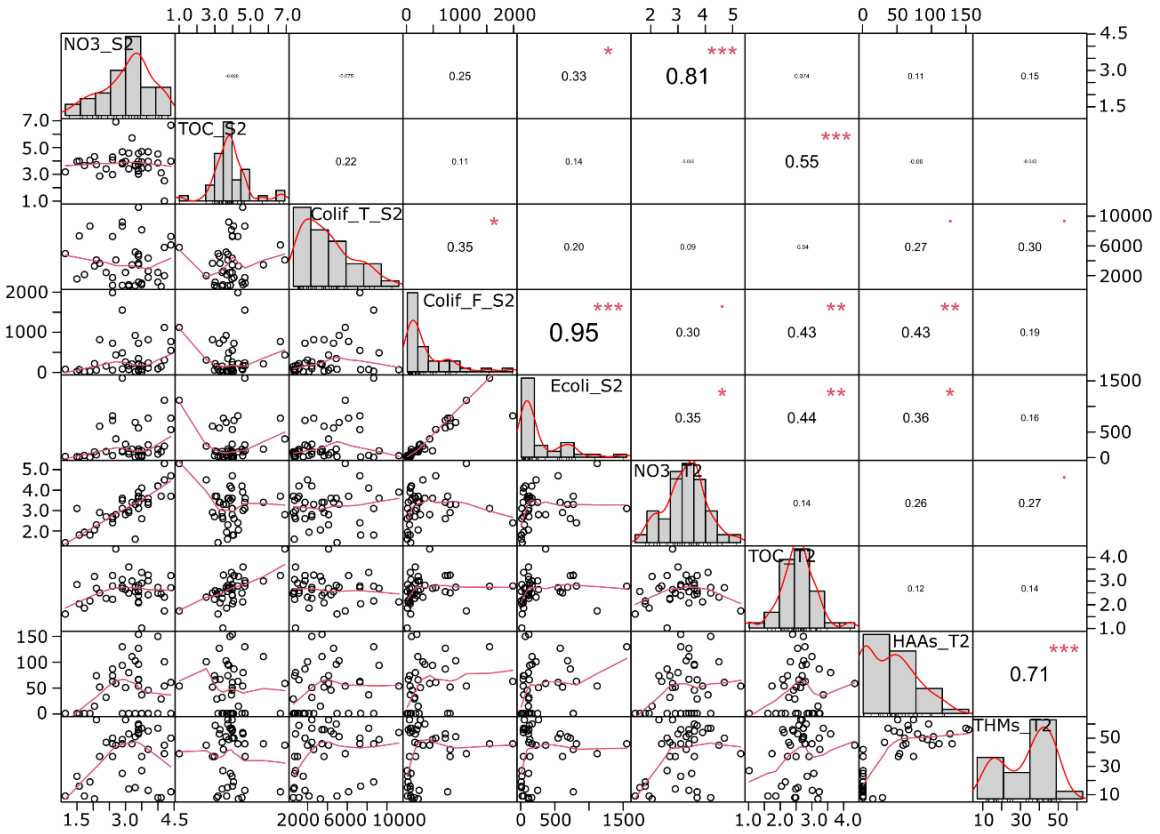

Figure 8. *Spearman correlation plots between the target monitored parameters between surface water source S2 and WTP T2 (System 2), over 6-year period (2014-2019) (n=41). * p values ≤ 0.05; ** ≤ 0.01; ***≤ 0.001.*

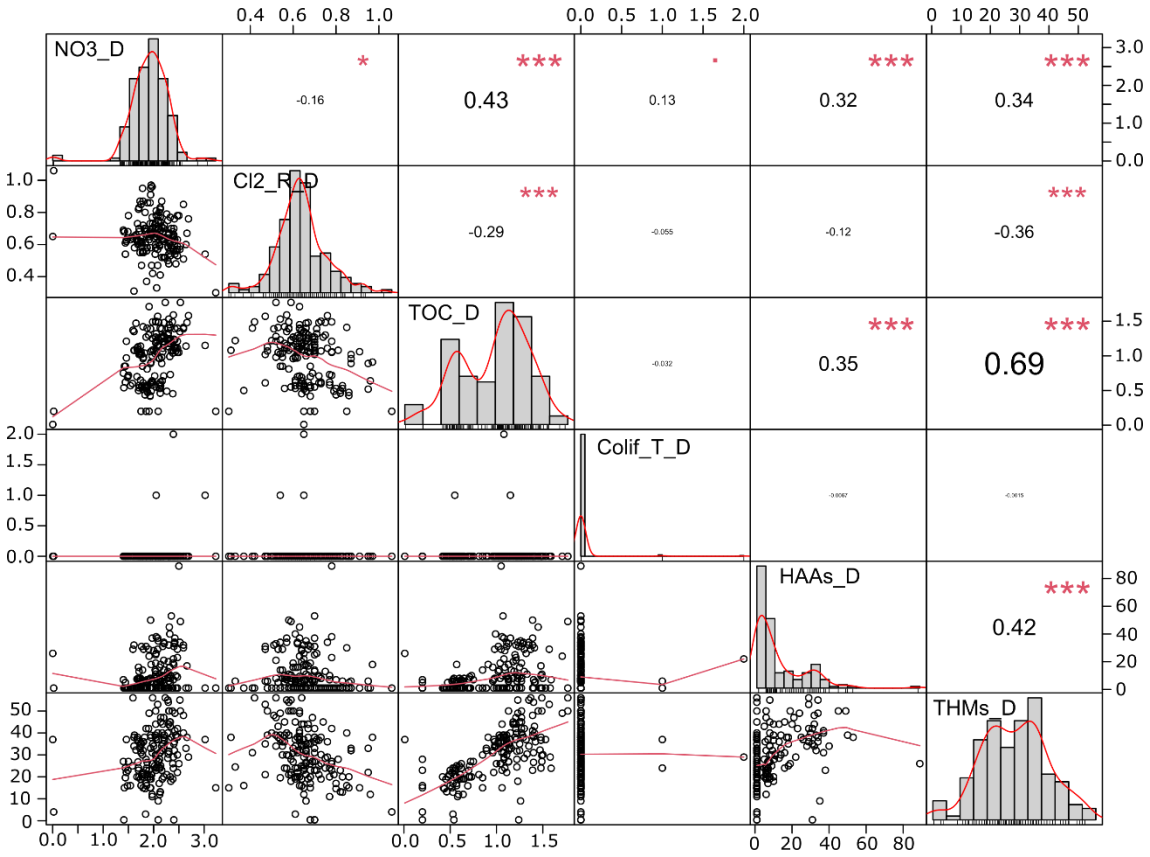

Figure 9. *Spearman correlation plots between the target monitored parameters in all the delivery points to the distribution network (D), over 6-year period (2014-2019) (n=171). * p values ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001.*

Considering System 2, a strong correlation between coliforms and *E. coli* (r = 0.95; p ≤ 0.001) was observed at S2 level (Figure 8).

When comparing surface water source S2 and the corresponding outlet of WTP T2 relations, fecal coliforms presented a moderate correlation (r = 0.43; p ≤ 0.01) with HAAs produced. This fact suggests that microbial contamination (anthropogenic occurring matter) is a potential precursor for HAAs formation, probably due to the disinfectant dosage necessary for fecal elimination. This reactivity with disinfectants will promote the DBPs formation, in particular in this system, for HAAs (Postigo et al., 2018). In addition, nitrates and, in a lesser extent, TOC also showed strong correlations (r = 0.81; p ≤ 0.001) and (r = 0.55, p ≤ 0.01), respectively, suggesting that their removal is limited due to the higher amounts in surface water.

At WTP level (T2), the organic compounds THMs and HAAs presented a strong correlation (r = 0.71; p ≤ 0.001). Both group of DBPs showed to be increased in the specific conditions

of S2, suggesting higher amounts of DBPs precursors, such as organic matter and microorganisms, and consequently requiring higher levels of disinfectants (Kim et al., 2014; Li et al., 2019; Richardson et al., 2015; Seçkin et al., 2018).

Considering System 1 (Figure s2, in supplementary information), nitrates (T1) presented a statistically significant correlation with HAAs in T1 ($r = 0.55$; $p \leq 0.001$) and with TOC in S1 ($r = 0.47$; $p \leq 0.001$).

Considering deliveries (D) (Figure 9), a strong correlation was observed between TOC and THMs production ($r = 0.69$; $p \leq 0.001$). TOC and HAAs ($r = 0.35$; $p \leq 0.001$) also presented a statistically significant correlation, although weaker when compared with THMs. This is consistent with current knowledge about DBPs production, since TOC is the measurement of natural organic matter (NOM) content, such as humic as fulvic compounds, which are precursors for DBPs formation (Chaukura et al., 2020; Mian et al., 2018; Postigo et al., 2018)**.** TOC also presented a statistically significant correlation with nitrates ($r = 0.43$; $p \leq 0.001$).

Nitrates presented moderate correlations with HAAs ($r = 0.32$, $p \leq 0.001$) and THMs ($r = 0.34$, $p \leq 0.001$), stressing the importance of inorganic content in the DBPs formation, regarding drinking waters.

Considering the DBPs group, a moderate correlation was identified for THMs and HAAs ($r = 0.42$; $p \leq 0.001$). This positive relationship is consistent with the correlation in T2, justified by their common formation mechanisms as chlorinated-DBPs (Du et al., 2017; Plewa et al., 2017; Richardson et al., 2015).

4. Conclusions

This work provides new knowledge regarding occurrence and seasonal behaviours of both microbiological and chemical parameters, essential to understand the water supply system and the technologies involved. The outcomes of this study can help water supplier companies to make a comprehensive analysis of water treatment plants, sampling campaigns and water safety plans. Understand, select and optimize the water treatment processes is crucial for the mitigation of human health risk, attending both regulated and non-regulated compounds, in drinking water.

Globally, the EPAL's water supply system presented improvements in water quality parameters control. Attending to the number of parameters in the legal context, the requirements have been accomplished in all the sampling points assessed. Comparing the two studied systems it was observed that System 2 presented higher levels of TOC and microorganisms, suggesting that this higher load led to the increased levels of DBPs in WTP2. After 2017, a sharp decrease of DBPs occurrence was identified in WTP2 due to treatment optimization in the pre-oxidation step by replacing chlorination for ozonation, reflecting improvements in the technologies employed in the WTPs. Correlations between the different measured parameters were established allowing to identify relevant occurrence relationships. Strong correlations were highlighted between TOC and THMs, at deliveries level; both chlorinated- DBPs (HAAs and THMs) at WTPs level; coliforms bacteria and *E. coli*, at catchments level; and nitrates content before and after treatment.

These outcomes play an important role in the establishment of a comprehensive evaluation of all the water supply system, allowing to identify potential improvements in both WTPs technologies and distribution network. These strategies are closely dependent also of the environmental modification, climate changes and anthropogenic pressure. In line, supporting water management decisions and production services, with more integrative and comprehensive tools is particularly relevant to face the challenges related with climate alterations. All these variables have been contributing to a growing awareness for compounds with emerging concern in drinking waters, such as a huge diversity of other unregulated DBPs.

Therefore, the local and continued monitoring of water supply systems combined with a comprehensive data assessment, are crucial to implement new approaches always guarantying the best quality of water for human consumption.

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CHAPTER III. Development of multi-residue gas chromatography coupled with mass spectrometry (GC-MS) methodologies for the measurement of 15 chemically different Disinfection By-products (DBPs) of emerging concern in drinking water from two different Portuguese water treatment plants.

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Abstract

Along Water Treatment Plants (WTPs), chemical agents, such as chlorine and ozone, might react with organic matter and anthropogenic contaminants, forming a high diversity of Disinfection By-products (DBPs). Due to the potential toxicological effects, the identification of unregulated DBPs (UR-DBPs) is critical to help water managers in the selection of effective water treatment processes, contributing to improve water safety plans. Given the limited validated analytical methods to detect UR-DBPs, here we developed new multi-residue gas chromatography coupled with mass spectrometry methodologies for the detection and quantification of 15 UR-DBPs, including aldehydes, haloketones (HK), nitrosamines and alcohols, in drinking water matrices. Solid-phase extraction (SPE), for nitrosamine group, and solid-phase micro extraction (SPME), for the remaining DBPs, were used as sample preparation method. The developed methodologies allowed the quantification of target UR-DBPS at trace concentration levels (ng/L), with method quantification limits (MQLs) ranging from 14.4 ng/L to 26.0 ng/L (SPE) and 2.3 ng/L and 1596 ng/L (SPME). The methods were applied to different drinking water matrices, considering distinct delivery points of EPAL - Empresa Portuguesa das Águas Livres WTPs. Overall, aldehydes group, represented by decanal, nonanal and 2-ethylheaxanal, showed the highest occurrence, followed by HKs and nitrosamines. The results of this study suggested that the formation of these UR-DBPs should be further monitored in WTPs.

1. Introduction

The use of anthropogenic, household, industrial or agricultural chemicals such as pharmaceuticals and personal care products (PPCPs), hormones, pesticides and herbicides, plasticizers, UV filters, is ubiquitous and they have been recognized as a source of environmental contamination (Torres et al. 2016; Macedo et al. 2017). In consequence, an increasing awareness of the regulatory sectors have arisen in order to minimize potential impacts in the environment and health. The majority of this chemicals co-exists with natural organic content, such as humic and fulvic acids, in the urban water cycle (Plewa et al. 2017; Chaukura et al. 2020). This topic has particular relevance at drinking water level because these precursors react with chemicals agents used in the water treatment process, forming Disinfection By-products (DBPs) (Chaves et al. 2019). Disinfected drinking water contains

a high diversity of DBPs, with their potential toxicological effects and hazard characterization being still unclear. In addition, few studies have been focused on the comprehensive non-target analyses of emerging/unregulated DBPs, towards a better characterization of the complete DBP exposure (the exposome), and their relationship with the diverse water treatment methodologies and process variables (Kimura et al. 2019).

The DBPs formation occur particularly in the disinfection and coagulation/flocculation steps of WTPs, with the addition of oxidant agents, such as chlorine, chlorine dioxide, chloramine, and ozone, with the purpose of inactivate microbial organisms and coagulate inorganic content. The characteristics of organic matter (OM), temperature, pH, disinfectant/coagulant type and applied dose, and disinfectant residual stability in drinking water distribution system were found to be among the most important factors to understand DBPs formation (Li et al. 2019; Chaukura et al. 2020).

Several hundreds of DBPs belonging to different classes of compounds have been identified in drinking water (Richardson et al. 2015; Postigo et al. 2017; Yang et al. 2018). However, just a few, including trihalomethanes (THMs), chlorite, bromate and a sum of five haloacetic acids (HAAs) are currently regulated in European Union by Drinking Water Directive 2020/2184 (EU) and in the United States by USEPA, under the Stage 1 and Stage 2 Disinfectants and DBPs Rules (EPA 2009; EU 2020). In addition, NDMA and 4 other nitrosamines are also indicated on the U.S. EPA's Contaminant Candidate List (CCL-4), a priority list of drinking water contaminants (EPA 2020). Word Health Organization (WHO) also reported NDMA, one of the most studied nitrosamine, in their guidelines for drinking water quality, establishing a guideline value of 100 ng/L (WHO 2011). In accordance three countries and territories have already set a regulatory/guideline value in drinking water (100 ng/L), namely Australia, California and Singapore (NHMRC 2011; Boards 2018; SEPH 2019).

Still, it is estimated that 50% of organic halogens formed during water treatment are unknown and their toxicological risk poorly characterized (Li et al., 2017a). Unregulated and emerging DBPs includes an array of classes of compounds such as halomethanes, halobenzoquinones, iodo-trihalomethanes, aldehydes, nitrosamines, among others (Richardson et al. 2015; Plewa et al. 2017; Mian et al. 2018; Florina et al. 2020).

DBPs are of particular interest due to their potential toxicological effects for human health and ecosystems, namely carcinogenicity and various non-carcinogenic effects, such as

endocrine disruption (Chaves et al. 2019). Importantly, several unregulated DBPs have been shown to be more genotoxic and cytotoxic than several regulated compounds. In fact, some studies support the need to improve knowledge in emerging and unregulated DBPs, highlighting that the toxicological effects of these unregulated DBPs seems to be potentially higher when compared with the already regulated ones (Chaves et al. 2020). Understanding the public health implications of these compounds of emerging concern in drinking water and their unclear biological mechanism, is crucial for societies, decision-makers and water safety plans (Mian et al. 2018).

Given the limited occurrence information and analytical protocols available to detect unregulated and emerging DBPs, in this work, we developed new gas chromatography (GC)-mass spectrometry (MS) methodologies for the detection and quantification of 15 UR-DBPs from different chemical groups: nitrosamines (N-nitrosodimethylamine (NDMA); N-nitrosodiphenylamine (NDPhA); N-nitrosomorpholine (NMOR); N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP)); aldehydes (2-ethyl-hexanal (2-EH); nonanal and decanal); ketones (1,1-dichloroacetone (1,1-DCA); 1,1,1-trichloroacetone (1,1,1-TCA); 1,3-dichloroacetone (1,3-DCA) and acetophenone); and alcohols (1,3-dichloropropanol; 3-chloro-2,2-dimethylpropanol and 2-ethyl-hexanol).

The selection of the target chemicals took in consideration (1) compounds identified in drinking waters by preliminary GC-MS untargeted analysis performed by EPAL - Empresa Portuguesa das Águas Livres, SA (unpublished data), the major Portuguese drinking water supplier, for which limited or no occurrence information is described in literature; and (2) compounds with toxicological data available, supporting the importance of their regular monitoring towards improving the knowledge about their occurrence in drinking water (Raksit and Johri 2001; Serrano et al. 2015b; Sieira et al. 2020).

2. Material and methods

2.1. Water samples

EPAL's water supply system includes the production, storage, adduction/ transport and the distribution systems. Currently, the company is responsible for the water supply to consumers in a total area of 7,095 km², which represents a population more than 2.8 million inhabitants. The local and continuous monitoring of water systems are crucial to implement

new approaches to guarantee the best quality of drinking water throughout the supply systems.

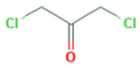
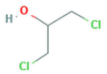
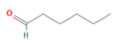
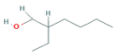
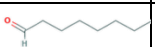
Twenty finished drinking water samples from different points of EPAL's distribution system, including two geographically different and conventional water treatment plants (WTPs), with distinct production capacities, Asseiceira and Vale da Pedra WTPs (Figure S1, supplementary information) were periodically sampled, between December 2019 and March 2020. The Asseiceira WTP treats the water from Castelo de Bode reservoir, that is one of the larger sub systems of EPAL's supply system representing about 75% of the company's production capacity. Asseiceira WTP has the capacity to produce around 625,000 m^3/day of water for human consumption and the water is treated through several steps: pre-oxidation with chlorine, remineralization, and correction of aggressiveness, coagulation/flocculation, filtration with sand filters, pH correction and disinfection with chlorine. Vale da Pedra WTP produces water for human consumption from Tagus surface water, with a daily capacity of 240,000 m^3 daily and water treatment includes several steps: pre-oxidation with ozone, pH adjustment, activated carbon adsorption, coagulation/flocculation, sedimentation, filtration with sand filters, pH correction and disinfection with chlorine. In emergency cases of increasing pollution in the Tagus river catchment, another pre-oxidation step using potassium permanganate is available in this WTP. In general, the technologies employed in the selected WTPs are used worldwide and the main difference between the conventional WTPs are the oxidant agent used during pre-oxidation step (chorine or ozone, for Asseiceira and Vale da Pedra WTPs, respectively). For SPE-GC-MS analysis, drinking water samples (one replicate per sample) were collected headspace-free in 1 L amber glass bottles containing sodium thiosulfate to quench the residual disinfectant. Prior to extraction and GC-MS analysis, samples were stored at 5±3 ℃ with storage times between <24 h and 3 days. For SPME-GC-MS the samples were collected in triplicate, directly in vials correctly sealed, to perform the analysis.

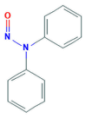
2.2. Standards and reagents

All analytical standards were of high purity (mostly 98%) and were provided by TCI America (1,1-trichloroacetone, CAS 918-00-3; 2-ethyl-1-hexanal, CAS 123-05-7; and 3-chloro-2,2-dimethyl-1-propanol, CAS 13401-56-4); by Dr. Ehrerstorfer (N-

nitrosodimethylamine, CAS 62-75-9; N-nitrosodiphenylamine, CAS 86-30-6; N-nitrosomorpholine, CAS 59-89-2; N-nitrosopyrrolidine, CAS 930-55-2; and N-nitrosopiperidine CAS 100-75-4); by Sigma-Aldrich (nonanal, CAS 124-19-6; decanal, CAS 112-31-2; 1,3- dichloroacetone, CAS 534-07-6 and 1,1-dichloroacetone, CAS 513-88-2); and by Chemservice (acetophenone, CAS 98-86-2; 1,3-dichloro-2-propanol, CAS 26545-73-3; and 2-ethyl-1-hexanol, CAS 104-76-7).

Table 1- Chemical characterization of target compounds

Compound	**Structure**	**Molecular Formula**	**CAS Nr**	**MW**	**quantification (qualifying) ions (m/z)**	**Ratio Q1/Q2 (CV, %)**
1,1-dichloroacetone (1,1-DCA)		$C_3H_4Cl_2O$	*513-88-2*	126.96	43 (83)	11.8 (2.7%)
1,1,1-tricloroacetone (1,1,1-TCA)		$C_3H_3Cl_3O$	*918-00-3*	161.41	125 (43)	4.78 (2.8%)
1,3-dichloroacetone (1,3-DCA)		$C_3H_4Cl_2O$	*534-07-6*	126.96	77 (49)	2.32 (8.0%)
1,3-dicloro-2-propanol (1,3- DP)		$C_3H_6Cl_2O$	*26545-73-3*	128.98	79 (81)	2.87 (2.3%)
3-cloro-2,2-dimetil-1-propanol (3-CDMP)		$C_5H_{11}ClO$	*13401-56-4*	122.59	56 (73)	2.31 (3.5%)
2-ethyl-1-hexanal (2-EH)		$C_8H_{16}O$	*123-05-7*	128.22	57 (72)	1.02 (6.4%)
2-ethyl-1-hexanol (2-HN)		$C_8H_{18}O$	*104-76-7*	130.23	41 (57)	2.42 (8.3%)
Acetophenone (ACPN)		C_8H_8O	*98-86-2*	120.15	77 (105)	1.36 (2.6%)
Nonanal		$C_9H_{18}O$	*124-19-6*	142.24	41 (57)	1.05 (5.9%)
Decanal		$C_{10}H_{20}O$	*112-31-2*	156.27	57 (43)	1.11 (3.9%)
N-Nitrosodimethylamine (NDMA)		$C_2H_6N_2O$	*62-75-9*	74.08	74 (42)	2.41 (1.0%)
N-Nitrosopyrrolidine (NPYR)		C_4H_8N2O	*930-55-2*	100.12	41 (100)	2.49 (7.2%)

N- Nitrosomorpholine (NMOR)		$C_4H_8N_2O_2$	*59-89-2*	116.12	56 (116)	4.72 (3.3%)
N- Nitrosopiperidine (NPIP)		$C_5H_{10}N_2O$	*100-75-4*	114.15	42 (114)	1.45 (6.6%)
N- Nitrosodiphenilamine (NDPhA)		$(C_6H_5)_2N_2O$	*86-30-6*	198.22	169 (51)	5.24 (6.9%)

Individual stock solutions of DBPs were prepared in dichloromethane (for SPE-GC-MS) and in methanol (for SPME-GC-MS). A standard mixture solution was prepared for each methodology by diluting each individual standard solution in the corresponding solvent to a concentration of around 45 mg/L to 75 mg/L and 2 mg/L (Std 1) for SPME-GC-MS and SPE-GC-MS methodologies, respectively. All solutions were prepared in glass material, stored at -20 ± 3 ˚C and in the absence of light.

Methanol (liquid chromatography grade, 99.9%) and dichloromethane (liquid chromatography grade, 99.9%) were provided by Merck (Germany).

2.3. SPE-GC-MS

SPE technique was performed by using an automated Autotrace 280 SPE workstation, Thermo Scientific Dionex (USA), equipped with an adjustable nitrogen stream to dry the cartridge before elution. Supelclean coconut charcoal bonding SPE (2g, 6mL) cartridges from Merck (Germany) were used for extraction.

The SPE method was optimized based on EPA 521 method (EPA, 2005). The coconut charcoal cartridge was conditioned by passing through 6 ml of dichloromethane, 12 ml of methanol and 15 ml of ultrapure water. Five hundred ml of sample was loaded through the cartridge at a flow rate lower than 20 mL/min. Then the cartridge was rinsed with 4.0 mL of ultra pure water and dried using a nitrogen stream during 80 min. Analytes were eluted with 12 ml of dichloromethane at a flow rate of 5 ml/min. The extract was evaporated to 1ml under a gentle stream of nitrogen (3 psi, 25 ˚C)

All samples were analyzed by gas chromatography coupled with mass spectrometry (GC-MS). A gas chromatography, GC System 7890B equipped with an Autosampler 7693, coupled to a mass spectrometer MSD, 5977A (Agilent Technologies, USA) was used. The

injection port worked at 250 ºC, in splitless mode for 3 min. The chromatographic separation was achieved with an Agilent J&W HP-5ms column (5% methylphenyl polysiloxane, 95% dimethylpolysiloxane; 60 m × 0,25 mm × 0,25 µm). A gradient oven temperature was used: held at 33 ºC during 6 min, increased at a rate of 4 ºC min^{-1} to a temperature of 85 ºC and held for 2 min, and then increased at a rate of 10 ºC min^{-1} to a final temperature of 250 ºC (held for 5 min).

Mass spectrometer operated in the electron impact (EI) mode at 70 eV, scanning the range m/z 20-350, at a quadrupole temperature of 150 ºC. The SPE-GC-MS parameters are summarized in table 2.

2.4. SPME-GC-MS

The used SPME device and fibers were purchased from Supelco (USA). The method optimization was performed in order to choose the best performing fiber, adsorption type, extraction temperature and time and desorption time. The following fibers were tested: poly(dimethylsiloxane)/ divinylbenzene (PDMS/DVB), 65 µm and divinylbenzene/ carboxen/ polydimethylsiloxane (DVB/CAR/PDMS), 50/30 µm (Kermani et al. 2013). The fibers were conditioned prior to use as recommended by the manufacturer. Both headspace and direct immersion of SPME fiber were accessed. A range of extraction temperatures, varying between 30 and 40 ºC, and extraction time, between 5 and 20 min, were optimized. Tests on desorption time were also performed, varying times between 2 and 10 minutes. Overall, the best performing conditions were selected as follow: (DVB/CAR/PDMS), 50/30 µm fiber (see figure S2 supplementary information), using headspace extraction at 40 ºC during 20 min. After the sorption process, the SPME fiber was desorbed at 250 ºC for 2 min on the GC injection port, in splitless mode (for 2 min).

Chromatographic determination was carried out using a GC system Agilent 6890N (USA) coupled to a mass spectrometer Agilent 5973N. GC separation was performed using an Agilent J&W DB-VRX fused silica capillary column (60 m length × 0,32 mm id × 1,8 µm film thickness), with oven programmed as follows: 35 ºC (held 5 min); increase of 8 ºC min-1 to 250 ºC (held 10 min), to a total run time of 42 min. Helium was used as carrier gas at a constant flow of 1.6 mL min-1. The MS interface temperature was set to 260 ºC. The selected ionization mode was electron ionization (EI) at 70 eV, scanning the range m/z 20-350, at a quadrupole temperature of 150 ºC. The SPME-GC-MS parameters are summarized in table 2.

Table 2- Summary of SPE-GC-MS and SPME-GC-MS parameters used for the analysis of target DBPs

Sample Preparation		
	SPE	SPME
Cartridge / Fiber	coconut charcoal bonding (2g)	DVB/CAR/PDMS headspace
Filter volume (ml)	6	
Conditioning	6mL (3x2) Methylene chloride, 12mL (3x4) MeOH, 15mL (3x5) Ultra pure H_2O	
Sample Load (mL)	500	15
Dry (min)	80	
Elution	12ml (4x3) Methylene chloride	
Evaporation	25 °C; N_2, 2-3 psi	
Extract final volume	1 mL	
Adsorption temperature (°C)		40
Extraction time (min)		20
Desorption time (min)		2
	GC conditions	
Injector Temperature	250 ºC	
Split/splitless valve flow rate	29 mL min^{-1}	20 mL/min
Splitless time	3 min	2 min
Pressure	15.51 psi	9.15 psi
Mobile phase	Helium	
Gas flow rate	1,0 mL/min	1,6 mL/min
Oven temperature program:		
Initial temperature	33 ºC (held 6 min)	35 ºC (held 5 min)
Ramps	4 ºC/min to 85 ºC (held 2min); 10 ºC/min to 300 ºC (held 5 min)	8 ºC/min to 250 ºC (held 10 min)
Total run time	48 min	42 min
MS conditions		
Interface temperature	270 ºC	260 ºC
Source temperature	250 ºC	
Ionization Mode	EI	
Ionization energy	70 eV	
MS quantification mode	SIM	

After optimization, the acquisition of the total ion chromatograms for the mixture standard solutions in full scan mode was performed. Target compounds peaks were identified by their retention time and mass spectra. The most abundant ion that showed no evidence of

chromatographic interference and had the highest signal-to-noise ratio was selected for quantification purposes (Q1). Qualifying ion (Q2) was also selected. For quantification, spectra acquisition was performed in selected ion monitoring (SIM) mode (table 1).

2.5. Validation studies

Linearity range (20-200 µg/L for all compounds in SPE method; and 1.6 - 120 µg/L in SPME method) was evaluated by the determination coefficient (r^2, values ≥ 0.9950) and coefficient of variation of the method (CVm, values ≤ 5%). Furthermore, several statistical tests, such as residual analysis (± 15%), Mandel test (VT ≤ F (1; N-3; 95%), RIKILT test (± 10%) and normalized areas test (± 15%), were also applied to assess the linearity in the selected concentration range. These results were treated in order to comply with all the established requirements for each statistical test. The final choice of the working range was made after evaluation of the mentioned tests (ISO 8466-1, 1990).

For SPE, method detection limit (MDL) was calculated using the equation MDL = IDL × 100 / (rec × Cf), where IDL corresponds to the instrumental detection limit; rec is the recovery percentage of target compound in a specific matrix; and Cf is the concentration factor of the specific SPE method (500, in this case). The method quantification limit (MQL) is calculated with the same equation but replacing IDL with the instrumental quantification limit (IQL) (Proctor et al., 2019). The IDL and IQL were both determined based on precision studies with repeatability conditions (n=10, IDL=3x standard deviation (SD); IQL=10XSD).

For SPME, MDL and MQL were determined using the minimum detectable amount of analyte with a signal-to-noise ratio (S/N) of 3 and 10, respectively (n=10).

The precision of the global methods was assessed under intermediate precision conditions at two concentration levels: low level (60 µg/L for SPE and 5 µg/L - 60 µg/L for SPME) and high level (200 µg/L for SPE and 17 µg/L – 200 µg/L for SPME); and expressed as a relative standard deviation, RSDI (n = 10).

The efficiency of the applied extraction methods was evaluated by recovery studies. In accordance, recovery studies were performed by spiking drinking water samples with two concentration levels (low and high) within the linearity range of the global methods (n=10): low concentration (around 60 µg/L for analytes using SPE and 5 µg/L - 60 µg/L for analytes using SPME); and high concentration (around 200 µg/L for SPE and 17 µg/L – 200 µg/L for SPME).

For both SPE and SPME methods, the quantification was performed by external calibration method (Cuadros-Rodríguez et al., 2007)

2.6. Data processing software

Water extracts processed with the Agilent GC-MS were analyzed with Mass Hunter software for quantification of target compounds.

3. Results

3.1. Validation Studies

3.1.1. SPE- GC-MS methods

The instrumental linearity range was evaluated by analysis of several nitrosamines standard solutions, n = 7 for all compounds except NMFD (n = 8), with equitably distributed concentration levels (20 µg/L - 200 µg/L). All compounds showed values of $r^2 \geq 0.9993$ and CVm ≤ 1.62 (table 3). Additionally, all the requirements of the statistical tests performed were fulfilled, namely Mandel test (VT ≤ F (0.05; 1; N-3), RIKILT test (± 10%) and Normalized values (± 15%).

The MQL and MDL were determined based on repeatability conditions. The MQL values varied between 14.4 ng/L and 26.0 ng/L for NDPhA and NPYR, respectively. The MDL values varied between 4.3 ng/L and 7.8 ng/L for NDPhA and NPYR, respectively.

Table 3- Linearity range studies and analytical thresholds of target DBPs analyzed by SPE-GC-MS method

Analyte	**Retention time (min)**	**Instrumental linearity range (µg/L)**	**N**	**Determination coeficient (r^2)**	**CV_m (%)**	**Mandel test ****	**RIKILT test (%) [90,110]**	**Normalized values (%) [85-115]**	**MQL (ng/L) ***	**MDL (ng/L) ***
NDMA	12.7	20-200	7	0.9993	1.62	4.48 < 7.71	[90,106]	[90-105]	16.0	4.8
NDPhA	35.7	20-200	7	0.9997	1.15	1.33 < 7.71	[90,105]	[87-101]	14.4	4.3
NMOR	26.3	20-200	8	0.9998	0.84	0.44 < 6.61	[98,109]	[93-102]	16.6	4.9
NPYR	26.1	20-200	7	0.9995	1.38	3.25 < 7.71	[95,110]	[90-104]	26.0	7.8
NPIP	27.3	20-200	7	0.9996	1.23	0.98 < 7.71	[97,109]	[90-101]	19.0	5.7

*Values corrected with a concentration factor of 500 due to the drinking water concentration by SPE technique; **VT ≤ F (0.05; 1; N-3).

Precision was evaluated under intermediate precision conditions at two fortification levels, obtaining RSD_I values lower than 25% for both concentration levels.

Figure 1 shows the recoveries of nitrosamines by SPE-GC-MS, considering both low and high concentration levels. For low concentration level, recoveries varied between 43% (NDPhA) and 115% (NMPD) and for high concentration level between 57% (NPYR) and 191% (NPIP). The recovery profiles presented similar behavior at both concentration levels except for NPIP which presented higher recovery values at the highest concentration level (above 190%). This may suggest the influence of matrix effects on the measurement of this specific compound. Although recoveries between 60 % and 140% are desirable, values above or below this range could be acceptable if its repeatability / intermediate precision present good performance. Thus, this method showed a satisfactory precision, with relative RSD_I lower than 25% for all the compounds.

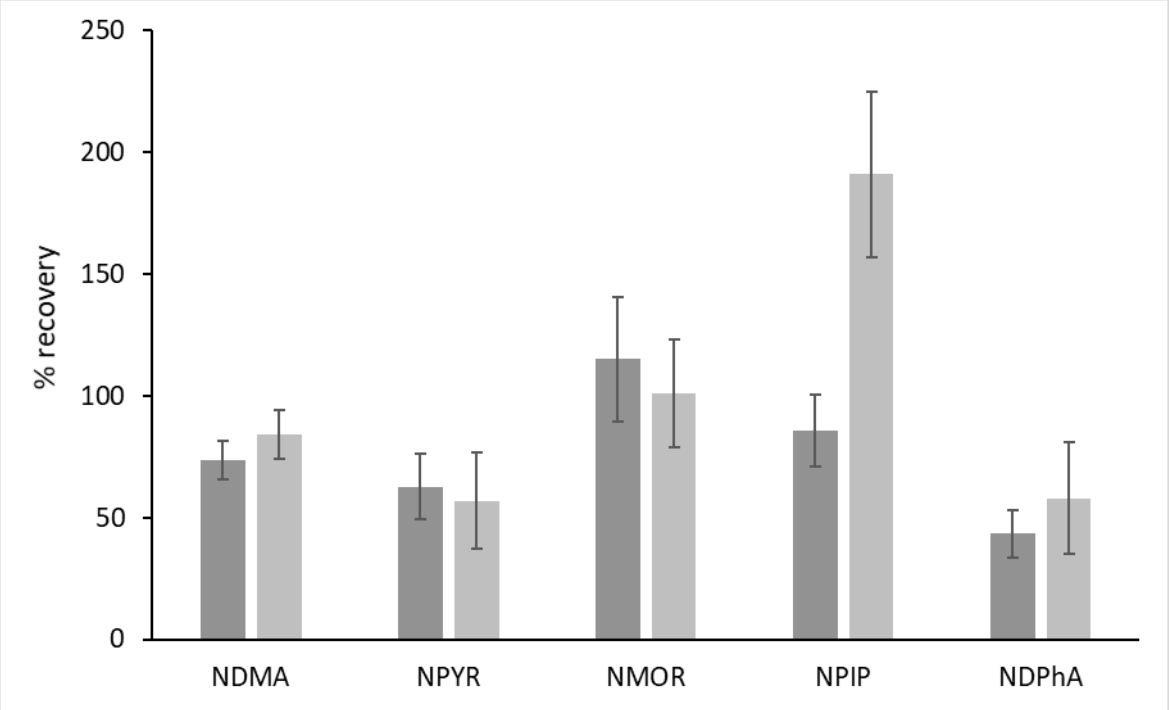


Figure 1- Recovery percentage of nitrosamines in drinking waters, analyzed by SPE- GC-MS, at two spinking levels, low and high concentrations (n = 10). Dark grey bars correspond to the low concentration and soft grey bars to the high concentration levels.

According with Chen et al., (2016) the MDLs measured by GC-MS for N-nitrosamines ranging from 17.4 ng/ L (NPIP) to 76.8 ng/ L (NDPhA), while GC-MS/MS yielded MDLs ranging from 1.1 ng/L (NDMA) to 3.1 ng/ L (NDBA), both using SPE sample preparation. The MDL values obtained in our study (GC-MS) are in the same order of magnitude of the MDLs values obtained by Chen et al. using a GC-MS/MS methodology. In other study performed by Jurado et al. (2007) the detection limits of a GC-MS method varied between 1- 13 ng/L also in the same order of magnitude of the MDLs values achieved in our study (Jurado-Sanchez et al. 2007).

Cheng et al. (2006), also adapted the EPA-521 method making use of a SPE and a GC-PCI-MS/MS system, reported MDLs in the range of 0.3–1.8 ng/L(Cheng et al. 2006). More recently, Sieira et al. (2020) assessed the ability of GC-MS/MS methods using both EI and PCI ionization, to measure 8 nitrosamines. Considering the same nitrosamines measured in our study (namely NDMA, NPIP, NPYR, and NMOR), in Sieira et al. (2020) the MDLs obtained using different ionization methods varied between 1.0 ng/L(NPIP) and 10 ng/L(NMOR) and between 0.4 ng/L (NDMA, NPIP, and NPYR) and 0.6 ng/L (NMEA), for

EI and PCI, respectively. Comparing these MDLs values with those obtained by our methodology (see table3), it is observed the same order of magnitude when compared with GC-EI-MS/MS values, whereas comparing with GC-PCI-MS/MS it is observed higher analytical thresholds in our methodology. Nevertheless, the MDLs in our methodology for the selected nitrosamines in drinking waters are below the 10 ng/L reporting level specified by the California, USA authorities and 10-fold below the 100 ng/L level considered as guideline value by WHO for NMDA in drinking water.

3.1.2 SPME- GC-MS methods

For SPME-GC-MS methodology, the instrumental linearity range was evaluated using 6 DBPs standard solutions, with equitably distributed concentration levels. Due to their physico-chemical properties, the studied DBPs presented different instrumental sensitivity. In this way, this group of compounds were divided in two different concentration ranges: (1,1-DCA), (1,1,1-TCA), (1,3-DCA), (1,3- DP), (3-CDMP) between 20 µg/L and 200 µg/L and 2-EH, 2-HN, ACPN, nonanal e decanal between 1.6 µg/L and 16.7 µg/L.

All compounds showed values of $r^2 \geq 0.9994$ and $CV_m \leq 1.44$ (table 4). Additionally, all the requirements of the statistical tests performed were fulfilled, namely Mandel test ($VT \leq F(0.05; 1; N-3)$), RIKILT test (± 10%) and Normalized values (± 15%).

The MDL and MQL varied between 0.8 and 2.3 ng/L (for 2-EH) and 532 and 1596 ng/L (for 1,3- DCA), respectively.

Table 4- Linearity range studies and analytical thresholds of target DBPs analyzed by SPME-GC-MS method.

Analyte	Retention time (min)	Linearity range (µg/L)	N	Determination coeficient (r^2)	CV_m (%)	Mandel test **	RIKILT test (%) [90,110]	Normalized values (%) [85-115]	MQL (ng/L)	MDL (ng/L)
1,1-DCA	15. 2	20-120	6	0.9999	0.89	0.68 < 10.13	[90,106]	[90-105]	52	17
1,1,1-TCA	18. 6	20-120	6	0.9995	1.33	8.39 < 10.13	[94,109]	[87-101]	36	12
1,3-DCA	19.1	20-120	6	0.9995	1.32	3.19 < 10.13	[96,106]	[93-102]	1596	532
1,3-DP	19.7	20-120	6	0.9997	1.09	2.13 < 10.13	[95,110]	[90-104]	880	293
3-CDMP	20.1	20-120	6	0.9997	1.02	0.93 < 10.13	[97,109]	[90-101]	279	93
2-EH	21.4	1.6-10	6	0.9994	1.44	2.74 < 10.13	[98,109]	[92-103]	2.3	0.8
2-HN	22.8	1.6-10	6	0.9997	1.01	0.90 < 10.13	[91,105	[98- 114]	11	3.8
ACPN	24.4	1.6-10	6	0.9998	0.8	0.70 < 1.13	[96,106]	[99-105]	23	7.7
Nonanal	24.7	1.6-10	6	0.9996	1.17	0.65 < 10.13	[98,109]	[89-100]	6.3	2.1
Decanal	26.8	1.6- 10	6	0.9995	1.37	5.28 < 10,13	[94,110]	[89-105]	10	3.4

** VT ≤ F (0,05; 1; N-3)

Precision was evaluated under intermediate precision conditions at two fortification levels, obtaining RSD_I values lower than 25% for both concentration levels.

Figure 2 shows the recoveries of all DBPs analyzed by SPME-GC-MS, considering the low and high concentrations level. Recoveries varied between 41% (1,1-DCA) and 67% (nonanal) and between 50% (1,3-DCA) and 95% (ACPN) for low and high concentration range, respectively. The exception verified was 1,1,1-TCA which presented low percentage of recovery in both concentration range (lower than 25%).

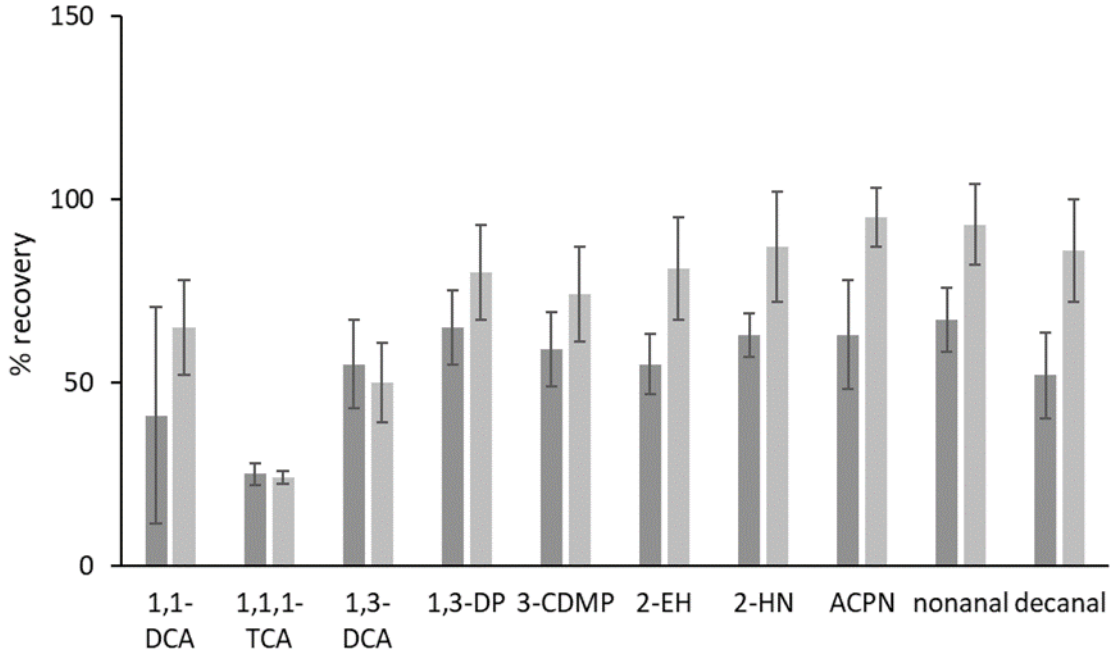


Figure 2- Recovery percentage of target DBPs in drinking waters, analyzed by SPME- GC-MS, at two spinking levels, low and high concentrations (n = 10). Dark grey bars correspond to the low concentration and soft grey bars to the high concentration levels.

When comparing both sample preparation methods (SPE vs SPME) for the target nitrosamines, the sensitivity of SPE in exploratory studies was higher. In accordance, we considered the US EPA 521 method (which describes the use of SPE technique for the extraction of nitrosamines from drinking water samples), with some necessary parameter's optimization. For the other target DBPs groups we selected the SPME preparation method. SPME is considered a green technique due to the reduction, or even the elimination, of organic solvents use; having also the advantage of simplicity of operation; possibility of full automation; easily coupled with GC allowing the reduction of potential contamination of the original samples and partial loss of analytes (Spietelun et al. 2013). However, disadvantages of SPME use could include, poor selectivity, limited number of commercially available fiber coatings/ materials, hindering the ability to extract some specific analytes; and potential problems with SPME device, (i.e., low mechanical resistance of fiber; and needle bending(Spietelun et al. 2013; Merkle et al. 2015; Eckert et al. 2018).

3.2 Sample analysis

The results of the target compounds measurements in the twenty drinking water samples are summarized in table 5. All measurements above MDL were considered a positive occurrence.

Table 5- Summary of the results obtained in the monitoring of the 15 selected DBPs in drinking water from Asseiceira and Vale da Pedra WTPs (n = 10 for each sampling point).

Class of DBPs	**Compound**	**Asseiceira WTP**		**Vale da Pedra WTP**	
		% Positives (nr occurrences/ total samples)	**Maximum measured concentration (ng/L)**	**% Positives (nr occurrences/ total samples)**	**Maximum measured concentration (ng/L)**
Nitrosamines	NDMA	30 (3/10)	< MQL	20 (2/10)	< MQL
	NDPhA	20 (2/10)	< MQL	0 (0/10)	n.d.
	NMOR	0 (0/10)	n.d.	0 (0/10)	n.d.
	NPYR	30 (3/10)	< MQL	30 (3/10)	< MQL
	NPIP	0 (0/10)	n.d.	0 (0/10)	n.d.
Ketones	1,1-DCA	60 (6/10)	< MQL	30 (3/10)	< MQL
	1,1,1-TCA	50 (5/10)	< MQL	30 (3/10)	< MQL
	1,3-DCA	0 (0/10)	n.d.	0 (0/10)	n.d.
	ACPN	0 (0/10)	n.d.	0 (0/10)	n.d.
Alcohols	3-CDMP	0 (0/10)	n.d.	0 (0/10)	n.d.
	1,3-DP	0 (0/10)	n.d.	0 (0/10)	n.d.
	2-HN	0 (0/10)	n.d.	0 (0/10)	n.d.
Aldehydes	2-EH	50 (5/10)	< MQL	50 (5/10)	< MQL
	Nonanal	70 (7/10)	< MQL	90 (9/10)	< MQL
	Decanal	80 (8/10)	< MQL	90 (9/10)	< MQL

MDL (method detection limit); n.d. (non-detected)

In terms of occurrence, the aldehydes group presented the higher percentage of occurrence, Decanal and nonanal were found in almost all samples (above 80% occurrence), and 2-EH with 50% of occurrence.

Aldehydes are widely present in aquatic and terrestrial environments derived from both natural and anthropogenic sources (Papageorgiou et al., 2014). The natural sources of aldehydes are related to photodegradation of dissolved organic matter and microbial oxidation, direct emission from some species of growing vegetation and biomass burning (Karnik et al., 2005; Serrano et al., 2015a). Anthropogenic sources of these carbonyl

compounds can include pollution from fuel combustion and manufacturing. Compounds such as 2-EH were also identified as a secondary metabolites of the degradation pathway of a common plasticizer, diethylhexyl phthalate (DEHP) (Beauchesne et al., 2008; Šalic et al., 2013). Particularly high level of carbonyls concentration was noted after periods of drought and in the warmer seasons. Due to their high water solubility, these compounds can be present during all the supply system, in surface water and also in drinking water (Dabrowska and Nawrocki, 2013; Serrano et al., 2015a).

Previous studies suggested that during conventional WTPs, the occurrence of aldehydes significantly increased after pre-ozonation step and chlorination process, decreasing gradually during steps such as coagulation/flocculation and sand or granular activated carbon filtration (Papageorgiou et al., 2014; Serrano et al., 2015a).

Besides aldehydes, target (halo) ketones (HKs) presented a heterogeneous behavior. 1,1-DCA (45%) and 1,1,1-TCA (40%) presented moderate occurrence levels whereas 1,3- DCA and ACPN were not detected in the analyzed samples.

Currently, there is a growing interest to evaluate HKs presence in drinking waters due to their potential health effects and unclear exposure doses. Despite the scarce information related with HKs occurrence in drinking water, 1,1-DCA and 1,1,1-TCA are the most common chlorinated ketones reported (Richardson and Postigo, 2011; Serrano et al., 2015b, 2014). Serrano et al. (2014) reported the presence of 1,1- DCA in Spanish drinking water in concentration between < 0.03 µg/L and 0.61 µg/L (using micro LLE extraction GC-MS method, with a LOD of 10 ng/L) (Serrano et al., 2014).

Haloketones can be formed in water treated with chlorine, chloramines, chlorine dioxide, as well as ozone–chlorine and ozone–chloramine combinations. Serrano et al., (2015b) studied the influence of the different steps involved in the WTPs on the occurrence of 1,1-DCA and 1,1,1-TCA and their seasonal behavior. It was found that both HKs were formed in the pre-oxidation step, and the next steps applied were ineffective in their removal. It is also suggested that 1,1-DCA could be a precursor of 1,1,1-TCA in the presence of residual chlorine and this reaction could also be driven by warmer temperatures (Deborde and Gunten, 2008; Serrano et al., 2015b; Shanks et al., 2013). Serrano and co-authors also compared the presence of HKs in tap water and in disinfected water from swimming pools, with the results suggesting that the HKs species formed were higher in the latter, potentially promoted by higher levels of residual chlorine and organic matter (Serrano et al., 2015b, 2014; Yang et al., 2018).

Nitrosamines were found in lower percentage of occurrence in the drinking water samples analyzed, with NPYRD, NDMA and NDPhA showing occurrences of 30%, 25% and 10%, respectively.

Nitrosamines have been of significant interest since they were identified in drinking water in 2002 (Choi and Valentine, 2002; Mitch and Sedlak, 2002). Nitrosamines are found in several food products and industrial environments, including tanneries, fish processing facilities, plasticizers, pesticides, surfactants, amines, and dyes manufacturing, Consumer products, including shampoos, laundry detergents, dish washing liquids, and fabric softeners, can also be precursors in the formation of nitrosamines (Richardson et al., 2015). Additionally, natural occurring precursors may include algal-derived organic matter, nitrates and nitrites, which may combine with certain amines to form these nitrogenous compounds, leading to their presence in soils and urban water cycle (Nawrocki and Andrzejewski, 2011; Zhao et al., 2008) (Gerecke and Sedlak, 2003). In WTPs context, recent studies suggested the importance of pre coagulation step in the production of some DBPs, particularly these nitrogenous disinfection byproducts (N-DBPs), through the addition of coagulants such as chitosan or cationic polymers such as polyacrylamide (PAM) and polydiallydimethyl ammonium chloride (polyDADMAC) (Chen et al., 2017; Z. Li et al., 2017; Wilczak et al., 2003).

NDMA is one of the most reported nitrosamines in drinking water (Mitch and Sedlak, 2002; Postigo et al., 2018; Wilczak et al., 2003). Due to its high miscibility in water and available toxicological data, it was classified as B2 (probable human) carcinogen, by the US Environmental Protection Agency (USEPA). NDMA is generally found at higher levels in chloraminated water systems (Richardson et al., 2007). A vast diversity of pharmaceuticals and personal care products (PPCPs) have been reported as potential precursors of NDMA in WTPs, especially during chloramine disinfection (Postigo and Richardson, 2014; Shen and Andrews, 2010). Chlorination can also form NDMA to some extent, specially promoted by the presence of nitrogen precursors in raw water (e.g., natural ammonia in the source water or nitrogen-containing coagulants or ion-exchange resins used in the water-treatment process) (Chen et al., 2017). Previous studies reported the presence of NDMA in drinking water in a wide range of concentration, varying between ND to 189 ng/L (Bei et al., 2016; Chen et al., 2016; Yin et al., 2019). For the other target N-DBPs, i.e. N-nitrosopyrrolidine, N- nitrosomorpholine, N- nitrosodiphenylamine and N- nitrosopiperidine, the information regarding their occurrence is limited, although some studies reported their presence in WTPs

and distribution system (Charrois et al., 2004; Wang et al., 2011; Yin et al., 2019; Zhou et al., 2009).

For the target alcohols-DBPs no occurrences were verified. The available information and occurrence data regarding this group of DBPs is scarce. In particular, 2-ethyl-hexanol is widely used as an industrial chemical, mainly employed in the production of polyvinyl chloride (PVC) plasticizers, such as di-2-ethylhexyl phthalate (D2EHP) and di-2-ethylhexyl adipate (Vitali and Leoni, 1993), being reported in bottled water and suggesting potential migration from these materials. It is also suggested that this alcohol is formed during WTP, in chlorination process and pre oxidation with chlorine dioxide, and may occur during water distribution system by migration from the pipe material (Richardson, 2011; Tomboulian et al., 2018).

For all the compounds, the occurrence values were lower than the method quantification limits (MQL). Even so, although they do not appear at quantification levels, it is possible to characterize the samples considering the presence of these target DBPs and their concentrations levels. The target class of DPBs most present in the drinking water was aldehydes, with occurrence percentage varying between 50% (2-EH) and 90% (nonanal and decanal), and alcohols were the DBPs class with lower occurrence, presenting no positive measurements.

Overall, the selected DBPs occurrence profile suggested a slightly higher occurrence percentage in Asseiceira WTP, when compared with Vale da Pedra WTP. Particularly, for the nitrosamines NDMA and NDPhA (30% and 20% in Asseiceira WTP and 20% and 0% in Vale da Pedra WTP, respectively), the ketones 1,1-DA and 1,1,1-TCA (60% and 50% in Asseiceira WTP and 30% and 30% in Vale da Pedra WTP, respectively). However, the aldehydes nonanal and decanal presented higher occurrence in Vale da Pedra WTP, when compared with Asseiceira (90% and 90% in Vale da Pedra WTP and 70% and 80% in Asseiceira, respectively).

All these compounds are UR-DBPs of emerging concern and occurs in a complex mixture of compounds in drinking water. Several unregulated DBPs displayed higher toxicity when compared with the available data for some already regulated, such as trihalomethanes (THMs),therefore it is crucial to understand their implications in public health and monitoring their occurrence (Starter et al., 2020). This work contributed to improve knowledge in drinking water characterization considering DBPs diversity, showing the first results on the occurrence of a large and chemically different group of UR-DBPs and reinforcing their potential interest for regular monitoring.

4. Conclusions

Globally, all the compounds analyzed in this study presented values bellow the MQL in the studied drinking water samples, expressing analytical thresholds in the low ng/L range. The most representative DBPs were ranked, from the highest to the lowest % of occurrence, as following: decanal > nonanal > 2-EH > 1,1- DCA > 1,1,1- TCA > NPYR > NDMA > NDPhA.

The development and validation of quantification methods to measure UR- DBPs in drinking water is the first step towards the implementation of regular monitoring, to establish the occurrence profiles and to help risk accessors and water managers to identify the critical DBPs.

The outcomes of this study contributed to improve knowledge in drinking water characterization, allowing to identify potential UR-DBPs to include in regular monitorization and to improve (local) water safety plans. Understand, select and optimize the water treatment processes and the technologies involved are crucial for the mitigation of human health risk, attending both regulated and non-regulated compounds, in drinking water.

Uncertainty Measurement

The uncertainty measurement for all target DBPs, based on both analytical methods, were also performed. The results are presented in supplementary information.

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CHAPTER IV. Hazard and Mode of Action of Disinfection By-Products (DBPs) in water for human consumption: evidences and research priorities

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Abstract

Disinfection of water system is an essential strategy to protect human health from pathogens and prevent their regrowth during water distribution, but the reaction of disinfectant agents with organic matter can lead to the formation of disinfection by-products (DBPs). Given their widespread occurrence, potential human health impacts and (eco)toxicity associated with exposure to DBPs are of particular interest due to their potential carcinogenicity and vary non-carcinogenic effects, such as endocrine disruption. Understanding the public health implications of this emerging issue is crucial for societies and decision-makers, supporting more effective water safety plans. Here, we review the recent literature on the effects of DBPs presented in drinking water and treated swimming pools water, focusing particularly in unregulated compounds and the putative underlying mode of action, linking the available data with adverse health outcomes. Overall, the majority of studies highlight the limited knowledge in the understanding of the underlying mode of action of DBPs. Yet, available evidences indicate that different signaling pathways seem to be involved in the adverse outcomes associated with distinct DBPs classes. The main knowledge gaps in this field are also identified, and future research priorities discussed.

1. Introduction

Water treatment plant (WTP) is a crucial tool to promote safe and clean drinking water. However, during this complex treatment process the raw water can react with chemical agents forming disinfection by-products (DBPs). These chemical reactions have been mainly reported in two potential steps of water treatment, coagulation and disinfection. However, pre-oxidation technologies using oxidants such as sodium hypochlorite or ozone are also potential factors affecting DBPs formation (Z. Li et al., 2017; Zheng et al., 2017).

The nature and quantity of DBPs formed depends on the physicochemical properties of the raw water. The WTP operational conditions parameters are particularly important, including disinfectant dose, temperature, pH, contact time and coagulant agent used in the process. Environmental conditions, such as climate, and the specific characteristics of the distribution system are also relevant parameters. (Plewa et al., 2017; Richardson, 2011; Richardson et al., 2015).

Humans are mostly exposed to water DBPs through direct ingestion of water, via inhalation and dermal absorption while showering, bathing and swimming in treated pools (Z. Li et al., 2017; Richardson and Postigo, 2011).

In this work, we review the recent literature on the effects of DBPs present in drinking water and treated swimming pools, focusing in particular in unregulated compounds and the putative underlying mode of action, linking the available data with adverse health outcomes. The main knowledge gaps in this field are also identified, and future research priorities discussed.

2. Forcing agents of water DBPs formation

The main precursors of DBPs present in raw water can include natural organic compounds, such as humic and fulvic acids, and anthropogenic contaminants of emerging concern including endocrine disrupting chemicals (EDCs), non-steroidal anti-inflammatory drugs (NSAIDs), pharmaceutical and personal care products (PPCPs), pesticides and herbicides, cyanotoxins, textile dyes, hormones, surfactants and UV-filters (Postigo et al., 2017; Richardson et al., 2015). In recent years, special attention has been given to this emerging contaminants due to theirs widespread and daily use, reactivity as DBPs precursors and poorly characterized biological effects (Park et al., 2016; Postigo et al., 2017; Richardson et al., 2015).

Raw waters with lower aromatic natural organic matter content have been associated with the preferential way to generate iodinated DBPs (I-DBPs) such as I- THMs and I- HAAs (Dong et al., 2017; Nihemaiti et al., 2016). High levels of bromide in raw water is associated with the formation of bromate; source water with high algal or wastewater content (rich in nitrogen) has been associated with N- DBPs (Komaki and Plewa, 2017). However, the formation scenarios and many reaction pathways are still unclear due to the poorly characterized nature of precursors.

Popular disinfectants of drinking water include chlorine, chloramines, ozone, chlorine dioxide and ultraviolet light (UV) (Richardson et al., 2015). Chlorine is the most ubiquitous disinfectant used worldwide, applied alone or in combination with other disinfectants. The most common approach involves the use of chlorine-based disinfectants in combination with

ozone in order to reduce the volume of some chlorine-based DBPs formed, such as THMs and HAAs. However, this combination results in the production of potential more reactive DBPs such as aldehydes, ketones, keto-aldehydes, carboxylic- acids, keto-acids, alcohols, among others. Disinfection with chloramines is usually associated with the formation of nitrogenated by-products such as nitrosamines (N-DBPs) (Kristiana et al., 2012). Even UV, considered as a clean technology, has the potential to produce aldehydes (Nikolaou et al., 1999; Richardson et al., 2015).

In addition to the nature of disinfectant agent, several physicochemical parameters are also important drivers for the formation of DBPs. The most relevant are pH and temperature, i.e., higher temperatures increases the amount of disinfectant used and the DBPs formed (Richardson et al., 2015). Basic pH promotes the formation of THMs but leads to the decrease of HAAs, haloacetamines and haloketones levels. In contrast, acidic pH increase the HAAs formation (Cortés and Marcos, 2018; Singer, 1995).

DBPs are also formed in disinfected swimming pools through reaction of the same chlorine-based disinfectants with precursors as urine, sweat, hair, sunscreens, lotions and personal care products (Yang et al., 2018). Chlorination is the most commonly used disinfection treatment in swimming pools. The higher formation of DBPs in swimming pools compared with drinking water is mainly associated with the high water temperature involved and the water recirculation. According to recent studies, the water of outdoor pools contained on average two times more DBPs, such as THMs (97,9 µg/L vs 67,7 µg/L) and HAAs (807,6 µg/L vs 412,9 µg/L) compared with indoor pools, during the same seasonal period (Cardador and Gallego, 2011; Simard et al., 2018). For outdoor pools, it is possible that the presence of higher levels of precursors requires the use of higher amounts of disinfectant and consequently higher levels of DBPs formed. Higher concentrations of DBPs such as total THMs (130 µg/L range vs 80 µg/L in average) were found in heated (26 ̊C) compared with non-heated (23 ̊C) outdoor pools (Simard et al., 2018). One of the most common DBPs in chlorinated swimming pools is trichloramine formed by reaction of nitrogenous compounds and chlorine (Villanueva and Cordier, 2015).

Coagulation is a commonly used process in water treatment to remove natural organic matter suspended in raw water. Recent studies suggested the importance of this treatment step in the production of some DBPs, namely N-DBPs, through the addition of coagulants such as chitosan or cationic polymers such as polyacrylamide (PAM) and polydiallydimethyl

ammonium chloride (polyDADMAC) (Z. Li et al., 2017; Nawrocki and Andrzejewski, 2011; Richardson and Postigo, 2011). The reaction mechanism of N- nitrosodimethylamine (N-DBP) formation is unclear but one possibility involves the amine group of PAM that can work as a precursor (Z. Li et al., 2017).

Due to the complex processes of DBPs formation, their high diversity and complexity, this analysis is challenging (table 1). However, understanding the public health implication of this issue is crucial for improving human health risk assessment and implementing effective water safety plans.

Table 1- Main classes of DBPs, average concentrations range and European legal context. Retrieved and adopted with legal information of EU legal context from (Cortés and Marcos, 2018)

Main classes of DBPs.	Average concentration range (µg/L)	Regulated (EU)
HALONITROMETHANES	**	
Chloronitromethane, dichloronitromethane, trichloronitromethane (chloropicrin), bromonitromethane, dibromonitromethane, tribromonitromethane (bromopicrin), bromochloronitromethane, bromodichloronitromethane, dibromochloronitromethane		
HALOACETIC ACIDS AND OTHER HALOACIDS	*****	
Chloroacetic acid, Dichloroacetic acid, Trichloroacetic acid. Bromoacetic acid, Dibromoacetic acid, Tribromoacetic acid, Iodoacetic acid, Diiodoacetic acid, Triiodoacetic acid, Bromochloroacetic acid, Bromodichloroacetic acid, Bromoiodoacetic acid, Dibromochloroacetic acid, Chlorodibromoacetic acid		
TRIHALOMETHANES	******	DW ; TSP
chloroform, bromoform, dibromochloromethane, bromodichloromethane, dichloroiodomethane, bromochloroiodomethane, dibromoiodomethane, chlorodiiodomethane, bromodiiodomethane, iodoform, dichloromethane, bromochloromethane, chlorodibromomethane, dibromomethane		
OXYHALIDES		DW
bromate (0.2 − 25.1), chlorate (up to 190), chlorite (up to 1100)		
HALOFURANONES	*	
mx, red-mx, ox-mx, emx, zmx, mucochloric acid, bmx-1, bmx-2, bmx-3, bemx-1, bemx-2, bemx-3		
HALOACETONITRILES	****	
chloroacetonitrile, dichloroacetonitrile, trichloroacetonitrile, bromoacetonitrile, dibromoacetonitrile, tribromoacetonitrile, bromochloroacetonitrile, bromodichloroacetonitrile, dibromochloroacetonitrile, iodoacetonitrile		
HALOKETONES	***	

Chloroacetones		
HALOAMIDES		
chloroacetamide, dichloroacetamide, trichloroacetamide, bromoacetamide, dibromoacetamide, tribromoacetamide, bromochloroacetamide, bromoiodoacetamide, bromodichloracetamide, dibromochloroacetamide, iodoacetamide, diiodoacetamide, chloroiodoacetamide		
HALOAMINES & OTHER AMINES	*****	
chloramines, nitrosamines (N-nitrosodimethylamine, N-nitrosodiphenylamine; N-nitrosopyroolidine; N-nitrosomorpholidine, N-nitrosopiperidine), heterocyclic amines		
ALDEHYDES	****	
formaldehyde, acetaldehyde, haloaldehydes, chloroacetaldehyde,haloacetaldehydes, dichloroacetaldehyde, bromochloroacetaldehyde, trichloroacetaldehyde (chloral hydrate), tribromoacetaldehyde		
OTHER DBPS : **Chloride**, quinones (benzoquinones), cyanogen halides, chlorophenols, aldoketoacids, carboxylic acids, haloacetates, halopyrroles, amoung others.		DW

main groups and occurrence (µg/L) (average) *0,08-0,8; ** 0.1-10; ***10-60; **** 0,4-300 ; ***** 1-2500;****** 0,05-400 (Krasner et al., 2006; Richardson et al., 2007; Simard et al., 2018; Weinberg et al., 2002). DW- Drinking Water; TSP- treated swimming pools. Individual regulated compounds are presented in bold.

2. Water DBPs: legal context

Over the past twenty years, more than 600 DBPs have been identified in water but just 11, including THMs, HAAs, bromate and chlorite are currently regulated by USEPA under the stage I and II Disinfectants and DBPs Rules (USEPA 2016b) (EPA, 2016). Still, it is estimated that over 50% of the halogenated material formed during chlorination are unknown and their toxicological risk poorly characterized. Importantly, several of these unregulated DBPs have been shown to be more genotoxic and cytotoxic than some regulated compounds. Unregulated and emerging DBPs includes different classes of compounds such as halomethanes, iodo-trihalomethanes, nitrosamines, halobenzoquinones among others (table 1) (Plewa et al., 2017; Richardson et al., 2015).

On the EU legal context, the Drinking Water Directive 98/83/CE sets a maximum water concentration of 100 ug/L for most prevalent THMs (THM4 - sum of chloroform BDCM, DBCM and bromoform) (The Council of the European Union, 1998). In comparison with the regulation in United States, the maximum level for THMs in drinking water is set at 80 µg/L. Bromate are regulated in both the EU and US with the limit of 10µg/L, chloride is

regulated with the limit of 250mg/L in EU and chlorite with 700µg/L in US. In addition, only 5HAAs (sum of monochloro-, dichloro-, trichloro, monobromo-, and dibromoacetic acids) and chlorate are regulated in US with the limites of 60µg/L and 700µg/L, respectively. Regarding the unregulated and emerging families DBPs, Word Health Organization (WHO) included compounds such as N-nitrosodimethylamine (NDMA) (0,1 µg/L), dichloroacetonitrile (20 µg/L) and dibromoacetonitrile (70 µg/L) in the drinking water guidelines, reinforcing their potential toxicological effect to humans (WHO, 2017).

Considering disinfectants for swimming pools water (SPW), only THMs have been regulated in some European countries (table 1). Based on DIN (national standards body for Germany) 19643, the maximum contaminant level (MCL) of THMs, in Germany, is set at 20 µg/L. In fact, Germany is the country with the lowest parametric value comparing with other European countries such as Finland, where the MCL for THMs is 100 µg/L (WHO, 2000d; Yang et al., 2018).

Given the limit information on the subject, it is crucial to improve risk assessment to support more effective regulatory limits and ensure the human health protection.

3. Exposure assessment and risk characterization of water DBPs

Given that DBPs are (semi-) volatile and skin permeable, the exposure pathways are numerous and include ingestion of water, inhalation and skin absorption while showering, bathing, swimming in treated pools, and hand dish-washing (Grellier et al., 2015; Richardson et al., 2015).

Toxicological studies, using cell cultures and animals, suggested potential genotoxicity, cytotoxicity, carcinogenicity, teratogenicity and endocrine disruption associated with the chronic exposure to DBPs, depending of the origin of water, disinfection procedure and disinfectant nature (Cortés and Marcos, 2018; Dong et al., 2017; Hanigan et al., 2017b; Richardson et al., 2007) (table 2). Neurotoxicity was also reported as a potential outcome based on bioassays. However only a few studies are available and therefore more knowledge is required (Guariglia et al., 2011; Moser et al., 2004).

Epidemiological studies involving exposure to DBPs reported cancer and non-cancer outcomes, i.e., bladder, lung and colon cancer, birth defects, asthma and skin rashes. These outcomes were associated with different contact routes such as respiratory, gastrointestinal tract and dermal contact. Most biological samples used in the former studies included blood,

urine and exhaled bread (Allen et al., 2017; Cantor et al., 2010; Rahman et al., 2010; Tardiff et al., 2006; Villanueva and Cordier, 2015; Wright et al., 2017)

Vlaanderen and colleagues (2017) studied the association between the exposure to THMs (measured in exhaled breath) in swimming pools, and respiratory effects, such as asthma, using several biomarkers of immune response, i.e., interleukin-1rA, C-X-C motif chemokine 10, C-C motif chemokine 11, among others. The study suggested a positive association between short-term exposure to DBPs and the selected markers, namely cytokines and chemokines. Yet, a fully demonstration of the association between exposure to DBPs and this acute modification on immune response awaits further evidences as the potential influence of the physical activity in the quantification of immune markers in swimmers serum should be further clarified (Vlaanderen et al., 2017)

The most common adverse outcome reported in embryo development is cardiovascular defects (CVDs) with an association with a sum of 4 THMs (chloroform, BDCM, DBCM, and bromoform),THM4, although the association between the risk of CVDs and individual DBPs remains unclear (Richardson et al., 2007; Wright et al., 2017).

Considering cancer outcomes, positive association have been found for bladder cancer and THM4, HAAs and TCM (table 2) (Grellier et al., 2015). It is important to highlight that some associations are not consistent across different epidemiological studies, given the complexity and diversity of this group of compounds. Some systematic reviews and meta-analysis dealing with cancers have been carried out but the evidence of association between humans DBPs exposure and cancer was not consistent to infer whether such association are causal (Grellier et al., 2015).

The potential genotoxic effects of DBPs, derived from *in vitro* studies, have been recent reviewed by Cortés and co-authors (2018). Considering bacterial and mammalian based cell tests, the authors concluded that the potential genotoxic action of DBPs could be ranked, from the highest to the lowest, as iodinated-brominated-chlorinated DBPs. Considering DNA damage after chronic exposure to different target families of DBPs the rank order suggested was (from the higher to the lower): haloacetic acids > haloacetamines > haloacetonitriles > halonitromethanes followed by haloacetaldehydes > nitrosamines and finally trihalomethanes. Considering cell growth inhibition after chronic exposure, the higher inhibition was as follow: haloacetamides > haloacetaldehydes > halonitromethanes > haloacetic acids > haloacetonitriles > trihalomethanes > nitrosamines (Cortés and Marcos, 2018)

These recent studies in emerging DBPs, including halonitromethanes, iodo-trihalomethanes, iodo-acids, haloamides, halofuranones, haloacetonitriles, haloacetaldehydes, nitrosamines and halobenzoquinones, suggest they can display higher genotoxicity and cytotoxicity potential than those that are currently regulated such as trihalomethanes and haloacetic acids. Based on *in vitro* studies, iodoacetic acid (IAA) is one of the most genotoxic DBP identified, to date. (Richardson et al., 2015, 2007; Wang et al., 2018).

In support of *in vitro* studies, several DBPs have been shown to be genotoxic, cytotoxic and carcinogenic in *in vivo* animal models, such as zebrafish *Danio rerio* and *Daphnia magna* (Park et al., 2016; Teixidó et al., 2015; Zheng et al., 2017). According to the *in vivo* tests, families of DBPs such as acetamines and acetic acids are more genotoxic than acetonitriles and nitrosamines. Based on mammalian development studies, exposure to DBPs led to retarded fetal development, spermatotoxicity, delayed sexual maturation, changes in reproductive organs/placenta and skeletal effects. (Tardiff et al., 2006; Wagner and Plewa, 2017; Wright et al., 2017). Recently, an association between DBPs and disruption of androgen signaling pathway was reported (Holmes et al., 2017). Based on this study, regulated and unregulated DBPs such as halobenzoquinones, haloacetonitriles, haloacids and halofuranones are able to binding to the androgen receptor as antagonist and subsequently altering gene transcription. Thus, some of these chemicals may disrupt normal endocrine function, mimicking or antagonizing endogenous hormones. However, the interaction of DBPs and nuclear receptors mediated signaling pathway (signaling and metabolic modules) needs further investigation (Holmes et al., 2017).

One of the main uncertainties relates on how the IC50 values derived for individual DBPs can be used to estimate the risk of complex mixtures. Since DBPs are present in waters as a complex mixture, studies with single compounds may not capture their potential synergistic, dose additive and antagonistic activity (Grellier et al., 2015).

One of the major knowledge gaps is related with the limit information available for many DBPs classes given that the majority of studies only address regulated DBPs such as THM4 and HAAs and only a few works have addressed emerging and unregulated DBPs, such as trichloromethane (TCM), bromodichloromethane (BDCM) and dibromochloromethane (DBCM) from BrTHMs class (Grellier et al., 2015). In addition, for the majority of DBPs, the underlying mechanism of action (MoA) is unknown and so more research on this topic is needed including the screening of additional adverse health effects.

Table 2- Summary of recent studies focusing mainly on the toxicological effects of emerging and unregulated DBPs.

DBP/ Hazard agent	Water Matrix	DBPs concentrations tested/ assessment	Endpoints Health outcome	Exposure assessment method/Model	Study reference
THM4; HAAs and TCM	chlorinated water	PFs/SFs/RfDs exposure pathway dependent*	Cancer for bladder, rectum, colon, others unspecified cancers; Birth defects	tap water and blood	Grellier et al., 2015 (review)*
THMs, HAAs, formaldehyde and acetaldehyde	chlorine and ozone	0.2, 0.5, 1.0 and 2.0 mg Cl2 L-1/ 24h	Ecotoxicity of THMs, HAAs, and formaldehyde	Daphnia Magna Exposure	Park et al., 2016
DBP9; DBCM; BDCM; DCAA;TCAA; HAA5; THM4; THMBr	chlorinated water	n.d	Birth defects (CVDs)	case control - levels in tap water compared with birth data	Wright et al., 2017
BDCM;BAM; 3-BPN; DBAA; DBAN; DCAN;DIAA; DCP; DCBQ; NDMA	Chemicals target	0.1 nM to 2 mM	Endocrine activity; disruption of endocrine system	ARBA - androgen receptor binding assay	Holmes et al., 2017
THMs	clorinated pools	Medium increase after swimming : 0.7 to 2.3 for MET(Metabolic equivalents), and 1.4 to 7.1 µg/m^3 for exhaled total THM (sum of the four THM)	Gene expression and DNA methylation	Sampling blood; exhaled breath before and after swimming activity- blood gene expression	Salas et al., 2017
Haloaldehydes	Disinfected water using PFA-performic acid	0.8 mg/L ; 0,6 mg/L; 1,5mg/L PFA dose	Chromosomal mutation and DNA damage	bacterial, plant, and human cells. Ames test (point mutation in Salmonella),the micronucleus (chromosomal damage) and Comet tests (primary DNA damage) on human hepatic cells (HepG2)	Ragazzo et al., 2017
Halomethanes; THM4;chlorinated, brominated and iodinated Haloacids; Haloacetonitriles: Haloaldehydes; Haloketones; Halonitromethanes; Haloamides	chlorinated water	LOQ (Limit of quantification) 0,1-2.0 ug/L	Skin rashes	Sampling shower water in homes	Allen et al., 2017

Chloroacetamide; Bromoacetamide; Iodoacetamide; Chloroaceticacid; Bromoaceticacid; Iodoaceticacid; Chloroacetonitrile; Dichloroacetonitrile; Trichloroacetonitrile; Bromoacetonitrile; Dibromoacetonitrile; Iodoacetonitrile; N-Nitrosodimethylamine; N-Nitrosodiphenylamine; N-Nitrosomorpholine	chorinated waste water	1-500 uM, compound depending	Mortality and morphology responses/ Zebrafish larval behavior	Zebrafish embryos exposure	Hanigan et al., 2017
Trihalomethanes, Haloacetic acids, Haloacetaldehydes, Haloacetamides, Hydroxyfuranone, N-Nitrosamines, Halonitromethanes, N- Chloramines, Haloacetonitriles, Halobenzoquinones, Iodoacids classes: TCM ,TBM,TIM, BDCM, DBCM, CDIM, BCIM, BDIM, DCIM, DBIM, CAA, BAA, IAA, DCAA, DBAA, DIAA, TCAA, TBAA, CDBAA, BCAA, BIAA, BDCAA,CAL,BAL, IAL, DCAL, DBAL ,TCAL, TBAL, BCAL, DBCAL, BDCAL, CAcAm, BAcAm,IAcAm, DCAcAm,DBAcAm DIAcAm TCAcAm TBAcAm BCAcAm, BDCAcAm, BIAcAm,DBCAcAm, MX, MBA, MCA, NDMA, NDEA, NPIP, NPYR, NMOR, NDPhA, NMEA,TCNM, BNM, Cl-Glycine, Cl-Histamine, Cl-Ethanolamine, Cl-Lysine, Cl-NaAcetyl lysine, CAN,BAN, IAN,DCAN, DBAN, TCAN,2-CBQ, TriCBQ,TetraCBQ ,2,5-DCBQ, 2,6-DCBQ, 2,5-DBBQ, 2,6-DBBQ, 2,3-DIBQ, Z3B3IPPA,Z3B3IPPA, E3B3IPPA, E3B2IPPA, E2I3MBDA	Chemical target	n.d	genotoxicity : SCGE (single cell gel electrophoresis, comet assay) genetic endpoint	mammalian cells	Cortés et al.,2018 (review)*
NDEA	chlorinated water	100, 150, 200, 250 and 300 mg/L	Mortality and morphology responses/Biochemical parameters	TK6 cells ; zebrafish	Zheng et al.,2017

Chloroform, Bromoform, BDCM, CDBM, DCA,TCA,DBA ;TBA; BCA	chemical target solutions	20–100 µg/mL	endpoints such as growth, hatching success, malformations and lethality/76h	Zebrafish	Teixidó et al., 2015

THM: Trihalomethanes; HAA: Holacetic acids; CF: Chloroform, BDCM: Bromodichloromethane, CDBM: chlorodibromomethane; DBCM: Dibromochloromethane ; DCA: dichloroacetic acid;TBM: Bromoform; Br-THM: Brominated trihalomethanes;TCA trichloroacetic acid; DBA dibromoacetic acid, TBA tribromoacetic acid and BCA bromochloroacetic acid; TTHM: Total trihalomethanes; .BAM, 2-bromoacetamide; 3-BPN: 3-bromopropionitrile; DBP9: sum of chloroform, bromodichloromethane (BDCM), dibromochloromethane (DBCM), bromoform, monochloroacetic acid (MCAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), monobromoacetic acid (MBAA), and dibromoacetic acid (DBAA); DBAN: dibromoacetonitrile ; DCAN: dichloroacetonitrile ; DCBQ: 2,6-dichloro-1,4-benzoquinone ; DCP- 2,3- dichloropropionamide ; DIAA- diiodoacetic acid; HAA5, sum of MCAA, DCAA, TCAA, MBAA, and DBAA; THM4, sum of chloroform, BDCM, DBCM, and bromoform; THMBr, sum of BDCM, DBCM, and bromoform.TCM: trichloromethane, TBM: tribromomethane, TIM: triiodemethane, BDCM: bromodichloromethane, DBCM: dibromochloromethane, CDIM: chlorodiiodomethane, BCIM: bromochloroiodomethane, BDIM: bromodiiodomethane, DCIM: dichloroiodomethane, DBIM: dibromoiodomethane, CAA: chloroacetic acid, BAA: [bromoacetic acid, IAA: iodoacetic acid, DCAA: dichloroacetic acid, DBBA: dibromoacetic acid, DIAA: diiodoacetic acid, TCAA: trichloroacetic acid, TBAA: tribromoacetic acid, CDBAA: chlorodibromoacetic acid, BCAA: bromochloroacetic acid, BIAA: bromoiodoacetic acid, BDCAA: bromodichloroacetic acid, CAL: chloroacetaldehyde, BAL: bromoacetaldehyde, IAL: iodoacetaldehyde, DCAL :dichloroacetaldehyde, DBAL: dibromoacetaldehyde, TCAL: tri- chloroacetaldehyde, TBAL: tribromoacetaldehyde, BCAL: bromochloroacetaldehyde, DBCAL: dibromochloroacetaldehyde, BDCAL: bromodichloroacetaldehyde, CAcAm: chloroacetamide, BAcAm: bromoacetamide, IAcAm: iodoacetamide, DCAcAm: dichloroacetamide, DBAcAm: dibromoacetamide, DIAcAm: diiodoa- cetamide, TCAcAm: trichloroacetamide, TBAcAm: tribromoacetamide, BCAcAm: bromochloroacetamide, BDCAcAm: bromodichloroacetamide, BIAcAm: bromoiodoacetamide, DBCAcAm: dibromochloroacetamide, MX: mutagen-X, MBA: mucobromic acid, MCA: mucochloric acid, NDMA: N-nitrosodimethylamine, NDEA: N-nitrosodiethylamine.

- For a detailed knowledge on the original studies regarding the target compounds presented, the reviews Grellier et al. (2015) and Cortés et al. (2018) should be consulted

According to the limit toxicological studies available concerning exposure to DBPs, modulation of several signaling pathways such as CYP2E1 gene, glutathione s-transferases GSTZ1 and GSTT1 have been associated with different adverse outcomes, such as bladder and lung cancers (Cantor et al., 2010; Grellier et al., 2015; Villanueva and Cordier, 2015) (Landi et al., 2003) and diseases at birth (Zhou et al., 2018) (table 3).

Lung epithelial cells, in *in vitro* models, were exposed to THMs to assess DNA damaging ability, through changes in the activity of GSTT1 (a glutathione S-transferase). The results showed that BrTHMs (BDCM and TBM) were more rapidly metabolized through this GSTT1 route comparing with their corresponding non-brominated THMs (TCM and DCM). Nevertheless, additional polymorphisms of GSTT1 may influence the response of human cells to the genotoxic effects of the THMs. In consequence, further studies are needed to assess this potential carcinogenic risk of THMs exposure (Landi et al., 2003).

In another study, a positive association between long-term exposure to THMs and risk of bladder cancer in presence of GSTT1 and GSTZ1 polymorphisms was also shown. A genotype assays using cases with confirmed urothelial carcinoma, found a significant association between bladder cancer and GSTZ1 and GSTT1 polymorphisms, depending on THMs concentrations in drinking water. This is consistent with the hypothesis that these genes are involved in the adverse outcomes of THMs. However, more scientific evidence is required to support this association (Cantor et al., 2010; Villanueva and Cordier, 2015)

Recent studies conducted by Zhou et al (2018) explored the association between CYP2E1 (gene rs2031920, rs3813867, and rs915906) and GSTZ1 (gene (rs7975) polymorphisms and adverse birth outcomes, through the exposure to selected THMs, namely BrTHM, and

trichloro-acetic acid (TCAA). Multivariate regression models were used to assess interactions between prenatal exposure to DBPs and newborns CYP2E1 and GSTZ1 polymorphisms expression in cord blood. Birth outcomes such as birth weight, birth length and gestational age were assessed. THMs were quantified in maternal blood and TCAA in maternal urine, including 426 pregnant women in a cohort study. The data were not consistent and given the sample sizes, the ability to effectively assess the effect was limited. The only potential interaction verified indicates that newborns with genetic variation of CYP2E1 gene rs2031920 could be correlated with the impact of prenatal BrTHM (sum of DBCM, BDCM and TBM) exposure on birth weight. Additional scientific evidence are needed in this case, but genetic susceptibility could play an important role, mediating the impacts of maternal exposure to DBPs on the birth outcomes (Zhou et al., 2018).

Nitrosamines metabolism and their potential carcinogenesis have also been suggested in association with human CYP2 family (Hanigan et al., 2017b; WHO, 2002). According with this pathway, NDMA is metabolized by the CYP2E1, producing secondary metabolites: α-hidroxy-NDMA, subsequently metabolized in N-nitroso-methylamine and finally in Methyldiazonium ion. This final metabolite binds DNA forming DNA adducts causing cancer such as liver cancer, lung cancer and renal cancer (Godoy et al., 2002). However, for a correct definition of the MoA of NDMA and risk assessment all the exposure routes should be crossed. Concerning NDMA, human exposure may also include food, tobacco, among others (World Health Organization, 2004).

Other biomarkers have been screened such as the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), that has been associated with exposure to DBPs and impairment, i.e., cytotoxy, genotoxy and neurotoxicity (table 3). Similarly, changes on the nuclear enzyme Topoisomerase II has been associated with cellular mitosis and DNA stability (Komaki and Plewa, 2017; Pals et al., 2011).

Monohaloacetic acids (MonoHAAs) were linked with the generation of reactive oxygen species (ROS) due to the inhibition of GAPDH. This association may play an important role in neurodegenerative diseases such as Alzheimer's disease. The mechanism of action of MonoHAAs involves the DNA damage by alkylating the thiol group of the cysteine residue in the active site of GAPDH (Dad et al., 2013; Pals et al., 2011). Considering the monoHAAs DBPs family, the effects reported were higher in IAA followed by BAA and CAA. Despite the positive correlation, more studies are needed to clarify this MoA.

In addition, in the same acetic acids group of DBPs, it was recently proposed a putative interaction of IAA with catalase, also linked with the oxidative stress. According to Wang

et al. (2018) the IAA binds the CAT through van der Waals and hydrogen bonding interactions in HIS 74 and TYR 357, that circles active sites. These interactions leads to changes in protein size and loss of protein skeletons (Wang et al., 2018).

Monohaloacetonitriles (monoHANs) were linked with the inhibition of decatenation activity of the enzyme topoisomerase II, in CHO cells, under acellular conditions. Topoisomerase is linked with cellular mitosis and DNA stability and replication. Komaki and colleagues (2017) recently suggested that monoHANs leads the hyperploidy induction in cells, as a consequence of mitosis inhibition, by suppression of the topoisomerase decatenation activity. However, the model proposed was only partially supported (table 3). Further investigations are needed to support the association between the hyperploidy verified with some monoHANS, namely chloroacetonitrile (CAN) and bromoacetonitrile (BAN), and tumorigenesis (Komaki and Plewa, 2017).

To evaluate oxidative damage, markers such as malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OHdG) were used (Du et al., 2013). Benzoquinones (HBQs) shown positive association with the production of intracellular ROS in T24 bladder cells though the measure of oxidative damage marker 8-OHdG. This association was concentration-dependent and suggested that DNA damage and protein carbonylation are involved in toxicity mechanism of this group of DBPs (Du et al., 2013).

Regarding endocrine disruption, nuclear receptors (NR) as androgen receptor (AR) was addressed (Holmes et al., 2017). In accordance, Halobenzoquinones and other DBPs classes such as haloacetonitriles, haloacids and halofuranes were linked with endocrine disruption by binding to nuclear receptors as the AR, leading to changes in the transcription of target genes. This hypothesis was recently proposed by Holmes et al. (2017) testing 21 suspected and known DBPs (Table 3). According to this study, 14 of 21 DBPs were found to bind AR at IC50 values ranging from 1,86 mM for 2,3-dichloropropionamide (DCP) to 13,5 µM for 3,4,5,6- tretachloro-1,2-benzoquinone (TCBQ). Benzoquinones have shown higher affinity with AR, comparing with the other compounds tested, in accordance, the higher effect was found with TCBQ and DCBQ (2,6-dichloro-1,4-benzoquinone). Given the large number of NRs families in mammalians, future studies should address the modulation of additional NR by DBPs (Gutierrez-Mazariegos et al., 2015; Holmes et al., 2017; Santos et al., 2018; Santos and Castro, 2014).

More recently, epigenetic changes, histone modification, miRNA modulation and DNA methylation have been described as susceptible biomarkers(Vrijens et al., 2015; Salas et al., 2017; Tao et al., 2004). THMs and HAAs have been described to modulate DNA

methylation and hypomethylation, which increases the mRNA expression of proto-oncogenes such as c-myc, c-jun (mice) and LINE-1 (human), linked with kidney and liver cancers, after long-term exposure (Pereira et al., 2001; Salas et al., 2017; Tao et al., 2004). The evidence in humans is still limited. Although, DNA methylation associated with changes in genetic elements, such as retrotransposons, seems to occur and folate metabolism seems to play a role in colorectal carcinogenesis, with folate deficiency associated with the increased formation of preneoplasic lesions in rodents, after exposure to DBPs (Geter et al., 2004; Villanueva and Cordier, 2015).

Hence, the use of epigenetics biomarkers and "omics" are expected to become important approaches to better understand the underlying molecular mechanisms and signature of disruption of DBPs.

Taken together, these results highlight the potential of DBPs to modulate many different signaling pathways potentially inducing adverse health effects. This is not surprising considering the large diversity of chemical structures. Hence, future studies should expand the screening to additional signaling pathways and untested DBPs. Scientific evidence in human effects of DBPs exposure in consumption water need to be more supported and specific exposure biomarkers should be taken in attention considering both genetics and lifestyle characteristics.

Table 3- Summary of recent studies addressing the putative mode of action of regulated and unregulated DBPs.

DBP/ Hazard agent	Endpoints	Molecular Ligand	Model	Concentration of DBP/ Treatment	Study reference
Monohaloacetic acids: IAA	oxidative stress /cytotoxicity	Catalase (CAT, EC 1.11.1.6)- HIS 74 and TYR 357	cell lines assay - mouse hepatocyes	(0, 1, 5, 10, 50, 100, 500, 1000, 2000, 400, 6000, 8000 mM) for 24 h	(Wang et al., 2018)
Monoacetonitriles: CAN and BAN	Hyperploidy induction /mitosis inhibition/disruption cell cycle (cell growth)	topoisomerase II	CHO cells	0.3, 0.5 and 0.8 µg (nuclear protein)	(Komaki and Plewa, 2017)
halobenzoquinones, haloacetonitriles, haloacids, and halofuranones: (DCBQ), (DBAA), (CSA), (TCP), (DBAN), (MCA), (TCBQ), (4-NP), (MX), (NDMA), (2-CP), (BDCM), (TCAN), (BAM), (DCAN, (IAA), (3-BPN), (DIAA), (DBPN), (DCP)	endocrine disruption	Androgen Receptor	ARBA, androgen receptor binding assay	0.1 nM to 2 mM	Holmes et al., 2017(Holmes et al., 2017)

Halobenzoquinones (HBQs): (DCBQ), (DCMBQ), (TCBQ), and (DBBQ)	bladder cancer/oxidative stress	8-hydroxydeoxyguanosine (8-OHdG) and malondialdehyde (MDA) adducts of proteins	T24 bladder cancer cells	25, 50, 75, 100, 125, 150 μM; IC50 values are 95 μM for DCBQ, 110 μM for DCMBQ, 151 μM for TCBQ, and 142 μM for DBBQ /24h exposure	(Du et al., 2013)
Monohaloacetic acids (monoHAAs): IAA; BAA; CAA	mitochondrial stress and genomic DNA damage	glyceraldehyde-3-phosphate dehydrogenase (GAPDH) - cytosolic enzyme.	CHO cells	25 mM IAA, 60 mM BAA or 6 mM CAA for 4 hr (Du et al., 2017); IAA (10- 50 μM), BAA (50 -150 μM) or CAA (1-10 mM) 10 to 60 min(Pals et al., 2011)	(Du et al., 2017); (Pals et al., 2011)
Thrihalomethanes: TCM;BDCM;DBCM, DCM and TBM	DNA damage	GSTT1 activity	lung epithelial cells	10, 100, and 1000μM	(Landi et al., 2003)
Thrihalomethanes and trichloroacetic acid: DBCM, BDCM, and TBM (BrTHMs) and TCM; TCAA	birth outcomes	CYP2E1 (rs2031920, rs3813867, and rs915906) and GSTZ1 (rs7975) polymorphisms	blood, urine and cord blood	cohort study/ The limit of detections (LOD) for blood BDCM, DBCM, TBM, and TCM were 0.45, 0.68, 2.00, and 1.95 ng/L / The LOD of TCAA in urine was 2.00 mg/L	(Zhou et al., 2018)

iodoacetic acid (IAA); chloroacetonitrile (CAN); bromoacetonitrile (BAN); 2,6-dichloro-1,4-benzoquinone, (DCBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ); 2,3,6-trichloro-1,4-benzoquinone (TCBQ); 2,6-dibromobenzoquinone (DBBQ); 2,6-dichloro-1,4-benzoquinone (DCBQ), dibromoacetic acid (DBAA), chlorosuccinic acid (CSA), 2,4,6- trichlorophenol (TCP), dibromoacetonitrile (DBAN), mucochloric acid (MCA), 3,4,5,6 tetrachloro-1,2-benzoquinone (TCBQ), 4-n-nonylphenol (4-NP), 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), N- nitrosodimethylamine (NDMA), 2-chlorophenol (2-CP), bromodichloromethane (BDCM), 2-bromopropionitrile (3-BPN), diiodoacetic acid (DIAA), 2,3- dibromopropionitrile (DBPN), 2,3- dichloropropionamide (DCP), trichloroacetonitrile (TCAN), 2- bromoacetamide (BAM), Dichloroacetonitrile (DCAN); bromodichloromethane (BDCM), dibromochloromethane (DBCM), bromoform(TBM), chloroform (TCM), dichloromethane (DCM) trichloroacetic acid (TCAA).

4. Conclusion and research priorities

According to Word Health Organization (WHO), the sixth goal for a sustainable development in accordance with Agenda 2030 is Water Quality (WHO, 2017). As a matter of fact, water safety and quality and sanitation are global priorities. Understanding the public health implications of chemical contamination of drinking water is important for societies, drinking water providers, risk assessors and their decision-makers due to their widespread occurrence and public health risks (Grellier et al., 2015).

The vast majority of the identified DBPs remains with unknown biological activities, which supports the need to understand how exposure to these compounds may impact the human health.

In summary, priority should be given to address the following knowledge gaps;

i) most available studies on DBPs health effects do not take in account individual characteristics, such as metabolic performance, genetics and lifestyle. Thus, these aspects should be integrated in the exposure assessments, considering also the MoA of individual and complex mixtures. Expanding the knowledge on the MoA of unregulated and poorly studied DBPs will improve risk assessment; ii) increase knowledge of new potential impacted signaling pathways and specific biomarkers should be identified to support a robust human health risk assessment. Ideally, adverse outcome pathways (APO) should be identified and characterized for target DBPs; iii) exposure routes are still not sufficiently addressed particularly for unregulated DBPs; other possible exposure routes such as tobacco, food industry and occupational disinfected environments, should also be considered to improve risk evaluation; iv) treated drinking water shows a complex mixture of DBPs. It is essential to increase the knowledge on the toxicity of DBPs mixtures. Future research should focus in the development of sensitivity analytical methods to identify new DBPs and improve the accuracy of the dose-response relationship measurements; v) increase knowledge on toxicity ranks considering emerging classes of DBPs will help prioritizing which DBPs should go for more detailed studies; vi) further investigation is needed to address forcing agents, during WTP processes ,i.e., how physicochemical parameters, such as temperature, pH, organic matter, pre-treatment and disinfectant agents and reactivity time, contribute to the formation of new DBPs, also including the reactions occurring during the distribution system, due to the residual disinfectant dose; vii) improve knowledge in filtration technologies after distribution system in order to minimized DPBs concentrations in consumption water; viii) conditions of lower rain-fall and high temperatures can contribute for increase formation of DBPs in drinking water due to the presence of more precursors (i.e., organic matter, toxins, etc.). This is particularly relevant considering the climate change scenarios which will hypothetically lead in some regions to alterations on the water cycle, temperature and extreme events. The importance of this association should be understood to improve the management of local water resources; ix) additional studies on treated swimming pools water are needed to improve exposure health assessment and effective parametric values.

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CHAPTER V. Toxicological Assessment of seven unregulated drinking water Disinfection By-products (DBPs) using the zebrafish embryo bioassay

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Abstract

Disinfection By-products (DBPs) are formed during the chemical treatment of water for human consumption, by the reaction of raw water with chemical agents used in the different steps of the process. Disinfection is one of the most important steps, inactivating pathogens and preventing their regrowth during water distribution. However, it is also involved in DBPs formation due to the use of disinfectant agents, such as chlorine, which reacts with dissolved precursors, such as pharmaceuticals, toxins, pesticides, among others. Given their widespread occurrence, potential human health and (eco)toxicological impacts DBPs are of particular interest due to their potential carcinogenicity and various non-carcinogenic effects, such as endocrine disruption. In this study, the developmental toxicity of chemically-different unregulated DBPs was evaluated using zebrafish embryo bioassay. Embryos were exposed to different concentrations of the target DBPs and multiple endpoints, including, mortality, morphological abnormalities and locomotor behavior were assessed at specific developmental stages (24, 48, 72 and 96 hpf). The different families of DBPs tested included nitrosamines, aldehydes, alcohols and ketones. The results show that the effects were compound dependent, with EC10 values varying between 0.04 mg/L (2-ethyl-1-hexanal) to 9.2 mg/L (hexachloroacetone). Globally, several of the tested unregulated DBPs displayed higher toxicity when compared with the available data for some already regulated, such as trihalomethanes (THMs), which highlights the importance of screening the toxicity of still untested and poorly characterized DBPs.

1. Introduction

Water Treatment Plant (WTP) is a crucial and integrated tool to promote safe and clean drinking water, inactivating pathogens of public water supplies and preventing their regrowth during the water distribution. However, during the chemical treatment, the water can react with disinfection and coagulant agents forming emerging compounds, such as Disinfection By-products (DBPs). The disinfectant nature, dose applied, and their residual stability in drinking water distribution system were found to be among the most important factors to understand DBPs formation (Li et al., 2019). Given that water DBPs are (semi-) volatile and skin permeable, humans can be exposed to DBPs through different contact routes, including direct ingestion of water, via inhalation and dermal absorption while showering, bathing, swimming in treated pools, among others (Chaves et al., 2019). In line

with that and attending to the diverse alternatives of water treatment methodologies and variables of the process, the hazard characterization of DBPs and the understanding of the implications for human health are key to make a risk-benefit balance.

The main precursors of DBPs present in raw water can include natural organic compounds, such as humic and fulvic acids, and contaminants of emerging concern including endocrine disrupting chemicals (EDCs), hormones, textile dyes, non-steroidal anti-inflammatory drugs (NSAIDs), pharmaceutical and personal care products (PPCPs), pesticides and herbicides, cyanotoxins, surfactants and UV-filters (Chaukura et al., 2020; Postigo and Richardson, 2014). In recent years, special attention has been given to this emerging contaminants due to theirs widespread and daily use, reactivity as DBPs precursors role and limit information on their biological effects (Chaves et al., 2019; Park et al., 2016; Postigo and Richardson, 2014; Richardson et al., 2015).

The reactivity and the amount of the DBPs formed depends on the physicochemical properties of raw water; on the WTP operational conditions parameters, such as disinfectant dose, temperature, pH, contact time and coagulant agent of the process; environmental conditions, such as climate, and distribution system conditions (Plewa et al., 2017; Richardson, 2011; Richardson et al., 2015).

Toxicological and epidemiological studies associated with the chronic exposure to DBPs pointed to potential genotoxicity and cytotoxicity, asthma and skin rashes, bladder and colon cancer in humans. However, some relations are not consistent across studies, namely in epidemiological studies, due to the many variables in study necessary to an accurate measurement of an exposure/dose-response relationship, and the complexity and diversity of this group of compounds (Chaves et al., 2019; Chen et al., 2019; Cortés and Marcos, 2018).

Importantly, the emerging and unregulated DBPs are of particular interest due to the potential toxicological and ecotoxicological effects, comparing with the already controlled ones (Mian et al., 2018). This analysis is challenging because DBPs present high diversity leading to complex mixtures (Postigo et al., 2018). Unregulated and emerging DBPs includes different classes of compounds such as halomethanes, haloketones, iodo-trihalomethanes, nitrosamines, halobenzoquinones, among others. Understanding the public health implication of this issue is crucial for societies and to support effective water safety plans.

In this study, we used the Zebrafish (*Danio rerio*) embryo bioassay (OECD 236), to evaluate the toxicity of seven unregulated DBPs, including Haloketones, Aldehydes, Alcohols and Nitrosamines: 1,1,1-trichloroacetone (TA); Hexachloroacetone (HA); nonanal; 2-ethyl-1-

hexanal (2-EH); 3-chloro-2,2-dimethyl-1-propanol (3-CDMP); N-nitrosodimethylamine (NDMA) and N-nitrosopyrrolidine (NPRD). Embryos were exposed to different concentrations of the target DBPs and multiple endpoints, including, mortality, morphological abnormalities and locomotor behavior were assessed, at specified developmental stages (24, 48, 72 and 96 hpf). The selection of the target chemicals took in consideration the drinking water non-target data analysis of EPAL- Empresa Portuguesa das Águas Livres, SA (unpublished data) and the international literature.

The lowest observed effect concentration (LOEC) and effective concentration 20 (EC20) and 10 (EC10) values were determined, and the findings discussed considering the current risk assessment framework for this group of compounds. The EC10 values ranked the studied DBPs, from the most toxic to the lowest, 2-EH > NDMA > TA > NPRD > 3- CDMP > Nonanal > HA. The target DBPs of this work showed higher toxicity values when compared with some already regulated ones, such as THMs.

This work provides meaningful toxicological data for unregulated DBPs, reinforcing the need of more comprehensive studies regarding the potential exposure effects in public health and ecological systems.

2. Material and Methods

2.1. Chemicals

The selected compounds represent different families of DBPs. The chemicals for the present study were provided by TCI America: ,1,1-trichloroacetone (CAS 918-00-3), 2-ethyl-1-hexanal (CAS 123-05-7) and 3-chloro-2,2-dimethyl-1-propanol (CAS 13401-56-4); by Dr. Ehrenstorfer: Hexachloroacetone (CAS 116-16-5); by Sigma-Aldrich: Nonanal (CAS 124-19-6) and NDMA- Nitrosodimethylamine (CAS 62-75-9) and by UltraChemical: N-nitrosopyrrolidine (CAS 930-55-2). All the chemical solutions were prepared daily according to the concentration range presented in table 1.

Table 2- physical chemical properties of the studied DBPs

Target Compounds	**Cas #**	**Range of concentration tested (mg/L)**	**pKa**	**Water solubility (mg/L)**	**Molecular mass (g/mol)**	**Log Kow**
1,1,1-trichloroacetone (TA)	918-00-3	10- 0.18	na	$7.45x10^{+3}$ (at 25°C)	161.4	1.12
Hexachloroacetone (HA)	116-16-5	10- 0.9	9.5 [b]	na	264.7	3.49
Nonanal	124-19-6	10- 0.4	15.6 [a] 6.9 [b]	96 (at 25°C)	142.2	3.27
2-ethyl-1-hexanal (2-EH)	123-05-7	2- 0.017	na	na	128.2	3.07
3-chloro 2,2-dimethylpropanol (3-CDMP)	13401-56-4	10- 0.18	na	na	122.6	na
N-nitrosodimethylamine (NDMA)	62-75-9	0.9-0.037	3.5	$1x10^{+6}$ (at 24°C)	74.1	-0.57
N- nitrosopyrrolidine (NPRD)	930-55-2	4.5- 0.18	3.3	$5.290x10^{+4}$	71.1	-0.19

[a] strongest acidic; [b] strongest basic; [na] non-available

2.2 Species Selection

Danio rerio has been used as a notable model vertebrate species in developmental genetics, toxicology and in a wide range of ecotoxicological test protocols (Oberemm, 2000; Scholz et al., 2008; Segner, 2009; Simmons et al., 2008) due to their small size, robustness, multiple progeny from a single mating, embryos transparency and easy maintenance under laboratory conditions. In addition, the close phylogeny of zebrafish and mammals, with highly conserved signaling pathways, makes this species ideal for performing toxicological studies. Hence, the zebrafish is increasingly used for assessing developmental toxicity of chemicals (Coimbra et al., 2015; Hanigan et al., 2017; Ribeiro et al., 2015; Teixidó et al., 2015).

2.2.1. Zebrafish maintenance

Wild-type zebrafish, 50 days age-old, were obtained from Singapore local suppliers. Animals were acclimated to controlled laboratory conditions, in 250 L aquarium with dechlorinated filtered and aerated water. During this period, fish were kept at 28 ± 1°C, under

a photoperiod of 14:10 h (light:dark) and fed, *ad libitum*, three times per day with commercial fish diet Tetramin (Tetra, Melle, Germany) until reaching adulthood.

2.2.2. Danio rerio fertilization and embryos collection

Adults of zebrafish were kept at a water temperature of 28 ± 1 °C under a photoperiod of 14:10 h (light:dark) in 150 liter aquaria with freshwater (dechlorinated and aerated tap water in a recirculation system with both mechanical and biological filters). The adults were fed ad libitum twice a day with commercial fish diet Tetramin (Tetra, Melle, Germany) supplemented with Artemia spp. In the afternoon before breeding, adult males and females (2:1) were housed in a cage with a bottom cover with glass marbles, within a 30 liters aquarium under the same water and photoperiod conditions as the stock, to allow the fall of the eggs to the bottom of the aquarium (Macedo et al., 2017; Ribeiro et al., 2015). Spawning and fertilization of the eggs were stimulated by the beginning of the light period. The fertilized eggs were collected from the bottom of the aquarium and were cleaned several times with water to remove detritus and avoid the proliferation of microorganisms throughout the experiments (Ribeiro et al., 2015).

2.2.3. Embryo bioassays

Static-water renewal toxicological tests with zebrafish embryo were performed according to the ecotoxicity test guidelines of the Organization for Economic Cooperation and Development (OECD), FET 236 (OECD, 2013).The incubation of the cleaned fertilized eggs was carried out in 24-wells plates. A magnifying glass was used for observation and 24 fertilized eggs, one per well, were placed in total by plate previously filled with 2 ml of fresh individual DBPs solution or experimental control. The solutions were prepared by dilution of the chemical standard in MilliQ water. The incubation medium was daily renewed.

The zebrafish embryo medium was composed of 14 mM NaCl, 0,5 mM KCl, 0,02 mM Na2HPO4, 0,04 mM KH2PO4, 1,36 mM CaCl2, 2,13 mM MgSO4, 4,34 mM NaHCO3, prepared in 100 mL dH2O (Oberemm, 2000). Exposure plates were sealed with film to prevent evaporation.

The 24-well plates were incubated at 26.5 °C during 96 h under the same conditions as the adults. The bioassays were repeated two independent times, under the same conditions, for a total of 48 embryos and 8 replicates per condition. The medium was daily renewed to

maintain the concentration of solutions and dissolved oxygen and to avoid the proliferation of microorganisms. In addition, dead embryos and chorions after the hatching were removed at each observation time-point. The effects of the exposure were evaluated considering different endpoints such as abnormal cell growth, embryo development, embryo abnormalities (eyes, head, yolk-sac, tail, pericardial edema, hemorrhages, and muscular involuntary contractions), mortality and absence of sensorimotor reflexes at four time-points, 24, 48, 72 and 96 hours post fertilization (hpf). Pictures of zebrafish embryos at each of these periods are presented in figure. 2. Mortality rate and morphological abnormalities were assessed at each of the above observation time-points. The developmental stages were compared with those described by Kimmel et al.(1995).

Furthermore, absence of sensorimotor reflexes was also evaluated at 96 h hpf, in accordance with previous studies in order to access the potential effect on the embryonic behavior (Cunha et al., 2017; Pinho et al., 2016). Each larvae was gently touched with a micropipette tip, in the head or in the tail, to register either positive (immediate swimming and reaction) or negative responses (no movement upon touch). Each larvae was just touched in head or in tail in order to not influence the positive reactions. Half of the replicates were touched in tail and the other half in head. This procedure was repeated 2 times in each, with 15 s rest between each touch, and the positive reactions were calculated.

The embryo development and abnormalities (figure 2) were observed using an inverted microscope (Nikon Eclipse 5100T) equipped with a digital camera (Nikon D5-Fi2) and a microscope camera controller (Nikon's Digital Sight DS-U3). Morphological abnormalities were rated as abnormalities in head, eyes, tail or yolk-sac, developmental delay, abnormal cells, pericardial edema, opaque chorion, excess or lack of pigmentation, lateral position, reduced mobility and involuntary movements. Then, the total abnormalities were expressed as the percentage of embryos with one or more abnormalities in comparison to the control.

2.2. Statistical analysis

All data was analyzed in SPSS Statistics software version 24.0. Homogeneity of variances and normality of data were performed using Levene's and Kolmogorov-Smirnov test, respectively. Significant differences among treatments were tested at the end of each assay: at 96 h post fertilization (hpf) by means of One-Way ANOVA. With the exception of morphological abnormalities, endpoint were square root and arcsine-square root transformed prior to ANOVA. Differences where considered statistically different when $p < 0.05$. Then,

comparisons between control groups and treatments were done using Student–Newman–Keuls multiple comparison test. Moreover, the non-parametric ANOVA Kruskal-Wallis followed by the Games- Howell test, was performed among individual treatments when homogeneity and normality were not achieved. EC10 and EC20 values (with 95 % confidence interval) at 96 hpf, were calculated using probit regression analysis, by modeling the number of individuals with positive response, on total per condition, at the different concentrations (OECD, 2012; Tanaka et al., 2018)

3. Results and discussion

In all sets of experiments, the control group showed a mortality, total abnormalities and negative reflexes response rates equal or lower than 10 %, which indicates that the zebrafish embryo assays were carried out in appropriate conditions. Embryo bioassays were performed upon exposure to a minimum of four different nominal concentrations, spaced by a constant factor of 2.2 (table 1) of the seven different DBPs. The percentage of mortality, total abnormalities and sensorimotor response of zebrafish embryos, at 96hpf, caused by the different concentrations of the tested DBPs is presented in figure1.

Figure 1- column (a) embryo mortality (%), column (b) total abnormalities (%) and column (c) absence of sensorimotor reflexes (%) of zebrafish exposed to different concentration of seven different DBPs, at 96 hpf. Data are expressed as mean ± standard error (n = 8). Different letters indicate significant differences ($p < 0.05$) among treatment.

As shown in figure 1 (a), 2-ethyl-1-hexanal (2-EH) and Hexachloroacetone (HA) were the compounds that induced higher mortality rate, 67 % at 2 mg/L nominal concentration and 60 % at 10 mg/L, respectively. All the other target compounds showed mortality rates lower than 40%. The compounds NDMA (40 % at 0.9 mg/L) and Nonanal (35 % at 4.5 mg/L) presented similar highest mortality rate, followed by NPRD with 29 % of mortality rate at 10 mg/L. In contrast, 1,1,1-trichloroacetone (TA) and 3- chloro- 2,2-dimethylpropanol (3-CDMP) showed no significant effect on embryos mortality rate ($p > 0.05$).

Considering morphological abnormalities (figure 1 (b)), the most sensitive endpoints observed were yolk sac edema, pericardia edema, scoliosis or caudal malformations. Examples of morphological abnormalities observed are presented in figure 2. The compound 2-EH (EC10=0.04 mg/L) was the one showing stronger effect on total morphological abnormalities, along the concentration range studied, with the maximum effect of 73% at 0.9 mg/L, followed by NPRD (max. 42 % at 2 mg/L), TA (max. 46 % at 10 mg/L) and NDMA (max. 38 % at 0.4 mg/L). For the remaining DBPs (Nonanal, HA and 3-CDMP) the abnormalities rates were lower than 30 % ($p<0.05$).

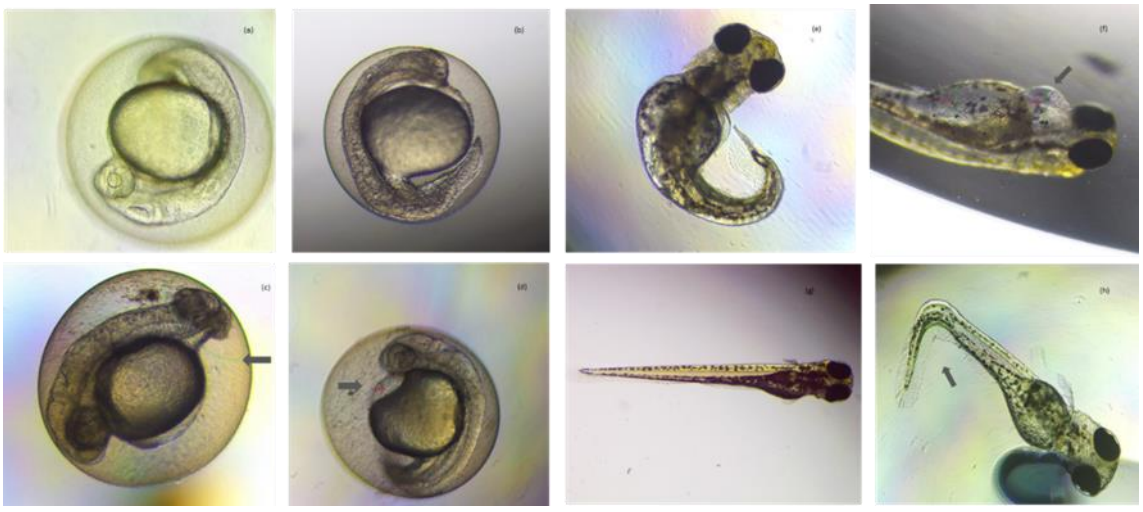

Figure 2- Examples of morphological abnormalities observed at different periods of embryo development with the tested compounds: (a) normal development at 24h hpf (control); (b) abnormal development at 24h hpf; (c) abnormal caudal development at 48h hpf (d) pericardia edema at 48h hpf (e) scoliosis and pericardia edema at 72hpf; (f) Yolk-sac and pericardia edema at 72h hpf; (g) normal development at 96h hpf (control); (h) scoliosis at 96h hfp..The embryo development and abnormalities were observed using an inverted microscope (Nikon Eclipse 5100T) equipped with a digital camera (Nikon D5-Fi2) and a microscope camera controller (Nikon's Digital Sight DS-U3).

Considering behavioral endpoint (figure 1 (c)), larvae exposed to 2-EH and NDMA displayed effects at the lowest concentration levels, particularly due to lack or even absence of sensorimotor reflexes after being gently touched. In some cases, the larvae also exhibited involuntary random contractions. These behaviors suggest that NDMA, and 2-EH could be neurotoxic, which is consistent with the findings of Hanigan et al.(2017), that tested chemicals with a similar structure. On the other hand, no statistically significant behavioral effect was observed for TA and HA ($p > 0.05$).

Based on the mortality rate, total morphological abnormalities and alterations on behavioral responses, the lowest observed effect concentration (LOEC) for each DBPs was measured and ranked, from the highest to the lowest toxicity, as following: NDMA (LOEC = 0.18 mg/L) > 2-ethyl-hexanal (LOEC= 0.4 mg/L) ≈ NPRD (LOEC= 0.4 mg/L) > 1,1,1-trichloroacetone (LOEC= 0.9 mg/L) ≈ 3-chloro-2,2, dimethylpropanol (LOEC= 0.9 mg/L) ≈ Nonanal (LOEC= 0.9 mg/L) > Hexachloroacetone (LOEC= 4.5 mg/L).

Two different DBPs presented similar LOEC value at 0.4 mg/L (2-EH and NPRD). However, they showed different rates of significant effect in the endpoints assessed, defining their overall position in the LOECs ranking. Accordingly, for total morphological abnormalities, at the same 0.4 mg/L nominal concentration, 2- EH (35 %) displayed higher mean abnormalities rate, when compared with the NPRD (27 %); for behavioral response, both showed 17 % of larvae lacking responses.

TA (21 %), Nonanal (15 %) and 3- CDMP (6 %) have LOEC at 0.9 mg/L, showing different effect on the total embryo morphological abnormalities at the same nominal concentration. However, 3- CDMP (21 %) presented higher significant effect in the behavior parameter against Nonanal (15 %) and TA (10 %).

The target DBPs belong to four different classes of compounds: aldehydes (2-EH and Nonanal), nitrosamines (NPRD and NDMA), ketones (HA and TA) and alcohols (3-CDMP). In terms of toxic effects, aldehydes and nitrosamines presented lowest LOEC values. However, 2-EH contrasts with the homologous aldehyde family member (Nonanal) since the first induced more significant effects. This could be related with the molecular structure differences affecting their electrophile characteristics.

***Table 3**- EC10 and EC20 values at 96 hpf, of target DBPs tested.*

DBPs	EC10 (mg/L)	EC20 (mg/L)
2-EH	0.04[a]	0.13 [a]
NDMA	0.06 [a]	0.18 [a]
TA	0.08 [a]	1.26 [a]
NPRD	0.11 [a]	0.33 [a]
Nonanal	0.50 [a]	1.84 [a]
3 CDMP	0.29 [b]	3.93 [b]
HA	9.20 [b]	na

[a] determined using morphological endpoint; [b] determined using behavior endpoint.

The results of this study showed EC10 values (table 2) for some of the DBPs that are 10-fold higher than the range expected in environmental waters (typically in the ng/L-µg/L) and the range reported in human body fluids, which is consistent with data already published for other unregulated DBPs classes (Hanigan et al., 2017b; Teixidó et al., 2015). In accordance with Zaitseva et al., (2018) NDMA has already been reported in children blood in concentration ranging between 0.1 and 0.9 µg/L, after consumption of drinking water (Zaitseva et al., 2018), which is approximately 80-fold lower than the EC10 reported here for this DBP (60 µg/L). Nevertheless, there is a lack of analytical studies reporting the levels of these emerging DBPs. The majority of the studies reports the already regulated DBPs, such as trihalomethanes (THMs) and haloacetic acids (HAAs), and there is a lack of data for unregulated DBPs. The emerging DBPs studied in this work shown EC20 values (table 2), for morphological endpoint, around 100-fold times lower when compared with EC20 for regulated ones, such as the THMs chloroform (84.7 mg/L) and chlorodibromomethane (CDBM) (23 mg/L) (Teixidó et al., 2015). Additionally, according to Teixidó et al., (2015), HAAs such as tribromoacetic acid (TBA) (EC20= 1,300 mg/L), have been described as potentially less toxic, when compared with those THMs reported. More recently, EC50 values attending to morphological abnormalities at 96h were measured for monohaloacetamides (monoHAcAms) and varied between 9.77 mg/L(BAcAm) and 21.10 mg/L (CAcAm), observing that monoHAcAms were potentially more toxic when compared

with the same regulated THMs and HAAs (Ding et al., 2020). Thus, the toxicological data presented reinforces the need of improve knowledge in emerging DBPs, their mode of action and production factors, during to the public distribution system.

While DBPs were tested here individually, most populations are exposed to a complex mixture of different DBPs, and thus combined effects cannot be excluded. Thus, the environmental and human health relevance of the present findings should be interpreted with caution.

4. Conclusions

The present work contributes to improve the available knowledge on the toxicity of seven unregulated DBPs. Importantly, the large number of emerging and unregulated DBPs are toxicologically poorly characterized and several of these have been shown to be potentially more toxic than some regulated ones.

Based on the current findings, special attention should be given to unregulated DBPs, particularly 2- ethylhexanal (EC10= 0.04 mg/L) and NDMA (EC10= 0.06 mg/L), since they showed the lowest ECs values. These results are consistent with the increasing awareness of the regulatory agencies, which are starting to give a special attention to the potential toxic effect of unregulated DBPs, such as NDMA and other chemically-similar nitrosamines.
In order to improve hazard assessment, further toxicological studies should be performed to clarify DBPs biochemical and molecular mode(s) of action. Whereas the zebrafish embryo bioassay is a sensitive high throughput toxicity test, the use of biochemical and molecular biomarkers to characterize the mode of action of DBPs could add additional insights into the toxicity of these chemicals and support hazard and risk assessment. Further, given that the most toxic DBPs tested here displayed EC10 values only 80-fold above concentrations detected in human fluids, chronic studies and mixture toxicity with model animals should be performed given that human population are likely exposed to a complex mixture of DBPs throughout their entire life-cycle.

Transcriptomic Analysis

Considering the results of this toxicological assessment, we selected the 2 DBPs (NDMA and 2-EH) for an exploratory transcriptomics analysis, since this 2 DBPs presented the lowest effective exposure concentrations (ECs), considering NOEC and EC10. Total RNA from zebrafish (n=8) was extracted, considering the exposure concentration (lower than) < NOEC and > (higher than) EC10 for each DBPs and respective controls. This additional study is presented in supplementary information.

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CHAPTER VI. General Discussion and Conclusions

Populations are exposed to multiple environmental stressors, which act combined and, in some cases, act synergistically, causing impacts on health. In 2012, 13 % of all deaths in the EU were attributable to environmental stressors (EEA, 2019, 2013). These deaths are preventable and can be significantly reduced through efforts to improve environmental quality. Non-communicable diseases represent the greatest burden of mortality and morbidity in the EU, representing 90% of deaths attributable to the environment, and includes cancers, stroke, cardiovascular diseases, asthma, among others (EEA, 2019). These diseases have multifactorial backgrounds that are not sufficiently understood. A wide range of chronic diseases is associated with exposure to hazardous chemicals, with the WHO estimating that 2.7 % of global deaths are attributable to chemical exposure (EEA, 2019; WHO, 2016).

Water has one of the most important roles in the regulation and mediation of the different environments, being essential to life and human consumption as well as an important vehicle in the spread of multiple chemicals and pollutants. The problem of many pollutant sources that affect the water quality and availability, and the presence of emerging pollutants that are not currently monitored in drinking water are current concerns.

Water quality monitoring is a fundamental tool in the management of freshwater resources, providing meaningful data to be used in catchment-wide decision making, and to ensure efficient operational control of the water treatment plants (WTPs) and the distribution network. A local and continued monitoring of water supply systems combined with a comprehensive data assessment, are crucial to guarantee the best quality of water for human consumption and effective water safety plans, also leading to the identification of new challenges.

Some of the current challenges addressed in this work include the maintenance of stable concentrations of the residual disinfectants throughout the supply system, preventing pathogens regrowth; and the formation of disinfection by-products (DPBs) mainly influenced by disinfectants dose/stability and the presence of natural and anthropogenic precursors/contaminants in raw waters that can interfere with final water quality.

In chapter II, the global characterization of EPAL's water supply system was made linking both unregulated and regulated water quality parameters considering a 6-year monitoring study. This analysis provides new knowledge regarding occurrence and seasonal behaviors

of both microbiological and chemical parameters, including regulated an UR-DBPs, essential to understand the water supply system and the technologies involved in two different WTPs.

Attending to the number of parameters in the legal context, the requirements have been accomplished in all the sampling points assessed. After 2017, a clear decrease of DBPs occurrence was identified in distribution system, reflecting improvements in the technologies employed in the WTPs. Correlations between the different measured parameters was stablished allowing to identify relevant occurrence relationships. A strong correlation ($r = 0.69$; $p \leq 0.001$) was identified between TOC and THMs, at delivery points; and between both chlorinated- DBPs (HAAs and THMs) at WTPs level (T2: $r = 0.71$; $p \leq 0.001$).

The findings presented in Chapter II give additional insights to the key parameters affecting water quality and may be of interest to other geographical areas with similar supply systems and conventional WTPs. The outcomes of this study contribute to the establishment of a framework to evaluate the quality of water supply system, providing meaningful information that decision makers can use for more effective water safety plans. Understand, select and optimize the processes is crucial for the mitigation of human health risk, attending both regulated and non-regulated compounds, in drinking water. This comprehensive study allowed the identification of improvements in the treatment technologies that led to the decrease of some contaminants, such as HAAs and THMs, in treated water. Despite these improvements, in recent years there has been an increased awareness for the presence of a diversity of emerging and unregulated DBPs in treated water.

Importantly, the emerging and UR- DBPs are of particular interest due to their potential toxicological and ecotoxicological effects, comparing with the already controlled ones (Mian et al., 2018). This analysis is challenging because DBPs present high diversity leading to complex mixtures, widespread occurrence in treated water (i.e., drinking water, swimming pool waters) and diversity of exposure routes (Postigo et al., 2018). The development and validation of quantification methods to measure UR- DBPs in drinking water is the first step towards the implementation of regular monitoring, to establish occurrence profiles and to help risk accessors and water managers to identify the critical DBPs.

In line, the outcomes of chapter III contributed to improve knowledge in drinking water characterization, allowing to identify potential UR-DBPs, such as aldehydes, nitrosamines, ketones, and alcohols, to potential inclusion in regular monitorization and to improve (local) water safety plans. Given the limited validated analytical methods to detect and quantify

UR-DBPs, in this study a new multi-residue gas chromatography coupled with mass spectrometry methodologies for the detection and quantification of 15 UR DBPs was validated, attending to four different classes of compounds, including aldehydes, alcohols, haloketones and nitrosamines. The selection of the target DBPs took in consideration the drinking water non-target data analysis of EPAL (unpublished data) and the toxicological data available. The validation process included two different samples preparation methods, SPE and SPME, depending of target DBPs classes.

From the 15 UR-DBPs analyzed, 8 of them were detected in the analyzed drinking water samples, and were ranked, from the highest to the lowest % of occurrence, as following: decanal > nonanal > 2-EH > 1,1- DCA > 1,1,1- TCA > NPYR > NDMA > NDPhA.

In terms of occurrence, the aldehydes group presented the higher % of occurrence. Decanal (85 %) and nonanal (80 %) were found in almost the samples, and 2-EH (50%) in half of samples analyzed. Besides aldehydes, target (halo) ketones (HKs) presented a moderate occurrence but a heterogeneous behavior, being 1,1-DCA (45%) and 1,1,1-TCA (40%) consistently detected, whereas 1,3- DCA and ACPN were not found in the analyzed samples. Nitrosamines were found in lower %occurrence in the drinking water samples analyzed, with NPYR, NDMA and NDPhA showing occurrences of 30%, 25% and 10%, respectively. For the target alcohols-DBPs no occurrences were verified.

Globally, all the compounds presented values bellow the MQL, expressing analytical thresholds in the low ng/L range. Even so, it is possible to characterize the samples considering the presence of the target UR- DBPs and their concentrations levels, establishing occurrence profiles, identifying critical DBPs and assessing their risk characterization. To our knowledge, this study presents the first insights regarding the measurement of some of the target DBPs in drinking water matrices, considering one of the biggest Portuguese public distribution systems. This study reinforced the need of better study new and emerging DBPs to support the establishment of a more robust water assessment framework. The findings of this study contributed to expand the list of compounds to potential include in future water quality assessments of EPAL, as the one performed in chapter II.

Several of UR- DBPs displayed higher toxicity when compared with the available data for some already regulated, such as trihalomethanes (THMs), so it is crucial to understand their implications in public health and monitoring their occurrence to promote more effective water safety plans.

In chapter IV, we review the recent literature on the toxicological effects of DBPs present in water for human consumption, considering drinking water and water for recreative use,

focusing on unregulated compounds and their putative underlying mode of action, linking the available data with adverse health outcomes. The main knowledge gaps in this field were also identified, and future research priorities discussed.

Overall, most studies highlighted the limited knowledge in the understanding of the underlying mode of action of DBPs and their environmental occurrence profiles. Toxicological and epidemiological studies associated with the chronic exposure to DBPs pointed to potential genotoxicity and cytotoxicity, asthma and skin rashes, bladder, and colon cancer in humans, among others. However, some relations are not consistent across studies, namely in epidemiological studies, due to the many variables in study necessary to an accurate measurement of an exposure/dose-response relationship, and the complexity and diversity of this group of compounds (Chaves et al., 2019; Chen et al., 2019; Cortés and Marcos, 2018).

In order to further contribute to improve risk assessment, seven of the most prevalent UR-DBPs were selected to be included in a toxicological approach.

In chapter V, the developmental toxicity of the chemically- different of 7 UR- DBPs was evaluated using zebrafish embryo bioassay, and considering morphological abnormalities, % mortality and behavior endpoints. This study contributed to improve the available knowledge on the toxicity of the target UR- DBPs. Based on the current findings, special attention should be given to unregulated DBPs, particularly 2- ethylhexanal (EC10 = 0.04 mg/L) and NDMA (EC10 = 0.06 mg/L), since they showed the lowest ECs values. These results are in line with the increasing awareness of the regulatory agencies, which increased the focus on the potential toxic effect of unregulated DBPs, such as NDMA and other chemically similar nitrosamines. The toxicological results showed EC10 values for some of the DBPs that are 10-fold higher than the range expected in environmental waters (typically in the ng/L-µg/L) and the range reported in human body fluids, which is consistent with data already published for other unregulated DBPs classes. The emerging DBPs studied in this work shown EC20 values, for morphological endpoint, around 100-fold times lower when compared with EC20 for regulated ones, such as the THMs chloroform (84.7 mg/L) and chlorodibromomethane (CDBM) (23 mg/L) (Teixidó et al., 2015). Given that the most toxic DBPs tested displayed EC10 values only 80-fold above concentrations detected in human fluids, chronic studies and mixture toxicity with model animals should be performed considering that human population are likely exposed to a complex mixture of DBPs throughout their entire life cycle.

While DBPs were tested individually, most populations are exposed to a complex mixture of different DBPs, and thus combined effects cannot be excluded. Thus, the environmental and human health relevance of the present findings should be interpreted with caution. The results presented in chapter V further supports the need to produce toxicological data in emerging DBPs and their mode of action to support risk assessment. To get further insights into DBPs MoA, two (NDMA and 2-EH) of the 7 target UR-DBPs were selected for an exploratory transcriptomic analysis. In supplementary information, the first results of this analysis are presented. The preliminary results pointed the potential KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.kegg.jp/) enriched pathways. Pathway enrichment analysis identifies significantly enriched metabolic pathways or signal transduction pathways associated with differentially expressed genes (DEGs), comparing the whole genome background. For NDMA the most enriched pathways (padj < 0.1) identified were ferroptosis with six DEGs (n = 6); steroid biosynthesis (n=4) apoptosis (n=10) and lysosome (n=9). For 2-EH the most enriched pathways (padj < 0.05) identified are related with necroptosis (n = 8) and ferroptosis (n= 5). These results set the foundations for more detail studies on the MoA of the most toxic DBPs identified in the present work.

Overall, the work developed here represents the first contributions for an integrative risk assessment considering the presence of target DBPs in drinking water, being accomplished the contaminants characterization, exposure assessment, hazard assessment and dose-response analysis, considering in vivo model and environmental relevant concentrations. Risk characterization combines the information on exposure, considering % of occurrence of target DBPs in drinking water, and dose–response into an overall estimation of likelihood of an adverse consequence. The occurrence profiles established, analytical thresholds (ng/L) and the ECs (ug/L) values measured will support risk accessors and water managers to identify the critical UR- DBPs.

In addition, the outcomes of this study play an important role in the establishment of a comprehensive evaluation of all the water supply system, allowing to identify crucial and emerging compounds, such as DBPs, potential improvements WTPs technologies and distribution network, always guarantying the best quality of water for human consumption. These strategies are closely dependent also of the environmental modification, climate changes and anthropogenic pressure. All these variables have been contributing to a growing awareness for compounds with emerging concern in drinking waters, such as a large diversity of unregulated DBPs. A local and continued monitoring of water supply systems combined with a comprehensive assessment of potential health risks and molecular

mechanisms associated with emerging and UR-DBPs are crucial to implement new approaches always guarantying the best quality of water for human consumption.

Gaps of Knowledge and Future perspectives

The benefits attributable to disinfection in terms of reducing the microbial diseases are indisputable. Water disinfection is one of the greatest achievements for health promotion. Although the water treatment and disinfection processes are well characterized and globally implemented, there is a continuous need of more comprehensive knowledge and technological improvements, leading to an overall better quality of drinking water thus fostering health. In the same way, understanding the public health implications of DBPs present in drinking water is important for societies and decision-makers, always assuming the precautionary principle.

In fact, the majority of studies related with toxicological data of DBPs are designed with regulatory and health-protective purposes, using *in vitro* and/or *in vivo* studies. These conventional strategies are recognized as the more adequate to assess compounds toxicity. However, the measured effects could represent an imprecise estimative or extrapolation of their real health impacts. Other limitations identified in DBPs risk assessment literature are related with the complex nature of DBPs and their occurrence as a complex mixture, limited characterization of exposure in space and time, limited identification of MoA, a poorly characterization of target populations not balancing potential risks of DBPs against the health benefits related with drinking water disinfection, and scarce uncertainties determination.

This thesis addresses several key questions in the field; based on the findings, several new questions emerged and should be the focus of future research. The molecular study should continued in order to complete the evaluation of putative MoA of the target DBPs. Overall, this project provides the first evidence of the molecular mechanisms and pathways affected by the exposure of specific UR-DBPs (NDMA and 2-EH). Thus, the results reported here highlight the importance of integrating chemical-induced effects in toxicity testing strategies and risk assessment.

Improvements on analytical performance can be assessed, considering specific steps, such as preparation/extraction methods, more selective GC columns, among others. The implementation of this methodologies for the regular monitoring of UR-DBPs should be applied to build robust occurrence profiles and seasonal behavior, enabling the establishment of more comprehensive knowledge integrating scientific, technical, ecological, and socio-economic outcomes.

The study of these emerging compounds is particularly relevant considering the climate change scenarios which will hypothetically lead in some regions to alterations on the water cycle, temperature and extreme events. The importance of this association should be understood to improve the management of local water resources.

This thesis represents the first insights for the complex process of risk/exposure assessment, considering the target group of unregulated DBPs selected. The results obtained may contribute for more comprehensive risk assessment considering these emerging compounds. In addition, this work supports the need to develop new approaches and to integrate new potential predictors (prediction factors), such as DBPs exposure, in multivariate models to predict the risk for human health. These prediction models are crucial to a more comprehensive and integrative prognosis studies regarding the relation between health and environment exposure, investigating variables associated with the potential health outcomes (Croft et al., 2015; Hemingway et al., 2010). In line with this, future epidemiologic studies should be also considered to better understand the potential public health outcomes and the positive association with some DBPs. It is important to highlight that some associations are not consistent across different epidemiological studies, given the complexity and diversity of this group of compounds and the heterogeneity of the cohorts. Some systematic reviews and meta-analysis assessing the potential relation between humans DBPs exposure and cancer and other toxicological outcomes have been carried out but the evidence of this association was not consistent to infer a causal association or the experimental design presented weakness that limited the conclusions (Grellier et al., 2015). Most of the reported studies highlights the need of future epidemiology and health studies, supporting this topic, before more definitive conclusion can be draw.

Expanding the knowledge on the MoA of unregulated and poorly studied DBPs will also improve global risk assessment. Crossing other possible exposure routes related with environment exposure, diet or lifestyle (such as tobacco, food industry and occupational

disinfected environments), should also be considered. A global assessment will lead to more comprehensive risk assessment approaches, producing knowledge on exposome, in order to understand how these different exposures, interact with individual characteristics, such as, genetics, physiology and epigenetics, impacting our health. A change towards a cumulative risk assessment, taking account of non-monotonic exposure-response relationships and the effects at low levels of exposure is necessary to avoid underestimation of the risks that might occur from exposure to chemicals present in the environment and in the diversity of consumption products. Since substances are presented in environment as mixtures, that may have synergistic, antagonist or even confounding effects, thus making more difficult to understand the real toxicity of individual compounds.

Widespread exposure to multiple pollutants and chemicals and the concerns about long-term damage to human health together imply the need for more integrative and precautionary approaches. Drinking water quality is presenting consistently high quality across the EU (EEA, 2019), according to the parameters currently monitored. However, the presence of emerging and unregulated pollutants that are not currently monitored in drinking water is a concern and should be further considered.

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Supplementary Information

Transcriptomics analysis

To gain additional insights into the biological functions and potential pathways disrupted by the exposure to 2-EH and NDMA, a comprehensive transcriptome assembly was produced and the transcriptomic profiles, comparing with both controls, were analyzed. After the toxicological measurements (see chapter V), the zebrafish embryos were preserved in RNA later and stored at − 80 °C until further use in RNA-sequencing. Total RNA from zebrafish (n=8) per group was extracted using the Illustra RNA spin Min RNA Isolation Kit (GE Healthcare), according to the manufacturer's protocol. RNA sample integrity and concentration were initially checked by agarose gel electrophoresis and then by an Agilent Bioanalyzer 2100 (Agilent Technologies, USA), with concentration values varying between 119 to 287 (ng/µL).

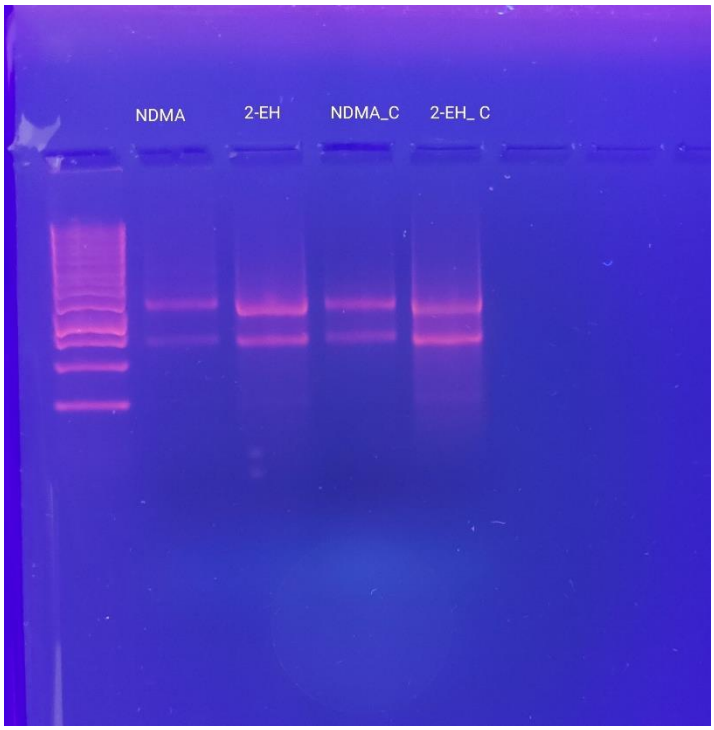

Figure S1- 1.5% agarose gel electrophoresis after RNA extraction.

Hierarchical clustering heatmap (figure. s2) revealed a distinct pattern of gene transcription between both DBPs and the respective controls. The Venn diagram corroborates this pattern identifying approximately 755 DEGs differentially expressed in 2-Ethylhexanal (EH) when

compared with the control (EH_C) (figure s1; C). In N-nitrosodimethylamine (NDMA), 1018 DEGs were differentially expressed, when compared with control (NDMA_C) (figure S1; F) (Anders and Huber, 2010; Neuparth et al., 2020).

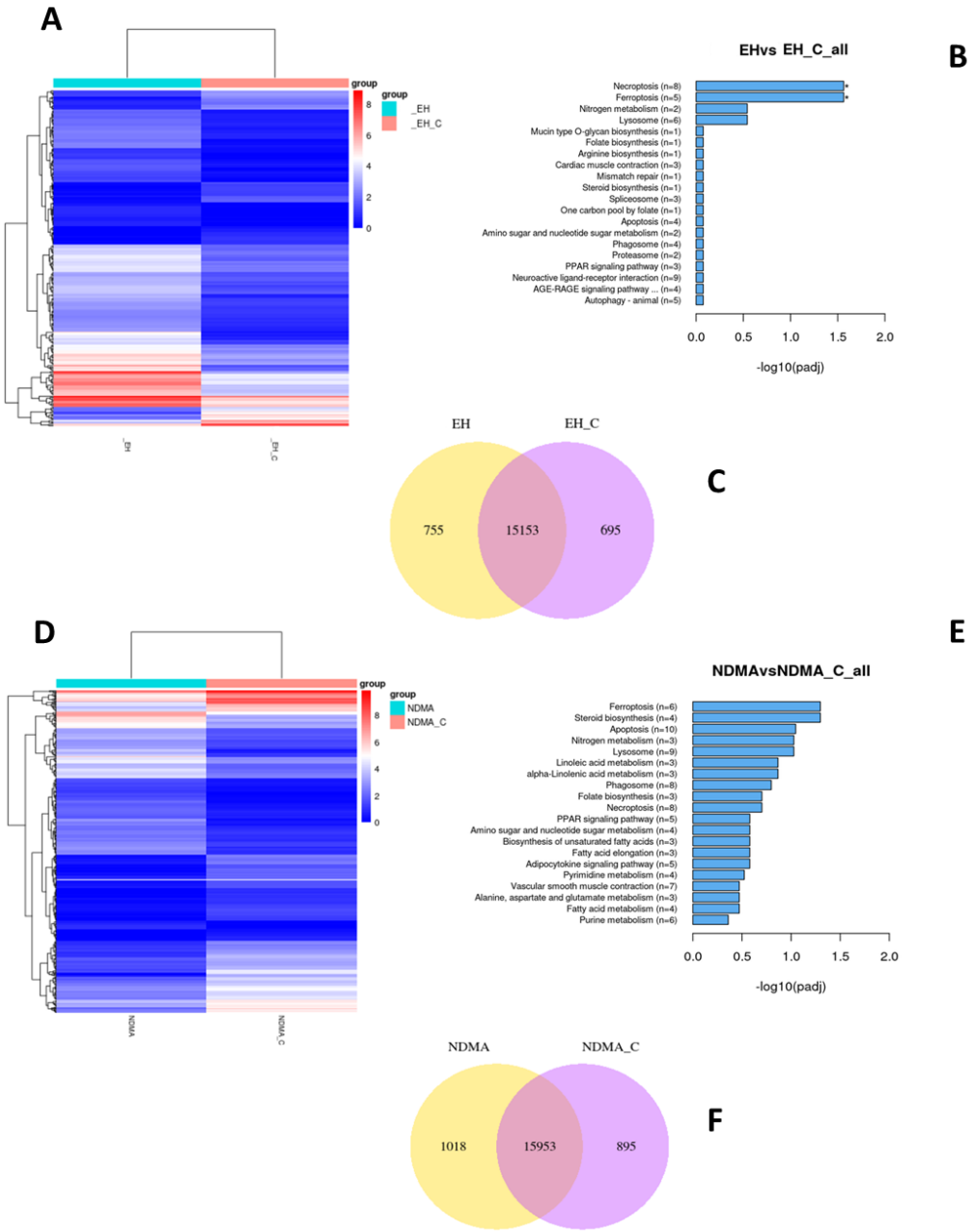

Figure S2: Transcriptomic analysis. (A) Hierarchical clustering heatmap depicting the patterns of gene transcription between 2-ethylhexanal (EH) and the respective control (EH_C). (B) KEGG Enrichment Histogram of top 20 significantly enriched terms for EH (n represents the number of differential gene expression (DEGs). (c) Venn diagram of the overlapping significantly ($p < 0.05$) modulated common genes to EH and control. (D) Hierarchical clustering heatmap depicting the patterns of gene transcription between N- nitrosodimethylamine (NDMA) and respective control (NDMA_C). (E) KEGG Enrichment Histogram of top 20 significantly enriched terms for NDMA (n represents the number of differential gene expression (DEGs). (F) Venn diagram of the overlapping significantly ($p < 0.05$) modulated common genes to NDMA and control

For NDMA the most enriched pathways (padj < 0.1) identified were ferroptosis with six DEGs (n = 6); steroid biosynthesis (n=4) apoptosis (n=10) and lysosome (n=9). For 2-EH the most enriched pathways (padj < 0.05) identified are related with necroptosis (n = 8) and ferroptosis (n= 5) (figure s2; C and E). Ferroptosis is the enriched pathway identified in common for both UR-DBPs.

Ferroptosis, apoptosis and necroptosis are pathways classified on cellular processes, cell growth and death class. Ferroptosis is characterized by a production of reactive oxygen species (ROS) from accumulated iron and lipid peroxidation. It is involved in multiple physiological and pathological processes, such as cancer cell death, neurodegenerative disease, tissue damage and acute renal failure. Apoptosis is a genetically programmed process for the elimination of damaged or redundant cells by activation of caspases (aspartate-specific cysteine proteases). The 'intrinsic' pathway is activated mainly by non-receptor stimuli, such as DNA damage, ER stress, metabolic stress, UV radiation or growth-factor deprivation. The central event in the 'intrinsic' pathway is the mitochondrial outer membrane permeabilization (MOMP), which leads to the release of cytochrome C. Necroptosis is a programmed form of necrosis. It can be initiated by different stimuli, such as tumor necrosis factor (TNF), viral DNA or RNA, DNA-damage agent, among others. Its execution involves ROS generation, calcium overload, the opening of the mitochondrial permeability transition pore, mitochondrial fission, inflammatory response and chromatinolysis. Necroptosis participates in many pathogenesis of diseases, including neurological diseases, retinal disorders, acute kidney injury, inflammatory diseases and microbial infections (Kanehisa and Goto, 2000). The steroid biosynthesis pathway is related with metabolism and lipid metabolism class. Cholesterol biosynthesis is one of the modules related with this pathway. Lysosome is a cellular processes, transport, and catabolism pathway. Lysosomes are membrane-delimited organelles in animal cells serving as the cell's main digestive compartment to which all sorts of macromolecules are delivered for degradation (Kanehisa and Goto, 2000).

Uncertainty Measurement

In accordance with the requirements of ISO 17025(IPAC, 2018) the component of uncertainties measurement is added to give combined uncertainty which is multiplied by a

coverage factor to give the expanded uncertainty. The measurement is expressed as following:

u_{pre}^2 = uncertainty due to precision studies (from recovery data in this study)

u_{std}^2= uncertainty due to preparation of calibration standards

u_{inter}^2= uncertainty due to interpolation of calibration curve

u_c = combined uncertainty

$$u_c = \sqrt{u_{pre}^2 + u_{std}^2 + u_{inter}^2}$$

$u_c \times 2$ (for 95% of confidence level) = U

U= expanded uncertainty

K = 2 (coverage factor for normally distributed data with 95% confidence level)

The table S1 present the uncertainty measurement considering to different approaches. The "bottom-up" approach aims to estimate the individual contribution of every step of the process to the overall uncertainty (U). The BIAS approach is based on the recovery studies.

Table S1- Uncertainty measurement of target DBPs, considering both SPE /GC-MS and SPME/GC-MS analytical methods

Analytical method	Compounds	U (%)	U BIAS (%)
SPME/ GC-MS	*1,1-dichloroacetone (1,1-DCA)*	16.3	27.7
	1,1,1-trichloroacetone (1,1,1-TCA)	17.3	25.4
	1,3- dichloroacetone (1,3-DCA)	19.1	33.6
	1,3-dichloro-2-propanol (1,3- DP)	16.8	29.9
	3-chloro-2,2-dimethyl-1-propanol (3-CDMP)	16.7	33.3
	2-ethyl-1-hexanal (2-EH)	19.2	30.9
	2-ethyl-1-hexanol	12.7	19.3

	(2-HN)		
	Acetophenone (ACPN)	15.0	29.6
	Nonanal	16.4	27.0
	Decanal	18.5	29.6
SPE/GC-MS	*N-Nitrosodimethylamine (NDMA)*	20.8	23.4
	N-Nitrosopyrrolidine (NPYR)	16.7	29.1
	N-Nitrosomorpholine (NMOR)	19.4	30.0
	N-Nitrosopiperidine (NPIP)	14.3	27.7
	N-Nitrosodiphenylamine (NDPhA)	17.9	29.5

All the expanded uncertainties (U) considering "bottom-up approach" presented values lower than 25%., for the target DBPs. The expanded uncertainties considering "BIAS" approach presented values slightly higher when compared with "bottom up" approach, but with values lower than 30%, in accordance with the requirements stablished in the legal context (DL 152/2017, 2017).